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EFFECTS OF PRIORITY AND EMERGING POLLUTANTS ON RIVER BIOFILMS

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Ph.D. Thesis

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PROGRAMA DE DOCTORAT EN CIÈNCIES EXPERIMENTALS I SOSTENIBILITAT

Dirigida per:

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Memòria presentada per optar al títol de doctor/a per la Universitat de Girona

Universitat de Girona

Institut d'Ecologia Aquàtica

El Dr. Sergi Sabater Cortés, catedràtic d'ecologia del departament de Ciències Ambientals de la Universitat de Girona i la Dra. Helena Guasch Padró, professora titular del departament de Ciències Ambientals de la Universitat de Girona

CERTIFIQUEN:

Que aquest treball, titulat "Effects of priority and emerging pollutants on river biofilms", que presenta Marta Ricart Viladomat per a l'obtenció del títol de doctor/a, ha estat realitzat sota la meva direcció i que compleix els requeriments per poder optar a Menció Europea.

Signatura

Dr. Sergi Sabater Cortés Dra. Helena Guasch Padró

Girona, 2010

A l'Albert, en Marçal, la Dolors i en Manel

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1. Ricart, M., Guasch, H., Barceló, D., Brix, R., Conceição, M.H., Geiszinger, A., López de Alda, M.J., López-Doval, J.C., Muñoz, I., Postigo, C., Romaní, A.M., Villagrassa, M., Sabater, S., 2010. Primary and complex stressors in polluted Mediterranean rivers: Pesticide effects on biological communities. Journal of Hydrology 383, 52-61.

Àrea temàtica: *Water Resources*

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2. Ricart, M., Barceló, D., Geszinger, A., Guasch, H., López de Alda, M., Romaní, A.M., Vidal, G., Villagrasa, M., Sabater, S. 2009. Effects of low concentrations of the phenylurea herbicide diuron on biofilm algae and bacteria. Chemosphere 76, 1392-1401.

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3. Ricart, M., Guasch, H., Alberch, M., Barceló, D., Bonnineau, C., Geiszinger, A., Farré, M., Ferrer, J., Ricciardi, F., Romaní, A.M., Morin, S., Proia, L., Sala, Ll., Sureda, D., Sabater, S., 2010. Triclosan persistence through wastewater treatment plant and its potential toxic effects on river biofilms. Aquatic Toxicology 100, 346-353.

Àrea temàtica: *Toxicology*

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4. Ricart, M., Guasch, H., Gillisen, F., Lürling, M., Sabater, S. Algal toxicity of diuron, propranolol, triclosan and their binary mixtures. *In prep.*

CONTENTS

RESUM

RESUM

L'activitat humana representa una de les majors causes d'entrada d'una gran varietat de substàncies en els ecosistemes fluvials. Conseqüentment, un ampli ventall de contaminants d'origen industrial, urbà i agrícola estan presents als ecosistemes fluvials. Els tòxics orgànics representen un dels problemes més importants en els sistemes aquàtics, ja que en la majoria de casos hi ha molt poca informació sobre la seva presència i toxicitat. Els sistemes aquàtics també es veuen afectats per altres pertorbacions físiques i químiques com per exemple alteracions en el cabal, en la temperatura de l'aigua o en la concentració de nutrients.

Els biofilms són communitats de microorganisms que viuen en superfícies, formats per bacteris, algues i fongs. Els biofilms són els primers en interaccionar amb les substàncies dissoltes de l'aigua (incloent els tòxics) i són capaços d'integrar les condicions ambientals al llarg del temps, la qual cosa els converteix en una eina valuosa a l'hora d'avaluar els efectes dels contaminants en els ecosistemes aquàtics.

Aquest treball pretén investigar els efectes que els tòxics orgànics poden exercir en els biofilms. L'estudi inclou una aproximació a diferents escales d'estudi, incloent diferents nivells d'organització biològica (de cultius a comunitats de biofilm), diferents escales experimentals (de microcosms a estudis de camp) i diferents tipus d'exposició (de tòxics individuals a barreges de tòxics).

CAPÍTOL II: Pertorbacions primàries i complexes en els rius Mediterranis contaminats: Efectes dels plaguicides en les comunitats biològiques. Aquest capítol pretenia examinar la presència de plaguicides al riu Llobregat, així com identificar els seus efectes en les comunitats bentòniques. El riu Llobregat constitueix la principal font d'aigua potable de la ciutat de Barcelona i de les seves rodalies. És un riu que ha estat sotmès a fortes pressions urbanístiques, industrials i urbanes, fet que l'ha portat a uns nivells elevats de contaminació. Les situacions de sequera i l'associada baixa capacitat de dilució augmenten l'impacte potencial dels contaminants, els quals es troben en

concentracions més elevades. En aquest estudi es van analitzar 22 plaguicides en l'aigua i en el sediment per tal de detectar els seus efectes en les comunitats de diatomees i invertebrats. Els plaguicides pertanyien a les famílies de les triazines, organofosfats, fenilurees, anilides, cloroacetanilides, herbicides àcids i tiocarbamats. Les màximes concentracions detectades en l'aigua corresponien a l'insecticida diazinon (785 ng/L) i a l'herbicida linuron (327 ng/L), mentre que en el sediment els herbicides diuró i metolaclor van registrar els valors més elevats (32 i 43 ng/g, respectivament). L'anàlisi estadístic multivariant va mostrar una influència dels plaguicides en la distribució de la comunitat de diatomees. Concretament, vàries espècies de diatomees estaven fortament associades a la presència d'herbicides pertanyents al grup de les triazines. No obstant, la distribució de les comunitats d'invertebrats no va resultar influenciada pels plaguicides. Una selecció de mètriques relacionades amb el biofilm va ser inclosa en l'anàlisi. Els resultats van suggerir efectes a nivell estructural i funcional deguts a la presència d'organofosfats i fenilurees. La sensibilitat de cada mètrica a un contaminant en particular, així com la causalitat potencial, s'ha de complementar amb aproximacions experimentals. L'ús d'ambdues aproximacions podria ser incorporada en l'avaluació del risc ambiental per tal de millorar la gestió ecològica dels ecosistemes fluvials.

CAPÍTOL III: Efectes de baixes concentracions de l'herbicida diuró (fenilurea) en les algues i els bacteris del biofilm. Aquest capítol tenia com a objectiu investigar els efectes a llarg termini (29 dies) de l'herbicida diuró sobre les comunitats algals i bacterianes del biofilm. Per aconseguir aquest objectiu, es va dissenyar un sistema de canals experimentals on el biofilm va ser exposat a concentracions creixents exponencials de diuró (de 0,07 a 7 µg/L). L'activitat fotosintètica es va veure afectada durant tota l'exposició, així com la biomassa algal. Després de 8 dies d'exposició, es van detectar canvis en la composició de la comunitat de diatomees. Algunes espècies van augmentar la seva abundància relativa amb el tòxic, mentre que en d'altres es va veure reduïda. El biovolum de la comunitat de diatomees també va resultar afectat, mostrant una dràstica reducció amb l'augment de concentracions de diuró. Els efectes de l'herbicida en la comunitat bacteriana van ser menys pronunciats que en la comunitat algal. No obstant, es va detectar un augment de l'activitat

leucina-aminopeptidasa i un augment remarcable de la mortalitat bacteriana. Els resultats d'aquest estudi van mostrar que l'entrada d'herbicides a baixes concentracions pot ocasionar una cadena d'efectes en els biofilms fluvials, els quals inclouen una inhibició en la comunitat algal i també efectes indirectes en la relacions entre els components dels biofilms.

CAPÍTOL IV: Persistència del triclosan a través de plantes de tractament d'aigües residuals i els seus efectes tòxics potencials en biofilms fluvials. Aquest estudi pretenia estudiar els efectes del bactericida triclosan en els biofilms fluvials. El triclosan és un bactericida àmpliament utilitzat que es caracteritza per la seva persistència a través de plantes de tractament d'aigües residuals, arribant així als ecosistemes fluvials. Per tal d'avaluar la seva toxicitat es va utilitzar un sistema de canals experimentals on es va portar a terme una exposició a concentracions creixents de triclosan (de 0,5 a 500 µg/L) durant 48 hores. El tòxic va causar un clar augment de la mortalitat bacteriana (NEC= 0,21 µg/L), arribant a un 85% de bacteris morts en la concentració més elevada. La toxicitat del triclosan va ser més baixa en el component algal dels biofilms. L'eficiència fotosintètica es va veure inhibida (NEC= 0,42 µg/L), així com els mecanismes de quenching no fotoquímic i la viabilitat de les diatomees. La toxicitat observada sobre el component algal dels biofilms podia ser atribuïble a un efecte indirecte del bactericida. No obstant, els clars efectes observats en les algues suggerien l'existència d'una afectació directa del component algal. Els efectes directes del triclosan en la comunitat bacteriana del biofilm, juntament amb els afectes detectats en les algues, fan que el triclosan representi un risc ambiental, especialment en zones Mediterrànies on les freqüents condicions d'escassetat d'aigua poden comportar una baixa capacitat de dilució del riu, i, conseqüentment, una concentració més elevada de tòxics en els ecosistemes fluvials.

CAPÍTOL V: Toxicitat algal dels compostos diuró, propranolol, triclosan i de les seves barreges binàries. Aquest estudi tenia com a objectiu avaluar la toxicitat de l'herbicida diuró, el producte farmacèutic propranolol i el bactericida triclosan, així com la toxicitat de les seves barreges utilitzant assajos amb monocultius algals (S*cenedesmus obliquus)*. També es pretenia comprovar

l'aplicabilitat dels conceptes "Concentration Addition" (CA) i "Independent Action" (IA), ambdós àmpliament usats per a predir la toxicitat de les barreges de tòxics.

El primer concepte s'atribueix habitualment a barreges de tòxics que tenen una acció similar. No obstant, quan la barreja està formada per tòxics que no tenen una acció similar, escollir quin dels dos conceptes és el més adequat és un tema que genera una certa controvèrsia.

Aplicats per separat, l'herbicida diuró era el compost que presentava una toxicitat més elevada, seguit del triclosan i del propranolol. El diuró i el propranolol afectaven principalment els processos fotosintètics, mentre que el triclosan reduïa el creixement algal.

La primera barreja (diuró i propranolol) va seguir les prediccions fetes pel model CA, principalment degut a l'acció similar dels dos components. La segona barreja (diuró i triclosan), va seguir les prediccions fetes pel model IA, probablement degut al diferent mecanisme d'acció dels dos tòxics. La toxicitat de la tercera barreja (propranolol i triclosan) va resultar ser més alta que la toxicitat predita per ambdós models, mostrant així un efecte sinèrgic.

Els resultats d'aquest estudi mostren que les barreges de tòxics poden tenir un impacte més gran del predit pels models, demostrant que la toxicitat d'una substància pot augmentar en combinació amb una altra.

SUMMARY

SUMMARY

Human activity is responsible for the entrance of many substances to the aquatic environment. A wide range of pollutants from industrial, urban and agricultural activities reach the aquatic systems. The occurrence of organic toxicants is one of the major problems affecting aquatic systems since information about their occurrence and toxicity is scarce for many of them. In addition, aquatic systems are also affected by physical and chemical disturbances such as alterations in water flow, water temperature or nutrient concentration. Biofilms are attached communities of bacteria, algae, and fungi. Biofilms are the first to interact with dissolved substances (including toxicants) and are able to integrate environmental conditions over extended periods of time. These conditions make them useful tools for monitoring the effects of pollutants in aquatic systems.

The present study aims to investigate the effects of various organic toxicants on fluvial biofilm communities. The study uses a multi-scale approach which includes different levels of biological organization (from cultures to biofilm communities), different experimental scales (from microcosms to field studies), and different types of exposures (from single toxicants to mixtures).

CHAPTER II: Primary and complex stressors in polluted Mediterranean rivers: Pesticide effects on biological communities. This chapter aimed to examine the presence of pesticides in the river Llobregat and identify their effects on benthic biological communities. The river Llobregat is a major drinking water resource for Barcelona. The river has been submitted to urban, industrial and agricultural pressures, and as such is a highly contaminated river. Water scarcity episodes and their lower dilution capacity increase the potential effect of pollutants, which are present in higher concentrations. In this study, 22 pesticides were analysed in the water and in the sediment for their effect on diatom and invertebrate communities. Pesticides were from the families of triazines, organophosphates, phenylureas, anilides, chloroacetanilides, acidic herbicides and tiocarbamates. The insecticide diazinon and the herbicide linuron registered the highest concentrations in the water (785 ng/L and 327

ng/L, respectively), whereas the herbicides diuron and metolachlor were the ones showing the highest levels in the sediment (32 ng/g and 43 ng/g, respectively).

The multivariate statistical analysis revealed that the diatom community was influenced by the presence of pesticides. Several diatom species were closely associated to the presence of triazine herbicides. However, the benthic invertebrate distribution was not influenced.

Several biofilm metrics were included in the analysis. Certain effects of organophosphates and phenylureas in both structural and functional aspects occurred. The sensitivity of each metric to particular stressors and the potential causality needs to be complemented with experimental approaches. The conjoint use of the two approaches could be incorporated into environmental risk assessment in order to improve the ecological management.

CHAPTER III: Effects of low concentrations of the phenylurea herbicide diuron on biofilm algae and bacteria. This chapter aimed to investigate the long-term effects (29 days) of the herbicide diuron on biofilm algae and bacteria. To this end, was designed a system of experimental channels where biofilm was exposed to a series of exponentially growing concentrations of diuron (from 0.07 to 7 µg/L). The photosynthetic efficiency of the algal community was affected during the whole exposure period, as well as the algal biomass. The composition of the diatom community was also affected after 8 days of exposure. Several diatom taxa increased in relative abundance with increasing concentrations of toxicants, whereas other taxa reduced its presence. The biovolume of the diatom community reduced with increasing concentrations of the toxicant. The effects on the bacterial community were less pronounced, but both an increase of the leucine-aminopeptidase activity, as well as a remarkable increase in bacterial mortality were detected. The results of this study showed that the input of low concentrations of herbicides may cause a chain of effects on biofilms, which include inhibitory effects on algae but also indirect effects on the relationships between biofilm components.

CHAPTER IV: Triclosan persistence through wastewater treatment plant and its potential toxic effects on river biofilms. This study aimed to investigate the effects of the bactericide triclosan on fluvial biofilms. Triclosan is a widely used bactericide that survives several degradation steps in waste water treatment plants, therefore reaching fluvial systems. A short-term exposure (48 hours) to increasing concentrations of triclosan (from 0.5 to 500 µg/L) was carried out in a system of experimental channels in order to evaluate its toxicity. Triclosan caused an increase of bacterial mortality (NEC= 0.21 µg/L)., Dead bacteria accounted up to 85% of the bacterial population at the highest concentration tested. Triclosan toxicity was lower in algae than in bacteria. Photosynthetic efficiency was inhibited (NEC= 0.42 µg/L), and the non photochemical quenching mechanisms and the diatom cells viability decreased. The observed toxicity on the algal component of biofilms could be an indirect effect of the bactericide. However, the clear effects detected on algae also suggested a direct effect of the toxicant on the algal compartment. Based on the effects described, the potential environmental risk of triclosan is high, especially in Mediterranean areas where the frequent water scarcity episodes results in lower dilution capacity and, as a consequence, higher concentration of toxicants that reach the fluvial systems.

CHAPTER V: Algal toxicity of diuron, propranolol, triclosan and their binary mixtures. This study aimed to evaluate the toxicity of the herbicide diuron, the pharmaceutical product propranolol, the bactericide triclosan and their binary mixtures using algal tests (*Scenedesmus obliquus*). We also aimed to test the applicability of the concepts “Concentration Addition” (CA) and “Independent Action” (IA), both widely used in mixture toxicity assessment.

The first concept is attributed to mixtures of chemicals having a similar action. However, when the mixture is composed by dissimilarly acting toxicants, the choice of the most appropriate concept is a controversial issue.

When applied as a single product, diuron was the compound showing the highest toxicity, followed by triclosan and propranolol. Diuron and propranolol affected photosynthetic processes, whereas triclosan effects were only detected on algal growth.

The mixture of diuron and propranolol followed the predictions done by the CA model, probably due to the similar mode of action of the two toxicants. The toxicity of the mixture of diuron and triclosan was however accurately predicted by the IA model. This result was attributed to the non capability of the two substances to cause the same ecotoxicological response. The toxicity of the mixture of propranolol and triclosan was higher than that predicted by the two models, and showed a synergistic effect.

The results of this study showed that mixtures of toxicants can have a greater negative impact than predicted by models, demonstrating that the toxicity of a single substance could increase strongly in combination with other toxicant.

CHAPTER I

General introduction

GENERAL INTRODUCTION

The European Water Framework Directive (WFD) aims to implement an optimal ecological integrity in European water bodies. Biofilms are amongst the biological compartments recognised by the WFD as a necessary target. Biofilms, also called periphyton or microphytobenthos in the literature, are attached communities of microorganisms on surfaces. In rivers and streams, biofilms are the first to interact with dissolved substances such as nutrients, organic matter and toxicants, and may be therefore affected and later used to detect the early effects these disturbances might cause on the ecosystem (Sabater et al., 2007). Biofilms integrate the influences of environmental conditions over extended periods of time, mainly because of the small size and rapid growth, species richness and physiological variety of the organisms that form them. For this reason they have been widely used for routine monitoring, since they may be very useful as "early warning systems" after disturbances (Sabater et al., 2007).

The interception of the toxicants by biofilms in the water phase may result in two biofilm responses which may differ in their temporal pattern: short-term physiological alterations and, long-term changes in the community structure. Both types of change can be transient or irreversible, but the responses occur rapidly, justifying the use of biofilms as good early warning indicators of toxicant exposure in aquatic ecosystems (Navarro et al., 2002).

Biofilms as a structural entity

Biofilms are biological structures made up of bacteria, algae, fungi and microfauna, located in close physical contact and embedded in a muccopolysaccharide matrix (Fig. 1). The biofilm community integrates several taxonomic kingdoms (e.g. prokaryotes, eukaryotes) thus covering most relevant metabolic pathways sustaining ecosystem functioning (e.g. anabolic and catabolic pathways). Biofilms are therefore complex entities made up of living and non-living components, each contributing to the mechanisms of uptake (of inorganic and organic nutrients) and retention (e.g. of heavy metals and

herbicides) that lead to self-depuration. Several factors affect the functioning and ecotoxicological response of biofilms. Some of these factors are physical (e.g. water current, temperature, light penetration), and chemical (pH, nutrient availability) while others are biological (relative contribution of autotrophs and heterotrophs, community composition, biomass thickness, grazing) (Stevenson, 1996).

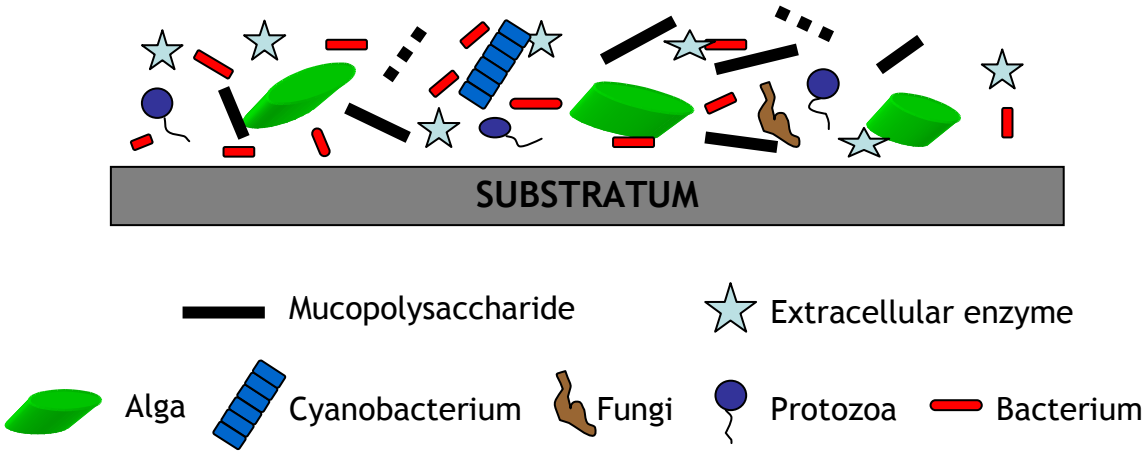

Fig. 1. Scheme of the river biofilm community structure. Adapted from Push et al., 1998.

Polysaccharide content, carbon, nitrogen, and phosphorus content, and density and diffusion properties of the biofilm change with time (Yallop et al., 2000). In the early stages of the biofilm there is a dominance of exopolymeric materials (EPS), which mainly consists of polysaccharides but also of proteins and lipids. EPS production may also reflect the physiological state of the biofilm (Sabater and Admiraal, 2005). Algae and bacterial biomass increase on later stages (Barranguet et al., 2004). The EPS improves cell attachment (Costerton et al., 1978) and is a crucial structural property affecting stability and architecture (Battin et al., 2003) which could determine rates of organic carbon uptake or release (Freeman and Lock, 1995) of the biofilm.

The ability of biofilms to act as interceptors of dissolved and particulate matter is based on their emerging physical and biological properties. The EPS offers

potential binding sites for a variety of colloidal, organic and inorganic compounds (Flemming, 1995). By physical adsorption, biofilms remove substances (for example heavy metals) from the flowing water (Kaplan et al., 1987), which can be later assimilated by the organisms. Large molecules are trapped in the EPS and remain bound by weak physico-chemical interactions (Flemming, 1995); they are progressively metabolised by extracellular enzymes. Because of their role as an interface between the overlying water and the sediments in the river ecosystem, biofilms are a suitable compartment for evaluating the effect of chemicals on river ecosystems. Biofilms integrate a variety of responses to chemical stressors of organisms from the heterotrophs (bacteria, fungi, protozoa) to the autotrophs (algae, cyanobacteria). By studying the effects of toxicants on biofilms, toxicity differences among populations can be evaluated, resulting in more ecological relevance.

Toxicants in fluvial ecosystems

During the last three decades, the impact of chemical pollution has focused almost exclusively on the “priority pollutants”, which have long been recognized as posing risks to aquatic ecosystems and human health, due to their toxicity, carcinogenic and mutagenic effects, and their persistence in the environment (Gros et al., 2008).

The Water Framework Directive (2000/60/EC) defines a strategy for protecting and restoring clean water across Europe. As a first step of this strategy, a list of priority substances was adopted in a Directive published in December 2008 (2008/105/EC), identifying 33 substances of priority concern and setting environmental quality standards (EQS) for each substance. Two types of EQS are defined, annual average concentrations and maximum allowable concentrations, one for protection against long-term and chronic effects, the other for short-term, direct and acute toxic effects.

Most of the compounds in the list are organic contaminants such us hydrocarbons, organochlorine compounds, organic solvents, pesticides and chlorophenols. The list also includes four toxic metals and one organometallic compound (Coquery et al., 2005).

Besides these recognized contaminants, numerous other chemicals are continuously released into the environment as a result of their use in industry, agriculture or household activities. With modern analytical techniques becoming more powerful -especially liquid chromatography (LC) combined with mass spectrometry (MS) and tandem MS (MS^2)-, more substances are being detected at trace levels (Barceló, 2008). The so-called emerging compounds are defined as compounds that are not currently covered by existing water-quality regulations, have not been studied before, and are thought to be potential threats to environmental ecosystems and human health and safety (Farré et al., 2008). Therefore, emerging contaminants may be candidates for future regulation, depending on research on their ecotoxicity, potential health effects and on monitoring data regarding their occurrence in the environment (Petrovic et al., 2008). The primarily source of emerging contaminants are waste water treatment plant effluents. Various groups of compounds are considered to be emerging, including drugs of abuse, flame retardants, gasoline additives, industrial additives and agents, personal care products, pharmaceuticals, steroids, hormones, surfactants and surfactant metabolites (Farré et al., 2008). The continual release of these contaminants by wastewater treatment plants makes them "pseudo-persistent" contaminants in fluvial ecosystems, therefore increasing their potential toxicity (Smital, 2008).

Some priority substances have been already investigated, providing information about their occurrence, mode of action and toxicity on biofilms (i.e. diuron (Pesce et al., 2006; Tlili et al., 2008), atrazine (Guasch et al., 2007), cadmium (Ivorra et al., 2002), isoproturon (Laviale et al., 2010)). The effect on biofilms of the so-called emerging compounds is still largely unknown. Moreover, for most emerging compounds, the mode of action, occurrence and ecotoxicological data are not available.

Several compounds have been investigated in this Thesis, covering both priority and emerging pollutants. The selected toxicants include the herbicide diuron, the pharmaceutical product propranolol and the personal care product triclosan (Fig. 2).

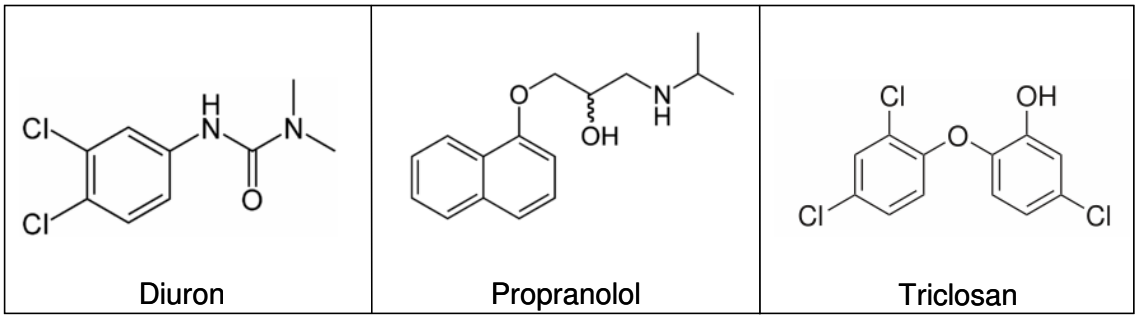

Fig. 2. Molecular structure of investigated toxicants in the Thesis.

Diuron was selected as a “classical” toxicant with a well-known mode of action on its target organisms (algae). This characteristic allowed us to investigate the direct and indirect effects on the main biofilm components (algae and bacteria), as well as to set-up a specific methodology based on a multiple-endpoint approach, to asses its toxic effects. Propranolol and triclosan were selected as emerging toxicants. The same multiple-endpoint approach as that developed for diuron was applied to assess their toxicity. Nor algae neither bacteria are the target organisms of propranolol, which allowed us to test the toxicity of a completely unknown mode of action toxicant. In the case of triclosan, the mode of action on bacteria was previously established, but as propranolol, scarce information was available about their toxicity and their non effect concentrations and effect concentrations values, thus giving the opportunity to provide ecotoxicological data about these emerging toxicants.

Diuron is a phenylurea herbicide mainly used for crop protection and also used for vegetation control in roads, railway lines and footpaths. Intensive agricultural areas are generally pesticide-dependent and generate water pollution due to the transfer of pesticide residues via runoff or leaching after being sprayed in agricultural fields (Montuelle et al., 2010). Diuron functions by inhibiting the Hill reaction in photosynthesis, limiting the production of high-energy compounds such as ATP, used in various metabolic processes. Diuron binds to the Q_B-binding niche on D1 protein of the photosystem II complex in chloroplast thylakoid membranes, thus blocking electron transport from Q_A to Q_B. This process prevents CO_2 fixation and the production of ATP and other high energy compounds (Hess and Warren, 2002). Diuron is included in the list of priority

pollutants of the WFD and has been regularly detected in surface waters (Rodríguez-Mozaz et al., 2004; Dorigo et al., 2007).

Propranolol is a human pharmaceutical product (β-blocker) used against hypertension and heart failure. It produces a reduction in cardiac index and an increase in systemic vascular resistance (Bristow, 1997). Human pharmaceuticals enter aquatic systems after ingestion and subsequent excretion through waste water treatment plants (Pérez and Barceló, 2007). If they are susceptible to passing through WWTPs, they can reach fluvial systems (Farré et al., 2008). It is considered an emerging contaminant and has been detected in waste water treatment plant effluents and surface waters (Roberts and Thomas, 2006; Muñoz et al., 2009). Propanolol's mode of action and toxicity to most non-target organisms such as algae is unknown.

Triclosan is a commonly used antiseptic agent. It is present in a wide range of personal care products, such as hand soaps, skin creams, toothphastes and household cleaners (Singer et al., 2002). As a bactericide, it inhibits the bacterial fatty acid synthesis. Triclosan binds to bacterial Enoyl-acyl carrier protein Reductase Enzyme (ENR), which catalyzes the terminal reaction in the fatty acid elongation cycle (Stewart et al., 1999). As a result of an incomplete removal in waste water treatment plants it reaches fluvial ecosystems, where has been detected (Kolpin et al., 2002). It is considered an emerging compound and the mode of action on algae is also unknown.

Biological and experimental organization in ecotoxicological studies

Since most potentially harmful compounds end up in fluvial ecosystems, evaluation of their toxicity is crucial in environmental risk assessment. Use of algae as ecotoxicological indicators has its origins in the analysis of the response of planktonic algae in cultures, in particular some well-known taxa such as *Scenedesmus quadricauda*, *Selenastrum capricornutum* or *Chlorella* sp. These taxa grow rapidly in the laboratory and are easily counted, so are suitable for toxicity bioassays. Because their growth may be affected by light, temperature and nutrient status, standard conditions are required and have been thoroughly implemented. Algal cultures have been used, for example, to

quantify effective doses of herbicides (Maule and Wright, 1984) and heavy metals (Bartlett et al., 1974) and, more recently, to assess the effects of emerging compounds such as surfactants (Lürling, 2006) and pharmaceuticals (Halling-Sørensen et al., 2000, Isidori et al., 2005) and in the assessment of the hazards of mixtures of chemicals (Faust et al., 2000, Geoffroy et al., 2002).

Although these approaches are reliable and thorough, they are based on single plankton species. Planktonic organisms are only dominant in a limited number of rivers (mostly large river systems); they are nearly absent from medium-sized and smaller fluvial systems. Basing diagnoses on single species and not in multi-species does not, moreover, enable the understanding of the effects of toxicants on the community. Biological community would integrate the responses of numerous species and their interaction and, therefore, the responses could be scaled up to the whole ecosystem level. Though tests for community responses are generally not as standardized as single specific tests species, for which international standards and guidelines are provided (i.e. OECD guidelines), they reflect the sensitivity of all potential species at risk and allow the detection of direct and indirect effects within the biological community (Mc.Clellan et al., 2008). Moreover, community experiments can include simple food chains (i.e. biofilms-grazers) where the magnified potential negative effects through the food web can be analyzed (Geiszinger et al., 2009).

In order to evaluate the potential impact of pollutants on the biota, the use of controlled exposures provides an excellent basis (Graney et al., 1984). Small scale laboratory experiments where algal cultures are exposed for short-time to toxicants have high experimental control and replication but lack of ecological realism.

Larger scale laboratory experiments (i.e. experimental channels) and field studies have greater ecological relevance but as the system increase in complexity and dimension, the experimental control and replication capacity decreases (Fig. 3) (Clements and Newman, 2002).

