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Life in an unsuspected antibiotics world: River biofilms

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2 Abstract

3 Waterborne bacteria that naturally live in biofilms are continuously exposed to pharmaceutical
4 residues, regularly released into the freshwater environment. At the source level, the discharge of
5 antibiotics into rivers has already been repeatedly linked to the development of antimicrobial
6 resistance. But what about biofilms away from the discharge point? Two rivers, with sites subject to
7 dispersed contamination of medium intensity, were studied as typical representatives of high- and
8 middle-income countries. The biofilms developed on rocks indigenous to rivers are perfectly
9 representative of environmental exposure. Our results show that away from the hotspots, the amount
10 of antibiotics in the biofilms studied favours the maintenance and enrichment of existing resistant
11 strains as well as the selection of new resistant mutants, and these favourable conditions remain over
12 a period of time. Thus, in this type of river, the environmental risk of selection pressure is not only
13 present downstream of urbanised areas but is also possible upstream and far downstream of
14 wastewater treatment plant discharges. Despite this, correlation analysis found no strong positive
15 correlation between antibiotic concentrations and the abundance of measured integrons and their
16 corresponding resistance genes. Nevertheless, this work highlights the need to consider the risks of
17 antibiotics beyond hotspots as well.

18 **Keywords:** antibiotics, antibiotic resistance, epilithic biofilm, freshwater environment, integrons, risk
19 quotient.

20 1. Introduction

21 The persistence of antimicrobial resistance (AMR) in the environment is among the biggest emerging
22 health threats, according to UN Environment (UNEP, no date). Many studies already link the misuse of
23 antibiotics in medicine and agriculture to increased AMR, but the effects of antibiotics in the
24 environment due to wastewater treatment plants (WWTPs) emissions and their relationship to local

25 microflora need to be deepened (Roche et al., 2016). In recent years, the role of the environment as a
26 reservoir of AMR and its dissemination pathway has become increasingly considered. Consequently,
27 growing attention is being paid to the presence of antibiotic-resistant bacteria (ARB) and antibiotic
28 residues (selectors of resistant bacteria) in the environment.

29 A recent study that surveyed 91 rivers around the world found antibiotics in the waters of nearly
30 two-thirds of all the studied sites, from the Thames to the Mekong (Boxall and Wilkinson, 2019). Typical
31 concentrations range from ng/L to µg/L, largely because of diffuse of WWTPs discharges, which are a
32 main source of antibiotics in the aquatic environment in France (Haenni *et al.*, 2022) and leachings from
33 livestock farms. Thus, both environmental and fecal bacteria (from animals or WWTPs) are constantly
34 exposed to antibiotics, which will probably affect microbial ecology and encourages the selection of
35 ARB or antibiotic resistant genes (ARGs) in rivers. Consequently, there is growing interest in
36 determining the potential of antibiotic concentrations in the environment that stimulate the
37 development of AMR (Chow, Ghaly and Gillings, 2021). Resistance to antibiotics is often carried by the
38 mobile genetic elements (MGEs) called integrons. These elements have a site-specific recombination
39 system that helps them capture, integrate, express, and exchange gene cassettes (Stalder *et al.*, 2014).
40 More than 130 resistance genes (RGs) have been described among the integron cassettes, including
41 genes conferring resistance to antibiotics (Gillings, 2014). The clinical class 1 integron-integrase gene
42 is now recognized as one of the indicators of “resistance” in the environment (Zheng *et al.*, 2020;
43 Haenni *et al.*, 2022). Several studies have highlighted the link between integrons (more specifically
44 class 1 and 2) and Gram-negative bacteria with multidrug resistance, even if resistance is due not only
45 to cassette-borne genes, but also to other ARGs located outside the integrons, and that the integron
46 marker has good negative predictive value for acquired resistance (Leverstein-Van Hall *et al.*, 2003;
47 Barraud *et al.*, 2014). Since bacteria, containing integrons, can be selected not only by antibiotics but
48 also by other pollutants (e.g. heavy metals, disinfectants, etc.) Gillings *et al.* (2015) proposed to use
49 the class 1 integron-integrase gene as a proxy for anthropogenic pollution.

50 As most bacteria live in communities known as biofilms, it seems important to consider this bacterial
51 lifestyle to evaluate the “true” exposure of bacteria to antibiotics circulating in rivers. Biofilms are
52 densely packed communities of microbial cells that grow on surfaces and secrete extracellular
53 polymeric substances (EPS). Bacteria inside biofilms may display properties significantly different from
54 free-living bacterial cells (Flemming *et al.*, 2016). The ecological success of biofilms lies in their
55 structure – this communal way of life provides bacteria physical protection from grazers and chemical
56 protection from toxic compounds (Raghupathi *et al.*, 2018; Sentenac *et al.*, 2022). Thus, several studies
57 show that the components of the biofilm (EPS, polysaccharides, lipids, DNA fragments) bind almost all

58 antibiotics (and other pollutants) present in water. This property makes the biofilms an attractive
59 indicator of water quality (Balcázar, Subirats and Borrego, 2015; Proia *et al.*, 2016; Pu *et al.*, 2019).
60 However, there are critical gaps in knowledge about the effect of antibiotics present in the biofilm on
61 bacterial viability and the spread of AMR in the environment (Larsson *et al.*, 2018). In order to
62 overcome the problem of antibiotic resistance, a global approach is required in human health, but also
63 in animal and environmental health (“One Health” concept) (Destoumieux-Garzón *et al.*, 2018). One
64 key area where research is considered urgent is the role of antibiotic contamination in the
65 environment.

66 Resistance, tolerance, and persistence are different responses to antibiotics exposure that lead to
67 increased survival compared to susceptible cells (Dincer, Uslu and Delik, 2020; Uruén *et al.*, 2021).
68 Clinical knowledge and experience show that strong antibiotic exposure can stimulate ARBs as well as
69 subpopulations of cells that can survive without being resistant (persisters) (Balaban *et al.*, 2019).
70 However, subinhibitory concentrations of antibiotics can also contribute to increased mutation rates,
71 horizontal gene transfer (HGT) and biofilm formation (structure and composition), which can lead to
72 the emergence and spread of AMR (Khan, Beattie and Knapp, 2017). Furthermore, resistant
73 populations selected at subinhibitory concentrations do not lose acquired resistance in the absence of
74 antibacterial agents (Hershberg, 2017). Thus, the accumulation of antibiotics in environmental biofilm
75 can have a significant impact on the evolution of AMR in bacterial populations in environmental
76 biofilms.

77 In France, as in other high-income countries, antibiotic pollution comes mainly from WWTP
78 discharges (i.e., hotspots). Most studies focus on the risk of AMR in such hotspot situations and few
79 focus on situations where pollution is more diluted/dispersed (even if these situations cover most of
80 the surface of the catchment areas). The aim of this work was to raise the question of the "real impact"
81 of antibiotics on environmental biofilm-producing bacteria and the possible consequences in terms of
82 antibiotic resistance. To this end, an in-situ approach was developed. Antibiotic concentrations in
83 freshwater biofilms (at dispersed pollution sites or hotspots, i.e. the WWTP discharges) were
84 investigated for one year. The measured concentrations were then compared with toxicological dose
85 descriptors as well as with the abundance of AMR markers (integrons and ARGs), also measured in
86 biofilms, to determine whether antibiotic concentrations could have a direct effect on AMR
87 development.

