Estudio de la presencia y comportamiento de las sulfamidas en el medio ambiente

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Estudio de la presencia y comportamiento de las sulfamidas en el medio ambiente

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Somos nuestros sueños; si no soñamos, estamos muertos. Si no se intenta, si los sueños se quedan en sueños, nunca vamos a conocer la persona que somos. Al final comprendemos que el sueño no es llegar a la cima, sino el camino para llegar a ella, y el fracaso no es no coronar una montaña o parar el crono unos minutos más tarde, sino no ser capaces de tomar este camino"

Kilian Jornet.

La cima es solo la mitad del camino

Ed Visteurs

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SUMMARY

Sulfonamides (SAs) are one of the most widely used antibiotics in human and especially in animal husbandry. So far, concern regarding the environmental presence of sulfonamides and other species of antibiotics has focused mainly on the potential spread of antimicrobial resistance. However, their biological activity and high resistance to biodegradation may lead to long residence times in both water and soil matrices. Long-term ecological risks and unpredicted effects can result from unintentional exposure of different organisms and even human health could be negatively affected. In this context, this PhD thesis aimed to study the environmental occurrence of the most relevant sulfonamides in all types of waters, sewage sludge and agricultural soils. In order to fully understand the fate and effects of this family of antimicrobials in the environment, further research on their resiliance and biodegradability has been carried out.

The need of developing new advanced analytical methodologies, fast, robust and with high sensitivity for the analysis of sulfonamides and their acetylated metabolites at environmental levels was the first and one of the main objectives of this PhD Thesis. The first method developed was based on off-line solid phase extraction followed by high performance liquid chromatography-tandem mass spectrometry (SPE-LC-MS/MS), for the analysis of 9 sulfonamides and 1 acetylated metabolite in wastewater, groundwater and surface water samples. This first method was further implemented by switchIng to on-line SPE, which reduced the sample handling by the user (and the implicit potential errors) to a minimum before HPLC-MS/MS analysis. Furthermore, the target analytes increased to a total of 16 sulfonamides and 5 acetylated metabolites. For the analysis of solid samples, a new method was developed based on pressurized liquid extraction (PLE) followed by HPLC-MS/MS analysis. In all cases, the methods were highly sensitive (limits of detection obtained were in the low picogram per liter level for all the matrices), highly selective (SRM scans) and reliable (the use of different deuterated compounds to act as surrogates and internal standards for quantification by internal standard calibration).

These analytical methodologies were succesfully applied for the analysis of real samples in different monitoring studies. The occurrence of sulfonamides was studied first along the Ebro River basin in two different campaigns carried out in autumn and summer; several samples were taken along the river course and from its main tributaries, together with influent

and effluent wastewaters sampled from 7 wastewater treatment plants (WWTPs) located in the proximities of relevant cities. Another sampling campaign was also carried out in the Llobregat River basin, with a characteristic hydrological regime from Mediterranean areas. Several groundwater samples from seven groundwater bodies under high agricultural pressure, including areas designated as nitrate vulnerable zones under the provisions of Directive 91/676/CEE, were also analyzed. And finally, two extensive sampling campaings were carried out in Germany, where a total of 54 wastewater effluents were analyzed, and in Catalonia, where influent and efluent samples together with sewage sludge were taken in 22 different WWTPs. Sulfonamides and their acetylated metabolites were ubiquitous in all the water samples studied, with levels ranging from the low pg L^{-1} to µg L^{-1} (mainly in wastewater influents, but also in some surface water samples from the Llobregat River). Maximum levels detected in sewage sludge were up to 139.2 ng g^{-1} (sulfamethazine).

All the data gathered from these analyses was used to perform multivariant statistical studies by means of principal component analysis (PCA), for each of the environmental matrices investigated. A more detailed interpretation of the results was achieved with the graphical representation of the scores and loadings obtained.

The levels of sulfonamides detected in the Ebro River basin and in the WWTPs from Catalonia did not pose an ecotoxicity risk for aquatic organisms according to the European Medicines Agency, except for sulfamethoxazole levels present in effluent wastewaters. Despite the dilution exerted from the receiving waters could diminish this risk, extended draught periods could also worsen the situation.

The last part of the PhD focused on the biodegradability and behaviour of the sulfonamides in the environment. The efficiency of conventional wastewater treatments, usually based on activated sludge (CAS), was evaluated and removal rates were estimated for the different sulfonamides studied. Results showed that sulfonamides were generally not fully eliminated and the removal rates obtained in the different WWTPs with the same CAS treatment were very diverse, not allowing for a final conclusion. Furthermore, concentrations detected in the effluent samples were higher than in the influent waters for some of the sulfonamides. Alternative treatments such as membrane bioreactors (MBR) were also considered and influent, effluent and sewage sludge samples from two different MBR pilot plants (using Kubota membranes and Koch membranes) were taken during a sampling campaing of three months. Removal efficiencies were only slightly better, rarely over 50%. The

amounts of SAs present in the different types of digested sludge did not contribute significantly to the overall removal (less than 3 %). In order to further study the behaviour of the sulfonamides, different fixed bed bioreactors (FBBR) were set to evaluate the biodegradability of sulfapyridine, one of the sulfonamides most frequently detected and in higher quantities in water, and its acetylated metabolite. Results showed that sulfapyridine was not fully degraded after 60 days of experiment, evidencing a low biodegradability. On the other hand, the acetylated metabolite was fully eliminated after 35 days, but half of its initial concentration reverted back into the original compound nearly from the start of the experiment. Complementary biodegradation batch studies, using activated sludge in aerobic conditions, confirmed this results, with a 10% of the initial concentration spiked remaining in the batches after 30 days of treatment for most of the sulfonamides.

The ability of white rot fungi *Trametes versicolor* to degrade sulfamethazine was evaluated. This organism has proved its capacity of degrading different clases of pharmaceuticals such as diclofenac or carbamazepine, due to the non-specificity of its enzymatic complex, constituted by peroxidases and laccases, and the intracelular activity of the cytochrome P450. It proved highly efficient to eliminate this sulfonamide to nearly 95% after 96 h. Ultra-performance liquid chromatography-quadrupole time of flight mass spectrometry (UPLC–QqTOF-MS) analysis allowed the identification and characterization of 4 different SMZ degradation intermediates. The degradative capacity of Trametes was also confirmed in solid media (sewage sludge) for sulfamethazine, sulfapyridine and sulfathiazole, enhancing its bioremediation potential.

In the last experiment of this PhD Thesis, we studied the photodegration kynetics of sulfapyridine, sulfamethazine and their respective acetylated metabolites in wastewater effluents and HPLC water under simulated irradiation. UV radiation levels similar to real environmental conditions were sufficient to efficiently remove high concentrations of the sulfonamides studied, requiring a much shorter time than microbial degradation. A total of 10 photodegradation products were identified and structurally characterized for SPY and 7 more for SMZ by means of UPLC-QqTOF-MS.

INDICE GENERAL

ÍNDICE DE ACRÓNIMOS Y TÉRMINOS

UE: Uníón Europea

EU: Estados Unidos

BOE: Boletín Oficial del Estado

DMA: Directiva Marco del Agua

EDAR: Estación depuradora de aguas residuales

CAFO: confined animal feeding operation

EPA: Agencia Europea del Medioambiente *(Environmental Protection Agency)*

EEUU: Estados Unidos

DBO: demanda biológica de oxígeno

FEDESA: Federación Europea de Sanidad Animal

SMX: sulfametoxazol

SDZ: sulfadiazina

PABA: ácido *p*-aminobenzoíco

SSX: sulfisoxazol

SMT: sulfametizol

CAS: fangos activos convencionales (conventional activated sludge)

SPY: sulfapiridina

STZ: sulfatiazol

AcSMX: N^4-acetilsulfametoxazol

SMZ: sulfametazina

AcSPY: N^4-acetilsulfapiridina

AcSDZ: N^4-acetilsulfadiazina

SMR: sulfamerazina

MBR: reactor biológico de membrana

Kd: coeficiente de distribución suelo-agua

SDM: sulfadimetoxina

SMR: sulfamerazina

EMEA: Agencia Europea del Medicamento

MRL: límites máximos de residuo

EC_{50}: concentración efectiva para el 50% de la población

ERA: Evaluación de riesgo medioambiental

PEC: concentración medioambiental prevista

PNEC: concentración estimada sin efecto

NOEC: concentración observada sin effecto

LC_{50}: concentración letal para el 50% de la población

MEC: concentración medioambiental observada

HQ: coeficiente de riesgo

CMI: concentración mínima de inhibición

ARG: reserva de genes resistentes

SCT: sulfacetamida

LC: cromatografía de líquidos

MS: espectrometría de masas

HRT: tiempo de retención hidraúlico

PET: polietileno tereftalato

SPE: extracción en fase sólida

Na_2EDTA: ácido etilendiaminotetraacético (sal sódica)

MIP: polímeros de imprenta molecular

USE: extracción mediante ultrasonidos

PLE: extracción mediante líquidos presurizados

LC-MS: cromatografía de líquidos acoplada a espectrometría de masas

LC-MS/MS: cromatografía de líquidos acoplada a espectrometría de masas en tandem

UHPLC: cromatografía de líquidos de ultra alta resolución

ESI: fuente de ionización electrospray

APCI : ionización química a presión atmosférica

APPI : fotoionización a presión atmosférica

Q : analizador de tipo cuadrupolo simple

LIT : analizador de tipo trampa de iones lineal

QqLIT : analizador híbrido tipo trampa de iones lineal-triple cuadrupolo

QqQ : analizador de tipo triple cuadrupolo

TOF: analizador de tipo tiempo de vuelo

QTOF: analizador híbrido de tipo tiempo de vuelo-triple cuadrupolo

IUPAC: Unión Internacional de Quimica Pura y Aplicada

EPA: Agencia de Protección del Medio Ambiente

RSD: desviación estándar relativa

SRM: selected reaction monitoring

FIA: flow injection analysis

HCOOH: ácido fórmico

MEOH: metanol

ACN: acetonitrilo

HPLC: cromatografía de líquidos de alta resolución

LOD: límite de detección

SNT: sulfanitran

PCA: análisis de componentes principales

PC: componente principal

FBBR: bioreactor de lecho fijo

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JUSTIFICACIÓN DE LA TESIS

La protección de los recursos hídricos para obtener agua potable, pero también para agua de riego, actividades lúdicas y reservas naturales debe ser prioritaria en cualquier legislación para el mantenimiento y la conservación del medioambiente y para un desarrollo sostenible. Durante la última década se han publicado un gran número de estudios de investigación sobre la ubicuidad de diferentes contaminantes orgánicos en todo tipo de matrices ambientales, principalmente en agua. Entre éstos, la presencia medioambiental de fármacos y en concreto de antibióticos ha sido investigada con especial interés, debido a sus altas tasas de consumo y elevada actividad biológica (promoción de resistencia bacteriana). A este deterioro de la calidad de los recursos hídricos se une además el problema de su escasez, sobre todo en las cuencas mediterráneas, de modo que la reutilización del agua se hace imprescindible. Sin embargo, la falta de eficacia de los sistemas convencionales de tratamiento de aguas residuales para eliminar estos contaminantes orgánicos pone de manifiesto su entrada continuada en las aguas superficiales receptoras mediante los efluentes de las depuradoras, y el riesgo que supondría esta reutilización. Así pues, queda patente la necesidad de disponer de información nueva y valiosa sobre la presencia medioambiental de las sulfamidas, familia de antibióticos de alto consumo y cuya presencia medioambiental tiene origen en un uso principalmente veterinario y agrícola, (sulfametazina, sulfadimetoxina), pero también humano (sulfametoxazol, sulfapiridina). Para ello, es necesario el desarrollo de diversas herramientas analíticas avanzadas que nos permitieran la determinación de esta familia de antibióticos en diferentes matrices de agua ambiental, así como en fango de depuradora y suelo agrícola. Con las diferentes metodologías analíticas se pretende evaluar la presencia y persistencia de estos antibióticos y de sus metabolitos mayoritarios en los diferentes ecosistemas acuáticos, evaluando la eficacia de las técnicas de tratamiento de aguas residuales convencionales aplicadas en la actualidad en diferentes EDARs, y también de varias alternativas como los MBR o FBBR. Asimismo, estudiando su biodegradabilidad y persistencia medioambiental, es posible conocer en mayor profundidad la naturaleza y comportamiento de estos compuestos. Todos estos nuevos conocimientos pueden ser de gran valor en la creación de nuevas normativas de carácter restrictivo-cautelar para el uso y consumo del agua y otras matrices ambientales.

OBJETIVOS GENERALES

Considerando la situación actual de contaminación de los recursos hídricos, la falta de eficacia de los tratamientos de agua residuales convencionales y la necesidad de nuevas metodologías analíticas capaces de detectar la presencia de estos contaminantes a niveles ambientales y de manera rápida y fiable, los objetivos de la presente Tesis han sido los siguientes:

1. El desarrollo de nuevas metodologías analíticas basadas en la cromatografía de líquidos acoplada a la espectrometría de masas para la determinación de sulfamidas y sus metabolitos acetilados en aguas (residuales, superficiales, subterráneas) y matrices sólidas (fangos de depuradora y suelos agrícolas).

2. La aplicación de dichas metodologías para la determinación de estos compuestos en todo tipo de aguas ambientales, principalmente aguas residuales pero también superficiales y subterráneas, así como fangos de depuradora y suelos agrícolas.

3. La evaluación de la eliminación de estos compuestos durante el tratamiento en las diferentes depuradoras, comparando la eficacia de diferentes tipos de tratamiento y su impacto en las zonas de vertido, comprendiendo así el destino de estos antibióticos en todo el ciclo del agua.

4. La evaluación de la biodegradabilidad de diferentes sulfamidas mediante el tratamiento con hongos en medio líquido y sólido, y la identificación de los productos de transformación derivados mediante cromatografía de líquidos de ultra-alta resolución-espectrometría de masas en tándem con un sistema híbrido cuadrupolo tiempo de vuelo (UPLC-QqTOF-MS/MS).

5. El estudio de la fotodegradación de diferentes sulfamidas y sus metabolitos acetilados en diferentes matrices acuosas y la identificación de los productos de fototransformación que se generan durante su fotólisis mediante UPLC-QqTOF-MS/MS
6. La evaluación del impacto medioambiental de las sulfamidas detectadas en los diferentes tipos de agua estudiados, estimando los coeficientes de riesgo asociados.

ESTRUCTURA DE LA TESIS

Esta Tesis está dividida en cinco capítulos principales. En el primer Capítulo y a modo de Introducción, se plantea la problemática actual derivada de la presencia de las sulfamidas en el medio ambiente, detallando sus características fisicoquímicas, hábitos de consumo y resaltando el posible impacto medoambiental generado en el medio receptor. Asimismo, se ha llevado a cabo una revisión bibliográfica detallada sobre los niveles detectados hasta la fecha en todo tipo de aguas ambientales y matrices sólidas, asi como de las metodologías analíticas más utilizadas para su análisis. El Capítulo 2 describe el desarrollo y la validación de los nuevos métodos analíticos desarrollados para el análisis de sulfamidas tanto en aguas como en suelos agrícolas y fangos, todos ellos basados en técnicas LC-MS/MS, incluyendo los 4 artículos producidos. El Capítulo 3 detalla la aplicación de dichos métodos para estudiar la presencia medioambiental de las sulfamidas. Este capítulo recoge 4 artículos donde se muestran los níveles de concentración detectados en los diferentes tipos de aguas, fangos y suelos estudiados e incluye además un estudio estadístico multivariante de estos datos para facilitar su interpretación. El Capítulo 4 describe los experimentos de biodegradación llevados a cabo en colaboración con el Profesor Thomas Knepper en la Hochschule Fresenius en Idstein (Frankfurt), y discute las eficacia de diferentes tipos de tratamiento de aguas residuales para la eliminación de las sulfamidas. También incluye el trabajo realizado conjuntamente con la Universidad Autónoma de Barcelona para evaluar la capacidad degradativa del hongo *Trametes versicolor* frente a estos antibióticos, y en el que además se caracterizaron los nuevos productos de transformación generados, e incluye los 3 artículos producidos. Para finalizar, el Capítulo 5 describe el trabajo realizado sobre fotodegradación bajo irradiación artificial o simulada, donde se estudió la cinética de degradación de diferentes sulfamidas y sus metabolitos acetilados y también se identificaron los diferentes productos de fototransformación derivados.

1. INTRODUCCION

1.1 EL RETO MEDIOAMBIENTAL DE LOS CONTAMINANTES EMERGENTES

En una sociedad que cada vez ejerce una presión mayor sobre los recursos naturales, prácticamente todas las actividades humanas (agricultura, industria, servicios, etc) generan y emiten sustancias nocivas al medio ambiente. La cantidad y tipo de estos contaminantes antropogénicos varía constantemente, siendo algunos de ellos, como plaguicidas, metales pesados o hidrocarburos aromáticos considerados altamente nocivos tanto para la salud humana como para el medio natural. Atmósfera, suelo y agua actúan irremediablemente como receptores de dichas emisiones, pero la importancia del agua como base fundamental de todo ecosistema ha hecho que este medio haya suscitado mayor interés científico que el resto. El incesante desarrollo económico y urbano ha hecho que tanto la cantidad como la calidad de este recurso haya disminuido de un modo alarmante, convirtiéndose en un bien escaso, contaminado y altamente vulnerable.

En España, la primera ley de protección de aguas fue redactada en 1879, derogada posteriormente al entrar en vigor la Ley 25/1985, de 2 de agosto (BOE num.189 de 8-8-1985), donde se puso de manifiesto la importancia y la vulnerabilidad de este recurso natural. Esta ley sería derogada posteriormente por la Ley 46/1999, de 13 de diciembre (BOE num. 298, de 14-12-1999) y finalmente por el Real Decreto 1/2001, de 20 de Julio, por el que se aprobaba el nuevo texto refundido de la Ley de Aguas (BOE num. 176, de 24-7-2001). A nivel europeo, se redactó la Directiva 2000/60/CE, también denominada Directiva Marco del Agua (DMA)(Directiva 2000/60/CE), que establece un marco comunitario de acción para la protección y gestión del agua. En dicha directiva se incluyen 33 sustancias de interés prioritario (recogidas en la lista de la Decisión num. 2455/2001/EC (Decisión num. 2455/2001/EC)). En España, esta directiva se adoptó mediante el Real Decreto 140/2003 de 7 de febrero, estableciendo también criterios sanitarios para la calidad del agua de consumo humano (RD 140/2003). Posteriormente, la DMA sería modificada en la nueva Directiva 2008/105/EC de 16 de Diciembre (Directiva 2008/105/EC); en ésta se establecían las concentraciones máximas y

medias anuales admisibles para estas sustancias prioritarias y otros contaminantes en aguas superficiales y biota. Recientemente se adoptó también la Directiva 2006/118/CE, de 12 de diciembre de 2006, para la protección de las aguas subterráneas contra la contaminación y el deterioro (Directiva 2006/118/CE). Respecto a los suelos, en España está vigente la Ley 22/2011, de 28 de julio, sobre residuos y suelos contaminados, pero no contiene ningún listado de sustancias prioritarias (BOE num. 181 de 29-7-2011). En la actualidad existe una propuesta de Directiva Marco para suelos similar a la existente para el agua que data del 2006, pero que aún no ha sido aprobada (http://ec.europa.eu/environment/soil/index_en.htm).

Los contaminantes prioritarios mencionados en la DMA son, sin embargo, una pequeña parte del amplio espectro de sustancias químicas que actualmente están siendo utilizadas y desechadas en el medio natural. Los recientes avances en el campo de la metodología analítica han permitido el estudio de gran cantidad de sustancias potencialmente perjudiciales para diferentes ecosistemas que previamente no eran detectadas o bien no eran consideradas de riesgo y, por tanto, no estaban incluidas en ningún tipo de legislación. Estas sustancias han sido denominadas ***contaminantes emergentes***, contaminantes que no habían sido reconocidos como tales y cuya presencia en el medio ambiente no es necesariamente nueva, pero si la preocupación por las posibles consecuencias de la misma. La Tabla 1.1 incluye algunas de las clases más destacadas, como fármacos, productos de cuidado personal, detergentes y retardantes de llama (Lopez de Alda *et al.* 2003). Una característica común a todos ellos es que, si bien son introducidos en el medio ambiente a concentraciones no muy elevadas, lo hacen de manera regular y continua, de modo que concentraciones consideradas inocuas pueden estar dando lugar a efectos negativos sin necesidad de que el contaminante o sustancia en cuestión sea persistente. Este es el principal rasgo que diferencia estas sustancias de otros contaminantes "clásicos" que llegan al medioambiente de manera puntual y en grandes cantidades, como los vertidos industriales (controlados o no) o los plaguicidas, aplicados de manera directa en suelos de cultivo. El creciente interés y número de trabajos de investigación publicados en los últimos años han proporcionado suficiente información para que algunos de éstos contaminantes emergentes, como los detergentes de tipo alquilfenol etoxilados (nonilfenoles y octilfenoles) y sus metabolitos, retardantes de llama polibromados o las parafinas cloradas, hayan sido incluidos en la lista de contaminanes prioritarios de la DMA.

Su alta frecuencia de detección junto con su potencial como disruptores endocrinos han sido sin duda decisivos en su inclusión en esta directiva.

Tabla 1.1. Clasificación de los contaminantes emergentes más comunes, adaptada de Lopez de Alda et al. 2003.

Fármacos	
Antibióticos (veterinarios y humanos)	Trimetoprima, eritromicina, lincomicina, sulfametoxazol
Analgésicos y antiinflamatorios	Codeina, ibuprofeno, acetaminofen, ácido acetilsalicílico,diclofenac, fenoprofen
Antidepresivos	Diazepam
Reguladores lipídicos	Bezafibrato, ácido clofíbrico, ácido fenofíbrico
Beta bloqueantes	Metaprolol, propanolol, timolol
Medios de contraste de rayos X	Iopromida, iopamidol, diatrizoato
Esteroides y hormonas	α-etinilestradiol(EE2), estradiol, estrona, estriol, dietilestilbestrol
Drogas de abuso	Metanfetaminas, metilenodioximetamfetamina (MDMA), morfina, 6-acetilmofina
Productos de cuidado personal	
Fragancias	Nitroalmizcles, musks policíclicos y macrocíclicos
Filtros solares	Benzofenona, metilbencililden canfor
Repelentes de insectos	N,N-dietilt-m-toluamida (DEET)
Antisépticos	Triclosan, clorofeno
Surfactantes y metabolitos	
Alkilfenol etoxilado, alkilfenol carboxilados, alkilfenoles (nonilfenol y octilfenol)	
Retardantes de llama	
Difenileter polibromado (PBDEs), tris(2-cloroetil) fostato	
Actividades industriales	
Antimonio, agentes quelantes (EDTA), sulfonas aromáticas	
Edulcorantes artificiales	
Sucralosa	
Retardantes de llama	
Difenileter polibromado (PBDEs), tris(2-cloroetil) fostato	
Nanomateriales	
Nanopartículas de plata, TiO_2, fulerenos	
Aditivos de la gasolina	
Dialkiléters, metil-t-butil eter (MTBE)	
Compuestos perfluorados	
Sulfonato perfluoroctano (PFOs), ácido perfluoroctánico (PFOA), ácidos perfluorocarboxílico (PFCAs)	

Otros contaminantes como los compuestos organoestannicos, subproductos de desinfección de agua potable y metabolitos de plaguicidas han sido legislados más recientemente (RD 140/2003). Los fármacos y productos de cuidado personal aún no han sido

incluidos en ningún tipo de legislación. Si bien ya se ha demostrado que algunos de ellos, como el estradiol, pueden actuar como disruptores endocrinos, el conocimiento sobre su presencia e impacto en los distintos compartimentos ambientales se considera aún insuficiente. Recientemente, los fármacos carbamazepina y diclofenaco fueron considerados para su inclusión en la lista de sustancias prioritarias de la nueva Directiva 2008/105/EC sobre estandares de calidad medioambientales, pero finalmente fueron descartados, mientras que otros contaminantes emergentes como el bisfenol A o la fragrancia musk xileno sí se incluyeron en el Anexo III de dicha Directiva para su revisión y posible clasificación como sustancias prioritarias (Directiva 2008/105/EC).

1.1.1 Los fármacos como contaminantes emergentes

De todos los contaminantes emergentes los fármacos son los que probablemente hayan generado una mayor preocupacion científica e impacto social en los últimos años debido principalmente a la cotidianeidad de su uso. El aumento en el consumo de fármacos tanto de uso humano como veterinario es consecuencia directa de la creciente población humana a nivel global y del aumento de la esperanza de vida, sobre todo en países occidentales. Paralelamente, el consecuente incremento de la demanda alimentaria y, por tanto, del numero de actividades ganaderas intensivas también contribuye de manera importante en la cantidad de fármacos consumidos, en este caso veterinarios. Su uso habitual y su inocuidad para el organismo en las dosis establecidas han hecho que las cantidades consumidas hayan sido consideradas irrelevantes medioambientalmente. Sin embargo, su detección en prácticamente todo tipo de aguas continentales ha suscitado el interés de la comunidad científica y como resultado, las investigaciones sobre su presencia y destino asi como el número de nuevas metodologías analíticas desarrolladas han aumentado y mejorado enormemente en los últimos años, especialmente en el estudio del agua y en el ámbito medioambiental, como refleja la Figura 1.1. En la actualidad, aproximadamente 3000 tipos de fármacos diferentes son utilizados en la UE, entre los que se encuentran antibióticos, beta bloqueantes, reguladores lipidicos, antidepresivos y analgésicos. Se estima que el consumo de fármacos en los paises de la UE es de varias toneladas por año, algunos empleándose en cantidades similares a las de los plaguicidas (Jones *et al.* 2001; Ternes *et al.* 2006). La Figura 1.2 muestra el aumento progresivo en el gasto en fármacos a nivel global a lo largo de la última década.

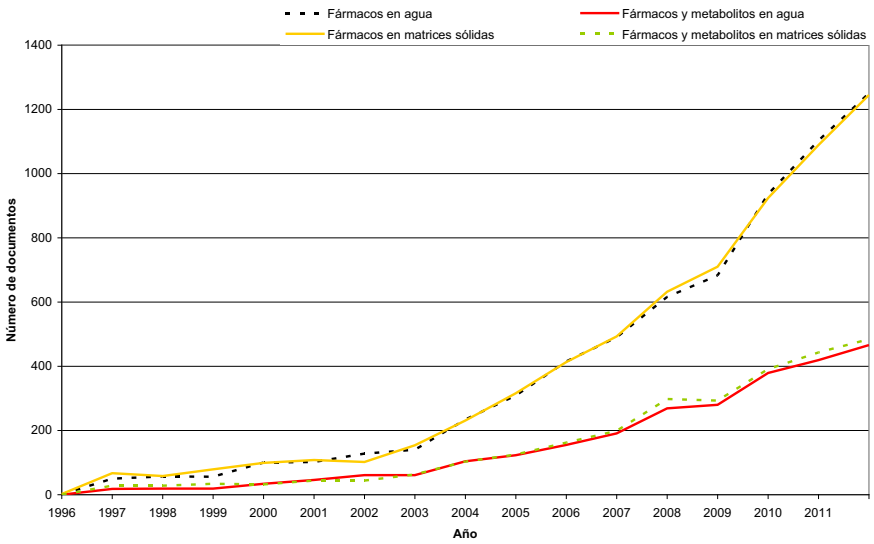

Figura 1.1. Número de publicaciones sobre la presencia de fármacos en el medio ambiente durante los últimos 15 años. Fuente: Scopus; fecha: 15.01.2012. Criterio de búsqueda: **1**. Occurrence + pharmaceuticals + surface waters or groundwaters or wastewaters. **2**. Occurrence + pharmaceuticals + metabolites +surface waters or groundwaters or wastewaters. **3**. Occurrence + pharmaceuticals + soil or sediment or sludge. **4**. Occurrence + pharmaceuticals + metabolites + soil or sediment or sludge.

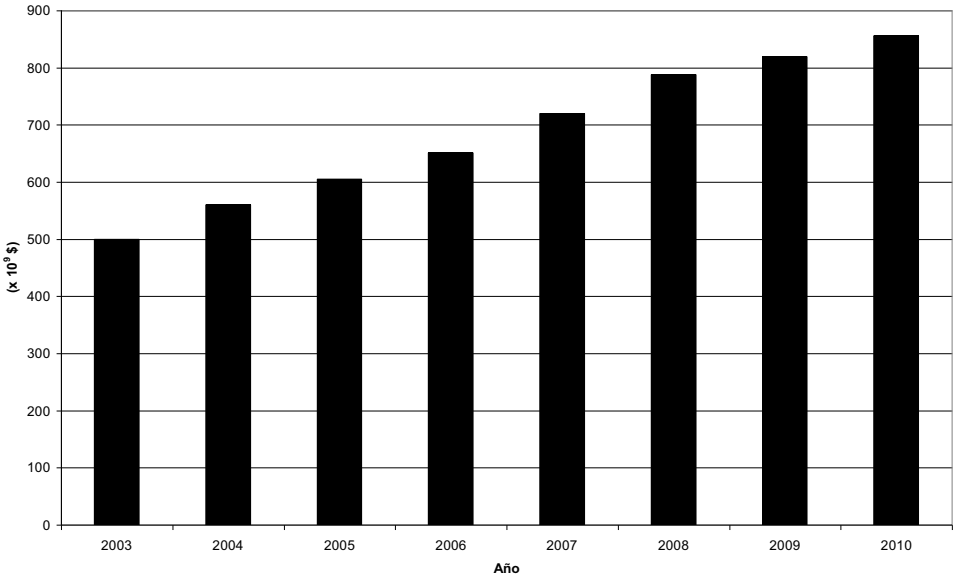

Figura 1.2. Gasto global en fármacos durante la última década. Fuente: www.imshealth.com

Como se observa en la Figura 1.3, España se encuentra entre los 10 paises con mayor gasto en fármacos del mundo.

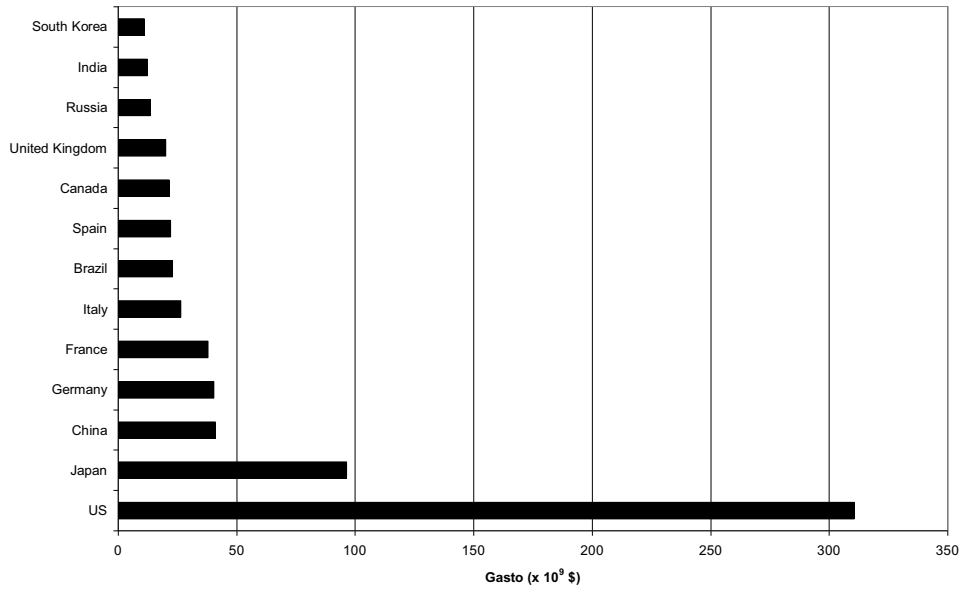

Figura 1.3. Gasto en fármacos por países. Fuente: www.imshealth.com

Los fármacos son sustancias biológicamente muy activas, diseñadas para causar una respuesta determinada en el organismo, y que una vez excretadas pueden mantener toda o parte de esa actividad en función del grado en que hayan sido metabolizadas, pudiendo causar efectos adversos en especies no diana (Sarmah *et al.* 2006). Un ejemplo reciente es el de la disminución drástica de la población de buitres en Pakistán, que se alimentaban de reses de ganado muertas que habían sido tratadas con el antiinflamatorio diclofenaco (Oaks *et al.* 2004). Así mismo, se ha documentado la aparición de cepas resistentes a determinados antibióticos, la bioacumulación de antidepresivos en diferentes tejidos de peces y fenómenos de feminizacion, hermafroditismo y disminucion de la fertilidad en éstos por la presencia de estrógenos o la inhibicion o estimulacion del crecimiento de organismos planctónicos, plantas acuaticas y algas (Nikolaou *et al.* 2007). Los posibles efectos cancerigenos y una mayor toxicidad debida a efectos sinérgicos son consecuencias que también deben ser consideradas.

1.1.2 Vías de entrada de los fármacos al medio natural

Tras su ingesta, los fármacos se transforman en mayor o menor medida en el organismo tras la ingesta (p.e. sólo un 4% del paracetamol es excretado en su forma original, mientras que la amoxicilina lo es en un 60%, (Lienert *et al.* 2007)), de modo que una combinación de estos compuestos y sus respectivos metabolitos y especies conjugadas es excretada regularmente en la orina y las heces. De hecho, las primeras evidencias de la presencia de fármacos en el medio ambiente hacían referencia a la detección del ácido clofibrico, metabolito activo de varios reguladores lipídicos (clofibrato, etofilin clofibrato y etofibrato), en aguas residuales de EEUU en la década de los 70 (Hignite *et al.* 1977). Las cantidades no asimiladas por el organismo y los correspondientes metabolitos son excretadas en aguas de desagüe (aguas residuales urbanas) que finalmente llegan a las estaciones depuradoras de aguas residuales (EDAR). Su eliminación durante el tratamiento suele ser insuficiente para la mayoria de fármacos, ya que entre los parámetros de control de eficiencia de las EDAR no se encuentra la determinación de ningún contaminante emergente y los objetivos de reducción de demanda biológica de oxígeno (DBO) marcados para las EDAR no son lo suficientemente efectivos. Por tanto, estos efluentes de EDAR suelen considerarse como las principales fuentes de vertido de estas sustancias en zonas urbanas (Kolpin *et al.* 2004; Miao *et al.* 2004; Gros *et al.* 2007). Por otro lado, la aplicación de biosólidos de EDAR como fertilizantes en campos de cultivo constituye tambien una importante via de entrada al medio natural de aquellos fármacos que durante el tratamiento han sido retenidos en los fangos biológicos. Una vez en el suelo, estos compuestos también pueden infiltrarse a través del terreno hasta alcanzar acuíferos o alcanzar cursos de agua superficial al ser arrastrados por la escorrentía (Edwards *et al.* 2009; Sabourin *et al.* 2009). Otras vías de entrada son los efluentes de hospitales y vertidos accidentales durante su proceso de fabricación (Brown *et al.* 2006; Lin *et al.* 2009; Chang *et al.* 2010), la eliminación de fármacos no usados o caducados a través de los sanitarios o WCs (denominado "*flushing*"), filtración de fosas sépticas y otros sistemas de almacenaje de residuos (Schwarzbauer *et al.* 2002; Bound *et al.* 2005). Además, una vez excretados tanto los fármacos como sus respectivos

metabolitos pueden continuar transformándose mediante procesos de degradación biológica (durante el tratamiento en las EDAR, o bien al llegar al suelo, vertedero u otros lugares de almacenaje de residuos) o abiótica (fotodegradación en los cursos de agua natural, en los tanques de aireación de las EDAR, etc).

En zonas rurales, los fármacos veterinario usados en los criaderos o cebaderos de ganadería intensiva (CAFOs, del inglés *confined animal feeding operations*) llegan al medio ambiente por la deposición directa de heces y orina de animales medicados en el suelo, su almacenaje o bien la posterior aplicación del estiércol derivado de éstos como abono en campos de cultivo, pudiendo llegar a alcanzar los acuíferos por infiltración o corrientes de agua superficiales tras eventos de precipitaciones fuertes y escorrentía. La Figura 1.4 esquematiza todas estas vías de entrada, interrelacionadas por el ciclo del agua.

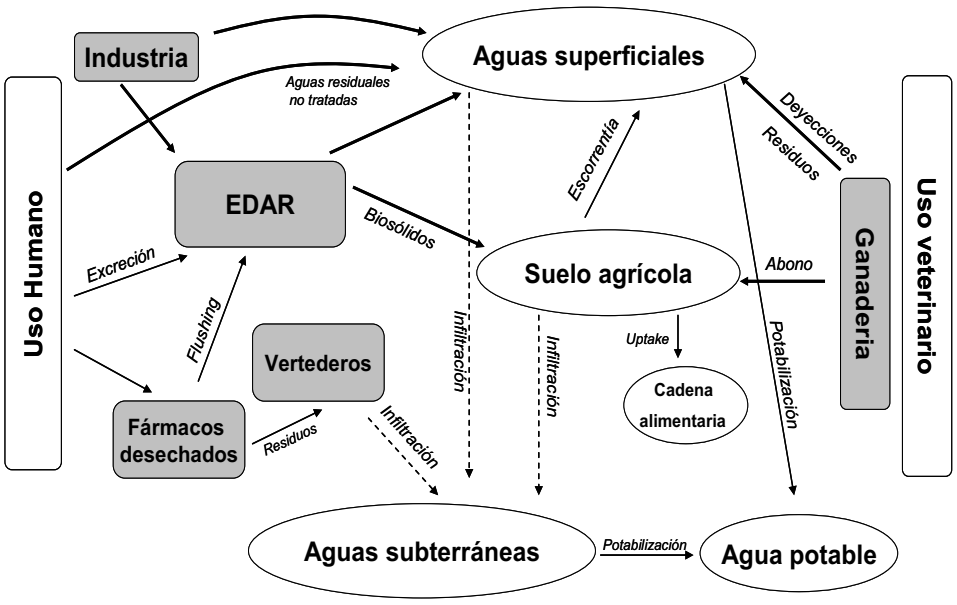

Figura 1.4. Rutas de entrada y destino de los fármacos en el medio ambiente.

1.2 ANTIBIÓTICOS. CARACTERÍSTICAS Y MECANISMOS DE ACCIÓN

De entre la amplia variedad de fármacos existentes en el mercado, los antibióticos son los que suscitaron inicialmente un mayor interés tanto a nivel científico como social. En general, se trata de moléculas complejas, con diferentes grupos funcionales y que, en algunos casos, pueden variar sus propiedades fisicoquímicas y también su actividad biológica en función del pH (Cunningham *et al.* 2008). En la actualidad se conocen unos 5500 antibióticos, de los que tan solo alrededor de unos 100 son sintéticos, entre ellos las sulfamidas, familia en la que se ha centrado esta Tesis. La gran mayoría son producidos de manera natural por diferentes comunidades de eubacterias y también mohos, si bien suelen ser modificados antes de su utilización para eliminar sus efectos tóxicos. El ejemplo más clásico es la penicilina, una de los primeras sustancias descubiertas con propiedades antibióticas que es producida de forma natural por los hongos del genero *Penicillium*. Se suelen agrupar según su estructura química. Así, pueden dividirse en diferentes grupos o familias, como los beta-lactámicos, quinolonas, tetraciclinas, macrólidos y sulfamidas, entre otros. Otras clasificaciones se basan en su mecanismo de acción. La mayoría de los antibióticos actúa inhibiendo la síntesis de compuestos celulares necesarios para mantener la estructura bacteriana, como ácidos nucleicos o proteínas. Por ejemplo, las penicilinas inhiben la síntesis de la pared celular bacteriana, y los beta-lactámicos inhiben la síntesis de los peptidoglicanos de dicha pared; las sulfamidas, como veremos en más detalle en la sección 1.3.1, inhiben la síntesis de proteinas celulares e impiden su crecimiento, compitiendo como análogos estructurales con el ácido *p*-aminobenzoico, mientras que las tetraciclinas y las quinolonas actúan impidiendo la síntesis correcta de moléculas de ARN y ADN. El uso de antibióticos no sólo se limita al tratamiento de infecciones en medicina humana y veterinaria. En las explotaciones ganaderas es muy común su consumo en tratamientos preventivos y como promotores de crecimiento (Gaskins *et al.* 2002), a pesar de la reciente prohibición de estas aplicaciones en la UE (UE 2003). Son también muy utilizados en acuicultura, aunque hoy en día son pocos los antibióticos autorizados, entre ellos oxitetraciclina, florfenicol, sarafloxacin, eritromicina y las sulfamidas en combinación con la trimetoprima (Reglamento CEE 2377/90). También se aplican en agricultura para tratar enfermedades de tipo bacteriano en árboles frutales (p.e. oxitetraciclina y estreptomicina) (McManus *et al.* 2000) .

1.2.1 Presencia medioambiental de los antibióticos.

En términos generales, la evaluación del riesgo ambiental derivado de la presencia de los antibióticos suele basarse en las siguientes consideraciones:

- Cantidades producidas y consumidas: el papel de los antibióticos en ganaderia hoy en dia es primordial, y este hecho se refleja en sus altas tasas de consumo. Aunque la información sobre ventas y cantidades usadas no está disponible para el publico general ni en EEUU ni en UE, calculos estimados indican ventas superiores a los 16000 t en EEUU en 2001, de los cuales 9300 t se emplearon en la cría de ganado intensivo (Sarmah *et al.* 2006). De acuerdo con la Federación Europea de Sanidad Animal (FEDESA), el consumo anual de antibióticos en la UE es de 13288 t con un 29% de este consumo destinado a la medicina veterinaria, un 6% utilizado como aditivos alimentarios y un 65% utilizado en medicina humana. Además, los medicamentos recetados se venden en cantidades un orden de magnitud por debajo de los medicamentos no recetados, dato a tener en cuenta al estimar las cantidades totales (Kümmerer 2004).
- Actividad biológica: como mencionábamos antes, todos los fármacos han sido diseñados para causar una respuesta de tipo biológico en el organismo receptor o al paciente, a concentraciones relativamente pequeñas. Propiedades físico-químicas tales como su alta polaridad o liposolubilidad les permiten atravesar membranas biológicas y también pueden favorecer su bioacumulación en diferentes tejidos.
- Biodegradabilidad: su resistencia a la biodegradación, oxidación, fotodegradación y termodegradación, entre otras, son las propiedades “deseables” para asegurar la efectividad del tratamiento, por ejemplo cuando se añaden antibióticos a los piensos alimenticios o al aplicar el tratamiento en los cultivos. Kümmerer *et al*. estimaron que un 70% de los antibióticos consumidos en hospitales y viviendas se excretaba en su forma original (Kümmerer *et al.* 2003). Estos residuos no metabolizados pueden mantener su actividad, al igual que sus metabolitos, causando efectos no previstos en organismos y especies no diana. Su mayor o menor biodegradabilidad es por tanto

uno de los principales indicadores de la tendencia de los fármacos a acumularse en el medioambiente.

1.2.2 Resistencia bacteriana a los antibióticos

La gravedad de la presencia medioambiental de los antibióticos radica principalmente en el desarrollo de cepas bacterianas patógenas resistentes después de una exposición prolongada al antibiótico en cuestión, dificultando su uso en posteriores tratamientos. Sin embargo, algunos autores como Ayscough *et al*. apuntan que es más probable que el motivo causante de la resistencia adquirida en los diferentes ecosistemas sea la excreción de bacterias ya resistentes por parte de los humanos o animales tratados y no la exposición a niveles ambientales (Ayscough *et al.* 2000). En ambos casos, el uso indiscriminado de antibióticos tanto en medicina humana como en la cría de animales de consumo son responsables directos del desarrollo de cepas resistentes e, indirectamente, de la imposibilidad de tratamiento antimicrobiano para determinadas enfermedades infecciosas. Los mecanismos de resistencia que pueden desarrollarse son variados. Las bacterias pueden disminuir la permeabilidad de la pared bacteriana con la pérdida o modificación de los canales de entrada o porinas; también puede tener lugar la expulsión activa del antibiótico a traves de la membrana, la producción de enzimas inactivantes de los antibióticos (inhibición de aminoglucósidos, penicilinas, cloranfenicol y de los beta-lactámicos) o la mutación y cambio de estructura de enzimas bacterianos sobre los que actuaba el antibiótico (tetraciclinas), (Iyobe 1997; Davies *et al.* 1998) . La *resistencia adquirida* se produce a través de mutaciones, transmitiéndose de forma vertical de generación a generación, pero también mediante la transmisión de material genético extracromosómico portador de dichos genes de resistencia (plásmidos o transposones); en este caso, la transmisión es horizontal, pasando tanto a miembros de nuevas generaciones como a otras especies de bacterias, de modo que éstas pueden adquirir la resistencia a uno o varios antibióticos sin necesidad de haber estado en contacto con estos (Guerra B *et al.* 2000). Así, el número de casos de enfermedades infecciosas como la tuberculosis, que parecía erradicada hacia la década de los 70, el paludismo o la malaria, han vuelto a aumentar sobre todo en algunos países de África o sureste asiático . En veterinaria a su vez, el aumento de casos de cepas de *Salmonella spp*. resistentes a los antibióticos ha sido alarmante en paises desarrollados a escala mundial (Threlfall 2002). Además de la baja efectividad de antibióticos

típicamente usados contra esta patología, como las fluoroquinolonas, las aves y productos destinados a la alimentación humana pueden estar contaminados con esta bacteria y afectar a los consumidores (Carramiñana *et al.* 2004).

Por último, cepas bacterianas beneficiosas como las descomponedoras de materia orgànica también pueden verse afectadas. En el medio ambiente urbano, el caso más evidente son los microorganismos que actúan en los procesos de tratamiento de aguas residuales. En el laboratorio se ha observado la disminución y alteración de estas comunidades microbianas en presencia de antibióticos de uso humano a concentraciones previamente detectadas en efluentes de hospitales (Kümmerer *et al.* 2000; Costanzo *et al.* 2005; Amin *et al.* 2006). El factor tiempo parece ser la variable más importante en estos experimentos, ya que normalmente los ensayos de laboratorio se desarrollan en períodos cortos y los datos obtenidos sólo se pueden interpretar como ecotoxicidad aguda. Se ha demostrado que exposiciones prolongadas (días) causaban efectos negativos a concentraciones de antibióticos un orden de magnitud más bajas que las utilizadas en ensayos de toxicidad aguda (Tomlinson *et al.* 1966) .

1.3 LAS SULFAMIDAS COMO CONTAMINANTES AMBIENTALES.

Las sulfamidas son antibióticos de efecto bacteriostático y con un amplio espectro de actuación frente a un gran número de microorganismos tanto grampositivos como gramnegativos, así como frente a diferentes protozoos. Constituyen una familia de agentes antimicrobianos sintéticos, derivados todos ellos de la sulfanilamida, y utilizados principalmente en acuicultura y en ganaderia para el tratamiento de especies destinadas a consumo humano. También son prescritos en medicina humana para el tratamiento, entre otras, de infecciones del tracto urinario, ojos y oídos, bronquitis crónicas, meningitis, así como algunos tipos de pneumonia y diarrea, y suelen aplicarse en combinación con diaminopirimidinas como la trimetoprima, para potenciar su actividad antibiótica (Pérez-Trallero *et al.* 2003). Hoy en día, sin embargo, la mayoría han perdido efectividad debido a la elevada resistencia bacteriana adquirida y su uso es escaso en medicina humana, siendo el sulfametoxazol (SMX) y la sulfadiazina (SDZ) en combinación con la trimetroprima las dos sulfamidas usadas con más frecuencia en este campo, seguidas de la sulfapiridina, (normalmente como sulfasalazina, unión de la sulfapiridina con mesalazina). En cambio, en

medicina veterinaria su uso es muy elevado (Hamscher *et al.* 2006; Cháfer-Pericás *et al.* 2010), ya que se trata de una familia de antibióticos de coste moderado, de amplio espectro y muy efectivas como promotores de crecimiento, aunque este uso ha sido prohibido legalmente (Regulación 1831/2003/EC). Por otro lado, el aumento en el número de CAFOs mencionado anteriormente ha llevado a un mayor uso de sulfamidas y fármacos veterinarios en general y, en consecuencia, a una mayor presencia medioambiental de dichas sustancias ya que estas instalaciones suelen carecer de una gestión de tratamiento de aguas residuales apropiada.

La acción antibiótica de las sulfamidas fue descubierta en la década de los 30 por Domagk y Tréfouel, siendo los primeros fármacos eficaces empleados para el tratamiento sistémico de infecciones bacterianas en humanos. La sulfanilamida como tal fue sintetizada en 1908 por primera vez como colorante para la tinción de bacterias, y en base a esta molécula se preparó la *p-((2,4-Diaminofenil)azo)bencenosulfonamida*, denominada sulfonamidocrisoidina o por su nombre comercial prontosil (CAS 103-12-8). Su acción bactericida contra los streptococos fue descubierta por el patólogo alemán Gerhard Domagk en 1932 en conejos y ratones, y posteriormente la utilizaría con éxito en humanos, convirtiéndose así en el primer antibiótico oral (Domínguez-Gil Hurlé *et al.* 2007). En la actualidad, las sulfamidas constituyen el segundo grupo de antibióticos más usado en veterinaria en la UE después de las tetraciclinas, con una media de un 23% de las ventas totales en paises como Dinamarca, Reino Unido y Alemania, mientras que en EEUU éstas representan un 2.3% (Sarmah *et al.* 2006).

1.3.1 Mecanismo de acción

Las sulfamidas son análogos estructurales del ácido *p*-aminobenzoico (PABA), y su mecanismo de acción se basa en la inhibición competitiva de la conversión de este ácido, actuando como su antagonista competivivo durante la síntesis del acido fólico bacteriano (Pérez-Trallero and Iglesias 2003). La baja especificidad de la enzima mediadora de esta conversión (pteridina) hace que tras la aplicación de sulfamidas, la enzima se una al antibiótico y no al PABA, formando análogos no funcionales, tal y como se muestra en la Figura 1.5. De este modo también es inhibida la dihidroteroato sintetasa, necesaria para la incorporación del PABA al ácido dihidroteróico, precursor del ácido fólico. El uso del PABA queda así anulado y la síntesis bacteriana del ácido fólico (vitamina B) y finalmente el desarrollo de la célula

bacteriana interrumpidos. La síntesis del ácido tetrahidrofólico, necesario para la síntesis de las bases púricas y del ADN, queda también bloqueada. Por otro lado, como se observa en la Figura 1.5, la trimetoprima es a su vez inhibidor competitivo de la dihidrofolato reductasa, enzima necesaria para el paso de dihidrofolato a tetrahidrofolato, cofactor necesario para la síntesis de ADN. Al actuar sobre la misma vía metabólica del ácido fólico su actividad es sinérgica con las sulfamidas. Además, la aparición de resistencia adquirida y de antibióticos de nueva generación hace que su utilización conjunta sea casi imprescindible para asegurar su eficacia. Las sulfamidas, por tanto, sólo son efectivas si las bacterias son metabólicamente activas. Generalmente al inicio del tratamiento se observa un periodo de ineficacia debido a que las bacterias pueden disponer de reservas de ácido fólico. A diferencia de las células eucarióticas de los mamíferos, que toman el ácido fólico en la dieta, las bacterias tienen que sintetizarlo, hecho que las hace más sensibles a la acción de las sulfamidas que el huesped.

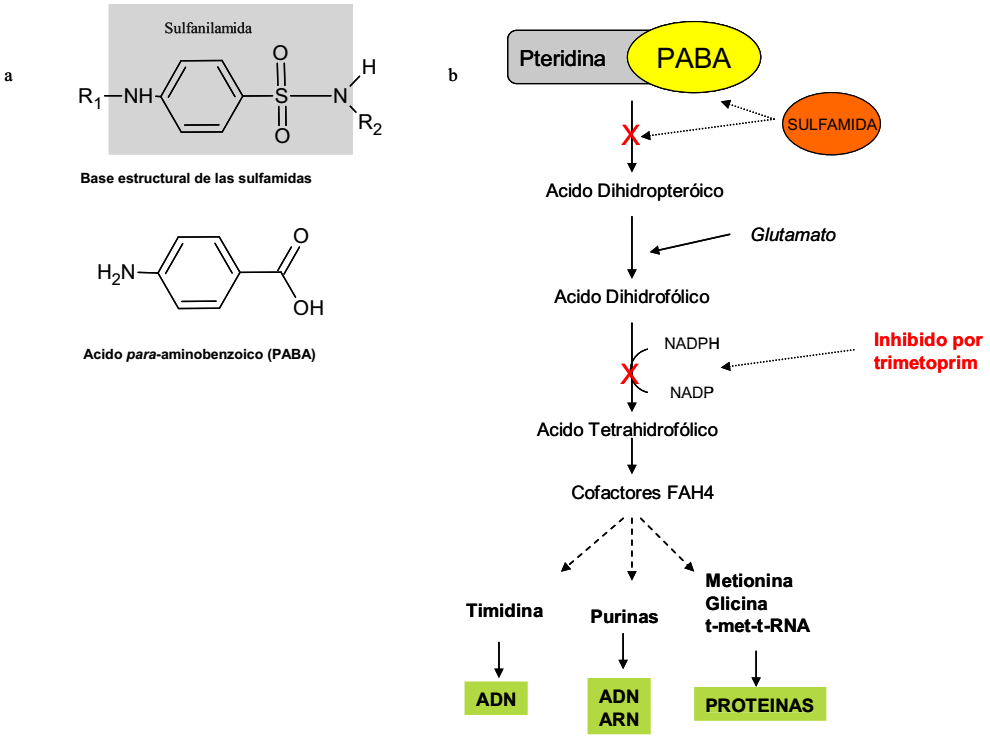

Figura 1.5. Comparación de las estructuras moleculares de las sulfamidas y el PABA (a) y mecanismo de acción de las sulfamidas y la trimetoprima (b).

1.3.2 Metabolitos y productos de degradación

El metabolismo de las sulfamidas depende del compuesto y del organismo en cuestión, aunque generalmente son metabolizadas en el hígado (con menor frecuencia en otros órganos como el pulmón) (Vree *et al.* 1995). La mayoría de los fármacos son detoxificados en este órgano en dos pasos, como indica la Figura 1.6. La primera fase consiste en hacer la molécula más polar o hidrofílica, añadiendo grupos funcionales como -OH, -SH, -NH_2, -COOH etc. Si bien estos metabolitos ya podrían ser eliminados sin mucha dificultad por el organismo, algunos son transformados aún más en una segunda etapa donde se conjugan con sustratos endógenos para aumentar aún más su solubilidad en agua, facilitando así la excreción. Esta conjugación suele realizarse añadiendo grupos sulfato, glucurónido o acetato, o incluso aminoácidos.

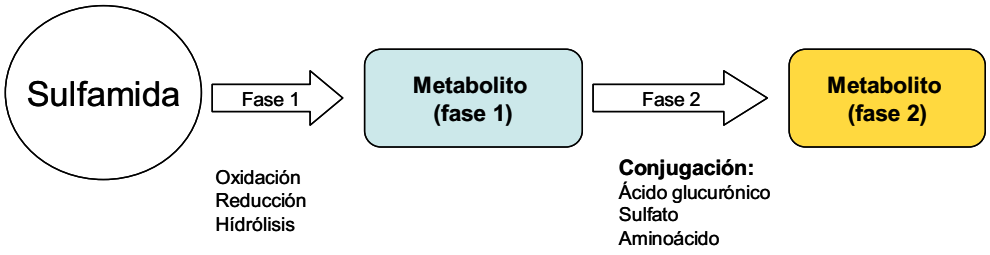

Figura 1.6. Esquema del metabolismo de las sulfamidas llevado a cabo en el hígado.

En el caso de las sulfamidas, su biotransformación consiste principalmente en una hidroxilación y/o una posterior acetilación del nitrógeno en posición N^4, o glucuronización del nitrógeno N^4 y N^1. La Publicación #1 que incluimos al final de esta sección recoge los trabajos de investigación más relevantes sobre la identificación de metabolitos y productos de transformación de las sulfamidas en matrices ambientales y los procesos de degradación biótica y abiótica que intervienen. En la Figura 1 de esta Publicación se muestran los metabolitos más comunes. Los productos hidroxilados conservan del 5 al 40% de la actividad de la sustancia original en algunos casos, mientras que los N^4-acetilados son inactivos terapéuticamente (Vree *et al.* 1990). La acetilación e hidroxilación aumentan la polaridad de la sulfamida y también su tasa de excreción. Sin embargo, los acetilados son generalmente menos solubles que el fármaco

original, pudiendo llegar a precipitar y formar cristales en los tubos renales. La glucuronización y la hidroxilación dan lugar a especies más solubles y de más fácil eliminación. Los metabolitos hidroxilados, además, pueden unirse posteriormente a un grupo glucurónido y sulfato (Papich *et al.* 2009). Normalmente, estos productos son eliminados a través de la orina junto con los restos no asimilados de la sustancia original, y en teoría abandonan el organismo con mayor rapidez que ésta. Los metabolitos acetilados son los más frecuentes en la orina de ganado vacuno, ovino y porcino, mientras que otras especies como los perros son incapaces de acetilar. El ser humano produce principalmente metabolitos acetilados y glucurónidos, como muestra la Tabla 1.2, variando en función de la sulfamida. Por ejemplo, en el caso de la sulfadimetoxina (SDM), el metabolito SDM-N^1-glucurónido es el más abundante para el ser humano y otros primates, constituyendo hasta un 80% del total excretado, mientras que perros o gatos excretan esta sulfamida hasta en un 82% en su estado original (Adamson *et al.* 1970). Una vez liberados al medio, no hay que olvidar que tanto la sustancia original como los metabolitos pueden sufrir otros procesos de biodegradación o de degradación abiótica.

Tabla 1.2. Metabolismo de diferentes sulfamidas en el ser humano. 1: porcentaje de excreción de la dósis durante las 24h posteriores a la medicación. 2: valores expresados como porcentaje de la cantidad total de sulfamidas excretadas durante el mismo período. Fuente: (Salvi and Plancher 1964).

	E% (1)	N^4-ACETILADO (2)	N^4-GLU (2)	N^1-GLU (2)	SULFAMIDA ORIGINAL (2)
Sulfametazina	64.7	70.3	4.8	11.8	12.8
Sulfamerazina	12.1	15.6	9.4	26.7	48.1
Sulfametoxidiazina	20.4	29.1	2.2	27.9	40.6
Sulfadimetoxina	12.8	0	10.1	80	9.7
Sulfametoxipiridazina	32.8	58.5	5.3	13.2	22.8
Sulfametoxipirazina	12.7	53.9	1.9	25.9	18.1
Sulfafenazol	52.6	3.4	5.8	83.6	7

1.3.3 Presencia medioambiental de las sulfamidas

El creciente número de publicaciones científicas sobre la presencia de las sulfamidas en el medioambiente durante las últimas dos décadas nos permite afirmar que las sulfamidas son ubicuas tanto en agua como en matrices sólidas (Campagnolo *et al.* 2002; Göbel *et al.* 2004; Hamscher *et al.* 2006; Cai-Ming *et al.* 2009; Tamtam *et al.* 2011). Como muestra la Figura 1.7 y siguiendo una tendencia parecida a la del resto de los fármacos, este aumento refleja la preocupación creciente generada por los datos obtenidos. Las sulfamidas son de especial interés dadas sus altas tasas de excreción y su elevada polaridad, que les confiere una gran movilidad en el medio una vez excretadas. Después de su administración, los restos del compuesto no metabolizado junto con los nuevos metabolitos formados pueden llegar al medio ambiente mediante las diferentes vías mostradas en la Figura 1.4 de la Sección 1.1.2.

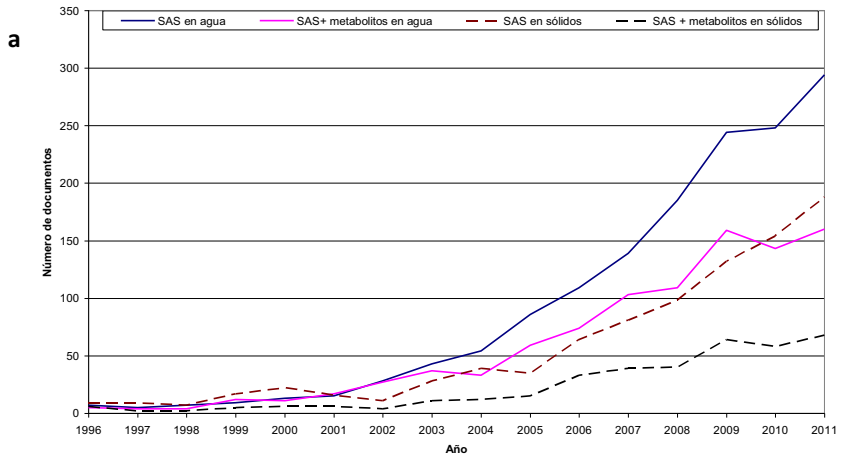

Figura 1.7.a. Número de documentos publicados durante los últimos 15 años. Fuente: Scopus. Fecha: 15.01.2012. Criterio de búsqueda: ocurrence/sulfonamide/metabolite/wastewater/groundwater/surface water). SAS: sulfamidas.

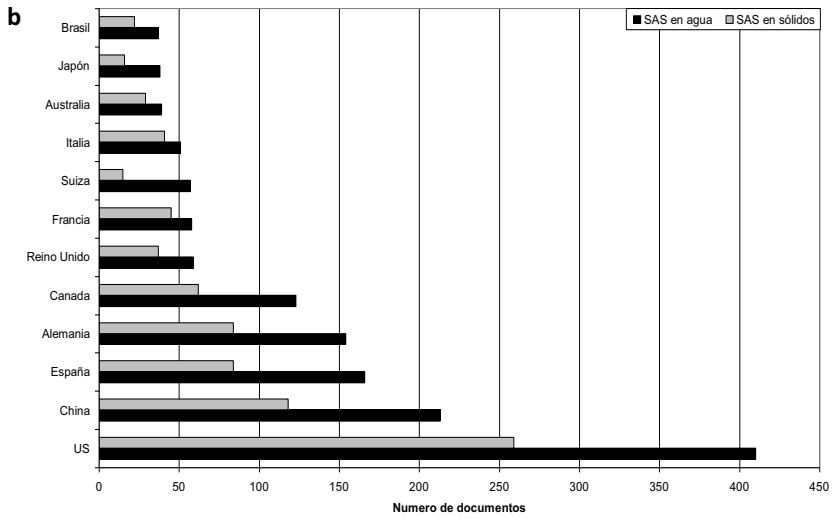

Figura 1.7.b. Países con mayor número de publicaciones durante los últimos 15 años. Fuente: Scopus. Fecha: 15.01.2012. Criterio de búsqueda: ocurrence/sulfonamide/metabolite/wastewater/groundwater/ surface water). SAS: sulfamidas.

1.3.3.1 Sulfamidas de consumo humano en aguas residuales urbanas

Las sulfamidas de consumo humano suelen clasificarse como de corta, media o larga actividad. Las de corta actividad se suelen utilizar para tratar infecciones urinarias, ya que generalmente son absorbidas y excretadas sin apenas metabolizarse, pudiendo así llegar al tracto urinario en su forma original. Este es el caso del sulfisoxazol (SSX), con tan sólo un 30% en forma acetilada detectada en la orina, y del sulfametizol (SMT), con un 80% de la dósis administrada detectada en la orina 8 h después del tratamiento. Las cantidades no asimiladas por el organismo y los correspondientes metabolitos son excretados en aguas de desagüe que finalmente llegaran a las EDAR. El tipo de tratamiento desarrollado en la depuradora será determinante para la eliminación del fármaco en cuestión. El tratamiento convencional llevado a cabo en las EDAR consiste en un tratamiento primario (desarenado, desengrasado, filtro de gruesos) seguido de un tratamiento biológico o secundario, que suele consistir en el tratamiento convencional o de fangos activos (CAS). En los últimos años, se han publicado numerosos trabajos de investigación dedicados a estudiar ya no sólo la presencia de las

sulfamidas en aguas de entrada y salida de EDAR, sino también en evaluar su eliminación y destino. El término eliminación en estos casos no hace referencia a la mineralización del contaminante orgánico investigado, sino a la transformación de su estructura molecular. Los datos obtenidos generalmente reflejan una eliminación incompleta durante los tratamientos de depuración convencionales, convirtiendo a las EDARs en las principales fuentes de vertido de estas sustancias en zonas urbanas. La aplicación de biosólidos provenientes de las EDAR como abono y aporte extra de nutrientes en campos de cultivo representa otro aporte relevante, ya que se ha demostrado que estos fangos contienen diferentes cantidades de sulfamidas y otros fármacos (Lapen *et al.* 2008; Topp *et al.* 2008; Sabourin *et al.* 2009), aunque en el caso de las sulfamidas, dada su alta polaridad y solubilidad, podría considerarse via de entrada secundaria en comparación con las cantidades introducidas mediante los efluentes de estas EDARs. La estimación de las tasas de eliminación (RE%) en las diferentes fases del tratamiento y la determinación de los niveles de concentración en aguas de salida se han convertido en fuentes de información imprescindibles a la hora de evaluar el impacto ambiental de dichos efluentes sobre la salud de los ecosistemas fluviales receptores o suelos, si estas aguas son utilizadas para regadío. La Tabla 1.3 muestra los valores de RE% encontrados en la literatura para las SAs hasta la fecha. Como se puede observar, estos valores no son muy abundantes pero si diversos, comprendiendo desde valores negativos hasta del 100% de eliminación, a pesar de la similitud estructural de las sulfamidas estudiadas. Estas RE% no siempre se pueden calcular, ya que la sulfamida en cuestión puede que no se detecte en aguas de entrada y de salida, o bien que no estén presentes en todas las depuradoras investigadas durante la campaña de muestreo, haciendo difícil llegar a conclusiones válidas. Generalmente, si el compuesto investigado no es detectado en aguas de salida y sí en aguas de entrada, se estima que la eliminación ha sido del 100%; por el contrario, en ocasiones las concentraciones detectadas en agua de salida de EDAR son mayores que las de agua de entrada, dando lugar a valores negativos de RE%. Dichas concentraciones en aguas de salida son atribuidas a la presencia de metabolitos o conjugados de las sulfamidas, no incluidas en el ámbito del estudio en cuestión, y que pueden transformarse en el compuesto original durante el tratamiento, explicando asi esas elevadas concentraciones (Göbel *et al.* 2007; Gros *et al.* 2010).

Tabla 1.3. Tasas de eliminación de diferentes sulfamidas durante el tratamiento de aguas residuales encontradas en la literatura. *: tratamiento seguido de filtración.

SULFAMIDA	TIPO DE TRATAMIENTO	ELIMINACIÓN (RE%)	REFERENCIA
Sulfametoxazol (SMX)	CAS	-279-66 57-67 18-100 20 93 -107-60 55.6 61.5 30-92 48	(Clara *et al.* 2005) (Carballa *et al.* 2005) (Karthikeyan *et al.* 2006) (Brown *et al.* 2006) (Choi *et al.* 2007) (Göbel *et al.* 2007) (Radjenovic *et al.* 2007) (Nakada *et al.* 2007) (Gros *et al.* 2007) (Chang *et al.* 2010)
	CAS+ UV/Cloro	35-65 81	(Xu *et al.* 2007); (Yang *et al.* 2005)
	MBR/ MBR+Ozono	78.3-80.8; 60.5; 95-100*; 90-99	(Radjenovic *et al.* 2007) (Radjenovic *et al.* 2007) (Senta *et al.* 2011) (Huber *et al.* 2003)
	Ozono	87.4 98	(Nakada *et al.* 2007) (Lin *et al.* 2009)
	Filtrado de arena	26.9	(Nakada *et al.* 2007)
N^4-acetilsulfametoxazol (AcSMX)	CAS	91-96	(Göbel *et al.* 2007)
	MBR+Ozono	90-99	(Huber *et al.* 2003)
Sulfadiazina (SDZ)	CAS	43-98 37	(Gros *et al.* 2007) (Chang *et al.* 2010)
	CAS + UV/Cloro	50	(Xu *et al.* 2007)
	CAS + cloro	97	(Peng *et al.* 2011)
	MBR/ MBR+Ozono	90-100 90-99	(Senta *et al.* 2011) (Huber *et al.* 2003)
	Ozono	95	(Garoma *et al.* 2010)
Sulfametazina (SMZ)	CAS	99	(Choi *et al.* 2007)
	CAS + UV/Cloro	50 80	(Xu *et al.* 2007) (Yang *et al.* 2005)
	MBR	95-100	(Senta *et al.* 2011)
	Ozono	98 99	(Lin *et al.* 2009) (Garoma *et al.* 2010)
Sulfadimetoxina (SDM)	CAS	93	(Choi *et al.* 2007)
	CAS + Cloro	57	(Yang *et al.* 2005)
	Ozono	98	(Lin *et al.* 2009)
Sulfametizol (SMT)	Ozono	90	(Garoma *et al.* 2010)

Tabla 1.3. (continuación)

SULFAMIDA	TIPO DE TRATAMIENTO	ELIMINACIÓN (RE%)	REFERENCIA
Sulfatiazol (STZ)	CAS	98	(Choi *et al.* 2007)
	MBR/MBR+ ozono	85-100* 90-99	(Senta *et al.* 2011) (Huber *et al.* 2003)
	Ozono	99	(Garoma *et al.* 2010)
Sulfapiridina (SPY)	CAS	-107 – 72; 4	(Göbel *et al.* 2007)
	MBR/MBR+ ozono	90-100* 90-99	(Senta *et al.* 2011) (Huber *et al.* 2003)
	Ozono	93.9	(Nakada *et al.* 2007)
Sulfamerazina (SMR)	CAS + cloro	98	(Peng et al. 2011)

El SMX es la sulfamida investigada con mayor frecuencia en estudios de presencia en aguas de EDAR, siendo también con frecuencia la única de esta familia de antibióticos incluida en estudios multiresiduo de fármacos. En los últimos años la sulfapiridina (SPY) ha empezado a ganar más relevancia ya que su incidencia es equiparable a la del SMX, mientras que la sulfadiazina (SDZ) y el sulfatiazol (STZ) son detectados habitualmente a niveles inferiores. Ambas SMX y SPY suelen ser detectadas con las mayores frecuencias en las muestras investigadas y representan la mayor proporción de la carga total de sulfamidas en las aguas de salida de las EDAR. Por ejemplo, en el trabajo presentado por Göbel *et al*. (Göbel *et al.* 2004), el SMX fue detectado a concentraciones de hasta 641 ng L^{-1} y 352 ng L^{-1} en aguas de entrada y salida, respectivamente.

La aplicación de tratamientos de aguas residuales avanzados como los reactores biológicos de membrana (MBR) se han introducido con fuerza durante la última decada con la finalidad de mejorar la eliminación de los microcontaminantes orgánicos, erigiéndose como una prometedora alternativa a los sistemas convencionales. Tratamientos terciarios como la nanofiltración o procesos de oxidación avanzada (AOPs) como la cloración o la ozonización han resultado ser muy eficaces en la eliminación de sulfamidas (Huber *et al.* 2003; Dodd *et al.* 2004; Kosutic *et al.* 2007; Nakada *et al.* 2007; Garoma *et al.* 2010), pero su utilización en las EDARs es aún limitada. La ozonización, que ha demostrado ser muy efectiva en la eliminación de sulfamidas y otros fármacos, es aplicado en escasas ocasiones (Lin *et al.* 2009; Garoma *et al.*

2010; Le-Minh *et al.* 2010). Además, la falta de información sobre la ecotoxicidad de los posibles productos de transformación no permite asegurar que estos procesos sean una alternativa viable a los tratamientos convencionales (Zwiener 2007; Rodayan *et al.* 2010).

Como muestra la Tabla 1.3, los valores de RE% encontrados en la literatura para SDZ se mueven en un rango positivo aunque variable en tratamientos convencionales, desde valores de tan sólo0 37% hasta casi el 100%. Los valores estimados para STZ son muy elevados incluso en tratamientos convencionales, mientras que para SPY y SMX estos valores son más difíciles de interpretar, ya que van desde tasas de eliminación negativas en numerosas EDARs hasta un 100%. Trabajos recientes han demostrado que, si bien las tasas de eliminación para SMX son mayores en el tratamiento con MBR que con CAS, esa eliminación era aún moderada ya que apenas supera el 50% (Radjenovic *et al.* 2007; Tambosi *et al.* 2010). Por otro lado, cada vez un mayor número de metabolitos acetilados son incluidos en el ámbito del estudio de presencia ambiental de las sulfamidas, si bien la mayoría de los estudios sólo incluyen el metabolito de SMX, N^4-acetilsulfametoxazol (AcSMX). Su exclusión en este tipo de estudios puede significar la subestimación de la concentración total de sulfamidas en la muestra. Göbel *et al*. detectaron concentraciones superiores de AcSMX que de SMX en aguas de entrada de dos depuradoras en Suiza (518 ng L^{-1} y 943 ng L^{-1} de AcSMX frente a 343 ng L^{-1} y 641 ng L^{-1} respectivamente) poniendo en evidencia la importancia de incluir metabolitos y productos de transformación en el ámbito de los estudios de presencia medioambiental (Göbel *et al.* 2004). Como se demuestra en el Capítulo 2 de esta Tesis, la presencia de diferentes metabolitos como N^4-acetilsulfapiridina (AcSPY) o N^4-acetilsulfadiazina (AcSDZ) ha sido demostrada y confirmada en los diferentes estudios de presencia medioambiental llevados a cabo tanto en aguas residuales como superficiales y subterráneas.

Por último, la Tabla 1.4 muestra las sulfamidas de consumo humano registradas en España. Son sólo 6 y en la mayoría de productos farmacéuticos éstas aparecen en combinación con otros antibióticos, como la trimetoprima o la tirotricina, para aumentar su actividad, y también formando complejos con Ag+ para obtener una actividad fungicida.

Tabla 1.4. Sulfamidas de consumo humano registradas en España. Fuente: www.aemps.es

PRINCIPIO ACTIVO	ACTIVIDAD (vida media, h)	NOMBRE COMERCIAL	INDICACIONES	FORMATO	PRESCRIPCIÓN	LABORATORIO
Sulfacetamida	Media (7-13h)	Colicursí	Infecciones oculares	Colirio	Si	Alcon Cusí, S.A.
Sulfacetamida +betametasona		Celestone		Coloide	Si	Schering Plough, S.A.
Sulfacetamida + tirotricina		Denticelso	Infecciones del tracto digestivo	Solución	No	Alcor, S.A.
Sulfadiazina argéntica Nitrato de cerio	Media (10 -17h)	Flammazine Cerio	Quemaduras	Pomada	Si	Solvay Pharma, S.A.
Sulfadiazina	Media (10-17h)	Sulfadiazina Reig Jofre S.A.	Infecciones oculares y del tracto digestivo; quemaduras	Cápsulas	Si	Reig Jofre, S.A.
Sulfadiazina argéntica	Media (10-17h)	Silvederma	Quemaduras	Crema Aerosol	Si	Aldo-Unión
		Flammazine		Crema	Si	Solvay Pharma
Sulfametizol +fenazopiridina	Corta (3h)	Micturol sedante Micturol sedante fuerte	Infeccion tracto urinario	Grageas	Si	S.I.T.
Sulfatiazol + triamcinolona	Corta (4h)	Cremsol	Dermatitis	Pomada	Si	Lab. Pérez Gimenez
Sulfatiazol + dihidro-estreptomicina	Corta (4h)	Salitanol Estreptomicina	Diarreas	Cápsulas	No	Quimpe S.L.

Tabla 1.4 (continuación).

PRINCIPIO ACTIVO	ACTIVIDAD (vida media, h)	NOMBRE COMERCIAL	INDICACIONES	FORMATO	PRESCRIPCIÓN	LABORATORIO
Sulfasalazina	Media (10-15h)	Salazopyrina	Colitis ulcerosa Enfermedad de Crohn	Comprimidos Supositorios	Si	Pfizer S.A. Pharmacia Spain, S.A.
Sulfametoxazol + trimetoprima + bromhexina	Media (11 h)	Bactopumon	Infeccion tracto respiratorio	Suspensión oral	Si	Cinfa S.A.
		Balsoprim		Comprimidos Suspensión oral	Si	Juste S.A.Q.F DESMA
		Bronco Aseptilex		Suspensión oral	Si	Chiesi España S.A.
		Broncovir		Suspensión oral	Si	Vir S.A.
		Bronquicisteina		Suspensión oral	Si	Iquinosa Farma S.A.
		Bronquidiazina CR		Suspensión oral	Si	Faes Farma
		Eduprim		Comprimidos Suspensión oral	Si	F5 Profas S.L.
Sulfametoxazol + trimetoprima	Media (11 h)	Septrin Septrin forte Septrin pediátrico		Comprimidos Suspensión oral	Si	UCB Pharma S.A.
		Soltrim	Infección tracto respiratorio y urinario	Inyección	Si	Almo-farma

1.3.3.2 Sulfamidas de uso veterinario

Las sulfamidas aplicadas en medicina veterinaria suelen ser clasificadas en función de su mayor o menor solubilidad. Aunque solo llegan a las EDARs en pequeñas cantidades, también han sido detectadas en aguas de entrada y salida con relativa frecuencia (Göbel *et al.* 2007; Gros *et al.* 2007; Ye *et al.* 2007). Pero su vía principal de entrada al medio ambiente es la

deposición de excrementos y orina de animales medicados, bien de manera directa por parte del ganado de pastoreo o bien mediante la aplicación de estiércol como abono en campos agrícolas. Después de su ingesta, el ganado puede llegar a excretar un 50–90% de la dosis administrada, bien en su forma original (9–30%) o bien como metabolito durante los días siguientes a la medicación [87-88]. En el caso de los metabolitos acetilados, los mayoritarios, se ha demostrado que pueden revertir en el compuesto original durante el almacenamiento del abono o una vez en el suelo (Zarfl *et al.* 2009). Las sulfamidas han sido detectadas en excrementos de animales tratados, a concentraciones que van desde los 8.7 mg kg^{-1} para la sulfametazina (SMZ) hasta 12.4 mg kg^{-1} para el sulfatiazol (STZ) (Haller *et al.* 2002). El estiércol es un fertilizante muy valorado ya que contiene nutrientes esenciales para el crecimiento de los cultivos, como nitrógeno, fósforo, carbón orgánico o potasio, y además su contenido en materia inorgánica mejora la estructura y la calidad del suelo. En suelos abonados con estiércol proveniente de animales tratados se han encontrado residuos de sulfamidas a niveles comparables a las tetraciclinas, antibióticos mucho menos polares (Thiele-Bruhn *et al.* 2004). Sin embargo, una vez en la capa superficial del suelo, su alta polaridad y coeficientes de distribución suelo-agua (K_d) bajos hacen que las sulfamidas se conviertan en contaminantes muy móviles, pudiendo ser arrastradas durante episodios de escorrentía superficial (lluvía, riego) y alcanzar ríos u otros cursos naturales de agua (Dolliver *et al.* 2008; Wei *et al.* 2011), o bien percolar a través de los diferentes estratos edáficos hasta alcanzar los acuíferos; esta posibilidad ya ha sido demostrada en diferentes publicaciones en las que se han detectado sulfamidas a diferentes concentraciones en aguas subterráneas ubicadas cerca de explotaciones ganaderas (Batt *et al.* 2006; Díaz-Cruz *et al.* 2006; Stoob *et al.* 2007). Es probable también que otras sustancias químicas como los plaguicidas sean aplicadas conjuntamente con estos abonos de origen animal y den lugar a efectos de antagonismo o sinergismo, modificando los posibles efectos tóxicos de estas sustancias sobre el ecosistema receptor (Boxall *et al.* 2003). Stoob *et al*. (Stoob *et al.* 2005) fueron los primeros en abordar este problema, estudiando la presencia conjunta de sulfamidas y plaguicidas en un área agrícola, aunque no profundizaron en la toxicidad generada por ambos tipos de compuestos. Este tipo de contaminación difusa desde los campos agrícolas es dificil de prevenir y de tratar debido a las enormes extensiones de terreno.

Otro escenario relevante para las sulfamidas veterinarias es el de la acuicultura, ya que los antibióticos se añaden directamente al agua y se ha estimado que hasta un 70–80% de la dosis administrada llega finalmente al ecosistema acuático (Hamscher *et al.* 2006; Thuy *et al.* 2011). Otras vías de entrada secundarias serían algunas de las ya mencionadas anteriormente: percolación desde fosas sépticas o almacenes de residuos en zonas agrícolas (Bjerg 1995), desecho de las medicinas no usadas o caducadas ("flushing"), infiltración desde vertederos (Ahel *et al.* 1998) o bien su arrastre desde éstos en episodios de inundación o fuertes lluvias y vertidos accidentales durante su manufactura (Lin and Tsai 2009). La Tabla 1.5 presenta las cinco sulfamidas veterinarias registradas por el Ministerio de Sanidad y Consumo. Sin embargo, el número de sulfamidas veterinarias actualmente en uso es más elevado, como se demuestra en el Capítulo 3 de esta Tesis. Según este registro, sólo SDZ y STZ son comunes en medicina humana y veterinaria. Al igual que con las sulfamidas de uso humano, las sulfamidas veterinarias suelen venderse en combinación con otros antibióticos como la trimetoprima o la neomicina, y también con agentes antiprotozoarios como la pirimetamina para aumentar la efectividad del producto.

Tabla 1.5. Sulfamidas de uso veterinario registradas en España. *: animales no destinados a consumo humano. Fuente: www.aemps.es.

PRINCIPIO ACTIVO	ACTIVIDAD (vida media, h)	NOMBRE COMERCIAL	ANIMAL	INDICACIONES	FORMATO	PRESCRIPCIÓN	LABORATORIO
Sulfaquinoxalina	Corta (11 h)	Quinoxiven	Aves	Coccidiosis cecal e intestinal	Solución oral	Si	Iven S.A.
Sulfaquinoxalina + pirimetamina		Lamons potenciado	Aves de jaula		Solución oral	Si	Lamons S.A.
		Coccigal PS	Ganado bovino, ovino, porcino, aves y conejos	Coccidiosis cecal, intestinal y hepática	Solución oral	Si	Ovejero S.A.
Sulfaquinoxalina sódica		Quinoxalina Chemical Solution	Aves	Anti-parasitario Coccidiosis	Solución oral	Si	Chemical Iberica Productos Veterinarios

Tabla 1.5 (continuación).

PRINCIPIO ACTIVO	ACTIVIDAD (vida media, h)	NOMBRE COMERCIAL	ANIMAL	INDICACIONES	FORMATO	PRESCRIPCIÓN	LABORATORIO
Sulfaquinoxalina sódica		Conejin CAG	Aves y conejos	Coccidiosis cecal, intestinal y hepática	Polvo hidro-soluble oral	Si	Agro-pecuaria de Guissona
		Lapisan Lamons Forte	Aves y conejos		Pre-mezcla	Si	Lamons S.A.
		Quinoxalina Syva	Aves	Coccidiosis cecal y duodenal	Comprimido	Si	Syva S.A.
Sulfadiazina sódica + trimetoprima	Corta (7.4 h)	Neumovex	Lechones	Diarrea neonatal	Solución oral	Si	S.P. Veterinaria
		Trimacrol	Bóvidos Óvidos Cápridos Porcinos Equinos	Neumonía Diarrea (…)	Solución para inyección	Si	S.P. Veterinaria
		Ultra-diazina	Bóvidos Óvidos Cápridos Porcinos	Neumonia Diarrea Metritis, (…)	Solución para inyección	Si	S.P. Veterinaria
Sulfadiazina + trimetoprima		Equibactin	Equinos	Infeccion (tracto digestivo, heridas)	Pasta oral	Si	Levet Pharma
		Tucoprim	Aves y conejos	Síndrome MMA, Metritis, Rinitis atrófica (…)	Mezcla para piensos	Si	Pfizer
		Sulfaprim	Porcino	Síndrome MMA, Rinitis, Diarrea	Pre-mezcla	Si	S.P. Veterinaria
		Tribisen anti-diarreico	Porcino (lechon)	Diarrea neonatal	Solución oral	Si	Schering Plough S.A.
		Duoprim 48	Bóvidos Porcino Equidos	Neumonia Diarreas, Metritis, …	Solución para inyección	Si	Schering Plough S.A.

Tabla 1.5 (continuación).

PRINCIPIO ACTIVO	ACTIVIDAD (vida media,h)	NOMBRE COMERCIAL	ANIMAL	INDICACIONES	FORMATO	PRESCRIPCIÓN	LABORATORIO
Sulfadiazina + trimetoprima	Corta (7.4 h)	Farcotrim plus cotridiazina		Neumonia, diarreas metritis, (…)	Solución para inyección	Si	C.Z. Veterinaria
		Intermax	Porcino	Síndrome MMA, Rinitis, Diarrea	Pre-mezcla	Si	Lamons S.A.
		Cenpremix		Síndrome MMA, Rinitis, Diarrea	Pre-mezcla	Si	Cenavisa S.A.
Sulfadiazina sódica + pirimetamina		Coccirex	Aves *	Coccidiosis cecal e intestinal	Solución oral	Si	S.P. Veterinaria
Sulfametazina	Media (12-22 h)	Hipramix-sulfa	Porcino	Rinitis atrófica	Pre-mezcla	Si	Hipra S.A.
		Apsamix 20%		Rinitis atrófica	Pre-mezcla	Si	Andrés Pintaluba S.A.
Sulfadoxina + trimetroprima	Corta (6-12 h)	Duoprim	Bóvidos Porcino Equidos Perros	Infeccion tracto urinario y digestivo	Solución para inyección	Si	Schering Plough S.A.
Sulfadimetoxina sódica	Larga (27-34 h)	Sulfadim	Aves, Ternero Conejos	Coccidiosis	Líquido oral	Si	S.P. Veterinaria
Sulfatiazol + neomicina sulfato	-	Framicas Polvo cutáneo	Perros y gatos	Dermatitis	Polvos	-	Laboratorios Ovejero S.A.

1.3.3.3 Presencia de sulfamidas en aguas superficiales.

El aumento global en la densidad de población ha incrementado el volumen de aguas residuales tratadas que finalmente alcanzan los cursos de agua natural, poniendo en riesgo el equilibrio ecológico del río receptor dado su alto contenido en nutrientes además de gran cantidad de microcontaminantes. Estos vertidos regulares pueden dar lugar a un gradiente de concentración de éstos desde la cabecera hasta la desembocadura del rio; en el caso de las sulfamidas, esto es más que probable ya que se trata de sustancias con baja adsorción en matrices sólidas (en este caso, el lecho del río) (Kolpin *et al.* 2002; Thiele-Bruhn *et al.* 2004) y baja fotodegradación y biodegradación. Al igual que ocurre con el resto de contaminantes orgánicos, los cambios estacionales deben ser considerados a la hora de interpretar los datos obtenidos. Por ejemplo, generalmente las concentraciones más altas de sulfamidas de consumo humano (SMX y SPY) son registradas en épocas secas, ya que la dilución ejercida por el curso del río es menor. Por ejemplo, Kim *et al*. (Kim *et al.* 2007) encontraron SMX a una concentración media maxima de 230 ng L^{-1} durante el invierno, y a 320 ng L^{-1} durante el verano, en la estación seca, en el río Cache La Poudre, en el estado de Colorado. Por otro lado, durante la estación lluviosa o bien tras episodios de fuertes lluvias, las concentraciones de sulfamidas de consumo humano suelen disminuir debido a la dilución mientras que la escorrentía procedente de los campos de cultivo puede causar el aumento observado en las concentraciones de sulfamidas de uso veterinario como SMZ o SDZ (Kolpin *et al.* 2004; Madureira *et al.* 2010; Zheng *et al.* 2011). También se ha comprobado que temperaturas bajas pueden contribuir a encontrar concentraciones algo más elevadas debido a un ritmo aún menor de biodegradación de los compuestos en agua (Kim and Carlson 2007). La Tabla 1.6 resume los niveles detectados en diferentes aguas superficiales desde 2005.

En Europa, diferentes trabajos de investigación han abordado el impacto de los aportes urbanos a la presencia de diferentes sulfamidas en aguas superficiales. En el río Duero a su paso por Portugal, se detectaron concentraciones máximas de SMX de 53.3 ng L^{-1} y una frecuencia de detección del 33% (Madureira *et al.* 2010). En el río Sena, en las cercanías de Paris, se detectó SMX en todas las muestras analizadas durante un periodo de seis meses en 2006,

Tabla 1.6. Niveles de sulfamidas detectados en aguas superficiales publicados en los últimos 10 años.

SULFAMIDA	PAÍS	CONCENTRACIÓN (ng L-1)	REFERENCIA
Sulfametoxazol (SMX)	Europa	5-169 <50 26 19.8 6 53.3 402; 70 22 5-118 26-60	(Gros *et al.* 2007) (Hilton *et al.* 2003) (Tamtam *et al.* 2008) (Tamtam *et al.* 2009) (Massey *et al.* 2010) (Madureira *et al.* 2010) (Perret *et al.* 2006) (Wiegel *et al.* 2004) (Pailler *et al.* 2009) (Meyer *et al.* 2011) (Kasprzyk-Hordern *et al.* 2007)
		300 80.7 3.1-33 564 5-7	(Brown *et al.* 2006) (Benotti *et al.* 2007) (Conley *et al.* 2008) (Campagnolo *et al.* 2002) (Arikan *et al.* 2008)
	Australia	2000	(Barker-Reid *et al.* 2010)
Sulfametoxazol (SMX)	China	8.9-93.4 173.2 15.5-58.3 560	(Zheng *et al.* 2011) (Jia *et al.* 2011) (Peng *et al.* 2011) (Wei *et al.* 2011)
	Japon	0.56 3 23	(Chang *et al.* 2008) (Managaki *et al.* 2007) (Managaki *et al.* 2007b)
	Asia	82 369 3847-4330 33 47-96	(Choi *et al.* 2007) (Lin and Tsai 2009) (Hoa *et al.* 2011) (Managaki *et al.* 2007) (Nageswara Rao *et al.* 2008)
N^4-acetilsulfametoxazol (AcSMX)	China	268.5	(Jia *et al.* 2011)
	Europa	239	(Hilton and Thomas 2003)
Sulfadiazina (SDZ)	China	60.5 50.7-316 10.5-55.3 17000 170 30.5 33.6	(Zheng *et al.* 2011) (Peng *et al.* 2011) (Zheng *et al.* 2011) (Wei *et al.* 2011) (Luo *et al.* 2011) (Jia *et al.* 2011) (Xu *et al.* 2007)
	Japon	0.05	(Chang *et al.* 2008)
	Europa	236	(Perret *et al.* 2006)

Tabla 1.6 (continuación).

SULFAMIDA	PAÍS	CONCENTRACIÓN (ng L^{-1})	REFERENCIA
N^4-acetilsulfadiazina (AcSDZ)	China	3.3	(Jia *et al.* 2011)
	Asia	28; 46.2-66.2	(Managaki *et al.* 2007)
Sulfametazina (SMZ)	Japon	0.14	(Chang *et al.* 2008)
	China	19.3-281 55.6-775.5 190 26.4	(Zheng *et al.* 2011) (Peng *et al.* 2011) (Luo *et al.* 2011) (Jia *et al.* 2011)
	Europa	19 5 9 10-1000	(Meyer *et al.* 2011) (Massey *et al.* 2010) (Perret *et al.* 2006) (Stoob *et al.* 2005)
N^4-acetilsulfametazina (AcSMZ)	China	11.5	(Jia *et al.* 2011)
Sulfamonometoxina (SMM)	China	31.1-49 35.1	(Zheng *et al.* 2011) (Jia *et al.* 2011)
Sulfadimetoxina (SDM)	China	1.9 0.8 1	(Peng *et al.* 2011) (Zheng *et al.* 2011) (Jia *et al.* 2011)
	Japón/Asia	0.17 47 – 96	(Chang *et al.* 2008) (Nageswara Rao *et al.* 2008)
	EUA	1–9 3 11-74	(Arikan *et al.* 2008) (Pailler *et al.* 2009) (Perret *et al.* 2006)
Sulfametizol (SMT)	China Japón	323 0.07	(Xu *et al.* 2007) (Chang *et al.* 2008)
Sulfacloropiridazina (SCP)	China	210 8.1	(Luo *et al.* 2011) (Jia *et al.* 2011)
Sulfatiazol (STZ)	China Japón	8.5 6.6	(Jia *et al.* 2011) (Chang *et al.* 2008)
	Australia	40	(Barker-Reid *et al.* 2010)
	Europa	2-5	(Meyer *et al.* 2011)
Sulfapiridina (SPY)	China Japón Asia	15.7 3 132 57.5	(Jia *et al.* 2011) (Chang *et al.* 2008) (Managaki *et al.* 2007) (Hoa *et al.* 2011)
	Australia	30	(Barker-Reid *et al.* 2010)
	Europa	8 – 39 66-121	(Kasprzyk-Hordern *et al.* 2007) (Perret *et al.* 2006)

con concentraciones medias en el intervalo 37 -140 ng L^{-1} (Tamtam *et al.* 2008). En este trabajo se indica que la concentración de SMX aumentó después de episodios de lluvia muy intensa, hecho atribuido en este caso al vertido de aguas sin tratar desde una EDAR con sus tanques desbordados y no a episodios de escorrentia desde campos de cultivo. Los mismos autores determinaron concentraciones más bajas de SMX en el rio Oise, el río Marne y de nuevo en el Sena (12 – 26 ng L^{-1}) (Tamtam *et al.* 2009). Concentraciones en el rango de 30-70 ng L^{-1} fueron detectadas también en diferentes puntos a lo largo del rio Elba a su paso por Alemania y la Republica Checa durante campañas de muestreo realizadas en 1999 y 2000 (Wiegel *et al.* 2004). Como veremos más adelante, en varios trabajos de esta Tesis se investigó la presencia de sulfamidas a lo largo de la cuenca de dos ríos peninsulares con diferentes ciclos hidrológicos, el Ebro y el Llobregat, incluyendo aguas de depuradora, de río y subterráneas.

La presencia de sulfamidas en las cuencas fluviales no se limita tan solo a la matriz acuosa, sino que también han sido detectadas en los sedimentos de sus lechos en diferentes trabajos (Löffler 2005; Kim *et al.* 2007; Tang *et al.* 2009; Yang *et al.* 2010; Tamtam *et al.* 2011; Zheng *et al.* 2011) aún tratándose de unos compuesto con valores de K_d muy bajos, haciendo evidente la regularidad de los vertidos en este ecosistema así como su vulnerabilidad ante este tipo de contaminación. También se han detectado metabolitos acetilados de sulfamidas en aguas de río. Ashton *et al*. publicaron los resultados de un muestreo en 2004 en el Reino Unido donde AcSMX fue detectado con más frecuencia que el fármaco original (Ashton *et al.* 2004), al igual que observaron Göbel *et al*. al analizar aguas de depuradora (Göbel *et al.* 2004). Por tanto, cada vez es más evidente la necesidad de incluir metabolitos en el ámbito de estudio y evaluar sus efectos en el ecosistema receptor. Como se discutirá más adelante en esta Tesis, hemos podido demostrar que AcSPY es más tóxico que la propia SPY frente a la bacteria marina *Vibrio fischeri* (ver Publicación #10 en el Capítulo 3).

Publicación científica 1

Identification and determination of metabolites and degradation products of sulfonamides

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Identification and determination of metabolites and degradation products of sulfonamide antibiotics

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Sulfonamide residues have been detected in all kinds of environmental water matrix. However, little attention has been so far paid to their metabolites and degradation products, and very few articles have included them in their analytical scope. The main goal of this article is to review those research studies in which both parent drugs and their respective metabolites have been considered together. We review the analytical methodologies used and assess biotic and abiotic degradation mechanisms of these antibiotics, and briefly evaluate their potential toxicity in the environment.

Keywords: Acetylation; Antibiotic; Degradation product; Elimination rate; Environmental analysis; Hydroxylation; Mass spectrometry; Metabolite; Sulfonamide; Toxicity

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1. Introduction

Sulfonamides are synthetic antimicrobial agents, derivatives of sulfanilamide, which are used in aquaculture, animal husbandry, and also as human medicines to treat many kinds of infection caused by bacteria and certain other microorganisms. Their mechanism of action is based on inhibiting the conversion of *p*-aminobenzoic acid, interrupting bacterial utilization of this compound in the synthesis of folic acid and ultimately of purine and DNA [1].

Sulfonamides were the first antibacterial agents successfully applied in human infectious diseases. They may be prescribed to treat urinary tract infections, ear infections, bronchitis, bacterial meningitis, certain eye infections, *Pneumocystis carinii* pneumonia, traveler's diarrhea, and a number of other kinds of infection. However, more relevant quantities are now being used in veterinary medicine to treat livestock herds and, at sub-therapeutic levels, as growth promoters and to improve feed efficiency. In the European Union (EU), sulfonamides are the second most widely used veterinary antibiotics. They accounted for nearly 21% of the sales in UK in 2000, and for 11–23% in several other European countries. Of the total of antibiotics used in the USA, 2.3% are sulfonamides (mainly sulfamethoxypyridazine, sulfachloropyridazine, sulfamethazine and sulfathiazole). Even in Kenya, where data are usually lacking, sulfonamides account for 22% of 14,600 kg of active antimicrobials used in animal-food production [2].

Increasing concern regarding sulfonamides (and antibiotics in general) in the past few years stems from the fact that these substances are continually being introduced into the environment and they may spread and maintain bacterial resistance in the different compartments. They may enter natural systems following different pathways [e.g., excreta, disposal ("flushing") of unused or expired drugs, medical waste, and discharge from wastewater-treatment plants (WWTPs), leakage from septic systems and agricultural waste-storage facilities and via application of human and agricultural waste to land to supplement fertilizers]. Animals out to pasture spread the drugs and respective metabolites via dung pats or urine in the fields. Similarly, farmyard manure is spread on the agricultural lands and, in both cases, run off into neighboring waters may take place.

Sulfonamides are weak acids, which are fairly soluble in water and polar, so they

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are likely to run off into surface water and groundwater after release into the environment. Furthermore, it is also likely that other chemical applications, such as pesticides, may be used at the same site. This could lead to additivity, antagonism, synergism, and eventual interactive effects on terrestrial and aquatic organisms, which could increase or decrease their effects in the ecosystem [3].

It is more evidently important to consider metabolites and degradation products of many drugs when studying their presence in the environment from the points of view therapeutic and toxicological viewpoints. Although, in many cases, metabolites are pharmacologically less active and less toxic than the parent compound, some of them are still bioactive and potentially more toxic, stable and mobile in the environment [2].

2. Metabolites of sulfonamides

The metabolism of sulfonamides is species dependent [4]. They are mainly metabolized in the liver, but also in other tissues. Biotransformation occurs mainly by phase I oxidation and phase II acetylation, giving the N^1 and N^4 derivatives shown in Fig. 1, with no microbial activity [5]. Glucuronide conjugation and aromatic hydroxylation also take place, the latter with 5–39.5% of the activity of the parent compound in some species.

Table 1 lists the main metabolites of sulfonamides, their molecular structures and some information concerning their analysis.

2.1. From humans

Studies on the fate and the distribution of metabolites of pharmaceuticals are generally scarce. It is known that the metabolism of sulfadiazine involves acetylation and oxidation at the N^4-nitrogen atom, leading to N^4-acetylsulfadiazine and N^4-hydroxysulfadiazine. The hydroxyl metabolites can be also conjugated with glucuronic acid and sulfate [4,5]. These products are eliminated in urine together with the remains of the parent compounds, and, in theory, these metabolites are cleared from the organism more quickly than the parent drug (i.e. 5-methylhydroxysulfamethoxazole, N^4-acetyl-5-methylhydroxysulfamethoxazole and sulfamethoxazole-N^1-glucuronide were isolated from human urine [6]). Once released, it should be noted that both parent and metabolites may undergo biotic as well as abiotic degradation processes, such as photodegradation.

Sulfonamides used to treat humans in hospitals or by prescription are ultimately excreted into domestic sewage and are discharged to WWTPs [7], which could therefore be considered as the main release sources of sulfonamides and their respective metabolites into the environment. Monitoring studies on the occurrence and the distribution of sulfonamides within sewage in WWTPs have been published quite often in recent years [8–15]. Although very few of them included metabolites as target compounds [10,12], they often reported elimination rates during the wastewater treatments, which could be understood as not only complete degradation of sulfonamides, but also transformation of the original drugs into other intermediate products.

2.2. From animals

The excretion of feces and urine from medicated animals and the subsequent application of the contaminated manure as fertilizer on agricultural land is among the major routes by which veterinary antibiotics enter the environment [3]. Following treatment, livestock will excrete 50–90% of the administered dose, usually within several days, the parent drug making up 9–30%. Acetic-acid conjugates comprise 5–60% of the excreted dose, although these may revert back to the parent compound during storage as the acetyl moiety is cleaved by bacteria [16]. The amounts of unchanged sulfonamides that are excreted vary, depending on the form of the drug and the animal age and species. As in humans, N^4-acetylation is the major metabolism pathway for sulfonamides in animals, and these metabolites usually possess weak, reduced antibacterial activity (see Table 1) (e.g., in pigs, sulfamethazine is known to metabolize into N^4-acetylsulfamethazine, desaminosulfamethazine and N^4-glucose conjugate).

Manure is regarded as a very valuable fertilizer, as it contains essential nutrients for plant growth [e.g., nitrogen (NH_4 and NO_3^-), phosphorous and potassium], this being the main reason for it being so widely applied on crop lands. Antibiotics have been found in manure-treated soils after periods ranging from days to months after application.

Residues of sulfonamides have been detected in manure in a range comparable to tetracyclines [17], which have reached a maximum concentration of 0.5 mg/kg. Concentrations of sulfathiazole of 12.4 mg/kg and of sulfamethazine up to 8.7 mg/kg have been reported [18]. However, while tetracyclines are immobile in soil under typical field conditions, sulfonamides have greater potential for leaching to groundwater because their sorption to soil is weaker [19]. This possibility has already been investigated and proved in several publications, showing the presence of sulfonamides at different concentrations in groundwater from various sites [10,20–24]. Results from a recent study showed sulfamethazine and sulfadimethoxine in groundwater from six private wells at concentrations in the range 0.046–0.22 μg/L [20]. These wells were located down gradient and in close proximity to a confined animal-feeding operation (CAFO). Analysis of nitrate and ammonia in these samples by isotopic ratio mass spectrometry (MS) indicated values characteristic of the source being animal waste.

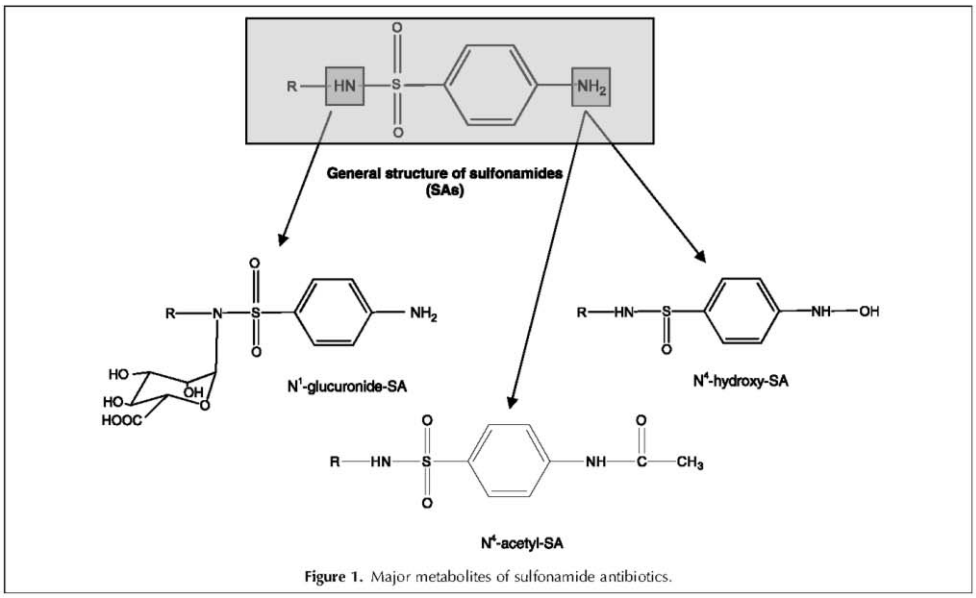


Figure 1. Major metabolites of sulfonamide antibiotics.

3. Degradation

3.1. Biological

3.1.1. In WWTPs. Various studies have indicated that the removal of sulfonamides during sewage treatment has been is incomplete [25–36]. Sulfamethoxazole is one of the most common sulfonamides to be administered and is more frequently studied in the context of urban sewage-treatment plants, as it is used very commonly in human medicine.

Hilton et al. [27] were the first to study the presence of N^4-acetylsulfamethoxazole in surface water and WWTP effluents, reporting concentrations up to 2.2 μg/L.

Ashton et al. [28] detected sulfamethoxazole and its acetylated metabolite in final effluent samples collected in five different WWTPs in the UK. Ternes et al. [29] detected sulfamethoxazole in sewage discharges at a mean of 0.62 μg/L and estimated an elimination rate of 94 ± 4% (anaerobic 95%) for it by comparing in-flowing and out-flowing loads of a German WWTP. Carballa et al. [30] observed an overall removal efficiency within a Spanish WWTP of 60%.

Similar results for this and some other sulfonamides were obtained by Perez et al. [31], who investigated the aerobic biodegradability in sewage sludge of sulfamethazine, sulfathiazole (of exclusive veterinary use in the USA), sulfamethoxazole and trimethoprim in biomass collected from a municipal WWTP at four stages: primary treatment, activated sludge treatment, aerobic nitrification process and after disinfection of treated sewage. The biodegradability tests were conducted in aerated batch reactors by spiking the test medium with 20 μg/L of each of the test substances. The three compounds followed a similar degradation pattern in primary and secondary treatment samples, which suggested that enzymes were class specific for primary degradation rather than compound specific.

Ingerslev and Hallig-Sorensen [32] also put that idea forward with respect to the low selectivity of the microorganisms involved in the biotransformation of sulfonamides. The highest biodegradation rates were discernable in the activated sludge where a steep exponential decline in concentrations resulted in the quick elimination of the assayed antimicrobials. In the nitrification process, sulfathiazole was degraded at a substantially faster rate than both sulfamethoxazole and sulfamethazine. While the concentration of sulfathiazole dropped to 8% of the initial level within 3 days, it took 13 days to achieve a similar reduction for sulfamethoxazole and about 30 days for sulfamethazine.

Drillia et al. [33] also studied the fate of sulfamethoxazole in the activated sludge process using a sequencing batch reactor. They found that the antimicrobial serves as both nitrogen and carbon source to the microbial community, as it was degraded whenever there was a depletion of carbon or nitrogen or both in

Table 1. Studies on sulfonamide metabolites: not reported

COMPOUND	MOLECULAR STRUCTURE	SAMPLE	ANALYTICAL METHOD	CONCENTRATION (μg/L)	REFERENCE
5-hydroxysulfadiazine		Plasma/urine	LC-MS	–	[4]
4-hydroxysulfadiazine		Plasma/urine	LC-MS	–	[4]
5-hydroxysulfadiazine glucuronide		Plasma/urine	LC-MS	–	[4]
5-hydroxysulfadiazine sulfate		Plasma/urine	LC-MS	–	[4]
N^4-acetylsulfadiazine		Surface water	LC-MS^2	0.01–1	[39]
Sulfadimethoxine-N^1-glucuronide		Plasma/urine	LC-MS	–	[5]

(continued on next page)

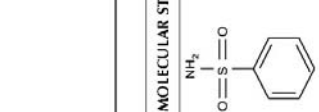
Table 1. (*continued*)

COMPOUND	MOLECULAR STRUCTURE	SAMPLE	ANALYTICAL METHOD	CONCENTRATION (μg/L)	REFERENCE
N^4-Sulfadimethoxine-N^1-glucuronide		Plasma/urine	LC-MS	–	[5]
N^4-acetylsulfadimethoxine		Plasma/urine	LC-MS LC-MS^2	– 0.01–1	[5] [39]
5-methylhydroxysulfamethoxazole		Plasma/urine	LC-MS	–	[6]
N^4-acetyl-5-methyl-hydroxysulfamethoxazole		Plasma/urine	LC-MS	–	[6]
Sulfamethoxazole-N glucuronide		Plasma/urine	LC-MS	–	[6]

N^4-acetylsulfamethoxazole	Waste water	LC-MS^2	0.85–1.6		[11], [12]
	Sewage sludge		2.2		[[27] [28]]
	Surface water	LC-MS^2	0.01–1		[39]
N^4-acetylsulfamethazine	Surface water	LC-MS^2	0.0007–0.317		[10]
	Wastewater		–		[10]
	Bottled water		0.0011		[10]
	Groundwater		0.0027		[10]
	Surface water	LC-MS^2	0.01–1		[39]
	HPLC water	LC-MS	–		[64]
	Top-soil				
N^4-acetylsulfathiazole	Surface water	LC-MS^2	0.01–1		[39]
Chloramine-T	Waste water	LC-MS^2	–		[35] [36]
	Surface water				
	Groundwater				
	Drinking water				
p-TSA	Waste water	LC-MS^2	5–50.8		[35] [36]
	Surface water		1.15		
	Groundwater		40.8		
	Drinking water		0.2		

(continued on next page)

Table 1. *(continued)*

COMPOUND	MOLECULAR STRUCTURE	SAMPLE	ANALYTICAL METHOD	CONCENTRATION (μg/L)	REFERENCE
BSA		Waste water Surface water Groundwater Drinking water	LC-MS^2	0.05–0.35 0.52 1.22 <0.05	[35] [36]
o-TSA		Waste water Surface water Groundwater Drinking water	LC-MS^2	<0.5 0.8 6.32 0.09	[35] [36]

the feeding medium, while, in the presence of acetate and NH_4-N (alternative carbon and nitrogen sources, respectively), it remained intact. Sulfamethoxazole is therefore expected to be detected in WWTP effluents, whenever there are easily biodegradable sources of carbon and nitrogen present in the wastewaters, as these would prevent initiation of pharmaceutical degradation.

By contrast, little sulfamethoxazole was found to be eliminated in Swedish WWTPs [34]. Removal rates seemed to depend strongly on the sewage-treatment process, but generally sulfonamides were resistant to biodegradation during the wastewater-treatment processes [8]. Göbel et al. [11] compared the elimination efficiency for sulfonamides of conventional activated sludge treatment with other wastewater technologies (e.g., fixed bed reactors, membrane bioreactors, sand filters and tertiary treatments). The elimination observed in primary treatment was generally low, with negative elimination rates for sulfamethoxazole. The presence of deconjugable metabolites of these compounds could explain that increase in sulfamethoxazole loads; as shown in a previous publication of the same author [12], slight elimination was observed for its main human metabolite, N^4-acetylsulfamethoxazole, in primary treatment (9–21%), which is presumably transformed into the parent compound.

Elimination in secondary treatment is due to not only transformation but also sorption processes. Again, for sulfamethoxazole, more than twice the concentration detected in the influent was in some cases detected in the respective secondary effluents of conventional activated sludge systems and the fixed-bed reactor. An elimination rate of up to 96% was determined for N^4-acetylsulfamethoxazole, which may imply the possible retransformation of N^4-acetylsulfamethoxazole to sulfamethoxazole. Similar results were obtained for sulfapyridine and, despite its main human metabolite, N^4-acetylsulfapyridine, not being included in the study, the results strongly suggested its presence in the influent and its potential transformation to sulfapyridine in biological treatment.

In a membrane bioreactor, despite the fact that of over 95% of N^4-acetylsulfamethoxazole was eliminated, no increase in sulfamethoxazole (or in sulfapyridine) was observed. Again, similar elimination rates for sulfapyridine and sulfamethoxazole were found.

All these data indicated the simultaneous elimination of the parent compounds and metabolites during this biological treatment, leading to a residual amount of 22% for sulfamethoxazole and its metabolite, compared to the 46% residual amount from the secondary treatment. The elimination rates proved to be independent of temperature and solid-retention times for any of the three secondary treatments.

Finally, during sand filtration, levels of sulfamethoxazole and its metabolite did not change significantly, again assuming that effective transformation of the human metabolite had already occurred in secondary treatment. However, higher loads of sulfapyridine were detected in the tertiary effluents compared to the respective influent concentrations (28% increase); again, a significant amount of potential sulfapyridine precursors (e.g., deconjugable human metabolites or sulfasalazine) are thought to pass through biological treatment and transform to sulfapyridine during this treatment step.

Concentrations of N^4-acetylsulfamethoxazole have also recently been investigated in surface waters from the UK; the metabolite was found at higher concentrations than the parent sulfamethoxazole (33% versus 9%, respectively) [28]. In this study, there was a very high correlation between the concentrations of acetylsulfamethoxazole found in effluents from WWTPs and those found downstream.

Recently, Richter et al. [35] studied the presence and the fate of chloramine-T (*N*-sodium-*N*-chloro-*p*-toluenesulfonamide) and its primary degradation products, *p*-TSA, *o*-TSA and BSA (toluene and benzene sulfonamides, respectively) in wastewater, surface water, groundwater and drinking water [36]. Chloramine-T is commonly used as an anti-microbial agent for bacterial gill diseases of a variety of fish species as well as a drug for treating different diseases of swine and poultry. It also has widespread applications in the food industry (farming, slaughterhouses and canteen kitchens) to disinfect surfaces, instruments and machinery. Its primary degradation product is *p*-TSA, a marker residue for this disinfectant, which has been described in a number of studies. It is also used as a plasticizer, an intermediate for pesticides and drugs, and as a fungicide in paints and coatings. BSA is applied as an intermediate for synthetic dyes, photo chemicals and disinfectants [16]. In 1998 in the USA, total production volumes were 50–230 tons of chloramine-T and 500–5000 tons of *p*-TSA. High concentrations of these substances were found in influent and effluent samples of four WWTPs and in groundwater below a former sewage farm. Lower concentrations were also detectable in surface-water and drinking-water samples in Berlin (see Table 1).

Results showed a significant decrease of *p*-TSA during wastewater treatment. For *o*-TSA, effluent concentrations exceeded influent concentrations for two of the WWTPs sampled. This may be due to a site-specific process during wastewater treatment enhancing the formation of *o*-TSA. Something similar happened with BSA, as it was found in small amounts (0.05 μg/L) in the influent, but higher concentrations above 0.35 μg/L appeared in the effluent. Biodegradation may be responsible for this transformation process. Previous studies indicated that BSA could be a metabolite of high-molecular-weight sulfonamides (e.g., phenylsulfonamides) [37].

3.1.2. In surface water. Sulfonamides appear to resist natural biodegradation rather strongly, as reflected in the high frequency of their detection in streams and rivers. A US Geological Service (USGS) analytical survey demonstrated this, detecting sulfamethoxazole in rivers and streams with frequencies up to 27% [38].

Besides, their low tendency to partition onto sediments enables them to be transported over long distances in flowing water.

As mentioned above, Perez et al. carried out an assessment of the degradability of sulfamethazine, sulfamethoxazole and sulfathiazole in surface-water samples [31]. After more than a month, these sulfonamides were not degraded by surface-water microorganisms in the batch reactor.

Five different sulfonamides and their respective acetyl metabolites were investigated in a little basin of Lake Greifensee in Switzerland [39], which is surrounded by intensive agricultural production, mainly grasslands. The results provided evidence of the absence of degradation products.

3.1.3. In manure and soils. Little is known about the biodegradation kinetics of sulfonamides in soil, manure, and manure-treated soil. The application of manure derived from treated animals as fertilizer in crop soils can be considered a principal cause of antibiotic contamination in soils and groundwater, so concentrated animal-feeding operations (CAFOs) are indirectly major sources for this type of contamination.

Wang et al. [40] proved that storage of manure before its application as fertilizer into tilt soil helps to eliminate the residue of sulfadimethoxine in manure due to there being more degrading microorganisms in manure than in soil [32]. However, storage for too long might enhance the sorption of sulfadimethoxine in the manure, thus impeding its degradation after the manure is spread onto soil. An increase in the moisture content was proved to accelerate sulfadimethoxine degradation in manure-treated soil, as sulfadimethoxine dissolved in water was readily available for the micro biota. Like many antibiotics, sulfadimethoxine also seems to hydrolyze more rapidly in alkaline soils.

Slowly degrading antibiotics can accumulate in the soil due to repeated application of the manure, and concentrations were at not only the sub-therapeutic level but also the level of the minimum inhibitory concentrations for relevant bacterial species in the environment [41]. Factors that affect mobility and persistence of sulfonamides in soil after application in manure are virtually unknown, with only a few studies demonstrating their persistence in manure and potential for leaching through clay soil [42–45].

Kotzerke et al. [46] studied the impact of sulfadiazine on the transformation of manure. Two types of soil were spiked with different concentrations of sulfadiazine (10 and 100 mg/kg). While the sulfadiazine concentrations in the treatments with only 10 mg/kg soil were below the limit of detection of the analytical method after 32 days of application, in those with 100 mg/kg, about 20% of the initial concentration was still present. This difference indicated that a huge amount of sulfadiazine had been either degraded or bound to the organic or mineral fraction of the soil, inactivating the drug. The different microbial activity of the soils studied indicated that most of the applied sulfadiazine had been adsorbed and was still undegraded. By adding manure in both soils, microbial activity was clearly induced. Although sulfadiazine-degradation products were not included in this study, the results highlighted their potential presence and negative effects on the microbial community in soil. They may accumulate, together with the parent drug, and affect organisms that are normally not directly targeted by the antibiotic. However, over time, there is assumed to be a reduction in the effects due to degradation and binding of the chemical compound, together with adaptation of soil microbes to the antibiotic by developing a resistant population. However, retransformation of N^4-acetylsulfamethazine during storage of manure has been reported. The retransformation of the other metabolites (e.g., N^4-acetylsulfameth-oxazole) in wastewater and natural water is considered possible.

The persistence and sorption of sulfamethazine and sulfachloropyridine in agricultural soil has also been investigated [47]. The authors estimated average half-life values of 18.6 days and 21.3 days, respectively, which were consistent with other reported data (10–30 days in soil) [40,42]. Again, there was visible enhancement of soil microbial activity due to the application of liquid slurry from pigs, and this led to a decrease in the persistence of sulfonamide in the soil studied (silty loam and sandy soils), compared to untreated soil, and to greater bioavailability of the sulfonamides for the degrading micro-organisms. The authors therefore correlated these results with increased biodegradation of the substances and attributed a major role for microbial processes in sulfonamide dissipation in soil. However, other publications stated that the higher pH in the soil solution due to the ammonia from the slurry was the factor responsible for a lower sorption of the sulfonamides [48–51]. But, phosphate anions, present in manure in high concentrations, also need to be taken into account, as they are known to form very strong complexes with iron oxides via ligand exchange and may compete for sorption places with the sulfonamides.

3.2. Photocatalytic

Photocatalytic degradation is regarded a means of elimination for sulfonamides, although studies on the production of photodegradation compounds in environmental settings have not been carried out. Andreozzi et al. [52] suggested that sulfonamides undergo degradation in a photocatalytic process similarly to many other organic compounds. Sulfamethoxazole was found to show a photodegradation half-life of 2.4 days. The presence of NO_3^- in aqueous solutions reduced this time and initiated the photodegradation process. Humic acids seemed to act as photosensitizers for it.

In another study by Lam et al. [53], the structural isomer of sulfamethoxazole, 3-amino-5-methylisoxazole, and sulfanilic acid were formed in pure water and solutions with 1 mM H_2O_2 after the irradiation with a sunlight simulator (actinic radiation, 290-800 nm). Reactive intermediates such as hydroxyl, alkyl peroxy radicals and singlet oxygen, were also taken into account in indirect photolytic processes, and, similarly to the work of Andreozzi et al. [52], dissolved organic matter absorbing radiation resulted in the production of most of these species. The persistence of the isomer and the parent compound were compared in pure water and in 1 mM H_2O_2 solution. The isoxazole ring was degraded in pure water at a rate slower than the parent compound, but it would degrade faster than sulfamethoxazole by OH-mediated reactions in irradiated surface water.

For all sulfonamides, sulfanilamide, sulfanilic acid, aniline, hydroquinone, quinine, carboxylic acid and dicarboxylic acid are some of the intermediate organic products detected after photodegradation takes place [54]. Fig. 2 shows a number of the potential photolysis-cleavage sites in sulfonamides as well as the structures of photodegradates.

Table 2 lists the major degradation products identified so far [e.g., sulfanilic acid was observed as a common degradation product (37–79% of occurrence) in a study on the photochemical fate of five sulfonamides with five-membered heterocyclic substituents (sulfamethoxazole, sulfisoxazole, sulfamethizole, sulfathiazole and sulfamoxole)] [55]. As long as these compounds are biodegradable, they can be removed during wastewater treatment via biological methods. If they are persistent or not readily biodegradable, complete mineralization of the pollutants is needed, meaning a much longer illumination period and, consequently, a more expensive process. But if the mineralization is not completed, the results may include risks of toxicity and the biodegradation resistance of organic intermediate products of sulfonamide photodegradation. To confirm this, toxicity tests were carried out with unicellular green alga *Chlorella vulgaris*. First, the toxicity of individual sulfonamides was investigated, the results showing that all of them were toxic to the algae, with sulfadiazine and sulfamethoxazole being the most toxic. Degradation products generated after various illumination times had inhibitory or stimulatory effect to the test cultures of the algae.

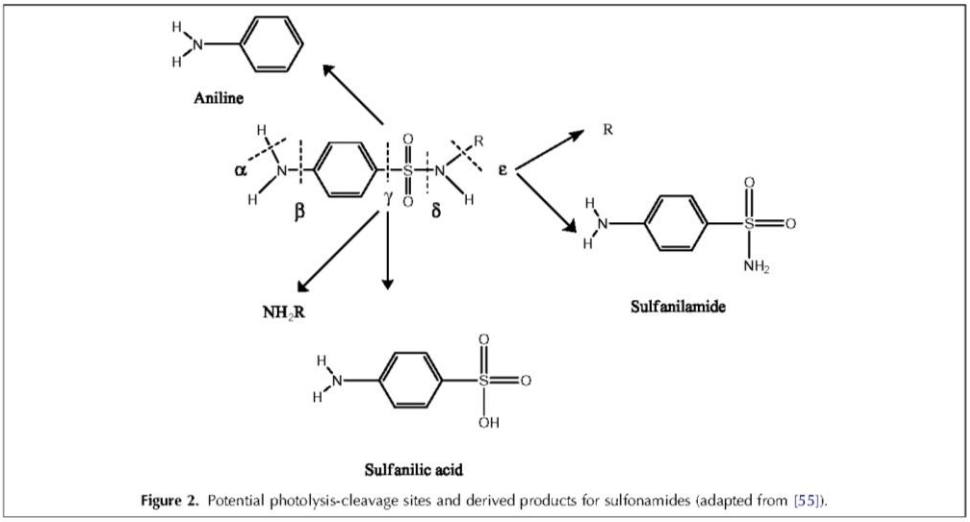


Figure 2. Potential photolysis-cleavage sites and derived products for sulfonamides (adapted from [55]).

Photodegradation products of sulfadiazine showed only inhibitory effects, but degradation products of sulfacetamide, sulfathiazole and sulfamethoxazole showed both effects. After UV illumination, growth inhibition was lower than before, that the toxicity of the intermediates was less than that of the parent compounds. The intermediate products were also biodegradable, in contrast with the sulfonamides investigated, and their mineralization could be accomplished through biological wastewater treatment, which is significantly cheaper than the photocatalytic process.

Finally, it was also proved that all the sulfonamides were photodegradable in the presence of TiO_2 in aqueous suspension during UV illumination [56]. However, this process is expensive, so its application is recommended for the degradation of only substances that otherwise resist biodegradation.

3.3. *Disinfection by-products*

Although aerobic degradation of sulfonamides is limited [32], recent studies showed that these substances are quite susceptible to chemical-oxidation processes (e.g., chlorination and ozonation). Chlorine-disinfection processes in municipal WWTPs turn out to be highly efficient at removing sulfonamides [57]. Chlorination of sulfamethoxazole with free available chlorine (FAC) yielded two main products, N-chlorinated and ring-chlorinated sulfamethoxazole. In natural waters, it was observed that N-chlorinated molecules were retransformed to the parent drug in the absence of reducing agents in only hours, as long as there was no more FAC present.

Dodd et al. [57] also considered that a substantial conversion of the parent compound could be expected for residence times typical of wastewater (5–30 min) and clear drinking-water wells (1–24 h). The decay of N-chlorinated sulfamethoxazole was typically followed by an increase in abundance of N-chloro-*p*-benzoquinonelmine (NCBQ). However, direct correlation between them could not be established, as NCBQ increased even after all N-chlorinated sulfamethoxazole had reacted, which meant that the appearance of NCBQ was probably due to formation and decay of an additional intermediate. It was then confirmed that sulfamethoxazole/FAC reactions contributed at least partial reduction or elimination of the antibacterial activity of parent compounds although some products, particularly NCBQ, possessed higher acute toxicity to aquatic organisms than the original compounds. Regarding aromatic ring-chlorination products, as this moiety is structurally common to all the compounds of the sulfonamide family, these reaction mechanisms, elucidated for sulfamethoxazole, could be extrapolated and applied to other sulfonamides.

