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UNIVERSITÉ DE GENÈVE
Section des Sciences de la Terre et de l'Environnement
Institut F.-A. Forel

FACULTÉ DES SCIENCES
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Exploring Research Frontiers in Aquatic Ecosystems: Role of Hospital and Urban Effluents in the Dissemination of Antibiotic Resistance and Metals to Fresh Water Ecosystems

THÈSE

présentée à la Faculté des Sciences de l'Université de Genève pour obtenir le grade de Docteur ès Sciences, mention *mention Sciences de l'environnement*.

par

Naresh DEVARAJAN

de

Tamil Nadu (India)

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Thèse de *Monsieur Naresh DEVARAJAN*

intitulée :

" Exploring Research Frontiers in Aquatic Ecosystems: Role of Hospital and Urban Effluents in the Dissemination of Antibiotic Resistance and Metals to Fresh Water Ecosystems "

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"*Arise, Awake, and Stop not Till the Goal is Reached*"

Swami Vivekananda, India

(1863-1902)

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Abstract

Since the middle of the 20th century, pollution of freshwater resource by different types of contaminants is a major environmental problem in many parts of the world. Industrial and anthropogenic activities including urban effluent waters from dredge systems or wastewater treatment plants (WWTP) constitute major sources of aquatic environment contamination by inorganic and organic micro-pollutants comprising toxic metals, hydrophobic organic compounds (PCBs, PAHs, OCPs), pathogenic multidrug resistant bacteria and antibiotic resistance genes (ARGs). On the other hand, in many developing countries, latrine and fecal contamination is also known to affect the quality of water associated with ponds and wells. Nonetheless, the role of hospital effluents discharged into water ecosystem in the above mentioned context remains largely unstudied in tropical countries, such as India. Consequently, during the recent past, increasing attention has been paid to the characterization and quantification of emerging contaminants in wastewaters, surface waters, ground waters, soil and sediment.

The main objective of this interdisciplinary research was to assess the emerging contaminants (metals, bacteria and antibiotic resistance genes) as well as the prevalence of antibiotic resistant *Pseudomonas* spp. in the sediments receiving treated/untreated wastewaters. In particular, the effects of contamination on the composition and the diversity of bacterial communities in the sediments were also performed.

In developing countries, channeling of untreated wastewaters to the fresh water reservoirs remains a major problem. For that, we studied the effects of urban and hospital effluents in a tropical river receiving system, Cauvery River basin in Tamil Nadu, India. The results indicate the high values and distribution of metals, ARGs and selected bacterial species (*E. coli*, ENT, and *Pseudomonas* spp.) in the sediment samples located downstream of the effluent discharge point than in the upstream (control site). The metal concentrations in the sampling sites largely exceeded the Sediment Quality Guidelines (SQGs) and the Probable Effect Concentration (PEC) for the Protection of Aquatic Life recommendation. The ecotoxicological analysis demonstrated the potential toxic effects on the aquatic biota.

The effects of wastewater treatment plant on the accumulation of emerging contaminants in the sediments from the Bay of Vidy, Lausanne, Switzerland were evaluated. Higher abundance of selected bacterial species including FIB (*E. coli* and *Enterococcus* spp.) and *Pseudomonas* spp. was found in sediments contaminated by WWTP when compared to the control site. The ARGs, especially the ESBLs and MBLs were identified in the surface sediments indicating their recent deposition in the lake sediments. A strong correlation existed with the ARGs and FIB ($r \geq 0.403$, $p < 0.05$, $n = 34$) with total organic matter and metal concentrations in the sediments. These results indicate a common and contemporary source of contamination and highlight sediment receiving treated/untreated wastewaters to act as reservoirs for these emerging contaminants.

We characterized the phenotypic and genotypic resistance profiles of *Pseudomonas* spp. isolated from the sediments receiving treated/untreated effluents under tropical and temperate climatic conditions. The environmental *Pseudomonas* spp. isolated from Switzerland (CH) were more susceptible to antipseudomonal antibiotics than the isolates from Democratic Republic of Congo (DRC) and India (IN). Multidrug resistant isolates were identified in all the three sampling locations. In this study, 7 and 2 isolates from IN and CH, respectively were resistant to 16 Abs tested. Sulfonamide resistant genes were identified only in the isolates from IN. Florfenicol resistant gene, *floR* was found abundant in CH isolates (65%), followed by IN (28%) and DRC (27%) isolates. Among the ß-lactamases, CTX-M was the most dominant in the CH isolates and VIM was dominant in DRC and IN isolates. The bla_{NDM} gene was identified in 8 and 34% of the isolates in CH and IN, respectively. Higher conjugation frequencies were observed at tropical temperature (30°C) than at the lower temperature (10°C). Chromosomal mediated efflux mechanisms conferred co-resistance to both metals and antibiotics.

Concerning the effects of contamination on the composition and the diversity of bacterial communities, the pyrosequencing results from distinct geographical study sites (Congo DR, India, Senegal and Switzerland) revealed significant effects induced by the metal contaminants on bacterial diversity. Several large shifts in the bacterial community were identified at the Class level with enriched amount of α, ß and γ-proteobacteria in the soil/sediments receiving contaminants from recreational shooting, mining and hospital effluents, respectively.

Contamination of water resources due to the release of treated/untreated wastewaters remains problematic in many parts of the globe. Results of our research highlight the release of emerging contaminants from urban and WWTP effluent waters. Consequently, the sediments receiving system can act as reservoir for biological emerging pollutants such as toxic metals, hydrophobic organic compounds and ARGs which could potentially be transferred to susceptible bacterial pathogens. The remobilization of these contaminants to the overlying water may place aquatic organisms and human at risks. Therefore, the reduction of contaminants from the sources is recommended for further improvement of water quality.

Résumé

La pollution des ressources en eau par les différents types de contaminants constitue un problème environnemental majeur dans le monde de puis le milieu du 20ème siècle. Les activités anthropogéniques et industrielles, notamment les systèmes d'évacuation d'eaux usées urbaines et les stations d'épurations (WWTP), constituent une source majeure de contamination pour le système aquatique par des micro-polluants organiques et inorganiques tel que les métaux toxiques, les composés organiques hydrophobiques (PCBs, PAHs, OCPs), les bactéries pathogènes multirésistantes et leurs gènes de résistance aux antibiotiques (ARGs). De plus, dans la plupart des pays en voie de développement, les latrines et la contamination fécale sont aussi connues comme affectant la qualité de l'eau des puits et des étangs. Cependant, le rôle des effluents hospitaliers rejetés dans des conditions similaires au sein des écosystèmes aquatiques reste un sujet peu étudié dans les pays tropicaux tel que l'Inde. En conséquence, une attention grandissante a été accordée ces dernières années en vue de la caractérisation et de la quantification de ces contaminants émergents dans les eaux usées, les eaux de surface, les nappes phréatiques ainsi que le sol et le sédiment.

Le principal objectif de cette recherche interdisciplinaire était d'évaluer la présence de ces contaminants émergents (métaux, bactéries et gènes de résistance aux antibiotiques), d'établir la prévalence de Pseudomonas spp. dans les sédiments soumis sous l'influence des eaux usées traitées ou non traitées, ainsi que d'évaluer les effets de la contamination sur la composition et la diversité des communautés bactériennes dans les sédiments.

Dans les pays en voie de développement, la canalisation des eaux usées non traitées vers les réservoirs d'eau douce constitue un problème majeur. Pour cela, nous avons étudiés les effets des effluents urbains et hospitaliers sur une rivière réceptrice, la rivière Cauvery dans le Tamil Nadu, Inde. Les résultats indiquent une forte quantité et une distribution importante des métaux, des ARGs et des espèces bactériennes étudiées (E.coli, ENT et Pseudomonas spp.) dans les sédiments échantillonnés en aval du point de décharge des effluents par rapport à ceux échantillonnés en amont (site contrôle). La concentration en métal aux sites d'échantillonnages excède largement les recommandations pour la qualité des sédiments (SQGs) et la concentration à effet probable (PEC) édités par la Protection de la Vie

Aquatique. Des analyses écotoxicologiques ont montré les potentiels effets toxiques sur la faune aquatique.

Les effets des stations d'épuration (WWTP) sur l'accumulation des contaminants émergents dans les sédiments de la Baie de Vidy, Lausanne, Suisse ont été évalués. Les quantités importantes des bactériennes analysées, dont les FIB (E. coli et Enterococcus spp.) et Pseudomonas spp. ont été détectées dans les sédiments contaminés par la WWTP par rapport aux sites de contrôle. Des ARGs, et plus spécifiquement les ESBLs et les MBLs ont été identifiés à la surface des sédiments, ce qui indique leurs récentes dépositions dans les sédiments lacustres de la baie. Une forte corrélation existe entre les ARGs et les FIB ($r \geq 0.403$, $p < 0.05$, $n = 34$) avec la matière organique totale et la concentration des métaux dans les sédiments. Ces résultats démontrent une source de contamination commune et contemporaine, ce qui indique que les sédiments qui sont soumis sous l'influence des eaux usées traités et ou partiellement traités jouent un rôle de stockage des contaminants émergents.

Nous avons caractérisés les profils de résistance phénotypique et génotypique de Pseudomonas spp. isolés des sédiments receveurs d'effluents traités ou non traités sous climat tropical et tempéré. Les souches environnementales de Pseudomonas spp. Isolés dans les échantillons de Suisse (CH) étaient plus susceptibles aux antibiotiques antipseudomonal que les isolats en provenance de République Démocratique du Congo (RDC) et de l'Inde (IN). Des isolats multirésistants ont étés identifiés dans chacun des trois sites d'échantillonnage. Dans cette étude, 7 et 2 isolats provenant respectivement d'IN et CH étaient résistants à 16 antibiotiques testés. Des gènes de résistance aux sulfonamides ont été identifiés uniquement dans les isolats provenant des échantillons de l'Inde. Le gène de résistance au florfénicol (floR) était abondant dans les isolats des échantillons de la suisses (65%), suivi par ceux de l'Inde (28%) et de la RDC (27%). Parmi les β-lactamases, CTX-M était le plus abondant dans les isolats suisses alors que le gène VIM était le plus abondant dans les isolats provenant de RDC et IN. Le gène blaNDM a été identifié dans 8 et 34% des isolats de CH et IN respectivement. Des fréquences de conjugaisons plus élevées ont été observées avec des températures tropicales (30°C) par rapport aux températures plus froides (10°C).

En ce qui concerne les effets de la contamination sur la composition et la diversité des communautés bactériennes, les résultats de pyroséquençage provenant de sites d'étude géographiquement différents (RD Congo, Inde, Sénégal et Suisse) ont révélé des effets

significatifs sur la diversité bactérienne induit par les métaux. Une forte modification de la communauté bactérienne, a été identifiée au niveau des groupe avec un enrichissement en α, β, γ-protéobactérie dans le sol/ sédiment recevant des contaminants provenant respectivement de stand de tir, de mines et d'effluents hospitalier.

La contamination des ressources en eau en raison du rejet des eaux usées non traitées ou partiellement traitées reste un problème majeur dans le monde. Les résultats de notre recherche démontrent le rejet des contaminants émergents par les effluents urbains et de WWTP. En conséquence, les sédiments soumis sous l'effet de ces effluents peuvent être considérés comme réservoir pour les polluants émergents tel que les métaux toxiques, les composés organique hydrophobes et les ARGs (qui peuvent être transférer génétiquement aux espèces bactériennes autochtones). La remobilisation de ces contaminants vers la colonne d'eau peut conduire à des risques pour la santé humaine et la santé de la faune aquatique. Donc, cette recherche recommande une réduction et élimination des contaminants à partir des sources polluantes pour une amélioration et préservation de la qualité de l'eau.

Abbreviation list

Abs: antibiotics
ACE: abundance-based coverage estimators
AHC: Agglomerative hierarchical clustering
AMA: advanced mercury analyzer
AMR: antimicrobial resistance
AR: antibiotic resistance
ARB: antibiotic resistant bacteria
ARG: antibiotic resistant gene
CH: Switzerland
CHUV: centre hospitalier universitaire Vaudois
CRB: Cauvery River Basin
CSQG: Canadian sediment quality guidelines
C_t: threshold cycle
DRC: democratic republic of Congo
E. coli: *Escherichia coli*
ENT: *Enterococcus*
ESBLs: extended spectrum ß-lactamases
FDA: food and drug administration
FIB: faecal indicator bacteria
HGT: horizontal gene transfer
HOP: hospital outlet pipes
ICP-MS: Inductively coupled plasma mass spectrometry
IN: India
LOD: limit of detection
MBLs: metallo ß-lactamases
MDR: multi drug resistance
MFA: multi factorial analysis
MLD: millions of liters per day

OCPs:	organochlorine pesticides
OM:	total organic matter
OTUs:	operational taxonomic units
P. spp:	*Pseudomonas* species
PAHs:	polycyclic aromatic hydrocarbons
PCA:	principle component analysis
PCBs:	polychlorinated biphenyls
PCR:	polymerase chain reaction
PEC:	probable effect concentration
PEL:	probable effect level
ppm:	parts per million
qPCR:	quantitative polymerase chain reaction
SI:	supporting information
SN:	Senegal
SQG:	Sediment Quality Guidelines
WWTP:	waste water treatment plant

CHAPTER 1

Introduction

Water is a natural resource vital for the survival of humanity and all species on earth. Fresh water compromises 3% of the total water on earth, and of that, less than 1% is available for human use in the form of lake, rivers, streams and aquifers (Hahn 2006). The organic/inorganic contaminants released in the environment often end up in the fresh water ecosystem and accumulate in the soil/sediment, or reach the groundwater (Wildi et al. 2004). Anthropogenic activities such as industrial, sewage, hospital, agriculture, landfills, mining are a leading source for the spread of these contaminants in the global environment (Thevenon and Pote 2012). Almost all human activities can and do have an adverse impact on water. Access to safe water is a basic human right and it is a precondition for human health and well-being to have access to clean water for drinking and sanitary purposes.

Unpolluted water is also essential for ecosystems. In lakes, rivers and seas, plants and animals react to changes in the environment induced by changes in chemical water quality and physical disturbance of their habitat (WMO 2013). The nature and concentration of chemical elements and compounds in a freshwater system are subject to change by various types of natural processes – physical, chemical, hydrological and biological – caused by climatic, geographical and geological conditions. Wastewaters change the water and bottom sediment chemical composition, destroy the biological balance of the self-cleaning processes and finally can cause unpredictable conversions in the receiving water ecosystem. Hence wastewaters entering receiving water bodies become one of the main factors of pollution of natural waters (Kazlauskiene et al. 2012).

Sources of water pollution are generally grouped into two categories based on their origin: point source and non-point source (Ouattara et al. 2011). Point source of pollution includes the contaminants that are released to a waterway through a discrete conveyance, such as outlet pipe and sewer channel. Examples of the point source of effluent discharge include; waste water treatment plants (WWTP), industrial and mining effluents outlet pipes. Non-point source are sources that cannot be traced to a single point of discharge. Examples of non-point source are: acid rain, automobiles, and runoffs in storm-water over an agricultural field. The constituents of wastewater varies widely and the contaminants leading to pollution include bacterial pathogens, viruses, parasitic worms, organic particles such as faces, organic soluble material such as urea, phosphorus, drugs, inorganic particles such as sand, silt, metals, plastics, gases such as methane, hydrogen sulfide, and other contaminants such as emulsions, paints, pesticides, toxins, herbicides and radioactive materials.

Water-quality monitoring provides an understanding of: (a) quality of water in streams, rivers, groundwater and aquatic systems; (b) variation of water quality conditions on native, regional and global scale; (c) does conditions change over period; (d) effect of natural features and anthropogenic activities on those conditions; and (e) where those effects are most pronounced. Hence, the monitoring of water quality to provide reliable and usable data involves many distinct activities and can be expensive. Thus, the first step in planning the establishment of such a system should be to define the objectives of monitoring (such as in support of management, research or policy) and what water quality issues are to be addressed. Water quality monitoring and assessment can be looked at from different perspectives in terms of basic variables and present status, time trends and spatial differences, uses, pollution impacts and management needs for information for decisions and action (UNEP/WHO 1996).

1.1. Chemical contaminants

One of the key environmental problems facing humanity is the increasing worldwide

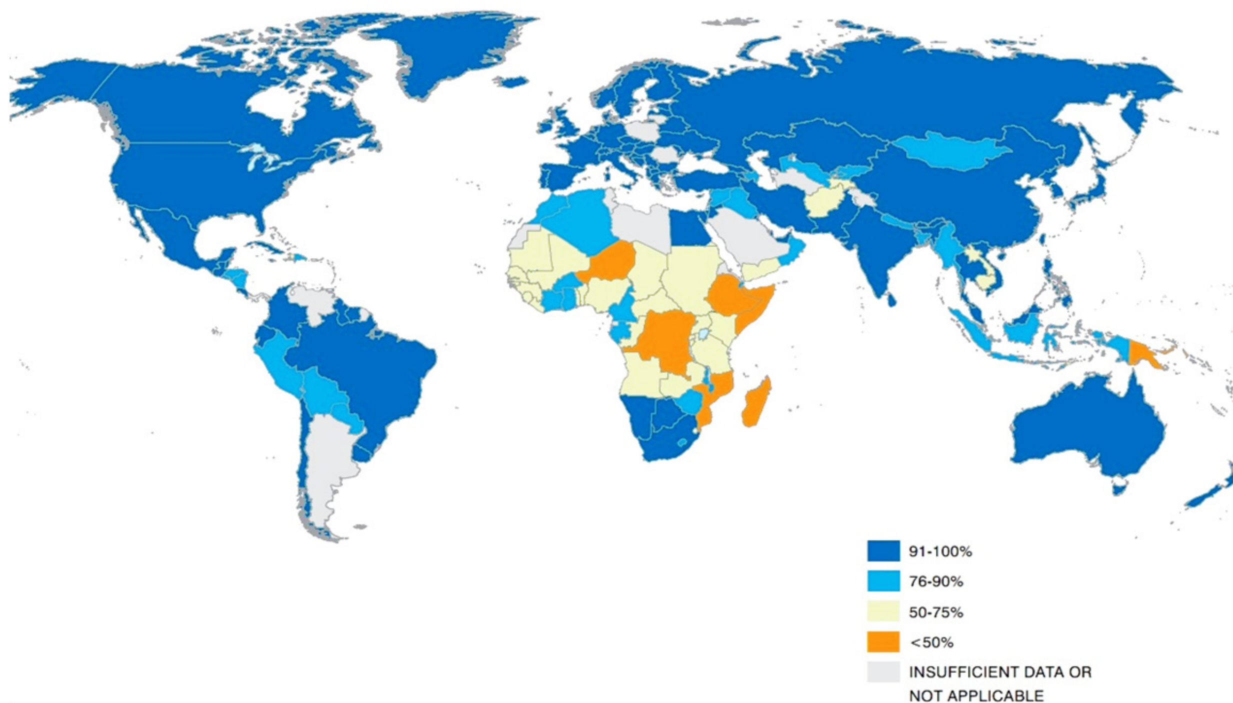


Figure. 1.1 Proportion of population using improved drinking water sources (%) 2010. Reference: (WHO 2012).

contamination of freshwater systems with thousands of industrial and natural chemical compounds. Their presence at low concentrations, raise considerable toxicological concerns as they are present as components of complex mixtures (Schwarzenbach et al. 2006).

Chemical contaminants in water include two subgroups namely: macro-pollutants and micro-pollutants.

1.1.1. Macro-pollutants in aquatic ecosystem

Macro-pollutants are chemical compounds, including organic compounds, such as acids, salts, nutrients and organic matter naturally present in the environment, which can become toxic at high concentrations (occurring at µg/liter or mg/liter). Enrichment of water sources with nitrates and phosphates results in eutrophication. Enhanced plant growth and depleted oxygen levels are the results of eutrophication. This issue often linked to municipal effluent releases and agricultural runoff containing soluble nitrates and phosphates. Deposited in large amounts these macro-pollutants may cause uncompromising effects such as oxygen depletion and results in the lack of decomposition of organic matter and unable to support many forms of aquatic life. Based on the level of eutrophication, severe effects on the water quality and aquatic ecosystem may occur. In most developing nations eutrophication remains a critical problem, whereas most of the developed nations have overcome this issue by installing waste water treatment plants (WWTP) (Dorioz et al. 1998). Again the challenges in eutrophication are to optimize treatment technologies and to develop integrated policies at the scale of aquatic basins (Jackson et al. 2001).

1.1.2. Micro-pollutants in aquatic ecosystem

Micro-pollutants are chemical compounds including inorganic (metals) and hydrophobic organic compounds, for example metals, polychlorinated biphenyls. Micro-pollutants differ from macro-pollutants based on: (1) their toxicity at very low concentrations (pg/liter or ng/liter), (2) very weak biodegradation, (3) inhibition of biological process and their negative effects on prokaryotes/eukaryotes, (4) potential bioaccumulation in the food chain. Additionally, it is far more difficult to assess the effect on the aquatic environment of the thousands of synthetic and natural trace contaminants that may be present in water at low concentrations (Schwarzenbach et al. 2006).

1.2. Metals in aquatic ecosystem

Metals such as iron, copper, manganese, magnesium, and zinc are essential for living organisms. However, excessive levels of the same induce damages to the organisms. Other heavy metals such as mercury and lead are toxic metals that have no beneficial biological role on organisms and their addition over time in the living system can cause/induce serious illness

(Salomons and Förstner 1984). Since Stone Age a total of 1150 million tons of heavy metals have been exploited and used for various activities. At an annual growth rate of 3.4%, it is estimated that an annual output of 14 million tons of metals being mined (Sheoran and Sheoran 2006). Anthropogenic activities such as industrial, sewage, hospital, agriculture, landfills, mining are a leading source for the spread of metals in terrestrial environment (Pote et al. 2008a, Niane et al. 2014, Devarajan et al. 2015). Among the quantitatively pollutant groups, metals are highly persistent in the soil/sediment environment and are capable to affect all groups of organisms and ecosystem process, which include the microbial community mediated process (Muller et al. 2001). The interface of biology and inorganic chemistry “Bioinorganic chemistry” emerged as a distinct discipline around 1962 with the first "Metals in Biology". Since this period, numerous expansions have been made in understanding the mechanisms and functions of metals in biological systems. In recent years, much progress has been made in the description of complication and phase-transfer processes of inorganic and organic micro-pollutants at the molecular level (Nature 2008).

1.3. Antibiotics in aquatic ecosystem

Antibiotics (Abs) are used extensively to treat bacterial infections in human and animal and also for prophylaxis. Most of these compounds administrated to humans/animals are only partially metabolized and are often discharged into the hospital sewage system or directly into sewer systems if used at residents. Along with feces, they flow with communal/municipal waste water and may pass by the sewer system and end up in the terrestrial environment, especially in the water compartment. Abs also used in aquaculture, and employed to promote more rapid growth of livestock. Abs used for livestock arrive at the terrestrial environment/water compartment when manure is applied to fields. Abs reaching the environmental waster bodies may either end up in soil or sediment or in surface/ground water (Allen et al. 2010, Kummerer 2004, Pachepsky et al. 2011).

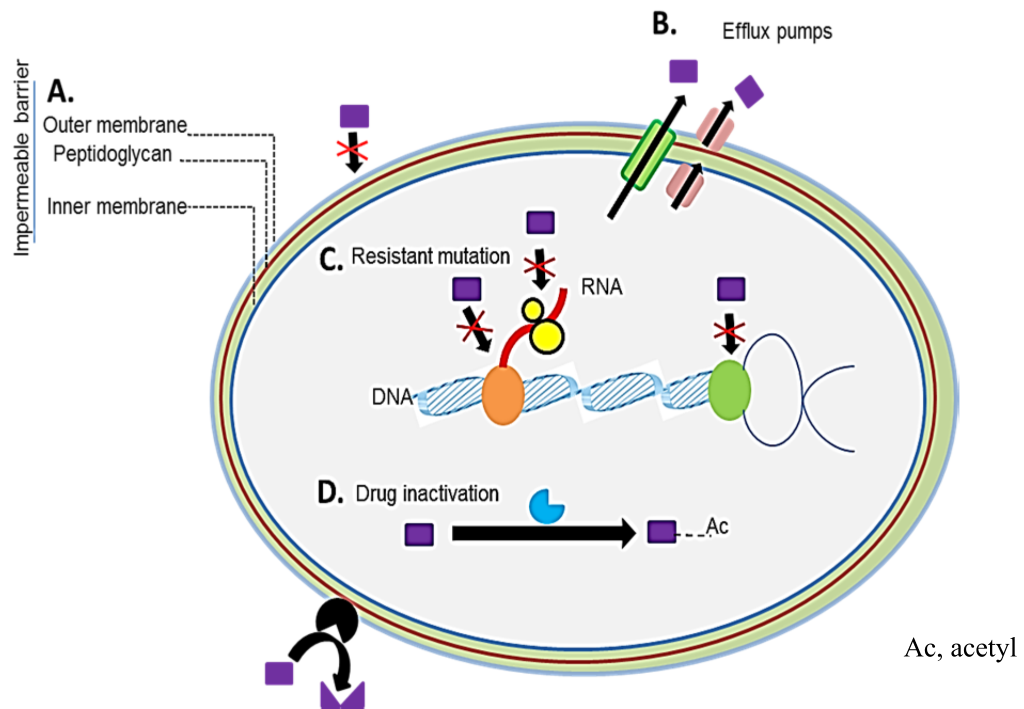
1.3.1. Antibiotic resistance

Bacterial antibiotic resistance (AR) is the capacity of bacteria to withstand the effects of Abs or biocides and the relative or complete ability of an bacterium, to counteract the desired bactericidal or bacteriostatic effect of one (antibiotic resistant bacteria-ARB) or more (multi drug resistance-MDR)) Abs or antibiotic classes. The potential development of tolerance or resistance to a therapeutic compound is thus compromised from the time it is first employed

(Davies and Davies 2010). Emergence of new resistant mechanisms and global spread are threating our ability to treat common infections, resulting in high mortality and disability of persons who until lately could continue a normal course of life. Infections caused by ARB/MDR often counteract to the standard prophylaxis, resulting in extended ailment/illness, higher expenditures, and increased risk of fatality. As an example, the fatality rate for patients treated in clinical settings with infections caused by resistant bacteria can be about twice that of patients with infections caused by the same non-resistant bacteria. MRSA (methicillin-resistant *Staphylococcus aureus*, a common source of severe infections in the community and in hospitals) are estimated to be 64% more likely to die than people with a non-resistant form of the infection (WHO 2014). In the natural environment, the presence of various potentially offensive compounds and conditions might select for exact or generic mechanisms of AR. Microbes (esp. bacteria) isolated from marine, air-water interface, soil, sediment have shown to be highly resistant to Abs than microbes cultured from bulk water. Several conditions, including radiation and pollution, that may act as a selective pressure for AR in terrestrial environment are been suggested (Allen et al. 2010).

1.3.2. Mechanisms of antibiotic resistance

The mode of action of some Abs and their targets in a bacterial cell and the mode of resistance by the bacterial cell are summarized in Table 1.1. For example in gram negative bacteria the following possible mechanisms of AR are observed (Figure 1.2.). A. Impermeable barriers. Some bacteria are naturally resistant to certain Abs (purple squares) due to impermeable membrane or lack the target of the Abs. B. Multidrug resistance efflux pumps. These pumps discharge Abs from the cell. Transporters, such as those of the resistance–nodulation–cell division family (green), can pump Abs precisely outside the cell, whereas others, such as those of the major facilitator superfamily (light red), pump them into the periplasm. C. Resistance mutations. Certain mutations modify the target protein, for example by disabling the Ab-binding site but leaving the cellular functionality of the protein intact. Specific examples include mutations in the gyrase (green), resulting in resistance to floroquinolones, in RNA polymerase subunit B (orange), resulting in resistance to rifampicin, and in the 30S ribosomal subunit protein S12 (encoded by *rpsL*) (yellow), resulting in resistance to streptomycin. (D) Inactivation of Abs. Inactivation can occur by covalent modification of the Abs, such as that catalyzed by acetyl-transferases (blue) acting on aminoglycoside Abs, or by degradation of the Abs, such as that catalyzed by ß-lactamases (black) acting on β-lactams.



Ac, acetyl

Figure 1.2. Mechanism of antibiotic resistance in gram negative bacteria adapted from (Allen et al. 2010)

Table 1.1 Mode of action and resistance mechanisms of commonly used antibiotics. Source Morar and Wright 2010

Antibiotic class	Example(s)	Target	Mode(s) of resistance
ß-Lactams	Penicillin's (ampicillin), cephalosporin's (cephamycin), penems (meropenem), monobactams (aztreonam)	Peptidoglycan biosynthesis	Hydrolysis, efflux, altered target
Aminoglycosides	Gentamicin, streptomycin, spectinomycin	Translation	Phosphorylation, acetylation, nucleotidylation, efflux, altered target
Glycopeptides	Vancomycin, teicoplanin	Peptidoglycan biosynthesis	Reprogramming peptidoglycan biosynthesis
Tetracycline's	Minocycline, tigecycline	Translation	Monooxygenation, efflux, altered target
Macrolides	Erythromycin, azithromycin	Translation	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Lincosamides	Clindamycin	Translation	Nucleotidylation, efflux, altered target
Streptogramins	Synercid	Translation	C-O lyase (type B streptogramins), acetylation (type A streptogramins), efflux, altered target
Oxazolidinones	Linezolid	Translation	Efflux, altered target
Phenicols	Chloramphenicol	Translation	Acetylation, efflux, altered target
Quinolones	Ciprofloxacin	DNA replication	Acetylation, efflux, altered target
Pyrimidines	Trimethoprim	C_1 metabolism	Efflux, altered target
Sulfonamides	Sulfamethoxazole	C_1 metabolism	Efflux, altered target
Rifamycins	Rifampin	Transcription	ADP-ribosylation, efflux, altered target
Lipopeptides	Daptomycin	Cell membrane	Altered target

1.4. ß-lactams

Bacterial cell wall, a primary structural feature, whose function is providing support for the maintenance of bacterial morphology, and its function, is absolutely indispensable for the organism. Lack of an effective cell wall, the bacteria would be capable of surviving only in media that match their internal osmotic pressure. A major component of the cell wall is the peptidoglycan, and families of enzymes (PBPs) are responsible for the assembly, maintenance and regulation of the function of peptidoglycan structure (Ghuysen 1991).

Approximately two-third of antibiotics administrated to humans are ß-lactams (Stoll et al. 2012). ß-Lactam Abs exhibits their bactericidal effects by inhibiting enzymes involved in cell wall synthesis. In a hypertonic and hostile environment, the integrity of the bacterial cell wall is essential in maintaining cell shape. The ß-lactam ring is sterically similar to the D-alanine–D-alanine of the NAM pentapeptide, and PBPs fallaciously use the ß-lactam as building components during cell wall synthesis. This results in acylation of the PBP, which renders the enzyme unable to catalyze further transpeptidation reactions. As cell wall synthesis slows to a halt, constitutive peptidoglycan autolysis continues. The breakdown of the murein sacculus leads to cell wall compromise and increased permeability. Thus, the ß-lactam-mediated inhibition of transpeptidation causes cell lysis (Bayles 2000, Drawz and Bonomo 2010).

1.4.1. Resistance to ß-lactam antibiotics

Four mechanisms are identified by which bacteria can overcome ß-lactam Abs.

- Production of ß-lactamase enzymes is the most common and important mechanism of resistance in Gram-negative bacteria.
- Changes in the active site of PBPs can lower the affinity for ß-lactam antibiotics and subsequently increase resistance to these agents.
- Decreased expression of outer membrane proteins such as *oprD* in *P. aeruginosa*.
- Efflux pumps, as part of either an acquired or intrinsic resistance phenotype, are capable of exporting a wide range of substrates from the periplasm to the surrounding environment

1.4.2. ß-lactamases

Genes in a microorganism which confer resistance to Abs are termed as antibiotic resistant genes (ARGs), for example these genes may by coding for enzymes which destroy it, by coding for surface proteins which prevent it from entering the microorganism, or by being a mutant form of the Abs target so that it can ignore it. The first ß-lactamase enzyme was identified in *Bacillus* (*Escherichia*) *coli* before the clinical use of penicillin. Currently, more than 850 ß-lactamases are identified (Drawz and Bonomo 2010), which include Amber and Bush-Jacoby-Medeiros classification system. For example the Ambler classification system in detail:

- Class A serine ß-lactamases and (e.g.: bla_{TEM} and bla_{SHV}) & Extended spectrum ß-lactamases (ESBLs) (eg: $bla_{\text{CTX-M}}$ and $bla_{\text{GES-1}}$)
- Class A serine carbapenemases (eg: bla_{KPC})
- Class B metallo- ß-lactamases (eg: bla_{VIM} and bla_{NDM})
- Class C serine cephalosporinases (eg: AmpC)
- Class D serine oxacillinases (eg: bla_{OXA})

The genes encoding ß-lactamases can be located on the bacterial chromosome, on plasmids, or on transposons. The genetic environment of the ß-lactamase (*bla*) gene dictates whether the ß-lactamases are produced in a constitutive or inducible manner. Recently, an increasing number of *bla* genes are being discovered on integrons. Mobile genetic elements (MGEs) that contain integrons are an important source for the spread of *bla* genes and for the dissemination of other resistance determinants in clinical and the environmental settings. Hence, integrons serves as a sink for ARGs are not mobile, but their integration in MGEs (plasmids, transposons) enables their movement to other bacterial cells (Gillings et al. 2015).

1.5. The global spread of antibiotic resistance

The global spread of AR is illustrated by the dissemination of New Delhi metallo-β-lactamase-1 ($bla_{\text{NDM-1}}$) rapidly from East to the West, and the spread of *Klebsiella pneumoniae* carbapenemase (KPC) from West to East (Figure 1.3.) (Molton et al. 2013). Recent studies of modern environmental and human commensal microbial genomes have a much larger concentration of ARGs has been previously recognized (D'Costa et al. 2011). Being identified in wild and remote human communities with no or little exposure to Abs,

antimicrobial genes are been identified in microbial (bacterial) DNA frozen for 30,000 years in Artic subterranean cave

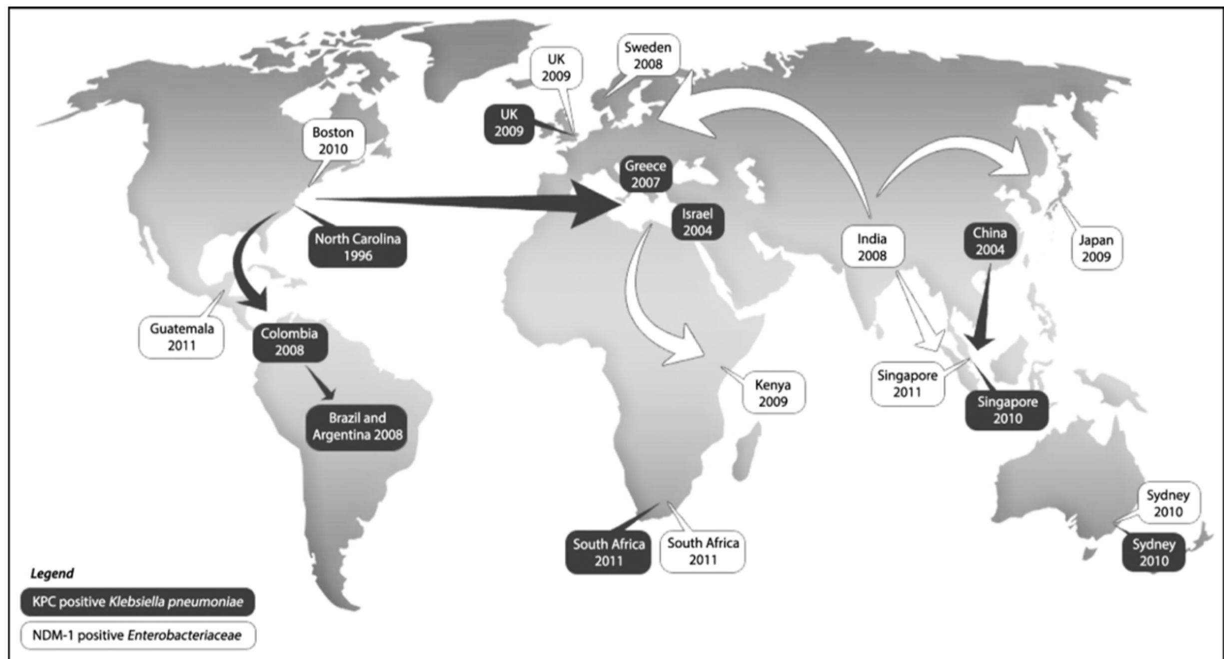


Figure 1.3 Global dissemination of ARGs: *Klebsiella pneumoniae* carbapenemase (KPC) –producing *K. pneumoniae* and New Delhi metallo-β-lactamase-1–producing Enterobacteriaceae. The initial reported cases in each continent are shown. Arrows indicate the significant international movements of these organisms. Abbreviations: KPC, Klebsiella pneumoniae carbapenemase; NDM-1, New Delhi metallo-β-lactamase–1; UK, United Kingdom. Adapted from Molton et al. (2013)

(Rolain et al. 2012). But whereas antimicrobial resistance clearly predates modern antibiotics, the advent and mass production of Abs since the 1940s have followed an unprecedented selection pressure. Most successive Ab in the clinical use has been followed by global emergence of resistance, with frequent identification in clinical settings. ARGs are often encoded in extra-chromosomal genetic elements (plasmids) or in segments that appear to have been recombined into the chromosome from other genomes (transposons). Plasmids are the largest extra-chromosomal elements, capable of self-replicating, double-stranded circles of DNA, some of these express mechanisms that are capable to transfer the extra-chromosomal elements to other bacterial cell (O'Brien 2002). Transposons are segments in plasmids which often contain the ARGs. Transposases are functional enzymes that enable the transposon to

recombine into other genomes. In vitro studies have demonstrated such recombination by transposons with identical nucleotide sequences on a variety of different plasmids (Negri et al. 2000). MGEs are segments of DNA that encode proteins that mediate the movement of DNA within genomes (intracellular mobility) or between bacterial cells (intercellular mobility). Intercellular movement takes place in three ways

(1) Transformation – gene transfer mediated by uptake of free DNA

(2) Conjugation – gene transfer mediated by certain plasmids and mobile genetic elements with relevant gene transfer genes. Cell – cell contact is required for conjugation

(3) Transduction – gene transfer that is mediated by certain bacteriophage

1.5.1. Gene transfer mechanisms

The MGEs are segments of DNA that encode enzymes and other proteins that mediate the movement of DNA within genomes (intracellular mobility) or between bacterial cells (intercellular mobility). Transformation was the first mechanism of prokaryotic horizontal gene transfer (HGT) to be discovered. On the other hand as shown in Figure 1.4., conjugation requires independently replicating genetic elements such as conjugative plasmids or chromosomally Integrated Conjugative Elements (ICEs), including a conjugative transposons (CTns). The genetic materials encode proteins which facilitate their own transfer and also the transfer of other cellular DNA from the donor to a recipient cell, lacking the plasmids or ICEs. Transduction is another form of DNA transfer mediated by independently replicating bacteriophages (Frost et al. 2005). For example as illustrated in Figure 1.4 (A), Transduction (1). The DNA genome (yellow) of a bacteriophage adapts into the chromosome (dark blue), additionally in later replicates, phage-infected bacterial cell, fragments of the host DNA are occasionally packaged into phage particles and can then be transferred to a recipient cell (generalized transduction) or with its own DNA (specialized transduction). The bacteriophage lyses the cell, and infects a naive recipient cell in which the novel DNA recombines into the recipient host cell chromosome (red). Conjugation (2). Conjugative plasmids (orange) use a protein structure (known as a pilus) to establish a connection between the recipient and donor cell to transfer themselves. Other mechanisms of conjugation include a copy of a small, multi-copy plasmid, defective genomic island or a replica of the entire bacterial chromosome can be transferred to a recipient cell. In the recipient cell these genetic elements either incorporate into the chromosome or multiply independently if compatible with the resident plasmids (light

green). Transposons (pink) and integrons (dark green) integrate into the new locations on the chromosome or plasmids by a method of non-homologous recombination. The mechanism of conjugation does not involve pili in gram positive bacteria. Transformation is demonstrated in Figure 1.4(B), Cells that are competent uptake free DNA from environment. It is important to note that DNA transfer from the donor will be maintained and expressed in the recipient only if they integrate into the genome of the recipient by recombination.

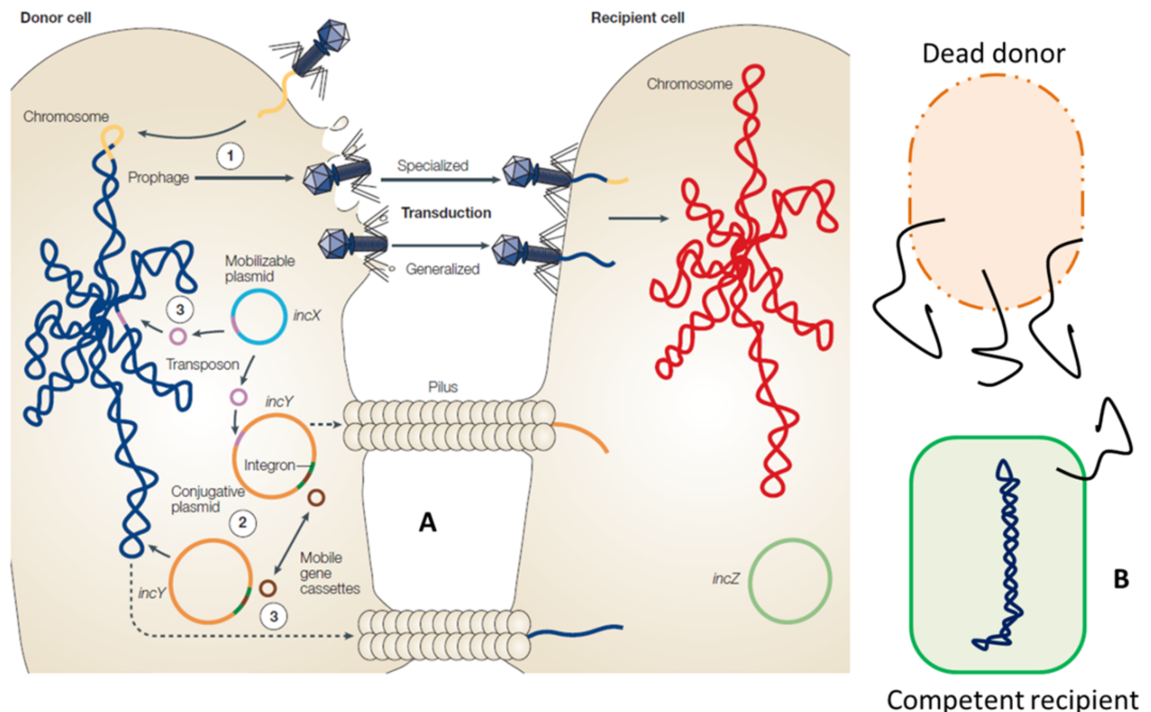


Figure 1.4 Method of gene transfer in bacterial cell (A) transduction and conjugation; (B) transformation. Source: Frost et al. 2005

1.5.2. Antibiotic resistance in the environment

Until recently the existence of Abs in the environment had very little attention. Studies have reported the presence of Abs in the low concentration (microgram per liter or the nanogram per liter) in fresh and waste water. Bacteria are responsible for the substance elimination in the environment, especially in sediment, soil, waste and fresh water. In the environment naturally occurring Abs from microbes control the dynamics of microbial populations. In contrast, recent inputs of semi-synthetic or synthetic compounds which are more stable and

not biodegradable by microbes are used widely around the globe. Furthermore, they often have a different, e.g. broader, activity spectrum (Kümmerer 2009). Bacteriostasis (cease growing) being the most common response by the cell to Abs, but for certain classes (ß-lactams), continued growth is permitted, with the inhibition of target in the cell, leading to cell lysis. In prophylaxis, bacteriostasis is often effective, as the cell death and elimination of the pathogen are mediated through host immune defenses. In the environment such augmentation is typically absent. Furthermore, in waste water, surface water, sediments, sludge and soil a mixture of different active compounds may be present in contrast to the medical and veterinary application of antibiotics and disinfectants (Kummerer 2004). In this respect, there is paucity of information in the environments (such as wastewater, sludge, surface water, and soil) in the use and effectiveness of Abs, when compared to clinical settings. Additionally concentrations of Abs in the environment are normally in lower orders of magnitude then of therapeutic use. Recently, a study in Netherlands, a country referred with little AMR, revealed the presence of ESBL producing Enterobacteriaceae in 8% of stool samples which is only a 2.6% lower that found in symptomatic patients of the same region (Reuland et al. 2013). In Brazil, a study on the untreated hospital effluents identified more than 50% of the isolates to be MDR with 41.9% of isolates carrying *intI1* (Spindler et al. 2012). High prevalence of $bla_{\text{CTX-M}}$ in *E. coli* isolated from different aquatic environment sources in Leon, Nicaragua were reported in 2012 (Amaya et al. 2012). A recent environmental prevalence study conducted by Walsh and team in New Delhi, India revealed the presence of NDM-1 in 4% of 7% of drinking water and sewage samples (Walsh et al. 2011). ARGs as well as ARB/MDR bacteria in the environment are increasingly seen as an ecological problem. The transmission of ARGs as well as ARB/MDR bacteria has been favored by the presence of sub-therapeutic concentrations of Abs in the environment and may play a major role in the stimulation of AR and transfer of ARGs in bacterial communities. But in general, the emergence of AR is the environment is a highly complex process which has not been fully understood in clinical and natural environments (Kummerer 2004). Hence, the gradual emergence of AR in pathogenic and nonpathogenic bacteria resulting from non-prudent use of antimicrobials has recently became a major global health concern (Cantas et al. 2013).

1.5.3. Coexistence of antibiotic and metal resistance

In recent years, the concern of metal contamination as a function of selective agent in the dissemination of AR is gaining attention in both clinical and environmental settings. Associations between the type of metals and the level of metal contamination and the precise

pattern of AR suggest that many mechanisms of AR underlie this co-selection process. The mechanisms of co-selection include co-resistance (resistant determinants of different types present in the same genetic element) and cross-resistance (the same genetic determinant acting for both Abs and metals). The best documented case of co-resistance is the presence of Tn21 and Tn21-like transposons, which contains Hg resistant operon and an integron with multiple ARGs (Baker-Austin et al. 2006).

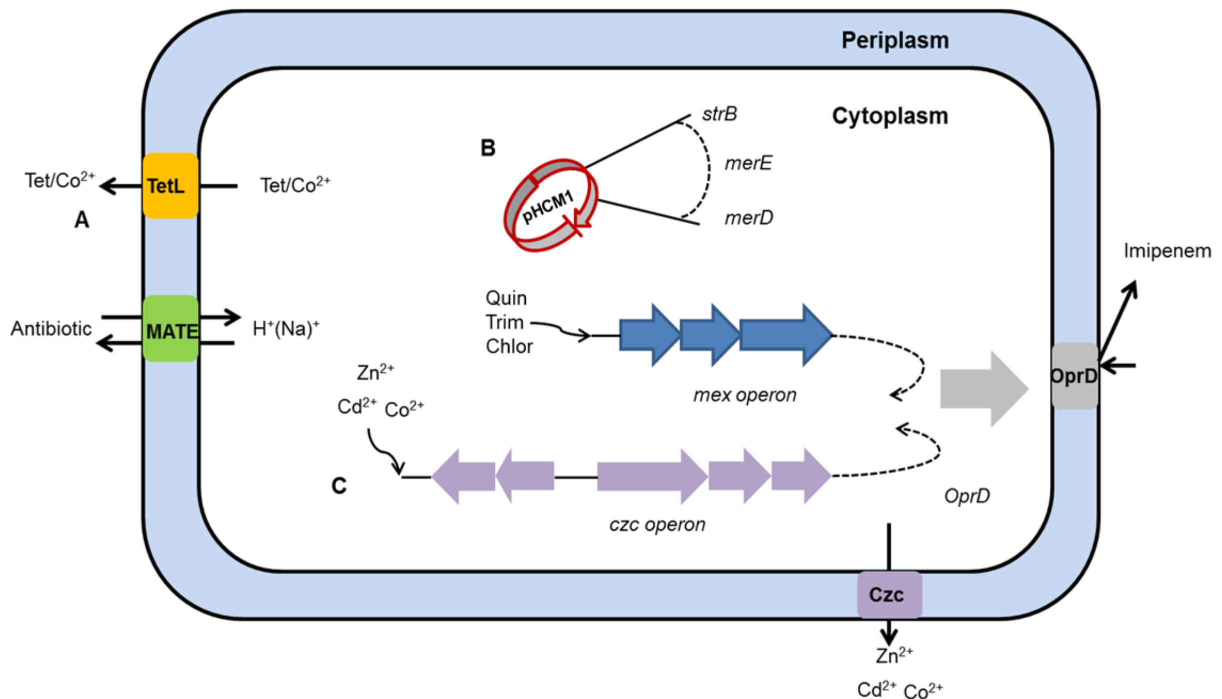


Figure 1.4 Examples of molecular mechanisms that include co-selection of metal and Abs
Source: Baker-Austin et al. 2006

Abbreviations: Chlor, chloramphenicol; MATE, multidrug and toxic compound extrusion; *merD*, gene encoding mer operon regulatory protein; *merE*, gene encoding Hg-efflux protein; pHCM1, Salmonella typhii CT18 resistance plasmid; Quin, quinolone (a class of antibiotics that inhibits DNA replication in bacteria); *strB*, gene encoding streptomycin-modifying enzyme; Tet, tetracycline; TetL, tetracycline efflux protein, Trim, trimethoprim.

Examples of molecular mechanisms that underlie metal and antibiotic co-selection are illustrated in Figure 1.4. (A) Cross-resistance, one biochemical system confers resistance to both Abs (tetracycline) and metal (cobalt). (B) Co-resistance a physical linkage of resistance determinants (streptomycin and Hg resistance gene). (C) Co-regulatory resistance, whereby several regulatory mechanisms are transcriptionally linked, thus, exposure to one can lead to

resistance to another as an unknown or unrecognized pathway. In this example, the linkage of the *mex* and *czc* operons leads to expression of metal efflux (Zn, Cd) and Ab (imipenem) resistance. Additionally, the potential for biofilm phenotypes as active mechanism of resistance to both Abs and metals are demonstrated by numerous reports.

1.5.4. Antibiotic resistance in *Pseudomonas* species.

Pseudomonas bacteria can be found in various different environments such as soil, sediment, water, wastewaters and plant and animal tissue. They are also one of the leading species of bacteria that cause nosocomial infections. Many different species of this bacterial genus are opportunistic pathogens that affect humans, animals, and plants. These bacteria have the ability to remain viable in aquatic environment for long periods and well known for its intrinsic resistance mechanisms. *Pseudomonas aeruginosa*, also called the "epitome" of opportunistic pathogens, almost never infects uncompromised tissues; however, it can infect practically any type of tissue if that tissue has compromised defense mechanisms. Large number of studies report and emphasize to *P. aeruginosa*, however other species belonging to this genus have already shown some relevance, since they can also be found in the environment, causing infections in humans and carrying ARGs and mobile genetic elements as well. A few studies have also reported ß-lactams resistance and relevant ARGs and mobile genetic elements in the in *Pseudomonas* spp. associated to wastewater. These opportunistic pathogenic bacteria can act as environmental reservoir of ARGs and contribute their dissemination to other bacterial species which can also be a source of human/animal infections (Spindler et al. 2012).