88 **2. Material and methods**

89 **2.1 Sampling area and sample collection**

90 Field experiments were conducted on two French rivers (Figure S1): the Clain river (CR) (upstream
91 (UPS) and downstream (DWS) from the WWTP of Poitiers = 160,000 population equivalent) and the
92 Vienne river (VR) (upstream from the WWTP of Châtelleraut = 93,000 population equivalent). These
93 rivers are typical of French catchment areas with moderate urbanisation and only 1 or 2 medium-sized
94 cities. These rivers are subject to moderate anthropogenic pressure and are not extremely polluted.
95 Pollution comes from discharges from WWTPs, which are considered in this study as hotspots, and
96 from individual sanitation systems, which generate dispersed pollution of medium intensity
97 throughout the catchment. It is interesting to study this type of river because the mechanisms
98 identified in the hotspots may "not necessarily" be the same as those involved in the dispersed
99 pollution sites. Furthermore, rivers with dispersed pollution of medium-intensity are widely present in
100 high- and middle-income European countries. The downstream biofilms collected near the discharge
101 pipe of the WWTP were, therefore, more intensively exposed to antibiotics released into the river
102 (local hotspots). Biofilms collected upstream of the WWTP may have encountered dispersed pollution
103 from upstream in the watershed. These UPS biofilms are probably not as contaminated with dead
104 bacteria, DNA residues, as the DWS biofilms.

105 Epilithic biofilms were grown on initially sterile rocks (about 1000 fragments of diorite per plot).
106 The sterile rocks were placed in rivers for 5 months and then gradually collected: a few rocks per
107 month, corresponding to one bulk sample. The biofilms were therefore cultivated for 5 months in the
108 river before the start of the experiments in order to have time to form and equilibrate
109 (microbiologically and chemically). Our biofilms developed on natural supports (rock) corresponding
110 to the rivers studied and not on artificial supports (as is widely the case: glass or plastic slides) which
111 very strongly modify/change the microbial populations and their capacity to sorb pollutants. All these
112 methodological precautions were taken to ensure that the biofilms were representative of their river
113 and the reality of their exposure condition.

114 Sampling campaign over one year was led from January to December 2018 (one sample per month).
115 The biofilm was carefully scraped off the rocks with a sterile toothbrush and MilliQ grade water. The
116 resulting solution was then gently centrifuged to remove the water. The obtained "fresh" biofilms were
117 stored in bottles at -80 °C before the analysis.

118 **2.2 Analytical methods for antibiotics quantification**

119 Twenty commonly used antibiotics (6 classes) (Table 1) were measured in all collected samples by
120 the procedure described in Aubertheau *et al.* (2017) and Matviichuk *et al.* (2022). Pressurized liquid
121 extraction (ASE™ 350, Thermo Scientific, Waltham - USA) was used to extract antibiotics from the

122 biofilm samples. Then, extracts were purified and pre-concentrated by solid-phase extraction (SPE)
123 (AutotraceTM 280, Thermo Scientific, Waltham – USA), using OASIS HLB 6cc cartridges, and finally
124 analysed by ultra-performance liquid chromatography coupled with triple quadrupole mass
125 spectrometry (Shimadzu 8060, Marne-la-Vallée – France). To overcome the matrix effect (changes
126 within the biofilm with the season), quantification was obtained based on the standard addition
127 method. Concentrations in biofilms are expressed in µg of the quantified antibiotic per L of “fresh”
128 biofilm (to compare with toxicological dose descriptors). More details on the method validation as well
129 as established limits of detection and quantification are given in the supplementary information.

130 **2.3 Resistance genes and integrons detection and quantification**

131 Three classes of integrons (*IntI1*, *IntI2* and *IntI3*) as well as eight ARGs (*sul1*, *sul2*, *ermB*, *ermC*, *qnrA*,
132 *qnrC*, *tolC*, *dfrF*), which were frequently found in freshwater compartments (water, biofilm, sediment)
133 as well as in WWTPs (Proia *et al.*, 2016; Huang *et al.*, 2019; Zhuang *et al.*, 2021) and represents the
134 main classes of antibiotics, were examined to assess the level of AMR in biofilm communities. First,
135 the total DNA was extracted using the Fast DNA[®] SPIN Kit for Feces (MP Biomedicals, Illkirch – France)
136 according to the manufacturer’s instruction and then stored at -80 °C until further analysis. To evaluate
137 the sample quality accurately, the 260/280 ratio was analysed using a NanoDrop[™] OneC Microvolume
138 UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). The results showed an average 260/280
139 ratio ranging from 1.92 for CR DWS to 2.04 for CR UPS samples. The number of *IntI1*, *IntI2*, and *IntI3*
140 genes was quantified using the qPCR method (MX3005P real-time detection system (Stratagene[®])) as
141 described by Barraud *et al.* (2010). The 16S rRNA encoding gene was quantified using two universal
142 primers (338F and 518R) (Table S6) by the SYBR green assay method, as described by Park and Crowley
143 (2006). Prior to qPCR, the concentration of the DNA samples was adjusted to a range of 0.5-1.5 ng/µL
144 to prevent qPCR inhibitor effects and each sample was analysed in triplicate. Then, for optimal
145 quantification of *IntI1*, *IntI2*, *IntI3* and 16S rRNA coding genes, each targeted sequence was cloned into
146 a single standard plasmid using the method described by Stalder *et al.* (2014). The number of copies
147 of this plasmid was adjusted to 0.2*10⁹ copies/µL. Then, it was successively diluted ten times until 10²
148 copy numbers to obtain complete standard curve that was run at the same time with DNA samples
149 and blank control during each analysis. The concentration of integrons and 16S rRNA genes was
150 calculated considering the mass of biofilm used for DNA extraction and the DNA dilution factor. Since
151 the average number of 16S rRNA-coding genes per bacterium is currently estimated to be 4.1
152 (Aubertheau *et al.*, 2017), the number of bacteria present in each sample was estimated by dividing
153 the determined number of 16S rRNA-coding genes by 4.1. Finally, the relative abundance of integrons

154 was calculated by dividing the average concentration of integrons (in number of copies per g of biofilm)
155 by the estimated average number of bacteria.

156 ARGs were analysed by nanolitre quantitative PCR. The assay was performed using 96.96
157 BioMark™ Dynamic Array for Real-Time PCR (Fluidigm Corporation, San Francisco, CA, USA) according
158 to the protocol described by Buelow *et al.* (2018, 2020). Assay results (threshold cycle (Ct) values) were
159 extracted using BioMark real-time PCR analysis software. The normalised gene abundance was
160 calculated from the 16S rRNA gene abundance using the following formula: $2^{-(Ct_{(RG)} - Ct_{(16S\ rRNA)})}$
161 given a detection limit of 20.