In a similar study [58], sulfamethoxazole, sulfamethazine, sulfapyridine and sulfathiazole were oxidized to more than 95% after contact time of only 30 min with ClO_2, with different reactivities. The authors attributed these differences to the fact that ClO_2 does not necessarily attack the aniline moiety, which is common to all sulfonamides.

Table 2. Studies on sulfonamide degradation products

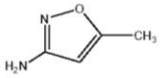
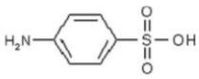
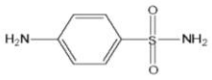
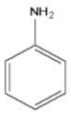
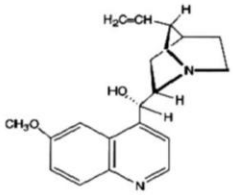
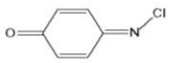
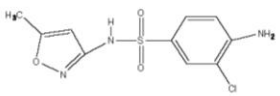
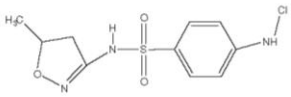
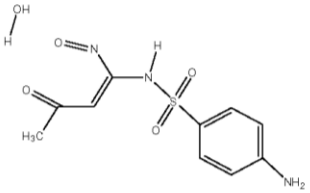
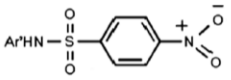
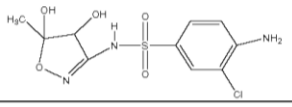
COMPOUNDS	MOLECULAR STRUCTURE	SAMPLE	ANALYTICAL METHOD	PROCESS	REFERENCE
3-amino-5-methyl-isoxazole		Water	LC-MS^2	Chlorination TiO_2 photocatalysis Photodegradation	[54][56][57]
Sulfanilic acid		Water	LC-MS^2	Photodegradation	[55]
Sulfanilamide		Water	HPLC-UV	Photodegradation	[55]
Aniline		Water	HPLC-UV		[55]
Hydroquinone		Water	HPLC-UV		[55]
Quinine		Water	HPLC-UV	Photodegradation	[55]
N-chloro-p – benzoquinonelmine (NCBQ)		Water	GC-MS	Chlorination	[57]
Sulfamethoxazole 1		Water	LC-MS	Chlorination	[57]
N-Chlorinated sulfamethoxazole		Water	LC-MS	Chlorination	[57]

Table 2 (*continued*)

COMPOUNDS	MOLECULAR STRUCTURE	SAMPLE	ANALYTICAL METHOD	PROCESS	REFERENCE
Plausible oxazole ring degradation-sulfamethoxazole		Water	LC-MS	Chlorination	[57]
Plausible aniline ring degradation-sulfamethoxazole		Water	LC-MS	Chlorination	[57]
Dihydroxilated SMX		Water	LC-MS^2	TiO_2 photocatalysis	[56]

Chemical oxidation with ferrate (VI) [$Fe^{VI}O_4^{2-}$, Fe (VI)] was studied for five sulfonamides (i.e. sulfisoxazole, sulfamethazine, sulfamethizole, sulfadimethoxine and sulfamethoxazole) [59]. Under acidic conditions, ferrate (VI) is one of the most powerful oxidizing agents, and, besides, spontaneous decomposition of Fe (VI) in water gives molecular oxygen and Fe (III), thus making Fe (VI) an environmentally-friendly chemical for disinfection and oxidation in water-treatment processes. At a stoichiometric ratio of 4:1 [Fe (VI):sulfamethoxazole], complete removal of sulfamethoxazole was achieved.

Analyses of oxidation products of the reaction of sulfamethoxazole suggested that, in the oxidation of both the isoxazole moiety and the aniline ring, Fe (VI) attacked with minimal preference. The reported results suggested that Fe (VI) had the potential to serve as a chemical oxidant for removing sulfonamides and converting them to relatively less toxic by-products in water.

The application of ozone for the treatment of surface water is widespread in Europe, whereas in the USA the number of plants is still quite low. Ozonation and advanced ozonation processes have proved to be effective in oxidizing pharmaceuticals, even when applied at low concentrations [29].

Regarding sulfonamides, Huber et al. [60] studied the ozonation of sulfamethoxazole, although no transformation products (TPs) could be found.

In another study, ozonation using 5–15 mg/L was enough to decrease the concentration of sulfamethoxazole from 0.62 μg/L to below 0.05 μg/L [61]. Although ozonation products could not be identified, it was thought that the polarity of the molecule and the number of functional groups increased, so the antibiotic properties were lost.

4. Analytical aspects

As reported, many sulfonamides undergo different transformation processes. It is therefore rational that, for their analysis, multi-residue methods are required, since not only the parent compounds but also their TPs are of relevance and concern. Metabolites and other TPs can be structurally diverse and are often present in relatively low concentrations, becoming a challenge for proper identification and quantification. In this review, we focus on the instrumental detection techniques, so extraction and purification procedures are not included. Detailed information on these aspects can be found in the cited literature.

MS is one of the most powerful techniques for detecting organic compounds in environmental matrices. Analytical methods are usually based on MS coupled to liquid chromatography (LC) or gas chromatography (GC) since multi-residue analysis requires the previous application of separation techniques. However, because most of the pharmaceutical residues, and especially their metabolites or degradation products, are highly polar and water soluble, LC-MS has become the method of choice for their determination. LC-MS has been widely accepted as the main tool in identification and structural

characterization of drug metabolites, due to its high sensitivity, selectivity and efficiency [62].

Traditional LC-MS methods using single-quadrupole MS can produce fragment spectra using in-source collision-induced dissociation (CID) (e.g., the two main chlorinated by-products of sulfamethoxazole, both with m/z of 288, were detected by LC-MS by Dodd et al. [57]. Fig. 3 shows their mass spectra as well as the identities of structural fragments. However, this technique can lead to unreliable analysis due to the co-fragmentation of matrix components other than those targeted. To face this shortcoming, tandem MS (MS/MS) techniques based on the fragmentation of the precursor ions produced in the ionization source, are currently preferred.

MS/MS is particularly useful for identification and determination of metabolites and TPs. Triple-quadrupole, ion traps (ITs) and quadrupole ITs are instruments more often employed in detecting sulfonamides and their TPs (see Tables 1 and 2). Performing precursor-ion scans and constant neutral-loss scans does not require previous knowledge about the molecular weight of metabolites. Since these molecules usually share a common structural part with the parent drug, precursor-ion scan can be used to search for metabolites structurally related to the parent compound. If the drug and metabolites have one or more common core structures that are lost in MS/MS experiments as neutral species, the mass spectrometer can be set to search for these metabolites using constant neutral-loss scan.

For the structural elucidation of metabolites and degradation products, high-resolution MS, by time-of-flight (TOF) instruments, is also a valuable tool to get better structural information. It provides the elemental formula of the molecule, which enables detection and identification of a priori unknown substances. As an example, Grant et al. [63] employed a matrix-assisted laser desorption/ionization (MALDI)-TOF-MS instrument to detect N^4-acetylsulfamethazine in environmental sam-

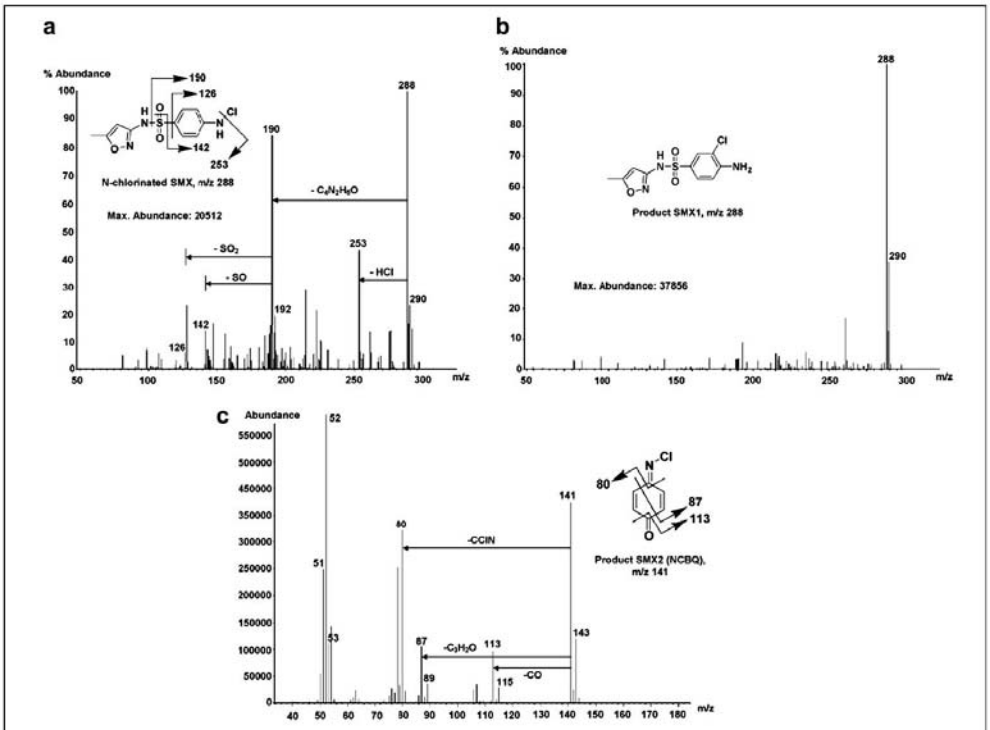


Figure 3. Mass spectra of the degradation products of sulfamethoxazole after chlorination with free available chlorine (FAC): a) N-chlorinated sulfamethoxazole; b) sulfamethoxazole-1; and, c) N-chloro-p-benzoquinoneimine (NCBQ). Spectra a) and b) were obtained by LC-(ESI+)-MS and spectrum c) was obtained by GC-MS. (extracted from [57]).

ples. Accurate-mass measurement and highly-sensitive MS can be combined in the quadrupole time-of-flight instrument (QqTOF), which has been already used to identify and to quantify pesticides and their metabolites in food [64–66].

The use of ITs has increased in recent years, as they possess higher sensitivity than triple-quadrupole instruments [10]. In addition, their MS^n-scan feature is highly efficient in the structural analysis of metabolites (provide better sensitivity and allowing clarification of the fragmentation process, thereby significantly facilitating interpretation of spectra). With built-in information-dependent-acquisition (IDA) experiments, one can collect the maximum amount of structural information with the minimum number of analytical runs [67]. The combination of QqTOF and a hybrid QqIT instrument with MS^3 capability has proved to be very powerful in the identification of unknown compounds [10,64].

5. Conclusions

Although not many studies have been published so far on sulfonamide metabolites and degradation products, and although there is generally a lack of data about biodegradation, we must mention that, wherever sulfonamides have been detected, metabolites have nearly always also been present, and sometimes at higher concentrations than the parent drugs. To omit metabolites and degradation products from studies may lead to erroneous estimations of the elimination rates, as the parent products are still present but transformed. It is also necessary to take into account the potential retransformation of these products back into the parent drug and their toxicity.

Sulfonamides resist biodegradation in wastewater-treatment processes and even in media with high microbial activity (e.g., activated sludge). Investigation of the patterns of concentration of parent compounds, metabolites and degradation products will become a relevant tool in evaluating the environmental fate of sulfonamides. In this scenario, there is a great need for new analytical methods capable of simultaneously determining parent compounds, their metabolites and degradation products.

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1.4 RIESGOS MEDIOAMBIENTALES DERIVADOS DE LA PRESENCIA DE SULFAMIDAS

1.4.1 Normativa vigente

La ubicuidad de las sulfamidas en todos los ecosistemas acuáticos ha suscitado una creciente preocupación respecto a su ecotoxicidad potencial en la biota, pero aún la información disponible es escasa. Hasta la fecha no hay ninguna normativa a nivel europeo que establezca límites de concentración de sulfamidas o de cualquier otro antibiótico en ninguno de los diferentes compartimentos medioambientales. En lo referente a medicina veterinaria, como se mencionó en la sección 1.2., sí existe la prohibición del uso de antibióticos como promotores de crecimiento desde 2003. Por otro lado, la Directiva Europea EC 2377/90 establece un procedimiento a través de la Agencia Europea del Medicamento (EMEA) para la estimación de límites máximos de residuos (MRLs) de medicamentos en productos alimenticios de origen animal (Reglamento CEE 2377/90). Para cualquier sulfamida, se ha establecido un MRL de 100 $\mu g\ kg^{-1}$ del compuesto original en determinados tejidos destinados al consumo tales como músculo, grasa, hígado y riñón, así como en leche de origen bovino, ovino y caprino. La suma de todos los residuos provenientes de las sulfamidas no debe sobrepasar los 100 $\mu g\ kg^{-1}$. Existen, además, otras directivas en referencia al uso de antibióticos en piensos alimenticios para ganado, como por ejemplo la Directiva EC 90/167, en las que no entraremos en detalle al no ser el objeto de esta Tesis.

La falta de legislación sobre la presencia medioambiental de las sulfamidas es probablemente el principal motivo para la falta de información sobre su ecotoxicidad, y viceversa. Si bien probablemente las concentraciones detectadas hasta el momento en los diferentes compartimentos medioambientales (normalmente a nivel de $ng\ L^{-1}$) no suponen un riesgo para el hombre, dichos niveles sí pueden resultar de riesgo para organismos inferiores "no diana" (p.e. algas, invertebrados acuáticos y organismos unicelulares).

1.4.2 Exposición en el medio acuático

Las sulfamidas llegan al medio natural de forma regular y constante, de modo que concentraciones consideradas como inocuas pueden suponer un riesgo ambiental a medio o largo plazo. Su demostrada resistencia a la biodegradación, incluso en medios con una alta actividad microbiana (en los fangos activos de las EDAR), hace suponer que son persistentes una vez excretadas. Como se comentaba en la sección 1.2.2 de esta Introducción, las poblaciones bacterianas de las plantas de tratamiento podrian considerarse como los primeros organismos afectados negativamente por las sulfamidas, principalmente las de consumo humano al ser las de mayor incidencia en estos casos. Se ha demostrado en dos estudios recientes que el SMX resultó tóxico para las colonias bacterianas de CAS, inhibiendo el crecimiento de las bacterias nitrificantes (Al-Ahmad *et al.* 1999) e inhibiendo parcialmente el proceso de metanogénesis (Fountoulakis *et al.* 2004). Una vez en las aguas superficiales, la presencia medioambiental de las sulfamidas puede afectar de manera diferente a distintos grupos taxonómicos de mayor o menor relevancia. Por ejemplo, en ecosistemas acuáticos la pérdida de los productores primarios (algas) supondría una desestructuración de toda la cadena trófica, ya que éstos representan una gran parte de toda la biomasa del ecosistema y son una fuente de carbono fundamental para el resto de la biosfera acuática. Por este motivo, diferentes especies de algas como *Pseudokirchneriella subcapitata* o *Selenastrum capricornotum* entre otras, son utilizadas con frecuencia como especies indicadoras del impacto ambiental derivado de la presencia de microcontaminantes. Los antibióticos tienen propiedades "anticloroplásticas" debido al orígen bacteriano de los cloroplastos, ya que estos orgánulos celulares evolucionaron a partir de las primeras cianobacterias; este hecho hace que las algas sean muy sensibles a la presencia de antibióticos en agua. A pesar de la falta de información antes mencionada, sí se ha podido demostrar que las microalgas son más sensibles que los crustáceos y peces a la presencia de antibióticos como triclosan y ciprofloxacina (Yang *et al.* 2008; Langdon *et al.* 2010). Las sulfamidas sin embargo han resultado ser bastante inocuas para las algas verdes (Pro *et al.* 2003; Yang *et al.* 2008). En otros niveles tróficos, la bacteria marina luminiscente *Vibrio fischerii*, los invertebrados de agua dulce *Daphnia magna* y *Moina macrocopa* y peces como la medaka japonesa (*Oryzias latipes*) son también muy utilizados como especies de referencia. La Publicación #2 incluida al final de esta Introducción recoge las publicaciones más destacadas hasta la fecha sobre la ecotoxicidad de las sulfamidas y sus metabolitos. Como se puede

observar en la Tabla 2 de esa Publicación, las concentraciones de inhibición estimadas son varios órdenes de magnitud superiores a las detectadas en aguas superficiales, de modo que la presencia medioambiental de las sulfamidas no se considera de riesgo para estos organismos. De nuevo ha sido SMX la sulfamida utilizada en un mayor número de ensayos de toxicidad. Sin embargo, es altamente probable que algas y demás organismos acuáticos estén expuestos, si bien a concentraciones menores, durante períodos de tiempo más largos que los ensayados en la mayoría de estudios en laboratorio, de modo que el riesgo que la presencia medioambiental de estos antibióticos supone para los diferentes taxones no queda definida con exactitud con parámetros de toxicidad aguda como el EC_{50} (concentración efectiva media), y nuevos ensayos en condiciones reales deberían ser propuestos (toxicidad crónica).

1.4.2.1 Evaluación de Riesgo Medioambiental siguiendo las directrices de la Agencia Europea del Medicamento

Como se ha mencionado anteriormente, hay muy poca información disponible sobre la ecotoxicidad de las sulfamidas y fármacos en general, ya que dichos trabajos de investigación no forman parte de los requisitos legales para la comercialización de estas medicinas. El único tipo de regulación relacionado es la obligatoriedad establecida por la EMEA de presentar una evaluación de riesgo ambiental (ERA) conjuntamente con la solicitud de autorización de comercialización de cualquier nuevo fármaco destinado al consumo humano (Breton *et al.* 2003). EMEA estableció estas directrices el año 2001. Al tratarse de las únicas pautas restrictivas bajo un marco legal que consideraban la ecotoxicidad potencial de fármacos, este procedimiento ha sido utilizado con relativa frecuencia en los últimos años para evaluar el riesgo medioambiental derivado de la presencia de fármacos de consumo humano que ya están siendo vertidos al medio ambiente acuático o terrestre (Ferrari *et al.* 2004; Huschek *et al.* 2004; Kim *et al.* 2007; Santos *et al.* 2007; Grung *et al.* 2008; Park *et al.* 2008; Gros *et al.* 2010). Este protocolo para el ERA se divide en dos fases: una primera fase que consiste en una estimación de la exposición medioambiental al fármaco tras su consumo, y una segunda etapa en la que se requiere información sobre sus propiedades fisicoquímicas, farmacológicas y ecotoxicológicas para poder evaluar dicho riesgo. La Figura 2 de la Publicación #2 muestra un esquema de ambas fases. La primera fase comienza con la estimación de la concentración medioambiental prevista o PEC del fármaco investigado en agua. El protocolo recomienda que si este valor supera los

0.01 µg L^{-1} en agua superficial, se lleve a cabo un estudio en mayor profundidad sobre la toxicidad del compuesto, pasando así a la Fase II del estudio. En esta segunda fase, se llevan a cabo diferentes test standard de toxicidad para estimar la concentracion estimada sin efecto o PNEC, o la concentración observada sin efecto o NOEC (EMEA-CHMP 2006). Estas variables de evaluación o *endpoints* ofrecen una estimación de la máxima concentración del fármaco para la que no se esperan efectos adversos. El PNEC normalmente se calcula utilizando datos disponibles sobre toxicidad del compuesto en cuestión. Sin embargo, cuando los valores de PNEC no pueden ser calculados a falta de algún parámetro, otro PNEC puede derivarse dividiendo datos sobre toxicidad aguda, como EC_{50} o LC_{50} (concentración letal media), por un factor de incertidumbre, de valor máximo 1000 (Sanderson *et al.* 2003); de este modo, esa información sobre la toxicidad aguda del compuesto queda convertida en valores que equivaldrían a una estimacion de toxicidad crónica, ya que estos no se suelen encontrar en la literatura disponible. De modo similar, las concentraciones detectadas en los diversos estudios publicados (MECs, del inglés *Measured Environmental Concentrations*) pueden utilizarse como valores de PECs siempre, eso si, esos MECs han de cumplir con el requisito de ser valores superiores a 0.01 µg L^{-1} como queda establecido en la fase 1 del ERA. Para establecer el escenario más desfavorable, se suele utilizar la mayor concentración ambiental detectada y el menor valor para EC_{50} o LC_{50} encontrados en la literatura. Los grupos taxonómicos estudiados han de ser representativos de las especies presentes en los ecosistemas acuáticos.

Por último, la relación entre el PEC y el PNEC, conocido como cociente de riesgo (HQ), es indicativo del impacto medioambiental potencial existente. Si HQ>1, se ha de realizar un estudio más detallado para refinar los valores de PEC y PNEC. Las directrices del EMEA también recomiendan que, si la suma del total de metabolitos de un compuesto es un 10% mayor que la concentración total del fármaco original excretado, la fase 2 del protocolo de ERA ha de realizarse también para ellos. El Comité de Medicamentos Veterinarios del EMEA ha establecido directrices similares para estimar el riesgo potencial de estos fármacos una vez vertidos o desechados al medio ambiente tanto acuático como terrestre (EMEA-CVMP 2004). La Tabla 1.7 resume los valores de HQ publicados hasta la fecha para sulfamidas. Como se puede observar, con la excepción del valor de HQ de STZ frente a la planta acuática *Lemna gibba*, solo se encontraron HQs > 1 para SMX, y solo en el caso de las microalgas.

Tabla 1.7. Valores de HQ estimados, según las directrices de la EMEA (EMEA-CHMP 2006) para concentraciones detectadas en efluentes de depuradora. Ag: agudo; cr: crónico;*: valores para aguas superficiales

SULFAMIDA	MEC	PEC	$REF_{MEC/PEC}$	TAXÓN	$PNEC_{aguda}$	HQ	REF
SMX	0.09	3.07 0.31*	(Ferrari *et al.* 2004)	Algae	0.027_{ag} 0.59_{cr}	11.4_{ag} 0.52_{cr}	(Ferrari *et al.* 2004)
	2 0.48*	16.02 1.60*	(Thomas A 2001)			59.3_{ac} 2.72_{cr}	
	0.40*	-	(Perret *et al.* 2006)		0.03	13.4	(Park and Choi 2008)
	-	0.95	(Kim *et al.* 2007)		0.146	6.3	(Kim *et al.* 2007)
	0.025	- - -	(Gros *et al.* 2010)	Peces	562	<0.02	(Gros *et al.* 2010)
				Microcrustáceos *(Daphnia magna)*	25.5	0.025	
				Algae	0.027	22.96 1.28*	
	-	0.06-11	(Grung *et al.* 2008)		118	0.57 97	(Grung *et al.* 2008)
	0.021	-			118	0.18	
STZ	0.13	-	(Metcalfe 2003)	Plantas *(Lemna gibba)*	0.10	1.30	(Park and Choi 2008)
SMZ	0.20	-	(Kolpin *et al.* 2002)	Algae	110.30	0.002	(Park and Choi 2008)
	0.18	-	(Gros *et al.* 2010)	Peces	562	<0.001	(Gros *et al.* 2010)
				Microcrustáceos *(Daphnia magna)*	25.5	<0.01	
				Algae	0.027	<0.02	
SDM	0.07	-	(Perret *et al.* 2006)	Algae	0.248	0.282	(Park and Choi 2008)

Por último, se ha de mencionar que recientemente se ha adoptado una nueva normativa referente al registro, la evaluación y autorización de sustancias químicas (EU REACH, CE 1907/2006) que las diferentes compañías tanto productoras como distribuidoras deberían adoptar. Su principal finalidad es que dichas compañías definieran ellas mismas los peligros y riesgos de todas aquellas sustancias producidas en cantidades iguales o superiores a una tonelada anual, y así proporcionar información toxicológica sobre el compuesto en cuestión.

1.4.3 Exposición en ecosistemas edáficos

En el medio edáfico, son las sulfamidas veterinarias y las consecuencias derivadas de su presencia las que se han estudiado en mayor profundidad. La aplicación reiterada de abono contaminado con sulfamidas (durante la época de siembra) puede llevar a su acumulación en esta matriz a concentraciones desde niveles subterapeúticos hasta niveles superiores a la concentración mínima de inhibición (CMI) para comunidades bacterianas de relevancia (Schmitt *et al.* 2005; Kotzerke *et al.* 2008; Heuer *et al.* 2011). La temperatura y la humedad en el suelo, los intervalos de la aplicación del abono así como las condiciones climáticas pueden determinar la movilidad y el destino final de las sulfamidas (Halling-Sørensen *et al.* 2003; Sengelov *et al.* 2003). Otros factores como los flujos preferenciales a traves de fracturas de desecación o galerías excavadas por algunos nemátodos u otros insectos también pueden ser determinantes en cuanto a su movilidad y traslocación (Kay *et al.* 2004; Kay *et al.* 2005; Kay *et al.* 2005b). Además, la actividad del antibiótico puede variar si se ve sometido a biodegradación en esta matriz. Se ha demostrado también que los valores de EC_{50} son mayores cuanto más bajo es el pH del suelo, siendo el efecto mayor para sulfamidas con pk_a más bajos (Tappe *et al.* 2008). El abono proveniente del ganado suele tener un pH alcalino o básico, de modo que cabe esperar que las sulfamidas esten presentes como especies aniónicas y que la actividad antibacteriana disminuya mientras el suelo se reajusta a las nuevas condiciones y recupera su pH de equilibrio (Tappe *et al.* 2008; Focks *et al.* 2010). Por tanto, la actividad antibacteriana de éstas en el ecosistema suelo no depende únicamente de la concentración introducida, sino también de su especiación, variable en función del pH y de su $pK_{a,}$ al igual que pueden variar su K_d y su biodisponibilidad. Por otro lado, la adsorción de las sulfamidas en el suelo es inversamente proporcional al pH (Zarfl *et al.* 2008), de modo que a pHs bajos, cuando su actividad es mayor, también lo es su K_d y son, por tanto, menos biodisponibles.

Tanto en agua como en suelo las sulfamidas, como el resto de fármacos, rara vez son detectadas como compuestos únicos en la matriz investigada, sino junto a muchos otros microcontaminantes que pueden haber llegado al medio por la misma vía (normalmente otras sulfamidas o fármacos), de modo que no sólo cabría esperar efectos negativos debidos a la presencia del antibiótico en cuestión, sino que también podrían tener lugar efectos sinérgicos de las diferentes sulfamidas o de éstas con otros compuestos (Eguchi *et*

al. 2004; Yang *et al.* 2008). Además, dada su similitud estructural y modos de actuación semejantes, un efecto acumulativo de concentraciones podría dar lugar a una toxicidad mayor. Los diferentes metabolitos y demás productos de transformación de las sulfamidas también pueden tener efectos negativos e influir en la toxicidad final, complicando la interpretación de los resultados. Uno de los pocos trabajos de investigación que trata sobre la ecotoxicidad de los metabolitos es el publicado en 2004 por Eguchi *et al*. (Eguchi *et al.* 2004), en el que los metabolitos acetilados de la SDM, el SMX y la SDZ mostraron por separado unos efectos inhibidores más bajos que sus correpondientes moléculas originales frente a microalgas. No obstante, estos efectos aumentaban cuando los tres metabolitos se encontraban simultáneamente en la matriz investigada, así como en presencia de trimetoprima. Como veremos en el Capítulo 3 de esta Tesis, en uno de los experimentos llevados a cabo se estimaron valores de EC_{50} para *Vibrio fischerii* superiores para AcSPY que para SPY. Según la Directiva Europea 447/93/67/EEC, que establece la toxicidad de un compuesto determinado en función de la EC_{50}, el metabolito debería considerarse tóxico, mientras que SPY quedaría clasificado en una categoría inferior, concretamente como perjudicial.

1.4.4 Resistencia bacteriana

La presencia medioambiental de los antibióticos ha tomado relevancia científica en las últimas décadas debido principalmente a la resistencia bacteriana derivada. La ubicuidad de los antibióticos en las diferentes matrices ambientales ha originado la proliferación de cepas resistentes en todas ellas (ARGs, *del inglés antibiotic resistant genes*). En el caso de las sulfamidas, de nuevo SMX, la sulfamida más ubicua en ecosistemas fluviales, ha resultado ser uno de los antibioticos menos efectivos contra bacterias pertenecientes a *Aeromonas spp*. (Hoa *et al.* 2011) y también en la familia de las *Enterobacteriaceae*, representativa de la flora intestinal humana y animal (Goñi-Urriza *et al.* 2000), debido a la aparición de cepas resistentes. En un estudio desarrollado por Hoa *et al.* en el 2011 (Hoa *et al.* 2011) se observó una mayor incidencia de cepas resistentes de *Aeromonas spp* al SMX (94%) que a macrólidos. El género *Acinetobacter* también se ha visto afectado por la presencia de SMX, y se han establecido correlaciones positivas entre la concentración detectada de SMX y la aparición de ARGs (Goñi-Urriza *et al.* 2000). Genes resistentes a las sulfamidas han sido detectados no sólo en el medio acuático. De hecho, la cantidad de genes resistentes en sedimentos de río se ha contabilizado

hasta 1200 veces más elevada que en agua, considerándose este medio como una reserva de ARGs (Luo *et al.* 2010). La presencia de sulfamidas en sedimentos puede afectar tanto al número de bacterias presentes en esta matriz como también a su estructura celular y funcionalidad, que a su vez puede afectar a la degradación de la materia orgànica. Bajo las instalaciones de piscifactorias, el problema se agrava ya que los antibióticos se añaden directamente al agua, y se estima que apenas un 20-30% es consumido eficazmente por los peces (Samuelsen *et al.* 1996). Los efluentes de las EDAR también se consideran como fuente u orígen de ARGs (Costanzo *et al.* 2005; Storteboom *et al.* 2010). La utilización de agua tratada o superficial para la irrigación de cultivos o zonas de recreo también podría significar la transferencia de estos ARGs a zonas agrícolas y demás ecosistemas (Barker-Reid *et al.* 2010). En casos en los que diferentes sulfamidas estan presentes en una misma área de estudio también se ha observado resistencia a todas ellas por parte de una misma cepa (Zheng *et al.* 2011).

1.4.5 Exposición humana a las sulfamidas

Como mencionamos antes, mientras que el riesgo por exposición directa a los residuos de antibióticos y otros fármacos encontrados en alimentos provenientes de animales medicados ha sido estudiado en detalle en la Directiva Europea CEE 2377/90 , el riesgo para la salud humana derivado de una exposición indirecta a las sulfamidas presentes en el medio ambiente aún no ha sido estimado y, por tanto, no se ha establecido ningún tipo de medida legal. La ya mencionada falta de información toxicologica sobre las sulfamidas es el principal motivo para la ausencia de legislación a este respecto. Ante esta falta de medidas regulatorias, diferentes estudios paralelos han pretendido establecer una jerarquía de riesgo para fármacos. Por ejemplo, Capleton *et al.* (Capleton *et al.* 2006) crearon un esquema para priorizar el riesgo causado por un fármaco en cuestión, basándose en la mayor o menor probabilidad de exposición de los individuos, su perfil toxicológico (información disponible sobre su toxicidad). En este trabajo, SDZ se consideró dentro de las sustancias que necesitaban una evaluación de riesgo prioritaria, mientras que SMZ se consideró como fármaco de riesgo leve. Existen otros trabajos similares como el de Kools *et al*. (Kools *et al.* 2008), que usaron la información sobre el consumo de las sulfamidas como indicador de su bioactividad y para predecir su ecotoxicidad. En este caso, de nuevo SDZ fue la única sulfamida con un índice de riesgo alto en ecosistemas edáficos cerca de instalaciones de ganadería intensiva y pasto, aunque no se destacó ningún

efecto adverso en humanos. Otros documentos, como el publicado por el Consejo de Estocolmo en 2009 (www.janusinfo.se/environment), consideraban no sólo la ecotoxicidad del compuesto, sino también su tendencia a bioacumularse en el medio y su biodegradabilidad. Dentro de esta nueva clasificación, las sulfamidas fueron consideradas como compuestos altamente tóxicos. Para el hombre, las principales vías de exposición a fármacos en el medio ambiente (principalmente suelo, agua y sedimentos) pueden resumirse en cuatro principales:

a. Cultivos que han crecido en suelos contaminados con fármacos y han acumulado dichas sustancias desde el suelo en sus tejidos. Boxall *et al*. (Boxall *et al.* 2006)demostraron que la exposición a productos alimenticios contaminados con restos de antibióticos u otros fármacos podría ser mucho más relevante que la exposición a agua contaminada. La estabilidad de las sulfamidas hace que puedan mantener su actividad y toxicidad potencial una vez excretadas. En el caso de los cultivos, el riego con aguas contaminadas podría suponer la absorción directa o indirecta de las sulfamidas por las plantas (Jjemba 2002). También hay que considerar la presencia de sulfamidas en el suelo debida a la aplicación de abono contaminado; aunque se ha demostrado que las sulfamidas son fuertemente adsorbidas en las primeras etapas en las que alcanzan el suelo (Stoob *et al.* 2007), estas cantidades son liberadas posteriormente a la solución intersticial pudiendo ser absorbidas por las raíces de las plantas. Sin embargo, diferentes estudios de absorción en zanahorias u hojas de lechuga demostraron que ninguna de ellas era vulnerable a la presencia de sulfamidas (Boxall *et al.* 2006). Migliore *et al*. (Migliore *et al.* 1993; Migliore *et al.* 1995; Migliore *et al.* 1997) estudiaron la toxicidad y la bioacumulación de la SDM en diferentes plantas terrestres, observando cómo su presencia influía negativamente en el desarrollo post-germinativo del centeno, mijo y maíz entre otros. También demostró que la SDM podía bioacumularse sobre todo en raíces, pero también en ramas. Sin embargo, las concentraciones ensayadas (300 mg L^{-1}) eran varios ordenes de magnitud superiores a las detectadas en diferentes suelos hasta la fecha, de modo que estos efectos negativos se consideraron poco probables en suelos bajo condiciones reales. Siguiendo con el estudio de la SDM, Forni *et al*. (Forni *et al.* 2002) demostraron que su presencia podía llegar a alterar la morfología de Azolla *spp*. (helecho acuático) acumulándose en sus tejidos a niveles de hasta mg g^{-1} (en peso seco de la planta). Durante las 5 semanas en las que se llevó a cabo el experimento, esta planta no sólo fue capaz de sobrevivir a la presencia de SDM, sino también de eliminar hasta un 89% de la concentración que inicialmente se había añadido al suelo

investigado. En un estudio similar, la SMZ fue absorbida por diferentes cultivos, en los que posteriormente se detectaría una concentración en sus tejidos entre 0.1 y 1.2 mg kg^{-1} en peso seco. Después de 45 días de cultivo, dicha concentración disminuyó hasta representar menos del 0.1% de la cantidad aplicada al suelo (Dolliver *et al.* 2007). Estos resultados demuestran que ciertas plantas son capaces no sólo de soportar el estrés y los efectos tóxicos de las sulfamidas a las que están expuestas, sino que también pueden actuar como como especies biorremediadoras. Períodos de exposición prolongados o la exposición simultánea a través de diferentes vías pueden tener efectos más perjudiciales para la planta. Sin embargo, todos los estudios hasta la fecha sugieren que los niveles de exposición de los consumidores superiores mediante el consumo de cultivos está muy por debajo de un posible umbral de toxicidad.

b. Exposición de peces en piscifactorías. La información sobre la bioacumulación de sulfamidas en diferentes tejidos de peces es escasa. Se demostró que la SDM, muy utlizada en acuicultura, tiende a acumularse en la gamba marina *Artemia spp.* con las consecuente implicaciones para el resto de la cadena alimentaria en ese ecosistema (Migliore *et al.* 1993). Won et al. (Won *et al.* 2011) detectaron niveles de SMX superiores a 5mg Kg-1en tejidos de un ejemplar de anguila común (*Anguilla anguilla*), pero no se detectó ningún residuo de estos antibióticos en más del 99% del total de muestras analizadas. Hou *et al*. (Hou *et al.* 2003) estudiarion la bioacumulación y eliminación de la SMZ en esturiones (*Acipenser schrenkii*). Los resultados indicaron que la bioacumulación de esta sulfamida era baja ysu presencia no era de esperar su presencia en tejidos del pez que posteriormente son consumidos por humanos, y tampoco seria probable que ocurriera una biomagnificación en otros peces depredadores superiores. Resultados similares se encontraron en el pez mosquito (*Gambusia spp*.) o en otras especies (carpa común) que habitan en aguas con alta influencia urbana (alto índice de vertido de aguas de salida de EDAR), en las que SMX no se pudo detectar en ninguno de los tejidos analizados (Ramirez *et al.* 2009).

c. Ganado que ha acumulado fármacos veterinarios en sus tejidos a través de la cadena alimentaria. Se han detectado concentraciones de hasta 75 mg Kg^{-1} de SDM en entrañas de ganado porcino y de 46 mg Kg^{-1} de SDZ en entrañas de ganado aviar en granjas Chinas (Chu *et al.* 2009). Hamscher *et al*. (Hamscher *et al.* 2003) también sugirieron la posibilidad de exposición por inhalación del polvo emitido desde instalaciones de ganadería intensiva.

d. Abstracción de aguas subterráneas y superficiales que contienen fármacos veterinarios para abastecimiento.

Sin embargo, y consdierando las cuatro vías de exposición comentadas, se considera que, hasta la fecha, los efectos tóxicos directos causados por la presencia medioambiental de las sulfamidas no constituyen una amenaza para la salud pública.

1.4.6 Ecotoxicidad de metabolitos y otros productos de transformación

El grado en el que las sulfamidas se degraden en el ecosistema receptor establece la base para proceder a una evaluación de impacto medioambiental completa. La degradación del compuesto puede suponer tanto su completa mineralización como su transformación en nuevos productos cuya actividad se desconoce, y que por tanto pueden suponer nuevos riesgos medioambientales. Todos estos metabolitos y productos de degradación deberían ser considerados siempre que se lleve a cabo esta evaluación. Para las sulfamidas, la degradación abiótica y los productos de transformación derivados han sido investigados en mayor detalle que los productos de biodegradación. La fotodegradación ha resultado muy eficaz en la eliminación de las sulfamidas como demuestran diferentes trabajos (Andreozzi *et al.* 2003; Boreen *et al.* 2004). Los diferentes productos de fotodegradación pueden biodegradarase rápidamente, por ejemplo durante el tratamiento convencional de aguas residuales, pero si por el contrario, se trata de productos apenas biodegradables y, por tanto, persistentes, deben considerarse también como potencialmente tóxicos. Baran *et al*. (Baran *et al.* 2006) demostraron que, tras su fotodegradación, diferentes sulfamidas (STZ, SMX, SDZ y SCM) podían tanto inhibir como potenciar el crecimiento de determinadas algas verdes. Se observó también un aumento en su toxicidad frente a la especie de microcrustáceo *Daphnia magna* bajo irradiación solar, sugiriendo la formación de productos intermedios de fotodegradacion más tóxicos que las sulfamidas originales (Jung *et al.* 2008). STZ resultó ser la sulfamida mas susceptible a la fotodegradación (39% de degradación después de 48 h de exposición), y también la que mostró un efecto inhibitorio del crecimiento de *D. magna* casi 10 veces más fuerte tras la irradiación, corroborando la teoría de una mayor toxicidad de los productos de transformación. Por el contrario, mientras que la sulfacetamida (SCT), STZ, SMX y SDZ

resultaron ser tóxicos para el alga *Chlorella vulgaris*, (C_0=0.01 mM), sus productos de fotodegradación tuvieron una incidencia negativa menor en el alga, registrándose de nuevo tanto efectos inhibidores como promotores del crecimiento (Baran *et al.* 2006). El ácido sulfanílico y el 3-amino-5-metilsoxazol fueron identíficados como principales productos de la fotodegradación del SMX por Nasuhoglu *et al*. (Nasuhoglu *et al.* 2011), que demostraron además que estos productos eran más tóxicos frente a *D. magna* que el compuesto original. Gonçalves *et al*. (Gonçalves *et al.* 2012) identificaron de nuevo el 3-amino-5-metilsoxazol y la *p*-benzoquinona como principales productos de transformación derivados de la ozonización del SMX, y demostraron que estos productos también eran más tóxicos que el SMX frente a *V. fischerii* durante los primeros 30 min del experimento. Otro estudio demostró también que los productos de transformación del SMX tras el proceso de ozonización eran tóxicos para *D. magna* y el alga *P. subcapicata* (Gómez-Ramos et al. 2011). Estos productos resultaron ser lo suficientemente activos para alterar la morfología de células de mamíferos en cultivo, aunque sin alterar seriamente la funcionalidad de éstas (Yargeau *et al.* 2008). Sin embargo, de nuevo las concentraciones utilizadas en este tipo de estudios estaban varios órdenes de magnitud por encima de las concentraciones ambientales, de modo que dicha toxicidad no sería registrada.

Publicación científica 2

"Combining chemical analysis and ecotoxicity to determine environmental exposure and to assess risk from sulfonamides"

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Combining chemical analysis and ecotoxicity to determine environmental exposure and to assess risk from sulfonamides

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In recent years, concern regarding the environmental presence of sulfonamides (SAs) and other species of antibiotics has increased considerably due mainly to the potential spread of antimicrobial resistance in the different bacterial communities. However, many other ecotoxicological effects due to the release of these drugs in the different ecosystems may also be taking place. Analytical chemistry plays an important role in evaluating ecotoxicity through unequivocal identification and precise quantification of target compounds even when concentrations are very small. We aim to review all the studies carried out to date on potential environmental hazards posed by the SAs and to comment on the usefulness of chemical analysis in this application.

Keywords: Antibiotic; Antimicrobial resistance; Chemical analysis; Drug release; Ecotoxicity; Environmental exposure; Environmental hazard; Environmental risk; Risk assessment; Sulfonamide

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1. Introduction

The sulfonamides (SAs) are among the most commonly used antibiotics in veterinary medicine and to a lesser extent in human medicine. They are used mostly in aquaculture and intensive livestock farming and are the most highly consumed antibiotics, after tetracyclines, in the European Union (EU) [1].

After administration, residues of antimicrobial agents that have not been completely metabolized can reach the environment by several pathways [2]. They may enter the environment indirectly through sewage-treatment plants, in the case of human intake, or directly through the application of manure from medicated animals on agricultural land. Antibiotics used in aquaculture are directly added to receiving waters, formulated as feed additives, with 70–80% of those administered entering the environment [3]. In soils, SA residues have been detected at concentrations comparable to those of tetracyclines, with values up to 11 ng/g [4,5]; values reported in manure range from 8.7 mg/kg for sulfamethazine to 12.4 mg/kg for sulfathiazole [6].

As SAs are both fairly water-soluble and polar, they have great potential for leaching to groundwaters or running off to surface waters [7]. Recently, several studies on the occurrence of SAs in wastewater effluents [8–10], surface waters [11], and groundwaters [12,13] have been published, and the analytical methodologies developed allowed their detection in these matrices at concentrations down to ng/L and pg/L levels. Although these levels are quite low, SAs are being continuously introduced into the environment and concentrations previously considered innocuous may pose a risk in certain environmental compartments. It has been demonstrated that some environmental bacteria develop antimicrobial resistance in soils and water matrices [14–16]. Most environmental research on antibacterial compounds has focused primarily on bacterial resistance, which has become the principal concern. However, although SAs are designed to target specific metabolic pathways [they competitively inhibit the conversion of *p*-aminobenzoic acid (*p*ABA)], by inhibiting the biosynthetic pathway of folate (an essential molecule required by all living organisms) [17], they can also have a number of often unknown effects on

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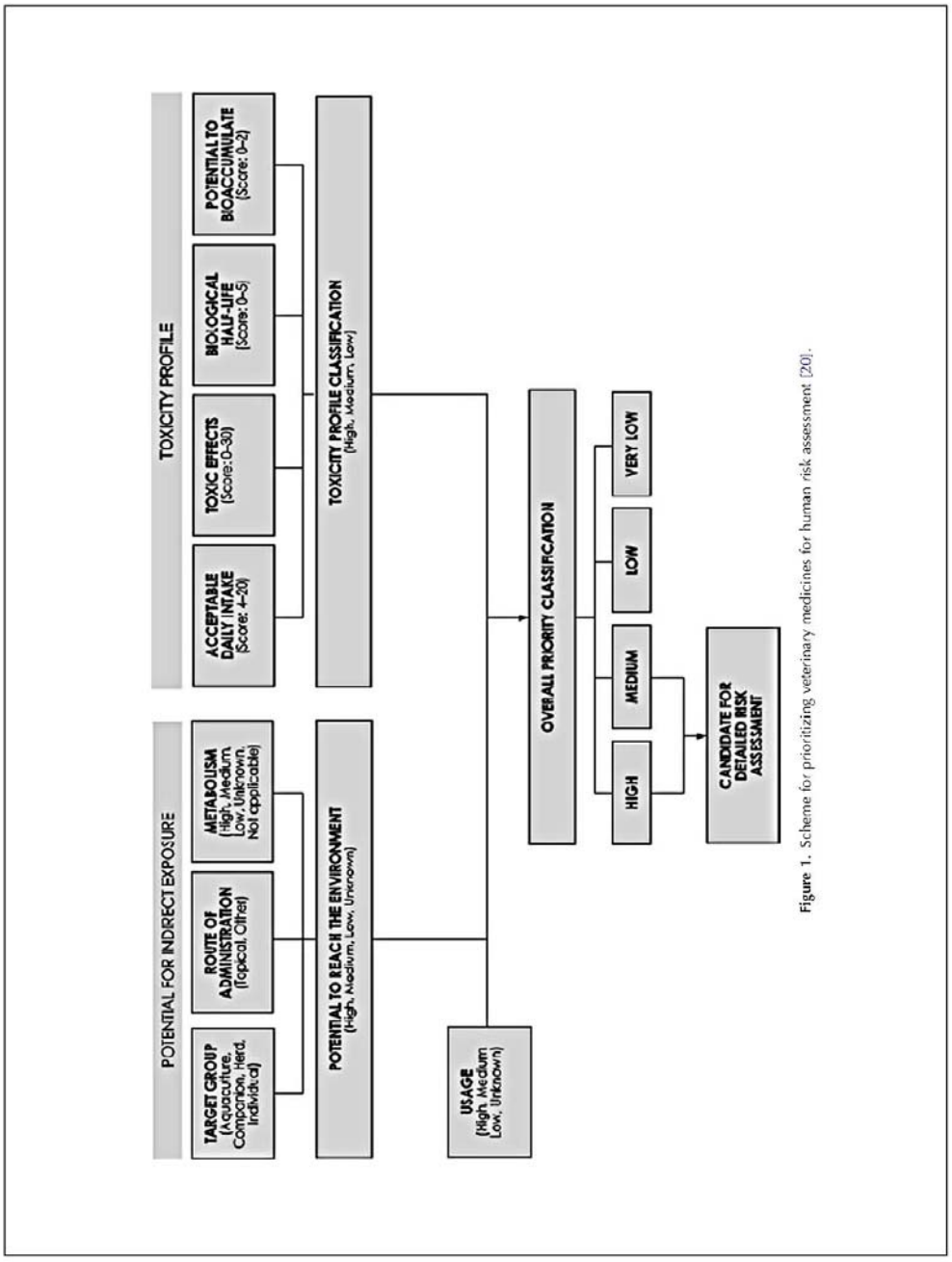

Figure 1. Scheme for prioritizing veterinary medicines for human risk assessment [20].

non-target organisms, which may be very relevant environmentally (e.g., autotrophs or photosynthetic organisms, such as phytoplankton and macrophytes).

It should also be mentioned that SAs are not fully metabolized after administration and different amounts of the parent compounds together with their metabolites are eliminated by the organism through urine and faeces. Besides, the formation and the presence of degradation products derived from SAs have been demonstrated by several authors [18], who performed studies of hydrolysis, photolysis, chemical oxidation, chemical complexation and also sorption to solids and other xenobiotics. The potential presence of these biotic and abiotic transformation products of SAs may also make environmental risk assessment more complex, and these compounds should therefore be considered from a toxicological point of view. Although metabolites are in many cases less active and less toxic than the parent SAs, some may still be active and potentially more toxic than the parent SAs.

2. Toxicity regulations in Europe

The risk to humans from direct exposure to veterinary medicine residues in food products from treated animals has been thoroughly assessed. EU Council Regulation EEC 2377/90 lays down a procedure through the European Medicines Evaluation Agency (EMEA, www.emea.eu.int) to establish maximum residue limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin [19]. For SAs, a definitive MRL has been established for parent drugs – 100 μg/kg in target tissues (muscle, fat, liver and kidney) and also in milk from bovine, ovine and caprine species. The combined residues of all substances in the SA group should not exceed 100 μg/kg (EU Commission Regulation EC 281/96). However, the potential extent of any indirect human exposure to veterinary medicine residues present in the environment, with the consequences for human health, has not been yet established. The only study on this matter was made by Capleton et al. [20], who created an interesting scheme to prioritize the risk posed by veterinary medicines on the basis of their potential for indirect human exposure via the environment and their toxicity profile (Fig. 1).

The lack of ecotoxicological data may be one of the main reasons for the absence of European regulation regarding adverse effects of SAs or other antimicrobial substances in any environmental compartment. The only restrictive measure established so far comes from the EMEA, which requires that an environmental risk assessment (ERA) should accompany an application for marketing authorization for a new medicinal product for human use [21]. Although they are designed as part of the process for registering new drugs, risk-assessment guidelines that include information on predicting levels of drugs in the environment can be used to prioritize the risk from drugs that are already in use and to assess the potential impact of drugs yet to be released [22–25]. The ERA protocol is a two-phase tiered process that

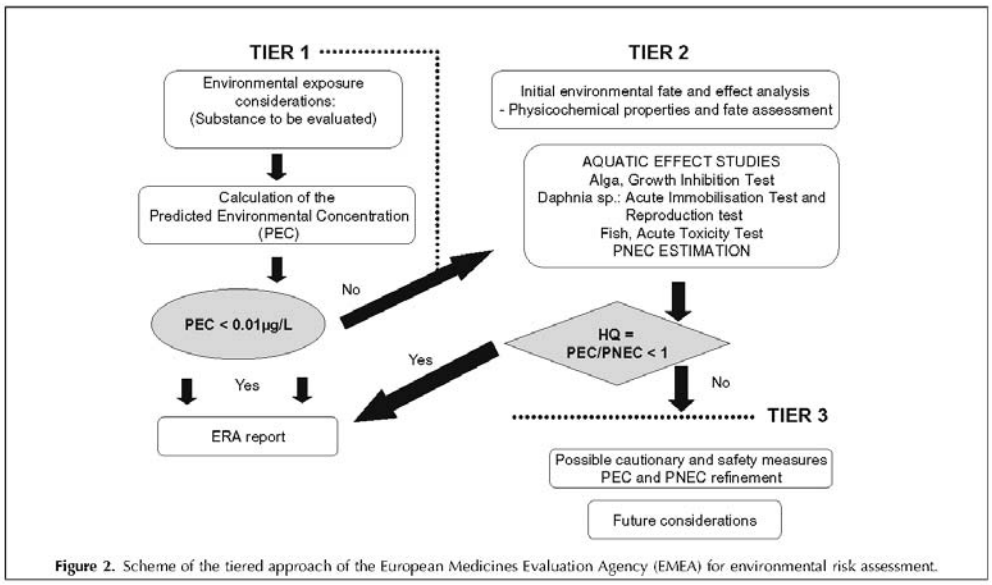

Figure 2. Scheme of the tiered approach of the European Medicines Evaluation Agency (EMEA) for environmental risk assessment.

Table 1. Summary of the analytical methodologies used in ecotoxicity studies

Compound	Matrix	Analytical method	Analytical conditions	Ref.
Sulfadimethoxine	Crop plants and weeds	HPLC-DAD	Stationary phase: 100 RP18 Lichrosphere column (250 × 4 mm, 5 µm) Mobile phase: 0.017 M H_3PO_4-diethylamine (0.5 g/L): acetonitrile Flow: 1 mL/min Injection volume: 50 µL Temperature: room temperature Detection at: 275 nm Spectra acquisition: 220–350 nm Quantitation: external standard LOD: 1 µg/g	[35,48,49]
Sulfadiazine	Soil samples	HPLC-DAD	Stationary phase: Nucleosil C18 RP (250 × 4.6 mm) Detection at: 254 nm LOD: 1 µg/L Quantitation: external standard	[38]
Sulfamethazine	Fish muscle Water residue	HPLC-DAD	Stationary phase: Inerstil C18 (250 × 4.6 mm, 5 µm) Mobile phase: acetonitrile:water:acetic acid (24:76:0.05, v:v) Flow: 1 mL/min Injection volume: 50 µL Temperature: room temperature.	[68]
Sulfadimethoxine	Plants	TLC Chromatography	10 µL of sample Mobile phase: chloroform:methanol (95:5, v:v) LOD: 20 ng	[50]
Sulfadimethoxine **Sulfamethoxazole** **Sulfadiazine**	Moderately hard water	HPLC-MS	Stationary phase: Luna C8 column (100 × 4.6 mm, 3 µm) Mobile phase: acetonitrile:water:acetic acid (24:76:0.05, v:v) Flow: 0.250 mL/min Temperature: (30°C column, 20°C sample)	[38]
Sulfadimethoxine **Sulfamethoxazole** **Sulfadiazine** **N^4-Acetylsulfadimethoxine** **N^4-Acetylsulfamethoxazole** **N^4-Acetylsulfadiazine**	Microalgae	HPLC-UV	Stationary phase: Capcell Pak C18 (UG120 S, 250 × 4.6 mm, 5 µm) Mobile phase: water:methanol:acetic acid (60:40:0.5, v:v:v) Flow: 1 mL/min Temperature: 40°C (column) Detection at: 270 nm	[56]
Sulfamethoxazole pABA	*Lemna gibba*	HPLC-MS^2	Stationary phase: Agilent Extended C18 column (15 cm × 2.1 mm, 5 µm) Mobile phase: water + 0.1% formic acid:methanol Flow: 350 µL/min Injection volume: 10 µL Temperature: 30°C (column) ESI + SRM mode	[33]
Ergosterol	Soil bacteria	HPLC-UV	Stationary phase: C18-100-5 reversed phase (250 × 4.6 mm) Mobile phase: methanol:water (95%:5%) Flow: 1.5 mL/min Temperature: 28°C (column) Detection at: 254 nm	[37]

(continued on next page)

Table 1. *(continued)*

Compound	Matrix	Analytical method	Analytical conditions	Ref.
Sulfachloropyridazine	Field drains	HPLC-UV	Stationary phase: GENESIS C18 end-capped, 4 μm Mobile phase: tetrahydrofuran, acetonitrile, 0.05% trifluoroacetic acid in water mobile phase. Injection volume: 20 μL Detector: 285 nm LOD: 250 ng/L	[25]
Sulfamethoxazole	WWTP effluent*	HPLC-MS	Stationary phase: GENESIS end-capped, base-deactivated RP-C18 (250 mm × 2.1 mm; 4 μm) Mobile phase: solvent A: 95% water; 5% acetonitrile; 0.1% HCOOH. Solvent B: acetonitrile + 0.1% HCOOH. Flow: 200 μL/min Temperature: 50°C (column) ESI+ SRM mode	[24]
Sulfacetamide **Sulfathiazole** **Sulfamethoxazole** **Sulfadiazine**	Distilled water	HPLC-UV	Stationary phase: Supelcosil Suplex pKB-100 LC-18, 250 × 4.6 mm, 5 μm Mobile phase: K_2HPO_4/acetonitrile (92:8, v:v) Injection volume: 20 μL Detector: 254 nm	[36]

HPLC-UV, High-performance liquid chromatography-ultraviolet detection; HPLC-DAD, High-performance liquid chromatography–diode array detection; TLC, Thin-layer chromatography; HPLC-MS, High-performance liquid chromatography-mass spectrometry; HPLC-MS^2, High performance liquid chromatography-tandem mass spectrometry; LOD, Limit of detection; ESI+, Electrospray ionization (positive mode); SRM, Selective reaction monitoring.

begins with an approximate calculation of the predicted environmental concentration (PEC) of the new drug in water (see Fig. 2). The guidelines recommend that any drug exceeding 0.01 mg/L in surface water should progress to Phase II. The recommended Phase II assessment is based on the ratio of the PEC to the predicted no-effect concentration (PNEC), known as the Hazard Quotient (HQ). PNEC is an estimation of the concentration of drug substances for which adverse environmental effects are not expected. PNEC is established using test data from relevant environmental testing. If HQ is >1, a potential environmental impact is indicated and further testing might be needed to refine PEC and PNEC values in Tier B.

The EMEA Committee for Medicinal Products for Veterinary Use (CVMP) also established similar guidelines to assess the potential for veterinary medicines to affect non-target species in the environment, including both aquatic and terrestrial species [26]. The taxonomic levels tested are intended to serve as indicators for the range of species present in the environment. SAs usually considered are for veterinary use, with the big exception of sulfamethoxazole, which is typically used in human medicine.

A very commonly used assessment factor for estimating the risk of toxicity of a given compound is lethal dose-50% (EC_{50}), which indicates the amount of the drug, in a single dose, which turns out to be lethal for the 50% of the exposed population of a given species. It is usually expressed as mg of the toxic substance divided by kg of the mass of the living organism. Other parameters used are LC_{50} (median lethal concentration) and NOEC (no-observed-effect concentration). However, for environmental risk assessment, these values may not be sufficient and a more holistic approach should be carried out.

3. Analytical considerations

Besides determination of acute-chronic toxicity by bioassays, usually derived from standardized methods (internationally recognized by, e.g., OECD, EPA and ISO) accurate determination of environmental concentrations of the pollutants is required in order to achieve a complete environmental hazard assessment. As mentioned above, environmental matrices are usually complex and the analytes are not present individually but in mixtures, so an appropriate analytical methodology is required (i.e. one able to detect components selectively and to measure them accurately and with the greatest sensitivity).

Sample-preparation procedure is fundamental in the development of the analytical methodology for environ-

mental matrices. One of the most common problems faced when extracting organic compounds from complex matrices (e.g., environmental waters or solids) is the large amount of natural organic matter in the samples. In the case of aqueous samples, commonly used methods for analysis usually include extraction for enrichment and clean up. Prior to analysis, filtration is often carried out on 0.45-μm or 0.2-μm glass-fiber filters. Matrix effects and possible interferences in the analysis are avoided by performing a clean-up step, which also results in a pre-concentration of the target SAs. This step is usually carried out by solid-phase extraction (SPE), in which the type of stationary phase and the elution solvent are critical parameters that can affect recoveries and limits of detection (LODs) of the individual SAs, so they need to be tested in order to optimize the extraction process. Oasis HLB cartridges from Waters (Milford, MA, USA) have yielded higher efficiencies in several studies [8,13,27].

Solid matrices are not as homogeneous as water. Various procedures have been described for extracting antimicrobials from soils, sediments and sewage sludge, going from liquid partitioning with sonication to the more advanced pressurized liquid extraction (PLE), more frequently used in recent years due to its better repeatability, lower solvent consumption and reduced time for sample pre-treatment [28]. A clean-up and enrichment step by SPE usually follows.

Liquid chromatography (LC) coupled to mass spectrometry (MS) has become an essential technique for the study of SAs, as they are polar and thermally labile contaminants, so they are not amenable to gas chromatography (GC) coupled to MS analysis (GC-MS). However, complex matrices (e.g., wastewaters and soils) often require efficient separation of the analytes from interfering compounds in the matrix. In these cases, LC coupled with tandem MS detection (MS^2) is particularly useful for unequivocal identification and quantification of SAs.

There are several studies in which triple-quadrupole and, more recently, quadrupole-linear ion trap (QqLIT) mass analyzers have been successfully employed [28]. The selectivity and the sensitivity of both instruments in selected reaction monitoring (SRM) mode makes them essential in both qualitative and quantitative analysis of SAs in different environmental matrices, yielding the low LODs required in environmental monitoring of SAs.

Regarding LC separation, typical mobile phases comprise water/ACN or water/MeOH mixtures where volatile organic modifiers (e.g., formic acid) are added to improve ionization efficiencies and control pH [29]. The LC-MS^2 analysis of SAs is usually performed with the electrospray-ionization (ESI) source operating in positive-ionization mode. The performance of the analysis may be affected by the ionizable impurities from the natural matrices that can interfere with the ionization processes (i.e. natural organic matter). This may result in a signal suppression or signal enhancement leading to low sensitivity and inaccurate results. There are several strategies in quantification to reduce matrix effects (e.g., external calibration using matrix-matched samples, standard addition or internal standard addition, as well as the dilution of

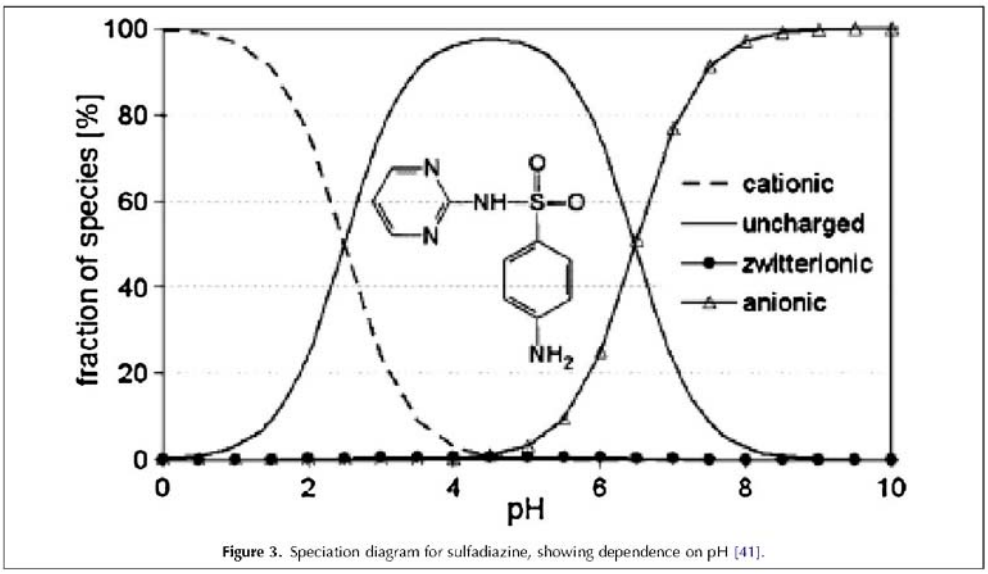

Figure 3. Speciation diagram for sulfadiazine, showing dependence on pH [41].

sample extracts). In analysis of water samples, instrumental and method LODs achieved for SAs reach the pg/L level [13]. For solid matrices, LODs are usually given as dry weight, and LODs below 2 μg/kg have been determined for sewage sludge [30] and below 5 μg/kg for matured agricultural soils [31] and sediments [32].

The use of ecotoxicity tests in combination with analytical methods relating toxicity to the chemical composition of the sample achieves a complete view of the exposure and the risks posed by the pollutants. However, there are no published studies evaluating the potential ecotoxicity of environmental samples in which SAs have been detected. Most of the publications included in this review deal with toxicity tests, bioassays and exposure studies, in which analytical chemistry is used as a complementary tool:

- to confirm the exposure concentrations of SAs used in the toxicity experiments [33,34];
- to analyze residual concentrations of these compounds in the soil or biota extracts after their application in the toxicity experiments [35]; or,
- to compare environmental levels with those applied in the assays.

LC techniques have been chosen in several ecotoxicity studies (Table 1). For example, before carrying out toxicity tests, Baran et al. [36] determined the concentration of SAs in spiked aqueous solutions before and after illumination by LC with ultraviolet detection (LC-UV). Boxall et al. [25] filtered samples from SA-treated soil-sorption studies and analyzed them by isocratic reversed-phase LC-UV. This technique was also applied by Thiele-Bruhn [37] to analyze ergosterol, an equivalent to cholesterol in animal cells and a useful end-point to detect antimicrobial activity. Extractable concentrations of the SAs were similarly analyzed in this study.

Jung et al. [38] used LC-MS to measure the photodegradation of the SAs analyzed under various UVB treatments over time. Brain et al. [33] used LC-MS^2 to verify the exposure concentrations of sulfamethoxazole in the culture dishes of the toxicity tests carried out.

4. Toxicity of sulfonamides in soil ecosystems

4.1. Bacterial tolerance

Most antimicrobial drugs enter the environment via application of manure, which is a valuable by-product in livestock farming, containing nutrients (e.g., nitrogen, phosphorus, and potassium), which are essential for plant growth [34]. It is a slow-release fertilizer and its organic material can improve soil quality. But manure also introduces high levels of ammonia that will increase the pH of the soil solution and it usually takes a few days before soil pH returns to its previous value [39]. Speciation of SAs and the rest of veterinary medicines can be altered, as can their tendency to sorp to soil, their bioavailability and their antimicrobial activity. Whether the antibacterial activity of an SA reaches an ecologically significant level will therefore depend on not only absolute concentration but also speciation.

Tappe et al. [40] studied to what extent pH affected sorption to soil, bioavailability and antibacterial effect of SAs. The dependence of pH on the activity of sulfadiazine, sulfathiazole, sulfadimethoxine, sulfamethazine, sulfaguanidine, sulfapyridine, sulfamethoxazole and sulfachloropyridazine was tested on two bacterial strains (i.e. *Pseudomonas aeruginosa* and the soil bacterial isolate *Pantoea agglomerans*). As a competitive process, sorption to soil increased with decreasing pH and resulted in decreased bioavailability.

SAs are characterized by two pK_a values:

- pK_{a1}, which describes the protonation of the amino group; and,
- pk_{a2}, which describes the deprotonation of the SO_2NH moiety.

SA speciation in solution would depend on the individual pK_a values and the pH of the medium [41]. As an example, Fig. 3 shows the speciation distribution of sulfadiazine as a function of pH. Because SAs enter the soil with manure and the pH of manure is usually alkaline, SAs would occur mainly as anionic species, resulting in a decrease in antibacterial activity as it takes a few days before soil adjusts and returns to the previous pH value [39]. SAs have to pass through the bacterial cell membrane in order to interfere with folate metabolism. The permeability of the cell membrane was assumed to be higher for neutral species than for ionic species, implying that pH below the pKa value would mean a greater concentration of the neutral species, a greater uptake by the bacteria and a greater inhibitory effect of the SA. The models used to predict the inhibition behavior of the tests were carried out assuming that speciation of SAs takes place inside and outside the cell and is in thermodynamic equilibrium. The bacterial intracellular pH value was set to pH 7.5 and extracellular pH varied according to the experimental set-up. The strongest inhibition was predicted for the lowest external pH and for SAs with the lowest pK_a values. Growth rates for the two bacterial strains depended on pH with an optimum at pH 7 for both strains, and cells grew freely suspended at all pH values between 5 and 8. *P. aeruginosa* was not as sensitive to the SAs as *P. agglomerans*, and showed greater inhibition the lower the pH, as predicted by the model. *P. agglomerans* was inhibited mostly at pH 7–8, the prevailing pH range in pig manure after storage and alkalinic fermentation. The poor regulation of intracellular pH of *P. agglomerans* may explain the weak inhibition at low soil pH. Bacteria with a strong control of intracellular pH (pH homeostasis) would therefore be strongly inhibited at low soil pH, especially by SAs with low pK_a values.

Regarding sorption to soil, Boxall et al. [25] demonstrated that neutral species of SAs seemed to be sorbed

Table 2. Summary of the ecological risk estimated for different sulfonamides

Taxon	Matrix/species	Ref	Time	Toxicity end-point	SPY	SDZ	SDM	SMX	SMT	SCP	STZ	SCT	N^4-acSMX	N^4-acSDM	N^4-acSDZ
Bacteria	Activated sludge	[45]	10 h	EC_{50}	-	16.8	-	-	-	-	-	-	-	-	-
	MilliQ Water	[45]	5 d	EC_{50} (exposed to light)	10.5	-	-	-	-	-	-	-	-	-	-
		[45]	5 d	EC_{50} (dark conditions)	14.6	-	-	-	-	-	-	-	-	-	-
	Soil microbial microcosm	[37]	24 h	ED_{10} (μg/g)	0.05	-	-	-	-	-	-	-	-	-	-
		[37]	24 h	ED_{50} (μg/g)	6.2	-	-	-	-	-	-	-	-	-	-
		[37]	24 h	EC_{10} (ng/g)	7.1	-	-	-	-	-	-	-	-	-	-
		[37]	24 h	EC_{50} (ng/g)	890	-	-	-	-	-	-	-	-	-	-
		[37]	2×24 h	ED_{10} (μg/g)	1.17	-	-	-	-	-	-	-	-	-	-
		[37]	2×24 h	ED_{50} (μg/g)	11.5	-	-	-	-	-	-	-	-	-	-
		[37]	2×24 h	EC_{10}(ng/g)	56	-	-	-	-	-	-	-	-	-	-
		[37]	2×24 h	EC_{50} (ng/g)	550	-	-	-	-	-	-	-	-	-	-
Diatom	*Cyclotella meneghiniana*	[24]	96 h	EC_{50}	-	-	-	2.4	-	-	-	-	-	-	-
		[24]	96 h	NOEC	-	-	-	1.25	-	-	-	-	-	-	-
Algae	*Mycrocistis aeruginosa*	[58]	7 d	EC_{50}	-	135	-	-	-	-	-	-	-	-	-
	Selenastrum capricornutum	[56]	72 h	EC_{50}	-	2.19	2.3	1.53	-	-	-	-	>100	>100	>100
		[56]	72 h	NOEC	-	<1.00	0.529	0.614	-	-	-	-	>100	80	>100
		[58]	7 d	EC_{50}	-	7.8	-	-	-	-	-	-	-	-	-
	Rhodomonas salina	[58]	7 d	EC_{50}	-	403*	-	-	-	-	-	-	-	-	-
	Chlorella vulgaris	[56]	72 h	EC_{50}	-	-	11.2	-	-	-	-	-	-	-	-
		[56]	72 h	NOEC	-	-	<20.3	-	-	-	-	-	-	-	-
		[57]	48 h	EC_{50}	-	-	-	-	-	>2000	-	-	-	-	-
		[35]	24-48 h	EC_{50}	-	0.0049**	-	0.0062**	-	-	0.064**	0.062**	-	-	-
	Pseudokirchneriella subcapitata	[24]	96 h	NOEC	-	-	-	0.09	-	-	-	-	-	-	-
		[24]	96 h	EC_{50}	-	-	-	0.146	-	-	-	-	-	-	-
		[55]	72 h	IC_{50}	-	-	-	0.0019–0.103	0.0087–0.103	-	-	-	-	-	-
		[55]	72 h	NOEC	-	-	-	<0.0005–0.103	0.001–0.103	-	-	-	-	-	-
		[55]	72 h	LOEC	-	-	-	0.0008–0.103	0.008–0.103	-	-	-	-	-	-
		[61]	30 min	EC_{50}	-	-	-	23.3	-	-	-	-	-	-	-
		[61]	3 d	EC_{50}	-	-	-	0.52	-	-	-	-	-	-	-
	Synechococcus leopoliensis	[24]	96 h	EC_{50}	-	-	-	0.0268	-	-	-	-	-	-	-
		[24]	96 h	NOEC	-	-	-	0.059	-	-	-	-	-	-	-
		[61]	3 d	EC_{50}	-	-	-	0.52	-	-	-	-	-	-	-
Aquatic plant	*Lemna gibba*	[65]	7 d	LOEC	-	-	-	1	-	-	0.1	-	-	-	-
		[65]	7 d	$EC_{50\text{-chlorophyll b}}$	-	-	3.552	0.682	>1	-	3552	-	-	-	-
		[65]	7 d	$EC_{50\text{-wet mass}}$	-	-	0.248	0.081	1.277	-	-	-	-	-	-
		[65]	7 d	$EC_{50\text{-frond number}}$	-	-	0.445	0.249	>1	-	-	-	-	-	-
		[33]	7 d	$EC_{50\text{-frond number}}$	-	-	-	0.132	-	-	-	-	-	-	-
		[33]	7 d	$EC_{50\text{-dry weight}}$	-	-	-	0.0616	-	-	-	-	-	-	-
		[33]	7 d	$EC_{50\text{-total pABA}}$	-	-	-	0.00336	-	-	-	-	-	-	-
		[33]	7 d	LOEC-frond number	-	-	-	0.108	-	-	-	-	-	-	-
		[33]	7 d	LOEC-fresh weight	-	-	-	0.028	-	-	-	-	-	-	-
		[33]	7 d	LOEC-total pABA	-	-	-	0.009	-	-	-	-	-	-	-
Bacterium	*Vibrio fischeri*	[25]	5 min	EC_{50}	-	-	>500	74.2	303	53.7	>1000	-	-	-	-
		[25]	15 min	EC_{50}	-	-	>500	78.1	344.7	26.4	>1000	-	-	-	-
		[24]	30 min	EC_{50}	-	-	-	0.084	-	-	-	-	-	-	-
		[61]	30 min	LC_{50}	-	-	-	23.3	-	-	-	-	-	-	-
Crustacean	*Daphnia magna*	[25]	48 h	EC_{50}	-	-	248	-	174.4	375.3	149.3	-	-	-	-
		[25]	96 h	EC_{50}	-	-	204.5	-	158.8	233.5	85.4	-	-	-	-
		[23]	24 h	EC_{50}	-	-	639.8	>200	506.3	-	616.7	-	-	-	-
		[23]	48 h	EC_{50}	-	-	-	123.1	215.9	-	-	-	-	-	-
		[23]	21 d	LOEC	-	-	-	-	-	-	35	-	-	-	-
		[23]	21 d	NOEC	-	-	-	-	-	-	11	-	-	-	-

(continued on next page)

Table 2. *(continued)*

Taxon	Matrix/species	Ref	Time	Toxicity end-point	SPY	SDZ	SDM	SMX	SMT	SCP	STZ	SCT	N^4-acSMX	N^4-acSDM	N^4-acSDZ
		[24]	48 h	EC_{50}	-	-	-	>100	-	-	-	-	-	-	-
		[24]	48 h	LC_{50}	-	-	-	>100	-	-	-	-	-	-	-
		[25]	48 h	EC_{50}	-	-	-	-	-	250	-	-	-	-	-
		[58]	24 h	LC_{50}	-	-	-	25.2	-	-	-	-	-	-	-
		[58]	24 h	EC_{50}	-	-	-	25.2	-	-	-	-	-	-	-
		[35]	48 h	EC_{50}	-	-	-	205.2	185.3	-	135.7	-	-	-	-
		[35]	96 h	EC_{50}	-	-	-	177.6	147.5	-	78.9	-	-	-	-
		[35]	48 h	EC_{50}-LVB	-	-	-	181	109.5	-	61.4	-	-	-	-
		[35]	96 h	EC_{50}-LVB	-	-	-	145.1	93.9	-	34.3	-	-	-	-
		[35]	48 h	EC_{50}-surviger	-	-	-	96.7	31.4	-	8.2	-	-	-	-
Crustacean	*Ceriodaphnia dubia*	[24]	48 h	EC_{50}	-	-	-	>100	-	-	-	-	-	-	-
		[24]	7 d	NOEC	-	-	-	2.50	-	-	-	-	-	-	-
		[24]	7 d	NOEC	-	-	-	0.25	-	-	-	-	-	-	-
		[61]	48 h	LC_{50}	-	-	-	15.51	-	-	-	-	-	-	-
		[61]	7 d	LC_{50}	-	-	-	0.21	-	-	-	-	-	-	-
		[61]	48 h	EC_{50}	-	-	-	15.51	-	-	-	-	-	-	-
	Thamnocephalus platyurus	[61]	24 h	LC_{50}	-	-	-	35.36	-	-	-	-	-	-	-
Rotifer	*Brachionus calyciflorus*	[24]	48 h	NOEC	-	-	-	>25	-	-	-	-	-	-	-
		[61]	24 h	EC_{50}	-	-	-	26.27	-	-	-	-	-	-	-
		[61]	48 h	EC_{50}	-	-	-	9.63	-	-	-	-	-	-	-
Invertebrate	*Artemia salina*	[67]	24 h	LC_{50}	-	-	1866	-	-	-	1866	-	-	-	-
		[67]	48 h	LC_{50}	-	-	851	-	-	-	851	-	-	-	-
		[67]	72 h	LC_{50}	-	-	537	-	-	-	-	-	-	-	-
		[67]	96 h	LC_{50}	-	-	19.5	-	-	-	-	-	-	-	-
	Moina macrocopa	[23]	24 h	EC_{50}	-	-	296.6	84.9	310.9	-	430.1	-	-	-	-
		[23]	48 h	EC_{50}	-	-	183.9	70.4	110.7	-	391.1	-	-	-	-
		[23]	8 d	LOEC	-	-	-	-	-	-	-	-	-	-	-
		[23]	8 d	NOEC	-	-	-	-	-	-	-	-	-	-	-
Fish	*Onchorynchus mykiss*	[60]	24 h	EC_{50}	-	-	-	***	-	-	-	-	-	-	-
		[60]	24 h	EC_{50} ache activity	-	-	108	-	-	-	-	-	-	-	-
		[67]	48 h	LC_{50}	-	-	-	-	-	-	>100	-	-	-	-
	Fish	[25]	96 h	LC_{50}	-	-	-	-	-	>1000	-	-	-	-	-
	Danio rerio	[24]	10 d	NOEC	-	-	-	>8	-	-	-	-	-	-	-
		[61]	96 h	LC_{50}	-	-	-	>1000	-	-	-	-	-	-	-
	Lepomis punctatus	[67]	24 h	EC_{50}	-	-	-	-	-	-	>100	-	-	-	-
	Lepomis macrochirus	[67]	48 h	EC_{50}	-	-	-	-	-	-	>100	-	-	-	-
	Salmo trutta	[67]	8 d	LOEC	-	-	-	-	-	-	>100	-	-	-	-
	Salvelinus fontinalis	[67]	8 d	NOEC	-	-	-	-	-	-	>100	-	-	-	-
	Salvelinus namaycush	[67]			-	-	-	-	-	-	>100	-	-	-	-
	Oryzias latipes	[22]	48 h	LC_{50}	-	-	>100	>750	>100	589.3	>500	-	-	-	-
		[22]	96 h	LC_{50}	-	-	>100	562.5	>100	535.7	>500	-	-	-	-
		[23]	48 h	LC_{50}	-	-	>500	-	>500	-	-	-	-	-	-
		[23]	96 h	LC_{50}	-	-	>500	-	>500	-	-	-	-	-	-

SPY, Sulfapyridine; SDZ, Sulfadiazine; SDM, Sulfadimethoxine; SMX, Sulfamethoxazole; SMT, Sulfamethazine; SCP, Sulfachloropyridazine; STZ, Sulfathiazole; SCT, Sulfacetamide; N^4acSMX, N^4-acetylsulfamethoxazole; N^4acSDM, N^4-acetylsulfadimethoxine; N^4acSDZ, N^4-acetylsulfadiazine; EC_{50} L = Median effective concentration (mg/L); LC_{50} = Median lethal concentration (mg/L); ED_{10} = Median effective dose (10% inhibition of microbial activity); ED_{50} = Median effective dose (50% inhibition of microbial activity); IC_{50} = Median inhibitory concentration (mg/L); NOEC = No-observed-effect concentration (mg/L); LOEC = Lowest-observed-effect concentration (mg/L); h = hours; min = minutes; d = days.
* = out of measured range.
** = milimol (mM).
*** = Not modellizable.

significantly more strongly than the anionic species. In this work, the EMEA guidelines were considered suitable for predictions regarding hydrophilic substances (e.g., SAs). The authors also studied the behavior of sulfachloropyridazine in two different soils (i.e. clay loam and sandy loam) to assess its potential to move from soil to surface waters and groundwaters. The solid-liquid distribution coefficient (K_d) values obtained were low, particularly under basic conditions. The results obtained from the clay field site supported these sorption studies and demonstrated that sulfachloropyridazine is highly mobile in soils and that, after application, it will be rapidly transported to field drains and ultimately enter surface waters. Finally, estimated PECs indicated that the SA was:

- non-persistent to moderately persistent in manure;
- moderately persistent to very persistent in sediments;
- moderately persistent to very persistent in water; and,
- non-persistent in activated sludge.

Schmitt et al. [42] investigated the pollution-induced community tolerance of bacteria in soils, which were spiked with sulfachloropyridazine. The tolerance effects were evaluated after three weeks' exposure. They observed an increase in the tolerance of bacterial inocula in soils with increasing SA concentration. Tolerance effects could be seen in the shifts in both the tolerance of the average of all metabolic activities and the sensitivity of each metabolic process individually. This could also be seen in smaller changes in the community-level physiological profile. Concentrations of 7 mg/kg triggered the first effects.

Sulfachloropyridazine was used again as a model compound to investigate the influence of time and nutrient status on pollution-induced community tolerance [43]. The antibiotic-induced tolerance of the microbial microcosms turned out to be greater if fresh pig slurry was applied as nutrient amendment. The effects of the SA could be seen after 7 days of exposure at 25°C. A longer soil exposure could increase the tolerance under certain nutrient conditions. It was not clear whether the sensitive species had disappeared through direct intoxication and more tolerant species had proliferated or simply the species had turned more resilient through physiological changes or genetic changes (e.g., acquisition of genetic material encoding for more resistance).

Generally, slowly degrading antibiotics can accumulate in the soil due to repeated application of the manure (i.e. during the growth period of the crops). Consequently, the antibiotic concentrations detected may not only be in the sub-therapeutic range but also reach the level of the minimum inhibitory concentrations for relevant bacterial species in the environment. Thiele-Bruhn et al. [37] added sulfapyridine and oxytetracycline to two different topsoils [i.e. Cambisol (sandy soil) and Luvisol (loamy soil)] in order to observe their effects on the microorganism communities. A nutrient substrate was simultaneously added to stimulate microbial activity and growth. Similarly to the results of Schmitt et al. [43] application of antibiotics together with a nutrient substrate turned out to be more environmentally relevant because antibiotics mostly reach the soils via manure, sludge and excreta from grazing livestock. Due to differences in soil organic matter, pedogenic oxides and clay minerals of both topsoils (2–3 times greater in Luvisol than in Cambisol), the calculated K_d of sulfapyridine in Luvisol was 3 times greater than in Cambisol. Correspondingly, the values for the effective doses resulting in 50% inhibition of microbial activity (ED_{50}) for this SA were about a factor of 2 greater in loamy Luvisol than in sandy Cambisol.

Kotzerke et al. [34] investigated the effects of manure containing different concentrations of antibiotic sulfadiazine on the microbial activities of Luvisol and Cambisol soils. By adding manure in both soils, with or without sulfadiazine, induction of microbial activity was observed through the increase in potential denitrification rates. However, both treatments in which sulfadiazine was added showed reduced denitrification rates. In the soils treated with sulfadiazine at 10 mg/kg, the antibiotic was under the LOD for the LC-MS^2 analysis 32 days after application, indicating that most of the compound was degraded or bound to the organic or mineral fraction of the soil, resulting in a bio-inactive form of the antibiotic. It is to be mentioned that these results were based on a single application of sulfadiazine, so cumulative effects were neglected. For example, it is possible that microbial populations that adapt will not be influenced by the SA after several applications.

In a similar study, Heuer et al. [44] mixed silt loam and loamy sand with manure containing sulfadiazine (10 mg/kg and 100 mg/kg) and compared them with untreated soil and manured soil without the SA over a 2-month period. In both soils, manure and sulfadiazine positively affected the appearance of sulfadiazine-resistant bacteria and also the frequency of transfer of plasmids conferring sulfadiazine resistance in soil bacteria and *Eschirichia coli* recipients.

The antibiotic potency of SAs could be diminished or changed when degradation takes place. Halling-Sørensen et al. [45] checked this possibility for the effects of sulfadiazine on bacteria from agricultural soil treated with manure. This SA, together with five other antimicrobials, was dissolved in environmentally-relevant matrices (e.g., soil interstitial water and sewage sludge under aerobic conditions). Samples were stored in the dark or exposed to light. Consistent with previous experiments [46], after 10-h exposure, the potency of sulfadiazine was reduced only 5.3% in the sludge experiment. This can be explained by the low K_d of SAs, which are not prone to sorb to solids. The potency of sulfadiazine was reduced by 56.2% and 38.1% in the

aerobic experiments in aqueous solution, which corresponded well with a similar reduction of the drug concentration.

4.2. Toxicity against plants as non-target soil organisms

It was recently demonstrated that, despite the low soil–water partition coefficient of SAs, which suggest a high mobility, fairly strong initial sorption of SAs to soil occurs under laboratory conditions and field experiments [39]. Temporarily, these sorbed amounts will be released to the soil solution and may be taken up by plants. Data regarding interaction of SAs with other soil macro-organisms (e.g., earthworms and insects) is lacking.

Because of their stability, these antibiotics are supposed to maintain significant residual activity and their potential toxicity in animal manure for long periods of time. After application of manure, SAs are very likely to reach surface waters and groundwaters. These contaminated waters may be used for irrigation and consequently taken up directly or indirectly by plants to meet their evapotranspiration and photosynthesis requirements [47]. Compounds with low octanol-water partition coefficient (K_{ow}) (e.g., SAs) are assumed to show low tendency to accumulate in cells and tissues. However, this consideration may not be completely true because K_{ow} is an accurate measure of chemical lipophilicity of a compound but not its biological lipophilicity, and K_{ow} does not take into consideration the life habits of the organisms under study (i.e. plants depend on water to survive). Therefore, K_{ow} may also indicate easy transfer between root and foliage and delivery in the constitutive parts of the plant.

Migliore et al. [35,48,49] evaluated the effects of sulfadimethoxine individually on different terrestrial plants [e.g., crop plants (*Hordeum distichum* L., Poaceae, Liliopsida, (barley seeds), *Panicum miliaceum*, *Pisum sativum* and *Zea mays*) [35,48] and cosmopolitan weeds (*Amaranthus retroflexus* L., *Plantago major* L. and *Rumex acetosella* L.)] [49]. Both toxicity and bioaccumulation were studied under laboratory conditions (in vitro tests) for all of them and also directly in soils for the barley seeds. At a concentration of 300 mg/L, sulfadimethoxine affected the post-germinative development of all the plants. These effects were observed in the barley plants on both synthetic medium and soils with different organic contents. These plants also showed high accumulation rates in soil but mostly in the synthetic medium, with bioaccumulation ratios (roots/foliage) in the range 3.30–4.40 for soils with different organic contents and 1.61 for the synthetic medium. For *Panicum miliaceum*, *Pisum sativum* and *Zea mays*, sulfadimethoxine was also toxic and the ratios between root and foliage bioaccumulation were 2-fold for *Pisum* and 20-fold for *Panicum* and *Zea*. Regarding the three weeds investigated, wet weight was lower in treated plants than in control plants for all of them, so sulfadimethoxine acted similarly to the two cases mentioned above.

Forni et al. [50] studied the toxicity of sulfadimethoxine in plants belonging to *Azolla sp*. and its efficiency in drug removal. Media with this SA at concentrations in the range 50–450 mg/L were prepared, and plant growth and morphology were observed. The alterations were obvious and increased with the concentration, but the plant survived to 5 weeks' treatment. After this period, a 56.3% and 88.5% of the total amount of sulfadimethoxine was degraded (media with 50 mg/L and 450 mg/L, respectively), proving the removal capability of this plant.

5. Toxicity of sulfonamides in aquatic ecosystems

Whether or not SAs are biodegraded in the aquatic environment would settle the very first step for a complete environmental risk assessment. It has already been demonstrated that SAs resist biodegradation in wastewater-treatment plants (WWTPs) and even in media with high microbial activity (activated sludge) [8,18,51,52]. Wastewater bacteria could be considered the first organisms to be potentially affected by antibiotics and SAs from human-medicine treatments. In order to check this possibility, growth-inhibition tests were carried out by Al-Ahmad et al. [53] for sulfamethoxazole together with other antibiotics. While no inhibition was measured, the colony-forming units monitoring revealed high toxicity for this SA.

Bacteria cultured from a sewage bioreactor and receiving waters from a WWTP were tested for resistance against six antibiotics [54]. The mixture sulfamethoxazole and trimethoprim was included, due to its very common use in veterinary medicine. Four types of bacteria were isolated from the bioreactor (three strains of *E. coli* and an unidentified bacterium), and two bacteria strains were isolated from the WWTP receiving waters (*E. coli* and *Xanthomonas maltophilia*). Of all of them, only the unnamed strain showed resistance against the mixture of sulfamethoxazole and trimethoprim.

Goñi-Urriza et al. [16] also evaluated the activity of some antibiotics on 138 different strains of *Aeromonas* spp., typical waterborne bacteria, isolated from two rivers. Sulfamethoxazole turned out to be one of the less active against these species, with a minimum inhibitory concentration (MIC) of 256 mg/L for 37 of the strains and >512 mg/L for 36 of the strains. In a different study by the same authors [15], 110 strains of *Enterobacteriaceae* (representative of the human and animal flora) and 118 of *Aeromonas* were selected for antibiotic-susceptibility testing. The most common resistances were to

quinolones, tetracyclines and β-lactams, and no significant mention was made of the SA group.

As mentioned above, SAs, due to their physicochemical properties including great mobility in the environment, have been detected in all kinds of water matrices [18]. While they are probably not pharmacologically active in humans at the concentrations detected so far (usually at the ng/L level, [28]), they might be potential micropollutants to key living organisms in aquatic ecosystems (e.g., fish, aquatic invertebrates and unicellular algae). These different taxonomic groups, belonging to different trophic levels, may be exposed and negatively affected to different extents. For example, severe toxic effects in primary producers may imply loss of the whole food-chain structure, as they represent a significant portion of the total biomass of the ecosystem and are important as a source of carbon for the rest of the aquatic biosphere. Table 2 provides an overview of the estimated values for the most usual toxicity indicators found in the literature.

Despite the lack of toxicity data available in the literature, it has been demonstrated that microalgae are more sensitive than crustaceans and fish to antibacterial agents (e.g., triclosan and ciprofloxacin). On these premises, Yang et al. [55] investigated the toxicity against freshwater green alga *P. subcapitata* of, among others, two SAs (i.e. sulfamethazine and sulfamethoxazole). Since, in the aquatic environment, antibiotics are not detected as isolated drugs but together with other similar compounds, the authors studied algal-growth inhibition in the presence of individual antibiotics, mixtures of 12 antibiotics (including the two SAs) and different binary mixtures. Test concentrations were 0.001–10 μg/L. SAs individually were the least toxic of all the antibiotics studied (i.e. macrolides, tetracyclines, fluoroquinolones, triclosan and trimethoprim). The median values of inhibitory concentration (IC_{50}) for SAs were much higher than those expected in surface waters or WWTP effluents (Table 2).

SAs were therefore considered unlikely to be toxic to algal growth at concentrations reported in fresh water. Synergistic effects were observed for the binary mixtures of SAs. Belonging to the same family of compounds implies similar molecular structure and modes of action, so "concentration addition" was to be expected. These effects were also observed for the binary mixtures of trimethoprim and SAs, commonly used in veterinary medicine, but also with the mixture of sulfamethazine and norfloxacin.

It is necessary to take into account that the degradation products of SAs may also be involved in the final toxic effects on the algae, making the interpretation of the toxic data more complex. Eguchi et al. [56] performed growth-inhibitory tests with sulfadimethoxine, sulfamethoxazole and sulfadiazine on freshwater green algae *Selenastrum capricornotum* and *Chlorella vulgaris*, also considering the synergetic effects of combined drugs of the parent compounds together with trimethoprim and including the metabolism products of the SAs. As acetylation has largely been proved to be the main metabolic route for SAs, mixtures of these together with the corresponding acetylated products were prepared at a ratio in line with the concentrations detected in the urine of medicated pigs. The IC_{50} values for individual SAs may indicate a certain risk to green algae, whereas their acetylated products were found to have a much weaker growth-inhibitory effect than the original substance. The addition of trimethoprim enhanced the inhibitory activity of sulfamethoxazole and sulfadiazine. As expected, the presence of the corresponding acetylated metabolites also enhanced the inhibitory effect of the three SAs plus trimethoprim on the algae, but to a lesser extent than addition of trimethoprim alone.

No toxicity was observed in a study by Pro et al. [57], in which the effects of sulfachloropyridazine on unicellular algae *Chlorella vulgaris* were studied. The end-point selected in this study was the total number of green fronds.

In a previous study by Lützhoft et al. [58], sulfadiazine was shown to inhibit chlorophyll content in blue-green algae *M. aeruginosa* and *S. capricornutum*, with EC_{50} values of 135 mg/L and 7.8 mg/L, respectively. The EC_{50} value of chlorophyll *b* for sulfamethoxazole was 682 mg/L, although sulfamethoxazole was the most toxic of all compounds tested with respect to wet mass, with an EC_{50} of 80 mg/L.

Besides algae, there are typical indicator species for toxicity at the different trophic levels. The most commonly used are marine luminescent bacterium *Vibrio fischeri*, freshwater zooplankton invertebrates *Daphnia magna* and *Moina macrocopa*, and fish Japanese medaka (*Oryzias latipes*). Recently, Kim et al. [59] carried out a toxicity study for five SAs (i.e. sulfamethoxazole, sulfachlorpyridazine, sulfathiazole, sulfamethazine and sulfadimethoxine) in the aquatic environment in Korea following the EMEA guidelines with the reference species mentioned above. None of the SAs were considered acutely toxic for any of the species tested, with the exception of sulfachloropyridazine and sulfamethoxazole, which showed acute median effective concentrations (E/LC_{50}) <100 mg/L for *V. fischeri*. When calculating PECs and PNECs, sulfamethoxazole showed the highest value of 0.95 μg/L for PEC and the lowest, 0.15 μg/L, for PNEC, yielding an HQ of 6.3, thereby suggesting potential ecological risks. These values were calculated considering only SAs used in human medicine, so there may be an underestimation of the parameters used, given the high consumption of SAs in veterinary medicine. Besides, another study showed that sulfamethoxazole lacked any cytotoxic effect in fish [60]. Table 3 summarizes the HQ values reported in the literature to date. Sulfamethoxazole, as one of the most

Table 3. Estimations of the environmental hazard posed by sulfonamides

Sulfonamide	MEC	PEC (μg/L)	PNEC	HQ	Comments	Ref.
Sulfamethoxazole		0.95	0.146	6.3		[22]
	0.4		0.03	13.4	Chronic toxicity MEC/PNEC ratio	[23]
		0.31	0.027	11.4	Acute toxicity test on surface waters in France	[24]
		0.31	0.59	0.52	Chronic toxicity test on surface waters in France	[24]
		1.6	0.027	59.3	Acute toxicity test on surface waters in Germany	[24]
		1.6	0.59	2.72	Chronic toxicity test on surface waters in Germany	[24]
				0.08*	Frond number	[27]
				0.2*	Fresh weight	[27]
				2.98*	*p*ABA	[27]
				0.01**	Frond number	[27]
				0.03**	Fresh weight	[27]
				0.56**	*p*ABA	[27]
				<1	MEC/PNEC ratio	[58]
Sulfathiazole	0.13		0.1	1.3	Chronic toxicity MEC/PNEC ratio	[23]
Sulfamethazine	0.2		110.3	0.002	Idem	[23]
Sulfadimethoxine	0.07		0.248	0.282	Idem	[23]
Sulfachloropyridazine	590	860			Soil pore water estimation	[25]

MEC = Measured environmental concentration (μg/L); PEC = Predicted environmental concentration; PNEC = Predicted no-effect concentration; HQ = Hazard quotient.
*= Based on the USFDA guidelines.
**= Raw values based on the USFDA guidelines.

consumed SAs in human medicine, has been reported most frequently and is usually considered ecologically harmful.

Taking into account the limitations mentioned above, Kim, Park and Choi [23] evaluated acute and chronic aquatic toxicities for sulfamethoxazole, sulfathiazole, sulfamethazine and sulfadimethoxine. Only sulfamethoxazole and sulfathiazole were considered of environmental concern. Sulfamethoxazole showed the most toxic effects in the acute toxicity test on *M. macrocapa* (54.8 mg/L and 70.4 mg/L). Sulfathiazole showed toxicity on *D. magna* but only after exposure for more than 48 h. With the toxicity data produced in this study and the measured environmental concentrations (MECs) reported in the literature, HQs were estimated and values >1 were obtained for these two SAs.

Isidori et al. [61] evaluated the ecotoxicity of six antibiotics (i.e. sulfamethoxazole representative of the SAs group) on non-target organisms. Acute toxicity tests were carried out on aquatic reducers, primary and secondary consumers [namely, *V. fischeri* (bacterium), *Brachionus calyciflorus* (rotifer), *Thamnocephalus platyurus* (crustacean anostaca), *D. magna* and *Ceriodaphnia dubia* (crustacean cladocera) and *Danio renio* (teleostei, cyprinidae)]. Chronic toxicity tests were performed on *P. subcapitata, B. calyciflorus and C. dubia.* and also mutagenesis/genotoxicity testing (AMES test) on *Salmonella typhimurium* and *E. coli* strains. In this study, data for acute toxicity tests were one order of magnitude lower than the data of Migliore [62]. EC_{50} values for algae and crustaceans were also quite relevant, 0.52 mg/L and 0.21 mg/L, respectively, indicating that the risk posed by this substance should not be excluded. The ecological risk for the aquatic environment was finally estimated following the EMEA guidelines, and sulfamethoxazole yielded HQ <1.

Abiotic factors (e.g., UV irradiation) may also be influential in the potential ecotoxicity of SAs. Photo-enhanced toxicity has been previously studied for polycyclic aromatic hydrocarbons (PAHs) and, to a lesser extent, for metals (e.g., copper, arsenic, and zinc) ([36] and refs. therein). These phototoxic effects would involve the generation of reactive oxygen species that interact with important macromolecules (e.g., DNA, proteins and lipids), resulting in the destruction of their structures and functions.

Jung et al. [38] studied the phototoxicity of sulfathiazole, sulfamethoxazole and sulfamethazine under four different lighting conditions [namely, no UVB, continuous UVB (96-h exposure), pulsed UVB (96-h exposure) and natural sunlight (48-h exposure)]. *D. magna* was the indicator species used in an acute immobilization toxicity test. The stability of the tested SAs under UV radiation was first investigated. Photo-enhanced toxicity was greatest under natural sunlight (likely because of the contribution of UVA light) followed by pulsed UV exposure, continuous UV exposure, and finally, fluorescent light only. Under natural sunlight, the toxicity of SAs increased up to 16.5-fold. Photo-enhanced toxicity was greatest for sulfathiazole (7.8-fold increase), even though this compound was the most vulnerable to photodegradation (39% degradation of the parent compound after 48-h exposure). This suggested that the photodegradation of the parent compound leads to the formation of toxic by-products. Another conclusion was that, compared to continuous UVB irradiation,

pulsed UVB irradiation resulted in greater phototoxicity in *D. magna*, with the dose of UVB being less important than the mode of irradiation. However, the concentrations of SAs that were acutely toxic to *D. magna*, even under sunlight, were much higher than levels detected in the environment [8,10,13] and the ecological risks associated were considered to be limited.

SAs undergo photocatalytic degradation [63,64], and the intermediate by-products should also be taken into account when evaluating their ecotoxicity. If the photodegradation products generated are biodegradable, they can be removed during wastewater treatment using biological methods. If they are persistent or not readily biodegradable, risks of ecotoxicity should be considered. On these premises, Baran et al. [36] carried out toxicity tests on algae *Chlorella vulgaris* to evaluate the effects of sulfacetamide, sulfathiazole, sulfamethoxazole and sulfadiazine and their respective photodegradation products. The results demonstrated that the four SAs were toxic for this algae (see Table 2) and the degradation products generated had both inhibitory and stimulatory effects on the algae cultures. For example, sulfadiazine-degradation products showed only inhibitory effects, whereas the derivatives of the other SAs showed both inhibitory and stimulatory effects. Nevertheless, the main conclusion of this study was that growth inhibition was lower after UV illumination than before, meaning that degradation products were less toxic than the parent compounds.

Brain et al. [65] assessed the phytotoxicity of sulfamethoxazole, sulfadimethoxine and sulfamethazine together with other antibiotic substances for aquatic higher plant *Lemna gibba* in one of the first studies dealing with this trophic level. Antibiotics in general are known to have antichloroplastic properties due to the cyanobacterial nature of the plastids, being consequently susceptible as potential antibiotic targets [66]. To measure phytotoxicity, these authors used wet mass, number of fronds, chlorophylls *a* and *b*, and carotenoids. A 7-day test was performed, and plants were treated with 0–1 g/L of pharmaceutical-containing growth media. The three SAs, with the exception of sulfamethazine, were all found to be highly phytotoxic to *L. gibba*. Sulfamethoxazole turned out to be the most toxic, followed by sulfadimethoxine and sulfamethazine. Growth inhibition was only clear on day 4 of the test for sulfamethoxazole in growth media of 100 mg/L.

In a later study, *Lemna gibba* was exposed only to sulfamethoxazole to measure the phytotoxic potency of this drug [33]. The feasibility of *p*-aminobenzoic acid (*p*ABA) as a biomarker of the negative effects was evaluated, and its concentration was measured as a response to increased exposure concentrations of sulfamethoxazole, and linked to effects at the morphological level. As mentioned above, SAs act as structural analogues of *p*ABA, inhibiting the biosynthetic pathway of folate. Folic acid is an essential molecule for the nucleic-acid-synthesis precursors (see Fig. 4). As ex-

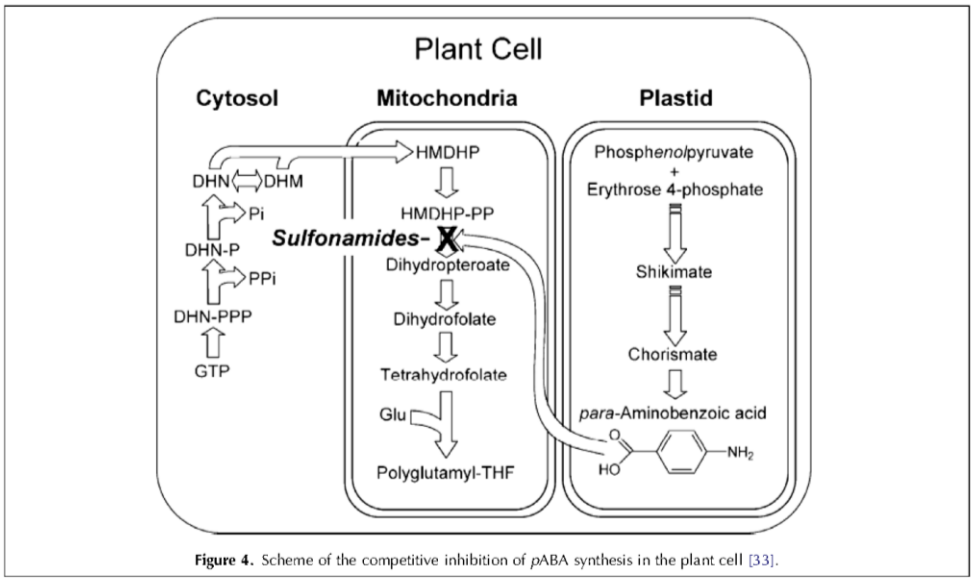

Figure 4. Scheme of the competitive inhibition of *p*ABA synthesis in the plant cell [33].

pected, *p*ABA levels increased upon exposure because SAs act as structural analogues of *p*ABA. The EC_{50} value estimated for *p*ABA (causing a 50% increase in the total *p*ABA content) was only 3.36 μg/L, 20 times lower than that of fresh weight (61.6 μg/L) and 40 times lower than frond number (132 μg/L) responses. It was concluded that *p*ABA could be considered as a highly sensitive biomarker of the effect of SAs. Due to several complications, folates turned out to be unsuitable for this purpose.

The toxic effects of sulfadimethoxine on brine shrimp *Artemia* were evaluated at two different life stages (nauplii and cysts) and at different concentration levels (75–1200 mg/L) [67]. The organisms showed a high sensitivity against this SA in both stages and, besides, bioaccumulation was also observed, with the potential implications for the rest of the food chain in the marine community.

Recently, Laville et al. [60] gave an overview on the toxicological potency of nine human pharmaceuticals, sulfamethoxazole being one, on fish as non-target species. Rainbow trout was the species studied and, due to the relevance of the liver in detoxification, two complementary hepatocyte models were tested. The cytotoxicity test (MTT assay) revealed that sulfamethoxazole appeared to be cytotoxic only at the highest tested concentration. Ethoxyresorufin-*O*-deethylase (EROD) activity, a highly sensitive indicator of contamination in fish, was inhibited from a concentration of 125 μM for the SA, but it was not cytotoxic enough to calculate EC_{50} values. Sulfamethoxazole did not produce any reactive oxygen species either.

Hou et al. [68] studied sulfamethazine bioconcentration and elimination in sturgeon (*Acipenser schrenkii*). Two groups of fish were exposed to concentrations of 1 mg/L and 0.10 mg/L of the SA, respectively, under flow-through aqueous conditions during an 8-day period. Rapid uptakes were observed in both groups. A bioconcentration factor was estimated for the muscle tissue of the fish, which was 1.19 for the low-concentration treatment level and 0.61 for the high-concentration treatment. Both values were considered to be of little environmental concern and the drug was therefore not expected to bioconcentrate in tissues consumed by humans or to biomagnify in fish consumed by fish predators. Similar results were found by Coats et al. in mosquito fish [69].

6. Conclusions

SAs are one of the most common water-polluting antibiotics. To date, little is known about the ecological risks posed by the presence of SAs in the different environmental compartments. Although designed to have specific effects on specific organisms, SAs have very high excretion rates and, as they are very mobile once released into the environment, they may affect many non-target organisms. As their presence in natural media is not as a single, isolated drug but usually with other compounds of the same family or the same type (antibiotics or pharmaceuticals, in general), accumulated concentrations or synergistic-antagonistic effects need to be considered. Besides, biotic and abiotic transformation products of the SAs also need to be taken into consideration when studying the fate and the effects of these antibiotics in the different ecosystems, as they may lead to a more complex environmental risk assessment. Potential toxicological implications have been investigated and reported in a few publications and toxicity effects have been proved for some SAs, but at test concentrations far higher than those expected in the environment.

Future studies need to consider more realistic environmental exposure scenarios. Approaches combining ecotoxicity tests and analytical methodologies should be applied to highly selective and sensitive determination of the target compounds and identification of potential ecotoxicological effects.

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1.5 PROPIEDADES FISICOQUÍMICAS DE LAS SULFAMIDAS

Las Figuras 1.8 y 1.9 presentan las dieciseis sulfamidas y cinco de sus metabolitos acetilados investigados en esta Tesis doctoral. Todas las sulfamidas son derivados de la sulfanilamida, que se caracteriza por tener libre el grupo amino en posición *para* y un grupo sulfona (-SO_2-) unido al anillo bencénico. Variando los grupos funcionales en la posición N^1 se obtienen un gran número de compuestos con diferentes propiedades farmacológicas. Se trata de compuestos muy polares y solubles que se comportan como ácidos débiles. Poseen dos valores de pK_a y su punto isoeléctrico se encuentra entre pH 4-5; a pHs bajos se convierten en especies catiónicas, ya que se protona el grupo amino (pK_a1); a pHs más altos su grupo amida pierde un protón (pK_a2), mientras que son especies neutras a pHs ligeramente ácidos. La Tabla 1.8 resume las principales propiedades físico-químicas de las sulfamidas estudiadas.

Tabla 1.8. Propiedades físico-químicas de las sulfamidas estudiadas más frecuentemente.

SULFAMIDA	Log P	pk_{a1}	pk_{a2}	K_{OC} pH 3	K_{OC} pH 7	K_{OC} pH 9
Sulfametizol	0.5	5.3	1.8	42.6	1.65	1
Sulfapiridina	0.54	8.8	3	22.4	23.4	15.4
Sulfadimetoxina	1.05	6.1	2.6	67.8	1.96	1.53
Sulfametazina	0.32	7.2	3.1	64.5	56.5	4.55
Sulfisomidina	0.4	7.7	2.6	1	1	1
Sulfabenzamida	1.53	4.7	1.8	102	2.13	1.06
Sulfacetamida	-0.75	5.3	1.8	7.01	1	1
Sulfadoxina	0.64	32.8	1	32.8	1	1
Sulfaquinoxalina	1.54	5.8	1.8	118	21	1.46
Sulfatiazol	0.69	7.5	2.3	21.4	16.1	1
Sulfametoxazol	0.84	5.81	1.39	68.6	9.6	1.72
Sulfisoxazol	1.01	4.9	1.8	79.8	4.47	1
Sulfadiazina	-0.07	6.5	2.1	20	7.51	1
Sulfametoxipiridazina	0.45	7.4	2.4	6.87	11.8	1
Sulfamerazina	0.11	7.2	2.1	35.2	24.2	1.05
Succinilsulfatiazol	0.19	4.6	2	0.59	51	1
N^4-acetilsulfametoxazol	1.02	5.1	-1.1	152	14.5	2.93
N^4-acetilsulfapiridina	0.82	8.2	2.4	50.9	47.9	5.72
N^4-acetilsulfadiazina	-0.77	5.9	1.1	39.6	5.93	1
N^4-acetilsulfametazina	-0.4	6.6	2.5	126	86.6	3.92
N^4-acetilsulfamerazina	-0.59	6.6	1.1	70.4	26.1	1.1

Log P:; pKa:; Koc: coeficiente de distribución octanol-agua;

Suelen ser clasificadas como sustancias no termolábiles y resistentes a la biodegradación. Su reactividad y coeficientes de partición son pH-dependientes, lo cual debe ser tenido en cuenta a la hora de evaluar y predecir su comportamiento en condiciones ambientales y también en el momento de llevar a cabo su determinación analítica.

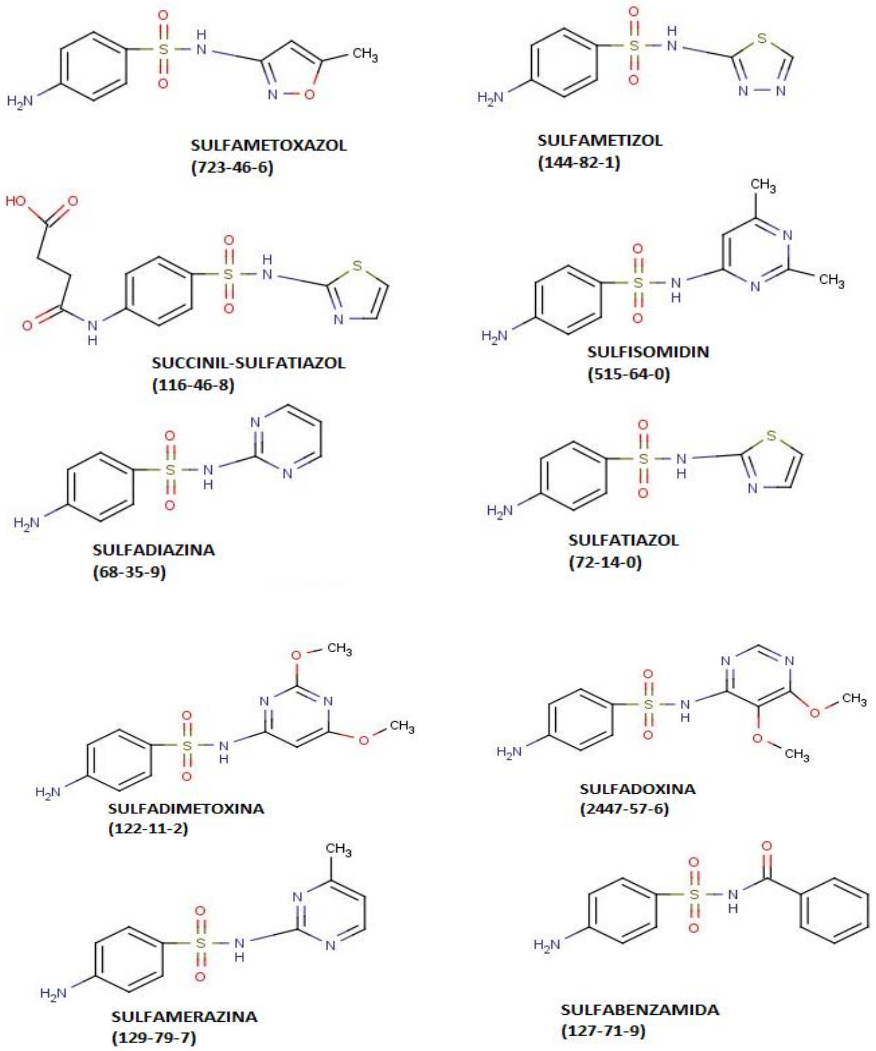


Figura 1.8. Estructura molecular de las sulfamidas estudiadas y sus respectivos números de registro (CAS).

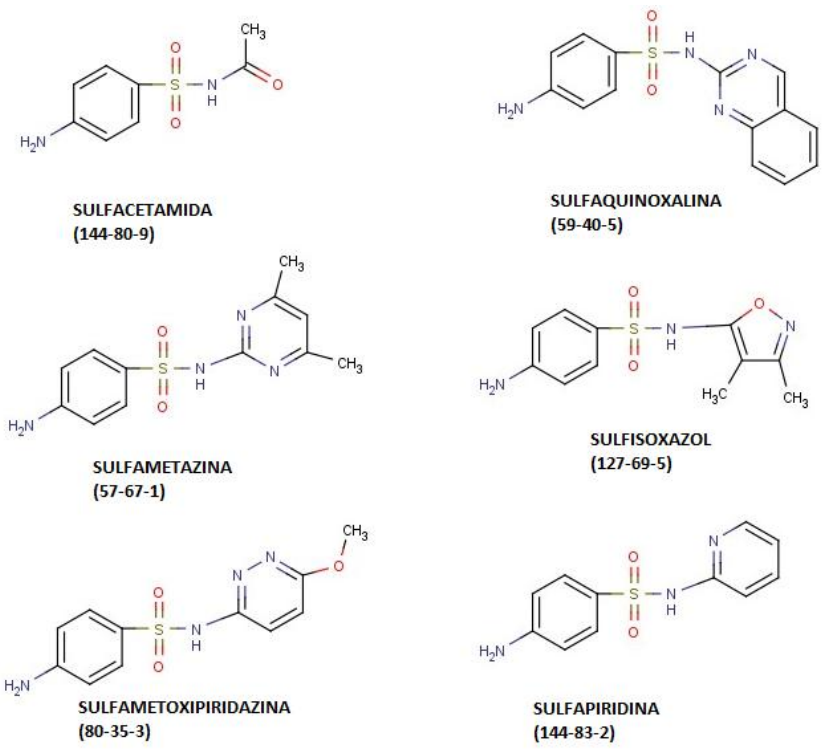


Figura 1.8 (continuación).

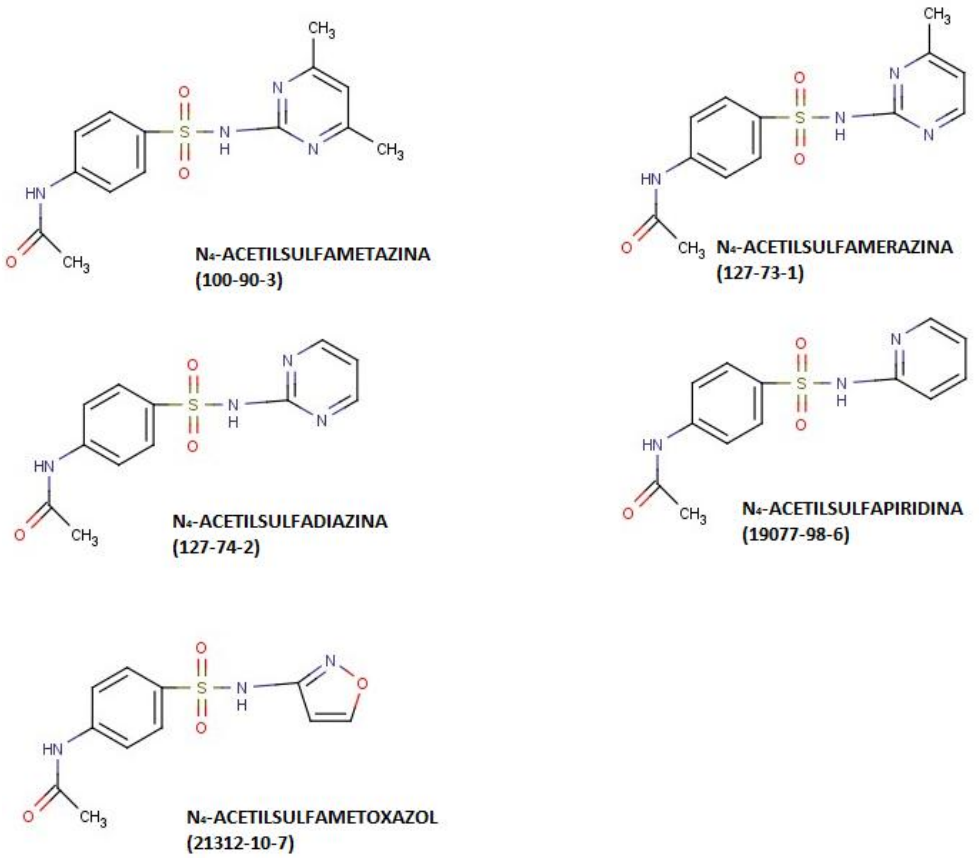


Figura 1.9. Estructura molecular de los metabolitos acetilados estudiados y sus respectivos números de registro (CAS).

1.6. ANÁLISIS DE SULFAMIDAS EN EL MEDIO AMBIENTE

El creciente interés durante los últimos años por la presencia medioambiental de las sulfamidas y otros antibióticos ha sido el principal motor del gran avance de las metodologías analíticas para su determinación. Su desarrollo engloba diferentes etapas, desde la toma de muestra hasta el análisis instrumental en el laboratorio. La mejora de la sensibilidad de los nuevos equipos ha permitido alcanzar límites de detección muy bajos (pg L^{-1}), obteniendo así gran cantidad de información sobre presencia y niveles medioambientales que antes eran

inaccesibles. Las técnicas de cromatografía de líquidos (LC) acopladas a espectrometría de masas (MS) son las más usadas para la detección de sulfamidas en las diferentes matrices medioambientales, precedidas por diferentes protocolos de toma de muestra y pretratamiento.

1.6.1. Toma de muestras ambientales y conservación

Al plantear un estudio de presencia medioambiental de contaminantes, se han de considerar las características tanto del medio a investigar como de los compuestos de interés. En primer lugar, el tipo de sistema ambiental (suelo, río, aguas subterráneas, aguas residuales) condiciona las características del muestreo. Generalmente, la toma de muestras de agua superficial y subterránea se realiza de manera puntual y con una periodicidad que dependerá de los objetivos del estudio. Estas muestras proporcionan información instantánea de la carga de contaminantes en el punto de muestreo, sin considerar las posibles fluctuaciones del sistema. Es un tipo de información no extrapolable y poco representativa a no ser que el muestreo se realice con una periodicidad lo suficientemente amplia para que los datos recogidos sean consistentes (por ejemplo, repitiendo el muestreo durante días sucesivos o semanas o cubriendo períodos relevantes desde un punto de vista hidrológico, como épocas de sequía y épocas de lluvia en un mismo año). El conocimiento de las vías de entrada del contaminante al medio natural facilita la elección de los puntos de muestreo más relevantes. En el caso de las sulfamidas, como se comentó en la introducción, localizaciones aguas abajo de las zonas de vertido de las EDAR o cercanas a campos de cultivo o ganadería son las más críticas en cuanto a carga de contaminantes en aguas superficiales y subterráneas, y también en sedimentos y suelos. Para la toma de muestra, pueden utilizarse puentes y otras infraestructuras que permitan el muestreo en la zona media del cauce, o bien pozos para aguas subterráneas.

Por el contrario, para aguas de entrada y salida de EDAR las muestras suelen tomarse de manera integrada durante 24 horas, recogiendo la misma cantidad de muestra a intervalos determinados y mezclando todas ellas en el mismo recipiente. Ya que estos estudios de presencia en EDARs suelen ir acompañados de la estimación de tasas de eliminación durante el tratamiento, es imprescindible, para una interpretación coherente de los resultados, que las muestras de salida se correspondan con las de entrada y, por tanto, parámetros del funcionamiento de la EDAR como el tiempo de retención hidraúlico (HRT) deben ser

considerados. También se han de considerar las variaciones estacionales en los caudales de entrada o acontecimientos puntuales como inundaciones y desbordamiento de los tanques, errores de funcionamiento de la planta y también externos como vertidos de industrias cercanas.

Para el transporte de las muestras líquidas se han de utilizar recipientes que no alteren la composición de la muestra, generalmente vidrio o polietileno tereftalato (PET) ambar (para evitar una posible fotodegradación) previamente lavadas con agua y disolvente orgánico y secadas en una estufa. Se transportan en frío (4 ºC) hasta el laboratorio, donde se filtran a través de discos de fibra de vidrio o nylon de hasta 0.45 µm de diámetro de poro y, en caso de no ser analizadas inmediatamente, son congeladas para su conservación. Las muestras sólidas (fangos de depuradora, suelos o sedimentos) suelen ser recogidas como muestras puntuales. Las muestras de suelos se limitan a los primeros 3-5 cm de suelo, recogidos con palas o herramientas similares. Los sedimentos se recogen con una draga en la parte más interior del lecho del río como sea posible en función de la accesibilidad y medios disponibles (ancho del cauce, profundidad del río, utilización de barcas) ya que estará sometida a menos turbulencia que los márgenes y tendrá lugar una mayor deposición de los materiales en suspensión. Las muestras se transportan en frío en recipientes de aluminio o vidrio, y una vez en el laboratorio, en función de su grado de humedad, se centrifugan para eliminar el agua retenida en la muestra. Una vez retirado el sobrenadante, la materia sólida se liofiliza para eliminar el agua intersticial y finalmente se congela para su conservación. Previamente a su análisis, se trituraran o tamizaran para homogeneizar y conseguir el menor tamaño de particulado posible, facilitando así el pasó y el contacto de disolvente con la muestra durante la extracción.

1.6.2 Pretratamiento de muestra

El pretratamiento de la muestra es fundamental para obtener una método analítico robusto y óptimo. Se estima que el mayor tiempo medio invertido de todo el método analítico es el empleado en el pretratamiento (más del 50% del tiempo total de análisis). Sin embargo, una manipulación excesiva de la muestra durante esta etapa puede constituir una fuente de error potencial, mayor cuanto más extenso y laborioso sea el pretratamiento. La tendencia actual en química analítica es, por tanto, reducir la manipulación o el número de etapas de pretratamiento de las muestras, ahorrando tiempo y utilización de recursos.

1.6.2.1 Extracción en fase sólida (SPE) de muestras líquidas

Las bajas concentraciones ambientales a las que se encuentran las sulfamidas y la complejidad de las matrices ambientales requieren que, de forma previa a su análisis, las muestras líquidas y los extractos de muestras sólidas sean preconcentrados. La extracción en fase sólida (SPE) es sin duda el procedimiento de purificación y preconcentración más utilizado en los estudios de presencia ambiental en aguas de estos antibióticos debido principalmente a la sencillez del procedimiento y a su bajo coste. La SPE consiste en pasar un volumen de muestra, previamente optimizado, de muestra a través de un lecho de material adsorbente contenido en un cartucho de plástico o vídrio. La SPE de fase inversa, con la fase estacionaria apolar y para muestras polares, es la más utilizada en estudios de presencia ambiental de sulfamidas. La Tabla 1.9 compila las diferentes metodologías analíticas para el análisis de sulfamidas en agua desarrolladas durante los últimos 10 años. De estos trabajos, sólo 3 se dedican exclusivamente a la familia de la sulfamidas (Malintan *et al.* 2006; Perret *et al.* 2006; Jia *et al.* 2011), mientras que el resto se desarrollaron para el análisis de diferentes familias de antibióticos y de fármacos. Como muestra esta tabla, el adsorbente más comúnmente utilizado es el copolímero de divinilbenzeno (lipofílico) y N-vinilpirrolidinona (hidrofílico), más conocido por su nombre comercial Oasis HLB®, material que ofrece un balance entre ambas fases apto para la retención de sustancias polares y apolares en un rango muy amplio de pH. Estos cartuchos se han utilizado tanto en estudios específicos para sulfamidas como para estudios multiresiduo de fármacos en los que éstas estaban incluidas. En diferentes trabajos se han utilizado otros polímeros de propiedades parecidas, como Strata X® (Phenomenex), o adsorbentes más clásicos como los de sílice modificada (octadecil C_{18} y octil C_8), y ocasionalmente materiales de intercambio iónico, más específicos para la extracción de compuestos susceptibles de ionización (p.e. SAX® de Supelco, Oasis MCX® de Waters). La extracción en tandem, utilizando dos tipos diferentes de cartuchos acoplados, también se ha llevado a cabo para el análisis de sulfamidas en muestras con mucha materia orgànica u otros interferentes y que requieren una purificación más intensa, aunque como muestra la Tabla 1.9, sólo el agua superficial ha sido extraida mediante este procedimiento. Así Luo *et al*. (Luo *et al.* 2011) extrajo muestras de agua superficial acoplando cartuchos SAX de intercambio iónico con

Tabla 1.9. Métodos analíticos para el estudio de la presencia medioambiental de las sulfamidas y otros antibióticos referenciados en la literatura en los últimos 10 años (los métodos multiresiduo han sido excluidos por brevedad.)

