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Title Page

Antibiotic resistance along an urban river impacted by treated wastewaters

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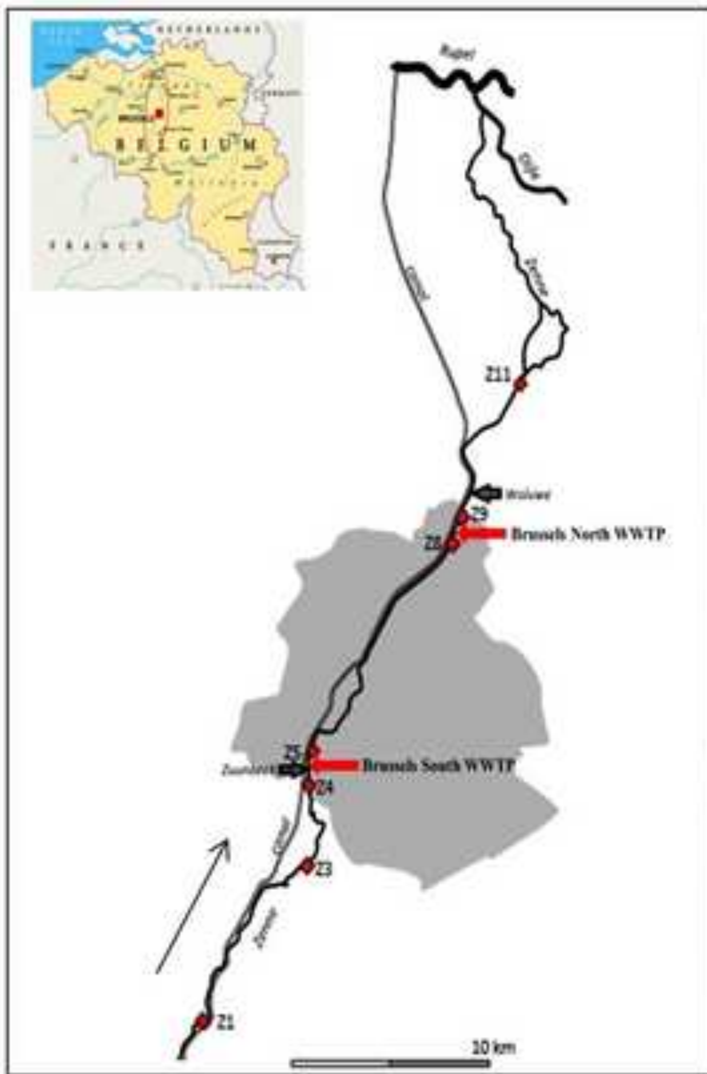
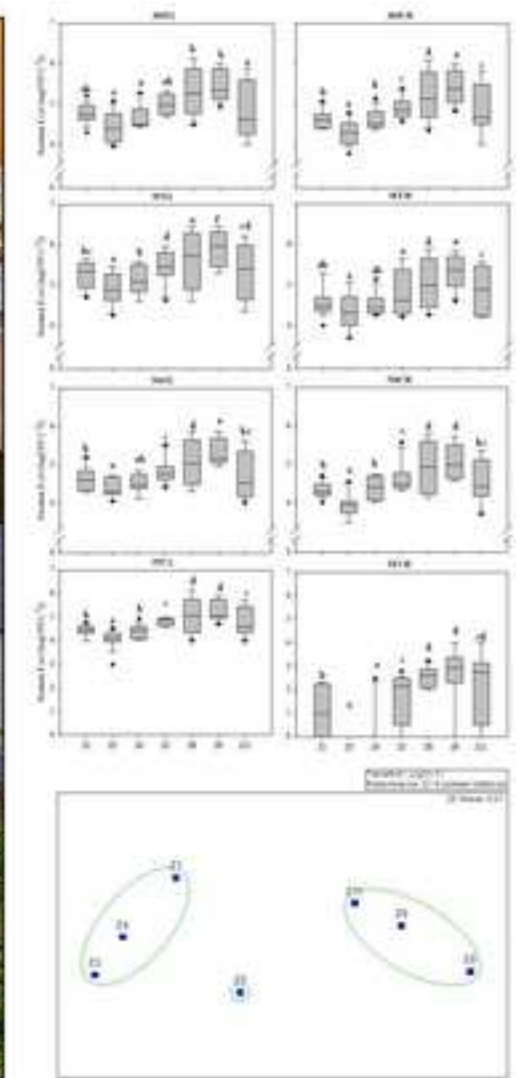
*Graphical Abstract





***Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

- Antibiotic resistant (AR) *E.coli* increased downstream the release of WWTP effluents
- Significant regression between AR *E. coli* and AR heterotrophic bacteria was found
- Tetracycline concentration significantly correlated with respective ARGs abundance
- Particle-attached bacteria showed higher levels of some ARGs than free-living ones

*Revised manuscript with no changes marked (double-spaced and continuously LINE and PAGE numbered)
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Abstract

Urban rivers are impacted ecosystems which may play an important role as reservoirs for antibiotic-resistant (AR) bacteria. The main objective of this study was to describe the prevalence of antibiotic resistance along a sewage-polluted urban river. Seven sites along the Zenne River (Belgium) were selected to study the prevalence of AR *Escherichia coli* and freshwater bacteria over a 1-year period. Culture-dependent methods were used to estimate *E. coli* and heterotrophic bacteria resistant to amoxicillin, sulfamethoxazole, nalidixic acid and tetracycline. The concentrations of these four antibiotics have been quantified in the studied river. The antibiotic resistance genes (ARGs), *sul*1, *sul*2, *tet*W, *tet*O, *bla*TEM and *qnr*S were also quantified in both particle-attached (PAB) and free-living (FLB) bacteria. Our results showed an effect of treated wastewaters release on the spread of antibiotic resistance along the river. Although an increase in the abundance of both AR *E. coli* and resistant heterotrophic bacteria was observed from upstream to downstream sites, the differences were only significant for AR *E. coli*. A significant positive regression was also found between AR *E. coli* and resistant heterotrophic bacteria. The concentration of ARGs increased from upstream to downstream sites for both particle-attached (PAB) and free-living bacteria (FLB). Particularly, a significant increase in the abundance of four among six ARGs analyzed was observed after crossing urban area. Although concentrations of tetracycline significantly correlated with tetracycline resistance genes, the antibiotic levels were likely too low to explain this correlation. The analysis of ARGs in different fractions revealed a significantly higher abundance in PAB compared to FLB for tetO and sul2 genes. This study demonstrated that urban activities may increase the spread of antibiotic resistance even in an already impacted river.

Keywords: Antibiotic resistance; Antibiotic resistance genes; Particle-attached bacteria; Free-living bacteria; Fecal bacteria; Urban Rivers.

1. Introduction

Indiscriminate use and overuse of antibiotics has led to an increase in the prevalence of antibiotic-resistant (AR) bacteria (Levy and Marshall, 2004). The use of antimicrobial agents and their subsequent release in aquatic environments may have consequences for autochthonous bacterial communities, especially in freshwater ecosystems. The direct effects of antibiotics can be detrimental to the ecosystem since autochthonous bacteria play key roles in biogeochemical processes (Costanzo et al., 2005). Moreover, recent studies have revealed that sub-inhibitory antibiotic concentrations, similar to those found in some aquatic environments (Kümmerer, 2009), may promote selection of AR bacteria (Gullberg et al., 2011). In addition, AR determinants may be considered as a form of pollution in sewage-impacted rivers (Martinez, 2009) given that they are introduced into the environment mainly by the release of enteric bacteria (Alonso et al., 2001).

During periods of treatment with antibiotics, bacteria from gastrointestinal tract are exposed to high concentrations of those compounds and develop resistance therein before being released into the aquatic environment, through treated or untreated wastewater, surface runoff and soil leaching (Servais and Passerat, 2009). Urban rivers are of the most involved environments, receiving both antibiotics and AR fecal bacteria from wastewater treatment plant (WWTP) effluents. Different studies reported the presence of AR opportunistic pathogens (Vancomycin-Resistant Enterococci, *Klebsiella pneumoniae*, *Acinetobacter, Psuedomonas* spp. and *Shigella* spp.) in urban rivers affected by treated and untreated wastewaters (Hladicz et al., 2017; Marathe et al., 2017; Nishiyama et al., 2017; Skariyachan et al., 2015). In general, these impacted ecosystems play an important role in driving the persistence and spread of AR bacteria (Taylor et al., 2011). In fact, urban rivers provide a setting in which the horizontal exchange of mobile genetic elements encoding antibiotic resistance between fecal and

105 freshwater bacteria can take place (Zhang et al., 2009). It is therefore of major
106 importance to investigate the main drivers of resistance behavior in freshwater bacteria
107 to identify possible management strategies able to control and reduce the dissemination
108 of antibiotic resistance in bacterial communities of freshwater environments.

109 Many works investigated the behavior of antibiotic residuals in freshwaters (Gibs et
110 al., 2013; Kümmerer, 2009; Zuccato et al., 2010) whereas many others focused on the
111 ARGs prevalence along sewage impacted rivers (Devarajan et al., 2016; Pruden et al.,
112 2012; Stoll et al., 2012). Moreover, several studies aimed to analyze the occurrence and
113 fate of antibiotic-resistant bacteria (ARB) in aquatic environments affected by WWTPs
114 release (Alm et al., 2014; Garcia-Armisen et al., 2013; Souissi et al., 2018). Despite
115 considerable amount of research have been carried out coupling the investigation of
116 antibiotics and ARGs behavior (Huerta et al., 2013; Khan et al., 2013; Rodriguez-
117 Mozaz et al., 2015) and that of ARB and ARGs (Guyomard-Rabenirina et al., 2017;
118 Zhang et al., 2014), comprehensive studies assessing at the same time the fate of
119 antibiotics, ARB and ARGs in urban rivers affected by wastewaters are still lacking.
120 One original study investigated the relationship between antibiotics, ARB and ARGs in
121 waters along a medical center−WWTP−river continuum (Oberlé et al., 2012).
122 Nevertheless, this study only considered the fecal indicator *E.coli* and mainly focused
123 on the sewage treatment system only sampling the river upstream from the release of the
124 WWTP effluent (Oberlé et al., 2012) .

125 The main objective of this study was then to describe the occurrence of antibiotic
126 pollution and the prevalence of ARB along a sewage-impacted urban river, focusing on
127 *Escherichia coli,* freshwater bacterial communities and ARGs. In the present study, *E.*
128 *coli* is used as a model of bacteria from enteric origin. We choose to use *E. coli* for such
129 a model as it is the most widely used fecal indicator bacteria to evaluate the level of

130 recent microbiological contamination in waters (Edberg et al., 2000). Enteric bacteria
131 can be exposed to high antibiotic concentrations in the human or animal gastrointestinal
132 tract and could acquire resistance before being released in the environment. These
133 bacteria can thus act as a source of resistance in natural environments because they can
134 disseminate antibiotic resistance genes (ARGs) to freshwater bacteria (Davison, 1999).
135 Considering that low antibiotic concentrations (lower than minimal inhibitory
136 concentration) are able to promote antibiotic resistance (Gullberg et al., 2011),
137 continuous release of low levels of antibiotics in river water could act as chronic
138 selective pressure on freshwater bacterial communities possibly contributing to the
139 spread of resistance in aquatic environments.

