Antibiotic resistant *Pseudomonas* spp. in the aquatic environment: a prevalence study under tropical and temperate climate conditions

Naresh Devarajan¹, Thilo Köhler^{2,3}, Periyasamy Sivalingam¹, Christian van Delden^{2,3}, Crispin K. Mulaji⁴, Pius T. Mpiana⁴, Bastiaan W. Ibelings¹ and John Poté^{1,4*}

¹*University of Geneva, Faculty of Sciences, Section of Earth and Environmental Science, Department F.-A. Forel for Environmental and Aquatic Sciences and Institute of Environmental Sciences, Blvd Carl-Vogt 66, CH-1205 Geneva, Switzerland.*

²*Department of Microbiology and Molecular Medicine, University of Geneva, 1, rue Michel Servet, CH-1211 Geneva 4, Switzerland.*

³*Transplant Infectious Diseases Unit, University Hospitals Geneva, 4, rue Gabrielle-Perret-Gentil, CH-1211 Geneva 14, Switzerland.*

⁴*University of Kinshasa (UNIKIN), Faculty of Science, Department of Chemistry, B.P. 190, Kinshasa XI, Democratic Republic of the Congo.*

* Corresponding author:

John Poté, PhD.
University of Geneva
Faculty of Sciences
Earth and Environmental Sciences
Department F.-A. Forel for environmental and aquatic sciences
Bld Carl-Vogt 66, CH-1205 Geneva, Switzerland.
Tel: (+41 22) 379 03 21
Fax: (+41 22) 379 03 29
E-mail: john.pote@unige.ch
<http://www.unige.ch/forel/index.html>

Abstract

Microbial populations which are resistant to antibiotics are an emerging environmental concern with potentially serious implications for public health. Thus, there is a growing concern in exploring the occurrence of antibiotic resistance in the environment with no limitations to the factors that contribute to their emergence. The aquatic environment is considered to be a hot-spot for the acquisition and spread of antibiotic resistance due to pollution with emerging contaminants derived from anthropogenic activities. In this study, we report on the isolation and characterization of 141 *Pseudomonas* spp. from aquatic sediments receiving partially (un)treated hospital and communal effluents from three distinct geographical locations: Democratic Republic of the Congo (DRC), India (IN), and Switzerland (CH). *P. putida* (42%) and *P. aeruginosa* (39%) were the dominant *Pseudomonas* species. The highest frequency of antibiotic resistance against eight anti-pseudomonas agents was found among IN isolates (35-60%), followed by DRC (18-50%) and CH (12-54%). CTX-M was the most frequent β -lactamase found in CH (47% of isolates), while VIM-1 was dominant in isolates from DRC (61%) and IN (29%). NDM-1 was found in 29% of the total IN isolates and surprisingly also in 6% of CH isolates. Chromosomally-encoded efflux mechanisms were overexpressed in *P. aeruginosa* isolates from all three geographic locations. *In vitro* conjugative transfers of antibiotic resistance plasmids occurred more frequently under tropical temperatures (30 and 37 °C) than under temperate conditions (10 °C). The presence of Extended Spectrum β -lactamases (ESBLs) and Metallo β -lactamases (MBLs) in the isolates from environmental samples has important implications for humans who depend on public water supply and sanitation facilities. To our knowledge, this is the first study to demonstrate a comparison between treated/untreated effluents from urban and hospital settings as a source of microbial resistance by evaluating the aquatic ecosystems

sediments from tropical and temperate climate conditions. Taken together, our findings demonstrate a widespread occurrence of antibiotic resistance in aquatic ecosystems sediments receiving untreated/treated wastewater and how these contemporary sources of contamination, contribute to the spread of microbial resistance in the aquatic environment. This research presents also useful tools to evaluate sediment quality in the receiving river/reservoir systems which can be applied to similar environments.

Key words: Urban wastewater, aquatic environment contamination, Extended Spectrum β -lactamases, *Pseudomonas* species, antibiotic resistance, climatic condition.

1. Introduction

Antibiotic resistance (AR) is not restricted to bacteria in a clinical setting but is also found in environmental bacteria. Several studies have documented the release of antibiotic resistance genes (ARGs) and multidrug resistant (MDR) bacteria from clinical and communal effluents to the receiving environmental water bodies (Czekalski et al. 2014; Devarajan et al. 2015a; Kummerer 2004; Walsh et al. 2011). Environmental bacteria resistant to antibiotics represent a major threat to humans, since they can act as a reservoir for the maintenance and spread of ARGs (Kummerer, 2004).

In most developing countries, effluents are discharged from the sewer systems directly into rivers and lakes. These untreated effluents may contain AR bacteria, which disseminate and potentially transfer their resistance genes (Spindler et al. 2012). In our previous studies (Laffite et al., 2016; Devarajan et al., 2015b; Mubedi et al., 2013), we showed that hospital effluents in developing countries are a major source of contamination of the aquatic environment. In most developed countries, waste water treatment plants (WWTPs) remove the majority of solid organic matter as well as nitrogen and phosphorous. However WWTP were not designed originally to remove AR bacteria or antimicrobial residues and the fate of these contaminants remains largely unknown (Novo et al., 2013; Czekalski et al., 2014; Mao et al., 2015; Devarajan et al. 2015a,), although their presence in sediments has been documented (Pote et al., 2010). The release of contaminants to the hydrosphere either by sediment re-suspension or by infiltration into the groundwater is considered as a potential human and environmental risk, since the remobilization can contaminate drinking water supplies and organisms in the food chain like fish, which may be harvested for human consumption (Wildi et al., 2004).

The presence of extended spectrum β -lactamase (ESBLs) and metallo β -lactamase (MBLs) genes among bacterial communities is of great concern, as they confer resistance to a large panel of beta-lactams (Walsh et al., 2011, Canton and Coque, 2006). Chromosomally encoded efflux mechanisms, abundant in gram negative bacteria and particularly in *Pseudomonas* spp., further contribute to raising the antibiotic resistance level (Li et al., 2015). Several studies have reported resistance genes in environmental samples, mainly carried by *Escherichia coli* and *Enterococcus* spp. (Hu et al., 2008; Laroche et al., 2009). However, few studies are available on presence of AR genes in other human pathogenic bacteria including *Pseudomonas* spp. (Spindler et al., 2012; Quinteira et al., 2005). *Pseudomonas* spp. are frequently isolated from environments rich in organic matter, where they contribute to biodegradation and bioremediation processes (Quinteira et al., 2005). Antibiotic resistant *Pseudomonas aeruginosa* have been found in hospital and communal effluents. However the presence of resistance genes in other *Pseudomonas* species, which could serve as donors or recipients in effluents and receiving systems, has not been studied thoroughly (Walsh et al., 2011; Spindler et al., 2012; Shah et al., 2012).

Although true in general, limited information is available concerning the true extent of AR and the presence of environmental MDR strains in effluents from Africa and India (WHO, 2014). The objective of this study was to identify the possible AR mechanisms prevailing in environmental bacteria, in particular in *Pseudomonas* spp., under tropical and temperate climatic conditions. This study provides baseline information for further investigations into the prevalence of AR bacteria in hospital and communal effluents and their spread into the aquatic ecosystem, representing potential threats to public health.

2. Materials and methods

94 The sediment samples were collected from two different climate conditions in 2012; (i)
95 Tropical conditions – sediments, receiving untreated hospital effluents, in the Democratic
96 Republic of the Congo (DRC) and India (IN) and (ii) temperate climatic conditions: sediments
97 from Lake Geneva receiving (partially) treated effluents from a waste water treatment plant
98 (WWTP) in Switzerland (CH). The sampling sites, sample collection protocols and the sediment
99 physicochemical properties are described in supporting information (SI) and in our previous
100 studies (Devarajan et al., 2015b; Mubedi et al. 2013; Thevenon et al., 2012). *Pseudomonas* spp.
101 from the sediment samples were isolated as previously described (Pote et al., 2010; Haller et al.,
102 2009). Briefly, the filter membranes were placed on *Pseudomonas* Selective Agar (PSA)
103 supplemented with the antifungal agent Nystatin (Sigma, MO, USA) at a final concentration of
104 100 mg/L, for the counting of colony forming units (CFU). The plates were incubated at 37 °C
105 for 24 h and the bacterial counts were expressed as CFU/100 g of fresh sediment. Approximately
106 10 isolates from each sampling site were randomly selected for further analysis. In total 141
107 isolates were taken for this study which include DRC (n=32), IN (n=85) and CH (n=26) isolates.
108 Antibiotic susceptibility testing was performed by the disk diffusion method on Müller –Hinton
109 (MH) agar (Oxoid Ltd., Basingstoke, U.K.) as described by Clinical and Laboratory Standards
110 Institute (CLSI) guidelines (CLSI, Jan, 2012). All isolated bacteria were tested against 16
111 antimicrobials; Piperacillin (PRL, 100 µg), Piperacillin-tazobactam (TZP, 110 µg), Ceftazidime
112 (CAZ, 30 µg), Cefepime (FEP, 30 µg), Aztreonam (ATM, 30 µg), Imipenem (IPM, 10 µg),
113 Meropenem (MEM, 10 µg), Cefoperazone (CFP, 30 µg), Ofloxacin (OFX, 5 µg), Norfloxacin
114 (NOR, 10 µg), Cefpirome (CPO, 30 µg), Ampicillin (AMP, 10 µg), Cefuroxime (CXM, 30 µg),
115 Streptomycin (STR, 10 µg), Cefotaxime (CTX, 30 µg) and Cotrimoxazole (SXT, 25 µg) (Oxoid,
116 Pratteln, Switzerland). In this study PRL, TZP, CAZ, FEP, IPM, MEM, OFX and NOR were

