



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



Naresh Devarajan^a, Thilo Köhler^{b, c}, Periyasamy Sivalingam^a, Christian van Delden^{b, c}, Crispin K. Mulaji^d, Pius T. Mpiana^d, Bastiaan W. Ibelings^a, John Poté^{a, d, *}

^a 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

^b Department of Microbiology and Molecular Medicine, University of Geneva, 1, rue Michel Servet, CH-1211 Geneva 4, Switzerland

^c Transplant Infectious Diseases Unit, University Hospitals Geneva, 4, rue Gabrielle-Perret-Gentil, CH-1211 Geneva 14, Switzerland

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

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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.

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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;

* Corresponding author. 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.

E-mail address: john.pote@unige.ch (J. Poté).

URL: <http://www.unige.ch/forel/index.html>

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 (Poté 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

The sediment samples were collected from two different climate conditions in 2012; (i) Tropical conditions – sediments, receiving untreated hospital effluents, in the Democratic Republic of the Congo (DRC) and India (IN) and (ii) temperate climatic conditions: sediments from Lake Geneva receiving (partially) treated effluents from a waste water treatment plant (WWTP) in Switzerland (CH). The sampling sites, sample collection protocols and the sediment physicochemical properties are described in supporting

information (SI) and in our previous studies (Devarajan et al., 2015b; Mubedi et al., 2013; Thevenon et al., 2012). *Pseudomonas* spp. from the sediment samples were isolated as previously described (Poté et al., 2010; Haller et al., 2009). Briefly, the filter membranes were placed on *Pseudomonas* Selective Agar (PSA) supplemented with the antifungal agent Nystatin (Sigma, MO, USA) at a final concentration of 100 mg/L, for the counting of colony forming units (CFU). The plates were incubated at 37 °C for 24 h and the bacterial counts were expressed as CFU/100 g of fresh sediment. Approximately 10 isolates from each sampling site were randomly selected for further analysis. In total 141 isolates were taken for this study which include DRC (n = 32), IN (n = 85) and CH (n = 26) isolates. Antibiotic susceptibility testing was performed by the disk diffusion method on Müller-Hinton (MH) agar (Oxoid Ltd., Basingstoke, U.K.) as described by Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, Jan, 2012). All isolated bacteria were tested against 16 antimicrobials; Piperacillin (PRL, 100 μ g), Piperacillin-tazobactam (TZP, 110 μ g), Ceftazidime (CAZ, 30 μ g), Cefepime (FEP, 30 μ g), Aztreonam (ATM, 30 μ g), Imipenem (IPM, 10 μ g), Meropenem (MEM, 10 μ g), Cefoperazone (CFP, 30 μ g), Ofloxacin (OFX, 5 μ g), Norfloxacin (NOR, 10 μ g), Cefpirome (CPO, 30 μ g), Ampicillin (AMP, 10 μ g), Cefuroxime (CXM, 30 μ g), Streptomycin (STR, 10 μ g), Cefotaxime (CTX, 30 μ g) and Cotrimoxazole (SXT, 25 μ g) (Oxoid, 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 $a/(b \times c)$, 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.

2.1. 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 recipient subsequently diluted in fresh LB media and grown 4 h 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 SL_S3). The ribosomal *rpsL* gene (housekeeping gene in *P. aeruginosa*) was chosen to correct for the differences in starting material.

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

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 2.03×10^5 – 34.33×10^5 , 0.22×10^5 – 0.48×10^5 and 1.4×10^5 – 13×10^5 CFU/100 g 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 Fig. 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

Table 1
Pseudomonas spp. colony forming units (CFU) counts and the multiple antibiotic resistance (MAR) index for 16 tested antibiotics.

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

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

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

Sampling site Reference: V4, V7 and G1 (Thevenon et al., 2012).

Sampling site Reference: C1, C2 and C3 and H1 to H10 (Mubedi et al., 2013).

