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DYNAMICS OF DRUG RESISTANCE IN ENVIRONMENTAL BACTERIA WITHIN AN AQUATIC ECOSYSTEM

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Dynamics of Drug Resistance in Environmental Bacteria within an Aquatic Ecosystem

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This is a thesis in which the chapters are written as a set of discrete research manuscripts submitted or intended for submission to internationally recognized peer-reviewed journals with an overall introduction and final summary.

This is to certify that the content of this thesis is the original research work of Mr Kelechi Chukwu, carried out under our supervision at the Antimicrobial Research Unit (ARU), Discipline of Pharmaceutical Sciences, School of Health Sciences, College of Health Sciences, Westville Campus, University of KwaZulu-Natal (UKZN), Durban, South Africa.

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DECLARATION

I, Mr Kelechi Chukwu, declare as follows:

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DEDICATION

This work is dedicated to the memory of my parents, Jerome and Theresa Chukwu, whose wishes were for me to finish this degree. Finally, I did it after many decades.

And to God Almighty, who made it possible.

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My special gratitude to my wife and strength, Dr Martina Chukwu, who stood by me and pushed me to finish this program. I also acknowledge my children, Jerdam, Angel and Zikora, as well as my sisters, Chika and Chinonyerem, and my brothers Martin, Iyke and Chigozie who continued to encourage me throughout.

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LIST OF ABBREVIATIONS AND ACRONYMS

AMR	Antimicrobial resistance
ANVIO	Advanced analysis and visualisation platform for omics data.
API	Active pharmaceutical ingredient
ARB	Antibiotic-resistant bacteria
ARG	Antibiotic resistance gene
ATCC	American Type Culture Collection
ATMAC	Alkyl trimethyl ammonium compounds
BAC	Benzalkonium chloride
BIT	1,2-benzisothiazolinone
BLAST	Basic l ºCal alignment search tool.
BP3	2-Hydroxy-4-methoxybenzophenone
BRG	Biocide resistance gene.
CCL2	chemokine (C-C motif) ligand 2
CEC	Contaminant of emerging concern.
CFU	Colony forming unit.
DADMAC	Diallyldimethyammonium chloride
DBPI	Dibromopropamidine isethionate
DDAC	Didecyldimethyl-ammonium chloride
DDBA	Didodecyldimethyl ammonium Bromide
DEET	N, N-diethyl-meta-toluamide
DICOIT	4, -dichloro-2-n-ocylisothiazoline
DTAB	Dodecyltrimethyl ammonium bromide
ESBL	Extended-spectrum β-lactamase
FAO	Food and Agriculture Organisation of the United Nations
FASTA	Fast alignment

HGT	Horizontal gene transfer
HIV	Human immune-deficiency virus
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
HSID	Hot surface induced desolvation
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectrophotometry
LMIC	Low- and middle-income countries
LOQ	Limit of quantification
MAEC	Minimum allowable environmental concentration
MDK	Minimum duration for killing
MDR	Multidrug resistance
MEM	2,5-dimethoxy-4-ethoxyamphetamine
MIC	Minimum inhibitory concentration
MPC	Mutant prevention concentration
MPN	Most probable number
MRM	Multiple reaction monitoring
MS/MS	Tandem Mass Spectrometry
MSC	Minimum selection concentration
MSW	Mutant selection window
NICD	National Institute for Communicable Diseases
NOEC	No-observed effect concentration
OIT	2-n-octyl-4-isothiazolinone
PAH	Polycyclic aromatic hydrocarbon
PATRIC	Pathosystems Resource Integration Centre
PCMX	Chroloxylenol or para-chloro-meta-xylenol
PEP	Phosphoenolpyruvate

PNBA	Poly (n-butyl acetate
PNEC	Predicted No-Effect concentration
$PNEC_{ENV}$	Environmental predicted no effect concentration
$PNEC_{RS}$	Resistance selective predicted no effect concentration
PPCP	Pharmaceutical and personal care product
PPM	Parts per million
PRP	Propylparabens
PTS	Phosphotransferase system
PWPTP	Pharmaceutical waste products treatment plant
QAC	Quantenery ammonium compound
QuEChERS	Quick easy cheap effective rugged and safe method
RND	Resistance-nodulated-cell Division
RNA	Riboxynucleic acid
ROS	Reactive oxygen stress
SANS	South African National Standard
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
SPE	Solid phase extractor
SPM	Suspended particulate matter
SPP	Superficially porous particles
SPSS	Statistical Package for the Social Sciences
TBL	Tolerance by lag
TSB	Tryptic Soy broth
USEPA	United States Environmental Protection Agency
WCX	Weak cation exchange
WGS	Whole genome sequencing

WHO	World Health Organization
WWTP	Wastewater treatment Plant

ABSTRACT

Recently there has been a rapid increase in the incidence and prevalence of drug-resistant bacteria and antimicrobial resistance genes in the environment, largely attributed to selection pressure from the environmental presence of antimicrobials such as antibiotics, biocides, and heavy metals, as well as other physicochemical stressors. such as Poly aromatic hydrocarbons, pH, temperature, and reactive oxygen. However, the concentrations at which these antimicrobials could elicit resistance are poorly understood. Such lack of information could hamper the development of standards for the environmental surveillance of antmicrobials with potential adverse effects on human, animal and environmental health.

In this study, Water samples were collected from all the points that impact the environment directly around the Darvill wastewater treatment plant, namely the treatment plant effluent discharge point, the upstream and downstream from the effluent discharge point.

Antibiotics, heavy metals, and biocides were identified and quantified from the water samples, and we ascertained the effect of environmental concentrations of some of these selected stressors on the antibiotic resistance in previously susceptible *Escherichia coli*.

Heavy metals concentrations were determined using the United States Environmental Protection Agency (US EPA) method 200.7. Biocide and antibiotic residue concentrations were determined using validated ultra-high-performance liquid chromatography with tandem mass spectrometry-based methods. *E. coli* was identified and quantified using the Colilert-18™ system from IDEXX, while antimicrobial susceptibility was performed using the disc diffusion method according to the Clinical and Laboratory Standards Institute guidelines.

The concentration of antibiotics observed ranged from sulfamethoxazole (286.180 µg/L) to penicillin (2.2 µg/L); for metals, sodium (27.734 mg/L) to iron (0.001 mg/L); and for biocides, benzalkonium chloride (BAC) 12 (7.805 µg/L) to BenthEZ (0.035 µg/L). There was observed increase in the pollutant concentrations in the effluent and downstream samples compared to the upstream samples, suggesting that the WWTP might be a potential source of interest, indicating that these pollutants, were not completely removed at the WWTP.

Thirty days' exposure of wholly susceptible *E. coli* ATCC 25922 strains, to environmental and sub-inhibitory concentrations of oxytetracycline, amoxicillin, zinc, copper, BAC 12 and DADMAC 10 was conducted but could not trigger phenotypic resistance. Genotypic analysis of the WGS on the exposed isolates, found only the macrolide resistance *mdf* (A) gene (which was also present in the control) and the disinfectant resistance gene *sit*ABCD. With further analysis for single nucleotide variants (SNV), mutations were detected for 19 genes compared to the control. Only one resistance gene was detected, *robA*, a member of the *ArcC/XylS* family, that regulates the *ArcAB-TolC* multi-drug efflux, that contributes

to multi-drug resistance. The other 18 genes we detected were tolerance conferring genes, *acnB, cusA, degQ, epmA, hsmP, mlc, purH, queG, srlE, tsaB, yddh* and *yqhH* genes, in all the exposed isolates. *filA* genes in only the oxytetracycline and BAC 12-exposed isolates, *mutM* gene in zinc exposed isolates, *nudK* gene in all the exposed isolates except the DADMAC 10 exposed isolates, *ptsG* gene in only the oxytetracycline-exposed isolates, and *ompD* in only DADMAC 12-exposed isolates. All the genes detected in the exposed isolates were also detected in the environmental isolates, except the *robA* gene. These genes detected encode for oxidative stress, DNA repair, membrane proteins efflux systems, growth and persister formations.

In addition, we observed that the 30-days exposed isolates developed increased tolerance to high (25 x MIC) concentrations of ampicillin by 30 to 50% when compared to unexposed control. BAC 12-exposed isolates had the highest tolerance increase. The increased tolerance seems to emanate from multi gene induced persister cells formations, as well as tolerance gene expressions. The MSW of the exposed isolates to ampicillin and amoxicillin, also slightly increased compared to the control indicating the amplification of persister cells during the 30-day exposure but the MSW remained same to oxytetracycline.

This indicates that exposure to sub-inhibitory concentrations of antibiotics, heavy metals and biocide residues, as observed in the aquatic environment, cannot induce phenotypic resistance but can encode for genes responsible for the development of persistence and tolerance in bacteria, which seems to be the pathway towards eventual antimicrobial resistance in environmental bacteria.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Recently there has been a rapid increase in the incidence and prevalence of drug-resistant bacteria and antimicrobial resistance genes (ARGs) in the environment, largely attributed to selection pressure from the presence of antimicrobials such as antibiotics, biocides and heavy metals as well as other stressors in the environment. However, the concentrations at which these antimicrobials could elicit resistance are poorly understood. Such lack of information could hamper the development of efficient guidelines for the environmental surveillance of antibiotics, with potentially severe effects on human, animal and environmental health (Berendonk et al., 2015; Gullberg et al., 2011; Nguyen et al., 2021; Pruden et al., 2018)

Antibiotics are pharmaceuticals that form part of the Pharmaceuticals and Personal Care Products (PPCP) family in the environment. Lately, the effects of this group of products in the environment have gained greater attention due to their ubiquity and the growing resistance to these chemicals by previously susceptible microorganisms (Bengtsson-Palme and Larsson, 2015). Antibiotic resistance significantly contributes to the increased rate and cost of hospitalization and over 700,000 deaths yearly (Dadgostar, 2019; O' Neil, 2014).

Antibiotics and bacteria in the environment, have always co-existed and maintained an eco-balance. Bacteria while maintaining this delicate balance also survived by developing antibiotic resistance genes, which enable them, to continue their metabolic and genetic purposes, even in the presence of these chemicals (Larsson and Flach, 2022). The number of bacteria in the environment is estimated to be about 10^{30}, and genetic mutation is integral to bacterial life and survival. Most resistance genes produced, are not expressed, but remain dormant unless the fitness cost is lowered (Bengtsson-Palme et al., 2018; Martínez, 2012).

The industrial-scale production of synthetic antibiotics used by humans and livestock, which subsequently find their way into the environment, has exposed environmental bacterial communities to unprecedented selection pressures, resulting in the rapid development of antibiotic resistance even to new drugs (Larsson, 2014; Martinez, 2009). Such exposure results in strains in the microbial community that can initiate gene mutation and transfer, spreading ARGs in the environment. This constitutes a hazard to environmental and human health with severe consequences if not mitigated (Larsson and Flach, 2022; Martinez, 2009).

Modern antibiotics are formulated to target pathogenic bacteria in human and animal health, but they have also been noticed in the environment. Their introduction into the environment is mostly via sewage discharge, runoffs from pharmaceutical industries, healthcare institutions, farms, fisheries, factories, landfill leachates,

and wastewater treatment plant (WWTP) discharges (Clarke et al., 2015; K'oreje et al., 2020). Their presence in the environment, which is not the target, coupled with the innate ability of bacteria to elicit genetic changes to survive and multiply, results in increased bacterial resistance to antibiotics in the environment, especially in effluents with PPCPs (Ebele et al*.,* 2017).

For a bacterial strain especially in the environment, where there might be multiple antimicrobials other than antibiotics, to be considered resistant, its minimum inhibitory concentration (MIC) to any antibiotics, must be higher than the MIC of the parental wild-type strain (Martínez et al., 2015). So it is expected that the strain must be susceptible at antibiotic concentrations above the MIC and start showing resistance below the MIC (Gullberg et al., 2011).

However, this has not been the case. On the contrary, it has been observed that sometimes, at concentrations below and beyond the MIC, there has been noticeable resistance, which is against the accepted concept. For antibiotics, there has been an observed trend that even at low concentrations, sub-MICs, bacteria can also select ARG and maintaining these selections, by altering the fitness cost, can subsequently lead to the entrenchment of resistant bacteria, even in pristine environments (Gullberg et al, 2011).

Above the MIC value is a new value known as the Mutant Prevention Concentration (MPC), which is the lowest concentration required to block the growth (MIC) of the least susceptible mutant in a high density bacterial population ($>10^{10}$ CFU) (Blondeau, 2009).This represents the concentration above which bacteria rarely select for resistant mutants and presently enhances our knowledge of microbial resistance development. So, antimicrobials must be administered above MPC concentrations for the desired outcome (Blondeau and Fitch 2019, Lazona-Huntelman et al 2020, Feng et al 2019, Kawamura et al 2019, Blondeau 2001; Dong et al., 2000). The concentration range between the MIC and the MPC is the Mutant Selection Window (MSW). This is the concentration range within which selective enrichment of resistant mutants may occur. So, the MIC, MPC, and MSW values should be considered, when identifying the effective antibiotic concentrations, to stop selection of resistant mutants (Lazona-Huntelman et al 2020, Feng et al 2019, Kawamura et al 2019, Drlica and Zhao, 2007; Pasquali and Manfreda, 2007).

Resistance gene development in environmental bacteria could be attributed to the selective pressure, arising from a combination of antimicrobial concentrations and environmental physicochemical factors, such as temperature, pH, and environmental pollutants, as they create opportunities for mutations, tolerance and other characteristics such biofilm formations, utilised for the survival and proliferation of the organism (Baquero et al., 1998). The concentration of the antimicrobials in the environment, is also affected by their bioaccumulation and biodegradability and the degree of exposure of the organism (Bengtsson-Palme et al., 2016).

Another factor that has been shown to contribute towards AMR is tolerance. Tolerance is the ability of microbes to survive in adverse environment, up to extremely high concentrations of antimicrobials, well above the MIC and MPC. It is mostly caused by the ability of bacteria to mutate to slow or non-growing phenotypes, called persisters, which can survive very high concentrations of antimicrobials, especially those that act on the growth processes of the microbes (Levin-Reisman et al., 2017). Tolerance has been observed in bacteria and is suggested to be, a first step towards ARGs acquisition, especially in biofilms. It has been observed, in most cases, that tolerance precedes the development of resistance genes, as most tolerant bacteria, start to express resistance genes, after further ($\geq 10^{th}$ generations) exposure to antimicrobials (Fridman et al., 2014, Levin-Reisman et al 2017).

The environment is not pristine but a mixture of many physical and chemical stressors that can contribute to the dynamics of AMR. These stressors are numerous and occur at varying concentrations, but have previously been accepted, within set limits (MIC and the predictive no-effect concentration (PNEC) levels), as being unable to select for resistance genes. However, recent studies as outlined above, have proven the enrichment for resistance beyond and below these limits. Furthermore, there is evidence that the presence of environmental stressors, contributes to the challenges surrounding bacteria survival (Martinez 2012) and generally reduces the fitness cost, thereby encouraging the selection of antimicrobial resistance. Therefore, more studies need to be undertaken for better insight into the dynamics of AMR at environmental concentrations of antibiotics, heavy metals and biocides. Also, there is a need to investigate how these dynamics contribute to the co-selection of antibiotic resistance in environmental bacteria. Such information could provide insight towards adequate legislation on the antibiotics and pollutants discharged into the environment (Bengtsson-Palme and Larsson, 2016; Tell et al., 2019).

1.2. Literature Review

Antibiotic resistance is a severe threat to human, veterinary and environmental health, and the continued excessive use of antimicrobials exacerbates the situation (Xia et al., 2019, Goethem et al 2018). The synthesis of modern antimicrobials, attributed to the invention of penicillin by Alexander Flemings in 1928, signified the advent of modern antimicrobial use. Its probable misuse and over-use was first warned about by same Dr Flemings in 1945 during his noble lectures (Inoue, 2019). It is estimated that infections due to antimicrobial resistance have caused over $20b and €2.5b in excess health care costs and the loss of over 8 and 2.5 million days in the US and EU, respectively and if not addressed globally, can result in 10m human deaths and over $100t economic loss by 2050 (Lau et al 2017; O'Neill 2016).

2015 marked the turning point for AMR, with the political focus on its threat and the inauguration of the Global Antimicrobial Surveillance System (GLASS). A lot of countries and WHO has since adopted comprehensive

approach towards AMR containment and there is need for increased research in this area (Chait et al., 2012; Huijbers et al., 2015; Tornimbene et al., 2018).

1.2.1. Literature Review Structure

This review is organised into three sections.

The first section introduces the sources and quantification of antimicrobials in the environment.

The second section presents a review of the effects of environmental antimicrobials on antibiotics resistance, looking at tolerance, mutant selection window and co-selection.

The third section is the conclusion.

1.2.2. Relevant Concepts

1. A couple of concepts will be of note as they are quite relevant to this study.
2. Environmental concentrations: The quantification of the chemical in the environment
3. Acceptable limits: The standard set for the chemical, that would not be injurious to organisms.
4. Minimum inhibition Concentration: The lowest concentration of an antimicrobial that completely inhibits growth of bacteria.
5. Maximum prevention concentration: The minimum concentration that inhibits the growth of the least susceptible strain in the bacteria population.
6. Mutant selection window: The concentration range between the MIC and the MPC.
7. Tolerance: The ability of bacteria to survive very high concentration of antimicrobials even above MPC.
8. Co-selection, Cross-and co-resistance: The ability of bacteria to select for multiple resistance either through same mechanism (Cross-resistance) or location of two genetic materials on same genetic element (Co-resistance).

SECTION ONE: Sources and Quantification of Antimicrobials in the Environment

1.2.3. Antibiotics in Nature

Antimicrobial resistance (AMR) predates modern antimicrobials and can be traced back to environmental bacteria, producing and releasing antimicrobial elements that enable them to grow and evolve under stressful conditions. These developments, enables them to out-compete other bacteria for food resources and survival (Fletcher, 2015). Thus, antibiotic resistance can be described as a natural involuntary and an unavoidable phenomenon, in line with the Darwinian principle of natural selection for survival. AMR dates to pre-historic era, as confirmed by the presence of ARGs (which encode resistance to even present day ß-lactams, tetracycline, and glycopeptide antibiotics), in 30,000-year-old Beringian permafrost sediments, which predates modern antibiotics usage (Cruz et al., 2019; D'Costa et al., 2011) One of the most prominent sources of antibiotics is soil microbes, which have been around for billions of years. Some present day synthesised antibiotics, such as vancomycin, erythromycin, and many β-lactams, were developed as derivatives of these natural antimicrobial products, from *Actinomyces* and fungi (Baltz, 2008; Fletcher, 2015). Thus, naturally occurring antibiotics, which were critical in the fight against pathogenic bacteria, may also have elicited the development of resistance genes in environmental bacteria (Allen et al., 2010; Bhullar et al., 2012; D'Costa et al., 2011; Lau et al., 2017). Present day antimicrobial resistance, may have evolved from the horizontal transfer of these natural genetic materials, between the environment and environmental bacteria and vertically between the bacteria and animals including humans (Agersø et al., 2019; Alanis, 2005; Fletcher, 2015; Lau et al., 2017).

However, humans have impacted the ecosystem through three main routes, habitat fragmentation, alteration of the community structure (e.g., via non-indigenous species), and chemical pollution (Daughton, 2001). While the first two are apparent, the effects of chemical pollution such as PPCPs like antibiotics, are sometimes not immediately noticeable despite being adverse and widespread. Antibiotics released into the environment, are among the major pharmaceuticals used in human and veterinary therapy and are quite widespread.

1.2.4. Antibiotics in the environment

Most of the studies on pharmaceuticals in Africa have been in South Africa, followed by Tunisia. A review by Madikizela et al. (2020) observed high concentrations of antibiotics in African water, which are far higher than observed in developed countries. They attributed this to very ineffective WWTPs with very low antibiotics removal efficiency, and direct deposition of antibiotics into the environment via open defecation, urination, and dumping of refuse into water bodies in Africa. Sulfamethoxazole (SMX) has been the most widely detected antibiotic in African surface waters with the highest detected concentrations of 33.6 µg/L but in effluents, tetracycline, and spiramycin, with concentrations of 33.6 µg/L, 45.38 µg/L respectively, has been detected in Tunisia (Azanu et al., 2018; Kanama et al., 2018; Madikizela et al., 2020; Olaniran et al., 2019).

People's economic condition and lifestyle of affect the kind of drugs prevalent in the environment. Bagnis et al. (2020) assessed the risk and prevalence of organic micro-pollutants in the Lake Victoria South Basin, Kenya, and detected 78 compounds, including antibiotics such as acetyl-sulfamethoxazole (A-SMX) at a concentration of up to 297 ng/L, in 80% of the samples and trimethoprim (TMP) that was detected at concentrations up to 110 ng/L. Furthermore, they observed that the cheaper sulfonamides and TMP were detected in higher concentrations than the more expensive macrolides and attributed these high concentrations to the low cost of the drugs and their heavy use for HIV treatment and prophylaxis. These observations were also in agreement with Madikizela et al. (2017) who stated that high environmental concentrations of antibiotics should be attributed to the low cost of these drugs, the culture of over-the-counter purchase of medication across Africa and LMIC countries, and ineffective WWTPs where available, coupled with the poor sanitary conditions that people live in. So, unlike in developed nations with better sanitation and effective WWTPs, this makes the water bodies in most LMIC a robust system for enrichment of ARGs.

Most antibiotics consumed are excreted unmetabolized or partially metabolized into the environment. Magwira et al. (2019) studied the fate, occurrence, and potential effects of TB drugs in South African waters. They observed that 20 to 50% of consumed first-line TB medications and up to 90% of second-line antibiotics were excreted unmetabolized into the environment. With up to 23,360 kg of rifampicin taken annually in Gauteng alone, this translates to about 5606.40 kg at 24% unmetabolized rate. For kanamycin, with an annual consumption of 35,040 Kg at 90% unmetabolized rate, potentially it will be about 31,536 Kg, and for trimethoprim at an annual consumption of 9344 Kg at 80% unmetabolized rate, it will be about 7475 Kg, which will be discharged into the Gauteng environment. This is a huge potential risk, considering that TB drug resistance in SA, stands at 3.4% of total TB patients, with an incidence level of 768/100,000 people (Garcia and South-Bodiford, 2017).

The effect of huge concentrations of antibiotics in the environment, is that it has the potential to increase the risk quotient (RQ) of most of the antibiotics, which will also increase the selective pressure in the environment for development of ARG and ARB selection. Risk quotient (RQ), is the ratio of the measured environmental concentration (MEC) or the predicted environmental concentration (PEC) to the predictive no-effect concentration, with values below 0.1 being low risk, below 1 being medium risk and above 1 being high risk (De Bruijn et al, 2003)

Danner et al. (2019), in their review looking at antibiotic pollution in surface waters, stated that over 100,000 tons of antibiotics were used yearly, and most of these substances are ubiquitous in the environment, impacting susceptible microorganisms and affecting the natural food web. Furthermore, they noted that the interactions between antibiotics and environmental stressors, such as metals, biocides, temperature, and pH, result in

increasing environmental challenges, like antibiotic resistance and ecological distortions. They further observed that the average antibiotic concentrations detected from multiple studies in the environment were in the range of > 15µg/L (Americas), >10 µg/L (Europe), >50 µg/L (Africa) and >450 µg/L (Asian Pacific). This shows that there is more antibiotic pollution in low- and middle-income countries (LMIC), especially in Africa and Asia, than the developed economies of Europe and America. Kovalakova et al. (2020), in their review also observed similar trends in an industrial area around Hyderabad in India. While ciprofloxacin was detected in the environment at concentrations of about 14,000 µg/L in WWTP effluents and 2500-6000 µg/L in surface water, its concentrations were in ng/L in Europe. Oxytetracycline (OTC) was also detected at 3611 µg/L in North China compared to 56.1 µg/L in Colorado. This could be attributed to the higher usage of antibiotics in LMIC, which can also be associated with increasing populations and increased agricultural usage (livestock and aquaculture) than in the developed world.

Furthermore, Fekadu et al. (2019), in a review comparing pharmaceuticals in freshwater aquatic environments in Africa and Europe, noted that the concentration of antibiotics detected in Africa and LMIC countries was about 20,000 times higher than that detected in Europe and attributed this to the high level of unprescribed antibiotic use. While 24 antibiotics were frequently reported in Africa, the authors observed that only 14 were reported in Europe and attributed this to their high consumption due to their relatively cheap cost and ease of purchase, especially in rural Africa. These drugs are excreted into the environment, mostly unmetabolized, polluting the African environment and favouring the emergence of ARG (Faleye et al., 2018).

1.2.5. Sources of antibiotics in the environment

Antibiotics are introduced into the environment, in both metabolized and unmetabolized forms, via urine, faeces, farm run-offs, and industrial discharge (Kovalakova et al., 2020). These chemicals, which are seen in wastewater treatment plants (WWTPs), surface waters and sediments, contribute to the proliferation of resistant bacteria and ARGs in the environment (Lee et al., 2017; McArthur and Tuckfield, 2000; Sharma et al., 2015).

1.2.5.1. Antibiotics from wastewater treatment plants

Wastewater treatment plants are major reservoirs and sources of ARGs into the environment, as most chemicals are discharged into them. These plants create an enabling environment where various antibiotic residues can effectively combine with several co-selection factors (such as metals, biocides, surfactants, pH and salinity) to elicit the proliferation of resistance genes, contributing to the development of resistant populations via gene transfers between resistant and non-resistant bacteria (Lee et al., 2017; Pruden et al., 2013; Rizzo et al., 2013). However, most African countries are poorly developed, and WWTPs, where they exist, are mostly inefficient. Necibi et al. (2021), in their paper on the occurrence, impact, and removal technologies of contaminants of

emerging concerns (CEC) in African wastewater effluents, noted that ineffective and obsolete WWTPs were a major pathway for the introduction of antibiotic residues into the environment.

South Africa has one of the most advanced and widespread WWTP systems in Africa, and many studies have been conducted on antibiotics from the WWTPs. Kanama et al. (2018) assessed 17 pharmaceuticals in two hospital sewage treatment plants in the Northwest region and detected three of the five targeted antibiotics in the water samples. The detected antibiotics included ciprofloxacin (0.06-9.11 µg/L; PNEC = 0.06 µg/L), norfloxacin (0.02-0.82 µg/L; PNEC = 0.50 µg/L), and tetracycline (0.48-75.81 µg/L; PNEC = 1.0 µg/L). There were also slight variations in the antibiotic concentrations between the influent and effluent samples, with the effluent samples showing lower concentrations. They noted a removal efficiency for fluoroquinolones and sulfamethoxazole of 34 and 56%, respectively, in one plant and 0% in the second plant, which could not remove the antibiotics. This indicates that these plants could be a constant source of these antibiotics and their residues in the environment (Hendricks and Pool, 2012; Tell et al., 2019).