considered as anti-pseudomonas antibiotics. The breakpoint values were interpreted according to the CLSI guidelines (CLSI, Jan, 2012). The multiple antibiotic resistance (MAR) index was calculated for the isolates as described by Shah et al. (2012) and Krumperman (1983). Briefly, the MAR index of a sediment sample is defined by the equation $a/(b*c)$, where a is the aggregate antibiotic resistance score of all the isolates from the sample, b is the number of antibiotics used (n=16 in this study) and c is the number of isolates originating from the sample. *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used for quality controls. The metal (Cadmium, Cobalt, Copper, Mercury, Nickel and Zinc) minimal inhibitory concentrations (MICs) for the selected *P. aeruginosa* isolates (n=15) were determined by the microdilution method on Tryptic Soy Broth (TSB) medium containing different concentrations of metal salts (1 – 35 mM). The maximum tolerance limit (MTC) is defined as the highest metal concentration at which growth was observed after 48 h of incubation at 30 °C (50). Total DNA from the bacterial isolates was extracted using Chelex Resin (Bio-Rad, Cressier, Switzerland) and Proteinase K (Sigma-Aldrich, Buchs SG, Switzerland) as described in Yang et al. (2008). All isolates (n=141) were screened for the presence of various ARGs with polymerase chain reaction (PCR) using the primers listed in Table-1S (Supporting Information). Amplified 16S rDNA and AR genes were sequenced and confirmed by blasting the DNA sequences (NCBI). The sequences are added to GenBank under numbers KY508304; KY511067 to KY511076; KP172294–KP172300.

Gene transfer by conjugation. Transfer of ARGs from *Pseudomonas* spp. to *E. coli* (DH α) susceptible to all 16 antibiotics (Abs) and negative for any ARGs was performed by filter mating as described earlier (Lampkowska et al. 2008). Thirty six strains of MDR *Pseudomonas* spp. were selected and screened for their potential to transfer ARGs. In a total volume of 2 mL, exponentially growing donor (*Pseudomonas* spp.) and recipient (*E. coli*) strains were mixed in a

ratio of 1:1. Filter mating experiments were conducted with over-night cultures of donor and receipt subsequently diluted in fresh LB media and grown 4 hours before mixing. The mixture was filtered, placed upright in a non-selective media (Muller Hinton Agar) and incubated overnight at 10, 30 or 37 °C, representing the average temperature of Lake Geneva (CIPEL, 2014), a tropical (DRC and IN) receiving system (Fig. 4), and the human gut, respectively. After incubation, the filters were recovered and washed vigorously with 2-3 mL of sterile peptone-saline. Serial dilutions of the washed bacterial suspension from the filters were spread on donor (PSA) and recipient (Tryptone Bile X-Gluc (TBX) agar). In all cases donor and recipient suspensions were treated in a similar way (diluted in sterile peptone-saline) and plated on TBX and PSA agar to assess the purity and determine colony counts. We used the following media to recover the β -lactamase positive trans-conjugants: Tryptone Bile X-Gluc agar amended with any one of the Abs, or a combination of two Abs at a final concentration of 100 mg/L (AMP), 0.5 mg/L (IMP/MER) and 6 mg/L (CTX/FEP). Trans-conjugants positive for *E. coli* (*uidA* gene) were selected and screened for the presence of ARGs by PCR using the primers used above. Transfer frequencies were expressed as the number of trans-conjugants colonies per recipient colony formed after the mating period and the reported values were the average of three different assays.

In this study 39.3% of the total isolates were identified as *P. aeruginosa*. A grouping was performed on their phenotypic and genotypic resistance patterns. Based on the grouping 15 isolates (a representative from each group) were screened for their efflux pump expressions. Total RNA isolation and cDNA synthesis were performed as described previously (Dumas et al. 2006). Four μ l of 10-times diluted cDNA was used as template for qPCR. The primers used are presented in Table 2S (Supporting Information). The expression of efflux mechanisms was

quantified by the Eco qPCR system (Illumina, Switzerland) using a SYBR FAST qPCR Universal Kit (KAPA Biosystems, USA). A melting curve was run at the end of the 40 cycles to test for the presence of a unique PCR reaction product (supporting Information SI_S3). The ribosomal *rpsL* gene (housekeeping gene in *P. aeruginosa*) was chosen to correct for the differences in starting material.

$$\text{Ratio} = (E_{\text{target gene}})^{\Delta \text{ct}_{\text{target(PA01-test strain)}}} / (E_{\text{rpsL}})^{\Delta \text{ct}_{\text{rpsL (PA01-test strain)}}}$$

The results are expressed as ratios between the target gene and the reference gene (*rpsL*). An effect on the gene expression was considered significant if the ratios were > 2.0 and < 0.4 (Pfaffl et al., 2002).

3. Results

3.1. *Pseudomonas* spp. and antibiotic susceptibility

The population densities of *Pseudomonas* spp. at our study sites are shown in Table 1. The total population of *Pseudomonas* spp. ranged between $2.03 \times 10^5 - 34.33 \times 10^5$, $0.22 \times 10^5 - 0.48 \times 10^5$ and $1.4 \times 10^5 - 13 \times 10^5$ CFU/100g of fresh sediment for the samples from IN, DRC and CH, respectively. *Pseudomonas* spp. phylogenetic likelihood is presented in Fig.1. From the total *Pseudomonas* spp., isolates 42.2% were identified as *P. putida* and 39.3 % as *P. aeruginosa*. These two species were dominant at all sampling sites, contributing 81.5% of the total samples. Other species identified in this study specific to the sampling sites are for DRC: *P. entomophila* 0.7% and *P. mosselii* 0.7 %, for IN: *P. fulva* 3.0 %, *P. mendocina* 0.7 %, *P. pseudoalcaligenes* 0.7 %, and *P. stutzeri* 0.7 %, for CH: *P. monteilii* 0.7 % and *P. moraviensis* 2.2 %, and for both DRC and IN: *P. plecoglossicida* 8.9 %. The phenotypic resistant profiles are presented in Fig. 2 and 1S (Supporting Information). The majority (81 %) of *Pseudomonas* spp. isolates from all

study sites were non-susceptible to AMP, CTX, CXM, SXT and ATM. Over 70% of the isolates from DRC and IN were resistant to STR and CFP. Among CH isolates, 38 and 58% of the isolates were resistant to STR and CFP, respectively. About 50% of the isolates from IN were also resistant to PRL, TZP and CAZ, which is higher than in the other two studied locations. On the other hand, 50 and 54% of isolates from DRC and CH, respectively were resistant to OFX. All isolates from DRC were susceptible to IPM, while 15 and 33% of the isolates from CH and IN, respectively, were resistant to IMP. Multidrug resistant isolates were identified in all 3 sampling areas. In this study, 7 isolates from IN and 2 isolates from CH were resistant to 16 Abs. Multiple antibiotic resistance (MAR) index values were highest in IN (0.79_H8), followed by DRC (0.48_C1) and CH (0.40_V4) sites. *Pseudomonas* spp. isolates were also more resistant to metals (Zn, Cu, Co, Cd and Hg) compared to the *P. aeruginosa* reference strain PAO1.