The alarming characteristic of these species of bacteria is their low susceptibility to multiple Abs due to their multidrug efflux pumps and the low permeability of their cellular envelopes. The frequent efflux mechanisms in *P. aeruginosa* include MexAB-MexXY-OprM, MexCD-OprJ, MexEF-OprN, *OprD* and *ampC*. ß-lactams are the mostly and widely used Abs. Carrying ARGs such as *ampC* and ß-lactamase enzymes that these bacteria would have the ability to fend off the beta-lactam Abs. Number of studies have characterized the resistance mechanisms in *Pseudomonas* spp. which include, characterizing ARGs gene expression (Dumas et al. 2006, Xavier et al. 2010), metal and Abs coresistance (Perron et al. 2004), ß-lactamases and mobile genetic elements (Spindler et al. 2012, Jovcic et al. 2011, Quinteira et al. 2005). ARGs expressing resistance to upper class of antibiotics such as carpabenems were

also identified in the environmental isolates of *P. pseudoalcaligenes* and *P. putida* in a recent study (Walsh et al. 2011).

1.6. Thesis rationale and structure

1.6.1. Rationale

Antimicrobials are substances that in small concentration can inhibit the growth or the existence of microbes without affecting the host. They comprise several classes of drugs of natural or seminatural origin (ß-lactams) or from synthetic sources (sulfonamides). The first report on the appearance of antimicrobials in the environment was published in 1983. Lack of sufficiently sensitive techniques compromised further research, and it was not until the late 1990s and early 2000 that sensitive methods of trace analysis were first introduced (Segura et al. 2009).

On a global scale research is documenting with increasing frequency that many chemical and microbial components that have not traditionally been considered as contaminants are present in the environment on a global scale. These "emerging contaminants" or “contaminants of emerging concern” are commonly derived from municipal, agricultural, and industrial wastewater sources and pathways. Emerging contaminants mostly related to unregulated contaminants/pollutants, may be candidates for future regulation depending on studies on their potential health effects and the results of monitoring of their presence in the terrestrial environment. They include drugs, pathogens, metals, personal care products, and many other groups of compounds. These newly identified contaminants represent a shift in the modern thinking as most of these contaminants are produced industrially and yet discarded to the terrestrial environment by domestic, commercial and industrial uses. Additionally these contaminants do not have to persist in the environment to induce the potential negative effects as their removal/degradation/transformation rates are compensated by their uninterrupted input to the native environment. Globalization, urbanization and the increase in population, intensification of agriculture to meet the growing human population and growth in industries result in increased freshwater pollution, particularly when coupled with inadequate sewage collection and treatment. The main threats to human health are from pathogens in sewage, nutrients, and toxic inorganic and organic chemicals used in communal, industry and agriculture. During the recent past, increasing attention has been paid to the identification and

quantification of emerging pollutants in wastewaters, surface waters, ground waters, soil and sediment (Pote et al. 2008a, Stoll et al. 2012, Walsh et al. 2011, Ngelinkoto et al. 2014, Mwanamoki et al. 2014, Thevenon et al. 2012a).

Hospital wastewaters carry a variety of toxic or persistent substances (pharmaceuticals, radionuclides, solvents and disinfectants for medical purposes) in widespread concentrations as a result of laboratory and research activities or excretion. A number of compounds in hospital wastewaters belong to the group of emerging contaminants. Referring to drugs, large amounts of various compounds have been used globally and their sales in the last decade have been increasing steadily (Van Boeckel et al. , Verlicchi et al. 2010). For example, in the USA, approximately 23,000 t of Abs are used annually (Ternes and Joss 2006). On the other side, the poor-quality drugs and medical products, both deficient and fake, cause preventable morbidity, mortality, drug resistance, especially in low-income and middle-income countries (Newton et al. 2014).

Microbial contamination of freshwater resources is still a major problem in many parts of the world. Faecal pollution originates from a variety of human and non-human sources, but faecal indicator bacteria (FIB) contamination from human faecal material is generally considered to be a greater risk to human health as it is more likely to contain human enteric pathogens. Many studies report on the microbial contamination of marine or freshwater beaches, due to partially or non-treated wastewater discharges, industrial inputs and non-point source surface runoffs. In developed countries, most faecal contamination has been contained by separating water cycles, WWTP and by disinfecting public water supplies. Most of the WWTPs were built and upgraded, with the primary role of removing carbon, nitrogen and phosphorus compounds, as well as microbes. These pollutants usually arrive at the WWTP in concentrations to the order of mg L^{-1} and at least 10^6 MPN/100 mL. These conventional WWTP not designed to be able to greatly remove emerging contaminants especially pharmaceuticals, are due to the fact that their concentrations are in the range 10^{-3}–10^{-6} mg L^{-1}, which is much smaller than those of conventional macropollutants (BOD5, COD, nitrogen and phosphorus compounds). Based on dilution of different discharges and most WWTPs does not provide an option for separation of emerging pollutants/contaminants, toxic compounds from the effluent which is then discarded into the environment (mostly water bodies). Hence, WWTP is not considered an adequate solution by many authors (Pote et al. 2008a, Thevenon et al. 2012a, Haller et al. 2009a, Haller et al. 2009b)). On the other hand in most developing nations the lack of WWTPs, sanitation facilities and the liquidation of

communal, industrial, hospital, agricultural and urban run-off to the receiving system without adequate treatment could cause major impacts on the quality of life. Contamination of freshwater resources may represent a source of toxicity to the indigenous bacterial communities, which are major players in biogeochemical processes, which could possible influence the natural biological balance as a whole.

Little is known regarding the epidemiological aspects of AMR in developing countries. Few studies have been conducted in India especially in northern parts Walsh et al. 2011, Ahammad et al. 2014, Castanheira et al. 2011, Lascols et al. 2011, Seema et al. 2011, which reported increased levels of AR in various environment including, rivers, sewage and drinking water. However, such data does not exist from southern part of India. Currently most European countries have established national and international surveillance of AMR, whereas countries in other parts of European region require strengthening of such systems (WHO 2014). Studies related to antimicrobial use, development/dissemination of AR, regional/global variation, and strategies according to existing health care situation in each part of the globe remain a big challenge.

1.6.2. Study site

1.6.2.1. Lake Geneva

Lake Geneva (Le Léman), is the largest freshwater body in Europe in terms of volume (89 km^3) and the second largest in terms of surface area (89 km^3). It is a monomictic temperate lake, with early spring overturn not occurring every year, and is classified in the warm monomictic category. The lake was considered eutrophic in the 70's and 80's, but is now mesotrophic after drastic reduction of phosphorus inputs. The lake provides drinking water but also receives the wastewater from urban developments. The largest WWTP is located in Lausanne and discharges treated wastewater and occasionally bypass water into Vidy Bay. The Bay of Vidy is located near the centre of the city of Lausanne, Switzerland, on the northern shore of Lake Geneva. There are three potential main inflows of contaminated water into the bay (Goldscheider et al. 2007): the urban sewage treatment plant, the Chamberonne River and the Flon River (Flon storm water outlet). Due to the combined treated and untreated wastewaters, Vidy Bay is a significant source of metals, organic micropollutants (Pote et al. 2008a) and faecal indicator bacteria (Haller et al. 2009a, Haller et al. 2009b, Thevenon et al. 2012b). Vidy Bay has been previously studied for circulation patterns (Goldscheider et al. 2007), ARGs and pathogen fate (Czekalski et al. 2012, Czekalski et al. 2014, Thevenon et al.

2012c), the spread of various contaminants in the sediment surfaces (Loizeau JL et al. 2004, Pote et al. 2008b) and the path and dynamics of the suspended sediments (Graham et al. 2014).

1.6.2.2. Cauvery River Basin

Cauvery River, located in the southern part of the Indian sub-continent is estimated to cover a drainage area of 81,555 km^2, with many tributaries generally flowing to the south and east of the states of Karnataka and Tamil Nadu. The river flows between 75°27' to 79°54' east longitude and 10°9' to 13°30' north latitude. From Coorg in the Western Ghats to the river mouth at the Bay of Bengal, the river flows through a densely populated area. The climate over the Cauvery River is tropical and its main tributaries include Hemavati, Kabini, Bhavani and Amaravati. Cauvery River is primarily supplied by monsoon rains, and the volume of this rain-fed river fluctuates seasonally. Cauvery River water is mostly used for irrigation, household consumption and the generation of electricity. The Cauvery is one of the most utilized rivers in India and it is considerably important for its irrigation. The river, on its course, receives a considerable amount of industrial effluents, untreated municipal sewage, urban runoff and agricultural runoff (Dekov et al. 1998, Dhanakumar et al. 2015). Studies have described in detail on the sediment transport (Vaithiyanathan et al. 1992), Radium levels (Dekov et al. 1998), chemical composition of sediment (Dekov et al. 1998), particle bound bacterial cell size (Harsha et al. 2007, Mahadevaswamy et al. 2008), metals (Dhanakumar et al. 2015, Begum et al. 2009, Raju et al. 2012, Mubedi et al. 2013) and one study reporting the AR in the upper part of the river (Skariyachan et al. 2015). Tiruchirappalli city is located in the central part of the state of Tamil Nadu between latitude of 10°10' and 11°20' north and a longitude of 78°10' and 29°0' east. Tiruchirappalli is the fourth largest municipal corporation in the state of Tamil Nadu, India. Tiruchirappalli's recorded history beings in the 3rd century BC when it was under the rule of Cholas. The city is supplied with 85 Mld (million liters per day) of water through four pumping stations located by the Cauvery River. The drainage system of the city is well designed to collect the rain which is conveyed by open drain, and sewage is removed by closed conduit. Seventy-seven percent of the historic Tiruchirappalli town is provided with underground sewer drainage and is well connected to the sewage treatment plant. Tiruchirappalli city generates an average of 68 Mld of waste water but only has facilities to collect 42 Mld in its existing sewage system with the end point being the nearby water sources (Cauvery River).

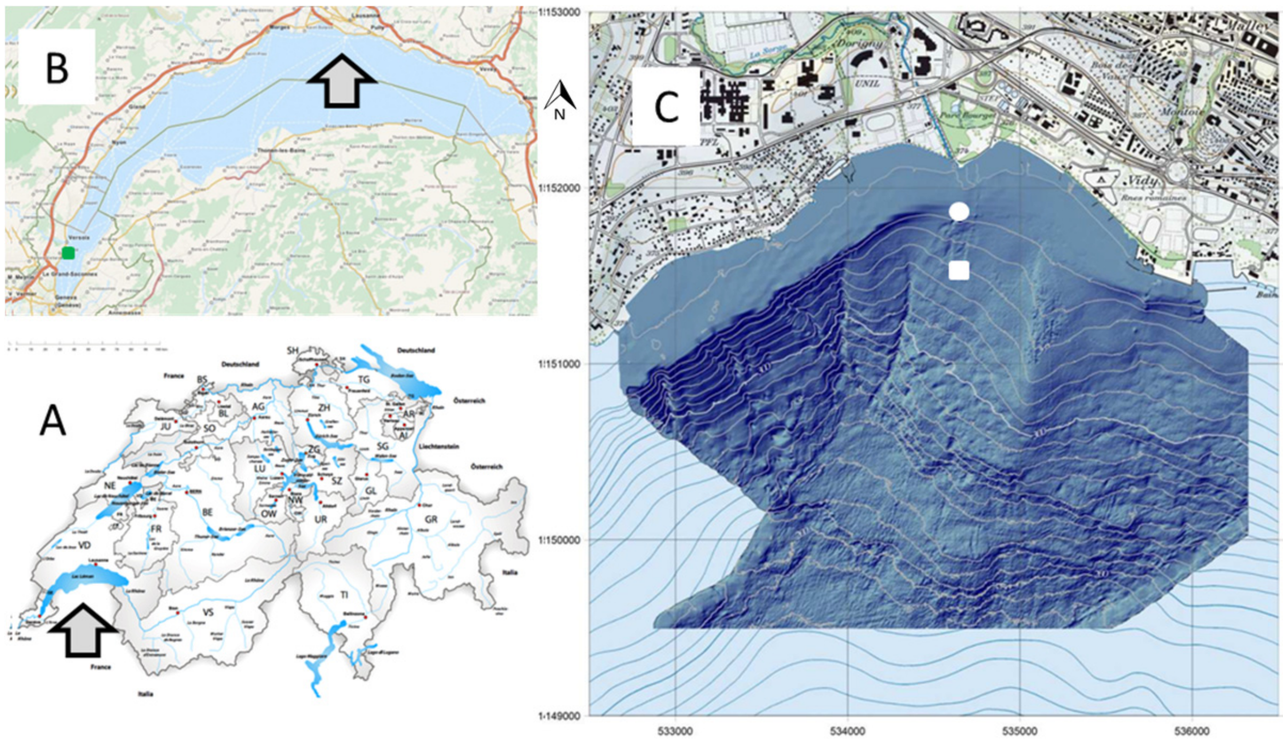


Figure 1.5 A topographic representation of Vidy Bay (C). The white square represents the approximate location of the WWTP outlet, while the white circle denotes the location of the outlet prior to 2001. Maps A and B show the location of Vidy Bay with respect to Lake Geneva and Switzerland as denoted by the grey arrows, green square represents the location of Greux de Genthod.

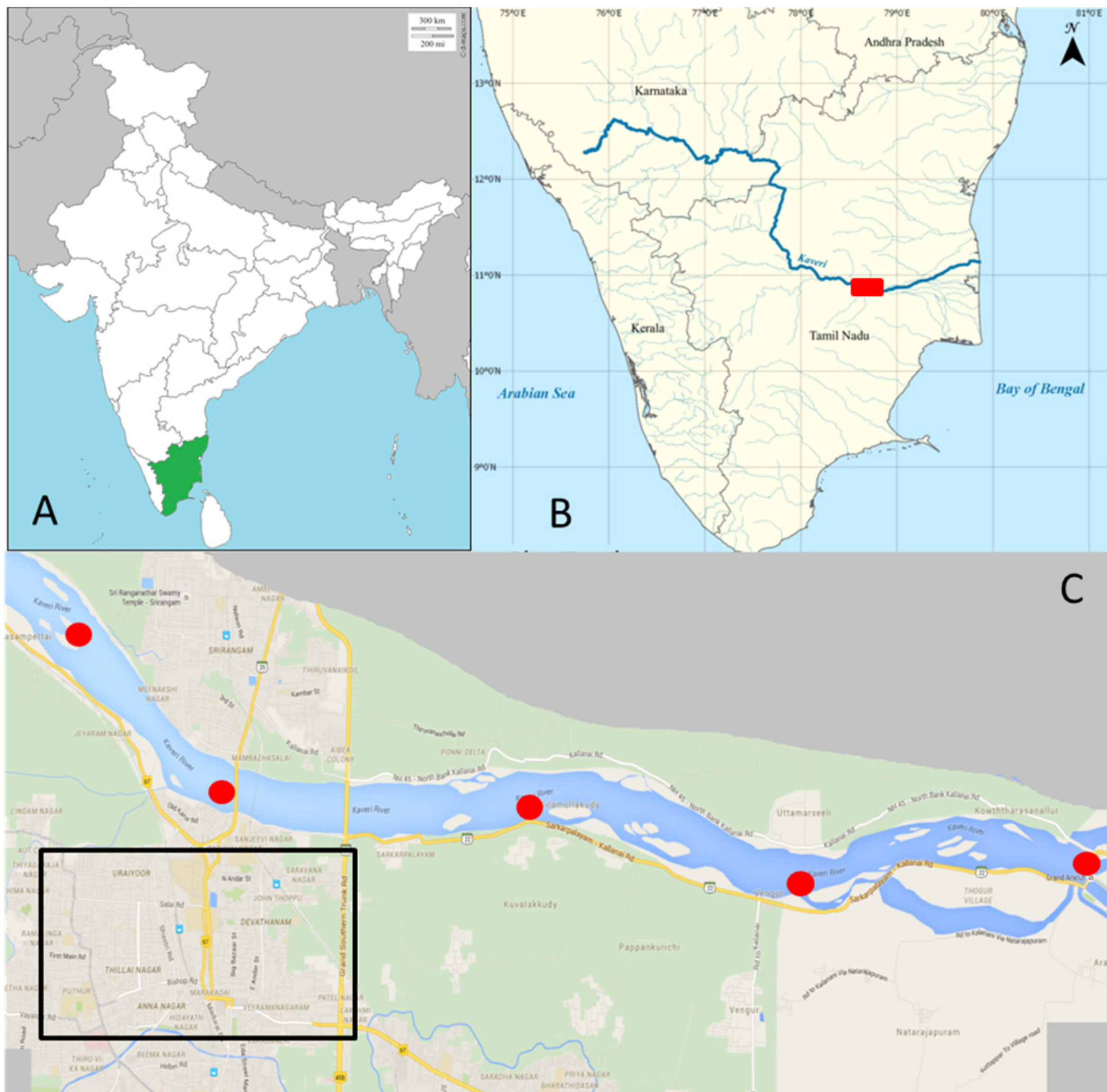


Figure 1.6 (A) Location map of Tamil Nadu (green shade), India. A topographic representation of Cauvery River (B). The red square represents the approximate location of the study area on the river. Map C show the location of study sites on Cauvery River (red circles) and the black square indicating the localization of hospital samples.

1.6.3. Objectives

Despite the numerous previous studies undertaken in Lake Geneva, and Cauvery River, in particular, no studies have been performed to explore the persistence of ARB/ARGs in these locations. This study further explores the correlation between AR levels to the other emerging contaminants including metals from urban and hospital effluents. The present study will provide insight on antibiotic resistance burden with special reference to ARB carrying multiple resistance genes discharged into the aquatic environment from effluents, which could be useful to establish the baseline information for environmentalists and healthcare providers. Each chapter addresses one of the main objectives of the overall study.

- To understand the role of untreated hospital and urban effluent waters as emerging source of metals and toxicity in tropic river receiving system
- To evaluate (to assess) the dissemination of emerging contaminants including faecal indicator bacteria and ARGs into the rivers receiving the effluents from various sources.
- To explore the effect of WWTP as source of emerging contaminates (metals and ARGs) into the fresh water ecosystem.
- To identify the bacterial phenotypic and genotypic antibiotic-resistance profiles and to compare the potential for conjugation in the isolates recovered from fresh water sediments receiving treated/untreated effluents.
- To identify the bacterial community structure related to the source and level of metal contaminants in soil and sediment from distinct geographical locations (India, CH, DRC and Senegal).

1.6.4. Thesis structure

The studies presented herein form part of an overall study which focuses on the quantification of metals, ARGs, the distribution of AR in *Pseudomonas* species and the influence of metal contamination on bacterial communities. In this study we address the key issues for the question of water quality under tropical and temperate river/lake receiving systems, using Lake Geneva, and Cauvery River Basin as a case study. Each chapter is self-containing and represents the form of a published article or article in the process of being submitted.

Chapter 1 contains the general introduction. Chapter 2, we quantified the metal load in the untreated hospital and urban effluents combined with ecotoxic effects under tropical conditions. Chapter 3, we quantified the bacterial load and ARGs in the sediments receiving untreated hospital effluents and urban wastewaters in Cauvery River, Tiruchirappalli, Tamil Nadu, India. Chapter 4, we studied the periodic accumulation of toxic metals, total bacterial load and clinically relevant ARGs in the sediment of Lake Geneva. Chapter 2, 3 and 4 provide the insight on the common and contemporary sources of contamination to the fresh water receiving systems in India and Switzerland. Chapter 5 we address the prevalence of MDR *Pseudomonas* species in hospital and communal effluents (treated/untreated) and their potential role in dissemination of AR to other bacterial species. Chapter 5: We address the effect of metal contamination on bacterial community structure by molecular approaches with inclusion from distinct geographical locations.

The introduction was written to provide a background for each chapter, with each chapter containing a brief and specific introduction to its study. A methods and discussion section are uniquely found in each chapter to which they pertain. This structure of the thesis allows the reader for a more concise description and understanding the findings without the need to refer to various sections of the thesis.

1.6.5. Institutional frame work

This interdisciplinary research was coined under the regulations of the University of Geneva, Faculty of Sciences, section of Earth Sciences and Environment, Institute F.-A. Forel.

The doctoral study was completed through the collaboration of the following institutions:

- University of Geneva (UNIGE), Faculty of Sciences, Earth and Environmental Sciences, Institute F. A. Forel and Institute of Environmental Sciences, Bd Carl-Vogt 66, CH-1211 Geneva
- Jamal Mohamed College (JMC), Bharathidasan University, Department of Zoology, Tiruchirappalli 620020, Tamil Nadu, India.
- University of Kinshasa (UNIKIN), Faculty of Science, B.P. 190, Kinshasa XI, Democratic Republic of the Congo.
- University Pedagogique Nationale (UPN). Quartier Binza/UPN, B.P. 8815 Kinshasa, Democratic Republic of the Congo.
- Ecole Polytechnique Fédérale de Lausanne (EPFL), Central Environmental Laboratory, School of Architecture, Civil and Environmental Engineering, Lausanne, Switzerland.
- University of Geneva (UNIGE), Department of Microbiology and Molecular Medicine, CH-1211 Geneva 4, Switzerland.

1.6.5. Scholarship and fundings

- Swiss Government Excellence Scholarship (FCS) for Foreign Scholars (Sep 2012 – June 2014)
- Ernst and Lucie Schmidheiny Foundation (Jun 2014 – Sep 2015)
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- Institute F.A. Forel, University of Geneva

1.6.6. List of publications relative to this research

- **Devarajan, N.**, Laffite, A., Graham, N., Meijer, M., Prabakar, K., Mubedi, J.I., Elongo, V., Mpiana, P.T., Ibelings, B.W., Wildi, W., and Pote-Wembonyama, J., 2015, Accumulation of Clinically Relevant Antibiotic-Resistance Genes, Bacterial Load, and Metals in Freshwater Lake Sediments in Central Europe: *Environmental Science & Technology*. 49: 6528-37.
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- 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-Wembonyama, J., 2014,Assessment of pathogenic bacteria in water and sediment from a water reservoir under tropical conditions (Lake Ma Vallée), Kinshasa Democratic Republic of Congo: *Environmental Monitoring and Assessment,* 186: 6821-30.
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The following are the list of publications in preparation

- **Devarajan, N.,** et al., Occurrence of Antibiotic Resistance Genes and Bacterial Markers in Tropical River Receiving Hospital and Urban Effluent Waters. *Submitted to the journal Environmental Science and Technology.*
- **Devarajan, N.,** et al., Dissemination of antibiotic resistant *Pseudomonas* spp., to the aquatic environment: An environmental prevalence study under tropical and temperate climatic conditions. Manuscript in preparation.
- **Devarajan, N.,** et al., Pyrosequencing based analysis of bacterial diversity in metal-contaminated sites: Case study of Congo DR, India, Senegal and Switzerland. Manuscript in preparation.

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CHAPTER 2

Hospital and urban effluent waters as a source of accumulation of toxic metals in the sediment receiving system of the Cauvery River, Tiruchirappalli, Tamil Nadu, India

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Abstract

Hospital and urban effluents contain a variety of toxic and/or persistent substances in a wide range of concentrations, and most of these compounds belong to the group of emerging contaminants. The release of these substances into the aquatic ecosystem can lead to the pollution of water resources and may place aquatic organisms and human health at risk. Sediments receiving untreated and urban effluent waters from the city of Tiruchirappalli in the state of Tamil Nadu, India, are analyzed for potential environmental and human health risks. The sediment samples were collected from five hospital outlet pipes (HOP) and from the Cauvery River Basin (CRB) both of which receive untreated municipal effluent waters (Tiruchirappalli, Tamil Nadu, India). The samples were characterized for grain size, organic matter, toxic metals, and ecotoxicity. The results highlight the high concentration of toxic metals in HOP, reaching values (mg kg−1) of 1851 (Cr), 210 (Cu), 986 (Zn), 82 (Pb), and 17 (Hg). In contrast, the metal concentrations in sediments from CRB were lower than the values found in the HOP (except for Cu, Pb), with maximum values (mg kg−1) of 75 (Cr), 906 (Cu), 649 (Zn), 111 (Pb), and 0.99 (Hg). The metal concentrations in all sampling sites largely exceed the Sediment Quality Guidelines (SQGs) and the Probable Effect Concentration (PEC) for the Protection of Aquatic Life recommendation. The ecotoxicity test with ostracods exposed to the sediment samples presents a mortality rate ranging from 22 to 100 % (in sediments from HOP) and 18–87% (in sediments from CRB). The results of this study show the variation of toxic metal levels as well as toxicity in sediment composition related to both the type of hospital and the sampling period. The method of elimination of hospital and urban effluents leads to the pollution of water resources and may place aquatic organisms and human health at risk.

2.1. Introduction

Hospitals perform a wide variety of activities (health care, hygiene, diagnostics, prophylaxis, etc.) for which they are a consumer of a variety of potentially toxic substances including drugs and their metabolites such as antibiotics, organic matter, radionuclides, solvents, metals, disinfectants, sterilization products, specific detergents for various instruments, radioactive markers, and iodinated contrast media (Mubedi et al. 2013, Verlicchi et al. 2010). Active substances in medicines are only metabolized to a certain extent on administration to patients. Following this, the mostly active un-metabolized substances are released through excretions into the effluents (Ternes and Joss 2006) and then released into the aquatic environment (Halling-Sorensen et al. 1998). Hospital effluents and urban runoff in most developing countries represent a significant source of many toxic elements in the aquatic environment because the effluents are discharged into drainage systems, rivers, and lakes without prior treatment, and then accumulate in sediments (Mubedi et al. 2013, Mwanamoki et al. 2014a, Mwanamoki et al. 2014b). The main human and environmental risk is remobilization of the contaminants and their return to the hydrosphere either by sediment re-suspension or by infiltration into groundwater (Wildi et al. 2004). The modification of environmental conditions such as pH, redox potential, bacterial activities, or ligand concentration leads to the release of contaminants from the sediment into the water column and increase their bioavailability (Cantwell et al. 2002, Gillan et al. 2005, Haller et al. 2011). As a result, polluted sediments also represent a significant source of contamination in fresh water organisms (Kang et al. 2000).

Tiruchirappalli city is the fourth largest city in the Tamil Nadu region of India with a population of 752,066 inhabitants (TCM and 2001). The city is located in the central part of Tamil Nadu, and the civic administration is divided into four areas and a total of 65 wards. The climate is tropical and the temperature usually 22–43 °C. Cauvery Basin is estimated to be 81,555 km2 with many tributaries generally flowing to the south and east of the states of Karnataka and Tamil Nadu. The water source of the river primarily acts as a major source of drinking water for the inhabitants of the Tiruchirappalli region and as irrigation for the Cauvery delta (Kalavathy et al. 2011). Agriculture, urbanization, industrialization, and vegetation along the river bank are part of the economy in the Indian sub-continent. On the other hand, the river also acts as a receiving system for most of the anthropogenic pollution in the locality. Continuous monitoring of the water sources to validate sustainable progress and to monitor environmental pollution is important, including in the case of the Cauvery River.

Little information is available on the assessment of contaminants in receiving systems in developing countries (under tropical conditions). A previous study (Mubedi et al. 2013) presents various characteristic aspects of sediment receiving hospital effluent waters. However, there is no quantitative information regarding the characterization of sediments receiving municipal effluent waters as well as about ecotoxicological aspects in the Cauvery River (Tamil Nadu Region). The aim of the research presented in this paper is to assess the effects of untreated hospital and urban effluent waters on the accumulation of toxic metals in the sediment receiving systems in the Cauvery River Basin, Tiruchirappalli, Tamil Nadu, India. This assessment was based on the sediment physicochemical characterization including sediment grain size, total organic matter (OM; loss on ignition), and toxic metals including Cr, Co, Ni, Cu, Zn, As, Cd, Pb, and Hg. Sediment samples were subject to ecotoxicological analysis in order to assess the potential risk to sediment living organisms.

2.2. Materials and methods

2.2.1. Study area and sample collection

Tiruchirappalli city is located in the central part of the state of Tamil Nadu between latitude of 10°10′ and 11°20′ north and a longitude of 78°10′ and 29°0′ east. The city is divided into four main areas namely Ariyamangalam, K.Abisekapuram, Golden Rock, and Sri Rangam. The city is supplied with 85 Mld (million liters per day) of water through four pumping stations located by the Cauvery River. (1) Kambarasampettai (26 Mld), (2) New Collector (32 Mld), (3) Ammamandapam (5.5 Mld), (4) Golden Rock (20Mld), and the remaining water supply (1.5 Mld) being sourced from ground water. The pumping stations of Kambarasampettai and New Collector are located 3 km upstream from the effluent discharge site (E1), and the Ammamandapam pumping station is 1 km upstream. The drainage system of the city is well designed to collect the rain which is conveyed by open drain, and sewage is removed by closed conduit. Seventy-seven percent of the historic Tiruchirappalli town is provided with underground sewer drainage and is well connected to the sewage treatment plant. However, newly built areas such as Golden Rock and the Sri Rangam localities are completely unsewered and the effluent released from this part of the city is released into the Cauvery River by surface channels. Tiruchirappalli city generates an average of 68 Mld of waste water but only has facilities to collect 42 Mld in its existing sewage system with the end point being the nearby water sources (Muthukumaran and Ambujam 2003). The corporation supports the small and medium-sized industries with 5 Mld of water and the large industries meet their

own water needs by being self-sustainable. The small and the medium-sized industries dump their effluent into the municipal sewage channels and the large industries discharge their effluent after initial treatment into the Uyakkondan irrigation canal which forms part of the Cauvery River. The Uyakkondan is a 1000-year-old canal built by Raja Raja Cholan and renovated by Kulothunga Cholan. The canal takes its flow from the Cauvery River and runs through Tiruchirappalli city and serves as a major source of water for more than 32,000 acres of agricultural land, 36 tanks, and also serves as the major conduit for receiving the effluent waters from the city. However, sampling of site E1 (effluent discharge point) found it free from effluents discharged by these large industries, but sewage from the municipality, the hospital, and small-scale industries was present.

The surface sediments were collected from (1) five selected hospital outlet pipes (HOP) labeled H1, H2, H3, H4, and H5. The collection points were adjacent to the hospital effluent outlet pipe before discharge into the municipal sewage for a period of 4 months (June to September 2013) and (2) in the Cauvery River basin (CRB) at Tiruchirappalli, Tamil Nadu, India (Figure 2.1.). The surface sediments (2–6 cm) in the CRB were collected at specific points and given the following names: R0, 5 km upstream from the effluent discharge point on the river (as a control sample); E1, in the vicinity of effluent discharge into the river; R1, 5 km downstream from the effluent discharge point; R2, 10 km downstream from the effluent discharge point; and R3, Grand Anicut (Kallanai Dam) located 15 km downstream from the effluent discharge point. The sampling took place in September 2013. The sediments were collected in sterile plastic bags and stored at 4 °C until they were shipped to the F.A. Forel Institute of the University of Geneva in Switzerland for further analysis.

2.2.2. Analysis of sediment grain size, water content, and total organic matter

The particle grain size was measured using a Laser Coulter® LS-100 diffractometer (Beckman Coulter, Fullerton, CA, USA), following 5 min ultrasonic dispersal in deionized water according to the method described by (Loizeau et al. 1994). The sediment total organic matter (OM) content was estimated by loss on ignition at 550 °C for 1 h in a Salvis oven (Salvis AG, Emmenbrücke, Lucerne, Switzerland).

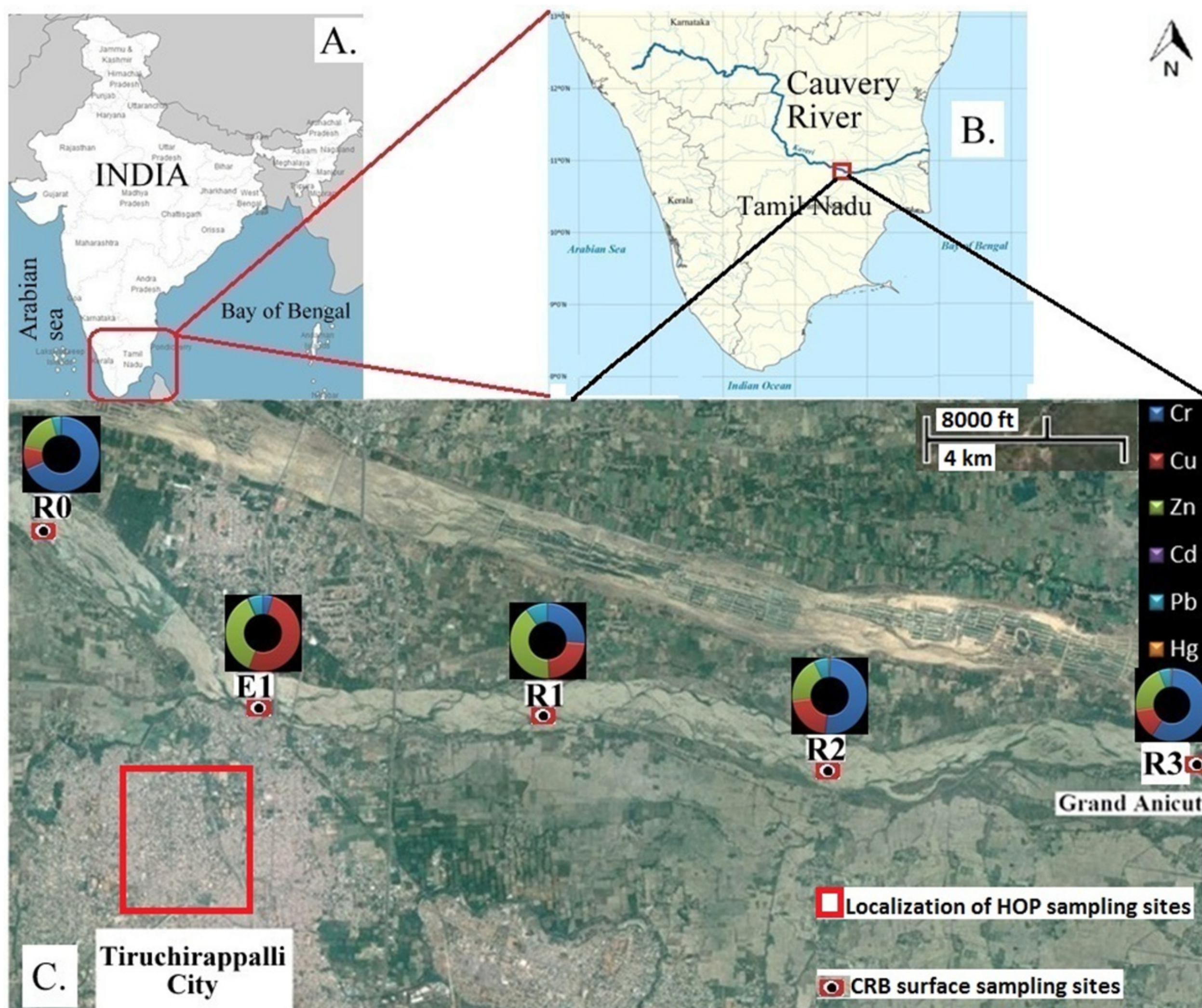

Figure 2.1. A. Location of study area Tamil Nadu, India. B. Flow of Cauvery River map. C. Location map of surface sediment sampling sites in HOP and CRB, Tiruchirappalli, Tamil Nadu, India. Graphical representation indicates the distribution of metals between the sampling sites. R0 (10°51'58" N; 78°39'57" E) 5 km upstream the river before the effluent discharge point (control site), E1 (10°50'17" N; 78°41'45" E) at the vicinity of effluent discharge into the river, R1 (10°50'13" N; 78°44'00" E) 5 km downstream the effluent discharge point, R2 (10°50'53" N; 78°44'23" E) 10 km downstream the effluent discharge point, R3 (10°49'54" N; 78°49'07" E) Grand Anicut located 15 km downstream the effluent discharge point.

2.2.3. Toxic metal analysis

Before being analyzed, sediment samples were lyophilized at −45 °C after homogenization and air-drying at ambient room temperature. Toxic metals including Cr, Co, Ni, Cu, Zn, As,

Cd, and Pb were determined by quadrupole-based inductively coupled plasma mass spectrometry (ICP-MS, Agilent model 7700 series) following the digestion of sediments in Teflon bombs heated to 150 °C in analytical grade 2 M HNO3 (Loizeau JL et al. 2004, Pardos et al. 2004, Pote et al. 2008). Multi-element standard solutions at different concentrations (0, 0.02, 1, 5, 20, 100, and 200 µg/L) were used for calibration. Total variation coefficients of triplicate sample measurements were under 10%and chemical blanks for the procedure were less than 2 % of the sample signal. The metal concentrations of sediments were expressed in ppm (mg kg^{-1} dry weight sediment).

Total Hg analysis was carried out using an atomic absorption spectrophotometer (AAS) for mercury determination (Advanced Mercury Analyzer; AMA 254, Altec s.r.l., Czech Rep.) following the method described by (Hall and Pelchat 1997) and (Roos-Barraclough et al. 2002). The method is based on sample combustion, gold amalgamation, and AAS. The detection limit (3 SD blank) was 0.005 mg kg−1 and the reproducibility better than 5 %.

2.2.4. Sediment toxicity test

The sediment sample toxicity test was performed using the TK36-Ostracodtoxkit F (MicroBio Tests Inc., Belgium) following the manufacturer's recommendations. In brief, the benthic ostracod crustacean Heterocypris incongruens cysts were hatched in standard fresh water at 25 °C with permanent illumination (approximately 3000–4000 lx) 54 h before the tests. The neonates were then measured for length and immediately placed in test wells. One test well consisted of 1 mL test sediment, 2 mL standard fresh water, 2 mL algal food suspension (provided with the kit), and ten living ostracods. The test plates containing six wells were sealed with parafilm, covered with a lid, and incubated at 25 °C in the dark for 6 days. At the end of the test, the mortality (%) of the organisms was determined using the formula % Mortality=B/A× 100, where B=total number of dead ostracods and A=total number of ostracods added to the test plate. In addition, the length of the surviving ostracods was measured using a micrometric strip placed at the bottom of the glass microscope plate. Growth inhibition was calculated using the following formula (Oleszczuk 2008):

Growth inhibition (%) = 100 – [(growth in test sediment/growth in reference sediment)*100]

2.2.5. Data analysis

Triplicate measurements were made for all analyses of sediment samples. Statistical processing of data (Spearman's rankorder correlation) was performed using SigmaStat 11.0 (Systat Software, Inc., USA).

2.3. Results and discussion

2.3.1. Sediment organic matter and particle grain size

Sediment characteristics including total organic matter measured by loss on ignition and particle grain size are shown in Table 2.1. The sediment samples fromall of the sites in the HOP were generally loamy-sandy sediments (Mubedi et al. 2013). The sediments were mainly composed of sand and silts, with values ranging from 22.5 to 79.8 % and 18.5–77.4 %, respectively. OM in sediments ranged from 0.6 to 10.7%. Sediments from CRB were mainly sandy sediments (with sand content ranging from 44.9 to 100 %) and presented the lowest values of organic matter content (maximum value 4.2 %). Furthermore, these sediments presented the lowest values of clay (maximum value 0.5 %).

Particle size distribution in the CRB was influenced by the hydrodynamic aspects of the river and probably by stream dynamics and the action of sewage inputs as well as wave action that can create a great deal of energy (Mwanamoki et al. 2014a, Mwanamoki et al. 2014b, Pote et al. 2008, Mwanamoki et al. 2015). With the exception of site E1, the coarser sediments were located in other sites with median values of 323.7, 278.4, 411.3, and 553.2 µm for R0, R1, R2, and R3, respectively. The median value at E1 was 53.5 µm. Compared to other sampling sites in the river, the sediments in this site had a low sand content (44.9 %). The total OMcontent of the sediments does not show considerable variation between sampling sites, ranging between 0.1 and 4.2 %. Previous studies by the authors have reported that distribution of sediment OM and grain size in freshwaters, lakes, rivers, and reservoirs display large variations (Pote et al. 2008, Haller et al. 2009). According to the results of these studies, the organic matter in non-contaminated freshwater sediments varied from 0.1 to 6.0 %. Consequently, the sediment of the CRB can be considered to be unpolluted by organic matter. For example, OM can reach more than 30 % in sediments contaminated by the municipal WWTP effluent waters (Pote et al. 2008). The low organic matter content in the sediment fromCRB can be explained by the hydrodynamic aspects of water flow and also by crops grown inside the watershed and by areas surrounding the river being forested.

Table 2.1 Water content, total organic matter, and particle grain size in sediment samples collected from HOP and CRB sampling sites

Sample.no	H_2O %	OM %	$CaCO_3$ %	Clay %	Silt %	Sand %	Median particle grain size (μm)
H1 June	10.8	5.4	1.6	0.7	41.7	57.6	92.5
H2 June	7.5	0.6	0.2	0.5	47.5	52.0	67.8
H3 June	20.0	3.6	0.8	0.3	42.1	57.6	115.8
H4 June	7.7	0.7	0.2	0.4	23.8	75.8	190.0
H5 June	9.7	10.7	2.5	1.0	55.7	43.3	46.3
H1 July	17.2	3.7	0.4	0.6	64.4	35.0	38.2
H2 July	17.1	2.9	0.3	0.3	68.5	31.2	33.7
H3 July	12.5	7.6	0.2	0.2	33.8	66.1	118.2
H4 July	8.6	0.8	0.3	1.7	79.8	18.5	18.6
H5 July	15.7	1.0	0.2	0.1	22.5	77.4	164.7
H1 Aug	16.9	6.2	1.3	1.4	72.2	26.5	24.0
H2 Aug	30.1	3.9	0.8	1.3	70.7	28.0	28.6
H3 Aug	3.3	3.1	0.9	0.4	48.1	51.6	66.2
H4 Aug	10.7	2.0	0.4	0.3	32.1	67.5	148.2
H5 Aug	15.9	3.7	0.6	0.8	51.6	47.7	57.8
H1 Sep	12.7	2.1	0.6	0.5	57.2	42.3	41.8
H2 Sep	9.8	0.7	0.2	0.4	38.0	61.6	172.4
H3 Sep	7.0	1.0	0.3	0.4	28.5	71.1	163.0
H4 Sep	7.5	0.6	0.2	0.2	38.2	61.6	153.4
H5 Sep	10.9	0.6	0.2	0.2	34.2	65.6	247.5
R0	6.3	4.2	0.0	0.1	1.5	98.5	323.7
E1	20.0	2.3	0.5	0.0	55.1	44.9	53.5
R1	7.7	0.9	0.2	0.5	16.0	83.4	278.4
R2	6.0	0.1	0.0	0.2	8.5	91.4	411.3
R3	7.3	0.4	0.1	0.0	0.0	100.0	553.2

All analyses were performed in triplicate and the standard deviation was less than 4.5 % of average Sediment samples: H1 hospital 1 sediment collection point, H2 hospital 2 sediment collection point, H3 hospital 3 sediment collection point, H4 hospital 4 sediment collection point, H5 hospital 5 sediment collection point

2.3.2. Toxic element concentrations in sediment samples

The results of the trace metal analysis are reported in Table 2.2. The concentration of metals in the sediments sampled from HOP sites varied considerably, ranging from 21–1851, 23–210, 66–987, 0.8–3.7, 9.6–82.5, and 0.18–16.8 mg kg^{-1} for Cr, Cu, Zn, As, Pb, and Hg, respectively. The same tendency was observed in sediment samples from the CRB, but the concentration values of all metals are generally low, except for site E1. Compared with other sampling points on the river, the maximum metal levels were generally observed in site E1. For example, values of 906, 650, and 111mg kg^{-1} for Cu, Zn, and Pb, respectively, were recorded in this site, which is characterized by the discharge of untreated municipal and hospital effluent waters. Effluent has no influence at site R0 (considered as a control site), located upstream of E1. However, the metal levels in sediments from this site were very low. An evaluation of the potential deleterious effects of the toxic metals on benthic fauna, applying the consensus-based guidelines for sediment quality (Long et al. 2006, MacDonald et al. 2000), estimates the hazard these sediment may represent to the biota. The concentration of toxic metal including Cr, Cu, Zn, As, Cd, Pb, and Hg obtained in this study were primarily compared with the Sediment Quality Guidelines for the Protection of Aquatic Life (CCME 1999) and sediments were also evaluated for their toxicity using the ostracod as the test organism. According to (Wildi et al. 2004), finegrained sediment with higher water content is unstable and mainly subject to erosion by currents and waves, gravity process, and human activities. In this case, the current of the river water may lead to erosion and resuspension of contaminated sediments and favor the uptake of contaminants by the aquatic living organisms present. Consequently, the metal concentration values from the sediment sample's fine fractions (<63 µm) need to be considered which was 20 × (Cr) to 35 × (Hg) higher in the HOP site and 5 × (Hg) to 13× (Cr) higher in the CRB (Figure 2.2.) than the PELs (probable effect levels) (CCME 1999). According to previous studies by the authors (Mubedi et al. 2013, Mwanamoki et al. 2014b, Haller et al. 2011, Pote et al. 2008, Mwanamoki et al. 2015, Ngelinkoto et al. 2014, Thevenon et al. 2011a, Thevenon et al. 2011b, Thevenon and Pote 2012) and compared to the results of this study, the sediment receiving systems from HOP and CRB (especially in site E1) can be considered to be heavily contaminated by toxic metals, especially Cr, Cu, Zn, Cd, Pb, and Hg, which are frequently associated with adverse biological effects.

Table 2.2 Metal content (mg kg^{-1} dry weight sediment)^{c} of surface sediments analyzed by ICP-MS and by AMA for total mercury

Sample site	Month	Cr	Co	Ni	Cu	Zn	As	Cd	Pb	Hg
H1	Jun - 2012#	**58.1**	7.95	28.32	**67.5**	**1652.2**	1.7	**1.4**	**45.5**	**2.46**
	Jun - 2013	**1851**	10.9	42.3	**183.6**	**986.9**	3.7	**1.9**	29.3	**1.62**
	Jul - 2013	**87.6**	13.7	51.7	**59.6**	**288.2**	1.6	0.4	30.4	**1.4**
	Aug - 2013	**978.9**	11.9	47.4	**112.2**	**573.7**	2.6	**1**	29.2	**1.67**
	Sep - 2012#	**85.4**	5.7	15.3	28.85	**313.9**	1.3	0.33	**36.3**	**0.71**
	Sep - 2013	**71.4**	11.2	38	34.2	**128.6**	1.4	0.2	29.2	**0.4**
H2	Jun - 2012#	29.41	4.22	12.78	9.84	44.47	0.6	0.06	12.73	**0.38**
	Jun - 2013	**74.9**	10.4	38.2	26.3	65.8	0.9	0.1	27.9	**0.26**
	Jul - 2013	**73.7**	11.8	45.9	**60.3**	**293**	1.5	0.4	27.5	**1.18**
	Aug - 2013	**131.8**	22.8	80.4	**75.9**	**321.9**	2.3	0.5	**82.5**	**1.37**
	Sep - 2012#	**40.6**	5.3	19.9	**45.1**	**263.2**	1.43	0.5	29.5	**1.83**
	Sep - 2013	**85**	13.6	49.6	**43.1**	**130.6**	1.3	0.2	**38.4**	**0.3**
H3	Jun - 2012#	**148.8**	1.78	10.73	**52.3**	**274.9**	0.9	**0.6**	16.6	**14.81**
	Jun - 2013	**53.1**	7.9	41.5	**155.8**	**505**	1.8	**1.4**	**42.7**	**16.81**
	Jul - 2013	21.3	2.4	12.4	**210.2**	**591.4**	1	0.4	9.6	**1.28**
	Aug - 2013	**96.5**	16.1	60	**86.6**	**344**	1.7	0.6	**52.1**	**3.62**
	Sep - 2012#	15.8	2.08	7.58	19.8	75.19	1.03	0.3	5.81	**2.27**
	Sep - 2013	**86.8**	13.6	49.4	**42.7**	**173.1**	1.3	0.2	**43.9**	**3.24**
H4	Jun - 2012#	16.6	2.1	6.87	13.89	88.84	1.8	0.08	5.98	**3.89**
	Jun - 2013	**74.1**	11.1	40.6	29	81.6	0.9	0.1	28.3	**0.39**
	Jul - 2013	**108.4**	15.5	57.6	**40.5**	97.7	1.5	0.1	**36.8**	**0.26**
	Aug - 2013	**69.6**	10.6	38.9	**81.1**	**138.7**	1.1	0.2	31.4	**0.67**
	Sep - 2012#	21.2	3.09	10.21	**71.57**	75.12	0.59	0.09	7.48	**2.67**
	Sep - 2013	**70.9**	10.6	36.7	27.6	98.2	2.7	0.2	25.8	**0.29**
H5	Jun - 2012#	22.93	3.23	10.48	25.11	**126.8**	1.01	0.22	17.2	**1.7**
	Jun - 2013	**1340.2**	5.9	25.6	**107.9**	**659.9**	2.3	**0.9**	14.4	**5.46**
	Jul - 2013	**56.7**	8.5	31.4	22.9	66.7	0.8	0.1	18.2	**0.18**
	Aug - 2013	**115.5**	20.2	71.2	**67.7**	**296.9**	2	0.5	**64.1**	**0.84**
	Sep - 2012#	19.78	3.13	10.16	23.59	109.78	0.79	0.21	8.9	**1.71**
	Sep - 2013	**108.6**	12.2	43.4	**39**	114.2	1.4	0.2	32.9	**0.21**
River	R0	18.9	2.7	7.2	2.7	4.9	0.2	0	1.1	0.04
	E1	**74.6**	9.7	51.4	**906.3**	**649.8**	2.8	**6.3**	**111.4**	**0.99**
	R1	34.4	5.5	17.4	30.4	53.6	0.6	0.1	12.4	**0.21**
	R2	13.9	2.7	6.4	5.6	5.4	0.2	0	1.7	0.16
	R3	31.7	4.3	12.4	7.3	11.6	0.4	0	2.8	**0.36**
Rec.[b] max conc[a]		**37.3**			**35.7**	**123**	**5.9**	**0.6**	**35**	**0.17**

The values in bold represent the concentration of the heavy metals above the recommended concentration according to the Sediment Quality Guidelines for the Protection of Aquatic Life recommendation (CCME 1999) Sediment samples: H1 hospital 1 sediment collection point, H2 hospital 2 sediment collection point, H3 hospital 3 sediment collection point, H4 hospital 4 sediment collection point, H5 hospital 5 sediment collection point
[a] Concentration unit: mg kg^{-1} dry weight sediment [b] Rec. BSediment Quality Guidelines for the Protection of Aquatic Life^ recommendations [c]All analyses were performed in triplicate and the standard deviation was less than 3 % of average #Data from 2012 (Mubedi et al. 2013)

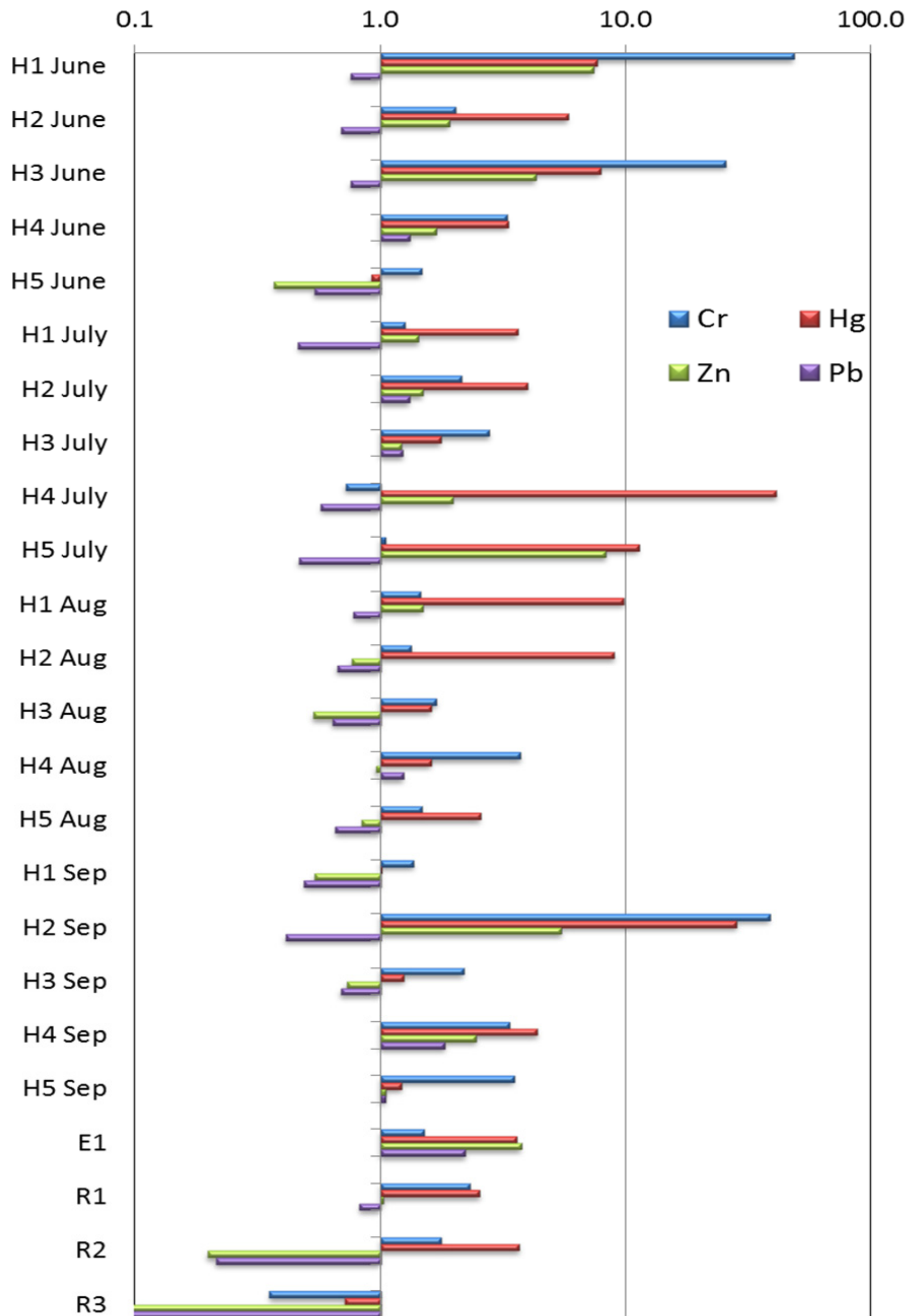

Figure 2.2. Graphical representation of probable effect levels (PEL) ratio calculated for Cr, Zn, Pb, and Hg in the fine sediments (<63 μm), and values above the recommended values are expressed using the $\log_{10}$ scale for the x-axis. *Sediment Quality Guidelines for the Protection of Aquatic Life recommendation (CCME 1999).