Antibiotics were detected from water, sediments and biosolids from the Umgeni River in KwaZulu-Natal Province in South Africa (Matongo et al., 2015). Sulfamethoxazole (SMX) at concentrations of 59.28 ± 0.68 µg/L (PNEC 16 µg/L) was the most detected in the WWTP, 6.01 ± 1.28 µg/L in surface waters, 50.73 ± 0.03 µg/L in sediments and 7.35 ± 5.87 µg/g in biosolids. Trimethoprim (TMP) was detected at very high concentrations of 0.16 ± 2.13 µg/L (PNEC = 0.5 µg/L) in the WWTP, but a low concentration of 0.87 ± 1.64 µg/L in surface water and was not detected in biosolids. Erythromycin was detected at 1.13 ± 0.49 µg/L (PNEC = 1.0 µg/L) in wastewaters, 0.26 ± 14.63 µg/L in surface water, 1.57 ± 0.45 µg/L in sediments, but not detected in biosolids. Sulfamethazine was detected at 1.10± 0.36 µg/L in wastewater, 1.24 ± 0.57 µg/L in surface water, Not detected (ND) in sediments and biosolids. In comparison, metronidazole was detected at 61.93 ± 0.31 µg/L (PNEC = 0.13 µg/L) in sediments and 87.48 ± 0.74 µg/L in biosolids but not detected in surface and wastewater. This shows that all the water system compartments were impacted and that the treatment plants were not 100% efficient. Most of these antibiotics were detected at concentrations above the PNEC value, indicating antimicrobial residue pollution in this river system (Matongo et al., 2015; Tell et al., 2019).

Faleye et al. (2019) assessed the presence of 13 antibiotics in 4 WWTPs and receiving water bodies in Durban, in the untreated wastewater and the receiving river water to determine the efficiency of the WWTPs. They observed that the efficiencies of the WWTPs ranged from 21-100% for different antibiotics and inferred that the WWTPs were highly efficient and were not a source of much impact for antibiotics pollution downstream. In the influent vs effluent samples concentrations, the observed a concentration of 1.3 vs 0 ng/L for azithromycin (PNEC = 0.25 µg/L) and 81.748 vs 70.76 µg/L for ciprofloxacin (PNEC = 0.06 µg/L) (Tell et al., 2019).

Although the studies mentioned above had different WWTP removal efficiencies, the detected antibiotic concentrations were mostly above the recommended PNEC values, posing a risk of probable ARG enrichment in these systems.

1.2.5.2. Antibiotics from agricultural activities

Agriculture is another major source of antibiotics in the environment, impacting directly on the soil, and water bodies and indirectly through leachates and WWTPs. In animal farming, veterinary antibiotics are a major source of antibiotic residues, as a large portion (up to 80% in the US) find their way into the environment. Reports indicate that up to 14,600 and 210,000 tons of antibiotics are used in the US and China, respectively (mostly of tetracyclines, macrolides and sulfonamides), and these pose significant ecotoxicological concern when discharged into the environment (Collignon and Voss, 2015; Du and Liu, 2012; Iwu et al., 2020).

Manure can be described as a hotbed for antibiotic residues and dissemination into the environment. Iwu et al. (2020) stated that over 70% of the antibiotics used in agriculture is released unmetabolized into the environment primarily through manures. Over 72% of unmetabolized tetracyclines and 90% of fluoroquinolones were detected in animals' urine and faeces, and these make animal manure a reservoir of antibiotics and a potential source and vector, for the emergence and transmission of antibiotic resistance in the environment. Other microbials, such as heavy metals and biocides, have also been detected in manures, potentially contributing to ARG enrichment directly and possible ARB co-selection (Iwu et al., 2020; Lu et al., 2014; Sukul et al., 2009; Winckler and Grafe, 2001).

Irrigation, especially with wastewater, is another agricultural activity contributing to antibiotic dissemination in the environment. Bougnom et al. (2020), in their study on the effect of raw wastewater irrigation in Ouagadougou (Burkina Faso), Ngaoundere and Yaoundé (Cameroon), detected very high concentrations of ENR, oxytetracycline, and SMX in irrigated fields, where the concentration ranged from 0.09-0.92 ng/g against 0.04-0.44 ng/g in non-irrigated fields. Antibiotics detected were SMX (0.18 ± 0.31 in irrigated and 0.04 ± 0.06 ng/g in non-irrigated field), TMP (0.25 ± 0.18 in irrigated and 0.31 ± 0.11ng/g in non-irrigated field), CIP (0.92 ± 1.24 in irrigated and 0.44 ± 0.44 ng/g in non-irrigated field), OXYTET (0.09 ± 0.15 ng/L in irrigated and ND in the non-irrigated field). These results indicate higher concentrations of antibiotics in irrigated fields compared to non-irrigated fields, and most of the detected antibiotic concentrations have the potential for AMR enrichment, as they were above the acceptable PNEC limit, indicating a high-risk factor as the Risk quotient will be higher than 1 (Bengtsson-Palme and Larsson, 2016; Tell et al., 2019).

Similarly, Azanu et al. (2018) detected 12 widely used antibiotics in hospital wastewaters and effluents from wastewater stabilization ponds used to irrigate vegetable farms in Kumasi, Ghana. Ciprofloxacin was detected

at concentrations as high as 11,352-15,733 ng/L, ERY 7944-10613 ng/L, metronidazole (MET) 247-420 ng/L, TMP 94-4826 ng/L, TET 58-116 ng/L, OXYTET 75-252 ng/L, CTC 16-24 ng/L, DOC 24-120 ng/L, AMX 2.0-6 ng/L, AMP 107-324 ng/L, SMX 2315-3590 ng/L and CXM1052-1557 ng/L. Antibiotics were also detected in the vegetables from the farm at concentrations of 13.5-44.0 ng/Kg for MET, CIP 28.5-92.8 ng/Kg, ERY 41.4-56.7 ng/Kg, TMP 32.7-104 ng/Kg, CFX 11.0-27.3 ng/Kg and SMX 11.2-21.4 ng/Kg, indicating a potential vertical transfer to man and possible enrichment within humans

Aquaculture is another agricultural activity that contributes immensely to antibiotics in the environment. Antibiotic use has been rampant and extensive in aquaculture, and these chemicals have been detected in the immediate environment and seafood, which sometimes act as vectors. Song et al. (2016) investigated antibiotics in 24 fishponds surrounding Tai Lake in China and detected 15 antibiotics in the water samples. The antibiotics detected were SDZ 404.40 ng/L, SMX 719.10 ng/L, sulfachloropyridiazine (SCP) 328.08 ng/L, sulfamonomethazone (SMM) 2723.10 ng/L, sulfaquinoxazole (SQX) 25.20 ng/L, NOR 210.57 ng/L, ENR 183.61 ng/L, CFX 163.26 ng/L, cefradine (CER) 177.22 g/l, cefotaxime (CEO) 361.54 ng/L, TET 250.63 ng/L, OXYTET 198.83 ng/L, CTC 187.35 ng/L, ERY 48.80 ng/L and florfenicol (FF) 2708.60 ng/L. In US-sold seafood collected from farms in 11 countries, only five out of the 47 targeted antibiotics were detected; the highest concentrations were OXYTET 8.56 ng/g, sulfadimethoxine 79.5 ng/g, ormetoprim 93.1 ng/g, virginiamycin 5.18 ng/g, and 4-epioxytetracycline 112.5 ng/g (Done and Halden, 2015). These residues are very high and above the PNEC values for these chemicals and can be attributed to the antibiotics not being easily biodegradable in the environment and making the ponds a very viable site for ARG enrichment.

Food processing is another agricultural activity that can contribute to antibiotics in the environment. This could be attributed to the fact that most of the abattoir waste, was directly discharged into the river and was not subjected to treatment. TET was detected at concentrations of 0.23 ± 0.01 µg/mL in the effluent and 0.85 ± 0.06 µg/L in the surface water (Olaniran et al., 2019). These indicate possible extraneous sources of TET introduction into the river water beside the abattoir. However, the concentrations detected at both effluent and river surface water were beyond acceptable PNEC limits and could select for ARG enrichment within the environment.

1.2.5.3. Antibiotics from landfills and leachates

Solid waste disposal is another source of antibiotics in the environment, especially landfills, as some unused antibiotics and antibiotics wastes are disposed of at households and health facilities, especially in LMIC countries. Studies have confirmed high concentrations of PPCPs, including antibiotics, in landfills (K'oreje et al., 2020). The fate of these antibiotics and their impact on ground and surface water depends on environmental factors that affect their transport, and they can exert selective pressure on environmental bacteria for ARG

development and AMR selection (Boy-Roura et al., 2018). Landfills impact not only the soil directly but also the underground water through leachates.

Clarke et al. (2015) studied leachates from five sites in the USA and detected antibiotics at different concentrations. These leachates, which resulted from containers of mostly unused or partially used products in municipal solid dumps, leaked into the groundwater and ran off into the surface waters and the WWTPs. Antibiotics detected include carbamazepine (CBZ) at a range of 23-282 ng/L, SMX <10-254 ng/L and TMP <10-64 ng/L. These concentrations were quite high and capable of exerting selective pressures for ARB enrichment, and the leachates could act as transport mediums for the dispersal of ARGs to another environment.

So, studies have shown the presence of antibiotics and their residues in different environmental matrices at varying concentrations from different sources. The fact that these chemicals are ubiquitous in the environment is a cause for concern, as they can exert selective pressure for ARB enrichment in the environmental. There have been calls for their regulations, and acceptable limits have been suggested. As most WWTPs are not designed to remove them, a huge proportion still find their way into the environment. From agriculture also, the fact that over 70% of ingested antibiotics are introduced directly into the environment via animals' urine and manure, so there is a need for further research into ways of remediating agricultural soils and manure before further use to reduce the proliferation of resistance amongst soil microorganisms. There is also a potential risk via plants (vegetables) for the intake and biomagnification along the food chain. Considering that the environment is a potpourri, with inter-relationships and different compounds, the total effects of these chemicals cannot be fully ascertained or wholly dependent on concentration. Their role in ARB enrichment and ARG selection in the environment may be impacted by other environmental variables, such as particulate size, temperature, pH, and the geochemical state of the soil, such as the presence of other chemicals, especially antimicrobials.

This study looked at these effects, alone at their concentrations and the possible interplay and dynamics between them and other environmental factors, especially the antimicrobials in the environment. Two major environmental antimicrobials of interest are heavy metals and biocides, and studies have shown that these pollutants are as ubiquitous in the environment as antibiotics.

1.2.6. Heavy metals in the environment

Heavy metals in the environment are from geological sources like weathering of rocks and soil deposition of particulates. Still, most observed elevated concentrations are mostly from anthropogenic sources such as mining, industrial and domestic discharges. Most of these heavy metals end up in surface waters (rivers and estuaries) or sediments, creating environmental reservoirs of pollutants (Fayiga et al., 2018).

Not all heavy metals are toxic. Some are important for physiological functions and are present as trace elements in the cell (e.g., nickel in urease and copper in cytochrome oxidase). However, others such as lead (Pb), cadmium (Cd), mercury (Hg), silver (Ag), and gold (Au) are not of any benefit to organisms and can be deleterious even in small concentrations (Nies, 1999; Souza et al., 2018; Ugur and Ceylan, 2003). Furthermore, heavy metals can bioaccumulate and potentially induce chronic toxicity. Chronic toxicity (such as antimicrobial resistance), though non-lethal, is more dangerous, as it affects the genetic wellbeing and integrity of organisms, affecting the microbial generation and causing changes in the population dynamics (Ghorab, 2018; Kamunda et al., 2016; Seiler and Berendonk, 2012). Also, environmental bacteria tolerance to heavy metals has been noted to elicit enrichment of antimicrobial genes, which can also trigger co-selection for ARGs in environmental bacteria (Wang et al., 2021).

Sources of heavy metals in the environment range from natural weathering of rocks, mining activities, industrial effluents, agricultural activities, and domestic sources. In this study, the WWTPs ere mostly impactedby natural, domestic, and agricultural activities.

1.2.6.1. Heavy metals from natural sources

Several studies, especially in South Africa and LMIC countries, have detected heavy metals from soil unaffected by anthropogenic sources. Many of these heavy metals in the environment seem to originate from natural sources under certain conditions like volcanic eruptions, sea-salt sprays, forest fires, rock weathering, biogenic sources, and wind-borne particles. Most of these heavy metals exist as hydroxides, oxides, sulphides, and sulphates of Pb, Ni, Cr, Cd, As, Hg, Zn, and Cu, with potentially severe health impacts (Bakare and Adeyinka, 2022; Edokpayi et al., 2018; Masindi and Muedi, 2018; Okereafor et al., 2020).

1.2.6.2. Heavy metals from mining activities

Mining tailings are a major source of toxic heavy metals like Zn, Pb, Cd, Ni, Fe, Mn, and As in the environment. Tailings are made up of natural oxides and chemically activated heavy metals, which were by-products of natural rocks and oxides exposed to the environment during mining. These tailings are usually large piles of rocks left over from the separation of minerals from ores and are either solids, liquids, or fine particulate slurry. In South Africa, gold tailings account for about 47% of all mineral wastes, and over 270 unprotected tailing dams that cover over 400 km^2 exist in the Witwatersrand Basin alone (Ebenebe et al., 2017). Similarly, Fosso-kankeu et al. (2017), in their study to predict the possible mobilisation and speciation of heavy metals from mining tailing samples collected near Krugersdorp in Gauteng, South Africa, detected major metal oxides, SiO_2, Fe_2O_3 and Al_2O_3. Hogarh and Foli (2009) analysed soil around the Obuasi gold mines in Ghana

and detected average concentrations of As (581 ± 130 mg/Kg), Cu (39.64 ± 3.02 mg/Kg), Pb (24.22 ± 2.62 mg/Kg) and Zn (72.64 ± 8.01 mg/Kg), all of which are very high and above acceptable WHO limits.

Mining tailings, chemicals and products of ineffective remediation actions on these tailings and deposits contribute to heavy metal pollution in Africa and LMIC environments (Okereafor et al., 2020). They also impact both surface and groundwater through run-offs and leaching, respectively, and are responsible for acid mine drainage, which is very catastrophic to environmental biota and creates a habitat for ARBs and a reservoir for resistance genes (Arsène-ploetze et al., 2017).

1.2.6.3. Heavy metals from agriculture activities

Heavy metals were detected in vegetables cultivated in gardens around Alice in the Eastern Cape, South Africa, at concentrations ranging from Cd (0.01-1.12 mg/Kg dw), Cu (0.92-9.29 mg/Kg dw), Mn (0.04-37338), Zn (4.27-89.88 mg/Kg dw). These metals were also detected in the soil samples in the garden at concentrations of Cd (0.01-0.08 mg/Kg dw), Cu (4.95-7.66 mg/Kg dw), Pb (5.15-14.01 mg/Kg dw), and Zn (15.58-53.01 mg/Kg dw). Even though the concentrations were higher in the soil than in the vegetables, run-offs, leaching and bioaccumulation through the vegetables can disperse these metals even to susceptible environment bacteria in other environments (Bvenura and Afolayan, 2012).

Similarly, in Johannesburg, Kootbodien et al. (2012), in their study on vegetables from school gardens, collected 20 soil samples from about 1.4 km around the school and 1.7 km from gold mine tailing dams and detected heavy metals in the vegetables. These included As (0.30-0.47 mg/Kg dw), Cr (0.92-1.71 mg/Kg dw), Cu (12.35-19.22 mg/Kg dw), Pb (0.66-1.46 mg/Kg dw), Hg (0.06-0.20 mg/Kg dw), Zn (53.73-97.60 mg/Kg dw). The soil samples contained As (23.20 mg/Kg dw), Cr (120.7-206.20 mg/Kg dw), Cu (0.00- 80.30 mg/Kg dw), Pb (27-100.20 mg/Kg dw), Zn (91.4-427.20 mg/Kg dw). These levels were high and mostly above the recommended PNEC values in the soil, with elevated lead and mercury concentrations in the vegetables, though still within acceptable limits. But like all metals, bioaccumulation can result in continuous intake and pose serious health challenges and possible selection pressure for AMR and ARG development in the environment.

1.2.6 .4. Heavy metals from domestic activities

Other sources of heavy metals that are often ignored in the environment, are domestic activities, offices and school dust particles. Areas like school playgrounds, small-scale industries and offices are potential heavy metal pollution sources. For example, Olowoyo et al. (2016) investigated the presence of trace heavy metals in 32 dust samples and soil from selected high schools in Pretoria. The metals detected in the classrooms were

Cr (25 ± 0.65-97.3 ± 2.33 µg/g Mn (39.2 ± 1.40 - 430.2 ± 1.88 µg/g), Co (0.02 ± 0.00 - 27.8 ± 0.36 µg/g), Ni (10.6 ± 0.22-57.8 ± 1.12 µg/g), Cu (6.01 ± 0.13- 90.9 ± 0.97 µg/g), Zn (41.1 ± 0.01-313.2 ± 3.11 µg/g), As (0.09 ± 0.00-3.79 ± 0.02 µg/g), Cd (0.08 ± 0.01-2.48 ± 0.18 µg/g), Sb (0.05 ± 0.01-8.48 ± 0.08 µg/g), Pb (7.37 ± 0.02-25.7 ± 5.29 µg/g) and uranium (U) 0.16 ± 0.01-3.00 ± 0.01 µg/g).

Therefore, heavy metal pollution from all these sources is potentially dangerous to environmental biota. Most of the heavy metals were detected at very high concentrations, especially on the road and in mining tailings and could be transported into surface water, where their concentrations can exert selection pressure for AMR development. Their detection in vegetation and agricultural products is of great concern due to the ability of heavy metals to bioaccumulate and biomagnify along the food chain. Their presence in higher vertebrates is also a source of concern, as they can also exert selection pressure on bacteria for heavy metal resistance genes (HMRG) development and can trigger the co-selection for antibiotic resistance.

So, the presence of heavy metals in the environment directly impacts man via biomagnification along the food chain and contributes to AMR through selection pressure and indirectly through co-selection and cross-selection with antimicrobials in microorganisms. For example, the abundance of ARGs like *tet*M, *tet*W, $bla_{OXA,}$ and *erm*F positively correlates to the presence of environmental Cu. The level of Ni, Fe, Cr, and Pb is also associated with the abundance of *tet*M and *tet*W, while *ern*(B) abundance is negatively correlated to the levels of Pb, Zn and Fe (Knapp et al., 2011; Martínez and Rojo, 2011; Zhu et al., 2013). So, there is a need for more mitigation, reduction, or elimination of these chemicals in the environment, as they can bioaccumulate along the food chain, even when detected at sub-MIC levels and exert selective pressure towards AMR in bacteria.

1.2.7. Biocides in the Environment

Unlike antibiotics and heavy metals that are sometimes naturally occurring, biocides are preparations containing one or more active substances used directly or in formulations used to destroy, render harmless, prevent the action of, or otherwise exert a controlling effect on harmful organisms by either chemical or biological means (Chen et al., 2012). They vary and act as lethal or inhibitory agents against a range of targets, from plants to vertebrates and are used in different processes such as in hygiene maintenance as disinfectants (e.g., sodium hypochlorite in pools), in agriculture (propoxur, an ant poison) and as preservatives (warfarin, a rat poison) (Hernández-Moreno et al., 2019; Kähkönen et al., 2010; Kahrilas et al., 2015; Rasmussen et al., 1999).

As they are toxic preparations, anxiety exists when they are detected outside the target, and their effects are enormous, from killing useful organisms to distorting the population dynamics in the environment to exerting

selective pressure for resistance gene development and, subsequently, antibiotics resistance in non-target organism and even man. Biocides vary in composition, and new formulations are developed to improve their toxicity and reduce environmental impact. Some of the most widely used biocides presently are the quaternary ammonium compounds (QACs), alcoholic and phenolic compounds, aldehydes, halogen-containing compounds, quinolines and iso-quinoline derivatives, heterocyclic compounds and peroxygenase (Hernández-Moreno et al., 2019).

Biocides are ubiquitous in the environment due to their extensive usage. For example, 99% of all leave-on and 77% of rinse-off cosmetics contain parabens, and over 1.81 million Kg of N, N-dimethyl1-3-methylbenzamide (DEET) and 0.45 million Kg of triclocarban are used annually in the USA alone (Chen et al. 2012). Also, about 75% of the QACs used globally are found in WWTPs, and the rest (several thousands of kilograms) are directly released into the environment, thereby compounding the hazard for man and the ecosystem (Mc Cay et al., 2010).

Studies have shown that even though biocides are usually applied at very high concentrations (400 and 500 mg/L) to achieve a lethal effect, their mean concentration in effluent is mostly between 0.5-0.005 mg/L, which is below the PNEC limit, indicating an efficient treatment process in most WWTPs and a treatment efficiency rate of 90 to 95%. This concentration gradient could also be partly from dilution effects and the biodegradable nature of most biocides and their possible transformation into other components, making them easily removable in treatment plants (Li and Brownawell, 2010; Tezel and Pavlostathis, 2015; Zhang et al., 2015). However, although WWTPs effectively remove biocides, a considerable amount is still detected in surface waters, probably due to direct run-off from agricultural soils and sewage effluents. Nevertheless, even these very low concentrations detected in the environment are still a problem, as studies have shown that they are enough to induce selective pressure and facilitate the evolution of resistance to biocides in environmental bacteria and possible co-selection for antibiotics resistance (Hernández-Moreno et al., 2019; Rasmussen et al., 1999; Tezel and Pavlostathis, 2015).

Although studies have shown that antimicrobials are present in all parts of the environment, water, soil, sediments, vegetation and even food items, there are no agreements on the real acceptable concentrations, as even sub-MIC concentrations have been observed to exert selective pressure for AMR (Andersson and Hughes, 2012; Chow et al., 2021; Gullberg et al., 2011). Thus, divergent views exist on the relationship between antimicrobials and resistance. While some studies suggest some associations (Gerba, 2015), other studies suggest no clear relationship between them and resistance, rather inferring that what occurs is mostly antimicrobial tolerance due to improper concentrations and applications (Gadea et al., 2017). Furthermore,

Russell (2003) postulates that the effects of biocides are non-specific in target and usually utilise mostly efflux pump induction mechanisms and non-specific, while antibiotics targets are mostly specific. There were also no observed significant differences between the MIC of bacteria in areas where biocides were applied and those that were not (Gerba, 2015; Russell, 2003).

However, other studies disagree with the above arguing that exposure to QACs results in the development of resistance genes in microbes (Gadea et al., 2017). They have also noted that bacteria exposed to sub-inhibitory concentrations of biocides develop loss of membrane osmoregulation, respiratory enzymes' inhibition, protein motive force's dissipation, oxidative stress, increased efflux genes and pump activities, altered fatty acid composition and enhanced biofilm formation, which are indicative of enhanced resistance (Blázquez et al., 2012; Gadea et al., 2017). So, there is a need to investigate the development of AMR in bacteria, especially in environments with a fluctuating range of antimicrobial concentrations.

Some important heavy metal and biocides, their acceptable environmental limits and human health effects are given in Table 1.1.

Table 1.1. Selected heavy metals and biocides, their acceptable environmental limits and human health effects

CHEMICAL	**Acceptable limit (µg/L) SANS 241**	**EFFECT ON HUMAN HEALTH**	**REFERENCES**
Iron (Fe)	2000	Induces DNA damage and decreases repairs, can induce cancer, coronary diseases and myocardial infractions, and atherosclerosis.	(Eid et al., 2017; Jomova and Valko, 2011)
Copper (Cu)	2000	Can induce cancer and neurological disorders such as Alzheimer's disease, diabetes, cardiovascular diseases, and atherosclerosis.	(Jomova and Valko, 2011; Taylor et al., 2020)
Chromium (Cr)	50	Causes breathing problems such as Asthma and cough, skin ulcers, lung cancer, renal damage, reproductive system damage.	(Zhitkovich, 2011)

Cobalt (Co)	500	Can induce oxidative stress leading to DNA damages, inhibits DNA repairs, Asthma, pneumonia and wheezing and congenital heart disease.	(Jomova and Valko, 2011; Zhang et al., 2020)
Cadmium (Cd)	3	Can cause testicular damage and necrosis, as well as cancer and Itai-Itai disease	(Godt et al., 2006; Jomova and Valko, 2011; Rahimzadeh et al., 2017)
Arsenic (As)	10	Induces distortion of hypoxia signalling pathway, carcinogenic and can cause cardiovascular disorders.	(Fatoki and Badmus, 2022; Kaur et al., 2024; Muzaffar et al., 2023)
Lead (Pb)	10	Carcinogenic, anaemia, hypertension, urinary tract impairment, immunotoxicity and toxicity of the reproductive organs.	(Collin et al., 2022)
BAC	0.01	Increases bacteria tolerance and antibiotics resistance. Nasal cavity bleeding, pulmonary toxicity and inflammation and lung damage,	(Choi et al., 2020; Kwon et al., 2019)
Bromodichloromethane	60	Causes liver damage, kidney failure and decreased immune response	(Leavens et al., 2007)
ADBAC & DDAC	0.01	Can cause respiratory and gastrointestinal tracks disruption. It affects mitrochondria function and ATP production, leading to cell death, inflammation, Dema and excess mucus.	(Osimitz and Droege, 2022)

SECTION TWO: Effects of Environmental Antimicrobials on Antibiotics Resistance

1.2.8. Antibiotic tolerance in bacteria

Bacteria and other microorganisms have continuously adapted to adverse stressors from the natural environment and anthropogenic activities. These resistances are exhibited phenotypically (persistence, biofilms, resistance, and tolerance) and genotypically through resistance and tolerance gene formations (Pompilio et al., 2021).

