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Corresponding Author: Dr. Lorenzo Proia, Ph.D.

Corresponding Author's Institution: Ecology of Aquatic Ecosystems - Université Libre de Bruxelles

First Author: Lorenzo Proia, Ph.D.

Order of Authors: Lorenzo Proia, Ph.D.; Adriana Anzil; Carles Borrego; Marinella Farrè; Marta Llorca; Josep Sanchis; Pierre Bogaerts; Jose Luis Balcázar; Pierre Servais

Abstract: This study aims to investigate the prevalence of clinically relevant carbapenemases genes (blaKPC, blaNDM and blaOXA-48) in water samples collected over one-year period from hospital (H), raw and treated wastewater of two wastewater treatment plants (WWTPs) as well as along the Zenne River (Belgium). The genes were quantified in both particle-attached (PAB) and free-living (FLB) bacteria. Our results showed that absolute abundances were the highest in H waters. Although absolute abundances were significantly reduced in WWTP effluents, the relative abundance (normalized per 16S rRNA) was never lowered through wastewater treatment. Particularly, for the PAB the relative abundances were significantly higher in the effluents respect to the influents of both WWTPs for all the genes. The absolute abundances along the Zenne River increased from upstream to downstream, peaking after the release of WWTPs effluents, in both fractions. Our results demonstrated that blaKPC, blaNDM and blaOXA-48 are widely distributed in the Zenne as a consequence of chronic discharge from WWTPs. To conclude, the levels of carbapenemases genes are significantly lower than other genes conferring resistance to more widely used antibiotics (analyzed in previous studies carried out at the same sites), but could raise up to the levels of high prevalent resistance genes.

Occurrence and persistence of carbapenemases genes in hospital and wastewater treatment plants and propagation in the receiving river

Lorenzo Proia [1], Adriana Anzil [1], Carles Borrego [2,3], Marinella Farrè [4], Marta Llorca [4], Josep Sanchis [4], Pierre Bogaerts [5], Jose Luis Balcázar [2], Pierre Servais [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), H2O Building, Scientific and Technological Park of the University of Girona, Emili Grahit 101, 17003 Girona, Spain

3Group of Molecular Microbial Ecology, Institute of Aquatic Ecology, University of Girona, Campus de Montilivi, 17003, Girona, Spain

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

[5] Laboratory of Clinical Microbiology, Belgian National Reference Center for Monitoring Antimicrobial Resistance in Gram-negative Bacteria, CHU UCL Avenue Docteur G. Thérasse, 1, 5530, Yvoir, Belgium.

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

This study aims to investigate the prevalence of clinically relevant carbapenemases genes (*bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$) in water samples collected over ~~one~~ one-year period from hospital (H), raw and treated wastewater ~~(WW)~~ of two wastewater treatment plants (WWTPs) as well as ~~at seven sites~~ along the Zenne River (Belgium). The genes were quantified in both particle-attached (PAB) and free-living (FLB) bacteria. Our results showed that ~~the~~ absolute abundances were the highest in H waters. Although ~~the~~ absolute abundances were significantly reduced in WWTP effluents, the relative abundance (normalized per 16S rRNA) was never lowered through ~~WW~~ wastewater treatment. Particularly, for the PAB ~~fraction~~ the relative abundances were significantly higher in the effluents respect to the influents of both WWTPs for all the genes. The absolute abundances along the Zenne River increased from upstream to downstream, peaking after the release of WWTPs effluents, in both fractions. Our results demonstrated that *bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ are widely distributed in the Zenne as a consequence of ~~the~~ chronic discharge from WWTPs. To conclude, the levels of carbapenemases genes are significantly lower than other genes conferring resistance to more widely used antibiotics (analyzed in previous studies carried out at the same sites), but could raise up to the levels of high prevalent resistance genes.

Keywords: Carbapenemases genes, Urban River; ARGs; Wastewaters; Hospital

Highlights

- Absolute carbapenemases genes (CGs) abundances are the highest in hospital waters
- CGs absolute abundances are significantly reduced in WWTP effluents
- WWTPs effluents show higher relative CGs abundances than influents in PAB fraction
- CGs abundances in the Zenne River peak downstream the release of WWTP effluents
- CGs occurrence in river and WW is significantly lower than other prevalent ARGs

1. Introduction

Antibiotics have saved millions of human lives since their discovery and application for treating bacterial infections. However, its extensive use has led to the emergence, and spread of antibiotic resistance among bacterial pathogens thus posing a risk to human health [1].

Carbapenems are β-lactam antibiotics with a broad spectrum of activity that are usually considered drugs of last resort because they can be effective against severe hospital- and community-acquired infections caused by multidrug-resistant Gram-negative pathogens [2,3]. However, an increasing number of reports indicate that some β-lactamases can efficiently hydrolyze carbapenems [3]. These enzymes, called carbapenemases, hydrolyze carbapenems and belong to molecular class A (KPC and some GES variants), B (IMP, NDM and IMP metallo-β-lactamases) or D (OXA-48-like) of beta-lactamases according to the classification of Ambler [4]. Several studies have demonstrated that carbapenemases-producing pathogens cause serious infections in immunocompromised patients associated with high mortality rates due to limited treatment options [5,6]. This alarming situation is even worsened by the lack of new antibiotics at/or near clinical approval that are active against multidrug-resistant pathogens [3].

Carbapenemases of most clinical relevance are those whose respective genes are carried on plasmids thus favoring their maintenance and spread among Gram-negative bacterial species [7]. Consequently, the genes encoding for those carbapenemases could easily spread conferring resistance to environmental bacteria, thus increasing the possibility of dissemination of resistance genes to pathogens [8]. In particular KPC, NDM, and OXA-48-type enzymes have been widely described as the clinically most important carbapenemases and the persistence of the respective genes in environmental

bacteria is of general concern due to its implications to public health [1,2]. In fact there is growing evidence that antibiotic resistance genes (ARGs) in clinical isolates are closely related to those found in their environmental counterparts [9].

Wastewaters treatment plants (WWTPs) are considered hotspots for the acquisition and spread of antibiotic resistance in aquatic systems and three major reasons are often put forward to sustain this idea: i) the chronic discharge of antibiotic residues, antibiotic-resistant bacteria (ARB), and ARGs collected in the municipal and clinical sewer systems; ii) the favorable conditions for both selection and/or spread of ARGs among bacterial cells in WWTPs; and iii) the widespread observation that WWTP effluents contain high concentrations of different ARGs conferring resistance to widely used and last-resort antibiotics [10–12] that are consequently detected in aquatic ecosystems [13]. As a consequence, WWTP effluents are among the most important conduits for the spread of ARGs in aquatic environments. Receiving systems, and particularly urban rivers, may play an important role in driving the persistence and spread of ARGs within bacterial communities. In fact, rivers (particularly urban ones) provide a setting in which the horizontal exchange of mobile genetic elements encoding antibiotic resistance between bacteria can take place [14,15].