2.3.3. Ecotoxicity test analysis

A bioindicator is a group of organisms that provide information on the environmental conditions, with effective variables being their mere presence or absence, abundance, age structure, or a statistical index applied to their populations (Rinderhagen M et al. 2000). Ostracods are usually included as useful bioindicators in the changing environmental conditions in recent and quaternary environments (Anadón et al. 2013, Boomer and Eisenhauer 2013). Previous studies have pointed out the almost instantaneous population response of these bioindicators to anthropogenic impacts such as oil spills or industrial sewages (Mostafawi 2001). The study presented in this paper used the potential of this group (ostracods) as possible bioindicators of environmental changes to the receiving systems induced by the hospital and urban effluent waters in tropical conditions (Ruiz et al. 2006). The percentage of growth inhibition and the mortality rate of ostracods are presented in Table 2.3. The mortality rate percentage in the sediment samples collected from the HOP ranged from 22.8 to 100 %, and the mortality rate in the sediment samples collected along CRB ranged from 18.18 to 87.5 %. The growth inhibition calculated for the samples with less than 30 % mortality rate (as per the manufacturer's instructions) ranged from 51.9 to 64.2 % in the 4 months of sample collection at the HOP and 13.2 to 75.4 % in the sediments collected in the CRB. The control site (R0) showed the smallest effect on the benthic ostracods with an 18.18 % mortality rate and 13.2 % growth inhibition whereas H4 July samples showed the highest mortality rate of 100 % and R3 sediment samples had the highest growth inhibition rate of 75.4 %. The ecotoxicological results indicate the potential risk of organisms exposed to the sediment samples studied. The impact on both the mortality and/or growth inhibition of the ostracods is notable. The mortality rate of sample H4 July is 100 %, which is explained by the fact that besides metals there are many other compounds present in the hospital effluent that could accumulate in the sediments. Remarkably, H4 July has metal values that are lower than H1 August, which would have been more toxic, but 100 % mortality was observed in H4 July and so only 28.90 % was observed in H1 August. These results indicate that sediment could carry other sources of contaminants/pollutants which could impact on the growth of the ostracods added to the test sediments. However, a detailed study should be carried out in order to fully understand the toxic effects of the hazardous substances present in the sediments by combining physicochemical and ecotoxicological studies (Mubedi et al. 2013, Verlicchi et al. 2010).

Table 2.3 Percentages of mortality and growth inhibition of ostracods (*Heterocypris incongruens*) exposed to the sediments from the HOP and CRB sampling sites.

sample	% Mortality	% Growth Inhibition
H1 June	92.00	n/a
H3 June	26.67	64.2 ± 9
H5 June	71.88	n/a
H5 July	82.30	n/a
H4 July	100.00	n/a
H3 Aug	91.67	n/a
H1 Aug	28.90	51.9 ± 9
H2 Sep	22.80	61.9 ± 9
H3 Sep	87.50	n/a
R0	18.18	13.2 ± 6
E1	87.50	n/a
R1	22.58	53.7 ± 6
R2	68.97	n/a
R3	24.32	75.4 ± 6

Sediment samples: H1 hospital 1 sediment collection point, H2 hospital 2 sediment collection point, H3 hospital 3 sediment collection point, H4 hospital 4 sediment collection point, H5 hospital 5 sediment collection point. n/a Growth inhibition not determined if the mortality rate is more than 30 % according to the manufacturer's recommendation

2.3.4. Correlation between metals, OM, and median grain size

The Spearman's rank-order correlation values are presented in Table 2.4. The positive mutual correlation of all of the metals is significant, and correlation coefficients ranged from 0.3 to 0.9.

Table 2.4 Spearman's rank-order correlation of selected parameters[a] analyzed in the surface sediments

	Median grain size	Cr	Cu	Zn	As	Cd	Pb	Hg
OM	**-0.531**	0.362	**0.688**	**0.734**	**0.476**	**0.664**	0.141	**0.626**
Median grain size		**-0.618**	**-0.576**	**-0.616**	**-0.684**	**-0.609**	**-0.475**	**-0.537**
Cr			**0.476**	**0.579**	**0.718**	**0.615**	**0.593**	**0.484**
Cu				**0.958**	**0.716**	**0.914**	**0.546**	**0.796**
Zn					**0.802**	**0.962**	**0.512**	**0.852**
As						**0.848**	**0.597**	**0.647**
Cd							**0.604**	**0.825**
Pb								**0.46**

[a] Parameters include toxic metals, median grain size, and total organic content [n=25, statistically significant coefficients ($p<0.05$) are in bold]

Furthermore, there is a positive correlation between the metals and OM in the sediment samples with values ranging from 0.4 to 0.7. These results indicate that metals and OM could be considered to originate from common sources and they are carried to the receiving system by common transporters. A negative correlation was found between particle grain size and the other parameters analyzed. The observation of the negative correlation with the metals and the particle grain size can probably be explained by the hypothesis that the grain size is not a strong factor influencing the transport of contaminants to receiving systems (Haller et al. 2009, Salomons and Förstner 1984). In general, the correlation between the sediment median grain size and the trace elements indicates that the metals are not of natural (geological) origin and that their deposition in CRB is linked to the transport of municipal sewage. This is also evident from the positive correlation between total organic content and the metals in sediments. This observation is also supported by the fact that the contaminants are attached to both large organic and small inorganic particles such as clay and they could behave in a

similar way in transporting contaminants to the receiving system (Pote et al. 2008, Zhao et al. 2015).

2.4. Conclusion

The results of this study demonstrate that the values of toxic metals from the HOP are higher than the values observed in sediments collected from CRB (with the exception of Cu and Pb). The higher concentration of Cu and Pb could be explained by input from other non-identified sources. These values vary according to the sampling period and it is highly likely that toxic metals released/accumulated in the sediments of the municipal sewage could be transported to the receiving system in large amounts during floods and periods of rain. The results presented in this paper suggest that the hospital effluents released into the municipal sewage without prior treatment could act as a potential source of pollutants in the CRB sediments. The ecotoxicological tests performed on the sediment samples confirm the potential risk to living organisms in the aquatic environment. According to previous studies by the authors (Mubedi et al. 2013, Mwanamoki et al. 2014b, Haller et al. 2011, Pardos et al. 2004), in addition to metals, the toxicity can be explained by the presence of a large number of compounds found in untreated or partially treated effluent waters,which can accumulate in sediments. Consequently, the results of this study recommend further studies be carried out to assess other parameters such as persistent organic pollutants, drugs, antibiotics, and other drugresistant microbial contaminations. The results of the study presented in this paper provide a strong argument for treating hospital effluents with technologies used in modern industrial and urban sewage treatment plants. Therefore, continuous monitoring of the accumulation of potential toxicmicro-pollutants in the receiving system should be considered and further studies to provide baseline information about other potential contaminants and bio-accumulation of toxic substances in the aquatic living organisms of the receiving system are also recommended.

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CHAPTER 3

Occurrence of antibiotic resistance genes and bacterial markers in Tropical River receiving hospital and urban effluent waters

Supporting Information available at the end of the chapter

A similar version of this chapter is submitted to the journal Environmental Science and Technology.

Devarajan, N., Laffite, A., Mulaji, C.K., Otamonga, J.P., Piana, P.T., Mubedi, J.I., Prabakar, K., Ibelings, B.W., & Pote-Wembonyama, J. Occurrence of antibiotic resistance genes and bacterial markers in tropical river receiving hospital and urban effluent waters. Submitted.

Abstract

The occurrence of emerging biological contaminants including antibiotic resistance genes (ARGs) and Faecal Indicator Bacteria (FIB) under tropical conditions is still little investigated. In this study, we quantified ARGs (*bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{NDM}$ and *aadA*), total bacterial load and selected bacterial species including *E. coli*, *Enterococcus* (ENT) and *Pseudomonas* spp. by targeting species specific genes using quantitative PCR (qPCR) in total DNA extracted from the sediments recovered from hospital outlet pipes (HOP) and Cauvery River Basin (CRB), in Tiruchirappalli, Tamil Nadu, India. The abundance of bacterial marker genes were 120, 104 and 89 fold higher for the *E. coli*, ENT and *Pseudomonas* spp., respectively at HOP when compared with CRB. The ARGs conferring resistance to aminoglycoside (*aadA*) and penicillins (*bla*$_{TEM}$) were most frequently detected in highest concentration in all the sampling sites. The *bla*$_{SHV}$ and *bla*$_{NDM}$ genes were identified in the samples downstream of the effluent discharge point at CRB and absent in the upstream control site. Results showed a positive correlation among total bacterial load (0.3 to 0.7, $P < 0.05$), *E. coli* ($r > 0.3$, $p < 0.05$) and selected ARGs. The results of this study suggest that the discharge of hospital and urban wastewaters into the aquatic systems leads to the dissemination of emerging microbial pollutants. Consequently, the river receiving systems can act as reservoir for biological emerging pollutants (ARGs), which could potentially be transferred to susceptible bacterial pathogens.

3.1. Introduction

Antibiotics (Abs) are extensively used in prophylaxis of human and veterinary medicine. A number of Abs administrated to human/animals are partially metabolized and discharged in the hospital/communal effluents, which end up in environmental water bodies either treated/untreated (Kummerer 2004). Antibiotic resistance (AR) is a growing global public threat with serious health, political and economic implications. New forms of AR could spread with ease in international boundaries and spread between continents (CDC 2013, WHO 2014). Antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) from human/animal sources, along with excessive use of Abs in the human, veterinary and agriculture settings are currently considered as serious environmental problem (Kummerer 2004). In the environment the increased dissemination of ARB is possibly caused by mechanisms involving; horizontal gene transfer (HGT) of ARGs, selective pressure induced by various contaminants (Abs, metals, biocides) in the environment, gene mutation and recombination (Berendonk et al. 2015). In the recent years, research studies outside the clinical settings on AR have begun to receive attention. This concern is based on the impression that understanding the diversity patterns and biological role of AR mechanisms may help to control its threats to human/animal health (Nardelli et al. 2012).

In many parts of the globe fresh water resources polluted by microbial contaminants is still a major problem (Haller et al. 2009a). Faecal indicator bacteria (FIB) (*Escherichia coli* (*E. coli*) and *Enterococcus* spp., (ENT)) residing in the gastrointestinal tract of warm blooded animals are generally used to monitor the microbial quality of water sources. Additionally, polluted surface waters and sediments can contain a variety of pathogenic microbes including bacteria, virus and protozoa (Haller et al. 2009a, Mwanamoki et al. 2014a). The choice of bacterial indicators is thus very important. Bacteria belonging to *Pseudomonas* genus (P. spp) are extensively disseminated in the environment, such as water soil and sediment. Being known for its innate resistance mechanisms, P.spp are capable of staying viable in the aquatic environments for long periods (Spindler et al. 2012), which has the ability to carry ARGs and mobile genetic elements and can cause infections in humans (Spindler et al. 2012, Quinteira et al. 2005). Sediments may contain 100 – 1000 fold higher bacteria than overlying water (Pote et al. 2010). Estimation of microbial contaminants in sediments may be a more stable index for long-term water quality (Haller et al. 2009a, Mwanamoki et al. 2014a, Pote et al. 2010, Devarajan et al. 2015a, Thevenon et al. 2012). Hence, sediments provide us the opportunity to

address the persistence of microbial contaminants/ARGs and the potential impact of the emergence of resistant bacteria from wastewaters to fresh water microbial community.

Effluents from hospital, industrial, communal, and urban/agricultural runoff in developing countries represent a significant source of emerging contaminants (metals, ARGs, ARB) in the receiving environment as the effluents are discharged to the sewer systems, river, lakes, and seas without prior treatment which then may accumulate in the sediments (Spindler et al. 2012, Mwanamoki et al. 2014a, Devarajan et al. 2015b). Rivers and Lakes are considered as putative reservoirs of emerging contaminants (drugs, metals, ARGs), since they collect waste waters containing various contaminants from different origins (Kummerer 2004, Pote et al. 2008, Allen et al. 2010). Studies investigating rivers as a potential reservoir of ARGs is still very limited in southern part of India (Skariyachan et al. 2015). However, a few studies have reported the prevalence of clinically relevant ARGs in the environmental samples from North India (Ahammad et al. 2014, Walsh et al. 2011).

The presence of emerging pollutants such as ARB/ARGs, metals in wastewaters and their dissemination to the environmental compartment are topics of scientific interest in the last few decades (Devarajan et al. 2015a, Czekalski et al. 2014, Verlicchi et al. 2010). In developing countries (under tropical conditions), little information is available on the assessment of contaminants in receiving systems. Few studies (Devarajan et al. 2015b, Mubedi et al. 2013) reported the physical-chemical characteristics of sediment receiving untreated hospital and urban effluent waters. However, there is paucity of information regarding the microbial water quality in the Cauvery River (Tiruchirappalli Region) as well as about the prevalence of clinically relevant ARGs with the contribution from untreated urban and hospital effluents. The aim of the research presented in this study report is to assess the role of untreated hospital and urban wastewaters on the accumulation of microbial contaminants in the sediment receiving systems in the Cauvery River Basin, Tiruchirappalli, Tamil Nadu, India. This assessment was based on quantitative polymerase chain reaction (qPCR) on ARGs (bla_{TEM}, $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{NDM} and *aadA*), total bacterial load, and selected bacterial marker genes FIB and P. spp. To our knowledge, this is first report on the accumulation of emerging microbial contaminants in the sediments of fresh water receiving system in Tiruchirappalli, Tamil Nadu, India.

3.2. Materials and methods

3.2.1. Study area and sample collection

Cauvery River, located in the southern part of the Indian sub-continent is estimated to cover a drainage area of 81,555 km^2, with many tributaries generally flowing to the south and east of the states of Karnataka and Tamil Nadu. The river flows between 75°27' to 79°54' east longitude and 10°9' to 13°30' north latitude. From Coorg in the Western Ghats to the river mouth at the Bay of Bengal, the river flows through a densely populated area. The climate over the Cauvery River is tropical and its main tributaries include Hemavati, Kabini, Bhavani and Amaravati. Cauvery River is primarily supplied by monsoon rains, and the volume of this rain-fed river fluctuates seasonally. Cauvery River water is mostly used for irrigation, household consumption and the generation of electricity. The river, on its course, receives a considerable amount of industrial effluents, untreated municipal sewage, urban runoff and agricultural runoff (Dekov et al. 1998, Dhanakumar et al. 2015). Located in the central part of Tamil Nadu, at a latitude of 10°10' and 11°20' north and a longitude of 78°10' and 29°0' east Tiruchirappalli has a sub-tropical climate with ca. 0.75 million inhabitants as of 2001 and the recent estimates measures to be ca. 1 million. The city generates an average of 68 Mld of waste water and facilitates to collect 42 Mld in its existing sewage system with the end point being the nearby water sources (Cauvery River). At Tiruchirappalli, large industries empty their wastewaters to Uyakkondan canal. This irrigation canal, which is a part of River Cauvery, serves as a major source of water for more than 32,000 acres of agricultural land, 36 tanks, and also serves as the major conduit for receiving the effluent waters from large industries. Hence the sampling site E1 (effluent discharge point) is free from waste water from large industries, and include the waste water from communal, hospital and small-scale industries (Devarajan et al. 2015b). The surface sediments were collected from: (1) five selected hospital outlet pipes (HOP) labelled H1, H2, H3, H4 and H5. The collection points were at the vicinity of HOP before released into the municipal sewers for a period of 4 months (June to September) during 2 calendar years (2012 and 2013) and (2) in the Cauvery River Basin (CRB),Tiruchirappalli, Tamil Nadu, India (Figure. 3.1). The surface sediments (2-6 cm) in the CRB were collected at 5 specific points labelled as R0 – 5 km upstream the effluent discharge point on the river as a control sample; E1 – effluent discharge point on the river; R1, R2, and R3 located 5, 10 and 15 km downstream on the river from E1 site. In CRB the sediments were retrieved from 1m water depth during September 2013. The sediments were

collected in sterile plastic bags and stored at -20 ˚C until they were shipped to Institute F.A. Forel, University of Geneva, Switzerland for further analysis.

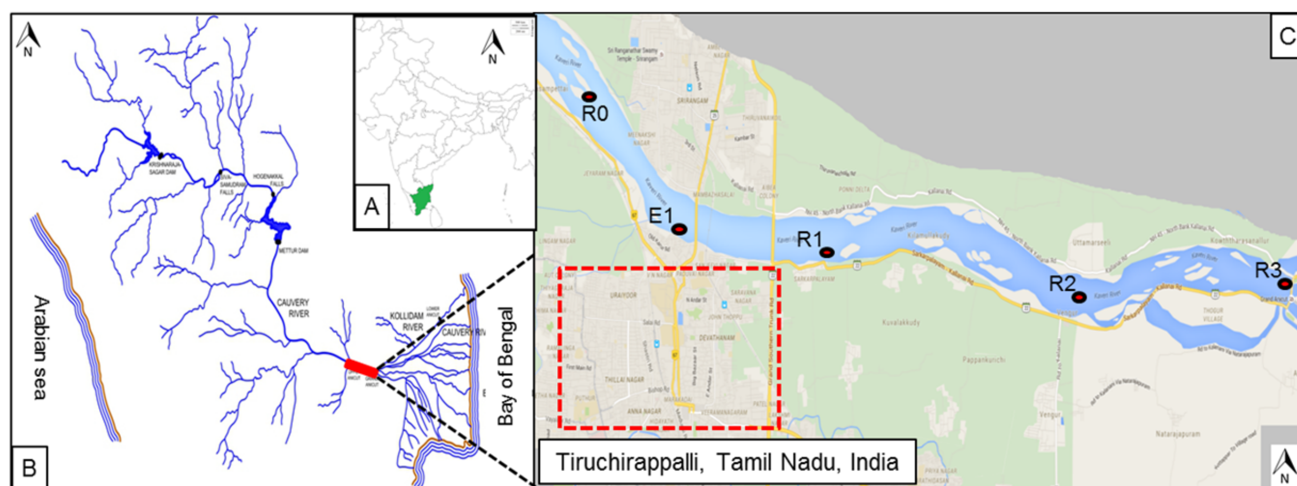


Figure 3.1. Location map of the sampling sites in Tiruchirappalli, Tamil Nadu, India. (A.) India outline map (green part representing Tamil Nadu region); (B.) Flow of River Cauvery to the Bay of Bengal and the approximate loaction of the study site (red box); (C.) Location map of the Cauvery River Basin and the sampling sites R0, E1, R1, R2, and R3 at the vicinity of the Tiruchirappalli city , the red box is the indicative representation of the hospital samples.

3.2.2. Total DNA extraction and quantification of FIB and ARGs

The total DNA from sediment samples were extracted using Ultraclean soil DNA Kit (Mo Bio Labs, Solana Beach, CA) according to the manufacturer's recommendations. The isolated DNA was stored at -20˚C until used. For each sample, DNA extraction was performed with two replicate samples to compensate for the heterogeneity. The DNA samples were diluted to 10x and 50x to avoid the inhibitors to the PCR reaction. Specific genes targeted include ARGs (bla_{TEM}, $bla_{CTX\text{-}M}$, bla_{SHV}, bla_{NDM} and *aadA*), and selected bacterial marker genes [*E. coli* (*uidA* gene); ENT and P. spp (16S rDNA)].The total abundance of 16S rDNA was also quantified to estimate total bacterial population size and used for the normalization of ARGs and selected bacterial marker genes abundance relative to total bacterial community size. The control plasmids, primers, qPCR reactions, calculation for absolute gene copy numbers (gene concentration) and the gene copy numbers normalized to 16S rDNA (abundance) were performed as described in previous study (Devarajan et al. 2015a). Briefly, all genes were quantified with KAPA SYBR® FAST qPCR Master Mix Universal Kit (KAPA Biosystems,

MA, USA) on Eco^{TM} Real-time PCR system (Illumina, Switzerland) using the primers for selected gene targets (Table 1S (Supplementary data). The assay was carried out in a final 10 µl reaction volume containing 2 µl of template DNA, 0.5 µl of each forward and reverse primer (10 pmol μl^{-1}), 2 µl of nuclease free water and 5 µl of qPCR master mix. The qPCR program was initial hold for 2 min at 50 ˚C for UDG incubation, 10 min at 95 ˚C for the polymerase activation; followed by 40 cycles of 95 ˚C for 30 s, optimal Tm (Table 1S (Supplementary data) for 30s and 72 ˚C for 30 s. Data were acquired at 72˚C. A melt curve was run at the end for the presence of unique PCR reaction product. All reactions included a negative and a positive control (10 x dilutions of control plasmids). Samples were considered to be below the limit of detection (LOD) or negative for a target gene if ≥2 out of 3 technical replicates were negative or if sample Ct values were ≥Ct of negative controls. For each reaction, the efficiency of the assay was calculated by using the measured slope of the standard curve ($E = 10^{(-1/slope)} - 1$). A reaction was considered effective if the slope (-3.1 to -3.6), efficiency (90-100 %) and R^2 (>0.99) values were within the recommended range.

3.2.3. Data analysis

A statistical treatment of data; Correlation matrix (Pearson), Principle Component Analysis (PCA) and Agglomerative hierarchical clustering (AHC) computation based on Ward's method were performed on XLSTAT (Version 2015.1.03.15828 ©Addinsoft 1995-2014). The 16S rDNA (total bacterial load) and the selected marker genes and ARGs in the samples are expressed as “gene copy numbers” in per gram of dry sediment weight normalized to the DNA extraction yield. The “relative abundance” of the selected genetic marker genes (normalized to 16S rDNA) were emphasised by the ratio = (copy number of a gene) / (copy number of 16S rDNA) for each sample (Czekalski et al. 2014, Devarajan et al. 2015a).

3. Results and Discussion

3.1. Bacterial population quantification

The total bacterial load in the sediment samples (16S rDNA gene copy numbers) were in the range of 9.9 to 11.4 and 10.02 to 12.6 log copy numbers per g^{-1} of dry sediment collected from CRB and HOP, respectively (Figure 3.2). The total average bacterial load at the HOP was ca.82 fold higher when compared to CRB. In CRB, the total bacterial load after the effluent discharge point were 0.3 to 16.5 fold higher than control site (R0). The average gene copy numbers of the bacterial marker genes for *E. coli*, ENT and P. spp in the sediment samples are presented in Figure. 3.1S (A) (Supporting Information). The bacterial density at HOP varied considerably, ranging from 5.9 to 8.5, 4.3 to 7.9, and 4.0 to 8.9 log for *E. coli*, ENT and P. spp., respectively. The similar inclination was observed in the sediment samples from CRB, but the abundance for the selected marker genes are generally low, except for the E1 site. Compared to the other sampling points on the river, E1 had higher abundance values of -3.3, -3.6 and -2.5 log values for *E. coli*, ENT and P. spp., respectively, which could possibly be influenced by the discharge of untreated urban and hospital effluents. Effluent has no influence at site R0 (control site in this study), located upstream of E1. However, the abundance levels for the selected bacterial marker genes were considerably low. The HOP site had 120, 104 and 89 fold higher bacterial load for *E. coli*, ENT and P.spp, respectively than CRB samples. Studies have suggested that ratio proportion of *E. coli* to ENT can be used to identify the source of fecal contamination in the aquatic systems and composition of fecal flora differ significantly in human and animal faeces (Ouattara et al. 2011). The ratio of *E. coli*/ENT is higher in human faeces than animal faeces (Geldreich 1976). In this study, the *E. coli*/ENT ratios estimated for CRB and HOP were 1.19 and 5.09, which is in complete agreement with the previous studies (Ouattara et al. 2011; Geldreich 1976). For example soil leaching and rain runoff brought by urban sewer channel floods FIB from human/animal origin in CRB while HOP, FIB are predominantly from human origin.

Environments such as water, soil and sediment have previously shown to represent as secondary habitant for FIB (*E. coli* and ENT) outside the gastrointestinal tract of warm blooded animals. Recent studies reported that *E. coli* can reproduce and persists in these secondary environments such as water and sediments in both temperate and tropical climates (Walk et al. 2007; Haller et al. 2009a, Haller et al. 2009b).

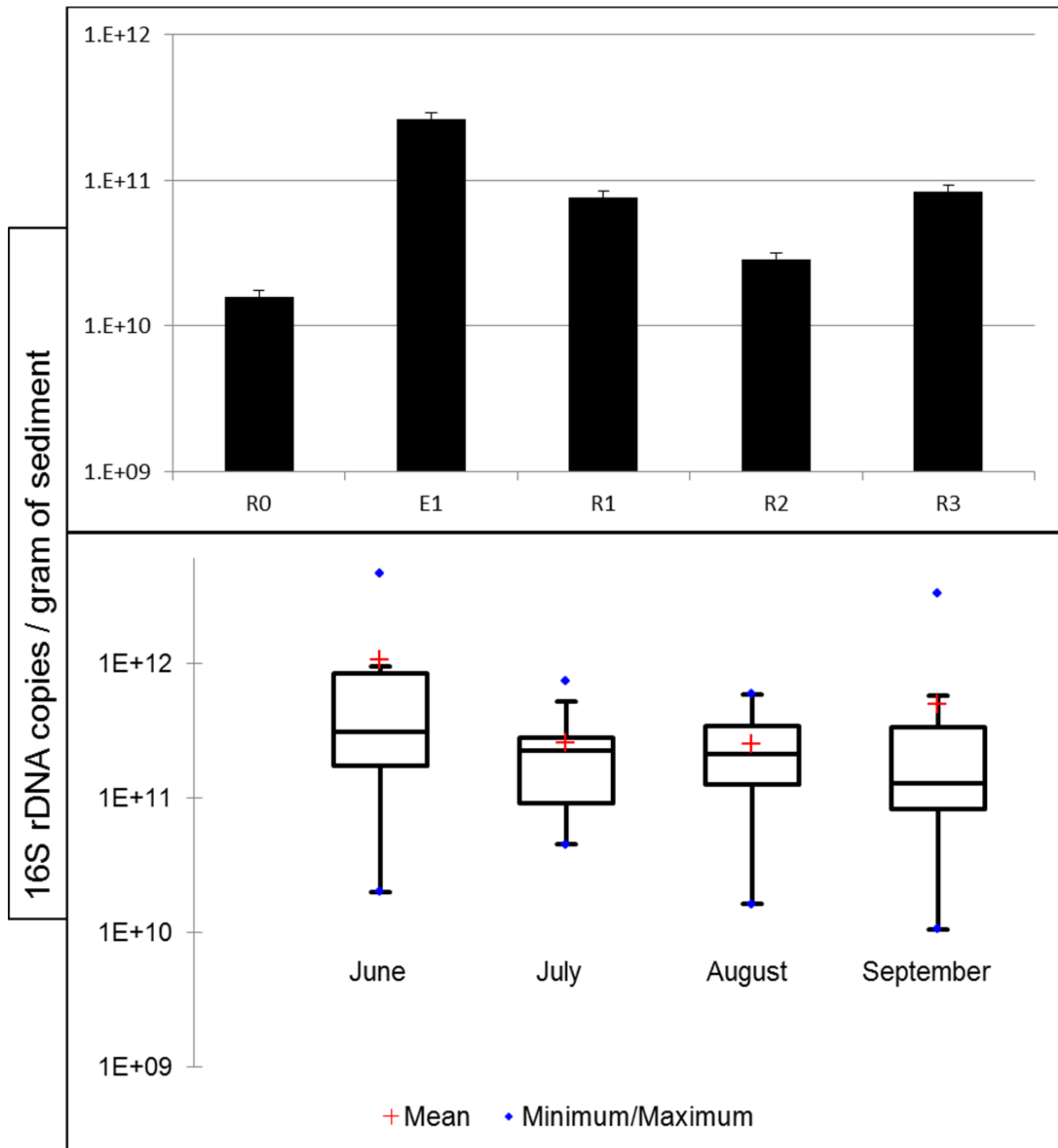


Figure 3.2. qPCR quantification of 16S rDNA in the surface sediment samples of CRB and HOP. Values (log transformed) are expressed for the copy number per gram of sediment dry weight normalized to the DNA extraction yield.

The presence of FIB in these secondary environments are used as an indicator of fecal pollution (Haller et al. 2009a). Sediments provide a favorable condition (temperature, nutrients) for growth and proliferation; shield them from sunlight inactivation and other parasite grazing. With additional protection from daylight inactivation, biofilm formation and enrichment of nutrients, sediments are hypothesized to act as reservoirs for the metabolically active bacteria (Mwanamoki et al. 2014a). Hence, it is evident that sediment in the effluent receiving system could act as a potential reservoir of bacterial populations from human and animal sources. Resuspension of these contaminants accumulated in the sediment could affect the water quality and pose a threat to human and animal health.

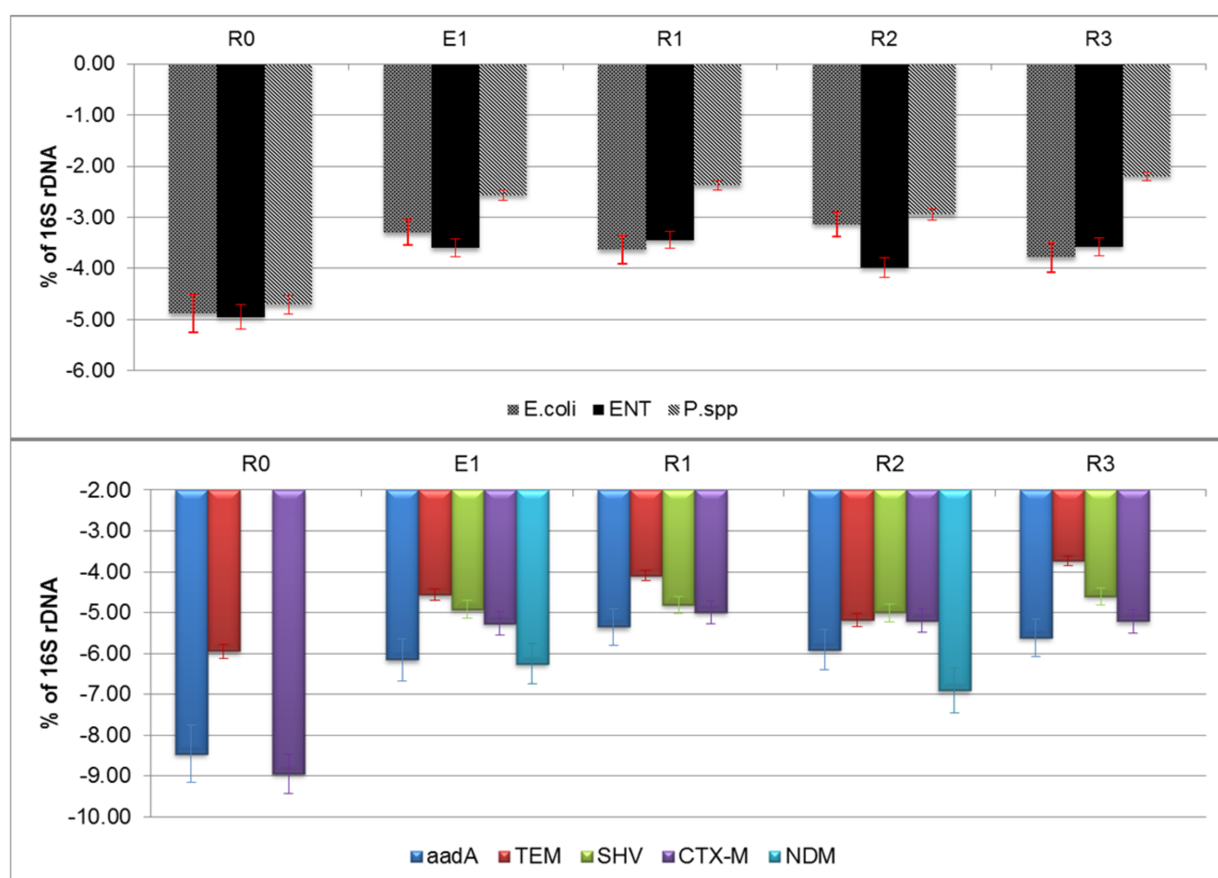


Figure 3.3. qPCR quantification of the bacterial population (A) for selected groups (*E. coli*, ENT, and P.spp.) at CRB surface sediemnts and selected antibiotic resistance genes (B) (*aadA*, bla_{TEM}, , $bla_{CTX\text{-}M}$, bla_{SHV}, and bla_{NDM}). Values (log transformed) are expressed as the percentage of 16S rDNA bacterial population.

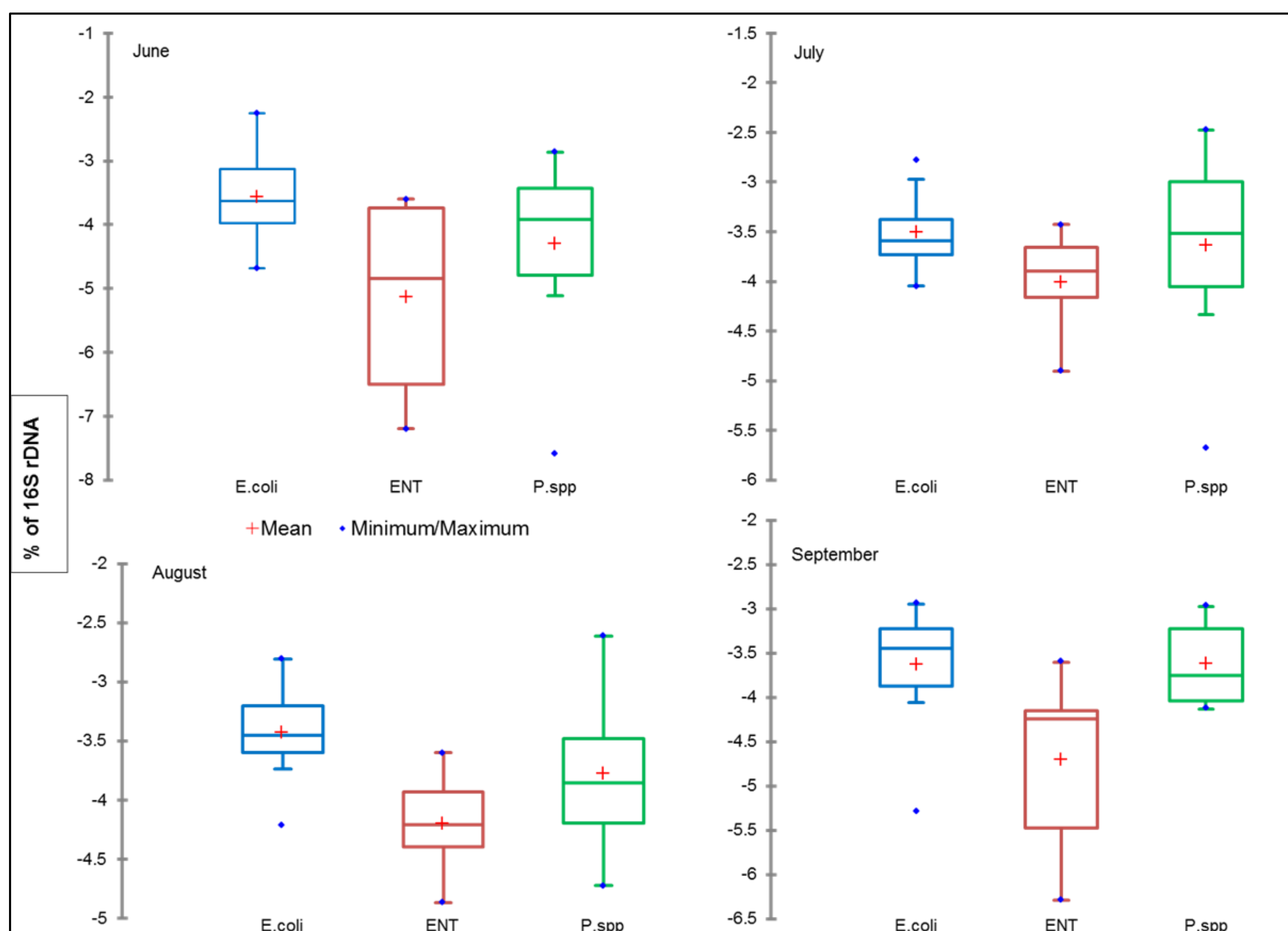


Figure 3.4. qPCR quantification of the bacterial population for selected groups (*E. coli*, ENT, and P.spp.) at HOP surface sediemnts. Values (log transformed) are expressed as the percentage of 16S rDNA bacterial population. The box plot represents the first and third quartile, the blue dots on maximum/minimum values and the red cross indicate mean.

3.2. Antibiotic resistance genes in sediments

The gene copy numbers of selected ARGs from CRB and HOP were quantified by qPCR and the results are presented in Fig 3.1S(B) and 4S (Supporting Information). The ARGs conferring resistance to ß-lactams were selected for the study based on the criteria that two-third of Abs administered to humans are ß-lactams (Stoll et al. 2012). The gene copy numbers at CRB varied from 4.7 – 6.1, 0 – 6.4, 1.7 – 6.1, 3.5 – 5.1 and 2.2 – 5.2 log for bla_{TEM}, bla_{SHV}, $bla_{\mathrm{CTX\text{-}M}}$, bla_{NDM} and *aadA*, respectively. Normalizing the ARGs and calculating for gene ratios serve as a proxy for the proportion of bacteria carrying ARGs (Pruden et al. 2012). In this study we use 16S rDNA normalized data for CRB and HOP samples (Figure.3.2(B) and

3.5). Assuming the ARGs and 16S rDNA extractions were analogous, normalizing with 16S rDNA supported for minor variations in DNA extraction efficiency. Additionally, the relative abundances of the studied ARGs provide a possibility to compare data among ARGs quantified in various other studies. In a previous study by Rodriguez-Mozaz et al. (2015), bla_{TEM} was one of the most frequently detected plasmid-borne gene, which confer resistance to penicillin's and extended spectrum Cephalosporin's. High prevalance of bla_{TEM} and *aadA* are also reported in the periodic sediments of Lake Geneva, from the start of twentieth century (Devarajan et al. 2015a, Thevenon et al. 2012) supporting the fact that these genes were carrried by bacteria as strucutral integrated reservoirs even before the inventions of Abs (Demaneche et al. 2008). Similarly, among sediment samples analyzed in this study, the ARGs bla_{TEM} and *aadA* were identified in all the sampling sites with relative abundance ranging from -6.8 to -3.7 and -8.4 to -4.5 log, respectively. These results emphasize that the older ARG like bla_{TEM} is likely endemic in the terrestrial environment (Ahammad et al. 2014, Laht et al. 2014).

Studies have highlighted that the amount of ARGs in the sediments close to metropolises is much higher than in pristine environments (Berglund et al. 2015, Devarajan et al. 2015a, Thevenon et al. 2012). In this study, bla_{SHV} and bla_{NDM} (metallo ß-lactamase - MBL) were absent in the control sample (R0) and identified in downstream of the effluent discharge point at CRB. The relative abundance of extended spectrum ß-lactamases (ESBLs) like bla_{CTX-M} was higher in sediment samples collected after the effluent discharge point than at the control site. These results indicate the role of urban and hospital effluents in the dissemination of ARGs to the terrestrial environment. The prevalence of microbial contaminants (e.g. bla_{CTX-M} 1.7 log) in the control site could be explained by the input from major activities like agricultural runoff, sand dredging, religious rituals, open defecation, urban discharges and other anthropogenic activities in the upper River Cauvery, which receives considerable amount of wastewaters on its course to Bay of Bengal (Dekov et al. 1998, Dhanakumar et al. 2015).

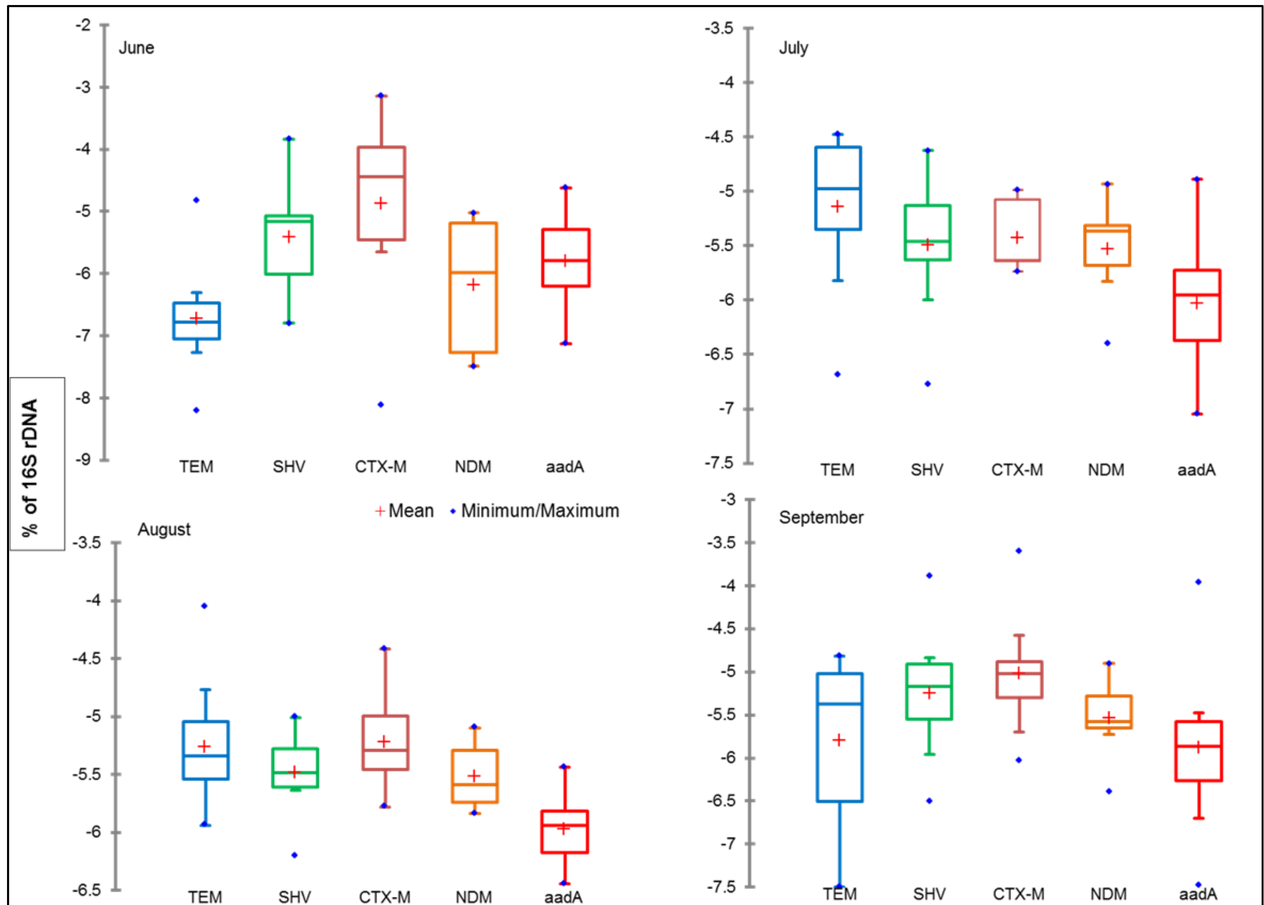


Figure 3.5. qPCR quantification of the selected antibiotic resistance genes (B) (*bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{NDM}$ and *aadA*) at HOP surface sediemnts. Values (log transformed) are expressed as the percentage of 16S rDNA bacterial population. The box plot represents the first and third quartile, the blue dots on maximum/minimum values and the red cross indicate mean.

Elevated levels of ARGs were observed in the sediments of HOP, than those found in the CRB. In a comparison between the CRB and HOP, the ARGs abundance were ca. 49.7, 50.9, 39.5, 32.8 and 40.3 fold higher for *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{NDM}$ and *aadA*, respectively at HOP. Most of the ARGs had a seasonal variation at HOP. These variations could be explained by the type of hospitals, sampling period and the disposal practices at the study sites to play a major role in the dissemination of clinically relevant ARGs. However the abundance of CTX-M and NDM were perpetually indicating the high prevalence of ESBL/MBL producing strains at HOP. The *bla*$_{CTX-M}$ gene is currently the most common ESBL worldwide, with

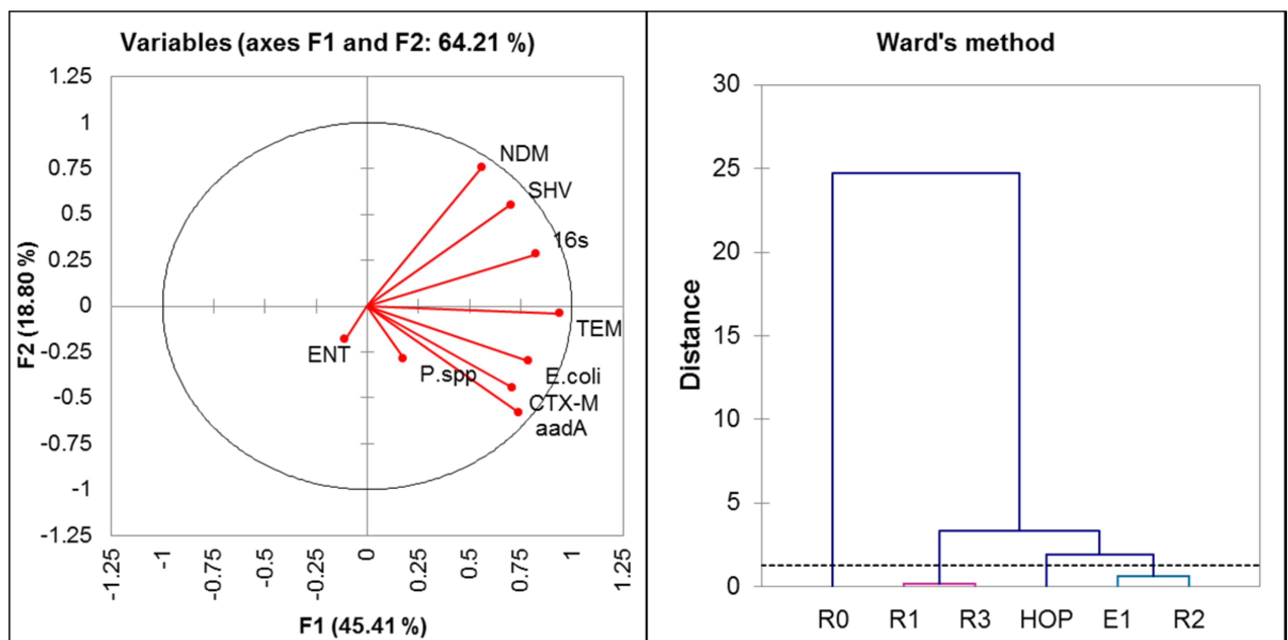
CTX-M-15 and CTX-M-14 predominantly invading humans and animals as well as environmental compartments (Canton et al. 2012). The spread of ESBL and MBL in the fresh water receiving system is highly alarming. Majority of ESBL/MBL producing strains are also resistant to other clinically relevant Abs. This is due to the fact that these ß-lactamases are commonly carried on conjugative plasmids that also harbor gene conferring resistance to other antibiotic classes (Zurfluh et al. 2013). The presence of ESBLs/MBLs genes in the fresh water sources are also reported in other countries, including France, Portugal, Finland, the United States, Brazil, Switzerland, China and Pakistan, (Zhang et al. 2013, Spindler et al. 2012, Devarajan et al. 2015b, Czekalski et al. 2014, Laht et al. 2014, Zurfluh et al. 2013, Khan et al. 2013,) indicating that a global environmental dissemination of ARGs is currently enduring.

3.3. Correlation among microbial contaminants

Inspection of Figure 3.6 (right pane) shows the contaminated sites at CRB (E1, R1-R3) are closely grouped with the HOP, and separately clustered to the control site on a greater distance, substantiating the high prevalence of ARGs and bacterial indicators at the HOP and in downstream of the effluent discharge point at CRB. Principal Component Analysis (PCA) on correlation is presented in Figure 3.6(left pane). The Pearson correlation matrix presented in Table 2s (supporting information). The positive mutual correlation of all of the ARGs is significant with total bacterial load (16S rDNA), and correlation ranged from 0.3 to 0.7 ($P < 0.05$). Likewise *E. coli* also had a positive correlation ($r > 0.3$, $p < 0.05$) with ARGs (except NDM). The bacterial species, ENT and P. spp were positively correlated ($r = 0.5$) between them. The *aadA* gene, frequently identified on the mobile genetic elements (class 1 integron genes) (Srinivasan et al. 2007) had a positive correlation ($r > 0.4$, $p < 0.05$) with 16S rDNA, *E. coli* and ARGs (bla_{TEM} and bla_{CTX-M}). This implies ß-lactamases association with the mobile genetic elements. This is also supported by a recent study on untreated hospital wastewaters in Brazil, reporting on high prevalence of ß-lactamases and class 1 integron genes (41.9%) (Spindler et al. 2012). The strong correlation among 16S rDNA and *E.coli* with ARGs leads to a conclusion that along with FIB other bacterial species also play a major role in the dissemination of ARGs from clinical settings to the terrestrial environment. Recent metagenomic studies on understanding the microbial populations in clinical environments highlight the differences in bacterial communities at various sections of the hospital (Poza et al. 2012) and in the sediments receiving urban effluents (Haller et al. 2011). In our recent study we have also observed presence of other bacterial species including *Acinetobacter*

(16%) in the study sites. This observation warrants further studies to understand the path and processes of selection of AR dissemination in the bacterial communities are required to palliate the risks associated with human and animal health.