Tolerance is not a recent phenomenon, as it was first advocated by Bigger in 1944, when he observed that after treatment with penicillin, viable bacteria cells could still grow from the plate, indicating that not all the cells were eliminated. He observed that these new cells, though unsusceptible to antibiotics, had the same MIC as the eliminated cells, indicating they were not mutants. These cells were called persisters, and the phenomenon termed tolerance (Balaban et al., 2013; Sulaiman and Lam, 2021). Thus, further studies have demonstrated that besides resistance, bacterial tolerance is a huge contributor to AMR, especially in chronic infections, such

as infections of catheters, orthopaedic devices, heart valves, urinary tract infections, and lungs of cystic fibrosis patients, particularly those involving biofilms (Balaban et al., 2013; Brauner et al., 2016; Sulaiman and Lam, 2019; Yan and Bassler, 2019).

Adverse factors like lack of nutrients and oxygen, and the accumulation of metabolic wastes, contribute to the development of these persister cells within the bacteria population. Besides the cells emanating due to adverse conditions, bacteria can stochastically generate these persister cells at a fixed rate in their populations, as a hedge population, to avoid eradication. Bergh et al. (2016) observed that 0.0001-0.1% of the bacteria population naturally switches from sensitive to a slow-/non-growing antibiotic-tolerant state, thereby trading viability for survival. These persister cells can survive adverse conditions, which will eliminate the normal cells and, with prolonged exposure, result in a persister cell-dominated population and a state of tolerance (Dhar and McKinney, 2007). The same was also observed when *E. coli* was subjected to cyclic daily exposures of ampicillin; insusceptibility occurred in the cells, and the acquired mutation extended their lag-phase (which is the phase before exponential growth is resumed, following a stationary phase). It was also observed that the culture could only be killed on growth resumption but remain unsusceptible as long as the tolerant strains were at the lag phase (Fridman et al., 2014; Mechler et al., 2015).

Most antimicrobials are designed to be very effective during active microbial growth, so non-/slow-growing bacteria tend to be unsusceptible to them. For example, Dhar and McKinney (2007) observed that the efficiency of most antibiotics is directly related to the growth rate of the bacteria, so if the bacteria are in the stationary phase, they become mostly unsusceptible to the antibiotics. So, antibiotics targeting growth processes are insignificant to these persister cells. The presence of these tolerant bacteria in a population, especially in the biofilm, is believed to be a major contributor to treatment failures. Biofilms are mostly refractive to antimicrobials due to their extracellular matrix, which prevents or limitedly allows the penetration of antimicrobials and the presence of these persister cells within the biofilm exacerbates the situation (Balcázar et al., 2015).

Tolerance is also the mechanism utilized by bacteria for antimicrobial insusceptibility at very high drug concentrations, which are far above the mutant prevention concentration (MPC) levels (such as biocides preparations in hospitals and treatment chemicals in WWTPs), as only these persister cells can survive. Since resistance mutations occur in microbial populations within the mutant selection window (MSW), any observed survival above MPC levels should be attributed to persistence and tolerance and may lead to the development of new resistant strains with a resultant extension of the resistance window (Levin-Reisman et al., 2017).

It has been observed that the evolution of persisters is inversely correlated to the duration of favourable conditions in their surrounding environment during treatment. So, the higher the unfavourable conditions, the

more the persister population developed. It was observed that persisters could adapt to antimicrobial treatment frequency, as the longer the unfavourable environment, the longer the lag phase of the persister bacteria (Bergh et al., 2016). Adverse conditions exert pressure for the activation of genetic programs that initiate the switch to persister cells, which down-regulates bactericidal activities like autolysis. (Cozens et al., 1989; Kussell and Leibler, 2005; Lewis, 2007).

According to Fridman et al. (2014), the greatest problem with persistence is selection of tolerance by lag (TBL) mutants. These TBL mutants are not drug-specific but can survive against a broad spectrum of stressors and drugs. They also observed that tolerance is time-dependent, and the time at which 99% of a culture is killed, is known as the MDK_{99} (minimum duration for killing 99%). This is opposed to resistance, which is concentration-dependent and is measured by the MIC, which is the concentration at which no growth is observed after 24 h of incubation. So, increased resistance will increase the MIC, while an increase in the tolerance will increase the MDK_{99}.

1.2.9. Antibiotic resistance and co-selection in bacteria

Antibiotics interrupt essential structures, including growth and reproduction, as well as resistance mechanisms in bacteria. As such, bacteria are either killed or stopped from multiplying. For antibiotics to be effective, they must be effectively introduced into the cells' interior, be able to avoid deactivation by the cells' intracellular enzymes, and must reach their target. So, any mechanism employed by bacteria to inhibit any of these activities makes the antibiotics ineffective and makes the bacteria resistant (Ghai and Ghai, 2018). However, bacteria have developed several antibiotic resistance mechanisms that aim to prevent or reduce access to the cells' cytoplasm for antibiotics, deactivate the antibiotics with their intracellular enzymes, reduce the concentration of antibiotics to an ineffective level in the intracellular fluid, and denature or prevent antibiotics from reaching their target site.

Two mechanisms are involved in controlling the effective concentration of antibiotics in bacteria: the efflux pump activities and the cell membrane activities. While the efflux pump controls the extrusion and expulsion of antibiotics out of the periplasm, the cell membrane functions through its lipid membranes and membrane proteins (OMP) (Alenazy, 2022; Sun et al., 2014). The bacterial cell membrane serves the dual purpose of protecting the cell and ensuring material exchange with the environment. While antibiotics and other toxic chemicals are removed from intracellular fluid through efflux pump activities to the extracellular environments, nutrients and signal molecules are fluxed across the membrane into the cell cytoplasm (Pagès et al., 2008). For most gram-negative bacteria, there are two membranes: a hydrophilic highly charged liposaccharide layer followed by a tight lipophobic layer with gate proteins known as porins. Hydrophobic antibiotics such as macrolides, diffuse across the lipid membrane while lipophilic antibiotics like β-lactams

utilize the porins to access the bacteria cytoplasm. So, specific changes in the bacterial membrane make it more difficult for antibiotics to adequately pass through, resulting in the bacteria being unsusceptible to the antibiotics (Winterhalter and Ceccarelli, 2015).

Efflux pump activities are another mechanism for antibiotic resistance in bacteria, first noted by McMurry et al. (1980). Efflux pumps are chromosomally encoded determinants, present in all known living cells (not just prokaryotes) and predates antibiotics use in man and agriculture (Blanco et al., 2016). There are five major classes of efflux pumps: adenosine (ATP)-binding cassette (ABC), superfamily, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS) and the multidrug and toxin compound extrusion (MATE) family. They use the proton motive force (except ABCs that utilize ATP hydrolysis as an energy source) to exude toxins, metabolites, and quorum sensory signals, out of the cell cytoplasm, thereby helping in cell homeostasis. They can be specific or exude multi-chemicals, such as two or more antimicrobials, contributing to AMR and cross-resistance (Blanco et al., 2016; Webber and Piddock, 2003).

Bacteria could enzymatically inactivate antibiotics. An example of such an enzyme is β-lactamase, which destroys penicillin's active component (the β-lactam ring). Bacteria can also modify the antibiotic by producing enzymes capable of adding different chemical groups to antibiotics, prohibiting binding between the antibiotic and its target in the bacterial cell. Furthermore, bacteria can also produce factors that break down antibiotics or change processes inside the cell to resist antibiotic action (Martínez and Baquero, 2014; Miller et al., 2014; Peterson and Kaur, 2018; Walsh, 2000).

Another mechanism involves camouflaging the target. This implies changing the composition or structure of the target in the bacterium resulting from mutations in the bacterial DNA. As such, the interaction between the antibiotic with the target is stopped. These target site alterations result from chromosomal changes induced by toxins such as antimicrobials. Most times, bacteria produce alternative proteins that could be used instead of those inhibited by antibiotics (Kyriakidis et al., 2021; Lambert, 2005; Peterson and Kaur, 2018). A typical example is the bacterium *Staphylococcus aureus,* which could acquire the resistance gene, *mecA*, and produce a new penicillin-binding protein. This protein is needed for bacterial cell wall synthesis and is the target of β-lactam antibiotics. The new penicillin-binding protein has a low affinity for β-lactam antibiotics and is thus resistant to the drugs, and the bacteria survive treatment. This resistance is the source of MRSA (methicillin-resistant *Staphylococcus aureus*) (Peterson and Kaur, 2018).

Some antibiotics target and destroy the bacterial cell wall. If a bacterium does not have a cell wall, the antibiotic will not affect it. This occurrence is known as intrinsic resistance, and when a bacterium previously susceptible

to an antibiotic evolves resistance, it is called acquired resistance (Martínez and Baquero, 2014; Miller et al., 2014; Peterson and Kaur, 2018; Walsh, 2000).

Heavy metals, biocides, and antibiotics have been effective over the years as antimicrobials, yet bacteria continue to develop resistance to them for survival. Microbial immunity to antimicrobials is exhibited as tolerance or resistance. Tolerance to an antimicrobial can be reversed by eliminating the stressor, but antimicrobial resistance is mostly permanent, even after the stress is removed (Keren et al., 2004). In the environment where these antimicrobials co-exist, their interactions should overlap, and bacteria need to evolve the ability to favour the selection of antimicrobial-resistant strains over the susceptible strains for survival (Squadrone, 2020). The primary genetic mechanisms leading to antimicrobial resistance are mutations and acquisitions of genetic materials intrinsically in the chromosomes or extrinsically from mobile genetic elements (MGEs), such as transposons and plasmids, via HGT. Resistance mutations manifest in reduced cell permeability (employed for Co, Ni, Cu, Zn, Al, Pb, meropenem, imipenem, chloramphenicol, gentamycin, ceftoprime and tetracycline resistance), over-expression of the efflux pumps (employed for Zn, Co, Cd, Ni, As and tetracycline resistance), alteration of the target site (employed for Hg, Zn, Cu, and carbapenem, ciprofloxacin, β-lactams, and trimethoprim-rifampicin resistance), or the expression of enzymes regulating antimicrobial deactivation (employed for As, Hg, gentamycin and β-lactams resistance) (Lin et al., 2015; Peterson and Kaur, 2018; Sarma et al., 2010).

Co-selection of resistance in bacteria occurs through two major processes: co-resistance and cross-resistance. Cross-resistance occurs mainly when a single resistance mechanism/morphological change (such as efflux actions and cell membrane changes) confers resistance to two or more different antimicrobials, e.g., the cross-resistance conferred by the DsbA-DsbB di-sulphide bond system, which affects five antibiotics and two heavy metals (Cd, Zn, β-lactams, kanamycin, erythromycin, novobiocin and ofloxacin), by the linkage of the max and cpc operon, leading to the efflux pump expression. But when two or more distinct genes present on the same MGE confer resistance to different compounds, it is termed co-resistance, e.g., multi-resistance to Ni and ampicillin in *Pseudomonas*, due to their resistance determinants being both physically present on plasmid pBC15 (Pal et al., 2017; Sarma et al., 2010).

Co-selection of resistance predates modern antimicrobial formulations. Dickinson et al. (2019), in their study of the palaeontological relationship between heavy metals and antibiotics in ancient environments, observed that ancient zinc-resistant isolates were positively associated with resistance to oxacillin, cefotaxime and trimethoprim. Furthermore, they noted that the zinc immunity mechanisms (such as reduced uptake due to cell wall modification, increased efflux activity, internal and external sequestration and transformation to less toxic forms) were also the same mechanisms utilised for antibiotic resistance, suggesting possible co-selection of

heavy metals and antibiotics in these ancient isolates. Therefore, they inferred that the co-selection of metal and antibiotic resistance predates modern antibiotics and industrialisation and seems to be a natural physiological mechanism bacteria employ for ecosystem homeostasis and enhanced survival.

1.2.9.1. Co-selection mechanisms

The environment, a reservoir of chemicals, including metals, biocides, and antibiotics, is a good matrix for co-selection. The association and possible selection of multi-resistance towards antimicrobials in different environments, e.g., surface water, wastewater, sediments, and domestic and agricultural soils, has been observed by studies in different parts of the world. According to Imran et al. (2019), the environment is a hotspot with different chemicals (e.g., antibiotics, heavy metals, biocides) and components such as micro-plastics, which can even become mini-ecosystems on their own and enhance the dissemination of resistance among distant but related microbes. For example, in an environment contaminated by heavy metals, long-term exposure of bacteria may result in the bioaccumulation of heavy metals such as Hg, Pb, Zn, Cu, and Cr to critical concentrations, which can promote heavy metal tolerance. These heavy metals can also induce antibiotic co-resistance, as the metal resistance genes may be present on the same genetic element as the ARGs. They also noted that these genes could be horizontally transferred to related bacteria in proximity, contributing to ARG proliferation and could also be transferred to related bacteria in other locations (even in distant environments) by movable components/mini ecosystems like micro-plastics, which abound in aquatic ecosystems. This same phenomenon was also observed by Fang et al. (2016), where the ST3-IncH12 plasmid, which harbours ARGs like the *bla* (β-lactams), *arm* (aminoglycoside), the *floR* (amphenicols) and *qnr* (quinolones) genes, as well as the HMRG, *pco* (copper) and *sil* (silver) genes. It also has the *oqx* efflux pump systems, which extrude the genes out of the bacteria cytoplasm, significantly contributing to HGT and dissemination of resistance genes into the surrounding environment and between *Enterobacteriaceae.* They also observed increased resistance to $CuSO_4$ and $AgN0_3$ among the isolates with the *pco* (copper resistance) and *sil* (silver resistance) genes on the IncH12 operon and a decreased susceptibility to β-lactams. In addition, they noted that the Tn-7-like transposon plays a role in transferring the *sil* and *pco* genes among *Enterobacteriaceae*.

Another mechanism is the expression of the *CpxAR* gene, which could upregulate the expression of the outer protein 5TM1530 and *ArcD* gene and the down-regulation of the outer protein *OmpC* and *ompD* genes, and also plays a very integral role in the aminoglycoside, ceftriaxone and β-lactam resistance in *Salmonella enterica* (Hu et al., 2011). The alteration of cell permeability facilitates resistance to heavy metals like As, Mn, Co, Cu, Ag and Zn. It is also one of the mechanisms bacteria utilise against chloramphenicol and tetracycline.

Lingzhi et al. (2018) noted that TCS-mediated alteration of cell proteins reduces cell membrane permeability and uptake of antibiotics and can result in cell damage.

1.2.9.2. Metal and antibiotics co-selection in water

The aquatic environment plays a major role, especially in the dissemination of wastes, and serves as a hotspot for ARG selection. As stated earlier, ESBL genes can be used as a bio-indicator for co-resistance, especially in the aquatic environment. River et al. (2020) studied the prevalence of ESBL-producing bacteria and the co-selection of ESBL genes and metal resistance genes from the highly polluted Yámana River in Delhi. They isolated 73 ESBL-producing bacteria and observed that they harboured the plasmid-mediated quinolone resistance (PMQR) genes, encoding β-lactams resistance. They also noted that 36.9% of them also harboured the silver resistance genes *silE*, *silP* and *silS*, and 60% harboured the *merB* gene. They observed co-existence and plasmid-mediated co-transfer of antibiotics and heavy metal resistance genes, indicating possible co-selection. A successful conjugation experiment showed that the trans-conjugants had antibiotic and metal resistance genes and similar immunity levels to their donors. This suggests that in heavily polluted aquatic environments, ESBL-producing bacteria may likely harbour both heavy metal resistance genes and ARGs on the same genetic elements, and the resultant microbial immunity is transferable between generations and among related species. This corroborated Ben Miloud et al. (2021), who, in their studies on the co-selection of silver and antibiotics in *K. pneumoniae*, isolates from hospital discharge and WWTPs in Tunisia, observed that all the isolates harboured the β-lactam and carbapenem resistance genes ($bla_{\text{CTX-M-15}}$, $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-1}}$), as well as the *si*lE gene, on the same plasmid, with a similar size to pMG101, indicating co-resistance.

Co-selection in the aquatic environment has also been affected by physical parameters such as water current and sediment depth. Manegabe et al. (2017) studied *Vibrio* spp. from sediments and water of the Kaliwa River in the Democratic Republic of Congo (DRC). They found a very strong association between the concentration of heavy metals in the environment and bacterial resistance and attributed this to the presence of both HMRG and ARG on the same plasmid. In addition, they observed an increased abundance of resistant bacteria from river water, which could be attributed to more rapid plasmid transport than in sediments (Nourhene et al., 2013).

1.2.9.3. Metal and antibiotics co-selection in agriculture

Metal and antibiotic co-selection has also been observed in agricultural environments. A study by Amachawadi et al. (2013), focusing on cattle fed with Cu^{2+}-infused feeds, observed an increased abundance of the transferable Cu resistance genes (*trcB*) in *Enterococcus* from faeces. Furthermore, they found that these *trcB*-

harbouring isolates also contained the *ermB* and *tetO* genes on the same conjugative Tn916/Tn1545 transposons, which is believed to contribute immensely to HGT among gut bacteria and a possible association between the Cu resistance genes and macrolide and tetracycline resistance in *Enterococcus* species. Similarly, Medardus et al. (2014) studied the effect of heavy metal micronutrients and their role in the persistence of multi-drug resistance in *Salmonella* from a swine farm. They observed relatively increased Cu^{2+} tolerance in faecal isolates compared to environmental isolates, suggesting possible gut selection for copper tolerance. In addition, they observed that over 60% of the faecal isolates were multi-resistant (the AmstTekm and AmClstSuTe strains) and that most of these MDR isolates also had the *pcoA* and *czcD* genes, associated with Zn and Cu tolerance, signifying a strong association between heavy metal and antimicrobial resistance. They attributed the co-selection to multi-resistance genetic determinants on the same genetic element.

Soils modified using metal-based fertilisers, such as Cu-derived compounds, are used in olive farms. Glibota et al. (2020), in their study of Cu2+-treated soil on an olive farm, detected high proportions of metal tolerance and β-lactam and tetracycline resistance genes. They also established an association between the *zntA* gene (responsible for the enzyme, Zn (11)-translocation P-type ATPase) and *tetC* gene, showing co-selection. Furthermore, they noted an association between the *copA* gene (responsible for Cu2+ tolerance) and the tetracycline resistance genes (*tetA, B, C, D*) and β-lactam resistance genes (bla_{PSE}, $bla_{CTX\text{-}M}$) and between the *czcD* and the tetracycline resistance genes (*tetC*). They attributed the associations to the coupled mechanism of resistance, such as the presentation of both copper resistance gene, *copA* and tetracycline resistance genes *tetA, B,* and *C* on the same plasmid and the activity of the cZeCBA and OprD porin proteins responsible for cell permeability, as well as the resistance modulated-cell division (RND) efflux pump activities. They inferred that Cu2+-infused soil, over time, becomes a source for ARG selection through co-selection and HGT of MGE.

1.2.9.4. Co-selection with biocides

Some studies have also noted biocide-antibiotics co-selection in bacteria. This is very troubling, considering the expansive use of biocides in homes and hospital environments. MRSA strains, when exposed to low biocide concentrations of dibromopropamidine isethionate (DBPI), chlorohexidine (CHX), cetylpyriridium chloride (CPC) and triclosan, exhibited low to high resistance to antibiotics gentamycin, kanamycin, tobramycin, and trimethoprim. It was observed that plasmid pSAS1 confers resistance to CHX and many antibiotics, while plasmid pSK1 confers co-resistance to the biocide QACs and the antibiotics, gentamycin, kanamycin, tobramycin, and trimethoprim. Also, pWG115, which encodes resistance to cationic surface-active agents, such as QACs and trimethoprim, can also confer resistance to gentamycin, and this happens when transposon Tn3851, from WG523 in MRSA, is transferred to pWG115 to generate an 18.0 megadalton pW53-like plasmid

that encodes gentamycin resistance, indicating an intrinsic mediated co-selection of resistance (Kampf, 2019; Suller and Russell, 1999; Townsend et al., 1984; Vazirian et al., 2019; Yamamoto et al., 1988).

The *qacA* and *qacB* genes have been reported to confer resistance to biocides (chlorohexidine, diamidines and other QACs) in MRSA. The genes are on the Psk1 plasmid, on which are also located other ARGs (*ermA, ermB, tetM* and *tetK*), which confers resistance to erythromycin and tetracycline and so contributes to the co-resistance of MRSA to both biocides and antibiotics. (Zmantar et al., 2012). As observed with heavy metals, co-selection in biocide is mostly mediated by cross-resistance mechanisms. Mann et al. (2019) studied the potential of cross-resistance in pathogens from a WWTP in Potchefstroom, South Africa. They detected biocide-resistant isolates at all sites, especially among the *Aeromonas*, *Pseudomonas*, *Bacillus* and *Klebsiella* species. The *P. aeruginosa* showed resistance to the biocides TCS and chloroxylenol (PCMX), and to antibiotics (vancomycin, amoxicillin, trimethoprim and oxytetracycline). They inferred that *P. aeruginosa* may have employed different mechanisms, such as enzyme ampC beta-lactamase and the membrane porin OprD. In *B. cereus*, MDR was exhibited towards TCS and PMCX, amoxicillin and trimethoprim through the Bur and Blt multidrug efflux pumps. Biocide-resistant *K. oxytoca* was also resistant to tetracycline, and they attributed this to the AcrAB efflux mechanism and Tolc-like protein. The detected biocide-resistant *Aeromonas* spp. were also resistant to trimethoprim due to the cassette-borne *drf*A and *drf*B resistance genes on the class1 operon.

Aulawi et al. (2018) observed increased resistance to amikacin in *P. aeruginosa* isolates exposed to biocides, especially those with reduced susceptibility to chlorhexidine gluconate (CHG). They attributed this to the multi-drug efflux systems, especially the MexAB-OpcM and MexCD-OprJ for CHG, and the MerEF-OprN and MexXY for aminoglycosides. They concluded that amikacin co-resistance must emanate from the expression of these different systems when *P. aeruginosa* is exposed to the biocide. They also observed a link in the reduced susceptibility to biocides in diverse bacteria species, such as *S. aureus*, *Listeria*, *Enterococcus*, *E. coli*, *Campylobacter* and *Salmonella*, as they employ similar mechanisms.

1.2.6.5. Co-selection in the agricultural and food industry

Biocides are extensively used in the meat industry, especially in slaughterhouses, to wash off blood and prevent the proliferation of bacteria and possible contamination of the meat. However, it is interesting to note that bacteria in slaughterhouses also exhibit insusceptibility to biocides and antibiotics. For example, Lavilla Lerma et al. (2015) observed that many *Pseudomonas* isolates from a slaughterhouse were immune to triclosan and multiple antibiotics. In addition, they observed an association in the resistance towards biocides and antibiotics, which they noted relied heavily on efflux pumps, such as the MexJK-opmH, MexAB-oprM, MexCD-OprJ, MexEF-OprN, triABC-OpmH and AcrABZ-TolC systems.

Sometimes, due to misapplication or dilution over time, biocides are greatly reduced to sub-inhibitory concentrations in most environments. A study by Molina-González et al. (2014) observed that *Salmonella enterica* isolates exposed to sub-inhibitory concentrations of biocides used in the food industry displayed decreased susceptibility to aminoglycosides and cephalosporins. Furthermore, they observed that the MDR could be attributed to efflux pump mechanisms like the AcrAB-TolC efflux pump utilised by QACs. They argued that the cross-resistance of bacteria induced by sub-inhibitory concentrations of biocides is strain-dependent, not species-based, and depends on the chemical involved.

Saleem et al. (2016) observed that CHD, which is extensively used in oral care products, can induce multidrug resistance in *Chryseobacterium cullis* and *Chryseobacterium indologenes* by upregulation of the Hly-D-like periplasmic adaptor protein of the CINOIS_RS05745 efflux pump gene, which facilitates multi-drug resistance to gentamycin, ampicillin, chloramphenicol, kanamycin and tetracycline. Interestingly, this non-specific efflux activity conferring co-selection of resistance was also observed by Guérin et al. (2021). They exposed *Listeria monocytogenes* to QACs and detected increased resistance to ciprofloxacin, which persisted in sub-cultures even in the subsequent absence of the biocides. They attributed this observation to the non-specific MdoL multidrug efflux system, which extrudes QACs, macrolides, cefotaxime, and heavy metals and the Lde efflux pump systems that export QACs, fluoroquinolones, acridine orange and ethidium. They also observed resistance of DDAC and BAC exposed strains to ciprofloxacin and attributed it to mutations affecting cell membrane permeability.

So, it could be argued that metal and biocide co-selection is more of tolerance than resistance, as the mechanisms utilised most times are non-specific and sometimes are not triggered primarily by the chemical but are stimulated as a reaction to adverse environmental conditions by the bacteria. So, co-selection can be argued to be more of a survival phenomenon than a mutation, and the likelihood of it being transferable is low even though it exists.

1.2.7. Mutant Prevention Concentration, Mutant Inhibition Concentration and Mutant Selection Window

Bacteria isolates are supposed to be susceptible to antimicrobials above the MIC, but microbial immunity has sometimes been observed in bacteria at concentrations above the MIC, up to a concentration value, where even the least susceptible cell is eradicated, and this is known as the mutant prevention concentration (MPC). The concentration range between the minimum inhibitory concentration (MIC) and the mutant prevention

concentration (MPC), is termed the mutant selection window (MSW), and it is within this range that bacteria can develop mutation to antimicrobials (Drlica and Zhao, 2007).

The mutant prevention concentration (MPC) is the lowest antimicrobial concentration that inhibits the growth of the least susceptible bacteria in a high-density population. It represents the threshold beyond which mutants of previously susceptible bacteria will rarely arise. This means organisms are expected to be completely eradicated beyond the MPC (Blondeau, 2009). Conversely, the MIC is the minimum antimicrobial concentration at which growth inhibition starts for susceptible bacteria after overnight incubation on the correct media. So, bacteria are not expected to survive at MIC, as the antimicrobial becomes bactericidal. But mutations can still occur, and this can be attributed to antimicrobial resistance, which is the ability of bacteria to survive bactericidal concentrations and retain their growth ability. Mutation must cease at MPC. So the concentration range that supports bacteria mutation beyond the MIC till it ceases at MPC is known as the MSW (Blondeau, 2009)

A major difference in the determination of MIC and MPC is the bacterial concentration. While the MIC is determined with a bacterial population of 10^5 CFU/mL, for the MPC, the bacterial population is $\leq 10^{10}$ CFU/mL, which represents the type of bacterial population concentration usually present during infection and in polluted environments like WWTPs and abattoirs. A study by Berghaus et al. (2013) suggested that adequate knowledge of the MPC can help predict and maintain the antimicrobial concentration high enough to prevent first-step mutation, thereby enhancing antimicrobial efficacy and eradicating bacteria (Blondeau, 2009; Drlica and Zhao, 2007).

