Universidade de Aveiro Departamento de Biologia
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Isabel Alexandre Teixeira da Silva

Role of metals in the selection of antibiotic resistance

O papel dos metais na seleção de resistência a antibióticos

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Isabel da Silva Henriques, Professora Auxiliar do Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia da Universidade de Coimbra, e sob coorientação da Doutora Marta Cristina Oliveira Martins Tacão Investigadora em pós-doutoramento do Departamento de Biologia da Universidade de Aveiro

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“Molecular biology has shown that even the simplest of all living systems on the earth today, bacterial cells, are exceedingly complex objects. Although the tiniest bacterial cells are incredibly small, weighing less than 10-12 gms, each is in effect a veritable micro-miniaturized factory containing thousands of exquisitely designed pieces of intricate molecular machinery, made up altogether of one hundred thousand million atoms, far more complicated than any machine built by man and absolutely without parallel in the nonliving world.”
— Michael Denton, Evolution: A Theory in Crisis

“Happiness and bacteria have one thing in common; they multiply by dividing!”
— Rutvik Oza

o júri

presidente

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palavras-chave

Resistência a antibióticos, metais, cobre, zinco, co-seleção, sistemas aquáticos, microcosmos, ensaios evolutivos, comunidades bacterianas

resumo

O ambiente tem vindo a ser reconhecido pelo seu papel na disseminação de resistência a antibióticos – uma das maiores ameaças para a saúde mundial. Para além dos antibióticos, vários outros contaminantes ambientais parecem contribuir para este problema. Este estudo teve como objetivo investigar o papel dos metais na seleção de resistência a antibióticos. Para tal, duas estratégias foram utilizadas: 1) uma abordagem de evolução experimental e 2) um ensaio baseado em microcosmos com comunidades bacterianas complexas.

Para tal, populações de *Escherichia coli* ATCC® 25922™ e *Aeromonas hydrophila* CECT 839^{T} foram expostas a concentrações crescentes de cobre e zinco durante 80 dias para investigar alterações genotípicas e fenotípicas. Alterações na tolerância destas populações a outros metais foram avaliadas, assim como a sua suscetibilidade a antibióticos pelo método de difusão em disco. A determinação de concentrações mínimas inibitórias (CMIs) de alguns antibióticos foi também realizada. Ensaios de crescimento foram realizados, para avaliar o efeito desta exposição no fitness bacteriano. Alterações genotípicas foram avaliadas por repPCR. A exposição a cobre e zinco levou ao aumento da tolerância destas populações a outros metais como crómio e níquel. Populações de *E. coli* evoluídas na presença de cobre aumentaram a sua tolerância à canamicina, imipenemo e sulfametoxazol; enquanto que populações de *E. coli* expostas a zinco aumentaram a tolerância ao sulfametoxazol. Populações de *A. hydrophila* evoluídas na presença destes metais aumentaram a tolerância à canamicina. Os resultados da determinação de CMIs nem sempre estiveram de acordo com os resultados do teste da suscetibilidade a antibióticos por difusão em disco. Ensaios de crescimento revelaram um custo de adaptação para populações de *E. coli* evoluídas com metal, o que não foi verificado para populações de *A. hydrophila*. Alterações genotípicas severas não foram detetadas.

Microcosmos foram implementados para confirmar a seleção de resistência a antibióticos em comunidades bacterianas aquáticas, imposta pela exposição a cobre ou zinco por 20 dias. Para tal, foram recolhidas amostras de água do rio Antuã. Depois da exposição ao metal, foi determinada a abundância bacteriana e a prevalência de resistência a vários antibióticos. Bactérias resistentes à cefotaxima e à canamicina, que tinham sido expostas a estes metais, foram identificadas. Análises de DGGE foram realizadas para avaliar os efeitos do metal na estrutura das comunidades bacterianas expostas. Os resultados mostraram um aumento da prevalência de bactérias resistentes a cefotaxima e tetraciclina, em comunidades expostas a cobre; enquanto que em comunidades expostas ao zinco se verificou um aumento significativo da prevalência de bactérias resistentes a cefotaxima e canamicina. Bactérias resistentes à cefotaxima e canamicina pertenciam a géneros intrinsecamente resistentes a estes compostos, por exemplo, *Pseudomonas* e *Sphingomonas*. O perfil de DGGE destas comunidades revelou que a exposição ao metal altera a sua estrutura diminuindo a sua riqueza e diversidade.

Este estudo confirmou que a exposição a metais induz alterações significativas nas estirpes bacterianas, levando a fenótipos de maior tolerância a metais e antibióticos. Em comunidades bacterianas do sistema aquático, a exposição a metais leva a uma maior prevalência de estirpes resistentes a antibióticos.

keywords

Antibiotic resistance, metals, copper, zinc, co-selection, aquatic systems, microcosms, experimental evolution assay, bacterial communities.

abstract

The environment is increasingly recognized for its role in the global dissemination of antibiotic resistance - one of the biggest threats to global health. Several non-antibiotic contaminants seem to increase this problem. This study aimed to investigate the role of metals in the selection of antibiotic resistance. To achieve this goal, two strategies were used: 1) an experimental evolution approach and 2) a microcosm-based assay with complex bacterial communities.

For this, *Escherichia coli* ATCC® 25922™ and *Aeromonas hydrophila* CECT 839^T populations were evolved in increasing concentrations of copper and zinc for 80 days and phenotypic and genotypic changes were investigated. Alterations in the tolerance of the evolved populations to other metals was assessed. Antibiotic susceptibility testing by disc diffusion and antibiotic MIC determination were performed. Growth assays were performed to evaluate the effect of metal exposure in bacterial fitness. Genotypic alterations were assessed by rep-PCR. Evolution in the presence of copper or zinc led to an increased tolerance against other metals such as chromium or nickel. Also, *E. coli* populations evolved with copper increased their MICs for kanamycin, imipenem and sulfamethoxazole; whereas MICs of populations evolved with zinc followed this trend for sulfamethoxazole. Concerning *A. hydrophila*, populations evolved with copper and zinc increased their MICs for kanamycin. In a few cases, results from disc diffusion tests were inconsistent with MICs determination. Growth assays revealed a fitness cost for *E. coli* populations evolved with metal, which was not verified for evolved *A. hydrophila* populations. Severe genotypic alterations were not detected.

For the microcosm-based experiment, lab scale microcosms were steed up to confirm selection of antibiotic resistance in aquatic bacterial communities imposed by exposure to copper and zinc for 20 days. Water samples were collected from Antuã river. After metal exposure, colony-forming units were counted in culture media with and without antibiotics. From microcosms exposed to copper and zinc, cefotaxime and kanamycin resistant bacteria were selected and identified. DGGE analyses were performed to assess metal effects in exposed bacterial communities' structure. Results showed a significant increase in the prevalence of bacteria resistant to cefotaxime and tetracycline in communities exposed to copper; whereas in communities exposed to zinc an increase in bacteria resistant to cefotaxime and kanamycin was verified. Cefotaxime and kanamycin resistant bacteria belonged to genera intrinsically resistant to these compounds, i.e. *Pseudomonas* spp. and *Sphingomonas* spp. DGGE profiling revealed that metal exposure altered the structure of bacterial communities while decreasing richness and diversity.

This study confirmed that metal exposure induces significant changes in bacterial strains, leading to higher metal and antibiotic tolerance. In aquatic system bacterial communities, metal exposure leads to a higher prevalence of antibiotic-resistant strains.

TABLE OF CONTENTS

LIST OF ABREVIATIONS

ARB: Antibiotic-resistant bacteria

ARGs: Antibiotic resistance genes

AST: Antibiotic susceptibility testing

AK: Amikacin

ATM: Aztreonam

BOX- PCR: BOX elements – polymerase chain reaction

CTX: Cefotaxime

C: Chloramphenicol

CIP: Ciprofloxacin

CFUs: Colony-forming units

DGGE: Denaturing Gradient Gel Electrophoresis

dNTP's: Deoxyribonucleotide triphosphates

Dt: Doubling time

EF-G: Elongation factor G

ERIC-PCR: enterobacterial repetitive intergenic consensus – polymerase chain reaction

ESBLs: Extended spectrum β-lactamases

FEP: Cefepime

H: Diversity index

ICEs: Integrative conjugative elements

IPM: Imipenem

ISCR: Insertion sequence common region

J: Pielou's evenness index

K: Kanamycin

LB broth: Luria-Bertani

MEM: Meropenem

$MgCl_2$: Magnesium chloride

MGE: Mobile genetic element

MIC: Minimum inhibitory concentration

MRSA: Methicillin-resistant *S. aureus*

NA: Nalidixic acid

NaCl: Sodium chloride

OMPs: Outer membrane proteins

OD: Optical density

PBPs: Penicillin-binding proteins

PCA: Plate Count Agar

PCR: Polymerase chain reaction

PRL: Piperacillin

QACs: Quaternary ammonium compounds

r: Growth rate

ROS: Reactive oxygen species

S: Sulfonamides

SMX: Sulfamethoxazole

SXT: Trimethoprim-sulfamethoxazole combination

S: Richness index

TA: Toxin-antitoxin systems

TAE: Tris-acetate-EDTA buffer

TE: Tetracycline

TGC: Tigecycline

TZP: Piperacillin-tazobactam combination

WWTPs: Waste water treatment plants

LIST OF FIGURES

Figure 10: Metal(oid) susceptibility profile of *A. hydrophila* populations after 20 transfers; A.A - stands for *A. hydrophila* ancestral population, A.20 01-20 05 - stands for populations evolved without the metal, A.20 Cu1-20 Cu5 - stands for populations evolved with copper and A.20 Zn1-20 Zn5 - refers to populations evolved with zinc. **Results, page 44;**

Figure 11: Metal(oid) susceptibility profile of *A. hydrophila* populations after 40 transfers; A.A - stands for *A. hydrophila* ancestral population, A.40 01-40 05 - stands for populations evolved without the metal, A.40 Cu1-40 Cu5 - stands for populations evolved with copper and A.40 Zn1-40 Zn5 - refers to populations evolved with zinc. **Results, page 45;**

Figure 12: Susceptibility profile of *E.coli* populations after 20 transfer (E.20) against piperacillin (PRL), piperacillin-tazobactam combination (TZP), cefepime (FEP), cefotaxime (CTX), aztreonam (ATM), imipenem (IPM), meropenem (MEM), tigecycline (TGC), tetracycline (TE), ciprofloxacin (CIP), nalidixic acid (NA), amikacin (AK), kanamycin (K), chloramphenicol (C), sulfonamides (S) and trimethoprim-sulfamethoxazole combination (SXT); R - resistant; I - intermediate resistance - populations not identified with a R or I were classified as susceptible to the antibiotics tested; E.A – *E. coli* ancestral population, E.20 01-20 05 - stands for populations evolved without the metal, E.20 Cu1-20 Cu5 - stands for populations evolved with copper and E.20 Zn1-20 Zn5 - refers to populations evolved with zinc. **Results, page 47;**

Figure 13: Susceptibility profile of *E.coli* populations after 40 transfer (E.40) against piperacillin (PRL), piperacillin-tazobactam combination (TZP), cefepime (FEP), cefotaxime (CTX), aztreonam (ATM), imipenem (IPM), meropenem (MEM), tigecycline (TGC), tetracycline (TE), ciprofloxacin (CIP), nalidixic acid (NA), amikacin (AK), kanamycin (K), chloramphenicol (C), sulfonamides (S) and trimethoprim-sulfamethoxazole combination (SXT); R - resistant; I - intermediate resistance - populations not identified with a R or I were classified as susceptible to the antibiotics tested; E.A – *E. coli* ancestral population, E.40 01-40 05 - stands for populations evolved without the metal, E.40 Cu1-40 Cu5 - stands for populations evolved with copper and E.40 Zn1-40 Zn5 - refers to populations evolved with zinc. **Results, page 48;**

Figure 14: Susceptibility profile of *A. hydrophila* populations after 20 transfer (A.20) against piperacillin (PRL), piperacillin-tazobactam combination (TZP), cefepime (FEP), cefotaxime (CTX), aztreonam (ATM), imipenem (IPM), meropenem (MEM), tigecycline (TGC), tetracycline (TE), ciprofloxacin (CIP), nalidixic acid (NA), amikacin (AK), kanamycin (K), chloramphenicol (C), sulfonamides (S) and trimethoprim-sulfamethoxazole combination (SXT); R - resistant; I - intermediate resistance - populations not identified with a R or I were classified as susceptible to the antibiotics tested; A.A – *A. hydrophila* ancestral population, A.20 01-20 05 - stands for

Mix - Thermo Fisher Scientific, USA). 20 stands for 20 transfers and 40 stands for 40 transfers, 01-05 - stands for populations evolved without the metal, Cu1- Cu5 - stands for populations evolved with copper and Zn1-Zn5 - refers to populations evolved with zinc. **Appendices, page 125;**

Figure 45: BOX-PCR DNA fingerprint patterns for *A. hydrophila* (A) populations. The lanes of the ends contained an external standard, a 1-kb molecular weight ladder (GeneRuler™ DNA ladder Mix - Thermo Fisher Scientific, USA). 20 stands for 20 transfers and 40 stands for 40 transfers, 01-05 - stands for populations evolved without the metal, Cu1- Cu5 - stands for populations evolved with copper and Zn1-Zn5 - refers to populations evolved with zinc. **Appendices, page 126;**

LIST OF TABLES

1. INTRODUCTION

Worldwide, antibiotic resistance remains a major threat to public health, increasing the mortality rates, duration of hospitalization and healthcare costs. Measures to control this problem have been implemented. However, it can be anticipated that antibiotic resistance will continue to develop faster than new agents to treat antibiotic-resistant bacteria (ARB) become available. That said, understanding the evolutionary mechanisms governing the spread of antibiotic resistance, as well as how anthropogenic activities might be causing its selection and/or evolution, is essential.

1.1 Antibiotic resistance

Emergence of antibiotic resistance is a natural phenomenon. However, the rate at which this process occurs has increased tremendously over the past few decades, bringing very serious consequences for public health. There are many different processes through which microorganisms can became resistant to antibiotics. Concerning bacteria, the acquisition of foreign DNA through horizontal gene transfer and gene mutation, are the two main mechanisms (Kumar et al., 2017).

1.1.1 Antibiotic resistance by horizontal gene transfer

Horizontal gene transfer seems to be the main driver of bacterial antibiotic resistance acquisition (Aminov, 2011). Microorganisms acquire external genetic information through three main mechanisms - transformation, transduction and conjugation – processes that clearly mediate bacterial evolution (Figure 1) (Munita and Arias, 2016; Furuya and Lowy, 2006).

First identified in the Gram-positive bacterium *Streptococcus pneumoniae*, transformation is characterized by the uptake, integration and expression of free DNA from the extracellular environment into a recipient cell (Johnston et al., 2014). Still, for this process to take place, cells must develop a state of competence that leads to the expression of a multi-component DNA uptake system (Juan et al., 2015). In addition, although the details vary widely among different species, most bacteria only develop this condition for a short period of time and under specific stressful situations, such as changes in growth conditions, nutrient access or high cell density (Thomas and Nielsen, 2005).

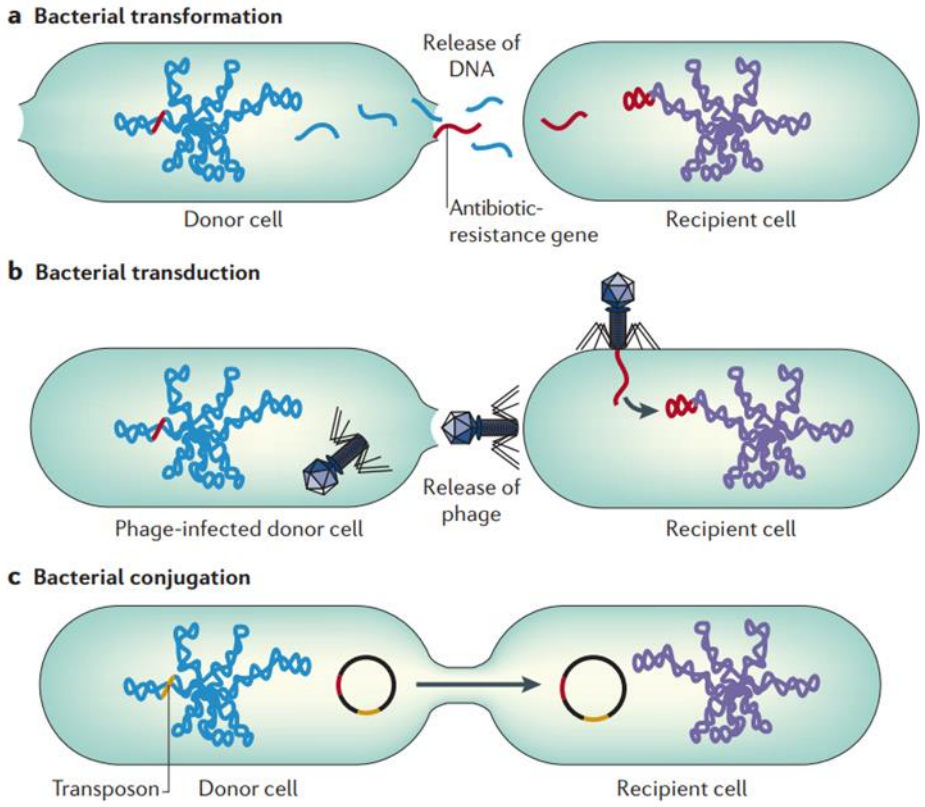

Figure 1: Horizontal gene transfer between bacteria (Furuya and Lowy, 2006);

The contribution of natural transformation to the dissemination of antibiotic resistance genes (ARGs) among bacteria is well documented (von Wintersdorff et al., 2016). As an example, using a microcosms experiment, Mao et al. (2014) observed high rates of transformation in bacteria from river sediments in China, when extracellular kanamycin resistance genes were added. In fact, the authors also found that, in these samples, extracellular DNA concentration was higher than intracellular DNA. Thus, since indigenous bacteria proved to be capable of competence, this amount of extracellular genetic material in these environments could serve as a large reservoir of ARGs, which may be incorporated by these bacteria trough transformation, facilitating antibiotic resistance dissemination (Mao et al., 2014). Several studies also reported that bacteria competence can be induced by antibiotic exposure, as demonstrated, for instance, by Charpentier and co-workers (2011) in *Legionella pneumophila*, a Gram-negative pathogen. These authors found that *omEA* expression - a reporter of competence development - was induced by chronic exposure to genotoxic molecules such as mitomycin C and antibiotics of the fluoroquinolone family (Charpentier et al., 2011).

Regarding the transduction mechanism, it is defined as the transfer of genetic material from one bacterial cell to another mediated by bacteriophages, a mechanism that promotes their

own survival. This can occur via generalized transduction, where any portion of the donor genome can be transferred with the phage to the recipient cell; or specialized transduction, in which only bacterial genes near the site of integration of phage DNA can be transferred (Soucy et al., 2015).

Recently, different studies suggested that the role of bacteriophages in the dissemination of antibiotic resistance is far more relevant than previously thought. In fact, bacteriophages have been isolated from very diverse environments such as oceans, lakes, marine systems, soils and sea ice; where concentrations of bacteriophages overcome those of bacteria, increasing the probability of bacteria-bacteriophages encounters and consequently the probability of transduction (Weinbauer, 2004). Additionally, distinct works also show that transduction might occur at frequencies several orders of magnitudes greater than previously recognized, as reported, for instance, by Kenzaka et al. (2010).

Numerous investigations demonstrate the role of transduction in the dissemination of antibiotic resistance determinants, as recently revised by Colavecchio et al. (2017) in foodborne pathogens of the *Enterobacteriaceae* family. The authors explain that several environmental and anthropogenic factors can influence the rate of transduction. The continuous flow of ARGs and mobile genetic elements (MGEs) to the environment through wastewater treatment plants, farm and slaughterhouse runoffs, hospital effluents, manure applications, and aquaculture, seems to increase this rate (Colavecchio et al., 2017). Ross and Topp (2015) reinforced this idea, studying the abundance of ARGs in bacteriophages after soil fertilization, the authors concluded, not only that soil-borne bacteriophages represent a substantial reservoir of ARGs in this environment, but also that the use of dairy manure, as soil fertilizer in agricultural settings, can promote bacteriophage-mediated horizontal gene transfer of ARGs.

All these studies addressed the significant contribution of transformation and transduction to the acquisition and mobilization of ARGs. However, conjugation is often considered as the most common mechanism responsible for this dissemination. One explanation seems to be the fact that nucleases and other contaminants, present in natural environments, can easily degrade naked DNA, impairing transformation (Norman et al., 2009). In addition, although during transduction the packaging of DNA into protein capsules provides a substantially better protection, conjugation seems to provide a more efficient way to do it, while having a broader host range (Norman et al., 2009).

Conjugation is the direct transfer of genetic material between cells, in close proximity, that occurs through the production of a pilus. The DNA to be transferred is supported by conjugative systems, encoded either by genes on plasmids or by integrative conjugative elements

(ICEs), that frequently carry additional determinants related to pathogenesis and/or antibiotic resistance (Ramsay and Firth, 2017; Wozniak and Waldor, 2010). The promptly spreading of extended spectrum β-lactamases (ESBLs) genes of the *bla*$_{\text{CTX-M}}$ family, across different bacterial species worldwide, is a good example of the contribution of conjugation and conjugative plasmids for the dissemination of antibiotic-resistant strains (Cantón et al., 2012). In fact, plasmids carrying ESBLs genes have been associated with aminoglycoside, tetracycline, sulfonamide and fluoroquinolone resistance determinants, allowing the relatively easy spread of multi-drug resistance (Cantón and Coque, 2006). This finding was corroborated by Tacão et al. (2014) that identified gene cassette arrays coffering resistance to aminoglycosides (*aadA*-type genes and *aacA4*), trimethoprim (*dfrA17*), chloramphenicol (*catB8*), fluoroquinolones (*qnrVC4*) and β-lactams (*bla*$_{\text{OXA-10}}$), among ESBL-producers harboring conjugative plasmids. Additionally, several studies support the idea that antibiotics can increase the rate at which conjugation occurs. As an example, the study of Schuurmans et al. (2014) concluded that a sub-inhibitory concentration of tetracycline can increase the rates of plasmid transfer between *Escherichia coli* strains, trough conjugation, even to higher rates than those achieved for higher concentrations of this antibiotic.

In summary, horizontal gene transfer has a crucial role in the acquisition and dissemination of ARGs. Of the three most well-known mechanisms, conjugation seems to have the greatest influence on this dissemination. However, recent discoveries suggest that the role of transformation and transduction may be larger than previously assumed. That said, understanding the extent of these mechanisms in the acquisition of ARGs, and how its mobilization to pathogenic bacteria takes place, is important to control the dissemination of these genes.

1.1.2 Antibiotic resistance acquisition by DNA mutation

Besides horizontal gene transfer, mutations have a striking role in the acquisition and dissemination of antibiotic resistance. Since bacteria have short generation times, mutations can easily emerge and accumulate rapidly. Consequently, some bacteria can become resistant to several antibiotic classes in a short time frame. Additionally, mutation in regulatory genes/regions can also alter the expression of several resistance genes, giving rise to different phenotypes, and thus playing a significant role in the evolution and diversification of bacterial strains.

Fluoroquinolones resistance, for instance, is mainly due to chromosomal mutations that alter the target enzymes - affecting the binding of the drug, or to reduced drug accumulation by amend efflux systems (Correia et al., 2017). The most common mechanism of resistance to this

group of antibiotics is due to mutations within *gyrA* and *gyrB* or *parC* and *parE* genes, which encode topoisomerase II and topoisomerase IV, respectively. These are essential enzymes for DNA replication (Redgrave et al., 2014). Mutations in these genes result in amino-acid substitutions that structurally change the target protein and, subsequently, their drug-binding affinity. Yet, higher levels of quinolone resistance usually require accumulation of mutations in one or both target enzymes. Several studies relate single mutations in *gyrA*, for instance, in isolates with low-level resistance, whereas higher MICs correspond to multiple mutations (Correia et al., 2017; Yanat et al., 2017). On the other hand, as the target of fluoroquinolones is intracellular, these molecules must have the ability to enter the bacterial cell. That said, mutations that result in downregulation or modification of the porin channels can also confer quinolone resistance (Correia et al., 2017). In Gram-negative bacteria, for instance, membrane permeability can be regulated by altering expression of outer membrane porin proteins that form channels for passive diffusion. In *E. coli*, mutations in the structural genes of the outer membrane proteins OmpF and OmpC can lead to reductions in cytoplasmic drug concentrations (Mayers, 2009). Concerning OmpF, a decrease in its expression is related to an increase of norfloxacin and ciprofloxacin resistance (Mayers, 2009).

Mutations are also the only responsible for antibiotic resistance in particular species. An example is the case of *Helicobacter pylori*, in which resistance to all therapeutic agents available is mediated by gene mutations. For this species, clarithromycin resistance, for instance, is the result of three-point mutations in 23S rRNA gene that results in an rRNA conformational change, leading to a decrease in binding of the drug (Mégraud, 2012). Besides clarithromycin, currently only five antibiotics are available for use in combined therapies to combat *H. pylori* infections: amoxicillin, tetracycline, rifampicin, metronidazole, and levofloxacin; and for all these antibiotics there are reports of mutations that lead to resistance (Alba et al., 2017). A mutation in the penicillin-binding protein 1A gene is the responsible for amoxicillin resistance (Kwon et al., 2017); tetracycline resistance is the result of a mutation in the 16S rRNA gene (Gerrits et al., 2003); rifampicin resistance is related with mutations in the RNA polymerase subunit B gene (Gong and Yuan, 2018); the acquisition of resistance to metronidazole is highly associated with mutational inactivation of the *rdxA* gene (Jenks and Edwards, 2002); and levofloxacin resistance is mainly due to mutations in the DNA gyrase gene *gyrA* (Miftahussurur et al., 2016).

Glycopeptide resistance in enterococci is another example of how mutations can shape antibiotic resistance evolution. In this genus, mutations can affect how resistance phenotypes are expressed. Glycopeptide antibiotics, vancomycin and teicoplanin, act by binding to D-Ala-D-Ala peptides and inhibiting peptidoglycan cross-linking. Resistance to these drugs is the result of *vanA* and *vanB* genes activation that encode a new pathway enzyme, leading to the production of D-

Ala-D-Lac peptides, which bind to vancomycin and teicoplanin with reduced affinity (Arthur and Quintiliani, 2001). In a normal situation, VanA-positive strains can resist to vancomycin and teicoplanin, while VanB-positive strains are only capable of vancomycin resistance. Yet, mutations in the regulators of these genes result in changes in the type of glycopeptide resistance phenotype expressed - mutations in the N-terminal sensor domain of the regulator of a VanA-positive strain can prevent it from resist to teicoplanin; while a six-residue deletion in the phosphatase domain of the regulator of a VanB-positive strain results in glycopeptide resistance, including to teicoplanin (Depardieu et al., 2007; Arthur and Quintiliani, 2001).

Another important role of mutations is their contribution for the evolution and diversification of acquired resistance determinants. Good examples of this influence are the genes encoding ESBLs. The TEM family of β-lactamases suffered a strong diversification due to mutations in the gene encoding TEM-1 - the first identified member of this family of enzymes. After the introduction of β-lactamase inhibitors and new generations of β-lactams, mutations arose, resulting in enzymes capable of hydrolyzing, e.g., cefotaxime and ceftazidime, or resisting the action of β-lactamase inhibitors, which gave rise to over 200 variants of the TEM family (http://www.bldb.eu/). A similar situation also occurred with other β-lactamase families such as those belonging to the CTX-M family. CTX-M enzymes preferentially hydrolyze cefotaxime, but an Asp240Gly substitution leads to an increased catalytic activity against ceftazidime in several subgroups of this family such as CTX-M-15, CTX-M-16, CTX-M-25, CTX-M-27 and CTX-M-32, when compared with CTX-M-3, CTX-M-9, CTX-M-26-like, CTX-M-14 and CTX-M-1 enzymes, respectively (Gniadkowski, 2008). Likewise, a substitution of Pro167 was observed in CTX-M-19 (for serine; Pro167Ser) and CTX-M-23 (for threonine; Pro167Thr), which are derivatives of CTX-M-14- and CTX-M-1-like enzymes, respectively (Gniadkowski, 2008); and a Ser130Gly mutation decreased the activity of CTX-M-9 against cefotaxime while significantly increased its resistance to β-lactamase inhibitors (Aumeran et al., 2003).

As demonstrated, mutation as a cause of antibiotic resistance has a great impact on antibiotic resistance evolution, being capable of influence, not only the resistance profile of specific pathogenic strains, but also having a profound impact in the diversification of acquired resistance genes. Furthermore, the existence of hypermutator strains of bacteria – strains with elevated mutation rates due to mutations in genes affecting DNA repair– is found to be one of the main drivers for the development of multi-drug resistance in many bacterial infections (Oliver and Mena, 2010).

1.2 Fitness cost associated with antibiotic resistance

Different studies have shown that the acquisition of antibiotic resistance might be associated with a reduced bacterial fitness (Maharjan and Ferenci, 2017; Melnyk et al., 2015; San Millan et al., 2015). However, it is well known that ARB are widely distributed in a wide variety of environments, even without the presence of a selective pressure, proving that some of these resistance traits can be permanently established among bacterial populations (Bengtsson-Palme et al., 2018).

The impact of antibiotic resistance acquisition in bacterial fitness seems to be different depending on the events that precede it. Concerning the acquisition of ARGs through MGEs, it seems to involve the use of novel metabolic resources, which is reflected in a fitness cost that can be very different, depending on the set of genes incorporated in the MGE (Hernando-Amado et al., 2017). In addition, although this potencial cost may be associated with the resistance function itself, it can also be the result of plasmid regulation or plasmid replication, and gene expression may interfere with bacterial growth in ways independent of any resistance functions carried by that plasmid (Dahlberg and Chao, 2003). As an example, San Millan et al. (2015) studies showed that the origin of the costs of horizontal gene transfer in a model of *Pseudomonas aeruginosa* were generated by the expression of the plasmid replication protein gene *rep*, which in turn, produced massive changes in the expression of genes in the genome of this organism. Namely, genes associated with the activation of the SOS response – the mechanism trough which bacteria responds to DNA damage; and chromosomal replication (San Millan et al., 2015).

On the other hand, several authors exposed that some horizontal gene transfer events could be associated with a fitness gain, which explains its maintenance in bacterial populations (Hernando-Amado et al., 2017). Schaufler and co-workers, for instance, investigated the fitness cost of ESBL-plasmids and their influence on chromosomally encoded features associated with virulence, and concluded that these elements, not only can increase the growth/metabolic fitness, but also the expression of genes with relevance for virulence, such as the *csgD* gene – a transcription factor involved in biofilm formation and bacterial motility (Schaufler et al., 2016). However, other mechanisms seem to be involved in the maintenance of MGEs, such as the selection of mutations that compensate their cost. An example of compensatory mutations is provided by the plasmid pBR322. This element contains a *tetA* tetracycline-resistance gene, whose fitness cost seems to be compensated by a chromossome mutation in the endogenous K^+ transport system Trk. The energy requirements make the TetA pump (encoded by the *tetA* gene) more efficient than Trk at taking up K^+. This makes the Trk system redundant and costly in the plasmid-bearing cell and drives its inactivation through mutation – the evolved strain is then

dependent on TetA (and the plasmid) to take up potassium (Hellweger, 2013). Another explanation for the maintenance of MGEs in bacterial populations is related to toxin-antitoxin systems (TA). TAs can be found on plasmids and the TA loci encode two-component systems that consist of a stable toxin and an unstable antitoxin. When a plasmid encoding this system is lost from a cell, the toxin is release killing plasmid-free cells that don't produce the antitoxin. Thus, TAs enhance plasmid maintenance by killing the plasmid-free daughter cells (Yang and Walsh, 2017).

Regarding the fitness cost associated to mutation-derived antibiotic resistance, different works have shown, not only that it depends on the mutation involved (Andersson and Hughes, 2010), but also on the genetic background of the bacterial strain (Hernando-Amado et al., 2017; Agnello et al., 2016; Melnyk et al., 2015). In addition, less costly mutations seem to be more frequently found in clinical antibiotic-resistant isolates (Shcherbakov et al., 2010). Several studies also report that it might be possible that some resistance mutations are cost-free (Wasels et al., 2015); and that, as in horizontal gene transfer driven resistance, a gain of fitness associated with the acquisition of resistance can also occur (Durão et al., 2015).

Like horizontal gene transfer -driven resistance, compensatory mutations are the most usual mechanism to compensate fitness costs of antibiotic resistance due to mutations - either by restoring the bacterial fitness, by reducing the need for a function that has been affected or by re-establishing its role (Hernando-Amado et al., 2017). Besier et al. (2005) work, for instance, exposed that individual amino acid exchanges within elongation factor G (EF-G) - that cause fusidic acid resistance in *Staphylococcus aureus* - are associated with fitness costs that are offset with compensatory EF-G mutations able to ameliorate these detrimental effects. More recently, Moura de Sousa et al. studies (2017) had similar findings. Using different approaches, the authors compared the cost of streptomycin and rifampicin double-resistance in *E. coli*, with that of a single resistance mutation, and concluded that low-fitness double-resistant bacteria compensate faster than single-resistant strains due to the acquisition of compensatory mutations, thereby potentially stabilizing costly multiple resistances in bacterial populations (Moura de Sousa et al., 2017).

A different way of bacteria to compensate fitness costs seems to be by increasing the number of copies of some genes. The work of Nielson et al. (2006), for instance, demonstrated that the decrease of the fitness costs associated to actinonin-resistant mutants in *Salmonella enterica* can be recouped, not only through additional mutations, but also with an increase in the copy number (from 4 to 40 copies) of genes involved in protein synthesis. Likewise, in *P. aeruginosa* secondary mutations aren't necessary, and the cost of resistance, that in this case results from an overexpression of an efflux pump, is compensated due to an overexpression of a secondary respiratory chain. According to Olivares et al. (2014), this alternative pathway,

normally expressed under anaerobic conditions, increases the respiration rate, preventing growth defects. The explanation seems to be the constant proton flow between the periplasm and the cytoplasm, necessary to the efflux pumps activity. According to the authors, this need might alter the proton gradient, which can decrease the internal pH and compromise the bacterial physiology, leading to fitness costs. Since the fastest way to eliminate protons is trough anaerobic respiration, Olivares et al. (2017) propose that this is the reason why mutants that overexpress these pumps show an overexpression of this secondary respiratory chain and higher oxygen consumption rates that their wild-type counterparts.

As has been pointed out, the impact of the acquisition of antibiotic resistance traits on the bacterial physiology by horizontal gene transfer or mutations could be different. Despite early studies stating that the acquisition of these traits is always associated with a fitness cost, recent works have shown that resistant bacteria are not always less fit than susceptible ones. In addition, fitness cost seems to be different according to the bacterial genetic background, and specific of the mutation or resistance gene involved. Further, several works also showed that some resistance mechanisms do not produce relevant cost or that this cost could be compensated. That said, it is possible that once a resistance mechanism has been selected, it may persist even in the absence of antibiotic pressure.

1.3 Aquatic systems as reservoirs of ARB and ARGs

Apart from clinical settings, the environment is increasingly being recognized for its role in the global dissemination of antibiotic resistance. Main contributors to this role seem to be the aquatic systems, serving both as natural reservoirs of antibiotic resistance and as conduits for the spread of clinical resistance traits of major concern (Silva et al., 2018; Suzuki et al., 2017; Manaia et al., 2016; Tacão et al., 2014). In fact, the increasing prevalence of ARGs in these systems, as a result of human activities, has led to the identification of these genes as emerging environmental contaminants with serious consequences for human health - due to their potential transfer from environmental bacteria to human pathogens, thereby lessening the efficacy of antibiotics (Tripathi and Cytryn, 2017). Aquatic bodies such as lakes, rivers, streams and cost lines, receive a diverse mixture of antibiotics and other pollutants and ARB, trough treated and untreated sewage, hospital waste, aquaculture discharges and agriculture runoff (Figure 2). Consequently, both water and sediments in these compartments may have a significant role in driving ARGs transfer and evolution (Araújo et al., 2017; Tacão et al., 2017, 2012a; Tripathi and Cytryn, 2017).

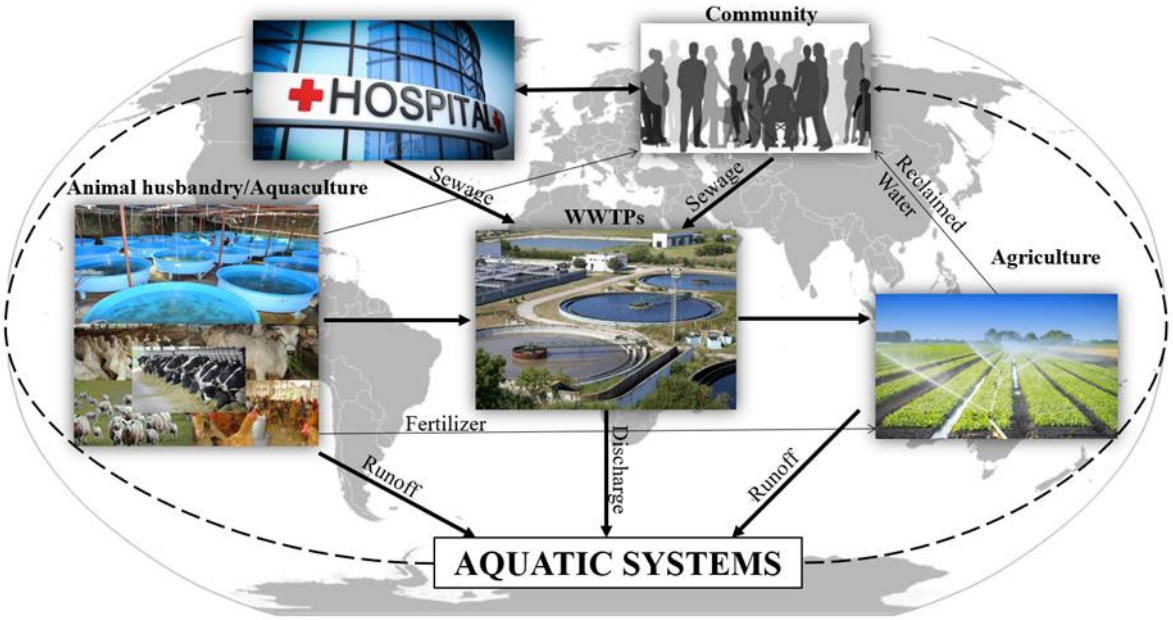

Figure 2: Different anthropogenic activities that result in the dissemination of antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARB) in aquatic environments, adapted from Tripathi and Cytryn, 2017;

Wastewater treatment plants (WWTPs) are among the main sources of antibiotic resistance to aquatic systems. The high bacterial densities present in these environments, together with the diversity of resistance genes in various forms – resistant bacteria able of conjugation, free plasmids/DNA and phage particles -are the main reasons (Silva et al., 2018; Rizzo et al., 2013; Moura et al., 2012). In addition, the presence of antibiotics and other pollutants, such as biocides, pharmaceuticals, and metals, further increases the selection of resistant strains in these facilities (Lood et al., 2017).

A wide variety of clinically important ARB have been detected in WWTPs, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* spp., and ESBL-producers (Karkman et al., 2018; Silva et al., 2018). Among the ARGs detected are plasmid-born resistance determinants representing genes conferring resistance to all common antibiotic classes. Resistance to ciprofloxacin, sulfamethoxazole, trimethoprim, quinolone, vancomycin, tetracycline or cefotaxime is frequently detected in effluents of urban residential areas, hospitals, and municipal WWTPs (Narciso-da-Rocha et al., 2018; Sharma et al., 2016; Varela et al., 2016). Amador et al. (2015) studies, for instance, studying the contribution of a WWTP to the spread of antibiotic resistance through treated wastewater released into the Mondego River, exposed not only the presence of trimethoprim/sulfamethoxazole, tetracycline and ciprofloxacin resistance, but also a high frequency of resistance to β-lactams such as cefoxitin, amoxicillin/clavulanic acid combination, cefotaxime, aztreonam, cefpirome, and ceftazidime. In addition, the authors also found that most of the isolates obtained from the sampled sites (affluent

and effluent of the WWTP, the wastewater collector boxes of the city urban area and of the hospital, the surface water of the Mondego River upstream and downstream of the WWTP treated effluent discharge) harbored ESBLs or AmpC β-lactamases, being *bla*$_{OXA}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{EBC}$ the most prevalent genes detected (Amador et al., 2015). Several studies also exposed the role of WWTPs in the dissemination of class 1 integrons (Moura et al., 2012). These genetic elements are capable of capturing, excising and expressing antibiotic resistance gene cassettes, being frequently associated with the development of multi-drug resistance in Gram-negative bacteria (Park et al., 2018). Additionally, some studies have suggested that common technologies that are being applied in WWTPs, such as the biological treatment based on activated sludge, provided an ideal environment for horizontal gene transfer- due to high bacterial densities, high oxygen and high nutrient concentrations - increasing both the prevalence of ARB and ARGs that are transmitted to aquatic ecosystems with treated effluents (Rizzo et al., 2013). Osińska et al. (2017), for instance, concluded that treated wastewater discharged to the aquatic environment increases the frequency of drug-resistant *E. coli* and determinants of antibiotic resistance. The authors exposed that the percentage of *E. coli* strains carrying genes encoding drug resistance, virulence and integrases from treated water samples was significantly higher than in untreated ones. In fact, the impact of tertiary treatments in municipal wastewaters on the diversity of several ARGs has also been investigated. As an example, Silva et al. studies (2018) demonstrated that, although UV-C disinfection removes ARB, isolates detected in the final effluent still represent different ESBL-producing strains with multi-drug resistance traits. Likewise, LaPara et al. (2011) experiments demonstrated that a tertiary-treated wastewater had 20-fold higher concentrations of various resistance determinants than before the treatment. That said, although advanced disinfection facilities can greatly reduce the danger of waterborne diseases, antibiotics, ARB and ARGs can still be selected and released to the environment in treated effluents. In addition, ARB and ARGs are known to occur in aquatic environments without antibiotic contamination and it has been demonstrated that they flow into rivers, groundwater and marine environments by influx of WWTPs effluents (Suzuki et al., 2017; Manaia et al., 2016).

Aquaculture practices are another major driver of the spread of antibiotic resistance in the aquatic environment. In Europe, the laws on the use of antibiotics in aquaculture are restricted and only a few compounds are authorized - amoxicillin, florfenicol, flumequine, oxolinic acid, oxytetracycline, sarafloxacin and sulfadiazine/trimethoprim (Pereira et al., 2015). However, 90% of the world aquaculture production is carried out in developing countries, which lack regulations on the use of antibiotics (Watts et al., 2017). In these countries, the use of prophylactic antibiotics is a widespread practice that exposes both sick and healthy individuals to high concentrations of these compounds. In addition, fish do not effectively metabolize antibiotics. As a consequence, some studies suggested that approximately 70–80% of the antibiotics applied in aquaculture are

dispersed into water systems allowing for bacteria to be exposed (Santos and Ramos, 2018). Additionally, these systems have been designated as "hotspots" for ARGs exchange between bacteria in the aquaculture environment and human pathogens. As an example, the work of Rhodes et al. (2000) exposed that specific resistance plasmids previously associated only with fish farm environments are similar to those associated with human pathogens. In addition, the authors also highlighted that fish pathogens such as *Aeromonas* can transfer and share determinants for resistance to antibiotics with pathogens such as *E. coli* isolated from humans (Rhodes et al., 2000).

