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Bacterial communities and antibiotic resistance in human-impacted water environments

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Front page picture: schematic figure representing the complex mixture of antibiotic residues, metals, pathogenic, non-pathogenic and antibiotic resistant bacteria as well as antibiotic resistance determinants in human-impacted water environments. Picture designed by Laura Castillo (castillo.kodama@gmail.com).

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Bacterial communities and antibiotic resistance in human-impacted water environments

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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♥To my mother and my husband with love♥

ABSTRACT

From a One-Health concept the health of humans, animals and the environment is interconnected and what happens to one of the domains will affect the other two simultaneously. This concept can be applied not only to the transmission of bacterial infectious diseases but also to the transmission of antibiotic resistance. While bacterial infectious diseases caused by water-borne pathogens are still a leading cause of morbidity and mortality worldwide, the development and spread of antibiotic resistant bacteria is threatening human health like never before. If we are not able to control the generation and spread of antibiotic resistant bacteria, in the near future thousands of people will die of infections that were treatable before. The relationship between human and animal health has been widely studied before but the environment is usually set aside minimizing the impact and effect of contaminated environments on human and animal health. Contaminated water environments represent a suitable place for the accumulation, spread and transmission of pathogenic and antibiotic resistant bacteria. In that sense, water environments are good interfaces where the transfer of antibiotic resistance determinants between bacteria might occur. Hence water environments might represent a good place for the surveillance of pathogenic bacteria and antibiotic resistance. In this thesis we aimed to characterize pathogenic and non-pathogenic bacterial communities as well as antibiotic resistant bacteria and antibiotic resistance genes in highly contaminated rivers in La Paz and Oruro in Bolivia as well as in a waster water pump at a suburban community in Oslo. We also aimed to evaluate the potential of bacterial communities from contaminated water environments to transfer antibiotic resistance determinants to *E. coli* and test the effect of heavy metals in the occurrence and transfer of antibiotic resistance. We found high prevalence of enterobacteria, pathogenic *E. coli* and other diarrheal bacteria in water, agricultural soil and vegetables from an urban-impacted basin in La Paz, Bolivia (Paper I). Moreover, we repeatedly found the globally distributed and multi-drug resistant *E. coli* sequence types ST131 in Norway and ST648 both in Bolivia and Norway showing the important role of the environment for the dispersion of pathogenic and antibiotic resistant bacteria (Paper I and II). Additionally, we proved a high capability of bacterial communities from contaminated water environments to transfer antibiotic resistance determinants to *E. coli.* We showed that presence of metals such as $ZnSO_4$ and $CuSO_4$ in conjugation experiments might favor the transfer/acquisition of more diverse phenotypic multi-drug resistance profiles and mobile genetic elements carrying higher diversity of genes including extended spectrum beta-lactamases and other relevant genes conferring important advantages to the bacterial host (Paper III). Bacterial donors from contaminated irrigation water transferred a high diversity of antibiotic resistance determinants at considerable levels showing the potential risk of transmission of antibiotic resistance to human populations by contaminated irrigation water and vegetables (Paper III). We did not find significant associations between metal composition, bacterial communities and the abundance of selected antibiotic resistance determinants in acid mine drainage contaminated watersheds in Oruro, Bolivia (Paper IV).

Keywords: Water environments, diarrheal bacteria, *Escherichia coli*, antibiotic resistance, heavy metals, acid mine drainage.

LIST OF SCIENTIFIC PAPERS

I. **Guzman-Otazo J**, Gonzales-Siles L, Poma V, Bengtsson-Palme J, Thorell K, Flach CF, Iñiguez V, Sjöling Å. Diarrheal bacterial pathogens and multi-resistant enterobacteria in the Choqueyapu River in La Paz, Bolivia. *PLoS One* 14, e0210735, doi:10.1371/journal.pone.0210735 (2019).

II. Paulshus E, Thorell K, **Guzman-Otazo J**, Joffre E, Colque P, Kuhn I, Möllby R, Sorum H, Sjöling Å. Repeated Isolation of Extended-Spectrum-beta-Lactamase-Positive Escherichia coli Sequence Types 648 and 131 from Community Wastewater Indicates that Sewage Systems Are Important Sources of Emerging Clones of Antibiotic-Resistant Bacteria. *Antimicrobial Agents and Chemotherapy* 63, doi:10.1128/aac.00823-19 (2019).

III. **Guzman-Otazo J,** Agramont J, Mamani N, Jutkina J, Boulund F, Hu Y O. O., Larsson D. G. J, Flach CF, Iñiguez V, Sjöling Å. Conjugative transfer of multi-drug resistance genetic elements from environmental water-borne bacteria to *Escherichia coli.* Submitted manuscript.

IV. **Guzman-Otazo J,** Hugerth LW, Agramont J, Östman M, Tysklind M, Joffré E, Calderon C, Gutierrez S, Flach CF, Larsson D.G.J, Iñiguez V, Sjöling Å. Metal concentrations do not correlate with microbial communities and antibiotic resistance determinants in rivers impacted by acid mine drainage in Oruro, Bolivia. Manuscript.

Paper not included in the thesis

Chuquimia O. D, Bergion V, **Guzman-Otazo J**, Sörén K, Rosén L, Pettersson T. J. R, Sokolova E, Sjöling, Å. Combining molecular analyses of fecal indicator bacteria and diarrheal pathogens with hydrodynamic modeling for microbial risk assessment of a drinking water source. Submitted manuscript.

CONTENTS

LIST OF ABBREVIATIONS

AMD	Acid Mine Drainage
AMP	Ampicillin
ARB	Antibiotic Resistant Bacteria
ARGs	Antibiotic Resistance Genes
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohaemorragic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ESBLs	Extended Spectrum β-Lactamases
ETEC	Enterotoxigenic *Escherichia coli*
ExPEC	Extra-intestinal Pathogenic *Escherichia coli*
Gfp	Green Fluorescent Protein
HGT	Horizontal Gene Transfer
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
IncN	Incompatibility Group N
InPEC	Intestinal Pathogenic *Escherichia coli*
KAN	Kanamycin
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LPS	Lipopolysacharides
MDRP	Multi-Drug Resistance Profile
MGE	Moblie Genetic Element
MIC	Minimal Inhibitory Concentration
MLST	Mutli Locus Sequence Typing
NA	Nalidixic Acid
ORG	Other Relevant Genes
OM	Outer Membrane
PBPs	Penicillin Binding Proteins
PCR	Polymerase Chain Reaction
RIF	Rifampicin
ROS	Reactive Oxygen Species
SMX/TMP	Sulfamethoxazol/Trimethoprim
ST	Sequence Type
TET	Tetracycline

UPEC	Uropathogenic *Escherichia coli*
WWTP	Waste Water Treatment Plant

1 INTRODUCTION

1.1 ONE-HEALTH: HUMAN-ANIMAL-ENVIRONMENT

The One-Health concept sustains the idea of an interconnection between the health of humans, animals and the environment where each interface affects the other two in a cyclic way. This interconnection has been highly associated with the dispersion and transmission of infectious diseases and antibiotic resistance in human populations[1,2]. For this reason, it is crucial to approach these public health issues studying the three different interfaces and their links. While the relationship between human and animal health has been commonly studied especially for infectious diseases[1,3,4], we know less about the impact of contaminated environments and ecosystems on human and animal health[2], in particular if we talk about antibiotic resistance[5,6].

In the environment, water, soil and air compartments can be contaminated with infectious and antibiotic resistance bacteria coming from human and animal discharges. Thus, contaminated drinking or irrigation water, vegetables and agricultural soils as well as aerosols can lead to transmission of infectious diseases and antibiotic resistant bacteria (ARB) to humans and animals[7,8]. Figure 1 depicts the complex transmission network of antibiotic resistance between the three main domains inside the One-Health concept. Moreover, in low- and middle-income countries the lack of hygiene and sanitization practices as well as lack of safe water and the unregulated agricultural practices exacerbate the problem[9,10]. This is a situation that is predicted to worsen due to climate change effects in the upcoming years[2]. For this reason, many countries have started to tackle antibiotic resistance with a One-Health overview, recruiting specialists from the clinical, veterinary and environmental areas to perform surveillance and prevention studies[11,12].

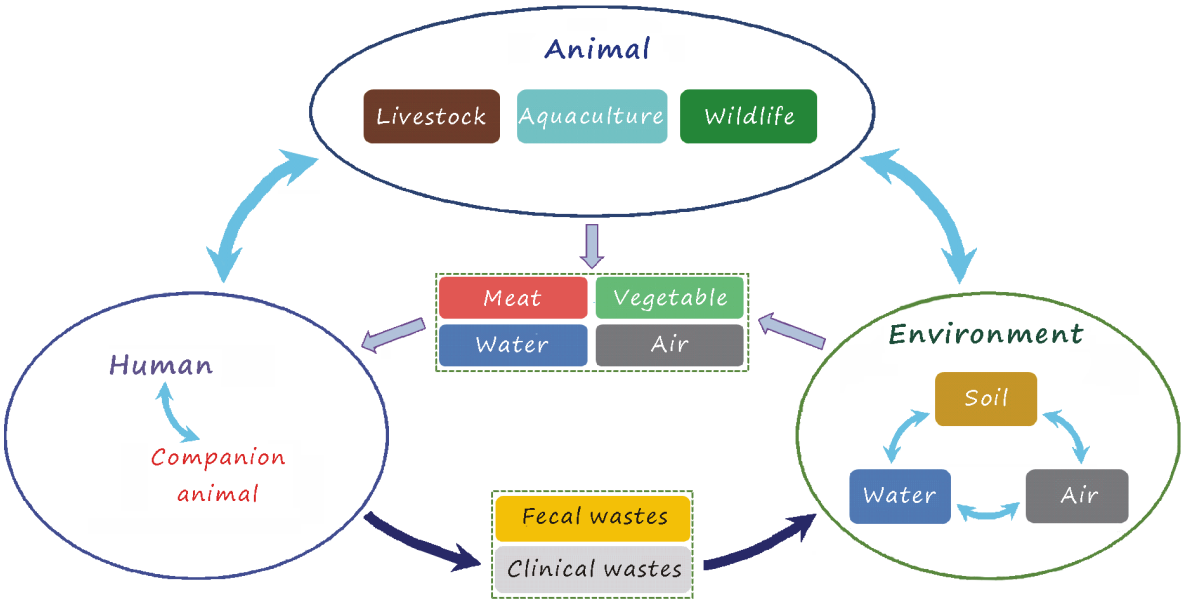

Figure 1. Antibiotic resistance transmission routes between humans, animals and the environment. Rights to reprint by Elsevier[7].

1.2 WATER AND HUMAN HEALTH

Water is a priceless natural resource, essential for the existence of all living organisms. In the case of humans, the access to safe water sources can make a significant difference between health and disease. Our daily activities including hygiene and sanitation practices, the preparation of food or simply drinking a cup of coffee depend on the access to clean water. Unfortunately, in many parts of the world the access to safe water is limited and many people struggle walking every day during several hours only to get and bring back home the amount of water they are able to carry[13].

This is a common situation in many low- and middle-income countries where the access to safe water is limited and rivers, lakes, wells and other water sources are highly contaminated with fecal discharges from animal and human origin and where also the correct treatment and disposal of waste is scarce[14]. Fecal contamination can include commensal as well as pathogenic microorganisms including parasites, protozoa, bacteria and viruses which are able to survive in the environment and be transferred to other humans or animals by the oral-fecal pathway[13,14]. This phenomenon has special relevance when the microorganism is able to cause disease in the new host. In the case of bacteria, they can be pathogenic and cause disease as well as carry antibiotic resistance genes (ARGs) causing antibiotic resistant infections. ARB can accumulate in the gut microbiome and they might be able to transfer antibiotic resistance determinants to other bacteria acting as vehicles to enrich the gut microbiota resistome[15,16]. At the same time the diverse members of the gut microbiota might be able to transfer ARGs to pathogenic or opportunistic bacteria inside our body[17,18]. Hence, the relevance of human and animal gut microbiota as reservoirs of ARB and ARGs is doubtless.

Water-associated infectious diseases occur worldwide causing hospitalization of patients and thousands of deaths annually. Approximately, 4% of total deaths around the world are caused by water, hygiene and sanitation associated infectious diseases[19-21]. According to the role of water in the transmission of the infectious disease they can be classified in five groups: water-borne, water-based, water-related, water-washed and water-dispersed[22]. The number and distribution of water-associated infectious diseases outbreaks around the world has been highly associated with social and environmental conditions of the region where all areas in the globe are differentially impacted by the different water-associated categories[19] (Figure 2).