Figure 3.6. Principle Component Analysis (PCA), on correlation biplot (left pane) indicating the corrlations among studied variables and bioplot scores on Axes 1 and 2 representing 64.21%. Agglomerative hierarchical clustering (AHC) computation based on Ward's method (right pane).



The emergence and spread of antibiotic resistance are complex mechanisms with many influential factors affecting co-selection and cross-selection of resistance. Metals (such as Cd, Cu, Hg and Zn) can selectively induce the co-selection of antibiotic resistance if they spread and accumulate in the environment at critical concentrations (Seiler and Berendonk 2012). From our previous study (Devarajan et al. 2015b) it is evident that the metal concentration in the studied locations (CRB/HOP) are above the probable effect levels as recommended by the sediment quality guidelines (CCME 1999). Horizontal gene transfer (HGT) mechanisms

between bacterial hosts are also considered as key factors behind the elevated levels of ARGs in the environment (Kummerer 2004, Srinivasan et al. 2008). A recent study by Walsh et al. (2011) reported an average temperature of 30 °C favor the HGT mechanisms than human gut temperatures. Hence the metal concentrations and the climatic factors may contribute for the elevated levels of ARGs in our tropical study site. Existence and proliferation of antibiotic resistance in pathogenic and zoonotic bacteria has intense associations with human/animal health as these bacteria could be transmitted via direct or indirect contact. The acquisition of resistance genes by bacteria (esp. ESBLs, MBLs) minimize the therapeutic options and probably lead to frequent infections (Stoll et al. 2012).

Molecular signature studies on ARGs from pristine and highly polluted sites provide evidence that transport of contaminants (ARGs) from the source is also an important factor for the dissemination of ARGs to the aquatic ecosystem (Chen et al. 2013, Storteboom et al. 2010). Molecules such as DNA are capable of interacting with the surfaces in complex ways, including multiple sorption methods (Keil and Mayer 2014, Pote et al. 2007). Sorbed to flocs, suspended solids, and/or organic matter these ARB/ARGs could be transported along the river water current (Pei et al. 2006). At CRB, the ARGs were detected at a distance of ca. 15 km from the effluent discharge point. This sample site (R3) "The Grant Anicut" (reservoir) serves the Cauvery Delta as a major source of irrigational water. While surface water contamination and cross contamination of irrigational water are suspected for some large outbreaks, increasing evidence of human gastrointestinal illness due to potential contamination by pathogenic microbes in fresh produce are been reported (Pachepsky et al. 2011). Nevertheless one may consider the fact that metagenomic studies that analyze the DNA, such as this study, may provide the information on the presence/absence and the quantitative data but do not provide information on the expression of these ARGs (Lachmayr et al. 2009). However, the expression of genes is not the central query of this study when the purpose of this study is to address the evaluation of the tropical aquatic environment to serve as reservoirs of ARGs that could be potentially transferred to bacterial communities.

4. Conclusion

Globally, rivers are not only part of the country's economy but also can act as a natural dumping ground for communities and industries situated along the bank. Discharge of wastewaters from various sources (industries, hospitals, and communities) has a direct influence on the proliferation and dissemination of emerging contaminants along the river

receiving system. Overall, this study supports the hypothesis that the natural environment (i.e., sediments) can act as a reservoir for emerging microbial contaminants such as FIB and ARGs. The relative abundance of ARGs vary according to the seasonal sampling period at HOP and it is likely that these ARGs released/accumulated in the sediments of the sewer systems could be transported to the receiving systems in large quantities during floods and periods of rain. Sediments receiving wastewaters from contemporary source offer the opportunity for restructuring pollution history and allow us to evaluate the potential impacts. The quantification of ARGs in the aquatic ecosystem will facilitate improved risk assessments for the prudent use of Abs in human, animal and agriculture, and provide baseline information for developing strategies to limit the spread of antibiotic resistance. Even added, changes to the ecosystem (input of emerging micro-contaminants) may support the emergence of unknown resistances in the bacterial community (Martinez 2008). The results of this study suggest that the discharge of hospital and urban wastewaters into the aquatic systems leads to the dissemination of emerging microbial pollutants. Therefore, the river receiving systems under tropical conditions such as CRB which has average daily peak temperature reaching 30°C, which could potentially favor the transfer frequencies of mobile genetic elements carrying ARGs to susceptible bacterial pathogens. Hence further studies are required to unleash the pathways involved in the spread of ARGs and FIB for assessing the human and environment potential risks in investigated and similar geographical locations.

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Supporting Information (SI)

Number of tables : 2

Number of figures : 3

Table.3.1S. Primers used in this study for quantification with RT-PCR

Target gene	Primer	Sequence (5' – 3')	Tm (˚C)	Size	References
Bacterial V3 region	338 F	ACTCCTACGGGAGGCAGCAG	55	197	(Ovreas et al. 1997)
	518 R	ATTACCGCGGCTGCTGG			
E. coli	Uida 405 F	CAACGAACTGAACTGGCAGA	55	121	(Chern et al. 2011)
	Uida 405 R	CATTACGCTGCGATGGAT			
ENT 16S	Ent376F	GGACGMAAGTCTGACCGA	55	221	(Ryu et al. 2013)
	Ent578R	TTAAGAAACCGCCTGCGC			
Pseudomonas spp.	Pse435F	ACTTTAAGTTGGGAGGAAGGG	54	251	(Bergmark et al. 2012)
	Pse686R	ACACAGGAAATTCCACCACCC			
bla_{TEM}	TEM-RT-F	GCKGCCAACTTACTTCTGACAACG	55	247	(Sidrach-Cardona et al. 2014)
	TEM-RT-R	CTTTATCCGCCTCCATCCAGTCTA			
bla_{CTX-M}	blaCTX-M-rt-f	ATTCCRGGCGAYCCGCGTGATACC	62	227	(Fujita et al. 2011)
	blaCTX-M-rt-r	ACCGCGATATCGTTGGTGGTGCCAT			
bla_{SHV}	blaSHV-rt-f	CGCTTTCCCATGATGAGCACCTTT	60	110	(Xi et al. 2009)
	blaSHV-rt-r	TCCTGCTGGCGATAGTGGATCTTT			
bla_{NDM-1}	NDM-F	TTGGCGATCTGGTTTTCC	58	195	(Zheng et al. 2013)
	NDM-R	GGTTGATCTCCTGCTTGA			
aadA	aadA-F	GCAGCGCAATGACATTCTTG	55	282	(Madsen et al. 2000)
	aadA-R	ATCCTTCGGCGCGATTTTG			

Table 3.2S. Correlation matrix (Pearson) performed for the analyzed parameters[a] in the surface sediment samples.

Variables	16s	E.coli	ENT	P.spp	aadA	TEM	SHV	CTX-M	NDM
16s	**1**	**0.529**	-0.090	0.248	**0.442**	**0.718**	**0.625**	**0.365**	**0.675**
E.coli		**1**	0.032	0.176	**0.686**	**0.722**	**0.362**	**0.565**	0.233
ENT			**1**	**0.555**	-0.069	-0.156	-0.107	-0.163	0.011
P.spp				**1**	0.145	0.146	-0.008	0.032	-0.008
aadA					**1**	**0.730**	0.198	**0.758**	-0.046
TEM						**1**	**0.611**	**0.653**	**0.492**
SHV							**1**	**0.301**	**0.749**
CTX-M								**1**	0.047
NDM									**1**

[a] Parameters include bacterial population (16s, *E. coli*, ENT, *Pseudomonas* spp. (P.spp), and the selected ARGs (*aadA*, bla_{TEM}, bla_{SHV}, $bla_{\mathrm{CTX\text{-}M}}$, and bla_{NDM}). Correlation coefficients have been calculated using the log transformed values to normalize their distribution. Statistically significant coefficients ($p < 0.05$) are in bold.

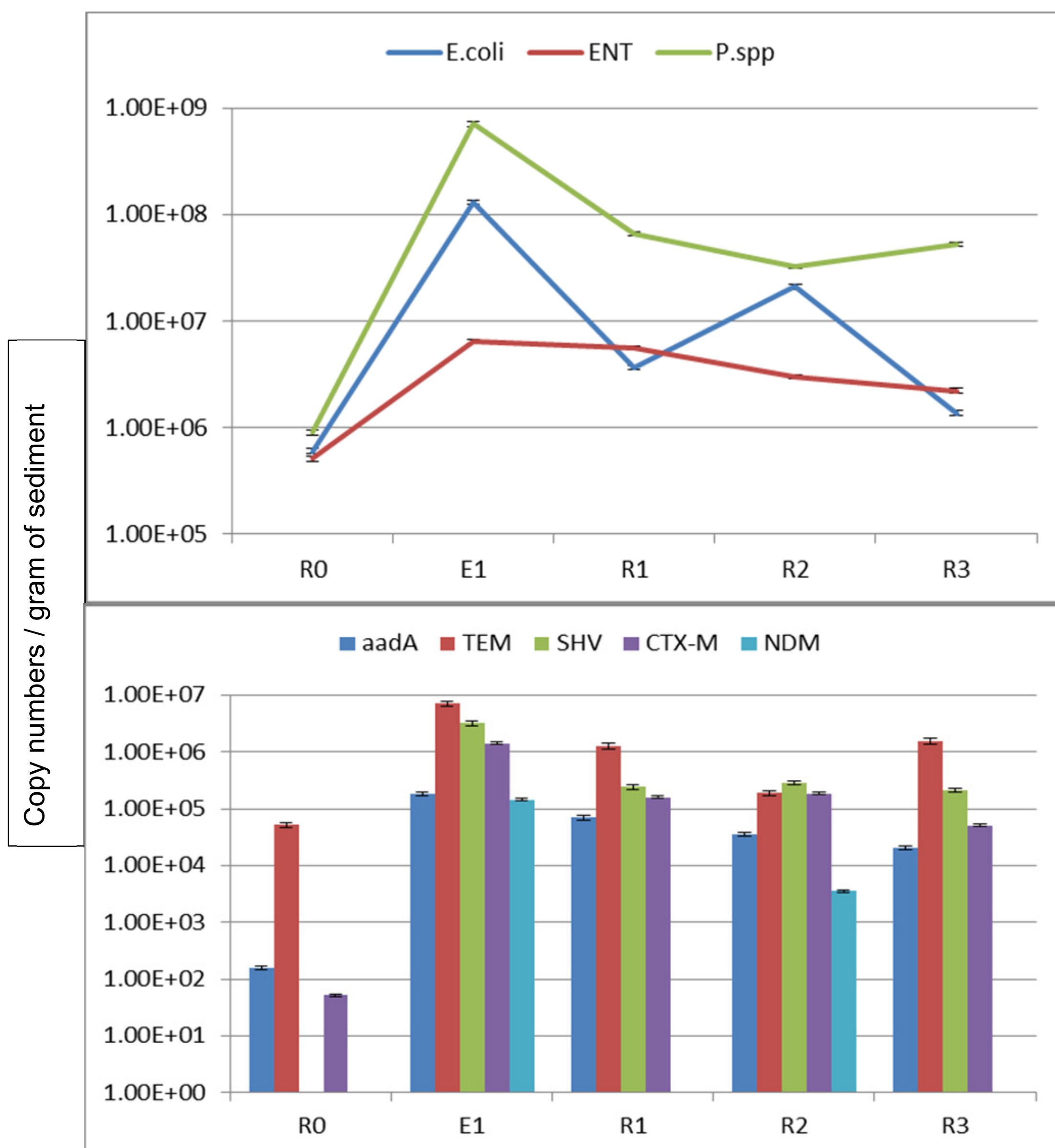

Figure 3.1S. . qPCR quantification of the bacterial population (A) for the selected bacterial species (*E. coli*, ENT and *Pseudomonas* spp.) and (B) antibiotic resistance genes (*aadA*, bla_{TEM}, bla_{SHV} bla_{CTX-M} and bla_{NDM}) in the surface sediments of Cauvery River Basin. Values are expressed as copy numbers in per gram of sediment.

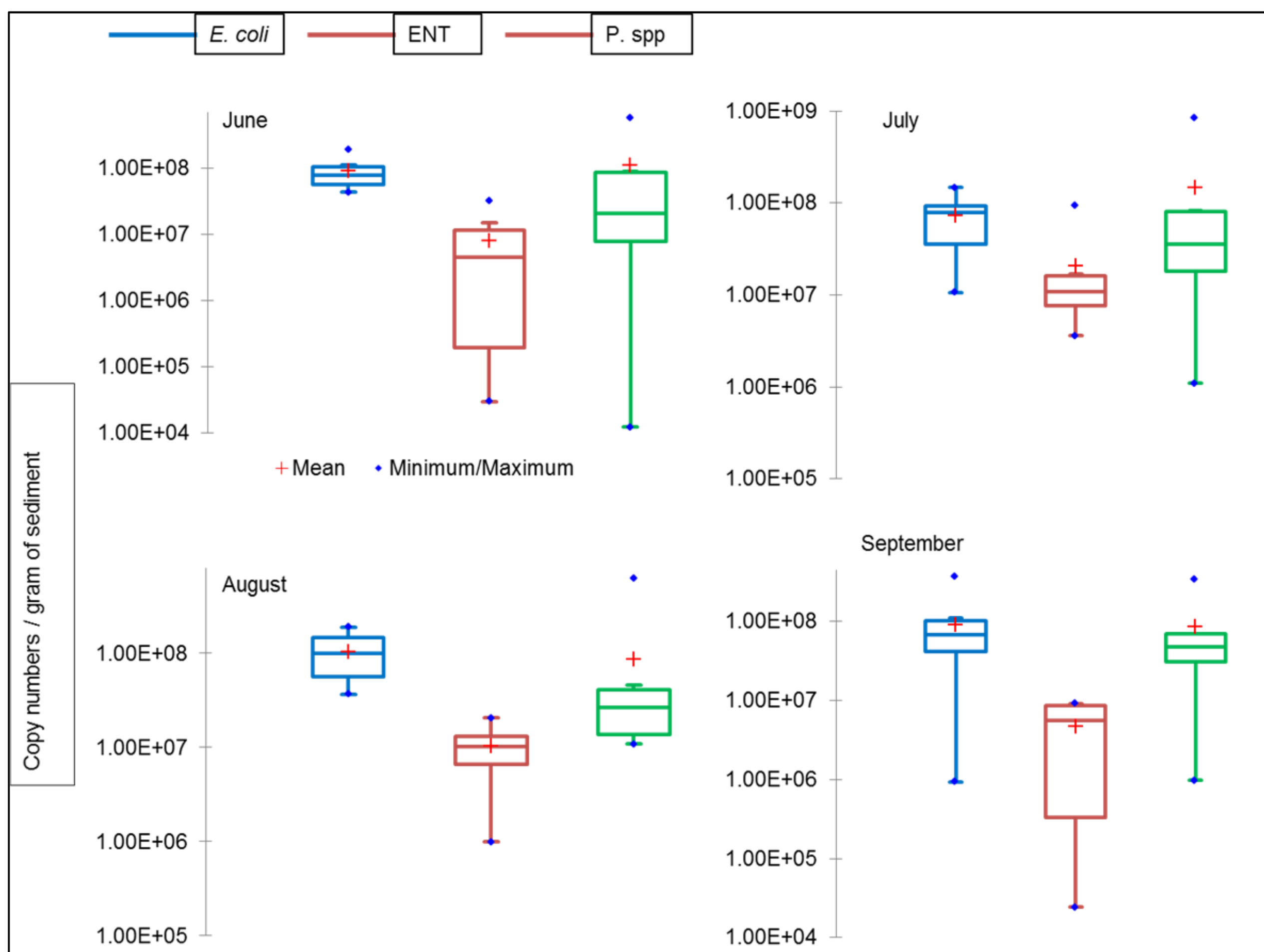

Figure 3.2S. qPCR quantification of the bacterial population for the selected bacterial groups (*E. coli*, ENT and *Pseudomonas* spp.) in the surface sediments of HOP samples. Values (log transformed) are expressed as copy numbers in per gram of sediment. The box plot represents the first and third quartile, the blue dots on maximum/minimum values and the red cross indicate standard errors

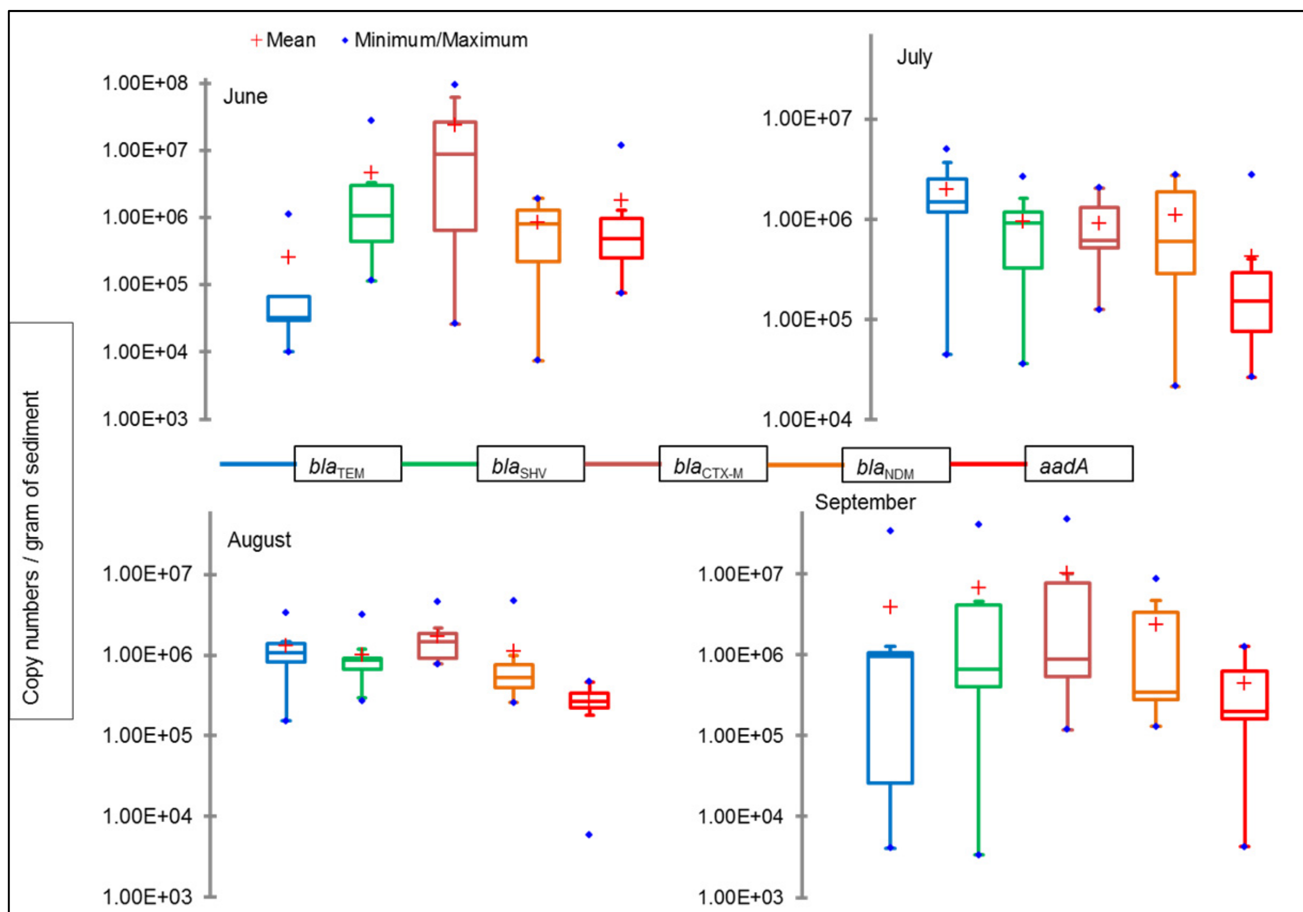

Figure 3.3S. qPCR quantification of the selected antibiotic resistance genes (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{NDM}$ and *aadA*) in the surface sediments of HOP samples. Values (log transformed) are expressed as copy numbers in per gram of sediment. The box plot represents the first and third quartile, the blue dots on maximum/minimum values and the red cross indicate standard errors

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CHAPTER 4

Accumulation of clinically relevant antibiotic-resistance genes, bacterial load, and metals in freshwater lake sediments in central Europe

Supporting Information available at the end of the chapter

A similar version of this chapter was published under the following reference:

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Abstract

Wastewater treatment plants (WWTP) receive the effluents from various sources (communities, industrial, and hospital effluents) and are recognized as reservoir for antibiotic-resistance genes (ARGs) that are associated with clinical pathogens. The aquatic environment is considered a hot-spot for horizontal gene transfer, and lake sediments offer the opportunity for reconstructing the pollution history and evaluating the impacts. In this context, variation with depth and time of the total bacterial load, the abundance of faecal indicator bacteria (FIB; *E. coli* and *Enterococcus* spp. (ENT)), *Pseudomonas* spp., and ARGs (bla_{TEM}, bla_{SHV}, $bla_{\mathrm{CTX\text{-}M}}$, bla_{NDM}, and *aadA*) were quantified in sediment profiles of different parts of Lake Geneva using quantitative PCR. The abundance of bacterial marker genes was identified in sediments contaminated by WWTP following eutrophication of the lake. Additionally, ARGs, including the extended-spectrum ß-lactam and aminoglycoside-resistance genes, were identified in the surface sediments. The ARG and FIB abundance strongly correlated ($r \geq 0.403$, $p < 0.05$, $n = 34$) with organic matter and metal concentrations in the sediments, indicating a common and contemporary source of contamination. The contamination of sediments by untreated or partially treated effluent water can affect the quality of ecosystem. Therefore, the reduction of contaminants from the source is recommended for further improvement of water quality.

4.1. Introduction

The question of the environmental and human risks of an increasing release of bacteria carrying antibiotic-resistance genes (ARGs) into the natural environment has been a subject of intense scientific and political debate over the past decade. The high concentration of antibiotics (Abs) found in water, sediments, and soils can be the consequence of shifts in the original use of Abs in hospitals and farms for treating or protecting against bacterial infection (Martinez 2008). The use of a wide variety of antimicrobials in human and veterinary medicine, including aquaculture, has led to the widespread emergence of antibiotic resistant pathogens (Shah et al. 2012). The waste water treatment plant (WWTP), which receives the mixture of effluent from hospitals, communities, and animal farming as well as industrial and agricultural runoff, is considered a major source of antibiotic resistant bacteria (ARB) and ARGs for the aquatic environment and a hot-spot for horizontal gene transfer (HGT) (Shah et al. 2012, Brechet et al. 2014, Graham et al. 2011, Thevenon et al. 2012a). The pathogenic bacteria are able to acquire the resistant genes from the environment and environmental bacteria through gene transfer mechanisms and could represent a potential threat to human and animal health (Zhu et al. 2013). ß-lactams are the most consumed Abs globally, and about 1000 ß-lactamase enzymes have been identified, conferring resistance to several ß-lactams. This indicates the rapid evolution of bacterial resistance to ß-lactams in soil and aquatic environments (Brechet et al. 2014, Davies and Davies 2010, Demaneche et al. 2008).

Freshwater sources (rivers and lakes) are a major public resource but also are typically the final destination of treated and untreated effluent water. WWTPs were not originally designed to have a specific impact on removal of resistant bacteria or antimicrobial residues, and little is known of the effects of WWTPs on microbial contaminants (Thevenon et al. 2011, Thevenon et al. 2012b).

Lake Geneva is a temperate lake with a surface area of 580.1 km^2, a volume of 89 km^3, and a maximum depth of 309.7 m. The lake provides drinking water but also receives the wastewater from urban developments. The lake was eutrophic during the 1970s, but after reduction of phosphorus inputs during the 1980s, the lake has become mesotrophic (Dorioz et al. 1998). The largest WWTP is located in the city of Lausanne, and it releases its effluent into Vidy Bay. From 1964 to 2001, the WWTP effluents were released into the lake about 300 m from the shore, at a water depth of 15 m. In 2001, the WWTP outlet pipe was extended by the authorities to a distance of 700 m from the shore at a 35 m water depth (Figure 4.1). The

reason for and the effect of the new and old outlet pipes are well described in (Pote et al. 2008). The release of effluents from this WWTP has made Vidy Bay a heavily polluted site (Pote et al. 2008). Long-term monitoring data are available on the composition and distribution of microbial and aquatic biota, including fecal indicator bacteria (FIB), as well as on the amount and distribution of organic matter, metals, micropollutants, and hydrophobic organic compounds in the lake (Thevenon et al. 2011, Pote et al. 2008, Arbouille et al. 1989, Haller et al. 2009a, Haller et al. 2009b, Pardos et al. 2004). In recent years, the surface sediments of the lake were studied for the presence of some ARGs (*sul1*, *sul2*, *tet*(*B*), *tet*(*M*), *tet*(*W*), and *qnrA*) and ARB (Thevenon et al. 2012a, Thevenon et al. 2012b, Czekalski et al. 2012, Czekalski et al. 2014). However, there is a paucity of information on the spatiotemporal variation in the distribution of ARGs (ß-lactam- and aminoglycoside-resistance genes) in the lake sediment.

To our knowledge, very little data are available on the concentration and periodic accumulation of ß-lactam-resistance genes in the lake sediments. Yet, the assessment of ARGs and their persistence overtime are critical for devising and evaluating strategies to mitigate ARG propagation (Thevenon et al. 2012a, Mao et al. 2014). ARB and ARGs are ubiquitous in nature and can occur in high concentrations in industrial, community, clinical, and farming wastewaters that are being released into freshwater ecosystems (Thevenon et al. 2012a, Thevenon et al. 2012b, Srinivasan et al. 2008). Studying the sediments allows us to address the persistence of ARGs and the potential impact of the emergence of resistant bacterial strains from WWTP to freshwater sediment microbes. Thus, the sediments offer a unique opportunity for reconstructing the pollution history and evaluating the impacts using quantitative data.

This research has been performed in Lake Geneva and has the following purposes: (i) to evaluate the sediment quality through physicochemical parameters including total organic matter (OM) content, particle grain-size distribution, and metal concentrations (Cr, Mn, Fe, Co, Ni, Cu, Zn, Ag, Cd, Pb, and Hg), and (ii) to quantify the FIB (*E. coli* and *Enterococcus* (ENT)), *Pseudomonas* spp. (P.spp), and ARGs (bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, bla_{NDM}, and *aadA*) in sediment profiles. The data were statistically analyzed and correlated in order to determine whether the WWTP effluent waters can affect the distribution of ARGs, FIB, and toxic metals in sediments and to interpret the time origin of pollution as well as the potential impact on lake water quality.

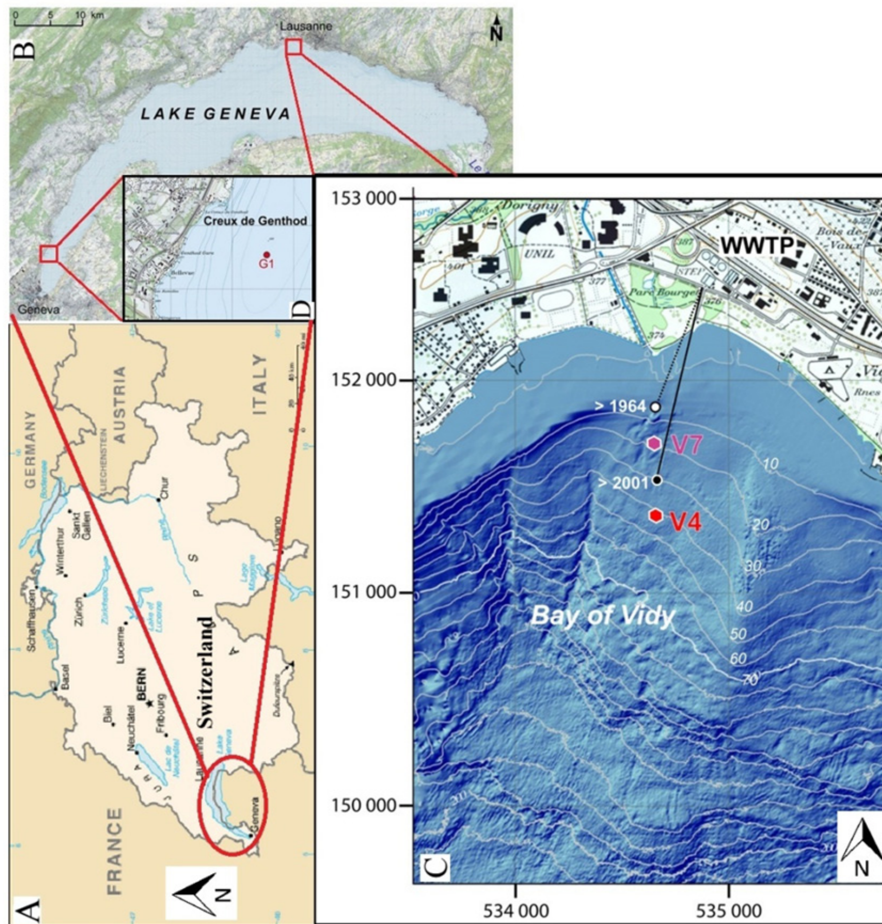


Figure 4.1. (A) Location of Lake Geneva at the border of France and Switzerland. (B) Location map of Lake Geneva with the cities of Lausanne and Geneva. (C) Vidy Bay and the position of cores V4 (red dot) and V7 (pink dot) and the 1964 (white circle) and 2001 (black circle) WWTP outlet pipe locations. (D) Creux de Genthod region (control site) with the location of core G1.

4.2. Materials and methods

4.2.1. Study site and sampling procedure

Vidy Bay is located at the northern shore of Lake Geneva at the city of Lausanne and accounts for the 0.3% of the total lake volume (Goldscheider et al. 2007). Approximately 1−3 $m^3 s^{-1}$ of urban wastewater is treated by this WWTP in Lausanne from ca. 214 000 inhabitants, including wastewater from several healthcare centers. The Centre Hospitalier Universitaire Vaudois (CHUV) is one of the largest and the most important facilities in terms of capacity and Abs consumption (Czekalski et al. 2012, Czekalski et al. 2014, Blanc 2010).

The main building accounts for 71% of the CHUV sewage outputs. On an average day, ca. 4.7 × 10^{-3} m3 s^{-1} of raw sewage is disposed from this building to the municipal sewer system of Lausanne (Blanc 2010). The WWTP receives on average 107 734 m^3 day^{-1} of wastewater and discharges 86 631 m^3 day^{-1} of treated sewage (typically 1−3 $m^3 s^{-1}$ and up to 5−6 m^3 s^{-1}) directly into Vidy Bay (Vioget et al. 2011). The other sources of contaminants to Vidy Bay include the Chamberonne River from its natural drainage basin and inputs of some untreated wastewater from damaged urban collectors (Haller et al. 2009b, Goldscheider et al. 2007). The other major input, the River Flon, also collects surface and wastewater from the western part of the city, which is usually treated at the WWTP but released into the lake at a 10 m depth during floods when the input to the WWTP exceeds 4−5 m^3 s^{-1}. One of the drinking water supplies for the city of Lausanne is lake water, taken at St-Sulpice, a pumping station located just 3.8 km downstream from the WWTP outlet. It provides 58% of drinking water for the city of Lausanne at an average rate of 385 L s^{-1} and taken from a 45 m depth. This water pumping station meets the water needs of 127 000 inhabitants (approximately) of the city of Lausanne (Pote et al. 2008, Czekalski et al. 2012, Vioget et al. 2011).

The boat La Licorne of the Institute F. A. Forel was used for the collection of core sediments from Lake Geneva in May 2014 at the following locations: (i) in Vidy Bay near the present WWTP outlet-pipe discharge (core V4, 60 m water depth, 55 cm in length, Swiss coordinates X = 534 682, Y = 151 410), (ii) in between the two outlet pipes (core V7, 35 m water depth, 40 cm length, Swiss coordinates X = 534 426, Y = 151 512), and (iii) in the Creux de Genthod area at a 51 m water depth (core G1, 45 cm length, Swiss coordinates X = 502 565, Y = 123 544). The Creux-de-Genthod area of Lake Geneva is a coastal area, but on the basis of the study by (Thevenon et al. 2012a) this area could be considered as WWTP-pollution-free. After collection, the cores were opened and sliced into 3 cm thick sections for the first 15 cm and into 5 cm thick sections for the remainder of the core. For chemical analysis, the sediment samples were frozen, freeze-dried, and ground into a fine powder. For the DNA extraction, the samples were stored in sterile plastic cups at 4 °C until used.

4.2.2. Sediment grain size and total organic matter and water content

The particle grain size was measured with a Laser Coulter LS-100 diffractometer (Beckman Coulter, USA) following the method described by (Loizeau et al. 1994). The sediment total OM content was estimated by the loss on ignition at 550 °C for 1 h in a Salvis oven (Salvis AG, Switzerland).

4.2.3. Metal analysis

Sediment samples were lyophilized at −45 °C after homogenization and air-drying at ambient temperature. The metals, including Cr, Co, Ni, Cu, Zn, As, Cd, and Pb, were determined by quadripole-based inductively coupled plasma mass spectrometry (ICP-MS, model 7700 series, Agilent, USA) following the method described previously (Pote et al. 2008, Pardos et al. 2004). Total variation coefficients of triplicate sample measurements were smaller than 10%, and chemical blanks for the procedure were less than 2% of the sample signal. The metal concentrations of sediments were expressed in ppm (mg kg^{-1} dry weight sediment). The total Hg analysis was quantified with an atomic absorption spectrophotometer (AAS; AMA 254, Altec s.r.l., Czech Republic) following the method described by (Roos-Barraclough et al. 2002). The detection limit (3 SD blank) was 0.005 mg kg^{-1}, and the reproducibility was better than 95%.

4.2.4. Total DNA extraction

Total DNA from sediment samples was extracted using Ultraclean Soil DNA Kit (Mo Bio Laboratories, USA) by following manufacturer's instructions. The isolated DNA was stored at −20 °C until used. DNA extraction was performed with two replicate samples (from the same section of the sediment core) to compensate for heterogeneity. The DNA samples were diluted to 10× and 50× to avoid inhibitors to the PCR reaction.

4.2.5. Positive control plasmid construction

The positive control target genes (*E. coli*, uidA gene; ENT and P.spp., 16S rRNA) were amplified from *E. coli* ATCC25922, *E. faecalis* ATCC29212, and *P. aeruginosa* ATCC27853 and ARGs (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{NDM}, and *aadA*) from well characterized laboratory strains available at Institute F. A. Forel, Universtiy of Geneva, using the primers described in Table 4.2S (Supporting Information). The PCR products were purified using Genelute Gel extraction kit (Sigma-Aldrich, USA), and cloned into the pGEM-T vector (Promega, USA) according to the manufacturer's instructions. Briefly, 4 µL of each ligated product was

transformed into chemically competent *E. coli* DHα cells. Plasmids from selected transformants were purified by Sigma-Aldrich Genelute HP plasmid extraction kit (Sigma-Aldrich, USA). The inserted PCR products in each plasmid were sequenced (GenBank numbers: KP172294−KP172300) for confirmation, and the concentration of plasmids was measured using Qubit assay kit (Life Technologies, Switzerland) following the manufacturer's instructions and used as standards for subsequent quantitative PCR (qPCR) reactions.

4.2.6. qPCR quantification of FIB and ARGs in sediments

The abundance of FIB and ARGs in sediments samples collected from Lake Geneva was quantified with an Eco qPCR system (Illumina, Switzerland) using KAPA SYBR FAST qPCR Master Mix Universal Kit (KAPA Biosystems, USA). The primer sequences and reaction conditions are provided in Table 4.2S (Supporting Information). The following cycling parameters were applied: 10 min at 95 °C for the polymerase activation; followed by 40 cycles of 95 °C for 30 s, optimal Tm (Table 2s, Supporting Information) for 30 s, and 72 °C for 30 s. The melting-temperature-curve profile was obtained using the following conditions: 95 °C for 30 s, optimal Tm (Table 2s, Supporting Information) for 30 s, followed by 95 °C for 30 s.

All the reactions included negative (with no template DNA) and positive controls (10-fold serial dilutions of pGEM-T plasmid with respective target gene insert). All negative controls resulted in either no amplification or a threshold cycle (Ct) higher than the most diluted standard (pGEM-T plasmid). A sample was considered to be below the limit of detection (LOD) or negative for a target gene if ≥2 out of 3 technical replicates were negative or if sample Ct values were ≥Ct of negative controls. Samples above LOD were considered to be below the limit of quantification when the standard deviation of Ct values of methodological triplicates was 40.5 and their Ct value was higher than the Ct of the most diluted standard whose standard deviation of Ct values was ≤0.5. For each reaction, the efficiency of the assay was calculated by using the measured slope of the standard curve ($E = 10^{(-1/\text{slope})} - 1$). The absolute copy number of each reaction was quantified by referring to the corresponding standard curve obtained by plotting the copy number of the constructed pGEM-T plasmid versus threshold cycles. The serial 10-fold dilutions of plasmid DNA containing the respective target gene copies were used for the standard curve. To emphasize the relative abundance of the resistance genes, the concentrations of the gene copy numbers were

presented as percentage of the ratio = (copy number of a gene)/(copy number of 16S rDNA) for each sample.

4.2.7. Data analysis

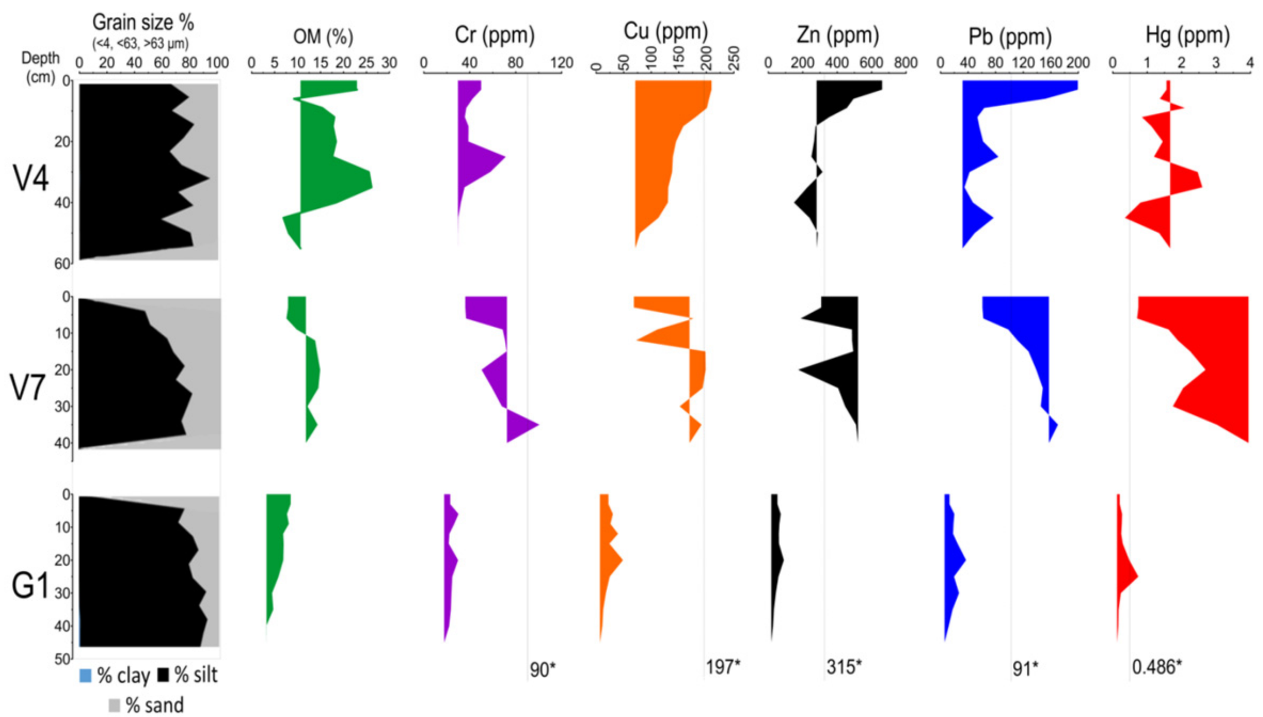
The target genes were normalized to sediment sample dry weight, and we refer this as "gene concentration" from here on. The gene copy numbers were normalized to 16S rDNA copy numbers and are reported as percentages as an indicator of relative abundance to bacterial/ARGs population. We refer to this as the "ARGs/bacterial 16S rDNA abundance" hence forth. For all measured parameters, triplicate measurements were performed on selected samples. The data was subjected to a Spearman Rank Correlation test to investigate possible relationships using SigmaStat 11.0 (Systat Software, Inc., USA). The data was subsequently subjected to a principle component analysis (PCA) using a correlation matrix performed with R Statistical Software, version 3.1.2 (The R core team) and the ade4 package (Dray and Dufour 2007). The correlation matrix form of PCA was used because the measured parameters had different scales of measurement. The correlation-matrix-based PCA normalizes each measured parameter allowing for direct comparison. The average of the qPCR efficiencies calculated from the slopes of standard curves for each assay and the representation of derivative melt curve analysis are presented in Figure 4.7s (Supporting Information). Previous studies have demonstrated that the copy number of 16S rDNA per bacterial genome can vary among bacteria, but in environmental samples, its quantification has previously been used to estimate the overall bacterial abundance and to normalize selected genes to the total bacterial population (Czekalski et al. 2014, Selvam et al. 2012).

4.3. Results and discussion

4.3.1. Grain size, organic matter and water content

The grain size distribution, total OM, and the water content are given in Table 1s (Supporting Information) and Figure 4.2. The sediments collected at all three sampling sites have a silty−sand composition with less than 1% of clay. The values of silt and sand ranged between 58.8−94.2/46.2−78.8/70.5−91.0 and 5.5−41.2/21.1−53.7/7.8−29.5 for the V4, V7, and G1 sites, respectively. Previous studies showed that total OM in the surrounding sediments varied between 4−8%, except for the sampling sites closer to the WWTP outlet (Thevenon et al. 2011, Pote et al. 2008).

Figure 4.2. Graphical representation of physical chemical parameters of the sediment cores including grain size, total OM content, and Cr, Cu, Zn, Pb, and Hg concentrations.* metal values above the recommended PEL (probable effect level) values as recommended by (CCME 1999).



Similar results were also observed in this study with the organic matter values ranging between 3.1−8.5% at control site (G1) and 6.9−26.1% at WWTP outlet site (V4). At this site, the organic matter content showed a similar pattern of variation with the lowest concentrations for the bottom part of the sediment core (preindustrial period) increasing values after the beginning of the WWTP discharge (in 1964) and values that tripled at a 40−45 cm depth during the eutrophication period (Thevenon et al. 2012b). The mean grain size of sediment particles in the upper part of core V4 ranged from 17.7−46.7 µm, which presents a slight decrease to 25.2−46.7 µm down from 50 cm; this difference can be linked to the installation of the WWTP in 1964 (Thevenon et al. 2012b, Haller et al. 2009b). However, the increase in grain size from the bottom of the cores to the surface (from 26 to 71 µm and from 14.4 to 40.7 µm) and the increase in sand (from 21 to 53% and from 1.8 to 40.7%) in cores from V7 and G1, respectively, indicates the coarser sediments (high-energy environment caused by wave action) and the source contributions (the Chamberonne River

delivers coarse sediments close to its mouth, V7 sample) (Goldscheider et al. 2007). The water content along the core samples depended on the total OM content because we observed an increase in water content with an increase in organic matter along the core samples depth.

4.3.2. Metal analysis

The values of metals are presented in Table 3s (Supporting Information) and Figure 4.2 (Cr, Cu, Zn, Pb, and Hg), ranked according to sampled core depth. The metal values (mg kg^{-1}) range between 17−100 (Cr), 155−319 (Mn), 7127−21130 (Fe), 3.2−6.2 (Co), 18.4−33.5 (Ni), 7−209.5 (Cu), 17−660.7 (Zn), 0.05−4.4 (Ag), 0.07−3.4 (Cd), 5.8−199.2 (Pb), and 0.12−3.9 (Hg). Deeper sediments were observed to have higher concentrations of metals at the V7 site compared to the surface of the core. The same is true for core V4, with the exception of its very surface layer having a greater concentration of metals. The concentrations of metals at the G1 site were compared to natural background values reported by (Arbouille et al. 1989). All the metals, except Hg, were comparable to natural background values, indicating that the accumulation of metals at the control site is a natural process. This observation indicates that the WWTP is a primary input source of metals to Vidy Bay. After the extension of the WWTP outlet pipe to the V4 site, the contaminants have started to accumulate more in the surface sediments of this area to concentrations that now exceed those at the bottom of the sediment core, corresponding to the time period before extension of the WWTP outlet. An evaluation of the potential deleterious effects of the metals toward benthic fauna, applied on consensus-based guidelines for sediment quality (MacDonald et al. 2000) gives an estimate of the potential hazard these sediment may represent to the biota. The metal concentrations were primarily compared to the Canadian Sediment Quality Guidelines (CSQG) for the Protection of Aquatic Life, (CCME 1999) and Cr, Cu, Zn, Pb, and Hg at V4 and V7 were always found to be above the CSQG guidelines. The values of the metals were one (Cr, Cu), two (Zn, Pb), and eight (Hg) times greater than the probable effect levels (PEL) recommended by the CSQG (CCME 1999).

The pattern and amplitude of Hg accumulation has changed since the prolongation of the WWTP outlet pipe, and the surface area of strongly contaminated sediments has decreased from 1.3 to 0.8 km^2 (Pote et al. 2008). Previous studies have shown higher Hg concentrations in the sediments of Vidy Bay, with maximum values of 27.18 mg kg^{-1} surrounding the WWTP outlet pipe, (Thevenon et al. 2011, Pote et al. 2008) in comparison with historical Hg values measured in Vidy Bay, which range from 4.28 mg kg^{-1} in 1996 (Thevenon et al. 2011)

to 8.64 mg kg^{-1} in 2005, (Pote et al. 2008) and 1.56 mg kg^{-1} (this study) in 2014. Hence, a reduction in Hg deposition with time is evident. However, the deeper, older sediments of Vidy Bay remain contaminated with high Hg concentrations (V7 at 40 cm = 3.9 mg kg−1). The fine sediment deposits, with higher water content, form bars and embankments, which are unstable and subject to erosion via waves, currents, movement by gravity, and anthropogenic activities (Pote et al. 2008, Arbouille et al. 1989). According to these parameters, it is evident that the sediments of Vidy Bay are heavily contaminated with metals and represent a significant potential threat to the ecosystem.

4.3.3. Quantification of bacterial population

The average gene copy numbers of the bacterial marker genes in the sediment samples are presented in Figure 4.8s (Supporting Information) for *E. coli*, ENT, and P. spp. The total bacterial load (on the basis of 16S rDNA gene copy numbers) in the sediment samples (Figure 4.3) were in the range of $1.01 \times 10^7 - 5.5 \times 10^{11}$ copy numbers per g^{-1} dry sediment. The bacterial density varied: $4.4 \times 10^4 - 1.8 \times 10^{10}$, $7.6 \times 10^3 - 1.1 \times 10^{10}$, and $8.6 \times 10^3 - 2.4 \times 10^9$ for *E. coli*, ENT, and P. spp., respectively. We repeat that in order to avoid inconsistencies among qPCR assays, including suboptimal efficiency, we use 16S-rDNAnormalized values (Figure 4.4). The highest abundance of bacterial populations was recorded in the core samples at the 40 and 35 cm depths of the V4 and V7 cores, respectively. This observation indicates that the bacterial load increased dramatically in the sediments during the 1970s in Vidy Bay, probably due to both eutrophication of the lake and the signature of the WWTP (Thevenon et al. 2012a).

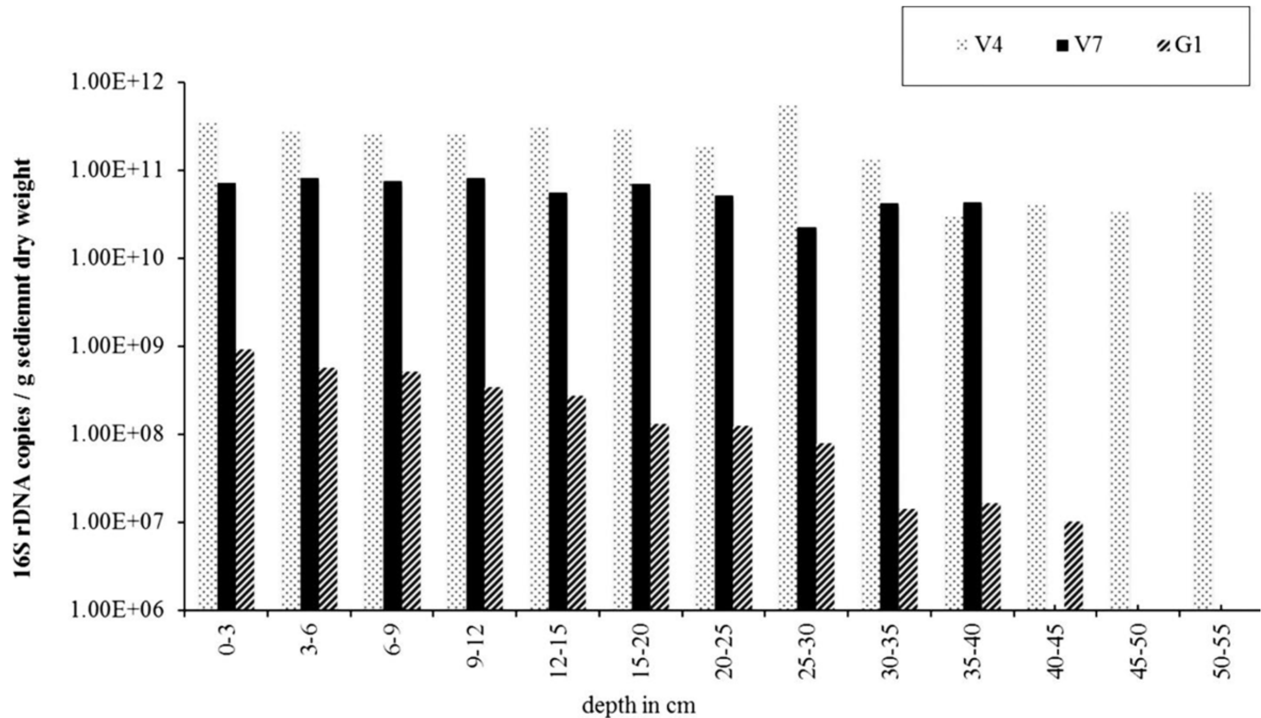


Figure 4.3. qPCR quantification of 16S rDNA in the sediment core samples at different depths. Values are expressed on a log scale for the copy number per gram of sediment dry weight normalized to the DNA extraction yield.

The vertical distribution of the *E. coli*, ENT, and P. spp. in the V4 and V7 cores shows the influence of the WWTP after 1964 and supports the impact of the eutrophication that influenced the increase the bacterial load in the sediments of Vidy Bay. *E. coli* and ENT are common indicators of pathogens in aquatic environments and can persist in the aquatic environment but preferably accumulate in the sediment rather than remaining in the watercolumn (Haller et al. 2009a, Haller et al. 2009b). Also, bacteria can survive longer in sediments, which provide favorable conditions for growth and protection from sunlight inactivation and protozoan grazing (Mwanamoki et al. 2014). Environments outside the gastrointestinal tract of warm-blooded animals (such as water, sediments, and soils) have previously been shown to represent a secondary habitat for FIB (Walk et al. 2007). Therefore, it can be hypothesized that sediments can act as reservoirs of metabolically active FIB. According to the metal analysis and the organic matter content data, it is evident that the G1 sampling site reflects the natural background values for Lake Geneva. In a comparison between the control site and the WWTP influenced site (V4) at the surface layer, there is a

720-, 700-, and 1080-fold increase in the bacterial load for the studied *E. coli*, ENT, and P. spp. bacterial markers, respectively. Hence, it is evident that the contaminated sediments of Vidy Bay constitute a reserve of bacterial populations that persist in certain areas of the bay. Resuspension of these sediments could affect water quality and could pose a potential health risk to humans and aquatic life.