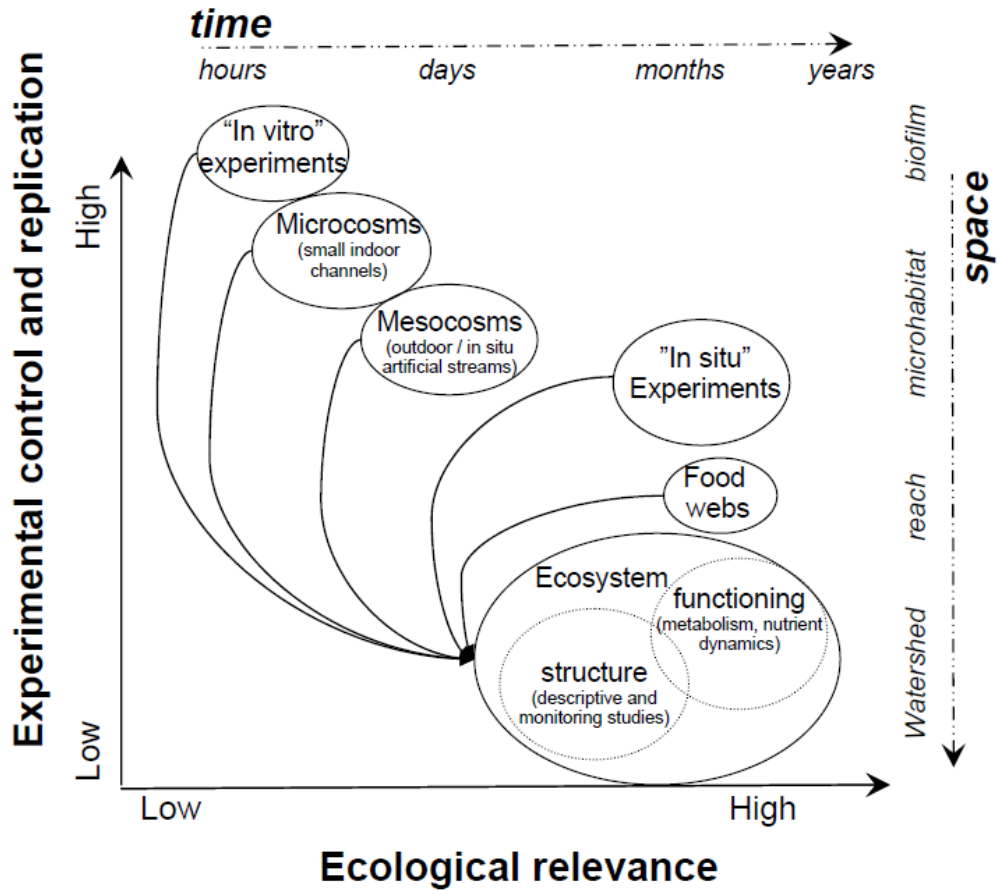

Fig. 3. Relationship between ecological relevance, experimental control and replication in fluvial ecology experimental approaches. Modified from Clements and Newman, 2002.

The use of experimental channels in which biofilm communities can develop may achieve the necessary compromise between simplification and standardisation of the natural system and the required replicability and repeatability (Fig. 4). Although smaller and less complex than real-world ecosystems, experimental channels provide the opportunity to perform ecosystem-level research in replicated test systems under conditions that are manageable in terms of costs and logistics (Roussel et al., 2007).

Experiments with biofilms in experimental channels have been used to investigate the effects of toxic substances (Navarro et al., 2008; Serra et al., 2009), and their dependence on specific environmental conditions (Guasch et al., 2004; Guasch et al., 2007; Serra et al., 2010), as well as effects of the

interaction between toxicants and grazers (Muñoz et al., 2001; Real et al., 2003; López-Doval et al., 2010).

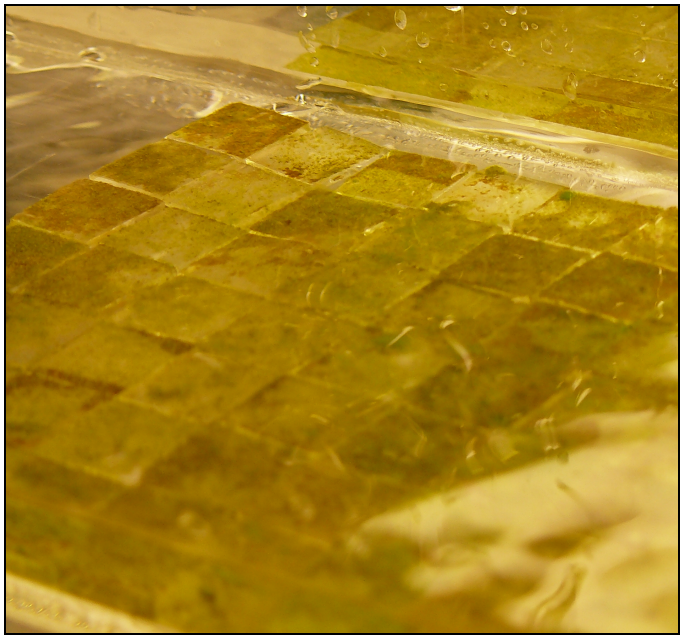

Fig. 4. Biofilm communities developed on glass substrata in experimental channels.

Field studies can provide support for a causal relationship between stressors and community responses; however, field studies alone cannot be used to show causation (Clements and Newman, 2002). Compared with laboratory experiments, they are more realistic, although have less control. Differences in pesticide sensitivity have been observed in a field survey conducted in a French river contaminated with pesticides (Pesce et al., 2010). Upstream-downstream gradients in biofilm metrics have been also detected with field studies due to increases in pollution (Montuelle et al., 2010; Morin et al., 2010a), as well as changes in community composition (Guasch et al., 2009).

The experiments included in this Thesis have been performed at different levels of biological and experimental organization. A field study that included benthic biological communities (invertebrates and biofilm) has been performed in the Llobregat river (Fig. 5).

Fig. 5. Llobregat river at Castellbell. June 2006

Effects of diuron and triclosan have been evaluated in microcosm studies (experimental channels) on biofilm communities (Fig. 6a, 6b) and in-vitro experiments with algal cultures of *Scenedesmus obliquus* have been used to address the effect of mixtures (Fig. 7). This multi-scale approach is appropriate to provide evidences of cause-effects relationships between toxic exposure and ecosystem damage.

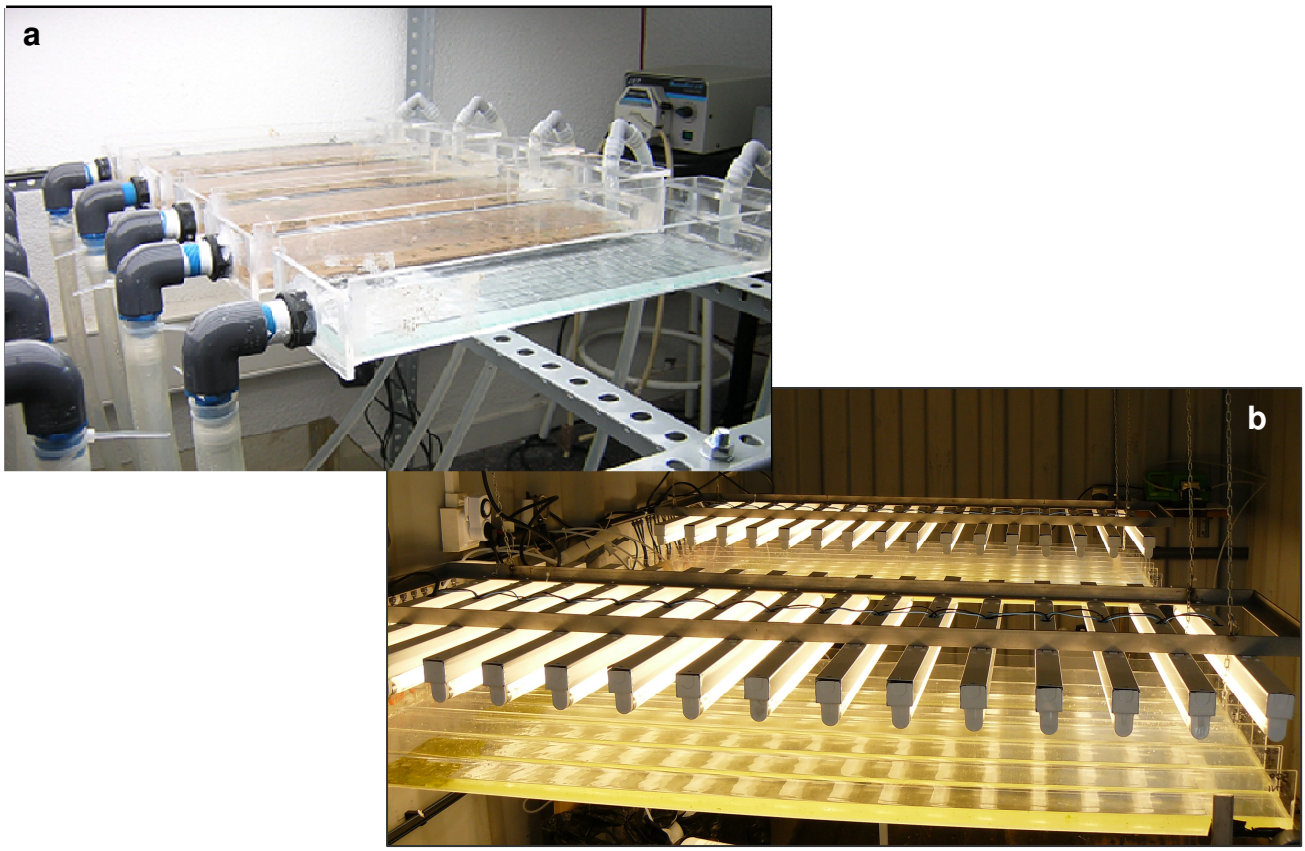

Fig. 6. Experimental channels used to evaluate the toxicity of the phenylurea herbicide diuron (a) and the antimicrobial agent triclosan (b) on biofilm communities.

Fig. 7 Algal cultures of *Scenedesmus obliquus* exposed to the phenylurea herbicide diuron during the mixture experiment.

Multi-endpoint approach in biofilm

Biofilm complexity provides a large panel of endpoints that can be used to detect the effect of toxicants. The endpoints range from function-based to structure-based and should account for a global status of biofilms, including their two most important compartments (algae and bacteria) (Bonnineau et al., 2010). The suitability of these endpoints for detecting the effects of toxicants depends on the potential effect of the toxicant(s) tested and on its mode of action. Acute effects may be easily detected by physiological endpoints. While some of these methods, e.g. photosynthesis, are specific, others, e.g. extracellular enzyme activity, are less specific. Selection of one endpoint or another may depend on the expected effect of the toxicant. Persistent or chronic effects should affect other biofilm endpoints, for example chlorophyll, or community composition. Among these, community composition should best reflect long-term effects of the toxicant(s), because the toxicant(s) may cause a shift from a sensitive to progressively tolerant community. Usually, however, community-composition-based approaches do not adequately reflect cause-effect relationships, and complementary analysis of the short-term, for example physiological, effects may be required (Sabater et al., 2007).

Use of biofilms for detection of the effects of toxicants cannot be reduced to simple tests, but implies the consideration of effects, and their interactions, in different biofilm compartments. Obtaining realistic results of the effects of chemicals on the fluvial ecosystem requires scaling up from physiological to structural responses. It has been shown that the combination between functional and structural endpoints may better reflect the adaptation of the community to a specific stressor (Tlili et al., 2008). This combination of approaches might enable detection of toxic substances affecting the “good ecological status” of fluvial systems.

A multi-endpoint approach has been used in this Thesis, covering both functional and structural aspects of the biofilm communities. The set of endpoints has been classified into four categories:

- Chlorophyll-*a* fluorescence measurements: photosynthetic efficiency (Yeff), photosynthetic capacity (Ymax), non-photochemical quenching (NPQ) and chlorophyll fluorescence (F).
- Extracellular enzyme activities: β-glucosidase, leucine-aminopeptidase and phosphatase.
- Biofilm structure: Chlorophyll-a content (Chl-a), Biovolume (BVL), Extracellular polysaccharide content (EPS), Bacterial abundance (distinguishing between live and dead cells), Diatom cell viability.
- Taxonomical composition of the community: diatoms and invertebrates.

The measurement of in vivo **chlorophyll-*a* fluorescence** of algae has been found to be one of the most sensitive tools for the rapid detection of compounds and environmental conditions that exhibit harmful effects on photosynthesis (Brack and Frank, 1998; Fai et al., 2007). Light energy absorbed by chlorophyll molecules in algae can be used to drive photosynthesis (photochemistry), can be dissipated as heat (i.e. excess energy), or it can be re-emitted as light (chlorophyll fluorescence). These three processes occur in competition, and any increase in the efficiency of one will result in a decrease in the yield of at least one of the other two. Hence, by measuring the yield of chlorophyll fluorescence, information about changes in the efficiency of photochemistry and heat dissipation can be obtained (Maxwell and Johnson, 2000). These endpoints based on photosynthetic activity are obviously adequate for toxicants affecting photosynthetic performance, either directly or indirectly. More recently, the multi-wavelenght excitation on pulse-amplified modulated (PAM) fluorimeters and deconvultion of the fluorescent signal from mixed samples has the potential to reveal the contribution of algal groups with different absorption spectra (Schreiber et al., 2002). Dorigo et al. (2001; 2004) noticed an increase of EC_{50} for river biofilms of the herbicides atrazine and isoproturon using photosynthetic efficiency as an endpoint. Similarly, Ivorra et

al. (2000) detected a reduction in photosynthetic efficiency in biofilms exposed to polluted stream water (Zn + Cd). Similar results have been obtained with copper (Guasch et al., 2002). Chronic effects of isoproturon, measured as chlorophyll-a fluorescence (F0) and photosynthetic capacity (Ymax) were detected by Schmitt-Jansen and Altenburger (2005) using periphyton communities in a microcosm study. Acute toxic effects of diuron have been detected as photosynthesis inhibition using PAM techniques by McClellan et al. (2008). In the same study, PAM measurements allowed to observe chronic effects of the herbicide in terms of changes in algal class composition. The energy that is dissipated in non-radiative processes to protect the photosynthetic apparatus (non photochemical quenching, NPQ) can also be indicative of toxic stress. During photosynthesis inhibition, NPQ increases in order to protect the photosynthetic apparatus from an excess of light reaching PSII that cannot be used for photosynthesis (Geoffroy et al., 2003). An inhibition of the NPQ is indicative of damage in the pigments where the NPQ takes place and has been observed with algal cultures exposed to metals (Juneau et al., 1999) and herbicides (Juneau et al., 2001; Fai et al., 2007). These techniques have been less applied to biofilm toxicity assessment, although Corcoll et al. (2010) reported a decrease of the NPQ mechanisms in natural periphyton communities chronically exposed to Zn. While most of the studies involving PAM techniques are performed with herbicides, studies involving other types of toxicants (i.e. emerging compounds) are less abundant. As an example, Bonnineau et al., (2010) assessed the toxicity of β-blockers to biofilm communities by analysing the sensitivity of the different groups of primary producers through their specific photosynthetic efficiencies.

Extracellular enzymatic activities are potentially useful for detecting the effects of toxicants on biofilm communities. Sensitivity and a direct relationship with organic matter make them relevant tools for assessing the toxicity of specific compounds. Exposure of a microbial community to a toxic compound may induce either an increase or a decrease in enzyme activity, depending on the possible relevance of the compounds as a carbon, nitrogen, or phosphorus source for microbial growth. Extracellular enzymatic activities have been used mainly in metal toxicity assessment, because of the interaction

of phosphate with metals. However, their use in studies with organic compounds is still rare. An increase of phosphatase activity after zinc exposure has been observed (Chapell and Goulder, 1994; Paulsson et al., 2002) and attributed to phosphorus depletion by precipitation of zinc phosphate and the related increase in phosphatase activity. Bonnineau et al. (2010) detected the effects of atenolol (pharmaceutical product) on the heterotrophic activity of biofilms by means of the extracellular enzyme activity leucine-aminopeptidase.

Biofilm structural endpoints may express the long term effect of toxicants on these communities. Chlorophyll-a content has commonly been used as a descriptor of biofilm biomass and effects of herbicides (Guasch et al., 2007) and heavy metals (Navarro et al., 2002) in natural biofilm communities. The protective role of EPS has been shown in studies of biocides (Samrakandi et al., 1997) and metals (Admiraal et al., 1999), where toxicants caused less damage in biofilms with higher EPS content. The biovolume of the cells has been mainly used in experiments with algal cultures (Lürling and Roessink, 2006) but less often applied in biofilm community toxicity assessment (Guasch et al. 2002). Within the biofilm structural endpoints, bacterial abundance (distinguishing between live and dead bacteria) and diatom cells viability have been more recently implemented in toxicological studies and, therefore, only a few examples are available in the literature. Bonnineau et al. (2010) found that bacterial mortality was significantly enhanced after exposure to β-blockers and Morin et al. (2010b) detected an increase of diatom mortality caused by the antimicrobial agent triclosan.

Communities react to toxicants by changing their **community composition**, usually favouring the most tolerant taxa. This effect might be detected at the class and order level and sometimes at the species level. Algae (diatoms) and invertebrate fauna are representatives of aquatic benthic communities inhabiting the sediment interphase, which makes them good indicators of the potential effects of pollutants (Muñoz et al., 2009). Changes in diatom species and invertebrates have been observed in laboratory experiments (Nebeker and Schuytema, 1998, Genter et al., 1987; Sabater et al., 2002; Gold et al., 2003) and in field studies. Sabater (2000) detected a

lasting effect on diatom communities after a mining spill accident. The diatom community composition was influenced by low concentrations of metals in a study performed by Guasch et al. (2009). Changes in the density of macroinvertebrate taxa have been detected between upstream and downstream zones of a river contaminated with endosulfan (Leonard et al., 1999). Muñoz et al. (2009) found a potential causal association between the concentrations of some anti-inflammatories and β-blockers and the abundance and biomass of several benthic invertebrates in a field study.

Multiple-stress approach in ecotoxicologial studies

Human activity is responsible for the entrance of many substances to the aquatic environment. Consequently, there is a great variety of scenarios, from one single contaminant or class of contaminants occurring at high concentrations (i.e. vineyard and mining areas) to others with a wide range of contaminants at low doses (i.e. WWTP-dominated rivers). In addition, aquatic systems are also affected by many disturbances such as changes in water flow, water temperature or nutrient concentration (Allan and Castillo, 2007). These multiple-stress situations include toxic stress (caused by toxic substances) and environmental stress (caused by the environmental conditions), their interaction to be considered in order to better predict environmental risk. Field studies include these situations with real exposures occurring directly in the field (Guasch et al., 2010). However, the amount of confounding factors interacting in the system complicates the assessment of causality (Culp et al., 2000). Multivariate statistical analyses have been used as a tool to assess the impact of toxicants on fluvial communities (Guasch et al., 2009; Muñoz et al., 2009). However, the absence of experimental approaches that confirm causality, and provide final links between chemical pollution and biological impairment hamper the conclusions that can be obtained. Therefore, an investigation that integrates experimental approaches at different scales is optimal for determining causation (Serra et al., 2010).

To transfer these complex scenarios to laboratory experiments is difficult, especially when various pollutants occur simultaneously. Experiments with

mixtures of toxicants are an attempt of reproducing the complexity of natural exposures. These experiments are usually performed with a reduced number of compounds which constitute an important step in mixture toxicity assessment but are still far from natural exposures. Moreover, most of these studies investigate the effects of mixtures of priority compounds (i.e. herbicides (Faust et al., 2001; Arrhenius et al., 2004), metals (Franklin et al., 2002) and pesticides (Deneer, 2000)), and information about the behaviour of mixtures composed by emerging compounds or the combination of priority and emerging compounds is still scarce.

Hypotheses

Based on the current knowledge in the ecotoxicology of priority and new emerging substances on fluvial ecosystems, the following hypotheses have been formulated in the present study:

- Pesticide pollution in rivers will influence the function and the structure of the benthic communities inhabiting these systems (adressed in chapter I).
- Exposure of fluvial biofilms to toxic substances will cause direct effects on their target organisms but also indirect effects on the non target ones, closely related in the biofilm matrix (addressed in chapters II and III).
- The joint toxicity of mixtures will be higher than single exposures due to the inter-activity of chemicals in producing a toxic response. This toxic response will be accurately predicted by the concepts used in mixture toxicity assessment (addressed in chapter IV).

Objectives

The present Thesis aims to investigate the effects that various priority and emerging compounds exert on fluvial communities, especially on biofilms. To achieve the main objective a multi-scale approach has been defined, including different levels of biological organization (from cultures to biofilm communities), different experimental scales (from microcosms to field studies), and different types of exposures (from single toxicants to mixtures).

The specific objectives are the following:

- To explore the cause-effect relationships between chemical pollution in fluvial systems and biological communities.
- To set up a specific methodology (derived from experimental channels) for the study of the effects of toxicants on biofilm communities at microcosms scale.
- To investigate the direct and indirect effects of priority and emerging compounds on the main biofilm components (algae and bacteria), by using a multi-endpoint approach.
- To assess the impact of chemical mixtures occurring in natural systems on algae as well as to test the applicability of the current models used for predictions of mixture toxicity.

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CHAPTER II

Primary and complex stressors in polluted Mediterranean rivers: Pesticide effects on biological communities

Ricart et al., 2010. Journal of Hydrology 383, 52-61

PRIMARY AND COMPLEX STRESSORS IN POLLUTED MEDITERRANEAN RIVERS: PESTICIDE EFFECTS ON BIOLOGICAL COMMUNITIES

ABSTRACT

We examined the presence of pesticides in the Llobregat river basin (Barcelona, Spain) and their effects on benthic biological communities (invertebrates and diatoms). The Llobregat river is one of Barcelona's major drinking water resources. It has been highly polluted by industrial, agricultural, and urban wastewaters, and-as a typical Mediterranean river-is regularly subjected to periodic floods and droughts. Water scarcity periods result in reduced water flow and dilution capacity, increasing the potential environmental risk of pollutants. Seven sites were selected, where we analysed the occurrence of 22 pesticides (belonging to the classes of triazines, organophosphates, phenylureas, anilides, chloroacetanilides, acidic herbicides and thiocarbamates) in the water and sediment, and the benthic community structure. Biofilm samples were taken to measure several metrics related to both the algal and bacterial components of fluvial biofilms.

Multivariate analyses revealed a potential relationship between triazine-type herbicides and the distribution of the diatom community, although no evidence of disruption in the invertebrate community distribution was found. Biofilm metrics were used as response variables rather than abundances of individual species to identify possible cause-effect relationships between pesticide pollution and biotic responses. Certain effects of organophosphates and phenylureas in both structural and functional aspects of the biofilm community were suggested, but the sensitivity of each metric to particular stressors must be assessed before we can confidently assign causality. Complemented with laboratory experiments, which are needed to confirm causality, this approach could be successfully incorporated into environmental risk assessments to better summarise biotic integrity and improve the ecological management.

INTRODUCTION

The levels of organic compounds found in surface waters have increased in the recent decades as a result of human activities. Of these organic compounds, pesticides are most commonly detected in flowing waters (Azevedo et al., 2000; Quintana et al., 2001, Nakamura and Daishima, 2005; Sáenz and Di Marzio, 2009). These compounds (insecticides, herbicides, fungicides, etc.) are mainly used for agricultural purposes. They enter the aquatic environment via runoff after being sprayed in agricultural fields and can potentially reach groundwater. They are also used in non-agricultural applications, such as weed control on railways, roads and golf courses (Planas et al., 1997), algaecides in paints, and protective agents in flat roof sealing (Rodríguez-Mozaz et al., 2004).

The contamination of water resources by pesticides has resulted in the publication of several regulatory documents. For example, the European Water Framework Directive, WFD, (Directive 2000/60/EC) requires a good ecological status for all European river systems by 2015. To achieve this goal, aquatic communities must be protected from chemical stress, which at the very least will require a progressive reduction in the influx of priority substances into European river systems.

Various pesticides are currently included in the list of priority substances (Decision 2455/2001/EC) and the European Union has recently established environmental quality standards (EQS), annual average (AA), and maximum allowable concentrations (MAC) for various priority pesticides (and other contaminants) in surface waters (European Parliament and Council, 2008). These EQS are very low for some compounds such as endosulfan (AA concentration of 5 ng/L in inland surface waters and 500 pg/L in other surface waters) but are less restrictive for other compounds such as alachlor, atrazine, diuron, and simazine, with AA concentrations of 0.3, 0.6, 0.2, and 1g/L, respectively, in both inland and other surface waters (Directive 2008/105/CE).

The WFD also stipulates that biological assessment must be an integral part both of water quality monitoring and of the evaluation of ecosystem health.

Benthic communities in rivers are continuously exposed to varying environmental conditions, which affect both community structure and function (Sabater et al., 2007). However, the diversity of the substances potentially

affecting these communities (e.g. nutrients, dissolved organic matter, and hazardous toxicants) and the variability of environmental conditions make it difficult to monitor the specific effects of certain toxicants and to differentiate them from the potential influences of other environmental parameters. Moreover, any disturbance in the river ecosystem might be buffered or enhanced by complex biological interactions (Geiszinger et al., 2009).

The Llobregat river is one of Barcelona's major drinking water resources (Catalonia, NE Spain). The major land use types in the study area (middle and lower sections of the river) are urban and industrial activities (38%) and farmlands (13%) (Muñoz et al., 2009). The Llobregat is highly polluted by industrial and urban wastewaters as well as by surface runoff from agricultural areas (Rodríguez-Mozaz et al., 2004). Nowadays, it receives inputs from various sewage treatment plants, which may be relevant during periods of water scarcity (Kuster et al., 2008a). These events result in reduced water flow and dilution capacity, increasing the potential environmental risk of pollutants to the immediate environment and potentially to the functioning of the entire ecosystem.

There has been a lot of research in recent decades aimed at developing methodological tools for bioassessment. Multivariate techniques (ter Braak and Verdonschot, 1995) have been widely used to assess the effects of pollution in aquatic ecosystems (Fore et al., 1996). These tools have been used to assess disturbance in the Llobregat and its tributary, the Anoia river, to determine potential relationships between the presence of pharmaceuticals and the structural composition of the biological communities (Muñoz et al., 2009).

The objective of the present work was to analyse the relevance of pesticides in the biological communities (benthic algae and invertebrate fauna) of a Mediterranean river basin, using multivariate analyses. Physical and chemical parameters were included in the data set, as well as the concentrations of 22 pesticides from seven chemical families found in water and sediment; both water and sediment can be sources of stress for benthic communities (Muñoz et al., 2009).

Several herbicides have been reported in the lower part of the Llobregat river (Planas et al., 1997; Lacorte et al., 1998; Kuster et al., 2008a), which could represent a toxicity threat to photosynthetic organisms. As micro-algae

comprise the largest fraction of biofilm biomass in rivers (Stevenson, 1996), we hypothesised that biofilms would be affected by herbicides. However, whether the pesticides can affect other biological groups, and to which degree, remains unknown. This question is particularly relevant in Mediterranean river systems due to their high hydrological and chemical variability. The respective effects of the environmental factors and pollutants in the community structure may be reflected in changes in the abundance of different taxa. Currently, this is assessed using multivariate techniques and various community metrics. Each metric represents a unique ecosystem attribute that responds to stress in a predictable way (Karr, 1993). The ecological condition of a site is assessed by considering different metrics (Plafkin et al., 1989). Both approaches have been followed in this study.

Our objectives were: (i) to identify the effects of pesticides in a typical Mediterranean basin, in this case the Llobregat, using invertebrate and diatom abundances as indicators; (ii) to identify indicative community metrics of these stressors; and finally, (iii) to compare the sensitivity of these two approaches with the aim of contributing to the improvement of ecological management systems.

MATERIALS AND METHODS

Study site and survey design

The Llobregat river is 156.5 km long (Tomàs and Sabater, 1985) and drains a catchment area of 4948 km^2. It has two main tributaries, the Cardener and the Anoia. The geological substratum of this river is mainly calcareous (Sabater et al., 1987). The mean annual discharge in the Llobregat River is 14 m^3/s, though monthly values range from <2 to 130 m^3/s. In dry years, the number of days below the average water flow ranges from 70-85%. Mostly in autumn, the torrential rain events can derive in catastrophic floods (exceptionally accounting for up to 1500-2000 m^3/s; Llasat et al., 2001).

Periodic floods and droughts have led to frequent morphological disturbances in the river bed and its banks. This is particularly true in the lower part of the Llobregat where the riparian vegetation has disappeared. The aquifers located

in the lower part of the basin are overexploited and since the river dries out every summer marine intrusion occurs into the aquifer. The ecological status of the Llobregat is also affected by the salt inputs deriving from the ancient salt mines of the Cardener watershed. This adds up to the high nutrient concentration and to the industrial and urban pollution reaching the river and the main tributary Anoia, causing a poor condition in the low part of the river.

There were seven sampling points selected along the watershed of the Llobregat. Four sites were established along the main course of the river (from its mid-to-lower part) and three sites from its tributary, the Anoia river (Fig. 1). The sites were selected in order to include a downstream pollution gradient. There were four samplings, which took place during two significant hydrological periods in the river system (spring and autumn 2005 and 2006). Water flow is usually low in spring (monthly average of 2.8-3.2 m^3/s during the study period) and higher in autumn (monthly average of 5.7-6.6 m^3/s).

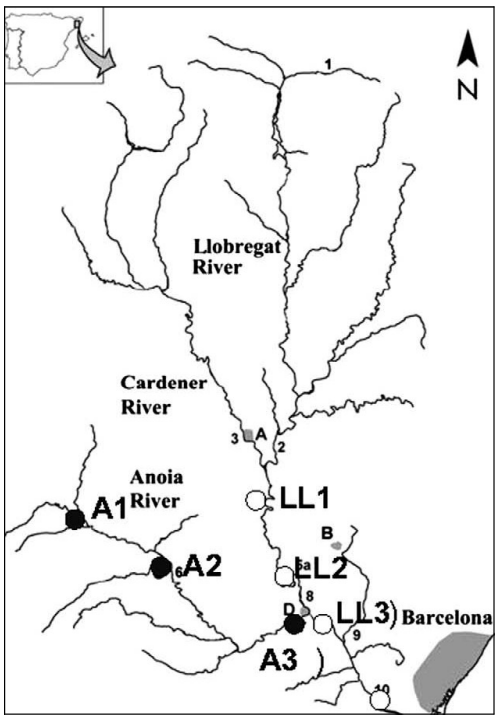

Fig. 1. Sampling sites at the Llobregat watershed. Sampling sites A1, A2, and A3 were located in the tributary Anoia River (A1: Jorba, A2: La Pobla de Claramunt, A3: Anoia River at Martorell). Sampling sites LL1, LL2, LL3, and LL4 were located along the main course of the Llobregat River (LL1: Castellbell, LL2: Abrera, LL3: Llobregat River at Martorell, LL4: Sant Joan Despí).

Physical and chemical parameters

Sampling parameters measured included oxygen (%), pH, conductivity, and temperature (WTW Meters, Weilheim, Germany), which were measured in the field during each sampling period. Water samples were collected in triplicate for nutrient analysis. Samples were filtered (Nylon Membrane Filters 0.2 µm, Whatman, Maidstone, UK) and frozen in the laboratory until analysis. Nitrate, sulphate, and chloride were determined by ion-chromatography (761 Compact IC, Metrohm, Herisau, Switzerland). Soluble reactive phosphorus (SRP) was determined according to Murphy-Riley's protocol (1992), while ammonium was measured following standard procedures (APHA, 1989).

Pesticides in water and sediment

A total of 22 pesticide compounds from seven chemical families were analysed in water and sediment. These families were triazines (deisopropylatrazine, desethylatrazine, simazine, cyanazine, atrazine and terbutylazine), organophosphates (fenitrothion, malathion, diazinon and dimethoate), phenylureas (chlortoluron, isoproturon, diuron and linuron), anilides (propanil), chloroacetanilides (alachlor and metolachlor), acidic herbicides (bentazone, MCPA, 2,4-D and mecoprop), and thiocarbamates (molinate).

Water samples were analysed by on-line solid phase extraction-liquid chromatography-electrospray-tandem mass spectrometry (SPE-LC-ESI-MS/MS) following previously described methods (Kampioti et al., 2005; Kuster et al., 2008b; Palma et al., 2009). In this method, water samples (5 mL) were preconcentrated with polymeric cartridges (HySphere Resin GP and PLRP-s from Spark Holland, Emmem, The Netherlands) and further LC-ESI-MS/MS analyses were performed using a hybrid triple quadrupole-linear ion trap mass spectrometer system (4000QTRAP from Applied Biosystems-Sciex, Foster City, CA). The reaction monitoring (SRM) mode was selected for this procedure. For analyte quantification and confirmation, two SRM transitions were monitored per compound. Six out of the twenty-two pesticides (propanil, fenitrothion 2,4-D, bentazone, MCPA, and mecoprop) were analysed in the negative ion (NI) mode (after preconcentration in HySphere Resin GP cartridges), and the remaining

sixteen compounds (triazines, phenylureas, anilines, organophosphates, and molinate) were analysed in the positive ion (PI) mode (after preconcentration in PLRP-s cartridges).

