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*Manuscript

1 **Response of biofilm bacterial communities to antibiotic pollutants in a Mediterranean river**

2

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Highlights (for review)

>We studied the effects of antibiotics detected in river waters on biofilm communities

>Biofilm switch to more polluted waters evidenced responses of bacteria communities

>Bacteria mortality increased and enzymes activity decreased in switched biofilms

>Actinobacteria abundance increased in switched biofilms

>Biofilm bacteria responses showed significant correlation with antibiotic levels

Abstract

Antibiotics are emerging contaminants, which wing to their bioactivity, may lead to short-term and long-term alterations of natural microbial communities in aquatic environment. We investigated the effects of antibiotics on biofilm bacterial communities in the Llobregat River (Northeast Spain). Three sampling sites were selected: two less polluted sites and one hotspot. River water was collected from each site and used both as inoculum and medium for growing biofilms in independent mesocosms. After 25 days of biofilm colonization, we exposed the colonized biofilms to river waters from the downstream sites (progressively contaminated by antibiotics). A control from each site was maintained where the growing biofilm was always exposed to water from the same site. The bacterial community composition, bacterial live/dead ratio and extracellular enzyme activities of the biofilms were measured before and 9 days after exposing the biofilms to increasing contaminated waters. Sixteen antibiotic compounds were detected in the water from the three sampling sites. At each site, the antibiotics present in the highest concentrations were sulfonamides, followed by quinolones and macrolides. Bacterial communities of biofilms grown with the three river waters differed markedly in their structure, but less so in terms of functional descriptors. After switching the medium water to increasing pollution, biofilms exhibited increased levels of actinobacteria (HGC), a trend that was associated to the higher antibiotic concentrations in the water. These biofilms also showed increased bacterial mortality, and decreased extracellular leucine-aminopeptidase and alkaline phosphatase. There was a significant correlation between antibiotic concentrations and biofilm responses. Our results indicate that the continuous entrance of antibiotics in running waters cause significant structural and functional changes in microbial attached communities.

Keywords:

Antibiotics, Biofilms, Mediterranean River, Bacteria, CARD-FISH, DGGE

53 **Introduction**

54 Anthropogenic activities are at the base of increasing levels of priority and emerging
55 contaminants, which mostly derive from point and diffuse sources that reach freshwater ecosystems.
56 Due to their strong bioactivity, antibiotics (*e.g.* ß-lactams, quinolones, tetracyclines, macrolides,
57 sulfonamides; Kümmerer et al., 2009) are among the most worrisome emerging classes of pollutants.
58 Given that 30% to 90% of any dose of most antibiotics administered to humans and animals is excreted
59 as an active substance (Rang et al., 1999), these drugs and their metabolites are commonly found in
60 aquatic environments (Gros et al., 2007, Luo et al., 2011; Managaki et al., 2007). Hirsch et al. (1998)
61 detected 18 compounds from four classes of antibiotics in German surface water samples, and
62 Watkinson et al. (2009) detected various antibiotics in 90% of freshwater, estuarine and marine samples
63 in six river catchments.

64 Antibiotics—either as single compounds or in mixtures—can have numerous detrimental effects
65 on aquatic life, including direct toxicity to aquatic microbes, even at low concentrations (Hernando et
66 al., 2006), accelerated acquisition of antibiotic resistance in several bacterial strains, including
67 pathogens (Kümmerer and Henninger, 2003; Obst et al., 2006), and widespread and persistent
68 contamination of water resources, since their mineralization is essentially due to the presence of
69 microorganisms with specific catabolic activities (Brain et al., 2004; Costanzo et al., 2005; Pomati et al.,
70 2006). Antibiotics are bioactive against natural bacterial communities, and their presence may lead to
71 short-term physiological alterations, including cell death and altered metabolic functions (*e.g.* biomass
72 production, respiration, and excretion of extracellular enzyme activities), as well as to long-term
73 changes in microbial biomass or in community composition (Bonnineau et al., 2010, Tlili et al., 2010).

74 To date, most studies concerning the effects of antibiotics in aquatic environments have focused
75 on planktonic microbial communities, while disregarding the response of river biofilms. However,
76 microbial attached communities constitute the major component for the uptake, storage and cycling of
77 carbon, nutrients (Pusch et al., 1998, Battin et al., 1999) and anthropogenic contaminants (Sabater et al.,

78 2007) in many river sections. Biofilms contain bacteria and other heterotrophs (*e.g.* fungi and protozoa),
79 and autotrophs (*e.g.* diatoms, green algae and cyanobacteria), all of which are embedded in an
80 extracellular polymeric matrix (Lock, 1993). The mutual benefits and the close spatial relationships
81 between organisms with distinct life-strategies closely mirrors the quality of the surrounding flowing
82 waters, generating a complex micro-ecosystem in which specific metabolic processes and interactions
83 may occur (*e.g.* use of algal exudates by bacteria, Murray et al., 1986). The major processes of biofilm
84 bacteria include their capability to mineralize organic molecules, a process in which extracellular
85 enzymes (Pusch et al., 1998; Romaní, 2010; Proia et al., 2012a), and microbial interactions within the
86 biofilm (Rier and Stevenson, 2002; Francoeur and Wetzel, 2003; Rier et al., 2007), play major roles.
87 Considering this complexity, the effects of antibiotics on the structure and function of microbial
88 communities in river biofilms remain unknown.

89 We had two objectives in this study: to investigate any structural and functional modifications of
90 biofilm bacterial communities along an antibiotic pollution gradient in the River Llobregat (Spain); and
91 to evaluate bacterial responses to biofilm translocation in river waters containing different levels of
92 antibiotic contamination. We hypothesized that biofilm communities inhabiting environments with
93 lower levels of antibiotics would be the most affected ones upon translocation to more polluted waters,
94 and that this effect would manifest itself in community composition changes. We tested this hypothesis
95 through translocation experiments performed under controlled conditions. Field translocation has been
96 previously used for assessing the effects of metal pollution and industrial discharge on river biofilms
97 (Ivorra et al., 1999; Victoria and Gómez, 2010; Tlili et al., 2011). In the present study, we analyzed the
98 response of biofilms grown in water from different river sites before and after the exposure to water
99 from sites with higher levels of antibiotics.