MATRIZ	V (mL)	SPE	COLUMNA LC CONDICIONES LC	DETECCIÓN MS/MS	REF
Agua superficial Agua mineral	1000 250	Oasis HLB (6 mL/500 mg) Elución: 10 mL MeOH + 50mM HCOOH	HPLC Alltech Alltima C_{18} 150 x 2.1 mm, 5 µm A: ACN + 1 mM HCOOH B: Agua + 1 mM HCOOH	ESI+ MS/MS (QqQ)	(Perret *et al.* 2006)
Agua residual	150	Oasis HLB Elución: 5 mL MeOH + NH_3 (19:1)	HPLC Supelcosil C_{18} 250 x 4.6 mm, 5 µm A: Agua + 0.5% HCOOH B: ACN	-	(Malintan and Mohd 2006)
Agua superficial Agua de mar	500 2000	Na_2EDTA 0.5 g L^{-1} Oasis HLB 500 mg/6 mL Elución: 6 ml DCM/MeOH (2:1, v:v) SPE adicional para agua superficial Sep-Pak Silica (500 mg/3 ml)	UPLC ACQUITY BEH C_{18} 100 X 2.1 mm, 1.7 µm 40 ºC A: MeOH B: Agua + 0.1% HCOOH	ESI+ MS/MS (QqQ)	(Jia *et al.* 2011)
Agua superficial	120	Oasis HLB 60 mg/3 mL Elución: 5 mL	HPLC Xterra MS C_{18} EC 50 x 2.1 mm,2.5 µm. 15 ºC Precolumna A: Agua + 0.1% HCOOH (pH 2.74) B: ACN + 0.1% HCOOH	(ESI+)-IT-MS	(Kim and Carlson 2007; Kim and Carlson 2007)
	500	pH 3 H_2SO_4 Na_2EDTA (0.5 mg) SPE en tandem: Oasis HLB-Oasis MCX, 200 mg, 6 mL	LC Luna C_{18} 150 x 3 mm, 3 µm. 50 ºC A: Agua +20 mM CH_3COONH_4/ACN (90/10) B:ACN	(ESI+)-MS	(Meyer *et al.* 2007)

Tabla 1.9 (continuación).

MATRIZ	V (mL)	SPE	COLUMNA LC CONDICIONES LC	DETECCIÓN MS/MS	REF
Agua residual	120	Na_2EDTA-ácido cítrico pH <3 (H_2SO_4) Oasis HLB 60 mg/3 mL Elución: 5mL MeOH	HPLC Xterra MS C_{18} 50 x 2.1 mm, 2.5 µm. 15 ºC A: Agua, 0.1% HCOOH B: ACN	(ESI+)-IT-MS	(Yang *et al.* 2005)
Agua superficial Agua residual[1]	10	Online-SPE Oasis HLB online	HPLC Luna C_{18} 150 x 3 mm, 3 µm A: Agua + 0.3% HCOOH B: ACN	(ESI+)-MS/MS	(Meyer *et al.* 2007) (Chang *et al.* 2010)[1] (Watanabe *et al.* 2010)
Agua superficial	100	pH 7(H_3PO_4) Oasis HLB (60 mg/3 mL) Elución: 5 mL MeOH	Acquity UPLC BEH C_{18} 100x 0.1 mm, 1.7 µm A: Agua + 0.01% HCOOH B: ACN + 0.01% HCOOH	(ESI+)-MS/MS (QqQ)	(Tamtam *et al.* 2008; Tamtam *et al.* 2009)
Agua residual Agua superficial Agua subterránea	50 250 500	pH 4 (HCl) Oasis HLB 60 mg/3 mL Elución: 2x3 mL MeOH + MTBE (1:9)	HPLC Dionex Acclaim C_{18} RP 150 x 2.1 mm, 4.6 µm A: ACN B: Agua + 0.1% HCOOH	(ESI+)-MS/MS (QqQ)	(Tong *et al.* 2009)
Agua residual[1] Efluente de granja porcina[3] Efluente de piscifactoria[3, 4]	20[1] 50[3] 250[2,4]	pH 4 (H_2SO_4) Oasis HLB 6 mL, 200 mg Elución: 2x1.5 mL MeOH-$C_4H_8O_2$ + 2x1.5 mL MeOH + 1% NH_4^+	HPLC YMC Pro C_{18} 150 x 2 mm, 3 µm Precolumna 10 x 2 mm A: agua + 1% HCOOH B: MeOH + 1% HCOOH	(ESI+)-MS/MS (QqQ)	(Göbel *et al.* 2004; Joss *et al.* 2005; Managaki *et al.* 2007; Hoa *et al.* 2011)
Agua residual Agua superficial Agua subterránea	500 1000	pH<3 (H_3PO_4) Na_2EDTA Oasis HLB (60 mg) Elución:8 mL ACN	HPLC BetaBasic-18 C_{18} 100 x 2.1 mm, 3 µm. Precolumna 30 ºC A: ACN; B: MeOH C: Agua + 0.3% HCOOH	(ESI+)-IT-MS	(Batt *et al.* 2005; Batt *et al.* 2006; Batt *et al.* 2007)

Tabla 1.9 (continuación).

MATRIZ	V (mL)	SPE	COLUMNA LC CONDICIONES LC	DETECCIÓN MS/MS	REF
Agua residual	1000	pH 3 (H_2SO_4) ENV+-200 mg/6mL Elución: 2 mL MeOH + (5 mL MeOH +5% TEA)	HPLC YMC Hydrospher C_{18} 150x4.6 mm, 5 µm. 25 ºC Precolumna A: Agua + 0.1% HCOOH B: ACN + 0.1% HCOOH	(ESI+)-IT-MS	(Lindberg *et al.* 2005)
Agua residual Agua superficial	1000	pH 3 + Na_2EDTA (0.2 g) Oasis HLB (500 mg, 6 mL) Elución: 2 x 3 mL MeOH	HPLC ODS-P 250 x 4.6 mm, 3.5 µm. 35 ºC A: ACN B: Agua + 0.2% HCOOH (v/v)	(ESI+)-MS/MS (QqQ)	(Xu *et al.* 2007; Xu *et al.* 2007b)
Agua residual Agua superficial[1] Agua de mar[1]	250 1000[1]	pH3 Oasis HLB (500 mg/6 mL) Elución: 8 mL MeOH	Acquity BEH™ C_{18} 50 × 2.1 mm, 1.7 µm 30 ºC A:Agua + 0.01% HCOOH B: ACN + 0.01% HCOOH	(ESI+)-MS/MS	(Li *et al.* 2009; Zou *et al.* 2011)
Agua superficial	1000	pH 3 (HCl) + Na_2EDTA (0.2 g) Oasis HLB 200 mg, 6 mL Elución: 6 mL MeOH	HPLC Inertsil ODS-SP 150 x 4.6 mm, 5 µm 40 ºC A :MeOH B : Agua + 5 mM CH_3COONH_4 + 0.1% HCOOH	(ESI+)-QLIT-MS	(Zhang *et al.* 2011)
Agua superficial	500	0.2 g Na_2EDTA SPE tándem SAX 200 mg/2 mL-Oasis HLB 500 mg/6 mL	HPLC Intersil ODS-3 250 × 2.1 mm, 5 µm 40 ºC A: ACN B: Agua + 0.3% HCOOH	MS/MS	(Luo *et al.* 2011)
Agua residual Águas superficiales	200	pH 7 H_2SO_4 Oasis HLB 500 mg/6mL Elución: 8 mL MeOH	HPLC ZORBAX RX-C_8, 150 x 2.1 mm, 5 µm Precolumna 30 ºC A: Agua + 1% HCOOH B: ACN + 0.1% HCOOH	(ESI+)-MS/MS (QqQ)	(Wei *et al.* 2011)

Tabla 1.9 (continuación).

MATRIZ	V (mL)	SPE	COLUMNA LC CONDICIONES LC	DETECCIÓN MS/MS	REF
Agua residual Aguas residual de hospitales	100	Centrifugado Na_2EDTA (1 mL) pH 3.5 (HCOOH) Oasis HLB 200 mg/6 mL Elución: 2 mL MeOH	HPLC Chromolith Performance RP-18e 100 x 4.6 mm. 30 ºC -	(ESI+)-MS/MS (QqQ)	(Brown *et al.* 2006)
Agua residual de hospitales e industria farmaceútica	250	pH 4 H_2SO_4 Oasis HLB 500 mg, 6mL Elución: 4 mL MeOH	HPLC ZORBAX Eclipse XDB-C_{18} A: Agua + 0.1% HCOOH B: MeOH + 0.1 HCOOH	(ESI+)-MS/MS (QqQ)	(Lin and Tsai 2009)
Agua residual Agua superficial	100 200 500	pH 3 (HCOOH) Oasis HLB (200 mg) Elución: MeOH + 1% NH3	HPLC YMC ProC_{18} 150 x2.1 mm, 3 µm Precolumna. 30 ºC A: Agua + 0.1% HCOOH B: MeOH + 0.1% HCOOH (v:v)	(ESI+)-MS/MS QqQ)	(Senta *et al.* 2008; Senta *et al.* 2011)
Agua residual Agua superficial	100 400	Na_2EDTA (0.5 g L^{-1}) Oasis HLB (200 mg) Elución: 5 mL MeOH	HPLC ZORBAX Eclipse XRD C_{18} 150 x 3.0 mm, 3.5 µm 25 ºC A: Agua + 0.1% HCOOH + 5 Mm $NH_4C_2H_3O_2$ B: MeOH.	(ESI+)-MS/MS (QqQ)	(Peng *et al.* 2008; Peng *et al.* 2011)
Agua superficial	1000	pH 3 H_2SO_4 0.5 g L^{-1} Na_2EDTA Oasis HLB 500 mg/ 6 mL Elución: 3x3 mL MeOH	HPLC TC C_{18} 150 × 4.6 mm, 5 µm. 30 ºC A: Agua + 0.01% HCOOH B: ACN + 0.01% HCOOH	(ESI+)-MS/MS	(Jiang *et al.* 2011)
Agua residual[1] Agua residual de hospitales Agua superficial Agua potable[2]	100/ 200[1] 100 500 1000[2]	Oasis HLB (60 mg[1], 200 mg) Oasis HLB (500 mg) Elución: 2 x 2 MeOH	HPLC Synergi Hydro RP 50 ×2 mm, 4 µm Precolumna: 4.0 × 2 mm A: Agua + 10 mM HCOOH B: MeOH	(ESI+)-MS/MS (QqQ)	(Watkinson *et al.* 2009) (Watkinson *et al.* 2007)

Tabla 1.9 (continuación).

MATRIZ	V (mL)	SPE	COLUMNA LC CONDICIONES LC	DETECCIÓN MS/MS	REF
Agua superficial		Oasis HLB	HPLC Agilent XRD C_{18} 150 x 3.0 mm, 3.5 µm. 25 ºC A: ACN B: Agua + 0.1% HCOOH	-	
Agua potable clorada	500	Oasis HLB 200 mg/6 mL Elución: 4x2 mL MeOH	HPLC Pursuit C_{18} 15 cm × 2 mm, 3 µm Precolumna A : Agua+ 0.1% HCOOH B : ACN	(ESI+)-MS/MS	(Ye *et al.* 2007)
Agua residual	100	pH 3 (HCl) Oasis HLB 500 mg, 6 mL Elución: 3 x 1.5 mL MeOH	HPLC Zorbax Eclipse XDB-C_{18} 3.0 × 150 mm, 3.5 µm Precolumna 2.0 × 4 mm A: Agua + 50 mM HCOOH + NH_4HCO_2 (pH 4.5) B: ACN	DAD-MS	(Peng *et al.* 2006)

HLB, con la finalidad de retener en esta primera etapa los ácidos húmicos de la muestra, cargados negativamente, y asi facilitar la extracción de los antibióticos de interés en el segundo cartucho.

El procedimiento de SPE consiste en cinco etapas. Se comienza por la activación del material adsorbente haciendo pasar un determinado volumen de disolventes orgánicos a través del cartucho. Generalmente se emplea una bomba de vacío para regular el flujo de carga en el cartucho, conectada a un sistema de vacío con diferentes válvulas de regulación de tipo manual (p.e. procesadores de columnas SPE J.T. Baker) o un sistema automático (Aspec^{TM}). A continuación se carga el volumen de muestra a analizar, haciendo que pase a través de la columna SPE a un flujo lento (3 mL min^{-1} aprox.), para permitir que los contaminantes de interés queden retenidos en la superficie del material y no sean arrastrados por un exceso de caudal. Los volúmenes de carga en el cartucho para un mismo tipo de agua y de material de extracción son muy diferentes. Así, como vemos en la Tabla 1.9, utilizando cartuchos de

extracción Oasis HLB, los volúmenes pretratados para agua de depuradora van desde 20 mL a 500 mL, para agua superficial de 100 mL a 1000 mL y para agua subterránea de 500 mL a 1000 mL. La adición de Na_2EDTA a la muestra es frecuente para el análisis de diferentes familias de antibióticos, ya que mejora la extracción de macrolidos, tetraciclinas y fluoroquinolonas. Estos compuestos tienden a unirse a metales presentes en la muestra o en el material que las contiene, disminuyendo la eficiencia de la extracción; el Na_2EDTA actua como agente quelante uniéndose a estos metales solubles y permitiendo la extracción de los analitos (Gros *et al.* 2008). En el caso de los análisis exclusivos de sulfamidas, la adición de Na_2EDTA no sería tan determinante. Igualmente, el rango de pH de las muestras previo a la extracción va desde pH 3 a valores neutros. El ajuste a pHs ácidos se realiza principalmente para evitar la degradación de tetraciclinas, incluidas en los estudios de presencia de antibióticos, y de nuevo no sería determinante para las sulfamidas. Posteriormente, la etapa de lavado elimina los componentes de la matriz que hayan podido ser retenidos durante la carga de muestra, sin afectar a los analitos ya adsorbidos, utilizándose normalmente agua HPLC o mezclas de disolventes orgánicos. Antes de la elución, los cartuchos SPE se secan con un flujo suave de aire durante un período corto de tiempo (10-30 min) para eliminar posibles trazas de disolventes. Por último, la etapa de elución es clave para la eficacia de todo el proceso SPE. El o los disolventes elegidos han de ser capaces, a su paso a través del cartucho, de contrarrestar la interacción analito-adsorbente y asi poder arrastrar el total de los analitos hacia el vial de recogida. Un flujo lento, normalmente por gravedad, facilitará que el eluente penetre bien en todos los poros del material adsorbente de la columna y arrastre los analitos. Este poder de arrastre, sin embargo, no debe afectar a las moléculas interferentes que pudieran haber quedado retenidas en la columna, para asi obtener un extracto limpio y que contenga sólo los analitos de interés. El MeOH es el disolvente utilizado en prácticamente todos los métodos que presenta la Tabla 1.9. El volumen final de elución (6-10 mL) se evapora total o parcialmente, reconstituyendo por último hasta el volumen final deseado (0.1 – 2 mL).

La SPE puede realizarse off-line, de manera independiente al posterior análisis por LC-MS, o bien on-line, mediante un sistema acoplado directamente al equipo de cromatografía en el cual el extracto de la muestra pasa directamente a la columna cromatográfica. La principal ventaja del procedimiento on-line es la automatización del

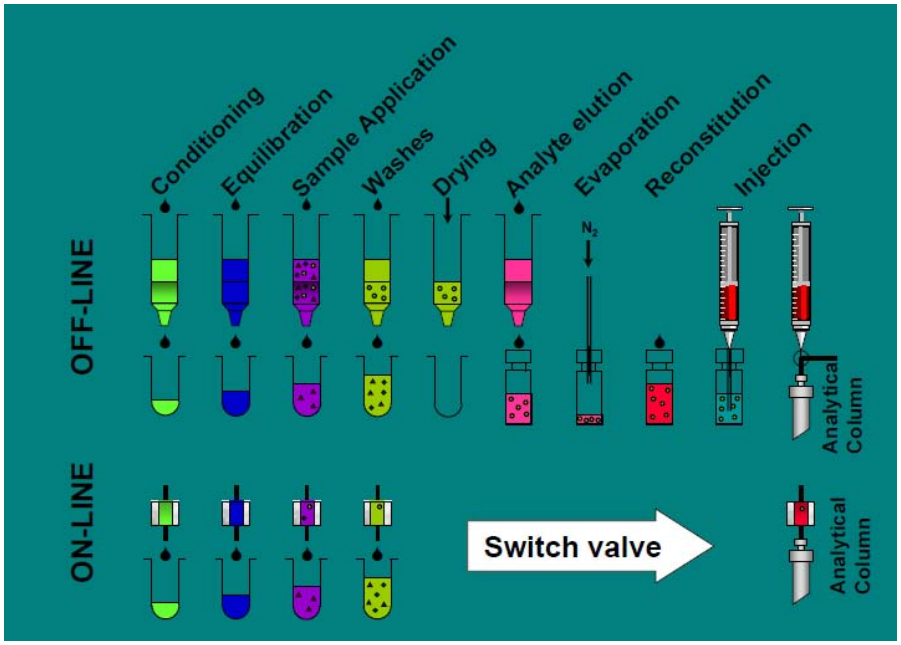


Figura 1.10. Comparación de las metodologías SPE offline y online. Fuente: Spark Holland. (Emmen, Países Bajos)

proceso, que reduce la manipulación de la muestra por parte del investigador a tan solo el filtrado inicial y al ajuste de pH de la muestra y/o la adición de modificadores como el Na_2EDTA en caso de que fuera necesario. Las etapas de activación del cartucho, carga de muestra, lavado y elución se realizan de forma automática, aumentando así la reproducibilidad y precisión del método (Rodriguez-Mozaz *et al.* 2007). Estos sistemas suponen una reducción considerable del volumen de disolventes utilizados y de muestra requerido, ya que el volumen del material adsorbente de los cartuchos de extracción online es mucho menor. Además, todo el volúmen de muestra extraido entra en el sistema de análisis, reduciendo asi la posibilidad de degradación de los analitos y consiguiendo límites de detección más bajos. En los protocolos de SPE offline, por el contrario, la inyección en el instrumento LC no suele ser superior a unos 5-30 µL del extracto de la muestra, y la etapa de extracción y su inyección pueden llevarse a cabo en días diferentes (Figura 1.10). Por último, los sistemas on-line suponen un ahorro considerable de tiempo de análisis, ya que la SPE tiene lugar simultáneamente con el análisis de la muestra previamente extraida. El principal inconveniente de estas metodologías de extracción online en

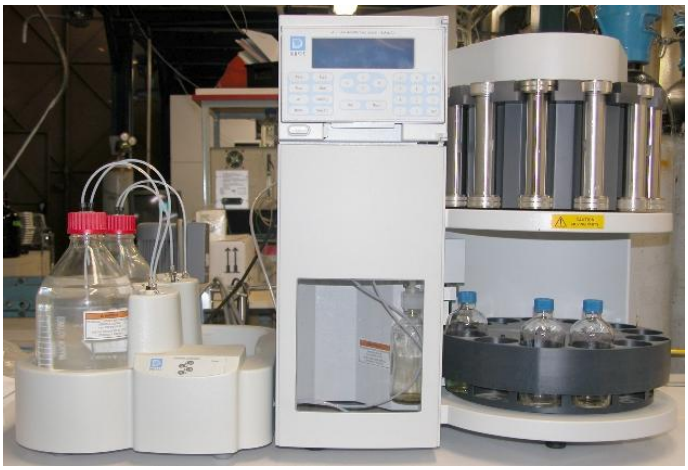
comparacion con la SPE off-line son el consumo total del extracto de la muestra, que elimina la posibilidad de futuras verificaciones o repeticiones del análisis, la imposibilidad de añadir patrón interno antes del análisis (sólo se puede adicionar como surrogate antes de la extracción) y también el elevado coste del instrumento. Los cartuchos de extracción suelen tener unas dimensiones de 1-2x10 mm, con unos 2.5-15 mg de material adsorbente, y suelen ser reutilizables. Stoob *et al*. (Stoob *et al.* 2005) fueron los primeros en utilizar este procedimiento online para estudiar la presencia de sulfamidas, junto con plaguicidas, en aguas superficiales. Posteriormente, Meyer *et al*. (Meyer *et al.* 2007) desarrollaría un protocolo de extracción on-line de diferentes familias de antibióticos utilizando cartuchos HLB, que ha sido utilizado por diferentes autores (Chang *et al.* 2010; Watanabe *et al.* 2010). Más recientemente, se han aplicado métodos on-line utilizando ya no cartuchos SPE, sino dos columnas de LC, una para preconcentrar la muestra y la segunda se utiliza como columna analítica. El sistema se conoce como Equan y ha sido comercializado por la casa Thermo-Scientific (Thermo-Scientific 2010). En el desaroollo de esta Tesis, se han utilizado diferentes sistemas SPE on-line, como el Prospekt2^{TM} o el Symbiosis^{TM} Pico, de Spark Holland.

Por último, cabría mencionar brevemente el desarrollo y utilización de polímeros de huella molecular o MIPs (del inglés *molecular imprinted polimers*) en varios trabajos para la separación de antibióticos en muestras medioambientales (Turiel *et al.* 2007; Chico *et al.* 2008; Gros *et al.* 2008b) , aunque en el caso de las sulfamidas su utilización hasta la fecha ha sido exclusiva para su análisis en alimentos o muestras biológicas. Se trata de materiales de extracción altamente selectiva, sintetizados para la extracción específica de un compuesto o familia de compuestos de estructura análoga. Esta alta especificidad se consigue durante la síntesis del polímero, en la que se introduce una molécula análoga a la de interés que actuará como un “molde”, uniéndose a monómeros funcionales y formando un complejo estable. La polimerización de este complejo dará lugar a unas cavidades complementarias en forma y funcionalidad química a la molécula “molde”, que será posteriormente eliminada mediante disolventes adecuados. De esta manera se crean sitios de reconocimiento o unión específicos en dichas cavidades entre el monómero y la molécula en cuestión. Los MIPs han significado un gran avance en el campo de la separación específica de los analitos de interés, aunque su aplicación medioambiental aún está en desarrollo.

1.6.2.2. Extracción de muestras sólidas

El procedimiento de extracción de sulfamidas en matrices sólidas suele dividirse en dos etapas, la extracción de la muestra propiamente dicha y la purificación y concentración del extracto, generalmente mediante un protocolo de SPE semejante al llevado a cabo para aguas. En referencia a las diferentes técnicas de extracción de sulfamidas en matrices sólidas, la literatura refleja una sustitución progresiva de métodos más tradicionales como la extracción por ultrasonidos (USE) o mediante Soxhlet, por la extracción con líquidos presurizados (PLE), técnica utilizada durante el desarrollo de esta Tesis. La PLE, cuyo instrumento se muestra en la Figura 1.11-a, combina temperaturas y presiones elevadas para mejorar la extracción de los analitos de interés, y supone un gran avance principalmente en cuanto a tiempo de preparación de muestra, cantidad de disolvente y automatismo. Parámetros como la temperatura, presión, número de ciclos de extracción y disolvente tienen que ser optimizados, si bien la presión no se considera un parámetro crítico en la eficacia del método (Lillenberg *et al.* 2009). Si se utiliza gran cantidad de disolvente orgánico, los extractos obtenidos son diluidos para reducir esta proporción por debajo del 5% y que posteriormente no afecte a la retención de los analitos de interés en el cartucho de SPE (ya que se suelen utilizar cartuchos Oasis HLB). La cantidad de muestra utilizada en procedimientos de PLE van desde <1 g para las muestras más complejas, como fangos de depuradora, hasta 5 – 10 g (suelos); las muestras son introducidas en las celda de extracción (ver Figura 1.11-b) junto a agentes dispersantes, como tierra de diatomeas, que evitan que las partículas se agreguen debido a las altas presiones, y generalmente se colocan filtros de celulosa en el interior de la base de la celda, para que el extracto se filtre durante su transferencia al vial de recogida. Aunque no hay ninguna referencia para el análisis de sulfamidas, para determinados compuestos, como plaguicidas o retardantes de llama, la extracción y purificación de la muestra se lleva a cabo simultáneamente dentro de la misma celda, añadiendo materiales como alumina o Fluorisil, de modo que el extracto obtenido no requerirà SPE.

a)



b)

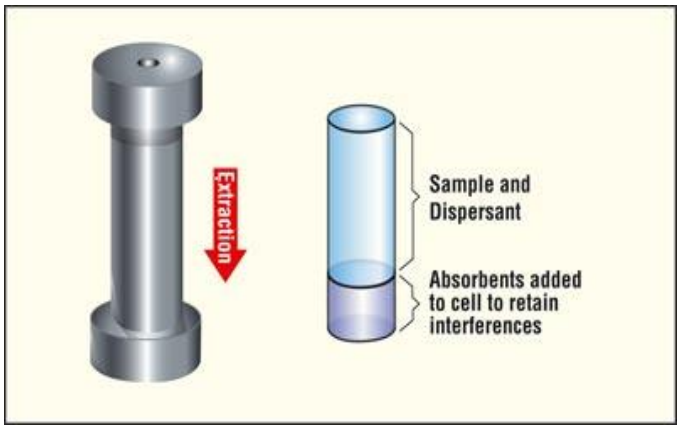


Figura 1.11. Imagen del instrumento ASE® de extracción por líquidos presurizados de Dionex (a), e imagen de las celdas de extracción utilizadas.

La Tabla 1.10 resume los estudios encontrados en la literatura sobre el análisis de sulfamidas en muestras sólidas.

Tabla 1.10. Protocolos de extracción para el análisis de sulfamidas y sulfamidas junto con otros antibióticos y fármacos en diferentes matrices sólidas.

MATRIZ	PROTOCOLO EXTRACCIÓN	ANALITOS/ ANÁLISIS	LODs	REF
Suelo	Muestra: 4 g + tierra de diatomeas PLE: agua + ACN (85:15, v/v) T: 200 ºC	Sulfamidas LC-MS/MS	5-15	(Stoob *et al.* 2006)
Suelo	Muestra: 5 g Extracción manual: 3 ciclos x 30 mL agua. SPE: Oasis HLB	Sulfamidas UPLC-MS/MS	0.01-0.34	(Shelver *et al.* 2010)
Abono	Muestra: 15 g Extracción líquido-líquido 1er ciclo: 15 mL n-hexano/ $C_4H_8O_2$, 95:5, v/v) 2º-3er ciclo: 45 mL $C_4H_8O_2$ SPE: aminopropil	Sulfamidas HPLC-MS/MS	0.4-1.3	(Pfeifer *et al.* 2002)
Sólidos en suspensión	Muestra: 1g Centrifugación-liofilización USE: 1er ciclo: 30 mL tampón ácido cítrico 0.2 M + 100 µL Na_2EDTA (5%) 2º ciclo: 10 mL MeOH/ ácido cítrico 0.2 M SPE: Oasis HLB	Antibióticos LC-MS	0.10-0.85*	(Pan *et al.* 2011)
Sólidos en suspensión	Muestra: 1 g + 1 g Hydromatrix PLE: 2 ciclos MeOH + tampón ácido cítrico/NaOH (50:50, v/v). T: 40 ºC. Extracción manual: 2 ciclos. 10 mL MeOH + tampón ácido cítrico/NaOH (50:50, v/v) SPE: online Oasis HLB	Antibióticos LC-MS/MS	-	(McKinney *et al.* 2010; Watanabe *et al.* 2010)
Sedimentos	Muestra: 1g Extracción manual: 2 ciclos. 20 mL bufer McIlvaine/tampón NaOH + 200 uL 5% Na_2EDTA SPE: Oasis HLB	Antibióticos HPLC-MS/MS	0.09-0.36	(Kim and Carlson 2007; Kim and Carlson 2007)
Sedimentos	Muestra: 2g USE: 3 ciclos. 10 mL ACN + 0.2 M ácido cítrico SPE: Oasis HLB	Antibióticos UPLC-MS/MS	0.58-1.90	(Yang *et al.* 2010)
Suelo	Muestra: 10 g + 10 g arena Ottawa. PLE: 2 ciclos 50% MeOH + 50% ácido cítrico SPE: tándem SAX + Oasis HLB	Antibióticos LC-MS/MS	7.7*	(Jacobsen *et al.* 2004)

Tabla 1.10 (continuación).

MATRIZ	PROTOCOLO EXTRACCIÓN	ANALITOS/ ANÁLISIS	LODs	REF
Sedimentos Suelos	Muestra: 2 g Soxhlet 1er ciclo: 50 mL MeOH + H_3PO_4 1M 2º ciclo: 50 mL MeOH + NaOH 5% (90:10, v/v) Dilución: 100 mL SPE: HLB	Antibióticos UPLC-MS/MS	1	(Tamtam *et al.* 2010; Tamtam *et al.* 2011)
Suelos	Muestra: 5g + hydromatrix PLE 2 ciclos agua/MeOH/Acetona (50:25:25, v/v/v) + 25 mM EDTA + 2% NaOH + 0.6 M NaCl Temperatura. ambiente	Antibióticos y estrógenos HPLC-MS/MS	0.01	(Tso *et al.* 2011)
Suelo y abono líquido	Muestra: 4 g suelo/2mL abono USE Suelo: MeOH + 0.1M EDTA + tampón McIlvaine (50:25:25, v/v/v) Abono: 8 mL 0.1M EDTA:tampón McIlvaine, 50:50, v/v) SPE: tàndem SAX + Oasis HLB	Antibióticos LC-UV	Suelo: 18 Abono: 140	(Blackwell *et al.* 2004)
Abono	Muestra: 1 g USE: 2 ciclos. 10 mL ACN + 10 mL 0.1 M EDTA + tampón McIlvaine	Antibióticos LC-UV	9-12*	(Hu *et al.* 2008)
Fango digerido y no digerido	Muestra: 9 g fango + arena PLE: 5 ciclos ACN + 0.35% H_3PO_4 + 0.01M ácido cítrico (pH 2.5) Temperatura: 100-110 ºC SPE: Oasis HLB	Antibióticos LC-MS	0.03	(Lillenberg *et al.* 2009)
Fango y sedimento	Muestra: 1g + Hidromatrix PLE: 3 ciclos MeOH + agua (1:2, v/v) Temperatura: 100 ªC SPE: Oasis HLB	Fármacos HPLC-MS/MS	0.14	(Jelic *et al.* 2009; Radjenovic *et al.* 2009; Silva *et al.* 2011)
Fango	Muestra: 1 g PLE: 2 ciclos Temperatura: 25 ºC MeOH/H_3PO_4 (50:50, v:v) SPE: Oasis HLB	Fármacos LC-MS/MS	2.9	(Edwards *et al.* 2009)

LOD: límite de detección del método (µg kg^{-1}); *: LOD en ng L^{-1}; USE: extracción por ultrasonidos; PLE: extracción por líquidos presurizados.

1.7. CROMATOGRAFÍA DE LÍQUIDOS ACOPLADA A ESPECTROMETRIA DE MASAS

La combinación de la elevada capacidad de separación de la cromatografía de líquidos (LC) con la gran selectividad y especifidad de la espectrometría de masas (MS) es hoy en día la técnica analítica por excelencia en el estudio de la presencia medioambiental de sulfamidas y muchos otros contaminantes orgánicos. Las versatilidad de las nuevas metodologías LC-MS ha permitido detectar niveles de concentración de tan solo pg L^{-1} con un alto poder de identificación (medición de masa exacta) para moléculas muy polares. Así mismo, la espectrometría de masas en tándem (MS/MS) proporciona gran cantidad de información estructural de la molécula, ayudando a establecer las rutas de fragmentación de los compuestos analizados .

1.7.1. Cromatografía de líquidos (LC)

La LC es la técnica más utilizada en el análisis de sulfamidas y antibióticos en el medio ambiente. Como reflejan las Tablas 1.9 y 1.10, todos los procedimientos de LC desarrollados para la separación de estos compuestos se basan en la LC de fase invertida. Las fases estacionarias más utilizadas son las de base sílice quimicamente modificada (cadenas de C_8 y C_{18}). La longitud y el tamaño de partícula son las variables a considerar para optimizar la eficacia en la separación. Normalmente, las columnas utilizadas suelen medir entre 10 y 25 cm, con diámetros internos variables entre 2.1 y 4.6 mm, y un tamaño de partícula entre 3 y 5 µm (Tabla 1.9). Recientemente, la disminución del tamaño de partícula (< 2 µm) ha permitido poder trabajar a flujos altos (300-800 µL min^{-1}) manteniendo una gran eficacia y proporcionando tiempos de análisis más cortos y una mayor resolución cromatogràfica. El principal problema de este tipo de columnas es el consecuente aumento de presión *o backpressure* en el sistema, asociado a la disminución del tamaño de partícula y a la utilización de estos flujos altos. Este tipo de cromatografía de se ha denominado *Ultra High Performance Liquid Chromatography* (UHPLC) y ofrece un mayor rendimiento de trabajo ya que permite analizar un mayor número de muestras en un tiempo mucho menor. Además, la mayor resolucion cromatografica que ofrece el UHPLC puede permitir la disminución del efecto matriz,

como se explica más adelante. Como muestran las Tablas 1.9 y 1.10, para el análisis de sulfamidas y otros antibióticos en muestras ambientales, varios autores han utilizado UHPLC como técnica de separación, aunque el uso de HPLC sigue siendo más frecuente.

Como fase móvil, se suele utilizar disolventes polares mixtos, normalmente agua con mezclas de MeOH o ACN. La adición de ácidos orgánicos volátiles y de soluciones tampón a la fase móvil es una práctica muy común, ya que ayudan a mejorar la forma de la señal cromatogràfica y regulan la ionización de los analitos. Suelen añadirse en concentraciones no superiores a 20 mM, ya que podrían reducir la intensidad de la señal (Gros Calvo 2009). Es importante utilizar soluciones volátiles como sales amónicas, ya que en caso contrario pueden precipitar tras la evaporación del disolvente, contaminando el sistema de espectrometría de masas y comprometiendo el análisis. Como muestra la Tabla 1.9, el ácido fórmico (HCOOH) es el más usado para el análisis de las sulfamidas.

1.7.2 Espectrometría de masas (MS) y espectrometría de masas en tandem (MS/MS)

Todas las metodologías de análisis de sulfamidas en muestras medioambientales descritas en las Tablas 1.9 y 1.10 utilizan la espectrometría de masas (MS) como técnica de detección. EL electrospray (ESI) es la técnica de ionización más utilizada en el análisis ambiental de las sulfamidas y de prácticamente todo tipo de contaminantes emergentes, incluyendo los antibióticos y las sulfamidas. En comparación a otras fuentes de ionización como la ionización química a presión atmosférica (APCI) o la fotoionización a presión atmosférica (APPI), la interfase ESI ha demostrado ser la técnica más efectiva y con determinaciones más sensibles para compuestos de polaridad moderada o alta dentro de un intervalo de masas moleculares muy amplio (entre 100 y 150000 Da), mientras APCI y APPI son más indicadas para compuestos menos polares. Las sulfamidas son detectadas en modo positivo como $[M+H]^+$. La formación de aductos con otras especies presentes en el medio como Na, K o NH_4^+ es común para este tipo de ionización. En esta Tesis se ha utilizado únicamente la interfase ESI. La espectrometría de masas en tandem (MS/MS) ha aumentado considerablemente el potencial que ofrece la técnica de LC-MS. La calidad y la cantidad de información proporcionada por LC- MS/MS es mucho mayor que la de los análisis MS, aumentando la especificidad especialmente en matrices complejas como aguas residuales o extractos de muestras sólidas. Tal y como reflejan las Tablas

1.9 y 1.10, para el análisis de sulfamidas se han utilizado casi exclusivamente los analizadores de tipo cuadrupolo (Q), los de trampa de iones (IT) y los de trampa de iones lineal (LIT), así como analizadores de tipo híbrido como el cuadrupolo-trampa de iones lineales, QLIT, o el triple cuadrupolo (QqQ). Este último es el espectrómetro de masas usado con mayor frecuencia en estudios de presencia medioambiental de sulfamidas (y de otros antibióticos) tanto en matrices de agua como en sólidos. Todos los trabajos realizados con estos analizadores de masas, resumidos en estas dos tablas, se basan en la determinación en diferentes matrices ambientales de diferentes sulfamidas de masa y características conocidas (*Target Analysis).* Sin embargo, los analizadores de tipo tiempo de vuelo (TOF), el híbrido cuadrupolo-tiempo de vuelo (QTOF) y en los últimos años el Orbitrap, son empleados para la elucidación o detección de nuevos productos de transformación o degradación cuya masa o estructura son desconocidas (*Non-target Analysis*) y, por tanto, su aplicación en estudios de presencia mediaombiental de contaminantes traza es algo más limitada que la de otros instrumentos como el QqQ, ya que la información obtenida más valiosa es cualitativa y no cuantitativa (Stolker *et al.* 2004). Por otro lado, hasta la fecha, los analizadores TOF han sido utlizados con más frecuencia para los estudios de identificación y elucidación de metabolitos y otros productos de transformación de las sulfamidas (Boreen *et al.* 2005; Trovó *et al.* 2009), que el QqTOF (Gómez-Ramos *et al.* 2011). En los últimos años, la aparición del Orbitrap por transformada de Fourier (FT-ORBITRAP) ha permitido aumentar tanto la sensibilidad como el intervalo de masas sobre el que se puede medir la masa exacta debido a una mayor transmisión de iones. Sin embargo, aún no se han utilizado para la detección de sulfamidas y/o sus productos de transformación en matrices ambientales. Durante el desarrollo de esta Tesis, hemos empleado un UHPLC-QTOF para la elucidación de productos de transformación de las sulfamidas derivados de su fotodegradación y de su biodegradación por hongos (ver Capítulo 3).

Como refleja la Tabla 1.9, en el análisis medioambiental de sulfamidas también se han utilizado otro tipo de analizadores como el IT y LIT (Lindberg *et al.* 2004; Batt and Aga 2005; Madureira *et al.* 2010), pero siempre realizando scans MRM o Product Ion scans. En los últimos años la aparición de analizadores híbridos como el QqTOF, pero también el cuadrupolo-trampa de iones lineal (QqLIT) han ganado rápidamente terreno frente a los analizadores convencionales. En el QqLIT, el tercer cuadrupolo puede funcionar como tal o como trampa de iones lineal, aumentando la versatilidad del instrumento. En modo trampa, los mismos tipos de barrido que se realizan en un QqQ ordinario aumentan enormemente su sensibilidad ya que el

QqLIT ofrece la posibilidad de una acumulación de los iones generados en la fuente., aumentando la señal en el detector. La alta sensibilidad de este instrumento híbrido se ha utilizado en diferentes estudios de presencia medioambiental de sulfamidas en aguas de río (Hao *et al.* 2006; Zhang *et al.* 2011) en los que era necesario trabajar con límites de cuantificación considerablemente bajos. Además, proporcionan valiosa información estructural y permiten la identificación de productos desconocidos efectuando barridos MS^n sin una pérdida significativa de sensibilidad. En el desarrollo de esta Tesis, se han llevado a cabo tanto *Target* como *Non-target analysis*, utilizando para ello analizadores de tipo QqQ, QLIT y QTOF.

1.8. VALIDACIÓN DEL MÉTODO ANALÍTICO

La validación del método de análisis desarrollado es imprescindible para asegurar la calidad y fiabilidad de los resultados obtenidos. En la UE, la Directiva 2002/657/EC establece los criterios de calidad necesarios para la validación de los métodos analíticos a utilizar en el control rutinario de confirmación de contaminantes orgánicos. Otros organismos internacionales como la IUPAC (International Union of Pure and Applied Chemistry) o la EPA (Environment Protection Agency) han desarrollado diferentes protocolos o criterios para evaluar la validez y fiabilidad de los métodos análiticos. Todas ellas coinciden en la necesidad de incluir información referente a la exactitud del método (ensayos de recuperación), la precisión (expresada como repetitividad y reproducibilidad), el intervalo de linealidad, especifidad y robustez

Por otro lado, la evaluación del efecto matriz en la validación del método analítico es fundamental, ya que compromete la exactitud y precisión de los resultados. La fuente de ionización ESI se ve más afectada por este efecto matriz que otras fuentes (APCI, APPI). Las alternativas que generalmente se aplican para evitar el efecto matriz en la cuantificación en LC-MS son de tipo preventivo o compensatorio. Las primeras pretenden corregirlo bien optimizando el pretratamiento de la muestra, diluyendo los extractos de las muestras o recurriendo a cromatografía de UHPLC. Las de tipo compensatorio lo corrigen durante la cuantificación y, por tanto, no lo eliminan. La utilización de patrones internos, con estructura molecular y tiempos de retención similares a las del analito de interés (generalmente el mismo compuesto marcado isotópicamente) es en la actualidad el procedimiento de corrección de

efecto matriz más aconsejable y el más expandido, ya que minimiza la etapa de procesamiento de la muestra. La preparación de una recta de calibrado en matriz también es un proceso muy común, aunque disminuye la sensibilidad del método. La Tabla 1.11 resume las ventajas e inconvenientes de cada una de estas técnicas.

Tabla 1.11. Ventajas e inconvenientes de los diferentes métodos de corrección del efecto matriz en muestras ambientales.

Medidas preventivas	**VENTAJAS**	**INCONVENIENTES**
Optimización del pretratamiento de la muestra	Mayor purificación del extracto.	Mayor manipulación de la muestra. Mayor inversión de tiempo y material.
Dilución del extracto	Mejora forma del pico. Mejor retención cromatográfica	Perdida de sensibilidad en estudios ambientales.
Cromatografía de alta resolución (UHPLC, LC-LC)	Evitar coelución de componentes. Mayor purificación (LC-LC).	Insuficiente para matrices muy complejas.
Medidas compensatorias		
Patrones internos	Elimina el efecto matriz durante la cuantificación.	Disponibilidad commercial. Alto precio.
Calibrado en matriz (*matrix matched calibration*)	El efecto matriz se iguala en la recta de calibrado para cada analito. Elimina el efecto matriz durante la cuantificación.	Disponibilidad de matriz blanco. Pérdida sensibilidad del método.

2. ANÁLISIS DE SULFAMIDAS EN MUESTRAS MEDIOAMBIENTALES. DESARROLLO DE METODOLOGÍAS ANALÍTICAS

2.1. INTRODUCCIÓN Y OBJETIVOS

Los bajos niveles ambientales de las sulfamidas en aguas y matrices sólidas, así como la complejidad de estas matrices ambientales, son las principales dificultades a la hora de desarrollar una metodología analítica lo suficientemente sensible y robusta como para que los resultados obtenidos sean fiables. Como ya se comentó en la Introducción, el pretratamiento de la muestra es una etapa crítica, ya que se deben aplicar procedimientos de purificación y preconcentración de la muestra lo suficientemente selectivos y eficaces. Como demuestra la bibliografía, las nuevas metodologías desarrolladas en los últimos años han priorizado no sólo la mejora de los límites de detección del método, sino también la reducción del tiempo tanto de pretratamiento de muestra como de análisis. La aplicación de SPE on-line ha logrado cumplir estos objetivos, ya que consigue una completa automatización del pretratamiento, permitiendo un ahorro considerable de tiempo, una menor manipulación de la muestra y el uso de un volumen mucho menor de ésta, sin que los límites de detección se vean afectados.

Muchas de las metodologías encontradas en la literatura se han desarrollado para el análisis multiresiduo de diferentes familias de fármacos, de propiedades muy dispares, con la finalidad de poder detectar el mayor número posible de compuestos en un solo análisis. A pesar de las ventajas obvias de estos métodos multiresiduo, la sensibilidad se puede ver comprometida durante la optimización de los parámetros comunes, como los de la fuente de ionización. Por otro lado, pocos autores han incluido metabolitos acetilados de las sulfamidas en sus trabajos sobre presencia ambiental de estos antibióticos (Hilton and Thomas 2003; Göbel *et al.* 2004; Stoob *et al.* 2005; Stoob *et al.* 2007), siendo casi siempre las aguas residuales el objetivo del estudio, excluyendo aguas superficiales y subterráneas. Sin embargo, trabajos

como el de Hilton o Göbel (Hilton and Thomas 2003; Göbel *et al.* 2004) evidenciaban la presencia frecuente de estos metabolitos a concentraciones superiores que las del compuesto original y la alta probabilidad de que alcanzaran el resto de matrices ambientales. Además, los límites de detección obtenidos para los metabolitos eran muy superiores a los obtenidos para las sustancias originales , evidenciando la necesidad de continuar y mejorar estas metodologías de análisis. Por todo esto, el objetivo principal de la presente Tesis fue la optimización y el desarrollo de nuevos métodos de análisis que permitieran la detección tanto de sulfamidas como de sus metabolitos acetilados a niveles ambientales y en diferentes tipos de muestras, solventando todos estos inconvenientes de una manera práctica. En este Capítulo se presentan las diferentes metodologías analíticas desarrolladas para el análisis de sulfamidas en distintos tipos de agua, suelos y fangos de depuradora. La disponibilidad del instrumental necesario para desarrollar SPE on-line ha determinado la elección de la extracción de muestras de agua off-line u on-line, de modo que se han desarrollado diferentes metodologías para ambos tipos de SPE. En el caso de las muestras sólidas, su extracción se ha desarrollado siempre mediante PLE, y el extracto ha sido purificado y preconcentrado mediante SPE off-line. Dado el carácter polar de estos antibióticos, LC ha sido elegida como técnica de separación, acoplada a detección MS/MS. Se ha utilizado un analizador hibrido QqLIT, que ha permitido trabajar en modo SRM con una sensibilidad mayor que un QqQ. Los principales objetivos planteados en este capítulo fueron los siguientes:

- Optimización de las diferentes etapas de la SPE off-line para el análisis de nueve sulfamidas y uno de sus metabolitos acetilados en agua de entrada y salida de EDAR, agua superficial, agua subterránea y agua de consumo.

- Desarrollo de una nueva metodología analítica para fangos de depuradora y suelos agrícolas, optimizando los diferentes parámetros de extracción PLE y posterior limpieza del extracto.

- Optimización de la metodología SPE on-line para la extracción y análisis de 17 sulfamidas y cinco metabolitos acetilados en los diferentes tipos de agua anteriormente mencionados.

- Optimización de la separación cromatográfica de los analitos, y de los diferentes parámetros del análisis MS/MS.

- Evaluación del efecto matriz en muestras sólidas.
- Validación de los diferentes métodos analíticos desarrolladas en términos de linealidad, sensibilidad, selectividad, exactitud y precisión.

2.2. PUBLICACIONES

Publicación científica 3

"Highly sensitive simultaneous determination of sulphonamide antibiotics and one metabolite in environmental waters by liquid chromatography-quadrupole linear ion trap-mass spectrometry"

M. Silvia Díaz-Cruz, María Jesús García Galán y Damià Barceló

Journal of Chromatography A (2008) 1193, 50-59

Publicación científica 4

"Determination of 19 sulfonamides in environmental water samples by automated on-line solid-phase extraction-liquid chromatography-tandemmass spectrometry (SPE-LC-MS/MS)"

María Jesús García Galán, M. Silvia Díaz-Cruz y Damià Barceló

Talanta (2010), Vol. 81, pp. 355-366.

Publicación científica 5

"Application of fully automated online solid phase extraction-liquid chromatography-electrospray-tandem mass spectrometry for the determination of sulphonamides and their acetylatd metabolites in groundwater"

María Jesús García Galán, Teresa Garrido, Josep Fraile, Antoni Ginebreda, M. Silvia Díaz-Cruz y Damià Barceló

Analytical and Bioanalytical Chemistry (2011), Vol. 399, pp. 795-806

Publicación científica 6

"Multiresidue trace analysisi of sulphonamides antibiotics and their metabolites in soils and sewage sludge by pressurized liquid extraction (PLE) followed by liquid chromatography-electrospray-quadrupole linear ion trap mass spectrometry (QqLIT-MS/MS)

María Jesús García Galán, M. Silvia Díaz-Cruz y Damià Barceló

Journal of Chromatography A (aceptado)

Journal of Chromatography A, 1193 (2008) 50–59

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Highly sensitive simultaneous determination of sulfonamide antibiotics and one metabolite in environmental waters by liquid chromatography–quadrupole linear ion trap–mass spectrometry

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ABSTRACT

The present work describes the development of a highly sensitive analytical method based on liquid chromatography–quadrupole linear ion trap–mass spectrometry (LC–QqLIT–MS) for the determination of nine sulfonamide antibiotics and one N^4-acetylated metabolite in environmental waters (wastewater, surface water and groundwater) and bottled mineral water. Special emphasis was devoted to the elimination of matrix components during solid-phase extraction (SPE) by the evaluation of three different extraction/purification strategies: single cartridges (Oasis HLB and Oasis MCX) and tandem (TD) extraction (combination of both). The method developed proved to be suitable for sulfonamide determination in all kinds of waters tested. The method was shown to be linear in a wide concentration range, with correlation coefficients higher than 0.999 for all compounds except for sulfadimethoxine (R^2 0.997). The overall instrumental repeatability was satisfactory, with the exception of the metabolite (RSD 34%). Method limits of detection achieved for sulfonamides were in the range 0.01–1.13 ng L^{-1} and for the metabolite 0.08–461 ng L^{-1}. Recovery rates were estimated at 500 ng L^{-1} spike level in the four water matrices selected. The highest recovery achieved in all matrices was that corresponding to the Oasis HLB cartridge. In environmental waters, recovery values obtained were higher than 61% for the surface water and, in general, higher than 90% for groundwater and wastewater. Bottled mineral water exhibited recovery rates higher than 92%, with the exception of sulfamethoxypiridazine (82%) and sulfapyridine (86%) In order to demonstrate the applicability of the developed method, several water samples were analyzed. Results evidenced the requirement for consideration of N^4-acetylated metabolites of sulfonamides in environmental residue analysis to avoid the underestimation of removal rates of such pharmaceutical compounds during wastewater treatments.

1. Introduction

A great variety of pharmaceutical residues have been detected in the different environmental compartments. The occurrence of these emerging contaminants has raised concern because they have the potential to negatively affect non-target living organisms, since they are designed to be biologically active, and are expected to be consumed in increasing amounts in the decades ahead. The presence of antibiotics is likely to be of most concern because it could lead to the development of bacteria with antibiotic resistance genes [1] and to the development of allergenic responses [2]. They are extensively used in both human and veterinary medicine against microbial infections; in addition a certain fraction of antibiotics is used to increase the rate of growth in animal farms and fisheries. Sulfonamides (SAs) are a widely consumed class of antibiotics due to their low cost, broad spectrum of activity and effectiveness in growth promotion. Several methods for the analysis of SA residues in water samples are described in the literature. However, none of them has proven to be applicable to a wide range of water matrices. So far, methods have been developed for wastewater [3–7] surface [8,9], surface and drinking waters [10,11], surface and wastewaters [12–14], and ground and surface water [15].

In order to better assess the occurrence of SAs in the environment the metabolites of dosed drugs should also be considered [16]. So far, few studies dealt with pharmaceutical metabolites in general, and sulfonamides in particular; the elimination of N^4-acetylsulfamethoxazol during wastewater treatment was tested and a tentative fragmentation process was presented [16]. In an earlier study Hilton and Thomas [12] included also

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N^4-acetylsulfamethoxazol among the pharmaceuticals investigated in effluent and surface water. Findings indicated that while sulfamethoxazole could not be quantified in any sample (<50 ng L^{-1}), N^4-acetylsulfamethoxazol was present in all samples and at quite high concentrations (<50–2200 ng L^{-1}). In a more recent study, five N^4-acetylated metabolites of sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethoxazole and sulfathiazole were analyzed. Results indicated that, in general, limits of detection (LODs) were 5-fold higher than those achieved for the related unmetabolized residues [8]. These early conclusions evidenced the need for analytical methods capable to simultaneously determine parent compounds as well as their metabolites at the low concentrations normally present in the environment. In order to allow the assessment of potential risks and effects on human health at high-risk locations, a reliable determination of sulfonamides and a precise prediction of likely concentrations of such compounds in drinking water is also needed.

Natural waters are complex matrices that contain high amounts of organic matter that due to their chemical properties are able to be co-extracted along with the analytes of interest from the sample, reducing the extraction efficiency and interfering detection. When mass spectrometric detection is to be performed, this natural organic matter (i.e. humic and fulvic acids) is responsible for matrix effects because it has surfactant properties that may affect the electrospray signal by promoting or hindering ionization in ESI working under positive ionization (PI) mode. Signal enhancement caused by surface and groundwater matrix components was observed by Furlong et al. [17] and by Lindsey et al. [15] in the analysis of herbicides and tetracycline antibiotics, respectively. Such effects depend on the analyte/sample binary combination; therefore a reliable approach followed to minimize matrix effects should take into account the variability of the sample matrix to be analyzed. Strategies to minimize those unwelcome effects together with improved calibration approaches are required. Matrix-matched standard calibration is an effective approach extensively used in antibiotic residue in food analysis. However, it cannot be successfully applied in environmental analysis due to the different origin and composition of the water samples, thus, the selection of a blank sample with matrix content similar to that of the set of samples is not feasible. The simplest solution commonly taken to correct matrix effects is the dilution of the sample; however this usually compromises the method sensitivity, a critical parameter due to the low LODs required in the environmental monitoring of SAs.

This paper presents a highly sensitive analytical method based on solid-phase extraction (SPE) and analysis by liquid chromatography–quadrupole linear ion trap–mass spectrometry (LC–QqLIT–MS) for the determination of nine sulfonamides and one N^4-acetylated metabolite in water samples. This is a general methodology capable to provide reliable results in a wide diversity of water matrices. Moreover, it is the first time, to the author's knowledge, that a SAs metabolite is analyzed in bottled mineral and groundwater. Especial emphasis has been paid to the isolation and purification procedures in order to minimize potential matrix effects. The approach followed in the present work to mitigate these undesired effects and to enhance sensitivity was to improve the selectivity of the extraction/purification during the sample pretreatment along with the use of an isotopically labeled sulfonamide as internal standard. The validation process of the method included an estimation of the following parameters: linearity, precision, recovery and limits of detection and quantification (LOQs). Finally, to prove the goodness of the new method, it was applied in the determination of the ten selected sulfonamides in surface, ground, and wastewater, and bottled mineral water.

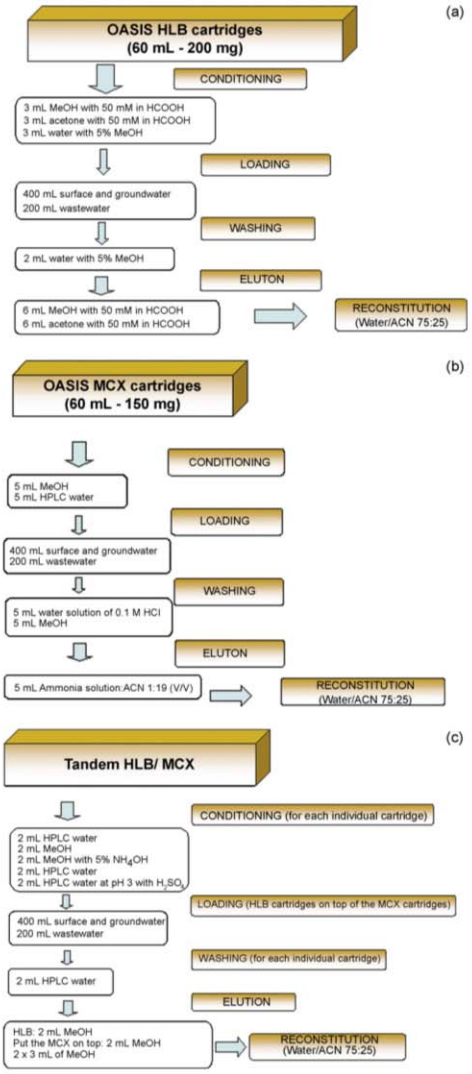

Fig. 1. Different solid-phase extraction strategies followed: (a) HLB cartridges, (b) MCX cartridges and (c) tandem extraction (HLB and MCX).

2. Experimental

2.1. Chemicals

Sulfamethizole (99.9%), sulfisoxazole (99.8%), sulfathiazole (99.9%), sulfadiazine (99.8%), sulfapyridine (98%) and sulfa-

Table 1
Optimized time-scheduled MRM transitions

Compounds	$[M+H]^+$	MRM transitions	Transitions ratio	DP (V)	CE (eV)	CXP (eV)
Sulfadiazine	251.1	251/156	1.5	46	27	10
		251/108		46	30	8
Sulfadimethoxine	311.1	311/156	4.8	76	31	8
		311/92		76	31	6
Sulfamethazine	279.1	279/156	1.3	26	30	10
		279/124		26	35	10
Sulfamethizole	271.1	271/156	8.9	36	23	12
		271/108		36	23	8
Sulfamethoxazole	254.1	254/156	2.5	56	25	10
		254/108		56	27	10
Sulfamethoxypyridazine	281.1	281/156	2.5	66	27	14
		281/126		66	27	12
Sulfapyridine	250.2	250/156	1.8	51	28	12
		250/92		51	31	6
Sulfathiazole	256.1	256/156	7.8	40	25	14
		256/92		40	25	10
Sulfasoxazole	268	268/156	1.5	71	21	10
		268/113		71	21	8
N^4-acetylsulfamethazine	321.1	321/134	1.2	86	35	4
		321/124		86	35	4
d_4-sulfathiazole	260	260/160	5.9	71	25	6
		260/96		71	25	6

Sulfonamides were analyzed in the ESI (PI) mode, taking $[M+H]^+$ as precursor ion in all cases. Compound dependent parameters: CE; collision energy; DP, declustering potential and CXP, collision cell exit potential.

methoxypyridazine (99.8%) were purchased from Riedel-de Haën (Seelze, Germany); N^4-acetylsulfamethazine (99.8%), sulfadimethoxine (99.9%) and sulfamethoxazole (99.9%) were purchased from Sigma–Aldrich (Seelze, Germany). Internal standard d_4-sulfathiazole (99.9%) was purchased from Toronto Research Chemicals (Ontario, Canada).

Individual antibiotic stock standard solutions were prepared at 100 μg mL^{-1} by dissolving 1 mg of the individual drug in 10 mL methanol (MeOH). For the internal standard, a solution at 5 μg mL^{-1} was prepared by dissolving 0.5 mg in 10 mL MeOH. A stock standard solution of the mixture of all compounds at a concentration of 5 and 1 μg mL^{-1} was made up by mixing, respectively, 5 and 1 mL of the individual antibiotic stock standard solutions with MeOH in 100 mL flasks. Working standard solutions of the mixture in the range 1–1000 ng mL^{-1} were freshly made by appropriate dilution of the stock standard mixture in MeOH. Solutions were transferred to amber bottles and stored in the dark at 4 °C to minimize potential analyte degradation.

Water, MeOH, acetone, *n*-hexane, acetonitrile (ACN) and dichloromethane (DCM) were of HPLC grade; formic acid and ammonium formate were of MS grade; HCl, H_2SO_4, acetic acid, ammonium hydroxide, ammonium acetate, citric acid monohidrate and Na_2HPO_4 of analytical grade. All they were purchased from Merck (Darmstadt, Germany). High quality N_2 was supplied by Abelló Linde (Barcelona, Spain).

2.7 μm glass fiber filters and 0.45 μm nylon membrane filters were supplied by Teknokroma (Barcelona, Spain). Oasis HLB cartridges of 200 mg (60 mL) and Oasis MCX cartridges of 150 mg (60 mL) used were from Waters (Milford, MA, USA).

Concerning safety, no particular measures had to be taken when handling with sulfonamides in the experimental procedures and analyses.

2.2. Instrumentation

Extraction and purification of SAs from waters was performed by SPE assisted by a 12-fold vacuum extraction box J.T. Baker (Phillipsburg, NY, USA). Extracts were evaporated under a gentle N_2 stream at 25 °C in a Turbo Vap LV Zymark evaporator (Hopkinton, MA, USA).

LC–tandem MS analyses were performed in a system consisting of an HP 1100 chromatograph from Agilent Technologies (Palo Alto, CA, USA) coupled to a 4000 QTRAP mass spectrometer from

Table 2
Instrumental parameters of the LC–QqLIT–MS method developed for the analysis of sulfonamides

Compound[a]	Retention time (min)	R^2	Calibration range (ng mL^{-1})	ILOD (pg)	Repeatability (RSD 500 ng L^{-1})
Sulfathiazole	4.58	0.9994	0.05–1000	0.3	18
Sulfadiazine	4.70	0.9996	0.05–1000	0.3	18
Sulfapyridine	4.88	0.9999	0.055–1000	0.1	20
N^4-acetylsulfamethazine	5.10	0.9999	0.05–1000	0.1	34
Sulfamethazine	6.21	0.9999	0.05–1000	0.3	29
Sulfamethoxypyridazine	6.49	0.9996	0.05–1000	0.1	26
Sulfamethizole	6.62	0.9996	0.05–1000	0.2	2
Sulfamethoxazole	12.56	0.9999	0.06–1000	0.3	19
Sulfasoxazole	14.10	0.9999	0.05–1000	0.1	24
Sulfadimethoxine	14.96	0.9971	0.05–1000	0.004	18

[a] Values have been obtained by injection of standard solutions.

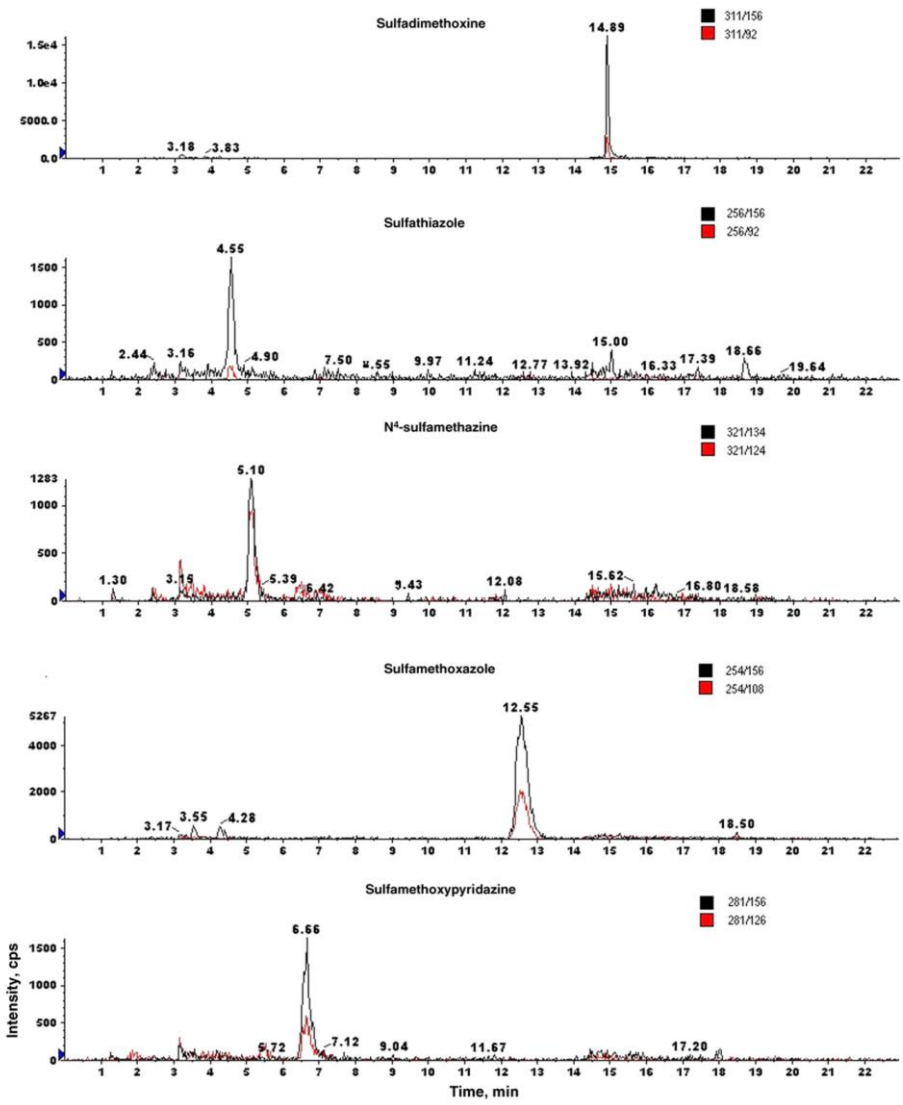

Fig. 2. Reconstructed ion chromatograms showing the two monitored MRM transitions for five of the sulfonamides studied in a spiked groundwater sample at 500 ng L^{-1}. The most intense is the one used for quantification, the second one for confirmation of the compound.

Applied Biosystems (Foster City, CA, USA) equipped with a turbospray electrospray interface. The LC analytical column employed was an Atlantis C18 (Waters) (3 μm, 150 mm × 2.1 mm) preceded by a guard column with the same packing material as the LC column.

2.3. Samples

Surface water samples were collected in the Llobregat River (Barcelona, Spain) in two different sampling campaigns (June 2005

and May 2006). Other surface water samples were taken from the Segre River (Lleida, Spain) and Anoia River (Barcelona) in June 2006. Samples from the wastewater treatment plant (WWTP) in Lleida were also sampled in June 2006. In May 2007 groundwater from the city of Barcelona was also sampled. Bottled mineral water was purchased from a supermarket.

Environmental water samples, around 1 L, were shipped to our laboratory under cool conditions in amber glass bottles pre-rinsed with HPLC grade water in a portable refrigerator, readily vacuum filtered through 2.7 μm glass fiber filters followed by 0.45 μm nylon membrane filters and stored at 4 °C until analysis (less than 2 days). Under such conditions any antibiotic activity in the samples was kept to the minimum. Three sample replicates were taken for each water matrix.

2.4. SPE extraction/purification

The analytical protocol followed for the SPE extraction was adapted from a methodology previously developed and applied by the authors in the purification of pressurized liquid extracts of sludge samples [18] for the analysis of SAs in sludge. Fig. 1 details the three approaches tested. The method using the HLB cartridges was shown to be the most efficient. Under the selected procedure, 400 mL of water samples (200 mL for wastewater) were loaded at 3 mL min^{-1} onto Oasis HLB cartridges preconditioned with 3 mL of MeOH in 50 mM HCOOH, followed by 3 mL of acetone in 50 mM HCOOH and 2 mL of HPLC grade water at neutral pH with a 5% of MeOH. After the sample extraction, the cartridges were rinsed with 3 mL of HPLC grade water at neutral pH with a 5% of MeOH, to remove potential interferences. Finally, cartridges were dried under vacuum (around 30 min.) and then eluted with 6 mL of MeOH in 50 mM HCOOH plus 6 mL of acetone in 50 mM HCOOH at 3 mL min^{-1}. The resulting eluates were brought to dryness under a gentle N_2 stream and reconstituted with 400 μL of MeOH containing d_4-sulfathiazole as internal standard, for further analysis (see Fig. 1).

2.5. LC–QqLIT–MS analysis

Separation was achieved using HPLC grade water with 1% formic acid (pH 2.2) (component A) and ACN with 1% formic acid (component B) as mobile phase. Different LC-gradients were evaluated during the optimization of the method. The best separation was obtained with a linear elution gradient programmed from 25% to 100% B in 11 min, hold for 2 min, and returned to the initial conditions in 10 min. The injection volume was set at 20 μL.

MS data acquisition was performed with the ESI source operating in PI mode under the time-scheduled multiple reaction monitoring (MRM) conditions listed in Table 1. The two most abundant MRMs between the precursor ion and two product ions were monitored per compound: the first one and more abundant was used for quantification and the second one for confirmation as shown in Fig. 2. The optimization of the MS/MS experimental conditions was performed first by infusion and afterwards by on-column injection of standard solutions of the individual compounds and a mixture solution of all of them. Identification of the precursor ions was performed in the full scan mode by recording mass spectra from *m/z* 50 to 500. The resulting optimized values were as follows: capillary voltage 3.5 kV; source temperature, 700 °C; desolvation temperature, 450 °C; extractor voltage 3 V; and RF lens 0.2 V. Nitrogen was used as both the nebulizing and the desolvation gas at 630 L h^{-1}. For operation in the MS/MS mode, argon was used as collision gas with a pressure of 2.6×10^{-3} mbar. Instrument control and data acquisition and evaluation were performed with the Analyst 1.4.2 software package purchased from Applied Biosystems. At the selected values (see Table 1), all compounds underwent soft fragmentation showing one predominant ion, which corresponded to the protonated molecular ion $[M+H]^+$. Further identification of the most abundant product ions and selection of the optimum collision energy, declustering potential (used to minimize the solvent clusters) and collision cell exit potential for each analyte were carried out in the MS/MS optimization mode (values from *m/z* 50 to the mass of the precursor ion plus 100 were tested in order to discard the formation of heavy adducts).

Identification of the target analytes was accomplished by comparing the LC retention time and the MS/MS signals of the target compounds in the samples with those of standards analyzed under identical conditions. For a positive identification, the following criteria had to be met: (i) LC retention time agreement within 2%; (ii) relative abundance of the two selected precursor ion-product ion transitions within a margin of ±20% [19]. Working under the conditions described, the requirements set by the EU regulation regarding the identification and confirmation of organic pollutants in the environment [20] (≥3 IP) was accomplished.

2.6. Method validation

For internal standard based quantification, eight-point calibration curves, based on peak areas, were constructed using a least-square linear regression analysis from the injection of standard mixtures of the analytes at concentrations ranging between 0.05 and 1000 ng L^{-1}.

For estimation of the method recovery (*R*%) three samples of each kind of water matrix were fortified at 500 ng L^{-1}, and were subjected to the entire analytical procedure mentioned before. The instrumental repeatability of the LC–QqLIT–MS equipment was calculated through six consecutive injections of a standard antibiotic mixture solution corresponding to a concentration of 500 ng L^{-1}. Three blank samples for each matrix were also evaluated to avoid overestimations in the calculation of the recovery.

Instrumental detection limits (ILODs) were experimentally calculated from the injection of the standard solution with a concentration corresponding to the lowest used to build the calibration curves (in this study, 0.05 ng L^{-1}). Method LODs and LOQs were experimentally calculated from the analysis of spiked water samples on the basis of a signal to noise ratio of 3 and 10, respectively.

3. Results and discussion

The calibration curves obtained for both the quantification and the confirmation MRMs were linear for all compounds in a wide range of concentrations, typically from 0.05 to 1000 ng L^{-1} with correlation coefficients (R^2) higher than 0.999 for all compounds except for sulfadimethoxine (R^2 0.997) (see Table 2). It has been recently demonstrated the requirement for a quite wide dynamic measuring range of the analytical method as well as high sensitivity in order to quantify base flow concentrations as well as pre-application samples in mass flux studies (temporal variation of SAs concentrations) in agricultural catchments [8].

An internal standard was decided to be added in order to avoid undesired matrix effects previously observed in previous studies on the analysis of SAs in sludge extracts by LC–ESI–MS/MS [18]. Isotopically labeled standards are the best option, however, there are very few commercially available for SAs. The internal standard, d_4-sulfathiazole was selected because it elutes within the same chromatographic time frame as the group of SAs studied shares a common ring structure with most of them, and responds satisfactorily in ESI (PI) mode.

Table 3
Comparison between the recovery rates obtained using HLB, MCX and tandem (TD) approaches

Compound	Segre River			WWTP effluent		Groundwater BCN		Bottled mineral water	
	HLB	MCX	TD	HLB	TD	HLB	MCX	HLB	MCX	TD
Sulfadiazine	128	69	–	106.6	–	105	99.4	102	128	–
Sulfadimethoxine	–	86.4	–	–	–	124	87.4	126	102.57	123
Sulfamethazine	109	70	75.6	102.6	127.7	124	134.7	147	140.97	148
Sulfamethizole	139	72.15	–	102.7	138.9	130	120	132	140.37	–
Sulfamethoxazole	–	58	–	–	–	125	77.4	138	113.78	136
Sulfamethoxypyridazine	102.9	–	92.49	101	102.6	75	62.9	82	73.72	94.7
Sulfapyridine	102.3	–	88.37	102.7	82.9	67	71.57	85.6	76.27	104.5
Sulfathiazole	97.10	–	77.1	93.7	67.5	129	116.27	–	141	86
Sulfasoxazole	124.4	55	–	97.8	148.07	89.92	61.26	97.8	71	141
N^4-acetylsulfamethazine	–	110	–	91.98	–	102.22	–	91.98	93.82	91.1

Recovery values below 50% or over 150% were neglected (–) and not included here. RSD for MCX and TD were in the range 1–44% and 4–16%, respectively. Samples from the Llobregat and Anoia rivers were processed only with HLB cartridges. For the recovery of Llobregat River samples see Table 4.

Table 4
Recovery rates (*R*%), obtained using HLB cartridges

Compound	Segre River				Llobregat River				Groundwater BCN				WWTP effluent				Bottled mineral			
	R%	RSD (%)	LOD ($ng\,L^{-1}$)	LOQ ($ng\,L^{-1}$)	*R*%	RSD (%)	LOD ($ng\,L^{-1}$)	LOQ ($ng\,L^{-1}$)	*R*%	RSD (%)	LOD ($ng\,L^{-1}$)	LOQ ($ng\,L^{-1}$)	*R*%	RSD (%)	LOD ($ng\,L^{-1}$)	LOQ ($ng\,L^{-1}$)	*R*%	RSD (%)	LOD ($ng\,L^{-1}$)	LOQ ($ng\,L^{-1}$)
Sulfadiazine	128	2	1.02	3.41	132	5	0.09	0.31	105	9	0.48	1.60	107	12	0.65	2.15	102	10	0.22	0.74
Sulfadimethoxine	–	–	0.31	1.02	–	–	0.04	0.12	124	5	0.01	0.05	–	–	0.19	0.63	126	6	0.02	0.05
Sulfamethazine	109	6	0.14	0.46	121	3	0.03	0.11	124	17	0.07	0.25	103	2	43.09	143.65	147	3	0.04	0.14
Sulfamethizole	139	4	0.42	1.41	97	7	0.02	0.06	130	5	0.07	0.22	103	35	0.16	0.53	132	12	0.04	0.12
Sulfamethoxazole	–	–	0.05	0.15	62	5	0.05	0.17	125	8	1.13	3.77	–	–	0.25	0.83	138	6	0.09	0.30
Sulfamethoxypyridazine	103	4	0.02	0.07	62	5	0.77	2.57	75	21	0.05	0.17	101	4	0.26	0.88	82	10	0.03	0.09
Sulfapyridine	102	5	0.07	0.22	–	–	0.18	0.62	67	3	0.05	0.18	103	5	0.18	0.59	86	5	0.04	0.14
Sulfathiazole	97	4	0.14	0.47	114	3	0.19	0.64	129	12	0.20	0.67	94	3	0.08	0.26	–	7	–	–
Sulfasoxazole	130	4	0.04	0.12	61	8	0.01	0.04	90	3	0.03	0.09	98	53	0.29	0.97	98	7	0.02	0.08
N^4-acetylsulfamethazine	100	–	0.24	0.80	140	7	0.22	0.73	102	8	0.11	0.35	92	–	460.90	1536.34	92	4	0.08	0.27

Precision, expressed as RSD (%), and method limits of detection and quantification for the different water matrices studied. Recovery values below 50% or over 150% were neglected (–).

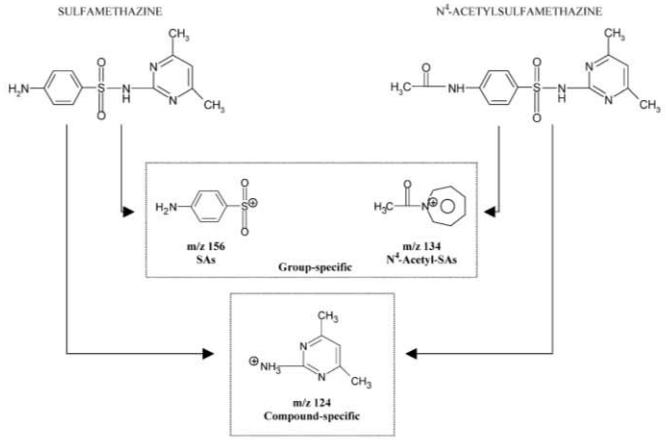

Fig. 3. Structures and fragmentation patterns of sulfamethazine and its N^4-acetylated metabolite.

Quantification was performed on standard in solvent, since matrix-matched standards is not viable due to the considerable number of different matrices analyzed.

The instrumental repeatability, expressed as RSD, was in general <20%. The metabolite showed the higher dispersion with a value of 34% (see Table 2).

The highest recovery achieved in all water matrices were those corresponding to the Oasis HLB cartridge strategy, and were similar to those previously reported in the literature. The results corresponding to the extraction using Oasis MCX cartridges were the ones showing the lowest recovery, as listed in Table 3, and the higher RSD values (1–44%). Recovery rates from the tandem were also quite good (see Table 3), but showed generally higher dispersion between replicates (RSD 4–16%). This may be due to the higher manipulation of the samples during the extraction step. These findings are in agreement with the results of Christian et al. [9], who used the polymeric SDB-2 cartridge followed by a HLB in the extraction of several antibiotics, including sulfamethazine and sulfamethoxazole from river water. In that study, good recovery for both sulfonamides were attained, 95% and 100%, respectively, at 100 ng L^{-1} spiking concentration; in contrast, at a lower concentration level (1 ng L^{-1}) recovery was unacceptable (154% and 203%, respectively). Another study using anion exchange cartridges in tandem with HLB to isolate the same two SAs from wastewater [6], evidenced the poor recovery in both secondary and final effluent samples, with values in the range 37–65% at 1000 ng L^{-1} spiking concentration with precision also not satisfactory (up to ± 31).

Concerning the different matrices tested, recovery corresponding to the effluent from the WWTP (using Oasis HLB cartridges) was the highest, >92% (see Table 4). Surface water taken from the Segre River showed quite high recovery, in the range 97% (sulfathiazole)–139% (sulfamethizole). Water from the Llobregat River, however, exhibited lower values, being the lowest one 61% (sulfasoxazole). Recovery for Barcelona groundwater and bottled mineral water were quite good, in general, higher than 90% and 92%, respectively. RSD were in most cases lower than 20%, with values usually between 2% and 10%, except for sulfasoxazole and sulfamethizole in WWTP effluent waters, with values of 53% and 35%, respectively.

Recovery values over 100% for some of the compounds may be explained by very low concentration levels of these compounds in the original surface water which could not be detected before spiking. This possibility, however, cannot be applied to bottled mineral water samples. In addition, small differences in the structure of the analytes with respect to the internal standard might account for a different ionization behavior, leading to slightly high recoveries for some of the SAs.

Low recovery rates may be attributed to the higher polarity and water solubility of these compounds. But as the other performance data, especially linearity and sensitivity (LOD < 0.01 ng L^{-1}) were excellent, the relatively low recovery were no drawback for a reliable determination of these compounds in water samples.

LODs were extremely low, in the pg L^{-1} level in all water matrices (see Table 4), with nearly all values in the range of 0.02–0.22 ng L^{-1} in bottled mineral water, 0.01–0.77 ng L^{-1} in surface water, 0.08–0.65 ng L^{-1} in wastewater and 0.01–1.13 ng L^{-1} in municipal ground water. In wastewater, however, LODs for sulfamethazine and the metabolite were relatively quite high, with values of 43 and 461 ng L^{-1}, respectively.

To establish comparisons with the LODs reported in the literature results very difficult, because the criteria and experimental methodologies followed for their estimation were different. Despite that, we can conclude that with the developed method LODs achieved are much lower than those reported to date; for instance, previous LODs achieved were 1–50 ng L^{-1} for surface water [8–13], and 1–90 ng L^{-1} for wastewater [3,6,7] around two orders of magnitude higher than those attained with the new method.

All sulfonamide antibiotics are derivatives of sulfanilamide. A typical sulfonamide's fragment loss, in ESI (PI) mode, is detected at m/z 156, which results from the cleavage of the S–N bond yielding the stable sulfanilamide moiety $[M-RNH_2]^+$. In our study, in most cases the transition $[M+H]^+ > m/z$ 156 was selected for quantification (see Table 1). The fragmentation process yields in addition other group-specific ions such as m/z 92 ($[M-RNH_2-SO_2]^+$), as well as a number of compound-specific ions from the variable amine substituent RNH_3 ($[M+H-155]^+$) [21]. Similarly, ^{4}N-acetylated sulfonamide metabolites show a number of group-specific product

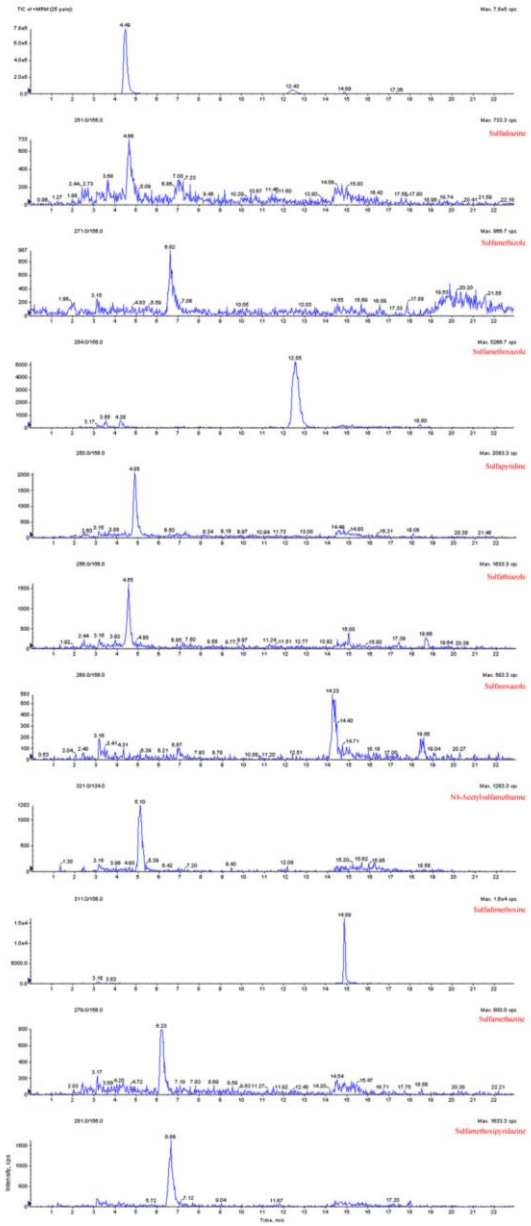

Fig. 4. Total ion current and ion chromatograms corresponding to the SPE extraction and LC–QqLIT–MS analysis of a natural surface water sample from the Llobregat River.

Table 5
Sulfonamide concentrations (ng L^{-1}) detected in the water samples analyzed

Compound	Segre River	Wastewater	Groundwater BCN	Bottled mineral water	Llobregat River		Anoia River	
					C1	C2	C1	C2
Sulfadiazine	16.2	34.3	<LOD	<LOQ	222.4	2312	<LOD	1.9
Sulfadimethoxine	12.1	12	0.2	0.164	182.4	62.7	1.5	3.4
Sulfamethazine	12	10.7	<LOQ	<LOQ	1336	6192	<LOD	1.7
Sulfamethizole	7	48.5	<LOQ	<LOQ	0.9	<LOQ	n.d.	<LOD
Sulfamethoxazole	70.9	241.6	9.9	<LOQ	1488	218.4	n.d.	6.4
Sulfamethoxypyridazine	4.4	–	<LOQ	<LOQ	31.6	3704	n.d.	n.d.
Sulfapyridine	6.5	28.8	<LOQ	<LOQ	2584	12000	11.8	1.2
Sulfathiazole	6.8	53.7	<LOQ	<LOQ	38.1	332	1.5	15.8
Sulfasoxazole	1.3	1.6	<LOD	<LOQ	0.5	2.8	n.d.	n.d.
N^4-acetylsulfamethazine	27.4	<LOD	2.7	1.1	30.7	316.8	<LOD	0.7

C1 correspond to the sampling of June 2005 and C2 to that of May 2006. n.d.: not detected, <LOD: below the limit of detection, and <LOQ: below the limit of quantification.

ions which indicates an identical fragmentation pattern as compared to those of the dosed compounds, i.e. at *m/z* 134 (92 + 42) and 198 (156 + 42) together with characteristic compound-specific product ions, such as *m/z* 65 for ^{4}N-acetylsulfamethoxazole and its related unmetabolized compound [8,12,16]. In this work, the *m/z* 124 was chosen for confirmation of the ^{4}N-acetylsulfamethazine as compound-specific ion and *m/z* 134 as the corresponding ^{4}N-acetylated group-specific fragment. Therefore, similarly to the case of the precursor ions for SAs, in which the *m/z* 156 common fragment provides the best basis for the MS identification and determination of the whole group, the *m/z* 134 common fragment would be the key for the ^{4}N-acetylated sulfonamide metabolite group to be mass spectrometrically detected and quantified. The structures of such fragments corresponding to sulfamethazine and its ^{4}N-acetylated metabolite are shown in Fig. 3.

4. Application to natural samples

The applicability of the method was assessed through the analysis of the selected SAs in several water samples, as shown in Table 5.

The highest SAs concentrations were those corresponding to surface water from the Llobregat River, likely due to the many agricultural areas located upstream of the sampling point, as well as to the proximity of Barcelona city. Fig. 4 shows the chromatograms corresponding to one of the three replicates of the surface waters taking during the first campaign (C1).

As expected, the lowest concentrations corresponded to the bottled mineral water and the groundwater of Barcelona, in which nearly all the sulfonamides detected were below the LOD. Sulfadimethoxine was the most frequently SAs residue detected; being present in all water samples, even in the mineral and groundwater (see Table 5). Sulfamethoxazole was also often detected in natural waters and at high concentrations (maximum 1488 ng L^{-1}), likely because it is used in both human (anti-acne agent) and veterinary medicine (antibiotic and growth promoter). Since it is administered orally and it is known to be metabolized (mainly undergoing acetylating) in the body to an extent >80% [22], its related metabolite, ^{4}N-acetylsulfamethoxazole, may be also present at relatively high concentration in the environment as pointed out in previous works [8–16]. Despite that, the highest concentration found in the natural water samples corresponded to sulfapyridine, which was found to be present in river water up to 12,000 ng L^{-1}.

5. Conclusions

A highly sensitive analytical method, based on SPE extraction and purification followed by LC–QqLIT–MS analysis in MRM mode, has been developed for the trace multiresidue analysis of sulfonamide antibiotics. Dosed compounds and one N^4-acetylated metabolite could be simultaneous determined with the required sensitivity (limits of detection below 1.13 ng L^{-1} for all compounds). Identification of the target analytes is based on the LC retention time and the monitoring of two MRM transitions per compound, which increases the selectivity and eliminates in great measure potential interferences, and therefore minimizes the possibility for erroneous identifications and allows accomplishing with EU regulation. The developed method was proved to be a powerful tool for the analysis of SAs and N^4-acetylated metabolites in complex natural waters, allowing determining concentrations down to the ng L^{-1} level in all kind of waters (wastewater, groundwater, surface and bottled mineral water). Results on groundwater and mineral bottled water constitute the first data reported on levels of SAs in these types of waters.

On the basis of these outcomes, the N^4-acetylated metabolites of sulfonamide antibiotics should be considered in environmental residue analysis to avoid the underestimation of the elimination rates of dosed SAs during wastewater treatments. In this study, N^4-acetylsulfamethazine was detected in all the samples at concentration up to 316 ng L^{-1} (in surface water).

Acknowledgements

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Determination of 19 sulfonamides in environmental water samples by automated on-line solid-phase extraction-liquid chromatography–tandem mass spectrometry (SPE-LC–MS/MS)

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ABSTRACT

The present study describes the development, validation and a practical application of a fully automated analytical method based on on-line solid-phase extraction-liquid chromatography–tandem mass spectrometry (SPE-LC–MS/MS) for the simultaneous determination of 19 sulfonamides, including one acetylated metabolite, in different water matrices. MS/MS detection was carried out in a quadrupole-linear ion trap (QqLIT) mass analyzer. Target compounds were identified in the selected reaction monitoring (SRM) mode, recording two transitions between precursor ions and the two most abundant product ions. The method developed was applied to evaluate the occurrence of the target antibiotics in different water samples: influent and effluent water from waste water treatment plants (WWTP), ground water and surface water. Under optimal conditions, the method detection limits achieved were in the range 0.05–7.84 ng/L for WWTP influent water, 0.01–6.90 ng/L for WWTP effluent water, 0.02–5.13 ng/L for ground water and 0.02–4.52 ng/L for surface water samples. The instrumental repeatability, expressed as RSD, was usually below 10% for the different water matrices. Results showed the wide presence of sulfonamides in the four types of water, including one acetylated metabolite, with maximum concentrations up to 855 ng/L corresponding to sulfapyridine in an influent waste water sample near a densely populated urban area.

1. Introduction

The continuous improvement of analytical methodologies has lowered the limits of detection for a wide array of trace xenobiotics, including pharmaceuticals, in environmental matrices, making their presence, despite at low concentrations, evident during the last few years [1].

Several tonnes of human and veterinary pharmaceuticals are used in Europe every year. Together with their metabolites, remnants of the active compounds (which have not been fully assimilated within the organism) end up in rivers and other natural systems. Waste water treatment plants (WWTPs) can be considered as main contributors to the presence of these substances in the environment, since all the residues from the different pharmaceutical discharges may gather in sewage waters. Besides, some of these compounds show low removal during waste water treatment processes, unspecific for these kind of molecules, and therefore are able to reach surface waters and other environmental matrices afterwards [2].

Since their discovery, antibiotics have been widely used in both human and veterinary medicine, farming and aquaculture, being the estimated total antibiotic market consumption world-wide between 100 000 tonnes and 200 000 tonnes [3]. Sulfonamides represent one of the most commonly used families of antibiotics in veterinary medicine. Although they were frequently applied as human medicines to treat many kinds of infection, nowadays much higher quantities are applied to treat and prevent infectious diseases in livestock and intensive cattle farming. The increase in the number of these confined animal feeding operations, which often lack proper waste management practices, is becoming a serious environmental problem as it constitutes one of the main release sources of these antibiotics in the natural media [4]. Previous studies have demonstrated that residues of sulfonamides (i.e., sulfathiazole and sulfamethazine) were present in manure in levels up to 12.4 mg/kg [5,6]. The excretion of faeces and urine from the medicated animals and the subsequent application of the contaminated manure as fertilizer into agricultural lands are among the major routes through which sulfonamides enter the environment. As they are weak acids

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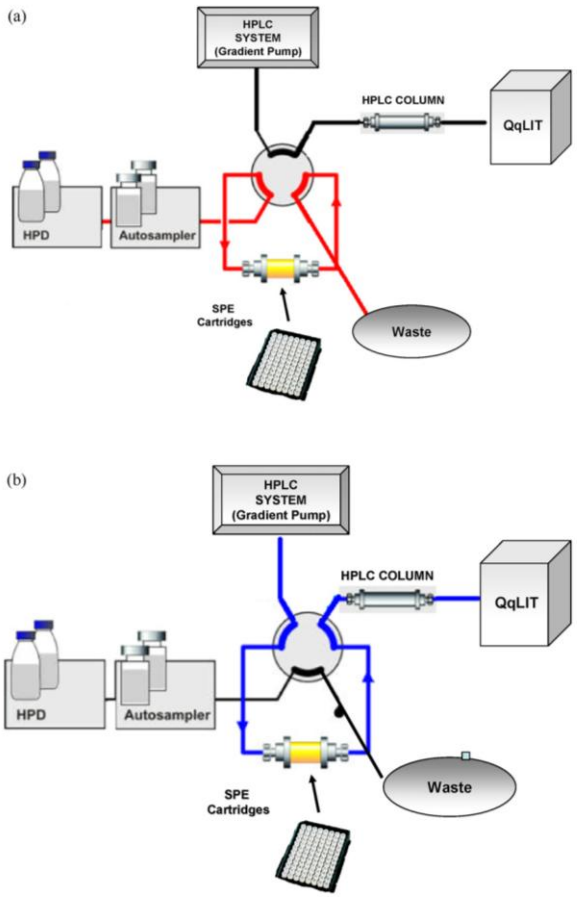

Fig. 1. On-line SPE procedure carried out with Prospekt 2™: (a) extraction of the analytes and (b) elution step.

and both fairly water-soluble and polar compounds, sulfonamides are retained weakly in soil systems, having a high potential for leaching or running off to ground waters and surface waters respectively after their release into the environment [7–11]. Intense rainfall events or the application of manure on irrigation crop lands accelerate these processes and, consequently, the diffuse contamination of ground waters and surface water by these compounds. Similarly, pasture animals may also spread the drugs and their respective metabolites via dung pats or urine into the fields. Aquaculture, hospital effluents, disposal of unused drugs and discharge from WWTPs are other sources to be considered when investigating the origin of the sulfonamides in the environment [12].

Sulfonamide antibiotics have been found in all kinds of water matrices [13]. Despite the low concentrations detected, they are being continuously introduced in the aquatic environment, and concentrations that were previously considered as harmless are leading to the emergence of antibiotic resistant bacteria strains and potential implications for human health and the environment.

Up to now, several studies on the environmental presence and analysis of sulfonamides have been published and different LC–MS/MS analytical methods have been developed for waste waters [14–18], surface water [14–17,19,20] and ground waters [7,9,10] separately or using the same methodology to analyze different matrices [9]. Richter et al. [21] analyzed all the water matrices mentioned before together with drinking water, being four the target sulfonamides and none of them within the scope of this study. Díaz-Cruz et al. [13] analyzed 10 sulfonamides in all the water matrices aforementioned, without affecting the sensitivity and the performance of the method and being equally suitable for all of them. The need to minimize the sample preparation, to improve sample throughput and to reduce analysis cost is a relevant issue, as usually methods for the extraction and quan-

Table 1
Optimized time scheduled SRM transitions used for the LC–MS/MS analysis of the sulfonamides studied (positive ionization mode).

Compounds	$[M+H]^+$	SRM transitions	RT	DP	CE	CXP	SRM Ratio ± STD
Sulfacetamide	215	215/156	3.2	46	21	10	1.40 ± 0.31
		215/92		46	35	6	
Sulfisomidin	279	279/124	3.3	76	33	8	2.02 ± 0.11
		279/186		76	23	14	
Succinyl-sulfathiazole	356	356/256	4.2	71	25	16	1.58 ± 0.22
		356/192		71	33	16	
Sulfathiazole	256	256/156	4.3	40	25	14	5 ± 0.26
		256/92		40	25	10	
d_4-Sulfathiazole	260	260/160	4.3	71	25	6	3.53 ± 0.27
		260/96		71	25	6	
Sulfaguanidine	215	215/156	4.3	56	13	10	2.33 ± 0.42
		215/92		56	31	4	
Sulfadiazine	251	251/156	4.5	46	27	10	1.30 ± 0.13
		251/108		46	30	8	
N^4-acetylsulfamethazine	321	321/134	4.6	86	35	4	1.45 ± 0.21
		321/124		86	35	4	
Sulfapyridine	250	250/156	4.7	51	28	12	1.17 ± 0.02
		250/92		51	31	6	
Sulfamerazine	265	265/92	5.4	61	47	6	1.30 ± 0.15
		265/156		61	27	8	
Sulfamethazine	279	279/156	6	26	30	10	1.48 ± 0.08
		279/124		26	35	10	
Sulfamethizole	271	271/156	6.3	36	23	12	6.85 ± 0.48
		271/108		36	23	8	
Sulfamethoxypyridazine	281	281/156	6.3	66	27	14	2.01 ± 0.09
		281/126		66	27	12	
Sulfadoxine	311	311/156	10.4	46	29	12	2.25 ± 0.40
		311/92		46	45	4	
Sulfamethoxazole	254	254/156	11.4	56	25	10	2.08 ± 0.24
		254/108		56	27	10	
Sulfisoxazole	268	268/156	12	71	21	10	1.45 ± 0.23
		268/113		71	21	8	
Sulfaquinoxaline	301	301/156	13	76	25	10	1.78 ± 0.14
		301/92		76	47	12	
Sulfabenzamide	277	277/156	13	56	17	10	1.71 ± 0.35
		277/92		56	41	6	
Sulfadimethoxine	311	311/156	13	76	31	8	4.37 ± 0.20
		311/92		76	31	6	
Sulfanitran	336	336/156	14.7	66	17	12	2.05 ± 0.25
		336/198		66	29	14	

RT: retention time (min); Compound dependent parameters: CE: collision energy (eV); DP: declustering potential (V); CXP: collision cell exit potential (eV). SRM ratio given with the standard deviation.

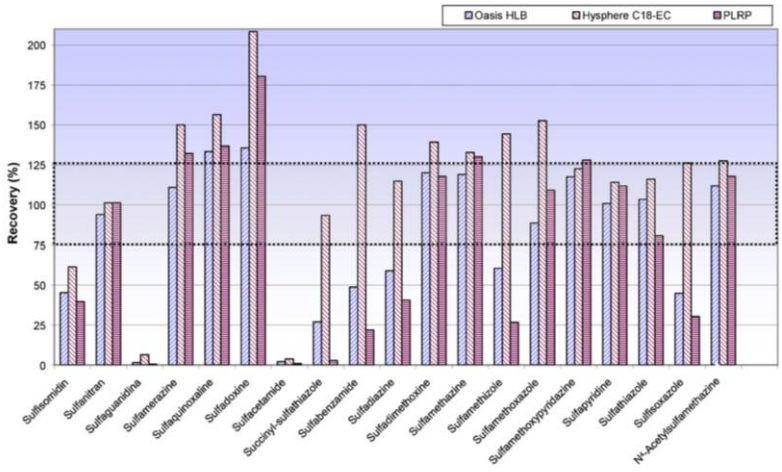

Fig. 2. Recovery values obtained from the evaluation of HLB Oasis, Hysphere C18 EC and PRLPs cartridges.

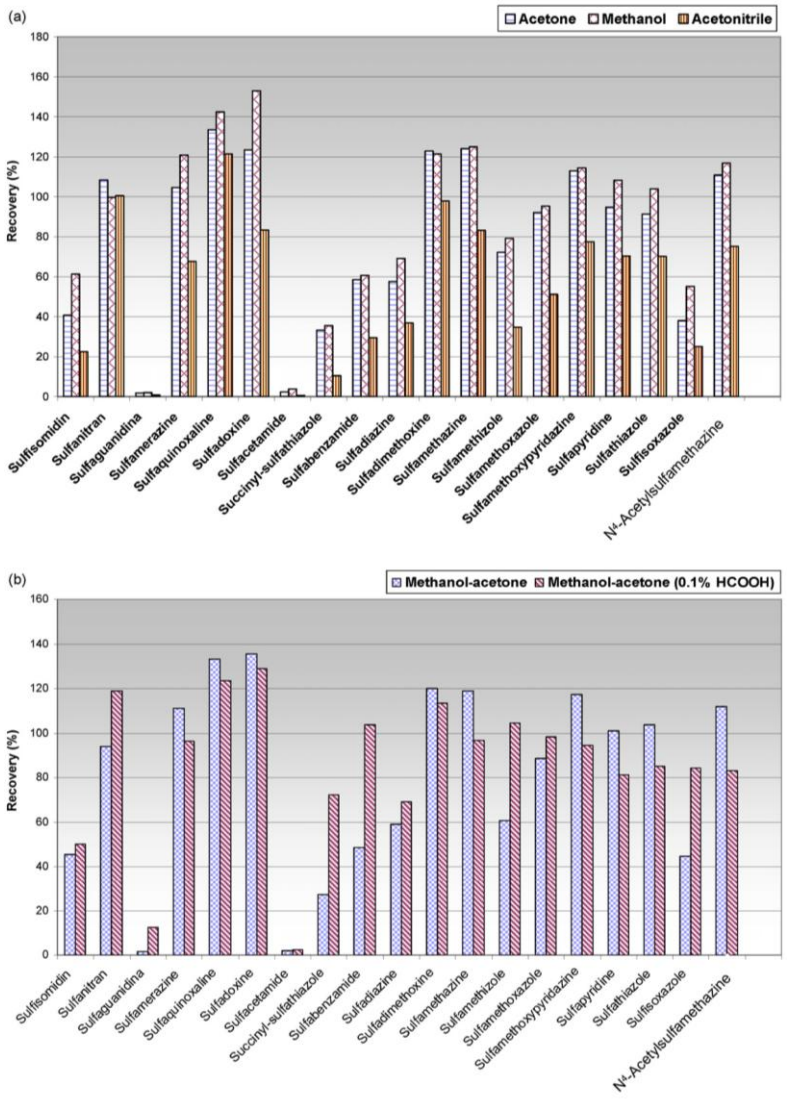

Fig. 3. Recovery values obtained from the evaluation of the recoveries using different equilibration solvents. (a) Acetonitrile, acetone and methanol. (b) Mixtures of methanol and acetone.

tification of antibiotics and pharmaceuticals in general in water matrices are time consuming and involve several steps (filtering and homogenization, clean-up of the sample and preconcentration and final analysis). Given the need of analytical methodology capable of detection at environmental levels (low picogram per liter level), and taking the environmental and analytical concerns mentioned into account, the aim of this work is to develop an automated multi-residue analytical method, based on on-line solid-phase extraction-liquid chromatography–tandem mass spectrometry (SPE-LC–MS/MS), for the simultaneous determination

of 19 selected sulfonamides in natural waters. This new method integrates LC–MS/MS analysis with on-line SPE, which is one of the most suitable sample preparation approaches available. Minimum sample manipulation, sample volume, time and solvents savings, and improved throughput are among the main advantages provided by this technique. Previous works account for the many advantages of this on-line SPE procedure [22–24] but, to the authors' knowledge, only one publication deals with on-line

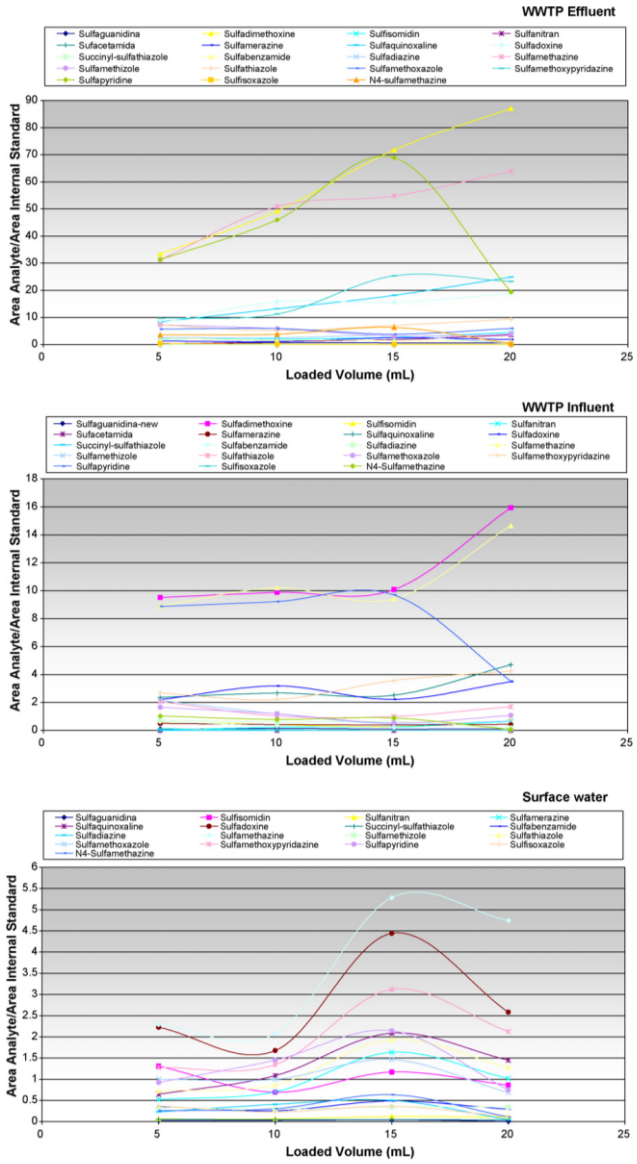

Fig. 4. Breakthrough curve representations for the different water matrices studied.

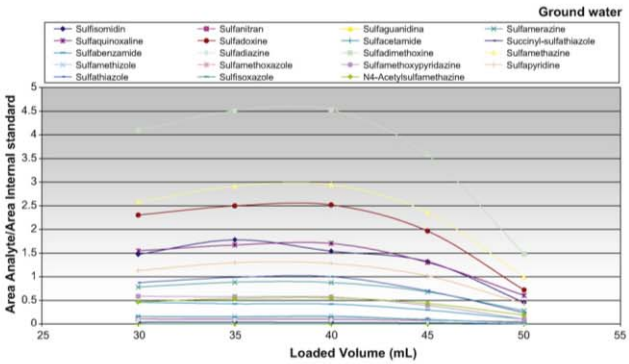

Fig. 4. (*Continued*).

SPE-LC–MS/MS analysis of sulfonamides [19], in which five sulfonamides and their corresponding acetylated metabolites were studied in surface waters.

The last part of our study covers the application of the new methodology to assess the occurrence and fate of 19 selected sulfonamides, including one of their metabolites, in WWTP influent and effluent water, surface water and ground water samples, taken all of them in the region of Catalonia and along the Ebro River Basin.

2. Experimental

2.1. Reagents and chemicals

HPLC-grade solvents (water, methanol, acetone and acetonitrile) and formic acid (98–100%) were supplied by Merck (Darmstadt, Germany).

High purity standards (>99%) of the 19 selected sulfonamides were purchased from Sigma (St. Louis, MO, USA). Stock standard solutions for each one of the analytes were prepared in methanol (MeOH) at 1 mg/mL and stored in the dark at −2 °C. Standard solutions of the mixtures of all compounds at concentrations ranging between 1 ng/mL and 500 μg/mL were prepared by appropriate dilution of the stock solutions in MeOH. The standard mixtures were used as spiking solutions for preparation of the aqueous calibration standards and in the recovery studies. Aqueous standard solutions contained <0.1% of MeOH.

Internal standard d_4-sulfathiazole (99.9%) was purchased from Toronto Research Chemicals (Ontario, Canada). Stock solutions were also prepared in methanol and stored at −2 °C until use.

2.2. Sample collection

Twenty-four hours-integrated samples of WWTP influent or effluent waters were taken in four different WWTPs. Surface water and ground water samples within agricultural areas were taken simultaneously. All the water matrices were collected in amber polyethylene terephthalate (PET) bottles and transported to the laboratory under cooled conditions (4 °C). Upon reception, samples were filtered through 0.45 μm Nylon filters (Whatman, Maidstone, UK) to eliminate suspended solid matter and then stored at 4 °C in the dark until analysis which was always carried out within 48 h of collection to avoid degradation.

2.3. Method development

2.3.1. On-line solid-phase extraction

Fully automated on-line preconcentration and purification of samples, aqueous standards and operational blanks was performed using an automated on-line SPE sample processor Prospekt-2™ (Spark Holland, Emmen, The Netherlands). This system consists of an automated cartridge exchange (ACE) module, which holds two trays of 96 extraction cartridges each, and a high pressure dispenser module (HPD) for handling of solvents by a 2 mL high pressure syringe. SPE solvents for conditioning, equilibration, sample application and clean up are provided by the HPD. The ACE module has two clamps and two high pressure valves. An aliquot of the raw sample is introduced by the autosampler and, when the SPE is completed, the cartridge is transferred to the elution clamp where the analytes will be eluted from the SPE cartridge directly onto the LC column by the HPLC. A scheme of the apparatus is represented in Fig. 1. The whole eluted volume gets to the chromatographic system instead of a final reconstituted extract as in off-line procedures, where usually volumes of 200 mL or bigger are reduced to approximately 0.5 mL and only around 20 μL will be injected in the mass analyzer [2,13]. During LC–MS/MS analysis, the extraction of the next sample is carried out on a new cartridge on the other clamp. Therefore, SPE is carried out entirely in parallel with the LC–MS/MS run. This configuration shortens the cycle times (in this case 23 min of sample analysis plus the conditioning and equilibration times only for the first sample). The Prospekt-2™ is controlled by means of the Sparklink software version 3.0 (Spark Holland).

2.3.2. LC–MS/MS analysis

LC-tandem MS analyses were carried out in a system consisting of an HP 1100 chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbospray electrospray (ESI) interface. The chromatographic separation was performed using an Atlantis C18 (Waters, 150 mm × 2.1 mm, 3 μm of particle size) LC-column preceded by a guard column with the same packing material. Sulfonamides were analyzed in the positive ionization mode (PI). The flow rate was set to 0.2 mL/min, being eluent A HPLC grade water slightly acidified with 0.1% of formic acid, and eluent B acetonitrile with 0.1% formic acid. The elution gradient started with 25% of eluent B, increasing to 80% in 10 min and 100% in 11 min. During the further 2 min the column was cleaned and readjusted to the initial conditions in 3 min, and equilibrated for 7 min.

Table 2
Quality parameters of the SPE-LC–MS/MS analytical method for all the water matrices studied: linear correlation coefficients (r^2). iLOD: instrumental limit of detection. iLOQ: instrumental limit of quantification. RSD: intraday repeatability expressed as relative standard deviation.

Compounds	WWTP influent			WWTP effluent			Ground water			Surface water		
	$(r^2)^a$	iLOD (pg injected)	RSD[b] (%)	$(r^2)^a$	iLOD (pg injected)	RSD[b] (%)	$(r^2)^a$	iLOD (pg injected)	RSD[b] (%)	$(r^2)^a$	iLOD (pg injected)	RSD[b] (%)
Sulfisomidin	0.9998	0.00104	3.23	0.9980	0.00056	2.01	0.9997	0.00083	3.23	0.9999	0.00059	4.37
Sulfanitran	0.9998	0.00418	2.59	>0.9999	0.00097	2.00	0.9996	0.00041	2.59	0.9996	0.00091	3.32
Sulfaguanidina	0.9998	0.11900	21.6	0.9948	0.07900	9.61	0.9986	0.09450	14.8	0.9999	0.01350	25.95
Sulfamerazine	0.9998	0.00296	1.77	>0.9999	0.00047	2.88	0.9998	0.00196	1.77	>0.9999	0.00086	2.06
Sulfaquinoxaline	0.9998	0.00049	0.51	0.9998	0.00049	0.59	0.9998	0.00030	0.51	0.9991	0.00024	1.43
Sulfadoxine	0.9948	0.00083	1.50	0.9988	0.00016	3.30	0.9999	0.00040	1.50	0.9999	0.00027	2.93
Sulfacetamide	0.9990	0.09530	8.23	0.9855	0.64300	7.24	0.9982	0.37700	11.0	0.9994	0.01910	8.08
Succinyl-sulfathiazole	0.9978	0.00430	9.53	0.9849	0.01490	12.9	0.9962	0.36600	9.53	0.9998	0.02730	2.39
Sulfabenzamide	0.9998	0.00277	4.79	>0.9999	0.00068	5.05	>0.9999	0.00244	4.79	0.9998	0.00079	0.07
Sulfadiazine	0.9998	0.00204	4.66	0.9996	0.00094	6.39	0.9998	0.00140	4.66	0.9998	0.00164	3.38
Sulfadimethoxine	>0.9999	0.00050	1.46	0.9998	0.00012	1.16	0.9998	0.00006	1.46	>0.9999	0.00027	2.78
Sulfamethazine	0.9998	0.00110	1.83	0.9994	0.00046	1.86	0.9996	0.00037	1.89	>0.9999	0.00018	2.88
Sulfamethizole	>0.9999	0.00933	3.84	0.9990	0.00364	5.65	0.9999	0.01780	6.61	0.9995	0.01040	5.13
Sulfamethoxazole	0.9996	0.00440	1.55	0.9996	0.00095	2.83	0.9998	0.00259	1.55	0.9998	0.00142	1.16
Sulfamethoxypyridazine	>0.9999	0.00070	1.54	>0.9999	0.00031	2.00	0.9999	0.00033	1.54	0.9998	0.00025	3.82
Sulfapyridine	0.9998	0.00190	3.04	>0.9999	0.00074	4.22	0.9997	0.00034	3.04	0.9999	0.00033	1.90
Sulfathiazole	>0.9999	0.00160	10.3	0.9998	0.00132	1.75	>0.9999	0.00210	10.3	>0.9999	0.00132	1.31
Sulfisoxazole	0.9998	0.00160	6.70	0.9982	0.00108	4.24	0.9998	0.01590	6.70	0.9999	0.00443	5.25
N^4-acetylsulfamethazine	0.9998	0.00280	4.42	0.9996	0.00074	3.85	0.9998	0.00112	4.42	0.9997	0.00009	1.00

[a] Calibration range between 0.05 ng/L and 1000 ng/L.
[b] Relative standard deviation ($n = 5$, spike concentration 100 ng/L).

For increased sensitivity and selectivity, MS/MS data acquisition was performed in the selected reaction monitoring (SRM) mode. For each analyte, two transitions between precursor ions and the two most abundant product ions were monitored; the more abundant one was used for quantitation and the other one for confirmation. Table 1 shows the optimized LC–MS/MS conditions used for the analysis of the target analytes. The optimization of the MS/MS experimental conditions was performed in a previous study [13] and were as follows: capillary voltage, 3.5 kV; source temperature, 700 °C; desolvation temperature, 450 °C; extractor voltage, 3 V; and RF lens, 0.2 V. Nitrogen was used as both the nebulizing and the desolvation gas at 630 L/h. For operation in the MS/MS mode, argon was used as collision gas with a pressure of 2.6×10^{-3} mbar. Instrument control and data acquisition and evaluation were performed with the Analyst 1.4.2 software package purchased from Applied Biosystems.

3. Results and discussion

3.1. On-line SPE optimization

Extraction efficiency of a SPE procedure is controlled mainly by (1) the nature of the adsorbing material, (2) the conditioning and elution solvents used and (3) the sample volume loaded.

3.1.1. Adsorbing material

The first stage of the SPE optimization was to determine the most appropriate adsorbing material. For this purpose, three different disposable trace enrichment cartridges were evaluated for their efficiency in the on-line SPE of the target sulfonamides from water: the polymeric cartridge Oasis HLB (macroporous polymer of divinylbenzene and *N*-vinylpyrrolidone, 30 μm particle size) from Waters (Barcelona, Spain), the polymeric phase PLRP-s (cross-linked styrenedivinylbenzene polymer, 15–25 μm particle size) from Spark Holland, and the silica-based cartridge Hysphere C18 EC (endcapped octadecyl phase, 8 μm particle size) also from Spark Holland. 10 mL of HPLC water spiked with a mixture of all the analytes at 100 ng/L were loaded at 1 mL/min onto the cartridges, previously conditioned with 1 mL of a mixture of methanol and acetone and 1 mL of water (flow rate 1 mL/min). Conditioning and equilibration were performed with the same solvents as in the off-line SPE procedure previously developed by the same authors [13]. Triplicates were run for each kind of cartridge. After sample loading and prior to elution, the cartridges were washed with 1 mL of water at a flow rate of 1 mL/min to improve the complete transfer of the sample and remove interferences. Recoveries were based on the ratio between the peak areas obtained with the on-line analysis and the results from a parallel off-line analysis of a standard mixture of the sulfonamides (same total mass injected in both cases). Recovery values are shown in Fig. 2, where a goodness range between 75% and 125% has been marked. As it can be seen, despite recoveries are higher for the Hysphere C18 cartridges, these values are for most of the analytes far above 100%, being this the main reason why this adsorptive material was discarded. Although PRLPs and Oasis HLB showed similar recoveries, the latter was finally selected as it has been previously proved to be suitable in on-line SPE [19] and off-line SPE procedures for the same family of compounds [2,13,18,25–26].

3.1.2. Solvents

Once the extraction cartridge has been selected, different solvents for the conditioning step of the cartridge were evaluated (acetonitrile, methanol and acetone separately). The same volume

Table 3
Method limits of detection (MLOD) and quantification (MLOQ) corresponding to the first SRM transition for all the sulfonamides.

Compounds	WWTP influent		WWTP effluent		Ground water		Surface water	
	MLOD	MLOQ	MLOD	MLOQ	MLOD	MLOQ	MLOD	MLOQ
Sulfisomidin	0.10	0.33	0.13	0.43	0.03	0.11	0.05	0.16
Sulfanitran	0.56	1.87	0.24	0.79	0.05	0.17	0.03	0.10
Sulfaguanidina	7.84	26.1	1.01	3.37	12.0	40.0	1.01	3.37
Sulfamerazine	0.25	0.83	0.19	0.62	0.12	0.40	0.21	0.70
Sulfaquinoxaline	0.55	1.83	0.04	0.15	0.02	0.08	0.19	0.63
Sulfadoxine	0.12	0.39	0.04	0.14	0.02	0.08	0.19	0.63
Sulfacetamide	–	–	6.90	23.0	5.13	17.1	4.06	13.5
Succinyl-sulfathiazole	7.23	24.1	2.73	9.10	3.29	11.0	2.58	8.60
Sulfabenzamide	0.05	0.17	0.11	0.38	0.22	0.74	0.41	1.38
Sulfadiazine	1.12	3.72	0.90	2.99	0.18	0.59	0.34	1.12
Sulfadimethoxine	0.16	0.54	0.01	0.02	0.02	0.08	0.02	0.07
Sulfamethazine	0.40	1.33	0.06	0.19	0.04	0.13	0.05	0.15
Sulfamethizole	3.01	10.0	1.04	3.47	3.10	10.3	4.52	15.1
Sulfamethoxazole	1.14	3.79	0.77	2.55	0.81	2.69	0.86	2.87
Sulfamethoxypyridazine	0.14	0.47	0.05	0.17	0.03	0.09	0.09	0.31
Sulfapyridine	0.32	1.08	0.13	0.42	0.02	0.05	0.06	0.20
Sulfathiazole	0.28	0.94	0.45	1.48	0.21	0.70	0.22	0.74
Sulfisoxazole	0.50	1.67	2.15	7.16	0.63	2.11	0.31	1.03
N^4-acetylsulfamethazine	2.17	7.25	0.14	0.48	0.02	0.08	0.02	0.06

Both are given in ng/L. Values for sulfacetamide in influent water could not be estimated, as this sulfonamide was not detected in any of the water samples.

of water spiked at 100 ng/L was loaded afterwards (triplicate analysis). As it can be seen in Fig. 3a, acetonitrile showed the lowest recoveries, whereas values for methanol and acetone were quite similar. Two mixtures of both solvents (1:1, v/v) were also studied, one of them at neutral pH and the second slightly acidified with formic acid at 0.1% (Fig. 3b). Differences between these two mixtures were hardly noticeable in the recoveries obtained; in order to make the procedure easier to handle, the mixture without acid was eventually selected.

3.1.3. Sample volume

Natural water samples were spiked with a mixture of the analytes at a concentration of 100 ng/L and volumes from 5 mL to 20 mL were loaded onto the HLB cartridges (5–50 mL for groundwater). Breakthrough curves were made for the different water matrices, where the sample volume extracted was represented against the integrated area obtained under the respective chromatogram peak (Fig. 4). The highest peak areas were generally bigger after extracting 15 mL of surface water and WWTP effluent, 5 mL for WWTP influent and 40 mL for ground water.

3.2. MS/MS detection optimization

The analytical method developed is based on a method previously described by the authors for the off-line SPE extraction and LC–MS/MS analysis of 10 sulfonamides (one of them a metabolite), in environmental water matrices [13]. Nine new sulfonamides were added to the method and correspondingly optimized, first by infusion and afterwards by on-column off-line injection of standard solutions of the individual compounds and a mixture solution of all of them. Identification of the precursor ions and optimum ionization conditions was performed in the full scan mode by recording mass spectra from m/z 50 to 500. Further identification of the most abundant fragment ions and selection of the optimum gas collision energies (CE) for each analyte were carried out in the product ion scan mode.

For the positive confirmation of the target analytes in the samples, strict criteria had to be met in order to avoid false positives. Following the European Commission Decision 2002/657/EC [27], a minimum of three identification points (IPs) is required for this purpose. Besides, the chromatographic retention time of the ana-

Table 4
Sulfonamide concentrations detected in the different WWTPs samples studied (given in ng/L).

Compounds	WWTP1		WWTP2		WWTP3		WWTP4	
	I	E	I	E	I	E	I	E
Sulfisomidin	–	–	–	–	–	<MLOQ	–	–
Sulfanitran	<MLOQ	–	<MLOD	–	<MLOQ	–	–	–
Sulfaguanidine	–	–	<MLOQ	1.88	–	–	–	–
Sulfamerazine	–	–	–	4.94	–	34.6	–	9.85
Sulfaquinoxaline	–	<MLOD	–	<MLOQ	–	<MLOQ	–	–
Sulfadoxine	–	<MLOQ	<MLOQ	<MLOQ	–	–	–	–
Sulfacetamide	–	–	–	–	–	–	–	–
Succinyl-sulfathiazole	–	<MLOQ	–	–	–	–	–	–
Sulfabenzamide	–	–	–	–	–	<MLOQ	–	–
Sulfadiazine	–	<MLOD	–	–	–	–	181	104
Sulfadimethoxine	–	–	–	–	–	–	20.1	10
Sulfamethazine	–	–	–	–	–	18	–	14.7
Sulfamethizole	–	–	247	<MLOD	–	<MLOQ	–	–
Sulfamethoxazole	–	12.4	–	302	–	77.4	89	133
Sulfamethoxypyridazine	–	–	–	–	–	–	–	–
Sulfapyridine	<MLOQ	<MLOQ	2.15	38.3	5.23	8.53	855	113
Sulfathiazole	–	–	–	5.12	–	9.21	37.5	7.46
Sulfisoxazole	–	<MLOQ	–	–	<MLOQ	<MLOQ	–	8.17
N^4-acetylsulfamethazine	<MLOD	<MLOD	<MLOD	<MLOQ	–	–	–	5.29

–: not detected; <MLOD: under the method limit of detection; <MLOQ: under the method limit of quantification.

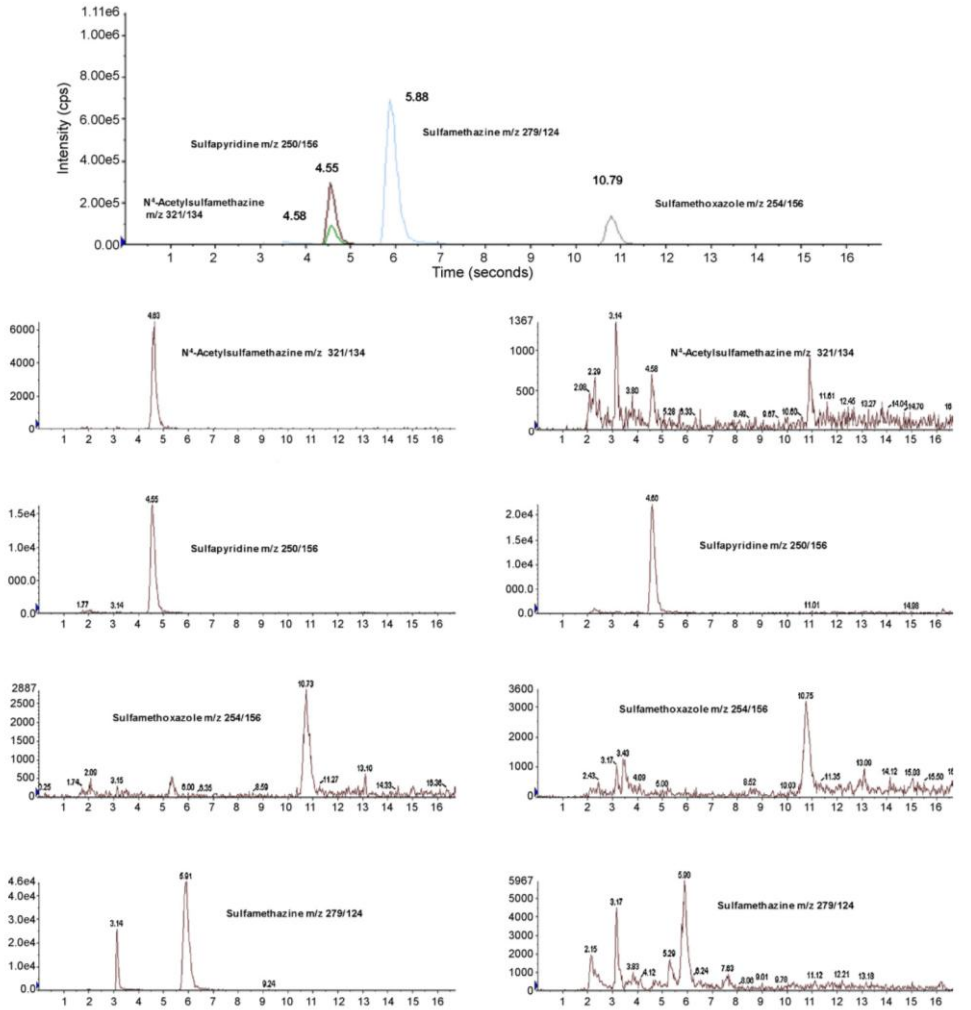

Fig. 5. Reconstructed chromatogram for four of the sulfonamides studied in surface and WWTP effluent water samples, and a reference chromatogram corresponding to an HPLC grade water spiked at 500 ng/L.

lyte in the sample should not vary more than 2% in comparison to the calibration standards', and the relative abundance of the two SRM transitions monitored must also be compared to the standards' corresponding values.

3.3. Method validation

After optimization, the analytical method developed was evaluated in terms of linearity, repeatability, accuracy, selectivity and sensitivity.

Quantification was performed based on peak areas and by the internal standard calibration method, crucial to correct potential matrix effects. Concentrations were estimated for the most abundant SRM transition selected. d_4-sulfathiazole was added to all the samples at a concentration of 500 ng/L right before analysis.