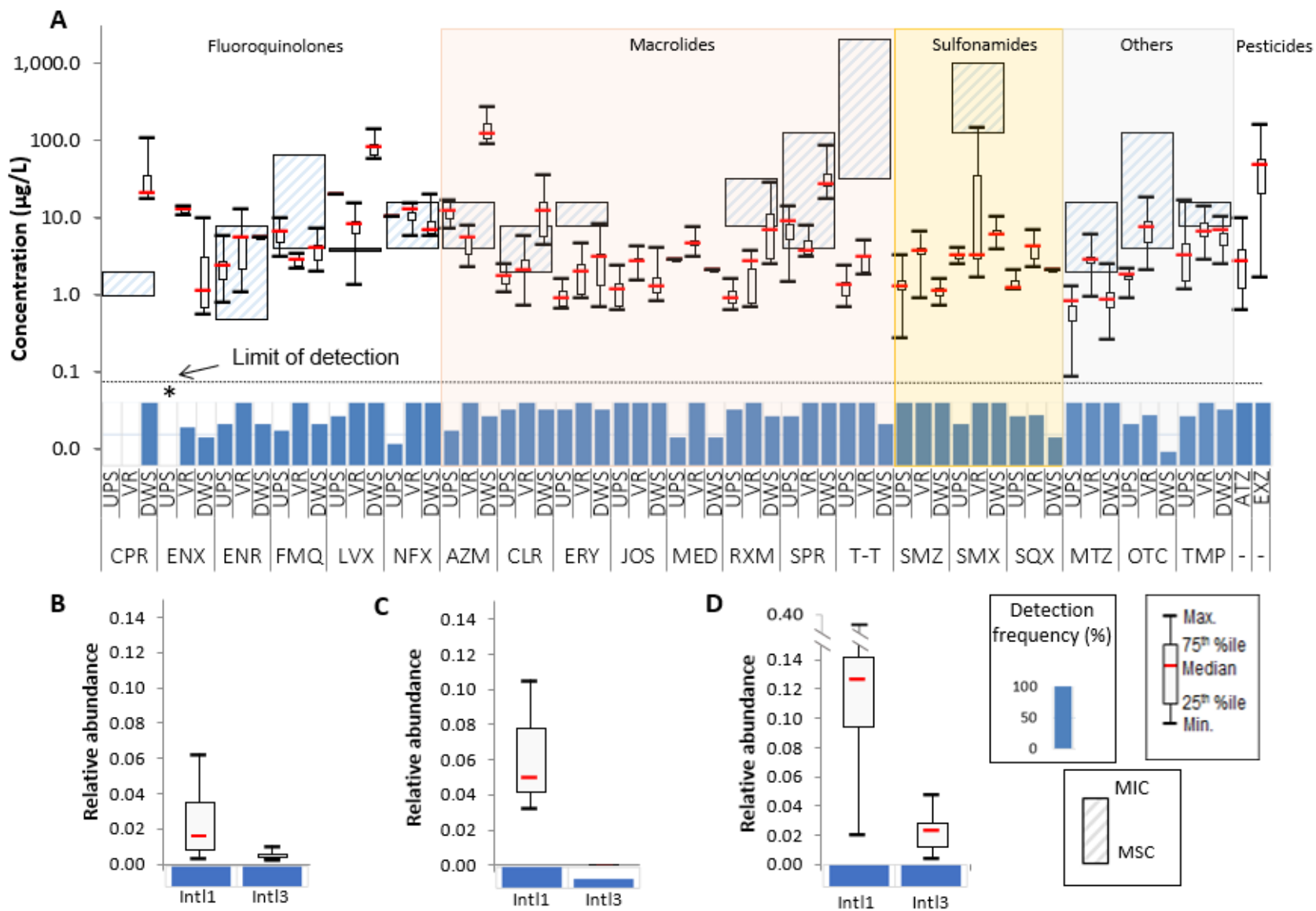
162 The Pearson correlation coefficient between antibiotic concentrations and abundance of
163 ARGs/integrons was calculated using the data analysis ToolPak. Principal component analysis (PCA)
164 was performed on RStudio (version 4.1106.0) using package “Rcmdr”.

165 **3. Results and discussion**

166 **3.1. Are there a lot of antibiotics in biofilms?**

167 Analysis of 20 antibiotics in biofilms collected monthly (over one year) reveal the huge occurrence
168 of these compounds: 18-20 and 19 antibiotics were detected in the two studied rivers (in CR and VR
169 biofilms, respectively – Figure 1). Differences of occurrence were observed depending on the
170 antibiotic’s class but on average, the most frequently detected in both upstream sites (CR UPS and VR)
171 were macrolides (in CR - 81 % and in VR - 100 % of all macrolides analysed). In VR, up to 16 antibiotics
172 were detected in 100% of biofilms. The other antibiotics were detected in over 50% of biofilms: OTC
173 and SQX in 80%, ENX in 60% of samples. In the CR UPS 4 compounds (JOS, T-T, SMZ and MTZ) were
174 detected in all analysed biofilms. The others were found in the half of the samples (except MED and
175 NFX detected at 44% and 33%, respectively). These findings confirm the data from other studies that
176 have reported the presence of antibiotics in biofilms (Aubertheau *et al.*, 2017; Guo *et al.*, 2018).

177 In the CR, results show a slight but statistically significant difference in antibiotic occurrences
178 between the upstream and the downstream points. This confirms the variability of antibiotics
179 contamination depending on the proximity of the pollution sources. Thus, 8 antibiotics (CPR, LVX, NFX,
180 JOS, SPR, SMZ, SMX, MTZ) were detected in 100% of biofilms collected downstream the WWTP. The
181 other antibiotics were found at more than 67% of samples analysed (except ENX, MED, SQX detected
182 at 44% and OTC detected in 22% of samples). The discharge of treated wastewater, however,
183 considerably modified the presence of certain antibacterial drugs such as CPR. Ciprofloxacin is known
184 for its strong affinity to particles (Zhang *et al.*, 2018), hence its absence in areas distant from the
185



186 **Figure 1. Distribution and superimposition of 20 antibiotics and 2 common pesticides concentrations in biofilms (in $\mu\text{g/L}$) grown and collected from two**
 187 **rivers with their mutant selection (MIC-MSC) windows (A) and relative abundance of *Int1* and *Int3* upstream (UPS) of the WWTP in the CR (B), in the VR**
 188 **(C), downstream (DWS) of the WWTP in the CR (D). MIC-MSC bars mark the boundaries of MSWs. * - concentration of ENX was <LOD in several UPS samples**

189 WWTPs can be explained by rapid adsorption on sludge or on suspended solids or sediments.

190 **3.2. What are the theoretical risks of so many antibiotics?**

191 Our results indicate that antibiotic concentration ranges (min, max, and mean) differ depending on
192 the river or the proximity to pollution sources. The highest concentrations (in $\mu\text{g/L}$) are observed for
193 LVX (20.85), AZM (16.64), and TMP (16.68) in CR UPS and SMX (145.70) in VR. The lowest
194 concentrations are observed for MTZ (0.09) and SMZ (0.28) in CR and RXM (0.69) and CLR (0.74) in VR.
195 The results also show that the maximum concentrations found downstream of the WWTP are higher
196 than upstream for certain compounds: LVX (141.05), AZM (274.25), SPR (85.82), and lower for SMZ
197 (1.61) and TMP (10.60). Several of the antibiotics analysed (CLR, ERY, and SMX) were also quantified
198 at similar concentrations in other studies (Zhang *et al.*, 2018). It should be noted, however, that the
199 concentrations remain far below from the values observed in the area polluted by drugs-producing
200 industries (Larsson, Pedro and Paxeus, 2007). Our results also point out that the concentrations of
201 antibiotics in biofilms are as high as the concentrations of certain pesticides. For instance, the
202 concentrations of atrazine (ATZ) (an herbicide banned in France since 2003 and in Europe since 2007)
203 varies from 0.04 to 14.45 $\mu\text{g/L}$ and that of epoxiconazole (EXZ) (a fungicide banned in France since May
204 2019) varies from 0.92 to 261.29 $\mu\text{g/L}$ (Figure 1) (note, ATZ and EXZ are used as examples to illustrate
205 pesticide concentration ranges in biofilms and for comparison with antibiotic concentrations).

