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 ($bla_{\text{CTX-M}}$), 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.

Capsule abstract

The study of biofilms in rivers revealed that small wastewater treatment plants can be a relevant source of antibiotic resistance genes to benthic communities of freshwater ecosystems.

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 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, 2009), 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 et al., 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 time-scale (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; Proia et al., 2013a&b). Antibiotic-resistant bacteria and resistance determinants may integrate into biofilms (Donlan et al., 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*$_{\text{CTX-M}}$), 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 1,035 PE and releases 207 m^3 day^{-1} to the Gualba stream. The Breda (BRE) plant treats 5,600 PE and releases 906 m^3 day^{-1} to the Repiaix stream. The Arbúcies (ARB) plant treats 9,000 PE and releases 2,400 m^3 day^{-1} to the Xica stream. Finally, the Santa Maria de Palautordera (SMP) plant treats 11,663 PE and releases 3,255 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 Analyzer (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 50–100 m stream section at each sampling site. The entire surface of each cobble was scrapped with sterile brushes and stored in filtered river water (0.2 µm Nylon Membrane Filters, Whatman, UK) to avoid the inclusion of suspended organisms. The surface of each cobble was measured by wrapping with aluminium foil. The foil was later dried and weighed, and cobble surface area estimated using a weight/area regression (McCreadie and Colbo, 1991). All variables were analysed in triplicate, and results expressed per unit surface area.

Each biofilm suspension was homogenized and two subsamples of known volume were filtered through pre-combusted (4 h at 450 °C) and pre-weighed 25 mm diameter GF/F Whatman glass fibre filters (0.7 µm pore size). One of the filters was used to

223 determine Ash Free Dry Mass (AFDW) after drying (72 h at 50 °C, for dry weight) and
224 combustion (4 h at 450 °C) and the remaining one was immersed in 10 ml of acetone
225 (90% v/v) for 12 h in the dark at 4 ºC for pigment extraction. Filters were then
226 homogenized, and the absorbance was measured with a Shimadzu UV-1800
227 spectrophotometer (Shimadzu, Japan). The Chl-*a* concentration was calculated by
228 means of the Jeffrey & Humphrey (1975) equation. The Autotrophic Index (AI) was
229 calculated for each replicate as the ratio between AFDW and Chl-*a* both expressed as
230 mg per cm^2 (Weber, 1973).

231 **2.4. Quantification of ARGs**

232 Biofilm samples were collected in triplicate from each sampling location, homogenized
233 in phosphate-buffered saline solution (PBS; 10 mM sodium phosphate, 150 mM sodium
234 chloride, pH 7.2) and centrifuged at 8,000 *g* for 10 min. Pellets were then re-suspended
235 in lysis buffer (20 mM Tris-Cl, pH 8.0; 2 mM sodium EDTA; and 1.2 % Triton X-100),
236 followed by enzymatic digestion with lysozyme (20 mg ml^{-1}) and proteinase K (10 mg
237 ml^{-1}). Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen;
238 Valencia, CA, USA), according to manufacturer's instructions. The DNA concentration
239 of each sample was measured using a Qubit 2.0 fluorometer (Life Technologies;
240 Carlsbad, CA, USA) and 260/280 ratios (Table S1) were determined using a NanoDrop
241 2000 spectrophotometer (Thermo Scientific; Wilmington, DE, USA). All DNA samples
242 were adjusted to 10 ng μl^{-1} for qPCR analysis.