140 To investigate the AR spread along a sewage impacted river, the Zenne River was
141 studied. The Zenne is a paradigm of sewage-impacted river because its discharge (on
142 annual average) is doubled after receiving the treated waters from the two WWTPs in
143 the city of Brussels (Brion et al., 2015); high levels of fecal contamination have been
144 already described in this river (Ouattara et al., 2014). Seven sites along the Zenne River
145 were sampled for 1 year to study the prevalence of AR *E. coli* and freshwater bacteria,
146 particularly focusing on the influence of treated sewage waters on the AR behavior
147 along the watercourse. Culture-dependent and -independent methods were used to
148 estimate the resistance of *E. coli* and heterotrophic bacteria by plate counts containing
149 or not containing antibiotics as well as by quantifying the abundance of six genes
150 conferring resistance to the main antibiotic families in both particle-attached (PAB) and
151 free-living (FLB) bacteria. We hypothesized that after the release of sewage waters into
152 the river the amount of resistant *E. coli* isolates would increase and that this increase
153 would be reflected on the freshwater bacteria and on the river resistome. Moreover, it
154 was expected to find higher levels of ARGs on PAB with respect to FLB because close

155 contact between cells attached to the same particle would increase the probability of
156 exchange of genetic material encoding resistance.

157 ## 2. Material and methods

158 ### 2.1. Study site and sampling strategy

159 The Zenne River is located in the Belgian part of the Scheldt watershed and is a
160 tributary of the Dijle River (Fig. 1). The Zenne watershed (991 km^2) is characterized by
161 agricultural activities in its upstream part and urbanization downstream. The population
162 density in the watershed is on average 1260 inhabitants per km^2 and mostly located in
163 Brussels region. The Zenne has a length of 103 km and crosses Brussels from south to
164 north over a distance of about 20 km. Its annual average discharge upstream from
165 Brussels is 4 $m^3 s^{-1}$ (Brion et al., 2015). Before the river reaches the Brussels area, it
166 already receives several effluents from small-scale WWTPs. In the Brussels area, the
167 Zenne receives effluents from two large WWTPs: the Brussels South WWTP (360,000
168 equivalent-inhabitants) and the Brussels North WWTP (1.2 million equivalent-
169 inhabitants). The Brussels South WWTP treatment line includes a primary settling stage
170 and a secondary biological treatment (activated sludge). At the Brussels North WWTP
171 the treatment includes a primary settling stage followed by a modern tertiary treatment
172 technology (removal of organic carbon, nitrogen and phosphorus through an activated
173 sludge process). The Zenne River also receives waters from two tributaries in the
174 Brussels area the Zuunbeek and the Woluwe Rivers which watersheds are mainly in
175 urban areas. Other small tributaries located in the Brussels area are diverted in the sewer
176 collectors so that their waters reach the Brussels WWTPs.

177 Seven stations were sampled along the Zenne River (Fig. 1) in the stretch located
178 downstream from the confluence with its major right-bank tributary the Sennette.
179 Accordingly, a kilometric scale along the river was defined; the zero is arbitrarily set at

station Z1 and increases from upstream to downstream. Stations Z1 (0 km) and Z3 (13 km) are located upstream from Brussels. Stations Z4 (19 km) and Z5 (20 km) are located upstream and downstream from the Brussels South WWTP effluent release. Stations Z8 (33 km) and Z9 (34 km) are located upstream and downstream from the Brussels North WWTP, respectively, and Station Z11 (41 km) is significantly downstream from the Brussels conurbation area.

Four sampling campaigns were conducted in 2016, one per season with different hydrological conditions (discharge recorded before the Brussels region). In particular, sampling campaigns were undertaken in January (4.9 $m^3 s^{-1}$), April (2.5 $m^3 s^{-1}$), July (3.0 $m^3 s^{-1}$) and November (1.3 $m^3 s^{-1}$). The samplings in each season were carried out during 2 subsequent days after at least 3 days of dry conditions in order to keep a steady flow state of the river thus avoiding any influence of different hydrological conditions on the results. Triplicate grab water samples were collected from the river channel and stored in sterile 2-L bottles kept at 4°C until analysis carried out in the laboratory within the following 4-6h.

2.2. Physicochemical analysis

Temperature, pH and conductivity were measured directly on-site using a portable WTW 340 multiprobe (WTW, Whatman). Dissolved oxygen was measured on the spot with a WTW oxi 323 field probe. Suspended particulate matter (SPM) was estimated as the weight of material retained on a Whatman GF/F glass fiber filter (diameter, 4.7 cm; particle retention size, 0.7 mm) per volume unit after drying the filter at 105°C.

2.3. Concentration of antibiotics

205 The concentrations of amoxicillin (AMX), sulfamethoxazole (SMX), nalidixic acid
206 (NAL) and tetracycline (TET) were determined in the last three campaigns by means of
207 liquid chromatography coupled to mass spectrometry in tandem (LC-MS/MS) after
208 sample clean-up and pre-concentration with solid phase extraction (SPE). The samples
209 were collected in amber sterile bottles and kept at 4°C in dark conditions until the pre-
210 treatment carried out within the 4h after collection. One hundred milliliters of blank
211 samples (consisting of Milli-Q water spiked with the mixture of native compounds at
212 100 ngL^{-1}) were used for traceability and cross-contamination monitoring. Then 100 mL
213 of blank samples and 100 mL of river samples were fortified with sulfamethoxazole-d_6
214 surrogate internal standard for a final concentration of 0.1 ng mL^{-1} in sample. After that,
215 the samples were homogenized and kept at −20°C before analysis in order to assure the
216 traceability of the results (Llorca et al., 2014).
217 Sample preparation was carried out following Gros et al. (2013). All the samples
218 were extracted in triplicate. More details are reported in Supplementary Material (A).
219
220 **2.4. Quantification of AR *Escherichia coli* and freshwater bacteria**
221 Resistance to AMX, STX, TET and NAL were tested in parallel in culturable *E. coli*
222 and freshwater bacteria. These antibiotics were chosen because they belong to four
223 different families with different mechanisms of action. Moreover, these antibiotics are
224 among the most used in Belgium for human (European Centre for Disease Prevention
225 and Control, http://ecdc.europa.eu) and veterinary medicine (Callens et al., 2017). For
226 this purpose, Chromocult Coliform agar (Merck Millipore, Darmstadt, Germany) was
227 used as specific culture medium to grow *E. coli* for 24 h at 37°C (Prats et al., 2008),
228 whereas heterotrophic bacteria were grown on nutrient broth diluted (DNB) 100 times
229 (Merck Millipore, Darmstadt, Germany) for 21-28 days at 20 °C. DNB was selected as

230 culture medium because a previous similar study demonstrated significantly higher
231 counts of resistant bacteria on this medium compared with richer ones (Garcia-Armisen
232 et al., 2013). Media were used as such (for total culturable *E. coli* and freshwater
233 bacteria) or supplemented with one of the four antibiotics. For each antibiotic, two
234 different concentrations (low and high) were tested: AMX (4 and 50 µg ml^{-1}), SMX (16
235 and 300 µg ml^{-1}), NAL (2 and 30 µg ml^{-1}) and TET (4 and 300 µg ml^{-1}) (Sigma
236 Chemical Company, St. Louis, USA). The lowest (L) are the breakpoint concentrations
237 established for *E. coli* by the French committee for antimicrobial standards (Comité de
238 l'Antibiogramme de la Société Française de Microbiologie) and the highest (H)
239 correspond to the values reported in previous studies dealing with antibiotic resistance
240 in environmental bacteria (Garcia-Armisen et al., 2013).
241 Two ten-fold serial dilutions were filtered (or spread) for each sample in order to obtain
242 a proper colonies number to ensure that at least one of them could be counted.
243 Triplicates were performed for each volume filtered or dilution spread. For each of the
244 combinations two plates were not inoculated and were incubated as negative controls.
245 All the controls were negative after incubation. The results were expressed in colony-
246 forming units (CFU) per liter. With these methods and considering the concentrations
247 used, we were able to quantify putative AR *E.coli* and freshwater bacteria. Thus, when
248 mentioning data of ARB enumerated using cultivation methods, we refer to putative
249 resistant bacteria all over the manuscript.

250 **2.5. DNA extraction**

251 The bacterial biomass was collected from the water and concentrated by filtration. An
252 aliquot (from 0.25 L to 1.5 L) of each sample was filtered to collect two different
253 bacterial fractions. Particle-attached bacteria (PAB) were collected filtering water on 5-
254 µm pore-size, 47-mm-diameter polycarbonate filters (Millipore, Billerica, MA, USA).

255 This pore size has been already used to distinguish the behavior of bacteria attached to
256 particles (which can settle) from that of free-living bacteria in river ecosystems (Garcia-
257 Armisen and Servais, 2009; Proia et al., 2016a). Filtrates were then filtered through
258 0.22-µm pore size 47-mm-diameter polycarbonate filters (Millipore) to retain free-
259 living bacteria (FLB). Filters were kept at −80°C until extraction. Extractions were
260 performed following García-Armisen et al. (2014). The details of the DNA extraction
261 are reported in the Supplementary Material (B).

262

263 **2.6. Quantification of ARGs using qPCR**

264 The number of copies of the selected ARGs (*sul*1, *sul* 2, *tet*W, *tet*O, bla_{TEM} and *qnr*S)
265 was quantified using qPCR assays. All qPCR assays were performed in duplicate using
266 SYBR green detection chemistry with a Step One Plus (Applied Biosystems,
267 ThermoFisher Scientific). Briefly, each reaction contained 8–9 µL of Power Up SYBR
268 Green master mix (Applied Biosystems, ThermoFisher Scientific), 200 nM each
269 forward and reverse primer(s) and 45 ng ofDNA template, and the final volume was
270 adjusted to 20 µL by adding DNase-free water. Each gene was amplified using specific
271 primer sets (Sigma Aldrich) and the PCR conditions included initial denaturation at
272 95°C for 3 min, followed by 40 cycles at 95°C for 15 s, then 20 s at the specific
273 annealing temperature depending on the gene (Table C.1), and finally two elongation
274 steps of 40 s at 72°C and 32 s at 78°C. The copy number of the bacterial 16S rRNA
275 gene was also quantified, and the amplification conditions included an initial
276 denaturation at 95°C for 3 min, followed by 35 cycles at 95°C for 15 s, then an
277 annealing temperature at 60°C for 1 min, 40 s at 72°C and 32 s at 78°C. A dissociation
278 curve was applied at the end of each run to detect nonspecific amplifications. Tenfold
279 dilutions of plasmid DNA containing known concentrations of the target gene, which

280 were generated as described by Proia et al. (2016), were used as standard curves. The
281 standards for each ARG were run in parallel with DNA samples and blank controls
282 (qPCR premix without a DNA template). The efficiency and sensitivity of each qPCR
283 assay was determined by the amplification of standard serial dilutions, as previously
284 described (Marti et al., 2013). Amplification efficiency (E) was calculated from the
285 resulting standard curves using the formula $E = 10^{(1/slope)} - 1$, and the analytical
286 sensitivity of the real-time PCRs was determined as the smallest DNA quantity detected
287 for each assay.
288

289 **2.7. Statistical analyses**

290 Resistance to each antibiotic at different concentrations was analyzed independently
291 using a one-way repeated measures analysis of variance (ANOVA) to test for the
292 differences among sampling sites during the year of sampling. The effects were
293 analyzed post hoc with Tukey's b test. Moreover, in order to test for the influence of
294 WWTPs on antibiotic resistance, the sites upstream and downstream from the release of
295 effluents into the Zenne River were grouped together respectively and tested using a
296 one-way analysis of variance (ANOVA) with location as a fixed factor (Up and Down).
297 Data were log-transformed to meet assumptions of normality and homogeneity of
298 variance when needed. Statistical significance was set at $p = 0.05$. Analyses were
299 performed using SPSS Version 15.0. Multidimensional scaling (MDS) was performed
300 using the PRIMER 6 software to visualize the similarity/dissimilarity among sampling
301 sites in terms of AR bacteria. The analysis was based on a Euclidean distance matrix
302 created from a log(X+1)-transformed abundance data set. Pearson correlation analyses
303 of antibiotic concentrations, culturable AR heterotrophic bacteria and ARGs were

304 performed using Sigma Plot software 11.0, as was regression analysis between the
305 resistant culturable *E. coli* and heterotrophic bacteria data sets.

