Universitat de Girona

OCCURRENCE OF ANTIBIOTIC RESISTANCE GENES IN AQUATIC MICROBIAL COMMUNITIES EXPOSED TO ANTHROPOGENIC ACTIVITIES

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PhD THESIS

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ELISABET MARTI SERRANO

2013

EXPERIMENTAL SCIENCES AND SUSTAINABILITY PhD PROGRAMME

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

El Dr. José Luis Balcázar Rojas, investigador del grup de Qualitat i Diversitat Microbiana de l'Àrea de Qualitat de l'Aigua de l'Institut Català de Recerca de l'Aigua, i el Dr. Joan Jofre Torroella, catedràtic de Microbiologia de la Facultat de Biologia de la Universitat de Barcelona.

CERTIFIQUEN:

Que el treball titulat "Occurrence of antibiotic resistance genes in aquatic microbial communities exposed to anthropogenic activities" que presenta l'Elisabet Marti Serrano per a l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció i que compleix els requeriments per poder optar a la Menció Internacional.

Dr. José Luis Balcázar Rojas

Dr. Joan Jofre Torroella

El doctorand
Elisabet Marti Serrano

Girona, novembre 2013

Als meus pares

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“Sólo si nos detenemos a pensar en las pequeñas cosas
llegaremos a comprender las grandes.”

José Saramago

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CONTENTS

SUMMARY

Antibiotic resistance is considered a natural phenomenon since a lot of microorganisms have intrinsic genes that encode resistance to the antibiotics that they produce themselves. However, an increasing number of studies have supported the idea that the overuse and misuse of antimicrobial agents has led to the selection of drug-resistant strains. Besides that, due to the anthropogenic pollution, the natural environment has become a reactor where bacteria from different origins, antibiotics, disinfectants and heavy metals are mixed, contributing to the evolution and spread of antibiotic resistance the environment. Therefore, although most of the research about antibiotic resistance has focused on resistance in clinically relevant human pathogens, it is currently well-known that environmental bacteria play an important role in the emergence and spread of antibiotic resistance genes (ARGs). In this context, the present thesis aimed to investigate the occurrence of ARGs in aquatic microbial communities influenced by anthropogenic activities. Accordingly, several experiments were carried out as follows.

First of all, we designed three qPCR assays to quantify the plasmid-mediated quinolone resistance (PMQR) determinants such as *qnrA*, *qnrB* and *qnrS*. Once these assays were validated in different environmental samples, we optimized existing qPCR assays to quantify ARGs conferring resistance to several antibiotic families. All these qPCR assays were employed to determine the prevalence of ARGs from bacterial communities from biofilms and sediments in a wastewater treatment plant (WWTP) discharge point and the receiving river. Moreover, we analysed our samples by high-throughput DNA sequencing to assess the shifts in the structure of bacterial communities.

Since recent investigations have been focused on the role of phages as mobile genetic elements, another study was conducted to evaluate the contribution of phages as a reservoir of ARGs in the environment. In this regard, ARGs were analysed in phage DNA fraction of water from two different WWTPs and final effluents from two hospitals.

Given that the increasing use of fluoroquinolones, particularly in our country, we developed an experiment to isolate and identify ciprofloxacin-resistant strains from the WWTP discharge point and the receiving river. We also examine the prevalence of PMQR determinants [*qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr*] in ciprofloxacin-resistant isolates and their association with extended-spectrum β-lactamases (ESBLs) such as bla_{TEM}, bla_{SHV} and bla_{CTX-M}. Ultimately, since *qnrS* were mostly found in *Aeromonas* spp. in the previous experiment, particularly the *qnrS2* allele, we further characterized a multidrug resistance-encoding plasmid from an *Aeromonas* sp. to gain a deep insight on the role of this species as a reservoir of ARGs.

In conclusion, ARGs were detected in many environments including different matrices (water, biofilm and sediment), different organisms (bacteriophages and bacteria) and different sources (rivers, effluents from several human and veterinary hospitals, subterranean water, chicken faeces and WWTP effluents), indicating that these emerging pollutants are widely distributed in the environments exposed to anthropogenic activities. Overall, the results obtained in this thesis represent one of the first approaches of ARG occurrence in our area since most of the studies about antibiotic resistance are still focused on clinical settings.

RESUM

La resistència als antibiòtics es considerada com un fenomen natural ja que molts microorganismes posseeixen gens intrínsecs que codifiquen resistències als antibiòtics que ells mateixos produeixen. Tot i això, cada vegada hi ha més estudis que defensen la idea que l'ús excessiu i el mal ús dels antibiòtics ha derivat amb la selecció de soques resistents. A més a més, degut a la contaminació antropogènica, el medi ambient ha esdevingut un reactor on es barregen bacteris de diferents orígens, antibiòtics, desinfectants i metalls pesants, contribuint així a l'evolució i disseminació de la resistència als antibiòtics a l'ambient. Així doncs, tot i que la majoria d'investigacions en aquest camp s'han enfocat als patògens humans d'importància clínica, actualment ja es reconeix que els bacteris ambientals poden jugar un paper important en l'aparició i disseminació de gens de resistència a antibiòtics. En aquest context, l'objectiu d'aquesta tesi és investigar la prevalença de gens de resistència a antibiòtics en comunitats microbianes aquàtiques influenciades per activitats antropogèniques. Per complir aquest objectiu, es van dur a terme els següents experiments.

En primer lloc, es van dissenyar tres assajos de qPCR per quantificar determinants de resistència a quinolones transmesos per plàsmids (PMQR, de l'anglès *plasmid-mediated quinolone resistance*), particularment els gens *qnrA*, *qnrB* i *qnrS*. Un cop aquests assajos van ser validats en mostres ambientals de diferent procedència, es va procedir a optimitzar qPCRs ja existents per quantificar gens de resistència a altres famílies d'antibiòtics. Tots els assajos de qPCR desenvolupats es van utilitzar per determinar la prevalença de gens de resistència a antibiòtics en les comunitats bacterianes de biofilms i sediments del punt de descarrega d'una estació depuradora d'aigües residuals i del riu receptor. A més a més, l'ADN d'aquestes comunitats bacterianes va ser analitzat mitjançant seqüenciació massiva per determinar els canvis en l'estructura de les comunitats.

Seguidament, degut a que estudis recents han remarcat el rol dels bacteriòfags com a elements genètics mòbils, es va fer un estudi per avaluar la capacitat d'aquests tipus de virus com a reservoris de gens de resistència a antibiòtics en el medi ambient.

En aquest sentit, els gens de resistència van ser determinats en la fracció d'ADN dels fags provinents de les aigües de diferents efluents de plantes depuradores i d'hospitals.

D'altra banda, degut a que l'ús de les fluoroquinolones ha augmentat en els últims temps, especialment al nostre país, es va desenvolupar un altre experiment per aïllar i identificar soques resistents a ciprofloxacina, una de les fluoroquinolones més utilitzades, en l'efluent d'una planta depuradora i en el riu receptor. També es va determinar la prevalença dels determinats PMQR, concretament els gens *qnrA*, *qnrB*, *qnrS* i *aac(6')-Ib-cr*, en les soques resistents a ciprofloxacina així com la seva associació amb β-lactamases d'ampli espectre tals com les codificades pels gens bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$. Finalment, com que el gen *qnrS* va ser majoritàriament identificat en *Aeromonas* spp., particularment la variant *qnrS2*, es va caracteritzar un plàsmid portador de diferents gens de resistència (entre ells el *qnrS2*) d'una *Aeromonas* sp.

En definitiva, els gens de resistència antibiòtics van ser detectats en molts tipus d'ambients, incloent diverses matrius (aigua, biofilm i sediment), diferents organismes (bacteriòfags i bacteris) i diferents fonts (rius, efluents d'hospitals, aigua subterrània, excrements de gallines i efluents de depuradora), indicant que aquests contaminants emergents es troben àmpliament distribuïts en ambients exposats a activitats humanes. En general, els resultats obtinguts en aquesta tesi representen una de les primeres aproximacions a la investigació de la prevalença de gens de resistència a antibiòtics en la nostra àrea, on molts dels estudis sobre aquest problema encara estan enfocats en ambients hospitalaris.

RESUMEN

La resistencia a los antibióticos esta considerada como un fenómeno natural ya que muchos microorganismos poseen genes que codifican resistencia a los antibióticos que ellos mismos producen. No obstante, cada vez más estudios respaldan la idea de que el uso excesivo y el mal uso de los antibióticos ha llevado a la selección de cepas resistentes. Además, debido a la contaminación antropogénica, el medio ambiente se ha convertido en un reactor dónde se mezclan bacterias con distintos orígenes, antibióticos, desinfectantes y metales pesados, contribuyendo así a la evolución y diseminación de la resistencia a antibióticos en el ambiente. Por lo tanto, aunque la mayoría de investigaciones sobre resistencia a antibióticos estén enfocadas en patógenos humanos de importancia clínica, actualmente está ya aceptado que las bacterias ambientales juegan un papel importante en la aparición i la diseminación de genes de resistencia a antibióticos. En este contexto, el objetivo de esta tesis fue investigar la prevalencia de genes de resistencia a antibióticos en comunidades microbianas acuáticas influenciadas por actividades antropogénicas. En consecuencia, se llevaron a cabo los estudios explicados a continuación.

En primer lugar, se diseñaron 3 ensayos de qPCR para cuantificar determinantes de resistencia a quinolonas mediados por plásmidos (PMQR, del inglés *plasmid-mediated quinolone resistance*), concretamente los genes *qnrA*, *qnrB* y *qnrS*. Una vez estos ensayos fueron validados en distintas muestras de procedencia ambiental, procedimos a optimizar ensayos de qPCR ya existentes para cuantificar genes que confieren resistencia a otras familias de antibióticos. Todos estos ensayos de qPCR fueron utilizados para determinar la prevalencia de genes de resistencia a antibióticos en las comunidades bacterianas de biofilms y sedimentos del punto de descarga de una estación depuradora de aguas residuales y del río receptor. Además, el ADN de estas comunidades bacterianas fue analizado por secuenciación masiva para determinar los cambios en la estructura de las comunidades.

Seguidamente, debido a que muchos estudios han resaltado el rol de los bacteriófagos como elementos genéticos móviles, se hizo un estudio para evaluar la capacidad de estos virus como reservorios de genes de resistencia a antibióticos en el medio ambiente. En este sentido, los genes de resistencia fueron determinados en la fracción de ADN de los fagos provinentes de las aguas de distintos efluentes de plantas depuradoras y hospitales.

Por otro lado, debido a que el uso de las fluoroquinolonas ha aumentado mucho en los últimos tiempos, especialmente en nuestro país, se desarrolló otro experimento para aislar i identificar cepas resistentes a ciprofloxacina, una de las fluoroquinolonas más utilizadas, en los efluentes de una planta depuradora y en el río receptor. También se determinó la prevalencia de los determinantes PMQR, concretamente los genes *qnrA*, *qnrB*, *qnrS* y *aac(6')-Ib-cr*, en las cepas resistentes a ciprofloxacina así como su asociación con las β-lactamases de amplio espectro tales como las codificadas por los genes bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$. Finalmente, como el gen *qnrS* fue mayoritariamente identificado en *Aeromonas* spp., en particular la variante *qnrS2*, se caracterizó un plàsmido portador de diferentes genes de resistencia (entre ellos el *qnrS2*) de una *Aeromonas* sp.

En definitiva, los genes de resistencia a antibióticos fueron detectados en muchos tipos de ambientes, incluyendo diferentes matrices (agua, biofilm y sedimento), diferentes organismos (bacteriófagos y bacterias) y distintas fuentes (ríos, efluentes de hospitales, agua subterránea, excrementos de gallinas y efluentes de depuradora), indicando que estos contaminantes emergentes se encuentran ampliamente distribuidos en ambientes expuestos a actividades humanas. En general, los resultados obtenidos en esta tesis representan una primera aproximación a el estudio de la prevalencia de genes de resistencia a antibióticos en nuestra área, dónde muchos de los estudios sobre este problema están aún enfocados en ambientes hospitalarios.

LIST OF PUBLICATIONS

List of publications in peer-reviewed journals derived from this doctoral thesis and included as chapters in the present thesis:

Marti E, Balcázar JL. 2012. Multidrug resistance-encoding plasmid from *Aeromonas* sp. strain P2G1. Clinical Microbiology and Infection 18: E366-368.
Impact factor: 4.58. 1st Quartile in Microbiology.

Marti E, Balcázar JL. 2013. Real-time PCR assays for quantification of *qnr* genes in environmental water samples and chicken feces. Applied and Environmental Microbiology 79: 1743-1745.
Impact factor: 3.68. 1st Quartile in Biotechnology and Applied Microbiology.

Marti E, Jofre J, Balzacar JL. 2013. Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a wastewater treatment plant. PloS ONE. 8: e78906.
Impact factor: 3.73. 1st Quartile in Multidisciplinary Sciences.

Marti E, Variatza E, Balzacar JL. 2013. Bacteriophages as a reservoir of extended-spectrum β-lactamase and fluoroquinolone resistance genes in the environment. Clinical Microbiology and Infection. Doi: 10.1111/1469-0691.12446
Impact factor: 4.58. 1st Quartile in Microbiology.

Marti E, Huerta B, Rodríguez-Mozaz S, Barceló D, Jofre J, Balzacar JL. 2013. Prevalence of plasmid-mediated quinolone resistance determinants and their association with extended-spectrum β-lactamases in ciprofloxacin-resistant strains isolated from a wastewater treatment plant and the receiving river. Submitted.

Additional publications related to this thesis:

Marti E, Monclús H, Jofre J, Rodriguez-Roda I, Comas J, Balcázar, JL. 2011. Removal of microbial indicators from municipal wastewater by a membrane bioreactor (MBR). Bioresource Technology 102: 5004-5009.
Impact factor: 4.75. 1st Quartile in Biotechnology and Applied Microbiology.

Huerta B, **Marti E**, Gros M, López P, Pompeo M, Armengol J, Barceló D, Balcazar JL, Rodríguez-Mozaz S, Marcé R. 2013. Exploring the links between antibiotic occurrence, antibiotic resistance and natural bacterial assemblages in water supply reservoirs. Science of the Total Environment 456-457: 161–170.
Impact factor: 3.26. 1st Quartile in Environmental Sciences.

Collado N, Buttiglieri G, **Marti E**, Ferrando-Climent L, Rodriguez-Mozaz S, Barceló D, Comas J, Rodriguez-Roda I. 2013. Effects on activated sludge bacterial community exposed to sulfamethoxazole. Chemosphere 93: 99-106.
Impact factor: 3.14. 1st Quartile in Environmental Sciences.

***Marti E**, Variatza E, Balzacar JL. 2013. The role of aquatic ecosystems as reservoirs of antibiotic resistance. Trends in Microbiology 22: 36-41.
Impact factor: 8.43. 1st Quartile in Microbiology.

***Marti E**, Balcázar JL. 2013. Antibiotic resistance in the aquatic environment. *In:* Analysis, Removal, Effects and Risk of Pharmaceuticals in the Water Cycle, 2nd Edition. Petrovic M., Barcelo D. and Pérez S. (Eds.). Elsevier, Amsterdam. pp. 671-684.

*Part of these publications has been included in the General Introduction.

LIST OF ABBREVIATIONS

ARB: antibiotic resistant bacteria

ARG: antibiotic resistance gene

DDD: defined daily dose

DNA: deoxyribonucleic acid

ECDC: European centre for disease prevention and control

ESBL: extended-spectrum beta-lactamases

HGT: horizontal gene transfer

IS: insertion sequence

IR: inverted repeats

MIC: minimum inhibitory concentrations

MGE: mobile genetic elements

NDM: New Delhi metallo-β-lactamase

ORF: open reading frames

PCR: polymerase chain reaction

PMQR: plasmid-encoded quinolone resistance

qPCR: quantitative PCR

RNA: ribonucleic acid

WWTP: wastewater treatment plant

WHO: World Health Organization

Chapter 1

General Introduction

1. What is antibiotic resistance?

Antibiotic resistance occurs when one microorganism is able to multiply in the presence of an antibiotic. This resistance is considered a natural phenomenon because many microorganisms have intrinsic genes that encode resistance to the antibiotics that they produce themselves. However, an increasing number of studies have supported the idea that the overuse and misuse of antimicrobial agents has led to the selection of drug-resistant microorganisms. In fact, the launch of nearly all new antibiotics has been accompanied by the subsequent appearance of resistant strains in clinical settings (Davies and Davies, 2010). As a consequence, the infectious agents responsible for illnesses such as acute respiratory infections, diarrhoeal diseases, measles, AIDS, malaria and tuberculosis, which account for more than 85% of the mortality from infection worldwide, are becoming resistant to first-line drugs and, in some instances, to second- and third-line treatments (WHO, 2001). Moreover, after the "golden age" of antibiotics, in the middle of the 20th century, the number of new licensed antibiotics has progressively declined due to the development of new agents is expensive and time-consuming, and the pharmaceutical industry has shifted its investment to the development of chronic disease therapies and lifestyle drugs that are more lucrative (Livermore, 2011). Furthermore, while developed countries can still afford the newest antibiotics to treat resistant pathogens, access to the latest generation drugs is often limited or totally absent in many developing countries. Antibiotic resistance is thus considered one of the main public health threats facing the world today and has led the World Health Organization (WHO) to establish a global strategy for the containment of antimicrobial resistance (WHO, 2001).

2. Mechanisms of antibiotic resistance among prokaryotes

The emergence of antibiotic resistance has revealed multiple and complex mechanisms by which the resistance trait arises and spreads among bacteria of the same species or even among different species. The most important mechanisms are as follows: (i) exclusion of the antibiotic by the cell membrane; (ii) modification and/or deactivation of the antibiotic; (iii) reduction in sensitivity of the cellular target; (iv)

extrusion from the cell; and (v) intracellular sequestration (Taylor et al., 2011). Sensitive bacteria may become resistant to antibiotics through mutation and further selection of the mutant or by acquiring genetic information that encodes resistance from a donor bacterial cell. The acquisition occurs through horizontal gene transfer (HGT; Figure 1), which is largely responsible for the spread of antibiotic resistance by various processes, such as conjugation, transduction and transformation (Bennett, 2008; Barlow, 2009).

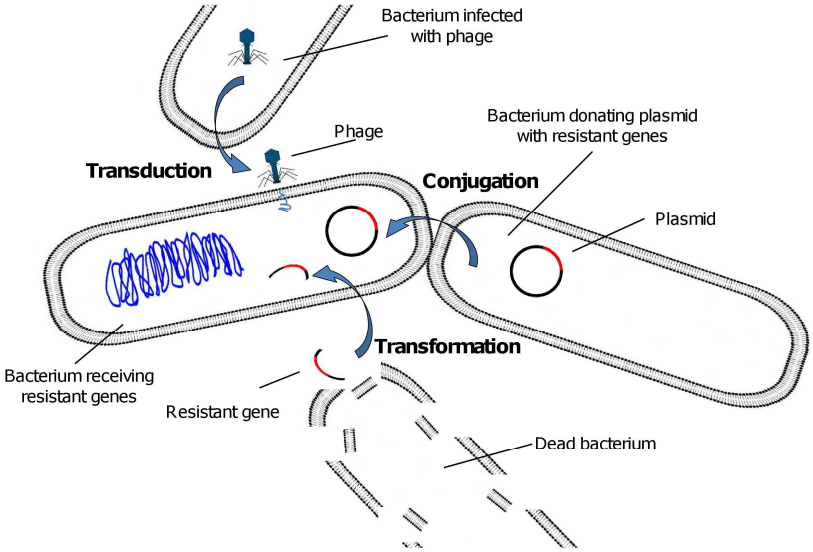

Figure 1. Mechanisms of HGT in bacteria. Exogenous nucleic acids can be obtained via cell-cell contact with other bacteria (conjugation), directly from the environment as naked DNA (transformation) or via phages (transduction). This figure has been adapted from Taylor et al., 2011.

Gene acquisition by HGT results from the successful transfer of genetic material followed by vertical inheritance of the transferred genetic material through succeeding generations. The presence of certain physical barriers to transfer, as well as the effect of different selective forces on the transferred genes, may explain the observed differences in the type of genes involved in HGT (Boto, 2010). Horizontal

transfer may be mediated by mobile genetic elements (MGEs), such as plasmids, transposons, integrons, insertion sequences and genomic islands, which are all involved in bacterial acquisition and the recombination of foreign DNA. Bacteriophages have also been considered as MGEs because they play a crucial role in mobilising genetic elements (Jackson et al., 2011). Table 1 summarises the main characteristics of MGEs.

2.1. Plasmids

Plasmids are extra-chromosomal, circular DNA molecules that replicate independently of chromosomal DNA. They are known to carry a variety of genes, including those that confer antibiotic resistance and those that provide virulence determinants and enhance the cell's capacity to repair DNA damage (Bennett, 2008). Plasmid-borne genes determine the probability of plasmid transfer from a donor to a recipient strain, the mechanism of such transfer (self-transmissible or mobilisable) and the host range for autonomous plasmid replication (Hoffmann et al., 1998).

Several studies have shown that genes encoding for extended-spectrum β-lactamases (ESBL) may be carried on plasmids (Yan et al., 2001; Boyd et al., 2004). ESBL are a group of enzymes that confer resistance to penicillins, cephalosporins, monobactams and oxyimino-cephalosporins. Since their first description in the early 1980s, more than 400 different ESBL genes have been identified (http://www.lahey.org/studies), with most belonging to the SHV, TEM and CTX-M families. Although ESBL have been mostly detected in members of the Enterobacteriaceae family, they have also been found in other bacterial genera, such as *Acinetobacter*, *Aeromonas* and *Pseudomonas*. For example, ESBL have been reported to occur in *Aeromonas* species, which are common inhabitants of aquatic environments, with $bla_{\text{PER-1}}$ identified in *Aeromonas media* from Switzerland (Picão et al., 2008a) and $bla_{\text{VEB-1a}}$, $bla_{\text{SHV-12}}$, $bla_{\text{PER-1}}$, $bla_{\text{PER-6}}$, $bla_{\text{TLA-2}}$ and $bla_{\text{GES-7}}$ in *Aeromonas* strains from the Seine River (Girlich et al., 2011). These results suggest that *Aeromonas* species may potentially act as reservoirs of antibiotic resistance genes (ARGs).