The mutant selection window represents the danger zone within which resistant mutants can emerge, so the narrower this window, the better the chances of preventing resistance enrichment by the bacteria to antimicrobial, a propensity to selectively enrich for resistant mutants (Drlica and Zhao, 2007). This is very important, especially in the environment, as it will entail using very high concentrations above MIC at treatment plants. This will lead to the development of some resistant (persister) strains that can survive above the MPC and may lead to the need for higher concentrations, far above the MPC, to eradicate them and further extension of the MSW (Fridman et al., 2014). These mutants escalate environmental AMR, spreading to other bacteria of the same or similar species and ultimately to man. So, monitoring the treatment effectiveness is extremely important to reduce the propagation of resistant bacteria.

There should be greater remediation monitoring at WWTPs. The concentration of each antimicrobial treatment and the interval must be maintained at levels that must exceed the MIC (% > MIC) and up to the MPC (% ≥ MPC). The treatment interval can be estimated by plotting mean drug concentration against time (Berghaus et al., 2013). This is especially applicable to treatments with biocides such as CHG, which is widely used in

hospitals and food industries. Maintaining the biocide concentration above the MPC ensures that the CHG concentration remains bactericidal and can even eradicate the persistent cells (Kawamura et al., 2019). A study by Cui et al. (2006) observed that *S. aureus* lost susceptibility to levofloxacin when the drug concentration fluctuated between the upper and lower limits of the MSW, and they concluded that the antimicrobial concentrations must be above the window to prevent the emergence of resistant mutant subpopulations. Similarly, Zhao and Drlica (2002) observed that resistance selection could be reduced if the MSW is narrowed. They noted that the selection index (ratio of MPC to MIC) of moxifloxacin, a C-8 methoxy fluoroquinolone, was 1/3 of its C-8hydrogen cognate, Bay y3114, indicating that the addition of the methoxy group, narrowed the MSW and that the narrower the MSW, the more efficient the drug. Moreso, Liu et al. (2005) noticed that during treatment (focused on the drug's MIC) of S. *aureus* with rifampicin, there was the killing of susceptible pathogens and the selective amplification of resistant mutants. This means that the acquisition of resistance occurred simultaneously with the eradication of the susceptible strains. This could lead to the continued flourishing of the resistant strains within the population. Since the drug's effect would have been eliminated, the resistant strains become the dominant strains. Therefore, the MSW must be minimal to reduce or eliminate the selection of mutants.

So, in the environment, MSW provides the guideline for antimicrobial concentration, as it provides evidence that antimicrobial concentration must be beyond the MPC and outside the window to ensure that the resistant mutants are not selected at all. This is against the old postulations suggesting that concentrations should be kept above the MIC to eradicate mutation, as studies have confirmed, the concurrent acquisition of resistance and eradication of susceptible populations within the MSW (Berghaus et al., 2013).

The proliferation of ARG in the environment and their detection in treated wastewater infers WWTP removal inefficiencies, highlighting the need for improved removal processes. Some current solutions include the pre-treatment hydrolysis technology with acid/base catalyst, hydrothermal treatment of fermentation by products of antibiotics manufacturing, membrane bioreactors, vermifiltration, advanced oxidation processes and membrane filtration processes.

SECTION THREE: Conclusion

1.2.8. Environmental factors and Antimicrobial Resistance

The rampant and indiscriminate use of antibiotics, coupled with their subsequent release into the environment, could be argued to be the leading cause of accelerated resistance in environmental bacteria. Like most PPCPs, antibiotics have many routes of entering the environment, ranging from the direct discharge of active pharmaceutical ingredients during manufacturing (which in most cases results in very high concentrations) to

the excretion of residues by humans and animals (which are mostly at concentrations lower than few ng/L), as well as the disposal of unused medications (Sarmah et al., 2006). Even at very low concentrations, persistent antibiotics in the environment promote increased proliferation of drug-resistant bacteria and ARGs, suggesting that antibiotics released into the environment are a significant contributor to the emergence and maintenance of resistance, highlighting the need for measures to reduce anthropogenic antibiotic pollution (Martinez, 2009; Pruden et al., 2018). While high concentrations could have acute effects on bacteria, sub-lethal antibiotic concentrations could easily alter microbial communities and population structures in nature and subsequently affect the ecology (Hirsch et al., 1999).

Water constitutes a major pathway for disseminating and proliferating antimicrobial resistance in the environment. It serves as the reservoir and channel for introducing antimicrobial materials to otherwise pristine habitats. The presence of antibiotics and other sources of antimicrobials such as detergents, disinfectants, and heavy metals in water bodies, combined with other environmental factors like dissolved oxygen and turbidity, has been observed to lead to the proliferation of the environmental resistomes (Baquero et al., 2008; Fletcher, 2015).

Temperature and salinity are some factors that also affect resistance in the environment. It has been observed that marginal increases in temperature, like 1 ºC, can be associated with a 1.14-fold and 1.06-fold increase in the growth of *K. pnuemoniae* and *P. aeruginosa* and increases ARG abundance and HGT rate in bacteria (Burnham, 2021; Li et al., 2023). Salinity also affects ARG abundance, but unlike temperature, an increase in salinity decreases the growth rate and ARG abundance of bacteria. It has even been suggested that NaCl can be used in WWTPs to reduce ARG abundance (Liu et al., 2018).

In the environment, unlike the laboratory, one of the major factors affecting the study of the effects of antibiotics is their ability to become contaminant mixes. In this complex mixture of contaminants, as seen in nature, presence does not necessarily infer effect, and determining effect is a very hard task. Nevertheless, the relationship between the exposure and effect of each contaminant in the environment on the dynamics of antibiotic resistance should provide the basis for ecological risk assessment modelling. Thus, chemical analysis, biomonitoring and microbiological assessments must always be employed (Oost et al., 2016). In addition, antibiotics tend to form complexes with other antibiotics and other elements, such as heavy metals and biocides in the environment. These complexes tend to have a synergistic effect through cross-resistance or collateral sensitivity, which can alter resistance, especially in the environment (Chait et al., 2012).

Antibiotics' presence in the environment, which is not the target, coupled with the innate ability of bacteria to elicit genetic changes for survival, ultimately increase ARGs, thus making the environment not just a dispersal

route but also a reservoir of resistant pathogens (Bengtsson-Palme et al., 2018; D'Costa et al., 2011; Forsberg et al., 2012; Larsson et al., 2018; Poirel et al., 2010; Taylor et al., 2011; Wellington et al., 2013).

Although the environment has been identified as a major reservoir and source of resistance genes, the mechanisms for developing resistance still need more understanding. Most times, antibiotic concentrations above the MIC value should elicit the selection of resistance genes, but it has also been observed (especially in complex conditions like the environment) that resistance genes are elicited even below the MIC (sub-MIC concentrations) (Andersson and Hughes, 2012). The mechanism for this keeps evolving with more studies, rendering mitigating strategies useless (Andersson and Hughes, 2012; Gullberg et al., 2014, 2011; Lee et al., 2017; Li et al., 2017). When exposed to antimicrobials at low concentrations in the environment, especially at sub-MIC values, bacteria have been observed to exhibit responses stimulated by both the concentration of the antibiotics and the environmental factors present. The selection pressure is determined by their concentration, chemical speciation (the distribution within the matrix, its toxicity, bioavailability, environmental fate and transport within the environment), period of exposure and the condition prevalent for bacterial growth (Baquero et al., 2009). Below MIC, factors like bioaccumulation, prolonged exposure and other environmental factors (such as pH, temperature and presence of other chemicals), the development of genetic mutations could trigger resistance against therapeutic agents (Gullberg et al., 2011).

Studies have also shown that the minimum selective concentration of antimicrobials, especially in complex systems like the environment, usually varies compared with laboratory studies, as the environment is subjected to extraneous conditions that it cannot control, unlike the laboratory with controlled parameters. (Quinlan et al., 2011). So, there is an urgent need to re-examine the predictive no-effect concentration (PNEC) and the emission limits for antimicrobials, as failure to do so will aggravate the already serious problem of ARG proliferation. Results of previous studies indicate that the PNEC depends not just on the concentration of the antibiotics but also on other environmental factors (Khan et al., 2017; Lee et al., 2017; Tello et al., 2012). These environmental stressors include physical and chemical parameters, such as temperature, turbidity, and pH, which impact the determination of water quality. In contrast, other factors, like the soil sorption and photodegradation of antibiotics in the environment, all affect the antibiotics' concentration detected in the environment. For example, in Kenya, it was observed that even though sulfamethoxazole and trimethoprim were administered together as co-trimoxazole at a mass ratio of 1:5 and excreted unchanged by humans, their detected concentrations in the water were at a ratio of 3:10, due to 99% photolytic degradation of trimethoprim (Abellán et al., 2009; Berendonk et al., 2015; Bergheim et al., 2010; K'oreje et al., 2012). Although these hypotheses have been advanced, minimal studies have been conducted, especially in Africa, to demonstrate the effects of concentrations and duration of antibiotic exposure required to induce antimicrobial resistance in the environment.

Environmental bacteria seem to have evolved a co-existence mechanism with ARGs in the environment, unlike what is obtained in clinical ARG developments, which shows a greater proliferation of ARGs. These phenomena can be attributed to the following factors: whereas antibiotics in the body are easily discharged and washed off, those in the environment persist over extended periods and, eventually, may decay into other components, which can be more toxic to previously susceptible bacteria. For example, tetracyclines break down in the environment into anhydrotetracycline, diminishing *tet*A-mediated tetracycline resistance and potentially resulting in tetracycline sensitivity (Markley and Wencewicz, 2018; Park et al., 2017).

Other factors that might be contributing to the maintenance of the ecological balance in the environment include the ability of antibiotics and toxins to form complexes with other chemicals in the environment and the ability of some bacteria to produce resistance suppressants (such as β-lactamase inhibitors and similar toxins), that can inhibit other bacterial populations (Chait et al., 2012).

Antibiotic resistance may have been developed for metabolic purposes or signal trafficking (physiological cell messaging using intracellular chemicals) within the bacterial community for survival. These can occur at low antibiotic concentrations and could lead to the emergence of transient resistant populations in the microbial community, generating a pool of resistance genes with gene-transfer units that can spread in nature, thus directly affecting the environmental microbiota and human health if ignored (Martinez, 2009).

Antimicrobial resistance genes are considered biological markers for survival by bacteria. Therefore, approaching and testing the effect of biomarkers of effect, which is the resistance gene acquisition, should be looked at from the point of the combined effect of the antibiotics and the other potential environmental antimicrobials, such as biocides and heavy metals. In addition, some physicochemical parameters, such as pH and salinity, must also be considered, which may play significant roles in the process, especially in the field.

According to Larsson et al. (2018), the four areas that should be considered in studies of environmental bacterial resistance and the proliferation of ARGs in the environment, which could be formatted to suit different studies, include:

1. The contributory roles of different environmental sources in antimicrobials and antibiotic-resistant bacteria into the environment (this could be observed at a WWTP ecosystem from 3 sources viz, upstream, discharge point and downstream of the WWTP).

2. the anthropogenic inputs and their contribution to the evolution of resistance (which could be observed by the possible effects of the stressors, heavy metals, biocides and antibiotics residues on the ARG development)

3. the possible health impacts caused by exposure to resistant bacteria from the environment (which could be observed by the antibiotics resistance profile of the bacteria from the ecosystem), and,

4. the efficacy of technological, social, economic, and behavioural interventions that can be implemented to mitigate against environmental antibiotic resistance (which could be observed by the efficiency of the WWTP in keeping the stressors within acceptable limits and the possible mitigating recommendations from the results from 1-3 above).

It is, therefore, imperative to understand the types and concentrations of stressors (in this study, heavy metals, biocides, and antibiotics residues) from different sources within the studied system, their routes of entry and exposure patterns, and path for action (which includes effects of exposure and any other factors that may aid bacterial growth and genetic expressions). In addition, there is also a need for knowledge of the development, diversity, characteristics and natural variability of the resistance genes and their mobile genetic materials (Ashbolt et al., 2013; Hunter et al., 2008; Larsson et al., 2018; Manaia, 2017; Pruden et al., 2013; Zhu et al., 2017).

So, it can be inferred that ARGs abound in nature but have increased due to the synthesis and disposal of synthesized and natural antimicrobials into the environment. These antimicrobial residues, combined with other environmental stressors, such as heavy metals, biocides, temperature, and pH, have contributed to increased antibiotic resistance in ways that need to be understood better. As mentioned earlier, the environment is a huge pot pouri and understanding the total effects of these stressors should come from understanding their individual and collective effects. This study, therefore, investigated how the presence and variable concentration of some heavy metals, biocides and antibiotic residues in the environment contribute to the dynamics of resistance, investigating their effect on the development of resistance and tolerance and their effect on the MSW of *E. coli* to selected antibiotics.

1.3. Aims of the research

1. To investigate the effects of environmental concentrations of selected antibiotics, heavy metals, and biocides on the dynamics of antibiotic resistance development in previously susceptible *E. coli*.

1.4. Objectives of the research

The specific objectives of the research were:

Determine the presence of selected environmental stressors, viz antibiotics, biocides and heavy metals, and their respective concentrations from water samples collected from a WWTP effluent and upstream and downstream from its receiving river.

1. Determine the antibiotic resistance profiles of *E. coli* from the WWTP and its receiving river.

2. Determine, via 30-day exposure experiments, the effect of environmental concentration of the selected antibiotics, biocides, and heavy metals on the development of ARGs in previously susceptible *E. coli* (ATCC 25922) strain phenotypically by AST and genotypically using WGS and SNV.

3. Determine the effect of 30-days exposure to selected stressors on the development of persistence and tolerance in exposed isolates, compared to previously susceptible *E. coli* (ATCC 25922) strain.

4. Determine the effect of 30-days exposure to selected stressors on the mutant selection window of exposed isolates compared to previously susceptible *E. coli* (ATCC 25922) strain.

1.5. Overview of the study design and methodology

1.6. Ethical considerations

This study was part of a larger project for which ethical approval had been received from the Biomedical Research Ethics Committee (Reference: BCA444/16) of the University of KwaZulu-Natal. The study is also recorded at the South African National Department of Agriculture, Forestry, and Fisheries (Reference: 12/11/1/5 (879).

1.7 General methodology

1.7.1. Field sample collection, analysis, and antimicrobial susceptibility test (AST)

Water samples were collected from 3 points, upstream, discharge point and downstream of a WWTP in KwaZulu-Natal and its receiving water body, in February and March 2020. The water samples were analysed for selected antibiotics, heavy metals, and biocide residues and the presence and antibiotic sensitivity of *E. coli* in the samples. Isolation and enumeration of *E. coli* were carried out using the Colilert®-18/Quanti-Tray® 2000 system (IDEXX, Maine USA). Ten randomly picked positive wells from the Colilert®-18/Quanti-Tray® 2000 system were streaked on Eosin Methylene Blue agar (Oxoid, Hampshire, England). AST was done by disc diffusion methods using the CLSI/EUCAST procedure (CLSI, 2017; EUCAST, 2022).

1.7.2. Laboratory exposure to determine ARG development

Fresh *E. coli* ATCC 25922 strains were exposed in the laboratory to sub-MIC concentrations of six chosen residues, two antibiotics (amoxicillin:2000, 136.38, 73.41 and 0.25 µg/L and oxytetracycline: 250, 37.84, 27.92 and 18 µg/L), two heavy metals (copper: 512000, 7, 1 and 2 µg/L and zinc: 512000, 78, 14 and 0.05 µg/L) and two biocides (BAC 12: 4000, 2.42, 1.24 and 0.01 µg/L and DADMAC 10: 16000, 0.83, 0.42 and

0.001 µg/L), and a combination of all the six residues, over 30 days. Fresh *E. coli* ATCC 25922 culture (10^6 CFU/mL) was placed in 1 mL Eppendorf tube containing a sub-MIC concentration of residue in Muller Hinton (MH) broth and incubated at 37 ºC for 24 h. After 24 h, 100 uL of the zero-day culture was transferred to a fresh tube containing the same sub-MIC concentration of residues in MH Broth for a second day and incubated as previously. Another 100 uL of the same zero-day overnight culture was plated on nutrient agar, incubated, and stored at -20 ºC in tryptic soy broth (Merck, Darmstadt, Germany) supplemented with glycerol for further experiments, AST and DNA analysis. This was repeated over 31 days to obtain 30-day exposure cultures.

The isolates were subjected to AST to determine if they developed phenotypic resistance. *E. coli* isolates were tested against a panel of 20 antibiotics as previously described (O'Halloran et al., 2018) using the disc diffusion method according to CLSI/EUCAST guidelines. Muller-Hinton medium was prepared according to the manufacturer's instructions, and plates were poured to achieve coverage. On each plate, 0.5 McFarlan isolates were inoculated using a micro-pipette and evenly spread using a cotton swab. Antibiotic discs were then applied using disc dispensers; a maximum of five antibiotic discs were placed on each plate, and incubated for 18-24 h at 36 ºC. Zone diameters were measured, and results were interpreted according to set values on the guidelines (CLSI, 2017; EUCAST, 2022).

1.7.3.DNA extraction

DNA was extracted and subjected to WGS on an Illumina MiSeq Machine (Illumina, San Diego, California, USA). All resultant sequences were analysed using bioinformatic pipelines previously described (Amoako et al., 2019) to determine the presence of any resistance or tolerance genes.

1.7.4. Laboratory exposure test for tolerance

The exposed 30th-day isolates were further exposed to a very high dose of ampicillin (25 x MIC) to determine the minimum duration to kill 99.99% ($MDK_{99.99}$) of the isolates, which is a measure of their tolerance. The stored 30th-day exposure sample was grown overnight in LB broth Lennox (LBL, Neogen Lasing Michigan USA) in 1 mL vials for a very concentrated culture. The next day, 1 mL of the 1 x 10^9 cultures was inoculated into a 50 mL LBL supplemented with 100g/mL ampicillin (about 25 times the *E. coli* ampicillin MIC value). Each culture mixture was prepared in 2 x triplicate sets for three different time intervals (T_a = 3, 5, and 8 hours), and the culture mixture was then incubated at 37 ºC in a shaker incubator at 200 rpm.

Depending on the time intervals (3, 5 and 8 h), the culture was brought out after the incubation and washed twice in 50 mL of LBL by centrifuging at 1400 g for 10 minutes. The pellets were resuspended in 1 mL of LBL and grown again for up to 24 h (3 h for 21, 5 h for 19 and 8 h for 16, respectively) at 37 ºC in a shaking incubator at 200 rpm. This enabled us to obtain fresh 24 h culture for the next day's exposures. After 24 h, the

plate is cell counted and recorded. The overnight culture was then subjected to the same exposure over three consecutive days, using the same cyclic periods.

For the confirmation of tolerance and not resistance, the surviving *E. coli* cells were subjected to MIC testing to confirm if there were changes in their MICs compared to the original stock (Fridman et al., 2014).

1.7.5. Laboratory test for mutant selection window

The MIC and MPC (MIC at $\geq 10^{10}$ CFU/mL) of the exposed isolates to amoxicillin, oxytetracycline and ampicillin, and the effect of the 30-day exposure on the mutant selection window was determined. Two-fold serial dilutions infused with test agents (ampicillin, amoxicillin and oxytetracycline) were dispensed into microdilution plates. With a sterile pipette, *E. coli* isolate suspensions were inoculated into the microdilution plates containing serially diluted antibiotics and incubated at 37 ºC for 24 h. Microdilution plates were then read and interpreted after 24 h. To obtain a bacteria culture of $\geq 10^9$ CFU/mL for MPC, an overnight culture of pure, wholly susceptible *E. coli* isolate was cultured on a nutrient agar plate in duplicate and incubated for 24 h at 37 ºC. After 24 h, the cultures in the nutrient agar plate were transferred into a 50 mL nutrient broth using a sterile swab and incubated for another 24 h at 37 ºC to achieve a bacteria density of $\geq 10^9$ CFU/mL. To ensure that the acceptable density was obtained, the inoculated broth was centrifuged at 3000 rpm for 20 mins, and the pellets were resuspended in a fresh, smaller volume of nutrient broth.

1.8. Outline of the thesis

The study is presented in the following five chapters:

Chapter 1. Introduction and literature review

This chapter provides a comprehensive background and the aim and objectives of the study

Chapter 2. Manuscript 1: Determining the abundance of antibiotic, heavy metal and biocide residues and antibiotic-resistant *E. coli* isolates in a wastewater treatment plant and its receiving water body.

This chapter describes the water sample residues and their concentrations. It also describes the presence and antibiotic resistance profile of the *E. coli* from the sample sites and addresses objectives 1 and 2.

Chapter 3. Manuscript 2: Environmental concentrations of antibiotics, biocides, and heavy metals fail to induce phenotypic antimicrobial resistance in *Escherichia coli*.

This chapter describes the effect of laboratory exposure to sub-MIC concentrations of antimicrobial residues, determined from earlier from the field to a wholly susceptible *E. coli* ATCC 25922 strain. This is to determine,

if 30-exposure exposure to determined sub-MIC concentrations of these anti-microbials, were able to elicit phenotypic or genotypic resistance & ARG development and addresses Objective 3.

Chapter 4. Manuscript 3: Impact of environmental sub-inhibitory concentrations of antibiotics, heavy metals, and biocides on the emergence of tolerance and effects on the mutant selection window in *E. coli.*

This chapter describes the determination of antibiotic tolerance and persistence development amongst the 30 days exposed isolates compared to the control and also the effect of the 30-day exposure on the mutant selection window (MSW) of three chosen antibiotics and addresses Objective 4 and 5.

Chapter 5. Conclusion, Limitations and Recommendations

This chapter summarises the research results, highlights the limitations and presents recommendations for future work.

1.9. REFERENCES

Abellán, M.N., Giménez, J., Esplugas, S., 2009. Photocatalytic degradation of antibiotics: The case of sulfamethoxazole and trimethoprim. Catal. Today 144, 131–136.

Agersø, Y., Bjerre, K., Brockmann, E., Johansen, E., Nielsen, B., Siezen, R., Stuer-Lauridsen, B., Wels, M., Zeidan, A.A., 2019. Putative antibiotic resistance genes present in extant Bacillus licheniformis and Bacillus paralicheniformis strains are probably intrinsic and part of the ancient resistome. PLoS One 14, 1–24.

Alanis, A.J., 2005. Resistance to antibiotics: are we in the post-antibiotic era? Arch. Med. Res. 36, 697–705.

Alenazy, R., 2022. Drug efflux pump inhibitors: A promising approach to counter multidrug resistance in gram-negative pathogens by targeting AcrB protein from AcrAB-TolC multidrug efflux pump from Escherichia coli. Biology. 11, 1328.

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J., Handelsman, J., 2010. Call of the wild: antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. 8, 251–259.

Amachawadi, R.G., Scott, H.M., Alvarado, C.A., Mainini, T.R., Vinasco, J., Drouillard, J.S., Nagaraja, T.G., 2013. Occurrence of the transferable copper resistance gene tcrB among fecal Enterococci of U.S. feedlot cattle fed copper-supplemented diets. Appl. Environ. Microbiol. 79, 4369–4375.

Amoako, D.G., Somboro, A.M., Abia, A.L.K., Allam, M., Ismail, A., Bester, L.A., Essack, S.Y., 2019. Whole-genome shotgun sequence of drug-resistant Staphylococcus aureus Strain SA9, isolated from a slaughterhouse chicken carcass in South Africa. Microbiol. Resour. Announc. 8, 9–11.

Andersson, D.I., Hughes, D., 2012. Evolution of antibiotic resistance at non-lethal drug concentrations. Drug Resist. Updat. 15, 162–172.

Arsène-Ploetze, F., Chiboub, O., Lièvremont, D., Farasin, J., Freel, K.C., Fouteau, S., Barbe, V., 2018. Adaptation in toxic environments: comparative genomics of loci carrying antibiotic resistance genes derived from acid mine drainage waters. Environ. Sci. Pollut. Res. 25, 1470–1483.

Ashbolt, N.J., Amézquita, A., Backhaus, T., Borriello, P., Brandt, K.K., Collignon, P., Coors, A., Finley, R., Gaze, W.H., Heberer, T., Lawrence, J.R., Larsson, D.G.J., McEwen, S.A., Ryan, J.J., Schönfeld, J., Silley, P., Snape, J.R., Van den Eede, C., Topp, E., 2013. Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. Environ. Health Perspect. 121, 993–1001.

Aulawi, K., Junko, N., Okada, S., 2018. Relationship between resistance to antibiotics and insusceptibility to biocides of Staphylococcus aureus and Pseudomonas aeruginosa isolated in Indonesian hospitals. Int. J. Res. Med. Sci. 6, 1890.

Azanu, D., Styrishave, B., Darko, G., Weisser, J.J., Abaidoo, R.C., 2018. Occurrence and risk assessment of antibiotics in water and lettuce in Ghana. Sci. Total Environ. 622–623, 293–305.

Bagnis, S., Boxall, A., Gachanja, A., Fitzsimons, M., Murigi, M., Snape, J., Tappin, A., Wilkinson, J. and Comber, S., 2020. Characterization of the Nairobi River catchment impact zone and occurrence of pharmaceuticals: Implications for an impact zone inclusive environmental risk assessment. Sci Total Environ. *703*, p.134925.

Bakare, B.F., Adeyinka, G.C., 2022. Evaluating the potential health risks of selected heavy metals across four wastewater treatment water works in Durban, South Africa. Toxics 10, 340.

Balaban, N.Q., Gerdes, K., Lewis, K., McKinney, J.D., 2013. A problem of persistence: Still more questions than answers? Nat. Rev. Microbiol. 11, 587–591. https://doi.org/10.1038/nrmicro3076

Balcázar, J.L., Subirats, J., Borrego, C.M., 2015. The role of biofilms as environmental reservoirs of antibiotic resistance. Front. Microbiol. 6, 1–9.

Baltz, R.H., 2008. Renaissance in antibacterial discovery from actinomycetes. Curr. Opin. Pharmacol. 8, 557–563.

Baquero, F., Alvarez-Ortega, C., Martinez, J.L., 2009. Ecology and evolution of antibiotic resistance. Environ. Microbiol. Rep. 1, 469–476.

Baquero, F., Martínez, J.L., Cantón, R., 2008. Antibiotics and antibiotic resistance in water environments. Curr. Opin. Biotechnol. 19, 260–265.

Baquero, F., Negri, M., Morosini, M., Blázquez, J., 1998. Antibiotic-selective environments. Clin. Infect. Dis. 27, S5–S11.