Carbapenem resistance has become a worldwide concern, and studies on the detection of carbapenemase-producing isolates in clinical settings are increasingly being reported [16]. Recently, several studies also started to investigate carbapenemase-producing bacteria and ARGs conferring resistance to carbapenems in non-clinical environments such as WWTPs [1,16,17], coastal recreational water [18], rivers [8,19,20] and lakes [21]. However none of these studies investigated at the same time the prevalence of the three most relevant genes conferring resistance to carbapenems

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(i.e. *bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$) in a complex, urban-impacted river. Our study aims to investigate the prevalence and spread of carbapenemases genes (CGs) from its source in hospital wastewater to the river water after passing through Brussels WWTPs [22] distinguishing particle-attached bacteria (PAB) from free-living bacteria (FLB). The different behaviors of the ARGs depending on the lifestyle of bacteria could have implications for the spread of resistance in aquatic ecosystems. In fact, PAB are 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, resistant bacteria colonizing particles 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 CGs in PAB and FLB could also provide useful information for wastewater treatment management in order to reduce the input of resistance determinants in aquatic ecosystems. To our knowledge this is the first study reporting the occurrence and abundance of ARGs conferring resistance to carbapenems in Belgian aquatic systems.

2. Material and Methods

2.1 Study site and sampling strategy

In this study, the two WWTPs located in the Brussels Capital Region (Belgium) and the Zenne River, in which they discharge treated sewage waters, were investigated as well as the sewage waters of the UZ Brussels Hospital. The Brussels South (BS) WWTP (360,000 equivalent-inhabitants) is in operation since the year 2000 whereas the Brussels North (BN) WWTP (1.1 million equivalent-inhabitants) operates since 2007 and receive, among others, the untreated waters from the UZ Brussels Hospital. 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). At the BN WWTP there is a biological line including 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 second treatment line only uses a primary settling process a and runs in parallel to the biological line when the volume of the influent is too high during storms. On an annual basis, the volume treated in the biological line accounts for roughly 90% of the total volume reaching the WWTP.

In the BS WWTP, average daily samples were collected with refrigerated automatic samplers, whereas at the BN WWTP grab samples were collected in the morning. Since sampling campaigns were conducted under dry weather conditions, all data from the BN WWTP presented in this paper concern the biological line. In addition, a grab sample was collected at the outlet of the UZ Brussels Hospital (H), which, according to the 2012 annual report, has 721 beds, 29,239 hospitalizations per year, and 23,692 day hospitalizations per year (excluding minimum flat rates). The effluents of both WWTPs are released in the Zenne River, a small urban river running through the city of Brussels [22,23].

Seven stations were sampled along the Zenne River. A kilometric scale along the river was defined; the zero is arbitrarily set at station Z1 and increased from upstream to downstream sites. 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 BS WWTP effluent discharge point, respectively. Stations Z8 (33 km) and Z9

(34 km) are located upstream and downstream from the BN WWTP, respectively, and Station Z11 (41 km) is downstream from Brussels area (Figure 1).

Four sampling campaigns were conducted in 2016, one per season (January, April, June, and November). During the sampling campaigns, triplicate samples were collected at all the sites described above, stored in sterile 1000 mL bottles and kept at 4°C until analysis.

2.2. Chemical analysis of carbapenems

The treatment of water samples was carried out as described elsewhere [23]. The final extracts were analyzed by means of liquid chromatography coupled to mass spectrometer triple quadrupole in tandem. The separation of 20 µL of extract was achieved with a Luna® (2 × 150 mm, 5 µm particle size, 100 Å pore size; Phenomenex) column with a mobile phase of water and acetonitrile flowing at 0.25 mL min^{-1}. Each gradient lasted 15 min. Detection was carried out using a spectrometer with a triple quadrupole analyser TSQ Quantiva (Thermo Fischer Scientific) equipped with a heated electrospray ionisation (H-ESI) source, operating in positive polarity with the following parameters: principal, auxiliary and sweep gas flows: 35 a.u., 9 a.u. and 1 a.u., respectively; voltage: 3,300 V; capillary and vaporizer temperatures: 325 ºC and 275 ºC. The selected transitions for quantification (in bold) and confirmation were **300 > 142** and 300 > 98, for imipenem, and **384 > 114** and 384 > 100, for meropenem. The entire system was controlled by Xcalibur software v.2.2. The recoveries of the entire process ranged from 77.9 % to 86.4 % for meropenem and from 70.3 % to 73.0 % for imipenem, with good limits of detection in each case (see Table 1).

2.3 Extraction of DNA

The bacterial biomass was collected from water samples and concentrated by filtration. An aliquot (from 0.25 L to 1.5 L) of each replicate was filtered to collect two

different bacterial fractions. Particle-attached 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 to retain free-living bacteria (FLB). Filters were kept at −80°C until extraction. Extractions were performed following the same protocol described in [24]. DNA concentration and purity were determined using a NanoDrop ND-2000 UV Vis spectrophotometer (ThermoFisher, Scientific Inc., Wilmington, Delaware, USA).

2.3. Quantification of CGs by qPCR

Copy numbers of CGs (*bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$) were quantified using quantitative PCR (qPCR). Standard curves were generated using known quantities of cloned target genes as described by [2].

All qPCR assays were performed in triplicate using SYBR green detection chemistry with a Step One Plus (Applied Biosystems, ThermoFisher Scientific). Briefly, each reaction contained 8–9 µL of Power Up SYBR Green master mix (Applied Biosystems, ThermoFisher Scientific), 200 nM each forward and reverse primer, and 45ng of DNA template, and the final volume was adjusted to 20 µL by adding DNase-free water.

Each gene was amplified using specific primer sets (Sigma Aldrich) and the optimal cycling protocol consisted of an initial cycle at95 °C for 3 min, followed by 40 cycles at 95 °C for 30 s and 60 °C for 45 s (*bla*$_{KPC}$ and *bla*$_{OXA-48}$-like genes) or 60 s (*bla*$_{NDM}$ genes) and finally two elongation steps lasting 40 s at 72°C and 32 s at 78°C. The number of copies of the 16S rRNA gene was also quantified, and the amplification conditions included an initial denaturation at 95°C for 3 min, followed by 35 cycles at 95°C for 15 s, then an annealing temperature at 60°C for 1 min 40 s at 72°C and 32 s at

78°C. In all cases, a dissociation curve was constructed in the range of 60 to 95 °C to verify the specificity of the amplified products.