306

307 # 3. Results

308 ## 3.1. Environmental variables and antibiotic concentrations

309 Conductivity gradually increased from 798 ± 58 µS cm^{-1} at Z1 to 1192 ± 68 µS cm^{-1}
310 at Z11 (Table 1). Dissolved oxygen tended to decrease downstream, whereas pH
311 remained fairly stable, close to neutrality, along the river course. The temperature
312 increased downstream, showing a peak at Z8 (14.3 ± 2.9°C) after crossing Brussels.
313 Suspended particulate matter did not show any clear pattern along the course of the
314 Zenne River (Table 1). In general, antibiotic concentrations were low along the river
315 course and did not show any clear pattern, except for TET, which increased from
316 upstream to downstream (Table 2). AMX was only detected at Z4 in two of the samples
317 analyzed (10%) whereas it was below the detection limit at all other sampling sites
318 (Table 2). STX was detected in 18 of the samples analyzed (86%). However, in 56% of
319 those samples STX was below the limit of quantification (LOQ = 5 ng L^{-1}).
320 Specifically, STX was detected at Z5 in all sampling campaigns, with a median
321 concentration of 227.9ng L^{-1} (Table 2). Similarly, NAL was detected in 15 of the
322 samples analyzed (71%). However, in 73% of those samples NAL was below the limit
323 of quantification (LOQ = 0.15 ng L^{-1}). Finally, TET was detected in 100% of the
324 samples analyzed and increased gradually from upstream to downstream with a peak
325 observed at Z9 (Table 2). In particular, TET concentrations clearly increased in the
326 Brussels area (Z5–Z9) and decreased at Z11, downstream of the city (Table 2).

327

328 ## 3.2. Antibiotic-resistant bacteria and *Escherichia coli*

329 3.2.1. *Antibiotic-resistant freshwater bacteria*

330 The average abundances of culturable freshwater bacteria along the Zenne River in
331 the four campaigns are shown in Fig. 2a. Bacterial abundance varied in the range of 10^8
332 CFU L^{-1} and no clear pattern was observed. The average abundance of resistant bacteria
333 to both concentrations of each antibiotic tested tended to increase downstream (Fig. 3).
334 The resistant bacteria were significantly higher for the lower concentrations tested (L)
335 for all the antibiotics tested (repeated measures ANOVA, $p<0.05$). The counts of
336 resistant bacteria highlighted significantly lower levels of resistance to TET compared
337 to the other antibiotics tested, resulting in a final order TET < AMX < STX, NAL.

338 The behavior of resistant bacteria along the Zenne River showed slight differences
339 and high variability among the sampling sites during the studied year. In general, the
340 resistance to the antibiotics tested tended to increase from upstream to downstream sites
341 but in most cases the increase was not significant. . Despite the high variability
342 observed among the sampling campaigns, Z9 was the site with the highest percentages
343 of resistant bacteria, independently of the antibiotic and concentration considered. For
344 STX, the increase was gradual from Z1 to Z9 and decreased at Z11 for both low and
345 high concentrations (Fig. 3b). A similar pattern was observed for NAL (Fig. 3c),
346 whereas for TET L the percentages of resistant bacteria were in general low, only
347 peaking at Z5 and Z9 (Fig. 3d). Resistance to a high concentration of TET was
348 extremely low in the Zenne River's bacteria (always below 1%, Fig. 3d). The
349 percentage of resistant bacteria slightly increased from Z4 to Z5 (upstream and
350 downstream from the outfall of Brussels South WWTP effluent to the river) except for
351 AMX H and STX L (Fig. 3a and b). In contrast, the increase between Z8 and Z9
352 (immediately upstream and downstream from the release of Brussels North WWTP
353 effluent to the river) was relevant (+40% on average) for all the antibiotics and

354 concentrations tested. The relative abundance of resistant bacteria after crossing the
355 Brussels-Capital region (from Z4 to Z9) increased approximately 60% on average.

356 To explore the relationships between antibiotic concentrations and the abundance of
357 culturable AR freshwater bacteria, a correlation analysis was performed. This analysis
358 showed a positive significant correlation between TET concentrations and the
359 abundance of bacteria resistant to the highest concentration of this antibiotic (Pearson
360 correlation; $r = 0.786$, $p = 0.036$).

361

362 3.2.2. *Antibiotic-resistant Escherichia coli*

363 The average abundances of culturable *E. coli* measured along the Zenne River in the
364 four campaigns are presented in Fig. 2b, demonstrating a significant increase from
365 upstream to downstream. In particular, higher abundances were observed at sampling
366 sites located after the discharge of the Brussels WWTPs into the river (Z5 and Z9, Fig.
367 2b). In contrast, the lowest abundances were observed upstream from Brussels (Z1–Z4,
368 Fig. 2b). The lowest value was observed at Z3 where *E. coli* abundance was on average
369 $1.76 \times 10^5 \pm 1.98 \times 10^5$ CFU L^{-1} and peaked at Z9 with values reaching $1.38 \times 10^6 \pm$
370 1.54×10^6 CFU L^{-1}.

371 Figure 4 shows box-plots of the abundance of *E. coli* resistant to both concentrations
372 of the four antibiotics tested. The abundance of resistant *E. coli* was significantly higher
373 for the lower concentrations tested (repeated measures ANOVA, $p<0.05$) for all
374 antibiotics except for AMX for which the difference between the two concentrations
375 was not statistically significant ($p>0.05$). The counts of resistant bacteria obtained with
376 TET were significantly lower than those obtained with AMX, STX and NAL (ANOVA,
377 $p<0.001$).

378 The behavior of resistant *E. coli* along the Zenne River followed the same pattern as
379 total *E. coli* abundance (Fig. 4). As a consequence, the percentages of resistant *E.coli*
380 among sites were not significantly different along the Zenne River for any of the
381 antibiotics and concentrations tested (Figure A1). Notably, Z3 was the sampling site
382 with the significantly lowest abundance of resistant *E. coli* independently of the
383 antibiotic and concentration considered. Similarly, the abundance of resistant *E. coli*
384 was significantly the highest at Z9 for all the antibiotics and concentrations tested (Fig.
385 4). In particular, the highest amount of resistant *E. coli* was observed at Z9 where
386 bacteria resistant to the lower concentration of STX were $1.18 \times 10^6 \pm 1.07 \times 10^6$ CFU
387 L^{-1}, corresponding to 78% of the total culturable *E. coli*. The increase of resistant *E. coli*
388 from Z4 to Z5 (upstream and downstream from the outfall of the Brussels South WWTP
389 to the river) was also significant, independently of the antibiotic and concentration
390 considered. Similarly, between Z8 and Z9 (upstream and downstream of the outfall of
391 the Brussels North WWTP to the river) resistant *E. coli* increased significantly except
392 for AMX L (Fig. 4a), NAL H (Fig. 4f) and TET both concentrations (Fig. 4g and h). In
393 general, downstream from the Brussels-Capital region (at Z11) the abundance of
394 resistant *E. coli* decreased with regard to the sampling sites located in the Brussels area
395 (Z5–Z9); however, only in a few cases (AMX L and NAL both concentrations) did the
396 values recover to levels similar to those upstream (Z1–Z4) from the city (Fig. 4a, e and
397 f).

398 The MDS performed to visualize the similarity among sites in terms of AR *E. coli*
399 clearly separates Z8 and Z9 from the rest of the locations sampled (Fig. 5). Furthermore,
400 the sites located upstream from the input of Brussels South WWTP effluent into the
401 river (Z1–Z4) are grouped together and are clearly separated from those located
402 downstream from the release of treated waters to the river (Z5–Z9, Fig. 5). Finally, Z11

403 is separated from the other sites, indicating that the levels of resistance to the antibiotics
404 tested differed here from all the other sites (Fig. 5).
405 To investigate the eventual role of AR fecal bacteria in the spread of resistance to
406 freshwater heterotrophic bacteria in the Zenne River, a linear regression analysis was
407 performed between the AR *E. coli* and AR heterotrophic bacteria data sets (Fig. 6). The
408 results of this analysis revealed a significant relationship between resistant fecal bacteria
409 and resistant culturable heterotrophic bacteria (r = 0.57; $p < 0.001$; $n = 198$)
410
411
412 **3.3. Abundance of ARGs along the river's course**
413 All the qPCR assays were performed with high R^2 values (average 0.997 ± 0.003),
414 high efficiencies (average 97.8 ± 2.7 %) and a dynamic range of at least 5 orders of
415 magnitude, indicating the validity of the resulting quantifications (Table A.3). Limit of
416 quantification was different depending on the gene and the run and all the details are
417 reported in table A.3.
418 In general, the absolute concentration of target ARGs increased from upstream to
419 downstream sites for both particle-attached (PAB) and free-living bacteria (FLB),
420 particularly increasing at Z8, Z9 and Z11 for all the genes analyzed (Fig. 7a-f). For most
421 of the ARGs studied, abundance peaked at Z8; nevertheless, the variability among the
422 sampling campaign was high and differences with the other downstream sites were not
423 significant considering the whole year. In contrast, an increase in the abundance of
424 ARGs after crossing the Brussels-Capital region was significant for the *sul*2 ($p = 0.004$),
425 *tet*W ($p = 0.024$), *qnr*S ($p = 0.002$) and *tet*O ($p = 0.004$) genes. The levels of ARGs
426 normalized to the 16s rRNA copies did not differ among sampling sites and varied
427 without showing any clear pattern along the Zenne River (Fig A.2).

428 The MDS performed to visualize the similarity of the sites in terms of ARG
429 abundance (Fig. 8) clearly separated the sites upstream from Brussels (Z1, Z3 and Z4)
430 from downstream sites (Z8, Z9 and Z11). Furthermore, the site located just downstream
431 from the Brussels South WWTP effluent release into the river (Z5) was separated from
432 all the other sites (Fig. 8), demonstrating the influence of treated waters released from
433 Brussels South WWTP in terms of ARG abundance along the Zenne River.

434 To determine the potential relations between the absolute abundance of ARGs and
435 the antibiotics to which they confer resistance, correlation analyses were carried out.
436 Significant positive correlations between the concentration of tetracycline and its
437 corresponding ARGs were observed. In particular, *tet*O abundances positively
438 correlated with TET concentrations ($r^2 = 0.87$, $p = 0.002$) as well as *tet*W ($r^2 = 0.87$, $p =$
439 0.002). For all the other ARGs, no significant correlation was found

440 To compare the levels of ARGs in the two fractions (FLB and PAB), the abundance
441 values were normalized to the 16S rRNA gene copy numbers. The results of this
442 comparison (Fig. 9) highlighted a significantly greater amount in PAB compared to
443 FLB for *tet*O ($p = 0.004$) and *sul*2 ($p = 0.038$). Moreover, the comparison of the genes
444 revealed that bla_{TEM} was significantly lower than other genes analyzed in both PAB and
445 FLB fractions along the Zenne River ($p < 0.001$, Fig. 9).

446

447 ## 4. Discussion

448 The Zenne is a small river known to be severely impacted by the release of
449 effluents of two large WWTPs in the Brussels area (Brion et al., 2015; Ouattara et al.,
450 2014). In this study, the concentration of four antibiotics, prevalence of ARB (*E.coli*
451 and freshwater) and abundance of ARGs were investigated along the Zenne River.