243 The copy numbers of the selected ARGs (*bla*$_{CTX-M}$, *qnrS*, *sul* I and *ermB*) were
244 quantified by qPCR assays. Ten-fold dilutions of plasmid DNA containing known
245 concentration of the target gene were used as standard curves, which were generated by
246 cloning the amplicon from positive controls into *Escherichia coli* using the pCR2.1-
247 TOPO vector system (Invitrogen, Carlsbad, CA, USA). All qPCR assays were

performed in duplicate using SYBR green detection chemistry on a MX3005P system (Agilent Technologies; Santa Clara, CA, USA), as previously described (Marti et al., 2013). Briefly, each reaction contained 12.5 µl of 2× SYBR Green QPCR master mix (Agilent Technologies, Palo Alto, CA, USA), 200 nM each forward and reverse primer, 1 µl of 10 ng μl^{-1} DNA template, and the final volume was adjusted to 25 µl by adding DNase-free water. Each gene was amplified using specific primer sets (Table S2) and the PCR conditions included an initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s and at the annealing temperature given in Table S1 for 20 s. Copy number of the bacterial 16S rRNA gene was also quantified for normalization of the data, and the amplification conditions included an initial denaturation of 35 cycles at 95 °C for 15 s, followed by an annealing temperature at 60 °C for 1 min. A dissociation curve was applied at the end of each run to detect nonspecific amplifications. Ten-fold serial dilutions of the standards for each ARG were run in parallel with DNA samples and blank controls (qPCR premix without DNA template). The efficiency and sensitivity of each qPCR assay was determined by the amplification of standard serial dilutions, as previously described (Marti et al., 2013). Amplification efficiency was calculated from the resulting standard curves using the formula $E = 10^{(-1/slope)} - 1$, and the analytical sensitivity of the real-time PCRs was determined as the smallest DNA quantity detected for each assay

2.5. Statistical analysis

The differences in biofilm descriptors and ARGs were analysed by two-way ANOVA in which Site and Stream were set as fixed factors. Post-hoc comparisons were done with Tukey test after passing the homogeneity of variance and normality (Kolmogorov-Smirnov) tests. The relation between biological and environmental variables was

analysed using Pearson correlation tests.. Statistical significance was set at $p < 0.05$. All analyses were carried out using Sigma Plot v.11.0 (Systat Software Inc. London, UK).

3. Results

3.1 Water physical and chemical parameters

The WWTP inputs influenced most parameters (conductivity, discharge and nutrient concentrations) downstream, and the influence extended up to 1 km downstream (Table 1). The relative increase caused by WWTP effluents ranged between 11% and 97% of the stream flow, being the highest in BRE stream (Table 2). Water conductivity increased between 7 and 34% at downstream sites, with patterns depending on the stream. In particular, conductivity increased from DW to DW1 in GUA and ARB streams and decreased in SMP and BRE streams. TOC concentration was usually 2 to 3-fold higher downstream, except in ARB where no increase was produced. Total nitrogen increased 2-fold downstream the WWTPs, expect in SMP where the difference was negligible. BRE was the stream most impacted by effluent discharge, the TN increased 20-fold in the DW site. Total phosphorus concentrations increased 3 to 11-times, and reached 20 times higher in BRE. Dissolved oxygen, temperature and pH did not vary significantly after the inputs of WWTPs effluents, except in BRE where temperature increased 1.8 °C at the DW site.

3.2 Characterization of streambed biofilms

Biofilm biomass (AFDW) was similar between the streams. Even though the significant effect of the stream factor (Table 3), the post hoc analysis did not reveal difference among streams. The AFDW was significantly higher at DW sites ($p = 0.012$), and especially in the DW1 sites ($p = 0.002$) compared to the UP ones. However, these

296 effects were not equal in all the systems, as confirmed by the significant interaction
297 between stream and site factors (Figure 1a, Table 3). This pattern could be attributed to
298 differences in nutrient concentrations at different sites. In fact, the Pearson correlation
299 analysis evidenced that AFDW was significantly correlated with both TN (r = 0.768; p
300 = 0.003) and TP (r = 0.753; p = 0.005).

301 Chlorophyll-*a* content was significantly higher in BRE than in ARB and SMP (p =
302 0.002; Table 3). Chl-*a* was generally higher at DW (p = 0.002) and DW1 (p = 0.001)
303 sites. The effects differed however depending on the studied systems (Figure 1b). The
304 highest Chl-*a* value was recorded at the DW site in BRE stream (Figure 1b; p<0.001).
305 The SMP and GUA streams had higher Chl-*a* measured at the DW1 than at the DW
306 (p<0.001 and p = 0.032 respectively) (Figure 1b). Pearson analysis revealed that Chl-*a*
307 was significantly and positively correlated with both TN (r= 0.887; p<0.001) and TP (r=
308 0.857; p<0.001).