Genes encoding quinolone resistance may be also present in plasmids. The *qnrS2* gene has been identified in a mobilisable IncQ-related plasmid that was isolated from a bacterial community in the activated sludge of a wastewater treatment plant in Germany (Bönemann et al., 2006). In addition to *qnr* genes, the *aac(6')-lb-cr* gene has also been identified in plasmids from aquatic species. Picão and colleagues detected two plasmid-encoded quinolone resistance (PMQR) determinants, *qnrS2* and *aac(6')-lb-cr*, along with four different antimicrobial resistance markers, on a single plasmid from *Aeromonas allosaccharophila* (Picão et al., 2008b). Some investigations suggest that the *qnr* genes could have originated in the chromosomes of environmental organisms and then entered into wider circulation on plasmids due to intensive quinolone pressure (Robicsek et al., 2006).

2.2. Genomic islands

Genomic islands are a collection of large, potentially mobile regions of DNA that are acquired via HGT and then contribute to rapid bacterial evolution and adaptation (Jackson et al., 2011). They encode complex biological functions, including functions involved in metabolism, antibiotic resistance, pathogenesis and symbiosis (Hacker and Kaper, 2000). In general, genomic islands have a different G+C content and codon usage from the rest of the genome and are usually flanked by direct repeat sequences and tRNA genes (Gal-Mor and Finlay, 2006). They often harbour functional or cryptic genes encoding factors involved in genetic mobility, such as integrases, transposases, insertion sequences, bacteriophage genes and origin of replication sites (Hacker and Kaper, 2000; Gal-Mor and Finlay, 2006). One of the first genomic islands containing an antibiotic resistance gene cluster was identified in *Salmonella enterica* and was thus named *Salmonella* genomic island 1 (SGI1). Subsequently, an SGI1 antibiotic-resistance gene cluster with evidence of two integron structures was identified in an isolate of *Salmonella enterica* serotype Paratyphi B from a tropical fish in Singapore (Meunier et al., 2002). The first integron carried the *aadA2* gene, which confers resistance to streptomycin and spectinomycin, and a truncated *sul1* resistance gene, and the second integron contained the β-lactamase gene bla_{PSE-1} and a complete *sul1* gene. Moreover, other authors identified a variant SGI1 antibiotic-resistance gene

cluster in a multidrug-resistant strain of *Salmonella enterica* serovar Albany isolated from food fish in Thailand (Doublet et al., 2003).

2.3. Insertion sequences and transposons

Insertion sequences (ISs) are the smallest (between 0.7 and 3 kb) and simplest autonomous MGEs that encode no functions other than those involved in their mobility (Siguier et al., 2006). Most ISs possess one or two open reading frames (ORF) that encode a transposase surrounded by linker regions that frequently end with short, imperfect inverted repeats (IR) ranging from 7 to 20 bp in length. As a result of their insertion, ISs generate a small duplication of the target DNA flanking the point of insertion that are called directed repeats. Although ISs do not harbour any genes other than one encoding a tranposase, after their insertion in a genome, they can interrupt the coding region of a gene or disrupt promoter regions and alter gene expression (Robinson et al., 2012).

Transposons are similar to ISs, but they possess one or more genes that confer a phenotype on the bacterial host, such as resistance to a specific antibiotic (Schaefer and Kahn, 1998). There are different types of transposons, which can be distinguished by structure, genetic relatedness and mechanism of transposition (Bennett, 2008). Most bacterial transposons correspond to the composite or non-composite forms. Composite transposons have two IS elements of the same type bracketing one or more genes, whereas in non-composite forms, the carrying genes are flanked with repeat sequences other than the IS elements (Schaefer and Kahn, 1998). In the environment, the $bla_{\text{PER-1}}$ gene was identified as part of a Tn*1213* composite transposon in an *Aeromonas* media isolate from Switzerland (Picão et al., 2008a). The truncated or complete transposon Tn*1721*, which contains the *tetA* gene that confers resistance to tetracycline, was detected in plasmids of *Aeromonas salmonicida* isolated from different geographical locations (Rhodes et al., 2000; Sørum et al., 2003).

Some transposons contribute to the spread of ARGs as part of class 1 integrons. These transposons include the Tn*3* family, the Tn*5053* family and Tn*402*-like transposons. Transposons belonging to the Tn*3* family have been found in a

diverse range of Gram-negative and Gram-positive bacteria. For instance, the Tn*21*-like transposon, which belongs to a subgroup of the Tn*3* family, has been detected in a multidrug-resistant *Aeromonas hydrophila* isolate that showed a macrolide inactivation gene cluster, *mphA-mrx-mphR*, adjacent to a class 1 integron (Poole et al., 2006). Another investigation demonstrated the presence of $bla_{\text{VIM-13}}$ associated with a Tn*1721*-class 1 integron structure in several metallo-β-lactamase-producing isolates from hospital sewage (Scotta et al., 2011).

2.4. Integrons

Integrons are genetic systems that allow bacteria to capture and express gene cassettes and can be found as part of plasmids, chromosomes and transposons. Integrons are composed of an *intI* gene that encodes an integrase that acts as a site-specific recombinase, an attachment site (*attI*) and one or two strong promoters that drive the expression of an inserted gene cassette (Moura et al., 2012). Gene cassettes are small mobile elements consisting of little more than a single promoter-less gene and a recombination site (Partridge et al., 2009). Gene cassettes can be inserted one after another into the integron insertion site (Bennett, 2008), producing long arrangements of ARGs that can then be transferred simultaneously between bacterial populations (Martinez, 2009). These MGEs can usually be found in clinical bacterial strains, possibly because most of the cassettes identified are associated with antibiotic resistance. In recent years, however, several studies have been conducted to determine the occurrence of integrons in bacteria from aquatic environments.

There are three main classes of integron structures, depending on their integrase, but most resistance integrons conform to a structure known as a class 1 integron (Bennett, 2008). Moura and co-authors detected genes encoding integrases belonging to class 1 and 2 integrons among Enterobacteriaceae and *Aeromonas* spp. in influents and effluents of a WWTP (Moura et al., 2012). These integrons harboured different gene cassettes conferring resistance to penicillins, fluoroquinolones and chloramphenicol, among others. Another recent investigation demonstrated that suspended aggregates of bacteria in natural aquatic systems (so-called flocs) contained class 1 integrons carrying clinically important ARGs (Drudge et al., 2012). All of these

studies showed that integrons make an important contribution to the dissemination of ARGs.

2.5. Bacteriophages

Transfer of ARGs between bacteria through MGEs has been widely studied and demonstrated. However, it took several decades to understand the contribution of bacteriophages in the transfer of genetic material. Bacteriophages, also known as phages, are viruses that infect prokaryotic cells (bacteria and archaea) and multiply in their cytoplasm, using the host's cellular machinery. Bacteriophages present two different life cycles, the lytic cycle and the lysogenic cycle. In the lytic cycle, the phage infects the host cell and immediately uses the host's cellular machinery to replicate and release newly produced viruses. These types of phages are known as virulent bacteriophages. In the lysogenic cycle, the genome of the phage can integrate into bacterial genome (where it is also called prophage) and remain latent until events such as ultraviolet radiation, nutrient deficiencies or some chemical compounds, such as quinolones, induce the lytic pathway (Muniesa et al., 2013). These types of phages are known as temperate bacteriophages.

One consequence of phage replication is bacterial DNA transfer from one bacterium to another, a process known as transduction. There are two different types of transduction: generalised and specialised. Generalised transduction is carried out by either temperate or virulent phages and occurs when genome fragments of the donor bacterial host cell are erroneously packaged into new phage particles, which are capable of infecting a recipient bacterial cell. This new host will incorporate the donor's DNA by genetic recombination. In contrast, specialised transduction is restricted to temperate phages that erroneously excise their genomes from the host chromosome, producing viral particles that carry hybrid genomes containing viral and bacterial genes. Because prophages contain sequence-specific integration sites, the number of genes that can be transferred is limited to those flanking the integration site; nevertheless, most temperate phages move frequently, thus increasing their transfer efficiency (Muniesa et al., 2011).

Diverse experiments have confirmed the in vitro transduction of antibiotic resistance determinants in *Pseudomonas aeruginosa* by phages F-116 or G-101 (Blahová et al., 1999), in *Actinobacillus actinomycetemcomitans* by the temperate bacteriophage AaΦ23 (Willi et al., 1997), in *Salmonella enterica* serovar Typhimurium by P22-like phages (Schmieger and Schicklmaier, 1999) and more recently among group A streptococci by phage Φm46.1 (Di Luca et al., 2010). In addition, some studies have demonstrated that bacterial 16S rRNA genes from Alpha-, Beta- and Gamma-Proteobacteria, Actinomycetes and Firmicutes were transduced by phages isolated from a wastewater treatment plant (Del Casale et al. 2011), indicating that transduction also occurs in these environments. Other studies have detected β-lactamase genes in phages occurring in sewage (Muniesa et al., 2004; Colomer-Lluch et al., 2011), verifying the contribution of phages in the spread of ARGs.

Table 1: Summary of mechanisms of antibiotic resistance among prokaryotes

Genetic element	Characteristics	Resistance determinants examples
Plasmid	Variable size (1 to > 100kb). Self-transmissible or mobilizable. Their transfer is called Conjugation.	Plasmid pCT:contain ESBL *bla*$_{\text{CTX-M-14}}$ Plasmid pMG252: contain PMQR gene *qnrA*
Insertion sequences	Small (<2.5 kb), contains terminal inverted repeats and encodes a transposase	IS*18* mediates overexpression of *bla*$_{\text{OXA-257}}$
Transposon	Large (until 12 kb aprox.), flanked by IS or inverted repeats. Encodes a transposase and other functional genes such as ARGs	Tn*1* and Tn*3*: confer resistance to β-lactams Tn*1721*: confer resistance to tetracycline
Integrons	Mediate the capture and expression of gene cassettes. Encodes for an integrase, attachments sites, and transcriptional elements.	Class 1: contain different cassettes conferring multidrug-resistance
Genomic island	Large mobile regions of DNA that encode complex biological functions	SGI1: confer resistance to streptomycin, spectinomycin, β-lactams and sulfonamides
Bacteriophage	Viruses infecting prokaryotic cells. Their replication allows the DNA transfer from one bacterium to another, known as Transduction	β-lactamase genes have been isolated in environmental phages

Previous studies based on microscopic enumeration of virus particles have determined that viruses represent one of the most abundant entities in nature (Danovaro et al., 2001). Moreover, recent studies based on metagenomics have

confirmed that phage genes dominate viral metagenomic libraries, providing an estimated phage abundance of approximately 10^{31} phages in the biosphere (Dinsdale et al., 2008). This large abundance and ubiquity, together with their distribution and mobility between environments, make phages one of the most efficient vectors for gene exchange (Muniesa et al., 2004). Furthermore, if the phage host range is not confined to a single bacterial species, as proposed by Hendrix et al. (Hendrix et al., 1999), phages would be able to move genetic material between phylogenetically unrelated species. Additionally, phages are known to be more persistent in the environment than their bacterial hosts (Muniesa et al., 2011). Taken together, these observations demonstrate that phages play an important role in the transfer of ARGs in natural habitats.

3. Antibiotic resistance as a global challenge

Emerging contaminants are defined as chemicals or microbiological constituents that were previously undefined or not recognised as being of concern to human health or the environment (Petrovic et al., 2004). Therefore, ARGs can be recognised as emerging pollutants (Pruden et al., 2006). In this era of globalisation, increased trade and human mobility has promoted the rapid spread of infectious agents, including those that harbour ARGs (Cars et al., 2011). Therefore, even if a nation has an effective strategy to contain resistance within its boundaries, it is very difficult to protect itself from the introduction of resistant pathogens through human travel and trade. In this sense, modern societies often ignore the consequences of individual actions, such as self-prescription or treatment interruption. A clear example of this phenomenon is the recent appearance of resistance to carbapenems, the latest β-lactams developed against Gram-negative pathogens. Resistance to this class of antibiotics is conferred in part by the New Delhi metallo-β-lactamase (NDM) enzyme, which was first described in 2008 in a *Klebsiella pneumoniae* isolated in Sweden from a patient coming from a New Delhi hospital (Nordmann et al., 2011b). Since its discovery, the NDM enzyme has been reported worldwide, mostly in bacteria from patients epidemiologically linked to the Indian subcontinent, where the NDM-containing bacteria are widespread in hospital and community infections and in

polluted urban water. India is characterised by overpopulation, poor hygiene and sanitation, difficulty in obtaining potable water, sale of non-prescribed antibiotics and deficient hospital antibiotic policies. These factors may have contributed to the spread of bacteria carrying NDM-1, and because millions of tourists visit India every year, uncontrolled NDM-1-related resistance may be expected in every part of the world (Nordmann et al., 2011a). Therefore, antimicrobial resistance poses a risk to individuals worldwide and urgent global action is needed.

4. Antibiotic consumption

To establish a strategy to contain the emergence of antibiotic resistance, it is crucial to know which antimicrobials are used and by whom (WHO, 2001). However, data on antibiotic consumption are scarce and very heterogeneous between countries (Kümmerer, 2009). Contrary to general expectations, the largest amounts of antibiotics are used in animals and not in humans. In Germany, for instance, veterinary practices use 85% of the total amount of antibiotics and only 15% is used in human health. Moreover, 85% of the antibiotics used in human medication are administered in the outpatient setting, and only 15% are used in hospitals (Meyer et al., 2013).

During the last decade, there has been an overall increase in antibiotic consumption in the European Union. The current use of antibiotics in the community (primary care sector), expressed as DDD (defined daily dose, according to the WHO), ranged from 11.1 DDD per 1000 inhabitants per day in Estonia to 39.4 DDD per 1000 inhabitants per day in Greece (ECDC, 2010). In particular, β-lactam antibiotics account for approximately 50-70% of the total antibiotic use, followed by sulfonamides, macrolides and fluoroquinolones. However, this consumption could be still higher considering that in some countries, antibiotics are partly sold over the counter, that is, without medical prescriptions (Kummerer, 2012). Although the vast majority of human antibiotic consumption occurs in the community, antibiotic use in hospitals is the main driver of the spread of antibiotic-resistant bacteria that cause nosocomial infections (ECDC, 2010).

Spain is one of Europe's largest antibiotic consumers, and wide-spectrum antibiotics are the most consumed, creating a high impact on the development and spread of antibiotic resistance. Nevertheless, the complete picture of consumption of antibiotics in Spain is difficult to ascertain because the Ministry of Sanitation and Social Aid reports that approximately 30% of the consumed antibiotics are obtained without a medical prescription (www.msssi.gob.es/campannas/campanas06/-antibioticos3.htm). However, the European Surveillance of Antimicrobial Consumption Network (ESAC-Net) interactive database (www.ecdc.europa.eu/en/healthtopics/-antimicrobial_resistance/esac-net-database) from the ECDC provides European reference data on antimicrobial consumption. In Spain, total antibiotic consumption was stable from 1997 until 2003, when a slight increase was detected. From 2007 to 2009, the consumption was steady again, but in 2010, a 4% increase in total consumption over the previous year was observed. The pattern of use has remained consistent over the years, with broad-spectrum penicillins accounting for 62% of the overall use in 2010. The use of quinolones as a group also appeared to be stable, although there has been a recent increase in the use of levofloxacin and a decrease in the use of moxifloxacin, norfloxacin and ofloxacin. In terms of DDD per 1000 inhabitants per day, the community consumption in 2010 of the most-used β-lactams, penicillins, was 12.64, followed by quinolones (2.54), macrolides (1.96), other β-lactams (1.56), tetracyclines (0.70) and sulfonamides (0.27) (ECDC 2010). In Catalonia, quinolone prescriptions increased from 13.3% to 15.7% from 1992-2007. However, the antibiotic family that increased the most was penicillin, which increased from 46.1% to 59.7% (Llor et al., 2009).

5. Resistance to fluoroquinolones

In recent years, some studies have demonstrated that Spain is one of the European countries with the highest use of fluoroquinolones outside of the hospital (Asensio et al., 2011). For this reason, the present work studies antibiotic resistance with special emphasis on fluoroquinolone resistance. Fluoroquinolones are a family of synthetic broad-spectrum antibiotics obtained by adding a fluorine and piperazinyl group to the core structure of quinolone (Sukul and Spiteller, 2007). Fluoroquinolones

interact with DNA gyrase (topoisomerase II) and topoisomerase IV, trapping these enzymes at the DNA cleavage stage and preventing strand rejoining. As a result, the DNA replication machinery becomes arrested at blocked replication forks, leading to the inhibition of DNA synthesis, bacteriostasis and, finally, cell death (Kohanski et al., 2010).

Quinolone resistance is mainly mediated by mutations in chromosomal genes encoding the quinolone targets (*gyrA* and *parC*, which encode a subunit of DNA gyrase and topoisomerase IV, respectively). However, more than one mutation is generally required to cause clinical resistance because wild-type organisms are highly susceptible. The discovery of PMQR in 1998 provided a satisfying explanation for the rising frequency of this resistance (Strahilevitz et al., 2009). The three PMQR mechanisms identified are as follows: (i) a quinolone-protective protein encoded by the *qnr* genes; (ii) a modifying enzyme encoded by the *aac(6')-Ib-cr* gene; and (iii) an efflux pumps encoded by *qepA* and *oqxAB* genes (Poirel et al. 2012). At present, five *qnr* families, *qnrA*, *qnrB*, *qnrS*, *qnrC*, and *qnrD*, have been described in plasmids disseminated among bacterial pathogens, but the presence of these genes in chromosomes of naturally occurring aquatic bacteria has also been reported, suggesting that these bacterial species may constitute the origin of and the environmental reservoir for these genes (Hernández et al., 2011). Although each of these mechanisms alone only confers a low level of resistance, the simultaneous presence of two or more of these mechanisms in the same microorganism exhibits a synergistic effect, increasing the minimum inhibitory concentration (MIC) to fluoroquinolones (Ruiz et al., 2012).

In addition, double-stranded DNA breaks resulting from fluoroquinolone action have been shown to induce the DNA stress response, also known as the SOS response (Kohanski et al., 2010). Because the SOS system promotes small local changes (mutations) in the nucleotide sequence and the conjugative transfer of genes, fluoroquinolones might influence the appearance of new antibiotic-resistant mutants and potentiate the horizontal dissemination of ARGs to a wide range of bacterial species (Beaber et al., 2004; López et al., 2007).

6. Link between clinical and environmental resistance

Although most of the literature on antibiotic resistance has focused on resistance in clinically relevant human pathogens, environmental bacteria are known to play an important role in the emergence and spread of ARGs (Wright, 2012). In the environment, bacteria have developed an ability to synthesise bioactive molecules to communicate with other members of the community in either a cooperative or antagonistic manner. The molecules developed to protect bacteria against the compounds from other microbes are encoded by genetic elements that constitute the "resistome" (Gaze et al., 2013). Moreover, the new high-throughput sequencing tools have revealed that there is an "intrinsic resistome", which includes myriad sequences normally belonging to bacterial metabolic networks that can eventually participate in resistance to antimicrobial agents. These pre-resistance genes can evolve to new resistance mechanisms if they encounter an environment with high concentrations of antibiotics. In this sense, antibiotics may act as selective agents for mechanisms of resistance and may also accelerate the evolution of resistance (Galán et al., 2013).

Several research groups have applied metagenomic approaches to explore antibiotic resistance genetic diversity in natural environments. The group led by Jo Handelsman at Yale University, for example, demonstrated that uncultured soil bacteria from a Wisconsin oak savannah harboured unknown aminoglycoside and tetracycline resistance genes that were significantly different from previously sequenced resistance genes (Riesenfeld et al., 2004). Another study from the same group found genes encoding for β-lactamases in one Alaskan soil sample, in which anthropogenic activities are minimal. These β-lactamases were more closely related to the ancestral β-lactamases than the β-lactamases isolated in clinical settings but were still capable of conferring resistance in *E. coli* (Allen et al., 2009).

Currently, there is no doubt that the natural environment is a huge reservoir of ARGs. However, evidence of the mobilisation of environmental resistance genes into clinical human pathogens has not been demonstrated until recently. Since 2001, various reports have shown the similarity of the gene encoding for CTX-M, an ESBL often found in clinical pathogens, with chromosomally encoded β-lactamases from

Kluyvera spp., a typical environmental bacterium (Humeniuk et al., 2002; Poirel et al., 2002; Rodríguez et al., 2004). Similarly, for *qnr* genes, the PMQR that confers a low level of resistance to quinolones, the origin of *qnrA* was identified in the chromosome of *Shewanella algae*, a Gram-negative species widely distributed in marine and freshwater environments (Poirel et al., 2005). The origins of the *qnrB* and *qnrS* genes remain unknown, but evidence appears to indicate that it may be close to some members of the Vibrionaceae family which possess chromosome-encoded Qnr-like determinants (Cattoir et al., 2007). More recently, Forsberg and colleagues created a platform to facilitate the rapid and efficient functional characterisation of metagenomic libraries, a platform called Parallel Annotation and Re-assembly of Functional Metagenomic Selections (PARFuMS). In applying PARFuMS to a collection of soil-derived cultures, the authors found ARGs in non-pathogenic soil bacteria representing all major mechanistic classes and having perfect nucleotide identity with many ARGs found in human pathogens. Moreover, the authors also found that these ARGs were located within long sequences flanked by MGEs, which suggests recent horizontal gene transfer HGT and the mechanism through which this exchange occurred (Forsberg et al., 2012).