452 ### 4.1. Antibiotic pollution

453 Antibiotic concentrations detected in Zenne's surface water were within the same
454 range as those found in other sewage-impacted European rivers (Fatta-Kassinos et al.,
455 2011). For example, even though AMX is a broad-spectrum antibiotic widely used in
456 human medicine, it was barely detected in our study (10% of samples), agreeing with
457 previous studies which demonstrated low persistence of beta-lactams in aquatic
458 environments (Andreozzi et al., 2004; Längin et al., 2009; Oberlé et al., 2012; Zuccato
459 et al., 2010). This low prevalence may be explained by its instability in aqueous media
460 (Gros et al., 2013; Hirsch et al., 1999) Similarly, although NAL was frequently detected
461 in most of the samples, its concentration was below the quantification limit of the
462 method applied (LOQ = 0.15 ng L^{-1}). This observation is expected considering that this
463 antibiotic is currently only used for livestock species in Belgium and agrees with the
464 results obtained in surface waters of sewage-impacted rivers worldwide (Gibs et al.,
465 2013; Gros et al., 2013; Komori et al., 2013). STX is one of the most widely detected
466 antibiotics in river waters. In our study, STX was found at a high frequency (86%) and
467 STX was detected in all the Z5 samples (directly downstream from the release of the
468 Brussels South WWTP effluent to the Zenne) at concentrations that were within the
469 range of those found in other impacted rivers (Fatta-Kassinos et al., 2011; Gros et al.,
470 2007; Oberlé et al., 2012; Proia et al., 2013; Tamtam et al., 2008). TET was the most
471 frequently detected antibiotic in the Zenne River, with concentrations increasing from
472 upstream to downstream sites and peaking at Z9 (immediately downstream from the
473 release of the Brussels North WWTP to the Zenne) at concentrations higher than those
474 found in other studies investigating antibiotic occurrence in surface waters (Fatta-
475 Kassinos et al., 2011; Gros et al., 2009; Proia et al., 2013). From the correlation analysis
476 performed to explore the relationships between antibiotic concentrations and the
477 abundance of culturable AR freshwater bacteria, only TET concentrations showed a

478 positive significant correlation with the abundance of bacteria resistant to the highest
479 concentration of this antibiotic. Even if low concentrations of TET could promote
480 resistance (Gullberg et al., 2011; Lundström et al., 2016), from the correlation found in
481 our study we cannot conclude about any causal relationship between TET levels
482 measured in the Zenne River and increasing TET resistant bacteria. In fact, the highest
483 TET concentrations measured in this study were still one order of magnitude lower than
484 those reported to promote resistance (Gullberg et al., 2011; Lundström et al., 2016).
485 Moreover, all the other antibiotics analyzed showed positive but non-significant
486 correlations with the abundance of culturable AR freshwater bacteria, thus highlighting
487 that some other factor must be the main driver of AR spread among resident bacterial
488 communities. This result was expected considering that they come from the same source
489 and also considering that antibiotics levels measured in the Zenne River did not follow
490 any clear pattern (except TET) and were several orders of magnitude lower than the
491 concentrations predicted to select for resistance.
492

493 **4.2 Antibiotic resistance of culturable bacteria**

494 Enteric bacteria from human and animal digestive tracks are found in surface urban
495 waters mainly brought into aquatic environments through treated or untreated
496 wastewater release (Servais and Passerat, 2009). The disappearance of fecal bacteria in
497 aquatic environments results from the combined actions of various biological (grazing
498 by protozoa, virus induced cell lysis and autolysis) and physico-chemical parameters
499 (stress due to osmotic shock (when released in seawater), nutrients depletion, sunlight
500 intensity and temperature decrease) and also to possible deposition to sediments
501 (Servais et al., 2007). Despite cryptic strains of *Escherichia* clades able to survive in
502 aquatic environments have been reported (Vignaroli et al., 2014), it has been

503 demonstrated that 90% of culturable *E. coli* would not survive more than 3 days in river
504 waters (Servais et al., 2007). Moreover, *E. coli* is still the most widely used indicator of
505 recent fecal contamination in aquatic environments (Edberg et al., 2000) and has been
506 chosen as a model in this study.

507 *E. coli* significantly increased from upstream to downstream sites in the Zenne
508 River, notably peaking after the release of the WWTP effluents into the main course
509 (Fig. 2b). In general, the abundance of *E. coli* observed in the present study (median
510 values at each station higher than 1×10^5 CFU L^{-1}), exceeded by more than one order of
511 magnitude those required for bathing activities in EU countries (EU, 2006). Moreover,
512 concentrations of *E. coli* measured in the present study were similar to those measured
513 in a previous study carried out on the same river in 2009–2010 (Ouattara et al., 2014).
514 Ouattara et al. (2014) already highlighted the impact of the Brussels WWTPs on the
515 abundance of fecal bacteria along the Zenne River. Furthermore, AR *E. coli* also
516 followed the same pattern (Fig. 4), suggesting that the main source of resistant fecal
517 bacteria to the Zenne is the discharge of treated effluents to the main river course.
518 However, fecal contamination (by both resistant and non-resistant *E. coli)* was already
519 high upstream of both WWTP discharges (Fig. 2b and Fig. 4). The origins of this
520 contamination can be ascribed to three main factors: i) the release of the effluents from
521 three relatively small WWTPs (with a total capacity of 103,300 equivalent inhabitants);
522 ii) the runoff on pastured areas and iii) the effluents from farms with intense breeding
523 activities in the upstream watershed (Ouattara et al., 2014). Despite the high levels of
524 antibiotic resistance found in *E. coli* upstream from the Brussels-Capital region, the
525 MDS confirmed a clear impact of urban activities on the occurrence of AR fecal
526 bacteria in the river (Fig. 5). Most particularly, our data highlighted a strong effect of
527 the Brussels South WWTP effluent, whereas the effect of the Brussels North WWTP on

528 AR *E. coli* was less pronounced (Z8 and Z9 grouped together). This observation could
529 be explained by the different efficiency of the two WWTPs in removing *E. coli* through
530 sewage water treatment and by others non-negligible sources like raw wastewaters
531 released from the Brussels old sewer system (Ouattara et al., 2014). In fact, a previous
532 study described significantly higher abundance of *E. coli* in Brussels South effluent
533 compared to Brussels North, suggesting that the tertiary treatment (applied only in
534 Brussels North WWTP) may be responsible for the lower amount of fecal bacteria
535 released into the Zenne River (Ouattara et al., 2014). As a consequence, the strong
536 effect of the higher amount of AR *E.coli* released by Brussels South effluent and the
537 additional effect of inputs from the Brussels old sewer system could have masked the
538 impacts of Brussels North effluent (no separation between Z8 and Z9).. Finally, the
539 significant decrease of *E. coli* (both resistant and non-resistant) at Z11 (Fig 2b and 4) is
540 probably explained by the high rates of mortality occurring in freshwater systems
541 (Servais et al., 2007).

542 . The linear regression analysis performed between AR *E. coli* and AR freshwater
543 bacteria revealed a significant positive relationship (Fig. 6). Few studies have
544 investigated the correlation between AR fecal and heterotrophic bacteria in sewage-
545 contaminated rivers. Garcia-Arminsen et al. (2011) found no significant correlation for
546 three of the four antibiotics investigated in the present study (AMX, NAL and TET).
547 However, one possible explanation for this different result is that these authors plotted
548 the percentages of resistant *E. coli* against the percentages of resistant freshwater
549 bacteria (Garcia-Armisen et al., 2011), whereas our significant regression was obtained
550 by plotting the absolute abundance values (Fig. 6). To verify that our relation was not
551 driven by the general increase of bacterial abundance (both *E. coli* and heterotrophic
552 bacteria), generally caused by the release of WWTP effluents into the rivers, the

553 regression analysis between culturable *E. coli* and culturable heterotrophic bacteria was
554 carried out and no significant correlation was found (r = 0.16, $p = 0.43$), confirming that
555 the relation existed only for AR bacteria. Despite the high variability detected along the
556 river and among campaigns, probably also related with the limited number of sampling
557 sites, these results suggest that the increase of resistance in freshwater bacteria could be
558 somehow related with the levels of sewage pollution but some role of fecal bacteria
559 released by wastewaters in the dissemination of AR determinants among freshwater
560 communities can be only hypothesized. In fact, without a characterization down to
561 species (and possibly strains), only specific controlled experiments can confirm the
562 possible primary active role of resistant enteric bacteria in the spread of antibiotic
563 resistance into freshwater bacterial communities.

564 **4.3. The river resistome**

565 **4.3.1 Effects of WWTP discharges on river resistome**

566 Many studies worldwide have reported higher levels of ARGs in response to
567 increased human activities in freshwater ecosystems (Huerta et al., 2013; Pei et al.,
568 2006; Pruden et al., 2012; Stoll et al., 2012). In particular, WWTPs have been widely
569 described as one of the main sources of ARGs to river ecosystem bacteria (Berglund et
570 al., 2015; Proia et al., 2016b; Rodriguez-Mozaz et al., 2015). Our study showed
571 increased levels of all the ARGs analyzed after crossing the Brussels-Capital region and
572 receiving the effluents of the city's two WWTPs. Notably, the MDS associated with
573 cluster analysis carried out with all the ARGs abundances highlighted the role of
574 WWTP effluents in the spread of ARGs along the Zenne River. In fact, the sampling
575 site located just downstream from the release of the Brussels South WWTP into the
576 river (Z5) was clearly separated from the upstream sites, demonstrating discontinuity in
577 terms of ARG abundance. Moreover, the downstream sites (Z8, Z9 and Z11) were

578 grouped together by the same analysis, thus highlighting the role of urban activities in
579 the spread of ARGs and indicating that the increased levels of ARGs in the river
580 (induced by the city) are maintained almost 8 km downstream (Z11). The combination
581 of this evidence with the increasing concentrations of some antibiotic downstream (i.e.
582 TET) suggests that some additional source of pollution could be present between the
583 release of Brussels North effluent to the Zenne and Z11. For example the tributary
584 Woluwe River could be a possible source of pollution between Z9 and Z11 explaining
585 this behavior. Nevertheless the limited number of sampling sites in this study does not
586 allow any conclusion about this hypothesis.

