Universitat de Girona

INFLUENCE OF ANTHROPOGENIC POLLUTION ON THE PREVALENCE, MAINTENANCE AND SPREAD OF ANTIBIOTIC RESISTANCE IN AQUATIC MICROBIAL COMMUNITIES

Jessica Subirats Medina

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Doctoral Thesis

Influence of anthropogenic pollution on the prevalence, maintenance and spread of antibiotic resistance in aquatic microbial communities

Jessica Subirats Medina

2018

Doctoral Programme in Water Science and Technology

Supervised by: Dr. Carles Borrego Moré and Dr. José Luis Balcázar Rojas

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Thesis submitted in fulfillment of the requirements for the degree of Doctor from the University of Girona.

El Dr. Carles Borrego Moré, professor titular de l'Àrea de Microbiologia de la Universitat de Girona i professor d'investigació de l'Institut Català de Recerca de l'Aigua (ICRA) i el Dr. José Luis Balcázar Rojas, investigador del grup de "Qualitat i Diversitat Microbiana" de l'Àrea de Qualitat d'ICRA.

D E C L A R E M :

Que el treball titulat **"Influence of anthropogenic pollution on the prevalence, maintenance and spread of antibiotic resistance in aquatic microbial communities"**, que presenta la llicenciada en Biologia **Jessica Subirats Medina** per la obtenció del títol de Doctora, ha estat realitzat sota la nostra supervisió i compleix tots els requisits per a poder optar a la Menció Internacional.

I perquè consti i tingui els efectes que corresponguin, siguem aquest document

Dr. Carles Borrego Moré   Dr. Jose Luis Balcázar Rojas

la doctoranda
Jessica Subirats Medina

Girona, Abril 2018

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List of publications

This doctoral thesis is a compendium of research articles. The publications presented as chapters of this PhD thesis are listed below:

I. Balcázar, J.L., J. Subirats and C.M. Borrego (2015) The role of biofilms as environmental reservoirs of antibiotic resistance. *Frontiers in Microbiology* 6: 1216. (IF: 4.07, Q1). DOI: 10.3389/fmicb.2015.01216

II. Subirats, J., E. Royo, J.L. Balcázar and C.M. Borrego (2017) Real-time PCR assays for the detection and quantification of carbapenemase genes (bla_{KPC}, bla_{NDM} and bla_{OXA-48}) in environmental samples. *Environmental Science and Pollution Research* 24: 6710-6714. (IF: 2.74, Q2). DOI: 10.1007/s11356-017-8426-6

III. Subirats, J., X. Triadó-Margarit, L. Mandaric, V. Acuña, J.L. Balcázar, S. Sabater and C.M. Borrego (2017) Wastewater pollution differently affects the antibiotic resistance gene pool and biofilm bacterial communities across streambed compartments. *Molecular Ecology* 26:5567-5581 (IF: 6.08, Q1). DOI: 10.1111/mec.14288

IV. Subirats, J., X. Timoner, A. Sànchez-Melsió, J.L. Balcázar, V. Acuña, S. Sabater and C.M. Borrego (2018). Emerging contaminants and nutrients synergistically affect the spread of class 1 integron-integrase (intI1) and sul1 genes within stable streambed bacterial communities. *Water Research* 138: 77-85 (IF: 6.94, Q1). DOI: 10.1016/j.watres.2018.03.025.

V. Subirats, J., A. Sànchez-Melsió, C.M. Borrego, J.L. Balcázar and P. Simonet (2016) Metagenomics analysis reveals that bacteriophages are reservoirs of antibiotic resistance genes. *International Journal of Antimicrobial Agents* 48: 163-167 (IF: 4.30, Q1). DOI: 10.1016/j.ijantimicag.2016.04.028.

List of acronyms

AMR: antimicrobial resistance
ARB: antibiotic-resistant bacteria
ARDB: antibiotic resistance genes database
ARG: antibiotic resistance gene
BOD: Biochemical oxygen demand
CARD: Comprehensive Antibiotic Resistance Genes Databases
CDC: Centre for Disease Control and Prevention
CFU: Colony Forming Units
DDD: daily doses
DNA: deoxyribonucleic acid
DWTP: drinking water treatment plant
ECOFFs: Epidemiological cut-off values
EPSs: extracellular polymeric substances
ESAC-Net: European surveillance of antimicrobial consumption network
EU: European Union
EUCAST: European Committee on Antimicrobial Susceptibility Testing
EVs: extracellular vesicles
EWFD: European Water Framework Directive
GI: genomic island
GTA: gene transfer agent
HGT: horizontal gene transfer
HRT: hydraulic retention time
ICEs: integrate conjugative elements
*intI*1: gene encoding the integrase of class 1 integrons
IRs: inverted repeats
IS: insertion sequences
KPC: *Klebsiella pneumoniae* carbapenemase
MGE: mobile genetic element
MIC: Minimum Inhibitory Concentration
MRSA: methicillin-resistant *Staphylococcus aureus*
NDM: New Delhi metallo-β-lactamase
OTU: Operational Taxonomic Unit
qPCR: quantitative PCR
Sub-MIC: sub-inhibitory concentration
WHO: World Health Organization
WWT: wastewater treatment
WWTP: wastewater treatment plant

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Table S2. Top 10 OTUs contributing the most to differences between biofilms collected at -1 and 28 day of experiment (A) and those collected in treatments with and without emerging contaminants (B).

Summary

Antibiotic resistance (AR) is a natural phenomenon that confers bacteria the ability to survive and multiply in the presence of antibiotics. In the last decades, the overuse and misuse of antibiotics have accelerated the evolution of AR and its dissemination among bacterial pathogens thus limiting the options for an efficient antibiotic therapy. The natural environment also contributes to the evolution and spread of AR because aquatic systems are main receptors of both antibiotic-resistance bacteria (ARB) and antibiotic resistance genes (ARGs) from animal and human origin. Once both contaminants are discharged into the environment, their fate and dissemination are ruled by complex interactions with indigenous bacteria and the variety and concentration of antibiotic residues, among other pharmaceutical compounds, that pollute natural habitats. In this work, we investigated the main factors contributing the maintenance and spread of AR in aquatic microbial communities subjected to different anthropogenic pollution sources.

Quantitative PCR (qPCR) is a technique widely used for quantifying ARGs in environmental samples. We have designed and optimized three qPCR assays to quantify three genes conferring resistance to carbapenems (bla_{NDM}, bla_{KPC} and $bla_{\mathrm{OXA\text{-}48}}$) in complex environmental samples (*e.g.* biofilms, sediments). This tool has allowed us to determine that genes bla_{NDM} and bla_{KPC} are widespread in environments receiving anthropogenic pollution such as raw and treated wastewater and hospital effluents. Moreover, our results showed that biofilms represent the major reservoir of carbapenemase genes.

Wastewater treatment plants (WWTPs) are considered the main source of contamination of aquatic environments by ARB, ARGs, emerging pollutants and nutrients. In this regard, we carried out a study to assess the impact of raw sewage and treated wastewater on the composition of biofilm bacterial communities and their associated resistome (the collection of ARGs in a given environment). Our study revealed that the microbiological quality of both effluents was the main factor contributing to the observed changes on the composition of bacterial communities. Bacteria from raw sewage, including potential pathogens, were able to colonize the streambed biofilms and alter their resistome. In turn, treated wastewater showed a minor impact on the abundance of the analyzed ARGs without noticeable effects on the bacterial community composition.

In order to elucidate the contribution of both nutrient and emerging contaminants on the maintenance and spread of AR in streambed biofilms, we designed a controlled manipulate experiment to assess the impact of a combined regime of nutrient and a mixture of emerging pollutants. The latter increased the prevalence of resistance genes and mobile genetic elements without altering the biofilm bacterial composition. Moreover, the experiment design allowed us to demonstrate that nutrients synergistically affect the effect of emerging contaminants on the spread of ARGs within streambed bacterial communities.

Finally, a metagenomic approach was used to evaluate the contribution of bacteriophages as a reservoir of ARGs in the environment. The phage DNA fraction was found to be enriched in ARGs conferring resistance to antibiotics widely used in human medicine. Besides, our results showed that studies on phage genomics can lead to the discovery of novel ARGs variants.

Resum

La resistència als antibiòtics (RA) confereix al bacteri l'habilitat de sobreviure y multiplicar-se en presencia d'aquests fàrmacs. En les últimes dècades, l'abús i el mal ús dels antibiòtics han accelerat l'evolució de la RA i la seva disseminació entre bacteris patògens, reduint l'eficàcia de la teràpia amb antibiòtics. El medi ambient també contribueix a l'evolució i disseminació de la RA ja que molts sistemes aquàtics reben residus d'origen antropogènic contaminats amb bacteris resistents a antibiòtics (ARB) i gens de resistència a aquests (ARG) tant d'origen animal com humà. En el medi ambient, els destí i la disseminació dels ARB i ARGs depèn de diversos factors, com ara la interacció amb els bacteris residents i la presencia i concentració de residus farmacèutics, inclosos els antibiòtics. En aquest treball doncs, s'han estudiat els principals factors que contribueixen a mantenir i disseminar la RA en les comunitats bacterianes d'ambients aquàtics afectats per contaminació antròpica.

La PCR quantitativa (qPCR) és una tècnica àmpliament utilitzada per a la quantificació d'ARGs en mostres ambientals. En un primer treball es van dissenyar i optimitzar diferents assajos de qPCR per a quantificar tres gens que confereixen resistència als carbapenems (bla_{NDM}, bla_{KPC} i $bla_{\mathrm{OXA\text{-}48}}$) en mostres complexes (ex. biofilms, sediments). Aquesta eina ens ha permès determinar que els gens bla_{NDM} i bla_{KPC} son ubics en ambients que reben contaminació antròpica. A més, els nostres resultats van demostrar que els biofilms representen el major reservori de gens de resistència a carbapenems.

Les estacions depuradores d'aigües residuals estan considerades la major font de contaminació dels sistemes aquàtics per ARB, ARGs, contaminants emergents i nutrients. En aquest context, es va realitzar un estudi per avaluar l'impacte de la descàrrega d'aigües residuals tractades i no tractades en la composició de la comunitat bacteriana dels biofilms i el seu resistoma (entès com el conjunt d'ARGs en un ambient). Els resultats van revelar que la qualitat microbiològica de l'aigua dels efluents estudiats va ser el factor principal que va contribuir als canvis detectats en la composició de les comunitats bacterianes. Els bacteris procedents de les aigües residuals no tractades, incloent potencials patògens, van colonitzar els biofilms del llit fluvial i van alterar el seu resistoma. En canvi, les aigües residuals tractades van tenir un impacte menor en l'abundància dels ARGs analitzats sense efectes notables en la composició de la comunitat bacteriana.

D'altra banda, es va dissenyar un experiment per avaluar l'impacte combinat dels nutrients i els contaminants emergents (cinc antimicrobians) en la prevalença i dispersió de la RA en els biofilms dels llits fluvials. Els antimicrobians van fer augmentar la prevalença dels gens de resistència i els elements genètics mòbils sense alterar la composició de la comunitat bacteriana dels biofilms tractats. A més, el disseny experimental va permetre demostrar que els nutrients potencien l'efecte dels antimicrobians en la dispersió dels ARGs dins la comunitat.

Finalment, es va realitzar un estudi per metagenòmica per determinar la contribució dels bacteriòfags com a reservori d'ARGs. Els resultats van demostrar que l'ADN de la fracció vírica estava enriquida en ARGs que confereixen resistència a antibiòtics utilitzats en la pràctica clínica. Aquest treball evidencia, a més, que la metagenòmica és útil per descobrir noves variants d'ARGs.

Resumen

La resistencia a los antibióticos (RA) confiere a las bacterias la capacidad de sobrevivir y multiplicarse en presencia de estos fármacos. En las ultimas décadas, el abuso y mal uso de los antibióticos han acelerado la evolución de la RA y su diseminación entre bacterias patógenas, reduciendo la eficacia de la terapia con antibióticos. El medio ambiente también contribuye a la evolución y dispersión de la RA y a que muchos sistemas acuáticos reciben residuos de origen antrópico contaminados con bacterias resistentes a antibióticos (ARB) y genes de resistencia (ARGs) tanto de origen animal como humano. En el ambiente, el destino y la diseminación de ambos contaminantes depende de diferentes factores, como la interacción con las bacterias residentes y la presencia y concentración de residuos farmacéuticos, incluidos los antibióticos. En este trabajo, se han estudiado los principales factores que contribuyen a mantener y diseminar la RA en las comunidades microbianas de ambientes acuáticos afectados por contaminación antrópica.

La PCR cuantitativa (qPCR) es una técnica ampliamente utilizada para cuantificar ARGs en muestras ambientales. Así pues, se diseñaron y optimizaron tres ensayos de qPCR para cuantificar tres genes que confieren resistencia a carbapenemasas (bla_{NDM}, bla_{KPC} y $bla_{\mathrm{OXA\text{-}48}}$) en muestras complejas (biofilms, sedimentos). Esta herramienta nos ha permitido determinar que los genes bla_{NDM} y bla_{KPC} son ubicuos en ambientes que reciben contaminación antropogénica. Además, los resultados nos han permitido mostrar que los biofilms representan un gran reservorio de genes resistencia a carbapenemasas.

Las estaciones depuradoras de aguas residuales son la mayor fuente de contaminación de los sistemas acuáticos por ARB, ARGs, contaminantes emergentes y nutrientes. Se llevo a cabo pues un estudio para evaluar el impacto de las aguas residuales tratadas y sin tratar en la composición de la comunidad bacteriana de los biofilms y su resistoma (el conjunto de ARGs en un ambiente dado). Los resultados revelaron que la calidad microbiológica del agua de los efluentes estudiados fue el principal factor que contribuyó a los cambios detectados. Las bacterias del agua residual sin tratar, incluidos algunos potenciales patógenos, colonizaron los biofilms del lecho fluvial y alteraron su resistoma. En cambio, los efluentes tratados tuvieron un impacto menor en la abundancia de los ARGs estudiados y no alteraron la composición de la comunidad bacteriana.

Por otro lado, se diseño un experimento para evaluar el efecto combinado de los nutrientes y los contaminantes emergentes (cinco compuestos antimicrobianos) en la presencia y dispersión de la RA en biofilms fluviales. Los compuestos antimicrobianos aumentaron la prevalencia de los ARGs y los elementos genéticos móviles sin alterar la composición de los biofilms. Además, el diseño experimental permitió demostrar que los nutrientes potencian el efecto de los antimicrobianos en la diseminación de los ARGs en la comunidad bacteriana.

Finalmente, se llevo a cabo un estudio por metagenómica para determinar la contribución de los bacteriófagos como reservorio de ARGs. Los resultados demostraron que la fracción del ADN vírico estaba enriquecida en ARGs que confieren resistencia a antibióticos ampliamente utilizados en la práctica clínica. Además, este trabajo evidencia que la metagenómica es una herramienta útil para descubrir nuevas variantes de ARGs.

Chapter 1

General introduction

The use of natural products to treat infections is probably as ancient as humans and it is at the dawning of modern medicine. Although more recent, the most illustrative example of the usefulness of natural compounds as therapeutic agents is the discovery of penicillin by Alexander Fleming in 1928. Few years after its deployment as an antibacterial drug, penicillin revolutionized medical science and, over the last 70 years, it has saved millions of lives. Being conscious of the extreme adaptability of microorganisms to environmental challenges, Fleming warned that antibiotics could become a double-edged sword and predicted a time when antibiotics could become less effective:

"*The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.*" (Alexander Fleming's Nobel Lecture in 1945).

1. What is antibiotic resistance?

Antibiotic resistance occurs when one microorganism is able to grow in the presence of antibiotics. Although resistance to antibiotics was recorded even before the first clinical use of penicillin in the early 1940s, the emergence of this phenomenon has been exacerbated by the overuse and misuse of antibiotics in the last decades (WHO, 2011). The poor application of regulations to prevent and control bacterial infections together with the inappropriate use of antibiotics have also led to the selection of multridrug-resistant microorganisms. In fact, there are very few antibiotics nowadays for which resistance does not exist (Hover et al., 2018; Ling et al., 2015). As a consequence, about 700,000 people worldwide die every year from drug-resistant strains of common bacterial infections, according to a recent report by the UK Review on Antimicrobial Resistance (https://amr-review.org). The low effectiveness of available antibiotics also jeopardizes the viability of interventions that leave the patient highly exposed to bacterial infections, such as those under cancer chemotherapy and organ transplantation. It has been estimated that, if action is not taken, the number of deaths caused by antibiotic-resistant microbes could reach 10 million by 2050, with a cumulative cost of around 100 trillion USD (https://amr-review.org). Moreover, after the golden era of antibiotics (from 1940 to the early 1970s), the rate of antibiotic discovery has dramatically fallen (https://amr-review.org). The reasons for this discontinuity are various, namely: *i)* the discovery of new antibiotics is expensive and time-consuming, *ii)* the erroneous perception that infection diseases are not really a challenge to public health due to the success of antimicrobial therapy in the past, *iii)* pharmaceutical companies have shifted their investments to commercially lucrative disease area, such as oncology

(https://amr-review.org). The scenario is even worse in developing countries where the access to the latest generation drugs and the implementation of strategies to tackle antimicrobial resistance is highly limited (WHO, 2011). Antibiotic resistance is thus recognized by the World Health Organization (WHO) as one of the principal threats to public health worldwide.

2. Mechanisms of antibiotic resistance

The genetic plasticity of bacteria has allowed them to respond to a comprehensive range of environmental threats, including the presence of drug molecules that put at risk their viability. Bacteria can be intrinsically resistant to some antibiotics but they can also acquire resistance through mutations or by horizontal gene transfer (HGT).

2.1. Intrinsic resistance

Intrinsic resistance is the natural resistance to a specific antibiotic that most bacterial species possess as a consequence of inherent structural or functional characteristics and it is independent of antibiotic selective pressure (Cox and Wright, 2013). The most basic example of intrinsic resistance is the absence of the susceptible target for a particular antibiotic. For instance, the intrinsic resistance of some Gram-negative bacteria to a wide range of drugs is caused by the inability of these compounds to overpass the outer membrane and, therefore, to gain access to the periplasm (Tsuchido and Takano, 1988). The structure of the cytoplasmic membrane thus compromises the effectiveness of therapeutic treatment of infections caused by Gram-negative pathogens (Cox and Wright, 2013). Several studies have identified many genes responsible for intrinsic resistance to antibiotics of different classes, including aminoglycosides, fluoroquinolones and β-lactams (Blake and Neill, 2013).

Environmental organisms are also intrinsically resistant to different classes of antibiotics. For instance, soil-dwelling bacteria possess a broad repertoire of intrinsic resistance genes that predates the clinical use of antibiotics (D'Costa et al., 2006, 2011). Although these organisms do not represent a clinical threat by themselves, soil could serve as a vast reservoir for resistance with the potential to emerge in clinically relevant pathogens and represents a major concern to human health. Under this point of view, it is crucial to undertake studies aimed to understand the genetic basis of intrinsic bacterial resistance to provide clues about the new mechanisms that may emerge in clinical settings and how to combat them (Cox and Wright, 2013).

In some cases, intrinsic resistance to antimicrobial agents relies on the lifestyle of the organisms, as is the case of microorganisms forming biofilms. Biofilms constitute a multicellular lifestyle in which microorganisms grow attached to an inert surface embedded within a self-produced matrix of extracellular polymeric substances (EPSs) (Costerton et al., 1978). The complex structure of the polysaccharide biofilm matrix impedes the free diffusion of antimicrobial compounds and allows bacteria to exhibit 10 to 100 times less susceptibility to a particular antimicrobial agent compared to free-living bacterial cells (Gilbert et al., 2002).

2.2. Acquired resistance

Acquired resistance is the consequence of successful gene modification (i.e. mutation) and/or gene exchange (through HGT). Mutation is the spontaneous modification in the DNA sequence of a given gene that can occur in the absence of antibiotic pressure. Once a bacterial cell becomes resistant by mutation, the selective pressure exerted by the antibiotic eliminates the susceptible population (*i.e.* those cells not carrying the mutation) whereas the resistant cells overgrow. Mutations resulting in antibiotic resistance may alter the mode of action of the antibiotic by several mechanisms, namely: *i)* those that decrease the sensitivity of the antibiotic target by genetic mutation or post-translational modification; *ii)* those that modify or inactivate the antibiotic by hydrolysis, and *iii)* those that attenuate the intracellular concentration of the antibiotic as a result of either a reduced permeability of the cell membrane or antibiotic efflux (Blair et al., 2015). For instance, resistance to fluoroquinolones, broad-spectrum antibiotics that have been used for the treatment of Gram-negative and Gram-positive pathogens since the late 1980s, is mainly caused by mutations in the genes encoding the type II topoisomerase, which is the main target of these antibiotics (Redgrave et al., 2014). A resistance mutation is thus of great value for the bacterium carrying it but the occurrence of mechanisms by which resistance may spread to neighboring cells is crucial under an evolutionary point of view for the whole community. HGT is an evolutionary process that spreads genes between, eventually taxonomically distant, bacterial species and is largely responsible for the development and spread of antibiotic resistance by various processes, such as conjugation, transduction and transformation (Bennett, 2008; Barlow, 2009) (Figure 1).

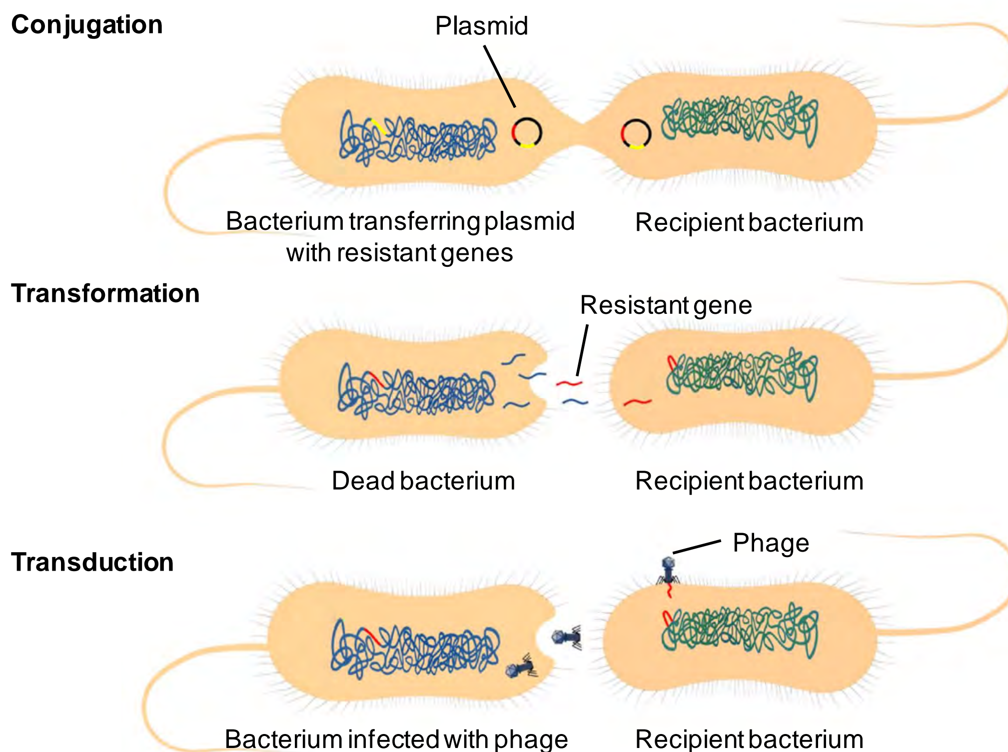

Figure 1. Main mechanisms of HGT in bacteria. Conjugation is the transfer of plasmid DNA from a donor to recipient via direct contact between cells. Transformation occurs when naked DNA from the environment is absorbed by a recipient bacterium. In transduction, genes are transferred from a donor to a recipient bacterium via bacteriophages (phage). This figure has been adapted from Furuya et al., 2006.

When the acquisition of a new trait by HGT confers a selective advantage to the host, the transferred gene has the potential both to spread rapidly within a bacterial population and to be transmitted by vertical inheritance to next generations. Mobile genetic elements (MGEs) such as plasmids, transposons, integrons, integrate conjugative elements, genomic islands and bacteriophages, play a crucial role in HGT because they are all involved in bacterial acquisition and the recombination of foreign DNA (Frost et al., 2005).

2.2.1. Plasmids

Plasmids are circular, double-stranded, extra-chromosomal DNA molecules that replicate independently of the bacterial chromosome. The plasmid size range size from 1 kb to more than 100 kb and are usually duplicated during cell division (Lodish et al., 2000). Plasmids do not carry essential genes for the host (*i.e.* involved in multiplication and growth) but accessory genes may provide some benefit to the host under certain conditions, thus enabling the cell to overcome environmental challenges (Bennett, 2008). These genes

include, among others, those that confer antibiotic and heavy metal resistance, those that provide virulence determinants, or those that enhance DNA repair (Bennett, 2008). Plasmid-encoded antibiotic resistance encompasses most of the resistance to antibiotics currently used in medicine, including last-resort antibiotics such as colistin (Torpdahl et al., 2017), third-generation cephalosporins (Kawahara et al., 2015) and carbapenems (Nordmann Patrice, Nass Thierry, 2011). This is particularly worrisome since plasmids can be transferred between phylogenetically distant species and the rate of transfer increases within heterogeneous bacterial communities (Svara and Rankin, 2011). Consequently, a single transfer event can provide multiple resistance traits to a sensitive cell. Carrying a plasmid, however, usually imposes a fitness cost in terms of growth-rate to the host bacterium, which will be counterbalanced only by the advantage that this plasmid confers under a given set of environmental conditions (Platt et al., 2012). Plasmids thus enhance the spread of resistance in niches characterized by the selective pressure exerted by antibiotics (Boto et al., 2010).

2.2.2. Insertion sequences and transposons

Insertion sequences (IS) and transposons are segments of DNA capable of moving from one site in the genome to another. Transposition can lead to the loss or rearrangement of DNA sequences, thus contributing to the evolution of bacterial genomes (Bennett, 2004).

IS are the simplest and smallest (between 1 and 1.5kb) type of transposable elements and they only carry genes required for their own transposition (Chandler 1998). The IS consist in two basic parts: a coding region for a transposase, the enzyme that mediates the transposition, and two non-coding sequences at the end of the element that are usually short inverted repeats (IRs) ranging from 15 to 40 bp in length. During transposition, a small duplication of the target DNA is created at the site of the insertion due to a staggered cut of the host target site that is later filled (Chandler 1998).

Transposons are larger and more complex in structure than IS-elements. They contain one or multiples functional genes that confer a given phenotype to the bacterial host, such as resistance to a specific antibiotic, in addition to those required for transposition (Schaefer and Kahn, 1998). The different types of transposons can be characterized by mechanisms of transposition, structure and genetic relatedness (Bennett, 2008). Most bacterial transposons correspond to the composite or non-composite forms. Composite transposons have a central sequence encoding the functional gene(s) which is flanked by IS elements. The IS element provides both the transposase and the sequences necessary for the DNA target recognition (Bennett, 2004). Instead of IS elements, the non-composite transposons,

are usually delimited by short IRs which flank genes that encode both those that confer a particular phenotype on the host cell and those needed for transposition (Bennett, 2004). Most bacterial transposons have great ability to spread to other bacteria since they have been usually found on plasmids (Reid et al., 2015). Both composite and non-composite transposons have been found in a number of plasmids in some clinical isolates encoding the so-called extended-spectrum β-lactamases, which can hydrolyze third-generation cephalosporins (Bennett, 2004). In recent years, these plasmid-associated extended spectrum β-lactamase genes have been detected worldwide thus threatening to the efficacy of antibiotic therapy. Moreover, transposons can also contribute to the spread of antibiotic resistance genes (ARGs) as part of integrons.

2.2.3. Integrons

Integrons are genetic systems that allow bacteria to acquire exogenous genes in the form of mobile gene cassettes. All integrons share three core features: the integron integrase gene, *intI*, encoding a site-specific recombinase; a recombination site, *attI*, where the integrase catalyzes the insertion of new genes as a part of genes cassette; and one or two strong promoters that drive the expression of an inserted gene cassettes (Gillings, 2014, 2017) (Figure 2). Integrons are ancient and they have been found in a wide diversity of natural environments. More than 15% of bacterial genomes harbor integrons (Boucher et al., 2007). There are hundreds of integron classes in environmental bacteria but only a few have become relevant in the clinical context, the class 1 integron being the most relevant (Gillings, 2017). The class 1 integron is an important player in the spread of antibiotic resistance and it is now found in 10 to 70% of Gram-negative pathogens from clinical isolates (Fluit et al., 1998; Essen-zandbergen et al., 2007). Moreover, 1 to 30 % of environmental bacteria from soil, freshwater and biofilms carry class 1 integron (Gaze et al., 2005; Hardwick et al., 2008). The success of the class 1 integron largely relies on its linkage to mobile DNA elements such as transposons and/or conjugative plasmids, therefore enhancing transfer between human commensal and pathogenic bacteria (Boucher et al., 2007; Gillings, 2014). Most class 1 integrons share common features: *i)* the three core features described above and *ii*) the carriage of the *qacE* and *sul*1 genes cassettes encoding resistance to disinfectants and sulfonamide, respectively (Figure 2) (Gillings, 2014, 2017). Moreover, class 1 integrons contain 0 to 6 cassettes which encode, most of them, a variety of resistance determinants (Gillings, 2014, 2017) (Figure 2). Gene cassette generate genomic plasticity, allow rapid adaptation and confer selective advantages under challenging environmental pressures. Thus, in last decades, the overuse of antibiotics has led to dissemination of class 1 integron and has vastly increased its abundance in natural

environments impacted by anthropogenic activities. As a consequence, class 1 integrons are being reported as "pollutants" of naturals environments and has been proposed as a proxy for anthropogenic pollution (Gillings et al., 2015).

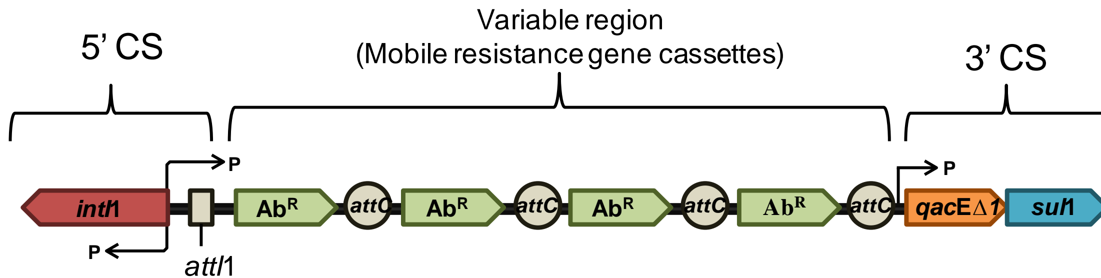

Figure 2. General structure of class 1 integrons. Integrons consist of integron integrase gene (*intI*1) and a recombination site (*attI1*) into which new genes (here conferring antibiotic resistance) harboring the recombination site *att*C are inserted through site-specific recombination. The gene cassettes constitute the variable region of the integron and they can vary among integrons. Figure adapted from Rajpara et al., 2015.

2.2.4. Integrate conjugative elements

Integrate conjugative elements (ICEs) are self-transmissible mobile genetic elements ranging from ~20 kb to >500 kb in size that are usually found integrated into the host chromosome (Johnson and Grossman, 2015). ICEs share features with plasmid and phages. Similar to plasmids, ICEs disseminate by conjugative transfer to new hosts and, like temperate phages, ICEs integrate into the host chromosome and replicate synchronously to assure their vertical inheritance (Burrus and Waldor, 2004). The main structure of ICEs consists of three modules containing the genes that catalyze its integration/excision, conjugation and regulation. Although the regulation of ICEs is still not completely elucidated, it seems that it requires certain conditions, such as the exposed to subinhibitory concentrations of antibiotics (Burrus and Waldor, 2004). This may be explained by the fact that many ICEs carry genes that allow the host bacterium to grow in hostile environments, such as in the presence of heavy metals and antibiotics (Burrus and Waldor, 2004; Johnson and Grossman, 2015).

2.2.5. Genomics islands

Genomic islands (GIs) are a collection of large (~10 kb to >200 kb) segments of DNA able to be integrated into the host chromosome and be transferred to a new host by HGT (Juhas et al., 2009). In general, GIs show different GC content and codon usage compared to the host genome and they are flanked by specific DNA sequences that contain direct repeats and tRNA genes (Jackson et al., 2011). GIs often carry genes encoding factors involved in

resistance, pathogenicity, metabolic pathways or symbiosis (Dobrindt et al., 2004; Kaper and Hacker, 2000), thus conferring a selective advantage for the bacteria. They also carry functional genes coding genetic mobility, including plasmid- and bacteriophage-encoded genes, IS elements, transposases and integrases (Jackson et al., 2011; Juhas et al., 2009).

2.2.6. Bacteriophages

Bacteriophages, or phages, are viruses that infect bacteria. They are the most abundant biological entities on Earth, with an estimated total abundance of 10^{30}–10^{32} (Dinsdale et al., 2008). Phages consist of a DNA or RNA genome packaged into a protective protein shell (the capsid). Phages are parasites that cannot reproduce by themselves but using the host's cellular machinery. Most phages have usually underwent a lytic cycle in which the phage infects a host cell and redirects the host machinery to produce new phages that are then released by lysis (the progeny). These phages are known as virulent or obligate lytic. Some phages, however, are able to undergo a different cycle in which the host cell is not lysed and the phage genome usually integrates into the host cell chromosome (as a "prophage"), where it remains quiescent and is replicated along with the host (lysogenic cycle). The prophage may remain for generations within the host cell or resume the lytic cycle after induction by certain conditions, such as ultraviolet radiation, nutrient limitation or some chemicals. Viruses able to undergo the lysogenic cycle are called temperate phages.

Phages may act as vectors for genetic exchange among different host bacteria in a process known as transduction. There are two types of transduction: generalized and specialized. Generalized transduction involves the transfer of any fragment of the donor genome to the recipient cell. The generalized transduction is caused by an error during the packaging of the phage genome into capsids. As a result, host cell DNA is erroneously packaged instead of phage DNA. Transduction particles can introduce bacterial DNA fragments into a new host after a successful infection event. This bacterial DNA fragment can be then inserted into the host genome by genetic recombination and expressed, thus conferring new capabilities to the host bacterium. Specialized transduction, in turn, produces viral particles that contain a hybrid-DNA molecule comprising both phage DNA and a specific fragment of bacterial DNA. Specialized transduction results from the faulty excision of the prophage genome during the induction of the lytic cycle. Specialized transduction can only transfer those genes flanking the integration site of the prophage and it is restricted to temperate phages. In general, the amount of DNA that can be transduced by phages is limited by the volume of the phage capsid, but it can reach up to 100 kb (Frost et al., 2005; Gogarten et al., 2009)

Although generalized transduction plays a more important role on the gene transfer in the environment compared to the specialized transduction (Bushman, 2001), it is considered a rare event, occurring approximately once every 10^7–10^9 phage infections (Penadés et al., 2015). However, the large concentration of phages and hosts found in many environments, makes transduction a significant event that may take place at higher frequencies than previously reported (Penadés et al., 2015). Moreover, phages are known to persist better than their bacterial hosts in the environment maybe due to their low susceptibility to be degraded by nucleases, radiation and temperature. Thus, owing to their abundance and persistence in the environment, phages have been considered key agents for gene transfer (Weinbauer, 2004).

Unexpectedly, several studies have reported the presence of ARGs in genomes from environmental phages (Lekunberri et al., 2017, 2016). For instance, multiple β-lactam (bla_{TEM}, bla_{CTX-M}, bla_{OXA-2}, bla_{PSE-1}, bla_{PSE-4}, *mec*A) as well as sulfonamide (*sul*1) and fluroquinolone (*qnr*S, *qnr*A) resistance genes have been found in phages from sewage (Calero-Cáceres et al., 2014, Colomer-Lluch et al., 2011, 2014, Muniesa et al., 2004), sludge (Calero-cáceres et al., 2014), water river (Calero-Cáceres et al., 2017, Colomer-Lluch et al., 2011) and sediment (Calero-cáceres et al., 2017) samples. Another study detected high concentrations of genes encoding resistance to β-lactams (bla_{TEM}, bla_{SHV}, bla_{CTX-M}) and fluoroquinolones (*qnr*S, *qnr*B and *qnr*A) in the phage DNA fraction isolated from hospital and WWTP effluents (Marti et al., 2013). In addition, ARGs and *intI*1 genes including bla_{TEM}, $bla_{OXA-2,}$ *tet*A, *tet*W *intI*1, *intI*2, *intI*3 have also been detected in phages isolated from soil, sewage and river water (Anand et al., 2016). This suggests that phages may contribute to the spread of antibiotic resistance in the environment. Moreover, transfer of ARGs mediated by phages has been demonstrated using laboratory phage strains (Kenzaka et al., 2007; Marinus and Poteete, 2016) and, most recently, using wild-type phages isolates from wastewater samples (Gunathilaka et al., 2017).

3. Antibiotic resistance: a global challenge

Antibiotic resistance is a major health concern not restricted to a specific geographical zone or social class. It is well known that "*the emergence and maintenance of antibiotic resistance anywhere is a threat to health everywhere*" (Palmer et al., 2013). The globalization of the markets and the increase of transcontinental trips in the recent years have increased the opportunities for resistant bacteria to be carried rapidly from one geographic location to another (Palmer et al., 2013). A clear example of this is the recent appearance of resistance to carbapenems, one of the last-resort antibiotics to treat infections caused by multidrug-resistant Gram-negative bacteria (Walsh, 2010). Resistance to carbapenems is conferred by carbapenemase enzymes encoded by different genes such as the bla_{KPC} (where KPC stands for *Klebsiella pneumoniae* carbapenemase), the bla_{NDM} (New Delhi metallo-β-lactamase) and the $bla_{\mathrm{OXA\text{-}48}}$ (which hydrolyze oxacillin antibiotic) (Walsh 2010). Despite the first occurrence of a KPC producer dates from 1996 in the United States, KPC-producing bacteria are currently considered endemic in many parts of the world, such as Puerto Rico, Colombia, Greece, Israel and China (Bij and Pitout, 2017). The NDM variant was first described in 2008 in a *K. pneumonia*e isolated from a Swedish patient who was previously hospitalized in New Delhi, India (Yong et al., 2009). Since its discovery, bacteria carrying the bla_{NDM} gene have subsequently been reported from different parts of the world, including several countries in Europe, the Middle East, Africa, Asia, Australia and North America, mostly in bacteria from patients epidemiologically linked to the Indian subcontinent (Nordmann et al., 2011). The OXA-48 variant was first identified from a *K. pneumoniae* strain isolated in Turkey in 2011 and since then, $bla_{\mathrm{OXA\text{-}48}}$ carrying bacteria have been isolated from patients hospitalized in several countries in Europe and North Africa (Bij and Pitout, 2017). The three genes (bla_{KPC}, bla_{NDM} and $bla_{\mathrm{OXA\text{-}48}}$) are located in plasmids that, occasionally, also carry genes conferring resistance to others antibiotics such as aminoglycosides, macrolides and sulfonamides (Nordmann et al., 2011). These features may explain their widespread occurrence among clinically relevant pathogens. ARGs reported to reside on MGEs such as plasmids in human bacterial pathogens have been considered resistance genes of high-risk to public health (Martínez et al., 2014). Therefore, it is necessary to implement strategic surveillances in order to reduce their dissemination and to minimize the factors leading to their maintenance within the community.