Once the link between clinical and environmental resistance has been demonstrated, it is essential to study how antibiotic resistance evolves in nature. Moreover, due to the introduction of antibiotics from human and veterinary sources into the environment, it has become a reactor in which bacteria from different origins, antibiotics, disinfectants and heavy metals, are mixed, thus contributing to the evolution and spread of antibiotic resistance (Baquero et al., 2008). While it is clear that most anthropogenic-impacted environments have elevated numbers of ARGs, there are two main hypotheses to explain this increase. The first hypothesis is that antibiotics released into the environment impose a selective pressure on bacteria, selecting resistant subpopulations and increasing the amount of ARGs. The second hypothesis is that ARGs selected in other environments, such as in human or animal gastrointestinal tracts, are transported mostly through runoff processes into the aquatic environment (Pruden and Arabi, 2012). In the case of the South Platte River basin study, the results supported the hypothesis of transport because the molecular

signatures between pristine and impacted sites were different and because ARGs were detected with greater frequency in suspended sediments than in streambed sediments, where the antibiotic concentrations were higher (Storteboom et al., 2010). Conversely, a metagenomic study which investigated microbial communities in river sediments exposed to wastewater discharges from an antibiotics production plant in India identified very high levels of ARGs, as well as elements for horizontal gene transfer. These results suggest that discharges of wastewater effluent contaminated with high concentrations of antibiotics promote the increase of ARGs and genetic elements for their mobility (Kristiansson et al., 2011). Another study on antibiotic resistance transfer from manure to soil also showed that persistence of ARGs in soil is mediated by horizontal gene transfer from the manure bacteria to indigenous soil bacteria because bacterial manure communities only survive for a few months due to their poor adaptation to the soil environment (Heuer et al., 2011).

6.1. Biofilm and sediment

Different environmental compartments, such as biofilm and sediment, might play a significant role in driving ARG transfer, ecology and evolution (Taylor et al., 2011). The majority of bacteria in natural aquatic ecosystems have been suggested to be organised in biofilms (Donlan and Costerton, 2002), which are complex assemblages of microorganisms that are embedded in a matrix of extracellular polymeric substances. The biofilm matrix facilitates structural organisation, protects the microbial community and plays a critical role in the spread of antibiotic resistance due to the high cell density, close proximity, increased genetic competence and accumulation of MGEs (Fux et al., 2005). Recent studies have shown that ARGs in aquatic systems may migrate rapidly to biofilms, where they may persist longer than in adjacent waters (Pruden et al., 2006; Engemann et al., 2008). These studies thus suggest that biofilms are important reservoirs for ARGs in the environment.

Aquatic sediments also represent an important environmental matrix for HGT because of the ability of these sediments to retain antimicrobial compounds (Taylor et al., 2011). Moreover, microbial communities inhabiting aquatic sediments are a complex and highly diverse assemblage of prokaryotic and eukaryotic organisms.

Several studies have shown the presence of ARGs in aquatic sediments, particularly those sediments exposed to anthropogenic activities (Pruden et al., 2006; Cummings et al., 2011). A recent study demonstrated that MGEs, such as class 1 integrons, were highly overrepresented in river sediments exposed to antibiotics, suggesting that aquatic sediments are important reservoirs for the acquisition and transfer of ARGs (Kristiansson et al., 2011).

6.2. The potential role of wastewater treatment plants (WWTPs)

The main goal of WWTPs is to protect the environment from adverse effects of suspended solids and organic matter loads from wastewater effluents. Municipal wastewater usually includes non-hazardous industrial waste and storm water. However, wastewater is considered the main reservoir of antimicrobial resistance because it contains gastrointestinal resistant microorganisms that have been selected in the gut of humans and animals receiving antibiotic therapy (LaPara and Burch, 2012). WWTPs are considered hotspots for antibiotic resistance spread because the treatment processes create a nutrient rich environment with a high cell density and recurrent contamination with both antibiotics and resistant bacteria (Rizzo et al., 2013). One study detected 140 different ARGs in bacteria from activated sludge and 123 in the final effluents of the WWTP (Szczepanowski et al., 2009). Moreover, most of the studies carried out in WWTPs worldwide, regardless of their efficiency or operating conditions, found that these plants produced final effluents containing antibiotic-resistant bacteria (ARB), occasionally at higher percentages than the raw influent (Novo et al., 2013). LaPara and co-workers investigated the impact of tertiary-treated municipal wastewater on the quantity of several ARGs in Duluth-Superior Harbor (USA) and determined that the treated water was a significant source point in the harbour for three tetracycline resistance determinants, as well as the class 1 integrase gene (LaPara et al., 2011). Another study observed that advanced wastewater treatment in a membrane biological reactor utility was more efficient at removing ARGs and ARB compared with conventional treatment (Munir et al., 2011). However, the same authors demonstrated that the disinfection process did little to reduce ARGs and ARB. Altogether, further research is needed to design a wastewater treatment facility that limits the spread of antibiotic resistance into the environment.

In conclusion, the high efficiency of MGE transferring antibiotic resistance determinants among phylogenetically distant bacteria from different environments makes it difficult to distinguish between naturally occurring resistance and the resistance promoted by antibiotics released from anthropogenic sources. However, the European Council concluded in 1998 that there was a relationship between the consumption of antimicrobial compounds and the prevalence of antibiotic-resistant bacteria. Since then, both the European Union and United States have launched many programs for antibiotic resistance surveillance to assess the actual risk for public health associated with the consumption of antimicrobial compounds. Nevertheless, the recent advances in metagenomics have shown that very little is known about the antibiotic resistome of the vast majority of environmental bacteria. Antibiotic resistance is by far better understood at the small scale (i.e., within an individual bacterium) than at the large scale (the natural environment). Given that antibiotics are the main weapon against bacterial pathogens, further research of environmental reservoirs is needed to better understand the mechanisms of antibiotic resistance.

Chapter 2

Objectives

The general aim of this thesis was to study the occurrence of ARGs in aquatic microbial communities influenced by anthropogenic activities.

To accomplish this main goal, the following specific objectives were defined:

- Develop and validate molecular techniques to quantify ARGs, particularly:
  - Design new qPCR assays to quantify *qnr* genes.
  - Optimise existing qPCR assays to quantify several ARGs.

- Assess the effect of WWTP effluent discharges on bacterial communities in the receiving river, expressly focusing on:
  - The quantification of several ARGs.
  - Shifts in the structure of bacterial communities.

- Evaluate the role of bacteriophages as a reservoir of ARGs in the environment.

- Characterise resistant strains from a WWTP discharge point and the receiving river, gaining a deeper insight into:
  - Isolation and identification of ciprofloxacin-resistant strains.
  - Detection of PMQR in ciprofloxacin-resistant strains.
  - Characterisation of a multidrug resistance-encoding plasmid from an *Aeromonas* sp.

Chapter 3

Real-time PCR assays for quantification of *qnr* genes in environmental water samples and chicken feces

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Real-Time PCR Assays for Quantification of *qnr* Genes in Environmental Water Samples and Chicken Feces

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Real-time PCR assays were developed for the enumeration of plasmid-mediated quinolone resistance (PMQR) determinants, such as the *qnrA*, *qnrB*, and *qnrS* genes, in different water samples and chicken feces. The results indicate that the developed assays are specific and sensitive for the quantification of *qnr* genes in complex samples.

Quinolones are broad-spectrum antibiotics of considerable importance in both human and veterinary medicine, but their extensive use has resulted in the development of antibiotic resistance. Moreover, several studies have suggested that clinical resistance may be intimately associated with antibiotic resistance genes that may have their origin in environmental bacteria (1, 2). It is important, hence, to study the antibiotic resistance in the environment because it could be a reservoir for antibiotic resistance genes that can be transferred to human pathogens. The main mechanisms of quinolone resistance are associated with mutations in the *gyrA* and *parC* genes encoding the A subunits of the DNA gyrase and topoisomerase IV, respectively (3). A number of plasmid-mediated quinolone resistance (PMQR) determinants have also been described, including the *qnr* genes encoding pentapeptide repeat proteins, which block the action of ciprofloxacin on bacterial DNA gyrase and topoisomerase IV, the AAC(6′)-Ib-cr aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin by *N*-acetylation of its piperazinyl amine, and the quinolone efflux pump QepA (4). Plasmid-mediated resistance encoded by *qnr* genes has been reported in *Enterobacteriaceae* and, to a lesser extent, in *Aeromonadaceae* and *Vibrionaceae* (1, 4, 5). In addition, there are currently different *qnr* genes described (*qnrA*, *qnrS*, *qnrB*, *qnrC*, *qnrD*, and *qnrVC*), but here we focus on *qnrA*, *qnrB*, and *qnrS* due to the fact that they are more prevalent (4). Since the culture-based methods cannot help us to distinguish the presence of specific resistance genes, it is necessary to turn to molecular techniques. Therefore, the aim of the present study was to develop real-time PCR assays for the rapid and specific quantification of *qnr* genes in environmental samples in order to understand the environmental distribution of such genes and how anthropogenic inputs affect their spread.

This study was conducted on the following types of samples: water and chicken feces. Water was obtained from four different sources, representing different grades of pollution, such as human and veterinary hospital wastewater effluents, subterranean water, and the Ter River, all of them located in the Autonomous Community of Catalonia. One-liter samples of subterranean and river water were collected and filtered through 0.45-μm-pore-size membranes. In the cases of human and veterinary hospital wastewater samples, only 50 ml was filtered due to the high particulate matter concentrations in the sample. The membranes were then resuspended in 1 ml of lysis buffer (1.2% Triton X-100, 20 mM Tris-Cl, 2 mM EDTA, and 20 mg/ml lysozyme) (6). Concerning the prevalence of *qnr* in poultry animals (7, 8), feces samples of chicken were also taken. These samples were weighted (around 25 mg each) and diluted directly with 1 ml of lysis buffer. In all cases, the samples were collected and analyzed in triplicate and DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

The plasmid-mediated quinolone resistance genes *qnrA*, *qnrB*, and *qnrS* were quantified in all environmental DNA samples using real-time PCR assays. Primers used (Table 1) were adapted from prior studies or designed using the Primer3Plus software (11), on the basis of the alignments of available sequences (http://www.lahey.org/qnrStudies). Primer pairs were designed to amplify all alleles of each known *qnr* gene until use, and the specificities and sensitivities were verified using the BLAST alignment tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (see Fig. S1 in the supplemental material). For the *qnrB* gene, the primers selected were degenerated due to the existence of a huge quantity of alleles. Real-time PCR assays were initially performed with various concentrations of primers and also the annealing temperature. Moreover, the DNA extracted from samples was diluted several times to determine which concentration was better and positive controls were spiked with our DNA samples in order to screen for PCR inhibition. The absence of PCR inhibitors was confirmed, except in the case of DNA from chicken feces, which presented some inhibitors that interfered with the quantification of *qnr* genes. However, this effect was avoided by doing serial dilutions (1:10) of the extracted DNA.

Once real-time PCR assays were optimized, analyses were performed to make an absolute quantification of selected genes in the samples. First, the copy number of the 16S rRNA gene was quantified to confirm the presence of bacteria in the samples and for normalization of the data. In this case, the real-time PCR was performed according to the conditions described previously (12). All real-time PCR assays were amplified in duplicate using SYBR green on a MX3005P system (Agilent Technologies, Santa Clara, CA). The optimal reaction component concentrations to amplify *qnr* genes were as follows: in a total volume of 30 μl, each reaction

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TABLE 1 Description of the primers and protocols used in developed real-time PCR assays

Target gene	Primer	Sequence	Source or reference	Thermal cycling	Calibration curve	% efficiency	Analytical sensitivity (copies/μl of DNA)
qnrA	qnrAf-RT qnrAr-RT	ATTTCTCACGCCAGGATTTG GCAGATCGGCATAGCTGAAG	Modified from reference 9	95°C for 3 min (1 cycle); 95°C for 15 s and 62°C for 20 s (40 cycles)	$y = -3.439 \log(x) + 39.39$; $R^2 = 0.994$	95.3	17
qnrB	qnrBmF qnrBmR	GGMATHGAAATTCGCCACTG TTYGCBGYYCGCCAGTCGAA	Modified from reference 10	95°C for 3 min (1 cycle); 95°C for 15 s and 62°C for 20 s (40 cycles)	$y = -3.379 \log(x) + 43.62$; $R^2 = 0.994$	97.7	27
qnrS	qnrSrtF11 qnrSrtR11	GACGTGCTAACTTGCGTGAT TGGCATTGTTGGAAACTTG	This study	95°C for 3 min (1 cycle); 95°C for 15 s and 62°C for 20 s (40 cycles)	$y = -3.282 \log(x) + 39.56$; $R^2 = 0.996$	101.7	18

mixture contained 1× the Brilliant III Ultra-Fast SYBR green qPCR master mix (including *Taq* DNA polymerase, deoxynucleoside triphosphate [dNTP], and $MgCl_2$) (Agilent Technologies), 200 nM each forward and reverse primer, and 1 μl of template and RNase- and DNase-free water to complete the 30-μl volume. The optimal cycling protocol consisted of an initial cycle at 95°C for 3 min, followed by 40 cycles at 95°C for 15 s and 62°C for 20 s. In all cases, after the PCR, a dissociation curve (melting curve) was constructed in the range of 60°C to 95°C to verify the specificity of the amplified products.

Escherichia coli strain 226 (*qnrA1*), *E. coli* strain J53 pMG301 (*qnrB2*), and *E. coli* strain J53 pMG306 (*qnrS1*) were used as positive controls to make the standards by transforming gene-bearing plasmids into *Escherichia coli* using the TOPO cloning kit (Invitrogen, Carlsbad, CA). Plasmids were extracted using the PureLink plasmid kit (Invitrogen), and the concentration was determined using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE). Ten-fold serial dilutions of purified plasmid DNA were obtained and amplified in triplicate for the generation of the standard curve. Gene copies were calculated as described previously (13). Qnr-positive strains were used as positive controls to test the sensitivity of each real-time PCR, which included the above-mentioned strains, *E. coli* strain J53 pMG289 (*qnrB1*), and *Aeromonas* sp. strain pP2G1 (*qnrS2*). The efficiency and sensitivity of the real-time PCRs were determined by the amplification of standard serial dilutions. Efficiencies were calculated using the resulting standard curves, by the formula $E = 10^{(-1/\text{slope})} - 1$, and the analytical sensitivity of the real-time PCRs was determined as the smallest DNA quantity detected for each assay. The obtained efficiency indicated that all real-time PCR assays were suitable (ranging from 95.3 to 101.7%). Moreover, the analytical sensitivity indicated that the reaction for *qnrA*, *qnrS*, and *qnrB* was able to detect 17, 18, and 27 gene copy numbers per reaction, respectively (Table 1). The correlation coefficient (R^2) obtained from the regression line of real-time PCR assays varied from 0.994 to 0.996, demonstrating that the quantification method was linear over a minimum range of 5 logs (see Fig. S2 in the supplemental material). Therefore, all these parameters indicate the validity of the three real-time PCR assays developed for the quantification of *qnr* genes.

A threshold cycle (C_T) average from the replicate samples was used for data analysis. To obtain the final result in gene copy numbers per sample volume, calculated gene copy numbers per reaction were divided by the template volume, multiplied by the total DNA elution volume, and the total divided by the sample volume (13). Statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL). The quantification results for the samples are summarized in Table 2. Briefly, the presence of bacterial DNA in all samples was confirmed by amplification of bacterial 16S rRNA gene, which ranged from 4.40×10^{10} copy numbers/mg in chicken feces samples to 1.20×10^7 copy numbers/ml in water from river samples. Regarding antibiotic resistance genes, not all *qnr* genes were detected in the samples, but we quantified a high copy number for *qnrA* (1.19×10^5 copies/mg) and *qnrB* (2.60×10^4 copies/mg) in chicken feces and for *qnrS* in hospital (5.29×10^4 copies/ml) and veterinary hospital (4.01×10^4 copies/ml) wastewater effluents. However, when these quantifications were normalized to 16S rRNA gene copies (see Table S1 in the supplemental material), the results showed that the resistance gene with the highest relative copy number was *qnrS* in the river

TABLE 2 Quantification of 16S rRNA, *qnrS*, *qnrB*, and *qnrA* in different analyzed samples

Sample	Gene copy no./ml[a]			
	16S rRNA	*qnrS*	*qnrB*	*qnrA*
Hospital effluent	1.85×10^9 ($\pm 6.97 \times 10^8$)	5.29×10^4 ($\pm 2.01 \times 10^4$)	2.50×10^3 ($\pm 2.55 \times 10^3$)	9.25×10^2 ($\pm 4.23 \times 10^2$)
River	1.20×10^7 ($\pm 2.73 \times 10^6$)	1.46×10^2 ($\pm 2.59 \times 10^1$)	ND	ND
Veterinary hospital effluent	5.23×10^7 ($\pm 5.84 \times 10^7$)	4.01×10^4 ($\pm 6.33 \times 10^4$)	2.05×10^2 ($\pm 1.08 \times 10^{2)}$	ND
Subterranean water	3.27×10^7 ($\pm 2.53 \times 10^6$)	ND	2.45×10^1 ($\pm 1.16 \times 10^1$)	ND
Chicken feces	4.40×10^{10} ($\pm 4.06 \times 10^9$)	ND	2.60×10^4 ($\pm 2.42 \times 10^4$)	1.19×10^5 ($\pm 7.42 \times 10^4$)

[a] Mean values and standard deviations of the means (in parentheses) are shown. Gene copy number/mg of sample for chicken feces. ND, not detected.

water samples and both hospital samples analyzed. This is in accordance with the observations of Poirel et al. (14), which stated that *qnrS* was the most commonly identified acquired *qnr* gene in the environment, because it is usually identified in waterborne species.

In conclusion, our results indicate that three real-time PCR assays represent a specific and sensitive tool for the rapid quantification of *qnrA*, *qnrB*, and *qnrS*. Moreover, we developed a method widely applicable in samples from different sources. Previous studies have demonstrated that real-time PCR is a highly sensitive and reliable tool to quantify genes encoding tetracycline (15), sulfonamide (16), and beta-lactam (17) resistance. However, to our knowledge, this is the first real-time PCR targeted to specifically quantify the most prevalent *qnr* genes, because all the earlier assays were carried out only for the detection of *qnr*.

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Chapter 4

Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a wastewater treatment plant

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Prevalence of Antibiotic Resistance Genes and Bacterial Community Composition in a River Influenced by a Wastewater Treatment Plant

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Abstract

Antibiotic resistance represents a global health problem, requiring better understanding of the ecology of antibiotic resistance genes (ARGs), their selection and their spread in the environment. Antibiotics are constantly released to the environment through wastewater treatment plant (WWTP) effluents. We investigated, therefore, the effect of these discharges on the prevalence of ARGs and bacterial community composition in biofilm and sediment samples of a receiving river. We used culture-independent approaches such as quantitative PCR to determine the prevalence of eleven ARGs and 16S rRNA gene-based pyrosequencing to examine the composition of bacterial communities. Concentration of antibiotics in WWTP influent and effluent were also determined. ARGs such as *qnrS*, bla_{TEM}, $bla_{CTX\text{-}M}$, bla_{SHV}, *erm*(B), *sul*(I), *sul*(II), *tet*(O) and *tet*(W) were detected in all biofilm and sediment samples analyzed. Moreover, we observed a significant increase in the relative abundance of ARGs in biofilm samples collected downstream of the WWTP discharge. We also found significant differences with respect to community structure and composition between upstream and downstream samples. Therefore, our results indicate that WWTP discharges may contribute to the spread of ARGs into the environment and may also impact on the bacterial communities of the receiving river.

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Introduction

Antibiotic resistance represents a significant global health problem due to the use and misuse of antibiotics, which favors the emergence and spread of resistant bacteria. Since the first warning of antibiotic resistance [1], this phenomenon has increased dramatically and as a result, 70% of all hospital-acquired infections in the United States are resistant to at least one family of antibiotics [2]. The treatment of these infections leads to higher healthcare costs because these therapies require longer hospital stays and more expensive drugs. To confront this increasing problem, it is necessary to understand the ecology of antibiotic resistance, including their origins, evolution, selection and dissemination [3].

Although antibiotic resistance has involved extensive research in clinically relevant human pathogens, environmental reservoirs of antibiotic resistance determinants and their contribution to resistance in clinical settings have only been considered in the last decade [4–6]. It has been shown that antibiotic resistance genes (ARGs) have environmental origins but the introduction and accumulation of antimicrobials in the environment facilitates their spread [7]. As a consequence, ARGs can be found in almost all environments and they are currently considered as emerging pollutants [8,9]. Therefore, identifying sources of resistance genes, their environmental distribution and how anthropogenic inputs affect their spread will aid in establishing strategies to combat antibiotic resistance.

The location of ARGs on genetic elements that can be mobilized, such as transposons, integrons and plasmids, facilitates the transfer of resistance to other organisms of the same or different species [10]. Although antibiotic resistance studies have been focused on cultivable bacteria and/or indicator organisms in treated wastewater, the vast majority of environmental bacteria cannot be cultured under standard laboratory conditions. As a result, there is little information

about how the discharge of wastewater effluents can affect bacterial communities and impact the prevalence of resistance genes in the environment.

It is well known that WWTPs reduce the total number of bacteria, especially coliforms, in their final effluent [11]. However, the treatment is not efficient enough to remove ARGs that are released to the receiving river [12,13]. In addition, WWTPs link human activities and the environment and may facilitate horizontal transfer of resistance determinants among a rich diversity of commensals, environmental microorganisms and clinically relevant pathogens [14]. In this regard, WWTP may contribute to the occurrence, spread and persistence of both antibiotic-resistant bacteria and antibiotic resistance determinants in the environment.