Five to eight point matrix matched calibration curves were constructed for each of the water types, using least-squares linear regression analysis at concentrations ranging from 0.05 ng/L to 1000 ng/L. Correlation coefficients (r^2) were higher than 0.999 for all of the sulfonamides studied.

Sensitivity is one of the method parameters enhanced when peforming on-line SPE analysis. Despite the low sample volumes required, it has been proved that sensitivity is not affected but, on the contrary, improved considerably. Table 2 shows the instrumental limits of detection (iLODs) for each of the four water matrices. Method limits of detection (MLOD) and quantification (MLOQ) were also calculated as the minimum detectable amount of analyte with a signal-to-noise ratio of 3 and 10, respectively. MLOD values were in the range of 0.03–8.44 ng/L for WWTP influent water, 0.21–7.31 ng/L for WWTP effluent water, 0.02–5.13 ng/L for ground water and 0.02–4.52 ng/L for surface water samples (Table 3).

The precision of the method was evaluated by analyzing five consecutive times the corresponding water matrices spiked with a standard mixture of the analytes at 100 ng/L. The relative standard deviations obtained varied from 0.02% to 26% (Table 2).

3.4. Practical application

The applicability of the method was assessed through the analysis of the target sulfonamides in the four different water matrices considered. Water from four WWTPs (influent and effluent samples) and surface water samples from four different rivers (Ebro River and three of its tributaries) were sampled in spring–summer 2008. Ground water samples from four wells in the same area were also taken that year and in October 2007. Fig. 5 shows the chromatograms of four of the sulfonamides most frequently detected in surface and WWTP effluent water samples. The reconstructed chromatogram corresponding to HPLC grade water spiked at 500 ng/L has been also included as a reference for the retention time of the analytes.

WWTP1, WWTP2 and WWTP3 were located in mountain and rural areas and served populations between 500 and 3200 inhabitants. As shown in Table 4, very few sulfonamides could be detected in these three plants, and the estimated concentrations usually remained below the MLOD or MLOQ. Their presence in influent water was usually less frequent and more arduous to determine than in effluents, probably due to matrix effects and the suppression of the signal intensity. For instance, sulfamethoxazole, sulfonamide typically applied in human medicine, was detected in effluent water of the three plants, but not in any of the influent samples. Sulfapyridine, also very common in human therapies to treat intestinal infections, was detected in both influent and effluent samples of WWTP2 and WWTP3, but concentrations in the effluent water were usually higher than that of the influent, which can be attributed also to intense strong matrix effects in the latter, which hampered the identification of the compounds. It could also be attributed to the fact that metabolites and conjugated forms, which are also present in the influent samples, may degrade and retransform into the parent compounds, being these sulfonamides released in the effluent [2]. For these three WWTPs, the highest concentration corresponded to sulfamethoxazole in effluent water of WWTP2 (302 ng/L) and the smaller to sulfaguanidine also in the effluent water sample of WWTP2, with a concentration of 1.88 ng/L.

WWTP4 served around 40 000 inhabitants in an urban area. As expected, the concentrations detected in this location were generally higher than those of the rural WWTPs, with values in influent water up to 855 ng/L for sulfapyridine. Sulfamethoxazole was detected in both influent and effluent waters at concentrations of 89 ng/L and 133 ng/L, respectively.

Ground water samples showed differences in the number of sulfonamides detected as well as in the concentrations estimated, depending on the location (see Table 5). Sulfisomidin, sulfamethazine and sulfamethoxazole were the sulfonamides detected with the highest frequency in both campaigns (Fig. 6) and with the highest concentrations. An occurrence study of these antibiotics in two ground water bodies of Catalonia (Spain) showed that these same sulfonamides were also repeatedly present in this water matrix (66.67%, 89.74% and 58.97% of the ground water samples, respectively) [28]. On the contrary, sulfaguanidine, sulfacetamide, succinyl-sulfathiazole and sulfathiazole were not detected in any of the samples. In those cases where sulfonamides were detected in the two campaigns in the same sampling locations, it could be observed that concentrations in 2007 were usually higher. A feasible explanation for this is that samples in 2008 were taken during the summer campaign which implies smaller infiltration rates to the ground water bodies due to the lack of rain events, meaning less sulfonamides being potentially leached down to the aquifers and, consequently, smaller concentration levels to be detected. However, with the exception of the sampling point GW4, generally a higher number of sulfonamides were detected during the 2008 summer campaign in all the ground water wells which had not been detected the previous year. In all cases, the highest concentrations corresponded to sulfamethoxazole, in a range from 14.8 ng/L to 53.9 ng/L. This data is relevant, as sulfamethoxazole is used mainly in humans and, therefore was not expected to be found so often in ground water from rural areas,

Table 5
Sulfonamide concentrations in the various ground water and surface waters collected (ng/L).

Compounds	GW1		GW2		GW3		GW4		R1	R2	R3	R4
	2007	2008	2007	2008	2007	2008	2007	2008				
Sulfisomidin	1.14	–	1.83		–	0.62	1.89	0.32	13.70	1.79	6.19	–
Sulfanitran	–	0.80	–	<MLOQ	–	0.82	–	–	–	–	–	–
Sulfaguanidina	–	–	–		–	–	–	–	–	–	–	–
Sulfamerazine	–	3.22	–	<MLOQ	–	–	0.77	–	15.50	–	10.10	3.18
Sulfaquinoxaline	<MLOQ	1.17	–	-	<MLOQ	–	0.32	–	20.80	–	5.89	–
Sulfadoxine	–	4.48	–	–	–	–	–	–	20.00	–	6.77	–
Sulfacetamide	–	–	–	–	–	–	<MLOD	–	–	–	–	–
Succinyl-sulfathiazole	–	–	<MLOD	–	<MLOD	–	–	–	–	–	–	–
Sulfabenzamide	–	–	–	3.41	–	–	–	–	–	1.78	–	–
Sulfadiazine	–	–	–	–	0.81	–	–	–	–	–	–	–
Sulfadimethoxine	0.11	1.65	–	–	–	–	0.51		18.10	–	5.02	0.52
Sulfamethazine	–	3.71	<MLOQ	–	0.16	0.43	0.88	0.16	20.10	–	8.04	2.52
Sulfamethizole	<MLOD	<MLOD	–	–	–	<MLOD	<MLOQ	–	2.65	–	–	–
Sulfamethoxazole	17.60	8.55	–	19.70	13.40	53.90	14.80	–	–	–	7.50	32.2
Sulfamethoxypyridazine	–	0.24	–	–	–	0.24	0.77	–	15.50	0.62	7.30	–
Sulfapyridine	–	0.70	–	0.05	–	0.75	1.11	–	11.20	0.16	4.98	0.79
Sulfathiazole	–		–	–	–	–	–	–	13.90	–	10.10	–
Sulfisoxazole	–	<MLOQ	–	<MLOQ	–	<MLOD	<MLOD	–	–	–	12.50	–
N^4-acetylsulfamethazine	–	2.18	–	0.14	–	–	0.38	–	–	0.25	5.32	–

–: not detected; <MLOD: under the method limit of detection; <MLOQ: under the method limit of quantification.

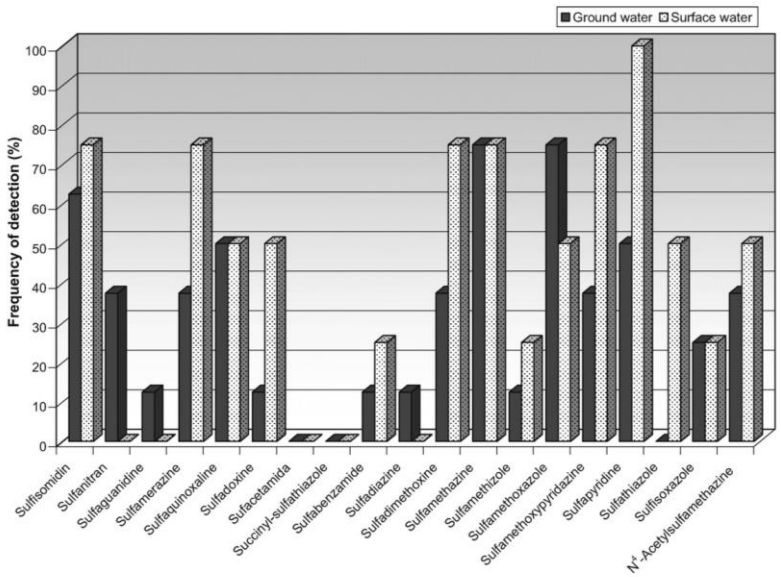

Fig. 6. Frequency of detection for the target sulfonamides studied in both ground water and surface water samples.

where veterinary sulfonamides such as sulfadiazine or sulfamethazine were more likely to be present (via direct deposition of excreta or discharges from cattle farms or via manure amendments in agricultural soil). The smallest concentration detected in the ground water samples corresponded to sulfamethazine in GW1, where only 0.11 ng/L of this compound was detected. The ground water sample which seemed to be less polluted by the sulfonamides studied was GW2, taken from a well located within an urban area. Only sulfisomidin was detected in 2007 in that location, and four other sulfonamides in 2008: sulfabenzamide, sulfamethoxazole, sulfapyridine (typically used in human medicine) and N^4-acetylsulfamethazine. The sample GW1, located in the same village surroundings, close to an agricultural field, showed a higher number of detected sulfonamides (4 in 2007 and 11 in 2008). These results would not allow us to establish a clear distinction between the origin of the sulfonamides present in these ground water samples, as sulfamethoxazole and sulfapyridine, widely use in human medicine, have been detected in frequencies similar to sulfamethazine, typically used in veterinary practices (Fig. 6).

Regarding surface water samples, concentrations ranged from 0.16 ng/L (sulfapyridine in R2) to 32.2 ng/L (sulfamethoxazole in R4). R1 and R3 were the sampling sites where a higher number of sulfonamides were detected (10 and 12, respectively). R1 was situated upstream the WWTP4, close to the same urban area, whereas R3 was close to a village of no more than 1204 inhabitants, where agriculture and cattle were the main economical activities. However, concentrations were slightly higher in R1, with values generally between 10 ng/L and 20 ng/L (see Table 5). Despite being located close to the biggest city of the sampling campaign, with approximately 660 000 inhabitants and a relevant industrial activity, only five sulfonamides could be detected in sampling site R2 and at quite low concentration. Sulfadiazine was not detected in any of the surface water samples analyzed. Except for sulfadiazine, MLODs obtained were slightly better than those previously calculated by Stoob et al. [19], especially for the acetylated metabolite.

4. Conclusion

For the fast and sensitive simultaneous determination of 19 selected sulfonamides and metabolites in natural waters, a new multi-residue analytical method based on on-line SPE-LC–MS/MS was developed. Compared to the existing methods, this analytical approach affords full automation, minimum sample handling by the analyst, low sample volume required (depending on the matrix, from 5 mL to 40 mL), high-throughput (23 min/sample), good reproducibility (with RSD values usually below 10%), improved accuracy (since aqueous calibration standards are processed in the same way as samples), high sensitivity (MLOD values usually <10 ng/L) and high selectivity. Differences are less obvious when comparing to a previous off-line SPE study [10], in which similar MLOD values and sometimes lower were achieved. However, it should be taken into account that the number of compounds to be analyzed by this new on-line SPE methodology is nearly two times higher, and therefore compromise in terms of optimization of the SPE procedure for all the target analytes had to be reached, which may somehow have slightly affected the performance of the methodology. Nevertheless, the application of the developed method to assess the contamination with sulfonamides of different water matrices evidenced the occurrence of most of the studied sulfonamides, even at pg/L level. Sulfonamides typically used in human medicine, such as sulfamethoxazole and sulfapyridine, were the most frequently found in all the water matrices studied, together with sulfamethazine and sulfisomidin. The recurrent presence of sulfamethoxazole in ground water samples of this and other studies by the same author make evident the

environmental health concern and the need of further investigation.

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ORIGINAL PAPER

Application of fully automated online solid phase extraction-liquid chromatography-electrospray-tandem mass spectrometry for the determination of sulfonamides and their acetylated metabolites in groundwater

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Abstract The present study describes an automated methodology based on a liquid chromatography-electrospray, tandem mass spectrometry method combined with online solid phase extraction (online SPE-LC-ESI-MS/MS) for the simultaneous analysis of 16 sulfonamides (SAs) and five of their acetylated metabolites in groundwater. The evaluation of the degree of SA pollution in groundwater was made through the analysis of a total of 39 samples taken in seven groundwater bodies of Catalonia (Spain). Recovery values obtained ranged from 34.3% (N^4-acetylsulfadiazine) to 134.4% (sulfabenzamide). The method limits of detection for all the analytes were 0.09–11 ng L^{-1}. Sulfamethoxazole was the SA detected more frequently (56.4% of the samples), with an average concentration of 2.3 ng L^{-1}, followed by sulfadimethoxine, present in 54% of the samples with an average concentration of 0.2 ng L^{-1}. It should be highlighted that the acetylated metabolites were ubiquitous in the different samples, with frequencies of detection up to 36% and maximum concentrations of 18 ng L^{-1} (N^4-acetylsulfamerazine).

Keywords Groundwater · Pharmaceuticals · Sulfonamides and metabolites · HPLC

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Introduction

The connection between agriculture and groundwater quality has become a highly relevant issue from a scientific and management point of view. The regular application of pesticides, fertilizers and organic amendments, together with the use of raw water for irrigation in agricultural land have compromised the quality of this water resource, as infiltration of rainwater or irrigation return flows are the main recharge sources that aquifers feed on [1]. As a result, nitrates (both of agricultural or cattle operations origin) and pesticide residues have been commonly detected in groundwater during the last decades [2]. Groundwater should be considered as an especially vulnerable water reservoir

because, despite presenting a bigger inertia to quality changes, once contaminated, the effects can hardly ever be reverted. Aquifers' quality preservation was established in the context of the Water Frame Directive (WFD, Directive 2000/60/EC) [3], in a specifically issued daughter Directive (Directive 2006/118/EC) [4]. Pesticides are included in this regulation, as they are considered one of the main contributors to diffuse contamination in aquifers together with nitrates, which had also been previously addressed in Directive 91/676/EEC [5]. Nowadays, the significant developments in analytical chemistry instrumentations have permitted not only to lower the limits of detection for these contaminants but also to confirm the presence of new organic pollutants. Veterinary drugs and especially veterinary antibiotics (VAs) are a clear and present example. They are used worldwide in intensive animal husbandry, and their presence in all kinds of environmental waters and matrices has been extensively demonstrated in several scientific studies [6–11]. Sulfonamides (SAs) are one of the families of VAs most often found. They are widely used in veterinary medicine, mainly in pig production, but also, to a lesser extent, in human therapies. SAs are generally poorly absorbed by the organism, being excreted in varying unmetabolized amounts or as metabolites (mainly *N*-acetylated and also *N*-glucuronidated) primarily via the urine and feces. They are generally highly polar and water soluble; besides, the adsorption coefficients (K_d) of SAs are small compared to those of other VAs, such as tetracyclines and fluoroquinolones. Because of their low K_d values, SAs are considered to be very mobile and weakly retained in soil, and therefore highly bioavailable and generally non-bioaccumulative. The application of liquid manure from livestock in agriculture (with the objective of producing sustainable nutrient recycling) is a common practice, a use that may represent the entrance of SAs and their metabolites to the different environmental compartments, together with other VAs contained in the manure [12, 13]. Grazing animals can also contribute to their environmental occurrence, through the direct depositions on the grassland. Hence, relevant amounts of SAs per hectare are being spread on agricultural soils annually [14–16]. Taking into consideration that their sorption to soil is weak, surface run-off and leaching into deeper soil layers will lead SAs to different environmental waters. It should not be forgotten that sewage sludge from wastewater treatment plants (WWTPs) is also commonly applied in agricultural fields as nutrient amendment, with the corresponding loads of pollutants that have not been fully eliminated during treatment. In WWTPs, SAs are most frequently detected in effluent waters due to its high polarity and little retention to solids; they are mainly SAs of human use [17–20].

So far, there is no legislation regarding the presence of SAs or any other pharmaceutical in groundwater or any environmental compartment. However, despite SAs are usually detected at low concentrations, they are being continuously introduced into the environment, and concentrations that were previously considered as harmless are leading to the emergence of antibiotic-resistant bacteria strains, and potential implications for human and the ecosystems health should be considered.

Within this context, an improved analytical methodology based on online solid phase extraction liquid chromatography-tandem mass spectrometry (online SPE-LC-MS/MS) was developed to investigate the presence of 16 SAs and five of their acetylated metabolites in a total of 39 groundwater samples taken in seven different groundwater bodies (GWBs) of Catalonia under a relevant agricultural pressure. Some of these metabolites, such as N^4-acetylsulfamethoxazole [21, 22] or N^4-acetylsulfadiazine [22], have been studied in environmental matrices before through online SPE-LC-MS/MS but, with the exception of N^4-acetylsulfamethazine [23], never in groundwater.

Experimental

Chemicals and materials

Four of the five acetylated metabolites, N^4-acetylsulfadiazine, N^4-acetylsulfapyridine, N^4-acetylsulfamethoxazole, N^4-acetylsulfamerazine and the deuterated d_4-sulfathiazole were purchased from Toronto Research Chemicals (Toronto, ON, Canada). High purity standards (>99%) of the remaining acetylated metabolite, N^4-acetylsulfamethazine, and the 16 selected SAs, namely sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethizole, sulfamethoxazole, sulfamethoxypyridazine, sulfapyridine, sulfisoxazole, sulfathiazole, sulfisomidin, sulfanitran, sulfamerazine, sulfaquinoxaline, sulfadoxine, succinyl-sulfathiazole and sulfabenzamide, were purchased from Sigma (St. Louis, MO, USA). High-performance liquid chromatography (HPLC)-grade solvents (water, methanol, acetone and acetonitrile) and formic acid (98–100%) were supplied by Merck (Darmstadt, Germany).

Stock standard solutions for each one of the analytes were prepared in methanol at 1 mg mL^{-1} and stored at −2 °C until use. Standard solutions of the mixtures of all compounds at appropriate concentrations were prepared by the corresponding dilution of the stock solutions in methanol. The standard mixtures were used as spiking solutions for preparation of the aqueous calibration standards, which always contained <0.1% of methanol.

Sampling area and sampling collection

A total of 39 different groundwater samples were taken from May till October 2009 in seven different GWBs of

Catalonia (see Fig. 1). The map codes are in accordance with those given by the Catalan Water Agency. Their main characteristics and quality situation are summarized in Table 1; further information is given in ESM 1. Following the WFD, all the GWBs investigated could be considered vulnerable in terms of contamination derived from agricultural practices, as they are all under a significant farming pressure. In fact, most of them are located in the so-called "vulnerable zones" by nitrate contamination, according to the provisions of the Directive 91/676/EEC [5]. Furthermore, WWTPs discharges are moderate or high in six out of the seven GWBs under study, contributing to increase the pressure on the quality of these reservoirs. Groundwater was sampled from surveillance and operative controls, including monitoring wells and natural springs, from depths ranging from 4 to 130 m (see ESM 1). Water samples were collected in amber glass bottles and transported under cooled conditions to the laboratory, where they were frozen until analysis.

Online SPE-LC-MS/MS analysis

Fully automated online pre-concentration and purification of samples, aqueous standards, and blanks were performed by means of a Symbiosis™ Pico System (Spark Holland, Emmen, The Netherlands). This automated system consists of three integrated parts: an HPLC system consisting of an Alias™ autosampler, a loop injector and two binary pumps with a four-channel solvent selector for each one; an automated cartridge exchange module, consisting of two clamps which can hold two trays of 96 extraction cartridges

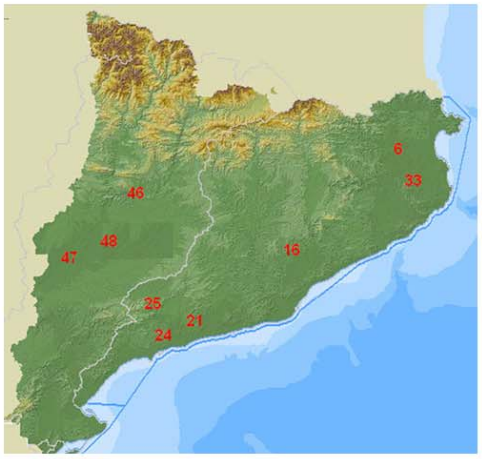

Fig. 1 Location of the different groundwater bodies studied [2]. The map codes' correspondences are given in Table 1

Table 1 Name, map code, sampling sites, and main environmental pressures exerted on the groundwater bodies studied (data provided by ACA [2])

Groundwater	Map code	Sampling sites	Geologic setting				Main use	Livestock waste	Intensive agriculture	Application of biosolids	WWTP discharges	Urban-industrial areas	Industrial discharges	Water catchment	Saline intrusion	Artificial refilling	Total pressure[a]	Vulnerability
			Lithology	Permeability (m/day)	Recharge coefficient (%)	Transmissivity (m^2/day)												
Al·luvial del Baix Segre	47	4	Alluvial	No data	No data	No data	Water supply	High	High	Low	Moderate	Moderate	High	Low	Null	Low	Moderate	High
Al·luvial del Vallès	16	6	Alluvial	40–300	11.2–14.5	No data	Water supply, industrial	High	Moderate	Null	High	High	Moderate	Moderate	Null	Null	High	High
Fluviodeltaic del Ter	33	8	Alluvial	100–1,000	15–20	2,500–11,000	Water supply	Moderate	High	Null	Low	Low	Null	Low	High	Moderate	High	High
Baix Francolí	24	4	Detritic (no alluvial), alluvial	100–2,500	5	Clays 10–50, 2,000–3,000 gravel	Water supply, cattle, irrigation	Low	High	Moderate	High	Moderate	High	Moderate	High	Low	High	High
Alt Camp	25	3	Detritic (no alluvial), alluvial	100–2,500	5	Clays 10–50, 2,000–3,000 gravel	Water supply, domestic use, irrigation	Low	High	Null	High	Null	Null	High	Null	Low	High	Moderate
Detritic neogen del Baix Penedès	21	5	Detritic (no alluvial)	No data	No data	90–360	Water supply	High	Null	Moderate	High	Moderate	Low	High	Null	Null	High	Low
Al·luvial d'Urgell	48	6	Alluvial	350–4,200	No data	No data	Industrial	Moderate	High	Low	High	Low	High	Moderate	Null	Moderate	High	High

[a] Pressure evaluated as chemical status of the site

each; and a high pressure dispenser module that provides solvents for SPE conditioning, equilibration, and clean up by means of a 2-mL high-pressure syringe. Briefly, after conditioning of the cartridge, the sample is transferred from the autosampler loop injector onto the conditioned cartridge. The loaded cartridge is then washed and transferred to the elution clamp where the analytes will be eluted from the SPE cartridge directly onto the LC column. During elution, a new cartridge is simultaneously placed in the conditioning clamp and undergoes conditioning, loading, and washing. Therefore, SPE is carried out entirely in parallel with the LC-MS/MS run, shortening the cycle times.

MS/MS analyses were carried out in a 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbospray electrospray (ESI) source, and using Analyst 1.4.2™ to acquire, process, and quantify the data. The chromatographic separation was performed using an Atlantis C18 (Waters, 150×2.1 mm, 3 μm of particle size) LC-column preceded by a guard column with the same packing material. SAs were analyzed in the positive ionization mode (PI). The flow rate was set to 0.2 mL min^{-1}, being eluent A HPLC-grade water slightly acidified with 10 mM of formic acid and eluent B acetonitrile with 10 mM formic acid. The elution gradient started with 25% of eluent B, increasing to 80% in 10 min, and to 100% in 11 min. During the next 2 min, the column was cleaned, readjusted to the initial conditions in 3 min, and equilibrated for 7 min. For increased sensitivity and selectivity, MS/MS data acquisition was performed in the selected reaction-monitoring mode (SRM). For each analyte, two transitions between precursor ions and the two most abundant product ions were monitored; the more abundant one was used for quantitation and the other one for confirmation. The optimization of the MS/MS experimental conditions was performed in a previous study [19] and was as follows: capillary voltage, 3.5 kV; source temperature, 700 °C; desolvation temperature, 450 °C; extractor voltage, 3 V; and RF lens, 0.2 V. Nitrogen was used as both the nebulizing and the desolvation gas at 630 L/h^{-1}. For operation in the MS/MS mode, argon was used as collision gas with a pressure of 2.6×10^{-3} mbar.

Results and discussion

MS/MS optimization

MS/MS optimization of the individual SAs was described by the authors in a previous work [24]. The four new metabolites included in this study were correspondingly optimized, first by infusion and finally by on-column offline injection of standard solutions of the individual compounds and a mixture solution of all of them. Identification of the precursor ions and optimum ionization conditions were performed in the full scan mode by recording mass spectra from *m/z* 50 to 500. Further identification of the most abundant fragment ions and selection of the optimum gas collision energies (CE) for each analyte were carried out in the product ion scan mode. Table 2 shows the optimized LC-MS/MS conditions used for the analysis of the target SAs and metabolites.

Online trace enrichment optimization

Sample cleanup and pre-concentration of the target analytes are critical and contribute most significantly to the final performance of the analytical method. The integration of SPE and LC on a fully automated platform (Symbiosis Pico™) increases the operative simplicity, as sample handling by the user is minimized to just one step before the automated analysis of the samples. It also has to be pointed out that in Pico™, samples are injected directly from individual glass vials onto the system instead of using channels to load the samples, which had to be rinsed whenever new samples were prepared. This way, potential carry over is also minimized compared to that in other online instruments like Prospekt™ used in previous works [23, 24]. Online Oasis HLB cartridges were employed for the extraction of the samples, as they showed the highest recovery rates in both offline and online SPE for SAs [21, 22, 24]. The optimal conditioning of the cartridges was obtained adjusting the elution solvent composition, cartridge washing, clump washing, and sample volume loaded. Conditioning finally consisted of 1 mL of methanol, 1 mL of acetone and 1 mL of HPLC water, loaded separatedly at a rate of 5 mL min^{-1}. Groundwater samples were spiked with a mixture of the analytes at a concentration of 100 ng L^{-1}, and volumes of 1, 2.5, and 5 mL were loaded onto the HLB cartridges at a rate of 1 ml/min^{-1}, following the full loop or partial loop configuration of the autosampler. The washing step was carried out with 1 mL of HPLC water loaded at 5 mL min^{-1}, and the clamp flush was carried out with 1 mL of acetonitrile followed by 1 mL of methanol and 1 mL of HPLC water. It should be mentioned that recoveries were calculated in each case dividing the peak areas obtained with the online analysis and those obtained from a parallel offline analysis of a standard mixture of the analytes in the matrix water, injecting the same total mass in the mass analyzer. As an example, Fig. 2 shows the total ion chromatograms of an SAs mixture containing the five acetylated metabolites and their respective parent compounds, analyzed both online and offline. Recovery rates for the different volumes tested are shown in Fig. 3. An extraction volume load of 5 mL yielded the highest recoveries for most of the SAs studied.

Table 2 Optimized time-scheduled SRM transitions

Sulfonamide	$[M+H]^+$	SRM 1, SRM 2	RT (min)	DP (V)	CE (eV)	CXP (V)	SRM 1/SRM 2±STD
Sulfisomidin	279	279/124	3.3	76	33	8	2.02±0.11
		279/186		76	23	14	
Succinyl-sulfathiazole	356	356/256	4.2	71	25	16	1.58±0.22
		356/192		71	33	16	
Sulfathiazole	256	256/156	4.3	40	25	14	5±0.26
		256/92		40	25	10	
d_4-sulfathiazole	260	260/160	4.3	71	25	6	3.53±0.27
		260/96		71	25	6	
Sulfadiazine	251	251/156	4.5	46	27	10	1.30±0.13
		251/108		46	30	8	
N^4-acetylsulfamethazine	321	321/134	4.6	86	35	4	1.45±0.21
		321/124		86	35	4	
Sulfapyridine	250	250/156	4.7	51	28	12	1.71±0.02
		250/92		51	31	6	
N^4-acetylsulfapyridine	292	292/134	4.9	70	30	8	1.21±0.11
		292/198		70	30	8	
N^4-acetylsulfadiazine	293	293/134	5.1	65	30	12	1.31±0.29
		293/198		65	30	12	
N^4-acetylsulfamerazine	307	307/134	6.4	60	35	8	1.56±0.15
		307/110		60	35	8	
Sulfamerazine	265	265/92	5.6	61	47	6	1.30±0.15
		265/156		61	27	8	
Sulfamethazine	279	279/156	6	26	30	10	1.48±0.08
		279/124		26	35	10	
Sulfamethizole	271	271/156	6.3	36	23	12	6.85±0.48
		271/108		36	23	8	
Sulfamethoxypyridazine	281	281/156	6.3	66	27	14	2.01±0.09
		281/126		66	27	12	
Sulfadoxine	311	311/156	10.4	46	29	12	2.25±0.40
		311/92		46	45	4	
Sulfamethoxazole	254	254/156	11.4	56	25	10	2.08±0.24
		254/108		56	27	10	
N^4-acetylsulfamethoxazole	296	296/134	11.6	60	30	10	1.57±0.22
		296/198		60	30	10	
Sulfisoxazol	268	268/156	12	71	21	10	1.45±0.23
		268/113		71	21	8	
Sulfaquinoxaline	301	301/156	13	76	25	10	1.78±0.14
		301/92		76	47	12	
Sulfabenzamide	277	277/156	13	56	17	10	1.71±0.35
		277/92		56	41	6	
Sulfadimethoxine	311	311/156	13	76	31	8	4.37±0.20
		311/92		76	31	6	
Sulfanitran	336	336/156	14.7	66	17	12	2.05±0.25
		336/198		66	29	14	

Compound-dependent parameters: *CE* collision energy, *DP* declustering potential, *CXP* collision cell exit potential

Method validation

The analytical method was evaluated in terms of calibration range and linearity, instrumental variation, accuracy, sensitivity and selectivity.

Matrix-matched calibration curves were built from the injection of seven aqueous standard mixtures of the analytes ranging from 0.01 to 500 ng L^{-1} and following a least-square linear regression analysis. Linearity is given as the regression coefficient (r^2) and was always equal or above 0.9992 (see Table 3). Quantitation was performed based on the internal standard approach by adding d_4-sulfathiazole to all the samples and aqueous standards for the calibration curve at a concentration of 100 ng L^{-1}

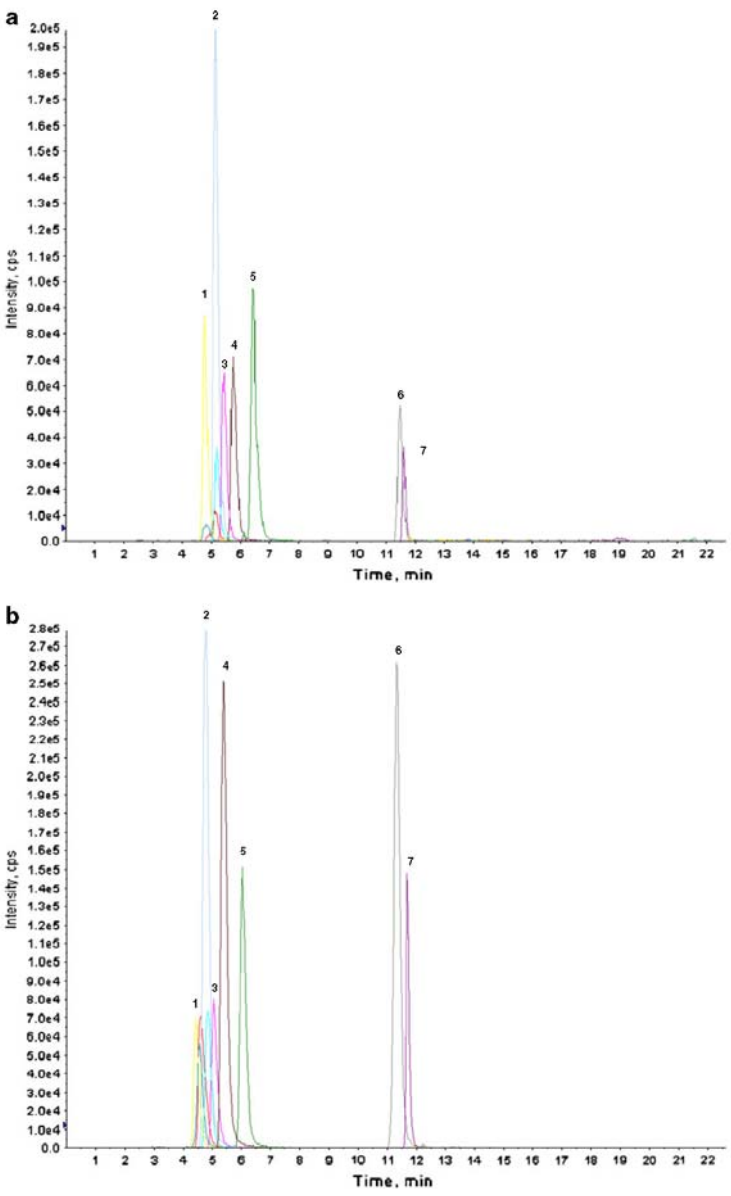

Fig. 2 Representative total ion chromatogram of a SAs mixture containing the five acetylated metabolites and their respective parents prepared in groundwater at 100 ng/L^{-1} and analyzed online (**a**) and offline (**b**). *Peak 1* N^4-acetylsulfapyridine/N^4-acetylsulfadiazine, *peak 2* sulfapyridine/N^4-acetylsulfamerazine/sulfadiazine, *peak 3* N^4-acetylsulfamethazine, *peak 4* sulfamerazine, *peak 5* sulfamethazine, *peak 6* sulfamethoxazole, *peak 7* N^4-acetylsulfamethoxazole

before extraction in order to correct potential matrix effects and losses during the SPE procedure and the analysis.

Instrumental variation is expressed as the relative standard deviation of five consecutive injections of an aqueous standard mixture at 100 ng L^{-1}. Results are given in Table 3, and values were below 10% for all the SAs but three.

Accuracy is given as the recovery values obtained, which ranged from 34.3% (N^4-acetylsulfadiazine) to

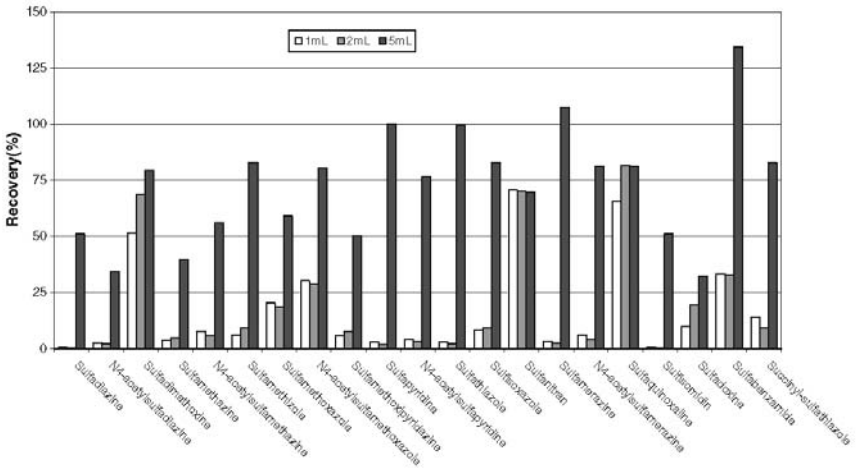

Fig. 3 Recoveries obtained for the different sulfonamides studied (using Oasis HLB cartridges)

134.4% (sulfabenzamide) for the SAs studied. N^4-acetylsulfamerazine presented the highest recovery of all the acetylated metabolites (81.2%) and N^4-acetylsulfadiazine the lowest (34.3%). Sulfapyridine and sulfathiazole showed the best recoveries for the parent SAs (100% and 99.5%, respectively). Sulfamethazine was the parent SA with the lowest recovery (39.7%).

Method limits of detection and quantification (MLODs and MLOQs, respectively) were calculated as the minimum detectable amount of analyte with a signal-to-noise ratio of

Table 3 Linearity (r^2), accuracy (expressed as recovery (percent) and standard deviation (STD), precision (expressed as RSD, percent), and method limit of detection (nanograms per liter) of the sulfonamides studied

Sulfonamide	Linearity (r^2)	Recoveries±STD (groundwater)	Precision (RSD, %)	M LOD (ng/L^{-1})	M LOQ (ng/L^{-1})
Sulfadiazine	0.9998	51±2.9	15.6	0.45	1.50
N^4-acetylsulfadiazine	0.9996	34.3±1.2	7.2	0.14	0.47
Sulfadimethoxine	0.9996	79.4±13.7	10.3	0.21	0.70
Sulfamethazine	0.9996	39.7±8.3	12.4	0.14	0.47
N^4-acetylsulfamethazine	0.9996	56±7.3	7.8	0.05	0.17
Sulfamethizole	0.9992	83±4.1	5.0	0.09	0.30
Sulfamethoxazole	0.9992	59.1±1.1	5.7	0.06	0.20
N^4-acetylsulfamethoxazol	0.9998	80.4±2.5	7.2	0.11	0.37
Sulfamethoxypyridazine	0.9996	50.2±6	7.2	0.03	0.10
Sulfapyridine	0.9998	100±3.7	9.6	0.07	0.23
N^4-acetylsulfapyridine	0.9992	76.6±2.7	8.6	0.09	0.30
Sulfathiazole	0.9998	99.5±3.5	8.5	0.06	0.20
Sulfisoxazole	0.9996	83±4.1	8.2	0.03	0.09
Sulfanitran	0.9998	69.8±8.3	7.1	1.99	6.63
Sulfamerazine	0.9992	107.6±2.5	6.8	0.05	0.17
N^4-acetylsulfamerazine	0.9988	81.2±3.4	9.7	0.18	0.60
Sulfaquinoxaline	0.9996	81.2±12.5	9.2	0.08	0.27
Sulfisomidin	0.9988	51±2.3	12.3	0.07	0.23
Sulfadoxine	0.9998	54.2±4.6	8.4	0.03	0.10
Sulfabenzamide	0.9992	134.4±0.8	9.3	1.04	0.13
Succinyl-sulfathiazole	0.9996	82.7±8.8	10.7	3.30	11.0

3 and 10, respectively, in the different samples analyzed. MLOD values were in the range of 0.03 (sulfadoxine and sulfisoxazole) to 3.30 ngL ^{1} (succinyl-sulfathiazole). These values are comparable to those obtained previously [24]. Regarding the selectivity of the method, requirements of the European Commission Decision 2002/657/EC [25] regarding a minimum of three identification points (IPs) for positive identification of the analytes and retention time variations were achieved.

SAs presence in groundwaters

Table 4 summarizes the results for each individual SA investigated. Sulfamethoxazole and sulfadimethoxine were the SAs detected more frequently, being present in 56% and 54% of all the samples and with average concentrations of 2.3 and 0.2 ng L ^{1}, respectively. These results agree with those obtained in a previous investigation carried out in two different GWBs in Catalonia, in which sulfadimethoxine also resulted as the most ubiquitous SA and sulfamethoxazole was very frequently detected [23]. These results could be explained in terms of high rates of consumption of these two SAs together with their low K_d (2.3 for sulfadimethoxine [26]) and high water solubilities (343 mg L ^{1} for sulfadimethoxine and 610 mg L ^{1} for sulfamethoxazole [26]). Conversely, sulfanitran was not detected in any of the samples, and sulfamethizole only in three out of the 39 samples analyzed, but always at levels below its MLOQ. Average concentrations are not given for sulfabenzamide and succinyl-sulfathiazole because their presence could be quantified only in one single sample each. Regarding the acetylated metabolites studied, N^4-acetylsulfamerazine was detected in 36% of the samples, occurring more frequently than its parent compound sulfamerazine (15%) and with an average concentration of 5 ng L ^{1}, higher than sulfamethoxazole's. N^4-acetylsulfamethoxazole and N^4-acetylsulfapyridine were detected in 25.6% and 23.1% of the samples, respectively, the latter also with a higher occurrence than sulfapyridine and in concentrations generally higher. From the 39 samples investigated, 32 of them were taken at depths no deeper than 43 m. The seven remaining samples were taken at depths from 80 to 130 m. No sampling was carried out between 43 and 79 m. Figure 4a shows the normalized number of positive detections related to depth. The number of SAs detected seems to increase until a depth of 40 m. In Fig. 4b, the findings at this depth can be observed in more detail. With the exception of sulfamethoxazole and sulfapyridine, used mainly in human therapies, all these SAs are usually employed in veterinary practices. The highest concentrations correspond to sulfamethoxazole and the acetylated

Table 4 Uses (human (H) or veterinary (V)), frequencies of detection (given as number of hits and relative frequency of detection (percent)), average and maximum concentrations for each of the sulfonamides studied

Sulfonamide	Use	Frequency of detection [no. of hits (%)] (n=39)	Average concentration (ng/L^{-1})±STD	Maximum concentration (ng/L^{-1})
Sulfamethoxazole	H	22 (56.4)	2.3±2.4	8.8
Sulfadimethoxine	V	21 (53.8)	0.2±0.1	0.6
N^4-acetylsulfamerazine	V	14 (35.9)	5.0±5.3	18.0
Sulfaquinoxaline	V	13 (33.3)	39.4±103.5	274.0
N^4-acetylsulfamethoxazole	H	10 (25.6)	1.4±1.7	5.5
N^4-acetylsulfapyridine	H–V	9 (23.1)	1.6±2.0	6.0
Sulfapyridine	H–V	8 (20.5)	0.7±0.9	2.0
Sulfisomidin	V	8 (20.5)	0.2	0.2
Sulfamethazine	V	7 (17.9)	2.7±5.2	13.4
Sulfamerazine	V	6 (15.4)	17.3±42	103.0
Sulfamethoxypyridazine	V	5 (12.8)	0.1±0.1	0.2
Sulfisoxazole	V	5 (12.8)	9.0±7.2	17.1
Sulfabenzamide	V	5 (12.8)	–	0.2
N^4-acetylsulfamethazine	V	4 (10.3)	1.8±2.3	4.4
Sulfathiazole	V	4 (10.3)	0.2±0.1	0.3
Sulfadiazine	H–V	3 (7.7)	4.1±4.2	2.2
N^4-acetylsulfadiazine	H - V	3 (7.7)	0.85±0.3	1.0
Sulfamethizole	V	3 (7.7)	–	–
Sulfadoxine	H–V	2 (5.1)	0.6	0.6
Succinyl-sulfathiazole	V	2 (5.1)	–	2.1
Sulfanitran	V	–	–	–

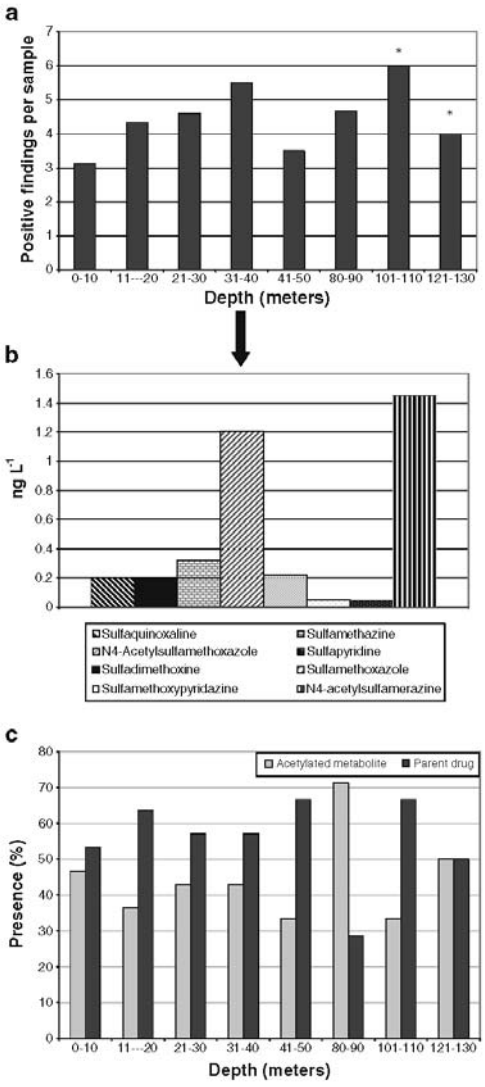

Fig. 4 **a** Number of positive SAs findings per sample. *Asterisks* only one sample analyzed. **b** Distribution of the SAs detected between 31 and 40 m. **c** Percentage of positive findings corresponding to acetylated metabolites and their respective parent drugs at each depth level

metabolite of sulfamerazine. A relevant percentage of these SAs positively detected at each depth were acetylated metabolites. Figure 4c shows the percentages of positive findings corresponding to the acetylated metabolites and their respective parent compounds at the different depths sampled. Frequencies are generally slightly higher for the parent SAs except at 80–90 m. Taking these results into account, the inclusion of metabolites of SAs has become crucial to get a complete picture of the occurrence and fate of these drugs. However, the amount of metabolized SAs released to the environment is compound dependent, and information on their stability in the different environmental compartments is lacking. It has been demonstrated through numerical models that the acetylated metabolite of sulfadiazine degrades to sulfadiazine in soil after manure application within 4 days [27] and that both compounds are highly mobile in this matrix [28]; this data could also apply for the rest of the SAs acetylated metabolites which could partly or totally revert back into the parent compounds in a relative short time and reach the groundwater matrix in both the metabolized and their original form.

When focusing in the results of each water body individually, the widest concentration range and highest total concentration corresponds to GWB 47, *Al·luvial del Baix Segre*, followed by GWB24, *Baix Francolí*, and GWB33, *Fluviodeltaic del Ter* (Table 5). Sulfamethoxazole, sulfisoxazole, and N^4-acetylsulfamerazine were the three compounds showing the highest contributions in most of the GWBs. In GWB 48, *Al·luvial d'Urgell*, the SAs with the highest contributions were both metabolites.

Total concentrations were generally below 30 ng L^{-1} with the exception of one sample corresponding to the GWB 47, *Al·luvial del Baix Segre*, taken at 6 m depth (see Electronic supplementary material), that presented concentrations up to 274 and 103 ng L^{-1} for sulfaquinoxaline and sulfamerazine, respectively (see Table 5). This aquifer's materials consist mainly of loose gravel within a matrix of thin components and therefore very permeable. Besides, the aquifer is located below a flat area scarcely covered with vegetation, and the unsaturated zone is not very relevant, favoring the infiltration of surface waters. Pressures exerted by livestock waste and intensive agriculture are also high in this area, which may contribute to the high concentrations detected, as these two SAs are generally used in veterinary practices. The rest of the samples taken in this area corresponded to water springs, and only three other SAs, sulfamethoxazole, sulfadimethoxine, and sulfapyridine, were detected at levels below 1 ng L^{-1}. None of the metabolites was detected.

In GWB 33, *Fluviodeltaic del Ter*, eight samples were analyzed, and SAs were detected in a concentration range between 0.14 and 18 ng L^{-1}. The most relevant information obtained in this sampling area is that four out of the five acetylated metabolites were present, with the exception of N^4-acetylsulfadiazine. N^4-acetylsulfapyridine was present in three of the samples at concentrations between 0.33 and 0.44 ng L^{-1}, but sulfapyridine was not detected in any of

Table 5 Concentration ranges, total (cumulative) concentration (given as the sum of concentrations of individual sulfonamides detected in the sample) and sulfonamides with the highest contribution for each of the samples and groundwater bodies investigated

Groundwater body	Sample (code)	Total concentration range (ng/L^{-1})	Concentration	Highest contribution
Al·luvial del baix Segre	25120-0020	0.30	0.07-0.23	Sulfaquinoxaline-sulfamerazine
	25102-0001	0.37	0.37[a]	
	25174-0001	0.31	0.13-0.18	
	25254-0004	377.00	103-274	
Al·luvials del Vallès	08108-0009	11.02	0.14-8.76	Sulfamethoxazole-sulfisoxazole
	08086-0049	1.27	1.27[a]	
	08088-0025	3.17	0.31-2.86	
	08106-0041	1.75	1.75[a]	
	08135-0021	12.95	0.17-6.70	
	08046-0016	1.11	0.15-0.56	
Fluviodeltaic del Ter	17018-0013	17.70	0.24-9.65	N^4-acetylsulfamerazine-sulfamethazine
	17191-0021	4.14	0.44-3.70	
	17199-0031	–	–	
	17199-0044	0.51	0.51[a]	
	17205-0012	23.63	5.63-18	
	17211-0025	3.98	0.20-2.56	
	17070-0049	9.64	0.14-8.97	
	17081-0008	15.07	0.12-13.40	
Baix Francolí	43047-0018	0.53	0.14-0.21	Sulfisoxazole-N^4-acetylsulfamerazine
	43047-0032	21.80	0.16-17	
	43047-0033	1.80	0.21-1.60	
	43095-0011	15.69	0.41-12	
Alt Camp	43161-0159	9.94	0.17-5.34	Sulfamethoxazole
	43005-0083	0.62	0.09-0.18	
	43161-0138	3.96	0.21-3.75	
	43034-0002	1.91	0.20-1.71	
	43119-0016	3.64	0.24-1.75	
	43161-0148	3.27	0.10-3.02	
Detrític neògen del Baix Penedès	08065-0003	3.60	0.16-3.20	N^4-acetylsulfamerazine-sulfamethoxazole
	43140-0056	6.28	0.541-3.05	
	43020-0056	2.76	0.05-0.79	
	43020-0074	2.84	0.16-1.62	
	43074-0056	3.29	3.29[a]	
Al·luvial d'Urgell	25048-0003	2.23	0.04-1.60	N^4-acetylsulfamethoxazole-N^4-acetylsulfapyridine
	25096-0008	4.76	0.14-2.45	
	25158-0004	2.01	0.89-1.13	
	25225-0003	8.11	1.03-6.03	
	25248-0002	6.93	1.41-5.52	
	25252-0003	2.49	0.39-2.10	

[a] Only one sulfonamide detected in that sample

them. The same applied for the rest of the metabolites detected: N^4-acetylsulfamerazine, the compound with the highest contribution to the total SA concentration in this water body, was present in four samples at the highest concentrations (from 2.56 to 18 ng L ^{1}), but sulfamerazine was not detected in any of the samples. N^4-acetylsulfamethoxazole was detected in two samples where sulfamethoxazole was not present, and in a third where both were detected, the latter at a slightly higher concentration (0.97 versus 0.63 ng L ^{1}). N^4-acetylsulfamethazine and N^4-acetylsulfamerazine were also present in this sample, at concentrations of 4.4 and 9.65 ng L ^{1}, respectively, whereas none of the corresponding parents was detected. The higher occurrence of the acetylated metabolites

compared to the parent compounds could be explained in terms of their solubility (as metabolites are usually more soluble in order to be excreted more easily by the organisms; this increased mobility may persist once they reach the natural media). A higher persistence of the acetylated compound could also explain this fact, although information regarding environmental half-lives is still lacking. Besides, the sandy lithology of the unsaturated zone together with the gravel materials of the saturated zone confers a high vulnerability to pollution on this area, which facilitates infiltration to the water table.

The presence of veterinary SAs was not very relevant in this area.

In GWB 24, *Baix Francoli*, sulfadimethoxine was detected in the four samples taken, but at concentrations below 0.41 ng L^{-1}. This SA, typically used in veterinary medicine, was also detected by Batt et al. [20] in groundwater samples taken from different wells located near cattle feeding operations, at concentrations ranging between 47 and 68 ng L^{-1}. Sulfisoxazole was the SA detected at the highest concentration, 17 ng L^{-1}, in this case. In that same sample, three metabolites, N^4-acetylsulfapyridine, N^4-acetylsulfamethazine, and N^4- acetylsulfamerazine, were detected at different concentrations (2.87, 0.41, and 12 ng L^{-1}, respectively), whereas none of the corresponding parent SAs was detected. The concentration range for all the SAs detected in this GWB was 0.14–17 ng L^{-1}.

In GWB 16, *Al·luvial del Vallés*, a higher number of SAs were detected, although at concentrations not higher than 10 ng L^{-1}. Sulfamethoxazole was detected in five out of the six samples analyzed, presenting the highest concentrations. Its acetylated metabolite, on the other hand, was not present in any of the samples. Its high occurrence could be attributed to urban contamination from both point sources (WWTPs discharges) or a diffuse source (losses from sewage systems) since this is a highly populated area. The occurrence of sulfamethoxazole, mainly used in human medicine, in groundwater has already been demonstrated in different works [23, 29–31]. Taking this data into consideration, although information is lacking regarding the SAs levels of the WWTP effluents in this area, sulfamethoxazole is likely to be present and, once in the water courses (rivers or streams), due to its high solubility and mobility, may infiltrate in the terrain and reach the water table.

A similar situation was found in GWB 25, *Alt Camp*, and GWB 21, *Detritic neògen del Baix Penedès*, in which sulfamethoxazole was the most frequently detected SA and one of the SAs yielding the highest contributions to the total concentrations. In GWB 25, it was detected in all the samples investigated and at the highest concentrations, ranging from 0.11 to 5.34 ng L^{-1}. However, its acetylated metabolite was not present in any of them. Both intensive agriculture and WWTP discharges are exerting a high pressure on the groundwater quality in this area, and it would be complex to say which activity is the main contributor to the ubiquity of sulfamethoxazole. In GWB 21, sulfamethoxazole was detected in four out of the five samples investigated, at depths down to 80–90 m, in which N^4-acetylsulfamethoxazole was also detected at lower concentrations than the parent substance (see Electronic supplementary material). This data highlights the high mobility and ubiquity of sulfamethoxazole and its metabolite, present even in water sampled from the deepest wells of the whole campaign and in an area considered not excessively vulnerable to pollution, as sand and clay layers in the saturated zone act as potential barriers to the percolation of contaminants [2]. The contribution of SAs of veterinary use was also relevant in these two GWB; sulfadimethoxine was also detected in all the samples in GWB 25, but at lower concentrations than sulfamethoxazole (see Electronic supplementary material). N^4-acetylsulfamerazine presented the highest concentration in both GWBs (between 2.04 and 3.29 ng L^{-1}, respectively).

Again, SAs contamination from urban origin was demonstrated in *Al·luvial d'Urgell*, GWB 48, but in this occasion, two acetylated metabolites were the most relevant compounds in terms of contribution to the total concentration detected: N^4-acetylsulfamethoxazole, which was detected in four out of the six samples at concentrations between 0.88 and 5.52 ng L^{-1} and N^4-acetylsulfapyridine, detected in two samples at concentrations of 1.41 and 6.03 ng L^{-1}, respectively.

Conclusions

The scientific interest in antimicrobial active compounds in manure and soil, but also in surface and groundwater, has increased during the last decade. The occurrence of 16 different SAs and five of their corresponding acetylated metabolites has been extensively investigated in seven GWBs of Catalonia for the first time. SAs have been detected ubiquitously in all of them, at levels generally below 30 ng L^{-1}. The lack of legislation setting up risk environmental levels for these substances does not allow establishing a definitive conclusion about the quality of the groundwater samples studied.

GWB 47 presented the highest load of SAs, with a total concentration of 378 ng L^{-1} that was detected nearly in its totality in one single sample. GWB 33 was next, with a total load of 75 ng L^{-1}; both GWBs are used basically for water supply and, despite the lack of toxicity data regarding these compounds, their presence in drinking water may entail a potential risk when consumed. Sulfamethoxazole, SA exclusively used in human medicine, has been the SA

detected more frequently (56.4% of the samples) and also at the deepest levels, with an average concentration of 2.3 ng L^{-1} and a maximum value of 8.8 ng L^{-1}, corresponding to GWB 16, in fair coincidence with the fact that this GWB supports a high population density. These results reveal that the higher levels of SAs detected are not only derived from livestock and agriculture practices as expected but also from urban contamination.

It needs to be highlighted the inclusion in the scope of this study of the acetylated metabolites of five of the SAs investigated and the confirmation of their presence in groundwater. N^4-acetylsulfamerazine was detected in 14 of the samples, occurring more frequently than its parent compound sulfamerazine (six samples) and with an average concentration of 5 ng L^{-1}, higher than sulfamethoxazole's. N^4-acetylsulfamethoxazole and N^4-acetylsulfapyridine were detected in 25.6% and 23.1% of the samples, respectively, the latter with a higher occurrence than sulfapyridine and in concentrations generally higher. These results could be explained in terms of higher solubilities than the parent SAs or also attributing to the metabolites a higher stability in the environment than the corresponding unmetabolized compound.

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Capítulo 2

"Multiresidue trace analysisi of sulphonamides antibiotics and their metabolites in soils and sewage sludge by pressurized liquid extraction (PLE) followed by liquid chromatography-electrospray-quadrupole linear ion trap mass spectrometry (QqLIT-MS/MS)

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ABSTRACT

The present study describes the development, validation and a practical application of a fully automated analytical method based on pressurized liquid extraction (PLE) followed by solid-phase extraction-liquid chromatography–tandem mass spectrometry (SPE-LC–MS/MS) for the simultaneous determination of 22 sulfonamides, including five acetylated metabolites, in sewage sludge and soil samples. Both matrix matched calibration curves and standard calibration curves were built in order to evaluate the potential matrix effects during analysis, and different internal standards were used to compensate these effects during quantification. The recovery efficiencies were found to be 60%-130% for the majority of the sulfonamides in both matrices and at two spike levels. The intra-day and inter-day precisions, expressed by the relative standard deviation (RSD), were below 23%. The method detection limits (MLODs) achieved were in the range 0.03–2.23 ng g^{-1} for sewage sludge and 0.01–4.19 ng g^{-1} for soil samples. The methodology was applied to evaluate the occurrence of the target sulfonamides in several sewage sludge and soil samples taken in different wastewater treatment plants (WWTPs) and agricultural areas. Results confirmed the wide presence of sulfonamides in both matrices, being sulfathiazole and sulfamethazine the sulfonamides most frequently detected in sewage sludge and soil samples, respectively. Maximum concentrations corresponded to sulfamethazine in both cases (139.2 ng g^{-1} and 8.53 ng g^{-1} for sewage sludge and soils respectively). Levels were generally lower in soils. Three of the five acetylated metabolites were detected in sewage sludge and two of them in soils, at concentrations not higher than 9.81 ng g^{-1}.

KEYWORDS
Sulfonamides; conjugated metabolites; sewage sludge; soils; pressurized liquids extraction; LC-MS/MS.

1. INTRODUCTION

Several studies have demonstrated the widespread presence of sulfonamides (SAs) in the environment and identified their main entrance pathways [1-5]. The occurrence of their main metabolites, the acetylated conjugates, at similar concentrations has become evident recently, especially in environmental waters [6,7]. It has been demonstrated that these metabolites, once excreted, can deconjugate and revert back to the original parent drug both in water and manure [2,8,9]. Therefore, the inclusion of these metabolites in environmental occurrence surveys for SAs is key to obtain more complete and reliable information on the actual levels, avoiding potential underestimations. Up to date, data on the presence of these metabolites in solid matrices such as soils or sludge from wastewater treatment plants (WWTPs) are scarce, and to our knowledge only one publication by Stoob et al. included acetylated metabolites in its scope [10]. However, the presence of the acetylated conjugates in groundwaters has been demonstrated in different works [1,6,11,12], indicating that these metabolites probably reach the aquifers percolating through the upper soil, in which residual concentrations may still be present. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been the analytical technique of choice in most of themonitoring studies carried out for SAs, due mainly to its versatility and high selectivity for the analysis of complex samples. However, despite the number of new analytical methodologies developed in the last decade for the detection of SAs in solid matrices, such as soils, sediments or sewage sludge, they have been detected only scarcely in these matrices, and generally at low concentrations [10,13-15]. This is mainly due to their high polarity and generally low tendency to adsorb onto solid particles [16,17], which make them very mobile contaminants once released into the environment. The method limits of detection (MLODs) of the method can be also compromised by the high organic content of the samples. Furthermore, despite the many advantages of LC-MS/MS analysis, matrix effects when using the electrospray ionization source (ESI) have become the main drawback during the analysis of environmental samples, leading to the potential suppression/enhancement of the signal. Solid matrices extracts usually have a high content of organic components such as humic acids, phenols, lipids etc, which increase the viscosity of the sample and the superficial tension of the droplets generated in the ESI source, hindering the evaporation efficiency of the target analytes. These interfering compounds can also contribute to the coprecipitation of the analytes, limiting their transfer to the gas phase, or even competing with them to reach the droplet surface for the maximum evaporation efficiency [18,19]. Different approaches can be followed to minimize matrix effects before analysis, based on more efficient clean up of the samples and improved chromatography with a better separation of the matrix compounds (i.e. ultra performance liquid chromatography, UPLC) to obtain a total separation of the analytes of interest from the interfering compounds. Compensating measures are also frequently used, such as

the use of internal standards or matrix matched calibration curves for quantification. The aim of the present study was to develop a new analytical method, based on pressurized liquid extraction (PLE) followed by LC-MS/MS analysis for the determination of 17 SAs and 5 of their acetylated metabolites in sewage sludge and soil samples. The potential matrix effects were evaluated in order to obtain the most accurated quantification approach and improve MLODs. After its validation, the new method was applied to the analysis of several agricultural soils and sewage sludge samples from different wastewater treatment plants (WWTPs) located in Catalonia and along the Ebro River basin.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

HPLC-grade solvents (water, methanol (MeOH), acetone and acetonitrile (ACN)) and formic acid (HCOOH, 98-100% purity) were supplied by Merck (Darmstadt, Germany). High purity standards (> 99%) of the 18 SAs studied, namely sulfisomidin (SSD), sulfamerazine (SZI), sulfacetamide (SCT), sulfadoxine (SDX), sulfabenzamide (SBZ), succinylsulfathiazole (SuSTZ), sulfaquinoxaline (SQX), sulfadiazine (SDZ), sulfadimethoxine (SDM), sulfamethazine (SMZ), sulfamethizol (SMT), sulfamethoxazole (SMX), sulfamethoxypyridazine (SMP), sulfapyridine (SPY), sulfathiazole (STZ), sulfisoxazole (SSX), sulfanitran (SNT) and N^4-acetylsulfamethazine (AcSMZ), were purchased from Sigma-Aldrich (St Louis, MO, USA). The standards of the remaining acetylated metabolites N^4-acetylsulfamethoxazole (AcSMX), N^4-acetylsulfapyridine (AcSPY), N^4-acetylsulfadiazine (AcSDZ) and N^4-acetylsulfamerazine (AcSZI), together with the isotopically labelled compounds d_4-sulfamethoxazole (d_4-SMX), d_4-sulfathiazole (d_4-STZ), d_4-sulfadiazine (d_4-SDZ) and d_4-sulfamethazine (d_4-SMZ), used as surrogate and internal standards, were purchased from Toronto Chemical Research (North York, Ontario, Canada). Stock standard solutions for each of the analytes were prepared in MeOH at 1 mg mL^{-1} and stored at -4 °C until use. Standard solutions of the mixtures of all compounds at appropriate concentrations were prepared by the corresponding dilution of the stock solutions in MeOH.

Oasis HLB solid phase extraction (SPE) cartridges (200 mg, 6 mL) were purchased from Waters (Milford, MA, USA).

2.2. Sampling site

Digested sludge samples were collected in June 2009 from different full-scale WWTPs located in Catalonia (Spain) and along the Ebro River basin. The water treatment applied in all the WWTPs consisted of sedimentation (primary settler) followed by biological treatment with P and/or N removal. Sludge from the primary and secondary settlers was thickened by gravity, digested under anaerobic conditions and finally dewatered by centrifugation. Soil samples were taken in rural areas under a significant farming pressure in Catalonia (6 samples) and also along the Ebro River basin (9 samples). Both sludge and soil samples were transported in cool conditions, freeze-dried upon arrival to the laboratory (-50 ºC, 0.044 bar vacuum) and kept at – 30 ºC until analysis.

2.3. Extraction and clean up

Soil and sludge samples were extracted by PLE using an ASE 300 accelerated solvent extractor (Dionex,

Sunnyvale, CA). Samples were grinded and homogenized in order to decrease the size particle and facilitate the PLE process. Prior to extraction, d^4-SMX was added as surrogate standard to the weighted samples at a concentration of 100 ng g^{-1}. The samples were mixed in the extraction cells with Hydromatrix dispersing agent, in order to prevent particle clumping of the sample particles and reduce interstitial volume in the cells [20]. Homogenized mixtures of the different soil and sludge samples, respectively, were used to optimize the extraction procedure in terms of sample weight, solvents and temperature. The optimized conditions for the PLE procedure were the following:

Sludge: 2 g, ACN-water (25:75, v:v), 50 ºC;

Soil: 5 g, MeOH-water (90:10, v:v), 100 ºC;

For both matrices, a preheating period of 5 min was chosen, and 3 static cycles of 5min each were carried out; a total flush volume of 100% and 60 s of nitrogen purge were applied. Pressure was set to 1,500 psi as it has been demonstrated that its variations are not decisive in the extraction efficiency [20]. The PLE extracts obtained were further purified by solid phase extraction (SPE), using a Baker vacuum system (J.T. Baker, The Netherlands). In all cases, the extracts (20 ml) were diluted with HPLC grade water (200 mL) to reduce the content of organic solvent to less than 5%, in order not to interfere in the SPE procedure, and filtrated through 0.45 µm nylon filters (Whatman, Maidstone, UK). Oasis HLB cartridges were conditioned with 3 mL of MeOH in 50 mM HCOOH, followed by 3 mL of acetone in 50 mM HCOOH and 2mL of HPLC grade water at neutral pH with 5% of MeOH [21]. After the sample loading, the cartridges were rinsed with 3mL of HPLC grade water at neutral pH with 5% of MeOH, to remove potential retained interferences. Cartridges were dried under vacuum (around 30 min.) and then eluted with 5mL of MeOH in 50mM HCOOH and 5mL of acetone in 50 mM HCOOH. SPE extracts were dried under a gentle N_2 stream and reconstituted with 1 mL of HPLC grade water-ACN (75:25, v:v). Before LC-MS/MS analysis, the corresponding internal standards were added to the reconstituted extracts at a concentration of 50 ng mL^{-1}.

2.4. Instrumental analysis

LC analysis was performed with a Symbiosis™ Pico System (Spark Holland, Emmen, The Netherlands), equipped with a HPLC system consisting of an Alias™ autosampler, a loop injector and two binary pumps with a four-channel solvent selector for each one. Chromatographic separation was performed using an Atlantis C_{18} LC-column (Waters, 150mm×2.1mm, 3µm of particle size) preceded by a guard column with the same packing material. The flow rate was set to 0.2 mL min^{-1}, being eluent A HPLC grade water acidified with 10 mM HCOOH, and eluent B ACN with 10 mM HCOOH. The elution gradient started with 25% of eluent B, increasing to 80% in 10 min and to 100% in 11 min. During the next 2 min the column was kept at 100% B, readjusted to the initial conditions in 3 min and equilibrated for 7 min. MS/MS analyses were carried out in a 4000 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbospray ionization source (ESI) working in the positive mode (ESI+). The optimization of the MS/MS experimental conditions was performed in a previous study [6]. For increased sensitivity and selectivity, MS/MS data acquisition was performed in the selected reaction monitoring (SRM)

mode, selecting the two most abundant product ions for each of the precursor ions.

2.5. Method Validation

The developed method was evaluated in terms of linearity, sensitivity, accuracy, repeatability, reproducibility and matrix effects.

Considering potential matrix effects, quantification was carried out following the internal standard calibration approach. Eight-point calibration curves (0.01 – 500 ng L^{-1}) were built for each of the analytes, following a least square linear regression analysis. Linearity was given as the regression coefficient (r^2) and was always equal or above 0.999. The corresponding deuterated compounds used as internal standards were added to all the samples and standard solutions for the calibration curve at a concentration of 50 ng mL^{-1} before LC-MS/MS analysis. Matrix matched calibration curves were also built by means of standard addition in soil and sludge extracts, which were spiked at the same concentration range as the standard's calibration curve. Both types of calibration curves were compared in order to evaluate the performance of the method, and response factors (RF) were calculated.

Instrumental variation was expressed as the relative standard deviation (RSD%) of five consecutive injections of a standard mixture at 50 ng mL^{-1}.

MLODs and method limits of quantification (MLOQs) were calculated as the minimum detectable amount of analyte with a signal-to-noise ratio of 3 and 10, respectively, in the different samples analyzed.

Accuracy was expressed in terms of the recovery values (R%) obtained after spiking the soils and sludge samples (n=3) with a standard mixture at two different concentrations, 50 ng g^{-1} and 100 ng g^{-1}. The surrogate was also added to the samples (100 ng g^{-1}) in order to evaluate the performance of the method, considering potential losses during extraction and clean up. Samples were stirred to facilitate the contact of the analytes to the solid matrix, and left overnight. Blank samples were also analyzed to check for SAs background concentrations in the calculation. R% were calculated comparing the chromatographic peak areas of the SAs obtained from the extracts ($Area\_SA_{sample}$) to those obtained from the solvent curve ($Area\_SA_{std}$) at the same concentration. Equation 1 was used for the calculations:

[1]

$$R\% = 100 \times \frac{(Area\_SA_{sample} - Area\_SA_{blank})}{Area\_surrogate_{sample}} : \frac{Area\_SA_{std}}{Area\_surrogate_{std}}$$

3. RESULTS AND DISCUSSION

3.1. PLE conditions

Different solvent mixtures and extraction temperatures were assayed simultaneously during the development of the method. For each of the solid matrices, four different solvent mixtures were used at three different temperatures, resulting in 24 preliminary experiments. Sample amounts of 10-15 g were used in previous studies for both sludge and soil samples [22-24]. In this study, 2 g of sewage sludge and 5 g of soil were sufficient to obtain good recoveries, avoiding an excess of matrix components in the extract and other problems related to larger amounts of sample [10,25].

3.1.1. Extracting Solvent

The selection of the solvent is decisive for the solubilisation of the analytes from the solid matrix. MeOH:water and ACN:water (25:75 and 10:90, (v/v) were evaluated, at a temperature of 50 ºC. As Figure 1 shows, the combination of ACN:water 25:75 (v:v) yielded

the best recoveries for sludge, whereas MeOH:water 90:10 (v:v) was the best approach for the soil samples.

3.1.2. Temperature

Temperature is critical in PLE extraction. High temperatures decrease the viscosity of solvent, obtaining a better penetration through the matrix. However, an excess of temperature may lead to thermal degradation of the target analyte [23] and also to a higher extraction of matrix components, which will compromise the selectivity and sensitivity of the method during the analysis [26]. Figure 2 shows the R% values obtained at the temperatures tested (50 ºC, 75 ºC and 100 ºC) for both matrices. For sludge, the best R% values were those obtained at an extraction temperature of 50 ºC, although results were similar to those obtained at 75 ºC. In contrast, an extraction temperature of 100 ºC led to extremely high recovery rates (frequently above the 200%). The signal enhancement observed can be attributed to an excess of matrix components co-extracted during PLE. On the contrary, 100 ºC resulted in the best R% values of SAs in soils. SSX and SCT were poorly recovered in both matrices.

The extraction was carried out in three different cycles of 5 min. each, in which solvent was newly introduced in the sample. Longer cycle times, which allow a better diffusion of the analytes in the extraction solvent, were not needed due to high polarity of SAs. Furthermore, it has been demonstrated that nearly all the analytes present in the sample were extracted during the first and second cycles, being the third cycle added as a reliability measure [23,27].

3.2. Method validation

Table 1 summarizes some of the validation results obtained for the different parameters considered. Instrumental variation, expressed as RSD%, ranged from 4 % to 24 % (SNT and SDZ, respectively). Except for three SAs, RSD% values were always below 20%. RSD% values for the acetylated metabolites ranged from 2% (AcSPY) to 21% (AcSMZ).

In general, the method yielded better MLODs for soil than for sludge samples. Average MLOD values for the sludge samples analysis ranged from 0.03 ng g^{-1} (SDM) to 2.23 ng g^{-1} for SDM and SuSTZ, respectively, and from 0.01 ng g^{-1} to 0.55 ng g^{-1} for SSD and SuSTZ, respectively, in soils. These results are similar, but occasionally lower for some of the sulfonamides, to those obtained in a recent study by Shelver et al. [25] by means of sonication extraction of soil samples and UPLC analysis. This could be attributed to the fact that, although sonication extraction requires a longer time than PLE, extracts are usually cleaner and UPLC is most efficient than HPLC to tackle with matrix effects during analysis. In both matrices in the present study, SNT yielded outlying MLOD values. Within those ranges, MLOD values for the acetylated metabolites were 0.37 ng g^{-1} -0.66 ng g^{-1} in sewage sludge and 0.07 ng g^{-1}-0.31 ng g^{-1} in soils. These values were improved compared to those obtained by Stoob *et al*. in the only previous method developed for the analysis of these metabolites in soil [10], which ranged from 5 to 15 ng g^{-1}.

The accuracy was given as R% values at two different spike levels. As shown in Table 1, results for R2% (higher spike level) were higher than R1% (lower spike level) for the majority of the analytes in both soil and sewage sludge. R2% values ranged from 56% to 130% in sewage sludge and from 47% to 133% in soil samples. Variations in the R% values were higher at the lowest spike level,

with R% values ranging from 19 % to 123% in sewage sludge and from 32%-130% in soil, although most of the values ranged from 40%-85%. SSD yielded the lowest R1% value in sewage sludge and SSX in soil, with only 19% and 32%, respectively. Considering an optimum range between 80% and 120%, it can be observed that results were slightly better in sewage sludge at both spike levels than in soil extracts.

3.2.1. Discerning matrix effects

As a first step to estimate the extent of matrix effects during analysis, response factors (RFs) were calculated for the matrix matched calibration curves and for the standard calibration curve in solvent, and given as the linear regression of the peak areas divided by the spike concentration (y axis) as a function of the spiked concentration (x axis). Figure 3 shows the RFs for four of the SAs. For the matrix curves, RFs values are about one order of magnitude below those obtained for the standard calibration curve. As observed, the variability of RFs is higher as the spiked concentration reaches the LOQs, but seems quite uniform in the rest of the concentration range. RFs are slightly higher in soil than in sludge matrix, indicating a lower load of organic or other interfering components in this matrix.

Different dilution assays were also performed in order to compare the signal intensities obtained. The highest point of the sludge curve was diluted several times, as showed in Figure 4 for some of the SAs investigated. Far from decreasing, the signal intensities obtained remained quite stable and, in most of the cases, increased as the sample was more diluted even to twice the initial intensity (SMX). Figure 4 demonstrates how successive dilutions of the final extracts contributed to the minimization of the matrix components, allowing for a better signal intensity of the target analytes.

3.2.2. Compensating matrix effects

Different approaches are commonly used to decrease the amount of matrix components during LC-MS/MS analyses, such as the improvement of the clean up of the extracts or the chromatographic separation. However, different authors consider these possibilities as time consuming and that they can also increase the risk of analyte losses or sample contamination due to prolonged handling [26]. As showed in Figure 4, dilution of samples decreases the amount of organic load entering the analyzer and improves the signal although it can reduce the sensitivity of the method considerably. This problem can also happen when quantification is carried out using matrix matched calibration curves. As an example, Figure 5 shows the worse peak shape and the lower signal obtained for the matrix matched calibration curves for both sludge and soil at 500 ng g^{-1} for two of the SAs investigated, compared to the signals obtained with the standard calibration curve. Undoubtedly, the use of isotopically labelled internal standards is the most versatile procedure to tackle with matrix effects in HPLC-ESI-MS/MS analysis of environmental samples, being the only inconvenient their usually high prices and their limited commercial availability. Considering the aforementioned, quantification was carried out following the internal calibration approach, using d_4-SDZ, d_4-STZ and d_4-SMZ as internal standards, whereas d_4-SMX was selected as surrogate. Figure 6a shows the standard calibration curves and the matrix matched calibration curves for sludge and soil extracts, for some of the SAs investigated. The notorious slope difference observed between the matrix matched and the solvent

curves highlighted the signal suppression effect. The extent of the matrix effects (*ME%*) were calculated as the difference of the slopes of the matrix matched and the standard calibration curves, following equation 2:

[2]

$$ME\% = 100 \times \left(1 - \frac{(Slope_{std\_addition})}{Slope_{solvent\_curve}} \right)$$

ME% values obtained are given in Table 2. Strong signal suppression was observed for both matrices, especially for the sludge samples, with *ME%* values ranging from 72% (SDX) to 98% (SCT). Lower but still relevant values were obtained in soil extracts, ranging from 49% (SDM) to 91% (STZ and SBZ). With the addition of the deuterated compounds, internal calibration curves were built for each of the matrices (Figure 6b). The difference in the slopes decreased considerably, contributing to a relevant reduction of the matrix effects during quantification for most of the SAs (see Table 2). It should be taken into account that solid matrices are not homogeneous, and neither are the matrix effects that may happen in the ESI source. This means that the ME% values given in Table 2 are mere indicators of the signal suppression or enhancement for our matrices, and that this *ME%* can differ in each individual sample.

4. APPLICATION OF THE METHOD

The method developed was applied in the analysis of dehydrated sewage sludge from 17 different WWTPs and agricultural soils located in Catalonia and also along the Ebro River basin.. LODs and LOQs were estimated individually for each sample and the average values are shown in Table 3, together with the recovery values obtained during the validation of the method, which were applied to calculate the concentrations. STZ, SDM and SPY were the two SAs most frequently detected in sewage sludge, and except for SDM, they also presented some of the highest concentrations, with values up to 122 ng g^{-1} (SPY). On the contrary, the acetylated metabolite, AcSPY, was present only in 2 of the sewage sludge samples and at lower concentrations (average value of 1.24 ng g^{-1}). Both parent and metabolite are one of the most commonly found SAs in WWTP effluents [2,8,28]. SMX, the most commonly detected SA in WWTPs, was only present in a 30% of the sewage samples analyzed, with an average concentration of 1.75 ng g^{-1}. Its metabolite was detected in the 24% of the samples at a higher average concentration than the parent compound (9.81 ng g^{-1}). AcSDZ was present in 1 sludge sample.

Occurrence in soils was less common for all the SAs investigated, with a maximum frequency of 47% (8 out of the 17 samples) for SMZ and AcSMX. The maximum concentration detected was also for SMZ, which is a SA commonly used in cattle farming; the application of the cattle wastes as organic amendment to agricultural soil is the most probable source of this SA in the soils analyzed. SDZ, a commonly used veterinary SAs and frequently investigated in agricultural soils [10,22,24], and its metabolite were scarcely present in these soils samples. The presence of SMX, typically used in human therapies, in soils is similar to that encountered in sewage sludge, and might be due to the application of biosolids from WWTPs as fertilizer or the use of blackwaters or treated wastewaters for irrigation in these agricultural areas. Its acetylated metabolite AcSMX has been detected more frequently than the parent

compound in the soils samples (47% of the samples) but at a lower concentration (0.95 ng g^{-1} average concentration).

5. CONCLUSIONS

A new PLE method was developed for the determination of 22 sulfonamides, including 5 of their acetylated metabolites, in both sewage sludge and soils. Clean up of the samples extracts was carried out using HLB cartridges. The evaluation of matrix effects has been prioritized, and to this purpose matrix matched calibration curves were built and extract dilutions carried out. The preferred compensating approach to tackle with the signal supression caused by these matrix effects was the use of different internal standards for quantification. The sensitivity achieved allowed the detection of the target sulfonamides at levels down to 0.03 ng g^{-1} in sewage sludge and 0.01 ng g^{-1} in soil samples. Application to both sewage sludge and soil samples with positive findings confirmed the method usefulness. Althgouh not at high concentrations, AcSDZ, AcSPY and AcSMX were detected in sewage sludge samples, and only AcSMX in soil samples, which made evident its antropogenic origin.

ACKNOWLEDGEMENTS

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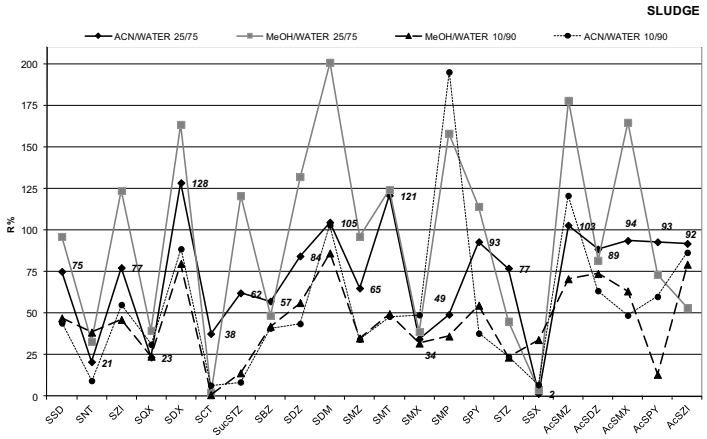
FIGURES AND TABLES



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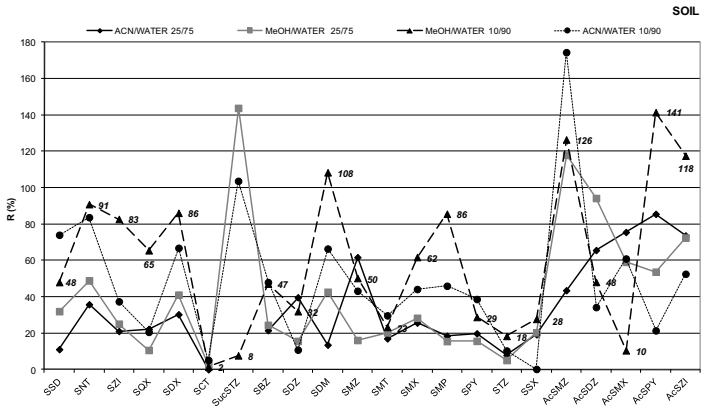
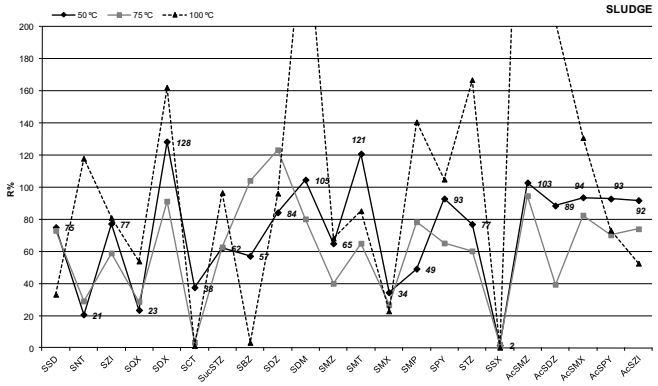


b.

Figure 1. Recoveries obtained for the sulfonamides investigated in a) sewage sludge and b) soils, after their PLE extraction using different solvent mixtures (T: 50 ºC; Pressure: 1500 psi)

Figure 1 (cont.)



a.

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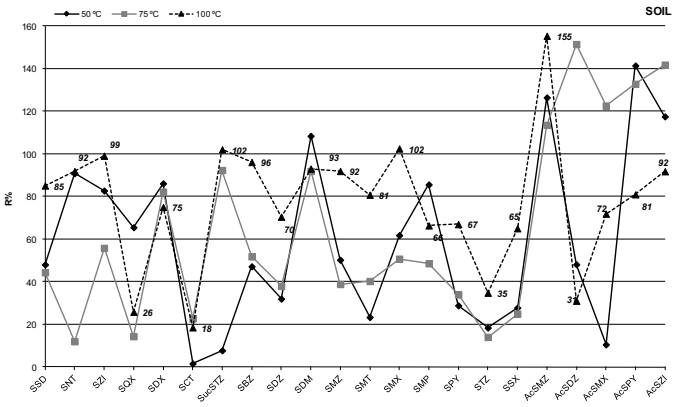

Figure 2. Recoveries obtained for the sulfonamides investigated in a) sewage sludge and b) soils, after their PLE extraction at different temperatures.

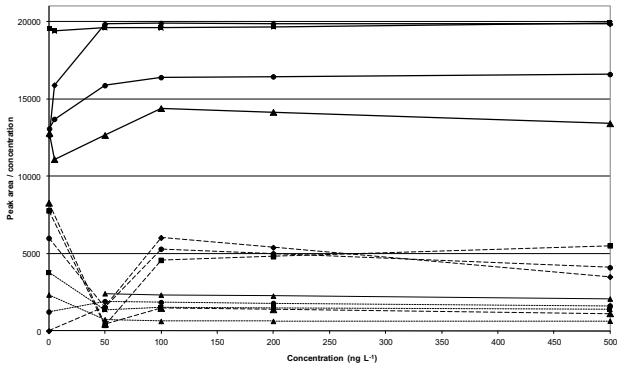

Figure 3. Response factors (RFs) of four of the investigated sulphonamides, estimated for sewage sludge and soil during LC-ESI-MS/MS analysis. ■: sulfamethoxazole; ●: sulfamethazine; ▲:N^4-acetyulsulfamethazine; (♦): sulfapyridine; ( ): standard curve;(**– –**): soil matrix; (·····): sludge matrix.

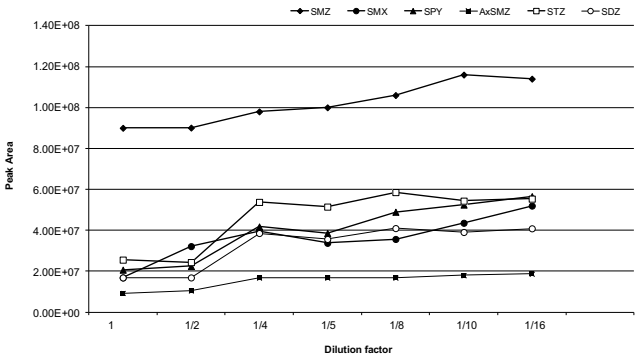

Figure 4. Dilution of the highest point of the matrix matched calibration curve for sewage sludge.

Figure 5. Chromatographic peaks of the 500 ng g^{-1} calibration point in solvent and in both sewage sludge and soil matrix matched calibration curves, for sulfapyridine (SPY) and sulfadiazine (SDZ).

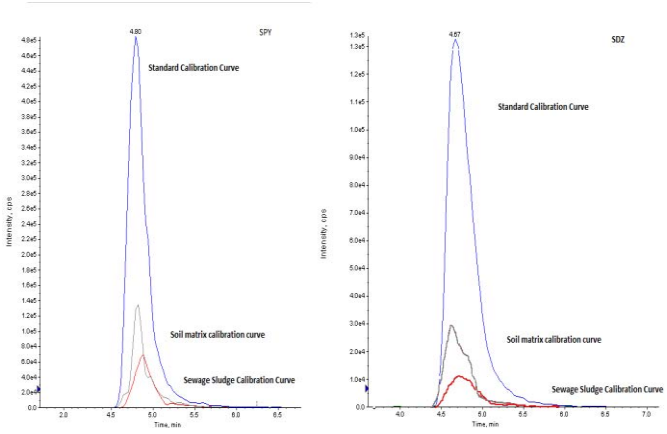

Figure 6. Standard addition calibration curves in (a) solvent, sewage sludge and soil extracts and (b) internal standard calibration curves for some of the sulfonamides investigated. SDZ: sulfadiazine; SMZ: sulfamethazine; SMX: sulfamethoxazole; STZ: sulfathiazole; (———): standard curve; (– – – –): soil matrix; (·····): sludge matrix.

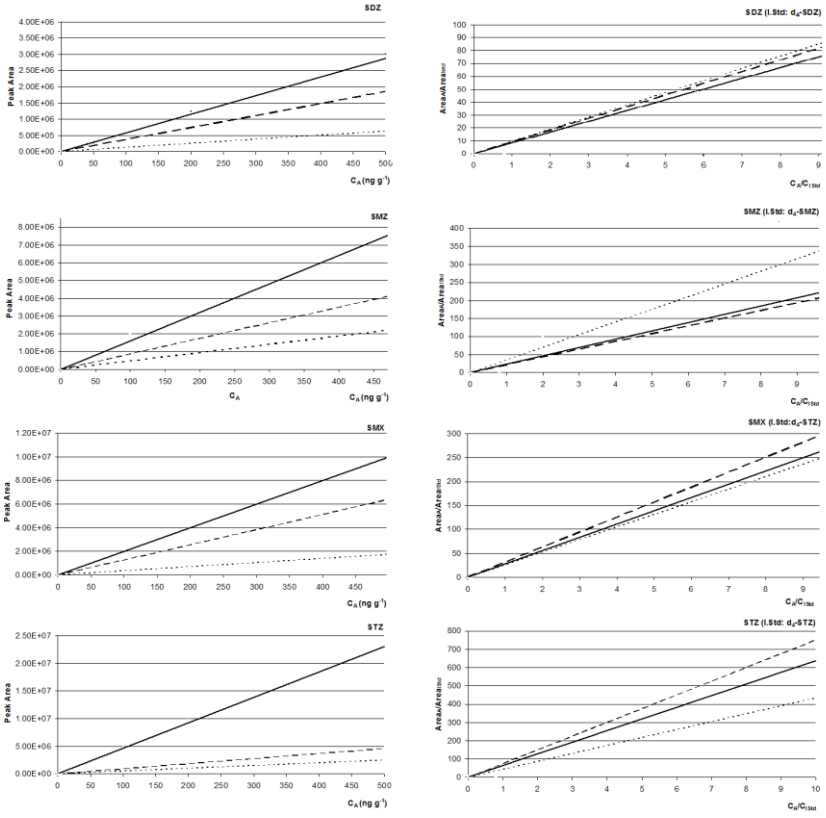

Table 1.Instrumental variation, given as relative standard deviation (RSD%), recovery values and standard deviation (n=3) at spike levels of 50 ng g^{-1} (R1%) and 100 ng g^{-1} (R2%), method limits of detection (MLOD) and quantification (MLOQ) (ng g^{-1}) for each of the analytes investigated. *: unique value.

		SLUDGE				SOIL			
	RSD(%)	R1% (50 ng g^{-1})	R2% (100 ng g^{-1})	MLOD	MLOQ	R1% (50 ng g^{-1})	R2% (100 ng g^{-1})	MLOD	MLOQ
SULFISOMIDIN	17	19 ± 8	86.2 ± 9	0.04	0.14	80 ± 12	66 ± 11	0.01	0.05
SULFAMERAZINE	18	106 ± 9	93.6 ± 8	0.05	0.18	62 ± 9	67 ± 11	0.04	0.13
SULFAQUINOXALINE	18	44 ± 11	111 ± 12	0.24	0.79	107 ± 9	121 ± 20	0.49	0.11
SUCCINYL-SULFATHIAZOLE	6	82 ± 20	104 ± 18	2.23	7.42	76 ± 3	70 ± 15	0.55	1.84
SULFADOXINE	6	56 ± 10	62 ± 4	0.07	0.23	99 ± 22	133.3 ± 7	0.02	0.08
SULFACETAMIDE	16	76 ± 8	101 ± 8	-	-	59 ± 11	122*	-	-
SULFABENZAMIDE	22	113 ± 13	77 ± 8	0.09	0.29	77 ± 8	77 ± 12	0.16	0.55
SULFADIAZINE	24	53 ± 10	89 ± 13	1.05	3.51	66 ± 5	104 ± 20	0.25	0.84
SULFADIMETHOXINE	11	81 ± 8	93 ± 9	0.03	0.10	76 ± 10	103 ± 14	0.01	0.05
SULFAMETHAZINE	9	66 ± 6	102 ± 8	0.40	1.32	72 ± 22	64 ± 9	0.34	1.13
SULFAMETHIZOLE	18	78 ± 10	56 ± 12	0.21	0.72	41 ± 3	112 ± 13	-	-
SULFAMETHOXAZOLE	18	58 ± 14	94 ± 12	0.18	0.60	79 ± 11	131 ± 26	0.05	0.17
SULFAMETHOXYPYRIDAZINE	17	68 ± 12	66 ± 5	0.15	0.50	41 ± 4	50 ± 5	0.08	0.28
SULFAPYRIDINE	15	123 ± 25	99 ± 4	0.44	1.45	57 ± 2	69 ± 11	0.28	0.93
SULFATHIAZOLE	8	42 ± 5	115 ± 12	0.37	1.24	43 ± 7	107 ± 14	0.06	0.21
SULFISOXAZOLE	18	48 ± 10	120 ± 20	0.17	0.57	32 ± 1	47 ± 1	0.11	0.35
SULFANITRAN	4	52 ± 8	108 ± 3	17.40	58.00	82 ± 20	84 ± 22	4.19	13.98
N^4-ACETYLSULFAMETHAZINE	21	103 ± 7	130 ± 31	0.47	1.55	85 ± 3	103 ± 23	0.25	0.82
N^4-ACETYLSULFADIAZINE	3	40 ± 1	85 ± 10	0.66	2.21	94 ± 1	59 ± 7	0.07	0.25
N^4-ACETYLSULFAMETHOXAZOLE	6	58 ± 1	127 ± 6	0.58	1.93	114 ± 11	90 ± 28	0.23	0.78
N^4-ACETYLSULFAPYRIDINE	2	80 ± 11	119 ± 3	0.30	1.00	137 ± 6	80 ± 17	0.31	1.05
N^4-ACETYLSULFAMERAZINE	5	84 ± 9	91 ± 8	0.39	1.30	130 ± 17	82 ± 10	0.20	0.67

Table 2. Matrix effects (ME%) calculated for each of the sulfonamides, with and withouth using internal standards, using equation [2] in section 3.2.2. Positive values indicate signal suppression and negative values signal enhancement. Istd: internal standards used.

		SLUDGE		SOIL	
	Istd	ME (%)	ME (Istd) (%)	ME (%)	ME (Istd) (%)
SULFISOMIDIN	d_4-sulfamethazine	95	70	77	-4
SULFAMERAZINE	d_4-sulfadiazine	89	24	84	26
SULFAQUINOXALINE	d_4-sulfamethoxazole	85	-14	75	11
SULFADOXINE	d_4-sulfamethoxazole	72	-7	59	28
SULFACETAMIDE	d_4-sulfamethazine	98	78	87	29
SUCCINYL-SULFATHIAZOLE	d_4-sulfadiazine	81	29	56	20
SULFABENZAMIDE	d_4-sulfamethoxazole	96	45	91	-12
SULFADIAZINE	d_4-sulfadiazine	90	-13	71	-9
SULFADIMETHOXINE	d_4-sulfamethoxazole	66	-3	49	-51
SULFAMETHAZINE	d_4-sulfamethazine	87	7	74	-52
SULFAMETHIZOLE	d_4-sulfamethazine	89	15	75	3
SULFAMETHOXAZOLE	d_4-sulfamethoxazole	91	5	68	-13
SULFAMETHOXYPYRIDAZINE	d_4-sulfamethoxazole	90	-15	76	10
SULFAPYRIDINE	d_4-sulfadiazine	89	37	72	48
SULFATHIAZOLE	d_4-sulfadiazine	94	32	91	-18
SULFISOXAZOLE	d_4-sulfamethoxazole	96	34	87	-16
SULFANITRAN	d_4-sulfamethoxazole	76	10	52	-30
N^4-ACETYLSULFAMETHAZINE	d_4-sulfamethazine	82	9	71	52
N^4-ACETYLSULFADIAZINE	d_4-sulfadiazine	-	-	-	-
N^4-ACETYLSULFAPYRIDINE	d_4-sulfadiazine	-	-	-	-
N^4-ACETYLSULFAMETHOXAZOLE	d_4-sulfamethoxazole	-	-	-	-
N^4-ACETYLSULFAMERAZINE	d_4-sulfadiazine	-	-	-	-

2.3. DISCUSIÓN DE LOS RESULTADOS

2.3.1. Optimización del método

Con el objetivo de poder analizar sulfamidas a niveles traza en muestras ambientales, matrices generalmente complejas, la necesidad de minimizar o eliminar falsos positivos hacía necesaria la utilización de equipos altamente selectivos y con alta sensibilidad. La Tabla 2.1 muestra los límites de detección instrumental obtenidos con diferentes tipos de analizadores. Los análisis LC-MS/MS nos permitirían detectar y diferenciar compuestos con el mismo ión precursor pero con diferentes iones producto, incluso si coeluían. Se desarrollo un nuevo método para triple cuadrupolo (QqQ), utilizando para ello el instrumento Quatro Waters Micromass ^{TM}. Para optimizar los parámetros que afectan a la señal en el analizador MS, como la energía de colisión y el voltaje de cono, realizamos diferentes inyecciones en columna de disoluciones de cada patrón por separado y posteriormente de una mezcla de todos ellos. Se utilizó una fuente ESI en modo positivo y para la identificación de los analitos se adquirieron los espectros de masas en modo full scan con un intervalo de masas entre m/z 100 y m/z 500. Como ión precursor se seleccionó la molécula protonada $[M+H]^+$ para cada una de las sulfamidas y no se observaron aductos como $[M+Na]^+$ o $[2M+Na]^+$, que si fueron detectados en análisis previos realizados con el cuadrupolo simple. Establecimos dos ventanas de tiempo, de 0 a 8 min para 7 de las sulfamidas, y de 8 a 23 min para el resto. Se eligieron dos patrones internos, d_4-sulfatiazol y d_4-sulfametoxazol, uno para cada ventana de tiempo. Se eligieron las dos transiciones SRM más abundantes para cada analito y para los compuesto deuterados: la primera se utilizó para la cuantificación, y la segunda como transición de confirmación (ver Tabla 1 de la Publicación #3). Como se puede observar también en esta tabla, dada la similitud estructural de las sulfamidas estudiadas, los iones fragmento obtenidos son los mismos para muchas de ellas. Por ejemplo, el fragmento m/z 156, derivado de la pérdida de la amina en la sulfanilamida, es común a todas ellas excepto para el metabolito acetilado. La Figura 2.1 muestra a modo de ejemplo como los iones fragmento m/z 156, m/z 108 y m/z 92 son comunes para STZ, SPY, SSX y SMZ. El fragmento característico del SSX es m/z 113 y de la SMZ el m/z 124, ya que contienen el grupo sustitutivo característico de cada una de ellas. Finalmente, el método MS/MS optimizado en QqQ se implementó para su utilización en el QqLIT. La intensidad de los

fragmentos de algunas sulfamidas varió entre el analizador QqQ al QqLIT, pero el patrón de fragmentación se mantuvo para todas ellas.

Tabla 2.1. Límites de detección instrumental (iLOD, pg) del cuadrupolo simple (Q), del triple cuadrupolo (QqQ) y de la trampa de iones lineal-cuadrupolo (QqLIT) para las diez primeras sulfamidas estudiadas.

SULFAMIDA	Q	QqQ	QqLIT
Sulfadiazina	1428	15.87	0.3
Sulfadimetoxina	166.7	5.88	0.004
Sulfametazina	545.5	15.18	0.26
Sulfametizol	3226	22.71	0.23
Sulfametoxazol	1765	15.15	0.26
Sulfametoxipiridazina	130.4	46.5	0.08
Sulfapiridina	250	26.55	0.11
Sulfatiazol	545.5	20.82	0.26
Sulfisoxazol	750	16.08	0.11
N^4-acetilsulfametazina	400	4.92	0.13

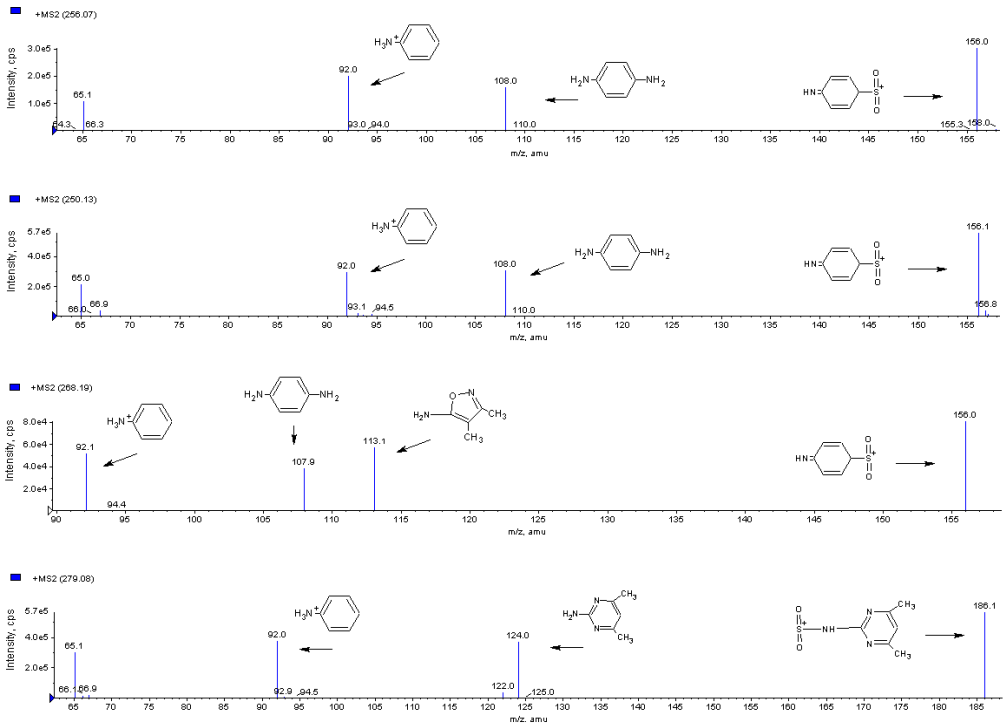

Figura 2.1. Fragmentos comunes y característicos obtenidos para sulfatiazol (STZ) (m/z 256), sulfapiridina (SPY) (m/z 250), sulfisoxazol (SSX) (m/z 268) y sulfametazina (SMZ)(m/z 279).

2.3.2. Extracción en fase sólida (SPE)

En todas las publicaciones presentadas en este capítulo, las metodologías desarrolladas se aplicaron en muestras ambientales. En el caso de las muestras de agua, para la optimización de la SPE, se extrajeron 400 mL de muestras de agua de río, subterránea y de efluente de depuradora, y 200 mL de agua residual no tratada, fortificadas con una mezcla standard de sulfamidas a una concentración de 500 ng L^{-1}. Se evaluaron diferentes estrategias de extracción, incluyendo SPE en tandem, que están resumidas en la Figura 1 de la Publicación #3. Finalmente, los cartuchos HLB Oasis fueron los elegidos ya que se obtuvieron mejores valores de recuperación de los analitos (ver Tabla 4 de la Publicación # 3). Si bien estos resultados fueron muy similares a los obtenidos tras la extracción SPE en tandem, como

muestra la Figura 2.2, para agilizar la metodología y reducir el tiempo de pretratamiento de muestra, el método de extracción con cartuchos HLB fue el seleccionado.

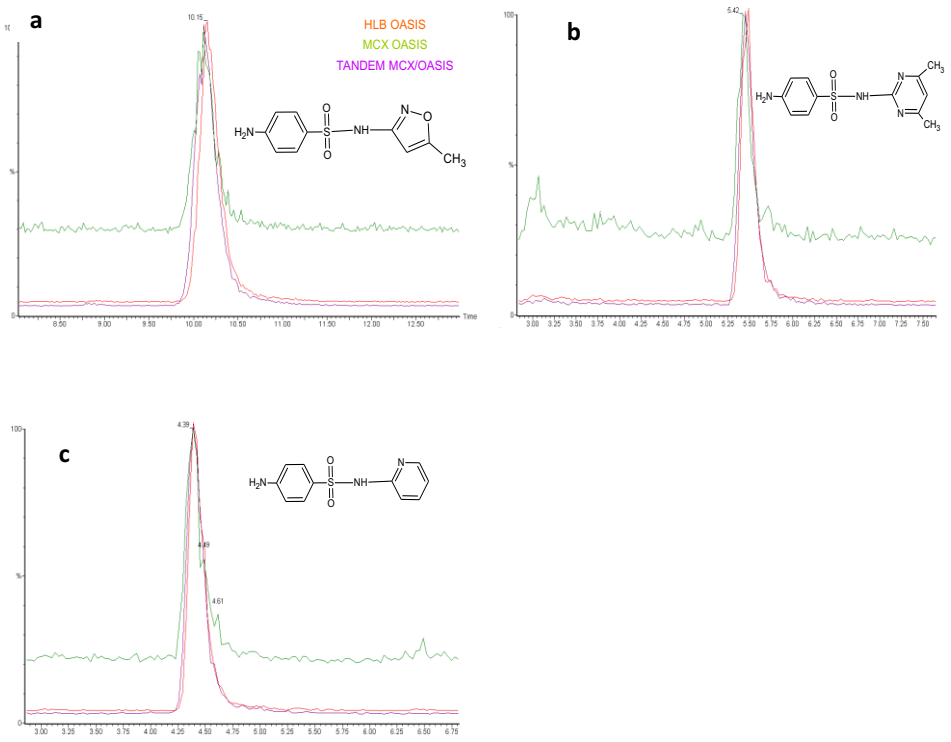

Figura 2.2. Picos cromatográficos obtenidos para a) sulfametoxazol (SMX), b) sulfametazina (SMZ) y c) sulfapiridina (SPY) con las tres estrategias de extracción en fase sólida evaluadas.

El paso de SPE off-line a SPE on-line significó una importante agilización de todo el método, ya que supuso la automatización de todas las etapas del SPE off-line y la utilización de volúmenes mucho menores tanto de muestra como de disolvente, además de prescindir de los pasos de evaporación del eluato y reconstitución en vial, ya que en la SPE on-line los analitos son eluidos directamente a la columna cromatogràfica (ver sección 1.7.1). Como se explica en la Publicación #4, el primer instrumento que utilizamos para desarrollar la SPE on-line fue un Prospekt-2^{TM} de Spark Holland. El volumen de muestra pasaba al cartucho SPE mediante unos

tubos de *peek* con superficies de absorción para la muestra (ver Figura 2.3). el método se optimizó en términos de volúmen de carga, tipo de cartucho SPE y disolventes de activación del cartucho, como muestran las Figuras 2, 3 y 4 de la Publicación #4.

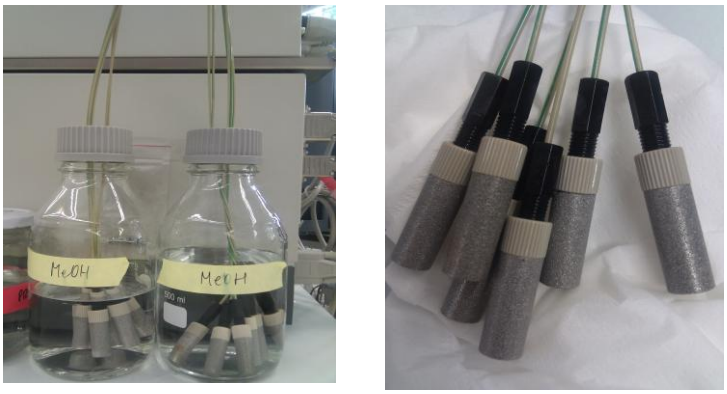

Figura 2.3. Tubos de peek y superficies de absorción para la captación de muestra durante la SPE on-line utilizando el Prospekt-2™

Para prever problemas de efecto memoria o *carry over* (presencia de concentraciones residuales de la muestra analizada previamente) en estos canales de *peek*, se realiza un lavado o *rinse* con MeOH y agua HPLC de dichos tubos y de la vàlvula de entrada, y también con un pequeño volumen de la nueva muestra que se extraerá a continuación. Los volúmenes de disolventes para la activación del cartucho y de las muestras llegaban al sistema con la ayuda de una jeringa de alta presión de 2 mL. Estimamos el efecto memoria utilizando los blancos que en la secuencia se analizaban después del punto más alto de la recta de calibrado, y observamos que, para todos los analitos y para cada matriz este efecto era despreciable (< 0.01%). Posteriormente, trasladamos este método a un instrumento nuevo para SPE on-line, un Symbiosis Pico™ también de Spark Holland. La principal diferencia respecto al Prospekt-2™ era que, si bien en éste primero el número limitado de tubos *peek* también limitaba el número de muestras que podrían analizarse en un mismo batch, el Symbiosis Pico™ disponía de un autosampler que permitía la inyección de cada muestra directamente desde su vial al equipo. De este modo, el número de muestras a inyectar era superior y dependía de la capacidad del autosampler. Además, el prescindir de estos canales también redujo la posibilidad de efecto

memoria. Otra diferencia importante era que la muestra era llevada hasta el cartucho mediante canales metálicos o *loops* , que muestra la Figura 2.4, con un volumen determinado. En función del volumen de muestra a extraer, se utilizaría un *loop* de volúmen adecuado que se cargaría con la muestra; ésta sería expulsada del loop hacia el cartucho haciendo pasar un volúmen adecuado de agua HPLC para llenar el loop que presionaría y expulsaría la muestra hacia le cartucho SPE. En el método desarrollado para el instrumento Symbiosis Pico ^{TM}, se incluyó una etapa de lavado intenso de la aguja de inyección y de la pinza transportadora de los cartuchos o *clump* como medida preventiva del posible efecto memoria, así como frecuentes muestras blanco con agua HPLC en la secuencia.

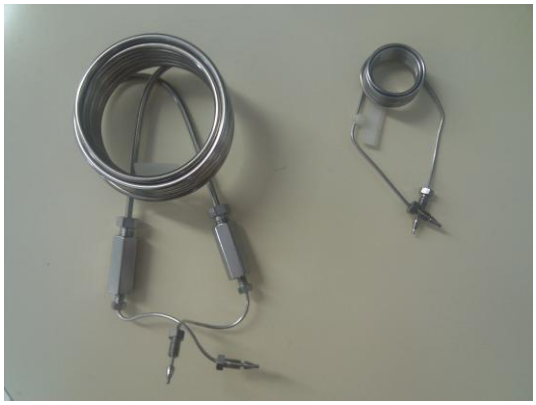

Figura 2.4. Loops de 5 y 1 mL del Symbiosis Pico^{TM} utilizados para cargar la muestra en los cartuchos de extracción on-line.

Por último, la Tabla 2.2 muestra una comparación de los valores de los límites de detección obtenidos para las metodologías off-line y on-line. Ambos procedimientos de extracción permitieron la detección a niveles muy similares, incluso en el caso de matrices algo más complejas como agua de río o de depuradora. Sulfamidas como SMZ o SMT mejoran notablemente sus LODs con la extracción on-line, al igual que AcSMZ, mientras que los límites para SSX son algo mayores en la metodología on-line. Para SDZ y SPY las diferencias entre los valores obtenidos son apenas evidentes, mientras que para SMX la extracción on-line parece más favorable sólo para aguas subterráneas.

Tabla 2.2. Límites de detección (ng L^{-1}) obtenidos en las metodologías off-line y on-line desarrolladas (Publicaciones #3 y #4) desarrolladas. Los valores para aguas de río son los valores medios obtenidos en las Publicaciones #3 y #7.

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	AGUA DE RÍO		AGUA SUBTERRÁNEA		AGUA DE SALIDA DE EDAR	
	Off-line	On-line	Off-line	On-line	Off-line	On-line
SULFADIAZINA (SDZ)	0.39	0.34	0.48	0.18	0.65	0.9
SULFADIMETOXINA (SDM)	0.15	0.02	0.01	0.02	0.19	0.01
SULFAMETAZINA (SMZ)	0.13	0.05	0.07	0.04	43.09	0.06
SULFAMETIZOL (SMT)	0.22	4.52	0.07	3.1	0.16	1.04
SULFAMETOXIPIRIDAZINA (SMP)	0.28	0.09	0.05	0.03	0.25	0.05
SULFAMETOXAZOL (SMX)	0.09	0.46	1.13	0.81	0.26	0.77
SULFAPIRIDINA (SPY)	0.14	0.06	0.05	0.02	0.18	0.13
SULFATIAZOL (STZ)	0.15	0.22	0.20	0.21	0.08	0.45
SULFISOXAZOL (SSX)	0.04	0.31	0.03	0.63	0.29	2.15
N^4-ACETILSULFAMETAZINA (AcSMZ)	0.19	0.02	0.11	0.02	460.9	0.14

2.3.3. Extracción de matrices sólidas

La complejidad de las matrices sólidas, en nuestro caso fangos de depuradora y suelos, planteaba un importante reto analítico. La extracción de las diferentes muestras sólidas se llevó a cabo mediante PLE. Se trata de una técnica rápida (aproximadamente 15-20 minutos por muestra, en función del número de ciclos), que requiere poca cantidad de muestra, usa volúmenes más pequeños de disolvente y permite operar automáticamente, y por eso es preferible a otras técnicas como USE, que si bien pueden conseguir extractos más limpios y LODs más bajos como demostraron Göbel *et al*. (Göbel *et al.* 2005), requieren mucho más tiempo para la extracción, cantidades mayores de muestra y de disolvente y una manipulación constante muestra a muestra. La purificación de los extractos mediante su filtrado y SPE ha de ser lo suficientemente intensa para poder eliminar todos los componentes de la matriz que pueden interferir en el análisis sin comprometer con ello la retención de los analitos de interés en el cartucho. En el trabajo presentado en la Publicación #6, los extractos obtenidos fueron diluidos con 200 mL de agua HPLC para obtener así un volumen equivalente al óptimo para la SPE off-line de aguas residuales no tratadas. Por tanto, estos extractos fueron previamente filtrados y preconcentrados siguiendo mismo procedimiento de SPE mencionado en la sección

2.3.2 La utilización de cinco sulfamidas deuteradas como patrones internos nos permitió cuantificar de forma más fiable, pudiendo así compensar el posible efecto matriz en la fuente ESI.

La principal novedad del nuevo método presentado en la Publicación #6 es la inclusión de cinco metabolitos acetilados, ya que hasta la fecha sólo existía un trabajo que había incluido estos compuestos en el análisis de suelos agrícolas (Stoob *et al.* 2006), pero no en fangos de depuradora. Se obtuvieron LODs metodológicos de hasta 0.03 ng g^{-1} para fangos y de 0.01 ng g^{-1} para suelos utilizando sólo 2 g de muestra de fangos y 5 g de suelos. Si comparamos dichos valores con métodos anteriores, como el de Stoob *et al*. (Stoob *et al.* 2006) o Shelver *et al*. (Shelver *et al.* 2010), comprobamos que son hasta dos órdenes de magnitud más bajos, también para los metabolitos.

3. DETERMINACION DE NIVELES AMBIENTALES

3.1. INTRODUCCIÓN Y OBJETIVOS

Como se comentó en la Introducción de esta Tesis, a pesar de tratarse de antibióticos mayoritariamente utilizados en prácticas veterinarias, la presencia de sulfamidas en aguas residuales urbanas es muy frecuente, ya que algunas de ellas como el SMX, el SPY o el SDZ también son utilizadas en medicina humana en terapias para el tratamiento del acné, en quemaduras y para diferentes infecciones. La baja eficacia de los diferentes tipos de tratamiento de aguas residuales en su eliminación explica su presencia en las aguas de salida de EDAR, en las aguas receptoras de estos emisores y en diferente medida en el resto de compartimentos ambientales, sin olvidar la aplicación de fangos de depuradoras como abono en campos de cultivo. En medios rurales, la deposición de excrementos de ganado de pastoreo o estabulado y la aplicación de éstos como estiércol y abono agrícola también constituye una vía de entrada relevante, en este caso para las sulfamidas veterinarias. Durante el desarrollo de esta Tesis hemos aportado nuevos datos sobre la presencia medioambiental de estos antibióticos, incluyendo el estudio de sus metabolitos y otros posibles productos de degradación. Las cinco publicaciones incluidas en este capítulo confirman su presencia en todos los tipos de aguas ambientales y matrices sólidas estudiadas, permitiendo dichos datos la evaluación de su posible toxicidad y efectos en los diferentes ecosistemas receptores. En definitiva, los objetivos de este capítulo son:

- La aplicación de las diferentes metodologías analíticas desarrolladas para la detección de niveles ambientales de sulfamidas.
- Obtener niveles concretos de las diferentes sulfamidas estudiadas en las diferentes matrices ambientales analizadas.
- Incluir los metabolitos acetilados en la evaluación medioambiental.
- Realizar un estudio estadístico multivariable para poder observar tendencias de contaminación en las zonas investigadas.

- Estudiar los parámetros más relevantes de diseño de EDAR, como tiempo de retención hidraúlico (HRT), ysu influencia en la eliminación de las sulfamidas.
- Evaluar el riesgo ambiental asociado a la presencia de sulfamidas en los diferentes tipos de aguas ambientales.

3.2. PUBLICACIONES

Publicación científica 7:

"LC-QqLIT MS analysis of nine sulfonamides and one of their acetylated metabolites in the Llobregat River basin. Quantitative determination and qualitative evaluation by IDA experiments"

María Jesús García Galán, Marta Villagrasa, M. Silvia Díaz-Cruz, y Damià Barceló.

Analytical and Bioanalytical Chemistry (2010), Vol. 397, pp. 1325-1334.

Publicación científica 8

"Simultaneous occurrence of nitrates and sulfonamide antibiotics in two ground water bodies of Catalonia (Spain)"

María Jesús García Galán, Teresa Garrido, Josep Fraile, Antoni Ginebreda, M. Silvia Díaz-Cruz, y Damià Barceló.

Journal of Hydrology (2010), Vol. 383, pp. 93-101.

Publicación científica 9

"Occurrence of sulfonamide residues along the Ebro river basin. Removal in wastewater treatment plants and environmental impact assessment"

María Jesús García Galán, M. Silvia Díaz-Cruz, y Damià Barceló.

Environment International (2011), Vol. 37, pp. 462-473.

Publicación científica 10

"Ecotoxicity evaluation and removal of sulfonamides and their acetylated metabolites during conventional wastewater treatment"

María Jesús García Galán, Susana González Blanco, Ramón López Roldán , M. Silvia Díaz Cruz, Damiá Barceló.

Science of the Total Environment (2012), Vol. 437, pp. 403-412

Anal Bioanal Chem (2010) 397:1325–1334
DOI 10.1007/s00216-010-3630-y

ORIGINAL PAPER

LC-QqLIT MS analysis of nine sulfonamides and one of their acetylated metabolites in the Llobregat River basin. Quantitative determination and qualitative evaluation by IDA experiments

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Abstract Robust instrumental analytical chemistry and the subsequent development of improved analytical methodologies and extraction procedures have enabled the detection at environmental levels of new emerging contaminants, for example pharmaceuticals. The objective of this study was to explore the potential of liquid chromatography–tandem quadrupole-linear ion trap (LC–QqLIT MS) for quantitative determination of nine sulfonamides (SAs) and one acetylated metabolite in surface water from the Llobregat River and one of its main tributaries, the Anoia River, in Catalonia (Spain). A total of 21 samples were taken in three sampling campaigns. Recoveries ranging from 26% to 123% were calculated for the SAs studied, and method limits of detection (MLODs) achieved were in the range 0.05–0.2 ng L^{-1}. Through the different campaigns, concentrations ranged from 0.74 ng L^{-1} (sulfamethizole) to 2,482 ng L^{-1} (sulfamethazine) in the Llobregat River, and from 0.27 ng L^{-1} (sulfamethizole) to 168 ng L^{-1} (sulfamethoxazole) in the Anoia River. Sulfamethoxazole and sulfapyridine were the two SAs most frequently detected (80% and 71% respectively). N^4-acetylsulfamethazine was detected in both rivers, but with different frequencies (4% of the samples from the Anoia River and 43% of those from the Llobregat River). Information-dependent acquisition (IDA) experiments were also developed in order to obtain enhanced product-ion spectra in surface water samples.

Keywords Sulfonamides · Antibiotics · Environmental analysis · Liquid chromatography · Mass spectrometry · IDA experiments

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Introduction

As a consequence of an increasing human population density and more intensive animal farming techniques, water systems are highly susceptible to be at risk of potential contamination by various pharmaceutical products from both human and veterinary use. Sulfonamides (SAs), some of the most commonly used antibacterial agents, especially in veterinary medicine, are amongst them. These antibiotics are widely used because they are inexpensive, effective against a broad spectrum of common bacterial infections, and have high effectiveness in growth promotion in veterinary applications, although this last use has been banned in the EU since 2006 for all antibiotics [1]. Concern has risen after the occurrence of SAs was reported in all kinds of water samples [2–6]; apart from the high quantities employed, this frequent detection can be explained on the basis of their low tendency to be adsorbed by soils. Another reason for greater occurrence may be found in their relatively low elimination efficiency during wastewater-treatment procedures [7], being present in the corresponding effluents and receiving streams, and in the increase in the number of

confined animal feeding operations, which often lack proper waste-management practices [8].

Liquid chromatography-tandem mass spectrometry (LC–MS) has become an essential tool to study trace organic environmental contaminants that are polar, thermally labile, and not amenable to GC–MS. SAs are among this category of compounds. LC–MS can be used for quantification purposes when analytes are present in simple matrices, such as tap water and bottled water, whereas LC–tandem MS is necessary to quantify with confirmation of identity of residues in complex matrices, such as wastewaters [9, 10], minimizing or eliminating false-positive detection. LC–tandem MS enables separation and detection of compounds having the same molecular mass but different product ions (isobaric ions), even if they co-elute. Therefore, although LC–MS has been used for quantification of antibiotics residues in environmental samples, tandem MS detection is preferred for enhanced sensitivity and selectivity in complex matrices. The methods published in the literature for analysis of antibiotics in water, sediment/soil, and sludge have recently been reviewed by Díaz-Cruz and Barceló [11, 12]. According to these studies, its versatility and high sensitivity and selectivity with complex matrices makes LC–tandem MS the technique of choice for environmental analysis of sulfonamide residues. One of the most important factors affecting the performance of LC–tandem MS-based methods is the type of mass analyzer employed, together with the sensitivity of the ion source [13–15]. The triple quadrupole (QqQ) mass spectrometer for liquid chromatography is considered a powerful mass analyzer for both qualitative and quantitative analysis, because of its selectivity, sensitivity in the selected reaction monitoring (SRM) mode, and versatility. However, its relative long duty cycle (slow scan speed) limits the number of scans that can be acquired simultaneously [12, 16, 17]. On the other hand, the hybrid quadrupole-ion trap (QqIT) mass spectrometer is becoming an important tool in environmental analysis. Unique features of an IT mass analyzer are that it can perform multiple stages of fragmentation (MS^n) and provide higher sensitivity in scan mode. Once the ions are generated in the source, a package of ions are trapped in the ion trap; however, quantification of the target analyte in complex matrices is hindered because of the low proportion of analyte ions compared with other undesired matrix component ions. Another drawback of IT mass analyzers is the limited dynamic mass range of product ions, because only ions with an *m/z* larger than ca. one third of the precursor ion can be successfully trapped [18]. To overcome these hindrances associated with QqQ and QqIT mass analyzers, a new hybrid quadrupole-linear ion trap (QqLIT) instrument has recently been developed [19, 20]. Essentially, this apparatus is a QqQ in which the third quadrupole can be operated as either a quadrupole or an ion trap. The QqLIT enables extraction of nearly an order of magnitude more ions into the linear ion trap compared with the conventional 3D ion traps aforementioned when both are operated at the same ion density. As a consequence, it enables higher sensitivity for product-ion scan and offers some extra scan possibilities such as enhanced full scan (EMS), enhanced product ion (EPI), and MS^3 (the term "enhanced" indicates that the data are generated in the ion-trap mode with improved performance). The configuration of the QqLIT also enables spatial separation of the fragmentation and trapping steps, so the precursor ion and the lowest *m/z* ion do not need to be simultaneously stable within the ion trap, which implies that there is no inherent low-mass cut-off in such mass analyzers [20]. So, in addition to all the capabilities of the triple quadrupole, the QqLIT instrument yields extra confirmative information. Recently some reports in the literature have highlighted these advantages and the feasibility of this kind of instrumentation for metabolite identification [21, 22].

This work describes the instrumental methodology for quantification and confirmation of trace levels of nine sulfonamide antibiotics and one acetylated metabolite in surface waters from the Llobregat and Anoia rivers (Catalonia, Spain) by LC–QqLIT-MS. To investigate the capability of the QqLIT instrument, some independent data acquisition experiments (IDA) were performed with surface water samples.

Experimental

Chemicals and materials

Sulfamethizole (99.9%), sulfisoxazole (99.8%), sulfathiazole (99.9%), sulfadiazine (99.8%), sulfapyridine (98%), sulfamethazine (99%), and sulfamethoxypyridazine (99.8%) were purchased from Riedel–de Haën (Seelze, Germany); sulfadimethoxine (99.9%), sulfamethoxazole (99.9%), and N^4-acetylsulfamethazine (99.8%), were purchased from Sigma–Aldrich (Seelze, Germany).

Internal standard d_4-sulfathiazole (99.9%) was purchased from Toronto Research Chemicals (Ontario, Canada).

Water, methanol (MeOH), acetone, and acetonitrile (ACN) were HPLC-grade; formic acid was MS-grade. They were all purchased from Merck (Darmstadt, Germany). High-quality nitrogen (N_2) and argon were supplied by Abelló Linde (Barcelona, Spain).

Individual antibiotic stock standard solutions were prepared at 100 μg mL^{-1} by dissolving 1 mg of the individual drug in 10 mL MeOH. A 5 μg mL^{-1} solution of the internal standard and stock standard solutions of mixtures of all the compounds at 5 and 1 μg mL^{-1} were prepared. Working standard solutions of the mixture were freshly made by appropriate dilution of the stock standard

mixture in MeOH. Solutions were transferred to amber bottles and stored in the dark at 4°C to minimize potential analyte degradation.