The use of antibiotics is a main driver for selection of resistance genes. Nowadays, antibiotics are still one of the most prescribed drugs, with more than 70 billion clinical doses administered in 2010 around the world (Crofts et al., 2017). Thus, surveillance and control of which antimicrobials are used and by whom is crucial to contain the emergence of

antibiotic resistance (WHO, 2001). However, precise data on antibiotic consumption are deficient and very diverse among countries (Kümmerer, 2009). Inappropriate prescription and use of antibiotics also lead to the development of resistance. It has been estimated that in 30% to 50% of cases, the dose or duration of the prescribed antibiotic treatment is not optimal (CDC Report, 2013). Moreover, in many countries, antibiotic supplies are not regulated and available without prescription. In fact, it has been estimated that more than 50% of antibiotics worldwide are purchased over the counter without prescription (Rasheed et al., 2016). As a result, patients are exposed to sub-therapeutic antibiotic concentrations thus promoting the development of antibiotic resistance by spontaneous mutations and HGT (Ventola, 2015).

Current surveillance of antibiotic consumption is mainly focused on data from human medicine, but the use of antibiotics in agriculture, aquaculture and veterinary medicine is also of major concern. Particularly, these sectors consume most of the 100,000 to 200,000 metric tons of antibiotics manufactured every year worldwide (Laxminarayan et al., 2013) resulting in a severe impact on the human and environment health. For instance, more than 35 years ago it was noted that the intestinal flora of both farmers and farm animals had high rates of antibiotic resistance, pointing out the transfer of resistant bacteria from farm animals to humans (Bartlett et al., 2013). Nowadays, molecular methods have showed that resistant bacteria in farm animals reach humans through consumption of contaminated food (Founou et al., 2016). The use of antibiotics in agriculture also represents a threat for the environment health because antibiotics are directly (by spraying antibiotics on fruit trees to act as pesticides (Levy, 2002)), or indirectly (by using manure that contains antibiotic residues and antibiotic resistant bacteria as fertilizer or soil conditioner, by dispersal into groundwater or by surface runoff) (Ventola, 2015). These practices contribute to the exposure of environmental microorganisms to sub-inhibitory concentrations of antibiotics, altering the environment ecology by favoring the selection of resistant bacteria.

It has been an overall increase in antibiotic consumption in the European Union (EU) during the last decade. In 2016, the European consumption of antibiotics in the community (outside hospitals) ranged from 10.4 (Netherlands) to 36.3 (Greece) defined daily doses (DDD) per 1000 inhabitants per day (ESAC-Net, 2017). These data correlate with the antibiotic resistance rate for methicillin-resistant *Staphylococcus aureus* (MRSA) and carbapenemase-producing *Klebsiella* for the Netherlands (10.6% and 0.20%, respectively) *versus* Greece (51% and 38%, respectively). It also reflects the policies and practices promoting careful antibiotic use and infection control in Dutch hospitals (Bartlett et al.,

2013). In European countries, β-lactam antibiotics are the most frequently used in human medicine, accounting for approximately 50% to 70% of total consumption, followed by tetracyclines, macrolides, quinolones and sulfonamides (Kümmerer, 2009). Although the community consumes most of antibiotics manufactured worldwide, the use of antibiotics in hospital settings is the main factor contributing to the emergence and dissemination of antibiotic-resistant bacteria responsible for relevant clinical infections (ESAC-Net, 2017).

4. Antibiotic resistance in the environment: a link to clinic?

Research on antibiotic resistance has mainly been focused on human bacterial pathogens occurring in clinical settings. In recent years, however, the magnitude of the problem has compelled researchers to broaden the scope and to investigate the origin and sources of resistance determinants. This new approach highlighted the role that the environment plays in the maintenance and spread of antibiotic resistance (Ashbolt et al., 2013; Martínez, 2008; Pruden et al., 2013; Wright, 2010). There are approx. 5×10^{30} bacteria on the planet and most of them are not pathogenic (Whitman et al., 1998). These organisms interact with a myriad of bioactive molecules synthesized by other bacteria, fungi, animals and plants, contributing to the natural selection and evolution of microbial genetic diversity. The exposure to environmental chemicals over millions of years has led the environmental microbes to develop complex machineries and regulatory networks to respond and counteract cytotoxic molecules, including antibiotics (Wright, 2010). Genome sequencing revealed that bacteria have genes to protect them from small molecules of external origin (Fraser et al., 1995). All ARGs constitute the so-called **resistome**, which includes all the ARGs in both pathogenic and non-pathogenic bacteria, their precursors and cryptic resistance genes in a giving environment (D'Costa et al., 2006). The resistome encompasses both intrinsic and acquired resistance genes, whose diversity rely in part on the selective pressure exercised by a specific environment. In this regard, antibiotics may act as selective agents for mechanisms of resistance and accelerate the evolution of resistance (Galán et al., 2013).

Many different ARGs are found in a wide variety of environments such as soil, sediments, water, as well as pristine habitats such as the Arctic and Antarctic archipelago (Miller et al., 2009; Sjölund et al., 2008). Natural environments represent reservoirs of ARGs and there is growing evidence that many clinically relevant ARGs carried by pathogenic bacteria have originated in environmental bacteria (Martínez, 2008). For instance, the CTX-M extended spectrum β-lactamase, often found in clinical pathogens, appears to have originated from chromosomal genes in *Kluyvera* spp., a typical environmental bacterium (Poirel et al., 2002).

Similarly, the *qnr*A gene located in plasmid-linked fluoroquinolone resistance has an environmental reservoir in the waterborne bacteria *Shewanella algae* (Poirel et al., 2005). The gene cluster conferring resistance to the glycopeptides antibiotic vancomycin in pathogenic bacteria has been identified in environmental strains (Perry and Wright, 2013; Wright, 2010). These examples suggest a link between clinical and environmental resistance and highlight the need to clarify which are the main factors contributing to their mobilization, maintenance and dissemination in environmental settings. A temporal study carried out in Netherlands showed that the concentration of genes conferring resistance to β-lactamases, tetracyclines and erythromycins in decades-old soil samples correlated with the industrial-scale production and use of these medicines, from the pre-antibiotic era to the present (Knapp et al., 2010). This study clearly showed the effect of the increased use of antibiotics in agriculture in the last years on the soil resistome. Likewise, it is well documented that the introduction of antibiotics and pharmaceutical compounds —the so-called emerging pollutants— from human and veterinary sources into the environment has stimulated the selection and evolution of antimicrobial resistance (Kümmerer and Henninger, 2003). A considerable fraction of antibiotics that are used in aquaculture, animal husbandry, industry, and human therapy end up in natural environments such as rivers, lakes and soils (Figure 3). As a consequence, bacteria are constantly exposed to sub-inhibitory concentration (sub-MIC) of antibiotics that can potentially select for resistance and stimulate HGT. Moreover, sub-MIC of antibiotics can also function as signaling molecules among cells, giving rise to a range of functional consequences, including quorum sensing, bacterial virulence and biofilm formation (Andersson and Hughes, 2014).

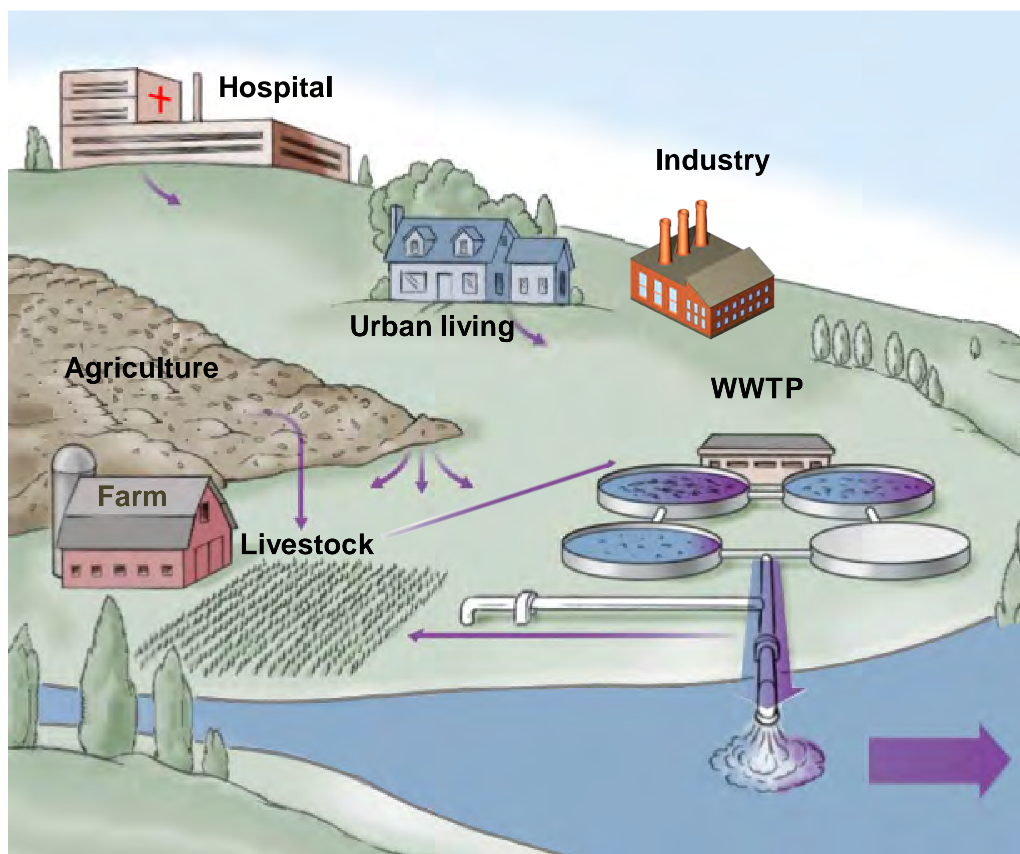

Figure 3. Main route of antibiotic-resistant bacteria (ARB) and ARGs dissemination from anthropogenic sources to the environment. This figure has been adapted from Cartwright 2017.

The aquatic systems can serve both as a natural reservoir of antibiotic resistance and as a platform for the spread of clinical resistant pathogens (Suzuki et al., 2017). Natural aquatic ecosystems, including rivers, lakes and streams receive effluent from wastewater treatment plants (WWTPs) and other anthropogenic inputs that may increase the natural background levels of ARGs and trigger their transfer among non-pathogenic and pathogenic bacteria (Michael et al., 2013). The success of gene transfer mostly depends on the proximity of donor and recipient, so the most likely place for gene exchange among pathogens and environmental organisms is expected to be a shared niche. In this regard, environments receiving anthropogenic pollutants and, especially, those receiving effluents from WWTPs have been an important focus of study in the last years, as these environments are meeting places for environmental bacteria and human bacterial pathogens in the presence of background concentrations of emerging pollutants (Szczepanowski et al., 2009).

5. WWTPs and antibiotic resistance

Antibiotics and other anthropogenic pollutants such as detergents, heavy metals and pesticides are chronically discharged into the environment through WWTP effluents (Michael et al., 2013; Rodríguez-Mozaz & Weinberg, 2010; Rodríguez-Mozaz et al., 2015).

WWTPs are the main link between human community and the environment, as sewage from households and hospitals contains high concentrations of both antibiotic residues and antibiotic-resistant bacteria (ARB) from human origin. The biological wastewater treatment process creates a nutrient rich environment with a high cell density, potentially suited for resistance development and spread under sub-MIC of antibiotics. Therefore, WWTPs have been considered among the main anthropogenic sources of antibiotics, ARB and ARGs release into the environment. Genes encoding resistance to all classes of antibiotics have been detected in WWTP effluents, which can discharge about 10^7-10^{10} Colony Forming Units (CFU) per day, per inhabitant equivalent carrying any type of antibiotic resistance trait into the environment (Rizzo et al., 2013). In fact, some resistance types, such as quinolone resistance in coliforms or enterococci, have been found to be more prevalent in the treated effluent than in the raw influent (Luczkiewicz et al., 2010; Novo et al., 2013). Although differences in treatment plant design and their operation may influence the fate of ARGs and ARB in wastewater (Guardabassi et al., 2002; Kim et al., 2007), a well-functioning wastewater treatment plant with secondary treatment will constantly discharge high load of ARGs and ARB to the receiving environment (Vaz-moreira et al., 2011). Probably, this burden exceeds the concentration that the environment is able to eliminate through natural biological purification processes. Many studies have reported the increase in ARB and ARGs abundance downstream of the wastewater discharge point (Pruden et al. 2006, 2012; Storteboom et al., 2010; Lapara et al. 2011; Huerta et al. 2013; Rizzo et al., 2013; Czekalski et al., 2014; Di Cesare et al., 2016). However, it is not clear if this effect is caused by the selective pressure exerted by antibiotic residues in the effluents, thereby promoting genetic exchange among bacteria *via* mobile genetic elements (*e.g.* plasmids, transposons) or by the ARB from the WWTP that are discharged into the environment and colonize the aquatic habitat. In fact, antibiotics and heavy metals have been pointed out as the major selective pressures causing dissemination of ARB in the environment through HGT. However, other factors such as water temperature and nutrient availability and scarcity should be included into the equation to completely understand the mechanisms contributing to the acquisition and spread of antibiotic resistance (Manaia et al., 2016). For instance, recent studies have pointed out that nutrient concentration may lead to an increase in the prevalence of ARGs (Lehmann et al., 2016; Zhao et al., 2016), maybe caused by higher HGT rates as a result of enhanced metabolic rates and cell mobility (Elsas et al., 2003).

6. Biofilms as environmental reservoir of antibiotic resistance

Most bacteria in natural aquatic bodies are organized in biofilms. A biofilm is a structured consortium of bacteria and other microorganisms (algae, fungi and protozoa) embedded in an extracellular matrix of polysaccharides that protect them from environmental hazards (Costerton et al., 1978). Biofilms have been intensively studied in clinical settings because they are responsible for several chronic diseases that are difficult to treat due to their higher tolerance to antibiotics and disinfectant chemicals (Høiby et al., 2010). Over the last few years, environmental biofilms have also shown to play a critical role in the acquisition and spread of ARGs due to high cell density and close contact among cells, increased genetic competence and accumulated MGEs within the biofilm matrix (Fux et al., 2005). Several studies have shown increased conjugation rates of plasmids in biofilms when compared to free-living bacterial cells (Ehlers and Bouwer, 1999) which may be favored when biofilms are exposed to sub-inhibitory concentrations of antibiotics (Taylor et al., 2014). Moreover, recent studies have observed that some ARGs may migrate from the water column towards biofilms where they persist longer (Engemann et al., 2008; Zhang et al., 2009). These aquatic compartments, such as water and biofilms, may therefore have a relevant role on driving ARG transfer, ecology and evolution. For an extended overview of the role of biofilms on the antibiotic resistance in the environment see the Chapter 3 of this thesis; a review entitled "*The role of biofilms as environmental reservoir of antibiotic resistance*".

7. Methods used to identify and quantify ARGs

For the last 70 years, research in antibiotic resistance has focused on the study of human bacterial pathogens using cultivation-dependent techniques. Traditional methods in clinical microbiology are based on conventional approaches such as plating sample on antibiotic-selective agar plates, isolating bacterial colonies and using disc diffusion among other methods to determine the Minimum Inhibitory Concentration (MIC) for resistance to antibiotics. MIC values are assessed against clinical breakpoints (databases available on EUCAST, www.eucast.org) or epidemiological cut-off values for resistance (ECOFFs) to determine if a bacterial isolate is either susceptible, intermediate or resistant to a given antibiotic (Turnidge and Paterson, 2007). Although this method is very informative, culturing and susceptibility testing have their limits with environmental bacteria, as it is estimated that only a small fraction of bacteria cells from environment (~ 0.1–2%) can be cultivated under laboratory conditions (Amann et al., 1995). Moreover, conventional methods give no information about the mechanism of resistance or if the resistance gene is able to disseminate to other bacterial species through HGT. To shed light on these topics a

molecular or genotypic characterization is required. While phenotypic antibiotic testing reports susceptibilities to an antibiotic, molecular analyses provide information about the resistance gene involved and its genetic context (i.e. if the gene is located in a plasmid or in the chromosome), which are valuable data to assess the potential capacity of the target gene to spread among community members (Heather K Allen et al., 2010). However, a general flaw of DNA-based methods is that they do not provide information about the functionality of the target gene. Besides, the lack of a gene that confers resistance to a given antibiotic does not necessarily imply that the host bacterium is susceptible to that particular antibiotic since resistance might be provided by many other resistance mechanisms (Tan, 2003). Therefore, the combination of both cultivation-based and molecular methods and an appropriate experimental design is required to study antibiotic resistance in the environment and to properly interpret the data under its ecological context.

7.1. PCR and quantitative PCR

PCR and quantitative PCR (qPCR) are the most widely methods for detecting ARGs in environmental samples because they are very sensitive and provide qualitative and quantitative information, respectively, about DNA sequences of interest within a relatively short time (2–3h). The process involves the use of oligonucleotide primers that are complementary to the flanking regions of the target gene and should be carefully designed after alignment of as many sequences of the target gene as possible. Many primer pairs for the specific amplification of ARGs using either standard PCR or qPCR are available in the specialized literature (e.g. Luby et al., 2016; Tan, 2003). While the conventional PCR provides qualitative information (presence or absence) about a given ARG in a sample, qPCR also yields quantitative information about the abundance of the target ARG. Both PCR and qPCR are dependent on DNA extraction, which may differ in efficiency among matrices (water, soil, biofilm, tissues). Also, resulting DNA extracts may contain inhibitors, such as humic acids or organics salts, that interfere in the amplification reaction and thus may lead to either false negatives or underestimated copy counts. Besides, both methods can lead to non-specific reactions resulting in false positives. Thus, it is important to be consistent in applying quality control measures, such as internal amplification controls or diluting extracted DNA to avoid false negatives (Luby et al., 2016). Besides, verifying expected product size by gel electrophoresis and sequencing the PCR or qPCR products is strongly advised to ensure the specificity of the amplification. However, the major inconvenient of PCR and qPCR is that the number of ARGs examined in one test is limited. Multiplex PCR and high throughput PCR arrays can solve these limitations but require additional control measures and careful primer design. Notwithstanding this, multiplex PCR and qPCR arrays

allow the simultaneous quantification of hundreds of genes in a single assay (Karkman et al., 2017).

7.2. Metagenomics

Metagenomics usually overcomes the limitations of cultivation-based methods and the bias associated with PCR-based techniques. Metagenomic techniques allow the sequencing of all genes present in a sample (*i.e.* the metagenome) and thus it is a powerful tool to describe the genetic potential of a natural community. The metagenome can then be screened for the presence of the gene or genes of interest, including all ARGs and MGEs (Yang and Zhang, 2017). Based on the experimental design, metagenomic approaches can be classified as descriptive and functional.

7.2.1. Descriptive metagenomics

Descriptive metagenomics involves extracting and random sequencing of DNA directly from the environment (*i.e.* water, sediments, biofilms, air). The resulting sequences are then compared directly to a reference database that encompasses sequences of known genes to assign taxonomic identity and functional potential. Particularly, ARGs can be identified by searching against public antibiotic-resistance gene databases, such as ResFinder (Zankari et al., 2012), ARG-ANNOT (Gupta et al., 2013), the Comprehensive Antibiotic Resistance Genes Databases (CARD) (Mcarthur et al., 2013), the Antibiotic Resistance Genes Database (ARDB) (Liu and Pop, 2009) and the expanded version of ARDB (ARDB+) (Port et al., 2012). CARD and ARDB are the two most widely used references databases in environmental studies related to ARGs because they accommodate most of the publicly available ARGs sequences (Yang and Zhang, 2017). Therefore, the whole resistome can be examined in a given sample through a single metagenomic analysis, as long as ARGs sequences are included in the reference database.

Besides, metagenomic data can also provide the genetic context of the identified genes. For example, sequences of heavy metal resistance or HGT markers, such as plasmids, integrons or transposons could also be identified using the appropriate reference databases (Franklin et al., 2016). This information can be useful to explain how ARGs spread among cells or from one environment to the other (Bengtsson-palme et al., 2014; Nesme and Simonet, 2014) or to resolve the links between ARGs in environmental bacteria and clinical pathogens (Gibson et al., 2015; Mcgarvey et al., 2012). Most metagenomic sequencing platforms generate short reads (100–400 bases) that give limited information about the sequenced gene (Karkman et al., 2017). Assembling short reads into longer sequences (*contigs*) provides valuable

information regarding which ARGs are present in which hosts and the genetic context of this gene (Henry et al., 2011). This knowledge is important to evaluate the dissemination potential of ARGs and its associated risk to public health. However, in most environments, the relative abundance of ARGs is low in comparison to other functional genes, and therefore a high sequencing depth is necessary to represent the whole diversity of ARGs (Bengtsson-Palme et al., 2016).

Descriptive metagenomic approaches have overcame common problems from PCR-based techniques such as the detection of false positives due to primer unspecificity or false negatives resulting from inhibitory compounds in DNA extracts. However, data analysis is a critical barrier in modern metagenomic studies due to the huge amount of data generated (from giga- to terabases), the computational resources needed and the expertise in bioinformatics required to make the most of the metagenomes. In fact, researchers are focusing efforts on standardizing bioinformatic pipeline to achieve better and comparable results across samples and sequencing technologies (Yang et al., 2014). As is the case with PCR and qPCR, descriptive metagenomics cannot confirm the functionality of a putative ARG. To do so, functional metagenomics is required.

7.2.2. Functional Metagenomics

Functional metagenomics is an useful tool not only in identifying ARGs but also in screening its phenotype (*i.e.* its function) (Mirete et al., 2016). Functional metagenomics is achieved by fragmenting extracted DNA with restriction enzymes, inserting these fragments into expression cloning vectors (such as plasmids, fosmids or bacterial artificial chromosomes, depending on the size of the DNA insert), and expressing these genes into a heterologous host, such as *Escherichia coli*. Hosts carrying the genes of interest (ARGs in this case) are identified by plating onto cultivation media supplemented with antibiotics (Luby et al., 2016; Yang and Zhang, 2017). Studies based on functional metagenomics have discovered genes encoding resistance to various antibiotic classes in samples from soil, animal manure and the gut microbiota (Donato et al., 2010; Su et al., 2014; Zhou et al., 2012). Despite these advantages, functional metagenomics is laborious and time consuming and it also suffers from biases in the gene expression that may be associated with the selection of the surrogate host (Luby et al., 2016).

The wide application of functional metagenomics together with high throughput sequencing will increase our knowledge of functionally annotated ARGs and their genetic context. This information will be highly valuable to design better strategies for antibiotic discovery and surveillance for new resistance variants.

Chapter 2

Objectives

The main objectives of this thesis were: *i)* to study the factors contributing to the maintenance and spread of antibiotic resistance genes (ARGs) in aquatic microbial communities subjected to anthropogenic impacts, and *ii)* to evaluate the role of biofilms as reservoirs of antibiotic resistance.

To accomplish these primary goals, the following secondary objectives were pursued:

I. To develop and validate robust qPCR assays to quantify ARGs conferring resistance to last-resort antibiotics, particularly three genes variants conferring resistance to carbapenems (bla_{NDM}, bla_{KPC} and $bla_{\mathrm{OXA\text{-}48}}$).

II. To assess the impact of raw sewage and treated wastewater on the composition of biofilm bacterial communities and their associated resistome

III. To evaluate the effect of a combined regime of nutrients and emerging pollutants on the composition and concentration of selected ARGs and class 1 integrons in streambed bacterial communities.

IV. To assess whether or not phage genomes contain ARGs.

According to these goals and on the basis of the published papers, the content of this thesis is structured in five chapters:

Chapter 3: The role of biofilms as environmental reservoirs of antibiotic resistance. (Review).

Chapter 4: Real-time PCR assays for the detection and quantification of carbapenemase genes (bla_{KPC}, bla_{NDM} and $bla_{\mathrm{OXA\text{-}48}}$) in environmental samples.

Chapter 5: Wastewater pollution differently affects the antibiotic resistance gene pool and biofilm bacterial communities across streambed compartments.

Chapter 6: Emerging contaminants and nutrients synergistically affect the spread of class 1 integron-integrase (*intI1*) and *sul*1 genes within stable streambed bacterial communities.

Chapter 7: Metagenomics analysis reveals that bacteriophages are reservoirs of antibiotic resistance genes.

Chapter 3

The role of biofilms as environmental reservoirs of antibiotic resistance

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The role of biofilms as environmental reservoirs of antibiotic resistance

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Antibiotic resistance has become a significant and growing threat to public and environmental health. To face this problem both at local and global scales, a better understanding of the sources and mechanisms that contribute to the emergence and spread of antibiotic resistance is required. Recent studies demonstrate that aquatic ecosystems are reservoirs of resistant bacteria and antibiotic resistance genes as well as potential conduits for their transmission to human pathogens. Despite the wealth of information about antibiotic pollution and its effect on the aquatic microbial resistome, the contribution of environmental biofilms to the acquisition and spread of antibiotic resistance has not been fully explored in aquatic systems. Biofilms are structured multicellular communities embedded in a self-produced extracellular matrix that acts as a barrier to antibiotic diffusion. High population densities and proximity of cells in biofilms also increases the chances for genetic exchange among bacterial species converting biofilms in hot spots of antibiotic resistance. This review focuses on the potential effect of antibiotic pollution on biofilm microbial communities, with special emphasis on ecological and evolutionary processes underlying acquired resistance to these compounds.

Keywords: aquatic ecosystems, biofilms, mobile genetic elements, antibiotic resistance genes, aquatic resistome

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ENVIRONMENTAL BIOFILMS

Nature is often unpleasant. It is then better to face environmental uncertainties under the principle of "strength through unity". In many habitats, either natural or artificial, microorganisms attach themselves to surfaces, either abiotic or biotic, forming a complex matrix of biopolymers known as biofilm that protect them from environmental hazards (Costerton et al., 1978). Biofilms may be composed of a single bacterial species (e.g., *Vibrio cholerae*, Teschler et al., 2015) but more frequently they are formed by a complex and diverse community of microorganisms (bacteria, algae, fungi and protozoa) embedded in an extracellular matrix of polysaccharides, exudates, and detritus (Costerton et al., 1978; Wimpenny et al., 2000). Many microbial species are able to change their lifestyle (free-living vs. attached) depending on their physiological status and the physicochemical conditions in their surroundings, taking advantage of the greater availability of organic matter in suspended particles and surfaces (Simon et al., 2002; Grossart et al., 2004; Grossart, 2010; Teschler et al., 2015). In aquatic habitats, biofilms develop not only in benthic substrata, such as streambed cobbles and sand (epilithic and epipsammic biofilms, respectively), but also on floating macro– and microaggregates (Simon et al., 2002). From an ecological perspective, microorganisms in environmental biofilms actively participate in organic matter decomposition, nutrient dynamics and biogeochemical cycling, being a key component of

ecosystem functioning (Sabater and Romaní, 1996; Sabater et al., 2002; Simon et al., 2002; Battin et al., 2007; Romaní, 2010). Moreover, streambed biofilms are considered as good indicators of the overall water quality and the ecological status of the system (i.e., ecosystem health) (Burns and Ryder, 2001; Sabater et al., 2007). It is then of special interest to assess how biofilm communities respond to anthropogenic pollution of aquatic environments (e.g., rivers, lakes, and reservoirs) considering the increasing amount of chemical compounds (metals, personal care products and drugs used in veterinary and human medicine) released into these waterbodies mainly through wastewater treatment plant (WWTP) effluents and agricultural run-off (Pruden et al., 2006; Sarmah et al., 2006; Baquero et al., 2008). This review focuses on the role of streambed biofilms as reservoirs of antibiotic resistant bacteria and resistance genes, providing a general overview of the causes and consequences of a chronic exposure of biofilm communities to sub-inhibitory concentrations of antibiotics and their role in the spread and persistence of antibiotic resistance.

BIOFILMS AND ANTIBIOTICS

Biofilms show an increased survival and resistance to environmental and chemical stressors (e.g., antibiotics) mainly, but not only, by the protection conferred by the extracellular polysaccharide matrix (Mah and O'Toole, 2001; Stewart and Costerton, 2001; Donlan, 2002; Donlan and Costerton, 2002; Stewart, 2002; Hall-Stoodley et al., 2004; Høiby et al., 2010). In biofilms, bacterial cells exhibit 10 to 1,000 times less susceptibility to specific antimicrobial agents compared with their planktonic counterparts (Gilbert et al., 2002). This reduced susceptibility is caused by a combination of different factors, namely: (i) a poor antibiotic penetration into the polysaccharide matrix; (ii) the arbitrary presence of cells showing a resistant phenotype (known as "persisters"); and (iii) the presence of either non-growing cells or cells that triggered stress responses under unfavorable chemical conditions within the biofilm matrix (Stewart and Costerton, 2001; Stewart, 2002). These protective mechanisms act synergistically to those responsible for conventional resistance linked to the presence of antibiotic resistance genes (ARGs) in bacterial genomes or extrachromosomal elements, yielding an overall increased resistance of biofilms to antimicrobial compounds. For instance, β-lactamase producing bacteria offered increased protection in biofilms because the β-lactam antibiotic, such as ampicillin, was inactivated by those β-lactamases (Anderl et al., 2000). Moreover, the *ampC* gene of *Pseudomonas aeruginosa* biofilms was strongly induced by exposure to antibiotics, such as imipenem (Bagge et al., 2004). Additionally, biofilm formation may result as a defensive reaction to the presence of antibiotics. Hoffman et al. (2005) found that sub-inhibitory concentrations of aminoglycosides induce biofilm formation as part of a defense response in *Escherichia coli* and *P. aeruginosa*. Similar results were described by Salcedo et al. (2014), who observed that sub-inhibitory concentrations of tetracycline and cephradine induce biofilm formation and enhance the transfer rate of the pB10 plasmid among the biofilm biomass (*E. coli* and *P. aeruginosa*) at rates 2–5 times faster than without antibiotic treatment. Since biofilm formation is also common for most bacterial pathogens, the enhanced resistance of biofilms to antibiotics is a serious concern for human health as many chronic infections are linked to biofilm growth on either natural surfaces (e.g., teeth, lungs) or foreign-body devices (e.g., pacemakers, catheters, prosthetic heart valves). The characteristics, composition, growth dynamics, and resistance mechanisms of clinically relevant biofilms have been reviewed in detail by several authors (Donlan and Costerton, 2002; Parsek and Singh, 2003; Hall-Stoodley et al., 2004; Høiby et al., 2010), and are out of the scope of this review. In clear contrast, lesser is known about the role of environmental biofilms as natural reservoirs of ARGs, their contribution to ARGs spreading among biofilm inhabitants and their transfer to free-living bacteria, increasing the risk for their transmission to aquatic microorganisms and potential human pathogens (Vaz-Moreira et al., 2014 and references therein).

ENVIRONMENTAL BIOFILMS UNDER CHEMICAL STRESS

Many aquatic systems (rivers, lakes, reservoirs) are affected by human activities such as continuous discharges from WWTP effluents. Under such conditions, macro- and microorganisms inhabiting these waterbodies are exposed to a low but constant concentration of a wide range of chemical pollutants (antibiotics but also analgesics, anti-inflammatory, and psychiatric drugs, β-blockers, pesticides, etc.) that alter their behavior at different levels, with consequences that we are only beginning to grasp (Bernier and Surette, 2013; Boxall, 2014). Several studies have demonstrated the effects of the so-called emerging pollutants on the composition, activity, and resilience of streambed biofilms (Bonnineau et al., 2010; Ricart et al., 2010; Proia et al., 2011, 2013a,b; Osorio et al., 2014), although the ecological implications of such background pollution are difficult to envisage. A serious drawback arises when comparing the environmental concentrations of antibiotics measured in polluted aquatic habitats (from ng/L to μg/L) to those used to treat bacterial infections (i.e., therapeutic concentrations, which are usually ≥1 mg/L). Since environmental concentrations of antimicrobial compounds are several orders of magnitude below the minimum inhibitory concentration (MIC) of most bacterial pathogens, their antibiotic effect is doubtful, if any (Waksman, 1961; Davies, 2006; Davies et al., 2006; Davies and Davies, 2010). Current data strongly suggest that antibiotics, at these sub-MIC concentrations, act as signaling molecules mediating a wide variety of cell processes (gene transcription and expression, quorum sensing, inter- or intra-species communication, biofilm formation, among others; Davies, 2006; Romero et al., 2011; Sengupta et al., 2013; Andersson and Hughes, 2014), instead of causing growth arrest or cell death. Moreover, low concentration of antibiotics may also trigger different stress responses that might accelerate horizontal gene transfer (HGT) and the spread of ARGs in a broad range of bacterial species (Beaber et al., 2004; Miller et al., 2004; Maiques et al., 2006). Under this perspective,

the chronic exposure to subinhibitory antibiotic concentrations that occurs in most aquatic ecosystems offers new avenues for research that deserve exploration. For instance, is the effect of this chronic exposure strong enough to shape the composition of microbial communities? Or is it buffered by the many other physico-chemical constraints that microbes face in their habitat? Is the antibiotic pollution adding a background noise that interferes with normal communication among bacterial cells in their habitats (e.g., biofilms)? If so, how can this noise effect be measured? And what about activity? Does antibiotic pollution have measurable effects on biogeochemical cycles at both local and global scales? In this regard, Roose-Amsaleg and Laverman (2015) have recently reviewed 31 articles dealing with the effects of antibiotics on microorganisms involved in biogeochemical cycles to ascertain if environmental concentrations of these compounds have side-effects on such cycles, with special focus on N cycling (anammox, denitrification, and nitrification). Despite the few studies available and the variability in terms of antibiotic types and conditions tested, conclusions of their work point to a clear alteration of microbial activity in key biogeochemical cycles, thus affecting ecosystem functioning at different levels.

Despite these considerations, it is now clear that chronic exposure to antibiotics, even at very low concentrations, promotes and maintains a pool of resistance genes in natural microbial communities (Séveno et al., 2002; Allen et al., 2010; Sengupta et al., 2013; Andersson and Hughes, 2014). It should be mentioned, however, that most of these genes, although conferring a resistant phenotype when expressed, are probably not “true” resistance genes (Martinez et al., 2015) thus having a function distantly related to that under therapeutic conditions (Allen et al., 2010; Martinez et al., 2015). Notwithstanding this, current data indicate that the extensive use of antibiotics over the last century has generated a selective pressure that has accelerated the acquisition and spread of ARGs among environmental bacteria posing a risk for human health assuming the striking capacity of microbes to share genes.

ACQUISITION AND SPREAD OF ARGs IN BIOFILMS

Susceptible bacteria may become resistant to antibiotics through chromosomal mutations or by HGT, being the latter the major contributor to the spread of antibiotic resistance determinants. The significance of HGT to microbial adaptation was initially recognized when antibiotic-resistant pathogens were identified (Sobecky and Hazen, 2009). HGT is mediated by mobile genetic elements (MGEs), which play an important role in the evolution and adaptation of bacterial species to new and/or changing environmental conditions (Frost et al., 2005). MGEs are segments of DNA encoding a variety of enzymes and proteins that mediate their movement within the host genome (intracellular mobility) or between bacterial cells (intercellular mobility). Interchange of DNA fragments between a cell donor and a receptor takes place through conjugation, transformation, or transduction, whereas intracellular movement is facilitated by integrons and transposons (Modi et al., 2014).

Together with phage transduction and natural transformation, the exchange of genetic material through conjugation is one of the most efficient pathways to disseminate antibiotic resistance among bacterial cells, where donor and recipient cells are in close contact. Conjugation is mainly mediated by the so-called “conjugative plasmids”, although “conjugative transposons” are also capable of triggering the process. One of the most important aspects of conjugative plasmids is that they can be exchanged among both related and phylogenetically distant bacteria (Dionisio et al., 2002). The high cell density and close contact among cells within the biofilm matrix together with increased genetic competence and accumulation of MGEs in these habitats convert them into an optimal scenario for the acquisition and spread of ARGs (Fux et al., 2005). Several studies have shown increased conjugation efficiencies in biofilms when compared to free-living bacterial cells. In fact, conjugation of the broad-host-range plasmid RP4 between two species of *Pseudomonas* occurred in a biofilm reactor at high frequencies (Ehlers and Bouwer, 1999). *In situ* assessment of gene transfer rates in biofilms using automated confocal laser scanning microscopy revealed conjugation rates 1,000-fold higher than those determined by classical plating techniques (Hausner and Wuertz, 1999). Molin and Tolker-Nielsen (2003) also showed that the efficiency of gene transfer seems to be correlated with the biofilm surface, suggesting that a high surface/volume ratios favor transfer within or between biofilm populations.