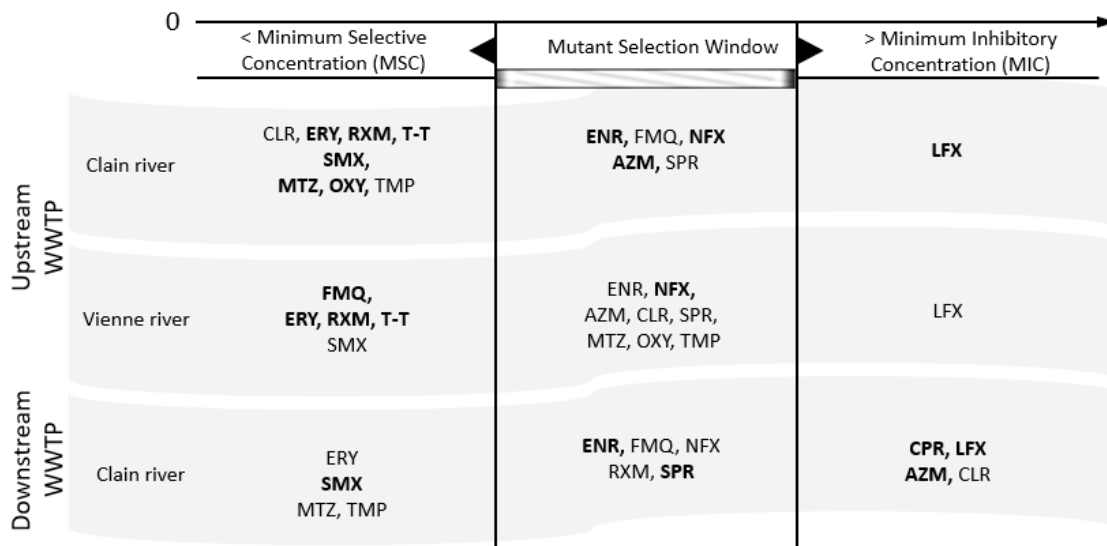
206 All these findings show that river biofilms may provide a “place” for the exposure of bacteria to
207 antibiotics. Considering this, the content of antibiotics found in biofilms was compared with the
208 toxicological dose descriptors to assess the potential effects expected depending on the range of
209 exposure. Thus, concentrations found in biofilms were compared first with their predicted no-effect
210 concentrations (PNEC_{ENV}) (Table 1) to determine the extent of their impact on the bacteria (based on
211 eco-toxicology). The corresponding PNEC_{ENV} was calculated using EC_{50} published in the literature for
212 the marine bacterium *V. fischeri* (see Table 1). For this purpose, the EC_{50} value was divided by an
213 estimate factor of 1000 as described in the EU Technical Guidance on Risk Assessment (De Bruijn *et*
214 *al.*, 2003). The results show that the maximum and average concentrations (Table S9) of four
215 fluoroquinolones (CPR, ENX, LVX, and NFX) and two macrolides (AZM, T-T) exceeded PNEC_{ENV} in at least
216 one of the studied sites. Thus, the average and maximum concentrations of CPR and LVX exceeded the
217 PNEC_{ENV} value of 11.5 $\mu\text{g/L}$ and 23 $\mu\text{g/L}$, respectively, in the CR DWS. For ENX, concentrations were
218 higher than the corresponding PNEC_{ENV} of 0.05 $\mu\text{g/L}$ in the VR and CR DWS. The concentrations of AZM
219 were above the PNEC_{ENV} of 11 $\mu\text{g/L}$ at both CR sites. For T-T, its average concentration exceeded
220 PNEC_{ENV} of 1.8 $\mu\text{g/L}$ only in the VR, while its maximum measured concentrations were above PNEC_{ENV}
221 in the VR and CR UPS. The concentrations of NFX exceeded the PNEC_{ENV} value of 0.02 $\mu\text{g/L}$ at all sites.

222 Consequently, potential hazardous effects are expected for these compounds. However, it should
223 be noted that the minimum concentrations (Table S9) of certain compounds (AZM, and CLR for the
224 upstream points) (Figure 1) are under their $PNEC_{ENV}$, suggesting a non-permanent threat level. We also
225 used the risk quotient (RQ) method to compare toxicity to environmental exposure (US EPA, 2020). RQ
226 is calculated by dividing a point estimate of exposure (i.e., median concentration measured in biofilms)
227 by a point estimate of ecological effects data ($PNEC_{ENV}$). If the resulting $RQ \geq 1$, this indicates an
228 environmental risk for bacteria; a $RQ = 0.1 - 1$ indicates an average risk, and from 0.01 to 0.1 indicates
229 a low risk (Patrolecco *et al.*, 2018). Since we found several values for $PNEC_{ENV}$ in the literature, which
230 sometimes vary considerably, we decided to review them all and focus especially on the lowest ones.
231 In addition, since the median concentration gives only a global value, which can “hide” values with
232 $RQ > 1$, we also calculated the percentage of samples with RQ above 1 (see Table 1, given in brackets
233 next to RQ values).

234 The calculated RQs show that the median concentration of six compounds (ENX, FMQ, NFX, SMX,
235 OTC and TMP) represents an environmental risk for aquatic bacteria ($RQ > 1$) in both rivers.
236 Concentrations of ENR, ERY, RXM, SPR, SMZ and MTZ pose no environmental risk in either river. Also,
237 median concentrations of FMQ and CLR (in both rivers), AZM (in the CR UPS and VR), T-T (in the CR –
238 both sites), SMX (in the CR UPS), and LVX (in the VR) have an average environmental risk. For the
239 remaining compounds, the median concentrations gave RQs greater than 1 in at least one of the
240 measured samples (i.e., AZM, T-T and SMX in the CR UPS and CLR in the CR DWS). It is worth noting
241 that the value of RQ showed a slight difference between the two studied rivers – 7 compounds have
242 $RQ > 1$ in the VR, 4 and 9 in the up and downstream points in the CR.