Sediment samples (5 g) were liophylised, homogenised, and sieved (125 µm) and were then extracted with a mixture of acetone/methanol (1:1, v/v) by pressurised liquid extraction (PLE) (temperature, 50 ºC; pressure, 1500 p.s.i.) using an accelerated solvent extraction system ASE 200 (Dionex, Sunnyvale, CA, USA). PLE extracts were dried under nitrogen and reconstituted in 20 mL water/methanol (95:5 acidified with formic acid until pH= 2-3). Further SPE clean-up of the extracts was performed in Carbograph Extract-Clean Columns (15 cc, 1000 mg) from Alltech Chromatography (Alltech, Deerfield, IL, USA). Pesticides measured in PI mode were eluted with 1 mL methanol followed by 8 mL dichloromethane:methanol (95:5); pesticides measured in NI mode were eluted with 10 mL of a mixture of dichloromethane:methanol (80:20) acidified with formic acid (pH 2-3). The purified extracts were blown down with nitrogen and reconstituted in 1 mL methanol for further LC-MS/MS analysis as above.

Both methodologies show satisfactory linearity (with correlation coefficients higher than 0.99), sensitivity (with method detection limits between 0.02 and 6.34 ng/L in water, and between 0.02 and 6.70 ng/g in sediments), accuracy, and repeatability (with recovery percentages above 70% and relative standard deviations below 20% for most compounds in both matrices).

If concentrations were below method detection limits, a value equal to half of the method detection limit was assigned to these data in the statistical analyses (Helsel, 1990).

Benthic communities

Biofilms growing on sediment (epipsammic biofilms) and on rocks (epilithic biofilms) were sampled. At each site, five rocks and five sediment samples were randomly collected. At the lower part of the Llobregat river (Sant Joan Despí) only sand was collected since the river bed was 100% covered by sediment. Epilithic biofilm samples were collected by scraping a known surface (1 or 4 cm^2) with a knife. Sediment samples (2-5 cm depth) were collected with a

polyvinyl sand corer, and sub-samples from the top sediment were collected by an untapped syringe, obtaining a final sediment volume of 1 mL per sample.

Diatom community composition

One replicate of epilithic biofilm (from each sampling site except in Sant Joan Despí, where only epipsammic biofilms were collected) was used for diatom identification and counting. Samples were cleaned, observed, and identified following the specifications of Tornés et al. (2007).

Biofilm metrics

Several biofilm metrics were analysed in both epilithic and epipsammic biofilms. Samples taken for autotrophic (chlorophyll extraction, in vivo fluorescence measurements, and extracellular polysaccharide content) and heterotrophic measurements (extracellular enzyme activities) were transported into the laboratory in a dark cool box. Samples for chlorophyll-a content and extracellular polysaccharide analyses were kept frozen until analysis.

Chlorophyll-a concentration. Three replicates per substratum (sediment and rocks) were used from each sampling site to determine the chlorophyll-a content. Extraction was done overnight in 90% acetone and analysed by spectrophotometry (Lambda UV/VIS spectrophotometer, Perkin-Elmer, Whaltham, Massachusetts, USA) following the Jeffrey and Humphrey method (1975).

Chlorophyll in vivo fluorescence measurements. The chlorophyll fluorescence emission was measured with the PhytoPAM (Pulse Amplitud Modulated) fluorometer (Heinz Walz GmbH), which uses a set of light-emitting diodes (LED) that excite chlorophyll fluorescence using four different wavelengths (470, 520, 645, and 665 nm). Five replicates for each substratum were analysed using this technique and all the measurements were based on the procedure described by Serra et al. (2009). The photosynthetic efficiency of photosystem II (PSII) (referred to as Yeff) and the photosynthetic capacity of PSII (referred to as Ymax) were estimated based on the fluorescence signal recorded at 665 nm and given as relative units of fluorescence. The minimum

fluorescence level of the dark adapted samples was used as an estimation of algal biomass. This estimation was based on the fluorescence recorded at the four different excitation wavelengths (F1 at 470 nm, F2 at 520 nm, F3 at 645 nm, and F4 at 665 nm). F1 is linked to the chlorophyll of green algae, whereas F2 is mostly related to that of diatoms. The F3 signal is related to cyanobacteria chlorophyll and the F4 signal is related to the chlorophyll of the whole algal community (Jakob et al., 2005). In this study, the ratio between F1 and F3 (green algae versus cyanobacteria) was calculated per each replicate and incorporated into the database.

Extracellular polysaccharide content (EPS). EPS extraction was done using a cation-exchange resin (Dowex Marathon C, Na^+ form, strongly acid, Sigma-Aldrich, St. Louis, Missouri, USA) following the procedure described by Romaní et al. (2008). After extraction, the extracellular polysaccharide content was determined following the methods described by Dubois et al. (1956). Three replicates of each substratum were analysed.

Extracellular enzyme activities. The extracellular enzyme activities of β-glucosidase (EC 3.2.1.21), leucine-aminopeptidase (EC 3.4.11.1), and phosphatase (EC 3.1.3.1-2) were measured following the methods of Romaní et al. (2001). Five replicates were used for each extracellular enzyme activity in each sampling site.

Invertebrate community

Five sediment samples were collected at each sampling site with a polyvinyl sand corer for invertebrate counting and identification, following the procedure described by Muñoz et al. (2009).

Data treatment

All the analyses were performed using the CANOCO software version 4.5 (ter Braak and Smilauer, 1998).

Relationship between diatom and invertebrate community structure and environmental conditions

Data from diatoms and invertebrates were used to determine the respective influence of pesticides and other environmental factors on their distribution. Invertebrate taxa with a relative proportion of >1% in at least two samples were included in the redundancy analysis (RDA) performed with those data. Diatom taxa accounting for at least more than 2% in two samples were included in a separate RDA. Taxa abundances were square-root transformed. The environmental dataset was reduced to fourteen variables, including pH, temperature, conductivity, NO_3^-, SO_4^{2-}, SRP, NH_4, and the concentrations of seven pesticides classes (Triazines, Organophosphates, Phenylureas, Acidic, Choroacetanilides, Thiocarbamates, and Anilides). Variables with a strong inter-correlation (oxygen, current velocity, Cl, Na, K, Ca, and Mg) were eliminated. Other than pH and dissolved oxygen (%), all environmental data were transformed by $\log_{10}(x+1)$ to reduce skewed distributions before further analysis.

The maximum gradient length for diatom and invertebrate data was determined using detrended correspondence analysis (DCA). The maximum amount of variation in the species data was 2.49 and 2.68 for the diatom and invertebrate data, respectively, indicating that linear methods would be appropriate (ter Braak and Smilauer, 2002). Consequently, we carried out various redundancy analyses (RDA) on both diatom and invertebrate data, whereby data were constrained by environmental variables. To avoid co-linearity, the variables were selected based on the inspection of variance inflation factors (VIF < 20) (ter Braak and Smilauer, 1998). Forward selection was used to reduce the environmental variables that significantly explained the distribution pattern of the diatoms and invertebrates at a cut-off point of p= 0.1. The significance of the RDA axes was assessed using the Monte Carlo permutation test (999

unrestricted permutations). Probabilities for multiple comparisons were corrected by applying the Bonferroni correction.

To separate the effects of pesticides from those of other chemical and physical variables on community distribution, the variance partitioning technique was applied. This technique enabled us to assess the fractions of the explained variance that are shared by two predictor variables, and to determine which of them could be uniquely attributed to each of them (Borcard et al., 1992). The explanatory variables were grouped into two subsets: (a) physical and chemical variables and (b) pesticides. The following sequences of RDAs and partial RDAs were performed for both datasets (invertebrate and diatom abundances): (a) RDA of the abundance data constrained with physical and chemical variables, (b) RDA of the abundance data constrained with pesticides, (c) partial RDA of the abundance data constrained with physical and chemical variables and using the pesticides as covariables and (d) partial RDA of the abundance data constrained with pesticides using the physical and chemical variables as covariables.

Relationship between biofilm metrics and environmental conditions

Metrics data were $\log_{10}(x+1)$ transformed before being included in the analysis. Once both datasets (metrics data obtained from epipsammic and epilithic biofilms) were submitted to the detrended correspondence analysis (DCA), the maximum length of the gradient (0.852 for cobbles samples and 0.833 for sediment samples) indicated that linear methods were also appropriate (ter Braak and Smilauer, 2002). Consequently, we carried out various redundancy analyses (RDA) in which metrics data obtained from epipsammic biofilms were constrained by environmental variables (including physical and chemical variables and pesticide concentrations in the sediment) and metrics data obtained from epilithic biofilms were constrained by environmental variables (including physical and chemical variables and pesticide concentrations in the water). Colinearity was defined based on variance inflation factors (VIF < 20) (ter Braak and Smilauer, 1998). Forward selection was used with a cut-off point of $p = 0.1$. The following steps were analogous to those described in the previous section.

To distinguish between the effects of pesticides on metric responses and those of other chemical and physical variables, a series of RDAs and partial RDAs were carried out following the variance partitioning technique (Borcard et al., 1992). These analyses were useful in evaluating whether these groups of variables were redundant or explained unique aspects of the metrics. The explanatory variables were grouped into two subsets: (a) physical and chemical variables and (b) pesticides. The following sequence of RDAs was performed: (a) RDA of the metrics matrix constrained by physical and chemical variables, (b) RDA of the metrics matrix constrained by pesticides, (c) partial RDA of the metrics matrix constrained by physical and chemical variables using the pesticides as covariables and (d) partial RDA of the metrics matrix constrained by pesticides using the physical and chemical variables as covariables.

RESULTS

Physical and chemical parameters

Water conductivity was high in most sites due to the presence of sulphates and chlorides resulting from the watershed lithology (Table 1). The salt mines around the Cardener (tributary of the Llobregat) and the large number of industrial facilities explain the high levels of salinity in the Llobregat. Our results show nutrient concentration to be high in most sampling sites (Table 1). High concentrations of soluble reactive phosphorus were found, especially in the Anoia river (A2) and at the mouth of the main course of the Llobregat river (LL3 and LL4). This is explained by the large amount of industrial activity and large population in the area (Barcelona city and its surroundings). Nitrate values were highest in the autumn, probably due to the increase in runoff after rain episodes (Table 1).

Site	pH	Cond (µS/cm)	NO_3 (mg/L)	SO_4 (mg/L)	SRP (µg/L)	NH_4 (mg/L)	Temperature (ºC)
A1	7.88	3142.5	6.82	630.05	30.38	0.29	13.88
	0.03	*112.8*	*2.45*	*229.43*	*7.46*	*0.14*	*0.92*
A2	7.66	3857.5	28.45	407.08	629.07	0.97	21.5
	0.08	*181.26*	*9.31*	*138.77*	*72.78*	*0.03*	*2.12*
A3	8.18	2240.75	5.72	281.77	335.82	0.48	20.05
	0.1	*135.7*	*1.89*	*101.82*	*34.78*	*0.17*	*2.69*
LL1	8.24	1460.5	6.33	106.97	213.23	0.43	18.65
	0.1	*27.02*	*2.62*	*33.60*	*21.88*	*0.24*	*2.54*
LL2	7.92	1674.5	7.52	111.18	187.15	1.24	20.28
	0.04	*76.25*	*1.72*	*34.22*	*23.99*	*0.8*	*2.2*
LL3	7.97	2144.5	8.87	278.98	493.12	1.07	20.13
	0.11	*399.14*	*3.14*	*29.35*	*111.64*	*0.46*	*1.96*
LL4	7.79	2765	8.04	420.32	429.48	0.54	20.6
	0.06	*158.35*	*2.07*	*213.98*	*143.06*	*0.13*	*2.61*
Autumn	7.90	2332.71	13.10	233.36	302.53	0.67	16.63
	0.08	*310.18*	*3.51*	*107.08*	*72.26*	*0.11*	*1.06*
Spring	7.99	2505.57	7.13	338.59	360.02	0.82	22.49
	0.10	*351.47*	*4.31*	*94.03*	*104.09*	*0.38*	*1.46*

Table 1. Physical and chemical parameters included in the analysis. Mean values (n=4) and standard error (in italics) of the four samplings are shown. The mean values obtained in autumn and spring (n = 14) are provided at the bottom of the table.

Pesticides in water and sediment

Twenty different compounds from seven families of pesticides were detected in the water (Table 2). Sites showing particularly high levels of certain pesticide classes were A1 (organophosphates and acidic herbicides), LL3 (chloroacetanilides and phenylureas), and LL4 (triazines and organophosphates) (Fig. 2). Concentrations of organophosphates and phenylureas were the highest in all the sampling sites (Table 2). From the organophosphates family, the presence of diazinon was highest, with a maximum of 785 ng/L. In the case of the phenylureas, linuron and diuron had the highest concentrations (up to 327 and 99.7 ng/L, respectively).

The concentration of pesticides in the sediment was lower than in water. In the sediment samples, 13 compounds from six families of pesticides were detected (Table 2), phenylureas and chloroacetanilides being the two with the highest levels. From the phenylurea group, diuron showed the highest concentration and from the chloroacetanilides group, metolachlor was found in the highest concentration. In the sediment, the following sites showed high levels of certain families of pesticides: A1 (acidic, anilide, and chloroacetanilides), LL2 (chloroacetanilides), and LL4 (phenylureas) (Fig. 2).

		Water		Sediment	
		Min	Max	Min	Max
Triazines	Atrazine	0.05	1.08	0.03	0.86
	Simazine	0.14	53.60	0.81	0.81
	Cyanazine	bdl	bdl	bdl	bdl
	Desethylatrazine	27.10	27.10	bdl	bdl
	Terbuthylazine	0.13	21.90	bdl	bdl
	Deisopropylatrazine	0.10	14.40	0.62	0.70
Organophosphates	Diazinon	0.83	785.00	0.09	1.29
	Dimethoate	0.65	87.80	bdl	bdl
	Fenitrothion	0.90	3.43	1.51	3.00
	Malathion	bdl	bdl	bdl	bdl
Phenylureas	Diuron	0.40	99.70	0.16	31.80
	Isoproturon	0.46	7.85	0.08	0.73
	Linuron	0.22	327.00	bdl	bdl
	Chlortoluron	0.48	3.12	bdl	bdl
Acidic	Mecoprop	0.41	7.02	0.23	1.79
	2.4-D	0.34	12.60	0.16	6.99
	Bentazone	0.71	9.60	0.17	8.59
	MCPA	0.11	67.40	0.46	1.96
Chloroacetanilides	Alachlor	2.17	17.10	bdl	bdl
	Metolachlor	7.37	7.37	7.59	43.20
Thiocarbamate	Molinate	0.96	3.78	bdl	bdl
Anilide	Propanil	0.03	0.39	0.11	24.70

Table 2. Minimum and maximum pesticide levels analysed in the water and sediment of the Anoia and Llobregat during the study period (n = 4). Values are expressed in ng/L (water) and ng/g (sediment).

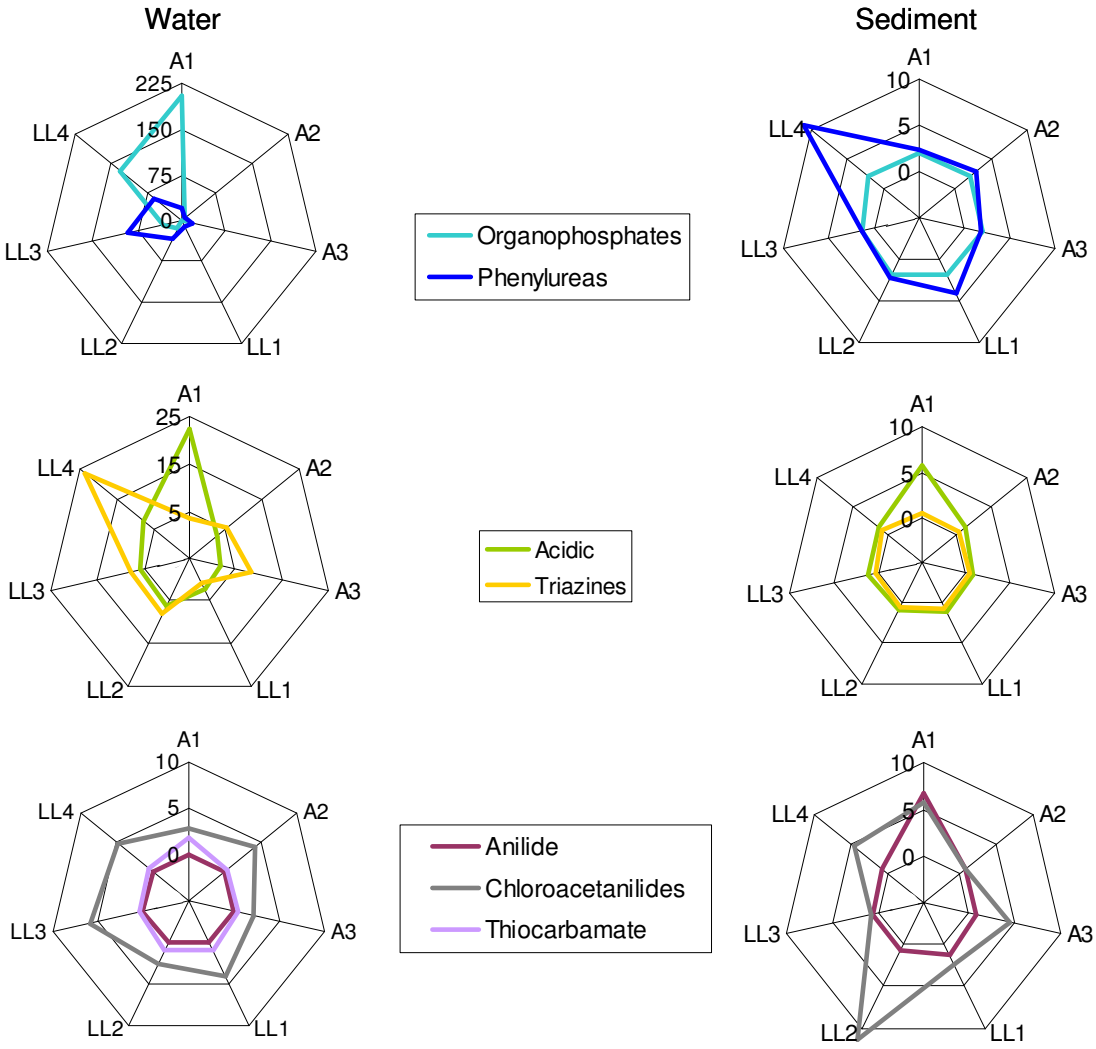

Fig. 2. Concentration of the pesticide families in water (ng/L) and sediment (ng/g) samples of the studied sites, grouped by class. Values show the mean of the four samplings.

Relationships between diatom and invertebrate community structure and environmental conditions

The RDA analysis for invertebrate fauna showed that temperature, conductivity, and NO_3^- significantly influenced the invertebrate community structure (Table 3). This analysis explains 42.4% of the variance (Fig. 3). The first axis shows the distribution of sites along a joint gradient of conductivity and NO_3^-. The abundance of *Chironomus* spp. and *Tanypodinae* were closely associated with sites with high conductivity and high NO_3^- content, particularly sites A2 and LL4. The second axis shows a temperature gradient. Abundances of *Stictochironomus spp*. and *Prodiamesa olivacea* were related to the coolest waters, which is characteristic of the upper part of the Anoia river (A1). The taxa *Caenis spp*. and *Polypedium spp.* were mainly found at the opposite end of the plot where temperature was higher. The rest of the taxa were related to lower conductivity levels. The presence of pesticides did not significantly contribute to the ordination of the faunal community.

Invertebrates		
	Axis 1	Axis 2
Temperature	0.546	- 0.909
Conductivity	0.638	0.545
NO_3	0.552	- 0.121
Eigenvalues	0.381	0.028
Diatoms		
	Axis 1	Axis 2
SRP	-0.912	-0.429
Triazines	-0.313	0.958
Eigenvalues	0.098	0.048

Table 3. Correlation between environmental variables and pesticides With the axes of the respective Redundancy Analysis (RDA) carried out with invertebrate and diatom abundance data.

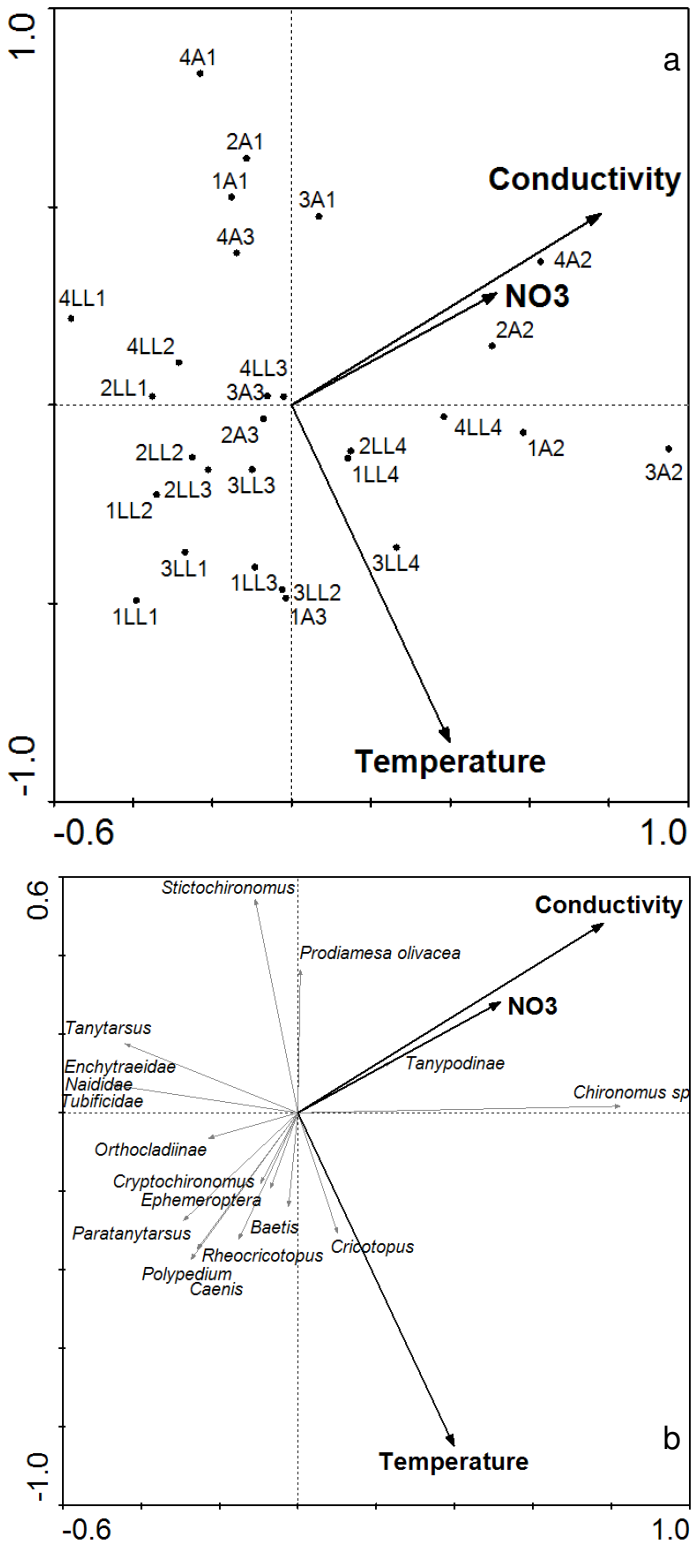

Fig. 3. Biplot based on redundancy analysis of invertebrate community in the Anoia (A) and Llobregat (LL): (a) ordination of sampling sites and (b) ordination of invertebrate taxa. Sampling period is also indicated: (1) June 2005; (2) October 2005; (3) June 2006; (4) October 2006.

The RDA analysis performed with the diatom taxa revealed that pesticides also influence the distribution of the community. Triazines and soluble reactive phosphorus significantly correlated with the RDA axes (Table 3), which explains 14.6% of the diatom community distribution (Fig. 4).

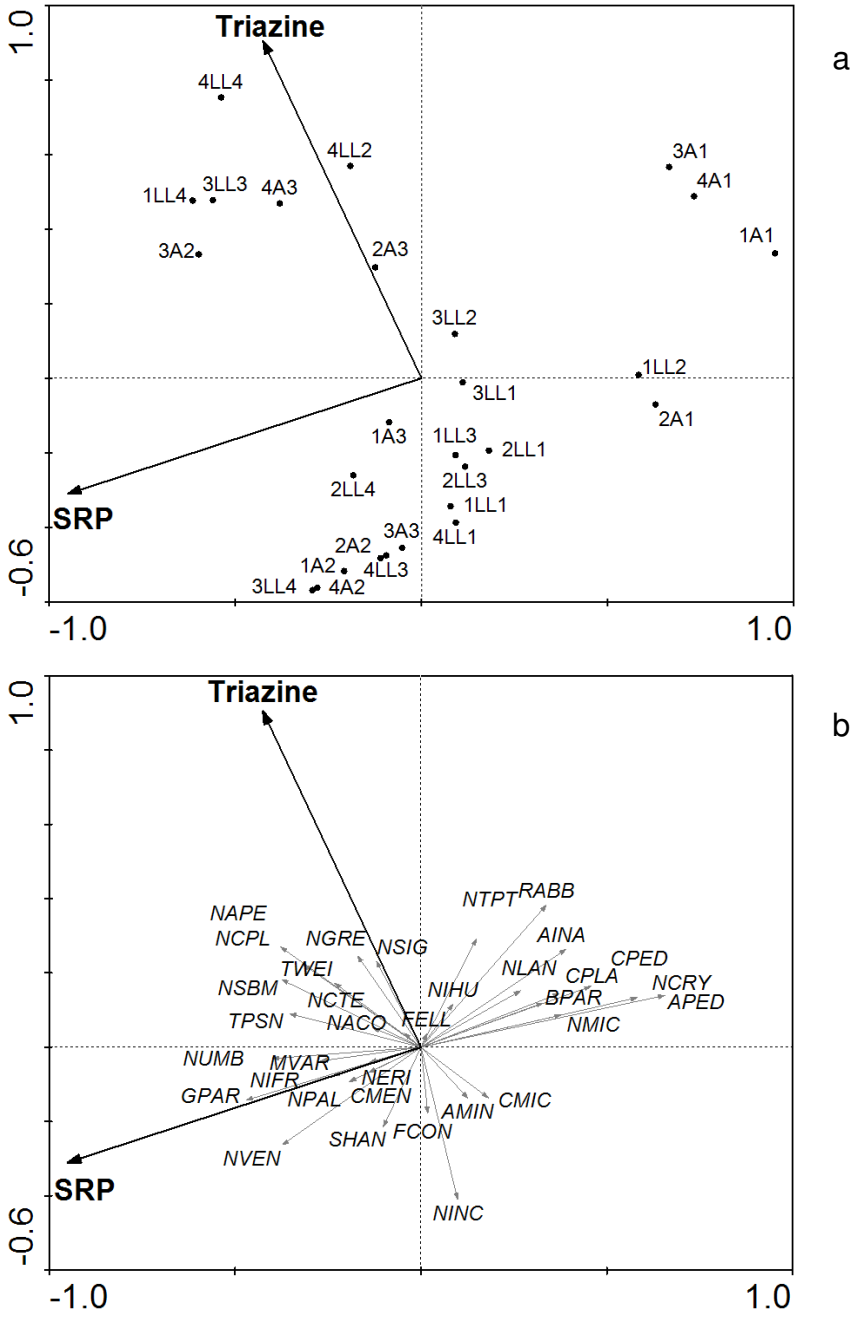

Fig. 4. Biplot based on redundancy analysis of diatom community in the Anoia (A) and Llobregat (LL): (a) ordination of sampling sites and (b) ordination of diatom species. Sampling period is also indicated: (1) June 2005; (2) October 2005; (3) June 2006; (4) October 2006.

Abbreviations used to identify the diatom taxa are shown in Table 4.

Code	Diatom taxa
AINA	*Amphora inariensis Krammer*
AMIN	*Achnanthes minutissima Kutzing v.minutissima Kutzing (Achnanthidium)*
APED	*Amphora pediculus (Kutzing) Grunow*
BPAR	*Bacillaria paradoxa Gmelin*
CMEN	*Cyclotella meneghiniana Kutzing*
CMIC	*Cymbella microcephala Grunow*
CPED	*Cocconeis pediculus Ehrenberg*
CPLA	*Cocconeis placentula Ehrenberg var. placentula*
FCON	*Fragilaria construens (Ehr.) Grunow f.construens (Staurosira)*
FELL	*Fragilaria elliptica Schumann (Staurosira)*
GPAR	*Gomphonema parvulum (Kützing) Kützing var. parvulum f. parvulum*
MVAR	*Melosira varians Agardh*
NACO	*Navicula accomoda Hustedt*
NAPE	*Navicula atomus (Kutz.) Grunow var.permitis (Hustedt) Lange-Bertalot*
NCPL	*Nitzschia capitellata Hustedt in A.Schmidt & al.*
NCRY	*Navicula cryptocephala Kutzing*
NCTE	*Navicula cryptotenella Lange-Bertalot*
NERI	*Navicula erifuga Lange-Bertalot*
NGRE	*Navicula gregaria Donkin*
NIFR	*Nitzschia frustulum(Kutzing)Grunow var.frustulum*
NIHU	*Nitzschia hungarica Grunow*
NINC	*Nitzschia inconspicua Grunow*
NLAN	*Navicula lanceolata (Agardh) Ehrenberg*
NMIC	*Nitzschia microcephala Grunow in Cleve & Moller*
NPAL	*Nitzschia palea (Kutzing) W.Smith*
NSBM	*Navicula subminuscula Manguin*
NSIG	*Nitzschia sigma(Kutzing)W.M.Smith*
NTPT	*Navicula tripunctata (O.F.Müller) Bory*
NUMB	*Nitzschia umbonata(Ehrenberg)Lange-Bertalot*
NVEN	*Navicula veneta Kutzing*
RABB	*Rhoicosphenia abbreviata (C.Agardh) Lange-Bertalot*
SHAN	*Stephanodiscus hantzschii Grunow in Cl. & Grun. 1880*
TPSN	*Thalassiosira pseudonana Hasle et Heimdal*
TWEI	*Thalassiosira weissflogii (Grunow) Fryxell & Hasle*

Table 4. Abbreviations used for the identification of the diatom taxa included in the analysis.

The first axis correlated mainly with the SRP and reflected a gradient of eutrophication, in which the downstream sites of both rivers were the most related to the axis. Diatom taxa associated with higher SRP levels were *Gomphonema parvulum* (GPAR), *Nitzschia umbonata* (NUMB), *Navicula atomus var. permitis* (NAPE), *Navicula subminuscula* (NSBM), and *Navicula veneta* (NVEN). In contrast, *Amphora pediculus* (APED) and *Navicula cryptocephala* (NCRY) were dominant in sites with lower SRP content.

The second axis correlated with triazine-type herbicide concentrations in the water. The highest concentrations of these compounds were found in sites A3, LL2, LL3, and LL4. Species *Rhoicosphenia abbreviata* (RABB), *Navicula tripunctata* (NTPT), and *Navicula atomus var. permitis* (NAPE) were most closely associated to the presence of this family of herbicide-acting compounds.

We did not establish the potential contribution of each of the two sets of variables (pesticides and physical and chemical variables) on the distribution of both invertebrates and diatoms. In both cases the second redundancy analysis did not show any significance after the Monte Carlo test.