As aquaculture, animal husbandry also plays a crucial role in antibiotic resistance spread to aquatic systems. In animal production, antibiotics are used not only for the treatment of diseases but also for disease prevention and growth promotion (Gebreyes et al., 2017). In Europe, the antibiotics use as growth promotors was banned in 2006 (Regulation 1831/2003/EC). However, this is a continuous practice in several non-European countries (Directorate-General for Health and Food Safety, 2017). In these countries, in cattle, sheep, pigs and poultry, antibiotics such as amoxicillin, colistin, erythromycin, florfenicol, oxytetracycline, penicillin, streptomycin, sulfamethazine and tetracycline are allowed as growth promotors (Directorate-General for Health and Food Safety, 2017). This frequent use of critically important antibiotics for human health in animal husbandry is extremely worrying. It exerts a strong selective pressure facilitating the emergence of ARB. In animal production samples, resistance to tetracycline, penicillins and sulphonamides has been commonly observed among bacterial isolates (Wall et al., 2016). Thus, farm animals may become potential reservoirs of resistant pathogens that can reach the aquatic systems. In fact, several studies demonstrate that antibiotic use in food animals influences the occurrence and distribution of ARB in humans. The work of Price et al, (2007) for instance, concluded that the occupational exposure to antibiotic-resistant *E. coli* from live-animal contact, in the broiler chicken industry, may be an important route for its entry into the community. The authors found that the load of ARB in the guts of farmers using antibiotics as animal growth promoters was higher than those not using them and the general population (Price et al., 2007). Likewise, the prevalence of multi-drug-resistant *Staphylococcus aureus* in humans has been directly associated with the time spent on animal farms as reported by Rinsky et al. (2013). Consequently, ARB and ARGs can be disseminated to aquatic systems not only through sewage discharges, but also through occasional leaching from animal farms or through residual antibiotics in post-treated animal wastes via runoff when utilized as fertilizers on farmlands (Zhang et al., 2014). Thus, agriculture also has a great influence on the amount of ARB and ARGs that reach the aquatic environments.

From aquatic systems, antibiotic resistance can reach humans through several routes. For instance, in developing countries, in the current situation of water demand, farmers often do not have alternative sources of irrigation water but to use treated wastewater (Intriago et al., 2018). As a consequence, crops and soil could be contaminated, serving as a potential route of both environmental and human exposure to antibiotic resistance. In fact, wastewater irrigated soils have been found to accumulate antibiotics in concentrations that are several folds higher than the ones found in the irrigation water (Christou et al., 2017). In addition, this contamination seems to endure even without the presence of the contaminant. As an example, Tamtam et al. (2011) sampled a site that had not been irrigated with wastewater for four years, and found high levels of antibiotic resistance in soil, suggesting that antibiotics can remain in these environment for a long time. In Portugal, Araújo et al. (2017) found evidences that contaminated irrigation water constitutes a source of *E. coli* multi-drug resistant strains that may enter the food chain through the consumption of raw vegetables.

When antibiotics are present in soil, they can easily infiltrate into groundwater systems. In fact, several studies exposed the presence of ARB, ARGs and MGEs in groundwater - which is extremely worrying since this is one of the main sources of drinking water. Szekeres et al. (2018), for instance, investigated the presence and prevalence of antibiotic residues, ARGs, MGEs, and microbial contamination in groundwater, at various distances from urban areas, and found relevant concentrations of cefepime, tazobactam, erythromycin, sulfamethoxazole and trimethoprim, also in remote areas.

As consequence of groundwater contamination, drinking water is also a source of antibiotic resistance. As demonstrated, groundwater becomes vulnerable to contamination with antibiotic residues, ARB and resistance genes from the above mentioned sources (Manyi-Loh et al., 2018). In Portugal, Henriques et al. (2012), for instance, exposed the presence of a diversity of carbapenem-resistant bacteria in untreated drinking water from fountains distributed in the North and Central regions of Portugal. On the other hand, although in drinking water disinfection systems these contaminants are usually low or below detection, the presence of antibiotic resistance in drinking water is exposed by several studies, as recently reviewed (O'Flaherty and Cummins, 2017). ARB have been found even in disinfected drinking water, such as those of the genera *Sphingobium*, *Sphingomonas*, *Pseudomonas* and *Acinetobacter* or nonfecal *Enterobacteriaceae* (Vaz-Moreira et al., 2014). Additionally, the occurrence of genes also detected in clinical isolates, encoding resistance to β-lactams, aminoglycosides, macrolides or sulfonamide has also been detected, even after the disinfection procedure (Vaz-Moreira et al., 2014). In fact, according to Vaz-Moreira et al., (2014) for some classes of antibiotics, antibiotic resistance may be more prevalent in tap water than in the groundwater sources. According to the

authors, one explanation could be the selective effect of the disinfection processes or the income of ARB downstream of the disinfection point (Vaz-Moreira et al., 2014). Thus, ARB and ARGs could be transmitted to humans, even by disinfected drinking water, with serious consequences for public health.

Also recreational waters are sources of antibiotic resistance. Since these environments are all interconnected (Figure 2), pathogenic agents found in recreational water are often the result of fecal pollution caused by inadequately treated sewage, storm water discharges, runoff from agricultural activities, inflow from contaminated rivers, discharges from ships, wildlife or domestic animals (O'Flaherty et al., 2019). Several studies reported the presence of ARGs of concern to public health in these waters. As an example, the work of Fernandes et al. (2017) described the occurrence of MCR-1-producing *E. coli* lineages – resistant to colistin - in recreational coastal waters of anthropogenically affected public beaches in Brazil. The authors hypothesized that residents, tourists, and wildlife could be exposed to this infectious threat directly from water exposure, contact with sand, or through food consumption on the beach (Fernandes et al., 2017). Likewise, Leonard et al. (2018) studies exposed the presence of *E. coli* harboring $bla_{\text{CTX-M}}$ in these environments. In addition, the authors also demonstrated an association between surfing and gut colonization by antibiotic-resistant *E. coli* - showing the role of recreational activities in the dissemination of antibiotic resistance. In Portugal, Alves et al. (2014) exposed that seawater of the Berlenga island is a reservoir of multi-drug-resistant *E. coli*, probably from seagull feces, including strains hosting plasmid-mediated quinolones resistance and ESBL genes.

In aquatic systems bacteria tend to form biofilms, which greatly contribute to their role as reservoirs of antibiotic resistance. Biofilms are aggregations of bacteria that live in a highly structured community, which serve as a protective mode of growth that allows microorganisms to survive in hostile environments (Gholami et al., 2017). Several studies suggested that biofilms serve as reservoirs of ARGs (Balcázar et al., 2015). In fact, biofilms harboring important resistance genes have already been detected in drinking water systems, representing a serious threat to human health (Zhang et al., 2019). In addition, according to Gilbert et al. (2002), in biofilms, bacterial cells can exhibit 10 to 1,000 times less susceptibility to specific antimicrobial agents. Stewart and Costerton hypothesized that poor antibiotic penetration into the polysaccharide matrix, the presence of cells harboring ARGs and triggered stress responses under unfavorable chemical conditions within the biofilm matrix, could explain this reduced susceptibility (Stewart and Costerton, 2001). Aggravating this problem, different studies also showed increased conjugation efficiencies in biofilms, when compared to free-living cells (Salcedo et al., 2015; Luo et al., 2005; Hausner and Wuertz, 1999). One explanation for the role

of biofilms in the enhanced acquisition and spread of ARGs, in aquatic systems, could be the high cell density and close contact among cells within the biofilm matrix, together with increased cell competence and accumulation of MGEs in these habitats (Fux et al., 2005).

Aquatic sediments also represent an environmental matrix where the dissemination of antibiotic resistance is prone to occur. These sediments can retain several antibiotics, used in a plethora of anthropogenic activities (Figure 2). In fact, according to Haller et al. (2009), bacteria can survive longer in sediments than in the water column, since sediments provide favorable nutrient conditions, which could facilitate the transfer, maintenance and dissemination of MGEs in this matrix. Numerous studies highlighted the importance of aquatic sediments in the dissemination of ARGs. Kristiansson et al. (2011), for instance, found high levels of ciprofloxacin in river sediments, downstream from a treatment plant, and MGEs, such as class 1 integrons and insertion sequence common regions (ISCR) elements, highly overrepresented in these sediments. Also Chen et al. (2013) studies demonstrated antibiotics accumulation in this environment. Studying the presence of ARGs in an estuary, the authors found that concentrations of tetracycline resistance genes in the sediments were at least 100 times higher than those in the water. Compared to freshwater sediments, the role of marine sediments in antibiotic resistance dissemination has not been well documented. However, some studies show that marine sediments also serve as a pool of resistance genes (Zhao et al., 2017, Yang et al., 2013). In marine samples, azithromycin, ciprofloxacin, erythromycin, sulfamethoxazole, triclocarban, and trimethoprim resistance were already detected (Scott et al., 2016).

As demonstrated, aquatic environments, which include surface water and groundwater bodies, are frequently impacted by anthropogenic activities and thus are important reservoirs of resistance. This continuous spread of antibiotic resistance is of growing environmental and public health concern. Contaminated water resources can impact human health through drinking water, agricultural/animal production, and even body contact, increasing the prevalence of waterborne infections caused by bacteria harboring important resistance genes, which lower the therapeutic options available. From the community, ARB and ARGs are released into the aquatic systems restarting this cycle.

1.4 Non-antibiotic contaminants that select for antibiotic resistance

Exposure to non-antibiotic compounds with antimicrobial activity, such as biocides and metals, can induce or select for bacterial adaptations that result in decreased susceptibility to one or more antibiotics. This may occur via non-specific mechanisms that confer resistance to both

compounds; or by selection of genetic determinants for resistance to non-antibiotic agents that are physically linked to those for antibiotic resistance (Wales and Davies, 2015).

The development of biocides with limited toxicity for animal tissues, such as quaternary ammonium compounds (QACs), has led to the increased use of such compounds daily - in antiseptics, disinfectants, preservatives, antifouling compounds, food preparation, among many other applications. However, soon it was realized that their use could select for antibiotic resistance (Wales and Davies, 2015). Different genes encoding reduced susceptibility to biocides and antibiotics can be linked on the same MGE. Consequently, the selection for one resistance gene will lead to the co-selection of the other, and multi-drug resistance may be transferred in a single event – resulting in co-resistance. In fact, *qac* genes have been found to be associated with different MGEs. According to the European Food Safety Authority, these genes are often together with *sul1* genes - encoding sulphonamide resistance - as part of class 1 integrons, which can harbor other resistance genes (EFSA, 2008). Naas et al. (2001) identified a *qacI* gene on a class 1 integron containing 9 different genes, in addition to the *qac* resistance gene, encoding resistance to β-lactams, aminoglycosides, phenicol, rifamycins and sulphonomides. Furthermore, the integron was located on a transposon residing in a plasmid, and therefore easily disseminated (Naas et al., 2001).

Besides co-resistance, several experiments also exposed the existence of cross-resistance to antibiotics and biocides, meaning that a single resistance mechanism (e.g. an efflux pump) confers resistance both to antibiotics and biocides (Blanco et al., 2016). The AcrAB-ToC efflux pump system, for instance, common in several clinically-relevant Gram-negative bacteria such as *E. coli* and *P. aeruginosa*, can confer resistance both to several antibiotics (e.g. chloramphenicol, fluoroquinolones, some β-lactams, tetracycline, rifampicin) and to biocides (acriflavine, ethidium bromide, cetrimide, and triclosan). Another example is the Mex efflux systems of *P. aeruginosa*, which are able to pump chloramphenicol, fluoroquinolones and tetracycline (antibiotics); as well as acriflavine, ethidium bromide and triclosan (biocides) (Piddock, 2006).

The above-mentioned examples clearly show the role of biocides in the antibiotic resistance problem. Consequently, concerns have been raised that reduced susceptibility to biocides may foster an elevated frequency of antibiotic resistance.

As in the case of biocides, the co- and cross-resistance of antibiotic and metals in bacteria populations, due to co-selection processes, can maintain and promote both antibiotic and metal resistance dissemination. In addition, co-regulatory mechanisms - that arise when multiple resistance genes, that confer resistance to different toxic compounds, are controlled by a single

regulatory mechanism – can also occur (Figure 3)(Henriques et al., 2016; Baker-Austin et al., 2006).

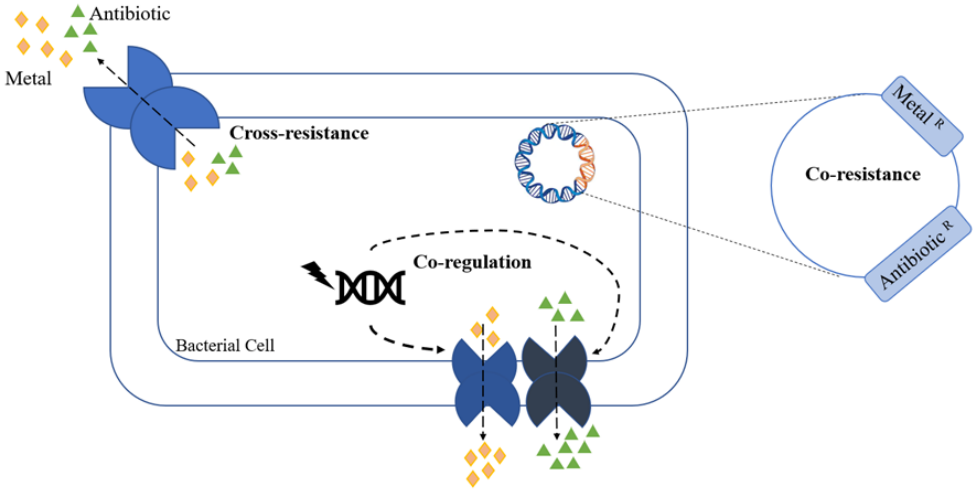

Figure 3: Mechanisms of cross-resistance, co-resistance and co-regulation of metal and antibiotic resistance, adapted from Pal et al., 2017;

1.4.1 Co-resistance to metals and antibiotics

Co-resistance to metal and antibiotic compounds occurs when different genes encoding both metal and antibiotic resistance are located together on a MGE. Thus, the selection of one gene leads to the co-selection of the other (Figure 3, Henriques et al., 2016; Baker-Austin et al., 2006). In 1993, the genetic linkage of mercury and antibiotic resistance traits on plasmids was demonstrated by Summer et al. (1993) when mercury resistance was co-transferred with antibiotic resistance. In fact, in 1995, the location of the *mer* operon on the *Tn*21 transposon was uncovered (Osbourn et al., 1995). This transposable element usually carries class 1 integrons that aren't mobile by themselves, but are responsible for the acquisition and expression of antibiotic resistance gene cassettes (Liebert et al., 1999).

This association between mercury and antibiotic resistance has been reported in other studies. McIntosh et al. (2008) portray the presence of *Aeromonas salmonicida* subsp. *salmonicida*, isolated from an aquaculture facility, carrying a plasmid harboring the *mer* operon and multiple ARGs (*aadA7*, *sul1*, *sul2*, *floR*, *tetA*, *tetR*, *strA*, *strB*, and bla_{CMY-2}). Also, in a plasmid recovered from animal samples, Schlüter et al. (2014) detected genes encoding resistance to aminoglycosides, penicillins, broad-spectrum β-lactams, tetracycline, sulfonamides, quinolones and trimethoprim, in addition to genes encoding resistance to mercury, tellurium, nickel, and quaternary ammonium. Sinegani et al. (2017), studying the antibiotic resistance profile

of bacteria isolated from metal-polluted soils in Iran, found not only a high rate of co-resistance towards Hg and amoxicillin, ampicillin, tetracycline and vancomycin, among the Gram-negative isolates; but also towards Zn, Ni, Hg, and the β-lactam antibiotics among the Gram-positive isolates. Knapp et al. (2017) examined 90 garden soils from Australia and found that even low concentration of metals, such as mercury, copper, zinc, arsenic, nickel and lead, can co-select for antibiotic resistance to β-lactams, tetracyclines and sulfonamides. Likewise, while investigating the distribution of sulfonamides and tetracycline resistance genes in agricultural soils across China, Zhou et al. (2017) exposed that the co-selection of resistance to metals and ARGs was significant for Cu, Hg and sulfonamides resistance genes.

Another example of the association of antibiotic and metal resistance genes on MGEs includes the copper resistance gene, *tcrB*. Several studies demonstrated the correlation between copper resistance and resistance to macrolides and glycopeptides. Different authors proposed that copper resistance (conferred by the *tcrB* gene) is physically linked to the *vanA* gene cluster (responsible for glycopeptide resistance) and *ermB* gene (responsible for macrolide resistance) on a single transferable plasmid (Hasman and Aarestrup, 2002). In fact, further support for this hypothesis was obtained by the repeated observation of the presence of both *tcrB* and *ermB* genes on a transferable plasmid containing the transposon *Tn*1546 (Hasman and Aarestrup, 2005). The association of *tcrB* gene and other ARGs was also found. Amachawadi et al. (2011), for instance, exposed the association of copper, erythromycin and tetracycline resistance genes in the same MGE. Additionally, other studies exposed the association of different copper and ARGs. As an example, the co-existence, on the same plasmid, of copper (*pco*) and silver (*sil*) resistance operons, *oqxA* gene, which confers resistance to QACs, and β-lactamase genes ($bla_{\text{CTX-M}}$) has also been reported. Fang et al. (2016), for instance, recently isolated plasmids carrying all these genes from food-producing animals.

Besides mercury and copper, the co-resistance of metals and antibiotics has already been described for other compounds. Osman et al. (2010) isolated a plasmid containing genes encoding resistance to antibiotics and metals such as chromium and cobalt, in an aquatic bacterium. The authors concluded that it is expected that these genes, in case of horizontal gene transfer, are transmitted at the same time due to its close arrangement in the plasmid (Osman et al., 2010). Similarly, Mahmud et al. (2016) study found an association between resistance to chromium and β-lactam antibiotics, such as imipenem, ceftazidime and amoxicillin, in bacteria isolated from tannery wastes. Metals, such as cadmium and zinc, driving the co-selection of methicillin resistance in *Staphylococcus aureus*- trough horizontal gene transfer of MGEs containing both *mec* and *czr* genes - was described by Cavaco et al. (2010). In fact, in another research, these authors also demonstrated the association of zinc resistance and MRSA from swine and veal

calves, suggesting that the use of zinc as a food additive might have contributed to the emergence of MRSA (Cavaco et al., 2011). Also Ghosh et al. (2000) proved the association of antibiotic and metal resistance genes on MGEs. In their studies, the authors described plasmids from *Salmonella* strains that confer resistance to ampicillin, arsenic, chromium, cadmium and mercury. In fact, the authors also demonstrated that, upon plasmid removal, the strains became sensitive to these toxicants (Ghosh et al., 2000). The resistance to arsenic, copper, mercury, silver, tellurium, chloramphenicol, kanamycin and tetracycline, carried by the resistance plasmid R478, was also described by Gilmour et al. (2004). In addition, a plasmid encoding resistance to β-lactams (harboring genes $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$ and $bla_{\text{OXA-1}}$), aminoglycosides [genes *aac-(6')-1b-cr* and *aadA2*], tetracyclines [*tet(A)* and *tetR*], trimethoprim (*dhfrXII*), sulphonamides (*sul1*), quaternary ammonium compounds (*qacEΔ1*), macrolides [*mph(A)-mxr-mphR(A)*] and metal ions (silver, copper and arsenic) was found in Sandegren et al. (2012) investigation.

As demonstrated, genes encoding resistance to metals such as copper, silver, cadmium, zinc and mercury frequently co-occur with resistance genes for many classes of antibiotics, e.g. sulphonamides, β-lactams, amphenicols, tetracyclines and aminoglycosides. In fact, as demonstrated, MGEs carrying resistance genes to antibiotics and metals/biocides are widely distributed in several environments.

1.4.2 Cross-resistance to metals and antibiotics

Cross-resistance can arise when a single mechanism, usually a membrane transport system, can confer resistance both to antibiotics and metals (Figure 3)(Pal et al., 2017). Cheng et al. (1996) demonstrated the cross-resistance to tetracycline and cobalt due to the TetA(L) protein that promotes the efflux of both compounds. Likewise, in 1998, Hernández et al. observed that the growth in the presence of vanadium induced multi-drug resistance phenotypes in *Escherichia hermannii* and *Enterobacter cloacae*. The authors proposed that these phenotypes could be the result of the expression of a membrane-bound efflux system (Hernández et al., 1998).

Several studies described the role of efflux systems in cross-resistance to metals and antibiotics. Mata et al. (2000), for instance, were the first to describe a multi-drug efflux pump in *Listeria*. In this organism, the MdrL protein encodes an efflux pump that reduces the efficacy of cefotaxime, clindamycin, erythromycin and josamycin while also eliminating metals, such as Zn, Co and Cr from the bacterial cell (Mata et al., 2000). Aendekerk and co-workers described a new efflux pump (MexGHI–OpmD) in *P. aeruginosa* responsible for cross-resistance to vanadium, ticarcillin and clavulanic acid (Aendekerk et al., 2002). The presence of efflux pumps that efflux both metals and antibiotics in *P. aeruginosa* was also described by Teixeira et al. (2016). The

studies of Nishino et al. (2007) described the regulation of an efflux system involved in multi-drug and metal resistance of *Salmonella enterica* serovar Typhimurium. The authors reported that a two-component regulatory system - BaeSR - increases antibiotic and metal resistance by overexpressing this efflux pump (Nishino et al., 2007). Also, the gold-transporting GesAB system in *E. coli* was exposed as capable of conferring cross-resistance to antibiotics and metals. The work of Conroy et al. (2010) demonstrated that the expression of this system leads to chloramphenicol, cloxacillin, nafcillin, thiamphenicol, amikacin, bleomycin, cefoxitin, cephalothin, chlortetracycline and oxacillin resistance. Moreover, the authors also concluded that the expression of copper and silver transporter CusCFBA leads to ethionamide, benserazide, sulfamethoxazole, cefmetazole, lincomycin and oxolinic acid resistance (Conroy et al., 2010). More recently, in 2017, the work of Flach et al. showed the co-selection of ARB by a copper and zinc-based antifouling paint in the marine environment via RND efflux systems (Flach et al., 2017).

Besides efflux systems, another mechanism was demonstrated responsible for cross-resistance to metal and antibiotic compounds – a disulfide bond formation system. The work of Hayashi et al. (2000) proposed that in *Burkholderia cepacia* the DsbA-DsbB disulfide bond formation system could increase bacterial resistance to antibiotics such as β-lactams, erythromycin, kanamycin, novobiocin and ofloxacin, along with metals such as Cd and Zn.

1.4.3 Co-regulation of resistance mechanisms to metals and antibiotics

When bacteria are exposed to a contaminant such as metals, the expression of antibiotic resistance-encoding genes can be altered forming a coordinated response to both compounds – co-regulation (Figure 3)(Baker-Austin et al., 2006). In 1995, Nakajima et al. demonstrated that the overexpression of the Rob protein - a binding protein encoded by the *robA* gene - increases resistance to metals, as well as to multiple antibiotics in *E. coli*, including silver, cadmium and mercury in addition to tetracycline, chloramphenicol and novobiocin (Nakajima et al., 1995).

This mechanism of co-regulation is frequently described in *P. aeruginosa*. As an example, the work of Canejo et al. (2003) aimed to investigate which of the siliconized latex urinary catheters components were responsible for the decreased activity of carbapenems observed for this organism. The authors concluded that the zinc present in this material led to the OprD loss, a protein that normally forms pores that allow permeability to carbapenems. Thus, the loss of this protein in the presence of zinc is responsible for reducing the activity of carbapenems against these bacteria (Conejo et al., 2003). These findings were corroborated by the work of

Perron et al. (2004) that studied the response of *P. aeruginosa* upon exposure to metals or antibiotics, to investigate whether common regulatory mechanisms govern resistance to both types of compounds. As Canejo et al. (2003), the authors found that *P. aeruginosa* isolates exposed to zinc were also resistant to other metals such as cadmium and cobalt, and to the carbapenem antibiotic, imipenem. This study went even further and proposed that this cross-resistance to zinc and imipenem was due to a co-regulation mechanism associated with a two-component regulatory system (CzcR-CzcS) that repressed OprD production (Perron et al., 2004). Later, in 2007, Caille et al. (2007) found that not only CzcR-CzcS could repress the production of this protein, but also a Cu-dependent regulatory system - CopR-CopS. The authors proposed that the regulator CopR links copper resistance to zinc tolerance by activating the czcRS operon coupling both CzcR-CzcS and CopR-CopS systems, leading to copper, zinc, cadmium, cobalt and imipenem resistance (Caille et al., 2007).

Co-regulation mechanisms resulting in metal and antibiotic resistance have also been described for other organisms. For instance, Lee et al. (2005) demonstrated that the *mdtABC* operon in *E. coli* – encoding for an efflux system – was upregulated in response to stress caused by zinc excess. This system has been implicated to resistance to certain antibiotics, including novobiocin, which is of concern, since findings suggest that *mdt* is upregulated in response to zinc in environmentally and clinically-relevant concentrations (Lee et al., 2005). Also, Hao et al. (2014) described this co-regulation mechanism in *E. coli*. According to the authors the *marRAB* operon - that regulates diverse genes involved in multi-drug resistance - could sense and bind Cu(II) leading to enhanced antibiotic resistance (Hao et al., 2014). Additionally, the upregulation of acrAB-tolC efflux pump due to the presence of chromate and copper was also described in *E.coli*, resulting in enhanced resistance to several antibiotics. The authors also demonstrated that arsenic, copper and zinc can induce tetracycline resistance in *Enterobacter* (Chen et al., 2015).

As has been shown, non-antibiotic compounds, such as biocides and metals, may contribute to the promotion of antibiotic resistance trough co-selection. That said, the decrease of antibiotic use does not necessarily prevent the spread and maintenance of antibiotic resistance in clinical or in natural environments. These observations have direct public health implications considering that some pathogenic strains of several bacterial genera, such as *Pseudomonas*, have established environmental reservoirs, and resistance genes can be laterally transferred from environmental organisms to human commensals. For this reason, it is essential to fully understand the drivers of the evolution and dissemination of antibiotic resistance.

1.5 The copper and zinc problem in the environment

Although metals are naturally-occurring elements, environmental contamination and human exposure to these compounds result from anthropogenic activities. Besides their use as antimicrobials, copper and zinc have been associated with industrial, agricultural and aquaculture settings. Concerning industrial activities, Kabir et al., (2012) for instance, while evaluating the status of trace metal pollution in soils of industrial areas, concluded that copper and zinc were among the most frequently found in smelter and metal industries. In fact, the authors also observed that levels of these metals frequently exceeded those set by the environmental legislation in the samples analyzed (Kabir et al., 2012). The relationship between these metals and agricultural activities has also been exposed by several studies. As an example, Tella et al. (2016) demonstrated that organic wastes application in agriculture increases zinc and copper availability in amended soil. This association was also demonstrated by Malan et al. (2015). Evaluating the concentrations of metals in soils and vegetables produced in horticultural areas, the authors found that copper and zinc concentrations exceeded the maximum permissible concentrations both for soils and vegetables, being farmyard manures the biggest agronomic contributor of these metals (Malan et al., 2015). In fact, a major agricultural source of these metals relates to their common use as feed additives, with copper and zinc ending up in the manures of food-producing animals (Seiler and Berendonk, 2012). Regarding the presence of copper and zinc in aquacultures, Cañedo et al. (2017) work found high levels of these metals in shrimp aquaculture and, according to the authors, shrimp feed was the main responsible for the increases in copper and zinc concentrations in this settings. In addition, fish farmers frequently use pharmaceuticals (such as antibiotics) and metal-containing products to prevent fouling, to feed and to treat fish and limit the spread of infections. For example, copper-containing materials are applied as anti-fouling agents for farm cages, nets and some cages are even made from copper alloys (Seiler and Berendonk, 2012).

The spread of copper and zinc in the environment is extremely worrying since there is a growing body of evidence supporting the hypothesis that these metals select for antibiotic resistance. This link was acknowledged in diverse environments that include industrial, clinical, aquatic and agricultural settings. Studying the effect of selective pressure of sub-lethal level of metals on the fate and distribution of ARGs in Xiangjiang River, China, Xu et al. (2017) demonstrated that the abundance of ARGs downstream of the Xiangjiang River was significantly higher, which was closely related to the anthropogenic activities, i.e. metal mining, in that area. Additionally, the authors found that levels of metals, such as copper and zinc, were significantly correlated with abundance of *sul3*, *tetA*, *tetM*, *qepA*, *qnrA*, *qnrB* and *qnrD* genes (Y. Xu et al., 2017). Likewise, Xu et al. (2017) studies on the distribution of tetracycline resistance genes and AmpC β-lactamase genes, in industrial sewage plants, showed a significant positive correlation

between abundance of ARGs and metals, particularly between *tet* and Zn, and bla_{AmpC} and Cu (Y.-B. Xu et al., 2017). An example of copper and zinc selection for ARB in aquatic environments is the work of Gao et al. (2015), which concluded that several metals, including copper and zinc, impose significant selections on the proliferation and dissemination of erythromycin resistance genes in wastewater. Finally, several studies focused their findings on the consequences of copper and zinc presence in agriculture settings. The work of Hu et al. (2016), for instance, demonstrated that copper contamination significantly affects the abundance, diversity and dissemination potential of a broad spectrum of ARGs in agricultural soils. Moreover, Lin et al. (2016) concluded that the upsurge of ARGs after the application of a mineral fertilizer in agriculture might be closely associated with the accumulation of copper and zinc in soil. In fact, copper presence in soils has been shown to increase the presence of ARGs, even at low levels (Knapp et al., 2017). Of concern is the metal selection of antibiotic resistance in food animals. The use of zinc in animal feed has been linked not only to the occurrence of MRSA in these animals (Cavaco et al., 2011), but also to an increase in MDR *E.coli* strains (Yazdankhah et al., 2014). Similarly, the use of copper as a feed additive selects for copper-resistant *Salmonella* spp. in swine, with an associated increase in multi-drug resistance (Medardus et al., 2014).

As described above, the dissemination of antibiotic-resistant organisms in copper and zinc contaminated environments can be explained by co-selection (Table 1) and co-regulation mechanisms (Table 2) (Poole, 2017). This is extremely worrying since levels of metals are currently higher than levels of antibiotics (Baker-Austin et al., 2006). In Europe, the concentrations of zinc in agricultural land exceed the threshold concentration of 200 mg kg^{-1} in more than 20% of the samples analyzed (Tóth et al., 2016). Concerning copper concentrations in European agricultural land, soil samples with high copper concentrations can be found in the countries of the Mediterranean being France, Italy, Portugal and Romania the agricultural lands more affected (Tóth et al., 2016). Concerning the release of these metals to water resources, urban wastewater treatment plants are responsible for 45.5% of the metal compounds that reaches aquatic systems, 19.3% is due to mining activities, agriculture contributes with 14% and thermal power stations are responsible for 6.4% of this release (https://www.eea.europa.eu). In Portugal, approximately 2979 kg per year of copper compounds are released in the water bodies, whereas the release of zinc compounds reaches 16560 kg per year (https://europa.eu). The Water Framework Directive (2000/60/EC) establishes good ecological practices to achieve god quality of European water being the reduction of metal concentrations included. However, specific legislation exists mainly to cadmium, mercury and lead (https://www.eea.europa.eu). Still, there is a more restricted legislation for the presence of copper and zinc in soils. Within this matrix, for copper, a value between 50 mg kg^{-1} and 200 mg kg^{-1} is permitted in soils where crops are grown for commercial purposes and intended solely for animal consumption; while a value between 150

mg kg^{-1} and 450 mg kg^{-1} is allowed for zinc compounds (https://europa.eu). These amount of copper and zinc compounds in the environment may have adverse effects. Unlike antibiotics, metals are not subjected to degradation and can subsequently represent a long-term selective pressure, particularly in soils (Baker-Austin et al., 2006). Thus, if indirect selection for bacteria and MGES carrying ARGs takes place in metal contaminated environments, this process may facilitate the spread of antibiotic resistance in human pathogens, which could lead to treatment failure.

Table 1: Examples of metal (copper and zinc) and antibiotic resistance co-selection **(**P=plasmid, C=chromossome) (Poole, 2017);

Metal	Metal^{R} gene(s)	Antibiotic^{R} gene(s)	Location	Organism
Cu	*mco*, *copA*	*ermT*, *tetL*, *dfrK*, *ermC*	P	MRSA (livestock, human)
	pcoA–D, *silABC*	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *bla*$_{OXA-1}$, *aac(6')-Ib-cr*, *aadA2*, *tetA*, *dhfrXII*, *sul1*, *mphR-mxr-mphA*, *qacEΔ1*	P	*Escherichia coli*, *Klebsiella pneumoniae* (human)
	copA, mco	*aadD*, *ermB*, *dfrK*, *tetL*, *apmA*	P	MRSA (livestock)
	tcrB, *cueO*	TC, ER, VN, AG, AP	P	*Enterococcus* spp. (human)
	pcoA–E	*aac(6')-Ib-cr*, *oqxAB*, *CM*, *rmtB*	P	*Escherichia coli* (livestock)
	pcoA–E, *silA–E*	Multiple	P, C	*Salmonella* spp. (livestock)
	pcoA–D, *silA–E*	*bla*$_{TEM-1}$, *strAB*, *sul2*, *tetB*	C	*Salmonella* Typhimurium (human)
	pcoE1, *pcoS*	*bla*$_{TEM-1}$, *bla*$_{SHV-1}$, *aac(6')-Ib*, *strAB*, *aac3*, *dfrA19*, *sul1*, *qacEΔ1*, *catA2*, *ereA*, *arr2*	P	*Klebsiella pneumoniae* (human)
	sil	*bla*$_{OXA-1}$, *bla*$_{SFO-1}$, *aadA*, *aacA4*, *armA*, *tetA*, *mrx-mphA*, *msrE-mphE*, *sul1*, *qacEΔ1*, *catB4*	P	*Leclercia adecarboxylata* (human)
	cusS, *pcoE*	*bla*$_{TEM-1}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *dhfrA14*, *strAB*, *aac(6')-Ib*, *aadA1*, *sul2*, *catA1*, *catB3*	P	*Salmonella* Typhimurium (human)
	silABC, *pcoS*, *pcoE*	*bla*$_{ACC-1}$, *bla*$_{VIM-1}$, *aacA4*, *aadA1*, *strAB*, *qacEΔ1*, *sul1*	P	*Escherichia coli*, *Salmonella* Typhimurium (livestock)
	copA, *cueO*, *pcoA-D*	TC, AG, CM, SP, BL	C	Not specified
	copB	*tetA*, *tetW*	P	*Bacillus megaterium* (aquatic)
	copA, *copB*	*tetA*	P	*Pseudomonas aeruginosa* (aquatic)
Zn	*czrC*	*mecA*	C	MRSA
	cad	*mphC*, *msrA*, *aph(3')-IIa*	P	Not specified
	czcD	*qnrA*, *qnrB*	P	*Shewanella oneidensis* (aquatic)

Table 2: Examples of metal-responsive antibiotic resistance and regulatory genes (Poole, 2017);

Metal	Antibiotic^R gene(s)	Antibiotic^R	Regulatory gene(s)	Organism
Cu	unknown	isoniazid	*Ms2173*	*Mycobacterium smegmatis*
	unknown	unknown	*cpxRA*	*Escherichia coli*
	oprD	carbapenem	*copRS, czcRS*	*Pseudomonas aeruginosa*
	unknown	unknown	*soxS*	*Escherichia coli*
Zn	*oprD*	carbapenem	*czcRS*	*Pseudomonas aeruginosa*
	arn	polymyxin	*basRS*	*Escherichia coli*
	unknown	polymyxin	*colRS*	*Pseudomonas aeruginosa*
	lmrA	unknown	Unknown	*Lactobacillus lactis*
Cu/Zn	*acrD, mdtABC*	unknown	*baeSR*	*Salmonella* Typhimurium
	unknown	unknown	*rpoE*	*Escherichia coli*

Indubitably, antibiotic resistance is a public health problem of increasing magnitude. Pathogenic bacteria can become resistant to all the treatment options both by mutation or horizontal gene transfer. Additionally, despite early claims that the acquisitions of these traits are always associated with a fitness cost, recent studies have shown that ARB are not always less fit, and that once a resistance mechanism has been selected, it may persist, even in the absence of antibiotic pressure. This fact is concerning, particularly in aquatic systems. The aquatic environment can serve both as a reservoir of ARB, ARGs, antibiotics and other contaminants, such as biocides and metals, that reach these systems due to anthropogenic activities, and that contribute for the dissemination of this problem due to co-selection mechanisms. This process is particularly worrying in the case of copper and zinc since several studies associated the presence of these metals, which are widely used, with antibiotic resistance and ARGs dissemination. That said, understanding the effect of copper and zinc in the selection of antibiotic resistance in aquatic systems, as well as how anthropogenic activity might be causing its evolution, is essential.

1.6 Aims of the study

As demonstrated, several studies associated the presence of copper and zinc with antibiotic resistance and ARGs dissemination. However, data demonstrating which alterations could be induced directly by copper and zinc exposure in bacterial strains are lacking. In addition, to the best of our knowledge, none of the previously studies confirm, using a microcosm approach, if metals can co-select for antibiotic resistance in complex bacterial communities.

That said, the main goal of this study was to investigate the role of non-antibiotic contaminants, such as metals, in the selection of antibiotic resistance, particularly in aquatic systems. Specific aims were: 1) to investigate genotype and phenotype alterations induced by the presence of copper and zinc on selected bacterial strains; 2) to confirm if metals select for antibiotic resistance in complex bacterial communities.

2. MATERIAL AND METHODS

2.1 Experimental evolution assay

2.1.1 Bacterial strains evolution

An experimental evolution approach was used to investigate genotype and phenotype alterations induced by the presence of copper ($CuSO_4.5H_2O$, Chem-Lab, Belgium) and zinc (ZnCl, Sigma, Germany). *Escherichia coli* ATCC® 25922™ and *Aeromonas hydrophila* CECT 839^T (Table 3 and 4) were evolved for 80 days, corresponding to 40 transfers to fresh medium. Populations of each strain (5 replicates per strain) were propagated in agar plates with a concentration gradient for each metal. To prepare each gradient, 90 mm petri plates with two layers of agar were used (Figure 4). The lower layer consisted of 10 mL of Plate Count Agar (Merck, Germany) poured with the plate slanted just sufficiently to cover the entire bottom. After placing the dish in the normal horizontal position, another 10 mL of PCA was added containing an appropriate concentration of the metal. The thickness ratio of agar layers, established a uniform concentration gradient (method described in Szybalski and Bryson, 1952).

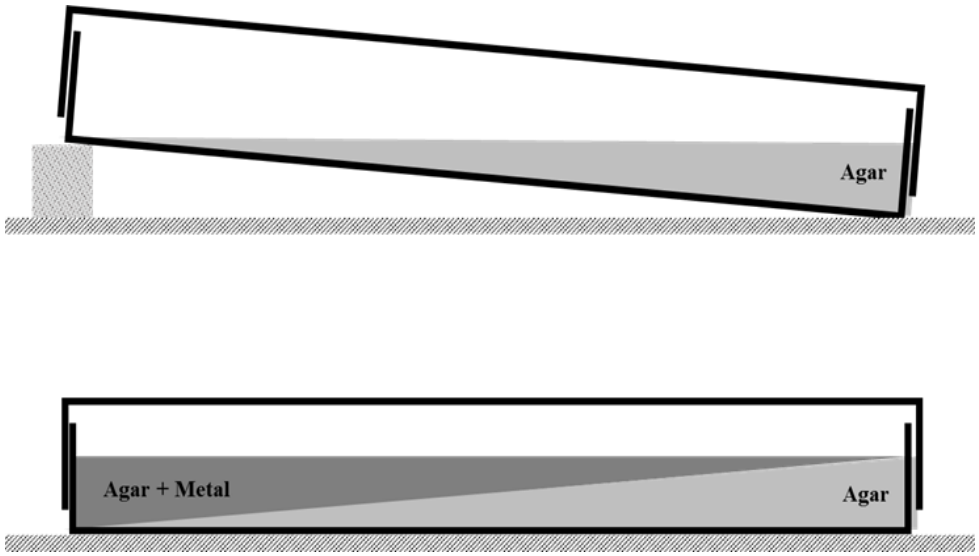

Figure 4: Preparation of a gradient plate (adapted from Szybalski and Bryson, 1952);

Bacterial colonies that were able to grow at the highest concentrations of each metal were transferred to a new plate each 48h. From each plate the number of transferred colonies was enough to achieve an O.D. between 0.3 and 0.5, at 600 nm, in 1 mL of LB. *E. coli* populations were grown at 37 ºC and *A. hydrophila* populations were grown at 30 ºC for 48h. The concentration of each metal was increased gradually according to each population adaptation. For further comparisons, 5 control populations for each strain were propagated in parallel without the contaminant. Ancestral populations were stored in 20% glycerol at -80 °C, as well as the evolved populations after 40 and 80 days (corresponding to 20 and 40 transfers, respectively). At the end of the assay, 31 populations of each species were obtained for further analysis: (a) *E. coli*: 1 ancestral (E..A), 15 populations after 20 transfers (E.20 1-5, E.20 Cu1-Cu5, E.20 Zn1-Zn5) and 15 populations after 40 transfers (E.40 1-5, E.40 Cu1-Cu5, E.40 Zn1-Zn5); and (b) *A. hydrophila*: 1 ancestral (A.A), 15 populations after 20 transfers (A.20 1-5, A.20 Cu1-Cu5, A.20 Zn1-Zn5) and 15 populations after 40 transfers (A.40 1-5, A.40 Cu1-Cu5, A.40 Zn1-Zn5).

Table 3: Features of *Escherichia coli* ATCC® 25922™ (ATCC: The Global Bioresource Center; EUCAST; Minogue et al., 2014);

***Escherichia coli* ATCC® 25922™ ***						
Source	**Growth conditions**	**Putative determinants of virulence**				
		Adhesion	Toxins	Iron acquisition	Antibiotic/Metal resistance	Other
Human clinical sample	Growth temperature (in °C): 37 Incubation time: 24h Atmospheric needs: aerobic	Type IV pilus secretin, PilQ Pilus class II adhesin Fimbrial-like adhesin exported protein Fimbrial regulatory protein, KS71A Fimbrial minor pilin protein, PAP Fimbrial adapter, PapK Fimbrial protein, PapE Type-1 fimbrial protein Fimbrial protein subunit SfaH, SfaS, SfaG, SfaA Fimbrillin, MatB Adhesin	Bacterial virulence factor hemolysin Toxin Ldr, type I toxin-antitoxin system Toxin YkfI, YhaV, YfjG, YeeV RTX toxin acyltransferase Enterotoxins Addiction module toxin, Txe/YoeB	Siderophore-interacting FAD-binding domain protein TonB-dependent siderophore receptor Siderophore-iron reductase, FhuF Catecholate siderophore receptor, *fiu* Bacterioferritin-associated ferredoxin, *bfd* Bacterioferritin, Bfr Ferrichrome-iron receptor, FhuA Ferric uptake regulation protein, Fur Ferrienterobactin-binding periplasmic protein, FepB Ferric enterobactin transport system permease protein, FepD, FepG Ferric enterobactin (Enterochelin) transport , FepE Ferrienterobactin receptor, FepA	Bicyclomycin resistance, Bcr Polymyxin resistance, ArnA MFS transporter, EmrB MDR protein, MdtL, D, MdtF, MdtE, B, MdtM, MdtK, MarA, MarR, MdlB, MdtD Tellurite resistance, TehB, TehA Multiple stress resistance, BhsA Peptide resistance, PagP Fosmidomycin resistance, Fsr Acriflavine resistance, A and B Zinc resistance, ZraP QAC - resistance, SugE β-lactamase, AmpC	ABC transporter, nickel/metallophore periplasmic binding protein ATP-dependent zinc metalloprotease, FtsH RIP metalloprotease, RseP Metal-binding protein, ZinT DNA adenine methyltransferase, YhdJ Nickel/cobalt efflux system, RcnA Magnesium and cobalt transport protein, CorA

***Other Collections:** CECT 434, CCM 3954, CCRC 14902, CCUG 7736, CCUG 17620, CCUG 21456, CIP 76.24, DSMZ 1103, IFO 15034, JCM 5491, LMG 8223, NCIMB 12210, NBRC 15034

Table 4: Features of *Aeromonas hydrophila* CECT 839^{T} (Colección Española de Cultivos Tipo – UV; Seshadri et al., 2006);

Aeromonas hydrophila* CECT 839^{T}						
Source	**Growth conditions**	**Putative determinants of virulence**				
		Adhesion	Toxins	Iron acquisition	Antibiotic resistance	Other
A tin of milk	Growth temperature (in °C): 30 Incubation time: 24h Atmospheric needs: aerobic	Tap pilus Pilus biosynthesis Type IV fimbria Type-IV pilus Fimbrillin Adhesin	RTX toxin Cytotonic enterotoxin; Ast Cytolytic enterotoxin; Act Metalloprotease Extracellular protease; EprA1 Extracellular hemolysin Hemolysin Phospholipase A1 Hyaluronidase Toxin transporter	Siderophore synthesis Ferric uptake regulator; Fur	β-lactamase CphA β-lactamase CepS β-lactamase AmpS Chloramphenicol acetyltransferases Bicyclomycin resistance Fosmidomycin resistance Aminoglycoside acetyltransferase Aminoglycoside phosphotransferase SapABCDF peptide intake transport	Invasin Enolase DNA adenine methyltransferase; Dam Glucose-inhibited division protein; GidA Autoinducer synthase; AhyI (Quorum sening) Quorum sensing regulon activator; AhyR Ribosylhomocysteine lyase Collagenase Mucin-desulfating sulfatase

***Other Collections:** ATCC 7966, BCRC 13018, CCUG 14551, CDC 359-60, CIP 76.14, IAM 12460, JCM 1027, LMG 2844, NCIMB 9240, NCTC 8049, DSM 301887, HAMBI 1847;

2.1.2 Determination of tolerance towards metal(loid)s

For each population, minimal inhibitory concentrations for each metal were determined in Plate Count Agar (Merck, Germany) supplemented with arsenic (As; 150, 200, 600, 700, 3000, 4000 and 4500 μg mL^{-1}, As as $Na_2AsO_4.7H_2O$), chromium (Cr III; 200, 300, and 350 μg mL^{-1} Cr as $CrCl_3 \cdot 6H_2O$), copper (Cu; 50, 100, 150, 200, 250, 300 and 350 μg mL^{-1} Cu as $CuSO_4 \cdot 5H_2O$), mercury (Hg; 5, 10, and 15 μg mL^{-1} Hg as $HgCl_2$), nickel (Ni; 200, 300, 400, 500, 600, 700 and 750 μg mL^{-1} Ni as $NiSO_4 \cdot 6H_2O$), and zinc (Zn; 50, 100, 150, 200, 250, 300, 350 and 400 μg mL^{-1} Zn as $ZnCl_2$). Metal(loid)s stock solutions were prepared in distilled water and sterilized. Experiments were conducted in triplicate. Results were registered after 5 days incubation at 37 ◦C for *E. coli* and 30 ºC for *A. hydrophila*.