3.2. Detection of antibiotic resistance genes (ARGs)

The selection of ARGs in this study was an evaluation of the degree to which sediments receiving treated/untreated effluents act as a reservoir of antibiotic resistance genes which could be potentially transferred to other susceptible bacteria. The screened ARGs include *aadA*, *strA* and *strB* which confer resistance to aminoglycosides, *sulI*, *sulII* and *sulIII* which confer resistance to sulfonamides, *floR* and *cmlA* genes which confer resistance to florfenicol and chloramphenicol, β -lactamases (TEM, SHV, CTX-M, VIM and NDM) which confer resistance to β -lactams, and the mobile genetic elements (*intl1*, *intl2* and *intl3*). The selection of these genes was based on previously published studies (Stoll et al., 2012; Czekalski et al., 2014; Devarajan et al., 2015a; Devarajan et al., 2016; Laffite et al., 2016) highlighting the co-selection and co-existence of resistance mechanisms in environmental settings.

208 The genotypic resistance profiles are presented in Fig 3 and 2S (Supporting Information).
209 Aminoglycoside resistance genes (*strA*, *strB* and *aadA*) were found in *Pseudomonas* spp. from
210 IN and CH. Eight percent of isolates from IN carried all three (*strA*, *strB* and *aadA*)
211 aminoglycoside ARGs, 33 % carried *strA* and *strB*, 14 % carried *strA* and *aadA*, and 2 % carried
212 *strA* alone. DRC isolates were negative for *strA* and *strB*, and carried only *aadA* (3%). Few
213 isolates (4%) from CH were PCR positive for all 3 aminoglycoside ARGs. Interestingly, 23 % of
214 the isolates were phenotypically resistant to STR but PCR results were negative for all three
215 screened aminoglycoside ARGs.

216 All the isolates from DRC and CH were PCR negative for the 3 *sul* genes tested (*sulI*, *sulII* and
217 *sulIII*). On the other hand, 25, 19 and 4% of IN isolates were found to carry *sulI*, *sulII* and *sulIII*,
218 respectively. The florfenicol resistance gene *floR* was found in many CH isolates (65%),
219 followed by IN (28%) and DRC (27%) isolates. The *cmlA* gene was identified in 12, 20 and 25%
220 of the isolates from CH, DRC and IN, respectively. We performed a PCR-based screening of all
221 isolates for the presence of *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{NDM}, *bla*_{VIM-1}, and *bla*_{VIM-2} β -lactamase genes.
222 The *bla*_{SHV} gene was identified in 3, 8 and 13% of the isolates from CH, DRC and IN,
223 respectively. The prevalence of *bla*_{CTX-M} gene was higher in CH (65 %), followed by DRC (27
224 %) and IN (19 %) isolates. On the other hand the metallo β -lactamases were found in the
225 majority of isolates from IN and DRC. Eight and 34% of the isolates from CH and IN
226 respectively were found to carry *bla*_{NDM}. Isolates from IN were also found to carry *bla*_{VIM-1} (26
227 %) and *bla*_{VIM-2} (16 %). However, isolates from DRC were negative for the *bla*_{NDM} and *bla*_{VIM-2},
228 but positive for *bla*_{VIM-1} (47 %). Integrase genes including those from classes 1, 2 and 3 were
229 screened in all the isolates. *IntI1* and *intI2* were identified in *Pseudomonas* spp., however, none of
230 the isolates carried *intI3*. The integrase genes, *intI1* was identified in 12 and 34 % of the isolates

from CH and IN, respectively. Isolates from DRC were PCR negative for *intl1* and 13% of the isolates carried *intl2*. All isolates from CH were PCR negative for *intl2*, while 14% of the isolates from IN were PCR positive for *intl2*.

3.3. Preferred conjugative mobilization

We studied the ability of the MDR *Pseudomonas* spp. isolates (n = 36) to transfer their ARGs into the *E. coli* recipient under three different temperature conditions. The transfer frequencies achieved at 30 °C were approximately 4 to 5 orders of magnitude higher when compared to 37 °C and 10 °C, respectively (Table 2). *P. putida* was the only species which had the ability to transfer their ARGs to the recipient under all 3 studied conditions. *P. fulva* was able to transfer ARGs only at 30 °C, while *P. aeruginosa* and *P. plecoglossicida* transferred plasmids at both 30 and 37 °C. The isolates from CH were able to transfer *bla*_{CTX-M} gene (n = 4), and one isolate was able to transfer the *bla*_{SHV} gene. DRC isolates were able to transfer *bla*_{VIM} in most of the cases (n= 4) followed by *bla*_{CTX-M} genes (n=1). On the other hand, isolates from IN were able to share most of their ARGs with the recipient, in this study we identified *bla*_{NDM}, *bla*_{VIM}, *bla*_{SHV} and *bla*_{CTX-M} in 3, 2, 1 and 5 recipients, respectively. In total, out of the 36 isolates selected for the conjugative experiments, 58% of the isolates were able to transfer their ARGs to the recipient *E. coli* with *bla*_{CTX-M} gene being the predominant ARG mobilized to the recipient cells.

3.4. Gene expression in *P. aeruginosa*

To identify the implication of efflux pumps in the resistance mechanisms, isolates (n = 15) from CH (n = 2), DRC (n = 3) and IN (n = 10) were screened for expression of the clinically relevant efflux pumps MexAB-OprM, MexCD-OprJ, MexXY/OprM and MexEF-OprN. The values were compared to the wild type strain PAO1 and the ratio of gene expression is presented in Table 3.

The threshold values are those expected to yield an impact on phenotypic resistance levels. The gene expression analysis showed that 5/14 (36%) isolates demonstrated increased *mexA* expression (> 2-fold). Five out of 14 isolates (36%) showed increased *mexX* expression (>4-fold) compared to the PAO1 reference strain. *MexC* expression was increased (>10-fold) in 6/15 isolates (40%), while *mexE* was overexpressed (>30-fold) in 5/15 (33%) isolates. Down-regulation of *oprD*, leading to decreased imipenem susceptibility, was not observed in any of the tested isolates.

4. Discussion

We report here on the prevalence of multidrug resistant *Pseudomonas* spp. from sediments receiving treated or untreated communal or hospital effluents in DRC, IN and CH. Upon comparison between the sampling locations the MAR index of sediments receiving WWTP effluent in CH (0.40) was within the range of sediments receiving untreated hospital effluent in DRC and IN. These results demonstrate that the potential impacts of both WWTP and untreated hospital effluents are comparable in terms of risk of spreading MDR *Pseudomonas* spp. in the environment. Our results are concurrent with previous studies (Czekalski et al., 2014; Devarajan et al., 2015a) showing that WWTP are not effective in removing MDR bacteria. These bacteria in the sediment of the receiving systems could be persistent, and may play a role in preserving and dispersing these MDR bacteria back to the human consumption via drinking water, fisheries or agricultural products using surface water for irrigation.

In this study, 20% of the *Pseudomonas* isolates carried both *strA* and *strB*, together with *aadA* (5%). Additionally, 8% of isolates carried a combination of *strA* and *aadA* genes. Interestingly, two aminoglycoside susceptible isolates were PCR positive for the *strA* gene alone. This

275 observation is concurrent with previous studies (Chiou and Jones, 1995; Srinivasan et al., 2007)
276 and confirms that both genes have to be present to obtain functional streptomycin resistance.
277 Conversely, few isolates in this study were PCR negative for all the 3 studied aminoglycoside
278 genes.

279 Sulfonamides act as competitive inhibitors of dihydropteroate synthase (DHPS), involved in folic
280 acid synthesis. The prevalence of *sulI*, *sulII* and *sulIII* genes in the environment is most likely
281 due to dissemination of these genes via mobile genetic elements (Stoll et al., 2012).
282 Dissemination of sulfonamide resistance genes in the aquatic environment has been reported
283 from other parts of the world (Hu et al., 2008; Hamelin et al., 2006). In this study, 93% of the
284 isolates were non-susceptible to SXT. The potency of sulfonamides against *P. aeruginosa* is
285 limited. The constitutive expression of the MexAB-OprM efflux pump is mainly responsible for
286 this intrinsic resistance of *P. aeruginosa* to sulfonamides (Kohler et al., 1996). Sulfonamide
287 resistant genes were identified in the isolates from IN sediments, while none of the DRC and CH
288 isolates carried sulfonamide resistance genes. In previous reports, *sulI* was linked to *intI1*, and in
289 our study 47% of the isolates positive for *sulI* genes were accompanied by a class 1 integrase
290 (*intI1*) gene. Hence, the *sul* genes are likely to be “bystander” genes which are co-transferred
291 with other resistance genes/cassettes in the mobile genetic elements. As reported in other studies
292 (Srinivasan et al., 2007; Baran et al., 2011), excessive amounts of sulfa drugs are introduced in
293 the biosphere from clinical and agricultural settings. Although the constitutive expression level
294 of MexAB-oprM would be sufficient to confer sulfonamide and trimethoprim resistance,
295 sulfonamides released into the environment could select for *mexA* overexpression which makes
296 strains more resistant to other antibiotics that are substrates of MexAB-OprM (Li et al., 2015;
297 Dumas et al., 2006).