Ben Miloud, S., Ali, M.M., Boutiba, I., Van Houdt, R., Chouchani, C., 2021. First report of cross resistance to silver and antibiotics in Klebsiella pneumoniae isolated from patients and polluted water in Tunisia. Water Environ. J. 35, 730–739.

Bengtsson-Palme, J., Hammarén, R., Pal, C., Östman, M., Björlenius, B., Flach, C.F., Fick, J., Kristiansson, E., Tysklind, M., Larsson, D.G.J., 2016. Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. Sci. Total Environ. 572, 697–712.

Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J.J., 2018. Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiol. Rev. 42, 68–80.

Bengtsson-Palme, J., Larsson, D.G.J., 2015. Antibiotic resistance genes in the environment: prioritizing risks. Nat. Rev. Microbiol. 13, 396–396.

Bengtsson-Palme, J., Larsson, D.G.J.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. Environ. Int. 86, 140–149.

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance: The environmental framework. Nat. Rev. Microbiol. 13, 310–317.

Bergh, B. Van Den, Michiels, J.E., Wenseleers, T., Windels, E.M., Boer, V., Kestemont, D., Meester, L. De, Verstrepen, K.J., Verstraeten, N., Fauvart, M., Michiels, J., 2016. Frequency of antibiotic application drives rapid evolutionary adaptation of Escherichia coli persistence. Nat. Microbiol. 1, 1–7.

Berghaus, L.J., Giguère, S., Guldbech, K., 2013. Mutant prevention concentration and mutant selection window for 10 antimicrobial agents against Rhodococcus equi. Vet. Microbiol. 166, 670–675.

Bergheim, M., Helland, T., Kallenborn, R., Kümmerer, K., 2010. Benzyl-penicillin (Penicillin G) transformation in aqueous solution at low temperature under controlled laboratory conditions. Chemosphere 81, 1477–1485.

Bhullar, K., Waglechner, N., Pawlowski, A., Koteva, K., Banks, E.D., Johnston, M.D., Barton, H.A.,

Wright, G.D., 2012. Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS One 7, 1–11.

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, 1–19.

Blázquez, J., Couce, A., Rodríguez-Beltrán, J., Rodríguez-Rojas, A., 2012. Antimicrobials as promoters of genetic variation. Curr. Opin. Microbiol. 15, 561–569.

Blondeau, J.M., 2009. New concepts in antimicrobial susceptibility testing: The mutant prevention concentration and mutant selection window approach. Vet. Dermatol. 20, 383–396.

Blondeau, J.M., Zhao, X., Hansen, G., Drlica, K., 2001. Mutant Prevention Concentrations of Fluoroquinolones for Clinical Isolates of Streptococcus pneumoniae 45, 433–438.

Bougnom, B.P., Thiele-Bruhn, S., Ricci, V., Zongo, C., Piddock, L.J.V., 2020. Raw wastewater irrigation for urban agriculture in three African cities increases the abundance of transferable antibiotic resistance genes in soil, including those encoding extended spectrum β-lactamases (ESBLs). Sci. Total Environ. 698, 134201.

Boy-Roura, M., Mas-Pla, J., Petrovic, M., Gros, M., Soler, D., Brusi, D., Menció, A., 2018. Towards the understanding of antibiotic occurrence and transport in groundwater: Findings from the Baix Fluvià alluvial aquifer (NE Catalonia, Spain). Sci. Total Environ. 612, 1387–1406.

Brauner, A., Fridman, O., Gefen, O., Balaban, N.Q., 2016. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat. Rev. Microbiol. 14, 320–330.

Burnham, J.P., 2021. Climate change and antibiotic resistance: a deadly combination. Ther. Adv. Infect. Dis. 8, 1–7.

Bvenura, C., Afolayan, A.J., 2012. Heavy metal contamination of vegetables cultivated in home gardens in the Eastern Cape. S. Afr. J. Sci. 108, 1–6.

Chait, R., Vetsigian, K., Kishony, R., 2012. What counters antibiotic resistance in nature? Nat. Chem. Biol. 8, 2–5.

Chen, Z.F., Ying, G.G., Lai, H.J., Chen, F., Su, H.C., Liu, Y.S., Peng, F.Q., Zhao, J.L., 2012. Determination of biocides in different environmental matrices by use of ultra-high-performance

liquid chromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 404, 3175–3188.

Choi, H.Y., Lee, Y.H., Lim, C.H., Kim, Y.S., Lee, I.S., Jo, J.M., Lee, H.Y., Cha, H.G., Woo, H.J., Seo, D.S., 2020. Assessment of respiratory and systemic toxicity of Benzalkonium chloride following a 14-day inhalation study in rats. Part. Fibre Toxicol. 17, 1–19.

Chow, L.K.M., Ghaly, T.M., Gillings, M.R., 2021. A survey of sub-inhibitory concentrations of antibiotics in the environment. J. Environ. Sci. 99, 21–27.

Clarke, B.O., Anumol, T., Barlaz, M., Snyder, S.A., 2015. Investigating landfill leachate as a source of trace organic pollutants. Chemosphere 127, 269–275.

CLSI, 2017. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; twenty-seventh informational supplement. CLSI Document M100-S27., Performance Standards for Antimicrobial Susceptibility Testing. 27th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2017.

Collignon, P., Voss, A., 2015. China, what antibiotics and what volumes are used in food production animals? Antimicrob. Resist. Infect. Control 4, 10–13.

Collin, M.S., Venkatraman, S.K., Vijayakumar, N., Kanimozhi, V., Arbaaz, S.M., Stacey, R.G.S., Anusha, J., Choudhary, R., Lvov, V., Tovar, G.I., Senatov, F., Koppala, S., Swamiappan, S., 2022. Bioaccumulation of lead (Pb) and its effects on human: A review. J. Hazard. Mater. Adv. 7, 100094.

Costa, V.M.D., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N., Wright, G.D., 2011. Antibiotic resistance is ancient. Nature 477, 457–461.

Cozens, R.M., Markiewicz, Z., Tuomanen, E., 1989. Role of autolysins in the activities of imipenem and CGP 31608, a novel penem, against slowly growing bacteria. Antimicrob. Agents Chemother. 33, 1819–1821.

Cruz, D.C.B., Santana, L.L., Guedes, A.S., De Souza, J.T., Marbach, P.A.S., 2019. Different ways of doing the same: Variations in the two last steps of the purine biosynthetic pathway in prokaryotes. Genome Biol. Evol. 11, 1235–1249.

Cui, J., Liu, Y., Wang, R., Tong, W., Drlica, K., Zhao, X., 2006. The mutant selection window in

rabbits infected with Staphylococcus aureus. J. Infect. Dis. 194, 1601–1608.

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N., Wright, G.D., 2011. Antibiotic resistance is ancient. Nature 477, 457–461.

Dadgostar, P., 2019. Antimicrobial Resistance: Implications and Costs. Infect. Drug Resist. 12, 3903–3910.

Danner, M.C., Robertson, A., Behrends, V., Reiss, J., 2019. Antibiotic pollution in surface fresh waters: Occurrence and effects. Sci. Total Environ. 664, 793–804.

Daughton, C.G., 2001. Pharmaceuticals and Personal Care Products in the Environment: Overarching Issues and Overview. In: ACS Symposium Series. pp. 2–38.

De Bruijn, J., Hansen, B., Johansson, S., Luotamo, M., Munn, S., Musset, C., Olsen, S., Olsson, H., Paya-Perez, A., Pedersen, F., Rasmussen, K., Sokull-Kluttgen, B., 2003. Technical Guidance Document on risk Assessment. Part 1. Part 2. EUR 20418 EN. 2002. JRC23785.

Decade, T.H.E.N., 2001. Pharmaceuticals and Care Products in the Environment, Environmental toxicology, ACS Symposium Series. American Chemical Society, Washington, DC.

Dhar, N., McKinney, J.D., 2007. Microbial phenotypic heterogeneity and antibiotic tolerance. Curr. Opin. Microbiol. 10, 30–38.

Dickinson, A.W., Power, A., Hansen, M.G., Brandt, K.K., Piliposian, G., Appleby, P., O'Neill, P.A., Jones, R.T., Sierocinski, P., Koskella, B., Vos, M., 2019. Heavy metal pollution and co-selection for antibiotic resistance: A microbial palaeontology approach. Environ. Int. 132, 105117.

Done, H.Y., Halden, R.U., 2015. Reconnaissance of 47 antibiotics and associated microbial risks in seafood sold in the United States. J. Hazard. Mater. 282, 10–17.

Dong, Y., Zhao, X., Kreiswlrth, B.N., Drlica, K., 2000. Mutant prevention concentration as a measure of antibiotic potency: Studies with clinical isolates of Mycobacterium tuberculosis. Antimicrob. Agents Chemother. 44, 2581–2584.

Drlica, K., Zhao, X., 2007. Mutant selection window hypothesis updated. Clin. Infect. Dis. 44, 681–688.

Du, L., Liu, W., 2012. Occurrence, fate, and ecotoxicity of antibiotics in agro-ecosystems. A review. Agron. Sustain. Dev. 32, 309-327

Ebele, A.J., Abou-Elwafa Abdallah, M., Harrad, S., 2017. Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment. Emerg. Contam. 3, 1–16.

Ebenebe, P.N., Shale, K., Sedibe, M., 2017. South African mine effluents: heavy metal pollution and impact on the ecosystem. Int. J. Chem. Sci. Res. 15, 198.

Edokpayi, J.N., Enitan, A.M., Mutileni, N., Odiyo, J.O., 2018. Evaluation of water quality and human risk assessment due to heavy metals in groundwater around Muledane area of Vhembe District, Limpopo Province, South Africa. Chem. Cent. J. 12, 1–16.

Eid, R., Arab, N.T.T., Greenwood, M.T., 2017. Iron mediated toxicity and programmed cell death: A review and a re-examination of existing paradigms. Biochim. Biophys. Acta - Mol. Cell Res. 1864(2):399-430.

EUCAST, 2022. Breakpoint tables for interpretation of MICs and zone diameters, The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 12.0, 2022. http://www.eucast.org.".

Faleye, A.C., Adegoke, A.A., Ramluckan, K., Bux, F., Stenström, T.A., 2018. Antibiotic Residue in the Aquatic Environment: Status in Africa. Open Chem. 16, 890–903.

Faleye, A.C., Adegoke, A.A., Ramluckan, K., Fick, J., Bux, F., Stenström, T.A., 2019. Concentration and reduction of antibiotic residues in selected wastewater treatment plants and receiving waterbodies in Durban, South Africa. Sci. Total Environ. 678, 10–20.

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, 1–8.

Fatoki, J.O., Badmus, J.A., 2022. Arsenic as an environmental and human health antagonist: A review of its toxicity and disease initiation. J. Hazard. Mater. Adv. 5, 100052.

Fayiga, A.O., Ipinmoroti, M.O., Chirenje, T., 2018. Environmental pollution in Africa. Environ. Dev. Sustain. 20, 41–73.

Fekadu, S., Alemayehu, E., Dewil, R., Van der Bruggen, B., 2019. Pharmaceuticals in freshwater

aquatic environments: A comparison of the African and European challenge. Sci. Total Environ. 654, 324–337.

Fletcher, S., 2015. Understanding the contribution of environmental factors in the spread of antimicrobial resistance. Environ. Health Prev. Med. 20, 243–252.

Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O.A., Dantas, G., 2012. The shared antibiotic resistome of soil bacteria and human pathogens. Science (80-.). 337, 1107–1111.

Fosso-kankeu, E., Barlow, B., Lemmer, N., Waanders, F., 2017. Geochemical Speciation of Metal Ions in the Leachate of Tailings Treated with Synthetic Rain Water. In: Nov. 27-28, 2017 South Africa ASETWM-17, RCABES-17 & EBHSSS-17. EARES.

Fridman, O., Goldberg, A., Ronin, I., Shoresh, N., Balaban, N.Q., 2014. Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. Nature 513, 418–421.

Gadea, R., Fernández Fuentes, M.Á., Pérez Pulido, R., Gálvez, A., Ortega, E., 2017. Effects of exposure to quaternary-ammonium-based biocides on antimicrobial susceptibility and tolerance to physical stresses in bacteria from organic foods. Food Microbiol. 63, 58–71.

Garcia, J., South-Bodiford, R., 2017. Hematology. In: Small Animal Internal Medicine for Veterinary Technicians and Nurses. pp. 161–192.

Gerba, C.P., 2015. Quaternary ammonium biocides: Efficacy in application. Appl. Environ. Microbiol. 81, 464–469.

Ghai, I., Ghai, S., 2018. Understanding antibiotic resistance via outer membrane permeability. Infect. Drug Resist. 11, 523–530.

Ghorab, M.A., 2018. Environmental pollution by heavy metals in the aquatic ecosystems of Egypt. Open Access J. Toxicol. 3, 555603.

Glibota, N., Grande, M.J., Galvez, A., Ortega, E., 2020. Genetic determinants for metal tolerance and antimicrobial resistance detected in bacteria isolated from soils of olive tree farms. Antibiotics 9, 1–11.

Godt, J., Scheidig, F., Grosse-Siestrup, C., Esche, V., Brandenburg, P., Reich, A., Groneberg, D.A., 2006. The toxicity of cadmium and resulting hazards for human health. J. Occup. Med. Toxicol. 1, 1–6.

Guérin, A., Bridier, A., Le Grandois, P., Sévellec, Y., Palma, F., Félix, B., Roussel, S., Soumet, C., Karpíšková, R., Pomelio, F., Skjerdal, T., Ricao, M., Bojan, P., Oevermann, A., Wullings, B., Bulawová, H., Castro, H., Lindström, M., Korkeala, H., Šteingolde, Ž., Szymczak, B., Gareis, M., Amar, C., 2021. Exposure to quaternary ammonium compounds selects resistance to ciprofloxacin in listeria monocytogenes. Pathogens 10, 1–11.

Gullberg, E., Albrecht, L.M., Karlsson, C., Sandegren, L., Andersson, D.I., 2014. Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. MBio 5, 19–23.

Gullberg, E., Cao, S., Berg, O.G., Ilbäck, C., Sandegren, L., Hughes, D., Andersson, D.I., 2011. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog. 7, 1–9.

Hendricks, R., Pool, E.J., 2012. The effectiveness of sewage treatment processes to remove faecal pathogens and antibiotic residues. J. Environ. Sci. Heal. - Part A Toxic/Hazardous Subst. Environ. Eng. 47, 289–297.

Hernández-Moreno, D., Blázquez, M., Andreu-Sánchez, O., Bermejo-Nogales, A., Fernández-Cruz, M.L., 2019. Acute hazard of biocides for the aquatic environmental compartment from a life-cycle perspective. Sci. Total Environ. 658, 416–423.

Hirsch, R., Ternes, T., Haberer, K., Kratz, K.-L., 1999. Occurrence of antibiotics in the aquatic environment. Sci. Total Environ. 225, 109–118.

Hogarh, J.N., Foli, G., 2009. Trace elements contamination of soils around gold mine tailings dams at Obuasi, Ghana. African J. Environ. Sci. Technol. 3, 353–359.

Hu, W.S., Chen, H.W., Zhang, R.Y., Huang, C.Y., Shen, C.F., 2011. The expression levels of outer membrane proteins STM1530 and OmpD, which are influenced by the CpxAR and BaeSR two-component systems, play important roles in the ceftriaxone resistance of Salmonella enterica serovar typhimurium. Antimicrob. Agents Chemother. 55, 3829–3837.

Huijbers, P.M.C., Blaak, H., De Jong, M.C.M., Graat, E.A.M., Vandenbroucke-Grauls, C.M.J.E., De Roda Husman, A.M., 2015. Role of the Environment in the Transmission of Antimicrobial Resistance to Humans: A Review. Environ. Sci. Technol. 49, 11993–12004.

Hunter, P.R., Wilkinson, D.C., Catling, L.A., Barker, G.C., 2008. Meta-analysis of experimental data

concerning antimicrobial resistance gene transfer rates during conjugation. Appl. Environ. Microbiol. 74, 6085–6090.

Imran, M., Das, K.R., Naik, M.M., 2019. Co-selection of multi-antibiotic resistance in bacterial pathogens in metal and microplastic contaminated environments: An emerging health threat. Chemosphere 215, 846–857.

Inoue, H., 2019. Strategic approach for combating antimicrobial resistance (AMR). Glob. Heal. Med. 1, 61–64.

Iwu, C.D., Korsten, L., Okoh, A.I., 2020. The incidence of antibiotic resistance within and beyond the agricultural ecosystem: A concern for public health. Microbiologyopen. 9(9):e1035.

doi: 10.1002/mbo3.1035.

Jomova, K., Valko, M., 2011. Advances in metal-induced oxidative stress and human disease. Toxicology 283, 65–87.

K'oreje, K.O., Demeestere, K., De Wispelaere, P., Vergeynst, L., Dewulf, J., Van Langenhove, H., 2012. From multi-residue screening to target analysis of pharmaceuticals in water: Development of a new approach based on magnetic sector mass spectrometry and application in the Nairobi River basin, Kenya. Sci. Total Environ. 437, 153–164.

K'oreje, K.O., Okoth, M., Van Langenhove, H., Demeestere, K., 2020. Occurrence and treatment of contaminants of emerging concern in the African aquatic environment: Literature review and a look ahead. J. Environ. Manage. 254, 109752.

Kähkönen, E., Soirinsuo, K., Nordstrom, K., 2010. Trends in EU biocides regulation 28, 19–23.

Kahrilas, G.A., Blotevogel, J., Stewart, P.S., Borch, T., 2015. Biocides in hydraulic fracturing fluids: A critical review of their usage, mobility, degradation, and toxicity. Environ. Sci. Technol. 49, 16–32.

Kampf, G., 2019. Antibiotic resistancecan be enhanced in gram-positive species by some biocidal agents used for disinfection. Antibiotics. 8(1):13. doi: 10.3390/antibiotics8010013.

Kamunda, C., Mathuthu, M., Madhuku, M., 2016. Health risk assessment of heavy metals in soils from witwatersrand gold mining basin, South Africa. Int. J. Environ. Res. Public Health 13, 663.

Kanama, K.M., Daso, A.P., Mpenyana-Monyatsi, L., Coetzee, M.A.A., 2018. Assessment of

pharmaceuticals, personal care products, and hormones in wastewater treatment plants receiving inflows from health facilities in North West Province, South Africa. J. Toxicol. 2018, 1–15.

Kaur, R., Garkal, A., Sarode, L., Bangar, P., Mehta, T., Singh, D.P., Rawal, R., 2024. Understanding arsenic toxicity: Implications for environmental exposure and human health. J. Hazard. Mater. Lett. 5, 100090.

Kawamura, M., Fujimura, S., Tokuda, K., Aoyagi, T., Endo, S., Kanamori, H., Watanabe, A., Kaku, M., 2019. Mutant selection window of disinfectants for Staphylococcus aureus and Pseudomonas aeruginosa. J. Glob. Antimicrob. Resist. 17, 316–320.

Keren, I., Shah, D., Spoering, A., Kaldalu, N., Lewis, K., 2004. Specialized persister cells and the mechanism of multidrug tolerance in Escherichia coli. J. Bacteriol. 186, 8172–8180.

Khan, S., Beattie, T.K., Knapp, C.W., 2017. The use of minimum selectable concentrations (MSCs) for determining the selection of antimicrobial resistant bacteria. Ecotoxicology 26, 283–292.

Knapp, C.W., McCluskey, S.M., Singh, B.K., Campbell, C.D., Hudson, G., Graham, D.W., 2011. Antibiotic Resistance Gene Abundances Correlate with Metal and Geochemical Conditions in Archived Scottish Soils. PLoS One 6, e27300.

Kootbodien, T., Mathee, A., Naicker, N., Moodley, N., 2012. Scientific Letter Heavy metal contamination in a school vegetable garden in Johannesburg. Sci. Lett. 102, 226–227.

Kovalakova, P., Cizmas, L., McDonald, T.J., Marsalek, B., Feng, M., Sharma, V.K., 2020. Occurrence and toxicity of antibiotics in the aquatic environment: A review. Chemosphere 251, 126351.

Kussell, E., Leibler, S., 2005. Ecology: Phenotypic diversity, population growth, and information in fluctuating environments. Science (80-.). 309, 2075–2078.

Kwon, D., Lim, Y.M., Kwon, J.T., Shim, I., Kim, E., Lee, D.H., Yoon, B. Il, Kim, P., Kim, H.M., 2019. Evaluation of pulmonary toxicity of benzalkonium chloride and triethylene glycol mixtures using in vitro and in vivo systems. Environ. Toxicol. 34, 561–572.

Kyriakidis, I., Vasileiou, E., Pana, Z.D., Tragiannidis, A., 2021. Acinetobacter baumannii antibiotic resistance mechanisms. Pathogens 10, 1–31.

Lambert, P.A., 2005. Bacterial resistance to antibiotics: Modified target sites. Adv. Drug Deliv. Rev.

57, 1471–1485.

Larsson, D.G.J., 2014. Antibiotics in the environment. Ups. J. Med. Sci. 119(2): 108–112.

Larsson, D.G.J., Andremont, A., Bengtsson-Palme, J., Brandt, K.K., de Roda Husman, A.M., Fagerstedt, P., Fick, J., Flach, C.F., Gaze, W.H., Kuroda, M., Kvint, K., Laxminarayan, R., Manaia, C.M., Nielsen, K.M., Plant, L., Ploy, M.C., Segovia, C., Simonet, P., Smalla, K., Snape, J., Topp, E., van Hengel, A.J., Verner-Jeffreys, D.W., Virta, M.P.J., Wellington, E.M., Wernersson, A.S., 2018. Critical knowledge gaps and research needs related to the environmental dimensions of antibiotic resistance. Environ. Int. 117, 132–138.

Larsson, D.G.J., Flach, C.-F., 2022. Antibiotic resistance in the environment. Nat. Rev. Microbiol. 20, 257–269.

Lau, C.H.-F., van Engelen, K., Gordon, S., Renaud, J., Topp, E., 2017. Novel Antibiotic Resistance Determinants from Agricultural Soil Exposed to Antibiotics Widely Used in Human Medicine and Animal Farming. Appl. Environ. Microbiol. 83, 1–18.

Lavilla Lerma, L., Benomar, N., Casado Muñoz, M. del C., Gálvez, A., Abriouel, H., 2015. Correlation between antibiotic and biocide resistance in mesophilic and psychrotrophic Pseudomonas spp. isolated from slaughterhouse surfaces throughout meat chain production. Food Microbiol. 51, 33–44.

Leavens, T.L., Blount, B.C., Demarini, D.M., Madden, M.C., Valentine, J.L., Case, M.W., Silva, L.K., Warren, S.H., Hanley, N.M., Pegram, R.A., 2007. Disposition of bromodichloromethane in humans following oral and dermal exposure. Toxicol. Sci. 99, 432–445.

Lee, J., Jeon, J.H., Shin, J., Jang, H.M., Kim, S., Song, M.S., Kim, Y.M., 2017. Quantitative and qualitative changes in antibiotic resistance genes after passing through treatment processes in municipal wastewater treatment plants. Sci. Total Environ. 605–606, 906–914.

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N., Balaban, N.Q., 2017. Antibiotic tolerance facilitates the evolution of resistance. Science 355, 826–830.

Lewis, K., 2007. Persister cells, dormancy and infectious disease. Nat. Rev. Microbiol. 5, 48–56.

Li, N., Sheng, G.-P., Lu, Y.-Z., Zeng, R.J., Yu, H.-Q., 2017. Removal of antibiotic resistance genes from wastewater treatment plant effluent by coagulation. Water Res. 111, 204–212.

Li, W., Liu, C., Ho, H.C., Shi, L., Zeng, Y., Yang, X., Huang, Q., Pei, Y., Huang, C., Yang, L., 2023. Association between antibiotic resistance and increasing ambient temperature in China: an ecological study with nationwide panel data. Lancet Reg. Heal. - West. Pacific 30, 100628.

Li, X., Brownawell, B.J., 2010. Quaternary ammonium compounds in urban estuarine sediment environments - A class of contaminants in need of increased attention? Environ. Sci. Technol. 44, 7561–7568.

Lin, J., Nishino, K., Roberts, M.C., Tolmasky, M., Aminov, R.I., Zhang, L., 2015. Mechanisms of antibiotic resistance. Front. Microbiol. 6, 2013–2015.

Lingzhi, L., Haojie, G., Dan, G., Hongmei, M., Yang, L., Mengdie, J., Chengkun, Z., Xiaohui, Z., 2018. The role of two-component regulatory system in β-lactam antibiotics resistance. Microbiol. Res. 215, 126–129.

Liu, M., Li, Q., Sun, H., Jia, S., He, X., Li, M., Zhang, X.X., Ye, L., 2018. Impact of salinity on antibiotic resistance genes in wastewater treatment bioreactors. Chem. Eng. J. 338, 557–563.

Liu, Y., Cui, J., Wang, R., Wang, X., Drlica, K., Zhao, X., 2005. Selection of rifampicin-resistant Staphylococcus aureus during tuberculosis therapy: Concurrent bacterial eradication and acquisition of resistance. J. Antimicrob. Chemother. 56, 1172–1175.

Lu, D., Wang, L., Yan, B., Ou, Y., Guan, J., Bian, Y., Zhang, Y., 2014. Speciation of Cu and Zn during composting of pig manure amended with rock phosphate. Waste Manag. 34, 1529–1536.

Madikizela, L.M., Ncube, S., Chimuka, L., 2020. Analysis, occurrence and removal of pharmaceuticals in African water resources: A current status. J. Environ. Manage. 253, 109741.

Magwira, C.A., Aneck-Hahn, N., Taylor, M.B., 2019. Fate, occurrence and potential adverse effects of antimicrobials used for treatment of tuberculosis in the aquatic environment in South Africa. Environ. Pollut. 254, 112990.

Manaia, C.M., 2017. Assessing the Risk of Antibiotic Resistance Transmission from the Environment to Humans : Non-Direct Proportionality between Abundance and Risk. Trends Microbiol. 25, 173–181.

Manegabe, B.J., Marie-Médiatrice, N.K., Barr Dewar, J., Christian, S.B., 2017. Antibiotic resistance and tolerance to heavy metals demonstrated by environmental pathogenic bacteria isolated from

the Kahwa River, Bukavu Town, Democratic Republic of the Congo. Int. J. Environ. Stud. 74, 290–302.