587 The absolute concentrations of ARGs in the Zenne River were higher than those
588 measured in other studies (Di Cesare et al., 2017; Jiang et al., 2013; LaPara et al., 2015;
589 Rodriguez-Mozaz et al., 2015). In fact, all the ARGs analyzed (PAB + FLB) in the
590 Zenne River varied within the range of 10^4 to 10^6 copies mL^{-1} except bla_{TEM}, which was
591 the lowest and varied between 10^3 and 10^4 copies mL^{-1} (Fig. 7). Jiang et al. (2013)
592 analyzed a large number of genes conferring resistance to tetracycline, sulfonamides
593 and β-lactams in a Chinese river crossing urban areas. They found the levels of *sul*1 and
594 *sul*2 comparable to the Zenne River (10^5 copies mL^{-1}) but much lower abundance (about
595 1000-fold) of *tet*W and *tet*O genes (Jiang et al., 2013). On other hands, Di Cesare et al.
596 (2016) studied the behavior of ARGs during rainfall events and reported peaks of *sul*1
597 and *qnr*S of about 10^2 copies mL^{-1} in a forested Italian river, 2-4 orders of magnitude
598 lower than those measured in the Zenne River. This huge difference is certainly
599 explained by the different nature of the watersheds. LaPara and colleagues (2015) also
600 reported lower levels of *tet*W and *sul* 1 respect to the Zenne, in a study assessing the
601 effects of multiple discharges of treated municipal wastewaters on the abundances of
602 ARGs in the upper Mississippi River (USA). Similarly, Rodriguez-Mozaz et al. (2015)

603 measured the absolute abundance of *tet*W, *bla*$_{TEM}$, *sul* 1 and *qnr*S genes, always below
604 10^4 copies mL^{-1} in a Spanish WWTP-affected river (Ter River), much lower than in the
605 Zenne. This could be explained by the lower impact of human activities and treated
606 sewage waters in the Ter River compared to the Zenne. Moreover, the same study also
607 found the absolute abundance of *bla*$_{TEM}$ to be the lowest of the genes analyzed, in
608 agreement with our finding. To conclude, these comparisons confirmed that even
609 though the Zenne River showed extremely high levels of ARGs also upstream from the
610 Brussels-Capital region, urban activities increased the spread of antibiotic resistance
611 determinants along the river with the effects still observed a few kilometers
612 downstream.
613

614 **4.3.2. Effects of antibiotics on river resistome**

615 The correlation analysis carried out between ARG abundances and antibiotic
616 concentrations only revealed significant correlation between *tet* genes and TET whereas
617 any significant correlation was found for the rest of measured ARGs mainly because the
618 concentrations of target antibiotics were generally low. Another study highlighted
619 significant positive correlations between ARGs and antibiotic concentrations in a
620 sewage-impacted river (Rodriguez-Mozaz et al., 2015). Nevertheless, TET was not
621 detected in the surface waters of the river they studied; therefore, no correlation analysis
622 with *tet* genes was performed (Rodriguez-Mozaz et al., 2015). In contrast, positive
623 correlations between *tet* genes and tetracycline concentrations were found in a study
624 analyzing ARGs and antibiotic levels in WWTP effluents and receiving surface waters
625 (Xu et al., 2015). . Most of the cited studies investigated these correlations in WWTPs
626 including surface water samples only upstream and downstream from the release of
627 treated sewage waters to the river. Hence, the present study is the first one reporting this

628 correlation for *tet* genes along a sewage-impacted river. Nevertheless, considering that
629 from a correlation analysis is not possible to conclude about causation and taking into
630 account that the measured TET concentrations were considerably lower than those
631 reported to promote resistance (Gullberg et al., 2011; Lundström et al., 2016) we cannot
632 conclude that TET could lead to selective pressure for the corresponding ARGs in
633 Zenne River waters. One possible reason of the significant correlation found in this
634 study is that the source of *tet* genes and TET would be the same (WWTP effluents) thus
635 explaining the positive correlation. However the role of the trace levels of antibiotics
636 detected in surface waters on the promotion and spread of AR in aquatic environments
637 is a matter of concern that need to be studied further under controlled conditions.
638

639 **4.3.3. ARGs in particle-attached vs. free-living bacteria**

640 Several studies have investigated the occurrence of ARGs in bacterial communities
641 inhabiting different compartments of freshwater ecosystems such as sediments
642 (Berglund et al., 2014; Czekalski et al., 2014; Marti et al., 2013), biofilms (Aubertheau
643 et al., 2017; Proia et al., 2016b; Schwartz et al., 2003; Subirats et al., 2017; Winkworth,
644 2013) and the water column (Czekalski et al., 2015; Rodriguez-Mozaz et al., 2015).
645 Nevertheless, to our knowledge the present study is the first investigating the
646 distribution of ARGs in bacterioplankton, distinguishing particle-attached bacteria
647 (PAB) from free-living bacteria (FLB). We hypothesized that PAB would show higher
648 levels of ARGs because their life style enhances the close contact between cells,
649 consequently increasing the probability of an exchange of genetic material encoding
650 resistance. The present study confirmed the hypothesis of higher ARGs levels in PAB
651 respect to FLB only for the *tet*O and *sul* 2 genes. Nevertheless, our data do not allow
652 identifying which mechanisms would be responsible for the observed increase.

Moreover it is also possible that bacterial communities living on particles are different from free living ones. this could lead to differences in ARG abundances because ARGs would be not equally abundant in all species. However this latest hypothesis could be only confirmed by a specific community structures analysis of both fractions that has not been carried out in this study. Anyway, the different behaviors of the ARGs depending on the lifestyle of freshwater bacteria could have implications for the spread of AR bacteria in aquatic ecosystems. In fact, PABs are more subjected to sedimentation processes and consequently, depending on the river flow, they are not expected to travel downstream as rapidly as FLBs are expected to do. However, particles with AR bacteria could both fall on the benthic compartment, favoring the spread of resistance in biofilms, and be re-suspended, as a consequence of flood events, consequently delivering resistance downstream. The study of the different behaviors of AR bacteria in PAB and FLB could also provide useful information for wastewater treatment management in order to reduce the input of AR determinants in aquatic ecosystems. This study provides the first evidence of differences in the behavior of some ARGs depending on the lifestyle of bacteria.

5. Conclusions

This study showed that urban activities may increase the occurrence of antibiotic resistance. Even if the levels of antibiotic resistance in the Zenne River were relatively high already upstream from Brussels, after crossing the city (and receiving the effluents of the two main WWTPs) antibiotic resistance increased significantly independently on the method used to quantify it (culture-dependent and –independent). Our results also suggest that the release of AR fecal bacteria through WWTP effluents could play some

role in the increased levels of AR heterotrophic culturable freshwater bacteria downstream even if transfer of resistance could be not demonstrated. Moreover, our findings highlighted that tetracycline levels positively correlated with the respective ARGs probably because they are released into river waters by the same sources. Finally, this is the first work investigating the distribution of ARGs in bacterioplankton distinguishing particle-attached from free-living bacteria and our hypothesis of higher ARGs levels in PAB respect to FLB was confirmed for two of the genes analyzed. To conclude, our study was conducted at ecosystem scale and does not allow conclusions about direct causality, nevertheless the evidences observed permit to generate valuable hypothesis about antibiotic resistance spread in real aquatic ecosystems strongly affected by human activities.

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934

Table 1
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Table 1. Environmental variables measured at each sampling site during the campaigns. Values are expressed as mean values and SD in *italics* between parentheses (n = 4). Cond = Conductivity; DO = dissolved oxygen; $satO_2$ = percentage of oxygen saturation; T = temperature and SPM = suspended particulate matter.

	Cond ($\mu S\ cm^{-1}$)	**pH**	**DO ($mg\ L^{-1}$)**	**$satO_2$ (%)**	**T (°C)**	**SPM ($mg\ L^{-1}$)**
Z1	798 *(58)*	6.9 *(0.7)*	9.5 *(2.1)*	82.3 *(4.9)*	9.2 *(5.1)*	31.3 *(24.7)*
Z3	814 *(75)*	7.4 *(0.6)*	10.1 *(2.1)*	87.4 *(4.7)*	9.4 *(5.2)*	26.4 *(25.2)*
Z4	849 *(68)*	7.6 *(0.3)*	9.8 *(1.1)*	85.9 *(2.4)*	9.9 *(5.1)*	37.8 *(33.0)*
Z5	931 *(90)*	7.5 *(0.4)*	8.5 *(0.4)*	82.1 *(4.2)*	12.9 *(3.7)*	32.4 *(25.8)*
Z8	1027 *(165)*	7.1 *(0.5)*	6.9 *(1.2)*	73.4 *(3.5)*	14.3 *(2.9)*	22.2 *(7.4)*
Z9	1022 *(31)*	7.2 *(0.3)*	8.6 *(1.9)*	81.1 *(2.8)*	12.6 *(3.8)*	25.5 *(17.8)*
Z11	1192 *(68)*	7.1 *(0.4)*	7.1 *(2.7)*	66.6 *(9.1)*	12.3 *(4.4)*	34.6 *(17.9)*

Table 2
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Table 2. Maximum, minimum and median antibiotic concentrations measured at each sampling site during the sampling campaigns. Values are expressed in ng L^{-1}. AMX = amoxicillin; STX = sulfamethoxazole; NAL = nalidixic acid and TET = tetracycline. When antibiotic concentration was detected but below the limit of quantification (LOQ) we considered the concentration as LOQ/2. (LOQ_{AMX} = 10 ng L^{-1}; LOQ_{STX} = 5 ng L^{-1}; LOQ_{NAC} = 0.15 ng L^{-1}; LOQ_{TET} = 50 ng L^{-1}).

	AMX			**STX**			**NAL**			**TET**		
	min	**max**	**median**	**min**	**max**	**median**	**min**	**max**	**median**	**min**	**max**	**median**
Z1	nd	nd	nd	nd	2930.1	146.0	nd	0.08	0.05	60.8	92.6	68.9
Z3	nd	nd	nd	nd	200.1	0.5	nd	0.08	0.05	73.1	89.3	82.8
Z4	nd	1729.7	283.6	2.5	128.5	2.5	0.08	0.65	0.08	54.7	107.6	59.7
Z5	nd	nd	nd	120.4	253.0	227.9	0.08	0.08	0.08	74.7	87.3	85.2
Z8	nd	nd	nd	2.5	2.5	2.5	nd	0.53	0.05	83.1	147.9	89.0
Z9	nd	nd	nd	2.5	2.5	2.5	0.08	0.08	0.08	87.1	137.9	116.5
Z11	nd	nd	nd	2.5	460.6	2.5	0.08	1.44	0.66	85.9	128.8	98.5

Figure 1
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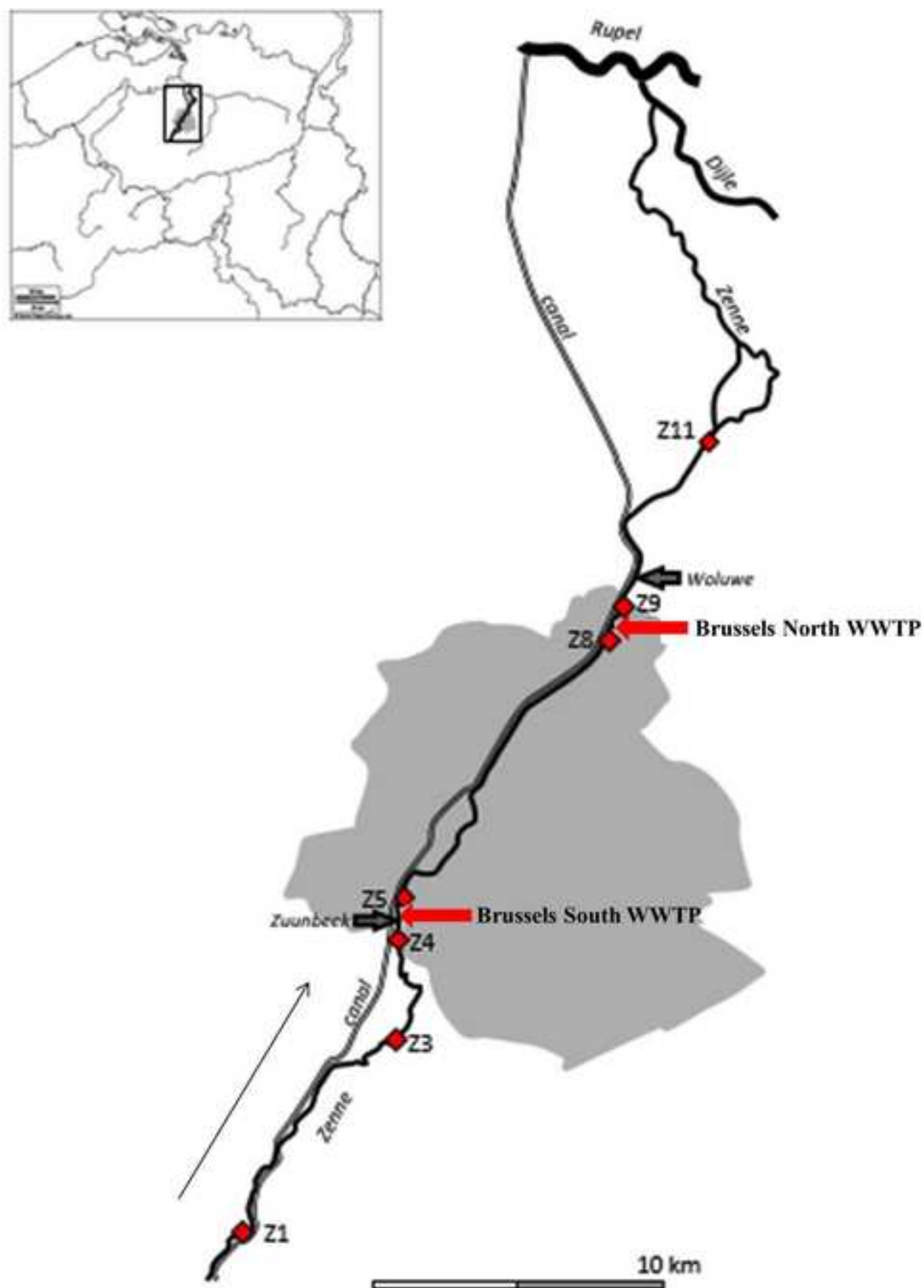

Figure 1. Study area with selected sampling sites (denoted with diamonds) along the Zenne River. The grey area indicates the Brussels-Capital region.