298 Decreased susceptibility to fluoroquinolones (FQ) was detected in almost 50% of isolates. FQ
299 resistance in *Pseudomonas* spp. is usually conferred by mutations in the topoisomerase genes
300 *gyrA* and *parC* and/or by efflux pump overexpression. We observed overexpression of *mexX*,
301 *mexC* or *mexE*, either alone or in combinations in isolates. In these isolates the expression ratios
302 compared to the FQ-susceptible strain PAO1, were increased to levels which previously have
303 been shown to confer a phenotypic decrease in FQ-susceptibility. It is likely that antibiotics
304 found at the different isolation sites have contributed to the selection of this type of mutants.

305 Florfenicol, a fluorinated structural analog of thiamphenicol and chloramphenicol was
306 approved by the Food and Drug administration (FDA) in 1996 for treatment of bovine
307 respiratory pathogens such as *Pasteurella* spp. in veterinary medicine and it is currently banned
308 for the treatment of cattle enteric diseases in USA (White et al., 2000). Since then, florfenicol
309 resistance has been detected in a wide variety of bacterial species and many of their resistance
310 determinants reside on mobile genetic elements (Kim et al., 1993; Schwarz et al., 2004).
311 Chloramphenicol resistance mechanisms include inactivation of chloramphenicol by
312 acetyltransferases (*cat* genes) and chloramphenicol efflux pumps (*cml* genes) and multidrug
313 transporters (Schwarz et al., 2004). Florfenicol used in veterinary medicine is related to
314 chloramphenicol and can select for cross-resistance among bacterial pathogens (Arcangioli et al.,
315 2000; Bolton et al., 1999; Keyes et al., 2000). *floR* genes have been reported in *E. coli* isolated
316 from humans and animals (Srinivasan et al., 2007), surface water samples in Germany and
317 Australia (Stoll et al., 2012). In this study, we found *floR* in 65 % of *Pseudomonas* isolates from
318 CH samples, while 25 % of the isolates from IN and 12% of the CH isolates were found to carry
319 the *cmlA* gene. CH samples were collected from Vidy bay (Lake Geneva), where the sediments
320 are receiving partially treated/untreated waste water from the WWTP (since 1964) and urban and

agriculture runoff from several sources (Poté et al., 2008). Consequently, the sediment from the studied site is heavily polluted and can act as reservoir of various contaminants including heavy metals, organic micro-pollutants, antibiotics, pathogens and ARGs over the period of time (Poté et al., 2008; Thevenon et al., 2012; Coutu et al., 2013; Devarajan et al., 2016). A recent study performed in the same Lake Geneva area reported the presence of chloramphenicol resistant bacteria (Czekalski et al., 2012). The prevalence *floR* in the Vidy bay sediment can be explained by the accumulation of ARGs and eventual dissemination into environmental bacteria (Devarajan et al., 2015a, 2016; Laffite et al., 2016).

In 1989, Germany, France and Italy recognized and reported new ESBLs. CTX-M, which confers resistance to CTX, was detected in many bacterial species from different environments. At present the CTX-M family includes 172 β -lactamases (as of August, 2015 <http://www.lahey.org/Studies/other.asp>). Unlike other β -lactamases (TEM and SHV) the population and diversity of CTX-M producing isolates is complex, due to its association with the spread of specific plasmids and/or mobile genetic elements rather than clonal epidemics (Canton, 2009; Mendonca et al., 2007). According to the reports from other countries, the prevalence of ESBL producing bacteria differs among geographical regions (Hawkey, 2008). Plasmid-borne *bla*_{CTX-M-15}, which is the global dominant ESBL gene, was first reported in India (Lascols et al., 2012). After CTX-M-15 (group 1), CTX-M-14 (a group of 9 genotypes) is dominant in China and has spread to become the second most reported CTX-M worldwide (Hawkey, 2008). In the past decade CTX-M enzymes have become the most prevalent ESBL in isolates originating from hospitals and municipal waste. CTX-M genes are often linked to the *ISEcpI* which carries other additional antibiotic resistance determinants. Hence. CTX-M can be disseminated by co-selection for other resistance markers (Walsh et al., 2007), including aminoglycoside,

344 tetracycline, sulfonamide or fluoroquinolone resistance genes (Canton, 2009). Indeed CTX-M
345 was found in all the sampling regions (DRC, IN and CH). In the present study, β -lactam resistant
346 isolates also conferred resistance to aminoglycosides and sulfonamides. Over 83% of the CTX-
347 M positive isolates from IN sediments were also positive for *strA*, *strB* and *sul* genes. To our
348 knowledge this is the first report on the prevalence of CTX-M genes in *Pseudomonas* spp.
349 isolated from environmental samples in DRC.

350 NDM-1 hydrolyses all β -lactam antibiotics except ATM, and over a decade different types of
351 carbapenemases have gradually been appearing in gram-negative bacteria (Struelens et al.,
352 2010). *bla*_{NDM-1} was initially identified in *E. coli* and *K. pneumoniae* and later reported in *A.*
353 *baumannii*, *C. freundii*, *M. morganii*, *P. mirabilis*, *Enterobacter* spp. and *P. aeruginosa*
354 (Struelens et al., 2010; Jovicic et al., 2011). Until recently, *bla*_{NDM-1} was identified only in clinical
355 settings, but there is a paucity of information on the presence of NDM-1 genes in the
356 environment. In a previous study, surface water samples collected in 2010 from India were
357 identified with MDR bacteria carrying the NDM-1 gene (Walsh et al., 2011). In our study,
358 NDM-1 was identified in 34 % of isolates from IN and 8 % from CH, and none from DRC, and
359 these isolates from CH and IN were also resistant to ATM. The ability of NDM-1 to spread is not
360 limited to *Enterobacteriaceae* and extends to other bacterial species including *Pseudomonas* spp.
361 This implies the possibility for emergence of new NDM-1 cases (Jovicic et al., 2011). In our
362 study we identified NDM-1 in various *Pseudomonas* species including, *P. putida* (n=7 IN), *P.*
363 *aeruginosa* (n=3 CH and n = 13 IN), *P. fulva* (n=4 IN) and *P. plecoglossicida* (n=2 IN).

364 Efficacy of carbapenems is compromised with the emergence of metallo- β -lactamases (MBL)
365 carried on mobile genetic elements (Walsh et al., 2005). In 2007, six out of 33 countries in the
366 European Antimicrobial Resistance Surveillance System (EARSS), reported carbapenem

367 resistance in 25% of *P. aeruginosa* isolates with the highest rate in Greece (51%) (Hawkey and
368 Jones, 2009). A recent review reports VIM-2 in 37 countries from 5 continents (Hawkey and
369 Jones, 2009). In the present study *bla*_{VIM-1} was found in IN and CH samples; and VIM-2 in DRC
370 and IN. The spread of MBLs among bacterial populations in the environment is of great concern,
371 not only because these enzymes confer resistance to carbapenems and other β -lactams, but also
372 because such bacteria are typically resistant to other antibiotics which limits treatment options.
373 Our study also emphasizes the extent to which MBLs can disseminate within environmental
374 bacterial communities.