Mann, B.C., Bezuidenhout, J.J., Bezuidenhout, C.C., 2019. Biocide resistant and antibiotic cross-resistant potential pathogens from sewage and river water from a wastewater treatment facility in the North-West, Potchefstroom, South Africa. Water Sci. Technol. 80, 551–562.

Markley, J.L., Wencewicz, T.A., 2018. Tetracycline-Inactivating Enzymes. Front. Microbiol. 9, 1–22.

Martinez, J.L., 2009. The role of natural environments in the evolution of resistance traits in pathogenic bacteria. Proc. R. Soc. B Biol. Sci. 276, 2521–2530.

Martínez, J.L., 2012. Bottlenecks in the transferability of antibiotic resistance from natural ecosystems to human bacterial pathogens. Front. Microbiol. 2, 1–6.

Martínez, J.L., Baquero, F., 2014. Emergence and spread of antibiotic resistance: Setting a parameter space. Ups. J. Med. Sci. 119, 68–77.

Martínez, J.L., Coque, T.M., Baquero, F., 2015. What is a resistance gene? Ranking risk in resistomes. Nat. Rev. Microbiol. 13, 116–123.

Martínez, J.L., Rojo, F., 2011. Metabolic regulation of antibiotic resistance. FEMS Microbiol. Rev. 35, 768–789.

Masindi, V., Muedi, K.L., 2018. Environmental Contamination by Heavy Metals. In: El-Din, H., Saleh, M., Aglan, R.F. (Eds.), Heavy Metals. InTech, London.

Matongo, S., Birungi, G., Moodley, B., Ndungu, P., 2015. Occurrence of selected pharmaceuticals in water and sediment of Umgeni River, KwaZulu-Natal, South Africa. Environ. Sci. Pollut. Res. 22, 10298–10308.

Mc Cay, P.H., Ocampo-Sosa, A.A., Fleming, G.T.A., 2010. Effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of Pseudomonas aeruginosa grown in continuous culture. Microbiology 156, 30–38.

McArthur, J. V, Tuckfield, R.C., 2000. Spatial patterns in antibiotic resistance among stream bacteria: effects of industrial pollution. Appl. Environ. Microbiol. 66, 3722–6.

McMurry, L., Petrucci, R.E., Levy, S.B., 1980. Active efflux of tetracycline encoded by four genetically different tetracyline resistance determinants in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. 77, 3974–3977.

Mechler, L., Herbig, A., Paprotka, K., Fraunholz, M., Nieselt, K., Bertram, R., 2015. A novel point mutation promotes growth phase-dependent daptomycin tolerance in Staphylococcus aureus. Antimicrob. Agents Chemother. 59, 5366–5376.

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, 2317–2325.

Miller, W.R., Munita, J.M., Arias, C.A., 2014. Mechanisms of antibiotic resistance in enterococci. Expert Rev. Anti. Infect. Ther. 12, 1221–1236.

Molina-González, D., Alonso-Calleja, C., Alonso-Hernando, A., Capita, R., 2014. Effect of sub-lethal concentrations of biocides on the susceptibility to antibiotics of multi-drug resistant Salmonella enterica strains. Food Control 40, 329–334.

Muzaffar, S., Khan, J., Srivastava, R., Gorbatyuk, M.S., Athar, M., 2023. Mechanistic understanding of the toxic effects of arsenic and warfare arsenicals on human health and environment. Cell Biol. Toxicol. 39, 85–110.

Mzukisi, L., Tawanda, N., Chimuka, L., 2017. Status of pharmaceuticals in African water bodies : Occurrence , removal and analytical methods. J. Environ. Manage. 193, 211–220.

Necibi, M.C., Dhiba, D., Hajjaji, S. El, 2021. Contaminants of emerging concern in african wastewater effluents: Occurrence, impact and removal technologies. Sustain. 13, 1–12.

Nguyen, A.Q., Vu, H.P., Nguyen, L.N., Wang, Q., Djordjevic, S.P., Donner, E., Yin, H., Nghiem, L.D., 2021. Monitoring antibiotic resistance genes in wastewater treatment: Current strategies and future challenges. Sci. Total Environ. 783, 146964.

Nies, D.H., 1999. Microbial heavy-metal resistance. Appl. Microbiol. Biotechnol. 51, 730–750.

Nourhene, S., Rihab, L., Fethi, B.A., Karima, B.R., Amina, B., 2013. Slime producing, heavy metals and antibiotics resistance in Aeromonas hydrophila isolated in Tunisia. African J. Microbiol. Res. 7, 5697–5708.

O' Neil, J., 2014. Review on Antibiotic resisitance. Antimicrobial Resistance : Tackling a crisis for the health and wealth of nations, Health and Wealth Nations. 2014, 1-16

O'Halloran, C., Walsh, N., O'Grady, M.C., Barry, L., Hooton, C., Corcoran, G.D., Lucey, B., 2018. Assessment of the comparability of CLSI, EUCAST and Stokes antimicrobial susceptibility profiles for Escherichia coli uropathogenic isolates. Br. J. Biomed. Sci. 75, 24–29.

Okereafor, U., Makhatha, M., Mekuto, L., Uche-Okereafor, N., Sebola, T., Mavumengwana, V., 2020. Toxic metal implications on agricultural soils, plants, animals, aquatic life and human health. Int. J. Environ. Res. Public Health 17, 1–24.

Olaniran, E.I., Sogbanmu, T.O., Saliu, J.K., 2019. Biomonitoring, physico-chemical, and biomarker evaluations of abattoir effluent discharges into the Ogun River from Kara Market, Ogun State, Nigeria, using Clarias gariepinus. Environ. Monit. Assess. 191, 44.

Olowoyo, J.O., Mugivhisa, L.L., Magoloi, Z.G., 2016. Composition of trace metals in dust samples collected from selected high schools in pretoria, South Africa. Appl. Environ. Soil Sci. 2016, 1–9.

Oost, R. van der, Beyer, J., Vermeulen, N.P.E., 2016. Soil Quality Field Kit : Part II. Environ. Toxicol. Pharmacol. 13, 57–149.

Osimitz, T.G., Droege, W., 2022. Adverse Outcome Pathway for Antimicrobial Quaternary Ammonium Compounds. J. Toxicol. Environ. Heal. - Part A Curr. Issues 85, 494–510.

Pagès, J.M., James, C.E., Winterhalter, M., 2008. The porin and the permeating antibiotic: A selective diffusion barrier in Gram-negative bacteria. Nat. Rev. Microbiol. 6, 893–903.

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, 1st ed, Advances in Microbial Physiology. Elsevier Ltd., Netherlands

Park, J., Gasparrini, A.J., Reck, M.R., Symister, C.T., Elliott, J.L., Vogel, J.P., Wencewicz, T.A., Dantas, G., Tolia, N.H., 2017. Plasticity, dynamics, and inhibition of emerging tetracycline resistance enzymes. Nat. Chem. Biol. 13, 730–736.

Pasquali, F., Manfreda, G., 2007. Mutant prevention concentration of ciprofloxacin and enrofloxacin against Escherichia coli, Salmonella Typhimurium and Pseudomonas aeruginosa. Vet.

Microbiol. 119, 304–310.

Peterson, E., Kaur, P., 2018. Antibiotic resistance mechanisms in bacteria: Relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. Front. Microbiol. 9, 1–21.

Poirel, L., Hombrouck-Alet, C., Freneaux, C., Bernabeu, S., Nordmann, P., 2010. Global spread of New Delhi metallo-β-lactamase 1. Lancet Infect. Dis. 10, 832.

Pompilio, A., Scribano, D., Sarshar, M., Di Bonaventura, G., Palamara, A.T., Ambrosi, C., 2021. Gram-negative bacteria holding together in a biofilm: The acinetobacter baumannii way. Microorganisms 9, 1–33.

Pruden, A., Alcalde, R.E., Alvarez, P.J.J., Ashbolt, N., Bischel, H., Capiro, N.L., Crossette, E., Frigon, D., Grimes, K., Haas, C.N., Ikuma, K., Kappell, A., LaPara, T., Kimbell, L., Li, M., Li, X., McNamara, P., Seo, Y., Sobsey, M.D., Sozzi, E., Navab-Daneshmand, T., Raskin, L., Riquelme, M.V., Vikesland, P., Wigginton, K., Zhou, Z., 2018. An Environmental Science and Engineering Framework for Combating Antimicrobial Resistance. Environ. Eng. Sci. 35, 1005–1011.

Pruden, A., Joakim Larsson, D.G., Amézquita, A., Collignon, P., Brandt, K.K., Graham, D.W., Lazorchak, J.M., Suzuki, S., Silley, P., Snape, J.R., Topp, E., Zhang, T., Zhu, Y.G., 2013. Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. Environ. Health Perspect. 121, 878–885.

Quinlan, E.L., Nietch, C.T., Blocksom, K., Lazorchak, J.M., Batt, A.L., Griffiths, R., Klemm, D.J., 2011. Temporal dynamics of periphyton exposed to tetracycline in stream mesocosms. Environ. Sci. Technol. 45, 10684–10690.

Rahimzadeh, Mehrdad Rafati, Rahimzadeh, Mehravar Rafati, Kazemi, S., Moghadamnia, A.A., 2017. Cadmium toxicity and treatment: An update. Casp. J. Intern. Med. 8, 135–145.

Rasmussen, K., Chemin, P., Haastrup, P., 1999. Regulatory requirements for biocides on the market in the European Union according to Directive 98/8/EC. J. Hazard. Mater. 67, 237–251.

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.

Russell, A.D., 2003. Biocide use and antibiotic resistance: The relevance of laboratory findings to clinical and environmental situations. Lancet Infect. Dis. 3, 794–803.

Saleem, H.G.M., Seers, C.A., Sabri, A.N., Reynolds, E.C., 2016. Dental plaque bacteria with reduced susceptibility to chlorhexidine are multidrug resistant. BMC Microbiol. 16, 1–9.

Sarma, B., Acharya, C., Joshi, S.R., 2010. Pseudomonads: a versatile bacterial group exhibiting dual resistance to metals and antibiotics. African J. Microbiol. Res. 4, 2828–2835.

Sarmah, A.K., Meyer, M.T., Boxall, A.B. a, 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 65, 725–59.

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, 1–10.

Sharma, V.K., Johnson, N., Cizmas, L., Mcdonald, T.J., Kim, H., 2016. Chemosphere A review of the in fl uence of treatment strategies on antibiotic resistant bacteria and antibiotic resistance genes. Chemosphere 150, 702–714.

Siddiqui, M.T., Mondal, A.H., Gogry, F.A., Husain, F.M., Alsalme, A., Haq, Q.M.R., 2020. Plasmid-Mediated Ampicillin, Quinolone, and Heavy Metal Co-Resistance among ESBL-Producing Isolates from the Yamuna River, New Delhi, India. Antibiotics 9, 826.

Song, C., Zhang, C., Fan, L., Qiu, L., Wu, W., Meng, S., Hu, G., Kamira, B., Chen, J., 2016. Occurrence of antibiotics and their impacts to primary productivity in fishponds around Tai Lake, China. Chemosphere 161, 127–135.

Souza, L.R.R., da Silva, V.S., Franchi, L.P., de Souza, T.A.J., 2018. Toxic and beneficial potential of silver nanoparticles: The two sides of the same coin. Adv. Exp. Med. Biol. 1048, 251–262.

Squadrone, S., 2020. Water environments: metal-tolerant and antibiotic-resistant bacteria. Environ. Monit. Assess. 192, 238.

Sukul, P., Lamshöft, M., Kusari, S., Zühlke, S., Spiteller, M., 2009. Metabolism and excretion kinetics of 14C-labeled and non-labeled difloxacin in pigs after oral administration, and antimicrobial activity of manure containing difloxacin and its metabolites. Environ. Res. 109,

225–231.

Sulaiman, J.E., Lam, H., 2019. Application of proteomics in studying bacterial persistence. Expert Rev. Proteomics 16, 227–239.

Sulaiman, J.E., Lam, H., 2021. Evolution of Bacterial Tolerance Under Antibiotic Treatment and Its Implications on the Development of Resistance. Front. Microbiol. 12, 1–9.

Suller, M.T.E., Russell, A.D., 1999. Antibiotic and biocide resistance in methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococcus. J. Hosp. Infect. 43, 281–291.

Sun, J., Deng, Z., Yan, A., 2014. Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations. Biochem. Biophys. Res. Commun. 453, 254–267.

Taylor, A.A., Tsuji, J.S., Garry, M.R., McArdle, M.E., Goodfellow, W.L., Adams, W.J., Menzie, C.A., 2020. Critical Review of Exposure and Effects: Implications for Setting Regulatory Health Criteria for Ingested Copper. Environ. Manage. 65, 131–159.

Taylor, N.G.H., Verner-Jeffreys, D.W., Baker-Austin, C., 2011. Aquatic systems: Maintaining, mixing and mobilising antimicrobial resistance? Trends Ecol. Evol. 26, 278–284.

Tell, J., Caldwell, D.J., Häner, A., Hellstern, J., Hoeger, B., Journel, R., Mastrocco, F., Ryan, J.J., Snape, J., Straub, J.O., Vestel, J., 2019. Science-based Targets for Antibiotics in Receiving Waters from Pharmaceutical Manufacturing Operations. Integr. Environ. Assess. Manag. 15, 312–319.

Tello, A., Austin, B., Telfer, T.C., 2012. Selective pressure of antibiotic pollution. Environ. Health Perspect. 120, 1100–1106.

Tezel, U., Pavlostathis, S.G., 2015. Quaternary ammonium disinfectants: Microbial adaptation,Degradation and ecology. Curr. Opin. Biotechnol. 33, 296–304.

Tornimbene, B., Eremin, S., Escher, M., Griskeviciene, J., Manglani, S., Pessoa-Silva, C.L., 2018. WHO Global Antimicrobial Resistance Surveillance System early implementation 2016–17. Lancet Infect. Dis. 18, 241–242.

Townsend, D.E., Ashdown, N., Greed, L.C., Grubb, W.B., 1984. Transposition of gentamicin resistance to staphylococcal plasmids encoding resistance to cationic agents. J. Antimicrob. Chemother. 14, 115–124.

Ugur, A., Ceylan, Ö., 2003. Occurrence of resistance to antibiotics, metals, and plasmids in clinical strains of Staphylococcus spp. Arch. Med. Res. 34, 130–136.

Vazirian, M., Hamidian, K., Noorollah, M., Manayi, A., Samadi, N., 2019. Enhancement of Antibiotic Activity and Reversal of Resistance in Clinically Isolated Methicillin-Resistant Staphylococcus aureus by Trachyspermum ammi Essential Oil. Res. J. Pharmacogn. 6, 1–10.

Walsh, C., 2000. Molecular mechanisms that confer antibacterial drug resistance. Nature 406, 775–781.

Wang, X., Lan, B., Fei, H., Wang, S., Zhu, G., 2021. Heavy metal could drive co-selection of antibiotic resistance in terrestrial subsurface soils. J. Hazard. Mater. 411, 124848.

Webber, M.A., Piddock, L.J.V., 2003. The importance of efflux pumps in bacterial antibiotic resistance. J. Antimicrob. Chemother. 51, 9–11.

Wellington, E.M.H., Boxall, A.B.A., Cross, P., Feil, E.J., Gaze, W.H., Hawkey, P.M., Johnson-Rollings, A.S., Jones, D.L., Lee, N.M., Otten, W., Thomas, C.M., Williams, A.P., 2013. The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. Lancet Infect. Dis. 13, 155–165.

Winckler, C., Grafe, A., 2001. Use of veterinary drugs in intensive animal production: Evidence for persistence of tetracycline in pig slurry. J. Soils Sediments 1, 66–70.

Winterhalter, M., Ceccarelli, M., 2015. Physical methods to quantify small antibiotic molecules uptake into Gram-negative bacteria. Eur. J. Pharm. Biopharm. 95, 63–67.

Xia, X., Wang, Z., Fu, Y., Du, X. dang, Gao, B., Zhou, Y., He, J., Wang, Y., Shen, J., Jiang, H., Wu, Y., 2019. Association of colistin residues and manure treatment with the abundance of mcr-1 gene in swine feedlots. Environ. Int. 127, 361–370.

Yamamoto, T., Tamura, Y., Yokota, T., 1988. Antiseptic and antibiotic resistance plasmid in Staphylococcus aureus that possesses ability to confer chlorhexidine and acrinol resistance. Antimicrob. Agents Chemother. 32, 932–935.

Yan, J., Bassler, B.L., 2019. Surviving as a Community: Antibiotic Tolerance and Persistence in Bacterial Biofilms. Cell Host Microbe 26, 15–21.

Zhang, C., Cui, F., Zeng, G. ming, Jiang, M., Yang, Z. zhu, Yu, Z. gang, Zhu, M. ying, Shen, L. qing,

2015. Quaternary ammonium compounds (QACs): A review on occurrence, fate and toxicity in the environment. Sci. Total Environ. 518–519, 352–362.

Zhang, N., Yang, S., Yang, J., Deng, Y., Li, S., Li, N., Chen, X., Yu, P., Liu, Z., Zhu, J., 2020. Association between metal cobalt exposure and the risk of congenital heart defect occurrence in offspring: A multi-hospital case-control study. Environ. Health Prev. Med. 25, 1–12.

Zhao, X., Drlica, K., 2002. Restricting the Selection of Antibiotic-Resistant Mutant Bacteria: Measurement and Potential Use of the Mutant Selection Window. J. Infect. Dis. 185, 561–565.

Zhitkovich, A., 2011. Chromium in drinking water: Sources, metabolism, and cancer risks. Chem. Res. Toxicol. 24, 1617–1629.

Zhu, Y.-G., Zhao, Y., Li, B., Huang, C.-L., Zhang, S.-Y., Yu, S., Chen, Y.-S., Zhang, T., Gillings, M.R., Su, J.-Q., 2017. Continental-scale pollution of estuaries with antibiotic resistance genes. Nat. Microbiol. 2, 16270.

Zhu, Y.-G.G., Johnson, T.A., Su, J.-Q.Q., Qiao, M., Guo, G.-X.X., Stedtfeld, R.D., Hashsham, S.A., Tiedje, J.M., 2013. Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc. Natl. Acad. Sci. USA 110, 3435–40.

Zmantar, T., Kouidhi, B., Hentati, H., Bakhrouf, A., 2012. Detection of disinfectant and antibiotic resistance genes in Staphylococcus aureus isolated from the oral cavity of Tunisian children. Ann. Microbiol. 62, 123–128.

CHAPTER 2

ANTIBIOTIC, HEAVY METAL, AND BIOCIDE CONCENTRATIONS IN A WASTEWATER TREATMENT PLANT AND ITS RECEIVINGWATER BODY EXCEED PNEC LIMITS: POTENTIAL FOR ANTIMICROBIAL RESISTANCE SELECTIVE PRESSURE AUTHOR CONTRIBUTIONS

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Kelechi B. Chukwu – As principal investigator, co-conceptualised the study, conducted the laboratory work, analysed the data, and drafted the manuscript.

Ovokeroye A. Abafe – Co-conceptualised the study, guided laboratory works, and critically revised the manuscript.

Daniel G. Amoako – Guided laboratory works, assisted in data analysis and critically revised the manuscript

Professor S.Y. Essack – As the Co-supervisor, co-conceptualized the study, guided the literature review and ethical clearance application, enabled data collection and analysis and undertook the critical revision of the manuscript.

Professor A.L.K. Abia – As the main supervisor, co-conceptualised the study, supervised the laboratory work and critically revised the manuscript.

Objectives met – Paper answers objectives 1 and 2

antibiotics

MDPI

Article

Antibiotic, Heavy Metal, and Biocide Concentrations in a Wastewater Treatment Plant and Its Receiving Water Body Exceed PNEC Limits: Potential for Antimicrobial Resistance Selective Pressure

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Abstract: Although the rise in antimicrobial resistance has been attributed mainly to the extensive and indiscriminate use of antimicrobials such as antibiotics and biocides in humans, animals and on plants, studies investigating the impact of this use on water environments in Africa are minimal. This study quantified selected antibiotics, heavy metals, and biocides in an urban wastewater treatment plant (WWTP) and its receiving water body in Kwazulu-Natal, South Africa, in the context of the predicted no-effect concentrations (PNEC) for the selection of antimicrobial resistance (AMR). Water samples were collected from the WWTP effluent discharge point and upstream and downstream from this point. Heavy metals were identified and quantified using the United States Environmental Protection Agency (US EPA) method 200.7. Biocides and antibiotic residues were determined using validated ultra-high-performance liquid chromatography with tandem mass spectrometry-based methods. The overall highest mean antibiotic, metal and biocide concentrations were observed for sulfamethoxazole (286.180 µg/L), neodymium (Nd; 27.734 mg/L), and benzalkonium chloride (BAC 12) (7.805 µg/L), respectively. In decreasing order per sampling site, the pollutant concentrations were effluent > downstream > upstream. This implies that the WWTP significantly contributed to the observed pollution in the receiving water. Furthermore, most of the pollutants measured recorded values exceeding the recommended predicted no-effect concentration (PNEC) values, suggesting that the microbes in such water environments were at risk of developing resistance due to the selection pressure exerted by these antimicrobials. Further studies are required to establish such a relationship.

Keywords: environmental stressors; antimicrobial resistance; selective pressure; biocides; heavy metals; antibiotic residues; aquatic environment; PNEC

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1. Introduction

The current high use of antimicrobials in humans, animals and plants has spilt over into the environment in the form of antimicrobial residues with the subsequent selection pressure for the emergence and proliferation of drug-resistant microorganisms and antimicrobial resistance genes (ARGs) [1,2]. Furthermore, emissions from industrial-scale production of antimicrobials expose environmental bacterial communities to unprecedented

selection pressures where concentrations exceed the predicted no-effect concentrations PNEC), resulting in the rapid development of antimicrobial resistance (AMR) [3].

Antimicrobials such as antibiotics, heavy metals and biocides are introduced into the environment via discharges from pharmaceutical industries, hospital effluents, direct excretion from humans and livestock, and runoff from farms [4]. Antibiotics that have been reported in wastewater and surface water bodies include sulfamethoxazole (SMX), tetracycline (TET), erythromycin (ERY), ciprofloxacin (CIP), amoxicillin (AMX), and trimethoprim (TMP) [4–9]. For example, in hospital wastewater in Ghana, the most detected antibiotics were CIP (15 µg/L) and SMX (7.2 µg/L) [10], while in Tunisia, over 1100 µg/L of erythromycin was detected [11].

Heavy metals in the environment could originate from geological sources like weathering of rocks and soil deposition of particulates. However, elevated concentrations usually come from anthropogenic sources such as industry, mining, and domestic discharges. Most of these heavy metals end up in surface waters (rivers and estuaries) or sediments, creating environmental reservoirs of these pollutants [12]. Many studies have reported varying concentrations of heavy metals in aquatic systems in different parts of Africa. For example, copper was detected at concentrations reaching 1070 µg/L in river water in Ethiopia [13]. Similarly, Diop et al. (2015) investigated the presence of heavy metals in coastal and estuary sediments in Dakar, Senegal, and recorded up to 1308 mg/kg of lead in sediments [14]. Furthermore, in South Africa, a study found aluminium concentrations ranging between 1.01–9.644 mg/L (water) and 4296–5557 mg/kg (sediments) in the Mvudi River [15], while another study in a wastewater treatment plant recorded high values of iron (69.789 mg/kg in sludge) and copper (6.588 mg/L in wastewater), exceeding local and international standards [16].

The Biocide Product Directive 98/8/EC of the European Commission describes biocides as "active substances or preparations containing one or more active substances used directly or in forms that can be applied or supplied to destroy, render harmless, prevent the action or otherwise exert a controlling effect on harmful organisms, by either chemical or biological means" [17–19]. While some biocides like hexachlorophene that causes skin disorder have been prohibited, others like triclosan are still widely used in diverse consumer and healthcare products, such as soaps, scrubs, gels, toothpaste, deodorants, hospital, and disinfectants. Similarly, benzalkonium chloride (BAC), a quaternary ammonium compound (QAC), is used extensively as an active ingredient in preservatives, medical disinfectants and ophthalmic systems [20–25]. These substances have also been reported in the environment. Studies have detected various biocides in varying concentrations in different parts of the world, ranging from 50 ng/L in Germany to 13 µg/L in South Africa [23,26–32].

Concentration limits have been set for these substances in different environments by governments and regulatory agencies, and their concentrations are expected to be below these acceptable limits. These limits are mostly set as predicted no-effect concentration (PNEC), which are levels presumed to exert no selection pressure on bacteria from the substances [1,33]. However, these substances are frequently discharged into the environment at concentrations greater than these limits, especially in most low- and middle-income countries, where treatment facilities function sub-optimally or are completely absent. The harmful effects and the potential to trigger antimicrobial resistance warrant the constant monitoring of these pollutants, especially in aquatic environments. Furthermore, studies addressing this topic are limited in Africa. With compromised sanitation facilities, these chemicals could easily find their way into different environments, including water bodies used by communities in resource-poor settings without access to potable water. Therefore, this study evaluated the presence and concentrations of selected antibiotics, heavy metals, and biocides in a WWTP's effluent and its receiving water body in KwaZulu-Natal, South Africa. KwaZulu-Natal is the second largest province in South Africa, with a population of close to nine million inhabitants, living in a mix of rural, periurban and highly industrialised areas. Thus, the province represents the typical demographics of the country.

In addition, the observed concentrations were compared to existing limits to infer their potential to exert selection pressure on environmental bacteria, leading to AMR.

2. Results

2.1. Distribution of Heavy Metals

Thirty-eight metals were detected across all the samples (Figure 1). Of these, the highest mean metal concentration was observed for Nd (27.734 mg/L), while the least was for Fe (0.001 mg/L) (Table S1; Supplementary Material). Summing up the metal concentrations at each of the three sampling points over the entire study period, there was an overall higher combined metal concentration in the effluent samples (178,473 mg/L) than in the downstream (105,354 mg/L) and upstream (35,071 mg/L) samples. However, only Ca ($p = 0.038$), Mg ($p = 0.002$), Ni ($p = 0.025$), Os ($p = 0.036$), Si ($p = 0.009$), Sr ($p = 0.022$), and Ti ($p = 0.017$) were statistically significantly different across the sites (Table S2; Supplementary Materials).