Tenfold serial dilutions of the standards for each target gene were run in parallel with DNA samples and blank controls (qPCR premix without DNA template). The efficiency and sensitivity of each qPCR assay were determined by the amplification of standard serial dilutions, as previously described [25]. Amplification efficiency (E) was calculated from the resulting standard curves using the formula $E = 10^{(1/slope)} - 1$, and the analytical sensitivity of the qPCRs was determined as the smallest DNA quantity detected for each assay.

2.4. Statistical analyses

Differences in absolute and relative abundances of CGs were analyzed independently using a one-way repeated measure analysis of variance (ANOVA) to test for the differences among sites during the entire year of sampling. River sites and wastewater samples were analyzed separately. 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. For all the analysis statistical significance was set at $\alpha = 0.05$.

3. Results and Discussion

3.1. Concentration of carbapenems in water

The concentration of two of the most used carbapenems in Belgium was measured in all samples collected (Table 1). The frequency of detection showed differences depending on the compound considered: Imipenem was detected in 90% and 53% of the

river and sewage samples whereas meropenem was detected in 71% and 27% of the river and sewage water samples, respectively.

In general, the concentrations of carbapenems in the river were in the range of ng L^{-1}. Particularly, meropenem increased from upstream to downstream sites (Table 1), peaking at Z11 with an average value of 266.7 ± 55.1 ng L^{-1}. A similar pattern was observed for imipenem which increased from upstream to downstream (Table 1), peaking at Z9 with an average value of 41.5 ± 2.8 ng L^{-1}. Surprisingly, carbapenems were never detected in the hospital sewage (Table 1). This could be explained by the method detection limit that was one order of magnitude higher for raw waters than in surface waters. Another possible co-explanation is about the pharmacokinetics of these compounds in human body. In fact both meropenem and ertapenem are only partially excreted as unchanged drug whereas different metabolites (not analyzed in this study) are normally released with human urines [26,27]. Despite the frequencies of detection were higher in river waters, the maximum concentrations were detected in WWTPs waters, as expected (Table 1). Meropenem was only detected in the effluent of BS whereas a reduction of about the 17% from the influent to the effluent was observed at BN. Finally, imipenem at BS was reduced on average by 30% from the influent to the effluent whereas at BN the concentration increased from 89 to 99 ng L^{-1} respectively (Table 1). To the authors' knowledge, this is the first study reporting chemical concentrations of these compounds in raw and surface waters

3.2 Occurrence of CGs in WWTPs and Hospital sewage

Several studies have investigated the occurrence and prevalence of the most relevant CGs in hospital sewage and through WWTPs applying different treatment technologies [1,2,16,28]. Nevertheless, to our knowledge, this is the first study investigating the abundance of CGs in PAB and FLB fractions in hospital, WWTPs and along the

receiving river. We expected to observe differences in the reduction of CGs levels in the two bacterial fractions and particularly we assumed that removal efficiencies during treatment would be higher for PAB compared to FLB because of the first settling stage applied in WWTP. However, considering absolute abundances of CGs, our results did not show any significant difference neither between the removal rates in PAB and FLB fractions nor between the two WWTPs (two-way ANOVA, $p>0.05$). Specifically, the log reduction from the influent to the effluent of the absolute CGs abundance ranged between 1.9 (bla_{KPC}) and 2.6 (bla_{NDM}) for PAB and between 2.2 (bla_{KPC}) and 2.4 (bla_{OXA-48}) for FLB, resulting on average 2.3 for both fractions. The log reductions reported herein are in agreement with those found by Yang and colleagues [14] for the bla_{KPC} gene and also in the same range of removal rates reported for others genes worldwide [29–31]. In particular, the log reductions of CGs measured in this study were in the same range than those reported for bacterial abundances (both resistant and not) and other ARGs in a study carried out at the same sites [32]. Moreover, the same study also reported similar log reductions for other parameters specifically targeted by wastewater treatment such as total suspended solids, chemical and biological oxygen demand [32].

The absolute abundances were significantly the highest in the hospital sewage for CGs analyzed in both bacterial fractions except for the bla_{NDM} in PAB than was the highest in BS influent (Figure 2). Considering that those genes are mainly carried by bacterial pathogens [33–35] that have been already reported in Belgian hospitals [36], this result was expected and confirms that sewage is the main source of carbapenemase genes to the aquatic environment. For all the genes, significantly lower numbers of copies per milliliter ($p < 0.05$) were detected in the effluents than in hospital effluent and WWTP influent samples (Figure 2). This is generally explained by the significant

reduction of the ~~number of fecal bacteria~~bacterial biomass normally observed during wastewater treatment, and already observed in the same WWTPs [32], that occurs independently from their resistance profile~~–~~. Consequently, we can conclude that the reduction of absolute CGs levels is an indirect effect of the normal functioning of both WWTPs which target specifically other parameters such as total suspended solids chemical and biological oxygen demand among others. ~~(Proia et al. *submitted)*~~.

Although WWTPs efficiently reduce the absolute copy number of CGs, important differences have been described when relative gene abundance (normalized to 16S rRNA copies) was considered [12,37,38]. This type of analysis makes it possible to quantify the relative changes in the abundance of ARGs, whether more or fewer genes appear in bacterial community [12,38,39]. In the present study, the variation in the relative abundance of CGs through wastewater treatment differed depending on the bacterial fraction, WWTP and gene considered (Figure 3). In general, in our study we never observed a significant reduction of the relative abundance for any of the genes analyzed. More particularly, in FLB fraction the relative abundance of the CGs showed different fates depending on the gene and WWTP considered. For example, the concentration of the bla_{KPC} gene in BS effluent was significantly higher than in the influent, whereas for bla_{NDM} and bla_{OXA-48} this increase from the influent to the effluent was only observed at BN WWTP (Figure 3). In contrast, for the PAB fraction the relative abundances of all the genes analyzed were significantly higher in the effluents respect to the influents of both WWTPs (Figure 3). Our results are in general agreement with those found in a Spanish WWTP in which a slight increase of the relative abundance of bla_{KPC} and bla_{NDM} genes was observed [2,31]. Several studies report similar findings for other ARGs and found positive correlation between antibiotic concentrations and ARGs through different wastewater treatment stages [31,39–41].