375 Integrons are gene exchange systems and play an important role in the acquisition, accumulation
376 and dissemination of antimicrobial resistance genes. In this study, 34 % of the studied isolates
377 were positive for integrons. Similarly, previous studies (Laroche et al., 2009; Hawkey and Jones,
378 2009; Skurnik et al., 2006) have also reported higher prevalence of class 1 integrons in the
379 environment as a result of clinical effluent discharge and high levels of urbanization (Nardelli et
380 al., 2012). Previous studies have demonstrated the prevalence of *intI1* variants in environmental
381 samples and the problem of detecting them in large numbers could be influenced by the large
382 numbers of genetically homogenous class 1 integrons being shed to the environment by clinical
383 and animal sources, human sewage, landfills and storm water arising from the urban
384 environment (Gillings et al., 2008; Gillings et al., 2015; Stalder et al., 2014). It is also possible
385 that integrase genes have evolved to acquire ARGs in pathogenic and commensal bacteria, which
386 could have serious implications for treating infections (Davies, 2007). There are large numbers
387 of mobile elements in the environment and potential resistance genes associated with them,
388 which continue to be a threat to the human welfare. Hence these gene pools are valuable to
389 predict future clinical cases potentially generated by gene transfer mechanisms from

environmental to clinical settings. Additionally, in this study MDR *Pseudomonas* spp. were able to transfer their ARGs to other bacterial species namely *E. coli*. Highest transfer frequencies were recorded at 30°C, followed by 37°C and 10°C, which suggests a complex relationship with environmental/climatic factors. The temperatures of sampling sites from DRC and IN are favorable for environmental transfer of ARGs as the average daily peak temperatures in Kinshasa, DRC and Tiruchirappalli, IN reaches 30 °C all around the year (Fig. 4). Study sites in DRC (Kinshasa) and IN (Tiruchirappalli) are also known for the poor sewage systems and the dissemination of urban effluents to the receiving systems (Devarajan et al., 2015b; Mwanamoki et al., 2014), along with monsoon, landfills and agricultural runoff potentially disseminating the ARGs and MDR bacteria. On the other hand, Lake Geneva which has a temperate climate with an average of 10 °C, has the potential to act as reservoir for preserving these ARGs and MDR bacteria (Devarajan et al., 2015b) with the possible threat of spread of ARGs following sediment re-suspension.

To investigate regulatory mechanisms governing resistance to both heavy metals and Abs, we examined the efflux mechanisms in *P. aeruginosa*. The over-expression of several efflux systems may impact on the survival of *P. aeruginosa* isolates in a selective environment, as they are potentially capable of pumping out many classes of contaminants, including Abs and metals. However the nature of the correlation between the increase in the transcriptional level of the efflux pumps and their contributions to resistance remains unclear (Dumas et al., 2006). The MexCD-OprJ system is generally not expressed in wild type strains but is inducible by membrane damaging agents (Li et al., 2015). Over-production of MexCD-OprJ will result in increased resistance to quinolones, macrolides, amphenicols, tetracyclines and certain β -lactams. The communal and hospital effluents constitute a mixture of membrane damaging agents

(Verlicchi et al., 2010) which could be responsible for the induction of MexCD-OprJ mechanisms in *P. aeruginosa*. Previous studies have reported the down regulation of *oprD* to select for antibiotic (carbapenem) resistance and/or metal (Zn) resistance (Dumas et al., 2006, Perron et al., 2004) implying the existence of common regulatory mechanisms for Zn and carbapenem resistance in *P. aeruginosa*. Hence, efflux over-expression and porin down regulation will favor bacterial survival in the environment, increasing the chances of acquiring new ARGs and enhancing resistance transmission.

5. Conclusion

Our study reports MDR *Pseudomonas* spp. in aquatic environmental samples, which could potentially be transferred to humans. The chromosomally encoded efflux pumps and their overexpression appear to be supporting mechanisms for antimicrobial resistance. Porin down-regulation and *ampC* could play a major role in phenotypic resistance in *P. aeruginosa*. The wide spread of ESBL/MBLs in communal settings may be a result of co-selection since most of the plasmids frequently carry multiple resistance genes. In this study both temperate (CH) and tropical conditions (IN and DRC) favored the exchange of genetic material among bacteria. Communal transmission of MDR bacteria is a global problem, but the risk level may vary with local standards of sanitation. With deficient sewer systems, in developing nations the transmission of MDR bacteria to drinking water may result in pressing public health problems. Additionally, community settings with proper sewer systems should consider the treatment options available for disinfecting the bacterial load/ARGs in the effluents before being discharged to the receiving system. Hence, we illustrate the requirement for surveillance of resistance, and the importance of the aquatic ecosystems for the dissemination of antibiotic

resistance determinants, as in many parts of the globe surface waters act as the end point for the treated/untreated effluents from waste water treatment systems.

Acknowledgments We are grateful to financial support from the Swiss National Science Foundation (grant no. 31003A_150163/1) and Bourse Augustin Lombard 2013 for field sampling. Naresh Devarajan as a Ph.D. student was supported by Ernst and Lucie Schmidheiny Foundation in Geneva. Periyasamy Sivalingam is a Postdoctoral fellow supported by Swiss Government Excellence Scholarship for Foreign Scholars. This study presents a tripartite collaboration among University of Geneva (Forel Department), University of Kinshasa and the Pedagogic National University of Congo (Democratic Republic of the Congo), and Jamal Mohamed College-Bharathidasan University (India).

Supplementary Information accompanies this paper on a separate file.

References

- Arcangioli, M.A., Leroy-Setrin, S., Martel, J.L. and Chaslus-Dancla, E. (2000) Evolution of chloramphenicol resistance, with emergence of cross-resistance to florfenicol, in bovine *Salmonella Typhimurium* strains implicates definitive phage type (DT) 104. Journal of Medical Microbiology 49(1), 103-110.
- Baran, W., Adamek, E., Ziemiańska, J. and Sobczak, A. (2011) Effects of the presence of sulfonamides in the environment and their influence on human health. Journal of Hazardous Materials 196, 1-15.
- Bolton, L.F., Kelley, L.C., Lee, M.D., Fedorka-Cray, P.J. and Maurer, J.J. (1999) Detection of multidrug-resistant *Salmonella enterica* serotype typhimurium DT104 based on a gene

457 which confers cross-resistance to florfenicol and chloramphenicol. *Journal of Clinical*
458 *Microbiology* 37(5), 1348-1351.

459 Canton, R. (2009) Antibiotic resistance genes from the environment: a perspective through
460 newly identified antibiotic resistance mechanisms in the clinical setting. *Clinical*
461 *Microbiology and Infection* 15, 20-25.

462 Canton, R. and Coque, T.M. (2006) The CTX-M beta-lactamase pandemic. *Current Opinion in*
463 *Microbiology* 9(5), 466-475.

464 CCME, EPC 98-E (Canadian Environmental Quality Guidelines, Canadian Council of Ministers
465 of the Environment). Canadian Sediment Quality Guidelines for the Protection of Aquatic
466 Life. 1999. <http://www.ccme.ca/>.

467 Chiou, C.S. and Jones, A.L. (1995) Expression and identification of the strA-strB gene pair from
468 streptomycin-resistant *Erwinia amylovora*. *Gene* 152(1), 47-51.

469

470 CLSI (Jan, 2012) Performance Standards for Antimicrobial Susceptibility Testing; Twenty-
471 Second Informational Supplement, M100-S22, Clinical and Laboratory Standards
472 Institute.

473 CIPEL (2014) THE SCIENTIFIC COUNCIL OF THE INTERNATIONAL COMMISSION,
474 General Conclusions. Campagne 2013 (ed), pp. 14-17.

475 Coutu, S., Rossi, L., Barry, D.A., Rudaz, S., Vernaz, N., 2013. Temporal Variability of
476 Antibiotics Fluxes in Wastewater and Contribution from Hospitals. *PLoS ONE* 8(1):
477 e53592. doi:10.1371/journal.pone.0053592.

478 Czekalski, N., Berthold, T., Caucci, S., Egli, A. and Burgmann, H. (2012) Increased levels of
 479 multiresistant bacteria and resistance genes after wastewater treatment and their
 480 dissemination into lake geneva, Switzerland. *Front Microbiol* 3, 106.

481 Czekalski, N., Gascon Diez, E. and Burgmann, H., 2014. Wastewater as a point source of
 482 antibiotic-resistance genes in the sediment of a freshwater lake. *Isme Journal* 8(7), 1381-
 483 1390.

484 Davies, J. (2007) Microbes have the last word. A drastic re-evaluation of antimicrobial treatment
 485 is needed to overcome the threat of antibiotic-resistant bacteria. *EMBO Reports* 8(7),
 486 616-621.

487 Devarajan, N., Laffite, A., Graham, N.D., Meijer, M., Prabakar, K., Mubedi, J.I., Elongo, V.,
 488 Mpiana, P.T., Ibelings, B.W., Wildi, W. and Pote, J. (2015a) Accumulation of clinically
 489 relevant antibiotic-resistance genes, bacterial load, and metals in freshwater lake
 490 sediments in central europe. *Environmental Science & Technology* 49(11), 6528-6537.

491 Devarajan, N., Laffite, A., Ngelikoto, P., Elongo, V., Prabakar, K., Mubedi, J.I., Piana, P.T.,
 492 Wildi, W. and Pote, J. (2015b) Hospital and urban effluent waters as a source of
 493 accumulation of toxic metals in the sediment receiving system of the Cauvery River,
 494 Tiruchirappalli, Tamil Nadu, India. *Environ Sci Pollut Res Int* 22(17), 12941-12950.

