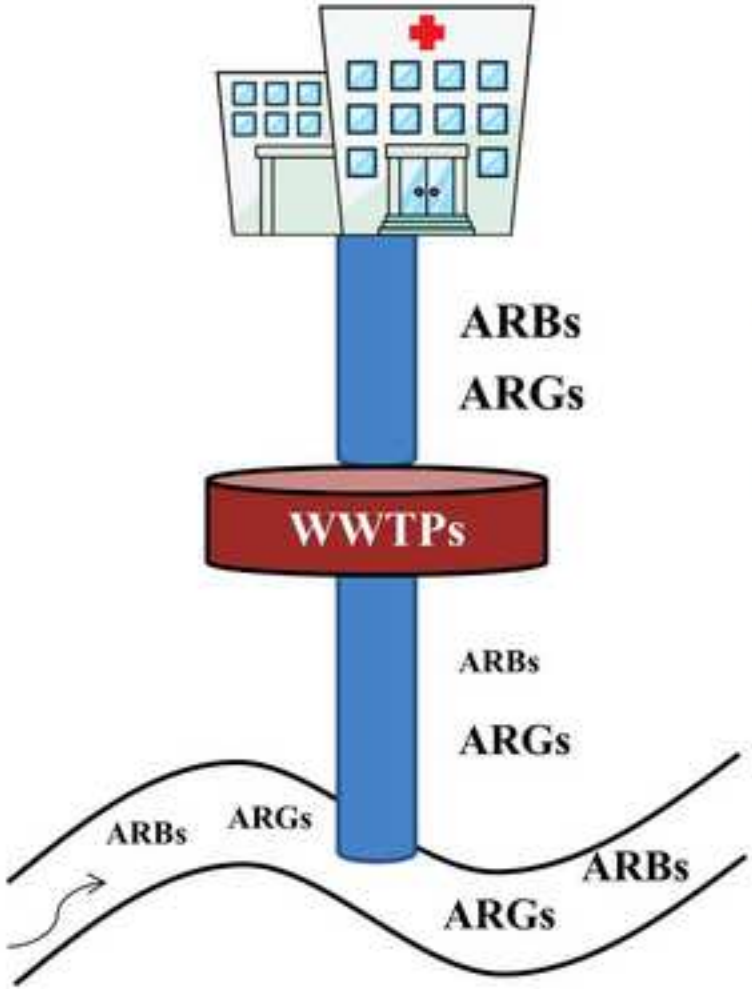
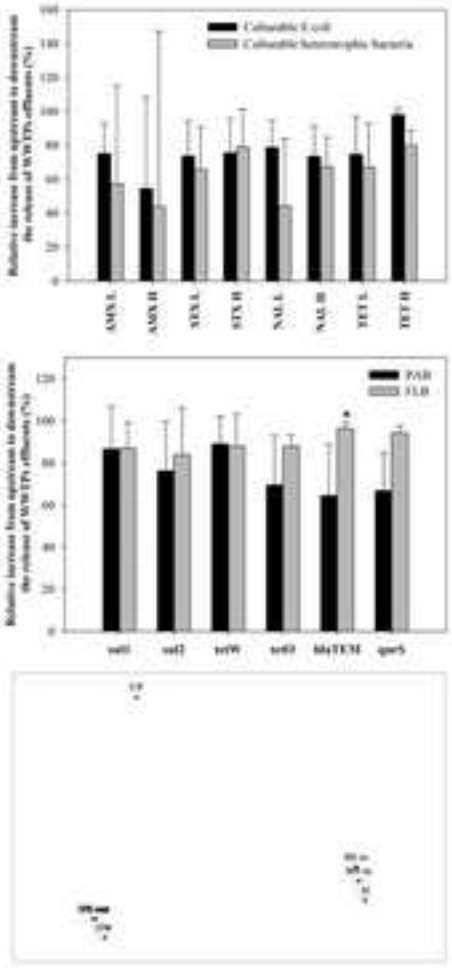
Graphical Abstract





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

- Brussels WWTPs efficiently remove antibiotic resistant bacteria (ARB)
- Significant increase of ARB was found downstream from the WWTPs outfalls
- Absolute ARGs abundances are reduced from influents to effluents of both WWTPs
- Significant increase of ARGs was found downstream from the WWTPs outfalls
- Some ARGs relative abundance significantly increased in the effluent of WWTPs

Antibiotic resistance in urban and hospital wastewaters and their impact on a receiving freshwater ecosystem

Proia Lorenzo[1], Anzil Adriana[1], Subirats Jessica[2], Borrego Carles[2], Farrè Marinella[3], Llorca Marta[3], Balcázar Jose Luis[2], Servais Pierre[1]

[1] Ecologie des Systèmes Aquatiques, Université Libre de Bruxelles, Campus de la Plaine, CP 221, Boulevard du Triomphe, 1050 Brussels, Belgium

[2]Catalan Institute for Water Research (ICRA), c/ Emili Grahit 101, 17003 Girona, Spain

[3]Water and Soil Quality Research Group, Department of Environmental Chemistry, IDAEA-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain

Corresponding author: Proia Lorenzo; Ecologie des Systèmes Aquatiques, Université Libre de Bruxelles, Campus de la Plaine, CP 221, Boulevard du Triomphe, 1050 Brussels, Belgium (proialorenzo@hotmail.it)

Abstract

The main objective of this study was to investigate the antibiotic resistance (AR) levels in wastewater (WW) and the impact on the receiving river. Samples were collected once per season over one year in the WW of a hospital, in the raw and treated WW of two wastewater treatment plants (WWTPs), as well as upstream and downstream from the release of WWTPs effluents into the Zenne River (Belgium). Culture-dependent methods were used to quantify *Escherichia coli* and heterotrophic bacteria resistant to amoxicillin, sulfamethoxazole, nalidixic acid and tetracycline. Six antibiotic resistance genes (ARGs) were quantified in both particle-attached (PAB) and free-living (FLB) bacteria. Our results showed that WWTPs efficiently removed antibiotic resistant bacteria (ARB) regardless of its AR profile. The ARGs levels were the highest in the hospital WW and were significantly reduced in both WWTPs. However, ARB and ARGs abundances significantly increased into the Zenne River downstream from the WWTPs outfalls. The variation in the relative abundance of ARGs through WW treatment differed depending on the WWTP, fraction, and gene considered. The *sul*1 and *sul*2 genes in PAB fraction showed significantly higher relative abundances in the effluent compared to the influent of both WWTPs. This study demonstrated that WWTPs could be hotspots for AR spread with significant impacts on receiving freshwater ecosystems. This was the first comprehensive study investigating at the same time antibiotics occurrence, fecal bacteria indicators, heterotrophic bacterial communities, and ARGs (distinguishing PAB and FLB) to assess AR levels in WW and impacts on the receiving river.

Keywords: antibiotic resistance; wastewaters; urban river; antibiotic resistance genes; particle-attached and free-living bacteria

1. Introduction

Antibiotics have saved millions of human lives since their discovery and application to treating bacterial infectious diseases. However, extensive use of antibiotics has led to an increased prevalence of antibiotic-resistant bacteria (ARB) (Levy and Marshall, 2004). Antibiotic resistance (AR) has been classified by the World Health Organization as one of the three greatest threats to public health in the 21st century and the latest report from the UK Review on Antimicrobial Resistance, published recently (O'Neill, 2016), estimates that the 700,000 annual deaths currently attributable to infections by drug-resistant pathogens will increase, if unchecked, to 10 million by 2050 (Robinson et al., 2016).

Wastewater treatment plants (WWTPs) receive sewage from various sources, including hospitals and households, which are both important sources of antibiotics and ARB (Laht et al., 2014). Hospital effluents, in particular, constitute a special category of waste that is highly hazardous because they contain a myriad of drug residues and infectious agents and are thus an important source of multidrug-resistant bacteria and antibiotics (Rodriguez-Mozaz et al., 2015). Between 20 and 97% of any dose of most antibiotics administered to humans and animals is excreted as an active substance, consequently reaching wastewaters (Jelic et al., 2015). On the other hand, during therapeutic treatment the human gut microbiota is exposed to high concentrations of antibiotics that may stimulate the generation of resistance phenotypes before being released into sewage via human excreta (Servais and Passerat, 2009). In fact, the presence of antibiotics, ARB, and antibiotic resistance genes (ARGs) has been confirmed in many WWTPs worldwide (Michael et al., 2013; Rizzo et al., 2013; Zanotto et al., 2016).

123 WWTPs are considered important hotspots for the acquisition and spread of
124 antibiotic resistance in the environment and three major reasons are often put forward to
125 sustain this idea: i) the heavy discharge of antibiotic residues, ARB, and ARGs
126 collected in the municipal sewage system; ii) the favorable conditions for both selection
127 and/or horizontal transfer of resistance genes among bacterial cells during the
128 wastewater treatment process; and iii) the widespread observation that WWTP effluents
129 contain high AR levels (sometimes higher than in the raw inflow) (Novo et al., 2013).
130 As a consequence, WWTP effluents are among the most important conduits for the
131 spread of AR to aquatic environments.

132 Many studies have investigated the fate of antibiotics through wastewater (WW)
133 treatment (Jelic et al., 2015; Michael et al., 2013), whereas many others have focused on
134 the ARG responses to WW treatment, sometimes considering the receiving environment
135 (Ben et al., 2017; Neudorf et al., 2017; Rafraf et al., 2016; Rizzo et al., 2013).
136 Moreover, several studies have analyze the occurrence and fate of AR bacteria in
137 WWTPs (Bouki et al., 2013; Łuczkiewicz et al., 2010). Despite a considerable amount
138 of research carried out combining the investigation of antibiotics and ARGs (Caucci et
139 al., 2016; Rodriguez-Mozaz et al., 2015; Subirats et al., 2017) as well as ARB and
140 ARGs (Yuan et al., 2015; Zanotto et al., 2016; Zhang et al., 2015) along the WW
141 treatment process, comprehensive studies assessing the fate of antibiotics, ARB, and
142 ARGs in WWTPs and its eventual influence on receiving water bodies are still lacking.

143 The main objective of this study was to fill this gap investigating the level of WW
144 contamination by antibiotics, the prevalence of AR in WW, and their effects on the
145 receiving river, focusing at the same time on the fecal bacteria *Escherichia coli*,
146 heterotrophic bacterial communities, and their ARGs distinguishing between particle-
147 attached (PAB) and free-living (FLB) bacterial fractions. *E. coli* was selected as a fecal

indicator bacterium that can be exposed to high antibiotic concentrations in the human or animal gastrointestinal tract and acquire resistance before being released into sewer systems and finally reaching WWTPs. The fecal bacteria can thus act as a source of resistance because they can disseminate ARGs to autochthonous bacteria (Baquero et al., 2008). Moreover, continuous release of antibiotics in WWTPs can act as chronic selective pressure able to promote AR (Gullberg et al., 2011).

The specific questions investigated in this study focused on:

- The concentration of antibiotic residues, ARB, and ARGs in raw and treated wastewaters (urban and hospital);
- The impact of WW treatment (secondary and tertiary treatments) on the abundance of antibiotics, ARB, and ARGs
- The eventual impacts of the WWTP effluent release into the receiving river
- The fate of ARGs in WW and receiving river depending on the bacterial fraction considered (PAB and FLB)

To reach these goals, samples were collected over 1 year (one sampling per season) in the WW of a hospital, in the raw and treated WW of the two Brussels (Belgium) WWTPs, as well as at two sites located upstream and downstream from the release of the two WWTP effluents into the receiving river. Culture-dependent and -independent methods were used to estimate the resistance of culturable *E. coli* and heterotrophic bacteria by plate counts containing antibiotics as well as by quantifying the abundance of six genes conferring resistance to the main antibiotic families. The selection of the antibiotics (and the respective genes) was done according to the specific objectives of the study, therefore including relevant clinical antibiotics for which resistance has been reported elsewhere. Since different behaviors are expected in response to the settling

stage applied as the first treatment stage in WWTPs, ARGs were analyzed in both PAB and FLB. Most particularly, higher removal rates were hypothesized for ARGs in PAB with respect to the FLB fraction. Finally, considering that the close contact between cells attached to the same particle would increase the probability of an exchange of genetic material encoding resistance, it is important to distinguish the fate of ARGs in these two fractions during WW treatment and in the receiving water bodies.