100

101 **Material and methods**

102 **Study site**

103 The Llobregat is a typical Mediterranean river that flows from the Pyrenees to the south of Barcelona

104 (Catalonia, Spain). The Llobregat 165 km long and drains a catchment area of 4,948 km^2 (Marcé et al.,

105 2012). Its water flow is characterized by high variability, including periodic flood and drought events

106 (Ricart et al., 2010). The mean annual precipitation in the area is 3,330 hm^3, and the River has an annual

107 average discharge of 693 hm^3 (Ginebreda et al., 2010). Nearly 30% of the discharge flowing in the

108 Llobregat basin is used for drinking water (Muñoz et al., 2009). Together with its two main tributaries,

109 the Cardener and the Anoia rivers, the Llobregat is a paradigm of overexploited Mediterranean rivers

110 (Muñoz et al., 2009; Marcé et al., 2012). The middle-lower part of the River is densely populated and

111 subjected to major industrial, agricultural and urban activities. In fact, its watershed is located in a

112 densely inhabited area (> 3 million people) and receives significant inputs of industrial and urban

113 wastewaters (~ 137 hm^3 annually, Ginebreda et al., 2010) as well as surface runoff from agricultural

114 areas (Kuster et al., 2008). Moreover, salt inputs deriving from salt mines in the Cardener watershed

115 cause an increase in water salinity downstream, worsening the poor conditions of the lower part of the

116 river. For the present study, we chose three sampling sites located in the middle-lower part of the

117 Llobregat river (Fig. 1): two of them were upstream of the entrance of the Anoia River (Castelbell, Mina

118 de Terrassa), and another was located downstream of its junction (Sant Joan Despí).

119

120 **Experimental design and biofilm translocation**

121 The two upstream sampling sites, Castellbell (A) and Mina de Terrassa (B), were selected as the less

122 polluted sites, and the one downstream, Sant Joan Despí (C), as the pollution hotspot. River water was

123 collected three times weekly from the three sites, and then used as inoculum to grow biofilms in 18

124 independent mesocosms. Biofilms were colonized on glass slides (1 cm^2 each) placed at the bottom of

125 each mesocosm (35 slides per mesocosm). The mesocosms comprised sterile glass jars (19 cm in

126 diameter x 9 cm high) filled with 1.5 L of river water, which was re-circulated using a submersible

127 pump (Hydor, Pico 300, 230 V 50 Hz, 4.5W). The water in each mesocosm was changed three times

128 weekly using fresh river waters of the corresponding sites. All mesocosms were maintained in an
129 incubator (SCLAB) under controlled temperature (18 °C) and light irradiance (150 to 180 µmol m^{-2} s^{-1};
130 dark/light cycle of 12 h/12 h). After 25 days of colonization, biofilms were exposed to river water from
131 more polluted sites switching the mesocosms medium water accordingly. Three water switches (with
132 three replicate jars each) were performed: from Castellbell to Mina de Terrassa (A→B,) from Castellbell
133 to Sant Joan Despì (A→C) and from Mina de Terrassa to Sant Joan Despì (B→C). Further, three
134 replicate glass jars per site (Castellbell, Mina de Terrassa or Sant Joan Despí) were maintained as
135 controls. The biofilms were sampled twice during the experiment: once before, and once 9 days after
136 switching the medium waters. Glass tiles from each mesocosm were randomly sampled.
137

138 **Physicochemical parameters**

139 Conductivity, temperature, pH and dissolved oxygen were measured with appropriate multi-parameter
140 sensor probes (HACH LANGE GMBH, Germany) in the field and in the jars, before and after each
141 water change. Water samples were collected for nutrient content measurement from the glass jars before
142 and after water renewals. All water samples were filtered (nylon membrane filters, 0.2 µm;
143 WHATMAN, Maidstone, UK) prior to analysis. Soluble reactive phosphate was measured following the
144 method of Murphy and Riley (1962). Samples for anions and cations analysis were stored frozen until
145 analysis by ion-chromatography (761 Compact IC, METROHM, Herisau, Switzerland).

146 **Antibiotics in the water**

147 Water samples (6 L) were collected from the sampling sites in the field, transported to the laboratory at
148 4ºC in dark conditions and immediately processed for antibiotics content measurement. The
149 concentrations of sixteen antibiotics representing different families were analyzed in surface waters
150 using a multiresidue analytical method based on solid-phase extraction and subsequent LC-MS/MS, as
151 described by Osorio et al. (2012).
152

153 **Catalyzed reported deposition-fluorescence *in situ* hybridization (CARD-FISH) analysis**

154 Biofilm replicates (1 cm^2 each) were scraped (sterile silicone cell scrapper, Nunc) and treated by
155 optimizing the density gradient centrifugation method described by Amalfitano and Fazi (2008). After
156 treatment, bacterial cells were fixed (formaldehyde, 2%) directly onto polycarbonate filters (Whatman).
157 Subsequently, CARD-FISH was performed according to the standard protocol (Pernthaler et al., 2002,
158 2004). Cells were immobilized on filter sections by embedding them with 0.2% agarose (Invitrogen Life
159 Technologies). Filters were permeabilized with lysozyme (1 h at 37 °C; 20 mg mL^{-1}, Fluka, Steinheim,
160 Germany) and Proteinase K (25 min at 37 °C; 0.034 U μL^{-1} - SIGMA-ALDRICH, Steinheim, Germany,
161 in Tris-EDTA buffer). Horseradish peroxidase-labeled oligonucleotide probes were used to hybridize
162 Bacteria (EUB I-III), Alpha-Proteobacteria (ALF968), Beta-Proteobacteria (BET42a), Gamma-
163 Proteobacteria (GAM42a), Cytophaga-Flavobacteria (CF319a) and Actinobacteria (HGC69a) at
164 appropriate stringency conditions (Loy et al., 2007).
165 The hybridized cells were washed, and were then double-stained with fluorescein-labeled tyramide (2
166 µL - SIGMA-ALDRICH, Germany - in 30% H_2O_2, PBS - pH 7.4, 5M NaCl, 10% w/v blocking reagent,
167 10% w/v dextran sulfate) and with 4'-6-diamino-2-phenylindole (DAPI, 1 µg mL^{-1}; Vector Laboratories,
168 USA). The filters were observed, and the number of cells quantified, by epifluorescence microscopy
169 (Leica DM LB 30, at 1000X magnification).
170

171 **Denaturing gradient gel electrophoresis (DGGE) analysis**

172 Biofilm replicates (1 cm^2 surface area) were scraped (sterile silicone cell scrapper, Nunc) in 1 mL of
173 pre-sterilized MilliQ water, and kept frozen at -80 °C. DNA was extracted by thermal shock, from
174 pellets of scraped biofilm obtained after centrifugation of samples (13 400 g for 30 min; Eppendorf
175 5415D centrifuge). Six to eight cycles of freezing (13 to 15 min at -80 °C) and defrosting (7 min at 60
176 °C) were performed to obtain the nucleic acids extracts. DNA concentration and purity were determined