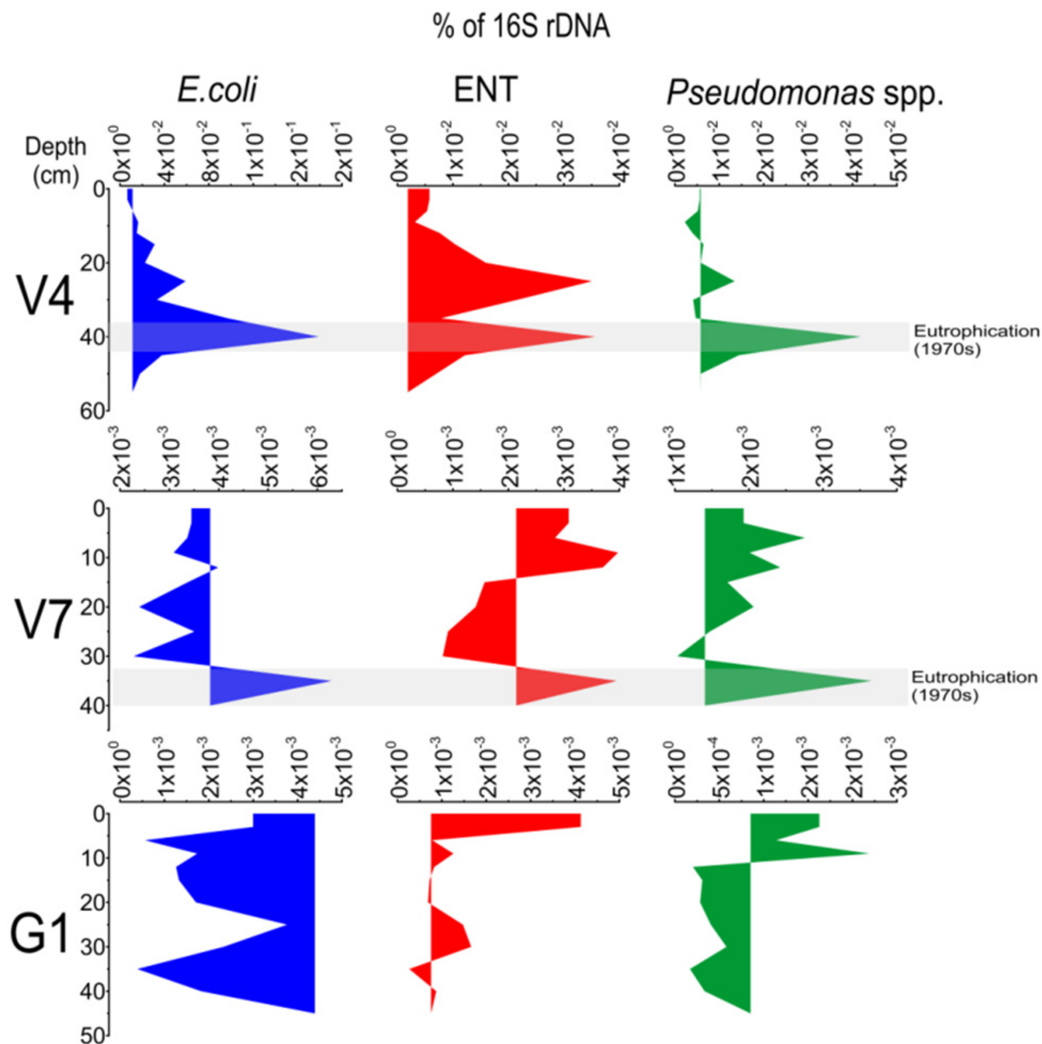


Figure 4.4. qPCR quantification of the bacterial population for selected groups (*E. coli*, ENT, and P.spp.) at different depths in the core sample. Values are expressed as the percentage of 16S rDNA bacterial population.

3.4. Quantification of antibiotic-resistance genes

The ARGs conferring resistance to ß-lactam (*bla_{TEM}*, *bla_{SHV}*, *$bla_{CTX\text{-}M}$*, and *bla_{NDM}*) and aminoglycoside (*aadA*) that were selected for the qPCR study were based on the following criteria: (i) clinically relevant genes (human risk); (ii) genes conferring resistance to frequently used Abs (penicillins and aminoglycosides) and newer extended-spectrum ß-lactams (carbapenems and cephalosporins); (iii) ARGs previously reported in mobile genetic elements; and (iv) ARGs not previously studied in Vidy Bay (Thevenon et al. 2012a, Czekalski et al. 2012, Czekalski et al. 2014).

The relative abundance of ARGs in the sediment core samples are presented in Figure 4.5. In the cores, bla_{TEM} and *aadA* genes were detected at all the sampling sites with relative abundances ranging from 2.6×10^{-5} to 2.8×10^{-3} and from 1.5×10^{-5} to 1.1×10^{-3}, respectively. These genes have previously been found to be abundant in sewage and effluent-receiving systems (Thevenon et al. 2012a). This enriched pattern of *aadA* and *bla_{TEM}* genes may be related to the long-term clinical usage of penicillin and aminoglycosides. The high abundance of *bla_{TEM}* genes at both the control and contaminated sites could be explained by their ubiquitous presence as housekeeping genes, which has previously been shown to occur frequently among soil bacteria (Demaneche et al. 2008). The relative abundance of *$bla_{CTX\text{-}M}$* and *bla_{SHV}* genes ranged from 3.2×10^{-6} to 1.7×10^{-3} and from 1.4×10^{-6} to 8.9×10^{-4}, respectively, and these genes were detected primarily in the top layers of the core samples (15−20 cm depth). The relative abundance of the *bla_{NDM}* gene ranged between 6.07×10^{-6} and 1.2×10^{-6} copy numbers and was identified only in the surface layer of the core samples (V4 and V7). The relative abundance of extended-spectrum ß-lactamases (ESBLs) was higher in sediment samples collected at the vicinity of WWTP-outlet-pipe discharge than at the control site. Likewise, elevated abundance of ARGs was observed in the surface layers of the V4 core, indicating the recent increase in ESBLs in Vidy Bay. The relative abundances of the studied ARGs provides a possibility to compare data between ARGs quantified in various other WWTPs (Czekalski et al. 2014, Selvam et al. 2012). According to (Lartigue et al. 2007) the spread of CTX-M-type ESBL producers in the community of acquired *E. coli* infections in Switzerland, in a manner similar to that which is observed in other countries, highlights the difficulties in controlling these resistance genes and their carriers in the environment. The persistence of ESBLs in the sediments of freshwater systems, as found in our study, could pose a further potential threat to the humans and aquatic life.

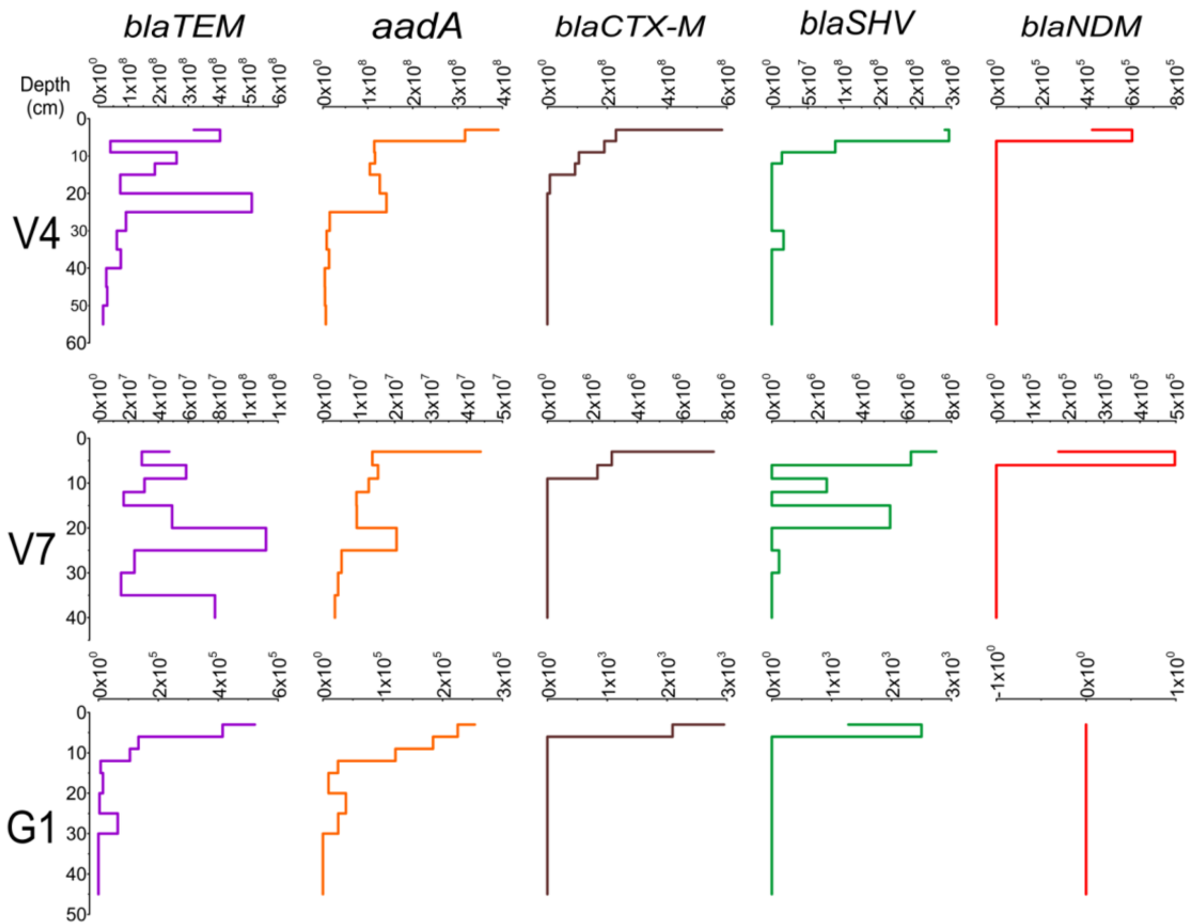


Figure 4.5. qPCR quantification of the selected antibiotic resistance genes (*bla_{TEM}*, *aadA*, *bla_{SHV}*, *$bla_{CTX\text{-}M}$* and *bla_{NDM}*) along the length of the sediment core, expressed as a percentage of 16S rDNA bacterial population.

4.3.5. Statistical correlations

Total organic matter content, metal concentrations (with the exception of Mn, Co, and Ni), bacterial-indicator genetic markers, and ARGs were, for the most-part, significantly, mutually, and positively correlated (Table 4. 4S, Supporting Information). Strong negative correlations were found among depth, grain size, total bacterial load, and the relative abundance of bacterial indicators and ARGs ($r \geq -0.1310$, $p < 0.05$, $n = 34$). Likewise, there were strong positive correlations among total OM, metal concentrations, and bacterial-

load/bacterial-indicator genetic markers/ARGs ($r \geq 0.403$ were observed, $p < 0.05$, $n = 34$). This implies that contaminants likely originate from a common source and were transported and deposited by a common carrier to the receiving lake ecosystem. The relationship between the contaminant content and the grain size is not as strong as would be expected from an existing general transport-and-deposition model of hydrophobic pollutants (Salomons and Förstner 1984). This supports the notion that contaminants, attached to both large organic and small inorganic particles, behave in a similar manner with respect to their mobility and sedimentation. A recent study in Vidy Bay has demonstrated that organic and inorganic particles at the sediment−water interface of Vidy Bay appeared to be unstable and play a role in the transport of contaminants over long distances (Graham et al. 2014).

Inspection of Figure 4.6(B) shows the contaminated sites (V4 and V7) to be oppositely and separately clustered to the control site on separate axes of the first-principle component, substantiating the high prevalence of metals, bacterial indicators, and ARGs at sites V4 and V7 as compared to the control site, G1. Figure 4.6(A) shows the relationship between the eigenvectors of each variable measured. Here, the segregation between metals is apparent because Co, Ni, and Mn are separated from the other metals analyzed. Also, grain size is noted to be segregated from both groups of metals and is directly opposed to the Co, Ni, and Mn eigenvectors along the second-principle component. This infers that as the grain size increases, the concentrations of Ni, Mn, and Co tend to decrease, whereas the other measured parameters (metals, bacterial marker genes, and ARGs) will increase with the total OM content at Vidy Bay.

4.3.6. Factors of ARGs contamination

In the natural ecosystem, the selective pressures to which bacteria are exposed may favor the emergence and spread of ARB. The discharge of Abs and ARB into the aquatic environment could favor the spread of ARB/ARGs to nonresistant bacterial communities through HGT (Davison 1999). A study by (Zuccato et al. 2010) reported an average of 352.5 g of Abs per 1000 inhabitants per year being discharged to the receiving lake by the WWTP of Lugano (located in Switzerland 300 km away from our study site). The study also averaged the penicillin discharge at 37 mg per 1000 inhabitants per day in the WWTP effluent. The total antibiotic consumption in Switzerland increased between 2004 and 2008, and Switzerland has an antibiotic usage close to the European mean (Pluss-Suard et al. 2011).

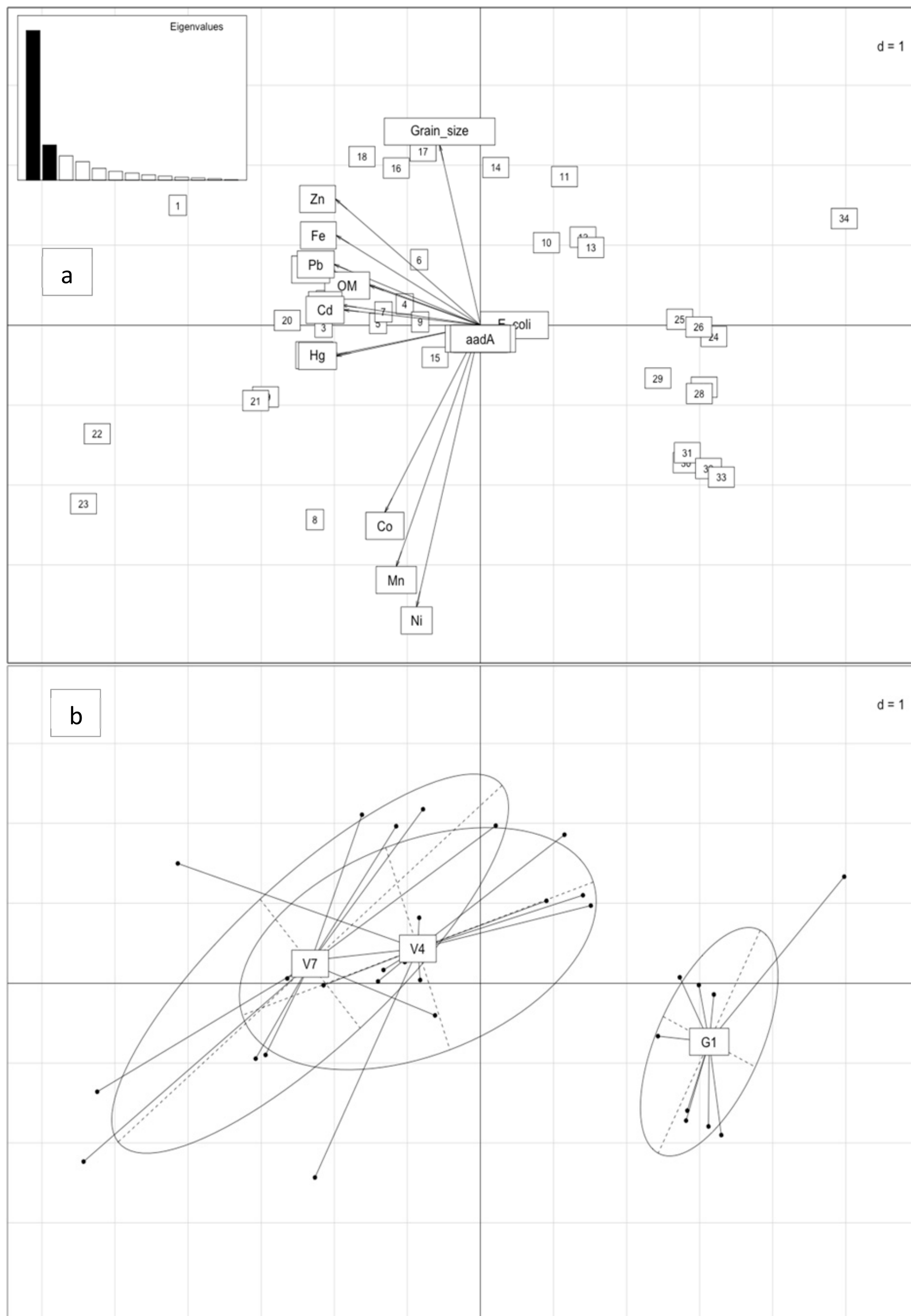


Figure 4.6. Scatter plot analysis on the eigenvectors/values for the studied parameters. Principle component is the x axis, with the second component being the y axis. Right (top) side is positive and the left (bottom) is negative. (a) Scatter of the PCA results. (b) Classification on the basis of the sampling site.

It is well-known that metals can coselect for ARGs because the mechanism often favors resistance to both and the genes are located on the same mobile elements (Knapp et al. 2011). The physiochemical results demonstrate a high contamination in the sediments of Vidy Bay with the metal concentrations being in excess of the natural background values. The correlation matrix (Table 3.4S, Supporting Information) shows the positive correlation values found among the bacterial indicators, ARGs, and the studied metals (expect Mn, Co, and Ni) with coefficient values ranging from 0.475 to 0.923. With positive correlation values and $p < 0.05$, one could argue that the bacterial load tends to increase with an increase in metal levels, and this coselection could be a relevant factor influencing the selection of ARGs in the sediment of Vidy Bay. However, the physicochemical sediment properties could also influence the accumulation and play an important role in the selection of ARGs. Sediments with a high content of fine particles (clay and silt) can bind DNA and protect it from degradation, aiding its transport to water saturated soils and groundwater (Pote et al. 2003). This is also suggested from the correlation between the total OM and ARGs with correlation coefficients ranging between 0.574 and 0.846. Sorbed to flocs, suspended solids, and/or activated sludge, Abs are partially removed from WWTPs. Using this sludge as fertilizers could favor the selection of antibiotic resistance with the release of antibiotic-laden sludges to the environment (Michael et al. 2013).

4.4. Conclusion

In this study, we investigated the accumulation of total OM, metals, abundance of bacterial population, and ARGs in sediment profiles from two different parts of Lake Geneva. The elevated levels of metals in Vidy Bay indicate the negative effects of effluent releases to the lake that would pose a threat to the ecosystem. Our study also supports the hypothesis that the natural environment (i.e., sediments) can act as a reservoir for ARGs, and as such, changes to the ecosystem may support the emergence of unknown resistances in the bacterial community (Martinez 2008, 2009). The identification of *aadA* and bla_{TEM} genes in the sediment layers deposited before the start of the twentieth century also supports the fact that ARGs have been an emerging contaminant in the aquatic ecosystem for more than a century (Thevenon et al. 2012a). It implies that these genes were carried by bacteria as structural integrated reservoir genes even before invention of ß lactams (Davies and Davies 2010, Srinivasan et al. 2008). According to European directive 271/1991/EC (EU) Switzerland and other European countries monitor the treatment efficiency of WWTPs by implementing selected water quality measures. These parameters, however, are not designed to estimate threats associated with the

spread of Abs/ARGs/ARB. In Switzerland, many of these WWTPs have been considered for, or are already undergoing, modernization. It is important to consider instituting advanced protocols for ARB/ARG removal from effluents when planning this modernization (Dodd 2012). Given the level of complexity, the evaluation of ARB/ARG removal or selection requires more intensive case studies (Thevenon et al. 2012a, Czekalski et al. 2014). It has been demonstrated that the contamination of surface as well as deep sediments by untreated or partially treated effluent water can potentially affect the water quality (Goldscheider et al. 2007, Graham et al. 2014). Therefore, the reduction of contaminants released to the receiving water system is highly recommended for further improvement of the water quality.

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CHAPTER 4

Supporting Information (SI)

Number of tables : 4

Number of figures : 3

Table.4.1S. Physical parameters of sediment analyzed in the core samples; including depth in the core, water content, total organic matter (OM) content, the proportion of clay, silt and sand, and median sediment grain size

Sample	Depth (cm)	Water content (%)	OM (%)	Clay (%)	Silt (%)	Sand (%)	Mean Grain size (μm)
V4	0-3	73.68	22.94	0.00	65.53	34.47	41.49
	3-6	77.49	25.51	0.01	79.13	20.86	27.78
	6-9	81.76	26.10	0.01	66.63	33.36	39.19
	9-12	79.95	18.00	0.01	82.77	17.22	26.22
	12-15	79.66	17.68	0.00	75.16	24.84	28.93
	15-20	79.45	18.36	0.00	64.98	35.02	43.46
	20-25	79.36	17.56	0.01	73.60	26.39	32.62
	25-30	68.59	9.22	0.20	94.22	5.58	17.70
	30-35	76.45	15.38	0.01	71.13	28.86	34.73
	35-40	77.95	18.36	0.00	82.19	17.81	27.21
	40-45	62.30	6.94	0.01	58.79	41.20	46.77
	45-50	65.59	8.10	0.05	80.03	19.92	25.42
	50-55	66.84	10.64	0.07	82.08	17.85	23.25
V7	0-3	57.45	7.93	0.00	46.23	53.77	71.47
	3-6	58.44	7.57	0.00	49.79	50.21	60.38
	6-9	63.36	9.80	0.02	61.53	38.46	44.35
	9-12	70.32	13.84	0.01	65.93	34.07	40.45
	12-15	62.75	14.24	0.08	73.66	26.26	31.07
	15-20	71.23	14.92	0.01	67.56	32.43	37.80
	20-25	72.48	14.54	0.03	78.89	21.08	26.61
	25-30	66.35	12.16	0.04	75.22	24.74	30.31
	30-35	70.92	14.37	0.03	71.36	28.61	32.83
	35-40	68.35	11.81	0.06	74.82	25.13	30.66
G1	0-3	81.19	8.50	0.00	74.99	25.01	36.89
	3-6	74.43	7.70	0.00	70.45	29.55	40.73
	6-9	77.81	8.10	0.00	80.78	19.22	32.72
	9-12	75.04	6.88	0.00	84.75	15.25	28.32
	12-15	73.85	6.99	0.01	77.94	22.05	33.28
	15-20	72.15	6.91	0.00	80.73	19.27	31.26
	20-25	69.70	5.86	0.16	90.24	9.60	21.41
	25-30	63.72	4.46	0.34	85.19	14.47	21.60
	30-35	59.41	4.72	1.11	91.02	7.87	14.42
	35-40	56.14	3.19	1.05	88.14	10.81	15.56
	40-45	55.23	3.23	1.04	85.96	13.00	16.94

Table 4.2S. Primers used in this study

Gradient PCR for plasmid construction					
Target organism/gene	Primer	Sequence (5' – 3')	Tm (˚C)	Size (bp)	References
E. coli (uidA)	298F	AATAATCAGGAAGTGATGGAGCA	53	587	(Ram et al. 2004)
	884R	CGACCAAAGCCAGTAAAGTAGAA			
ENT (16S rDNA)	ENT-151-F	ACACTTGGAAACAGGTGC	53	458	(Ryu et al. 2013)
	Ent-578-R	TTAAGAAACCGCCTGCGC			
Pseudomonas spp. (16S rDNA)	PAGS-F	GACGGGTGAGTAATGCCTA	54	618	(Spilker et al. 2004)
	PAGS-R	CACTGGTGTTCCTTCCTATA			
bla_{CTX-M}	blaCTX-M-f	CGCTTTGCGATGTGCAG	52	593	(Bonnet et al. 2001)
	blaCTX-M-r	ACCGCGATATCGTTGGT			
bla_{SHV}	blaSHV F	ATGCGTTATATTCGCCTGTG	56	865	(Paterson et al. 2003)
	blaSHV R	GTTAGCGTTGCCAGTGCTCG			
bla_{NDM-1}	NDM-Fm	GGTTTGGCGATCTGGTTTTC	53	621	(Nordmann et al. 2011)
	NDM-Rm	CGGAATGGCTCATCACGATC			

qPCR for quantification					
Bacterial 16S rDNA	338 F	ACTCCTACGGGAGGCAGCAG	55	197	(Ovreas et al. 1997)
	518 R	ATTACCGCGGCTGCTGG			
E. coli (uidA)	Uida 405 F	CAACGAACTGAACTGGCAGA	55	121	(Chern et al. 2011)

	Uida 405 R	CATTACGCTGCGATGGAT			
ENT (16S rDNA)	Ent376F	GGACGMAAGTCTGACCGA	55	221	(Ryu et al. 2013)
	Ent578 R	TTAAGAAACCGCCTGCGC			
Pseudomonas spp. (16S rDNA)	Pse435F	ACTTTAAGTTGGGAGGAAGGG	55	251	(Bergmark et al. 2012)
	Pse686 R	ACACAGGAAATTCCACCACCC			
bla_{TEM}	TEM-RT-F	GCKGCCAACTTACTTCTGACAACG	55	247	(Sidrach-Cardona et al. 2014)
	TEM-RT-R	CTTTATCCGCCTCCATCCAGTCTA			
bla_{CTX-M}	blaCTX-M-rt-f	ATTCCRGGCGAYCCGCGTGATACC	62	227	(Fujita et al. 2011)
	blaCTX-M-rt-r	ACCGCGATATCGTTGGTGGTGCCAT			
bla_{SHV}	blaSHV-rt-f	CGCTTTCCCATGATGAGCACCTTT	60	110	(Xi et al. 2009)
	blaSHV-rt-r	TCCTGCTGGCGATAGTGGATCTTT			
bla_{NDM-1}	NDM-F	TTGGCGATCTGGTTTTCC	58	195	(Zheng et al. 2013)
	NDM-R	GGTTGATCTCCTGCTTGA			
aadA	aadA-F	GCAGCGCAATGACATTCTTG	55	282	(Madsen et al. 2000)
	aadA-R	ATCCTTCGGCGCGATTTTG			

Table 4.3S. Metal content (mg kg^{-1} dry weight sediment)[a] 35 of sediment core samples V4, V7 and G1.

		mg kg^{-1}										
Sample	Depth (cm)	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ag	Cd	Pb	Hg
V4	0-3	**49.89**	168.88	17790.95	6.20	25.63	**209.52**	**660.76**	2.32	3.46	**199.27**	**1.56**
	3-6	**42.43**	185.33	16466.07	4.78	29.97	**205.00**	**495.10**	4.08	1.07	**151.93**	**1.37**
	6-9	36.95	278.30	21130.36	4.77	27.97	**201.15**	**455.93**	2.17	0.84	**63.41**	**2.09**
	9-12	35.68	215.57	19884.40	5.34	25.32	**180.59**	**354.38**	2.13	0.72	**53.58**	**0.85**
	12-15	**38.89**	213.69	18233.15	4.98	27.68	**158.27**	**274.11**	2.13	3.04	**56.25**	**1.12**
	15-20	**38.82**	208.43	17926.52	4.87	26.00	**145.16**	**265.35**	1.98	1.01	**61.81**	**1.45**
	20-25	**71.38**	208.09	18462.50	4.80	28.88	**139.39**	**250.39**	1.57	1.26	**84.08**	**1.20**
	25-30	**57.84**	239.96	18356.15	6.08	33.53	**137.96**	**315.44**	1.54	0.66	**42.25**	**2.46**
	30-35	35.46	226.10	16259.79	5.36	24.87	**131.00**	**226.89**	0.95	0.71	**34.76**	**2.60**
	35-40	32.27	159.44	16146.03	4.67	24.77	**130.29**	**148.91**	0.83	0.66	**47.03**	**0.81**
	40-45	30.01	157.54	15740.98	4.59	24.27	**113.15**	**239.23**	0.65	1.18	**77.27**	**0.35**
	45-50	29.78	156.10	15307.75	4.57	24.21	**79.78**	**287.65**	0.56	0.66	**49.61**	**1.35**
	50-55	29.71	155.74	14707.34	4.55	24.20	**71.09**	**280.43**	0.46	0.48	**32.03**	**1.67**
V7	0-3	36.07	202.30	15217.74	4.26	25.74	**68.63**	**307.00**	1.86	2.05	**60.85**	**0.74**
	3-6	36.70	217.92	15430.22	5.20	32.81	**177.15**	**187.81**	1.00	2.37	**62.10**	**0.71**
	6-9	**68.64**	155.29	17506.47	4.34	27.41	**111.15**	**485.86**	0.86	2.54	**98.27**	**1.62**
	9-12	**70.74**	168.65	17603.06	4.10	25.29	**72.39**	**486.39**	1.10	0.82	**111.71**	**1.89**
	12-15	**72.02**	175.93	17615.22	4.08	25.00	**198.34**	**493.34**	0.99	1.00	**127.79**	**2.25**
	15-20	**50.22**	209.76	15994.21	5.19	32.90	**198.82**	**172.77**	3.14	2.98	**138.96**	**2.69**
	20-25	**59.43**	213.58	16836.41	4.46	30.96	**193.24**	**405.24**	1.97	3.10	**148.43**	**2.04**
	25-30	**67.94**	285.60	17195.81	5.22	29.12	**151.68**	**445.97**	2.68	2.51	**145.50**	**1.75**
	30-35	**100.75**	315.80	17964.05	5.45	30.43	**191.37**	**509.21**	3.04	3.28	**170.50**	**3.02**
	35-40	**72.39**	319.20	18538.11	5.40	33.56	**169.54**	**520.84**	4.40	3.44	**157.31**	**3.94**
G1	0-3	22.92	193.58	9270.09	4.33	27.40	22.37	53.07	0.27	0.28	13.01	**0.20**
	3-6	30.20	193.95	11625.32	4.40	27.61	30.51	72.08	0.08	0.38	20.02	**0.27**
	6-9	26.48	201.68	10469.09	4.45	25.54	26.08	64.25	0.21	0.34	18.83	**0.26**
	9-12	22.22	203.50	9269.56	4.51	28.28	**39.87**	61.35	0.11	0.35	18.18	**0.25**
	12-15	21.66	205.06	8659.78	4.69	28.56	24.17	62.01	0.14	0.38	24.82	**0.29**
	15-20	29.85	196.85	10551.86	4.69	28.87	**48.80**	90.23	0.08	0.48	**37.07**	**0.48**
	20-25	24.77	210.89	9301.51	4.70	30.74	24.73	58.99	0.14	0.28	19.59	**0.74**

	25-30	24.03	219.46	10694.98	5.32	27.48	17.77	43.90	0.10	0.25	26.81	**0.24**
	30-35	23.50	199.97	11457.13	4.71	30.98	13.02	32.83	0.13	0.18	17.52	**0.17**
	35-40	22.02	199.99	10782.10	4.86	30.77	11.36	27.55	0.07	0.12	11.90	0.16
	40-45	17.70	189.24	7127.79	3.28	18.48	7.09	17.02	0.05	0.07	5.81	0.12
*CQSG		37.3					35.7	123		0.6	35	0.17
#Nat-back		30					20	50	0.2	0.2	20	0.03

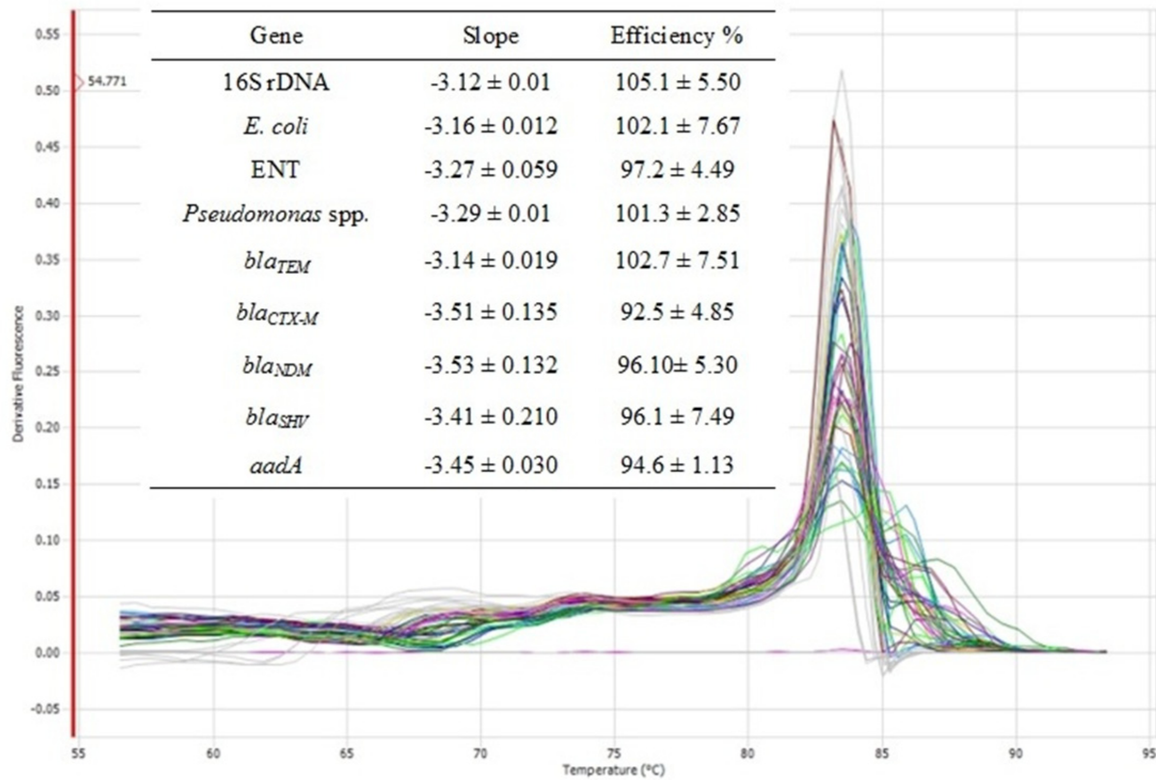
Cr – Chromium, Mn – Manganese, Fe – Iron, Co – Cobalt, Ni – Nickel, Cu – Copper, Zn – Zinc, As – Arsenic, Cd – Cadmium, Pb – Lead, Hg – Mercury.
[a] Total variation coefficients for triplicate measurements is less than 5% for both ICP-MS and AMA (Hg) measurements. [b] Detection limit of Hg 0.005 mg kg^{-1} according to manufacturer. #Natural Background values based on (Arbouille et al. 1989)

The values in bold represent the concentration of the heavy metals above the recommended concentration according to Canadian Sediment Quality Guidelines (*CSGQ) for the Protection of Aquatic Life recommendation (CCME 1999)

Table 4.4S. Spearman Rank Order correlation matrix performed for the analyzed parametersa in the sediment core samples V4, V7 and 1.

sample	OM	Grain	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ag	Cd	Pb	Hg	16s	*E.coli*	ENT	P.spp	TEM	aadA	CTX-M	SHV
Depth	-0.10	**-0.57**	-0.14	0.02	-0.05	0.16	-0.09	-0.22	-0.18	-0.20	-0.19	-0.14	0.08	**-0.35**	-0.04	-0.14	-0.12	-0.20	**-0.39**	**-0.49**	**-0.42**
OM		0.23	**0.66**	0.24	**0.79**	**0.40**	-0.08	**0.72**	**0.62**	**0.71**	**0.59**	**0.55**	**0.74**	**0.81**	**0.85**	**0.82**	**0.80**	**0.81**	**0.77**	**0.56**	**0.57**
Grain Size			**0.34**	-0.07	0.20	-0.10	-0.13	0.31	0.31	0.32	**0.51**	**0.41**	0.21	**0.40**	0.25	0.29	0.28	0.28	**0.42**	**0.49**	**0.40**
Cr				0.26	**0.84**	0.29	0.25	**0.78**	**0.85**	**0.81**	**0.84**	**0.89**	**0.83**	**0.67**	**0.52**	**0.54**	**0.53**	**0.67**	**0.64**	**0.41**	**0.60**
Mn					**0.34**	**0.66**	**0.59**	0.27	0.08	**0.40**	0.24	0.15	0.31	0.15	0.07	0.01	-0.01	0.12	0.06	-0.05	0.16
Fe						**0.48**	0.13	**0.80**	**0.80**	**0.81**	**0.72**	**0.74**	**0.75**	**0.80**	**0.73**	**0.74**	**0.72**	**0.78**	**0.74**	**0.56**	**0.65**
Co							**0.47**	**0.44**	0.24	**0.49**	**0.36**	0.32	**0.36**	**0.36**	0.31	0.31	0.32	**0.36**	0.24	0.14	0.30
Ni								0.18	0.01	0.26	0.22	0.19	0.15	-0.01	-0.19	-0.18	-0.17	-0.01	-0.09	-0.11	0.11
Cu									**0.80**	**0.88**	**0.83**	**0.86**	**0.77**	**0.77**	**0.64**	**0.66**	**0.68**	**0.77**	**0.78**	**0.65**	**0.70**
Zn										**0.81**	**0.80**	**0.88**	**0.81**	**0.71**	**0.57**	**0.60**	**0.58**	**0.68**	**0.68**	**0.56**	**0.70**
Ag											**0.86**	**0.86**	**0.77**	**0.75**	**0.63**	**0.65**	**0.63**	**0.77**	**0.75**	**0.62**	**0.76**
Cd												**0.92**	**0.72**	**0.67**	**0.54**	**0.57**	**0.58**	**0.73**	**0.70**	**0.59**	**0.60**
Pb													**0.77**	**0.62**	**0.48**	**0.51**	**0.52**	**0.67**	**0.66**	**0.50**	**0.62**
Hg														**0.65**	**0.59**	**0.53**	**0.52**	**0.61**	**0.56**	0.33	**0.56**
16s															**0.87**	**0.91**	**0.90**	**0.86**	**0.91**	**0.76**	**0.75**
E.coli																**0.98**	**0.97**	**0.88**	**0.84**	**0.70**	**0.60**
ENT																	**0.99**	**0.89**	**0.89**	**0.76**	**0.63**
P.spp																		**0.89**	**0.88**	**0.76**	**0.62**
TEM																			**0.91**	**0.76**	**0.68**
aadA																				**0.84**	**0.70**
CTX-M																					**0.75**

Parameters include depth in the sediment core (cm), mean grain size, toxic metal concentration, total organic matter content (OM), bacterial population (16s, *E. coli*, ENT, *Pseudomonas* spp. (P.spp), and the selected ARGs (*bla*$_{TEM}$, *aadA*, *bla*$_{CTX-M}$, *bla*$_{SHV}$). Correlation coefficients have been calculated using the log transformed values to normalize their distribution. Statistically significant coefficients ($p < 0.05$) are in bold



Gene	Slope	Efficiency %
16S rDNA	-3.12 ± 0.01	105.1 ± 5.50
E. coli	-3.16 ± 0.012	102.1 ± 7.67
ENT	-3.27 ± 0.059	97.2 ± 4.49
Pseudomonas spp.	-3.29 ± 0.01	101.3 ± 2.85
bla_{TEM}	-3.14 ± 0.019	102.7 ± 7.51
$bla_{CTX\text{-}M}$	-3.51 ± 0.135	92.5 ± 4.85
bla_{NDM}	-3.53 ± 0.132	96.10± 5.30
bla_{SHV}	-3.41 ± 0.210	96.1 ± 7.49
aadA	-3.45 ± 0.030	94.6 ± 1.13

Figure.4.7S. Average of qPCR efficiencies calculated from the slopes of the standard curves for each assay and the representation of derivative melt curve analysis

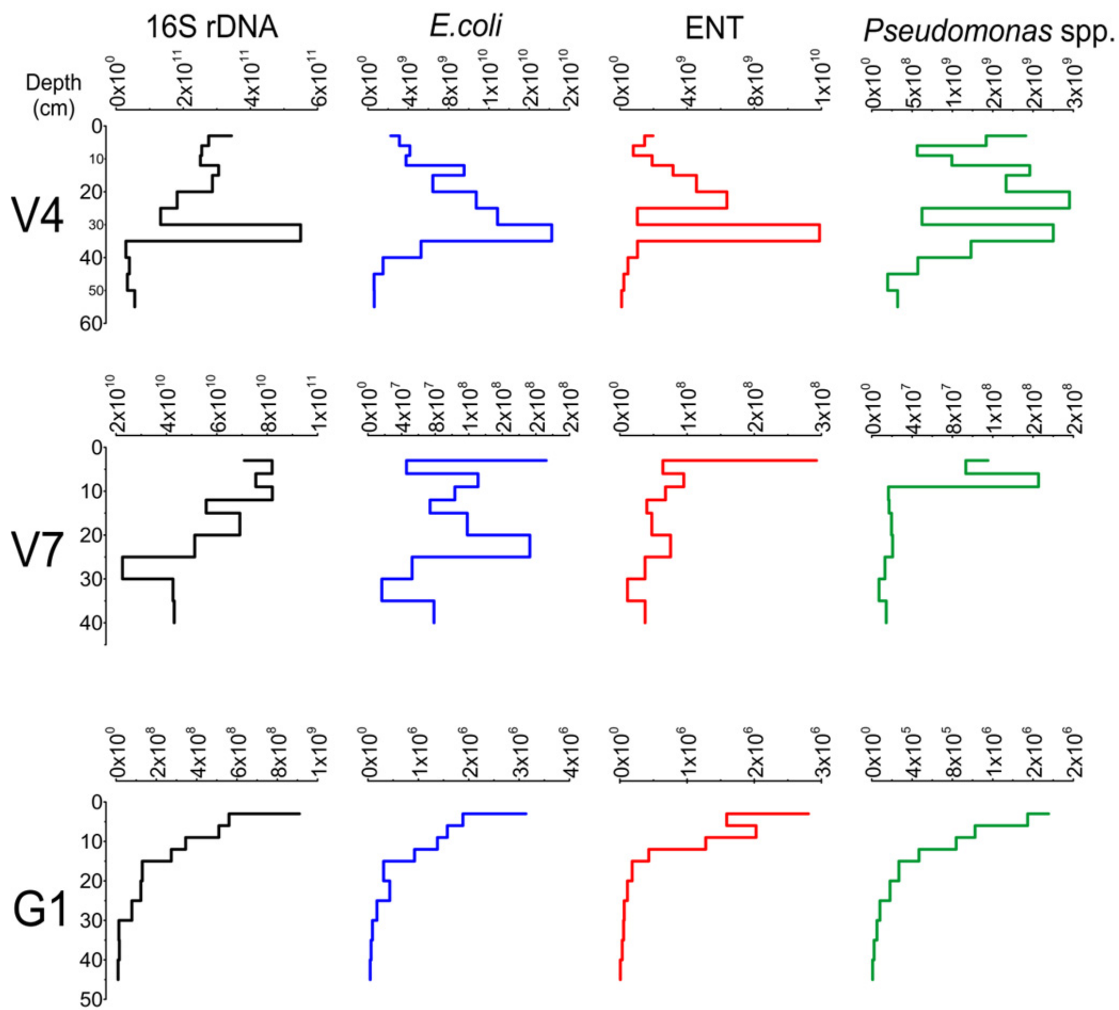


Figure.4.8S. qPCR quantification of the bacterial population and the selected bacterial groups (*E. coli*, ENT and *Pseudomonas* spp.) according to the depth in the sediment core. Values are expressed in copy numbers per gram of dry sediment

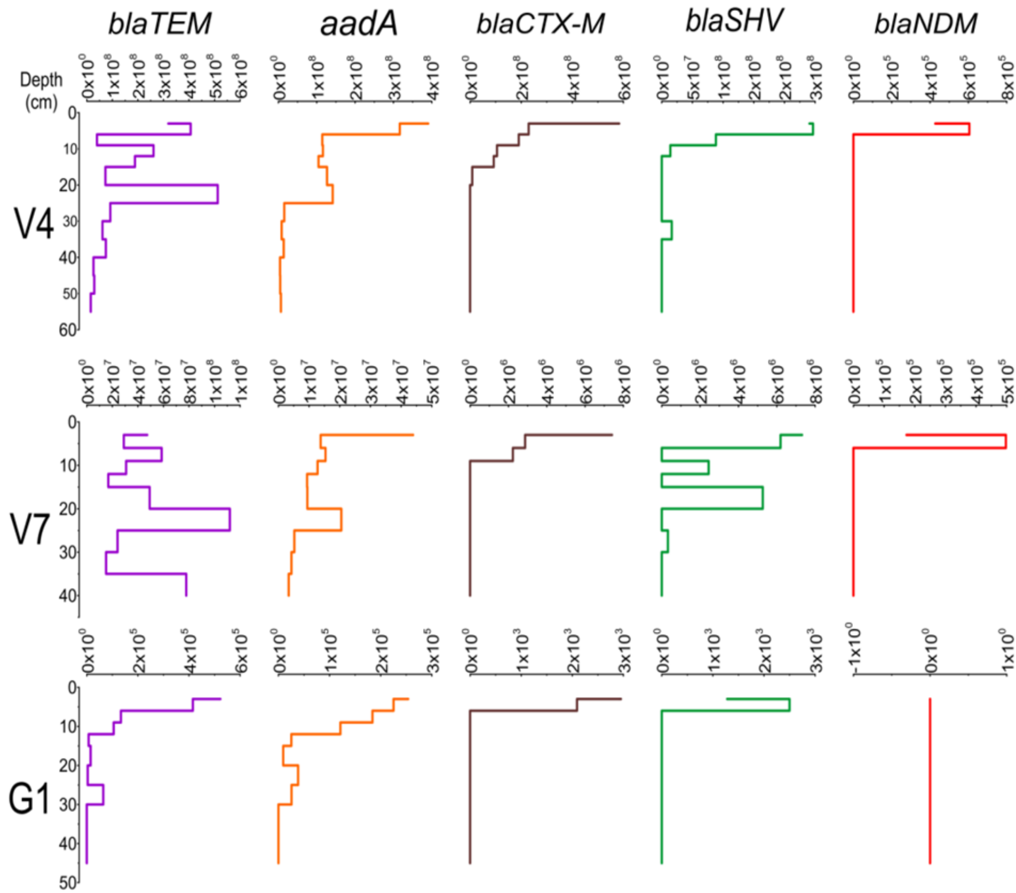


Figure.4.9S. qPCR quantification of the selected antibiotic resistance genes (*bla*$_{TEM}$, *aadA*, *bla*$_{CTX\text{-}M}$, *bla*$_{SHV}$, *bla*$_{NDM}$) along the depth of the core samples. The values are expressed in copy numbers per gram of dry sediment.

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CHAPTER 5

Dissemination of antibiotic resistant *Pseudomonas* spp., to the aquatic environment: An environmental prevalence study under tropical and temperate climatic conditions

Supporting Information available at the end of the chapter

Manuscript in preparation

Abstract

In this study, the prevalence of antibiotic resistance in *Pseudomonas* spp., isolated from sediments receiving hospital and urban wastewaters in three distinct geographical locations; Switzerland (CH), India (IN) and Democratic Republic of Congo (DRC), was evaluated for their phenotypic and genotypic resistance profiles. Antipseudomonal antibiotics including TZP, CAZ, FEP, IMP, MER and NOR were more effective on the isolates collected from CH sediments when compared to DRC and IN. Among the ß-lactamases CTX-M was the most dominant in the CH isolates with VIM being dominant for the isolates from DRC and IN samples. Conjugative experiment represents the ability of bacteria to acquire resistant genes under tropical temperatures (DRC, IN) when compared to temperate conditions (CH). The chromosomal mediated efflux mechanism in *P. aeruginosa* could play as adjuvant mechanism of bacterial resistance which benefits the bacteria to withstand the environmental selective pressure and acquire more resistant genes. Additionally *oprD* in *P. aeruginosa* may exhibit the co-selection of Zinc and Carbapenem (IMP) antibiotic. The data suggest that irrespective of geographical boarders, wastewaters act as a source of antibiotic resistant bacteria disseminating into the aquatic ecosystem.

5.1. Introduction

Antibiotic resistance (AR) is not only of medical concern but it is also relevant to ecology, as AR is not restricted to pathogenic organisms, but also disseminated among environmental bacteria. During the last few years there are many studies relevant to AR in the environment, with antibiotic resistant genes (ARGs) and multidrug resistant (MDR) bacteria released from the clinical and communal effluents to the receiving environmental water bodies (Czekalski et al. 2014, Devarajan et al. 2015a, Walsh et al. 2011). The presence of these AR bacteria possesses a threat to the environment as they might act as a reservoir for the contribution of maintenance and spread of ARGs. Resistance genes from environmental commensal bacteria are able to mobilize into the genomes of pathogenic bacteria through gene transfer mechanisms and could be a potential threat to the human and animal health (Kummerer 2004).

In most developing countries, the effluents are discharged to the sewer systems, rivers and lakes and these untreated/treated effluents contribute the high rates of resistant bacteria (Spindler et al. 2012). In our previous studies(Devarajan et al. 2015b, Mubedi et al. 2013), the hospital effluents in developing countries are a major source of various contaminants in the aquatic environment as the effluents are discharged to the receiving system without prior treatment. On the other hand in most of the developed countries the WWTPs were not originally designed to have a specific impact on the resistant bacteria or the antimicrobial residues and their effects on these microbial contaminants are widely unknown (Czekalski et al. 2014, Devarajan et al. 2015a). Bacteria are the most ubiquitous organisms and are found in environments including soils and sediments, while the latter being an important reservoirs of microbial diversity (Pote et al. 2010). The possibility of appearance to toxic effects after organism's exposure to hazardous substances and the release of contaminants to the hydrosphere either by sediment re-suspension or by infiltration into the groundwater are considered as potential human and environmental risks, with remobilization of these contaminants and their possible return to the food chain or in drinking water (Wildi et al. 2004).

Extended spectrum ß-lactamases (ESBLs) and Metallo ß-lactamases (MBLs) genes among bacterial communities are of great concern, as they confer resistant to multiple Abs and allowing very limited options for treatment (Walsh et al. 2011, Canton and Coque 2006). Additionally the chromosomal encoded efflux mechanisms, abundant in gram negative

bacteria (esp. in *P. aeruginosa*) may contribute to AR and also involve in the other functions, which include the bacterial stress response (Li et al. 2015). Studies on antibiotic resistance in the environmental sources have been carried on fecal indicator bacteria (FIB) including coliforms, *E. coli* and *Enterococcus* spp., (Hu et al. 2008, Laroche et al. 2009), and very few studies are available on other human pathogenic bacterial community in the non-clinical environments, including *Pseudomonas* spp. (Spindler et al. 2012, Quinteira et al. 2005). The concern on spread of resistance from indigenous environmental bacteria to pathogenic bacteria and thereby compromising the treatment of pathogenic organisms has also been raised (Walsh et al. 2011, Spindler et al. 2012, Shah et al. 2012). In the environment *Pseudomonas* spp. are widely distributed, easily isolated from different sources and mostly related to biodegradation and bioremediation process (Quinteira et al. 2005). Antiobiotic resistant *Pseudomonas aeruginosa* have been commonly found in hospital/communal effluents, and it is important to consider the presence of resistant genes in other *Pseudomonas* species (P. spp.,), since they may represent a possible reservoir of resistance determinants in the effluents, and the receiving systems as shown by previous studies (Walsh et al. 2011, Spindler et al. 2012).