309 The AI of biofilms was significantly different among streams (Table 3), being higher
310 in GUA than in the others streams (p<0.05). The AI increased moderately after
311 receiving the WWTP effluents, except in the BRE stream (Figure 1c; Table 3).

312 **3.3. Prevalence of ARGs**

313 All the qPCR assays were performed with high R^2 values (average 0.99), high
314 efficiencies (89.6 to 99.6 %) and a dynamic range of at least 5 orders of magnitude,
315 indicating the validity of the resulting quantifications. The results from qPCR analyses
316 showed that the total copy number of bacterial 16S rRNA genes were consistent in all
317 samples and ranged from 3.88×10^5 to 1.16×10^6 copies ng^{-1} DNA. The concentrations of
318 ARGs copies were normalized to the bacterial 16S rRNA gene copy number to avoid an
319 underestimation of the abundance that can be caused by the presence of eukaryotic
320 DNA (*i.e.* protozoa, fungi and algae). Statistical analysis confirmed that normalized

ARG gene copies were usually higher in biofilms at DW sites than those from UP sites (Figure 2 and Table 3).

Regarding particular genes, the relative concentration of the $bla_{\text{CTX-M}}$ gene was higher in the DW biofilms in the SMP stream ($p<0.05$; Figure 2). Remarkably, $bla_{\text{CTX-M}}$ was below detection limit at upstream sites of BRE and GUA streams, but it was detected downstream (Figure 2).

The *qnrS* gene abundance was also higher in SMP biofilms ($p<0.001$; Table 3). This gene was significantly more abundant in biofilms from DW ($p = 0.003$) and DW1 ($p<0.001$) than in those from upstream (Table 3). DW and DW1 biofilms showed significantly higher concentrations of *qnrS* gene (respect to samples collected upstream) in the BRE stream ($p = 0.005$ and $p<0.001$, respectively; Figure 2), and changes were only significant in the DW site of the GUA stream ($p = 0.043$) and in the DW1 site of SMP (Figure 2).

The relative concentration of the *sul* I gene was more abundant in SMP and BRE than in ARB and GUA streams ($p<0.001$; Table 3). This gene resulted significantly more abundant in DW ($p<0.001$) and DW1 ($p = 0.003$) sites (Table 3). In ARB and GUA streams, both DW ($p = 0.001$ and $p = 0.016$, respectively) and DW1 ($p = 0.002$ and $p = 0.001$, respectively) biofilms had significantly higher concentrations of the *sul* I gene than in upstream sites (Figure 2). In BRE stream, the difference was only observed at the DW site ($p<0.001$; Figure 2).

The relative concentration of the *ermB* gene was the highest in SMP stream ($p<0.001$; Table 3). The *ermB* gene abundance was significantly higher at the DW and DW1 sites ($p<0.001$; Table 3). The biofilms in ARB, SMP and BRE streams showed significantly higher abundance of *ermB* gene in the DW and DW1 sites than in UP ($p<0.02$; Figure 2). Only in GUA stream the significant increase of *ermB* gene relative concentration

346 observed at DW (p = 0.003) was not observed at the DW1 site (Figure 3). Pearson
347 correlation analysis evidenced the significant and positive correlation between *ermB* and
348 *qnrS* gene abundances (r=0.840, p<0.001).

349 4. Discussion

350 WWTP effluents strongly modified the hydrology, physico-chemistry and biological
351 characteristics of the receiving low order streams and increased the abundance of ARGs
352 in microbial benthic communities. It was also shown that the magnitude of the effect
353 depended on the relative contribution of each WWTP to the receiving system.