The diversity and abundance of ARGs in environmental biofilms have been investigated by several authors to unveil differences in the concentration of target genes between planktonic and benthic compartments. Less information is available, however, on the contribution of MGE to the acquisition and spread of ARGs among biofilm inhabitants and between them and free-living bacteria. **Table 1** summarizes some relevant studies dealing with the presence, diversity and abundance of ARGs in biofilms from different environmental settings such as rivers exposed to WWTP effluent discharges, WWTP and drinking water network pipelines, experimental mesocosm, and sand filters. Although not exhaustive, **Table 1** provides a general overview of results obtained by different research groups studying the role of environmental biofilms as hot spots for the accumulation and transfer of ARGs. Schwartz et al. (2003) demonstrated that the *vanA* gene, which confers a high-level resistance to vancomycin, was detected in drinking water biofilms in the absence of any vancomycin-resistant enterococci, suggesting a potential gene transfer from them to autochthonous bacteria in drinking water systems. Gillings et al. (2008) investigated the presence of a MGE, the class 1 integrase (*intI1*) gene, in bacterial isolates collected from diverse environmental samples near Sydney. Authors found that 1 to 3% of bacterial isolates from lake sediments were *intI1* positive, while in biofilms from a groundwater treatment plant, the number of *intI1*-positive isolates reached 30% despite no antibiotics were used as selective agents for culturing. Moreover, Engemann et al. (2008) found that the abundance of six genes conferring resistance to tetracycline

TABLE 1 | Studies on antibiotic resistance and related genes in environmental biofilms.

Type of biofilm	Sampling Point†	Target ARG	Organism	Method	Pollution source†	Main findings	Reference
River bed Wastewater pipeline	DWN HWP Upstream WWTP WWTP effluent	*vanA* *mecA* *ampC*	Enterococci Staphylococci *Enterobacteriaceae*	Cultivation, PCR	UWW HWW	• All target genes were amplified from hospital wastewater biofilms. • *van*A and *amp*C genes were detected in all wastewater biofilms.	Schwartz et al., 2003
GWTP	GACF	*Intl1*	Multi-species biofilm	PCR, CE-SSCP	GWP	• In biofilms from the groundwater treatment plant, the number of *intl1*-positive colonies reached 30%.	Gillings et al., 2008
Experimental Mesocosms	Peripheral biofilms grown in mesocosm	*tetO, tetW, tetM, tetQ, tetB* and *tetL*	Multi-species biofilm	qPCR	CWS	• *tet(W)* gene showed the highest migration from the water column to biofilms. • Only 15% of ARGs disappearance rate was caused by migration to biofilms.	Engemann et al., 2008
River bed Wastewater pipes	WWTP HWP	*aac*(6')-*Ie* +*aph*(2") *mecA, tetA, tetB*	Multi-species biofilm	qPCR, PCR	UWW HWW	• The highest concentration of all genes was observed in the hospital pipeline.	Börjesson et al., 2009
Experimental mesocosms	Peripheral biofilms grown in mesocosm	*tet(O), tet(W), tet(M), tet(Q), tet(B)* and *tet(L)*	Multi-species biofilm	qPCR	PWS (periodic pulse addition)	• Studied genes migrate rapidly from water to biofilms, where they persisted longer than in adjacent water.	Zhang et al., 2009
Horizontal subsurface constructed wetland	Influent Wetland biofilm Effluent	*tetA, tetB, tetM, sul1, ermB, ampC, qnrS*	Multi-species biofilm	qPCR	UWW	• All genes were detected in the three studied compartments • ARGs concentration in the biofilm and in the effluent were affected by system operational parameters.	Nõlvak et al., 2013
Drinking water treatment plant	Clarifier sand filter	*Intl1, sul1* *qacEΔ1*	Multi-species biofilm	PCR	UWW	• All class 1 integron genes detected were positive for the *qacEΔ1*gene. In turn, only 37.5% of class I integron genes were positive for *sul1*.	Farkas et al., 2013
River bed	Upstream river waters WWTP discharge Downstream river waters	*qnrA, qnrB, qnrS* bla_{TEM}, bla_{CTX-M}, bla_{SHV} *ermB, sulI, sulII, tetO, tetW*	Multi-species biofilm	qPCR	UWW	• Relative abundance of target ARG's was significantly higher in biofilm samples collected downstream the WWTP discharge point than in biofilms collected in upstream waters.	Marti et al., 2013
River bed	Six sites along the river (Upstream-downstream)	*vanA, vanB* aacA-aphD, *mecA* *ermA, ermB* *tetA, tetB, tetK, tetM*	Multi-species biofilm	PCR	LF	• Only three antibiotic resistance genes (ARG) were detected within the 147 samples collected.	Winkworth, 2013
River bed	Upstream river waters WWTP discharge Downstream river waters	*qnrA, qnrB, qnrS* *aac*(6')-*Ib-cr*	Multi-species biofilm	PCR	UWW	• The *qnr*S gene was the most prevalent among *qnr* genes in the environment.	Marti et al., 2014b

†*WW, Wastewater; WWTP, Wastewater treatment plant; GWTP, Groundwater treatment plant; DWN, Drinking water network; HWP, Hospital wastewater pipeline; HWW, Hospital wastewater; UWW Urban wastewater; CWS, cattle waste slurry; LF, Livestock farming; PWS, Piglet waste slurry; GACF, Granulated activated charcoal filter; GWP, Groundwater pollution; CE-SSCP, Capillary electrophoresis single strand conformation polymorphism.*

was reduced at different rates in the water column, and some genes, particularly *tetW*, readily migrated into biofilms. Transfer to biofilms did not, however, completely explain disappearance of *tet* genes from the planktonic compartment and other factors such as sunlight and potential microbial degradation would probably contributed (Engemann et al., 2006, 2008). In a similar experimental approach but using periodical piglet waste loadings, Zhang et al. (2009) observed that *tet* genes migrate rapidly to biofilms, where they persist longer than in adjacent waters. Recently, Farkas et al. (2013) also observed that 9.4% of isolates from drinking water biofilms harbored class 1 integrons, which were mainly detected in bacteria (e.g., *Enterobacteriaceae*) that may be associated with microbiological contamination.

Because biofilms play an important role as reservoirs for ARGs, they could be considered as biological indicators of antibiotic resistance pollution in the same way as river ecologists use streambed biofilms as indicators of the overall "ecological status" of the river ecosystem (Sabater et al., 2007). The chronic exposure to sub-MIC concentration of antibiotics exerts a selective pressure on biofilm bacterial communities that may stimulate the emergence and spread of antibiotic resistance (Allen et al., 2010; Andersson and Hughes, 2014; Marti et al., 2014a; Chow et al., 2015). The presence of other pollutants, such as heavy metals from feed additives, organic, and inorganic fertilizers, pesticides and anti-fouling products, also contributes in the co-selection of antibiotic resistance because the close location of genes encoding for these resistance phenotypes in the same MGE (Seiler and Berendonk, 2012). Such exposures may eventually have consequences on the selection and abundance of MGEs, thereby facilitating the spread of ARGs among different species; different biofilm compartments (e.g., epilithic, epipsammic, and hyporheic streambed); or even between different prokaryotic communities as recently assessed by plasmid metagenomics (Sentchilo et al., 2013). Besides, several studies provided evidence that ARGs tend to accumulate in biofilms rather than in the planktonic compartment. In this regard, Börjesson et al. (2009) found a high proportion of genes encoding resistance to aminoglycosides and tetracyclines in biofilm samples collected at a WWTP. Winkworth (2013) demonstrated that, while the levels of ARGs in biofilm samples collected along the Taieri River were low, sites subjected to combined influences of greater human activity and intensive dairy farming showed an increased level of ARGs. Likewise, a study carried out by our research group clearly showed the effect of WWTP effluents on the prevalence of several ARGs in the Ter River, accompanied by a significant increase in their relative abundance in biofilm samples collected downstream the WWTP discharge point (Marti et al., 2013). Moreover, we have investigated the prevalence of plasmid-mediated quinolone resistance (PMQR) determinants in ciprofloxacin-resistant strains isolated in biofilm and sediments from a WWTP discharge point and its receiving river (upstream and downstream sites). We observed that, while the number of strains harboring PMQR determinants was higher in sediments, PMQR-positive strains were also detected in biofilm samples, especially in those from the WWTP discharge point and downstream sites (Marti et al., 2014b). In a study carried out in a horizontal subsurface flow constructed wetland, Nõlvak et al. (2013) found that copy numbers of *tetA* and *sul1* genes in the wetland biofilms were one order of magnitude higher than in the effluent water, despite the fact that this facility had a similar efficiency to conventional WWTP in removing ARGs from wastewater. Altogether, these studies undoubtedly demonstrate the contribution of biofilms in the acquisition and spread of ARGs.

ANTIBIOTIC RESISTANCE IN BIOFILMS ASSESSED BY METAGENOMICS

Until the last decade our knowledge of antibiotic resistance has largely depended on data provided by traditional culture-based methods (Cockerill, 1999). Although useful, these data are limited and biased towards cultivable members of the community. Recent advances in genomics and metagenomics are now providing new avenues for understanding evolutionary processes controlling antibiotic resistance mechanisms and their spreading among microbial populations.

To date, several thousand metagenomes have already been sequenced from a large variety of environments, and this number is set to grow rapidly in the forthcoming years. Most of these metagenomes are publically available through various databases and annotation platforms, such as MG-RAST (Meyer et al., 2008), CAMERA (Sun et al., 2011), and IMG/M (Markowitz et al., 2012), which provide additional insight in the function of complex microbial communities through comparative analyses. Moreover, the availability of specialized databases such as the ARG Database (ARDB; Liu and Pop, 2009), the Comprehensive Antibiotic Resistance Database (CARD; McArthur et al., 2013), the Integron Database (INTEGRALL; Moura et al., 2009), the Bush, Palzkill, and Jacoby's collection of curated β-lactamase proteins (http://www.lahey.org/Studies/), and the implementation of high-throughput sequence analysis tools such as BLAT (Kent, 2002), USEARCH (Edgar, 2010), and DIAMOND (Buchfink et al., 2015), provide a comprehensive molecular toolbox that allow a better understanding of the evolution, ecology, and spread of antibiotic resistance in different organisms and ecosystems.

We have conducted a comparative analysis of selected metagenomes corresponding to several projects and environments publically available in the MG-RAST database (http://metagenomics.anl.gov/) to provide an overall insight on the prevalence of MGEs and ARGs in environmental biofilms. This analysis showed that MGEs-related sequences, such those from phages and plasmids, were found in a lower proportion in metagenomes from river biofilms than those from WWTPs and river water environments. Remarkably, transposons were detected in a higher proportion in WWTPs and river biofilms than those from river water environments (**Figure 1**). Similarly, sequences related to genes conferring resistance to β-lactam antibiotics were also detected more frequently among microbial communities from WWTPs and streambed river biofilms than those from river water environments. Sequences related to genes conferring resistance to tetracyclines were also abundant in WWTPs and river biofilms, but to a lesser extent than β-lactams. Finally, no differences in the proportion of genes conferring

resistance to sulfonamides were observed among the examined environments.

Interestingly, the analysis of the selected metagenomes also showed that two acid mine drainage biofilm samples from the Richmond Mine (4441138.3 and 4441137.3) yielded a high proportion of sequences related to genes conferring resistance to β-lactam antibiotics (5.7 to 7.2%). These relatively high values of β-lactamases might be related to the higher proportion of transposons in these acidophilic biofilms (0.5 to 1.6%) than those detected in environments close to neutral pH such as riverbed biofilms, WWTPs and freshwater systems (**Figure 1**).

A recent study revealed a remarkable abundance and diversity of genes encoding transposases in the metagenome of a hydrothermal chimney biofilm (Brazelton and Baross, 2009). The comparative analysis between this metagenome (4461585.3) and the metagenomes mentioned above confirmed these observations (8.1% of transposase sequences), but similar proportions were observed for β-lactamases between the hydrothermal vent biofilms and those from river water environments. The high relative proportion of transposases may favor an enhanced gene transfer between bacterial genomes that confer new and useful accessory functions, including resistance to heavy metals or antimicrobial compounds. The presence of genes conferring resistance to β-lactams in environments not subjected to antibiotic pollution such as deep sea vents or pristine systems raises interesting questions not only about the origin and ecological function of these genes in nature but also the criteria that researchers adopt when defining a resistance gene (Martinez et al., 2015).

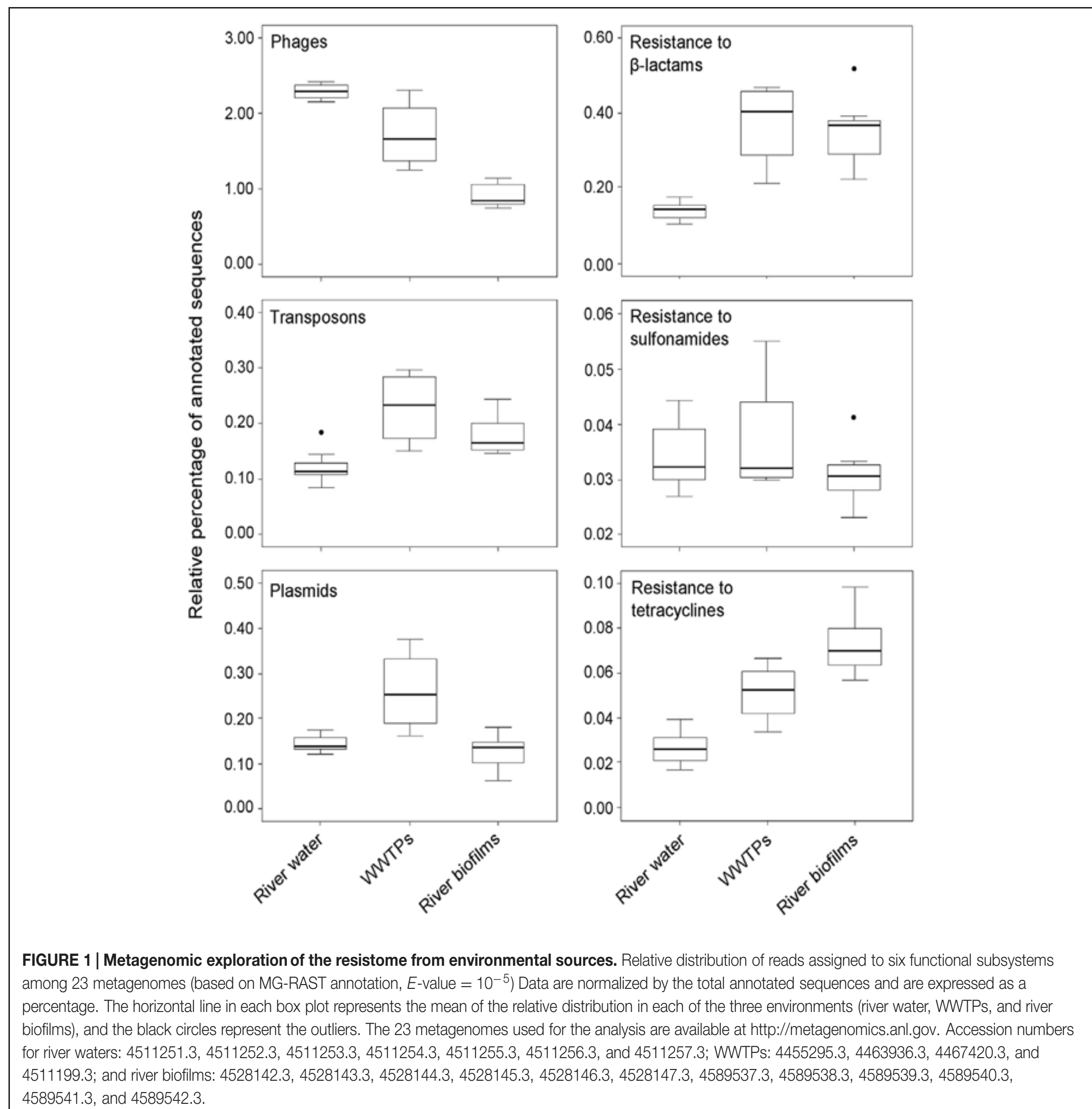

FIGURE 1 | Metagenomic exploration of the resistome from environmental sources. Relative distribution of reads assigned to six functional subsystems among 23 metagenomes (based on MG-RAST annotation, E-value = 10^{-5}) Data are normalized by the total annotated sequences and are expressed as a percentage. The horizontal line in each box plot represents the mean of the relative distribution in each of the three environments (river water, WWTPs, and river biofilms), and the black circles represent the outliers. The 23 metagenomes used for the analysis are available at http://metagenomics.anl.gov. Accession numbers for river waters: 4511251.3, 4511252.3, 4511253.3, 4511254.3, 4511255.3, 4511256.3, and 4511257.3; WWTPs: 4455295.3, 4463936.3, 4467420.3, and 4511199.3; and river biofilms: 4528142.3, 4528143.3, 4528144.3, 4528145.3, 4528146.3, 4528147.3, 4589537.3, 4589538.3, 4589539.3, 4589540.3, 4589541.3, and 4589542.3.

FINAL REMARKS AND FUTURE PROSPECTS

Biofilms occur in almost any submerged surface in both natural and man-made systems providing a suitable and optimal environment for the growth, activity, and interaction of different bacterial species. Biofilms also provide a shelter where to cope with transient or permanent stress conditions, also favoring metabolic interactions and genetic interchange between different bacterial species struggling for survival in a changing environment. Punctual or continuous discharges of pharmaceutical compounds into aquatic systems might constitute not only a selective pressure on aquatic bacterial communities that stimulate the transmission and spread of ARG, but also a chronic source of background biochemical noise that may potentially interfere the communication networks that microbes finely tuned during evolution. Although little information is available on the actual capacity of aquatic bacteria to transfer antibiotic-resistance determinants to potential human pathogens, current data corroborate that environmental biofilms are true reservoirs of ARGs. Further research is needed; however, to elucidate to which extent such hot spots of antibiotic resistance may constitute a serious concern for human health, how the diversity and abundance of ARG change between different biofilm compartments, how this resistance genetic pool moves among communities and how this gene transfer varies in response to the amount of chemical pollution (antibiotics but also other stressors such as heavy metals and xenobiotic compounds) in the receiving waters. The continuous refinement of sequencing technologies (e.g., metagenomics, metatranscriptomics) and bioinformatic tools and the availability of specialized and properly curated databases may help to reach these goals and hit new research targets. Answering these (and other) questions will provide a better knowledge of the transfer dynamics of resistance genes at ecosystem level (between species, communities, and/or habitats), yielding clues to fight against antibiotic resistance and the threat that it poses to the environment and to the human health.

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Chapter 4

Real-time PCR assays for the detection and quantification of carbapenemase genes in environmental samples

Subirats, J., E. Royo, J.L. Balcázar and C.M. Borrego. "Real-time PCR assays for the detection and quantification of carbapenemase genes (*bla*KPC, *bla*NDM and *bla*OXA-48) in environmental samples". *Environmental Science and Pollution Research.* Vol. 24, issue 7 (March 2017) : 6710-6714.

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Abstract

In this study, we have developed real-time PCR assays using SYBR Green chemistry to detect all known alleles of bla_{KPC}, bla_{NDM}, and bla_{OXA-48}-like carbapenemase genes in water, sediment, and biofilm samples collected from hospital and wastewater treatment plant (WWTP) effluents and rivers receiving chronic WWTP discharges. The amplification of bla_{KPC}, bla_{NDM}, and bla_{OXA-48} DNA was linear over 7 log dilutions (R^2 between 0.995 and 0.997) and showing efficiencies ranging from 92.6% to 100.3%. The analytical sensitivity indicated that the reaction for bla_{KPC}, bla_{NDM}, and bla_{OXA-48}-like genes was able to detect 35, 16, and 19 copy numbers per assay, respectively. The three carbapenemase genes were detected in hospital effluents, whereas only the bla_{KPC} and bla_{NDM} genes were detected in biofilm and sediment samples collected from wastewater-impacted rivers. The detection of bla_{KPC}, bla_{NDM}, and bla_{OXA-48}-like genes in different matrices suggests that carbapenem-resistant bacteria occur in both planktonic and benthic habitats thus expanding the range of resistance reservoirs for last-resort antibiotics. We believe that these real-time PCR assays would be a powerful tool for the rapid detection and quantification of bla_{KPC}, bla_{NDM}, and bla_{OXA-48}-like genes in complex environmental samples.

Keywords

bla_{KPC} genes; bla_{NDM} genes; bla_{OXA-48}-like genes; Real-time PCR; Environmental biofilm

Chapter 5

Wastewater pollution differently affects the antibiotic resistance gene pool and biofilm bacterial communities across streambed compartments

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ORIGINAL ARTICLE

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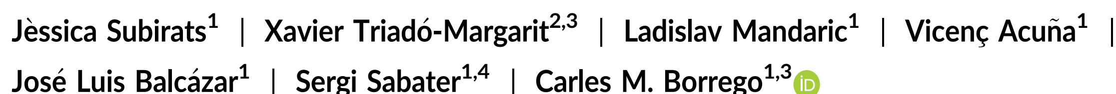
Wastewater pollution differently affects the antibiotic resistance gene pool and biofilm bacterial communities across streambed compartments



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Abstract

Wastewater discharges introduce antibiotic residues and antibiotic-resistant bacteria (ARB) into surface waters. Both inputs directly affect the streambed resistome, either by exerting a selective pressure that favour the proliferation of resistant phenotypes or by enriching the resident communities with wastewater-associated ARB. Here, we investigated the impact of raw and treated urban wastewater discharges on epilithic (growing on rocks) and epipsammic (growing on sandy substrata) streambed biofilms. The effects were assessed by comparing control and impact sites (i) on the composition of bacterial communities; (ii) on the abundance of twelve antibiotic resistance genes (ARGs) encoding resistance to β-lactams, fluoroquinolones, sulphonamides, tetracyclines, macrolides and vancomycin, as well as the class 1 integron-integrase gene (*intI1*); (iii) on the occurrence of wastewater-associated bacteria, including putative pathogens, and their potential linkage to target ARGs. We measured more pronounced effects of raw sewage than treated wastewater at the three studied levels. This effect was especially noticeable in epilithic biofilms, which showed a higher contribution of wastewater-associated bacteria and ARB than in epipsammic biofilms. Comparison of correlation coefficients obtained between the relative abundance of both target ARGs and operational taxonomic units classified as either potential pathogens or nonpathogens yielded significant higher correlations between the former category and genes *intI1*, *sul1*, *sul2* and *ermB*. Altogether, these results indicate that wastewater-associated micro-organisms, including potential pathogens, contribute to maintain the streambed resistome and that epilithic biofilms appear as sensitive biosensors of the effect of wastewater pollution in surface waters.

KEYWORDS
antibiotic pollution, antibiotic resistance genes, Aquatic Resistome, Ebre river, raw sewage, streambed biofilms, wastewater effluents

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1 | INTRODUCTION

Sewage discharges impair the chemical and microbiological quality of surface waters and may lead to outbreaks of different waterborne diseases (Pandey, Kass, Soupir, Biswas, & Singh, 2014). This is particularly worrisome when polluted waters are used as a drinking water source, for re-creational purposes or crop irrigation, a condition that is unfortunately common in less affluent countries (Ashbolt, 2004). In developed countries, sewage is usually treated in urban wastewater treatment plants (UWWTPs) where nutrients and pathogens are reduced to acceptable levels before discharging into the environment. However, UWWTPs are not well-suited to effectively remove pharmaceutical compounds (including antibiotics), antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs). Effluents from UWWTPs are known to cause a chronic antibiotic pollution of surface waters (Michael et al., 2013; Rodríguez-Mozaz & Weinberg, 2010; Rodríguez-Mozaz et al., 2015) despite the fact that dilution into the receiving system largely reduce antibiotic concentrations far below those used in clinical practices (Davies, Spiegelman, & Yim, 2006). These environmental subinhibitory concentrations are still sufficient to select for ARB and to favour the spread of ARGs between environmental bacteria and clinically relevant micro-organisms (Chow, Waldron, & Gillings, 2015; Tello, Austin, & Telfer, 2012). Similarly, the efficiency of UWWTPs in the removal of ARB and ARGs greatly depends on the inclusion of advanced treatments (e.g., sand and membrane filtration, ozonation) or disinfection steps (e.g., chlorination, UV radiation) after conventional activated sludge (CAS) treatment (Rizzo et al., 2013). Many UWWTPs do not incorporate these final polishing treatments and only rely on biological CAS for wastewater treatment. Several studies have demonstrated that effluents from CAS-UWWTPs contain a high abundance of ARB and ARGs that are continuously released into the environment (Manaia, Macedo, Fatta-Kassinos, & Nunes, 2016; Vaz-Moreira, Nunes, & Manaia, 2014). For instance, UWWTP secondary effluents discharge an average of $1.6 \times 10^{13} \pm 3.7 \times 10^{13}$ ciprofloxacin-resistant coliforms into the environment per day, whereas raw sewage effluents increase these figures by two orders of magnitude (Vaz-Moreira et al., 2014). Considering that these numbers only refer to a small fraction of cultivable ARB, the total number of ARB discharged into aquatic environments on a daily basis would probably be overwhelming.

Streambed microbiota forms biofilms on surfaces of rocks and cobbles (epilithic), gravels and sands (epipsammic), and even of aquatic plants, clays or submerged wood (Romaní, 2010). These biofilms are key players in the stream biogeochemistry, contributing to nutrient dynamics, primary production, CO_2 emission and organic matter decomposition (Battin et al., 2016). Assuming this ecological role, anthropogenic pollution (i.e., wastewater discharges) may have important consequences on the composition and functioning of the streambed biofilms (Proia et al., 2013a; Ricart et al., 2010). The intrinsic characteristics of biofilms (heterogeneous composition, close contact between cells, fast growth rates, reduced mobility, among others) make them highly responsive to alterations caused by environmental or chemical stress (Sabater et al., 2007), including antibiotic pollution (Aubertheau et al., 2016; Proia et al., 2013b, 2016). Recently, low levels of several ARGs have been detected in freshwater biofilms receiving faecal contamination (Winkworth-Lawrence & Lange, 2016). Several studies highlighted that streambed biofilms act as environmental reservoirs of ARGs (see Balcázar, Subirats, & Borrego, 2015 for a review on this topic) but less information is available on how these resistance determinants distribute between different streambed compartments or how they associate with wastewater-associated micro-organisms, including potential pathogens.

In this study, we assess the impact of raw and treated wastewater on the prevalence of ARGs encoding resistance to β-lactams (*bla*$_{TEM}$, *bla*$_{KPC}$, *bla*$_{CTX-M}$, *bla*$_{NDM}$ and bla$_{OXA48}$), fluoroquinolones (*qnrS*), glycopeptides (*vanA*), macrolides (*ermB*), sulphonamides (*sul1*, *sul2*) and tetracyclines (*tetM*, *tetW*) in streambed biofilms. We aimed to assess whether or not the pollution source has a determinant effect on the streambed resistome. We evaluated differences in the abundance of ARGs between control and impact sites (i.e., upstream and downstream the discharge point, respectively) and streambed compartments (i.e., epilithic and epipsammic biofilms). Moreover, we assessed to what extent wastewater-associated bacteria (including potential pathogens) may end up in streambed biofilm communities and contribute to maintain the resistance gene pool. Our results point to a clear effect of the pollution source on the composition of bacterial communities and their associated resistome, especially in epilithic biofilms that appear as sensitive biosensors to gauge the effect of wastewater pollution in surface waters.

2 | MATERIALS AND METHODS

2.1 | Study site, sampling and collection of environmental variables

Two tributary streams (Montsant and Matarranya) to the Ebro River (NE Spain) were selected according to the different pollution sources they receive. The Montsant stream at La Bisbal de Falset (41.288623 latitude, 0.724773 longitude) receives continuous discharges from the nearby UWWTP that treats water from a 541 population equivalents (PE) and receives an average daily flow of about 55 m^3 primarily made up of domestic wastewater. The plant operates using CAS at a very high Hydraulic Retention Time of 80 hr and at a Sludge Retention Time of 7 days allowing a reduction of 95% in BOD_5 and a concentration of N-NH_4^+ $\leq$10 mg/L in the final effluent. The plant does not include any treatment for phosphorous removal or additional disinfection steps. The Matarranya stream receives raw sewage from Vall-de-roures (40.857559 latitude, 0.1307797 longitude), a village with 7,015 PE. The location of both study sites is shown in Fig. S1. Discharge, water velocity, width and depth were measured at each section directly in the field with an acoustic-Doppler velocity meter (Sontek, YSI, USA). Water temperature, pH and electrical conductivity were measured in situ using hand-held probes (WTW, Weilheim, Germany).

In both streams, epilithic and epipsammic biofilm samples were collected in triplicate at 100 m upstream the discharge point, and at 250 and 500 m downstream the discharge point in the case of the Montsant and the Matarranya, respectively. These distances varied among the two case studies because of the differences in river width and hydrology, which determine the length of the mixing reach (Kilpatrick & Cobb, 1985). Epilithic biofilms were obtained by randomly selecting streambed cobbles that were then collected in sterile containers. Biofilms growing on cobble surface was scraped in situ using sterile brushes, and collected biomass was stored in sterile 15-ml polypropylene conical tubes (Falcon™, Fisher Scientific, Wilmington, DE, USA). Streambed sediment of sands and gravels (containing epipsammic biofilms) was collected from the top layer fraction (0–5 cm in depth) of the streambed and placed into sterile 15-ml polypropylene conical tubes (Falcon™, Fisher Scientific, Wilmington, DE, USA). Water samples (1 L) from the WWTP effluent (Montsant stream) and from the raw sewage (Matarranya stream) were collected for comparative purposes. After collection, biofilm and water samples were transported to the laboratory in a refrigerated isothermal container (−20°C). Once in the laboratory, biofilms were pelleted by centrifugation at 4,600 × *g* for 15 min, and 0.5 g of pelleted biomass were weighted using a precision scale (Metter Toledo, MS-S/MS-L model), transferred into sterile Eppendorf tubes and stored at −30°C until DNA extraction. For water samples, 40 ml was filtered through 0.22-μm nylon membrane filters (Whatman, Maidstone, UK) using a filtration device and a vacuum pump. Filters were stored at −30°C until DNA extraction.

Water samples for nutrient analysis (ammonium and phosphorous) were collected in triplicate upstream and downstream the discharge point at each stream, filtered through 0.7 μm GF/F filters (Whatman Intl. Ltd., Maidstone, UK) and kept at −20°C until analysis. Ammonium concentration was determined in a Dionex ICS-5000 ion chromatograph (Dionex Co., Sunnyvale, USA, Hach Chemical Company, 2002), and phosphate concentration was determined colorimetrically using an Aliance-AMS Smartchem 140 spectrophotometer (AMS, Frepillon, France) after (Murphy & Riley, 1962).

2.2 | Analyses of antibiotics in water samples

Water samples (1 L) for the quantification of antibiotics were collected in 1-L amber polyethylene bottles from the effluent and at upstream and downstream sites of both streams and stored at −20°C until analyses. Samples were transported to the laboratory in a refrigerated isothermal container (−20°C) and stored at −20°C until extraction.

All chemical standards used were of high purity grade (>90%), and they are listed in Table S1. Antibiotics were purchased from Sigma-Aldrich, while fresh stock solutions were prepared every month due to their limited stability. Isotopically labelled compounds, used as internal standards, were Erythromycin-N, *N*-dimethyl-$^{13}C_2$, Ronidazole-d_3 and Ofloxacin-d_3 from Sigma-Aldrich. Azithromycin-d_3 and Sulfamethoxazole-d4 were purchased from Toronto Research Chemicals (Ontario, Canada). All standards were stored at −20°C after preparation.

The analysis of antibiotics in water samples was carried out in triplicate using an offline solid phase extraction (SPE) followed by ultra-high-performance liquid chromatography coupled to triple quadrupole-linear ion trap tandem mass spectrometry (UHPLC-QqLIT-MS^2) according to Gros, Rodríguez-Mozaz, and Barceló (2012). Chromatographic separations were performed with a Waters Acquity Ultra-Performance™ liquid chromatography system, coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source. Target compounds were eluted from the column into the chromatograph with the LC-mobile phase, and the separation was performed with the binary pump systems (Milford, MA, USA), using an Acquity HSS T3 column (50 mm × 2.1 mm, i.d. 1.8 μm particle size) purchased from Waters Corporation. Electrospray Ionization (ESI) and selected reaction monitoring (SRM) modes were selected for the MS2 detection. Quantification was performed based on the internal standard approach. Method performance parameters of antibiotics including the limits of detections (LODs), limits of quantifications (LOQs), and recovery rates are compiled in Table S2.

2.3 | Extraction of DNA

Extraction of DNA from filters (water samples) and biofilms was performed using the FastDNA® SPIN kit for soils (MP Biomedicals) according to manufacturer's instructions. The DNA concentration in each sample was measured using Qubit 2.0 fluorometer (Life Technologies; Carlsbad, CA, USA), and purity was determined by measuring A_{260}/A_{230} and A_{260}/A_{280} absorbance ratios using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific; Wilmington, DE, USA).

2.4 | Quantification of antibiotic resistance genes

Real-time PCR (qPCR) was used to quantify copy numbers of eleven genes encoding resistance to the main antibiotic families used in human and veterinary such as β-lactams (bla_{TEM}, bla_{CTX-M}, bla_{KPC}, bla_{NDM} and bla_{OXA-48}), fluoroquinolones (*qnr*S), sulphonamides (*sul1* and *sul2*), tetracyclines (*tet*M, *tet*W), macrolides (*erm*B) and vancomycin (*van*A). Copy numbers of the class 1 integron-integrase gene (*intI1*) were also quantified as a proxy for anthropogenic pollution and horizontal gene transfer (Gillings et al., 2015; Stalder et al., 2014). Copy numbers of each target ARG in a sample were normalized to the copy numbers of the 16S rRNA gene, which was used as a proxy for the bacterial abundance in that sample. All genes were quantified using primers and conditions compiled in Table S3. All qPCR standard curves were obtained after serial dilutions of DNA extracts containing known concentration of target gene ranging from 10^9 to 10^2 gene copies per μl (see details in the Supporting Information). All qPCR assays were performed using SYBR green detection chemistry on a MX3005 system (Agilent Technologies; Santa Clara,

CA, USA), as previously described (Marti, Jofre, & Balcázar, 2013). Samples were analysed in duplicate with a standard curve and a negative control included in each run. Specificity of amplification was determined by analysis of the melting curves and gel electrophoresis of amplified products.

2.5 | High-throughput sequencing and sequence processing

High-throughput multiplexed 16S rRNA gene sequencing with the Illumina MiSeq System (2 × 250 PE) was carried out using primer pair 515f/806r (Caporaso et al., 2011) targeting the V4 region of the 16S rRNA gene complemented with Illumina adapters and sample-specific barcodes at the genomics core facilities and methods of the Research Technology Support Facility Michigan State University, USA (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). The analysis yielded high sequencing quality (73% of reads averaged ≥Q30 scores). Details on the raw sequence processing, quality filtering, Operational Taxonomic Unit (OTU) clustering and filtering, construction of OTU table and downstream analyses are provided as Supporting Information.

2.6 | Sequence-based identification of potential pathogens

The presence of putative pathogenic bacteria in both biofilm and water samples was analysed by comparing the similarity of representative OTU sequences against a custom made database of 16S rRNA gene sequences from 283 bacterial species of both obligate and opportunistic pathogens using BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990). The searched database was constructed from the list of pathogenic species inventoried in a previous work (Ecker et al., 2005) and further refined by online searches of the ascribed taxa (Triadó-Margarit et al., 2017). Only those OTU sharing sequence identity values and alignment coverage values above 98% to listed pathogens were considered for downstream analysis. OTUs showing high sequence identity to well-known pathogenic bacteria (≥98%) were further classified according to their host and categories related to affectation or pathogenic potential (obligate or opportunistic) as previously described (Triadó-Margarit et al., 2017).

2.7 | Statistical analyses

Differences in the abundance of target ARGs, *intI1* and *16S rRNA* genes were analysed using two-way ANOVA in which sites (upstream/downstream) and streambed compartments (epilithic/epipsammic biofilms) were set as fixed factors. Increases in the relative concentration of ARGs at downstream sites were calculated using the corresponding concentration at upstream sites as reference. Accordingly, the increase was calculated as the log10 of the ratio between the downstream and the upstream concentration of the target ARG (i.e., $\log10([ARG_{downstream}/[ARG_{upstream}]])$. Thus, positive values indicate an increase in the concentration of the target ARG after the discharge, whereas negative values indicate higher concentrations upstream the discharge point. Statistical significance of differences in ARG abundance between upstream and downstream sites was assessed by multivariate Hotelling's T^2 test after transforming data (logarithmic transformation) to ensure normality. Multivariate Hotelling's T^2 tests were also run to assess for differences on the overall resistome between effluents (raw/treated) or streams (Montsant/Matarranya). When the abundance of a gene was below detection limit (*b.d.l.*), the value corresponding to the detection limit of the qPCR was used to allow data analysis. Statistical tests were carried out using SPSS version 21.0 (IBM Corp), and statistical significance was determined at $\alpha = 0.05$.

For community composition analyses, we constructed a similarity matrix using the Bray–Curtis distance (Legendre & Legendre, 1998) based on the relative abundance (square root transformed) of each bacterial taxon. Ordination of samples according to Bray–Curtis distance matrices was performed using nonmetric multidimensional scaling (nMDS) plots. A stress level <0.10 was considered acceptable for nMDS ordination. Analysis of similarity between communities based on their taxonomic composition was performed after grouping samples by habitat (epilithic vs. epipsammic) or by site (upstream vs. downstream) for each stream (Montsant and Matarranya), which were used as factors in PERMANOVA. Also, the contribution of each OTU to the Bray–Curtis distance between samples grouped by site was calculated using the analysis of similarity percentages (SIMPER). All these analyses were run in the PRIMER-6 statistical package with the PERMANOVA+ add-on (PRIMER-E, Plymouth Marine Laboratory, UK).

Significant differences in the relative abundance of OTUs ascribed to potentially pathogenic bacteria in epilithic biofilms between sampling sites (upstream and downstream from discharge point) were assessed in R version 3.3.1 (R Development Core Team, 2011; http://www.R-project.org) using Student's *t* test (package STATS) or the nonparametric equivalent when normality was not achieved. *p*-values were adjusted for multiple testing using the false discovery rate correction (FDR, Benjamini & Hochberg, 1995) with a significance cut-off of 0.05. In addition, correlation analyses based on Spearman's rank order coefficient were carried out to investigate whether the association between the relative abundance of potentially pathogenic OTUs and ARGs could be different, in average, to the degree of association observed for OTUs not identified as pathogens. Comparison of Spearman's *r* distributions between abundance of ARGs and these two OTU categories (nonpathogens and potential pathogens) was carried out through Welch's *t* test, and *p*-values were also adjusted for multiple comparisons using FDR (see above). R package GGPLOT2 was used for data visualization (Wickham, 2009).