2.1.3 Antibiotic susceptibility testing

Antimicrobial resistance patterns were determined for the 62 populations (31 for each species) by the agar disc diffusion method on Mueller-Hinton agar (Oxoid, UK), against 16 antibiotics from 6 classes: beta-lactams (penicillins, cephalosporins, monobactams and carbapenems), quinolones, aminoglycosides, phenicols, tetracyclines, sulfonamides and the sulfamethoxazole/trimethoprim combination. Discs (Oxoid, UK) containing the following antimicrobial agents were used: piperacillin (30 μg), piperacillin-tazobactam (36 μg), cefepime (30 μg), cefotaxime (5 μg), aztreonam (30 μg), imipenem (10 μg), meropenem (10 μg), tigecycline (15 μg), tetracycline (30 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), amikacin (30 μg), kanamycin (30 μg), chloramphenicol (30 μg), sulfonamides (300 μg) and trimethoprim-sulfamethoxazole (25 μg). *E. coli* ATCC® 25922™ was used as quality control. Isolates were classified as sensitive, intermediate or resistant according to the EUCAST recommendations (http://www.eucast.org) after 18-24h incubation at 37 ºC for *E. coli* and 30 ºC for *A. hydrophila* populations. For the interpretation of results for tetracycline, nalidixic acid and sulfonamides, the CLSI guidelines were followed (https://clsi.org/).

2.1.4 Minimum inhibitory concentrations (MICs) determination

The minimum inhibitory concentration for several antibiotics was determined for 10 populations of each species – ancestral, 3 populations exposed to copper, 3 exposed to zinc and 3 control populations, frozen at the 80th day (40 transfers). For *E. coli* populations, MICs for piperacillin, nalidixic acid, kanamycin, sulfamethoxazole, imipenem, tigecycline, ciprofloxacin, aztreonam and cefotaxime were determined. For *A. hydrophila* populations, MICs were determined only for piperacillin, kanamycin, sulfamethoxazole and cefotaxime. For MIC determination, cells were resuspended in 0.9% NaCl solution to a 0.5 McFarland standard to inoculate in Mueller–Hinton agar (Oxoid, UK) plates. In each plate a strip with a concentration gradient of each antibiotic (Liofilchem®, Italy) was placed. Plates were incubated for 18-24h at 37 ◦C for *E. coli* and 30 ºC for *A. hydrophila* populations. MICs were defined as recommended by the manufacturer. *E. coli* ATCC® 25922™ was used as quality control.

2.1.5 Growth assay

To evaluate the fitness of each population, growth assays were performed in Luria-Bertani broth (LB, NZYtech, Portugal), LB supplemented with copper ($CuSO_4.5H_2O$, Chem-Lab, Belgium), LB supplemented with zinc (ZnCl, Sigma, Germany) and LB supplemented with sulfamethoxazole (Fluka, USA) (Table 5), as previously described (Hall et al., 2014). Growth assays were performed in 96 well plates covered with a breath-easier sealing membrane (Sigma-Aldrich, USA) using the Synergy HT multi-detection microplate reader (BioTek, USA). Each well was inoculated with 100 µL of fresh medium and 100 µL of an overnight culture (0.05< OD <0.1, 600 nm). Measurements were made in triplicate for each population. The cultures were grown for 24h in LB, 30h in LB supplemented with metal and for 40h in LB supplemented with antibiotic. Optical density was measured every 30 minutes. Assays were performed at 37 ºC for *E. coli* and 30 ºC for *A. hydrophila* populations. Growth rates (r) and doubling time (Dt) were determined for each population as followed: r= ln (OD2/OD1)/(T2-T1) in exponential phase and Dt= ln2/r.

Table 5: Concentrations of the compounds (metals and antibiotic) used in each condition for each population;

Populations	LB+Cu	LB+Zn	LB+Sulfamethoxazole
Escherichia coli ATCC® 25922™	50 µg mL^{-1}	50 µg mL^{-1}	16 µg mL^{-1}
Aeromonas hydrophila CECT 839^{T}	100 µg mL^{-1}	100 µg mL^{-1}	4 µg mL^{-1}

2.1.6 DNA extraction and molecular typing by rep-PCR fingerprinting

Total genomic DNA was extracted from 31 populations of each species – the ancestral, 5 populations exposed to copper, 5 populations exposed to zinc and 5 populations evolved without contaminant, frozen at the 40^{th} day (20 transfers); and 5 populations exposed to copper, 5 populations exposed to zinc and 5 populations evolved without contaminant frozen at the 80^{th} day (40 transfers) – using Wizard® Genomic DNA Purification Kit (Promega, USA). Molecular typing was performed by PCR amplification of the regions between repetitive elements of the bacterial genomes, namely box elements (BOX-PCR) and enterobacterial repetitive intergenic consensus sequences (ERIC-PCR) as previously described (Table 6, Araújo et al., 2017; Versalovic et al., 1994, 1991). The reaction mixtures (25 µL) used for the two sets of primers consisted of 6.25 µL NZYTaq 2 × Green Master Mix (2.5 mM $MgCl_2$; 200 µM dNTPs; 0.2 U $µL^{-1}$ DNA polymerase, NZYtech, Portugal), 15.75 µL of ultrapure water and as for the primers, 2 µL of a 10 µM BOX A1R primer solution, and 1 µL of 10 µM solutions of ERIC primers. PCR reactions were performed using a MyCycler Thermal Cycler (Bio-Rad, California, USA). PCR products were loaded in 1.5% agarose gel for electrophoresis and a molecular weight marker DNA (GeneRuler™ DNA ladder Mix - Thermo Fisher Scientific, USA) was used as an external reference standard. Electrophoresis were run at 60V, in 1xTAE for 2h. At the end of the run the gel was stained for 20 min in a solution of 0.5µg mL^{-1} ethidium bromide (Sigma, USA) and washed in distilled water. Gel images were captured under UV light with the imaging Molecular Imager® Gel Doc™ XR+ System (Bio-Rad, USA).

Table 6: Primers and PCR conditions;

Primer	Target	Amplicon size (bp)	Sequence (5'-3')	Annealing T(ºC)
BOXA1R	BOX elements	Variable	CTACGGCAAGGCGACGCTGACG	53
ERIC1	ERIC elements	Variable	AAGTAAGTGACTGGGGTGAGC	52
ERIC2			ATGTAAGCTCCTGGGGATTCAC	

Program for PCR reaction: 95ºC for 7 min, 30 cycles of 94ºC for 1 min, 1 min at annealing temperature, 65ºC for 8 min, and 65ºC for 16 min;

2.2 Microcosm assay

2.2.1 Sample collection

Water samples were collected from Antuã river included in the Vouga River basin, located in Aveiro, Portugal (Figure 5). Water was collected in sterile bottles and kept on ice for transportation.

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Figure 5: Sampling site in the Antuã River (40º 44.580 N, 08º 34.173 W);

2.2.2 Microcosm experiment setup

To assess metal effects on antibiotic resistance evolution, a lab-scale microcosm was steed up (Figure 6). For this, 9 Erlenmeyer were prepared, each with 150 mL of water sample. After 1 day of rest, 3 replicates were exposed to 50 µg L^{-1} and 3 replicates were exposed to 100 µg L^{-1} of copper ($CuSO_4.5H_2O$, Chem-Lab, Belgium) and 3 replicates were maintained without contaminant. Microcosms were incubated for 20 days at room temperature. The same experimental set up and metal concentrations were used for zinc exposure (ZnCl, Sigma, Germany). Water samples from each microcosm were collected after 10 and 20 days of exposure and used for culture-dependent analysis.

Figure 6: Microcosm experiment setup, 3 L of water (150 mL for each erlenmeyer) was collected at April 11, 2018 for copper assay; and at June 22, 2018 for zinc experiment;

2.2.3 Plate counts on antibiotic-selective media

To determine bacteria abundance and the prevalence of resistance to several antibiotics, water samples were collected from each microcosm after 10 and 20 days of exposure and filtered through a 0.45 µm grid (PALL, Mexico) (Table 7). Membranes were placed on Plate Count Agar (PCA, Merck, Germany) and PCA supplemented with cefotaxime (8 µg mL^{-1}), tetracycline (16 µg mL^{-1}) and kanamycin (64 µg mL^{-1}) for microcosms exposed to copper. For samples exposed to zinc, PCA supplemented with imipenem (8 µg mL^{-1}) was also tested. For each condition, 3 replicates were incubated at 30 ºC. After 48h of incubation colony-forming units (CFUs) on each plate were counted.

Table 7: Summary of the filtration assay, CTX stands for cefotaxime, TET stands for tetracycline, KAN stans for kanamycin, IMI stands for imipenem, volumes filtered after 10 (T10) and 20 days (T20);

Condition		Medium	Filtered volume (µL)	
			T10	T20
Copper assay	Control	PCA	100 (1/100)	100 (1/100)
		PCA+CTX	100 (1/10)	100 (1/10)
		PCA+TET	100 (1/10)	500
		PCA+KAN	100 (1/10)	100 (1/10)
	Cu 50/100 µg L^{-1}	PCA	100 (1/10)	100 (1/10)
		PCA+CTX	100 (1/10)	100 (1/10)
		PCA+TET	500	500
		PCA+KAN	500	500
Zinc assay	Control	PCA	100 (1/100)	100 (1/10)
		PCA+CTX	100 (1/10)	100 (1/10)
		PCA+TET	500	1000
		PCA+KAN	100 (1/10)	100 (1/10)
		PCA+IMI	1000	1000
	Zn 50/100 µg L^{-1}	PCA	100 (1/10)	100 (1/10)
		PCA+CTX	100 (1/10)	100 (1/10)
		PCA+TET	500	1000
		PCA+KAN	500	100 (1/10)
		PCA+IMI	1000	1000

2.2.4 Identification by 16S rRNA gene sequencing

After the maximum exposure time (20 days), colonies were selected from plates supplemented with cefotaxime (8 µg mL^{-1}), for microcosms exposed to 100 µg L^{-1} of Cu and 100 µg mL^{-1} of Zn. For microcosms exposed to Zn, colonies from plates supplemented with kanamycin (64 µg mL^{-1}) were also selected. Each colony was picked and resuspended in 20 µL ultra-pure distilled water and 1 µL of the suspension was used as DNA template for the PCR reaction. The reaction mixture (25 µL) consisted of 0.75 µL of each primer solution, namely 27F 10 µM and 1492R 10 µM (27F: 5'-AGAGTTTGATCCTGGCTCAG-3'/1492R: 5'-GGYTACCTTGTTAACGACTT-3', Weisburg et al., 1991), 6.25 µL NZYTaq 2× Green Master Mix (2.5 mM $MgCl_2$; 200 µM dNTPs; 1.25U DNA polymerase; NZYtech, Portugal) and 16.25 µL of sterile ultra-pure water. A control reaction mixture containing 1 µL of sterile water instead of culture was also included as a negative control. The 16S rRNA gene amplification conditions

consisted of an initial denaturation at 94 °C for 3 min, followed by 30 amplification cycles consisting of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and a final extension step at 72 °C for 10 min. PCR products were purified using NZYtech Gelpure purification kit following the manufacturer's instructions; and sent to GATC Biotech (Germany) for sequencing.

Online similarity searches were performed with BLAST software against the GenBank database at the National Center for Biotechnology Information web site (https://blast.ncbi.nlm.nih. gov/Blast.cgi) and against the EzTaxon database at EzBioCloud (http://www.ezbiocloud.net) for confirmation.

2.2.5 DGGE analysis of bacterial communities

According to the experiment described above, samples exposed to the highest concentration (100 µg L^{-1}) were filtered after 10 and 20 days of exposure for the bacterial communities' analysis. Water samples were filtered through 0.22 µm pore-size filters (Merck, US), and total DNA was extracted as follows: cells were washed from the filters with 2 mL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and collected by centrifugation (13,000rpm, 15 min); pellet was resuspended in 200 µL TE buffer containing 2 mg mL^{-1} of lysozyme and incubated for 1h at 37 ºC followed by 10 min at 65ºC; after the described lysis steps, DNA was extracted and purified using the Genomic DNA purification kit #K0512 (Thermo scientific, US) according to the manufacturer's instructions.

The V3 region of the bacterial 16S rRNA gene was amplified with primers 338F (5'-GACTCCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3'), with a GC clamp attached to the forward primer (Muyzer et al., 1993). The amplification reaction mixture (final volume of 25 µL) contained 1 µL of the DNA template (50 to 100 ng of DNA), 0.75 µL of each primer solution at 10 µM, 6.25 µL NZYTaq 2× Green Master Mix (2.5 mM $MgCl_2$; 200 µM dNTPs; 1.25U DNA polymerase; NZYtech, Portugal) and 16.25 µL of ultra-pure water. Temperature profile included 5 min at 94°C, followed by 30 cycles of 30 sec at 92°C, 30 sec at 55°C and 30 sec at 72°C, and a final extension for 7 min at 72°C. A negative control was included. PCR amplification products were loaded into 8% (w/v) polyacrylamide gels (37.5:1, acrylamide:bisacrylamide) gels with denaturing gradient ranging from 35% to 60% [100% denaturant corresponded to 7 M urea and 40% (v/v) formamide]. A DGGE marker composed by 8 bands was included in the extremities of each gel (Henriques et al., 2004). The electrophoresis was performed on a D-Code Universal Mutation Detection System (Bio-Rad) with in 1X TAE buffer (Sigma-Aldrich, Germany) at 60ºC in two steps, (1) for 15 min at 20 V and (2) 70 V for 16

h. Gels were stained in a solution of Ethidium bromide (0.5 μg/mL) during 5 minutes and rinsed in water distilled (20 min) and their images captured in a UV transillumination with Molecular Imager FX system (Bio-Rad).

For DGGE analysis, banding patterns similarity was analyzed with the Bionumerics Software (Applied Maths, Belgium). A dendrogram was constructed using Jaccard coefficient (based on presence/absence of fingerprint bands) and clustered according to the unweight pair group mean average (UPGMA) algorithm. Bacterial diversity was estimated by the richness index (S), Shannon index of diversity (H) (Shannon and Weaver, 1949) and Pielou's evenness index (J), calculated using PRIMER v6 software (Primer-E Ltd., Plymouth, UK) as previously described (Alves et al., 2016).

2.2.6 Statistical analysis

For DGGE, statistical analysis was used to determine if metal exposure had significant influence ($P < 0.05$) in the diversity of bacterial communities. Statistical significance of variance indexes (S, H and J) was evaluated with Student's t-tests when data had a normal distribution (checked using the Shapiro-Wilk test) and equal variance (checked using Levene's test).

One-way analyses of variance (ANOVA) were performed on plate counts to assess significant differences ($p \leq 0.05$) between metal exposure and control. Data normal distribution and equal variance was analyzed as previously described. For that, the colony forming unit values were transformed as followed: log (PCA+ antibiotic)/log PCA.

3. RESULTS

3.1 Experimental evolution assay

3.1.1 Bacterial strains evolution

Populations of *Escherichia coli* ATCC® 25922™ and *Aeromonas hydrophila* CECT 839^{T} were exposed to a gradient of copper and zinc for 80 days, being transferred to fresh medium every 48h (40 transfers in total). Concerning *E. coli* populations, the initial gradient contained a maximum concentration of 150 µg mL^{-1} for both metals. This concentration reached 250 µg mL^{-1} for copper and 300 µg mL^{-1} for zinc, after 20 transfers. After 40 transfers, *E. coli* populations were able to grow on a gradient whose maximum concentration was 400 µg mL^{-1} for both metals. Regarding *A. hydrophila* populations, a maximum concentration of 100 µg mL^{-1} of both metals was used for the initial gradient. These concentrations reached 350 µg mL^{-1} for both metals at the 20th transfer. At the end of the 40th transfer, *A. hydrophila* populations were able to grow on a gradient whose maximum concentration was 400 µg mL^{-1} for both metals. *A. hydrophila* populations needed less time to adapt to a given metal concentration (appendix 7.1).

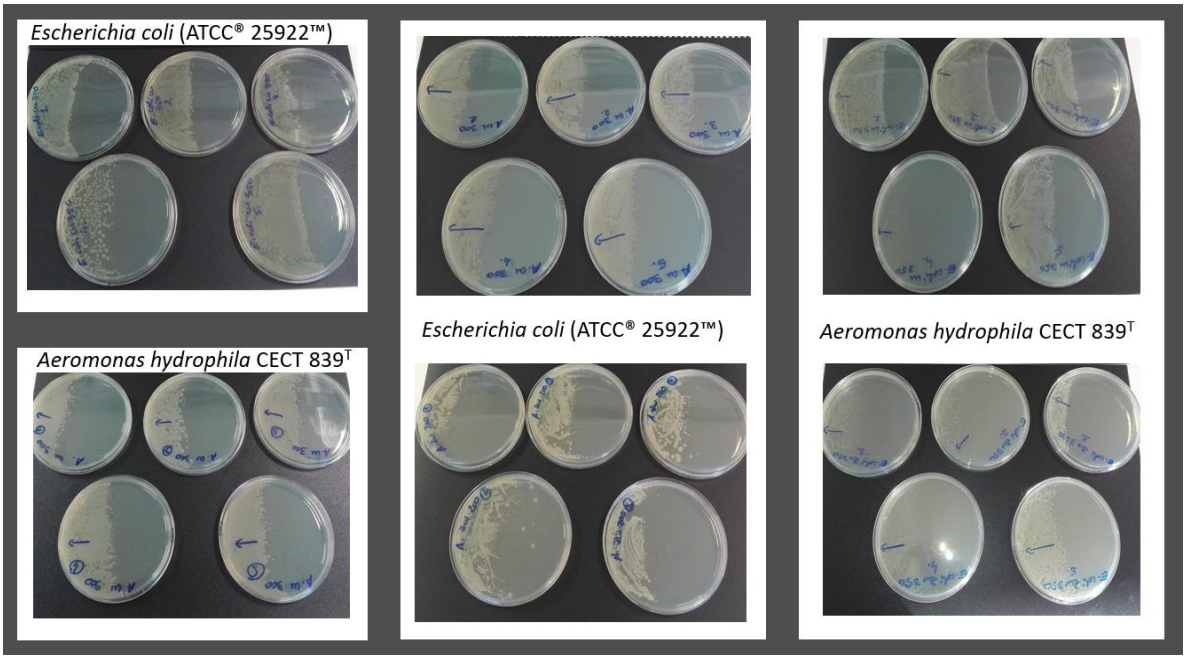


Figure 7: Examples of *E. coli* and *A. hydrophila* populations growing on copper and zinc gradients;

3.1.2 Populations tolerance to metal(loid)s

The tolerance of each population to five metals (Cr, Ni, Cu, Zn, and Hg) and the metalloid As was determined for *Escherichia coli* ATCC® 25922™ and *Aeromonas hydrophila* CECT 839^{T}, after 20 and 40 transfers. Results showed that, for both species, exposure to copper and zinc influenced the metal(oid)s susceptibility profile of these populations, already after the 20th transfer (Figures 8-11).

Concerning *E. coli*, results showed that, after 40 transfers (Figure 9), populations evolved without the metal (populations E.40 01 to E.40 05) maintained a metal(oid)s tolerance profile close to that of the ancestral, except for nickel. Towards this metal most populations increased their tolerance from 200 µg mL^{-1} to 300 µg mL^{-1}. *E. coli* populations evolved with copper (E.40 Cu1 to E.40 Cu5) augmented their tolerance, not only to copper, for which the minimum inhibitory concentration increased from 50 µg mL^{-1} to 150 µg mL^{-1} (for populations E.40 Cu1, E.40 Cu2. E.40 Cu3 and E.40 Cu5) or to 300 µg mL^{-1} (population E.40 Cu4)*;* but also to chromium – minimum inhibitory concentration for some *E. coli* populations increased from 200 µg mL^{-1} to 300 µg mL^{-1} (populations E.40 Cu1, E.40 Cu2 and E.40 Cu4), an increase already verified after 20 transfers (Figure 8). Populations growing with zinc augmented their tolerance to zinc, copper and nickel. Zinc tolerance increased from 50 µg mL^{-1} to 200 µg mL^{-1} (population E.40 Zn2) or to 300 µg mL^{-1} (populations E.40 Zn1, E.40 Zn3, E.40 Zn4 and E.40 Zn5); copper tolerance increased from 50 µg mL^{-1} to 100 µg mL^{-1} (populations E.40 Zn2, E.40 Zn3 and E.40 Zn4) or 300 µg mL^{-1} (population E.40 Zn5); and nickel tolerance increased from 200 µg mL^{-1} to 300 µg mL^{-1} (populations E.40 Zn1 and E.40 Zn5) or to 400 µg mL^{-1} (population E.40 Zn3). Concerning zinc and copper, this increase had already been observed after 20 transfers. *E. coli* populations evolved with copper or zinc decreased their tolerance to mercury already after 20 transfers (appendix 7.2).

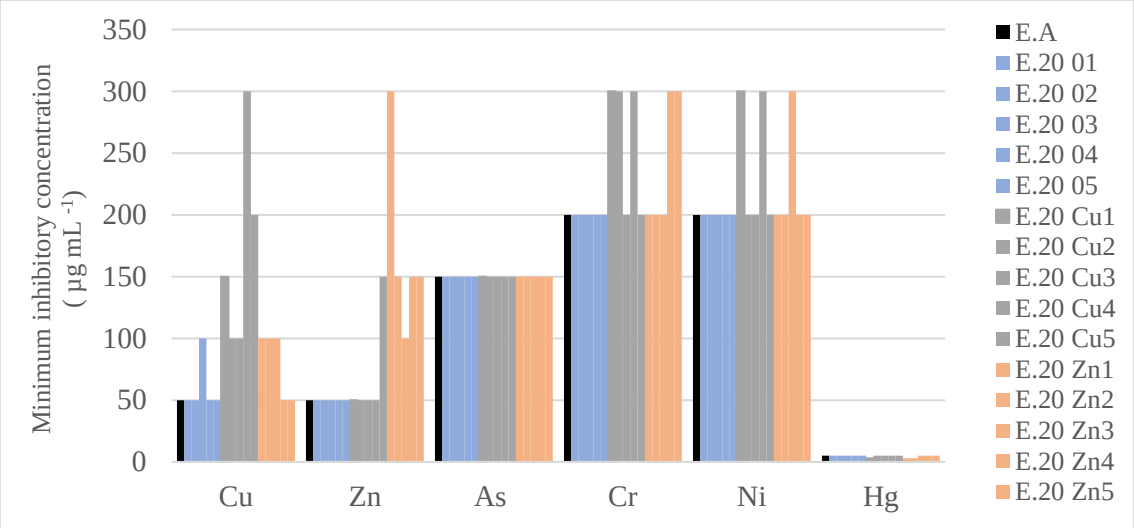


Figure 8: Metal(oid) susceptibility profile of *E. coli* populations after 20 transfers; E.A - stands for *E. coli* ancestral population, E.20 01-20 05 - stands for populations evolved without the metal, E.20 Cu1-20 Cu5 - stands for populations evolved with copper and E.20 Zn1-20 Zn5 - refers to populations evolved with zinc;

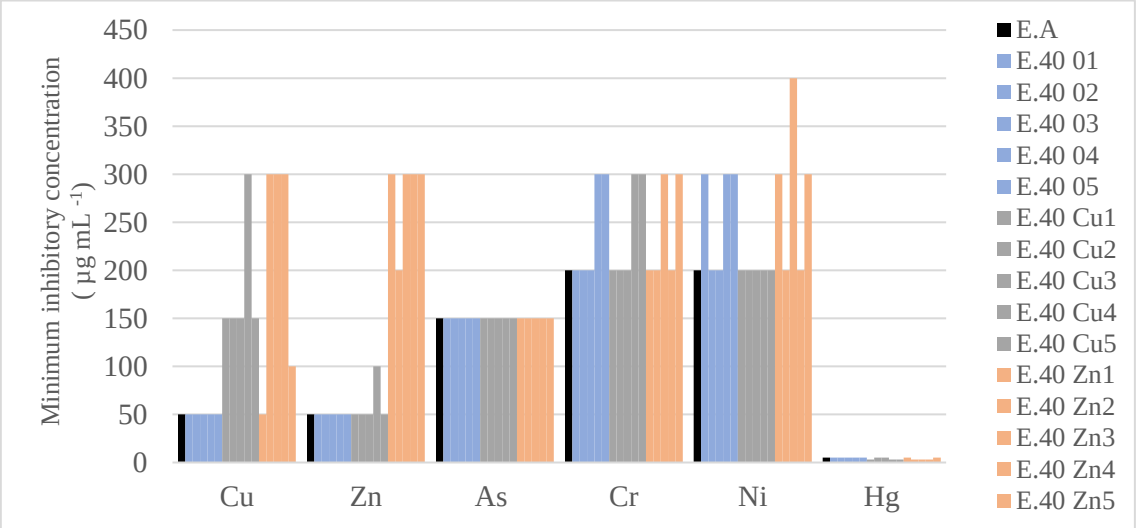


Figure 9: Metal(oid) susceptibility profile of *E. coli* populations after 40 transfers; E.A - stands for *E. coli* ancestral population, E.40 01-40 05 - stands for populations evolved without the metal, E.40 Cu1-40 Cu5 - stands for populations evolved with copper and E.40 Zn1-40 Zn5 - refers to populations evolved with zinc;

After 40 transfers, the metal(oid) susceptibility profile of *A. hydrophila* populations evolved without metal (populations A.40 01 to A.40 05) remained identical to that of the ancestral, except for copper and nickel (Figure 11). Towards copper, minimum inhibitory concentration for all populations increased from 100 µg mL^{-1} to 150 µg mL^{-1}, an alteration already observed after 20 transfers (Figure 10). To nickel, minimum inhibitory concentration increased from 600 µg mL^{-1} to 700 µg mL^{-1} for most populations. *A. hydrophila* populations evolved with copper (A.40 Cu) augmented their tolerance to chromium – minimum inhibitory concentration increased from 200 µg mL^{-1} to 250 µg mL^{-1} - an increase already observed in the 20th transfer. Population growing with zinc (A.40 Zn) increased their tolerance, not only towards this metal, but also to chromium, already after 20 transfers. Minimum inhibitory concentration of these populations increased from 100 µg mL^{-1} to 350 µg mL^{-1} to zinc; and from 200 µg mL^{-1} to 250 µg mL^{-1} to chromium.

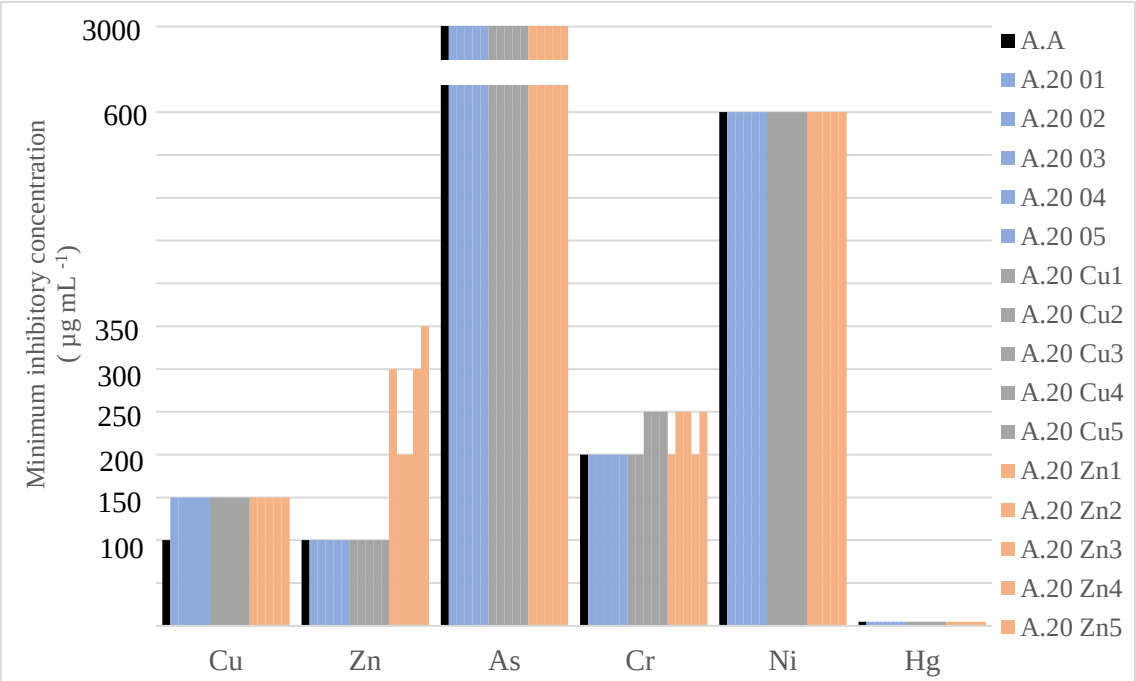


Figure 10: Metal(oid) susceptibility profile of *A. hydrophila* populations after 20 transfers; A.A - stands for *A. hydrophila* ancestral population, A.20 01-20 05 - stands for populations evolved without the metal, A.20 Cu1-20 Cu5 - stands for populations evolved with copper and A.20 Zn1-20 Zn5 - refers to populations evolved with zinc;

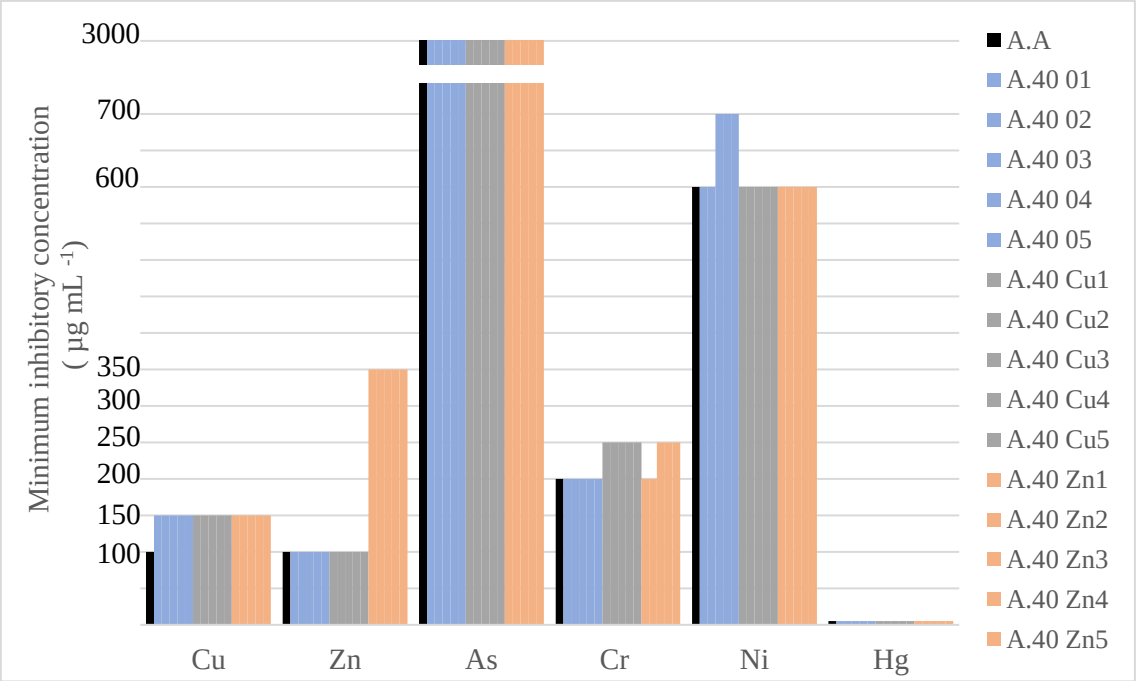
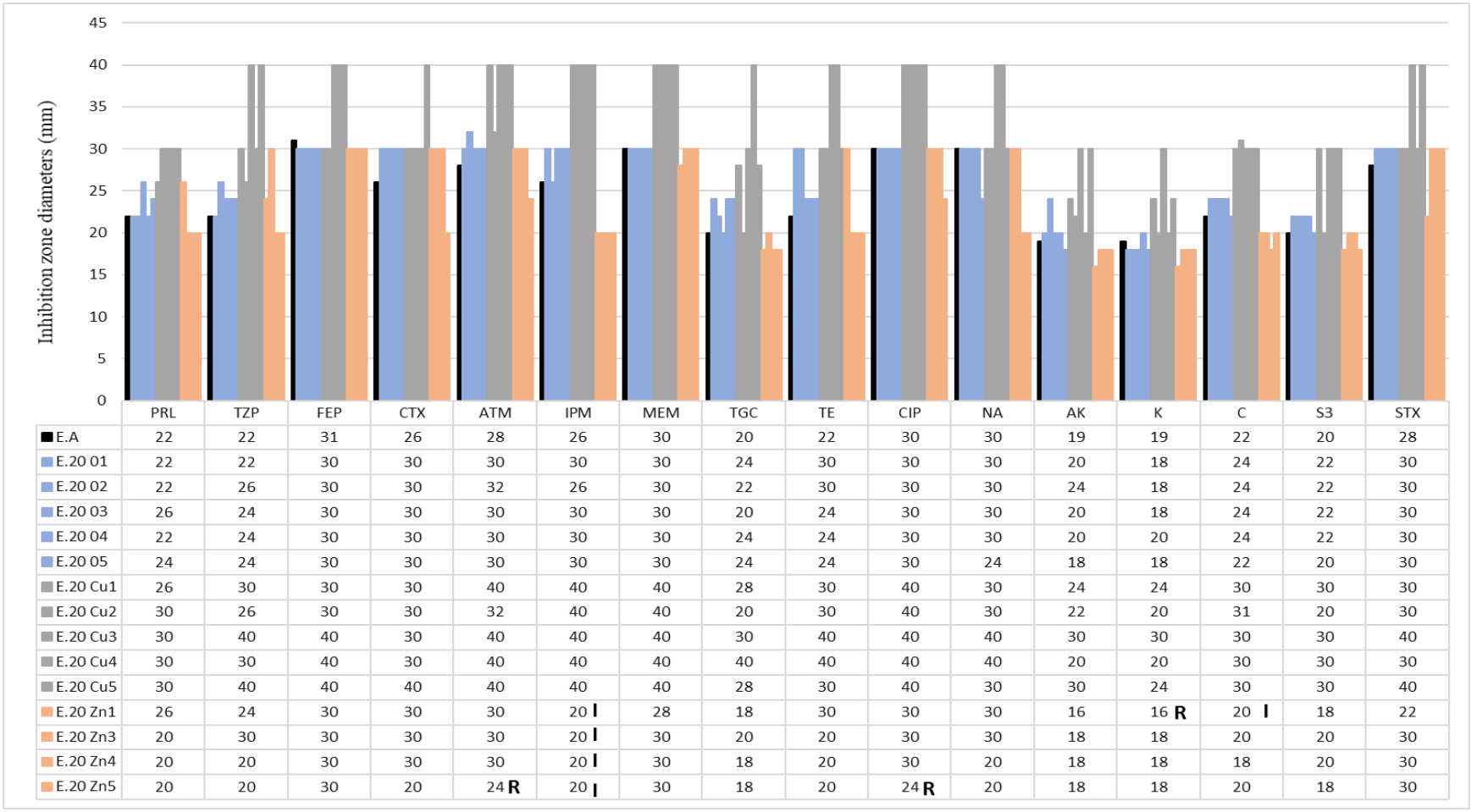


Figure 11: Metal(oid) susceptibility profile of *A. hydrophila* populations after 40 transfers; A.A - stands for *A. hydrophila* ancestral population, A.40 01-40 05 - stands for populations evolved without the metal, A.40 Cu1-40 Cu5 - stands for populations evolved with copper and A.40 Zn1-40 Zn5 - refers to populations evolved with zinc;

3.1.3 Changes in antibiotic susceptibility profiles assessed by disc diffusion assays

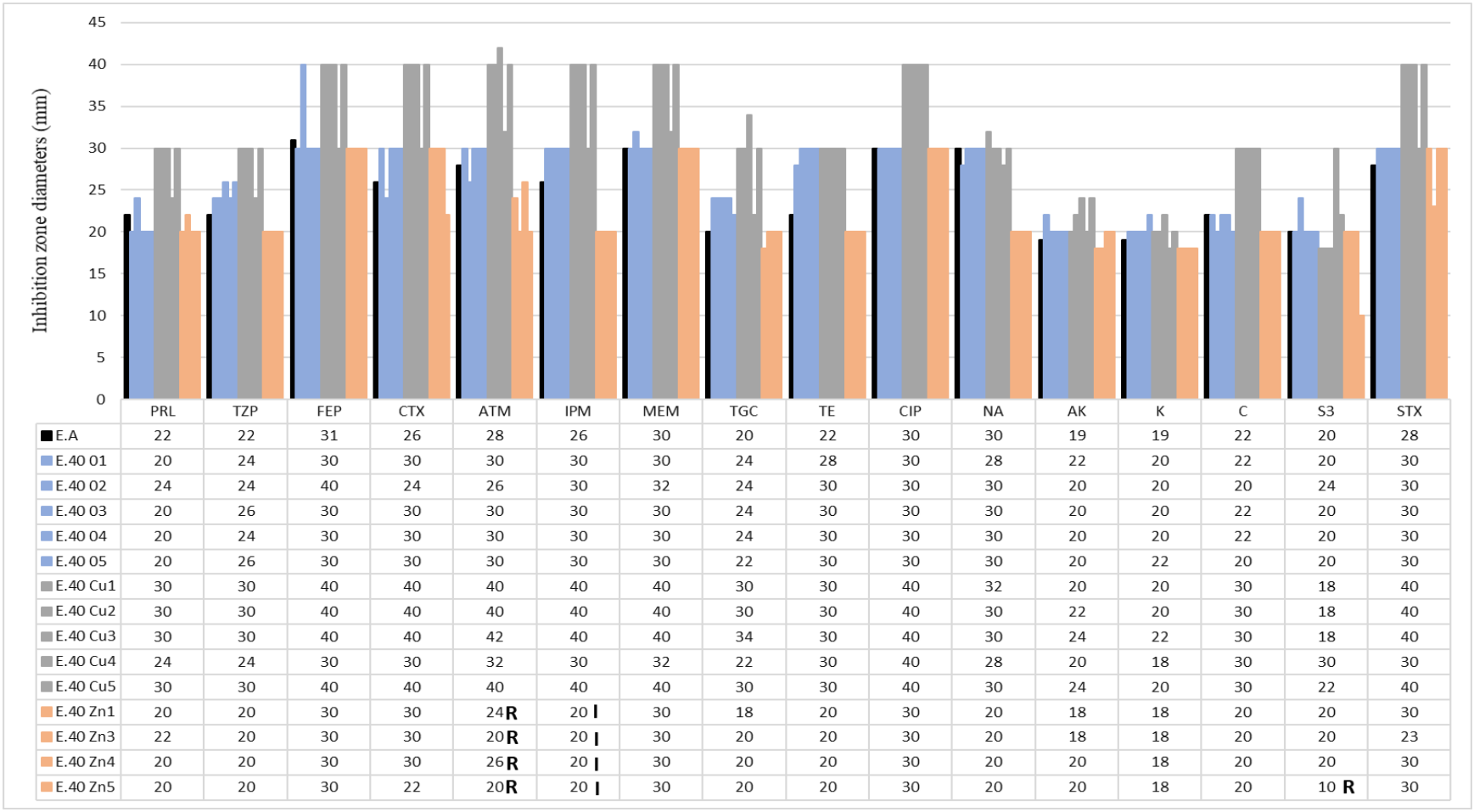
Antibiotic susceptibility testing was performed to evaluate changes in sensitivity of these populations to several clinically-important antibiotic classes. Results showed that, for both species, exposure to copper and zinc influenced the results of the antibiotic susceptibility test (Figures 12-15).

After 40 transfers (Figure 13), the antibiotic susceptibility profile of *E. coli* populations evolved without the contaminant (E.40 01-05) remained similar to the ancestral, except for tetracycline. For this antibiotic, the inhibition zone diameter was slightly higher for these populations. For populations exposed to copper (E.40 Cu) the diameter zones measured for most antibiotics increased slightly in comparison with the ancestral and the populations evolved without metal. This increase was already verified after 20 transfers (Figure 12). Exceptions were tetracycline, nalidixic acid, amikacin, kanamycin and sulfonamides, to which no relevant variation was observed. For imipenem, for instance, inhibition zone diameters increased from 30 mm, for populations evolved without the metal, to 40 mm, for populations evolved with copper. For populations growing in the presence of zinc (E.40 Zn) a slight decrease in the inhibition zones diameter was verified for aztreonam and imipenem after 40 transfers, which was sufficient to alter the classification of the populations from susceptible to resistant in the case of aztreonam or from susceptible to intermediate resistant in the case of imipenem, according to the classification proposed by the EUCAST rules (http://www.eucast.org/). Towards imipenem, inhibition zone diameters decreased from 30 mm, for populations evolved without metal, to 20 mm, for populations evolved with zinc - a characteristic already acquired by the populations after 20 transfers (E.20 Zn). Populations evolved with zinc also exhibited smaller inhibition zone diameters for nalidixic acid, when compared to populations evolved without the metal.