354 The increase in nutrient concentrations after the WWTP discharge was proportional and
355 consistent with the increase of river flow caused by the effluents (Table 2). These
356 alterations on the hydrology and water chemistry were also observed in studies carried
357 out in larger rivers (Acuña et al., 2015; Aristi et al., 2015), and were also reflected in the
358 structural properties of microbial benthic communities. In fact, the biofilm biomass
359 (both AFDW and Chl-*a*) increased downstream from the WWTP discharge point
360 mirroring the nutrients concentration pattern. This was confirmed by the significant
361 positive correlation between biomass-related parameters (AFDW and Chl-*a*) and both
362 TN and TP. Similar increases in biofilm biomass and nutrient concentrations
363 downstream the WWTP were described by Aristi et al. (2015). In our study, the effect
364 of the WWTP effluent was particularly evident in the case of BRE stream, where the
365 contribution of the treated water to total downstream flow was the highest (97%, Table
366 2). Thus, BRE was the stream where biofilms at downstream sites (mainly at DW)
367 received the highest nutrient loading (and probably of antibiotic resistant elements)
368 from the WWTP because the negligible dilution of the effluent. As a consequence, BRE
369 was the stream in which the most significant changes were observed, and the only
370 stream where all ARGs increased downstream from the discharge point (Figure 2). The

371 magnitude and pattern of downstream increase of target ARGs also mirrored those of
372 nutrients and water flow, stressing the important role of WWTP effluents as a source of
373 resistance genes to streambed biofilm communities.

374 Although previous studies have demonstrated that WWTPs reduce the concentration of
375 antibiotics and ARGs from raw sewage (Rodriguez-Mozaz et al., 2015), our results
376 indicate that WWTP discharges significantly increase the prevalence of antibiotic
377 resistance in the aquatic environment, and in particular in the streambed microbial
378 biofilms. ARGs are currently considered as emerging pollutants because they are
379 introduced into the environment due to the inefficient treatment of domestic and
380 hospital wastewater, favouring the presence of antibiotic resistant bacteria in WWTP
381 effluents and, thus, in the receiving systems (Alonso et al., 2001; Martinez, 2009;
382 Pruden et al., 2012). Nevertheless, risk assessment procedures to properly quantify the
383 levels of ARGs that represent a threat for human and ecosystem health are still not
384 available (Berendonk et al., 2015). Baquero and colleagues, (2008) describe genetic
385 reactors as the places in which high biological connectivity, generation of variation and
386 presence of specific selection favour genetic evolution. In particular four main reactors
387 in the evolution of antibiotic resistance were highlighted: human and animal microbiota,
388 hospital and farms, wastewater treatment systems and receiving systems in which
389 environmental organisms may be in continuous contact with organisms originated in the
390 previous reactors (Baquero et al., 2008). In fact, bacteria can be exposed to high doses
391 of antibiotic compounds due to its therapeutic use in both human and veterinary
392 medicine, and thereby develop resistance before being released into the aquatic
393 environment through faeces (i.e. sewage), surface runoff and soil leaching (Servais and
394 Passerat, 2009). We found a significant increase in the concentration of ARGs,
395 particularly the *ermB* and *sul* I genes, in biofilms collected downstream of the WWTP

discharge points. These observations are consistent with previous studies, suggesting that WWTP discharges may contribute to an increase of antibiotic resistance (Berglund et al., 2015; Marti et al., 2013; Rodriguez-Mozaz et al., 2015). Interestingly, biofilms collected upstream the WWTP discharge point showed lower but detectable levels of almost all the ARGs analysed (with some exceptions, see Figure 2). This is in agreement with previous studies analysing ARGs in different river compartments (biofilms, sediments and water column) up and downstream the WWTPs inputs to the river channel (Marti et al., 2013; Pei et al., 2006; Pruden et al., 2006; Rodriguez-Mozaz et al., 2015). The idea of an existing background level of antibiotic resistance naturally occurring in the environment has been pointed out by several authors (Allen et al., 2010; Berglund, 2015; D'Costa et al., 2011) and our observation of ARG levels in UP biofilms supports this hypothesis. In the absence of point WWTP discharges, several factors may account for the maintenance of this background resistance level in streambed biofilms collected at upstream sites, namely: livestock rearing, agricultural runoff and soil leaching, which may represent diffuse sources of both antibiotic residues and ARGs into the streams.