Other studies suggested that antibiotics, poorly removed during primary treatment processes, could be responsible for a selective pressure on bacteria within wastewater treatment systems [41]. In our study, however, carbapenems were barely detected in sewage (Table 1). Consequently, factors other than the selection pressure exerted by carbapenem residues may apply although co-selection of CGs by other antibiotics or metals could not be formally ruled out. One possible explanation could be the horizontal transfer of resistance genes favored by the conditions met in biological treatment stages used in WWTP~~in the WWTP digesters~~, especially in activated sludge tanks, where the concentration of bacterial cells, organic matter and nutrients are very high [10,42]. However, this theory has not been fully demonstrated and ~~Another~~ another possible hypothesis would be that the bacterial community composition, which has been demonstrated to be different in PAB and FLB [43–45], may shape the diversity and abundance of the resistome [46–48]. Unfortunately, we did not analyze the composition of bacterial communities and thus this hypothesis could not be either confirmed or refuted. To conclude, our study demonstrated that although the studied WWTPs efficiently reduced the concentration of CGs in effluents, their prevalence within the bacterial community was still sufficient to potentially impact the receiving river and their indigenous bacterial communities.

3.2. Occurrence and persistence of CGs in Zenne River

In order to verify the eventual influence of WWTP effluents in the spread of CGs in freshwaters, we collected surface water samples at different locations along the Zenne River that were further analyzed to assess variations in the abundance of CGs across space. In particular, $bla_{\text{OXA-48}}$ and bla_{KPC} genes were detected in 96% and 93% of the samples analyzed, respectively, whereas bla_{NDM} was detected in 68% of Zenne River samples. These detection frequencies reflect the epidemiology reported in Belgian

hospitals for carbapenemases-producing pathogens with $bla_{\text{OXA-48}}$ and bla_{NDM} being the most and the less gene found, respectively [36]. Remarkably, no significant differences between PAB and FLB fraction for any of the genes analyzed were observed ($p>0.05$).

The average absolute abundances of all CGs measured in PAB and FLB fractions along the Zenne River in the four campaigns are presented in figure 4 and demonstrate a general increase from upstream to downstream. Particularly, the absolute abundances (copy number ml^{-1}) were significantly the highest at Z9 in both fractions with the exception of $bla_{\text{OXA-48}}$ in PAB (Figure 4). This site is located just downstream from the release of the BN WWTP effluent to the Zenne and few km downstream from BS WWTP. Previous studies already demonstrated significantly higher amounts of total and resistant fecal indicator bacteria at this site [23,49]. Moreover, other ARGs have been shown to peak at Z9 suggesting a strong effect of the discharge of treated effluents on the resistome of the Zenne River [23].

In general, the absolute abundance of CGs significantly increased in both bacterial fractions from upstream to downstream the release of both WWTPs effluents into the river ($p<0.05$, Figure 4). In fact, Z5 showed significantly higher levels of CGs than Z4 as well as Z9 values were significantly higher than those at Z8 (Figure 4) thus confirming the effect of WWTP effluent release to the river. In contrast, different behavior was observed for PAB and FLB fractions between Z5 and Z8 depending on the gene considered. Indeed, for bla_{KPC} and $bla_{\text{OXA-48}}$, although in PAB fraction the levels of at Z8 recovered to values similar to those measured at Z4 (upstream from BS WWTP), the FLB levels remained unvaried between Z5 and Z8 (Figure 4). This pattern could be explained by the different fate of particle-attached bacteria that are more likely to settle under steady flow conditions whereas free-living cells could be easily transported downstream. The significant decrease in the absolute abundance of the three

targeted genes measured few kilometers downstream (at Z11) in both fractions (Figure 4), could be explained by the dilution effect occurring in the river after their introduction by WWTP effluents. This phenomenon has been already described for other genes and may account for a reduction of up to one order of magnitude in the absolute abundance for a given gene within the first 1000 m after the WWTP discharge into the river [20]. Another possible explanation could be the low survival of bacterial human pathogens and commensal bacteria (either resistant or not) in freshwater systems [50]. In fact, a similar behavior between Z9 and Z11 has been observed for total and resistant fecal indicator bacteria [23].

The absolute abundances of bla_{KPC} along the Zenne River were in the same order of magnitude than those measured in WWTP effluents worldwide (Table 2). However, the relative abundances (normalized to 16S rRNA copies) were from one to three orders of magnitude lower than those reported in treated wastewater (Table 2). The average absolute abundance of bla_{NDM} measured in Zenne River water was 5.8×10^1 copies ml^{-1}, which is much lower than values found in Indian and Chinese rivers (Table 2) differently affected by anthropogenic pollution [8,20]. Same trend was observed after comparing the relative abundance of bla_{NDM} between the Zenne river and the aforementioned sites (Table 2, [8,20]). These results could be explained by the prevalence of bla_{NDM} in India [51] and its current widespread distribution [17]. The average abundance of bla_{OXA-48} measured in Zenne River waters was 2.4×10^2 copies ml^{-1}, in the same range than values found in low and medium impacted sites of upper Gange River and three orders of magnitude lower than values observed in the Yamuna River, crossing the city of Delhi, India [8]. Moreover, the concentrations measured in our study were in the same range than those reported in the Almendares River, Havana, Cuba [52]. In contrast, the relative abundances of bla_{OXA-48} measured in the Zenne River

were 4 order of magnitude lower than those reported in two Indian Rivers [8] and one order of magnitude lower than the values reported in a Cuban River [52] (Table 2). This gene was not found in Chinese and Spanish WWTPs [2,16].

Our results demonstrated that *bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ are already widely distributed in the Zenne River as a consequence of the chronic discharge of WWTP effluents into the river course.

3.3. Comparison of CGs with other ARGs

The relative abundances of CGs detected in this study have been compared with those of other ARGs measured during the same sampling campaigns at the same sites ([23,32], ~~; [21] (~~Figure 5). Remarkably, CGs were always significantly lower than the other ARGs analyzed in both PAB and FLB fractions (Figure 5, ~~[20~~[23,32]~~]~~; ~~Proia et al. *submitted)*~~ with the exception of the β-lactamase *bla*$_{CTX-M}$ (Figure 5). CTX-M coding genes are very often associated with NDM and OXA-48 gene variants. In contrast, *bla*$_{TEM}$ has been detected at similar concentrations to those of the other more abundant ARGs (Figure 5). This different behavior could be explained by the different nature of those β-lactamases which belong to two different subgroups [4]. In fact, *bla*$_{TEM}$ pertains to the subgroup which includes the most common plasmid-mediated β-lactamases identified in the 1970s and early 1980s whereas CTX-M enzymes originated as chromosomally-encoded β-lactamases [4] and spread later on the plasmids. This difference clearly determines their different spread among aquatic bacteria. The differences on the relative abundances of CGs with the other ARGs could be also explained by the recent and limited use of carbapenems in human medicine in comparison to other type of antibiotics. Particularly, the analysis of the consumption data in Belgium in the last five years (2012-2016) revealed that carbapenems are only used in hospitals with a defined daily dose (DDD) per 1000 inhabitants per day of