495 Devarajan, N., Laffite, A., Mulaji, C.K., Otamonga, J.P., Mpiana, P.T., Mubedi, J.I., Prabakar,
 496 K., Ibelings, B.W. and Pote, J. (2016) Occurrence of Antibiotic Resistance Genes and
 497 Bacterial Markers in a Tropical River Receiving Hospital and Urban Wastewaters. *PLoS*
 498 *One* 11(2).

499 Dumas, J.L., van Delden, C., Perron, K. and Kohler, T. (2006) Analysis of antibiotic resistance
 500 gene expression in *Pseudomonas aeruginosa* by quantitative real-time-PCR. Fems
 501 Microbiology Letters 254(2), 217-225.

502 Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M. and Zhu, Y.-G. (2015) Using
 503 the class 1 integron-integrase gene as a proxy for anthropogenic pollution. Isme Journal
 504 9(6), 1269-1279.

505 Gillings, M.R., Krishnan, S., Worden, P.J. and Hardwick, S.A. (2008) Recovery of diverse genes
 506 for class 1 integron-integrases from environmental DNA samples. Fems Microbiology
 507 Letters 287(1), 56-62.

508 Haller, L., Pote, J., Loizeau, J.L. and Wildi, W. (2009) Distribution and survival of faecal
 509 indicator bacteria in the sediments of the Bay of Vidy, Lake Geneva, Switzerland.
 510 Ecological Indicators 9(3), 540-547.

511 Hamelin, K., Bruant, G., El-Shaarawi, A., Hill, S., Edge, T.A., Bekal, S., Fairbrother, J.M.,
 512 Harel, J., Maynard, C., Masson, L. and Brousseau, R. (2006) A virulence and
 513 antimicrobial resistance DNA microarray detects a high frequency of virulence genes in
 514 *Escherichia coli* isolates from Great Lakes recreational waters. Appl Environ Microbiol
 515 72(6), 4200-4206.

516 Hawkey, P.M. (2008) Prevalence and clonality of extended-spectrum beta-lactamases in Asia.
 517 Clinical Microbiology and Infection 14, 159-165.

518 Hawkey, P.M. and Jones, A.M. (2009) The changing epidemiology of resistance. Journal of
 519 Antimicrobial Chemotherapy 64(4), 3-10.

520 Hu, J., Shi, J., Chang, H., Li, D., Yang, M. and Kamagata, Y. (2008) Phenotyping and
 521 Genotyping of Antibiotic-Resistant *Escherichia coli* Isolated from a Natural River Basin.
 522 Environmental Science & Technology 42(9), 3415-3420.

523 Jovicic, B., Lepsanovic, Z., Suljagic, V., Rackov, G., Begovic, J., Topisirovic, L. and Kojic, M.
 524 (2011) Emergence of NDM-1 metallo-beta-lactamase in *Pseudomonas aeruginosa*
 525 clinical isolates from Serbia. Antimicrob Agents Chemother 55(8), 3929-3931.

526 Keyes, K., Hudson, C., Maurer, J.J., Thayer, S., White, D.G. and Lee, M.D. (2000) Detection of
 527 florfenicol resistance genes in *Escherichia coli* isolated from sick chickens. Antimicrobial
 528 Agents and Chemotherapy 44(2), 421-424.

529 Kim, E.H., Yoshida, T. and Aoki, T. (1993) Detection of R Plasmid-Encoded with Resistance to
 530 Florfenicol in *Pasteurella-Piscicida*. Fish Pathology 28(4), 165-170.

531 Kohler, T., Kok, M., MicheaHamzehpour, M., Plesiat, P., Gotoh, N., Nishino, T., Curty, L.K.
 532 and Pechere, J.C. (1996) Multidrug efflux in intrinsic resistance to trimethoprim and
 533 sulfamethoxazole in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy
 534 40(10), 2288-2290.

535 Krumperman, P.H. (1983) Multiple Antibiotic-Resistance Indexing of *Escherichia coli* to
 536 Identify High-Risk Sources of Fecal Contamination of Foods. Applied and
 537 Environmental Microbiology 46(1), 165-170.

538 Kummerer, K. (2004) Resistance in the environment. Journal of Antimicrobial Chemotherapy
 539 54(2), 311-320.

540 Laffite, A., Kilunga, P.I., Kayembe, J.M., Devarajan, N., Mulaji, C.K., Giuliani, G., Slaveykova,
 541 V.I., and Poté, J. (2016). Hospital Effluents Are One of Several Sources of Metal,

Antibiotic Resistance Genes, and Bacterial Markers Disseminated in Sub-Saharan Urban Rivers. *Frontiers in Microbiology* 7, 1128.

Lampkowska, J., Feld, L., Monaghan, A., Toomey, N., Schjørring, S., Jacobsen, B., van der Voet, H., Andersen, S.R., Bolton, D., Aarts, H., Krogfelt, K.A., Wilcks, A. and Bardowski, J. (2008) A standardized conjugation protocol to assess antibiotic resistance transfer between lactococcal species. *International Journal of Food Microbiology* 127(1–2), 172-175.

Laroche, E., Pawlak, B., Berthe, T., Skurnik, D. and Petit, F. (2009) Occurrence of antibiotic resistance and class 1, 2 and 3 integrons in *Escherichia coli* isolated from a densely populated estuary (Seine, France). *FEMS Microbiology Ecology* 68(1), 118-130.

Lascols, C., Hackel, M., Hujer, A.M., Marshall, S.H., Bouchillon, S.K., Hoban, D.J., Hawser, S.P., Badal, R.E. and Bonomo, R.A. (2012) Using nucleic acid microarrays to perform molecular epidemiology and detect novel beta-lactamases: a snapshot of extended-spectrum beta-lactamases throughout the world. *Journal of Clinical Microbiology* 50(5), 1632-1639.

Li, X.Z., Plesiat, P. and Nikaido, H. (2015) The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clin Microbiol Rev* 28(2), 337-418.

Mao, D., Yu, S., Rysz, M., Luo, Y., Yang, F., Li, F., Hou, J., Mu, Q. and Alvarez, P.J. (2015) Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. *Water Res* 85, 458-466.

Mendonca, N., Leitao, J., Manageiro, V., Ferreira, E. and Canica, M. (2007) Spread of extended-spectrum beta-lactamase CTX-M-producing *Escherichia coli* clinical isolates in

community and nosocomial environments in Portugal. *Antimicrobial Agents and Chemotherapy* 51(6), 1946-1955.

Mubedi, J.I., Devarajan, N., Le Faucheur, S., Mputu, J.K., Atibu, E.K., Sivalingam, P., Prabakar, K., Mpiana, P.T., Wildi, W. and Pote, J. (2013) Effects of untreated hospital effluents on the accumulation of toxic metals in sediments of receiving system under tropical conditions: case of South India and Democratic Republic of Congo. *Chemosphere* 93(6), 1070-1076.

Mwanamoki, P.M., Devarajan, N., Thevenon, F., Atibu, E.K., Tshibanda, J.B., Ngelinkoto, P., Mpiana, P.T., Prabakar, K., Mubedi, J.I., Kabele, C.G., Wildi, W. and Pote, J. (2014) Assessment of pathogenic bacteria in water and sediment from a water reservoir under tropical conditions (Lake Ma Vallee), Kinshasa Democratic Republic of Congo. *Environ Monit Assess* 186(10), 6821-6830.

Nardelli, M., Scalzo, P.M., Ramirez, M.S., Quiroga, M.P., Cassini, M.H. and Centron, D. (2012) Class 1 integrons in environments with different degrees of urbanization. *PLoS One* 7(6), e39223.

Novo, A., Andre, S., Viana, P., Nunes, O.C. and Manaia, C.M. (2013) Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. *Water Res* 47(5), 1875-1887.

Perron, K., Caille, O., Rossier, C., van Delden, C., Dumas, J.L. and Kohler, T. (2004) CzcR-CzcS, a two-component system involved in heavy metal and carbapenem resistance in *Pseudomonas aeruginosa*. *Journal of Biological Chemistry* 279(10), 8761-8768.

585 Pfaffl, M.W., Horgan, G.W. and Dempfle, L. (2002) Relative expression software tool (REST)
 586 for group-wise comparison and statistical analysis of relative expression results in real-
 587 time PCR. *Nucleic Acids Research* 30(9), e36.