We also showed that the presence of ARGs in biofilms was noticeable far downstream (*i.e.* DW1) of the WWTP discharge. This observation points out that some ARGs genes can persist in the environment, even in absence of additional punctual pollution sources such as WWTP effluents. This trend was particularly relevant in the Breda stream where the concentration of *ermB* and *qnrS* genes 1 km downstream of the WWTP discharge were significantly higher than in DW biofilms (Figure 2). This could be explained either by the presence of unknown diffuse sources of ARGs between DW and DW1 (not directly investigated in this study), or by the drift of either antibiotic resistant bacteria or ARGs originated upstream that may settled into riverbed biofilms after travelling with

421 flowing water far from the emission point. This latter possibility is unlikely in this
422 particular case, as the "drift effect" is expected to be more relevant closer to the point
423 source of resistant bacteria and decreases travelling downstream. In fact, a controlled
424 spike experiment in a low-order stream showed that more than 67% of the *E. coli* added
425 to the channel were retained in the first 200 meters (Drummond et al., 2015). The
426 relevant decrease of water conductivity we observed between DW and DW1 at BRE
427 (from 667 µS cm^{-1} down to 208 µS cm^{-1}) evidenced that different waters probably
428 entered the main stream channel within this river reach. Unfortunately our results can
429 not discriminate between both hypothesis since no analyses were carried out to identify
430 potential diffuse sources of pollution between DW and DW1 sites..

431 The pattern observed in BRE stream with *ermB* and *qnrS* is, however, not a general
432 trend since other ARGs in other streams decreased their abundance with stream dilution,
433 similarly to results observed by Czekalski et al. (2014) downstream of the Lake Geneva
434 (Switzerland). Although antibiotics released from the WWTP are diluted into river
435 water and their concentrations are expected to decrease considerably downstream, these
436 compounds can also exert a selective pressure on autochthonous bacteria, thereby
437 increasing horizontal transfer of resistance genes (Balcázar et al., 2015; Gullberg et al.,
438 2011). This can be especially relevant in streams with low dilution, a common situation
439 in semiarid streams such as the Mediterranean ones selected here, where the relative
440 contribution of the WWTP effluent may become seasonally dominant (Kuster et al.,
441 2008; Osorio et al., 2012). Alternatively, environmental and chemical stressors other
442 than antibiotics (*i.e.* metals) can trigger stress-responses that stimulate ARGs transfer
443 among prokaryotes (Baker-Austin et al., 2006).

444 In conclusion, WWTP effluents can deeply modify the characteristics of low order
445 streams, being a relevant source of ARGs to benthic microbial biofilms. 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 excellent biological indicators of anthropogenic pollution by pharmaceutical active compounds. However, further studies will be needed to determine if the detected increase of ARGs in biofilm microbial communities is caused either by the release of resistant bacteria from WWTPs or by a resistance response of indigenous biofilm communities to antibiotic residues discharged into the stream. A better understanding of the mechanisms and pathways involved in the acquisition and spread of antibiotic resistance will be essential to reach these goals.

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573 **Figure captions**

574

575 **Figure 1.** Differences among biofilms at sampled streams and sites in: a) Ash Free Dry
576 Weight; b) Chl-*a* content and c) Autotrophic Index. Post-hoc Tukey analysis results are
577 shown with letters when differences resulted significant. Statistical significance was set
578 at $p<0.05$ (two-way ANOVA).

579

580 **Figure 2.** Relative concentrations of the ARGs measured in the biofilms from the
581 different sites of the studied streams. Post-hoc Tukey analysis results are shown with
582 letters when differences resulted significant. Statistical significance was set at $p\leq0.05$
583 (two-way ANOVA).