0.0063 which represent less than the 0.4% of the total amount of antibiotics supplied in clinical settings (www.ecdc.europa.eu). Nevertheless, carbapenems are only used in Belgian hospitals and therefore it seems obvious that its possible selective pressure on CGs is expected to be lower than that exerted by other antibiotics over other ARGs. In fact, others widely used antibiotics (i.e. amoxicillin, sulfamethoxazole) have been reported at concentrations one to two orders of magnitude higher than those of the carbapenems measured in this study at the same WWTPs [32]~~(Proia et al., *submitted*)~~. It is consequently logical to hypothesize that the spread in the aquatic environment (mainly occurring via sewage waters) somehow reflects this situation. However, considering that our study did not aimed to measure the selective pressure of antibiotics on ARGs, this remains a hypothesis that needs to be confirmed (or not) with further investigations. To conclude, even if the levels of CGs are still significantly lower than other ARGs conferring resistance to more used antibiotics, the increasing number of multiresistant pathogens carrying those genes that has recently been reported in Belgium [36] allow us to predict that the carbapenems consumption will tend to increase in the next years. As a consequence, the spread of CGs will be probably stimulated at short term, also in aquatic environment thus increasing the threat for human health. In fact, aquatic environments play a crucial role because water constitutes one of the main routes by which resistance is spread among natural bacteria and, at the same time, a possible direct and indirect way of dissemination for antibiotic-resistant organisms among human and animal populations [53]. Consequently, in absence of a well-structured and coordinated national surveillance plan to limit the dissemination of carbapenemase-producing bacteria in Belgian hospitals, and without any improvement of wastewater treatment technologies aiming to reduce CGs spread, the concentration of genes conferring resistance to last-resort antibiotics is expected to raise up to the levels

of high prevalent resistance genes such as *bla*$_{TEM}$, *sul*, and *tet*. Innovative technologies aiming to reduce the levels of ARBs and ARGs in WWTPs have been investigated by numerous studies lately [54], being ozonation, chlorination and UV disinfection, among the most tested and showing different efficiencies depending on the target considered [55–59]. Coagulation technology to achieve ARGs reduction in WWTPs effluents has been also investigated obtaining promising results [60] and graphene-based TiO_2 composite photocatalysts under solar radiation showed good removal as well [61]. Finally, by applying TiO_2 photocatalysis under UV irradiation in combination with H_2O_2, good removal efficiencies of resistant bacteria and ARGs (both intracellular and extracellular forms) from aqueous solution have recently been reported [62]. However, most of these studies have been performed in laboratory conditions focusing on reduced number of target AR determinants and not specifically investigating CGs. Therefore, further research is needed to improve the removal efficiency of carbapenemase-producing bacteria and CGs through WW treatment steps in order to mitigate the risk for human and animal health of the uncontrolled spread of carbapenem resistance among environmental bacteria.

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Figure captions

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. Total abundance of CGs in H and WWTPs waters during the four sampling campaigns (log scale). Black bars show the results for particle-attached bacteria (PAB) and grey bars for free-living bacteria (FLB). Tukey's b analysis results are shown with letters when differences among sampling sites were significant. **Bold** and *italic* letters are for PAB and FLB, respectively. Statistical significance was set at $p \leq 0.05$ (one-way repeated measures analysis of variance, ANOVA).

Figure 3. Relative abundance (normalized × 16S rRNA copies) of CGs in particle-attached (PAB, left) and free-living (FLB, right) bacteria in H and WWTPs waters during the four sampling campaigns (log scale). 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 4. Abundances of CGs at the different sampling sites along the Zenne River during the four campaigns. Black bars show the results for particle-attached bacteria (PAB, left) and grey bars for free-living bacteria (FLB, right). Tukey's b analysis results are shown with letters when differences among sites were significant. Statistical significance was set at $p \leq 0.05$ (one-way repeated measures analysis of variance, ANOVA).

Figure 5. Comparison of relative abundances of carbapanemases genes with others ARGs measured at the same sites during the same sampling campaigns in the Zenne River (top), WWTPs and H (bottom) for both PAB (left) and FLB (right) fractions. 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).

Tables legend

Table 1. Maximum, minimum and median carbapenem concentrations measured at each sampling site during the campaigns. Values are expressed in ng L^{-1}. bdl = below detection limit. Method detection limits (MDLs) for meropenem: Zenne water = 17 ng L^{-1}; raw waters = 180 ng L^{-1}; treated waters = 110 ng L^{-1}. MDLs for imipenem: Zenne water = 2.3 ng L^{-1}; raw waters = 9 ng L^{-1}; treated waters = 7.2 ng L^{-1}

Table 2. Comparison among different studies measuring absolute (copies/ml) and relative (copies/16S) abundances of $bla_{KPC,}$ bla_{NDM} and bla_{OXA-48} genes in WWTPs, H and river waters worldwide. bdl = below detection limit; na = not analyzed.

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Table 1

Sampling sites	Meropenem			Imipenem		
	Median	Max	Min	Median	Max	Min
Z1	36	73	bdl	27	32	24
Z3	111	111	bdl	29	30	bdl
Z4	95	95	bdl	36	36	bdl
Z5	163	183	93	32	34	29
Z8	195	204	131	33	35	31
Z9	225	241	bdl	41	45	39
Z11	242	330	131	42	44	36
H	bdl	bdl	bdl	bdl	bdl	bdl
BS in	bdl	bdl	bdl	105	105	bdl
BS out	636	646	bdl	75	82	65
BN in	950	950	bdl	89	105	bdl
BN out	731	731	bdl	99	104	bdl