Glass fiber (2.7 μm) and Nylon membrane (0.45 μm) filters were supplied by Teknokroma (Barcelona, Spain). Oasis HLB cartridges (60 mg, 3 mL) were from Waters (Milford, MA, USA).

Concerning safety, no particular measures had to be taken when handling with SAs in the experimental procedures and analyses.

Instrumentation

Extraction and purification of the SAs from water samples were performed by solid phase extraction (SPE) assisted by a 12-fold vacuum extraction box (J.T. Baker, Phillipsburg, NY, USA). Extracts were evaporated under a gentle N_2 stream at 25°C in a Turbo Vap LV Zymark evaporator (Hopkinton, MA, USA).

LC MS MS analyses and IDA experiments were carried out with an HP 1100 chromatograph from Agilent Technologies (Palo Alto, CA, USA) coupled to a 4000 QTRAP mass spectrometer from Applied Biosystems (Foster City, CA, USA) equipped with a turbospray electrospray (ESI) source.

Sampling site and sample collection

The Llobregat River (NE Spain) is 156 km long and covers a catchment area of about 4,957 km^2 and 3,089,465 inhabitants (data from 1999). This basin follows the hydrological pattern of Mediterranean rivers, with low winter and summer discharges and periodic floods in spring and autumn. The Llobregat is an illustrative example of an overexploited river because its middle and lower sections run through very densely populated and industrialized areas. In this part of the basin it also receives brine leachates from natural salt formations and mining operations, which have caused an increase in water salinity downstream. Together with its two main tributaries, the Cardener River and the Anoia River, the Llobregat is subjected to heavy anthropogenic pressure, receiving extensive urban and industrial wastewater discharges (137 Hm^3 $year^{-1}$; 92% from wastewater-treatment plants), which are only partially diluted by its natural flow (0.68 6.5 m^3s^{-1} basal flow). Forty-eight percent of these point sources are located in the studied area. Furthermore, 30% of the annual discharge of the river (693 Hm^3) is used for drinking water supply, including the city of Barcelona.

Four sampling sites were selected from the middle and lower parts of the Llobregat main channel and another three were selected in the Anoia tributary (Fig. 1). These sites were part of a pollution gradient: sites LL1 and A1 were the least polluted but receive some industrial effluents and

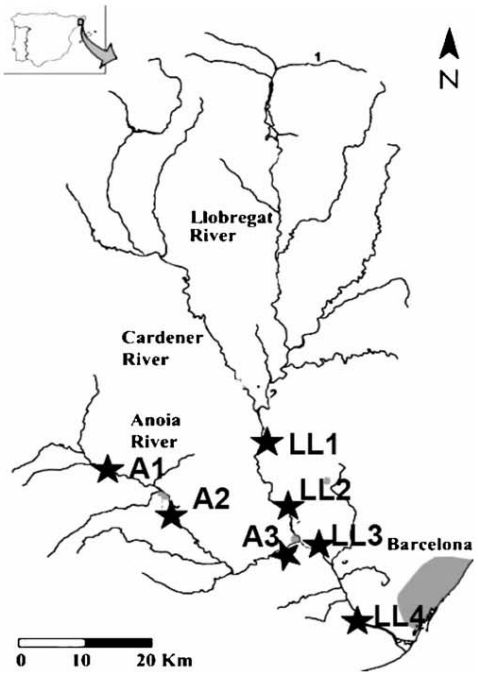

Fig. 1 Selected sampling sites along the Llobregat and Anoia rivers

surface runoff from agricultural areas. Sites A3 and LL4 were located in the last section of the two rivers, and were the most polluted sites. Site A2 was located in a highly polluted area receiving waste waters from tannery, textile and paper industries together with the contribution from urban areas. Sites LL2 and LL3 were located in a densely inhabited area and receive urban and industrial wastewater inputs. Sampling was performed in early June 2005, late May 2006, and November 2006 (campaigns 1, 2, and 3 respectively). These three periods covered two of the most relevant periods (spring and autumn) in the system in terms of its hydrology. A total of 21 samples were taken during the three sampling campaigns. The samples were shipped to our laboratory under cool conditions (4°C) in amber glass bottles pre-rinsed with HPLC-grade water in a portable refrigerator, readily vacuum filtered through 2.7-μm glass fiber filters followed by 0.45-μm Nylon membrane filters, and stored at 4°C until analysis (less than two days).

Solid phase extraction and purification

The extraction method was based on that used by Gros et al. [23]. Briefly, 500 mL surface water, previously filtered,

were preconcentrated on Oasis HLB cartridges previously conditioned with 5 mL MeOH and 5 mL HPLC water. After the sample loading, the cartridges were further rinsed with 5 mL HPLC-grade water, dried under vacuum for 15–20 min, and eluted with 2×4 mL MeOH. The extracts were brought to dryness under a gentle nitrogen stream and finally reconstituted with 1 mL of ACN–water (25:75, *v/v*). d_4-sulfathiazole was added as internal standard prior to analysis.

LC-tandem MS analysis

This method with the QqLIT MS operating as a QqQ was developed by Díaz-Cruz et al. in previous work [3]. Separation was always carried out on an Atlantis C18 (Waters) (3 μm, 150×2.1 mm) LC analytical column, preceded by a guard column with the same packing material, using HPLC-grade water at pH 2.2 (A) and ACN (B), both containing 10 mmol L^{-1} formic acid, as

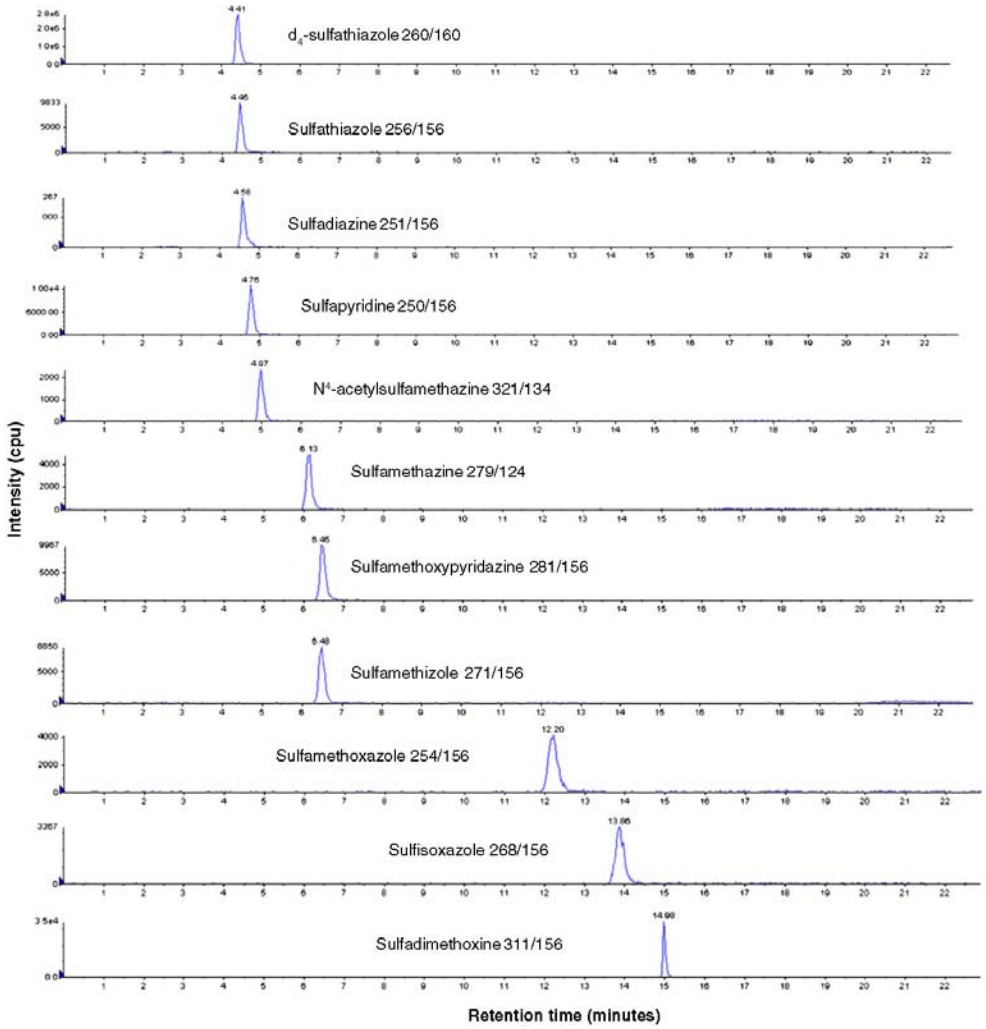

Fig. 2 Reconstructed ion chromatogram showing the most intense precursor ion → product ion transition for each of the sulfonamides studied

mobile phase. The best separation was obtained with a linear elution gradient programmed from 25% to 100% B in 11 min, hold for 2 min, and returned to initial conditions in 3 min and equilibrated for 7 min. Seven more minutes were spent re-equilibrating the column before the next injection. The flow rate was 0.2 mL min ^{1}, and the injection volume of standards and sample extracts was always set at 20 μL.

Two SRM transitions were monitored for each compound, the most abundant for quantification and the second for confirmation. Figure 2 shows a reconstructed chromatogram with the most intense transitions for each of the target analytes, including the internal standard, for the lowest point of the calibration curve (0.05 ng L ^{1}).

In order to compare the performance characteristics of the two operational modes of the QqLIT (QqQ and LIT), a built-in IDA method was developed using the LIT configuration. Four different experiments were looped. The first experiment or survey scan was an SRM which included the most abundant transitions of the nine SAs studied, and that had been previously predefined. The intensity threshold was set to 3,000 cps. When the intensity of the ions reached this preset threshold, three different EPI scans (dependent scans) were triggered at different collision energies (25, 30, and 35 eV). The cycle was repeated throughout the acquisition in order to obtain a large amount of informative data. For the SRM transitions, the same conditions that were optimized for the QqQ SRM were applied. The dwell time was optimized to 10 ms. For the EPI scan (see the Introduction), the same precursor ion as in the SRM was used. The optimized fill time of the LIT was 50 ms; the scan rate was 4,000 amu s ^{1} and the collision energies tested were 25 eV, 30 eV, and 35 eV.

Method validation

Quantitation was performed by using internal standard calibration. Curves were estimated using least-squares linear regression analysis after injection of seven standard mixtures of the analytes at concentrations from 0.05 ng L ^{1} to 5,000 ng L ^{1}.

In order to calculate the recovery values for the different SAs, six replicates of surface water were spiked at 100 ng L ^{1} and subjected to the same SPE procedure as the natural samples (see the section "Solid phase extraction and purification"). Instrumental repeatability was calculated by making six consecutive injections of a standard mixed solution of 500 ng L ^{1}. Blank samples were also evaluated to check for background concentrations in the calculation of the recoveries.

Results and discussion

Method performance

The corresponding performance parameters are listed in Table 1. Calibration curves were linear for all compounds in the range 0.01–5,000 ng L ^{1} with the exception of sulfadimethoxine, for which the range was 0.01 to 100 ng L ^{1}. In all cases the regression coefficient (r^2) was higher than 0.999. Recoveries for the SAs studied ranged

Table 1 Instrumental data obtained with the QqLIT SRM and EPI scan

	R % (n=6)	QqLIT							
		SRM SCAN					EPI SCAN		
		Repeatability %(RSD)	iLOD (pg injected)	MLOD (ng L^{-1})	MLOQ (ng L^{-1})	r^2	Repeatability %(RSD)	iLOD (pg injected)	r^2
Sulfadiazine	38	6.1	0.001	0.05	0.17	0.9991	4.1	0.006	0.9928
Sulfadimethoxine	108	3.3	0.002	0.11	0.37	0.9999	1.9	0.003	0.9901
Sulfamethazine	110	8.4	0.004	0.22	0.73	0.9999	2.1	0.004	0.9963
Sulfamethizole	41	6.3	0.004	0.21	0.70	0.9999	5.8	0.003	0.9906
Sulfamethoxazole	86	5.8	0.003	0.17	0.57	0.9999	1.5	0.003	0.9981
Sulfamethoxypyridazine	96	7.8	0.001	0.05	0.17	0.9995	1.8	0.002	0.9927
Sulfapyridine	94	5.6	0.003	0.16	0.53	0.9996	1.6	0.003	0.9929
Sulfathiazole	123	6.5	0.003	0.13	0.43	0.9995	2.2	0.003	0.9960
Sulfisoxazole	26	5.5	0.001	0.06	0.20	0.9998	2.0	0.003	0.9984
N^4-Acetylsulfamethazine	115	7.5	0.002	0.12	0.40	0.9997	1.6	0.002	0.9902

Repeatability was calculated at 500 ng L^{-1}

iLOD, instrumental limit of detection; MLOD, method limit of detection; MLOQ, method limit of quantification

from 26% for sulfisoxazole to 123% for sulfathiazole. Recovery values for the same water matrix were estimated by the authors in previous work [3]. Differences can be attributed to the different SPE method applied in this work, which was designed for analysis of a wide range of pharmaceuticals [23] and not specifically for SAs [3].

Precision was given by the repeatability of the instrument, expressed as the relative standard deviation (% RSD)

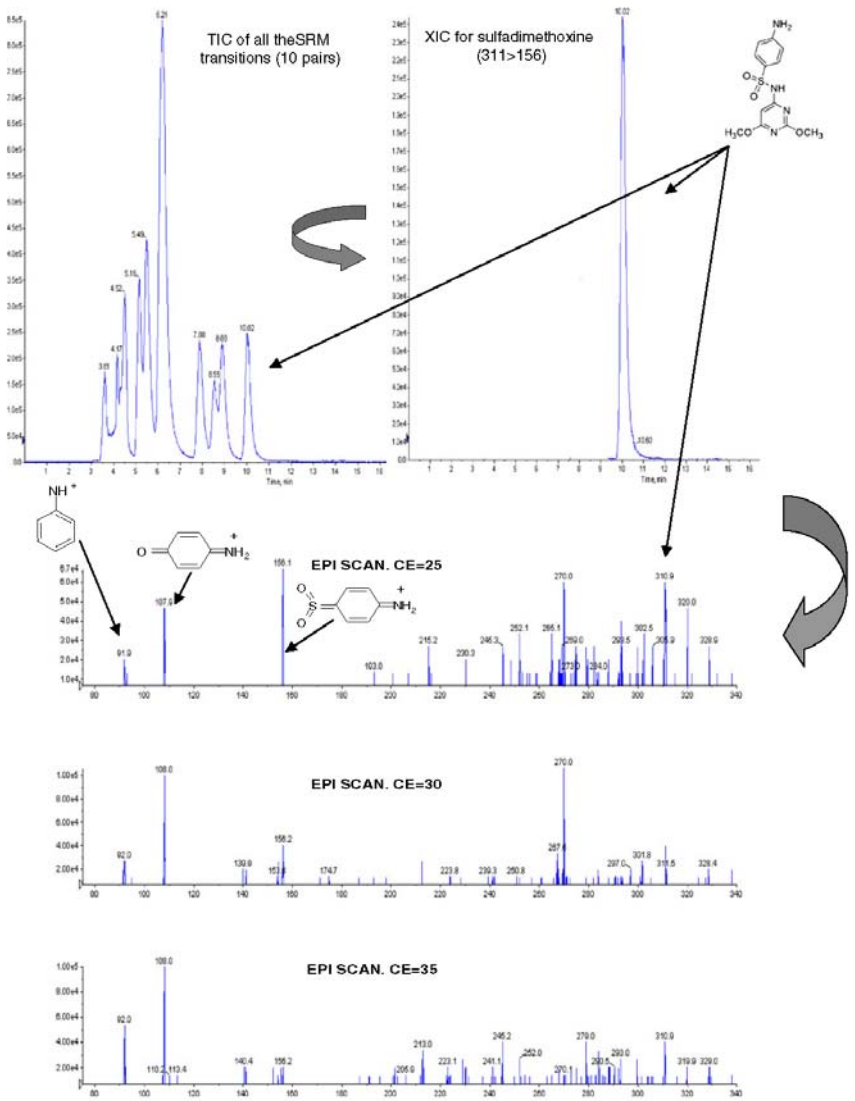

Fig. 3 LC-QqLIT MS determination of sulfadimethoxine in: (a) a standard solution at 50 ng mL^{-1} and (b) a surface water sample (0.5 ng L^{-1}). The recorded SRM transition and the three EPI scans at different collision energies are shown. TIC, total ion chromatogram; XIC, extracted ion chromatogram

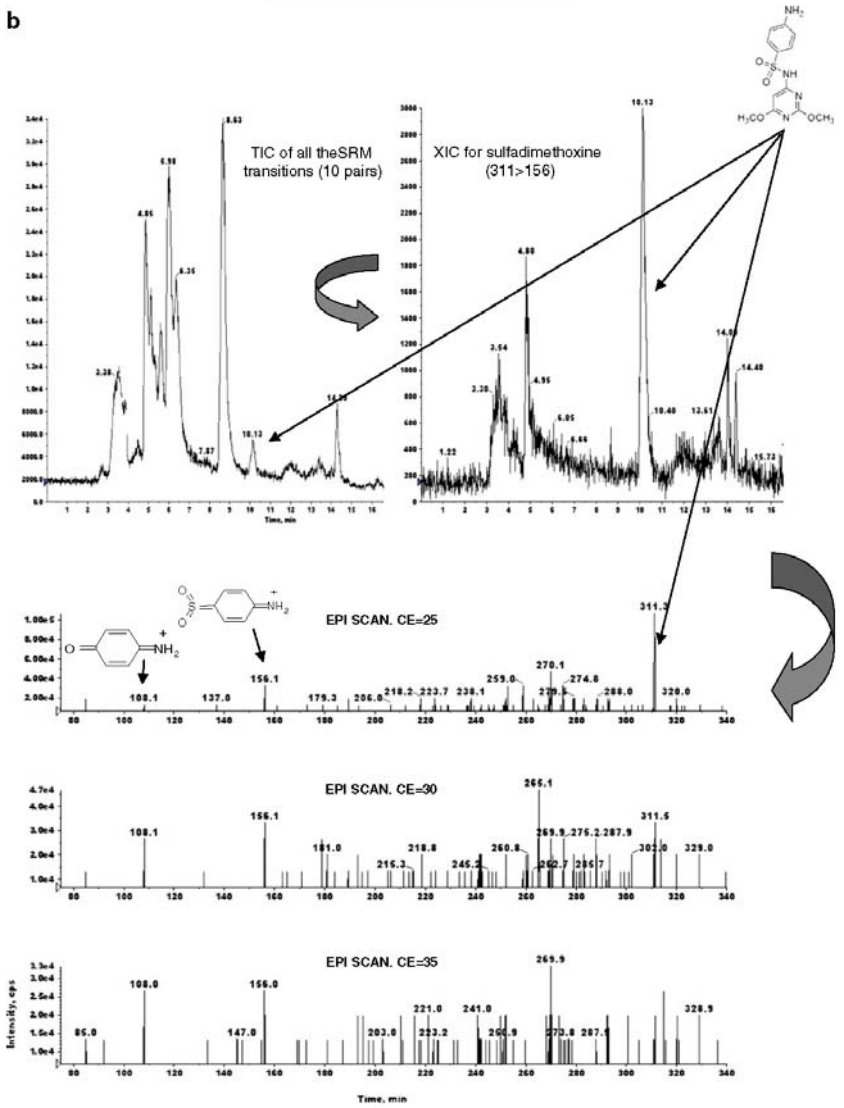

Fig. 3 (continued)

from six consecutive injections of a standard mixture at 500 ng L^{-1} and was not higher than 8%. The chromatogram shown in Fig. 2 demonstrates the high selectivity and specificity of the method for each of the sulfonamides under study.

Qualitative analysis. IDA method

Figure 3a shows the determination of sulfadimethoxine in a standard solution at 50 ng mL^{-1} following the IDA experiment. Instrumental LODs for the SRM mode and the

EPI scans are shown in Table 1. Because SRM is the most commonly used approach in quantification, and sensitivities and linearity were better than in the IDA experiment for the studied compounds, this was eventually the operational mode of choice. Similar results were reported by Xia et al. [24], who found that SRM gave a better sensitivity than the EPI mode for analysis of the drug propranolol in biological fluids.

When performing IDA experiments and following the EU criteria established by Directive 96/23/EC and Commision Decision 2002/657/EC [25], the number of identification points (IPs) was seven for each compound, taking into account the SRM transition (1 IP for the precursor ion and 1.5 for the product ion) and the three EPI scans carried out (1.5 IP for each), *versus* the 4 IPs yielded by SRM acquisition with the QqQ configuration. According to Annex 2 of the EU Directive, only three IPs are required for the confirmation of veterinary and human drugs. Therefore, the inclusion of an IDA experiment would provide an unequivocal identification of the target SAs. However, for some other substances such as illegal drugs, the required number of IPs is higher for valid, reliable identification, and the use of IDA experiments including MS^n scans may become essential.

The applicability of the new LC-QqLIT MS method developed for quantitative purposes was tested on natural water samples (surface water). In this case, sample preparation of extracts was performed as described by Díaz Cruz et al. [3]. As an example, the determination of sulfadimethoxine is shown in Fig. 3b. Similarly to Fig. 3a, the enhanced product ion scans showed the most abundant fragments (*m/z* 156 and 108) at different collision energies.

Quantitative analysis. Sulfonamide concentrations in the Llobregat River and Anoia River

The absolute and relative frequencies of detection for the different SAs studied in the Llobregat River and Anoia River are depicted in Fig. 4. Concentrations detected in the Llobregat and Anioa rivers, after correction by applying the corresponding recovery values for each analyte, are shown in Table 2. The application of these recovery corrections to the results compensates for the application of a different SPE method. Differences from the results shown here and those published by Díaz-Cruz et al. [3] can be explained because the sampling locations along the Llobregat River were randomly chosen in the former.

As it can be seen in Fig. 4, sulfamethoxazole and sulfapyridine were the two SAs found more frequently in the two water courses. Both are mainly used in human medicine to treat acute urinary infections and some types of dermatitis, respectively. These results are in agreement with those obtained in a sampling campaign along the Ebro river basin, in which these two SAs were detected in the highest concentrations in the effluent water of all the wastewater-treatment plants sampled (a total of seven), and were also very frequently present in 15 different surface water samples analyzed along this basin (100% presence for sulfamethoxazole, 60% for sulfapyridine) [26]. It could be concluded that this high presence has more to do with the high quantities employed than with physicochemical properties such as the solubility of the compound in water. Sulfamethoxazole and sulfapyridine are both soluble compounds (610 and 268 mg L^{-1} respectively), and therefore are expected to be found in the aqueous media, but others such

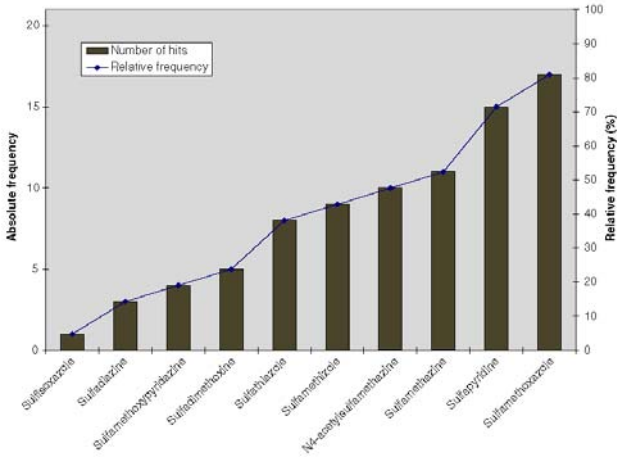

Fig. 4 Absolute and relative frequencies of detection for the sulfonamides studied

Table 2 Sulfonamide concentrations (ng L^{-1}) detected in the Llobregat River and Anoia River

		Sulfadiazine	Sulfadimethoxine	Sulfamethazine	Sulfamethizole	Sulfamethoxazole	Sulfamethoxypyridazine	Sulfapyridine	Sulfathiazole	Sulfisoxazole	N⁴-acetylsulfamethazine
RIVER	S.S.	Sampling Campaign 1									
ANOIA	A1	n.d.	n.d.	n.d.	n.d.	6.4	n.d.	n.d.	n.d.	n.d.	20.2
	A2	n.d.	n.d.	n.d.	3.7	26.5	n.d.	17.1	1.6	n.d.	n.d.
	A3	n.d.	5.4	n.d.	10.3	43.9	n.d.	n.d.	n.d.	n.d.	n.d.
LLOBREGAT	LL1	n.d.	n.d.	22.6	n.d.	7.0	n.d.	n.d.	n.d.	n.d.	13.3
	LL2	n.d.	n.d.	26.4	1.4	6.7	n.d.	n.d.	n.d.	n.d.	14.0
	LL3	n.d.	n.d.	n.d.	n.d.	9.9	n.d.	4.2	1.7	n.d.	n.d.
	LL4	n.d.	n.d.	2481.8	5.6	4297.3	95.8	>CR	960.6	n.d.	76.0
		Sampling Campaign 2									
ANOIA	A1	n.d.	1.1	11.5	n.d.	n.d.	n.d.	2.8	<LOQ	n.d.	n.d.
	A2	n.d.	n.d.	n.d.	n.d.	167.9	n.d.	n.d.	3.0	n.d.	n.d.
	A3	n.d.	n.d.	n.d.	0.3	6.4	n.d.	9.2	n.d.	n.d.	n.d.
LLOBREGAT	LL1	n.d.	n.d.	23.2	n.d.	n.d.	n.d.	4.7	n.d.	n.d.	15.1
	LL2	n.d.	n.d.	47.8	n.d.	7.2	n.d.	22.3	n.d.	n.d.	100.5
	LL3	n.d.	3.8	28.1	n.d.	7.6	n.d.	33.3	n.d.	n.d.	12.8
	LL4	13.4	n.d.	n.d.	4.7	19.8	164.9	91.8	n.d.	n.d.	n.d.
		Sampling Campaign 3									
ANOIA	A1	n.d.	n.d.	15.7	n.d.	<LOQ	n.d.	4.5	<LOQ	n.d.	n.d.
	A2	n.d.	4.5	n.d.	n.d.	18.7	n.d.	39.7	0.8	n.d.	n.d.
	A3	n.d.	n.d.	n.d.	0.4	6.0	n.d.	10.8	n.d.	n.d.	n.d.
LLOBREGAT	LL1	n.d.	n.d.	3.1	1.7	1.2	n.d.	1.9	n.d.	n.d.	98.4
	LL2	n.d.	n.d.	17.0	0.7	4.3	0.4	5.5	n.d.	n.d.	n.d.
	LL3	1.7	n.d.	n.d.	n.d.	n.d.	n.d.	15.4	n.d.	n.d.	11.4
	LL4	>CR	136.0	>CR	n.d.	652.7	>CR	n.d.	523.5	24.7	695.1

S.S., sampling site; n.d., not detected; <LOQ, below the limit of quantification; >CR, above the maximum limit of the calibration range

as sulfamethazine and sulfamethizole with higher solubilities (1,500 and 1,050 mg L^{-1}) are detected less frequently.

In the Anoia River, the highest concentrations corresponded also to sulfamethoxazole and sulfapyridine (168 ng L^{-1} and 40 ng L^{-1} respectively), which were detected in the same location, sampling site A2 in the second and third campaigns, respectively (Table 2). Concentrations in the Anoia ranged from 0.07 ng L^{-1} (sulfathiazole) to 168 ng L^{-1} (sulfamethoxazole), with a median value of 6.2 ng L^{-1}. Sulfadiazine, sulfamethoxypyridazine, and sulfisoxazole were not detected in the Anoia River in any of the campaigns.

In the Llobregat River, in contrast, all the SAs were detected in at least one of the sampling sites during the three campaigns. In this case, sulfamethazine was detected with the same frequency as sulfapyridine, and at slightly higher concentrations. For all the SAs, concentrations ranged from 0.74 ng L^{-1} (sulfamethizole) to 2,482 ng L^{-1} (sulfamethazine). However, the median value estimated in this case is 14 ng L^{-1}, and, as can be seen in Table 2, most of the concentrations detected in the Llobregat River were around this value Regarding the highest concentrations detected, sulfamethoxazole was found at a concentration of 860 ng L^{-1} in the first sampling campaign, and at 653 ng L^{-1} in the third. Sulfamethazine was detected at the ppb level in the first campaign (2,482 ng L^{-1}) and its acetylated metabolite was present at a concentration of 695 ng L^{-1} in the third campaign. As expected, these high concentrations corresponded to sampling site LL4 (Fig. 1), in the low course of the river and near its mouth, where, in addition to these four SAs mentioned previously, estimated values for sulfamethoxypyridazine, sulfadiazine, and sulfamethazine were out of the calibration range (>5,000 ng L^{-1}) in campaign 3 and for sulfapyridine in campaign 1 (these values are not included in Table 2). As SAs seem to resist natural biodegradation [27], these high concentrations may be due not only to regular maximum concentrations but also to cumulative effect throughout the water course, following a pollution gradient. This holds true for the Anoia River in the first campaign, but some of the SAs could not be detected in the following two campaigns in all the sampling sites and this concentration trend could not be confirmed.

Only one sulfonamide, sulfamethoxazole, was included in the study carried out by Muñoz et al. [28] dealing with the presence of active pharmaceuticals in the Llobregat and Anoia rivers. Results are in agreement with those obtained in this study.

Conclusions

The requirement for quantitative data at environmentally relevant concentrations (ng L^{-1} level) reinforces the need of powerful analytical methodologies which ensure low limits of detection and are certain to confirm analyte identities. LC–tandem MS fulfils these criteria. Moving from LC–MS analysis to LC–tandem MS and finally to analysis by LC-QqLIT MS results in definitive and meaningful improvement in sensitivity and accuracy when analyzing natural water samples in which very low concentrations are expected. In future studies, the inclusion of an IDA experiment would contribute to avoiding false positives in cases where transitions are recorded at low intensities or the compound has poor fragmentation. In the example illustrated here, for reliable determination of sulfonamide antibiotic residues in environmental waters an LC–QqLIT instrument was employed, working with the

QqQ configuration. Concentration values found ranged from 0.27 ng L^{-1} to 2,482 ng L^{-1}, although median values showed that most of the concentration values were not usually higher than 50 ng L^{-1} for both rivers, except for some outlier values at the sampling site LL4. Higher concentrations were also detected near the mouth of the Llobregat River, where in some cases concentrations were higher than 5,000 ng L^{-1}.

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Simultaneous occurrence of nitrates and sulfonamide antibiotics in two ground water bodies of Catalonia (Spain)

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SUMMARY

In the present work the occurrence of 19 selected sulfonamides, including one acetylated metabolite, was investigated in ground water samples taken from two ground water bodies in Catalonia (Plana de Vic and La Selva). Both include areas designated as nitrate vulnerable zones, according to Directive 91/676/EEC. A fully automated analytical methodology based on on-line solid-phase extraction–liquid chromatography-tandem mass spectrometry (on-line SPE–LC–MS/MS) was developed for this purpose. The high selectivity and sensitivity achieved (limits of detection between 0.005 and 0.8 ng/L) permitted to demonstrate the ubiquity of these antibiotics in both ground water bodies. Results showed a wide range of concentrations, from 0.01 ng/L up to 3460.57 ng/L. Since sulfonamides are related to livestock veterinary practices, they can be used as a specific indicator of manure contamination. However, the presence of sulfonamides appeared not to be directly related to the concentration of nitrates, as it is reflected on the low correlation coefficients found.

Introduction

Ground water systems are dynamic and adjust continually to short-term and long-term changes in climate, ground water withdrawal, and land use. In Catalonia, ground water represents the 35% of the water resources, being of high importance in drinking water, industry and agriculture supply. Its use in these sectors equals approximately 900 hm^3 per year, only a part of the total ground water resources available; for instance, the average use for drinkable water equals 200 hm^3, corresponding to 30–35% of the total ground water supply, a high value considering only this single use. The kind of use depends on the location, being the areas with higher demands (i.e. coastal and touristic areas) not necessarily placed in regions with enough ground water resources, and the other way round. It should also be considered that part of the flow in streams, lakes and wetlands is sustained by the discharge of ground water.

When talking about ground water bodies, we are referring to a more protected water matrix. The soil system above acts as 'protective shield', providing inertia to quality changes and a slowed propagation of the contaminants. But for this same reason, however, ground water bodies are especially vulnerable to pollution because once they are contaminated, the effects are often irreversible, or, at least, of difficult and expensive remediation. Point sources of contamination such as sewage treatment plants discharge, industrial facilities and storm water drains contribute with a wide variety of contaminants to rivers and streams which eventually may also affect ground water quality, especially where rivers are the main source of ground water recharge, where ground water withdrawals induce seepage from streams and where floods cause stream water to become bank storage (www.groundwater.water.ca.gov/other_references/index.cfm).

Application of inorganic fertilizers and manure to cropland can also result in significant sources of contaminants to ground water resources. Agriculture is the main cause of diffuse pollution, which is quite difficult to deal with and prevent, as pesticides and fertilizers are spread on very extense areas. Intensive cattle farming is another relevant source of not-point pollution, since a wide variety of veterinary pharmaceuticals and specially antibiotics, are used in the prevention and treatment of microbial infections and also as growth promoters (Díaz-Cruz and Barceló, 2008). Following treatment, livestock will excrete 50–90% of the administered dose, the parent drug making up for 9–30%. These amounts of the unchanged substance vary depending on the form of the drug and the animal age and species (Parfitt, 1999). Manure is regarded as a very valuable fertilizer, as it contains essential nutrients for plant growth such as nitrogen, phosphorous, organic carbon, potassium etc. The extensive use of manure from medicated animals in crop

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fields is among the major routes by which veterinary antibiotics enter the environment (Boxall et al., 2003) and, eventually, the ground water systems. Once on the topsoil, the excreted residues may percolate and contaminate the aquifers directly or reach surface waters during run off and contaminate the ground water bodies indirectly.

Sulfonamides are one of the most widely used antibiotics in human and especially in veterinary medicine. They have been detected in all kind of water matrices (Díaz-Cruz et al., 2008), not only because of their high consumption rates, but also due to their amphoteric properties, rather poor chelating ability and low sorption to soils tendency (i.e. partition coefficient values (K_d) of 0.22 for sulfamethoxazole, 2.5 for sulfadiazine, 1.6 for sulfapyridine and 1 for sulfamethazine). They are usually classified as short-acting sulfonamides, with half lives (in serum) of 5–10 hours (i.e. sulfisoxazole, sulfadiazine and sulfamethizole); intermediate-acting sulfonamides, with half-lives of 10–12 h (i.e. sulfamethoxazole, sulfadimethoxine and sulfamethazine); and finally long-acting sulfonamides, with half lives from 40 h onwards (i.e. sulfadoxine and sulfamethoxypyridazine) (Scholar and Pratt, 2000). Their relatively low elimination efficiency during sewage treatment procedures and the increase in the number of confined animal-feeding operations, which often lack proper waste management practices, are other reasons for greater occurrence of these substances (Carballa et al., 2004). Residues of sulfonamides have been detected in manure (Kotzerke et al., 2008; Schmitt et al., 2005) and, as their sorption to soil is weak, they could leach to ground waters. This possibility has already been proved in several publications, showing the presence of sulfonamides at different concentrations in ground water from various sites close to animal farming facilities (Batt et al., 2006; Blackwell et al., 2004; Diaz-Cruz and Barcelo, 2008; Karthikeyan and Meyer, 2006; Lindsey et al., 2001; Sacher et al., 2001). A recent study showed sulfamethazine and sulfadimethoxine in ground water from wells located down gradient and in close proximity to a confined animal-feeding operation (CAFO) (Batt et al., 2006).

A special protection figure is therefore needed for these vulnerable water systems. The so called Ground Water Directive (Directive 2006/118/EC) offspring of the Water Frame Directive (WFD, 2000/60/CE) establishes how to manage and protect the different ground water resources from pollution. Under the provisions of such directives, 53 different ground water bodies have been typified in Catalonia in order to evaluate together anthropogenic and non-anthropogenic pressures on the different water systems and environmental impacts, of which 29 are considered under risk of non compliance of good status, as it is precluded by the WFD. So far, there is no legislation regarding the presence of sulfonamides or any other antibiotic in any of the environmental compartments. On the other hand, nitrates from animal farming have been thoroughly studied and regulated in Directive 91/676/EEC and six nitrate vulnerable zones have been established in Catalonia following this Directive. Nitrogen in its different species (organic, ammonium, nitrite and nitrate) is a major constituent of manure, and, similarly to sulfonamides, is very soluble in water. Increased concentrations of nitrate that result from both nitrification of ammonium or direct introduction from mineral fertilizers are commonly present in both ground water and surface water associated with ammended agricultural lands.

The aim of this work is to assess the occurrence of 19 selected sulfonamides, including one acetylated metabolite, in ground water samples taken from surveillance and operational monitoring networks located in two ground water bodies (Plana de Vic and La Selva); both of them include areas designated as nitrate vulnerable zones under the provisions of Directive 91/676/CEE.

2. Experimental section

2.1. Chemicals and reagents

HPLC-grade solvents (water, methanol, acetone and acetonitrile) and formic acid (98–100%) were supplied by Merck (Darmstadt, Germany).

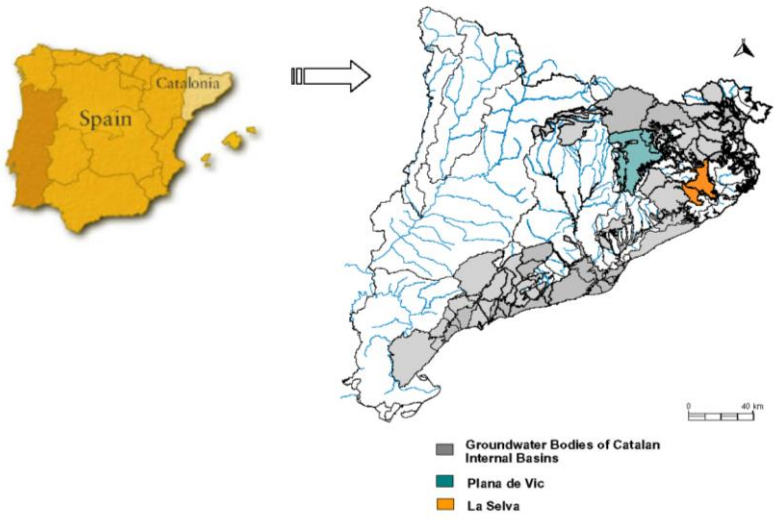

Fig. 1. Location of La Plana de Vic and La Selva ground water bodies.

High purity standards (>99%) of the 19 selected sulfonamides were purchased from Sigma (St. Louis, MO, USA). Stock standard solutions for each of the analytes were prepared in methanol (MeOH) at 1 mg/mL and stored in the dark at −2 °C. Standard solutions of the mixtures of all compounds at concentrations ranging between 1 ng/mL and 500 μg/mL were prepared by appropriate dilution of the stock solutions in MeOH. The standard mixtures were used as spiking solutions for preparation of the aqueous calibration standards and in the recovery studies. Aqueous standard solutions contained <0.1% of MeOH.

Internal standard d_4-sulfathiazole (99.9%) was purchased from Toronto Research Chemicals (Ontario, Canada). Stock solutions were also prepared in methanol and stored at −2 °C until use.

2.2. Sample collection and preparation

Ground water samples were taken in spring 2008 in two ground water bodies in Catalonia: Plana de Vic and La Selva (see Fig. 1). Water was sampled from 39 sampling sites, including monitoring wells and natural springs. In Plana de Vic, which comprises 740 km^2, 26 samples were taken from different wells, a natural spring and a mine. Ground water was sampled from depths ranging from 3 to 200 m. In La Selva, with an extension of 291 km^2, 13 wells were studied. Similarly, ground water was sampled from depths going from 10 to 206 m. Land and ground water use in the areas where these two ground water bodies are located are shown in Fig. 2.

Water samples were collected in amber polyethylene terephthalate (PET) bottles and transported to the laboratory under cooled conditions (4 °C). Once there, samples were filtered through 0.45 μm Nylon filters (Whatman, Maidstone, UK) to eliminate suspended solid matter and then stored at 4 °C in the dark until analysis which was always carried out within 48 h of collection to avoid microbial degradation.

2.3. On-line SPE–LC–MS/MS analysis

The fully automated SPE–LC–MS/MS analytical method used to determine the sulfonamide occurrence in the ground water samples was recently developed by the authors (García-Galán et al., 2009) to analyse sulphonamide residues in several aqueous matrices. Briefly, the on-line preconcentration of samples, aqueous standards and operational blanks was performed using an automated on-line SPE sample processor Prospekt 2™ (Spark Holland, Emmen, The Netherlands). 40 mL of ground water samples were extracted using Oasis HLB cartridges (divinylbenzene and *N*-vinylpyrrolidone polymer, 30-μm particle size) from Waters (Barcelona, Spain).

This system consists of an automated cartridge exchange (ACE) module, which holds two trays of 96 extraction cartridges each, and a high pressure dispenser module (HPD) for handling of solvents by a 2-mL high pressure syringe. SPE solvents for conditioning, equilibration, sample application and clean up are provided by the HPD. The ACE module has two clamps and two high pressure valves. An aliquot of the raw sample is introduced by the autosampler and, when the SPE is completed, the cartridge is transferred to the elution clamp and the analytes are eluted from the SPE cartridge directly onto the LC column by the HPLC. During LC–MS/MS analysis, the extraction of the next sample is carried out on a new cartridge in the other clamp. Therefore, SPE runs entirely in parallel with the LC–MS/MS run. This configuration shortens the cycle times (in our case 23 min of sample analysis plus the conditioning and equilibration times only for the first sample). The Prospekt-2 TM is controlled by means of Sparklink version 3.0 (Spark Holland).

LC–MS/MS analyses were carried out in a system consisting of an HP 1100 chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbospray electrospray (ESI) interface. The chromatographic separation was performed using an Atlantis C18 (Waters, 150 mm × 2.1 mm, 3 μm of particle size) LC-column preceded by a guard column with the same packing material. Sulfonamides were analyzed in the positive ionization mode (PI). The flow rate was set to 0.2 mL/min, being eluent A HPLC grade water slightly acidified with 0.1% of formic acid, and eluent B acetonitrile with 0.1% formic acid. The elution gradient started with 25% of eluent B, increasing to 80% in 10 min and 100% in 11 min. During the further 2 min, the column was cleaned and readjusted to the initial conditions in 3 min, and equilibrated for 7 min.

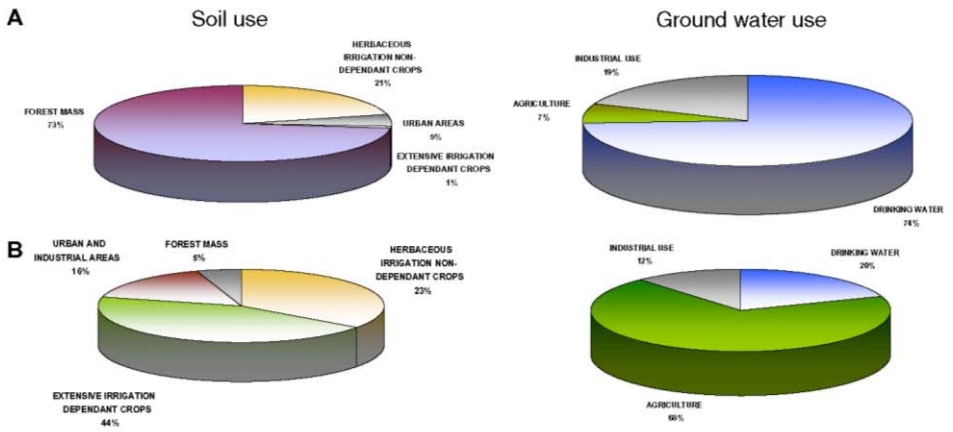

Fig. 2. Soil and ground water use in the areas corresponding to the two ground water bodies studied: (A) La Plana de Vic and (B) La Selva.

The MS/MS experimental conditions were as follows: capillary voltage 3.5 kV; source temperature, 700 °C; desolvation temperature, 450 °C; extractor voltage 3 V; and RF lens 0.2 V. Nitrogen was used as both the nebulizing and the desolvation gas at 630 L h^{-1}. For operation in the MS/MS mode, argon was used as collision gas with a pressure of 2.6×10^{-3} mbar. Instrument control and data acquisition and evaluation were performed with the Analyst 1.4.2 software package purchased from Applied Biosystems.

2.3.1. Quantification quality assurance

For increased sensitivity and selectivity, MS data acquisition was performed in the selected reaction monitoring (SRM) mode. For each analyte, two transitions between precursor ions and the two most abundant product ions were monitored, the most abundant transition was used for quantitation and the other one for confirmation. Table 1 shows the optimized LC–MS/MS conditions used for the target analytes. A strict criteria has to be followed in order to ensure positive confirmations of the target analytes in the samples and avoid false positives. The European Commission Decision 2002/657/EC establishes that a minimum of three identification points (IPs) are required for this purpose. Two different SRM transitions, together with the precursor ion, yield four identification points. Other specific criteria applied in LC–MS/MS is that the chromatographic retention time of the analyte in the sample should not vary more than 2% in comparison to the calibration standards and the relative abundance of the two SRM transitions monitored must also be compared to the standards corresponding values [22].

The performance of the method applied is shown in Table 2.

2.4. Method validation

The analytical method developed was evaluated in terms of linearity, accuracy, selectivity and sensitivity as presented in Table 2.

Quantification was performed based on peak areas and using an internal standard calibration method, crucial to correct potential matrix effects. Concentrations were estimated for the most abundant SRM transition selected. d_4-sulfathiazole was added to all the samples at a concentration of 500 ng/L right before analysis.

Table 1
Optimized time-scheduled SRM transitions [M+H$^+$]. Compound dependent parameters: CE, collision energy (eV); DP, declustering potential (V) and CXP, collision cell exit potential (eV). Retention time is given in minutes.

Compounds	[M+H]$^+$	Transitions	Retention time	DP	CE	CXP
Sulfacetamide	215	215/156	3.2	46	21	10
		215/92		46	35	6
Sulfisomidin	279	279/124	3.3	76	33	8
		279/186		76	23	14
Succinyl-sulfathiazole	356	356/256	4.2	71	25	16
		356/192		71	33	16
Sulfathiazole	256	256/156	4.3	40	25	14
		256/92		40	25	10
d_4-sulfathiazole	260	260/160	4.3	71	25	6
		260/96		71	25	6
Sulfaguanidina	215	215/156	4.3	56	13	10
		215/92		56	31	4
Sulfadiazine	251	251/156	4.5	46	27	10
		251/108		46	30	8
N^4-acetylsulfamethazine	321	321/134	4.6	86	35	4
		321/124		86	35	4
Sulfapyridine	250	250/156	4.7	51	28	12
		250/92		51	31	6
Sulfamerazine	265	265/92	5.4	61	47	6
		265/156		61	27	8
Sulfamethazine	279	279/156	6.0	26	30	10
		279/124		26	35	10
Sulfamethizole	271	271/156	6.3	36	23	12
		271/108		36	23	8
Sulfamethoxypyridazine	281	281/156	6.3	66	27	14
		281/126		66	27	12
Sulfadoxine	311	311/156	10.4	46	29	12
		311/92		46	45	4
Sulfamethoxazole	254	254/156	11.4	56	25	10
		254/108		56	27	10
Sulfisoxazole	268	268/156	12.0	71	21	10
		268/113		71	21	8
Sulfaquinoxaline	301	301/156	13.0	76	25	10
		301/92		76	47	12
Sulfabenzamide	277	277/156	13.0	56	17	10
		277/92		56	41	6
Sulfadimethoxine	311	311/156	13.0	76	31	8
		311/92		76	31	6
Sulfanitran	336	336/156	14.7	66	17	12
		336/198		66	29	14

Table 2
Performance of the on-line SPE–LC-QqLIT–MS method applied. r^2, correlation coefficient; LOD, method limit of detection; LOQ, method limit of quantification and RSD, relative standard deviation (%).

Sulfonamide	r^2	LOQ (ng/L)	LOD (ng/L)	RSD
Sulfisomidin	>0.999	0.012	0.042	3.01
Sulfanitran	0.981	0.058	0.195	3.32
Sulfaguanidine	0.994	0.796	2.653	5.24
Sulfamerazine	0.998	0.086	0.286	2.18
Sulfaquinoxaline	0.998	0.016	0.055	1.60
Sulfadoxine	0.999	0.019	0.064	1.50
Sulfacetamide	0.986	8.876	29.586	6.51
Succinyl-sulfathiazole	0.999	0.065	0.218	2.29
Sulfabenzamide	0.983	0.019	0.062	8.19
Sulfadiazine	0.999	0.021	0.069	3.78
Sulfadimethoxine	0.999	0.039	0.131	3.26
Sulfamethazine	0.998	0.034	0.113	1.83
Sulfamethizole	0.998	0.366	1.221	6.61
Sulfamethoxazole	>0.999	0.050	0.167	1.41
Sulfamethoxypyridazine	0.999	0.036	0.118	4.60
Sulfapyridine	0.999	0.023	0.077	2.59
Sulfathiazole	>0.999	0.005	0.018	2.15
Sulfisoxazole	0.997	0.042	0.140	6.02
N^4-acetylsulfamethazine	0.999	0.049	0.162	1.12

Five to eight point matrix matched calibration curves were made, using least-squares linear regression analysis at concentrations ranging from 0.05 to 1000 ng/L. Correlation coefficients (r^2) were equal or higher than 0.999 for the majority of the sulfonamides. The best limit of detection (LOD) value for the method was achieved for sulfathiazole (0.005 ng/L), whereas sulfacetamide and sulfaguanidine were the compounds investigated with the lowest sensitivity.

The intraday precision of the method was evaluated by analyzing five consecutive times water spiked with a standard mixture of the analytes at 100 ng/L and estimating the relative standard deviation (RSD). Values ranged from 1.1% to 8.1% for all the sulfonamides.

3. Results and discussion

3.1. Nitrates

As aforementioned, a total of 38 wells and a natural spring were sampled in two different ground water bodies in Catalonia. Both are at risk of not reaching WFD environmental objectives in 2015 due to nitrate pollution.

Box plots representing the nitrate concentrations measured in different aquifers from La Plana de Vic and La Selva ground water bodies in the last few years are shown in Fig. 3. The boxes have lines at the lower quartile, median and upper quartile values. The distribution of the nitrate concentrations hardly shows significant differences between seasons or years, with values slightly skewed both to high and low values in the different sampling dates.

In the case of La Plana de Vic ground water body, high concentrations of nitrate have been documented during the period 1996–2007, with average nitrate concentrations ranging between 39.5 and 99.7 mg/L. The highest amounts were detected in 2004 and 2006 (Fig. 3A). It should be taken into account that Directive 91/676/EEC establishes that a ground water is considered polluted when concentrations of 50 mg/L or higher of nitrates are detected and that, in this area, a 74% of the ground water extractions is used for drinking water uses with the consequent health risks (i.e. gastric problems due to the formation of nitrosamines, blue baby syndrome, etc. Human health risk is out of the scope of this study and will not be dealt with in this study) as it can be observed in Fig. 2, only a 22% of the total soil area is dedicated to agriculture (both irrigated and non-irrigated), being most of the area covered by forest. On the other hand, this region is one of the most important of Catalonia regarding livestock, being the 15% of the total pig farming activity and the 13% of bovine farming located here. Therefore, the pressure derived from cattle droppings is very high, with an estimation of 13.170 tons N/year generated.

Nitrate levels are much lower in La Selva, with peak values no higher than 100 mg/L, and concentrations between 12.1 and 78.5 mg/L have been registered from 1998 to 2007. The only data available for Fig. 3B dated from 2007 onwards. The nitrogen quantity from cattle droppings is more moderated than that in Plana de Vic, and the pressure on the aquifers is distributed homogeneously over the whole area. Agriculture activity, higher than in La Plana de Vic (see Fig. 2) may also exert a moderated pressure on the chemical quality of the ground waters.

3.2. Sulfonamide concentrations

Table 3 shows the frequencies of detection of each sulfonamide studied in both ground water bodies, considering the whole data set and the two sampling sites individually (succinyl-sulfathiazole has been excluded in this table, as it could not be detected in any sampling site). In the first case (Plana de Vic), frequencies range from 8.16% (sulfamethizole) to 89.74% (sulfadimethoxine and sulfamethazine). In this ground water body, sulfamethizole and sulfaguanidine are the compounds with the lowest frequency of detection (12.12%) and sulfadimethoxine and sulfamethazine are the two sulfonamides with highest frequencies (69.7%). In La Selva, sulfamethizole was not detected in any of the sampling sites, sulfisoxazole was detected only in 6% of the cases, and again sulfadimethoxine and sulfamethazine were the compounds detected more frequently (75%). As it can be observed, the frequency values for these two sulfonamides are slightly higher in the area of La Selva, but this trend does not follow for all the analytes. However, the highest concentrations do not correspond to these two sulfonamides, but to sulfacetamide (3460.57 ng/L) and sulfamerazine (744.73 ng/L). Whereas sulfacetamide possessed also high frequencies of detection (79.49% for all the sampling sites), sulfamerazine has been detected only in 20.15% of the samples. It should be pointed out that for 14 of the 19 sulfonamides studied; their correspondent highest concentration values were detected in La Selva ground water body.

For the whole data set, the comparison between the mean and the median values of the concentrations showed that the median was lower than the average for all the sulfonamides, indicating that concentrations are skewed to low values. Standard deviation values (SD) had the same order of magnitude or bigger than the mean values for all the sulfonamides, pointing out the big differences of concentration in the different sampling sites.

Box plots shown in Fig. 4A and B have been used to summarize the results obtained in the sampling points from both ground water bodies, and show the distribution of the concentration values for each of the sulfonamides in the different sampling points. As mentioned, succinyl-sulfathiazole has been excluded in both graphs, as it could not be detected in any sampling site. In Fig. 4A, corresponding to La Plana de Vic, the distribution of the values was similar for all the sulfonamides except for sulfamerazine (S4), which showed a higher dispersion of the values (as could be expected from its SD value, see Table 2). For all the other compounds, concentrations were skewed to very low values, near the limit of detection. There were also several values out of the box plot range, all of them above the box plot, highlighting again the big dispersion of the data. A similar picture could be seen for the box plot corresponding to La Selva ground water body. Again, concentration values were generally low and the most disperse concentration values corresponded to sulfamerazine, and in this case also to the acetylated metabolite.

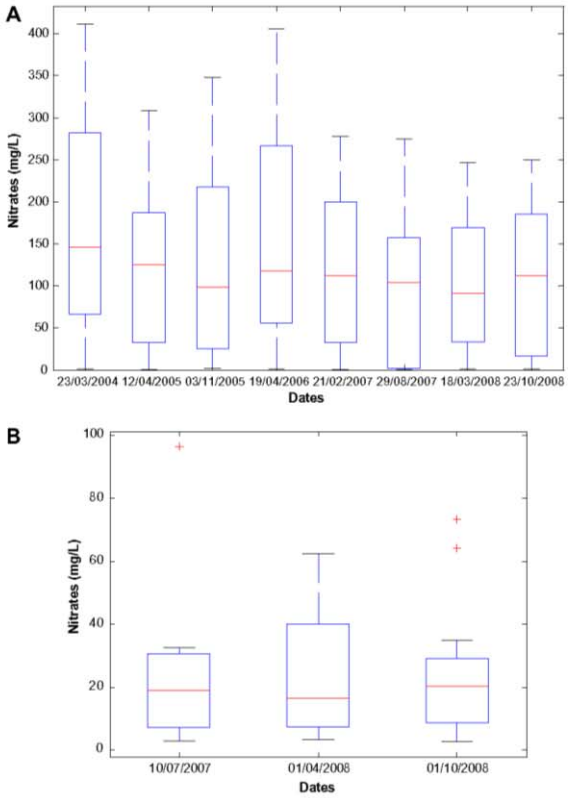

Fig. 3. Box plots showing the distribution through time of the nitrate concentrations in: (A) La Plana de Vic (39 samples) and (B) La Selva ground water bodies (13 samples).

Table 3
Descriptive univariate statistics of the data. SD, standard deviation and *, municipalities and ground water bodies correspond to the location of the maximum concentration value detected for each sulfonamide.

Compound	Frequency of detection (%) (total)	Frequency of detection (%) (Plana de Vic)	Frequency of detection (%) (La Selva)	Median (ng/L)	Mean (ng/L)	SD	Maximum value (ng/L)	Minimum value (ng/L)	Municipality*	Ground water body
Sulfisomidin	66.67	51.52	56.25	2.80	11.62	15.18	64.40	0.01	Ruidellots de la Selva	La Selva
Sulfanitran	48.72	42.42	31.25	12.36	44.76	127.7	568.8	0.04	Campllong	La Selva
Sulfaguanidine	20.51	12.12	25.00	10.61	22.59	29.85	91.78	3.30	Campllong	La Selva
Sulfamerazine	79.49	66.67	56.25	14.83	49.02	132.9	744.7	0.11	Campllong	La Selva
Sulfaquinoxaline	74.36	63.64	50.00	1.44	7.13	20.69	112.1	0.01	Campllong	La Selva
Sulfadoxine	84.62	66.67	68.75	2.33	6.21	12.30	53.63	0.02	Campllong	La Selva
Sulfacetamide	20.51	15.15	18.75	80.45	631.8	1205	3461	1.77	Campllong	La Selva
Sulfabenzamide	35.90	33.33	18.75	1.57	1.94	2.56	10.32	0.09	Balenyà	La Plana de Vic
Sulfadiazine	23.08	21.21	12.50	0.38	1.25	2.21	6.98	0.14	Malla	La Plana de Vic
Sulfadimethoxine	89.74	69.70	75.00	4.18	11.39	19.95	91.48	0.01	Campllong	La Selva
Sulfamethazine	89.74	69.70	75.00	3.68	8.20	18.74	106.8	0.03	Campllong	La Selva
Sulfamethizole	8.16	12.12	0.00	0.81	2.78	4.35	9.29	0.22	Les Masies de Roda	La Selva
Sulfamethoxazole	58.97	51.52	37.50	4.57	23.40	66.40	312.2	0.08	Cassa de la Selva	La Selva
Sulfamethoxypyridazine	35.90	21.21	43.75	1.00	8.96	18.65	68.70	0.02	Campllong	La Selva
Sulfapyridine	56.41	48.48	37.50	2.26	6.31	15.33	72.45	0.07	Campllong	La Selva
Sulfathiazole	56.41	45.45	43.75	2.62	4.50	5.25	16.78	0.01	Balenyà	La Plana de Vic
Sulfisoxazole	20.51	21.21	6.25	0.51	1.55	1.75	4.43	0.21	Vic	La Plana de Vic
N^4-acetylsulfamethazine	82.05	63.64	68.75	1.59	9.88	16.38	56.95	0.02	Campllong	Selva

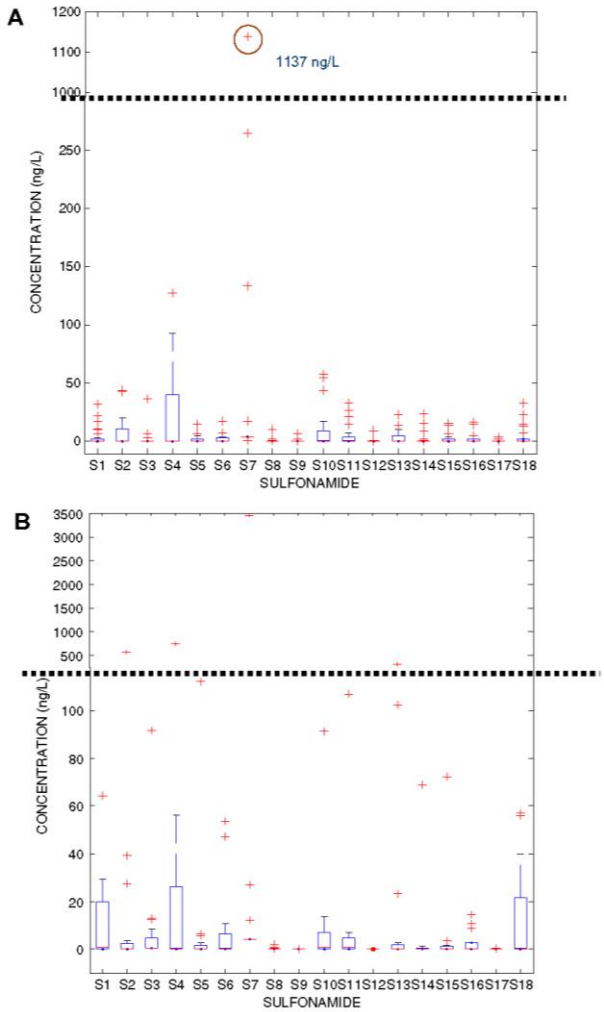

Fig. 4. Box plots for the distribution of the concentrations of the corresponding sulfonamides in the sampling sites of Plana de Vic (A) and La Selva (B). In A, each variable has 26 measured values. In B, each variable has 13 measured values. S1, sulfisomidin; S2, sulfanitran c; S3, sulfaguanidine; S4, sulfamerazine; S5, sulfaquinoxaline; S6, sulfadoxine; S7, sulfacetamide; S8, sulfabenzamide; S9, sulfadiazine; S10, sulfadimethoxine; S11, sulfamethazine; S12, sulfamethizole; S13, sulfamethoxazole; S14, sulfamethoxypyridazine; S15, sulfapyridine; S16, sulfathiazole; S17, sulfisoxazole and S18, N^4-acetylsulfamethazine.

Sulfonamides commonly used in human medicine, such as sulfadiazine and mainly sulfamethoxazole, have also been detected in both ground water bodies and the latter at relatively high frequency and concentrations (peak concentration of 312 ng/L and total frequency of detection of 59% for sulfamethoxazole, see Fig. 4 and Table 3, respectively). As the origin of these sulfonamides residues is not manure application on agricultural practices, alternative pathways for entering this environmental compartment should be considered. Waste water treatment plants (WWTPs) discharges into surface waters should be taken into account, as the removal efficiency for sulfonamides in these facilities has proved to be inefficient (Carballa et al., 2004; Miao et al., 2004). There are 13 WWTPs in Plana de Vic and nine in La Selva. Despite the fact that sulfonamides are highly soluble substances and possess low soil–water partition coefficient (K_d), biosolid applications on agricultural soils could also represent the main entrance to the ground water matrix. In La Selva, biosolids from the nine WWTPs are applied in soils from the different municipalities. High concentrations

Table 4
Physico-chemical parameters for each of the ground water bodies studied. TOC, total organic carbon.

La	La Plana de Vic	La Selva
Depth of the sampling sites (m)	3–200	10–206
Nitrates (mg/L)	0.60–264	5–60.8
Conductivity (μS/cm)	303–1906	290–711
TOC (mg/L)	0.6–8.5	1.3–5.2
Maximum sulfonamide concentration (ng/L)	3322	1137

detected in the municipality of Casà de La Selva, for instance, could be attributed to this practice. However, biosolids are seldom applied in La Plana de Vic, where both sulfadiazine and sulfamethoxazole have also been detected, but at lower concentrations.

3.3. Relationship between the presence of sulfonamides and nitrates

A brief summary of the main physico-chemical parameters that characterize both ground water bodies studied is shown in Table 4. As nitrates and sulfonamides detected in ground waters may share a common origin (extensive and intensive cattle farming and manure application in crop lands), the possibility of establishing a correlation between both parameters was worth to consider. The depth of the sampling site was also considered to play an important role in the concentration distribution of both nitrates and sulfonamide concentrations. With this aim, pair wise correlations between nitrates concentration, depth of the well and sulfonamides concentration were estimated and are given in Table 5. Despite its high solubility and potential to percolate through soil, there is a lack of correlation between nitrates and depth. Similarly, there was no apparent relationship between any sulfonamide concentration and this parameter, as it is reflected on their low correlation coefficients, being sulfisomidin and sulfadoxine the two compounds showing the highest ones (0.39 and 0.38, respectively). Regarding nitrates and sulfonamides, a clear correlation between the occurrence of both could not be established from the data obtained. Sulfadiazine and sulfamethizole are the two sulfonamides with the highest correlation coefficients regarding nitrates concentration (0.37 and 0.33, respectively). However both sulfadiazine and sulfamethizole were the compounds with the lowest frequencies of detection in both ground water bodies (not detected in any site of La Selva, and only in 12% of the sampling sites in La Plana de Vic, see Table 3). Therefore, the relationship between both nitrates and these antibiotics should be investigated in further detail. Regarding conductivity and total organic carbon (TOC) measured in the sampling sites, no clear relation between those parameters and the concentrations of the sulfonamides detected could be settled, and these data was eventually not included in Table 5. As an example, TOC seemed to be inversely correlated with the presence of three sulfonamides, sulfisoxazole, sulfacetamide and sulfabenzamide, although the corresponding correlation coefficients were low (−0.40, −0.42 and −0.42, respectively).

Sulfonamides are usually applied in mixtures and not as single antibiotics. Consequently, correlation amongst them turned out to be quite high for some of the combinations. For instance, sulfadimethoxine was detected together with sulfamethoxypyridazine, sulfapyridine and sulfathiazole very frequently, with correlation coefficients of 0.90, 0.86 and 0.80, respectively. Something similar happened with the presence of sulfamethazine and the same three sulfonamides. It should be highlighted that N^4-acetylsulfamethazine was detected simultaneously with 12 of the 19 sulfonamides studied; the correlation coefficients estimated for the simultaneous presence of the metabolite and each of the 12 sulfonamides were higher than 50%.

Conclusions

Contaminants such as sulfonamides and nitrates, highly soluble in water, may reach the water table and be transported by the slowly moving ground water, widening its presence through very extensive ground water systems. 18 out of the 19 target sulfonamides have been detected in two ground water bodies from Catalonia, being sulfadimethoxine and sulfamethazine, commonly used in veterinary practices, those occurring more frequently. It should be highlighted, however, the high frequency of detection for the acetylated metabolite N^4-acetylsulfamethazine, comparable to the highest frequencies aforementioned. The need for the inclusion of this and other metabolism products in future monitoring studies is unquestionable.

Despite the peak concentration values detected in different sampling sites, the average detected concentrations of sulfona-

Table 5
Relationship between depth, nitrate and sulphonamide concentrations, expressed as pairwise correlation coefficients. The variables are the concentrations of the corresponding sulfonamides (see legend of Fig. 4). Higher pairwise correlations are marked in bold.

	Depth	Nitrates	S1	S2	S3	S4	S5	S6	S7	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19
Depth	**1**																			
Nitrates	−0.19	1.00																		
S1	**0.39**	−0.01	1.00																	
S2	0.08	−0.08	0.27	1.00																
S3	0.20	−0.20	0.29	0.02	1.00															
S4	0.08	0.01	0.32	0.97	−0.06	1.00														
S5	0.08	−0.0001	0.30	0.98	−0.06	**0.99**	1.00													
S6	**0.38**	−0.01	**0.78**	**0.69**	0.09	**0.73**	**0.73**	1.00												
S7	0.20	−0.15	0.27	0.03	0.97	−0.05	−0.05	0.09	1.00											
S8	**−0.09**	0.11	0.14	−0.02	0.06	0.08	0.09	0.16	0.05	1.00										
S9	**−0.10**	**0.37**	−0.03	−0.03	−0.06	−0.02	−0.03	−0.05	−0.04	−0.02	1.00									
S10	0.12	0.24	0.34	0.72	−0.02	**0.83**	**0.83**	**0.68**	−0.01	**0.42**	0.04	1.00								
S11	0.11	0.14	0.36	**0.91**	0.00	**0.97**	**0.96**	**0.74**	0.01	0.17	−0.01	0.91	1.00							
S12	0.24	0.33	0.18	−0.03	**−0.04**	0.13	0.09	0.18	−0.03	−0.04	0.03	0.38	0.24	1.00						
S13	0.00	−0.11	0.08	0.05	0.25	0.04	0.03	0.04	0.27	0.04	−0.03	0.04	0.08	0.03	1.00					
S14	0.09	0.03	0.35	**0.91**	**−0.04**	**0.96**	**0.97**	**0.75**	−0.04	0.26	−0.03	**0.90**	**0.97**	0.19	0.04	1.00				
S15	0.09	0.01	0.33	**0.96**	−0.02	**0.99**	**0.99**	**0.75**	−0.01	0.16	−0.03	**0.86**	**0.98**	0.15	0.04	**0.99**	1.00			
S16	0.20	0.17	0.49	0.46	0.37	**0.58**	**0.56**	**0.59**	0.34	**0.48**	−0.07	**0.80**	**0.71**	**0.45**	0.12	**0.68**	**0.63**	1.00		
S17	0.04	0.27	0.22	−0.04	−0.02	0.07	0.08	0.18	0.03	**0.64**	−0.01	**0.36**	0.19	**0.34**	0.09	0.23	0.14	**0.47**	1.00	
S18	0.19	0.01	**0.47**	**0.54**	**0.55**	**0.59**	**0.57**	**0.56**	**0.53**	0.12	−0.05	**0.65**	**0.66**	0.27	0.25	**0.60**	**0.61**	**0.79**	0.17	1

mides are generally below 50 ng/L. Sensitivity is therefore one of the most critical parameters in order to obtain unequivocal and reliable determination for he compounds investigated. When performing on-line SPE analysis, its fully automation and the minimum sample manipulation requirements permits the enhancement of sensitivity, as the whole sample volume (40 mL) gets to the chromatographic system instead of a final reconstituted extract as in off-line procedures, where usually volumes of 200 mL or bigger are reduced to approximately 0.5 mL and only around 10 μL will be injected in the mass analyzer. Despite the low sample volumes required in on-line procedures, it has been proved that sensitivity is not affected but, on the contrary, improved considerably, with limits of detection down to the pg/L level. Besides, LC–MS/MS allows for an unequivocal identification of the target sulfonamides.

From the results obtained, no strong correlation between sulfonamides and nitrates concentrations could be established. Whereas nitrates in ground water are originated from fertilizers of both animal and mineral origin, sulfonamides could be specifically considered as potential indicators of pollution from animal origin. For this reason, and because data on nitrates is historically richer and more consistent, the presence of sulfonamides in ground water matrices should be investigated in further detail in order to propose these substances as reference points to indicate pollution from animal farm and agriculture practices.

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Occurrence of sulfonamide residues along the Ebro river basin Removal in wastewater treatment plants and environmental impact assessment

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ABSTRACT

Sulfonamides (SAs) have become one of the antibiotic families most frequently found in all kind of environmental waters. In the present work, the presence of 16 SAs and one of their acetylated metabolites in different water matrices of the Ebro River basin has been evaluated during two different sampling campaigns carried out in 2007 and 2008. Influent and effluent samples from seven wastewater treatment plants (WWTPs), together with a total of 28 river water samples were analyzed by on-line solid phase extraction-liquid chromathography-tandem mass spectrometry (on-line SPE-LC-MS/MS). Sulfamethoxazole and sulfapyridine were the SAs most frequently detected in WWTPs (96–100%), showing also the highest concentrations, ranging from 27.2 ng L^{-1} to 596 ng L^{-1} for sulfamethoxazole and from 3.7 ng L^{-1} to 227 ng L^{-1} for sulfapyridine. Sulfamethoxazole was also the SA most frequently detected in surface waters (85% of the samples) at concentrations between 11 ng L^{-1} and 112 ng L^{-1}. In order to assess the effectiveness of the wastewater treatment in degrading SAs, removal efficiencies in the seven WWTPs were calculated for each individual SA (ranging from 4% to 100%) and correlated to the corresponding hydraulic retention times or residence times of the SAs in the plants. SAs half-lives were also estimated, ranging from to 2.5 hours (sulfadimethoxine) to 128 h (sulfamethazine). The contribution of the WWTPs to the presence of SAs depends on both the load of SAs discharging on the surface water from the WWTP effluent but also on the flow of the receiving waters in the discharge sites and the dilution exerted; WWTP4 exerts the highest pressure on the receiving water course. Finally, the potential environmental risk posed by SAs was evaluated calculating the hazard quotients (HQ) to different non-target organisms in effluent and river water. The degree of susceptibility resulted in algae>daphnia>fish. Sulfamethoxazole was the only SA posing a risk to algae in effluent water, with an HQ>7.

1. Introduction

At present, approximately 3000 different pharmaceutical ingredients are used in the European Union (EU), including antibiotics, beta-blockers, lipid regulators, antidepressants and many more (Ternes et al., 2006). Being designed to have specific biologic effects within a given species, some of them may remain as bioactive substances and/or are not easily degradable once they are excreted and released into the environment. In these cases, they could also pose a toxicological risk to different non-target organisms, altering the ecosystem dynamics (Boxall et al., 2002; Daughton and Ruhoy, 2009). A very clear example is the presence of antibiotic residues in the natural media and the consequent inducement of resistance in bacterial strains (Costanzo et al., 2005; Goñi-Urriza et al., 2000; Schmitt et al., 2005; Thiele-Bruhn and Beck, 2005). Due to low cost and relative efficiency against many common bacterial infections, sulfonamides (SAs) and their combination with diaminopyrimidines are some of the most commonly used antibiotics (Pérez-Trallero and Iglesias, 2003). They are used for humans but mostly in veterinary medicine, especially in animal husbandry and fish farms (Chafer-Pericas et al., 2010; Hamscher et al., 2006). After their usage, their high solubility and excretion rates make them potential pollutants to environmental waters; their presence in different aquatic systems has been demonstrated in different works (Díaz-Cruz et al., 2008; Lindsey et al., 2001; Managaki et al., 2007; Peng et al., 2008).

River basins and catchment areas can therefore be considered vulnerable systems regarding SA contamination. Data on the removal efficiency (RE%) of these compounds during wastewater treatment is still quite scarce and WWTP could be considered as point sources of SAs contamination, as their effluents commonly discharge into natural

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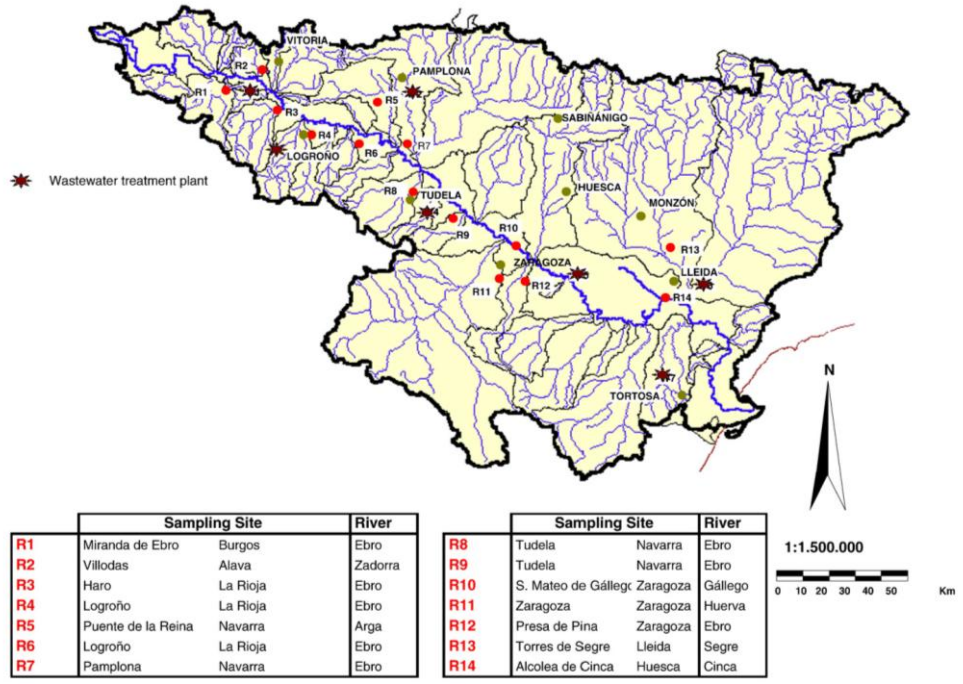

	Sampling Site		River
R1	Miranda de Ebro	Burgos	Ebro
R2	Villodas	Alava	Zadorra
R3	Haro	La Rioja	Ebro
R4	Logroño	La Rioja	Ebro
R5	Puente de la Reina	Navarra	Arga
R6	Logroño	La Rioja	Ebro
R7	Pamplona	Navarra	Ebro

	Sampling Site		River
R8	Tudela	Navarra	Ebro
R9	Tudela	Navarra	Ebro
R10	S. Mateo de Gállego	Zaragoza	Gállego
R11	Zaragoza	Zaragoza	Huerva
R12	Presa de Pina	Zaragoza	Ebro
R13	Torres de Segre	Lleida	Segre
R14	Alcolea de Cinca	Huesca	Cinca

Fig. 1. Locations of the WWTPs and surface waters monitored along the Ebro River basin.

water courses (Gobel et al., 2007; Gros et al., 2007; Ye et al., 2007). Manure application on agricultural soils is also a relevant entrance pathway, as SAs may reach surface waters by runoff and even percolate and reach groundwater bodies (Campagnolo et al., 2002; De Liguoro et al., 2007; Kay et al., 2005; Schmidt et al., 2008; Stoob et al., 2007; García-Galán et al., 2010a). The lack of ecotoxicological data regarding adverse effects of SAs is possibly one of the main reasons for the absence of European regulation on maximum levels of this family of antibiotics (or any other pharmaceutical product) in any environmental compartment. The only restrictive measure established so far comes from the European Medicines Agency (EMEA), which requires that an environmental risk assessment (ERA) accompanies the application for marketing authorization for a new medicinal product for human use (EMEA CVMP, 2004; Huschek et al., 2004). Although

Table 1
Characteristics of the WWTPs studied. HRT and SRT values refer to the residence times of the SAs in the plant. –: no data available (not facilitated by the WWTP).

WWTP	Date	Population served	Population equivalent	Average flow (m^3/day)	Receiving surface water	Type of wastewater treated	HRT (h)	SRT (days)	Primary treatment	Secondary treatment
WWTP1	7/10/2007	42,000	52,700	11,181	Ebro	Urban-industrial	32	–	–	Activated
	1/7/2008			11,062						sludge
WWTP2	7/10/2007	171,024	–	60,000	Ebro	Urban-industrial	8	6	Primary	Activated
	1/7/2008			60,000					settling	sludge
WWTP3	8/10/2007	37,300	110,000	22,640	Ebro	Urban	18	9	Primary	Biologic
	30/6/2008		65,000	24,680					settling	Filters
WWTP4	8/10/2007	355,688	721,829	102,124	Arga	Urban-industrial	21	20	Primary	Activated
	2/7/2008			126,144					settling	sludge
WWTP5	31/01/2008	650,000	800,000	152,800	Ebro	Urban	10	4	Primary	Activated
	29/06/08			194,600					settling	sludge
WWTP6	10/10/2007	141,977	222,049	69,114	Segre	Urban	10	6	Primary	Activated
	3/7/2008		191,675	54,175					settling	sludge
WWTP7	10/10/2007	40,937	178,932	10,064	Ebro	Urban	24	19	Primary	Activated
	3/7/2008		179,609	5227			46		settling	sludge

they are designed as part of the process for registering new drugs, risk-assessment guidelines have been used in a few occasions to prioritize the risk from drugs that are already in use and to assess the potential impact of drugs yet to be released (Boxall et al., 2002; Ferrari et al., 2004; Kim and Carlson, 2007; Park and Choi, 2008). The ERA protocol is a two-phase tiered process that begins with an approximate calculation of the predicted environmental concentration (PEC) of the new drug in water. The guidelines recommend that any drug exceeding 10 ng L^{-1} in surface water should progress to Phase II, where standard acute toxicity tests will be carried out in order to estimate a predicted no-effect concentration (PNEC) or a non-observed effect concentration (NOEC). These end points are the estimation of the concentration of the drug for which adverse environmental effects are not expected. The recommended Phase II assessment is based on the ratio of the PEC to PNEC, known as the Hazard Quotient (HQ). If HQ is >1, a potential environmental impact is indicated and further testing might be needed to refine PEC and PNEC values in Phase II. If HQ<1, no further testing is required. The EMEA Committee for Medicinal Products for Veterinary Use (CVMP) also established similar guidelines to assess the potential for veterinary medicines to affect non-target species in the environment, including both aquatic and terrestrial species (EMEA CVMP, 2004).

The aim of this work is to assess the presence of 16 SA antimicrobials and one of their acetylated metabolites in different water matrices along the Ebro River basin, the most extensive of the Spanish fluvial system. Influent and effluent samples from WWTPs were taken, together with surface water upstream and downstream of the WWTP's discharge areas. RE% and half-lives ($t_{1/2}$) were estimated for each compound when possible, in order to correlate the biodegradability of the SAs with the removal efficiency of the WWTP. In addition, the potential ecotoxicological risk posed by the SAs residues detected was estimated and expressed as HQs.

2. Materials and methods

2.1. Chemicals

HPLC-grade solvents (water, methanol (MeOH), acetone and acetonitrile) and formic acid (98–100% purity) were supplied by Merck (Darmstadt, Germany). High purity standards (>99%) of the 17 selected SAs, namely sulfadiazine (SDZ), sulfadimethoxine (SDM), sulfamethazine (SMZ), N^4-acetylsulfamethazine (AcSMZ), sulfamethizole (SMT), sulfamethoxazole (SMX), sulfamethoxypyridazine (SMP), sulfapyridine (SPY), sulfisoxazole (SSX), sulfathiazole (STZ), sulfisomidin (SSD), sulfanitran (SNT), sulfamerazine (SZI), sulfaquinoxaline (SQX), sulfadoxine (SDX), succinyl-sulfathiazole (SuSTZ) and sulfabenzamide (SBZ), were purchased from Sigma (St Louis, MO, USA). Stock standard solutions for each of the analytes were prepared in MeOH at 1 mg mL^{-1} and stored at −2 °C until use. Standard solutions of the mixtures of all compounds at appropriate concentrations were prepared by the corresponding dilution of the stock solutions in MeOH. The standard mixtures were used as spiking solutions for preparation of the aqueous calibration standards, which always contained <0.1% of MeOH. Internal standard d_4-sulfathiazole (99.9% purity) was purchased from Toronto Research Chemicals (Ontario, Canada). The corresponding stock solution at 2.5 mg mL^{-1} was also prepared in MeOH and stored at −2 °C until use.

2.2. Sampling area and sample collection

The Ebro River basin (northeast Spain) drains an area of c.a. 85,000 km^2, which equals to 17.3% of the surface area of Spain, representing the most relevant irrigated land of the country. The Ebro River runs throughout 930 km from its source to its mouth (NW–SE) and is fed by 347 major tributaries. The agricultural sector (including grazing livestock) makes up for the 58% of the land use and for the 8% of the employed population. Irrigation is a relevant economic tool in the Spanish agriculture, as the productivity of irrigated land is 7.3 fold higher on average than that of non-irrigated areas. In the Ebro River basin up to a 9.16% of agricultural land is irrigated, particularly in the central areas of the basin (especially in Aragon and Catalonia). Population in the basin is close to 3 million inhabitants (AQUATERRA, 2004). The sampling area is shown in Fig. 1.

Both river and WWTP samples were taken on the same dates during two different campaigns carried out in October 2007 and July 2008, in order to analyze and compare results from samples taken in a hot and a wet season (July and October respectively). 24 h-integrated samples of influent and effluent wastewaters were taken in the seven WWTPs corresponding to the most relevant cities along the basin. Table 1 summarizes the characteristics of the WWTPs studied. With the exception of WWTP3, with a secondary treatment consisting of biologic filters, all of them perform the same type of water treatment, based on a primary settling and conventional activated sludge (CAS). The main differences, on the other hand, lie in the hydraulic and solid retention times (HRT and SRT respectively) across the WWTP.

A total of 28 surface water samples (14 samples in each sampling campaign) were taken to determine the presence of the SAs in the different river waters along the basin, in order to establish a spatial concentration gradient (from the source to the mouth of the river) but

Table 2
Method limits of detection (LOD), method limits of quantification (LOQ) and precision of the method, expressed as a relative standard deviation ($n=5$).

SA	WWTP INFLUENT			WWTP EFFLUENT		SURFACE WATER		
	LOD (ng L^{-1})	LOQ (ng L^{-1})	RSD (%)	LOD (ng L^{-1})	LOQ (ng L^{-1})	LOD (ng L^{-1})	LOQ (ng L^{-1})	RSD (%)
SSD	0.10	0.32	3.23	0.02	0.05	0.03	0.11	4.37
SNT	0.46	1.53	2.59	0.24	0.79	0.03	0.10	3.32
SZI	1.53	5.11	1.77	0.20	0.66	0.09	0.30	2.06
SQX	0.59	1.97	0.51	0.04	0.13	0.03	0.11	1.43
SDX	0.21	0.69	1.50	0.02	0.08	0.03	0.09	2.93
SuSTZ	2.21	7.37	9.53	0.95	3.15	0.31	1.03	2.39
SBZ	0.05	0.15	4.79	0.03	0.11	0.03	0.11	0.07
SDZ	0.39	1.29	4.66	0.09	0.32	0.06	0.22	3.38
SDM	0.13	0.45	1.46	0.01	0.05	0.02	0.05	2.78
SMZ	0.12	0.40	1.83	0.06	0.19	0.05	0.17	2.88
SMT	0.37	1.24	3.84	0.81	2.71	0.58	1.95	5.13
SMX	1.11	3.69	1.55	0.15	0.48	0.08	0.27	1.16
SMP	0.11	0.38	1.54	0.05	0.16	0.04	0.12	3.82
SPY	0.27	0.91	3.04	0.10	0.33	0.05	0.17	1.90
STZ	0.23	0.77	5.08	0.24	0.79	0.08	0.27	1.31
SSX	0.51	1.71	6.70	0.12	0.41	0.04	0.12	5.25
N4-AcSMZ	0.89	2.95	4.42	0.14	0.48	0.02	0.06	1.00

RSD values for both effluent and surface waters are the same, as the same volume is extracted during on-line SPE for both matrices.

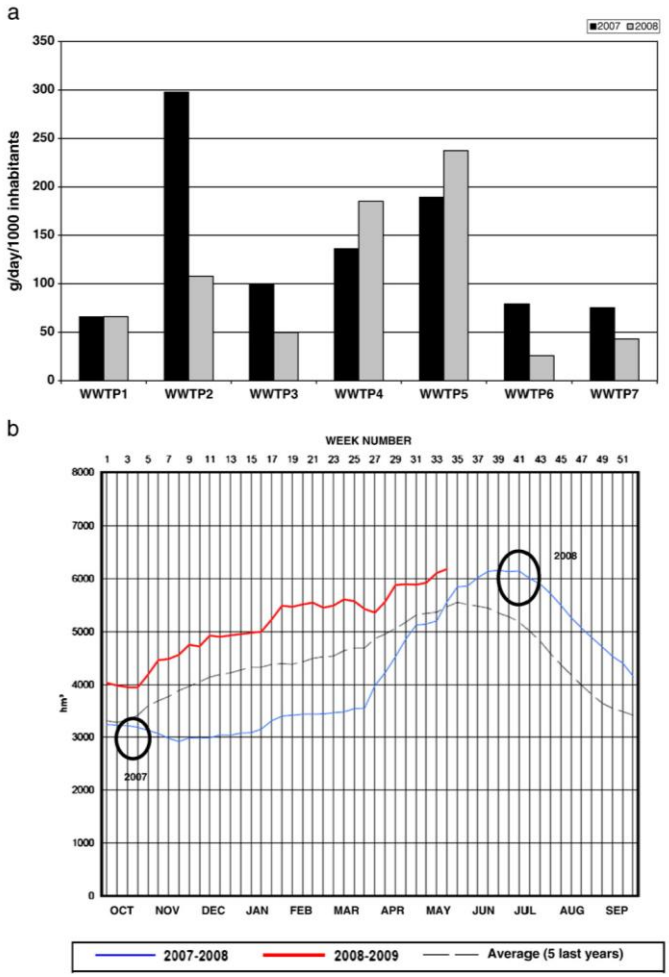

Fig. 2. Total loads, given as g/day/1000 inhabitants, of the17 SAs studied in each WWTP effluent (a) and water reserve in the Ebro River basin during 2007 and 2008.

also to evaluate the impact of the WWTP effluents in the receiving waters, downstream the treatment facilities. With this purpose, river waters were taken upstream and downstream the WWTPs, and at different distances to them depending on the ease of access to the water bed. Samples were taken from the Ebro River (R1, R3, R4, R6, R8, R9, R12 and R15) and from some of its tributaries: Zadorra River (R2), Arga River (R5–R7), Gállego River (R10), Huerva River (R11), Segre River (R13) and Cinca River (R14). Sample R2 was taken only in the 2007 campaign. River water was collected as grab samples in the middle part of the rivers, using for this purpose a clean amber glass bottle placed inside a stainless steel cage. All water samples were transferred to amber polyethylene terephthalate (PET) bottles and transported to the laboratory under cooled conditions (4 °C). Upon reception, samples were filtered through 0.45 μm Nylon filters (Whatman, Maidstone, UK) to eliminate suspended solid matter and then frozen until analysis (usually no more than a week).

2.3. Analytical method

The analytical methodology for the measurement of SAs in surface water and wastewater has been previously described (García-Galán et al., 2010b). Sulfacetamide and sulfaguanidine were not part of the scope of this study as the optimum method limits of detection (MLODs) were not low enough for the concentrations expected in

the environmental samples investigated. Regarding the on-line SPE procedure, pre-concentration of the samples was performed using an automated on-line SPE sample processor Prospekt-2 (Spark Holland, Emmen, The Netherlands). On-line SPE of all samples, aqueous standard solutions and blanks was performed by loading 15 mL of surface water and WWTP effluent and 5 mL of WWTP influent, at a rate of 1 mL min^{-1}, onto Oasis HLB online cartridges, previously conditioned with 1 mL of a mixture of methanol and acetone. After sample loading and prior to elution, the cartridges are washed with 1 mL of HPLC grade water at a flow rate of 1 mL min^{-1} to complete the transfer of the sample and remove interferences. Analytes are eluted directly to the LC column by means of the HPLC system.

LC-MS/MS analyses were carried out in a system consisting of an Agilent HP 1100 pump (Agilent Technologies, Palo Alto,CA) connected to a 4000 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (QqLIT) equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, CA). Further LC-MS/MS experimental conditions were already discussed in a previous study (Díaz-Cruz et al., 2008).

2.4. Method validation

The analytical method described was evaluated in terms of linearity, repeatability, accuracy, selectivity and sensitivity for this study. Quantification was carried out based on internal standard calibration, by adding d_4-sulfathiazole to all the samples, aqueous standards for the calibration curve and blanks at a concentration of 500 ng L^{-1} before extraction in order to correct potential matrix effects and losses during the SPE procedure and the analysis. Five to eight point matrix matched calibration curves were constructed for each of the water types at concentrations ranging from 0.01 ng L^{-1} to 1000 ng L^{-1}. They were all previously corrected with the corresponding blanks. Correlation coefficients (r^2) were higher than 0.999 for all of the SAs studied. Precision was given by the repeatability of the instrument, expressed as the relative standard deviation (%RSD) of five consecutive injections of a standard aqueous solution (in HPLC water in this case) at 100 ng L^{-1}. The obtained relative standard deviation was generally below 6% (see Table 2). Method limits of detection (LOD) and quantification (LOQ) were also calculated as the minimum detectable amount of analyte with a signal-to-noise ratio of 3 and 10 respectively. LOD values were in the range of 0.05–2.21 ng L^{-1} for WWTP influent wastewaters, 0.01–0.95 ng L^{-1} for WWTP effluent wastewaters and 0.02–0.58 ng/L for surface water samples.

3. Results and discussion

3.1. Occurrence of SAs in wastewaters

Total loads in the effluents of the different WWTPs, which afterwards would discharge into river waters, were calculated multiplying the total concentration of SAs in each WWTP by the corresponding effluent flow rate and then dividing by the corresponding population served (Fig. 2a). They ranged from 75 to 297.5 g/day/1000 inhabitants in campaign 2007 and from 25.7 to 237 g/day/1000 inhabitants in 2008. Despite that the sampling was carried out during October (rainy season) in 2007, in most of the WWTPs, loads were higher this year than in 2008; the hydrological reserve in 2007 was lower than in 2008, which means less rainfall and runoff converging in the WWTPs influents, and therefore a lesser dilution of the target SAs (Fig. 2b). In both campaigns, the highest loads corresponded to WWTP2 and WWTP5, which serve two of the biggest populations of the entire basin.

SMX, SPY and SDZ presented the highest frequencies of detection in both influent (85–100%) and effluent samples (79–100%) and in both sampling campaigns (Fig. 3). They are all used typically in human medicine (although SDZ is used in both human and veterinary therapies), being this the main reason why they are recurrently detected in urban wastewaters. In the case of SMX and SPY, they also presented the highest concentration levels detected in both matrices, as shown in Fig. 4. Whereas SMX was found in the effluent wastewater of WWTP5 at concentrations of 596 ng L^{-1} and 650 ng L^{-1} in 2007 and 2008, respectively, SPY was detected at its highest levels in influent wastewaters of WWTP1, at concentrations of 320 ng L^{-1} in 2007 and 226 ng L^{-1} in 2008. These results agree with those presented by the same authors in a previous work (García-Galán et al., 2010b), in which four different WWTPs from different rural and urban areas were monitored. In that study, SMX and SPY were again the two compounds detected more often and in the highest concentrations. SMX was present in the four WWTP effluents investigated, at concentrations up to 302 ng L^{-1} in effluent samples, and SPY was present in all influent and effluent samples, presenting the highest concentration also in one of the influent wastewaters (855 ng L^{-1}).

3.2. Removal efficiencies of SAs in WWTPs

The removal of each individual SA was calculated comparing the concentrations detected for each of the SAs individually in the influent and effluent samples of the WWTPs. RE% are presented as percentage values of the campaigns of 2007 and 2008 in Table 3. Values of 100% removal were obtained when the SA was detected only in the influent water. In some other cases, the target compound was detected only in effluent water, and RE% could not be determined. Negative RE% were obtained when the SA was present at higher concentrations in the effluent waters. These three situations can be explained in terms of the HRT of the WWTP and the sampling procedure. Influent and effluent samples were taken simultaneously as composite samples throughout 24 h. This means that the effluent waters investigated at the end of the treatment process did not fully correspond to the influent wastewaters treated. Besides, the development of the water treatment may not be fully homogeneous, which could also contribute to the non-correspondence of the influent–effluent concentrations found. Negative removals could also be explained by the presence of SA conjugates and metabolites that reverted back during treatment into the original compound (García-Galán, submitted).

It is difficult to assess which of the SAs showed the highest or lowest elimination, as they were not regularly present in all the WWTPs. WWTP1, WWTP3, WWTP4 and WWTP6 presented the highest number of SAs being removed, ranging from 9 to 11 out

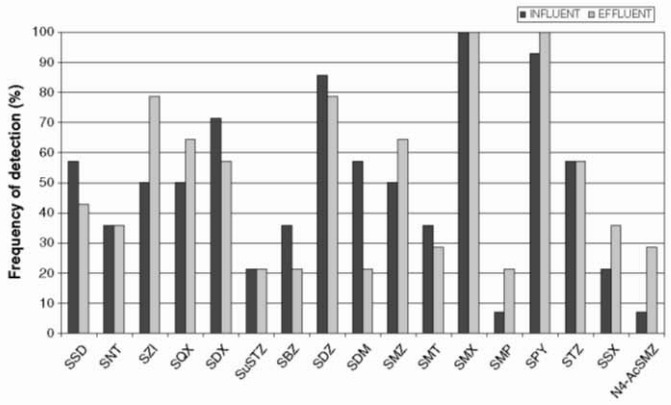

Fig. 3. Frequencies of detection (%) of SAs in the seven WWTPs studied.

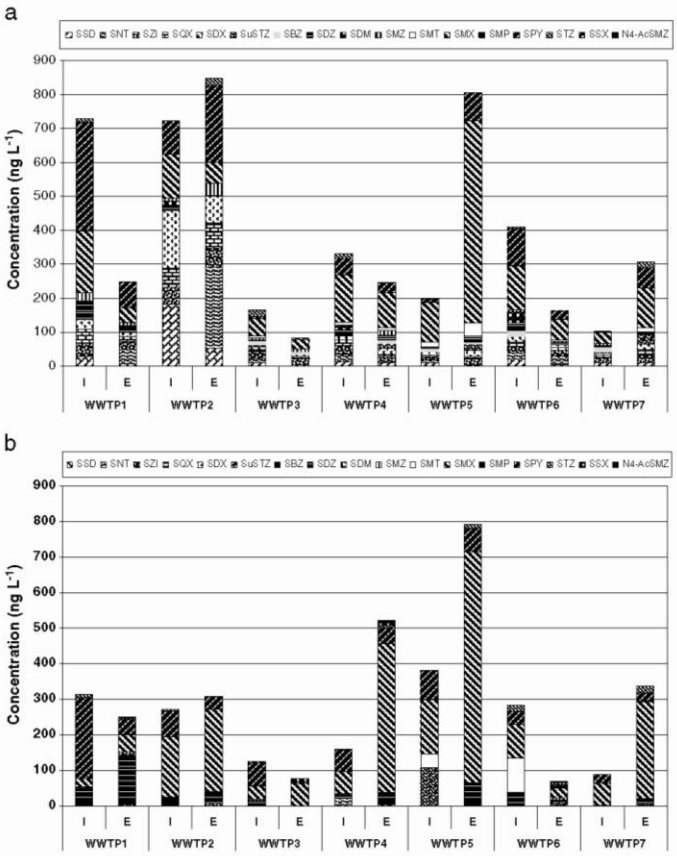

Fig. 4. Cumulative levels (ng L^{-1}) of SAs in the different WTPs investigated during (a) 2007 and (b) 2008.

of the 17 SAs monitored, and with RE% going from 4% to 100%. In WWTP1, WWTP3 and WWTP6, most of them were also removed at rates higher than 50%. SSD was the only SA detected and positively removed in the seven WWTPs during the first campaign, but was not present in any of the samples in 2008. In WWTP2 and WWTP5, the two WWTPs with highest effluent SA loads, many of the SAs were detected at higher concentrations in the effluent than in the influent. Besides, these two plants presented low RE% for most of the SAs, justifying these high loads.

As mentioned in the previous section, SMX, SPY and SDZ were the SAs most frequently detected and at highest concentrations. Focusing in these three SAs, their RE % values were higher than 50% in most of the WWTPs, but also presented negative values, especially SMX, which was detected at higher concentrations in the effluent than in the influent of five of the seven WWTPs in 2008; WWTP5 and WWTP7 presented the most relevant concentration differences between influent and effluent, as can be seen in Fig. 4. The presence of N^4-acetylsulfamethoxazole in this WWTP was not within the scope of this work, but previous studies demonstrated its occurrence in WWTP influent and effluent wastewaters (Gobel et al., 2004); the presence of the acetylated metabolite and its degradation or back transformation into the parent substance could explain the increase in the concentration of SMX in the effluent samples, together with the inefficiency of the water treatment to eliminate this antibiotic. SDZ was in average the SA eliminated most efficiently, and SPY showed intermediate to high RE% values. On the contrary, SZI presented the lowest RE%. If we correlate these results with the treatment procedures of each WWTP, it can be observed that HRT seemed to play an important role in the RE% of the plant, being this latter higher as HRT increases. However, WWTP7 presents also high HRT and, whereas RE% of 100% were estimated for five of the SAs, there were also negative RE% for six of them. A similar situation is found in WWTP5, where the concentration of seven of the SAs was higher in the effluent samples than in the influent.

In order to have more comprehensive information regarding the removal at the different WWTPs and to draw a more complete picture of the persistence of SAs in this matrix, half-lives ($t_{1/2}$) were estimated for the target SAs. Similar to what happens with many drugs in the human body after intake, we assumed a decrease of the concentration through time proportional to the concentration remaining in the matrix (pseudo-first order kinetics). Half-lives were estimated following the equation:

$$t_{1/2} = \frac{\ln 2}{k}$$

where k is the rate loss constant. k was calculated for each SA following the pseudo-first order kinetics equation:

$$-\frac{d[A]}{d[t]} = k[A]$$

where A is the concentration of the target compound. Fig. 5 shows RE% and $t_{1/2}$ in WWTP1, which operates at the highest HRT of all the WWTPs monitored, and in WWTP6, with a low HRT. Values of $t_{1/2}$ ranged from 2.8 h (SSD) to 46.6 h (SZI) in WWTP1, and from 2 h (SDM)

Table 3
Removal efficiencies (%) o
**: concentrations found i

SA	Remova
	WWTP1
	2007
SSD	100
SNT	*
SZI	38
SQX	69
SDX	45
SuSTZ	–
SBZ	–
SDZ	74
SDM	*
SMZ	62
SMT	–
SMX	76
SMP	–
SPY	77
STZ	100
SSX	–
N4-AcSMZ	–

Table 4
Frequencies of detection (FQ) ($n = 14$), total sulfonamide concentrations, average values, median values and concentration ranges for the SAs studied in surface waters during the two sampling campaigns.

SA	2007					2008				
		Concentrations (ng L^{-1})					Concentrations (ng L^{-1})			
	FQ (%)	Total	Average	Median	Range	FQ (%)	TOTAL	Average	Median	Range
SSD	100	111.2	8.6	7.5	2.4–23.8	54	70.0	5.0	3.5	1–40.4
SNT	93	119.6	8.5	7.7	17.8–127	0	0.0	0.0	–	–
SZI	100	610.7	47.0	32.9	13.1–42.2	62	90.2	6.4	8.9	1–35.4
SQX	100	216.4	72.1	72.7	4.5–40.4	23	54.6	3.9	13.3	5.9–27.9
SDX	100	316.9	22.6	21.0	2.7–43.3	38	59.8	4.3	5.1	3.3–26.4
SuSTZ	71	50.0	3.8	2.2	1.1–37	15	0.0	0.0	–	–
SBZ	100	203.1	14.5	13.1	6–14.6	15	1.8	0.1	1.8	1.8
SDZ	21	27.1	3.9	3.5	1.3–6.4	15	0.7	0.1	0.7	0.7
SDM	93	10.9	3.6	3.2	1–23.1	69	48.1	3.4	1.1	0.5
SMZ	100	259.3	18.5	15.9	3–65.2	69	74.3	5.3	3.7	2.5
SMT	14	25.7	5.1	1.3	2.3–4.6	23	2.7	0.2	2.7	2.7
SMX	100	356.7	89.2	89.8	1.9–35.6	69	357.1	25.5	30.3	0.2
SMP	57	154.0	11.0	6.5	0.6–18.1	38	41.3	3.0	7.3	0.6
SPY	100	109.8	11.0	7.4	1.1–42.5	62	38.1	2.7	0.8	0.1
STZ	50	6.9	3.5	3.5	1–9.6	23	25.5	1.8	10.1	1.5
SSX	29	146.8	10.5	3.8	0.1–0.7	8	12.5	0.9	12.5	12.5
N4-AcSMZ	36	159.3	11.4	5.5	0.8–20.2	15	5.6	0.4	2.8	0.2

to 128 h (SMZ) in WWTP6.
(32 h); in consequence, th
treatment procedure and b

appeared to be unaffected by HRT. A similar conclusion could be reached for STZ and SDX. On the other hand, SMZ seemed to be the SA more affected by the different HRT, presenting the highest difference of $t_{1/2}$ more than two fold the $t_{1/2}$ of WWTP1 in WWTP6.

3.3. Presence of SAs in surface waters

Frequencies of detection and concentrations of SAs estimated in the different campaigns are given in Table 4. Eight SAs were ubiquitous in all the samples taken during 2007, amongst them SMX and SPY, which were also the most frequently found in the WWTPs monitored. Its high occurrence could be attributed to urban contamination from both point sources (discharges from WWTPs where SAs have not been fully eliminated during the treatment process) or diffuse sources (losses from sewage systems) since the nearby cities were highly populated. However, SMZ, SZI and SSD, which are typically used in veterinary practices, were also often detected, indicating that SAs present in surface water may also come from livestock waste accumulation (or storage) nearby the river course or its application in crop lands, from which SAs would have been displaced by run off or percolation. These possibilities have been demonstrated in previous works, proving that SAs are usually highly mobile in soils (Boxall et al., 2002; Campagnolo et al., 2002; Thiele-Bruhn and Aust, 2004), and therefore likely to reach surface and groundwaters (Batt et al., 2006; García-Galán et al., 2010a; Stoob et al., 2007; Tamtam et al., 2008). A similar situation is observed for the samples taken in 2008.

An increase in the SA concentration would be expected towards the end part of the basin; urban pressure becomes more intense close to the mouth of the river. Furthermore, at this point the Ebro River and its tributaries have received SAs inputs, both agricultural and urban, all along the basin; the big resilience of SAs to degradation and low tendency to adsorb to solid matrices (from the river bed) (Kolpin et al., 2002; Thiele-Bruhn and Aust, 2004) would contribute also to a marked concentration gradient from the source to the mouth of the river. However, Fig. 6 shows that, although there is a slight increase in the concentration of the SAs closer to the final stretch of the river basin, a concentration gradient could not be established for any of the SAs, as levels vary frequently and do not show a clear trend. In 2007, samples R10, taken from the Gállego River, and R11, taken in the Huerva River, presented the highest total concentration of SAs. Sample R11 was sampled close to the city of Zaragoza, the most populated city of the basin. It should also be considered that flow rates of the Ebro tributaries are much lower than those of the Ebro River itself and therefore, exert a lower dilution on the SAs input in these water courses (Table 5). It is also observed in sample R7, taken from the Arga River and downstream of the WWTP4 discharge area, which also presented a high total concentration. The flow of this tributary is not very large, and therefore the dilution is not especially relevant. In 2008, the input from WWTP4 also reflected in the concentrations detected at R7, but R1 and R8 were the locations with the highest total concentrations. Both samples were taken from the Ebro River (upstream WWTP1 and WWTP3, respectively). Generally, levels were lower than those detected the previous year. Not all the flow rate values for 2008 where available when writing this article (only for four of the six WWTPs), but as mentioned before, the hydrological reserve in 2008 was higher than in 2007, which means higher flow rates in the different rivers and higher dilutions of the target SAs (Fig. 2b).

Regarding the environmental impact of WWTPs in surface waters, Fig. 7 depicts the different concentrations detected in the WWTP effluents and the river waters sampled upstream and downstream. WWTP3 and WWTP6 discharge in the Arga River and Segre River respectively, whereas the rest of the WWTPs do it in the Ebro River waters (see Table 5). Dilution factors have been calculated dividing the flow rate of the receiving water course (downstream the WWTPs discharge point) by the corresponding WWTP's discharge flow (SAIHEbro 2009). Values ranged from 26 to 123 for those WWTPs discharging in the Ebro River, and from 19 to 62 for those WWTPs discharging onto the Ebro tributaries. Dilution factors were higher in 2008 due to the highest rainfall (Fig. 2b). The dilution factor could not be calculated for WWTP7, as the water downstream the plant was not sampled. Different cases could be established:

a) The SAs concentration downstream the WWTPs are similar or lower than upstream: the impact of the WWTPs effluents is minimum in the water course; the concentration in the effluent is not relevant and does not increase the levels already present in the river flow; also the flow of the receiving river in the sampling point is higher than upstream the WWTP and SAs concentrations of the effluent are diluted enough as not to add up to the concentrations upstream; WWTP6 exemplifies this case in campaign 2008: the effluent loads for this WWTP were pretty low, and the flow of the receiving river, the Segre, was quite high as it's located in the final part of the basin. (The loads for WWTP6 are higher in 2007 and the impact in the river course downstream is more pronounced). This situation is also observed in WWTP3 and WWTP5 in 2008, and in WWTP1, WWTP2 and WWTP5 during the 2007 campaign. The exceptions are those SAs with peak concentrations in the WWTP effluents, such as SMX, for which the dilution is not high enough and the concentration increases downstream the plant.
b) The SA concentrations downstream the WWTP are higher than upstream: this would be the expected case, as the input concentration from the WWTPs would account for an increase in the SA load in the river downstream. As we mentioned before, this generally happens for those SAs that present peak concentration in the WWTP effluents, such as SDZ, SMX and SPY; this is clearly observed in WWTP1 (2008). Low dilution factors also contribute to these results (Table 5). For instance, in WWTP4 during both campaigns (2007–2008), as it discharges onto the Arga River, tributary with a low flow; the impact is lower in 2008 due to the frequency of rainfall events (Fig. 2b). WWTP5 would be the exception: in this case, despite the high concentrations in the effluent, the high flow of the receiving water course (the Ebro River) dilutes the concentrations enough as to make them lower than the upstream levels. However, the dilution factor estimated for this plant is not very high, so there is the possibility that the sample site did not exactly corresponded with the downstream area of the WWTP. Different sources of SAs pollution could also be considered; for instance, in 2007 and downstream WWTP3, concentrations were higher than those detected upstream and higher than the effluents' for SNT, SMZ, SQX and SuSTZ; this could also be observed for WWTP4 in both campaigns (2007–2008). These two plants are located in rural areas of the basin, with a high agricultural activity, explaining the high concentration for these 4 SAs (all of them typically used in veterinary medicine).

4. Environmental impact of the SAs studied

Based on the EMEA guidelines, HQ have been calculated to estimate the potential adverse effects of the SAs levels detected in both surface and WWTP effluents on non target organisms. This quotient is usually calculated as the ratio between the PEC and the PNEC or NOEC. When PNEC values are not available, an alternative PNEC can be derived by dividing the lowest EC_{50} or LC_{50} values (50% effect concentration or 50% lethal concentration, respectively) by an

Fig. 5. Rer

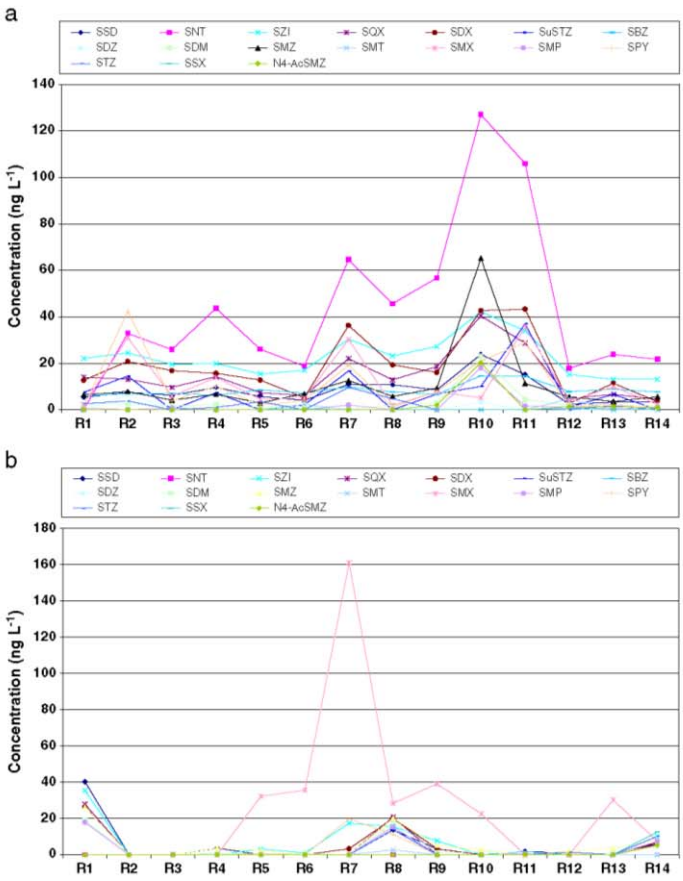

Fig. 6. Concentration gradient established for the SAs monitored in the different surface waters in (a) 2007 and (b) 2008 (from the farthest point to the mouth of the Ebro River to the closest).

Table 5
River flows for the different water courses monitored, expressed as monthly average values corresponding to September 2007 and June 2008 (www.saihebro.com). Dilution factors estimated in the waters sampled downstream the WWTPs (i.e. DW1 corresponds to the surface water sampled downstream WWTP1). N/A: information not available. –: values could not be calculated.

			2007		2008	
	Sampling site	Surface water	Flow of the receiving water ($m^3\ s^{-1}$)	Dilution factor	Flow of the receiving water ($m^3\ s^{-1}$)	Dilution factor
Downstream	R3-DW1	Ebro	15.2	118	N/A	–
	R6-DW2	Ebro	37.0	33	48.0	69.1
	R7-DW3	Arga	22.3	19	N/A	–
	R9-DW4	Ebro	27.2	104	35.2	123.2
	R12-DW5	Ebro	45.1	26	54.0	24.0
	R13-DW6	Segre	49.4	62	52.0	82.9
Upstream	R1	Ebro	15.2	–	–	–
	R2	Zadorra	24.6	–	–	–
	R4	Ebro	23.0	–	–	–
	R5	Arga	22.3	–	–	–
	R8	Ebro	27.2	–	–	–
	R10	Gállego	9.1	–	–	–
	R11	Huerva	0.8	–	–	–
	R14	Cinca	28.2	–	–	–

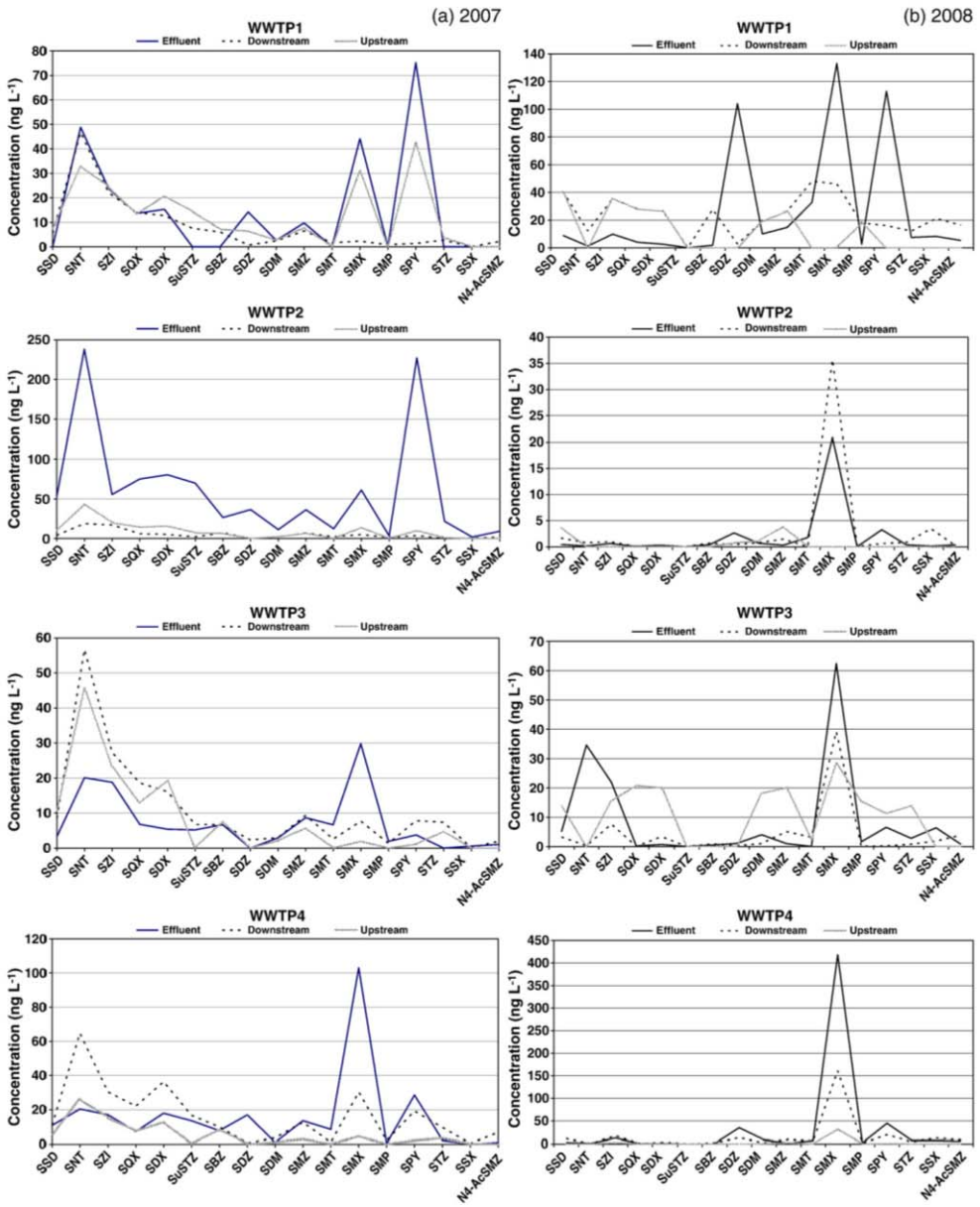

Fig. 7. SAs concentrations detected in the effluents, upstream and downstream of each of the WWTPs studied.

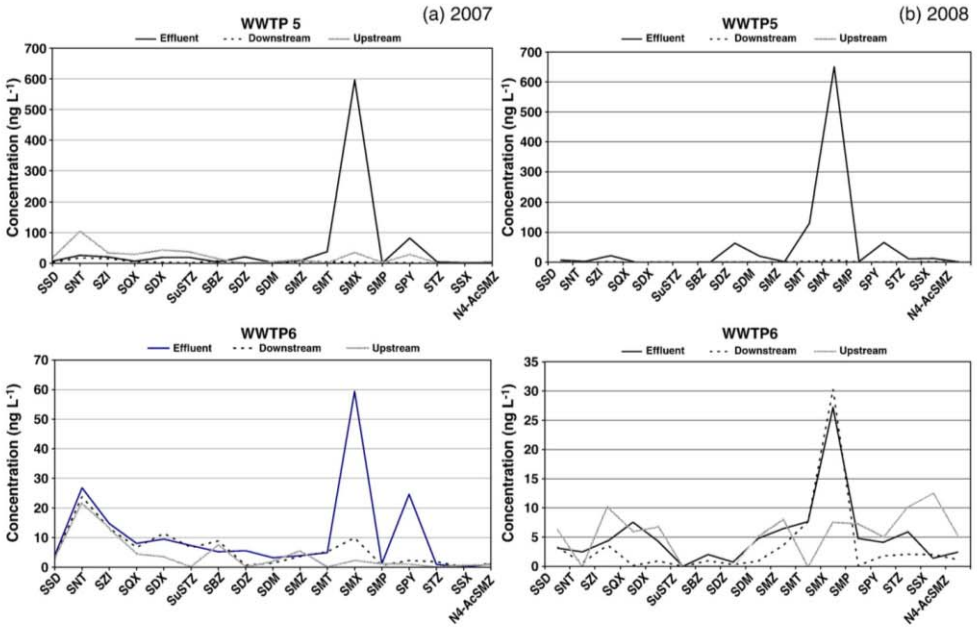

Fig. 7 (*continued*).

uncertainty factor of up to 1000 (Sanderson et al., 2003). It has to be taken into consideration that EC_{50} or LC_{50} values usually result from a laboratory acute toxicity test, typically of 24–96 h, and therefore may not be representative enough of the real situation in the natural media. But since data on chronic toxicity for SAs was lacking, EC_{50}–LC_{50} values taken from the literature (García-Galán et al., 2009) were used and normalized by the arbitrary uncertainty factor of 1000. Likewise, measured environmental concentrations (MECs) were used in the calculation instead of PECs (Ginebreda et al.,; Gros et al., 2010; Santos et al., 2007). In order to set up a worst case scenario, MEC values used corresponded to the maximum values detected in this study whereas EC_{50}–LC_{50} values used were the lowest found in the literature. In all cases, the MECs were higher than the boundary value of 10 ng L^{-1} established by EMEA in Tier 1. In order to cover different trophic levels of the aquatic ecosystems, HQ were estimated for fish, daphnids and algae. Table 6 summarizes the EC_{50}–LC_{50} values taken and the HQ calculated. It can be observed that values were generally far below the boundary of 1 established by the EMEA guidelines, indicating that no risk can be derived from the presence of the detected levels of SAs in both matrices. There is only one exception, SMX, with a HQ of 7.25 for algae in WWTP effluent (corresponding to the concentration detected in WWTP 5 in the 2008 campaign). This SA also shows the highest value for algae in surface water (0.425), although below the cut off value of 1. SDM presented the lowest HQ in both matrices and for all the selected bioindicators in contrast to SMX, which usually presented the highest values. In conclusion, algae was the most sensitive taxa against SAs in both water matrices, followed by daphnids and fish. These results agree with those reported by Gros et al. (Gros et al., 2010) for different pharmaceutical classes, which included SMZ and SMX, detected in the same water courses.

5. Conclusions

The present work reports the environmental occurrence of SAs along the Ebro River basin and the potential ecotoxicological implications. The different pattern of occurrence found for the SAs in

Table 6
EC_{50} (in mg L^{-1}) used to calculate PNEC (dividing by an uncertainty factor of 1000) and hazard indexes (HQs) for fish, Daphnia and algae for those SAs with acute toxicity indexes available (García-Galán et al., 2009). Values in bold correspond to the highest indexes estimated. *: Data not available.

SA	Fish	Daphnids	Algae	HQ (river waters)			HQ (WTP effluent)		
	EC_{50}	EC_{50}	EC_{50}	Fish	Daphnids	Algae	Fish	Daphnids	Algae
SDZ	*	*	1	–	–	3.60E−03	–	–	3.20E−02
SDM	100	204.5	2.3	3.80E−05	1.86E−05	1.65E−03	3.69E−05	1.80E−05	1.60E−03
SMX	562.5	25.2	0.0268	2.03E−05	4.52E−04	**0.43**	3.46E−04	7.71E−03	**7.25**
SMZ	100	31.4	*	1.10E−04	3.50E−04	–	9.86E−05	3.14E−04	–
STZ	100	8.2	*	3.90E−05	4.76E−04	–	1.10E−04	1.34E−03	–

wastewater and river water evidenced the different sources of contamination related to urban and veterinary consumption. River waters seemed to have an additional input of SAs typically used in veterinary medicine, probably from livestock waste storage or its application in crop fields. According to the results, SAs were ubiquous in both surface and WWTP waters, and RE% estimated ranged from 4% to 100%; as the treatment carried out was the same in nearly all the WWTPs, the RE% varied mainly depending on the SA. No risks could be associated to their presence in surface waters, with the exception of SMX, whose estimated HQ equaled 7.25 in WWTP effluent and against algae. However, taking into consideration that these waters are considerably diluted once they reach the receiving waters, this risk could be lessened. Furthermore, there is an important lack of information regarding chronic toxicity of this family of antibiotics, fact that hindered the estimation of HQs for a number of the SAs studied. It should not be forgotten that the different metabolism and degradation products of SAs may also be present at different levels in the water matrices studied, which could add new environmental risk factors that should be considered in future works.

Acknowledgments

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Ecotoxicity evaluation and removal of sulfonamides and their acetylated metabolites during conventional wastewater treatment

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HIGHLIGHTS

- The removal of selected sulfonamides was studied in 22 wastewater treatment plants.
- Five different acetylated metabolites were detected in both water matrices and sewage sludge.
- Hydraulic retention times and removals of sulfonamides were not correlated.
- EC_{50} was calculated for N^4-acetylsulfapyridine and sulfapyridine for the first time.
- N^4-acetylsulfapyridine was more toxic than its parent drug, sulfapyridine.

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ABSTRACT

The present study describes the evaluation of the risk posed by the occurrence of sulfonamides (SAs) in wastewaters. A fully automated analytical method based on on-line solid-phase extraction-liquid chromatography–tandem mass spectrometry (on line SPE-LC–MS/MS) was developed, validated and applied for the analysis of sixteen SAs and, for the first time in wastewaters and sewage sludge, five of their acetylated metabolites. Influent and effluent samples from twenty two different wastewater treatment plants (WWTPs) in Catalonia (Spain) and sewage sludge from fifteen of them were analyzed; removal rates (RE%) and half-lives ($t_{1/2}$) for each SA were calculated. The estimated correlations between RE% and to the hydraulic retention time (HRT) of the different plants indicated no clear influence of HRT on removals. Sulfamethoxazole (SMX), sulfapyridine (SPY) and their corresponding acetylated metabolites were detected with the highest frequencies of detection and at the highest concentrations. The ecotoxicity of both SPY and AcSPY was evaluated for the first time through bioluminescent inhibition assays, resulting in a higher toxicity being attributed to the metabolite. Finally, the potential environmental risk posed by the levels of SAs detected was evaluated calculating the hazard quotients (HQ) to different non-target aquatic organisms in treated wastewaters. SMX was the only SA posing a risk to algae, with an HQ > 10.

1. Introduction

Awareness of the presence of pharmaceuticals in wastewaters and aquatic ecosystems is growing as investigations regarding new pollutants increase and analytical techniques for detecting these chemicals improve. Antibiotics could be considered as pharmaceuticals of priority concern since they are produced in large quantities, are biologically very active and many of them resist biodegradation and can bioaccumulate (Jones et al., 2005; Kümmerer, 2009). Sulfonamides (SAs) are one of the most widely used antibiotics in humans and especially in animal husbandry and fish farming (Chafer-Pericas et al., 2010; Hamscher et al., 2006). However, although veterinary antibiotics such as SAs would be expected to reach wastewater treatment plants (WWTPs) only to a limited extent, they have been detected often in influent and most importantly, in effluent wastewaters (García-Galán et al., 2011; Gobel et al., 2007; Gros et al., 2007; Ye et al., 2007) together with SAs of human use, which are predominant in these matrices. Data on removal efficiencies (RE%) of SAs during wastewater treatment are still quite scarce, and elimination rates reported so far are usually low (Chang et al., 2008; García-Galán et al., 2011; Gobel et al., 2007; Gros et al., 2007).