Figure
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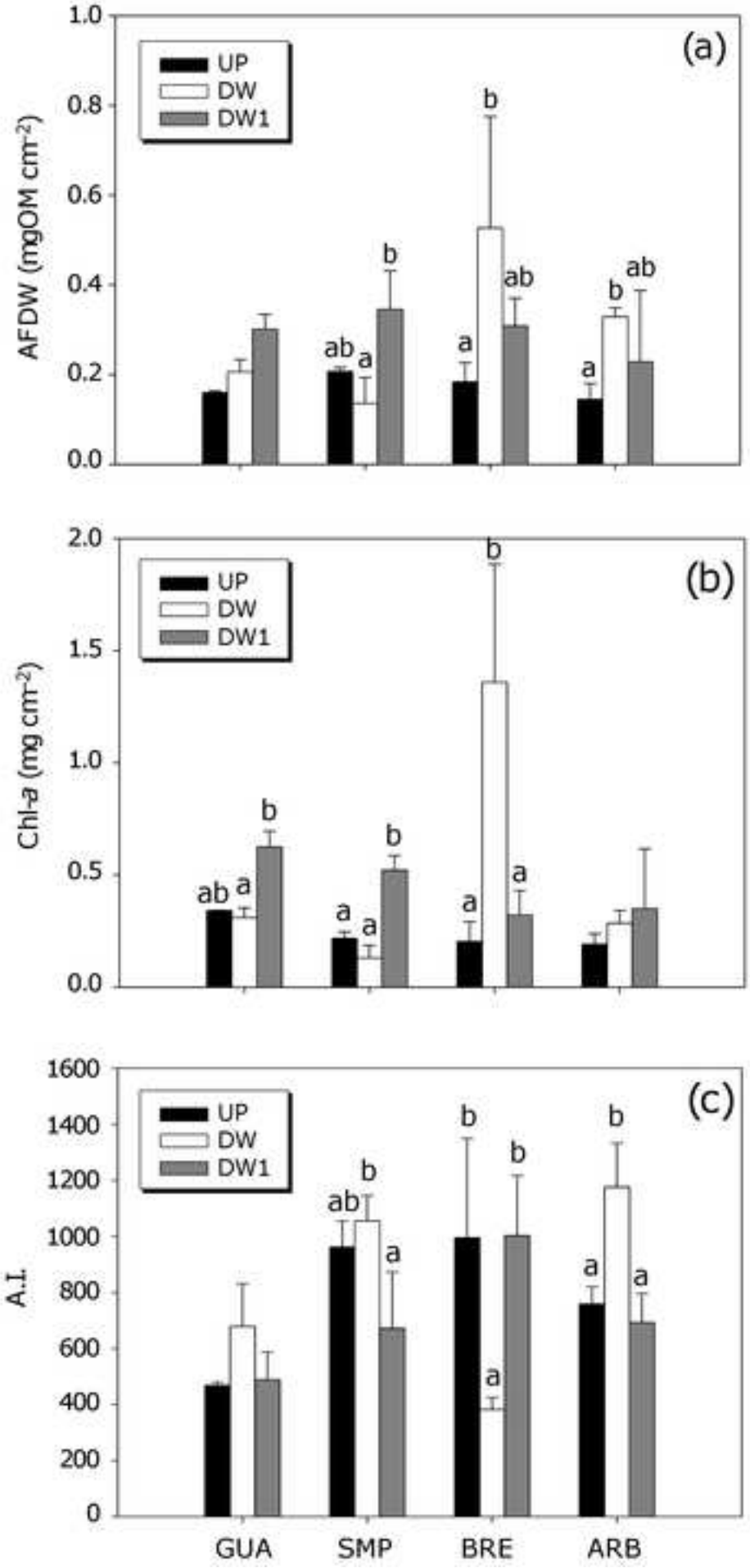