Relationship between biofilm metrics and environmental conditions

It was not possible to determine the potential contribution of each of the two sets of variables (pesticides and physical and chemical variables) to the variance of the epilithic biofilm metrics because the second partial RDA did not reveal any significance after the Monte Carlo test. However the covariance explained by the two sets of variables obtained from the biological metrics in the epipsammic biofilms accounted for 50%. In terms of physical and chemical variables, temperature and SO_4^{2-} had a significant effect on the metric responses, explaining 17% of the total variance. Pesticides alone accounted for 6.1% of the explained variation, with organophosphates and phenylureas being the statistically significant variables. Shared variance represented 26.9%.

The percentage of variance explained by pesticides and physical and chemical variables differed between the biofilm metrics (Table 5). Chlorophyll-a, photosynthetic efficiency, and photosynthetic capacity responses were explained mainly by the presence of pesticides. Pesticides explained 91.57% of variance of the chlorophyll-a response. Extracellular enzymatic activities were

determined mainly by physical and chemical variables. Up to 97.43% was explained by these variables in the leucine-aminopeptidase, and ca. 90% in the other two activities (Table 5). The F1/F3 ratio and EPS were influenced by the two sets of variables.

	Fraction of total variance		Fraction of explained variance (%)	
Biofilm metric	Physical and chemical variables	Pesticides	Physical and chemical variables	Pesticides
Chl-a	2.36	**25.63**	8.43	**91.57**
EPS	6.14	7.05	46.55	53.45
Ymax	2.57	8.98	22.25	**77.75**
Yeff	7.68	**11.98**	39.06	**60.94**
F1/F3	**14.47**	**17.71**	44.97	55.03
Glucosidase	**23.39**	2.27	**91.15**	8.85
Peptidase	**22.33**	0.59	**97.43**	2.57
Phosphatase	**21.04**	2.83	**88.14**	11.86

Table 5. Results of the partial Redundancy Analysis (RDA) using the epipsammic biofilm metrics, pesticides, and physical and chemical variables. On the left the fraction of total variance is shown; bold is used to indicate the total variance higher than 10%. On the right, the fraction of explained variance of each variable amounted to percentage is shown; bold is used to indicate values that were clearly biased towards certain group of variables.

DISCUSSION

A total of 20 pesticides were detected in the lower part of the Llobregat river at levels similar to or higher than those reported in the literature (Planas et al., 1997; Quintana et al., 2001; Kitada et al., 2008; Kuster et al., 2008a). These contaminants were triazines, phenylureas, acidics, anilides, chloroacetanilides, and thiocarbamates, which act as herbicides, and organophosphates, which are insecticides. In water, the concentrations of organophosphates and phenylureas were highest, whereas in sediment chloroacetanilides and phenylureas were most abundant. Of the twenty-two pesticides investigated, five are classified as priority substances in the EU in the field of water policy and are subject to environmental quality standards in surface waters (European Parliament and

Council, 2008). These pesticides are alachlor, diuron, isoproturon, atrazine, and simazine. However, the maximum admissible concentrations (MAC) for these compounds in inland surface waters (0.7, 1.8, 1.0, 2.0, and 4 µg/L, respectively) are much higher than those detected in the Llobregat.

The same European Directive (2008/105/EC) includes a list of substances subject to review for possible identification as priority substances or priority hazardous substances. This list contains two compounds which were investigated in the present study: bentazon and mecoprop. Environmental quality standards for these compounds remain undefined.

In the main course of the Llobregat river, the most polluted sites in terms of total concentration of pesticides were LL3 (Martorell) and LL4 (the mouth of the river located at Sant Joan Despí). In these two sites, high nutrient concentrations seemed to appear in relation to high pesticide concentrations. Other pollutants such as analgesics, anti-inflammatories, lipid regulators, and antibiotics were also found in high concentrations, particularly at the lower section of the two rivers (Muñoz et al., 2009). The fact that other types of toxicants co-occur in the river with pesticides, suggests that experimental approaches to investigate the response of the communities submitted to a toxicant mixtures are needed, as well as the evaluation of their persistence in aqueous or sediment matrix. Furthermore, the Llobregat river suffers extreme flow fluctuations due to its Mediterranean character and the exploitation of its resources (Sabater and Tockner, 2009). The combined environmental threat from pollution, high nutrient concentrations, and water scarcity in the Llobregat complicates the assessment of its ecological status. Consequently, determining the impact of any specific stressor is a complex process, as is discussed by Culp et al. (2000). Under these circumstances, multivariate statistical techniques are useful in determining spatial and temporal relationships between stressors and their effects along a gradient (Muñoz et al., 2009).

In the present study, the multivariate analyses revealed that although the diatom community was affected by the presence of pesticides, the benthic invertebrate distribution was not influenced. Indeed some authors have reported effects of herbicides on invertebrates but only at high non-realistic concentrations (e.g. for diuron, in Nebeker and Schuytema, 1998, and for atrazine in Solomon et al., 1996). The fact that fauna and primary producers

differed in their response to pesticides can be explained in that the detected compounds work differently depending on their target organism (Duke, 1990; DeLorenzo et al., 2001). Of the 20 chemicals found, 15% are insecticide-acting compounds and the rest are herbicides, which are nearly always found in higher concentrations than insecticides. Benthic algae share similarities with the target organisms of herbicides (weeds) and can therefore be easily affected by herbicides (Dorigo et al., 2004).

Triazine-type herbicides, especially atrazine, act as a PSII inhibitor on algae (Van Rensen, 1989). Short-term physiological tests have shown that atrazine levels of between 86.3 µg/L and 172.6 µg/L can inhibit photosynthesis by up to 50% (Guasch et al., 1997). In another study, Guasch et al. (2007) found that chlorophyll-a content was reduced by up to 40% after exposure to 100 µg/L of atrazine over a period of three weeks (Guasch et al., 2007). Various studies have reported on the effects of herbicides on benthic communities. Acting in mixture, tryazine-type herbicides can cooperate to produce a severe joint effect (Faust et al., 2001). Although triazines played a relevant role in structuring diatom assemblages in the polluted sites of the Llobregat, the multivariate analyses revealed that other factors such as nutrient enrichment also contribute to the distribution of these communities (Sabater et al., 1987). The combination of soluble reactive phosphorus and triazine herbicides explained 14.6% of the total variance. SRP defined an enrichment gradient and determined the presence of several taxa tolerant to nutrient enrichment (Kovács et al., 2006; Tornés et al., 2007). Guasch et al. (1998) showed that in rivers across Europe, the presence of both atrazine and nutrients were most influential in the distribution of periphyton communities (Guasch et al., 1998). Though the diatom community was influenced by pesticides and phosphorus, only a small portion of the variance could be explained. Indeed, it has been shown that as well as nutrient levels other environmental factors governing diatom assemblages are associated with a variety of physical and chemical characteristics (Pan et al., 1996; Potatova and Charles, 2002) and probably that diminishes the predictability of the detected variables.

In the present study, the metrics obtained from epipsammic biofilms complemented the results based on diatom community composition. The variance conjointly explained by the two sets of explanatory variables was 50%

and it was possible to carry out a variance partitioning analysis. Water temperature and SO_4^{2-} content were the most statistically significant environmental variables (17% of the variance), while a smaller proportion of the variance was explained by organophosphates and phenylureas (6.1%).

This tool has been used in some studies to assess the effect of toxicants in benthic communities. Guasch et al. (2009) analysed the effects of metal pollution on diatom communities and determined that the best results were found when both water and biofilm metal concentrations were included in the analysis. Rogers et al. (2002) determined the influence of metals on the biological condition of the invertebrate communities as an indicator of the ecological status of the area.

In this study, the three extracellular enzymatic activities had the strongest relationship with the physical and chemical variables. The metrics most responsive to the presence of pesticides were chlorophyll-a, photosynthetic capacity, and photosynthetic efficiency. These metrics encompass both structural and functional attributes of the biofilm. Several studies have demonstrated that some of these metrics are affected by pesticides. Torres and O'Flaherty (1976) reported that 1 µg/L of malathion (organophosphate insecticide) had a 100% inhibitory effect on chlorophyll production in *Stigeoclonium*, *Tribonema*, and *Vaucheria*. Other authors reported that dimethoate, another organophosphate insecticide, completely inhibited *Scenedesmus incrassatulus* growth at concentrations above 0.075 mg/L (Jampani and Kumari, 1988). Diuron (phenylurea herbicide) has been shown to cause a drop in photosynthesis in algal communities at concentration of 1.5 µg/L (Ricart et al., 2009). McClellan et al. (2008) reported EC_{50} values with diuron concentrations of between 2.6 and 15.2 µg/L, using photosynthesis as an endpoint.

Although a range of periphyton metrics for the diagnosis of cause-effect relationships between stressors and biofilm structure and function have been reported using laboratory experiments (Schneider et al., 1995), field approaches are rare. Fore and Grafe (2002) included several diatom metrics in a multimetric index (River Diatom Index, RDI). The RDI could reliably detect three different categories of biological condition in large rivers in Idaho, USA. Griffith et al. (2002) compared the sensitivity of diatom species abundances with that of

community metrics for periphyton assemblages (diatom species richness, non–diatom-genera richness, total number of algal cells, etc.). While species abundance was sensitive to the effects of nutrients, substrates, and riparian vegetation, the periphyton metrics were also sensitive to toxicological effects associated with metals.

The effective detection of primary stressors requires a selection of metrics that includes functional and structural biological attributes as well as different biological levels of organisation, which should encompass a wide range of responses to many types of disturbances. As noted by Carlisle and Clements (1999), efforts to identify ecologically-relevant endpoints require systematic testing with toxicants and mixtures of known metric sensibilities to a variety of ecosystem stressors. Despite the fact that laboratory experiments can often lack ecological relevance, which does not allow for extrapolation of the results to natural systems, they are crucial in attribute the effects noticeable at ecosystem level to specific stressors.

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CHAPTER III

Effects of low concentrations of the phenylurea herbicide diuron on biofilm algae and bacteria

Ricart et al., 2009. Chemosphere 76, 1391-1401

EFFECTS OF LOW CONCENTRATIONS OF THE PHENYLUREA HERBICIDE DIURON ON BIOFILM ALGAE AND BACTERIA

ABSTRACT

A system of recirculating channels was used in this study to examine the long-term effects (29 d) of environmentally realistic concentrations of the herbicide diuron (from 0.07 to 7 µg/L) on biofilm communities. The autotrophic activity of biofilms was affected by this herbicide, as reflected by a marked decrease in the photosynthetic efficiency. Diuron exposure also increased chlorophyll-a content and reduced the biovolume of diatom taxa at low concentrations. The effects on bacteria were also remarkable. Bacterial abundance was reduced after a week of exposure to the herbicide at a range of concentrations. Effects were on the number of live bacteria and on the increase in the leucine-aminopeptidase activity. It is suggested that inputs of herbicides to the river ecosystem at low concentrations may cause a chain of effects in the biofilm, which include inhibitory effects on algae but also indirect effects on the relationships between biofilm components.

INTRODUCTION

Herbicides used for crop protection enter the aquatic ecosystem via runoff, leaching, spray drift or accidental spills and have become one of the most regularly detected substances in surface waters. The herbicide diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is included in the list of priority pollutants of the EU Water Framework Directive (European Commission, 2000). Diuron inhibits photosynthesis via blockage of the electron transport in the photosystem II (PSII) (Van Rensen, 1989), although high diuron concentrations inhibit photosystem I (PSI) and even cause the photodestruction of pigments (Ridley and Horton, 1984). Several studies have reported the presence of diuron in surface waters. Blanchoud et al. (2004) found more than 0.1 µg/L of the herbicide in all of the study sites in two semi-urban catchments. Rodríguez-Mozaz et al. (2004) detected average diuron concentrations of 0.239 µg/L in the lower part of the River Llobregat (NE Spain). Diuron has also been found in

samples from a pilot study in rivers in Portugal (Azevedo et al., 2000), where concentrations ranged from 0.01 to 1.24 µg/L.

PSII inhibitors are expected to have direct effects on algae, and ecotoxicological assessments commonly address the direct effects of herbicides on primary producers. Molander and Blanck (1992) detected photosynthesis inhibition in marine phytoplankton communities at 0.7 µg/L of diuron. Chronic effects on freshwater periphyton were reported by McClellan et al. (2008), who found changes in biomass and community composition after exposure to 0.08-2 µg/L of diuron for three months. Acute toxicity tests reported EC_{50} values that ranged from 4 to 9 µg/L, using photosynthesis as an endpoint. Knowledge about whether these toxicants affect other groups of organisms by altering the relationship between the different components of the biofilm is still relatively limited. It has been recently shown that bacterial community diversity can be affected by diuron (Pesce et al., 2006; Tlili et al., 2008). Shifts in the bacterial community composition (Pesce et al., 2008) were related to direct toxic effects of diuron (effects on sensitive species, or development of species able to degrade diuron), as well as to trophic links between algae and bacteria.

Biofilms comprise a combination of phototrophic and heterotrophic microorganisms embedded in a mucilage matrix (Lock, 1993). Interactions between the living components within the biofilm characterize the community structure and function. In well-illuminated environments, microalgae make up the greatest fraction of the biofilm biomass, though bacteria also account for a large amount. These two main components of biofilms interact both positively and negatively (Rier and Stevenson, 2002; Sabater et al., 2006). While these interactions have been studied extensively, the effect of toxicants has received little attention.

In rivers and streams, biofilms are biological layers inhabiting the sediment interface and in contact with the flowing water. Since both water and sediments can act as sources of pollutants for benthic communities (Muñoz et al., 2009), biofilms are the ones being first affects, and they can therefore be used to detect the early effects on the ecosystem (Sabater et al., 2007).

Our main objective was to examine the effect of realistic concentrations of diuron at biofilm community level, when submitted to the entrance of pulses of

herbicide that occurs in natural systems. Specific objectives were to study the effects of the herbicide between species of the same trophic level (algae), but also to examine these effects among different trophic levels (algae and bacteria). Both the characterization of the overall exposure and a multi biomarker approach allowed us to study the effect in the algal (target) and the bacterial (non-target) components of biofilms, as well as on their biological interactions. We hypothesised that biofilm response would be dose-dependant for direct effects, allowing us to estimate EC_{50} values for those endpoints who were directly affected by the presence of the herbicide. However, a non dose-dependant response was expected for indirect effects, motivated mainly by changes in the competitive relations within the biofilm and not by the specific concentration of the toxicant. The experiment was carried out with a biofilm growing on high water conductivity. Tests using communities derived from natural ecosystems can simulate the ecology of a natural system (Cairns and Niederlehner, 1987) and lead to more conclusive analysis. Many river systems in calcareous environments are affected by extremely high alkalinity and conductivity. This situation is reinforced in Mediterranean rivers, where low water flow results in reduced dilution ability. A paradigmatic example of this confluence of circumstances is the River Llobregat (Sabater et al., 1987). This water course is highly polluted by surface runoff from agricultural areas and by industrial and urban wastewaters. Furthermore, the occurrence of natural salt formations and the corresponding mining exploitations in the basin have increased the salinity of its water.

MATERIALS AND METHODS

A chronic toxicity test (29 d) was carried out using artificial channels. These types of mesocosms provide a logistically feasible and easily replicated tool for ecosystem research under controlled conditions (Navarro et al., 2000). The effects of the herbicide on young (few days) as well as on mature biofilms (several weeks older) were examined.

The mesocosm setup

The experiment was conducted using ten rectangular perspex recirculating channels (40 cm long x 10 cm wide). The bottom of each channel was covered with sandblasted glass substrata (1.2 x 1.2 cm each). Biofilm colonisation was achieved by introducing an aliquot of the microbenthic community obtained from a reference site in the River Llobregat (the Anoia River at Jorba, UTM Coordinates X 378982; Y 4606241). The biofilms were collected by scraping the surface of five cobbles chosen at random from the riverbed. Once in the laboratory, the aliquot was homogeneously distributed and allowed to adapt in the channels for 7 d at a pre-established water velocity. Water used to fill the channels was pumped from the respective aquaria (4.5 L) using soft silicone tubes. The temperature of the system (17ºC) was controlled by placing the aquaria in a refrigerated water bath.

Four of the channels were used as controls, one of which was established as an abiotic control (without biofilm). The remaining channels received increasing concentrations of diuron (0.07, 0.2, 0.7, 2, 5 and 7 µg/L). Herbicide addition followed exponential concentrations that allowed estimation of EC_{50} values and comparison between early effects (days 1 and 8) and later effects (days 22 and 29) of the compound. Replicate control channels (n=3) were also used to monitor temporal changes in the non-exposed biofilm.

During the experiment, the water used to feed the channels was renewed twice a week from the reference site. Nutrients (addition of a solution of KH_2PO_4 in each aquarium to obtain a final concentration of 80 µg/L PO_4) and toxicant (when appropriate) were added at that time to avoid their potential depletion in the channels. Water depth in the channels ranged between 1.5 and 2 cm and the flow rate was maintained at 1.5 L/min. Photosynthetically available radiation was provided by fluorescent tubes (L 36W/965 Biolux, OSRAM, Munich, Germany). The irradiance level was 120 µE/m^2 s and the light regime was 12:12 h light:dark.

Physical-chemical parameters

Water temperature, conductivity, pH and dissolved oxygen were measured with appropriate probes (WTW METERS, Weilheim, Germany) in each channel at each water change. Light was measured twice a week with an underwater cell (LI-COR Inc., Lincoln, Nebraska, USA) placed above the colonised glass substrata. Water samples were collected for nutrient content from three channels in triplicate before and after water renewals. All the water samples were filtered (Nylon Membrane Filters 0.2µm, WHATMAN, Maidstone, UK) prior to their analysis. Ammonium was determined following standard methods (American Public Health Association, 1989). Soluble reactive phosphate was measured following Murphy and Riley (1992) and nitrate, sulphate and chloride were determined by ion-chromatography (761 Compact IC, METROHM, Herisau, Switzerland).

Diuron analysis

Water samples for measuring diuron concentration were taken from three of the channels on each water change, both before and after water renewals. Diuron analysis in these samples was performed by on-line solid phase extraction-liquid chromatography-electrospray-tandem mass spectrometry (SPE-LC-ESI-MS/MS) following the method described in Kampioti et al. (2005). This was a fully automated on-line trace analysis, where enrichment was performed with an automated on-line SPE sample processor Prospekt-2 (SPARK HOLLAND, Emmen, the Netherlands) by passing 5 mL of the samples and aqueous standards through previously conditioned PLRP-s cartridges (SPARK HOLLAND, Emmen, the Netherlands). Elution of the trapped compound to the LC-MS/MS (triple quadrupole) system was done with a gradient acetonitrile/water chromatographic mobile phase. Chromatographic separation was performed in a reversed-phase Purospher STAR-RP-18e analytical column (125 × 2 mm, 5 µm particle diameter). ESI-MS/MS detection was performed in the positive ion mode by monitoring two precursor ion-product ion selected reaction monitoring (SRM) transitions: *m/z* 233 followed by 72 for quantification and *m/z* 235 and 72 for confirmation. Under these conditions, the total analysis

time per sample was 25 min, the limits of detection and quantification were 0.80 and 2.22 ngL, respectively, the relative standard deviation (RSD) of the method was 2% and the recovery percentage (%R) 96%.

Initial concentrations of diuron in each aquarium were determined using water samples taken after each water change (n=30). These values were also used to calculate the deviation from the nominal concentrations and to estimate the EC_{50} for each channel in each sampling day. Samples taken before each water renewal (n=30) were used to calculate the rate of loss from measured initial concentration within three days in each aquarium.

Biofilm parameters

To monitor the evolution of the control channels over time as well as to evaluate the effects of the herbicide on the treated channels, a set of endpoints were used. Algal related endpoints were: photosynthetic efficiency at PSII, algal biomass and composition and biovolume of the diatom community. Bacterial related endpoints included the extracellular leucine-aminopeptidase enzymatic activity and the bacterial density.

Algal biomass

On each sampling day, three glass tiles from each channel were collected at random and the chlorophyll-a content was extracted with 90% acetone for 12 h. Sonication (40 W power, 40 kHz frequency, SELECTA, Abrera, Spain) after 2 min improved the pigment extraction and the chlorophyll-a concentration was subsequently estimated from spectrophotometric measurements (Lambda UV/VIS spectrophotometer, PERKIN-ELMER, Waltham, Massachusetts, USA) following the method described in Jeffrey and Humphrey (1975). The values obtained in the diuron treatments were used to estimate the Non-Effect Concentration (NEC) and the Effective Concentration of diuron that had a 50% of effect (EC_{50}).

Diatom community composition and biovolume estimation

Biofilm samples for diatom examination were collected in duplicate from each channel on each sampling date and fixed with 4% formaldehyde. Thereafter

diatoms were cleaned with concentrated sulphuric acid and potassium dichromate. Washed frustules were mounted on slides using Naphrax (r.i.:1.74) in slides and 400 valves from each slide were counted on random transects with a light microscope (Nikon Eclipse 600W) using phase-contrast and Nomarski differential interference contrast optics at a magnification of 1000X. Diatom identification was done following Krammer and Lange-Bertalot (1991-1997) and Lange-Bertalot (2001). The relative abundances were then calculated. Data are given as the means of the two glass substrata from each channel. Diatom biovolume was obtained following the procedure described in Hillebrand et al. (1999). At least ten randomly selected individuals of each taxon were measured and their biovolume was calculated. The values were transformed to the assemblage cell density by using the surface area of the sample, the necessary dilutions and the number of cells counted. Data are expressed as $\mu m^3/cm^2$. The values obtained in the different treatments were used to calculate the effective concentration of diuron that reduced biovolume by 50% (EC_{50}) and the Non-Effect Concentration (NEC).

Bacterial abundance

The abundance of live and dead bacteria in the biofilm samples was measured using the double staining Live/Dead *Bac*Light Bacterial Viability kit (MOLECULAR PROBES, Carlsbad, California, USA). This double staining consists of a mixture of 3.34 mM SYTO® 9 (which stains all cells) and 20 mM propidium iodide (which penetrates cells that have damaged cell membranes) (Freese et al., 2006). Three colonised glass tiles were selected at random from each channel in each sampling day. Twenty random microscopy fields were counted for each sample. Data are expressed as number of live bacteria cm^2.

Photosynthetic efficiency

Effective PSII quantum yield (efficiency of PSII) and maximal PSII quantum yield (potential efficiency of PSII) were measured with the PhytoPAM (Pulse Amplitude Modulated) fluorometer (HEINZ WALZ, Effeltrich, Germany) and calculated following Genty et al., 1989. PSII quantum yield is defined as a measure of the photosynthetic efficiency of the community (Schreiber, 2004). Five randomly selected colonised glass tiles from each channel were used to

obtain the average effective PSII quantum yield of algae on each sampling day. The same protocol described in Serra et al. (2009) was followed during measurements. The values obtained in the treatments were used to estimate the effective concentration of herbicide that reduced the PSII fluorescence yield by 50% and the Non-Effect Concentration (NEC).

Extracellular enzyme activities

The extracellular enzyme activity leucine-aminopeptidase (EC 3.4.11.1) was analysed in the biofilm samples. Five colonised glass tiles were collected from the experimental channels each sampling day and placed in vials filled with 4ml of channel water passed through 0.2 µm filters (nylon membrane filters, WHATMAN, Maidstone, UK). Samples were immediately incubated with the specific artificial substrate for the enzyme (Leucine-AMC, from SIGMA-ALDRICH, St. Louis, Missouri, USA) at the water temperature of the system. The artificial substrate was added at a final concentration of 0.3 mM (saturation concentration, (Romaní et al., 2004). Standards of AMC (0, 0.25, 2.5, 5, 10, 50, 100µM) were also incubated with the samples, blanks and controls. Fluorescence was measured by the SFM25 KONTRON fluorometer (Basel, Switzerland) at 364/445 nm excitation/emission.

Data analysis

Differences in the endpoints in the control channels over time were tested using analysis of variance (one-way ANOVA). Statistical significance was set at $p=0.05$. Effects were analysed *post hoc* with a Tukey test. The relation between biological and environmental variables was analysed for each sampling day using Pearson correlation tests. Data were log-transformed before the analyses. Statistical analyses were performed using the SPSS programme (Version 15.0). The NEC and the EC_{50} of the respective parameters was estimated from concentration/effect curves, using the three control and six measured diuron concentrations. Data were transformed in natural logarithms. EC_{50} values were determined using the four parameter logistic curve and, in the cases in which the fit was not significant, a linear regression curve was used. The analysis was done using the SIGMAPLOT programme (Version 8.0). NEC values and the

range of NEC were determined by linear regression and by inverse regression, respectively, as described by Liber et al. (1992). The analysis was done using R 2.6.2 (R development Core Team, 2008). The statistical significance for all the analyses was set at $p < 0.05$.

RESULTS

Experimental conditions

Measured initial diuron concentrations (µg/L) were 0.05±0.01 (n=4), 0.16±0.03 (n=4), 0.51±0.08 (n=4), 1.59±0.25 (n=4), 4.08±0.64 (n=4) and 5.42±0.85 (n=4) in each aquarium. The overall deviation from the nominal concentrations was 22.6%. Diuron concentrations declined after 3-4 d in the recirculating channels between 31% and 67% of the measured initial concentrations.

Water temperature, conductivity and pH were stable over the exposure period. pH values remained close to 8.4, water temperature was nearly 17ºC and mean conductivity was 2.02 mS/cm in treated and control channels. Table 1 summarises nutrient and oxygen concentration during the experiment.

	SRP (µg/L) (n=3)	NH_4 (mg/L) (n=3)	NO_3 (mg/L) (n=3)	Oxy (%) (n=10)
Control 1	81.57 (10.82)	93.00 (77.92)	5.74 (5.91)	96.36 (8.90)
Control 2	76.83 (12.30)	79.90 (72.21)	4.55 (3.26)	97.24 (7.93)
Control 3	83.50 (0.57)	98.65 (45.28)	5.02 (3.77)	97.21 (8.51)
C1	77.15 (6.43)	83.70 (14.01)	4.46 (5.60)	98.46 (5.90)
C2	77.57 (8.28)	62.00 (44.73)	5.45 (4.22)	98.93 (7.27)
C3	72.40 (8.91)	96.63 (81.06)	4.71 (3.12)	99.72 (7.65)
C4	83.35 (2.05)	61.03 (65.54)	4.20 (4.05)	98.17 (5.90)
C5	77.78 (4.70)	38.51 (41.53)	4.48 (3.54)	98.81 (7.64)
C6	81.75 (1.91)	86.55 (107.33)	3.25 (2.70)	97.15 (6.50)
Abiotic Control	86.80 (13.29)	78.94 (62.37)	3.90 (3.30)	97.01 (4.89)

Table 1. Average and standard deviation (in parentheses) of soluble reactive phosphate, ammonium, nitrate and dissolved oxygen in each channel

Temporal changes in the control channels

The autotrophic activity and algal biomass of the biofilms were relatively constant during the experiment (Fig.1). However, at day eight there was a significant increase in the chlorophyll-a content (ANOVA, Tukey test, $p < 0.05$). The photosynthetic efficiency was slightly lower at day 22 (ANOVA, Tukey test, $p < 0.05$). The bacterial community showed remarkable temporal variability (Fig. 1). The number of live bacteria decreased at the end of the treatment (ANOVA, Tukey test, $p < 0.05$). Extracellular enzyme activity leucine-aminopeptidase increased over time when standardized per number of live bacteria (ANOVA, Tukey test, $p < 0.05$).

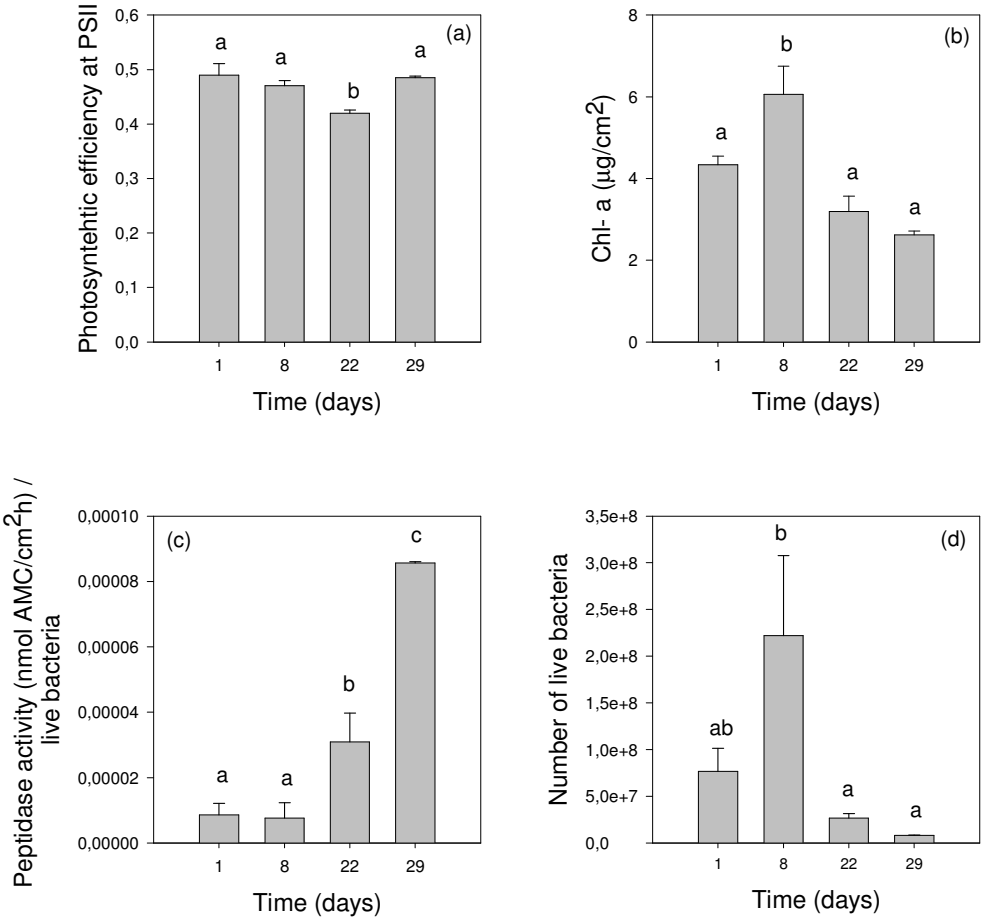

Fig. 1. Temporal changes in the control channels in the following endpoints: (a) Photosystem II fluorescence yield; (b) Chlorophyll-a content; (c) Extracellular leucine-aminopeptidase enzyme activity per live bacteria; (d) Number of live bacteria. Values are mean and standard error (n=3).

Diuron effects

The response of the biofilm community to the addition of diuron was visible after one day. Diuron affected the autotrophic activity of biofilms (Fig. 2).