We used culture-independent approaches to determine the prevalence of ARGs and to examine how bacterial communities from biofilms and sediments respond to the discharge of WWTP effluents in the receiving river. ARGs and bacterial community composition in the upstream river were also analyzed to determine the contribution of wastewater discharge to antibiotic resistance in the downstream river samples. Biofilm and sediment were selected rather than water because they are substrates with a high bacterial density where the frequency of physical contacts between bacteria increase the possibility for horizontal gene transfer [15]. Actually, some studies have proposed aquatic biofilms as long-term reservoirs for ARGs in environment [9,16].

Materials and Methods

Study site and sampling

The study was carried out in the Ter River upstream and downstream of the Ripoll WWTP discharge. This river is the water supply for most cities in Catalonia. The WWTP has a primary and secondary treatment operating with conventional activated sludge and was planned for a population of 45,000 inhabitants. It receives an average daily flow about 8000 m^3 made up primarily of domestic wastewater and a small amount of industrial and hospital wastewater, which are not pre-treated. Samples were obtained in June 2010, the end of the spring season, when water flow is maximum. Biofilm and sediment samples were collected in duplicate at the WWTP discharge point and at 100 m upstream and downstream of the WWTP. Both sample types were collected manually, scraping the surface of submerged stones for epilithic biofilm and collecting the top layer (0-5 cm) of sediment. Water samples for antibiotics quantification were taken from influent and effluent of the WWTP. All samples were stored on ice and transported to the laboratory for immediate processing.

Ethics statement

Permission for the WWTP samples was granted by the Ripoll Treatment Plant (Girona, Spain), specifically Angel Maderiano de Pastor (Supervisor, Wastewater Treatment Division). No specific permits or permissions were required for the samples collected in the Ter River.

Antibiotics quantification

To determine the efficiency of WWTP on antibiotics removal, some of these substances were quantified in WWTP influent and effluent. Quantification of antibiotics was carried out following the analytical methodology previously described [17]. Briefly, water samples were filtered through 1 µm glass microfibre filters followed by 0.45-µm nylon membrane filters (Whatman Maidstone, UK). Target compounds were extracted by solid-phase extraction using Oasis HLB cartridges (60mg, 3ml; Waters, Milford, MA, USA). Cartridges were loaded with 200 mL of water samples and a Baker vacuum system (J.T. Baker, Deventer, The Netherlands) was used to preconcentrate samples. The extracts were evaporated under a gentle nitrogen stream and reconstituted with 1 mL of methanol/water (25:75, v/v). Extracts were then analyzed by high-performance liquid chromatography tandem mass spectrometry using an Agilent HP 1100 HPLC (Agilent Technologies, Palo Alto, CA, USA) connected with a QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems/ MDS SCIEX, Foster City, CA, USA).

DNA extraction

Biofilm and sediment samples were homogenized in phosphate-buffered saline solution (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2) and the supernatants were then resuspended in lysis buffer (20 mM Tris-Cl, pH 8.0; 2 mM sodium EDTA; 1.2 % Triton X-100; and 20 mg/ml lysozyme). Genomic DNA was extracted using a standard phenol-chloroform method and the final concentration and purity were determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA was extracted in duplicate from each independent sample, obtaining 4 analytical replicates, and all DNA samples were stored at -20 °C until further analysis.

Quantification of antibiotic resistance genes

Quantitative PCR was used to quantify eleven genes encoding resistance to the main antibiotic families used for treating human and animal infections such as beta-lactams (bla_{TEM}, bla_{CTX-M} and bla_{SHV}), fluoroquinolones (*qnrA*, *qnrB* and *qnrS*), tetracyclines [*tet*(O) and *tet*(W)], sulfonamides [*sul*(I) and *sul*(II)] and macrolides [*erm*(B)]. The 16S ribosomal RNA (rRNA) gene was also analyzed to quantify the total bacterial load and to normalize the abundance of ARGs in the collected samples. All qPCR assays were performed on an Mx3005P system (Agilent Technologies) using SYBR Green detection chemistry. Each reaction was carried out in a total volume of 30 µl, containing 1 µl of template, the corresponding concentration of each primer (from 0.2 to 0.6 µM) and 2× Brilliant III Ultra Fast QCPR Master Mix (Stratagene, La Jolla, CA, USA), except for the bla_{TEM} gene, which was amplified using the SYBR® Green Master Mix (Applied Biosystems). Primers and thermal cycling conditions for each gene are given in supplementary material, Table S1. In all cases, DNA extracted from samples was diluted 10- and 100-fold and positive controls were spiked with our DNA samples in order to screen for PCR inhibition by environmental matrices. Moreover, after

the PCR a dissociation curve was constructed in the range of 60°C to 95°C to verify the specificity of the amplified products.

Standard curves were generated using known quantities of cloned target genes. Briefly, amplicons from positive controls were ligated into pCR2.1-TOPO cloning vectors (Invitrogen, Carlsbad, CA, USA) and transformed into *Escherichia coli* competent cells following the manufacturer's protocol (Invitrogen). Plasmids were extracted using the PureLink Plasmid kit (Invitrogen), and the concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific). Copy number was then calculated as described previously [18]. Tenfold serial dilutions of plasmid DNA were amplified over seven orders of magnitude and in triplicate to generate a standard curve for each qPCR assay.

Mean values (copy number of each ARG) of four analytical replicates for each sample were compared using the one-way analysis of variance (ANOVA) or Kruskal-Wallis test as appropriate, because most data were not normally distributed. Data were analyzed using SPSS for Windows version 17.0 (SPSS, Chicago, IL, USA).

Sequence analysis and phylogenetic classification

DNA extraction replicates from each sample were pooled and submitted to the Research and Testing Laboratory (Lubbock, TX, USA) for tag-pyrosequencing. Samples were amplified with primers 27F (3'-GAG TTT GAT CNT GGC TCAG-5') and 519R (3'-GTN TTA CNG CGG CKG CTG-5'), and the amplicons were sequenced using Roche 454 GS-FLX Titanium technology [19]. Sequences were quality trimmed using the MOTHUR software package [20]. Briefly, we removed sequences that did not perfectly match the PCR primer at the beginning of a read, sequences that contained more than one ambiguous base, sequences having a homopolymer stretch longer than 8 bp, and sequences with an average quality score below 30. We also included only the first 250 bp after the proximal PCR primer, because the quality of sequences degrades beyond this point. Then, sequences were aligned using the SILVA reference database [21] and potential chimeric sequences were detected and removed by using chimera.uchime incorporated into MOTHUR. Qualified sequences were assigned to operational taxonomic units (OTUs) based on a 97% sequence similarity. The Shannon diversity index (H') and the Chao1 richness estimator were also calculated. The Bray-Curtis distance, which incorporates both membership and abundance, was used to compare beta diversity among samples. The parsimony test, as implemented by MOTHUR, was used to assess whether two or more communities have the same structure. A Bonferroni correction was applied to adjust the significance level for multiple pairwise comparisons ($p \leq 0.05/15$ [0.0033]). The Ribosomal Database Project (RDP) pipeline and Classifier function were used to align and assign identities at a confidence threshold of 50% [22]. The sequences from this study have been deposited in the NCBI Short Read Archive under accession number SRA067245.

Table 1. Concentrations of antibiotics determined in WWTP influent and effluent water samples.

Antibiotic	Influent (ng/L)	Effluent (ng/L)
Clarithromycin	181.9	166.0
Trimethoprim	22.0	20.8
Metronidazole	161.0	43.3
Sulfamethoxazole	136.0	57.8
Ciprofloxacin	913.0	231.0

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Results

Antibiotic concentrations

Antibiotic compounds, such as clarithromycin, sulfamethoxazole, trimethroprim, metronidazole and ciprofloxacin were detected in WWTP influent and effluent samples at concentrations ranging from 20.8 to 913 ng/L (Table 1). Although concentrations found in the WWTP influent were higher than those in the effluent, relatively high levels of antibiotic compounds were detected in treated water from the WWTP.

Quantification of ARGs

ARGs, including *qnrA*, *qnrB*, *qnrS,* bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, *erm*(B), *sul*(I), *sul*(II), *tet*(O) and *tet*(W), and the 16S rRNA gene were quantified by qPCR in the biofilm and sediment samples. High R^2 values (average 0.995) and high efficiencies (from 95 to 103%) were obtained over at least 5 orders of magnitude in all qPCR assays, indicating the validity of these quantifications (data not shown). Results revealed that the total copy numbers of bacterial 16S rRNA genes were consistent in all samples and ranged from 1.45×10^9 to 1.21×10^{11} copy numbers per gram. Relative concentrations of ARGs (normalized to the 16S rRNA gene copy number) are shown in Figure 1. From this figure it can be seen that, except *qnrA* and *qnrB*, all ARGs were detected in the samples analyzed. It is noteworthy that relative abundances of the *qnrB*, *qnrS,* bla_{TEM}, bla_{SHV}, *erm*(B), *sul*(I), *sul*(II), *tet*(O) and *tet*(W) genes were significantly higher ($p<0.05$) in the downstream biofilm samples than those found in the upstream samples. Regarding sediment samples, no significant differences in relative concentrations of ARGs were observed among them, except for the *erm*(B) gene.

Bacterial community composition

A total of 77,056 reads from biofilm and sediment samples were obtained after quality trimming and filtering the initial reads. The library size of each sample was then normalized to the smallest number of sequences obtained from biofilm and sediment samples (4,328 and 7,587 sequences, respectively) in order to minimize any bias due to the difference in the total number of sequences. The number of OTUs observed at a 97% taxonomic cutoff ranged from 262 (in the upstream biofilm) to 2,527 (in sediment from the WWTP discharge point). Shannon diversity index and Chao richness estimators were

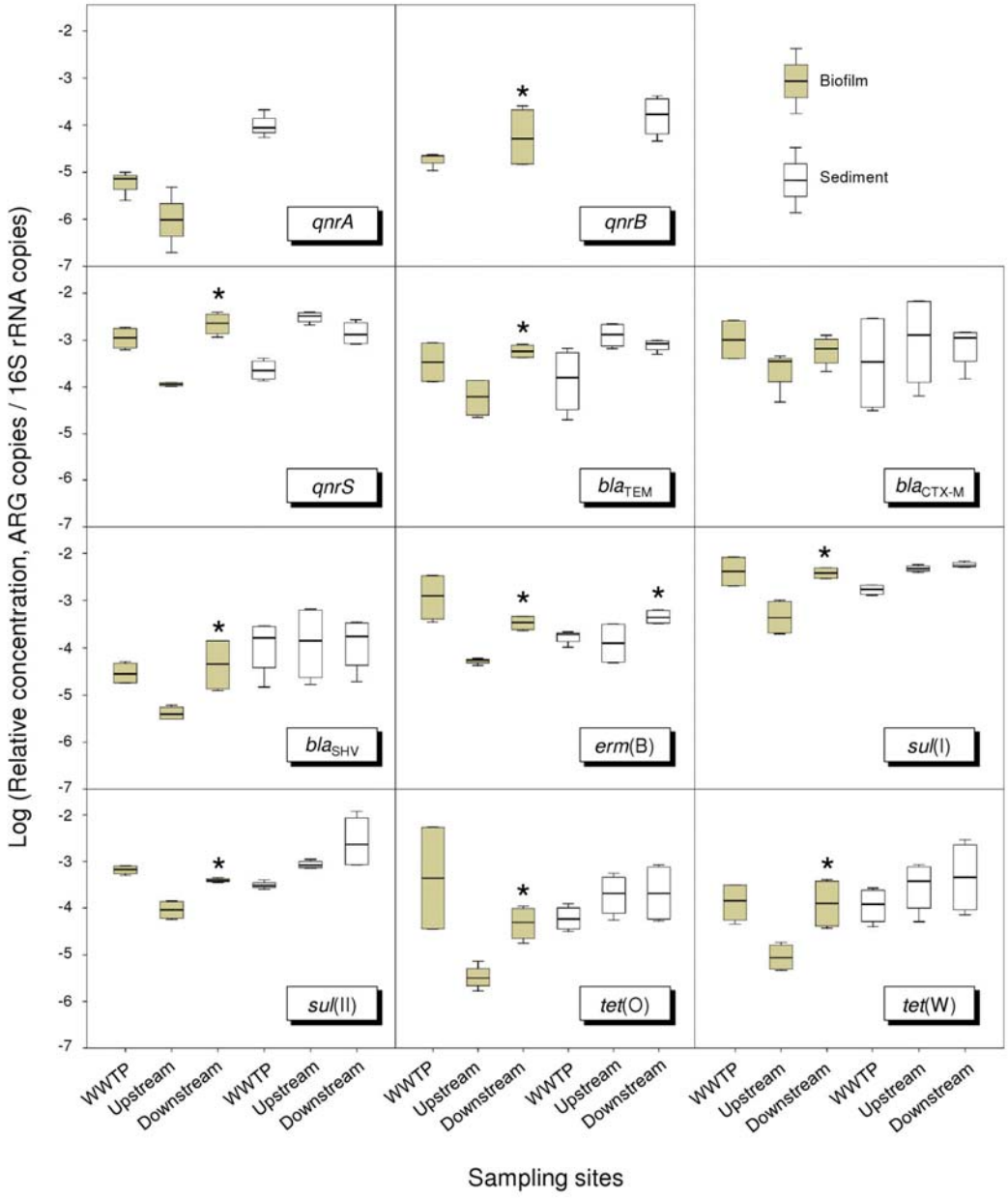

Figure 1. Relative concentration of ARGs in biofilm and sediment samples. Within the box plot chart, the crosspieces of each box plot represent (from top to bottom) maximum, upper-quartile, median (black bar), lower-quartile, and minimum values. An asterisk (*) denotes a statistically significant difference between upstream and downstream sites (P<0.05).

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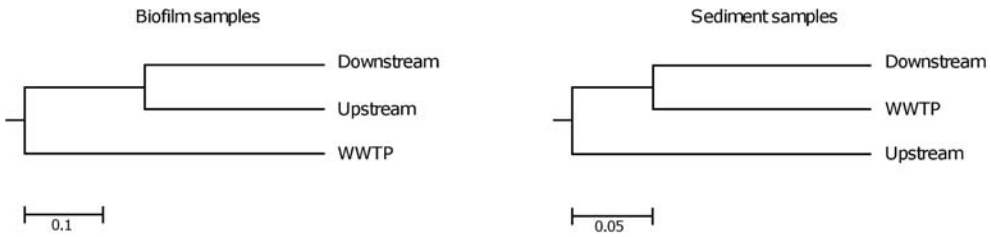

Figure 2. The dendrograms represent the similarity among the samples based on the Bray-Curtis coefficient. Scale bars indicate the similarity obtained from calculated matrices.

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Table 2. Measures of α diversity for the biofilm and sediment samples.

	Biofilm			Sediment		
	WWTP	Upstream	Downstream	WWTP	Upstream	Downstream
No. of sequences	4328	4328	4328	7587	7587	7587
OTUs	560	262	740	2527	1795	2202
H'	4.75	1.53	3.97	7.06	5.81	6.70
Chao1	1145	852	1988	6372	4378	5649

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also determined (Table 2), demonstrating that the sediment samples had a higher bacterial diversity and richness than the biofilm samples.

To compare the bacterial community structure and determine the effect of WWTP discharges into the environment, we used the phylogeny-based parsimony test which showed a significant difference (p<0.001) in community structure between all analyzed samples. When the pairwise distances for all samples were calculated using the Bray-Curtis distance metric, the results revealed the impact of the WWTP effluents on the bacterial community structure in the sediment of receiving river, as visualized by the terminal branch lengths (Figure 2).

Phylogenetic classification of sequences was determined using the RDP Classifier tool with a bootstrap cutoff of 50% (Figure 3). Overall taxonomic characterization of the bacterial community was conducted at the phylum level, and only Proteobacteria were classified at the class or order level. Biofilm sequences showed great differences between WWTP and river samples. Although the biofilm from the WWTP discharge point was dominated by *Cyanobacteria* and Proteobacteria, the most abundant groups in both upstream and downstream biofilms were Firmicutes. Gammaproteobacteria were mainly represented by the genera *Aeromonas* (16%) and *Acinetobacter* (8%) in the biofilm from the WWTP discharge point, whereas *Exiguobacterium* was the most predominant genus (data not shown), accounting for 85% of the observed OTUs in upstream samples and 46% in downstream samples. On the other hand, members of the Proteobacteria and Actinobacteria phyla were abundant in all sediment samples. Sequences from sediment samples were dominated by common genera such as *Aeromonas*, *Exiguobacterium*, *Piscinibacter*, *Pseudohodoferax*, *Acinetobacter* and *Pseudomonas*.

Discussion

In this study we investigated the prevalence of eleven ARGs and the bacterial community composition in biofilm and sediment samples from a river influenced by a WWTP. It has been some years since Iwane and colleagues [23] showed that the increase of antibiotic resistant bacteria in the Tama River was associated with WWTP effluent discharges. Since then, a wide range of genetic methods have been developed and some culture-independent studies have been performed to explore the impact of antibiotics in the environment; however, most of these studies have been limited to a few ARGs [24–26].

Although WWTPs efficiently reduce high nutrient concentrations from raw sewage, our study demonstrates, to a certain extent, that antibiotics are not completely degraded during wastewater treatment. Previous studies have shown that antibiotic levels in treated wastewater are typically in the nanogram per liter range [15] and WWTP effluents are the major pathway for pharmaceuticals to reach the aquatic environment [27,28]. Effluents are diluted once they reach the river, and even though antibiotic concentrations in the environment are low, in general below the minimum inhibitory concentration for most sensitive bacteria, they may still exert a selection pressure [29,30] and impact on the microbial community [31].

Regarding ARG concentrations, we identified nine of the eleven ARGs studied in all samples analyzed. Detection of these genes in upstream biofilm and sediment samples supports the idea of an existing background level of antibiotic resistance naturally occurring in the environment [10]. However, other anthropogenic activities in the river upstream, such as livestock rearing, may have perturbed the bacterial community. We also report that there was a significant increase in the relative concentration for almost all ARGs studied in the biofilm samples after the WWTP effluent discharges. This is consistent with the observations of Engemann et al. [9] which suggested the migration of ARGs from the water column to

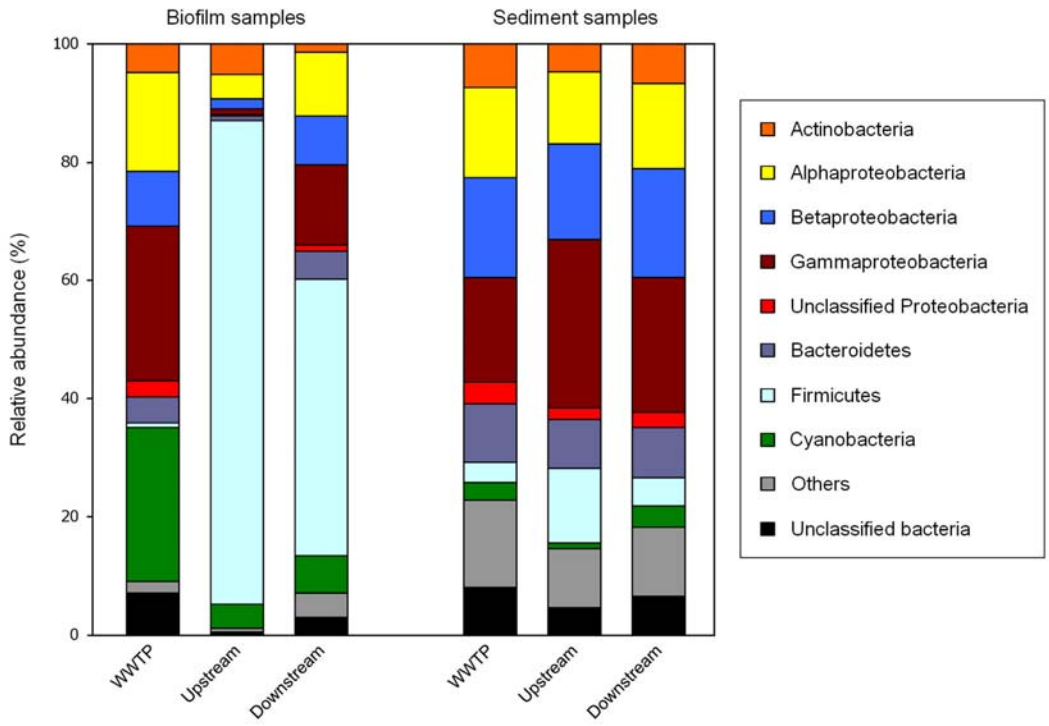

Figure 3. Relative abundance of major bacterial lineages (phyla; and classes for Proteobacteria) found in the biofilm and sediment samples.

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biofilms. Our findings thus, together with those previously reported, suggest that biofilms could be considered as good indicators of antibiotic resistance acquisition. Moreover, our results reinforce the view that environmental compartments directly impacted by anthropogenic activities, such as wastewater discharges, show a higher concentration of ARGs [8,24,32].

Previous studies have also suggested that aquatic sediments could be important reservoirs for ARGs due to their ability to retain antibiotic compounds as well as representing an important environmental matrix within which horizontal gene transfer can occur [33]. LaPara et al. [32] detected ARGs encoding resistance to tetracyclines in water and sediments samples in locations influenced by WWTP effluents. Although ARGs were found in most of the sediment samples analyzed, we could not determine the effect of WWTP in the receiving river as some ARG levels in the discharge point had similar values in terms of relative concentration to those found in the upstream and downstream sediment samples.

We also investigated the potential effect of WWTP effluents on bacterial communities associated with the receiving river by using 454-pyrosequencing technology. Based on the Chao1 and Shannon indices, the bacterial communities in the sediment samples had higher richness and diversity than those in the biofilm samples. This could be because, although biofilms can be composed of multiple microbial species, they may also be dominated by a few genera in the primary colonization phase [34].