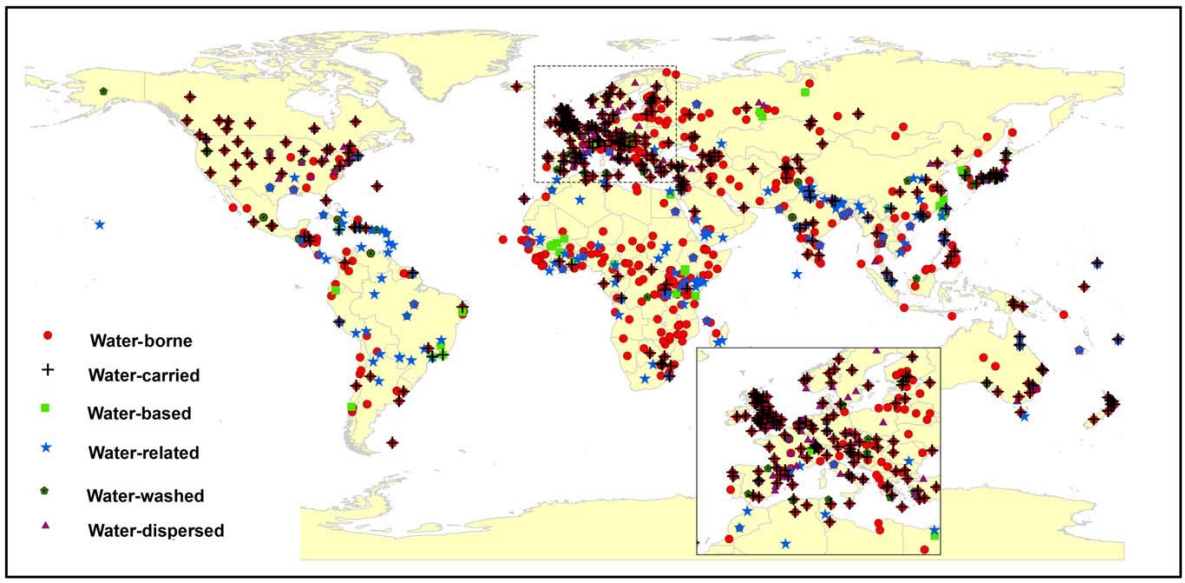

Figure 2. Map with the geographic distribution of water-associated infectious diseases outbreaks reported around the world between 1991 and 2008. Rights to reprint by PLOS Neglected Tropical Diseases[19].

1.2.1 Water-borne bacterial diseases

In the case of water-borne diseases, the pathogen uses water as a passive vehicle to spread and infect the host. Common water-borne infectious diseases include: Typhoid fever, Bacillary dysentery, Cholera, Hepatitis, Leptospirosis and Gastroenteritis among others[14]. Gastrointestinal infections are commonly caused by pathogenic enterobacteria such as pathogenic *Escherichia coli, Vibrio cholerae, Shigella spp.* and *Salmonella enterica* among others. The way of transmission is generally through contaminated drinking water and food causing dehydration, diarrhea and even death as the final outcome[23]. Diarrheal diseases are one of the leading causes of morbidity and mortality worldwide. Only in 2010, 1.9 billion cases of diarrheal infections were reported around the globe. Pathogenic bacteria were responsible for more than 700 millions of cases and pathogenic *E. coli* itself caused approximately 320 millions of infections in different age groups where children under five years of age were highly affected[24]. In the year 2016 more than 1 million of deaths were caused by diarrheal diseases around the world[25].

While *E. coli* can be a harmless and a common commensal in the gut of humans and animals, other types of *E. coli* strains that acquired colonization factors and virulence determinants can infect humans producing intestinal and extra-intestinal infectious diseases. Intestinal pathogenic *E. coli* (InPEC) is the group of strains able to cause watery, bloody and non-bloody diarrhea in humans and is divided in five pathotypes according to their virulence properties including: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC) and enteroinvasive *E. coli* (EIEC)[26]. ETEC and EPEC are the most commonly reported in diarrheal patients world-wide[24]. Extra intestinal pathogenic *E. coli* (ExPEC) group includes the strains producing urinary tract infections, septicemia and meningitis[27]. The ExPEC sequence type ST131 has been identified as a pandemic lineage causing diverse extra-intestinal infections typically multi-resistant to antibiotics[28-30].

During the last decades, diverse strains of *E. coli* and other enterobacteria carrying multiple antibiotic resistance determinants have emerged causing serious infection cases in the clinical and community settings[31]. Enterobacteria producing extended-spectrum β-lactamases (ESBLs), such as the world-wide distributed CTX-M group of enzymes are of particular concern and also considered pandemic being isolated from human, animal and environmental samples[32-34].

Once bacteria face an oligotrophic water environment, they can remain viable and persist for extended periods of time. In water compartments, bacteria are able to survive and even grow[35-37]. Such bacteria might remain infectious and could potentially transfer genetic material to other bacteria. Many studies have probed the presence of diarrheal pathogens such as ETEC in drinking water tanks in countries with high incidence of diarrheal diseases like Bangladesh[35,38]. ETEC has also been detected tightly attached to vegetables by means of flagella reflecting the risk of transmission through contaminated drinking and irrigation water[39].

1.2.2 Environmental surveillance of pathogenic and antibiotic resistant bacteria

The value of clinical epidemiological surveillance of infectious diseases in human populations is very well known. However, taking into account the One-Health concept and the environment as an important and interconnected domain for the health of humans and animals the environmental surveillance of pathogenic and antibiotic resistant bacteria might be a useful tool to evaluate and make inferences about the prevalence of infectious diseases, pathogenic bacteria and ARB in human populations.

Many studies have found potential associations between bacterial pathogens found in municipal wastewater[40], drinking water[38] and surface waterbodies[41] and the prevalence of disease in the associated human populations. An association between antibiotic resistant coliforms in children and their environment (animals and water) has also been reported[42]. However, the environmental surveillance of ARB and antibiotic resistance has many knowledge gaps and limiting factors that need to be addressed in order to be able to make appropriate inferences to improve the health of humans, animals and ecosystems[43]. Among the most important, we can mention the need to determine appropriate markers and sampling areas for the evaluation of the transmission risk of ARB from the environment to humans, the risk for antibiotic resistance evolution, and the prevalence of antibiotic resistance in human populations[43]. Antibiotic resistance is underestimated in many places. For instance, in low and middle-income countries the lack of equipment and resources for diagnosis together with the lack of surveillance systems mask the real status of resistance. This situation makes it difficult to draw a picture about the epidemiology of ARGs, including resistance to last generation of antibiotics[44]. Interestingly, if correctly addressed the environmental surveillance of antibiotic resistance might offer special advantages for low-and middle-income settings contributing to the evaluation of resistance epidemiology with low resources[43].

1.3 ANTIBIOTIC RESISTANCE

1.3.1 Origin and evolution

Antibiotics are a group of molecules able to kill bacteria or stop their growth. They have been widely used since their discovery to treat many bacterial infectious diseases in humans and animals saving millions of lifes and enabling us to prevent and control infectious outbreaks, epidemics and pandemics that otherwise would have depleted human populations[45]. However, the use of antibiotics and the generation of resistance go hand in hand and the generation of resistance to every new discovered or developed antibiotic is inevitable. Antibiotic resistance occurs when bacteria use metabolic or other defenses to survive in presence of antibiotics and continue growing. The release of antibiotic residues to the environment, the over use and the non-therapeutic use of antibiotics have accelerated the generation and evolution of antibiotic resistance causing a worldwide public health problem[46].

Antibiotic resistance and ARGs are ancient[47,48] and ubiquitously present in the environment, especially in soils where diverse bacteria and fungi produce antimicrobials to protect themselves from other microorganisms causing also a selection pressure for the transfer and acquisition of ARGs in these microenvironments[45,49]. Thereby, antibiotic-producing microorganisms also need a way to survive to their own attack so they commonly carry ARGs that allow them to survive in presence of these toxicants[49,50]. Since resistance genes are an intrinsic and natural system in many bacteria, it is very common to find resistant bacteria even in places characterized for having low amount of antibiotic usage/residues[51]. Once ARGs are inside the ecosystems they are easily kept with the potential of being transferred between non-pathogenic and pathogenic bacteria of the same or closely related species by means of horizontal gene transfer (HGT)[51,52], reflecting the implications of this phenomena for infectious diseases in humans.

Antibiotic resistance can be acquired in bacteria by mutations in target proteins and regulatory genes, which can be transferred in a vertical way to daughter cells. However, antibiotic resistance can also be acquired by horizontal transfer of antibiotic resistance determinants between similar and different species of bacteria by means of mobile elements such as plasmids, integrons and transposons[45]. HGT has been widely recognized as one of the most important and crucial mechanisms for the mobilization and spread of ARGs[53]. The mobilization of genetic elements carrying ARGs includes processes such as: a) conjugation by bacterial plasmids and conjugative transposons, b) transformation by acquisition of free DNA from the environment and c) transduction by bacteriophages[53] (Figure 3). The occurrence of these HGT mechanisms depends on different constraints and rate factors where the conjugation and transfer of bacterial plasmids is dominant[54] (Table 1). Furthermore, multidrug resistance in bacteria has evolved by co-resistance mechanisms (co-selection of multiple resistance genes within a mobile genetic element (MGE) and/or by cross-resistance mechanisms (presence of resistance genes with a broad substrate range)[45,55].

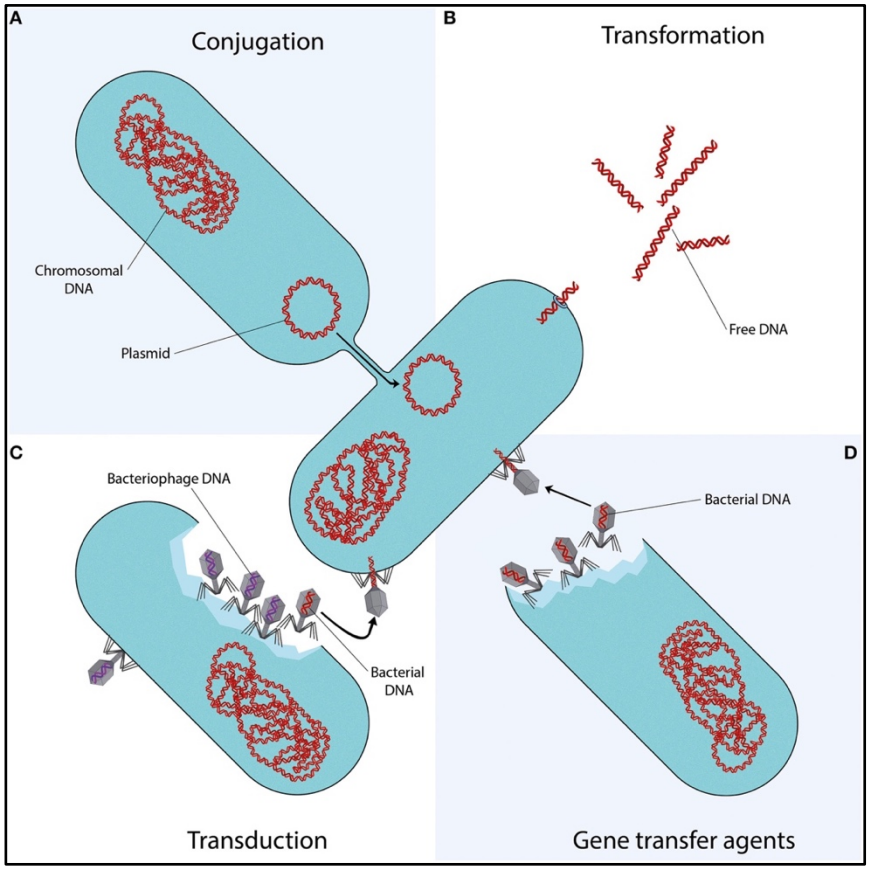

Figure 3. Horizontal gene transfer mechanisms for the acquisition of antibiotic resistance in bacteria. Rights to reprint by Frontiers in Microbiology[53].

Table 1. Constraints and rate factors for different horizontal transfer mechanisms of antibiotic resistance. Table extracted from Nazarian *et al.* 2018 with permission from Frontiers in Microbiology[54].

Mechanism	Vector	Constraints	Rate Factors
Conjugation	Direct cell-to-cell contact	Only possible for conjugative and mobilizable plasmids	Pairwise compatibility of donor and recipient
Transformation	Free extracellular DNA	Approximately 1% of species are naturally transformable (i.e. able to become competent and act as recipients)	Competence rate of recipient
Transduction	Transducing phages	Donor and recipient must both be in the host range of the transducing phage	Size of phage population common to donor and recipient
Vesicle-mediated transfer	Extracellular vesicles	Vesicles production and uptake low in some species	Efficiency of donor and efficiency of recipient

The acquisition of antibiotic resistance often comes with a price that is called fitness cost. In many cases, resistant bacteria present lower virulence and lower capability to growth compared to the susceptible equivalents when the antibiotic is not present[56]. The cost is highly dependent on the resistance mechanism involved where commonly target site and chromosomal mutations tend to have a higher fitness cost for bacteria[45]. Nevertheless, bacteria have the ability to evolve with compensatory mutations in order to reduce the fitness cost and keep the acquired antibiotic resistance determinant[56].

1.3.2 Antibiotics: mechanisms of action

Antibiotics generally target specific bacterial characteristics such as the cell wall, the cell membrane as well as processes such as nucleic acid and protein synthesis (Figure 4).