588 Poté, J., Bravo, A.G., Mavingui, P., Ariztegui, D. and Wildi, W.. 2010. Evaluation of
 589 quantitative recovery of bacterial cells and DNA from different lake sediments by
 590 Nycodenz density gradient centrifugation. *Ecological Indicators* 10(2), 234-240.

591 Pote, J.; Haller, L.; Loizeau, J. L.; Garcia Bravo, A.; Sastre, V.; Wildi, W., 2008. Effects of a
 592 sewage treatment plant outlet pipe extension on the distribution of contaminants in the
 593 sediments of the Bay of Vidy, Lake Geneva, Switzerland. *Bioresour Technol* 99, 7122-
 594 31.

595 Quinteira, S., Ferreira, H. and Peixe, L. (2005) First isolation of blaVIM-2 in an environmental
 596 isolate of *Pseudomonas pseudoalcaligenes*. *Antimicrob Agents Chemother* 49(5), 2140-
 597 2141.

598 Schwarz, S., Kehrenberg, C., Doublet, B. and Cloeckert, A. (2004) Molecular basis of bacterial
 599 resistance to chloramphenicol and florfenicol. *FEMS Microbiol Rev* 28(5), 519-542.

600 Shah, S.Q., Colquhoun, D.J., Nikuli, H.L. and Sorum, H. (2012) Prevalence of antibiotic
 601 resistance genes in the bacterial flora of integrated fish farming environments of Pakistan
 602 and Tanzania. *Environmental Science & Technology* 46(16), 8672-8679.

603 Skurnik, D., Ruimy, R., Andremon, A., Amorin, C., Rouquet, P., Picard, B. and Denamur, E.
 604 (2006) Effect of human vicinity on antimicrobial resistance and integrons in animal faecal
 605 *Escherichia coli*. *Journal of Antimicrobial Chemotherapy* 57(6), 1215-1219.

606 Spindler, A., Otton, L.M., Fuentefria, D.B. and Corcao, G. (2012) Beta-lactams resistance and
607 presence of class 1 integron in *Pseudomonas* spp. isolated from untreated hospital
608 effluents in Brazil. *Antonie Van Leeuwenhoek* 102(1), 73-81.

609 Srinivasan, V., Gillespie, B.E., Nguyen, L.T., Headrick, S.I., Murinda, S.E. and Oliver, S.P.
610 (2007) Characterization of antimicrobial resistance patterns and class 1 integrons in
611 *Escherichia coli* O26 isolated from humans and animals. *International Journal of*
612 *Antimicrobial Agents* 29(3), 254-262.

613 Stalder, T., Barraud, O., Jove, T., Casellas, M., Gaschet, M., Dagot, C. and Ploy, M.-C. (2014)
614 Quantitative and qualitative impact of hospital effluent on dissemination of the integron
615 pool. *Isme Journal* 8(4), 768-777.

616 Stoll, C., Sidhu, J.P.S., Tiehm, A. and Toze, S. (2012) Prevalence of Clinically Relevant
617 Antibiotic Resistance Genes in Surface Water Samples Collected from Germany and
618 Australia. *Environmental Science & Technology* 46(17), 9716-9726.

619 Struelens, M.J., Monnet, D.L., Magiorakos, A.P., O'Connor, F.S., Giesecke, J. and Participants,
620 E.N.-S. (2010) New Delhi metallo-beta-lactamase 1-producing Enterobacteriaceae:
621 emergence and response in Europe. *Eurosurveillance* 15(46), 9-16.

622 Thevenon, F., Adatte, T., Wildi, W. and Pote, J., 2012. Antibiotic resistant bacteria/genes
623 dissemination in lacustrine sediments highly increased following cultural eutrophication
624 of Lake Geneva (Switzerland). *Chemosphere* 86(5), 468-476.

625 Verlicchi, P., Galletti, A., Petrovic, M. and Barcelo, D., 2010. Hospital effluents as a source of
626 emerging pollutants: An overview of micropollutants and sustainable treatment options.
627 *Journal of Hydrology* 389(3-4), 416-428.

628 Walsh, T.R., Toleman, M.A. and Jones, R.N., 2007. Comment on: Occurrence, prevalence and
629 genetic environment of CTX-M β -lactamases in Enterobacteriaceae from Indian
630 hospitals. *Journal of Antimicrobial Chemotherapy* 59(4), 799-800.

631 Walsh, T.R., Toleman, M.A., Poirel, L. and Nordmann, P., 2005. Metallo-beta-lactamases: the
632 quiet before the storm? *Clin Microbiol Rev* 18(2), 306-325.

633 Walsh, T.R., Weeks, J., Livermore, D.M. and Toleman, M.A. (2011) Dissemination of NDM-1
634 positive bacteria in the New Delhi environment and its implications for human health: an
635 environmental point prevalence study. *Lancet Infectious Diseases* 11(5), 355-362.

636 White, D.G., Hudson, C., Maurer, J.J., Ayers, S., Zhao, S.H., Lee, M.D., Bolton, L., Foley, T.
637 and Sherwood, J. (2000) Characterization of chloramphenicol and florfenicol resistance
638 in *Escherichia coli* associated with bovine diarrhea. *Journal of Clinical Microbiology*
639 38(12), 4593-4598.

640 WHO (2014) World Health Organization, ANTIMICROBIAL RESISTANCE Global Report on
641 Surveillance, WHO Library Cataloguing-in-Publication Data, Geneva, Switzerland
642 <http://www.who.int/drugresistance/documents/surveillancereport/en/>.

643 Wildi, W., Dominik, J., Loizeau, J., Thomas, R.L., Favarger, P., Haller, L., Perroud, A. and
644 Peytremann, C. (2004) River, reservoir and lake sediment contamination by heavy metals
645 downstream from urban areas of Switzerland. *Lakes & Reservoirs: Research and*
646 *Management* 9, 75-87.

647 Yang, J.L., Wang, M.S., Cheng, A.C., Pan, K.C., Li, C.F. and Deng, S.X. (2008) A simple and
648 rapid method for extracting bacterial DNA from intestinal microflora for ERIC-PCR
649 detection. *World Journal of Gastroenterology* 14(18), 2872-2876.

Figure legends

Fig. 1. (A). Distribution of *Pseudomonas* spp. and their phylogenetic relationship based on 16S rDNA sequences used to construct a maximum likelihood phylogenetic tree. Numbers at the branch nodes represent the boot strap values. (B) Total percentage of *Pseudomonas* spp. identified in this study.

Fig. 2. Comparison of phenotypic resistance in the studied *Pseudomonas* spp. between DRC (n=30), IN (n=85) and CH (n=26). Left panel: percentage of isolates resistant to the antipseudomonal agents. Right panel: percentage of isolates resistant to multiple number of antibiotics (n=16) in this study.

Fig note:

CH – Switzerland, DRC – Congo Democratic Republic, IN – India.

Piperacillin (PRL, 100 µg), Piperacillin-tazobactam (TZP, 110 µg), Ceftazidime (CAZ, 30 µg), Cefepime (FEP, 30 µg), Imipenem (IPM, 10 µg), Meropenem (MEM, 10 µg), Ofloxacin (OFX, 5 µg) and Norfloxacin (NOR, 10 µg).

Fig. 3. Comparison and ratio distribution among β -lactamases in the studied *Pseudomonas* spp., isolated from the sediments receiving treated/untreated effluent in (A) Switzerland, (B) DRC – Congo Democratic Republic and (C) IN – India.

Fig. 4. Mean high and low temperature of Tiruchirappalli, Tamil Nadu, India and Kinshasa, Democratic Republic of the Congo; Tiruchirappalli Grey bar, Pink line representing mean high and low, respectively. Kinshasa – Blue bar, Yellow line representing mean high and low, respectively. Green line indicating the temperature of 30 °C.

Table.1. *Pseudomonas* spp. colony forming units (CFU) counts for the studied samples isolated on *Pseudomonas* selective agar, and the multiple antibiotic resistance (MAR) index calculated for each sampling site for 16 antibiotics.

Climate	Region	Sample	<i>P. spp.</i> CFU x 10 ⁵ 100 g ⁻¹ wet weight	MAR Index
Temperate	CH	V4	13 ± 4.3	0.4
		V7	2.7 ± 0.9	0.28
		G1	1.4 ± 0.9	0.2
Tropical	DRC	C1	0.22 ± 0.04	0.48
		C2	0.48 ± 0.26	0.44
		C3	0.23 ± 0.10	0.28
	IN	H1	3.6 ± 1.6	0.39
		H2	4.87 ± 2.2	0.67
		H3	1.78 ± 0.9	0.48
		H4	2.03 ± 0.8	0.66
		H5	5.77 ± 2.7	0.33
		H6	3.23 ± 2.7	0.32
		H7	6.73 ± 2.0	0.34
		H8	25.67 ± 8.3	0.79
		H9	34.33 ± 17.3	0.61
		H10	4.07 ± 1.3	0.59

CFU values are expressed for mean of 3 samples ± standard deviation.