It has also been reported that sediments from aquatic environments have a complex and dynamic community of microorganisms [35]. In this study, the bacterial communities in the biofilm and sediment samples from the WWTP discharge point and downstream river had a higher diversity than those in the upstream samples, suggesting that WWTP effluent discharges may have promoted bacterial growth by supplying nutrients [36].

Despite the high degree of similarity among the bacterial communities from river and WWTP sediment samples at the phylum level, differences with respect to community structure and composition were significant at the genus level (97% similarity) as revealed by the phylogeny-based parsimony test. Similarly, Kristiansson et al. [6] found differences in the distribution of bacterial genera between the upstream and downstream sediments from an Indian river influenced by WWTP effluents. Moreover, our results also agreed with the observation of Kristiansson et al. [6] that all sediment

communities were dominated by Proteobacteria, Bacteroidetes and Firmicutes. With regard to the biofilm samples, differences in community structures were evident even at the phylum level. *Cyanobacteria* and Proteobacteria were dominant in the WWTP discharge point, whereas Firmicutes was the dominant group in both upstream and downstream samples. A high representation of *Cyanobacteria* was found in biofilm samples from the WWTP, which may be a consequence of light intensity, as the WWTP effluent canal is less deep than the river, promoting the growth of phototrophic microorganisms. Concerning the relationship between changes in ARG concentrations and bacterial community composition, we found a higher proportion of Gammaproteobacteria in downstream biofilm samples compared with upstream samples, which could explain the increase of ARGs as several members of this class harbour ARGs [37,38]. A computer search using the Antibiotic Resistance Genes Database [39] also revealed that most of the bacterial species harboring ARGs belong to this class. In this sense, the similarity among the proportion of Gammaproteobacteria in bacterial communities from sediment samples could be related with the similar values of ARGs found in the different points analysed.

Additionally, we could detect and estimate the number of bacterial genera in each sample, which have been previously identified as harbouring ARGs. The genus *Exiguobacterium*, which was the dominant OTU in biofilm river samples and also appeared in sediment samples, has been recently characterized as a carrier of some ARGs encoding resistance to beta-lactams and sulphonamides [40]. The genus *Aeromonas*, which was detected in all samples, has been widely studied because most of ARGs we analyzed have been detected in several species of this genus [41,42]. Moreover, members of the genus *Acinetobacter*, which were present in high percentages in biofilm and sediment samples, have been also described as multidrug-resistant microorganisms encoding resistance to beta-lactams, aminoglycosides, fluoroquinolones and carbapenems [43]. Given this, microorganisms belonging to these genera may have contributed to the occurrence, spread and persistence of ARGs.

In conclusion, eleven different ARGs encoding resistance to the most important antibiotic families were analyzed using a culture-independent method, which contributes to a better understanding on the spread of antibiotic resistance in the environment. Of special concern is that our findings, together with reports from other settings, demonstrate that WWTP discharges may increase the prevalence of ARGs and bacterial community composition of the receiving river. However, further research is needed to evaluate if the increase of ARGs in aquatic ecosystems is due to the release of resistant bacteria from WWTP or due to antibiotics discharged in their effluents promoting horizontal gene transfer once they reach the river.

Supporting Information

Table S1. Primer sequences and qPCR conditions used in this study.
(DOC)

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Author Contributions

Conceived and designed the experiments: EM JJ JLB. Performed the experiments: EM. Analyzed the data: EM JLB. Contributed reagents/materials/analysis tools: JLB. Wrote the manuscript: EM.

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Supplementary Table S1. qPCR primer sequences, targets and conditions of reactions.

Target gene	Primers	Sequence	Conditions	Reference
16S rRNA	F1048	GTGSTGCAYGGYTGTCGTCA	95°C 3 min (1 cycle); 95°C 15 s and 60°C 1 min (35 cycles)	[1]
	R1194	ACGTCRTCCMCACCTTCCTC		
qnrA	qnrAf-RT	ATTTCTCACGCCAGGATTTG	95°C 3 min (1 cycle); 95°C 15 s and 64°C 20 s (40 cycles)	Modified from Robicsek *et al.* [2]
	qnrAr-RT	GCAGATCGGCATAGCTGAAG		
qnrB	qnrBmF	GGMATHGAAATTCGCCACTG	95°C 3 min (1 cycle); 95°C 15 s and 60°C 20 s (40 cycles)	Modified from Cattoir *et al.* [3]
	qnrBmR	TTYGCBGYYCGCCAGTCGAA		
qnrS	qnrSf-RT	ATGCAAGTTTCCAACAATGC	95°C 3 min (1 cycle); 95°C 15 s and 62°C 20 s (40 cycles)	[4]
	qnrSr-RT	CTATCCAGCGATTTTCAAACA		
bla_{TEM}	bla-TEM, FX	GCKGCCAACTTACTTCTGACAACG	95°C 3 min (1 cycle); 95°C 15 s and 60°C 20 s (40 cycles)	[5]
	bla-TEM, RX	CTTTATCCGCCTCCATCCAGTCTA		
bla_{CTX}	RTCTXM-F	CTATGGCACCACCAACGATA	95°C 3 min (1 cycle); 95°C 15 s and 60°C 20 s (40 cycles)	Modified from Kim *et al.* [6]
	RTCTXM-R	ACGGCTTTCTGCCTTAGGTT		
bla_{SHV}	RTblaSHVF	CGCTTTCCCATGATGAGCACCTTT	95°C 3 min (1 cycle); 95°C 15 s and 64°C 30 s (40 cycles)	[5]
	RTblaSHVR	TCCTGCTGGCGATAGTGGATCTTT		
sul(I)	Sul(I)-FW	CGCACCGGAAACATCGCTGCAC	95°C 3 min (1 cycle); 95°C 15 s and 65°C 20 s (40 cycles)	[7]
	Sul(I)-RV	TGAAGTTCCGCCGCAAGGCTCG		
sul(II)	Sul(II)-FW	TCCGGTGGAGGCCGGTATCTGG	95°C 3 min (1 cycle); 95°C 15 s and 58°C 20 s (40 cycles)	[7]
	Sul(II)-RV	CGGGAATGCCATCTGCCTTGAG		
tet(O)	tet(O)-FW	ACGGARAGTTTATTGTATACC	95°C 3 min (1 cycle); 95°C 15 s and 50°C 20 s (40 cycles)	[8]
	tet(O)-RV	TGGCGTATCTATAATGTTGAC		
tet(W)	tet(W)-FW	GAGAGCCTGCTATATGCCAGC	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	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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Chapter 5

Bacteriophages as a reservoir of extended-spectrum β-lactamase and fluoroquinolone resistance genes in the environment

Bacteriophages as a reservoir of extended-spectrum β-lactamase and fluoroquinolone resistance genes in the environment

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Abstract

Six antibiotic resistance genes (*bla*$_{CTX}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *qnrA*, *qnrB* and *qnrS*) were quantified by qPCR in both phage and bacterial DNA fractions of environmental water samples in order to determine the contribution of phages to the dissemination of antibiotic resistance genes (ARGs) in the environment. Although the highest copy numbers (p<0.05) of ARGs were detected in the bacterial DNA fraction, *qnrS* and *bla*$_{SHV}$ genes were found in the phage DNA from all samples analysed, reaching up to 4 log10 copy numbers/ml in hospital samples. These results indicate that bacteriophages are a potential reservoir of resistance genes and may act as efficient vehicles for horizontal gene transfer.

Keywords:

Bacteriophages, antibiotic resistance genes, hospital and WWTP effluents, public health.

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The wide use of antibiotics have contributed to the spread of antibiotic resistance owing to the release of antibiotic compounds, antibiotic-resistant bacteria, and antibiotic resistance genes (ARGs) into the environment [1]. Aquatic environments constitute large reservoirs of antibiotic resistant bacteria carrying ARGs which could be spread among bacteria through horizontal gene transfer [2]. This phenomenon is mediated by the transfer of mobile genetic elements such as plasmids, genomic islands, transposons and integrons by conjugation, transformation or also by bacteriophage transduction [3].

Several studies have demonstrated that transduction takes place in natural settings such as freshwater and wastewater environments and they further identified bacterial 16S ribosomal DNA sequences in the viral fraction of the wastewater, showing the ability of phages to carry bacterial genes [4,5]. In fact, recent studies have been focused on the role of phages as potential candidates for the spread of ARGs [6], as they may act as efficient vehicles for horizontal gene transfer and recombination [7,8] more frequently than previously believed [9]. Given the fact that up until now the number of studies regarding the contribution of bacteriophages to the acquisition of ARGs in the environment is limited, the aim of this study was focused on the detection of $bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM} genes, which confer resistance to β-lactams, and *qnrA*, *qnrB* and *qnrS* genes, which confer reduced susceptibility to fluoroquinolones, in different environmental settings. These ARGs were quantified by real-time PCR (qPCR) in the phage DNA fraction of environmental water samples. Moreover, we also quantified the selected ARGs in bacterial fraction to compare the results obtained in phages.

Five water samples were obtained from two different wastewater treatment plants (WWTPs) and from two hospitals, all located in Girona except one WWTP which was located in Ripoll, all of them in the Autonomous Community of Catalonia, Spain. In the case of both WWTPs, samples were collected from the treated effluents. Regarding the hospitals, in Josep Trueta (JT) hospital, samples from the untreated effluent containing all the sewage from the hospital were taken, while in Santa Caterina hospital we had the opportunity to collect water samples from two untreated effluent outlets, the first one containing the wastes derived from hospitalized patients (SC) and the second one representing wastewater which came exclusively from the emergency department (ED-SC). Four replicates of one-liter samples were collected, kept at 4 °C and transported immediately to the laboratory for analysis.

All samples were initially evaluated for faecal contamination by enumerating *Escherichia coli*, total coliforms and somatic coliphages. *E. coli* and coliforms were detected and quantified using Chromocult coliform agar (Merck, Darmstadt, Germany), whereas somatic coliphages were enumerated by standardized methods, as previously described [10]. The four replicates of each sample were subsequently filtered through low protein-binding 0.22-μm-pore-size membranes (Millipore, Bedford, MA), which allowed the passage of phage particles in the filtrate, while bacteria were retained on the membrane surface. Bacteria were resuspended in lysis buffer for the following DNA extraction, which was performed using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The filtrates which contained viruses were concentrated to 1 ml using 100 kDa Amicon Ultra centrifugal filter units (Millipore) and were treated with DNase (100 U/ml) to eliminate free DNA outside the phage particles. Phage DNA was then extracted and purified as described previously [6].

The six selected ARGs were quantified in the bacterial and phage DNA fractions by qPCR. All qPCR assays were performed in duplicate on an Mx3005P system (Agilent Technologies, Santa Clara, CA) using SYBR Green detection chemistry. Each reaction was carried out in a total volume of 30 μl, containing 1 μl of template, the corresponding concentration of each primer (from 0.2 to 0.6 μM) and 2× Brilliant III Ultra Fast QCPR Master Mix (Agilent Technologies). Primers used in each qPCR assay are given in Table S1. Standard curves were generated using known quantities of cloned target genes. Briefly, PCR amplicons from positive controls were cloned using TOPO TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Plasmids were then extracted using the PureLink Plasmid kit (Invitrogen), and copy number was calculated as described previously [11]. Tenfold serial dilutions of plasmid DNA were amplified in triplicate to establish the standard curve for each qPCR assay. Statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL).

Plate counts showed that faecal indicators levels, both bacterial and viral, were significantly higher ($p<0.05$) in the hospital effluent samples than those from WWTP discharge points (Table 1), as expected, since it has been demonstrated that wastewater treatment processes can significantly reduce the microbial load [12].

However, there is evidence that some treatment plants may increase the proportion of resistant bacteria at the end of the process [13].

Table 1. Faecal coliforms and *E. coli* as bacterial indicators and somatic coliphages as viral indicators detected in five different sampling points.

Samples	Log (CFU/ml)*		Log (PFU/ml)*
	Faecal coliforms	*E. coli*	Somatic coliphages
Girona WWTP	2.87 (0.05)	2.19 (0.10)	1.65 (0.33)
Ripoll WWTP	1.42 (0.15)	0.58 (0.68)	1.31 (0.23)
JT Hospital	4.95 (0.07)	4.49 (0.05)	4.00 (0.05)
SC Hospital	5.80 (0.10)	4.88 (0.11)	4.61 (0.08)
ED – SC Hospital	4.29 (0.13)	3.14 (0.05)	2.15 (0.17)

*Mean values (n = 4) and standard deviations (in parentheses) are shown.

The quantification results for all ARGs analysed are shown in Figure 1. Overall, higher copy numbers (p<0.05) of ARGs were detected in the bacterial DNA fraction than in the phage DNA fraction. However, ARGs such as *qnrS* and bla_{SHV} were found in the phage DNA from all samples analysed, reaching up to 4 log10 copy numbers/ml in the SC hospital and JT hospital effluent samples, respectively. Comparing the results among different sampling sites, wastewater samples from hospitals showed high prevalence of ARGs, both in bacterial and viral DNA, than those samples from WWTP effluents. These values were in accordance with culture results, since we found more ARG quantities where there was more faecal pollution and consequently, the microbial load was higher. Moreover, it is likely that microorganisms would have acquired ARGs in the gastrointestinal tract of hospitalized patients under antibiotic treatment and then excreted to hospital wastewater. Phage results agree with previous studies which have reported the presence of these viruses in sewage carrying various β-lactamase genes [14]. The samples collected at the emergency department showed a lower prevalence

of all ARGs than the hospital effluents. This could be explained by the fact that the faecal input to wastewater from emergency department is smaller than the one from long-stay hospitalization area. In Ripoll WWTP, the bla_{SHV} and *qnrS* genes were only detected in the phage DNA fraction. Interestingly, the culture results from this sampling point showed the highest proportion of somatic coliphages in relation to bacterial faecal indicators in comparison to other sampling points, suggesting that in this case, phages have persisted better in the environment than their bacterial hosts, as previously reported [3].

In conclusion, we demonstrated that phages may be efficient vectors for the acquisition and dissemination of ARGs [6,14]. Taking into account that the viruses of prokaryotes are highly diverse and constitute the majority of organisms on the planet [15], the potential reservoir of genes that can be transferred by phages is immense [16]. Thus, considering the growing evidence that clinical resistance is closely associated with antibiotic resistance in environment, the role of bacteriophages in ARGs transfer cannot be overlooked.

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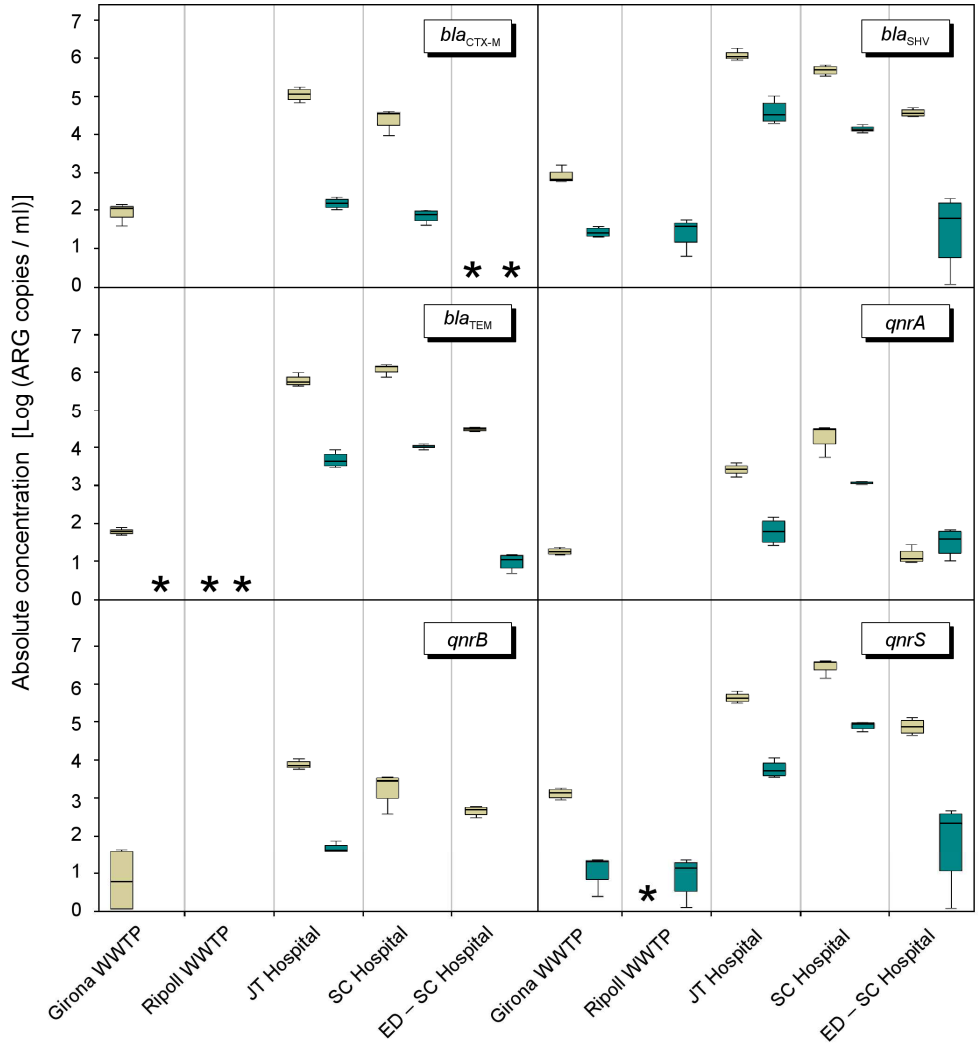

Figure 1. Absolute concentration of ARGs in the bacterial (light brown) and phage (green) DNA fraction of different environmental samples. Within the box plot chart, the crosspieces of each box plot represent (from top to bottom) maximum, upper-quartile, median (black bar), lower-quartile, and minimum values. Asterisks (*) indicate values below the detection limit.

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Supplementary Table S1. Primer sequences used in this study.

Target gene	Primers	Sequence	Reference
qnrA	qnrAf-RT	ATTTCTCACGCCAGGATTTG	Modified from [1]
	qnrAr-RT	GCAGATCGGCATAGCTGAAG	
qnrB	qnrBmF	GGMATHGAAATTCGCCACTG	Modified from [2]
	qnrBmR	TTYGCBGYYCGCCAGTCGAA	
qnrS	qnrSrtF11	GACGTGCTAACTTGCGTGAT	[3]
	qnrSrtR11	TGGCATTGTTGGAAACTTG	
bla_{TEM}	bla-TEM, FX	GCKGCCAACTTACTTCTGACAACG	[4]
	bla-TEM, RX	CTTTATCCGCCTCCATCCAGTCTA	
$bla_{\text{CTX-M}}$	RTCTXM-F	CTATGGCACCACCAACGATA	Modified from [5]
	RTCTXM-R	ACGGCTTTCTGCCTTAGGTT	
bla_{SHV}	RTblaSHVF	CGCTTTCCCATGATGAGCACCTTT	[4]
	RTblaSHVR	TCCTGCTGGCGATAGTGGATCTTT	

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Chapter 6

Prevalence of plasmid-mediated quinolone resistance determinants and their association with extended-spectrum β-lactamases in ciprofloxacin-resistant strains isolated from a wastewater treatment plant and the receiving river

Prevalence of plasmid-mediated quinolone resistance determinants and their association with extended-spectrum β-lactamases in ciprofloxacin-resistant strains isolated from a wastewater treatment plant and the receiving river

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Keywords: antibiotic resistance, *qnr* genes, β-lactamases, wastewater treatment plant, biofilm, sediment.

Abstract

The prevalence of the *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr* genes was evaluated in 252 ciprofloxacin-resistant isolates from biofilm and sediment samples collected at the discharge point of a wastewater treatment plant (WWTP) and the receiving river. We also determined whether these isolates harboured plasmid-encoded β-lactamases, such as TEM, SHV and CTX-M. Moreover, the phylogenetic affiliation of ciprofloxacin-resistant bacteria and antibiotic concentrations were also determined. Most of the ciprofloxacin-resistant isolates belonged to the *Gammaproteobacteria* class and 17 of them carried a *qnr* gene. In particular, 15 isolates carried the *qnrS* gene and 2 carried the *qnrB* gene. Among the *qnr*-positive isolates, 12 harboured the *aac(6')-Ib-cr* gene and 2 of them also carried a β-lactamase on the same plasmid, indicating that they may be transferred simultaneously. It is also noteworthy that all *qnr*-positive isolates identified as *Aeromonas* species harboured the same *qnrS* allele, namely the *qnrS2*. Due to the low prevalence of the *qnr* genes in ciprofloxacin-resistant isolates we could not determine if the prevalence of these genes in the environment is influenced by WWTP discharges. However, our findings highlight the role of environmental bacteria as vehicles for dissemination of antibiotic resistance genes.

Introduction

Antibiotic resistance is not a new phenomenon as the first penicillin-resistant strain was isolated from a hospitalised patient in the 1940s, just a few years after the introduction of this antibiotic (Levy, 2002). However, this first warning was ignored because the resistant pathogens could be treated with the new antimicrobials developed throughout the 1950s and 1960s. Today, due to the increased appearance of resistant bacteria and the difficulties in producing new antimicrobials, the antibiotic resistance has become a significant global health concern (World Health Organization, 2001).