Very limited information is available concerning the true extent of AR problem in central Africa and south India as very few studies have carried the surveillance on MDR bacteria in the environment (WHO 2014). The objective of this study was to identify the prevalence of antibiotic resistant *Pseudomonas* species in the sediments receiving wastewaters from various sources under tropical and temperate climatic conditions. The sediment were sampled from tropical (DRC& IN) and temperate (CH) climatic conditions to differentiate the pollutants based on the method of wastewater elimination and the probable influence of temperature. The phenotypic resistance profiles were characterized with 16 Abs, and their respective genotypes along the mobile genetic elements. Additionally selected isolates (*P. aeruginosa*) were characterized for their chromosomal mediated efflux resistance mechanisms.

5.2. Materials and methods

The sediment samples were collected from two different climate conditions in 2012; (1) Tropical condition - sediments receiving untreated hospital effluents from Democratic Republic of Congo (DRC) and India (IN); (2) Temperate climatic conditions - sediments receiving treated/partially treated effluents after the waste water treatment plant (WWTP)

from Switzerland (CH). The sampling sites description, sample collection protocols and the metal concentrations are described in our previous studies (Mubedi et al. 2013, Thevenon et al. 2012). *Pseudomonas* spp., from the sediment samples were isolated as described in our previous studies ((Pote et al. 2010, Haller et al. 2009). Briefly, the filter membranes were placed on *Pseudomonas* Selective Agar (PSA) supplemented with antifungal agent Nystatin (Sigma, MO, USA) at a final concentration of 100 mg/L for colony forming units (CFU) count. 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 was 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 CLSI guidelines (CLSI Jan, 2012). All isolated were tested against 16 antimicrobials; Piperacillin (PRL, 100 μg), Piperacillin-tazobactam (TZP, 110 μg), Ceftazidime (CAZ, 30 μg), Cefepime (FEP, 30 μg), Aztreonam (ATM, 30 μg), Imipenem (IPM, 10 μg), Meropenem (MEM, 10 μg), Cefoperazone (CFP, 30 μg), Ofloxacine (OFX, 5 μg), Norfloxacine (NOR, 10 μg), Cefpirome (CPO, 30 μg), Ampicillin (AMP, 10 μg), Cefuroxime (CXM, 30 μg), Streptomycin (STR, 10 μg), Cefotaxime (CTX, 30 μg), and Cotrimoxazole (SXT 25 μg) (Oxoid, Pratteln, Switzerland). The breakpoint values were interpreted according to the CLSI guidelines (CLSI Jan, 2012). *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used for quality controls. The heavy metal (Cadmium, Cobalt, Copper, Mercury, Nickel and Zinc) minimal inhibitory concentration (MICs) for the selected *P. aeruginosa* (n=16) were determined by 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 (Perron et al. 2004). The MAR index was calculated for the isolates as described by Krumperman (1983) and Shah et al. (2012). Total DNA from the bacterial isolates was extracted using Chelex Resin (Bio-Rad, Cressier, Switzerland) and Proteniase K (Sigma-Aldrich, Buchs SG, Switzerland) as described by (Yang et al. 2008). All isolates (n=141) were screened for the presence of various ARGs with Polymerase Chain reaction (PCR) using the primers mentioned in Table-5.1S (Supporting Information). Amplified 16S rDNA and AR genes were sequenced and confirmed by blasting the resulted sequences in the NCBI Blast page.

Transfer of ARGs from P. spp to *E. coli* (DHα) (Forel Institute, Geneva) (susceptible to all 16Abs studied and negative for ARGs) was performed by filter matting approach as described earlier (Boguslawska et al. 2009). Thirty six strains of MDR P. spp were selected and screened for their potential to transfer ARGs. A total volume of 2 mL, exponentially growing donor (P. spp) and recipient (*E. coli*) strains were mixed in a ratio of 1:1. The mixture was filtered, placed upright in a non-selective media (MHA) and incubated overnight at 10, 30 and 37 ˚C symbolizing the average temperature of Lake Geneva (CIPEL 2014), tropical (DRC and IN) receiving system (Figure. 5.3) and the human gut temperatures, respectively. Control of recipient and donor were also treated in the same manner. 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, recipient and trans-conjugant selective 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). The trans-conjugants were screened for the presence of ARGs by PCR using the primers used above.

Total RNA isolation, cDNA synthesis from selected *P. aeruginosa* strains (n=16) were performed as described by previous study (Dumas et al. 2006). Four µl of 10-times diluted cDNA was used in a total volume of 20 µL reaction. The primers used are presented in Table. 5.2S (Supporting Information). The expression of efflux mechanisms were quantified by Eco qPCR system (Illumina, Switzerland) using KAPA SYBR FAST qPCR Master Mix Universal Kit (KAPA Biosystems, USA). Negative (without reverse transcriptase), a blank (no template reaction mix) and positive (PA01 genomic DNA dilutions) control was included in all the reactions. All negative control resulted in no amplification or a threshold cycle (Ct) higher than the blank. The ribosomal *rpsL* gene (housekeeping gene in *P. aeruginosa*) was chosen to correct for the differences in starting material. The results of the expression of the efflux pump were expressed as ratio between the target (target gene expression) and the reference gene (*rpsL*), using the following equation:

Ratio = $(E_{\text{target gene}})$ ΔCt Target (PA01-test strain) / (E_{rpsL}) ΔCt rpsL(PA01-test strain).

E = RT-PCR efficiency for a reaction and Ct the crossing point of the amplification curve with the threshold. A reaction is considered effective if the values of slope (-3.1 to -3.6), efficiency (90-100 %) and R2 (>0.99) values were within the recommended range by the

manufacturer. An effect on the gene expression was considered to be significant if the ratios were > 2.0 and < 0.4 (Pfaffl et al. 2002).

5.3. Results

5.3.1. *Pseudomonas* spp. and antibiotic susceptibility

The population density of the *Pseudomonas* spp. at our study sites are described in Table 5.1. The total population of *Pseudomonas* spp. ranged between 2.03 – 34.33, 0.22 – 0.48 and 1.4 – 13 x 105 CFU for the samples from IN, DRC and CH, respectively. *Pseudomonas* spp. phylogenetic likelihood is presented in Figure.5.1S (Supporting Information). From the total isolates 42.2 and 39.3 % of isolates were identified as *Pseudomonas putida* and *Pseudomonas aeruginosa*, respectively. These two species were predominant in all the sampling sites, contributed to 81.5% of the total isolation. Other species identified in this study specific to the sampling sites are *Pseudomonas entomophila* 0.7% (DRC), *Pseudomonas fulva* 3.0 % (IN), *Pseudomonas mendocina* 0.7 % (IN), *Pseudomonas monteilii* 0.7 % (CH), *Pseudomonas moraviensis* 2.2 % (CH), *Pseudomonas mosselii* 0.7 % (DRC), *Pseudomonas plecoglossicida* 8.9 % (DRC, IN), *Pseudomonas pseudoalcaligenes* 0.7 % (IN), and *Pseudomonas stutzeri* 0.7 % (IN). The phenotypic resistant profiles are presented in Figure. 5.1 and Figure 5.2S (Supporting Information). Majority (> 81 %) of 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 other studied locations. On the other hand, 50 and 54% isolates from DRC and CH, respectively were resistant to OFX. All the isolated from DRC were susceptible to IPM, while 15 and 33% of the isolated from CH and IN, respectively were resistant to IMP. Multidrug resistant isolates were identified in all the 3 sampling areas. In this study, 7 and 2 isolates were resistant to 16 Abs in the sediments collected from IN and CH, respectively. Additionally MAR index values were higher in IN (0.79), followed by DRC (0.48) and CH (0.40) sites.

Table.5.1. *Pseudomonas* spp. colony forming units (CFU) count for the studied samples isolated on *Pseudomonas* selective agar, and the MAR index calculated for the sampling site for 16 antibiotics.

Climate	Region	Sample	*P.* spp. CFU x 10^5 100 g^{-1} wet weight	MAR Index
Temperate	CH	V4	13 ± 4.3	0.40
		V7	2.7 ± 0.9	0.28
		G1	1.4 ± 0.9	0.20
Tropical	DRC	C1	0.22 ± 0.04	0.48
		C2	0.48 ± 0.26	0.44
		C3	0.23 ± 0.10	0.28
	IN	I1	3.6 ± 1.6	0.39
		I2	4.87 ± 2.2	0.67
		I3	1.78 ± 0.9	0.48
		I4	2.03 ± 0.8	0.66
		I5	5.77 ± 2.7	0.33
		I6	3.23 ± 2.7	0.32
		I7	6.73 ± 2.0	0.34
		I8	25.67 ± 8.3	0.79
		I9	34.33 ± 17.3	0.61
		I10	4.07 ± 1.3	0.59

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

5.3.2. Antibiotic resistant genes (ARGs)

Most of the phenotypically resistant *Pseudomonas* isolates carried the respective resistance genes Figure 5.2 & Figure. 5.3S (Supporting Information). The higher prevalence of all three aminoglycoside resistance genes (*strA*, *strB* and *aadA*) was found in *Pseudomonas* spp. from IN (30 %) followed by CH (4 %). Among IN isolates, 8 % carried *strA*, *strB* and *aadA* together, 33 % carried *strA* and *strB*, and 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 resistance genes. However, interestingly few isolates (23 %) were phenotypically resistant to STR and PCR negative for all three ARGs *strA*, *strB* and *aadA*.

All the isolates from DRC and CH were PCR negative for all the 3 *sul* genes, *sulI*, *sulII* and *sulIII*. On the other hand, 25, 19 and 4% of IN isolates were found to carry *sulI*, *sulII* and *sulIII*, respectively. Florfenicol resistant 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 have PCR screened five ß-lactamase genes including bla_{SHV}, bla_{CTX-M}, bla_{NDM}, bla_{VIM-1}, and bla_{VIM-2} in all test isolates. The bla_{SHV} gene was identified in 3, 8 and 13% of the isolates from CH, DRC and IN, respectively. 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 ß-lactamases were found in high number of isolates from IN and DRC. In this study, 8 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 %). Integrase genes including class 1, 2 and 3 were screened in all the isolates. In this study, *intl1*and *intl2* were identified in *Pseudomonas* spp. however, none of isolates carried *intl3*. 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 *intl1* and 13% of the isolates carried *intl2*. Isolates from CH were PCR negative for *intl2* and 14% of the isolates from IN were PCR positive for *intl2*.

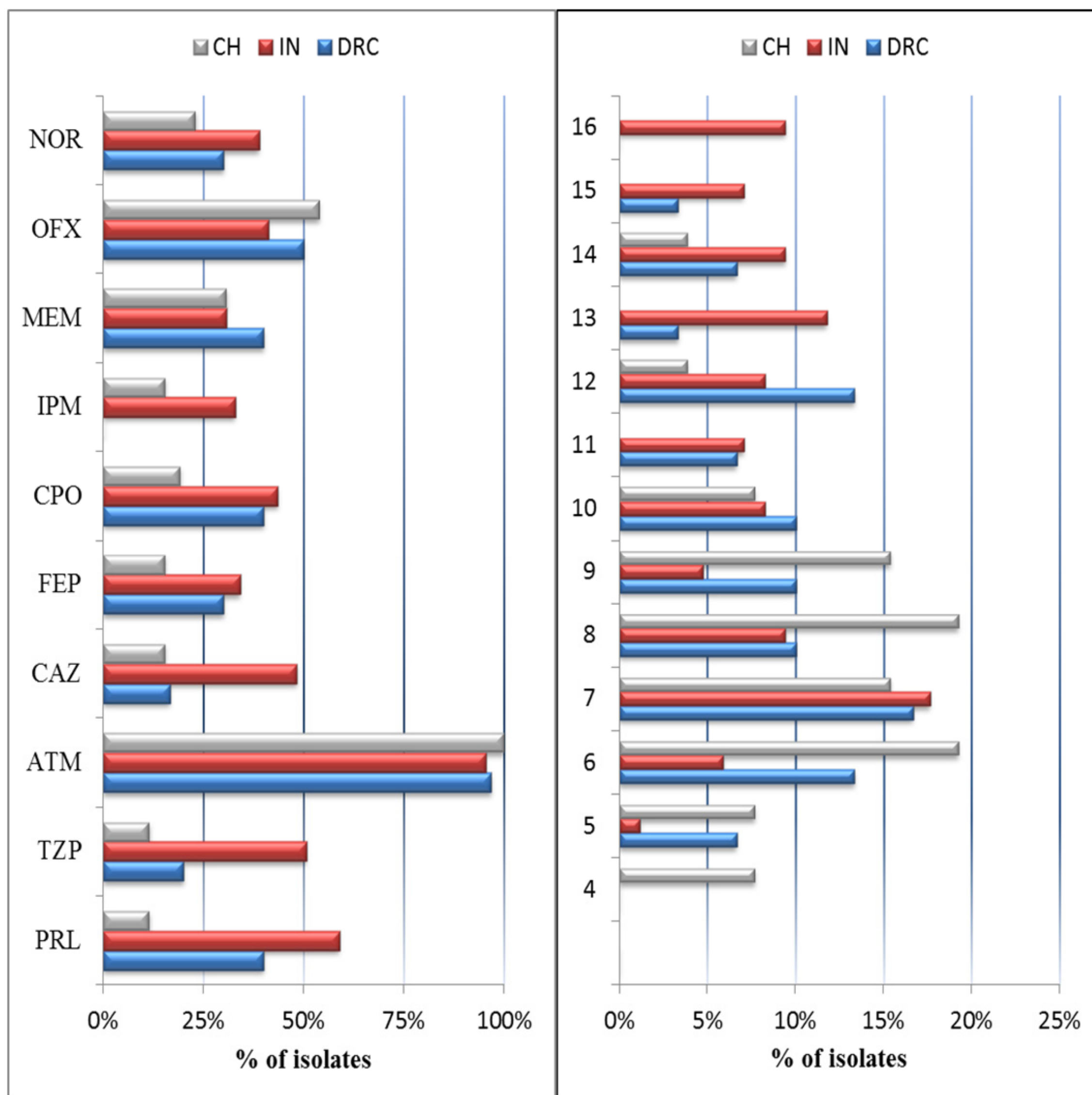

Figure.5.1. Comparison of phenotypic resistance in the studied *Pseudomonas* spp. between DRC, IN and CH. Left panel:, percentage of isolates resistant to the antipseudomonal agents. Right panel: percentage of isolates resistant to multiple antibiotics (n=16) in this study.

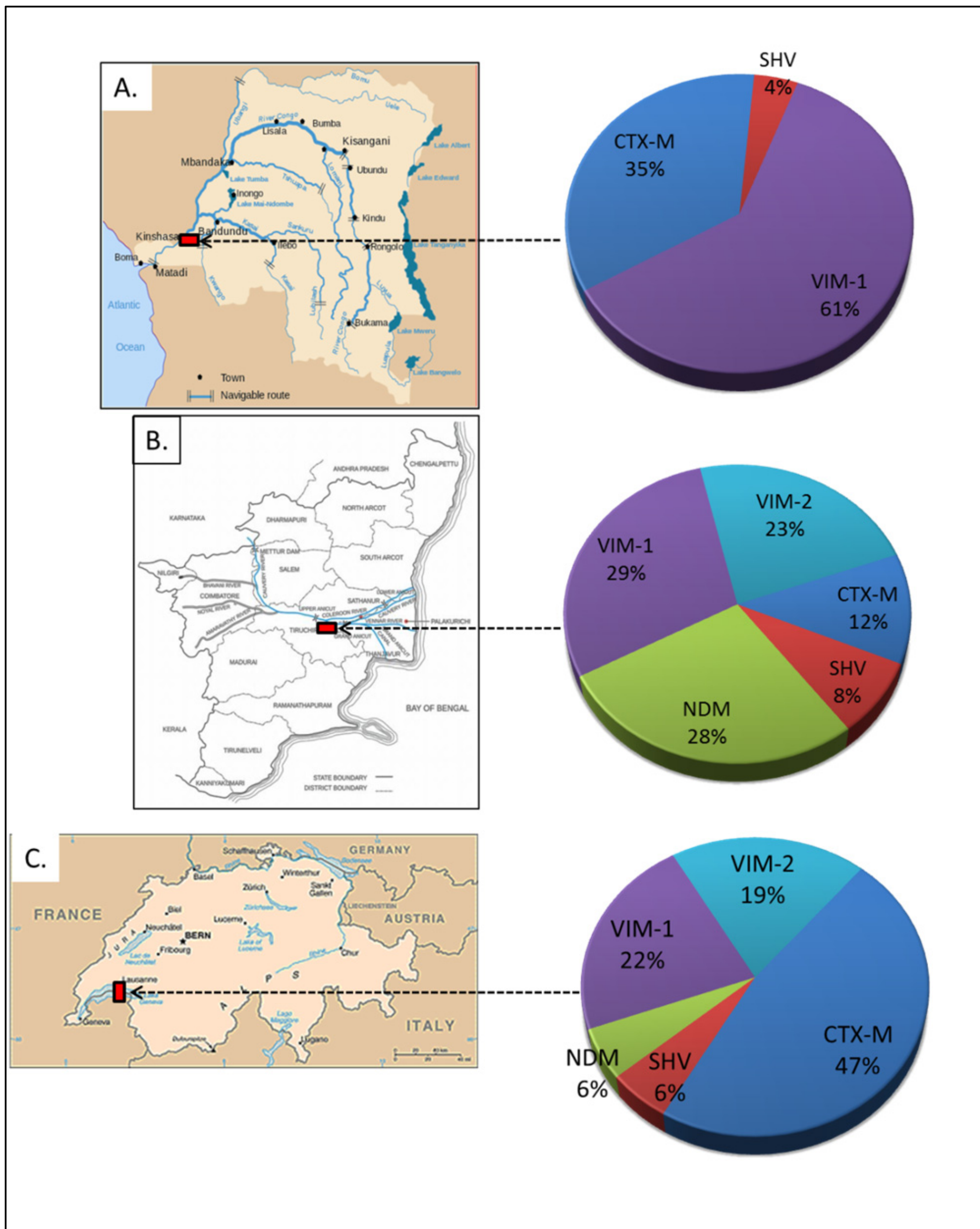

Figure. 5.2. Comparison of genotypic resistance to ß-lactams in the studied *Pseudomonas* spp., isolated from the sediments receiving treated/untreated effluent in DRC, IN and CH.

Table. 5.2. Transfer frequency of ARGs from environmental *Pseudomonas* isolates to *E. coli* cells, and the role of temperature in the studied geographical locations. n/a – no transfer of ARGs observed.

	Transconjugants		Transfer frequency		
Donor (n = 36)	Recipient	ARGs identified (n =21)	10 ˚C	28 ˚C	37 ˚C
P. aeruginosa	*E. coli*	*CTX-M* (4); *VIM* (3); *NDM* (2)	n/a	0.5×10^{-5}	6.2×10^{-8}
P. putida	*E. coli*	*SHV* (2); *CTX-M* (3); *VIM* (3); *NDM* (1)	1.2×10^{-9}	0.9×10^{-4}	0.53×10^{-8}
P. pseudoalcaligenes	*E. coli*		n/a	n/a	n/a
P. fulva	*E. coli*	*CTX-M* (1)	n/a	1.7×10^{-7}	n/a
P. mendocina	*E. coli*		n/a	n/a	n/a
P. monteilii	*E. coli*		n/a	n/a	n/a
P. moraviensis	*E. coli*		n/a	n/a	n/a
P. mosselii	*E. coli*		n/a	n/a	n/a
P. plecoglossicida	*E. coli*	*CTX-M* (2)	n/a	8.3×10^{-5}	$< 1\times10^{-9}$
P. entomophila	*E. coli*		n/a	n/a	n/a
P. stutzeri	*E. coli*		n/a	n/a	n/a

Table. 5.3. Relative expression of selected gene expression in *P. aeruginosa* determined by quantitative RT-PCR.

Isolate	mexR	mexA	oprM	mexZ	mexX	mexY	nfxB	mexC	oprJ	mexT	mexE	oprN	ampC
PA01	1	1	1	1	1	1	1	1	1	1	1	1	1
CH 19	**2.3**	*0.5*	*0.3*	**4.09**	1.51	**4.09**	**7.49**	**5.99**	**4.27**	**3.41**	**8.78**	**7.91**	*0.1*
CH 21	**4.21**	*0.6*	*0.3*	**10.36**	**3.77**	**10.36**	**6.11**	**11.42**		**13.19**	**27.15**	**20.59**	**15.15**
DRC 27	*0.1*	*0.2*	0.07	*0.05*	*0.2*	*0.2*	*0.2*	*0.2*	*0.2*	*0.2*	*0.2*	*0.2*	*0.1*
DRC 38	*0.3*	*0.5*	0.24	*0.02*	*0.3*	*0.3*	*0.3*	*0.3*	*0.3*	*0.3*	*0.3*	*0.3*	*0.1*
DRC 39	**2.84**	1.55	0.89	**5.35**	**2.76**	**5.35**	**3.42**	**5.31**	**5.59**	**4.17**	**3.41**	**4.14**	*0.3*
IN 85	**5.5**	**2.03**	0.27	**3.9**	**2.08**	**3.9**	**4.56**	**6.43**	**6.2**	**7.1**	**15.49**	**13.27**	**6.9**
IN 87	**4.16**	**3.43**	1.9	**8.3**	**5.3**	**8.3**	**4.57**	**5.44**	*0.08*	**4.93**	**6.55**	**8.17**	*0.09*
IN 96	*0.3*	*0.5*	*0.3*	*0.03*	*0.09*	*0.09*	*0.09*	*0.09*	*0.09*	*0.09*	*0.09*	*0.09*	*0.1*
IN 100	*0.3*	*0.5*	*0.3*	*0.05*	*0.03*	*0.03*	*0.03*	*0.03*	*0.03*	*0.03*	*0.03*	*0.03*	*0.2*
IN 103	*0.3*	*0.5*	*0.3*	*0.07*	*0.4*	*0.4*	*0.4*	*0.4*	*0.4*	*0.4*	*0.4*	*0.4*	*0.4*
IN 109	**2.4**	0.79	*0.3*	**3.11**	*0.05*	**3.11**	**2.42**	**2.26**	**3.25**	**3.12**	**4.01**	**3.76**	*0.1*
IN 117	0.27	*0.6*	*0.3*	*0.2*	*0.1*	*0.1*	*0.08*	*0.02*	*0.1*	*0.1*	*0.1*	*0.1*	*0.1*
IN 120	0.43	*0.5*	*0.3*	*0.09*	*0.09*	*0.09*	1.75	1.42	*0.09*	*0.09*	*0.09*	*0.09*	**6.86**
IN 125	**4.01**	*0.5*	*0.3*	**6.01**	1.52	**6.01**	**2.48**	**4.22**	**5.07**	**4.61**	**4.11**	**4.48**	*0.1*
IN 128	**2.61**	0.79	0.11	**4.15**	**3.12**	**4.15**	**3.22**	**4.6**	**4.41**	**3.93**	**5.97**	**5.52**	*0.1*
IN 129	**4.81**	**2.68**	1.37	**8.18**	**3.39**	**8.18**	**4.8**	**6.84**	**8.13**	**6.53**	**6.32**	**7.17**	*0.1*

Values represent the fold change in comparison with the transcription level in PA01; values above 2.0 are shown in bold, values less than 0.4 are shown in italics. In all cases standard deviation of duplicates was less than 13% of the mean.

Table. 5.4. Metal MIC values (mM) and Carbapenem (IMP) susceptibilities profile by disk diffusion method (mm) and *oprD* expression by RT-PCR in selected *P. aeruginosa* isolates

Isolate	Zn	Cu	Co	Cd	Ni	Hg	IPM (mm)	oprD
PA01	12	7	3	3	4	1	22	1.0
CH 19	**31**	**8**	1	**7**	3	1	6	**-4.54**
CH 21	**21**	**8**	2	**8**	3	1	6	**-1.14**
DRC 27	**23**	**10**	**4**	**5**	2	**2**	22	**-0.79**
DRC 38	**21**	**8**	3	**8**	3	**2**	22	**-2.21**
DRC 39	**15**	**8**	3	**8**	3	1	23	1.54
IN 85	**17**	7	**4**	**8**	4	1	6	**1.00**
IN 87	**17**	**11**	**4**	**9**	4	**2**	6	2.23
IN 96	**27**	**12**	3	**7**	3	**2**	30	**-1.22**
IN 100	**29**	**13**	3	**9**	4	**3**	28	**-1.31**
IN 103	**21**	**13**	3	**11**	4	**3**	6	**-1.10**
IN 109	**15**	**8**	**4**	**7**	3	1	28	1.78
IN 117	**21**	**8**	2	**7**	3	**5**	18	**0.39**
IN 120	**19**	**10**	2	3	4	**2**	6	1.45
IN 125	**19**	7	3	**7**	4	1	6	1.02
IN 128	**23**	**10**	3	**8**	3	1	6	2.50
IN 129	9	7	1	3	3	1	6	3.65

5.3.3 Conjugative experiments

The ability of the MDR *Pseudomonas* spp. isolates (n = 36) to transfer their ARGs into the recipient species, *E. coli* was studied under three different temperatures. The values of conjugative transfer frequencies are presented in Table 5.2. Generally the transfer frequencies were higher at 30 ˚C, when compared to 10 and 37 ˚C. The transfer frequencies achieved at 30 ˚C were ca. 10,000 and 100,000 times higher when compared to 37 and 10 ˚C, respectively. *P. putida* was the only species which had the ability to transfer their ARGs to the recipient at all the 3 studied conditions. *P. fulva* was able to transfer ARGs only at 30 °C; *P. aeruginosa* and *P. plecoglossicida* were at 30 and 37 ˚C, respectively. The isolates from CH were able to transfer $bla_{\text{CTX-M}}$ 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 $bla_{\text{CTX-M}}$ genes

(n=1). On the other hand, isolates from IN were able to share most of their ARGs to 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.

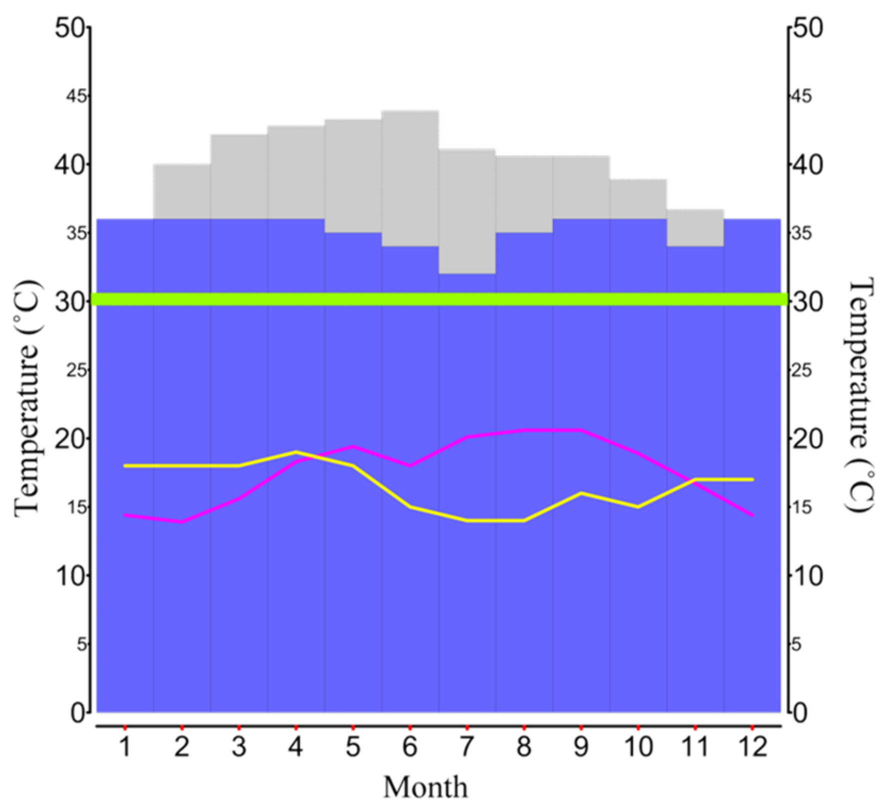

Figure. 5.3.
Mean high and low temperature of Tiruchirappalli, Tamil Nadu, India and Kinshasa, Democratic Republic of Congo; Tiruchirappalli Grey bar, Pink line representing mean high and low, respectively. Kinshasa – Blue bar, Yellow line representing mean high and low, respectively. Green line indicating the temperature of 30 ˚C.

5.3.4. Gene expression in *P. aeruginosa*

To identify the expression of efflux pump mechanisms, random selected isolates from CH (n = 2), DRC (n = 3) and IN (n = 11) were screened for clinically relevant efflux pump resistant mechanisms. The values were compared to the wild type strain PA01 and the ratio of gene expression is presented in Table. 5.3. The gene expression analysis showed that 50% of the isolates demonstrated increased *mexR* (2.3 to 5.5 fold) and 38 % of these were identified with increased *mexA* (from 2.0 to 3.4 fold). Overexpression MexXY-OprM was observed in 38 and 56% of the isolates with *mexX* (from 2.0 to 5.3 fold) and *mexZ* (from 3.1 to 10.3 fold), respectively. MexCD-OprJ hyper expression was observed in 56, 56 and 44 % of the total isolates with increased *nfxB* (from 2.4 to 7.0 fold), *mexC* (from 2.2 to 11.4 fold) and *oprJ* (from 3.2 to 8.1 fold), respectively. MexEF-OprN regulation was overproduced in 56% of the isolates with increased *mexT* (from 3.1 to 13.1 fold), *mexE* (from 3.4 to 27.1 fold) and *oprN* (from 3.7 to 20.6 fold). The hyper expression of *ampC* was identified in 19 % of the isolates and *oprD* down regulation was identified in 44 % of the isolates. Isolates non-susceptible to the studied 16 Abs [IN (n=4), CH (n=2)], had hyper expression of all the studied efflux genes, and the down regulation of *oprD* was observed only in the isolates from CH sediments. The isolates from IN sediments either had a neutral or increased *oprD* expression values (from 1 to 3.6 fold). To investigate the possible link between the heavy metal resistance and the Abs resistance we examined selected *P. aeruginosa* (n=16) isolates resistance to 6 heavy metals (Table. 5.4). Except for Ni 94, 80, 25, 90, and 50 % of the selected *P. aeruginosa* isolates were resistant to Zn, Cu, Co, Cd and Hg when compared to PA01. There existed a mutual relationship between *oprD* and Zn as we could observe substantial increase in the MIC values of Zn with the down-regulation of *oprD*. Additionally in this study we identified the down regulation of the selected efflux pumps in isolates collected from DRC (n =1) and IN (n = 3) when compared to the PA01 wild type strain.

5.4. Discussion

We report the prevalence of multidrug resistant *Pseudomonas* spp., from sediments receiving treated/untreated effluents from DRC, IN and CH. On comparison between the sampling locations MAR index of sediment receiving WWTP effluent CH_V4 (0.40) had higher MDR bacteria than sediment receiving untreated hospital effluent IN_I6 (0.32) (Table 5.1). According to the previous study (Kummerer 2004), the MAR index between 0.2 and 0.25 are in the range of ambiguity and requires careful scrutiny. Hence the presence of MDR bacteria

in the CH_V4 sediment, demonstrates the effect of the WWTP effluent water on the distribution of MDR *Pseudomonas* spp. to the environment. Our studies are concurrent with the previous studies (Czekalski et al. 2014, Devarajan et al. 2015a) that the WWTP are not effective in complete removal of MDR bacteria during the treatment and could act as a potential reservoir for the positive selection of MDR bacteria within other bacterial community. On the other hand the samples from tropical regions (DRC and IN) had a broader range of MDR *Pseudomonas* spp., in the receiving system. MAR index range for the IN and DRC sampling sites ranged from 0.32 to 0.79 and 0.28 to 0.48, respectively. The results represent that, the effluent released to the receiving system are possibly diluted, but the presence of MDR bacteria could be persistent in the sediment of the receiving systems, and might play a role in preserving and dispersing these MDR bacteria back to the food chain via drinking, agriculture and recreational activities.

Among all isolates, some (20%) carried both *strA* and *strB* together and also *aadA* (5%) in subset of those isolates. Additionally, 8% isolates were carrying a combination of *strA* and *aadA* gene, interestingly, two aminoglycoside susceptible isolates were positive for *strA* gene alone. This observation is concurrent with previous studies (Chiou and Jones 1995, Srinivasan et al. 2007, Lanz et al. 2003) and also confirms the observation that both genes have to be present to obtain functional streptomycin resistance. However, few isolates in this study were PCR negative for all the 3 studied aminoglycoside genes. This could be possible explained by the existence of the MexXY proteins forming functional tri-partite efflux machinery with outer membrane component OprM as reported in previous studies (Aires et al. 1999, Hocquet et al. 2003, Mine et al. 1999) as other unknown mechanisms of resistance. The results are also concurrent with the efflux pump mechanisms analyzed in this study were 56% of the selected *P. aeruginosa* isolates represent for the hyper expression of *mexY* gene.

In the folic acid pathway of the bacterial cells, Sulfonamides act as competitive inhibitors of enzyme dihydropteroate synthase (DHPS). The prevalence of *sulI, sulII* and *sulIII* genes in the environment is most likely due to easy dissemination of these genes via mobile genetic elements, especially *sulI* and *sulII* are located in the 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). The prevalence of sulfonamide resistant genes in aquatic environment could also be the result of the high usage of this antibiotic in human and veterinary medicine (Srinivasan et al. 2007). In our study SXT resistant isolates were found abundant in all three sampling regions. The presence

of sulfonamide resistant genes was identified in the resistant isolates collected from IN sediments. However, none of the DRC and CH isolates carried sulfonamide resistance genes. Hence, the presence of phenotypic resistance in our studied isolates and the absence of the respective genes could be explained by the potency of trimethoprim and sulfonamides are limited against *Pseudomonas* spp., and these drugs MIC values are usually in the resistant range, with respect to the concentration of the drug used in this study (Eliopoulos and Huovinen 2001). *mexABoprM* multidrug efflux system is mainly responsible for the intrinsic resistance of *P. aeruginosa* to sulfonamides (Köhler et al. 1996) and this study 38% of the isolates were identified with increased *mexA* expression. Resistance to this inexpensive drug group like sulfonamides will be a serious problem in developing nations with regard to prophylaxis of respiratory infections.

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). 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). High prevalence of *floR* genes was reported in *Listeria monocytogenes* (66%) isolated from dairy environment (Stoll et al. 2012, Srinivasan et al. 2005). In this study, we found *floR* gene in 65 % of *Psuedomonas* isolates from CH samples. Chloramphenicol resistance mechanisms include chloramphenicol acetyltransferases (*cat* genes) and chloramphenicol efflux pumps (*cml* genes) and multidrug transporters (Schwarz et al. 2004). In this study the isolates from IN (25 %) were found to carry *cmlA* gene followed by the CH isolates (12%). The high prevalence of *cmlA* resistance genes in IN samples than in CH could be due to the fact that these Abs were banned for veterinary medicine since 1994 in the European countries (Stoll et al. 2012).

In 1989, Germany, France and Italy have recognized and reported new ESBL enzyme, CTX-M which confers resistance to CTX. Since then CTX-M ESBLs were detected in many bacterial species from different environments. At present CTX-M family includes more than 180 ß-lactamases (http://arpcard.mcmaster.ca/). The population structure of CTX-M producing isolates is complex in association with the spread of specific plasmids and/or

mobile genetic elements rather than clonal epidemics (Mendonca et al. 2007). It has also been well documented that specific CTX-M types are associated with geographical regions (Hawkey 2008). Since 2005, the prevalence of CTX-M enzymes have been increased dramatically all over the world. Plasmid-borne $bla_{\text{CTX-M-15}}$ gene was first reported from India, which is the global dominant ESBL (Lascols et al. 2012). After CTX-M-15 (group 1), CTX-M-14 (a group 9 genotype) is the dominant type in China and has spread to become the second most reported CTX-M worldwide (Hawkey 2008). In the past decade both in nosocomial and in community settings CTX-M enzymes have become the most prevalent ESBLs. Often located in variable transposons containing multridrug resistance determinants and these CTX-M genes are often linked to IS*Ecp1* transposon. The possible dissemination of CTX-M genes are by co-selection process as these genes are frequently carried in the large plasmids (> 100 kb) (Walsh et al. 2007)) along with aminoglycoside, tetracycline, sulfonamide or fluoroquinolone resistance genes (Canton 2009). Similarly, we have also detected higher prevalence of CTX-M genes in *Pseudomonas* spp. isolated from all the sampling regions (DRC, IN and CH). In the present study, ß-lactam resistant isolates from this study 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 the environmental samples of DRC.

NDM-1 hydrolyses all ß-lactam Abs except for ATM (which is hydrolyzed by *ampC)*, and over a decade different types of carbapenemases are gradually appearing in the gram negative bacteria (Struelens et al. 2010). $bla_{\text{NDM-1}}$ was initially identified in *E. coli* and *K. pneumoniae* and later reported in *A. baumannii, C. freundii, M. morganii, P. mirabilis, Enterobacter* spp., and *P. aeruginosa* (Struelens et al. 2010, Jovcic et al. 2011). Until recently, $bla_{\text{NDM-1}}$ was identified only in clinical settings, but there is a paucity of information on the presence of NDM-1 gene in the environmental bacterial community. In a previous study, surface water samples collected in 2010 from India were identified with MDR bacteria carrying NDM-1 gene (Walsh et al. 2011). In our study NDM-1 was identified in 34 % of isolates from IN and 7 % from CH, and none from DRC, and these isolates from CH and IN were also resistant to ATM with hyper production of chromosomal *ampC* in 18% of the studied *P. aeruginosa* isolates. The ability of NDM-1 to spread not limited to *Enterobacteriaceae* but also to other bacterial species including P. spp., implies the possibility for emergence of new NDM-1 cases to be detected in the future (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).

Carbapenems efficacy are compromised with the emergence of mobile metallo-ß-lactamases (MBL) since it was identified in *P. aeruginosa* during 1988 in Japan (Walsh et al. 2005). In 2007, six countries among 33 countries in 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). The prevalence of VIM genes in *P. aeruginosa* is a clinical threat, and a recent review reports the VIM-2 in 37 countries from 5 continents (Hawkey and Jones 2009). In the present study $bla_{\text{VIM-1}}$ was found in IN and CH samples; and VIM-2 in DRC and IN. The spread of MBLs among bacterial population 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 on the extent to which MBL can disseminate to the environmental bacteria.

Integrons are gene exchange systems and play an important role in the acquisition and dissemination of antimicrobial resistance genes. In this study, 34 % of the total studied isolates were positive for the integrons. Similarly, previous studies (Laroche et al. 2009, Hawkey and Jones 2009, Skurnik et al. 2006) have also reported higher prevalence of class 1 integrons in the environment as a result of clinical effluent discharge and high level of urbanization (Nardelli et al. 2012). On the other hand, there are studies which describe the prevalence of *intl1* variants in the environmental samples and the problem of detecting them in large numbers could be influential by the large numbers of genetically homogenous class 1 integrons 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). It is also possible that integrase genes have evolved to acquire ARGs in pathogenic and commensals bacteria could be the crisis for infectious diseases management (Davies 2007). There are possible large numbers of mobile elements in the environment and potential resistance genes associated with them, which continue to be a threat to the human/animal welfare. Hence these gene pools are highly valuable to predict the future clinical cases that could be 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, *E. coli*. Higher transfer frequencies were recorded at the 30˚C, followed by 37 and 10˚C, which suggests a positive relationship between 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 (Figure. 5.3). 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 the Lake Geneva 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. 2015a) with the possibility threat of sediment suspension.

To investigate the common regulators mechanisms governing resistance to both heavy metals and Abs we examined the efflux mechanisms of *P. aeruginosa*. The over expression of the efflux systems may impact on the survival of *P. aeruginosa* isolates in the competitive environment as they are potentially capable of pumping out many classes of contaminants (including Abs & metals). However to understand the correlation between the increase in the transcriptional level of the efflux coding region and their potential resistance to the contaminants remain unclear (Dumas et al. 2006). MexCD-OprJ system is generally not over-expressed in wild type strains but is inducible by a membrane damaging agent (Li et al. 2015). Over production of MexCD-OprJ will result in increased resistance to quinolones, macrolides, amphenicols, tetracyclines and ß-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*. Another interesting fact observed was the down regulation of *oprD* either selected for carbapenem resistance or for the metal (Zn) resistance and in few isolates for both. The results observed with *oprD* regulation are in agreement with previous studies (Perron et al. 2004, Dumas et al. 2006) implying the existence of common regulators mechanisms for Zn and carbapenem in *P. aeruginosa.* Hence, efflux over expression, porin down regulation will favor the bacteria in the environment to withstand the selective pressure, increasing the chances of acquiring new ARGs and enhancing further resistance to Abs.

5.5. Conclusion

This study reports MDR *Pseudomonas* spp from sediments receiving different effluent wastewaters. The chromosomal mediated efflux pump mechanisms and its overexpression appear to be adjuvant mechanisms for antimicrobial resistance, with ß-lactamase production

increase the resistance to Abs. However, in *P. aeruginosa* porin down-regulation and ampC could play a major role in resistant phenotypes (Xavier et al. 2010). The wide spread of ESBL/MBLs in the communal settings may be a result of co-selection as most of the plasmids frequently carry multiple resistant genes. Compared to temperate conditions such as the samples from the bay of Vidy, the sediments from tropical conditions (IN and DRC) favor the exchange of genetic materials in bacteria. With deficient sewer systems, in developing nations the transmission of MDR bacteria to drinking water can be a pressing problem in the future. Additionally, community settings with proper sewer systems should consider the treatment options available for disinfecting the bacterial load/ARGs in the effluents before been discharged to the receiving system (Devarajan et al. 2015a). Hence, we illustrate the requirement for the surveillance of resistance and the importance of environment in the dissemination of resistant genes/bacteria, as in most parts of the globe water sources act as the end point for the treated/untreated effluents.

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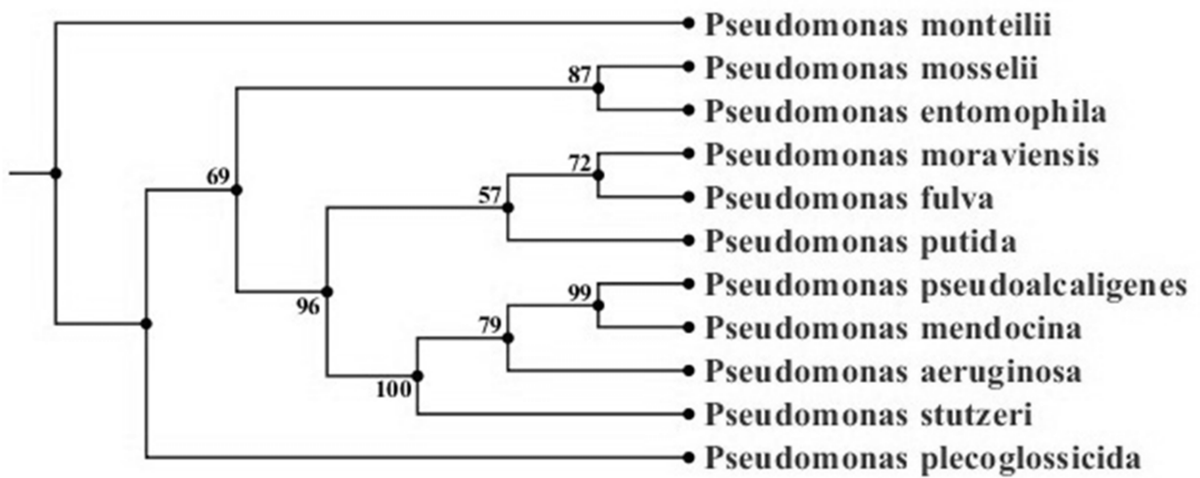
CHAPTER 5

Supporting Information (SI)

Number of tables : 2

Number of figures : 3



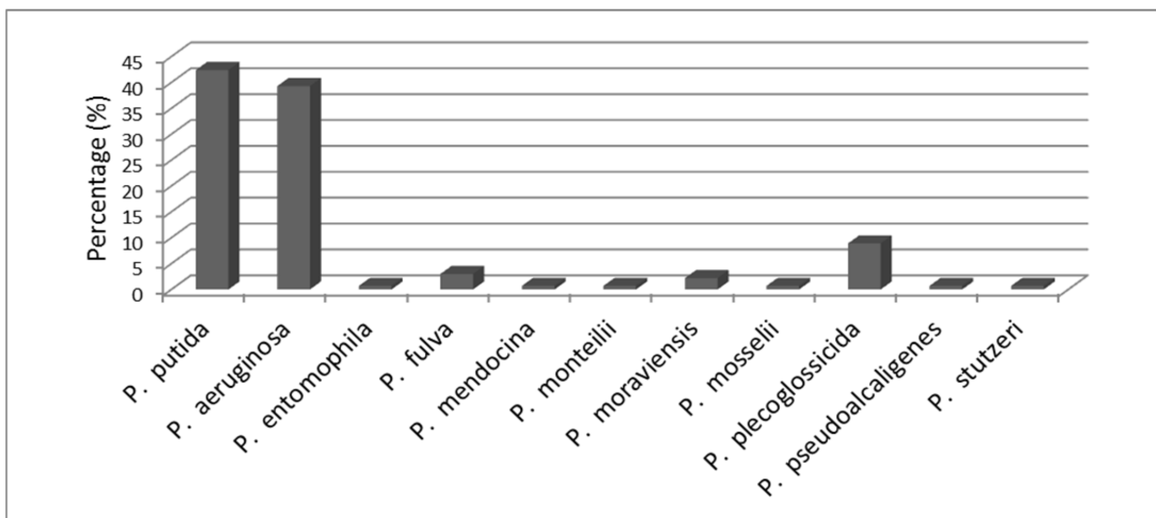


Figure. 5.1s. Distribution of *Pseudomonas* spp. and their phylogenetic relationship based on 16s rDNA sequences used to construct a maximum likelihood phylogenetic tree. Numbers at the branch nodes represent the boot strap values. Length of the branch not displayed as the topology layout for the tree was used.

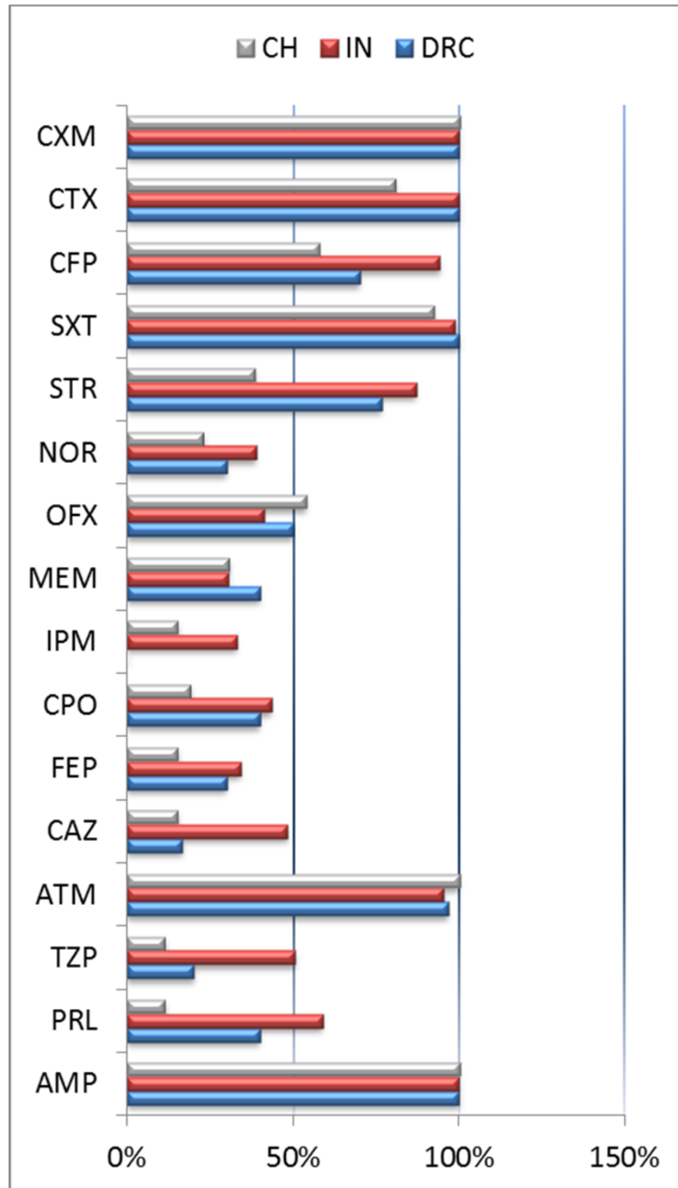


Figure. 5.2S. Comparison of phenotypic resistance in the studied *Pseudomonas* spp. between DRC, IN and CH.