Table 2

Study	Sample	Country	*bla*$_{KPC}$		*bla*$_{NDM}$		*bla*$_{OXA-48}$	
			copies/ml	copies/16S	copies/ml	copies/16S	copies/ml	copies/16S
This study	WWTP influent	Belgium	1.06×10^{5}	-6.52	1.31×10^{4}	-6.43	5.37×10^{4}	-5.81
	WWTP effluent		2.07×10^{2}	-5.63	4.38×10^{1}	-5.84	6.13×10^{1}	-4.98
	H effluent		1.27×10^{8}	-3.41	1.00×10^{3}	-6.80	3.19×10^{5}	-4.78
	River water		9.02×10^{2}	-5.75	5.78×10^{1}	-6.27	2.45×10^{2}	-5.30
Subirats et al., 2017 [2]	H effluent	Spain	4.40×10^{7}	-1.39	7.43×10^{5}	-3.16	1.59×10^{6}	-2.83
	H effluent		5.39×10^{4}	-4.49	1.23×10^{6}	-3.14	6.65×10^{4}	-4.40
	WWTP influent		3.13×10^{3}	-4.92	3.35×10^{4}	-3.89	bdl	-
	WWTP effluent		3.91×10^{2}	-4.80	1.84×10^{4}	-3.12	bdl	-
Yang et al., 2016b [18]	River downstream WWTPs	China	na	na	7.61×10^{2}	-4.20	na	na
Luo et al., 2014 [15]	WWTP effluent	China	na	na	1.32×10^{3}	-4.31	na	na
	WWTP effluent		na	na	1.43×10^{3}	-4.09	na	na
Yang et al., 2016a [14]	WWTPs influent	China	2.20×10^{5}	-4.29	na	n.a	bdl	-
	WWTPs effluent		1.50×10^{3}	-4.87	na	n.a	bdl	-
Nasri et al., 2017 [1]	H effluent	Tunisia	8.51×10^{5}	-3.47	3.96×10^{6}	-2.81	6.39×10^{4}	-4.60
	H effluent		1.14×10^{5}	-3.96	3.79×10^{6}	-2.44	1.38×10^{6}	-2.88
	H effluent		9.31×10^{4}	-4.23	3.70×10^{6}	-2.63	5.31×10^{6}	-2.47
	H effluent		bdl	–	2.57×10^{7}	-1.96	5.69×10^{5}	-3.61
	H effluent		bdl	–	2.29×10^{7}	-1.67	5.49×10^{5}	-3.29
	H effluent		bdl	–	6.18×10^{6}	-2.25	6.90×10^{6}	-2.20
	H effluent		bdl	–	8.30×10^{6}	-2.29	1.61×10^{7}	-2.00
Lamba et al., 2017 [24]	WWTP effluent	India	na	na	6.98×10^{5}	-6.07	5.30×10^{5}	-6.19
	WWTP effluent		na	na	5.08×10^{5}	-4.56	2.01×10^{5}	-4.97
	WWTP effluent		na	na	3.49×10^{4}	-5.68	8.11×10^{3}	-6.31
	WWTP effluent		na	na	4.37×10^{5}	-4.50	2.63×10^{5}	-4.72
	WWTP effluent		na	na	2.54×10^{4}	-6.57	1.15×10^{5}	-5.91
	H effluent		na	na	1.99×10^{13}	-1.05	2.77×10^{6}	-7.91
	H effluent		na	na	1.58×10^{14}	-1.74	2.17×10^{6}	-9.60
	H effluent		na	na	1.26×10^{13}	-2.22	2.89×10^{5}	-9.86
	H effluent		na	na	2.51×10^{14}	-1.84	4.45×10^{6}	-9.59
	H effluent		na	na	1.58×10^{14}	-2.24	7.06×10^{6}	-9.59
	H effluent		na	na	3.97×10^{13}	-1.84	1.01×10^{6}	-9.44
	H effluent		na	na	1.58×10^{15}	-1.71	7.84×10^{6}	-10.01
	H effluent		na	na	1.58×10^{13}	-2.34	3.43×10^{5}	-10.01
	H effluent		na	na	2.51×10^{13}	-0.51	5.97×10^{5}	-8.13
	H effluent		na	na	1.58×10^{14}	-1.71	1.06×10^{7}	-8.89
	H effluent		na	na	1.26×10^{7}	-3.02	9.25×10^{4}	-5.15
	H effluent		na	na	1.99×10^{8}	-2.46	4.00×10^{5}	-5.16
Ahammad et al., 2014 [8]	River Water low impacts	India	na	na	4.59×10^{2}	-1.17	1.44×10^{2}	-1.67
	River Water medium impacts		na	na	8.69×10^{2}	-0.99	2.81×10^{2}	-1.48
	River Water high impacts		na	na	3.13×10^{5}	-1.58	2.16×10^{5}	-1.75
Knapp et al., 2012 [46]	River water column	Cuba	na	na	na	na	7.59×10^{2}	-4.75

Figure 1

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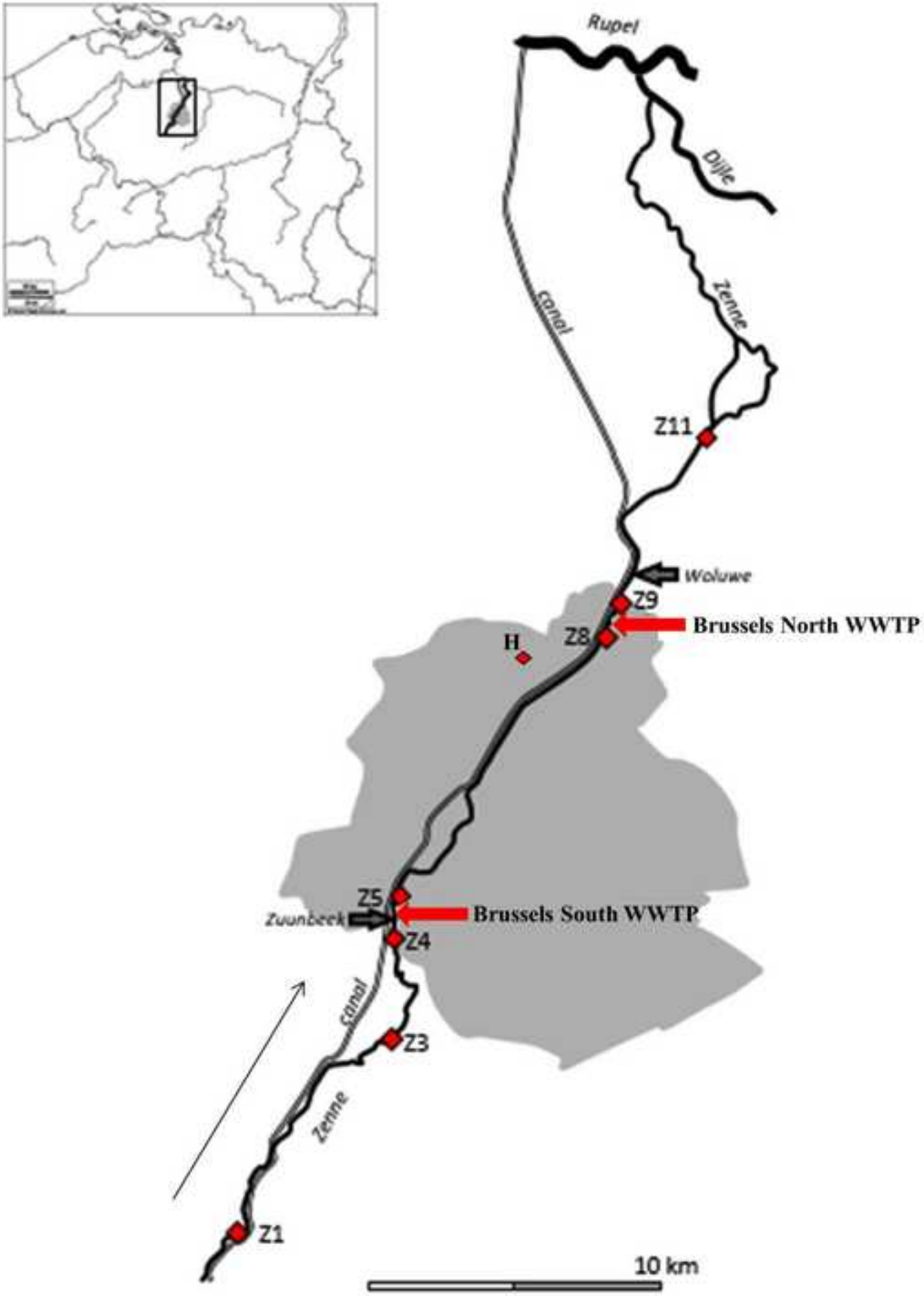