243 To consider the resistance risk, concentrations of antibiotics in biofilms were also compared with
244 $PNEC_{MIC}$, which represents the antibiotic concentrations over which several effects may occur, leading
245 to resistance selection. This comparison showed that the median concentrations of almost all
246 antibiotics in both rivers (except ERY and RXM in CR UPS and SMX) exceeded $PNEC_{MIC}$, meaning that
247 antibiotic levels in river biofilms can lead to the development of antibiotic resistance in biofilm
248 bacterial communities. Moreover, five to eight (depending on the site) of studied antibiotics are
249 present in the biofilms at concentrations sufficient to obtain resistance, according to the mutant
250 selection window (MSW) hypothesis, which states that the selection of resistant mutants occurs in the
251 concentration range between the minimum selective concentration (MSC) and the minimum inhibitory
252 concentration (MIC) (Gullberg *et al.*, 2011). There are several compounds systematically present in the
253 MSC-MIC window: ENR, NFX and SPR (Figure 2). Others, such as AZM are found in the MSC-MIC window
254 only at the CR upstream point and the VR. FMQ is present also in the MSC-MIC window only in the
255 samples collected in the CR. Thus, all these antibiotics are at concentrations that theoretically inhibit

256 the growth of susceptible strains, enrich existing resistant mutants, and can even select for resistant
 257 mutants from non-resistant populations. The risk analysis points out that several antibiotics are found
 258 in biofilms at concentrations higher than MIC (LVX) or lower than MSC (ERY and SMX). It is believed
 259 that if the obtained concentration of an antibiotic exceeds the MIC, the growth rate of non-resistant
 260 strains will be inhibited in favour of already resistant bacteria or (at far above MIC concentrations)
 261 even can lead to the death of the bacteria. If the obtained concentration of antibiotic is below the
 262 MSC, the risk of developing/acquiring resistance is negligible. Recent studies show that sub-MSC
 263 increase persistence mechanisms in species that have not yet developed/acquired resistance but does
 264 not promote the acquisition of resistance through other mechanisms (i.e., mutagenesis), nor does it
 265 disrupt vital functions in bacterial cells (Stanton *et al.*, 2020). In our study, RXM, T-T and SMX are lower
 266 than MSC for the CR UPS and the VR.



267 **Figure 2. Schematic representation of ATBs concentrations measured in biofilms compared to the mutant**
 268 **selection window. Whether an antibiotic belongs to a specific zone is determined by the position of its median**
 269 **value. Bold - means that all measured concentrations are within the range of the corresponding window,**
 270 **unbolded- means that median concentration is within the range of the corresponding window**

271 Thus, based on Figure 2, it is possible to conclude that the upstream positions (CR, VR) are
 272 characterized by the presence of sub-MIC and sub-MSC antibiotic concentrations in biofilms, while the
 273 downstream position (CR) is characterized by sub-MIC and over-MICs, favourable for the maintenance
 274 of resistant bacteria.

275 3.3. Are there any witnesses of the resistance?

276 Resistant integrons are frequently studied in bacteriology due to their ability to acquire, exchange,
 277 and express genes encoding resistance to antibiotics. Thus, class 1, class 2 and class 3 integrons were

278 evaluated in all samples as indicators of antibiotic resistance, as suggested by Haenni *et al.* (2022). In
279 addition, eight ARGs (*sul1*, *sul2*, *ermB*, *ermC*, *qnrA*, *qnrC*, *tolC*, *dfrF*) were also analysed to see if the
280 appearance of integrons could be directly related to the presence of antibiotics and, if so, used as AMR
281 indicators in the environment.

282 The results showed the presence of class 1 (*Int1*) in the biofilms of both rivers (Figure 1). Class 2
283 integrons were not detected and Class 3 (*Int3*) integrons were quantified in the CR biofilms and
284 detected but not quantified in the VR biofilms. *Int1* was detected in all downstream-collected samples
285 and in 78 % of the upstream samples. For *Int3*, these rates were 89 and 56 %, respectively. In samples
286 collected from the VR, *Int1* was detected in only half of the analysed biofilms. The relative abundance
287 (mean value) of these two classes of integrons is higher in the biofilms collected from the CR
288 (downstream point) than in the others: CR DWS: *Int1* - 0.14, *Int3* - 0.02; CR UPS: *Int1* - 0.02, *Int3* -
289 0.01; VR: *Int1* - 0.06, *Int3* - 0. The presence of class 1 and 3 integrons in polluted water and their
290 enrichment in WWTP discharges has already been described in various studies (Gillings *et al.*, 2015;
291 Bengtsson-Palme and Larsson, 2016; Aubertheau *et al.*, 2017; Cacace *et al.*, 2019; Matviichuk *et al.*,
292 2022). The CR and VR biofilms exhibit a high abundance of *Int1*, close to the values observed in
293 urbanized areas and/or exposed to WWTP effluents (see a comparison of results with data from the
294 literature - Figure 3). There is little environmental data on class 3 integrons (compared to two other
295 classes) but the abundance found in the CR is consistent with values from other river biofilms
296 (Aubertheau *et al.*, 2017). In most cases, the enrichment of integrons in wastewater-exposed
297 environments is associated with mechanisms of acquired resistance, in particular through HGT
298 (Karkman *et al.*, 2016; An *et al.*, 2018). The fact is that WWTPs create unique conditions for this. The
299 increased density of bacteria, presence of many stressors (antimicrobials, heavy metals, reduced
300 nutrients, organic pollutants, etc.), as well as a large variety of MGEs and ARBs contribute to the
301 horizontal transfer of integrons. Diverse MGEs (including plasmids, integrative and conjugative
302 elements, transposons, and integrons) are present in wastewater and can be captured and integrated
303 into the genome of biofilm community cells. A study by Che *et al.* (2019) revealed that most of the
304 ARGs found in WWTP compartments were carried by plasmids, known to be one of the main carriers
305 of integrons, which have no self-replication or transfer systems (Domingues, da Silva and Nielsen,
306 2012). Several studies have illustrated the enrichment of *Int1* in WWTP effluent (Che *et al.*, 2019), in
307 sediments near the discharge site (Karkman *et al.*, 2016) or in the freshwater biofilm impacted by
308 sewage effluent (Lehmann *et al.*, 2016). All these studies linked this enrichment to HGT, so it can be
309 assumed that the same mechanism led to an increase in *Int1* in CR DWS and VR biofilms (since several
310 WWTPs are located upstream of this sampling site).