Antibiotics targeting the synthesis of the cell wall include the large beta-lactam group including antibiotics used for decades such as penicillin, and ampicillin and more recent derivates such as cephalosporins, carbapenems and monobactams. β-lactams in general bind to penicillin binding proteins (PBPs) in the cell wall and inhibit the synthesis of new peptidoglycan leading to bacterial lysis. Glycopeptides such as vancomycin also inhibit cell wall synthesis by interacting with peptidoglycan precursors which obstructs their binding to PBPs[57,58].

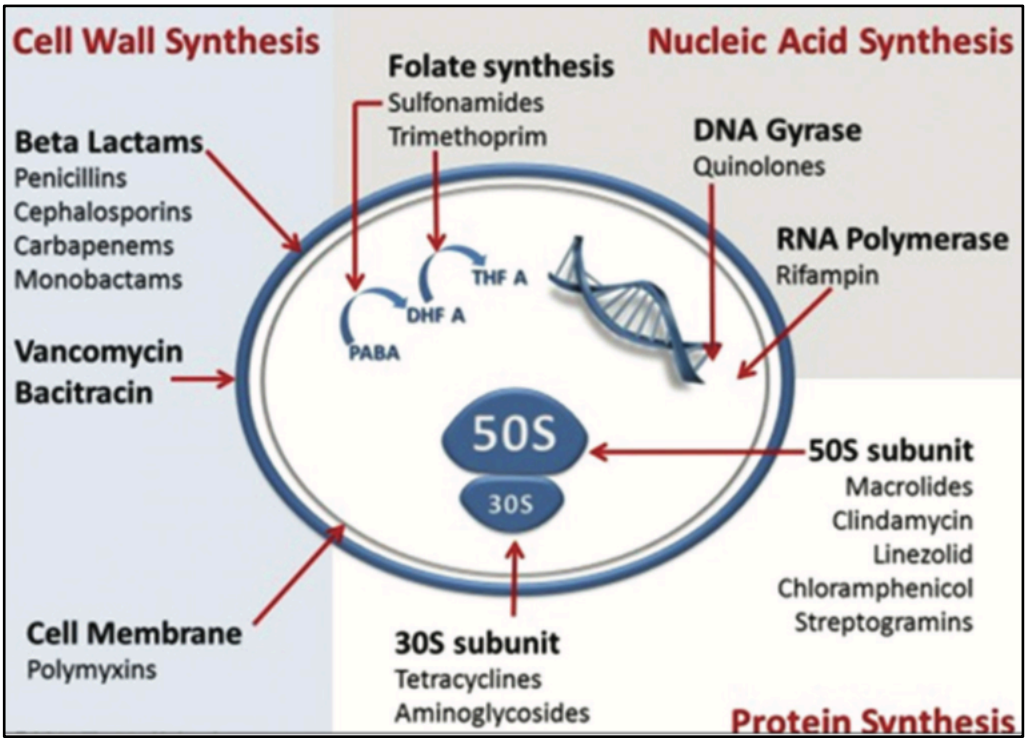

Figure 4. Mechanisms of action for the different antibiotic groups. Rights to reprint by Journal of Anaesthesiology Clinical Pharmacology[58].

Antibiotics including tetracyclines, aminoglycosides, macrolides and chloramphenicol among others interact with the bacterial machinery for protein synthesis, the ribosome. They target the big (50S) or small (30S) subunits at the ribosome causing inhibition of protein synthesis by interacting with t-RNA, stopping translation, and causing the release of unfinished peptide chains in the bacterial cells[58].

Quinolones are able to inhibit DNA synthesis by binding to the subunit A of DNA gyrase and also to topoisomerase IV in bacteria and thereby disturbing and stoping the DNA replication process. DNA replication in bacteria needs these two key enzymes to cut, and release supercoiling of DNA during replication and re-join the DNA chains to continue with the process[58,59]. Finally, sulfonamides and trimethoprim act by inhibiting the enzymes dihydropteroate synthase and dihydrofolate reductase respectively, which inhibits folic acid metabolism in bacteria[58].

1.3.3 Antibiotics: mechanisms of resistance

The main bacterial mechanisms for antibiotic resistance can be grouped in three main categories including changes in membrane permeability and efflux pumps (Figure 5a and b), modification of the target (Figure 5c) and inactivation of the antibiotic (Figure 5d).

In Gram-negative bacteria antibiotics need to penetrate the outer membrane (OM) composed of phospholipids and lipopolysaccharides (LPS). Antibiotics can go trough the OM using porins, this is the case of many β-lactams, fluoroquinolones and chloramphenicol. The aminoglycoside group of antibiotics use the mechanism of self-promoted uptake to penetrate through the OM. Changes in porin number or size can promote antibiotic resistance by decreasing the penetration of antibiotics and thereby limit their ability to find their targets[57]. Efflux pumps can be present in Gram-negative and Gram-positive bacteria. These are membrane proteins able to pump out of the bacterial cell different kind of molecules such as antibiotics, metals and other drugs before they reach toxic concentrations in the host. Efflux pumps can vary in specificity and while some have very specific substrates to pump out of the cell some other have a broad range of action and are able to transport a big variety of molecules. Such efflux pumps are denominated multi-drug efflux pumps. Changes in regulatory genes and overexpression of efflux pumps proteins can promote antibiotic resistance in bacteria especially for antibiotics such as fluoroquinolones, macrolides and tetracycline which need to get into the cell to be able to exert their action[57,58].

Modification of the target is a very effective resistance mechanism in bacteria that occur by mutations that cause significant changes in the binding sites and prevent the affinity between the antibiotic and the target. Sometimes bacteria can express these mutations without altering the normal functioning of the cell but is also common the need of extra compensatory mutations to maintain all bacterial systems running and express antibiotic resistance at the same time[57]. This mechanism promote resistance to β-lactams by alterations in the active domain of PBPs, to antibiotics interfering with protein synthesis (tetracyclines, aminoglycosides, macrolides, etc), by mutations in the subunits (30S and 50S) of the ribosome to prevent binding and finally to quinolones by mutations in the enzymes involved in DNA replication, DNA gyrase and topoisomerase IV, hence preventing their inhibition and protecting the acid nucleic metabolism in bacteria[57,58].

Antibiotic inactivation is a widely spread mechanism of antibiotic resistance in Gram-positive and Gram-negative bacteria. This mechanism consists in the production of enzymes capable to inactivate the antibiotic by hydrolysis or by group transfer. The widely known enzymatic group of β-lactamases hydrolyze the β-lactam ring thus causing the degradation and complete inactivation of beta-lactams (penicillins, cephalosporins, monobactams and carbapenems)[57]. More than 300 different β-lactamases have been reported and they are divided in four groups A (penicillinases), B (metallo-β-lactamases), C (cephalosporinases) and D (oxacillinases)[58]. The presence of β-lactamases such as ESBLs and carbapenemases especially in members of the Enterobacteriaceae family is causing serious problems worldwide when treating bacterial infections since they are able to inactivate almost all available beta-lactams including cephalosporins and carbapenems, which are considered last resort antibiotics[32,60]. The inactivation by group transfer is caused by transferases that are able to transfer adenylyl, phosphoryl or acetyl groups to the chemical structure of antibiotics such as aminoglycosides, macrolides, chloramphenicol and others. In this way bacteria get resistance because the modified antibiotic is unable to work and bind to the target.[57]

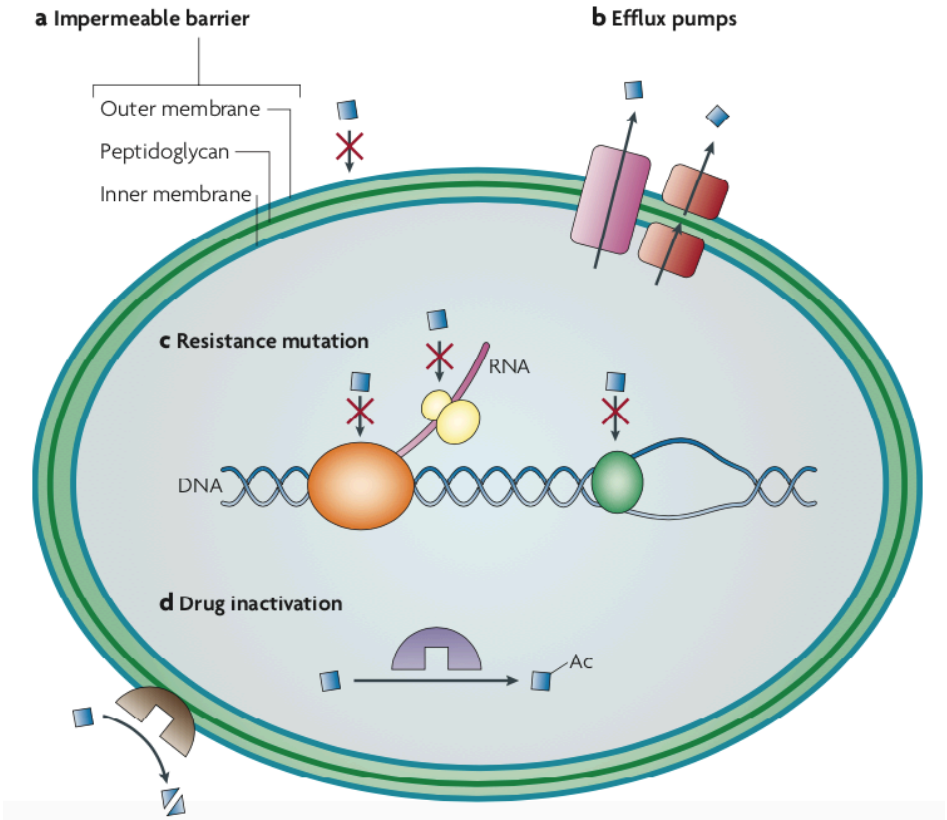

Figure 5. Antibiotic resistance mechanisms in Gram-negative bacteria. Rights to reprint by Springer Nature[6].

1.3.4 Metals and antibiotic resistance

Metals and biocides have a long history of antibacterial usage in the human civilization. However, even if high levels of biocides and metals may initially suppress bacterial growth it could actually increase the spread of ARGs resulting from co-resistance and cross-resistance mechanisms[45,55] (Figure 6). Metals such as copper (Cu), zinc (Zn), mercury (Hg), arsenic (As) and silver (Ag) have been associated at different levels with the occurrence and mobilization of ARGs in the environment[45,61]. Due to the possibility of co-existence of antibiotic and metal resistance genes in the same MGE; there might be a possibility of increased antibiotic resistance occurrence and mobilization by means of metals selection[62]. There is an overlap between known mechanisms for metals and antibiotic resistance, such as those for copper and tetracycline, copper and ciprofloxacin, and arsenic and β-lactams[55]. A study of the coexistence of antibiotic residues, metals and ARGs in manure and agricultural soils in China showed that the occurrence of ARGs for sulfonamides and tetracycline was strongly correlated with metal content indicating the effect of long-term selection pressure by metals since they are able to accumulate in ecosystems and persist during long periods of time[62].

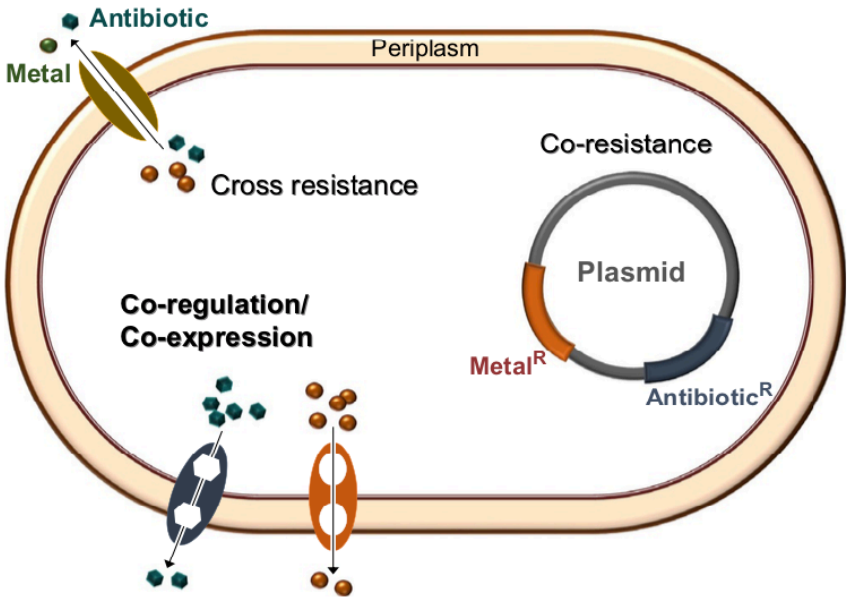

Figure 6. Co- and cross-resistance mechanisms promoting the co-selection and spread of antibiotic and metal resistance in bacteria. Co-regulation and co-expression occur when the expression of antibiotic and metal resistance is regulated by common regulatory units. Rights to reprint by Elsevier[45].