Figure 2
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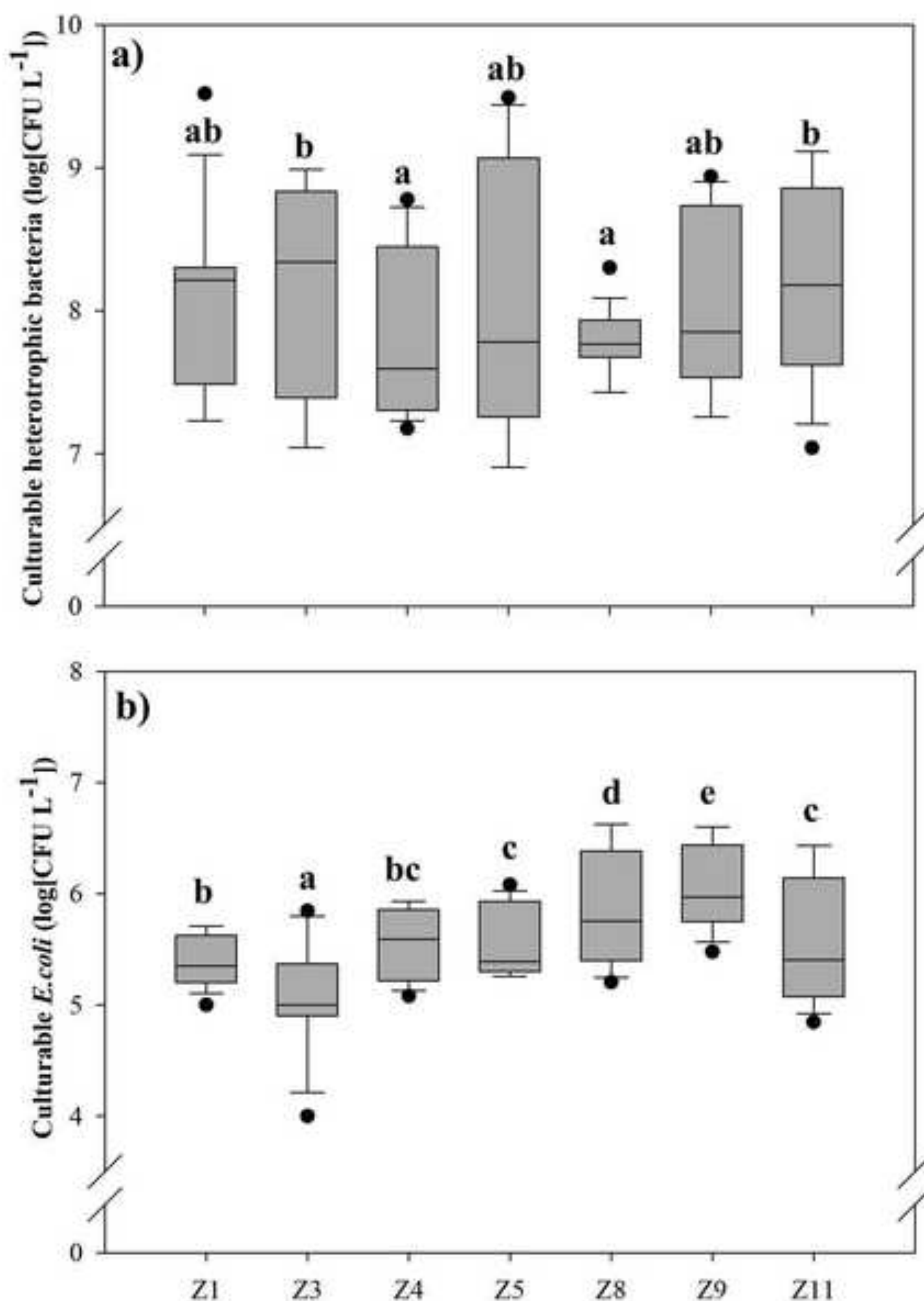

Figure 2. Box-plots in log units of the abundance of culturable heterotrophic bacteria (a) and culturable *E. coli* (b) measured at the different sites along the Zenne River during the four sampling campaigns. Box plots represent the median (horizontal line in the box), the lower and upper quartiles (bottom and top box lines), the 10th and 90th percentiles (bottom and top whiskers) and the outliers (black circles). Post-hoc Tukey's b analysis results are shown with letters when differences among sampling sites were significant. Statistical significance was set at $p \leq 0.05$ (one-way repeated measures analysis of variance, ANOVA).

Figure 3
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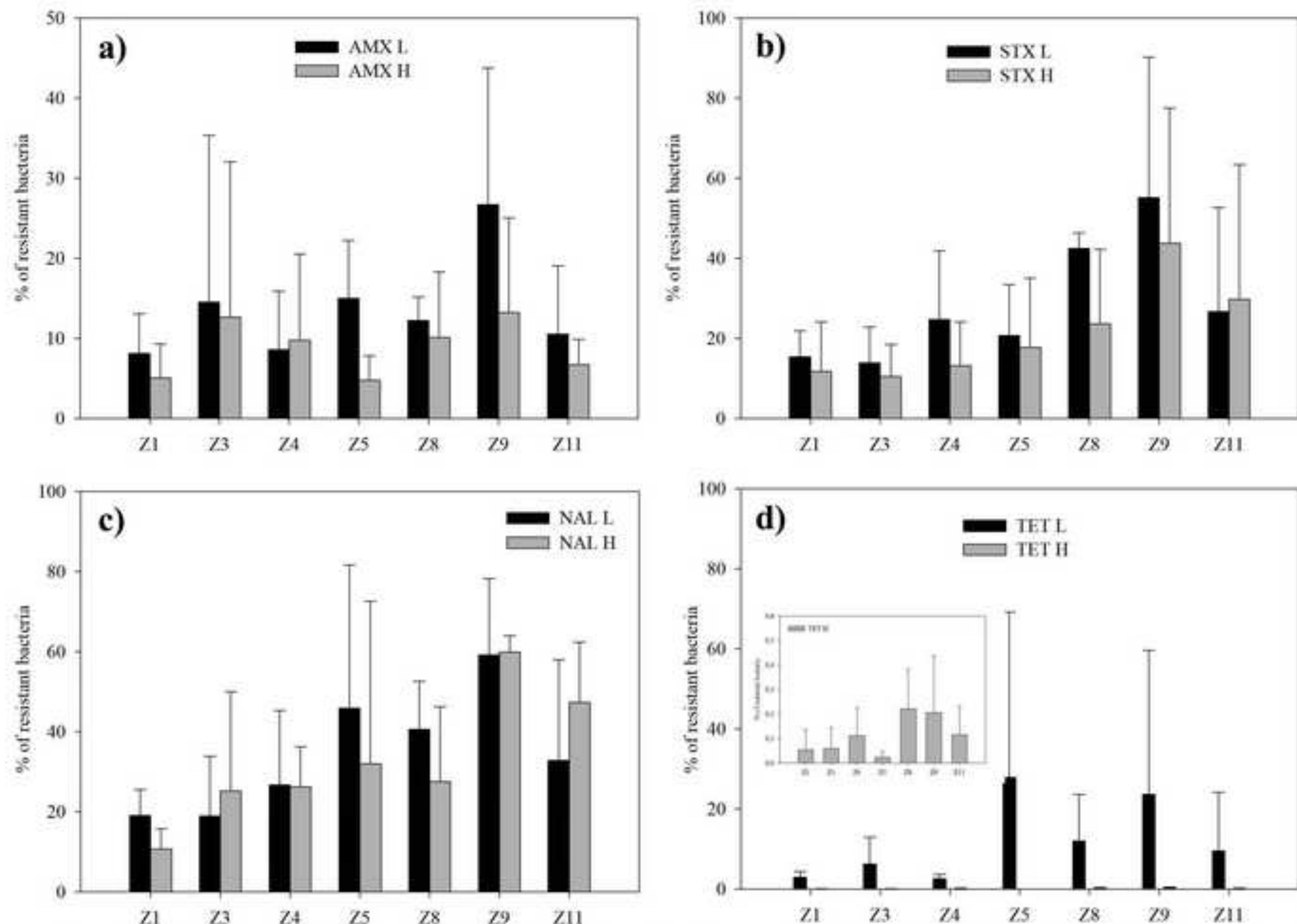

Figure 3. Percentages of culturable heterotrophic bacteria resistant to amoxicillin (a), sulfamethoxazole (b), nalidixic acid (c) and tetracycline (d) measured at the different sites along the Zenne River during the four sampling campaigns. Black bars show the results for lower concentrations (L) and grey bars for higher ones (H).

Figure 4

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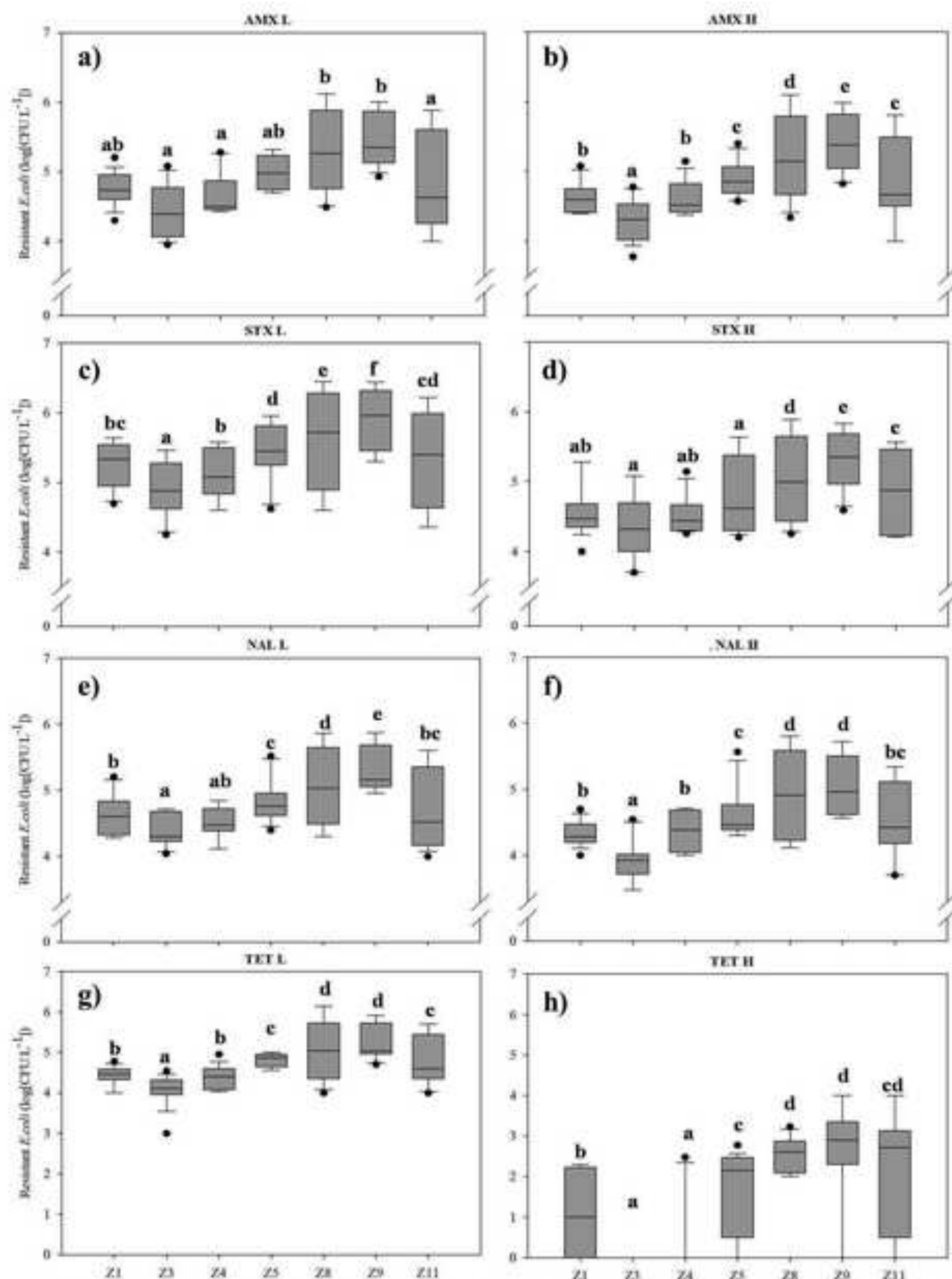