311 Regarding the presence of ARGs, similar observations to the profiles of integrons were made: the
312

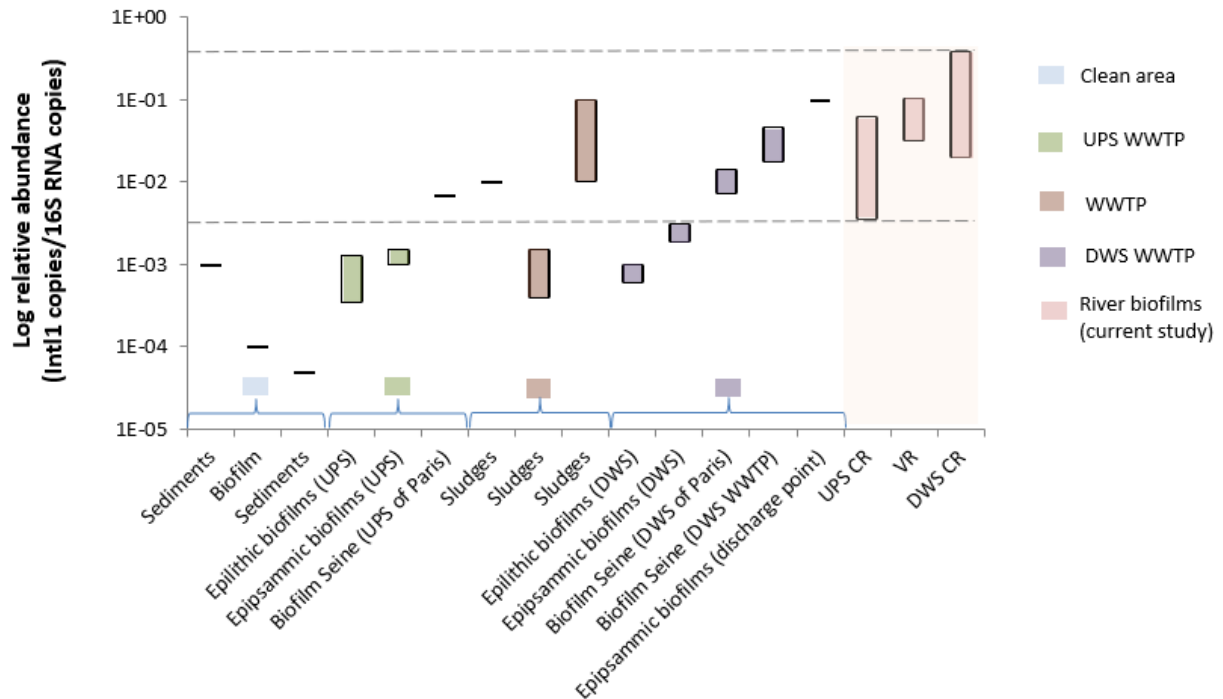
313 **Table 1. Median concentrations of antibiotics (C_{med}) detected in benthic biofilms of the Clain river (CR) upstream (UPS) and downstream (DWS) of the WWTP and the**
 314 **Vienne river (VR) (in µg/L), their PNEC_{ENV} and PNEC_{MIC}, and corresponding RQs (based on the median concentration of antibiotics).**

Class	Compound	Abbr.	Ecotoxicological incidence				Resistance selection			Combined median concentrations			References for PNEC _{ENV}	
			PNEC _{ENV} ^a	RQ Clain UPS	RQ VR	RQ Clain DWS	PNEC _{MIC} ^b	MSC	MIC	C _{med} CR UPS	C _{med} VR	C _{med} CR DWS		
Fluoroquinolones	Ciprofloxacin	CPR	11.50	-	-	1.81 (100%)	0.06	1.00	2.00	-	-	21.39	(Martins <i>et al.</i> , 2012)	
	Enoxacin	ENX	0.05	-	252.1 (60%)	22.16 (44%)	-	-	-	-	12.60	1.11	(Backhaus, Scholze and Grimme, 2000)	
	Enrofloxacin	ENR	326.80	0.01	0.02	<0.01	0.06	0.50	8.00	2.4	5.53	5.5	(Park and Choi, 2008)	
	Flumequine	FMQ	11.00	0.10	0.22	0.23	0.25	4.00	64.00	6.52	2.84	4.17	(Zounková <i>et al.</i> , 2011)	
				0.02	54.74 (56%)	118.67 (100%)	126.83 (67%)							(Backhaus, Scholze and Grimme, 2000)
	Levofloxacin	LVX	23.00	0.09	0.37	3.7 (100%)	0.25	4.00	4.00	20.85	8.49	85.07	(Le Page <i>et al.</i> , 2017)	
	Norfloxacin	NFX	0.02	195.16 (33%)	296.97 (100%)	320.4 (100%)	0.50	4.00	16.00	10.59	13.03	6.89	(Backhaus, Scholze and Grimme, 2000)	
Macrolides	Azithromycin	AZM	NE 10*	0.66 (22%)	0.35	11.3 (78%)	0.25	4.00	16.00	12.35	5.58	124.2	(Harada <i>et al.</i> , 2008)	
	Clarithromycin	CLR	NE 100*	0.01	0.02	0.06	0.25	2	8.00	1.77	2.12	11.87	(Isidori <i>et al.</i> , 2005)	
			NE 10 *	0.13	0.20	0.52 (33%)								
	Erythromycin	ERY	NE 100*	0.01	0.02	0.03	1.00	8.00	16.00	0.91	2.02	3.17	(Isidori <i>et al.</i> , 2005)	
	Josamycin	JOS	-	-	-	-	-	-	-	1.21	2.75	1.33	-	
Midecamycin	MED	-	-	-	-	-	-	-	2.88	4.91	2.12	-		

	Roxithromycin	RXM	> 1000	<0.01	<0.01	<0.01	1.00	8.00	32.00	0.93	2.65	6.93	(Choi <i>et al.</i> , 2008)
	Spiramycin	SPR	8263	<0.01	<0.01	<0.01	0.50	4.00	125.00	8.97	3.7	28.02	(Lofrano <i>et al.</i> , 2018)
	Tylosine tartrate	T-T	1.8	0.46 (22%)	1.75 (100%)	0.58	4.00	32.00	2000	1.35	3.16	<LOQ	(van der Grinten <i>et al.</i> , 2010)
Sulfonamides	Sulfamethazine	SMZ	344.70	<0.01	0.01	<0.01	-	-	-	1.33	3.78	1.13	(Białk-Bielińska <i>et al.</i> , 2011)
			>100	0.01	0.04	0.01							
	Sulfamethoxazole	SMX	78.1	0.02	0.04	0.05	16.00	125.00	1000	3.26	3.38	6.19	(Kim <i>et al.</i> , 2007)
			23.3	0.05	0.15	0.17							(Isidori <i>et al.</i> , 2005)
			1.5	0.66 (22%)	2.26 (100%)	2.59 (89%)							(van der Grinten <i>et al.</i> , 2010)
	Sulfaquinoxaline	SQX	-	-	-	-	-	-	-	1.21	4.31	2.09	-
Others	Metronidazole	MTZ	243.00	<0.01	0.01	<0.01	0.13	2.00	16.00	0.81	2.83	0.87	(Kołodziejska <i>et al.</i> , 2013)
	Oxytetracycline	OTC	108.00	0.01	0.07	0.02	0.50	4.00	125.00	1.89	7.8	<LOQ	(Isidori <i>et al.</i> , 2005)
			64.50	0.01	0.12	0.04							(Kołodziejska <i>et al.</i> , 2013)
			0.1	9.22 (78%)	75.78 (80%)	22.93 (22%)							(van der Grinten <i>et al.</i> , 2010)
	Trimethoprim	TMP	176.70	0.01	0.04	0.02	0.50	8.00	16.00	3.36	6.37	7.03	(Kim <i>et al.</i> , 2007)
			0.28	6.59 (89%)	22.77 (100%)	12.31 (89%)							(van der Grinten <i>et al.</i> , 2010)

315 NE 10/100- no acute effect (EC50) at 10/100 mg/L; a - PNEC_{ENV} was calculated from the EC50, found in the literature, divided by the appropriate assessment factor; b – given by
316 (Bengtsson-Palme and Larsson, 2016); RQ was calculated by dividing median concentration (C_{med}) of antibiotic by its related PNEC_{ENV}; values in brackets represent the percentage of samples
317 among all samples analysed with RQs > 1. Cells stained in red highlight compounds with RQ>1 and orange highlight compounds with RQ>1 in at least one analysed biofilm.