Metals exert their bactericidal effect on bacteria by producing reactive oxygen species (ROS), altering membrane permeability and function as well as causing DNA damage by their genotoxic action. Bacteria have developed mechanisms of resistance/tolerance to metals, some of them similar to antibiotic resistance mechanisms. The most importan mechanisms for metal resistance in bacteria include reduced uptake, efflux pumps, intra- and extra-cellular sequestration, damage repair, metabolic by-pass and chemical modification[63] (Figure 7).

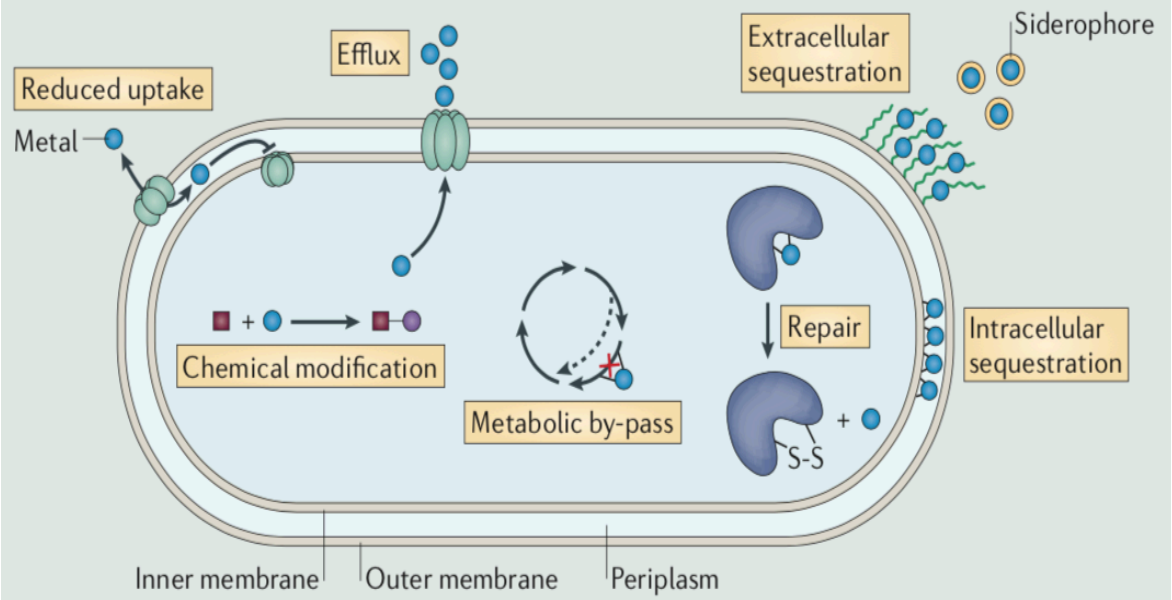

Figure 7. Mechanisms for metal resistance in Gram-negative bacteria. Rights to reprint by Springer Nature [63].

1.4 ANTIBIOTIC RESISTANCE IN THE ENVIRONMENT

Antibiotic resistance development has been potentiated by the release of antibiotic residues and ARB from municipal, agricultural and pharmaceutical sectors to the environment including watersheds and soils. ARB might also be transferred from the environment to human and animal populations by consumption of contaminated water or food[64] (Figure 8). Many studies have shown that the release of antibiotics at sub-lethal concentrations into the environment might promote the transfer and dissemination of antibiotic resistance determinants[64-66]. Besides, metals and bioides are also released to the environment at sub-lethal levels and this might impose a risk of selection and transfer of antibiotic resistance by co- and cross-resistance mechanisms[55,66].

Different environmental compartments have been recognized as important reservoirs of ARB and ARGs including urban watersheds and sewage systems, agricultural soils and heavy metal contaminated environments. However, a better understanding of the evolution, mobilization, transfer and dissemination of ARB and ARGs in the environment is needed in order to understand in depth the role of the environment in the appearance of antibiotic resistance in clinical and community settings[67]. Prevent the formation of environments that promote the selection and dissemination of ARB and ARGs as well as avoid the transmission of ARB and ARGs to human and animal microbiota are crucial to mitigate the development and dissemination of antibiotic resistance[67].

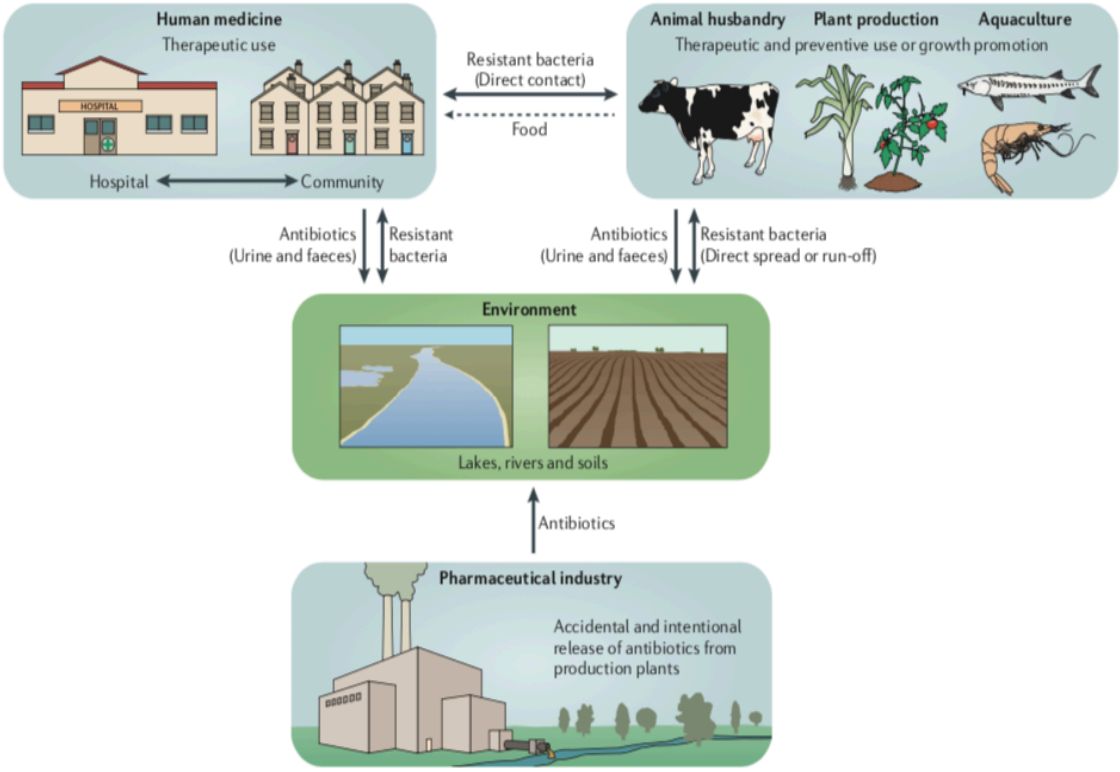

Figure 8. Ecology of antibiotics and antibiotic resistance in humans, animals and the environment. Rights to reprint by Springer Nature[64].

1.4.1 Antibiotic resistance in urban watersheds and wastewater

Urban watersheds, specially in low- and middle-income countries, might face contamination problems associated with sewage discharge and runoff from surrounding contaminated areas. This can result in high levels of fecal bacteria, which might affect the quality of recreational and drinking water, deteriorate the quality of ecosystems, and cause high risk for waterborne infection diseases in humans[68]. In many urban settings, watersheds and soils receive a heavy discharge of contaminants including municipal, agricultural and industrial residues. As expected, a big part of the contaminants are bacteria from human and non/human origin. In that sense, urban watersheds and aquatic sediments are good places for the dissemination and accumulation of pollutants, ARB and ARGs. They also represent environmental compartments where transfer of antibiotic resistance might occur[5,69].

Waste water and waste water treatment plants (WWTPs) have been largely recognized as reservoirs of ARB and ARGs as well as a good scenario for HGT. Municipal and hospital waste waters are an important supplier of ARB and ARGs to the environment, and in WWTPs they merge with higher numbers of other pathogenic/non-pathogenic bacteria from different origins and generally in presence of sub-lethal levels of antibiotics, biocides and metals. This complex mixture between bacteria and agents that might promote or select for antibiotic resistance transfer and dissemination as well as the possibility for ARGs to be released from WWTPs to the environment show the importance to study these unique interfaces[70].

1.4.2 Antibiotic resistance in agroecosystems

In agricultural practices like animal husbandry, high amounts of antibiotics are commonly used involving the release of residues to the environment. This agricultural practice involves the usage of tons of antibiotics in order to treat and prevent animal diseases[62]. Countries like China, Turkey and The United States report very high amounts of antibiotics used annually, for animal feeding and growth promotion purposes, going from 8000 to 16000 tons respectively[71-73]. Once antibiotics are administered to animals, residues of different nature and ARB are excreted in feces and urine, which in turn will be used as manure to prepare the fields for cultivation of diverse vegetables and crops for human consumption. This is how plant agricultural products might also be affected due to an accumulation of antibiotic residues and ARB from animal husbandry[74]. The irrigation of agricultural soils and crops with contaminated water or reclaimed waster water is also considered an important way to transfer antibiotic residues, ARB and ARGs to crops and agricultural soils. This transfer not only affect the health of soil ecosystems but also pose the risk of transmission of ARB and ARGs to human and animal populations by consumption of contaminated food[75].

It is clear that the misuse and overuse of antibiotics in animal husbandry together with the use of contaminated water for the irrigation of crops are increasing the release of antibiotics, ARB and ARGs to the environment and the food chain and this is a general and serious problem around the world. This problem is highly associated with the global food demand, which is predicted to rise in the upcoming years[75]. As an example, studies about the occurrence and accumulation of antibiotic residues in soil, manure and vegetables specimens from cultivated lands in China suggest that irrigation water and manure are among the main contaminant sources posing a risk of transmission of antibiotic residues and ARB to humans by direct consumption of vegetables[74].

1.4.3 Antibiotic resistance in extreme environments: acid mine drainage

Extremely acidic environments are rare, and they are usually associated with mineral exploitation activities. In mining places, the formation of highly acidic waters containing metals and acid mine drainage (AMD) occurs due to the action of extremophile bacteria and Archaea that oxidize metal sulphides keeping these extreme conditions[76,77]. Comparative studies of bacterial communities in acidic waters, lakes and rivers, associated with the Iberian Pyrite Belt in Spain found genera such as *Leptospirillum*, *Acidiphilium*, *Metallibacterium*, *Acidithiobacillus*, *Ferrimicrobium* and *Acidisphaera* as the most prevalent in highly acidic ecosystems[78].

These extreme environments are also good places for the formation of natural biofilms which are exclusively a product of environmental conditions[79,80]; these complex structures allow the sub-formation of micro-conditions inside the biofilm to offer habitats for microorganisms less adapted[81]. If we talk about water bodies, biofilms allow the survival of microorganisms that are not adapted to conditions present in the water column[82]. Microbial phyla associated with photosynthetic biofilms in the highly acidic river Rio Tinto in Spain included Alpha, Beta and Gammaproteobacteria, Nitrospira, Actinobacteria, Acidobacteria, Firmicutes and Archaea[82]. Diversity studies showed that depending on the thickness, biofilms present different prokaryotic diversity. Thick biofilms (>200 um) usually contain higher diversity of microorganisms because they may create more diverse micro-niches for less adapted individuals that are not able to survive in extreme conditions outside the natural biofilm[82].

Environments with high metal content have been proposed as potential reservoirs of ARB and ARGs that might be transferred to other non-pathogenic and pathogenic bacteria by means or co- and cross-resistance mechanisms[45,55]. Due to the high levels of metals in AMD-impacted watersheds, microbial populations residing in these extreme environments might use resistance mechanisms such as multi-drug efflux pumps to express resistance to metals, antibiotics and a variety of other chemicals such as disinfectants. If genes encoding multi-drug efflux pumps and/or other genes associated with metal an antibiotic resistance are transferred to bacterial species able to colonize humans or animals, AMD contamination might facilitate the spread of ARB and ARGs. Indeed, a number of bacterial genera such as *Acinetobacter* and *Pseudomonas* are recognized human pathogens as well as emerging carriers of ARGs but they are also very well adapted to survive and live in extreme environments such as AMDs[83].

2 AIMS

This thesis had the general aim to characterize pathogenic and non-pathogenic bacterial communities as well as ARB and ARGs and its potential to be transmitted to other bacteria in polluted water environments. We also aimed to evaluate the potential impact of metals in the occurrence and transfer of antibiotic resistance determinants in human-impacted water environments.