3 | RESULTS

3.1 | Influence of wastewater discharges on stream water chemistry and antibiotic pollution

The physical characteristics of water did not show large variations downstream of the discharge of both raw and treated effluent.

However, there was an effect of both type of effluents on nutrient concentrations, especially in the Matarranya where the discharge of raw sewage caused a substantial increase in the concentration of ammonium and phosphate (Table S4).

Using a multiresidue analysis, we identified and quantitated eight antibiotics in water samples collected at control and impact sites as well as in the studied effluents (Table 1). Particularly, sulfamethoxazole, clarithromycin, ofloxacin, dimetridazole, metronidazole-OH, metronidazole, trimethoprim and ronidazole were detected at concentrations ranging from less than 3 to 645 ng/L in the raw sewage effluent (Matarranya, Table 1). In the receiving stream, however, only ofloxacin showed concentrations above the limit of quantification (19.7 and 53.7 ng/L in water samples collected upstream and downstream the discharge point, respectively). The treated effluent discharging into the Montsant stream contained sulfamethoxazole, clarithromycin, ofloxacin, trimethoprim and ronidazole at highly variable concentrations (from <6.0 to 459 ng/L). Ofloxacin was again the sole antibiotic detected over the limit of quantification in water samples collected upstream and downstream the impact point (Table 1).

3.2 | Abundance of antibiotic resistance genes across streambed compartments

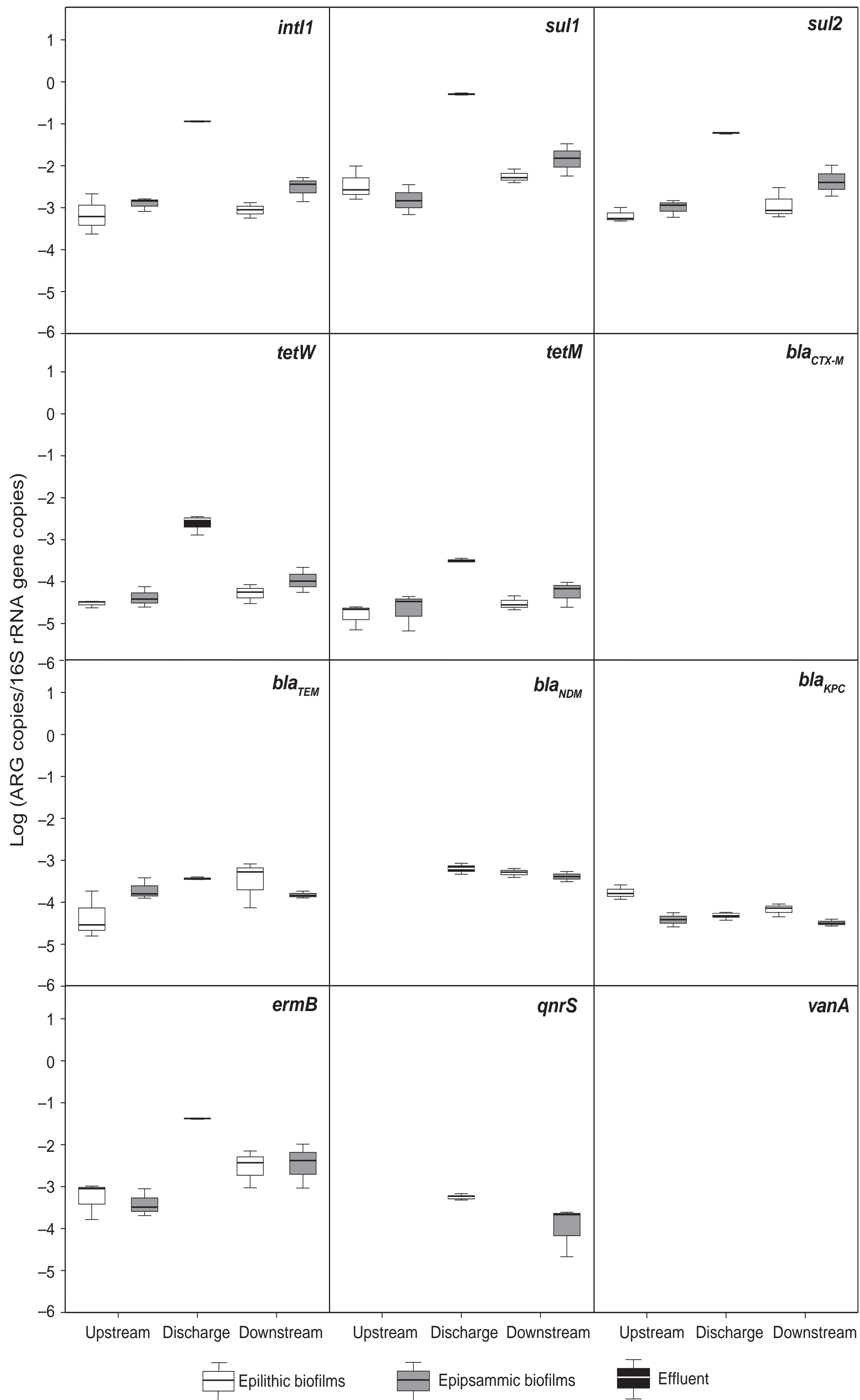
All qPCR assays were performed with high R^2 values (0.998 in average), high efficiencies (90.2–100%) and a dynamic range of at least five orders of magnitude, thus validating the gene quantification. The abundance of bacterial 16S rRNA genes ranged from $4.73 \times 10^9 \pm 9.24 \times 10^8$ to $7.59 \times 10^9 \pm 2.78 \times 10^8$ copy numbers per gram of biofilm in the Montsant stream and from $8.54 \times 10^7 \pm 6.49 \times 10^7$ to $1.19 \times 10^9 \pm 1.75 \times 10^9$ copy numbers per gram of biofilm in the Matarranya stream. Copy numbers of bacterial 16S rRNA gene were $2.44 \times 10^7 \pm 4.13 \times 10^6$ copies/ml in the treated effluent (Montsant) and $2.4 \times 10^8 \pm 1.88 \times 10^7$ copies/ml in the raw sewage (Matarranya). In the Montsant stream, most of the target genes except *van*A and $bla_{CTX\text{-}M}$ were detected in almost all samples (Figure 1). Remarkably, the bla_{NDM} gene was only detected in the effluent and downstream samples, whereas the *qnr*S gene was only detected in the effluent and downstream epipsammic biofilms. In the Matarranya, the normalized concentration of all detected genes was between one and two orders of magnitude higher than those in the Montsant, particularly downstream of the raw sewage discharge point (Figure 2). In this stream, the $bla_{CTX\text{-}M}$ gene was only detected in the untreated effluent, whereas the *van*A gene was detected only in epilithic biofilms but not in the sewage. The concentration of the $bla_{OXA\text{-}48}$ gene was below detection limit in both streams. As a general trend, the normalized concentration of most ARGs was higher in both effluents (raw and treated) than in the streambed biofilms, with the exception of three genes encoding resistance to β-lactams (bla_{TEM}, bla_{NDM} and bla_{KPC}), which were equally or more abundant in samples collected downstream the discharge points (Figures 2 and 3). Remarkably, the comparison of the overall resistome between raw and treated effluents resulted in a significant difference (Hoteling's T^2 = 46,412.7, p = .007), although the collection of ARGs contributing to this difference was not the same for the treated effluent (*sul1*, *sul2*, *intI1*, bla_{NDM} and bla_{KPC}) than for the raw sewage (*tet*W, *tet*M, $bla_{CTX\text{-}M}$, bla_{TEM}, *erm*B and *qnr*S).

In the Montsant, *sul1*, *sul2*, *tet*W, $bla_{CTX\text{-}M}$, bla_{NDM}, *erm*B and *qnr*S genes were significantly more abundant in biofilms collected downstream the discharge than in those from upstream samples (Figure 2; Table S5). Notwithstanding this, no significant differences existed in the abundance of ARGs in epilithic or epipsammic biofilms, except for the bla_{KPC} gene, which was more abundant in the former. In this stream, increases in the relative concentration of each ARG after the discharge were positive for both type of biofilms, although only the *sul1* gene in epipsammic biofilms and the bla_{NDM} gene in both biofilms resulted in significant differences (Hoteling's T^2 post hoc test, p = .033 and p < .001, respectively) (Figure 3). A reduction in the relative concentration of bla_{TEM} and bla_{KPC} genes in epipsammic biofilms and the bla_{KPC} gene in epilithic biofilms occurred in the Montsant stream, although only the latter decrease was significant (Hoteling's T^2 post hoc test, p = .039).

TABLE 1 Concentration of antibiotics detected in the studied effluents and sites from both streams

	Montsant			Matarranya		
Antibiotic	Upstream (ng/L)	WWTP effluent (ng/L)	Downstream (ng/L)	Upstream (ng/L)	Raw sewage effluent (ng/L)	Downstream (ng/L)
Sulphamethoxazole	ND	30.7 ± 3.28	ND	ND	37.4 ± 1.91	< 1.4
Clarithromycin	ND	459 ± 11.6	< 2.9	< 2.9	424 ± 12.9	< 2.9
Ofloxacin	15.8 ± 0.06	213 ± 6.44	18.6 ± 0.88	19.2 ± 3.26	645 ± 32.2	53.7 ± 5.23
Dimetridazole	ND	ND	ND	ND	79.6 ± 2.78	<18.7
Metronidazole-OH	ND	ND	ND	ND	180 ± 8.91	<3.0
Metronidazole	ND	ND	ND	ND	261 ± 17.1	<2.2
Trimethoprim	<3.1	8.81 ± 0.54	<3.1	<3.1	<3.1	<3.1
Ronidazole	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0

ND, not detected.



intI1
sul1
sul2
tetW
tetM
blaCTX-M
blaTEM
blaNDM
blaKPC
ermB
qnrS
vanA
Log (ARG copies/16S rRNA gene copies)
1
0
−1
−2
−3
−4
−5
−6
Upstream
Discharge
Downstream
Epilithic biofilms
Epipsammic biofilms
Effluent

FIGURE 1 Boxplots showing the relative concentrations of ARGs in effluent and biofilm samples collected from the Montsant stream (N = 3 in all cases except effluent samples where N = 1). The lower and upper edges of each boxplot are the first and third quartiles, the mid-line shows the median and the whiskers extend from the minimal and maximal values

In the Matarranya, all target genes except bla_{TEM}, bla_{KPC}, bla_{NDM} and *van*A showed an increase in their abundance in samples collected after the raw sewage discharge (Figure 2; Table S5). Moreover, all genes except *sul2* were more abundant in epilithic than epipsammic biofilms. The effect of the raw sewage discharge on the abundance of ARGs was clearly observed in the Matarranya, where significant differences were obtained when comparing the increases in ARG abundance at downstream sites (Figure 3). Genes conferring resistance to β-lactam antibiotics behaved differently due to their high prevalence in upstream samples, thus resulting in nonsignificant increases and even decreases in both streambed habitats.

3.3 | Composition of biofilm bacterial communities

The composition and structure of bacterial communities inhabiting epilithic and epipsammic biofilms were clearly different according to pollution source. The treated wastewater effluent had a more diverse community where Actinobacteria (12.2% of total reads), Bacteroidetes (16.6%) and Betaproteobacteria (17.6%) were the most abundant taxa (Fig. S2A). In contrast, the raw sewage was dominated by Firmicutes (49.7% of total reads) and, to a lesser extent, by Gammaproteobacteria (24.3%) (Fig. S2B). Substantial differences also existed between bacterial communities of epilithic and epipsammic biofilms. Sequences affiliated to algal chloroplasts, Cyanobacteria and Alphaproteobacteria were prevalent in the epilithic biofilms, whereas epipsammic communities were characterized by a more even taxa distribution (Fig. S2). The difference in diversity between epilithic and epipsammic communities was evident when comparing the Shannon diversity index (Fig. S3). In both streams, this comparison yielded significant differences between streambed compartments (p = .04 and p = .06 for Montsant and Matarranya, respectively). In turn, no significant differences were observed for each compartment between control and impact sites although the diversity of epilithic bacterial communities in the Matarranya clearly dropped (Shannon index, 7.5 ± 0.08 and 6.5 ± 0.19 for upstream and downstream epilithic biofilms, respectively).

The ordination of samples according to their similarity in community composition (Bray–Curtis distance) showed different patterns in the two streams. Bacterial communities in the Montsant formed individual clusters for the epilithic and epipsammic compartments, without clear separation between upstream and downstream samples (Fig. S4A). Such a distinct distribution was corroborated with a PERMANOVA, which yielded highly significant differences in community composition across streambed compartments (p = .003), but marginally significant differences between control and impact sites (p = .045). In the Matarranya, however, epilithic biofilms at the impact site (downstream) segregated from those collected upstream and distributed closer to the raw sewage effluent (Fig. S4B). This pattern was not followed by the epipsammic biofilms, which formed a tight cluster irrespective of their collection site. The PERMANOVA yielded highly significant p values for streambed compartments and sampling site (p = .004 and p = .003, respectively) and also identified a significant interaction between biofilm type and sampling site in this stream (p = .011).

SIMPER analyses helped on the identification of bacterial taxa that contributed the most to differences between control and impact sites and biofilm compartments (Tables S6 and S7). In the Montsant, upstream communities were mainly composed of OTUs affiliated to common freshwater bacterial taxa such as Actinobacteria, Bacteroidetes, Alpha-, Beta-, Gammaproteobacteria and Verrucomicrobia (Table S6). In the Matarranya, downstream biofilms had a higher relative abundance of Gammaproteobacteria and Firmicutes in epilithic biofilms, plus Actinobacteria and Alphaproteobacteria in epipsammic biofilms (Table S7). Gammaproteobacteria and Firmicutes were the most prevalent taxa in the raw sewage effluent (Fig. S2B). Besides, both taxa encompass most of the bacterial pathogens putatively identified in streambed biofilms communities (see next).

3.4 | Identification of potential pathogens in the streambed

After comparing representative OTU sequences against a database containing 283 obligate and opportunistic pathogens, 81 OTUs showed high sequence similarity (≥98%) to well-known bacterial pathogens. From this initial subset, 46 OTUs showing relatives abundances <0.01% were considered rare and discarded, thus retaining only 35 OTUs for downstream analyses. This subset of potential pathogenic OTUs showed variations in their relative abundance depending on the effluent type (Figure 4a). In both effluents, the analysis identified OTUs with high sequence identity to several well-known bacterial pathogens such as *Arcobacter cryaerophilus*, *Yersinia pseudotuberculosis*, *Shigella flexneri*, *Staphylococcus saprofiticus*, *Granulicatella adiacens*, *Mycobacterium fortuitum*, *Aeromonas caviae* and several species within genera *Acinetobacter*, *Pseudomonas* and *Acidovorax*. Remarkably, their relative abundance was substantially higher in the raw sewage effluent (Matarranya). Moreover, several of these putative pathogenic OTUs (i.e., *Granulicatella adiacens*, *Acinetobacter baumannii* and *A. haemolyticus*) were significantly enriched in downstream samples from the Matarranya (Figure 4b) but not in those collected at the Montsant stream (data not shown). A global BLAST analysis of the 35 retained OTUs against a general nucleotide database was also carried out to assess if top-ten hits matched the previous identification conducted using the pathogen database. In most cases (28 out of 35 OTUs, 80%), first BLAST hits affiliated to sequences of the same genera of the target pathogen and even some matched sequences of well-known bacterial pathogens (e.g.,

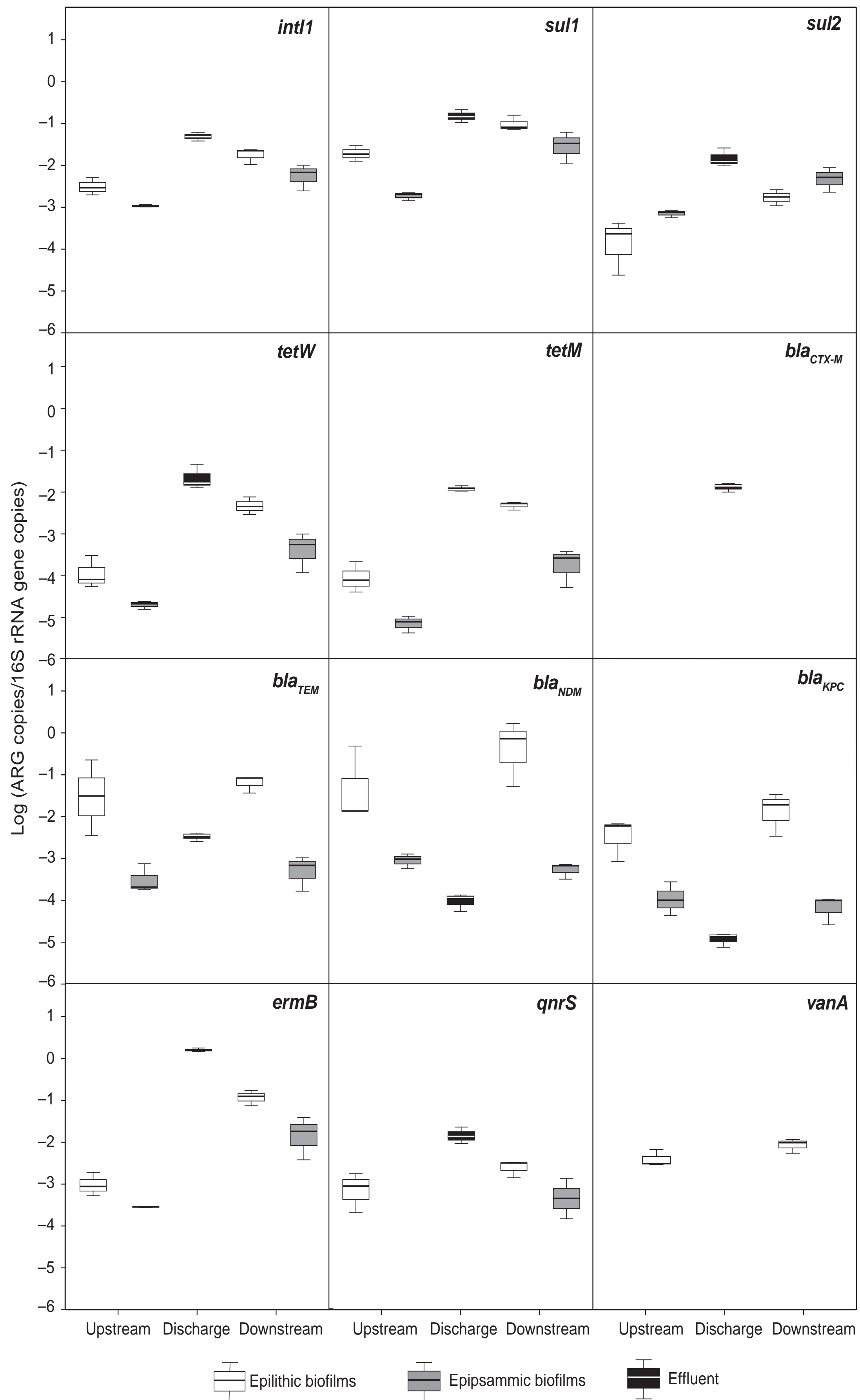

intI1
sul1
sul2
tetW
tetM
blaCTX-M
blaTEM
blaNDM
blaKPC
ermB
qnrS
vanA
Log (ARG copies/16S rRNA gene copies)
1
0
−1
−2
−3
−4
−5
−6
Upstream Discharge Downstream
Epilithic biofilms
Epipsammic biofilms
Effluent

FIGURE 2 Boxplots showing the relative concentrations of ARGs in effluent and biofilm samples collected from the Matarranya stream (N = 3 in all cases except effluent samples where N = 1). The lower and upper edges of each boxplot are the first and third quartiles, the midline shows the median and the whiskers extend from the minimal and maximal values

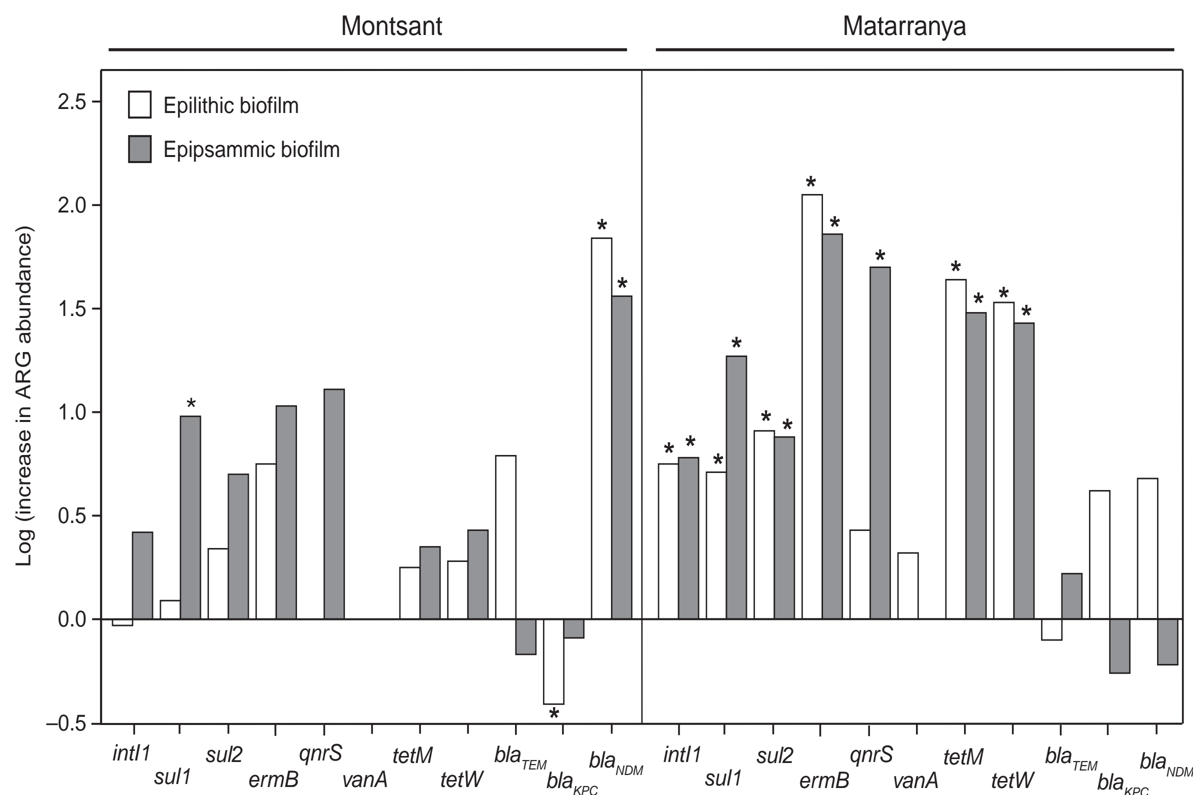


FIGURE 3 Logarithmic increases in ARG abundance in epilithic (white bars) and epipsammic (grey bars) compartments in Montsant (left panel) and Matarranya (right panel) streams. Increases were calculated using the relative concentration of the corresponding ARG in upstream samples as reference (see Section 2 for details). Asterisks indicate significant differences in gene abundance between upstream and downstream sites (multivariate Hotelling's T^2 test, $\alpha = 0.05$)

Clostridium perfringens, *Pseudomonas stutzeri*, *Aeromonas enterica*) (Table S8).

To assess whether or not these wastewater-associated putative pathogens were able to colonize the streambed, the mean relative abundance of the identified OTUs in each streambed compartment was plotted according to their collection site (i.e., upstream/downstream) (Figure 5). These biplots revealed an increase in the abundance of potential pathogens in epilithic biofilms collected after the raw sewage discharge (Figure 5c). The impact on the epipsammic compartment was less pronounced according to the low number and abundance of putative pathogens in this habitat (Figure 5d). Besides, many low-abundant OTUs showing high identity to sequences of well-known pathogens distributed evenly across streambed compartments regardless of their collection site (upstream/downstream) and stream (1:1 line in Figure 5). Finally, the comparison of Spearman's Rank Order correlation values between the relative abundance of ARGs and OTUs belonging to both categories (putative pathogens and nonpathogenic ones) yielded significant results (Welsh T test, $p < .01$) for *sul1*, *sul2* and *ermB* (Fig. S5). The bla_{TEM} gene also yielded significant differences (Welsh T test, $p < .01$), but the mean of their correlation coefficients was close to 0 (i.e., variables were weakly associated). OTUs potentially identified as pathogens were also significantly correlated to the relative abundance of gene *intI1*.

4 | DISCUSSION

Stream biofilms are considered an ecological boundary that controls the fluxes of matter and energy between the flowing water and the streambed, thus contributing to the proper functioning of the ecosystem (Battin et al., 2016). Alterations on the integrity of this boundary, such as those caused by wastewater discharges, may have severe consequences at different scales. Although several studies have reported the effects of the chronic discharge of WWTP effluents on the antibiotic resistance profile of river biofilms (Aubertheau et al., 2016; Marti et al., 2013; Proia et al., 2016), none provided

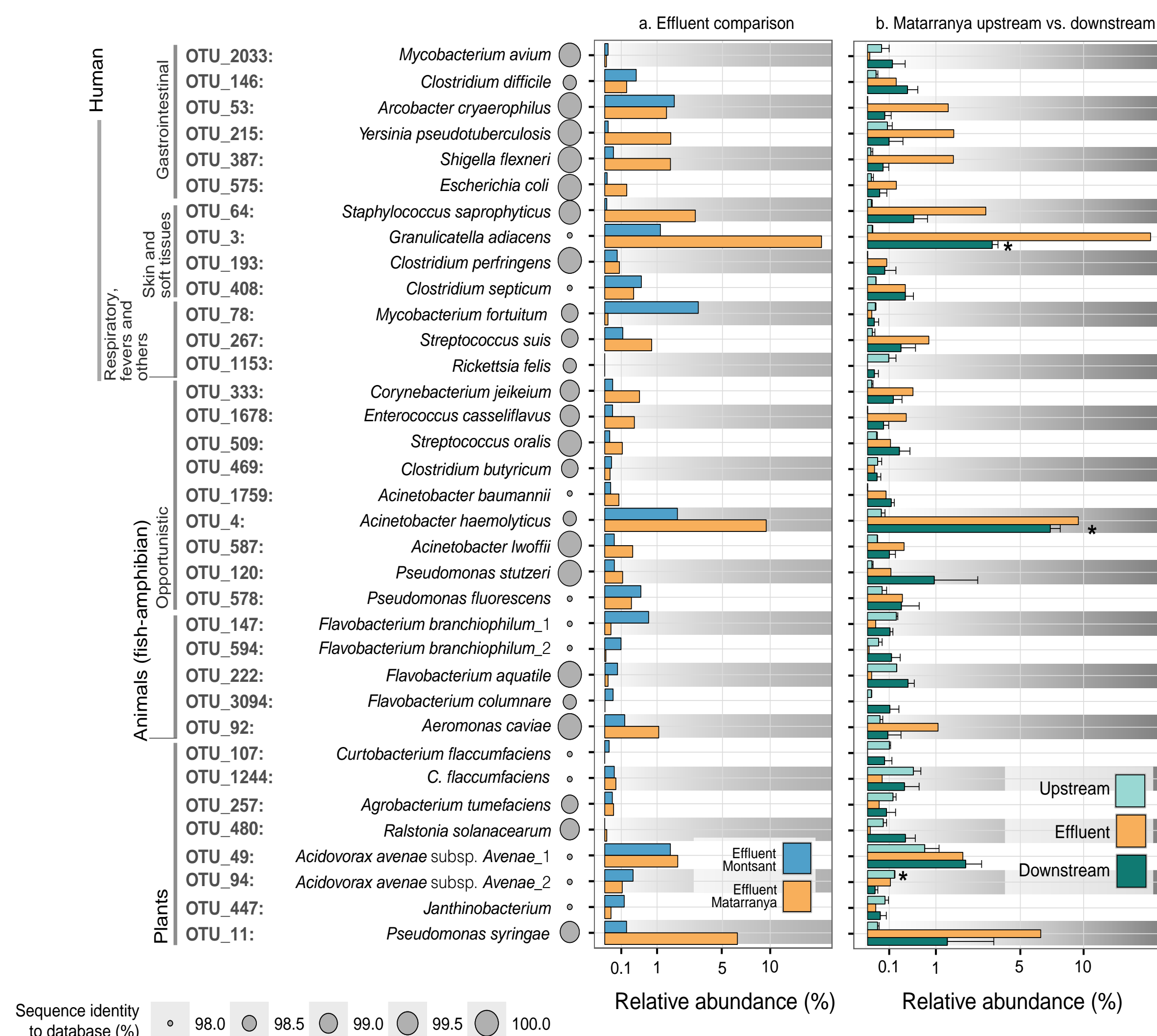


FIGURE 4 Relative abundance (% of total reads) of OTUs closely related to 16S rRNA gene sequences of bacterial pathogens (sequence similarity >98%, see Section 2 for details) in (a) effluents discharging into the Montsant and Matarranya streams, and (b) upstream and downstream samples at the Matarranya stream. Only OTUs with relative abundances >0.01% were retained for the analysis (see main text for details). The size of the circles next to labels varies according to sequence identity in BLAST. Putative pathogenic OTUs are ordered according to their host and pathogenic potential as specified in labels on the left. Asterisks label significant differences (* <0.05) in the relative abundance of the correspondent OTU between upstream and downstream epilithic biofilms

clues to discern both how wastewater-associated micro-organisms, including potential pathogens, segregate among streambed compartments and how they contribute to maintain the streambed resistome.

4.1 | Effect of pollution on the streambed resistome

Biofilms collected downstream of the pollution source showed a higher prevalence of ARGs, regardless of the type of effluent they received (raw or treated). These results agree with previous studies carried out in surface waters subjected to anthropogenic pollution (Aubertheau et al., 2016; Marti et al., 2013; Proia et al., 2016; Winkworth, 2013). The relative abundance of *sul1*, *sul2*, *ermB*, bla_{TEM} and *tetM* genes in biofilms receiving treated effluents was similar to those reported for epilithic biofilms collected from the Ter river after a WWTP discharge (Marti et al., 2013). Similar values for *sul1* and *ermB* genes were also measured in biofilms exposed to WWTP effluents in four low-order streams (Proia et al., 2016). In turn, the relative concentration of most ARGs at sites receiving the raw sewage discharge (Matarranya) was one order of magnitude higher than those exposed to WWTP effluents. This result point to a more severe impact of untreated wastewater on the streambed resistome, although the higher PE in Vall-de-roures (7,015) compared with La Bisbal de Falset (541) may also contribute to the differences observed. Current data do not allow to discriminate if the higher abundance of ARGs in the Matarranya streambed is solely caused by (i) the lack of wastewater treatment, (ii) the higher pollution burden

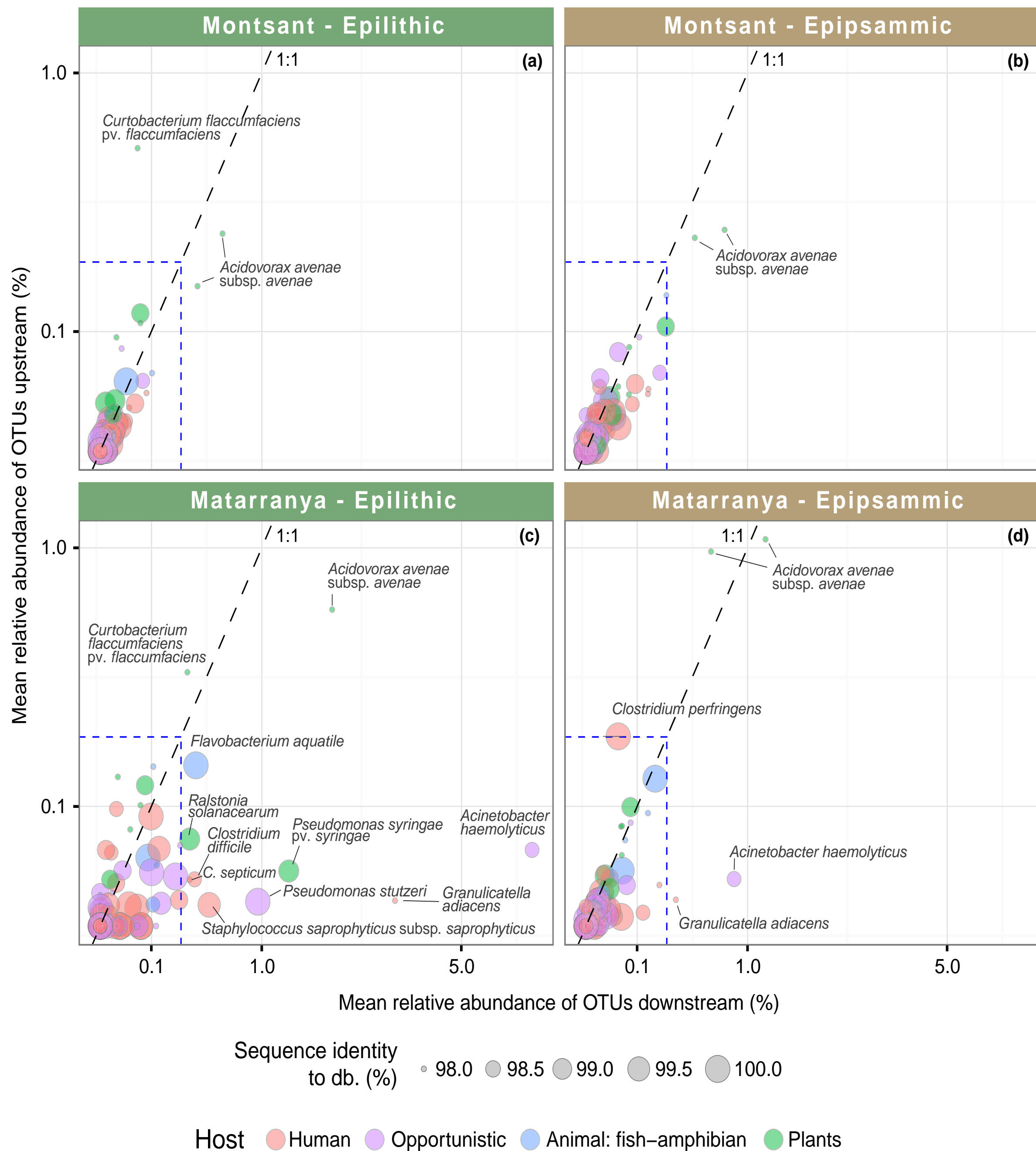


FIGURE 5 Biplot representation of the relative abundance (% of total reads) of OTUs closely related to 16S rRNA gene sequences of bacterial pathogens (sequence similarity >98%, see Section 2 for details) in epilithic (left panels) and epipsammic (right panels) compartments and collection sites (upstream (Y-axis) and downstream (X-axis)) for both streams (Montsant, upper panels) and Matarranya (bottom panels). The size of the circles next to labels varies according to sequence identity in BLAST. Putative pathogenic bacterial OTUs are coloured according to their host and pathogenic potential. OTUs with relative abundances >0.25% are labelled using their taxonomic affiliation

caused by the large PE or, more probably, (iii) the combination of both.

Despite these general differences, the effect of the discharge differed between streambed compartments. Considering the higher prevalence of ARGs in epilithic than in epipsammic biofilms from the Matarranya stream, the former communities were probably more enriched in ARB. This habitat segregation may result from either active colonization or passive trapping of wastewater-associated microbes into the polysaccharide matrix of epilithic biofilms in comparison with the thinner, less structured matrix of epipsammic biofilms (Romaní, 2010). Once there, these immigrant bacteria may interact with other community members and, under favourable conditions, transfer their genes via mobile genetic elements thus increasing their prevalence. Indeed, several studies underlined that

the structured matrix of freshwater biofilms provides an optimal environment for gene transfer among drug-resistant bacteria (Gillings, Holley, & Stokes, 2009; Molin & Tolker-Nielsen, 2003). The prevalence of gene *intI1* in the epilithic biofilms at the Matarranya supports this idea considering that this gene encodes the integrase of mobile Class I integrons that usually contain genes conferring resistance to antibiotics, disinfectants and heavy metals (Di Cesare et al., 2016; Gillings et al., 2009). Moreover, the high prevalence in epilithic biofilms of genes encoding resistance to beta-lactams and vancomycin (i.e., bla_{TEM}, bla_{KPC}, bla_{NDM}, *van*A) might be taken as a further evidence that gene exchange is favoured in this habitat considering that these genes are usually carried on transmissible plasmids (Hawkey & Jones, 2009).

An unexpected finding of our study was the high relative concentration of bla_{TEM}, bla_{KPC} and bla_{NDM} in epilithic biofilms from the Matarranya in both control and impact sites. Remarkably, their concentrations in the streambed were even higher than those measured in the raw sewage effluent (Figure 2). The presence of multiple copies of $bla_{NDM\text{-}1}$ either in the carrying plasmid (Huang et al., 2013) or even in the genome (Jovčić, Lepšanović, Begović, Filipić, & Kojić, 2014; Strauß et al., 2015; Wu, Espedido, Feng, & Zong, 2016) may provide a plausible explanation for its prevalence, but their occurrence in upstream samples suggests the presence of pollution sources other than the studied effluent. In this regard, the Matarranya stream receives untreated domestic sewage from the municipality of Beceite (>2,000 PE) at approximately 7 km upstream the studied site. The presence of this second source of pollution, together with other diffuse inputs, may exert the long-term selective pressure needed to maintain the resistance gene pool along the streambed (Subbiah, Top, Shah, & Call, 2011). The Montsant stream also receives pollution discharges other than the studied effluent, but of lower magnitude (domestic sewage from Margalef de Montsant, a small village of only 50 PE, discharge into the stream 4 km upstream the studied site). In this case, upstream biofilm samples yielded negative results for the bla_{NDM} gene, thus suggesting that the treated effluent is likely to be responsible for the presence of this gene at downstream locations. This is consistent with findings by Yang and coworkers, who found that the prevalence of $bla_{NDM\text{-}1}$ in river water is directly linked to WWTP effluent discharges (Yang, Mao, Zhou, Wang, & Luo, 2016).