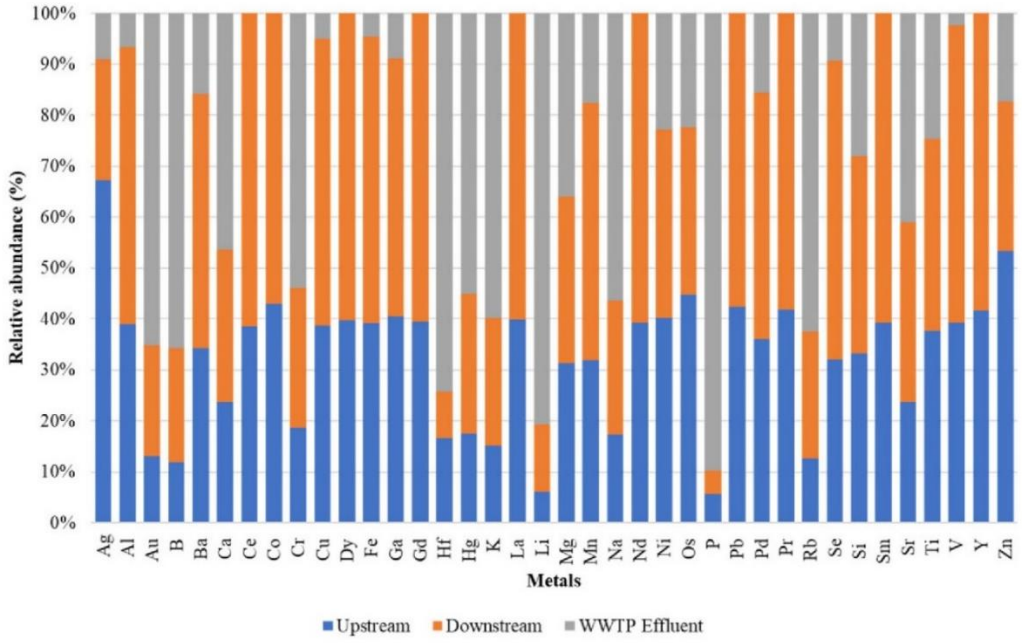

Figure 1. Heavy metal distribution across sampling points.

2.2. Distribution of Antibiotics

A total of 13 antibiotics were detected across the samples, with sulfamethoxazole being the most detected (286.180 µg/L) and penicillin being the lowest (2.2 µg/L) (Table 1; Table S3; Supplementary Materials).

Overall, the highest total antibiotics concentration was observed in the WWTP effluent samples (1335.400 µg/L), followed by downstream (846.830 µg/L) and upstream (724.120 µg/L) (Table S3, Supplementary Materials), with the difference being statistically significant for all the antibiotics identified except for Sulfamonomethoxine ($p = 0.131$) and penicillin ($p = 0.131$) (Table S4; Supplementary Materials). Some antibiotics, like tetracycline and sulfapyridine, were only detected in effluent and downstream samples.

2.3. Distribution of Biocides

Benzalkonium chloride (BAC) 12 had the highest mean concentration (7.805 µg/L), while the least detected was BenthEZ (0.035 µg/L) (Table S5; Supplementary Materials). However, the single highest concentration (29.58 µg/L) was recorded in the upstream

sample in the afternoon sampling period. Additionally, BAC14 was only detected in the downstream samples, while BAC14 and DDAC were not detected in the upstream samples.

Table 1. Antibiotic concentrations across sampling sites in the study.

ANTIBIOTICS	PNEC (Resistance Selection) #	Mean Environmental Concentrations (µg/L)		
		Upstream	Effluent	Downstream
Sulfadimidine	NA	0.95	3.92	1.01
Sulfamethazine	NA	0.79	1.74	0.73
Sulfamonomethoxine	NA	0.00	0.00	3.25
Sulfamethoxazole *	16	11.63	144.41	32.33
Sulfapyridine	NA	0.00	5.17	0.32
Oxytetracycline *	0.5	18.79	18.70	18.71
Tetracycline *	1	0.00	10.05	19.73
Doxycycline *	2	5.75	6.68	5.60
Lasalocid A	NA	3.42	1.69	3.34
Monensin	NA	0.80	1.81	1.28
Lincomycin *	2	3.91	7.95	7.81
Penicillin	NA	0.00	0.00	0.55
Amoxycillin *	0.25	62.31	59.73	52.45

Values below the limit of detection are represented as zero. NA represents antibiotics for which PNEC values are unavailable. * Antibiotics with at least one mean value above the PNEC recommended limit. # [1].

3. Discussion

This study quantified selected antibiotics, heavy metals, and biocides in an urban WWTP and its receiving water body in Kwazulu-Natal, South Africa, comparing them to the PNEC for the selection of AMR. Heavy metals were identified and quantified using the US EPA method 200.7. Biocides and antibiotic residues were determined using validated ultra-high-performance liquid chromatography with tandem mass spectrometry-based methods. The highest antibiotic, metal and biocide concentrations were observed for sulfamethoxazole, neodymium, and BAC 12, respectively. In decreasing order per sampling site, the pollutant concentrations were effluent > downstream > upstream. Most of the pollutants measured recorded values exceeding the recommended PNEC values.

3.1. Heavy Metals

A total of 69 heavy metals were screened, of which 26 heavy metals were detected, with seven having concentrations above the recommended World Health Organization (WHO) (WHO 2008) and South African National Standard (SANS) [34] limits. Although Al (3.43 mg/L) and Zn (0.078 mg/L) were higher than the SANS limits (Al—0.3 mg/L; Zn—0.005 mg/L), other heavy metals (As, Ag, Au, Ba, Be, Cd, Co, Cr, Hg, Mo, Ni, Pb, Sb, Se, U, V) were detected at very low concentrations (Table S2). Previous South African studies had reported low heavy metal concentrations in some South African aquatic ecosystems and suggested that the studied water bodies were not heavily impacted, especially by industries and metal works [15,35]. However, for some heavy metals, like Cd, Pb, Al, Fe, and As, the concentrations reported were lower than those reported in previous studies [36,37]. The differences could be due to the anthropogenic activities surrounding the various water bodies in the different studies. High Cd and As environmental concentrations, for example, have been associated with the heavy use of agricultural chemicals like in fish farming [38], which were not practised in our study area. Additionally, this study only focused on surface water, while Letsoalo et al. [37], for example, analysed both surface and sediment samples (sediments can absorb more heavy metals), which could have increased their chances of isolating higher metal concentrations.

Comparing the different sampling sites in this study revealed that the WWTP effluent samples had a statistically significantly higher heavy metal concentration than the downstream and upstream samples. This could be associated with reduced treatment plant efficiency in removing inorganic pollutants. Similar findings had previously been reported in South Africa. For example, Agoro et al. [16], in a study conducted on the evaluation of heavy metals in wastewater and sludge in selected WWTPs in the Eastern Cape Province,

South Africa, reported that the removal efficiency for heavy metals like Cu and Zn was very low in all studied WWTPs, resulting in poor wastewater effluent quality regarding heavy metals. The authors concluded that this resulted in contamination of the downstream sites and suggested that the effluent was unsuitable for irrigation. The downstream site in this study also had a higher metal concentration than the upstream site, indicating that the WWTP significantly influenced the presence of pollutants in the receiving water body. Although in our study, we observed low concentrations of most heavy metals downstream compared to the SANS values, several authors have indicated the ability of sub-inhibitory concentrations of heavy metals to select for resistance genes in microbes and that they could still be toxic to humans and other aquatic organisms [16,37,39]

Heavy metals, even at low concentrations, can alter bacterial efflux pump system expression, promoting cross-resistance to antibiotics and contributing to the development of multidrug-resistant bacterial species [39]. For example, in a laboratory experiment to determine the effect of sub-lethal concentrations of two heavy metals (Cu and Zn) on the development of multiple antibiotic resistance in bacteria in liquid media, Xu et al. [39] observed that exposure of *E. coli* to these heavy metals resulted in the upregulation of efflux pump genes that also favoured antimicrobial resistance. Similarly, an earlier study on river water exposed to nickel or cadmium stressors resulted in increased antibiotic-resistant bacteria [40]. Li et al. [41] observed that sub-minimum inhibitory concentrations of silver (Ag^{2+}), zinc (Zn^{2+}) and copper (Cu^{2+}) increased the mutation rates of exposed microbes, and the mutants exhibited significant resistance to multiple antibiotics. Furthermore, Chen et al. [42] observed that the *sul*2 gene responsible for resistance to sulphonamides co-occurred with metal resistance genes in their studied bacteria, suggesting a co-selection of resistance. Based on the above studies, it could be speculated that the sub-lethal heavy metal concentrations recorded in this study might present a potential for antimicrobial resistance development in the studied environment. However, such speculations could be verified through exposure experiments to the observed environmental concentrations to ascertain if such exposure would induce phenotypic and genotypic AMR in environmental bacteria within the studied aquatic ecosystem.

3.2. *Antibiotics*

Antibiotics are extensively used in human and animal medicine for treatment and prophylaxis. However, approximately 75 to 95% of the antibiotics used in food animals are excreted unmetabolised or partially metabolised [43]. As a result, these antibiotics and their residues end up in waterways, mainly through WWTPs, and it has been shown that WWTPs are hotspots for the dissemination of antibiotics into the environment, especially aquatic ecosystems [44–46]. Due to their potential to adversely affect human, animal and environmental health, international (PNEC) and local (SANS) guideline values have been set for allowable environmental concentrations. In this study, a total of 24 antibiotics were targeted, and we detected 14 across all samples, with the WWTP effluent samples recording the overall highest total antibiotic concentration compared to the other sites. This confirms the role of WWTPs as hotspots for the discharge of antibiotics into receiving water, as previously mentioned. This argument is further strengthened by the higher antibiotic concentrations observed in the downstream samples compared to the upstream samples (Figure 2; Table S3).

Sulfapyridine is a sulphonamide with allergenic properties and a by-product of sulfasalazine. The use and manufacture of this drug ended in 1990, and it is a known environmental pollutant [47]. However, the drug is still used in veterinary medicine to treat diarrhoea in dogs [48]. Sulfapyridine is not registered for use in South Africa (www.sahpra.org.za), indicating that its identification in this study, like in another recent South African study, suggests possible illegal use within the country [49]. Sulfamethoxazole is widely used in treating chronic bronchitis, urinary tract infections, enteric infections as well as pneumocystis pneumonia (PCP), which is an opportunistic infection in immunocompromised people such as HIV patients; it is also used in livestock animals treatment [50].

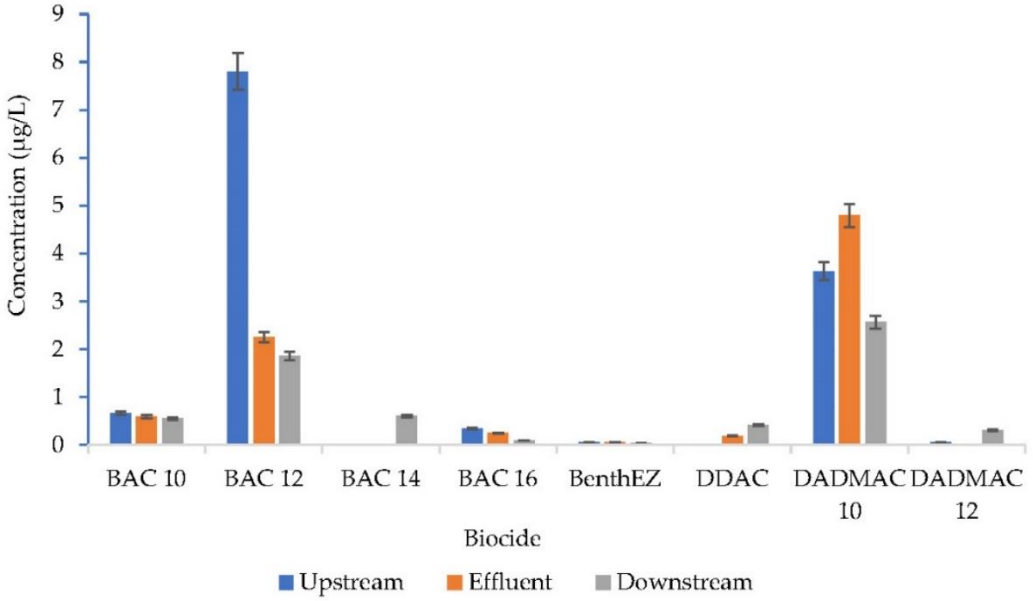

Figure 2. The overall distribution of biocide across sampling sites. Error bars represent the standard error of the mean.

In reviews conducted on antibiotics in African waters [7,50,51], it was reported that sulfamethoxazole was the most detected drug, as was the case in this study. This can be explained by the fact that South Africa has a high HIV prevalence rate, estimated at over 13%, translating to over 8 million people living with the infection [52], thus the extensive use of the drug within the country for PCP prophylaxis.

Most of the antibiotic concentrations detected in our study were comparable to those detected across Africa [9,50,53–56]. However, other antibiotics like tetracycline, doxycycline, amoxicillin, and lincomycin were detected at concentrations higher than those reported in other African countries [7,57,58]. Most importantly, some of these concentrations were higher than the recommended PNEC values, as observed for sulfamethoxazole tetracycline, oxytetracycline, lincomycin, and amoxicillin (Table 1). These high values indicate that the water environment may facilitate the selection of antimicrobial resistance in microbial communities. However, further studies would be required to establish such risk.

3.3. Biocides

Biocides have been widely used in different applications such as human medicine [59], water distribution pipelines to prevent microbially associated corrosion [60], water chemistry [61], antibiofilm coatings [62], and domestic/household products [63]. This has therefore led to their continuous discharge into the environment. Eight of the 10 targeted biocides detected in this study had quantifiable values (Table S5). Benzalkonium chloride (BAC) 12 had the highest detected concentration, while BenthEZ was the most detected in 75% of the samples. BAC 14 and DDAC were only detected in the effluent and downstream samples, again pointing to the role of WWTPs in surface water pollution. Additionally, the BAC 12 concentrations were higher than those reported in other studies [32,64–68]. This can be explained by the extensive use of BAC and its derivatives in detergents, disinfectants, hair shampoos and most personal care products [20–25]. Due to their potential deleterious effects, biocides have been included with detergents and regulated in South Africa by the National Regulator for Compulsory Specifications (NRCS) [69].

The concept of co-resistance is relatively broad depending on the specific study, although in its basic forms, it would imply the ability of an organism to harbour multiple resistance traits [70]. On the other hand, cross-resistance is resistance to several distinct

antimicrobials mediated by the same or one molecular mechanism [71]. Despite their usefulness, biocides have been associated with the induction and co-selection of antimicrobial resistance through co- and cross-resistance mechanisms [72], leading to the use of many of them being discouraged. For example, triclosan and triclocarban had values below the limits of quantification, indicating that these products are now being replaced as recommended by WHO in most household products in South Africa, even though they are not banned in South Africa. However, a previous study had reported higher values in South African water bodies [28], although it has been reported that QACs are more widely used in products within the WWTP catchment area. So the low concentration can be due to the effectiveness of the WWTP and the lack of particulate matter, as most biocides are not entirely solubilised in the aqueous state but can adhere to particulate matter and carbon-rich sediments [73,74]. Nevertheless, given that chemical adhesion to particulate matter was not investigated in the current study, further research is needed to understand the dynamics of these pharmaceuticals in the aquatic environment.

Notably, most of the biocides identified in the current study had higher concentrations than the recommended PNEC value of 0.01 µg/L [75]. The WWTP effluent point has the higher detection concentrations and serves as the point of greatest impact into the WWTP catchment area. This indicates that more effort needs to be applied, especially in the treatment plant, to reduce/remove them from the water.

4. Materials and Methods

4.1. Study Area and Sampling Site

The study was conducted at a WWTP serving the uMsunduzi Municipality that discharges water directly into the uMsunduzi River. The study site and a description of the study area have previously been described [76]. Samples were collected from the final effluent discharged into the river and upstream and downstream from the WWTP discharge point (Figure 3). These points were selected to provide insight into the potential role of the WWTP on the abundance of pollutants in the receiving water body.

4.2. Sample Collection, Processing, and Analysis

Samples were collected in February and March 2020. Triplicate grab water samples were collected hourly between 8 am and 2 pm on each sampling day into sterile 500 mL sample bottles. Samples were then pooled into the morning (8–11 am) and evening (1–4 pm) composite samples per sampling round. These samples were immediately frozen and shipped to the Agricultural Research Council, Pretoria (antibiotics and biocides analysis) and WaterLab (Pty) Ltd., Pretoria, South Africa (heavy metals analysis).

4.3. Metal Analysis

4.3.1. Digestion of Water Samples for Metal Analysis

The United States Environmental Protection Agency, US EPA method 2007:7 [77] for the digestion of water samples was used with slight modifications. Briefly, 3 mL of concentrated nitric acid (HNO_3) was added to 50 mL of the water sample in a beaker. The solution was heated at approximately 85 °C to less than 10 mL on a hot plate in a fume hood. The solution was allowed to cool, after which 5 mL of concentrated HNO_3 was added, and the mixture was heated until digestion was complete. Following cooling, 10 mL of 1:1 HCl: HNO_3 and 15 mL of deionised water were added. The solution was then heated for 15 min. The walls of the beaker and watch glass were rinsed with deionised water. The solution was filtered using a Whatman No 1 filter paper (Cytiva, MA, USA) into a 100 mL volumetric flask and made up to 100 mL mark with distilled water.

4.3.2. Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) Analysis of Heavy Metals

The digested samples were analysed for heavy metals using inductively coupled plasma-atomic emission spectrometry (ICP-OES) iCAP 6500 DUO (Thermo Scientific,

Waltham, MA, USA). The ICP-OES operating conditions used for the determination were 0.7 L/min nebuliser flow, 0.5 L/min auxiliary flow, 1.5 mL/ min sample uptake rate, 1150 W Radio Frequency power, plasma stabilisation time of 10 min and 12 L/min coolant gas flow. Heavy metal determination was achieved using Duo view, while 2-point background correction and three replicates were used to measure the analytical signal. The emission intensities were obtained for the most sensitive lines free of spectral interference. The calibration standards were prepared by diluting the 50 mg/L stock solution of a multi-element standard solution. The detection limit for each metal was obtained following the procedure outlined in the US EPA method 200.7 [77].

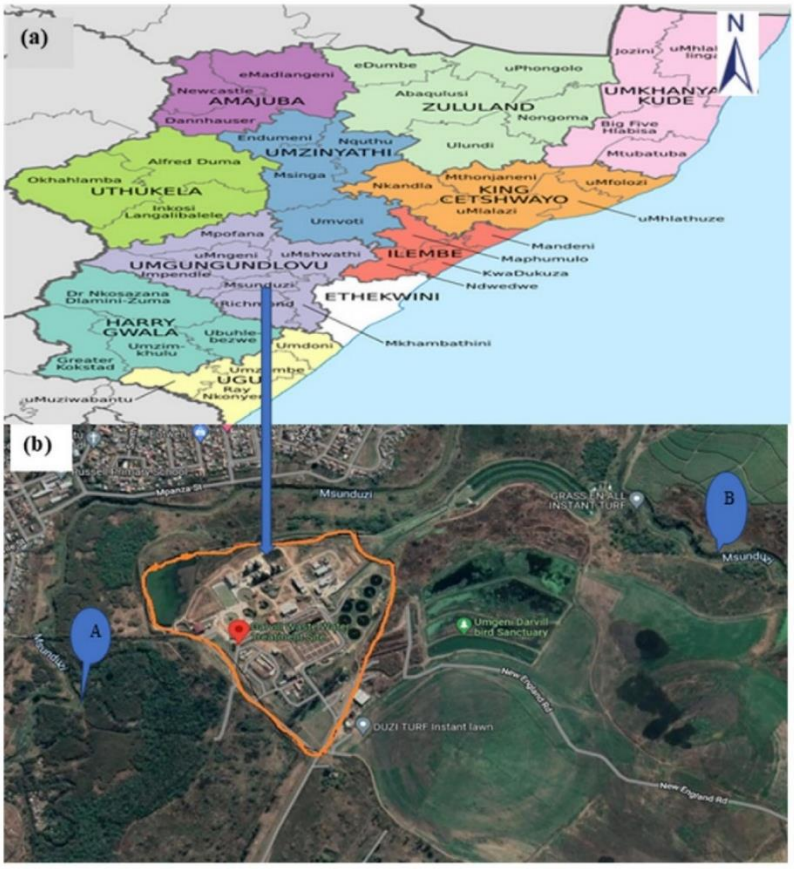

Figure 3. Map of the Wastewater Treatment Plant serving the uMsunduzi Municipality. (**a**) Study area and (**b**) Sampling points for this study (A) Upstream (29°36′02.60″ S 30°25′49.61″ E) (B) Downstream (29°36′27.54″ S 30°27′0.76″ E) (Afzelia Environmental Consultants cc. https://sahris.sahra.org.za/sites/Darvill/WWTP.pdf) (Location of Msunduzi Local Municipality within KwaZulu-Natal Coordinates: 29°37′ S 30°23′ E).

4.3.3. Antibiotics and Biocides Analysis

High purity (>97%) analytical standards of targeted analytes were used in this study. All methods were developed and optimised in-house.

4.3.4. Standard Preparation

Water samples were tested for the following sulphonamides: sulfadiazine, sulfathiazole, sulfamethazine, sulphapyridine, sulfamerazine, sulfamethoxypyridazine, sulfachloropyridazine, sulfadoxine, sulfaquinoxaline, sulfamethoxazole and sulfadimethoxine; four tetracyclines including, tetracycline, oxytetracycline, chlortetracycline and doxycy-

cline; two ionophores: lasalocid-A, monesin; one lincosamide: lincomycin; three β-lactams: penicillin, ampicillin and amoxicillin; two anthelmintics: oxfendazole and fenbendazole and one beta agonist: Clenbuterol (Sigma Aldrich, Kempton Park South Africa). For biocides, ammonium acetate and ten biocide standards were used, which included benzyldimethyldecyl-ammonium chloride (BAC-C10), benzyldimethyldodecyl-ammonium chloride (BAC-C12), benzyldimethyltetradecyl-ammonium chloride (BAC-C14), benzyldimethylhexadecyl-ammonium chloride (BAC-C16), benzethonium chloride (BenzEth), didecyldimethyl-ammonium chloride (DDAC), didodecyldimethyl-ammonium bromide (DDAB), dodecyltrimethyl-ammonium bromide (DTAB), triclosan and triclocarban (Sigma-Aldrich, Kempton Park, South Africa).

A 1.0 mg/L multi-residue stock solution containing a mixture of all antibiotic standards was prepared in methanol and kept at −20 °C throughout the analysis. From the stock solution, working standard solutions in the range of 0.5–100 μg L^{-1} were prepared and stored at 4 °C throughout the analysis [78]. A 10-mg/L stock solution containing the combination of the ten biocide standards was prepared in methanol. The solution was kept at −20 °C and was used to prepare 10 μg/L and 1000 ng/L working standard solutions in methanol. These operational standards were further used to prepare an 8-point calibration curve of 1 μg/L to 100 μg/L concentrations.

4.3.5. Sample Preparation and Extraction

Sample preparation and extraction of antibiotics followed a modified solid-phase extraction (SPE) method described previously [78]. Briefly, 500 mL of each water sample was first filtered through a 0.45 μm non-pyrogenic syringe filter (Sarstedt, Numbrecht, Germany) and then centrifuged at 8000 rpm at 4 °C for 10 min. After that, Hydrophile Lipophile Balance (HLB) Solid Phase Extractor (SPE) cartridges (Waters, Milford, MA, USA) were activated and conditioned using 5 mL of methanol, followed by 5 mL deionised water at an approximate flow rate of 5 mL/minute. The samples were passed through the cartridges at a slow flow rate of approximately one drop per second. Next, the cartridges were eluted with 5 mL methanol and 3 mL ethyl acetate. The eluents were then evaporated under a gentle stream of nitrogen to incipient dryness and reconstituted with 1 mL of methanol. These were then filtered into an autosampler vial through a 0.22 μm nylon membrane syringe filter. Three matrix blanks were prepared using deionised water, and a six-point matrix-matched calibration curve, fortified with 0.5 ng/mL, 2 ng/mL, 10 ng/mL, 50 ng/mL, 100 ng/mL, 150 ng/mL, 200 ng/mL and 400 ng/mL were included and processed like the samples.

Five hundred millilitres (500 mL) of water sample were filtered through a 0.45 μm non-pyrogenic syringe filter into a separating funnel to extract biocides. The samples were extracted by solid phase extraction using 6 mL Oasis WCX cartridges (500 mg, 60 μm) (Waters, Milford, MA, USA). First, the cartridge was conditioned with 5 mL each of methanol and deionised water (resistivity of 18.2 Ω). Then, the samples were loaded onto the cartridge at a steady flow rate of approximately 5 mL/min. Next, the cartridge was dried under a vacuum and eluted with 5 mL of 1% formic acid in acetonitrile. The eluate was then concentrated to incipient dryness under a gentle stream of nitrogen at 40 °C and reconstituted in 1 mL of 10 mM ammonium acetate in methanol and filtered through 0.22 μm nylon syringe filter into an HPLC vial.

Deionised water was used to prepare three matrix blanks and an eight-point matrix-matched calibration curve, fortified with 1 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, 30 ng/mL, 40 ng/mL, 50, ng/mL and 100 ng/mL, following the same procedure as the sample preparation.

4.3.6. Instrumental Analysis

The chromatographic separation of target analytes was achieved using a PerkinElmer® LX-50 UHPLC system (Perkin Elmer, Waltham, MA, USA), equipped with a PerkinElmer® Brownlee SPP (Superficially Porous Particles) C18 (2.7 μm: 100 × 2.1 mm) column. The

column oven temperature was set at 40 °C. Separation was attained using 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) as the mobile phases at a constant flow rate of 0.4 mL/min and a sample injection volume of 10 µL. The total run time was 17.2 min, with an equilibration time of 3.5 min between runs.

Chromatographic separation of biocides was achieved using the same PerkinElmer LX-50 UHPLCequipped with a 2.1 × 100mm, 1.7 µm Phenomenex® Kinetex® C_{18} column (Phenomenex, Torrance, CA, USA). The column oven temperature was set at 50 °C, while 10 µL of the sample was injected. The biocides were separated using a linear gradient of 10 mM ammonium acetate in water (solvent A) and methanol (solvent B). These were eluted at a constant flow rate of 0.8 mL/minute. An initial flow of 90% A was held for 5 min, then decreased to 20% for 4.1 min. Following this, the flow was held at 100% B for 3.1 min and then changed to 90% A for 0.1 min, bringing the total run time to 12.3 min. This was followed by 3.5 min of equilibration.