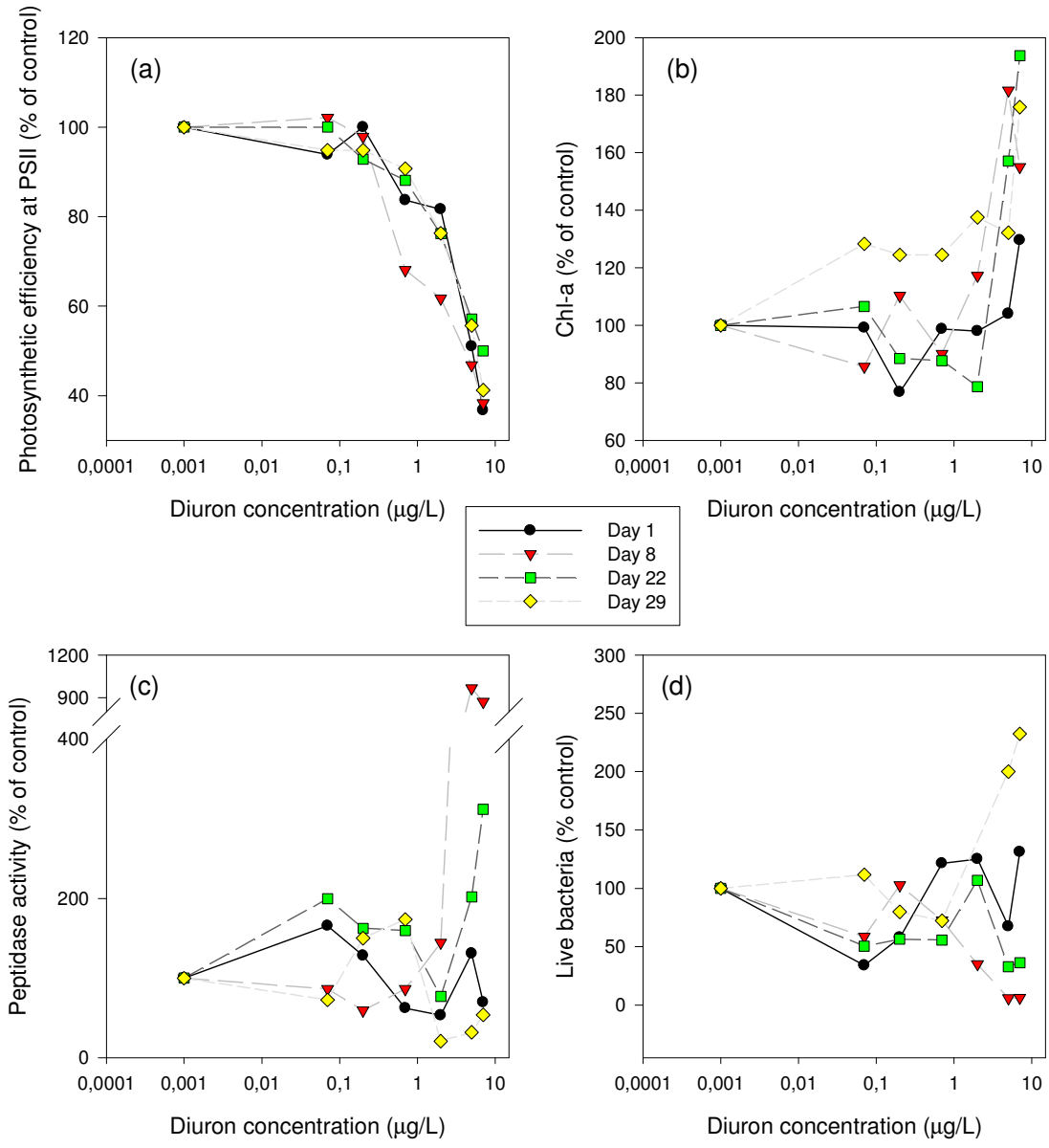

Fig. 2. Diuron effects on the following endpoints: (a) Photosystem II fluorescence yield; (b) Chlorophyll-a content; (c) Extracellular leucine-aminopeptidase enzyme activity per live bacteria; (d) Number of live bacteria. Values are given as percentage of control.

Photosynthetic efficiency was affected from the beginning and throughout the experiment. The maximal fluorescence quantum yield and the effective fluorescence quantum yield showed the same tendency (r=0.85, Pearson correlation test). Since the information provided by the two variables was essentially the same, only the effective fluorescence quantum yield was considered. Estimated NEC and EC_{50} values during the experiment are shown on Table 2. The effects of the herbicide on algal biomass were detectable on all the sampling days. However, chlorophyll-a content increased with increasing diuron concentrations. Obtained NEC and EC_{50} on each sampling date are shown in Table 2.

		Efficiency of PSII	Chlorophyll-a	Biovolume
Day 1	EC_{50}	5.22 ± 0.9	13.3* ± 2.1	n.a.
	NEC	0.1 [0.02-0.38]	n.d.	n.a.
Day 8	EC_{50}	3.64* ± 1.5	4.8* ± 1.2	0.087 ± 0.01
	NEC	0.07 [0.02-0.19]	n.d.	0,02 [0.0001-0.18]
Day 22	EC_{50}	4.61* ± 0.7	4.6* ± 0.9	n.a.
	NEC	0.07 [0.02-0.2]	n.d.	n.a.
Day 29	EC_{50}	4.3 ± 0.9	4.8* ± 1.3	0.054 ± 0.009
	NEC	0.08 [0.02-0.27]	n.d.	$9.5x10^{-6}$ [$3.3x10^{-18}$-0.004]

n.a. not available

n.d. not determinable

(*) EC_{50} values obtained by linear regression

Table 2. Effective diuron concentrations values (EC50, in µg/L) and the corresponding confidence interval (±) and Non Effect Concentrations values (NEC in µg/L) and the corresponding range (in brackets). The different values are obtained from dose response relationships of diuron (six concentrations) versus the photosynthetic efficiency at PSII, the chlorophyll-a concentration and the biovolume of the diatom taxa on the four sampling days

The composition of the diatom community was also affected by diuron (Tables 3 and 4). Several diatom taxa showed relevant changes in their relative abundances. After 8 d of diuron exposure, two trends were detected. *Diploneis oblongella* decreased (r= -0.68, Pearson correlation test), whereas *Navicula atomus var. permitis*, *Nitzschia inconspicua* and *Navicula menisculus* (r= 0.77; r= 0.69; r= 0.95 Pearson correlation test, respectively) were favoured by increasing concentrations of the toxicant. At the end of the treatment (after 29 d) the proportion of *Navicula cryptotenella* increased (r= 0.68, Pearson correlation test) with diuron, whereas *Navicula gregaria* decreased (r= -0.85, Pearson correlation test).

Day 8	Diuron concentration (µg/L)								
	0	**0**	**0**	**0.07**	**0.2**	**0.7**	**2**	**5**	**7**
Amphora inariensis Krammer	0.8	1.1	0.7	1.2	0.9	0.2	1.0	0.8	0.4
Achnanthes lanceolata (Breb.) Grunow	2.2	0.7	2.6	0.6	1.4	0.7	2.0	1.7	1.6
Achnanthes lanceolata (Breb.) Grun. ssp. frequentissima Lange-Bertalot	2.6	2.1	2.4	1.4	2.0	1.2	2.0	3.1	1.9
Achnanthes minutissima Kützing	2.2	3.2	3.8	3.1	4.5	3.9	5.5	4.2	4.8
Amphora pediculus (Kützing) Grunow	4.1	6.5	2.7	3.9	4.0	3.8	4.1	5.1	10.3
Cyclotella meneghiniana Kützing	0.1	0.0	0.0	0.3	0.0	0.1	0.0	0.0	0.0
Cymbella microcephala Grunow	0.0	0.2	0.0	0.5	0.1	0.2	0.5	0.0	0.1
Cymbella minuta Hilse ex Rabenhorst	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.2
Cocconeis pediculus Ehrenberg	0.8	1.5	1.4	0.4	0.4	0.1	0.2	0.4	0.5
Cocconeis placentula Ehrenberg	1.1	0.9	1.4	0.9	0.4	0.5	0.3	0.4	0.7
Diploneis oblongella (Naegeli) Cleve-Euler	1.5	2.3	1.4	1.7	0.6	0.7	0.4	0.5	0.1
Diatoma vulgaris Bory 1824	0.7	6.9	0.1	1.4	0.2	0.1	0.0	0.1	0.2
Fragilaria capucina Desmazières var. gracilis (Oestrup) Hustedt	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Fragilaria capucina Desm. var. rumpens (Kütz.) Lange-Bert.	0.0	0.2	0.0	0.0	0.1	0.0	0.1	0.0	0.0
Fragilaria capucina Desmazières var. vaucheriae (Kützing) Lange-Bertalot	0.0	0.0	0.0	0.0	0.0	0.2	0.1	0.0	0.0
Fragilaria construens (Ehrenberg) Grunow f. venter (Ehr.) Hustedt	0.0	0.0	0.1	0.0	0.1	0.1	0.0	0.0	0.0
Fragilaria ulna (Nitzsch.) Compère	0.4	0.5	0.3	0.7	0.6	0.2	0.5	0.1	0.0
Gomphonema angustatum (Kutzing) Rabenhorst	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Gomphonema olivaceum (Hornemann) Brébisson	0.2	1.2	0.3	0.1	0.2	0.1	0.1	0.1	0.1
Gomphonema parvulum Kützing	0.1	0.5	0.1	0.1	0.2	0.1	0.2	0.0	0.0
Gyrosigma acuminatum (Kutzing) Rabenhorst	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Melosira varians Agardh	0.1	0.4	0.4	0.6	0.6	0.4	0.4	0.2	0.5
Nitzschia amphibia Grunow	0.0	0.2	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Navicula atomus var. permitis (Hustedt) Lange-Bertalot	0.0	0.0	1.4	0.9	0.5	1.1	1.0	0.8	6.8
Navicula capitata (Ehr.) Lange-Bert. Metzeltin & Witkowski	0.5	0.0	0.0	0.1	0.1	0.4	0.1	0.0	0.2
Nitzschia communis Rabenhorst	0.1	0.0	0.0	0.1	0.0	0.2	0.0	0.0	0.0
Nitzschia constricta (Kützing) Ralfs	2.1	3.0	2.3	3.2	2.1	2.1	3.0	2.5	3.2
Nitzschia capitellata Hustedt	0.4	0.5	1.1	2.0	0.5	1.8	1.0	0.4	0.6
Navicula capitatoradiata Germain	0.2	1.8	1.5	1.7	0.8	1.0	0.5	1.4	0.6
Navicula cryptocephala Kützing	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Navicula cryptotenella Lange-Bertalot	0.2	2.3	2.3	1.0	0.7	1.4	0.0	1.4	0.9
Navicula cuspidata Kutzing	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Nitzschia dissipata (Kützing) Grunow	0.9	0.5	0.8	1.1	1.1	1.7	1.0	0.7	1.2
Nitzschia fonticola Grunow	0.2	0.5	0.5	0.1	0.1	0.1	0.5	0.4	0.0
Navicula gregaria Donkin	48.1	18.6	30.1	27.4	41.3	43.6	38.0	39.2	27.1
Nitzschia hantzschiana Rabenhorst	0.0	0.0	0.3	0.1	0.3	0.1	0.0	0.0	0.1
Nitzschia frustulum (Kützing) Grunow	0.7	1.0	0.9	1.9	1.8	0.9	1.4	1.1	3.3
Nitzschia gracilis Hantzsch	0.5	0.3	0.0	0.2	0.0	0.0	0.0	0.0	0.0
Nitzschia inconspicua Grunow	3.0	2.6	4.5	3.7	4.6	2.7	5.6	3.5	9.0
Navicula lanceolata (Agardh) Ehrenberg	11.2	20.8	11.7	8.6	9.4	10.7	8.2	9.5	4.1
Nitzschia levidensis (W.Smith) Grunow in Van Heurck	0.0	0.6	0.1	0.1	0.0	0.1	0.7	0.1	0.1
Navicula menisculus Schuman var. grunowii Lange-Bertalot	0.0	0.0	0.0	0.0	0.2	0.0	0.6	1.1	1.1
Nitzschia microcephala Grunow in Cleve & Moller	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.2	0.0
Nitzschia paleacea (Grunow) Grunow in van Heurck	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Nitzschia palea (Kützing) W. Smith	2.1	3.3	4.5	5.6	4.1	3.7	5.0	4.5	3.7
Navicula pelliculosa (Brébisson ex Kützing) Hilse	0.1	0.0	0.0	0.0	0.2	0.4	0.0	0.4	0.2
Navicula phyllepta	0.5	1.1	1.8	0.9	1.2	1.0	1.8	0.2	0.2
Navicula reichardtiana Lange-Bertalot	0.5	0.7	1.8	3.1	2.2	1.2	0.9	0.8	0.9
Navicula subminuscula Manguin	0.0	0.0	0.1	0.4	0.1	0.0	0.4	2.3	0.6
Navicula seminulum (Grunow) D.G. Mann	0.0	0.0	0.0	0.0	0.0	0.0	0.6	1.7	0.4
Navicula tripunctata (O.F.Müller) Bory	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.1
Navicula veneta Kützing	3.1	1.3	3.3	4.0	3.3	3.0	3.5	4.0	2.0
Nitzschia supralitorea Lange-Bertalot	0.5	0.9	2.8	1.8	0.4	1.4	1.4	0.5	1.0
Rhoicosphenia abbreviata (C.Agardh) Lange-Bertalot	4.5	6.3	3.8	4.1	3.1	3.3	1.4	2.5	2.7
Surirella brebissonii Krammer & Lange-Bertalot	3.0	5.1	6.1	10.9	4.5	4.4	5.9	4.1	8.2
Thalassiosira pseudonana Halse et Heimdal	0.0	0.2	0.8	0.0	0.6	0.0	0.0	0.0	0.0

Table 3. Diatom community composition (relative abundance) after 8 days of exposure to various diuron concentrations. Values are mean of two replicates.

Day 29	Diuron concentration (µg/L)								
	0	**0**	**0**	**0.07**	**0.2**	**0.7**	**2**	**5**	**7**
Amphora inariensis Krammer	2.4	1.3	2.2	1.8	1.4	1.9	1.0	4.7	2.0
Achnanthes lanceolata (Breb.) Grunow	2.4	4.3	1.8	1.7	2.3	3.7	2.2	4.8	3.6
Achnanthes lanceolata (Breb.) Grun. ssp. frequentissima Lange-Bertalot	5.0	6.3	3.0	1.7	4.2	5.6	2.5	9.6	6.8
Amphora libyca Ehr.	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Achnanthes minutissima Kützing	14.0	22.9	14.9	13.0	8.6	13.0	15.3	15.5	10.0
Amphora pediculus (Kützing) Grunow	10.7	11.5	6.1	5.8	8.5	7.7	5.1	15.7	9.5
Cymbella affinis Kutzing var.affinis	0.2	0.0	0.1	0.6	0.1	0.0	0.0	0.0	0.0
Cyclotella meneghiniana Kützing	0.5	0.0	0.5	0.3	0.3	0.1	0.2	0.1	0.3
Cymbella microcephala Grunow	0.4	1.4	0.6	1.4	0.1	0.9	0.7	0.7	0.9
Cymbella minuta Hilse ex Rabenhorst	0.6	0.3	0.1	0.0	0.0	0.6	0.0	0.1	0.1
Cocconeis pediculus Ehrenberg	0.5	0.1	0.0	0.3	0.0	0.4	0.0	0.2	0.1
Cocconeis placentula Ehrenberg	1.0	0.5	0.7	0.1	0.6	0.6	0.2	0.7	0.8
Diatoma tenuis Agardh	0.7	0.6	1.1	0.4	0.3	0.1	0.0	0.5	0.3
Diploneis oblongella (Naegeli) Cleve-Euler	1.1	2.2	0.5	1.9	1.9	2.8	2.1	3.0	1.6
Diatoma vulgaris Bory 1824	1.0	0.5	0.5	0.3	0.2	0.7	0.2	0.5	0.9
Fragilaria biceps (Kutzing) Lange-Bertalot	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Fragilaria capucina Desmazieres var.capitellata (Grunow) Lange-Bertalot	0.0	0.3	0.5	0.0	0.0	0.0	0.1	0.0	0.0
Fragilaria capucina Desmazieres var.distans(Grunow) Lange-Bertalot	0.0	0.0	0.0	0.0	0.3	0.0	0.5	0.2	0.0
Gomphonema olivaceum (Hornemann) Brébisson	0.0	0.1	0.3	0.3	0.1	0.3	0.0	0.0	0.0
Gomphonema parvulum Kützing	0.6	0.3	0.0	0.0	0.4	0.1	0.0	0.0	0.1
Melosira varians Agardh	0.0	0.4	0.8	0.6	1.6	0.8	0.7	0.2	1.2
Nitzschia acuminata (WM.Smith) Grunow	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Nitzschia amphibia Grunow	0.6	0.9	0.3	0.4	0.1	0.4	0.0	0.5	0.3
Navicula capitata (Ehr.) Lange-Bert. Metzeltin & Witkowski	1.1	0.3	0.0	0.0	1.0	0.8	0.6	0.5	0.4
Nitzschia constricta (Kützing) Ralfs	3.8	1.1	3.5	3.6	2.8	5.2	6.7	3.1	3.5
Nitzschia capitellata Hustedt	0.2	0.3	0.0	0.0	0.0	0.5	0.0	0.0	0.4
Navicula cryptocephala Kützing	2.0	0.4	2.6	0.0	0.0	1.2	0.0	0.5	0.2
Navicula cryptotenella Lange-Bertalot	1.1	0.6	0.0	0.3	0.7	0.7	0.2	0.0	0.4
Navicula cuspidata Kutzing	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Nitzschia dissipata (Kützing) Grunow	2.1	1.1	1.8	1.4	1.5	1.2	1.7	1.4	1.4
Nitzschia dubia W.M.Smith	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Nitzschia fonticola Grunow	0.1	0.0	0.8	0.4	0.0	0.0	0.0	0.0	0.0
Navicula gregaria Donkin	12.6	13.6	16.3	28.0	16.8	13.8	16.9	10.6	17.5
Navicula halophila (Grunow) Cleve	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Nitzschia hantzschiana Rabenhorst	0.0	0.1	0.1	2.3	0.3	0.4	1.0	0.5	0.0
Nitzschia frustulum (Kützing) Grunow	4.4	4.6	5.8	5.1	5.5	5.6	5.1	4.1	4.1
Nitzschia inconspicua Grunow	5.9	3.3	10.1	3.9	6.9	3.9	3.6	5.8	6.8
Navicula lanceolata (Agardh) Ehrenberg	5.5	2.4	1.1	3.5	1.8	3.7	1.7	2.6	4.5
Nitzschia levidensis (W.Smith) Grunow in Van Heurck	0.0	0.1	0.1	0.4	0.0	0.3	0.2	0.5	0.1
Nitzschia microcephala Grunow in Cleve & Moller	3.9	1.1	4.5	3.7	5.9	3.3	2.8	0.7	3.2
Nitzschia paleacea (Grunow) Grunow in van Heurck	2.1	1.1	1.4	0.9	0.5	0.8	2.1	0.6	1.5
Nitzschia palea (Kützing) W. Smith	5.4	1.6	6.2	3.7	5.4	4.6	6.8	3.1	4.9
Navicula phyllepta	0.0	0.0	1.8	2.0	3.3	1.8	3.3	0.0	2.6
Navicula tripunctata (O.F.Müller) Bory	1.4	2.0	0.0	1.3	1.5	1.7	1.9	2.1	1.4
Navicula veneta Kützing	1.5	7.2	4.4	4.9	6.7	4.6	6.2	3.1	3.6
Nitzschia supralitorea Lange-Bertalot	1.5	1.5	0.9	0.0	4.1	1.0	2.5	1.4	1.1
Rhoicosphenia abbreviata (C.Agardh) Lange-Bertalot	1.6	1.8	0.8	1.5	2.1	1.4	1.4	1.1	1.6
Surirella brebissonii Krammer & Lange-Bertalot	1.7	1.9	3.7	2.8	2.1	3.7	3.6	1.2	2.2

Table 4. Diatom community composition (relative abundance) after 29 days of exposure to various diuron concentrations. Values are mean of two replicates.

The biovolume of the diatom community was also estimated after 8 and 29 d (Fig. 3). Biovolume decreased in response to the presence of the toxicant, even in the channels receiving the lowest concentrations. Estimated NEC and EC_{50} values were about two orders of magnitude lower than those based on photosynthesis inhibition (Table 2).

The effects of diuron on heterotrophs were less pronounced than those on primary producers and were not dose-dependant (Fig. 2). The number of live bacteria decreased exponentially after 1 week, and dead bacteria accounted for up to 90% of the bacterial population. The leucine-aminopeptidase activity increased with diuron concentration (r=0.933; r=0.714, Pearson correlation test after 8 and 22 d respectively). This pattern was transient and did not remain up to the end of the treatment.

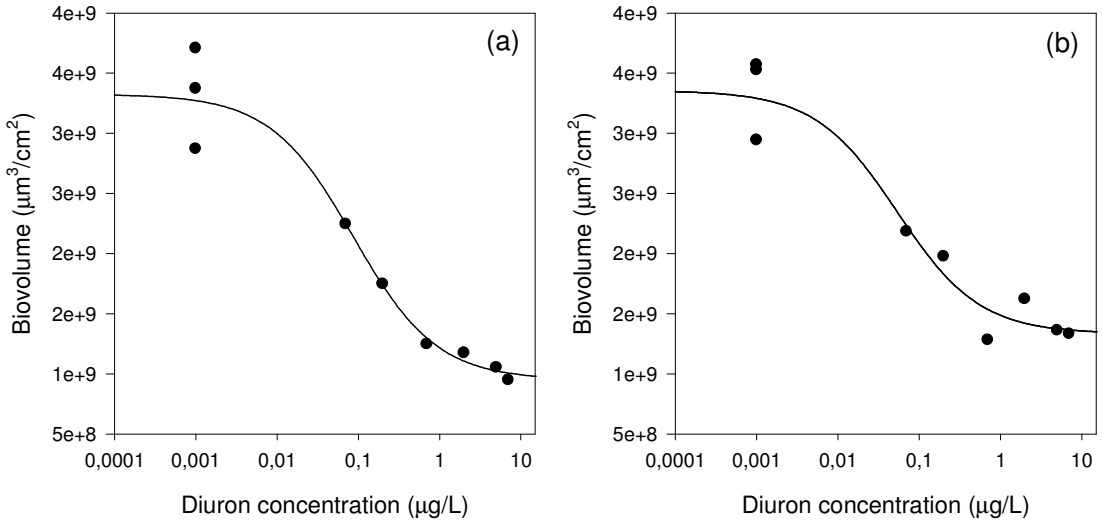

Fig. 3. Biovolume of the diatom taxa after 8 days (a) and after 29 days (b) of diuron exposure. Values are obtained from two analytical replicates of each channel

DISCUSSION

The non-exposed biofilms (developing in the control channels) followed a usual transition from exponential to stationary phases (Sabater and Admiraal, 2005) during the experiment. Since biofilms are a complex community of active and inactive heterotrophic and autotrophic organisms in close contact (Rier and Stevenson, 2002), interactions are expected to be intense and, as such, variable over time. After the initial phase (in terms of algal biomass; from days 1 to 8), the interaction between algae and bacteria expressed as the increase of peptidase activity and reduction in the number of live bacteria was obvious.

Environmentally realistic diuron concentrations produced responses both in target (algae) and non-target (bacteria) organisms. Direct effects were noticeable in all the algal related endpoints. The algal community represents potential primary targets for diuron-type herbicides, because of the mode of action of this toxicant. Diuron inhibits photosynthesis by preventing oxygen production (Wessels and Van der Veen, 1956) and blocking the electron transfer at PSII (Giacomazzi and Cochet, 2004). Previous studies on the effects of diuron at the community level have described decreases in algal community photosynthesis (McClellan et al., 2008). Community photosynthesis was strongly and rapidly affected after one day of exposure to the herbicide and these effects remained visible until the end of the experiment. That the EC_{50} did not significantly change with time was indicative that the algal community did not adapted to the diuron presence. Nevertheless, examples exist in the literature where communities develop tolerance to diuron-type herbicides (Schmitt-Jansen and Altenburger, 2005) or to diuron at concentrations similar to the ones used in this study (McClellan et al., 2008). McClellan et al. (2008) reported a tolerance increase on periphyton communities pre-exposed at 0.08 µg/L during seven weeks, using inhibition of photosynthesis as an endpoint. However, the low EC_{50} obtained using the biovolume as an endpoint revealed that this structural parameter can turn out to be equal or more sensitive than the functional ones. Changes in the biovolume demonstrated the direct effect of a chronic exposure of this herbicide on the competitive interactions between species of the same trophic level.

A direct effect of the herbicide was also detected in the chlorophyll-a, though in a different manner than expected due to growth inhibition, given that chlorophyll-a increased with high diuron concentrations. Diuron toxicity proceeds in two stages. The disruption of electron flow in PSII during the light reaction of photosynthesis occurs at low concentrations and induces the formation of shade-type chloroplasts. Microalgae may adjust their intracellular concentration of photosynthetic pigments in response to environmental conditions (Kana et al., 1997). The so-called "shade-adaptation" response consists of an increase in photosynthetic pigments in response to a decrease in light intensity (Falkowski and LaRoche, 1991). This adaptation may compensate for the reduction in photosynthetic efficiency in spite of the energy cost that implies for photosynthetic autotrophs (Chesworth et al., 2004). Higher diuron concentrations inhibit PSI and can achieve the photodestruction of pigments (Ridley and Horton, 1984). Though the second inhibition was not detected in our study, results consistently point to the formation of shade-type chloroplasts and the associated increase in chlorophyll-a with higher diuron concentrations.

Changes in algal physiology have been reported to have the capacity to influence the toxicity of PSII herbicides. Guasch and Sabater (1998) found an increase in tolerance to atrazine (PSII inhibitor) in shade-adapted natural biofilm communities. The decrease we detected in the biovolume contrasts with the increase in algal biomass, supporting this hypothesis and indicating that chlorophyll-a concentration is not a reliable indicator of the herbicide effects on algal biomass, since it is directly and dose-dependant affected by diuron.

The relative abundance of certain taxa was altered by the presence of the herbicide. It is well known that some species are more sensitive than others. Pérès et al. (1996) reported differences in the impact of the herbicide on various diatom species, attributed to the specific herbicide uptake and their accessibility to the binding sites on the thylakoid membranes. In an experiment with periphyton communities, Goldsborough and Robinson (1986) provided evidence that *Cocconeis placentula* was partially tolerant to two triazine-type herbicides (simazine and terbutryn), mainly because of its colonising character. In a study performed for 20 stream and river sites on a latitudinal gradient across Europe (Sweden, The Netherlands, Spain), Guasch et al. (1998) detected that *Navicula*

menisculus was one of the taxa most closely associated with high nutrient content and atrazine concentration.

The decrease in the diatom biovolume is associated with the predominance of smaller growth forms in the biofilms (Sabater and Admiraal, 2005). The EC_{50} values for the biovolume calculated from this parameter were extremely low, indicating that this was a highly sensitive parameter to diuron exposure, and that effects might be significant in nature. NEC and EC_{50} values obtained with the biovolume were lower than the ones obtained in most of the previous works using short-term single-species tests, registered in the US EPA database (i.e. Gatidou and Thomaidis, 2007 (EC_{50}=27 µg/L, *Navicula forcipata*, growth); Ma et al., 2001 (EC_{50}=4.3 µg/L, *Chlorella vulgaris*, growth); Podola and Melkonian, 2005 (EC_{50}=6.4 µg/L, *Cryptomonas sp.*, chlorophyll-a fluorescence)). This difference emphasises the relevance of using natural communities to approach environmental relevance.

The River Llobregat records average monthly values of diuron from 0.064 to 0.239 µg/L (Rodriguez-Mozaz et al., 2004), and it has been reported in the range of 0.0013-0.11 µg/L in the Thames Estuary (UK) and the Brisbane River (Australia) (Bengtson et al., 2004). Blanchoud et al. (2004) detected a peak of 8.7 µg/L diuron in the Morbras catchment after a rain event.

Diuron affected several parameters of the bacterial components of biofilms. In this case, responses were not always dose-dependant and thus, attributable to changes in the interactions within the biofilm components. After one week of exposure to diuron, 1.7 µg/L of this herbicide was enough to reduce the number of live bacteria by 50%, whereas 4 µg/L was required to achieve a reduction of 90%. Though 0.239 µg/L of diuron had previously been detected in the River Llobregat (Rodriguez-Mozaz et al., 2004), extrapolations from our results indicate that this concentration would result in a 22% decrease in the number of live bacteria. However, direct effects of diuron to bacteria were not expected, since PSII inhibitors are only weakly toxic to non-photosynthetic organisms and very high LOEC has been obtained in laboratory tests (Fernandez-Alba et al. (2002); BioTox test, LOEC = 7.6 mg/L). Probably, the direct diuron damage of the autotrophic compartment was affecting the bacterial compartment, strongly linked to the algae (Rier and Stevenson, 2002). However, bacteria recovered at the end of the experiment, suggesting that a change could happen in the

bacterial community. It has been observed that natural periphytic communities were able to use the herbicide as energy source (Pesce et al., 2009). Similarly, long-term effects of phenylurea herbicides on soil bacteria cause changes in the community structure and metabolic potential (El Fantroussi et al., 1999). Changes in the heterotrophic compartment were also observed in our study. Algal material released because of cell lysis might provide organic proteinaceous compounds for bacteria. This is shown by the increase in the peptidase activity that occurs under the highest diuron concentrations. Similar increases in peptidase activity have been reported following the spring phytoplankton bloom in lakes, related to the degradation and lysis of senescent algal cells and release of proteinaceous compounds (Middelboe et al., 1995).

In conclusion, we have shown that the function and structure of a biofilm community are modified by diuron at environmentally realistic concentrations. These effects are apparent on the biovolume, chlorophyll and photosynthetic efficiency at PSII of the algal communities of natural biofilms. Furthermore, because of the close link between bacteria and algae in the biofilm, indirect effects of the herbicide may also arise, as reflected both by the number of live bacterial cells as well as by their capacity to process organic matter.

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CHAPTER IV

Triclosan persistence through wastewater treatment plants and its potential toxic effects on river biofilms

Ricart et al., 2010. Aquatic Toxicology 100, 346-353.

TRICLOSAN PERSISTENCE THROUGH WASTEWATER TREATMENT PLANTS AND ITS POTENTIAL TOXIC EFFECTS ON RIVER BIOFILMS

ABSTRACT

Triclosan is a commonly used bactericide that survives several degradation steps in WWTP (wastewater treatment plants) and potentially reaches fluvial ecosystems. In Mediterranean areas, where water scarcity results in low dilution capacity, the potential environmental risk of triclosan is high. A set of experimental channels was used to examine the short-term effects of triclosan (from 0.05 to 500 µg/L) on biofilm algae and bacteria. Environmentally relevant concentrations of triclosan caused an increase of bacterial mortality with a no effect concentration (NEC) of 0.21 µg/L. Dead bacteria accounted for up to 85% of the total bacterial population at the highest concentration tested. The toxicity of triclosan was higher for bacteria than algae. Photosynthetic efficiency was inhibited with increasing triclosan concentrations (NEC= 0.42 µg/L), and non-photochemical quenching mechanisms decreased. Diatom cell viability was also affected with increasing concentrations of triclosan. Algal toxicity may be a result of indirect effects on the biofilm toxicity, but the clear and progressive reduction observed in all the algal-related endpoints suggest the existence of direct effects of the bactericide. The toxicity detected on the co-occurring non-target components of the biofilm community, the capacity of triclosan to survive through WWTP processes and the low dilution capacity that characterizes Mediterranean systems extend the relevance of triclosan toxicity beyond bacteria in aquatic habitats.

INTRODUCTION

Though WWTP (wastewater treatment plants) function as partial barriers to pollutants, they are not specifically designed for the effective removal of organic compounds such as pharmaceuticals, detergents or personal care products. Thus, WWTP effluents can potentially alter the water quality of fluvial ecosystems when some of these chemical compounds reach the aquatic environment as a result of an incomplete removal during wastewater treatment

processes (Adolfsson-Erici et al., 2002; Coogan and La Point, 2008). These potential effects derived from WWTP inputs on fluvial ecosystems are aggravated under water scarcity conditions (Paul and Meyer, 2001), which are especially relevant in arid and semi-arid areas such as the Mediterranean region (Guasch et al., 2010). Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) has been used as an antimicrobial agent for more than 20 years and is present in effluents from waste water treatment plants and in the receiving river systems (Kolpin et al., 2002; Bester, 2005). Triclosan is added as a preservative or as an antiseptic agent in a variety of consumer products of daily use, such as hand soaps, skin creams, toothpastes, household cleaners and even textiles (Singer et al., 2002). It is a stable lipophilic compound with a relatively high octanol water partition coefficient (Kow= 4.8 at pH= 7) that can be bioaccumulated. In aqueous solution, triclosan has the ability to produce several types of polychlorinated dibenzo-p-dioxins under exposure to sunlight, especially at high pH values (Mezcua et al., 2004). Chalew and Halden (2009) summarized that triclosan concentrations in WWTP effluents were in the range of 0.027-2.7 µg/L, with the maximum amount detected in rivers being 2.3 µg/L.