4.2 | Identification of potential wastewater-associated pathogens across streambed compartments and links to antibiotic resistance

Remarkably, bacterial communities in epilithic biofilms collected at impact sites in the Matarranya were largely enriched in Gammaproteobacteria and Firmicutes, two signature groups for untreated wastewater (Shanks et al., 2013) that were prevalent in the raw sewage effluent discharging into this stream (Fig. S2). Aubertheau et al. (2016) recently reported a similar enrichment in members of the order Clostridiales (phylum Firmicutes) in river biofilms exposed to WWTP effluents. This result together with the occurrence of a diverse and abundant resistance gene pool in the streambed led us to consider whether or not these ARGs were linked to wastewater-associated micro-organisms, including potential pathogens that end up into the stream. We addressed this issue by comparing the recovered OTU sequences to an in-house database of 283 bacterial species of obligate and opportunistic pathogens using an approach that has recently been applied to bioareosols from the Barcelona subway system (Triadó-Margarit et al., 2017). Being solely based on sequence similarity, the following results must only be regarded as indicative for the potential presence of pathogenic bacteria (i.e., similar to the concept of indicator species in microbiological analysis of water quality), and thus, they should be treated with caution. Indeed, even at sequence identity values of 100% and full alignment coverage, additional confirmatory tests (e.g., studying specific virulence markers) would be necessary to unequivocally assign the true pathogenicity to a given OTU.

Being aware of these considerations, we might expect the higher relative abundance of potential pathogens in the raw sewage considering the effectiveness of wastewater treatment on the removal of pathogens (Wen, Tutuka, Keegan, & Jin, 2009). The release of sewage-associated micro-organisms, including pathogens, into the stream challenges resident bacterial communities, especially in epilithic biofilms where most OTUs tentatively identified as putative pathogens preferentially accumulate (Figure 5). Remarkably, most of these OTUs affiliated to bacterial taxa already identified by SIMPER analysis as top contributors to differences between control and impact sites (i.e., OTUs within families *Moraxellaceae*, *Pseudomonadaceae* and *Vibrionaceae* within the class Gammaproteobacteria, and to families *Carnobacteriaceae* and *Peptostreptococcaceae* within phylum Firmicutes). Among them, two OTUs (OTU-3 and OTU-4) were significantly more abundant at impact sites compared to control ones. OTU-3 showed a 98% sequence identity to *Granulicatella adiacens*, a member of the family Carnobacteriaceae that is particularly abundant in the oral cavity and the gastrointestinal tract of humans. This bacterium has consistently been associated with invasive infections in humans and considered as an opportunistic pathogen carrying resistance to β-lactams, tetracyclines, macrolides and fluoroquinolones (Cargill, Scott, Gascoyne-Binzi, & Sandoe, 2012; Gardenier, Hranjec, Sawyer, & Bonatti, 2011; Shailaja, Sathiavathy, & Unni, 2013). OTU-4 showed high sequence identity to *Acinetobacter haemolyticus* (98.5%). The genus *Acinetobacter* encompasses several multidrug-resistant human pathogens usually associated with nosocomial infections (Peleg, Seifert, & Paterson, 2008), but the presence of *Acinetobacter* species in the streambed is expected considering their widespread distribution in natural environments (e.g., soil and surface waters), artificial systems (e.g., activated sludge) and even in the human body (Peleg et al., 2008). Our results then suggest that wastewater-associated bacteria showing high sequence identity to well-known human pathogens are able to persist in the streambed and eventually being part of the resident microbiota. The precise contribution of these invading micro-organisms on the streambed resistome is difficult to envisage, but the significant correlations obtained between the relative abundance of these potential

pathogenic OTUs and some of the studied genes (*intI1*, *sul1*, *sul2* and *erm*B) (Fig. S5) seem to support this claim. The source and identity of bacteria carrying resistance to tetracyclines, fluoroquinolones and β-lactams could not be resolved from the data and deserves future investigation.

5 | CONCLUSIONS

Considering that the transmission of ARB to humans after exposure to the natural environment is plausible (Huijbers et al., 2015), the environmental resistome pose a risk to human health if ARGs end up into bacterial pathogens (Ashbolt et al., 2013; Martinez, 2009). Our work provides evidences that streambed biofilms, especially those growing on rock surfaces, are environmental reservoirs of ARGs. Moreover, micro-organisms released by wastewater effluents, including some species closely related to well-known human and animal pathogens, may persist in the streambed contributing to maintain a diverse resistance gene pool. In biofilms, the selection pressure exerted by antibiotic pollution and the close contact between cells favour the maintenance and spread of resistance genes, including those encoding resistance to last-resort antibiotics. Our results expand the usefulness of epilithic biofilms as biosensors of wastewater discharges on the prevalence of antibiotic resistance in surface waters.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA ACCESSIBILITY

Raw sequencing data of this study have been deposited in the NCBI database under Accession no. SRP099542. qPCR data for all target genes (*16S rRNA*, *intI1* and ARGs) have been deposited on Dryad Digital Repository (https://doi.org/10.5061/dryad.8kb4m).

AUTHOR CONTRIBUTIONS

The design of the study was conducted by S.S., V.A. and C.B. The field sampling was carried out by J.S., V.A. and L.M. All molecular analyses were carried out by J.S. The analysis of high-throughput sequencing data sets was carried out by C.B. The analysis of antibiotics was conducted by L.M. The molecular identification of pathogens and the statistical analysis of the correspondent data set were carried out by X.T.-M. The data were analysed and the manuscript was written by J.S., J.-L.B. and C.B.M. All authors reviewed the manuscript and contributed to the discussion of results.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Chapter 6

Emerging contaminants and nutrients synergistically affect the spread of class integron-integrase (intI1) and sul1 genes within stable streambed bacterial communities

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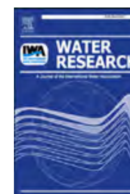
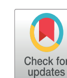


Emerging contaminants and nutrients synergistically affect the spread of class 1 integron-integrase (*intI1*) and *sul1* genes within stable streambed bacterial communities



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A B S T R A C T

Wastewater effluents increase the nutrient load of receiving streams while introducing a myriad of anthropogenic chemical pollutants that challenge the resident aquatic (micro)biota. Disentangling the effects of both kind of stressors and their potential interaction on the dissemination of antibiotic resistance genes in bacterial communities requires highly controlled manipulative experiments. In this work, we investigated the effects of a combined regime of nutrients (at low, medium and high concentrations) and a mixture of emerging contaminants (ciprofloxacin, erythromycin, sulfamethoxazole, diclofenac, and methylparaben) on the bacterial composition, abundance and antibiotic resistance profile of biofilms grown in artificial streams. In particular, we investigated the effect of this combined stress on genes encoding resistance to ciprofloxacin (*qnrS*), erythromycin (*ermB*), sulfamethoxazole (*sul1* and *sul2*) as well as the class 1 integron-integrase gene (*intI1*). Only genes conferring resistance to sulfonamides (*sul1* and *sul2*) and *intI1* gene were detected in all treatments during the study period. Besides, bacterial communities exposed to emerging contaminants showed higher copy numbers of *sul1* and *intI1* genes than those not exposed, whereas nutrient amendments did not affect their abundance. However, bacterial communities exposed to both emerging contaminants and a high nutrient concentration (1, 25 and 1 mg L^{-1} of phosphate, nitrate and ammonium, respectively) showed the highest increase on the abundance of *sul1* and *intI1* genes thus suggesting a factors synergistic effect of both stressors. Since none of the treatments caused a significant change on the composition of bacterial communities, the enrichment of *sul1* and *intI1* genes within the community was caused by their dissemination under the combined pressure exerted by nutrients and emerging contaminants. To the best of our knowledge, this is the first study demonstrating the contribution of nutrients on the maintenance and spread of antibiotic resistance genes in streambed biofilms under controlled conditions. Our results also highlight that nutrients could enhance the effect of emerging contaminants on the dissemination of antibiotic resistance.
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1. Introduction

Sewage and treated wastewater effluents release nutrients and a myriad of pharmaceutical residues, including antibiotics, into the environment (Batt et al., 2006; Selvaraj et al., 2013; Galbet-Giraud et al., 2014; Rodriguez-Mozaz et al., 2014). These pharmaceutical compounds —the so-called emerging contaminants— not only cause adverse effects on the biodiversity and ecosystem functioning (Hernando et al., 2006; Proia et al., 2013) but may also stimulate the selection and evolution of antimicrobial resistance within resident bacterial communities (Kümmerer and Henninger, 2003; Salem-milani et al., 2013; Mirsonbol et al., 2014). Susceptible bacteria can acquire resistance to antibiotics under the appropriate selective pressure either by genomic mutations or by horizontal gene transfer, the latter being the major contributor to the

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acquisition and spread of antibiotic resistance genes (ARGs) *via* mobile genetic elements (MGEs) such as plasmids, transposons, bacteriophages or *via* genetic elements linked to MGEs such as integrons (Frost et al., 2005; Bennett, 2008; Subirats et al., 2016). Emerging contaminants favor the maintenance of MGEs within bacterial communities and stimulate their dissemination (Gaze et al., 2013). Moreover, sewage and agricultural discharges provide ideal conditions for gene mobilization between environmental bacteria and potential pathogens (Obst et al., 2006; Kümmerer, 2009).

Contaminants reach aquatic environments in which biofilms develop and, consequently, microorganisms inhabiting biofilms are constantly exposed to those pollutants (Lock 1993). Streambed biofilms are able to accumulate, recycle or transform most solutes (Battin et al., 2016). Whereas most emerging contaminants decrease either the microbial metabolic rates (e.g. bacterial respiration, gross primary production) or the diversity of bacterial communities (Rosi-Marshall et al., 2013; Shaw et al., 2015; Subirats et al., 2017), nutrients in wastewater usually exhibit a subsidy effect (Wagenhoff et al., 2011; Dunck et al., 2015) on microbial populations characterized by a hump-shape response of their biomass and activity (Paerl et al., 2003; Rubin and Leff, 2007; Yergeau et al., 2012; Liu et al., 2015).

Previous studies have also shown that nutrient load may favor horizontal gene transfer among microorganisms (Elsas et al., 2003; Amos et al., 2015). This observation suggests that nutrients may thus promote the spread of ARGs. However, information on the combined effect of nutrients and emergent contaminants on the composition of resident bacterial communities and their antibiotic resistance profile is limited (Zheng et al., 2017). We therefore conducted an experiment using artificial streams under controlled conditions to assess the role of nutrients and their combined effect with emerging contaminants on the composition of biofilm bacterial communities and their associated resistome. The experiment followed a factorial design with 2 levels of a mixture of emerging contaminants (absence/presence) and three levels of nutrient concentrations (low, medium and high) (Aristi et al., 2016). For the compounds in the mixture, we selected three antibiotics (ciprofloxacin, erythromycin, sulfamethoxazole) and two pharmaceuticals with antimicrobial activity: the anti-inflammatory drug diclofenac (Dastidar et al., 2000; Salem-milani et al., 2013) and the preservative methylparaben, both largely used in food industry and cosmetics (Moir and Eyles, 1992; Mirsonbol et al., 2014). The selection of the five compounds was based on their high ecotoxicological relevance and their widespread occurrence in anthropogenic polluted rivers in the Mediterranean region (Kuzmanovi et al., 2014). The working concentrations of these five compounds were selected to mimic the worst scenario reported in the lower Llobregat river (González et al., 2012; Gorga et al., 2015). Our goal was to evaluate the combined effect of nutrients and the selected pharmaceuticals on the prevalence of six genes enconding resistance to sulfamethoxazole (*sul1*, *sul2*), ciprofloxacin (*qnr*A, *qnr*B and *qnr*S) and erythromycin (*erm*B). The class 1 integron-integrase gene (*intI1*) was also analyzed to assess if treatment exposure favors the potential dissemination of ARGs located in MGEs since *intI1* is usually linked to MGEs and has been considered to play an important role in the acquisition and dissemination of ARGs among bacteria (Ma et al., 2017; Stalder et al., 2014). Our hypotheses were that: *i*) the abundance of the target ARGs and *intI1* gene would increase with emerging contaminants, reaching the maximum increase at the highest nutrient concentrations (i.e. producing a synergistic interaction between nutrients and emerging contaminants); *ii*) the bacterial abundance would increase at medium nutrient concentration (subsidy effect) but decrease in the presence of emerging contaminants regardless of the nutrient concentration; and *iii)* the mixture of emerging contaminants and nutrients would alter the composition of streambed biofilm bacterial communities.

2. Methods

2.1. Experimental design

The experiment was performed in the indoor Experimental Streams Facility of the Catalan Institute for Water Research (Girona, Spain). The treatments followed a factorial design based on three nutrient concentrations [low (L), medium (M) or high (H)] and two levels for the mixture of emerging contaminants [no emerging (NE) or emerging (E)]. Thus, the experimental design was based on the following six treatments: treatments with no emerging contaminants at low (NE_L), medium (NE_M) or high (NE_H) nutrient concentrations; and treatments with emerging contaminants at low (E_L), medium (E_M) or high (E_L) nutrient concentrations. The experimental streams facility consisted in eighteen artificial streams, each artificial stream was assigned to one of six treatments following a randomized complete block design (with three replicates per treatment; and one replicate per block of six artificial streams). Nutrient treatment was based on a mixture of phosphate (P-PO_4^{3-}), nitrate (N–NO_3^-) and ammonium (N–NH_4^+) at different concentrations, whereas the treatment with emerging contaminants consisted in a mixture of the 5 previously described contaminants (ciprofloxacin, erythromycin, sulfamethoxazole, diclofenac, and methylparaben). Treatment exposure lasted for 28 days thus allowing the assessment of short- (14 days) to long-term effects (28 days) of both the separate effects of nutrients and emerging contaminants and their interaction.

2.2. Experimental settings

Each stream consisted of an independent methacrylate channel (L x W x D = 200 cm - 10 cm - 10 cm) containing 5 L of sterile sand particles (d_{50} = 0.74 mm) collected from an unpolluted segment of the Llémena River, a small calcareous tributary of the Ter River (Girona, Spain) to mimic a streambed that allow the growth of biofilms (Aristi et al., 2016). Each channel was fed by a 70 L rainwater that provided a constant flow of 50 mL s^{-1}, and operated under a scheme of combined flow-recirculation (118 min) and flow-open (2 min) every 2 h. The sand particles were sterilized with a Presoclave-II 30L autoclave (120 °C for 2 h) (JP Slecta S.A., Barcelona, Spain) and the rainwater was filtered through activated carbon. Water of each artificial stream was renewed once a day (exchange rate per hour 4.28%), the average water flow was 0.88 ± 0.03 cm s^{-1} and water depth over the streambed ranged between 2.2 and 2.5 cm (Aristi et al., 2016). Daily cycles of photosynthetic active radiation (PAR) were defined as 12 h daylight +12 h darkness, and were simulated by LED lights (Lightech, Girona, Spain). PAR was held constant at 173.99 ± 33 μE m^{-2} s^{-1} during the daytime, and was recorded at 10-min intervals using 4 quantum sensors located across the whole array of streams (sensor LI-192SA, LiCOR Inc, Lincoln, USA). Water temperature was held constant at 19.80 ± 0.42 °C by means of a cryo-compact circulator (Julabo CF-31, Seelbach, Germany), and recorded at 10-min intervals using VEMCO Minilog (TR model, AMIRIX Systems Inc, Halifax, NS, Canada) temperature data loggers (−5–35 °C, ±0.2 °C). Overall, physico-chemical conditions in the artificial streams (water velocity, temperature, and light cycles) emulated those of the Llémena River during late spring and under low flow conditions.

Biofilm was inoculated twice per week during the colonization period using combined inoculum from epilithic (growing on surface of rocks) and epipsammic (growing on the surface of sand)

biofilms from an unpolluted segment of the Llémena River. Briefly, biofilm inoculum was obtained after scrapping epilithic biofilms from 10 to 12 cobbles and washing approximately 10 L of fine sediments. The colonization period in the artificial streams lasted for 20 days before the exposure to treatments. Nutrients during the colonization period were held constant at 0.040, 1.7 and 0.040 mg L^{-1} of phosphate, nitrate and ammonium by means of injection of concentrated solutions (KH_2PO_4, $NaNO_3$, and NH_4Cl, respectively) and using a peristaltic pump (IPC pump, Ismatec, Switzerland). These concentrations were maintained in the L treatment, and increased to 0.2 mg L^{-1} of phosphate, 5 mg L^{-1} of nitrate and 0.2 mg L^{-1} of ammonium in the M treatment and to 1, 25 and 1 mg L^{-1} of phosphate, nitrate and ammonium, respectively, in the H treatment (Aristi et al., 2016). The highest concentrations mimicked those observed during low flow conditions in the Llobregat River (Aguilera et al., 2012).

We used a mixture of 5 emerging contaminants at constant concentrations of 0.1 μg L^{-1} of methylparaben and 1 μg L^{-1} of ciprofloxacin, diclofenac, erythromycin and sulfamethoxazole. The working standard mixture of emerging contaminants was prepared every 2 days at a concentration of 100 mg L^{-1} in 10% methanol: water (v: v) and added to the artificial streams using a peristaltic pump (IPC pump, Ismatec, Switzerland). The total concentration of methanol reaching the artificial streams was 400 ng L^{-1}. The same concentration of methanol was added to treatments without emerging contaminants. High purity (>97%) standard solutions of the target compounds and their deuterated counterparts (used as surrogate standards) were obtained from Sigma-Aldrich (St Louis, U.S.A.), Aldrich (Milwaukee, U.S.A.), Dr. Ehrenstorfer (Wesel, Germany) Fluka (Buchs, Switzerland) and CDN Isotopes (Pointe-Claire, Quebec, Canada).

2.3. Water chemistry

Dissolved oxygen, pH and specific conductivity were measured weekly by noon in each artificial stream using WTW (Weilheim, Germany) hand-held probes. Background concentrations of nutrient and emerging contaminants were measured weekly from water collected from the channel outlet to assess the possible differences between nominal and observed concentrations (see Aristi et al., 2016 for details).

Emerging contaminants were analyzed using a method based on an online pre-concentration with EQuan MAXTM technology coupled to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific, San José, U.S.A.) (on-line SPE-LC/MS/MS) equipped with an electrospray ionization source (ESI) (see Aristi et al., 2016 for details). Note that the limits of detection (LODs) and quantification (LOQs) were estimated experimentally from real samples as the concentration of analyte that provides a signal-to-noise ratio of 3 and 10, respectively.

2.4. Sampling and sample processing

Biofilm samples from each artificial stream were collected before treatment started (at day −1), as well as after 14 and 28 days. The top sand layer (1 cm) was collected with a core sampler (diameter = 1.2 cm), placed in sterile 15-mL polypropylene tubes (Fisher Scientific; Wilmington, DE, USA) and stored at −80 °C until DNA extraction. Biofilms were detached from sand particles by vortexing for 5 min × 3 times after adding 5 ml of 10 mM Tris-HCL buffer at pH 7.5 to each sterile 15-mL polypropylene tubes. Biofilm biomass was then pelleted by centrifugation at 6000 rpm for 15 min and genomic DNA was extracted using the PowerSoil DNA isolation Kit (MoBio Laboratories, USA) according to manufacturer's instructions. The DNA concentration was measured using Qubit 2.0 fluorometer (Life Technologies; Carlsbad, CA, USA) and purity was determined by measuring A_{260}/A_{230} and A_{260}/A_{280} absorbance ratios using a NanoDrop 2000 spectrophotometer (Thermo Scientific; Wilmington, DE, USA).

2.5. Quantification of antibiotic resistance genes

Copy numbers of six genes encoding resistance to the antibiotics added in the mixture of emerging contaminants, namely: *sul1* and *sul2* genes conferring resistance to sulfamethoxazole; *qnr*A, *qnr*B and *qnr*S genes conferring resistance to ciprofloxacin and the *erm*B gene conferring resistance to erythromycin were determined in triplicate using real-time PCR (qPCR) assays. Moreover, copy numbers of the class 1 integron-integrase gene (*intI1*) and the bacterial 16S rRNA gene were also determined. The abundance of the latter was used as a proxy for bacterial abundance and to normalize the concentration of target ARGs to minimize variations due to different DNA extraction efficiency across samples and to provide an indication of the proportion of total bacterial carrying ARGs (Heuer et al., 2011; Pruden et al., 2006).

All genes were quantified using primers and conditions compiled in Supporting Information Table S1. The standard curves were carried out as previously described (Subirats et al., 2017). All reactions were performed using SYBR green detection chemistry on a MX3005 system (Agilent Technologies; Santa Clara, CA, USA), as previously described (Marti et al., 2013). Samples were analyzed in duplicate with a standard curve and a negative control included in each run. The specificity of the reaction was verified by melting curve analysis and by electrophoresis run. All qPCR assays were performed with high R^2 values (0.998 in average), high efficiencies (91.86 ± 3.18%) and a dynamic range of at least 5 orders of magnitude, thus validating the gene quantification. The limit of quantification of each gene was 18, 100, 80, 56, 250 copies μL^{-1} for *qnr*S, *sul1*, *sul2*, *erm*B and *intI1* genes, respectively.

2.6. High-throughput sequencing and sequence processing

For community analysis, equimolar amounts of DNA extracted from each artificial stream under same treatment conditions were combined to obtain a composite sample for that treatment. These composite samples were then subjected to high-throughput multiplexed 16S rRNA gene sequencing with the Illumina MiSeq System (2 × 250 PE) using primer pair 515f/806r (Caporaso et al., 2011) complemented with Illumina adapters and sample-specific barcodes at the Research Technology Support Facility Michigan State University, USA (Kozich et al., 2013). Pair merging, quality filtering, chimera checking, clustering into Operational Taxonomic Units (OTU, 97% cutoff) and construction of OTU table were carried out using default parameters in UPARSE (Edgar 2013). The resulting OTU table was converted to Biological Observation Matrix (BIOM) format (Mcdonald et al., 2012) and then imported and analyzed in QIIME (Caporaso et al., 2010). Sequencing depth ranged between 167 and 97,540 sequences per sample. The sample with the lowest sequence number (14NE-H, 167 sequences) was removed from downstream analysis. OTUs affiliated to eukaryotes (6; 0.2%), algal chloroplasts (83; 3.3%), archaea (8; 0.3%) and unclassifieds (175; 6.5%) were filtered from the original OTU table using appropriate scripts in QIIME. After filtering, we obtained a total number of 2435 bacterial OTUs. For community analysis (alpha and beta diversity), the number of sequences in each sample was normalized by randomly selecting a subset of 27,700 sequences per sample to minimize bias due to different sequencing depth across samples. The sequence data set was deposited in the NCBI Sequence Read Archive (SRA) database under accession number SRP128512.

2.7. Data analysis

We used one way-analysis of variance (ANOVA) with blocks as fixed factor to test for differences among experimental arrays for all variables before treatment onset. After that, differences in the abundance of target ARGs, *intI1* and 16S rRNA genes were analyzed using a mixed effects model with time, nutrients and emerging pollutant treatments as fixed factors, and arrays as random factor ($n = 36$). Interactions between the considered fixed factors were also tested, and Post-hoc Tukey tests were done for each sampling day. Normality of all variables was checked with the Shapiro-Wilk test and statistical significance was determined at $\alpha = 0.05$. All analyses were performed with R software (version 3.1.1; R Development Core Team, Vienna, Austria).

For community composition analyses, we constructed a similarity matrix using the Bray–Curtis distance (Legendre and Gallagher, 2001) based on the relative abundance (square root transformed) of each bacterial OTU. Ordination of samples according to Bray-Curtis distance matrices was done using principal coordinates analysis (PCoA). Analysis of similarity between communities based on their taxonomic composition was done after grouping samples by sampling day either (−1, 14 and 28 days) or (14 and 28 days), by nutrient (low, medium and high concentration) and by emerging contaminants (presence *vs.* absence), which were used as factors in PERMANOVA. Also, the contribution of each OTU to the Bray-Curtis distance between samples grouped by time was calculated using the analysis of similarity percentages (SIMPER). All these analyses were run in the PRIMER-6 statistical package with the PERMANOVA + add-on (PRIMER-E, Plymouth Marine Laboratory, UK).

3. Results

3.1. Water chemistry

Environmental conditions (dissolved oxygen, pH and specific conductivity) showed no statistically significant differences between arrays before the onset of the treatments (data not shown). Dissolved oxygen was steady throughout the whole experiment and among treatments, with values ranged between 9.99 and 10.41 mg L^{-1}. Conductivity increased with nutrient concentration, from 206 ± 19 mS cm^{-1} at low, 233 ± 29 mS cm^{-1} at medium, and 350 ± 64 mS cm^{-1} at high nutrient concentration (Aristi et al., 2016).

The concentration of the five selected chemicals was similar throughout the entire experiment among those channels exposed to the emerging contaminants treatment (Table 1). However, measured concentrations were between 15 and 40% lower than the nominal ones, except for ciprofloxacin, which was approximately 24% higher (Table 1). These differences may be caused by natural attenuation within the artificial streams due to phototransformation and biotransformation. The higher concentration of ciprofloxacin measured, however, might be due to its high sorption capacity to sediments (Marques et al., 2013).

Regarding nutrients, phosphate and ammonium concentrations were lower (25–80% and 80–90% less, respectively) than the nominal concentrations in all studied levels (L, M and H). The concentration of nitrate in L and M was similar to the nominal one, but not in H, which was 10% lower (Table 1) (Aristi et al., 2016).

3.2. Bacterial abundance

Copy numbers of bacterial 16S rRNA gene was used as a proxy for bacterial abundance. It should be noted, however, that 16S rRNA copy numbers cannot be directly converted to cell numbers due to the large variation in the number of rRNA operons among bacterial species (Farrelly et al., 1995; Stoddard et al., 2015).

Before treatment started, concentration of bacterial 16S rRNA genes ranged from 9.75×10^7 to 5.92×10^8 copies cm^{-2} with no significant differences among replicates (data not shown). We then assessed the separated effect of nutrients and emerging contaminants on the 16S rRNA abundance over time. The copy numbers of bacterial 16S rRNA gene increased along time as a result of bacterial growth ($p < 0.001$, Table 2) (Fig. 1). Whereas the presence of emerging contaminants did not affect the 16S rRNA gene abundance ($p = 0.425$, Table 2), nutrients had a clear effect on its concentration ($p < 0.001$, Table 2) (Fig. 1). Particularly, copy numbers of the 16S rRNA gene were significantly higher in biofilms exposed to medium nutrient concentrations in comparison to those grown at low and high nutrient concentrations. This short-term (14 days) effect occurred regardless of the presence of emerging contaminants (Fig. 1). After 28 days of exposure, the abundance of the 16S rRNA genes was similar between medium and high nutrient concentrations but significantly higher when compared to low nutrient conditions (Fig. 1). The differential effect of nutrient on the 16S rRNA abundance over time was corroborated with the significant interaction between the factors Nutrient and Time ($p < 0.001$, Table 2).

3.3. Composition of biofilm bacterial communities

After colonization of artificial channels and one day before treatment started (day −1), biofilm bacterial communities were dominated by Firmicutes (48.63% ± 1.13% of total reads), Alphaproteobacteria (20.26% ± 1.49%) and Betaproteobacteria (9.07% ± 3.01%) (Fig. 2). The ordination of samples according to their similarity in community composition showed that "Time" was the main factor causing the observed differences in bacterial community composition throughout the experiment, regardless of the treatment nutrients or emerging contaminants (Fig. 3A). At day −1, bacterial communities clustered together and significantly segregated from those at days 14 and 28 (PERMANOVA test,

Table1
Average (±SD) of nutrients and emerging contaminants in each treatment (n = 18), resulting from 3 replicates per treatment and 6 surveys over the experiment. (Data from Aristi et al., 2016).

Compound	Low	Medium	High
Ammonium (mg N–NH_4^+ L^{-1})	0.008 ± 0.003	0.050 ± 0.018	0.194 ± 0.015
Nitrate (mg N–NO_3^- L^{-1})	1.720 ± 0.230	5.099 ± 1.143	22.89 ± 0.608
Phosphate (mg P –PO_3^{4+} L^{-1})	0.014 ± 0.006	0.113 ± 0.046	0.779 ± 0.133
Erythromycin (μg L^{-1})	0.625 ± 0.104	0.635 ± 0.087	0.636 ± 0.131
Sulfamethozaxole (μg L^{-1})	0.805 ± 0.141	0.849 ± 0.125	0.756 ± 0.193
Diclofenac (μg L^{-1})	0.812 ± 0.115	0.834 ± 0.111	0.754 ± 0.166
Ciprofloxacin (μg L^{-1})	1.234 ± 0.133	1.336 ± 0.144	1.171 ± 0.216
Methylparaben (μg L^{-1})	0.037 ± 0.032	0.039 ± 0.034	0.032 ± 0.034

Table 2
Results for the type III test of fixed-effect in 16S rRNA, *sul*2, *sul*1, and *intI1* gene copy number, indicating the Chisq and its significance (*p*-values). N, nutrients; E, emerging contaminants; D, day of experiment; N*E, N*D, E*D and N*E*D indicate the interactions between factors.

Factor	16S rRNA	*intI*1	*sul*1	*sul*2
N	22.9 (<0.001)	5.43 (0.066)	4.65 (0.097)	0.30 (0.858)
E	0.06 (0.794)	16.9 (<0.001)	28.8 (<0.001)	0.63 (0.425)
D	18.4 (<0.001)	28.6 (<0.001)	6.79 (0.009)	10.8 (0.001)
N*E	2.39 (0.305)	12.2 (0.002)	6.02 (0.049)	0.02 (0.989)
N*D	17.8 (<0.001)	9.61 (0.008)	2.60 (0.272)	3.63 (0.162)
E*D	0.69 (0.403)	0.32 (0.571)	5.00 (0.025)	0.18 (0.664)
N*E*D	1.66 (0.434)	9.71 (0.007)	2.43 (0.296)	3.43 (0.179)

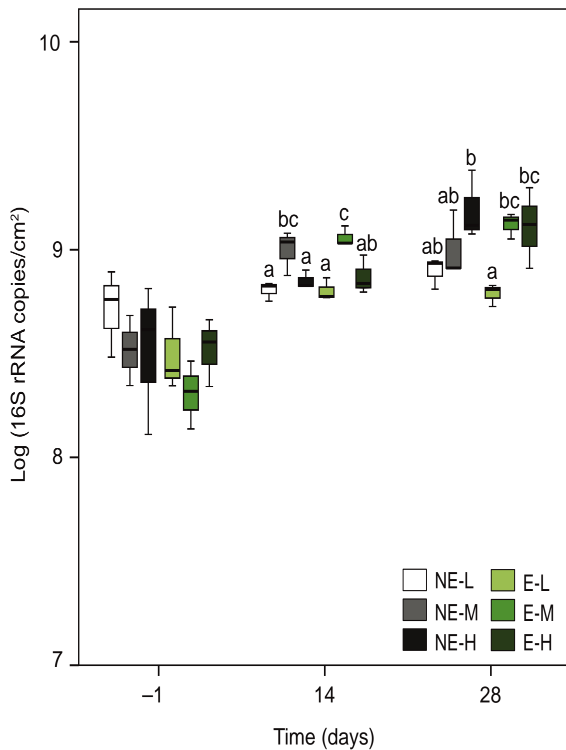

Fig. 1. Boxplots showing the relative concentration of 16S rRNA gene copies in biofilms samples collected before (day −1) and after (14 and 28 days) the implantation of treatments (N = 3 in all cases). NE: Treatment without emergent contaminants; E: Treatment with emergent contaminants; L, M and H indicate treatments with low, medium and high nutrient concentrations (see Materials and Methods for details). Different superscripts denote significant differences between treatments for each day (Post-hoc Tukey test, $p < 0.05$).

$p < 0.001$), (Fig. 3A). A SIMPER analysis was then carried out to identify which taxa were responsible of the observed differences between biofilm communities at days −1 and 28. SIMPER revealed that families contributing the most to these differences were mainly affiliated to phyla Firmicutes, Cyanobacteria and Proteobacteria (particularly classes Alpha-, Beta-, and Gammaproteobacteria) (Table S2A). Firmicutes were largely represented by sequences affiliated to order *Bacillales* (98.04% of reads) at day −1 but they decreased to 38.47% after 28 days (data not shown). In turn, sequences affiliated to Alpha- and Betaproteobacteria grouped into several orders that exhibited slight shifts in their relative abundance during the experiment, particularly *Bradyrhizobiaceae* and *Acetobacteraceae* (Alphaproteobacteria) and *Oxalobacteraceae* (Betaproteobacteria) (Table S2A). Sequences affiliated to phylum Cyanobacteria and class Gammaproteobacteria accounted to 0.76% ± 0.17 and 4.73% ± 0.25, respectively, before treatment implementation (day −1) but their relative abundances increased and decreased to 21.11% and 2.67%, respectively, after 28 days (data not shown).

Instead, the treatments nutrients and emerging contaminants did not have a clear effect on the composition of biofilm bacterial communities (Fig. 3B). A principal coordinate analysis (PCoA) indicated that nutrients did not change bacterial communities while only 14.1% of the variability was accounted for the presence of emerging contaminants (Fig. 3B). This minor effect of the emerging contaminants on community composition was further confirmed by SIMPER analysis (Suppl. Table S2B), which showed that the differential abundance of OTUs contributing the most to the observed differences between biofilms exposed or not to emerging contaminants did not exceed 1.6%, except for Cyanobacteria. This distribution was also corroborated with a PERMANOVA test, which yielded significant differences in community composition by time ($p = 0.005$) but neither by nutrient concentration ($p = 0.613$) nor emerging contaminants ($p = 0.171$).

3.4. Abundance of ARGs and the intI1 gene

The genes conferring resistance to sulfamethoxazole (*sul1* and *sul2*) as well as the *intI1* gene were detected in biofilm samples at day −1 and during treatment (day 14 and 28). Overall, the relative abundance of the *sul2* gene was lower than *sul*1 and *intI1* genes (Fig. 4). The concentration of genes conferring resistance to fluoroquinolones (*qnr*A, *qnr*B and *qnr*S) and macrolides (*erm*B) were always below the detection limit of the essay (data not shown) although antibiotics belonging to these families were present in the treatment mixture (ciprofloxacin and erythromycin, respectively).

Nutrients did not have any effect on the relative concentration of

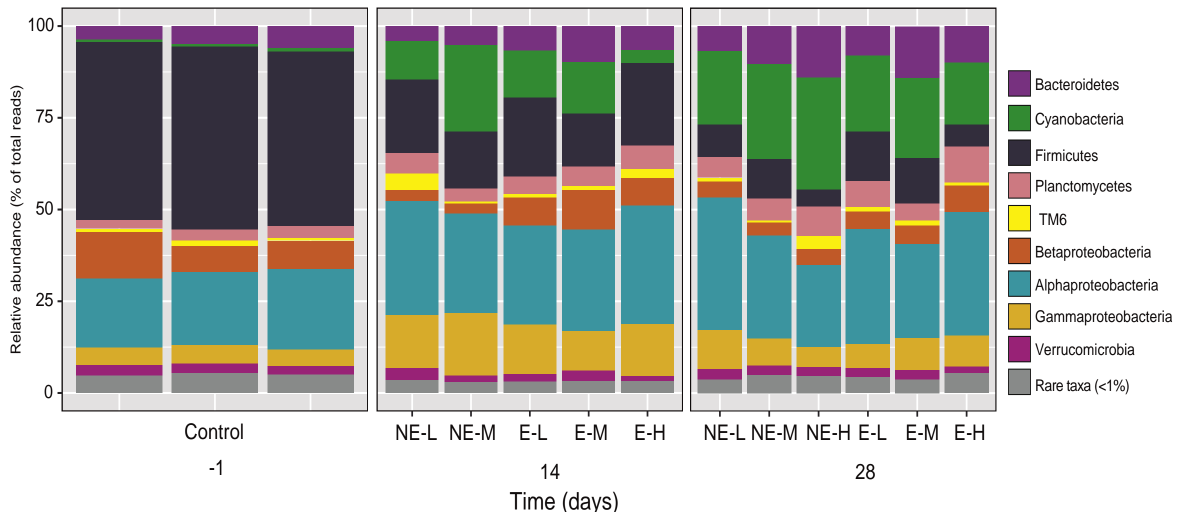

Fig. 2. Composition of biofilm bacterial communities at Phylum level (Class for the Proteobacteria) collected in artificial streams before (day −1) and after (14 and 28 days) the implantation of treatments. NE: Treatment without emergent contaminants; E: Treatment with emergent contaminants; L, M and H indicate treatments with low, medium and high nutrient concentrations (see Materials and Methods for details).

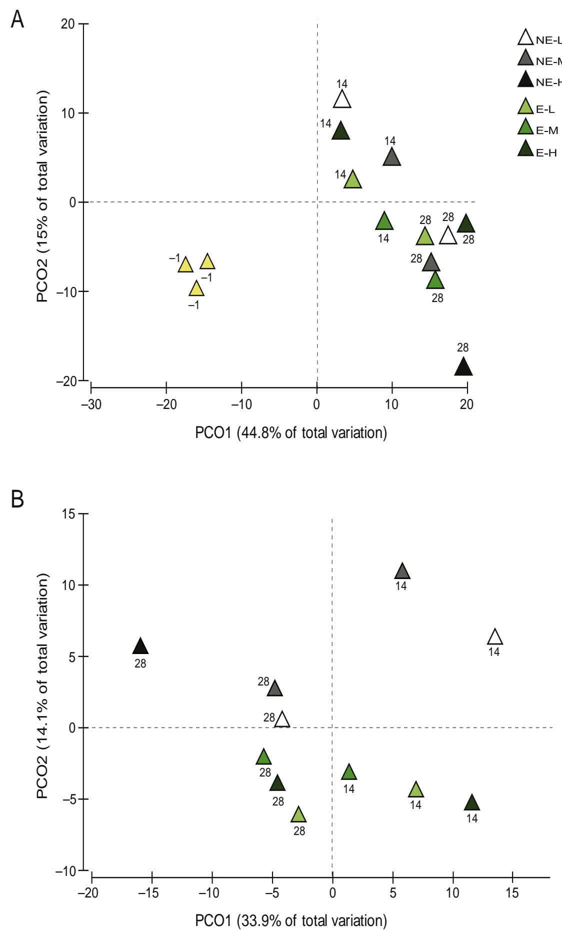

Fig. 3. PCO ordination of samples according to the relative abundance of different bacterial taxa (Bray-Curtis dissimilarity distance). (A) Samples collected before (day −1) and after (14 and 28 days) the implantation of treatments. (B) Ordination of samples collected after 14 and 28 days of treatment implantation. NE: Treatment without emergent contaminants; E: Treatment with emergent contaminants; L, M and H indicate treatments with low, medium and high nutrient concentrations (see Materials and Methods for details).

intI1, *sul1* and *sul2* genes ($p = 0.066$, $p = 0.097$, $p = 0.858$, respectively), while the presence of emerging contaminants significantly increased the relative abundance of both *intI1* and *sul1* ($p < 0.001$) (Fig. 4, Table 2). These genes increased further when nutrient concentration co-occurred with the emerging contaminants (Fig. 4). The combined stress caused by emerging contaminants and high nutrient concentrations resulted in higher concentrations of genes *sul1* and *intI1* when compared to biofilms incubated without the presence of contaminants at the same nutrient concentration (Fig. 4). This differential effect was corroborated further by the significant interaction measured between the corresponding factors “Emerging contaminants” and “Nutrients” (Table 2).