Although antibiotic resistance is common in naturally occurring bacteria, even in pristine environments (Huerta *et al*., 2013), previous studies suggest that when the resistance determinants reach the environments related to human activities, the evolution of antibiotic resistance is remarkably accelerated (Galán *et al*., 2013). Municipal wastewater treatment plants (WWTPs) represent one of the environments with a high anthropogenic contribution. WWTPs have been described as reservoirs of bacteria with a large content of mobile genetic elements, such as plasmids, transposons or integrons harbouring antibiotic resistance genes (Moura *et al*., 2012) and the presence of antibiotics has also been reported in both influents and effluents of municipal WWTPs (Hirsch *et al*., 1999; Miao *et al*., 2004; Karthikeyan & Meyer, 2006). Although the antibiotic concentrations reported in WWTPs are well below the minimum inhibitory concentration (MIC) for most bacteria, they are sufficient to produce effects in the bacteria such as transcription changes (Davies *et al*., 2006) and modulation of the interactions within microbial communities (Martínez, 2008). Recent reports have shown that several antibiotic resistance genes (ARGs) may have been transferred between environmental bacteria and clinical pathogens because their nucleotide sequences were identical (Szczepanowski *et al*., 2009; Forsberg *et al*., 2012). Considering that the presence of antibiotics may contribute to the dissemination of antibiotic resistance genes (Martinez, 2009; Marti *et al*., 2013a), further investigation on the effects of releasing WWTP secondary effluents into the aquatic environment is required.

Quinolones were clinically introduced in the 1960s and years later, they were chemically modified to obtain the fluoroquinolones, a more potent antibiotic that had a wider spectrum of activity than its predecessors. These antibiotics interact with DNA gyrase and topoisomerase IV, blocking the DNA replication, which leads to cell death (Robicsek *et al*., 2006). Following the hypothesis that antibiotic producers are the origin of antibiotic resistance genes as mechanism to self-protect, environmental bacteria were not thought to harbour quinolone resistance genes because these antimicrobials are synthetic (Hernández *et al*., 2011). Nevertheless, resistance to quinolones can be achieved by chromosomal mutations in the genes encoding DNA gyrase or topoisomerase IV, by efflux systems which reduce the antibiotic concentration in the cytoplasm, and by acquiring quinolone resistance determinants through horizontal transfer (Ruiz, 2003). Different plasmid-mediated quinolone resistance (PMQR) determinants have been identified, such as the Qnr proteins, the aminoglycoside acetyltransferase AAC(6')-Ib-cr, and the efflux pumps QepA and OqxAB (Poirel *et al*., 2012). Qnr proteins are pentapeptide repeat proteins that block the action of ciprofloxacin on bacterial DNA gyrase and topoisomerase IV, whereas the cr variant of the gene *aac(6')-Ib* encodes an aminoglycoside acetyltransferase with the amino acid changes Trp102Arg and Asp179Tyr, that can inactivate ciprofloxacin by N-acetylation of its piperazinyl amine (Park *et al*., 2006). Despite the fact that PMQR determinants confer low-level resistance, they can be disseminated between bacteria and the simultaneous presence of two or more determinants in the same microorganism has an additive effect, increasing the MIC values of quinolones (Ruiz *et al*., 2012). In addition, the association of *qnr* and *aac(6')-Ib-cr* with genes encoding extended-spectrum β-lactamases (ESBLs) has been reported, indicating that resistance to quinolones and β-lactams may be transferred simultaneously (Briales *et al.*, 2012; Dobiasova *et al.,* 2013).

This study was carried out in a WWTP area previously selected for a broad investigation into the occurrence of several ARGs in the aquatic environment using culture-independent methods (Marti *et al*., 2013b). Results from our previous study demonstrated that WWTPs act as a reservoir of ARGs that may be transferred to bacteria from the receiving river. Thus, the present

study aimed to examine the prevalence of PMQR determinants in ciprofloxacin-resistant isolates from the WWTP discharge point and from the receiving river and to determine whether these isolates also harboured plasmid-encoded β-lactamases such as TEM, SHV and CTX-M. A combination of culture-dependent and culture-independent methods was therefore used to obtain information on the resistance phenotypes conferred by the detected genes and the phylogenetic affiliation of bacteria carrying those genes. Antibiotic concentrations were also measured to evaluate any possible association between the detected ARGs and antibiotics discharged from the WWTP. Knowing which bacteria harbour ARGs in environmental settings may help to define strategies to prevent the transfer of these genes to human pathogens.

Materials and Methods

Study site and sample collection

Samples were collected at the discharge point of the Ripoll WWTP and the receiving river, the Ter river. This river is located in the north-east of Spain and it supplies 43% of raw drinking water for the city of Barcelona and its surrounding area (www.aiguesdebarcelona.cat). The Ter river receives the wastewater effluents from several WWTPs, including the discharges from the Ripoll WWTP. Samples from the final effluent of this WWTP and 100 m upstream and downstream of the treated wastewater discharge point were collected during two sampling events (June and September, 2010). Biofilm samples were obtained from submerged stones by scraping the surface and sediment samples were collected from the top layer (0-5 cm) from each location. Water and sediment samples were also taken to determine antibiotic concentrations. All samples were stored at 4°C until they were processed in the laboratory.

Antibiotic concentration

A method based on solid phase extraction (SPE) and analysis by ultra performance liquid chromatography (UPLC) was used to measure antibiotic concentrations in water and sediment samples. The antibiotics analysed are shown in Table 1. A Baker vacuum system (J.T. Baker, Deventer, The Netherlands) was used to pre-concentrate the water samples using a method

previously described (Gros *et al*., 2013). Briefly, water sample was pH-adjusted to 3 with 1.0 M HCl and 10 ml of 1 M EDTA (4 %, v/v) was added. Oasis HLB cartridges (60 mg, 3 ml) (Waters, Milford, MA, USA) were loaded with 250 ml of water samples. Cartridges were eluted with 10 ml of methanol and the extracts were then evaporated under a gentle nitrogen stream and reconstituted with 1 ml of a methanol-water mixture (50:50, v/v). Sediment samples were extracted following a procedure previously described (Yang *et al*., 2010). Briefly, freeze-dried sediment samples (1.0 g) were sonicated in 10 ml of 0.2 M citric buffer (pH 4.0) and acetonitrile solution (50:50, v/v) for 15 min. Each extract was centrifuged and the supernatant was then diluted to 500 ml and purified with SPE following the procedure mentioned above. Water and sediment extracts were analysed using an UPLC chromatographic system (Waters) coupled to a Qtrap 5500 mass spectrometer (Applied Biosystems, Foster City, CA, USA), as previously described (Gros *et al*., 2013). Chromatographic separation was performed at 30°C on an ACQUITY UPLC HSS T3 column (1.8 µm, 2.1 × 50 mm) supplied by Waters.

Isolation and phylogenetic identification of the isolates

Each sample (approximately 20 g of sediment and 3 g of biofilm) were homogenized with an equal volume of sterile phosphate-buffered saline solution (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2), and serially diluted with the same solution. Several dilutions were plated on different culture media supplemented with ciprofloxacin (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 5 mg L^{-1}. This concentration was selected according to the CLSI guidelines, which consider ciprofloxacin-resistant strains those that grow in the presence of a concentration greater than or equal to 4 mg $L^{-1}$of ciprofloxacin (CLSI, 2007). Tryptic soy agar (TSA; Scharlau, Barcelona, Spain), R2A (Scharlau), Chromocult® (Merck, Darmstadt, Germany), Pseudomonas agar (Conda laboratories, Madrid, Spain) and Cytophaga agar (0.05% tryptone, 0.05% yeast extract, 0.02% sodium acetate and 1.5% agar, adjusted to pH 7.2) were the selected culture media used to isolate different bacterial species. All plates were incubated at 22°C for 48 h, except for the Chromocult® plates, which were incubated overnight at 37°C. After incubation, morphologically different colonies were isolated and subcultured for further analysis. Selected

ciprofloxacin-resistant isolates were subjected to DNA extraction by boiling method, followed by amplification of the 16S rRNA gene by PCR using universal primers 27F and 1492R as previously described (Lane 1991). Additionally, two housekeeping genes, *gyrB* (encoding the β-subunit of DNA gyrase) and *rpoD* (encoding the σ70 subunit of RNA polymerase), were amplified to establish the taxonomic affiliation of some ciprofloxacin-resistant isolates belonging to the genus *Aeromonas*, following the conditions previously described (Soler *et al*., 2004). PCR products were checked by 1.5% (w/v) agarose gel electrophoresis (Bio-Rad, Hercules, CA, USA) at 100 V for 30 min.

Sequencing of the purified PCR products was performed using the BigDye Terminator cycle sequencing kit on an ABI 3730XL DNA analyser (Applied Biosystems) according to the manufacturer's instructions. The obtained sequences were edited using the Sequence Scanner Software v1.0 (Applied Biosystems) and compared against the sequences available in the GenBank, EMBL and DDBJ databases obtained from the National Center for Biotechnology Information using the BLAST program and the Eztaxon-e database (Kim *et al*., 2012). A phylogenetic tree was also constructed using the software MEGA version 5.0 (Tamura et al., 2011), in order to define the evolutionary relationship among ciprofloxacin-resistant isolates belonging to the genus *Aeromonas*.

Detection of PMQR and ESBL genes

All ciprofloxacin-resistant isolates were screened by PCR for the presence of PMQR determinants, namely the *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr* genes. PCR detection for the *qnr* genes and the *aac(6')-Ib cr* gene was performed as previously described (Park *et al*., 2006; Cattoir *et al*., 2007). Positive controls were included for *qnrA1* (*Escherichia coli* strain 226), *qnrB2* (*E. coli* strain J53 pMG301), *qnrS1* (*E. coli* strain J53 pMG306) and *aac(6')-Ib-cr* (*E. coli* strain J53 pMG298). The *qnr*-positive isolates were also investigated for the presence of $bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM} using the primers described previously (Perilli *et al*., 2002; Mazzariol *et al*., 2003; Boyd *et al*., 2004). *E. coli* strain J53 pMG298, *E. coli* strain J53 pMG301 and *E. coli* strain 488 were used as positive controls for $bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM}, respectively. PCR products

were checked by agarose gel electrophoresis, which were further confirmed by direct sequencing as mentioned above.

Plasmid analysis

Transfer of the *qnr*-positive plasmids from isolates was attempted by using transformation and conjugation techniques, using *E. coli* TOP10 and azide-resistant *E. coli* J53 strains as recipient strains, respectively. Transformants were selected on Luria Bertrani (Sigma-Aldrich) 2x agar plates containing ciprofloxacin (0.6 mg L^{-1}) and transconjugants were selected in the same media containing ciprofloxacin (0.6 mg L^{-1}) plus azide (150 mg L^{-1}). Plasmid extraction from each *qnr*-positive isolate and its corresponding transformant or transconjugant was performed by alkaline lysis method (Sambrook & Russell, 2001). Extracted plasmids from transformants and transconjugants were run on a 0.7% agarose gel together with molecular weight markers, and plasmid sizes were then determined using PyElph software (Pavel & Vasile, 2012). Plasmids from transformants or conjugants were confirmed to be *qnr* positive by multiplex PCR described above. Moreover, the presence of ESBL genes was also screened by PCR when required.

Antibiotic susceptibility testing

All *qnr*-positive isolates were evaluated for resistance to different antibiotic agents by using the disk diffusion susceptibility method on Mueller-Hinton agar (Scharlau). Eight antibiotics were selected, including the β-lactam and quinolone families, such as amoxicillin (AML, 25 μg), ampicillin (AM, 10 μg), penicillin (P, 6 μg), carbenicillin (CB, 100 μg), cefalotin (CF, 30 μg), nalidixic acid (NA, 30 μg), norfloxacin (NOR, 10 μg) and pipemidic acid (PI, 20 μg) (Bio-Rad). After incubation at 22-37°C for 24-48h, zones of bacterial growth inhibition were measured and the results were interpreted according to Clinical and Laboratory Standards Institute guidelines.

Results

Antibiotic concentrations

The highest antibiotic concentrations were found in water samples from WWTP effluents, and the most abundant antibiotic families found were macrolides and quinolones, with spiramycin

and ofloxacin as the main representatives, respectively (Table 1). In contrast, very low concentrations were found in water samples from the river, both upstream and downstream from the WWTP, where most of antibiotics were not detected or their concentrations were below the quantification limit. For antibiotic concentrations in the sediment samples, fewer compounds were detected compared with the water samples but, some compounds, such as ciprofloxacin and ofloxacin were detected at higher concentrations in the river samples than at the WWTP discharge point. For ofloxacin, its concentration in river sediments was the highest found for all antibiotics measured (40.4 ng g^{-1}), even though it was not found in river water samples collected at the same sampling point.

Identification of the isolates

From all ciprofloxacin-resistant isolates obtained from the different sampling points and different culture media (see Table S1), a total of 252, morphologically different isolates (105 from the first sampling and 147 from the second) were selected to be further identified and to study the prevalence of the *qnr* genes. Analysis of 16S rRNA gene sequences showed that most of the sequences displayed high similarity to those of known species and all isolates were identified at least to the genus level (Table 2). Most of the ciprofloxacin-resistant isolates belonged to the *Gammaproteobacteria* and were closely related to the *Enterobacteriaceae* family, primarily the *Escherichia* and *Shigella* genera, and to the *Acinetobacter* and *Arthrobacter* genera. For *qnr*-positive isolates, the phylogenetic tree based on concatenated sequences of the 16S rRNA, *gyrB* and *rpoD* genes revealed that most of those isolates belonging to the genus *Aeromonas* were closely related to *A. media*, *A. caviae* and *A. hydrophila* subsp. *hydrophila* (Figure 1).

The 16S rRNA gene sequences from this study have been deposited in the EMBL nucleotide sequence database under the accession numbers from HF936801 to HF937052. Nucleotide sequences of the two housekeeping genes (*gyrB* and *rpoD*) are available as supplementary material.

Detection of PMQR and β-lactamase genes

All ciprofloxacin-resistant isolates were examined by PCR for the presence of *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr*. The *qnr* genes were detected in 8 isolates (7.6%) from the first sampling and 9 isolates (6.1%) from the second sampling, mostly at the WWTP discharge point and in downstream samples. Among the 17 isolates, 15 carried the *qnrS* gene (14 *Aeromonas* sp. and one *Raoutella terrigena*), and two carried the *qnrB* gene (*Citrobacter freundii* and *Klebsiella oxytoca*). The *qnrA* gene was not detected in any of the studied isolates. Sequence analysis of PCR products revealed the specific variant of each *qnr*, except for the *qnr* genes from the P1B7, P1E10 and 2P1B12 strains, in which the variant could not be determined because the nucleotides of the amplified fragment were homologous between two different variants. It is remarkable that all *qnr*-positive isolates identified as *Aeromonas* species harboured the *qnrS2* allele. The 17 *qnr*-positive isolates were also screened for the *aac(6')-Ib* gene, and 13 of them carried this gene (Table 3). DNA sequence analysis indicated that 12 of these 13 sequences correspond specifically to the *aac(6')-Ib-cr* variant. All sequences of the *qnr* genes and *aac(6')-Ib-cr* gene are available as supplementary material. Furthermore, PCR results for ESBL showed that three *qnr*-positive isolates were also positive for the bla_{SHV} gene, and one isolate was positive for the bla_{TEM} gene; another isolate carried both the bla_{TEM} and $bla_{\text{CTX-M}}$ genes. Regarding the sensitivity patterns to tested antibiotics, including β-lactam antibiotics, cephalosporins and quinolones, no specific pattern was observed in relation to the detected *qnr*, *aac(6')-Ib-cr*, and β-lactamase genes (Table 3).

Association between the PMQR and ESBL genes

Plasmid analysis revealed that most of the *qnr* genes were located on plasmids ranging from 48 to 54 kb, except plasmids from isolates P1B7, P1E10 and 2P1B12, which had a molecular size greater than 80 kb, plasmid from P2G1 isolate, which had a size of 27 kb, and the plasmids from P2B5 and 2P1E3 showed a size of 7 kb (Figure 2). Moreover, it was also determined that among the isolates that harboured both *qnr* and ESBL genes, only the P1B7 and 2P1B12 isolates showed an association between *qnr* determinants and ESBL on the same plasmid. In particular, P1B7

harboured the *qnrS* and *bla*$_{TEM}$ genes, whereas 2P1B12 carried the *qnrB* and *bla*$_{CTX-M}$ genes. In contrast, all of the *aac(6')-Ib-cr* genes detected were present on the same plasmid with the *qnr* gene for each *qnr*-positive isolate.

Discussion

Antibiotic concentrations found in WWTP effluents support the hypothesis that these substances are not completely eliminated by the WWTP, and thus, some end up in the environment, i.e., in water compartments and sediments (Kümmerer, 2009; Michael *et al*., 2013). However, the concentrations detected in the river were much lower, indicating that a significant dilution factor occurs once antibiotics reach the river. In our study, most of the detected antibiotics in WWTP effluents were in the range of ng L^{-1}, which is comparable to the occurrence of antibiotics in other WWTP effluents (Karthikeyan & Meyer, 2006; Gros *et al.*, 2012). The presence of some fluoroquinolones and macrolides in downstream samples confirms that these antibiotics show the highest persistence and are frequently detected in wastewater and surface waters (Brown *et al*.. 2006). Moreover, both families include antibiotics with high concentrations in sediment samples, indicating that quinolones and macrolides released by WWTP can be retained in the river sediments. Quinolones are well-known to be able to bind to soil and sediments, which delays their biodegradation (Martinez, 2009). On the other hand, the presence of antibiotics in the upstream river samples may indicate that, in addition to the WWTP discharges, the river receives other antibiotic inputs, such as runoff from nearby fields fertilised with contaminated manure (Jechalke *et al*., 2013). Our failure to detect tetracyclines and β-lactams may be due to their susceptibility to photodegradation and hydrolysis, respectively (Kümmerer, 2009).

The second aim of this work was to study the diversity of ciprofloxacin-resistant bacterial groups. Although the results obtained are based on culture-dependent methods, which are known to underestimate the actual microbial diversity, this is the only method to identify and characterise bacteria that harbour genes of interest. To increase the diversity of bacterial isolates, different culture media were used, allowing the isolation of ciprofloxacin-resistant

bacteria belonging to different taxonomic groups. This methodology was unlike several earlier studies that only considered members of the family *Aeromonadaceae* or/and *Enterobacteriaceae* (Goñi-Urriza *et al*., 2000; Ferreira da Silva *et al*., 2007; Akiyama & Savin, 2010), which are known to possess intrinsic mechanisms of antibiotic resistance. Thus, in addition to *Aeromonas*, *Escherichia* and *Shigella* species, we also detected many *Acinetobacter* and *Arthrobacter* species, indicating that resistance to quinolones is not exclusive to a few taxonomic groups, which agrees with previous findings (Takasu *et al*., 2011). Moreover, our results showed that ciprofloxacin-resistant strains were ubiquitous in both sediment and biofilm from aquatic environments, even at sites where no quinolone or fluoroquinolone was detected. In contrast, because of the results of bacterial counts and percentages of resistant isolates did not follow the same pattern between sampling events (Table S1), we could not compare the number of ciprofloxacin-resistant isolates regarding the culture media and the sampling point.

Focusing on the detection of PMQR determinants, the low prevalence of *qnr* genes (6.9% as the average for two samplings) among ciprofloxacin-resistant isolates could be explained since other mechanisms such as chromosomal mutations or efflux systems may be conferring resistance to fluoroquinolones. However, the higher prevalence of the *qnrS* gene compared with the *qnrB* gene among *qnr*-positive isolates and the non-detection of the *qnrA* gene in this study was consistent with other studies from environmental settings (Takasu *et al*., 2011; Chen *et al*., 2012). Although the prevalence of these determinants is mostly studied in clinical isolates, our results are in accordance with previous studies that have shown that the *qnrS* variant is the most prevalent among *qnr* genes in the environment (Poirel *et al*., 2012). Moreover, we found that the *qnrS* gene, specifically the *qnrS2* allele, is commonly carried by *Aeromonas* spp., which has also been previously described (Cattoir *et al*., 2008; Picão *et al*., 2008). The detection of the *qnr* genes in different-sized plasmids demonstrates that these genes were located in different vehicles of dissemination. Moreover, the results from the phylogenic tree together with the plasmid analyses showed that all of the *Aeromonas* identified were not clonally related. Members of the genus *Aeromonas* are frequent inhabitants of aquatic environments and primary fish pathogens,

as well as opportunistic pathogens responsible for gastroenteritis in humans (Han *et al*., 2012). These observations emphasise the importance of this genus in the spread of antibiotic resistance determinants. However, the detection of the *qnrB* gene in *Citrobacter freundii* and *Klebsiella oxytoca* agrees with the idea that the prevalence of this gene is high among *Enterobacteriaceae* species (Poirel *et al*., 2012).

Regarding the fluoroquinolone-modifying enzyme gene *aac(6')-Ib-cr*, 12 of 17 *qnr*-positive isolates co-harboured this gene, which is known to act additively with other resistance mechanisms, such as *qnr* genes (Robicsek *et al*., 2006). The prevalence of studied ESBLs among isolates carrying PMQR was 29.4%, which reinforces the previously association found in other studies (Cattoir *et al*., 2007; Wang *et al*., 2008). In addition, two isolates harboured a *qnr* gene and *bla* gene on the same plasmid, indicating that they can be transferred simultaneously. The presence of different ARGs in the same vehicle for dissemination is a matter of concern because the selective pressure exerted by only one antibiotic may contribute to the spread of resistance determinants to several antibiotics. Results obtained from antibiograms confirm the presence of multidrug resistant bacteria in the environment. However, since we only analysed some ARGs among the great number of mechanisms that confer antibiotic resistance, we were unable to establish a relationship between resistance phenotypes and the presence of the PMQR and ESBL studied. Most of the *qnr*-positive isolates in this study belonged to the genus *Aeromonas*, which are known to possess a chromosomal AmpC that may be responsible for the β-lactam resistance observed (Henriques *et al*., 2006).

In summary, the low prevalence of the *qnr* genes among the analysed isolates could be due to our study was not restricted to *Enterobacteriaceae* and *Aeromonadaceae,* families with a high prevalence of *qnr* genes according to reported data. Moreover, to determine if WWTP discharges increase the *qnr* genes prevalence in the environment, more studies should be done since we did not find notable differences between the proportions of *qnr* genes among the sites analysed. However, the high prevalence of the *qnrS* gene in *Aeromonas* spp., which are water-borne bacteria, and the high number of multiresistant isolates detected suggest that aquatic

environments are important reservoirs for ARGs. These findings highlight the importance of studying antibiotic resistance in both the environment and clinical settings. Overall, further investigation is required because reports demonstrating the dissemination of ARGs from environmental bacteria to human pathogens are limited.