Figure 2

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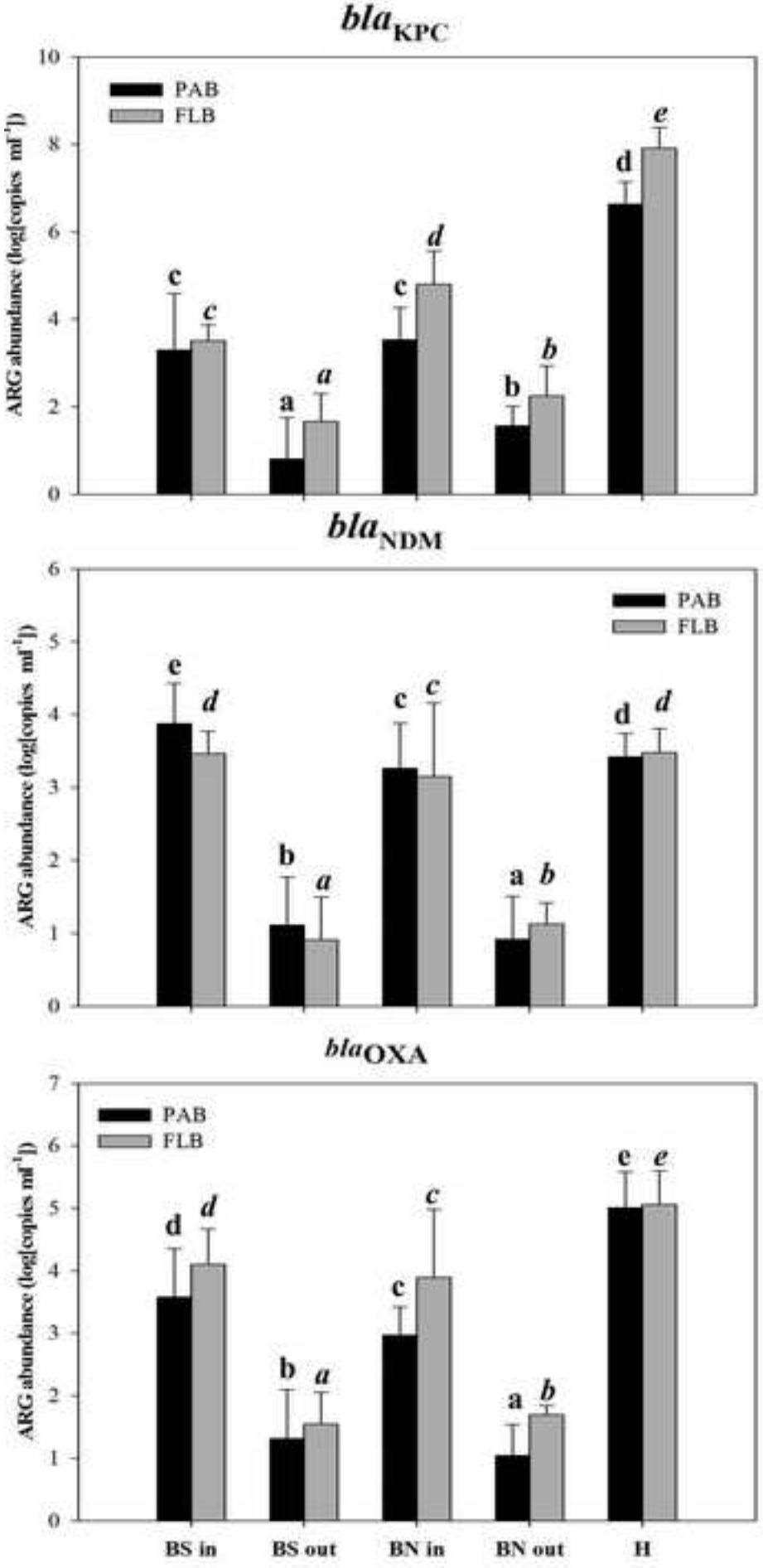