2. Material and methods

2.1. Study sites and sampling strategy

In this study, the two WWTPs located in the Brussels Capital Region (Belgium) were investigated: the Brussels South (BS) WWTP (360,000 equivalent-inhabitants) in operation since the year 2000 and the Brussels North (BN) WWTP (1.1 million equivalent-inhabitants) in operation since 2007. The two WWTPs function on different technologies. The BS WWTP treatment line includes a primary settling stage (to remove suspended solids) and a secondary biological treatment (an activated sludge process to remove biodegradable organic matter; there is presently no tertiary treatment to remove nitrogen and phosphorus in this plant). At the BN WWTP, there are two treatment lines. The first one (biological line) includes a primary settling stage followed by a modern tertiary treatment technology (simultaneous removal of biodegradable organic carbon, nitrogen, and phosphorus by an activated sludge process; Azenit P® technology). The other treatment line (rain line) runs parallel to the biological line when the discharge reaching the WWTP is too high in wet weather situations; this rain line uses only a primary settling process. On an annual basis, the volume treated in the biological line accounts for roughly 90% of the total volume reaching the WWTP. The effluents of both WWTPs are released in the Zenne River, the small urban river running through the city of Brussels (Brion et al., 2015). The Zenne is a paradigm of sewage-

198 impacted river because its discharge (on annual average) is doubled after receiving the
199 treated waters from the two WWTPs in the city of Brussels (Brion et al., 2015). We also
200 collected surface water from two sites along the Zenne River: one just upstream from
201 BS WWTP (Up) and the second one downstream from the release of BN effluent to the
202 river (Dw). However, the Zenne River is already impacted upstream from the release of
203 BS WWTP by human activities carried out in the upstream watershed as: i) the release
204 of the effluents from three relatively small WWTPs (with a total capacity of 103,300
205 equivalent inhabitants); ii) the runoff on pastured areas and iii) the effluents from farms
206 with intense breeding activities in the upstream watershed (Ouattara et al., 2014).
207 Four sampling campaigns were conducted in 2016, one per season with different
208 hydrological conditions of the receiving river. Discharge recorded just upstream from
209 the Brussels region during the sampling campaigns were respectively 4.9 m^3 s^{-1} in
210 January, 2.5 m^3 s^{-1} in April, 3.0 m^3 s^{-1} in July and 1.3 m^3 s^{-1} in November. During the
211 sampling campaigns, samples were collected in the inlet and outlet of both Brussels
212 WWTPs. In the BS WWTP, average daily samples were collected with refrigerated
213 automatic samplers in order to integrate the daily fluctuations in sewage quality, while
214 at the BN WWTP grab samples were collected in the morning. Since sampling
215 campaigns were conducted in dry weather conditions, data concerning the treated waters
216 of the BN WWTP presented in this paper concern the biological line. In addition, during
217 each sampling campaign a sample was also collected in the sewer at the outlet of the UZ
218 Brussels Hospital (H), which, according to the 2012 annual report, has 721 beds, 29,239
219 hospitalizations per year, and 23,692 day hospitalizations per year (excluding minimum
220 flat rates). The sewer of the UZ Brussels Hospital discharges untreated wastewaters to
221 the BN WWTP. All samples were collected in triplicate, stored in sterile 2-L bottles and
222 kept at 4°C until analysis. 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 receiving river and a functioning of WWTPs characteristics of dry weather conditions, thus avoiding any influence of climatic conditions on the results.

2.2. Physicochemical analysis

The chemical oxygen demand (COD), biological oxygen demand (BOD), suspended particulate matter (SPM), total nitrogen (Ntot), and total phosphorus (Ptot) were analyzed daily during 2015 and 2016 in the laboratories of the two WWTPs following the standard methods for water and wastewater (APHA,2012). Data were obtained from the SBGE (Société Bruxelloise de Gestion des Eaux), which is in charge of collecting the auto-control data from WWTPs in the Brussels Capital area.

2.3. Concentration of antibiotics

The concentrations of amoxicillin (AMX), sulfamethoxazole (SMX), nalidixic acid (NAL), and tetracycline (TET) were determined during the last three campaigns by means of liquid chromatography coupled to mass spectrometry in tandem (LC-MS/MS) after sample clean-up and pre-concentration with solid-phase extraction (SPE). These antibiotics were analyzed because they had been tested for resistance (see section 2.4). One hundred milliliters of blank samples (consisting of Milli-Q water spiked with the mixture of native compounds at 100 ngL^{-1}) were used for traceability and cross-contamination monitoring. Then 100 mL of blank samples and 100 mL of river samples were fortified with sulfamethoxazole-d_6 surrogate internal standard for a final concentration of 0.1 ng mL^{-1} in sample. After that, the samples were equilibrated and kept at −20°C before analysis to ensure the traceability of the results (Llorca et al., 2014). The samples were prepared following Gros et al. (2013). All the samples were extracted in triplicate. Further details about sample preparation, antibiotic

247 quantification, quality control and quality assurance parameters are reported in
248 Supplementary Material (A, Table A.1 and Table A.2).

249 **2.4. Quantification of AR *Escherichia coli* and heterotrophic bacteria**

250 Resistance to AMX, STX, TET, and NAL were tested simultaneously in culturable *E.*
251 *coli* and heterotrophic bacteria. These antibiotics were chosen because they belong to
252 four different families and have different mechanisms of action. Moreover, these
253 antibiotics are among the most widely used in Belgium for humans (European Centre
254 for Disease Prevention and Control, http://ecdc.europa.eu), except NAL, which today is
255 used only in veterinary medicine (Callens et al., 2017). NAL was selected to compare
256 resistance levels to antibiotics currently used in human medicine with those no longer
257 consumed by humans. For that purpose, *E. coli* was grown on Chromocult Coliform
258 agar (Merck Millipore, Darmstadt, Germany) for 24 h at 37°C, whereas heterotrophic
259 bacteria were grown on R2A agar (Merck Millipore, Darmstadt, Germany) for 7 days at
260 20°C. Media were used as such (for total culturable *E. coli* and heterotrophic bacteria)
261 or supplemented with one of the four antibiotics. For each antibiotic, two different
262 concentrations (low and high) were tested: AMX (4 and 50 µg mL^{-1}), SMX (16 and 300
263 µg mL^{-1}), NA (2 and 30 µg mL^{-1}), and TET (4 and 300 µg mL^{-1}) (Sigma Chemical
264 Company, St. Louis, MO, USA). The lowest (L) are the breakpoint concentrations
265 established for *E. coli* by the French committee for antimicrobial standards (Comité de
266 l'Antibiogramme de la Société Française de Microbiologie) and the highest (H)
267 correspond to the values reported in previous studies dealing with antibiotic resistance
268 in environmental bacteria (Garcia-Armisen et al., 2013).
269 Two tenfold serial dilutions were filtered (or spread) for each sample to obtain an
270 accurate number of colonies to ensure that at least one of them could be counted.
271 Triplicates were performed for each volume filtered or dilution spread. For each of the

combinations two plates that were not inoculated with samples were incubated as negative controls. All the controls were negative after incubation. The results were expressed in colony-forming units (CFU) per liter. With these methods and considering the concentrations used, we were able to quantify putative AR *E.coli* and heterotrophic bacteria. Thus, when mentioning data of ARB enumerated using cultivation methods, we refer to putative resistant bacteria all over the manuscript.

2.4. DNA extraction

The bacterial biomass was collected from the water fraction and concentrated by filtration. An aliquot (from 0.25 L to 1.5 L) of each sample was filtered in triplicate to collect two different fractions of bacteria. PAB were collected by filtering water on 5-µm pore-size, 47-mm-diameter polycarbonate filters (Millipore, Billerica, MA, USA). Filtrates were then filtered through 0.22-µm pore-size 47-mm-diameter polycarbonate filters (Millipore) to retain FLB. Filters were kept at −80°C until extraction. Extractions were performed following García-Armisen et al. (2014). Briefly, all filters were cut and placed in 3-mL lysis buffer (10 mM tris, 1 mM EDTA, pH 8.0) with 1.5 U mL^{-1} mutanolysin and 50 mg mL^{-1} lysozyme (both enzymes from Sigma Aldrich) and incubated at 37°C for 30 min. We added 500 µL of sodium dodecyl sulfate (25%) and 100 µL of proteinase K (23 mg mL^{-1}), and the tubes were incubated 30 min at 60°C followed by three cycles of freezing/heating at −80°C/65°C. DNA was extracted from each pellet by adding 5 mL of phenol-chloroform-isoamyl alcohol (25:24:1) pre-warmed to 60°C (Sigma Aldrich). Phases were separated by centrifugation (13,000 rpm at 4°C for 5 min) using Phase Lock Gel tubes (Eppendorf AG, Hamburg, Germany). The DNA was precipitated by adding sodium acetate (0.3 M final concentration, pH 5.2) and 0.7 (v/v) isopropanol followed by centrifugation for 30 min at 4°C. The supernatant was removed and 1 mL of ice-cold 70% ethanol was added to the pellets;

297 the solution was then mixed and centrifuged at 14,000 rpm for 5 min (4°C). Each pellet
298 was air-dried (15–30 min) and suspended in 100 µL of sterile TE buffer (10 mM tris, 1
299 mM EDTA, pH 8.0). DNA concentration and purity were further determined using a
300 NanoDrop ND-2000 UV Vis spectrophotometer (NanoDrop, ThermoFisher).

301 **2.5. Quantification of ARGs using qPCR**

302 The copies of targeted ARGs conferring resistance to sulfonamides (*sul* 1, *sul* 2),
303 tetracyclines (*tet*W, *tet*O), β-lactams (bla_{TEM}), and quinolones (*qnr*S) were quantified
304 using qPCR assays. Tenfold dilutions of plasmid DNA containing known
305 concentrations of the target gene were used as standard curves, which were generated by
306 cloning the amplicon from positive controls into *E. coli* using the pCR2.1-TOPOvector
307 system (Invitrogen, Carlsbad, CA, USA) (Proia et al., 2016). All qPCR assays were
308 performed in duplicate using SYBR green detection chemistry with a Step One Plus
309 (Applied Biosystems, ThermoFisher Scientific).

310 Briefly, each reaction contained 8–9 µL of Power Up SYBR Green master mix
311 (Applied Biosystems, ThermoFisher Scientific), 200 nM each forward and reverse
312 primer, and 9 µL of 5-ng μL^{-1} DNA template, and the final volume was adjusted to 20
313 µL by adding DNase-free water. Each gene was amplified using specific primer sets
314 (Sigma Aldrich) and the PCR conditions included initial denaturation at 95°C for 3 min,
315 followed by 40 cycles at 95°C for 15 s, then 20 s at the specific annealing temperature
316 depending on the gene (Table B.1), and finally two elongation steps lasting 40 s at 72°C
317 and 32 s at 78°C. The number of copies of the bacterial 16S rRNA gene was also
318 quantified, and the amplification conditions included an initial denaturation at 95°C for
319 3 min, followed by 35 cycles at 95°C for 15 s, then an annealing temperature at 60°C
320 for 1 min 40 s at 72°C and 32 s at 78°C. A dissociation curve was applied at the end of
321 each run to detect nonspecific amplifications. Tenfold serial dilutions of the standards

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

2.6. **Statistical analyses**

Resistance to each antibiotic at different concentrations was analyzed independently using a one-way repeated measure analysis of variance (ANOVA) to test for the differences among sampling sites during the entire year of sampling. The effects were analyzed post hoc with Tukey's b test. Data were log-transformed prior to statistical analyses to meet assumptions of normality and homogeneity of variance when needed. Analysis was performed using SPSS Version 15.0. Kruskal-Wallis one-way analysis of variance on ranks was performed when data did not meet assumptions of normality. Regression analysis between the resistant culturable *E. coli* and heterotrophic bacteria data sets were performed using Sigma Plot software 11.0. Statistical significance was set at $p = 0.05$.

3. Results and Discussion

3.1. Physicochemical variables and antibiotic concentrations

The concentrations in the inlet waters of the variables measured were quite similar for both WWTPs and were generally low, especially for COD and BOD_5 (Table 1), with regards to what is usually expected for domestic WW in Europe. This could be explained by the fact that Brussels is equipped with a combined sewer system in

347 which, during rainy periods, runoff waters are mixed with domestic WW, decreasing
348 thus the concentration of COD and BOD_5 at the entrance of BS and BN treatment
349 plants.

350 The two Brussels WWTPs, based on an annual average, efficiently reduce the
351 concentrations of all the physicochemical variables analyzed (removal efficiency
352 >75% and >0.7 log, Table 1) with only the exception of total nitrogen (Ntot) at BS. In
353 fact, although the thresholds established by the Council Directive for wastewater
354 treatment (91/271/EEC) were generally respected for all the variables considered in
355 this study, the BS effluent annual average exceeded the Ntot concentration required by
356 the directive for sensitive zones (91/271/EEC). This is explained by the design of BS,
357 which does not yet include a specific treatment to remove nitrogen. Moreover, the log
358 removal on Ntot at BN was significantly higher than that of BS ($p > 0.05$; Kruskal-
359 Wallis one-way analysis of variance) whereas for all the other variables differences in
360 log removal were slight and in general not significant (Table 1).