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

Donor (n = 36)	Transconjugants		Transfer frequency		
	Recipient	ARGs identified (n = 21)	10 °C	30 °C	37 °C
<i>P. aeruginosa</i>	<i>E. coli</i>	CTX-M (4); VIM (3); NDM (2)	n/a	0.5×10^{-5}	6.2×10^{-8}
<i>P. putida</i>	<i>E. coli</i>	SHV (2); CTX-M (3); VIM (3); NDM (1)	1.2×10^{-9}	0.9×10^{-4}	0.53×10^{-8}
<i>P. pseudoalcaligenes</i>	<i>E. coli</i>		n/a	n/a	n/a
<i>P. fulva</i>	<i>E. coli</i>	CTX-M (1)	n/a	1.7×10^{-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>	CTX-M (2)	n/a	8.3×10^{-5}	$<1 \times 10^{-9}$
<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.

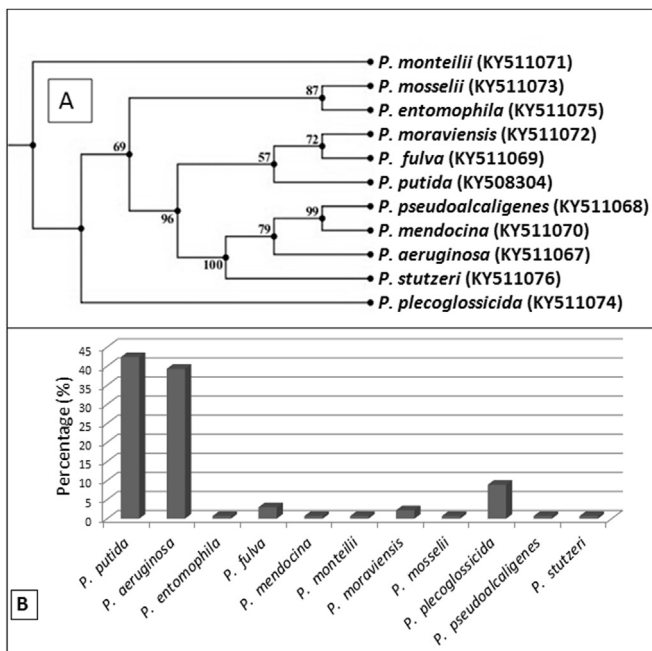


Fig. 1. (A). Distribution of *Pseudomonas* spp. and their phylogenetic relationship based on 16S rDNA sequences used to construct a maximum likelihood phylogenetic tree. Numbers at the branch nodes represent the boot strap values. (B) Total percentage of *Pseudomonas* spp. identified in this study.

ARGs include *aadA*, *strA* and *strB* which confer resistance to aminoglycosides, *sull*, *sullI* and *sullIII* 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, 2016; Laffite et al., 2016) highlighting the co-selection and co-existence of resistance mechanisms in environmental settings.

The genotypic resistance profiles are presented in Figs. 3 and 2S (Supporting Information). Aminoglycoside resistance genes (*strA*, *strB* and *aadA*) were found in *Pseudomonas* spp. from IN and CH. Eight percent of isolates from IN carried all three (*strA*, *strB* and *aadA*) aminoglycoside ARGs, 33% carried *strA* and *strB*, 14% carried *strA* and *aadA*, and 2% carried *strA* alone. DRC isolates were negative for *strA* and *strB*, and carried only *aadA* (3%). Few isolates (4%) from CH were PCR positive for all 3 aminoglycoside ARGs. Interestingly,