In general, Spanish WWTPs apply primary and secondary treatments, the latter usually based on conventional activated sludge.

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Tertiary treatments such as ozonation, which have demonstrated to be highly efficient in the removal of different pharmaceuticals including SAs, are seldom applied (Garoma et al., 2010; Le-Minh et al., 2010; Lin et al., 2009). As these effluents commonly discharge into natural water courses, river basins and catchment areas are directly exposed to SAs contamination amongst others. Furthermore, biosolids from WWTPs can also be applied as organic amendment in agriculture (Topp et al., 2008), representing one of the major routes by which SAs may enter the soil ecosystems and eventually the different natural waters.

In this context, it is superlative to consider the potential risks derived of the environmental presence of SAs. Up to date, data on SAs toxicity is scarce and based mainly on acute toxicity tests which hardly represent environmental real conditions (Boxall et al., 2002; Ferrari et al., 2004; Kim et al., 2007; Migliore et al., 1993; Park and Choi, 2008) Regarding metabolites, only one work by Eguchi et al. (2004) provided toxicity endpoint values for N^4-acetylsulfamethoxazole (AcSMX), N^4-acetylsulfadiazine (AcSDZ) and N^4-acetylsulfadimethoxine (AcSDM). Due to this lack of information, the risk assessment guidelines set up by the European Medicines Agency (EMEA) for the marketing authorization of new medicinal products are frequently used to evaluate environmental risk from drugs that are already consumed and entering the aquatic or terrestrial environment (Ferrari et al., 2004; Ginebreda et al., 2010; Gros et al., 2010; Park and Choi, 2008). These guidelines consider that concentrations of the investigated drugs higher than 10 ng L^{-1} in surface water should lead to a further toxicity evaluation in order to estimate their effects on different non-target organisms upon exposure (predicted no-effect concentration (PNEC) or non-observed effect concentration (NOEC)). It is also recommended that, when the total concentration of metabolites is 10% greater than the concentration of the corresponding parent drug, the metabolites are also to be further investigated (phase II tier B) in order to determine their ecotoxicological effects. Risk evaluation is based on the calculation of hazard quotients (HQs), which estimate the potential adverse effects of the drug concentration detected in the studied environmental matrix, on non target organisms. This quotient is usually calculated as the ratio between the predicted environmental concentration (PEC) and PNEC. HQ>1 implies a potential environmental impact which should be investigated in further detail.

In this study, a new analytical methodology based on on-line solid phase extraction liquid chromatography tandem mass spectrometry (on-line SPE-LC–MS/MS) was optimized and applied to investigate the presence of 16 SAs and 5 of their acetylated metabolites in influent and effluent waters and sewage sludge of several WWTPs located in Catalonia (Spain), focusing in their removal and overall mass balance during treatment. The detected concentrations were used to estimate the environmental risk posed by SAs and finally, the 50% effective concentrations (EC_{50}) for the two most frequently found compounds, SPY and AcSPY, were calculated for the first time.

2. Experimental

2.1. Chemicals and materials

The 5 acetylated metabolites, N^4-acetylsulfadiazine (AcSDZ), N^4-acetylsulfapyridine (AcSPY), N^4-acetylsulfamethazine (AcSMZ), N^4-acetylsulfamethoxazole (AcSMX), N^4-acetylsulfamerazine (AcSMR) and the isotopically labeled internal standards d_4-sulfathiazole, d_4-sulfamethoxazole, d_4-sulfamethazine and d_5-sulfadiazine were purchased from Toronto Research Chemicals (Ontario, Canada). Dimethyl sulfoxide (DMSO) and high purity standards (>99%) of the remaining acetylated metabolite, N^4-acetylsulfamethazine (AcSMZ), and the 16 selected SAs, namely sulfadiazine (SDZ), sulfadimethoxine (SDM), sulfamethazine (SMZ), sulfamethizole (SMT), sulfamethoxazole (SMX), sulfamethoxypyridazine (SMP), sulfapyridine (SPY), sulfisoxazole (SSX), sulfathiazole (STZ), sulfisomidin (SSD), sulfanitran (SNT), sulfamerazine (SMR), sulfaquinoxaline (SQX), sulfadoxine (SDX), succinyl-sulfathiazole (SuSTZ) and sulfabenzamide (SBZ), were purchased from Sigma (St. Louis, MO, USA). HPLC-grade solvents (water, methanol (MeOH), acetone and acetonitrile) and formic acid (HCOOH) (98–100%) were supplied by Merck (Darmstadt, Germany). Stock standard solutions of each of the analytes were prepared in MeOH at 1 mg mL^{-1} and stored at −2 °C until use. Standard solutions of the mixtures of all compounds were prepared at appropriate concentrations by the corresponding dilution of the stock solutions in MeOH. The standard mixtures were used as spiking solutions for preparation of the aqueous calibration standards, which always contained <0.1% of MeOH.

For the inhibition assays, freeze dried *Vibrio fischerii* bacteria, cultivation media and NaCl 20% solution were obtained from microLAN (Waalwijk, Nederlands). Stock solutions of SPY and AcSPY were prepared in HPLC water at 160 mg L^{-1} and 48 mg L^{-1}, respectively. To enhance the solubility of the two compounds 0.4% DMSO was added. Previous control experiments (Hernando et al., 2007) and control experiments carried out in this study with 0.4% DMSO in HPLC water exhibited no observable toxic effects in TOXcontrol assays.

2.2. Sampling area and sampling collection

Influent and effluent wastewater samples were collected from 22 full-scale WWTPs located in Catalonia (Spain) during 2009. Table S1 in the Supporting Information (SI) summarizes their functional characteristics. The water treatment applied in all the WWTPs was similar and consisted of sedimentation (primary settler) followed by biological treatment with P and/or N removal. Sludge from the primary and secondary settlers was thickened by gravity, digested in anaerobic conditions and finally dewatered (centrifuged). Only three of the WWTPs applied also a tertiary treatment (microfiltration and chlorination). Populations served ranged from 42,076 inhabitants (W5) to 1,444,884 inhabitants (W10), with average daily inflows of 13,500 m^3 day^{-1} and 50,000 m^3 day^{-1}, respectively (http://aca-web.gencat.cat). The main differences, however, resided in the hydraulic retention times (HRTs) and solid retention times (SRTs) of each WWTP, ranging from 4 h (W14) to 40 days (W5) for HRTs and from 4 days (W17) to 17 days (W3) for SRTs.

Wastewater and sludge samples were taken in June 2009, during the dry weather period. 24 h composite influent and effluent samples were collected in amber glass bottles and transported under cool conditions. Upon reception, all water samples were filtered through 0.45 μm Nylon filters (Whatman, Maidstone, UK) to eliminate suspended solid matter and then frozen until analysis (within the following week).

Dehydrated sewage sludge was collected in 15 of the 22 WWTPs. Sludge samples were transported also in cool conditions, freeze-dried upon arrival (−50 °C, 0.044 bar vacuum) and kept at −30 °C until analysis.

2.3. Samples pre-treatment and analysis

2.3.1. Water samples. On-line SPE-LC–MS/MS analysis

Fully automated on-line preconcentration and purification of aqueous samples, aqueous standards and blanks was performed by means of a Symbiosis™ Pico System (Spark Holland, Emmen, The Netherlands). This automated system consisted of three integrated parts: an HPLC system with an Alias™ autosampler, a loop injector and two binary pumps with a four-channel solvent selector for each one; an automated cartridge exchange (ACE) module, consisting of two clamps which hold two trays of 96 extraction cartridges each, and a high pressure dispenser module (HPD), that provides solvents for SPE conditioning, equilibration and clean up by means of a 2 mL high pressure syringe. Briefly, after conditioning of the cartridge, the sample is transferred and loaded from the autosampler loop injector onto the conditioned cartridge, where the target analytes will be retained. The loaded cartridge is then washed and transferred to the

elution clamp where the analytes will be eluted from the SPE cartridge directly onto the LC column. During elution, a new cartridge is simultaneously placed in the conditioning clamp and undergoes conditioning, loading and washing. Therefore, SPE is carried out entirely in parallel with the LC–MS/MS run, shortening the cycle times.

2.3.1.1. On-line trace enrichment optimization. On-line Oasis HLB cartridges were employed for the extraction of the samples, as it has been demonstrated in previous studies that these yielded the highest recovery rates for SAs (García-Galán et al., 2011; García-Galán et al., 2010). The optimal conditioning of the cartridges was obtained adjusting the elution solvent composition, cartridge washing, clump washing and sample volume loaded. Conditioning finally consisted of 1 mL of MeOH, 1 mL of acetone and 1 mL of HPLC water with 5% MeOH, loaded at a rate of 5 mL min^{-1}. In order to optimize the load volume, influent and effluent wastewater samples were spiked with a mixture of the analytes at a concentration of 100 ng L^{-1} and volumes of 1 mL, 2.5 mL and 5 mL were loaded onto the cartridges at 1 mL min^{-1}. The washing step was carried out with 1 mL of HPLC water and the clamp flush was carried out with 1 mL of ACN followed by 1 mL of MeOH and 1 mL of HPLC water.

2.3.2. Sludge samples

Sludge samples were extracted by pressurized liquid extraction (PLE) using an accelerated solvent extractor (ASE) 300 (Dionex, Sunnyvale, CA) with a water–ACN mixture (75:25, v:v) at 50 °C. Grinded sludge (2 g) was mixed with Hydromatrix dispersing agent and placed within 11 mL stainless steel cells. A mixture of the deuterated standards was added to the weighted samples as surrogates at a concentration of 100 ng g^{-1}, and samples were left overnight. Extraction was carried out at a pressure of 1500 psi and at a temperature of 50 °C, using as extraction solvent a mixture of HPLC water/ACN (75/25, v/v). The extracts were diluted to 200 mL HPLC water to reduce the proportion of organic solvent content, and then were pre-concentrated and purified following the off-line SPE protocol previously described (Díaz-Cruz et al., 2008). The optimization of this extraction method is given elsewhere (unpublished results).

2.4. Instrumental analysis

Chromatographic separation was performed using an Atlantis C_{18} (Waters, 150 mm × 2.1 mm, 3 μm particle size) LC-column preceded by a guard column with the same packing material. SAs were analyzed in the positive ionization mode (PI). The flow rate was set to 0.2 mL min^{-1}, with eluent A being HPLC water acidified with 10 mM of HCOOH, and eluent B being ACN with 10 mM HCOOH. The elution gradient started with 25% of eluent B, increasing to 80% in 10 min and to 100% in 11 min. During the next 2 min the column was cleaned, readjusted to the initial conditions in 3 min and equilibrated for 7 min. MS/MS analyses were carried out in a 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbospray electrospray (ESI) source working in the PI mode. The optimization of the MS/MS experimental conditions was performed in a previous study (Díaz-Cruz et al., 2008). For increased sensitivity and selectivity, MS/MS data acquisition was performed in the selected reaction monitoring (SRM) mode, selecting the two most abundant product ions for each of the precursor ions.

2.5. Ecotoxicity evaluation

The determination of the EC_{50} for SPY and AcSPY was performed using TOXcontrol (microLAN, Waalwijk, Nederlands), which allowed the procedure indicated in EU-EN ISO 11348 (EU, 2007) to be fully automated. The system is prepared to work with a variety of *Vibrio fischeri* bacteria that only need to be changed once a week. The freeze dried bacteria needs to be cultivated for at least 5 days in a separate unit called TOXBioshaker, (microLAN, Waalwijk, Nederlands), for automated and controlled cultivation before starting the measures in TOXcontrol. Then the freshly cultivated light emitting bacteria are placed in the bacteria storage chamber, where they are maintained at 5 °C for 1 week.

The total period of the measurements was 25 min, including 15 min of incubation time. It started with the mixing of 50 μL of bacteria with 2% NaCl during an adaptation time of 5 min to bring the bacteria from 5 to 15 °C. NaCl was needed to adjust the osmolarity of the samples. Then the bacteria were mixed with a known concentration of the selected SA for a contact time of 15 min. Increasing volumes (from 0 to 4.5 mL) of SPY at 160 mg L^{-1} and AcSPY at 48 mg L^{-1} were diluted in the mixture of bacteria in 2% NaCl till a total volume of 10 mL. All analyses were done in triplicate. At time 0 and during the incubation time the luminescence was measured every minute, so not only information at the beginning and at the end of the luminescence measurement, but also data about the changes of the measurement of sample and reference sample were obtained. Luminescence was measured with a photomultiplier placed into a light-closed housing.

At the same time, a parallel "blank" measure was done with reference water to correct the possible changes occurred during the incubation period. A Correction Factor (CF) is calculated based on the change of the luminiscence intensity in the reference blank. According to the ISO regulation, the test is valid when CF is between 0.6 and 1.3. The final luminescence intensity of the sample was corrected by this factor. At the end of the measurement, the sample chambers were rinsed with reference water, and the inhibition in accordance with the ISO regulation was calculated.

3. Results and discussion

3.1. Method validation

The analytical method was evaluated in terms of accuracy, calibration range and linearity, instrumental variation, sensitivity and selectivity.

The recovery values obtained are shown in Fig. 1, and were calculated as the ratio between the peak areas obtained from the on-line analysis of a spiked water sample and the peak areas obtained from a parallel off-line analysis of a standard mixture at the same concentration prepared in the chromatographic gradient initial conditions; the same total mass was injected in the mass analyzer in both cases. Loads of 5 mL, 2.5 mL and 1 mL were tested to select the optimum extraction volume. As can be observed in Fig. 1, an extraction volume load of 5 mL yielded the best recoveries for most of the SAs studied in effluent water, whereas volumes of 2.5 mL gave the best results for influent waters.

Matrix matched calibration curves were built from the injection of seven aqueous standard mixtures of the analytes ranging from 0.01 ng L^{-1} to 500 ng L^{-1} and following a least-square linear regression analysis. Linearity was given as the regression coefficient (r^2) and was always equal or above 0.9992 (see SI, Table S2). Quantitation was performed based on the internal standard approach, in order to correct potential matrix effects and losses during the on-line SPE procedure. The deuterated compounds were added as internal standards before extraction to all the samples and aqueous standards of the calibration curve at a concentration of 100 ng L^{-1}.

Instrumental variation, expressed as the relative standard deviation (RSD), ranged from 2% to 13% for influent water, and from 1% to 8% in effluent water (see SI, Table S2).

Method limits of detection and quantification (LODs and LOQs, respectively) were calculated as the minimum detectable amount of analyte with a signal-to-noise ratio of 3 and 10, respectively, in the different samples analyzed. LOD values were in the range of 0.01 ng L^{-1}

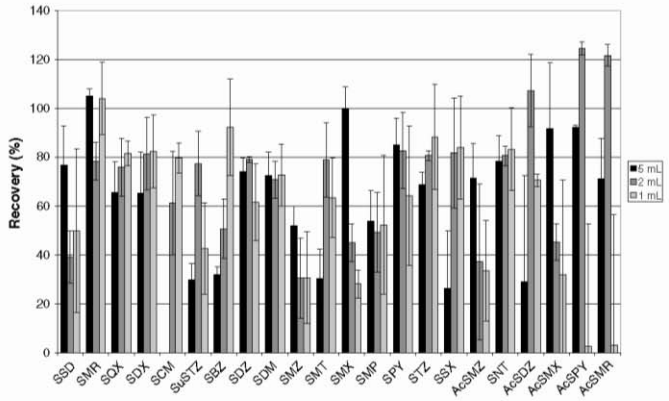

Fig. 1. Optimization of the volume load on the SPE cartridges of the two water matrices (5 mL, 2 mL and 1 mL). Recovery values obtained for each of the SAs studied.

(SuSTZ and SBZ) to 2.35 ng L^{-1} (SMZ) for influent water, and in the range of 0.01 ng L^{-1} (SDM) to 1.38 ng L^{-1} (SDZ) for effluent water.

Last of all, the selectivity of the method was evaluated following the requirements of the European Commission Decision 2002/657/EC (Decission, 2002) regarding a minimum of three identification points (IPs) for positive identification of the analytes and retention time variations.

3.2. Occurrence of SAs in wastewater and sludge

The total loads of SAs in the influent and effluent samples were calculated for each WWTP following Eq. (1):

$$TL_w = 1000 \times \frac{V \times \sum C_w}{inhab} \quad (1)$$

where TL_w is the influent or effluent total load (g day^{-1} 1000 $inhabitants^{-1}$); V is the water flow rate per day (L day^{-1}), C_w is the sum of all the SAs concentrations detected in the influent or effluent samples of each WWTP (g L^{-1}) and inhab is the population served by the corresponding WWTP. The calculated values are shown in Fig. 2. The highest influent loads corresponded to big populations in the case of W8 and W20, but also high loads were estimated in W5, where the population served was the smallest of all, and in W15, average within the population spectrum studied (see Table S1). This denoted either a higher consumption of SAs in these urban areas or most probably the contribution of not urban inputs such as runoff from animal feeding operations or agricultural fields nearby in which biosolids have been applied. Effluent loads were higher than influent loads only in 2 of the WWTPs, and in the other 3, SAs were present only in the effluents.

Table 1 summarizes the frequencies of detection and concentration ranges of the individual SAs detected in the wastewater and

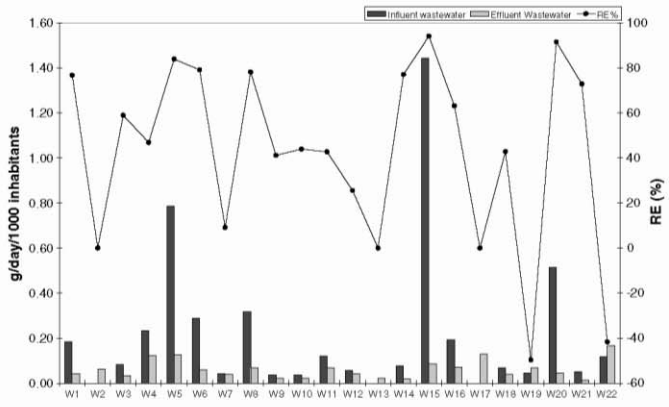

Fig. 2. Influent and effluent loads of SAs detected in each of the WWTPs studied, and water removals estimated (RE%).

Table 1
Frequencies of detection and concentration ranges in each matrix for all the SAs studied and aqueous removals and overall removals (RE% and $RE\%_{total}$) estimated. *: only one value detected.

	Frequencies of detection (%)			Concentration range					
	Influent	Effluent	Sludge	Influent (ng/L)	Effluent (ng/L)	Sludge (ng/g)	Aqueous removal (%)	Overall removal (%)	Removal overestimation (%)
SSD	0	18	20	–	1.7–6.52	0.06–0.6	–	–	
SMR	14	18	20	2.13–7.37	0.205–1.93	0.2–0.5	99.1	99.0	0.07
SQX	14	45	20	3.81–210	0.246–14.6	0.25–13.25	63.8	51.9	11.94
SDX	18	9	0	1	0.1–0.47	–	100*	100*	–
SuSTZ	0	23	13	–	1.65–107	0.35–0.46	–	–	–
SBZ	14	14	40	0.4–1.17	29.9*	0.09–2.5	100.0	100.0	–
SDZ	27	41	20	49.1–1240	8.75–286	0.38–14.875	93.0	93.0	–
SDM	41	50	33	0.35–4.92	0.08–1.58	0.037–0.3	74.0	74.0	–
SMZ	23	32	40	3.36–145	0.458–36.4	0.4–22.975	87.5	81.5	5.93
SMT	18	41	7	2.92–33.7	1.06–70.7	0.042*	69.0	69.0	–
SMX	86	95	7	17.4–665	10.8–284	0.108*	64.8	64.8	–
SMP	14	27	20	39.6–518	0.223–47	0.0675–12.85	87.5	87.0	0.49
SPY	82	100	60	12.2–3270	3.35–177	0.1975–37.5	72.1	71.9	0.25
STZ	50	73	87	7.31–142	0.7–73	1–14.95	71.0	58.5	12.55
SSX	27	41	0	0.607–2.86	0.3–1.18	–	70.4	70.4	–
AcSMZ	9	23	0	8.6–18.2	0.43–16.4	–	100*	100*	–
SNT	14	18	13	32.1–62.3	17–512	1.85–39.25	100.0	100.0	–
AcSPY	68	100	13	1.71–244	10.1–522	0.09–0.35	45.8	45.2	0.64
AcSDZ	14	27	20	8.85–28.2	3.23–67.4	0.10–0.15	86.5	86.5	–
AcSMX	64	73	20	24.9–412	4.63–94.6	0.4–1.1	84.2	84.2	–
AcSMR	18	5	7	16.8*	–	0.07*	100.0	99.9	0.08

sludge samples investigated. SAs were ubiquitous in the 22 WWTPs studied, but SMX and SPY were markedly the two SAs detected most frequently in both influent and effluent wastewaters, followed by their corresponding acetylated metabolites and STZ. Both SMX and SPY are the SAs most commonly applied in human therapies and therefore, the most ubiquitous in urban wastewaters, together with their transformation products (García-Galán et al., 2011; Gobel et al., 2004; Pedrouzo et al., 2011). The highest contributions to the total SAs loads in both WWTP influents and effluents also corresponded to both compounds and their metabolites in the majority of the WWTPs, although the presence of SDZ and SMZ was also remarkable (Fig. 3). SDZ is used mainly in veterinary therapies, but also as dermatologic treatment for skin burns. However, SMZ is nearly exclusively of veterinary use, so its presence can only be justified by diffuse contamination from confined animal feeding operations or the presence of agricultural fields where either contaminated manure or biosolids are applied. The maximum concentrations were detected in the influent wastewater and corresponded to SPY (3270 ng L^{-1}) and SDZ (1240 ng L^{-1}). As observed, in several occasions concentrations detected in the effluents were higher than in the influents for SMX, SPY and some other SAs. This event has been already discussed in different studies, and is usually attributed to the presence of conjugate compounds which revert back to their original compound during treatment (Garcia-Galan et al., 2012; Göbel et al., 2007). Average aqueous removals of 84% and 46% (see Section 3.3) after the water treatment were calculated for AcSMX and AcSPY respectively; this elimination of both acetylated compounds could partly consist of their deconjugation, yielding the parent compounds and contributing to the increase of the SMX and SPY concentrations in the effluents. Likewise, in some of the WWTPs studied, the acetylated metabolites were present more frequently and in higher amounts in the effluent wastewaters than in the influents, which could be attributed to the biodegradation and/or acetylation of the parent compounds during biologic treatment.

It should also be considered that influent and effluent samples were taken simultaneously as composite samples throughout 24 h. Depending on the HRT of the WWTP, effluent waters investigated at the end of the treatment process did not fully correspond to the influent wastewaters sampled, and this could also explain negative removals or the presence of SAs only in effluent wastewaters. WWTPs also work as continuous systems and, therefore, can be subjected to oscillations in both water and sludge treatment lines, and different parameters such as inflow or HRT do not necessarily remain invariable all the time and could also contribute to these variations in the concentrations detected.

Sewage sludge daily loads of SAs for the 15 WWTPs investigated were never higher than 1.1 g day^{-1}. Nevertheless, STZ, SPY and SMZ were the SAs detected more frequently in the sludge samples, and the highest concentrations detected corresponded also to SPY and SMZ, and to SNT (37, 23 and 39 ng g^{-1}, respectively). Despite its high occurrence in water influents and effluents, SMX was detected only in one sample of sludge (0.108 ng g^{-1}). Regarding the acetylated metabolites, the maximum concentration corresponded to AcSMX (1.1 ng g^{-1}), which was actually higher than that of the parent drug.

3.3. Removal of SAs from the influent water

Whereas the loads detected in the influent waters give a valuable information on the consumption rate of SAs in the area of study, loads detected in both effluent waters and also in sludge samples are the most environmentally relevant, as they will enter the environment on a regular basis when those effluents are discharged onto surface waters or when treated sewage sludge (biosolids) is applied in agricultural field as nutrient amendment. Therefore, the estimation of the SAs removal during treatment yields valuable information on the environmental impact of the effluent.

Removal of contaminants during WWTP treatment comprises mainly biodegradation and adsorption, although it also depends on the nature of the pollutant. (i.e. photodegradation can also play a relevant role, whenever the water is exposed to sunlight, i.e. clarifiers). Concerning SAs, distribution coefficient (K_d) values found in the literature are usually low (K_d<500 L kg^{-1}) and they are considered to be eliminated by less than 10% through adsorption (Ternes et al., 2004). However, recent studies have demonstrated that adsorption should not be completely neglected, as the aromatic amine common to all SAs is likely to covalently cross-couple to natural organic matter, diminishing their presence in environmental water matrices and also reducing their bioavailability and biological activity (Bialk et al., 2005). Air stripping and volatilization in the

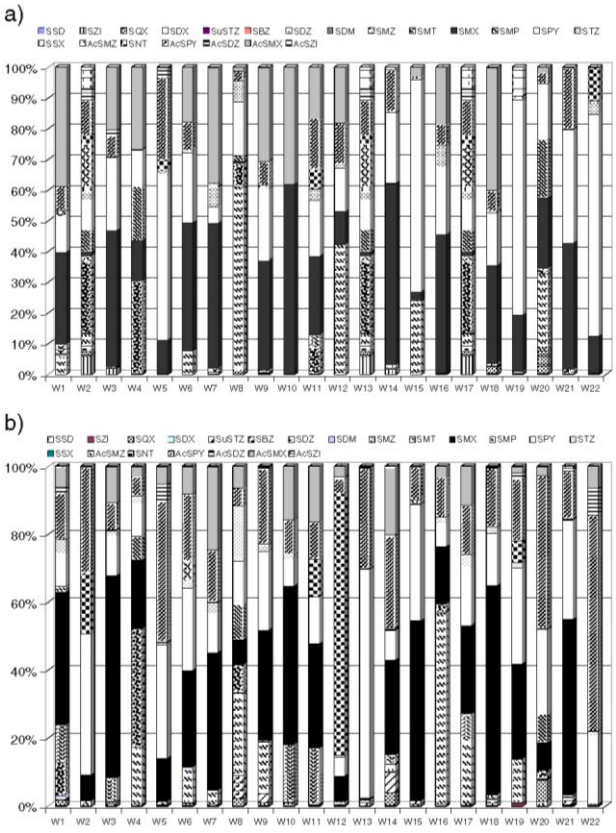

Fig. 3. Cumulative levels (ng L^{-1}) of SAs in the different WWTPs investigated. (a) Influent wastewater; (b) effluent wastewater.

different biological reactors were neglected, as SAs are polar substances with very low Henry constants (Li and Zhang, 2010).

Although WWTP performance parameters are hardly fully homogeneous as mentioned in Section 3.1, RE% were calculated using a mass balance throughout the water treatment. Assuming that wastewater inflow and outflow rates remain the same in all WWTPs, influent and effluent daily loads (g day^{-1}) of each SA were calculated as follows:

$$L_w = V \times C_w \tag{2}$$

where L_w is the influent or effluent daily load (g day^{-1}). In this occasion, the population served was not included in the calculation. The water removal (RE%) in the water matrix of each individual SA was calculated using Eq. (3):

$$RE\% = 100 \times \left(1 - \frac{L_{inf}}{L_{eff}}\right) \tag{3}$$

where L_{inf} and L_{eff} are the loads estimated in the influent and effluent samples, respectively. Similarly, SAs daily loads in the sludge (g day^{-1}) were calculated applying Eq. (4):

$$L_{sludge} = M_{sludge} \times C_{sludge} \tag{4}$$

where M_{sludge} is the amount of sludge produced in the WWTP (g day^{-1}) and C_{sludge} is the concentration detected in the sludge sample. In order to obtain a more accurate calculation of the SA's biological transformation during treatment, Eq. (3) should include the amount of SA retained in the sludge, L_{sludge}, and therefore:

$$RE\%total = 100 \times \left(1 - \frac{L_{eff} + L_{sludge}}{L_{inf}}\right). \tag{5}$$

RE% values in water for the individual SAs are shown in Fig. 4. RE% values could not be established for all SAs in all the 22 WWTPs, as they were seldom detected or were present only in effluent waters. Values of 100% removal were assumed when the SA was

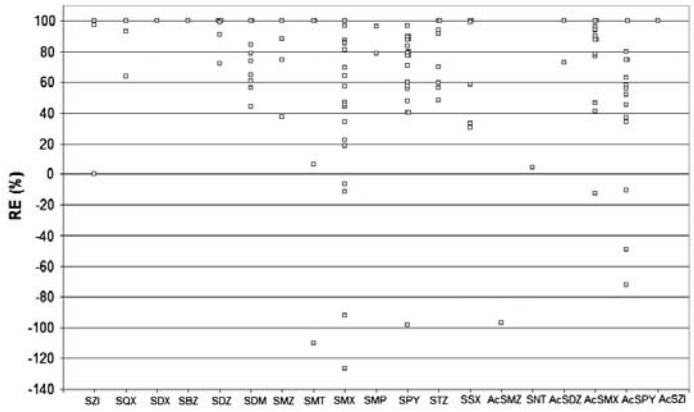

Fig. 4. Aqueous removals (RE%) obtained for each SA in all the WWTPs.

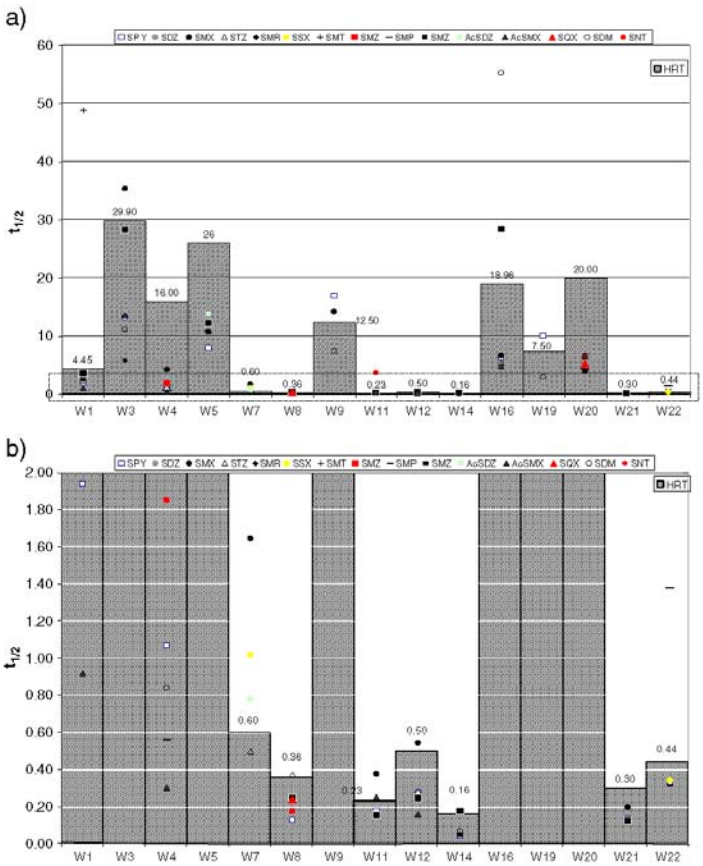

Fig. 5. a) Hydraulic retention times (RTs, days) and half lives ($t_{1/2}$, days) for each of the WWTPs. b) Zoomed figure for HRTs between 0 and 2 days.

Table 2
EC_{50} (in mg L^{-1}) used to calculate PNEC (dividing by an uncertainty factor of 1000) and hazard indexes (HQs) for *V. fischerii*, Daphnia, fish and algae for those SAs with acute toxicity indexes available. Values in bold correspond to the EC_{50} values estimated in this work.

SA	MEC (ng L^{-1})	Taxa	EC50 (mg L^{-1})	HQ	Ref
SMX	284	Blue green algae	0.027	**10.52**	Ferrari et al., 2004
		V. fischerii	78.1	3.64E−03	Boxall et al., 2002
		Fish	562.5	5.05E−04	Kim et al., 2007
		Daphnids	25.2	1.13E−02	Lutzhoft et al., 1999
AcSMX	94.6	Blue green algae	101	9.37E−04	Eguchi et al., 2004
SPY	177	*V. fischerii*	**27.4**	6.46E−03	–
ACSPY	522	*V. fischerii*	**8.2**	6.37E−02	–
SMZ	36.4	*V. fischerii*	303	1.20E−04	Yang et al., 2008
		Fish	101	3.60E−04	Kim et al., 2007
		Daphnids	147.5	2.47E−04	Migliore et al., 1993
STZ	73	Blue green algae	16.32	4.47E−03	Migliore et al., 1993
		V. fischerii	1001	7.29E−05	Boxall et al., 2002
		Daphnids	78.9	9.25E−04	Migliore et al., 1993
		Invertebrates	391.1	1.87E−04	Park and Choi, 2008
		Fish	101	7.23E−04	Migliore et al., 1993
SDZ	286	Blue green algae	1.225	**0.23**	Migliore et al., 1993
AcSDZ	67.4	Blue green algae	101	6.67E−04	Eguchi et al., 2004
SDM	1.58	Blue green algae	2.3	6.87E−04	Eguchi et al., 2004
		V. fischerii	501	3.15E−06	Boxall et al., 2002
		Daphnids	204.5	7.73E−06	Boxall et al., 2002
		Invertebrates	19.5	8.10E−05	Migliore et al., 1993
		Fish	101	1.56E−05	Kim et al., 2007

H.C.H. Lutzhoft, B. Halling-Sorensen, S.E. Jorgensen, Arch. Environ. Contam. Toxicol. 36 (1999) 1.
L.H. Yang, G.G. Ying, H.C. Su, J.L. Stauber, M.S. Adams, M.T. Binet, Environ. Toxicol. Chem. 27 (2008) 1201.

detected only in the influent water. General statements are hard to establish as RE% values were distributed both above and below 50%. For SMX, RE% values were higher than 50% only in 9 WWTPs. On the contrary, its acetylated metabolite was eliminated in rates higher than 75% in 8 of the 12 WWTPs where it was detected. Better RE% were obtained for SPY than for SMX and rates estimated were higher than 50% in 14 of the 19 WWTPs (10 of them higher than 75%). AcSPY was removed to a lesser extent than AcSMX, with average RE% near 50%.

To have a more complete picture of the persistence of SAs in wastewaters, half-lives ($t_{1/2}$) were estimated for each SA in each WWTP. Similar to what happens with many drugs in the human body after intake, pseudo-first order kinetics was assumed, (decrease of the concentration through time proportional to the concentration remaining in the matrix). Half-lives were estimated following the equation:

$$t1/2 = \frac{\mathrm{Ln}2}{k} \quad (6)$$

where k is the rate loss constant and was calculated for each SA following the pseudo-first order kinetics equation:

$$\mathrm{Ln}\frac{C_{eff}}{C_{inf}} = -kt. \quad (7)$$

As C_{eff} is the final concentration of the drug after treatment, the corresponding HRTs are used as t in the equation. The estimated $t_{1/2}$ values are shown in Fig. 5. No average values are given due to the huge variability of the results. For instance, SMX yielded $t_{1/2}$ values going from 0.05 to 35.3 days in the different WWTPs. As observed, some SAs have $t_{1/2}$ higher than the HRT of the corresponding WWTP, meaning that they are being discharged from the WWTP onto receiving waters at concentrations higher than 50% of the input. This is the case of SMX in four of the WWTPs: W7, W11 and W12, which had HRT shorter than 1 day, and W9, which had a HRT of 12.5 days. On the contrary, in those WWTPs with low HRTs most of the SAs detected showed shorter $t_{1/2}$ than HRT and therefore were removed by 50–100% before discharge. Up to date, the influence of HRT on the RE% of SAs during biological treatment is not well defined; some authors state that higher HRTs usually mean higher RE% for many pharmaceuticals, (including SMX) (Gros et al., 2007), whereas others found no clear correlation between HRT, sludge age or temperature and RE% of SMX (Joss et al., 2005), considering that HRT is not a determinant parameter for the interpretation of results. In the present work, no positive correlation was obtained when calculating the Pearson's correlation factor ($P<0.0001$; $r=0.17$) between the HRTs of the 22 WWTPs and the RE% obtained. Likewise, no clear correlation could be established either between the elimination of SAs and the SRT of the different WWTPs, and SRTs varying from to 4 to 17 days did not show a significant influence on RE% values obtained. This data may indicate that these design parameters of the reactor do not play a significant role in the degradation of the SAs, and that other factors such as influent loads or the physico-chemical properties of the drug may be more relevant (Göbel et al., 2007). For instance, the highest individual RE% for SMX and SPY were registered in W20, which had an SRT of only 6 days but presented high loads of these drugs in the influent wastewater.

For the 15 WWTPs where sewage sludge was sampled, RE% values and total RE% values (Eqs. (5) and (7)) are given and compared, and the average values are given in Table 2. Only small variations were observed and RE% overestimations due to adsorption in the sludge were usually below 5%, with the exception of SQX and STZ, where RE% in water could be overestimated to 12%. In any case, as shown in Table S2, those values are in the range of the analytical error of the method, given as RSD, and could be neglected. Therefore, it could be concluded that SAs' adsorption to the sludge did not play a relevant role in their elimination along the different WWTPs. Mineralization assays have not been

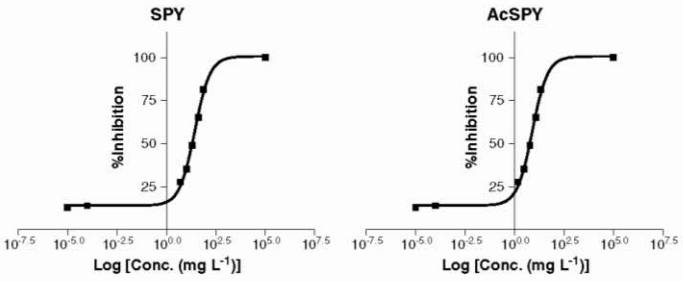

Fig. 6. *Vibrio fischerii* inhibition curves for SPY and AcSPY after 15 min of exposure. The percentage of inhibition is shown vs. the logarithm of the concentration, given in mg L^{-1}.

performed in this study and consequently, full mineralization of the SAs cannot be assured when RE% values of 100% were obtained.

3.4. Ecotoxicity evaluation for sulfapyridine and its acetylated metabolite

The percentage of *V. fischerii* inhibition was calculated from the results obtained with the different solutions of SPY and AcSPY. A sigmoidal curve was fitted and the EC_{50} was calculated with the help of the Prism 4 software (GraphPad Software Inc.) (Fig. 6). EC_{50} is one of the most commonly used assessment factor for the risk of toxicity, indicating the amount of drug, in a single dose, which turns out to be lethal for the 50% of the exposed population of a given species.

EC_{50} was observed for concentrations of 27.4 mg L^{-1} and of 8.2 mg L^{-1} for SPY and ACSPY respectively after 15 min exposure. According to the EU legislation (Directive 93/67/EEC) that categorizes the toxicity to aquatic organisms depending on the EC_{50}, SPY would be classified as harmful, and Ac-SPY as toxic. To the author's knowledge, the only reference regarding harmful effects of acetylated SAs is that by Eguchi et al. (2004), in which the metabolites showed much weaker growth inhibitory effects than the corresponding parent SA against microalgae, usually the more sensitive taxa. In our study, however, *V. fischerii* was found to be more sensitive to the presence of the metabolite than to the original drug.

4. Environmental risk assessment

When the parameters required for the calculation of HQ (PEC and PNEC) are not available, alternative PNECs can be derived by dividing EC_{50} or LC_{50} values found in the literature (50% effect concentration or 50% lethal concentration, respectively) by an uncertainty factor of up to 1000 (Sanderson et al., 2003), in order to make these acute toxicity values more representative of the real situation in the natural media (longer periods of exposure). Likewise, measured environmental concentrations (MECs) are used in the calculation instead of PECs (García-Galán et al., 2011; Ginebreda et al., 2010; Gros et al., 2010; Santos et al., 2007). In order to set up a worst case scenario, MEC values used corresponded to the maximum values detected in this study whereas EC_{50}–LC_{50} values used were the lowest found in the literature. HQs were calculated only for the SA concentrations detected in the wastewater effluents, as they eventually discharge onto surface waters and enter the natural media. Calculations were subjected to the availability of the toxicity data, which is still quite scarce regarding SAs, and HQs could be calculated for only 9 of the 16 SAs investigated. As shown in Table 2, maximum MECs were higher than the boundary value of 10 ng L^{-1} established by EMEA in all cases, and the concentration of the corresponding acetylated metabolites was >10% of the parent concentration. Different trophic levels of the aquatic ecosystems could be covered and HQs were estimated for blue-green algae, the aquatic bacteria *V. fischerii*, invertebrates, daphnids and fish. The EC_{50} values calculated in Section 3.4 were used. HQ values were far below the boundary of 1 established by the EMEA guidelines, indicating that no risk could be derived from the presence of SAs in the effluent wastewaters. The only exception was SMX against blue-green algae. SMX concentrations from 284 ng L^{-1} (HQ = 10.5) down to 30 ng L^{-1} (HQ = 1.1) would be in the toxicity range for this Taxa. Daphnids and *V. fischerii* were the other most sensitivity taxa against SMX, the degree of susceptibility resulting in algae > daphnia > fish, although their HQ values were far below the cut off value of 1. HQ > 1 has been previously estimated for SMX against blue-green algae in previous works (Ferrari et al., 2004; García-Galán et al., 2011; Kim et al., 2007). It should be mentioned that the detected concentrations are subjected to dilution once the WWTP effluents discharge onto the natural water courses. Therefore, these concentrations, and also the risk derived from exposure, would be considerably lower.

5. Conclusions

This work reports the results of an extensive monitoring campaign in 22 WWTPs in Catalonia, Spain, evidencing the widespread presence of SAs in both influent and effluent wastewater and their incomplete removal after conventional wastewater treatment. SAs' degradability was evaluated in terms of $t_{1/2}$ and results suggested that HRT was not a decisive parameter in SA removal, but more likely a parameter related to the nature of the drug studied. Again SMX and SPY where the two SAs more frequently detected and in higher amounts, together with their respective metabolites.

Regarding the adverse effects against the different ecosystems exposed, the ecotoxicological studies up to date showed different results but limited solely to SMX. To the author's knowledge, it is the first time that EC_{50} values are published for SPY and AcSPY. They were classified as harmful and toxic respectively, according to the EU legislation. The lack of ecotoxicologal data for SPY and AcSPY does not allow the establishment of a comparison of the inhibitory effects of this SA either with different taxa or with different SAs. Furthermore, studies performed with realistic environmental concentrations are scarce and the results reported unclear, and so the ecological risks associated can be considered limited. Future studies should focus on biodegradation pathways of these ubiquitous metabolites together with further research regarding their potential ecotoxicological effects in realistic environmental conditions.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2012.08.038.

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SUPPORTING INFORMATION #10

TABLE S1. Operational parameters of the different wastewater treatment plants (WWTPs) investigated.

WWTP	Type of treatment	HRT (d)	SRT (d)	Designed Treatment Capacity (m3/day)	Average Flow (m3/day)	Population served	Equivalent inhabitants (design)	Equivalent inhabitants	Sludge Treatment	Disposal of sludge	Sludge production (t/year)
W1	Biologic with P + N removal	4.45	-	30000	21000	92576	121500	182000	Anaerobic digestion	Soil application (10.6%) Thermal drying (89.4%)	2325
W2	Biologic with P + N removal	-	-	30000	14700	100856	220000	-	Anaerobic digestion	-	-
W3	Biologic	29.9	17	60000	38000	195160	500000	165000	Anaerobic digestion	Soil application (98.2%) Composting (1.8%)	3787.2
W4	Biologic with P + N removal	1.27	16	34560	23000	44590	204166	399000	-	Thermal drying (99%) Landfill (1%)	2864.8
W5	Biologic with tertiary treatment	26 - 40	10	47500	36500	42076	210583	74000	-	Disposal to soil. Agricultural use	-
W6	Biologic with P + N removal	-	-	35000	25500	118675	296333	-	Anaerobic digestion	-	-
W7	Biologic	0.6	-	22500	15000	82756	131250	84000	-	Composting (99%) Thermal drying (1%)	1512.4
W8	Biologic with P + N removal	0.36	12	64000	43000	279959	373333	198000	Anaerobic digestion	Soil application (43%) Thermal drying (50.7) Composting (6.2%)	3789.4
W9	Biologic with P + N removal + Tertiary	12.5	-	55000	37000	143975	206250	147000	Anaerobic digestion	Soil application (97%) Composting (2%)	3162
W10	Biologic	-	-	525000	350000	1444884	2843750	-	-	-	-
W11	Biologic	0.23	-	57000	27000	177818	451250	154000	Anaerobic digestion	Soil application (37.2%) Thermal drying (62.8%)	2797
W12	Biologic with P + N removal + Tertiary	0.5	-	420000	270000	1092573	2275000	1142000	Anaerobic digestion	Soil application (24.3%) Thermal drying (75.7%)	14954.8

Tabla S1. (cont.)

WWTP	Type of treatment	HRT (d)	SRT (d)	Designed Treatment Capacity (m3/day)	Average Flow (m3/day)	Population served	Equivalent inhabitants (design)	Equivalent inhabitants	Sludge Treatment	Disposal of sludge	Sludge production (t/year)
W13	Biologic with P removal	0.5	-	43000	33000	138714	358333	205000	Anaerobic digestion	Landfill (7%) Thermal drying (93%)	2142
W14	Biologic with P + N removal	0.16	6	87500	65000	117673	186666	177000	Anaerobic digestion	Soil application (100%)	2045
W15	Biologic with P + N removal	-	-	53500	28000	85224	196.167	-	Anaerobic digestion	-	-
W16	Biologic	18.96	-	25000	16500	108595	195833	124000	Anaerobic digestion	Soil application (78.6%) Composting (21.4%)	1954.3
W17	Biologic with N removal	0.3	4	27000	23000	77994	135000	142000	Anaerobic digestion	Thermal drying (83.5%) Composting (16.5%)	1844.5
W18	Biologic with N removal	-	-	40000	28000	66620	300000	-	Anaerobic digestion	-	-
W19	Biologic with P + N removal	7.5	9	14400	13500	52177	192000	87000	Anaerobic digestion	composting (1.4%) Soil application (98.6%)	991.9
W20	Biologic	20	6	35000	27000	143884	175000	170000	Anaerobic digestion	Disposal to soil. Incineration	-
W21	Biologic	0.3	-	72600	50000	253364	423500	229000	Activated sludge	Dehydration (100%)	6155
W22	Biologic with P + N removal	0.44	10	64000	35000	172208	384000	272000	Anaerobic digestion	Soil application (100%)	2750

TABLE S2. Method precision (RSD), limits of detection (LOD), limits of quantification (LOQ) and linearity for each SA studied in each of the water matrices analyzed.

	INFLUENT				EFFLUENT			
	PRECISION RSD (%) INFLUENT	LOD (ng L^{-1})	LOQ (ng L^{-1})	LINEARITY	PRECISION RSD (%) EFFLUENT	LOD (ng L^{-1})	LOQ (ng L^{-1})	LINEARITY
SSD	7	-	-	0.9994	3	0.05	0.18	0.9996
SZI	6	0.61	2.05	0.9998	5	0.12	0.39	0.9998
SQX	6	0.15	0.49	0.9996	7	0.04	0.14	0.9994
SDX	10	0.16	0.54	1.0000	6	0.11	0.38	0.9998
SuSTZ	11	0.01	0.04	0.9994	8	1.37	4.58	1.0000
SBZ	7	0.01	0.03	0.9996	6	0.20	0.66	1.0000
SDZ	8	0.40	1.32	0.9994	4	1.38	4.58	0.9998
SDM	6	0.02	0.08	1.0000	8	0.01	0.02	0.9990
SMZ	5	2.35	7.83	1.0000	5	0.19	0.62	1.0000
SMT	13	0.16	0.53	1.0000	4	0.57	1.91	0.9994
SMX	4	0.16	0.54	0.9998	3	0.19	0.64	0.9996
SMP	8	0.71	2.38	0.9998	2	0.08	0.27	1.0000
SPY	5	0.98	3.27	0.9996	2	0.24	0.79	1.0000
STZ	10	0.24	0.81	0.9998	3	0.21	0.69	0.9990
SSX	8	0.18	0.60	0.9996	5	0.13	0.44	0.9998
AcSMZ	6	1.40	4.66	0.9998	2	0.28	0.93	0.9998
SNT	12	0.26	0.86	1.0000	1	0.06	0.20	0.9998
AcSDZ	3	0.38	1.28	0.9998	1	0.36	1.19	0.9998
AcSMX	6	0.03	0.09	0.9998	1	0.17	0.58	0.9998
AcSZI	5	0.67	2.25	0.9992	4	0.46	1.52	0.9994
AcSPY	2	1.16	3.85	0.9996	3	0.44	1.46	0.9998

3.3. DISCUSIÓN DE LOS RESULTADOS.

3.3.1. Niveles ambientales de sulfamidas en el medio ambiente.

Dada la la cantidad de muestras analizadas y la diversidad de matrices ambientales estudiadas durante el desarrollo de esta Tesis, con el fin de obtener conclusiones válidas se han analizado por separado los datos correspondientes a cada tipo de matriz. La Figura 3.1. nos muestra la contribución de cada una de las sulfamidas estudiadas a las concentraciones totales detectadas en las diferentes matrices ambientales.

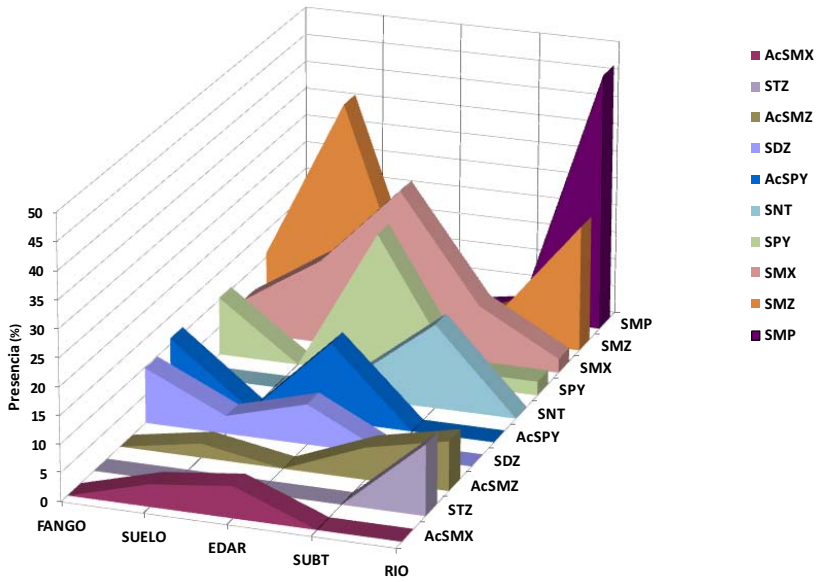

Figura 3.1. Presencia medioambiental de las diez sulfamidas encontradas con mayor frecuencia y a mayor concentración en las diferentes matrices ambientales estudiadas.

En efluentes de EDAR, son el SMX y la SPY las sulfamidas con mayor contribución (30% y 26%) seguidas de sus dos metabolitos acetilados y de la SDZ. La distribución en los fangos de depuradora es diferente, ya que la presencia del SMX es tan sólo del 6% y del 10% para SPY. La SDZ mantiene una contribución del 10%, y el AcSPY de un 11%, valores similares a los encontrados en efluentes de EDAR para ambas; aparecen además otras sulfamidas como el SSX y la SMZ, minoritarias en agua de salida. En agua de río, vemos que la mayor contribución

corresponde a sulfamidas veterinarias como la SMP (46%), el STZ (12%) o la SMZ y su metabolito acetilado (23% y 9% respectivamente). En aguas subterráneas predominan también sulfamidas veterinarias, en este caso el SNT o la SMR, que no fueron detectadas en aguas superficiales o urbanas, indicando un orígen claramente agrícola. Por último en suelos agrícolas, la SMZ y la SSX son las sulfamidas predominantes (40% y 26% respectivamente), pero destaca también la presencia de sulfamidas de orígen humano como el SMX y su metabolito acetilado con una contribución conjunta del 19%. Si bien los datos obtenidos en las diferentes matrices pertenecen a diferentes localizaciones y tiempo, éstos permiten tener una idea consistente de los patrones de consumo y la influencia de las diferentes actividades humanas en la distribución de estos antibióticos en el medioambiente.

3.3.1.1. Niveles de sulfamidas y sus metabolitos en aguas residuales y tasas de eliminación.

Para estudiar la presencia de sulfamidas en aguas de entrada y salida de depuradora, se analizaron muestras de un total de 47 EDARs, de las que 29 estaban situadas en la zona septentrional de España el resto en Alemania, más concretamente en el estado de Hesse, en las cercanías de la ciudad de Frankfurt. Se tomaron muestras integradas de agua de entrada y salida en todas las EDARs españolas, mientras que en las alemanas sólo se tomaron muestras de salida. Ya que uno de los principales objetivos de esta Tesis es investigar la presencia y destino ambiental de las sulfamidas, en este apartado sólo discutiremos los resultados obtenidos en las aguas de salida de las EDAR, que son la que finalmente son vertidas a aguas naturales. Los datos provenientes de las aguas de entrada han sido utilizados para estimar valores de eliminación durante el tratamiento, como se explica en la sección 3.2.5 de este Capítulo.

La ubicación de las EDARs es el principal factor a considerar para obtener una interpretación coherente de los datos. Así, mientras todas las muestras tomadas a lo largo de la cuenca del Ebro y en Cataluña correspondían a EDARs ubicadas cerca de núcleos urbanos muy poblados (desde 35000 habs. hasta 674725 habs.), las depuradoras del estado de Hesse servían a poblaciones más pequeñas y ubicadas en una región agrícola. La Tabla 3.1 recoge los descriptores estadísticos de los datos obtenidos (concentraciones máximas, mínimas, media, mediana y frecuencia de detección) para cada sulfamida. Los casos en los que el compuesto no

haya sido detectado o esté por debajo del límite de cuantificación no han sido considerados en el cálculo final.

Tabla 3.1. Níveles máximos y mínimos (ng L^{-1}), valor medio, mediana y frecuencia de detección de las concentraciones de sulfamidas detectadas en aguas de salida de EDAR (ver Publicaciones #9 y #10).

SULFAMIDA	NUM. DE MUESTRAS	MEDIA	MEDIANA	VALOR MAXIMO	VALOR MINIMO	FRECUENCIA DE DETECCIÓN (%)
Sulfisomidina	n=55	8.1	5.8	52.8	0.4	33
Sulfadiazina		47.1	28.3	286	0.7	**73**
Sulfadimetoxina		10.1	4	84	0	44
Sulfametazina		13.3	7.4	102	0.3	51
N^4-acetilsulfametazina		3.2	1.6	16.4	0.4	35
Sulfametizol		41.0	8.5	426	1	40
Sulfametoxazol		144.4	100.7	650	10.8	**96**
Sulfapiridina		120.3	64.6	590	3.3	**100**
Sulfatiazol		8.6	5.9	73	0.4	56
Sulfisoxazol		2.1	0.8	13	0	42
Sulfamerazina		12.6	11.2	55.6	0.1	36
Sulfabenzamida		5.1	1.8	29.9	0.3	35
Sulfanitran		62.5	23.1	512	0.1	33
Sulfaquinoxalina		7.5	3.5	75.1	0.1	47
Sulfadoxina	n=37	11	3.6	80.4	0.1	43
Sufacetamida		306.1	150	22.2	1280	38
Succinil-sulfatiazol		23.7	10.4	107	1.7	32
Sulfametoxipiridazina		6	1.9	47	0.02	54
N^4-acetilsulfapiridina	n=41	93.73	60.60	522	10.1	**100**
N^4-acetilsulfadiazina		43.6	34.9	67.4	0.6	26
N^4-acetilsulfametoxazol		46.3	19.8	94.6	4.6	**70**
N^4-acetilsulfamerazina		0	-	0.8	-	4

Como muestra esta tabla, tanto la SPY como su metabolito acetilado AcSPY fueron detectados en todas las aguas de salida investigadas, mientras que SMX y AcSMX fueron detectados en un 96% y 70% de las muestras, respectivamente. Los valores de la mediana son muy inferiores a los valores de la media para la mayoría de las sulfamidas, indicando una distribución con tendencia hacía los valores más pequeños entre el máximo y el mínimo. Por el contrario, la SMR presenta unos valores muy parecidos de mediana y media, indicando una distribución más equitativa de las concentraciones detectadas. La Tabla 3.2 muestra la matriz de correlación entre variables que permite visualizar mejor las posibles asociaciones entre

éstas. Para simplificar la matriz se eliminó el AcSMR, ya que sólo fue detectada en 1 de las muestras. Los colores más fuertes indican una mayor correlación y viceversa:

	SSD	SMR	SQX	SDX	SuSTZ	SBZ	SDZ	AcSDZ	SDM	SMZ	AcSMZ	SMT	SMP	SMX	AcSMX	SPY	AcSPY	STZ	SSX	SNT
SSD	1	**0.867**	**0.929**	**0.959**	0.525	**0.643**	-0.030	-0.193	0.097	0.228	0.462	0.144	0.282	-0.010	-0.128	-0.021	-0.081	0.266	0.246	0.384
SMR	**0.867**	1	**0.784**	**0.854**	0.485	0.587	-0.087	-0.246	0.097	0.187	0.399	0.082	0.501	0.104	-0.164	-0.131	-0.122	0.226	0.405	0.313
SQX	**0.929**	**0.784**	1	**0.947**	0.561	**0.726**	-0.036	-0.183	0.102	0.276	0.423	-0.012	0.378	-0.145	-0.102	-0.029	-0.081	0.328	0.032	0.370
SDX	**0.959**	**0.854**	**0.947**	1	0.567	**0.675**	-0.056	-0.152	0.056	0.245	0.421	0.018	0.515	-0.035	-0.101	0.002	-0.074	0.231	0.051	0.389
SuSTZ	0.525	0.485	0.561	0.567	1	0.367	0.434	-0.177	0.149	0.237	0.202	-0.003	0.206	-0.089	-0.101	-0.092	-0.127	0.172	-0.038	0.214
SBZ	**0.643**	0.587	**0.726**	**0.675**	0.367	1	0.070	-0.179	0.287	0.137	0.256	-0.009	0.379	-0.147	-0.089	-0.073	-0.133	**0.771**	0.016	0.243
SDZ	-0.030	-0.087	-0.036	-0.056	0.434	0.070	1	0.262	0.253	-0.024	-0.029	-0.013	-0.178	0.128	0.053	0.270	0.050	0.222	0.050	-0.124
AcSDZ	-0.193	-0.246	-0.183	-0.152	-0.177	-0.179	0.262	1	-0.194	-0.219	-0.201	-0.155	0.000	0.279	0.240	**0.890**	0.590	-0.221	-0.181	-0.165
SDM	0.097	0.097	0.102	0.056	0.149	0.287	0.253	-0.194	1	-0.032	0.037	0.115	-0.032	-0.026	-0.109	-0.194	-0.190	0.420	0.193	-0.025
SMZ	0.228	0.187	0.276	0.245	0.237	0.137	-0.024	-0.219	-0.032	1	0.067	0.277	0.058	0.052	0.087	-0.162	-0.132	0.120	-0.023	0.130
AcSMZ	0.462	0.399	0.423	0.421	0.202	0.256	-0.029	-0.201	0.037	0.067	1	0.152	-0.032	0.043	-0.108	-0.098	-0.111	0.192	0.255	0.126
SMT	0.144	0.082	-0.012	0.018	-0.003	-0.009	-0.013	-0.155	0.115	0.277	0.152	1	-0.080	0.362	-0.035	-0.155	-0.084	0.302	0.403	-0.021
SMP	0.282	0.501	0.378	0.515	0.206	0.379	-0.178	0.000	-0.032	0.058	-0.032	-0.080	1	-0.239	0.000	-0.246	0.000	-0.043	-0.110	0.163
SMX	-0.010	0.104	-0.145	-0.035	-0.089	-0.147	0.128	0.279	-0.026	0.052	0.043	0.362	-0.239	1	0.293	0.361	0.096	-0.037	0.449	-0.141
AcSMX	-0.128	-0.164	-0.102	-0.101	-0.101	-0.089	0.053	0.240	-0.109	0.087	-0.108	-0.035	0.000	0.293	1	0.319	0.417	-0.072	-0.119	-0.066
SPY	-0.021	-0.131	-0.029	0.002	-0.092	-0.073	0.270	**0.890**	-0.194	-0.162	-0.098	-0.155	-0.246	0.361	0.319	1	0.581	-0.187	-0.149	-0.075
AcSPY	-0.081	-0.122	-0.081	-0.074	-0.127	-0.133	0.050	0.590	-0.190	-0.132	-0.111	-0.084	0.000	0.096	0.417	0.581	1	-0.113	-0.052	-0.105
STZ	0.266	0.226	0.328	0.231	0.172	**0.771**	0.222	-0.221	0.420	0.120	0.192	0.302	-0.043	-0.037	-0.072	-0.187	-0.113	1	0.184	0.075
SSX	0.246	0.405	0.032	0.051	-0.038	0.016	0.050	-0.181	0.193	-0.023	0.255	0.403	-0.110	0.449	-0.119	-0.149	-0.052	0.184	1	-0.017
SNT	0.384	0.313	0.370	0.389	0.214	0.243	-0.124	-0.165	-0.025	0.130	0.126	-0.021	0.163	-0.141	-0.066	-0.075	-0.105	0.075	-0.017	1

Tabla 3.2. Matriz de correlación de Pearson para las variables estudiadas.

Como se puede observar, la presencia de las sulfamidas veterinarias SSD, SMR, SQX y SDX está altamente correlacionada, dato que nos indica una procedencia común de todas ellas. Esta correlación no se observó entre las sulfamidas de consumo humano SMX, SPY y SDZ, indicando un consumo desigual de dichas sustancias en los diferentes núcleos urbanos, con la excepción de la correlación entre SPY y AcSDZ, mayor incluso que el valor correspondiente a SPY y AcSPY.

Para poder evaluar el conjunto de todos los resultados obtenidos, se ha realizado un análisis de componentes principales (PCAs) para poder así sintetizar toda la información. Un PCA nos permite identificar los factores que contribuyen de forma significativa a la variabilidad de los datos. Así mismo, nos permite observar la correlación entre las diferentes variables del estudio (que en nuestro caso son las sulfamidas) y reducir su número con la pérdida mínima de información. Obtendremos así un número menor de variables o componentes principales (PCs), que serán combinación lineal de las originales, y que recogen la mayor cantidad de variabilidad original para así poder observar tendencias generales. Para el análisis de PCAs utilizamos el programa estadístico XLSTAT para Excel.

En un primer paso del tratamiento estadístico, organizamos los datos en una matriz con 14 columnas correspondientes a cada una de las variables y 55 filas, por cada una de las EDARs muestreadas. A todos aquellos valores por debajo de límite de cuantificación se les asignó el valor del límite de detección correspondiente, y a aquellos por debajo del límite de detección o no detectados se les asignó un valor arbitrario igual a la mitad del límite de

detección (Navarro *et al.* 2006). Hay que tener en cuenta que los metabolitos acetilados, excepto AcSMZ, fueron añadidos al método general de sulfamidas a posteriori, y por tanto el número de muestras en las que se han analizado estos compuestos es menor (ver Tabla 3.1). El software XLSTAT estimará estos valores no determinados o "perdidos" en la matriz de datos con una aproximación de vecino más próximo. En primer lugar, autoescalamos los datos de la matriz aplicando a todos ellos la siguiente fórmula:

$$X'_{n,m} = \frac{X_{n,m} - X_m}{SD_m}$$

donde $X'_{n,m}$ es el dato autoescalado, X_m es la media aritmética de la variable y SD_m es su desviación estándar.

La Figura 3.2 muestra los PCs obtenidos tras el análisis. Estos componentes siempre se extraen de forma que el primer PC explica la mayor parte de la variabilidad de los datos, siendo esta progresivamente menor para el resto de PCs. En este caso, la variabilidad explicada por el primer PC es de un 30%, y de un 14% para el segundo PC. El número de PCs a utilizar es arbitrario, y un criterio utilizado es retener los PCs con valor propio superior a 1, que en nuestro caso nos llevaría a retener 6 PCs que explicarían un 75% de la variabilidad, perdiendo el 25% de la información original. Si consideramos los 10 primeros PCs, el 90% de la variabilidad de los datos quedaría explicada. Sin embargo, no resulta estadísticamente significativo aumentar en 4 componentes para poder explicar únicamente un 15% más de la variabilidad. Así pues, pasaríamos de 21 variables a sólo 6.

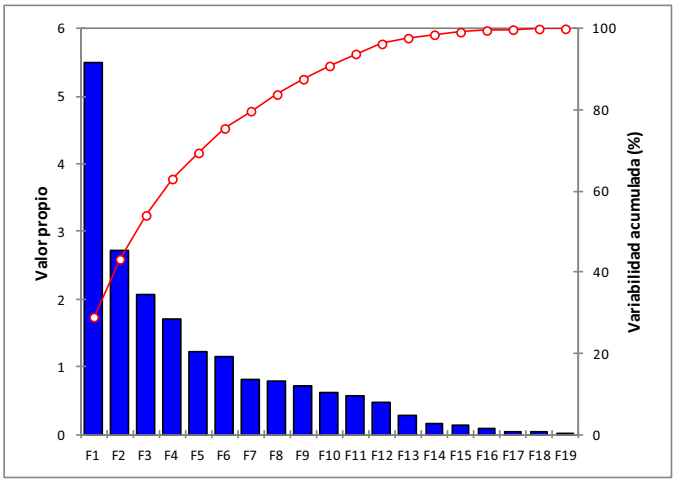

Figura 3.2. Valores propios y varianza acumulada de los componentes principales obtenidos.

A continuación se caracterizarán los 4 primeros PCs identificados en función de las variables originales. Para ello, establecemos una matriz de correlación entre estos nuevos factores y las variables, también denominada matriz de *loadings* o de carga factorial. Esta matriz indica la contribución de cada una de las variables originales a los nuevos PCs. La Figura 3.3 presenta los *loadings* para los primeros 4 PCs.

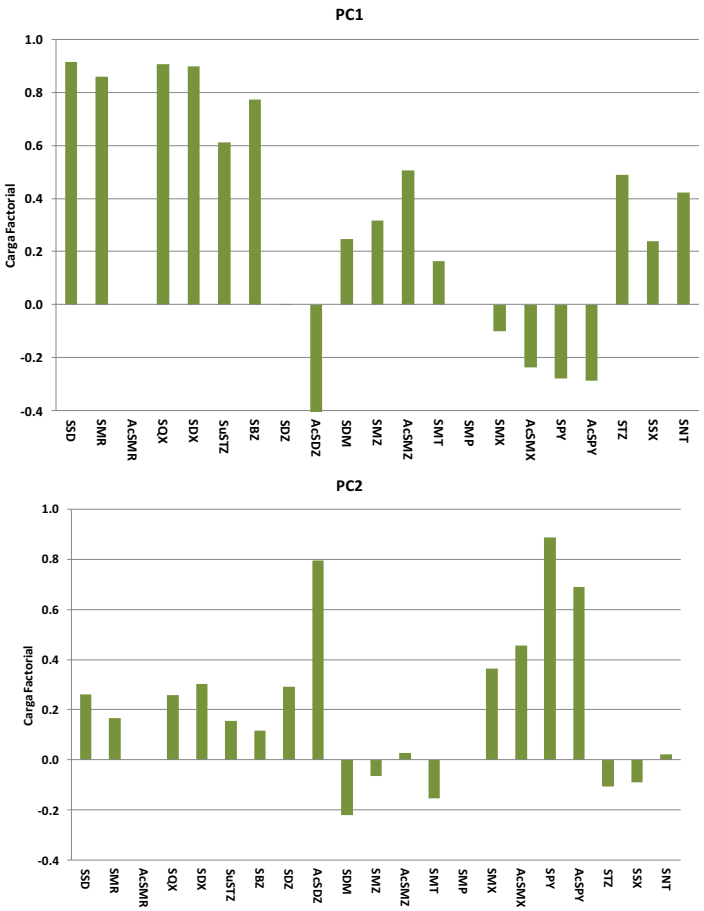

Figura 3.3. Diagramas de loadings para los cuatro primeros componentes principales.

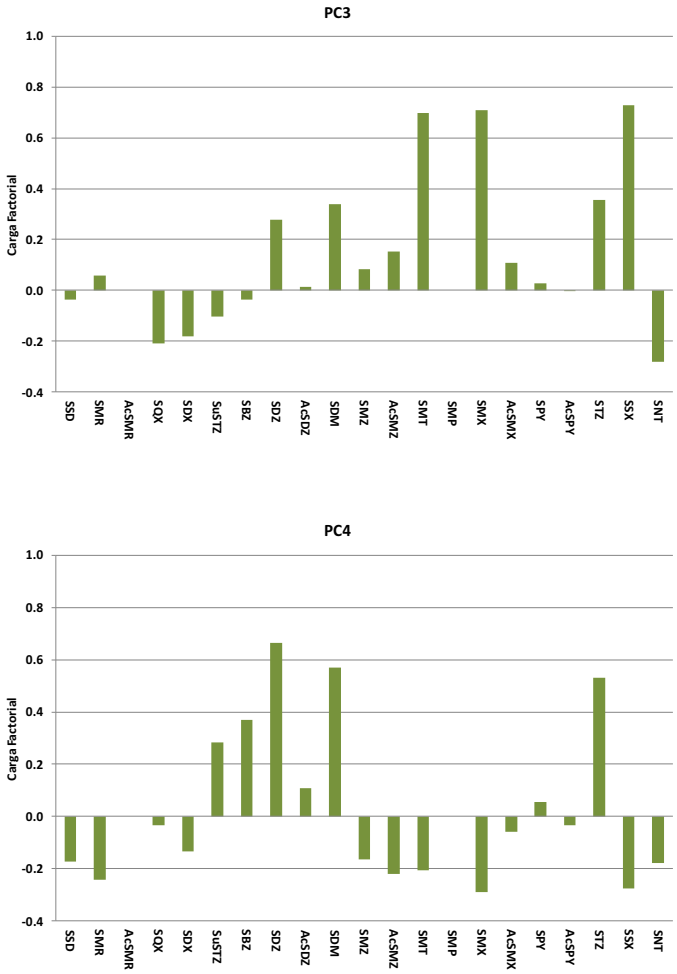

Figura 3.3 (continuación).

Como se puede observar, 16 de las 21 variables presentan correlaciones positivas para el primer PC, siendo SSD, SDX y SQX las variables con una mayor contribución (valores más cercanos a 1). Podríamos considerar por tanto este PC como posible indicador de una fuente importante de contaminación dada la correlación positiva de la mayoría de las sulfamidas; sin embargo, son las sulfamidas de uso humano (SMX, SDZ, SPY y sus correspondientes

metabolitos acetilados) las que presentan correlaciones negativas en este PC1. Ya que el SMX y la SPY son dos de las sulfamidas detectadas con mayor frecuencia y a mayor concentración, este PC finalmente no representaría una fuente de contaminación relevante, ya que toda muestra que quede representada en su espacio factorial contendrá la mayoría de las sulfamidas excepto las más significativas. Los *loadings* para el PC2, que explica un 14% de variabilidad de los datos, presentan valores negativos muy bajos para 5 de las variables, mostrando el resto correlaciones positivas. Este PC si podría considerarse como fuente de contaminación ya que, al contrario que el PC1, las muestras representadas en su espacio factorial contendrán las sulfamidas más relevantes cuantitativamente (SMX y SPY). Este PC2 representará también la fuente principal de los metabolitos acetilados, principalmente el AcSPY y el AcSDZ. Las variables en PC4 y PC5 presentan valores de correlación tanto positivos como negativos casi al 50%, haciendo su interpretación gráfica algo más complicada.

Por último, construimos el plano factorial con PC1 en abcisas y PC2 en ordenadas. En este plano representamos en primer lugar las antiguas variables, obteniendo un gráfico denominado círculo de correlación (Figura 3.4). La proximidad de cada una de las variables a uno de los componentes significa una contribución importante a dicho componente.

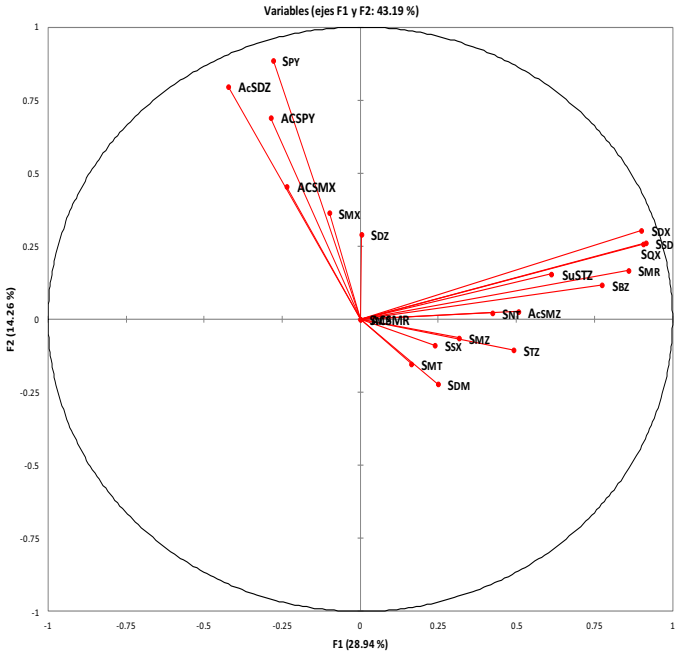

Figura 3.4. Circulo de correlación de variables

Para finalizar añadimos las muestras analizadas al plano factorial con el fin de poder observar tendencias (Figura 3.5). De este modo, podremos identificar la contribución de cada una de las antiguas variables a la concentración total de la muestra en función de su ubicación en el gráfico. Por ejemplo, si la muestra se sitúa cerca del eje positivo de ordenadas implicará que ésta contiene una concentración importante de SMX, SPY, SDZ y sus respectivos metabolitos.

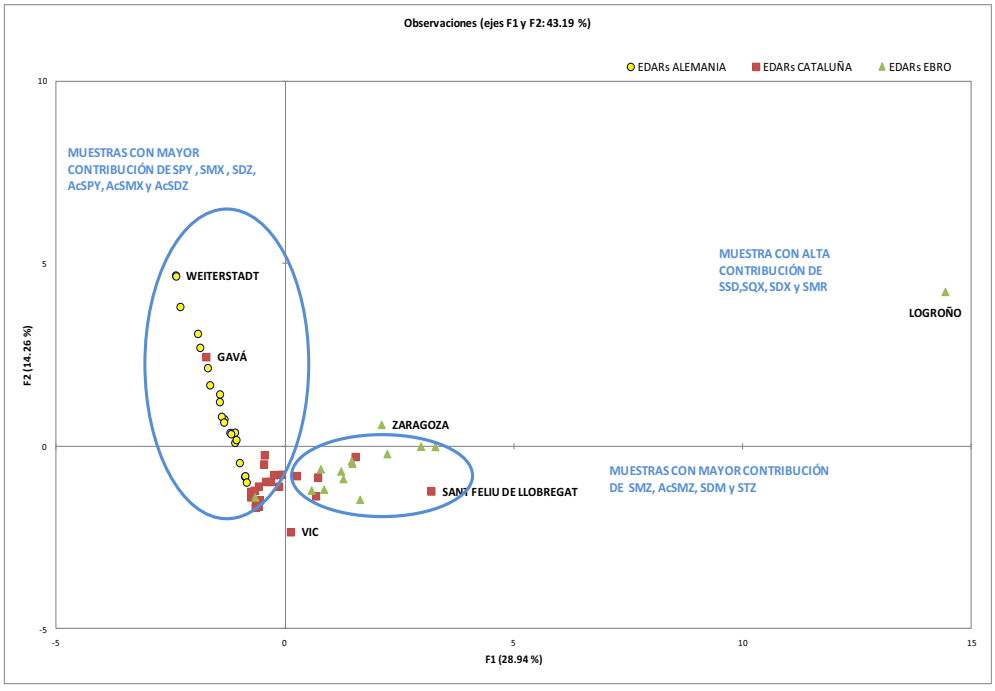

Figura 3.5. Plano factorial PC1-PC2 con todas las muestras de agua de salida de EDAR analizadas.

A pesar de que todas las sulfamidas estudiadas son muy similares en estructura y peso molecular, podemos observar como las muestras de EDARs alemanas aparecen alineadas en el gráfico en el sector factorial correspondiente a las muestras con una mayor concentración de SMX, SPY, SDZ y sus metabolitos acetilados. La mayoría de las muestras correspondientes a las EDAR de Cataluña han quedado ubicadas en el sector inferior, lo que indica que también contienen estas sulfamidas y metabolitos aunque en menor concentración. Respecto a las

muestras de las EDAR del Ebro, todas se sitúan en el segundo cuadrante, indicando alta contribución de SMZ, AcSMZ, SDM y STZ en dichas muestras. Tan solo la muestra de salida de la EDAR de Logroño no presenta una tendencia similar a la del resto de las EDARs del Ebro; su ubicación en el primer cuadrante del plano factorial indica la presencia mayoritaria de SSD, SQX y SMR, sulfamidas veterinarias, y por tanto evidencia la ubicación de esta EDAR en una zona de mayor actividad agrícola-ganadera que el resto. Podríamos concluir que las muestras tomadas a lo largo de la cuenca del Ebro presentan una proporción mayor de sulfamidas de uso veterinario que las tomadas en Cataluña. Aunque ambos grupos se localizan en su mayoría en el eje negativo de PC2, indicando una presencia baja de sulfamidas de consumo humano, la ubicación en el gráfico de las muestras catalanas en el eje negativo de PC1 contienen también estas sulfamidas y sus metabolitos, pero sin duda son las depuradoras alemanas las que contienen una mayor proporción de éstos últimos.

3.3.1.2. Niveles de sulfamidas y sus metabolitos en aguas superficiales

Las Publicaciones #7 y #9 presentadas en este Capítulo presentan los resultados obtenidos durante diferentes campañas de muestreo de aguas de río llevadas a cabo entre 2005 y 2008. En la Publicacion #7 se tomaron muestras del río Llobregat y de su afluente el río Anoia durante tres campañas diferentes llevadas a cabo entre 2005 y 2006. Posteriormente, Se analizaron muestras procedentes de toda la cuenca del Ebro durante dos campañas consecutivas (otoño del 2007 y verano del 2008) incluyendo afluentes y localizaciones aguas arriba y aguas debajo de puntos de vertido de diferentes EDAR mencionadas en el apartado anterior. En total, se recogieron 36 muestras de aguas superficiales. Excepto el AcSMZ, ningún otro metabolito acetilado pudo ser incluido en estos estudios en el momento de su realización. La Tabla 3.3 muestra los descriptores estadísticos para todas ellas.

Tabla 3.3. Níveles máximos y mínimos (ng L^{-1}), valor medio, mediana y frecuencia de detección de las concentraciones de sulfamidas detectadas en aguas de río (ver Publicaciones #7 y #9).

SULFAMIDA	NUM. DE MUESTRAS	MEDIA	MEDIANA	VALOR MAXIMO	VALOR MINIMO	FRECUENCIA DE DETECCIÓN (%)
Sulfadiazina	n= 36	4.07	1.2	13.4	0.5	13.9
Sulfadimetoxina		14.2	2.5	136	0.5	41.7
Sulfametazina		144.8	15.7	2481.8	2.1	**58.3**
N^4-acetilsulfametazina		88.5	14.5	695.0	0.3	36.1
Sulfametizol		3.2	2.2	10.3	0.3	30.6
Sulfametoxipiridazina		53.9	18.7	652.7	0.3	**75.0**
Sulfametoxazol		13.65	5.2	91.8	0.1	**66.7**
Sulfapiridina		37.8	11.4	164.9	0.2	27.8
Sulfatiazol		137.9	1.7	960.6	0.2	33.3
Sulfisoxazol		18.6	18.6	24.7	12.5	8.3
Sulfisomidina	n=15	10	3.7	40.4	1.0	**53.3**
Sulfamerazina		11.3	8.86	35.4	0.6	**60.0**
Suilfaquinoxalina		18.2	20.8	27.3	5.9	26.7
Sulfadoxina		10	5.1	26.4	3.3	46.7
Succinil-sulfatiazol		-	-	-	-	-
Sulfabenzamida		1.2	1.2	1.8	0.6	20.0
Sulfanitran		-	-	-	-	-

Las concentraciones medias y valores máximos más elevados corresponden a la SMZ y al STZ, sulfamidas de uso veterinario cuya presencia en estos ríos puede deberse principalmente a la escorrentía desde instalaciones de ganadería intensiva o desde campos de cultivo abonados. Si bien esperábamos que el papel de estas sulfamidas veterinarias fuese el más relevante en este tipo de agua frente a otras de consumo humano como el SMX o la SPY, estas últimas fueron detectadas con las mayores concentraciones, alcanzando niveles de µg L^{-1}, un orden de magnitud superior a los valores medios detectados en otros ríos (ver Publicación #7). Estas concentraciones se detectaron principalmente en la desembocadura del río Llobregat, donde los valores se situaban incluso por encima del rango de cuantificación (> 5000 µg L^{-1}). Los ríos de la franja mediterránea, como el Llobregat, se presentan como casos de especial interés ya que su cauce suele presentar un flujo basal muy bajo debido a los largos períodos de sequía característicos del clima mediterráneo y no ejercen el efecto de dilución esperado sobre los aportes de contaminantes desde las diferentes fuentes. En muchos casos

además, los vertidos de aguas residuales desde las EDARs constituyen una alta proporción del caudal total del río, hecho que explicaría la alta frecuencia de detección del SMX tanto en el Llobregat como en los ríos de la cuenca del Ebro. Por el contrario, la frecuencia de detección de la SPY es bastante menor en este caso, siendo otras sulfamidas veterinarias como la SMZ y su metabolito AcSMZ, o la SMP más ubicuas. De nuevo los valores de la mediana son por lo general inferiores a los de la media, indicando que la mayoría de las concentraciones detectadas tienden hacia valores inferiores en el rango. Para poder identificar posibles asociaciones entre la presencia de las variables, desarrollamos la matriz de correlación de

	SSD	SMR	SQX	SDX	SBZ	SDZ	SDM	SMZ	AcSMZ	SMT	SMP	SMX	SPY	STZ	SSX
SSD	**1**	**0.667**	**0.900**	**0.884**	-0.126	0.195	0.185	0.216	0.036	0.423	0.213	0.120	0.390	0.213	0.060
SMR	**0.667**	**1**	**0.649**	**0.755**	-0.323	0.171	0.163	0.165	0.072	0.296	0.173	0.427	0.289	0.170	0.073
SQX	**0.900**	**0.649**	**1**	**0.985**	-0.190	0.325	0.156	0.346	0.040	**0.699**	0.344	0.296	0.555	0.329	0.050
SDX	**0.884**	**0.755**	**0.985**	**1**	-0.238	0.324	0.164	0.341	0.051	**0.682**	0.344	0.356	0.546	0.328	0.061
SBZ	-0.126	-0.323	-0.190	-0.238	**1**	-0.080	-0.096	-0.063	-0.064	-0.104	-0.083	-0.199	-0.107	-0.093	-0.090
SDZ	0.195	0.171	0.325	0.324	-0.080	**1**	-0.051	-0.040	-0.052	0.291	-0.042	**0.826**	**0.842**	-0.050	-0.048
SDM	0.185	0.163	0.156	0.164	-0.096	-0.051	**1**	-0.041	**0.951**	-0.044	0.100	-0.091	-0.004	0.439	**0.884**
SMZ	0.216	0.165	0.346	0.341	-0.063	-0.040	-0.041	**1**	0.069	0.376	**0.987**	0.024	0.474	**0.875**	-0.040
AcSMZ	0.036	0.072	0.040	0.051	-0.064	-0.052	**0.951**	0.069	**1**	-0.039	0.208	-0.078	0.023	0.532	**0.864**
SMT	0.423	0.296	**0.699**	**0.682**	-0.104	0.291	-0.044	0.376	-0.039	**1**	0.369	0.252	0.462	0.304	-0.099
SMP	0.213	0.173	0.344	0.344	-0.083	-0.042	0.100	**0.987**	0.208	0.369	**1**	0.011	0.470	**0.935**	0.089
SMX	0.120	0.427	0.296	0.356	-0.199	**0.826**	-0.091	0.024	-0.078	0.252	0.011	**1**	**0.726**	-0.023	-0.098
SPY	0.390	0.289	0.555	0.546	-0.107	**0.842**	-0.004	0.474	0.023	0.462	0.470	**0.726**	**1**	0.420	-0.012
STZ	0.213	0.170	0.329	0.328	-0.093	-0.050	0.439	**0.875**	0.532	0.304	**0.935**	-0.023	0.420	**1**	0.399
SSX	0.060	0.073	0.050	0.061	-0.090	-0.048	**0.884**	-0.040	**0.864**	-0.099	0.089	-0.098	-0.012	0.399	**1**

Pearson (Tabla 3.4)

Tabla 3.4. Matriz de correlación de Pearson para las variables estudiadas.

En esta matriz se han eliminado el SuSTZ y el SNT, ya que no fueron detectadas en ninguna de las muestras. Al igual que para las aguas de depuradora, son las sulfamidas veterinarias SSD, MSR, SQX y SDX las que presentan un mayor índice de correlación, de modo que la presencia de cualquiera de ellas indica una alta probabilidad de un uso y presencia conjunto con las demás. En cuanto a las sulfamidas de consumo humano, SMX, SPY y SDZ, en este caso sí están altamente correlacionadas, indicando un origen común, probablemente de la descarga de aguas residuales urbanas al curso del río. Al igual que en el apartado anterior realizamos un análisis de PCA para facilitar la interpretación de los datos. En este caso, con seis PCs queda explicado 95% de la variabilidad, como muestra la Figura 3.6.

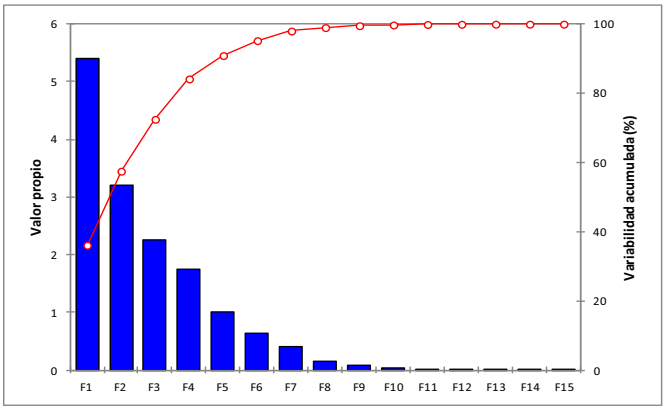

Figura 3.6. Valores propios y varianza acumulada de los componentes principales obtenidos

El primer PC explica un 36% de esta variabilidad. Si miramos su diagrama de *loadings* (Figura 3.7) este PC podría indicar una fuente general de contaminación ya que todas las variables, excepto SBZ, presentan correlaciones positivas y elevadas. La mayor contribución a este PC la presentan sulfamidas típicamente veterinarias como SSD, SQX o SMR. El PC2 explica a su vez un 21% de la variabilidad de los datos. En este caso, los *loadings* negativos se corresponden con las sulfamidas de uso humano como el SMX, la SDZ y la SPY. Suponemos por tanto que, ya que el SMX tiene una alta frecuencia de detección en las muestras analizadas y su correlación con este PC es negativa, la mayoría de las muestras se situaran en el segundo o tercer cuadrante del plano factorial.

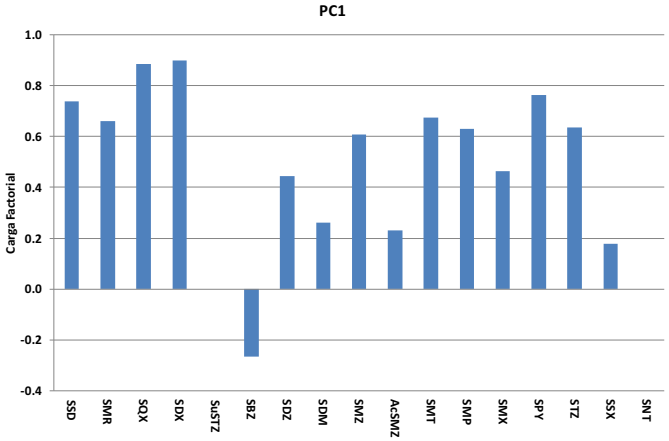

Figura 3.7. Diagramas de loadings para los cuatro primeros componentes principales.

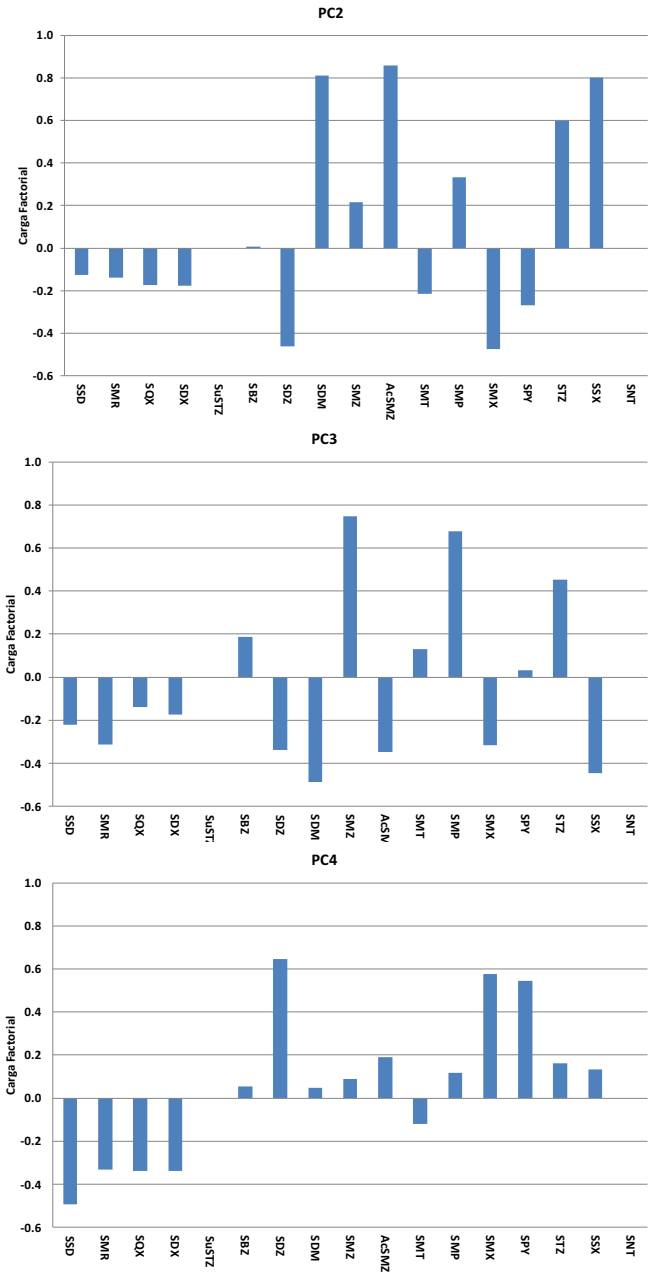

Figura 3.7. (continuación).

De nuevo creamos el plano factorial representando PC1 en el eje de abcisas y PC2 en ordenadas, y esta vez representaremos un biplot con las muestras y las variables en el mismo gráfico (Figura 3.8). Observamos que la mayoria de las muestras se sitúan en el eje negativo de ambos componentes, en el tercer cuadrante del plano factorial, indicando la contribución, aunque moderada, de sulfamidas de consumo humano y también la presencia de cuatro sulfamidas típicamente veterinarias (SBZ, SSD, SMR y SQX) mientras que ni la SMZ ni su metabolito estarían presentes. Sin embargo, ya que hemos considerado el eje positivo de PC1 como posible fuente de contaminación, son las muestras situadas en el primer y sobre todo en el segundo cuadrante las que mostraran una mayor concentración total de sulfamidas, principalmente de las veterinarias. En este úultimo encontramos una muestra tomada en la desembocadura del Llobregat, junto con dos muestras del Ebro y dos muestras tomadas en al Anoia, una en su desembocadura y la otra a su paso por una zona que recibe vertidos tanto industriales como urbanos. En concreto, las muestras del Ebro fueron tomadas aguas abajo de puntos de descarga de depuradoras de ciudades con alta densidad poblacional como Miranda de Ebro y Tudela.

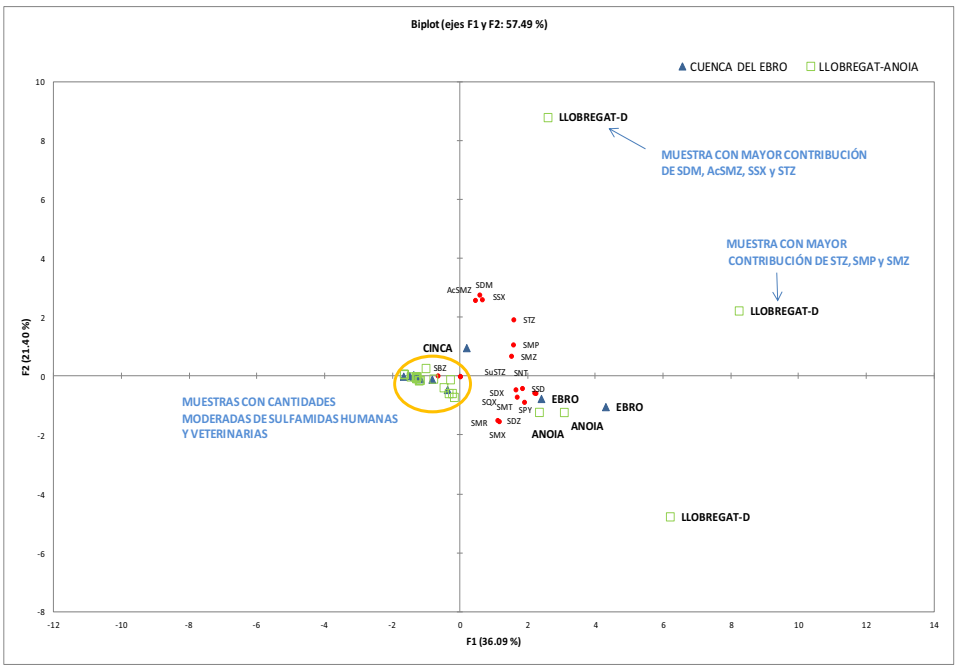

Figura 3.8. Plano factorial PC1-PC2 con todas las muestras de agua de salida de EDAR analizadas.

Vemos también que en el primer cuadrante se localizan tan solo 3 muestras, las que contienen una mayor concentración de SMZ y de su metabolito acetilado, junto con STZ y SSX. Se trata de la muestra del río Cinca, tomada en una zona rural, y de 2 muestras tomadas en la desembocadura del Llobregat en dos campañas consecutivas. En particular las muestras de la desembocadura del Llobregat presentan valores extremadamente altos de prácticamente todas las sulfamidas estudiadas, y por tanto aparecen muy alejadas del resto de muestras en la representación.

3.3.1.3. Niveles de sulfamidas y sus metabolitos en aguas subterráneas.

Durante el 2008 y 2009 y en colaboración con la Agencia Catalana del Aigua (ACA) se realizaron dos muestreos de aguas subterráneas en áreas rurales bajo una alta presión agrícola y ganadera. Estos acuíferos ya habían sido muestreados previamente para la detección de plaguicidas y además estaban considerados como altamente vulnerables a la contaminación por nitratos procedentes de los purines de ganadería y abonos. Ya que purines y sulfamidas comparten la misma vía de entrada en el medio natural (deposición en el suelo y posterior infiltración y percolación hacia estratos inferiores), en las nuevas campañas se decidió incluir el análisis de las sulfamidas en representación de antibióticos veterinarios, como posible indicador de contaminación por nitratos. En total, se recogieron 79 muestras de aguas subterráneas. La Tabla 3.5 muestra los descriptores estadísticos para todas ellas.

Tabla 3.5. Níveles máximos y mínimos (ng L^{-1}), valor medio, mediana y frecuencia de detección de las concentraciones de sulfamidas detectadas en aguas subterráneas (ver Publicaciones #5 y #8).

SULFAMIDA	MUESTRAS	MEDIA	MEDIANA	VALOR MAXIMO	VALOR MINIMO	FRECUENCIA DE DETECCIÓN (%)
Sulfisomidina		8.9	1.7	32.1	0.01	43.0
Sulfamerazine		43.9	1.3	744.7	0.11	46.8
Suilfaquinoxaline		11.2	0.5	274.0	0.01	**54.4**
Sulfadoxina		5.9	2.3	53.6	0.02	44.3
Succinil-sulfatiazol		3.8	3.8	5.5	2.10	2.5
Sulfabenzamida		1.4	0.5	10.3	0.02	25.3
Sulfadiazina		1.8	0.5	9.0	0.14	16.5
Sulfadimetoxina		7.5	0.7	91.5	0.01	**70.9**
Sulfametazina	N=79	7.1	2.1	106.8	0.03	**54.4**
N^4-acetilsulfametazina		8.9	1.3	57.0	0.02	45.6
Sulfametizol		1.5	0.2	9.3	0.05	10.1
Sulfametoxipiridazina		7.4	0.8	68.7	0.02	24.1
Sulfametoxazol		13.0	1.8	312.2	0.08	**57.0**
Sulfapiridina		5.0	1.6	72.5	0.07	38.0
Sulfatiazol		3.0	0.6	16.8	0.01	32.9
Sulfisoxazol		3.0	0.6	17.1	0.05	16.5
Sulfanitran		44.8	12.4	568.8	0.04	24.1
N^4-acetilsulfamerazina		4.5	2.7	18.0	0.30	35.9
N^4-acetilsulfadiazina		0.6	0.6	1.0	0.24	7.7
N^4-acetilsulfametoxazol	N=39	1.3	0.9	5.5	0.14	25.6
N^4-acetilsulfapiridina		1.4	0.4	6.0	0.15	23.1

La SMR y el SNT fueron las sulfamidas con valores máximos y una concentración media superior, aunque las detectadas con mayor frecuencia fueron la SDM, la SQX y la SMZ (veterinarias) y también el SMX. Los valores de la mediana vuelven a ser muy inferiores a los de la media en la mayoría de los casos, indicando de nuevo una tendencia de las concentraciones hacia valores inferiores. Si observamos la matriz de correlaciones que nos muestra la Tabla 3.6, al contrario que en el caso de aguas de salida de EDAR y aguas superficiales, no se aprecia una asociación destacable entre sulfamidas ni humanas ni veterinarias. La SMZ es la sulfamida que presenta asociaciones más elevadas con un mayor número de sulfamidas, principalmente veterinarias, como la SMP, la SMR o el STZ y también con la SPY, indicando un posible uso veterinario de esta última o una mayor estabilidad y resistencia a la biodegradación desde su vertido hasta alcanzar el acuífero. Además, como veremos en el siguiente capítulo de esta

Tesis, la presencia de su metabolito acetilado en esta matriz puede significar un aumento del compuesto original debido a su deconjugación.

	SSD	SMR	AcSMR	SQX	SDX	SuSTZ	SBZ	SDZ	ACSDZ	SDM	SMZ	AcSMZ	SMZ	SMP	SMX	ACSMX	SPY	ACSPY	STZ	SSX	SNT
SSD	**1**	0.340	0.160	0.086	**0.796**	-0.056	0.194	-0.040	-0.066	0.397	0.394	0.511	0.194	0.373	0.110	0.155	0.352	-0.134	0.535	0.024	0.296
SMR	0.340	**1**	-0.081	0.484	**0.731**	-0.035	0.110	-0.029	-0.042	**0.827**	**0.951**	0.601	0.140	0.954	0.059	0.060	**0.981**	-0.084	0.588	-0.007	**0.961**
ACSMR	0.160	-0.081	**1**	-0.094	0.089	-0.017	-0.040	-0.115	-0.106	-0.081	-0.096	0.168	-0.027	-0.095	-0.030	-0.026	-0.083	0.288	0.043	-0.075	-0.052
SQX	0.086	0.484	-0.094	**1**	0.253	-0.027	0.019	-0.036	-0.032	0.286	0.340	0.189	0.026	0.356	-0.002	-0.027	0.362	-0.063	0.183	-0.023	0.362
SDX	**0.796**	0.731	0.089	0.253	**1**	-0.045	0.204	-0.047	-0.054	**0.703**	**0.748**	0.591	0.196	**0.754**	0.069	0.098	**0.751**	-0.104	0.619	0.020	**0.697**
SuSTZ	-0.056	-0.035	-0.017	-0.027	-0.045	**1**	-0.040	-0.034	0.045	-0.053	-0.039	-0.053	-0.021	-0.028	-0.022	-0.064	-0.032	-0.036	-0.055	-0.034	-0.025
SBZ	0.194	0.110	-0.040	0.019	0.204	-0.040	**1**	-0.026	-0.048	0.451	0.206	0.171	-0.023	0.278	0.065	-0.059	0.183	-0.093	0.509	0.170	0.004
SDZ	-0.040	-0.029	-0.115	-0.036	-0.047	-0.034	-0.026	**1**	-0.041	0.008	-0.023	-0.049	0.026	-0.026	-0.026	0.009	-0.034	-0.020	-0.059	-0.041	-0.029
ACSDZ	-0.066	-0.042	-0.106	-0.032	-0.054	0.045	-0.048	-0.041	**1**	-0.063	-0.051	-0.063	-0.025	-0.034	-0.033	0.028	-0.037	0.543	-0.066	-0.041	-0.030
SDM	0.397	**0.827**	-0.081	0.286	**0.703**	-0.053	0.451	0.008	-0.063	**1**	**0.905**	**0.672**	0.390	**0.895**	0.073	0.055	**0.863**	-0.100	**0.818**	0.071	**0.719**
SMZ	0.394	**0.951**	-0.096	0.340	**0.748**	-0.039	0.206	-0.023	-0.051	**0.905**	**1**	**0.673**	0.246	**0.963**	0.100	0.068	**0.969**	-0.093	**0.720**	0.023	**0.907**
AcSMZ	0.511	**0.601**	0.168	0.189	0.591	-0.053	0.171	-0.049	-0.063	**0.672**	0.673	**1**	0.279	0.613	0.272	0.139	0.618	-0.118	**0.809**	0.011	0.547
SMZ	0.194	0.140	-0.027	0.026	0.196	-0.021	-0.023	0.026	-0.025	0.390	0.246	0.279	**1**	0.196	0.042	0.038	0.159	-0.049	0.456	0.091	-0.019
SMP	0.373	0.954	-0.095	0.356	**0.754**	-0.028	0.278	-0.026	-0.034	**0.895**	**0.963**	0.613	0.196	**1**	0.060	0.045	**0.985**	-0.067	0.686	0.051	**0.912**
SMX	0.110	0.059	-0.030	-0.002	0.069	-0.022	0.065	-0.026	-0.033	0.073	0.100	0.272	0.042	0.060	**1**	-0.007	0.061	-0.071	0.147	0.013	0.070
ACSMX	0.155	0.060	-0.026	-0.027	0.098	-0.064	-0.059	0.009	0.028	0.055	0.068	0.139	0.038	0.045	-0.007	**1**	0.047	0.141	0.099	-0.086	0.050
SPY	0.352	**0.981**	-0.083	0.362	**0.751**	-0.032	0.183	-0.034	-0.037	**0.863**	**0.969**	0.618	0.159	**0.985**	0.061	0.047	**1**	-0.076	**0.639**	0.020	**0.958**
ACSPY	-0.134	-0.084	0.288	-0.063	-0.104	-0.036	-0.093	-0.020	0.543	-0.100	-0.093	-0.118	-0.049	-0.067	-0.071	0.141	-0.076	**1**	-0.117	-0.043	-0.058
STZ	0.535	0.588	0.043	0.183	0.619	-0.055	0.509	-0.059	-0.066	**0.818**	**0.720**	**0.809**	0.456	0.686	0.147	0.099	0.639	-0.117	**1**	0.100	0.473
SSX	0.024	-0.007	-0.075	-0.023	0.020	-0.034	0.170	-0.041	-0.041	0.071	0.023	0.011	0.091	0.051	0.013	-0.086	0.020	-0.043	0.100	**1**	-0.031
SNT	0.296	**0.961**	-0.052	0.362	**0.697**	-0.025	0.004	-0.029	-0.030	**0.719**	**0.907**	0.547	-0.019	**0.912**	0.070	0.050	**0.958**	-0.058	0.473	-0.031	**1**

Tabla 3.6. Tabla de correlación de Pearson para las variables estudiadas.

Los resultados del PCA nos ofrecen 8 PCs que explican un 80% de la variabilidad de los datos, como muestra la Figura 3.9. Representaremos como siempre los loadings de los cuatro primeros PCs.

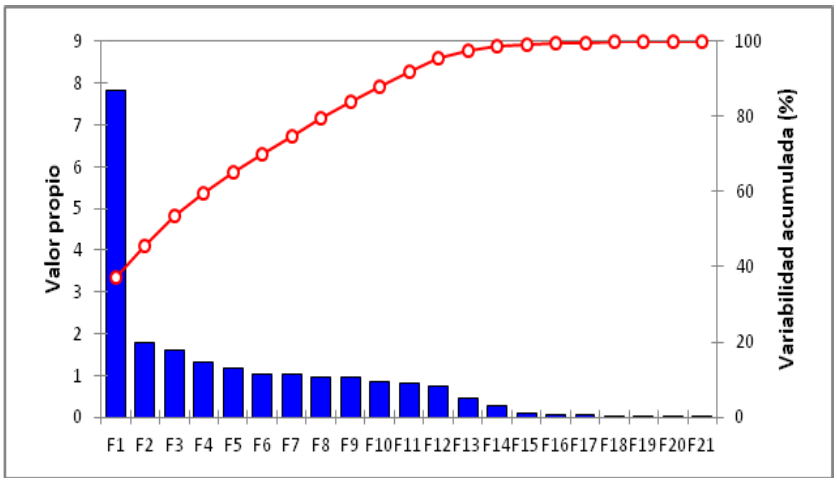

Figura 3.9. Valores propios y varianza acumulada de los componentes principales obtenidos

El primer PC explica un 37% de toda la varianza. Como se observa en la Figura 3.8, la mayoría de las variables poseen una correlación positiva elevada con el PC1, principalmente sulfamidas veterinarias como la SMR, el SNT, la SDM y la SMZ y su metabolito. Contribuyen negativamente los metabolitos acetilados AcSDZ, AcSMR y AcSPY, mientras que el SMX y el AcSMX tienen una correlación positiva, aunque baja. El PC2 explica un 8.3% de la variabilidad y las antiguas variables presentan por igual *loadings* positivos y negativos. El SNT, la SPY y la SMR presentan correlaciones negativas en este caso, al igual que la SMZ, aunque su valor es más pequeño.

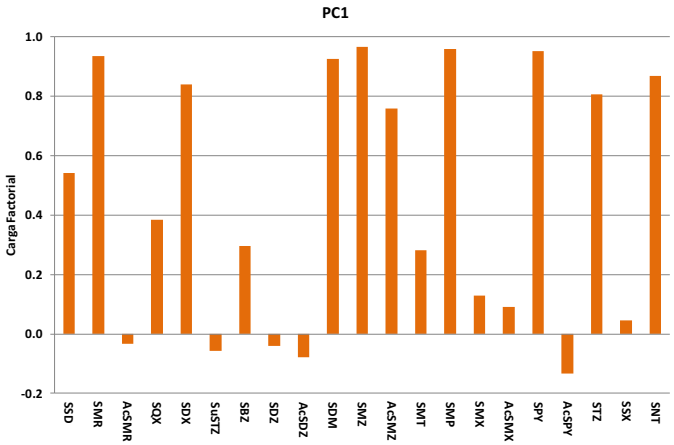

Figura 3.10. Diagramas de loadings para los cuatro primeros componentes principales.

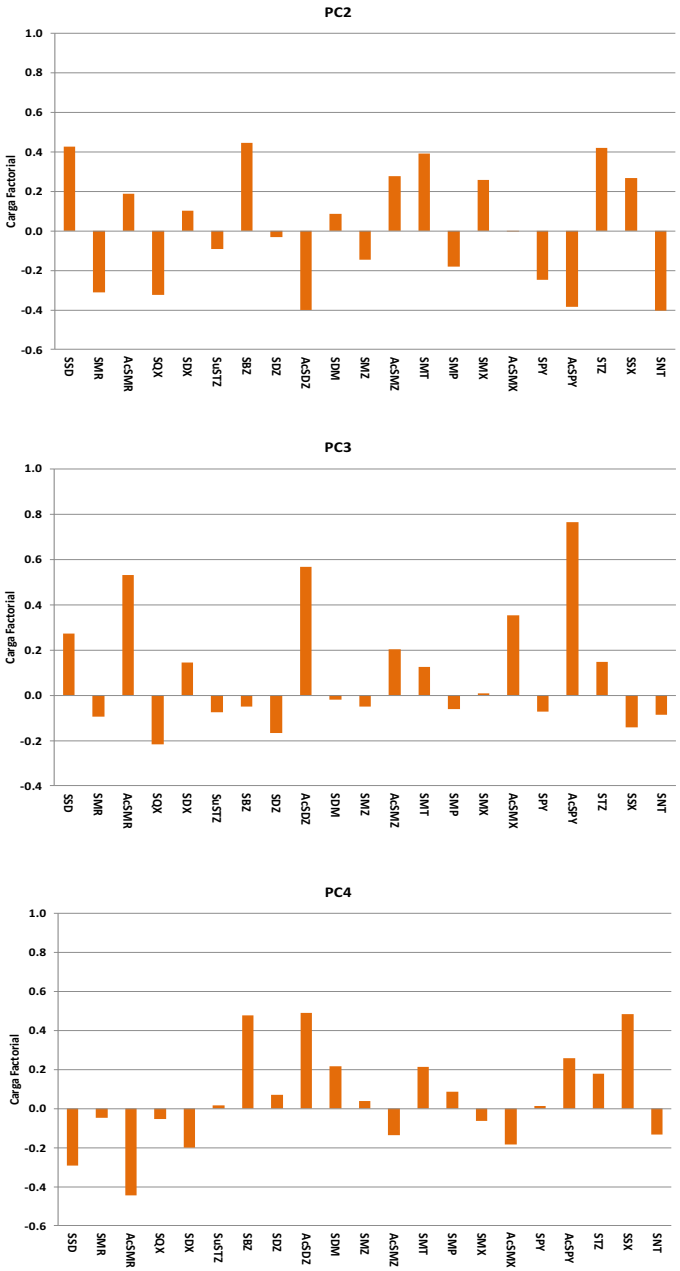

Figura 3.10. (continuación)

Representamos PC1 en abcisas y PC2 en ordenadas. Como vemos en la Figura 3.11, la mayoría de las muestras se ubican en los ejes negativos de ambos PCs, siendo AcSPY, AcSDZ y SDZ las sulfamidas con mayor presencia en estas muestras. Las cinco muestras que se localizan en el cuarto cuadrante, en el eje positivo de PC2, contienen principalmente AcSMR y no SMR. El resto de las muestras se localizan en el primer y segundo cuadrante del plano factorial. En el primer cuadrante las muestras presentan mayor proporción de AcSMZ, STZ, SMX y SSD, entre otros, mientras que las del segundo cuadrante contienen mayoritariamente SMR, SMP, SMZ y SPY entre otras.

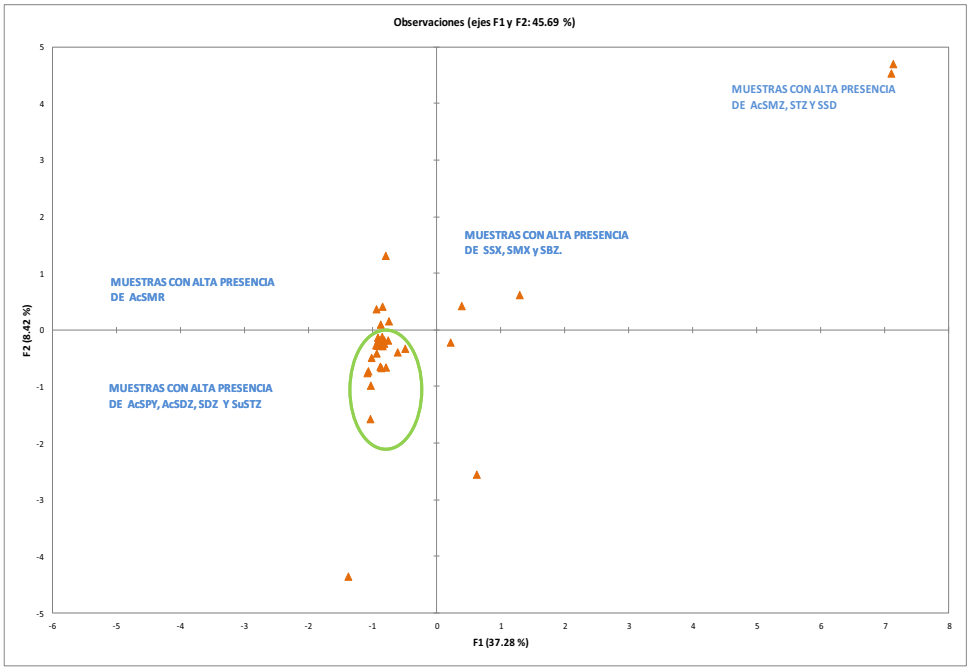

Figura 3.11. Plano factorial PC1-PC2 con todas las muestras de agua subterránea.

3.3.1.4. Presencia de sulfamidas en matrices sólidas

En tres de las publicaciones presentadas en esta Tesis (Publicación #10 de este Capítulo, Publicación #6 del Capítulo 2 y la Publicación #12 del Capítulo 4) se presentan los niveles de sulfamidas encontrados en fangos de depuradora y también en suelos. Se analizaron un total de 31 fangos de depuradora, 15 de ellas correspondientes a EDARs situadas en Cataluña y el resto provenientes de EDARs de la cuenca del Ebro, donde también se tomaron 15 muestras de suelo agrícola. Como muestra la Tabla 3.7, la frecuencia de detección y las concentraciones fueron por lo general más altas en los fangos de EDAR que en los suelos. El valor máximo fue para la SMZ y el AcSMR en ambos casos. El STZ y la SPY fueron las sulfamidas detectadas con mayor frecuencia en las muestras de fango y la SMZ yel AcSMX en los suelos agrícolas. La presencia de sulfamidas de consumo humano como el SMX y su metabolito AcSMX en suelos agrícolas (detectadas en el 40% y 33% de las muestras, respectivamente) puede atribuirse a la aplicación continuada de dichos fangos de depuradora como aporte orgánico de nutrientes al suelo, aunque los datos indican que su presencia en fangos es baja. La presencia de la SMZ y otras sulfamidas veterinarias como la SDM procede probablemente de residuos ganaderos aplicados como abono al suelo. Los valores de las medianas son por lo general inferiores a los de la concentración media para todas las sulfamidas, indicando una mayor incidencia de las concentraciones más bajas. Al estudiar la correlación entre la presencia de las variables, no se destaca ningún tipo de relación de éstas en suelo agrícola, de modo que la Tabla 3.8 muestra la matriz de correlaciones entre las variables sólo para fangos de depuradora. La SQX y la SDZ presentan las relaciones más fuertes con otras sulfamidas veterinarias como la SBZ, la SMZ o la SMP, y la presencia de la SPY y el STZ, a las que se les atribuye un mayor consumo humano que veterinario, también está relacionada con las anteriores.

Tabla 3.7. Niveles máximos y mínimos (ng L^{-1}), valor medio (Xn), mediana (Mn) y frecuencia de detección (F%) de las concentraciones de sulfamidas detectadas en fangos de depuradora (n=31) y en suelos agrícolas (n=15).

SULFAMIDA	FANGOS EDAR					SUELOS AGRÍCOLAS				
	Xn	Mn	MAX	MIN	F%	Xn	Mn	MAX	MIN	F%
SSD	1.27	0.15	6.63	0.02	19	0.11	0.11	0.22	0.01	13
SMR	0.32	0.25	0.50	0.20	10	0.06	0.06	-	-	7
SQX	27.89	3.20	120.45	0.25	16	0.43	0.43	0.56	0.30	13
SDX	0.16	0.14	0.25	0.09	10	0.02	0.02	-	-	7
Su-STZ	1.12	0.47	2.53	0.35	10	1.52	1.21	3.01	0.64	27
SBZ	1.90	0.50	8.85	0.09	23	0.34	0.30	0.51	0.22	20
SDZ	17.89	3.86	112.26	0.39	26	1.07	0.29	4.26	0.21	33
SDM	0.25	0.12	1.48	0.04	42	0.18	0.15	0.39	0.09	40
SMZ	20.95	1.13	139.28	0.33	26	9.69	5.76	20.42	3.32	**47**
SMT	1.06	1.17	1.85	0.04	13	-	-	-	-	-
SMP	1.49	0.74	4.95	0.11	16	1.01	0.83	2.58	0.34	33
SMX	12.87	0.40	75.59	0.07	23	4.30	0.71	15.59	0.15	40
SPY	9.02	0.89	121.95	0.20	**61**	0.25	0.22	0.57	0.09	33
STZ	8.53	2.86	76.99	0.02	**71**	0.72	0.72	-	-	7
SSX	1.03	0.71	2.50	0.35	16	1.74	1.38	2.47	1.38	20
AcSMX	0.60	0.79	0.90	0.11	10	0.29	0.29	-	-	7
AcSDZ	16.53	0.15	81.75	0.11	16	0.07	0.07	-	-	7
AcSMX	1.63	1.22	3.67	0.40	13	0.87	0.87	1.38	0.28	**60**
AcSPY	0.54	0.42	1.76	0.09	32	0.19	0.19	0.27	0.11	20
AcSMR	89.61	48.08	301.92	1.86	13	9.20	5.95	17.07	4.56	7
SNT	0.38	0.31	0.81	0.07	16	0.15	0.15	-	-	20

	SSD	SMR	SQX	SDX	SuSTZ	SBZ	SDZ	SDM	SMZ	SMT	SMX	SMP	SPY	STZ	SSX	AcSMZ	AcSDZ	AcSMX	AcSPY	SNT	ACSMR
SSD	1	-0.017	-0.009	0.103	0.053	-0.036	-0.028	0.053	-0.038	-0.040	-0.045	-0.034	-0.030	-0.047	-0.060	0.011	-0.021	-0.052	0.092	-0.023	0.018
SMR	-0.017	1	-0.044	0.188	0.006	0.006	-0.045	-0.100	-0.051	-0.051	-0.077	-0.057	-0.048	0.054	-0.083	0.026	-0.035	-0.038	-0.096	0.020	0.065
SQX	-0.009	-0.044	1	0.101	0.081	**0.977**	**0.997**	**0.920**	**0.976**	-0.025	-0.044	**0.973**	**0.981**	**0.915**	-0.059	0.027	-0.022	-0.054	0.212	-0.025	**0.827**
SDX	0.103	0.188	0.101	1	0.499	0.103	0.117	0.101	0.027	0.199	0.002	0.050	0.129	0.210	-0.107	0.451	0.102	-0.006	0.216	0.183	0.486
SuSTZ	0.053	0.006	0.081	0.499	1	0.096	0.089	0.050	0.017	0.127	-0.038	0.028	0.097	0.155	-0.131	0.254	0.079	0.096	0.169	0.126	0.324
SBZ	-0.036	0.006	**0.977**	0.103	0.096	1	**0.977**	**0.899**	**0.963**	-0.053	-0.061	**0.959**	**0.989**	**0.872**	-0.077	0.002	-0.031	-0.073	0.233	0.014	**0.797**
SDZ	-0.028	-0.045	**0.997**	0.117	0.089	**0.977**	1	**0.920**	**0.973**	-0.030	-0.042	**0.971**	**0.983**	**0.917**	-0.060	0.028	-0.023	-0.055	0.218	-0.025	**0.831**
SDM	0.053	-0.100	**0.920**	0.101	0.050	**0.899**	**0.920**	1	**0.896**	0.165	-0.026	**0.895**	**0.909**	**0.824**	-0.118	0.172	0.009	-0.025	0.227	-0.057	**0.759**
SMZ	-0.038	-0.051	**0.976**	0.027	0.017	**0.963**	**0.973**	**0.896**	1	-0.057	-0.009	**0.980**	**0.966**	**0.877**	-0.035	-0.028	-0.033	-0.050	0.178	-0.043	**0.774**
SMT	-0.040	-0.051	-0.025	0.199	0.127	-0.053	-0.030	0.165	-0.057	1	-0.054	-0.053	-0.045	-0.026	-0.063	0.790	0.306	0.008	0.148	-0.037	0.053
SMX	-0.045	-0.077	-0.044	0.002	-0.038	-0.061	-0.042	-0.026	-0.009	-0.054	1	-0.011	-0.030	0.052	0.435	-0.029	-0.039	0.043	-0.031	-0.054	-0.030
SMP	-0.034	-0.057	**0.973**	0.050	0.028	**0.959**	**0.971**	**0.895**	**0.980**	-0.053	-0.011	1	**0.964**	**0.878**	-0.076	-0.020	-0.029	-0.049	0.186	-0.038	**0.791**
SPY	-0.030	-0.048	**0.981**	0.129	0.097	**0.989**	**0.983**	**0.909**	**0.966**	-0.045	-0.030	**0.964**	1	**0.885**	-0.053	0.018	-0.026	-0.063	0.205	-0.029	**0.807**
STZ	-0.047	0.054	**0.915**	0.210	0.155	**0.872**	**0.917**	**0.824**	**0.877**	-0.026	0.052	**0.878**	**0.885**	1	-0.018	0.064	-0.015	-0.070	0.178	-0.006	**0.819**
SSX	-0.060	-0.083	-0.059	-0.107	-0.131	-0.077	-0.060	-0.118	-0.035	-0.063	0.435	-0.076	-0.053	-0.018	1	-0.120	0.035	-0.092	-0.119	-0.050	-0.008
AcSMZ	0.011	0.026	0.027	0.451	0.254	0.002	0.028	0.172	-0.028	**0.790**	-0.029	-0.020	0.018	0.064	-0.120	1	-0.104	-0.095	0.219	0.042	0.212
AcSDZ	-0.021	-0.035	-0.022	0.102	0.079	-0.031	-0.023	0.009	-0.033	0.306	-0.039	-0.029	-0.026	-0.015	0.035	-0.104	1	0.214	0.103	-0.018	0.037
AcSMX	-0.052	-0.038	-0.054	-0.006	0.096	-0.073	-0.055	-0.025	-0.050	0.008	0.043	-0.049	-0.063	-0.070	-0.092	-0.095	0.214	1	-0.089	-0.056	-0.047
AcSPY	0.092	-0.096	0.212	0.216	0.169	0.233	0.218	0.227	0.178	0.148	-0.031	0.186	0.205	0.178	-0.119	0.219	0.103	-0.089	1	**0.839**	0.266
SNT	-0.023	0.020	-0.025	0.183	0.126	0.014	-0.025	-0.057	-0.043	-0.037	-0.054	-0.038	-0.029	-0.006	-0.050	0.042	-0.018	-0.056	**0.839**	1	0.074
ACSMR	0.018	0.065	**0.827**	0.486	0.324	**0.797**	**0.831**	**0.759**	**0.774**	0.053	-0.030	**0.791**	**0.807**	**0.819**	-0.008	0.212	0.037	-0.047	0.266	0.074	1

Tabla 3.8. Tabla de correlación de Pearson para las variables estudiadas.

Aplicamos un PCA a todos los datos obtenidos y, como muestra la Figura 3.12, los 10 primeros PCs explican hata el 95% de la variabilidad de las datos estudiados. El primer PC explica 40% de toda la varianza,y el segundo un xxx.

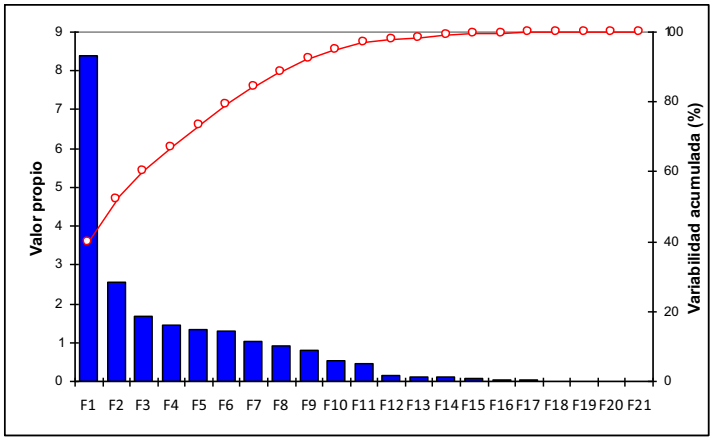

Figura 3.12. Valores propios y varianza acumulada de los componentes principales obtenidos

En la representación de los *loadings* que muestra la Figura 3.13, observamos que las sulfamidas detectadas con mayor frecuencia y a mayor concentración, como la SPY, el STZ o la SMZ, presentan una correlación positiva elevada con el PC1, mientras que pocas contribuyen negativamente, como es el caso de SMX y AcSMX. El PC2 explica un 12% de la variabilidad y en

este caso pocas variables presentan *loadings* positivos, aunque si lo hacen los metabolitos acetilados excepto AcSMX.