	PRL	TZP	FEP	CTX	ATM	IPM	MEM	TGC	TE	CIP	NA	AK	K	C	S3	STX
E.A	22	22	31	26	28	26	30	20	22	30	30	19	19	22	20	28
E.20 01	22	22	30	30	30	30	30	24	30	30	30	20	18	24	22	30
E.20 02	22	26	30	30	32	26	30	22	30	30	30	24	18	24	22	30
E.20 03	26	24	30	30	30	30	30	20	24	30	30	20	18	24	22	30
E.20 04	22	24	30	30	30	30	30	24	24	30	30	20	20	24	22	30
E.20 05	24	24	30	30	30	30	30	24	24	30	24	18	18	22	20	30
E.20 Cu1	26	30	30	30	40	40	40	28	30	40	30	24	24	30	30	30
E.20 Cu2	30	26	30	30	32	40	40	20	30	40	30	22	20	31	20	30
E.20 Cu3	30	40	40	30	40	40	40	30	40	40	40	30	30	30	30	40
E.20 Cu4	30	30	40	30	40	40	40	40	40	40	40	20	20	30	30	30
E.20 Cu5	30	40	40	40	40	40	40	28	30	40	30	30	24	30	30	40
E.20 Zn1	26	24	30	30	30	20 I	28	18	30	30	30	16	16 R	20 I	18	22
E.20 Zn3	20	30	30	30	30	20 I	30	20	20	30	30	18	18	20	20	30
E.20 Zn4	20	20	30	30	30	20 I	30	18	20	30	20	18	18	18	20	30
E.20 Zn5	20	20	30	20	24 R	20 I	30	18	20	24 R	20	18	18	20	18	30

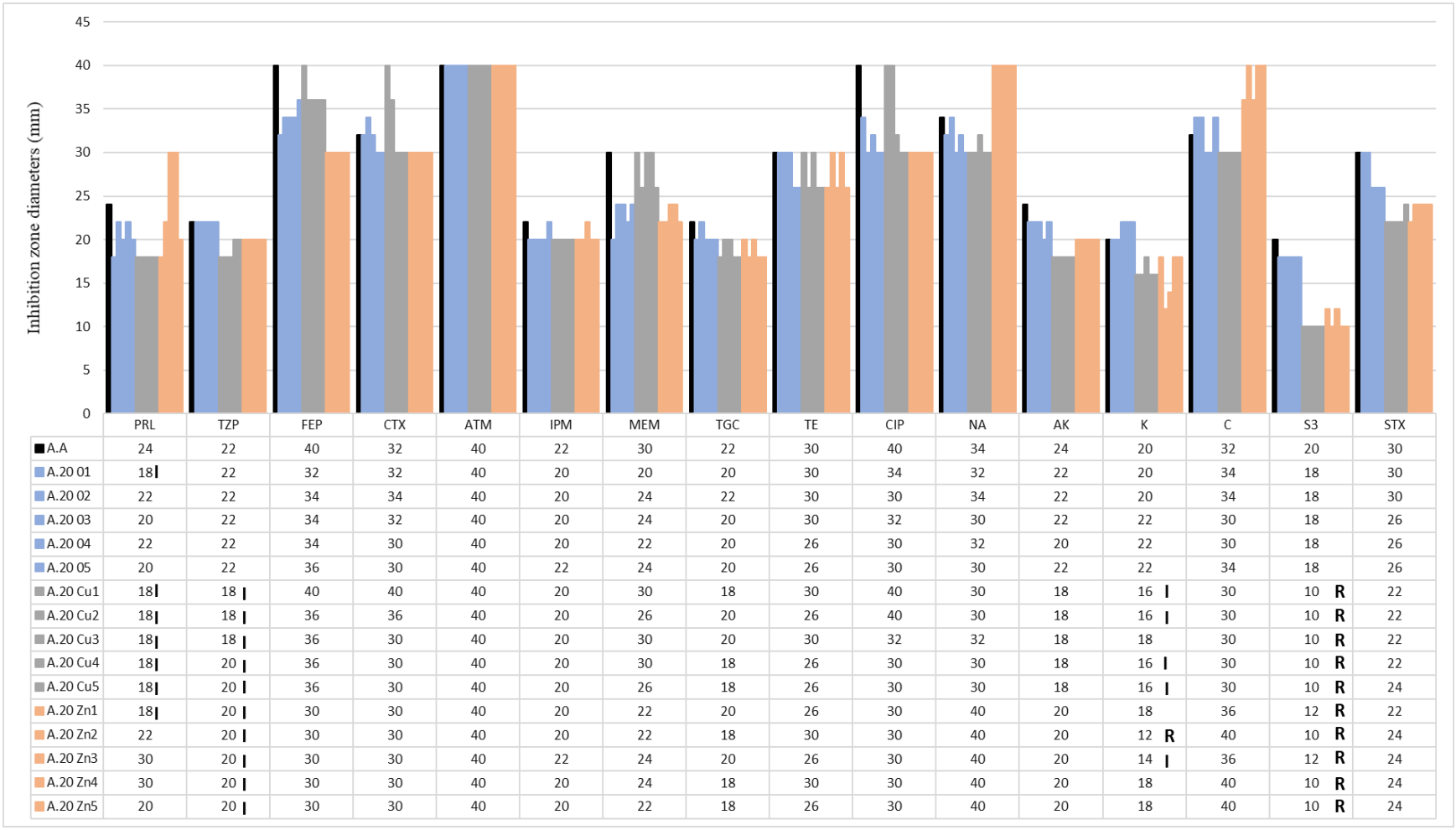
Figure 12: Susceptibility profile of *E.coli* populations after 20 transfer (E.20) against piperacillin (PRL), piperacillin-tazobactam combination (TZP), cefepime (FEP), cefotaxime (CTX), aztreonam (ATM), imipenem (IPM), meropenem (MEM), tigecycline (TGC), tetracycline (TE), ciprofloxacin (CIP), nalidixic acid (NA), amikacin (AK), kanamycin (K), chloramphenicol (C), sulfonamides (S) and trimethoprim-sulfamethoxazole combination (SXT); R - resistant; I - intermediate resistance - populations not identified with a R or I were classified as susceptible to the antibiotics tested; E.A – *E. coli* ancestral population, E.20 01-20 05 - stands for populations evolved without the metal, E.20 Cu1-20 Cu5 - stands for populations evolved with copper and E.20 Zn1-20 Zn5 - refers to populations evolved with zinc;



	PRL	TZP	FEP	CTX	ATM	IPM	MEM	TGC	TE	CIP	NA	AK	K	C	S3	STX
E.A	22	22	31	26	28	26	30	20	22	30	30	19	19	22	20	28
E.40 01	20	24	30	30	30	30	30	24	28	30	28	22	20	22	20	30
E.40 02	24	24	40	24	26	30	32	24	30	30	30	20	20	20	24	30
E.40 03	20	26	30	30	30	30	30	24	30	30	30	20	20	22	20	30
E.40 04	20	24	30	30	30	30	30	24	30	30	30	20	20	22	20	30
E.40 05	20	26	30	30	30	30	30	22	30	30	30	20	22	20	20	30
E.40 Cu1	30	30	40	40	40	40	40	30	30	40	32	20	20	30	18	40
E.40 Cu2	30	30	40	40	40	40	40	30	30	40	30	22	20	30	18	40
E.40 Cu3	30	30	40	40	42	40	40	34	30	40	30	24	22	30	18	40
E.40 Cu4	24	24	30	30	32	30	32	22	30	40	28	20	18	30	30	30
E.40 Cu5	30	30	40	40	40	40	40	30	30	40	30	24	20	30	22	40
E.40 Zn1	20	20	30	30	24 **R**	20 **I**	30	18	20	30	20	18	18	20	20	30
E.40 Zn3	22	20	30	30	20 **R**	20 **I**	30	20	20	30	20	18	18	20	20	23
E.40 Zn4	20	20	30	30	26 **R**	20 **I**	30	20	20	30	20	20	18	20	20	30
E.40 Zn5	20	20	30	22	20 **R**	20 **I**	30	20	20	30	20	20	18	20	10 **R**	30

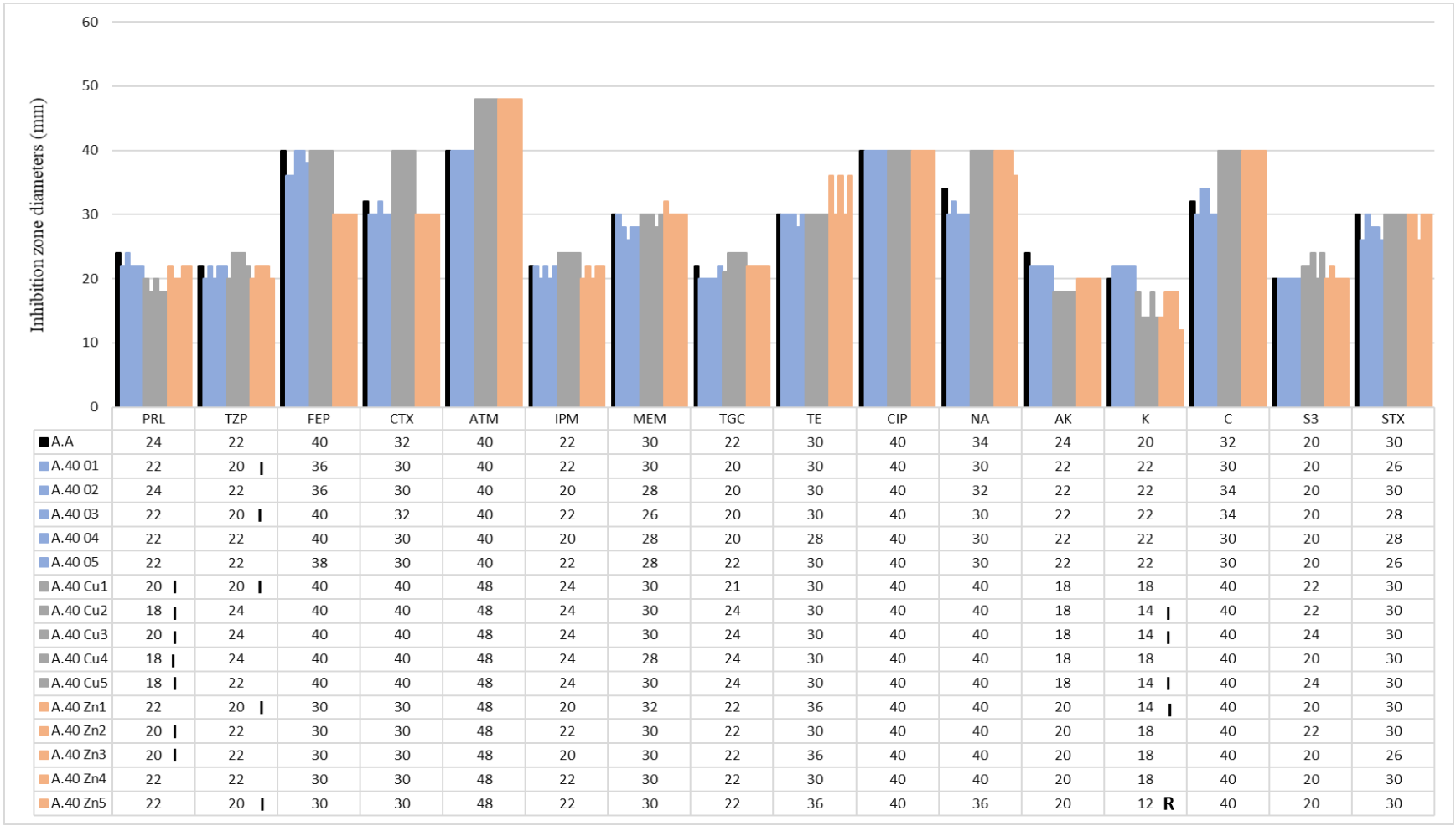
Figure 13: Susceptibility profile of *E.coli* populations after 40 transfer (E.40) against piperacillin (PRL), piperacillin-tazobactam combination (TZP), cefepime (FEP), cefotaxime (CTX), aztreonam (ATM), imipenem (IPM), meropenem (MEM), tigecycline (TGC), tetracycline (TE), ciprofloxacin (CIP), nalidixic acid (NA), amikacin (AK), kanamycin (K), chloramphenicol (C), sulfonamides (S) and trimethoprim-sulfamethoxazole combination (SXT); R - resistant; I - intermediate resistance - populations not identified with a R or I were classified as susceptible to the antibiotics tested; E.A – *E. coli* ancestral population, E.40 01-40 05 - stands for populations evolved without the metal, E.40 Cu1-40 Cu5 - stands for populations evolved with copper and E.40 Zn1-40 Zn5 - refers to populations evolved with zinc;

The antibiotic susceptibility profile of *A. hydrophila* populations evolved without metal (A.40 01-05) remained close to that of the ancestral, after 40 transfers (Figure 15). *A. hydrophila* populations evolved with copper (A.40 Cu) exhibited smaller inhibition zones for piperacillin, amikacin and kanamycin. Towards piperacillin and kanamycin, the change observed resulted in a classification alteration from susceptible to intermediate resistant (I). This tendency was already verified after 20 transfers (A.20 Cu), for these populations (Figure 14). For sulfonamides, a phenotype alteration observed after 20 transfers (from susceptible to resistant) was reverted after 40 transfers. Populations growing in the presence of zinc for 40 transfers (A.40 Zn), exhibited slightly smaller inhibition zones for cefepime, amikacin and kanamycin. For instance, for cefepime, the inhibition zone diameter decreased from 40 mm to 30 mm. One population became resistant to kanamycin - inhibition zone diameter decreased from 20 mm to 12 mm (A.40 Zn5). For nalidixic acid, chloramphenicol and aztreonam a slight increase in the diameter of inhibition zones was observed for these populations.



	PRL	TZP	FEP	CTX	ATM	IPM	MEM	TGC	TE	CIP	NA	AK	K	C	S3	STX
A.A	24	22	40	32	40	22	30	22	30	40	34	24	20	32	20	30
A.20 01	18 I	22	32	32	40	20	20	20	30	34	32	22	20	34	18	30
A.20 02	22	22	34	34	40	20	24	22	30	30	34	22	20	34	18	30
A.20 03	20	22	34	32	40	20	24	20	30	32	30	22	22	30	18	26
A.20 04	22	22	34	30	40	20	22	20	26	30	32	20	22	30	18	26
A.20 05	20	22	36	30	40	22	24	20	26	30	30	22	22	34	18	26
A.20 Cu1	18 I	18 I	40	40	40	20	30	18	30	40	30	18	16 I	30	10 R	22
A.20 Cu2	18 I	18 I	36	36	40	20	26	20	26	40	30	18	16 I	30	10 R	22
A.20 Cu3	18 I	18 I	36	30	40	20	30	20	30	32	32	18	18	30	10 R	22
A.20 Cu4	18 I	20 I	36	30	40	20	30	18	26	30	30	18	16 I	30	10 R	22
A.20 Cu5	18 I	20 I	36	30	40	20	26	18	26	30	30	18	16 I	30	10 R	24
A.20 Zn1	18 I	20 I	30	30	40	20	22	20	26	30	40	20	18	36	12 R	22
A.20 Zn2	22	20 I	30	30	40	20	22	18	30	30	40	20	12 R	40	10 R	24
A.20 Zn3	30	20 I	30	30	40	22	24	20	26	30	40	20	14 I	36	12 R	24
A.20 Zn4	30	20 I	30	30	40	20	24	18	30	30	40	20	18	40	10 R	24
A.20 Zn5	20	20 I	30	30	40	20	22	18	26	30	40	20	18	40	10 R	24

Figure 14: Susceptibility profile of *A. hydrophila* populations after 20 transfer (A.20) against piperacillin (PRL), piperacillin-tazobactam combination (TZP), cefepime (FEP), cefotaxime (CTX), aztreonam (ATM), imipenem (IPM), meropenem (MEM), tigecycline (TGC), tetracycline (TE), ciprofloxacin (CIP), nalidixic acid (NA), amikacin (AK), kanamycin (K), chloramphenicol (C), sulfonamides (S) and trimethoprim-sulfamethoxazole combination (SXT); R - resistant; I - intermediate resistance - populations not identified with a R or I were classified as susceptible to the antibiotics tested; A.A – *A. hydrophila* ancestral population, A.20 01-20 05 - stands for populations evolved without the metal, A.20 Cu1-20 Cu5 - stands for populations evolved with copper and A.20 Zn1-20 Zn5 - refers to populations evolved with zinc;



	PRL	TZP	FEP	CTX	ATM	IPM	MEM	TGC	TE	CIP	NA	AK	K	C	S3	STX
A.A	24	22	40	32	40	22	30	22	30	40	34	24	20	32	20	30
A.40 01	22	20 I	36	30	40	22	30	20	30	40	30	22	22	30	20	26
A.40 02	24	22	36	30	40	20	28	20	30	40	32	22	22	34	20	30
A.40 03	22	20 I	40	32	40	22	26	20	30	40	30	22	22	34	20	28
A.40 04	22	22	40	30	40	20	28	20	28	40	30	22	22	30	20	28
A.40 05	22	22	38	30	40	22	28	22	30	40	30	22	22	30	20	26
A.40 Cu1	20 I	20 I	40	40	48	24	30	21	30	40	40	18	18	40	22	30
A.40 Cu2	18 I	24	40	40	48	24	30	24	30	40	40	18	14 I	40	22	30
A.40 Cu3	20 I	24	40	40	48	24	30	24	30	40	40	18	14 I	40	24	30
A.40 Cu4	18 I	24	40	40	48	24	28	24	30	40	40	18	18	40	20	30
A.40 Cu5	18 I	22	40	40	48	24	30	24	30	40	40	18	14 I	40	24	30
A.40 Zn1	22	20 I	30	30	48	20	32	22	36	40	40	20	14 I	40	20	30
A.40 Zn2	20 I	22	30	30	48	22	30	22	30	40	40	20	18	40	22	30
A.40 Zn3	20 I	22	30	30	48	20	30	22	36	40	40	20	18	40	20	26
A.40 Zn4	22	22	30	30	48	22	30	22	30	40	40	20	18	40	20	30
A.40 Zn5	22	20 I	30	30	48	22	30	22	36	40	36	20	12 R	40	20	30

Figure 15: Susceptibility profile of *A. hydrophila* populations after 40 transfer (A.40) against piperacillin (PRL), piperacillin-tazobactam combination (TZP), cefepime (FEP), cefotaxime (CTX), aztreonam (ATM), imipenem (IPM), meropenem (MEM), tigecycline (TGC), tetracycline (TE), ciprofloxacin (CIP), nalidixic acid (NA), amikacin (AK), kanamycin (K), chloramphenicol (C), sulfonamides (S) and trimethoprim-sulfamethoxazole combination (SXT); R - resistant; I - intermediate resistance - populations not identified with a R or I were classified as susceptible to the antibiotics tested; A.A – *A. hydrophila* ancestral population, A.40 01-40 05 - stands for populations evolved without the metal, A.40 Cu1-40 Cu5 - stands for populations evolved with copper and A.40 Zn1-40 Zn5 - refers to populations evolved with zinc;

3.1.4 Alterations in antibiotics minimum inhibitory concentrations (MICs)

Based on antibiotic susceptibility test results, several antibiotics were selected for MIC determination. For this, 10 populations evolved for 40 transfers were selected: ancestral, 3 populations evolved without metal, 3 populations evolved with copper, and 3 populations evolved with zinc.

MIC determination for *E. coli* populations evolved with copper (E.40 Cu) showed that these populations became less susceptible to kanamycin, imipenem and sulfamethoxazole. For kanamycin, an increase of 2.6 X in the average MIC value was observed, compared with populations evolved without the metal. For imipenem, this increase was 5.5 X; and for sulfamethoxazole, an increase of 34 X was observed. *E. coli* populations evolved with copper became more susceptible to nalidixic acid (NA) and to aztreonam (ATM). For these compounds it was observed a reduction of 2.6 X (NA) and 4 X (ATM) in the average MIC value, compared with the MIC of populations evolved without the metal. *E. coli* populations growing in the presence of zinc (E.40 Zn) became less sensitive to sulfamethoxazole (SMX). For this compound an increase of 2.5 X in the average MIC values of these populations, when compared with populations evolved without the contaminant, was observed. Contrariwise, average MIC values decreased for imipenem (2X) and cefotaxime (6.5X).

Table 8: MIC values (μg mL^{-1}) for *E. coli* populations against piperacillin (PRL), nalidixic acid (NA), kanamycin (K), sulfamethoxazole (SMX), imipenem (IPM), tigecycline (TGC), ciprofloxacin (CIP), aztreonam (ATM) and cefotaxime (CTX). E.40 01-40 03 - stands for populations evolved without the metal, E.40 Cu1-40 Cu3 - stands for populations evolved with copper and E.40 Zn1, E.40 Zn3 and E.40 Zn5 - refers to populations evolved with zinc;

Antibiotic	Ancestral (*E. coli*)	Without metal			Mean*	Copper			Mean*	Zinc			Mean*
		E.40 01	E.40 02	E.40 03		E.40 Cu1	E.40 Cu2	E.40 Cu3		E.40 Zn1	E.40 Zn3	E.40 Zn5	
PRL	3	2	3	2	2.3 ±0.5	2	2	2	2	1	1	1	1
NA	1.5	3	2	1.5	2.2 ±0.6	1	0.75	0.75	0.83 ±0.1	1	1	1	1
K	1.5	1	1.5	1	1.2 ±0.2	3	3	3	3	1	1	1	1
SMX	32	24	32	32	29 ±4	>1024	>1024	>1024	1024	96	96	32	74 ±30
IPM	0.0944	0.094	0.19	0.125	0.14 ±0.04	0.75	0.75	0.75	0.75	0.064	0.064	0.064	0.064
TGC	0.125	0.19	0.125	0.19	0.17 ±0.03	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
CIP	0.008	0.008	0.012	0.012	0.011 ±0.002	0.008	0.016	0.016	0.013 ±0.004	0.047	0.012	0.012	0.024 ±0.02
ATM	0.19	1	0.125	0.047	0.4 ±0.4	0.094	0.094	0.094	0.09	0.75	0.064	0.064	0.29 ±0.3
CTX	0.094	0.064	0.125	0.125	0,.10 ±0.03	0.19	0.064	0.016	0.09 ±0.07	0.016	0.016	0.016	0.016

* ± standard deviation

For *A. hydrophila* populations (Table 9), MICs were determined for piperacillin, sulfamethoxazole, kanamycin and cefotaxime. MIC values were similar between the ancestral and populations evolved without contaminant (A.40 01-03). Populations evolved with copper (A.40 Cu) became more sensitive to sulfamethoxazole, to which a reduction of 3.4 X in the average MIC value was observed – compared with those observed for populations evolved without the contaminant; For kanamycin, a 4 X increase in the average MIC value was observed. The same was observed for populations evolved with zinc (A.40 Zn) - a reduction of 2 X in the average MIC value for sulfamethoxazole; and an increase of 5.3 X for kanamycin.

Table 9: MIC values (µg mL^{-1}) for *A. hydrophila* populations against piperacillin (PRL), sulfamethoxazole (SMX), cefotaxime (CTX) and kanamycin (K); A.40 01-40 03 - stands for populations evolved without the metal, A.40 Cu1-40 Cu3 - stands for populations evolved with copper and A.40 Zn1 - 40 Zn3 - refers to populations evolved with zinc;

Antibiotic	Ancestral (*A. hydrophila*)	Without metal			Mean*	Copper			Mean*	Zinc			Mean*
		A.40 01	A.40 02	A.40 03		A.40 Cu1	A.40 Cu2	A.40 Cu3		A.40 Zn1	A.40 Zn2	A.40 Zn3	
PRL	1	0.75	0.75	0.75	0.75	1	1	1	1	1	1	1	1
SMX	16	16	16	16	16	6	4	4	4.7 ±0.9	8	8	8	8
CTX	0.016	<0.016	<0.016	<0.016	<0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
K	2	1.5	1.5	1.5	1.5	6	6	6	6	8	8	8	8

* ± standard deviation

3.1.5 Fitness cost associated to evolution in the presence of metals

The fitness of bacterial strains was determined growing the different populations in diverse conditions: in LB, in LB supplemented with a metal (copper or zinc) and in the presence of an antibiotic (sulfamethoxazole)(Figure 16-23). Growth rate (Tables 10 and 12) and doubling time (Tables 11 and 13) were determined for each population/growth condition.

For all the conditions tested, *E. coli* populations evolved without the metal for 40 transfers presented a higher fitness than the ancestral population and than populations evolved with metal (Figure 16-19). When cultivated in LB, populations evolved with copper for 40 transfers demonstrated an average growth rate 1.4 X lower than those evolved without any contaminant (Table 10). The average doubling time of these populations increased 1.5 X (Table 11). In LB supplemented with copper, populations evolved in the presence of this metal still presented a lower fitness than populations evolved without metal, although growth rates and doubling time differences between all populations were not so evident. When growing in LB supplemented with zinc, populations evolved without metal and populations evolved with zinc presented a similar fitness and a similar average growth rate and doubling time, both after 20 and 40 transfers (appendix 7.3). When cultivated in LB supplemented with sulfamethoxazole, populations evolved without metal and populations evolved with zinc presented a similar fitness, while populations evolved with copper presented a lower one. However, under these growth conditions, the average growth rate of populations evolved with both metals was 2 X lower than populations evolved without a contaminant and the average doubling time was 2 X higher.

Table 10: Grow rates [r = ln (OD2/OD1)/(T2-T1)] of *E. coli* populations after 40 transfers, per minute; growing in LB, LB supplemented with copper, LB supplemented with zinc and LB supplemented with sulfamethoxazole; E.A - stands for *E. coli* ancestral population, E.40 01-40 05 - stands for populations evolved without the metal, E.40 Cu1-40 Cu5 - stands for populations evolved with copper and E.40 Zn1-40 Zn5 - refers to populations evolved with zinc;

Growth rates after 40 transfers								
***E. coli* populations**	**LB**	**Mean***	**LB+Cu**	**Mean***	**LB+Zn**	**Mean***	**LB+SMX**	**Mean***
E.A	0.017	-	0.004	-	0.011	-	0.017	-
E.40 02	0.017	0.015 ±0.002	0.008	0.009 ±0.0005	0.013	0.018 ±0.005	0.016	0.015 ±0.001
E.40 03	0.017		0.008		0.016		0.014	
E.40 05	0.013		0.009		0.025		0.014	
E.40 Cu1	0.015	0.011 ±0.003	0.014	0.010 ±0.003	-	-	0.014	0.009 ±0.004
E.40 Cu4	0.011		0.007		-		0.004	
E.40 Cu5	0.007		0.008		-		0.008	
E.40 Zn2	0.015	0.013 ±0.001	-	-	0.014	0.014 ±0.006	0.007	0.008 ±0.002
E.40 Zn3	0.012		-		0.007		0.006	
E.40 Zn4	0.013		-		0.021		0.011	

* ± standard deviation

Table 11: Doubling time [Dt = ln2/r] in minutes of *E. coli* populations after 40 transfers; growing in LB, LB supplemented with copper, LB supplemented with zinc and LB supplemented with sulfamethoxazole; E.A - stands for *E. coli* ancestral population, E.40 01-40 05 - stands for populations evolved without the metal, E.40 Cu1-40 Cu5 - stands for populations evolved with copper and E.40 Zn1-40 Zn5 - refers to populations evolved with zinc;

Doubling time after 40 transfers								
***E. coli* populations**	**LB**	**Mean***	**LB+Cu**	**Mean***	**LB+Zn**	**Mean***	**LB+SMX**	**Mean***
E.A	42.0	-	182.4	-	63.6	-	39.8	-
E.40 02	41.8	46.4 ±6	83.5	80.9 ±5	54.6	42.4 ±11	43.1	46.7 ±3
E.40 03	42.0		84.5		44.7		48.5	
E.40 05	55.5		74.5		27.8		48.5	
E.40 Cu1	45.3	69.6 ±23	51.0	76.5 ±19	-	-	51.0	100.3 ±46
E.40 Cu4	63.0		95.0		-		161.2	
E.40 Cu5	100.5		83.5		-		88.9	
E.40 Zn2	45.0	52.2 ±5	-	-	50.2	61.0 ±28	101.9	91.9 ±22
E.40 Zn3	57.8		-		99.0		111.8	
E.40 Zn4	53.7		-		33.8		61.9	

* ± standard deviation

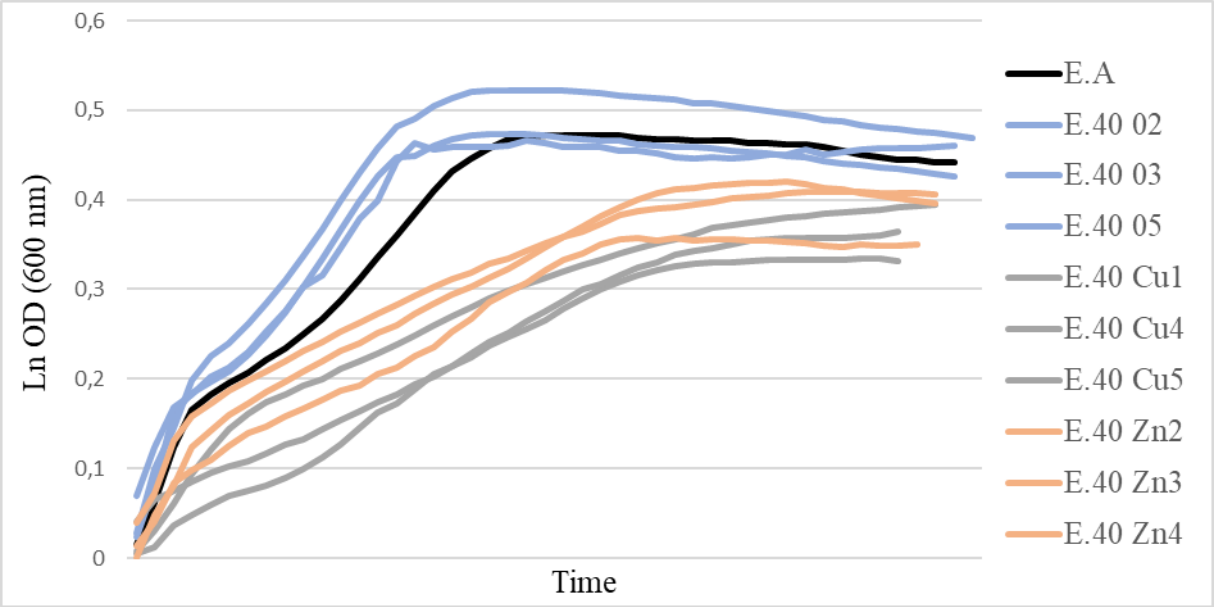


Figure 16: Growth of *E. coli* populations after 40 transfers in LB for 24h; E.A - stands for ancestral, E.40 01-40 05 - stands for populations evolved without the metal, E.40 Cu1-40 Cu5 - stands for populations evolved with copper and E.40 Zn1-40 Zn5 - refers to populations evolved with zinc;

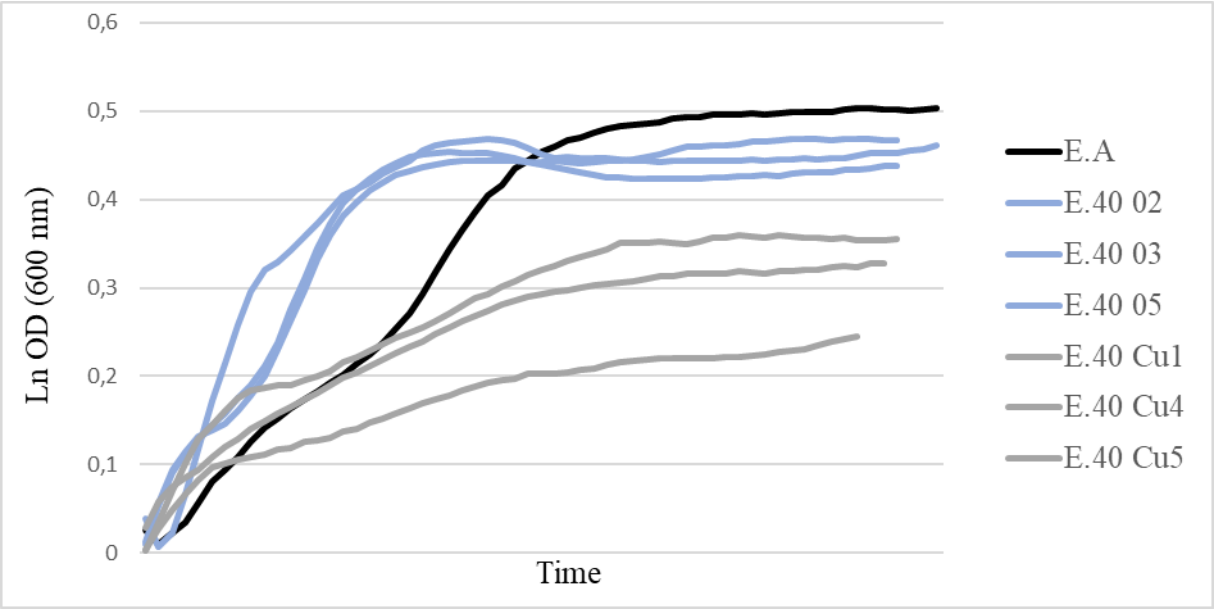


Figure 17: Growth of *E. coli* populations after 40 transfers in LB supplemented with copper, for 30h; E.A - stands for ancestral, E.40 01-40 05 - stands for populations evolved without the metal and E.40 Cu1-40 Cu5 - stands for populations evolved with copper;

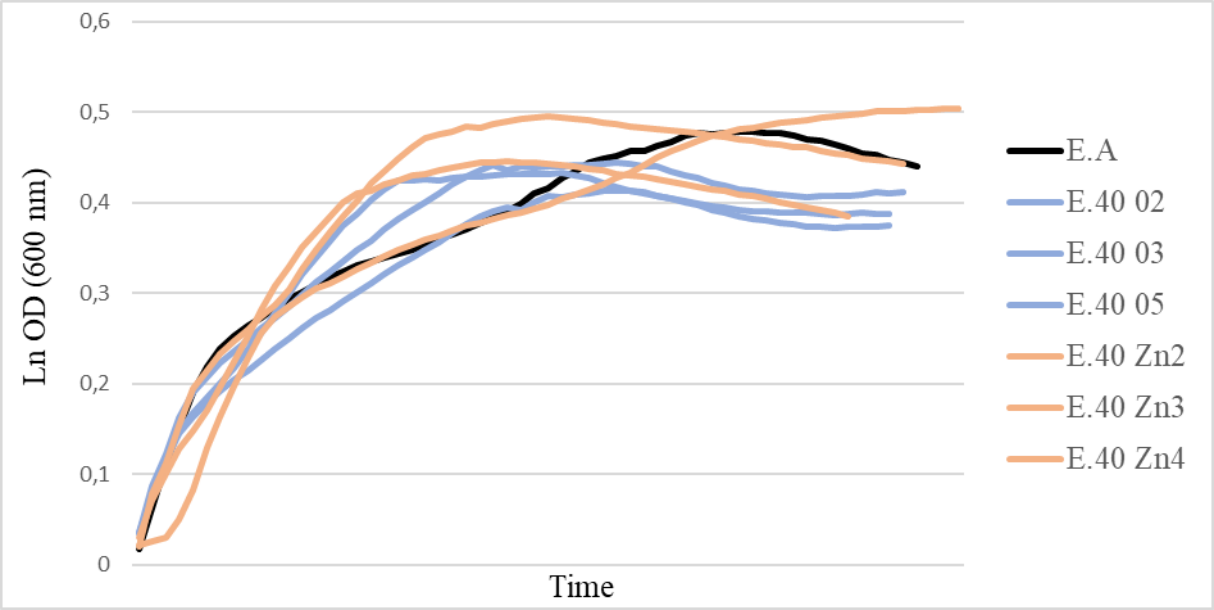


Figure 18: Growth of *E. coli* populations after 40 transfers in LB supplemented with zinc, for 30h; E.A - stands for ancestral, E.40 01-40 05 - stands for populations evolved without the metal and E.40 Zn1-40 Zn5 - refers to populations evolved with zinc;

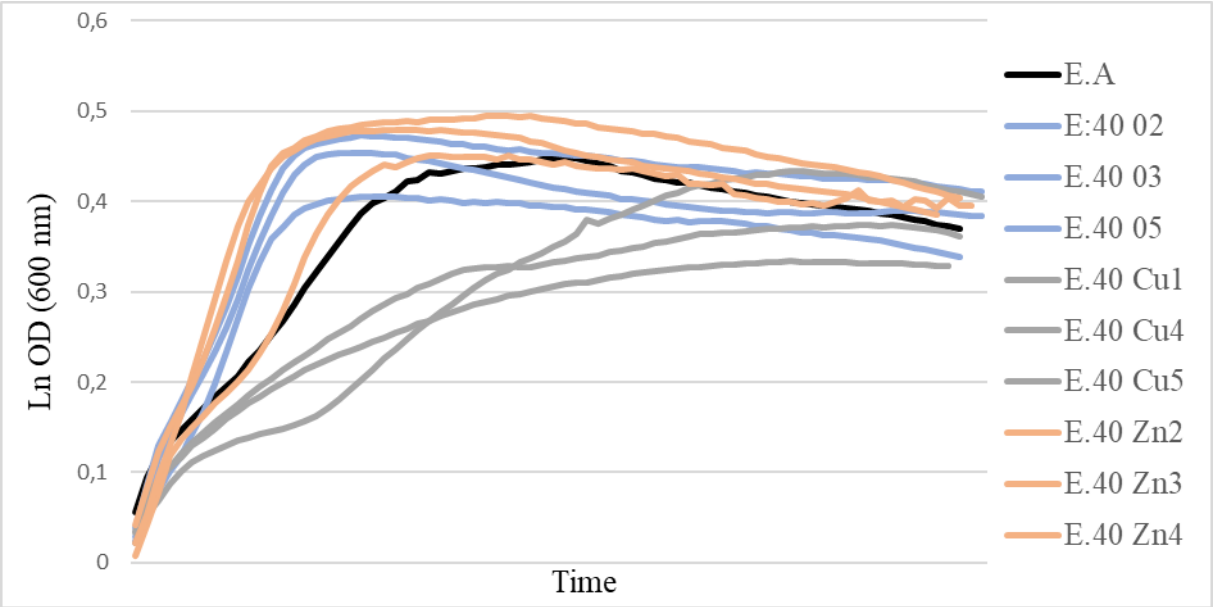


Figure 19: Growth of *E. coli* populations after 40 transfers in LB supplemented with sulfamethoxazole, for 40h; E.A - stands for ancestral, E:40 01-40 05 - stands for populations evolved without the metal, E.40 Cu1-40 Cu5 - stands for populations evolved with copper and E.40 Zn1-40 Zn5 - refers to populations evolved with zinc;

For all the conditions tested, the fitness of *A. hydrophila* populations after 20 transfers and 40 transfers was similar (appendix 7.3). After 40 transfers (Figure 20-23), differences between populations growth rates and doubling times were observed particularly when cultivated in LB supplemented with metal. In LB supplemented with copper, populations evolved with copper presented an average growth rate 1.5 X lower than populations evolved without the contaminant and average doubling times 1.8 X higher. Contrarily, in LB supplemented with zinc, populations evolved with this metal presented an average growth rate 1.5 X higher than populations growing without metal and average doubling time 1.5 X lower.