As a bactericide, triclosan inhibits bacterial fatty acid synthesis by inhibiting the enzyme ENR (enoyl-acyl carrier protein reductase) and thus blocking the FabI step (Heat et al., 1999). However, triclosan is a broad-spectrum antimicrobial agent, and as such, it might generate effects in sensitive species and also on co-occurring non-target components of the fluvial community (Wilson et al., 2003).

Bacteria coexist in biofilms with other phototrophic and heterotrophic microorganisms (Lock, 1993). Biofilm communities are linked to the whole aquatic ecosystem and integrate the influences of environmental conditions over extended periods of time, and they represent a reliable indicator for detection of ecosystem stress (Sabater et al., 2007). Biofilm sensitivity has been demonstrated for a large number of toxicants, including priority (Guasch et al., 2003; Tlili et al., 2008) and emerging pollutants (Lawrence et al., 2005; Bonnineau et al., 2010). Interactions between bacteria and algae characterise the functioning of biofilms (Rier and Stevenson, 2002), though toxicant effects on these interactions are less known.

Several ecotoxicological studies have demonstrated that triclosan is toxic to bacteria (DeLorenzo et al., 2008; Farré et al., 2008), while others have observed effects on algal cultures (Orvos et al., 2002). The analysis of triclosan effects on biofilm communities has shown effects on photosynthesis at high concentrations (EC_{50}= 900 µg/L, Franz et al., 2008), as well as effects of concentrations of 10 µg/L on microbial community composition, algal biomass, architecture and activity of biofilms (Lawrence et al., 2009). In this study the response of microbial communities to triclosan was investigated by means of flow-through experimental channels. The occurrence of triclosan photodegradation and the associated production of dioxins, which have even higher toxicity, do not recommend the use of recirculating channels, which could result in the accumulation of dioxins in the water. The objective of this study was to mimic the effects of triclosan in WWTP-dominated rivers by examining the effects of triclosan at the biofilm community level, including effects on its target (bacteria) and non-target (algae) organisms. We also aimed to determine the non-effect concentrations, which could be useful from a regulatory perspective, especially in Mediterranean regions where water scarcity and the associated low dilution capacity can lead to an increase of concentrations that could enhance the potential effects of triclosan.

MATERIALS AND METHODS

A system of continuous flow channels was installed in a waste water treatment plant (WWTP). In order to obtain purified water without toxicants at the plant effluent, a part of it was redirected to a pilot plant equipped with a microfiltration and reverse osmosis system. This facility guaranteed a continuous supply of water to the channels without toxicants. The pilot plant also eliminated the salts from the water, and re-mineralization was needed to provide water suitable for biofilm growth. Therefore water reached the channels after passing through the pilot plant and the re-mineralization step. Triclosan toxicity was investigated in the channels by deploying 6 exponentially increasing concentrations (from 0.5 to 500 µg/L). After the complete passage through the channels outflowing water was redirected to the inlet of the WWTP.

Purification efficiency (WWTP plus reverse osmosis)

Triclosan occurrence throughout the whole system (WWTP and pilot plant) was analysed to test the applicability of the system as a supplier of purified water for ecotoxicological studies. Five points along the operating system were monitored twice during the experiment, as follows: (1) influent of the WWTP, (2) effluent of the WWTP (equivalent to the entrance of the pilot plant), (3) permeate MF (located after the microfiltration system), (4) concentrate RO (residual of the reverse osmosis step) and (5) permeate RO (located after the reverse osmosis system and water supply to the experimental channels) (Fig. 1).

Triclosan was analysed using the Triclosan IA kit (Abraxis LLC), a commercially available magnetic particle-based enzyme immunoassay that has been proved to be a sensitive and accurate technique for testing waste water samples (Kantiani et al., 2008). The kit does not distinguish between triclosan and methyl-triclosan, but the rapidity and simplicity of the test makes it a useful tool for monitoring studies. The analyses were done following the protocol described in Kantiani et al. (2008).

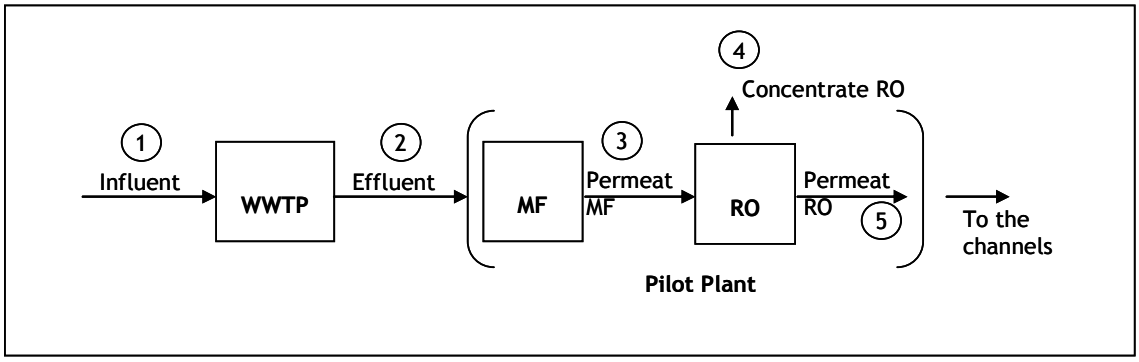

Fig. 1. Sampling sites for triclosan analyses throughout the waste water treatment plant and the pilot plant in Torroella de Montgrí (NE Spain). WWTP, wastewater treatment plant; MF, microfiltration; RO, reverse osmosis.

Experimental study of triclosan toxicity

Experimental set-up

The channel system (8 perspex channels, 170 cm long, 10 cm wide) was located in the waste water treatment plant of Torroella de Montgrí (NE Spain). The experimental design of this experiment included two control channels, one solvent control channel and 6 channels with increasing concentrations of triclosan. Before the exposure, one control channel was eliminated for technical reasons. Thus, 8 channels were used during the exposure (1 control, 1 solvent control and 6 channels with triclosan addition). The water supply was provided from a pilot plant equipped with microfiltration and reverse osmosis systems used to purify the effluent of the WWTP. After passing through the WWTP and the pilot plant, the purified water was mineralized to obtain suitable water for the growth of the biofilm community. Conductivity and pH were regulated up to similar values of the river Llèmena (biofilm source during colonization) and nutrients were added ($(NH_4)_3PO_4$ and NH_4NO_3) to avoid nutrient limitation. The water was distributed to the channels by using a set of droppers (Netafim, Regaber), which guaranteed a constant flow of 1.2 L/min in each channel. Water conductivity, pH and dissolved oxygen were measured with appropriate probes (WTW Meters). The main anions and cations were analysed by ion-chromatography (761 Compact IC, Metrohm). Triclosan solutions were placed in dark carboys and pumped continuously to the head of the treated channels using a set of peristaltic pumps (FCO electromagnetic pumps LFCO0703). After passing through the channel system, water was redirected to the entrance of the WWTP. Water depth in the channels was between 1 and 2 cm. Available radiation for photosynthesis was provided by fluorescent tubes (L 36W/965 Biolux, Osram). The irradiance level was 120 $\mu E/m^2$ s and the light regime was 12:12 h light:dark.

Biofilm colonization

Biofilm colonization was carried out on sandblasted glass substrata placed in crystallizing dishes filled with 1.5 L of tap water (previously dechlorinated using an active carbon filter) following the same procedure described in Bonnineau et al. (2010). A microbenthic community from the river Llèmena (NE Spain) was used as a biofilm source and was inoculated once a week for 3 weeks. After this period, the biofilms were transferred to the channel system and acclimated for 3 days before the onset of the experiment. The photosynthetic efficiency (Yeff) and the minimum fluorescence yield (F0) of the colonized substrata were analysed 1 day before the exposure started. This step was performed in order to check that the initial biofilm community was the same in all the channels. These parameters provide information of the physiological status of the community, are non destructive and they account for a global condition of the biofilm community (Sabater et al., 2007). Details are provided below (Biofilm measurements).

Acute triclosan toxicity test

Test chemical

Triclosan, 5-chloro-2-(2,4-dichloro-phenoxy)phenol (CAS number 3380-34-5), 97%, was purchased from Sigma-Aldrich. The effect of triclosan was tested at concentrations of 0, 0.05, 5, 25, 125, 250 and 500 µg/L using methanol as a solvent. The final concentration of methanol in the channels was 0.05% (v/v).

Triclosan and methyl-triclosan levels during exposure were determined by high performance liquid chromatography (HPLC). The standards used to quantify the compounds were of the highest purity available (HPLC grade, Sigma-Aldrich). Stock solutions (1 mg/mL) of individual standards and standard mixtures were prepared by dissolving pure standards in methanol. Working solutions were obtained by further dilution of stock solutions. Water samples for triclosan and methyl-triclosan analyses were collected from all of the channels during the toxicant exposure. Samples were filtered through 0.45-µm nylon membrane filters (Whattman) and immediately loaded onto C18 SPE cartridges (Waters) that were previously conditioned with 5 mL of HPLC water, acetone and, finally,

methanol, at a flow rate of 1 mL/min. Samples (500 mL) were loaded at a flow rate of 5 mL/min. After preconcentration, the cartridges were dried completely by vacuum for 20 minutes to avoid hydrolysis and kept frozen until analyses. Thereafter, cartridges were eluted with 4 mL of methanol and injected directly. The HPLC system consisted of a Waters 717 autosampler and a Waters 1525 binary pump. The HPLC separation was achieved on a 5-µm, 150 x 4 mm i.d. C_{18} reversed-phase column (SunFire, Waters). The injection volume was set at 50 µL, and the flow rate was 1 mL/min of 90:10 methanol:water with isocratic flow. Detection was carried out using a UV-vis detector (Waters 2489) at a 280 nm wavelength. Triclosan and methyl-triclosan peaks were quantified against an absolute standard using Empower 2 Chromatography Software (Waters).

Biofilm measurements

Six channels with biofilms received increasing concentrations of triclosan (from 0.05 to 500 µg/L). The remaining two, one was left as a control and another as a solvent control (0.05% of methanol). Biofilms were sampled after 48 hours of continuous toxicant exposure. Glass substrata were collected at random from each channel for chlorophyll-a fluorescence measurements (4 glass substrata), diatom cell viability (4 glass substrata), and bacterial abundance (live/dead bacteria, 4 glass substrata). The values obtained from the two non-exposed channels were used to calculate the mean value of the non-exposed communities. The % of deviation from this mean value in all the parameters measured was calculated (referred to as % of control) and used for graphical representation.

Chlorophyll-a fluorescence measurements. Chlorophyll-a in vivo fluorescence measurements were determined with the PhytoPAM fluorometer (Heinz Walz). The samples were dark adapted for 20 minutes, and the minimum fluorescence yield was recorded (F0). The minimum fluorescence yield of a dark adapted cell is proportional to its chlorophyll-a concentration (Serodio et al., 1997; Rysgaard et al., 2001). Thereafter, the samples were light adapted for 20 minutes and then exposed to saturating pulses of actinic light, provided by the instrument, in order to obtain the maximum fluorescence yield (Fm'). The effective PSII quantum yield was then calculated following Genty et al. (1989)

(Yeff = (Fm'-F0)/Fm'). PSII quantum yield is defined as a measure of the photosynthetic efficiency of the community (Schreiber, 2004). Using the parameters obtained, non-photochemical quenching (NPQ) was calculated following Bilger and Björkman (1990) (NPQ = (Fm-Fm')/Fm'). This parameter represents the energy that is dissipated in non-radiative processes to protect the photosynthetic apparatus.

Diatom cell viability. The colonized glass tiles were ultrasonicated to separate the aggregated cells without destroying the frustules following the method described in Morin et al., (2010). Counting of the samples was done using light microscopy at a 100X magnification. Data were recorded as cells per unit area of sampled substratum (number of cells/cm^2) and separated into empty cells (considered as dead) and cells with chloroplasts (considered as alive), whatever their colour (from pale yellow to green or brown) (Cox, 1996). Data are expressed as the ratio between live and dead diatom cells (LD diatoms ratio).

Bacterial abundance. The double staining Live/Dead BacLight Bacterial Viability Kit (Molecular Probes) was used to measure the abundance of live and dead bacteria in the biofilm samples. This double staining consists of a mixture of 3.34 mM SYTO® 9, which stains all the cells, and 20 mM propidium iodide, which only penetrates cells that have damaged cell membranes (Freese et al., 2006). Twenty random microscopy fields were counted for each sample using epifluorescence microscopy at a magnification of 1000X. Data are expressed as the ratio between live and dead bacterial cells (LD bacterial ratio).

Data analysis

Differences in photosynthetic efficiency and minimum fluorescence yield among the channels were tested using analysis of variance (one-way ANOVA). The colonised substrata were analysed before the exposure to check for the uniformity of the initial biofilm community. During the exposure, the control and the solvent control were compared statistically by a Student's *t*-test to determine if the measures were affected by the methanol. The relationship between triclosan concentrations and each endpoint was analysed by a Pearson correlation test. These analyses were performed using SPSS software (Version 17.0).

Following analyses were done using R 2.6.2 (R development Core Team, 2008). Concentration-effect curves were fit to data from the acute triclosan toxicity test and therefore used to calculate the non-effect concentration (NEC) and the effect-Concentrations (EC_{10} and EC_{50}, with the latter only being used when the inhibition of the endpoint was higher than 50%) of the respective parameters. A best-fit method was applied for the determination of the best regression model for each endpoint. Six different types of functions suitable to describe concentration-response data were selected and independently fitted to each set of data (package drc, Ritz and Streibig, 2005). In a first selection step, unreliable models were excluded based on the Akaike Information Criterion (AIC) (Akaike, 1974) as a measure of the goodness of fit of an estimated statistical model. All the models were ranked according to their AIC, with those with the lowest value being the best. The final selection of the model was based on the analysis of residuals. To detect departures from the fitted model, the residuals were plotted against the mean effects estimated from the fitted regression model. This analysis provides insight into the validity of a regression model and the error assumption (Scholze et al., 2001). The effect concentrations were determined by inverse regression. The NEC and the range of the NEC were determined by linear regression and by inverse regression, respectively, as described by Liber et al. (1992). The statistical significance for all of the analyses was set at $p < 0.05$.

RESULTS

Purification efficiency (WWTP plus reverse osmosis)

Triclosan concentrations decreased through the WWTP and the pilot plant (Table 1). The WWTP removed an important fraction of triclosan, although it was still detected after passing through the WWTP (from 0.488 µg/L in the influent to 0.071 µg/L in the effluent). Of the two systems installed in the pilot plant, the reverse osmosis system (RO) was the most efficient in terms of triclosan removal. The microfiltration system (MF) practically did not contribute to the removal of the toxicant, whereas the RO eliminated approximately 40% of the triclosan present.

Sampling site	Triclosan (µg/L)
Influent (1)	0.488 (0.084)
Effluent (2)	0.071 (0.002)
Permeate MF (3)	0.066 (0.003)
Concentrate RO (4)	0.122 (0.041)
Permeate RO (5)	0.029 (0.002)

Table 1. Triclosan concentrations throughout the wastewater treatment plant and the pilot plant. The concentrate of the RO system was rejected and returned to the head of the plant. Values are means of two replicates and standard error (in parenthesis).

Experimental study of triclosan toxicity

The water obtained from the pilot plant was mineralized, and nutrients were added. After these two modifications, the water was more similar to the water from the river Llèmena and thus was suitable for biofilm maintenance (Table 2).

	Output from the reverse osmosis	Input to the channels
Cond. (µs/cm)	198.5	355.8
	8.2	*10.9*
pH	5.9	7.5
	0.38	*0.22*
DO (mg/L)	5.2	8.4
	0.5	*0.7*
$PO4^{3-}$ (µg/L)	20.8	110.3
	1.6	*1.7*
NO^{3-} (mg/L)	3.4	4.8
	0.9	*1.2*
$SO4^{2-}$ (mg/L)	2.29	22.3
	0.49	*3.6*
Cl^{-} (mg/L)	49.3	90.5
	5.6	*3.4*
K^{+} (mg/L)	3.1	7.1
	1.7	*0.9*
Na^{+} (mg/L)	19.3	58.9
	2,8	*3.47*
Ca^{+} (mg/L)	7.7	21.7
	2.6	*3.4*
Mg^{+} (mg/L)	1.35	6.52
	0.5	*0.7*

Table 2. Physicochemical characteristics of the water at the output from the reverse osmosis system and at the input to the channels (after mineralization and addition of nutrients). Average values (n=3) and standard error (in italics) are given. (Cond: conductivity; DO: dissolved oxygen).

Differences were not occurring between the initial biofilm community before the addition of the toxicant ($ANOVA_{Yeff}$, p= 0.142, F= 1.870; $ANOVA_{F0}$, p= 0.378, F= 1.158). The photosynthetic efficiency of the initial biofilm community was 0.34 ± 0.05 (n=24) and the minimum fluorescence yield was 193.5 ± 15.93 (n=24).

During exposure, both triclosan and its main degradation product, methyl-triclosan, were measured in the channels. Methyl-triclosan concentrations were always below detection limits, and the measured concentrations of triclosan were more than 80% of the target concentrations. Therefore, nominal concentrations were used in further analysis.

The means in the control and solvent control channels were not statistically different (Student's test ; Yeff, p=0.105; NPQ, p=0.295; LD diatoms, p=0.681; LD bacteria, p=0.684), thus confirming the null effect of the 0.05% methanol used as solvent.

The short-term exposure to triclosan affected both the algal and bacterial components of the biofilm. Results obtained per each measured parameter are shown in Table 3.

	Control	Solvent control	0.5 µg/L	5 µg/L	25 µg/L	125 µg/L	250 µg/L	500 µg/L
Chl-a fluorescence measurements								
Photosynthetic efficiency (Yeff)	0,413	0,359	0,390	0,317	0,331	0,276	0,319	0,290
	0,030	*0,046*	*0,056*	*0,015*	*0,064*	*0,042*	*0,039*	*0,061*
Non-photochemical quenching (NPQ)	0,509	0,534	0,234	0,428	0,433	0,261	0,190	0,163
	0,016	*0,020*	*0,181*	*0,052*	*0,453*	*0,231*	*0,166*	*0,077*
Bacterial abundance								
Live bacteria (cells/cm^2)	8,34E+07	1,09E+08	1,15E+08	7,76E+07	5,83E+07	5,26E+07	7,03E+07	4,66E+07
	1,10E+07	*2,46E+07*	*1,93E+07*	*1,71E+07*	*1,30E+07*	*4,91E+06*	*2,61E+07*	*1,32E+07*
Dead bacteria (cells/cm^2)	1,18E+08	1,51E+08	1,71E+08	1,49E+08	1,54E+08	1,79E+08	2,76E+08	2,34E+08
	1,86E+07	*3,86E+07*	*2,82E+07*	*4,28E+07*	*2,62E+07*	*2,92E+07*	*7,24E+07*	*5,05E+07*
Live-dead bacterial ratio	0,712	0,723	0,672	0,525	0,377	0,299	0,249	0,197
	0,019	*0,022*	*0,023*	*0,037*	*0,051*	*0,049*	*0,041*	*0,014*
Diatom cells viability								
Live diatom cells (cells/cm^2)	1,32E+05	1,28E+05	1,12E+05	1,70E+05	1,15E+05	9,87E+04	1,11E+05	9,14E+04
	3,43E+04	*5,06E+04*	*3,37E+04*	*6,40E+04*	*3,71E+04*	*1,07E+04*	*3,95E+04*	*1,60E+04*
Dead diatom cells (cells/cm^2)	2,18E+04	2,04E+04	1,81E+04	2,76E+04	2,18E+04	2,02E+04	2,32E+04	2,68E+04
	6,24E+03	*7,90E+03*	*4,89E+03*	*8,73E+03*	*7,69E+03*	*2,88E+03*	*7,32E+03*	*7,19E+03*
Live-dead diatom ratio	6,097	6,273	6,180	6,070	5,448	4,976	4,767	3,505
	0,433	*0,692*	*0,667*	*0,410*	*0,925*	*1,021*	*0,784*	*0,591*

Table 3. Triclosan effects on the biofilm measured parameters. Values are mean and standard deviation (in italics) of 4 replicates.

The number of live bacteria decreased exponentially from the control channels to the most contaminated channels, where dead bacteria accounted for up to 85% of the bacterial population. A decrease was observed in the ratio between live and dead bacterial cells (Pearson correlation test, r= -0.791, p=0.0001). At the highest concentration, triclosan caused the live/dead bacteria ratio to decrease to 73 ± 2% with respect to the control (Fig. 2).

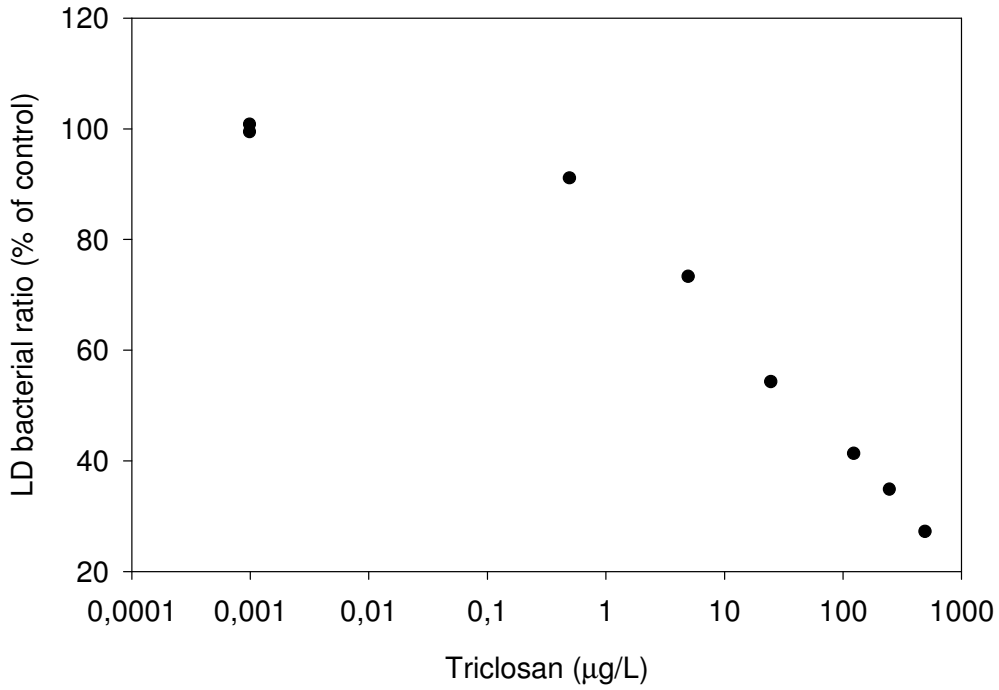

Fig. 2. Triclosan effects on bacterial cell viability, expressed as the ratio between live and dead cells. Values are given as percentage of the control.

Photosynthetic efficiency (Fig. 3a) was progressively reduced with increasing toxicant concentrations (Pearson correlation test, r= -0.443, p=0.011), with a maximal inhibition of 25 ± 15% at the highest concentration. A reduction was also observed in non-photochemical quenching (Pearson correlation test, r= -0.504, p= 0.020), which was reduced approximately 70 ± 15% of controls at the highest concentration (Fig. 3b). There were also effects on diatom cell viability. Live diatom densities decreased from approximately 130,000 cells/cm^2 in the controls (n=8) to 91,000 cells/cm^2 (n=4) at the highest triclosan concentration.

The ratio between live and dead diatom cells (LD diatom ratio) decreased with increasing triclosan concentration (Pearson correlation test, r= -0.792, p= 0.0001), with a reduction of approximately 55 ± 9% with respect to the controls at the most contaminated channel (Fig. 3c).

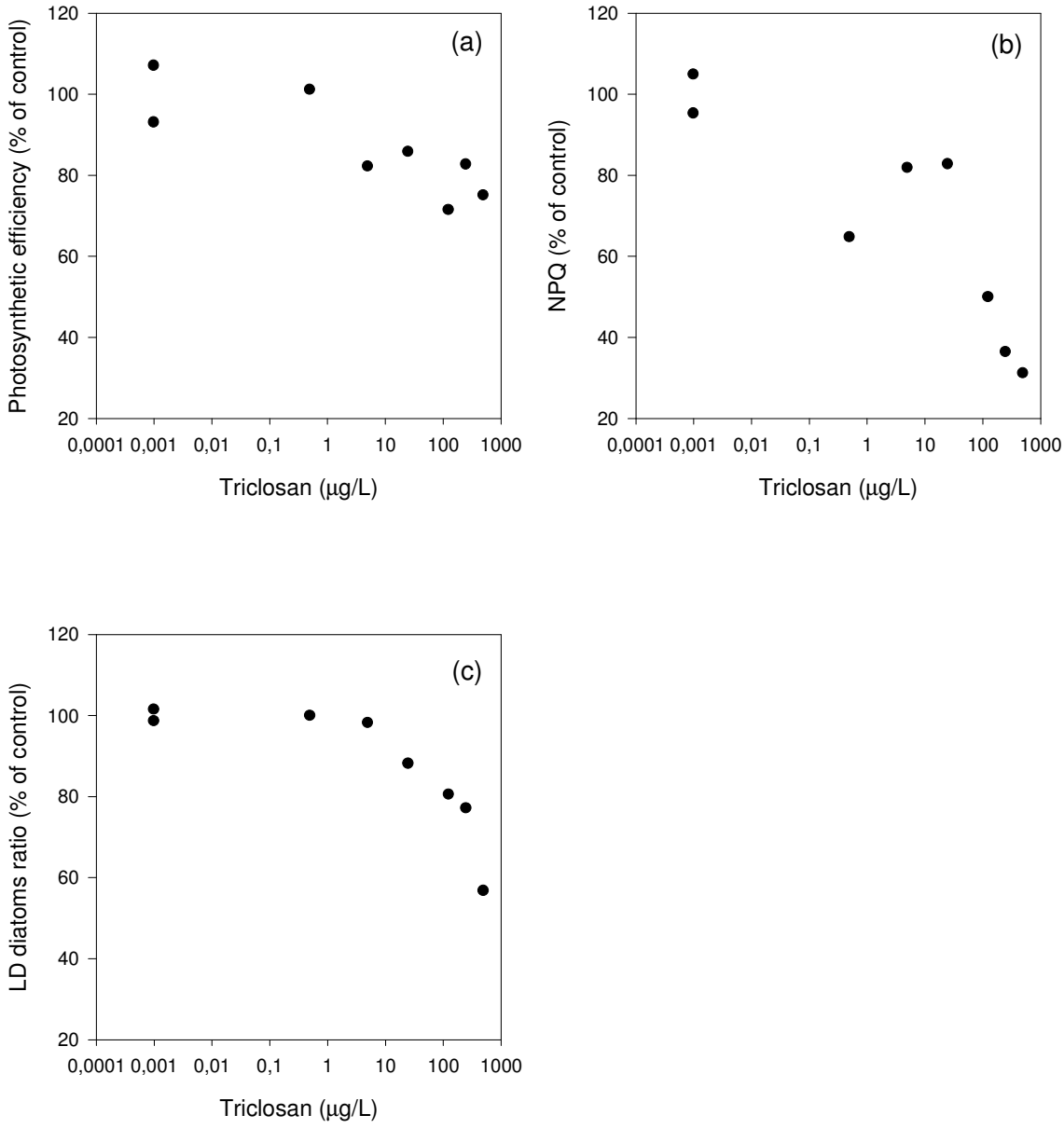

Fig. 3. Triclosan effects on: (a) photosynthetic efficiency (Yeff.), (b) non-photochemical quenching and (c) diatom cells viability (expressed as the ratio between live and dead cells). Values are given as percentage of control.

Estimated non-effect concentrations and effect concentrations (EC_{10} and EC_{50}) showed that the effects of triclosan on heterotrophs were the most pronounced (Table 4). The NEC and the EC obtained with the bacteria-related endpoint were all lower than those with algal-related endpoints. Non-photochemical quenching (NPQ) was the most affected of the algal-related endpoints, followed by photosynthetic efficiency (Yeff) and the ratio between live and dead diatom cells (LD diatom ratio). The EC_{50} was not determined for Yeff and the LD diatom ratio because inhibition of these endpoints was not higher than 50%. The EC_{50} obtained for the NPQ was over two times higher (110.97 µg/L) than the one obtained for the LD bacterial ratio (43.76 µg/L).

Endpoints	NEC (µg/L)	EC_{10} (µg/L)	EC_{50} (µg/L)	Best-fit model	Model parameters		
					φ_1	φ_2	φ_3
Phot. efficiency (Yeff)	0.42 [$9.1x10^{-8}$-84.3]	3.37 ± 4.74	-	Five-parameter log-logistic	-0.13	0.01	5.74
Non-phot. quenching (NPQ)	n.s.	1.31 ± 5.53	110.97 ± 29.42	Two-parameter log-logistic	-0.49	4.77	
LD diatom ratio	1.49 [0.006-26.5]	3.70 ± 0.64	-	Two-parameter log-logistic	-0.71	6.82	
LD bacterial ratio	0.21 [0.077-0.47]	0.56 ± 0.15	43.76 ± 4.75	Five-parameter log-logistic	-0.32	0.18	4.37

Table 4. Non-effect Concentrations (NEC) and effect concentrations (EC_{10} and EC_{50}) and the corresponding range (in brackets, for the NEC) and standard error (for the EC_{10} and EC_{50}), obtained by each endpoint. The best fit model and the estimated model parameters (φ_1, φ_2 and φ_3) of the each concentration-response function are given for concentrations expressed in µg/L.

DISCUSSION

The experimental design used in this study was based on flow-through experimental channels (Crossland et al., 1991; Navarro et al., 2000) that avoided the depletion of toxicant concentrations during the experimental period (Brooks et al., 1996). This design was particularly relevant for triclosan exposure because dioxins accumulate in the water due to the triclosan photodegradation (Aranami and Readman, 2007). The flow-through channel system permitted a controlled and persistent exposure and the detection of toxicity and calculation of NECs and ECxs values. The flow-through system avoided potential triclosan degradation that could affect the real exposure conditions and lead to higher NECs and ECxs values. The pilot plant efficiently removed triclosan from the effluent of the WWTP before redirecting this water to the channels. The majority of the removal of triclosan was achieved by the reverse osmosis system, which presented an effective barrier for triclosan, similar to the depletion that has been observed with a wide range of contaminants such as heavy metals (Qdais and Moussa, 2004) or pharmaceuticals (Kim et al., 2007).

Environmentally realistic concentrations of triclosan produced responses both in target (bacteria) and non-target (algae) organisms of the biofilm. The lower NEC obtained in this study was 0.2 µg/L. Therefore, effects should not be expected below this concentration. However, within the range of concentrations in which triclosan has been detected in rivers and WWTP effluents (0.027-2.7 µg/L, Chalew and Halden, 2009), it still would produce effects on biofilm communities. Direct effects of triclosan were detected on the bacterial community, as was expected due to the mode of action of the toxicant. Triclosan destroys enzymes involved in the synthesis of fatty acids in bacteria cell walls (Heat et al., 1999). Triclosan blocks lipid synthesis in *Escherichia coli* by specifically inhibiting the enzyme enoyl-acyl carrier protein reductase (ENR) (McMurry et al., 1998; Levy et al., 1999). Our results are in accordance with most publications that focus on the acute effects of triclosan on standard test organisms, such as the bacterium *Vibrio fisheri*. DeLorenzo et al. (2008) found an EC_{50} of 53 µg/L using this bacterial bioluminescence assay, whereas Farré et al. (2008) obtained an EC_{50} of 280 µg/L. Moreover, the mortality observed in the present study at low

concentration (NEC= 0.21 µg/L) may lead to changes in bacterial community composition in the river ecosystem. Similar results have been observed by Lawrence et al. (2009) in biofilms exposed to 10 µg/L of triclosan.