Figure 3

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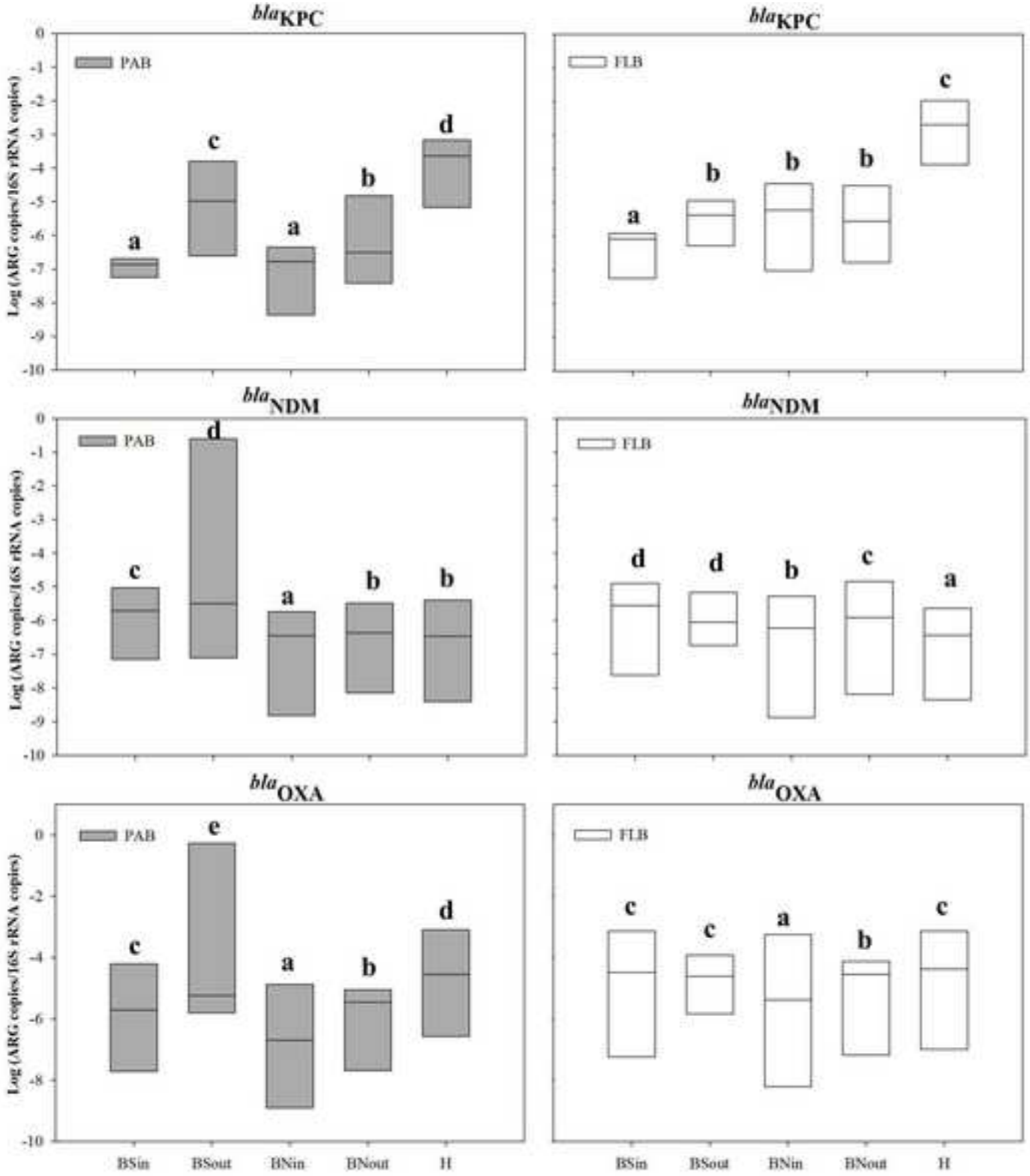

Figure 4

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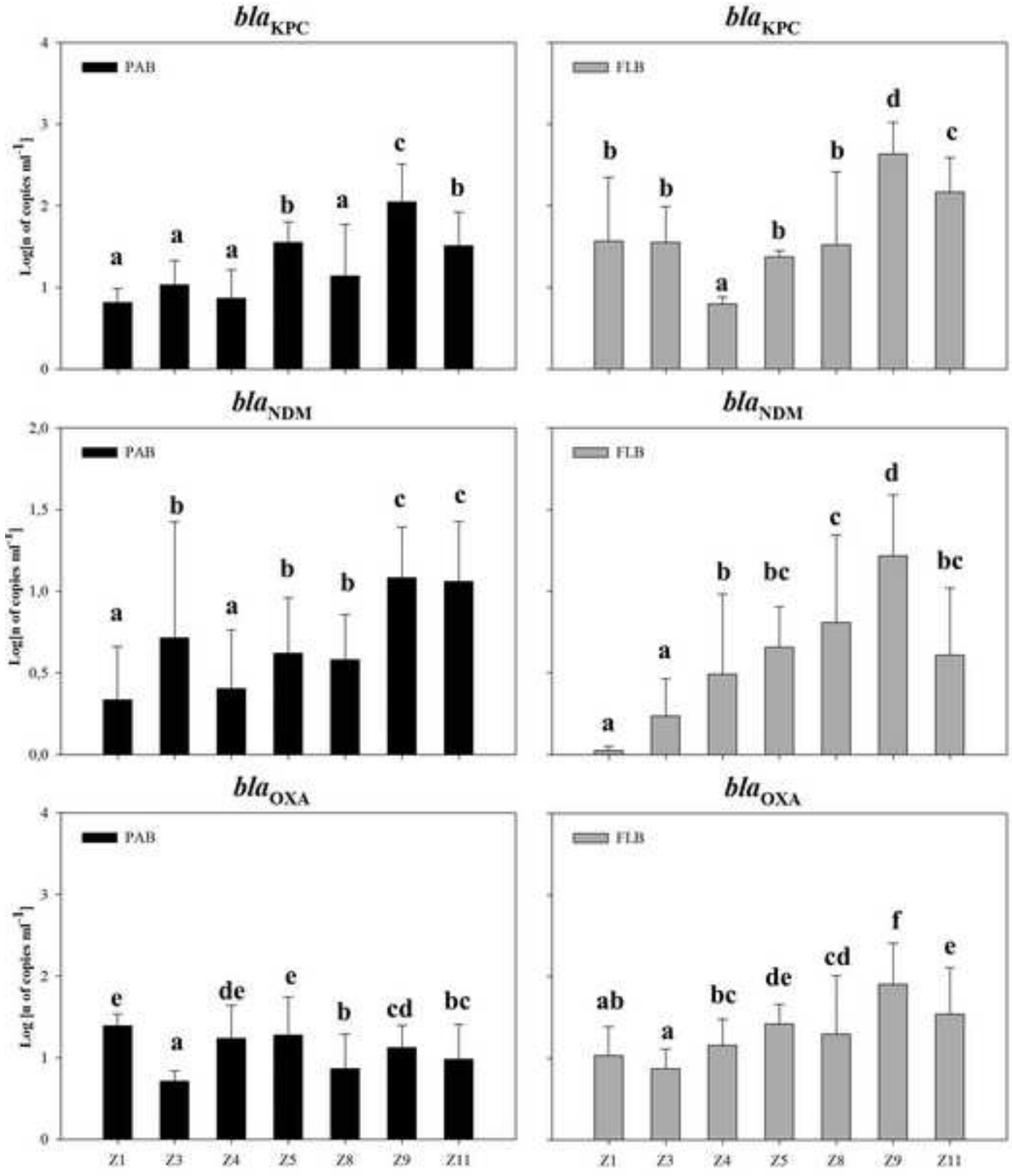

Figure 5

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