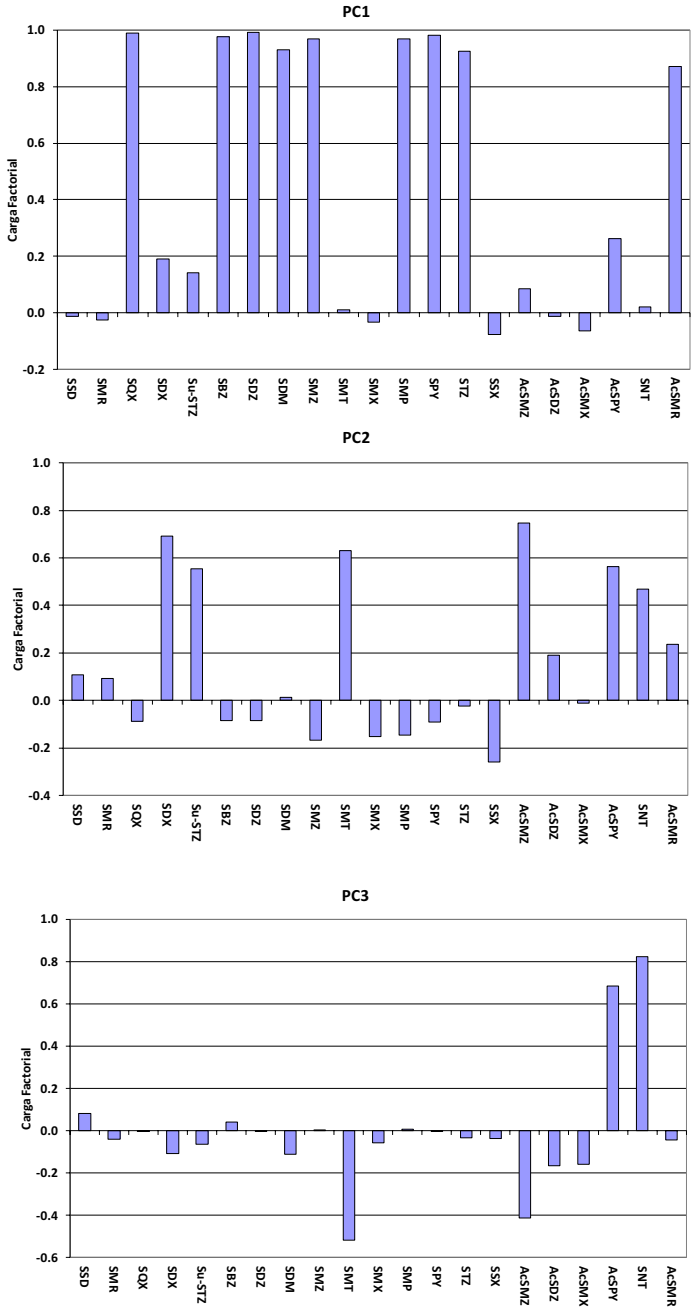

Figura 3.13. Diagramas de loadings para los cuatro primeros componentes principales.

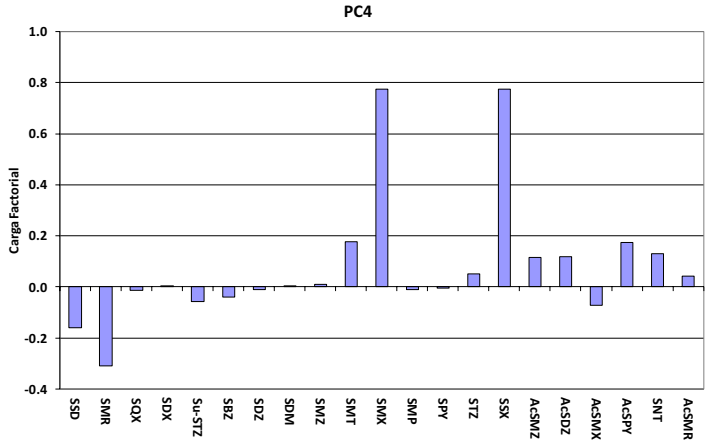

Figura 3.13 (continuación).

Por último, representamos las muestras analizadas en el plano factorial creado por PC1 en abcisas y PC2 en ordenadas. Como vemos en la Figura 3.14, la mayoría de las muestras de fango se ubican en el cuarto cuadrante donde las variables SSD, SMR y AcSDZ tienen un peso mayor. Algunos de estos fangos se desplazan al primer y segundo cuadrante debido al peso de concentraciones puntuales muy elevadas de SNT o AcSMR. Todas las muestras de suelo se localizan en el tercer cuadrante, destacando la presencia del SMX y su metaboito acetilado.

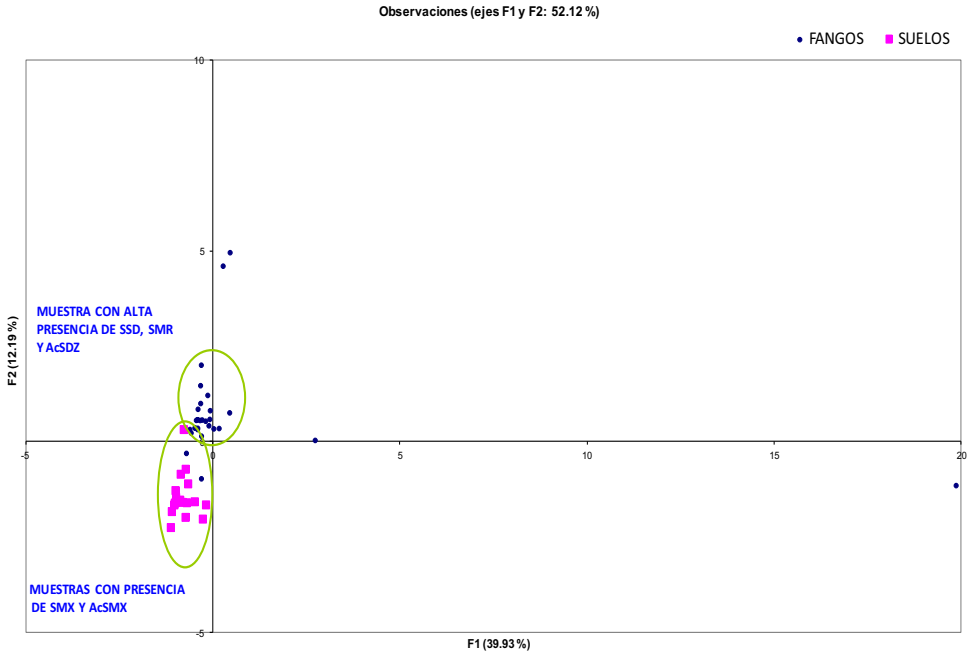

Figura 3.14. Plano factorial PC1-PC2 con todas las muestras de fangos de EDAR y suelos agrícolas analizadas.

3.3.2. Eliminación de las sulfamidas y sus metabolitos en las EDARs

En tres de las publicaciones presentadas en este Capítulo, el análisis de muestras de entrada de EDAR nos permitió estimar la eliminación de las sulfamidas a su paso por las estaciones depuradoras y también calcular, en función del HRT de cada EDAR, su vida media. La Figura 3.15 muestra los valores de eliminación para cada una de las sulfamidas estudiadas.

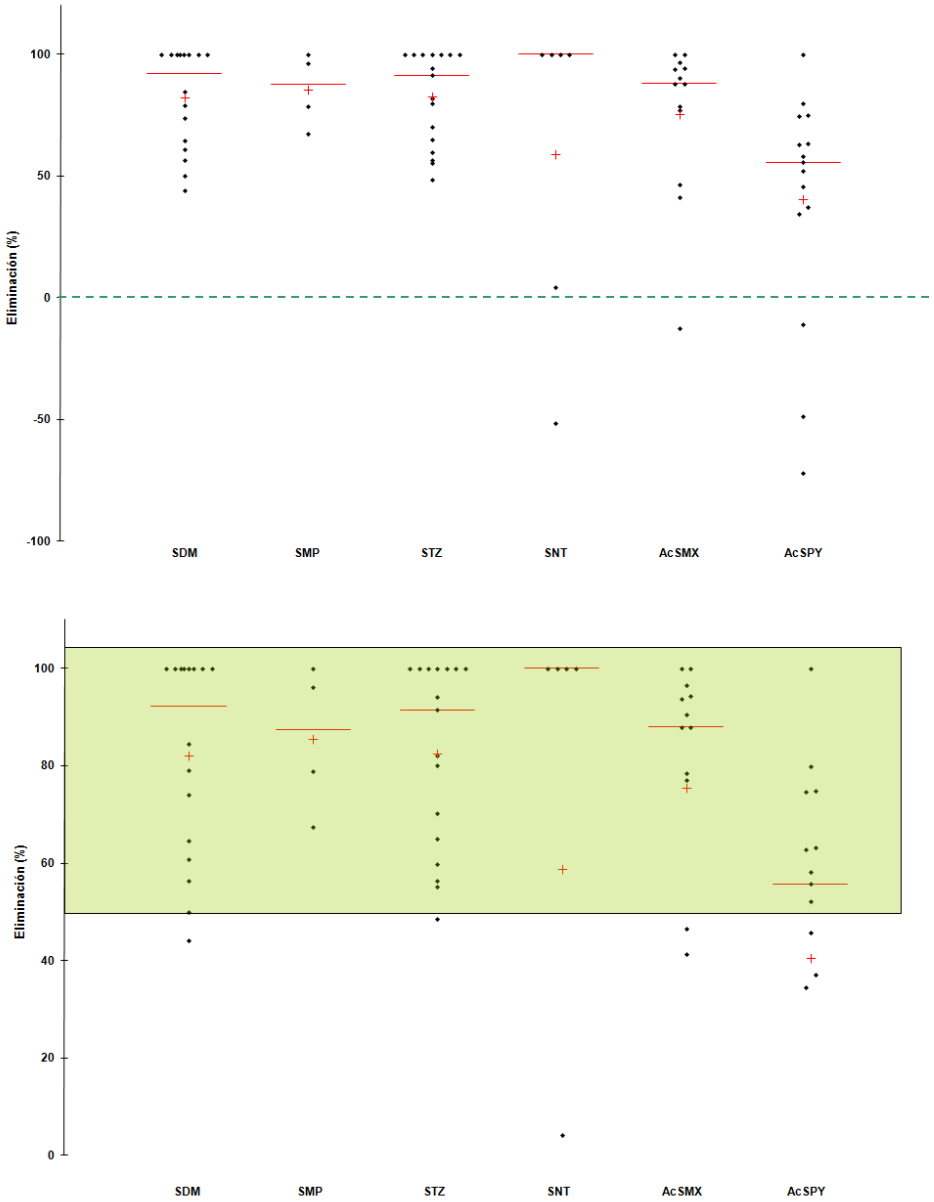

Figura 3.15. Diagramas de dispersión de los valores de eliminación de las sulfamidas estudiadas durante el tratamiento en las estaciones depuradoras.

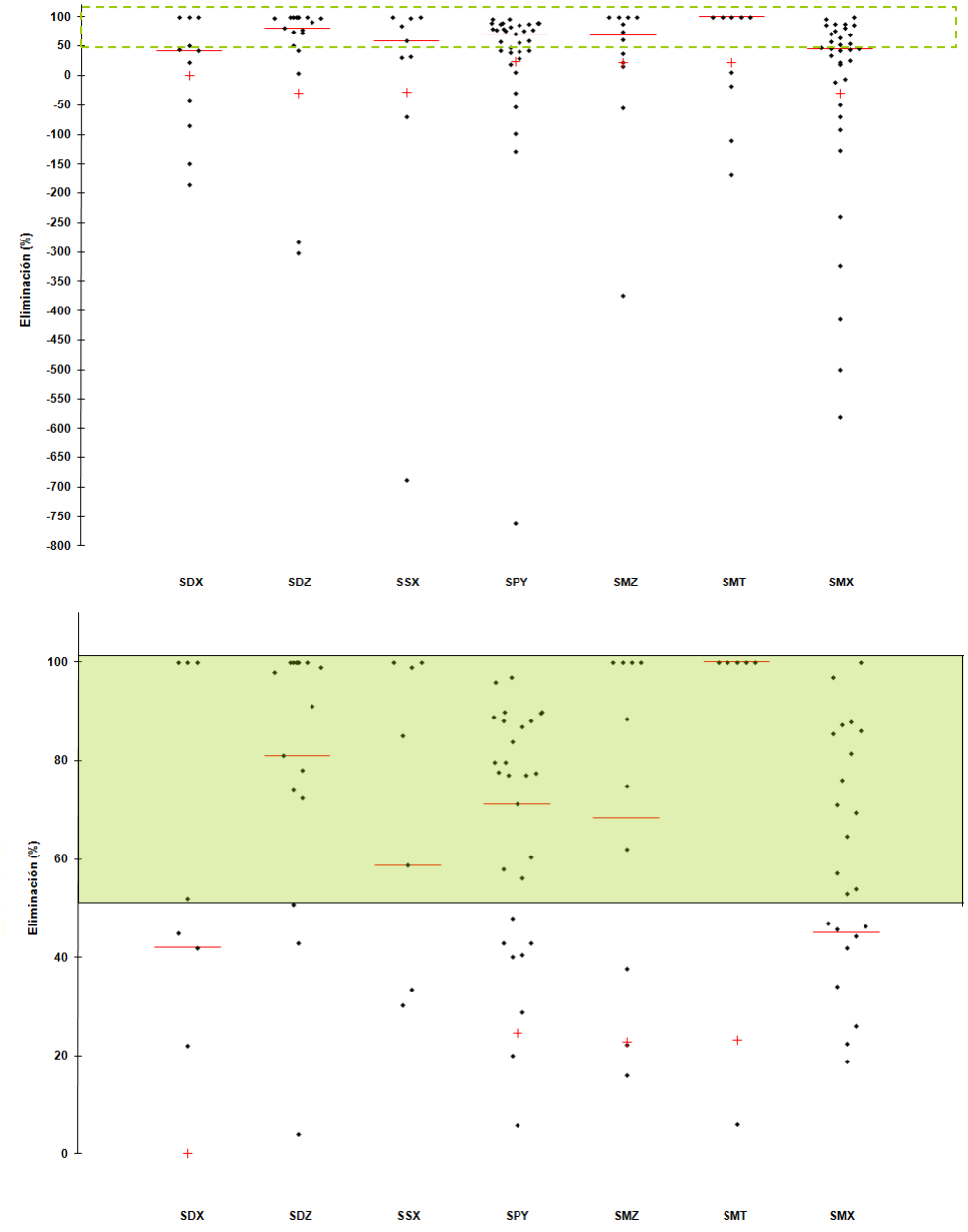

Figura 3.15 (continuación)

La zona del eje de ordenadas comprendida entre los valores 0% y 100% ha sido aumentada en los diagramas inferiores, y la zona de eliminación comprendida entre 50% y 100%, que podemos considerar como óptima, ha sido destacada. Podemos diferenciar tres casos concretos:

- *No se detecta concentración del compuesto en el agua de salida de la EDAR, pero si en el agua de entrada:* en este caso se asume una eliminación del 100%.
- *La concentración del compuesto en el agua de salida es mayor que en la del agua de entrada*: en este caso obtenemos valores de eliminación negativos.
- *Detectamos sólo concentración de la sulfamida en el agua de salida de la EDAR*: no es posible estimar la eliminación del compuesto.

En general, la dispersión de los valores de eliminación es muy amplia, haciendo la discusión de los datos más complicada. SDM, SMP y STZ son las únicas sulfamidas que no presentan eliminación negativa en ninguna de las EDAR estudiadas, mostrando unos valores en su mayoría comprendidos entre el 50% y el 100%. Las sulfamidas con mayor número de RE% negativos son SDX, SMT y SMX. Si bien los valores negativos no son muy frecuentes, sus valores suelen superar el -100%, indicando que las concentraciones detectadas a la salida son varios ordenes de magnitud superiores a los de la entrada. Para SMX y SPY, las sulfamidas detectadas con mayor frecuencia y con una mayor contribución a la carga de masa total en las EDARs, se han construido histogramas de distribución. Como muestra la Figura 3.16, la mayoría de los EF% calculados para SMX se encuentran en el rango 25%-53% (23%), 53%-82% (20%) y 82%-100% (18%). Para SPY, sin embargo, el 52% de los valores calculados son superiores al 64% de eliminación. Respecto a los metabolitos acetilados, AcSDZ y AcSMZ han sido detectadas en sólo dos EDAR y AcSMR sólo en una. AcSMX y AcSPY por el contrario han sido detectadas con mucha mayor frecuencia y a mayores concentraciones, al igual que los respectivos compuestos originales. La mayoría de los valores de RE% de AcSMX son superiores al 77% (en 10 de 13 EDARs). Ya que esta eliminación no significa una completa mineralización y eliminación del compuesto, sino que también puede significar su transformación en un compuesto diferente, podemos asumir su desconjugación con la pérdida del grupo acetilado, generando de nuevo SMX. Esta transformación justificaría las concentraciones más elevadas de SMX a la salida que a la entrada de la planta, y de hecho ya ha sido considerado por diferentes autores y demostrado recientemente en la Publicación #12 presentada en esta Tesis.

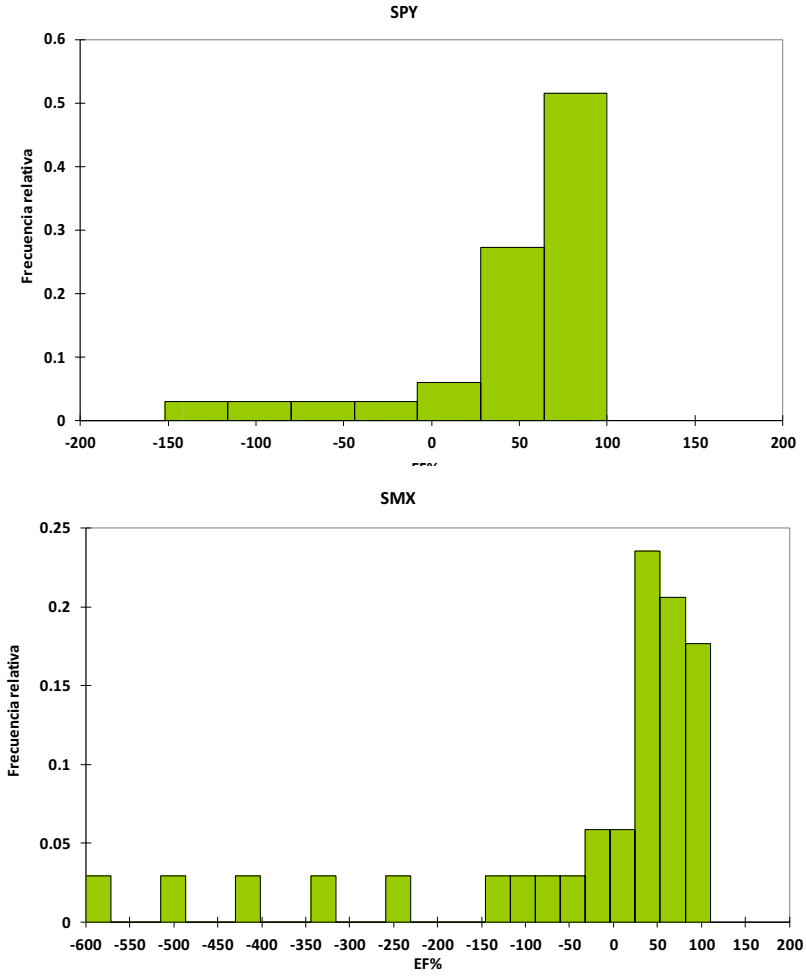

Figura 3.16. Histogramas para sulfapiridina (SPY) y sulfametoxazol (SMX). El valor de EF%= 700 de SPY ha sido excluido por practicidad, al tratarse del único valor negativo para esta sulfamida.

En el caso de AcSPY el rango de valores de eliminación es más amplio, y sólo 4 de los 15 valores está por encima del 75%. Por último, valores de eliminación negativos para estos metabolitos podrían significar la acetilación del compuesto original durante el tratamiento biológico. En los diferentes estudios de eliminación en las EDARs presentadas en este capítulo, la no correspondencia temporal de las muestras de entrada y salida dificulta la corroboración de dicha hipótesis y, como demuestra la Figura 3.17, no existe la correlación negativa esperada entre los valores de RE% del

compuesto original y del metabolito. Recientemente, diferentes publicaciones han intentado justificar la disparidad de los valores de RE% que su suelen observar en los estudios de eliminación en depuradoras mediante la modelización y caracterización de los flujos de entrada y salida de la EDAR independientemente de la presencia y naturaleza de los metabolitos presentes en el agua residual. Así, Ort *et al*. (Ort *et al.* 2010) explican que las aguas de entrada son masas de composición muy heterogénea y que como mínimo sería necesario un muestreo en intervalos de 5 min o menos para poder realizar una buena caracterización de las variaciones temporales que se registran en el influente diariamente. Asimismo, Majewsky *et al.* (Majewsky *et al.* 2011) afirmaron que el HRT de la EDAR proporciona una información muy escasa, ya que no considera las mezclas y la distribución de los contaminantes en los diferentes tanques. Demostraron también que la carga de contaminantes del agua de entrada se distribuye en el efluente de la EDAR durante más de un día, y que las muestras integradas de 24 h de este influente, tomadas considerando el retraso debido al HRT de la EDAR, contendrían menos de la mitad de la carga total del influente. Sin embargo, la mayoría de las publicaciones hasta la fecha han considerado el HRT de las EDAR como parámetro fundamental en la eliminación de diferentes fármacos, concluyendo que un mayor HRT significaba una mayor eficiencia de la EDAR. La Figura 3.18 muestra la correlación de HRT con los valores EF% obtenidos para las sulfamidas en los diferentes trabajos presentados en este Capítulo. Las EDAR estudiadas trabajaban con HRT desde 4 h hasta 30 días, de modo que se tendría que observar una tendencia ascendente de los valores a medida que aumenta HRT. Sin embargo, si observamos los valores de EF% comprendidos entre 70%-100%, vemos que en su mayoría se encuentran en HRT inferiores a 5 días. Igualmente, para EDARs con HRT < 5 d vemos que los valores de EF% van desde 4% hasta el 100%. SPY se elimina con EF% superiores al 70% tanto en EDARs con HRT inferior a 1 día y en EDARS con HRT de más de 20 días. Por tanto, consideramos que HRT no es un parámetro crítico en la eliminación de las sulfamidas durante el tratamiento de aguas residuales.

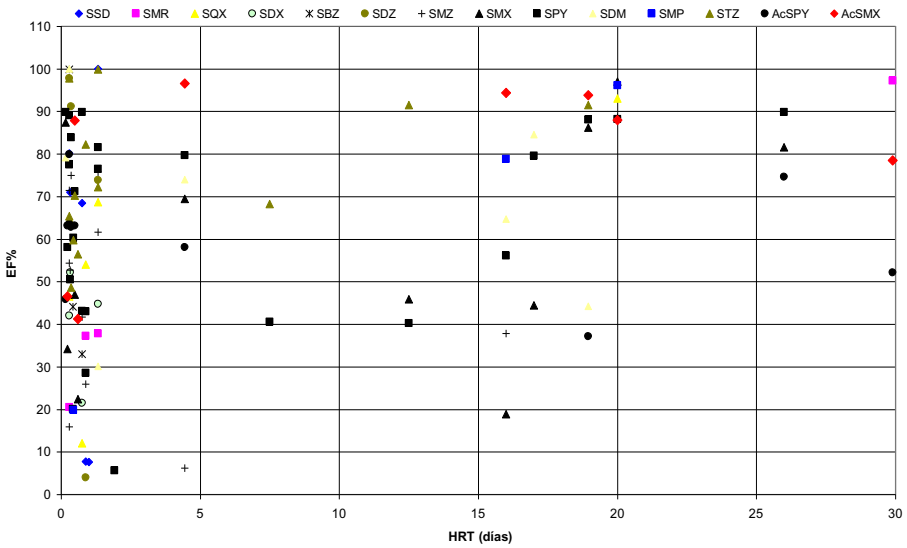

Figura 3.17. Correlación entre los valores de eliminación (EF%) obtenidos en las diferentes estaciones depuradoras con sus respectivos tiempos de retención hidráulicos (HRT).

3.3.3. Evaluación de toxicidad

En dos de las publicaciones presentadas en este Capítulo se han estimado coeficientes de riesgo (HQ) derivados de la presencia de sulfamidas en aguas naturales, siguiendo las directrices de la EMEA (EMEA-CVMP 2004; EMEA-CHMP 2006). Sólo SMX obtuvo valores de HQ superiores a 1 para las concentraciones detectadas en efluentes de EDAR, utilizando para la estimación del HQ las concentraciones medioambientales detectadas en aguas de salida de EDAR y superficiales, y únicamente para algas verdeazuladas (Publicación #9), mientras que el resto de las sulfamidas estudiadas obtuvieron valores hasta varios ordenes de magnitud por debajo del valor límite de 1. En referencia a la toxicidad de los metabolitos mayoritarios, tan solo Eguchi *et al*. han aportado datos de toxicidad para metabolitos acetilados de las sulfamidas y siempre trabajando con valores de concentración mucho más elevados que la de los respectivos compuestos originales (Eguchi *et al.* 2004). Ya que no existía ningún valor de EC_{50} en la literatura para AcSPY, aún siendo una de las sulfamidas detectadas con mayor frecuencia, realizamos el estudio para evaluar su toxicidad junto a la de SPY. En la Publicación #10 se

demostró que el metabolito era más tóxico que SPY para *V. fischerii*, demostrando de nuevo la importancia de incluir metabolitos en los futuros estudios de presencia ambiental.

Sin embargo, para calcular valores de HQ>1 para SPY y AcSPY a partir de estos nuevos EC_{50} (ver Tabla 2 de la Publicación #10) se utilizarón concentraciones reales detectadas en aguas de salida de depuradora. Estos efluentes son diluidos en mayor o menor medida al desembocar en los ríos receptores, de modo que el riesgo teórico se vería disminuido. Por otro lado, ya que estos cálculos se realizan utilizando valores de EC_{50} o LC_{50}, normalmente derivados de ensayos de toxicidad aguda, son considerados tan sólo como valores de referencia ya que se consideran poco representativos de las condiciones ambientales. Estudios de toxicidad crónica serían por tanto muy necesarios para establecer con mayor solidez riesgos ambientales derivados de la presencia de las sulfamidas y demás contaminantes orgánicos.

4. BIODEGRADACIÓN AERÓBICA EN FANGOS Y AGUAS RESIDUALES

4.1. INTRODUCCIÓN

La ya demostrada ubicuidad de las sulfamidas en los diferentes ecosistemas, tanto acuáticos como edáficos, ha generado la necesidad de evaluar su mayor o menor biodegradabilidad una vez vertidas al medio natural para poder completar así el estudio sobre su presencia y destino en el medioambiente. En el caso de aguas superficiales receptoras de los vertidos de las EDARs, el transporte, destino y efectos biológicos de las sulfamidas dependen de las condiciones hidrológicas reales del río, incluyendo variables tanto físicas (dilución, dispersión), como químicas (volatilización, adsorción, fotólisis) y biológicas (transformación, asimilación). Sin embargo, la mayoría de los estudios llevados a cabo en el laboratorio suelen centrarse en un único proceso y bajo condiciones controladas, de modo que si bien proporcionan una información muy valiosa sobre el comportamiento y la persistencia del compuesto, ésta podría considerarse poco representativa de los procesos que realmente están sucediendo en condiciones ambientales. Los estudios de atenuación natural en ríos han supuesto un gran avance en este ámbito. Su objetivo es observar el comportamiento del contaminante en cuestión a lo largo de un tramo del río, combinando la toma de muestras en diferentes puntos a lo largo de la cuenca con la medida del *"travel time"* o tiempo de desplazamiento del contaminante entre puntos de muestreo (utilizando para ello trazadores naturales como el bromuro, tintes, etc), y considerando diferentes parámetros físicoquímicos tanto del compuesto como del agua del río. Con frecuencia se utilizan los puntos de vertido de las EDARs en los ríos receptores como origen de la contaminación y zona de inicio del tramo del río a investigar y se intentan establecer gradientes de concentración con toda la información obtenida.

En referencia a la mejora de los procedimientos de depuración de aguas residuales para mejorar la eliminación de contaminantes orgánicos como los fármacos, los biorreactores

de membrana (MBR) han sido la alternativa mejor considerada al CAS y utilizada con frecuencia creciente en la última década. Estos biorreactores ofrecen la particularidad de combinar el tratamiento convencional con filtración a través de unas membranas que se encuentran generalmente sumergidas en el reactor. Mediante presión, el agua pasa a través de estas membranas obteniendo un efluente de alta calidad sin necesidad de sedimentación y filtrado posterior. Ya que el filtrado tiene lugar dentro del reactor, se consigue también una retención de sólidos mayor, trabajando con cantidades de sólidos totales disueltos (TDSS) hasta cuatro veces más altas que los reactores convencionales. Las comparaciones entre la efectividad de CAS y MBR para eliminar diferentes fármacos ha sido objeto de estudio en diferentes trabajos científicos, si bien las sulfamidas aparecen en pocos de ellos, siendo normalmente el SMX la única incluida en el análisis (Clara *et al.* 2005; Göbel *et al.* 2007; Radjenovic *et al.* 2007; Tambosi *et al.* 2010). Los reactores de lecho fijo (FBBR, del inglés *fixed bed bioreactor*) por su parte ofrecen un soporte fijo para los microorganismos que intervienen en la biodegradación, favoreciendo su desarrollo y actividad. Son sistemas que permiten ejemplificar con facilidad la biodegradación biológica en diferentes tipos de aguas debido principalmente a la sencillez de su montaje. Ambos tipos de tecnologías se utilizan como alternativa al tratamiento convencional o bien como tratamiento terciario.

En todo caso, las comunidades bacterianas presentes en los reactores de CAS son el elemento fundamental del buen funcionamiento del proceso de depuración en las EDARs. Como ejemplo, la peroxidasa *Bjerkandera adusta* es capaz de oxidar el SMX, mientras que bacterias como *Rhodococcus rhodochrous o Pseudomonas aeruginosa* pueden utilizarlo como cosubstrato (Larcher *et al.* 2011; Bouju *et al.* 2012; Gauthier *et al.* 2012). Sin embargo, hasta el momento no se ha demostrado que ninguna cepa bacteriana sea capaz de mineralizar completamente ninguna sulfamida. Recientemente, la gran capacidad degradativa de determinados hongos para la eliminación de contaminantes recalcitrantes como el benceno o el tolueno (Aranda *et al.* 2010) ha llevado a su aplicación en la eliminación de diferentes fármacos que apenas se degradan en las EDARs, como la carbamazepina, el diclofenaco o el naproxeno entre otros, consiguiendo resultados experimentales favorables para todos ellos (Blánquez *et al.* 2008; Marco-Urrea *et al.* 2009; Marco-Urrea *et al.* 2010; Marco-Urrea *et al.* 2010b; Marco-Urrea *et al.* 2010c; Jelic *et al.* 2012). *Trametes versicolor*, una especie de hongo de la familia de los basidiomicetes, ha sido el hongo estudiado en un mayor número de ocasiones, ya que posee sistemas enzimáticos oxidativos muy potentes, tanto intracelulares (citocromo P450) como

extracelulares (principalmente lacasas y peroxidasas), que le permiten utilizar una gran variedad de substratos como fuente de carbono, dada su inespecificidad. Demuestran, por tanto, un gran potencial para ser utilizadas en proyectos de bioremedación.

Los objetivos de los trabajos desarrollados en este capítulo son los siguientes:

- Comparar la eliminación de diferentes sulfamidas durante tratamiento convencional y MBR.
- Estudiar la cinética de degradación de la sulfapiridina y de su metabolito acetilado en reactores FBBR.
- Evaluar la degradación de SMZ, SPY y STZ con el hongo *Trametes versicolor*, e identificar y confirmar los posibles productos de transformación.

4.2. PUBLICACIONES

Publicación científica 11

"Removal of sulfonamide antibiotics upon conventional activated sludge and advanced membrane bioreactor treatment"

María Jesús García Galán, M. Silvia Díaz-Cruz, Damià Barceló

Analytical and Bioanalytical Chemistry (2012), Vol. 404, PP. 1505-1515.

Publicación científica 12

"Biodegradation studies of N^4-acetylsulfapyridine and N^4-acetylsulfamethazine in environmental water by applying mass spectrometry techniques"

María Jesús García Galán, Tobias Frömel, Jutta Müller, Manuela Peschka, Thomas Knepper, M. Silvia Díaz-Cruz, Damià Barceló

Analytical and Bioanalytical Chemistry (2012), Vol. 402, PP. 2885-2896

Publicación científica 13

"Biodegradation of sulfamethazine by *Trametes versicolor*: Removal from sewage sludge and identification of intermediate products by UPLC–QqTOF-MS"

María Jesús García Galán, Carlos E. Rodríguez-Rodríguez, Teresa Vicent , Gloria Caminal, M. Silvia Díaz-Cruz, Damià Barceló

Science of the Total Environment (2011), Vol. 409, pp.5505-5512

Anal Bioanal Chem (2012) 404:1505–1515
DOI 10.1007/s00216-012-6239-5

ORIGINAL PAPER

Removal of sulfonamide antibiotics upon conventional activated sludge and advanced membrane bioreactor treatment

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Abstract This work reports the removal efficiencies of nine sulfonamides (SAs) and one of their acetylated metabolites during conventional activated sludge (CAS) and membrane bioreactor (MBR) treatments. Two different types of membranes were studied, hollow-fiber membranes and flat-sheet membranes, in two separate pilot plants operating in parallel to a full-scale CAS treatment. A total of 48 water samples and 16 sewage sludge samples were analyzed by liquid chromatography–tandem mass spectrometry. We obtained 100 % elimination in the MBR effluents for three SAs (sulfadiazine, sulfadimethoxine, and sulfamethoxypyridazine) and the metabolite. For the rest of the SAs, the removal efficiencies during CAS and MBR treatments were similar and usually below 55 %. Sulfamethizole was the most recalcitrant SA, exhibiting negative removal efficiencies in all the treatments investigated. The concentrations of SAs in the different sewage sludge types were also calculated and ranged from 0.01 to 11 ng g^{-1}. Furthermore, adsorption and biodegradation of SAs in activated sludge were investigated in two sets of batch reactors, which were spiked at high and low concentration (1,000 and 50 ng mL^{-1}, respectively). All SAs followed a similar trend and, with the exception of sulfathiazole, were not fully eliminated after 25 days of treatment.

Electronic supplementary material The online version of this article (doi:10.1007/s00216-012-6239-5) contains supplementary material, which is available to authorized users.

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Keywords Sulfonamides · Wastewater · Conventional wastewater treatment · Membrane bioreactor · Removal efficiencies

Introduction

During the last few years several studies have clearly demonstrated that nearly all therapeutic classes of pharmaceuticals are widely present in the different environmental compartments. Special attention is devoted to the aquatic environment as most of these substances are biologically active and may inflict serious adverse effects on the diverse aquatic organisms. The best documented cases are pharmaceuticals with endocrine disruption properties, such as 17β-estradiol and estrone [1], and also the resistance developed by some environmental bacteria in the presence of certain antibiotics [2]. Adverse effects have also been demonstrated for sulfonamides (SAs), a family of antibiotics commonly used in cattle-rearing facilities and also in human medicine. SAs have been detected in basically all kinds of environmental water matrices [3–5]. In urban ecosystems, wastewater treatment plants (WWTPs) are considered to be the main entry pathways for SAs and pharmaceuticals in general, and several works have been devoted to evaluating their presence and fate in influent and effluent wastewaters [6–8]. The incomplete removal of some of these antibiotics during conventional activated sludge (CAS) treatment can be directly linked to their presence not only in surface waters and sediments but also in soils and groundwater bodies. Furthermore, the application of WWTP sewage sludge as organic amendment to croplands represents another environmental input for these compounds [9, 10].

The potential ecotoxicity derived from the environmental presence of pharmaceuticals (including SAs) makes the search for alternative treatment technologies a priority. Besides, the

low concentrations at which they are present in WWTPs make their elimination a complicated issue. Membrane bioreactors (MBRs) have become a promising wastewater treatment technology in the last few years [11]. Their main advantage over CAS treatment is that these bioreactors integrate biological degradation and membrane filtration in one step. In MBR systems, there is no secondary clarifier, and separation of the treated sewage and the sludge is achieved via membrane filtration, producing much higher retention of suspended solids within the reactor and thus creating richer mixed liquors, which are capable of degrading a wider range of organic pollutants [12–14]. This also means longer solids retention times (SRTs), which allow the development of slow-growing bacteria, such as nitrifiers, the adaptation of some microorganisms to degrade certain persistent pollutants, and the retention of exocellular enzymes or soluble oxidants, which are usually eliminated in CAS treatments and which can contribute to creating a much more active mixture liquor. These longer SRTs correlate with improved effluent quality parameters such as chemical oxygen demand and levels of nitrogen, phosphorous, and suspended solids.

In this work, the removal of nine SAs and one acetylated metabolite in a full-scale CAS treatment and in two different pilot-scale MBRs was studied. Total input and output loads of SAs were calculated and removal efficiencies (RE) were established for each treatment. The presence of SAs in the different types of sewage sludge was investigated. Simultaneously, the biodegradability of some of the selected SAs in sewage collected from the CAS reactor was assessed in the laboratory.

Materials and methods

Chemicals

High-performance liquid chromatography (HPLC)-grade solvents (water, methanol, acetone, and acetonitrile) and formic acid (98-100 % purity) were supplied by Merck (Darmstadt, Germany). High-purity standards (greater than 99 % purity) of the nine SAs and one acetylated metabolite studied, namely, sulfadiazine (SDZ), sulfadimethoxine (SDM), sulfamethazine (SMZ), sulfamethizole (SMT), sulfamethoxazole (SMX), sulfamethoxypyridazine (SXP), sulfapyridine (SPY), sulfathiazole (STZ), sulfisoxazole (SSX), and N^4-acetylsulfamethazine (AcSMZ), were purchased from Sigma-Aldrich (St Louis, MO, USA). Isotopically labeled compounds d_4-sulfamethoxazole (d_4-SMX) and d_4-sulfathiazole (d_4-STZ), used as a surrogate and as internal standard, respectively, were purchased from Toronto Research Chemicals (North York, ON, Canada). Further information regarding stock solutions and solvents is given in Díaz-Cruz et al. [15]. Oasis HLB solid-phase extraction (SPE) cartridges (200 mg, 6 mL) were from Waters (Milford, MA, USA).

Sampling site

Terrassa is located 30 km to the northwest of Barcelona. Its WWTP serves 195,160 inhabitants, and it has been designed for a capacity of 500,000 equivalent inhabitants, although its actual capacity is devoted to 277,000. It receives domestic waters (80 %) and industrial waters, mainly from the pharmaceutical and textile industries (20 %). The average daily flow at the time of the sampling was 46,447 m^3 day^{-1}. Treatment consisted of a physicochemical primary treatment (grit and grease removal), followed by a primary clarifier or sedimentation unit, and a CAS treatment and secondary clarifier as secondary treatment. An aeration tank and an anaerobic digester were used for the biological treatment (Fig. 1). The average hydraulic retention time (HRT) and the SRT for the CAS treatment are given in Table 1. Part of the secondary sludge is recirculated to the aeration tank, and the rest is combined with the primary sludge and treated further (dewatered and digested in anaerobic conditions). Biosolids in the anaerobic digester are stabilized for 30 days at approximately 34 °C. The average amount of treated sludge produced in this plant is 40 $t day^{-1}$.

The two pilot-scale MBRs operated in parallel with the CAS treatment. Both were inoculated with the activated sludge and fed with the same wastewater as the conventional biological treatment. Two different types of submerged membranes were employed: hollow-fiber ultrafiltration membranes (Koch), from Koch Membrane Systems (Wilmington, MA, USA), and flat-sheet microfiltration membrane modules (Kubota), from Kubota (Osaka, Japan).

Sample collection

The sampling was done during 4 weeks from March to April 2007, 3 days a week. A total of 48 water samples were taken, 12 samples for each kind of water matrix: WWTP influent (after the primary clarifier), CAS effluent, Koch MBR effluent, and Kubota MBR effluent. All of them were sampled as 24-h composites, and were transported in cool conditions to the laboratory, where they were immediately filtered through 0.45-μm nylon membrane filters (Whatman, UK). Triplicates of 100 mL of WWTP influent and 200 mL of the three different effluents were extracted through Oasis HLB cartridges. The SPE procedure is described elsewhere [15]. Before SPE, d_4-SMX was added to each water sample as a surrogate at 100 ng mL^{-1}(final concentration in the vial). d_4-STZ was added to the vials immediately before injection at a concentration of 50 ng mL^{-1}.

During the same period, sewage sludge samples were taken weekly as grab samples. Five different types of sludge were sampled: primary sludge (from the first clarifier), activated sludge (from the aeration basin of the CAS

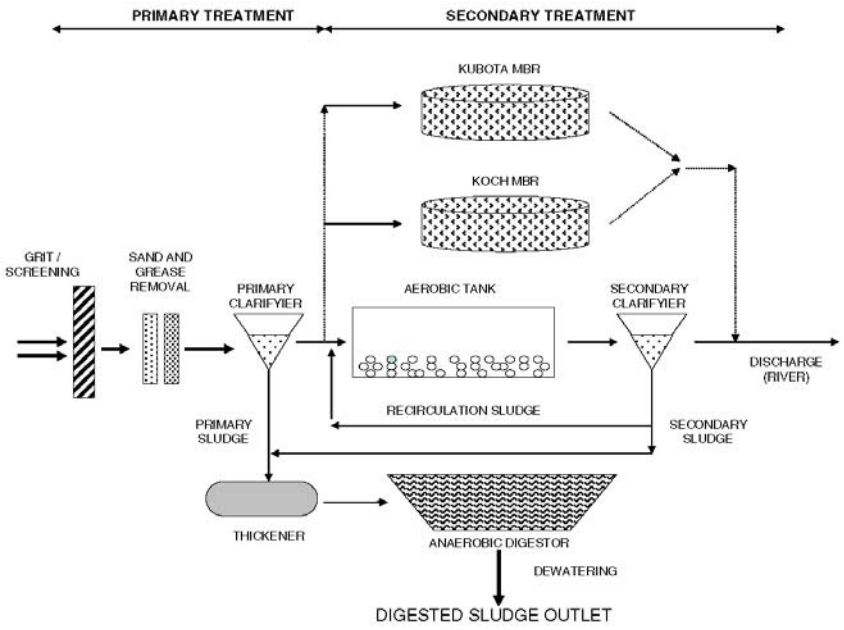

Fig. 1 The design of the wastewater treatment plant (WWTP) studied. *MBR* membrane bioreactor

treatment), treated dehydrated sludge, Koch sludge, and Kubota sludge. They were transported in cool conditions and centrifuged upon arrival at the laboratory, and the solid fraction was freeze-dried (−40 °C and 0.044-mbar vacuum). All samples were extracted in triplicate by pressurized liquid extraction using an ASE 200 accelerated solvent extractor (Dionex, Sunnyvale, CA, USA) with a water–acetonitrile mixture (75:25, v/v) at 50 °C. Optimized parameters are given elsewhere [16]. Prior to extraction, the surrogate was added at a concentration of 50 ng g^{-1}. The approximately 15 mL extracts were diluted with HPLC-grade water (100 mL) to reduce the proportion of organic solvent content, and then were preconcentrated and purified following SPE as described for the water samples.

Biodegradation experiments

A modified Zahn–Wellens test [17] was applied in two sets of batch reactors to evaluate the biodegradability of the target SAs during CAS treatment. Briefly, each batch was set up with 1 L of activated sludge and the target analyte was added at high concentration (1,000 ng mL^{-1}) and low concentration (50 ng mL^{-1}) in separate reactors. The impact of antibiotics on the biological activity of the sludge at the

Table 1 Operational parameters of the different reactor systems: conventional activated sludge (*CAS*) treatment and membrane bioreactors (MBRs)

	WWTP	CAS	Koch	Kubota
Average inflow (m^3 h^{-1})	2000	-	-	-
Flow (L m^{-2} h^{-1})	-	-	17	10-20
Reactor volume (m^3)	-	25,000	3.6	2×3.75
MLSSC (mg L^{-1})[a]	-	2,000	2,700	14,500
HRT (h)	-	11.5	7.2	10-20
SRT (days)	-	10	30-40	65-75
Membrane nominal porosity (μm)	-	-	0.05	0.4
Effective membrane area (m^2)	-	-	30	40

WWTP wastewater treatment plant, *MLSSC* mixed liquor suspended solids concentration, *HRT* hydraulic retention time, *SRT* solids retention time

[a]Mixed liquor suspended solids registered in the reactor

assayed concentrations was considered negligible [18, 19]. All batches were continuously aerated by means of Teflon tubes. Abiotic removal (adsorption or hydrolysis) was tracked by setting up a control batch, which was biologically inactivated by adding sodium azide at 2 % vol. The blank value of the activated sludge was also evaluated. Aliquots of 1 mL were taken at regular intervals, and were immediately inactivated by adding sodium azide before storage and analysis. Water losses due to the aeration were compensated for by adding HPLC-grade water. SMX was not included in the experiment as its biodegradation profiles had been previously studied in detail [19, 20].

Chemical analysis and method validation

Liquid chromatography–tandem mass spectrometry analyses were performed with an HP 1100 chromatograph from Agilent Technologies (Palo Alto, CA, USA) coupled to a 4000 Q TRAP mass spectrometer from Applied Biosystems (Foster City, CA, USA) equipped with a turbospray electrospray ionization source. Details of the optimization of the analytical method were described in previous work [15]. Separation was performed on an Atlantis C_{18} (Waters) (150 mm × 2.1 mm, 3 μm) liquid chromatography analytical column preceded by a guard column with the same packing material using HPLC-grade water at pH 2.2 (solvent A) and acetonitrile (solvent B), both with 1 % formic acid, as the mobile phase. The elution gradient program was as follows: from 25 % to 100 % solvent B in 11 min, hold for 2 min, and return to the initial conditions in 10 min. The flow rate was 0.2 mL min^{-1}, and the injection volume of standards and sample extracts was always set at 20 μL. Quantification was performed following the internal standard calibration approach. Calibration curves were built at concentrations ranging from 0.1 to 500 μg L^{-1}.

The calculation of the SPE recoveries was performed through the analysis of six replicates of each kind of water matrix containing all the SAs at a concentration of 500 ng L^{-1}. Blank samples were also evaluated to check for background concentrations. Limits of quantification of the method were calculated as the minimum detectable amount of analyte with a signal-to-noise ratio of 10 (Table 2).

Results and discussion

REs of CAS and MBRs

The values of different biological parameters were measured in the effluents of the three different reactors during the sampling campaign in order to obtain an evaluation of their REs (Table 3). During the sampling period, the Koch MBR had to be purged on different occasions owing to severe foaming. Therefore, the suspended solids (mixed liquor suspended solids, MLSS) concentration registered for this reactor (2.7 gL^{-1}) was below the desired concentration [14] and was similar to the MLSS concentration registered in the CAS reactor. The MLSS concentrations registered in the Kubota MBR, however, were high as expected (14.5 gL^{-1} on average). Nevertheless, the quality of the Koch and Kubota MBR effluents was very similar in terms of chemical oxygen demand, biological oxygen demand, and free ammonia (NH_4^+). The REs for these parameters in both MBRs ranged between 80 % and 99 % of the influent content and the values were always higher than those obtained in the CAS effluent, especially for NH_4^+, of

Table 2 Method limits of quantification (*LOQ*), solid-phase-extraction recovery values (*R*), and precision expressed as the relative standard deviation (*RSD*; *n*=6) obtained for each of the water matrices analyzed

	LOQ (ng L^{-1})	CAS influent		CAS effluent		Koch effluent		Kubota effluent	
		R (%)	RSD (%)	*R* (%)	RSD (%)	*R* (%)	RSD (%)	*R* (%)	RSD (%)
SDZ	1.08-5.21	83	5	84	6	63	8	50	7
SDM	0.58-2.37	96	23	70	18	80	11	80	14
SMZ	1.01-7.91	64	5	110	2	127	8	96	3
SMT	0.44-6.86	96	1	77	18	65	5	69	6
SMX	2.97-15.15	118	12	125	4	80	6	127	4
SXP	1.14-3.31	97	9	58	23	86	1	113	12
SPY	1.77-5.53	77	4	81	12	69	8	108	2
STZ	2.08-6.47	82	4	78	14	111	6	83	12
SSX	0.56-2.58	91	6	98	5	64	2	53	6
AcSMZ	2.17-4.60	115	21	112	10	91	8	131	9

SDZ sulfadiazine, *SDM* sulfadimethoxine, *SMZ* sulfamethazine, *SMT* sulfamethizole, *SMX* sulfamethoxazole, *SXP* sulfamethoxypyridazine, *SPY* sulfapyridine, *STZ* sulfathiazole, *SSX* sulfisoxazole, *AcSMZ* N^4-acetylsulfamethazine

Table 3 Biological performances of the CAS and MBR treatments during the sampling campaign in terms of chemical oxygen demand (*COD*), biological oxygen demand (*BOD*), free ammonia (NH_4^+), and total suspended solids (*TSS*) measured in the effluents. Values are given as averages with the standard deviation in *parentheses*. Removal efficiencies (*RE*) are given as percentages

	WWTP influent	CAS effluent	RE (%)	Koch effluent	RE (%)	Kubota effluent	RE (%)
TSS (mg L^{-1})	264 (2)	20 (11)	92	1.3 (0.4)	99	1.85 (0.9)	99.0
COD (mg L^{-1})	651 (57)	88 (26)	87	48 (34)	97	42 (33)	94.0
BOD (mg L^{-1})	363 (36)	15 (11)	96	4.1 (3.4)	99	3.36 (1.6)	99.0
NH_4^+ (mg L^{-1})	40 (3)	30 (9)	25	1.4 (3.2)	97	7.85 (13)	80.0

which only 25 % could be eliminated in the conventional treatment.

Removal of SAs from the influent water

Biodegradation and adsorption are considered the two main removal mechanisms during secondary treatments in WWTPs, although the removal mechanism also depends on the nature of the pollutant. Likewise, other nonbiological removal processes such as photodegradation (whenever the water is exposed to sunlight, i.e., clarifiers) should be considered. For SAs, the distribution coefficient (K_d) values found in the literature are usually low (K_d<500 Lkg^{-1}), and these antimicrobials have generally been considered to be eliminated at a level of less than 10 % through this mechanism [21]. Mineralization assays were not performed in this study and, consequently, full mineralization of the SAs cannot be assured. Air stripping and volatilization in the different biological reactors were also not employed, as SAs are polar substances with very low Henry constants [18].

The average daily loads of SAs in the WWTP influent were calculated using Eq. 1:

$$\text{Load}_{\text{inf}} = VC_{\text{inf}}, \quad (1)$$

where V is the average wastewater inflow of the plant (L day^{-1}) and C_{inf} is the SA concentration measured in the influent. The total influent load for the SAs was 194 g day^{-1} (ranging from 0.153 g day^{-1} for SSX to 171 g day^{-1} for SMX; see "Presence of SAs in sewage sludge"). More than 93 % of this load corresponded to SMX and SPY. Thus, the elimination of these two SAs during the different wastewater treatments is critical to reduce the concentration of SAs in the effluents and to evaluate the performance of the different reactors. In this work, unless indicated otherwise, RE refers to a water matrix, and was calculated on the basis of the influent and effluent concentrations detected (Table 4).

The higher MLSS concentrations and SRTs of both MBRs theoretically favor the adorption to sludge and biodegradation of polar and hydrophilic substances such as SAs. As shown in Table 4, the REs are generally higher than in CAS treatment, and although different, they are not remarkable. In previous studies, rather inconsistent values were obtained for the elimination of SMX [3, 7, 8], with both positive and negative REs in CAS treatment. In the present study, 46 % of SMX was removed during CAS treatment, 52 % in the Koch MBR, and 55 % in the Kubota MBR. Although the REs were slightly higher in the two MBRs, elimination was only partial as nearly

Table 4 Mean RE in water (n=4) of the sulfonamides studied and their RSDs after CAS, Koch MBR, and Kubota MBR treatments in the Terrassa WWTP

	CAS		MBR			
			Koch		Kubota	
	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)
SDZ	50.7[a]	-	100[b]		100[b]	
SDM	<−100	5.8	100[a]		<−100	29.9
SMZ	22.3[b]	3.8	46.0	31.6	49.1	14.5
SMT	<−100	21.8	<−100	35.9	<−100	135.3
SMX	46.4	8.7	51.8	11.2	54.6	12.6
SMP	67.4[a]	-	-	-	-	-
SPY	−53.2	49.0	39.1	53.1	20.6	118.8
STZ	55.3	27.4	59.3	20.2	73.3	58.5
SSX	85.2	24.6	100[a]	-	100[a]	-
AcSMZ	53.6[a]	-	100[a]	-	100[a]	-

[a]Unique values (no RSD)

[b]Removal of 100 % assumed. Calculation performed for n=3

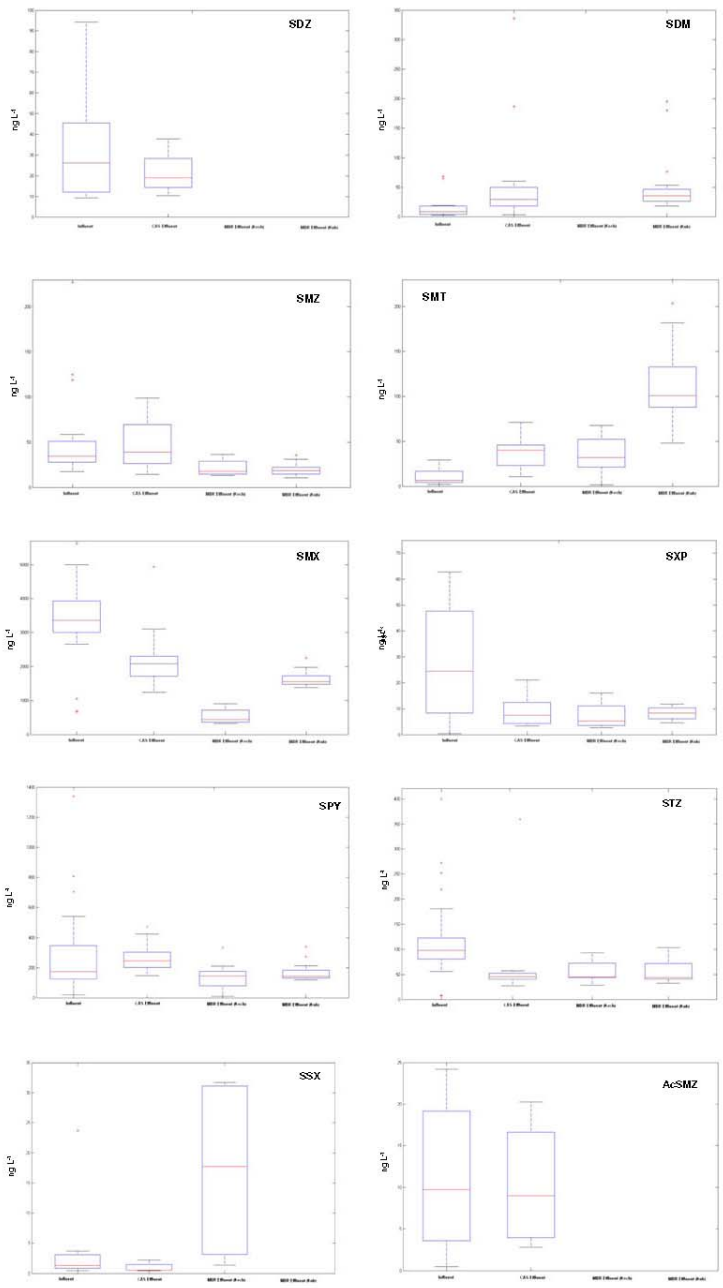

Fig. 2 Box plots showing the distribution of the concentrations of the corresponding sulfonamides (SAs) in the Terrasa WWTP. *SDZ* sulfadiazine, *SDM* sulfadimethoxine, *SMZ* sulfamethazine, *SMT* sulfamethizole, *SMX* sulfamethoxazole, *SXP* sulfamethoxypyridazine, *SPY* sulfapyridine, *STZ* sulfathiazole, *SSX* sulfisoxazole, *AcSMZ* N^4-acetylsulfamethazine, *Kub* Kutota

half of the SMX input could still be detected in the respective effluents. Similar results were obtained in two Koch MBRs with different SRTs by Tambosi et al. [22], with REs of 55 % and 64 % for SRTs of 15 and 30 days, respectively. Radjenovic et al. [23, 24] reported different REs for SMX in Kubota membranes (60-81 %) and in Koch membranes (78 %). Sahar et al. [25] obtained an RE of 69.6 % after MBR treatment, explaining this low removal in terms of the lower pK_a of SMX (5.8) which made the molecule slightly more acidic and reduced the potential for its biodegradation. However, SPY, with a pK_a of 8.8, was also poorly removed in MBRs, whereas SSX, with a pK_a of 4.9, was completely removed after treatment, so this hypothesis would not fit with the results presented in this article. Different studies considered the cleavage of N^4-acetylsulfamethoxazole as a feasible reason for the low removal of SMX reported in CAS treatment [3, 26], which could be extrapolated to MBRs. The highest RE reported for SMX was greater than 95 % after 2 weeks using a Zenon MBR without sludge removal [27]. Nevertheless, our study demonstrates that the effectiveness of both MBRs, despite the higher MLSS content and longer SRT (which favors the removal rates), was not especially good for SMX. In contrast, MBRs were more effective than CAS for STZ, with slightly better REs in the MBRs (Table 4). We assumed 100 % removal for SDZ, SSX, and AcSMZ as they were not detected in any of the MBR effluents. SSX was also removed quite efficiently by CAS treatment (85.2 %); however, only 51 % and 53 % of SDZ and AcSMZ, respectively, were removed during the CAS treatment. The parent drug of the latter, SMZ, exhibited low REs in both MBRs (36 % and 50 % for the Koch and Kubota MBRs, respectively), but 68 % removal in the CAS effluent.

As observed in Fig. 2, the concentrations in the CAS effluent were higher than in the influent for SDM, SMT, and SPY, and negative REs were obtained. This could be a consequence of the deconjugation of different metabolites of these SAs also present in the influent (beyond the scope of this study), which could yield the parent compound as a product. Göbel et al. [3] assumed that sulfasalazine (a human pharmaceutical containing SPY) and N^4-acetysulfapyridine (the human metabolite of SPY) could be present in the biological treatment inflow and could be transformed into the parent substance during the secondary treatment. Furthermore, the deconjugation of N^4-acetysulfapyridine has recently been demonstrated in a laboratory-scale fixed-bed bioreactor, showing that up to 50 % of the initial concentration of the acetylated metabolite was transformed back to the parent drug [26]. The concentrations obtained for SDM showed the biggest differences on average, with values in influent water samples not higher than 11 ng L^{-1} and levels detected in CAS effluent four times higher, resulting in extremely negative REs. Regarding the fate of these three SAs in the MBRs, SMT also exhibited negative REs in Kubota and

Table 5 Method LOQ, concentration range, and frequency of detection (*F*) for each of the sludge matrices analyzed

	Primary sludge			Secondary sludge			Koch sludge			Kubota sludge			Digested sludge		
	LOQ (ng g^{-1})	Concentration (ng g^{-1})	*F* (%)	LOQ (ng g^{-1})	Concentration (ng g^{-1})	*F* (%)	LOQ (ng g^{-1})	Concentration (ng g^{-1})	*F* (%)	LOQ (ng g^{-1})	Concentration (ng g^{-1})	*F* (%)	LOQ (ng g^{-1})	Concentration (ng g^{-1})	*F* (%)
SDZ	1.07	1.17	25	-	ND	-	-	ND	-	0.01	0.06-0.14	50	0.04	0.14-0.27	75
SMD	0.03	0.02-2.37	100	-	ND	-	0.40	1.45	25	0.01	0.07	25	1.9	<LOQ	50
SMZ	0.24	3.13	50	-	ND	-	-	ND	-	0.21	0.74-2.24	100	-	-	-
SMT	0.05	0.06-0.08	75	0.02	0.02	25	2.40	<LOQ	25	0.02	0.03-18	75	0.03	0.03	75
SMX	0.12	1.02	25	0.11	1.23	25	1.13	2.04	25	0.03	0.03-1.12	75	-	ND	-
SXP	0.003	0.01-2.17	50	0.02	0.08	25	0.27	0.48	25	0.02	0.06-0.17	75	0.01	0.01-0.07	75
SPY	0.02	0.07-1.63	75	0.01	0.4	25	0.02	0.26	25	0.03	0.04-0.99	75	0.02	0.04-0.05	75
STZ	0.03	1.72-11.3	100	0.02	0.12-1.63	50	0.01	0.02-0.21	50	0.01	0.03-0.12	50	0.12	0.21-2.08	100
SSX	0.01	0.01-0.04	50	0.01	0.08	25	0.001	0.003-1.5	50	0.01	0.03-0.06	50	0.01	0.02-0.22	75
AcSMZ	0.03	0.07-0.88	50	0.02	0.42-0.88	50	0.03	0.85	25	0.02	0.14-4.30	75	0.06	0.08-1.83	100

ND not determined

Koch effluents, as did SDM in Kubota effluents. In contrast, SDM was not detected in the Koch effluent, assuming 100 % elimination. After SMX, SPY was the most relevant SA in terms of concentration and frequency of detection; MBRs were more efficient than CAS regarding SPY, but the REs obtained were still below 50 % (39 % in Koch MBR and 21 % in Kubota MBR).

Presence of SAs in sewage sludge

Residual concentrations of SAs in the different sludge types were calculated in order to obtain a broader picture of the RE of the CAS reactor and both MBRs. The concentrations detected were very low, with a maximum value of 11.3 ng g^{-1} for STZ (primary sludge). (Table 5). STZ was detected with the highest frequency and in the highest concentrations in the primary, secondary, and digested sludge. For most of the SAs, the highest concentrations were also detected in the primary sludge, and the trend primary sludge > secondary sludge > digested sludge was confirmed. Despite the longer SRTs, the levels detected in the Koch and Kubota sludges were similar to those detected in the digested sludge.

In general, data on SA levels in sludge are scarce in the literature, with SMX again being the SA most frequently investigated. The concentrations are usually in the low nanogram per gram range [28, 29], although values as high as 68 μg g^{-1} for SMX and 28 μg g^{-1} for SPY have also been reported [4]. In our case, however, SMX was not detected at concentrations higher than 2 ng g^{-1} in any of the sludges studied. Jelic et al. [30] found SMZ levels in digested sludge from three different WWTPs similar to those presented in this study. Factors such as the location of the WWTP, the SRT, the HRT, and the sedimentation capacity of the reactor should be considered when aiming to establish a comparison with the different results in the literature. Besides, most of the values reported in the literature correspond to CAS or digested sludge, and only few correspond to MBRs [24].

Average daily input and output wastewater mass loads and the output load in the digested sludge were calculated following Eqs. 2–5.

$$\mathrm{Load}_{\mathrm{eff}} = VC_{\mathrm{eff}}, \tag{2}$$

$$\mathrm{Load}_{\mathrm{sludge}} = PC_{\mathrm{sludge}}, \tag{3}$$

where C_{eff} is the concentration measured in the CAS effluent, P is the average digested sludge production (40 $tday^{-1}$), and C_{sludge} is the concentration measured in the sewage sludge. These values were used to estimate the RE in the water matrix (RE_{water}) and in the whole treatment process considering the load retained in the sewage sludge (RE_{total}). The following equations were used:

$$\mathrm{RE}_{\mathrm{water}}(\%) = 100 \times \frac{(\mathrm{load}_{\mathrm{inf}} - \mathrm{load}_{\mathrm{eff}})}{\mathrm{load}_{\mathrm{inf}}}, \tag{4}$$

$$\mathrm{RE}_{\mathrm{total}}(\%) = 100 \times \frac{\left[\mathrm{load}_{\mathrm{inf}} - (\mathrm{load}_{\mathrm{eff}} + \mathrm{load}_{\mathrm{sludge}})\right]}{\mathrm{load}_{\mathrm{inf}}}. \tag{5}$$

Figure 3 shows the SA fraction removed during treatment, the fraction discharged into the environment, and the fraction sorbed onto the sludge (estimated values are given in Table S1). Values for those SAs with negative RE_{water} values are not shown. As observed, the amount of SAs retained in the sludge represented a maximum of 3 % of the SA input load (value corresponding to AcSMZ). This

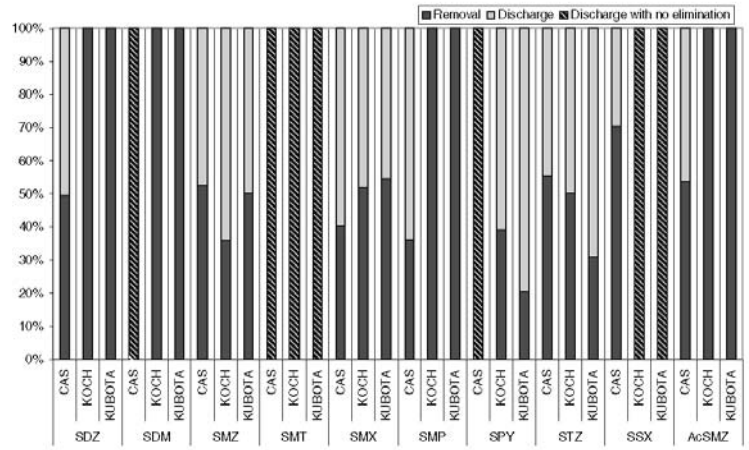

Fig. 3 Overall removal efficiency during treatment, fraction sorbed to sludge, and discharge in the WWTP effluent of the SAs investigated. *CAS* conventional activated sludge

percentage could be considered negligible compared with the concentration still detected in the effluent, reinforcing the conclusion that the role of sorption–desorption of the SAs to the different sludges did not play a relevant role in their elimination in the treatment plant. The treated sludge from the Terrassa WWTP is regularly applied as nutrient amendment in agriculture, but does not represent a relevant entry pathway for SAs to the soil ecosystems.

Biodegradation of SAs

The concentration profiles of the different SAs in the batch reactors spiked at high and low concentrations are shown in Fig. 4. The control batch did not show any relevant variation in the concentration of SAs during the experiment (data not shown). A relevant decrease during the first 2–3 days was observed for all the analytes at both spiking concentrations. It was especially steep for SDM, SMZ, and STZ in the batch spiked at 1,000 ng mL^{-1}, reaching levels below 30 % of the initial concentration. As discussed in "Removal of SAs from the influent water," this initial decrease registered during the first few days should not be attributed to adsorption onto the dissolved organic matter in the reactor in terms of K_d. However, in a similar study, Yang et al. [31] attributed a 19 % decrease of SDM content during the first 12 h to adsorption onto the activated sludge, which later would be desorbed almost completely on the aqueous phase. The acetylated metabolite showed the steepest decline, with only 8 % of the initial concentration present after 5 days. This metabolite was the compound showing the highest sorbed

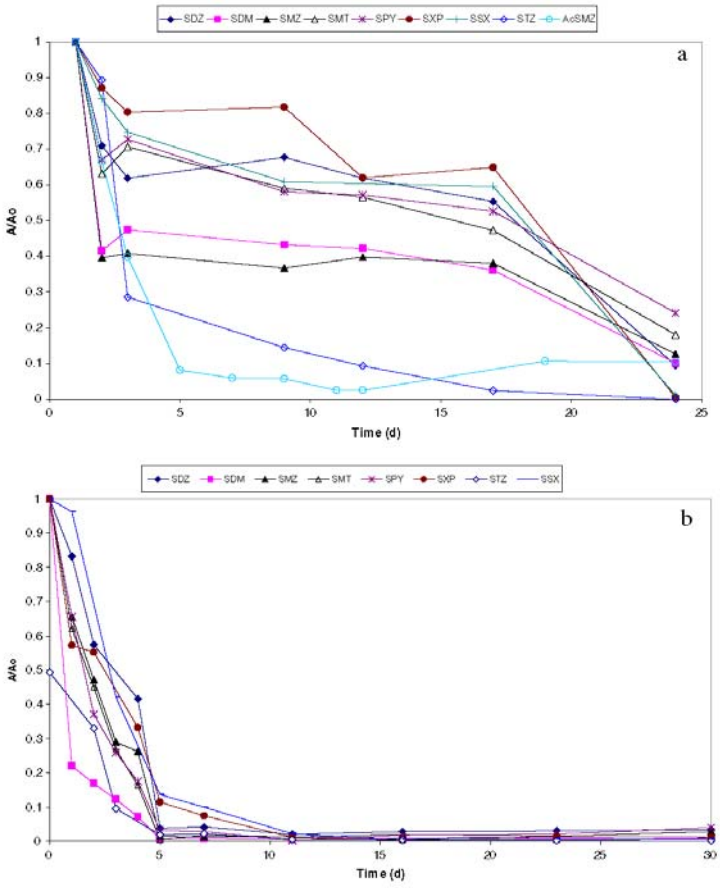

Fig. 4 Degradation profile of the nine SAs investigated in the batch reactors spiked at **a** 1,000 ng mL^{-1} and **b** 50 ng mL^{-1}

fraction in digested sludge (see Fig. 3), despite it not being present at very high concentrations in either water or sludge (see Fig. 2, Table 5). Although deconjugation is a more feasible explanation, the concentration of SMZ did not increase during the experiment and this possibility was not confirmed. Furthermore, previous experiments in a fixed-bed bioreactor demonstrated that this acetylated metabolite did not deconjugate after 90 days of experiment [26]. More likely, transformation and disappearance of SAs from the mixed liquor could be due to the covalent cross-coupling to natural organic matter, which is present at high concentration in the liquor [32]. However, analysis of the solid phase of the liquor was not considered and an evaluation of the resulting transformation products generated was not done and, consequently, this mechanism cannot be confirmed. As samples were taken daily and not hourly in this study, the initial faster dissipation of SAs was considered to be due to a higher amount of nonspecific degraders in the fresh sludge. Likewise, the presence of already acclimatized biota could also be a feasible explanation, as SAs are frequently detected in the different stages of wastewater treatment. After that initial decrease, the concentrations remained fairly stable until day 17, from which again an exponential decrease was observed. This lag phase, followed by the accelerated degradation, was attributed to the time required by the microbial community to adapt their enzymatic systems to use the antimicrobial as a nutrient, as observed previously [33]. The excess concentration in these batches may have required more numerous degrading microbiota adapted to the antibiotics. Only STZ showed a slightly different trend, with apparently no lag phase and a constant decrease. As mentioned in "Presence of SAs in sewage sludge," STZ was the SA detected in sludge most frequently and at the highest concentrations, and adsorption onto the solid particles of the liquor may have been more relevant in this case. This SA, together with SSX and SXP, was fully removed from the batch after 25 days, whereas the rest of the SAs still remained at residual concentrations of 10 % of the initial concentration or higher.

In the batch spiked at 50 ng mL^{-1}, a different trend was observed. Again, the concentrations of all the SAs seemed to decrease constantly from day 1 until day 6, reaching by then levels below 10 % of the initial concentration (11 % for SMP, less than 1 % for SMT and SDM). The decrease is more even marked because of the lower concentrations added. The aforementioned lag phase for the high-concentration batch reactors could not be clearly established in this case. After 30 days of experiment, all the SAs were still present in the batch reactors at levels between 3-4 % of the initial concentration.

The concentration decrease was similar for all the SAs tested at each concentration, which suggested that the enzymes involved in the degradation were class-specific rather than compound-specific, as suggested by Ingerslev et al. [33], who reported shorter lag phases for adaptation of SA degraders. Li and Zhang [18] reported a low RE for SDZ, which still remained in the batch reactor after 50 days of experiment (50 % of the initial concentration). Pérez et al. [19] also demonstrated that STZ and SMZ remained in the reactors after 50 days of aeration (around 5 % of the residue).

Conclusions

The behavior of ten different SAs (including one acetylated metabolite) during different wastewater treatments was evaluated in this study. The REs in two MBRs were higher than those observed for CAS in most cases, with 100 % removal for four of the SAs investigated. However, for SMX and SPY, the two most relevant SAs in terms of the frequencies of detection and amounts, the results were only slightly better in MBRs than in CAS for SMX, and the REs for SPY were not higher than 45 %. Negative REs were found for both CAS and MBRs effluents. The different REs observed in each of the wastewater treatments investigated cannot be explained easily, as SAs are very similar in molecular structure and physico-chemical properties. The amounts of SAs present in the digested sludge do not contribute significantly to the overall removal of the WWTP (less than 3 %), and could be disregarded in the overall calculations. Different biodegradation assays also demonstrated that SAs were not fully removed after 25–30 days of aeration in different batch reactors, which could imply that the average HRTs in WWTPs are generally too short to allow complete elimination of SAs. Although MBRs are becoming a promising alternative to overcome this and other drawbacks of CAS, these systems should be optimized further (i.e., assays with different membrane materials or more specialized microorganism populations) to achieve the desired results.

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ORIGINAL PAPER

Biodegradation studies of N^4-acetylsulfapyridine and N^4-acetylsulfamethazine in environmental water applying mass spectrometry techniques

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Abstract This work evaluates the biodegradation of N^4-acetylsulfapyridine (AcSPY) and N^4-acetylsulfamethazine (AcSMZ), metabolites of two of the most commonly used sulfonamides (SAs) in human and veterinary medicine, respectively. Aerobic transformation in effluent wastewater was simulated using aerated fixed-bed bioreactors (FBBRs). No visible changes in concentration were observed in the AcSMZ reactor after 90 days, whereas AcSPY was fully degraded after 32 days of experiment. It was also demonstrated that AcSPY was transformed back to its parent compound sulfapyridine (SPY). The environmental presence of these two metabolites in wastewater effluent had been previously investigated and confirmed, together with three more SA acetylated metabolites and their corresponding parent compounds, in 18 different wastewater treatment plants in Hesse (Germany). Sulfamethoxazole (SMX) and SPY were the two SAs detected most frequently (90% and 89% of the samples, respectively) and in the highest concentrations (682 ng L^{-1} for SMX and 532 ng L^{-1} for SPY). To conclude, hazard quotients were calculated whenever toxicity data were available. None of the SAs studied posed an environmental risk.

Keywords Sulfonamides · Metabolites · Biodegradation · HPLC-MS/MS analysis · Wastewater

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Introduction

During the last decade, scientific interest has shifted from studies dealing with the environmental occurrence of pharmaceuticals to their fate and especially the presence of their transformation products (TPs) [1, 2]. Although metabolites are usually pharmacologically less active and less toxic than the parent compound, some of them may still be biologically active when entering the environment and pose a potential risk to the ecosystem [3]. Nevertheless, their environmental presence has become made evident through different studies [4, 5].

Sulfonamides (SAs) represent one of the most frequently used families of antibiotics in Europe, after tetracyclines [6]. They are mainly used in veterinary treatments but also, to a lesser extent, in human medicine, although nowadays the increasing number of resistant bacteria against SAs has made their use inappropriate in human therapies [7]. However, several SAs are still being detected in different monitoring studies in wastewater treatment plants (WWTPs) at different concentrations, indicating that some of these antibiotics are still prescribed to treat different human infections. Their high polarity and solubility in water makes them very mobile once released into the environment and have led to their detection in all kinds of environmental waters

and also in solid matrices [8–10]. The environmental presence of their metabolites has also been published recently in different works [11–13].

Although the metabolism of SAs is species-dependent [14, 15], they are generally metabolized in the liver by phase I oxidation and phase II acetylation; glucuronide conjugation and aromatic hydroxylation can also take place yielding the N^1 and N^4 derivatives [15, 16]. The amounts of metabolites and unchanged SAs that are excreted depend on the form of the drug and the animal age and species. Livestock will usually excrete up to the 50–90% of the administered dose, usually within several days after treatment; the parent drug makes up for the 9–30% of the excreted amount, and usually acetic acid conjugates comprise 5–60% of the dose [17]. SAs used in human therapies are ultimately excreted into domestic sewage and discharged to WWTPs. Usually, removal efficiencies for SAs and the majority of other pharmaceuticals are calculated measuring the difference of concentration between the levels detected in influent and effluent wastewaters, without considering the formation of metabolites or other TPs [18–20]. The presence of acetylated metabolites such as N^4-acetylsulfamethoxazole (AcSMX) or N^4-acetylsulfamethazine (AcSMZ) in WWTP effluents [8, 11, 13, 21] reinforces the possibility that, during wastewater treatment, these and other different acetylated metabolites are totally or partially cleaved by bacteria and reverted back to the original drug. This deconjugation could explain the higher SAs concentrations in the output than in the input of the WWTPs, which were encountered in recent works [12, 19]. AcSMX was the first SA metabolite to be detected in WWTP effluents in concentrations up to 2.2 μg L^{-1} [22].

As a consequence of its recent inclusion in the scope of monitoring studies, there is a substantial lack of ecotoxicological data regarding adverse effects of these acetylated metabolites (and of SAs in general). However, the risk assessment guidelines set up by the European Medicines Agency (EMEA) for the marketing authorization of new human medicinal products have been used in different occasions to establish the environmental risk posed by drugs that are already being consumed and present in aquatic or terrestrial environments [23, 24]. Briefly, the environmental risk assessment protocol is a two-phase tiered process that begins with an approximate calculation of the predicted environmental concentration (PEC) of the drug in water. These guidelines [25] recommend that any drug with a PEC value > 10 ng L^{-1} in surface water should progress to phase II. Standard acute toxicity tests will be carried out in this stage in order to estimate predicted no-effect concentration (PNEC) or non-observed effect concentration (NOEC). Finally, the ratio of the PEC to PNEC, known as the hazard quotient (HQ), indicates whether a potential environmental impact is implicit and further testing might be needed (HQ> 1). It is also recommended that, when the total concentration of metabolites is a 10% greater than the concentration of the corresponding parent drug, the metabolites are also to be further investigated (phase II tier B) in order to determine their ecotoxicological effects. The EMEA Committee for Medicinal Products for Veterinary Use also established similar guidelines [26].

The aim of this study is to investigate the occurrence and fate of five acetylated metabolites of SAs and the respective parent drugs in 18 different WWTPs in the state of Hesse (Germany) during the year 2008. The biodegradability of sulfapyridine (SPY) and N^4-acetylsulfapyridine (AcSPY), together with AcSMZ, was further studied using a fixed-bed bioreactor (FBBR). Finally, the potential environmental risk posed by each of the studied SAs and metabolites was considered, following the EMEA guidelines.

Experimental section

Chemicals and materials

High purity standards (>99%) of the five selected SAs, namely sulfadiazine (SDZ), sulfamethazine (SMZ), AcSMZ, SMX, SPY, and sulfamerazine (SZI), were purchased from Sigma Aldrich (St Louis, MO, USA). Analytical standards for N^4-acetylsulfadiazine (AcSDZ), AcSMX, AcSPY, and N^4-acetylsulfamerazine (AcSZI) and the internal standard, d_4-sulfathiazole (99.9% purity), were purchased from Toronto Research Chemicals (ON, Canada). High-performance liquid chromatography (HPLC)-grade solvents (water, methanol (MeOH), acetone, and acetonitrile (ACN)) and formic acid (98–100% purity) were supplied by Merck (Darmstadt, Germany). Stock standard solutions for each of the analytes were prepared in MeOH at 1 mg mL^{-1} and stored at −2 °C until use. Standard solutions of the mixtures of all compounds at appropriate concentrations were prepared by the corresponding dilution of the stock solutions in MeOH.

Oasis HLB cartridges (60 mg, 3 mL) were provided by Waters (Milford, MA, USA).

Monitoring study

Sampling area and sample collection

WWTP effluent samples were taken from 18 different WWTPs located in the rural district of Darmstadt-Dieburg, in the state of Hesse (Germany). The land use in this district is mainly agriculture. The proportion of urban/industrial water is approximately 90:10. All the WWTPs followed a similar treatment scheme: pretreatment, primary treatment (phosporous elimination), and conventional activated sludge (CAS) as secondary treatment. Sampling was carried out

every 3 weeks, from 11 April to 12 June of 2007. Three weekly composited samples (volume of 1 L) were prepared collecting hourly small volumes during that period. The mixed samples were stored in amber glass bottles in the dark and frozen until analysis.

Sample preparation

WWTPs effluent samples were extracted and purified by means of a 12-fold vacuum extraction box J.T. Baker (Phillipsburg, NY, USA). The extraction method was based on that used by Buttiglieri et al. [27]. Briefly, 200 mL of WWTP effluent, previously filtered through 0.45-μm glass fiber filters, were preconcentrated on Oasis HLB cartridges; these had been previously conditioned with 2 mL of *n*-hexane, 6 mL of MeOH, and 10 mL of HPLC water. After the sample loading, the cartridges were dried under a gentle nitrogen flow for 40 min, and eluted with 3×1.5 mL acetone/ethyl acetate (1:1; *v/v*). The extracts were evaporated to dryness under a gentle nitrogen stream and finally reconstituted with 500 μL of a mixture of H_2O/MeOH (80/20; *v/v*) with 5 mM ammonium formate. d_4-sulfathiazole was used as internal standard and was added to each sample before extraction at a concentration of 500 ng L^{-1}.

Liquid chromatography-tandem mass spectrometry

Reversed-phase HPLC was performed using two Series 200 Micro Pumps (Perkin-Elmer, Norwalk, CT, USA). Separation was carried out on an Atlantis C18 (Waters) (150×2.1 mm, 3 μm) LC analytical column, preceded by a guard column with the same packing material. The mobile phase consisted of HPLC-grade water (A) and ACN (B), both with 10 mM formic acid. The initial conditions of the elution gradient programmed were A/B 75:25%. From 0 to 11 min, the eluent B was increased to 100% held for 2 min and returned to initial conditions in 3 min. Finally, the column was equilibrated for further 7 min before the next injection. The flow rate was 0.2 mL min^{-1}, and the injection volume of standards and sample extracts was set at 10 μL.

Tandem mass spectrometry (MS/MS) analysis was performed on a 3200 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbospray electrospray (ESI) interface in the positive ionization mode. For increased sensitivity and selectivity, MS/MS data acquisition was performed in the selected reaction monitoring mode. For each analyte, the two most abundant product ions were monitored: The more abundant was used for quantitation and the other one for confirmation. Table 1 summarizes the MS/MS conditions for the SAs investigated. The temperature of the source was set at 700 °C, and a positive capillary voltage of 3.5 kV was applied; nitrogen was used as both nebulizing and collision gas (when performing MS/MS experiments).

Degradation experiments

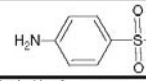
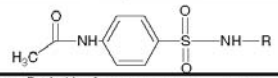
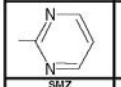
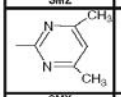
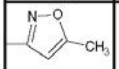
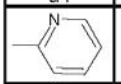
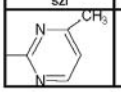
FBBRs consisted of a tank or storage bottle connected to a fixed bed, consisting of a glass column filled with glass beads (18 cm filling level, 350 mL volume); the effluent wastewater was circulated on a closed loop through this column by means of a vacuum pump, allowing the microorganisms of the water matrix to accumulate on the surface of the glass beads [28]. The flow rate was 16 mL min^{-1} on average. A second membrane pump aerated the water in the storage bottle. Samples were taken through a three-way valve at the top of the fixed bed. A scheme of the experimental design is depicted in Fig. 1.

Four different FFBRs were set up in order to assess the biodegradability of SPY, AcSPY, and AcSMZ. The fourth reactor corresponded to a blank control. Each of them were run with 5 L of WWTP effluent from the municipal WWTP Beuerbach (Hesse, Germany) and spiked with the corresponding analyte at 200 μg L^{-1}. Toxicity of the SAs investigated against the microorganisms of the reactors was considered negligible, as SAs concentrations up to 0.5 mg L^{-1} exert no negative effects on aerated reactors containing richer bacterial populations [29]. The measured pH of the wastewater was 6.9 (average value). The experiment ran for 60 days in the case of SPY, AcSPY, and the blank and for 92 days for AcSMZ, at room temperature and neutral pH. The reactors were kept in the dark to avoid photodegradation. Volume losses due to evaporation were compensated with the addition of equal volumes of the same water matrix. FBBR samples were taken periodically along the experimental period, filtered through a 0.45-μm membrane filter and stored at −20 °C until analysis.

UPLC-QqTOF-MS

Chromatographic separation followed by accurate MS analyses of each of the target analytes and their corresponding TPs was carried out quadrupole tandem time of flight (QqTOF) coupled to a Waters Acquity ultra-performance liquid chromatography (UPLC) system (Micromass, Manchester, UK). Regarding chromatography, a Waters Acquity BEH C18 column (10×2.1 mm, 1.7 μm particle size) was employed. Flow rate was set at 0.3 mL min^{-1}, with eluent A, HPLC-grade water, and eluent B, ACN, both containing 10 mM of formic acid. The elution started at 5% B for 2 min; it was then linearly increased to 60% of B in 7 min, further increased to 95% of B in the following 2 min, and then returned to initial conditions. Total run time, including the conditioning of the column and back to initial conditions, was 13 min. The injection volume of the sample was set to 5 μL.

Table 1 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) parameters for the different SAs and their respective acetylated metabolites analyzed

	Sulfonamide						N^4-Acetylsulfonamide					
												
R	Precursor Ion	Product Ion 1 Product Ion 2	RT (min)	DP (V)	CE (V)	CXP (V)	Precursor Ion	Product Ion 1 Product Ion 2	RT (min)	DP (V)	CE (V)	CXP (V)
SDZ							AcSDZ					
	251	251/156 251/108	4.89	46 46	27 30	10 8	293	293/134 293/198	4.77	65 65	30 30	12 12
SMZ							AcSMZ					
	279	279/156 279/124	6.24	26 26	30 35	10 10	321	321/134 321/124	5.18	86 86	35 35	4 4
SMX							AcSMX					
	254	254/156 254/108	11.20	56 56	25 27	10 10	296	296/134 296/298	11.40	60 60	30 30	10 10
SPY							AcSPY					
	250	250/156 250/92	4.99	51 51	28 31	12 6	292	292/134 292/198	4.63	70 70	30 30	8 8
SZI							AcSZI					
	265	265/92 265/156	5.61	61 61	47 27	6 8	307	307/134 307/110	5.02	60 60	35 35	8 8

Compound-dependent parameters: *CE*, collision energy (V); *DP*, declustering potential (V); and *CXP*, collision cell exit potential (eV)

MS analyses were performed in the positive ionization mode, and the operating conditions were as follows—desolvation gas flow, 350 L h^{-1}; source and desolvation temperatures, 120 °C and 350 °C, respectively; capillary voltage, 3,000 V; sample voltage, 30 V; and extraction cone voltage, 1 V. The collision energy was held at 4 V for MS analyses, and the applied collision gas was argon. For continuous internal mass calibration, an independent reference (valine–tyrosine–valine) was used as a lock mass, with *m/z* 380.2185 and was acquired in all the measurements. MS data was recorded scanning from *m/z* 50 to *m/z* 500.

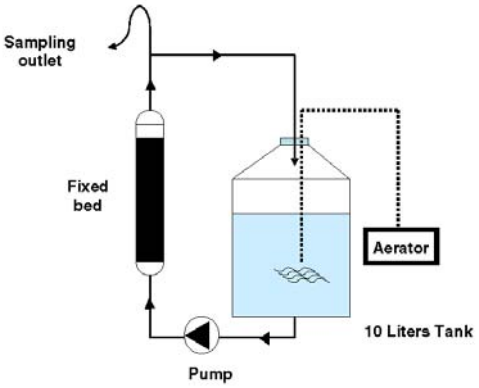


Fig. 1 Schematic setup of the FBBR

Results and discussion

Monitoring study

Method validation

Performance parameters are listed in Table 2. Calibration curves were linear for all compounds in the range 0.5–100 ng L^{-1}. Linearity is expressed as the regression coefficient (r^2) and was always equal or above 0.998. Accuracy of the method is given by the recovery values of the SAs studied and ranged from 85% to 137%. Instrumental variation is expressed as the relative standard deviation of three consecutive injections of a standard mixture at 5 ng L^{-1}. Values ranged from 2.8 (SMZ) to 16.5 (SDZ). Quantitation was performed based on the internal standard approach, adding d_4-sulfathiazole to all the samples and aqueous

Table 2 Performance parameters of the offline solid-phase extraction–LC-MS/MS method applied in the monitoring study and results from the monitoring study

Method performance						Concentration				
SAS	R% (± SD)	r^2	RSD	MLOD	MLOQ	Maximum (ng L^{-1})	Minimum (ng L^{-1})	Average (ng L^{-1})	Median	Relative frequency (%)
SDZ	85.3 (14.08)	0.9986	7.2	3.79	12.63	152	10.1[a]	54.9	42.6	72.0
AcSDZ	92.8 (14.16)	0.9996	6.5	2.16	7.19	25.3	3.66[b]	14.2	13.3	39.0
SMZ	114.9 (3.25)	0.9998	9.4	0.56	1.87	27.4	0.91[c]	8.2	1.9	13.0
AcSMZ	109.8 (6.02)	0.9976	5.5	1.20	4.00	–	–	–	–	–
SMX	137.5 (19.18)	0.9996	5.4	1.51	5.04	532	55.1	229.6	207	91.0
AcSMX	105.4 (9.00)	0.9998	11.5	0.85	2.84	190	2.8	44.3	26.3	59.0
SPY	123.3 (8.40)	0.9996	4.6	2.14	7.14	682	47	309.3	233	89.0
AcSPY	115.5 (12.46)	0.9986	8.9	2.97	9.90	415	11	134.7	119.5	74.0
SZI	125.3 (6.63)	0.9986	4.4	0.12	0.41	–	–	–	–	–
AcSZI	122.3 (11.21)	0.999	4.9	1.29[d]	4.30[d]	–	–	–	–	–

R% recovery values, R^2 coefficient of determination, *RSD* relative standard deviation (%), *MLOD* method limit of detection (average value), *MLOQ* method limit of quantification (average value)

[a] MLOQ value for this sample=4.27

[b] MLOQ value for this sample=3.10

[c] MLOQ value for this sample=0.25

[d] Values obtained from the lowest calibration point of the curve, as it was not detected in any of the samples

standards for the calibration curve at a concentration of 500 ng L^{-1} before SPE. Method limits of detection and quantification (MLODs and MLOQs, respectively) were calculated as the minimum detectable amount of analyte with a signal-to-noise ratio of 3 and 10, respectively, in the different samples analyzed. MLOD values were in the range of 0.12 ng L^{-1}–3.8 ng L^{-1} (for SZI and SDZ, respectively). Values given for AcSZI correspond to the lowest point of the corresponding calibration curve, as it was not present in any of the samples.

Results

As shown in Table 2, SMX and SPY, typically used in human medicines, were the two SAs most frequently detected (90% and 89% of the samples, respectively) and in the highest concentrations (682 ng L^{-1} for SMX, 532 ng L^{-1} for SPY); these data agree with previous publications in which these two SAs were also the most commonly detected not only in WWTPs, but also in river and groundwaters [12, 30]. AcSMX and AcSPY were also the most ubiquitous metabolites and were detected in the highest concentrations. On the contrary, neither AcSMZ nor AcSZI was detected in any of the effluent samples. SZI was also not detected in any of the samples, whereas SMZ was detected in seven of them, although, in three occasions, levels were below MLOQ.

Figure 2 shows the box plots for SMX, SDZ, SPY, and their respective acetylated metabolites, using the average concentration values of the three samples taken in each of the WWTPs. Medians indicate that concentrations were generally skewed to lower values, except for AcSPY, which shows an even distribution of the concentrations. All the metabolites exhibit a narrower distribution of their concentration levels in contrast with their respective parents, which show a higher concentration range, especially at levels above the median. However, the metabolites also presented the three only values out of the box plot range: AcSMX showed two concentrations out of the interquartile range and AcSPY, one.

Degradation study

The biodegradability under aerobic conditions of SPY and AcSPY was evaluated using two FBBRs containing the parent drug and the acetylated metabolite, respectively, at 200 μg L^{-1}. The selection of SPY is justified by the fact that, as mentioned in the previous section, it is one of the most commonly detected SAs (together with SMX), whereas the latter is commonly within the scope of different research studies regarding WWTPs [32–34]; to the authors' knowledge, both SPY and AcSPY have barely been investigated. Another FBBR was set up for AcSMZ, at the same concentration. Although it was not present in any of the German WWTPs monitored in this study, this metabolite has been detected in WWTP effluents as well as in river waters

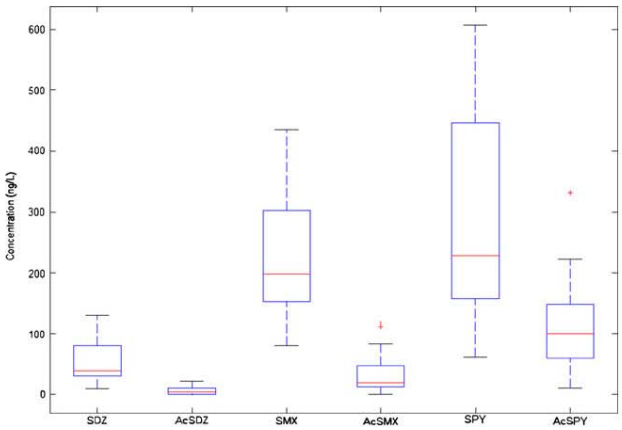


Fig. 2 Box *plots* showing the distribution of the concentrations of the corresponding SAs and their acetylated metabolites in the 18 German WWTPs studied. Concentrations are given as the average values of the three samples taken in each WWTP

and groundwaters in Spain [12, 30]. Although the spike concentrations used are not environmentally realistic, these levels were required for the identification of degradation intermediates (see section "Identification of degradation products of SPY and AcSPY"). SPY is usually detected at the highest concentrations in WWTP effluents, together with SMX, and usually in the nanogram-per-liter range.

The concentration profiles of the assayed compounds in the FBBRs are displayed in Figs. 3 and 4. No background concentrations for any of the SAs under study were detected in the blank FBBR (data not shown).

The initial concentration of SPY was unaltered till the 20th day. From then on, concentration was reduced to 50% within only 7 days and down to 1% by the end of the experiment, after 60 days. Total depletion of the initial concentration was not accomplished within the length of the experiment. It has been demonstrated that, in general, biodegradation is initially negligible when a new compound is introduced in the system till the specific degrading microbiota grow enough to make degradation relevant [29]. Then, SAs can serve as nitrogen and carbon sources for the microorganisms [35]. The decrease of SPY concentration in the FBBR could therefore be attributed mainly to biodegradation, with an initial lag phase required by the microbial community to adapt their enzymatic systems to use the antimicrobial as a nutrient and the following accelerated degradation.

As shown in Fig. 4, the degradation profile for AcSPY is quite different. T_0 corresponded to the first sample taken on the same day after the spike, and in the case of AcSPY, mixing was not fully accomplished, and the initial total concentration was detected on the following day. Concentration seemed to diminish gradually and irregularly during the first 25 days, till only the 50% of the initial concentration remained. At this point, concentration dropped quickly, and the metabolite was fully removed from the reactor in 7 days. However, dissolved organic carbon measurements were not carried out, and the full degradation of either SPY or AcSPY in the FBBRs cannot be confirmed. The slow decrease of AcSPY from the first day, without presenting a lag period for the degrading microbiota to acclimatize and/or develop, may indicate that no biodegradation was taking place during that first stage, but abiotic processes such as hydrolysis or adsorption onto biomass. The latter was not considered as biomass is present in the reactor in low amounts if compared, for instance, to CAS processes. However, the glass beads of the fixed bed could represent a significant adsorption site [36], and AcSPY, despite its high polarity, could have been gradually retained in the bed. Nevertheless, the marked decline in the profile from day 25 till day 32, similar to the decline of SPY in Fig. 3, suggests that, in fact, adaptation took place for the microorganisms during an initial lag phase (simultaneous to the slow disappearance of the metabolite). The concentration of SPY in the same reactor was detected from the first day and started to increase simultaneously to the decrease of the metabolite, reaching a peak concentration on day 39, when AcSPY was fully depleted (Fig. 4). Bacteria degrading the metabolite could break the amidic bond between the SA and the acetyl group, yielding the parent compound. But, as SPY was detected from the first day of the experiment, both abiotic and biotic processes should be regarded. It is also noticeable that the maximum concentration reached by this newly formed SPY equaled the concentration of AcSPY at which biodegradation started (half of its initial concentration); this correspondence suggests that, before day 23, the transformation of the metabolite yielded different byproducts and SPY only in small quantities, whereas, from that day on, the increase of SPY was more relevant. However, SPY remained only briefly in the FBBR and started

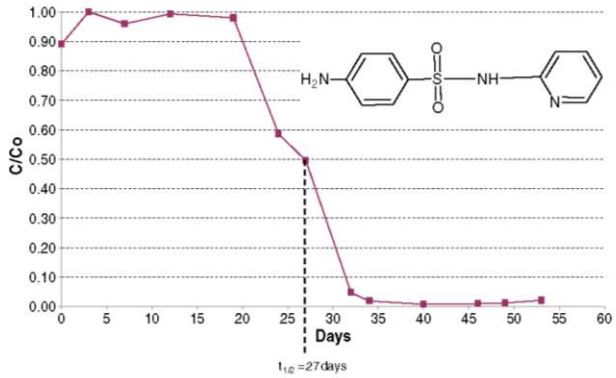


Fig. 3 Degradation profile of SPY on a FBBR containing effluent wastewater spiked at 200 μg L^{-1}

decreasing right after reaching the maximum concentration. Then, it was degraded at a similar rate as the spiked SPY in the first FBBR, and the concentration decreased to its half in 7 days. No lag phase for adaptation could be determined in this case, suggesting two possibilities:

a): The degraders involved in the depletion of the metabolite were capable of degrading not only the amidic bond of the metabolite but also different structural parts of the parent compound; Ingerslev et al. demonstrated that the specific degraders developed during exposure to four different SAs could easily degrade four other SAs in activated sludge and attributed this capacity to the hydrolysis of common structural parts [29]. The observation of the degradation of AcSPY and SPY on the same FBBR could demonstrate that SAs can be vulnerable to their metabolites degrading bacteria, as these acquire general properties needed for degradation of both metabolite and parent drug during the lag period.

b): Different types of bacteria might be devoted to the degradation of both species separately. In this case, a lag phase could be inferred from the moment the newly formed SPY was present in the reactor at enough concentration for bacteria to start their adaptation until the degradation started.

The newly formed SPY, similar to what could be observed in the FBBR for SPY, was not fully depleted by the end of the experiment.

In order to check if a different acetylated metabolite followed this behavior, a third FBBR was set up containing AcSMZ at the same initial concentration (200 μg L^{-1}). However, the metabolite concentration remained almost unaltered during the whole experiment. The length was extended to 90 days, but no changes were observed in the profile (Fig. 5). Similar results were achieved in different laboratory-scale FBBRs set up for other pharmaceuticals such as carbamazepine or clotrimazole [37, 38], which were considered as recalcitrant. In these studies, the spike concentration of the target compound was lower and the possibility of surpassing the toxicity threshold of the degrading bacteria disregarded. A plausible explanation for the outcome of the AcSMZ FBBR could be that the toxicity threshold for AcSMZ has been exceeded. The lack of data

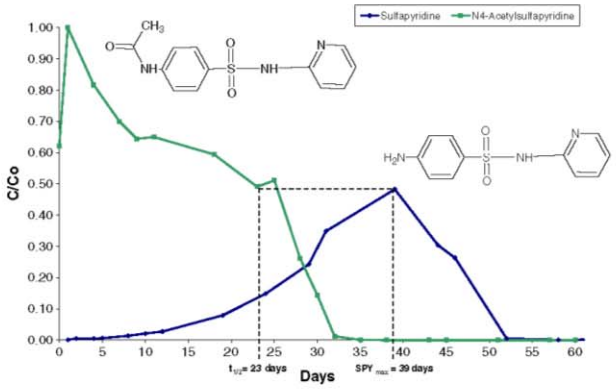


Fig. 4 Degradation profile of AcSPY on a FBBR containing effluent wastewater spiked at 200 μg L^{-1} and simultaneous appearance of newly formed SPY

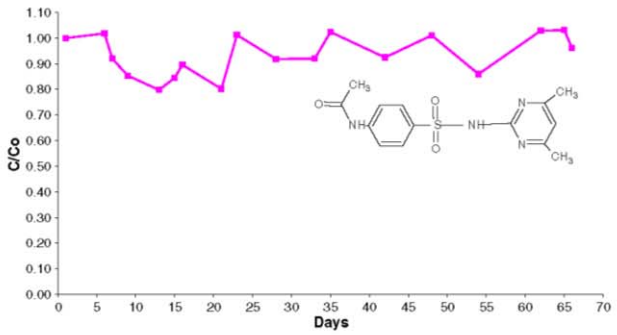
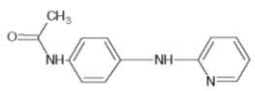
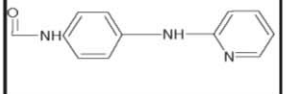
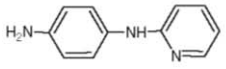


Fig. 5 Degradation profile of AcSMZ on a FBBR containing effluent wastewater spiked at 200 μg L^{-1}

regarding ecotoxicity of SAs and their metabolites hinders a clear conclusion for this result.

Despite lag phases observed, the FBBRs for SPY and AcSPY in this study are much longer than the average WWTPs hydraulic retention times (usually below 24 h [19]), the continuous exposure to different SAs and metabolites could lead to the adaptation of microbiota to these drugs. Besides, the content of biomass in CAS is much higher, and therefore the biodegradation should be accelerated. However, several studies on the presence and fate of SAs in WWTPs show rather incomplete elimination rates. In the particular case of SPY, these rates are diverse and hard to interpret. In a recent study carried out in seven different WWTPs during two consecutive years [12], the estimated elimination rates for SPY were dissimilar; in 2007, the elimination rate varied from 43% to 77% in four of the WWTPs; SPY concentration in the effluent was higher than in the influent in two of the WWTPs, and in one of them, SPY was only detected in the effluent. In 2008, the elimination rate ranged from 6% to 96%. Although AcSPY was not included in the scope of this study, these results strongly suggested its presence in the influent wastewaters and its deconjugation during the aerobic treatment. Göbel et al. reached the same conclusion when observing the SMX concentrations in the effluents, which were more than twice the inflowing load in some cases [39]. Elimination rates for AcSMX were usually above 80%, whereas those for SMX were often negative. A relevant factor to bear in mind regarding degradation rates is the temperature of the WWTP. Lower temperatures usually mean longer lag phases for the microbiota and slower degradation rates. For instance, in the study by García et al. previously mentioned [12], the variability in the elimination rates of each year could be also due to the fact that the sampling campaign in

Table 3 Accurate mass measurements of the degradation products determined by UPLC/ESI (+)-QqTOF in MS mode

Fragment (m/z)	Samples	Observed mass	Calculated mass	Elemental composition	Error (mDa)	DBE	Proposed structural composition
228	AcSPY-38 AcSPY-65	228.1142	228.1137	$C_{13}H_{14}N_3O$	2.3	8.5	
214	AcSPY-32 AcSPY-38 AcSPY-65 SPY-32 SPY-35 SPY-38	214.0984 214.0963	214.0980	$C_{12}H_{12}N_3O$	1.3 2.6	8.5	
186	AcSPY-32 AcSPY-65 SPY-32 SPY-35 SPY-38	186.1039 186.1030	186.1031	$C_{11}H_{12}N_3$	2.3 1.4	7.5	

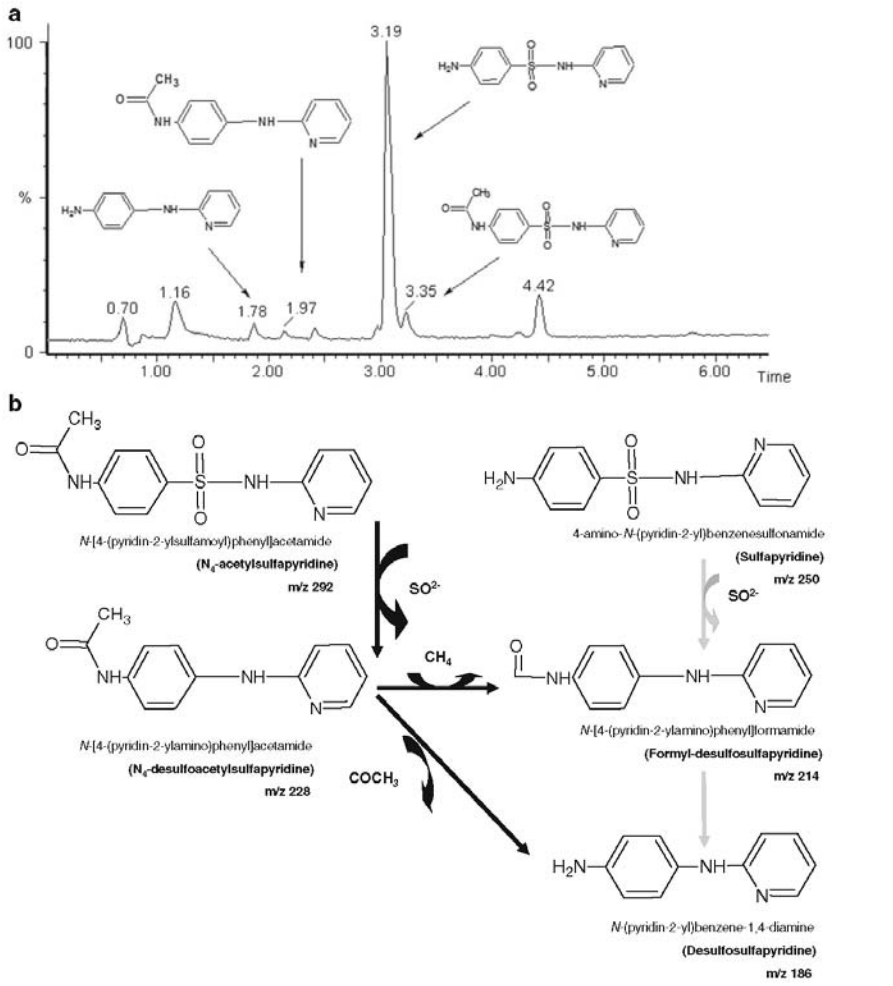


Fig. 6 Chromatogram corresponding to AcSPY degradation experiment (day 38) (**a**) and proposed transformation products of AcSPY and SPY detected during the FBBR experiment (**b**)

2007 was carried out in autumn, whereas in 2008, samples were taken in summer. Also, the amount of nutrients in the water matrix should be considered when estimating elimination rates. Drillia et al. demonstrated that the degradation of different pharmaceuticals started only when carbon or nitrogen were depleted, and then microbiota used up these molecules as alternative nutrient source [35]. Higher amounts of organic matter in the wastewater would lead to longer lag phases or an inefficient elimination.

Identification of degradation products of SPY and AcSPY

As shown in Fig. 4, the decrease of AcSPY in the FBBR partly corresponded to its back-transformation in SPY. The remaining concentration may have been fully mineralized but also transformed into different metabolites. A similar case was presented in the SPY FBBR. Samples corresponding to days 30, 32, 38, and 65 of the AcSPY degradation experiment, in which AcSPY reached its minimum, and similarly, samples

Table 4 Percentage of the acetylated metabolites concentration with respect to the concentration of their respective parents, MECs (values corresponding to the maximum concentration detected, in mg L^{-1}), PNECs (value corresponding to the EC_{50} values divided by a safety factor of 1,000), and HQs estimated for the SAs metabolites investigated

SAS	Metabolites percentage	Ec_{50}	MEC	PNEC	HQ
SDZ	–	–	1.52E-04	–	–
AcSDZ	25.9	–	2.53E-05	–	–
SMZ	–	344.7	2.74E-05	3.45E-01	0.00008
AcSMZ	–	–	–	–	–
SMX	–	78.1	5.32E-04	7.81E-02	0.00681
AcSMX	19.3	–	1.90E-04	–	–
SPY	–	27.4*	6.82E-04	2.74E-02	0.02489
AcSPY	43.5	8.2*	4.15E-04	8.20E-03	0.05061
SZI	–	–	–	–	–

EC_{50} values given are for *V. fischerii* after an exposition time of 15 min [31]
–: values not available. *: unpublished results

of days 32, 35, and 38 in the case of the SPY FBBR were individually investigated in order to identify different possible intermediate compounds. The use of the tandem quadrupole time-of-flight (QqTOF) instrument in MS full-scan mode provided the exact masses of the potential unknowns. After chromatographic separation with UPLC, two new unidentified peaks appeared in the chromatogram of both AcSPY and SPY samples. A full confirmation with product ion scans (MS/MS) could not be performed because the concentrations of the intermediate products were not high enough to reach the LODs of the QqTOF. The chemical structures proposed were confirmed with the double-bond equivalents (DBE) obtained.

AcSPY transformation products A new chromatographic peak showed after 1.98 min in sample AcSPY-38, with a base peak *m/z* 228.1182 in the corresponding mass spectra. The proposed elemental composition for this mass is $C_{13}H_{14}N_3O$ (theoretical *m/z* 228.1137; Table 3) and corresponds to the loss of the sulfonate group in AcSPY (see Fig. 6). This finding agrees with previous studies in which both degradation and photodegradation products of different SAs such as sulfamethazine or sulfadiazine were defined as SO_2 extrusion products [40–42]. This new product was also detected after 65 days in the AcSPY, when AcSPY was not longer detectable.

A second peak appeared at a chromatographic retention time of 5.7 min in both the AcSPY and SPY samples. It corresponded to the mass *m/z* 214.1006, and its elemental composition was attributed to $C_{12}H_{12}N_3O$; the molecular structure is proposed in Fig. 6 as N^4-formyl-SPY-desulfonate and could be identified in AcSPY samples of days 32, 38, and 65, and in SPY samples of days 35 and 38.

SPY transformation products Besides the peak attributed to N^4-formyl-SPY-desulfonate, a second peak was detected in the three SPY samples investigated, at a chromatographic retention time of 1.78 min; it corresponded to an *m/z* of 186.1039. In this case, the elemental composition that best fitted was $C_{11}H_{12}N_3$, and the molecular structure proposed corresponds to the desulfonated SPY (Fig. 6).

Environmental risk assessment

Following the EMEA guidelines, HQs were calculated to estimate the potential adverse effects of the SAs levels detected on non-target organisms. HQs were also estimated for the metabolites, altough their average concentrations were not a 10% greater than the levels detected for the corresponding parents (Table 4). This quotient is usually calculated as the ratio between the PEC and the PNEC or NOEC. When PNEC values are not available, an alternative PNEC can be derived by dividing the lowest EC_{50} or LC_{50} values (50% lethal concentration) by a safety factor of up to 1,000 [43, 44]. Chronic toxicity data would be more representative of the environmental conditions, but this information was lacking for SAs metabolites and acute toxicity test values (EC_{50} or LC_{50}) were used up as chronic toxicity data after being normalized by a safety factor of 1,000. Likewise, measured environmental concentrations (MECs) were used in the calculation instead of PECs [18–20]. In order to set up a worst-case scenario, MEC values used corresponded to the maximum values detected in the monitoring study whereas EC_{50}–LC_{50} values used were the lowest found in the literature. In all cases, the MECs were higher than the boundary value of 0.01 μg L^{-1} established by EMEA in tier 1. Only two publications showed ecotoxicity data for other SAs metabolites and only against the green algae *Selenastrum capricornotum* [45] and against the marine bacteria *Vibrio fischerii* (unpublished results). Table 4 summarizes the EC_{50}–LC_{50} values taken and the HQ calculated. For AcSPY, the corresponding HQ value was below the boundary of 1 established by the EMEA guidelines, and therefore the levels detected in the effluents would pose no ecological risk (Table 4). Similar results were obtained for the rest of SAs, which HQs could be estimated. In previous studies, only SMX yielded HQs higher than 1 in the same matrix [12, 19, 24] but only against green and blue–green algae, which are generally the most sensitive taxa.

Conclusions

Nowadays, SAs are one of the most common water-polluting antibiotics. The presence of their metabolites has become relevant only recently. As demonstrated in the results of the monitoring study carried out, three of the five acetylated metabolites investigated were present at detection frequencies and concentrations similar to their parent compounds. Aerobic degradation experiments, simulated in laboratory scale FBBRs, have demonstrated that AcSPY, the acetylated metabolite detected at the highest frequency and concentrations, is completely eliminated after 35 days but not fully mineralized as half of its initial concentration reverts back to the parent compound, SPY. The rest is probably transformed to different degradation products. Two of them could be identified in this work but not fully confirmed due to the low concentrations. It could be concluded that biodegradation of both SPY and AcSPY was carried out by the same adapted microorganisms, although different bacteria communities could have also degraded the different molecules separately. In this and a second FBBR, in which the fate of SPY was studied, it was not fully degraded and was still present at the end of the experiments, although at very low concentrations. Despite the low biomass content, it seems that adsorption may play a role in the initial decrease of AcSPY in the reactor, as the glass beads of the fixed bed could retain the metabolite. On the contrary, the concentration of AcSMZ was invariable after 90 days of experiment.

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Biodegradation of sulfamethazine by *Trametes versicolor*: Removal from sewage sludge and identification of intermediate products by UPLC–QqTOF-MS

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ABSTRACT

Degradation of the sulfonamide sulfamethazine (SMZ) by the white-rot fungus *Trametes versicolor* was assessed. Elimination was achieved to nearly undetectable levels after 20 h in liquid medium when SMZ was added at 9 mg L^{-1}. Experiments with purified laccase and laccase-mediators resulted in almost complete removal. On the other hand, inhibition of SMZ degradation was observed when piperonilbutoxide, a cytochrome P450-inhibitor, was added to the fungal cultures. UPLC–QqTOF-MS analysis allowed the identification and confirmation of 4 different SMZ degradation intermediates produced by fungal cultures or purified laccase: desulfo-SMZ, N^4-formyl-SMZ, N^4-hydroxy-SMZ and desamino-SMZ; nonetheless SMZ mineralization was not demonstrated with the isotopically labeled sulfamethazine-phenyl-$^{13}C_6$ after 7 days. Inoculation of *T. versicolor* to sterilized sewage sludge in solid-phase systems showed complete elimination of SMZ and also of other sulfonamides (sulfapyridine, sulfathiazole) at real environmental concentrations, making this fungus an interesting candidate for further remediation research.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) have been the focus of recent concern and research due to their widespread occurrence in the environment (Onesios et al., 2009). Among these emerging contaminants, antibiotics are extensively used not only in human and veterinary medicine but also as growth promoting agents in factory farming and aquaculture (Sarmah et al., 2006). Their environmental impact is linked to adverse effects on ecological systems and the generation and persistence of microbial strains resistant to antibiotics (Kümmerer, 2009).

Sulfonamides (SAs) constitute a widely consumed class of antibiotics, due to their low cost, broad spectrum of activity and effectiveness in growth promotion. Although they were used for human application, currently higher amounts are applied to treat or prevent infectious diseases in livestock and cattle farming (Boxall et al., 2003). The excretion of unmetabolized SAs in feces and urine from human and animals is therefore one of the main sources for the introduction of these antibiotics into the environment. A big fraction of these residues is eventually discharged into the sewage, where monitoring data of treated sewage in wastewater treatment plants indicates that the techniques nowadays applied are not effective to remove antibiotics, as they are detected in the effluents and the sludge produced (Garcia-Galan et al., 2011; Gros et al., 2010; Jelic et al., 2009). The discharge of these effluents in natural waters together with the application of sludge in croplands as nutrients amendment represent the entrance of SAs in the different environmental compartments (Boxall et al., 2003).

Some laboratory-scale studies have been performed to evaluate the biodegradability of PPCPs contained in sewage. However, all of them relied on bacterial communities originated from activated sludge, which is the base of WWTP processes (Perez et al., 2005). Recently, *Trametes versicolor*, a member of the group of white rot fungi (WRF), has received attention due to its ability to degrade in liquid medium some PPCPs, such as nonsteroidal antiinflammatory drugs, lipid regulators or antiepileptics (Marco-Urrea et al., 2010a; Marco-Urrea et al.,

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2009). This organism is able to simultaneously attack a wide range of pollutants due both to the non-specificity of its ligninolytic enzymatic complex constituted by peroxidases and laccases, and to the intracellular activity of the cytochrome P450 system (Doddapaneni and Yadav, 2004; Gianfreda et al., 1999; Martinez et al., 2005), features especially desirable for bioremediation purposes in complex matrixes such as sludge. In this respect, the degradation of some spiked PPCPs (naproxen and carbamazepine) demonstrated by *T. versicolor* in sludge cultures (Rodriguez-Rodriguez et al., 2010) revealed promising results for potential applications.

In this work, the ability of the *T. versicolor* to degrade sulfamethazine (SMZ), one of the most commonly used veterinary SAs, was studied in defined liquid medium at high concentrations in order to identify the metabolites released during its degradation and some of the possible enzymes involved in the process. The occurrence of SAs contained in sewage sludge from a wastewater treatment plant (WWTP), and their fate after a fungal treatment with *T. versicolor* were also investigated.

2. Materials and methods

2.1. Fungal strain

The strain *T. versicolor* ATCC 42530 was acquired from the American Type Culture Collection, and maintained by subculturing every 30 days on 2% malt extract agar slants (pH 4.5) at 23 °C. *T. versicolor* pellets were produced by inoculating a 1 L Erlenmeyer flask containing 250 mL malt extract medium with 1 mL blended mycelium suspension and shaking (135 rpm) at 25 °C for 5 days (Font Segura et al., 1993).

2.2. Chemicals and reagents

SMZ (4-amino-*N*-(4,6-dimethylpyrimidin-2-yl)benzenesulfonamide, 99%), sulfathiazole (STZ, 4-amino-*N*-(1,3-thiazol-2-yl)benzenesulfonamide, 99%), sulfapyridine (SPY, 4-amino-*N*-(pyridin-2-yl)benzenesulfonamide, 99.9%), sulfamethazine-phenyl-$^{13}C_6$ hemihydrate (SMZ-phenyl-$^{13}C_6$), piperonil butoxide (PB, ≥90%), 1-hydroxybenzotriazole (HOBT, ≥98.0%), 3,5-dimethoxy-4-hydroxyacetophenon (DMHAP, 97%), 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS, ~98%) were obtained from Sigma-Aldrich (St. Louis, MO). Internal standard d4-sulfathiazole (99.9%) was purchased from Toronto Research Chemicals (Ontario, Canada). HPLC grade acetonitrile, water and formic acid 98% were provided by Merck (Darmstadt, Germany). 0.45 μm Nylon filters were purchased from Whatman (Maidstone, UK). Hydromatrix dispersing agent was purchased from Agilent (Santa Clara, CA, USA). Purified laccase from *T. versicolor* was obtained from Sigma-Aldrich (St. Louis, MO).

2.3. Experimental procedures

2.3.1. In vivo degradation experiments

Degradation experiments were performed in 250 mL Erlenmeyer flasks containing 10 g of fungal pellets (wet weight) in a total volume of 50 mL of a chemically defined medium. Defined aqueous medium (pH 4.5) contained per liter: 8 g glucose, 498 mg nitrogen as ammonium tartrate, 10 and 100 mL micro- and macronutrient solution (Kirk et al., 1978), respectively and 1.168 g 2,2-dimethylsuccinate as a buffer. Uninoculated flasks containing 50 mL defined medium and autoclaved (121 °C, 15 min) cultures cultivated under identical conditions to the experimental cultures were employed as abiotic controls and heat-killed controls, respectively. All samples were tested in triplicate.

SMZ was added to the flasks to give the desired concentration (approximately 9 mg L^{-1}) from a stock solution in methanol. Flasks were incubated in the dark on an orbital shaker (135 rpm) at 25 °C. In time-course experiments 1 mL samples were periodically withdrawn, filtered (0.22 μm Millex-GV filters, Millipore, Billerica, MA) and subsequently analyzed by high performance liquid chromatography (HPLC). Degradation was calculated by comparing the SMZ concentration in the abiotic controls with that in the experimental flasks. All the concentration values were corrected for the adsorption determined in the heat-killed controls.

Mineralization experiments were performed in 125 mL amber serum bottles (Wheaton, Millville, NJ) sealed with Teflon-coated gray butyl rubber stoppers, with the same proportion fungal pellets/medium, in a final volume of 10 mL. Isotopically labeled SMZ-$^{13}C_6$ was added to a final concentration of 10 mg L^{-1}. After 7 days, samples were analyzed for the determination of $^{13}C/^{12}C$ ratios in the CO_2 contained in the headspace air.

2.3.2. Experiments with cytochrome P450 inhibitors and enzymatic degradation with laccase

Laccase-mediated degradation experiments were performed in Erlenmeyer flasks containing 50 mL of a purified laccase solution at an initial activity concentration of 343 ± 24 activity units (U) L^{-1} (pH 4.5). The effect of laccase-mediators was evaluated by adding either 1 mM HOBT, 0.8 mM DMHAP or 0.8 mM ABTS to the reaction mixture. Controls containing milli-Q water at pH 4.5 were included. SMZ was added at a concentration of 20 mg L^{-1}. Flasks were incubated in the dark on an orbital shaker (135 rpm) at 25 °C. At designated time-points, 1 mL samples were withdrawn and 100 μL acetic acid was added to stop the reaction prior to HPLC analysis.

To determine the effect of cytochrome P450 inhibitor, PB was added to a final concentration of 5 mM (Marco-Urrea et al., 2009) in experiments performed as described in Section 2.3.1. SMZ was added at 9 mg L^{-1}. Heat-killed and inhibitor-free controls were included in triplicate.

2.3.3. SAs degradation by T. versicolor in solid-phase sewage sludge systems

Removal of SAs at environmental concentrations contained in sludge was evaluated in sewage sludge collected from the WWTP of El Prat de Llobregat, in Barcelona, Spain. The plant has a total treatment capacity of two million equivalent inhabitants. It is a typical biological activated sludge plant with sludge anaerobic digestion and thermal dehydration. Dry sludge obtained from the final stage of processing, i.e., after thermal dehydration (~10% water content) was employed in sterile solid-phase, biopile-like treatments inoculated with *T. versicolor* previously grown on wheat straw pellets (Rodríguez-Rodríguez et al., 2011). Biopiles were incubated at 25 °C and continuously moisturized and homogenized for 42 days. To act as a control, raw sludge (i.e., fungus free) received the same processing as the treated sludge.

2.4. Analytical procedures

2.4.1. Analysis of SMZ in liquid medium

SMZ determinations were performed using a Dionex 3000 Ultimate HPLC (Sunnyvale, CA) equipped with a UV detector at 264 nm. Chromatographic separation was achieved by injection of 20 μL samples on a Grace Smart RP18 column (250 × 4 mm, 5 μm particle size) and a mobile phase consisting 65% of 40 mM ammonium acetate buffer (pH 7) plus 35% methanol, added isocratically at 1 mL min^{-1} (de Zayas-Blanco et al., 2004). Column temperature was kept at 30 °C. Under these experimental conditions, retention time for SMZ was 5.2 min.

2.4.2. Gas chromatography–combustion-isotope ratio mass spectrometry (GC–CIRMS) analyses

$^{13}C/^{12}C$ ratios of headspace CO_2 were determined by a GC–combustion-isotope ratio mass spectrometry system in samples from mineralization experiments, as previously described (Marco-Urrea et al., 2008). The GC–CIRMS consisted of a GC (Agilent 6890N) coupled to a Thermo-Finnigan (Bremen, Germany) Delta Plus mass spectrometer through a FinniganMat GC combustion-III interface. Ratios were expressed as relative deviations δ‰ (delta per mille). The $\delta^{13}C$ value is

defined as $\delta^{13}C = (R_s/R_r - 1) \times 1000$, where R_s and R_r are the $^{13}C/^{12}C$ ratios in the sample and the international standard Vienna Peedee Belemnite, respectively.

2.4.3. Identification of degradation products in liquid samples

MS and tandem MS (MS/MS) analyses of SMZ and its degradation products were performed using a Waters/Micromass QqTOF-Micro system coupled to a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system (Micromass, Manchester, UK). A Waters Acquity BEH C18 column (10×2.1 mm, 1.7 μm particle size) was employed. Flow rate was set up at 0.3 mL min^{-1}, with eluent A acetonitrile and eluent B HPLC grade water, both acidified with 10 mM of formic acid (pH 3.5). The elution started at 5% B for 2 min, it was linearly increased to 60% of B in 7 min, further increased to 95% of B in the following 2 min, and then returned to initial conditions in 2 min. The injection volume of the sample was 5 μL.