4. Discussion

Microbial communities thriving in aquatic environments receiving wastewater effluents are challenged by a large variety of anthropogenic-derived compounds but, at the same time, their growth and activity are fueled by nutrients also released by these discharges. Few studies have been designed, however, to elucidate the effects of both stressors (Aristi et al., 2016; Zheng et al., 2017) and none have addressed the effects of both type of pollutants on the composition and antibiotic resistance profile of resident bacterial communities under controlled conditions.

The nutrient concentrations measured during the experiment were, in most cases, lower than the nominal ones. Notwithstanding this discrepancy, previous studies have shown that concentrations of ammonium and nitrate similar to those measured in the high nutrient treatment caused toxic effects in aquatic ecosystems (Camargo and Alonso, 2006). In the present study, the observed increase in 16S rRNA gene copies (here used as proxy for bacterial abundance) in biofilms exposed to medium nutrient concentrations after 14 days of treatment agrees with the hump-shaped response previously observed in aquatic biofilms subjected to increasing nutrient loads (Dunck et al., 2015; Niyogi et al., 2007). The lack of this differential response at long term (28 days of treatment) may respond to the adaptation of bacterial communities to the nutrient excess, either by the overgrowth of copiotrophic species (Koch, 2001) or the enrichment of bacterial taxa possessing a high copy number of rRNA operons (Eichorst et al., 2007;

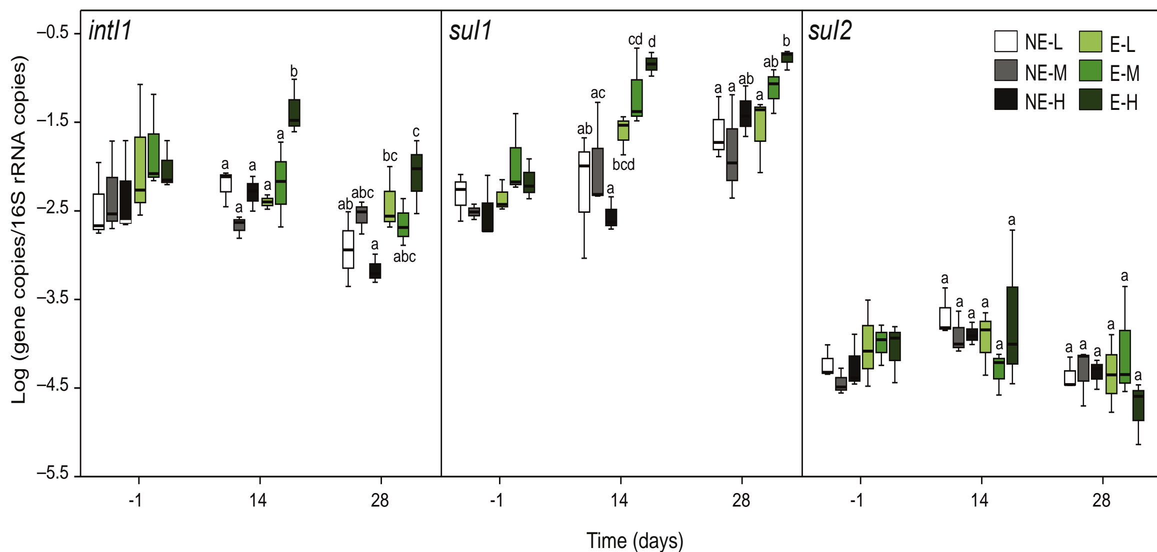

Fig. 4. Boxplots showing the relative concentrations of *intI1*, *sul1* and *sul2* in biofilms samples collected before (day −1) and after (14 and 28 days) the implantation of treatments (N = 3 in all cases). NE: Treatment without emergent contaminants; E: Treatment with emergent contaminants; L, M and H indicate treatments with low, medium and high nutrient concentrations (see Materials and Methods for details). Different superscripts denote significant differences between treatments for each day (Post-hoc Tukey test, $p < 0.05$).

Klappenbach et al., 2000). Remarkably, emerging contaminants did not produce clear effects on bacterial abundance at any time suggesting that bacterial growth was not affected at the concentrations used. Although the five antimicrobials used in our study are known to inhibit bacterial growth at sub-inhibitory concentrations (Mirsonbol et al., 2014; Pankey and Sabath, 2004; Salem-milani et al., 2013; Silva et al., 2011), the polymeric biofilm matrix and its structural complexity probably lessened their inhibitory action (Steve et al., 2013). Also, the higher resistance of bacterial cells within biofilms (Gilbert et al., 2002) may account to the reduced susceptibility of the overall community to the emerging contaminants used.

Likewise, emerging contaminants did not significantly change bacterial community composition. Similar results were found when analyzing the effect of low-level concentrations of sewage effluent on the bacterial community composition of river biofilms (Lehmann et al., 2016). Other studies also reported that low concentrations of emerging contaminants had low to no effect on the aquatic biota (Brausch and Rand, 2011; Grung et al., 2008). In contrast, shifts in the bacterial community composition have been observed under environmental stress, including selection for more resistant bacteria with an associate change in overall diversity (Ford, 2000). This difference might be related to the existence of minimum inhibitory concentrations (MICs) of antibiotics that can select for resistant bacteria (Gullberg et al., 2011, 2014; Liu et al., 2012). The predicted no-effect concentrations that select for resistance (PNEC) for erythromycin, sulfamethoxazole and ciprofloxacin have been estimated to be 1, 16 and 0.064 $\mu g\,L^{-1}$, respectively (Bengtsson-palme and Larsson, 2016). The concentrations used in our experiment were 1.6 and 20 times lower than the estimated PNEC for erythromycin and sulfamethoxazole and only ciprofloxacin was present in a concentration higher than its PNEC value ($1.247 \pm 0.083\,\mu g\,L^{-1}$, ≈20 times higher) (Table 1). Assuming than only ciprofloxacin could select for resistance under our experimental conditions, it is surprising that genes conferring resistance to this antibiotic (*qnr*S, *qnr*A and *qnr*B) were below the detection limit of the essay in all treatments and time points. These results suggest that the combination of the selected pharmaceuticals at the concentrations used together with the incubation time (28 days) was not sufficient to generate resistant genotypes to ciprofloxacin.

The increase of the relative abundance of *sul1* and *intI1* genes (normalized to 16S rRNA copies) in biofilm communities exposed to emerging contaminants evidenced a clear impact on bacterial communities, even in the absence of significant relevant shifts in their composition. Changes on the composition of bacterial communities have been invoked as crucial to explain changes in the resistome of sewage sludge (Jia et al., 2015) and drinking water (Su et al., 2014). In our study, the variation in the abundance of *intI1* and *sul1* may be caused by others mechanisms rather than compositional changes. The increase in the relative abundance of both *intI1* and *sul1* only when biofilms were exposed to emerging contaminants suggest that these genes were selectively enriched within the community. Actually, concentration of antibiotics below the MIC are known to stimulate horizontal gene transfer *via* MGE and thus favor the development of resistance within a given community (Andersson and Hughes, 2014). In this regard, the class I integron is a gene capture platform usually located in MGEs such as transposons and plasmids (Gillings et al., 2015). The prevalence of *intI1* in biofilm bacterial communities exposed to emerging contaminants may thus be the consequence of the potential mobilization of Class I integrons within the biofilm community. Several studies have shown that the structured matrix of biofilms provides an optimal environment for gene transfer among bacteria (Gillings et al., 2009; Molin and Tolker-Nielsen, 2003). Similarly, the increase in the relative abundance of *sul1* may be also related with integron mobilization since this gene is usually linked to Class I integrons (Gillings et al., 2015). After 14 days of experiment, the relative abundance of the *intI1* gene showed a significant correlation with the abundance of the *sul1* gene (Spearman's rho 0.750, $p = 0.02$) only in biofilms exposed to emerging contaminants (Fig. S1). These results showed that the abundance of the *intI1* gene, which is usually linked to several resistance genes, can rapidly change in response to environmental pressures, supporting the idea of using the *intI1* gene as a generic marker for anthropogenic pollution (Gillings et al., 2015).

Previous studies have shown that nutrient concentration has a key role in stimulating horizontal gene transfer rates in biofilms as a result of enhanced metabolic rates and cell mobility (Elsas et al., 2003). Remarkably, *intI1* and *sul1* genes reached their maximum abundance in those biofilms exposed to the combined effect of emerging contaminants and high nutrient concentration at both short- and long-term. This suggests a synergistic effect of emerging contaminants and nutrients on the spread of *intI1* and *sul1*genes. Moreover, our results are consistent with those by Zhao and coworkers, who found that nutrients might directly contribute to the dissemination of ARGs in sediments collected at different sampling sites along a mariculture drainage ditch (Zhao et al., 2016). Likewise, a study carried out in the Dongjiang River basin found that antibiotics, heavy metals and nutrients explained 21% of the cumulative percentage variance in ARG data (Su et al., 2014). Overall, these results confirm that nutrients lead to an increase in the prevalence of ARGs and the *intI1* gene under the combined effect of emerging contaminants (Lehmann et al., 2016).

5. Conclusions

The main conclusions of this work are summarized as follows:

- Environmental concentrations of emerging contaminants favor the spread of class I integrons and *sul*1 genes without significant shifts in the composition of streambed bacterial communities.
- Nutrients have a significant contribution to the spread of class I integrons and *sul*1 genes but only when combined with emerging contaminants, showing a synergistic effect.
- Considering that WWTP effluents constantly release both nutrients and emerging contaminants into surface waters, future efforts should be focused on *i)* the combined effect of these stressors on the spread of antibiotic resistance and, *ii)* the implementation of new wastewater treatments to efficiently reduce the concentration of emerging contaminants and nutrients to minimum values.

Conflict of interest statement

The authors declare no conflict of interest.

Author contributions

S.S. and V.A designed the experiment. V.A and X.T carried out the experiment and the sampling; J.S and A.S carried out all molecular analyses; C.B carried out the analysis of the high-throughput sequencing datasets; J.S. analyzed the data; J.S., J-L.B. and C.B wrote manuscript; all authors reviewed the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.03.025.

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Chapter 7

Metagenomic analysis reveals that bacteriophages are reservoir of antibiotic resistance genes

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Metagenomic analysis reveals that bacteriophages are reservoirs of antibiotic resistance genes

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ABSTRACT

A metagenomics approach was applied to explore the presence of antibiotic resistance genes (ARGs) in bacteriophages from hospital wastewater. Metagenomic analysis showed that most phage sequences affiliated to the order Caudovirales, comprising the tailed phage families Podoviridae, Siphoviridae and Myoviridae. Moreover, the relative abundance of ARGs in the phage DNA fraction (0.26%) was higher than in the bacterial DNA fraction (0.18%). These differences were particularly evident for genes encoding ATP-binding cassette (ABC) and resistance–nodulation–cell division (RND) proteins, phosphotransferases, β-lactamases and plasmid-mediated quinolone resistance. Analysis of assembled contigs also revealed that bla_{OXA-10}, bla_{OXA-58} and bla_{OXA-24} genes belonging to class D β-lactamases as well as a novel bla_{TEM} (98.9% sequence similarity to the bla_{TEM-1} gene) belonging to class A β-lactamases were detected in a higher proportion in phage DNA. Although preliminary, these findings corroborate the role of bacteriophages as reservoirs of resistance genes and thus highlight the necessity to include them in future studies on the emergence and spread of antibiotic resistance in the environment.
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1. Introduction

Although antibiotic therapy has been extremely useful in combating infectious diseases, the emergence of resistance amongst bacterial populations threatens to undermine this success. Susceptible bacteria may develop resistance to antibiotics through multiple and complex mechanisms, such as mutation and horizontal gene transfer (HGT). The latter represents an important evolutionary process by which organisms acquire new traits that can be beneficial under challenging conditions. These include genes that confer virulence or resistance to antibiotics and other antimicrobial agents [1]. HGT may be mediated by mobile genetic elements (MGEs) such as insertion sequences, transposons, integrative conjugative elements, plasmids and bacteriophages, which are involved in bacterial acquisition and recombination of foreign DNA [2].

Although the transfer of antibiotic resistance genes (ARGs) amongst bacteria through these MGEs has been widely studied and demonstrated, especially in clinically relevant isolates [3,4], the role of bacteriophages as reservoirs of ARGs has not been extensively explored. Bacteriophages (phages), viruses that infect bacteria, can act as vehicles for horizontal exchange of genetic information, can genetically modify their host by insertion of their DNA into the bacterial genome, and can carry genes that encode new functions or modify existing ones [1]. The study of phages has traditionally relied upon culture-dependent techniques and their further characterisation using molecular methods. Although useful, these data are limited and biased towards particular phages that efficiently multiply in well characterised hosts under controlled laboratory conditions. Recent advances in genomics and metagenomics are now providing opportunities to improve our understanding of the structure and function of naturally occurring viral communities. The aim of this study was therefore to explore and compare the presence of ARGs both in phage and bacterial DNA fractions using Illumina-based high-throughput sequencing, thus allowing a complete survey of potential ARGs in phage genomes.

2. Materials and methods

2.1. Sampling and DNA extraction

Two wastewater samples (250 mL each) were collected in sterile glass bottles from Josep Trueta Hospital (Girona, Spain). Samples were stored in the dark at 0 °C in a portable icebox and were transported to the laboratory within 1 h. Upon arrival, samples were

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Table 1
Summary of data generated from the analysed metagenomes.

Feature	Phage DNA	Bacterial DNA
No. of raw reads (×10^6)	21.50	18.90
No. of filtered reads (×10^6)	21.33	18.39
No. of 16S rRNA gene reads	9,419	45,711
16S rRNA gene reads (%)	0.04	0.25
No. of ARG reads	55,761	33,839
ARG reads (%)	0.26	0.18

ARG, antibiotic resistance gene.

immediately filtered through low-protein-binding 0.22-μm pore size membranes, which allowed the passage of phage particles in the filtrate whilst bacteria were retained on the membrane surface. Phages were then concentrated to 1.0 mL using 100 kDa Amicon® Ultra Centrifugal Filter Units (EMD Millipore, Darmstadt, Germany) and were treated with DNase (100 U/mL) to eliminate free DNA. Phage DNA was extracted and purified as described previously [5]. For comparative analysis, bacterial cells were immediately collected from the membrane surface and genomic DNA was extracted and purified using a standard phenol–chloroform method [6].

2.2. Metagenome sequencing and analysis

DNA samples were pooled for each fraction and were sequenced on an Illumina HiSeq™ 2000 platform (Illumina Inc., San Diego, CA) using a paired-end library, with a read length of 100 bp and an insert size of 350 bp. A total of 40.4 million Illumina sequencing reads were generated. Low-quality reads were filtered using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) with a quality cut-off of 20, resulting in 21.3 million and 18.4 million reads in the viral and bacterial metagenomes, respectively (Table 1). The presence of 16S rRNA gene sequences in each DNA fraction was determined using METAXA2 [7] and was used to assess potential contamination of the viral DNA fraction by bacterial DNA.

2.3. Detection and abundance of phage taxa and phage–bacterial associations

Phage sequences were compared with the National Center for Biotechnology Information (NCBI) non-redundant (nr) protein database and the ACLAME protein database [8] using BLASTX (*E*-value < 10^{-3}). Classification of phage hosts was based on host information in the ACLAME database. Classification of viruses was performed according to the NCBI viral classification system, as proposed by the International Committee on Taxonomy of Viruses. MEGAN software was used to assign taxonomic groups to phage sequences [9]. Contigs were assembled using the Velvet assembly package [10], and phage–bacterial associations were then determined by computing the combination of phage and bacterial phylogenetic annotations on a given contig as previously described [11]. BiMat implemented within MATLAB [12] was used for analysis and visualisation of the associations obtained.

2.4. Annotation of antibiotic resistance genes

BLASTX implemented in DIAMOND software [13] was employed to align reads of each metagenome against an in-house database containing sequences from the Antibiotic Resistance Genes Database (ARDB) [14], the Comprehensive Antibiotic Resistance Database (CARD) [15], and the Bush, Palzkill and Jacoby collection of curated β-lactamase proteins (http://www.lahey.org/Studies/). A read was annotated as a resistance gene if the best BLAST hit (BLASTX) had ≥90% amino acid identity over >90% of the length of the target sequence.

This stringent threshold was chosen to minimise false positives [16,17]. ARGs were then grouped according to their resistance mechanism [18]. The relative abundance of reads annotated as ARGs was then calculated in relation to the total number of reads in each metagenome.

High-quality reads, previously assembled in contigs, were also characterised through a similarity search of their predicted genes using MetaGeneMark [19] against an in-house database and the NCBI non-redundant (nr) protein database. Contigs were considered to contain ARG sequences when the BLASTP *E*-value was <10^{-5}. Contigs of less than 250 bp or with less than 3× coverage were discarded. This assembly strategy was chosen to identify the predominant variants of ARGs. Metagenomics data sets have been deposited in the NCBI Short Read Archive (SRA) under accession no. **SRX1092351**.

3. Results and discussion

This preliminary study suggests that hospital wastewater can be an important source of phages harbouring ARGs to the environment. We are aware, however, that the low percentage of sequences from viral origin in the phage DNA fraction (3.4%) is a serious limitation to draw general conclusions, although our results appear consistent with previous reports. In fact, an extensive study using metagenomics approaches revealed that few sequences (2–5%) were assignable to virus or phage origin in swine faecal viromes, suggesting that phages harbour more bacterial genes than previously expected [20]. On the other hand, the low abundance of 16S rRNA gene sequences in the phage DNA fraction (0.04%) compared with that in the bacterial DNA fraction (0.25%) rejected major contamination of the phage DNA sample and thus confirmed the validity of our observations (Table 1).

3.1. Phage taxa and phage–bacterial associations

Most of the phage sequences in the phage DNA fraction affiliated to the order Caudovirales, comprising the tailed phage families Podoviridae (14.6%), particularly represented by the species *Escherichia* phage N4 and *Salmonella* phage FSL SP-058, Siphoviridae (26.5%), represented by the species *Enterobacteria* phage HK022 and *Burkholderia* phage phiE125, and Myoviridae (32.9%), which was dominated by the species *Aeromonas* phage phiO18P, *Ralstonia* phage RSY1 and *Enterobacteria* phage P1. Previous studies have demonstrated that most members of these families have a temperate life cycle, whereby they may contribute to the evolution and ecology of bacteria by, for example, promoting the transfer of virulence and resistance determinants through either transduction or phage conversion [21].

We also constructed a bipartite ecological network between bacteria and known phages to predict phage–bacterial associations. The resulting network revealed 92 phage–bacterium interactions (Fig. 1). We find that some phages can infect multiple hosts (average of 2.30) and some bacterial hosts are infected by multiple phages (average of 2.63). The proportion of specialist phages (47.5%) was similar to that of generalist phages (52.5%). In fact, some phage taxa appear to have a specific host range, such as *Phikmvlikevirus* genus, which is only connected to species of the genus *Pseudomonas*, or *Twortlikevirus* genus, which only interacts with members of the genus *Staphylococcus*. Other phages showed a broader host range, such as the *Lambdalikevirus* genus that connects with bacterial taxa from two different phyla (Firmicutes and Proteobacteria).

3.2. Abundance and diversity of antibiotic resistance genes

We initially compared sets of unassembled reads, not assembled contigs, because making quantitative comparisons amongst assembled metagenomes is not straightforward. The metagenomic

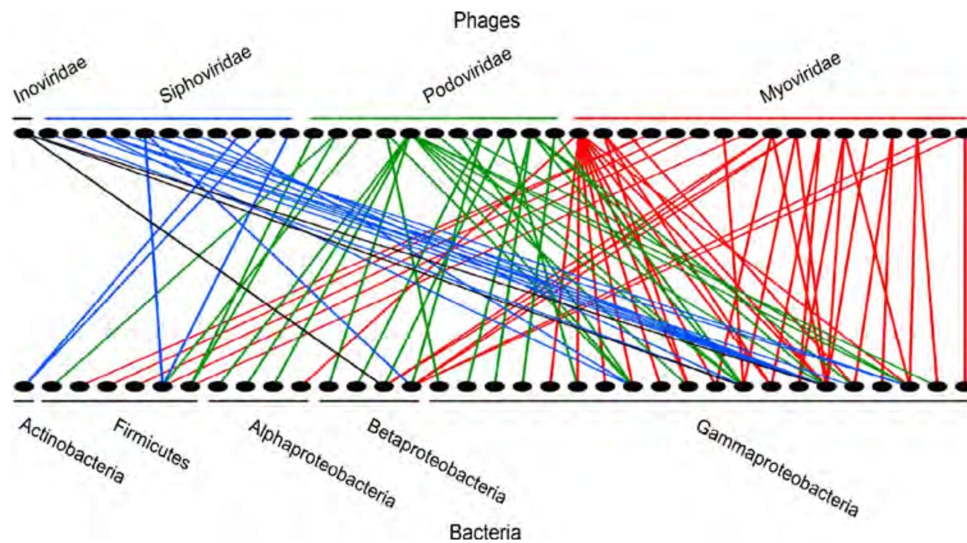

Fig. 1. Phage–bacterial ecological network. Coloured lines indicate the association between the most abundant phage families (Siphoviridae, Podoviridae and Myoviridae) belonging to the order Caudovirales and their bacterial hosts. Black lines denote the association between members of the *Inovirus* genus (Inoviridae family) and their bacterial hosts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

analysis showed that the relative abundance of ARGs was higher in the phage DNA fraction (0.26%) than in the bacterial DNA fraction (0.18%). These differences were particularly evident amongst genes encoding ATP-binding cassette (ABC) and resistance–nodulation–cell division (RND) proteins, phosphotransferases, β-lactamases and plasmid-mediated quinolone resistance (Fig. 2a). Previous studies have demonstrated that genes encoding efflux pumps, especially ABC and RND transporters, were predominant amongst genes detected in swine faecal viromes [20]. Moreover, genes encoding efflux pumps and, to a lesser extent, β-lactamases were dominant in the virome obtained from an experimental aquaculture facility [22].

Because sequences related to β-lactamases were detected in a high proportion both in phage and bacterial DNA fractions and because of their clinical relevance, a more detailed analysis was carried out to determine the prevalence of different β-lactamase classes in both metagenomes. Interestingly, sequences related to class D β-lactamases were detected more frequently in the phage DNA fraction than in the bacterial DNA fraction (Fig. 2b). Likewise, the relative proportion of class A and class C β-lactamases was higher in the phage DNA fraction, although to a lesser extent than class D β-lactamases.

These results may be of special importance because whilst class A and class C β-lactamases have been considered as the most

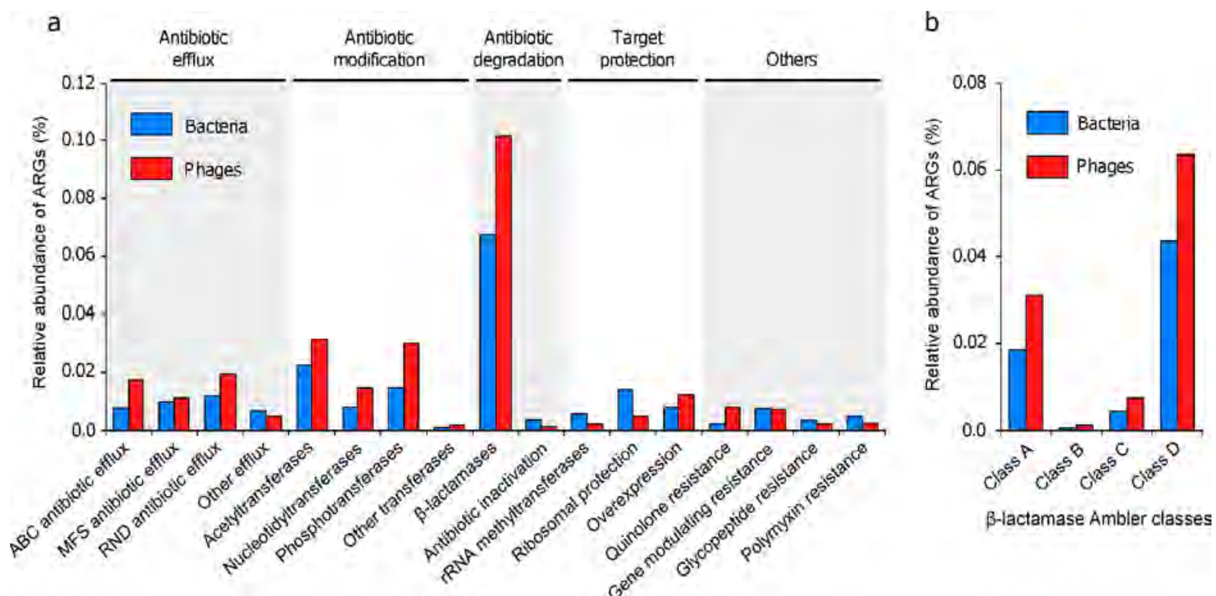

Fig. 2. Relative abundance of antibiotic resistance genes (ARGs) in the bacterial and phage DNA fractions from hospital wastewater samples. The distribution of ARGs is based on (a) all mechanisms and (b) Ambler classification. Counts were normalised to the total number of reads per metagenome. Overexpression mechanism includes the *sul* and *dfr* genes. ABC, ATP-binding cassette; MFS, major facilitator superfamily; RND, resistance–nodulation–cell division.

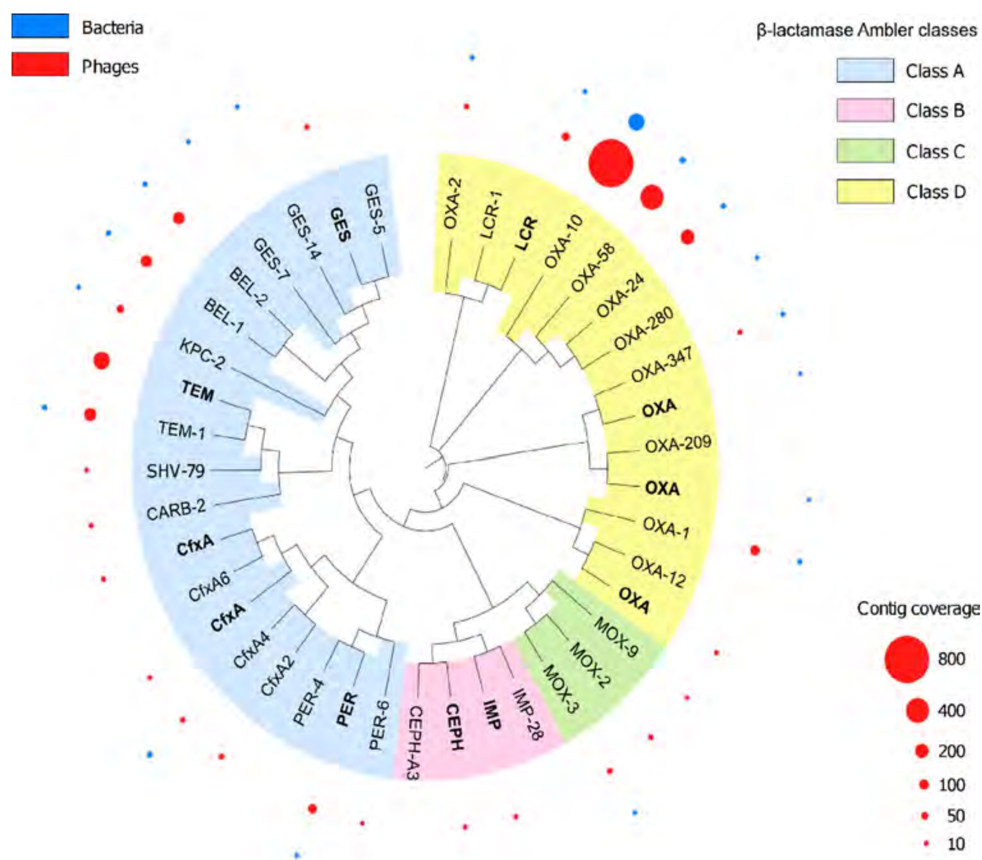

Fig. 3. Phylogenetic relationships amongst β-lactamases identified in the assembled contigs from the bacterial and phage DNA fractions. The phylogenetic tree was constructed based on aligned amino acid sequences of β-lactamases identified in this study and the most closely related known β-lactamases using the neighbour-joining method with 1000 bootstrap replicates in MEGA v.5.0 [25]. The tree was plotted using Interactive Tree of Life software [26]. Circle size indicates the coverage of contigs, and novel variants are shown in boldface type.

clinically important classes of β-lactamases [23], some class D β-lactamases confer resistance to last-resort antibiotics such as carbapenems, thus severely limiting therapeutic alternatives [24]. These observations were further supported by the analysis of assembled contigs (Fig. 3). Based on the coverage of contigs, OXA-58 and OXA-24 variants belonging to class D β-lactamases that confer resistance to carbapenems [27,28] were detected in high proportions in the phage DNA. It should be noted that the OXA-10 variant was the most prevalent in the phage DNA fraction; however, this variant confers resistance only to penicillins and some early cephalosporins [24].

The comparative analysis between the assembled contigs and known ARGs described in public databases revealed the occurrence of novel variants of β-lactamases both in phage and bacterial DNA fractions (Fig. 3). Specifically, five novel variants closely related to CfxA6, GES-5, PER-4 and TEM-1 variants belonging to class A β-lactamases, two novel variants closely related to CEPH-A3 and IMP-28 variants belonging to class B β-lactamases, and four novel variants closely related to LCR-1, OXA-12, OXA-209 and OXA-347 belonging to class D β-lactamases were detected in phage and/or bacterial DNA fractions.

4. Conclusions

Although preliminary, these results highlight the role of bacteriophages as reservoirs of resistance genes, including novel variants of ARGs with clinical relevance. In the current scenario of the global threat posed by multidrug-resistant pathogens (http://www.who.int/drugresistance/), this role should not be underestimated considering that phages are the most abundant biological entities on our planet and efficiently transfer genes amongst bacterial hosts. In this regard, a complete understanding of the factors contributing to the acquisition and environmental spread of antibiotic resistance would only be possible if phages are also included into the equation.

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Chapter 8

General discussion

The current strategy of the World Health Organization to tackle the problem of antimicrobial resistance (AMR), the so-called One-Health approach, is based on the idea that only a holistic action encompassing human and animal medicine and the environment would be helpful (So et al., 2015). Under this perspective, researchers must go beyond hospital walls and investigate AMR where microbes live, multiply and adapt, that is, the soil, the water and the air. In fact, experts say that the spread of AMR could only be contained after gaining a better knowledge on its origin, abundance and dissemination routes (Wright, 2012). Natural environments play a key role on the origin and evolution of antibiotic resistance (Martínez, 2008). In particular, aquatic environments can serve both as reservoirs of ARB and ARGs and as hotspots for the transfer of resistance genes among pathogenic and non-pathogenic bacteria under favorable conditions (Suzuki et al., 2017). Aquatic systems, including rivers, lakes and streams receiving chronic discharges of anthropogenic wastes (*e.g*. WWTP effluents, hospital and antibiotic production plants sewage) harbor high concentration of both ARB and ARGs due to the selective pressure exerted by pollutants, including pharmaceutical compounds such as antibiotic residues (Szczepanowski et al., 2009).

The risk of transmission of antibiotic resistance from the environment to humans is on the spotlight of a large number of investigations during the last decade. However, there are still many knowledge gaps to fill in to clearly define the risk that the exposure of humans to environmental ARGs and ARB may pose to public health. In this regard, the studies carried out during this thesis would help to evaluate the main factors contributing to the occurrence and maintenance of ARGs in aquatic microbial communities, especially in streambed biofilms which have been recognized as a major reservoir of antibiotic resistance in the environment (Balcázar et al., 2015).

Our investigations have particularly focused on the detection and quantification of several genes (bla_{NDM}, bla_{OXA} and bla_{KPC}) conferring resistance to carbapenems, which are considered antibiotics of last resort (**Chapter 4, 5 and 7**). They are considered as resistance genes of high-risk because: *i)* they are usually found in transferable genetic elements hosted by human bacterial pathogens, and *ii)* they contribute to the failure of antibiotic treatment of many infectious diseases (Hawkey and Jones, 2009; Martínez et al., 2014). Our studies demonstrated that these genes are carried by both environmental bacteria and bacteriophages and thus, they are widespread in different compartments of aquatic habitats (water, biofilms and sediments) and sites (*e.g*. rivers, raw sewage, treated wastewater, hospital effluents). Of special concern is the detection of these three genes at high concentrations in hospital sewage thus indicating their prevalence in human pathogens

circulating among community members (Bij and Pitout, 2017; Yong et al., 2009). Our results also agree with previous studies highlighting that hospital effluents are one of the main sources of both ARB and ARGs that are released into the environment (Barancheshme and Munir, 2018; Laffite et al., 2016) but also reveal that, in this waste, bacteriophages contain ARGs and may contribute to their dissemination. Accordingly, our results point to the urgent implementation of special treatment processes to hospital effluents to mitigate their environmental impact. In this regard, we also encourage authorities to define new standard guidelines and policies to regulate the treatment of hospital wastewater since urban WWTPs are not optimized to efficiently remove medical and biological waste (Krarup, 2015).

One of the main risks associated with the environmental resistome relies on the diverse composition of bacterial communities (Manaia, 2017). While strict environmental bacteria, such as many members of phyla Actinobacteria, Proteobacteria, or Bacteroidetes, can be considered reservoirs of ARGs with low risk of transmission to humans, ARB associated to anthropogenic waste may be considered at high risk for transmission (Manaia, 2017). The major route of transmission of a resistant pathogen to a human being is through direct contact with infected individuals. In contrast, transfer from the environment is less probable because of the rigid dispersal barriers existing in natural habitats (Bengtsson-palme et al., 2018). One of these barriers is the reduced survival time of the target ARB outside its ecological niche. Since most ARB are human-associated bacteria and, particularly, commensal bacteria inhabiting the human gut, they are anaerobes exhibiting low survival rates after being discharged into surface waters (Shanks et al., 2013). Other bacteria, however, including opportunistic and emerging pathogens, can temporally use the environment as a surrogate habitat where to persist and grow (Berg et al., 2005). If those bacteria have the opportunity to interact with the human microbiome (*e.g.* through the food chain or consumption of polluted water (H K Allen et al., 2010; De Boeck et al., 2012; Ghaly et al., 2017)) they may pose a risk to human health.

A well-functioning WWTP with secondary treatment will constantly discharge high loads of ARGs and ARB into the environment (Vaz-moreira et al., 2011). This scenario is even worse if we consider that nearly 80% of wastewater produced worldwide is discharged into the environment without any treatment (UN World Water Development Report 2017). In **Chapter 5** we aimed to asses both the impact of raw and treated wastewater on the prevalence of twelve ARGs in streambed biofilms and the contribution of wastewater-associated bacteria (including putative pathogens) to the dissemination of antibiotic resistance. The main difference between raw and treated wastewater mainly relied on the

microbiological quality of the produced effluent. This quality is strongly influenced by the hydraulic retention time (HRT) in the WWTPs (Novo and Manaia, 2010). Authors stated that a HRT of 24 h contributes to the homogenization of the bacterial community and allows a removal of up to >99% of all bacterial groups (Novo and Manaia, 2010). In our study, the WWTP operated using conventional activated sludge at very high HRT of 80 h and at a sludge retention time of 7 days thus allowing a reduction in BOD_5 of 95%, which indicates a high reduction of bacterial load in the final effluent. Our results showed that bacteria released with the treated effluent did not cause noticeable changes in the bacterial composition of streambed biofilms but altered the streambed resistance profile. In turn, the raw sewage caused a remarkable change in both the composition of epilithic biofilms and the concentration of the measured ARGs. These epilithic biofilms were enriched in Gammaproteobacteria and Firmicutes, two signature groups for the raw sewage (Shanks et al., 2013) that were also prevalent in the sewage effluent discharged into the studied stream. Within these phyla, genera such as *Aeromonas*, *Acinetobacter*, *Pseudomonas*, and *Enterococcus* have frequently been described as potential carriers and vectors for antibiotics resistance transmission to humans (Vaz-moreira and Nunes, 2014; WHO, 2014). Interestingly, we identified two OTUs with 98.5% and 98% sequence identity to *Acinetobacter haemolyticus* and *Granulicatella adiacens,* two opportunistic pathogens that have been associated with invasive infections in humans and usually develop resistance to a wide range of antibiotics (Cargill et al., 2012; Gardenier et al., 2011; Lapara et al., 2011; Peleg et al., 2008; Shailaja et al., 2013). Moreover, these potential pathogenic OTUs significantly correlated with some of the studied genes (*intI*1, *sul*1, *sul*2 and *erm*B) thus highlighting their potential contribution to the streambed resistome.