177 spectrophotometrically from these extracts, using a Nanodrop ND-1000 UV-Vis spectrophotometer
178 (Nanodrop, DE).
179 Bacterial community structure and composition were determined by DGGE analysis. Two universal
180 primers were used for amplification of a 566-bp long fragment of the 16S rDNA of Eubacteria: 27F (5'-
181 AGA GTT TGA TCM TGG CTC AG-3'), with a degenerate base pair at one position, with a GCclamp
182 (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-5') spanning
183 *Escherichia coli* positions 8–27; and 517R (5'-ATT ACC GCG GCT GCT GG-3') spanning *E. coli*
184 positions 518–534. The PCR was performed as follows: an activation step for the polymerase (15 min at
185 95 °C), 35 cycles with initial denaturation (30 s at 94 °C), annealing (30 s at 54 °C) and elongation (1.5
186 min at 72 °C), followed by a final elongation step (7 min at 72 °C). The PCR mix contained 2 μL of the
187 template, 1.25 U of the HotStar DNA Taq polymerase (PeqLab, Erlangen, Germany), 1 μL of dNTPs
188 (0.2 mM final concentration per vial and dNTP), 1.5 μL of each primer (20 pM final concentration per
189 vial), and 5 μL of reaction buffer (10X), such that the total volume was 50 μL. DGGE analysis of the
190 PCR products (15 to 25 μL) was performed using the D-Code-System (BioRadLaboratories GmbH,
191 Munich, Germany), in polyacrylamide gels containing a 40% to 70% urea gradient. The DGGE gels
192 were run in 1 x TAE buffer (40 mmol l^{-1}Tris, 20 mmol l^{-1} acetate, 1 mmol l^{-1} EDTA) at 70 V and 60 °C
193 for 16 h. The gels were stained with SYBR®Gold (Invitrogen, Karlsruhe, Germany). The stained gels
194 were immediately analyzed using the Lumi-Imager Working Station (Roche Diagnostics, Mannheim,
195 Germany). DGGE fingerprints were scored by the presence or absence of DNA bands. The DNA bands
196 (19 in total) were excised and sequenced for taxonomic identification. The bands were selected by
197 comparing DGGE profiles of different samples: those bands commonly observed in all samples, and
198 bands characteristics of specific biofilms, were selected. The selected bands were excised from the
199 DGGE gel, and the slices were equilibrated in 15 mL of sterile water overnight at room temperature.
200 The DNA extract was re-amplified by PCR and re-subjected to DGGE to verify the purity of the PCR
201 re-amplification product. PCR products were purified using a ExoSap kit (USB, Staufen, Germany). The

202 sequencing reaction was done using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied
203 Biosystems), and sequence detection was done using the ABI Prism 310 genetic analyzer (Applied
204 Biosystems), according to the manufacturer's protocol. DNA was identified by comparing the nucleic
205 acid sequences with GenBank sequences using the BLAST program (http:// www.ncbi.nlm.nih.gov).
206
207 **Bacterial cell viability**
208 Live and dead bacteria, identified as intact cells and membrane-compromised cells, respectively, were
209 stained using the LIVE/DEAD® Bacteria Viability Kit L7012 (BacLight™, Molecular Probes,
210 Invitrogen L7012). Colonized glass substrata were sonicated (< 60 s, sonication bath at 40 W and 40
211 kHz, Selecta) and scraped (sterile silicone cell scrapper, Nunc) to obtain a biofilm suspension. Samples
212 were then diluted with pre filtered-sterilized water from mesocosms, and 2 mL subsamples were
213 incubated with 3 μL of 1:1 mixture of SYTO 9 and propidium iodide, for 15 to 30 minutes in the dark.
214 At the end of the incubation, samples were filtered through a 0.2 μm black polycarbonate filters
215 (Nuclepore, Whatman). Filters were then dried, placed on a slide with mounting oil (Molecular Probes)
216 and counted by epifluorescence microscopy (Nikon E600, 1000x in immersion oil). Green and red (live
217 and dead, respectively) bacteria cells were counted in 20 random fields per filter.
218
219 **Extracellular enzyme activities**
220 The extracellular activities of the enzymes leucine-aminopeptidase (EC 3.4.11.1), alkaline phosphatase
221 (EC 3.1.3.1-2) and β-D-1,4-glucosidase (EC 3.2.1.21) in the biofilms were measured
222 spectrofluorometrically immediately after collection, by using the fluorescent-linked substrates L-
223 leucine-4-methyl-7-coumarinylamide (Leu-AMC, Sigma-Aldrich), 4-methylumbelliferyl -phosphate
224 (MUF-P, Sigma-Aldrich) and 4-methylumbelliferyl β-D-glucopyranoside (4-MUF β-D-glucoside,
225 Sigma-Aldrich), as described by Proia et al. (2012b).
226

227 **Statistical analysis**

228 The differences between biofilms before switching the medium waters , and the responses of biofilm
229 metrics to each water switch , were analyzed by ANOVA with sampling site and treatment as fixed
230 factors. Effects were analyzed *post hoc* with Tukey's b test. Analysis was performed using SPSS
231 Version 15.0. Cluster analyses of bacterial community composition determined by DGGE were
232 performed with PRIMER 6.0 using Bray-Curtis similarity matrix. The relation between biofilm bacterial
233 metrics and antibiotics concentrations was analyzed using Spearman correlation tests. Data were log-
234 transformed before the analyses.

235 The multi-group SIMilarity PERcentage test (SIMPER), using the Bray-Curtis similarity measure, was
236 run to identify the antibiotics that were primarily responsible for the observed differences among
237 samples before and after water switch. In the output table, the antibiotics were sorted in descending
238 order of their contribution to group difference.

239 Canonical Correspondence Analysis (CCA) was used to visualize how antibiotic concentration varied
240 with the distribution patterns of the major bacterial groups in the analyzed samples, before and after the
241 water switch experiments.

242 A Mantel test was applied to identify the degree of correlation among five different Bray-Curtis
243 similarity matrices (5,000 randomized runs), computed by the combination of the antibiotic
244 concentrations; the bacterial structural parameters (CARD-FISH and DGGE bands); and the bacterial
245 functional parameters (live/dead ratio, and enzymes). For the multivariate analyses, all data were log-
246 transformed and processed using the PAST software package (PAlaeontological STatistics, v. 1.80).

247

248 **Results**

249 *Physical-chemical water characterization*

250 Dissolved oxygen (DO) levels, pH and temperature did not differ among the three sites (A, B and C).
251 Conductivity was relatively high in all the sampling sites (Table 1) and was significantly higher in C (p