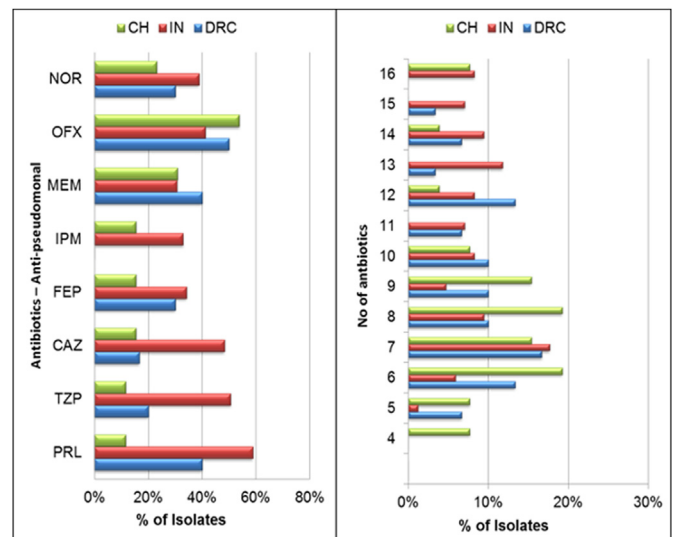


Fig. 2. Comparison of phenotypic resistance in the studied *Pseudomonas* spp. between DRC (n = 30), IN (n = 85) and CH (n = 26). Left panel: percentage of isolates resistant to the antipseudomonal agents. Right panel: percentage of isolates resistant to multiple number of antibiotics (n = 16) in this study. Fig note: CH – Switzerland, DRC – Congo Democratic Republic, IN – India. Piperacillin (PRL, 100 μ g), Piperacillin-tazobactam (TZP, 110 μ g), Ceftazidime (CAZ, 30 μ g), Cefepime (FEP, 30 μ g), Imipenem (IPM, 10 μ g), Meropenem (MEM, 10 μ g), Ofloxacin (OFX, 5 μ g) and Norfloxacin (NOR, 10 μ g).

23% of the isolates were phenotypically resistant to STR but PCR results were negative for all three screened aminoglycoside ARGs.

All the isolates from DRC and CH were PCR negative for the 3 *sul* genes tested (*sull*, *sullI* and *sullIII*). On the other hand, 25, 19 and 4% of IN isolates were found to carry *sull*, *sullI* and *sullIII*, respectively. The florfenicol resistance gene *floR* was found in many CH isolates (65%), followed by IN (28%) and DRC (27%) isolates. The *cmlA* gene was identified in 12, 20 and 25% of the isolates from CH, DRC and IN, respectively. We performed a PCR-based screening of all isolates for the presence of *bla_{SHV}*, *bla_{CTX-M}*, *bla_{NDM}*, *bla_{VIM-1}* and *bla_{VIM-2}* β -lactamase genes. The *bla_{SHV}* gene was identified in 3, 8 and 13% of the isolates from CH, DRC and IN, respectively. The prevalence of *bla_{CTX-M}* gene was higher in CH (65%), followed by DRC (27%) and IN (19%) isolates. On the other hand the metallo β -lactamases were found in the majority of isolates from IN and DRC. Eight and 34% of the isolates from CH and IN respectively were found to carry *bla_{NDM}*. Isolates from IN were also found to carry *bla_{VIM-1}* (26%) and *bla_{VIM-2}* (16%). However, isolates from DRC were negative for the *bla_{NDM}* and *bla_{VIM-2}*, but positive for *bla_{VIM-1}* (47%).