Most of these wastewater-associated ARGs can be transferred to environmental bacteria and eventually to human pathogens (Manaia, 2017). In this regard, the potential transfer of ARGs by HGT is another risk to take into account. As mentioned above, the transfer of ARGs from environmental bacteria to human pathogens is modulated by several bottlenecks. The first one is the "ecological connectedness", which is defined as the necessity that both the donor and the recipient cell have to share the same niche and interact for a successful transfer to occur (Bengtsson-palme et al., 2018). The second bottleneck that determines the spread of a given ARG is the presence of antibiotic residues in the environment since antibiotic pollution may help to reduce the fitness cost associated with ARGs located in MGEs such as plasmids (Bengtsson-palme et al., 2018; Martínez et al., 2014). This is because only those genes with a low fitness cost, and thus maintained in the natural environment, will be able to disseminate to the human microbiome. However, mobile resistance genes

may also be favored in the absence of antibiotics by cross-resistance (Baker-austin et al., 2006) or by the nutrient load in aquatic system (Elsas et al., 2003; Amos et al., 2015).

To gain a better understanding of the effects of both nutrients and emerging pollutants on bacterial communities and their associated resistome, we conducted a manipulative experiment in the laboratory to assess the impact of a combined regime of a mixture of five antimicrobials (ciprofloxacin, erythromycin, sulfamethoxazole, diclofenac, and methylparaben) (**Chapter 6**). This controlled experiment allowed us to exclude the influence of physical and microbiological variables and to shed light on the sole contribution of both nutrient and emerging pollutants on the spread of antibiotic resistance. Our results showed that the mixture of pollutants significantly increased the prevalence of *intI*1 and *sul*1 genes in streambed biofilms without causing a noticeable variation in the composition of biofilm bacterial communities. Moreover, the exposure to high nutrient concentrations enhanced the effect of emerging contaminants on the spread of *intI*1 and *sul*1 genes within the community. We hypothesized that the emerging contaminants and high nutrient concentration favored the spread of both genes within bacterial population via HGT. This hypothesis was based on different reasons: *i*) the lack of variations in the bacterial community composition after treatment exposure, which suggests that other mechanisms were responsible for the prevalence of both genes in those biofilms; *ii*) the fact that *intI1* gene is often carried in MGEs, including transposons and plasmids and it is usually linked to *sul*1 (Gillings et al., 2015); *iii*) the significant correlation found between the abundance of both genes only in biofilms exposed to emerging contaminants. This last result is especially relevant because it suggests that gene *sul*1 may be part of the class 1 integron structure, whose abundance rapidly changed in response to emerging contaminants pressure. This behavior supports the idea proposed by Gillings and co-workers of using *intI1* gene as a proxy of anthropogenic pollution (Gillings et al., 2015). Beside this, biofilms usually harbor a dense and diverse bacterial community thus facilitating successful gene transfer events due to the close contact between biofilm inhabitants (Martinez, 2009; Fux et al., 2005). Overall, we can conclude that when streambed biofilms are only exposed to abiotic factors such as emerging contaminants and nutrients, the HGT mechanism may be the main responsible on the dissemination of ARGs within the community. However, these results may vary depending on the concentration of pollutants and the exposure time.

Results from **Chapter 5** also showed that wastewater-associated ARB, including potential human pathogens, are able to colonize the streambed and disseminate their genes to indigenous bacterial populations. However, the likelihood of both the maintenance and the spread of those ARGs within the community may be influenced by the background antibiotic

concentration in the system. Thus, aquatic habitats receiving chronic WWTP discharges may be considered high-risk environments because they harbor both potential carriers and vectors of antibiotic resistance while maintaining favorable conditions for their transmission to human commensals and/or pathogens. It should be noted that rivers are usually used for recreational activities or as water supplies for irrigation or consumption and thus may potentially transmit ARB to humans (Bengtsson-palme et al., 2018). However, as we showed in Chapter 5, raw sewage poses a higher risk than treated effluents since wastewater treatment significantly reduces the total bacterial load in the final effluent (Novo and Manaia, 2010). Moreover, raw sewage also contributes to increasing of nutrient concentration in the receiving river, which enhances the effect of antibiotics on the maintenance and spread of resistance traits within the community (**Chapter 6**).

Recent investigations have also highlighted that bacteriophages contribute to the dissemination of antibiotic resistance in the environment (Balcazar, 2014; Muniesa et al., 2013; Colomer-Lluch et al., 2011). The study of phages has frequently relied on culture-dependent techniques and their downstream characterization using molecular methods (Calero-cáceres and Muniesa, 2016; Colomer-lluch et al., 2014; Marti et al., 2013). However, these data are highly biased toward phages capable to multiply under controlled conditions in the laboratory. To perform a complete analysis of potential ARGs in phage genomes, we carried out a study using a metagenomic approach to explore and compare the presence of ARGs both in phage and bacterial DNA fractions from hospital effluent samples (**Chapter 7**). The metagenomic analysis showed that the abundance of ARGs identified in phage genomes was higher than that in bacterial DNA. Both DNA fractions were largely enriched with sequences related to clinically important classes of β-lactamases. Concretely, the variants OXA-58 and OXA-24 conferring resistance to last resort antibiotics such as carbapenems were detected in high proportion in the phage DNA. Both genes have been previously described in relevant clinical isolates and the variant OXA-24 was part of an outbreak in Spain in 1997 (Evans and Amyes, 2014). These results highlighted that phages can serve as a reservoir of clinically relevant ARGs although more research is needed to unequivocally demonstrate that these phage-encoded ARGs can be successfully transferred to environmental bacteria (Gunathilaka et al., 2017).

Bacteriophages are the most abundant viruses in most environments and they can transfer genes to bacteria through either generalized or specialized transduction (Frost et al., 2005; Gogarten et al., 2009). Although the transduction is a rare event under laboratory conditions, the large abundance of these entities in nature makes gene transfer by transduction highly significant in the environment (Penadés et al., 2015). Our results

showed that more than 50% of phages in a hospital effluent are polyvalent bacteriophages since they can infect multiples host, even bacterial taxa from different phyla. A broad host range may provide better survival rates to phages that, together with the high abundance of bacteria and phages found in raw sewage (Cantalupo et al., 2011), may guarantee the phages-bacteria encounters needed for infection. Moreover, stress conditions, such as the presence of antibiotics, have been found to increase the number of phages as a result of the induction of prophages in lysogenic bacteria (Wang and Wood, 2016) and enhance transduction events (Modi et al., 2013). Phages can reach aquatic environments through WWTP effluents (Lood et al., 2017; Purnell et al., 2015), and thus may contribute to the background level of antibiotic resistance by spreading ARGs to new bacteria via transduction. This is of importance because, as our results showed in Chapter 7, the bacterial resistome in the hospital effluent is mirrored in the phage resistome from the same sample. Once discharged into the environment, the structural characteristics of phage particles allow them to resist challenging environmental conditions (Weinbauer 2004) and propagate among different biomes (Sano et al., 2004). This implies that phages can easily transfer DNA fragments, including ARGs, among cells and between environments (Hamilton, 2006). A recent study has demonstrated the successful transfer of ARGs by coliphages isolated from wastewater (Gunathilaka et al., 2017) thus suggesting that phages are not only reservoirs but also spreaders of resistance genes into environmental bacteria.

Chapter 9

Future perspectives

The growing concern on antibiotic resistance has drove the scientific community to invest their efforts in the comprehension of mechanisms and factors that facilitate the spread of ARGs in natural environments receiving anthropogenic pollution. Our studies have shed light on how resistance genes are disseminated and maintained in aquatic systems and, particularly, in streambed biofilms. The magnitude of the problem, however, requires further investigations and the commitment of public and private institutions at all society levels.

Deep knowledge on the mechanisms involved in the evolution of antibiotic resistance is crucial to predict the emergence of a resistance pathogen. Further studies should unravel the selection forces promoting both resistance phenotypes and the reduction of the fitness cost associated with carrying resistance genes, especially in heterogeneous communities where complex interactions between species occur (*i.e.* biofilms). To better understand the mechanisms involved in the spread of ARGs, it is essential to assess the combined effect of factors exerting selective pressure over microorganisms, rather than carrying out studies addressing a single factor. Since the maintenance and spread of ARBs in natural environments are considered the main risk to the environment, it would be appropriate to build a model to predict the effects of both biotic and abiotic factors on: *i)* the survival of the target ARB in the environment; *ii)* the fitness cost associated with the carriage of a given ARG; and *iii)* the gene transfer rates. These data would be helpful to assess the risk of dissemination of a given ARG between environmental bacteria and both human-associated microbiota (including opportunistic and primary pathogens). In order to develop this model and its risk-assessment framework, it is necessary to establish a quantitative long-term monitoring of ARB and ARGs in environments receiving chronic anthropogenic impacts. In this regard, this surveillance is still lacking despite the wealth of information regarding the risk that environmental ARB and ARGs pose to human health (Dulio et al., 2018). It is then recommended that both ARB and ARGs should be included as priority pollutants within the European Water Framework Directive (EWFD) that should also establish reliable and suitable monitoring methods. Moreover, the EWFD should also provide quality standards and guidelines reporting the maximum concentrations allowed in water bodies for both contaminants according to their associated risks (Berendonk et al., 2015). Those genes that have been described to reside in MGEs and hosted by human pathogens (*e.g.* bla_{NDM}, bla_{OXA} and bla_{KPC} genes) must be considered as high-risk resistance genes (Martinez, 2014) and thus restrictive quality parameters should be applied. This guideline may be included in the regulation of practices such as wastewater reuse.

To develop successful mitigation strategies for the dissemination of antimicrobial resistance it is also desirable to minimize the establishment of conditions favoring the mobilization and selection of ARGs in environmental settings. In this regard, we strongly recommended both to implement innovative processes for the treatment of wastewater with high levels of pollutants, such as hospital sewage and industrial effluents, and the improvement of current wastewater treatment (WWT) technologies. A recent study highlighted that the incorporation of UV systems to a drinking water treatment plant (DWTP) significantly reduced the antibiotic-resistant (AR) *E.coli* concentrations in tap water thus minimizing the likelihood of human exposure to this agent (Flaherty et al., 2017). However, this study also pointed out that the risk of human exposure to AR *E.coli* through drinking water also depends on the water collection site, water supply systems and the amount of water consumed by humans (Flaherty et al., 2017). The new WWT technologies should also be efficient in removing bacteriophages. The application of new technologies such as membrane filtration and reverse osmosis or chemical treatments such as ozone, has been found to be efficient against viral particles (Lood et al., 2017; Purnell et al., 2015). Besides developing better treatment technologies, it is also crucial to improve the current regulation of wastewater management because nearly 80% of wastewater produced worldwide is discharged in the environment without any treatment.

Under a social point of view, the rational use of antibiotics in both hospital settings and the community is imperative to reduce the generation of new resistance gene variants and to mitigate their spread. We consider that the first step to achieve this goal is education since all community members should be aware of the consequences of antibiotic misuse and the causes of the spread of antibiotic resistance among human bacterial pathogens. In many countries, antibiotics can be obtained without medical prescription and without any accurate information on how they work and how they should be used (Hawkings et al., 2008). The implementation of adequate educational programmes would surely help to reduce common practices such as consumption of leftover antibiotics, the sharing of remaining drugs with other people, to stop the prescribed antibiotic course too early or to purchase drugs over the counter without prescription. These programmes should be initiated in schools, making antibiotic resistance as a part of professional education targeting different settings including households, pharmacies, and drug vendor outlets.

Hospital settings should reduce the use of antibiotics. In this regard, a better regulation of practices such as drug-promotion incentives or fee-for-service remuneration scheme (Masiero et al., 2010) and the application of the appropriate diagnosis test will help to accomplish this goal (WHO, 2001). For instance, it has been estimated that the prescription

of unnecessary antibiotics could decrease by 2.4% for every additional minute that a doctor spends with a patient during a visit (Imanpour et al., 2017). Besides, hospital settings should have access to appropriate sanitation facilities and infection control practices to minimize the incidence of infection with ARB (WHO, 2001).

The use of antibiotics for non-therapeutic purposes must be banned. The use of antibiotics in human medicine, veterinary practices, food production, agriculture or aquaculture must be totally restricted. Instead, practices to prevent infections, such as vaccination of animals or keeping animal housing clean, should be reinforced to improve animal health and thus reduce the necessity of antimicrobial therapy.

Finally, the discovery of new antimicrobials is crucial to efficiently combat resistance. It is essential that governments encourage pharmaceutical companies to develop new antibiotics by: *i)* funding research initiatives aimed to the discovery and refinement of new antibiotics, and *ii*) applying tax incentives to those pharmaceutical companies investing in the discovery of new drugs. Some of the reasons argued by pharmaceutical companies to discontinue research on the discovery of new antibiotics are related to the low economic counter-benefits and the appearance of resistance shortly after drug deployment into the market. Nowadays, the discovery of new antibiotics is harder than it was in the golden era of antibiotics (1950–1970). Natural products from cultivable soil microorganisms have been the main source of antibiotics currently used in human and veterinary medicine. However, the low cultivability of most microorganisms (≈1%) (Amann et al., 1995) is the biggest barrier that has impeded researchers to discover new antibiotics. In fact, all the available antibiotics in the last 30 years have been derivatives of existing drugs discovered until 1984 (Lewis, 2012). Fortunately, recent investigations have shed light on the hidden microbiome with the discovery of the two first new antibiotics in 30 years by growing soil microorganisms using an electronic chip (teixobactin; Ling et al., 2015) and using culture-independent methods (malacidins; Hover et al., 2018). Both antibiotics are active against multidrug-resistance pathogens, such as methicillin- resistance *Staphylococcus aureus* or *Mycobacterium tuberculosis* (Hover et al., 2018; Ling et al., 2015). In this regard, new metagenomic tools may help researchers to identify potentially new antibiotic modes of action and resistance genes in the environment that will help to predict the emergence of future resistance. This, together with more severe regulation management and use of antibiotics will increase the lifespan of our antibiotic arsenal and avoid the advent of a “pre-antibiotic” era.

Chapter 10

General conclusions

The main conclusions of this thesis are:

I. The optimized qPCR assays for the quantification of genes *bla*$_{NDM}$, *bla*$_{KPC}$ and *bla*$_{OXA-48}$ are widely applicable in different environmental matrices, including complex habitats such as streambed biofilms.

II. The *bla*$_{NDM}$, *bla*$_{KPC}$ and *bla*$_{OXA-48}$ genes, conferring resistance to carbapenems, are widely spread in aquatic environments subjected to anthropogenic pollution such as rivers receiving chronic discharges of raw sewage and treated wastewater or hospital effluents.

III. Raw sewage has a larger impact on the composition of biofilm bacterial communities and their associated resistome than treated wastewater.

IV. Wastewater-associated antibiotic-resistant bacteria, including human pathogens, are able to colonize streambed biofilms where they may persist and potentially disseminate their genes among their bacterial counterparts.

V. A mixture of emerging contaminants common in wastewater treatment plant effluents caused an increase in the abundance of *intI*1 and *sul*1 genes in streambed biofilms without significant changes in community composition. This observation suggests that the observed enrichment was caused by the dissemination of target ARGs rather than by the selection of resistant taxa.

VI. Nutrients enhanced the effect of emerging pollutants on the maintenance and dissemination of resistance genes within biofilm bacterial communities.

VII. Streambed biofilms are reservoirs of antibiotic resistance genes and mobile genetic elements, especially in aquatic habitats receiving chronic wastewater discharges. Biofilms are thus a suitable biosensor of both anthropogenic pollution and environmental antibiotic resistance.

VIII. In hospital sewage, bacteriophages constitute a large and unexplored reservoir of genes conferring resistance to antibiotics widely used in human medicine.

Chapter 11

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ANNEX

Supplementary information

Chapter 4

Supplementary Data

KPC-type β-lactamases

Primers	**Sequence (5'→3')**
Kpc_rtF	CAGCTCATTCAAGGGCTTTC
Kpc_rtR	GGCGGCGTTATCACTGTATT

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                                  Forward
KC433553.1|KPC-15      TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
JX524191.1|KPC-14      TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
KC465199.1|KPC-16      TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
HQ342889.1|KPC-13      TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
EU447304.1|KPC-4       TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
EU400222.2|KPC-5       TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
HQ641421.1|KPC-12      TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
EU729727.1|KPC-7       TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
KJ775801.1|KPC-19      TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
FJ624872.1|KPC-9       TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
KR052099.1|KPC-24      TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
KM379100.1|KPC-22      TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
LN609376.1|KPC-21      TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
KP681699.1|KPC-18      TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
KC465200.1|KPC-17      TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
HM066995.1|KPC-11      TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
GQ140348.1|KPC-10      TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
FJ234412.1|KPC-8       TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
EU555534.1|KPC-6       TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
AF395881.1|KPC-3       TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
AY034847.1|KPC-2       TGCAGCTCATTCAAGGGCTTTCTTGCTGCCGCTGTGCTGGCTCGCAGCCAGCAGCAGGCC
                       ************************************************************

KC433553.1|KPC-15      GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCGGTGGTCACCCATC
JX524191.1|KPC-14      GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCCGTGGTCACCCATC
KC465199.1|KPC-16      GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCCGTGGTCACCCATC
HQ342889.1|KPC-13      GGCTTGCTGGGCACACCCATCCGTTACGGCAAAAATGCGCTGGTTCCGTGGTCACCCATC
EU447304.1|KPC-4       GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCGGTGGTCACCCATC
EU400222.2|KPC-5       GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCGGTGGTCACCCATC
HQ641421.1|KPC-12      GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCCGTGGTCACCCATC
EU729727.1|KPC-7       GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCCGTGGTCACCCATC
KJ775801.1|KPC-19      GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCCGTGGTCACCCATC
FJ624872.1|KPC-9       GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCCGTGGTCACCCATC
KR052099.1|KPC-24      GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCCGTGGTCACCCATC
KM379100.1|KPC-22      GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCCGGGGTCACCCATC
LN609376.1|KPC-21      GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCCGAGGTCACCCATC
KP681699.1|KPC-18      GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCCGTGGTCACCCATC
KC465200.1|KPC-17      GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCCGTGGTCACCCATC
HM066995.1|KPC-11      GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCTGTGGTCACCCATC
GQ140348.1|KPC-10      GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCGGTGGTCACCCATC
FJ234412.1|KPC-8       GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCCGTGGTCACCCATC
EU555534.1|KPC-6       GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCCGTGGTCACCCATC
AF395881.1|KPC-3       GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCCGTGGTCACCCATC
AY034847.1|KPC-2       GGCTTGCTGGACACACCCATCCGTTACGGCAAAAATGCGCTGGTTCCGTGGTCACCCATC
                       ********** ********************************** * ************
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ᵏAll alignments were generated using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/)

NDM-type β-lactamases

Primers	**Sequence (5'→3')**
Ndm_rtF	GATTGCGACTTATGCCAATG
Ndm_rtR	TCGATCCCAACGGTGATATT

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                                                                              Forward
KF361506.1|NDM-10     GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT
KP862821.1|NDM-16     GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT
KP735848.1|NDM-15     GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT
KM210086.1|NDM-14     GCGCATCAGGACAAGATGGGCGGTATGGGCGCGCTGCATGCGGCGGGGATTGCGACTTAT
LC012596.1|NDM-13     GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT
AB926431.1|NDM-12     GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT
KP265939.1|NDM-11     GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT
KC999080.2|NDM-9      GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT
AB744718.1|NDM-8      GCGCATCAGGACAAGATGGGCGGTATGGGCGCGCTGCATGCGGCGGGGATTGCGACTTAT
JX262694.1|NDM-7      GCGCATCAGGACAAGATGGGCGGTATGAACGCGCTGCATGCGGCGGGGATTGCGACTTAT
JN967644.1|NDM-6      GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT
JN104597.1|NDM-5      GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT
JQ348841.1|NDM-4      GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT
JQ734687.1|NDM-3      GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT
FN396876.1|NDM-1      GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT
JF703135.1|NDM-2      GCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTAT
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KF361506.1|NDM-10     GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC
KP862821.1|NDM-16     GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC
KP735848.1|NDM-15     GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGCTGGTTGCGGCGCAACACAGC
KM210086.1|NDM-14     GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC
LC012596.1|NDM-13     GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGCTGGTTGCGGCGCAACACAGC
AB926431.1|NDM-12     GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGCTGGTTGCGGCGCAACACAGC
KP265939.1|NDM-11     GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGGTGGTTGCGGCGCAACACAGC
KC999080.2|NDM-9      GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAAAGGGGATGGTTGCGGCGCAACACAGC
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JX262694.1|NDM-7      GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGCTGGTTGCGGCGCAACACAGC
JN967644.1|NDM-6      GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC
JN104597.1|NDM-5      GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGCTGGTTGCGGCGCAACACAGC
JQ348841.1|NDM-4      GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGCTGGTTGCGGCGCAACACAGC
JQ734687.1|NDM-3      GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC
FN396876.1|NDM-1      GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC
JF703135.1|NDM-2      GCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGC
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KF361506.1|NDM-10     CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC
KP862821.1|NDM-16     CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC
KP735848.1|NDM-15     CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC
KM210086.1|NDM-14     CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC
LC012596.1|NDM-13     CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC
AB926431.1|NDM-12     CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC
KP265939.1|NDM-11     CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC
KC999080.2|NDM-9      CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC
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JQ348841.1|NDM-4      CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC
JQ734687.1|NDM-3      CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC
FN396876.1|NDM-1      CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC
JF703135.1|NDM-2      CTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTC
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OXA-48-like β-lactamases

Primers	Sequence (5'→3')
Oxa_rtF	AGGCACGTATGAGCAAGATG
Oxa_rtR	TGGCTTGTTTGACAATACGC

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                                                                         Forward
KP410734.1|OXA-438      CCTGTTTATCAAGAATTTGCCCGCCAAATTGGCGAGGCACGTATGAGCAAGATGCTACAT
JX893517.1|OXA-247      CCTGTTTATCAAGAATTTGCCCGCCAAATTGGCGAGGCACGTATGAGCAAGATGCTACAT
HQ700343.1|OXA-163      CCTGTTTATCAAGAATTTGCCCGCCAAATTGGCGAGGCACGTATGAGCAAGATGCTACAT
KP727573.1|OXA-439      CCTGTTCATCAAGAATTTGCCCGCCAAATTGGCGAGGCACGTATGAGCAAGATGCTACAT
JQ809466.1|OXA-204      CCTGTTTATCAAGAATTTGCCCGCCAAATTGGCGAGGCACGTATGAGCAAGATGCTACAT
KM589641.1|OXA-405      CCTGTTTATCAAGAATTTGCCCGCCAAATTGGCGAGGCACGTATGAGCAAGATGCTACAT
JN704570.1|OXA-199      CCTGTTTATCAAGAATTTGCCCGCCAAATTGGCGAGGCACGTATGAGCAAGATGCTACAT
KF900153.1|OXA-370      CCTGTTTATCAAGAATTTGCCCGCCAAATTGGCGAGGCACGTATGAGCAAGATGCTACAT
JX438001.1|OXA-245      CCTGTTTATCAATACTTTGCCCGCCAAATTGGCGAGGCACGTATGAGCAAGATGCTACAT
KU531433.1|OXA-505      CCTGTTTATCAAGAATTTGCCCGCCAAATTGGCGAGGCACGTATGAGCAAGATGCTACAT
JX438000.1|OXA-244      CCTGTTTATCAAGAATTTGCCCGCCAAATTGGCGAGGCACGTATGAGCAAGATGCTACAT
AY236073.2|OXA-48       CCTGTTTATCAAGAATTTGCCCGCCAAATTGGCGAGGCACGTATGAGCAAGATGCTACAT
HM015773.1|OXA-162      CCTGTTTATCAAGAATTTGCCCGCCAAATTGGCGAGGCACGTATGAGCAAGATGCTACAT
                        ****** ***** * *********************************************

KP410734.1|OXA-438      GCTTTCGATTATGGTAATGAGGACATTTCGGGCAATGTAGACAGTTTCTGGCTCGACGGT
JX893517.1|OXA-247      GCTTTCGATTATGGTAATGAGGACATTTCGGGCAATGTAGACAGTTTCTGGCTCGACGGT
HQ700343.1|OXA-163      GCTTTCGATTATGGTAATGAGGACATTTCGGGCAATGTAGACAGTTTCTGGCTCGACGGT
KP727573.1|OXA-439      GCTTTCGATTATGGTAATGAGGACATTTCGGGCAATGTAGACAGTTTCTGGCTCGACGGT
JQ809466.1|OXA-204      GCTTTCGATTATGGTAATGAGGACATTTCGGGCAATGTAGACAGTTTCTGGCTCGATGGT
KM589641.1|OXA-405      GCTTTCGATTATGGTAATGAGGACATTTCGGGCAATGTAGACAGTTTCTGGCTCGACGGT
JN704570.1|OXA-199      GCTTTCGATTATGGTAATGAGGACATTTCGGGCAATGTAGGCAGTTTCTGGCTCGACGGT
KF900153.1|OXA-370      GCTTTCGATTATGGTAATGAGGACATTTCGGGCAATGTAGACAGTTTCTGGCTCGACGGT
JX438001.1|OXA-245      GCTTTCGATTATGGTAATGAGGACATTTCGGGCAATGTAGACAGTTTCTGGCTCGACGGT
KU531433.1|OXA-505      GCTTTCGATTATGGTAATGAGGACATTTCGGGCAATGTAGACAGTTTCTGGCTCGACGGT
JX438000.1|OXA-244      GCTTTCGATTATGGTAATGAGGACATTTCGGGCAATGTAGACAGTTTCTGGCTCGACGGT
AY236073.2|OXA-48       GCTTTCGATTATGGTAATGAGGACATTTCGGGCAATGTAGACAGTTTCTGGCTCGACGGT
HM015773.1|OXA-162      GCTTTCGATTATGGTAATGAGGACATTTCGGGCAATGTAGACAGTTTCTGGCTCGACGGT
                        **************************************** *************** ***

KP410734.1|OXA-438      GGTATTCGAATTTCGGCCACGGAGCAAATCAGCTTTTTAAGAAAGCTGTATCACAATAAG
JX893517.1|OXA-247      GGTATTCGAATTTCGGCCACGGAGCAAATCAGCTTTTTAAGAAAGCTGTATCACAATAAG
HQ700343.1|OXA-163      GGTATTCGAATTTCGGCCACGGAGCAAATCAGCTTTTTAAGAAAGCTGTATCACAATAAG
KP727573.1|OXA-439      GGTATTCGAATTTCGGCCACGGAGCAAATCAGCTTTTTAAGAAAGCTGTATCACAATAAG
JQ809466.1|OXA-204      GGTATTCGAATTTCGGCCACTGAGCAAATCAGCTTTTTAAGAAAGCTGTATCACAATAAG
KM589641.1|OXA-405      GGTATTCGAATTTCGGCCACGGAGCAAATCAGCTTTTTAAGAAAGCTGTATCACAATAAG
JN704570.1|OXA-199      GGTATTCGAATTTCGGCCACTGAGCAAATCAGCTTTTTAAGAAAGCTGTATCACAATAAG
KF900153.1|OXA-370      GGTATTCGAATTTCGGCCACGGAGCAAATCAGCTTTTTAAGAAAGCTGTATCACAATAAG
JX438001.1|OXA-245      GGTATTCGAATTTCGGCCACGGAGCAAATCAGCTTTTTAAGAAAGCTGTATCACAATAAG
KU531433.1|OXA-505      GGTATTCGAATTTCGGCCACGGAGCAAATCAGCTTTTTAAGAAAGCTGTATCACAATAAG
JX438000.1|OXA-244      GGTATTCGAATTTCGGCCACGGAGCAAATCAGCTTTTTAAGAAAGCTGTATCACAATAAG
AY236073.2|OXA-48       GGTATTCGAATTTCGGCCACGGAGCAAATCAGCTTTTTAAGAAAGCTGTATCACAATAAG
HM015773.1|OXA-162      GGTATTCGAATTTCGGCCACGGAGCAAATCAGCTTTTTAAGAAAGCTGTATCACAATAAG
                        ******************** ***************************************
                                                     Reverse
KP410734.1|OXA-438      TTACACGTATCGGAGCGCAGCCAGCGTATTGTCAAACAAGCCATGCTGACCGAAGCCAAT
JX893517.1|OXA-247      TTACACGTATCGGAGCGCAGCCAGCGTATTGTCAAACAAGCCATGCTGACCGAAGCCAAT
HQ700343.1|OXA-163      TTACACGTATCGGAGCGCAGCCAGCGTATTGTCAAACAAGCCATGCTGACCGAAGCCAAT
KP727573.1|OXA-439      TTACACGTATCGGAGCGCAGCCAGCGTATTGTCAAACAAGCCATGCTGACCGAAGCCAAT
JQ809466.1|OXA-204      TTACACGTATCGGAGCGCAGCCAGCGTATTGTCAAACAAGCCATGCTGACCGAAGCCAAT
KM589641.1|OXA-405      TTACACGTATCGGAGCGCAGCCAGCGTATTGTCAAACAAGCCATGCTGACCGAAGCCAAT
JN704570.1|OXA-199      TTACACGTATCGGAGCGCAGCCAGCGTATTGTCAAACAAGCCATGCTGACCGAAGCCAAT
KF900153.1|OXA-370      TTACACGTATCGGAGCGCAGCCAGCGTATTGTCAAACAAGCCATGCTGACCGAAGCCAAT
JX438001.1|OXA-245      TTACACGTATCGGAGCGCAGCCAGCGTATTGTCAAACAAGCCATGCTGACCGAAGCCAAT
KU531433.1|OXA-505      TTACACGTATCGGAGCGCAGCCAGCGTATTGTCAAACAAGCCATGCTGACCGAAGCCAAT
JX438000.1|OXA-244      TTACACGTATCGGAGCGCAGCCAGCGTATTGTCAAACAAGCCATGCTGACCGAAGCCAAT
AY236073.2|OXA-48       TTACACGTATCGGAGCGCAGCCAGCGTATTGTCAAACAAGCCATGCTGACCGAAGCCAAT
HM015773.1|OXA-162      TTACACGTATCGGAGCGCAGCCAGCGTATTGTCAAACAAGCCATGCTGACCGAAGCCAAT
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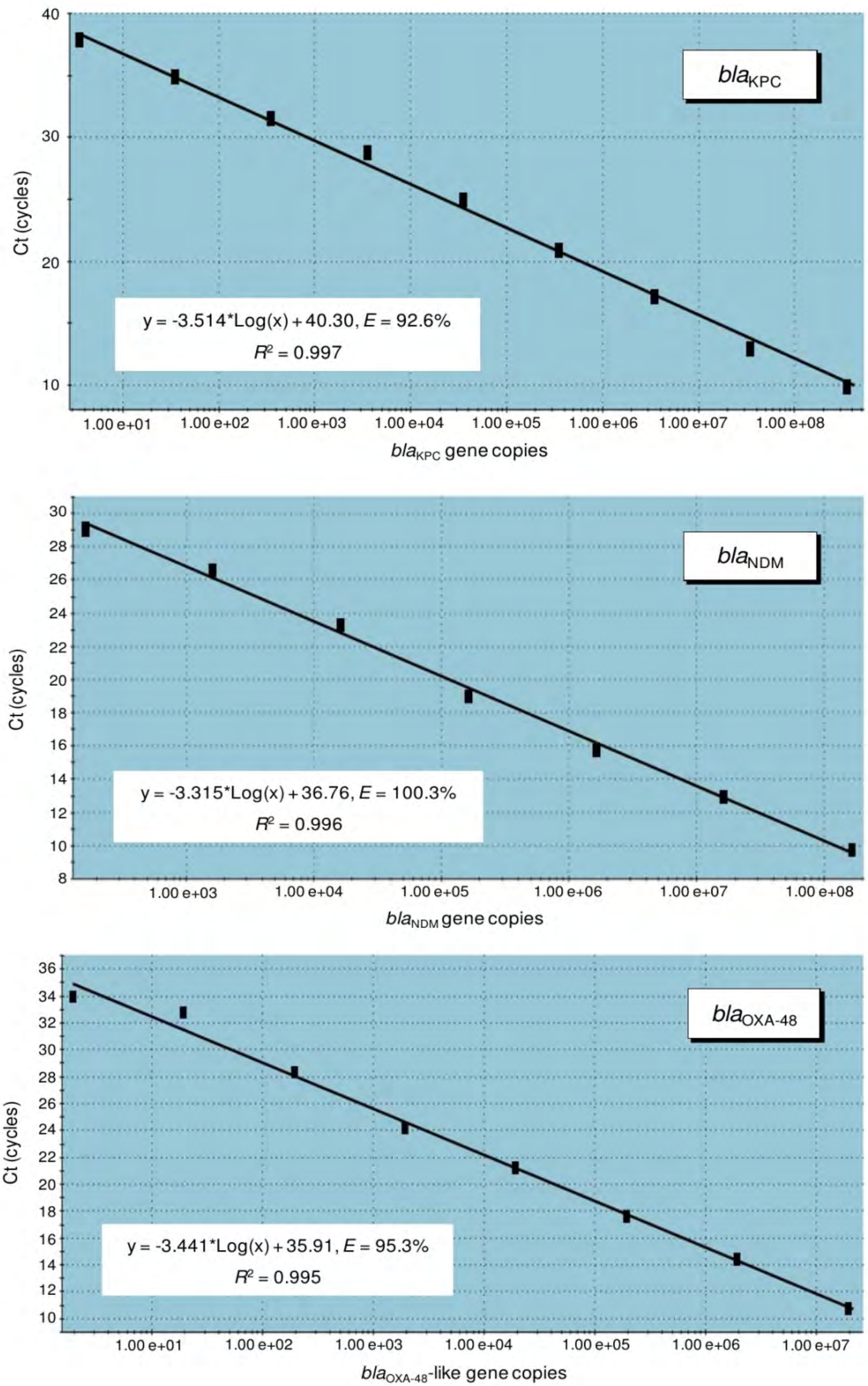
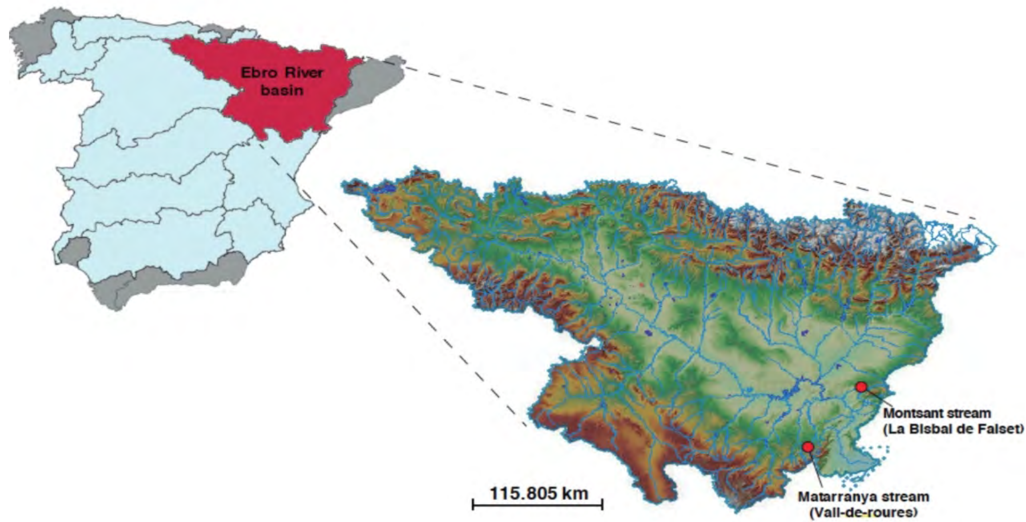
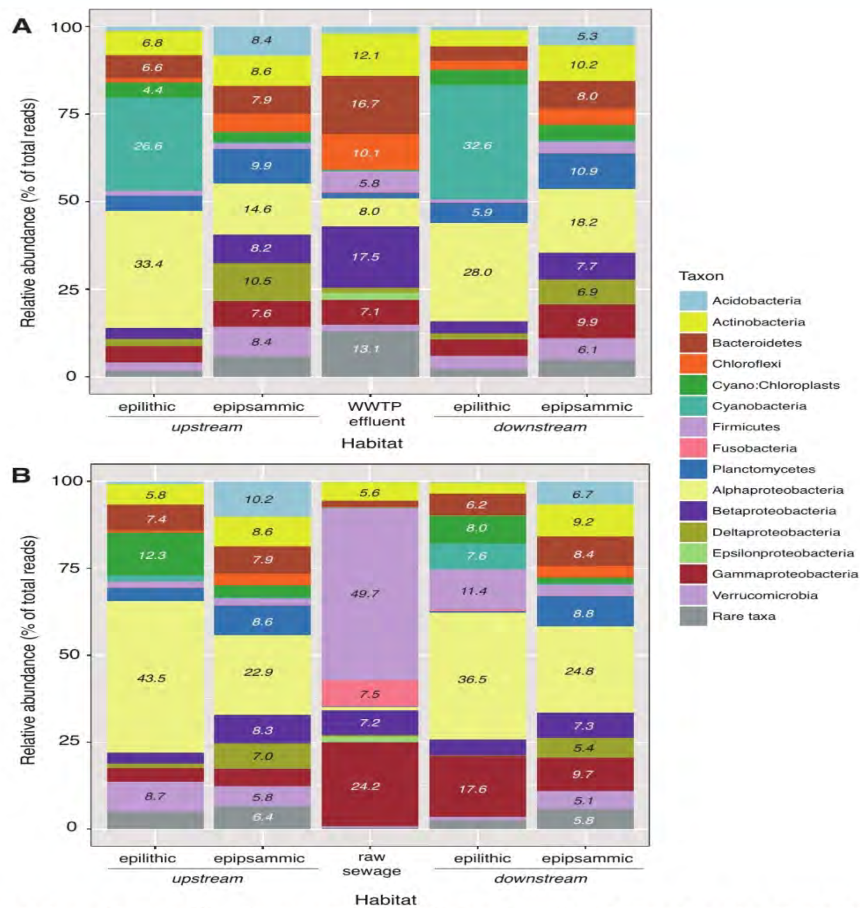


Supplementary Figure S1. Standard curves for bla_{KPC}, bla_{NDM}, and bla_{OXA-48}-like genes by plotting initial quantities of tenfold serial dilutions of plasmid DNA against the observed *Ct* value. The equations for the regression line and correlation coefficient (R^2) are also shown.