252 < 0.05). Soluble reactive phosphorus (SRP) levels gradually increased downstream while nitrate (NO_3)
253 levels were similar in A and B but were much higher in C ($p < 0.05$, Table 1). Discharge during the
254 study period was generally low ($< 7m^3 s^{-1}$), with significant flow decrease from A to C ($p < 0.0001$,
255 Table 1) because of water abstraction along the river.
256
257 *Antibiotics in the water*
258 A total of sixteen antibiotic compounds were detected in river water from the three sampling sites. The
259 antibiotics comprised fifteen compounds from four different families of antibiotics (seven macrolides,
260 five quinolones, two sulfonamides and one tetracycline; see Table 2), plus the bacteriostatic antibiotic
261 trimethoprim (a chemotherapeutic agent), which was detected at low concentrations. Concentrations in
262 Sant Joan Despí (C) were on average 6.1 times higher than in Castellbell (A) or in Mina de Terrassa (B).
263 At each sampling site the most abundant antibiotic class was sulfonamides, followed by quinolones and
264 macrolides; the most abundant drugs from each of these classes were sulfamethoxazole (with a peak of
265 1576 ng L^{-1} at Site C), ofloxacin and clarithromycin, respectively (Table 2). The broad-spectrum
266 antibiotic tetracycline was detected at high concentrations in C (avg. conc. = 336.61 ng L^{-1}), whereas the
267 concentrations at sites A and B were below 10 ng L^{-1}. A SIMPER test indicated that four out of the
268 sixteen measured antibiotics were chiefly responsible for the average dissimilarity among all sampling
269 points (19.9). These were respectively enrofloxacin (2.7), tetracycline (2.1), roxithromycin (1.7), and
270 ofloxacin (1.6).
271
272 *Responses of biofilm bacterial community structure to water switch*
273 Before water switches the bacterial communities differed in their abundance and composition
274 according to sampling site. The bacterial abundance significantly increased from A to C ($p < 0.05$),
275 shifting from $3.28 \pm 0.81 \times 10^7$ cells cm^{-2} in A to $4.45 \pm 1.48 \times 10^7$ cells cm^{-2} in B and $5.70 \pm 2.28 \times 10^7$
276 cells cm^{-2} in C. Alpha- Proteobacteria were more abundant in C (Fig. 2a) than in A and B ($p < 0.05$),

277 whereas Beta and Gamma- Proteobacteria were significantly more abundant in B and C than in A ($p < 0.05$, Fig. 2a). Canonical correspondence analysis stressed the bacterial community differences among
278
279 sites, and their respective relationships to antibiotic levels (Fig. 3a). In particular, the Actinobacteria
280 group (HCG) was highly associated to the sixteen antibiotics in the C biofilms (Fig. 3a). Differences in
281 bacterial community composition of the biofilms before water switch were confirmed by the cluster
282 analysis of DGGE gels (Fig. 4a). Three out of the 18 partial 16S sequences retrieved from DGGE gels
283 matched with referenced 16S sequences of the BetaProteobacteria *Variovorax paradoxus*
284 (Burkholderiales; Comamonadaceae) (Table A1, Fig A.1). These bands were common in the community
285 fingerprints of site A, B and C. Two bands only observed in fingerprint of site A had partial 16S
286 sequences that matched with referenced sequences of the Alpha-Proteobacteria *Roseomonas lacus*
287 (Rhodospirillales; Acetobacteraceae) and the Gamma-Proteobacteria *Legionella pneumophila*
288 (Legionellales; Legionellaceae). Two of the partial 16S sequences retrieved from B fingerprint matched
289 with referenced 16S sequences of the Alpha-Proteobacteria *Rhodobacter* sp. (Rhodobacterales;
290 Rhodobacteraceae) and the Beta-Proteobacteria *Limnobacter* sp. (Burkholderiales; Burkholderiaceae).
291 Finally, two bands only observed in fingerprinting of site C had partial 16S sequences that matched with
292 referenced sequences of the cyanobacteria *Cyanobium* sp. (Chroococcales) and the Beta-Proteobacteria
293 *Acidovorax* sp. (Burkholderiales; Comamonadaceae). The two complementary analyses of the bacterial
294 community structure confirmed the differences between communities, which were especially marked
295 between site C and other two sites, A and B.

296 Nine days after switching the medium water to the more polluted ones the bacterial community
297 structure changed. The biofilms switched from A to B showed a significant increase in levels of Beta-
298 Proteobacteria, Cytophaga-Flavobacteria and Actinobacteria ($p < 0.05$, Fig. 2b). In the biofilms
299 switched from A to C, Gamma-Proteobacteria, Cytophaga-Flavobacteria and Actinobacteria increased
300 significantly ($p < 0.05$, Figure 2b). However, in those switched from B to C, the only significant
301 increase was in the levels of Actinobacteria ($p = 0.001$, Fig. 2b). The Mantel test confirmed the

302 significant correlation between antibiotic concentrations and bacterial community composition, as
303 analyzed by CARD-FISH and DGGE (Table 3). Canonical correspondence analysis of the bacterial
304 community composition studied by CARD-FISH revealed differences among the distinct control and
305 switched biofilm communities and highlighted the role of antibiotics (Fig. 3b). In particular, the higher
306 levels of Actinobacteria (HCG) in all the biofilms exposed to more polluted waters were associated to
307 the higher levels of antibiotics in each destination. The Spearman test confirmed the positive correlation
308 between Actinobacteria abundance and concentrations for all antibiotic families ($R \geq 0.63$, $p \leq 0.005$).
309 The similarity analysis with the DGGE results showed that the switched communities become similar in
310 composition to the autochthonous communities of the destination site (Fig. 4b). In other words, the
311 bacterial community in the biofilms switched from sites B to C became similar to that of the site C
312 biofilms (Fig. 4b).
313
314 *Functional responses of biofilms to water switch*
315 The biofilm communities from the sampling sites differed in their bacterial viability. Before switching
316 the medium water, the biofilms from site C had a significantly lower proportion of live bacteria than did
317 those from site A or B ($p < 0.001$, Fig. 5a). However, there were no significant differences in the
318 extracellular enzyme activities among the biofilms from the different sites (Fig. 5a). Mantel test analysis
319 revealed a significant correlation between antibiotic levels and functional parameters (Table 3).
320 Bacterial viability and community functioning were affected by the switch of the medium
321 waters.. The biofilms showed a significant increase in bacterial mortality ($p < 0.001$, Fig. 5b). The most
322 pronounced increase in mortality was observed in the biofilms switched from site A to site C (Fig. 5b).
323 The Spearman test revealed a significant negative correlation between tetracycline concentrations and
324 the live/dead bacteria ratio ($R = -0.551$ $p = 0.018$). Furthermore, the biofilms showed a significant
325 decrease in their extracellular peptidase activity, particularly those that were exposed to water from the
326 site C ($p < 0.05$, Fig. 5b). Also, a significant decrease in extracellular phosphatase activity was observed

327 in the biofilms switched from site A to site C (p = 0.008, Fig. 5b). However, β-Glucosidase activity was
328 not affected by translocation. The Mantel test confirmed the significant correlation between antibiotic
329 concentrations and functional biofilm metrics after translocation (Table 3).
330
331 **Discussion**
332 Antibiotics were detected in more than 90% of the water samples analyzed in the selected Llobregat
333 sites, and concentrations (in the range of ng L^{-1}) were comparable to those observed in other impacted
334 areas (Costanzo et al., 2005; Luo et al., 2011; Managaki et al., 2007; Watkinson et al., 2009). The inputs
335 of antibiotics in running waters may occur via point or non-point sources (Watkinson et al., 2009).
336 Antibiotics for human use are continuously released into aquatic environments via point-source
337 wastewater, whereas those for veterinary and agricultural use enter by non-point sources, mainly after
338 rainfall events. Consequently, higher concentrations of antibiotics entering from point sources should
339 occur in densely populated areas during low-flow dry periods (because of reduced dilution capacity),
340 whereas peaks of compounds entering from non-point sources are expected in more rural zones during
341 floods after rainfall.
342 The higher concentrations of antibiotics that we found at Sant Joan Despí (site C) were related both to
343 the low flow recorded during the sampling period (Table 1) and to the fact that this site is the final
344 reception point for a densely populated area. The most abundant antibiotics in this area
345 (sulfamethoxazole, clarithromycin and ofloxacin) are all for human use, thereby confirming that most of
346 the antibiotics in the lower Llobregat enter via urban wastewater effluents.
347 It can be argued that the distinct bacterial community structures observed at the three sampling
348 sites result from a combination of their respective pollution gradients and their local physicochemical
349 characteristics. Bacterial mortality increased in biofilms from the most polluted site, and significantly
350 correlated with concentrations of quinolones and sulfonamides. These findings are consistent with the
351 effects of sulfamethoxazole, since it inhibits the synthesis of dihydrofolic acid, a compound which