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Table 1. Antibiotic concentrations in water and sediment samples from two samplings[a].

Antibiotics		Antibiotic concentration in water (ng/L)						Antibiotic concentration in sediment (ng/g)					
		Upstream		Downstream		WWTP[b]		Upstream		Downstream		WWTP	
		S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
Macrolides	Erythromycin					51.2	49.9						1.48
	Azythromycin			1.46	13.07	42.5	34.5		1.44		1.38		16.97
	Clarithromycin				bdl[c]	25.74	83.83			bql	1.47	1.89	0.90
	Spyramycin					158.9	173.3						
β-lactams	Cefotaxime				bdl		14.77						
Quinolones & Fluoroquinolones	Pipemidic Acid			bql	bql	4.59	6.04						
	Ciprofloxacin	bql[d]		bql	6.26	54.33	70.89		1.06	5.26	14.45		
	Difloxacin					bql	bql						
	Enrofloxacin												2.43
	Flumequine								bql				
	Ofloxacin			bql		81.84	80.01		4.19	17.1	40.4		
	Norfloxacin					24.12	34.84					15.04	19.26
Sulfonamides	Sulfamethoxazole					12.42	29.16					bql	bql
	Sulfathiazole					bql	bql	0.40	10.3			14.56	3.38
	Sulfapyridine					bql	2.71						
Tetracyclines	Chlorotetracycline		bdl				bdl						
Lincosamides	Clindamycin					6.45	6.42						
Other	Trimethoprim					bdl	2.63					3.70	0.44

[a] S1, first sampling; S2, second sampling.
[b] WWTP denotes samples collected at the WWTP secondary effluent.
[c] bdl, below detection limit.
[d] bql, below quantification limit

Table 2. Phylogenetic affiliation of ciprofloxacin-resistant isolates from the WWTP discharge point, upstream and downstream river samples[a]

		No. of isolates from[b]:						
		Biofilm			Sediment			
Phylum	Genus or species	WWTP	UP	DOWN	WWTP	UP	DOWN	Total
α-proteobacteria	*Brevundimonas naejangsanensis*				1			1
	Brevundimonas terrae		1		1			2
	Novosphingobium spp.				1			1
	Phenylobacterium sp.	1						1
β-proteobacteria	*Achromobacter spanius*	2	1	1	2		1	7
	Comamonas spp.	2					1	3
γ-proteobacteria	*Acinetobacter bouvetii*			1				1
	Acinetobacter johnsonii	4	5	3	1	3	7	23
	Acinetobacter oryzae	1	2					3
	Acinetobacter parvus					1		1
	Acinetobacter spp.	9	5	11	4	10	7	46
	Aeromonas caviae			1		1		2
	Aeromonas hydrophila subsp. hydrophila			1				1
	Aeromonas media	2					4	6
	Aeromonas spp.	3	1	2	2	7	1	16
	Citrobacter freundii	1	1			1	2	5
	Escherichia coli	5	3	2	4	2	2	18
	Escherichia fergusonii		2	1	1	2	2	8
	Klebsiella oxytoca			1				1
	Pseudomonas fuscovaginae	1						1
	Raoutella terrigena			1	1			2
	Rheinheimera texanensis	2						2
	Salinibacterium spp.	1						1
	Shigella boydii						1	1
	Shigella dysenteriae				1			1
	Shigella flexneri	1	4	3	2	4	4	18
	Shigella sp.			1				1
	Stenotrophomonas terrae	1						1
	Stenotrophomonas maltophilia		2				1	3
	Stenotrophomonas humi				2			2
	Stenotrophomonas rhizophila	1		1	3		1	6
	Stenotrophomonas spp.						2	2
	Streptococcus anginosus	1						1
Actinobacteria	*Agrococcus jejuensis*		1			1		2
	Arthrobacter defluvii					1		1
	Arthrobacter kerguelensis				2			2
	Arthrobacter nicotinovorans		1		2			3
	Arthrobacter nitroguajacolicus		1	2	3	5	3	14
	Arthrobacter sulfureus					1		1

	Arthrobacter spp.		1	1		1		3
	Microbacterium testaceum		2		1			3
	Microbacterium spp.	1		1				2
Bacteroidetes	*Cloacibacterium normanense*						2	2
	Cloacibacterium rupense					1	1	2
	Flavobacterium oncorhynchi	2		1				3
	Flavobacterium spp.		2	1				3
	Myroides odoratimimus					1		1
	Myroides spp.					1		1
	Pedobacter agri			1				1
	Pedobacter alluvionis			1				1
	Pedobacter caeni			1				1
	Pedobacter roseus					1		1
	Pedobacter soli		1			1		2
	Pedobacter steynii			2			1	3
	Pedobacter spp.		2	1	3	1	1	8
	Wautersiella spp.					1		1
Firmicutes	*Enterococcus casseliflavus*				1			1
	Lactobacillus pentosus			1				1
Other	*Cellulosimicrobium funkei*	1						1
TOTAL		42	38	43	38	47	44	252

[a] For each sampling site, bacterial isolates of two sampling times were combined.

[b] WWTP, WWTP discharge point; UP, upstream from the WWTP discharge; DOWN, downstream from the WWTP discharge.

Table 3. Distribution of ESBLs and *aac(6')-Ib* genes among *qnr*-positive isolates, including their phylogenetic affiliation and their resistance phenotype.

Isolate	Species	*qnr* alleles	*aac(6')-Ib*	ESBLs	Source	Resistance phenotypes[a]
First sampling						
P1B7	*Raoutella terrigena*	S1/S3	–	TEM	Sediment (WWTP[b])	NA, PI, AM,P,CB, AML
P1D3	*Aeromonas* sp.	S2	+, cr[c]	SHV	Sediment (upstream)	NA, NOR, PI, CF, AM, P,CB,AML
P1E10	*Citrobacter freundii*	B14/B18	–	SHV	Sediment (downstream)	NA, NOR, PI, CF, AM, P, AML
P4E3	*Aeromonas media*	S2	+, cr	–	Sediment (downstream)	NA, NOR, PI, CF, AM, P,CB,AML
P2A2	*Aeromonas media*	S2	+, cr	–	Biofilm (WWTP)	NA, NOR, PI, CF, AM, P,CB,AML
P2G1	*Aeromonas* sp.	S2	+, cr	–	Biofilm (WWTP)	NA, NOR, PI, CF, AM, P,CB,AML
P2A12	*Aeromonas* sp.	S2	+, cr	SHV	Biofilm (upstream)	NOR, PI, AM, P, AML
P2B5	*Aeromonas hydrophila* subsp. *hydrophila*	S2	–		Biofilm (downstream)	NA, NOR, PI, CF, AM, P,CB,AML
Second sampling						
2P1F9	*Aeromonas caviae*	S2	+, cr	–	Sediment (upstream)	NA, NOR, PI, CF, AM, P,CB,AML
2P2E7	*Aeromonas* sp.	S2	+, cr	–	Sediment (upstream)	PI, CF, AM, P,CB,AML
2P1G3	*Aeromonas media*	S2	–	–	Sediment (downstream)	NA, PI, CF, AM, P,CB,AML
2P2A9	*Aeromonas media*	S2	+, cr	–	Sediment (downstream)	PI, CF, AM, P,CB,AML
2P2A10	*Aeromonas* sp.	S2	+, cr	–	Sediment (downstream)	PI, CF, AM, P,CB,AML
2P2F1	*Aeromonas media*	S2	+, cr	–	Sediment (downstream)	NA, NOR, PI, CF, AM, P,CB,AML
2P1F1	*Aeromonas media*	S2	+	–	Biofilm (WWTP)	NA, NOR, PI, CF, AM, P,CB,AML
2P1B12	*Klebsiella oxytoca*	B6/B17	+, cr	TEM, CTX-M	Biofilm (downstream)	NA, AM, P, AML
2P1E3	*Aeromonas caviae*	S2	+, cr	–	Biofilm (downstream)	NA, PI, CF, AM, P,CB,AML

[a] NA, nalidixic acid; NOR: norfloxacin; PI, pipemidic acid; CF, cefalotin; AM, ampicillin; P, penicillin; CB, carbenicillin; AML, amoxicillin.

[b] WWTP, WWTP discharge point

[c] cr, cr variant.

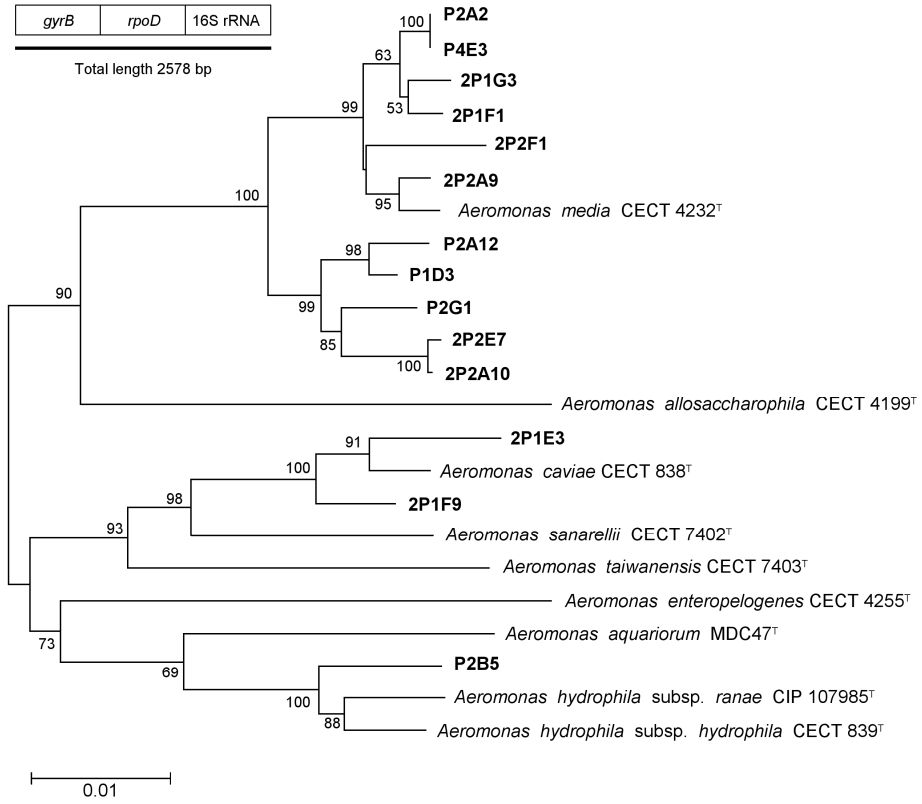

Figure 1. Phylogenetic tree of some ciprofloxacin-resistant isolates harboring *qnr* genes with the most closely related *Aeromonas* strains, based on 16S rRNA, *gyrB* and *rpoD* gene sequences and constructed by the neighbour-joining method. Bootstrap percentages (>50%) based on 1,000 replications are shown at branch nodes. Bar, 1% estimated sequence divergence.

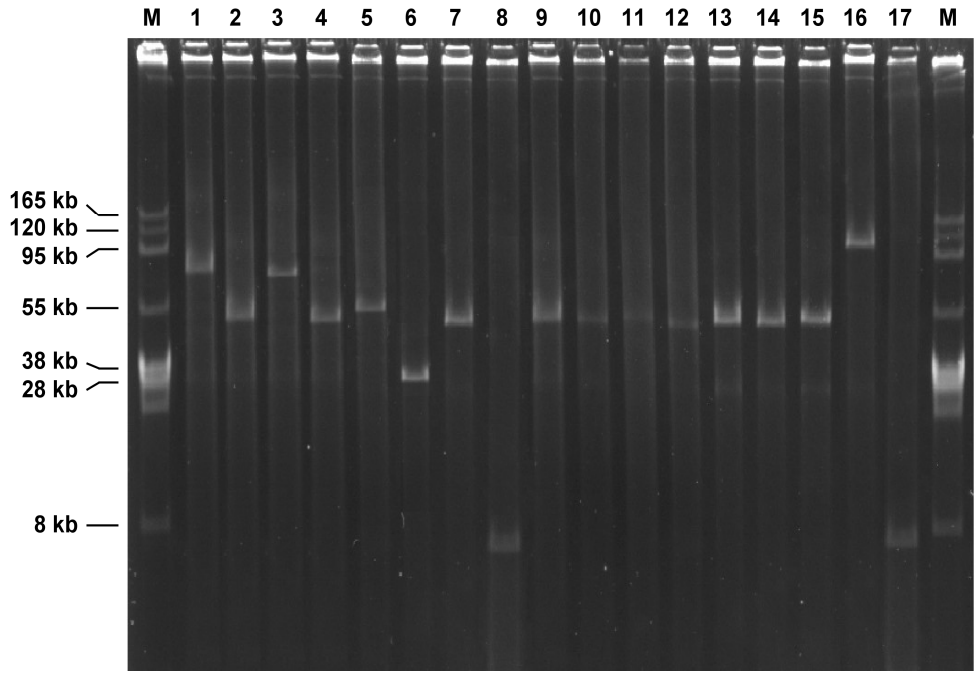

Figure 2. Plasmid profiles. Lanes: M, BAC-Tracker Supercoiled DNA ladder (Epicentre); 1, *E. coli*-pP1B7 transformant; 2, *E. coli*-pP1D3 transformant; 3, *E. coli*-pP1E10 transformant; 4, *E. coli*-pP4E4 transformant; 5, *E. coli*-pP2A2 transformant; 6, *E. coli*-pP2G1 transformant; 7, *E. coli*-pP2A12 transformant; 8, *E. coli*-pP2B5 transformant; 9, *E. coli*-p2P1F9 transformant; 10, *E. coli*-p2P2E7 transformant; 11, *E. coli*-p2P1G3 transformant; 12, *E. coli*-p2P2A9 transformant; 13, *E. coli*-p2P2A10 transformant; 14, *E. coli*-p2P2F1 transformant; 15, *E. coli*-p2P1F1 transformant; 16, *E. coli*-p2P1B12 transformant; 17, *E. coli*-p2P1E3 transformant.

Supplementary information

Table S1. Bacterial counts (BC) on different culture media (expressed in CFU g^{-1}) and the percentage of ciprofloxacin-resistant strains (% Resistants).

		Biofilm				Sediment			
		First sampling		Second sampling		First sampling		Second sampling	
Sampling area	Culture medium	BC	% Resistants	BC	% Resistants	BC	% Resistants	BC	% Resistants
WWTP	Chromocult agar	7.00E+04	1.09	1.00E+04	1.60	5.50E+04	0.26	1.40E+03	3.14
	R2A	5.34E+06	0.38	8.90E+05	1.46	5.46E+06	1.98	8.20E+05	0.12
	TSA	2.92E+06	9.38	1.00E+06	1.40	3.10E+06	10.84	2.20E+05	0.18
	Cytophaga agar	1.00E+06	2.88	5.00E+05	28.00	6.60E+05	11.52	6.20E+05	0.29
	Pseudomonas agar	2.40E+04	39.17	1.17E+05	6.24	4.00E+05	4.00	3.80E+04	2.11
Upstream	Chromocult agar	4.02E+04	1.84	2.60E+04	1.27	7.60E+03	0.97	3.20E+04	2.81
	R2A	5.40E+07	1.26	1.00E+06	0.40	6.92E+05	12.43	2.88E+06	0.31
	TSA	5.98E+07	1.24	5.00E+05	1.80	4.16E+05	7.26	1.60E+06	0.74
	Cytophaga agar	6.44E+07	1.24	1.20E+05	70.83	2.30E+05	2.87	3.80E+05	18.95
	Pseudomonas agar	1.10E+06	42.00	3.40E+04	20.59	4.80E+04	7.92	8.80E+03	15.91
Downstream	Chromocult agar	2.18E+04	1.74	2.20E+04	1.05	1.76E+05	0.17	4.20E+03	0.70
	R2A	2.10E+06	1.54	1.63E+06	0.25	5.16E+06	0.60	4.00E+04	2.10
	TSA	2.72E+06	7.35	8.20E+05	1.05	1.60E+06	52.50	1.32E+05	0.70
	Cytophaga agar	9.60E+05	3.29	3.00E+05	34.67	1.04E+06	5.77	1.16E+05	3.10
	Pseudomonas agar	6.80E+04	26.47	2.60E+04	6.54	5.00E+04	32.00	1.60E+03	1.25

Sequences of the *gyrB* gene

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Sequences of the *rpoD* gene

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Sequences of the *qnr* genes:

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CCAGCCGTGTACATGGTTCAAACACGCGG

Chapter 7

Multidrug resistance-encoding plasmid from *Aeromonas* sp. strain P2G1

Multidrug resistance-encoding plasmid from *Aeromonas* sp. strain P2G1

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Abstract

A plasmid (pP2G1), which confers multidrug resistance in an environmental *Aeromonas* species, was completely sequenced using a shotgun approach. Plasmid pP2G1 encoded resistance to aminoglycosides and quinolones [*aac(6′)-Ib-cr*], β-lactams (bla_{OXA-1}), chloramphenicol (*catB3*), macrolides [*mphA-mrx-mphR*], quaternary ammonium compounds (*qacEΔ1*), quinolones (*qnrS2*), rifampicin (*arr-3*) and sulphonamides (*sul1*). These findings suggest that *Aeromonas* species may potentially act as reservoirs of antibiotic resistance genes.

Keywords: *Aeromonas* species, antibiotic resistance genes, environment, multidrug resistance, plasmid-mediated antibiotic resistance

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Antibiotic resistance has become a major public health concern because the organisms that cause infections are becoming less sensitive to antibiotic treatment. Antibiotic resistance traditionally has been studied in bacterial pathogens, limiting efforts to only clinically identified mechanisms [1]. However, antibiotic resistance can also arise in non-pathogenic bacteria as a result of horizontal gene transfer. Moreover, antibiotics are released daily into the natural environment with treated wastewater effluents and through use in animal husbandry. Aquatic bacteria could, therefore, provide a reservoir for antibiotic resistance genes. In this study, we investigated a multidrug-resistant *Aeromonas* sp. strain P2G1, isolated from the Ter River in Ripoll, Spain.

Strain P2G1 was identified by 16S rRNA gene sequencing (corresponding to positions 27–1492 in the *Escherichia coli* gene) as *Aeromonas* sp., with 99.4% sequence similarity to its closest relatives, *Aeromonas hydrophila* subsp. *ranae* LMG 19707^T and *Aeromonas caviae* ATCC 15468^T. Antibiotic susceptibility tests were determined using the broth microdilution method according to the CLSI guidelines [2]. The strain was resistant to amoxicillin and ciprofloxacin (MICs of >64 $\mu g/mL^{-1}$ and 16 $\mu g/mL^{-1}$, respectively).

An initial screening, using a multiplex PCR assay for simultaneous detection of *qnr* genes [3], showed the presence of a *qnrS* gene. Subsequently, plasmid DNA extraction was performed using a PureLink Quick plasmid miniprep kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations. *Escherichia coli* TOP10 was used as a recipient strain for transformation assays, and transformants were selected on Mueller-Hinton (MH) agar plates containing nalidixic acid (3 $\mu g/mL^{-1}$). As expected, no growth was observed on antibiotic control plates when plasmid DNA was not added to the cells. Further analysis identified a single 26.6-kb plasmid (pP2G1) from *E. coli* transformants, which conferred increased MIC values of several antibiotics (Table 1).

The complete nucleotide sequence of pP2G1 was determined using a shotgun sequencing approach (Macrogen, Seoul, Korea). Briefly, randomly sheared plasmid fragments of 3–4 kb were cloned and transformed into *E. coli* DH10B. Inserts were sequenced by dye terminator chemistry. The sequences were then assembled with phrap (phred/phrap/consed; available from http://www.phrap.org). Nucleotide and amino acid sequence analyses were performed with NCBI (http://www.ncbi.nlm.nih.gov) and European Bioinformatics Institute (http://www.ebi.ac.uk) analysis tools.

The nucleotide sequence of plasmid pP2G1 (EMBL-Bank accession number HE616910) consisted of 26 645 bp in length and belonged to the IncU incompatibility group. Plasmids belonging to this incompatibility group have been isolated from *Aeromonas* species [4–6], as well as from clinical isolates of *E. coli* [7]. Annotation of the pP2G1 nucleotide sequence revealed the presence of a *qnrS2* gene inserted within the *mpR* gene encoding a putative zinc-metalloprotease. The nucleotide sequence identified downstream of the *qnrS2* gene was identical to that found in *qnrS2*-positive *Aeromonas* strains from France and Switzerland [5,6]. However, sequence analysis of the regions flanking the 5′ end of the *qnrS2* gene revealed that an IS element, IS*Kpn9*, was present upstream of *qnrS2* in strain

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TABLE 1. Antibiotic susceptibility profiles for *Aeromonas* sp. strain P2G1 and *E. coli* TOP10 harbouring natural plasmid pP2G1

Strain	MIC (mg/L)[a]												
	AMX	CAZ	CIP	NAL	NOR	ENR	LEV	OFX	SMX	RIF	GEN	KAN	ERY
Aeromonas sp. P2G1	>64	<0.06	16	64	32	16	4	16	>128	16	0.5	32	>32
E. coli TOP10 + pP2G1	64	<0.06	0.24	4	1	0.72	0.24	0.48	>128	>128	0.25	2	>32
E. coli TOP10	4	<0.06	<0.01	1	0.03	<0.01	<0.01	0.01	16	16	0.25	1	<0.1

[a]AMX, amoxicillin; CAZ, ceftazidime; CIP, ciproflocaxin; NAL, nalidixic acid; NOR, norfloxacin; ENR, enrofloxacin; LEV, levofloxacin; OFX, ofloxacin; SMX, sulphamethoxazole; RIF, rifampicin; GEN, gentamicin; KAN, kanamycin; ERY, erythromycin.