361 BN's greater efficiency removing Ntot is explained by the different technologies
362 applied to the two WWTPs. In fact, BN has a modern treatment technology
363 associating the removal of C, N, and P, whereas there is no tertiary treatment in BS. In
364 general, the implementation of the two WWTPs has increased the water quality of the
365 receiving river since their operation started (Brion et al., 2015).

366 The fate of antibiotic residuals in WWTPs differed depending on the compound
367 considered (Table 2). Verlicchi and colleagues (2012) reviewed the occurrence of
368 antibiotics in WWTPs worldwide and showed a huge range of concentrations and
369 removal efficiencies for different compounds, as well as for the same substance
370 (Verlicchi et al., 2012).

In our study, AMX was detected only in the 33% of samples analyzed and the concentrations were always below the detection limit in the hospital effluent (Table 2). These results are in general agreement with those found by other studies and the low prevalence of AMX may be explained by its instability in aqueous media (Andreozzi et al., 2004; Gros et al., 2013; Rodriguez-Mozaz et al., 2015). AMX at BS was only detected during one sampling campaign; its concentration was reduced by about 48% between the influent and the effluent. In contrast, AMX was detected only in the BN effluent while data were below the detection limit (LOD) in the influent for all the samplings carried out (Table 2). This behavior has already been reported in a study carried out in Spanish WWTPs (Gros et al., 2013), and how certain metabolites of antibiotics can be transformed back to the parent compound as a result of enzyme-catalyzed reactions occurring in activated sludge is also known (Plósz et al., 2010).

STX is one of the most widely detected antibiotics in sewage waters (Fatta-Kassinos et al., 2011; Kümmerer, 2009). In the present study, the highest median concentration was observed in the H effluent (Table 2); this value is much higher than those found in similar studies conducted on hospital sewage waters (Gros et al., 2013; Rodriguez-Mozaz et al., 2015). STX concentrations were below the quantification limit (LOQ = 4 and 2 ngL^{-1} for influent and effluent, respectively) in most of the samples (83%) taken at BN (Table 2). In contrast, STX was detected in all the samples analyzed at BS, making it possible to calculate an average reduction of about 53% between the influent and the effluent. The average STX concentrations in both the influent and effluent of BS were three times higher than those reported in a study reviewing 31 papers investigating the fate of STX (among others) in WWTPs worldwide (Verlicchi et al., 2012).

Although NAL was detected in 100% of the samples analyzed, its concentration was below the LOQ of the method applied (LOQ = 0.4 and 0.2 ng L^{-1} for influent and effluent, respectively) in 67% of the cases (Table 2). These findings are in general agreement with those reported in other studies considering the differences in the analytical method used (Gros et al., 2013; Rodriguez-Mozaz et al., 2015).

In our study, TET was the most frequently detected antibiotic, with concentrations above the LOQ in all the samples analyzed. The TET concentrations measured in this study were 2 and 3.5 times higher at BN and BS, respectively, than those reported in a study reviewing the fate of TET in WWTPs worldwide (Verlicchi et al., 2012). Although in the present study the highest concentrations were observed at BS for both influent and effluent (Table 2), the average removal of TET was higher at BN (63%) compared to BS (45%). The TET concentration in the H sewage waters was in the same range as that of BS influent. Despite the significant reduction of TET concentration along WW treatment lines, the levels of this antibiotic significantly increased in surface waters after the release of BS and BN effluents into the Zenne River ($p < 0.001$, ANOVA one-way analysis of variance). In particular, the TET concentration was on average 57.2 ± 2.5 ng L^{-1} upstream from the WWTP effluent outfalls and rose to 127.2 ± 10.2 ng L^{-1} downstream. Increasing the TET concentration along a WWTP-impacted river has already been described (Proia et al., 2013), and the concentrations measured in the Zenne surface waters were within the same range as those reported in four human-impacted Spanish rivers (Osorio et al., 2016).

3.2. Antibiotic-resistant culturable *Escherichia coli* and heterotrophic bacteria

3.2.1. *Antibiotic-resistant Escherichia coli*

The abundance of culturable *E. coli* was reduced from 6.0 ± 4.4 × 10^7 CFU L^{-1} to 7.1 ± 7.0 × 10^5 CFU L^{-1} and from 8.7 ± 3.0 × 10^7 CFU L^{-1} to 7.1 ± 7.0 × 10^5 CFU L^{-1} in BS

and BN, respectively (Figure C1a; Table 3). The values measured in this study are in agreement with those reported in previous studies on the same (Ouattara et al., 2014) and other WWTPs (Lucas et al., 2014; Ouattara et al., 2011; Servais et al., 2007). The abundance of culturable *E. coli* in raw waters was quite similar for both WWTPs. Removal of *E. coli* close to two log units (average log reduction, 2.0 ± 0.6 and 2.3 ± 0.4 in BS and BN, respectively) was observed in both BS and BN WWTPs despite the different technologies applied (Figure C1a). Even though both WWTPs removed culturable *E. coli* efficiently, the concentration of culturable *E. coli* into the river increased on average from 4.1 ± 2.9 × 10^5 CFU L^{-1} at the upstream site to 7.5 ± 2.7 × 10^5 CFU L^{-1} at the downstream site (after the discharge point), although this increase was not significant ($p > 0.05$). A previous study highlighted that the major reason for this lack of significance was the high fecal pollution occurring upstream from the two Brussels WWTPs (Ouattara et al., 2014).

The behavior of culturable *E. coli* resistant to the different antibiotics and concentrations tested followed the same pattern as total culturable *E. coli.* Specifically, the absolute abundance of resistant *E. coli* was significantly reduced in both WWTPs, whereas the AR levels did not differ in untreated waters (Figure 1). Furthermore, the magnitude of AR-*E. coli* reduction varied slightly around 2 log values and did not differ between BS and BN (Figure C2a). These results are in agreement with those found in other studies (Lüddeke et al., 2015; Osińska et al., 2017) and in general demonstrate that conventional wastewater treatment reduces the absolute concentration of *E. coli* regardless of its antibiotic resistance profile. In fact, there was no significant difference between the log reduction of AR-*E. coli* and that of total *E. coli* in any of the WWTPs studied (Figure C2a). Despite their high removal efficiency, BS and BN effluents still release on average 1.6 ± 1.2 × 10^5 CFU L^{-1} and 1.3 ± 1.2 × 10^5 CFU L^{-1} of AR-*E. coli* to

445 the Zenne River, respectively. As a consequence, the abundance of AR-*E. coli* increased
446 significantly (one-way ANOVA; $p < 0.05$) from upstream to downstream of the WWTP
447 outfall into the Zenne River for all the antibiotics and concentrations considered in this
448 study. Most particularly, the relative increase from upstream to downstream sites ranged
449 between 54% (AMX L) and 98% (TET H) and was on average approximately 75%
450 (Figure 3).

451 The different effects of WWTP effluent release on total and AR *E. coli* levels in the
452 river could be explained by two main factors: i) the behavior of the relative abundance
453 of AR-*E. coli* through wastewater treatment and ii) the lower absolute and relative
454 abundance of AR-*E. coli* measured at upstream sites with respect to those measured in
455 the WWTP effluents (Table 3). In fact, the percentages of AR-*E. coli* were not
456 significantly reduced from the influent to the effluent and sometimes increased,
457 especially at BN (Table 3). Actually, several studies reported unvaried or increased
458 percentages of AR-*E. coli* in WWTP effluents compared to the influent sewage
459 (Koczura et al., 2012; Łuczkiewicz et al., 2010; Osińska et al., 2017). In addition, the
460 absolute abundance and the percentage of AR-*E. coli* in the effluents were much higher
461 than those measured at upstream sites in the Zenne River (Table 3). As a consequence,
462 the abundance of AR-*E. coli* in the Zenne increased on average about seven times from
463 upstream to downstream of the release of WWTP effluents to the river, also causing an
464 increase of the percentage of AR for all the antibiotics considered (Table 3). This result
465 confirms that despite the reduction of total AR-*E. coli* during the wastewater treatment
466 process, BN and BS effluents may still be the main source of resistant fecal bacteria to
467 the Zenne, causing the significant increase in surface waters observed.

468 #### 3.2.2. *Antibiotic-resistant heterotrophic bacteria*

469 The abundance of total culturable heterotrophic bacteria were significantly reduced
470 (on average) from $5.1 \pm 3.3 \times 10^{10}$ CFU L^{-1} to $2.5 \pm 3.0 \times 10^{9}$ CFU L^{-1} and from $3.5 \pm$
471 1.3×10^{10} CFU L^{-1} to $1.0 \pm 1.3 \times 10^{9}$ CFU L^{-1} in BS and BN, respectively (Figure C1b;
472 Table 3). The bacterial abundance in H raw waters did not differ from those of WWTP
473 influents (Figure C1b).

474 The behavior of ARB was the same for all the antibiotics and concentrations studied
475 and followed the same pattern as the total culturable heterotrophic bacteria,
476 demonstrating that conventional wastewater treatment reduces bacterial abundance
477 independently from antibiotic resistance. In fact, the abundance of ARB was
478 significantly reduced in both WWTPs, whereas the ARB levels were no different in raw
479 waters, except for NAL L, which was significantly higher in the influent of BN, and
480 TET H, which was significantly lower in BS influent (Figure 2). The ARB abundance in
481 H raw waters did not differ from those of WWTP influents (Figure 2), confirming that
482 although they are considered as hotspots for ARB dissemination, the actual evidence of
483 their role is not strong in the present study (Karkman et al., 2017). Furthermore, the
484 magnitude of ARB reduction varied slightly around 1.5–2 log values except for AMX H
485 and STX H, which showed an average reduction of about 4 log with relevant variation
486 among the sampling campaigns (Figure C2b). The comparison between ARB removal
487 efficiency in both WWTPs revealed nonsignificant differences except for AMX L and
488 TET H, which were more efficiently reduced at BN compared to BS ($p < 0.05$; Figure
489 C2b). The abundance of ARB measured in this study closely agree with those of other
490 studies investigating the behavior of ARB in WWTPs (Gao et al., 2012; Munir et al.,
491 2011) and confirmed that, in general, WWTPs efficiently remove ARB through the
492 different treatment steps. Nevertheless, despite the high removal efficiency, BS and BN
493 effluents still release on average $4.5 \pm 5.8 \times 10^{7}$ CFU L^{-1} and $2.3 \pm 2.5 \times 10^{7}$ CFU L^{-1} of

ARB to the Zenne River, respectively. As a consequence, the abundance of ARB increased significantly (one-way ANOVA; $p < 0.05$) from upstream to downstream from the WWTP outfall into the Zenne River for all the antibiotics and concentrations considered in this study (Table 3). Most particularly, the relative increase from upstream to downstream sites ranged between 44% (AMX H and NAL L) and 80% (TET H) and was on average around 63% (Figure 3), confirming that despite the high removal efficiency occurring through sewage water treatment steps, WWTPs are still one of the main important sources of ARB to the river (Rizzo et al., 2013).