Figure 4. Box-plots in log units of the abundance of culturable *E. coli* resistant to amoxicillin (a and b), sulfamethoxazole (c and d), nalidixic acid (e and f) and tetracycline (g and h) measured at the different sites along the Zenne River during the four sampling campaigns. Box plots represent the median (horizontal line in the box), the lower and upper quartiles (bottom and top box lines), the 10th and 90th percentiles (bottom and top whiskers) and the outliers (black circles). Left, the results for the lowest concentration tested (a, c, e and g); right (b, d, f and h) the results for the highest concentration. Post-hoc Tukey's b analysis results are shown with letters when differences among sampling sites were significant. Statistical significance was set at $p \leq 0.05$ (one-way repeated measures analysis of variance, ANOVA.

Figure 5
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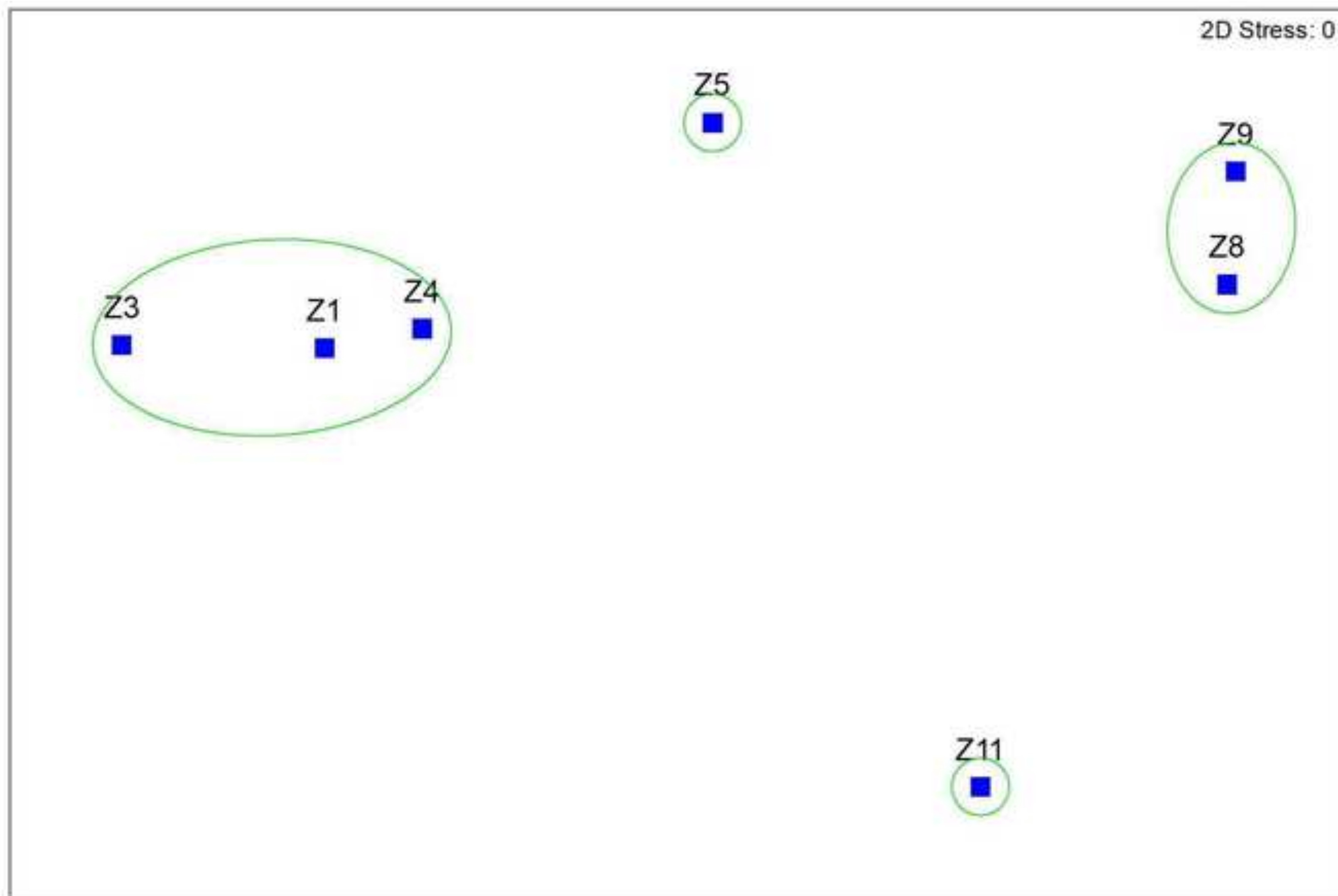

Figure 5. Multidimensional scaling (MDS) ordination of the culturable resistant *E. coli* dataset for the four sampling campaigns along the Zenne River, based on log(x+1)-transformed abundance and Euclidean distance. The circles represent the results of the cluster analysis carried out on the same data set and demonstrate groups of sampling sites depending on their similarity in terms of resistant culturable *E. coli* abundance.

Figure 6
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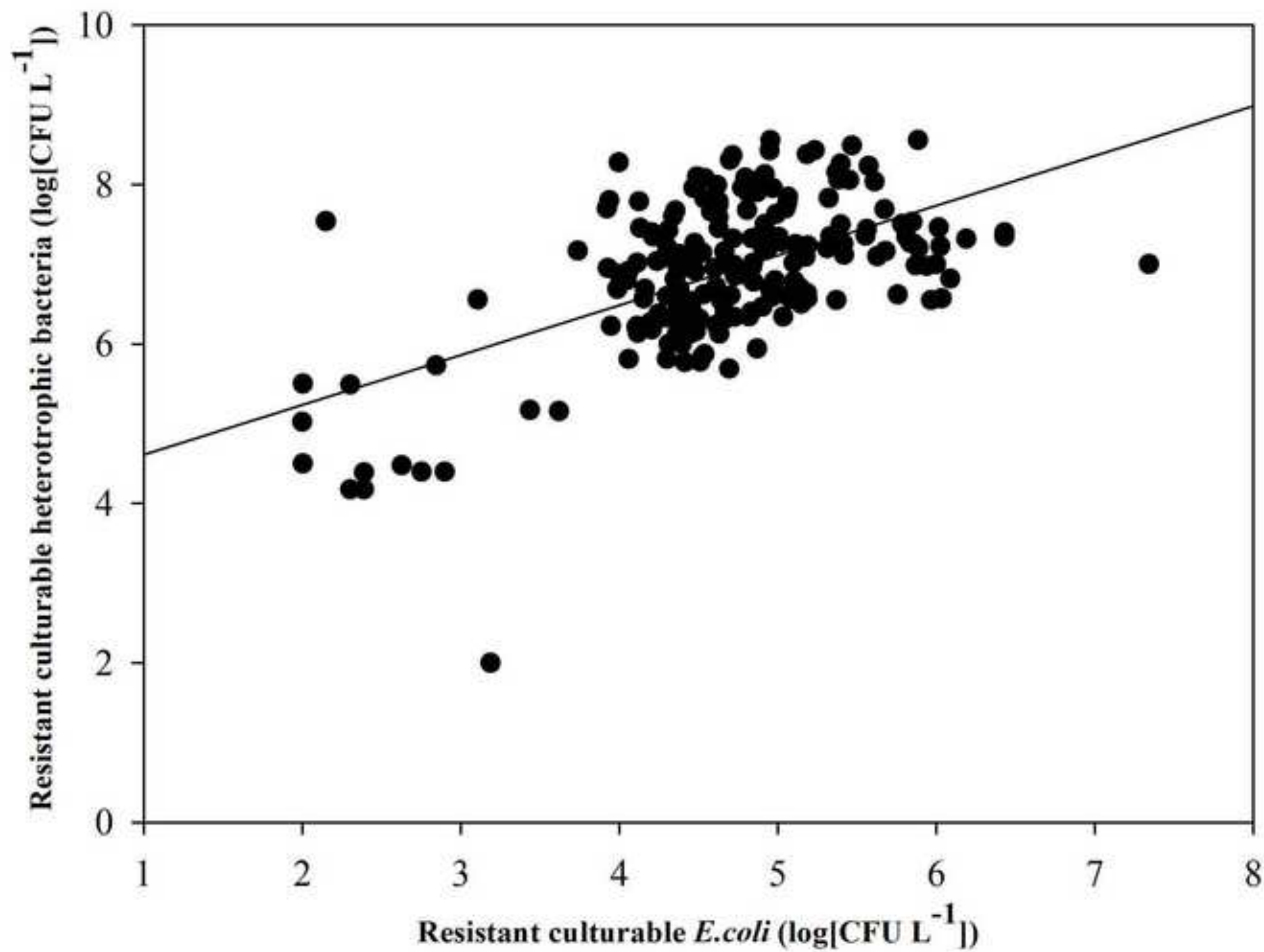

Figure 6. Regression analysis between culturable resistant *E. coli* and culturable resistant heterotrophic bacteria.