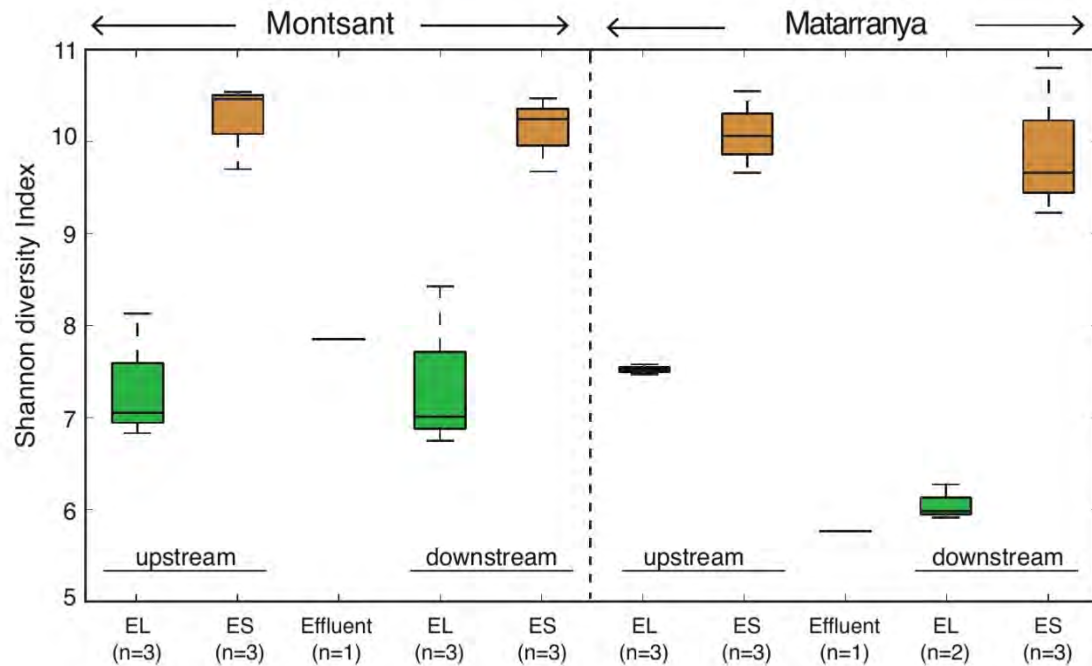
Chapter 5



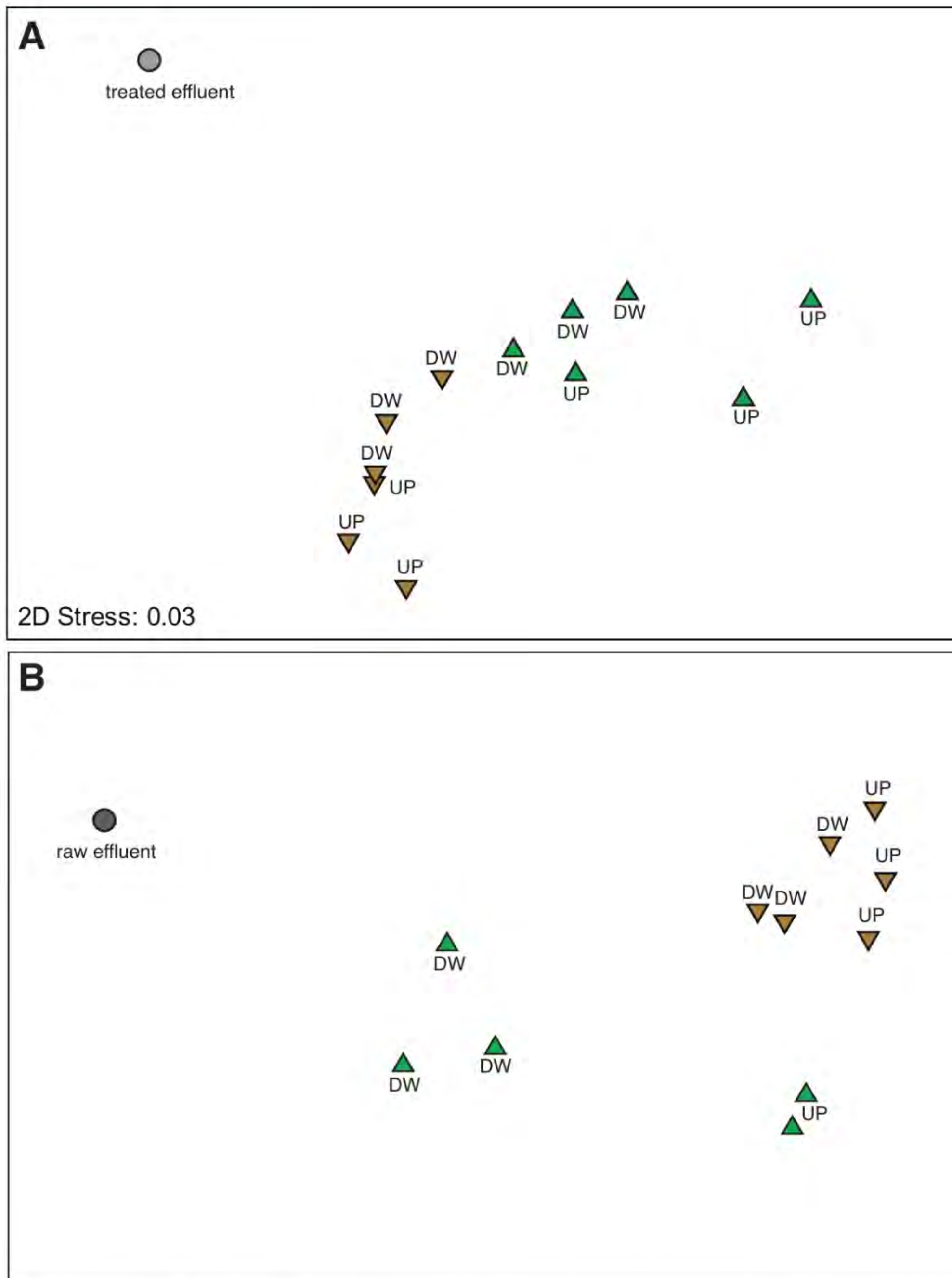
Supplementary Figure S1. Location of the studied streams (source: http://www.chebro.es).



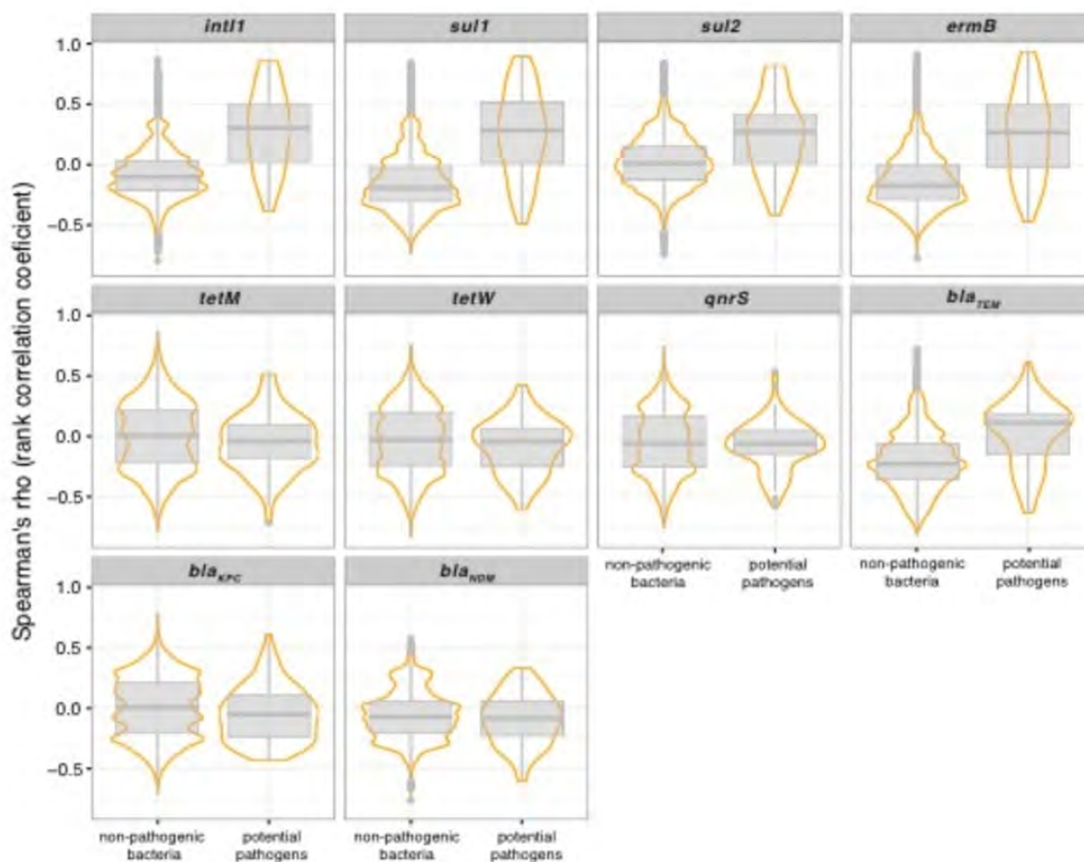
Supplementary Figure S2. Composition (at Phylum level and Class for the Proteobacteria) of effluent and biofilm bacterial communities in (A) the Montsant and (B) the Matarranya streams. Values are the mean of triplicate measurements (except for effluents where N=1) after normalizing to the same number of reads per sample (40.000). Values inside bars indicate the relative abundance of the corresponding taxa (to improve clarity only values >5% of total reads are shown).



Supplementary Figure S3. Shannon diversity index calculated for effluents, epilithic (green) and epipsammic (brown) bacterial communities at each collection site in Montsant (left panel) and Matarranya (right panel) streams. The lower and upper edge of each boxplot are the first and third quartiles, the red line shows the median and the whiskers extend from the 10th to the 90th percentile. The number of replicas (n) is also indicated. Calculations were carried out in QIIME after rarefying the number of sequences per sample to 40.000 (see Supplementary Methods for details).



Supplementary Figure S4. nMDS ordination of samples according to the relative abundance of different bacterial taxa (Bray-Curtis dissimilarity distance). (A) Montsant stream; (B) Matarranya stream. Epilithic biofilms (green), epipsammic biofilms (brown), UP: upstream samples; DW: downstream samples.



Supplementary Figure S5. Distribution of Spearman's r coefficients for correlation analysis between the relative abundance of the measured ARGs and OTUs identified as potential pathogens and those non-ascribed to pathogens (see main text for details). Violin plots show the probability density of the data (i.e. correlation coefficients, orange line). Boxplots showing the summary statistics for the obtained values are also shown in gray. The lower and upper edge of each boxplot are the first and third quartiles, the mid line shows the median, the whiskers extend from the hinges to the minimal and maximal values that are within 1.5x inter-quartile range and circles represent the outliers. The number of OTUs in each category was 16.482 and 81 for non-pathogenic and putative pathogens, respectively.

Preparation of standard curves for qPCR

Each standard curve was generated by cloning a fragment of the target gene previously amplified by conventional PCR and purified using the QIAquick®PCR Purification Kit (Qiagen Inc., Valencia, CA). Purified amplicons of each target gene were then transformed into *E. coli* using pCR2.1-TOPO vector system (Invitrogen, Carlbad, CA, USA) following manufacturer's instructions (Invitrogen). Plasmids containing the target insert were extracted using the PureLink Plasmid kit (Invitrogen) and their concentrations were then quantified using Qubit 2.0 (Life Technologies; Carlsbad, CA, USA). Gene copy numbers for each standard curve were calculated as previously described (Ritalahti *et al.* 2006) and three replicates of each dilution were added to each reaction. All qPCR assays were performed using SYBR green detection chemistry on a MX3005 system (Agilent Technologies; Santa Clara, CA, USA), as previously described (Marti *et al.* 2013) . Briefly, the qPCR reactions were assembled in a final volume of 30 μL containing either SYBR®Green Master Mix (Applied Biosystems) for bla_{TEM} or 2x Brilliant III UltraFast QPCR Master Mix (Stratagene, La Jolla, CA, USA) for the rest of the genes analyzed, 200 nM each forward and reverse primer and 10 ng of the DNA template. The final volume was adjusted to 30 μL by adding DNase-RNase-free water. Cycling conditions and primer sets for each gene are provided in Supplementary Table S1. All real-time qPCR analysis followed the MIQE guidelines (Bustin *et al.* 2009) and all essential information has been included in this section.

High-throughput sequencing and sequence processing

High-throughput multiplexed 16S rRNA gene sequencing with the Illumina MiSeq System (2×250 PE) was carried out using primer pair 515f/806r (Caporaso *et al.* 2011) targeting the V4 region of the 16S rRNA gene complemented with Illumina adapters and sample-specific barcodes at the genomics core facilities and methods of the Research Technology Support Facility Michigan State University, USA (Kozich *et al.* 2013). Analysis yielded high sequencing quality (73% of reads averaged ≥ Q30 scores). Raw sequences were analyzed using default parameters in USearch v8.1 (Edgar 2010) and after merging and quality filtering we ended with a total of 2.2 million reads. De-novo and reference-based chimera checking, clustering into Operational Taxonomic Units

(OTU, 97% cutoff), identification of representative OTU sequences and construction of OTU table were also carried out in UPARSE. Singletons were removed to avoid inflation of diversity caused by spurious OTUs. The resulting OTU table was converted to Biological Observation Matrix (BIOM) format (McDonald *et al.* 2012) and then loaded and analyzed into QIIME (Caporaso *et al.* 2010a). Sequencing depth ranged between 41,000 and 109,000 sequences per sample. Representative sequences from each OTU were aligned to the Greengenes imputed core reference alignment (DeSantis et al., 2006) using PyNAST (Caporaso *et al.* 2010b). Taxonomical assignments for each OTU were done in QIIME using the BLAST method and the QIIME-formatted version of the SILVA 119 reference database (Quast *et al.* 2013). For community analysis, the number of sequences in each sample was normalized by randomly selecting a subset of 40,000 sequences from each sample to minimize bias due to different sequencing effort across samples. QIIME was also used to calculate α-diversity indicators of richness (Observed Species and Chao1), diversity (Shannon Index) and Phylogenetic Diversity (Faith 1992) and to calculate community similarity between sites and substrates (β-diversity) using UniFrac distances (Lozupone & Knight 2005). Significance of the difference in community composition between sample categories (*e.g.* site, biofilm type) was assessed using the Adonis method implemented in QIIME.

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Supplementary Table S1. Isotopically labeled internal standards assigned for quantification of antibiotics.

Therapeutic group	Analyte	Number	CAS number	Corresponding internal standard
Antibiotics (10)	Erythromycin	1	59319-72-1	Erythromycin-N.N$^{13}C_2$
	Clarithromycin	2	81103-11-9	Azithromycin-d3
	Ofloxacin	3	82419-36-1	Ofloxacin-d3
	Sulfamethoxazole	4	723-46-6	Sulfamethoxazole-d4
	Trimethoprim	5	738-70-5	Sulfamethoxazole-d4
	Metronidazole	6	443-48-1	Ronidazole-d3
	Metronidazole-OH[a]	7	4812-40-2	Ronidazole-d3
	Dimetridazole	8	551-92-8	Ronidazole-d3
	Ronidazole	9	7681-76-7	Ronidazole-d3
	Cefalexin	10	15686-71-2	Sulfamethoxazole-d4

Supplementary Table S2. Performance parameters of the method used for the quantification of antibiotic concentration in water samples. Recoveries (%), Relative Standard Deviation (RSD% for n=3), Limits of Detection (LOD, water ng L^{-1}), Limits of Quantification (LOQ, water ng L^{-1}).

Therapeutic group	Analyte	WATER			
		LOD (ng L^{-1})	LOQ (ng L^{-1})	Recovery (%) (n=3)	RSD (%) (n=3)
Antibiotics (10)	Erythromycin	5.4	18.0	117	2.5
	Clarithromycin	0.9	2.9	104	14.9
	Ofloxacin	4.3	14.3	43.4	11.8
	Sulfamethoxazole	0.4	1.4	43.6	2.7
	Trimethoprim	0.9	3.1	71.7	2.8
	Metronidazole	0.7	2.2	76.5	2.6
	Metronidazole-OH[a]	0.9	3.0	7.84	2.9
	Dimetridazole	5.6	18.7	96.8	5.2
	Ronidazole	1.8	6.0	69.2	16.1
	Cefalexin	1.4	4.6	22.7	5.4

[a]Metabolite

Supplementary Table S3. Information of qPCR primers and conditions used in the study.

Gene	Primers	Sequence (5'–3')	Product size (bp)	Conditions	Reference
16S rRNA	F1048	GTGSTGCAYGGYTGTCGTCA	146	95°C 3 min (1 cycle); 95°C 15 s and 60°C 1 min (35 cycles)	[1]
	R1194	ACGTCRTCCMCACCTTCCTC			
Intl1	intl1-LC1	GCCTTGATGTTACCCGAGAG	196	95°C 3 min (1 cycle); 95°C 30 s and 60°C 1 min (40 cycles)	[2]
	Intl1-LC5	GATCGGTCGAATGCGTGT			
vanA	vanAF	CATGGCAAGTCAGGTGAAGA	187	95°C 3 min (1 cycle); 95°C 15 s and 58°C 30 s (40 cycles)	This study
	vanAR	CCACCGGCCTATCATCTTT			
qnrS	qnrSf-RT	ATGCAAGTTTCCAACAATGC	240	95°C 3 min (1 cycle); 95°C 15 s and 62°C 20 s (40 cycles)	[3]
	qnrSr-RT	CTATCCAGCGATTTTCAAACA			
bla_{TEM}	bla-TEM, FX	GCKGCCAACTTACTTCTGACAACG	247	95°C 3 min (1 cycle); 95°C 15 s and 60°C 20 s (40 cycles)	[4]
	bla-TEM, RX	CTTTATCCGCCTCCATCCAGTCTA			
bla_{CTX_M}	RTCTXM-F	CTATGGCACCACCAACGATA	103	95°C 3 min (1 cycle); 95°C 15 s and 60°C 20 s (40 cycles)	Modified from Kim *et al.* [5]
	RTCTXM-R	ACGGCTTTCTGCCTTAGGTT			
bla_{KPC}	KPC-rtF	CAGCTCATTCAAGGGCTTTC	196	95°C 3 min (1 cycle); 95°C 30 s and 60°C 45 s (40 cycles)	[6]
	KPC-rtF	GGCGGCGTTATCACTGTATT			
bla_{NDM}	Ndm-rtF	GATTGCGACTTATGCCAATG	189	95°C 3 min (1 cycle); 95°C 30 s and 60°C 60 s (40 cycles)	[6]
	Ndm-rtF	TCGATCCCAACGGTGATATT			
bla_{OXA-48}	Oxa-rtF	AGGCACGTATGAGCAAGATG	189	95°C 3 min (1 cycle); 95°C 30 s and 60°C 45 s (40 cycles)	[6]
	Oxa-rtR	TGGCTTGTTTGACAATACGC			
sul1	Sul(I)-FW	CGCACCGGAAACATCGCTGCAC	162	95°C 3 min (1 cycle); 95°C 15 s and 65°C 20 s (40 cycles)	[7]
	Sul(I)-RV	TGAAGTTCCGCCGCAAGGCTCG			
sul2	Sul(II)-FW	TCCGGTGGAGGCCGGTATCTGG	190	95°C 3 min (1 cycle); 95°C 15 s and 58°C 20 s (40 cycles)	[7]
	Sul(II)-FW	TCCGGTGGAGGCCGGTATCTGG			
tet(M)	tetMF	GCTCATGTTGATGCAGGAAA	204	95°C 3 min (1 cycle); 95°C 15 s and 60°C 30 s (40 cycles)	This study
	tetMR	TCCTGGCGTGTCTATGATGT			
tet(W)	tet(W)-FW	GAGAGCCTGCTATATGCCAGC	168	95°C 3 min (1 cycle); 95°C 15 s and 60°C 20 s (40 cycles)	[8]
	tet(W)-RV	GGGCGTATCCACAATGTTAAC			
erm(B)	erm(B)-91f	GATACCGTTTACGAAATTGG	364	95°C 3 min (1 cycle); 95°C 15 s and 58°C 20 s (40 cycles)	[9]
	erm(B)-454r	GAATCGAGACTTGAGTGTGC			

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Supplementary Table S4. Physical, chemical and hydrological variables measured upstream (UP) and downstream (DW) the studied streams. T = Temperature, DO = Dissolved Oxygen.

Stream	Population Service	Site	Cond. (μS cm^{-1})	Discharge (L s^{-1})	Temp (ºC)	DO (mg L^{-1})	pH	N-NH_4 (mg N L^{-1})	P-PO_4 (mg P L^{-1})
Montsant	227	UP	427	0.79	14.4	9.23	7.94	0.005 ± 0.004	0.004 ± 0.002
		DW	430	0.77	14.3	9.51	8.04	0.023 ± 0.012	0.014 ± 0.006
Matarranya	2335	UP	551	0.97	15.7	10.12	8.18	0.003 ± 0.003	0.003 ± 0.001
		DW	556	1.09	12.9	10.30	8.07	0.412 ± 0.143	0.028 ± 0.006

Supplementary Table S5. Results from the two-way ANOVA on the effect of stream site (upstream/downstream) and streambed compartment (epilithic/epipsammic) on the abundance of target genes in the Montsant stream.

Gene	Factor	F	*p* value
*intI*1	Site	1.897	0.206
	Biofilm	5.032	0.055
	Site x Biofilm	0.578	0.469
sul1	Site	9.816	**0.018**
	Biofilm	0.016	0.904
	Site x Biofilm	3.769	0.088
sul2	Site	6.856	**0.031**
	Biofilm	4.991	0.056
	Site x Biofilm	1.225	0.301
*erm*B	Site	10.812	**0.011**
	Biofilm	0.017	0.900
	Site x Biofilm	0.160	0.699
*tet*M	Site	3.448	0.100
	Biofilm	1.129	0.319
	Site x Biofilm	0.105	0.754
*tet*W	Site	19.197	**0.037**
	Biofilm	16.296	0.120
	Site x Biofilm	0.199	0.667
bla_{TEM}	Site	2.396	0.160
	Biofilm	0.459	0.517
	Site x Biofilm	4.156	0.076
bla_{KPC}	Site	7.773	**0.024**
	Biofilm	31.301	**0.001**
	Site x Biofilm	3.765	0.088
bla_{NDM}*	Site	927.040	**0.001**
	Biofilm	0.659	0.440
	Site x Biofilm	6.143	**0.038**

*bla_{NDM} gene was below detection limit in upstream biofilms and the value of the detection limit (normalized by its corresponding 16S rRNA value) was used as reference to carried out the statistical analyses. Detection limit of bla_{NDM} (Epilithic biofilms) = $7.45 \times 10^{-6} \pm 6.27 \times 10^{-7}$, bla_{NDM} (Epipsammic biofilms) = $1.15 \times 10^{-5} \pm 2.45 \times 10^{-6}$.

Supplementary Table S5 (continued). Results from the two-way ANOVA on the effect of stream site (upstream/downstream) and streambed compartment (epilithic/epipsammic) on the abundance of target genes in the Matarranya stream.

Gene	Factor	F	*p* value
*intI*1	Site	1.944	**0.001**
	Biofilm	4.256	**0.005**
	Site x Biofilm	0.647	0.856
sul1	Site	7.638	**0.001**
	Biofilm	16.034	**0.001**
	Site x Biofilm	4.744	0.121
sul2	Site	89.407	**0.001**
	Biofilm	33.147	**0.027**
	Site x Biofilm	5.549	**0.522**
ermB	Site	32.082	**0.001**
	Biofilm	10.254	**0.001**
	Site x Biofilm	0.087	0.295
*tet*M	Site	12.189	**0.001**
	Biofilm	15.667	**0.001**
	Site x Biofilm	0.085	0.362
*tet*W	Site	19.197	**0.001**
	Biofilm	16.296	**0.001**
	Site x Biofilm	0.199	0.416
bla_{TEM}	Site	0.001	0.404
	Biofilm	30.644	**0.001**
	Site x Biofilm	0.285	0.832
bla_{KPC}	Site	0.046	0.475
	Biofilm	25.431	**0.001**
	Site x Biofilm	0.097	0.152
bla_{NDM}	Site	0.021	0.331
	Biofilm	18.801	**0.001**
	Site x Biofilm	0.283	0.135
*qnr*S*	Site	24.070	**0.001**
	Biofilm	9.050	**0.001**
	Site x Biofilm	23.565	**0.042**

**qnr*S gene was below detection limit in upstream epipsammic biofilms and the value of the detection limit (normalized by its corresponding 16S rRNA value) was used as reference to carried out the statistical analyses. Detection limit of *qnr*S (Epipsammic biofilms) =$1.25 \times 10^{-5} \pm 2.65 \times 10^{-6}$.

Supplementary Table 6. Top 10 OTUs contributing the most to differences between upstream (UP) and downstream (DW) biofilm communities in (A) epilithic and (B) epipsammic samples from the Montsant stream. n.d. Not detected.

(A) Epilithic biofilms

Phylum (or Class for *Proteobacteria*)	Family / Order	High Abund. UP	High Abund. DW	% difference	Rel. abund. in the WWTP effluent (%)*
Actinobacteria	*Norcadioidaceae*	X		1.88	0.17
	Pseudonocardiaceae	X		1.50	n.d.
Bacteroidetes	*Cytophagaceae*	X		0.84	1.33
	Sphingobacteraceae	X		2.53	0.005
Alphaproteobacteria	*Caulobacteraceae*	X		4.76	0.13
	Rhodobacteraceae		X	8.30	2.26
	Sphingomonadaceae	X		4.26	0.71
Deltaproteobacteria	*Polyangiaceae*	X		1.01	n.d.
Gammaproteobacteria	*Alteromonadaceae*		X	1.60	0.79
Verrucomicrobia	*Verrucomicrobiaceae*		X	2.79	0.19

(B) Epipsammic biofilms

Phylum (or Class for *Proteobacteria*)	Family / Order	High Abund. UP	High Abund. DW	% difference	Rel. abund. in the WWTP effluent (%)*
Acidobacteria	*Acidobacteria Subgroup6*	X		3.12	0.04
	Acidobacteria PeM15		X	1.22	2.29
Cytophaga	*Cytophagaceae*	X		2.04	1.32
Alphaproteobacteria	*Rhodobiaceae*	X		1.50	0.17
	Rhodobacteraceae		X	4.72	2.26
Betaproteobacteria	*B1-7BS*	X		1.39	0.02
	Nitrosomonadaceae	X		1.71	0.82
Gammaproteobacteria	*Xanthomonadaceae*	X		0.93	0.9
Spartobacteria	*Xiphinematobacte-raceae*		X	1.74	0.02
Verrucomicrobia	*Verrucomicrobiacea*	X		8.22	0.19

* Relative abundance (% of total reads) of OTUs affiliated to each taxon.

Supplementary Table 7. Top 10 OTUs contributing the most to differences between upstream (UP) and downstream (DW) biofilm communities in (A) epilithic and (B) epipsammic samples from the Matarranya stream. n.d. Not detected.

(A) Epilithic biofilms

Phylum (or Class for *Proteobacteria*)	Family / Order	High Abund. UP	High Abund. DW	% difference	Rel. abund. in raw sewage effluent (%)*
Alphaproteobacteria	*Erythrobacteraceae*	X		5.8	0.01
	Sphingomonadaceae	X		7.4	0.05
Bacteroidetes	*Sapropiraceae*	X		2.4	n.d.
Chlamydiae	*Parachlamydiaceae*	X		3.5	0.003
Gammaproteobacteria	*Moraxellaceae*		X	12.6	14.3
	Pseudomonadaceae		X	3.4	8.31
	Vibrionaceae		X	4.1	n.d.
Verrucomicrobia	*Verrucomicrobiaceae*	X		11.4	0.53
Firmicutes	*Carnobacteriaceae*		X	4.8	21
	Peptostreptococcaeae		X	2.05	10.3

(B) Epipsammic biofilms

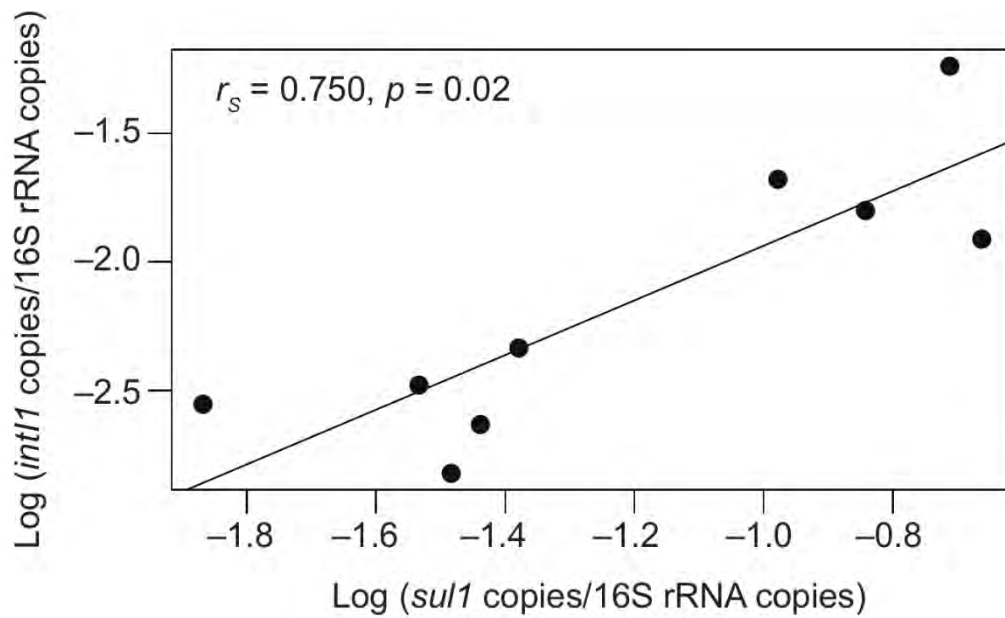
Phylum (or Class for *Proteobacteria*)	Family / Order	High Abund. UP	High Abund. DW	% difference	Rel. abund. in raw sewage effluent (%)*
Acidobacteria	*Acidobacteria Subgroup-6*	X		2.01	n.d.
Alphaproteobacteria	*Rhodobacteraceae*		X	6,1	0.63
Actinobacteria	*Acidimicrobiales Incerta Sedis*		X	0.78	0,006
Firmicutes	*Carnobacteriaceae*		X	0,5	21
	Clostridiaceae	X		0.14	2.1
	Peptostreptococcaeae		X	0.35	10.3
Gammaproteobacteria	*Moraxellaceae*		X	2.08	14.3
	Xanthomonadaceae		X	2.00	0.03
Planctomycetes	*Planctomycetaceae*		X	1.25	n.d.
Verrucomicrobia	*Verrucomicrobiaceae*	X		1.13	0.53

*Relative abundance (% of total reads) of OTUs affiliated to each taxon.

Table S8. Summary of results from a global BLAST analysis of the 35 retained OTUs tentatively identified as potential pathogens against a general nucleotide database. ID refers to the sequence identity of the OTU representative sequence against the first blast hit (FBH) based on percentage of matchings on a pairwise sequence alignment.

OTU_ID	Same genus of the FBH?	ID to FBH (%)	Taxonomic identity of the FBH (GenBank Acc.)	Position of the hit affiliated to the same genus (% ID)
OTU_3	False	100	*Trichococcus pasteurii* (NR036793.2)	12 out of 40 (99.2)
OTU_4	True	100	*Acinetobacter johnsonii* (NR117624)	1 (100)
OTU_11	True	100	*Pseudomonas psychrophila* (KX186965)	1 (100)
OTU_49	True	100	*Acidovorax defluvii* (NR026506)	1 (100)
OTU_53	True	100	*Arcobacter faecis* (KC551780)	1 (100)
OTU_64	True	100	*Staphylococcus equorum* (NR027520)	1 (100)
OTU_78	True	100	*Mycobacterium flavescens* (NR_115311)	1 (100)
OTU_92	True	100	*Aeromonas enterica* (LT630760)	1 (100)
OTU_94	False	99.2	*Pseudorhodoferax aquaterrae* (NR_108842)	3 out of 53 (98.8)
OTU_107	False	100	*Naasia aerilata* (NR_109606)	21 de100 (98.4)
OTU_120	True	100	*Pseudomonas stutzeri* (NR_103934)	1 (100)
OTU_146	False	100	*Intestinibacter bartlettii* (NR_027573)	2 out of 18 (98.4)
OTU_147	True	100	*Flavobacterium succinicans* (NR_118478)	1 (100)
OTU_193	True	100	*Clostridium perfringens* (KP326373)	1 (100)
OTU_215	True	100	*Yernisia pekkanenii* (KY606578)	1 (100)
OTU_222	True	100	*Flavobacterium aquatile* (NR_118482)	1 (100)
OTU_257	False	100	*Rhizobium azooxidifex* (NR_144599)	8 out of 51 (98.8)
OTU_267	True	100	*Streptococcus salivarius* (LC071828)	1 (100)
OTU_333	True	100	*Corynebacterium stationis* (CP009251)	1 (100)
OTU_387	True	100	*Citrobacter murliniae* (KY178281)	1 (100)
OTU_408	True	99.6	*Clostridium celatum* (AB971795)	1 (99.6)
OTU_447	False	100	*Massillia namucuonensis* (NR_118215)	18 out of 30 (98)
OTU_469	True	100	*Clostridium saccarobutylicum* (NR_122051)	1 (100)
OTU_480	True	100	*Ralstonia syzygii* (NR_134149)	1 (100)
OTU_509	True	100	*Streptococcus infantis* (LC096227)	1 (100)
OTU_575	True	100	*Escherichia coli* (KY655119)	1 (100)
OTU_578	True	100	*Pseudomonas fluorescens* (KX186944)	1 (100)
OTU_587	True	100	*Acinetobacter lwoffii* (NR_113346)	1 (100)
OTU_594	True	99.2	*Flavobacterium terrigena* (NR_044006)	1 (99.2)
OTU_1153	True	98.8	*Rickettsia hoogstraalii* (FJ767735)	1 (98.8)
OTU_1244	False	100	*Pseudoclavibacter terrae* (NR_145621)	29 out of 38 (98)
OTU_1678	True	100	*Enterococcus eurekensis* (NR_114649)	1 (100)
OTU_1759	True	98.4	*Acinetobacter brisouii* (NR_115871)	1 (98.4)
OTU_2033	True	99.6	*Mycobacterium colombiensis* (CP020821)	1 (99.6)
OTU_3094	True	99.2	*Flavobacterium* sp. (LN849948)	1 (99.2)

Chapter 6



Supplementary Figure S1: Spearman correlation between the relative abundance of *sul1* and *intI1* under emerging contaminants conditions at 14 days of the experiment. Spearman r and *p*-value are also indicated.

Supplementary Table S1. Information of qPCR primers and conditions used in the study.

Gene	Primers	Sequence (5'–3')	Conditions	Reference
16S rRNA	F1048	GTGSTGCAYGGYTGTCGTCA	95ºC 3 min (1 cycle); 95ºC 15 s and 60ºC 1 min (35 cycles)	[1]
	R1194	ACGTCRTCCMCACCTTCCTC		
Intl1	intl1-LC1	GCCTTGATGTTACCCGAGAG	95ºC 3 min (1 cycle); 95ºC 30 s and 60ºC 1 min (40 cycles)	[2]
	Intl1-LC5	GATCGGTCGAATGCGTGT		
qnrS	qnrSf-RT	ATGCAAGTTTCCAACAATGC	95ºC 3 min (1 cycle); 95ºC 15 s and 62ºC 20 s (40 cycles)	[3]
	qnrSr-RT	CTATCCAGCGATTTTCAAACA		
qnrA	qnrAf-RT	ATTTCTCACGCCAGGATTTG	95ºC 3 min (1 cycle); 95ºC 15 s and 64ºC 20 s (40 cycles)	Modified from Robicsek *et al*. [4]
	qnrAr-RT	GCAGATCGGCATAGCTGAAG		
qnrB	qnrBmF	GGMATHGAAATTCGCCACTG	95ºC 3 min (1 cycle); 95ºC 15 s and 60ºC 20 s (40 cycles)	Modified from Cattoir *et al*. [5]
	qnrBmR	TTYGCBGYYCGCCAGTCGAA		
*sul*1	Sul(I)-FW	CGCACCGGAAACATCGCTGCAC	95ºC 3 min (1 cycle); 95ºC 15 s and 65ºC 20 s (40 cycles)	[6]
	Sul(I)-RV	TGAAGTTCCGCCGCAAGGCTCG		
*sul*2	Sul(II)-FW	TCCGGTGGAGGCCGGTATCTGG	95ºC 3 min (1 cycle); 95ºC 15 s and 58ºC 20 s (40 cycles)	[6]
	Sul(II)-RV	CGGGAATGCCATCTGCCTTGAG		
erm(B)	erm(B)-91f	GATACCGTTTACGAAATTGG	95ºC 3 min (1 cycle); 95ºC 15 s and 58ºC 20 s (40 cycles)	[7]
	erm(B)-454r	GAATCGAGACTTGAGTGTGC		

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Supplementary Table S2. Top 10 OTUs contributing the most to differences between biofilms collected at -1 and 28 day of experiment (A) and those collected in treatments with and without emerging contaminants (B).

A)

Phylum (or Class for *Proteobacteria*)	Family / Order	Higher abundance		% difference
		Day -1	Day 28	
Firmicutes	*Alicyclobacillaceae*	X		3.05
	Bacillaceae	X		24.7
	Paenibacillaceae	X		6.74
	Planococcaceae	X		4.02
Alphaproteobacteria	*Bradyrhizobiaceae*		X	3.42
	Acetobacteraceae		X	1.36
Betaproteobacteria	*Oxalobacteraceae*	X		4.76
Gammaproteobacteria	*NKB5*		X	1.58
	Litoricolaceae		X	1.08
Cyanobacteria	*Subsection III*		X	21.1

B)

Phylum (or Class for *Proteobacteria*)	Family / Order/Class	Higher abundance		% difference
		With Emerging contaminants	Without emerging contaminants	
Firmicutes	*Bacillaceae*	X		0.56
	Paenibacillaceae	X		1.49
Gammaproteobacteria	*NKB5*		X	1.56
	Litoricolaceae	X		0.98
Planctomycetes	*OM190*		X	1.49
Bacteroidetes	*Chitinophagaceae*	X		0.74
	Cytophagaceae		X	1.58
TM6	*Uncultured sediment bacterium*		X	0.78
Cyanobacteria	*Subsection III*		X	7.10