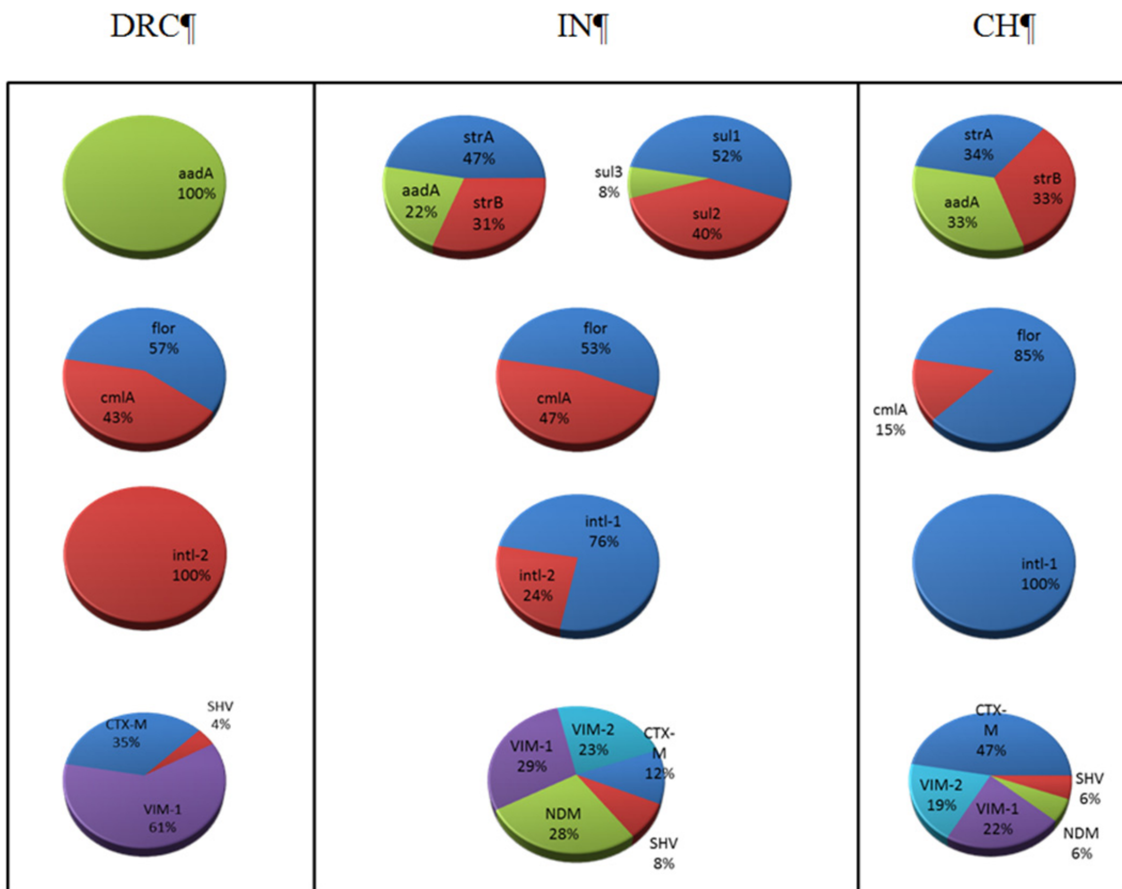


Figure. 5.3s. Distribution of antibiotics resistant genes expressed as ratio among the resistance type in the studied isolates, collected from the sediments receiving treated/untreated effluent at the studied geographical locations

Table. 5.1S. Primers used in this study to identify bacterial 16S rDNA, and antibiotic resistant genes.

gene	primer	sequence 5' - 3'	Temp (°C)	Size (bp)	Reference
universal bacterial 16s	27f	AGAGTTTGATCMTGGCTCAG	58	1500	Wang X. et al. 2003
	1492r	TACGGYTACCTTGTTACGACTT			
strA	StrA-F	CTTGGTGATAACGGCAATTC	55	548	Gebreyes W.A. and Altier C. et al. 2002
	StrA-R	CCAATCGCAGATAGAAGGC			
strB	StrB-F	ATCGTCAAGGGATTGAAACC	55	509	Gebreyes W.A and Altier C. 2002
	StrB-R	GGATCGTAGAACATATTGGC			
aadA	AadA-F	GTGGATGGCGGCCTGAAGCC	62	525	Srinivasan et al. 2008
	AadA-R	AATGCCCAGTCGGCAGCG			
sulI	SulI-F	GTGACGGTGTTCGGCATTCT	59	779	Srinivasan et al. 2007
	SulI-R	TCCGAGAAGGTGATTGCGCT			
sulII	SulII-F	CGGCATCGTCAACATAACCT	57	721	(Srinivasan et al. 2007)
	SulII-R	TGTGCGGATGAAGTCAGCTC			
sulIII	Sul3F	GAGCAAGATTTTTGGAATCG	55	789	Perreten and Boerlin 2003
	Sul3R	CTAACCTAGGGCTTTGGA			
floR	FloR-F	TATCTCCCTGTCGTTCCAG	55	399	Srinivasan et al. 2007
	FloR-R	AGAACTCGCCGATCAATG			
cmlA	CmlA-F	CCGCCACGGTGTTGTTGTTATC	60	698	
	CmlA-R	CACCTTGCCTGCCCATCATTAG			
blaTEM	TEM-C	ATCAGCAATAAACCAGC	54	516	Shah et al. 2012
	TEM-H	CCCCGAAGAACGTTTTC			
blaCTX-M-1	CTX-M-U1	ATGTGCAGYACCAGTAARGTKATGGC	59	593	Boyd et al. 2004
	CTX-M-U2	TGGGTRAARTARGTSACCAGAAYCAGCGG			
blaNDM-1	NDM1-F	GGGCAGTCGCTTCCAACGGT	60	475	Boyd et al. 2004
	NDM1-R	GTAGTGCTCAGTGTCGGCAT			

blaNDM-1	NDM-Fm	GGTTTGGCGATCTGGTTTTC	53	621	Nordmann et al. 2011
	NDM-Rm	CGGAATGGCTCATCACGATC			
blaSHV	F	ATGCGTTATATTCGCCTGTG	56	865	Babini and Livermore 2000
	R	GTTAGCGTTGCCAGTGCTCG			
blaVIM-1	F	AGTGGTGAGTATCCGACAG	57	261	Shibata et al. 2003
	R	ATGAAAGTGCGTGGAGAC			
blaVIM-2	F	ATGTTCAAACTTTTGAGTAAG	52	801	Shibata et al. 2003
	R	CTACTCAACGACTGAGCG			
intM1-U	intM1-U	ACGAGCGCAAGGTTTCGGT	54	565	Su et al. 2006
	intM1-D	GAAAGGTCTGGTCATACATG			
intM2-U	intM2-U	GTGCAACGCATTTTGCAGG	52	403	
	intM2-D	CAACGGAGTCATGCAGATG			
intM3-U	intM3-U	CATTTGTGTTGTGGACGGC	56	717	
	intM3-D	GACAGATACGTGTTTGGCAA			

Table. 5.2S. Primers used in RT-PCR to quantify the gene expression in *Pseudomonas aeruginosa* (Dumas et al., 2005)

Gene	Primer	5' -sequence-3'	Length (bp)	Position in gene	Product length (bp)
ampC	ampC1	CGGCTCGGTGAGCAAGACCTTC	22	264	218
	ampC2	AGTCGCGGATCTGTGCCTGGTC	22	481	
mexA	mexA1	CGACCAGGCCGTGAGCAAGCAGC	23	375	316
	mexA2	GGAGACCTTCGCCGCGTTGTCGC	23	668	
mexC	mexC1	ATCCGGCACCGCTGAAGGCTGCG	23	284	344
	mexC2	CGGATCGAGCTGCTGGATGCGCG	23	605	
mexC	mexC3	GTACCGGCGTCATGCAGGGTTC	22	1101	164
	mexC4	TTACTGTTGCGGCGCAGGTGACT	23	1164	
mexE	mexE4	CCAGGACCAGCACGAACTTCTTGC	24	944	114
	mexE5	CGACAACGCCAAGGGCGAGTTCAC C	25	831	
mexR	mexR1	CGCGAGCTGGAGGGAAGAAACC	22	217	150
	mexR2	CGGGGCAAACAACTCGTCATGC	22	366	
mexX	mexX1	TGAAGGCGGCCCTGGACATCAGC	23	302	326
	mexX2	GATCTGCTCGACGCGGGTCAGCG	23	605	
mexZ	mexZ1	GCATGGGCTTTCTCCGCCAGTGC	23	266	364
	mexZ2	GCGTCCGCCAGCAACAGGTAGGG	23	629	
mexT	mexT1	CAGCACCGCGGTGTTCCGCATCG	23	420	216
	mexT2	ACGGTCTTGCGCTTGGCGTTGGC	23	635	
oprD	oprD1	ATCTACCGCACAAACGATGAAGG	23	772	156
	oprD2	GCCGAAGCCGATATAATCAAACG	23	927	
oprD	oprD3	CTCGACGGCACCTCCGACAAGAC	23	287	232
	oprD4	AGCCCTTCGAATTCGCTGCTCTG	23	518	
oprJ	oprJ1	GTTCCGGGCCTGAATGCCGCTGC	23	345	305
	oprJ2	TCGCGGCTGACCAGGGTCTGACG	23	627	
oprM	oprM1	GATCCCCGACTACCAGCGCCCCG	23	60	247
	oprM2	ATGCGGTACTGCGCCCGGAAGGC	23	265	
oprN	oprN1	CAACCGGGAGTGACCGAGGACCG	23	358	235
	oprN2	TGCTCAGGGCAATCTTCTCGCGC	23	570	
nfxB	nfxB1	CGCCTGATCAAGGAACACCTCACC	24	244	164
	nfxB2	CGAAACACGCCTTTCTGCTGTCC	23	407	
rpsl	rpsL-F	GCAAGCGCATGGTCGACAAGA	21	35	201
	rpsL-R	CGCTGTGCTCTTGCAGGTTGTGA	23	235	

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CHAPTER 6

Pyrosequencing based analysis of bacterial diversity in metal-contaminated sites: Case study of Congo DR, India, Senegal and Switzerland

Supporting Information available at the end of the chapter

Manuscript in preparation

Abstract

We studied the influence of metal concentrations on structure and diversity of bacterial communities using pyrosequencing in soil/sediments collected from distinct geographical study sites (Congo DR, India, Senegal and Switzerland). Ecological tools were used to access the combined effect on the bacterial communities and their metal concentrations. Statistical clustering of samples revealed that bacterial communities were structured and closely related to the source of the contaminants. Results revealed significant effects induced by the metal contaminants on bacterial community in the study sites. Several large shifts were identified at the Class level with enriched amount of α, ß, γ-proteobacteria in the soil/sediments receiving contaminants from recreational shooting, mining and hospital effluents, respectively. At the Order level *Clostridiales* and *Actinomycetales* had significant negative correlations (r^2 - 0.7, $p < 0.05$) with total organic matter, Cr, Cu, and Pb. In this study we identified 107 (37%) genus in common among the study sites from distinct geographical locations. On the other hand the *Chloroflexi* phyla contributed to 41% of the total population in Pb contaminated site and *Acinetobacter* (16%) was the most dominant group in the sediments receiving hospital effluents. Metals accumulated in the terrestrial environment from various sources as a result of anthropogenic activities have the potential impact on bacterial communities in the effluent receiving ecosystems.

6.1. Introduction

Since Stone Age a total of 1150 million tons of metals have been exploited. At an annual growth rate of 3.4%, it is estimated that an annual output of 14 million tons of metals being mined per year (Sheoran and Sheoran 2006). Anthropogenic activities such as industrial, sewage, hospital, agriculture, landfills, fire arms, mining are a leading source for the spread of metals in terrestrial environment (Pote et al. 2008, Niane et al. 2014a, Devarajan et al. 2015). As an example in Switzerland; there are nearly 6000 bullet proof mounds. Annually 400 tons of Lead, 20 tons of Antimony and other metal components of bullets are introduced in the soil by shooting society which is the main source of Pb in the environment (Kettler and Schenk 2006). Among the quantitatively pollutant groups, metals are highly persistent in the soil/sediment environment and are capable to affect all groups of organisms and ecosystem processes, which include the microbial community mediated process (Muller et al. 2001). Recently the toxic effect of the metals on microorganisms has received more attention, as microbes play a key role in the recycling of nutrients. The effect of metals in the soil/sediment matrix is complex and strenuous to study as the diversity of the microbes is complex in these systems (Hu et al. 2007). On the other hand microorganisms can tolerate the toxicity effect induced by the metals and their resistances to the metals are not well known. Previous studies have demonstrated that microbes have the adeptness to remove metals in the polluted environments and metals concentration at probable effective levels may be toxic to one species, induce growth stimulation for the others (Khan et al. 2010, Sheik et al. 2012, Drury et al. 2013). Hence a wide spread environmental change will promote the geographic expansion of some species and reduction of others (McKinney and Lockwood 1999).

A pronounced function of the soil/sediment microbial diversity is therefore a prerequisite for the ecological balance (Chodak et al. 2013). Soil/Sediment microbial community is markedly different between systems, across geographical boarders and macroscopically homogenous landscapes. Studies on whole bacterial community across board geographical scales have revealed that there is a degree of prokaryotic endemism at higher taxonomic resolutions (Fulthorpe et al. 1998, Wawrik et al. 2007, Fulthorpe et al. 2008). Factors for the development of prokaryotic endemism are, (i) the strong selective pressure of local conditions on a ubiquitous base of current diversity (Fulthorpe et al. 2008); (ii) the evolution and extinction of endemic populations at rates that outstrip the pace of global mixing (Martiny et al. 2006) and transportation of exotic species (McKinney and Lockwood 1999). A large number of studies

supports that biogeographic patterns are observed in free living microorganisms, and the environmental conditions, are accountable for the spatial variation in microbial diversity (Martiny et al. 2006).

Advances in molecular techniques have drastically increased our adeptness to identify these microbial community structures in the natural environments. Microorganisms those are challenging to culture (99% of microbes in the natural environment) could now be performed by these modern methods (Fulthorpe et al. 2008, Lozupone and Knight 2007, Poza et al. 2012). In the last few decades analysis of amplified and sequenced 16S rDNA has evolved as an important method to understand the microbial structure and diversity (Chodak et al. 2013). Previous studies have highlighted the use of this method may provide a better insight into the microbial community structure (Poza et al. 2012, Golebiewski et al. 2014, Junemann et al. 2012, Xu et al. 2014).

The objective of this study was to assess the influence of wastewaters from various sources in distinct geographical locations on structure and diversity of microbial communities in soils/sediments contaminated with various concentrations of metals. To identify the effects induced by various sources across geographical boarders we studied the soil/sediment bacterial community structure receiving contaminants from (1) recreational shooting (stop butt) and (2) waste water treatment plant (WWTP) in Switzerland (CH). (3) Artisanal Small scale Gold Mining (ASGM) Senegal, (4) hospital (Congo DR and India).

6.2. Materials and Methods

6.2.1. Study sites and sampling procedure

Samples were collected from 4 different geographical locations (Table 6.1); (1) The stop butt of the recreational shooting ranges surface soil samples were selected to represent the high contamination of Pb in these environments. (Kettler and Schenk 2006). Soil (0 – 3 cm) samples were collected, behind the targets at 3 shooting ranges Nyon (A1), Cartigny (A2), Versoix (A3). In Switzerland; Lake Geneva is the largest fresh water reservoir with a surface area of 580.1 km^2. At the location of Vidy Bay, on the northern shore of the lake a waste water treatment plant (WWTP) was built in 1964. In 2001, the WWTP outlet pipe was extended further to a distance of 700 m from the lakeshore, at 35 m water depth (Pote et al. 2008). At this new WWTP outlet pipe (G1) the surface sample (0-5 cm) was collected using

the boat "La Licrone" of Institute F. A. Forel. Creux-de-Genthod (G3) region located approximately 70 km away from the G1 site is a coastal area. At this site the surface sediment samples were collected at 50m water-depth. Wettingen reservoir (G2) located on the bank of the River Limmat is approximately 200 km away from the G1 site. Located downstream from Zurich this reservoir is contaminated with metals from the urban colonisation and the nearby industry. (3) In Senegal; the main gold deposits are located in the Kedougou region. This is an emerging gold camp, with more than 10 million ounces of gold resources discovered. Miners handle Hg and burn amalgam to extract gold at the ASGM sites. During this process a large volume of Hg is released to the environment. At the ASGM sites there are noticeable environmental impacts with the abundant use of Hg over the past decade (Niane et al. 2014b). Surface sediment samples (0-3 cm) were collected at the ASGM sites (Tinkoto (B2), Bantako (B3) and along the Gambia River bank (Samekouta (B1) at 1m water depth. (4) In India; the surface sediment samples were collected from 3 principle hospitals (N1, N2, N3) at Tiruchirappalli, Tamil Nadu, India. The collection points were adjacent to the hospital effluent outlet pipe before been discharged into the municipal sewage. (5) In Congo DR; the surface sediment sample was collected adjacent to the effluent outlet pipes of hospital Kintambo (N4) before been released to River Makelele. The sediment/soil samples were collected in sterile plastic bags and stored at 4 ˚C until they were shipped to Institute F.A. Forel, University of Geneva, Switzerland for analysis.

6.2.2. Total Organic Matter and metal analysis

Total organic matter content (OM) was estimated by loss on ignition at 550 ˚C for 1 h in a Salvis oven (Salvis AG, Emmenbrücke, Luzern, Switzerland). The particle grain size was measured using a Laser Coulter® LS-100 diffractometer (Beckman Coulter, Fullerton, CA, USA), following 5 min ultrasonic dispersal in deionized water according to the method described by (Loizeau et al. 1994). Samples (Soil/Sediment) were lyophilized at 45 ˚C, followed by homogenization and air-drying at room temperature. The total metal concentrations of Cr, Cu, Zn, As, Cd, Sb, and Pb were determined by by quadrupole-based inductively coupled plasma mass spectrometry (ICP-MS, Agilent model 7700 series) following the digestion of sediments in Teflon bombs heated to 150 ˚C in analytical grade 2 M HNO_3 as described by (Pote et al. 2008, Pardos 2004, Mubedi et al. 2013). The total Hg analysis was carried out using the Atomic Absorption Spectrophotometer (AAS) for mercury determination (Advanced Mercury Analyser; AMA 254, Altec s.r.l., Czech Rep.) following

the method described previously (Roos-Barraclough et al. 2002). The detection limit (3 SD blank) was 0.005 mg kg^{-1} and the reproducibility better than 95%. The metal concentrations of soil/sediment samples were expressed in ppm (mg kg^{-1} dry weight sediment).

6.2.3. Barcoded Pyrosequencing

Total DNA from sediment samples was extracted using Ultraclean soil DNA Kit (Mo Bio Labs, USA) by following manufacturer's instructions. For each sample, DNA extraction was performed with replicate samples to compensate for heterogeneity. The isolated DNA was stored at -20 ˚C until shipped to Research and Testing Laboratory, Texas, USA. An average of 10K reads was obtained on the amplification of the V3-V5 region of the bacterial 16S rDNA using a forward and reverse fusion primer. The forward primer was constructed with (5'-3') the Roche A linker (CCATCTCATCCCTGCGTGTCTCCGACTCAG), an 8-10bp barcode, and the 341F primer (5'-CCTACGGGAGGCAGCAG-3'). The reverse fusion primer was constructed with (5'-3') a biotin molecule, the Roche B linker (CCTATCCCCTGTGTGCCTTGGCAGTCTCAG), and the 907R primer (5'-CCGTCAATTCCTTTGAGTTT-3') (Bernard et al. 2012). Amplifications were performed in 25 µl reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, California), with 1ul of each 5uM primer, and 1ul of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, California) under the following thermal profile: 95˚C for 5 min, followed by of 94˚C for 30 sec, 54˚C for 40 sec, 72˚C for 1 min, for 20 cycles and additional one cycle of 72˚C for 10 min. Amplified products were visualized with eGels (Life Technologies, NY, USA). Products were then pooled equimolar and each pool was cleaned with Diffinity RapidTip (Diffinity Genomics, NY, USA), and size selected using Agencourt AMPure XP (Beckman Coulter, IN, USA) following Roche 454 protocols (454 Life Sciences, CT, USA). Size selected pools were then quantified and 150 ng of DNA were hybridized to Dynabeads M-270 (Life Technologies, CT, USA) to create single stranded DNA following Roche 454 protocols. Single stranded DNA was diluted and used in emPCR reactions, which were performed and subsequently enriched. Sequencing following established manufacture protocols (454 Life Sciences, CT, USA).

6.2.4. Sequence analysis

Sequencing reads were downloaded as .sff files from the Research and Testing Laboratory, Texas, USA server and were processed on BioLinux 7, (Field et al. 2006). A desktop

computer T3600 2 Ghz (Dell, USA), equipped with a 12 cores processor array and 32 GB of RAM was used to run the Linux Ubuntu platform. MOTHUR (Schloss et al. 2009) was used to check the reads for different quality criteria. The selection of reads (Trimming for quality and length) was based on (i) the size of the amplicons (>150 and <600 bp), (ii) the absence of ambiguous base (N) and an average of Phred quality score >25, (iii) the absence of homopolymers longer than 8 bp. Following the above selection the reads were aligned with the Greengenes database (DeSantis et al. 2006) and de-noised with the Single Linkage Preclustering (SLP) method (Huse et al. 2010). During the SLP process, reads with a pairwise distance smaller than 2% were clustered and merged while the cluster size were retained as counts (Junemann et al. 2012). UCHIME algorithm implemented in MOTHUR (Edgar et al. 2011) and the SILVA database (Quast et al. 2013) was used to screen for artificial chimeric formations. The resulted high quality reads were used for Hierarchical clustering using Esprit-Tree (Cai and Sun 2011) and multi-classifying to the lowest possible rank in the Greengenes database and the removal of *Archaea* domain. Statistical analysis and numerical ecology were performed on R (R Developemt Core Team 2009).

6.3. Results and Discussion

6.3.1. Geochemistry overview in study sites

Sediment/soil samples were evaluated to establish an overview of metal distribution in comparison to basic sediment/soil characteristics. Contaminated sites differed in the presence and concentrations of metals and the values are summarized in Table 6.1. Total organic matter was abundant in the surface soils of shooting range samples (A3 – 30.8%), followed by ASGM site (B2 – 21.8 %) and sediments receiving WWTP effluent (G1 – 15.9%). The sediment samples from DRC were identified as sandy sediments (> 91% sand) and measured the least values for OM (2.6%). Samples from Indian receiving system were generally loamy – sand sediments. The shooting range samples were mostly composed of clay and presented the lowest values for mean grain size (A2 – 10 μm). Cr, Cu and Zn were present at both contaminated and control sites with higher concentrations at contaminated sites as seen in B2 (1555 mg kg^{-1}), A3 (3459 mg kg^{-1}) and B2 (1048 mg kg^{-1}). As and Cd were detected only in trace amounts with exception at B3 (45 mg kg^{-1}) and G1 (3.5 mg kg^{-1}) samples. The shooting range sites A1 (27,517 mg kg^{-1}), A2 (67,222 mg kg^{-1}) and A3 (372,678 mg kg^{-1}) contained massive amounts of Pb and trace amounts of Hg. On the other hand, the ASGM sites and the

hospital sites presented the higher concentrations for Hg 14.8, 7.4 and 4.2 mg kg^{-1} in samples N1, B2 and B3, respectively.

6.3.2. Bacterial diversity indices.

Taxon richness and evenness were analyzed for diversity indices (Table 6.2), and the results indicate that both the parameters were highest in fresh water sediments (B2, G1) and lowest in the hospital sites (N4). The volumes of OTUs estimated at 5% clustering level varied substantially among the samples. OTU numbers varied from 859 to a maximum of 6038, for samples N4 and B2, respectively. The specificity of these samples were confirmed with the values for Abundance-based Coverage Estimators (ACE) and CHA01 estimators and rarefaction analysis (Figure 6.1). The highest values for the ACE (13286) and CHA01 (12133) were obtained in the sample B2 followed by sample G1 [ACE (4595) and CHA01 (10182)], indicating the highest taxon richness in these fresh water effluent receiving system. The shape of the rarefaction curves provided by most of the samples (except B2 and G1) verified that the analysis was able to discourse almost the entire bacterial diversity present in the samples. The Fisher's α index provided that the control site samples (G3 74.3) was highly diverse in species when compared to the contaminated site (G1). On the other hand the Shannon index (measuring both abundance and evenness) identified that the samples from the Pb polluted sited had the lowest (A3 – 2.93) value.

6.3.3 Microbial Community Response to Contamination Revealed by Pyrosequencing

After a quality screening, an average of 10,172 reads per sampling site were obtained from a total of 132,245 sequences sampled from 4 distinct geographical locations. Several large shifts in the relative abundance of the bacterial phyla were observed on a comparative analysis between the sampling sites. The bacterial community was composed by a dominant number of phyla (n=14, Figure 6.2.) out of the total identified phyla (n=59) in this study. Among these, *Proteobacteria* showed the highest contributions in all the samples with an average of 42%. However sample A3 and N3 had the least contribution from *Proteobacetria* and were enriched by *Chloroflexi* (49%) and *Firmicutes* (47%), respectively. Uncontaminated site (G3) had a balanced bacterial taxa structure with contributions from *Proteobacteria* (34%), *Bacteroidetes* (34%), *Firmicutes* (10%), *Spirochaetes* (5%) and unclassified (7%). This pattern is similar to the phylum level profiles generated by previous studies (Haller et al. 2011, Wang et al. 2012).

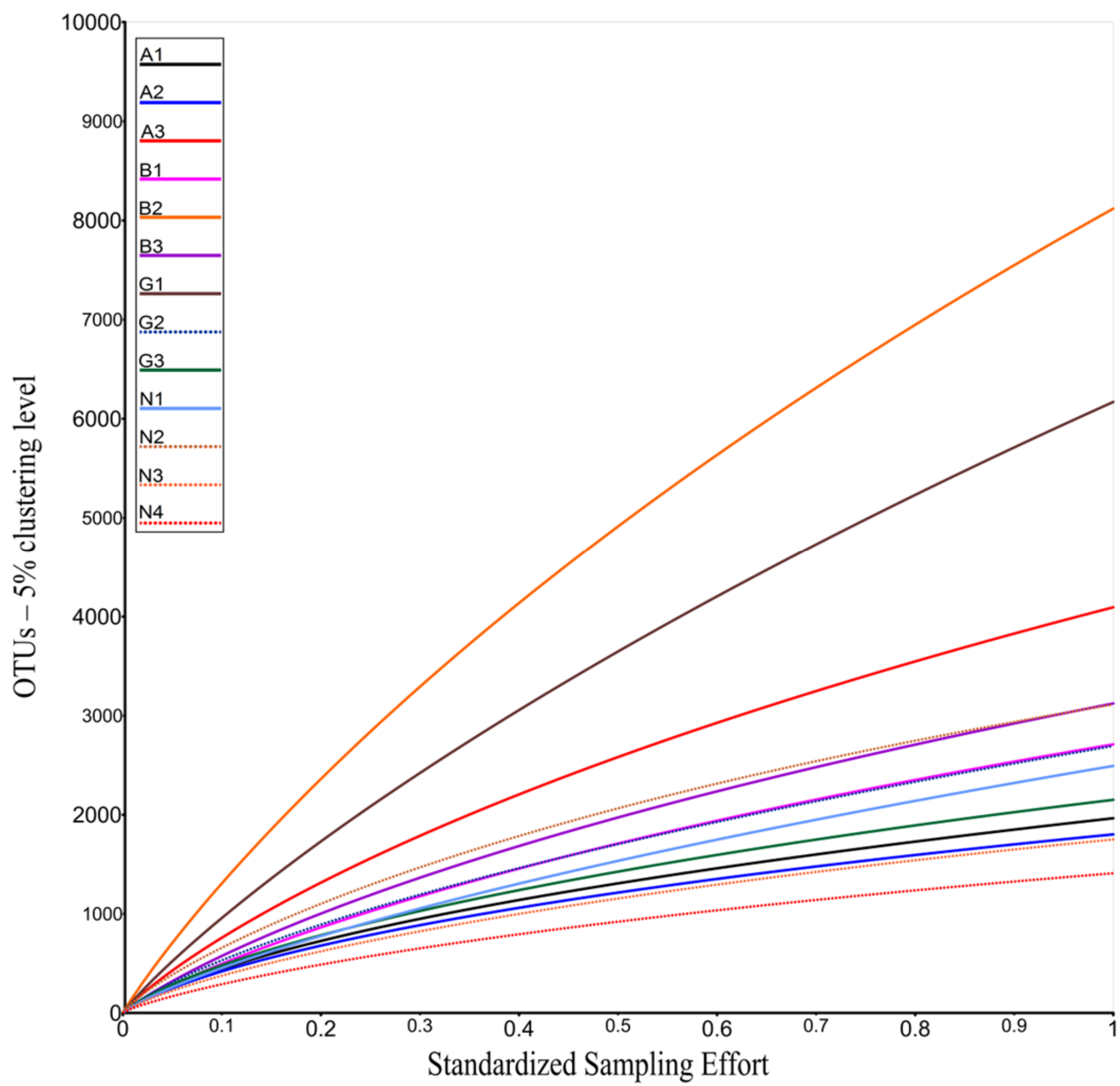


Figure. 6.1. Rarefaction analysis carried out on the bacterial communities sampled from Congo DR, India, Senegal and Switzerland using ESPRIT-Tree (5% clustering level)

Table. 6.1. Source of the metal contamination, geographical region of sample collection, analysis of total organic matter (OM) and metal content from the sampling sites of Congo DR, India, Senegal and Switzerland

					mgkg^{-1}							
Region – Climate	Sample Name	Sample site	Grain size (µm)	OM (%)	Cr	Cu	Zn	As	Cd	Pb	Hg	Reference
CH – Temperate	A1	Nyon	27	4.5	42.8	535.3	52.3	2.0	0.1	27517.0	0.0	
CH – Temperate	A2	Cartigny	10	5.1	19.6	963.9	55.0	6.9	0.2	67222.0	0.1	This study
CH – Temperate	A3	Versoix	50	30.8	41.1	3459.3	197.7	0.0	0.2	372678.0	0.1	
SN – Tropical	B1	Samekouta	60	5.6	363.0	30.0	49.0	8.0	n/a	19.0	0.2	
SN – Tropical	B2	Sabodala	95	21.8	1550.0	92.0	1048.0	2.5	n/a	17.0	7.4	Niane et al. 2014b
SN – Tropical	B3	Bantako	73	4.9	1123.0	33.0	58.0	45.0	n/a	19.0	4.2	
CH – Temperate	G1	Vidy	41	15.9	79.0	260.8	535.6	n/a	3.5	199.2	1.5	
CH – Temperate	G2	Wet	80	7.8	34.1	42.0	126.3	n/a	n/a	21.8	0.1	This study
CH – Temperate	G3	CDG	75	2.1	52.9	50.0	113.1	n/a	0.2	28.8	0.2	
IN – Tropical	N1	H3	363	12.6	148.8	52.3	274.9	0.9	0.6	16.6	14.8	
IN – Tropical	N2	H4	432	2.1	16.6	13.9	88.8	1.8	0.1	5.9	3.9	Mubedi et al. 2013
IN – Tropical	N3	H5	94	2.9	22.9	25.1	126.8	1.0	0.2	17.2	1.7	
DRC – Tropical	N4	C1	335	2.6	3.3	4.0	48.8	0.6	0.1	9.9	0.5	

n/a – below detection limit; CH – Switzerland; SN – Senegal; IN – India; DRC – Democratic Republic of Congo

In an average *Bacteroidetes* (12%), *Chloroflexi* (9%), *Firmicutes* (8%), *Actinobacteria* (7%), *Acidobacteria* (5%) and unclassified (7%) were the most common shared phyla after *Proteobacteria* in the studied samples (Figure. 6.1). On comparison with the 3 Pb contaminated sites, sample A3 was less diverse than A2 and A1. At this study site, *Chloroflexi* was more abundant than any other phyla with one sequence (Species/Genus unclassified, and identified as *S47* (Family); *A31* (Order); *Anaerolineae* (Class); *Chloroflexi* (Phylum)) contributing to 41% of the total population, indicating a clear shift in the bacterial diversity. Additionally the other phyla groups *Armatimonadetes*, *Chlamydiae*, *Planctomycetes*, *SPAM*, *TM7*, *Verrucomicrobia* and *ZB2* showed a significant increase in sample A3. On the other hand there was a decrease in the phyla *Acidobacteria*, *Actinobacteria*, *Gemmatimonadetes*, and *Nitrospirae* in the sample A3 when compared to sample A1 and A2. The phyla groups were compared between the control site and the contaminated site in the samples from Senegal and Switzerland. Phyla *AC1*, *Chlorobi*, *Chloroflexi*, *Cyanobacteria*, *Gemmatimonadetes*, *NC10*, *Nitrospirae*, *OP8*, *Verrucomicrobia*, and *WS3* showed a clear increase in the contaminated sites when compared to the control sites. *Firmicutes* was the only phyla that decreased in both the contaminated regions and had higher values in the control sites of Senegal and Switzerland. Hospital samples from India and DRC were dominated by 5 major phyla groups representing 94% of the sequences, including *Proteobacteria* (45%), *Firmicutes* (21%), *Bacteroidetes* (13%), *Actinobacteria* (12%) and *Chloroflexi* (3%). The remaining 6% of the phyla groups were composed of 4% (unidentified group) and 2% (include 25 phyla groups). *Proteobacteria* is the most abundant phylum as typically observed in soil libraries. They are composed of enormous level of morphological, physiological and metabolic diversity and play a major role in global carbon, nitrogen and sulfur cycling (Janssen 2006, Spain et al. 2009). The vast majority of the *Proteobacteria* are yet to be cultivated and in this study we take a closer look on the composition of this phyla group at the Class level (Figure. 6.3). The vast majority of the Pb contaminated samples were enriched with *Alphaproteobacteria* (47%) followed by *Beta*- (23%), *Gamma*- (15%) and *Delta- proteobacteria* (11%). The most abundant order in *Alphaproteobacteria* was *Rhizobiales* and contributed for 61% of the total *Proteobacteria* reads. On the other hand *Burkholderiales* (59%) and *Rhodocyclales* (40%) were the most common order in the samples from Senegal and Switzerland, respectively. *Betaproteobacteria* was the most abundant class in the samples from Senegal (53%) and Switzerland (38%), followed by *Delta-, Gamma-* and *Alphaproteobacteria*. The class *Epsilonproteobacteria* was identified only in the control site

(G3 – 1.6%) of Lake Geneva and (N1 – 0.9%) hospital sample from India. The hospital samples were enriched with *Gammaproteobacteria* (56%) followed by Beta-(25%), Alpha-(15%), and *Deltaproteobacteria* (0.5%). *Gammaproteobacteria* include a number of important pathogens *Pseudomonadales* (68%) was the most abundant order identified in these samples followed by *Xanthomonadales* (23%) and *Enterobacteriales* (4%). At genus level *Acinetobacter*, contributed 16% of the total reads from the hospital samples.

Table 6.2. Estimators computed with high quality reads at 5% clustering level using Esprit-Tree and R.

Samples	Read Numbers	Phylum Richness	Genus numbers	OTUs	ACE	CHAO1	Shannon	Pielou's Eveness	Fisher's α
A1	9323	18	254	1284	2444	2270	4.37	0.79	51.81
A2	10734	22	254	1197	2171	2164	4.04	0.73	50.87
A3	29945	24	223	2733	5243	4979	2.93	0.54	36.35
B1	12392	28	276	1860	3543	3226	4.32	0.77	55.26
B2	22541	35	396	6038	13286	12133	4.13	0.69	73.14
B3	13421	35	275	2166	4213	3810	3.78	0.67	53.75
G1	16946	38	364	4595	11371	10182	3.99	0.68	69.97
G2	11878	39	266	1989	4120	3739	4.06	0.73	51.94
G3	13659	39	358	1492	2734	2671	3.83	0.65	74.31
N1	11596	21	276	1735	3992	3738	3.90	0.69	55.60
N2	20502	17	307	1949	3052	2903	3.83	0.67	56.09
N3	14380	16	259	1103	1833	1688	3.96	0.71	50.59
N4	15110	20	278	859	1475	1390	3.38	0.60	55.42
Mean	**15571.30**	**27.07**	**291.23**	**3199.61**	**8005.19**	**7203.23**	**3.89**	**0.69**	**56.54**

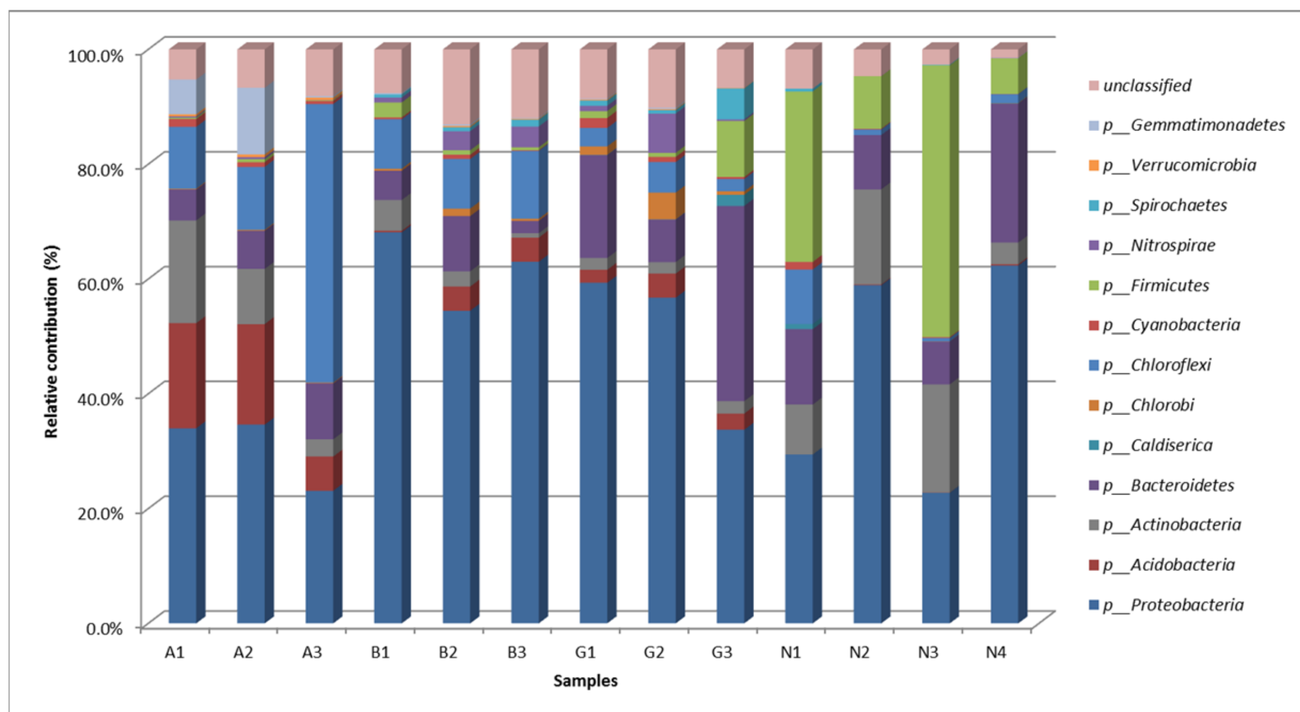
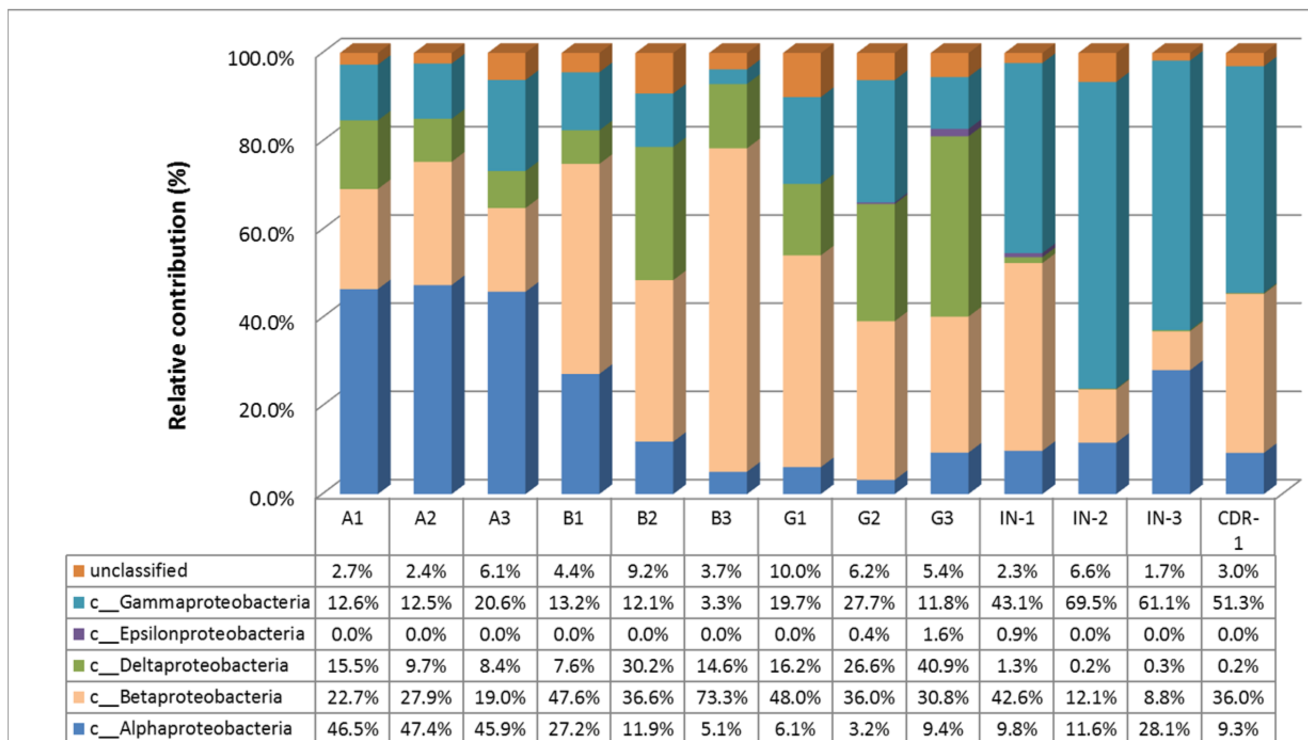


Figure. 6.2. Relative contribution of selected Phyla (n=14) in the studied samples.



	A1	A2	A3	B1	B2	B3	G1	G2	G3	IN-1	IN-2	IN-3	CDR-1
unclassified	2.7%	2.4%	6.1%	4.4%	9.2%	3.7%	10.0%	6.2%	5.4%	2.3%	6.6%	1.7%	3.0%
c__Gammaproteobacteria	12.6%	12.5%	20.6%	13.2%	12.1%	3.3%	19.7%	27.7%	11.8%	43.1%	69.5%	61.1%	51.3%
c__Epsilonproteobacteria	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.4%	1.6%	0.9%	0.0%	0.0%	0.0%
c__Deltaproteobacteria	15.5%	9.7%	8.4%	7.6%	30.2%	14.6%	16.2%	26.6%	40.9%	1.3%	0.2%	0.3%	0.2%
c__Betaproteobacteria	22.7%	27.9%	19.0%	47.6%	36.6%	73.3%	48.0%	36.0%	30.8%	42.6%	12.1%	8.8%	36.0%
c__Alphaproteobacteria	46.5%	47.4%	45.9%	27.2%	11.9%	5.1%	6.1%	3.2%	9.4%	9.8%	11.6%	28.1%	9.3%

Figure. 6.3. Relative contribution of selected Class (alpha, beta, delta, epsilon, gamma and unclassified) in the Phyla *Proteobacteria*

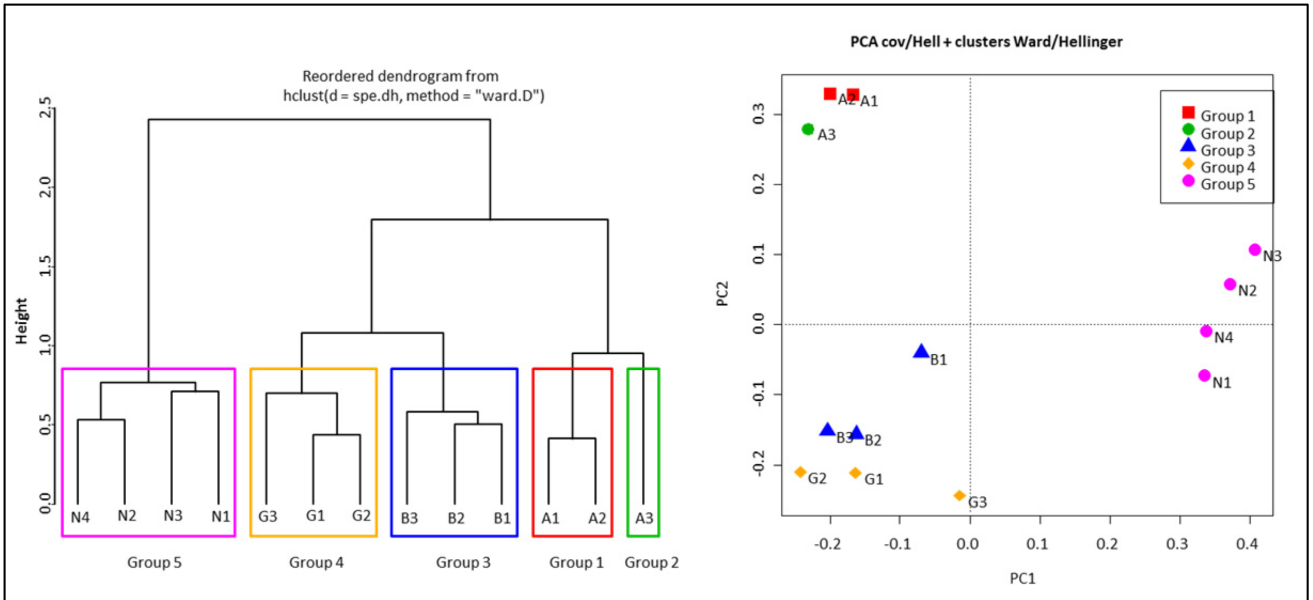


Figure. 6.4. Re-orderd dendrogram at the Order level showing the 5 different clusters (left). PCA analysis using colors for each cluster (right)

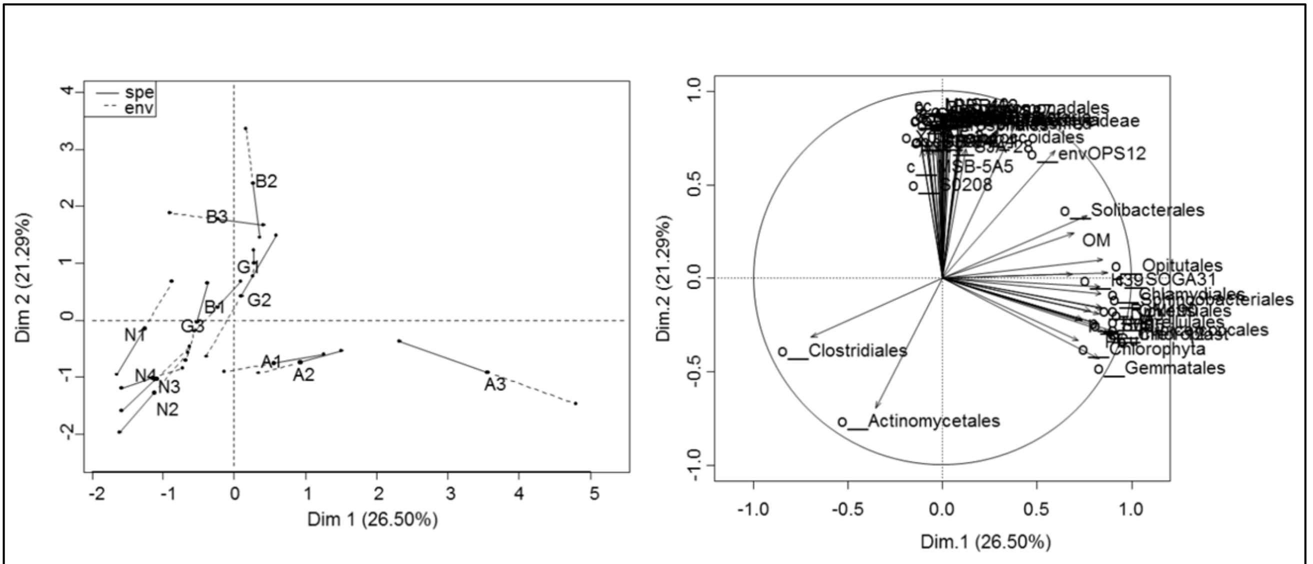


Figure. 6.5. Left: Multi Factorial Analysis (MFA) site scores using bacterial phylogeny at the order level (closed lines) and environmental (OM & metals) data sets (open lines). Right: Correlations between environmental (OM & metals) data and the bacterial Order level. Variables showing statistically significant RV coefficients ($p<0.05$) are only displayed.

6.3.4. Environmental factors and Bacterial Community correlation

The clustering of the sampling sites were performed on the reads at Order level (Figure. 6.4) using the Ward's method. Five groups were identified, Group 1 (A1, A2) and Group 2 (A3), represent the samples from the recreational shooting sites. These results highlight that bacterial communities are structure based on the type and concentrations of the metals even from the same source. The hospital samples from India and DRC were identified in the same cluster groups at all the taxonomic units, indicating the uniqueness in the bacterial diversity released from the hospital effluents at various geographical locations. In addition, bacterial communities from each sample type grouped closely indicating that bacterial communities from the most leading to the unique species were selected by their habitants rather than physical location (Wang et al. 2012). Multifactorial analysis (MFA) was carried out on both environmental and sequencing data sets (Figure 6.5, right panel). MFA exposes correlative structures without any reference to a directionality of possible causal relationship (Borcard et al. 2011). Results of the analysis showed that the first two axes represented more than 48% of the total variance. Four environmental variables Cu, Pb, OM (axis 1) and Cr (axis 2) had close association of vectors. All the four variables had high correlation values (Table 6.2 Supporting Information (SI)). Computed correlation values indicate that most identical variables were significantly correlated at Order level, and adding a strong link between very specific biological activities and bacterial factors representing deep origins of phylogeny. *Clostridiales* vector was almost co-linear with *Actinomycetales* vector and showed a correlation values of -0.69 (axis 1) and -0.68 (axis 2), respectively. In a recent study, a group of variables (Cr, As, OM and Na) were identified to deeply influence the bacterial community structures, and appeared to be the predominant factors that structured the composition of bacterial communities along a contaminant gradient (Sheik et al. 2012). In our study, Cu (0.91), Pb (0.88) and Cr (0.85) had a strong correlation with axis 1 and axis 2 of the MFA, respectively. Additionally the OM also had a correlation value of 0.69 with the axis 1 of the MFA. The MFA analysis identified equally the evolution with the communities using the site scores computed with bacterial community at species level and the analyzed geochemical parameters (Figure. 6.5 left panel). The samples are presented in the center between the projected phylogeny and geochemical sets. Sample A3 displayed a large distance between both sets of variables indicating the fewer adequacies between the community structures and the geochemical variables. This is also evident from the initial community constituting by a large dominance of *Chloroflexi*, and showing low population evenness in sample A3.

Distance between the projected communities and the geochemical variables were minimal in the other sampling groups when compared to the sample A3. The heat-map (Figure 6.6) reflects the correlation between the selected geochemical parameters and the taxa at order level displaying the significance with the first two axis of the MFA (Table 6.1s. supporting information). This analysis presents the evolution in the bacterial shift, with remarkably low amount of taxa being involved in correlation with geochemical parameters. The geochemical parameters Cu and Pb were in the same cluster, with Cr and OM forming an individual cluster. The clustering of the bacterial taxa (at Order level) formed 5 distinct groups, composed of Orders significantly correlated either with the geochemical group Cu and Pb or the individual group OM and Cr. The heat-map showed that the taxa cluster A and B were linked with the metals Cu and Pb, and includes the taxa only from the axis 1 of the MFA analysis. On the other hand Cr was strongly linked with the cluster D, and includes the taxa from axis 2 of the MFA analysis. Two taxa groups *Clostridiales* and *Actinomycetales* formed a separate group (taxa group E) and had a strong affinity and negative correlation values for the studied geochemical parameters. A strong negative correlation explains that, if one parameter increases the other decreases. The relative contributions of these two groups are presented in (Figure. 6.3s supporting information). This could be explained from a recent study that demonstrated the role of *Clostridium* spp., (contributing to 38% of the *Clostridiales* order in our samples) initiating the metal bio-removal by sulfide generation when sulfate reducing bacteria were inhibited by a mixture of Cu, Zn and Fe (Alexandrino et al. 2014).

6.3.5. Bacterial diversity Genus level distribution

The top 10 most abundant genera differed among the sample groups (Table 6.2s Supporting information) and one rank id (0.2.85.1.1.1.1) from the unclassified bacterial taxa group was found common in all the sample groups. This Genus contributed to 7% of the total reads in an average. Significant amount of the genera in all the samples groups were unclassified and hence we have used the next higher level taxa to identify meaningful information. Pb polluted samples contained; *Gemmatimonas* – a rare to cultivate bacteria was one of the dominant genus in the Group 1 indicating the advantages of metagenomic approaches to identify the bacterial community in polluted environments. Additionally the genus *Rhodoplanes* and *Cytophaga* involved in the sulfur and carbon cycles respectively were also found to be predominant in these samples. *Candidatus Solibacter* a bacteria equipped with large amount of anion:cation symporters were also identified in the A3 sampling site.