352 bacteria must produce in order to survive (Isidori et al., 2005). Nevertheless biofilms grown under
353 certain levels of antibiotics concentration are expected to develop a bacterial community more tolerant
354 to the direct stressors (antibiotic). Moreover, the higher autotrophic biomass and thickness of biofilms in
355 Sant Joan Despí water (observed in this and others studies, Ricart et al., 2010) could limit the
356 penetration of antibiotics into the biofilm (Stewart and Costerton, 2001). More abundant extracellular
357 materials in thick biofilms can act as a trap for dissolved substances and limit the interaction and
358 subsequent effects on target and non-target organisms (Sabater et al., 2007). Therefore, although the
359 direct effect of antibiotics on non-resistant bacteria cannot be totally discarded, other factors can also
360 account for the reduced viability of the bacterial community in the most polluted site. The high biofilm
361 thickness can also account for the higher proportion of dead bacteria in these communities. Several
362 studies have positively related the biomass or production of algae and bacteria in natural biofilms
363 (Haack and McFaters,1982; Rier and Stevenson, 2002; Romaní and Sabater, 1999; Romaní and Sabater,
364 2000) while others have shown that these relationships may be uncoupled (Sobczak, 1996; Ylla et al.
365 2009), especially when bacteria compete with algae for inorganic nutrients (Jansson, 1988). It might
366 happen that the strong competition for resources, combined with lower exchange rates with the flowing
367 water column, both resulting from high biofilm thickness, can generate unfavorable conditions for
368 bacteria, reducing the physiological state of cells, and resulting in higher mortality rates. Under these
369 conditions, the direct effects of antibiotics on bacteria could be reduced because of the low interaction
370 between biofilms and antibiotics in the water, as well as because of the reduced metabolic activity of
371 cells. Furthermore, the higher proportion of dead bacteria at the most polluted site could be also related
372 to the effects of multiple stressors on local biofilms (Ricart et al., 2010, Muñoz et al., 2009, Proia et al.,
373 2011). The presence of many priority and emerging compounds along the Llobregat river gradient (Gros
374 et al., 2007; Osorio et al., 2012, Ricart et al., 2010) may cause indirect stress for bacteria in more
375 polluted sites. The negative direct effects of chemicals on biofilm autotrophs can also affect indirectly
376 biofilm bacteria (Bonninneau et al., 2010; Proia et al., 2012a). For example, the effect of Diuron on

377 bacterial communities has been attributed to the indirect action of the herbicide on heterotrophs via
378 direct action on autotrophs (Ricart et al., 2009; López-Doval et al., 2009; Pesce et al., 2006; Tlili et al.,
379 2008).

380 Antibiotics in river water could represent a selective pressure during the first phases of biofilm
381 development, when bacteria are early colonizers and attach to mineral surfaces via their polysaccharide
382 glycocalyx (Bärlocher and Murdoch 1989). Some role of the experimental setup used in this work for
383 the selection of species in batches cannot be discarded, nevertheless biofilm behavior and responses
384 during colonization and after water switch highlighted that different water quality influenced biofilm
385 bacterial communities assemblage. Different bacterial strains may be selected according to their
386 resistance to antibiotics and other environmental stressors, resulting in different compositions at once
387 biofilm colonization has ended. Has been widely described the capacity of bacteria to develop resistance
388 to antibiotics (Luo et al., 2010; Manivasagan et al., 2011; Storteboom et al., 2010; Schwartz et al.,
389 2003), as well as the innate natural resistance of bacteria isolated from the aquatic environments (Kelch
390 and Lee,1978; Ash et al., 2002). The significant positive correlations between certain antibiotics and the
391 abundances of certain bacterial groups corroborate these possibilities.

392 The significantly higher levels of the main bacterial groups (*Alpha-, Beta and Gamma* -
393 Proteobacteria) in the most polluted site were probably associated to the general increase in bacterial
394 density downstream, which roughly coincides with the trophic state. Multivariate analysis (CCA)
395 revealed that despite the higher levels of all the Proteobacteria groups in Sant Joan Despí, only the
396 difference in the HCG (Actinobacteria) group was related to the increasing concentrations of antibiotics
397 (Figure 3a).

398 Major changes in bacterial composition occurred in the biofilms that were switched from less to
399 more polluted waters respect to their controls. Particularly, HCG and CF were associated with switching
400 changes and the antibiotics concentration between sites (Figs. 2b and 3b, respectively). High CG-
401 content bacteria are Gram-positive bacteria of the class of Actinobacteria, a group that includes some of

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402 the most common soil, freshwater and marine life microbes. The increased abundance of this group
403 could be directly and indirectly related to the selective pressure of the antibiotics, as suggested by the
404 positive correlation between this increase and all the antibiotic families ($R > 0.63$; $p \leq 0.005$). Species of
405 this bacterial group—especially those from the genus Streptomyces—produce hundreds of naturally
406 occurring antibiotics (*e.g.* tetracycline, streptomycin) (Watve et al., 2001) and are therefore intrinsically
407 resistant to them. A recent study performed on DNA from 30,000-year-old Beringian permafrost
408 sediments focused on 16S rRNA sequences of the Actinobacteria group, found a highly diverse
409 collection of genes encoding the resistance to several antibiotics, therefore showing that resistance is a
410 natural ancient phenomenon in bacteria (D'Costa et al., 2011). Moreover, antibiotics can act directly on
411 no resistant bacteria, thereby rapidly eliminating the most sensitive species and consequently promoting
412 the development of the resistant taxa. Given a lack of strong competition, these fledgling taxa can
413 ultimately achieve high cell density (Fleeger et al., 2003; Le Jeune et al., 2007). A different process may
414 account for the increase in Cytophaga Flavobacterium in the biofilms switched to more polluted waters.
415 This group is one of the most abundant bacteria groups in aquatic environments (Kirchman et al., 2002).
416 The positive correlation with tetracycline ($R = 0.491$; $p = 0.038$) suggests that resistant strains of this
417 group of bacteria may be favored by high concentrations of the antibiotic in C. This result contrasts with
418 that of Kelch and Lee (1978), who observed resistance to tetracycline in only the 1% of the planktonic
419 CF in the Tillamook Bay (Oregon, US). The enhanced resistance of bacteria to antibiotics, once they are
420 organized in biofilms have been widely described (Stewart and Costerton, 2001), but cannot be the only
421 reason to justify our results. The higher conductivity, nitrate and SRP levels observed downstream in
422 our study also may explain the aforementioned Cytophaga Flavobacterium trend. In fact, CF bacteria are
423 known to be involved in organic matter (OM) uptake and degradation because of their specialized role
424 and abundance in river ecosystems (Kirchman, 2002). It can be therefore possible that higher inputs of
425 OM (co-occurring with increased conductivity, SRP and nitrates) derived from increasing WWTPs
426 effluents downstream could explain the CF increase in biofilms switched to C waters. Olapade and Leff