Specifically we aimed to:

- Determine the presence and quantity of diarrheal bacterial pathogens in a highly urban-impacted basin in La Paz, Bolivia and to characterize multi-drug resistant bacterial isolates extracted from this basin in a previous study for presence of ESBLs and carbapenem resistance genes. (Paper I)

- Characterize the phenotype, antibiotic resistance profile and genotype of *E. coli* isolates extracted from a waste water pump station in a suburban community in Norway by the PhP-AREB method and whole genome sequencing. (Paper II)

- Evaluate the potential of bacterial communities from contaminated water in an urban and agricultural area to transfer antibiotic resistance determinants to an easy traceable lab recipient. Evaluate the impact of metals such as $ZnSO_4$ and $CuSO_4$ on the transfer frequency of antibiotic resistance and on the diversity, richness and abundance of unique multi-drug resistance profiles (MDRPs) obtained from conjugation experiments. (Paper III)

- Characterize the microbial communities and the presence of metals, antibiotics and biocides in AMD-impacted water environments in Bolivia to evaluate the association of all tested parameters with a focus on metals with the ocurrence and quantity of selected antibiotic resistance determinants. (Paper IV)

3 MATERIALS AND METHODS

The materials and methods used in this thesis are explained in detail in each paper. An overview is presented below.

3.1 DESCRIPTION OF THE STUDY AREA

The contamination of fresh water threats food security and human health especially in low and middle-income countries where the high population growth, increased urbanization and high malnutrition status potentiate the increased demand for sanitized water[84]. Annually in Bolivia, around 870.000 diarrheal patients are in need of medical help, from which a very representative group include children under five years of age[85].

Bolivia is a low and middle-income country with watersheds around all its territory. However, some of them are strongly polluted with chemical and biological agents. One of the most populated cities in Bolivia is La Paz, with the principal river called Choqueyapu as a part of the La Paz river basin. The Choqueyapu river receives discharge of different kind of contaminants including metals and chemical compounds from factories and urban residues such as detergents, garbage, fecal waste and hospital residues[86]. In the La Paz River basin, contaminated river effluents and sludge are used for the production of crops that later on will supply of fresh produce including vegetables like lettuce and chard to the nearby La Paz and El Alto cities[87].

The territory of Oruro in Bolivia is also highly contaminated due to the exploitation of lead, silver, zinc and gold among other minerals. This activity generates a permanent damage in the ecosystems and the degradation of the whole environment due to the release of different metals and the formation of AMD[88]. Mine tails contain sulphide minerals and organic products that end up in nearby waterways and cause long-term pollution problems, even after the exploitation activities have ceased[89,90]. In Oruro, some of these water bodies contaminated with mine residues are the rivers Tagarete, Poopo and Sora Sora[88].

3.2 SAMPLE COLLECTION

Water, soil, rinse water of vegetables, sediment and biofilms were collected in the La Paz River basin and in the Oruro mining district. Both sampling areas are located at the highlands of the Andean Altiplano in Bolivia where samples were collected from several different sampling points.

The La Paz river basin was analyzed including its main river “Choqueyapu”. Samples were taken along the basin at four locations once a month over one year in 2014. For the conjugation experiments described in Paper III, water samples were again taken from two of the same sampling points located at the urban and agricultural areas at three occasions in 2016 and 2018. In Oruro samples were taken from AMD-impacted sites at one occasion in April 2014. Samples were also collected from a community waste water pump station outside Oslo, Norway. These samples were collected by continuous sampling over 24 hours in tubes and then pooled. Three consecutive days were sampled each month over a period of 15 months in 2016-2017.

The collected water samples were either filtered onto 0.45 um filters and used directly for DNA extraction (Paper I and Paper IV) or used for culturing on selective agar plates with subsequent collection of colonies[87] (Paper II). Water samples for Paper III were centrifuged

and the pellet containing concentrated environmental bacteria was used directly for mating experiments. A small volume was used for selective culture as a control. DNA was also extracted from sediment and biofilm samples (Paper IV) and from isolated colonies/transconjugants prior to whole genome sequencing (Paper I, Paper II and Paper III).

For water samples temperature, conductivity, pH and redox potential were measured at the time of collection (Papers I to IV). In the case of sediments and biofilms from AMD-impacted rivers the concentration of metals, antibiotics and biocides were analyzed using inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma optical emission spectrometry (ICP-OES) and liquid chromatography tandem mass spectrometry (LC-MS/MS)[91] (Paper IV).

3.3 MOLECULAR BIOLOGY METHODS

The DNA samples were used for molecular studies using qPCR to quantify presence of pathogenic and coliform bacteria (Paper I). 16S sequencing was performed to analyze resident environmental microbiota (Paper IV). Isolates obtained from Choqueyapu, Norwegian waste water, and transconjugants from paper III were subjected to whole genome sequencing. For all sequencing applications we used protocols and bioinformatic pipelines established at the Centre for Translational Microbiome Research (CTMR) at Karolinska Institutet[92] with minor modifications. The genomes were assembled, annotated and analyzed for presence of antibiotic resistance genes and additional features including *in silico* multi locus sequence typing (MLST). Bioinformatic analyses[93] and programs available at Center for Genomic Epidemiology (CGE), Denmark (http://www.genomicepidemiology.org) as well as manual blasting of contigs were used to further characterize contigs and putative plasmids in detail.

3.4 ANTIBIOTIC SUSCEPTIBILITY TESTING

Isolates, including transconjugants, were analyzed for antibiotic resistance using the Kirby-Bauer Disk Diffusion Susceptibility Test[94] (Paper I and III). In paper II the PhP-AREB method was used for typing of *E. coli* like colonies and to determine phenotypic resistance[95]. The 96-well AREB plates contain liquid media supplemented with ampicillin, cefotaxime, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, cefpodoxime, tetracycline, and trimethoprim. Inoculated bacterial colonies are recorded for growth or no growth to assess resistance and susceptibility, respectively. The PhP method simultaneously analyze ability to ferment eleven different carbon sources in liquid media supplemented with a pH indicator. The analysis allows a phylogenetic grouping of the analyzed isolates based on the fermentation patterns. The resolution seems to be comparable to MLST[95].

3.5 METAL TOLERANCE TESTING

Transconjugants obtained from conjugation experiments and ST648 and ST131 isolates from Norway were evaluated for tolerance to $ZnSO_4$ and $CuSO_4$ using the agar dilution MIC determination method for metals[96] with some minor modifications (Paper II and Paper III). A significant increase in the MIC value for tested isolates or transconjugants compared to the MIC of the original recipient or control strain was defined as acquired metal tolerance.

3.6 CONJUGATION EXPERIMENTS

The ability of complex bacterial communities from the La Paz River basin to transfer antibiotic resistance MGEs to an easy traceable recipient, the lab strain of *E. coli* CV601 was evaluated by conjugation experiments in LB media[65] (Paper III). Bacterial communities from an urban and agricultural site at the La Paz River basin were used as donors for conjugation experiments. The lab strain of *E. coli* CV601 is characterized for markers such as kanamycin (KAN) and rifampicin (RIF) resistance as well as *gfp* expression[97]. Donors and recipients were mixed in equal amounts and placed in a Millipore filter (0.2 um) onto solid LB media for 3 hours[65]. After mating incubation, the filters were washed, ten-fold diluted and plated in Mueller-Hinton media supplemented with KAN, RIF and the antibiotic mix sulfamethoxazole/trimethoprim (SMX/TMP) in order to select for all recipients that got SMX/TMP resistance from environmental communities now called transconjugants. Alternatively, conjugation experiments were performed in presence of $ZnSO_4$ and $CuSO_4$ at 0.5 and 1 mM in order to tests the effect of metals on the transfer of antibiotic resistance MGEs (paper III).

3.7 BIOFILM ANALYSIS

The isolates were analyzed for biofilm formation using Crystal violet assays, detection of extra polymeric substances (EPS), and by colony formation on Congo red LB agar plates without salt. Biofilm characteristics were analyzed after growth in 28 °C and 37 °C (Paper II).

4 RESULTS AND DISCUSSION

Pathogenic bacterial species isolated in the La Paz river basin water samples

The presence of putative pathogenic bacterial species at different locations along the La Paz river basin in Bolivia (Figure 9) was quantified by qPCR (paper I) and simultaneously by traditional culture methods[87]. For the qPCR, we used a standard curve in each run which allowed us to determine the concentration of gene copies per volume of collected water. This method has been used by us and others in several publications[35,98,99]. In general, levels of coliform bacteria (indicative of anthropogenic contamination) determined by both analysis of thermotolerant coliforms and qPCR in water environments are within the range of 10^2-10^4 genomes per 100 ml[99-101]. In the La Paz river basin, and in particular at the Choqueyapu River in the urban area and located close to hospital outlets (SP2), the levels were in the range of 10^5-10^6 genomes per 100 ml (Paper I) (Figure 10). Previous studies showed that the levels of thermotolerant coliforms obtained along the different sites in the La Paz river basin were in the same range[87]. Thus, the urban site had relatively high levels of both enterobacteria (*gapA*) and pathogens. The sampling sites in an agricultural area downstream of the hospital (SP3) and from an additional tributary (SP4) also had considerable levels of enterobacteria and pathogenic *E. coli* (Figure 10). *Klebsiella spp*. were found at levels around 10^5 genome copies per 100 ml at all sampling locations including a pristine area (SP1) located in the Altiplano above the La Paz city (Figure 11).

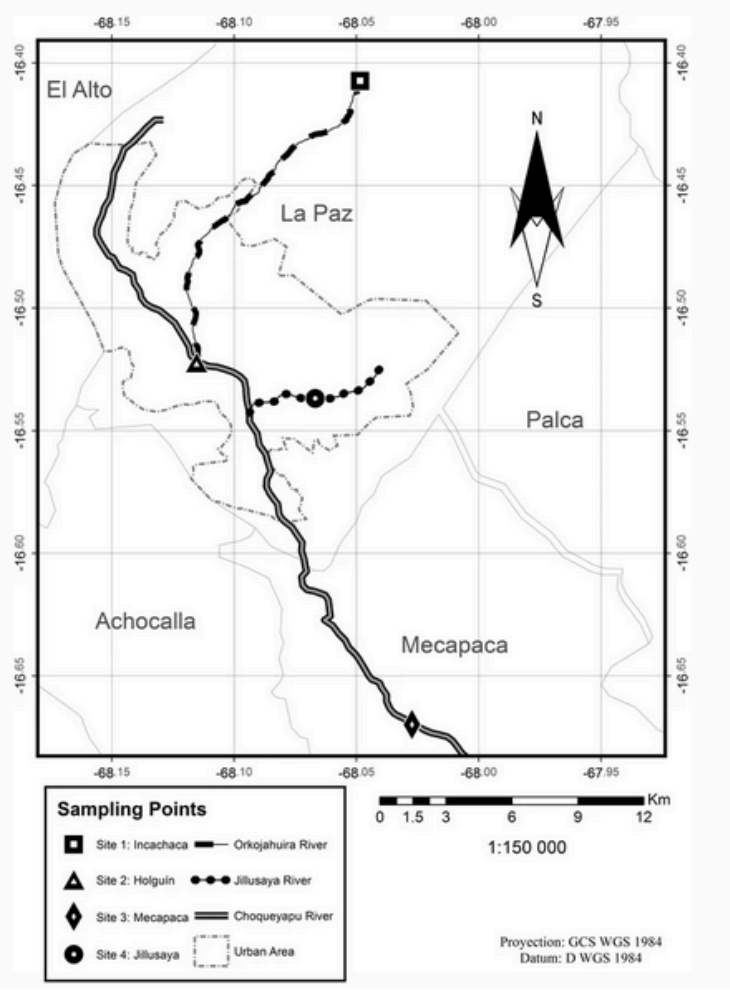

Figure 9. Sampling sites along the La Paz river basin for papers I and III. Rights to reprint by SpringerPlus[87].

One of the most abundant pathogens determined by qPCR at the urban site was ETEC carrying the LT toxin gene *eltB* (Figure 10). PCR analysis of colonies grown on MacConkey plates from the same water sample collection time points confirmed high levels of ETEC in the samples and ETEC together with Salmonella were the most frequently found by culture[87]. *Salmonella enterica* was detected in lower numbers by qPCR in this study (Figure 11). ETEC and other pathogenic *E. coli* such as EAEC and EPEC are frequently detected in environmental and irrigation water and are believed to be water-borne pathogens[102-104]. We detected all *E. coli* pathotypes in water from at least one of the sampling points along the La Paz river basin (Figures 10 and 11) and our results have been corroborated by previous bacteriological and culture studies performed in the La Paz river basin[87,105]. We also found that soil and vegetables from the agricultural area (SP3) harbored the same pathogens as the irrigation water, with enterobacteria and ETEC as the most abundant. In addition, a high incidence of diarrheagenic *E. coli*, including ETEC, EPEC and EAEC was reported in clinical isolates from diarrheal patients in La Paz[106] supporting that contaminated and irrigation water might be a transmission route.