3.3. Antibiotic Resistance Genes

Numerous studies have investigated the occurrence and prevalence of ARGs through wastewater treatment applying different technologies (Conte et al., 2017; Di Cesare et al., 2016; Du et al., 2015; Gao et al., 2012; Laht et al., 2014; Lee et al., 2017). Nevertheless, to our knowledge, the present study is the first one investigating the abundance of ARGs in PAB and FLB bacterial fractions in WWTPs and receiving river waters. We expected to observe differences in the removal of ARGs in the two bacterial fractions and particularly we assumed there would be higher removal efficiencies for PAB compared to FLB because of the settling stage applied as the first treatment stage in WWTP. However, our results did not show any difference in the absolute abundance of ARGs at both BS and BN WWTPs between the two fractions (Figure 4). Specifically, the log reduction of the total ARG abundance did not show any significant difference between WWTPs (Figure C3) and ranged between 1.5 (*tet*O) and 3.3 (*tet*W) for PAB and between 1.6 (*tet* O) and 2.4 (*sul* 2) for FLB, resulting in an average 2.4 and 2.3, respectively. Considering the sum of both fractions, the results reported herein are in agreement with those reported by other studies analyzing the removal of the same genes through wastewater treatment (Gao et al., 2012; Munir et al., 2011; Rodriguez-

519 Mozaz et al., 2015). The H sewage waters always showed the highest levels of ARGs in
520 both fractions but only for *sul*1 ($6.4 \pm 5.8 \times 10^8$ copies mL^{-1}) and bla_{TEM} ($3.2 \pm 4.4 \times$
521 10^8 copies mL^{-1}); in the FLB fraction the abundance levels were significantly higher
522 than those measured in WWTP influents (Figure 4). For all the genes, greater numbers
523 of copies per milliliter ($p < 0.05$) were detected in hospital effluent and WWTP influent
524 samples than those found in the effluents (Figure 4). The levels of ARGs in raw and
525 treated waters measured in this study are similar to those reported worldwide (Gao et
526 al., 2012; Laht et al., 2014; Rodriguez-Mozaz et al., 2015). Despite the significant
527 reduction of ARG abundance from raw to treated waters, BS and BN effluents still
528 released a high amount of resistance genes to the Zenne River. In fact, the ARG
529 abundance in the effluents (PAB + FLB) ranged on average between $1.3 \pm 2.3 \times 10^4$
530 copies mL^{-1} (bla_{TEM}) and $8.3 \pm 4.3 \times 10^5$ copies mL^{-1} (*sul*1). As a consequence, the
531 abundance of ARGs increased significantly ($p < 0.05$) from upstream to downstream
532 from the WWTP outfall into the Zenne River for all the genes in both bacterial fractions
533 (Figure 5). The significant increase of ARGs into the Zenne River after the release of
534 WWTP effluents was on the same order of magnitude for both fractions except for the
535 bla_{TEM} gene, which showed a significantly higher increase in FLB compared to PAB
536 (Figure 5). Greater abundance of ARGs downstream of the discharge of WWTP
537 effluents to the river have been reported in many studies assessing how AR spreads in
538 different freshwater bacterial communities: planktonic (Czekalski et al., 2015; Pruden et
539 al., 2012; Rodriguez-Mozaz et al., 2015; Storteboom et al., 2010), epilithic (Aubertheau
540 et al., 2017; Proia et al., 2016; Subirats et al., 2017), and epipsammic (Czekalski et al.,
541 2014; Graham et al., 2011; Marti et al., 2013). All these studies suggested that WWTPs
542 are the main source of ARGs and may be responsible for the observed increase. In our
543 study, the abundance of ARGs in the BS and BN effluents ranged between 10^4 and 10^6

544 copies mL^{-1} concentrations, which were at least one order of magnitude greater than
545 those detected in upstream river waters (10^3–10^4 copies mL^{-1}) for all the genes analyzed.
546 This result confirms that WWTPs are a relevant source of ARGs to the Zenne River,
547 provoking a significant increase of AR along the river course.

548 Although WWTPs efficiently reduce the absolute number of copies of ARGs,
549 important differences have been described when relative gene abundance (normalized to
550 16S rRNA gene copies) is considered (Karkman et al., 2017). This type of analysis
551 makes it possible to quantify the relative changes in the abundance of ARGs, whether
552 more or fewer ARGs appear per microbial genome (Laht et al., 2014). In the present
553 study, the variation in the relative abundance of ARGs through wastewater treatment
554 differed depending on the WWTP (BS or BN), fraction (PAB or FLB), and gene
555 considered (Figure 6). The only gene whose relative abundance was significantly
556 reduced in the effluent was *tet*W and only at BN (Figure 6). In most of the other cases,
557 no significant differences between influent and effluent were observed, whereas several
558 cases showed a significant increase of relative ARG abundance from the influent to the
559 effluent at either BN and BS. In particular, the relative abundance of sulfonamide
560 resistance genes (*sul*1 and *sul*2) in the PAB fraction was significantly higher in the
561 effluent compared to the influent of both WWTPs investigated (Figure 6). Moreover,
562 the relative abundance of the *tet*O gene in the BS effluent was significantly higher than
563 in the influent for both the PAB and FLB fractions (Figure 6). Finally, the relative
564 abundance of the *tet*W gene in the FLB fraction was significantly higher in the BS
565 effluent than in the influent (Figure 6). Several studies reported similar findings (Laht et
566 al., 2014; Mao et al., 2015; Neudorf et al., 2017; Rodriguez-Mozaz et al., 2015). Mao et
567 al. (2015) showed a positive correlation between antibiotic concentrations
568 (sulfonamides and tetracyclines) and ARGs (*sul* and *tet)* through different wastewater

569 treatment steps. Other studies suggested that antibiotics that were poorly removed
570 during primary treatment processes could be placing a selective pressure on bacteria
571 within wastewater systems (Neudorf et al., 2017). In this study, STX was barely
572 detected and TET concentrations were extremely low (Table 1). Consequently, even if
573 the presence of other antibiotics of the same families may not have been discarded,
574 some other factor should have played a role in the enrichment of some genes measured
575 in the effluents. One possible explanation could be the co-action of both selection
576 and/or horizontal transfer of resistance determinants favored by the conditions generated
577 in WWTP environments, mostly in activated sludge (Novo et al., 2013; Rizzo et al.,
578 2013). Another hypothesis would be the bacterial community composition, which has
579 been demonstrated to be different in PAB and FLB (Jackson et al., 2014; Mohit et al.,
580 2014; Tang et al., 2009) and could have relevant impacts on the resistome (Lekunberri
581 et al., 2018). Nevertheless bacterial communities' structures were not analyzed in this
582 study and it is therefore impossible to confirm these hypotheses.

583 **4. Conclusions and perspectives**

584 To conclude, this study has demonstrated that even if WWTPs efficiently remove
585 absolute abundance of ARB and ARGs, they could still be hotspots for AR resistance
586 spread to nonresistant bacteria, and they significantly increase the AR levels in
587 receiving freshwater ecosystems (Figure C4). Our results are thus confirming what was
588 observed worldwide about the major role of wastewaters release for the dissemination
589 of AR in freshwater ecosystems. In order to preserve the water quality of the receiving
590 systems, thus limiting the risk for human health, the application of new technologies in
591 wastewater treatment and the implementation of watershed management strategies
592 aiming to control the AR spread is a big challenge that needs to be faced in next years.
593 Numerous studies investigated new technologies to reduce the levels of ARBs and

ARGs in WWTPs in last years (Sharma et al., 2016). Among others ozonation, chlorination and UV disinfection, used independently or in combination, are of the most investigated and showed different efficiencies depending on the target considered in different studies (Czekalski et al., 2016; Guo et al., 2015; Sousa et al., 2017; Zhang et al., 2015; Zheng et al., 2017). Promising results have been obtained by using coagulation technology to achieve ARGs reduction in wastewater treatment plants effluents (Li et al., 2017). Moreover, graphene-based TiO_2 composite photocatalysts under solar radiation also showed good efficiency removing antibiotics, ARBs and ARGs from sewage waters (Karaolia et al., 2018). In addition, by applying TiO_2 photocatalysis under UV irradiation in combination with H_2O_2 good removal efficiencies of ARBs and ARGs (both intracellular and extracellular forms) from aqueous solution have been recently reported (Guo et al., 2017). However, most of these studies have been performed in laboratory conditions focusing on reduced number of target AR determinants. Therefore, further research is needed to improve the WWTPs removal efficiency of ARBs and ARGs reducing the costs of advanced technologies and strategies for the mitigation of AR spread in river ecosystems should be implemented at watershed scale possibly including the limitation of the antibiotics use in agriculture and livestock farming.

Acknowledgments

During the present study, Lorenzo Proia worked under a FNRS (Fonds National de la Recherche Scientifique, Belgium) postdoctoral grant. The authors thank David Pireaux (Société Bruxelloise de Gestion des Eaux) for providing the physicochemical data measured in the inlet and outlet waters of both Brussels WWTPs and the workers of both WWTPs for the samples collection. The authors thank Natacha Motteu, Aurore Abbe and Amandine Lafitte for their participation in the sampling campaigns and lab work.

5. References

Andreozzi, R., Caprio, V., Ciniglia, C., De Champdor??, M., Lo Giudice, R., Marotta, R., Zuccato, E., 2004. Antibiotics in the environment: Occurrence in Italian STPs, fate, and preliminary assessment on algal toxicity of amoxicillin. Environ. Sci. Technol. 38, 6832–6838. doi:10.1021/es049509a

Aubertheau, E., Stalder, T., Mondamert, L., Ploy, M.-C., Dagot, C., Labanowski, J., 2017. Impact of wastewater treatment plant discharge on the contamination of river biofilms by pharmaceuticals and antibiotic resistance. Sci. Total Environ. 579, 1387–1398. doi:http://dx.doi.org/10.1016/j.scitotenv.2016.11.136

Baquero, F., Martínez, J.-L., Cantón, R., 2008. Antibiotics and antibiotic resistance in water environments. Curr. Opin. Biotechnol. 19, 260–265. doi:10.1016/j.copbio.2008.05.006

Ben, W., Wang, J., Cao, R., Yang, M., Zhang, Y., Qiang, Z., 2017. Distribution of antibiotic resistance in the effluents of ten municipal wastewater treatment plants in China and the effect of treatment processes. Chemosphere 172, 392–398. doi:10.1016/j.chemosphere.2017.01.041

Bouki, C., Venieri, D., Diamadopoulos, E., 2013. Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: a review. Ecotoxicol. Environ. Saf. 91, 1–9. doi:10.1016/j.ecoenv.2013.01.016

Brion, N., Verbanck, M. a., Bauwens, W., Elskens, M., Chen, M., Servais, P., 2015. Assessing the impacts of wastewater treatment implementation on the water quality of a small urban river over the past 40 years. Environ. Sci. Pollut. Res. 22, 12720–12736. doi:10.1007/s11356-015-4493-8

Callens, B., Sarrazin, S., Cargnel, M., Welby, S., Dewulf, J., Hoet, B., Vermeersch, K., Wattiau, P., 2017. Associations between a decreased veterinary antimicrobial use and resistance in commensal Escherichia coli from Belgian livestock species (2011–2015). Prev. Vet. Med. doi:https://doi.org/10.1016/j.prevetmed.2017.10.013

Caucci, S., Karkman, A., Cacace, D., Rybicki, M., Timpel, P., Voolaid, V., Gurke, R., Virta, M., Berendonk, T.U., 2016. Seasonality of antibiotic prescriptions for outpatients and resistance genes in sewers and wastewater treatment plant outflow. FEMS Microbiol. Ecol. 92, 1–10. doi:10.1093/femsec/fiw060

Conte, D., Palmeiro, J.K., da Silva Nogueira, K., de Lima, T.M.R., Cardoso, M.A., Pontarolo, R., Degaut Pontes, F.L., Dalla-Costa, L.M., 2017. Characterization of CTX-M enzymes, quinolone resistance determinants, and antimicrobial residues from hospital sewage, wastewater treatment plant, and river water. Ecotoxicol. Environ. Saf. 136, 62–69. doi:10.1016/j.ecoenv.2016.10.031

Czekalski, N., Gascón Díez, E., Bürgmann, H., 2014. Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. ISME J. 8, 1381–90. doi:10.1038/ismej.2014.8

Czekalski, N., Imminger, S., Salhi, E., Veljkovic, M., Kleffel, K., Drissner, D., Hammes, F., Bürgmann, H., von Gunten, U., 2016. Inactivation of Antibiotic Resistant Bacteria and Resistance Genes by Ozone: From Laboratory Experiments to Full-Scale Wastewater Treatment. Environ. Sci. Technol. 50, 11862–11871. doi:10.1021/acs.est.6b02640

667 Czekalski, N., Sigdel, R., Birtel, J., Matthews, B., Bürgmann, H., 2015. Does human
668 activity impact the natural antibiotic resistance background? Abundance of
669 antibiotic resistance genes in 21 Swiss lakes. Environ. Int. 81, 45–55.
670 doi:10.1016/j.envint.2015.04.005

671 Di Cesare, A., Fontaneto, D., Doppelbauer, J., Corno, G., 2016. Fitness and Recovery of
672 Bacterial Communities and Antibiotic Resistance Genes in Urban Wastewaters
673 Exposed to Classical Disinfection Treatments. Environ. Sci. Technol. 50, 10153–
674 10161. doi:10.1021/acs.est.6b02268

675 Du, J., Geng, J., Ren, H., Ding, L., Xu, K., Zhang, Y., 2015. Variation of antibiotic
676 resistance genes in municipal wastewater treatment plant with A2O-MBR system.
677 Environ. Sci. Pollut. Res. 22, 3715–3726. doi:10.1007/s11356-014-3552-x

678 Fatta-Kassinos, D., Meric, S., Nikolaou, A., 2011. Pharmaceutical residues in
679 environmental waters and wastewater: Current state of knowledge and future
680 research. Anal. Bioanal. Chem. 399, 251–275. doi:10.1007/s00216-010-4300-9