427 (2004) made similar findings when studying the epilithic biofilms of a stream in the USA: they showed
428 that this bacterial group clustered with conductivity, nitrates and SRP. Finally, the co-occurrence of low
429 concentrations of a huge number of priority and emerging pollutants, not measured in this study but
430 occurring in Llobregat surface waters (Osorio et al., 2012; Ricart et al., 2010), may also interfere with
431 the observed result.

432 The magnitude of the bacterial community response in each sampling site was associated with
433 the local levels of antibiotics. The greatest responses were observed in communities grown with water
434 from the least polluted site (A: Castellbell) when switched in in water of the most polluted site (C: Sant
435 Joan Despí). Even though the antibiotic concentrations were very low, their reactivity, as well as their
436 continuous influx into the River, could affect bacterial viability and community composition. In the
437 most polluted site total antibiotic concentration was 6.1 times higher, and the level of the protein
438 synthesis inhibitor Tetracycline was 36.8 times higher. Bacterial mortality could be associated to the
439 presence of antibiotics in the water. Indeed, there was a significant correlation between the tetracycline
440 concentration and the proportion of dead bacteria in the biofilms switched to waters from C site.

441 Our study reveals that the changes in the biofilm bacteria community structure before the
442 experimental manipulations were not associated with differences in the corresponding heterotrophic
443 extracellular enzyme activities. However, switch to more polluted waters caused a decrease in the
444 extracellular activities of peptidase and phosphatase–enzymes that have been associated to the capacity
445 of heterotrophic bacteria to degrade high molecular weight molecules into small peptides, amino acids
446 and inorganic nutrients for easy uptake (Rosso and Azam, 1987; Chróst, 1991). Francoeur and Wetzel
447 (2003) suggested that area-specific enzyme activity may be altered by changes in three factors: the
448 abundance of enzyme-producing organisms; the amount of enzyme produced per organism; and the
449 activity of individual enzyme molecules. The decrease in phosphatase activity in the most polluted site
450 could be related to the higher concentration of inorganic phosphorus that may inhibit phosphatase
451 expression (Chróst and Overvebck, 1987). However, phosphatase activity negatively correlated with

452 tetracycline after switching the waters (R = -0.621; p = 0.006). Also, the increase in bacterial mortality
453 in the switched communities could provoke the decrease in extracellular phosphatase and peptidase
454 activities.. Moreover, two of the most abundant antibiotics in the Llobregat River (tetracycline and
455 clarithromycin) are protein synthesis inhibitors, which may limit the amount of enzymes (*e.g.* peptidase
456 and phosphatase) that each cell can produce.

457 Our findings in the Llobregat River indicate that the continuous entrance of antibiotics into river
458 ecosystems may lead to significant changes in microbial attached communities. In particular, higher
459 levels of antibiotics induced changes in the bacterial community structure of river biofilms by favoring
460 the antibiotic-resistant bacteria (Actinobacteria). Moreover, the levels of antibiotics detected in the most
461 polluted site of Llobregat River induce bacterial mortality and reduce the biofilm capacity to mineralize
462 organic matter. These changes may have consequences in terms of loss of biodiversity and alteration of
463 biogeochemical cycles at ecosystem level. Microbial attached communities dominate the metabolism of
464 most river ecosystems and are a major component for the uptake, storage, and cycling of nutrients.
465 Although polluted rivers are affected by many co-occurring factors, the presence of antibiotics in urban
466 areas must be considered as a relevant risk factor for bacterial biofilm communities in aquatic
467 ecosystems.

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471

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Figure captions

Figure 1. Study area with selected sampling sites (denoted with stars) in the Llobregat River.

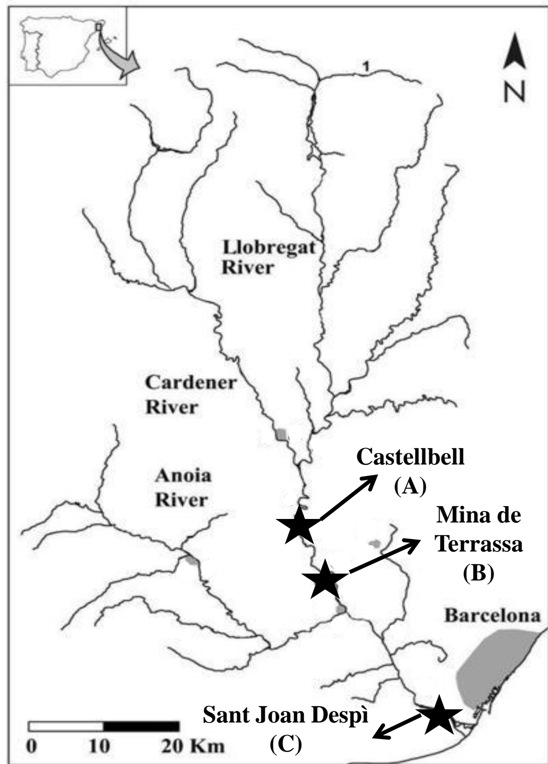
Figure 2. Abundances of the bacterial groups analysed by CARD-FISH before (a) and after (b) the translocation experiments. Values are means +/- standard deviations (n = 3).

Figure 3. Responses of the biofilm bacterial groups to antibiotic contamination in river water before (a) and after (b) the translocation experiments as expressed by canonical correspondence analysis (CCA).