318 normalised gene abundance increased with increasing anthropogenic pressure (lowest in CR UPS and
 319 highest in CR DWS) (Figure S3). Moreover, the distribution of ARGs in CR UPS differs from that in VR
 320 and CR DWS. Whereas in the latter two, *sul2* is the most represented, followed by *dfrF*, in CR UPS *dfrF*
 321 predominates and *sul2* comes second.

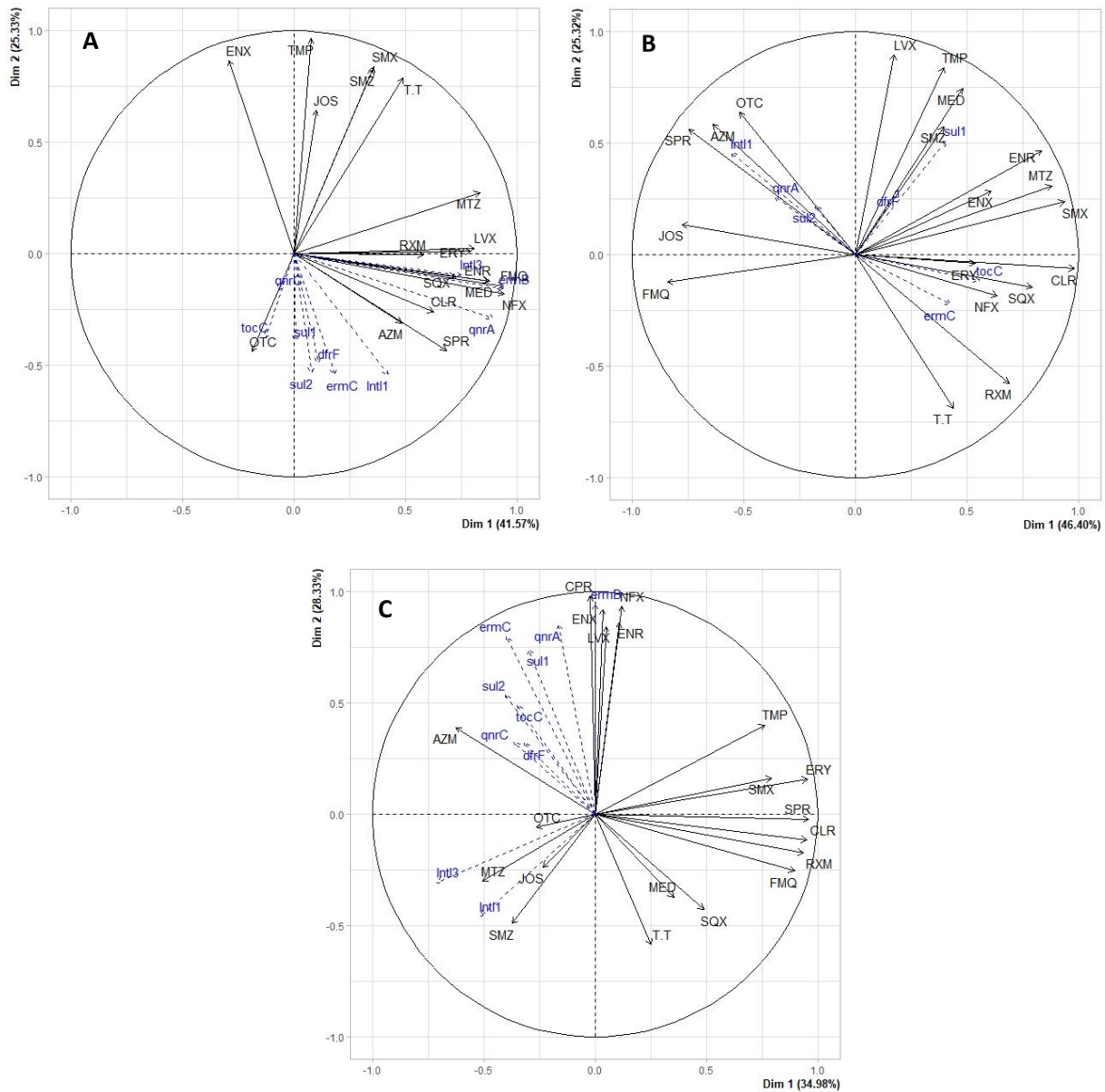


322 **Figure 3. Relative abundance (copies/16S RNA) of *Int1* in studied river biofilms and different solid matrices**
 323 **reported in the literature (Stalder *et al.*, 2012; Subirats *et al.*, 2017; Labadie *et al.*, 2020), UPS – upstream, DWS –**
 324 **downstream, CR – Clain river, VR – Vienne river.**

325 The present data show that the quantity of resistance integrons does not directly correlate with
 326 antibiotic concentrations (considered separately or as a sum by family) measured in the CR or VR
 327 biofilms. The values of R^2 are less than 0.5. Only the sum of fluoroquinolones (FQ) shows a high
 328 correlation with *Int13* ($r^2 = 0.885$, p -value <0.001) in the biofilms collected in CR UPS (Table S10). No
 329 correlation between FQs and *Int13* was found in CR DWS, but it was observed for MTZ and JOS with
 330 *Int11*. No significant correlation patterns were found between antibiotic content and the abundance of
 331 integrons in VR biofilms.

332 A similar pattern was observed between antibiotics and associated RGs. In CR UPS, macrolide (MRL)
 333 and fluoroquinolone (FQ) RGs (*ermB*, *ermC* and *qnrA*) were significantly cross correlated with members
 334 of both these classes of antibiotics simultaneously (Figure 4, Table S10). For instance, ENR, FMQ and
 335 NFX (fluoroquinolones) appeared to be significantly (p -value >0.001) correlated with macrolide RG
 336 (*ermB*) and fluoroquinolone RG (*qnrA*). Whereas the other genes seem to appear uninfluenced by the
 337 presence of antibiotics. The same situation, but even more pronounced, is observed in CR DWS (ENX,

338 ENR and NFX significantly (p -value >0.001) correlated with *ermB*), suggesting that the occurrence of
 339 ARGs is probably strongly influenced by WWTP discharges. In the VR biofilms correlation coefficient
 340 showed no association between antibiotics and ARGs, even though PCA (Figure 4B) demonstrated the
 341 potential for some correlations (i.e., SMZ and *sul1*). This sampling point is not directly in an urbanised
 342 area and is not located in the immediate vicinity of a WWTP, therefore the explanation for the
 343 observations made may be that the observed evidence of resistance is the result of previous exposure,
 344 which is maintained due to the persistence of antibiotics in the environment.