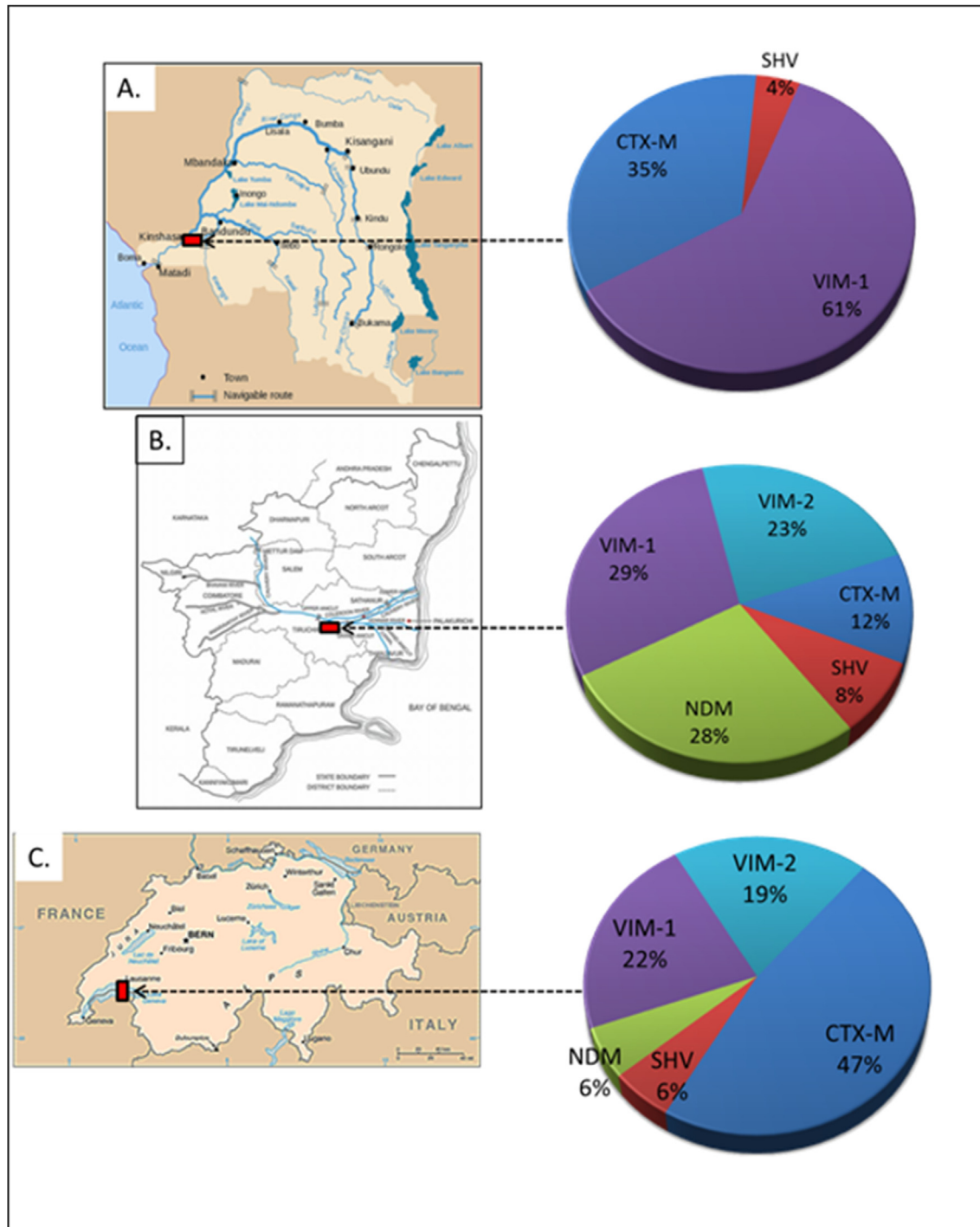


Fig. 3. Comparison and ratio distribution among β -lactamases in the studied *Pseudomonas* spp., isolated from the sediments receiving treated/untreated effluent in (A) Democratic Republic of the Congo; (B) India; (C) Switzerland.

Integrase genes including those from classes 1, 2 and 3 were screened in all the isolates. *Int1* and *int2* were identified in *Pseudomonas* spp., however, none of the isolates carried *int3*. The integrase genes, *int1* was identified in 12 and 34% of the isolates from CH and IN, respectively. Isolates from DRC were PCR negative for *int1* and 13% of the isolates carried *int2*. All isolates from CH were PCR negative for *int2*, while 14% of the isolates from IN were PCR positive for *int2*.