Figure

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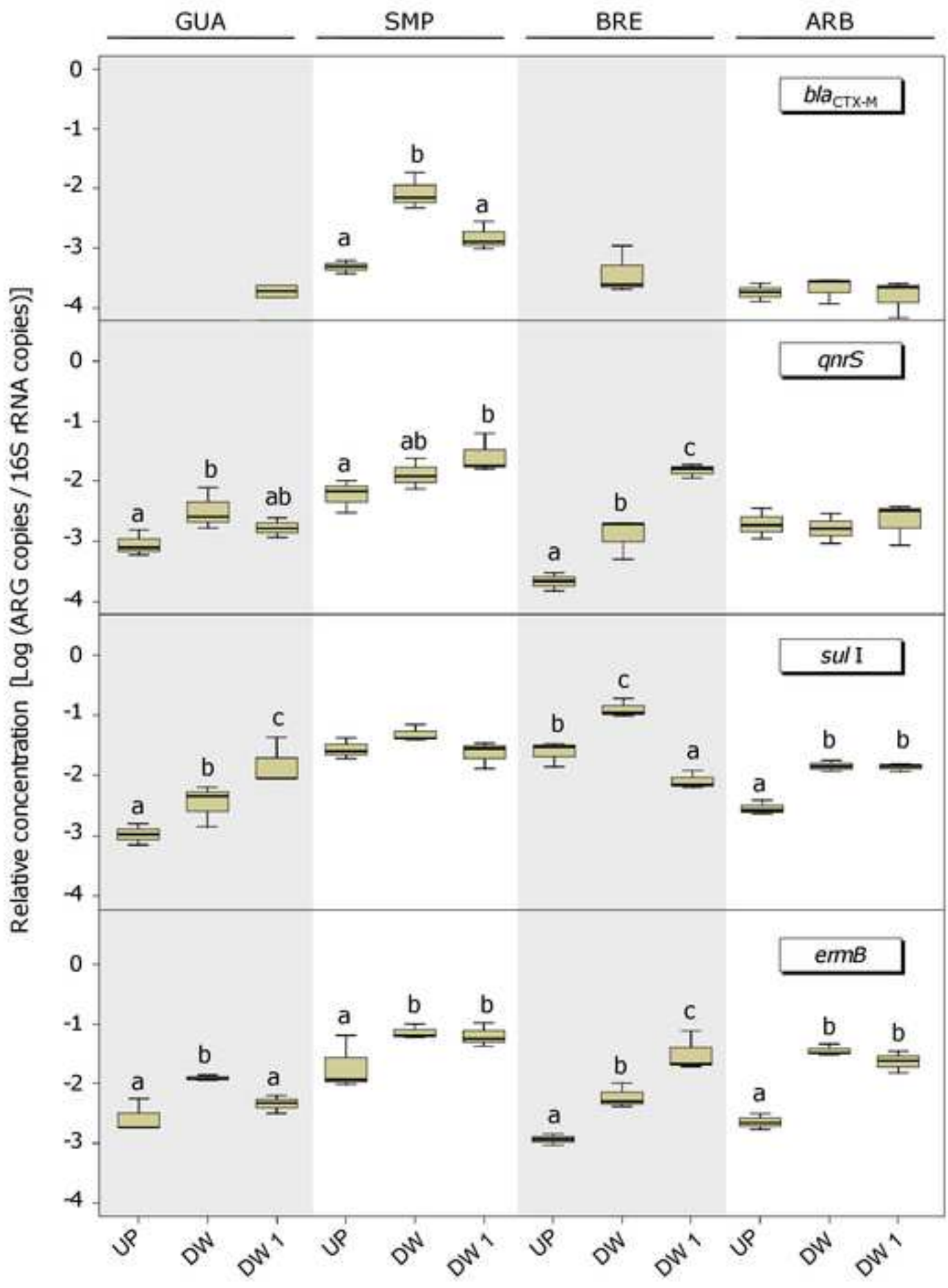

Table

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Table 1. Location of sampling sites. Physical, chemical and hydrological variables measured at the different sampling sites of the different streams studied. GUA = Gualba; BRE= Breda, ARB= Arbúcies, SMP= Santa Maria Palautordera. Cond = Conductivity, T = Temperature, DO = Dissolved Oxygen, TOC = Total Organic Carbon, TN =Total Nitrogen, TP = Total Phosphorus.