Table 12: Grow rates [r = ln (OD2/OD1)/(T2-T1)] of *A. hydrophila* populations after 40 transfers, per min; growing in LB, LB supplemented with copper, LB supplemented with zinc and LB supplemented with sulfamethoxazole; A.A - stands *A. hydrophila* ancestral population, A.40 01-40 05 - stands for populations evolved without the metal, A.40 Cu1-40 Cu5 - stands for populations evolved with copper and A.40 Zn1-40 Zn5 - refers to populations evolved with zinc;

Growth rates after 40 transfers								
***A. hydrophila* populations**	**LB**	**Mean***	**LB+Cu**	**Mean***	**LB+Zn**	**Mean***	**LB+SMX**	**Mean***
A.A	0.006	-	0.013	-	0.004	-	0.019	-
A.40 01	0.009	0.008 ±0.002	0.010	0.009 ±0.003	0.005	0.06 ±0.001	0.025	0.019 ±0.007
A.40 04	0.005		0.005		0.006		0.023	
A.40 05	0.010		0.012		0.007		0.010	
A.40 Cu1	0.005	0.007 ±0.001	0.006	0.005 ±0.001	-	-	0.015	0.014 ±0.002
A.40 Cu3	0.008		0.005		-		0.017	
A.40 Cu5	0.008		0.004		-		0.012	
A.40 Zn1	0.012	0.010 ±0.002	-	-	0.010	0.009 ±0.001	0.019	0.019 ±0.001
A.40 Zn2	0.012		-		0.009		0.021	
A.40 Zn5	0.008		-		0.007		0.018	

* ± standard deviation

Table 13: Doubling time [Dt = ln2/r] of *A. hydrophila* populations after 40 transfers, per minute; growing in LB, LB supplemented with copper, LB supplemented with zinc and LB supplemented with sulfamethoxazole; A.A - stands *A. hydrophila* ancestral population, A.40 01-40 05 - stands for populations evolved without the metal, A.40 Cu1-40 Cu5 - stands for populations evolved with copper and A.40 Zn1-40 Zn5 - refers to populations evolved with zinc;

Doubling time after 40 transfers								
***A. hydrophila* populations**	**LB**	**Mean***	**LB+Cu**	**Mean***	**LB+Zn**	**Mean***	**LB+SMX**	**Mean***
A.A	117.5	-	55.5	-	182.4	-	35.9	-
A.40 01	75.3	91.8 ±30	72.2	92.9 ±41	144.4	121.8 ±21	27.9	43.4 ±21
A.40 04	133.3		150.7		126.0		30.0	
A.40 05	66.6		55.9		95.0		722	
A.40 Cu1	130.8	102.5 ±20	108.3	141.3 ±24	-	-	46.2	49.1 ±8
A.40 Cu3	87.7		150.7		-		41.3	
A.40 Cu5	88.9		165.0		-		59.8	
A.40 Zn1	59.8	68.2 ±12	-	-	72.2	82.7 ±13	36.3	36.0 ±2
A.40 Zn2	60.3		-		75.3		33.5	
A.40 Zn5	84.5		-		100.5		38.3	

* ± standard deviation

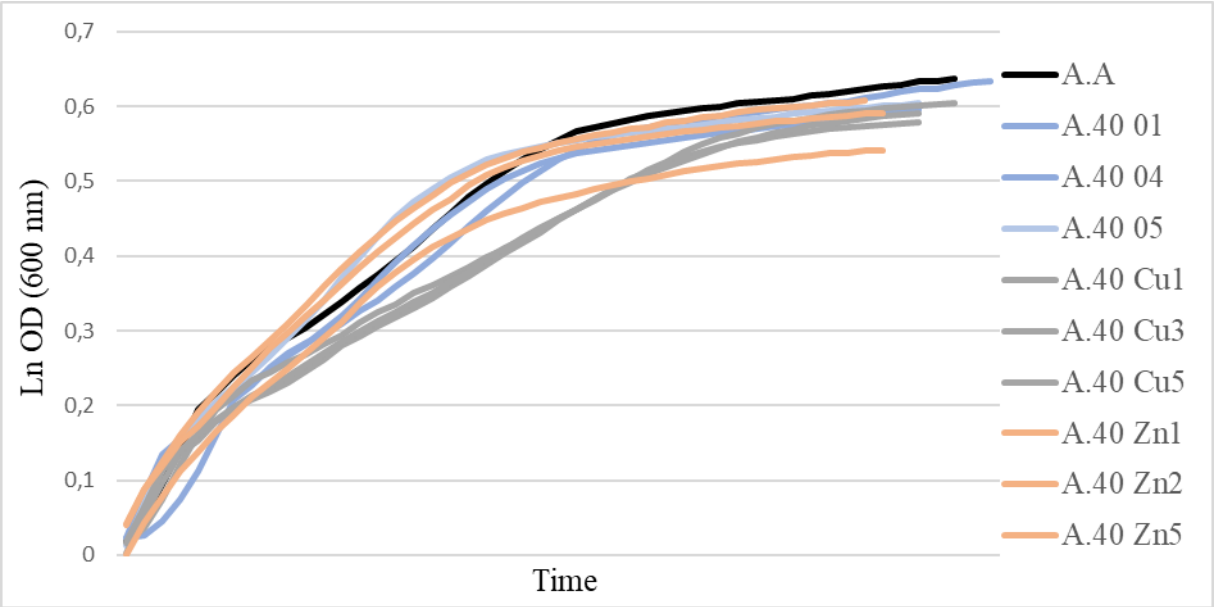


Figure 20: Growth of *A. hydrophila* populations after 40 transfers in LB for 24h; A.A - stands for ancestral, A.40 01-40 05 - stands for populations evolved without the metal, A.40 Cu1-40 Cu5 - stands for populations evolved with copper and A.40 Zn1-40 Zn5 - refers to populations evolved with zinc;

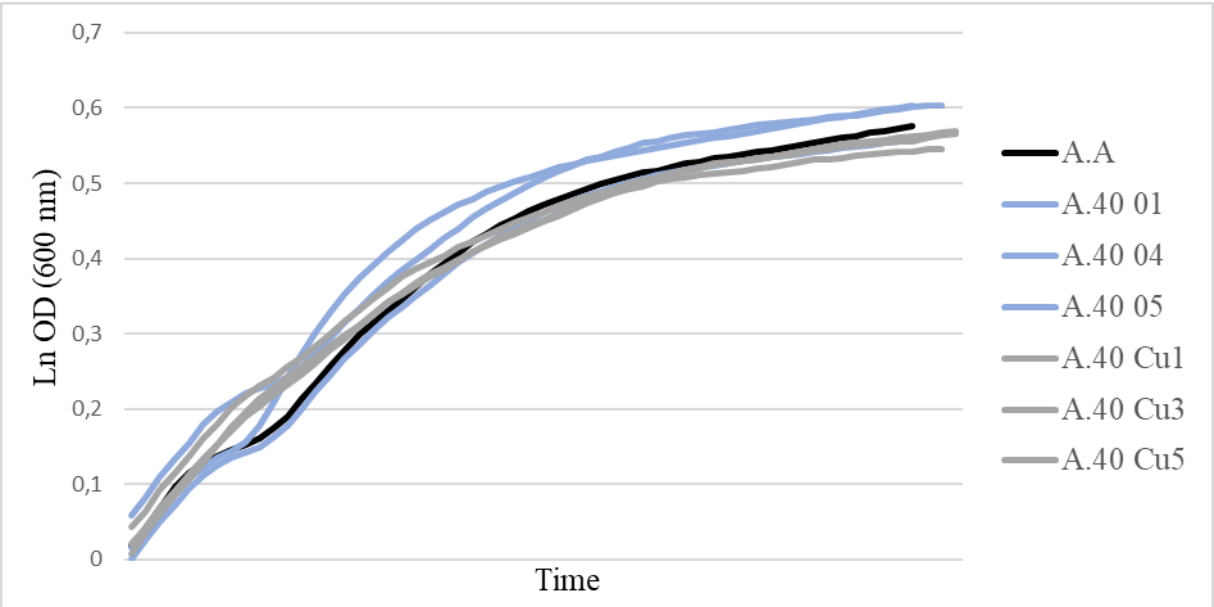


Figure 21: Growth of *A. hydrophila* populations after 40 transfers in LB supplemented with copper, for 30h; A.A - stands for ancestral, A.40 01-40 05 - stands for populations evolved without the metal and A.40 Cu1-40 Cu5 - stands for populations evolved with copper;

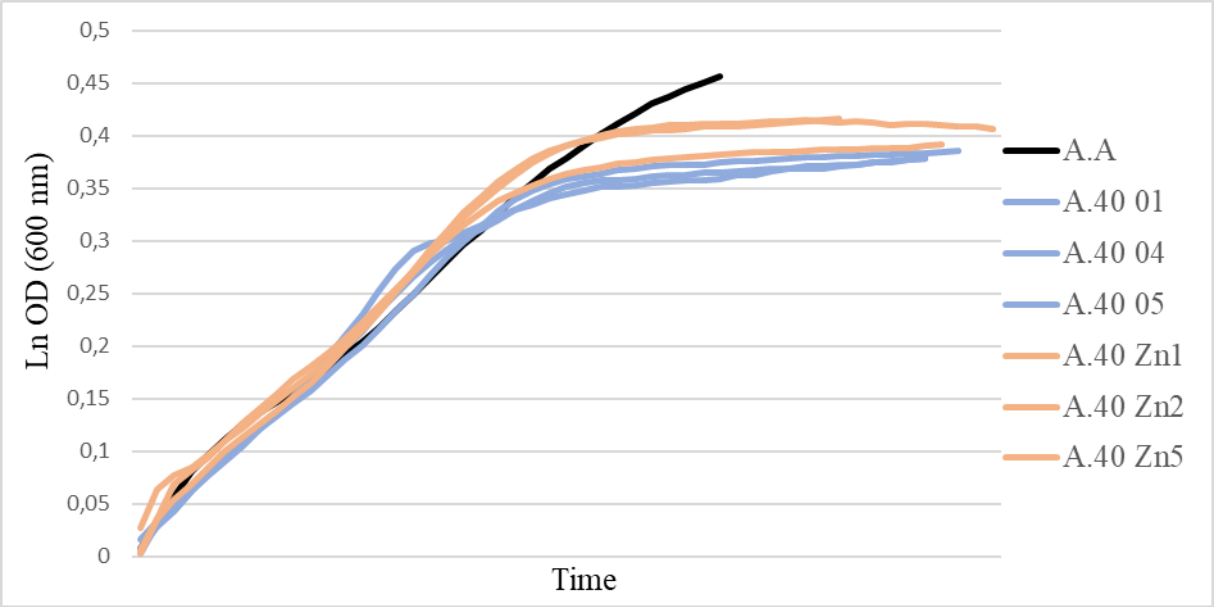


Figure 22: Growth of *A. hydrophila* populations after 40 transfers in LB supplemented with zinc, for 30h; A.A - stands for ancestral, A.40 01-40 05 - stands for populations evolved without the metal and A:40 Zn1-40 Zn5 - refers to populations evolved with zinc;

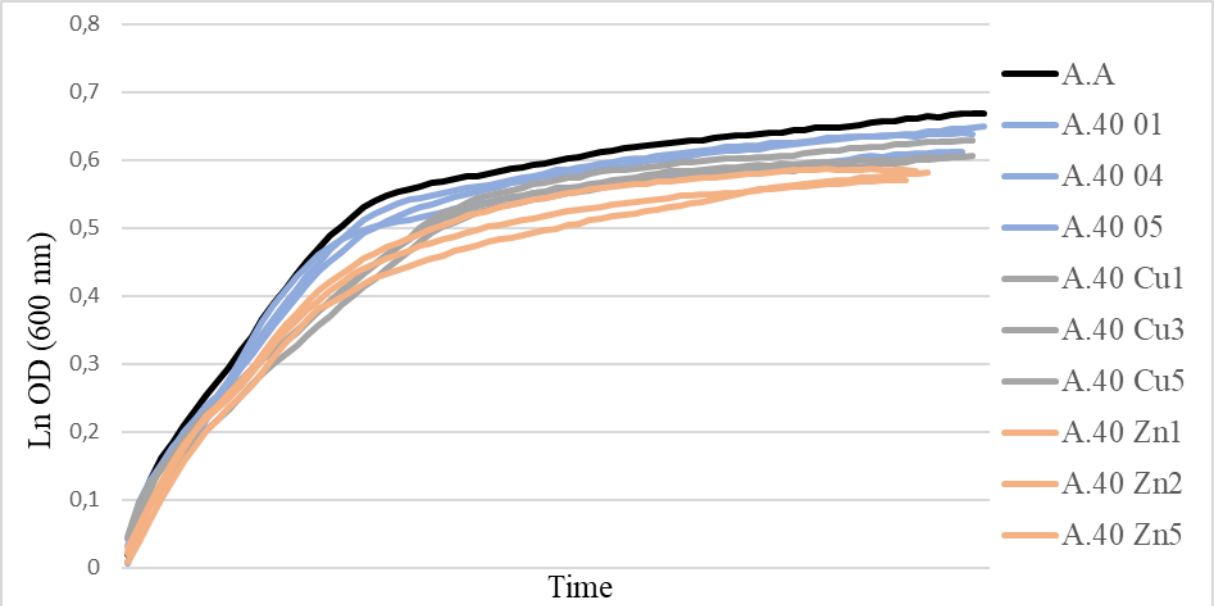


Figure 23: Growth of *A. hydrophila* populations after 40 transfers in LB supplemented with sulfamethoxazole, for 40h; A.A - stands for ancestral, A.40 01-40 05 - stands for populations evolved without the metal, A.40 Cu1-40 Cu5 - stands for populations evolved with copper and A.40 Zn1-40 Zn5 - refers to populations evolved with zinc;

3.1.6 Comparison of genotypic profiles

For both species, the analysis of BOX and ERIC-PCR profiles did not show genotypic differences between the ancestral and the populations exposed to copper and zinc, both after 20 and 40 transfers (Figure 24; appendix 7.4). Comparing BOX and ERIC-PCR methodologies, for *E.coli* populations, the amplification of box elements produced more complex band patterns, allowing a better discrimination of possible differences between isolates. On the other hand, for *A. hydrophila* populations, the amplification of enterobacterial repetitive intergenic consensus sequences (ERIC-PCR) produced more complex profiles.

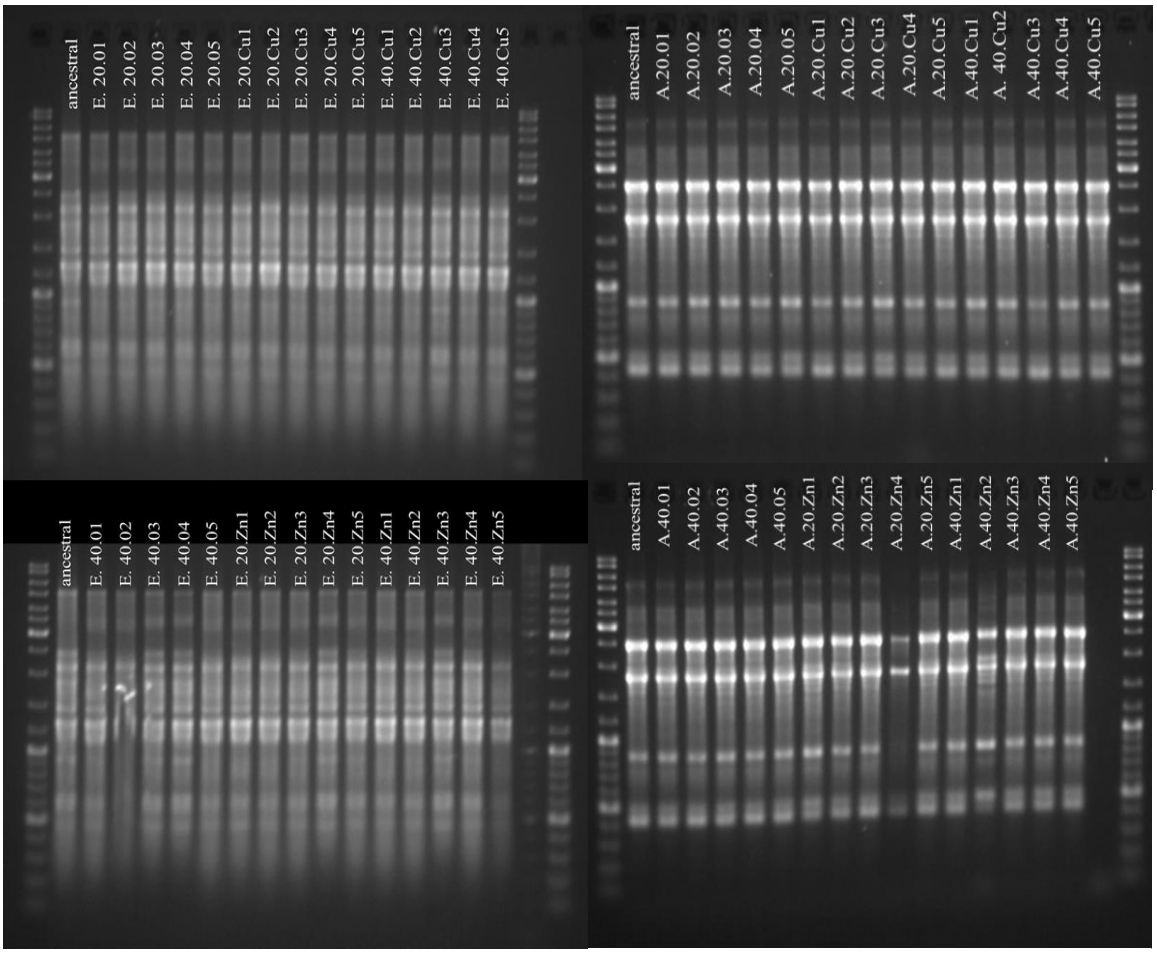


Figure 24: BOX-PCR DNA fingerprint patterns for *E. coli* (E; left) and ERIC-PCR DNA fingerprint for *A. hydrophila* populations (A; right). The lanes of the ends contained an external standard, a 1-kb molecular weight ladder (GeneRuler™ DNA ladder Mix - Thermo Fisher Scientific, USA). 20 stands for 20 transfers and 40 stands for 40 transfers, 01-05 - stands for populations evolved without the metal, Cu1-Cu5 - stands for populations evolved with copper and Zn1-Zn5 - refers to populations evolved with zinc.

3.2 Microcosms assay

3.2.1 Plate counts on antibiotic-selective media

To assess metal effects on antibiotic resistance selection, a lab-scale microcosm was steed up. The number of culturable bacteria resistant to different antibiotics was determined, as well as the percentage of antibiotic resistant bacteria [(CFUs mL $^{-1}$ in PCA supplemented with antibiotic/CFUs mL^{-1} in PCA)*100], after 10 and 20 days of exposure to copper and zinc.

After 10 days of exposure, no significant differences were detected in the number of bacteria resistant to cefotaxime, between the populations exposed to copper and the control populations (Figure 25). Percentage of cefotaxime resistance was 19.94% for control, 23.22% for bacterial communities exposed to 50 µg L^{-1} of copper and 29.50% for communities exposed to 100 µg L^{-1} (Table 14). After 20 days of exposure, the number of cefotaxime-resistant bacteria increased significantly in communities exposed to copper. Percentage of bacteria resistant to this compound increased from 2.35% in control to 9.49% for bacterial communities exposed to 50 µg L^{-1} of copper and to 16.75% in that exposed to 100 µg L^{-1} of this metal.

For tetracycline, a significant decrease in the number of bacteria resistant to this compound was observed after 10 days of exposure to copper at 50 µg L^{-1} (Figure 26). Percentage of tetracycline resistant bacteria decreased from 0.67% in communities growing without the metal to 0.59% in bacterial communities exposed to 50 µg L^{-1} of the metal (Table 14). The scenario was different after 20 days of exposure. Communities exposed to 100 µg L^{-1} of copper suffered a significative increase in the number of bacteria resistant to this compound. Percentage of tetracycline resistant bacteria increased from 0.03% in control to 0.23% among bacterial communities exposed to 100 µg L^{-1} of copper.

A significative decrease in the number of kanamycin resistant bacteria was observed after 10 days of exposure, for both copper concentrations tested (Figure 27). Percentage of bacteria resistant to this compound decreased from 3.85% in control to 3.11% and 1.96% for communities exposed to 50 and 100 µg L^{-1}, respectively (Table 14).

Table 14: Percentage of resistant bacteria for bacterial communities exposed to copper, CT stands for bacterial communities growing without a contaminant; Cu50 stands for communities exposed to 50 µg L^{-1} of copper and Cu100 stands for exposure to 100 µg L^{-1}, after 10 (T10) and 20 days (T20) of exposure;

Antibiotic	T0			T10			T20		
	CT	Cu50	Cu100	CT	Cu50	Cu100	CT	Cu50	Cu100
CTX	30,53	30,40	32,96	19,94	23,22	29,50	2,35	9,49	16,75
TET	1,28	1,17	1,12	0,67	0,59	0,71	0,03	0,11	0,23
KAN	16,52	12,17	12,40	3,85	3,11	1,96	1,26	1,69	0,97

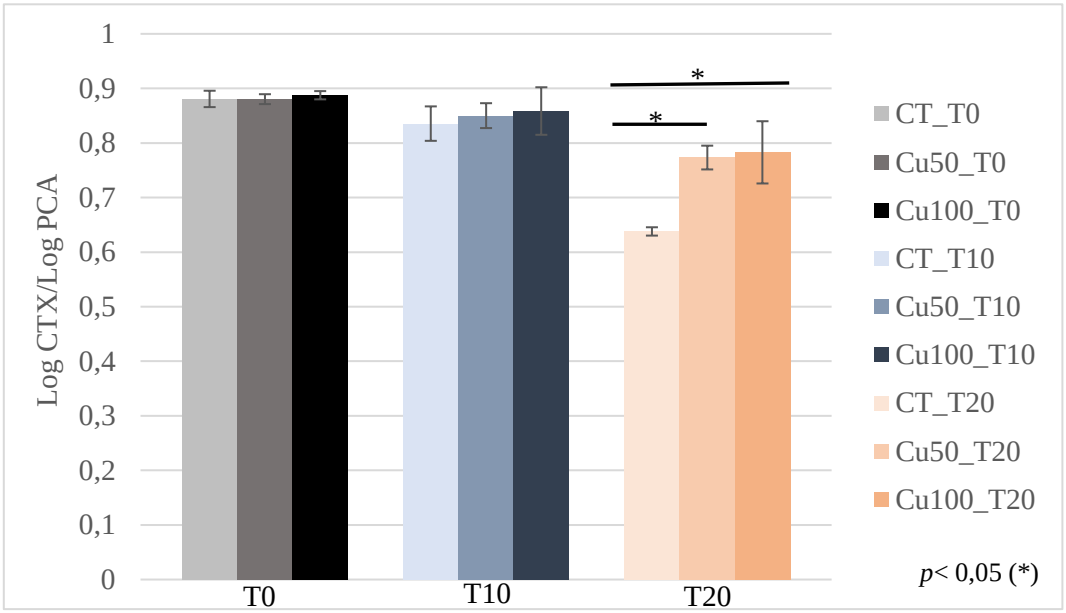


Figure 25: Prevalence of bacteria resistant to cefotaxime over time measure as followed: log of the number of bacteria per mL in PCA supplemented with CTX/ log of the number of bacteria per mL in PCA, when exposed to copper; CT stands for bacterial communities growing without a contaminant; Cu50 stands for communities exposed to 50 µg L^{-1} of copper and Cu100 stands for exposure to 100 µg L^{-1}, after 10 (T10) and 20 days (T20) of exposure;

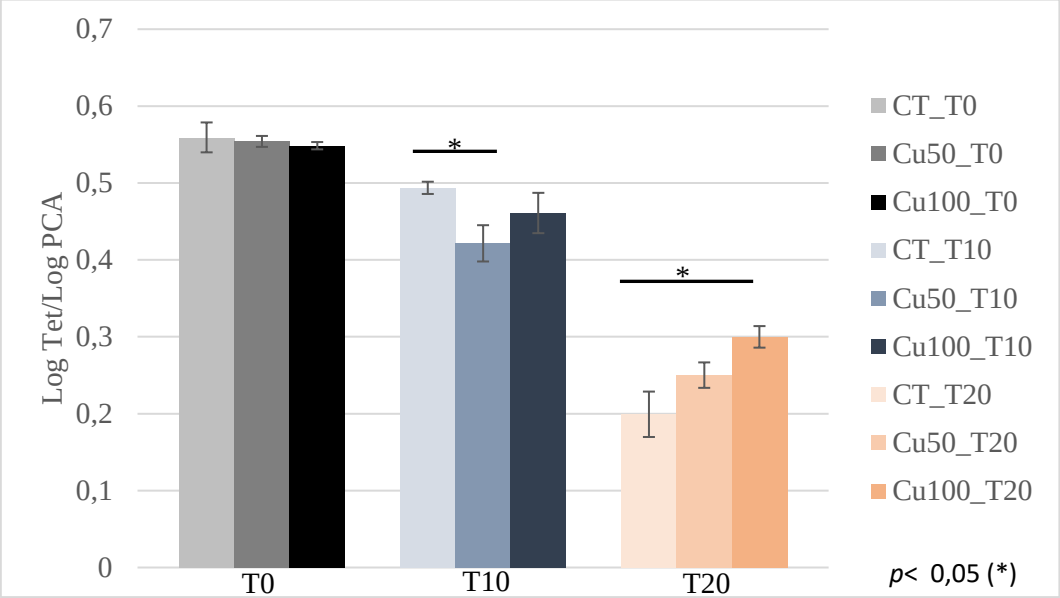


Figure 26: Prevalence of bacteria resistant to tetracycline over time measure as followed: log of the number of bacteria per mL in PCA supplemented with Tet/ log of the number of bacteria per mL in PCA, when exposed to copper; CT stands for bacterial communities growing without a contaminant; Cu50 stands for communities exposed to 50 µg L^{-1} of copper and Cu100 stands for exposure to 100 µg L^{-1}, after 10 (T10) and 20 days (T20) of exposure;

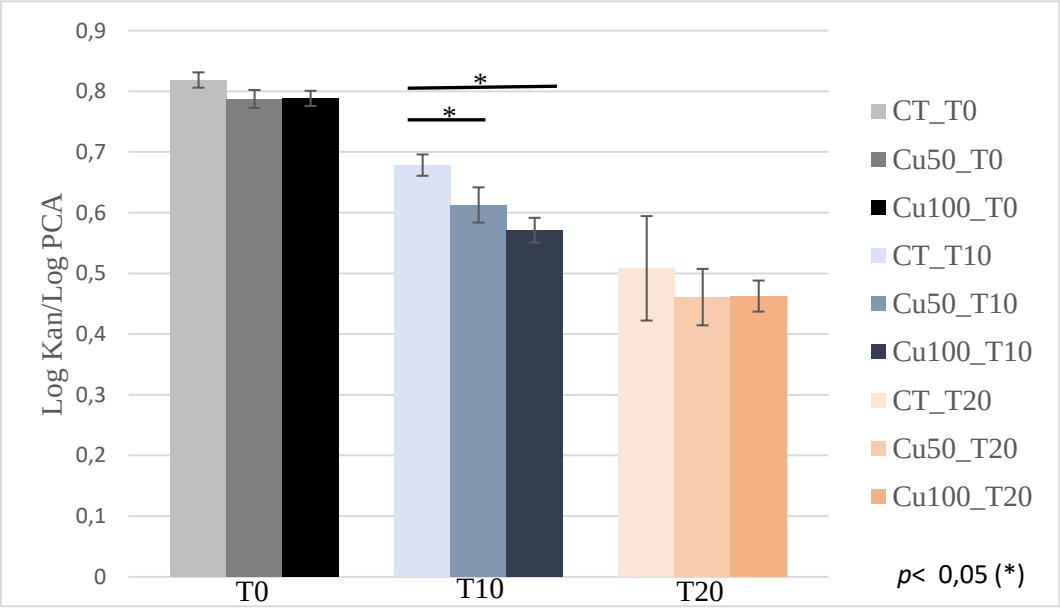


Figure 27: Prevalence of bacteria resistant to kanamycin over time measure as followed: log of the number of bacteria per mL in PCA supplemented with Kan/ log of the number of bacteria per mL in PCA, when exposed to copper; CT stands for bacterial communities growing without a contaminant; Cu50 stands for communities exposed to 50 µg L^{-1} of copper and Cu100 stands for exposure to 100 µg L^{-1}, after 10 (T10) and 20 days (T20) of exposure;

For bacterial communities exposed to zinc, the number of cefotaxime-resistant bacteria increased for both concentrations, and after 10 and 20 days of exposure (Figure 28). Percentage of cefotaxime-resistant bacteria increased from 41.98% in the control communities to 76.80% and 70.84% in bacterial communities exposed for 10 days to 50 and 100 µg L^{-1}, respectively (Table 15). This increase was even higher after 20 days of exposure. Percentage of cefotaxime-resistant bacteria increased from 24.59% in the control communities to 91.28% in communities exposed to 50 µg L^{-1} of zinc, and to 72.43% for communities exposed to 100 µg L^{-1} of this metal.

For kanamycin, a significative increase in the number of bacteria resistant to this compound was observed after 20 days of exposure to both concentrations of zinc tested (Figure 29). After 20 days, percentage of kanamycin-resistant bacteria increased from 6.08% in control to 24.11% in communities exposed to the lower concentration, and to 42.97% in bacterial communities exposed to 100 µg L^{-1} of this metal (Table 15).

Bacterial communities exposed to zinc demonstrated an increased resistance to tetracycline for both concentrations tested, after 10 days of exposure (Figure 30). Percentage of tetracycline-resistant bacteria increased from 0.01% in communities growing without the contaminant to approximately 0.6% in bacterial communities exposed to both concentrations (Table 15).

For imipenem, the number of resistant bacteria increased in communities exposed for 10 days to both concentrations (Figure 31). Percentage of imipenem-resistant bacteria increased from 0.02% in control to 0.31% in communities growing in 50 µg L^{-1} of zinc, and to 0.37% in communities exposed to 100 µg L^{-1} of this metal (Table 15).

Table 15: Percentage of resistant bacteria for bacterial communities exposed to zinc, CT stand for bacterial communities growing without a contaminant; Zn50 stands for communities exposed to 50 µg L^{-1} of zinc and Zn100 stands for exposure to 100 µg L^{-1}, after 10 (T10) and 20 days (T20) of exposure;

Antibiotic	T0			T10			T20		
	CT	Zn50	Zn100	CT	Zn50	Zn100	CT	Zn50	Zn100
CTX	30,62	29,62	25,87	41,98	76,80	70,84	24,59	91,28	72,43
TET	0,86	0,88	0,87	0,01	0,06	0,06	0,00	0,04	0,04
KAN	2,74	3,01	2,88	4,71	9,18	5,37	6,08	24,11	42,97
IMI	1,00	1,12	1,09	0,02	0,31	0,37	0,06	0,07	0,04

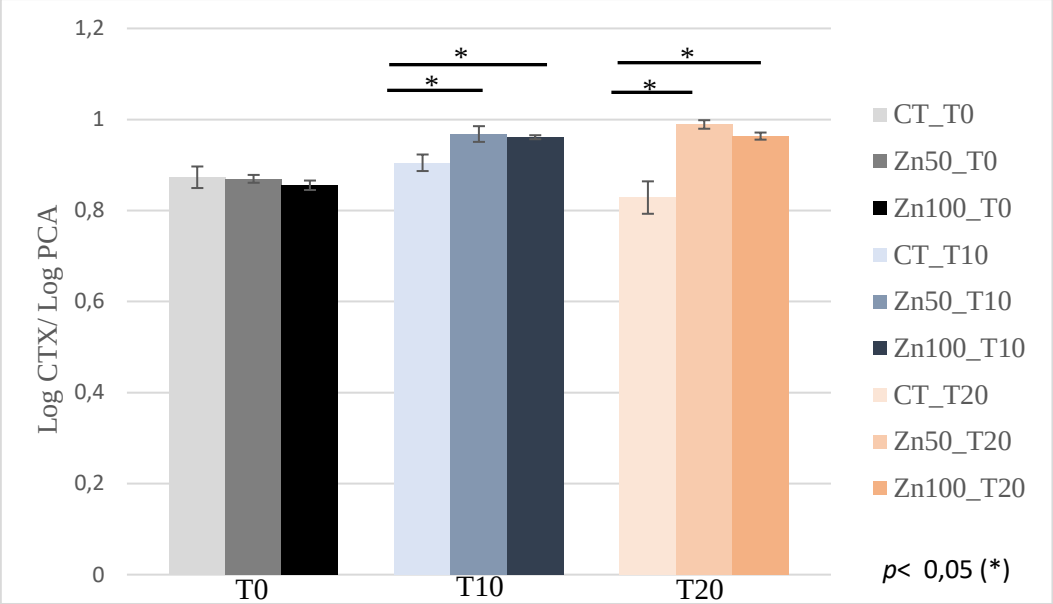


Figure 28: Prevalence of bacteria resistant to cefotaxime over time measure as followed: log of the number of bacteria per mL in PCA supplemented with CTX/ log of the number of bacteria per mL in PCA, when exposed to zinc; CT stands for bacterial communities growing without a contaminant; Zn50 stands for communities exposed to 50 µg L^{-1} of zinc and Zn100 stands for exposure to 100 µg L^{-1}, after 10 (T10) and 20 days (T20) of exposure;

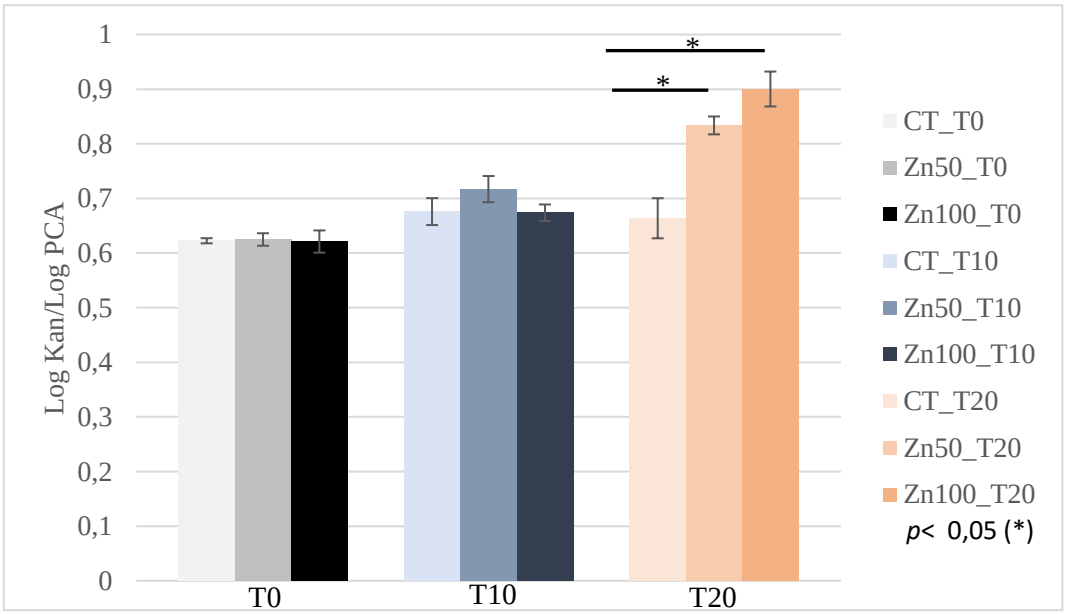


Figure 29: Prevalence of bacteria resistant to kanamycin over time measure as followed: log of the number of bacteria per mL in PCA supplemented with Kan/ log of the number of bacteria per mL in PCA, when exposed to zinc; CT stands for bacterial communities growing without a contaminant; Zn50 stands for communities exposed to 50 µg L^{-1} of zinc and Zn100 stands for exposure to 100 µg L^{-1}, after 10 (T10) and 20 days (T20) of exposure;

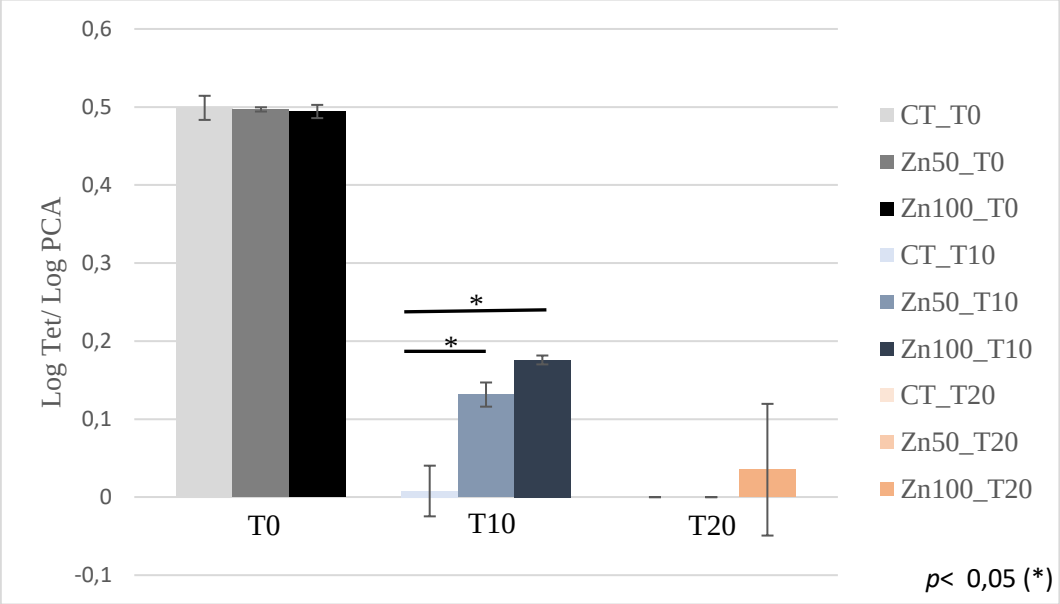


Figure 30: Prevalence of bacteria resistant to tetracycline over time measure as followed: log of the number of bacteria per mL in PCA supplemented with Tet/ log of the number of bacteria per mL in PCA, when exposed to zinc; CT stands for bacterial communities growing without a contaminant; Zn50 stands for communities exposed to 50 µg L^{-1} of zinc and Zn100 stands for exposure to 100 µg L^{-1}, after 10 (T10) and 20 days (T20) of exposure;

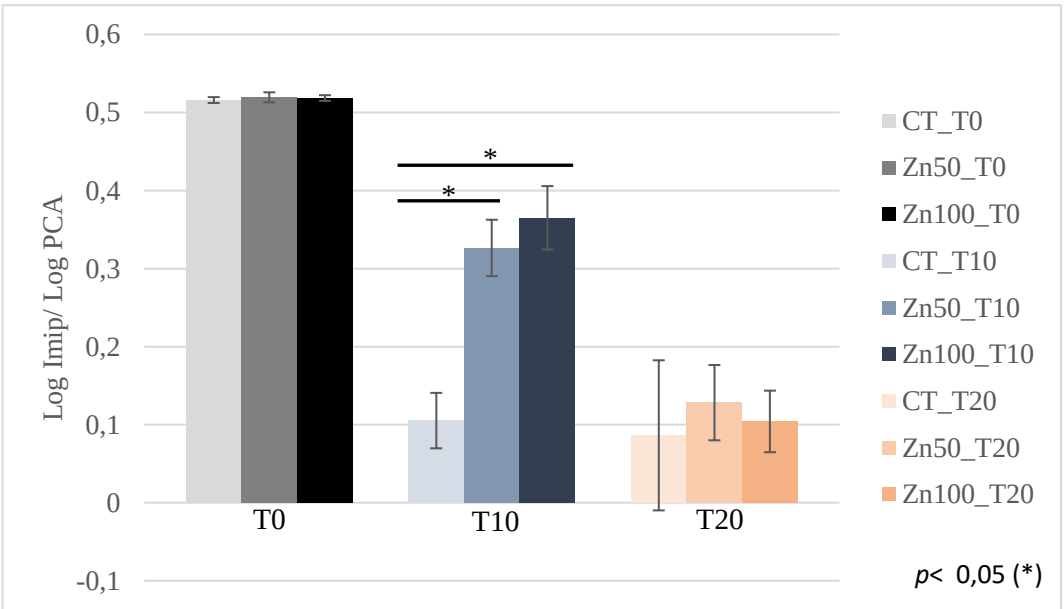


Figure 31: Prevalence of bacteria resistant to imipenem over time measure as followed: log of the number of bacteria per mL in PCA supplemented with Imip/ log of the number of bacteria per mL in PCA, when exposed to zinc; CT stands for bacterial communities growing without a contaminant; Zn50 stands for communities exposed to 50 µg L^{-1} of zinc and Zn100 stands for exposure to 100 µg L^{-1}, after 10 (T10) and 20 days (T20) of exposure;

3.2.2 Taxonomic affiliation of strains selected during the microcosm experiment

After 20 days of exposure, several colonies were selected from plates supplemented with cefotaxime (8 µg mL^{-1}), from microcosms exposed to 100 µg L^{-1} of Cu, for identification. For microcosms exposed to 100 µg L^{-1} of Zn, colonies from plates supplemented with kanamycin (64 µg mL^{-1}) were also selected. 16S rRNA gene sequences were compared with sequences deposited in GenBank (NCBI, USA) and in EzBioCloud databases (appendix 7.5).

Among bacterial communities exposed to copper for 20 days, colonies resistant to cefotaxime belong to the following genera: *Bacillus* (n=2), *Delftia* (n=1), *Janthinobacterium* (n=2), *Pseudomonas* (n=17) and *Sphingobium* (n=2), being the genus *Pseudomonas* the most represented (17 out of 24). Concerning bacterial communities exposed to zinc, the selected colonies from plates supplemented with cefotaxime affiliated to *Ensifer* (n=3), *Sphingomonas* (n=9), *Pseudomonas* (n=1), *Pedobacter* (n=2) and *Pseudacidovorax* (n=1) genera. Among these, *Sphingomonas* (9 out of 16) was the most represented genus. Bacteria resistant to kanamycin belongs mainly to *Ensifer* (n=13) followed by *Pedobacter* (n=3) genus.

3.2.3 DGGE analysis of bacterial communities

DGGE analysis of 16S rRNA gene fragments was used to investigate the effect of metal exposure on the bacterial community structure after 10 and 20 days of exposure. DGGE profiling revealed that metal exposure altered the structure of these communities. These changes were dependent on the exposure time and the metal (Figure 32).

The dendrogram resulting from the DGGE banding profiles clearly showed that bacterial communities were different after 10 and 20 days of exposure – these communities were separated into two main clusters sharing approximately 40% of similarity (Figure 32). After 10 days of exposure, communities were separated in two different clusters – one composed by control communities and one composed by communities exposed to metals (approximately 45% of similarity). Among these, bacterial communities exposed to copper and zinc showed approximately 60% of similarity. After 20 days of exposure, two clusters were formed with approximately 55% of similarity – one composed by communities exposed to copper and the other composed by control and communities exposed to zinc. Control and communities exposed to zinc shared 60% of similarity.

PCoA analyses of the DGGE profiles (Figure 33) demonstrated that 28.4% of the total variation verified among the bacterial communities may be explained by the time of exposure.

The exposition to contaminants seems to explain a significant part of the variation observed for communities exposed for 10 days (distributed along the axis that represents 20.7% of the variation). However, after 20 days of exposure the variation between bacterial communities was lower.

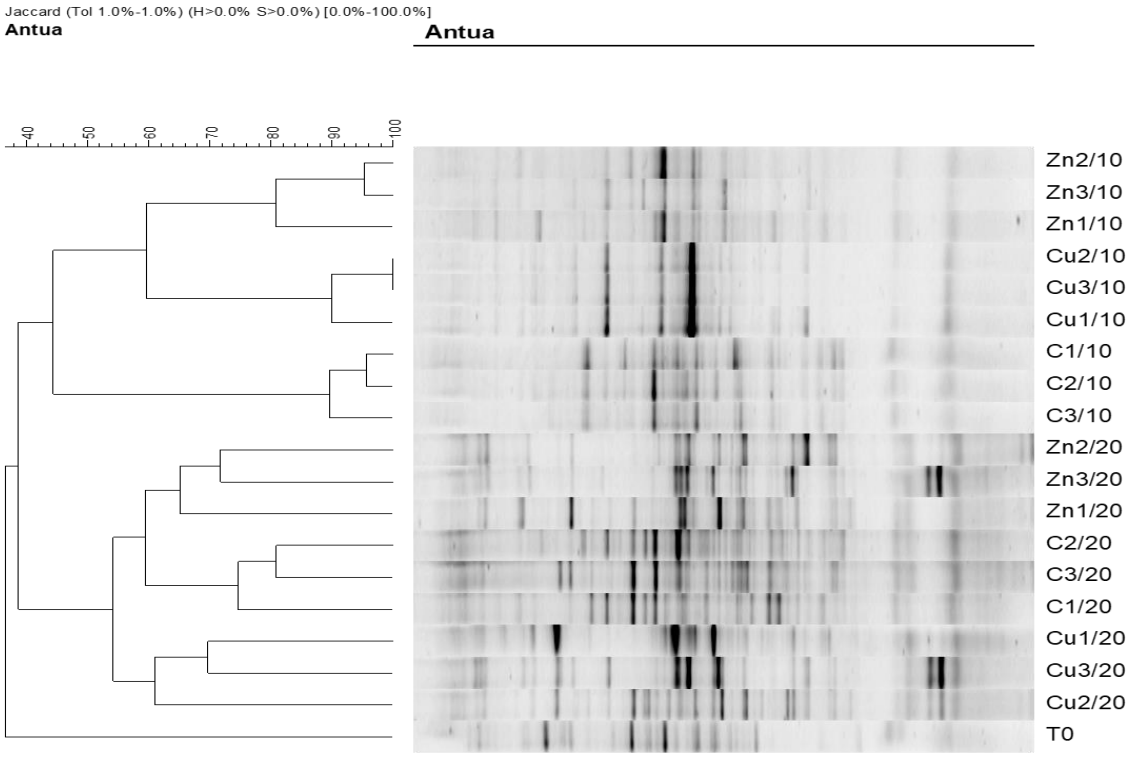


Figure 32: Bacterial community structure exposed to 100 µg L^{-1} of copper and zinc over time (10 and 20 days) analyzed by DGGE profiling of 16S rRNA gene fragments;

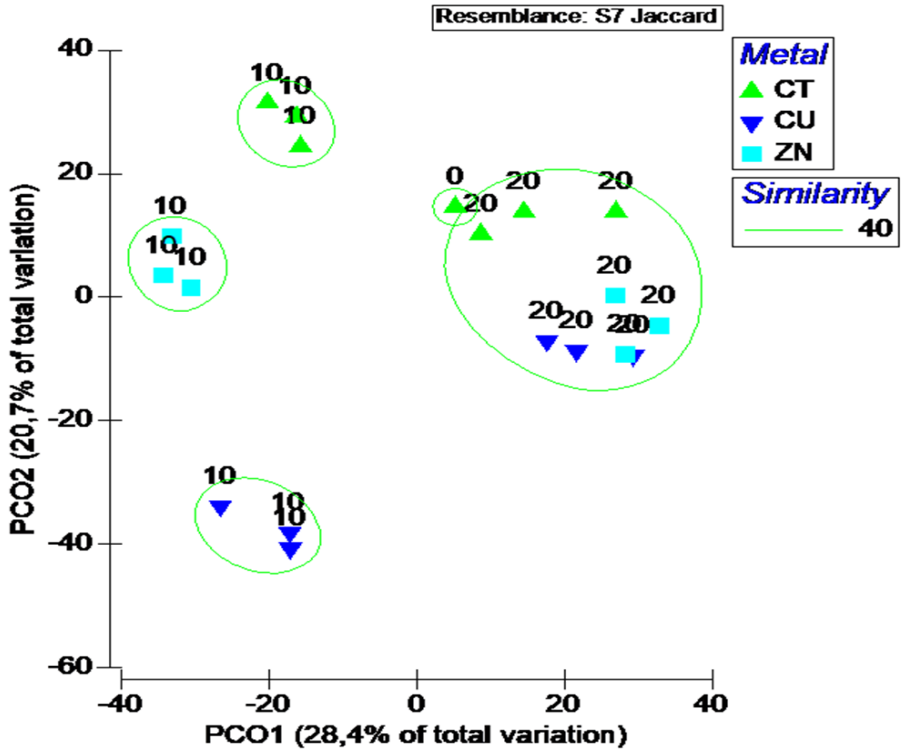


Figure 33: PCoA of bacterial communities exposed to 100 µg L^{-1} of copper and zinc after 10 and 20 days of exposure;

Diversity indexes calculated based on DGGE profiles showed significative differences between control communities and communities exposed to copper, concerning richness index (S) (Table 16). Richness of communities exposed to copper for 10 days decreased from 23 ±0.47 to 18 ±0.47. After 20 days of exposure to this metal, the trend was similar – richness of bacterial communities exposed to copper decreased from 41 ±3.86 in control to 30 ±2.94. Concerning diversity, significative differences were observed between control and communities exposed to copper for 10 days - Shannon index of diversity (H) decreased from 2.77 ±0.10 in control to 2,25 ±0,10 in populations exposed to copper. The same was observed for communities exposed to zinc for 20 days. Diversity index (H) decreased from 3,33 ±0,10 in populations growing without metal to 3,03 ±0,07 in communities exposed to zinc. Concerning Pielou's evenness index (J), a significative decrease was observed from 0,89 ±0,03 in control to 0,77 ±0,03 in populations exposed to copper for 10 days.