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 4–5 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

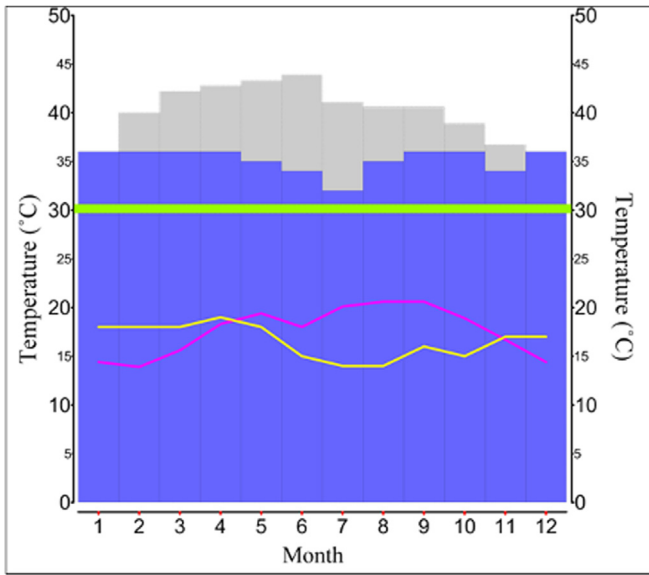


Fig. 4. Mean high and low temperature of Tiruchirappalli, Tamil Nadu, India and Kinshasa, Democratic Republic of the Congo; Tiruchirappalli Grey bar, Pink line representing mean high and low, respectively. Kinshasa – Blue bar, Yellow line representing mean high and low, respectively. Green line indicating the temperature of 30 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 (Table 4).

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

Table 3 Relative expression of selected genes in *P. aeruginosa* determined by quantitative PCR.

Isolate	Gene																									
	Origin	PRL	TZP	CAZ	FEP	ATM	IPM	MEM	CPP	CTX	OPX	NOR	STR	mexR	mexA	oprM	mexZ	mexY	mexX	nfxB	mexC34	mexT	mexE	oprD34	ARGs present	
PAO1	25	26	25	25	26	22	22	25	16	16	19	25	14	1	1	1	1	1	1	1	1	1	1	1	1	Not studied
CH_19	11	13	11	14	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	1	<i>bla</i> _{CTX-M} , <i>floR</i>
CH_21	13	18	10	14	10	6	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	1	<i>bla</i> _{NDM} , <i>aacA</i> , <i>int1</i>
DRC_27	24	22	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	1	<i>bla</i> _{CTX-M} , <i>cmIA</i>
DRC_38	26	24	20	18	6	22	26	22	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	1	<i>bla</i> _{CTX-M} , <i>aacA</i> , <i>int2</i>
DRC_39	26	26	21	17	6	23	25	21	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	1	<i>bla</i> _{VIM-1} , <i>floR</i>
IN_85	6	8	9	14	10	6	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	1	<i>bla</i> _{VIM-2} , <i>sull</i> , <i>strA</i> , <i>int1</i>
IN_87	10	11	16	17	15	6	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	1.5	1	<i>bla</i> _{NDM} , <i>sull</i> , <i>strA</i> , <i>strB</i> , <i>int1</i>
IN_96	19	21	19	23	6	30	19	16	9	19	24	12	12	6.98	2.44	0.88	9.51	9.51	2.29	4.23	3.17	7.72	6.67	1.5	1	<i>bla</i> _{VIM-2}
IN_100	22	21	21	23	10	28	21	18	10	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	1	<i>bla</i> _{CTX-M} , <i>strA</i> , <i>strB</i> , <i>aacA</i> , <i>int1</i>
IN_103	18	20	20	11	11	6	18	14	11	20	24	10	10	3.06	1.27	0.56	7.13	7.13	1.62	2.73	1.98	5.31	6.03	1.49	1	<i>bla</i> _{VIM-1} , <i>sull</i> , <i>floR</i>
IN_109	19	22	21	18	9	28	23	18	11	11	21	25	13	3.87	1.09	0.88	6.82	6.82	0.35	3.96	3.46	6.62	14.48	2.38	1	<i>bla</i> _{CTX-M} , <i>sull</i> , <i>cmIA</i> , <i>int2</i>
IN_117	23	21	21	23	10	18	12	18	12	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	1	<i>bla</i> _{VIM-1} , <i>sull</i> , <i>cmIA</i> , <i>aacA</i>
IN_125	13	18	10	14	10	6	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	1	<i>bla</i> _{NDM} , <i>floR</i> , <i>sull</i>
IN_128	10	13	11	13	10	6	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	1	<i>bla</i> _{NDM} , <i>sull</i> , <i>strA</i> , <i>strB</i> , <i>int1</i>
IN_129	10	13	11	13	10	6	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	1	<i>bla</i> _{NDM} , <i>sull</i> , <i>strB</i> , <i>int2</i>