Stream	Site	Location		Discharge	Cond	T	DO	pH	TOC	TN	TP
		Long 2°E	Lat 41°N	(L s^{-1})	(µS cm^{-1})	ºC	(mg L^{-1})		(mg C L^{-1})	(mg N L^{-1})	(mg P L^{-1})
GUA	UP	43' 47"	30' 33"	42	140	17.3	9.42	7.83	2.90 ± 0.23	0.43 ± 0.01	0.035 ± 0.004
	DW	43' 42"	30' 38"	56	152	17.3	9.22	7.83	2.79 ± 0.29	0.99 ± 0.10	0.133 ± 0.007
	DW1	43' 22"	31' 05"	63	156	17.7	8.96	7.82	3.41 ± 0.23	1.05 ± 0.07	0.129 ± 0.004
SMP	UP	41' 04"	27' 26"	176	164	15.7	8.93	7.33	1.65 ± 0.27	1.51 ± 0.03	0.059 ± 0.014
	DW	41' 02"	27' 32"	195	217	17.9	8.73	7.42	2.57 ± 0.19	1.43 ± 0.01	0.178 ± 0.003
	DW1	40' 45"	28' 07"	197	204	18.1	8.84	7.52	2.45 ± 0.12	1.47 ± 0.02	0.195 ± 0.0003
BRE	UP	44' 12"	34' 10"	1	624	19.7	5.42	7.68	6.98 ± 0.41	1.23 ± 0.03	0.678 ± 0.016
	DW	44' 09"	34' 08"	9	667	21.5	6.3	7.74	10.37 ± 0.21	21.58 ± 0.02	2.64 ± 0.027
	DW1	43' 40"	34' 13"	7	208	17.5	7.89	7.48	3.00 ± 0.29	1.56 ± 0.004	0.313 ± 0.014
ARB	UP	48' 50"	31' 16"	151	238	17.4	9.58	8.3	1.76 ± 0.18	1.04 ± 0.001	0.054 ± 0.002
	DW	48' 52"	31' 22"	230	291	16.9	9.23	8.1	4.25 ± 0.09	2.37 ± 0.03	0.306 ± 0.021
	DW1	48' 42"	31' 53"	269	318	16.8	8.82	8.06	7.84 ± 0.32	5.43 ± 0.03	0.589 ± 0.029

Table 2. Relative contribution of WWTP effluents to the studied streams at DW in terms of water flow and nutrient concentration.

Stream	Contribution of WWTP effluents at DW (%)	
	Flow	Nutrients
GUA	25.0	42.0
SMP	9.7	32.2
BRE	93.6	67.1
ARB	34.5	65.7

Table 3. Results from the two-way ANOVA using Stream (GUA, SMP, BRE and ARB) and Site (UP, DW and DW1) factors on the measured biofilm variables. Significant effects are marked in bold.

		Stream	**Site**	**Site × Stream**
AFDW	d.f.	3	2	6
	MS	0.29	0.82	0.51
	F	3.05	8.55	5.28
	P level	**0.048**	**0.002**	**0.001**
Chl-*a*	d.f.	3	2	6
	MS	0.00009	0.00012	0.00016
	F	8.33	10.37	12.18
	P level	**<0.001**	**<0.001**	**<0.001**
AI	d.f.	3	2	6
	MS	233667	38656	235666
	F	9.27	1.53	9.35
	P level	**<0.001**	0.240	**<0.001**
ermB	d.f.	3	2	6
	MS	1.62	2.51	0.31
	F	33.09	51.21	6.24
	P level	**<0.001**	**<0.001**	**<0.001**
***sul* I**	d.f.	3	2	6
	MS	1.77	0.90	0.58
	F	41.00	20.81	13.36
	P level	**<0.001**	**<0.001**	**<0.001**
qnrS	d.f.	3	2	6
	MS	1.69	1.51	0.55
	F	23.99	21.37	7.81
	P level	**<0.001**	**<0.001**	**<0.001**