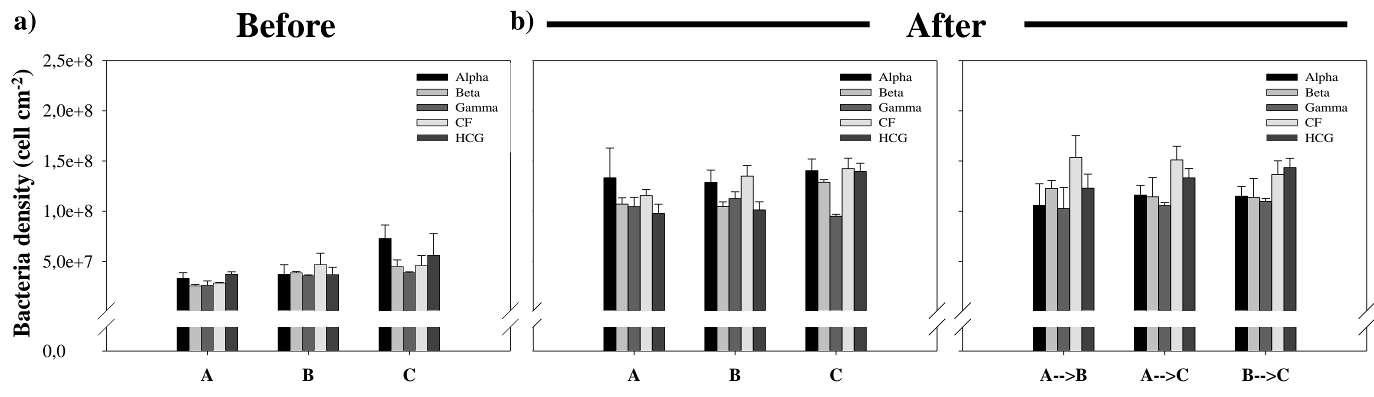
Figure 4. Cluster analysis based on Bray-Curtis similarity of biofilm bacterial community analysed by DGGE in translocation from sites A to B (a), B to C (b), and A to C (c).

Figure 5. Functional responses of biofilm before (a) and after (b) the translocation experiments. Values are means +/- standard deviations (n = 3).