CH – Switzerland, DRC – Democratic Republic of the Congo, IN – India

Sampling site Reference: V4, V7 and G1 [Thevenon et al., 2012].

C1, C2 and C3 and H1 to H10 [Mubedi et al., 2013].

Table. 2. Effect of different temperature on transfer frequency of ARGs from environmental *Pseudomonas* spp. isolates to *E. coli* cells

Donor (n = 36)	Recipient	Transconjugants	Transfer frequency		
		ARGs identified (n =21)	10 °C	30 °C	37 °C
<i>P. aeruginosa</i>	<i>E. coli</i>	<i>CTX-M</i> (4); <i>VIM</i> (3); <i>NDM</i> (2)	n/a	0.5 x 10 ⁻⁵	6.2 x 10 ⁻⁸
<i>P. putida</i>	<i>E. coli</i>	<i>SHV</i> (2); <i>CTX-M</i> (3); <i>VIM</i> (3); <i>NDM</i> (1)	1.2 x 10 ⁻⁹	0.9 x 10 ⁻⁴	0.53 x 10 ⁻⁸
<i>P. pseudoalcaligenes</i>	<i>E. coli</i>		n/a	n/a	n/a
<i>P. fulva</i>	<i>E. coli</i>	<i>CTX-M</i> (1)	n/a	1.7 x 10 ⁻⁷	n/a
<i>P. mendocina</i>	<i>E. coli</i>		n/a	n/a	n/a
<i>P. monteilii</i>	<i>E. coli</i>		n/a	n/a	n/a
<i>P. moraviensis</i>	<i>E. coli</i>		n/a	n/a	n/a
<i>P. mosselii</i>	<i>E. coli</i>		n/a	n/a	n/a
<i>P. plecoglossicida</i>	<i>E. coli</i>	<i>CTX-M</i> (2)	n/a	8.3 x 10 ⁻⁵	< 1×10 ⁻⁹
<i>P. entomophila</i>	<i>E. coli</i>		n/a	n/a	n/a
<i>P. stutzeri</i>	<i>E. coli</i>		n/a	n/a	n/a

n/a – no transfer of ARGs observed

723 Table. 3. Relative expression of selected gene expression in *P. aeruginosa* determined by quantitative qRT-PCR.

Isolate	Break point values (mm)												Gene											
origin	PRL	TZP	CAZ	FEP	ATM	IPM	MEM	CFP	CTX	OFX	NOR	STR	mexR	mexA	oprM	mexZ	mexY	mexX	nfxB	mexC34	mexT	mexE	oprD34	ARGs present
PA01	25	26	25	26	22	22	25	16	16	19	25	14	1	1	1	1	1	1	1	1	1	1	1	Not studied
CH_19	11	13	11	14	10	6	6	6	6	6	6	6	18.26	5.68	3.09	27.89	27.89	15.76	0.64	43.51	23.43	86.19	3.65	bla _{CTX-Mr} floR
CH_21	13	18	10	14	10	6	6	6	6	6	6	6	3.28	1.27	0.84	10.59	10.59	3.26	4.73	12.87	17.33	271.43	1.19	bla _{NDM} , aadA, int11
DRC_27	24	22	22	22	19	22	26	20	15	19	26	6	5.27	1.91	1.18	15.22	15.22	1.33	3.12	2.91	6.63	1.21	0.76	bla _{CTX-M} cmlA
DRC_38	26	24	20	18	6	22	26	22	6	16	19	9	6.94	2.27	1.57	14.76	14.76	7.9	9.86	23.97	13.5	52.76	2.28	bla _{CTX-Mr} aadA, int12
DRC_39	26	26	21	17	6	23	25	21	6	15	20	6	4.78	1.83	1.1	31.88	31.88	5.6	7.48	30.59	12.37	7.56	1.8	bla _{VIM-1} floR
IN_85	6	8	9	14	10	6	9	6	6	6	6	6	7.23	2.16	1.16	4.16	4.16	2.37	5.26	10.04	12.23	257.78	1.5	bla _{VIM-2} , sull, strA, int11
IN_87	10	11	16	17	15	6	6	10	6	6	6	6	1.24	0.97	0.57	5.3	5.3	2.25	1.44	1.91	1.58	2.94	0.63	bla _{NDM} , sulll, strA, strB, int11
IN_96	19	21	19	23	6	30	19	16	9	19	24	12	6.98	2.44	2.24	9.51	9.51	2.29	4.23	3.17	7.72	6.67	1.5	bla _{vim-2}
IN_100	22	21	21	23	10	28	21	18	10	21	27	13	5.65	2.08	1.34	14.07	14.07	2.41	3.47	3.21	8.43	8.8	1.38	bla _{CTX-Mr} strA, strB, aadA, int11
IN_103	18	20	20	11	11	6	18	14	11	20	24	10	3.06	1.27	0.88	7.13	7.13	1.62	2.73	1.98	5.31	6.03	1.49	bla _{VIM-1} , sulll, floR
IN_109	19	22	21	18	9	28	23	18	11	21	25	13	3.87	1.09	0.56	6.82	6.82	0.35	3.96	3.46	6.62	14.48	2.38	bla _{CTX-Mr} sulll, cmlA, int12
IN_117	23	21	21	23	10	18	12	18	12	22	25	14	1.67	1.1	1.64	18.51	18.51	62.71	4.46	18.3	1.74	55.78	1.09	bla _{VIM-1} , sull, cmlA, aadA
IN_125	13	18	10	14	10	6	6	6	6	6	6	6	6.77	1.78	1.51	13.24	13.24	3.12	4.08	3.21	8.07	7.12	2.5	bla _{NDM} , floR, sull
IN_128	10	13	11	13	10	6	6	6	6	6	6	6	2.63	0.84	0.54	7.02	7.02	4.48	3.91	9.27	5.85	23.08	2.47	bla _{NDM} , sulll, strA, strB, int11
IN_129	10	13	11	13	10	6	6	6	6	6	6	6	4.37	1.86	1.09	17.26	17.26	2.84	4.4	9.96	8.44	8.34	2.74	bla _{NDM} , sulll, strB, int12

724 Values represent fold change (mean of duplicate samples) in comparison with the transcription level in wild-type strain PA01. In all cases the standard deviation
725 for duplicate samples was less than 15% of the mean. Values ≥ 2.0 are shown in bold. qRT-PCR, quantitative real-time-PCR chain reaction. CH – Switzerland,
726 DRC – Democratic Republic of the Congo, IN – India.
727 PRL to STR: Values represent the break point values (mm) interpreted according to the CLSI guidelines (CLSI, Jan, 2012).
728 In this study all the selected *P. aeruginosa* was resistant to Cotrimoxazole (SXT, 25 µg).
729 Abbreviations: Piperacillin (PRL, 100 µg), Piperacillin-tazobactam (TZP, 110 µg), Ceftazidime (CAZ, 30 µg), Cefepime (FEP, 30 µg), Aztreonam (ATM, 30
730 µg), Imipenem (IPM, 10 µg), Meropenem (MEM, 10 µg), Cefoperazone (CFP, 30 µg), Cefotaxime (CTX, 30 µg), Ofloxacin (OFX, 5 µg), Norfloxacin (NOR,
731 10 µg) and Streptomycin (STR, 10 µg).

Table. 4. Metal MIC values (mM) in *P. aeruginosa* isolates and wild type PA01 isolate.

Isolate	Zn	Cu	Co	Cd	Ni	Hg
PA01	12	7	3	3	4	1
CH 19	31	8	1	7	3	1
CH 21	21	8	2	8	3	1
DRC 27	23	10	4	5	2	2
DRC 38	21	8	3	8	3	2
DRC 39	15	8	3	8	3	1
IN 85	17	7	4	8	4	1
IN 87	17	11	4	9	4	2
IN 96	27	12	3	7	3	2
IN 100	29	13	3	9	4	3
IN 103	21	13	3	11	4	3
IN 109	15	8	4	7	3	1
IN 117	21	8	2	7	3	5
IN 125	19	7	3	7	4	1
IN 128	23	10	3	8	3	1
IN 129	9	7	1	3	3	1

MIC values above PA01 isolate are shown in bold.

CH – Switzerland, DRC – Democratic Republic of the Congo, IN – India