681 Gao, P., Munir, M., Xagoraraki, I., 2012. Correlation of tetracycline and sulfonamide
682 antibiotics with corresponding resistance genes and resistant bacteria in a
683 conventional municipal wastewater treatment plant. Sci. Total Environ. 421–422,
684 173–183. doi:10.1016/j.scitotenv.2012.01.061

685 Garcia-Armisen, T., Anzil, A., Cornelis, P., Chevreuil, M., Servais, P., 2013.
686 Identification of antimicrobial resistant bacteria in rivers: Insights into the
687 cultivation bias. Water Res. 47, 4938–4947. doi:10.1016/j.watres.2013.05.036

688 García-Armisen, T., İnceoğlu, Ö., Ouattara, N.K., Anzil, A., Verbanck, M. a., Brion, N.,
689 Servais, P., 2014. Seasonal Variations and Resilience of Bacterial Communities in
690 a Sewage Polluted Urban River. PLoS One 9, e92579.
691 doi:10.1371/journal.pone.0092579

692 Graham, D.W., Olivares-Rieumont, S., Knapp, C.W., Lima, L., Werner, D., Bowen, E.,
693 2011. Antibiotic resistance gene abundances associated with waste discharges to
694 the Almendares river near Havana, Cuba. Environ. Sci. Technol. 45, 418–424.
695 doi:10.1021/es102473z

696 Gros, M., Rodríguez-mozaz, S., Barceló, D., 2013. Rapid analysis of multiclass
697 antibiotic residues and some of their metabolites in hospital , urban wastewater and
698 river water by ultra-high-performance liquid chromatography coupled to
699 quadrupole-linear ion trap tandem mass spectrometry. J. Chromatogr. A 1292,
700 173–188. doi:10.1016/j.chroma.2012.12.072

701 Gullberg, E., Cao, S., Berg, O.G., Ilbäck, C., Sandegren, L., Hughes, D., Andersson,
702 D.I., 2011. Selection of Resistant Bacteria at Very Low Antibiotic Concentrations.
703 PLoS Pathog. 7, e1002158. doi:10.1371/journal.ppat.1002158

704 Guo, C., Wang, K., Hou, S., Wan, L., Lv, J., Zhang, Y., Qu, X., Chen, S., Xu, J., 2017.
705 H2O2and/or TiO2photocatalysis under UV irradiation for the removal of antibiotic
706 resistant bacteria and their antibiotic resistance genes. J. Hazard. Mater. 323, 710–
707 718. doi:10.1016/j.jhazmat.2016.10.041

708 Guo, M.T., Yuan, Q. Bin, Yang, J., 2015. Distinguishing effects of ultraviolet exposure
709 and chlorination on the horizontal transfer of antibiotic resistance genes in
710 municipal wastewater. Environ. Sci. Technol. 49, 5771–5778.
711 doi:10.1021/acs.est.5b00644

712 Jackson, C.R., Millar, J.J., Payne, J.T., Ochs, C.A., 2014. Free-living and particle-

associated bacterioplankton in large rivers of the Mississippi River basin demonstrate biogeographic patterns. Appl. Environ. Microbiol. 80, 7186–7195. doi:10.1128/AEM.01844-14

Jelic, A., Rodriguez-Mozaz, S., Barceló, D., Gutierrez, O., 2015. Impact of in-sewer transformation on 43 pharmaceuticals in a pressurized sewer under anaerobic conditions. Water Res. 68, 98–108. doi:10.1016/j.watres.2014.09.033

Karaolia, P., Michael-Kordatou, I., Hapeshi, E., Drosou, C., Bertakis, Y., Christofilos, D., Armatas, G.S., Sygellou, L., Schwartz, T., Xekoukoulotakis, N.P., Fatta-Kassinos, D., 2018. Removal of antibiotics, antibiotic-resistant bacteria and their associated genes by graphene-based TiO2composite photocatalysts under solar radiation in urban wastewaters. Appl. Catal. B Environ. 224, 810–824. doi:10.1016/j.apcatb.2017.11.020

Karkman, A., Do, T.T., Walsh, F., Virta, M.P.J., 2017. Antibiotic-Resistance Genes in Waste Water. Trends Microbiol. doi:https://doi.org/10.1016/j.tim.2017.09.005

Koczura, R., Mokracka, J., Jabłońska, L., Gozdecka, E., Kubek, M., Kaznowski, A., 2012. Antimicrobial resistance of integron-harboring Escherichia coli isolates from clinical samples, wastewater treatment plant and river water. Sci. Total Environ. 414, 680–685. doi:10.1016/j.scitotenv.2011.10.036

Kümmerer, K., 2009. Antibiotics in the aquatic environment – A review – Part I. Chemosphere 75, 417–434. doi:10.1016/j.chemosphere.2008.11.086

Laht, M., Karkman, A., Voolaid, V., Ritz, C., Tenson, T., Virta, M., Kisand, V., 2014. Abundances of Tetracycline , Sulphonamide and Beta- Lactam Antibiotic Resistance Genes in Conventional Wastewater Treatment Plants (WWTPs) with Different Waste Load 9, 1–8. doi:10.1371/journal.pone.0103705

Lee, J., Jeon, J.H., Shin, J., Jang, H.M., Kim, S., Song, M.S., Kim, Y.M., 2017. Quantitative and qualitative changes in antibiotic resistance genes after passing through treatment processes in municipal wastewater treatment plants. Sci. Total Environ. 605–606, 906–914. doi:10.1016/j.scitotenv.2017.06.250

Lekunberri, I., Balcázar, J.L., Borrego, C.M., 2018. Metagenomic exploration reveals a marked change in the river resistome and mobilome after treated wastewater discharges. Environ. Pollut. 234, 538–542. doi:https://doi.org/10.1016/j.envpol.2017.12.001

Levy, S.B., Marshall, B., 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nat.Med. 10, S122–S129. doi:10.1038/nm1145

Li, N., Sheng, G.P., Lu, Y.Z., Zeng, R.J., Yu, H.Q., 2017. Removal of antibiotic resistance genes from wastewater treatment plant effluent by coagulation. Water Res. 111, 204–212. doi:10.1016/j.watres.2017.01.010

Llorca, M., Gros, M., Rodríguez-mozaz, S., Barceló, D., 2014. Sample preservation for the analysis of antibiotics in water. J. Chromatogr. A 1369, 43–51. doi:10.1016/j.chroma.2014.09.089

Lucas, F.S., Therial, C., Gonçalves, A., Servais, P., Rocher, V., Mouchel, J.M., 2014. Variation of raw wastewater microbiological quality in dry and wet weather conditions. Environ. Sci. Pollut. Res. 21, 5318–5328. doi:10.1007/s11356-013-2361-y

Łuczkiewicz, A., Jankowska, K., Fudala-Ksiazek, S., Olańczuk-Neyman, K., 2010. Antimicrobial resistance of fecal indicators in municipal wastewater treatment

759 plant. Water Res. 44, 5089–5097. doi:10.1016/j.watres.2010.08.007
760 Łuczkiewicz, A., Jankowska, K., Fudala-Książek, S., Olańczuk-Neyman, K., 2010.
761 Antimicrobial resistance of fecal indicators in municipal wastewater treatment
762 plant. Water Res. 44, 5089–5097. doi:https://doi.org/10.1016/j.watres.2010.08.007
763 Lüddeke, F., Heß, S., Gallert, C., Winter, J., Güde, H., Löffler, H., 2015. Removal of
764 total and antibiotic resistant bacteria in advanced wastewater treatment by
765 ozonation in combination with different filtering techniques. Water Res. 69, 243–
766 251. doi:http://dx.doi.org/10.1016/j.watres.2014.11.018
767 Mao, D., Yu, S., Rysz, M., Luo, Y., Yang, F., Li, F., Hou, J., Mu, Q., Alvarez, P.J.J.,
768 2015. Prevalence and proliferation of antibiotic resistance genes in two municipal
769 wastewater treatment plants. Water Res. 85, 458–466.
770 doi:10.1016/j.watres.2015.09.010
771 Marti, E., Jofre, J., Balcazar, J.L., 2013. Prevalence of Antibiotic Resistance Genes and
772 Bacterial Community Composition in a River Influenced by a Wastewater
773 Treatment Plant. PLoS One 8, e78906. doi:10.1371/journal.pone.0078906
774 Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., Dagot,
775 C., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for the
776 release of antibiotics in the environment: A review. Water Res. 47, 957–995.
777 doi:10.1016/j.watres.2012.11.027
778 Mohit, V., Archambault, P., Toupoint, N., Lovejoy, C., 2014. Phylogenetic Differences
779 in Attached and Free-Living Bacterial Communities in a Temperate Coastal
780 Lagoon during Summer, Revealed via High-Throughput 16S rRNA Gene
781 Sequencing. Appl. Environ. Microbiol. 80, 2071–2083. doi:10.1128/AEM.02916-
782 13
783 Munir, M., Wong, K., Xagoraraki, I., 2011. Release of antibiotic resistant bacteria and
784 genes in the effluent and biosolids of five wastewater utilities in Michigan. Water
785 Res. 45, 681–693. doi:10.1016/j.watres.2010.08.033
786 Neudorf, K.D., Huang, Y.N., Ragush, C.M., Yost, C.K., Jamieson, R.C., Truelstrup
787 Hansen, L., 2017. Antibiotic resistance genes in municipal wastewater treatment
788 systems and receiving waters in Arctic Canada. Sci. Total Environ. 598, 1085–
789 1094. doi:10.1016/j.scitotenv.2017.04.151
790 Novo, A., André, S., Viana, P., Nunes, O.C., Manaia, C.M., 2013. Antibiotic resistance,
791 antimicrobial residues and bacterial community composition in urban wastewater.
792 Water Res. 47, 1875–1887. doi:10.1016/j.watres.2013.01.010
793 O'Neill, J., 2016. Tackling drug-resistant infections globally: final report and
794 recommendations. Rev. Antimicrob. Resist. 84. doi:10.1016/j.jpha.2015.11.005
795 Osińska, A., Korzeniewska, E., Harnisz, M., Niestępski, S., 2017. The prevalence and
796 characterization of antibiotic-resistant and virulent Escherichia coli strains in the
797 municipal wastewater system and their environmental fate. Sci. Total Environ.
798 577, 367–375. doi:10.1016/j.scitotenv.2016.10.203
799 Osorio, V., Larrañaga, A., Aceña, J., Pérez, S., Barceló, D., 2016. Concentration and
800 risk of pharmaceuticals in freshwater systems are related to the population density
801 and the livestock units in Iberian Rivers. Sci. Total Environ. 540, 267–277.
802 doi:10.1016/j.scitotenv.2015.06.143
803 Ouattara, N.K., Garcia-Armisen, T., Anzil, A., Brion, N., Servais, P., 2014. Impact of
804 Wastewater Release on the Faecal Contamination of a Small Urban River: The

Zenne River in Brussels (Belgium). Water, Air, Soil Pollut. 225, 2043. doi:10.1007/s11270-014-2043-5

Ouattara, N.K., Passerat, J., Servais, P., 2011. Faecal contamination of water and sediment in the rivers of the Scheldt drainage network. Environ. Monit. Assess. 183, 243–257. doi:10.1007/s10661-011-1918-9

Plósz, B.G., Leknes, H., Thomas, K. V, 2010. Impacts of Competitive Inhibition, Parent Compound Formation and Partitioning Behavior on the Removal of Antibiotics in Municipal Wastewater Treatment. Environ. Sci. Technol. 44, 734–742. doi:10.1021/es902264w

Proia, L., Lupini, G., Osorio, V., Pérez, S., Barceló, D., Schwartz, T., Amalfitano, S., Fazi, S., Romaní, a. M.M., Sabater, S., 2013. Response of biofilm bacterial communities to antibiotic pollutants in a Mediterranean river. Chemosphere 92, 1126–1135. doi:10.1016/j.chemosphere.2013.01.063

Proia, L., Von Schiller, D., Sànchez-Melsió, A., Sabater, S., Borrego, C.M., Rodríguez-Mozaz, S., Balcázar, J.L., 2016. Occurrence and persistence of antibiotic resistance genes in river biofilms after wastewater inputs in small rivers. Environ. Pollut. 210, 121–128. doi:10.1016/j.envpol.2015.11.035

Pruden, A., Arabi, M., Storteboom, H.N., 2012. Correlation between upstream human activities and riverine antibiotic resistance genes. Environ. Sci. Technol. 46, 11541–11549. doi:10.1021/es302657r

Rafraf, I.D., Lekunberri, I., Sànchez-Melsió, A., Aouni, M., Borrego, C.M., Balcázar, J.L., 2016. Abundance of antibiotic resistance genes in five municipal wastewater treatment plants in the Monastir Governorate, Tunisia. Environ. Pollut. 219, 353–358. doi:https://doi.org/10.1016/j.envpol.2016.10.062

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. 447, 345–360. doi:10.1016/j.scitotenv.2013.01.032

Robinson, T.P., Bu, D.P., Carrique-Mas, J., Fèvre, E.M., Gilbert, M., Grace, D., Hay, S.I., Jiwakanon, J., Kakkar, M., Kariuki, S., Laxminarayan, R., Lubroth, J., Magnusson, U., Thi Ngoc, P., Van Boeckel, T.P., Woolhouse, M.E.J., 2016. Antibiotic resistance is the quintessential One Health issue. Trans. R. Soc. Trop. Med. Hyg. 377–380. doi:10.1093/trstmh/trw048

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. 69, 234–242. doi:10.1016/j.watres.2014.11.021

Servais, P., Garcia-Armisen, T., George, I., Billen, G., 2007. Fecal bacteria in the rivers of the Seine drainage network (France): Sources, fate and modelling. Sci. Total Environ. 375, 152–167. doi:10.1016/j.scitotenv.2006.12.010

Servais, P., Passerat, J., 2009. Antimicrobial resistance of fecal bacteria in waters of the Seine river watershed (France). Sci. Total Environ. 408, 365–372. doi:10.1016/j.scitotenv.2009.09.042

Sharma, V.K., Johnson, N., Cizmas, L., McDonald, T.J., Kim, H., 2016. A review of the influence of treatment strategies on antibiotic resistant bacteria and antibiotic resistance genes. Chemosphere 150, 702–714.