Values represent fold change (mean of duplicate samples) in comparison with the transcription level in wild-type strain PAO1. In all cases the standard deviation 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, DRC – Democratic Republic of the Congo, IN – India. PRL to STR: Values represent the break point values (mm) interpreted according to the CLSI guidelines (CLSI, Jan, 2012). In this study all the selected *P. aeruginosa* was resistant to Cotrimoxazole (SXT, 25 µg). Abbreviations: Piperacillin (PRL, 100 µg), Piperacillin-tazobactam (TZP, 110 µg), Ceftriaxime (CAZ, 30 µg), Cefepime (CEP, 30 µg), Aztreonam (ATM, 30 µg), Imipenem (IPM, 10 µg), Meropenem (MEM, 10 µg), Cefoperazone (CFP, 30 µg), Cefotaxime (CTX, 30 µg), Ofloxacin (OPX, 5 µg), Norfloxacin (NOR, 10 µg) and Streptomycin (STR, 10 µg).

Table 4
Metal MIC values (mM) in *P. aeruginosa* isolates and wild type PAO1 isolate.

Isolate	Zn	Cu	Co	Cd	Ni	Hg
PAO1	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 PAO1 isolate are shown in bold.

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

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

Sulfonamides act as competitive inhibitors of dihydropteroate synthase (DHPS), involved in folic acid synthesis. The prevalence of *sull*, *sullII* and *sullIII* genes in the environment is most likely due to dissemination of these genes via mobile genetic elements (Stoll et al., 2012). Dissemination of sulfonamide resistance genes in the aquatic environment has been reported from other parts of the world (Hu et al., 2008; Hamelin et al., 2006). In this study, 93% of the isolates were non-susceptible to SXT. The potency of sulfonamides against *P. aeruginosa* is limited. The constitutive expression of the MexAB-OprM efflux pump is mainly responsible for this intrinsic resistance of *P. aeruginosa* to sulfonamides (Kohler et al., 1996). Sulfonamide resistant genes were identified in the isolates from IN sediments, while none of the DRC and CH isolates carried sulfonamide resistance genes. In previous reports, *suI* was linked to *intl1*, and in our study 47% of the isolates positive for *suI* genes were accompanied by a class 1 integrase (*intl1*) gene. Hence, the *suI* genes are likely to be “bystander” genes which are co-transferred with other resistance genes/cassettes in the mobile genetic elements. As reported in other studies (Srinivasan et al., 2007; Baran et al., 2011), excessive amounts of sulfa drugs are introduced in the biosphere from clinical and agricultural settings. Although the constitutive expression level of MexAB-oprM would be sufficient to

confer sulfonamide and trimethoprim resistance, sulfonamides released into the environment could select for *mexA* overexpression which makes strains more resistant to other antibiotics that are substrates of MexAB-OprM (Li et al., 2015; Dumas et al., 2006).