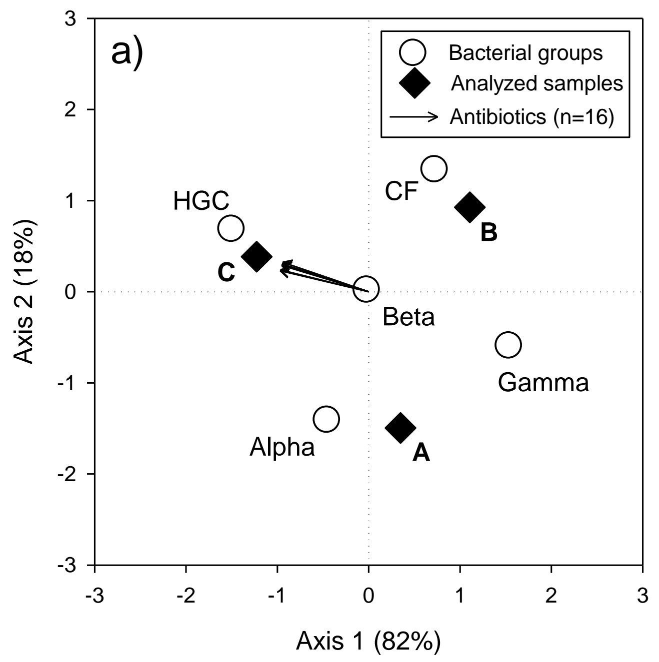
Figure

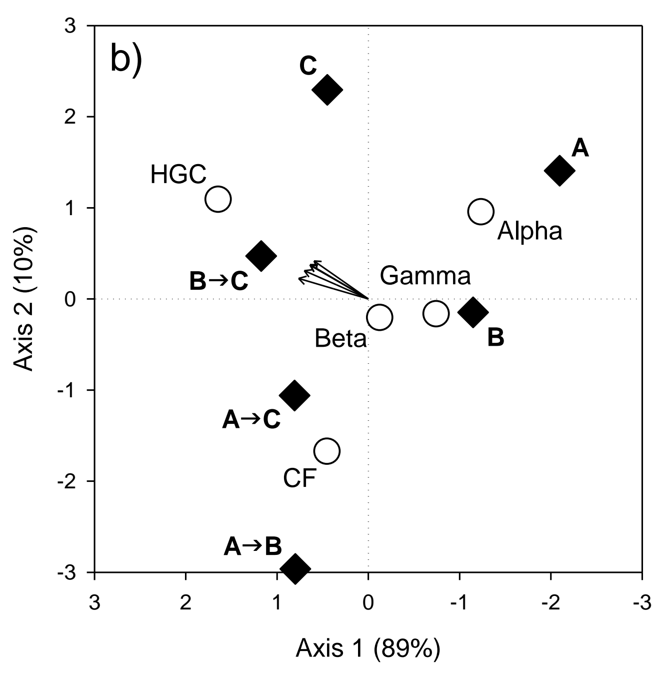


Figure



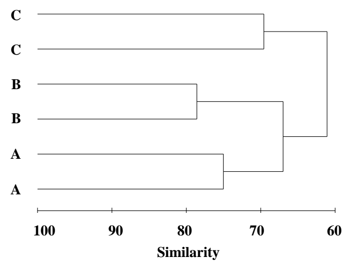
Figure



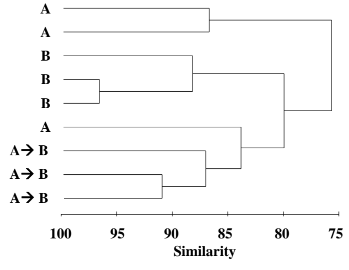


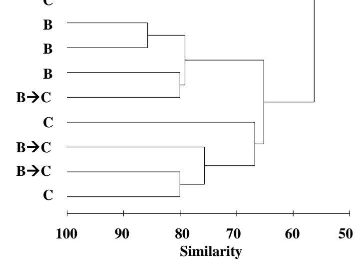
Figure

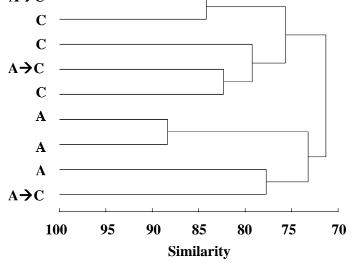
a) **Before**



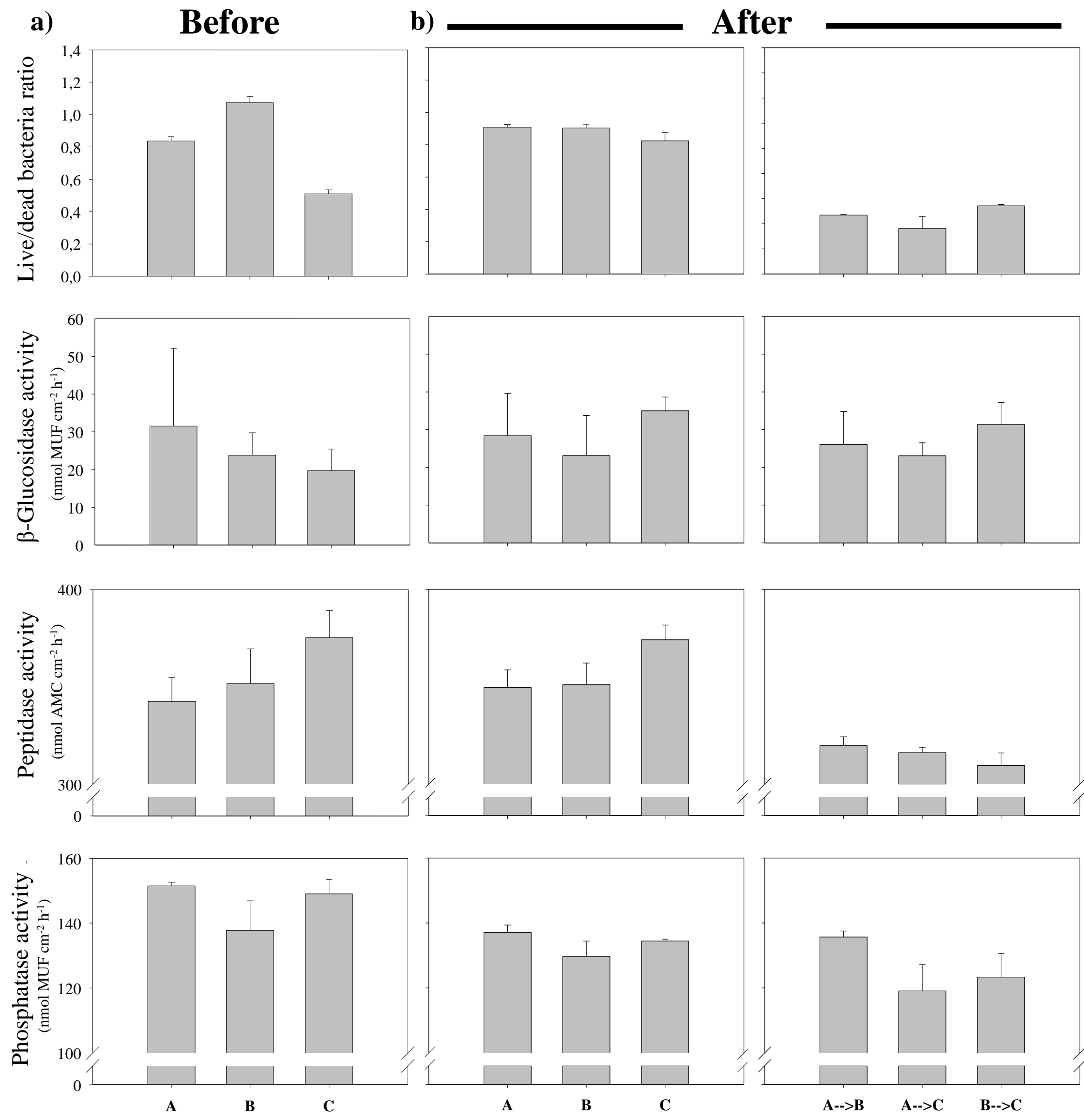
b) **After**







Figure



Table
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	Discharge ($m^3 s^{-1}$)	**Conductivity (µS cm^{-1})**	**pH**	**Oxygen (mg L^{-1})**	**T (°C)**	**SRP (µg L^{-1})**	**$N\text{-}NH_4$ (mg L^{-1})**	**$N\text{-}NO_3$ (mg L^{-1})**
A	6.06 *(0.50)*	1649.5 *(22.6)*	8.52 *(0.11)*	10.89 *(1.37)*	13.5 *(2.7)*	44. 0 *(57.18)*	0.09 *(0.10)*	7.40 *(0.87)*
B	4.85 *(0.72)*	1695.8 *(38.7)*	8.43 *(0.06)*	9.77 *(1.09)*	14.5 *(2.8)*	70.85 *(50.73)*	0.13 *(0.03)*	7.10 *(0.83)*
C	2.91 *(1.04)*	2045.0 *(67.4)*	8.44 *(0.13)*	10.29 *(1.38)*	16.1 *(2.5)*	104.63 *(38.04)*	0.07 *(0.04)*	10.84 *(2.62)*

Table 1. Results of physical-chemical variables measured at each sampling site. Values are expressed as mean values with SD in parenthesis (n= 16).

Table
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		A	B	C
Macrolides	**Eritromycin**	4.00 *(0.23)*	3.89 *(1.47)*	32.27 *(14.21)*
	Azithromycin	6.99 *(0.02)*	6.97 *(0.02)*	7.18 *(0.04)*
	Roxithromycin	0.69 *(0.22)*	0.58 *(0.07)*	5.98 *(2.33)*
	Clarithromycin	47.43 *(4.00)*	36.84 *(1.91)*	162.01 *(67.03)*
	Tylosin	3.68 *(0.38)*	2.95 *(1.05)*	18.77 *(12.60)*
	Josamycin	0.52 *(0.12)*	0.39 *(0.08)*	3.08 *(0.60)*
	Spiramycin	8.00 *(2.02)*	5.55 *(0.74)*	40.13 *(14.04)*
	Tilmicosin	93.19 *(154.64)*	24.64 *(39.68)*	2.24 *(0.37)*
Quinolones	**Ofloxacin**	29.96 *(6.20)*	20.75 *(3.69)*	207.60 *(72.73)*
	Ciprofloxacin	25.43 *(4.28)*	21.60 *(2.19)*	59.16 *(23.88)*
	Enoxacin	11.38 *(2.65)*	9.48 *(0.51)*	27.49 *(4.28)*
	Enrofloxacin	3.58 *(0.28)*	2.87 *(0.78)*	154.34 *(28.17)*
	Flumequine	0.30 *(0.05)*	0.35 *(0.05)*	0.47 *(0.11)*
Sulfonamides	**Sulfamethoxazole**	234.10 *(13.51)*	213.70 *(17.25)*	907.60 *(312.43)*
	Sulfadiazine	6.56 *(0.90)*	9.02 *(4.10)*	29.17 *(4.86)*
Tetracyclines	**Tetracycline**	7.96 *(2.67)*	11.29 *(2.86)*	148.71 *(43.31)*
Chemotherapeutic	**Trimethoprim**	7.60 *(1.02)*	6.01 *(0.44)*	27.38 *(6.62)*

Table 2. Results of antibiotics concentrations measured at each sampling site. Values are expressed as mean values with SD in parenthesis (n= 18).

Table
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	Antibiotics	Structure	Function
Antibiotics	-	R = 0.003 p = 0.388	**R = 0.403** **p = 0.013**
Structure	**R = 0.108** **p = 0.041**	-	R = 0.015 p = 0.354
Function	**R= 0.274** **p = 0.023**	**R = 0.578** **p = 0.0002**	-

Table 2. Mantel tests between three different Bray-Curtis similarity matrices: (1) the antibiotic concentrations; (2) the bacterial community structure (CARD-FISH and DGGE), and (3) function (live/dead ratio and enzyme activities) of biofilms before and after translocation (the upper and lower parts of the table, respectively). The Pearson's correlation coefficient (R) was calculated among all the entries in the two matrices and compared in 5000 random permutations. The reported p-values are one-tailed.