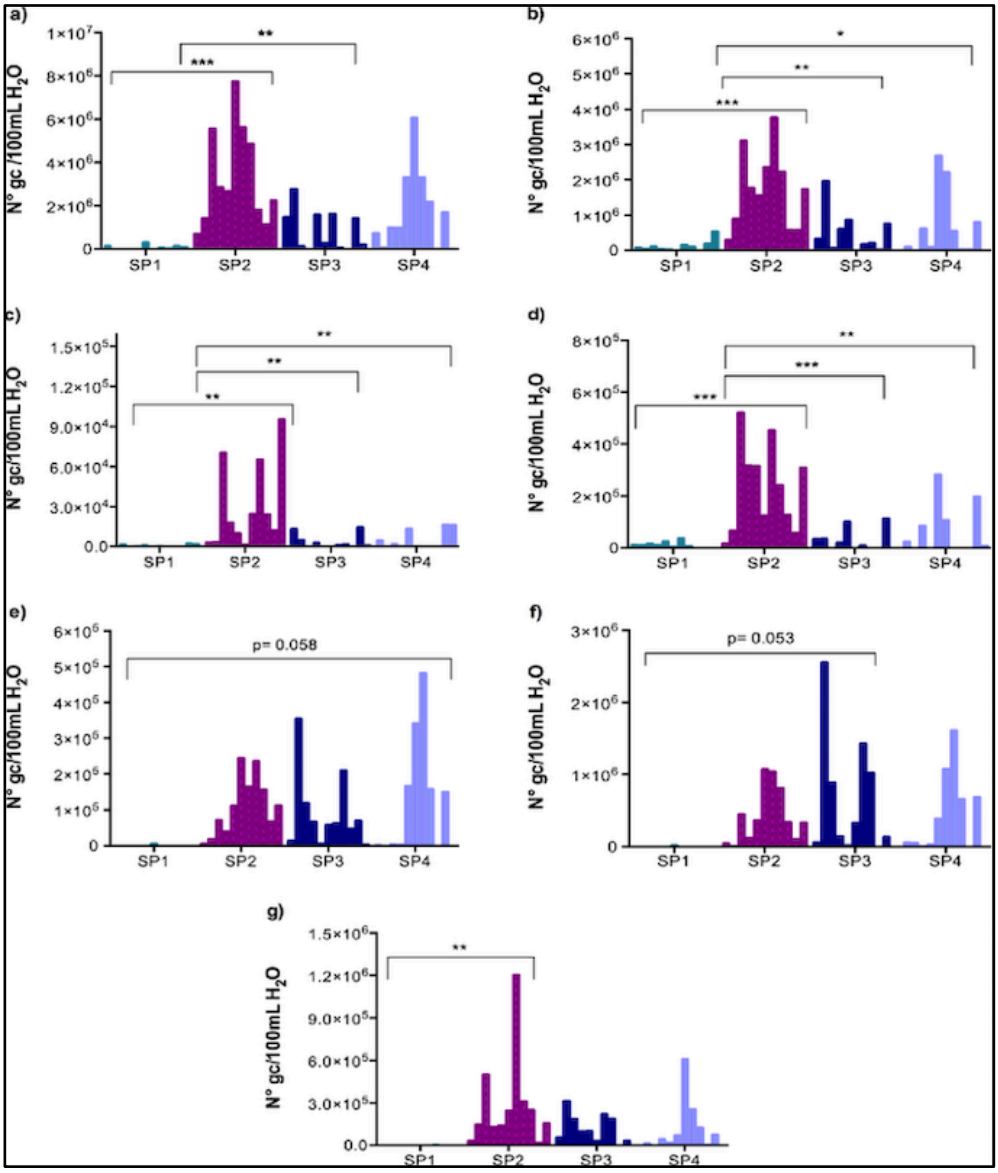

Figure 10. Quantification of enterobacteria and diarrheal bacterial pathogens in water samples from the La Paz river basin over one year April to March. a) enterobacteria (*gapA*), b) ETEC (*eltB*), c) ETEC (*estA1*), d) ETEC (*estA2-4*) e) EPEC/EHEC (*eae*), f) EAEC (*aggR*) and g) *Shigella spp.*/EIEC (*ipaH*). Rights to reprint by PLOS ONE[34]

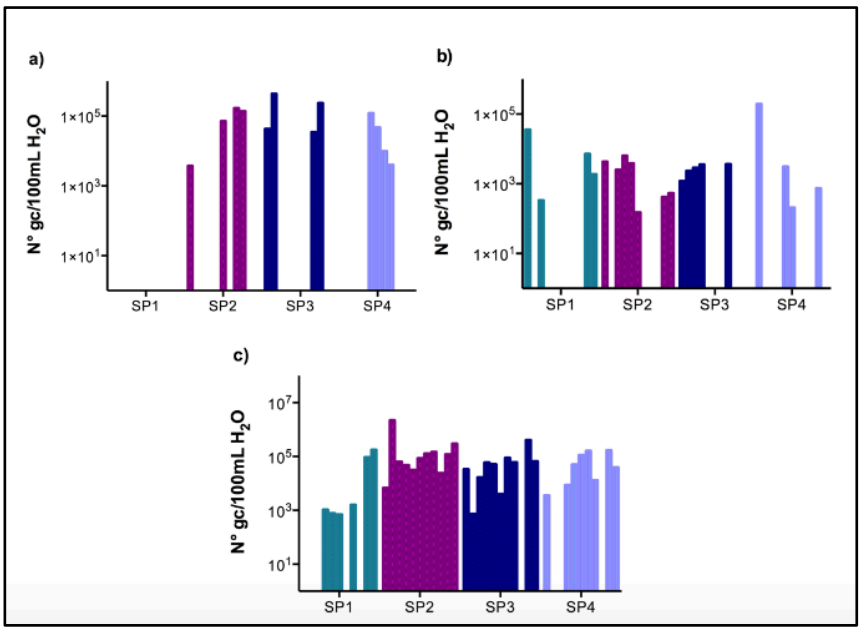

Figure 11. Quantification of pathogenic bacteria in water samples from the La Paz river basin. a) EHEC (*stx1*), b) *S. enterica* (*invA*) and c) *K. pneumoniae* (*ntrA*).

Multi-resistant enterobacteria are present in the La Paz river basin

The cultured isolates from the river were further analyzed for antibiotic resistance by the Kirby-Bauer disc diffusion test. Resistance against ampicillin, nalidixic acid, trimethoprim–sulfamethoxazole and tetracycline was frequently detected[87]. The resistance pattern was similar to previously reported resistance studies in pathogenic *E. coli* isolated from water in the La Paz river basin[105] and from clinical diarrheagenic *E. coli* isolates in the La Paz area[106,107].

From the previous bacteriological study with water from the La Paz river basin[87], five lactose positive colonies and five lactose negative colonies were collected from MacConkey plates at each sampling occasion. A subset of these isolates that were resistant to three or more of the antibiotics tested were further analyzed by PCR using primers designed to detect ESBL and carbapenemase genes (Paper I).

PCR detected presence of ESBL genes in 5 out of 101 tested isolates. Of these five, one isolate was lost but the remaining four were subjected to whole genome sequencing to further characterize their genotype. Three of the isolates were *E. coli* while the fourth was an *Enterobacter cloacae*. Analysis of the genomes by ResFinder indicated presence of several ARGs particularly in the *E. cloacae* isolate and in an *E. coli* identified to belong to the sequence type ST648. These two isolates were both collected from the Choqueyapu River at the urban site (SP2). The other two *E. coli* isolates were assigned to ST162 and ST410 and were isolated from soil samples in the agricultural site of the study (Paper I). Resistance genes for tetracycline (*tetA*), sulfamethoxazole (*sul1* and *sul2*), quinolones (*qnrS1, qnrB1* and *AAC(6')-lb-cr*), trimethoprim (*dfrA17*), β-lactams (bla_{TEM}, bla_{OXA-1} and $bla_{CTX-M-3}$) were found in these isolates but no carbapenem resistance genes were found.

ST648, ST410 and ST162 all belong to established or emerging multi-resistant *E. coli* clones known to spread globally and to be prone to carry ESBL and carbapenemase resistance genes such as bla_{CTX-M}, bla_{OXA} and bla_{NDM} on transmissible plasmids[108-110]. ST648 is a uropathogenic *E. coli* (UPEC) and similar to ST131, an infamous UPEC with

increasing multi-resistance, it often carries plasmid-borne ESBL resistance mediated by *bla*$_{CTX\text{-}M\text{-}15}$[108,111,112]. The uropathogenic *E. coli* ST648 has also been isolated from healthy animals and animals with urinary tract infection[113,114] indicating that this sequence type can affect both humans and animals and since we also found it in environmental samples both in Bolivia and in sewage in Norway (Paper II) we show the importance of a One-Health approach. The presence of these multi-resitant *E. coli* clones in the La Paz River water indicated that transfer of antibiotic resistance genes might occur in these environments and this was addressed in paper III (discussed below).

Emerging multi-resistant *E. coli* sequence types are present globally

The findings of high levels of both *E. coli*-like colonies and pathogenic *E. coli* in environmental water samples as well as in waste water have been described in numerous studies[115-117]. A quick and affordable method to test both the phylogenetic structure of isolated *E. coli* colonies and simultaneously their antibiotic resistance pattern is to use the PhP-AREB system developed by researchers at Karolinska Institutet[95,115] (www.phplate.com). In a collaboration project with Norwegian researchers we initiated analysis of two pheneplate types with suspected ESBL resistance that were repeatedly found over 15 months of sampling in a waste water pump station in a community outside Oslo, Norway. Whole genome sequencing was performed on a subset of the isolates and analysis of multi locus sequence type (MLST), plasmid content, antibiotic resistance genes and putative virulence genes revealed that the two phenotypes belonged to ST648 and ST131 respectively (Paper II). All Norwegian isolates harbored the *bla*$_{CTX\text{-}M\text{-}15}$ gene located on a putative plasmid conserved in all isolates and 99% identical to *bla*$_{CTX\text{-}M\text{-}15}$ plasmids recovered in *E. coli* from India and US. The HN80 ST648 isolate from the La Paz River did not contain CTX-M-15 encoding genes (Paper I) and phylogenetic analyses indicated that it grouped far from the Norwegian ST648 within this ST type (Figure 12).

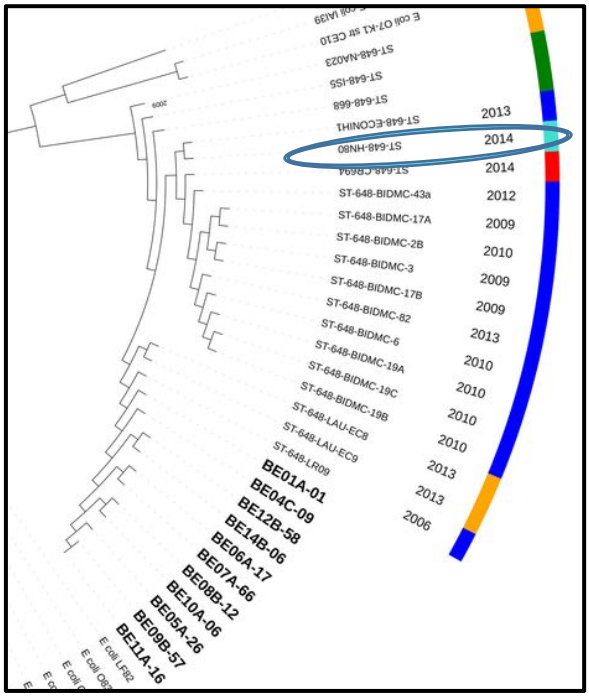

Figure 12. Section of the core phylogenetic tree for ST648 isolates showing the *E. coli* isolate HN80-ST648 from the La Paz river in Bolivia (light blue circle) was grouped distantly from the group of ST648 isolates from the waste water pump in Norway (in bold) (Paper II). Rights to reprint by Antimicrobial Agents and Chemotherapy[111].

Norwegian isolates belonging to the sequence types ST131 and ST648 showed tolerance to $CuSO_4$ comparable to the *E. coli* control and a slight increase in the tolerance to $ZnSO_4$. In general, ST131 and ST648 isolates showed high capability to form biofilms and to produce extracellular polymeric substances (EPS) specially at 28°C (Figure 13), an observation that might suggest that both sequence types use biofilm adaptation to survive and persist in sewage systems and in the environment. Furthermore, ST131 isolates showed a conserved *rdar* morphotype (pdar and bdar) while ST648 isolates showed more variable phenotype including saw and bdar morphotypes (Paper II).