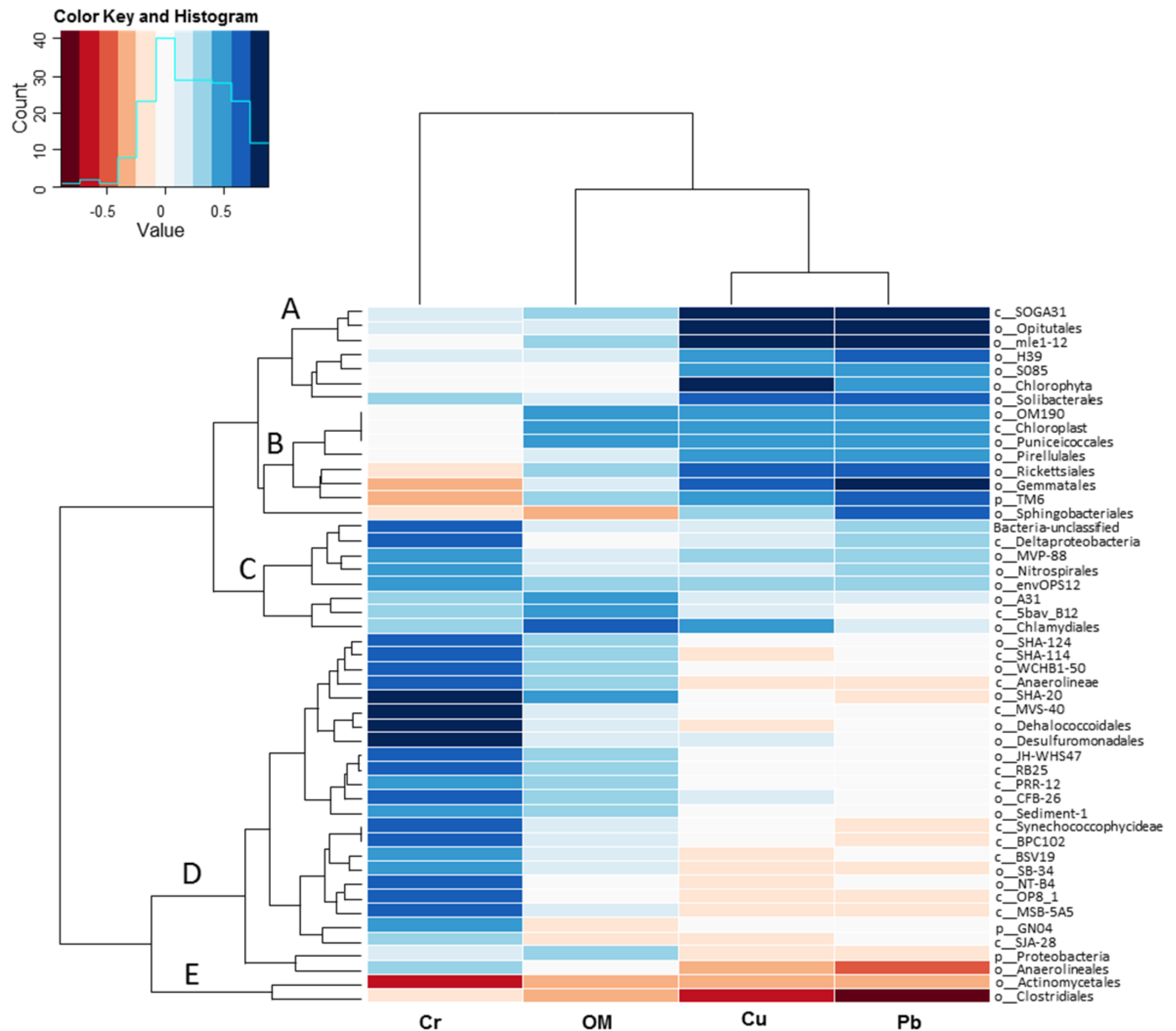


Figure. 6.6. Heat-map built by the joined analysis of bacterial data (at Order level) and the environmental data sets. Contribution of both data sets was selected by MFA analysis and the variables statically correlated with Axes 1 and 2 were used. When unknown orders were found significant, the higher phylogenetic level (Class or Phylum) providing meaningful information was added.

In ASGM sites, *Thiobacillus, Phenylobacterium, Geobacter, Anaeromyxobacter, Methylotenera* identified indicate the survival of the bacteria capable of bioremediation process. These capabilities of these bacterial species were able to reduce and oxidize the metals available from the mining effluents. On comparing to the control site *Nevskia* (3% in

the total reads) an identical genus identified commonly in the air water interface of the fresh water bodies was reduced in the contaminated site (0.5%). In communal effluent, *Dechloromonas, Syntrophus,* and *Geobacter* were the most common genera identified in the communal effluent. The first are responsible to catabolize and biodegrade benzene and other organic contaminants, while the latter responsible for the degradation of metals. *Clostridium, Acinetobacter, Stenotrophomonas, Flavobacterium, Pseudomonas, Chryseobacterium, Enterococcus*, and *Massilia* were the most common genera in the hospital effluents. This observation is similar to those of the Phylum results, an indication of the pathogens enriched in the hospital effluent samples. The sample groups (Figure. 6.4) were used to prepare a Venn diagram (Figure. 6.7) to represent the common number of common Genus shared between sample groups. In this study we identified 107 genus shared between all the five sample groups which contributes to an average of 37% of the genus shared in common between the sampling groups. The top 10 most abundant genera shared by all the five groups varied among the sample groups. The contribution of these reads varied significantly from 0.003% to 40.8%. On the other hand the Group 5 had the highest individual score of 134 genus identical to them and Group 2 had the least individual score of 10 genus, identical to them. Group 2 had the least interaction with the other group of samples sharing 17, 5, 1 and 0 Genus with Group 1, 3, 4 and 5, respectively. More than half a century ago MacArthur (Macarthur 1955), concluded that diversity increase should increase the system stability as a result of the redundancy of the species function. Again functionally similar species which react to changes in the environment differently may increase the functional stability in certain conditions (Chapin et al. 1997). Hence the number of species needed to stabilize a system and the extent of damage the ecosystem can withstand is unknown.

The present study provides a detailed investigational comparison of bacterial communities in four different geographical locations contaminated with metals from various sources. The high concentrations of the Pb, Cu in the soil of group 2 samples seem to have markedly affected the soil microbial community at many levels of the bacterial taxa. This is evident from the species richness, evenness and diversity index calculated for the group 1 and 2 samples. However group 1 samples had higher concentration of Pb and had no significant changes. Similar observations were made by other studies demonstrating that various metals at various compositions affect the bacterial communities differently (Chodak et al. 2013, Berg et al. 2012). In this study, Hg has not significantly correlated with bacterial taxa. However,

studies have demonstrated the significant impact on the bacterial communities in the Hg contaminated areas. The Hg contamination and the biotransformation to methylated-Hg (MeHg) by indigenous bacteria are important to understand the Hg interaction with the soil microbes (Muller et al. 2001, Vishnivetskaya et al. 2011).

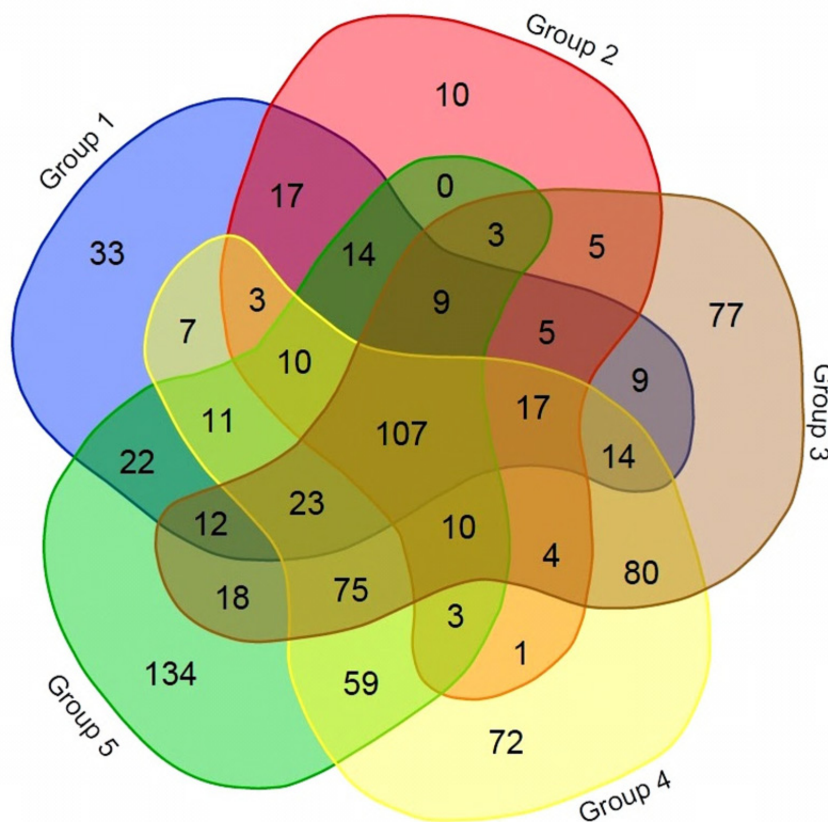


Figure. 6.7. Venn diagrams of shared Genus between each group.

Number of studies have reported on the community composition in sediments/soils, indicating the same major phyla identified regardless of the sample location and indicating the bacterial community structure at the phylum level is nonetheless highly site specific (Dunbar et al. 1999, Hackl et al. 2004, Lamarche et al. 2007). Additionally, studies on homogenous landscapes prove that community phyla structures are seasonally dynamic and highly heterogeneous (Franklin and Mills 2003, Sliwinski and Goodman 2004). Studies have looked on the correlated community structure to ecological parameters (Lozupone and Knight 2007, Rossi et al. 2012, Fierer and Jackson 2006) and few studies have considered finer

phylogenetic levels in sample, and have proven that the levels of resolution become more restricted at species level. These studies and our results prove that geological location and physical-chemical parameters provide strong selection pressure on bacterial composition. In this study, a large amount of genus identified in common among the sample groups explain that the taxa that dominate the community can change rapidly with changing environmental conditions. On the other hand in this study more than 7% of the reads belong to the unclassified group, and it is possible that the long tail of the unidentified species, and those which were not detected could harbor a large amount of ubiquitous bacteria.

The quality of information extracted from the source environments and the accessibility of information in a public data base provide the ability to compare the 16S rDNA data with the physical chemical factors and to identify their effects. In this study we find clear patterns of distribution between the samples groups based on the source of contamination, but the other influencing factors remain unclear, as detailed measurements were not available for many environmental variables and the limitations in the molecular methods used in this study (Fulthorpe et al. 2008, Lozupone and Knight 2007). As metagenomic study includes the following drawbacks; (A) the inclusion of preserved genomic material of inactive/dead bacteria, (B) the number of sequence reads we obtained, (C) the tendency of PCR to prefer and amplify the more numerically dominant sequences even with a low number of cycles, (D) bias in incomplete cell lysis and DNA extraction procedure. Additionally process such as horizontal gene transfer (HGT), salinity are also important factors for the adaptation to the new environments, as bacteria can make genomic exchange and prevent major changes in way of life by simple acquisition of new gene (Lozupone and Knight 2007). Hence for future studies to identify the microbial diversity influenced by physical chemical factors, the required quality and availability of physical/chemical information and structured robotic machine-readable fields in the database will be a key requirement.

6.4 Conclusion

To our knowledge this is first report on the bacterial community composition in the sediments receiving untreated hospital effluents under tropical conditions. The extensive use of metals and the lack of environmental regulation have left the soils/sediments near metropolitan areas inundated with metal concentrations influencing the structure of the bacterial diversity. These contaminants released to the terrestrial environment have the potential to reduce the natural

variability of bacterial communities that exists among the receiving ecosystems. In this study, among many driving forces soil/sediment geochemical compositions precede in shaping the structure and diversity of bacterial communities with additional influencing heralds from source of the contaminants. Further studies involving higher number of sampling sites and precise control sites along the contaminated gradient are required to explore further the long term impact of metal exposure to soil/sediment microbial community.

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Supporting Information (SI)

Number of tables: 3

Number of figures: 3

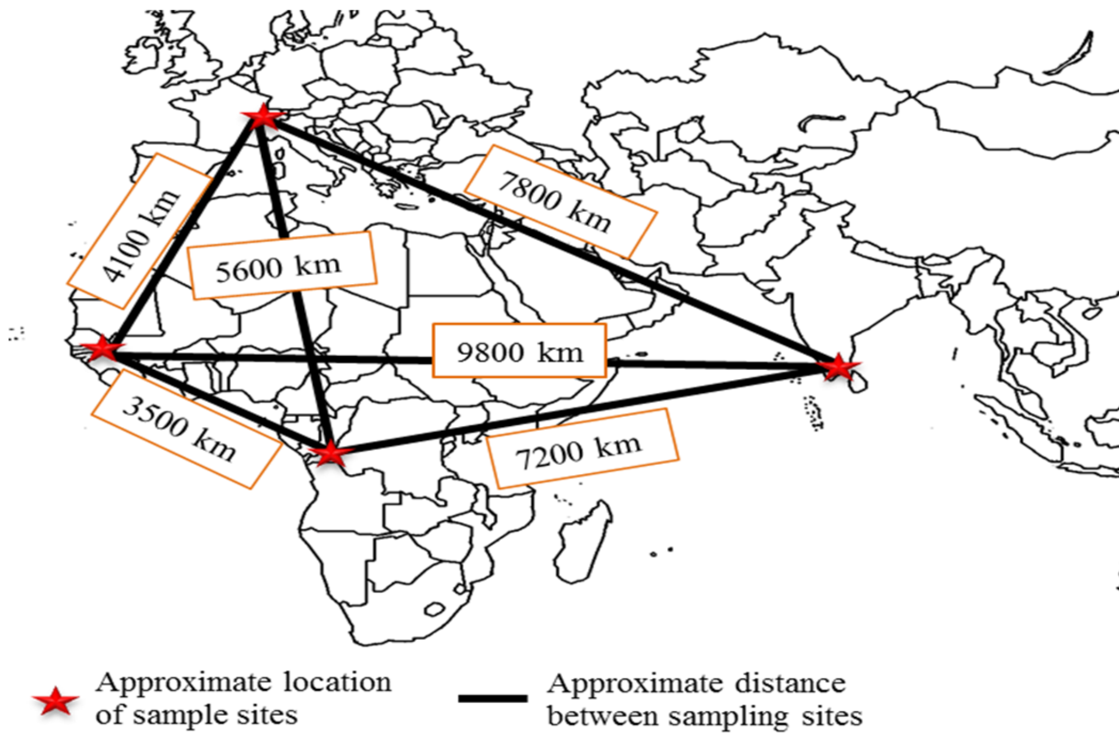


Figure.6.1S. Geographical location of the sampling area and the approximate distances between the sampling sites.

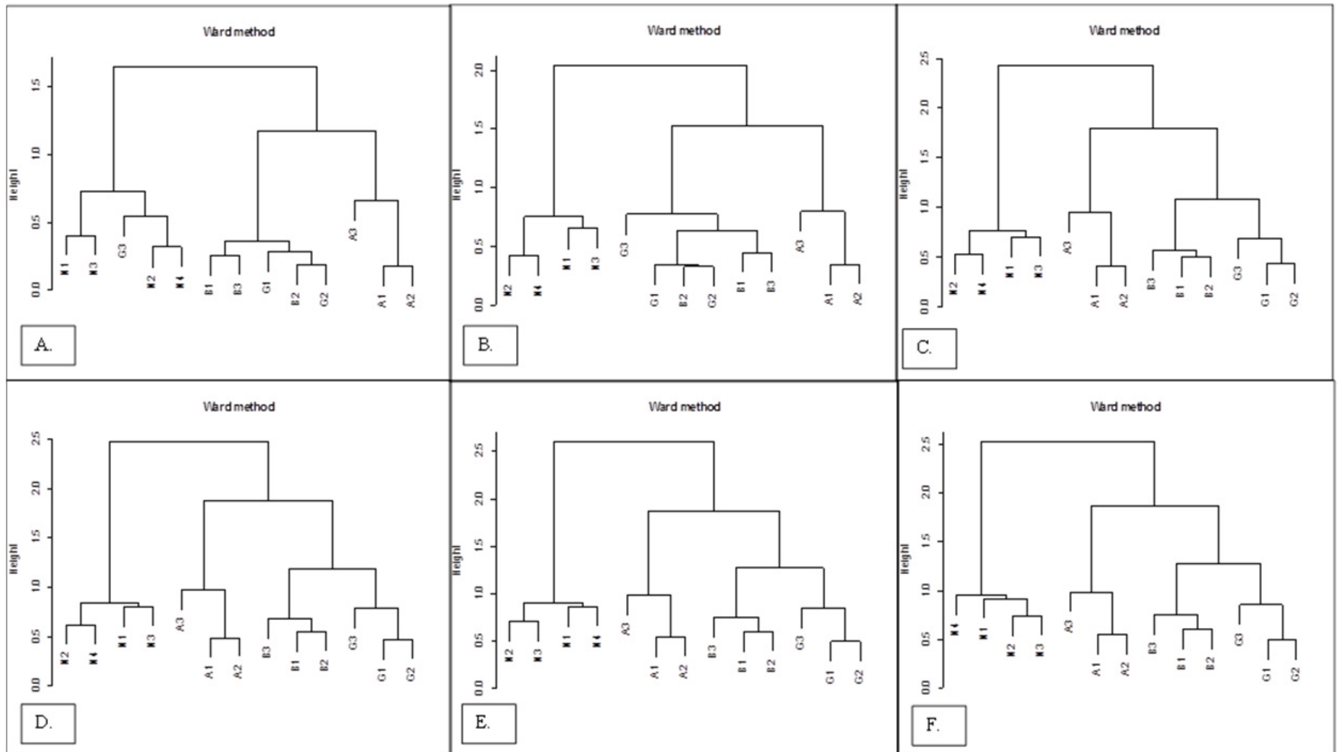
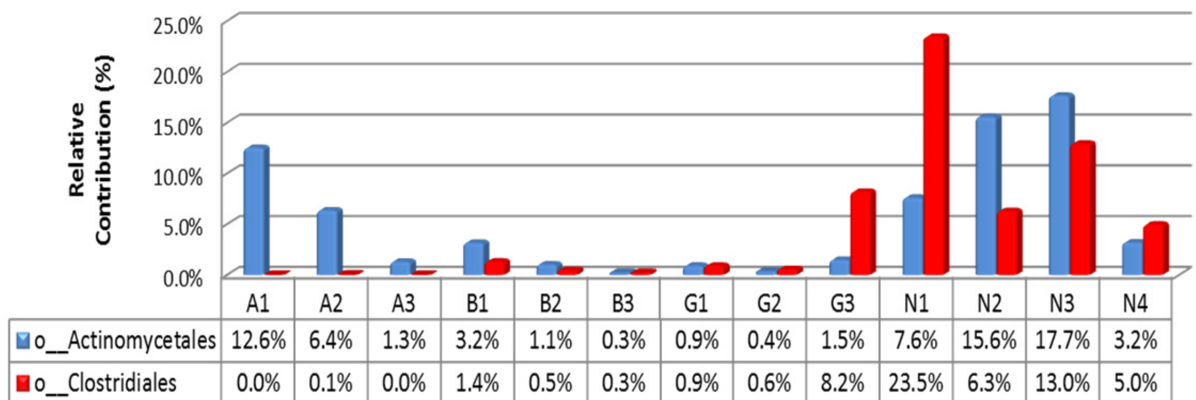


Figure. 6.2S. R-based clustering computation carried out on Hellinger-transforemd bacterial data sets using the Ward technique at A. Phyla level; B. Class level; C. Order level; D. Family level; E. Genus level; F. Species level



	A1	A2	A3	B1	B2	B3	G1	G2	G3	N1	N2	N3	N4
o__Actinomycetales	12.6%	6.4%	1.3%	3.2%	1.1%	0.3%	0.9%	0.4%	1.5%	7.6%	15.6%	17.7%	3.2%
o__Clostridiales	0.0%	0.1%	0.0%	1.4%	0.5%	0.3%	0.9%	0.6%	8.2%	23.5%	6.3%	13.0%	5.0%

Fig. 6.3S. Relative contribution of *Actinomycetales* and *Clostridiales*(Orders with negative correlation) in the studied samples

Table. 6.1S. MFA analysis at Order level: statistically significant variables ($p<0.05$). When unknown orders were found significant, the higher phylogenetic level (Class or Phylum) providing meaningful information was added.

Dim.1 Quantities	
Parameter	Correlation
Cu	0.9154284
Pb	0.8840781
c__SOGA31	0.8745172
o__A31	0.8491160
o__Opitutales	0.8482256
o__mle1-12	0.8424157
o__Sphingobacteriales	0.8409422
o__Chlamydiales	0.8335100
o__Puniceicoccales	0.8324462
c__Chloroplast	0.8324462
o__OM190	0.8324462
o__Pirellulales	0.8302396
o__Gemmatales	0.8267764
o__Rickettsiales	0.7824799
o__Solibacterales	0.7665065
o__S085	0.7418573
P__TM6	0.7337363
o__Chlorophyta	0.7182347
OM	0.6951143
o__H39	0.6852448
o__Clostridiales	-0.6958677

Dim.2 Quantities	
Parameter	Correlation
c__MVS-40	0.9047700
o__Desulfuromonadales	0.8892109
c__PRR-12	0.8882622
c__Deltaproteobacteria	0.8878583
c__SHA-114	0.8800516
Cr	0.8506619
c__Anaerolineae	0.8419946
c__BSV19	0.8417234
Bacteria_unclassified	0.8245792
c__RB25	0.8109415
o__CFB-26	0.8062814
o__Sediment-1	0.7950524
o__WCHB1-50	0.7913711
c__Synechococcophycideae	0.7799923
o__Nitrospirales	0.7723556
o__JH-WHS47	0.7715907
o__SHA-20	0.7341834
o__Dehalococcoidales	0.7310327
c__BPC102	0.7291458
p__GN04	0.7266711
c__SJA-28	0.7231407
c__OP8_1	0.7149016
o__A31	0.7041863
c__5bav_B12	0.7040580
o__S0208	0.7036963
o__MVP-88	0.6997763
o__SHA-124	0.6948455
p__Proteobacteria	0.6930665
c__MSB-5A5	0.6912596
o__NT-B4	0.6850005
o__envOPS12	0.6837519
o__Actinomycetales	-0.6887481

Table 6.2S. Most abundant Genus identified in the sampling groups

Genus	Group 1	Group 2	Group 3	Group 4	Group 5
Acinetobacter				0.01%	16.33%
Anaeromyxobacter	0.01%		1.77%	0.13%	
Aquamonas			1.98%	0.01%	0.01%
Arthrobacter	0.88%	0.25%	0.54%	0.01%	0.43%
CandidatusSolibacter	2.00%	2.17%	1.33%	0.24%	
Chryseobacterium	0.01%			0.01%	1.54%
Clostridium	0.03%	0.01%	0.25%	0.43%	4.75%
Comamonas		0.01%			2.61%
Cytophaga	2.25%	4.79%	0.35%	0.39%	0.02%
Dechloromonas	0.06%		0.09%	3.25%	0.01%
Enterococcus	0.01%				1.08%
Flavobacterium	0.11%	0.02%	0.07%	1.06%	2.05%
Gemmatimonas	3.29%	0.06%	0.03%	0.04%	0.01%
Geobacter			3.19%	1.63%	
Haloanella					2.00%
Lactobacillus		0.01%			3.76%
LCP-6			0.61%	1.94%	
Massilia	0.11%		1.65%	0.01%	1.44%
Methylotenera	0.06%	0.01%	2.23%	0.23%	
Methyloversatilis	0.15%	0.03%	0.42%	1.26%	0.01%
Nevskia			2.22%		
Novosphingobium	0.28%	0.12%	1.06%	0.21%	0.19%
Pedomicrobium	0.49%	1.07%	0.02%	0.02%	0.01%
Phenylobacterium	0.32%	0.19%	0.81%	0.02%	0.14%
Propionicimonas				0.04%	2.04%
Pseudomonas	0.06%		0.66%	0.18%	1.35%
Pseudonocardia	1.31%	0.16%	0.01%	0.01%	0.06%
Rhodoplanes	2.78%	1.36%	0.42%	0.07%	0.06%
SA-8			0.01%	1.02%	0.04%
Simplicispira			0.03%	0.01%	0.65%
Stenotrophomonas	0.06%				4.11%
Syntrophus			0.06%	2.03%	0.01%
T78				0.02%	1.03%
Thiobacillus	0.02%	0.03%	1.15%	1.12%	0.01%
Total	14.32%	10.29%	20.97%	15.40%	45.72%

Table. 6.3S. List of common Genus shared between each group (grouping based on the R-statistical) analysis (Figure 6.5). When unknown Species were found in common, the higher phylogenetic level (Family or Order or Class or Phylum) providing meaningful information was added.

Names	total	elements
Group 1 Group 2 Group 3 Group 4 Group 5	107	g__Hyphomicrobium c__TM7-1 g__Sphingopyxis o__Actinomycetales g__Phycicoccus o__0319-7L14 f__Sinobacteraceae o__Rhodospirillales o__mle1-48 g__Rhodopseudomonas o__MIZ46 f__Burkholderiaceae c__Betaproteobacteria f__Rhodocyclaceae o__Sphingomonadales c__Alphaproteobacteria c__CH21 g__Novosphingobium g__Chondromyces g__Frateuria g__Agrobacterium f__Caulobacteraceae g__Nitrospira o__Bdellovibrionales g__Phenylobacterium f__Iamiaceae p__Cyanobacteria g__Afipia o__HN1-15 o__Rhizobiales f__Phyllobacteriaceae f__Microbacteriaceae o__Acidobacteriales f__Comamonadaceae g__Arthrobacter g__Clostridium f__Nocardioidaceae f__Cystobacteraceae g__Marmoricola f__Polyangiaceae f__Oxalobacteraceae o__Chromatiales f__Acidobacteriaceae o__Acidimicrobiales c__Chloracidobacteria o__Solirubrobacterales f__Micromonosporaceae g__Sphingomonas p__Bacteroidetes o__Myxococcales o__MC47 c__Gammaproteobacteria c__OPB56 o__DS-18 f__Bradyrhizobiaceae f__Hyphomicrobiaceae o__A31 g__Bradyrhizobium f__Flammeovirgaceae g__Bacillus g__Pseudonocardia f__Alicyclobacillaceae g__Cytophaga f__Erythrobacteraceae f__Xanthomonadaceae g__Roseomonas o__Sphingobacteriales o__Clostridiales g__Devosia c__SOGA31 g__Caulobacter o__Burkholderiales f__Acetobacteraceae g__Lutibacterium g__Lysobacter p__TM7 o__A4b unclassified g__Methyloversatilis f__Microthrixaceae f__Patulibacteraceae g__Azohydromonas f__Rhodospirillaceae g__Acidovorax g__Thiobacillus f__Rhodobacteraceae g__Pedomicrobium g__Rhodobacter f__EB1017 f__Haliangiaceae p__Proteobacteria g__Nocardioides g__Gemmatimonas f__S47 c__Anaerolineae f__Sphingomonadaceae f__Gemmatimonadaceae g__Flavobacterium o__Gemmatimonadales o__S085 o__Roseiflexales g__Rhodoplanes f__Saprospiraceae c__0319-6G9 f__Methylocystaceae f__CL500-29 f__Coxiellaceae
Group 1 Group 2 Group 3 Group 4	17	g__Nitrobacter c__Acidobacteria-5 g__CandidatusSolibacter f__Syntrophobacteraceae g__Methylotenera p__ZB2 o__mle1-12 f__Verrucomicrobiasubdivision3 g__Ramlibacter g__Opitutus o__Elusimicrobiales f__Opitutaceae f__Nitrosomonadaceae o__Verrucomicrobiales o__Stramenopiles f__Flexibacteraceae c__PAUC37f
Group 1 Group 2 Group 3 Group 5	9	f__Trebouxiophyceae f__Micrococcaceae g__Methylosinus f__Dolo_23 g__Geodermatophilus g__Methylobacterium p__SM2F11 g__Paenibacillus g__Kaistobacter
Group 1 Group 2 Group 4 Group 5	10	g__Steroidobacter c__SJA-4 g__Iamia g__Lautropia g__Dokdonella o__OPB54 o__Chloroflexales g__Bosea g__Mesorhizobium g__Salinibacterium
Group 1 Group 3 Group 4 Group 5	23	g__Fluviicola g__Anaeromyxobacter g__Rubrobacter g__Herbaspirillum g__Arenimonas g__Rhodococcus g__Aeromicrobium f__Rhizobiaceae g__Haematobacter g__Skermanella f__Cryomorphaceae g__Balneimonas f__Intrasporangiaceae g__Massilia g__Pseudomonas g__Pseudoxanthomonas f__Beijerinckiaceae g__Sphingobium f__Erysipelotrichaceae f__Porphyromonadaceae f__Sphingobacteriaceae g__Caldilinea g__Dechloromonas
Group 2 Group 3 Group 4 Group 5	10	g__Erythromicrobium o__SJA-36 f__Flavobacteriaceae f__BSV43 f__ClostridialesFamilyXIII.IncertaeSedis g__Olsenella g__Mycobacterium g__Comamonas g__Thioclava g__Sporichthya
Group 1 Group 2 Group 3	5	o__Rhodocyclales c__SM1B09 p__Acidobacteria f__AKYH910 f__FFCH4570
Group 1 Group 2 Group 4	3	p__TM6 g__Nitrosovibrio f__Gemmataceae

Group 1 Group 2 Group 5	14	f__Geodermatophilaceae g__Aquicella g__Blastococcus g__Deinococcus g__Yonghaparkia f__Kouleothrixaceae g__Oscillochloris f__Pseudonocardiaceae g__Legionella g__Adhaeribacter g__Brevundimonas g__Porphyrobacter o__Thermomicrobiales f__Legionellaceae
Group 1 Group 3 Group 4	14	g__Rhodoferax f__211ds20 o__CTD005-82B-02 f__NB1-i o__FAC88 o__Sva0725 g__Actinomadura p__SC3 g__Nitrosomonas f__Streptomycetaceae g__Nitrosospira g__Bdellovibrio o__MVP-88 p__Verrucomicrobia
Group 1 Group 3 Group 5	12	f__Ectothiorhodospiraceae f__Myxococcaceae g__Nocardia g__Rhizobium g__Streptomyces g__Lysinibacillus o__Streptophyta g__Agromyces g__Shinella g__Rubellimicrobium g__Leptolyngbya g__Azospirillum
Group 1 Group 4 Group 5	11	f__AKIW874 g__Amaricoccus f__Planococcaceae f__Enterobacteriaceae g__Microbacterium f__Chlamydomonadaceae g__Chryseobacterium g__Microlunatus g__Mycoplana f__Nakamurellaceae g__Pimelobacter
Group 2 Group 3 Group 4	4	c__vadinHA49 c__TM7-3 g__Desulfobacca c__SBRH58
Group 2 Group 3 Group 5	3	f__Bacillaceae g__Alicyclobacillus g__Lactobacillus
Group 2 Group 4 Group 5	3	g__Lewinella c__Mollicutes g__Collinsella
Group 3 Group 4 Group 5	75	o__Desulfuromonadales g__PSB-M-3 g__Aeromonas g__Anaerolinea g__Syntrophobacter p__NKB19 g__Desulfobulbus o__HMMVPog-54 g__E6 g__Trichococcus g__C1_B004 o__Bacteroidales f__Methylococcaceae o__Flavobacteriales g__Pelotomaculum f__Fusobacteriaceae g__Levilinea o__GN14 f__Clostridiaceae g__Aquamonas o__GCA004 f__Ignavibacteriaceae g__Acetivibrio p__Firmicutes f__DMPU-92 g__Acidaminobacter f__Anaerolinaceae g__Paracoccus o__NT-B4 f__Dehalococcoidaceae g__Turicibacter p__Tenericutes f__Catabacteriaceae g__Rhodocyclus f__Marinilabiaceae g__Simplicispira c__BSV19 f__SHA-31 o__Oceanospirillales o__Pseudomonadales g__Runella g__Leucobacter f__Pseudomonadaceae g__SA-8 c__OP8_2 f__Aeromonadaceae g__Treponema g__Thauera o__SHA-124 g__Coprococcus f__125ds10 g__Caloramator c__GW-22 g__Crenothrix f__Coriobacteriaceae f__Holophagaceae g__Geothrix g__Syntrophus f__Desulfobacteraceae c__SJA-28 g__Thermomonas c__Bacilli f__SHA-116 f__Veillonellaceae f__Bacteriovoracaceae g__Thermosinus g__WCHB1-05 g__Hydrogenophaga c__Dehalococcoidetes g__Syntrophomonas f__Rubrobacteraceae g__Acinetobacter f__At425_EubF1 g__Zoogloea g__Pelomonas
Group 1 Group 2	17	g__A17 o__Gemmatales g__Acrocarpospora o__SM1D11 g__Lentzea g__Sporocytophaga g__Rickettsia f__Rhodobiaceae g__Gemmata f__Actinosynnemataceae g__Spirosoma c__PW285 g__Actinoplanes o__Chlorophyta o__Pirellulales g__Rickettsiella g__Kineosporia
Group 1 Group 3	9	g__Frankia c__S15B-MN24 g__Actinocorallia f__Pseudanabaenaceae g__Rhodomicrobium g__Cohnella g__Kribbella g__Hydrocarboniphaga g__Methylocystis
Group 1 Group 4	7	g__Zymomonas f__Solirubrobacteraceae c__Gemmatimonadetes g__Polaromonas g__Tatlockia g__Roseococcus g__Chthoniobacter
Group 1 Group 5	22	f__Isosphaeraceae g__Micromonospora g__Brevibacillus g__Sphingobacterium g__Averyella g__Pedobacter g__Actinomycetospora g__Nannocystis g__Roseiflexus g__Chloroflexus g__Virgisporangium g__Myxococcus g__Stenotrophomonas g__Hymenobacter g__Amycolatopsis g__Aurantimonas g__Weissella g__Escherichia g__Chelatococcus c__Chloroflexi f__Promicromonosporaceae g__Enterococcus
Group 2 Group 3	5	g__Acidobacterium g__Planctomyces o__Chlamydiales g__Pirellula g__Leptospira
Group 2 Group 4	1	g__Allobaculum
Group 3 Group 4	80	o__LD1-PA13 o__CV106 f__MND4 g__Alkanindiges o__JH-WHS47 g__Desulfomonile c__GN15 c__OS-K g__Methylococcus o__Caldithrixales g__Methylobacillus g__LCP-6 p__HDBW-WB69 o__Synechococcales g__TM3 g__Desulfovibrio g__Herminiimonas g__Haliscomenobacter f__Gallionellaceae o__32-20 o__Methylococcales g__Ignavibacterium o__Sediment-1 p__Elusimicrobia f__Syntrophaceae f__Methylophilaceae c__MVS-40 g__Desulfuromonas o__ZB1 g__Desulfatibacillum

		c__Elusimicrobia g__Malikia f__Desulfobulbaceae c__Endomicrobia c__5bav_B12 g__Dehalococcoides g__Methylophilus f__FCPT525 p__LCP-89 g__Gallionella o__GN09 g__Geobacter c__LC-1 g__Sporotalea g__Azovibrio g__Pelobacter g__Thermobaculum c__RB384 p__Spirochaetes p__GN04 f__Rhodothermaceae g__Giesbergeria o__CFB-26 g__Cystobacter c__M1NP2-04 f__OM60 o__Chroococcales f__HTCC2089 c__BD4-9 g__Desulfococcus c__RB25 c__BB34 g__GOUTA19 c__SHA-114 o__SSS58A f__KNA6-EB22 f__Spirochaetaceae g__Cupriavidus f__Thermodesulfovibrionaceae c__PBS-25 g__Desulfobacterium g__Prochlorococcus f__PRR-10 g__4-29 p__MVP-15 c__Ignavibacteria o__wb1_H11 c__OP8_1 p__Chlorobi g__Methylomonas
Group 3 Group 5	18	g__Xenophilus g__Actinotalea g__Corynebacterium g__SHD-14 p__GAL15 g__Tolumonas o__OPB95 g__Sporanaerobacter g__Methylocaldum g__Methylocella c__Oscillatoriophycideae g__Aneurinibacillus f__Neisseriaceae g__Achromobacter g__Methylovorus g__Janibacter g__Limnohabitans g__Parvibaculum
Group 4 Group 5	59	g__Faecalibacterium f__ML635J-28 f__Synergistaceae f__Cellulomonadaceae g__Psychrobacter g__Blautia g__vadinCA02 f__Alcaligenaceae g__Fusobacterium f__Moraxellaceae g__Longilinea f__Chromatiaceae g__Sedimentibacter g__Cellvibrio g__Dehalobacterium g__Eubacterium g__Arcobacter g__SHD-231 g__PD-UASB-13 f__SHA-4 f__Propionibacteriaceae f__Beutenbergiaceae g__Fusibacter g__Chroococcidiopsis g__Pseudochrobactrum g__Lachnobacterium g__Aquimonas g__Acetobacterium g__Propionicimonas g__Wautersiella g__Parabacteroides g__Ruminococcus g__Streptococcus g__Acidaminococcus g__T78 g__Bacteroides g__Sulfuricurvum o__WCHB1-03 g__Tessaracoccus g__Thiothrix g__Pseudoclavibacter g__Anaerofustis g__Demequina p__Hyd24-12 g__Oscillospira g__W22 c__Clostridia g__Dietzia o__SHA-98 o__RF39 f__ClostridialesFamilyXI.IncertaeSedis g__Subdoligranulum g__Prevotella g__Knoellia c__PRR-11 f__Nocardiaceae g__CandidatusAzobacteroides f__Actinomycetaceae o__Lactobacillales
Group 1	33	f__Streptosporangiaceae g__Plesiocystis c__Opitutae g__Cryptosporangium g__Corallococcus o__Legionellales g__Microcoleus f__Kineosporiaceae f__Nannocystaceae g__Promicromonospora g__Labrys g__Solirubrobacter f__5B-12 g__Nostoc g__Collimonas c__Bljii12 g__Couchioplanes o__CL500-15 f__Nostocaceae g__Variovorax g__Friedmanniella g__Modestobacter g__Blastomonas f__Cyclobacteriaceae g__Methylopila g__Blastochloris g__Euzebia g__Archangium g__Verrucosispora f__wb1_P06 g__Sporosarcina g__Dyadobacter p__SC4
Group 2	10	g__CandidatusProtochlamydia o__OM190 f__Puniceicoccaceae c__Chloroplast g__Acidisphaera g__Inquilinus g__CandidatusRhabdochlamydia g__Prosthecobacter g__Singulisphaera g__Epulopiscium
Group 3	77	f__Leptospiraceae g__Pandoraea g__Symbiobacterium g__Zhihengliuella f__Paenibacillaceae g__Arcicella g__Burkholderia g__Curvibacter c__VHS-B5-50 c__MSB-5A5 g__Oscillatoria g__Arthronema g__GOUTA7 g__Pseudanabaena g__Polynucleobacter c__TK-SH13 g__Bellilinea g__Vogesella g__Rhodospirillum g__Desulfosporosinus g__Sutterella g__Aromatoleum g__Azoarcus g__Thermincola o__MSB-4E2 o__BPC110 o__J-1 p__GOUTA4 g__Caldimonas g__Desulfitobacterium g__Acidisoma g__Phormidium c__FFCH6980 p__SPAM g__Asticcacaulis f__FW g__Dyella c__RA13C7 g__Edaphobacter c__Synechococcophycideae g__Schlegelella f__ACK-M1 g__Ammoniphilus g__Desulfosporomusa c__12-24 g__Ralstonia g__CandidatusMethylomirabilis g__Terriglobus g__Magnetospirillum g__Nevskia o__Xanthomonadales g__Meiothermus p__49S1_2B f__Thermomonosporaceae o__Oscillatoriales g__Methylosarcina c__Chloroflexi-4 g__Anaerospora c__BPC102 g__Chromobacterium g__Terracoccus g__Kouleothrix g__HB118 g__BD2-6 p__GN12 c__Ktedonobacteria g__Azospira g__Planktothrix f__Phormidiaceae g__Spirochaeta o__Euglenozoa g__Telmatospirillum g__Paucimonas c__KD3-113 g__CandidatusRhodoluna f__ClostridialesFamilyXII.IncertaeSedis g__Erythrobacter
Group 4	72	g__Haliangium g__P30-6 o__Phycisphaerales c__ML615J-28 p__Planctomycetes o__WCHB1-07 o__Nitrospirales g__Desulfocapsa o__Z20 g__Sphingosinicella f__JTB38 o__KD3-145 c__KSB3 g__SJA-88 c__MSB-5B5 g__Holdemania g__Desulfobacter f__Thiotrichaceae c__WM88 g__Zplanct13 g__Fibrobacter o__GIF10 g__Desulfotomaculum g__Xanthobacter f__Nitrospiraceae g__Desulfosarcina o__BA129 c__Phycisphaerae g__Congregibacter c__iii1-8 g__u114 g__Cyanobacterium g__Desulfomicrobium c__09D2Y74 g__Bordetella g__RFN20 f__JdFBGBact c__FFCH393 c__TP21 c__Spirochaetes g__Desulfobacula o__GN03 c__OPB80 o__PBS-III-9

		o__Rickettsiales f__Helicobacteraceae c__GN13 o__Erysipelotrichales g__Kaistia g__Emticicia g__Sulfurimonas g__Brachyspira o__YS2 g__Deefgea f__Hyphomonadaceae f__Verrucomicrobiaceae g__Rhodanobacter g__Halochromatium o__Haptophyceae o__Campylobacterales g__Propionigenium c__R76-B18 c__Rs-J96 g__Alistipes f__kpj58rc g__Luteolibacter p__CCM11b g__Arsenicicoccus g__Sorangium o__Rhodobacterales f__Chlorobiaceae o__PBS-18
Group 5	134	g__Isoptericola g__Catenibacterium g__Veillonella g__Subtercola g__Tindallia g__Moraxella o__I025 g__Citrobacter g__Vitreoscilla g__Sejongia g__Allochromatium g__Diaphorobacter o__SHA-1 g__Sulfurospirillum g__Planomicrobium g__Rheinheimera g__Ensifer g__CandidatusCloacamonas g__Slackia g__Luteimonas g__Anaerofilum g__Roseivivax g__Brachybacterium g__Alkaliphilus g__Alkalibacterium f__Thermotogaceae g__Azotobacter g__Kaistella g__Leclercia g__Enhydrobacter g__Agrococcus g__Pontibacter g__Janthinobacterium g__Tetrathiobacter g__Peptococcus g__Dialister o__Synergistales g__Kocuria f__Carnobacteriaceae g__Bifidobacterium f__Lactobacillaceae g__Kurthia g__Roseburia g__Acetobacter f__Halomonadaceae g__Anaerotruncus g__Klebsiella g__Delftia o__LF045 g__Herpetosiphon g__Halomonas g__Leuconostoc g__Succinivibrio g__TG5 g__Lactococcus g__Gordonia g__Pediococcus g__Ochrobactrum g__Hylemonella g__Facklamia f__SHBZ1169 g__Pannonibacter o__Nostocales g__Pseudaminobacter g__Sarcina g__Marinobacter g__Dysgonomonas g__Oligella g__Dermacoccus g__Catellibacterium o__BA021 f__Brachyspiraceae g__Oerskovia g__Bergeyella g__Haloanella g__Azonexus g__Rhodobaca g__Exiguobacterium g__Nonomuraea f__Dethiosulfovibrionaceae g__HA73 g__Saccharopolyspora g__Ornithinicoccus g__Solibacillus g__Denitrobacter g__Brevibacterium g__Nitratireductor g__Shigella g__Propionibacterium g__Azorhizophilus g__Erysipelothrix g__Enterobacter g__Jonesia g__Kosmotoga g__Chromohalobacter g__Actinomyces g__Aestuariimicrobium g__Muricauda g__Soehngenia f__Desulfitobacteraceae g__Alicycliphilus g__Patulibacter g__Adlercreutzia g__Salinicoccus g__Cellulomonas f__Brucellaceae g__Micrococcus g__Tissierella g__Shuttleworthia g__Georgenia g__Rummeliibacillus g__Cryocola g__Laribacter g__Idiomarina g__Cellulosimicrobium g__Elizabethkingia g__Dermatophilus g__Luteococcus g__Vagococcus o__DRC31 f__Enterococcaceae g__Eggerthella g__Aminobacter g__Butyrivibrio p__WPS-2 g__Aminiphilus g__Ethanoligenens g__Brachymonas g__Aerococcus o__Alteromonadales g__Salegentibacter g__Serinicoccus g__Trabulsiella f__Hydrogenophilaceae

CHAPTER 7

Conclusion and perspectives

7.1. Conclusion

The main objective of this study was to assess the emerging contaminants including metals, bacteria and antibiotic resistance genes in sediment receiving wastewaters. Additionally, the prevalence of antibiotic resistant *Pseudomonas* spp. in the sediments receiving treated/untreated wastewaters was evaluated. In particular, the effects of metal contamination on the composition and the diversity of bacterial communities in the sediments were also performed. The major conclusions of this research are summarized below;

7.1.1. Accumulation of emerging contaminants in the sediments of Cauvery River Basin

The results of this task demonstrate the elevated levels of emerging contaminants (metals, ARGs and bacterial load) in the sediments collected from the hospital outlet pipe (HOP) and Cauvery River Basin (CRB). In all sampling sites the metal concentrations largely exceeded the Sediment Quality Guidelines (SQGs) and the Probable Effect Concentration (PEC) for the Protection of Aquatic Life recommendation. A mortality rate ranging from 22 to 100 % (HOP) and 18–87% (CRB) were observed with ecotoxicological tests in the sediment samples. We identified a poor microbial water quality in the sediment samples collected from downstream of the effluent discharge point in CRB. The abundance of bacterial species was 120, 104 and 89 fold higher for the *E. coli*, ENT and *Pseudomonas* spp., respectively in HOP than CRB. The extended-spectrum ß-lactamases - ESBLs (bla_{SHV}) and metallo ß-lactamases – MBLs (bla_{NDM}) genes were identified in the samples collected from downstream of the effluent discharge point in CRB whereas those genes were not detected in the upstream of CRB (control site). This observation suggests that the untreated urban and hospital effluents released into the municipal sewage without prior treatment carry ARGs and could act as a potential source of microbial pollutants in the receiving system. The levels of these emerging pollutants varied depending on the sampling periods. Hence, during the event of rain and floods, it is highly likely that these emerging contaminants released/accumulated in the sediments of the municipal sewage could be transported to the receiving system in large amounts. A continual monitoring on the accumulation of these emerging contaminants is highly needed.

7.1.2. Effect of WWTP on the accumulation of emerging contaminants in the sediments of Vidy Bay, Lake Geneva

We quantified metals, bacteria and antibiotic resistant genes in the sediments from Vidy Bay receiving WWTP effluent waters. The results suggest that the WWTP is a primary input source of emerging contaminants into the bay. Our results were concurrent with previous studies with reduction of Hg deposition at the surface layers. However the deeper older sediments remain contaminated with higher Hg concentrations. In a comparison between the control site and the WWTP influenced site at the surface layer, there is a 720, 700, and 1080-fold increase in the bacterial load for the studied *E. coli*, ENT, and *Pseudomonas* spp., respectively. The identification of *aadA* and bla_{TEM} genes in the sediment deposited before the start of the twentieth century supports the fact that ARGs have been an emerging contaminant in the aquatic ecosystem for more than a century. Elevated levels of ESBLs and MBLs identified in the surface layers indicate the recent increase of clinically relevant ARGs at the bay. Strong positive correlations existed among total OM, metal concentrations, and bacterial-load/bacterial-species markers/ARGs ($r \geq 0.403$ were observed, $p < 0.05$) indicating a common and contemporary source of contamination. Pollution reduction at the source will be necessary for further improvement of water quality.

7.1.3. Prevalence of antibiotic resistant *Pseudomonas* spp. in the sediments receiving treated/untreated effluents

The sediments receiving urban/hospital wastewaters could act as a potential reservoir of metals, ARGs and antibiotic resistant bacteria. Here, we studied the prevalence of antibiotic/metal resistance in environmental *Pseudomonas* spp., isolated from sediments of three distinct geographical locations. The phenotypic and genotypic resistance profiles of isolated *Pseudomonas* spp. were evaluated. *Pseudomonas* spp. isolated from Switzerland (CH) were sensitive to antipseudomonal antibiotics including TZP, CAZ, FEP, IMP, MER and NOR when compared to Democratic Republic of Congo (DRC) and India (IN). In this study, 9 of 141 isolates (7 from IN and 2 from CH) were phenotypically resistant to 16 Abs tested. Additionally MAR index values were higher in IN (0.79), followed by DRC (0.48) and CH (0.40) study sites. Among three countries, IN isolates carried sulfonamide (21%) resistance genes. Isolates carrying Florfenicol resistant gene (*floR)* was abundant in CH (65%), followed by IN (28%) and DRC (27%) isolates. The CTX-M gene was the most dominant in the CH isolates, and MBL (VIM) was dominant in the DRC and IN isolates. The

bla$_{NDM}$ gene was identified in 8 and 34% of the isolates in CH and IN, respectively but not found in DRC isolates. Mobile genetic elements were also identified in all the three study sites. The integrase gene, *intl-1* was identified in IN (37%) and CH (3%) isolates; whereas *intl-2* was found only in DRC (12%) and IN (4%) isolates. Higher conjugation frequencies were observed at tropical temperature (30°C) than at the lower temperature (10°C). The chromosomal mediated efflux mechanisms in *P. aeruginosa* were identified to play as adjuvant mechanism of bacterial resistance which benefits the bacteria to withstand the environmental selective pressure and acquire more resistant genes. The co-selection of antibiotic and metal resistance are well highlighted by *oprD* expression in *P. aeruginosa* which exhibit co-resistance to metal (Zn) and Carbapenem (IMP) antibiotic.

Wastewaters a potential source for multidrug and integron positive bacteria can pollute the receiving environment significantly with higher concentrations of resistant bacteria than occur naturally. The main concern resides in the fact that their mobile nature and capacity to accumulate resistance genes could lead to a wide dissemination of antibiotic resistance in the environment.

7.1.4. Impact of metal pollution on bacterial community

The pyrosequencing study provides a detailed investigational comparison of bacterial communities in four different geographical locations (Congo DR, India, Senegal and Switzerland) contaminated with metals from various sources. Several large shifts were identified in the bacterial community structure at the Class level enriched amount of α, ß, γ-proteobacteria were observed in the soil/sediments receiving contaminants from recreational shooting, mining and hospital effluents, respectively. Statistical analysis of the data revealed that *Clostridiales* and *Actinomycetales* had significant negative correlations with total organic matter, Cr, Cu, and Pb. This negative correlation indicates the metal bio-removal by sulfide generation when sulfate reducing bacteria were inhibited by a mixture of Cu, Zn and Fe. Based on the concentrations of the metals the *Chloroflexi* phyla contributed to 41% of the total population in Pb contaminated site and *Acinetobacter* (16%) was the most dominant group in the sediments receiving hospital effluents. Hence, metals accumulated in the terrestrial environment from various sources as a result of anthropogenic activities have the potential impact on bacterial communities in the effluent receiving ecosystems.

This study sheds more light on awareness of emerging contaminants discharged into the aquatic environment, which can be helpful to establish the baseline information for further studies in the area.

7.2. Perspectives

- Metals, especially in organometallic form contribute for toxicity in the aquatic environments due to its ability to biomagnify in food chains. In the present study, we report new data from pristine geographical locations on the concentration of metals. Further studies to understand the role of metal toxicity in aquatic living organisms as well as exposure of the local population to metals are warranted.
- Input of emerging contaminants to the aquatic ecosystem may support the emergence of new resistance mechanisms in bacterial community; additionally the selection criteria involved in the acquisition of resistance genes remain unclear in the environment. Hence, further studies to identify the selective pressure that favors microbial resistance in the environment are to be addressed.
- Antibiotic resistant pathogens are profoundly important to public health, but demonstrating the casual link between the antibiotic use and resistance is challenging with the environmental reservoirs being poorly understood. Hence understanding the prevalence and polymorphism of antibiotic resistance genes in the environment and their potential to transfer horizontally are required. These data will help us to evaluate the likelihood of transfer of these resistance genes from environment to clinical settings.
- With the available data on the metal concentrations the co-selection of metal and antibiotic resistance requires further attention. Studies to investigate the combination of metals and antibiotics which likely co-select during gene transfer mechanisms are required. Studies investigating co-selection of antibiotic and metals in the environment with specific interests to identify the metal concentrations that potentially induce a selection pressure are warranted.
- The wastewaters from various sources released to the environment do have an impact on the bacterial community, but the ecological functions of these diverse bacterial

populations remain unknown. Detailed insight on the specific functions and ecology of microorganisms inhabiting freshwater ecosystem are to be explored.

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