Triclosan toxicity to bacteria was higher than it was to algae ($EC_{50\ (LD\ Bacterial\ ratio)}$= 43.97 µg/L; $EC_{50\ (NPQ)}$= 110.97 µg/L). The LD bacterial ratio was also the one with the lower NEC and EC_{10}, confirming its higher toxicity.

Photosynthetic efficiency was the endpoint with the lowest NEC in algae, though it was not possible to determine the NEC for all the algal-related endpoints. The EC_{10} showed that the most affected algal endpoint was the NPQ, followed by Yeff and the LD Diatom ratio, confirming that photosynthetic parameters were the most sensitive to triclosan.

Triclosan toxicity toward photosynthetic efficiency in biofilm communities has barely been investigated. Franz et al. (2008) found effects of triclosan on algal photosynthetic efficiency, with the EC_{50} values ranging from 3.7 µg/L (chlorophyte culture) to 900 µg/L (periphyton communities).

The effects observed on non-photochemical quenching mechanisms suggested that triclosan produced damage in the photosynthetic apparatus. The decrease detected in the photosynthetic efficiency can be a precursor of a toxic effect that could take place at the structural level (Fai et al., 2007). Non-photochemical quenching is a collection of mechanisms designed to avoid photodamage of the photosynthetic apparatus under an excess of light. During photosynthesis inhibition, the NPQ increases in order to protect the photosynthetic apparatus from an excess of light reaching PSII that cannot be used for biochemical reactions such as photosynthesis (Juneau et al., 2001, Geoffroy et al., 2003). If an inhibition of the NPQ mechanism occurs, damage in the pigments where the NPQ takes place is expected. Fai et al. (2007) observed this inhibition when exposing a culture of *Selenastrum capricornutum* to increasing concentrations of the herbicide paraquat. Corcoll et al. (2010) reported a decrease of the NPQ mechanisms in natural periphyton communities chronically exposed to Zn. This was also observed in our experiments, especially at the highest concentrations tested, where the NPQ was approximately 70% lower than in the controls.

Structural effects on algae resulted from increasing concentrations of toxicant and were also observed from the decrease in the ratio between live and dead diatom cells. In the present study, NEC value for LD diatom ratio (1.49 µg/L)

was close to environmental concentrations and in accordance with the low triclosan concentrations found to cause changes in algal community during long-term exposure. Lawrence et al. (2009) found a reduction in algal biomass together with a shift in algal composition in biofilms exposed to concentrations of 10 µg/L during 8 weeks. Changes in algal communities exposed to triclosan have been also reported by Wilson et al. (2003), who found a consistent decline in final richness of algae in an experiment with natural algal assemblages exposed to low concentrations of triclosan (from 0.015 to 1.5 µg/L). Changes in the algal community in our study were reflected in the ratio between live and dead diatom cells. Though LD diatom ratio is not widely used in ecotoxicological studies, the present study confirmed that this technique can provide reliable results and it is easier to apply than the relative abundances of species or staining protocols (Morin et al., in press).

The common toxicity of triclosan to algae and bacteria might be attributed to the strong bacterial-algal link that exists in fluvial biofilms. Though triclosan directly damages the heterotrophic compartment, the algal component is strongly linked to bacteria (Stock and Ward, 1989; Rier and Stevenson, 2002) and might be affected because of the common use of space and resources within the biofilm (Carr et al., 2005).

However, direct effects of triclosan on algae are also possible. It is known that triclosan inhibits the formation of fatty acids by specifically inhibiting the enzyme enoyl-acyl carrier protein reductase (ENR). This enzyme has been found in bacteria, fungi and also in higher plants (Franz et al., 2008). Though it is unknown if algae have this specific target enzyme, several studies have investigated the effects of this compound on axenic algal cultures (Capdevielle et al., 2008) and observed that the EC_{50} values for 5 microalgal species ranged from 0.7 to > 66 µg/L. The absence of bacteria in these studies would suggest that the adverse effects were directly caused by triclosan. Moreover, in the case of triclosan there is an apparent baseline toxicity or narcosis (Verhaar et al., 1992) that could contribute to the toxicity acting non-specifically by disturbing the functioning of cell membranes (van Wezel and Opperhuizen, 1995; Franz et al., 2008).

Triclosan toxicity at environmentally realistic concentrations exerted significant effects on bacteria, which can be directly attributed to the mode of action of the

bactericide. Potential indirect effects on the algal component of the biofilm were also possible, although direct effects on algae need to be considered.

Triclosan survives several microbial degradation steps in WWTPs (Adolfsson-Erici et al., 2002) and, therefore, potentially reaches fluvial systems. Concentrations of triclosan in the receiving rivers can be similar or even higher than the NECs obtained in this study (Chalew and Halden, 2009), so the potential environmental risk of triclosan can be high. The presence of this compound in WWTP-dominated rivers justifies the growing concern about triclosan toxicity, especially in rivers frequently subjected to water scarcity, where the inputs of WWTP effluents can have a greater negative impact due to the low dilution capacity that characterizes these systems.

CONCLUSIONS

This study demonstrates the persistence of triclosan through WWTPs. The pilot plant equipped with microfiltration and reverse osmosis system was able to eliminate triclosan, but the reverse osmosis was the most effective in triclosan removal. Triclosan affected both bacterial and algal communities within the biofilm, although triclosan toxicity was higher for bacteria than algae. Effects on bacteria can be attributed to a direct mode of action of the bactericide, but effects on algae might be a result of indirect interactions within the biofilm community. However, direct effects on algae cannot be disregarded, as well as the potential of triclosan to act by narcosis on them. Based on the described effects, the potential environmental risk of triclosan is high especially in rivers where water scarcity results in low dilution capacity.

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CHAPTER V

Algal toxicity of diuron, propranolol, triclosan and their binary mixtures

ALGAL TOXICITY OF DIURON, PROPRANOLOL, TRICLOSAN AND THEIR BINARY MIXTURES

ABSTRACT

Two different concepts have been developed to evaluate the toxicity of a mixture: Concentration Addition (CA) and Independent Action (IA). While CA is generally accepted for mixtures of similarly acting chemicals, the most appropriate model for analysing mixtures of dissimilarly acting chemicals is a controversial issue. We studied the toxicity of diuron (D), propranolol (P), triclosan (T) and their binary mixtures on Scenedesmus obliquus. Applied singly, diuron was the most toxic compound ($EC_{50\text{-}48h}$ ~ 4 µg/L), followed by triclosan ($EC_{50\text{-}48h}$ ~ 40 µg/L) and propranolol ($EC_{50\text{-}48h}$ ~ 1300 µg/L). Diuron and propranolol mainly affected photosynthesis, whereas triclosan affected algal growth. The observed mixture toxicities were compared with the model predictions. The first mixture, (D+P), was accurately predicted by the CA model; the additive effects of this mixture was attributed to the similar actions of the compounds. The second mixture, (D+T), followed the predictions of the IA model, likely due to the inability of the compounds to cause the same toxicological effects. Both models underestimated the toxicity of the third mixture, (P+T), which showed a synergistic effect. Our study reveals that mixtures of chemicals can have a greater negative impact than predicted by models and demonstrates that the toxicity of a single substance can increase strongly in combination with others.

INTRODUCTION

One of ecotoxicology's primary goals within the last decade has been to clarify the cause-effect relationships between chemical pollution and ecological alterations in aquatic ecosystems (Halling-Sørensen, 2000; Cleuvers, 2005). Several publications focus more on the effect of single compounds on a single species and less on the effects of mixtures of chemical compounds. Considering that organisms are exposed to a multitude of toxicologically and structurally different chemical compounds in polluted freshwater systems (Junghans et al., 2006), it is essential to fill this knowledge gap by experimentally testing the combined effects of toxicants on biota.

Chemical pollution comprises a wide range of substances often released into the environment. Complex mixtures with varying constituents, concentrations and ratios are frequently detected in polluted rivers. Experimental testing of all possible combinations is simply impossible. Hence, modelling approaches for the prediction of mixture toxicity are needed (Arrhenius et al., 2004). Two different concepts have been developed to predict the toxicity of a mixture: Concentration Addition (CA) (Loewe and Muischnek, 1926; Loewe, 1927) and Independent Action (IA) (Bliss, 1939). The first concept assumes that each component contributes to the mixture toxicity, which is attributed to chemicals having the same mode of action (i.e., same biological pathway or same molecular target). The second concept assumes that only the components present in the mixture at a dose provoking and effect if applied separately contribute to the mixture toxicity.

Most of the current studies focus on the effects of mixtures of similarly acting compounds (Faust et al., 2001; Junghans et al., 2003a; Cleuvers, 2005) These studies demonstrate a good predictability with the CA model. Whether CA is more appropriate for mixtures of dissimilarly acting chemicals is still under discussion (Backhaus et al., 2000). Although numerous studies focus on the predictive power of CA, studies on the capabilities of IA are less abundant. There are some reports in the literature of IA applicability in mixtures composed strictly of dissimilarly acting chemicals (Faust et al., 2003; Backhaus et al., 2004). It has also been demonstrated that IA tends to calculate a lower combined effect compared to CA (Drescher and Bödeker, 1995). Thus,

irrespective of the similar or dissimilar actions of compounds in a mixture, several authors recommend using CA as a default approach for risk assessment (Faust et al., 2003; Backhaus et al., 2004).

This study aimed to understand the effects of three toxicants with differential modes of action and potential targets that are commonly found in polluted rivers (Chalew and Halden, 2009; Muñoz et al., 2009; Ricart et al., 2010a) on the green algae *Scenedesmus obliquus*. The investigated compounds were diuron (phenylurea herbicide), propranolol (β-blocker) and triclosan (antimicrobial agent). Diuron inhibits photosynthesis via the blockage of electron transport at the level of photosystem II (Van Rensen, 1989). Propranolol is widely used in human therapy for hypertension and heart failure. Though the mode of action of propranolol on algae is unknown, it has been found to be one of the most toxic β-blockers, primarily affecting photosynthetic processes (Beate et al., 2006). Triclosan is used as an antiseptic agent in a wide range of personal care products. Its mode of action on algae remains unknown, although some studies have reported a toxic effect on growth (Orvos et al., 2002, Ricart et al., 2010b).

The main objective of this study is to evaluate the toxicity of three compounds when applied singly and in binary mixtures and to test the applicability of CA and IA in mixtures composed of chemicals from different classes (herbicides, pharmaceuticals and personal care products). Mixtures of chemicals from different classes have gained little attention in ecotoxicological studies. These studies are mostly focused on mixtures of chemicals with a common mechanism of action. The three toxicants in this study do not have similar mechanisms of action. However, the term *similar action* is used in the broadest sense and is inclusive of all substances able to cause the same ecotoxicological response (Faust et al., 2001). Most of the published literature agrees that substances able to produce the same response follow the predictions of the CA model. It is also known that joint effects of chemicals that are not able to produce the same response are more accurately predicted by the IA model (De Zwart and Posthuma, 2005). Therefore, we hypothesise that the toxicity of the first mixture (diuron and propranolol, both affecting mainly photosynthetic processes), would be better predicted by the CA model. Furthermore, we also hypothesise that in the second (diuron and triclosan) and third mixtures (propranolol and triclosan), triclosan would mostly impact algal growth, while

diuron and propranolol would impact photosynthesis. Thus, we believe that both mixtures will follow the predictions of the IA model.

MATERIALS AND METHODS

Organisms

The green algae *Scenedesmus obliquus* SAG 276/3a was obtained from the culture collection of the University of Göttingen, Germany, and maintained in a 1.0 l chemostat system on a standard algal growth medium (Lürling and Beekman, 1999). The medium supply was set at a dilution rate of 1.0 d^{-1}. The chemostat was aerated with sterilised (0.2-µm membrane filter) and moistened air flowing from the bottom to the top of the vessel, ensuring optimal mixing of the algal population. The chemostat was illuminated with a constant incident irradiance of approximately 100 µmol quanta/m^2 s. Aliquots of exponentially growing unicellular green algae were derived from an overflow vessel connected to the chemostat and transferred into Erlenmeyer flasks containing 50 ml of algal growth-medium (Lürling and Beekman, 1999) for dose-response assays (*see below*).

Test chemicals

Diuron, propranolol and triclosan were tested for toxicity both separately and in binary mixtures. Diuron and propranolol produce analogous ecotoxicological responses (inhibition of photosynthesis), whereas triclosan effects algal growth. All of the compounds were of the highest purity available. Diuron (3-(3,4-Dichlorophenyl)-1,1-dimethylurea; CAS number: 330-54-1 with the structure formula C9H10Cl2N2O,) was purchased from Sigma-Aldrich (USA). The β-blocker propranolol (Propranolol hydrochloride; CAS number: 3506-09-0 with the linear formula C16H21NO2) was purchased from Dr. Ehrenstorfer (Germany), and triclosan, commercially named as Irgasan (5-Chloro-2-(2,4-dichlorophenoxy)phenol, CAS number: 3380-34-5 with the structure formula C12H7Cl3O2,), was purchased from Sigma-Aldrich (USA). Stock solutions of diuron and triclosan were prepared using methanol as a solvent. The maximum

amount of methanol in the flasks was 0.04%. Propranolol was diluted without the use of additional solvents.

Nominal concentrations of exposure concentrations under the conditions of the algal toxicity test were checked by LC-MS/MS.

Amber glass bottles pre-rinsed with ultra-pure water were used for sample collection. In each dose-response test, 25 mL of each concentration were collected.

The LC analyses were performed using an Agilent 1200 LC coupled to an Agilent G6410A QQQ mass spectrometer equipped with an ESI source. Chromatographic separation was achieved with a Zorbax Eclipse AAA column (4.6 x 75 mm., particle size 3.5 µm) supplied by Agilent (USA), by following the procedure described by Vanderford et al., (2003), Gros et al. (2006) and Mezcua et al. (2006).

The analyses were performed using water with 0.1% (v/v) of formic acid as eluent A and acetonitrile with 0.1% (v/v) of formic acid as eluent B. The elution gradient started as 65% of eluent B while keeping isocratic conditions for 3 minutes. Then eluent B was increased to 80% in two minutes and was held for five minutes. Finally, initial conditions were reached again in two minutes and maintained for three minutes. The sample injection volume was 10 µL. The flow rate was 0.4 ml/min, and the temperature of the column was 35ºC.

The LC-MS/MS was operated with an ESI source. Nitrogen was used as the drying and collision gas. Calibration standards were dissolved in methanol for the analysis, and the measured amount was calculated against a standard curve. Table 1 shows the parameters of the LC-MS/MS analysis.

Target compound	Time (min)	Scan type	Polarity	Delta (mV)	Precursor Ion	MS1 Res	MS2 Res	Dwell	Frag-mentor (V)	Collision Energy (V)
Diuron	2.9	MRM	Positive	400	233	Unit	Widest	200	74	28
					233	Unit	Widest	200	74	24
Propranolol	0	MRM	Positive	0	260	Unit	Unit	200	81	16
					260	Unit	Unit	200	81	16
Triclosan	6	MS2Sim	Negative	0			Unit	200	70	

Table 1. Detector settings for the LC-MS/MS analysis of diuron, propranolol and triclosan.

Dose-response tests

Algal cells were inoculated in 100-mL Erlenmeyer flasks containing 50 mL of the standard algal growth medium. Flasks were closed with a cellulose plug. The chlorophyll-*a* content of the initial algal cultures was 24.3 ± 3.5 µg/L (n=18). The photosynthetic efficiency was 0.46 ± 0.05 (n=18), and the total biovolume was $4.8 \cdot 10^6 \pm 1.4 \cdot 10^6$ μm^3/mL (n=18).

The dose-response assays were performed in the same way for both individual toxicants (Diuron (D), Propranolol (P) and Triclosan (T)) and the mixtures (Mix 1: D+P, Mix 2: D+T and Mix 3: P+T). In each binary mixture, the components were present in the molar ratio of their individual EC_{50} values. The resulting composition of the first mixture was 1.26% diuron and 98.74% propranolol. The second mixture was composed of 6.12% diuron and 93.88% triclosan, and the third mixture was 95.05% propranolol and 4.95% triclosan.

Each dose-response consisted of six increasing toxicant concentrations. Diuron was tested at the concentrations of 0.1, 0.25, 0.75, 2.5, 7.5 and 25 µg/L. Propranolol was applied at 0.5, 50, 250, 750, 2000 and 5000 µg/L, and triclosan toxicity was evaluated at the concentrations of 0.1, 0.3, 1, 2.5, 25 and 150 µg/L. For the mixtures, a fixed-ratio design was used in which the mixture ratio was kept constant and the overall concentration of the mixture was systematically varied (Arrhenius et al., 2004). Concentration-response data presented in this study always refer to analytically validated initial conditions.

In each test, controls, solvent controls and treatments were run for 48 hours in triplicate. The flasks were placed on a rotating shaking device at 24ºC in continuous light (100 µmol quanta/m^2

s). After 48 hours of toxicant exposure, a set of endpoints was used to assess toxicity: photosynthetic efficiency, chlorophyll-*a* concentration and biovolume concentration.

Photosynthetic efficiency. *In vivo* chlorophyll fluorescence measurements were done with the PhytoPAM (Pulse Amplitud Modulated) fluorometer (Heinz Walz, Germany) at room temperature. Samples were exposed to actinic light provided by the instrument, reaching a steady-state of electron transport. The application of saturating light pulses gave F'm (maximum fluorescence in the

light) and F (steady-state value of fluorescence). The efficiency of PSII (effective PSII Quantum Yield) was then estimated, following Genty et al. (1989): Yeff = (Fm'-F) / Fm'. The percentage of inhibition of photosynthetic efficiency was calculated and used to estimate the EC_{50}.

Chlorophyll-a *content*. A total of 25 mL of each sample were filtered (GF/C filters, Whatman, UK) for chlorophyll-*a* analysis. The filters were stored frozen (-20ºC) prior to analysis (maximum one month). Afterward, chlorophyll-*a* was extracted with 80% ethanol. The samples were placed in a water bath (Memmert W350, Germany) at 75ºC for five minutes. Immediately, the samples were cooled down using ice water and finally centrifuged for five minutes (3000 rpm at 5ºC, Harrier 18/80r, UK). Subsequently, the chlorophyll-*a* concentration was estimated from spectrophotometric measurements. We followed the Dutch standard protocol (NEN6520) based on the ethanol extraction spectrophotometric method with a phaeopigment correction as described by Moed and Hallegraeff (1978). The percentage of inhibition with respect to the controls was then calculated and used to estimate the EC_{50}.

Total biovolume. A total of 100 µL of each sample were diluted into a 10 mL electrolyte solution (Casyton, Innovatis, Germany), and the total number of particles (particles/mL), the mean particle volume (μm^3) and the total biovolume (μm^3/mL) were determined in the size range of 0 to 50 µm by the Casy Counter (capillary 150 µm orifice width, CASY. Model TT, Innovatis, Germany). The mean particle volumes (MPVs) were calculated from the number of particles and the total biovolume determined by the Casy Counter. The MPV reflects the morphological appearance of the test organism, which varies from being unicellular to colonial (Lürling, 2006). Because no differences were detected in the MPV, this endpoint was not further addressed in this study. Only the total biovolume was included in the analysis, instead of the total number of particles, because it reflects biomass more accurately. The percentage of inhibition of the total biovolume (BVL) was calculated and used to estimate the EC_{50}.

Data analysis

Toxicant effects were tested for each dose-response using analysis of variance (ANOVA, one single factor (toxicant concentration) with three replicates). Statistical significance was set at $p<0.05$. These analyses were performed using the SPSS program (v.17.0).

The following analyses were done using R 2.6.2 (R development Core Team, 2008). Concentration-effect curves were fit to data from each dose-response test and were used to calculate the EC_{50}. A best fit method was applied for the determination of the best regression model for each endpoint. Six different types of functions suitable to describe concentration-response data were selected and fitted independently to each set of data (package drc, Ritz and Streibig, 2005). In the first selection step, unreliable models were excluded based on the Akaike Information Criterion (AIC) (Akaike, 1974) as a measure of the goodness of fit to an estimated statistical model. All of the models were ranked according to their AIC, with those having the lowest value being the best models. The final selection of the model was based on the analysis of residuals. To detect departures from the fitted model, the residuals were plotted against the mean effects estimated from the fitted regression model. This analysis provides insight into the validity of a regression model as well as the error assumption (Scholze et al., 2001). The EC_{50s} were determined by inverse regression. Confidence intervals for the effect concentrations were estimated using the bootstrap approach (Davison and Hinkley, 1997; R package Bootstratp R (S-Plus) Functions version 1.2-41, Canty and Ripley, 2009). The statistical significance for all of the analyses was set at $p<0.05$.

RESULTS

Effects of individual toxicants

The measured concentrations of diuron, propranolol and triclosan were close to the nominal concentrations. The maximal % of deviation below the nominal value was 21% for diuron, 22.1% for propranolol and 15.9% for triclosan.

The toxicity of the three compounds varied considerably with EC_{50} values and ranged over three orders of magnitude (Table 2). As expected from its mechanism of action, diuron was highly toxic to algae and had low EC_{50} values. All of the measured endpoints (photosynthetic efficiency (Yeff), total biovolume (BVL) and chlorophyll-*a* (Chl)) were progressively inhibited by increasing the concentration of diuron (ANOVA, Yeff: $p<0.001$, $F=413.6$; BVL: $p<0.001$, $F=40.79$ and Chl: $p<0.001$, $F=12.4$). Propranolol showed the least toxicity, although all of the endpoints were progressively affected with increasing concentrations of toxicant (ANOVA, Yeff: $p<0.001$, $F=1241$; BVL: $p<0.001$, $F=30.46$ and Chl: $p<0.001$, $F=7.741$). Differences in sensitivity were detected, and the most affected parameter was the photosynthetic efficiency. Triclosan did not affect the photosynthetic efficiency of algae, whereas the rest of the endpoints (total biovolume and chlorophyll-a) were inhibited (ANOVA, BVL: $p=0.038$, $F=2.865$; Chl: $p=0.044$, $F=2.756$).

Toxicant	EC_{50} (µmol/L)	EC_{50} (µg/L)
Diuron		
Photosynthetic efficiency	0.019 [0.004-0.075]	4.4 [0.9-17.5]
Total biovolume	0.014 [0.003-0.052]	3.3 [0.7-12.1]
Chlorophyll-*a*	0.023 [0.004-0.07]	5.4 [0.9-16.3]
Propranolol		
Photosynthetic efficiency	2.922 [0.42-7.636]	756.8 [108.8-1977.7]
Total biovolume	6.811 [4.254-9.983]	1764.0 [1101.8-2585.6]
Chlorophyll-*a*	5.260 [2.687-8.169]	1363.3 [695.9-2115.78]
Triclosan		
Photosynthetic efficiency	-	
Total biovolume	0.103 [0.059-0.324]	29.8 [17.1-93.8]
Chlorophyll-*a*	0.183 [0.037-0.589]	53.0 [10.7-170.5]

Table 2. Effective concentration values (EC_{50}) and the corresponding 95% confidence intervals in brackets.

Effects of pollutant mixtures

Measured concentrations of the toxicants were close to the nominal values. In the first mixture, the maximal deviations below the nominal concentration were 19.2% for diuron and 18% for propranolol. The concentrations of the toxicants present in the second mixture had maximal deviations from the nominal values of 19% for diuron and 8.1% for triclosan. For the last mixture, the deviations were 13.5% and 17.3% for triclosan and propranolol, respectively.

The first mixture was composed of diuron and propranolol. Statistical analyses confirmed a significant reduction in all of the measured endpoints (ANOVA, Yeff: $p<0.001$, $F=437.0$; BVL: $p<0.001$, $F=185.3$ and Chl: $p<0.001$, $F=27.87$). The mixture toxicity on the photosynthetic efficiency, total biovolume and chlorophyll-*a* content was accurately predicted by the CA model, while IA underestimated the toxicity (Fig. 1). This result was confirmed with the EC_{50} obtained for each endpoint (Table 3), where 95% confidence intervals overlapped with those predicted by the CA and differed from the ones predicted by IA.

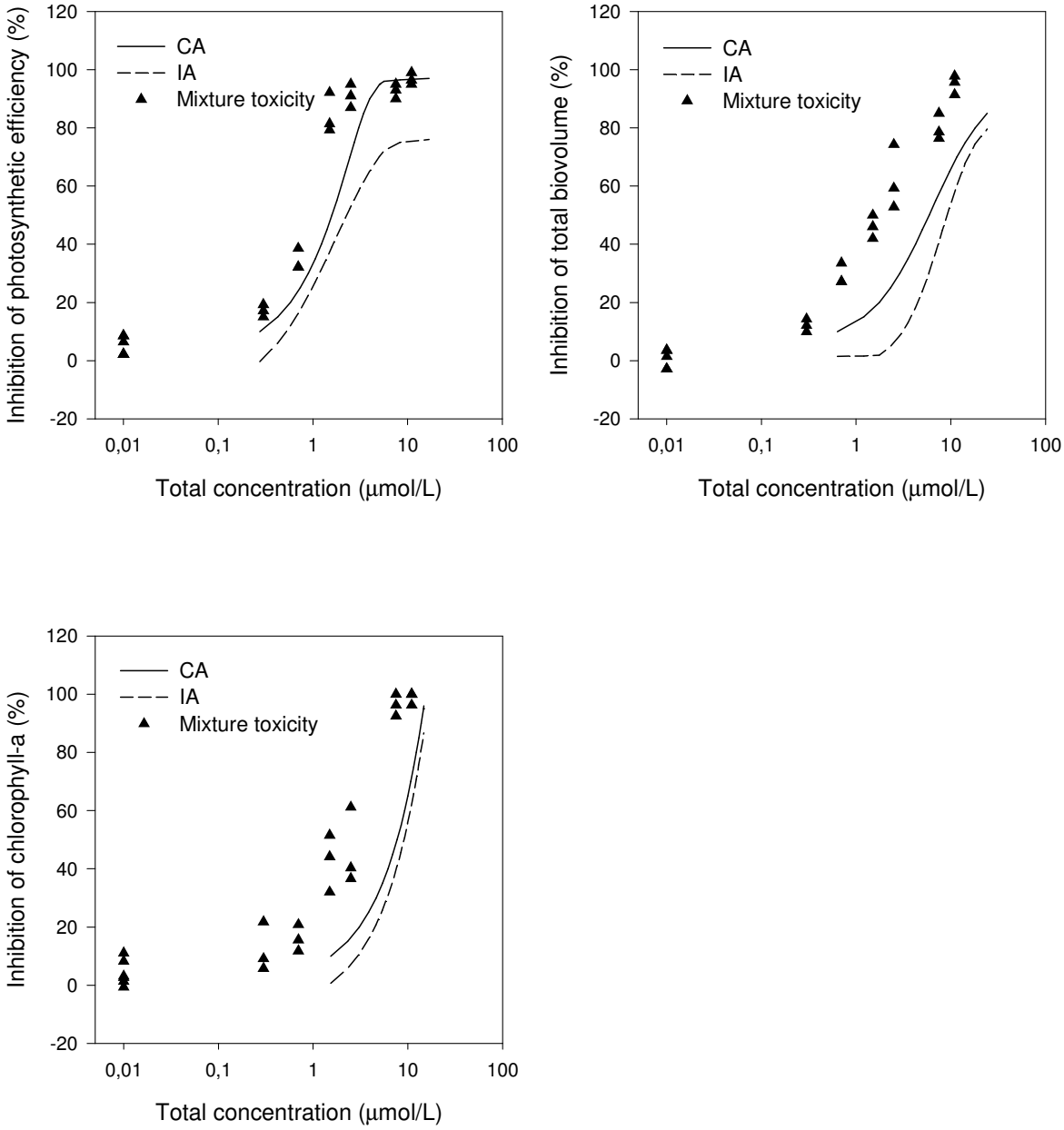

Fig. 1. Observed toxicity of diuron and propranolol compared to the predicted toxicity by the concentration addition (CA) and independent action (IA) models on the following endpoints: photosynthetic efficiency, total biovolume and chlorophyll-*a* content.

Endpoint	Predicted toxicity EC_{50} (µmol/L)		Observed toxicity EC_{50} (µmol/L)
	CA	IA	
Mix 1: D+P			
Photosynthetic efficiency	0.9 [0.2-2.7]	1.7 [1.1-2.7]	0.4 [0.1-0.9]
Total biovolume	5.5 [2.9-9.6]	7.4 [4.2-13.8]	1.4 [0.1-3.8]
Chlorophyll-*a*	7.4 [5.3-8.8]	9.3 [8.6-10.7]	6.7 [5.2-8.2]
Mix 2: D+T			
Total biovolume	0.4 [0.2-0.7]	0.7 [0.4-1.0]	0.6 [0.4-0.9]
Chlorophyll-*a*	0.2 [0.1-0.3]	0.4 [0.3-0.6]	0.5 [0.3-0.7]
Mix 3: P+T			
Total biovolume	6.4 [4.0-9.5]	7.4 [5.2-10.7]	1.8 [0.7-3.3]
Chlorophyll-*a*	10.2 [7.4-14.4]	12.2 [7.8-14.6]	3.1 [1.6-6.9]

Table 3. Predicted and observed effective concentrations (EC_{50} in µmol/L) and the corresponding 95% confidence interval for each mixture.

The combinatorial effect of diuron and triclosan (mixture 2) showed an important reduction of all of the endpoints with increasing doses of the mixture (ANOVA, Yeff: $p<0.001$, $F=487.2$; BVL: $p<0.001$, $F=79.92$ and Chl: $p<0.001$, $F=81.44$). When triclosan was in the mixture (mixtures 2 and 3), we could not predict its joint toxicity on photosynthetic efficiency because no effect of triclosan was detected when applied singly. The toxicity in the second mixture could be well calculated for the total biovolume and chlorophyll-*a* content using the IA model, which predicted lower toxicity than CA (Fig. 2). The 95% confidence intervals obtained for each endpoint overlapped with those predicted by IA and were different than the ones obtained by CA, thus confirming that IA accurately predicted the toxicity of the second mixture (Table 3).

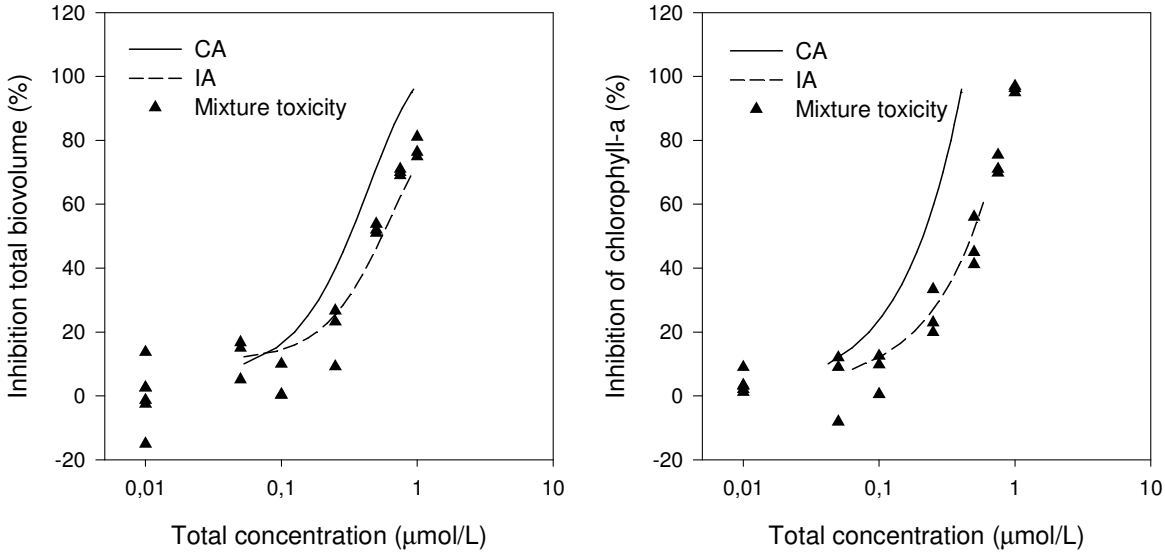

Fig. 2. Observed toxicity of diuron and triclosan compared to the predicted toxicity by the concentration addition (CA) and independent action (IA) models on the total biovolume and chlorophyll-*a* content.