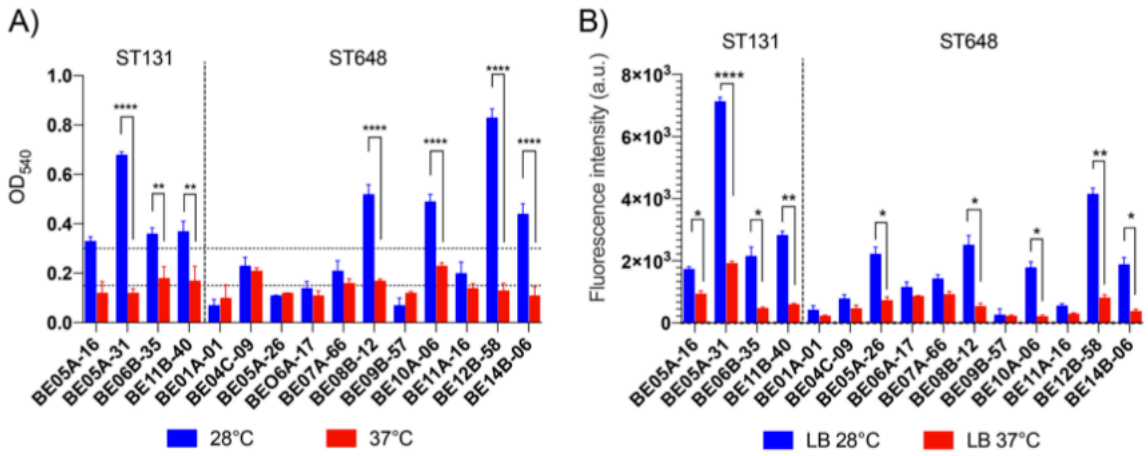

Figure 13. Biofilm formation and production of EPS by *E. coli* ST131 and ST648. Stain of bacterial biofilms with Crystal violet (A) and FITC-CoA (B) after incubation for 48 h at 28 or 37°C. Rights to reprint by Antimicrobial Agents and Chemotherapy[111].

Bacterial communities from the La Paz river basin exhibit high potential to transfer antibiotic resistance determinants to *E. coli*

In paper III, we used filtrated water samples from the urban and agricultural sites (SP2 and SP3) in the La Paz river basin as source of bacterial donors for conjugation experiments in LB media using the lab strain of *E. coli* CV601 as recipient. Bacterial communities from both sampling sites presented high capability to transfer SMX/TMP resistance determinants to *E. coli* with an average frequency of one event per one thousand cells (10^{-3}) and the highest frequencies detected were as high as one event per one hundred cells (10^{-2}) (Figure 14). Other conjugation studies using bacterial donors from contaminated water in India and Sweden testing the transfer of SMX resistance to *E. coli* CV601 have reported maximum frequencies of 10^{-4} and 10^{-5} events per recipient respectively[65,118]. Our findings indicate that bacterial communities from the La Paz river basin have a remarkable potential to transfer antibiotic resistance determinants to commensal and pathogenic bacteria like *E. coli*.

Bacterial donors from the urban site were able to transfer SMX/TMP resistance elements at significantly higher frequencies compared with donors from the agricultural area (Figure 14), suggesting that the urban site might possess higher number of suitable donors. *E. coli* and *Klebsiella spp*. have been widely recognized as important carriers of ARGs among Enterobacteriaceae family members[32,60]. In paper I, we reported constant levels of *K. pneumoniae* in all sampling sites along the La Paz river basin (Figure 11). However, the levels of enterobacteria and pathogenic *E. coli* especially ETEC were significantly higher at the urban site (Figure 10) confirming the high levels of anthropogenic contamination at this site and hence a higher number of suitable donors.

In paper I, we showed the presence of enterobacteria and diarrheal pathogens in irrigation water, soil and vegetables from the agricultural area in the La Paz river basin and later in paper III we confirmed the transfer of antibiotic resistance at considerable levels with donors from the same agricultural area (Figure 14). Thus, we clearly show the potential transmission risk of ARB and ARGs to the population by contaminated irrigation water and vegetables.

In the conjugation experiments we used the mix SMX/TMP to select for all recipients who got any MGE carrying SMX/TMP resistance genes from environmental communities in the La Paz river basin. The reason to use this specific mix to select for transconjugants was because in general a big variety of donors and MGEs are characterized to carry sulfamethoxazole resistance genes[65]. Additionally, sulfamethoxazole/trimethoprim, commonly known as cotrimoxazole, is widely used to treat gastrointestinal and urinary tract infections in Bolivia so the presence of associated antibiotic residues and ARGs has been already reported in Bolivian watersheds[119-121]. Resistance to SMX/TMP has also been reported in bacterial strains isolated from the La Paz river basin[87,105].

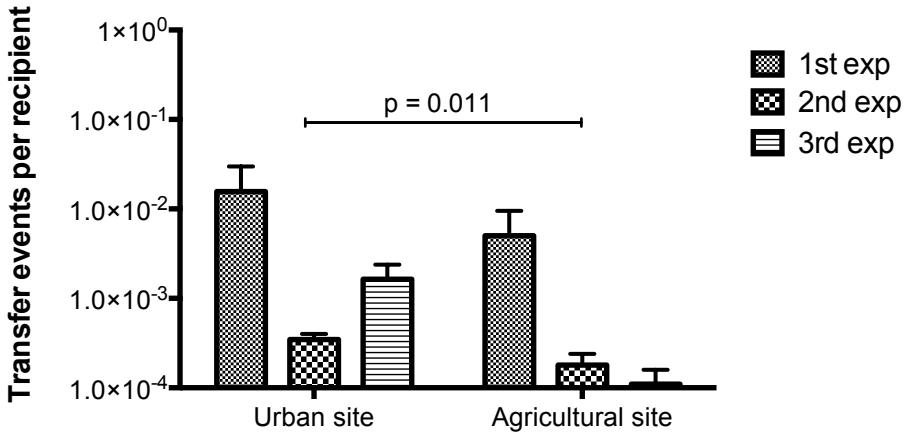

Figure 14. Transfer frequency of SMX/TMP resistance from conjugation experiments using donor communities from water in urban and agricultural areas in the La Paz River basin and *E. coli* CV601 as recipient.

Higher diversity, richness and number of unique phenotypic MDRPs were transferred from donors in the agricultural area in presence of metals

In order to test the effect of metals on the transfer of antibiotic resistance, we performed conjugation experiments in presence of $ZnSO_4$ and $CuSO_4$ at 0.5 and 1 mM using bacterial donors from the urban and agricultural areas in the La Paz river basin (Paper III). For both donor sites, the addition of $ZnSO_4$ and $CuSO_4$ to conjugation experiments at the concentrations tested in this study did not significantly increase the transfer frequency of SMX/TMP resistance compared to the control in LB media without metals. The effect of metals on the transfer frequency of antibiotic resistance has been evaluated previously by other authors but in general contradictory results have been reported with positive or negative effects highly dependent on the type and concentration of metal used [122-124].

Although the addition of $ZnSO_4$ and $CuSO_4$ did not cause a significant effect on antibiotic resistance transfer frequencies, the presence of these metals in experiments with donors from the agricultural site promoted the transfer of more diverse gene cassettes (Paper III). Twenty-eight different MDRPs (P1-P28) were obtained from the antibiotic susceptibility

testing by the Kirby Bauer test for 13 antibiotics of 150 selected transconjugants from all experimental treatments. Every different combination of susceptibility, intermediate resistance and resistance to the antibiotics tested was defined as a single phenotypic MDRP. A significantly higher diversity and number of unique phenotypic MDRPs were obtained from donors in the agricultural area when $ZnSO_4$ and $CuSO_4$ were added to conjugation experiments (Figure 15). The observed phenotypic MDRPs commonly presented intermediate resistance or resistance to ampicillin (AMP), nalidixic acid (NA) and tetracycline (TET) and all obtained transconjugants were resistant to SMX/TMP. These patterns of resistance coincide with previously reported resistance studies in pathogenic *E. coli* isolated from water in the La Paz river basin[87,105] and from clinical diarrheagenic *E. coli* isolates in the La Paz area[106,107].

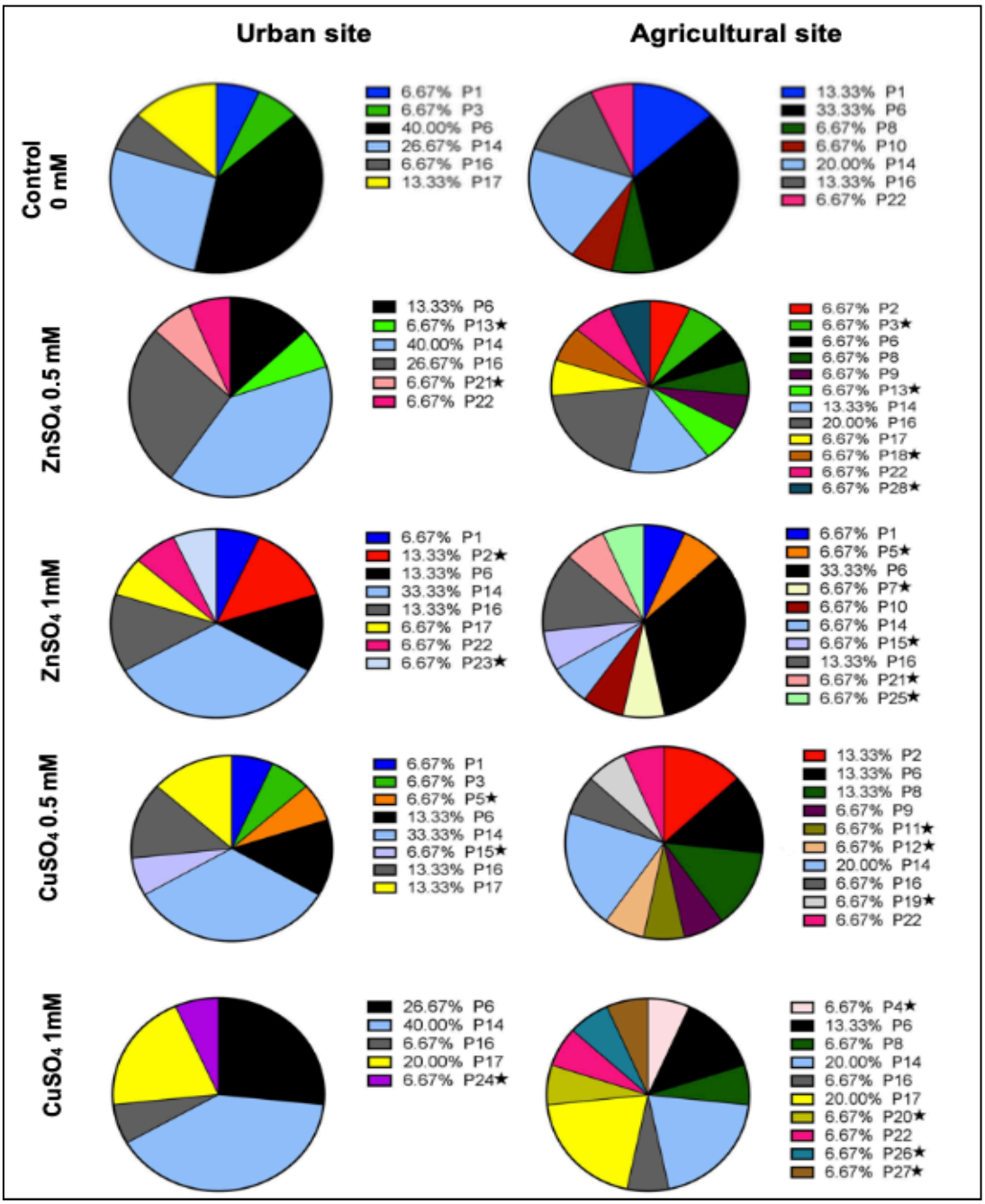

Figure 15. Multi-drug resistance profiles (MDRPs) obtained from conjugation experiments in absence and presence of $ZnSO_4$ and $CuSO_4$ using water from urban and agricultural areas in the La Paz River basin as source of donors. Stars show unique MDRPs per site.

The co-selection of antibiotic and metal resistance genes in transconjugants obtained in paper III was evaluated trough the tolerance testing to $ZnSO_4$ and $CuSO_4$. None of the transconjugants presented increased tolerance to these metal salts compared to the original recipient suggesting that the co-transfer of antibiotic and metal resistance genes to *E. coli* from La Paz River communities is less likely to occur. These findings are corroborated by the fact that biocide, metal and antibiotic resistance genes were rarely found to co-localize in plasmids from environmental isolates (<0.7%), while this co-localization was found to be more common in clinical isolates[61].

ESBLs and higher diversity of other relevant genes (ORGs) were transferred from the La Paz river bacterial communities to *E. coli* in presence of metals

A subgroup of 47 selected transconjugants representing every phenotypic MDRP observed in paper III were whole genome sequenced and further analyzed using ResFinder and BacMet databases as well as the identification of Incompatibility group (Inc), pMLST and virulence genes. Manual analysis and blasting of sequences was also performed to identify restriction system genes (RSGs), transferred gene contigs and the number of transferred base pairs. Higher number of ARGs and ORGs associated with resistance to disinfectants and antimicrobial peptides, multi-drug transporters, biofilm formation and persister state in bacteria, survival in stress conditions and virulence determinants were transferred from donors in the urban and agricultural area in presence of $ZnSO_4$ and $CuSO_4$ (paper III). ESBLs such as $bla_{CTX\text{-}M}$, bla_{SHV}, and bla_{OXA} were found in unique MDRPs which were obtained only in presence of metals. The majority of studies evaluating the effect of metals on the conjugative transfer frequency are performed with a established donor, recipient and transferable element[122-125]. Our approach uses complex bacterial communities from contaminated environments as donors so besides testing the effect of metals on the transfer frequency of antibiotic resistance, we were able to evaluate the diversity, richness and kind of phenotypic MDRPs, ARGs and ORGs transferred from environmental bacterial donors to *E. coli* in absence and presence of metals. It has been proposed that the possible effect of metals on antibiotic resistance transfer might involve the generation of ROS, the activation of SOS responses and the increase in permeability of bacterial membranes[123]. While this effect is mainly associated with an increase in the transfer frequency of antibiotic resistance, we hypothesize that these mechanisms might also favor in some way the selection and transfer of bigger MGEs carrying higher diversity and number of ARGs including ESBLs as well as higher number of ORGs that confer important advantages to the survival of the host.