Table 16: Bacterial diversity of the bacterial communities exposed to 100 µg L^{-1} of copper and zinc after 10 and 20 days of exposure (based on DGGE profiles);

Time of exposure	Sample	Richness index (S)	Shannon index of diversity (H)	Pielou's evenness index (J)
10 days	CT	23 ±0,5[a]	2,77 ±0,1[a]	0,89 ±0,03[a]
	Cu	18 ±0,5[a]	2,25 ±0,1[a]	0,77 ±0,03[a]
	Zn	21 ±0,8	2,60 ±0,1	0,85 ±0,04
20 days	CT	41 ±4[a]	3,33 ±0,1[a]	0,90 ±0,01
	Cu	30 ±3[a]	2,95 ±0,2	0,87 ±0,03
	Zn	32 ±3	3,03 ±0,07[a]	0,87 ±0,01

[*]Values are means of three replicates ± standard deviation

[a]Significantly different ($p < 0,05$)

4. DISCUSSION

The main goal of this study was to investigate the role of metals in the selection and evolution of antibiotic resistance in aquatic systems. Since this compartment is a large reservoir of both metals and antibiotics, in addition to antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Suzuki et al., 2017), it plays a key role in antibiotic resistance dissemination. Thus, in this system, the risk of antibiotic resistance transfer to and among pathogens is extremely high and worrying (Manaia, 2017). To achieve our goal, an experimental evolution approach was used to investigate phenotype and genotype alterations induced by long-term exposure to copper and zinc, in two strains representing different species - *E. coli* ATCC® 25922™ and *Aeromonas hydrophila* CECT839^{T}. These strains were selected since *E. coli* is a common commensal inhabitant of the gastrointestinal tract of humans and a frequent agent of several human infections (Allocati et al., 2013); and *Aeromonas* sp. are ubiquitous waterborne bacteria frequently present in contaminated environments with potential to cause human diseases (Seshadri et al., 2006). Additionally, a microcosm approach was used to confirm selection of antibiotic resistance in aquatic bacterial communities imposed by exposure to copper and zinc. These metals were selected since several studies associated the presence of copper and zinc with antibiotic resistance and ARGs dissemination (Poole, 2017; Wales and Davies, 2015). Additionally, high levels of these compounds reach the aquatic systems as the result of a plethora of anthropogenic activities (https://europa.eu). The concentrations of copper and zinc that have been reported in surface water ranged from 0.7 μg L^{-1} to 162 μg L^{-1} (Liu et al., 2018). Thus, in this study bacterial communities were exposed to environmentally relevant concentrations of copper and zinc (50 μg L^{-1} and 100 μg L^{-1}).

4.1 Phenotype and genotype alterations induced by the presence of metals

Populations of *Escherichia coli* ATCC® 25922™ and *Aeromonas hydrophila* CECT 839^{T} were exposed to a gradient of copper and zinc for 40 transfers. Although populations of both strains evolved to grow in a gradient with a maximum concentration of 400 μg mL^{-1} for both metals, *A. hydrophila* populations seem to adapt more swiftly to the presence of these metals. *E. coli* is a human commensal organism (Minogue et al., 2014) while *A. hydrophila* is an environmental isolate (Seshadri et al., 2006). In natural environments, bacterial isolates are constantly subjected to environmental stresses that can contribute to a faster adaptation to different environmental conditions. *A. hydrophila* CECT 839^{T} is considered one of the most pathogenic species among the genus *Aeromonas* (Janda and Abbott, 2010; Seshadri et al., 2006),

which could be the result of an array of genes that allows its adaptation, more easily, to different conditions. Several studies also reported that *A. hydrophila* survives in polluted waters, being resistant to various disinfectants, insecticides, and chemicals. As an example, Tacão et al. (2012) compared the antibiotic resistome within polluted and unpolluted aquatic systems and found that 21.7% of the isolates resistant to cefotaxime found in polluted waters affiliated with *Aeromonas* spp.. On the other hand, being a commensal isolate, *E. coli* ATCC® 25922™, in theory, is less prepared to adapt to changes in environmental conditions. Nevertheless, both species had the ability to grow in gradients of increasing concentrations of both metals, showing their adaptability.

For both species, populations evolved with copper or zinc demonstrated phenotype alterations when compared to the ancestral or to the populations evolved without a metal. Changes in tolerance towards metal(loid)s, antibiotic susceptibility and fitness were observed.

Exposure to a long-term selective pressure imposed by copper or zinc led to alterations in the susceptibility profile to other metals. Concerning *E. coli*, populations evolved with copper increased their tolerance not only to this metal, but also to chromium and, for some populations, to nickel. *E. coli* populations evolved with zinc increased their tolerance to zinc and copper, and, for some populations, to chromium and nickel. *A. hydrophila* populations evolved both with copper and zinc became more tolerant to chromium. The most probable explanation for this increase in tolerance to different metals is the expression of genes encoding for efflux pumps that efflux several metals. It has been demonstrated that tolerance to high levels of copper and zinc is associated with efflux pumps that reduce the intracellular amount of these compounds (Choi et al., 2017; Franke et al., 2003). The same is true for other metals such as chromium (Viti et al., 2014). Additionally, it is known that zinc is an important co-factor for several enzymes that contribute to the proper functioning of the antioxidant defense system being, for instance, a potent metallothionein inducer (Marreiro et al., 2017). Metallothioneins are proteins effective in reducing hydroxyl radicals and sequestering reactive oxygen species (ROS) produced in stressful situations (Marreiro et al., 2017). In fact, metallothionein has the ability to effectively bind metal ions, such as zinc, copper and chromium (Marreiro et al., 2017). An additional explanation could be the overexpression of genes related with SOS DNA repair system, due to copper and zinc exposure, that could lead to increased tolerance to other metals. Indeed, a mechanism of resistance to chromium compounds in *E. coli*, already described by several studies, is the activation of genes related to SOS response to repair DNA damages (Ramírez-Díaz et al., 2008). The decreased tolerance observed against mercury for *E. coli* populations evolved with copper and zinc could be the result of the need for these populations to direct their energy to the efflux of high levels of copper and zinc, decreasing the expression of genes encoding for mercury detoxification. Still,

our results showed that exposure to copper and zinc increase bacterial tolerance not only to these metals, but also to additional ones.

Antibiotic susceptibility testing was performed to evaluate the metal effect on the sensitivity of these populations to several clinically-important antibiotic classes. For *E. coli* and *A. hydrophila* populations evolved with metals, results confirmed alterations in terms of susceptibility to antibiotics, with an increase in MICs for some. For instance, the MIC of kanamycin, an aminoglycoside antibiotic that inhibits protein synthesis (Garneau-Tsodikova and Labby, 2016), was increased in populations of *E. coli* exposed to copper and populations of *A. hydrophila* exposed both to copper and zinc. Bacterial phenotypes corresponding to decreased susceptibility to antibiotics could be the result of mutations due to metal exposure. In fact, it has been described that mutations can be responsible for aminoglycoside resistance, such as kanamycin. Mutations that altered efflux pumps that further efflux these compounds; modifications leading to the fortification of cell wall, which serve as a natural barrier for these molecules; or, although rare, mutations in the ribosomal target of aminoglycosides, have been reported (Garneau-Tsodikova and Labby, 2016).

The most drastic phenotypic change was observed for sulfamethoxazole, to which an increase of 34X in the MIC of *E. coli* populations evolved with copper was registered. For *E. coli* populations evolved with zinc, a smother increase was also observed. However, the opposite (an MIC decrease) was observed for *A. hydrophila* populations evolved with metals. Sulfametoxazole is used to treat several infections, namely urinary tract infections, and act by blocking the synthesis of folic acid – essential for bacterial growth and reproduction (Guneysel et al., 2009). It is possible that the chromosomal mutation in *folP* gene, necessary for folic acid synthesis, results in lower affinity of sulfametoxazole to its target, decreasing the susceptibility of bacteria to this drug (Buwembo et al., 2013). In fact, mutations in *folP* gene leading to sulfonamide resistance have already been reported in *E. coli*, among other species (Sköld, 2000). However, *A. hydrophila* resistance to this compound is normally the result of two plasmid-borne genes, *sul1* and *sul2*, which could be an explanation for the results obtained for this species (Kadlec et al., 2011; Sköld, 2000).

E. coli populations evolved with copper demonstrated an increase in inhibition zone diameters to imipenem. However, an opposite result was obtained by MIC determination (MIC increased about 5.5X in populations evolved with copper). Differences in both methodologies could be the result of variations usually associated with disc diffusion test. In fact, several studies have been reporting discrepancies in disc diffusion performance and other antibiotic susceptibility testing (AST) methodologies (Badger et al., 2018; Jean et al., 2017; Lehtopolku et al., 2012). β-lactam resistance, can also be achieved by mutations that alter the target enzymes (Zapun et al.,

2008). However, in the case of β-lactams resistance, mutation of the target (PBPs) is less common in Gram-negative bacteria (Munita and Arias, 2016). Nevertheless, several mutations related to carbapenem resistance, for instance, have been described. As an example, the work of Yamachika et al. identified PBP2 mutations that conferred resistance to carbapenems in *E. coli* (Yamachika et al., 2013). Although this result should be further confirmed, the fact that copper exposure may induce phenotypes of decreased susceptibility to imipenem is extremely worrying since it is a last-resort antibiotic used for managing multidrug-resistant bacterial infections (Osei Sekyere, 2016). Additionally, the use or incorporation of copper in hospital surfaces, due to their supposed inherent antimicrobial properties, is a common practice (Sandle, 2017). Therefore, if copper can induce decreased susceptibility to these last resort antibiotics, this may have serious consequences for public health.

Beside mutations, other mechanisms can be responsible for bacterial phenotypes corresponding to decreased susceptibility to antibiotics such as changes in the expression of genes owing to direct effect of metals (activating or repressing genes). To survive high concentrations of copper or zinc, these populations may need to overexpress genes related with efflux pumps, that effluxes both metals and antibiotics. In fact, this association has been reported in *E. coli* by several studies, as reviewed by Anes et al. (2015). According to the authors, in *E. coli*, the MdtABC-TolC efflux system, for instance, is responsible for the extrusion of several substrates including both zinc and antibiotics (Anes et al., 2015).

The different responses to metal exposure observed between the two species tested can be the result of their genetic background. Although the mechanisms that led to resistance could be the same, the consequences can be different. For example, the same mutation, occurring in two different individuals, can have very different consequences for phenotype and for fitness (Wong, 2017). Nevertheless, both species became less susceptible to several clinically-important antimicrobial compounds due to metal exposure, which is extremely worrying. Both species are known to cause human infections and could be present in polluted aquatic systems (Tacão et al., 2012), where metal compounds are present in sub-inhibitory concentrations. Our results suggest that this exposure can lead to reduced antibiotic susceptibility in both species.

Growth assays revealed a high variability between the evolved populations for all the conditions tested. This result was expected since genetic changes among replicate populations, during adaptive evolution, depend both on the number of different possible adaptive mutations and on the frequency at which they occur (Nakatsu et al., 1998; Barrick and Lenski, 2009). Despite variability, results showed that for *E.coli*, populations evolved with metal presented lower fitness than populations evolved without the contaminant, for all conditions tested. An explanation could be the fact that metal exposure leads to mutations that can influence the bacterial fitness. It is

known that chromosomal mutations often have a negative impact on the host bacterium and these effects are often associated with a reduction in the bacterial growth rate (Melnyk et al., 2015). Mutations that alter the channel properties or levels of expression of outer membrane proteins to reduce the metal uptake through the outer membrane, for instance, can also reduce uptake of other, beneficial compounds, including nutrients, which can consequently cause a reduced growth rate (Knopp and Andersson, 2018). The opposite can also occur. The efflux of metals through transporters or efflux pumps is one of the main mechanisms for bacteria to tolerate high levels of these compounds, which can impose a metabolic burden. The activation of these mechanisms consumes cell energy resources and indiscriminately remove some useful metabolic substances from the cell (Wales and Davies, 2015). Nevertheless, several studies demonstrated that even if a fitness cost is associated with a resistance mechanism, compensatory genetic evolution can rapidly reduce this cost and thereby maintain this resistance mechanism in the environment (Andersson and Hughes, 2011). In our studies, the exposure time may not have been enough for the populations to be able to compensate for the cost of resisting the metal. However, *A. hydrophila* populations evolved with copper or zinc presented a similar fitness to populations evolved without the contaminant in all conditions tested, including in the presence of a contaminant (metal and antibiotic). In fact, in LB supplemented with zinc, populations evolved with this metal presented an average growth rate 1.5 X higher than populations growing without metal and average doubling time 1.5 X lower. These results are extremely worrying since they suggest that, in the environment, strains carrying resistance traits can be selected and prevail.

This study provides, for the first time using experimental evolution assays, evidence of decreased susceptibility to antibiotics in strains subjected to environmentally-relevant concentrations of metals. In this study, the contribution of MGEs was excluded, and the observed effects are probably associated with mutations in genes involved in resistance to these compounds or changes in the expression of these genes. A generalized response of the cell to the stress imposed by the presence of the metal may also justify the observed results. In terms of genotype, no severe changes were identified. However, this result is not surprising given that the technique used does not have enough resolution to detect mild changes as single mutations. Further studies should be conducted to identify possible mutations or alterations in expression, for example through whole-genome and transcriptome sequencing.

4.2 Impact of metals exposure on natural bacterial communities

A microcosm approach was used to confirm selection of antibiotic resistance in aquatic bacterial communities imposed by copper and zinc exposure. The number of cefotaxime-resistant

bacteria increased significantly in communities exposed to copper and zinc, for both concentrations used. Copper exposure also resulted in a significant increase of tetracycline-resistant bacteria and zinc exposure resulted in a significant increase of kanamycin-resistant bacteria.

In the environment, the occurrence of co-selection mechanisms could explain the results obtained. Co-resistance to metals and antibiotics, for instance, is an explanation that cannot be excluded. Several studies reported the presence of MGEs linking genes conferring resistance to antibiotics to genes encoding for copper and zinc resistance, particularly in polluted waters (Henriques et al., 2016; Seiler and Berendonk, 2012; Baker-Austin et al., 2006). Thus, the selection for genes conferring metal resistance, due to the presence of copper or zinc, can lead to the co-selection of genes conferring antibiotic resistance. The work of Fang et al., for instance, reported the association of $bla_{\text{CTX-M}}$ gene, that confers cefotaxime resistance, with the *pco* operon, that confer resistance to copper, in IncHI2 plasmids of *E. coli* isolates (Fang et al., 2016). The work of Martins et al., on the other hand, exposed the presence of co-resistance to tetracycline and copper in a conjugative plasmid harbored by *Pseudomonas aeruginosa* isolates from an aquatic environment close to industries and a hospital (Martins et al., 2014). Pal et al. showed that zinc resistance genes occasionally co-occur with resistance genes to antibiotics such as aminoglycosides, as kanamycin (Pal et al., 2015). Co-resistance has brought concern among scientists due to its contribution to the dissemination of this co-selection in the environment. When genes encoding both metal and antibiotic resistance are located together on a mobile genetic element, they can easily disseminate between bacterial strains. In fact, a correlation between the richness of ARGs and the concentration of metals such as copper and zinc has been reported (Zhou et al., 2016). Moreover, it has been demonstrated that in aquatic systems the rates of conjugation are high due to the tendency of bacteria to form biofilms in these systems (Stalder and Top, 2016). The work of Salcedo *et al.*, for instance, observed that the biofilm biomass enhance the transfer of a particular plasmid at rates 2-5 times faster (Salcedo et al., 2015). In fact, water samples used for this microcosm assay were collected from Antuã river, integrated in the Vouga River basin, located in Aveiro, Portugal, whose water body is exposed to different anthropogenic impacts such as industrial and urban discharges, runoffs from agricultural fields and discharges from animal farming (http://www.apambiente.pt/). So, the presence of bacteria harboring plasmids encoding genes for both metal and antibiotic resistance in this water is very likely. The work of Tacão et al., for instance, found high levels of $bla_{\text{CTX-M}}$ genes, encoding for cefotaxime resistance, in Antuã River, which is in agreement with the high levels of cefotaxime resistance found in this study (Tacão et al., 2012). This co-selection of metal and antibiotic resistance is extremely worrying.

Besides co-resistance, reports of cross-resistance and co-regulation mechanisms conferring resistance to these compounds can also be found. An example of cross-resistance to copper, zinc, β-lactams and tetracyclines is the reduction of membrane permeability. Metal exposure can induce changes in membrane permeability that may lead to both metal and antibiotic resistance (Baker-Austin et al., 2006; Ruiz et al., 2003). Another example, as previously mentioned, includes the simultaneous efflux of the metal and antibiotic. Efflux pumps expelling both copper and zinc and antibiotics such as tetracyclines and β-lactams have been described (Baker-Austin et al., 2006; Nies, 2003). The multidrug efflux pump MdrL found in *Listeria monocytogenes*, for instance, confers resistance to metals such zinc and antibiotics such as cefotaxime (Yu et al., 2017; Mata et al., 2000). Concerning co-regulation mechanisms, the two-component regulatory system, encoded by *cscRS* gene found in *Pseudomonas aeruginosa*, is a good example. In the presence of zinc, this two-component system influences the transcription of the *czcCBA* operon that encodes an efflux pump conferring resistance to zinc; while reducing the expression of OprD specific porin through which imipenem enters the bacterium, conferring also imipenem resistance (Yu et al., 2017; Caille et al., 2007).

Bacteria exposed to copper and zinc for 20 days and resistant to cefotaxime and kanamycin were identified. Bacteria exposed to copper and resistant to cefotaxime belonged manly to *Pseudomonas* genus. Bacteria exposed to zinc and resistant to cefotaxime belonged mainly to *Sphingomonas* spp., while the ones resistant to kanamycin belonged manly to *Ensifer* genus. Among *Pseudomonas* spp., several studies already reported the link between metal and antibiotic resistance (Devarajan et al., 2017). Teixeira et al., for instance, analyzed the resistome of a *Pseudomonas aeruginosa* strain isolated from a metal-contaminated estuary, and found an arsenal of genes related to metal and antibiotic resistance. However, the authors didn't find physical links between metal and ARGs, therefore suggesting that a predominance of cross-resistance associated with multidrug efflux pumps was the most probable explanation for both metal and antibiotic resistance in this strain (Teixeira et al., 2016). Other mechanisms could be the reason why this genus can tolerate both metals and β-lactam antibiotics in the environment such as the presence of both β-lactamases and genes conferring metal resistance or, as previously mentioned, reduced drug and metal uptake owing to loss of outer membrane porin proteins (Poole, 2011). In fact, Class D OXA enzymes, that confer resistance to cefotaxime, occur predominantly in *P. aeruginosa* (Poole, 2011). The most predominant genus found among isolates exposed to zinc and resistant to cefotaxime was *Sphingomonas* spp. These bacteria have a remarkable capacity to cope with stress conditions and to adapt to new habitats being associated, for instance, to metal corrosion of plumbing systems and biofouling of drinking water and industrial water distribution systems (Yim et al., 2010). Some *Sphingomonas* species, however, have been associated with contaminated hospital environment such as tap and distilled water, and among these isolates

resistance to several antibiotics, including cefotaxime has been reported (Hardjo Lugito et al., 2016). Bacteria exposed to zinc and resistant to kanamycin belonged manly to *Ensifer* genus. In fact, a recent study reported the potencial of *Ensifer adhaerens* for heavy metal bioaccumulation, biosorption, and phosphate solubilization under metal stress condition, which could be an explanation for the high prevalence of these strains in microcosms exposed to zinc (Oves et al., 2017). In fact, the association between *E. adhaerens* and resistance to numerous antibiotics, including kanamycin, has been described (diCenzo et al., 2018). Since most antibiotic resistance mechanisms reported for the identified genera are intrinsic, the most probable explanation for these association between antibiotic and metal resistance is cross-resistance, although other resistance mechanisms can't be excluded. The quantification of acquired ARGs across microcosms would be a valuable result to assess the co-resistance hypothesis.

The selection of metal-resistant bacteria and ARB may result in strong alterations in terms of bacterial community structure. Alterations were in fact confirmed by DGGE. Observed changes were different when the communities were exposed for 10 or 20 days and were dependent on the metal. These results are in agreement with other studies related to metal exposure. The work of Ahmed et al., for instance, concluded that exposure to copper leads to a rapid marked effect on the structure and on most of the functions of the exposed communities in soils (Mahamoud Ahmed et al., 2018). Additionally, the work of Mertens et al. concluded that the bacterial community structure in uncontaminated and long-term contaminated soil samples was different and could be related to soil zinc concentrations (Mertens et al., 2006). Our results also showed that copper exposure seems to cause more changes in the bacterial communities than zinc exposure. One explanation could be the fact that, although both copper and zinc are essential metals for bacteria, copper toxicity is higher than that of zinc. For instance, copper has more affinity for cellular components and biomolecules competing with important functional groups (Hobman and Crossman, 2015). Diversity indexes calculated based on DGGE banding profiles revealed significative differences between control communities and communities exposed to copper and zinc. Diversity of bacterial communities exposed to a metal significantly decreased when compared with that of control populations. One explanation could be the fact that metal exposure selects for specific bacterial groups while eliminating others. In fact, the Pielou's evenness index (J) suffered a significative decrease in populations exposed to copper for 10 days, showing that copper exposure could increase the predominance of certain bacterial groups.

5. FINAL CONSIDERATIONS

The study here presented aimed to explore the role of metals in the selection and evolution of antibiotic resistance in aquatic systems investigating genotype and phenotype alterations, induced by the presence of copper and zinc, on selected bacterial strains; and to confirm if metals select for antibiotic resistance in complex bacterial communities.

Populations of *Escherichia coli* ATCC® 25922™ and *Aeromonas hydrophila* CECT 839^{T} evolved in the presence of copper and zinc became more tolerant to other metals and less susceptible to some antibiotics such as sulfametoxazole and kanamycin. These changes depended on the metal and on the exposed bacterial strain. For *A. hydrophila* there was no detectable fitness cost associated to the evolution in the presence of metals. No severe genotypic alterations were detected in any of the evolved populations.

Metal exposure selected for higher prevalence of antibiotic-resistant bacteria in bacterial communities of an aquatic system. Cefotaxime- and tetracycline-resistant bacteria increased significantly in communities exposed to copper, while communities exposed to zinc increased the prevalence of bacteria resistant to cefotaxime and kanamycin, showing that the selection effect is metal- and antibiotic-dependent. Moreover, metal exposure selected genera intrinsically resistant to antibiotics, suggesting the occurrence of cross-resistance mechanisms. In fact, metal exposure decreased the richness and diversity of exposed communities, showing that metal exposure can select for specific bacterial groups.

The increasing contamination of aquatic systems with metals due to anthropogenic activities, is extremely worrying, favoring the prevalence of antibiotic-resistant bacteria and probably the transfer of antibiotic resistance genes. The potencial transfer of these genes from environmental bacteria to human pathogens can then bring serious consequences for public health by lessening the efficacy of antibiotic treatment.

6. REFERENCES

Aendekerk, S., Ghysels, B., Cornelis, P., Baysse, C., 2002. Characterization of a new efflux pump, MexGHI-OpmD, from *Pseudomonas aeruginosa* that confers resistance to vanadium. Microbiology 148, 2371–2381. https://doi.org/10.1099/00221287-148-8-2371

Agnello, M., Finkel, S.E., Wong-Beringer, A., 2016. Fitness cost of fluoroquinolone resistance in clinical isolates of *Pseudomonas aeruginosa* differs by type III secretion genotype. Front. Microbiol. 7, 1591. https://doi.org/10.3389/fmicb.2016.01591

Alba, C., Blanco, A., Alarcón, T., 2017. Antibiotic resistance in *Helicobacter pylori*. Curr. Opin. Infect. Dis. 30, 489–497. https://doi.org/10.1097/QCO.0000000000000396

Allocati, N., Masulli, M., Alexeyev, M.F., Di Ilio, C., 2013. *Escherichia coli* in Europe: an overview. Int. J. Environ. Res. Public Health 10, 6235–54. https://doi.org/10.3390/ijerph10126235

Alves, M., Pereira, A., Matos, P., Henriques, J., Vicente, C., Aikawa, T., Hasegawa, K., Nascimento, F., Mota, M., Correia, A., Henriques, I., 2016. Bacterial community associated to the pine wilt disease insect vectors *Monochamus galloprovincialis* and *Monochamus alternatus*. Sci. Rep. 6, 23908. https://doi.org/10.1038/srep23908

Alves, M.S., Pereira, A., Araújo, S.M., Castro, B.B., Correia, A.C.M., Henriques, I., 2014. Seawater is a reservoir of multi-resistant *Escherichia coli*, including strains hosting plasmid-mediated quinolones resistance and extended-spectrum beta-lactamases genes. Front. Microbiol. 5, 426. https://doi.org/10.3389/fmicb.2014.00426

Amachawadi, R.G., Shelton, N.W., Shi, X., Vinasco, J., Dritz, S.S., Tokach, M.D., Nelssen, J.L., Scott, H.M., Nagaraja, T.G., 2011. Selection of fecal enterococci exhibiting *tcrB*-mediated copper resistance in pigs fed diets supplemented with copper. Appl. Environ. Microbiol. 77, 5597–603. https://doi.org/10.1128/AEM.00364-11

Amador, P.P., Fernandes, R.M., Prudêncio, M.C., Barreto, M.P., Duarte, I.M., 2015. Antibiotic resistance in wastewater: Occurrence and fate of *Enterobacteriaceae* producers of Class A and Class C β-lactamases. J. Environ. Sci. Heal. Part A 50, 26–39. https://doi.org/10.1080/10934529.2015.964602

Aminov, R.I., 2011. Horizontal gene exchange in environmental microbiota. Front. Microbiol. 2, 158. https://doi.org/10.3389/fmicb.2011.00158

Andersson, D.I., Hughes, D., 2011. Persistence of antibiotic resistance in bacterial populations. FEMS Microbiol. Rev. 35, 901–911. https://doi.org/10.1111/j.1574-6976.2011.00289.x

Andersson, D.I., Hughes, D., 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? Nat. Rev. Microbiol. 8, 260–271. https://doi.org/10.1038/nrmicro2319

Anes, J., McCusker, M.P., Fanning, S., Martins, M., 2015. The ins and outs of RND efflux pumps in *Escherichia coli*. Front. Microbiol. 6, 587. https://doi.org/10.3389/fmicb.2015.00587

Araújo, S., A.T. Silva, I., Tacão, M., Patinha, C., Alves, A., Henriques, I., 2017. Characterization of antibiotic resistant and pathogenic *Escherichia coli* in irrigation water and vegetables in household farms. Int. J. Food Microbiol. 257, 192–200. https://doi.org/10.1016/J.IJFOODMICRO.2017.06.020

Arthur, M., Quintiliani, R., 2001. Regulation of VanA- and VanB-type glycopeptide resistance in enterococci. Antimicrob. Agents Chemother. 45, 375–81. https://doi.org/10.1128/AAC.45.2.375-381.2001

Aumeran, C., Chanal, C., Labia, R., Sirot, D., Sirot, J., Bonnet, R., 2003. Effects of Ser130Gly and Asp240Lys substitutions in extended-spectrum beta-lactamase CTX-M-9. Antimicrob. Agents Chemother. 47, 2958–61. https://doi.org/10.1128/AAC.47.9.2958-2961.2003

Badger, S., Abraham, S., Saputra, S., Trott, D.J., Turnidge, J., Mitchell, T., Caraguel, C.G.B., Jordan, D., 2018. Relative performance of antimicrobial susceptibility

assays on clinical *Escherichia coli* isolates from animals. Vet. Microbiol. 214, 56–64. https://doi.org/10.1016/J.VETMIC.2017.12.008

Baker-Austin, C., Wright, M.S., Stepanauskas, R., McArthur, J. V, 2006. Co-selection of antibiotic and metal resistance. Trends Microbiol. 14, 176–82. https://doi.org/10.1016/j.tim.2006.02.006

Balcázar, J.L., Subirats, J., Borrego, C.M., 2015. The role of biofilms as environmental reservoirs of antibiotic resistance. Front. Microbiol. 6, 1216. https://doi.org/10.3389/fmicb.2015.01216

Barrick, J.E., Lenski, R.E., 2009. Genome-wide mutational diversity in an evolving population of *Escherichia coli*. Cold Spring Harb. Symp. Quant. Biol. 74, 119–29. https://doi.org/10.1101/sqb.2009.74.018

Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J., 2018. Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiol. Rev. 42. https://doi.org/10.1093/femsre/fux053

Besier, S., Ludwig, A., Brade, V., Wichelhaus, T.A., 2005. Compensatory adaptation to the loss of biological fitness associated with acquisition of fusidic acid resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. 49, 1426–31. https://doi.org/10.1128/AAC.49.4.1426-1431.2005

Blanco, P., Hernando-Amado, S., Reales-Calderon, J.A., Corona, F., Lira, F., Alcalde-Rico, M., Bernardini, A., Sanchez, M.B., Martinez, J.L., 2016. Bacterial multidrug efflux pumps: much more than antibiotic resistance determinants. Microorganisms 4. https://doi.org/10.3390/microorganisms4010014

Buwembo, W., Aery, S., Rwenyonyi, C.M., Swedberg, G., Kironde, F., 2013. Point mutations in the *folP* gene partly explain sulfonamide resistance of *Streptococcus* mutans. Int. J. Microbiol. 2013, 367021. https://doi.org/10.1155/2013/367021

Caille, O., Rossier, C., Perron, K., 2007. A copper-activated two-component system interacts with zinc and imipenem resistance in *Pseudomonas aeruginosa*. J. Bacteriol. 189, 4561–8. https://doi.org/10.1128/JB.00095-07

Cantón, R., Coque, T.M., 2006. The CTX-M β-lactamase pandemic. Curr. Opin. Microbiol. 9, 466–475. https://doi.org/10.1016/j.mib.2006.08.011

Cantón, R., González-Alba, J.M., Galán, J.C., 2012. CTX-M enzymes: origin and diffusion. Front. Microbiol. 3, 110. https://doi.org/10.3389/fmicb.2012.00110

Cavaco, L.M., Hasman, H., Aarestrup, F.M., Members of MRSA-CG:, Wagenaar, J.A., Graveland, H., Veldman, K., Mevius, D., Fetsch, A., Tenhagen, B.-A., Concepcion Porrero, M., Dominguez, L., Granier, S.A., Jouy, E., Butaye, P., Kaszanyitzky, E., Dán, A., Zmudzki, J., Battisti, A., Franco, A., Schwarz, S., Gutierrez, M., Weese, J.S., Cui, S., Pomba, C., 2011. Zinc resistance of *Staphylococcus aureus* of animal origin is strongly associated with methicillin resistance. Vet. Microbiol. 150, 344–348. https://doi.org/10.1016/j.vetmic.2011.02.014

Cavaco, L.M., Hasman, H., Stegger, M., Andersen, P.S., Skov, R., Fluit, A.C., Ito, T., Aarestrup, F.M., 2010. Cloning and occurrence of czrC, a gene conferring cadmium and zinc resistance in methicillin-resistant *Staphylococcus aureus* CC398 isolates. Antimicrob. Agents Chemother. 54, 3605–8. https://doi.org/10.1128/AAC.00058-10

Charpentier, X., Kay, E., Schneider, D., Shuman, H.A., 2011. Antibiotics and UV radiation induce competence for natural transformation in *Legionella pneumophila*. J. Bacteriol. 193, 1114–21. https://doi.org/10.1128/JB.01146-10

Chen, B., Liang, X., Huang, X., Zhang, T., Li, X., 2013. Differentiating anthropogenic impacts on ARGs in the Pearl River Estuary by using suitable gene indicators. Water Res. 47, 2811–2820. https://doi.org/10.1016/J.WATRES.2013.02.042

Chen, S., Li, X., Sun, G., Zhang, Y., Su, J., Ye, J., 2015. Heavy metal induced antibiotic resistance in bacterium LSJC7. Int. J. Mol. Sci. 16, 23390–404. https://doi.org/10.3390/ijms161023390

Choi, S.-H., Lee, K.-L., Shin, J.-H., Cho, Y.-B., Cha, S.-S., Roe, J.-H., 2017. Zinc-dependent regulation of zinc import and export genes by Zur. Nat. Commun. 8, 15812. https://doi.org/10.1038/ncomms15812

Christou, A., Agüera, A., Bayona, J.M., Cytryn, E., Fotopoulos, V., Lambropoulou, D., Manaia, C.M., Michael, C., Revitt, M., Schröder, P., Fatta-Kassinos, D., 2017. The potential implications of reclaimed wastewater reuse for irrigation on the agricultural environment: The knowns and unknowns of the fate of antibiotics and antibiotic resistant bacteria and resistance genes – A review. Water Res. 123, 448–467. https://doi.org/10.1016/J.WATRES.2017.07.004

Colavecchio, A., Cadieux, B., Lo, A., Goodridge, L.D., 2017. Bacteriophages contribute to the spread of antibiotic resistance genes among foodborne pathogens of the *Enterobacteriaceae* family - A Review. Front. Microbiol. 8, 1108. https://doi.org/10.3389/fmicb.2017.01108

Conejo, M.C., García, I., Martínez-Martínez, L., Picabea, L., Pascual, A., 2003. Zinc eluted from siliconized latex urinary catheters decreases OprD expression, causing carbapenem resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 47, 2313–5. https://doi.org/10.1128/AAC.47.7.2313-2315.2003

Conroy, O., Kim, E.-H., McEvoy, M.M., Rensing, C., 2010. Differing ability to transport nonmetal substrates by two RND-type metal exporters. FEMS Microbiol. Lett. 308, 115–22. https://doi.org/10.1111/j.1574-6968.2010.02006.x

Correia, S., Poeta, P., Hébraud, M., Capelo, J.L., Igrejas, G., 2017. Mechanisms of quinolone action and resistance: where do we stand? J. Med. Microbiol. 66, 551–559. https://doi.org/10.1099/jmm.0.000475

Dahlberg, C., Chao, L., 2003. Amelioration of the cost of conjugative plasmid carriage in *Eschericha coli* K12. Genetics 165, 1641–9.

Depardieu, F., Podglajen, I., Leclercq, R., Collatz, E., Courvalin, P., 2007. Modes and modulations of antibiotic resistance gene expression. Clin. Microbiol. Rev. 20, 79–114. https://doi.org/10.1128/CMR.00015-06

Devarajan, N., Ohler, T.K.€, Sivalingam, P., Van Delden, C., Mulaji, C.K., Mpiana, P.T., Ibelings, B.W., Pot, J., 2017. Antibiotic resistant *Pseudomonas* spp. in the aquatic environment: A prevalence study under tropical and temperate climate conditions. https://doi.org/10.1016/j.watres.2017.02.058

diCenzo, G.C., Debiec, K., Krzysztoforski, J., Uhrynowski, W., Mengoni, A., Fagorzi, C., Gorecki, A., Dziewit, L., Bajda, T., Rzepa, G., Drewniak, L., 2018. Genomic and biotechnological characterization of the heavy-metal resistant, arsenic-oxidizing bacterium *Ensifer* sp. M14. Genes (Basel). 9, 379. https://doi.org/10.3390/genes9080379

Directorate-General for Health and Food Safety (European Commission), 2017. Non-EU countries' national policies and measures on antimicrobial resistance. https://doi.org/10.2772/60954

Durão, P., Trindade, S., Sousa, A., Gordo, I., 2015. Multiple resistance at no cost: rifampicin and streptomycin a dangerous liaison in the spread of antibiotic resistance. Mol. Biol. Evol. 32, 2675–2680. https://doi.org/10.1093/molbev/msv143

EFSA, 2008. Assessment of the possible effect of the four antimicrobial treatment substances on the emergence of antimicrobial resistance - Scientific Opinion of the Panel on Biological Hazards. EFSA J. 6, 659. https://doi.org/10.2903/j.efsa.2008.659

Fang, L., Li, X., Li, L., Li, S., Liao, X., Sun, J., Liu, Y., 2016. Co-spread of metal and antibiotic resistance within ST3-IncHI2 plasmids from *E. coli* isolates of food-producing animals. Sci. Rep. 6, 25312. https://doi.org/10.1038/srep25312

Fernandes, M.R., Sellera, F.P., Esposito, F., Sabino, C.P., Cerdeira, L., Lincopan, N., 2017. Colistin-resistant *mcr-1*-positive *Escherichia coli* on public beaches, an infectious threat emerging in recreational waters. Antimicrob. Agents Chemother. 61. https://doi.org/10.1128/AAC.00234-17

Flach, C.-F., Pal, C., Svensson, C.J., Kristiansson, E., Östman, M., Bengtsson-Palme, J., Tysklind, M., Larsson, D.G.J., 2017. Does antifouling paint select for antibiotic resistance? Sci. Total Environ. 590–591, 461–468. https://doi.org/10.1016/j.scitotenv.2017.01.213

Franke, S., Grass, G., Rensing, C., Nies, D.H., 2003. Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. J. Bacteriol. 185, 3804–

12. https://doi.org/10.1128/JB.185.13.3804-3812.2003

Furuya, E.Y., Lowy, F.D., 2006. Antimicrobial-resistant bacteria in the community setting. Nat. Rev. Microbiol. 4, 36–45. https://doi.org/10.1038/nrmicro1325

Fux, C.A., Costerton, J.W., Stewart, P.S., Stoodley, P., 2005. Survival strategies of infectious biofilms. Trends Microbiol. 13, 34–40. https://doi.org/10.1016/j.tim.2004.11.010

Gao, P., He, S., Huang, S., Li, K., Liu, Z., Xue, G., Sun, W., 2015. Impacts of coexisting antibiotics, antibacterial residues, and heavy metals on the occurrence of erythromycin resistance genes in urban wastewater. Appl. Microbiol. Biotechnol. 99, 3971–3980. https://doi.org/10.1007/s00253-015-6404-9

Garneau-Tsodikova, S., Labby, K.J., 2016. Mechanisms of resistance to aminoglycoside antibiotics: overview and perspectives. Medchemcomm 7, 11–27. https://doi.org/10.1039/C5MD00344J

Gebreyes, W.A., Wittum, T., Habing, G., Alali, W., Usui, M., Suzuki, S., 2017. Spread of antibiotic resistance in food animal production systems. Foodborne Dis. 105–130. https://doi.org/10.1016/B978-0-12-385007-2.00004-8

Gerrits, M.M., Berning, M., Van Vliet, A.H.M., Kuipers, E.J., Kusters, J.G., 2003. Effects of 16S rRNA gene mutations on tetracycline resistance in *Helicobacter pylori*. Antimicrob. Agents Chemother. 47, 2984–6. https://doi.org/10.1128/AAC.47.9.2984-2986.2003

Gholami, S., Tabatabaei, M., Sohrabi, N., 2017. Comparison of biofilm formation and antibiotic resistance pattern of *Pseudomonas aeruginosa* in human and environmental isolates. Microb. Pathog. 109, 94–98. https://doi.org/10.1016/j.micpath.2017.05.004

Ghosh, A., Singh, A., Ramteke, P.W., Singh, V.P., 2000. Characterization of large plasmids encoding resistance to toxic heavy metals in *Salmonella abortus* equi. Biochem. Biophys. Res. Commun. 272, 6–11. https://doi.org/10.1006/BBRC.2000.2727

Gilbert, P., Maira-Litran, T., McBain, A.J., Rickard, A.H., Whyte, F.W., 2002. The physiology and collective recalcitrance of microbial biofilm communities, in: Advances in Microbial Physiology. Academic Press, Inc., pp. 203–256. https://doi.org/10.1016/S0065-2911(02)46005-5

Gilmour, M.W., Thomson, N.R., Sanders, M., Parkhill, J., Taylor, D.E., 2004. The complete nucleotide sequence of the resistance plasmid R478: defining the backbone components of incompatibility group H conjugative plasmids through comparative genomics. Plasmid 52, 182–202. https://doi.org/10.1016/J.PLASMID.2004.06.006

Gniadkowski, M., 2008. Evolution of extended-spectrum β-lactamases by mutation. Clin. Microbiol. Infect. 14, 11–32. https://doi.org/10.1111/J.1469-0691.2007.01854.X

Gong, Y., Yuan, Y., 2018. Resistance mechanisms of *Helicobacter pylori* and its dual target precise therapy. Crit. Rev. Microbiol. 44, 371–392. https://doi.org/10.1080/1040841X.2017.1418285

Guneysel, O., Onur, O., Erdede, M., Denizbasi, A., 2009. Trimethoprim/Sulfamethoxazole resistance in urinary tract infections. J. Emerg. Med. 36, 338–341. https://doi.org/10.1016/j.jemermed.2007.08.068

Hall, B.G., Acar, H., Nandipati, A., Barlow, M., 2014. Growth rates made easy. Mol. Biol. Evol. 31, 232–238. https://doi.org/10.1093/molbev/mst187

Haller, L., Poté, J., Loizeau, J.-L., Wildi, W., 2009. Distribution and survival of faecal indicator bacteria in the sediments of the Bay of Vidy, Lake Geneva, Switzerland. Ecol. Indic. 9, 540–547. https://doi.org/10.1016/J.ECOLIND.2008.08.001

Hao, Z., Lou, H., Zhu, R., Zhu, J., Zhang, D., Zhao, B.S., Zeng, S., Chen, X., Chan, J., He, C., Chen, P.R., 2014. The multiple antibiotic resistance regulator MarR is a copper sensor in *Escherichia coli*. Nat. Chem. Biol. 10, 21–28. https://doi.org/10.1038/nchembio.1380

Hardjo Lugito, N.P., Cucunawangsih, Kurniawan, A., 2016. A Lethal case of

Sphingomonas paucimobilis bacteremia in an immunocompromised patient. Case Rep. Infect. Dis. 2016, 1–4. https://doi.org/10.1155/2016/3294639

Hasman, H., Aarestrup, F.M., 2005. Relationship between copper, glycopeptide, and macrolide resistance among *Enterococcus faecium* strains isolated from pigs in Denmark between 1997 and 2003. Antimicrob. Agents Chemother. 49, 454–6. https://doi.org/10.1128/AAC.49.1.454-456.2005

Hasman, H., Aarestrup, F.M., 2002. *tcrB*, a gene conferring transferable copper resistance in *Enterococcus faecium*: occurrence, transferability, and linkage to macrolide and glycopeptide resistance. Antimicrob. Agents Chemother. 46, 1410–6.

Hausner, M., Wuertz, S., 1999. High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. Appl. Environ. Microbiol. 65, 3710–3.

Hayashi, S., Abe, M., Kimoto, M., Furukawa, S., Nakazawa, T., 2000. The dsbA-dsbB disulfide bond formation system of *Burkholderia cepacia* is involved in the production of protease and alkaline phosphatase, motility, metal resistance, and multi-drug resistance. Microbiol. Immunol. 44, 41–50.

Hellweger, F.L., 2013. *Escherichia coli* adapts to tetracycline resistance plasmid (pBR322) by mutating endogenous potassium transport: *in silico* hypothesis testing. FEMS Microbiol. Ecol. 83, 622–631. https://doi.org/10.1111/1574-6941.12019

Henriques, I., Tacão, M., Leite, L., Fidalgo, C., Araújo, S., Oliveira, C., Alves, A., 2016. Co-selection of antibiotic and metal(loid) resistance in gram-negative epiphytic bacteria from contaminated salt marshes. Mar. Pollut. Bull. 109, 427–434. https://doi.org/10.1016/J.MARPOLBUL.2016.05.031

Henriques, I.S., Almeida, A., Cunha, Ã., Correia, A., 2004. Molecular sequence analysis of prokaryotic diversity in the middle and outer sections of the Portuguese estuary Ria de Aveiro. FEMS Microbiol. Ecol. 49, 269–279. https://doi.org/10.1016/j.femsec.2004.04.003

Henriques, I.S., Araújo, S., Azevedo, J.S.N., Alves, M.S., Chouchani, C., Pereira, A., Correia, A., 2012. Prevalence and diversity of carbapenem-resistant bacteria in untreated drinking water in Portugal. Microb. Drug Resist. 18, 531–537. https://doi.org/10.1089/mdr.2012.0029

Hernández, A., Mellado, R.P., Martínez, J.L., 1998. Metal accumulation and vanadium-induced multidrug resistance by environmental isolates of *Escherichia hermannii* and *Enterobacter cloacae*. Appl. Environ. Microbiol. 64, 4317–20.