851 doi:10.1016/j.chemosphere.2015.12.084

852 Sousa, J.M., Macedo, G., Pedrosa, M., Becerra-Castro, C., Castro-Silva, S., Pereira,
853 M.F.R., Silva, A.M.T., Nunes, O.C., Manaia, C.M., 2017. Ozonation and
854 UV254nmradiation for the removal of microorganisms and antibiotic resistance
855 genes from urban wastewater. J. Hazard. Mater. 323, 434–441.
856 doi:10.1016/j.jhazmat.2016.03.096

857 Storteboom, H., Arabi, M., Davis, J.G., Crimi, B., Pruden, A., 2010. Tracking antibiotic
858 resistance genes in the south platte river basin using molecular signatures of urban,
859 agricultural, and pristine sources. Environ. Sci. Technol. 44, 7397–7404.
860 doi:10.1021/es101657s

861 Subirats, J., Triadó-Margarit, X., Mandaric, L., Acuña, V., Balcázar, J.L., Sabater, S.,
862 Borrego, C.M., 2017. Wastewater pollution differently affects the antibiotic
863 resistance gene pool and biofilm bacterial communities across streambed
864 compartments. Mol. Ecol. doi:10.1111/mec.14288

865 Tang, X., Gao, G., Qin, B., Zhu, L., Chao, J., Wang, J., Yang, G., 2009.
866 Characterization of Bacterial Communities Associated with Organic Aggregates in
867 a Large, Shallow, Eutrophic Freshwater Lake (Lake Taihu, China). Microb. Ecol.
868 58, 307–322. doi:10.1007/s00248-008-9482-8

869 Verlicchi, P., Al Aukidy, M., Zambello, E., 2012. Occurrence of pharmaceutical
870 compounds in urban wastewater: Removal, mass load and environmental risk after
871 a secondary treatment-A review. Sci. Total Environ. 429, 123–155.
872 doi:10.1016/j.scitotenv.2012.04.028

873 Yuan, Q., Guo, M., Yang, J., 2015. Fate of Antibiotic Resistant Bacteria and Genes
874 during Wastewater Chlorination : Implication for Antibiotic Resistance Control 1–
875 11. doi:10.1371/journal.pone.0119403

876 Zanotto, C., Bissa, M., Illiano, E., Mezzanotte, V., Marazzi, F., Turolla, A., Antonelli,
877 M., De Giuli Morghen, C., Radaelli, A., 2016. Identification of antibiotic-resistant
878 Escherichia coli isolated from a municipal wastewater treatment plant.
879 Chemosphere 164, 627–633. doi:10.1016/j.chemosphere.2016.08.040

880 Zhang, S., Han, B., Gu, J., Wang, C., Wang, P., Ma, Y., Cao, J., He, Z., 2015. Fate of
881 antibiotic resistant cultivable heterotrophic bacteria and antibiotic resistance genes
882 in wastewater treatment processes. Chemosphere 135, 138–145.
883 doi:10.1016/j.chemosphere.2015.04.001

884 Zheng, J., Su, C., Zhou, J., Xu, L., Qian, Y., Chen, H., 2017. Effects and mechanisms of
885 ultraviolet, chlorination, and ozone disinfection on antibiotic resistance genes in
886 secondary effluents of municipal wastewater treatment plants. Chem. Eng. J. 317,
887 309–316. doi:10.1016/j.cej.2017.02.076

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Figure legends

Figure 1.
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 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 10^{th} and 90^{th} 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 2.
Box plots in log units of the abundance of culturable heterotrophic bacteria 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 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 10^{th} and 90^{th} 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 3.
Relative increase of culturable AR-*E. coli* (black bars) and heterotrophic bacteria (grey bars) from the river site upstream of the site downstream of the release of the two WWTP effluents to the Zenne.

Figure 4.
Total abundance (n of copies mL^{-1}) of the six ARGs analyzed at the different sampling sites during the four sampling campaigns (log scale). Black bars show the results for particle-attached bacteria (PAB) and grey bars free-living bacteria (FLB). Asterisks are shown 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.
Relative increase of ARGs in particle-attached (PAB, black bars) and free-living (FLB, grey bars) bacteria from the river site upstream of the site downstream of the release of the two WWTP effluents to the Zenne.

Figure 6.
Relative abundance (normalized × 16s rRNA copies) of the six ARGs analyzed in particle-attached (PAB, top) and free-living (FLB, bottom) bacteria at the influent (In) and effluent (Out) of Brussels south (BS, left) and Brussels north (BN, right) WWTPs.

Table 1

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		BS			BN		
		Influent	**Effluent**	**Log Removal**	**Influent**	**Effluent**	**Log Removal**
COD (mg l^{-1})	**Median**	526	36	1.12	491	35	1.11
	25% Perc	408	31		386	41	
	75% Perc	593	41		566	31	
BOD_5 (mg l^{-1})	**Median**	218	3.0	1.75	207	3,0	1.78
	25% Perc	167	3.0		155	3,0	
	75% Perc	254	3.2		244	3,3	
SPM (mg l^{-1})	**Median**	250	6.6	1.40	237	8	1.48
	25% Perc	203	8.1		195	6	
	75% Perc	289	10.7		284	9	
N tot (mg l^{-1})	**Median**	39	19	0.28	84	9	0.94
	25% Perc	29	15		65	9	
	75% Perc	46	23		96	10	
P tot (mg l^{-1})	**Median**	4.9	0.7	0.70	5.6	0.8	0.80
	25% Perc	3.6	0.5		4.3	0.6	
	75% Perc	5.9	1.0		6.4	1.0	

Table 1. Annual average of variables measured daily at each wastewater treatment plant (WWTP) during 2015 and 2016. Median, 25% percentile and 75% percentile are reported. COD = Carbon oxygen demand; BOD_5 = Biological Oxygen Demand; SPM = Suspended Particulate Matter; N tot = Total Nitrogen; P tot = Total Phosphorus. BS = Brussels south WWTP; BN = Brussels north WWTP.

Table 2

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	AMX (µg L^{-1})			STX (µg L^{-1})			NAL (µg L^{-1})			TET (µg L^{-1})		
	median	**min**	**max**	**median**	**min**	**max**	**median**	**min**	**max**	**median**	**min**	**max**
BS inf	33.8	nd	33.8	1.9	1.5	5.1	0.005	<loq	0.007	0.97	0.32	1.96
BS eff	17.5	nd	17.5	0.6	0.5	1.8	0.002	<loq	0.003	0.24	0.22	1.29
BN inf	nd	nd	nd	<loq	<loq	<loq	<loq	nd	<loq	0.72	0.66	0.79
BN eff	46.0	29.2	116.4	0.9	<loq	0.9	0.001	<loq	0.001	0.27	0.25	0.28
H	nd	nd	nd	18.4	8.5	66.4	<loq	<loq	<loq	0.96	0.70	1.18

Table 2. Maximum. minimum and median antibiotic concentrations measured at each sampling site during the sampling campaigns. Values are expressed in µg L^{-1}. AMX = amoxicillin; STX = sulfamethoxazole; NAL = nalidixic acid and TET = tetracycline. BS = Brussels south wastewater treatment plant; BN = Brussels north wastewater treatment plant; H = Hospital. Inf = influent; Eff = effluents; nd = not detected; loq = limit of quantification.

Table 3

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	Ctr		AMX L				AMX H				STX L				STX H				NAC L				NAC H				TET L				TET H			
	E.coli	HB	*E.coli*		HB		*E.coli*		HB		*E.coli*		HB		*E.coli*		HB		*E.coli*		HB		*E.coli*		HB		*E.coli*		HB		*E.coli*		HB	
	CFU L^{-1}	CFU L^{-1}	*CFU L^{-1}*	*%*	CFU L^{-1}	%	CFU L^{-1}	%	CFU L^{-1}	%	CFU L^{-1}	%	CFU L^{-1}	%	CFU L^{-1}	%	CFU L^{-1}	%	CFU L^{-1}	%	CFU L^{-1}	%	CFU L^{-1}	%	CFU L^{-1}	%	CFU L^{-1}	%	CFU L^{-1}	%	CFU L^{-1}	%	CFU L^{-1}	%
H	*4.4 x10^{7}*	3.4 x10^{10}	*2.3 x10^{7}*	*60*	2.4 x10^{9}	17	*2.1 x10^{7}*	*52*	8.4 x10^{8}	7	3.6 *x10^{7}*	*84*	3.7 x10^{9}	20	*1.3 x10^{7}*	*34*	2.6 x10^{9}	14	*2.8 x10^{7}*	*60*	7.0 x10^{9}	32	*1.5 x10^{7}*	*39*	2.4 x10^{9}	20	*1.1 x10^{7}*	*22*	4.8 x10^{8}	1	*6.3 x10^{3}*	*0.02*	1.3 x10^{7}	0.05
BS in	*6.0 x10^{7}*	5.1 x10^{10}	*2.1 x10^{7}*	*34*	1.7 x10^{9}	5	*1.5 x10^{7}*	*32*	3.2 x10^{8}	2	4.6 *x10^{7}*	*82*	2.7 x10^{9}	9	*1.4 x10^{7}*	*33*	1.3 x10^{9}	3	*3.3 x10^{7}*	*43*	4.9 x10^{9}	22	*1.3 x10^{7}*	*20*	3.0 x10^{9}	15	*1.3 x10^{7}*	*22*	4.9 x10^{8}	2	*0*	*0.00*	6.3 x10^{5}	0.01
BS out	*7.1 x10^{5}*	2.5 x10^{9}	*1.8 x10^{5}*	*26*	2.6 x10^{7}	9	*1.4 x10^{5}*	*18*	5.9 x10^{6}	2	4.3 *x10^{5}*	*72*	4.2 x10^{7}	10	*1.4 x10^{5}*	*27*	1.8 x10^{7}	1	*1.7 x10^{5}*	*26*	1.6 x10^{8}	24	*7.8 x10^{4}*	*13*	1.0 x10^{8}	6	*1.4 x10^{5}*	*21*	4.1 x10^{6}	1	*8.2 x10^{2}*	*0.06*	1.4 x10^{5}	0.09
BN in	*8.7 x10^{7}*	2.4 x10^{10}	*2.6 x10^{7}*	*30*	1.8 x10^{9}	23	*1.6 x10^{7}*	*19*	6.5 x10^{8}	9	5.9 *x10^{7}*	*70*	6.0 x10^{9}	37	*2.4 x10^{7}*	*24*	1.3 x10^{9}	10	*2.4 x10^{7}*	*26*	6.5 x10^{9}	21	*1.3 x10^{7}*	*14*	4.3 x10^{9}	14	*1.6 x10^{7}*	*18*	6.4 x10^{8}	2	*3.4 x10^{4}*	*0.04*	2.7 x10^{6}	0.01
BN out	*5.6 x10^{5}*	1.0 x10^{9}	*1.6 x10^{5}*	*31*	1.7 x10^{7}	8	*1.1 x10^{5}*	*25*	2.7 x10^{6}	3	3.9 *x10^{5}*	*81*	3.7 x10^{7}	19	*9.7 x10^{4}*	*19*	6.3 x10^{6}	5	*1.7 x10^{5}*	*42*	7.0 x10^{7}	21	*5.7 x10^{4}*	*10*	4.5 x10^{7}	11	*7.2 x10^{4}*	*16*	3.7 x10^{6}	2	*4.9 x10^{2}*	*0.09*	7.5 x10^{4}	0.04
Up	*4.1 x10^{5}*	1.4 x10^{8}	*5.9 x10^{4}*	*18*	8.1 x10^{6}	8	*5.0 x10^{4}*	*16*	1.3 x10^{8}	1	1.8 *x10^{5}*	*50*	1.5 x10^{7}	11	*4.3 x10^{4}*	*13*	5.8 x10^{6}	4	*3.6 x10^{4}*	*13*	4.0 x10^{7}	30	*3.0 x10^{4}*	*12*	1.4 x10^{7}	10	*2.9 x10^{4}*	*8*	2.2 x10^{6}	2	*1.1 x10^{2}*	*0.07*	6.5 x10^{4}	0.05
Dw	*7.5 x10^{5}*	3.7 x10^{8}	*4.0 x10^{5}*	*28*	4.7 x10^{7}	23	*3.5 x10^{5}*	*20*	1.3 x10^{7}	5	1.2 *x10^{6}*	*79*	6.2 x10^{7}	24	*2.9 x10^{5}*	*20*	2.9 x10^{6}	17	*2.8 x10^{5}*	*19*	8.0 x10^{7}	51	*1.7 x10^{5}*	*10*	6.4 x10^{7}	30	*2.8 x10^{5}*	*16*	1.1 x10^{7}	5	*3.0 x10^{3}*	*0.21*	3.5 x10^{5}	0.26

