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Occurrence and persistence of antibiotic resistance genes in river biofilms after wastewater inputs in small rivers

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ABSTRACT

The extensive use of antibiotics in human and veterinary medicine and their subsequent release into the environment may have direct consequences for autochthonous bacterial communities, especially in freshwater ecosystems. In small streams and rivers, local inputs of wastewater treatment plants (WWTPs) may become important sources of organic matter, nutrients and emerging pollutants, such as antibiotic resistance genes (ARGs). In this study, we evaluated the effect of WWTP effluents as a source of ARGs in river biofilms. The prevalence of genes conferring resistance to main antibiotic families, such as beta-lactams ($bla_{CTX\text{-}M}$), fluoroquinolones (*qnrS*), sulfonamides (*sul* I), and macrolides (*ermB*), was determined using quantitative PCR (qPCR) in biofilm samples collected upstream and downstream WWTPs discharge points in four low-order streams. Our results showed that the WWTP effluents strongly modified the hydrology, physico-chemistry and biological characteristics of the receiving streams and favoured the persistence and spread of antibiotic resistance in microbial benthic communities. It was also shown that the magnitude of effects depended on the relative contribution of each WWTP to the receiving system. Specifically, low concentrations of ARGs were detected at sites located upstream of the WWTPs, while a significant increase of their concentrations was observed in biofilms collected downstream of the WWTP discharge points (particularly *ermB* and *sul* I genes). These findings suggest that WWTP discharges may favour the increase and spread of antibiotic resistance among streambed biofilms. The present study also showed that the presence of ARGs in biofilms was noticeable far downstream of the WWTP discharge (up to 1 km). It is therefore reasonable to assume that biofilms may represent an ideal setting for the acquisition and spread of antibiotic resistance determinants and thus be considered suitable biological indicators of anthropogenic pollution by active pharmaceutical compounds.

1. Introduction

Antibiotics are widely used to treat or prevent bacterial infectious diseases in both human and veterinary medicine, but overuse and misuse has led to the increase of antibiotic-resistance among microbes (Servais and Passerat, 2009). This has caused antibiotic resistance to become a global health concern. Infectious microorganisms are becoming resistant to the commonly prescribed antibiotics, resulting in prolonged illness and greater risk of death (Cosgrove, 2006). Recent data from the European Centre for Disease Prevention and Control and the European Medicines Agency shows that every year approximately 25,000 European citizens die from infections caused by bacteria that developed antibiotics resistance (Borg, 2011). Moreover, it is estimated that more than 70% of bacteria causing these infections are resistant to at least one of the antibiotics commonly used to treat them (Muto, 2005).

Some bacteria are intrinsically resistant to antibiotics because they have either an impermeable membrane or they lack the antibiotic target, whereas others can actively pump antibiotics outside the cell by membrane efflux pumps. In other cases, bacteria

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acquire resistance to antibiotics through gene mutations that alter the target protein (e.g. the antibiotic binding-site) without affecting its functionality (e.g. mutations in *gyrA* and *parC* genes that encode DNA gyrase and topoisomerase IV conferring resistance to fluoroquinolones). Bacteria can also be resistant through the production of enzymes that inactivate antibiotics both by modification (e.g. covalent modification of aminoglycoside antibiotics catalysed by acetyltransferases) or degradation (e.g. such as that catalysed by β-lactamases acting on β-lactam antibiotics) (Allen et al., 2010). Susceptible bacteria may furthermore become resistant to antibiotics by acquiring resistance genes through horizontal gene transfer, which is largely, although not exclusively, responsible for the spread of antibiotic resistance among bacteria (Frost et al., 2005; Taylor et al., 2011).

Despite growing concerns on antibiotic resistance, this phenomenon has not been fully explored in environmental settings, possibly because antibiotic concentrations in non-clinical settings are generally very low (Marti et al., 2014). However, recent studies have revealed that sub-inhibitory concentrations of antibiotics, similar to those found in some aquatic environments (Kümmerer, 2009a, 2009b), may promote antibiotic resistance and select for resistant bacteria (Chow et al., 2015; Gullberg et al., 2011). Moreover, the extensive use of antibiotics in human and veterinary medicine and their subsequent release into the environment, via treated or untreated wastewater discharges, increasing use of recycled water in agricultural practices, and agricultural runoff, may have direct consequences for autochthonous bacterial communities, especially in freshwater ecosystems. Previous studies have shown the detrimental effect of antibiotics on the environment because of their effects on autochthonous bacteria and their impairment of biogeochemical processes or the degradation of organic pollutants (Buesing and Gessner, 2006; Garcia-Armisen et al., 2011; Proia et al., 2013a; Roose-Amsaleg and Laverman, 2015).