Hernando-Amado, S., Sanz-García, F., Blanco, P., Martínez, J.L., 2017. Fitness costs associated with the acquisition of antibiotic resistance. Essays Biochem. 61, 37–48. https://doi.org/10.1042/EBC20160057

Hobman, J.L., Crossman, L.C., 2015. Bacterial antimicrobial metal ion resistance. J. Med. Microbiol. 64, 471–497. https://doi.org/10.1099/jmm.0.023036-0

Hu, H.-W., Wang, J.-T., Li, J., Li, J.-J., Ma, Y.-B., Chen, D., He, J.-Z., 2016. Field-based evidence for copper contamination induced changes of antibiotic resistance in agricultural soils. Environ. Microbiol. 18, 3896–3909. https://doi.org/10.1111/1462-2920.13370

Intriago, J.C., López-Gálvez, F., Allende, A., Vivaldi, G.A., Camposeo, S., Nicolás Nicolás, E., Alarcón, J.J., Pedrero Salcedo, F., 2018. Agricultural reuse of municipal wastewater through an integral water reclamation management. J. Environ. Manage. 213, 135–141. https://doi.org/10.1016/J.JENVMAN.2018.02.011

Janda, J.M., Abbott, S.L., 2010. The genus *Aeromonas*: taxonomy, pathogenicity, and infection. Clin. Microbiol. Rev. 23, 35–73. https://doi.org/10.1128/CMR.00039-09

Jean, S.-S., Liao, C.-H., Sheng, W.-H., Lee, W.-S., Hsueh, P.-R., 2017. Comparison of commonly used antimicrobial susceptibility testing methods for evaluating susceptibilities of clinical isolates of *Enterobacteriaceae* and nonfermentative Gram-negative bacilli to cefoperazone–sulbactam. J. Microbiol. Immunol. Infect. 50, 454–463. https://doi.org/10.1016/J.JMII.2015.08.024

Jenks, P.J., Edwards, D.I., 2002. Metronidazole resistance in *Helicobacter pylori*. Int. J. Antimicrob. Agents 19, 1–7. https://doi.org/10.1016/S0924-8579(01)00468-X

Johnston, C., Martin, B., Fichant, G., Polard, P., Claverys, J.-P., 2014. Bacterial transformation: distribution, shared mechanisms and divergent control. Nat. Rev. Microbiol. 12, 181–196. https://doi.org/10.1038/nrmicro3199

Juan, P.-A., Attaiech, L., Charpentier, X., 2015. Natural transformation occurs independently of the essential actin-like MreB cytoskeleton in *Legionella pneumophila*. Sci. Rep. 5, 16033. https://doi.org/10.1038/srep16033

Kabir, E., Ray, S., Kim, K.-H., Yoon, H.-O., Jeon, E.-C., Kim, Y.S., Cho, Y.-S., Yun, S.-T., Brown, R.J.C., 2012. Current status of trace metal pollution in soils affected by industrial activities. Sci. World J. 2012, 916705. https://doi.org/10.1100/2012/916705

Kadlec, K., von Czapiewski, E., Kaspar, H., Wallmann, J., Michael, G.B., Steinacker, U., Schwarz, S., 2011. Molecular basis of sulfonamide and trimethoprim resistance in fish-pathogenic *Aeromonas* isolates. Appl. Environ. Microbiol. 77, 7147–50. https://doi.org/10.1128/AEM.00560-11

Karkman, A., Do, T.T., Walsh, F., Virta, M.P.J., 2018. Antibiotic-resistance genes in waste water. Trends Microbiol. 26, 220–228. https://doi.org/10.1016/j.tim.2017.09.005

Kenzaka, T., Tani, K., Nasu, M., 2010. High-frequency phage-mediated gene transfer in freshwater environments determined at single-cell level. ISME J. 4, 648–659. https://doi.org/10.1038/ismej.2009.145

Knapp, C.W., Callan, A.C., Aitken, B., Shearn, R., Koenders, A., Hinwood, A., 2017. Relationship between antibiotic resistance genes and metals in residential soil samples from Western Australia. Environ. Sci. Pollut. Res. Int. 24, 2484–2494. https://doi.org/10.1007/s11356-016-7997-y

Knopp, M., Andersson, D.I., 2018. Predictable phenotypes of antibiotic resistance mutations. MBio 9, e00770-18. https://doi.org/10.1128/mBio.00770-18

Kristiansson, E., Fick, J., Janzon, A., Grabic, R., Rutgersson, C., Weijdegård, B., Söderström, H., Larsson, D.G.J., 2011. Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. PLoS One 6, e17038. https://doi.org/10.1371/journal.pone.0017038

Kumar, P., Bag, S., Ghosh, T.S., Dey, P., Dayal, M., Saha, B., Verma, J., Pant, A., Saxena, S., Desigamani, A., Rana, P., Kumar, D., Sharma, N.C., Hanpude, P., Maiti, T.K., Mukhopadhyay, A.K., Bhadra, R.K., Nair, G.B., Ramamurthy, T., Das, B., 2017. Molecular insights into antimicrobial resistance traits of multidrug resistant enteric pathogens isolated from India. Sci. Rep. 7, 14468. https://doi.org/10.1038/s41598-017-14791-1

Kwon, Y.H., Kim, J.Y., Kim, N., Park, J.H., Nam, R.H., Lee, S.M., Kim, J.-W., Kim, J.M., Park, J.Y., Lee, D.H., 2017. Specific mutations of penicillin-binding protein 1A in 77 clinically acquired amoxicillin-resistant *Helicobacter pylori* strains in comparison with 77 amoxicillin-susceptible strains. Helicobacter 22, e12437. https://doi.org/10.1111/hel.12437

LaPara, T.M., Burch, T.R., McNamara, P.J., Tan, D.T., Yan, M., Eichmiller, J.J., 2011. Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior Harbor. Environ. Sci. Technol. 45, 9543–9549. https://doi.org/10.1021/es202775r

Lee, L.J., Barrett, J.A., Poole, R.K., 2005. Genome-wide transcriptional response of chemostat-cultured *Escherichia coli* to zinc. J. Bacteriol. 187, 1124–34. https://doi.org/10.1128/JB.187.3.1124-1134.2005

Lehtopolku, M., Kotilainen, P., Puukka, P., Nakari, U.-M., Siitonen, A., Eerola, E., Huovinen, P., Hakanen, A.J., 2012. Inaccuracy of the disk diffusion method compared with the agar dilution method for susceptibility testing of Campylobacter spp. J. Clin. Microbiol. 50, 52–6. https://doi.org/10.1128/JCM.01090-11

León-Cañedo, J.A., Alarcón-Silvas, S.G., Fierro-Sañudo, J.F., Mariscal-Lagarda, M.M., Díaz-Valdés, T., Páez-Osuna, F., 2017. Assessment of environmental loads of Cu and Zn from intensive inland shrimp aquaculture. Environ. Monit. Assess. 189, 69.

https://doi.org/10.1007/s10661-017-5783-z

Leonard, A.F.C., Zhang, L., Balfour, A.J., Garside, R., Hawkey, P.M., Murray, A.K., Ukoumunne, O.C., Gaze, W.H., 2018. Exposure to and colonisation by antibiotic-resistant *E. coli* in UK coastal water users: Environmental surveillance, exposure assessment, and epidemiological study (Beach Bum Survey). Environ. Int. 114, 326–333. https://doi.org/10.1016/J.ENVINT.2017.11.003

Liebert, C.A., Hall, R.M., Summers, A.O., 1999. Transposon Tn21, flagship of the floating genome. Microbiol. Mol. Biol. Rev. 63, 507–22.

Lin, H., Sun, W., Zhang, Z., Chapman, S.J., Freitag, T.E., Fu, J., Zhang, X., Ma, J., 2016. Effects of manure and mineral fertilization strategies on soil antibiotic resistance gene levels and microbial community in a paddy–upland rotation system. Environ. Pollut. 211, 332–337. https://doi.org/10.1016/J.ENVPOL.2016.01.007

Liu, X., Jiang, J., Yan, Y., Dai, Y., Deng, B., Ding, S., Su, S., Sun, W., Li, Z., Gan, Z., 2018. Distribution and risk assessment of metals in water, sediments, and wild fish from Jinjiang River in Chengdu, China. Chemosphere 196, 45–52. https://doi.org/10.1016/J.CHEMOSPHERE.2017.12.135

Lood, R., Ertürk, G., Mattiasson, B., 2017. Revisiting antibiotic resistance spreading in wastewater treatment plants - bacteriophages as a much neglected potential transmission vehicle. Front. Microbiol. 8, 2298. https://doi.org/10.3389/fmicb.2017.02298

Luo, H., Wan, K., Wang, H.H., 2005. High-frequency conjugation system facilitates biofilm formation and pAMbeta1 transmission by *Lactococcus lactis*. Appl. Environ. Microbiol. 71, 2970–8. https://doi.org/10.1128/AEM.71.6.2970-2978.2005

Mahamoud Ahmed, A., Lyautey, E., Bonnineau, C., Dabrin, A., Pesce, S., 2018. Environmental concentrations of copper, alone or in mixture with arsenic, can impact river sediment microbial community structure and functions. Front. Microbiol. 9, 1852. https://doi.org/10.3389/fmicb.2018.01852

Maharjan, R., Ferenci, T., 2017. The fitness costs and benefits of antibiotic resistance in drug-free microenvironments encountered in the human body. Environ. Microbiol. Rep. 9, 635–641. https://doi.org/10.1111/1758-2229.12564

Mahmud, J., Chowdhury, M.K., Sami, A.B., Akhand, A.A., Ahsan, N., 2016. Co-resistance to chromium and antibiotics in bacteria isolated from tannery wastes. Dhaka Univ. J. Pharm. Sci. 14, 193. https://doi.org/10.3329/dujps.v14i2.28510

Malan, M., Müller, F., Raitt, L., Aalbers, J., Cyster, L., Brendonck, L., 2015. Farmyard manures: the major agronomic sources of heavy metals in the Philippi Horticultural Area in the Western Cape Province of South Africa. Environ. Monit. Assess. 187, 708. https://doi.org/10.1007/s10661-015-4918-3

Manaia, C.M., 2017. Assessing therisk of antibiotic resistance transmission from the environment to humans: non-direct proportionality between abundance and risk. Trends Microbiol. 25, 173–181. https://doi.org/10.1016/j.tim.2016.11.014

Manaia, C.M., Macedo, G., Fatta-Kassinos, D., Nunes, O.C., 2016. Antibiotic resistance in urban aquatic environments: can it be controlled? Appl. Microbiol. Biotechnol. 100, 1543–1557. https://doi.org/10.1007/s00253-015-7202-0

Manyi-Loh, C., Mamphweli, S., Meyer, E., Okoh, A., Manyi-Loh, C., Mamphweli, S., Meyer, E., Okoh, A., 2018. Antibiotic use in agriculture and its consequential resistance in environmental sources: potential public health implications. Molecules 23, 795. https://doi.org/10.3390/molecules23040795

Mao, D., Luo, Y., Mathieu, J., Wang, Q., Feng, L., Mu, Q., Feng, C., Alvarez, P.J.J., 2014. Persistence of extracellular DNA in river sediment facilitates antibiotic resistance gene propagation. Environ. Sci. Technol. 48, 71–78. https://doi.org/10.1021/es404280v

Marreiro, D. do N., Cruz, K.J.C., Morais, J.B.S., Beserra, J.B., Severo, J.S., de Oliveira, A.R.S., 2017. Zinc and oxidative stress: current mechanisms. Antioxidants (Basel, Switzerland) 6. https://doi.org/10.3390/antiox6020024

Martins, V.V., Zanetti, M.O.B., Pitondo-Silva, A., Stehling, E.G., 2014. Aquatic

environments polluted with antibiotics and heavy metals: a human health hazard. Environ. Sci. Pollut. Res. 21, 5873–5878. https://doi.org/10.1007/s11356-014-2509-4

Mata, M.T., Baquero, F., Pérez-Díaz, J. C., 2000. A multidrug efflux transporter in *Listeria monocytogenes*, FEMS Microbiology Letters, Volume 187, Issue 2, Pages 185–188, https://doi.org/10.1111/j.1574-6968.2000.tb09158.x

Mayers, D., 2009. Antimicrobial drug resistance. Volume 1, Mechanisms of drug resistance. Humana.

McIntosh, D., Cunningham, M., Ji, B., Fekete, F.A., Parry, E.M., Clark, S.E., Zalinger, Z.B., Gilg, I.C., Danner, G.R., Johnson, K.A., Beattie, M., Ritchie, R., 2008. Transferable, multiple antibiotic and mercury resistance in Atlantic Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida* is associated with carriage of an IncA/C plasmid similar to the *Salmonella enterica* plasmid pSN254. J. Antimicrob. Chemother. 61, 1221–8. https://doi.org/10.1093/jac/dkn123

Medardus, J.J., Molla, B.Z., Nicol, M., Morrow, W.M., Rajala-Schultz, P.J., Kazwala, R., Gebreyes, W.A., 2014. In-feed use of heavy metal micronutrients in U.S. Swine production systems and its role in persistence of multidrug-resistant *Salmonellae*. Appl. Environ. Microbiol. 80, 2317 LP-2325.

Mégraud, F., 2012. The challenge of *Helicobacter pylori* resistance to antibiotics: the comeback of bismuth-based quadruple therapy. Therap. Adv. Gastroenterol. 5, 103–9. https://doi.org/10.1177/1756283X11432492

Melnyk, A.H., Wong, A., Kassen, R., 2015. The fitness costs of antibiotic resistance mutations. Evol. Appl. 8, 273–83. https://doi.org/10.1111/eva.12196

Mertens, J., Springael, D., De Troyer, I., Cheyns, K., Wattiau, P., Smolders, E., 2006. Long-term exposure to elevated zinc concentrations induced structural changes and zinc tolerance of the nitrifying community in soil. Environ. Microbiol. 8, 2170–2178. https://doi.org/10.1111/j.1462-2920.2006.01100.x

Miftahussurur, M., Shrestha, P.K., Subsomwong, P., Sharma, R.P., Yamaoka, Y., 2016.

Emerging *Helicobacter pylori* levofloxacin resistance and novel genetic mutation in Nepal. BMC Microbiol. 16, 256. https://doi.org/10.1186/s12866-016-0873-6

Minogue, T.D., Daligault, H.A., Davenport, K.W., Bishop-Lilly, K.A., Broomall, S.M., Bruce, D.C., Chain, P.S., Chertkov, O., Coyne, S.R., Freitas, T., Frey, K.G., Gibbons, H.S., Jaissle, J., Redden, C.L., Rosenzweig, C.N., Xu, Y., Johnson, S.L., 2014. Complete genome assembly of *Escherichia coli* ATCC 25922, a Serotype O6 Reference Strain. Genome Announc. 2. https://doi.org/10.1128/genomeA.00969-14

Moura, A., Jové, T., Ploy, M.-C., Henriques, I., Correia, A., 2012a. Diversity of gene cassette promoters in class 1 integrons from wastewater environments. Appl. Environ. Microbiol. 78, 5413–6. https://doi.org/10.1128/AEM.00042-12

Moura, A., Pereira, C., Henriques, I., Correia, A., 2012b. Novel gene cassettes and integrons in antibiotic-resistant bacteria isolated from urban wastewaters. Res. Microbiol. 163, 92–100. https://doi.org/10.1016/j.resmic.2011.10.010

Moura de Sousa, J., Balbontín, R., Durão, P., Gordo, I., 2017. Multidrug-resistant bacteria compensate for the epistasis between resistances. PLoS Biol. 15, e2001741. https://doi.org/10.1371/journal.pbio.2001741

Munita, J.M., Arias, C.A., 2016. Mechanisms of antibiotic resistance. Microbiol. Spectr. 4. https://doi.org/10.1128/microbiolspec.VMBF-0016-2015

Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.

Naas, T., Mikami, Y., Imai, T., Poirel, L., Nordmann, P., 2001. Characterization of In53, a class 1 plasmid- and composite transposon-located integron of *Escherichia coli* which carries an unusual array of gene cassettes. J. Bacteriol. 183, 235–49. https://doi.org/10.1128/JB.183.1.235-249.2001

Nakajima, H., Kobayashi, K., Kobayashi, M., Asako, H., Aono, R., 1995.

Overexpression of the *robA* gene increases organic solvent tolerance and multiple antibiotic and heavy metal ion resistance in *Escherichia coli*. Appl. Environ. Microbiol. 61, 2302–7.

Nakatsu, C.H., Korona, R., Lenski, R.E., de Bruijn, F.J., Marsh, T.L., Forney, L.J., 1998. Parallel and divergent genotypic evolution in experimental populations of *Ralstonia* sp. J. Bacteriol. 180, 4325–31.

Narciso-da-Rocha, C., Rocha, J., Vaz-Moreira, I., Lira, F., Tamames, J., Henriques, I., Martinez, J.L., Manaia, C.M., 2018. Bacterial lineages putatively associated with the dissemination of antibiotic resistance genes in a full-scale urban wastewater treatment plant. Environ. Int. 118, 179–188. https://doi.org/10.1016/J.ENVINT.2018.05.040

Nies, D.H., 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol. Rev. 27, 313–39.

Nilsson, A.I., Zorzet, A., Kanth, A., Dahlström, S., Berg, O.G., Andersson, D.I., 2006. Reducing the fitness cost of antibiotic resistance by amplification of initiator tRNA genes. Proc. Natl. Acad. Sci. U. S. A. 103, 6976–81. https://doi.org/10.1073/pnas.0602171103

Nishino, K., Nikaido, E., Yamaguchi, A., 2007. Regulation of multidrug efflux systems involved in multidrug and metal resistance of *Salmonella enterica* serovar Typhimurium. J. Bacteriol. 189, 9066–75. https://doi.org/10.1128/JB.01045-07

Norman, A., Hansen, L.H., Sørensen, S.J., 2009. Conjugative plasmids: vessels of the communal gene pool. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 364, 2275–89. https://doi.org/10.1098/rstb.2009.0037

O'Flaherty, E., Cummins, E., 2017. Antibiotic resistance in surface water ecosystems: Presence in the aquatic environment, prevention strategies, and risk assessment. Hum. Ecol. Risk Assess. An Int. J. 23, 299–322. https://doi.org/10.1080/10807039.2016.1247254

O'Flaherty, E., Solimini, A., Pantanella, F., Cummins, E., 2019. The potential human

exposure to antibiotic resistant-*Escherichia coli* through recreational water. Sci. Total Environ. 650, 786–795. https://doi.org/10.1016/J.SCITOTENV.2018.09.018

Olivares, J., Álvarez-Ortega, C., Martinez, J.L., 2014. Metabolic compensation of fitness costs associated with overexpression of the multidrug efflux pump MexEF-OprN in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 58, 3904–13. https://doi.org/10.1128/AAC.00121-14

Olivares Pacheco, J., Alvarez-Ortega, C., Alcalde Rico, M., Martínez, J.L., 2017. Metabolic compensation of fitness costs is a general outcome for antibiotic-resistant *Pseudomonas aeruginosa* mutants overexpressing efflux pumps. MBio 8, e00500-17. https://doi.org/10.1128/mBio.00500-17

Oliver, A., Mena, A., 2010. Bacterial hypermutation in cystic fibrosis, not only for antibiotic resistance. Clin. Microbiol. Infect. 16, 798–808. https://doi.org/10.1111/j.1469-0691.2010.03250.x

Osbourn, S.E.V., Turner, A.K., Grinsted, J., 1995. Nucleotide Sequence within Tn3926 confirms this as a Tn21-like transposable element and provides evidence for the origin of the *mer* operon carried by plasmid pKLH2. Plasmid 33, 65–69. https://doi.org/10.1006/PLAS.1995.1008

Osei Sekyere, J., 2016. Current state of resistance to antibiotics of last-resort in south Africa: A review from a public health perspective. Front. public Heal. 4, 209. https://doi.org/10.3389/fpubh.2016.00209

Osińska, A., Korzeniewska, E., Harnisz, M., Niestępski, S., 2017. The prevalence and characterization of antibiotic-resistant and virulent *Escherichia coli* strains in the municipal wastewater system and their environmental fate. Sci. Total Environ. 577, 367–375. https://doi.org/10.1016/J.SCITOTENV.2016.10.203

Osman, O., Tanguichi, H., Ikeda, K., Park, P., Tanabe-Hosoi, S., Nagata, S., 2010. Copper-resistant halophilic bacterium isolated from the polluted Maruit Lake, Egypt. J. Appl. Microbiol. 108, 1459–1470. https://doi.org/10.1111/j.1365-2672.2009.04574.x

Oves, M., Khan, M.S., Qari, H.A., 2017. *Ensifer adhaerens* for heavy metal bioaccumulation, biosorption, and phosphate solubilization under metal stress condition. J. Taiwan Inst. Chem. Eng. 80, 540–552. https://doi.org/10.1016/J.JTICE.2017.08.026

Pal, C., Asiani, K., Arya, S., Rensing, C., Stekel, D.J., Larsson, D.G.J., Hobman, J.L., 2017. Metal resistance and its association with antibiotic resistance. Adv. Microb. Physiol. 70, 261–313. https://doi.org/10.1016/BS.AMPBS.2017.02.001

Pal, C., Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J., 2015. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. BMC Genomics 16, 964. https://doi.org/10.1186/s12864-015-2153-5

Park, J.-H., Kim, Y.-J., Binn-Kim, Seo, K.-H., 2018. Spread of multidrug-resistant *Escherichia coli* harboring integron via swine farm waste water treatment plant. Ecotoxicol. Environ. Saf. 149, 36–42. https://doi.org/10.1016/J.ECOENV.2017.10.071

Pereira, A.M.P.T., Silva, L.J.G., Meisel, L.M., Pena, A., 2015. Fluoroquinolones and tetracycline antibiotics in a Portuguese aquaculture system and aquatic surroundings: Occurrence and environmental impact. J. Toxicol. Environ. Heal. Part A 78, 959–975. https://doi.org/10.1080/15287394.2015.1036185

Perron, K., Caille, O., Rossier, C., Van Delden, C., Dumas, J.-L., Köhler, T., 2004. CzcR-CzcS, a two-component system involved in heavy metal and carbapenem resistance in *Pseudomonas aeruginosa*. J. Biol. Chem. 279, 8761–8. https://doi.org/10.1074/jbc.M312080200

Piddock, L.J. V., 2006. Multidrug-resistance efflux pumps ? not just for resistance. Nat. Rev. Microbiol. 4, 629–636. https://doi.org/10.1038/nrmicro1464

Poole, K., 2017. At the nexus of antibiotics and metals: The impact of Cu and Zn on antibiotic activity and resistance. Trends Microbiol. 25, 820–832. https://doi.org/10.1016/j.tim.2017.04.010

Poole, K., 2011. *Pseudomonas aeruginosa*: resistance to the max. Front. Microbiol. 2, 65. https://doi.org/10.3389/fmicb.2011.00065

Price, L.B., Graham, J.P., Lackey, L.G., Roess, A., Vailes, R., Silbergeld, E., 2007. Elevated risk of carrying gentamicin-resistant *Escherichia coli* among U.S. poultry workers. Environ. Health Perspect. 115, 1738–42. https://doi.org/10.1289/ehp.10191

Ramírez-Díaz, M.I., Díaz-Pérez, C., Vargas, E., Riveros-Rosas, H., Campos-García, J., Cervantes, C., 2008. Mechanisms of bacterial resistance to chromium compounds. BioMetals 21, 321–332. https://doi.org/10.1007/s10534-007-9121-8

Ramsay, J.P., Firth, N., 2017. Diverse mobilization strategies facilitate transfer of non-conjugative mobile genetic elements. Curr. Opin. Microbiol. 38, 1–9. https://doi.org/10.1016/J.MIB.2017.03.003

Redgrave, L.S., Sutton, S.B., Webber, M.A., Piddock, L.J.V., 2014. Fluoroquinolone resistance: mechanisms, impact on bacteria, and role in evolutionary success. Trends Microbiol. 22, 438–445. https://doi.org/10.1016/J.TIM.2014.04.007

Rhodes, G., Huys, G., Swings, J., McGann, P., Hiney, M., Smith, P., Pickup, R.W., 2000. Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments: implication of Tn1721 in dissemination of the tetracycline resistance determinant tet A. Appl. Environ. Microbiol. 66, 3883–90.

Rinsky, J.L., Nadimpalli, M., Wing, S., Hall, D., Baron, D., Price, L.B., Larsen, J., Stegger, M., Stewart, J., Heaney, C.D., 2013. Livestock-associated methicillin and multidrug resistant *Staphylococcus aureus* is present among industrial, not antibiotic-free livestock operation workers in North Carolina. PLoS One 8, e67641. https://doi.org/10.1371/journal.pone.0067641

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. 447, 345–360. https://doi.org/10.1016/J.SCITOTENV.2013.01.032

Ross, J., Topp, E., 2015. Abundance of antibiotic resistance genes in bacteriophage following soil fertilization with dairy manure or municipal biosolids, and evidence for potential transduction. Appl. Environ. Microbiol. 81, 7905–13. https://doi.org/10.1128/AEM.02363-15

Ruiz, N., Montero, T., Hernandez-Borrell, J., Viñas, M., 2003. The role of *Serratia marcescens* porins in antibiotic resistance. Microb. Drug Resist. 9, 257–264. https://doi.org/10.1089/107662903322286463

Safari Sinegani, A.A., Younessi, N., 2017. Antibiotic resistance of bacteria isolated from heavy metal-polluted soils with different land uses. J. Glob. Antimicrob. Resist. 10, 247–255. https://doi.org/10.1016/J.JGAR.2017.05.012

Salcedo, D.E., Lee, J.H., Ha, U.H., Kim, S.P., 2015. The effects of antibiotics on the biofilm formation and antibiotic resistance gene transfer. Desalin. Water Treat. 54, 3582–3588. https://doi.org/10.1080/19443994.2014.923206

San Millan, A., Toll-Riera, M., Qi, Q., MacLean, R.C., 2015. Interactions between horizontally acquired genes create a fitness cost in *Pseudomonas aeruginosa*. Nat. Commun. 6, 6845. https://doi.org/10.1038/ncomms7845

Sandegren, L., Linkevicius, M., Lytsy, B., Melhus, Å., Andersson, D.I., 2012. Transfer of an *Escherichia coli* ST131 multiresistance cassette has created a *Klebsiella pneumoniae*-specific plasmid associated with a major nosocomial outbreak. J. Antimicrob. Chemother. 67, 74–83. https://doi.org/10.1093/jac/dkr405

Sandle, T., 2017. Antimicrobial copper surfaces in hospitals, The Clinical Services Journal, 16 (6): 47-51

Santos, L., Ramos, F., 2018. Antimicrobial resistance in aquaculture: Current knowledge and alternatives to tackle the problem. Int. J. Antimicrob. Agents 52, 135–143. https://doi.org/10.1016/J.IJANTIMICAG.2018.03.010

Schaufler, K., Semmler, T., Pickard, D.J., de Toro, M., de la Cruz, F., Wieler, L.H., Ewers, C., Guenther, S., 2016. Carriage of extended-spectrum beta-lactamase-plasmids does not reduce fitness but enhances virulence in some strains of

pandemic *E. coli* lineages. Front. Microbiol. 7, 336. https://doi.org/10.3389/fmicb.2016.00336

Schlüter, A., Nordmann, P., Bonnin, R.A., Millemann, Y., Eikmeyer, F.G., Wibberg, D., Pühler, A., Poirel, L., 2014. IncH-type plasmid harboring $bla_{\text{CTX-M-15}}$, $bla_{\text{DHA-1}}$, and *qnrB4* genes recovered from animal isolates. Antimicrob. Agents Chemother. 58, 3768–73. https://doi.org/10.1128/AAC.02695-14

Schuurmans, J.M., van Hijum, S.A.F.T., Piet, J.R., Händel, N., Smelt, J., Brul, S., ter Kuile, B.H., 2014. Effect of growth rate and selection pressure on rates of transfer of an antibiotic resistance plasmid between *E. coli* strains. Plasmid 72, 1–8. https://doi.org/10.1016/j.plasmid.2014.01.002

Scott, G.I., Porter, D.E., Norman, R.S., Scott, C.H., Uyaguari-Diaz, M.I., Maruya, K.A., Weisberg, S.B., Fulton, M.H., Wirth, E.F., Moore, J., Pennington, P.L., Schlenk, D., Cobb, G.P., Denslow, N.D., 2016. Antibiotics as CECs: an overview of the hazards posed by antibiotics and antibiotic resistance. Front. Mar. Sci. 3, 24. https://doi.org/10.3389/fmars.2016.00024

Seiler, C., Berendonk, T.U., 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front. Microbiol. 3, 399. https://doi.org/10.3389/fmicb.2012.00399

Seshadri, R., Joseph, S.W., Chopra, A.K., Sha, J., Shaw, J., Graf, J., Haft, D., Wu, M., Ren, Q., Rosovitz, M.J., Madupu, R., Tallon, L., Kim, M., Jin, S., Vuong, H., Stine, O.C., Ali, A., Horneman, A.J., Heidelberg, J.F., 2006. Genome sequence of *Aeromonas hydrophila* ATCC 7966T: jack of all trades. J. Bacteriol. 188, 8272–82. https://doi.org/10.1128/JB.00621-06

Shannon, C.E. and Weaver, W. ,1949. The Mathematical Theory of Communication. The University of Illinois Press, Urbana, 117 p.

Sharma, V.K., Johnson, N., Cizmas, L., McDonald, T.J., Kim, H., 2016. A review of the influence of treatment strategies on antibiotic resistant bacteria and antibiotic resistance genes. Chemosphere 150, 702–714. https://doi.org/10.1016/J.CHEMOSPHERE.2015.12.084

Shcherbakov, D., Akbergenov, R., Matt, T., Sander, P., Andersson, D.I., Böttger, E.C., 2010. Directed mutagenesis of *Mycobacterium smegmatis* 16S rRNA to reconstruct the in vivo evolution of aminoglycoside resistance in *Mycobacterium tuberculosis*. Mol. Microbiol. 77, 830–840. https://doi.org/10.1111/j.1365-2958.2010.07218.x

Silva, I., Tacão, M., Tavares, R.D.S., Miranda, R., Araújo, S., Manaia, C.M., Henriques, I., 2018. Fate of cefotaxime-resistant *Enterobacteriaceae* and ESBL-producers over a full-scale wastewater treatment process with UV disinfection. Sci. Total Environ. 639, 1028–1037. https://doi.org/10.1016/j.scitotenv.2018.05.229

Sköld, O., 2000. Sulfonamide resistance: mechanisms and trends. Drug Resist. Updat. 3, 155–160. https://doi.org/10.1054/drup.2000.0146

Soucy, S.M., Huang, J., Gogarten, J.P., 2015. Horizontal gene transfer: building the web of life. Nat. Rev. Genet. 16, 472–482. https://doi.org/10.1038/nrg3962

Stalder, T., Top, E., 2016. Plasmid transfer in biofilms: a perspective on limitations and opportunities. npj Biofilms Microbiomes 2, 16022. https://doi.org/10.1038/npjbiofilms.2016.22

Stewart, P.S., William Costerton, J., 2001. Antibiotic resistance of bacteria in biofilms. Lancet 358, 135–138. https://doi.org/10.1016/S0140-6736(01)05321-1

Summers, A.O., Wireman, J., Vimy, M.J., Lorscheider, F.L., Marshall, B., Levy, S.B., Bennett, S., Billard, L., 1993. Mercury released from dental "silver" fillings provokes an increase in mercury- and antibiotic-resistant bacteria in oral and intestinal floras of primates. Antimicrob. Agents Chemother. 37, 825–34.

Suzuki, S., Pruden, A., Virta, M., Zhang, T., 2017. Editorial: Antibiotic resistance in aquatic systems. Front. Microbiol. 8, 14. https://doi.org/10.3389/fmicb.2017.00014

Szekeres, E., Chiriac, C.M., Baricz, A., Szőke-Nagy, T., Lung, I., Soran, M.-L., Rudi, K., Dragos, N., Coman, C., 2018. Investigating antibiotics, antibiotic resistance genes, and microbial contaminants in groundwater in relation to the proximity of urban areas. Environ. Pollut. 236, 734–744.

https://doi.org/10.1016/J.ENVPOL.2018.01.107

Szybalski, W., Bryson, V., 1952. Genetic studies on microbial cross resistance to toxic agents. I. Cross resistance of *Escherichia coli* to fifteen antibiotics. J. Bacteriol. 64, 489–99.

Tacão, M., Correia, A., Henriques, I., 2012. Resistance to broad-spectrum antibiotics in aquatic systems: anthropogenic activities modulate the dissemination of *bla*$_{\text{CTX-M}}$-like genes. Appl. Environ. Microbiol. 78, 4134–40. https://doi.org/10.1128/AEM.00359-12

Tacão, M., Moura, A., Correia, A., Henriques, I., 2014. Co-resistance to different classes of antibiotics among ESBL-producers from aquatic systems. Water Res. 48, 100–107. https://doi.org/10.1016/J.WATRES.2013.09.021

Tacão, M., Silva, I., Henriques, I., 2017. Culture-independent methods reveal high diversity of *OXA-48*-like genes in water environments. J. Water Health 15, 519–525. https://doi.org/10.2166/wh.2017.260

Tamtam, F., van Oort, F., Le Bot, B., Dinh, T., Mompelat, S., Chevreuil, M., Lamy, I., Thiry, M., 2011. Assessing the fate of antibiotic contaminants in metal contaminated soils four years after cessation of long-term waste water irrigation. Sci. Total Environ. 409, 540–547. https://doi.org/10.1016/j.scitotenv.2010.10.033

Teixeira, P., Tacão, M., Alves, A., Henriques, I., 2016. Antibiotic and metal resistance in a ST395 *Pseudomonas aeruginosa* environmental isolate: A genomics approach. Mar. Pollut. Bull. 110, 75–81. https://doi.org/10.1016/J.MARPOLBUL.2016.06.086

Tella, M., Bravin, M.N., Thuriès, L., Cazevieille, P., Chevassus-Rosset, C., Collin, B., Chaurand, P., Legros, S., Doelsch, E., 2016. Increased zinc and copper availability in organic waste amended soil potentially involving distinct release mechanisms. Environ. Pollut. 212, 299–306. https://doi.org/10.1016/J.ENVPOL.2016.01.077

Thomas, C.M., Nielsen, K.M., 2005. Mechanisms of and barriers to, horizontal gene transfer between bacteria. Nat. Rev. Microbiol. 3, 711–721.

https://doi.org/10.1038/nrmicro1234

Tóth, G., Hermann, T., Da Silva, M.R., Montanarella, L., 2016. Heavy metals in agricultural soils of the European Union with implications for food safety. Environ. Int. 88, 299–309. https://doi.org/10.1016/J.ENVINT.2015.12.017

Tripathi, V., Cytryn, E., 2017. Impact of anthropogenic activities on the dissemination of antibiotic resistance across ecological boundaries. Essays Biochem. 61, 11–21. https://doi.org/10.1042/EBC20160054

Varela, A.R., Nunes, O.C., Manaia, C.M., 2016. Quinolone resistant *Aeromonas* spp. as carriers and potential tracers of acquired antibiotic resistance in hospital and municipal wastewater. Sci. Total Environ. 542, 665–671. https://doi.org/10.1016/j.scitotenv.2015.10.124

Vaz-Moreira, I., Nunes, O.C., Manaia, C.M., 2014. Bacterial diversity and antibiotic resistance in water habitats: searching the links with the human microbiome. FEMS Microbiol. Rev. 38, 761–778. https://doi.org/10.1111/1574-6976.12062

Versalovic, J., Koeuth, T., Lupski, J.R., 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 19, 6823–31.

Versalovic, J., Schneider, M., Lupski, J.R., 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol. Cell. Biol. 5, 25–40.

Viti, C., Marchi, E., Decorosi, F., Giovannetti, L., 2014. Molecular mechanisms of Cr(VI) resistance in bacteria and fungi. FEMS Microbiol. Rev. 38, 633–659. https://doi.org/10.1111/1574-6976.12051

von Wintersdorff, C.J.H., Penders, J., van Niekerk, J.M., Mills, N.D., Majumder, S., van Alphen, L.B., Savelkoul, P.H.M., Wolffs, P.F.G., 2016. Dissemination of Antimicrobial resistance in microbial ecosystems through horizontal gene transfer. Front. Microbiol. 7, 173. https://doi.org/10.3389/fmicb.2016.00173

Wales, A.D., Davies, R.H., 2015. Co-selection of resistance to antibiotics, biocides and

heavy metals, and its relevance to foodborne pathogens. Antibiot. (Basel, Switzerland) 4, 567–604. https://doi.org/10.3390/antibiotics4040567

Wall, B.A., Mateus, A., Marshall, L., Pfeiffer, D.U., Lubroth, J., Ormel, H.J., Otto, P., Patriarchi, A., 2016. Drivers, dynamics and epidemiology of antimicrobial resistance in animal production. Food and Agriculture Organization of the United Nations, Rome.

Wasels, F., Kuehne, S.A., Cartman, S.T., Spigaglia, P., Barbanti, F., Minton, N.P., Mastrantonio, P., 2015. Fluoroquinolone resistance does not impose a cost on the fitness of *Clostridium difficile* in vitro. Antimicrob. Agents Chemother. 59, 1794–1796. https://doi.org/10.1128/AAC.04503-14

Watts, J.E.M., Schreier, H.J., Lanska, L., Hale, M.S., 2017. The rising tide of antimicrobial resistance in aquaculture: sources, sinks and solutions. Mar. Drugs 15. https://doi.org/10.3390/md15060158

Weinbauer, M.G., 2004. Ecology of prokaryotic viruses. FEMS Microbiol. Rev. 28, 127–181. https://doi.org/10.1016/j.femsre.2003.08.001

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703.