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

Florfenicol, a fluorinated structural analog of thiamphenicol and chloramphenicol was approved by the Food and Drug administration (FDA) in 1996 for treatment of bovine respiratory pathogens such as *Pasteurella* spp. in veterinary medicine and it is currently banned for the treatment of cattle enteric diseases in USA (White et al., 2000). Since then, florfenicol resistance has been detected in a wide variety of bacterial species and many of their resistance determinants reside on mobile genetic elements (Kim et al., 1993; Schwarz et al., 2004). Chloramphenicol resistance mechanisms include inactivation of chloramphenicol by acetyltransferases (*cat* genes) and chloramphenicol efflux pumps (*cml* genes) and multi-drug transporters (Schwarz et al., 2004). Florfenicol used in veterinary medicine is related to chloramphenicol and can select for cross-resistance among bacterial pathogens (Arcangioli et al., 2000; Bolton et al., 1999; Keyes et al., 2000). *floR* genes have been reported in *E. coli* isolated from humans and animals (Srinivasan et al., 2007), surface water samples in Germany and Australia (Stoll et al., 2012). In this study, we found *floR* in 65% of *Pseudomonas* isolates from CH samples, while 25% of the isolates from IN and 12% of the CH isolates were found to carry the *cmlA* gene. CH samples were collected from Vidy bay (Lake Geneva), where the sediments are receiving partially treated/untreated waste water from the WWTP (since 1964) and urban and agriculture runoff from several sources (Pote 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 (Pote 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 *ISEcp1* 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, tetracycline, sulfonamide or fluoroquinolone resistance genes (Canton, 2009). Indeed CTX-M was found in all the sampling regions (DRC, IN and CH). In the present study, β -lactam resistant isolates also conferred resistance to aminoglycosides and sulfonamides. Over 83% of the CTX-M positive isolates from IN sediments were also positive for *strA*, *strB* and *sul* genes. To our knowledge this is the first report on the prevalence of CTX-M genes in *Pseudomonas* spp. isolated from environmental samples in DRC.

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

Efficacy of carbapenems is compromised with the emergence of metallo- β -lactamases (MBL) carried on mobile genetic elements (Walsh et al., 2005). In 2007, six out of 33 countries in the European Antimicrobial Resistance Surveillance System (EARSS), reported carbapenem resistance in 25% of *P. aeruginosa* isolates with the highest rate in Greece (51%) (Hawkey and Jones, 2009). A recent review reports VIM-2 in 37 countries from 5 continents (Hawkey and Jones, 2009). In the present study *bla*_{VIM-1} was found in IN and CH samples; and VIM-2 in DRC and IN. The spread of MBLs among bacterial populations in the environment is of great concern, not only because these enzymes confer resistance to carbapenems and other β -lactams, but also because such bacteria are typically resistant to other antibiotics which limits treatment options. Our study also emphasizes the extent to which MBLs can disseminate within environmental bacterial communities.

Integrations are gene exchange systems and play an important role in the acquisition, accumulation and dissemination of antimicrobial resistance genes. In this study, 34% of the studied isolates were positive for integrations. Similarly, previous studies (Laroche et al., 2009; Hawkey and Jones, 2009; Skurnik et al., 2006) have also reported higher prevalence of class 1 integrations in the environment as a result of clinical effluent discharge and high levels of urbanization (Nardelli et al., 2012). Previous studies have demonstrated the prevalence of *int1* variants in environmental samples and the problem of detecting them in large numbers could be influenced by the large numbers of genetically homogenous class 1 integrations being shed to the environment by clinical and animal sources, human sewage, landfills and storm water arising from the urban environment (Gillings et al., 2008, 2015; Stalder et al., 2014). It is also possible that integrase genes have evolved to acquire ARGs in pathogenic and commensal bacteria, which could have serious implications for treating infections (Davies, 2007). There are large

numbers of mobile elements in the environment and potential resistance genes associated with them, which continue to be a threat to the human welfare. Hence these gene pools are valuable to 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 over-expression 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.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2017.02.058>.

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