Analyses were carried out with electrospray ionization in the positive ionization (ESI+) mode, and the operating conditions were as follows: desolvation gas flow 350 L h^{-1}; source and desolvation temperatures 120 °C and 350 °C, respectively; capillary voltage 3000 V; sample voltage 30 V; and extraction cone voltage 1 V. The collision energy (CE) was held at 5 V for MS analyses, and varied from 10 to 35 V to obtain the fragmentation patterns when performing product ion scans. The applied collision gas was argon at a pressure of −20 psi. For continuous internal mass calibration, an independent reference (Valine–tyrosine–valine) was used as a lock mass, with m/z 380.2185, and was acquired in all the measurements. For the MS analyses on the QqTOF instrument, MS data was obtained by scanning from m/z 50 to m/z 500.

2.4.4. Pressurized liquid extraction and clean up of sludge samples

All samples of dehydrated sludge from the biopile were previously freeze-dried at −35 °C and at 0.045 bar vacuum. They were extracted by pressurized liquid extraction (PLE) using and accelerated solvent extractor ASE 300 (Dionex, Sunnyvale, CA). Grinded sludge (2 g) was mixed with Hydromatrix dispersing agent and placed within 11 mL stainless steel cells. Samples were extracted at a pressure of 1500 psi, a temperature of 50 °C and using as extraction solvents a mixture of water/acetonitrile (75/25, v/v).

Clean up was carried out after the extraction of the samples; the extracts obtained in PLE (20 mL) were diluted in 200 mL of HPLC water, to decrease the organic solvent amount below 5%, and preconcentrated using Oasis HLB cartridges (200 mg, 6 mL, Waters Corporation, Milford, MA). Recovery values of 65% were obtained for SMZ, with a relative standard deviation (RSD) of 5%. Both the optimization of the extraction and the solid phase extraction procedures (SPE) are described in a different work (Garcia-Galan et al., submitted for publication).

2.4.5. Determination of SAs in sludge samples

LC–MS/MS analyses were carried out following the method already published by the authors (Diaz-Cruz et al., 2008), which was adapted for the sole analysis of SMZ. The system consisted of an Agilent HP 1100 pump (Agilent Technologies, Palo Alto, CA) connected to a 4000QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (QqLIT-MS) equipped with a turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, CA). Chromatographic separation was carried out using an Atlantis C18 (Waters, 150×2.1 mm, 3 μm particle size) LC-column preceded by a guard column with the same packing material. The flow rate was set to 0.2 mL min^{-1}, using HPLC grade water (eluent A) and acetonitrile (eluent B) both 10 mM in formic acid (pH 3.5). The elution gradient started with 25% eluent B, increasing to 80% in 10 min and 100% in 11 min, remained there for 2 min, readjusted to the initial conditions in 3 min, and finally equilibrated for 7 min. MS/MS data acquisition was performed in the selected reaction monitoring (SRM) mode, in the PI mode.

2.4.6. Other analyses

Laccase activity was measured using a modified version of the method for manganese peroxidase determination (Wariishi et al., 1992); the reaction mixture consisted of 200 μL sodium malonate (250 mM, pH 4.5), 50 μL 2,6-dimethoxyphenol (DMP, 20 mM) and 600 μL sample. DMP is oxidized by laccase even in the absence of a cofactor. Changes in the absorbance at 468 nm were monitored for 2 min at 30 °C. Results were expressed as U per liter. One U was defined as the number of micromoles of DMP oxidized per min. The DMP extinction coefficient was 24,800 M^{-1} cm^{-1}.

Glucose was analyzed by the glucose oxidase method in an YSI 2700 analyzer (Yellow Springs, OH). Mycelia dry weight was determined by vacuum filtering the cultures through preweighed glass filters (Whatman GF/C). Filters containing the biomass were dried at 100 °C to constant weight.

3. Results and discussion

3.1. Degradation of SMZ by T. versicolor

In order to demonstrate SMZ degradation by *T. versicolor* and to favor the identification of its metabolites, *in vivo* transformation was tested during a 96 h period with high concentration of the SA. Time-course results of degradation in defined medium showed a steep decrease in SMZ concentration in the first 20 h (>95%, Fig. 1). Adsorption was determined by comparing the concentration in the heat-killed controls to that in the uninoculated flasks. By the end of the experiment adsorption seemed negligible, though the concentration in the uninoculated controls was reduced by approximately 25%. This reduction in the SMZ concentration could be explained by the formation of N^4-glycosyl-SMZ as a by-product, as determined by MS and tandem MS analyses (see Section 3.3); it was found also in the controls, indicating that it was produced merely by chemical affinity. The N^4-glycosyl-SMZ remained constant in both the heat-killed and the uninoculated controls, but disappeared in the reaction flasks, suggesting its consumption by the fungus. Anyhow, the finding of degradation metabolites in the reaction flasks together with their absence in the heat-killed and uninoculated controls (Section 3.3) demonstrates that SMZ disappearance by active fungus is due to degradation and not merely sorption. The experimental degradation rate was 120 ng h^{-1} mg^{-1} dry weight biomass.

3.2. Role of laccase and cytochrome P450 inhibition in degradation of SMZ

The role of laccase has been demonstrated in the degradation of a wide range of pollutants and interesting reviews are available in this respect (Couto and Herrera, 2006; Gianfreda et al., 1999). In this case, a continuously increasing extracellular laccase activity was detected

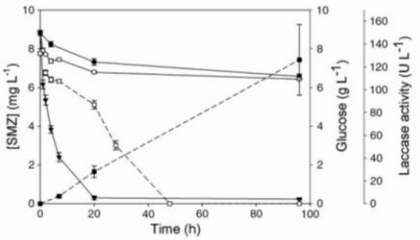

Fig. 1. Time-course of SMZ degradation (9 mg L^{-1}, initial concentration) by *T. versicolor*. Symbols: uninoculated controls (●), heat-killed controls (○), experimental cultures (▼), glucose (□) and laccase activities (■). Values plotted are means ± standard deviations for triplicate cultures. The initial mycelial-pellet dry weight added to the flasks was 380.4 ± 2.9 mg.

throughout *in vivo* degradation of SMZ, reaching a maximum value of 126 UL^{-1} by the end of the experiment. Therefore investigation was conducted to determine whether purified laccase and laccase-mediator systems could be able to degrade SMZ. HOBT, DMHAP and ABTS are known to enhance oxidation of non-phenolic compounds by acting as mediators in the laccase reaction system as diffusible electron carriers between the substrate and the enzyme (Bourbonnais et al., 1998; Niku-Paavola and Viikari, 2000); molecules produced by the fungus may play an analogous role in active cultures. Results in Fig. 2A show that solely laccase at 343 UL^{-1} degraded 22% of SMZ, while removal with the mediators ranged from 93% to 100% after 48 h. Similar results have been reported for other pharmaceuticals such as naproxen (Marco-Urrea et al., 2010a) and diclofenac (Marco-Urrea et al., 2010b), although in the latter almost complete transformation was achieved even without the addition of mediators.

Another enzymatic complex usually involved in the transformation of environmental pollutants is the cytochrome P450 system. This intracellular mechanism of degradation has been associated to depletion of diverse compounds by WRF, ranging from polycyclic aromatic hydrocarbons (Bezalel et al., 1997) and chlorinated hydrocarbons (Marco-Urrea et al., 2008) to pharmaceuticals (Marco-Urrea et al., 2009). To assess its possible relation with SMZ transformation, the cytochrome P450 inhibitor PB was applied to *in vivo* experiments. As Fig. 2B reveals, a similar pattern of elimination occurred both in the PB-containing and the inhibitor-free cultures during the first hours. However, after 24 h an important delay in the elimination of SMZ (32%) was noticed in the flasks with inhibitor. The initial disappearance of SMZ with or without PB might be ascribed to the active transport of the antibiotic into the cell, as it has been hypothesized for other pharmaceuticals (Marco-Urrea et al., 2009), and the effect of incipient laccase. Meanwhile, the posterior delay in the degradation rate of SMZ in PB-containing cultures compared to that in the inhibitor-free controls indicates the effect of the PB on the cytochrome P450, thus suggesting that this enzymatic system could be involved in the transformation of SMZ. The reduction in the antibiotic concentration at 48 h in the presence of PB could be due to i) only partial cytochrome P450 inhibition or ii) the action of laccase (based on the aforementioned results) or other enzymatic systems.

3.3. Identification of SMZ products from in vivo experiments

Given that mineralization of some pollutants by *T. versicolor* has been previously reported (Marco-Urrea et al., 2008; Tuomela et al., 1998), this capability was tested in cultures spiked with SMZ-phenyl-$^{13}C_6$ in order to calculate the $^{13}C/^{12}C$ ratios in the headspace CO_2. Yet, data from GC–CIRMS analyses did not demonstrate mineralization. Relative concentration of $\delta^{13}C$ in CO_2 in 7 day-cultures with SMZ-phenyl-$^{13}C_6$ was -25.3 ± 0.2, whereas in control cultures with non-labeled SMZ it was -25.2 ± 0.3. Results corresponded to a natural composition of ^{13}C, thus indicating the absence of a mineralization process.

Nonetheless, identification of metabolites produced during the degradation of SMZ by *T. versicolor* and purified laccase was achieved through UPLC–QqTOF-MS analyses. Fig. 3 shows the different intermediate products detected in the *in vivo* and enzymatic degradation experiments. After chromatographic separation with UPLC, SMZ was detected at a retention time of 3.52 min. At reaction time 0, a peak at 2.38 min appeared in both abiotic and heat-killed control samples, with a base peak m/z 441.1433 in the corresponding mass spectra (observed mass). The elemental composition for this mass is $C_{18}H_{24}N_4O_7S$ (theoretical m/z 441.1444; see Supplementary data), and corresponds to the by-product N^4-glycosyl-SMZ, which is formed due to the presence of glucose in the defined culture medium described in Section 2.3.1. It was also detected in the *in vivo* experiments at times 8 h and 24 h, until its complete disappearance after 96 h. In order to confirm and also to gain further structural information, product ion scans at different CEs were performed. The spectra obtained showed the original molecule of SMZ as a fragment, and also fragments at m/z 254.1039, characteristic for this by-product, and fragments m/z 186.0359 and m/z 124.0885, also common to the SMZ fragmentation pattern (Díaz-Cruz and Barceló, 2004).

A second peak appeared at a chromatographic retention time of 2.02 min, which corresponded to a m/z of 215.1298. Its elemental composition was elucidated as $C_{12}H_{15}N_4$ and associated to the loss of the sulfonate group in the molecule, as shown in Fig. 3. This finding agrees with previous studies in which enzymatic degradation, physicochemical oxidation and photodegradation products of SAs were defined as SO_2 extrusion products (Neafsey et al., 2010; Schwarz et al., 2010; Unold et al., 2009a, 2009b). The peak intensity of this molecule in the chromatogram, increased with the reaction time and reached 43% of the initial intensity of the parent SMZ after 24 h (Fig. 4), decreasing again to 11% at 96 h. The product ion spectrum (CE of 30 eV) for confirmation is shown in Fig. 5. A second signal corresponding to the estereoisomer of this intermediate product was present at RT 1.78 min. The intensity of this signal was however much lower than that registered at RT 2.02 min, and not considered in Fig. 4. This desulfonated product of SMZ was the only common metabolite present also in the enzymatic degradation experiments.

A third chromatographic peak appeared after 3.18 min in the fungal cultures, with an observed m/z of 307.0864. The elemental composition for this mass is $C_{13}H_{15}N_4O_3S$ and may correspond to the addition of a formyl group to SMZ. The corresponding product ion spectrum shows

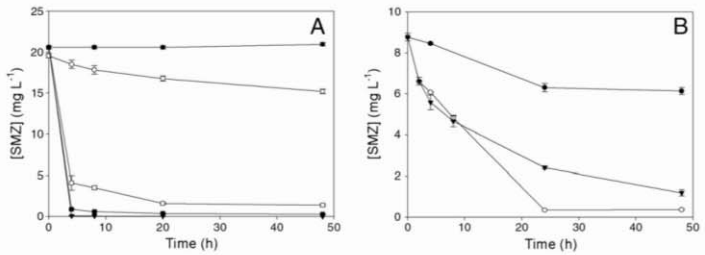

Fig. 2. Role of laccase and cytochrome P450 on SMZ degradation. (A) Time-course degradation of SMZ (20 mg L^{-1}) by purified laccase at an initial activity of 343.4 ± 24.4 UL^{-1}. Symbols: laccase-free controls (●), laccase without mediators (○), ABTS 0.8 mM as mediator (▼), DMHAP 0.8 mM as mediator (□) and HOBT 1 mM as mediator (■). (B) Influence of the cytochrome P450 inhibitor piperonil butoxide (5 mM) on the degradation of SMZ (9 mg L^{-1}, initial concentration). Symbols: uninoculated controls (●), inhibitor-free controls (○) and cultures containing piperonil butoxide (▼). The initial mycelial-pellet dry weight added to the flasks was 299.1 ± 0.2 mg. Values plotted are means ± standard deviations for triplicate cultures.

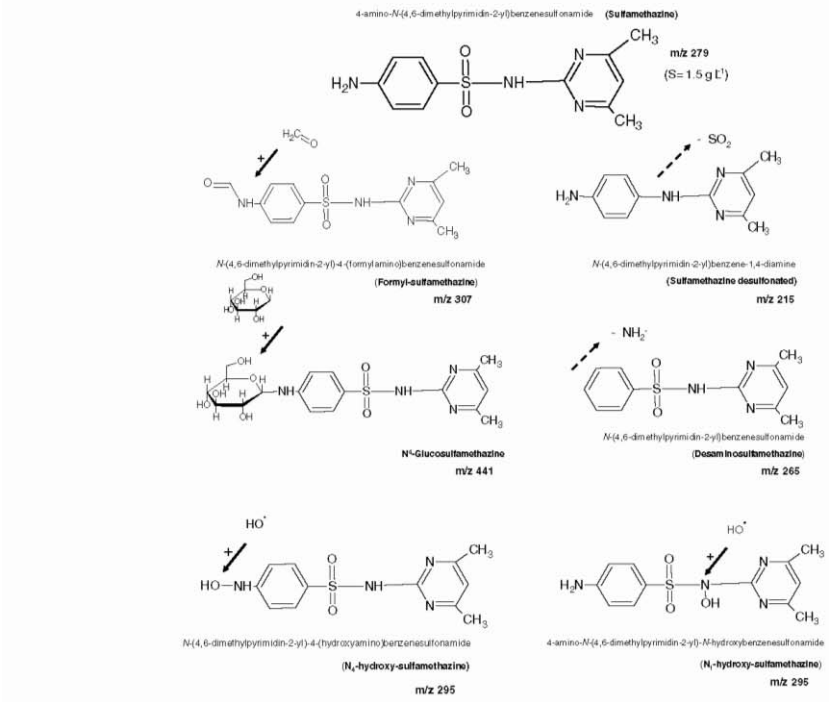

Fig. 3. Proposed transformation products of SMZ after the *in vivo* and enzymatic degradation experiments. S: solubility at 29 °C.

the different fragments identified for this molecule (see Supplementary data). Similar formyl metabolites were proposed during metabolism of sulfadiazine in mammals, photodegradation of sulfadiazine in water and laccase-mediated degradation of sulfapyridine (Lamshöft et al., 2007; Schwarz et al., 2010; Sukul et al., 2008). The presence of formic acid in the UPLC eluents used could have led to the formation of this molecule as a by-product in the samples. However, it was not detected either in the abiotic or in the heat-killed control samples, which were analyzed with the same UPLC eluents and gradient.

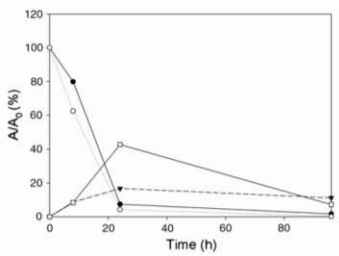

Fig. 4. Time-course degradation of SMZ by *T. versicolor*, and time course evolution of N^4-glycosyl-SMZ, N^4-formyl-SMZ and desulfonated-SMZ. Symbols: SMZ (●), N^4-glycosyl-SMZ (○), desulfonated-SMZ (□) and N^4-formyl-SMZ (▼).

3.4. Identification of SMZ products from enzymatic degradation experiments

After 48 h of reaction, two new chromatographic peaks appeared and were only identified in the enzymatic degradation assays (Fig. 6). The first of them showed a retention time of 3.5 min and a base peak of m/z 295.0878, corresponding to the hydroxylated metabolite of SMZ. The product ion spectra obtained, however, could not provide the required information regarding the location of the hydroxyl group in the molecule. The QqLIT-MS instrument was also employed to elucidate the structure of this metabolite, due to its higher sensitivity compared to the QqTOF, and enhanced product ion scans at different CE were obtained. However, the results were similar (see Fig. 6) and the fragments obtained were only those characteristic for SMZ. The proposed structures for the hydroxylated metabolite are shown in Fig. 3. This is all set on the basis that, although phenol-like compounds are the typical substrates of laccase-mediated oxidation, aromatic amines can also act as a substrate (Nyanhongo et al., 2007); moreover, the primary amine seems to be the most reactive site in the case of the SMZ molecule. To the author's knowledge, this metabolite is reported for the first time for SMZ degradation. Other hydroxylated metabolites, 6-hydroxymethyl-SMZ and 5-hydroxy-SMZ, have been reported in plasma and urine of mammals, however, this oxidation is due to cytochrome P450 activity (Witkamp et al., 1993) rather than laccase. 4-Hydroxy- sulfadiazine was detected by Lamshöft et al. (2007) and was considered in the proposed hydroxylated moieties for SMZ in Fig. 3.

The cleavage of the N^4 amine bond yielded a new transformation product, a desaminated metabolite with m/z 264.0794, which was

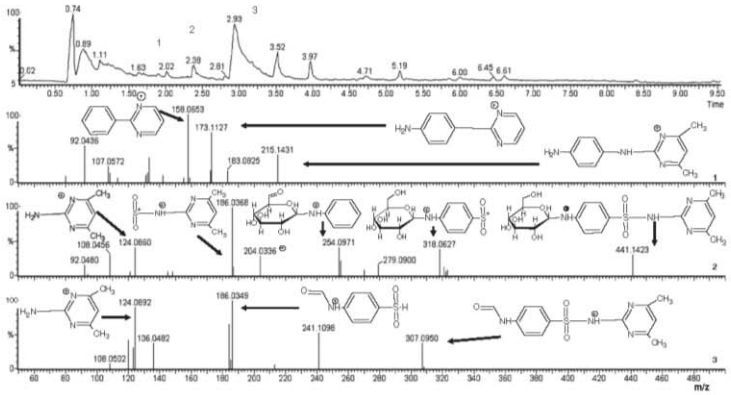

Fig. 5. Total ion chromatogram (TIC) and product ion spectra obtained from ESI (+)MS/MS experiments with UPLC–QqTOF for the fungal cultures after 24 h of reaction: 1) desulfonated-SMZ (CE 30 eV); 2) N^4-glycosyl-SMZ (CE 25 eV); and 3) N^4-formyl-SMZ (CE 20 eV) (cone voltage: 30 V for all the experiments).

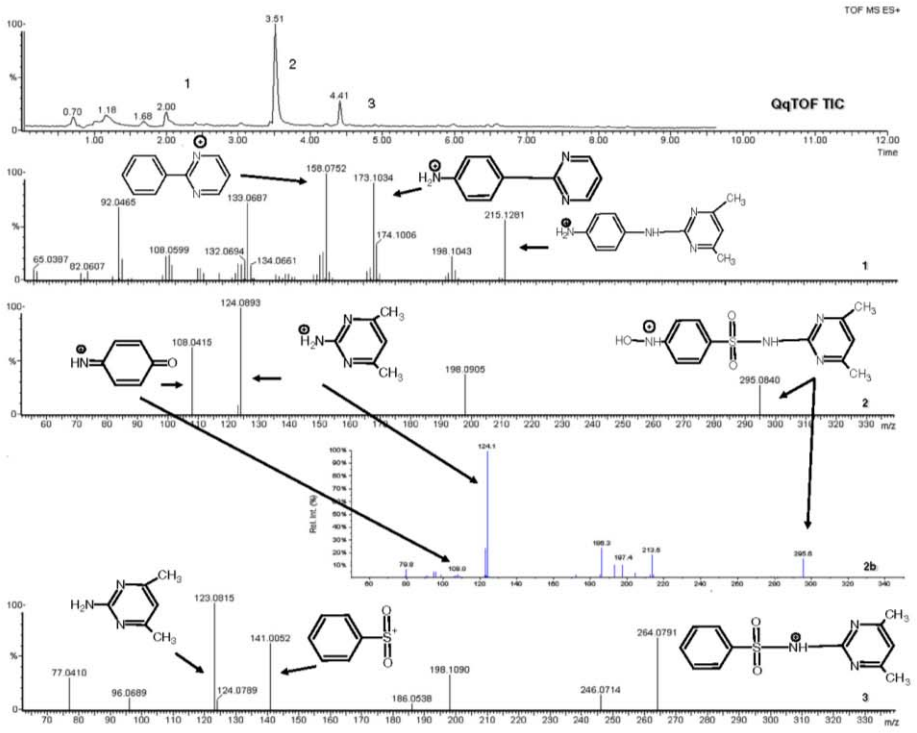

Fig. 6. Total ion chromatogram (TIC) and product ion spectra obtained from ESI (+)MS/MS experiments with UPLC–QqTOF for the enzymatic degradation assays after 48 h of reaction: 1) desulfonated-SMZ (CE 30 eV); 2) hydroxyl-SMZ (CE 20 eV) ; 3) desaminated-SMZ (CE 20 eV) (cone voltage: 30 V for all the experiments). Spectrum 6.2.b corresponds to an enhanced product ion experiment carried out with HPLC-QqLIT (CE 25 eV).

Table 1
Removal of sulfonamides from sewage sludge after fungal solid-phase treatment with *T. versicolor*.

Sulfonamide	Internal standard	LOD (ng g^{-1})[a]	Raw sludge (ng g^{-1}) ± RSD[b], (%)	Treated sludge (ng g^{-1}) ± RSD[b], (%)	Removal (%)
Sulfamethazine	d4-Sulfamethazine	0.12	19.1 (±25.4)	n.d.[c]	100
Sulfapyridine	d4-Sulfamethazine	0.02	29.4 (±15.6)	n.d.	100
Sulfathiazole	d4-Sulfamethazine	0.01	71.1 (±9.0)	n.d.	100

[a] Limit of detection of the method.
[b] Relative standard deviation of mean concentration ($n=3$).
[c] n.d.: not detected.

identified at a chromatographic retention time of 4.59 min. Product ion scans resulting from an optimum fragmentation at CE of 30 eV confirmed the proposed structure (Fig. 6). Interestingly, this metabolite is known to be present, among others, in mammal tissues as part of SMZ metabolism (Fischer et al., 1992). Besides these two new products, the desulfonated metabolite previously identified in the *in vivo* assays (see Section 3.3) was also present in the enzymatic degradation experiments. The intensity of the corresponding peak was at least one order of magnitude higher than the obtained in the 24 h fungal culture (when the maximum formation of the desulfonated metabolite was registered). The presence of a common intermediate metabolite with *in vivo* experiments supports a possible role of laccase in SMZ transformation by *T. versicolor*.

3.5. Fungal SAs degradation in sewage sludge systems

In this work the demonstrated ability of *T. versicolor* to degrade SMZ, and presumably other SAs was tested for a potential application. Considering both that the previous conditions of degradation in liquid wastes would be unfeasible in real systems (e.g. glucose addition), and that degradation needs to be demonstrated at environmentally relevant concentrations, the elimination of SAs was tested in a real complex matrix such as sludge and using a solid waste material as the substrate for the fungus. Thus, removal of SAs at their preexistent concentrations in sewage sludge was assessed in solid-phase biopile-like systems (Rodríguez-Rodríguez et al., 2011), consisting of sterilized sludge supplemented with a fungal inoculum previously grown on a lignocellulosic substrate (wheat straw pellets).

Initial characterization of the sludge is presented in Table 1. Concentrations of SAs ranging from 19.1 to 71.1 ng g^{-1} were found in the raw sludge, which again highlights the fact that important concentrations might be released in the environment if biosolids are applied as organic amendments in cropland (Kim et al., 2011). Reports of SAs removal in WWTPs usually refer to elimination without considering the compounds sorbed to biosolids, while other have demonstrated important degradation rates of SAs in different stages of sewage treatments (Perez et al., 2005). In the present work, after the fungal-mediated treatment the degradation of preexistent SAs was highly efficient (around 100%), since none of them was found in the treated sludge. Dissipation of the SAs forming sequestered residues was ruled out, as removal was determined by comparison of same-old raw and treated sludge, thus ascribing elimination to the action of the fungus. Reports of analogous treatments applied to solid wastes for the removal of antibiotics are scarce, with different levels of success depending on the SA. In an anaerobic fermentation process of swine manure, some SAs including sulfadiazine, sulfamerazine, sulfamethoxypyridazine, sulfamethoxazole and sulfamethoxine were eliminated at rates over 70%, however, SMZ and sulfathiazole were not removed at all after 34 days (Mohring et al., 2009). Similarly, in a process of natural attenuation in biosolids, sulfamethoxazole was completely degraded, whereas SMZ was reduced to a 40% (Wu et al., 2009). Though more experiments should be conducted in order to optimize this solid-phase process, the remarkable results suggest that *T. versicolor* may be an interesting bioremediation agent for the elimination of SAs in sewage sludge.

4. Conclusions

The degradation of SMZ by *T. versicolor* was demonstrated. Laccase seemed to play a key role in the transformation process, and three metabolites were elucidated from enzymatic degradation assays. Two other degradation products were identified during *in vivo* experiments, and though their concentration decreased after 96 h, their full mineralization was not achieved after 7 days. The degradation capacity of *T. versicolor* was additionally demonstrated in sewage sludge sterilized systems, where 100% removal was accomplished for SMZ, sulfapyridine and sulfathiazole, demonstrating the potential application of the fungus for bioremediation purposes.

Supplementary materials related to this article can be found online at doi:10.1016/j.scitotenv.2011.08.022

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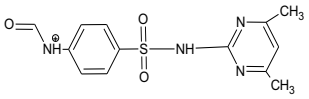
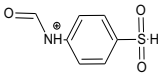
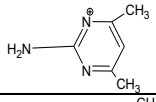
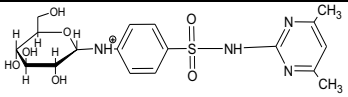
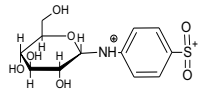
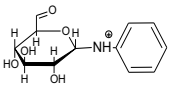
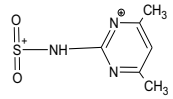
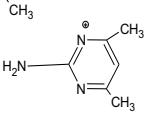
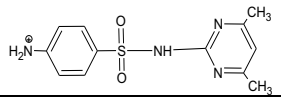
SUPPLEMENTARY INFORMATION #13

Table 1. Accurate mass measurement of sulfamethazine biodegradation products as determined by UPLC/ESI-QqToF in MS^2 mode and optimized cone voltage (25 V) and collision energies (25-35 eV).

	Fragment (m/z)	Elemental composition	Observed mass	Calculated mass	Error (mDa)	Proposed structural composition
PRODUCTS IN REACTION SAMPLES	**307**	**$C_{13}H_{15}N_4O_3S$**	**307.0864**	**307.0865**	**0.1**	
	186	$C_7H_8NO_3S$	186.0217	186.0225	0.8	
	124	$C_6H_{10}N_3$	124.086	124.0875	1.5	
	441	**$C_{18}H_{25}N_4O_7S$**	**441.1433**	**441.1444**	**1.1**	
	318	$C_{12}H_{16}NO_7S$	318.0675	318.0647	2.8	
	254	$C_{12}H_{16}NO_5$	254.1039	254.1028	1.1	
	186	$C_6H_8N_3O_2S$	186.0359	186.0337	2.2	
	124	$C_6H_{10}N_3$	124.0885	124.0875	1.5	
	94	C_6H_8N	94.0645	94.0657	1.2	
	279	$C_{12}H_{15}N_4O_2S$	279.09	279.0916	1.5	

4.3. DISCUSIÓN

4.2.1. Eliminación de sulfamidas en bioreactores de membrana

En el Capítulo 3 de esta Tesis se presentaron valores de eliminación (RE%) durante el tratamiento de aguas residuales, que se estimaron utilizando los datos de presencia de sulfamidas en aguas de entrada y salida de depuradoras en las que se llevaba a cabo un tratamiento de CAS. La disparidad de dichos valores para algunas de las sulfamidas no permitió alcanzar una conclusión clara sobre su mayor o menor biodegradabilidad, ya que para algunas de ellas como SMX o SDZ el rango de RE% cubría valores tanto negativos como positivos. Para poder contrastar estos datos y evaluar la eficacia de los tratamientos alternativos al CAS, se realizó una nueva campaña de muestreo en la depuradora de Terrassa (Barcelona), que contaba con dos MBRs diferentes, de tipo Koch y Kubota. Los valores de RE% obtenidos en los efluentes de ambos MBRs fueron más elevados que los obtenidos a la salida de CAS de la misma planta (ver Tabla 3 de la Publicación #11) para la mayoría de las sulfamidas estudiadas. Aún así, los RE% correspondientes a las sulfamidas más relevantes (SMX y SPY) fueron apenas superiores a los obtenidos con CAS en esa misma depuradora. A pesar de ello, el dato más destacable fue que los rangos de valores de RE% obtenidos en los dos MBRs para estas dos sulfamidas eran más acotados en comparación a los obtenidos en los efluentes de CAS. La Figura 4.1 muestra dichos rangos, calculados a partir de los valores de RE% obtenidos en todos los efluentes de CAS analizados en las Publicaciones #9 y #10, (n=29) y en los efluentes de MBR Koch y MBR Kubota de la Publicación #11 (n=12). También para la SMZ los rangos de RE% en los bioreactores fueron más pequeños, pero no así para la SDZ (sólo con valores de Koch) y el STZ. En ningún caso se observó una eliminación completa a la salida de los MBR. Algunos autores indican que indican que pHs ligeramente ácidos (4.3-5) permiten una degradación mucho más eficiente en estos reactores para compuestos ácidos(Urase *et al.* 2005). En función del pH de la muestra, las sulfamidas pueden encontrarse protonadas, en estado neutro o con carga negativa. A pH neutro, que es el normal en las muestras de efluente de EDAR (p.e. las muestras de salida de los MBR tenían un pH de 7.2 a 7.8 durante los diferentes días de muestreo) las sulfamidas suelen encontrarse cargadas negativamente, de modo que su

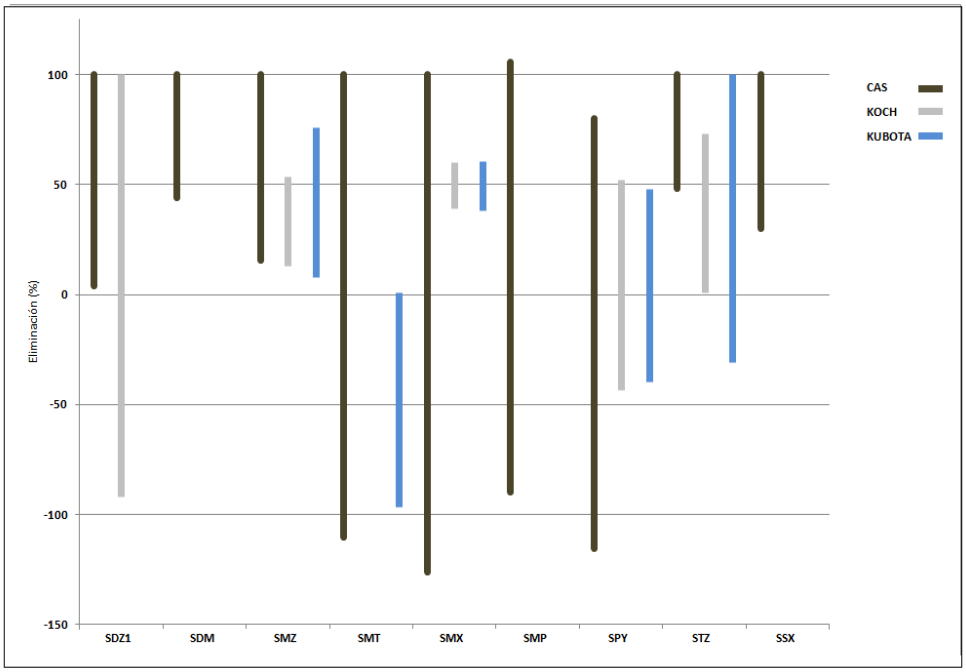


Figura 4.1. Rangos para los valores de eliminación (RE%) obtenidos para las diferentes sulfamidas durante el tratamiento con CAS y con MBRs.

adsorción a los sólidos del reactor es poco probable, ya que éstos también tienen una superficie con carga negativa. En el caso del SMX, otros autores sin embargo atribuyen esta baja eficacia de los MBR a su resistencia a la biodegradación, comparándola con otros fármacos muy recalcitrantes como la carbamazepina e incluso proponiéndo su uso como trazador de contaminación orgánica en aguas residuales (Benotti *et al.* 2009). Sin embargo, la capacidad degradativa de los MBR sigue considerándose una buena alternativa al CAS. En un trabajo reciente, Bouju *et al*. consiguieron aislar cinco cepas bacterianas procedentes de un MBR, previamente aclimatizadas a SMX, carbamazepina y diclofenaco, y demostraron su capacidad para mineralizar completamente estos compuestos (Bouju *et al.* 2012).

4.2.2. Eliminación de SPY, SMZ y sus metabolitos acetilados utilizando reactores de lecho fijo

Los FBBRs actúan de forma similar a los reactores de CAS, con la diferencia de que los microorganismos no se encuentran en suspensión en el reactor sino en un soporte fijo; en nuestro caso unas pequeñas bolas de vídrio colocadas en una columna ofrecieron la superficie necesaria a los microorganismos para establecerse y desarrollarse. En la literatura aún no existe ninguna referencia sobre la eliminación de sulfamidas en reactores de tipo FBBR. En nuestro estudio, utilizamos varios FBBRs a escala de laboratorio para simular el comportamiento de SPY, AcSPY y AcSMZ en aguas ambientales. En la Tabla 4.1 hemos incluido los valores medios de RE% para SPY y AcSPY en los FBBRs y los obtenidos en las EDAR con tratamiento CAS que aparecen en las Publicaciones #9 y #10 del Capítulo 3. Ya que los reactores tipo FBBR estuvieron funcionando unos 60 días, tiempo que excede el tiempo de retención hidraúlico (HRT) de las EDAR consideradas, se ha utilizado como referencia el tiempo de vida media obtenido en los FBBR. No obstante, la comparación de los resultados es complicada no sólo porque los HRT son distintos en cada tipo de tratamiento, sino que la matriz también es distinta, ya que para FBBR se empleó efluente de EDAR y no licor mezcla. Sin embargo hay resultados llamativos. Por ejemplo, la Figura 3 de la Publicación #12 nos muestra que la eliminación completa de SPY no se consiguió tras 60 días de experimento, mientras que el 50% fue eliminado tras unos 27 días aproximadamente.

Tabla 4.1. Valores de RE% para SPY y AcSPY después de tratamientos CAS y FBBR con tiempos de retenció hidraúlico (HRT) semejantes.

	HRT *(días)*	SPY	AcSPY
FBBR	**27**	**50**	
	23		**50**
CAS	19	88	37
	20	88	-11
	30	80	52

El metabolito acetilado AcSPY fue eliminado completamente tras 32 días, con un tiempo de vida media algo inferior que el de SPY (23 días). Como muestra la Tabla 4.1, los valores de RE% obtenidos con tratamientos de CAS en EDAR con HRT similares son bastante superiores para SPY, hecho que puede explicarse en base a que el licor mezcla es mucho más activo microbiológicamente que el agua de salida de EDAR. Sin embargo, los RE% obtenidos para

AcSPY fueron por lo general inferiores en CAS que en FBBR. Otro resultado a destacar es la evidencia de la conversión parcial de este metabolito en el compuesto original durante su eliminación en el FBBR (ver Figura 4 de la Publicación #12), hecho que ayudaría a explicar las eliminaciones negativas para algunas de las sulfamidas, registradas en varias de las EDAR de los trabajos anteriores. Göbel *et al*. (Göbel *et al.* 2007), ya habían considerado esta posibilidad para SMX, pero sin demostrarlo mientras que este comportamiento sí se había observado en abono proveniente de granjas ganaderas que contenía sulfadiazina (Kotzerke *et al.* 2008). Por último, cabe destacar la detección del producto de degradación desulfonado de AcSPY, común en trabajos de degradación biótica y abiótica. El metabolito AcSMZ permaneció, por el contrario, inalterado durante todo el experimento.

4.2.2. Biodegradación de sulfamidas mediante hongos

En la Publicación #13 se investigó la capacidad degradativa de *Trametes versicolor* frente a tres sulfamidas, una de consumo típicamente humano y detectada con frecuencia y en concentraciones relevantes en aguas de depuradora (SPY), STZ y SMZ, sulfamidas típicamente de uso veterinario pero también detectadas en ecosistemas urbanos. Se evaluó la biodegradación de cada una de ellas aplicando *T. versicolor* en medio liquido controlado, dopado a una concentración de 9 mg L^{-1}, y en muestras reales de fango y fango tratado, donde se evaluó la capacidad degradativa del hongo a concentraciones ambientales. La Tabla 4.2 muestra un resumen los resultados obtenidos en medio líquido.

Tabla 4.2. Biodegradación de tres de las sulfamidas estudiadas mediante la aplicación de *Trametes versicolor* (*pellets*) en medio liquido.

	C_0 (mg L^{-1})	T (h)	BIOD (%)	ADSORCIÓN	DEGR. EXTRA CELULAR	DEGR. INTRA CELULAR	OTROS	REF
SMZ	9	96	95	No	✓	✓	-	*
SPY	9	48	100	No	✓	✓	-	**
STZ	9	170	100	No	✓	✓	-	**

*C_0: concentración inicial; T: tiempo en horas o días; BIOD(%): biodegradación; REF: referencia bibliográfica. SMZ: sulfametazina; SPY: sulfapiridina; STZ: sulfatiazol; *: Publicación #13; **: Anexo A2, Publicación 1.*

Con el fin de poder diferenciar entre la actividad degradativa extracelular o intracelular, se llevaron a cabo dos experimentos más en paralelo: el primero sólo con la enzima lacasa purificada (sin el inóculo del hongo) y con mediadores que potencian su actividad oxidativa. Y un segundo experimento en el que se añadió el inóculo del hongo e inhibidores del citocromo P-450, responsable del metabolismo intracelular de los fármacos. De las tres sulfamidas, el STZ y la SPY fueron eliminadas completamente en los experimentos *in vivo*, mientras que concentraciones residuales de la SMZ fueron detectadas pasadas 96 h. En función de los tiempos obtenidos para SMZ, el experimento con el STZ se prolongó para así poder conseguir su completa eliminacion (después de 170 h). Mientras que las concentraciones de la SMZ y la SPY comenzaron a disminuir casi inmediatamente tras el comienzo del experimento, la degradación del STZ presentó un lag time o retraso de 24h. La presencia de un grupo sustitutivo diferente en la amina secundaria (N^1) podria explicar esta diferencia, ya que mientras la SMZ y la SPY tienen anillos heterocíclicos de 6 carbonos (piridina y piridimidina), el STZ posee un anillo tiazol (5 carbonos) que podría dificultar la asimilación de la molécula ya que podría significar un proceso de adaptación diferente por parte del sistema enzimático del hongo. Como muestra la Figura 1 de la Publicación #1 del Anexo A2, la SPY fue eliminada en menos de 48 h. Si atribuimos la biodegradación de la SPY principalmente a la actividad oxidativa de la lacasa, esta mayor rapidez de eliminación podría quedar explicada, ya que si la degradación tiene lugar intracelularmente, la molécula ha de atravesar la pared celular del hongo y su degradación puede comenzar con un retraso de varias horas, como se ha demostrado que sucede con el ketoprofeno (Marco-Urrea *et al.* 2010). Sin embargo, experimentos con lacasa purificada y potenciadores de esta muestran una tasa de degradación mucho más lenta para SPY que en los experimentos *in vivo*, de modo que la actuación conjunta de ambos sistemas degradativos es la que proporciona la alta eficiencia en la eliminación de esta sulfamida. Para la SMZ y el STZ, los datos obtenidos no permitieron una diferenciación definitiva entre biodegradación intracelular o extracelular, ya que se observó que tanto la lacasa como el citocromo P-450 intervenían en su biodegradación (ver Publicacion #12 y Publicación #1 del Anexo A2). Si comparamos estos resultados con los obtenidos para otros fármacos, vemos como el tiempo para la completa eliminacion de STZ (170 h) es equiparable al de la carbamazepina, uno de los fármacos más persistentes de los detectados en el medio ambiente y que no fue degradado completamente (94%) tras el mismo tiempo de ensayo (Clara *et al.* 2004).

Respecto a los productos de transformación genderdos, a pesar de tratarse de un hongo con alta capacidad oxidativa, en el caso de las sulfamidas los metabolitos hidroxilados fueron los productos minoritarios frente a otros productos como los desulfonados o los desaminados. Para la SMZ y el SPY los metabolitos hidroxilados fueron detectados sólo en los experimentos *in vivo*, pero con una intensidad de señal demasiado baja para poder confirmar su presencia mediante MS/MS, mientras que no fue detectado en ninguno de los experimentos para el STZ. Para las tres sulfamidas estudiadas, el principal producto de degradación fue su correspondiente molécula desulfonada. Estas se detectaron en las muestras después de 8 h y, en el caso de la SMZ y el SPY, fue un producto de transformación común en ambos experimentos *in vivo* y con lacasa purificada, resultado que sugiere la participación de ambos sistemas degradativos del hongo. Para el STZ, el compuesto desulfonado sólo se detectó en los experimentos realizados con lacasa purificada, si bien la participación del citocromo P-450 en su biodegradación también habia quedado demostrada previamente. A diferencia de los metabolitos hidroxilados, que también se detectan como productos del metabolismo humano y animal de las sulfamidas, estos productos desulfonados no son característicos del metabolismo humano, si bien se han registrado, como veremos en el siguiente Capítulo de esta Tesis, en experimentos de fotodegradación y también en FBBR, como producto de transformación de AcSPY.

La aplicación de *Trametes versicolor* para la eliminación de estas tres sulfamidas también se investigó en dos tipos de matrices sólidas, en fango de EDAR tomado a la salida del reactor anaerobio y en fangos deshidratados térmicamente. Para los primeros se crearon reactores de 8 L donde se añadió fango esterilizado (5.4 L aprox.) y se inoculó el hongo, mezcla denominada *bioslurry*, mientras que para los fangos deshidratados se inoculó directamente el hongo en el sustrato sólido (ver más detalles en la Publicación #1 del Anexo A2). La Tabla 4.3 resume los resultados obtenidos.

Tabla 4.3. Eliminación (E%) de la sulfamidas estudiadas mediante la aplicación de *Trametes versicolor* (*pellets*) en diferentes tipos de fango de depuradora.

	BIO SLURRY (ng g^{-1})	FANGO TRATADO CON T. *versicolor* (ng g^{-1})	E (%)	REF	FANGO DESHIDRATADO TÉRMICAMENTE (ng g^{-1})	FANGO TRATADO CON T. *versicolor* (ng g^{-1})	E(%)	REF
SMZ	6.1	0.5	91	*	19.1	n.d.	100	*
SPY	21.4	n.d.	100	***	29.4	n.d.	100	**
STZ	141	143	85.9	***	71.1	n.d.	100	**

*SMZ: sulfametazina; SPY: sulfapiridina; STZ: sulfatiazol; REF: referencia bibliográfica. ; *: Publicación #13; **: Anexo A2, Publicación 1; ***: Anexo A2, Publicación 2.*

Como se puede observar, la eliminación de la SMZ y el STZ es ligeramente mayor en la aplicación directa de *T. versicolor* en el sistema sólido, mientras que SPY fue eliminada completamente en ambos sistemas. La amplia presencia de enzimas oxidoreductasas en sistemas edáficos, incluyendo lacasas y similares, junto con el potencial degradativo de *T. versicolor* en fangos hacen que la aplicación potencial de este tipo de hongo en biorremediación de suelos contaminados con sulfamidas y otros contaminantes orgánicos, así como el tratamiento de fangos de EDAR antes de su aplicación como biosólidos sea una posibilidad a considerar. Sería sin embargo imprescindible llevar a cabo una evaluación exhaustiva de la toxicidad potencial de los productos de degradación derivados de la biodegradación de estos compuestos

5. FOTODEGRADACIÓN BAJO IRRADIACIÓN ARTIFICIAL

5.1. INTRODUCCIÓN

La baja biodegradabilidad de las sulfamidas y, por tanto, la escasa eficiencia de los tratamientos convencionales para su eliminación durante el tratamiento de aguas residuales ha llevado a multitud de autores a evaluar la eficacia de diferentes tratamientos terciarios, desde la nanofiltración y ósmosis inversa hasta procesos de oxidación avanzados como la cloración o la ozonización. Los resultados obtenidos con los primeros son bastante variables, con valores comprendidos entre 15%-95% de eliminación (Homem and Santos 2011). La ozonación y la cloración, por el contrario, han resultado en RE% muy altos en diversos estudios realizados, aunque plantean el problema sobre la ecotoxicidad de los productos de transformación derivados. Además, el principal inconveniente de estos procesos es su elevado coste, debido al elevado consumo energético y a la utilización de reactivos como H_2O_2 o O_3. En España, la implantación de estos tratamientos terciarios en las EDAR no está muy extendida debido no sólo a estos costes elevados, sino también a que los objetivos de rendimiento de las EDAR se basan fundamentalmente en alcanzar valores de DBO y DQO establecidos en diferentes normativas y no en la eliminación de microcontaminanes orgánicos.

Por otro lado, la fotocatálisis o fotolisis mediada por un catalizador ha demostrado ser altamente eficaz en la eliminación de diferentes sulfamidas, como se muestra en la Tabla 5.1.

Tabla 5.1. Degradación de diferentes sulfamidas con tratamientos terciarios, adaptada de Homem et al. (Homem and Santos 2011). C_0: concentración inicial; RE%: eliminación; T: tiempo (horas).

SULFAMIDA	MATRIZ	C_0	Tratamiento	RE%	T	Observaciones
SDM, STZ	Agua destilada	15 mg L^{-1}	Fotocatálisis TiO_2 200 mg L^{-1} 340-400 nm UV	100%	0.5	-
SDZ, SMR				80-90%		
SMZ	Agua destilada	10-70 mg L^{-1}	Fotocatálisis 0- 4 mg L^{-1} TiO_2Na-ZnO 0-800 mg H_2O_2	95%	2	TiO2 más efectivo que ZnO para eliminación de DOC
				100%	1.6	H_2O_2 aceleró la degradación
SMZ	Agua desionizada	50 mg L^{-1}	Fotofenton 176-1024 mg L^{-1} H_2O_2 12-68 mg L^{-1} Fe^{2+}	100%	0.03	La toxicidad de los productos intermedios es mayor al comienzo de la reacción
SMT, SMX, STZ, SSX	Agua destilada Agua superficial	100 µM	Luz natural 175W Fotofenton pH 3 30% H_2O_2 40 µm Fe^{2+}			Dependiente de pH
SMX	Agua desionizada con materia orgánica y bicarbonatos	5-500 µM	Fotocatalisis UV 324-400 nm 0.01-1 g L^{-1} TiO_2	100%	1	Influencia de la matriz en la reactividad; eliminación favorecida a pH más altos; poca biodegradabi-lidad en efluentes
	Agua destilada	25-200 mg L^{-1}	Fotocatálisis UV 240-310 nm pH 2-11 0-2 g L^{-1} TiO_2	90%	6	
SMX	Agua destilada	200 mg L^{-1}	Fotofenton pH 2.8 Luz negra 365 nM 10 mg L^{-1} Fe^{2+}			

Tabla 5.1. (continuación)

SULFAMIDA	MATRIZ	C0	Tratamiento	RE%	T	Observaciones
SMX	Agua destilada Agua marina	10 mg L^{-1}	Fotólisis 290 nm UV	98%	30	Solo 14% en agua marina Alta toxicidad de los productos de transformación generados
	Agua destilada Agua marina	50 mg L^{-1}	Fotofenton 290 nm pH 2.5-9 30-210 mg L^{-1} H_2O_2 2.6-10.4 mg L^{-1} Fe^{2+}	80% agua destilada 50% agua marina		Mejora la eliminación a mayor concentración de hierro. El aumento de H_2O_2 disminuyó la toxicidad de la muestra

Sulfamidas: SDZ: sulfadiazina; SMX: sulfametoxazol; STZ: sulfatiazol; SDM: sulfadimetoxina; SMT: sulfametizol; SMZ: sulfametazina; SSX: sulfisoxazol; SMR: sulfamerazina.

Se diferencian dos tipos de fotocatálisis:

- Fotocatálisis homogénea: procesos conocidos también como procesos Fenton o foto-Fenton. Se denomina homogénea porque tanto los reactivos como los catalizadores se encuentran en la misma fase. La combinación de H_2O_2 y Fe^{2+} (conocido como reactivo Fenton) en medio ligeramente ácido da lugar a la formación de radicales hidroxilo (OH·), con un poder oxidativo superior al del ozono o el cloro , y a la oxidación de Fe^{2+} a Fe^{3+}. A su vez, la fotorreducción del Fe^{3+} puede generar más radicales OH·.
- Fotocatálisis heterogénea: se utiliza como catalizador de la reacción un óxido o sulfuro (TiO_2, ZnO, CnS) que es irradiado con luz UV generando una excitación. La fase fluida permite a su vez la tranferencia de los electrones excitados hacia la molecula aceptora, que reaccionara generando los diferentes productos de transformación.

La radiación natural también puede jugar un papel importante en la eliminación de las sulfamidas una vez vertidas desde las EDARS a aguas naturales expuestas a la radiación. La fotólisis directa se produce por absorción de la radiación solar directamente por la molécula, mientras que en la fotólisis indirecta compuestos naturales como ácidos húmicos o nitratos pueden intervenir en la degradación de la molécula. Se ha demostrado que las sustancias húmicas, debido a su estructura química, son "fotoactivadas" por la radiación UV y producen especies reactivas como radicales OH· con alta capacidad degradativa (ref). Por tanto, la fotodegradación directa depende tanto de la intensidad lumínica de la radiación (época del año, latitud) como de la naturaleza y composición del agua que las contiene (cantidad de materia orgánica disuelta, turbidez, dureza, pH, etc). En el trabajo que presentados en este apartado, muestras de efluente de EDAR y agua HPLC fortificadas con diferentes sulfamidas fueron expuestas a una radiación simulada, equivalente a la radiación natural en el momento del experimento, logrando así evitar las posibles fluctuaciones naturales. Los objetivos principales de este trabajo fueron los siguientes:

- Evaluar la cinética de fotodegradación de las diferentes sulfamidas expuestas y en las diferentes matrices acuosas estudiadas.
- Comparar la fotodegradación de las sulfamidas estudiadas con las de sus respectivos metabolitos acetilados.
- Elucidación y confirmación de diferentes productos de transformación generados.

5.2. PUBLICACIONES

Publicación científica 14

"Kinetic studies and characterization of photolytic products of sulfamethazine, sulfapyridine and their acetylated metabolites in water under simulated solar irradiation"

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Kinetic studies and characterization of photolytic products of sulfamethazine, sulfapyridine and their acetylated metabolites in water under simulated solar irradiation

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ABSTRACT

Sulfapyridine (SPY), sulfonamide (SA) typically used in human therapies, and veterinary SA sulfamethazine (SMZ), are amongst the two SAS most frequently detected in effluent wastewater and surface water respectively. Within this context, this study reports the behaviour of both SAs and their acetylated metabolites, AcSPY and AcSMZ, under artificial irradiance conditions in both high performance liquid chromatography (HPLC) water and in reclaimed wastewater, in order to compare the influence of dissolved organic matter (DOM) and also inorganic matter in the photolysis kinetics. Estimated degradation rate constants (k) ranged from 0.063 h^{-1} (SPY) to 2.808 h^{-1} (AcSPY), both in HPLC water, with corresponding half-lives ($t_{1/2}$) of 10.93 h and 0.25 h, respectively. A total of 10 different photodegradation products were identified during the photolytic transformation of SPY and 7 for SMZ, through ultra-performance liquid chromatography-quadrupole time of flight mass spectrometry analyses (UPLC-QqTOF-MS), which allowed for exact mass measurements. Regarding the acetylated metabolites, 3 photoproducts were generated for AcSMZ and one for AcSPY. The desulfonated products of each of the four analytes under study were the most relevant photodegradation products identified.

1. Introduction

Sulfonamides (SAs) are a family of antimicrobials widely used in both veterinary and human medicine. They have been detected in all kinds of environmental waters, and also in solid matrices (Diaz-Cruz et al., 2008; Gobel et al., 2005; Shelver et al., 2010). The occurrence of their metabolites (mainly acetylated and hydroxylated moieties) has also become relevant in recent years, and the presence of both parent drugs and their respective metabolites has been considered together and demonstrated within the scope of an increasing number of research studies (Diaz-Cruz et al., 2008; García Galán et al., 2011a,b,c; Gobel et al., 2004; Radke et al., 2009; Stoob et al., 2006). However, the environmental fate of SAs and their metabolites is currently unclear. Their full elimination during conventional wastewater treatment has not been demonstrated yet (García-Galán et al., 2011b; Göbel et al., 2007; Perez et al., 2005).

Together with sulfamethoxazole (SMX), the environmental presence of sulfapyridine (SPY) has gained relevance as

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different studies have demonstrated that it is one of the SAs most frequently detected and in the highest concentrations, mostly in urban wastewater treatment plant (WWTP) effluents (ranging from 70 ng L^{-1} (Gobel et al., 2004) to 227 ng L^{-1} (García-Galán et al., 2011b)) but also in surface waters (91.8 ng L^{-1}, (Garcia-Galan et al., 2010)). Considering that hydraulic retention times in WWTPs are usually not longer than 40 h (Gros et al., 2010; Radjenovic et al., 2009), residues of SPY and AcSPY will be discharged into surface waters, as it was recently demonstrated that 32 days were required to eliminate AcSPY, and more than 60 days for SPY during aerobic biodegradation experiments (García Galán., submitted for publication). Regarding veterinary SAs, they can reach environmental waters through direct deposition of livestock or application of cattle farms waste as organic amendment in crop fields. Sulfamethazine (SMZ) is one of the most commonly used SAs to treat bacterial infections in cattle, and has been frequently detected in both surface waters (Garcia-Galan et al., 2010) and groundwaters (Batt et al., 2006; García-Galán et al., 2011a) in concentrations up to 2482 ng L^{-1} and 220 ng L^{-1}, respectively.

Photocatalytic degradation with photo-Fenton and titanium dioxide (TiO_2) has proved to be a successful alternative to conventional water treatments to eliminate non-biodegradable compounds such as SAs and other pharmaceuticals (Trovo et al., 2008, 2009a). Their efficiency is based on the formation of reactive species (i.e. •OH) through the combination of oxidant agents (i.e. hydrogen peroxide) with ultraviolet or visible irradiation and different catalysts (metal ions such as Fe^{2+}). Direct photodegradation may also play a key role on the fate of SAs and metabolites once they have been released into the environment. Previous studies have demonstrated that different SAs can be degraded through direct photolysis (by exposure to UV light) or indirect photodegradation via sensitized photoprocesses such as reactions with dissolved organic matter (DOM) or other reactive species that can be formed in sunlit natural waters (Guerard et al., 2009; Lester et al., 2010, 2008). Direct or indirect photolysis processes yield a number of different intermediates or transformation products (TPs) which, similarly to any metabolite, can be more stable than the parents and also have negative effects at the different trophic levels of the ecosystem (Trovo et al., 2009a, 2009b). The identification of these compounds and their environmental fate should be clarified and fully understood. The use of the hybrid detector quadrupole time of flight (QqTOF-MS) instrument provides the exact masses of the potential unknowns and also, through mass spectrometry in tandem (MS/MS) experiments, their elemental composition and structure can be elucidated.

The objectives of the present study were to evaluate the photodegradation kinetics of SPY, SMZ and their respective acetylated metabolites by irradiating water solutions by means of simulated sunlight. Two different matrices were investigated, WWTP effluent and high performance liquid chromatography (HPLC) water. The variations in concentration of the different target analytes were followed up through liquid chromatography (LC) coupled to MS/MS, and predicted half-lives ($t_{1/2}$) were calculated. The study also focused on the identification and further structural characterization of the different photodegradation products formed by means of QqTOF-MS analyzer coupled to ultra-performance liquid chromatography (UPLC). Finally, tentative photolytic pathways were proposed based on the structural elucidation of these photoproducts. To the author's knowledge, it is the first time that the behaviour of SPY or SMZ under simulated solar radiation has been investigated.

2. Materials and methods

2.1. Chemicals and reagents

High purity standards (>99%) of SMZ, AcSMZ and SPY were purchased from Sigma (St Louis, MO, USA). Analytical standards for AcSPY and the internal standard d_4-sulfathiazole (99.9% purity), were purchased from Toronto Research Chemicals (Ontario, Canada).

HPLC grade solvents (water, methanol (MeOH), acetone, acetonitrile) and formic acid (98–100% purity) were supplied by Merck (Darmstadt, Germany). Stock standard solutions for each of the analytes were prepared in MeOH at 1 mg mL^{-1} and stored at −4 °C until use.

A volume of 500 mL of WWTP effluent was sampled from the WWTP of Terrassa (Barcelona) in March 2010 (pH 7.8; chemical oxygen demand (COD): 93 mg L^{-1}). The COD value was below the maximum boundary agreed on the EU Directive on Urban Waste Water Treatment (91/271/EEC) (Jacobsen and Warn, 1999).

2.2. Experimental set up

HPLC grade water and WWTP effluent were used as media for the photodegradation experiments. Pre-treatment of the water samples was avoided to prevent possible losses of transformation products. Twenty mL-aliquots of each water matrix were spiked with the corresponding target SA or metabolite at 40 mg L^{-1}. The selected initial concentration was higher than those usually found in the different environmental water matrices (usually in the low $\mu g \cdot L^{-1}$ level) in order to permit the direct analysis and elucidation of the potential TPs formed.

Solutions were transferred into quartz reaction tubes and clamp-sealed. Before irradiation, aliquots of 1 mL of every spiked sample were taken and put in the dark (aluminium wrapped vials) at room temperature (26 °C). Simulation of natural sunlight was carried out using a SunTest CPS instrument from Atlas Material Testing Technology (Chicago, Illinois, USA), equipped with a xenon arc Lamp (UV light simulator). Irradiance was set at 450 W m^{-2}, and the emitted wavelengths ranged from 200 to 800 nm, nearly the wavelength spectrum of natural sunlight. Samples were irradiated in the SunTest over a period of 100 h. Aliquots of 0.5 mL were withdrawn regularly for analysis at scheduled time intervals.

2.3. Analytical methods

2.3.1. LC-MS/MS and LC-QqLIT-MS/MS analysis

LC-MS/MS analyses were carried out following the method already published by García Galán et al. (Diaz-Cruz et al., 2008), which was adapted for the analysis of the four target

analytes solely. The system consisted of an Agilent HP 1100 pump (Agilent Technologies, Palo Alto, CA) connected to a 4000QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (QqLIT-MS) equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, CA). Chromatographic separation was carried out using an Atlantis C18 (Waters, 150 mm × 2.1 mm, 3 μm particle size) LC-column preceded by a guard column with the same packing material. The flow rate was set to 0.2 mL min^{-1}, being eluent An HPLC grade water and eluent B acetonitrile, both 10 mM in formic acid. The initial conditions of the elution gradient programmed were 75% A: 25% B. From 0 to 11 min the eluent B was increased to 100%, held for 2 min and returned to initial conditions in 3 min. The column was then equilibrated during 7 more minutes before the next injection. The injection volume of standards and sample extracts was set at 10 μL.

2.3.2. *Transformation products analysis*

Chromatographic separation followed by accurate MS and MS/MS analyses of each of the target analytes and their corresponding TPs was carried out with a Waters/Micromass QqTOF-Micro system coupled to a Waters Acquity ultra-performance LC (UPLC) system (Micromass, Manchester, UK). Regarding chromatography, a Waters Acquity BEH C18 column (10 mm × 2.1 mm, 1.7 μm particle size) was employed. Flow rate was set at 0.3 mL min^{-1}, with eluent A acetonitrile and eluent B HPLC grade water, both containing 10 mM of formic acid. The elution started at 5% B for 2 min, it was then linearly increased to 60% of B in 7 min, further increased to 95% of B in the following 2 min, and then returned to initial conditions. Total run time, including the conditioning of the column and back to initial conditions, was 13 min. The injection volume of the sample was set to 5 μL.

MS and MS/MS analyses were performed in the positive ionization (PI) mode, and the operating conditions were as follows: desolvation gas flow 350 L h^{-1}; source and desolvation temperatures 120 °C and 350 °C, respectively; capillary voltage 3000 V; sample voltage 30 V; and extraction cone voltage 1 V. The collision energy (CE) was held at 4 V for MS analyses, and varied from 10 to 35 V to obtain the fragmentation patterns when performing product ion scans. The applied collision gas was argon. For continuous internal mass calibration, an independent reference (valine-tyrosine-valine) was used as a lock mass, with *m/z* 380.2185, and was acquired in all the measurements. For the MS analyses on the QqTOF instrument, MS data was recorded scanning from *m/z* 50 to *m/z* 500.

Table 1 – Pseudo first order kinetic parameters estimated during the photolysis of the sulfonamides investigated. k: rate constant; r^2: correlation coefficient; $t_{1/2}$: Half life.

Analyte	*m/z*	Water matrix	*k* (h^{-1})	r^2	$t_{1/2}$ (h)	Removal (%)		
						5 h	30 h	70 h
AcSMZ	321	HPLC	0.724	0.998	0.92	97.5	100	100
		EFFL	0.348	0.995	2.03	69.8	100	100
AcSPY	292	HPLC	2.808	0.981	0.25	99.9	100	100
		EFFL	0.809	0.992	0.86	98.7	100	100
SMZ	279	HPLC	0.110	0.997[a]	6.30	49.6	96.3	100
		EFFL	0.138	0.998	5.03	40.5	97.6	100
SPY	250	HPLC	0.063	0.979	10.93	7.5	72.6	98.5
		EFFL	0.282	0.996[b]	2.46	74.0	99.9	99.9

HPLC: high performance liquid chromatography water.
EFFL: wastewater treatment plant effluent.
a Calculated after 30 h under irradiation.
b Calculated after 20 h under irradiation.

3. Results

3.1. *Direct photolysis kinetics*

Dark controls showed no relevant variations in concentration of any of the target analytes in HPLC water or WWTP effluent (Fig. S1-Supplementary Information). Fluctuations were not greater than 0.1% in any case, which could be attributed to the response variability of the instrument. The samples were submitted to UV radiation in the SunTest instrument which was slightly lower than the environmental UV radiation registered in the same location and at the same time of the year where the experiments were carried out ((Barcelona, latitude 41° 23′ 12″ N; longitude: 2° 12′ 4″ E; altitude: 25 m) (http://www.infomet.fcr.es/). Data obtained from the meteorological station at the University of Barcelona showed an average global irradiance during the month of September 2010 of 430 W m^{-2} and average UV radiation of 541 W m^{-2} at the maximum insolation hours (from 14.00 h to 16.00 h). Therefore, results from experiments under both irradiation sources would be perfectly comparable, and then, the use of the sunlight simulator appropriate.

The degradation pattern of the two SAs and their respective acetylated metabolites was adjusted to the pseudo-first-order kinetic model, which assumes a decrease of the concentration through time proportional to the concentration remaining in the matrix and follows equation (1):

$$-\frac{d[A]}{d[t]} = k[A] \quad (1)$$

where A is the concentration of the target compound (in our case integrated areas of the chromatographic peaks), *t* is the time of the experiment (h) and *k* is the rate constant (h^{-1}). Half-lives were calculated following equation (2):

$$t_{1/2} = \frac{\ln 2}{k} \quad (2)$$

The behavior of all the selected analytes fitted very well with the model shown in equations (1) and (2), with correlation coefficients (r^2) usually higher than 0.99 (Table 1). However, a deviation from the initial rates was observed for SPY and SMZ before the end of the experiment (Fig. 1a). In the case of SMZ, the change is observed in the HPLC water matrix and happened after 30 h of irradiation, when more than 96% of the initial concentration of the analyte had already been degraded, therefore *k* and $t_{1/2}$ values given correspond to those of the first 30 h. On the contrary, for SPY the change in *k* was observed in the WWTP effluent matrix, in which the degradation rate also slowed down considerably after 30 h of irradiation, when a 99% of the SA had already been eliminated;

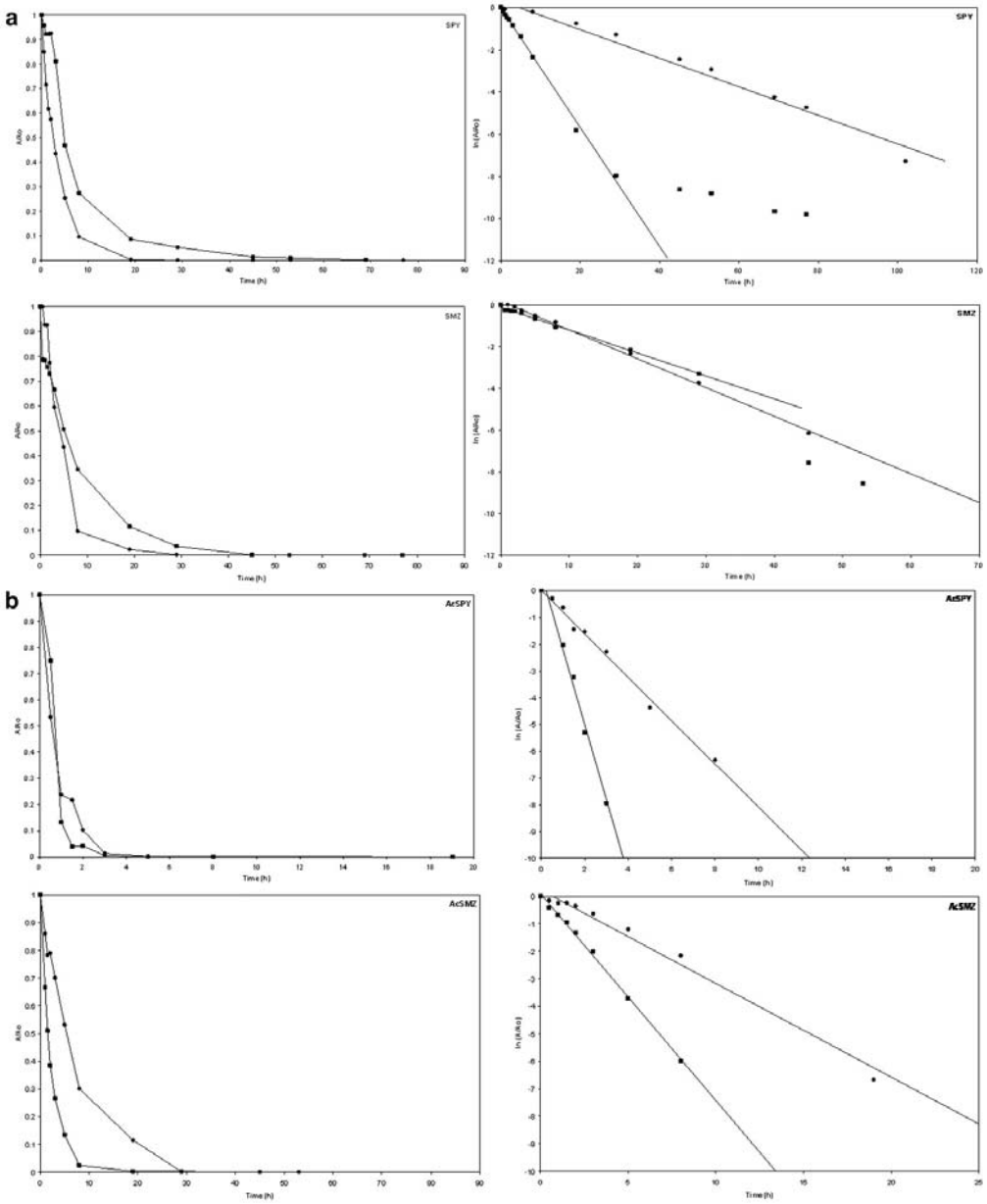

Fig. 1 – Time course photodegradation of SPY and SMZ (a) and their acetylated metabolites (b) in HPLC water (■) and WWTP effluent (●).

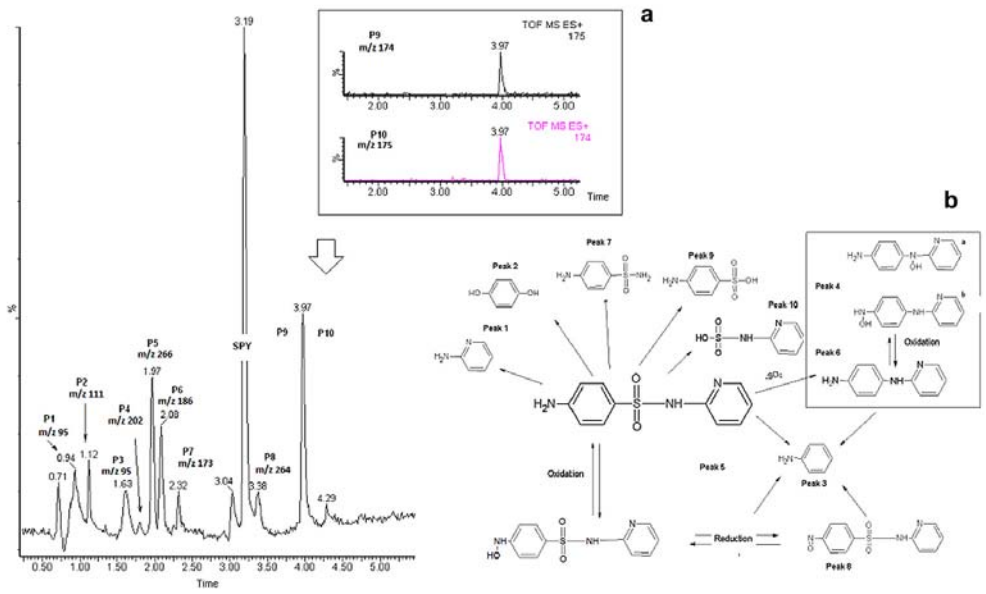

Fig. 2 – Total ion chromatogram (TIC) of SPY photolysis upon irradiation for 30 h in HPLC water (a), and proposed TPs (b).

calculations were made for that time interval. A feasible explanation for these variations in k would be the presence of newly formed TPs, which may absorb the radiation, and thus, screen the remaining parental analyte from the irradiation source (Kariem et al., 2000). As described in Section 3.2.3, photodegradation of SMZ in HPLC water yielded 7 different TPs, and the two most abundant were at its maximum concentration after 30 h of experiment, when the degradation rate changed. Ten different TPs were identified for SPY in HPLC water (see Section 3.2.1) and, although the attenuation effect on k was observed only in WWTP effluent water, we could consider that similar TPs were present in the WWTP effluent matrix. The highest concentrations for most of these TPs were also registered after 30 h of irradiation, exactly when k decreased.

SPY presented the highest $t_{1/2}$ of the SAs investigated (HPLC water). On the contrary, k values calculated in WWTP effluent showed a photodegradation fivefold faster in this matrix, being SPY removed in a 99.9% after 19 h compared to a removal of 53% in HPLC water, where residues of SPY could still be detected after 102 h of irradiation (99% removal). For SMZ, k values for both water matrices were rather similar, but again photodegradation was faster in WWTP effluent samples. DOM contained in this matrix could have acted as a catalyst for both SAs, as it has been documented for other SAs containing six-membered heterocyclic ring groups (Boreen et al., 2005). On the other hand, it should be considered that, despite the fact that sorption to particles is usually disregarded as a photodegradation impeding factor for SAs, due to their low distribution coefficients (K_d) (Ternes et al., 2004), recent studies state that the adsorption should not be completely neglected; it has been demonstrated that the aromatic amine common to all SAs is likely to covalently cross-couple to natural organic matter, diminishing their

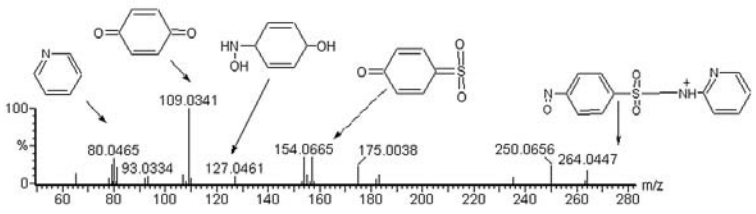

Fig. 3 – ESI(+)-MS/MS spectrum of sulfapyridine photoproduct *m/z* 264 at QqToF instrument (cone voltage 25 V, collision energy 30 eV).

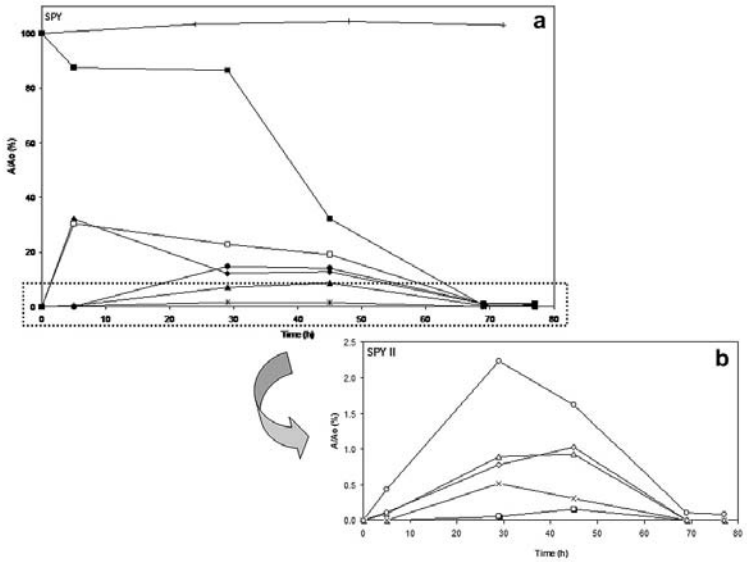

Fig. 4 – Time course photodegradation of SPY and evolution of its major transformation products (a) and minority transformation products (b). Symbols (a): Dark control SPY (+); SPY (■); *m/z* 186 (□); *m/z* 95 (●); *m/z* 266 (◆); *m/z* 264 (▲); *m/z* 202(✱). Symbols (b): *m/z* 94 (○); *m/z* 174 (△); *m/z* 175 (◇); *m/z* 173 (×); *m/z* 111 (□).

presence in environmental water matrices and also reducing their bioavailability and biological activity (Bialk et al., 2005). So partial adsorption to DOM in the effluent would also play a role in the faster degradation rates observed. On the contrary, a lower adsorption tendency to DOM of the acetylated metabolites, due to their higher polarity, could explain the lower transformation rates of both compounds in wastewater effluent. The fastest photodegradation rates were registered in HPLC water for both compounds, as depicted in Fig. 1b. It was found that 50% of the initial concentration of AcSPY was removed after 0.2 h in HPLC water and after 0.8 h in WWTP effluent. Similarly, AcSMZ showed a quick decrease in concentration, with $t_{1/2}$ of 0.9 h in HPLC water and 2 h in WWTP effluent.

3.2. *Identification and structural characterization of photolytic products*

3.2.1. *Sulfapyridine*

Photolysis of SPY produced 4 major TPs, and other 6 which were considered as minority products (relative abundances below 5%). Fig. 2a shows the chromatogram with the different signals recorded for each of the 10 different TPs identified for SPY upon irradiation for 30 h. The structural similarity of some of the TPs led occasionally to their coelution in the chromatogram (i.e. peaks P9 and P10 in Fig. 2a). In order to overcome this problem and obtain clearer MS/MS spectra, the molecular ions were extracted individually from the chromatogram.

The TP detected at the highest intensity appeared at a retention time (RT) of 1.97 min, with a base peak of *m/z* 266.0599 in the corresponding mass spectrum (P5). Since hydroxylation reactions are typical of photolytic processes, the best fit for the elemental composition was chosen to be $C_{11}H_{12}N_3O_3S$, and corresponded to the hydroxylated metabolite of SPY (Fig. 2b). Fragmentation of this molecule yielded fragments with *m/z* 108, *m/z* 93 and *m/z* 186, this last one common to a second TP detected. However, on the basis of the MS/MS fragmentation patterns alone, an unequivocal assignment of a structure was not possible, as the position of the hydroxyl (•OH) group could not be elucidated from any of these fragments, not even when low CEs were applied to obtain potential fragmentation patterns. This hindrance has already been reported in similar studies of the photodegradation products of ranitidine (Radjenovic et al., 2007) and also in the biodegradation of SMZ by a white-rot fungus (García Galán et al., 2011c). Finally, the location of the •OH group was determined based on the MS/MS spectral information of the reduced form of this metabolite, of *m/z* 264, which demonstrated that the •OH group was present in the N^4 position (see Fig. 3).

The second most intense peak appeared at RT 2.08 min and corresponded to the SO_2 extrusion product, N-(pyridin-2-yl)benzene-1,4-diamine, with an *m/z* of 186.1031 and an elemental composition of $C_{11}H_{12}N_3$ (Fig. 2b). Desulfonated products have been previously reported as characteristic of indirect photodegradation studies of SAs (Boreen et al., 2005), but it is also a common product in biodegradation processes,

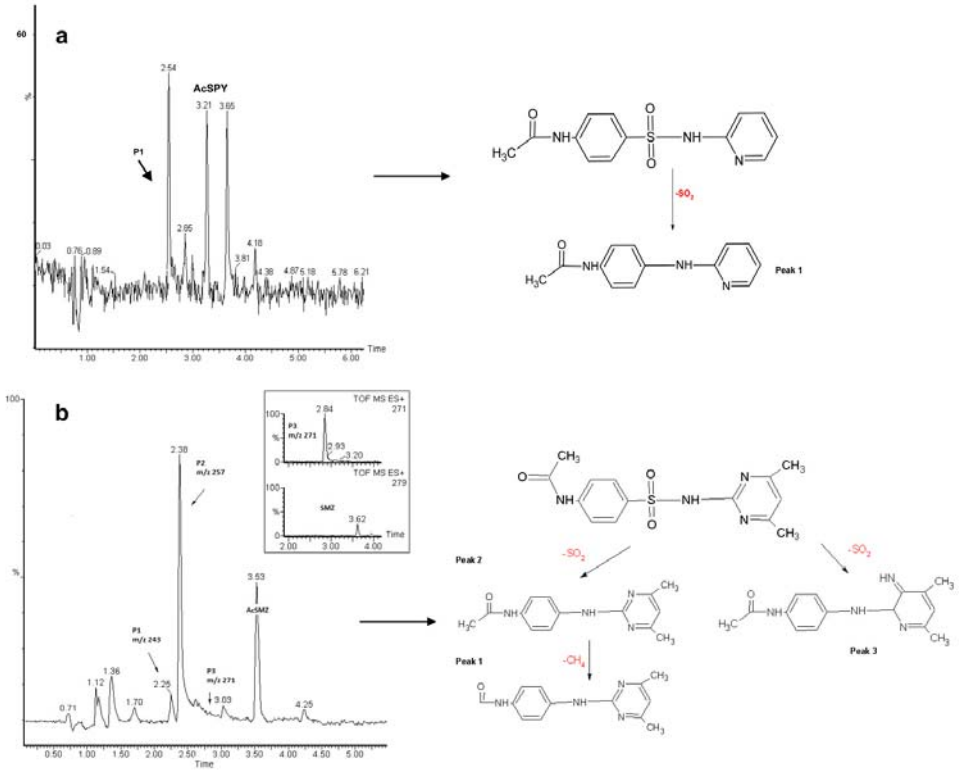

Fig. 5 – Total ion chromatogram (TIC) of AcSPY (a) and AcSMZ (b) photolysis in HPLC water upon irradiation for 1 h and 5 h, respectively, and proposed TPs.

as shown after degradation of SPY, SMZ or sulfadimethoxine (SDM) with white-rot fungus (García Galán et al., 2011c; Schwarz et al., 2010), and in aerobic biodegradation simulation in fixed-bed bioreactors (FBBRs) (García Galán et al., submitted for publication). The hydroxylated moiety of this desulfonated product, N-hydroxy-N-(pyridin-2-yl)benzene-1,4-diamine, was detected as an intermediate product at an RT of 1.93 min with a base peak of *m/z* 202.0980 and an elemental composition of $C_{11}H_{12}N_3O$. Its concentration evolution was therefore paired to that of *m/z* 186. It was present as a minority product (P4), and its fragmentation pattern indicated that the •OH could be placed in either the N^1 or N^4 position.

The third peak in intensity appeared at an RT of 3.38 min, and was detected at *m/z* 264.0443. Its elemental composition was elucidated as $C_{11}H_{10}N_3O_3S$, and its presence was associated to the reduction of the hydroxylamine in the N^4 position of the hydroxylated product. Furthermore, Fig. 4 shows how its concentration increased simultaneously to the decrease of the hydroxylated product during the first 30 h of experiment, till equilibrium was reached. This information together with that obtained from the MS/MS fragmentation of this new product indicated that the •OH was attached to the N^4 position of the parental molecule (Fig. 3).

The fourth major signal appeared at an RT 0.94 min, with an *m/z* of 95.0609 (P1). The best fitting elemental composition for this mass was $C_5H_7N_2$ and corresponded to pyridine-2-amine.

The products whose relative intensities (A/A_0) were lower than 5% throughout the photodegradation experiment were considered minority photoproducts, and are shown in Fig. 2a denoted as P2, P3, P7 and P9, which would correspond to hydroquinone, aniline, sulfanilamide and sulfanilic acid, respectively (these four compounds are considered as common photodegradation products of SAs (Boreen et al., 2004)); P4, which corresponded to the molecule of mass *m/z* 202.0980 mentioned above; and P10, which was detected at RT 3.97 min with *m/z* of 175.0177. The elemental composition proposed for this molecule was $C_5H_7N_2O_3S$, and was assigned to an aminosulfonic acid (pyridin-2-ylsulfamic acid), which

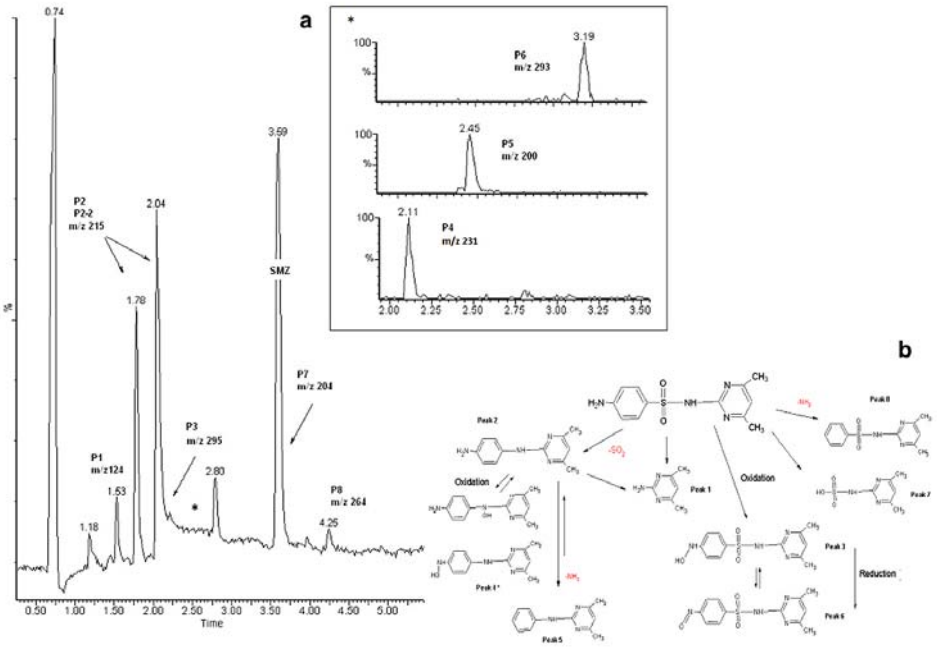

Fig. 6 – Total ion chromatogram (TIC) of SMZ photolysis upon irradiation for 30 h in HPLC water (a), and proposed TPs (b).

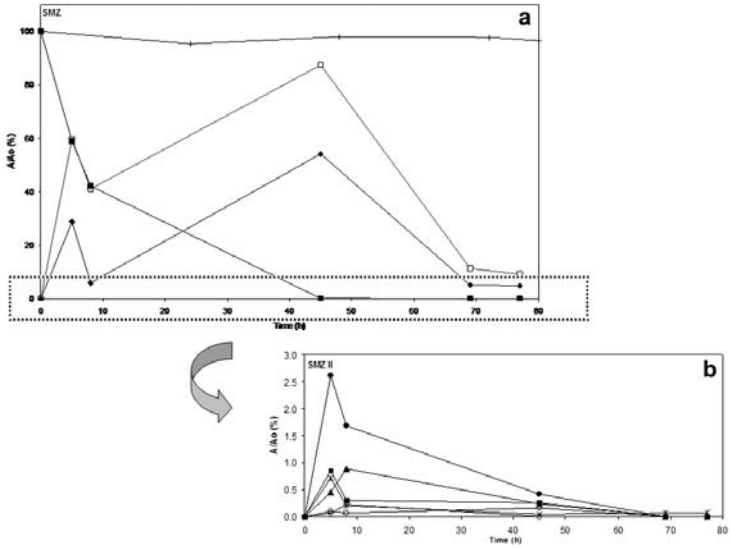

Fig. 7 – Time course photodegradation of SMZ and evolution of the corresponding TPs. Symbols (a): Control-SMZ (+); SMZ (■); m/z 215 (□); m/z 124 (◆). (b): m/z 204 (●); m/z 295 (▲); m/z 231 (△) m/z 200 (✱); m/z 293 (○); m/z 264 (◇).

resulted from the cleavage of the bond between the aniline ring and the sulfonic group, followed by an •OH addition to the SO_2 group. However, with the exception of *m/z* 202, limited mass spectral information was obtained for these photoproducts, due mainly to their low molecular masses and also to the low concentrations in which they were present. Although the relative mass errors were good enough for identification, no more than a single fragment could be obtained for each of them. The chemical structures proposed were confirmed with the double bond equivalents (DBE) obtained and the sodium adducts detected for some of them (Table S1-Supplementary Information).

The concentration profile of all the TPs identified *versus* time is shown in Fig. 4. Both the desulfonated and the hydroxylated products reached their maximum concentration upon irradiation for 5 h, dropping down slowly onwards. Correspondingly to the highest decrease in SPY concentration, nearly all the minority photoproducts (Fig. 4b) reached a maximum between 30 and 45 h of irradiation.

3.2.2. N^4-Acetylsulfapyridine

The loss of the sulfonate group in AcSPY yielded a new molecule of *m/z* 228.1137. Its elemental composition was elucidated as $C_{13}H_{14}N_3O$, and appeared in the chromatogram at an RT of 2.55 min (Fig. 5a). It reached a maximum signal intensity after 2 h, exactly when AcSPY reaches its minimum intensity (Fig. S2-Supplementary Information), and its MS/MS fragmentation yielded daughter ions at *m/z* 186 and *m/z* 170 (CE 30). SPY was not detected throughout the experiment length as a photolytic product. The parental compound, however, had been identified in previous biodegradation experiments of AcSPY, as a transformation product (García Galán et al., submitted for publication).

3.2.3. Sulfamethazine

Eight different TPs were identified for SMZ (Fig. 6a). Similarly to what happened with SPY, the desulfonated product of SMZ was the TP detected along the experiment at the highest concentration. In this case, however, two different signals

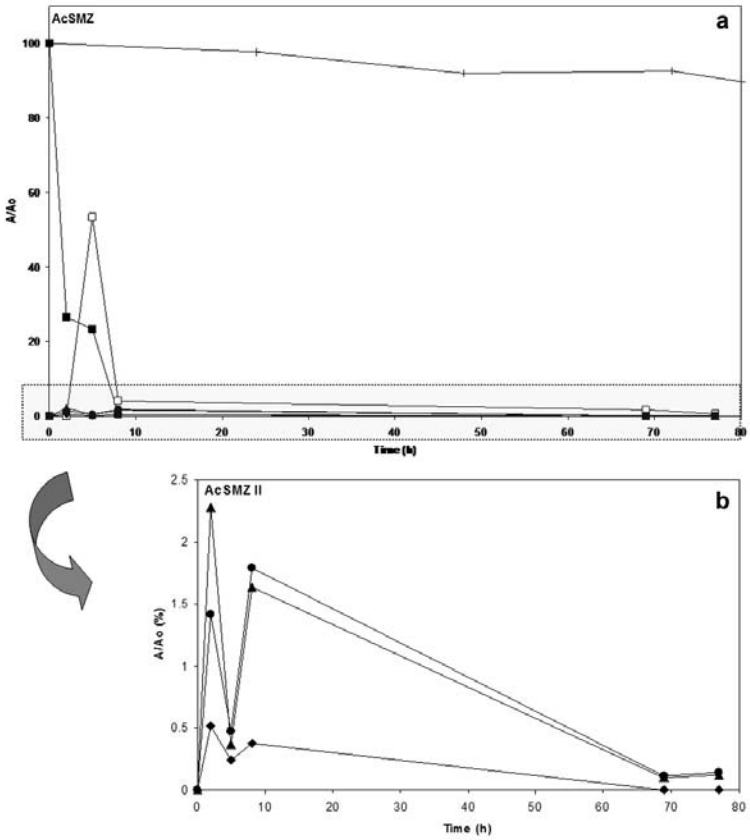

Fig. 8 – Time course photodegradation of AcSMZ and evolution of their corresponding TPs. Symbols (a): AcSMZ (■); *m/z* 257 (□). (b): *m/z* 271 (●); *m/z* 279 (◇); *m/z* 243 (▲).

corresponded to the same mass and yielded the same mass spectra: P2 and P2-2. Both stereoisomers appeared in the chromatogram at an RT 1.78 and 2.04 min, respectively, with a base peak of *m/z* 215.1297 and corresponding elemental composition of $C_{12}H_{15}N_4$. The maximum concentration of the strongest signal (RT 2.04) was equivalent to the 87.5% of the SMZ initial concentration, and was recorded after irradiation for 45 h (Fig. 7). The same desulfonated product of SMZ was detected for the first time, as only molecule, in a biodegradation experiment of SMZ with the fungus *Trametes versicolor* (García Galán et al., 2011c). Two similar intermediates formed simultaneously, as can be observed in Fig. 6: its hydroxylated moiety,

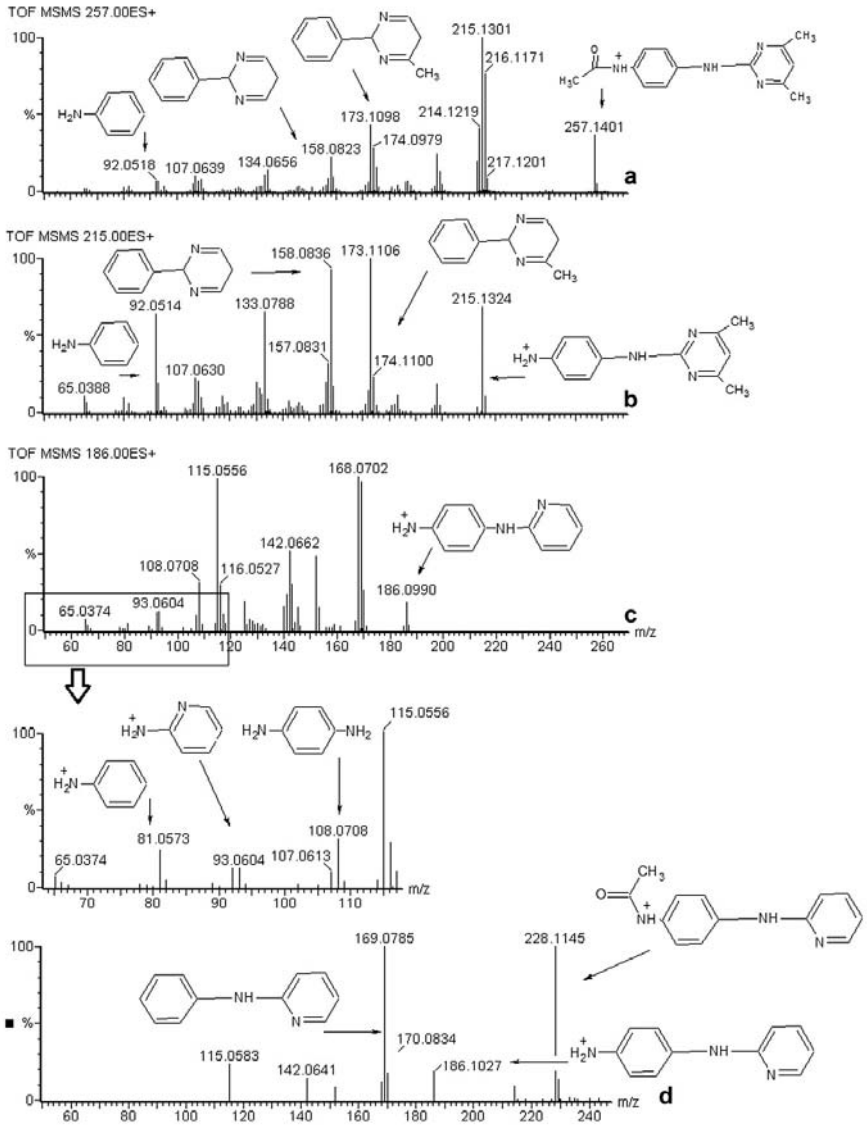

Fig. 9 – Spectra obtained in ESI(+)-MS/MS experiments at QqToF instrument (cone voltages 25 V, collision energies 15–25 eV) for the SO_2 extrusion products of N^4-acetylsulfamethazine (a), sulfamethazine (b), sulfapyridine (c) and N^4-acetylsulfapyridine (d).

with an elemental composition of $C_{12}H_{15}N_4O$ and a base peak of *m/z* 231.1246, and the desaminated product, with an elemental composition of $C_{12}H_{14}N_3$ and base peak of *m/z* 200.1188. They appeared in the chromatogram at RT of 2.11 min and 2.45 min respectively (Fig. 6b). However, its concentration decreased after 5 h upon irradiation.

The second most intense signal was registered at an RT 1.53 min in the chromatogram, with a base peak of *m/z* 124.0875 in the mass spectra. The best fitting elemental composition was $C_6H_{10}N_3$, which would correspond to 4,6-dimethylpyrimidin-2-amine. As can be observed in Fig. 6, its concentration profile was analogous to that of the desulfonated product.

The remaining 5 TPs detected were minority products, and their structures could only be confirmed through their Na adducts and DBE values (Table S2-Supplementary Information). Similarly to the TPs detected in the study with the fungus *T. versicolor*, the hydroxylated product (*m/z* 295.0865) and desaminated moiety (*m/z* 264.0807) of SMZ were also observed (P3 and P8, respectively in Fig. 6a). Like it happened to SPY, the reduction of the hydroxylamine in the N^4 position of the hydroxylated product yielded a less polar intermediate which eluted at RT 3.19 min; its observed mass was *m/z* 293.0708 and the elemental composition assigned was $C_{12}H_{13}N_4O_3S$.

Similarly to the case of SPY, a new aminosulfonic acid was present at an RT of 3.61 min. Its base peak in the mass spectra was *m/z* of 204.0443 and corresponded to (4,6-dimethylpyrimidin-2-yl) sulfamic acid, with a molecular structure of $C_6H_{10}N_3O_3S$.

3.2.4. N^4-Acetylsulfamethazine

Photodegradation of AcSMZ yielded 3 TPs that appeared in the chromatograms within 3 min (see Fig. 5b). Apart from those, the parent molecule, SMZ, was detected at low concentrations (below 0.5% of the initial AcSMZ) throughout the experiment (RT 3.61 min).

The first photoproduct (P1) showed up at RT 2.22 min with a nominal mass of *m/z* 243. The best fitting elemental composition was $C_{13}H_{14}N_4O$, and was the result of the loss of SO_2 and the methyl group from the acetylated moiety. A different signal (P2) was registered very close to this TP in the chromatographic run (RT 2.38 min) and was identified as the desulfonated molecule of AcSMZ (*m/z* 257). This was again the TP registered at a maximum intensity. Its concentration reached a maximum after 5 h of irradiation (Fig. 8) and then decreased and remained nearly constant throughout the experiment. Its MS/MS fragmentation is shown in Fig. 9. The third new product (P3) showed in the chromatogram at an RT 2.84 min and a base peak in the spectra of *m/z* 271. The best fitting elemental composition was $C_{15}H_{19}N_4O$ and was attributed to a structural rearrangement of the desulfonated photoproduct. Further structural information and accurate mass measurements are given in Table S3 (Supplementary Information).

4. Conclusions

The photodegradation of SPY, SMZ and their two corresponding N^4-acetylated metabolites was investigated in WWTP effluents and HPLC water, under an artificial UV light source. It has been demonstrated that high concentrations of the SAs studied could be eliminated under UV radiation levels similar to those registered in the environment, representing an alternative route to ordinary microbial degradation, which require substantially longer times. At concentrations of 40 mg L^{-1}, the $t_{1/2}$ estimated suggested that at least 30 h would be required to fully degrade the analytes except for SPY, which required exposition times longer than 100 h for both WWTP effluent and HPLC water matrices. It should be taken into account that the expected environmental concentrations for these SAs are much lower, in the range of ng L^{-1} and probably shorter times would be required for direct photodegradation; therefore, it could be considered as a feasible degradation pathway of SAs in the environment in natural water courses or lakes/storage lagoons; in WWTPs, it would depend also on the HRTs, Regarding the acetylated metabolites in HPLC water, 99% of degradation was achieved in only 5 h (AcSPY) and 8 h (AcSMZ) of irradiation. DOM contained in the WWTP effluent matrix seemed to act as a weak catalyst during the photodegradation of the parental compounds, and appeared, on the contrary, to slow down slightly the photolysis of the acetylated metabolites.

Whereas the determination of photodegradation kinetics is relevant for environmental fate modelling, the identification of the photolytic products is also significant in terms of ecotoxicity. QqTOF MS analyses were employed as the best tool to identify the different unknown transformation products generated. Identification was achieved through full scan spectra, and finally confirmation and structural characterization for most of them was elucidated by means of MS/MS spectra. These results constitute the first data on photodegradation of these two SAs and their acetylated metabolites in aqueous solution under simulated solar radiation.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2011.11.035.

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SUPPLEMENTARY INFORMATION #14

Figure S1. Dark controls time evolution for sulfapyridine (m/z 250), sulfamethazine (m/z 279) and N^4-acetylsulfamethazine (m/z 321) in high performance liquid chromatography water (HPLC) . (Data for N^4-Acetylsulfapyridine not available).

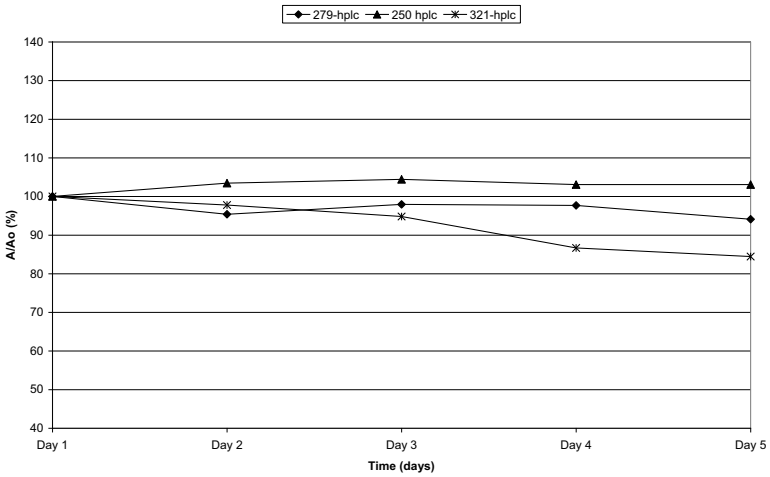


Figure S2. Time course photodegradation of AcSPY and evolution of its desulfonated TP. Symbols (a): AcSPY (■); m/z 228(□).

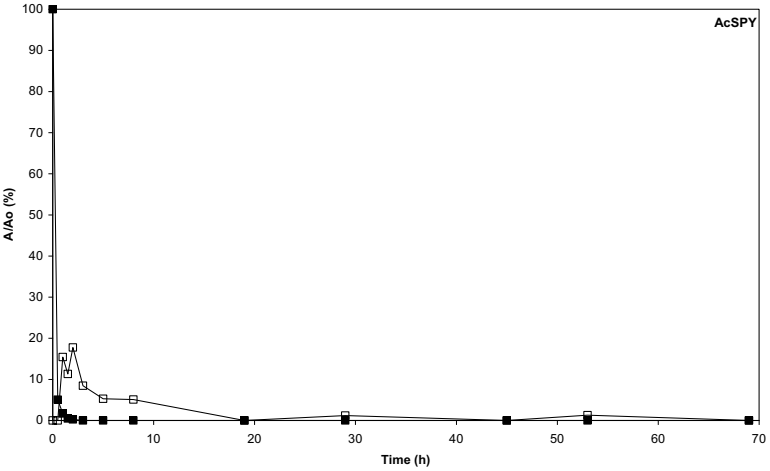


Table S1. Accurate mass measurement of sulfapyridine majority and minority photodegradation products as determined by UPLC/ESI-QqToF in MS^2 mode and optimized cone voltage (25 V) and collision energies (25-35 eV). RT, chromatographic retention time. DBE, double bond equivalent.

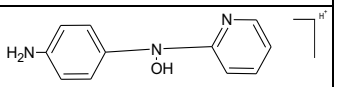
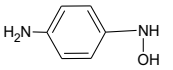
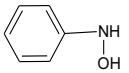
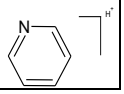
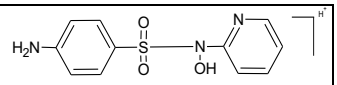
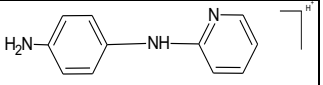
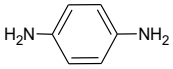
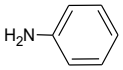
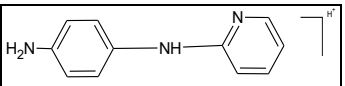
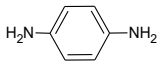
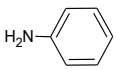
Fragment (m/z)	RT (min)	Elemental composition	Observed mass	Calculated mass	Error (mDa)	Error (ppm)	DBE	Proposed structural composition
202	**1.93**	**$C_{11}H_{12}N_3O$**	**202.0979**	**202.098**	**0.9**	**0.2**	**7.5**	
124		$C_6H_8N_2O$	124.0646	124.0637	0.9	7.6	4	
109		C_6H_7NO	109.0501	109.0528	2.7	24.4	4	
80		C_5H_6N	80.0521	80.05	2.1	25.9	3.5	
266	**1.98**	**$C_{11}H_{12}N_3O_3S$**	**266.0599**	**266.0599**	**0.1**	**0.1**	**7.5**	
186		$C_{11}H_{12}N_3$	186.1035	186.1031	0.5	1.7	7.5	
108		$C_6H_8N_2$	108.0701	108.0687	1.7	15.7	4	
93		C_6H_7N	93.0577	93.0578	1.3	13.9	4	
186	**2.1**	**$C_{11}H_{12}N_3$**	**186.1030**	**186.1031**	**0.7**	**1.1**	**7.5**	
108		$C_6H_8N_2$	108.0698	108.0687	1.1	9.75	4	
93		C_6H_7N	93.0578	93.0578	0.1	0	4	
81		C_5H_7N	81.05835	81.0578	0.4	4.95	3	

Table S1 (cont.)

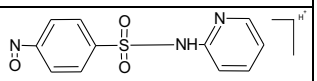
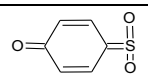
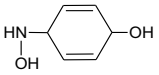
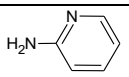
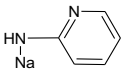
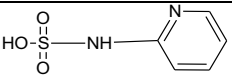
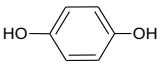
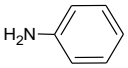
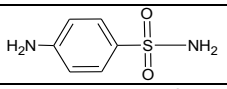
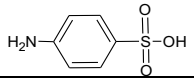
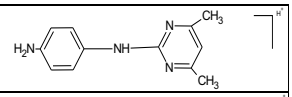
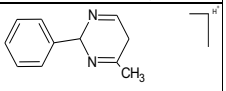
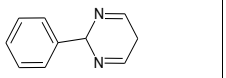
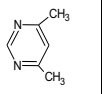
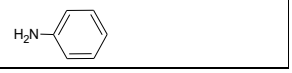
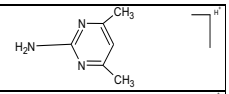
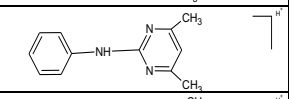
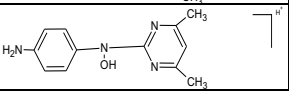
Fragment (m/z)	RT (min)	Elemental composition	Observed mass	Calculated mass	Error (mDa)	Error (ppm)	DBE	Proposed structural composition
264	**3.38**	**$C_{11}H_{10}N_3O_3S$**	**264.0443**	**264.0443**	**1.5**	**5.7**	**8.5**	
157		$C_6H_5O_3S$	156.9960	156.9959	0.5	3.5	4.5	
127		$C_6H_9NO_2$	127.0629	127.0633	0.4	3.4	3	
109		$C_6H_5O_2$	109.0309	109.029	1.7	15.3	4.5	
80		C_5H_6N	80.0510	80.05	1	12.2	3.5	
95	**1.12**	**$C_5H_7N_2$**	**95.0616**	**95.0609**	**6.65**	**0.65**	**3.5**	
117		$C_5H_6N_2Na$	117.0426	117.0429	2	0.2	3.5	
175	**3.9**	**$C_5H_7N_2O_3S$**	**175.0155**	**175.0177**	**12.9**	**2.3**	**3.5**	
111	**1.12**	**$C_6H_7O_2$**	**111.0432**	**111.0446**	**12.4**	**1.4**	**3.5**	
94	**1.56**	**C_6H_8N**	**94.0664**	**94.0657**	**7.8**	**0.733333**	**3.5**	
173	**2.61**	**$C_6H_9N_2O_2S$**	**173.0371**	**173.0385**	**7.7**	**1.3**	**3.5**	
174	**3.97**	**$C_6H_8NO_3S$**	**174.0234**	**174.0228**	**5.1**	**0.9**	**3.5**	

Table S2. Accurate mass measurement of sulfamethazine majority and minority photodegradation products as determined by UPLC/ESI-QqToF in MS^2 mode and optimized cone voltage (25 V) and collision energies (25-35 eV). RT, chromatographic retention time. DBE, double bond equivalent.

Fragment (m/z)	RT (min)	Elemental composition	Observed mass	Calculated mass	Error (mDa)	Error (ppm)	DBE	Proposed structural composition
215	**2.02**	**$C_{12}H_{15}N_4$**	**215.1291**	**215.1297**	**1.28**	**6.04**	**7.5**	
173		$C_{10}H_{11}N_3$	173.10975	173.1079	1.85	10.75	6.5	
158		$C_{10}H_9N_2$	158.0840667	158.0844	0.43	3.4	7	
108		$C_6H_8N_2$	108.0698	108.0687	1.1	10.2	4	
93		C_6H_7N	93.0587	93.0578	0.95	9.95	4	
124	**1.53**	**$C_6H_{10}N_3$**	**124.087525**	**124.0875**	**0.725**	**5.925**	**3.5**	
200	**2.45**	**$C_{12}H_{14}N_3$**	**200.12015**	**200.1188**	**1.4**	**6.95**	**7.5**	
231	**2.11**	**$C_{12}H_{15}N_4O$**	**231.1238333**	**231.1246**	**1.3**	**5.53**	**7.5**	

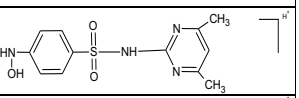
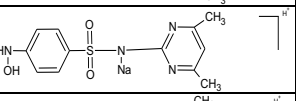
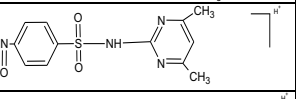
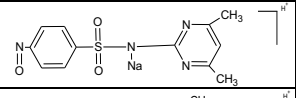
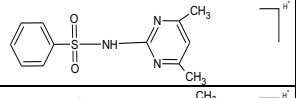
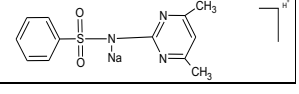
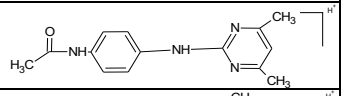
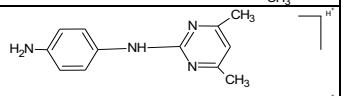
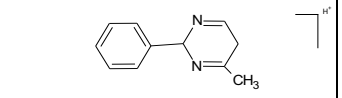
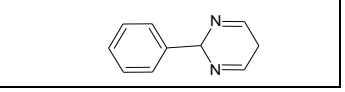
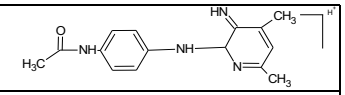
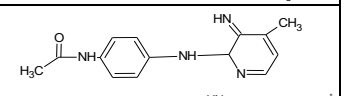
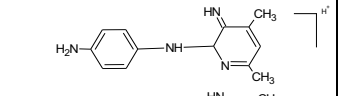
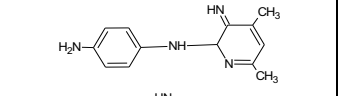
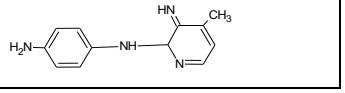
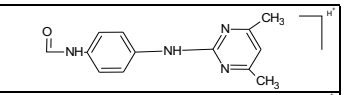
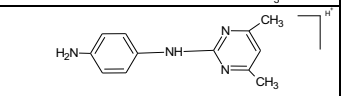
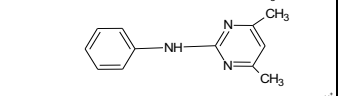
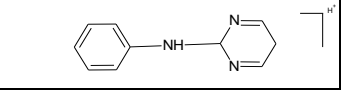
Fragment (m/z)	RT (min)	Elemental composition	Observed mass	Calculated mass	Error (mDa)	Error (ppm)	DBE	Proposed structural composition
295	**2.22**	**$C_{12}H_{15}N_4O_3S$**	**295.0868667**	**295.0865**	**1.3**	**4.45**	**7.5**	
317		**$C_{12}H_{14}N_4O_3NaS$**	**317.070033**	**317.0684**	**1.66**	**5.3**	**7.5**	
293	**3.19**	**$C_{12}H_{13}N_4O_3S$**	**293.0703**	**293.0708**	**1.06**	**3.63**	**8.5**	
315		**$C_{12}H_{12}N_4O_3NaS$**	**315.0531**	**315.0528**	**2.9**	**9.15**	**8.5**	
264	**4.25**	**$C_{12}H_{14}N_3O_2S$**	**264.07995**	**264.0807**	**0.75**	**2.9**	**7.5**	
286		**$C_{12}H_{13}N_3O_2NaS$**	**286.0599**	**286.0592**	**2.7**	**9.6**	**7.5**	

Table S3. Accurate mass measurement of N^4-acetylsulfamethazine photodegradation products as determined by UPLC/ESI-QqToF in MS^2 mode and optimized cone voltage (25 V) and collision energies (25-35 eV). RT, chromatographic retention time. DBE, double bond equivalent.

Fragment (m/z)	RT (min)	Elemental composition	Observed mass	Calculated mass	Error (mDa)	Error (ppm)	DBE	Proposed structural composition
257	**2.35**	**$C_{14}H_{17}N_4O$**	**257.1399**	**257.1402**	**0.27**	**1.07**	**8.5**	
215		$C_{12}H_{15}N_4$	215.1301	215.1297	0.43	2.17	7.5	
173		$C_{11}H_{13}N_2$	173.1098	173.1079	1.70	9.97	6.5	
158		$C_{10}H_{10}N_2$	158.0835	158.0844	1.13	7.13	7	
271	**2.84**	**$C_{15}H_{19}N_4O$**	**271.1577**	**271.1559**	**1.83**	**6.80**	**8.5**	
256		$C_{14}H_{16}N_4O$	256.1339	256.1324	0.55	2.15	9	
229		$C_{13}H_{17}N_4$	229.1463	229.1453	1.07	4.67	7.5	
228		$C_{13}H_{16}N_4$	228.1366	228.1375	0.87	3.80	8	
214		$C_{12}H_{14}N_4$	214.1208	214.1218	8.33	1.77	8	
243	**2.22**	**$C_{13}H_{15}N_4O$**	**243.1254**	**243.1246**	**1.17**	**4.77**	**8.5**	
215		$C_{12}H_{15}N_4$	215.1287	215.1297	1.70	7.90	7.5	
199		$C_{12}H_{13}N_3$	199.1108	199.1109	0.10	0.70	8	
173		$C_{11}H_{13}N_2$	173.1518	173.1079	0.55	9.00	6.5	

5.3. DISCUSIÓN

Considerando que diferentes trabajos han documentado la fotodegradación de las sulfamidas mediante Fotofentón o bajo radiación solar directa (natural o simulada), junto con la reciente detección de sus metabolitos acetilados con prácticamente la misma ubicuidad que las sustancias originales, nos llevó a centrar nuestro estudio en evaluar la posible fotodegradación de estos metabolitos acetilados. Algunos trabajos como el de Boreen *et al.* sugerían que la interacción de la radiación con la materia orgánica disuelta en agua aumenta la degradación de determinadas sulfamidas. Para evaluar el papel de la materia orgánica disuelta durante la fotodegradación, utilizamos dos tipos diferentes de matriz acuosa, agua HPLC y agua de salida de EDAR. Elegimos la SMZ y la SPY como sulfamidas veterinaria y humana de alta incidencia ambiental respectivamente, y sus correspondinetes metabolitos acetilados. Los resultados de la Tabla 1 de la Publicación #15 nos muestran que en agua HPLC, el tiempo necesario para eliminar completamente la SMZ fue de 70 h y algo superior para SPY. Otros f Podemos considerar por tanto que ambos son vulnerables a la radiación solar, ya que en ambos casos sus tasas de eliminación son cercanas al 100%. En el caso de sus metabolitos acetilados, en apenas 5 h ambos se degradaron en su totalidad. Cabe recordar que nos referimos a la desaparición de la molécula como tal, no a su completa mineralización. La hidrólisis y pérdida del grupo acetilado no parece ser la causa de esta rápida disminución de los metabolitos en ninguno de los dos casos, ya que la sustancia original no fue detectada en ninguna de las muestras irradiadas tras 5 h, por lo que cabía esperar la presencia de diferentes productos de fototransformación.

Si observamos de nuevo la Tabla 1 de la Publicación #15, vemos que los efectos de la presencia de materia orgánica en el agua apenas afectaron la tasa de fotodegradación de la SMZ, mientras que la vida media del SPY en la muestra se redujo de 11 h a apenas 2.5 h. Para los metabolitos acetilados, por el contrario, el efecto de la materia orgánica fue el inverso, y la tasa de fotodegradación de ambos fue más lenta, especialmente la del AcSMZ. Parece ser, por tanto, que la capacidad oxidativa de la materia orgánica depende de la estructura molecular del compuesto en cuestión. Boreen et al. (Boreen *et al.* 2004; Boreen *et al.* 2005) ya observaron diferencias entre las sulfamidas con anillos heterocíclicos de cinco y seis carbonos y destacó la i

influencia de pequeñas variaciones estructurales en la tasa de fotodegradación de estos compuestos. El hecho de que los metabolitos acetilados estudiados sean más resistentes a la fotodegradación en matrices ambientales (con materia orgánica) que las respectivas sustancias originales destaca de nuevo la relevancia de su presencia medioambiental y la necesidad de su estudio.

Respecto a los productos de fotodegradación, quizás el dato más relevante es la identificación del producto desulfonado, mayoritario tanto para las sulfamidas originales como para sus metabolitos. La identificación de este compuesto también en los trabajos de biodegradación con *T. versicolor* y en reactores de lecho fijo, presentados en el Capítulo 4, refuerza la posibilidad de su presencia medioambiental. Como vemos en las Figuras 4 y 7 de la Publicación #15, la molécula desulfonada de la SPY fue detectada unas 5 h tras el comienzo del experimento, y desapareció después de 70 h de irradiación, casi simultáneamente con la SPY. Sin embargo, el producto desulfonado de la SMZ seguía presente en la muestra tras 76 h de irradiación, mientras que la propia SMZ había sido fotodegradada en 45 h. Si bien estas curvas de presencia en el tiempo se realizaron con los ensayos realizados en agua HPLC, la baja influencia que la materia orgánica tenía en la degradación de la SMZ hace pensar que en aguas superficiales observaríamos una tendencia parecida tanto para ésta como para su producto desulfonado. En el estudio de degradación de la SMZ realizado con *T. versicolor*, el producto de transformación desulfonado seguía presente después de 80 h de experimento (ver Figura 4 de la Publicación #13). Para los metabolitos acetilados, el producto desulfonado del AcSMZ fue detectado en la muestra hasta después de 70 h de experimento, si bien a niveles inferiores al 5% (ver Figura 8a de la Publicación #15) . El producto desulfonado de AcSPY no fue detectado después de 20 h de experimento (ver Figura 1 de la Publicación 1 del Anexo A2), aunque se siguieron detectando nilveles traza intermitentemente hasta el final del experimento (70 h). En el estudio realizado con el reactor de lecho fijo presentado en la Publicación #12, este mismo producto de transformación se detectó hasta la finalización del experimento después de 62 días. Todos estos resultados llevan a pensar en la necesidad futura de evaluar la toxicidad crónica de estos productos de transformación mayoritarios, ya que su resistencia a la degradación y su presencia medioambiental ya han quedado demostradas.

CONCLUSIONES GENERALES

Los diferentes estudios llevados a cabo durante el desarrollo de esta Tesis nos permiten llegar a las siguientes conclusiones:

1. La necesidad de desarrollar metodologías analíticas lo suficientemente sensibles y robustas para detectar niveles traza de diferentes contaminantes en muestras ambientales ha motivado el desarrollo y validación de tres nuevos métodos, todos ellos basados en LC-QqLIT-MS, para el análisis de sulfamidas y varios de sus metabolitos acetilados en diferentes tipos de matrices ambientales. Su alta reproducibilidad, selectividad y sensibilidad ha sido demostrada para todos ellos.

2. Mediante la aplicación de estas nuevas metodologías para el estudio de muestras ambientales se ha demostrado la ubicuidad de esta familia de antibióticos en aguas residuales, superficiales e incluso subterráneas, así como en fangos de depuradora y suelos agrícolas. Cabe destacar la inclusión de cinco metabolitos acetilados dentro de los objetivos de estudio, y la confirmación de su presencia en prácticamente todas las matrices ambientales estudiadas, en ocasiones a niveles similares o superiores a los del compuesto original.

3. Todas las sulfamidas estudiadas, incluyendo los metabolitos acetilados, han sido detectadas en diferentes aguas de salida de depuradora a niveles de hasta $\mu g\ L^{-1}$. El sulfametoxazol (SMX) y la sulfapiridina (SPY), junto con sus metabolitos acetilados, han sido con diferencia las sulfamidas detectadas a mayor concentración. Se han estimado las tasas de eliminación (RE%) de las sulfamidas en las diferentes plantas depuradoras estudiadas, y los valores obtenidos han cubierto rangos de valores demasiado amplios, no permitiendo alcanzar conclusiones definitivas a pesar del gran número de muestras analizadas. La generalmente baja eficacia de los tratamientos de depuración convencionales para la eliminación de sulfamidas, basados en tratamientos biológicos por fangos activados, hace que los efluentes de dichas plantas

de tratamiento sean considerados como la vía principal de entrada de las sulfamidas de consumo humano en el medio natural.

4. La falta de legislación sobre la presencia medioambiental de las sulfamidas y sus metabolitos, así como concentraciones máximas de riesgo o su ecotoxicidad, no nos ha permitido hasta la fecha definir la calidad de las muestras estudiadas.

5. Debido al punto anterior, hemos evaluado métodos de depuración alternativos como los bioreactores de membrana (MBR). Las tasas de eliminación obtenidas fueron mejores para las sulfamidas con mayor contribución a la carga total de sulfamidas (SMX y SPY), pero aún así los valores de eliminación fueron sólo ligeramente superiores al 50%.

6. Mediante un bioreactor de lecho fijo (FBBR) estudiamos la biodegradabilidad de la SPY y su metabolito acetilado. Observamos como el SPY seguía presente en el reactor después de 60 días de tratamiento, mientras que su metabolito acetilado era eliminado tras 32 días. Comprobamos como este último revertía en un 50% en el compuesto original. Se demostró así no sólo la baja biodegradabilidad de las sulfamidas, sino la importancia de incluir sus metabolitos acetilados dentro de los objetivos de los estudios de presencia medioambiental, ya que su exclusión podría suponer una importante subestimación de las concentraciones reales.

7. La escasa biodegradabilidad de las sulfamidas hace que mecanismos de degradación abiótica como la fotodegradación se consideren alternativas factibles para su eliminación. Se estudió el comportamiento de la SPY, la sulfametazina (SMZ) y sus respectivos metabolitos acetilados bajo radiación solar simulada, en agua de salida de depuradora. Se logró su completa eliminación en 30 h de irradiación excepto para la SPY, que aún pudo ser detectada tras 70 h. La fotodegradación podría así considerarse como una alternativa más eficaz y rápida que la biodegradación para la eliminación de las sulfamidas. Sin embargo, el gran número de productos de transformación generados, identificados mediante UPLC-QqTOF-MS, plantea un nuevo problema

sobre el desconocimiento de las propiedades fisicoquímicas de éstos y su posible ecotoxicidad.

8. La aplicación del hongo *Trametes versicolor* para eliminar sulfamidas en medio líquido y sólido fue demostrado para la SMZ, SPY y STZ. Su alta capacidad oxidativa fue capaz de eliminar completamente las tres sulfamidas en plazos relativamente cortos (20 h-170 h). La inespecifidad de su sistema enzimático, exocelular e intracelular, convierten a este hongo en un gran instrumento de biorremediación para sitios fuertemente contaminados por sulfamidas. Se identificaron a su vez diferentes productos de transformación derivados de la biodegradación de las sulfamidas estudiadas. Al igual que con los fotoproductos, será imprescindible evaluar la naturaleza y ecotoxicidad de estos nuevos productos antes de su apliación medioambiental.

9. El producto desulfonado de las sulfamidas estudiadas se ha identificado como producto de transformación detectado más abundante y común en los estudios de fotodegradación, FBBR y biodegradación mediante *Trametes versicolor*. La escasa información sobre la biodegradabilidad y sobre la ecotoxicidad potencial de estos nuevos productos de transformación, así como de los metabolitos generados y excretados por el organismo, una vez vertidos al medio natural, hace necesario el planteamiento de futuros estudios que proporcionen dicha información.

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ANEXOS

A1. LISTA DE PUBLICACIONES PRESENTADAS EN ESTA TESIS

1. M.J. García-Galán, M.S. Díaz-Cruz, D. Barceló. " Identification and determination of metabolites and degradation products of sulfonamide antibiotics". Trends in Analytical Chemistry , Vol. 27, Nº 11 (2008) 1008 - 1022

2. M.J. García-Galán, M.S. Díaz-Cruz, D. Barceló. "Combining chemical analysis and ecotoxicity to determine environmental exposure and to assess risk from sulfonamides". Trends in Analytical Chemistry, Vol 28 (2009) 804-819.

3. M.S. Díaz-Cruz, M.J. García-Galán, D. Barceló. "Highly sensitive simultaneous determination of sulfonamide antibiotics and one metabolite in environmental waters by liquid chromatography-quadrupole linear ion trap-mass spectrometry". Journal of Chromatography A, 1193 (2008) 50-59.

4. M.J. García-Galán, M.S. Díaz-Cruz, D. Barceló. “Determination of 19 sulfonamides in environmental waters by automated on-line solid-phase extraction-liquid chromatography–tandem mass spectrometry (SPE-LC-MS/MS)”. Talanta, Vol 81 (2010) 355-366

5. M.J. García-Galán, Teresa Garrido, Josep Fraile, Antoni Ginebreda, M.S. Díaz-Cruz, D. Barceló. “Simultaneous occurrence of nitrates and sulfonamide antibiotics in two groundwater bodies of Catalonia (Spain)”. Journal of Hydrology, Vol 383 (2010) 93-101.

6. M.J. García-Galán, M. Villagrasa, M.S. Díaz-Cruz, D. Barceló. “LC-QqLIT MS analysis of nine sulfonamides and one of its acetylated metabolites in the Llobregat river basin. Quantitative determination and qualitative evaluation by IDA experiments”. Analitycal and Bioanalitycal Chemistry, Vol 397 (3) (2010) 1325-1334

Anexos

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