Figure 7

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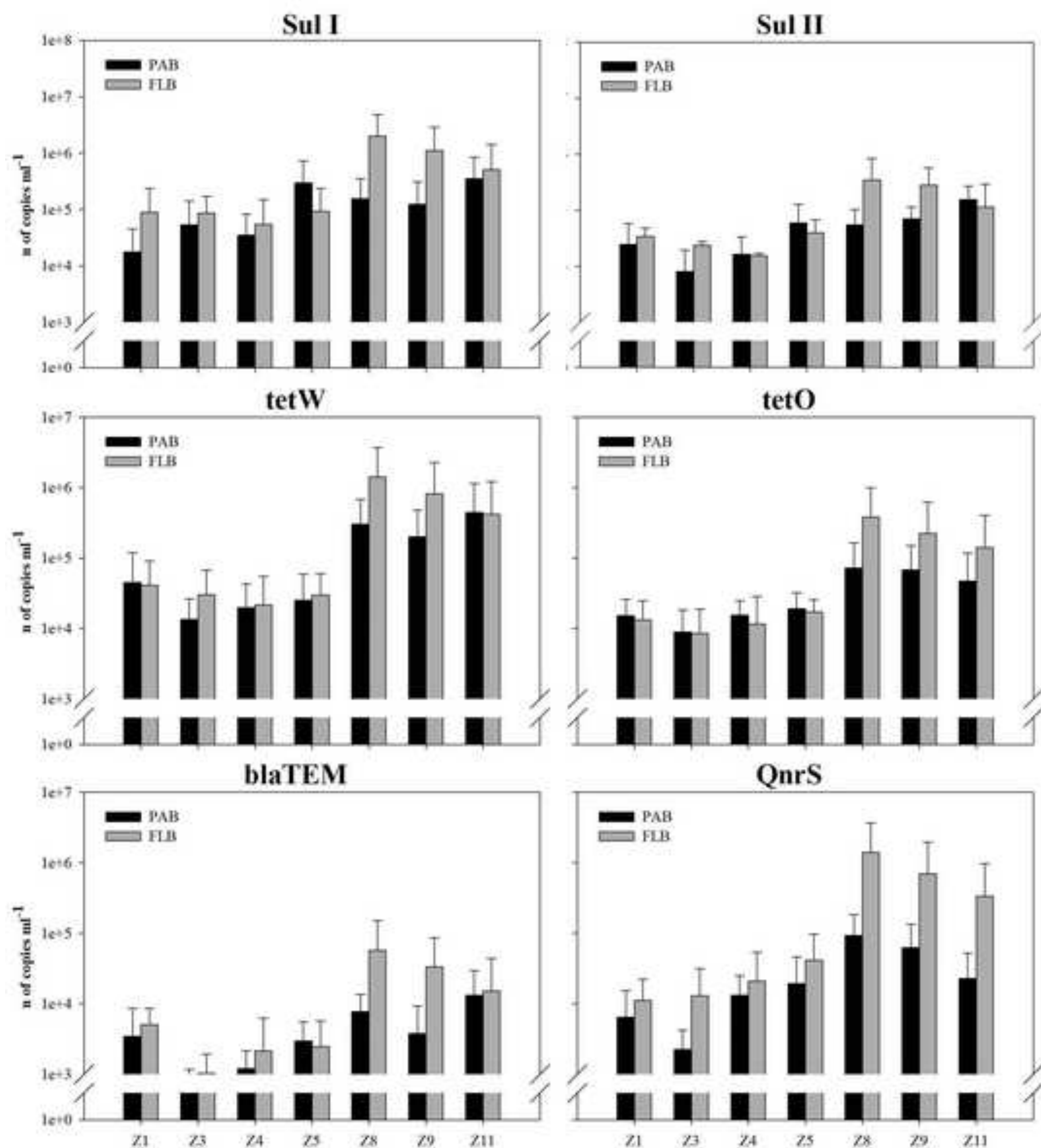

Figure 7. Abundances of ARGs at the different sampling sites along the Zenne River during the four sampling campaigns (log scale). Black bars show the results for particle-attached bacteria (PAB) and grey bars free-living bacteria (FLB).

Figure 8
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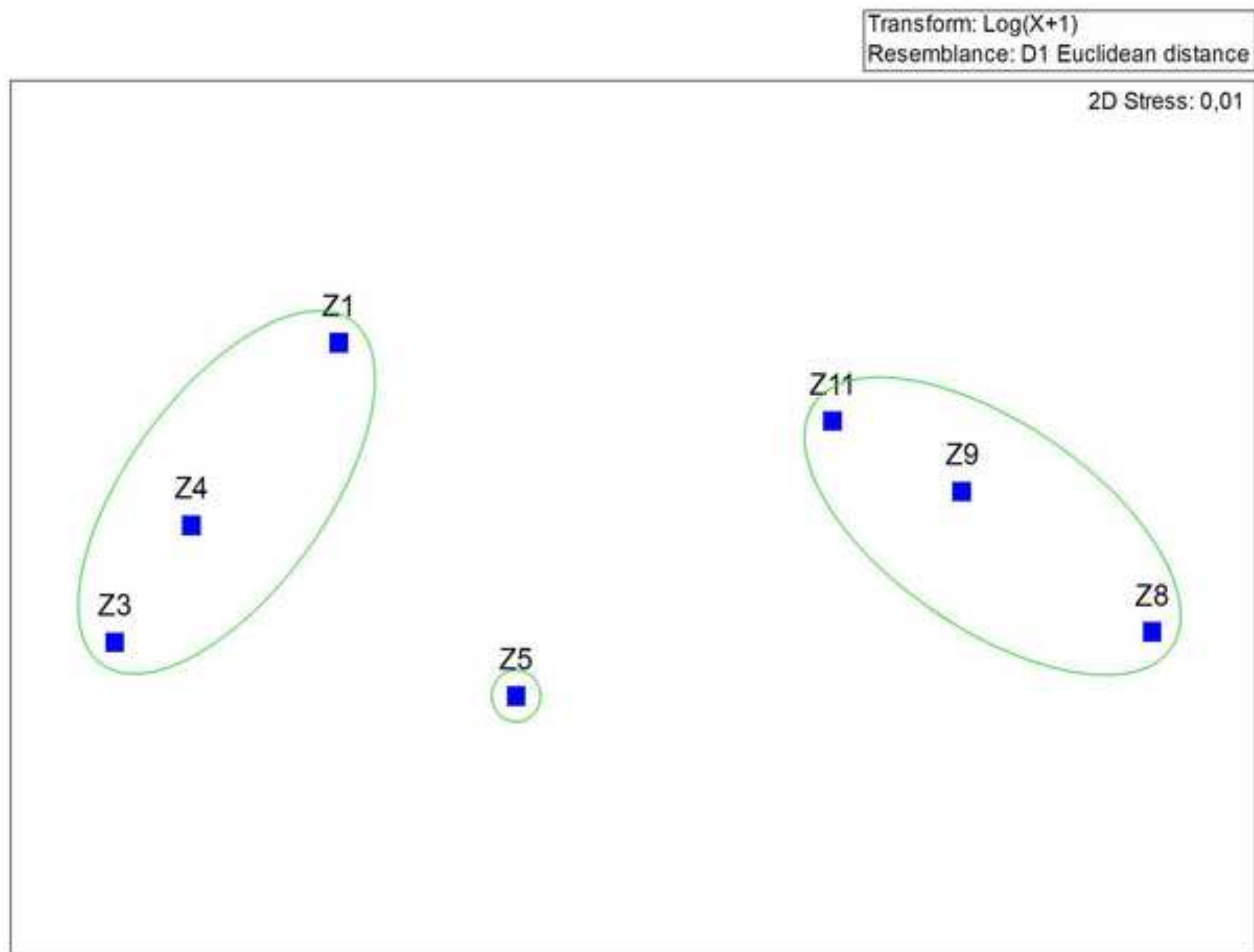

Figure 8. Multidimensional scaling (MDS) ordination of the ARG data set for the four sampling campaigns along the Zenne River, based on log(x+1)-transformed abundance and Euclidean distance. The circles represent the results of the cluster analysis carried out on the same data set and demonstrate groups of sampling sites depending on their similarity in terms of ARG abundance.

Figure 9
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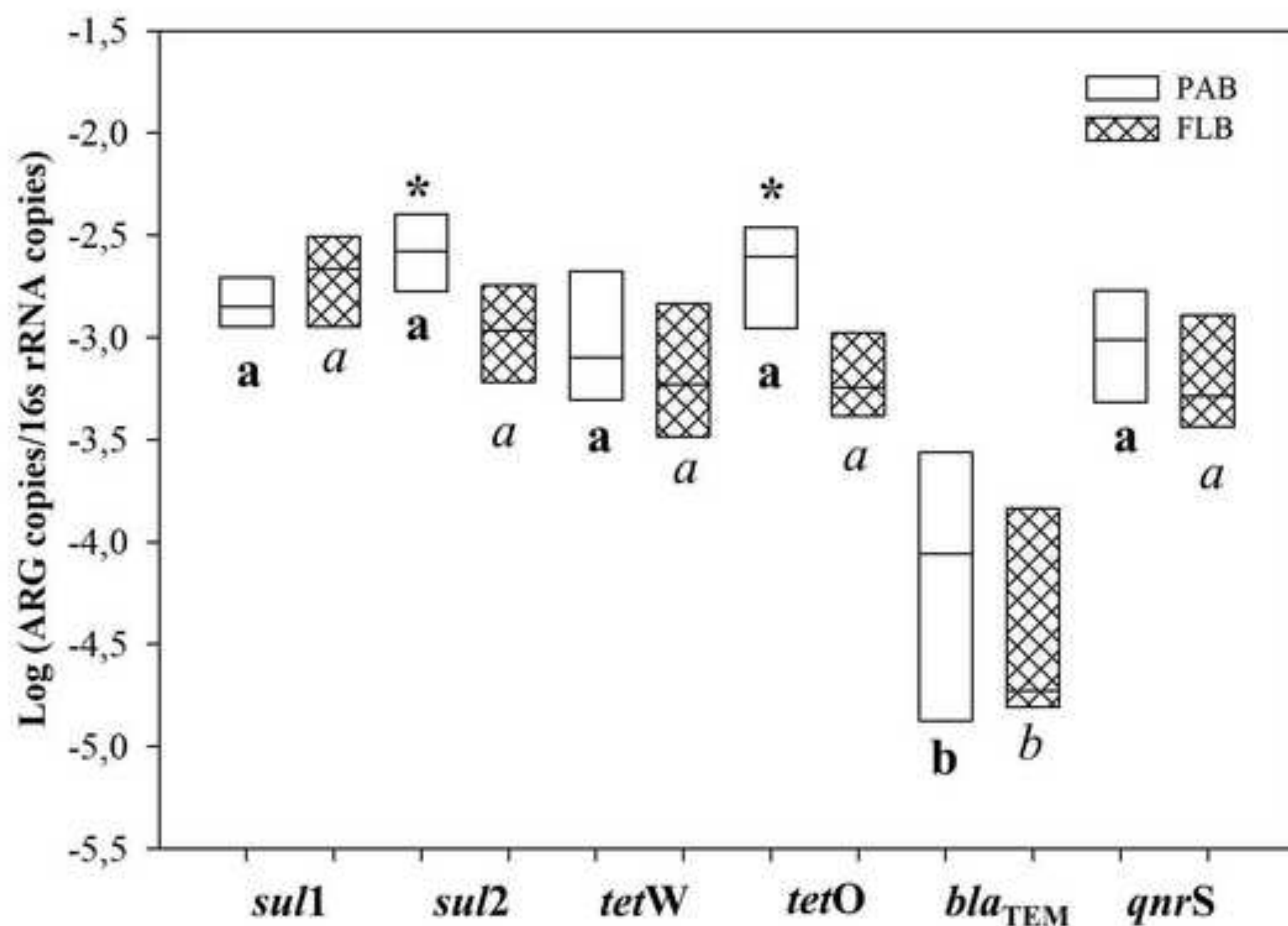

Figure 9. Comparison among ARGs and between PAB (Empty boxes) and FLB (filled boxes). Values are log units of ARG normalized to 16s rRNA copies. Box plots represent the median (horizontal line in the box), the lower and upper quartiles (bottom and top box lines), the 10th and 90th percentiles (bottom and top whiskers). Results of statistical analyses are reported. Asterisks (*) represent significant differences between PAB and FLB fractions. **Bold** letters represent the results of post-hoc Tukey's b analysis for PAB fraction when differences among ARG were significant. *Italic* letters represent the results of post-hoc Tukey's b analysis for FLB fraction when differences among ARG were significant Statistical significance was set at $p \leq 0.05$ (one-way analysis of variance, ANOVA).

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