The last mixture was composed of propranolol and triclosan. This mixture caused an inhibition of all of the measured endpoints (ANOVA, Yeff: $p<0.001$, $F=36.19$; BVL: $p<0.001$, $F=17.21$ and Chl: $p<0.001$, $F=62.59$). The observed toxicity of this combination was stronger than predicted by the models (Fig. 3). No overlap was detected between the 95% confidence intervals obtained by IA and CA. The obtained EC_{50}s were three times lower than the ones predicted by both models (Table 3). Thus, a significant difference between the observed mixture toxicity and both predictions was detected, indicating a synergistic effect between the two compounds.

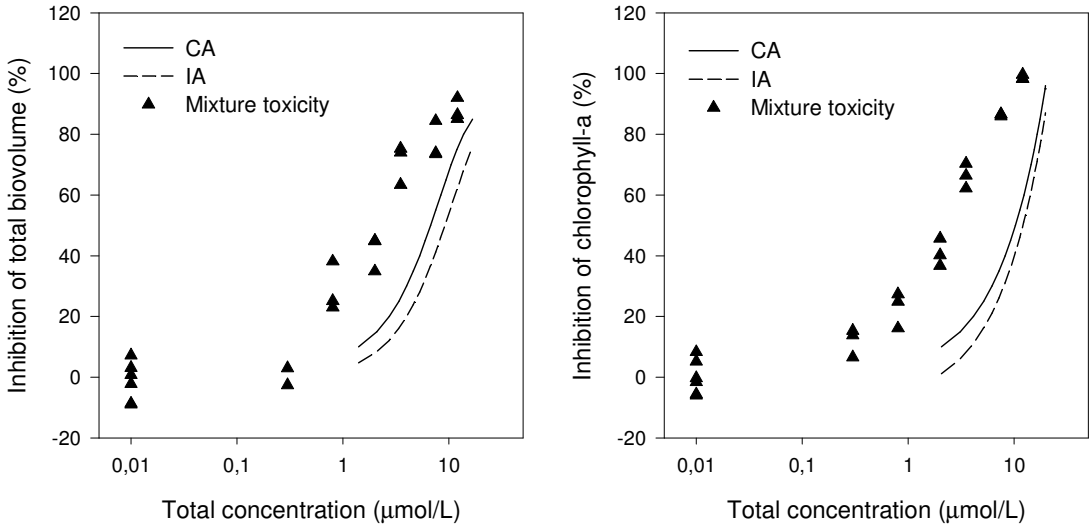

Fig. 3. Observed toxicity of propranolol and triclosan in compared to the predicted toxicity by the concentration addition (CA) and independent action (IA) models on the total biovolume and chlorophyll-*a* content.

DISCUSSION

The algal toxicity of the three compounds differed considerably. Diuron was the most toxic compound, followed by triclosan and propranolol. Increased diuron toxicity can be attributed to the mode of action of the diuron-type herbicides, which inhibits photosynthesis by preventing oxygen production (Wessels and Van der Veen, 1956) and blocking the electron transport chain in Photosystem II (PSII) of phototrophic microorganisms and higher plants (Moreland, 1967). Previous studies have shown effects on algal photosynthesis and growth, with similar EC_{50} values to the ones obtained in this study (Backhaus et al., 2004, Mc. Clellan et al., 2008).

The measured toxicity of triclosan and propranolol differed markedly between endpoints. Triclosan affected chlorophyll-*a* and total biovolume, indicative of effects on algal growth. Obtained EC_{50}s are in concordance with others reported elsewhere (EC_{50} of algal growth between 0.7 and 66 µg/L, Orvos et al., 2002). Propranolol primarily affected the photosynthetic efficiency, whereas total biovolume and chlorophyll-*a* were less affected. Cleuvers (2005) found that

propranolol inhibited algal growth with an EC_{50} of 0.7 mg/L (within the range of our results), and Beate et al. (2006) concluded that propranolol affected algal photosynthesis. Our experiments confirmed the similarity of diuron and propranolol toxicity (inhibition of photosynthesis) and the dissimilarity between triclosan and the other two compounds, which affected algal growth but not photosynthesis. The experiment therefore confirmed the different modes of action of triclosan versus diuron and propranolol.

Environmental concentrations of diuron can be close to the determined EC_{50}s (Azevedo et al., 2000; Ricart et al., 2010a), making relevant the effects of this compound in aquatic environments. However, environmental concentrations of pharmaceuticals and personal care products are below 1 µg/L (Fent et al., 2006; Santos et al., 2010), revealing the unrealistic potential acute effects of these compounds when tested in the laboratory. However, their continuous discharge into surface waters implies multigenerational exposure of aquatic non-target organisms to constant low doses of these products. Moreover, the inter-activity of chemicals present in the environment might play an important role in producing a toxic response that could increase the potential risk of these compounds. The combinatorial effect of diuron and propranolol could be calculated well with the CA model, which is appropriate for substances with a similar action (Pöch, 1993). Because the mode of action of propranolol on algae is unknown, it is not possible to attribute the behaviour of the mixture to a hypothetical identical mechanism of action for the two toxicants. However, assuming that all of the substances that cause the same toxicological response can be included under the term similar action (Faust et al., 2001), the effects of diuron and propranolol exposure on photosynthesis may explain their additive behaviour. Predictions according to IA were also evaluated for each endpoint. Toxicity was underestimated with IA by a factor of 4.3 for photosynthetic efficiency, 5.3 for total biovolume and 1.4 for chlorophyll-*a*. These differences are in the same range as reported by other studies (Faust et al., 2003; Arrhenius et al., 2004) and confirm that the toxicity of these compounds is better predicted by the CA concept. Mixtures of herbicides have been widely used in toxicity assessment, showing that predictions of CA are more accurate for similarly acting compounds (Faust et al., 2001; Junghans et al., 2003a; Junghans et al., 2003b; Arrhenius et al., 2004). Other studies using mixtures of

single class chemicals (i.e., β-blockers, Cleuvers, 2005; anti-inflammatory drugs Cleuvers, 2004) achieve similar conclusions. The algal toxicity of the diuron and triclosan mixture was accurately predicted by the IA model, according to their dissimilar action (Greco et al., 1995). Diuron primarily affects the electron flow in photosynthesis, while triclosan does not affect this process. The CA model overestimated the toxicity of the mixture of diuron and triclosan by a factor of 1.5 for total biovolume and 2.5 for chlorophyll-*a*. Faust et al. (2003) was able to demonstrate the predictability of a mixture of 16 dissimilarly acting chemicals (herbicides, antibiotics, disinfectants and fungicides) by IA. A mixture of priority pollutants, mostly with unknown modes of action, has also been reported to follow the predictions of IA (Walter et al. 2002).

We predicted that IA would be a more appropriate model for the mixture of propranolol and triclosan due to their different modes of action (Greco et al., 1995). However, a synergistic effect was observed, and both the CA and IAmodels predicted lower toxicity than was observed. Effects on total biovolume and chlorophyll-*a* were underestimated by a factor of 3.5 by the predictions of the CA model, while IA underestimated the observed toxicity by a factor of 4.1 for total biovolume and 3.9 for chlorophyll-*a*. These results confirm that dissimilarly acting chemicals can exhibit synergy. Synergistic effects are attributable to the interactions between the toxicants (Munkegaard et al., 2008) and have been reported mainly in mixtures of similarly acting compounds. Yang et al. (2008) observed synergistic interactions with a mixture of antibacterial agents, whereas Cleuvers (2005) detected an underestimation of both models in a mixture of three β-blockers when applied at the range of EC_5 to EC_{20}. Synergistic interactions were also detected in a study with mixtures of antifouling biocides (Fernández-Alba et al., 2002).

It is accepted that all chemicals, regardless of whether they have a specific mode of action, exert non-specific toxic effects. This baseline toxicity (Verhaar et al., 1992) exhibits a common mechanism, called narcosis, to *a priori* dissimilarly acting chemicals (Altenburger et al., 2004) that exert their influence non-specifically via membrane perturbation (De Zwart and Posthuma, 2005). It is well known that narcotic pollutants act additively in mixtures (Broderius et al., 1995; Loon et al., 1997). Both triclosan and propranolol may exert narcotic

effects as a common mode of action, which may result in a concentration additive behaviour (Altenburger et al., 2003).

A more specific mode of action of one of the components of the mixture (greater toxicity), in addition to the narcotic additive effects, may result in synergy (Altenburger et al., 2005). As a bactericide, triclosan inhibits bacterial fatty acid synthesis (Heat et al., 1999). Though the mode of action of this compound on algae is not clear, toxicity via membrane perturbation has been suggested (Franz et al., 2008; Ricart et al., 2010b). Thus, the specific effects of triclosan on biological membranes in addition to the narcotic action exerted by both triclosan and propranolol may explain their synergism.

The results of this study confirm that CA is a good model for predicting the toxicity of mixtures composed of similarly acting compounds. The results also show that the IA model accurately predicts the toxicity of mixtures composed of dissimilarly acting toxicants. However, the synergistic effects detected in the mixture of propranolol and triclosan provide evidence that the toxicity of dissimilarly acting chemicals is not always accurately predicted by IA. Additionally, their toxicity can be higher than expected from the available model predictions, which assumes that there are no interactions between chemicals (Munkegaard et al., 2008). Therefore, additional knowledge of a toxicant's mode of action as well as potential interactions between toxicants is necessary to design better models as a basis for improved risk assessment.

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CHAPTER VI

General discussion

GENERAL DISCUSSION

Pesticides in fluvial systems and their effects on biofilm communities

Different approaches have been used in the present investigation to better understand the relationships between pesticides occurrence and their effects on biological communities. Field and laboratory experiments have been used as a tool for investigating the functional and structural responses of biofilm communities to pesticides in fluvial systems. Field investigations have been focused on the lowest part of the river Llobregat, highly polluted by industrial and urban wastewaters, and by surface runoff from agricultural areas (Kuster et al., 2008a; Muñoz et al., 2009). Due to their Mediterranean climate influence, it experiences periodic floods and droughts (Sabater and Tockner, 2010). In this study 20 pesticides were detected in the river, and their presence affected the biofilm community in a variety of ways. The toxicity of pesticides depends on the type of pesticide and the species exposed (DeLorenzo et al., 2001). The majority of pesticides detected in the river Llobregat were herbicides, which are generally more toxic to phototrophic organisms due to their shared similarities with the target organisms of herbicides (weeds). Herbicide occurrence (triazines) was related with the distribution of the diatom communities, but not the invertebrate community.

The complexity and abundance of stressors that co-occur in the lower part of the Llobregat were efficiently approached by multivariate statistical analyses. These are reliable tools to determine the relationships between stressors and their effects on biofilm communities, as has been already shown in other studies (Muñoz et al., 2009; Guasch et al., 2009). The simultaneous sampling of chemical (pesticides) and biological parameters helped to elucidate the potential relationships between the two, not only by contrasting the presence/absence of species but also by including other characteristics of the community structure and function. Because the occurrence of indicator species is influenced by numerous factors other than contaminants, presence/absence data alone are insufficient for assessing the most severe forms of pollution. By selecting metrics that respond to different classes of stressors, results of

multivariate analyses may be useful for identifying specific stressors in systems receiving multiple perturbations (Clements and Newman, 2002). The inclusion of several biofilm metrics provided interesting results showing structural and functional changes in the algal component of biofilms. Specifically, chlorophyll-a, photosynthetic efficiency, and photosynthetic capacity responses were explained mainly by the presence of pesticides (phenylureas and organophosphates), thus indicating the existence of toxicological effects of these compounds under real-world conditions.

Field approaches on their own cannot be used to demonstrate causation. Experimental approaches can confirm the results obtained in the field. In this study, a system of experimental channels was designed to examine the long-term effects of realistic concentrations of diuron -one of the pesticides influencing biofilm communities in the river Llobregat- at biofilm community level. Diuron was selected as a "classical" toxicant with a known mode of action (inhibition of photosynthesis, Van Rensen, 1989) and widely reported to cause effects on algae (Pesce et al., 2006; Mc. Clellan et al., 2008; Tlili et al., 2008).

Effects of diuron on biofilms occurred at concentrations presently exceeded those reported in many river systems (Fig. 1). These effects were, as shown in Chapter III, most likely due to diuron mode of action. As hypothesised, biofilm response was dose-dependant for direct effects, allowing us to estimate the NEC and the EC_{50} of those endpoints directly affected by the presence of the herbicide (photosynthetic efficiency, chlorophyll-a and biovolume of the diatom community). The maximum diuron concentration detected during the Llobregat field study (Chapter II) was 1 µg/L (Fig. 1). Taking into account that the EC_{50s} obtained in the experimental study of diuron (Chapter III) ranged from 0.05 to 13.3 µg/L, direct effects of the herbicide are likely to occur in the river Llobregat. Waters with similar or higher concentration of diuron could therefore be at risk if exposed concentrations are higher than the NECs obtained in the experimental study (from $9.5 \cdot 10^{-6}$ to 0.1 µg/L). Actually, there are fluvial systems that have diuron concentrations within the NEC and the EC_{50} range detected in this study (Fig. 1). Diuron has been detected in rivers from Europe at a wide range of concentrations. Rodríguez-Mozaz et al. (2004) detected average diuron

concentrations of 0.24 µg/L in the river Llobregat. In a study in rivers in Portugal diuron concentrations ranged from 0.01 to 1.24 µg/L. Similar concentrations of diuron have been measured in Germany (Mc. Clellan et al., 2008). Higher levels of diuron have only been reported in some Frensh rivers surrounding vineyard areas (from 0.05 to 36 µg/L, Pesce et al., 2006; Tlili et al., 2008) and seem therefore particularly at risk.

The environmental quality standards for priority substances established that the maxim allowable concentration (MAC) of diuron in surface waters is 1.8 µg/L and the annual average (AA) should not exceed 0.2 µg/L (Fig. 1). These environmental quality standards are clearly set too high for protection of fluvial biofilms since the NEC range is $9.5 \cdot 10^{-6}$ – 0.1 µg/L.

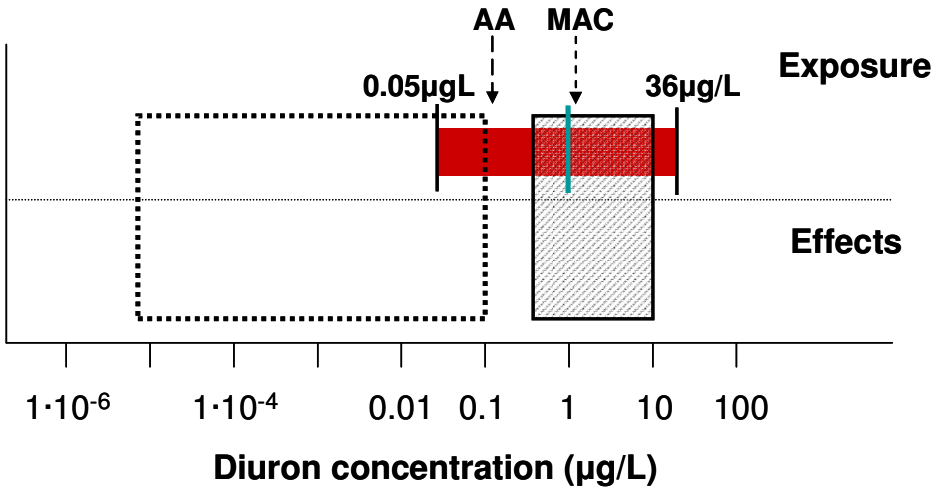

Fig. 1 Diuron concentrations in river systems and effects on algae. *Exposure*: The red horitzontal bar represents the range of diuron concentrations from several European rivers (Rodríguez-Mozaz et al., 2004; Pesce et al., 2006; Mc. Clellan et al., 2008; Kuster et al., 2008b; Tlili et al., 2008). The arrows represent the AA (Annual Average) and the MAC (Maximum Allowable Concentration) established by the EU Directive 2008/105/EC on environmental quality standards. The vertical blue line indicates the maximum concentration of diuron detected in the Llobregat field study (Chapter II). *Effects*: The open bar represents the range of the NECs obtained in the diuron study (Chapter III) and the dashed bar represents the range of the EC_{50s} obtained in the same study.

Although photosynthesis-inhibitors are only weakly toxic to non-photosynthetic organisms (DeLorenzo et al., 2001) indirect effects may exist. This was shown in the present Thesis with the non-target effects of diuron on bacteria. Indirect effects are an expression of the complex relationships within the biofilm and may be motivated by changes in the competitive relations within algae and bacteria. After one week of exposure 1.7 µg/L diuron was enough to reduce the number of live bacteria by 50%. Though 1 µg/L diuron was detected in the Llobregat field study, extrapolations from our results indicate that this concentration would result in a 30% decrease in the number of bacteria. These findings highlight the relevance of including the assessment of direct and indirect effects in pesticide toxicity assessment. With this information, the maximum allowable concentrations (MAC) of pesticides in the water can be established taking into account the potential for indirect effects on non-target components since it improves the necessary knowledge for the ecological management of fluvial systems.

Combining field observations with experimentation in the laboratory allows the application of the observation to field situations. Microcosm results provided a link between these two scenarios and, together with a multi biomarker approach allowed to confirm the field observations. Though it may not be possible to identify all possible field effects from laboratory-derived data, microcosm studies have the ability to integrate controlled and realistic exposure regimes with the assessment of endpoints at high levels of biological organization. Microcosm approaches also allow studying species interactions and indirect effects, which should provide better laboratory to field toxicity extrapolations.

Emerging pollutants in fluvial systems and its effects on biofilm communities

Natural waters still carry toxicants after receiving waste water treatment plant effluents (i.e. low concentrations of pesticides and pharmaceutical products have been detected in WWTP effluents; Kuster et al., 2008a). After evidences in experimental channels where a set of algal and bacterial endpoints allowed the

detection of toxicant effects at biofilm community level (Chapter III), the same experimental approach was applied to triclosan (Chapter IV). However, triclosan characteristics required some modifications in the experimental system. The need of water free of triclosan implied the installation of the channels system in a WWTP equipped with microfiltration and reverse osmosis that continuously supplied purified water to the channels. Also, the channels were based in a flow-through system that avoided the depletion of the toxicant, its photodegradation and the accumulation of dioxins in the water and permitted a controlled and persistent exposure of triclosan. Whit these modifications, it was possible to obtain data about Non Effect Concentrations (NEC) and Effect Concentrations (EC_x).

Triclosan effects on biofilms at level of EC_{10} occurred at concentrations that are present in the field (Fig. 2), while triclosan effects at level of EC_{50} (43 – 110 µg/L) are far from real world exposure conditions. Triclosan concentrations in the environment have been recently reviewed by Chalew and Halden (2009). They conclude that triclosan can be found in fluvial systems in a range of 0.03 – 2.3 µg/L (Fig. 2). According to the range of NECs obtained in this study (0.21 – 1.49 µg/L), some fluvial systems have higher concentrations than the NECs, therefore existing a potential risk for biofilms developing in those systems. Two studies have been recently performed in the Llobregat river and have shown that triclosan concentrations did not exceed 0.045 µg/L (Kuster et al., 2008a; Kantiani et al., 2008). The fact that this value is lower than the lowest NEC suggests that no potential effects could be expected due to triclosan in this river. However, the existence of other stressors and their potential interactions with triclosan should be taken into account in predicting the environmental risk.

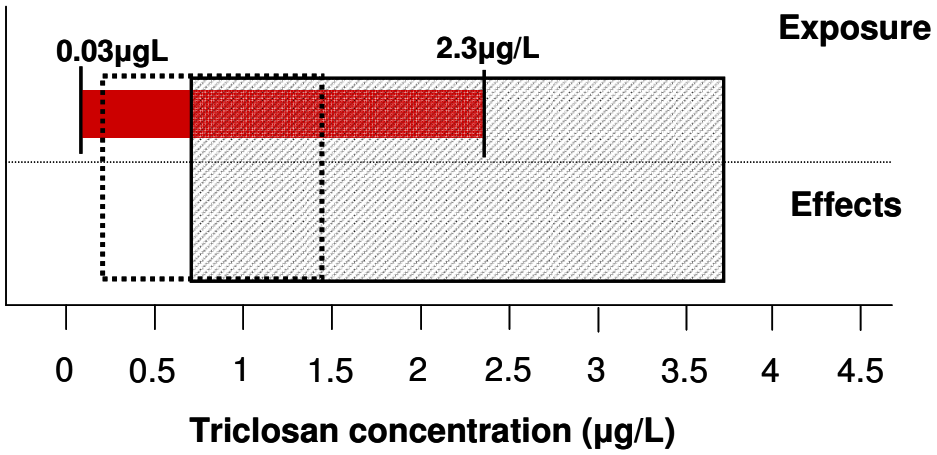

Fig. 2. Triclosan concentrations in river systems and effects on algae. *Exposure*: The red horitzontal bar represents the range of triclosan concentrations in fluvials systems reported in the literature (Chalew and Halden, 2009). *Effects*: The open bar represents the range of the NECs obtained in the triclosan study (Chapter IV) and the dashed bar represents the range of the EC_{10s} obtained in the same study.

Environmentally relevant triclosan concentrations affected both algal and bacterial components of the biofilm, though toxicity was higher in bacteria than in algae. In this case, bacterial toxicity was attributed to the triclosan mode of action, which inhibits the bacterial fatty acid synthesis (Heat et al., 1999). Although less pronounced, effects on the autotrophic component of biofilms were also remarkable, being the photosynthetic parameters the most sensitive to triclosan exposure. All the algal-related endpoints were affected, being progressively inhibited with increasing concentrations of triclosan, what would suggest a direct effect of the bactericide. However, the lack of information on the mode of action of triclosan on algae prevents from concluding that direct effects of triclosan on algae were detected. The verification of whether the high sensitivity of the algae results from direct or indirect effects demands future work.

To date, these gaps of knowledge that characterise emerging toxicants impede the evaluation and mitigation of the causes for an insufficient ecological status in many aquatic ecosystems, thus highlighting the need of more experimentation on their toxicity.

Another important element related to emerging compounds is the lack of regulation in the current legislation. In order to establish the acceptable contamination limits of these substances on fluvial systems, additional data are required. Ecological hazard of emerging compounds has to be predicted with relevant ecological tests. The experimental approach used in this study represents a first step, but experiments including long-term community exposures where direct and indirect effects are assessed are also needed.

Approaching multiple-stress situations in fluvial systems and their effects on algae with toxicant mixtures

Although there has been significant improvement in our ability to quantify the effects of chemical stressors over the past 20 years, we generally have a poor understanding of the effects of multiple-stressors and their interaction. In this study, 20 pesticides were detected at the lower part of the river Llobregat (Chapter II). In a simultaneous monitoring, 21 pharmaceutical products were found (Muñoz et al., 2009). Detergents (Petrovic et al., 2002) and heavy metals (Modamio, 1986) have been also detected. Thus, the biofilm communities developing in the river Llobregat are exposed to a multitude of different compounds with different modes of action.

The joint toxicity of toxicants with a similar action has been accurately predicted by the concentration addition (CA) model in a variety of studies (Faust et al., 2001; Arrhenius et al., 2004), which predicts an additive behaviour within the compounds in a mixture and therefore greater negative impact than single toxicants exposure (Loewe and Muischnek, 1926). This has been confirmed in this study with the mixture of diuron and propranolol, the two toxicants affecting photosynthetic processes on algae (Chapter V). However, in systems like the

river Llobregat where several toxicants with different mode of action or even unknown mode of actions co-occur, the assessment of their joint toxicity is extremely difficult. The independent action (IA) model has been found to be able to predict the toxicity of dissimilarly acting mixtures of chemicals (De Zwart and Posthuma, 2005), though the number of examples is still limited compared with the numerous studies focussing on the predictive power of CA. The results obtained in this Thesis with a mixture of diuron and triclosan (Chapter V) confirmed the ability of this model in predicting the joint toxicity of substances that do not cause the same ecotoxicological response.

Experimental designs where the effects of chemicals are investigated in combination can be used to identify potential interactions among stressors. The presence of one compound might enhance the presence of the other. That is the rationale behind synergisms, which have serious implications for risk assessment since they result in a higher toxic effect than predicted by models (Cedergreen and Streibig, 2005). Although rare, synergisms have been mainly reported for mixtures of similarly acting chemicals (Fernández-Alba et al., 2002; Cleuvers, 2005). The results obtained with the mixture of propranolol and triclosan (Chapter V) demonstrate that dissimilarly acting chemicals can exhibit synergistic influence beyond their individual influences. Thus, it might be concluded from our results that better models that consider the potential interactions between the toxicants that may lead to synergistic effects have to be developed.

An improved understanding of the effects of mixtures is necessary if we aim to predict responses to anthropogenic disturbances. However, mixture experiments are complex since they require exhaustive chemical analysis and several tests (including single and mixture exposures) conducted with exactly the same biological community. Due to this complexity, most of the mixture experiments are conducted with single-species tests, which provide interesting results but need to be complemented with community approaches. The composition of the mixture to be tested is an element that adds complexity to the already complex mixture experiments. Natural scenarios are changing and the composition of the mixtures is changing too. Changes in rainfall can

influence the presence of pesticides that reach fluvial systems by runoff. The human activity around the fluvial system may determine the entrance of compounds via WWTP. Water scarcity episodes may change the concentration of each toxicant present in a mixture due to less dilution capacity, which makes particularly relevant the assessment of mixture toxicity in Mediterranean fluvial systems.

Perspectives

Clarify the cause-effect relationships between toxicants and biological communities has been one of the main goals of ecotoxicology by the combined use of field and laboratory approaches. Nevertheless, field studies are still scarce. The simultaneous sampling of chemical and biological data and its later use in multivariate statistical analysis has been proved as an efficient tool in exploring the relationships between stressors and their effects. Since fluvial ecosystems are exposed to many stressors, it is necessary to investigate the influence of other toxicants to the biological communities. That might be potentially achieved using the field approach used in this Thesis. However, the need of searching for causality determines that laboratory approaches to investigate the response of the communities submitted to toxicants are also needed, especially with those toxicants that have not been yet investigated but they are detected in fluvial systems, such us emerging compounds.

Effects of diuron and triclosan on biofilm communities have been explored using experimental channels and a multiple-endpoint approach, but some questions are still open. Diuron was selected as a “classical” toxicant with known effects on its target organisms (algae). However, indirect effects on non target organisms (bacteria) were also detected. Thus, this approach could be used to evaluate the potential indirect toxicity of toxicants that are well known but, as happened with diuron, they are able to indirectly damage non target organisms. Triclosan was selected as an emerging toxicant, with less information available on its toxicity. Direct effects on bacteria have been reported, but algae were also affected. Whether the effects on algae were direct or a result of indirect interactions within the biofilm community remains unknown. Taking into account

the high persistence of triclosan through waste water treatment plants, it is essential to investigate the mode of action of triclosan on algae.

The fact that toxicants co-occur in fluvial systems implies that not only single exposures of toxicants have to be performed in the laboratory, but also multiple exposure experiments with complex mixtures of toxicants. The two models currently available to predict the toxicity of a mixture have shown a high level of predictability but still there are some conditions that cannot be predicted with these models, for example synergisms. These situations of higher toxicity than predicted are, of course, of great concern and have serious implications for risk assessment. For that reason, it is essential to develop models that include the chemical interactions between toxicants that lead to synergisms. It is also true that in contaminated environments, highly diverse biological communities are exposed to highly diverse chemical mixtures. Consequently, the assessment of mixture toxicity should also be applied on a community level rather than at single species level. The effects of mixtures on non target organisms should also be addressed. This approach would allow assessing if the effects of the mixture on non target organisms may even increase if those are added to the indirect effect that some chemicals may exert on complex communities, as has been shown in this Thesis. That would, of course, contribute with higher ecological realism and would improve the ecological management of fluvial systems.

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CONCLUSIONS

CONCLUSIONS

Primary and complex stressors in polluted Mediterranean rivers: pesticide effects on biological communities

1. The lowest part of the river Llobregat was highly polluted by pesticides, organophosphates and phenylureas being the groups detected at higher concentrations in the water. Although in lower concentrations, phenylureas and chloroacetanilides were the two groups with the highest levels detected in the sediment.

2. The diatom community was influenced by pesticides. Specifically, several diatom species were closely associated to the presence of triazine herbicides. Pesticides did not contribute to the ordination of the invertebrate fauna.

3. Pesticides determined structural and functional changes in the algal component of the biofilms. Chlorophyll-a, photosynthetic efficiency and photosynthetic capacity responses were explained mainly by the presence of phenylureas and organophosphates .

4. Multivariate statistical analysis allowed the assessment of the fractions of variance explained by environmental variables to perform a complete diagnosis of the stressor effects. However, experimental approaches are needed to confirm causality.

5. The inclusion of biofilm metrics complemented the results, although the sensitivity of each metric to particular stressors must be assessed with laboratory experiments before assigning causality.

Effects of low concentrations of the phenylurea herbicide diuron on biofilm algae and bacteria

6. Direct effects of diuron on target organisms (algae) as well as on non-target organisms (bacteria) could be well detected and quantified using experimental channels and a multiple-endpoint approach that included both functional and structural algal and bacterial endpoints.

7. Diuron exposure caused direct and persistent effects on algae throughout the exposure. These effects included an inhibition of the photosynthetic efficiency, an increase in chlorophyll-a levels, changes in the diatom community composition and a reduction in the biovolume of the diatom community.

8. Indirect effects of the herbicide were detected on the bacterial community. These effects included an increase of the bacterial mortality and an increase in the leucine-aminopeptidase extracellular enzyme activity.

9. Biofilm response was dose-dependant for direct effects, thus making possible the estimation of EC_{50} values for those endpoints who were directly affected by the presence of the herbicide (photosynthetic efficiency, chlorophyll-a and biovolume of the diatom community).

10. The responses of the bacterial community were transient, non dose-dependant and did not remain up to the end of the experiment; therefore they were motivated mainly by changes in the competitive relations within algae and bacteria and not by the specific concentration of the toxicant.

Triclosan persistence through wastewater treatment plants and its potential toxic effects on river biofilms

11. A flow-through system of experimental channels was an efficient tool for investigating triclosan toxicity on biofilms. It avoided the depletion of the toxicant in the channels and ensured a controlled and persistent exposure.

12. The pilot plant efficiently removed triclosan from the effluent of the waste water treatment plant. The majority of the removal was achieved with the reverse osmosis system.

13. Triclosan toxicity was higher in bacteria than in algae. Environmentally relevant concentrations of triclosan caused a marked increase in bacterial mortality.

14. The effects of triclosan on the algal component of biofilms were also remarkable. Photosynthetic efficiency was inhibited and non photochemical mechanisms decreased. Diatom cells viability was also affected with increasing concentrations of triclosan.

15. The multiple-endpoint approached allowed us to detect direct effects of triclosan on target organisms (bacteria) and non target organisms (algae), although the verification of whether the high sensitivity of the algae results from direct effects or not requires future work.

Algal toxicity of diuron, propranolol, triclosan and their binary mixtures

16. Diuron was the most toxic compound when applied as a single toxicant, followed by triclosan and propranolol. Diuron and propranolol toxicity was similar (inhibition of photosynthesis), whereas triclosan affected algal growth.

17. The joint toxicity of diuron and propranolol was accurately predicted by the concentration addition model.

18. The toxicity of the mixture composed by diuron and triclosan followed the predictions of the independent action model.

19. Both concentration addition and independent action models underestimated the toxicity of the mixture of propranolol and triclosan. This mixture had a synergistic effect and confirmed that mixtures of dissimilarly acting chemicals can have a greater negative impact than that predicted by the models.

20. Models that incorporate synergistic interactions should be developed and its predictions should be considered in establishing the acceptable contamination limits for aquatic ecosystems.