345 **Figure 4. Principal component analysis (PCA) of antibiotics, their related resistance genes and integrons: A –**
 346 **CR upstream, B – VR, C – CR downstream**

347 The co-occurrence of integrons (as a carriers of different resistance genes), ARGs and antibiotics is
 348 likely to be causal, as co-selection of integrons and ARGs occurs in environments polluted with drugs,

349 disinfectants and heavy metals (Gillings *et al.*, 2015). This co-selection is most likely due to the physical
350 location of class 1 integrons on a series of transposons and plasmids that also carry various RGs
351 (including ARGs). In the Wales and Davies's review (2015), several examples illustrate the increased
352 frequency of ARB in river water microcosms exposed to heavy metals or in agriculture and aquaculture
353 where heavy metals have been used. This increase can be due to the mechanism, which is called cross-
354 resistance and refers to the phenomenon where ARGs multiply even in the absence of their selective
355 agents (Pal *et al.*, 2017; Haenni *et al.*, 2022). This is because MGEs (e.g., plasmids, integrons or
356 transposons) often carry several groups of RGs (e.g., biocides, metals and ARGs), so selection of
357 integrons can occur not only through antibiotic exposure, but also through co-selection. For instance,
358 Gaze *et al.* (2011) investigated the effect of quaternary ammonium compounds (QACs) (disinfectants,
359 sanitizers, detergents and biocides) in co-selecting ARGs and showed their enrichment due to the
360 predominance of *Int1* as a result of QACs exposure. The phenomenon of co-selection is also closely
361 related to HGT, as well as to the efflux pump mechanism, which is one of the mechanisms responsible
362 for cross-resistance and a frequently used "defence" mechanism in bacterial biofilms (Wales and
363 Davies, 2015; Pal *et al.*, 2017; Cheng *et al.*, 2019; Matviichuk *et al.*, 2022).

364 Besides, the lack of correlation can be explained by several other factors. It may be related to the
365 structure of biofilms. EPS, which envelops bacteria and keeps them on the surface, is one of the
366 mechanisms to protect bacteria within biofilms (Khatoun *et al.*, 2018; Karygianni *et al.*, 2020). The
367 presence of specific components (such as proteins or polysaccharides) and certain physicochemical
368 properties (such as electrical impedance) reduce the diffusion of chemicals inside the biofilm and thus
369 limit the exposure of bacteria (Hathroubi *et al.*, 2017; Gebreyohannes *et al.*, 2019; Karygianni *et al.*,
370 2020). Only some antibiotics (such as tetracycline and ciprofloxacin) are reportedly able to penetrate
371 the biofilm matrix (Gebreyohannes *et al.*, 2019). The ability of bacteria to communicate with each
372 other (a mechanism called quorum sensing) may help regulate their EPS production when the presence
373 of antibiotic is detected (Gebreyohannes *et al.*, 2019; Uruén *et al.*, 2021). Likewise, bacteria are capable
374 of producing degrading enzymes which reduces the antibiotics content in the immediate vicinity of
375 cell (Cairns *et al.*, 2018). Consequently, concentration gradients or hot spots probably exist in the
376 biofilm (Figure S2), thus, bacterial cells are presumably exposed to local concentrations that would be
377 lower than the bulk concentration (Haenni *et al.*, 2022). This configuration would limit the possibilities
378 of maintaining/acquiring resistance. Indeed, the measured amount of antibiotics corresponds to bulk
379 concentrations, which probably do not reflect the exact concentration of antibiotics around the
380 bacterial cell. Secondly, the enrichment of integrons may be related to co-selection mechanisms
381 between resistance to antibiotics and heavy metals and/or biocides that are commonly present in the
382 human-exposed environment (Matviichuk *et al.*, 2022).

383

3.4.A world of antibiotics that raises many questions

384 Despite the apparent lack of correlation, the coexistence of integrons and antimicrobials makes
385 river biofilms a microenvironment that must be monitored to effectively "fight" against antibiotic
386 resistance. This uncharted world of bacterial communities, containing such a diversity of antibiotics
387 and genes, also highlights the interconnectedness of urban/anthropogenic and natural ecosystems,
388 which can play a crucial role in the emergence and spread of resistance. A recent reformulation of the
389 classic "One Health" approach highlights the importance of such microbial communication between
390 humans, animals, plants, and the external environment.

391 The results presented also call into question the limitations of global measurements, given the
392 structural complexity of the biofilm. A similar issue was also raised by Haenni *et al.* (2022), which
393 reinforces the importance of this question. It would be crucial to access concentrations near the
394 bacterial cells to determine true exposure. If the gradient hypothesis is confirmed, the bulk
395 concentrations would not allow us to anticipate the potential for selection in the biofilm. In addition,
396 the MSC and MIC values found in the literature were determined for bacteria isolated in hospitals or
397 planktonic bacteria, not for bacteria living in a biofilm. Thus, it is possible that the MSC and MIC values
398 we referred to are different from the concentrations that can select for resistance in bacteria living in
399 biofilms.

400 Another important point concerns the assessment of antibiotic resistance. Quantification of
401 integrons (and few ARGs) provides a first estimate of the presence of resistance markers, but the
402 diversity of antibiotics and potential co-selectors present in the biofilm makes it difficult to
403 demonstrate direct correlation. Thus, RNA sequencing (metatranscriptomics) will be required to assess
404 the expression levels and diversity of functionally viable resistance genes, which will allow analysis of
405 specific correlations with the presence of certain antibiotics or families. Moreover, it is necessary to
406 complement the conducted research with metagenomics, which will provide a global view of the
407 population dynamics within the biofilm and evaluate how microbial communities are structured under
408 the influence of various antimicrobial agents.

409 **Conclusion**

410 This study highlights that dispersed pollution is sufficient to contaminate biofilms to levels capable
411 of posing a risk for the acquisition and maintenance of antibiotic resistance. Specifically, our results
412 have shown that conditions favourable to the "selection pressure of resistant bacteria" are not only
413 present downstream of urbanised areas but are also possible upstream and far downstream of WWTP
414 discharges. Furthermore, our results also show that these "favourable conditions" are sustainable over

415 time as the antibiotic concentrations in biofilms are within or exceed the MSC-MIC window several
416 months of the year. However, this risk seems to be lower in upstream biofilms (less antibiotics at
417 concentrations > MIC) than in biofilms in hotspots downstream of WWTPs. These results underline the
418 need to consider the risk of antibiotics and AMR also outside the hotspots, i.e., in areas of dispersed
419 pollution of medium intensity, which are common patterns in high- and middle-income countries.

420 As in other studies, the relationship between the presence of antibiotics and antibiotic resistance
421 descriptors could not be demonstrated. Thus, no direct correlation was found between antibiotic
422 concentrations and the abundance of integrons (Int1, Int3) or specific resistance genes, both
423 upstream of the WWTP discharge and in its direct vicinity (local hotspot). However, to better
424 understand the mechanism of AMR in environmental biofilms, further studies covering the study of
425 internal and external biofilm parameters are needed. In particular, this work highlights the need to
426 study the bioaccessibility and bioavailability of antibiotics within biofilms, which will help to fill gaps in
427 the assessment of the role of the environment in antimicrobial resistance and other ecotoxicological
428 effects on microbial communities.

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