Table 3. Absolute (CFU L^{-1}) and relative (%) abundances of culturable *E. coli* and Heterotrophic Bacteria (HB) at the different sampling sites. H = Hospital; BS in = Influent of Brussels South WWTP; BS out = Effluent of Brussels South WWTP; BN in = Influent of Brussels North WWTP; BN out = Effluent of Brussels North WWTP; Up = Zenne River site located upstream the release of WWTPs effluents; Dw = Zenne River site located downstream the release of WWTPs effluents

Figure 1

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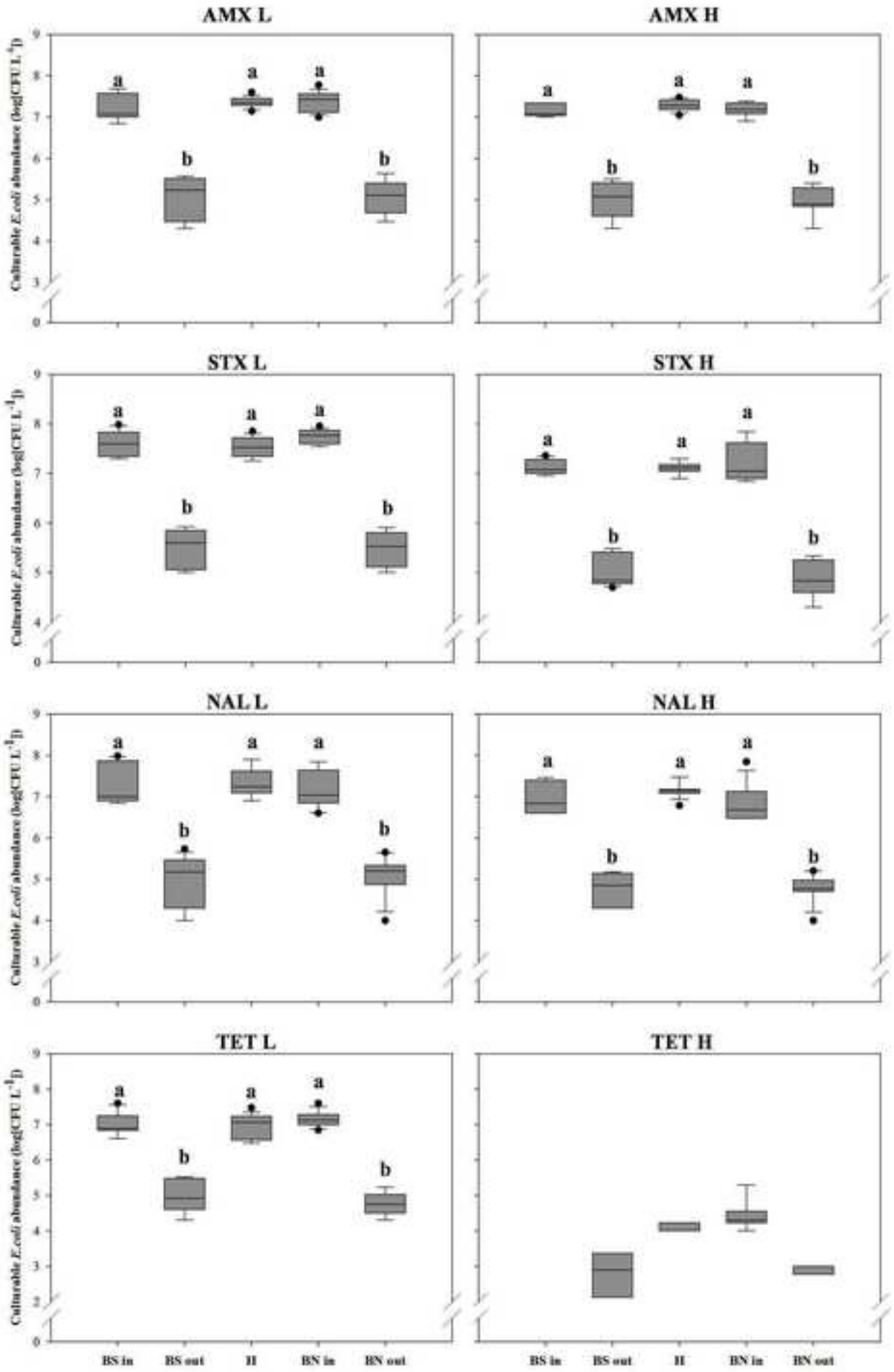

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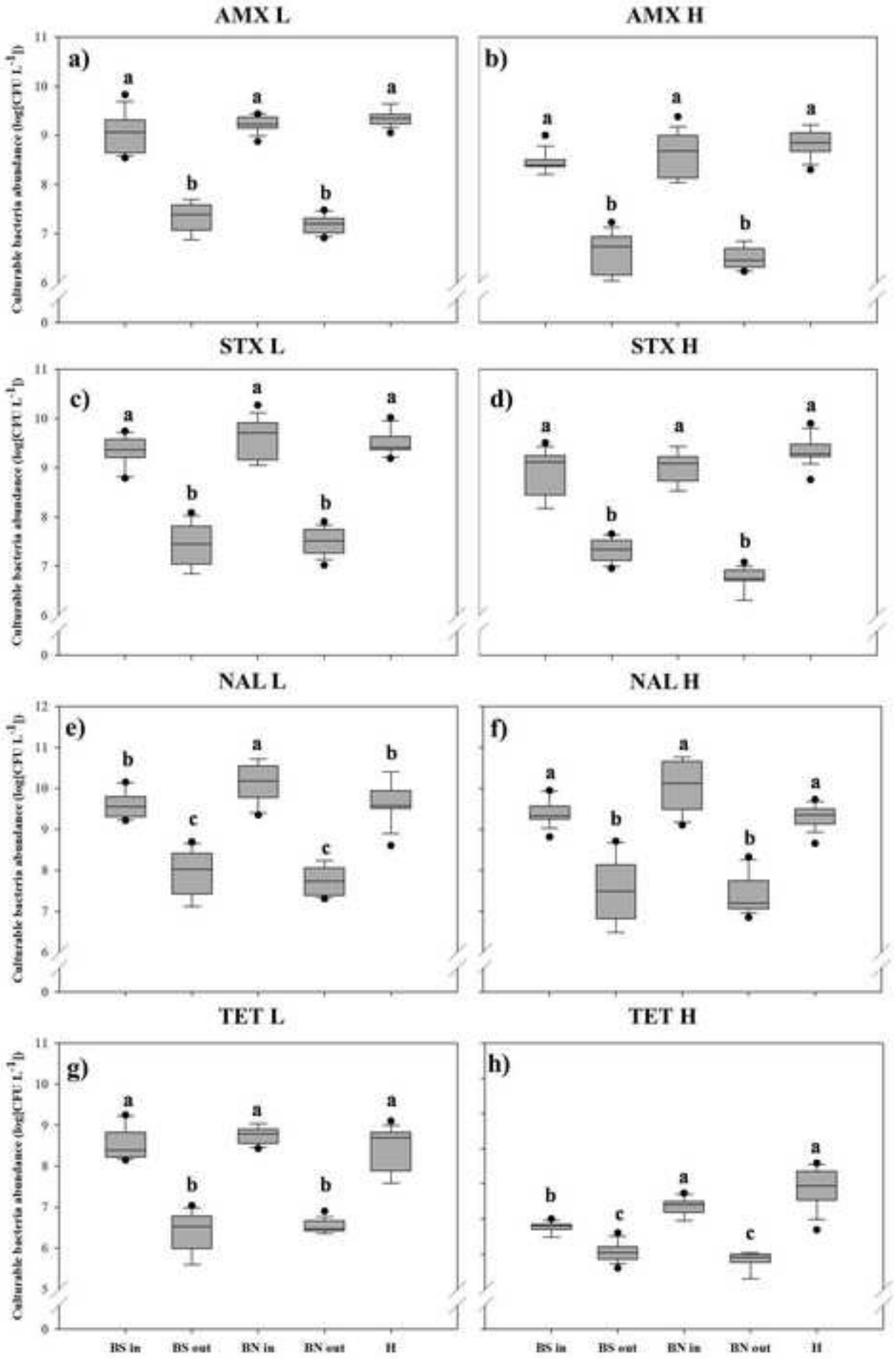

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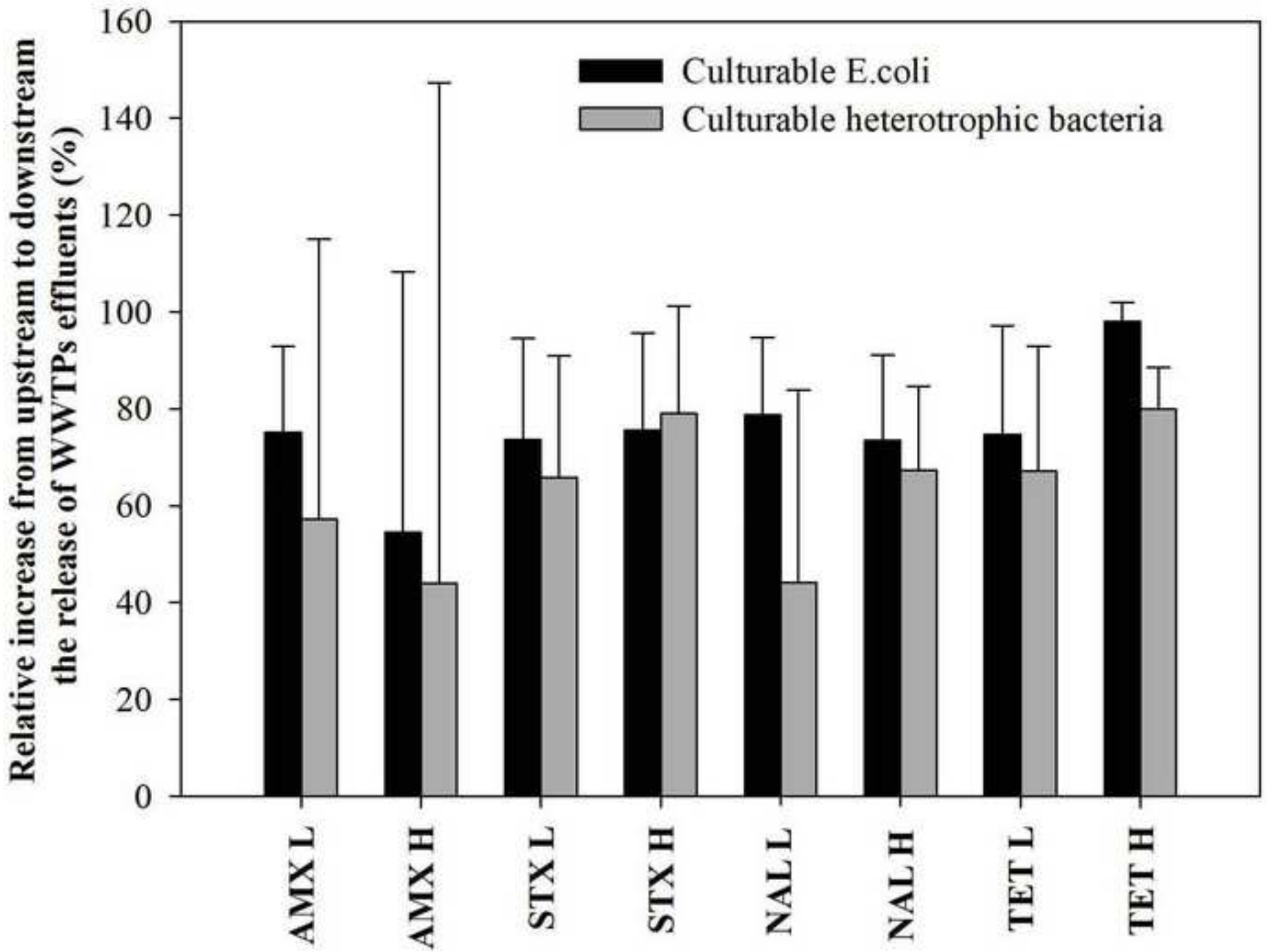