Additionally, four plasmid like-structures were repeatedly found in transconjugants from the urban and agricultural area in the La Paz river basin. These plasmid-like structures were found to belong to the Inc groups IncN, IncN2 and IncN3 that are recognized to be self-conjugative and a have broad range of hosts in Enterobacteriaceae family members. The IncN group of plasmids have been highly associated with antibiotic resistance dispersion since they were commonly found carrying ESBLs, oxacillinases, carbapenemases, quinolone, aminoglycoside and sulfonamide resistance genes[126-128]. The four multi-drug resistance isolates from the La Paz river basin sequenced in Paper I share some of the antibiotic resistance genes for β-lactams, quinolones, sulfamethoxazole and tetracycline observed in transconjugants from Paper III. However, none of these four isolates carried IncN group of plasmids so it is less likely that they were the donors of the resistance elements isolated and observed in paper III.

Bacterial communities and selected ARGs do not correlate with metal content in AMD-impacted rivers in Oruro, Bolivia

Since heavy metals might be involved in the selection and transfer of antibiotic resistance driven by co- and cross-resistance mechanisms and this might occur in environments heavily contaminated with metals[55,129,130], we decided to have a look at the bacterial communities and ARGs residing in AMD-impacted rivers in the mining area of Oruro in Bolivia (Paper IV). We evaluated river water and sediments from three rivers in the Uru Uru-Poopó basin, an still active mine and even biofilms from hot spring wells used for mineral exploitation. We jointly evaluated metals, antibiotic and biocide concentrations in sediment and biofilm samples.

We found high levels of iron (Fe), aluminium (Al), lead (Pb), arsenic (As), zinc (Zn), copper (Cu) and tin (Sn) in sediments from all sampling points. Our results are corroborated by previous studies evaluating the chemical composition of waterbodies in the Uru Uru-Poopó basin in Bolivia, reporting high levels of metals[88,131] and a high associated health-risk for human populations[132-134]. Antibiotics and biocides were mainly found in places close to human activity being this the first study evaluating the presence of antibiotic and biocide residues in the Uru Uru-Poopó basin.

In general, 16S sequencing revealed the dominance of Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes phyla in the three rivers at the Uru Uru-Poopó basin. Other microbial phyla such as Euryarchaeota, Thaumarchaeota, Deinococcus-Thermus, Acidobacteria and Acetothermia including many extremophile species were detected in places with low pH (2.2-3.5) and high temperature (>70°C). The archaeal family Ferroplasmaceae including iron-oxidizing microorganisms such as *Ferroplasma spp.* was found in water samples from the still active mine San José. Microbial phyla such as Cyanobacteria and Chloroflexi were also commonly found in water, sediments and biofilms from the Uru Uru-Poopó basin. Overall, our results widely coincide with previous reports of microbial communities in acidic and AMD-contaminated environments[135-140].

We found the presence of environmental bacteria such as *Arcobacter* and *Acinetobacter* that are known to frequently carry *intI1* and ARGs relevant to humans[141-143] and the fecal marker crAssphage in the sampling sites with closer contact to anthropogenic contamination (Figure 16). The class 1 integron has been widely reported in clinical and environmental samples being associated with the transmission of antibiotic resistance[144], anthropogenic contamination[145] and correlated with the presence of Proteobacteria[146] and genera such as *Acidovorax, Cloacibacterium, Sulfurospirillum and Tolumonus*[143]. While crAssphage is the most abundant virus in the human gut[147,148] and it has been used as a marker of human fecal contamination in the environment in a number of studies[149]. Class 1 integrase (*intI1*) and diverse ARGs for B-lactams (*acc-3, bla$_{IMP-12}$, bla$_{OXA-2}$*), sulfonamides (*sul1* and *sul2*) and tetracycline (*tetA*) were detected in water, sediment and biofilm samples from AMD-impacted rivers in Oruro (Figure 16). Resistance genes such as *sul1, sul2* and *tetA* were positively correlated with the presence of *intI1* (Paper IV). The presence of the human fecal marker coincided with the highest abundances for *intI1* and ARGs in water and sediment samples suggesting that fecal pollution is highly linked to the abundance of ARGs as has been previously reported in human-impacted environments[150,151]. Interestingly, the most acidic place (San José mine) was devoid of human fecal marker, *intI1* and contained low levels of ARGs. We thus hypotesize that the mine site might be too extreme to be a reservoir for human associated ARGs.

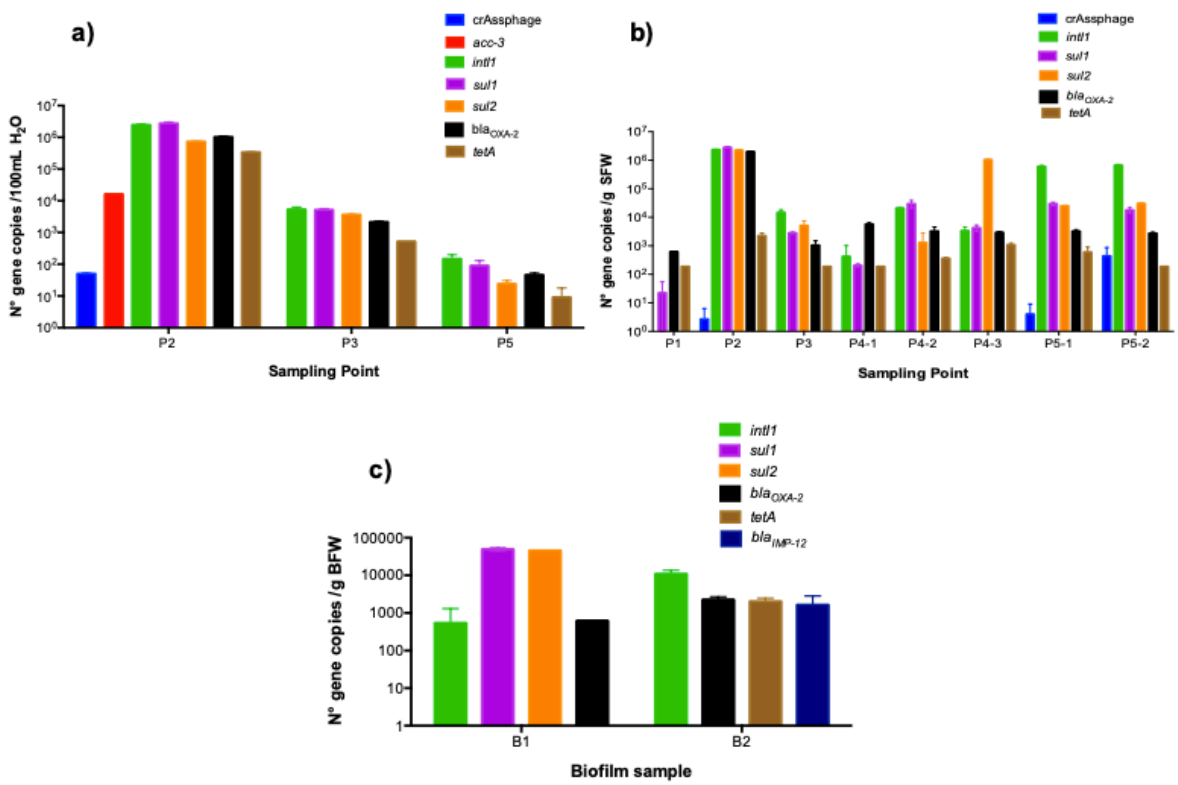

Figure 16. Abundance of ARGs in water (a), sediment (b) and biofilm (c) samples from five different sampling points (P1-P5) along the AMD-impacted Uru Uru-Poopó basin in Oruro, Bolivia.

Previous studies in AMD-impacted environments have reported that parameters such as temperature, pH, conductivity and the concentration of different metals significantly influenced the diversity and structure of microbial communities [152,153] and this variations were also influenced by seasonality[154,155] However, in paper IV metal composition in sediment and biofilm samples from the Uru Uru-Poopó basin did not significantly influence the composition of microbial communities nor the enrichment of *intI1* and the ARGs evaluated in this study. We only detected a significant correlation between the presence of mercury (Hg) in biofilms and the presence of the resistance gene bla_{IMP-12} that was first identified in Italy in a *Pseudomona putida* isolate from a clinical setting and might confer resistance to cephalosporins and carbapenems[156]. We think that since the samples in paper IV were few and taken at one occasion additional studies might be needed to assess if significant associations occur.

5 CONCLUSION

This thesis work aimed to evaluate pathogenic and non-pathogenic bacterial communities as well as ARB and ARGs and its potential to be transmitted to other bacteria in polluted water environments. We showed high prevalence of enterobacteria, pathogenic *E. coli* and other diarrheal bacteria in water, agricultural soil and vegetables from an urban-impacted basin in La Paz, Bolivia (Paper I). Moreover, we repeatedly found and characterized multi-drug resistance *E. coli* sequence types ST131 in Norway and ST648 both in Bolivia and Norway showing that these successful lineages are distributed globally carrying ESBLs and posing an important role of the environment for the dispersion of pathogenic bacteria and ARB (Paper I and II). We proved the high capability of bacterial communities from contaminated environments to transfer antibiotic resistance determinants to other bacteria like *E. coli* where higher contamination in urban areas might be associated with higher number of suitable donors and thus higher antibiotic resistance transfer frequencies (Paper III).

We also aimed to evaluate the potential impact of metals in the occurrence and transfer of antibiotic resistance determinants in human-impacted water environments and we showed that the presence of metals such as $ZnSO_4$ and $CuSO_4$ might favor transfer/acquisition of more diverse phenotypic MDRPs and bigger MGEs carrying higher diversity of ARGs including ESBLs and ORGs conferring important advantages to the host (Paper III). However, this effect is highly dependent of the donor community since we observed a stronger effect when using bacterial donors from contaminated irrigation water in the agricultural area in the La Paz river basin and in that way we also show the potential risk of transmission of ARB to human populations by irrigation water and probably contaminated vegetables (Paper III). We finally investigated the bacterial communities and ARGs abundances in AMD-contaminated watersheds in Oruro Bolivia but we could not find significant and strong associations between metal composition in the sampling area and the composition of microbial communities and/or the abundance of antibiotic resistance determinants in the samples (Paper IV). However, further sampling events and metagenomic analysis will be needed to finally conclude about the risk that these environments pose for human and animal health.

The three main domains in a One-Health approach: humans, animals and the environment, are closely interconnected. The study and surveillance of contaminated environments is as important and urgent as the studies on clinical and community settings.

6 FUTURE PERSPECTIVES

Our data reveal that is is important to recognize that not only direct use of antibiotics and closeness to *e.g* hospital discharges are responsible for dissemination of ARB. We found that bacteria carrying ARGs are resisdent in the environment over long time periods. Our data in Papers I, and II indicate that genetically identical clones of *E. coli* carrying ARGs on plasmids that are seemingly conserved, are found repeatedly in water environments. This either indicate that these *E. coli* are resident and able to survive for long periods perhaps in the form of biofilms, or even grow in these environments. It could also be that human contamination constantly feeds new ARB into the water environments. Perhaps driven by use and misuse of antibiotics or simply because the human and/or animal population in the area is colonized by these bacteria at a high rate. We need to explore the mechanisms behind these findings.

We could show in paper III that there is a real risk of transmission of ARGs to pathogenic *E. coli* since we found high frequencies of transmission to a model *E. coli* strain from environmental water residing bacteria. Further studies need to evaluate the risk of transfer to known pathogenic bacterial species.

Since presence of metals have been suggested to drive transfer of ARGs due to co- and cross resistance we looked at the water microbiota and presence of selected ARGs in the Oruro district in Bolivia, we found no strong correlations in the present study but it is important to expand these studies and use metagenome sequencing and more samples collected over time to be able to draw any rigid conclusions about the risk.

Regarding the impact of our studies, in the future we plan to continue studying the prevalence and transmission risk of ARB and ARGs in contaminated environments. There are future plans for the implementation of a WWTP for the Choqueyapu River in Bolivia so we think it will be important to evaluate the effect of this implementation on the occurrence of ARB and ARGs. We also think that it is very important to work together with the government, health and environmental authorities in Bolivia to evaluate in depth the current situation and implement adequate surveillance methods and risk mitigation measures to prevent the dissemination of ARB and ARGs.

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