Wong, A., 2017. Epistasis and the evolution of antimicrobial resistance. Front. Microbiol. 8, 246. https://doi.org/10.3389/fmicb.2017.00246

Wozniak, R.A.F., Waldor, M.K., 2010. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. Nat. Rev. Microbiol. 8, 552–563. https://doi.org/10.1038/nrmicro2382

Xu, Y.-B., Hou, M.-Y., Li, Y.-F., Huang, L., Ruan, J.-J., Zheng, L., Qiao, Q.-X., Du, Q.-P., 2017. Distribution of tetracycline resistance genes and AmpC β-lactamase genes in representative non-urban sewage plants and correlations with treatment processes and heavy metals. Chemosphere 170, 274–281. https://doi.org/10.1016/j.chemosphere.2016.12.027

Xu, Y., Xu, J., Mao, D., Luo, Y., 2017. Effect of the selective pressure of sub-lethal

level of heavy metals on the fate and distribution of ARGs in the catchment scale. Environ. Pollut. 220, 900–908. https://doi.org/10.1016/J.ENVPOL.2016.10.074

Yamachika, S., Sugihara, C., Kamai, Y., Yamashita, M., 2013. Correlation between penicillin-binding protein 2 mutations and carbapenem resistance in *Escherichia coli*. J. Med. Microbiol. 62, 429–436. https://doi.org/10.1099/jmm.0.051631-0

Yanat, B., Rodríguez-Martínez, J.-M., Touati, A., 2017. Plasmid-mediated quinolone resistance in *Enterobacteriaceae*: a systematic review with a focus on Mediterranean countries. Eur. J. Clin. Microbiol. Infect. Dis. 36, 421–435. https://doi.org/10.1007/s10096-016-2847-x

Yang, Q.E., Walsh, T.R., 2017. Toxin-antitoxin systems and their role in disseminating and maintaining antimicrobial resistance. FEMS Microbiol. Rev. 41, 343–353. https://doi.org/10.1093/femsre/fux006

Yazdankhah, S., Rudi, K., Bernhoft, A., 2014. Zinc and copper in animal feed - development of resistance and co-resistance to antimicrobial agents in bacteria of animal origin. Microb. Ecol. Health Dis. 25. https://doi.org/10.3402/mehd.v25.25862

Yim, M.-S., Yau, Y.C.W., Matlow, A., So, J.-S., Zou, J., Flemming, C.A., Schraft, H., Leung, K.T., 2010. A novel selective growth medium-PCR assay to isolate and detect *Sphingomonas* in environmental samples. J. Microbiol. Methods 82, 19–27. https://doi.org/10.1016/J.MIMET.2010.03.012

Yu, Z., Gunn, L., Wall, P., Fanning, S., 2017. Antimicrobial resistance and its association with tolerance to heavy metals in agriculture production. Food Microbiol. 64, 23–32. https://doi.org/10.1016/J.FM.2016.12.009

Zapun, A., Contreras-Martel, C., Vernet, T., 2008. Penicillin-binding proteins and β-lactam resistance. FEMS Microbiol. Rev. 32, 361–385. https://doi.org/10.1111/j.1574-6976.2007.00095.x

Zhang, M., Wang, L., Xu, M., Zhou, H., Wang, S., Wang, Y., Bai, M., Zhang, C., 2019. Selective antibiotic resistance genes in multiphase samples during biofilm growth

in a simulated drinking water distribution system: Occurrence, correlation and low-pressure ultraviolet removal. Sci. Total Environ. 649, 146–155. https://doi.org/10.1016/J.SCITOTENV.2018.08.297

Zhang, X., Li, Y., Liu, B., Wang, J., Feng, C., Gao, M., Wang, L., 2014. Prevalence of veterinary antibiotics and antibiotic-resistant *Escherichia coli* in the surface water of a livestock production region in northern China. PLoS One 9, e111026. https://doi.org/10.1371/journal.pone.0111026

Zhao, Z., Wang, J., Han, Y., Chen, J., Liu, G., Lu, H., Yan, B., Chen, S., 2017. Nutrients, heavy metals and microbial communities co-driven distribution of antibiotic resistance genes in adjacent environment of mariculture. Environ. Pollut. 220, 909–918. https://doi.org/10.1016/J.ENVPOL.2016.10.075

Zhou, Y., Niu, L., Zhu, S., Lu, H., Liu, W., 2017. Occurrence, abundance, and distribution of sulfonamide and tetracycline resistance genes in agricultural soils across China. Sci. Total Environ. 599–600, 1977–1983. https://doi.org/10.1016/J.SCITOTENV.2017.05.152

7. APPENDICE

7.1 Bacterial strains evolution

Table 17: Evolution of the maximum concentrations of the copper and zinc gradient over time (in µg mL^{-1});

Number of transfers	*E. coli*		*A. hydrophila*	
	Cu	Zn	Cu	Zn
0	150	150	100	100
5	200	200	200	200
10	250	250	300	300
15	250	250	300	300
20	250	300	350	350
25	350	350	400	400
30	350	350	400	400
35	350	400	400	400
40	400	400	400	400

7.2 Populations tolerance to metal(loid)s

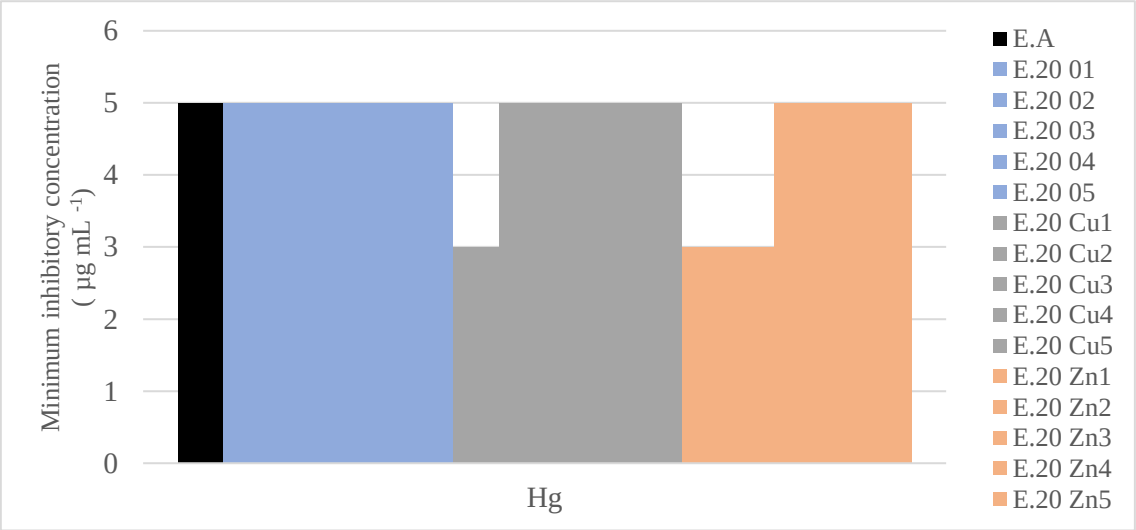


Figure 34: Hg susceptibility of *E. coli* populations after 20 transfers; E.A - stands for ancestral, E.20 01-20 05 - stands for populations evolved without the metal, E.20 Cu1-20 Cu5 - stands for populations evolved with copper and E.20 Zn1-20Zn5 - refers to populations evolved with zinc;

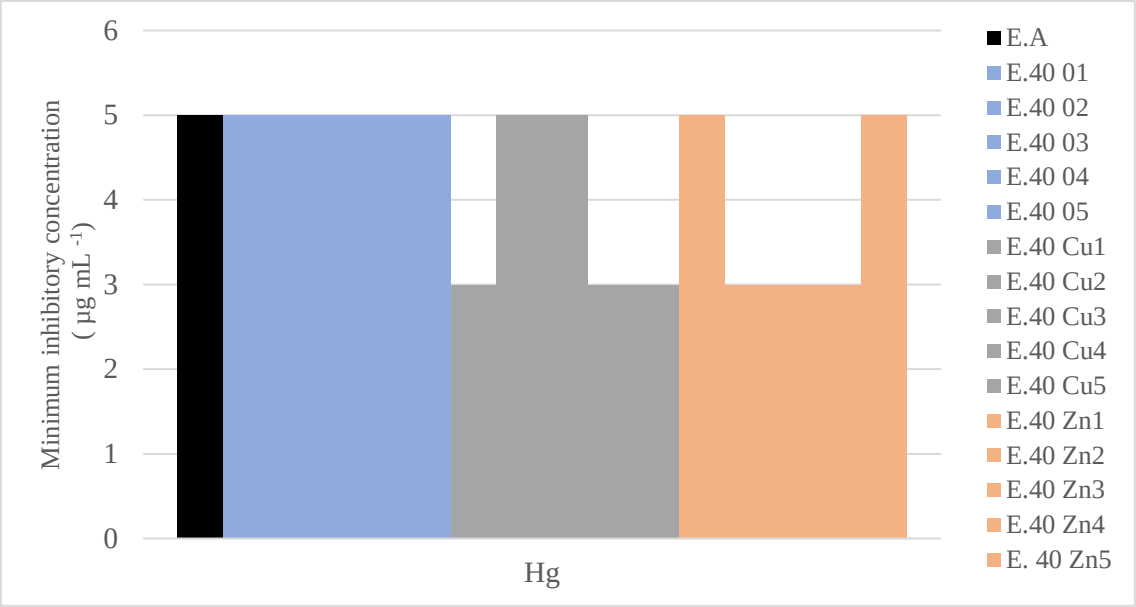


Figure 35: Hg susceptibility of *E. coli* populations after 40 transfers; E.A - stands for ancestral, E.40 01-40 05 - stands for populations evolved without the metal, E.40 Cu1-40 Cu5 - stands for populations evolved with copper and E.40 Zn1-40Zn5 - refers to populations evolved with zinc;

7.3 Fitness cost associated to evolution in the presence of metals

Table 18: Grow rates [r = ln (OD2/OD1)/(T2-T1)] of *E. coli* populations after 20 transfers, per minute; growing in LB, LB supplemented with copper, LB supplemented with zinc and LB supplemented with sulfamethoxazole; E.A - stands for *E. coli* ancestral population, E.20 01-20 05 - stands for populations evolved without the metal, E.20 Cu1-20 Cu5 - stands for populations evolved with copper and E.20 Zn1-20 Zn5 - refers to populations evolved with zinc;

Growth rates after 20 transfers								
***E. coli* populations**	**LB**	**Mean***	**LB+Cu**	**Mean***	**LB+Zn**	**Mean***	**LB+SMX**	**Mean***
E.A	0.017	-	0.004	-	0.011	-	0.017	-
E.20 03	0.026	0.025 ±0.004	0.011	0.010 ±0.003	0.017	0.015 ±0.003	0.009	0.010 ±0.003
E.20 04	0.030		0.006		0.018		0.014	
E.20 05	0.020		0.013		0.012		0.006	
E.20 Cu1	0.015	0.015 ±0.0001	0.011	0.010 ±0.004	-	-	0.005	0.010 ±0.007
E.20 Cu2	0.015		0.015		-		0.020	
E.20 Cu3	0.015		0.005		-		0.004	
E.20 Zn2	0.011	0.012 ±0.002	-	-	0.010	0.013 ±0.002	0.012	0.013 ±0.002
E.20 Zn3	0.010		-		0.016		0.015	
E.20 Zn5	0.014		-		0.014		0.011	

* ± standard deviation

Table 19: Doubling time in minutes [Dt = ln2/r] of *E. coli* populations after 20 transfers; growing in LB, LB supplemented with copper, LB supplemented with zinc and LB supplemented with sulfamethoxazole; E.A - stands for *E. coli* ancestral population, E.20 01-20 05 - stands for populations evolved without the metal, E.20 Cu1-20 Cu5 - stands for populations evolved with copper and E.20 Zn1-20 Zn5 - refers to populations evolved with zinc;

Doubling time after 20 transfers								
***E. coli* populations**	**LB**	**Mean***	**LB+Cu**	**Mean***	**LB+Zn**	**Mean***	**LB+SMX**	**Mean***
E.A	42	-	182.4	-	63.6	-	39.8	-
E.20 03	26.7	28.3 ±5	64.2	76.1 ±25	41.8	46.8 ±9	73.7	79.5 ±27
E.20 04	23.5		111.8		38.9		49.2	
E.20 05	34.8		54.2		59.8		115.5	
E.20 Cu1	47.8	47.4 ±0.3	60.8	86 ±46	-	-	150.7	115.6 ±57
E.20 Cu2	47.2		46.5		-		34.8	
E.20 Cu3	47.2		150.7		-		161.2	
E.20 Zn2	64.8	61.2 ±9	-	-	67.3	53.5 ±10	59.2	56.8 ±9
E.20 Zn3	70.0		-		43.1		45	
E.20 Zn5	48.8		-		50.2		66	

* ± standard deviation

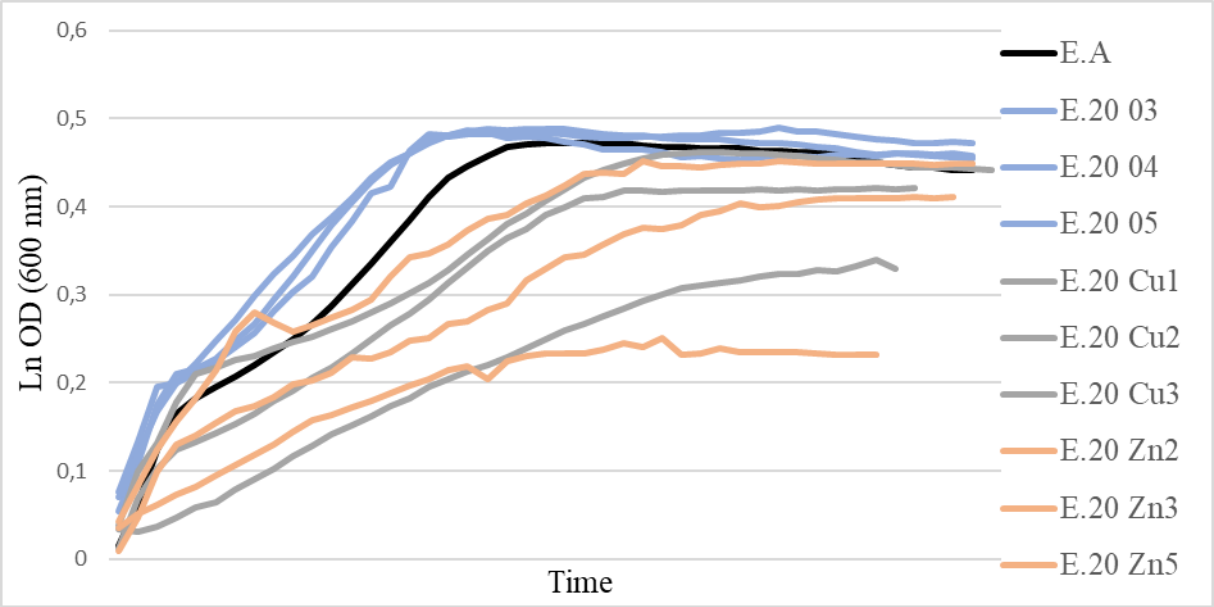


Figure 36: Growth of *E. coli* populations after 20 transfers in LB, for 24h; E.A - stands for ancestral, E.20 01-20 05 - stands for populations evolved without the metal, E.20 Cu1-20 Cu5 - stands for populations evolved with copper and E.20 Zn1-20 Zn5 - refers to populations evolved with zinc;

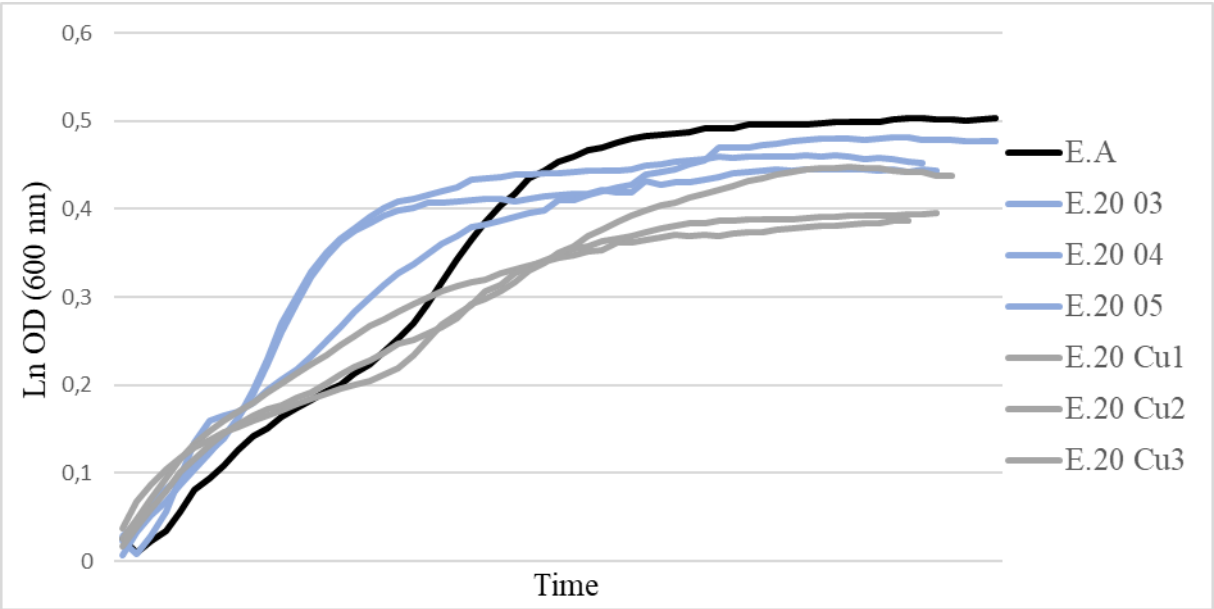


Figure 37: Growth of *E. coli* populations after 20 transfers in LB supplemented with copper, for 30h; E.A - stands for ancestral, E.20 01-20 05 - stands for populations evolved without the metal and E.20 Cu1-20 Cu5 - stands for populations evolved with copper;

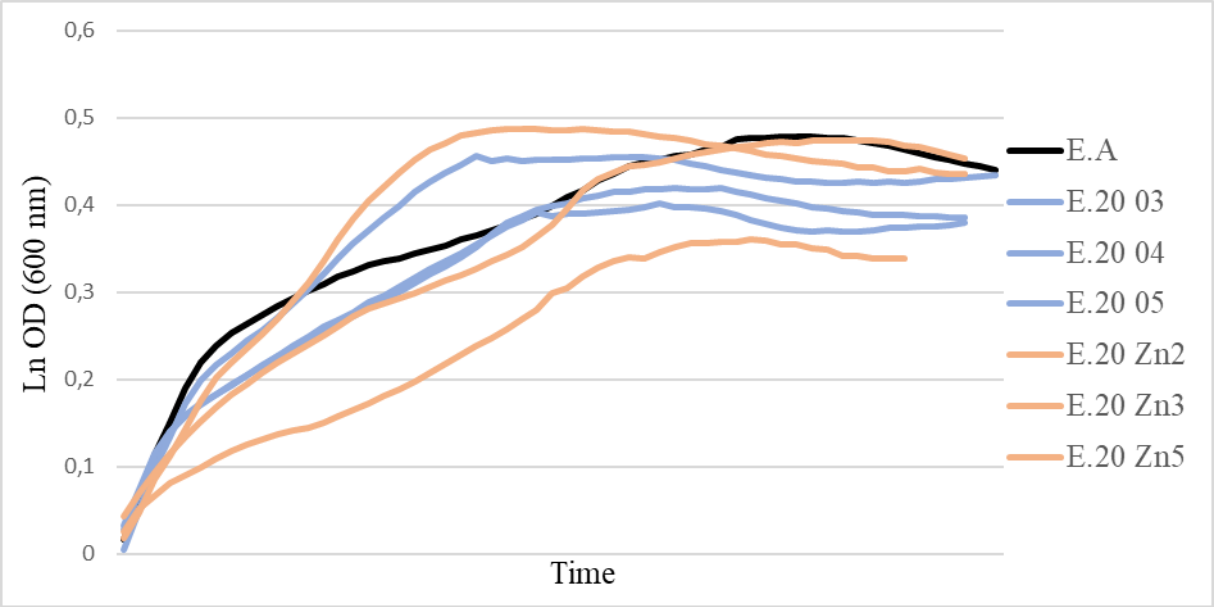


Figure 38: Growth of *E. coli* populations after 20 transfers in LB supplemented with zinc, for 30h; E.A - stands for ancestral, E.20 01-20 05 - stands for populations evolved without the metal and E.20 Zn1-20 Zn5 - refers to populations evolved with zinc;

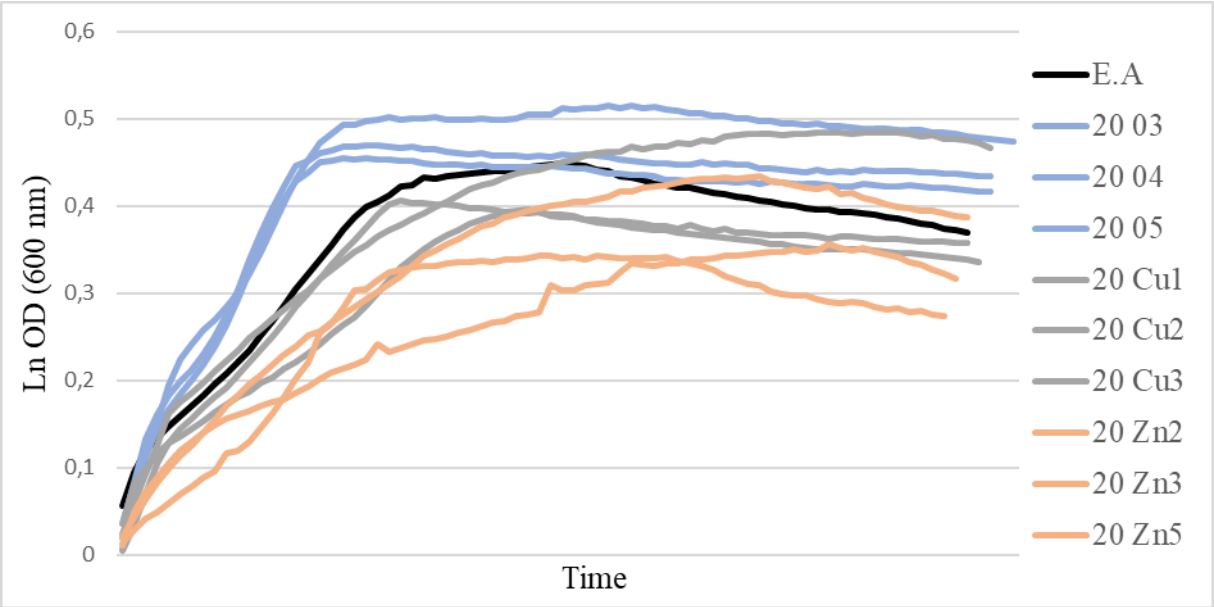


Figure 39: Growth of *E. coli* populations after 20 transfers in LB supplemented with sulfamethoxazole, for 40h; E.A - stands for ancestral, E.20 01-20 05 - stands for populations evolved without the metal, E.20 Cu1-20 Cu5 - stands for populations evolved with copper and E.20 Zn1-20 Zn5 - refers to populations evolved with zinc;

Table 20: Grow rates [r = ln (OD2/OD1)/(T2-T1)] of *A. hydrophila* populations after 20 transfers, per minute; growing in LB, LB supplemented with copper, LB supplemented with zinc and LB supplemented with sulfamethoxazole; A.A - stands *A. hydrophila* ancestral population, A.20 01-20 05 - stands for populations evolved without the metal, A.20 Cu1-20 Cu5 - stands for populations evolved with copper and A.20 Zn1-20 Zn5 - refers to populations evolved with zinc;

Growth rates after 20 transfers								
A. hydrophila populations	**LB**	**Mean***	**LB+Cu**	**Mean***	**LB+Zn**	**Mean***	**LB+SMX**	**Mean***
A.A	0.006	-	0.013	-	0.004	-	0.019	-
A.20 01	0.010	0.015 ±0.005	0.008	0.010 ±0.004	0.018	0.017 ±0.001	0.015	0.024 ±0.009
A.20 02	0.021		0.016		0.017		0.021	
A.20 03	0.015		0.007		0.016		0.036	
A.20 Cu2	0.009	0.014 ±0.008	0.018	0.017 ±0.002	-	-	0.020	0.018 ±0.001
A.20 Cu3	0.008		0.014		-		0.016	
A.20 Cu5	0.026		0.018		-		0.018	
A.20 Zn1	0.013	0.016 ±0.005	-	-	0.019	0.018 ±0.002	0.017	0.016 ±0.001
A.20 Zn2	0.024		-		0.020		0.015	
A.20 Zn4	0.011		-		0.015		0.017	

* ± standard deviation

Table 21: Doubling time [Dt = ln2/r] of *A. hydrophila* populations after 20 transfers, per minute, growing in LB, LB supplemented with copper, LB supplemented with zinc and LB supplemented with sulfamethoxazole; A.A - stands *A. hydrophila* ancestral population, 20 01-20 05 - stands for populations evolved without the metal, 20 Cu1-20 Cu5 - stands for populations evolved with copper and 20 Zn1-20 Zn5 - refers to populations evolved with zinc;

Doubling time after 20 transfers								
A. hydrophila populations	**LB**	**Mean***	**LB+Cu**	**Mean***	**LB+Zn**	**Mean***	**LB+SMX**	**Mean***
A.A	117.5	-	55.5	-	165.0	-	35.9	-
A.20 01	73.0	50.5 ±17	88.9	78.8 ±27	39.6	41.8 ±2	45.9	32.8 ±11
A.20 02	33.0		42.5		41.5		33.3	
A.20 03	45.6		105.0		105.0		19.3	
A.20 Cu2	80.6	65.5 ±27	37.7	42.0 ±5	-	-	35.5	38.9 ±3
A.20 Cu3	88.9		49.2		-		43.3	
A.20 Cu5	27.0		39.4		-		37.9	
A.20 Zn1	55.0	48.6 ±14	-	-	37.7	39.4 ±5	41.8	43.6 ±3
A.20 Zn2	29.5		-		49.2		47.5	
A.20 Zn4	61.3		-		39.4		41.5	

* ± standard deviation

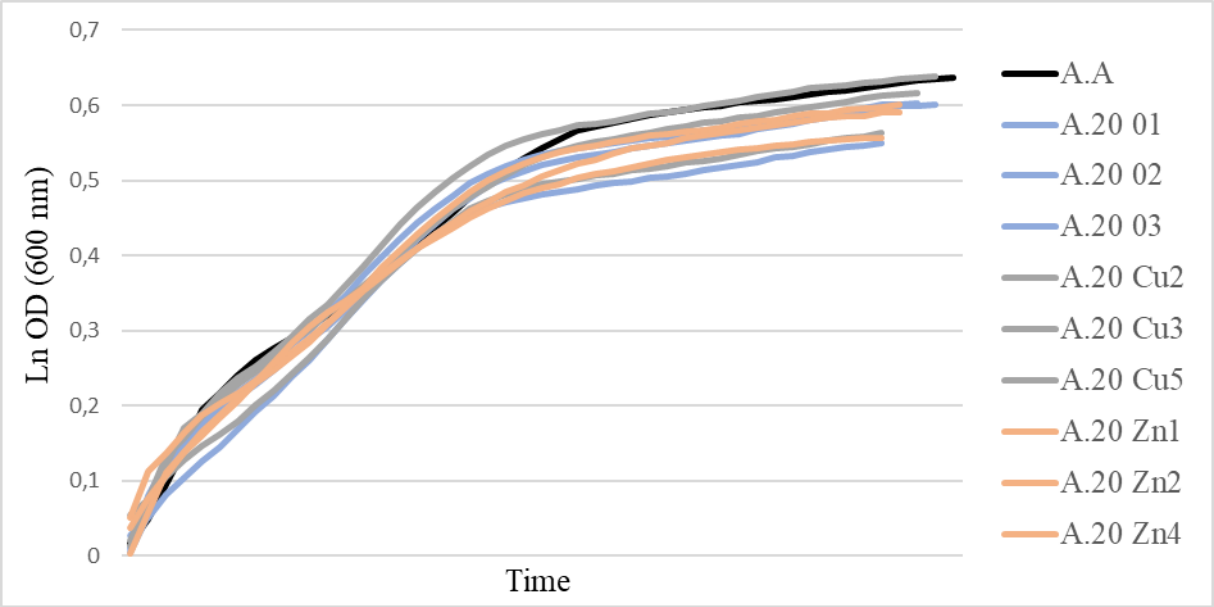


Figure 40: Growth of *A. hydrophila* populations after 20 transfers in LB, for 24h; A.A - stands for ancestral, A.20 01-20 05 - stands for populations evolved without the metal, A.20 Cu1-20 Cu5 - stands for populations evolved with copper and A.20 Zn1-20 Zn5 - refers to populations evolved with zinc;

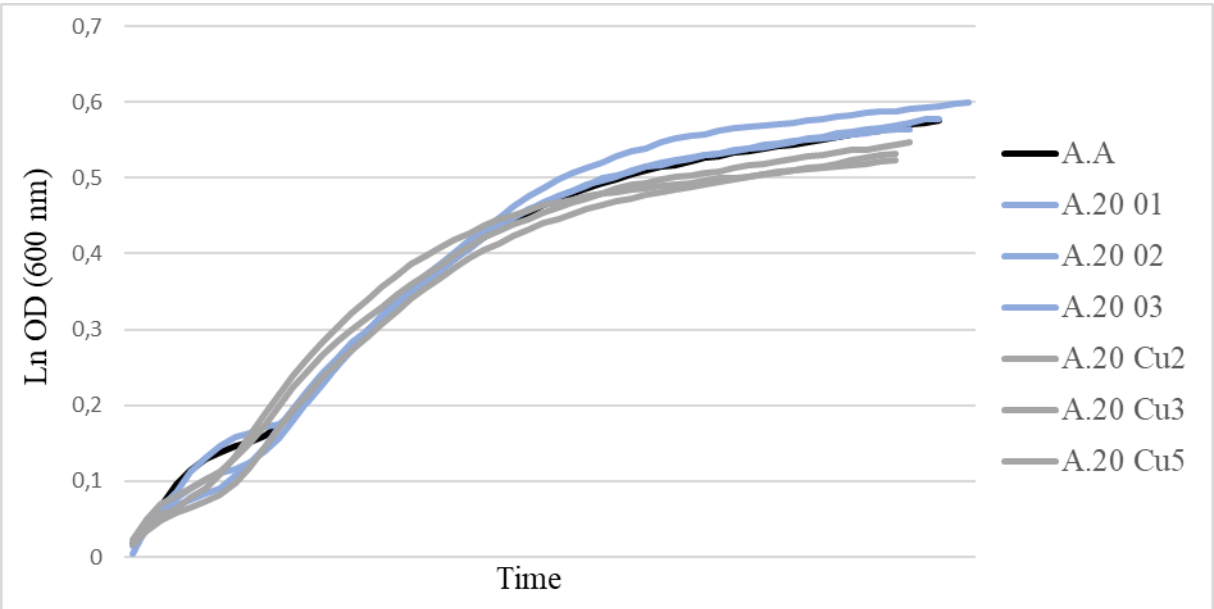


Figure 41: Growth of *A. hydrophila* populations after 20 transfers in LB supplemented with copper, for 30h; A.A - stands for ancestral, A.20 01-20 05 - stands for populations evolved without the metal and A.20 Cu1-20 Cu5 - stands for populations evolved with copper;

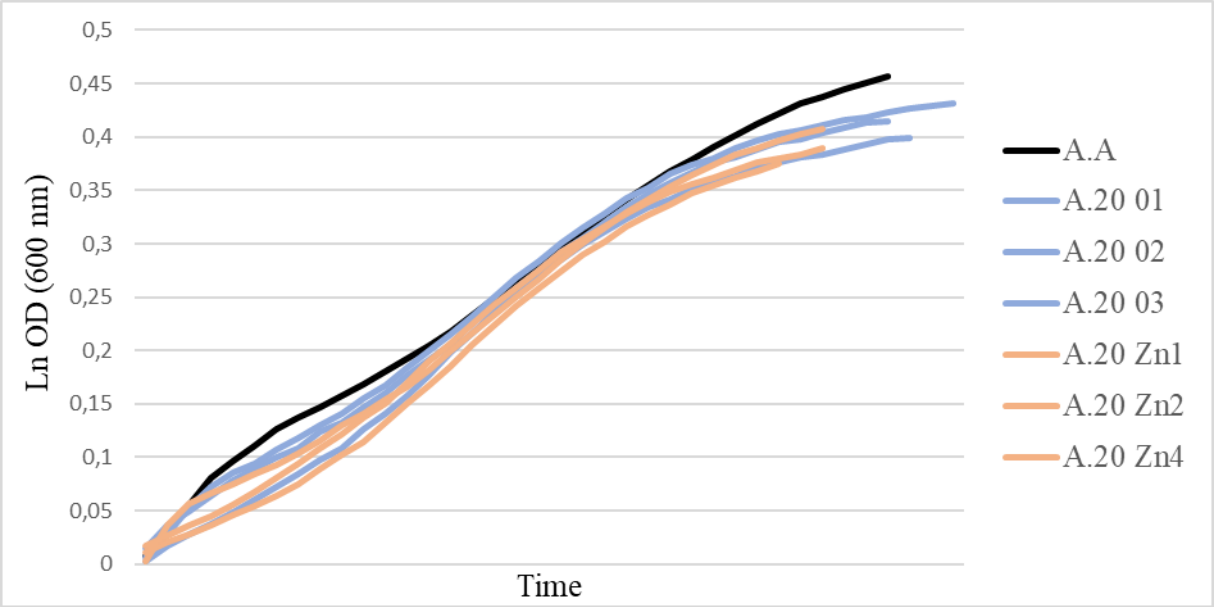


Figure 42: Growth of *A. hydrophila* populations after 20 transfers in LB supplemented with zinc, for 30h; A.A - stands for ancestral, A.20 01-20 05 - stands for populations evolved without the metal and A.20 Zn1-20 Zn5 - refers to populations evolved with zinc;

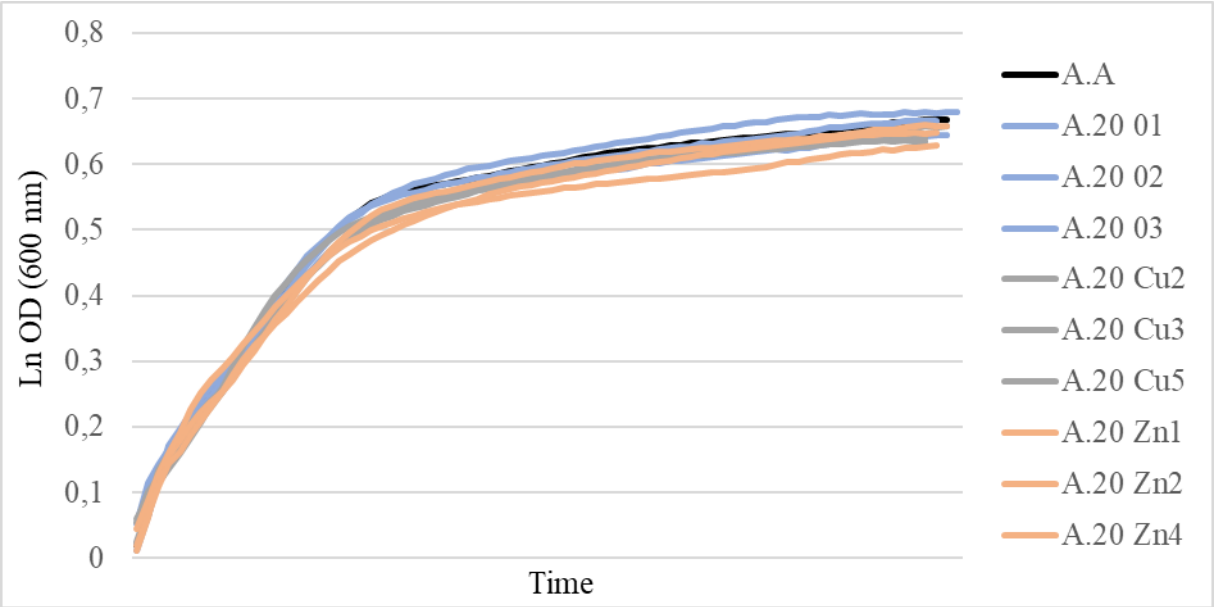


Figure 43: Growth of *A. hydrophila* populations after 20 transfers in LB supplemented with sulfamethoxazole, for 40h; A.A - stands for ancestral, A.20 01-20 05 - stands for populations evolved without the metal, A.20 Cu1-20 Cu5 - stands for populations evolved with copper and A.20 Zn1-20 Zn5 - refers to populations evolved with zinc;

7.4 Comparison of genotypic profiles

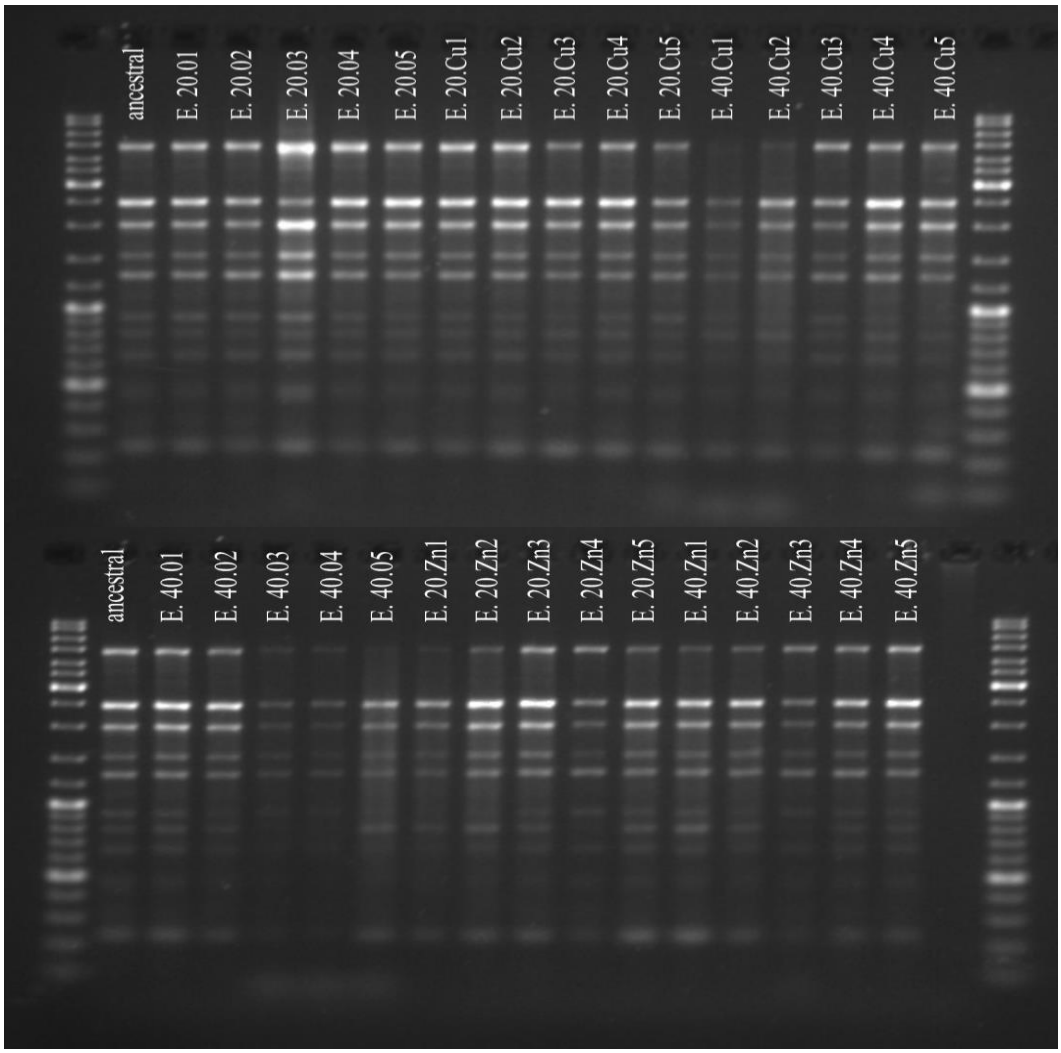


Figure 44: ERIC-PCR DNA fingerprint patterns for *E. coli* (E) populations. The lanes of the ends contained an external standard, a 1-kb molecular weight ladder (GeneRuler™ DNA ladder Mix - Thermo Fisher Scientific, USA). 20 stands for 20 transfers and 40 stands for 40 transfers, 01-05 - stands for populations evolved without the metal, Cu1- Cu5 - stands for populations evolved with copper and Zn1-Zn5 - refers to populations evolved with zinc;

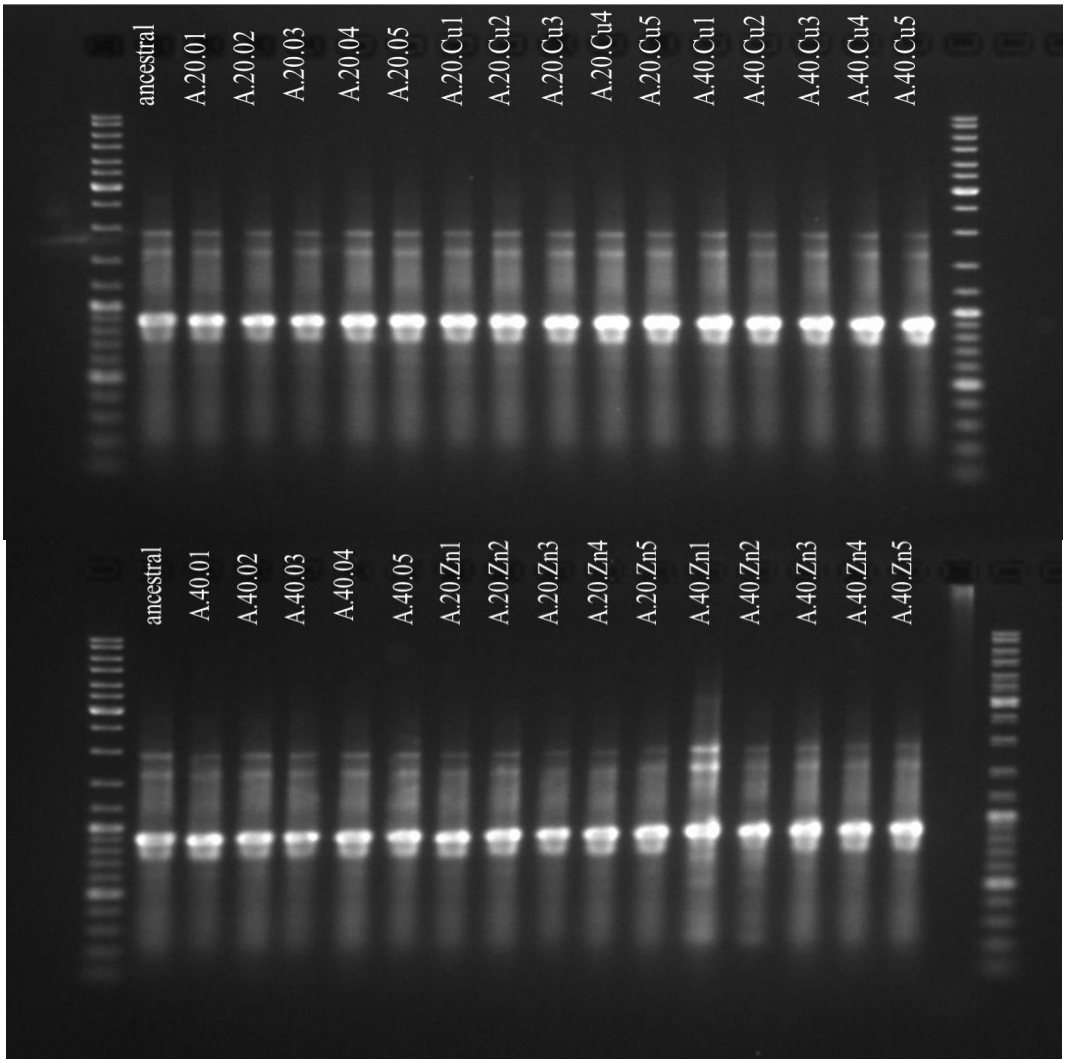


Figure 45: BOX-PCR DNA fingerprint patterns for *A. hydrophila* (A) populations. The lanes of the ends contained an external standard, a 1-kb molecular weight ladder (GeneRuler™ DNA ladder Mix - Thermo Fisher Scientific, USA). 20 stands for 20 transfers and 40 stands for 40 transfers, 01-05 - stands for populations evolved without the metal, Cu1- Cu5 - stands for populations evolved with copper and Zn1-Zn5 - refers to populations evolved with zinc;

7.5 Taxonomic affiliation of strains selected during the microcosm experiment

Table 22: Identification of bacteria resistant to cefotaxime exposed to copper for 20 days by 16S rRNA gene sequencing;

NCBI	% similarity	EzBioCloud	% similarity
Bacillus pseudomycoides	100	*Bacillus pseudomycoides*	100
Bacillus wiedmannii	100	*Bacillus wiedmannii*	100
Delftia lacustris	100	*Delftia lacustris*	100
Janthinobacterium lividum	100	*Janthinobacterium lividum*	100
Janthinobacterium lividum	100	*Janthinobacterium lividum*	100
Pseudomonas grimontii	99	*Pseudomonas grimontii*	100
Pseudomonas grimontii	99	*Pseudomonas grimontii*	100
Pseudomonas grimontii	99	*Pseudomonas grimontii*	99.87
Pseudomonas koreensis	100	*Pseudomonas koreensis*	100
Pseudomonas lurida	100	*Pseudomonas lurida*	99
Pseudomonas lurida	99	*Pseudomonas lurida*	99.38
Pseudomonas mucidolens	99	*Pseudomonas gessardii*	99.03
Pseudomonas rhodesiae	99	*Pseudomonas rhodesiae*	99.19
Pseudomonas trivialis	99	*Pseudomonas trivialis*	99.90
Pseudomonas umsongensis	99	*Pseudomonas umsongensis*	99.68
Pseudomonas umsongensis	99	*Pseudomonas moorei*	99.72
Pseudomonas umsongensis	99	*Pseudomonas umsongensis*	99.67
Pseudomonas umsongensis	99	*Pseudomonas moorei*	100
Pseudomonas vancouverensis	100	*Pseudomonas vancouverensis*	100
Pseudomonas vancouverensis	100	*Pseudomonas vancouverensis*	100
Pseudomonas vancouverensis	99	*Pseudomonas vancouverensis*	99.72
Pseudomonas veroni	100	*Pseudomonas veronii*	99.84
Sphingomonas bisphenolicum	99	*Sphingobium amiense*	98.37
Sphingobium rhizovicinum	99	*Sphingobium rhizovicinum*	99.50

Table 23: Identification of bacteria resistant to cefotaxime exposed to zinc for 20 days by 16S rRNA gene sequencing;

NCBI	% similarity	EzBioCloud	% similarity
Ensifer adhaerens	99	*Ensifer adhaerens*	99,79
Ensifer adhaerens	99	*Ensifer adhaerens*	99,63
Ensifer adhaerens	99	*Ensifer adhaerens*	99,61
Pedobacter jejuensis	99	*Pedobacter jejuensis*	99,37
Pedobacter jejuensis	99	*Pedobacter jejuensis*	99,53
Pseudacidovorax intermedius	100	*Pseudacidovorax intermedius*	100
Pseudomonas sesami	99	*Pseudomonas sesami*	99,88
Sphingomonas aquatilis	99	*Sphingomonas melonis*	100
Sphingomonas aquatilis	100	*Sphingomonas melonis*	100
Sphingomonas aquatilis	99	*Sphingomonas melonis*	100
Sphingomonas aquatilis	100	*Sphingomonas melonis*	100
Sphingomonas melonis	99	*Sphingomonas melonis*	100
Sphingomonas melonis	99	*Sphingomonas melonis*	99.91
Sphingomonas melonis	100	*Sphingomonas melonis*	100
Sphingomonas melonis	100	*Sphingomonas melonis*	100
Sphingomonas melonis	100	*Sphingomonas melonis*	100

Table 24: Identification of bacteria resistant to kanamycin exposed to zinc for 20 days by 16S rRNA gene sequencing;

NCBI	% similarity	EzBioCloud	% similarity
Acinetobacter lwoffii	100	*LSZI_s*	100
Ensifer adhaerens	99	*Ensifer adhaerens*	99,79
Ensifer adhaerens	99	*Ensifer adhaerens*	99,79
Ensifer adhaerens	99	*Ensifer adhaerens*	99,69
Ensifer adhaerens	99	*Ensifer adhaerens*	99.57
Ensifer adhaerens	99	*Ensifer morelensis*	99,64
Ensifer adhaerens	99	*Ensifer adhaerens*	99,61
Ensifer adhaerens	100	*Ensifer adhaerens*	100
Ensifer adhaerens	99	*Ensifer adhaerens*	99,34
Ensifer adhaerens	99	*Ensifer adhaerens*	99,61
Ensifer adhaerens	99	*Ensifer morelensis*	99,64
Ensifer adhaerens	99	*Ensifer adhaerens*	9,71
Ensifer adhaerens	99	*Ensifer adhaerens*	99,48
Ensifer sesbaniae	99	*Ensifer sesbaniae*	99,8
Pedobacter jejuensis	99	*Pedobacter jejuensis*	99,52
Pedobacter jejuensis	99	*Pedobacter jejuensis*	98,78
Pedobacter ginsengiterrae	99	*Pedobacter ginsengiterrae*	99,18