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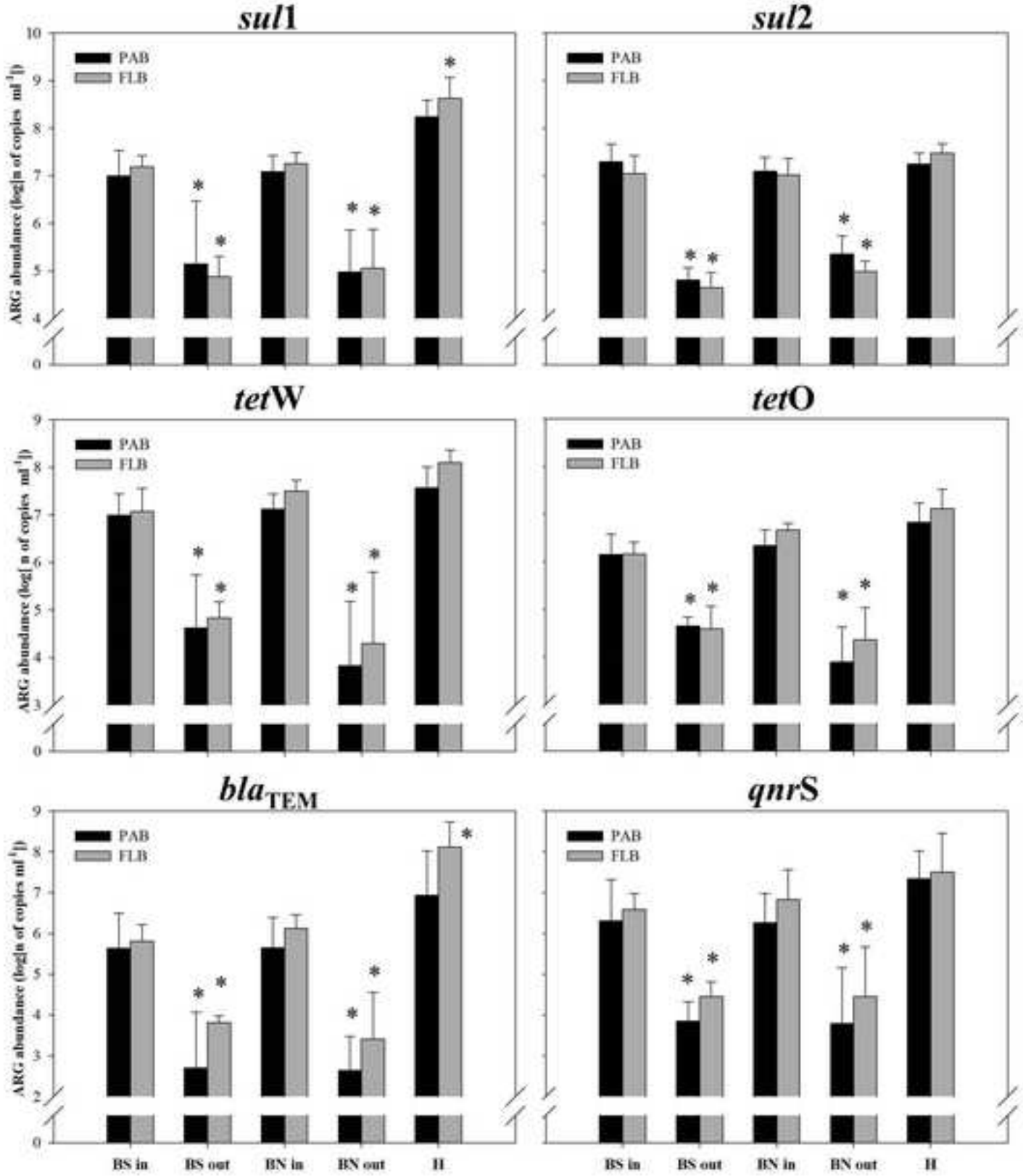

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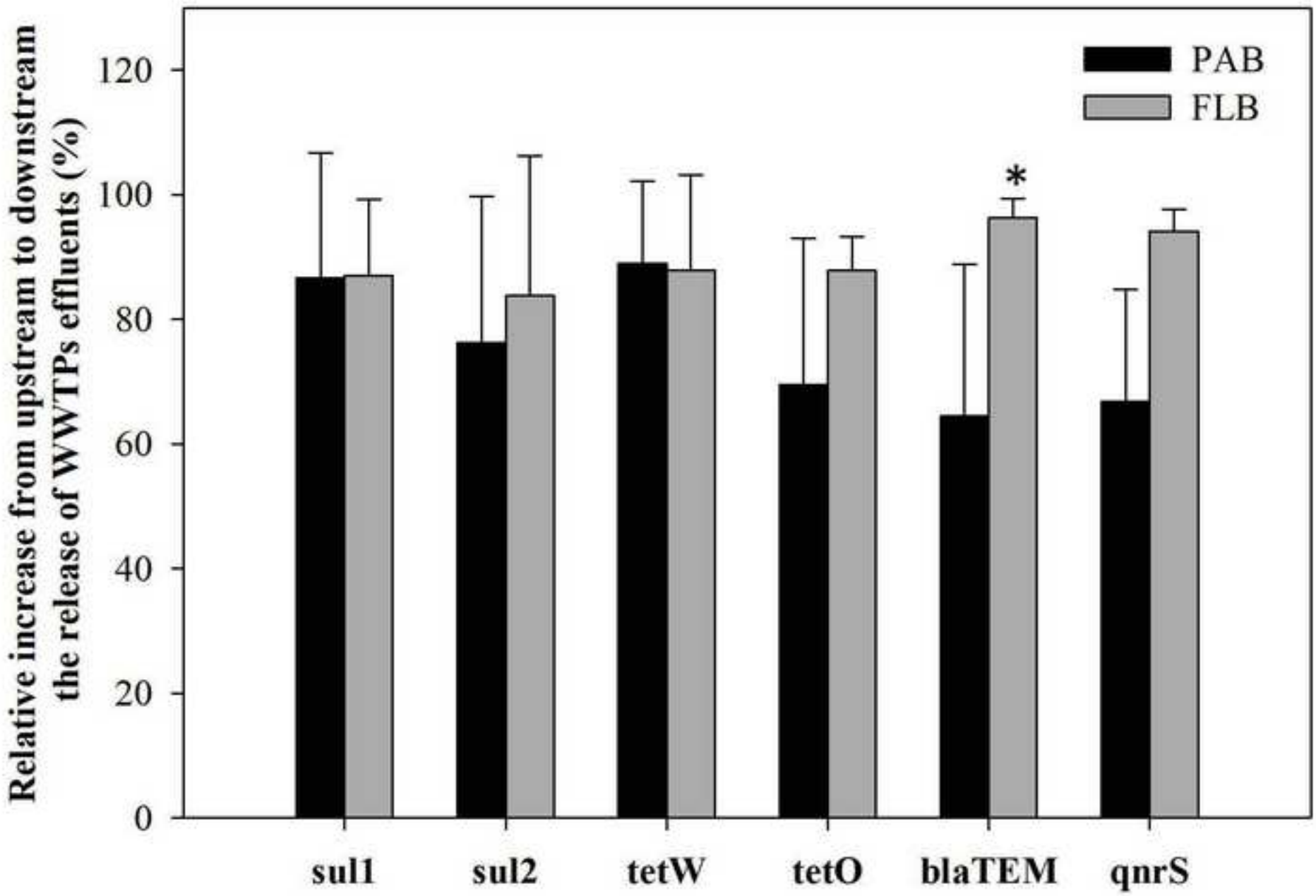

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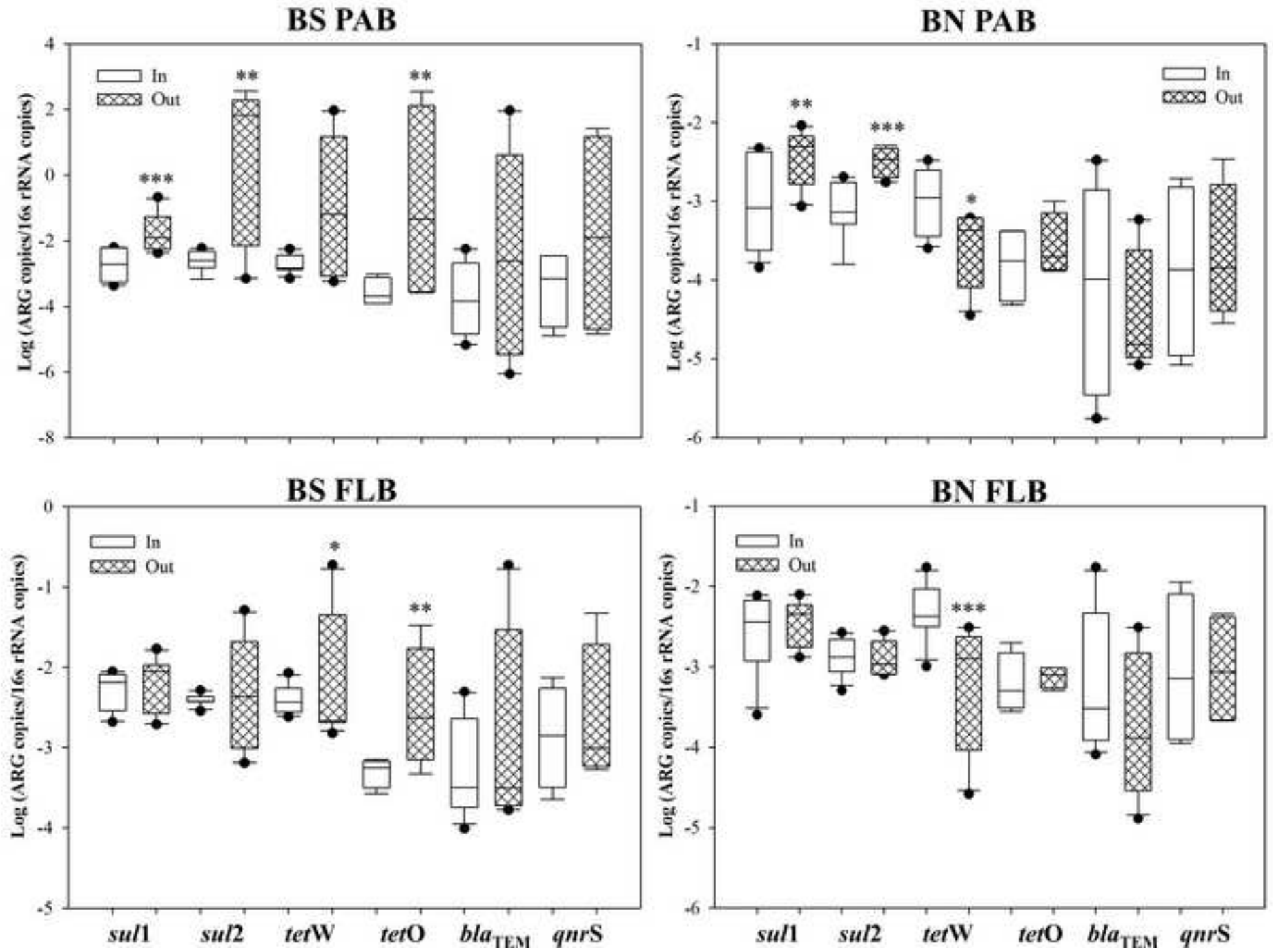

Supplementary Material

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