P2G1 (Fig. 1). This IS element belongs to the IS*As1* family, which has been identified in a *Klebsiella pneumoniae* clinical strain [8]. Analysis of the IS*Kpn9* insertion sites revealed a target site duplication (CTATTTTACC) in strain P2G1, in which the *qnrS2* gene was plasmid located. This suggests that transposition of IS*Kpn9* occurs independently of *qnrS2* gene acquisition. Moreover, putative promoter sequences were found in the 125-bp sequences that separated the IRL of IS*Kpn9* from the ATG site of the *qnrS2* gene, which suggests that IS*Kpn9* is not involved in *qnrS2* expression.

A class 1 integron was also identified (Fig. 1), which included four integrated resistance gene cassettes, namely *aac(6′)-Ib-cr*, bla_{OXA-1}, *catB3* and *arr-3*, encoding an aminoglycoside acetyltransferase, an oxacillinase conferring resistance to β-lactams (penicillins and cephalosporins), an acetyltransferase conferring resistance to chloramphenicol and an ADP-ribosylating transferase conferring resistance to rifampicin, respectively [6]. This class 1 integron showed an identical structure to the In37 integron [9], and the 3′-conserved segment included the *qacEΔ1* and *sul1* genes, which provide resistance to quaternary ammonium compounds and sulphonamide, respectively. The In37 integron has been described in an *E. coli* isolate from China [9] and in a *K. pneumoniae* clinical isolate from Argentina [10]. However, the integron identified in plasmid pP2G1 was not associated with an ISCR1 element, as previously reported in those

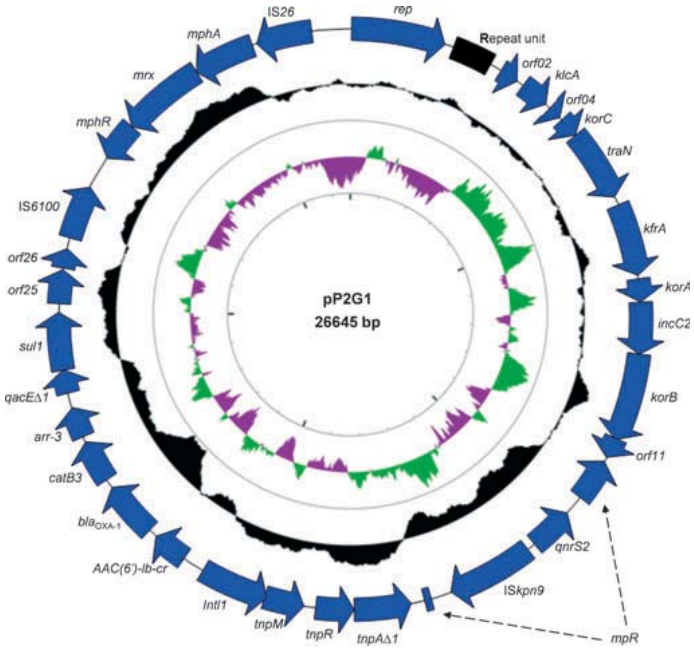

FIG. 1. Genetic map of the multidrug-resistant plasmid pP2G1. Coding regions are shown by arrows indicating the direction of transcription. The two inner circles represent the G+C content plotted against the average G+C content of 50% (black circle) and GC skew information (green and purple circles). The G+C plot was generated by using the CGView tool [17].

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isolates from Argentina and China [9,10]. In addition, it is important to note that *aac(6′)-Ib-cr*, another plasmid-mediated quinolone determinant, was found in association with the *qnrS2* gene on the same plasmid, which may have contributed to reduced ciprofloxacin and norfloxacin susceptibility in both the donor and the transformant strains (Table 1).

Interestingly, a macrolide resistance operon *mphA-mrx-mphR* was located downstream and in the opposite orientation from an IS element, IS*6100*. The macrolide-resistance operon encodes a macrolide phosphotransferase (MphA), a protein required for MphA expression (Mrx) and a negative transcriptional regulator (MphR), and confers high-level erythromycin resistance [11,12]. In fact, as a result of the acquisition of the macrolide resistance operon, the susceptibility of *E. coli* transformants harbouring pP2G1 to erythromycin decreased substantially (Table 1). The macrolide-resistance operon *mphA-mrx-mphR* and its surrounding regions have been previously found on a *K. pneumoniae* $bla_{CTX\text{-}M\text{-}62}$-encoding plasmid, pJIE137 [13], and on an *E. coli* $bla_{CTX\text{-}M\text{-}15}$-encoding plasmid, pEK499 [14].

Antibiotic resistance genes have been detected in several *Aeromonas* species [5,6,11]. Moreover, those species have been isolated from different sources and geographical areas [15,16]. Thus, our findings contribute to highlighting the role of environmental *Aeromonas* species as reservoirs of antibiotic resistance genes, which may have important public health implications because of the evolution and emergence of antibiotic resistance genes.

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Transparency Declaration

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Chapter 8

Main Results and Discussion

This PhD thesis has been prepared as a compendium of publications. Each research article from chapters 3 to 7 comprises its own results and discussion. Therefore, the aim of the present chapter is to summarise the main results of the thesis and offer a more global discussion. Moreover, a future perspectives section has been included to present recommendations for further research on antibiotic resistance in the environment to counteract this major and growing problem.

1. Main Results

The first objective of this thesis was to develop and validate molecular techniques to quantify ARGs. Quantitative methods are needed to monitor the prevalence of the genes in the environment to study differences over time, differences between compartments and to determine whether ARG concentrations increase due to anthropogenic effects. At the beginning of this project, no well-established quantitative method was available to quantify the *qnr* genes. Therefore, three qPCR assays were designed to quantify the PMQR determinants *qnrA*, *qnrB* and *qnrS* (chapter 3). The efficiency and sensitivity obtained indicated the validity of the three qPCR assays. Moreover, the qPCR assays were widely applicable for samples from different sources, as the assays provided accurate quantification using several types of water samples and chicken faeces. Similarly, qPCR assays to quantify genes encoding resistance to the main antibiotic families, such as beta-lactams (bla_{TEM}, $bla_{\text{CTX-M}}$ and bla_{SHV}), tetracyclines [*tet*(O) and *tet*(W)], sulfonamides [*sul*(I) and *sul*(II)] and macrolides [*erm*(B)], were optimised (chapter 4). Each qPCR assay was improved in terms of efficiency and specificity because the previously designed primers did not operate conveniently with our equipment and reactives.

The second objective was to assess the effect of WWTP effluent discharges on bacterial communities in the receiving river, focusing on the quantification of several ARGs and the shifts in the structure of bacterial communities. For the quantification of ARGs, the qPCR assays that were previously developed and optimised were used to quantify the selected ARGs in biofilm and sediment samples from the discharge point of a WWTP, as well as from downstream and upstream areas of the receiving river.

Except for *qnrA* and *qnrB*, all ARGs were detected in the samples analysed. For the *qnrB*, *qnrS*, bla_{TEM}, bla_{SHV}, *erm*(B), *sul*(I), *sul*(II), *tet*(O) and *tet*(W) genes in biofilm samples, we found a higher copy number of these ARGs at the WWTP discharge point than in the river. It is also noteworthy that the relative abundances of these genes were significantly higher (p<0.05) in the downstream biofilm samples than in the upstream samples. To look at shifts in the structure of bacterial communities among samples, their total DNA was analysed using the 454-pyrosequencing technology. The analysis using the phylogeny-based parsimony test showed a statistically significant difference (p<0.001) in community structure between all analysed samples (chapter 4).

Another specific objective was to evaluate the role of bacteriophages as a reservoir of ARGs in the environment. In this sense, the genes $bla_{CTX\text{-}M}$, bla_{SHV}, bla_{TEM}, *qnrA*, *qnrB* and *qnrS* were quantified by qPCR in the phage DNA fraction of environmental water samples and in the bacterial fraction. Although the highest copy numbers (p<0.05) of ARGs were detected in the bacterial DNA fraction, *qnrS* and bla_{SHV} genes were found in the phage DNA from all samples analysed, reaching up to 4 log10 copy numbers/ml in hospital samples. Moreover, the amount of ARGs in the different samples analysed was proportional to faecal pollution (chapter 5).

The last specific aim of this thesis was to characterise resistant strains from a WWTP discharge point and the receiving river. First, 252 ciprofloxacin-resistant, morphologically different isolates were obtained from biofilm and sediment samples from the WWTP discharge point and the downstream and upstream river. Analysis of the 16*S* rRNA gene sequence showed that most of the ciprofloxacin-resistant isolates belonged to the Gammaproteobacteria and were closely related to the Enterobacteriaceae family, mainly to the *Escherichia* and *Shigella* genera, and were also related to the *Acinetobacter* and *Arthrobacter* genera (chapter 6). All ciprofloxacin-resistant isolates were examined by PCR for the presence of PMQR.

The *qnr* genes were detected in 18 isolates (7.2% on average for 2 samplings), mostly at the WWTP discharge point and in downstream samples. Among these resistant isolates, 16 carried the *qnrS* gene (15 *Aeromonas* sp. and 1 *Raoutella*

terrigena) and 2 carried the *qnrB* gene (*Citrobacter freundii* and *Klebsiella oxytoca*). The *qnrA* gene was not detected in any of the isolates studied. Moreover, 12 of the 18 *qnr*-positive isolates carried the *aac(6')-lb-cr* variant, and five *qnr*-positive isolates were also positive for one ESBL. Further analysis of the isolates that harboured both the *qnr* and ESBL genes revealed that two isolates showed an association between PMQR and ESBL in the same plasmid (chapter 6). As *qnrS* were mostly found in *Aeromonas* spp., particularly the *qnrS2* allele, we investigated a multidrug resistance-encoding plasmid from an *Aeromonas* sp. to gain a deeper insight into the role of this species as a reservoir of ARGs. Plasmid pP2G1 conferred resistance to aminoglycosides and quinolones [*aac(6')-Ib-cr*], β-lactams (bla_{OXA-1}), chloramphenicol (*catB3*), macrolides (*mphA-mrx-mphR*), quaternary ammonium compounds (*qacEΔ1*), quinolones (*qnrS2*), rifampicin (*arr-3*) and sulphonamides (*sul1*) (chapter 7).

2. Discussion

Previous studies have suggested that ARGs naturally occur in microbial populations localised in environments with little human influence. In fact, a highly diverse collection of genes encoding resistance to β-lactam, tetracycline and glycopeptide antibiotics was detected in DNA sequences recovered from 30,000-year-old Beringian permafrost sediments (D'Costa et al., 2011). However, the natural environment has broadly changed under human stewardship and is now influenced by numerous pollutants that result from anthropogenic activities. Environments with different degrees of human influence can be observed, ranging from hospitals that have a strong antibiotic selective pressure to mountain lakes that have minimal human influence on the ecology of antimicrobial resistance. Nevertheless, due to the movement of antibiotics and antibiotic resistance genes between different habitats, mainly through water pathways but also through humans and animals, it may be questionable that any environment can be considered absolutely pristine (Kallenborn et al., 2007). As a consequence, there is growing concern that the aquatic environment may act as a reservoir for drug resistant bacteria and ARGs, thereby contributing to the widespread dissemination of antibiotic resistance. Therefore, the goal of this thesis

was to gain knowledge about the occurrence of ARGs in aquatic microbial communities influenced by anthropogenic activities.

2.1. Prevalence of ARGs in the environment

In this thesis, ARGs were detected in many environs, including different matrices (water, biofilm and sediment), different organisms (bacteriophages and bacteria) and different sampling sites (rivers, effluents from several human and veterinary hospitals, subterranean water, chicken faeces and WWTP effluents), indicating that these emerging pollutants are widely distributed in environments exposed to human activities. This observation is consistent with studies conducted in tap water (Xi et al., 2009), municipal wastewater (Volkmann et al., 2004; Gao et al., 2012), a river (Pei et al., 2006), a river estuary (Henriques et al., 2006b; Chen et al., 2013), a fish farm (Di Cesare et al., 2013), a harbour (LaPara et al., 2011) and swine feedlot wastewater (Li et al., 2012), among others. Collectively, these studies suggest that there is a large concentration of ARGs in anthropogenic-impacted sites. In our studies, we particularly observed a higher amount of ARGs in WWTP and hospital effluents than in the rivers, which mirrored the pattern we observed for antibiotic concentrations. Although we did not have sufficient data to establish correlations, the results obtained in this thesis represent one of the first approaches to environmental ARG occurrence in our area because most of the studies of antibiotic resistance in Spain are still focused on clinical settings.

Furthermore, the development and optimisation of quantitative techniques, such as qPCR, allowed us to compare the concentrations of ARGs associated with anthropogenic impacts, providing a more direct measure than previous culture-dependent methods. However, high concentrations of ARGs do not necessarily imply selection by antibiotics in a specific site because ARGs can be selected in other sources and then reach the environment through waste discharges (Martinez and Olivares, 2012). In this sense, this thesis, similar to most of the studies conducted at present, fails to distinguish the process by which ARGs increase in human-impacted sites. Further studies on the genetic environment of ARGs will be useful to determine the environmental origin of ARG proliferation.

2.2. Bacteriophages as a reservoir of ARGs

Although bacteriophages are accepted to play a crucial role in mobilising genetic elements between bacteria, there are surprisingly few studies investigating the transfer of ARGs through phages in the environment. Our experiments, similar to previous studies (Muniesa et al., 2004; Colomer-Lluch et al., 2011a, Colomer-Lluch et al., 2011b,), conclude that there are many ARGs circulating between environmental bacteriophages, making bacteriophages a potential reservoir for ARGs. We also observed a large amount of ARGs in phage DNA from samples that were highly contaminated with faecal pollution, suggesting that the ARGs detected were from phages excreted by human or animals. Because phages persist longer than their bacterial hosts in aquatic environments (Lucena et al., 2003) and because a high frequency of transduction of ARGs has been demonstrated in vitro (Blahova et al., 1993; Di Luca et al., 2010), bacteriophages feasibly mediate ARG transfer in natural environments. Moreover, a study using metagenomic approaches revealed that resistance to antibiotics was enriched in phage metagenomes from antibiotic-treated mice (Modi et al., 2012). Thus, the transfer of ARGs by phages from environmental anthropogenic-impacted sites could likely also increase due to the presence of antibiotics released into aquatic habitats.

2.3. Antibiotic-resistant bacteria

Because molecular techniques did not allow identification of the bacterial strains harbouring ARGs, culture-dependent methods were used to isolate ciprofloxacin-resistant bacteria. As mentioned in the Introduction section, ciprofloxacin was chosen for the study of resistant strains because Spain has one of the highest usage rates of fluoroquinolones in Europe (Asensio et al., 2011). Most of the ciprofloxacin-resistant strains found belonged to the Proteobacteria phylum, which comprises only Gram-negative bacteria. Gram-negatives are characteristically highly efficient at up-regulating or acquiring genes that code for mechanisms of antibiotic drug resistance, especially in the presence of antibiotic selection pressure (Peleg et al., 2010). This property could explain the capability of the isolates to grow in the presence of ciprofloxacin.

The low prevalence of *qnr* genes among ciprofloxacin-resistant isolates could be explained by the criteria used for selecting these strains (5 mg/L of ciprofloxacin) because this favours the growth of strains with mechanisms conferring high-level resistance. Because PMQR is well known to confer low-level resistance, the actual prevalence of Qnr determinants could be underestimated in this study. However, the relatively high prevalence of the *qnrS* gene with respect to the *qnrB* gene and the low or non-detection of the *qnrA* gene was consistent with other studies performed in environmental settings (Chen et al. 2012; Kaplan et al., 2013). In fact, our results are consistent with a recent review (Poirel et al. 2012) that stated that the *qnrS* variant is the most prevalent *qnr* gene in the environment.

Although the study of the prevalence of *qnr* genes has traditionally focused on clinical enterobacterial strains, progressively more studies have found these ARGs in other environmental Gram-negative bacteria, such as *Aeromonas* (Cattoir et al., 2008), *Vibrio* (Cattoir et al, 2007) and *Pseudomonas* (Ahmed et al., 2007; Tran et al., 2011). Our data support the hypothesis that environmental bacteria may be a reservoir of PMQR because we mostly found *qnr* genes in *Aeromonas* spp. Because all *Aeromonas* isolated carried the *qnrS2* allele, one plasmid carrying the *qnrS* from a positive strain (P2G1) was further characterised. The same plasmid, which carried the *qnrS2* gene, also harboured genes conferring resistance to aminoglycosides, β-lactams, chloramphenicol, macrolides, quaternary ammonium compounds, rifampicin and sulphonamides. Our results, together with studies that identified other plasmids containing ARGs in *Aeromonas* (Cattoir et al., 2008; Picao et al., 2008), indicate that these bacterial species may act as a reservoir of antibiotic resistance in the aquatic environment. Because HGT from naturally occurring bacteria to clinical pathogens has been indirectly demonstrated through metagenomic studies (Forsberg et al., 2012), environmental bacteria, such as *Aeromonas*, are a potential source of ARGs.

2.5. Future perspectives

Due to the increasing problem of antibiotic resistance, there is a need to improve our understanding of the potential role of aquatic environments subjected to

anthropogenic impacts in the amplification of antibiotic resistance levels. Although the results from this study contribute to a better comprehension of the occurrence of ARGs in different human-impacted sites, environmental studies on antibiotic resistance remain scarce. Advances in this field are needed to develop strategies to combat the dissemination of resistance. These strategies should focus on both the environmental and clinical perspectives of the problem to be successful.

Because many studies have demonstrated that ARGs are primarily transported into aquatic environments, best management practices for limiting runoff should be taken into account. Contact between both ARB and other types of microbiota from different ecosystems increases the potential for genetic variation and the possible emergence of novel resistance mechanisms that are consequently reintroduced into the human environment (Baquero et al., 2008). Thus, the release of residues from hospitals containing ARB and antibiotics should be minimised to avoid exchange of genetic material (Martinez et al., 2009). Government regulations should be implemented to control the wastewater discharges, either treated or untreated, from urban, industrial and farming activities.

Future investigations focused on studying the flanking regions of ARGs are also required. Knowing the genetic environment of ARGs would be useful to track the dissemination of ARGs and determine if the increasing amount of ARGs in impacted sites is due to their transport from other sources or if they are selected on-site. Moreover, gaining information about the MGEs where the ARGs are located would provide knowledge about the sources and spread of ARGs among clinically relevant pathogens and naturally occurring bacteria.

Similarly, an examination of the wastewater treatment processes should be considered because WWTPs are one of the predominant sources of both antibiotics and ARGs. For example, water disinfection by chlorine may reduce the release of ARB. However, the application of chlorine occasionally results in the additional production of carcinogenic disinfection by-products. As an alternative, sand filtration could be used to achieve the same objective, with the advantage that it does not require the

addition of chemical products and does not generate harmful by-products (LaPara and Burch, 2012). Disinfection using ultraviolet light is also worth consideration because it is effective, although expensive (Baquero et al., 2008). In any case, it is important to find a balance between an efficient wastewater treatment technology that produces acceptable quality water and technology that has low energy requirements and operating costs.

Since most of society's healthcare gains are sustained by the use of antibiotics, the development of new substances that are effective against resistant pathogens is crucial to reduce the future impact of resistance. It is essential that governments encourage pharmaceutical companies to invest resources into research of new antimicrobials, although the development of other types of medicinal products may be more profitable. Knowing that resistance has subsequently developed for all new antibiotics introduced up to now, it would be interesting to test the risk for emerging resistance before launching a new antibiotic. In this regard, the new metagenomic tools and phylogenetic analysis can help us to identify resistance (or pre-resistance) genes in the environment that would be useful to predict the emergence of resistance to future antibiotics.

In conclusion, proliferation of antibiotic resistance is a continuous process because there is a constant introduction of ARGs and ARBs into the environment, together with both nutrients and stressors, such as antibiotics, heavy metals and disinfectants (McVey and Montforts, 2012). Moreover, it is now evident that naturally occurring microorganisms harbour a previously underestimated amount of ARGs. Therefore, our surrounding environment is a part of the ecosystem that we interact with, and its role in the dissemination of antibiotic resistance cannot be neglected.

Chapter 9

Conclusions

The main conclusions of the present thesis can be listed as follows:

- Three qPCR assays were developed for the rapid and specific quantification of the *qnrA*, *qnrB* and *qnrS* genes. Each assay was widely applicable in samples from different sources. The qPCR assays to quantify bla_{TEM}, $bla_{\text{CTX-M}}$, bla_{SHV}, *tet*(O), *tet*(W), *sul*(I), *sul*(II) and *erm*(B) were also optimised.

- The significant increase in the relative concentration for nearly all of the ARGs studied in the biofilm river samples after the WWTP effluent discharges and the significant differences with respect to community structure and composition at the genus level indicates an effect of WWTP discharges into the receiving river.

- Bacteriophages constitute a reservoir of ARGs in the environment. The detection of large amounts of ARGs in phage DNA from environments with high inputs of faecal pollution, suggests that the ARGs came from phages excreted by human or animals.

- Most of the ciprofloxacin-resistant strains isolated from a WWTP discharge point and from the receiving river belonged to the Gammaproteobacteria and were closely related to the Enterobacteriaceae. Altought the prevalence of *qnr* genes among the ciprofloxacin-resistant isolates was low, most of the *qnr* genes were detected in naturally occurring bacteria, supporting the idea that the natural environment is a potential reservoir of ARGs.

- Some *qnr*-positive strains harboured the gen *aac(6')-lb-cr* and also a gene encoding for an ESBL, indicating that there may exist an association between resistance to quinolones and resistance to β-lactams.

- A plasmid encoding multidrug resistance from an *Aeromonas* sp. was characterised, indicating that these environmental species may act as a reservoir of ARGs.

Chapter 10

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