In small streams and rivers, local inputs of wastewater treatment plants (WWTPs) may become sources of organic matter, nutrients and emerging pollutants including antibiotic resistance genes (ARGs) (Pruden et al., 2006; Rysz and Alvarez, 2004). Many of these WWTPs receive inputs from municipal, clinical, agricultural, and industrial sources providing an optimal setting for the emergence and selection of antibiotic resistant bacteria (Amos et al., 2014). As a consequence, ARGs and resistant bacteria are released to the receiving water bodies through WWTP effluents (Marti et al., 2013; Rodriguez-Mozaz et al., 2015). Although the prevalence of ARGs has been studied in aquatic systems worldwide, most data focused on their abundance in the water column and the sediment of anthropogenic impacted systems (Huerta et al., 2013; Lapara et al., 2011; Pruden et al., 2012, 2006) whereas few studies address the role of biofilms in the acquisition and spread of ARGs in aquatic environment (Balcázar et al., 2015).

Streambed biofilms play a fundamental role in the trophic web and biogeochemical cycles (Battin et al., 2003; Lock, 1993), acting as an interface between the water and the riverbed (Sabater et al., 2007; Romaní, 2010). The short life cycle of biofilm microorganisms, the microbial interactions occurring among them and their reduced mobility allow for the detection of direct and indirect effects on the biofilm consortia on both short and long-term timescale (Proia et al., 2012). River biofilms can therefore be useful in determining the early effects of pollutants on freshwater ecosystems (Sabater et al., 2007) thus triggering their extensive use as indicators to assess the effects of priority and emerging compounds both in the field and in the laboratory (Bonnineau et al., 2010; Morin et al., 2010; Proia et al., 2011, 2013a, b). Antibiotic resistant bacteria and resistance determinants may integrate into biofilms (Donlan and Costerton, 2002; Engemann et al., 2008), together with other autotrophic and heterotrophic organisms. In contrast to the planktonic lifestyle, biofilms provide a more efficient environment for genetic exchange due to high cell density, proximity, and accumulation of mobile genetic elements (Gillings et al., 2009; Sorensen et al., 2005).

The occurrence of ARGs in river biofilm, sediments or water column has been reported along anthropogenic impacted riverine systems (e.g. Marti et al., 2013; Pruden et al., 2006; Rodriguez-Mozaz et al., 2015). However, this is the first extensive study evaluating the effect of WWTP effluents on the prevalence of ARGs in river biofilms. The abundance of genes conferring resistance to main antibiotic families, such as beta-lactams ($bla_{CTX\text{-}M}$), fluoroquinolones (*qnrS*), sulfonamides (*sul* I), and macrolides (*ermB*), was determined using quantitative PCR (qPCR) in biofilm samples collected upstream and downstream of WWTP discharge points in four small Mediterranean streams. The target ARGs were selected because these confer resistance to antibiotics commonly used in hospital and community settings in our region. Moreover, previous studies have demonstrated a higher prevalence of those antibiotics — considered to select our resistance genes — in water samples collected from Mediterranean rivers impacted by WWTP discharges (Gros et al., 2012; Rodriguez-Mozaz et al., 2015).

We hypothesize that WWTP effluent inputs into streams would favour increased levels of downstream biofilm biomass and ARGs. We theorize that the magnitude of the increase would be related to the relative contribution of the WWTP effluents to the stream flow: the higher the percentage of WWTP water, the greater the effects observed. Finally, we assumed that the alterations associated to the WWTPs would persist downstream of the effluents.

2. Materials and methods

2.1. Study sites

This study was performed on four streams within the Tordera River Basin (Catalonia, Spain), selected considering the domestic sewage contribution through WWTP discharges (Table 1). The streams had limited anthropogenic activity upstream of the WWTPs, and were selected for the WWTPs to include a gradient of population-equivalent (PE) treated and discharge released. The Gualba plant (GUA) treats 1035 PE and releases 207 m^3 day^{-1} to the Gualba stream. The Breda (BRE) plant treats 5600 PE and releases 906 m^3 day^{-1} to the Repiaix stream. The Arbúcies (ARB) plant treats 9000 PE and releases 2400 m^3 day^{-1} to the Xica stream. Finally, the Santa Maria de Palautordera (SMP) plant treats 11,663 PE and releases 3255 m^3 day^{-1} to the Tordera stream. In all the streams, three sites were selected i) 100 m upstream (UP); ii) 50–100 m downstream (DW), and; iii) 1 km downstream (DW1).

2.2. Water physical and chemical parameters

Discharge, water velocity, width and depth were measured at each section directly in the field with an acoustic-Doppler velocity meter (Sontek, YSI, USA). Conductivity, temperature, pH and dissolved oxygen were measured with sensor probes (Hach Lange, Germany) directly in the field. Water samples ($n = 3$ per site) for total organic carbon (TOC), phosphorus (TP) and nitrogen (TN) were collected and stored in polyethylene bottles. TOC was analysed with a Total Organic Carbon Analyser (TOC-V CSH, Shimadzu, Japan) using the catalytic oxidation method Total N and P were determined after alkaline digestion and subsequent spectrophotometric determination (Grasshoff et al., 1983).

2.3. Biofilm descriptors

Biofilm samples were randomly collected from cobbles along a