Target antibiotics were identified and quantified using a PerkinElmer® Qsight™ 220 triple quadruple mass spectrometer (TQMS) (PerkinElmer South Africa (Pty) Ltd., Midrand, South Africa) in the positive and negative electrospray ionisation mode. The electrospray voltage was set at 5000 V, with nitrogen as the nebuliser and drying gas at 200 and 120, respectively. The ion source temperature was set at 400 °C, while the Hot-Surface Induced Desolvation (HSID) temperature was set at 320 °C. A time-managed multiple reaction monitoring (MRM) mode was used to acquire the different analytes. At least two MRM transitions were used to positively identify and confirm the presence of the analytes in a sample. The entrance voltages (EV), collision energies (CE) and cell exit potential (CXP) were optimised for each analyte individually. Instrumental data were acquired using Perkin Elmer Simplicity™ 3Q software (version 1.4.1806.29651) (PerkinElmer, Buckinghamshire, UK).

Each biocide was identified and confirmed with a PerkinElmer® Qsight™ 220 TQMS (Waltham, MA, USA) operated in both the positive and negative electrospray ionisation modes. Nitrogen was used as the nebuliser and drying gas for both the positive and negative modes, and these were set at 60 and 120 arbitrary units, respectively. The ion source temperature was kept at 450 °C, and the optimised HSID temperature was set at 320 °C. The electrospray voltage was set at −3000 V for the negative and 2500 V for the positive ionisation modes. A time-managed MRM mode was used to acquire biocides. The CE, EV and CXP were individually optimised in-house for each analyte.

4.4. Statistical Analysis

A one-way analysis of variance (ANOVA) with a Games-Howell posthoc test was performed to compare the mean concentrations of the various parameters between the sampling points. Concentrations below the limits of quantification (LOQ) were treated as zero. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) Version 27 and were considered significant at $p \leq 0.05$.

5. Conclusions

This study evaluated the concentration of antibiotics, heavy metals, biocides, and antibiotic-resistant bacteria in a WWTP and its receiving water body and observed that most of the chemical pollutants were higher than the maximum allowable environmental concentrations and the PNEC as applicable. In addition, sulphonamides, β-lactams and tetracyclines were the most detected antibiotics and targeted QACs were detected at significant concentrations, indicating anthropogenic pollution. Furthermore, higher concentrations were observed in the effluent and downstream samples compared to the upstream, suggesting that the WWTP significantly contributed to the downstream pollution. However, while the current study provides valuable information regarding the presence of metals, antibiotics and biocides in the environment, some of which had concentrations above acceptable levels, these results should not be generalised as the sampling regime was not robust enough. For example, some unexplained occurrences like the presence of some pollutants like penicillin in the downstream samples, albeit their absence in the

upstream and effluent samples. These could be due to natural occurrences in the environment, although the extremely low concentrations could be due to the sensitivity of the instrumentation used. Nevertheless, more robust studies involving extensive sampling and exposure experiments are needed to ascertain if the observed environmental pollutant concentrations could induce resistance to antimicrobials in previously susceptible bacteria.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics12071166/s1, Table S1: Overall distribution of metals at the study site; Table S2: Comparison of metal concentrations between sampling points; Table S3: Antibiotics distribution across all sampling points; Table S4: Comparison of antibiotic concentrations between sampling points; Table S5: Concentration of biocides at sampling sites.

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References

1. Bengtsson-Palme, J.; Larsson, D.G.J. Concentrations of Antibiotics Predicted to Select for Resistant Bacteria: Proposed Limits for Environmental Regulation. *Environ. Int.* **2016**, *86*, 140–149. [CrossRef] [PubMed]
2. Martinez, J.L. The Role of Natural Environments in the Evolution of Resistance Traits in Pathogenic Bacteria. *Proc. R. Soc. B Biol. Sci.* **2009**, *276*, 2521–2530. [CrossRef]
3. Larsson, D.G.J. Antibiotics in the Environment. *Ups. J. Med. Sci.* **2014**, *119*, 108–112. [CrossRef] [PubMed]
4. Ebele, A.J.; Oluseyi, T.; Drage, D.S.; Harrad, S.; Abou-Elwafa Abdallah, M. Occurrence, Seasonal Variation and Human Exposure to Pharmaceuticals and Personal Care Products in Surface Water, Groundwater and Drinking Water in Lagos State, Nigeria. *Emerg. Contam.* **2020**, *6*, 124–132. [CrossRef]
5. Ergie, A.A.; Leng, Y.; Wang, J. Antibiotics and Resistance Genes in Awash River Basin, Ethiopia. *Ecohealth* **2019**, *16*, 441–453. [CrossRef]
6. Kandie, F.J.; Krauss, M.; Beckers, L.M.; Massei, R.; Fillinger, U.; Becker, J.; Liess, M.; Torto, B.; Brack, W. Occurrence and Risk Assessment of Organic Micropollutants in Freshwater Systems within the Lake Victoria South Basin, Kenya. *Sci. Total Environ.* **2020**, *714*, 136748. [CrossRef]
7. Madikizela, L.M.; Ncube, S.; Chimuka, L. Analysis, Occurrence and Removal of Pharmaceuticals in African Water Resources: A Current Status. *J. Environ. Manag.* **2020**, *253*, 109741. [CrossRef]
8. Matongo, S.; Birungi, G.; Moodley, B.; Ndungu, P. Occurrence of Selected Pharmaceuticals in Water and Sediment of Umgeni River, KwaZulu-Natal, South Africa. *Environ. Sci. Pollut. Res.* **2015**, *22*, 10298–10308. [CrossRef]
9. Segura, P.A.; Takada, H.; Correa, J.A.; El Saadi, K.; Koike, T.; Onwona-Agyeman, S.; Ofosu-Anim, J.; Sabi, E.B.; Wasonga, O.V.; Mghalu, J.M.; et al. Global Occurrence of Anti-Infectives in Contaminated Surface Waters: Impact of Income Inequality between Countries. *Environ. Int.* **2015**, *80*, 89–97. [CrossRef]

10. Azanu, D.; Styrishave, B.; Darko, G.; Weisser, J.J.; Abaidoo, R.C. Occurrence and Risk Assessment of Antibiotics in Water and Lettuce in Ghana. *Sci. Total Environ.* **2018**, *622–623*, 293–305. [CrossRef]
11. Moslah, B.; Hapeshi, E.; Jrad, A.; Fatta-Kassinos, D.; Hedhili, A. Pharmaceuticals and Illicit Drugs in Wastewater Samples in North-Eastern Tunisia. *Environ. Sci. Pollut. Res.* **2018**, *25*, 18226–18241. [CrossRef]
12. Fayiga, A.O.; Ipinmoroti, M.O.; Chirenje, T. *Environmental Pollution in Africa*; Springer: Dordrecht, The Netherlands, 2018; Volume 20, ISBN 1066801698944.
13. Akele, M.L.; Kelderman, P.; Koning, C.W.; Irvine, K. Trace Metal Distributions in the Sediments of the Little Akaki River, Addis Ababa, Ethiopia. *Environ. Monit. Assess.* **2016**, *188*, 389. [CrossRef] [PubMed]
14. Diop, C.; Dewaelé, D.; Cazier, F.; Diouf, A.; Ouddane, B. Assessment of Trace Metals Contamination Level, Bioavailability and Toxicity in Sediments from Dakar Coast and Saint Louis Estuary in Senegal, West Africa. *Chemosphere* **2015**, *138*, 980–987. [CrossRef] [PubMed]
15. Edokpayi, J.; Odiyo, J.; Popoola, O.; Msagati, T. Assessment of Trace Metals Contamination of Surface Water and Sediment: A Case Study of Mvudi River, South Africa. *Sustainability* **2016**, *8*, 135. [CrossRef]
16. Agoro, M.A.; Adeniji, A.O.; Adefisoye, M.A.; Okoh, O.O. Heavy Metals in Wastewater and Sewage Sludge from Selected Municipal Treatment Plants in Eastern Cape Province, South Africa. *Water* **2020**, *12*, 2746. [CrossRef]
17. European Commission. *Directive 98/5/EC of the European Parliament and of the Council of 16 February 1998*; European Commission: Brussels, Belgium, 1998.
18. De Bruijn, J.; Hansen, B.; Johansson, S.; Luotamo, M.; Munn, S.; Musset, C.; Olsen, S.; Olsson, H.; Paya-Perez, A.; Pedersen, F.; et al. *Technical Guidance Document on Risk Assessment. Part 1. Part 2*; EUR 20418 EN. 2002. JRC23785; European Commission: Luxembourg, 2002.
19. Chen, Z.F.; Ying, G.G.; Lai, H.J.; Chen, F.; Su, H.C.; Liu, Y.S.; Peng, F.Q.; Zhao, J.L. Determination of Biocides in Different Environmental Matrices by Use of Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry. *Anal. Bioanal. Chem.* **2012**, *404*, 3175–3188. [CrossRef]
20. Schweizer, H.P. Triclosan: A Widely Used Biocide and Its Link to Antibiotics. *FEMS Microbiol. Lett.* **2001**, *202*, 1–7. [CrossRef]
21. Du, S.; McLaughlin, B.A.; Pal, S.; Aizenman, E. In Vitro Neurotoxicity of Methylisothiazolinone, a Commonly Used Industrial and Household Biocide, Proceeds via a Zinc and Extracellular Signal-Regulated Kinase Mitogen-Activated Protein Kinase-Dependent Pathway. *J. Neurosci.* **2002**, *22*, 7408–7416. [CrossRef]
22. Zhao, J.L.; Zhang, Q.Q.; Chen, F.; Wang, L.; Ying, G.G.; Liu, Y.S.; Yang, B.; Zhou, L.J.; Liu, S.; Su, H.C.; et al. Evaluation of Triclosan and Triclocarban at River Basin Scale Using Monitoring and Modeling Tools: Implications for Controlling of Urban Domestic Sewage Discharge. *Water Res.* **2013**, *47*, 395–405. [CrossRef]
23. Chen, Z.F.; Ying, G.G.; Liu, Y.S.; Zhang, Q.Q.; Zhao, J.L.; Liu, S.S.; Chen, J.; Peng, F.J.; Lai, H.J.; Pan, C.G. Triclosan as a Surrogate for Household Biocides: An Investigation into Biocides in Aquatic Environments of a Highly Urbanised Region. *Water Res.* **2014**, *58*, 269–279. [CrossRef]
24. Nuñez, O.; Moyano, E.; Galceran, M.T. Determination of Quaternary Ammonium Biocides by Liquid Chromatography-Mass Spectrometry. *J. Chromatogr. A* **2004**, *1058*, 89–95. [CrossRef]
25. Montaseri, H.; Forbes, P.B.C. A Review of Monitoring Methods for Triclosan and Its Occurrence in Aquatic Environments. *TrAC-Trends Anal. Chem.* **2016**, *85*, 221–231. [CrossRef]
26. Healy, M.G.; Fenton, O.; Cormican, M.; Peyton, D.P.; Ordsmith, N.; Kimber, K.; Morrison, L. Antimicrobial Compounds (Triclosan and Triclocarban) in Sewage Sludges, and Their Presence in Runoff Following Land Application. *Ecotoxicol. Environ. Saf.* **2017**, *142*, 448–453. [CrossRef] [PubMed]
27. Juksu, K.; Zhao, J.L.; Liu, Y.S.; Yao, L.; Sarin, C.; Sreesai, S.; Klomjek, P.; Jiang, Y.X.; Ying, G.G. Occurrence, Fate and Risk Assessment of Biocides in Wastewater Treatment Plants and Aquatic Environments in Thailand. *Sci. Total Environ.* **2019**, *690*, 1110–1119. [CrossRef]
28. Lehutso, R.F.; Daso, A.P.; Okonkwo, J.O. Occurrence and Environmental Levels of Triclosan and Triclocarban in Selected Wastewater Treatment Plants in Gauteng Province, South Africa. *Emerg. Contam.* **2017**, *3*, 107–114. [CrossRef]
29. Li, X.; Brownawell, B.J. Quaternary Ammonium Compounds in Urban Estuarine Sediment Environments—A Class of Contaminants in Need of Increased Attention? *Environ. Sci. Technol.* **2010**, *44*, 7561–7568. [CrossRef] [PubMed]
30. Liu, W.R.; Zhao, J.L.; Liu, Y.S.; Chen, Z.F.; Yang, Y.Y.; Zhang, Q.Q.; Ying, G.G. Biocides in the Yangtze River of China: Spatiotemporal Distribution, Mass Load and Risk Assessment. *Environ. Pollut.* **2015**, *200*, 53–63. [CrossRef] [PubMed]
31. Mao, H.; Li, H.; Li, Y.; Li, L.; Yin, L.; Yang, Z. Four Typical Personal Care Products in a Municipal Wastewater Treatment Plant in China: Occurrence, Removal Efficiency, Mass Loading and Emission. *Ecotoxicol. Environ. Saf.* **2020**, *188*, 109818. [CrossRef] [PubMed]
32. Martínez-Carballo, E.; González-Barreiro, C.; Sitka, A.; Kreuzinger, N.; Scharf, S.; Gans, O. Determination of Selected Quaternary Ammonium Compounds by Liquid Chromatography with Mass Spectrometry. Part II. Application to Sediment and Sludge Samples in Austria. *Environ. Pollut.* **2007**, *146*, 543–547. [CrossRef]
33. AMR Industry Alliance List of Predicted No-Effect Concentrations (PNECs). *AMR Industry Alliance Antibiotic Discharge Targets*; AMR Industry Alliance: Geneva, Switzerland, 2021; pp. 1–5.
34. SANS South African National Standard (SANS). *Drink. Water SANS* **2015**, *241*, 1–2.

35. Chetty, S.; Pillay, L. Assessing the Influence of Human Activities on River Health: A Case for Two South African Rivers with Differing Pollutant Sources. *Environ. Monit. Assess.* **2019**, *191*, 168. [CrossRef]
36. Awofolu, O.R.; Mbolekwa, Z.; Mtshemla, V.; Fatoki, O.S. Levels of Trace Metals in Water and Sediment from Tyume River and Its Effects on an Irrigated Farmland. *Water SA* **2005**, *31*, 87–94. [CrossRef]
37. Letsoalo, M.R.; Godeto, T.W.; Magadzu, T.; Ambushe, A.A. Quantitative Speciation of Arsenic in Water and Sediment Samples from the Mokolo River in Limpopo Province, South Africa. *Anal. Lett.* **2018**, *51*, 2761–2775. [CrossRef]
38. Perera, P.C.T.; Sundarabarathy, T.V.; Sivananthawerl, T.; Kodithuwakku, S.P.; Edirisinghe, U. Arsenic and Cadmium Contamination in Water, Sediments and Fish Is a Consequence of Paddy Cultivation: Evidence of River Pollution in Sri Lanka. *Achiev. Life Sci.* **2016**, *10*, 144–160. [CrossRef]
39. Xu, Y.; Tan, L.; Li, Q.; Zheng, X.; Liu, W. Sublethal Concentrations of Heavy Metals Cu^{2+} and Zn^{2+} Can Induce the Emergence of Bacterial Multidrug Resistance. *Environ. Technol. Innov.* **2022**, *27*, 102379. [CrossRef]
40. Stepanauskas, R.; Glenn, T.C.; Jagoe, C.H.; Tuckfield, R.C.; Lindell, A.H.; King, C.J.; McArthur, J.V. Coselection for Microbial Resistance to Metals and Antibiotics in Freshwater Microcosms. *Environ. Microbiol.* **2006**, *8*, 1510–1514. [CrossRef] [PubMed]
41. Li, X.; Gu, A.Z.; Zhang, Y.; Xie, B.; Li, D.; Chen, J. Sub-Lethal Concentrations of Heavy Metals Induce Antibiotic Resistance via Mutagenesis. *J. Hazard. Mater.* **2019**, *369*, 9–16. [CrossRef]
42. Chen, J.; Li, J.; Zhang, H.; Shi, W.; Liu, Y. Bacterial Heavy-Metal and Antibiotic Resistance Genes in a Copper Tailing Dam Area in Northern China. *Front. Microbiol.* **2019**, *10*, 1916. [CrossRef]
43. Marshall, B.M.; Levy, S.B. Food Animals and Antimicrobials: Impacts on Human Health. *Clin. Microbiol. Rev.* **2011**, *24*, 718–733. [CrossRef]
44. Michael, I.; Rizzo, L.; Mcardell, C.S.; Manaia, C.M.; Merlin, C.; Schwartz, T.; Dagot, C.; Fatta-kassinos, D. Urban Wastewater Treatment Plants as Hotspots for the Release of Antibiotics in the Environment: A Review. *Water Res.* **2012**, *47*, 957–995. [CrossRef]
45. Batt, A.L.; Bruce, I.B.; Aga, D.S. Evaluating the Vulnerability of Surface Waters to Antibiotic Contamination from Varying Wastewater Treatment Plant Discharges. *Environ. Pollut.* **2006**, *142*, 295–302. [CrossRef]
46. Mutiyar, P.K.; Mittal, A.K. Occurrences and Fate of Selected Human Antibiotics in Influents and Effluents of Sewage Treatment Plant and Effluent-Receiving River Yamuna in Delhi (India). *Environ. Monit. Assess.* **2014**, *186*, 541–557. [CrossRef] [PubMed]
47. National Center for Biotechnology Information PubChem Compound Summary for CID 5336, Sulfapyridine. Available online: https://pubchem.ncbi.nlm.nih.gov/compound/sulfapyridine (accessed on 4 December 2021).
48. Marks, S.L. Diarrhea. In *Canine and Feline Gastroenterology*; Elsevier: Amsterdam, The Netherlands, 2013; pp. 99–108, ISBN 9781416036616.
49. Ncube, S.; Nuapia, Y.B.; Chimuka, L.; Madikizela, L.M.; Etale, A. Trace Detection and Quantitation of Antibiotics in a South African Stream Receiving Wastewater Effluents and Municipal Dumpsite Leachates. *Front. Environ. Sci.* **2021**, *9*, 733065. [CrossRef]
50. Fekadu, S.; Alemayehu, E.; Dewil, R.; Van der Bruggen, B. Pharmaceuticals in Freshwater Aquatic Environments: A Comparison of the African and European Challenge. *Sci. Total Environ.* **2019**, *654*, 324–337. [CrossRef]
51. K'oreje, K.O.; Okoth, M.; Van Langenhove, H.; Demeestere, K. Occurrence and Treatment of Contaminants of Emerging Concern in the African Aquatic Environment: Literature Review and a Look Ahead. *J. Environ. Manag.* **2020**, *254*, 109752. [CrossRef]
52. Statistics South Africa (Stats SA). *Statistical Release P0302: Mid-Year Population Estimates 2021 (Embargoed until 19th July 2010 10:00)*; Statistics South Africa: Pretoria, South Africa, 2021.
53. Agunbiade, F.O.; Moodley, B. Occurrence and Distribution Pattern of Acidic Pharmaceuticals in Surface Water, Wastewater, and Sediment of the Msunduzi River, Kwazulu-Natal, South Africa. *Environ. Toxicol. Chem.* **2016**, *35*, 36–46. [CrossRef]
54. Archer, E.; Petrie, B.; Kasprzyk-Hordern, B.; Wolfaardt, G.M. The Fate of Pharmaceuticals and Personal Care Products (PPCPs), Endocrine Disrupting Contaminants (EDCs), Metabolites and Illicit Drugs in a WWTW and Environmental Waters. *Chemosphere* **2017**, *174*, 437–446. [CrossRef] [PubMed]
55. Ngumba, E.; Gachanja, A.; Tuhkanen, T. Occurrence of Selected Antibiotics and Antiretroviral Drugs in Nairobi River Basin, Kenya. *Sci. Total Environ.* **2016**, *539*, 206–213. [CrossRef]
56. K'oreje, K.O.; Vergeynst, L.; Ombaka, D.; De Wispelaere, P.; Okoth, M.; Van Langenhove, H.; Demeestere, K. Occurrence Patterns of Pharmaceutical Residues in Wastewater, Surface Water and Groundwater of Nairobi and Kisumu City, Kenya. *Chemosphere* **2016**, *149*, 238–244. [CrossRef]
57. Kairigo, P.; Ngumba, E.; Sundberg, L.R.; Gachanja, A.; Tuhkanen, T. Occurrence of Antibiotics and Risk of Antibiotic Resistance Evolution in Selected Kenyan Wastewaters, Surface Waters and Sediments. *Sci. Total Environ.* **2020**, *720*, 137580. [CrossRef]
58. Tahrani, L.; Mehri, I.; Reyns, T.; Anthonissen, R.; Verschaeve, L.; Khalifa, A.B.H.; Van Loco, J.; Abdenaceur, H.; Mansour, H. Ben UPLC-MS/MS Analysis of Antibiotics in Pharmaceutical Effluent in Tunisia: Ecotoxicological Impact and Multi-Resistant Bacteria Dissemination. *Arch. Microbiol.* **2018**, *200*, 553–565. [CrossRef]
59. SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks). Assessment of the Antibiotic Resistance Effects of Biocides. *Environment* **2009**, *68*, 1–87.
60. Javaherdashti, R.; Akvan, F. Microbiologically Influenced Corrosion (MIC). In *Failure Modes, Effects and Causes of Microbiologically Influenced Corrosion*; Elsevier: Amsterdam, The Netherlands, 2020; pp. 43–104, ISBN 9780128184486.
61. Ceng, G.S. Water and Effluents. In *Plant Engineer's Reference Book*; Reed Educational and Professional Publishing Ltd.: Boston, MA, USA, 2002; pp. 1–16.

62. Ünal, H. Antibiofilm Coatings. In *Handbook of Antimicrobial Coatings*; Elsevier: Amsterdam, The Netherlands, 2018; pp. 301–319, ISBN 9780128119822.
63. Kim, S.; Seo, M.; Na, M.; Kim, J. Investigation on Combined Inhalation Exposure Scenarios to Biocidal Mixtures: Biocidal and Household Chemical Products in South Korea. *Toxics* **2021**, *9*, 32. [CrossRef]
64. Ferrer, I.; Furlong, E.T. Identification of Alkyl Dimethylbenzylammonium Surfactants in Water Samples by Solid-Phase Extraction Followed by Ion Trap LC/MS and LC/MS/MS. *Environ. Sci. Technol.* **2001**, *35*, 2583–2588. [CrossRef]
65. Olkowska, E.; Polkowska, Z.; Namieśnik, J. A Solid Phase Extraction-Ion Chromatography with Conductivity Detection Procedure for Determining Cationic Surfactants in Surface Water Samples. *Talanta* **2013**, *116*, 210–216. [CrossRef]
66. Berendonk, T.U.; Manaia, C.M.; Merlin, C.; Kassinos, D.F.; Cytryn, E.; Walsh, F.; Bürgmann, H.; Huovinen, P.; Stefani, S.; Schwartz, T.; et al. Tackling Antibiotic Resistance: The Environmental Framework. *Nat. Rev. Microbiol.* **2013**, *13*, 310–317. [CrossRef] [PubMed]
67. Kreuzinger, N.; Fuerhacker, M.; Scharf, S.; Uhl, M.; Gans, O.; Grillitsch, B. Methodological Approach towards the Environmental Significance of Uncharacterized Substances—Quaternary Ammonium Compounds as an Example. *Desalination* **2007**, *215*, 209–222. [CrossRef]
68. Santos, M.A.d.O.; Vianna, M.F.; Nishino, L.K.; Lazarini, P.R. Vestibular Disorders in Bell's Palsy: A Prospective Study. *Rev. Laryngol. Otol. Rhinol.* **2015**, *136*, 29–31.
69. NRCS Chemical Registration in South Africa, Hazardous Substances Act, GHS. Available online: https://csra.freyrsolutions.com/chemical-regulatory-services-in-south-africa (accessed on 15 April 2023).
70. Wong, P.H.P.; von Krosigk, M.; Roscoe, D.L.; Lau, T.T.Y.; Yousefi, M.; Bowie, W.R. Antimicrobial Co-Resistance Patterns of Gram-Negative Bacilli Isolated from Bloodstream Infections: A Longitudinal Epidemiological Study from 2002–2011. *BMC Infect. Dis.* **2014**, *14*, 393. [CrossRef] [PubMed]
71. Colclough, A.; Corander, J.; Sheppard, S.K.; Bayliss, S.C.; Vos, M. Patterns of Cross-Resistance and Collateral Sensitivity between Clinical Antibiotics and Natural Antimicrobials. *Evol. Appl.* **2019**, *12*, 878–887. [CrossRef]
72. Ruiz, L.; Alvarez-Ordóñez, A. *The Role of the Food Chain in the Spread of Antimicrobial Resistance (AMR)*; Elsevier Inc.: Amsterdam, The Netherlands, 2017; ISBN 9780323417372.
73. Tezel, U.; Pavlostathis, S.G. Quaternary Ammonium Disinfectants: Microbial Adaptation, Degradation and Ecology. *Curr. Opin. Biotechnol.* **2015**, *33*, 296–304. [CrossRef] [PubMed]
74. Mc Cay, P.H.; Ocampo-Sosa, A.A.; Fleming, G.T.A. Effect of Subinhibitory Concentrations of Benzalkonium Chloride on the Competitiveness of Pseudomonas Aeruginosa Grown in Continuous Culture. *Microbiology* **2010**, *156*, 30–38. [CrossRef]
75. European Chemical Agency (ECHA). *Guidance on the Biocidal Products Regulation: Volume I: Identity of the Active Substance/Physico-Chemical Properties/Analytical Methodology—Parts A+B+C: Information Requirements, Evaluation and Assessment*; European Chemicals Agency: Helsinki, Finland, 2022; Volume III, ISBN 9789290205029.
76. Mbanga, J.; Abia, A.L.K.; Amoako, D.G.; Essack, S.Y. Quantitative Microbial Risk Assessment for Waterborne Pathogens in a Wastewater Treatment Plant and Its Receiving Surface Water Body. *BMC Microbiol.* **2020**, *20*, 346. [CrossRef] [PubMed]
77. EPA (US Environmental Protection Agency). *METHOD 200.7—Determination of Elements and Trace Elements in Water and Wastes by Inductively Coupled Plasma-Atomic Emmission Spectropemtry*; US Environmental Protection Agency: Washington, DC, USA, 1994; Volume EPA/600/4.
78. Abafe, O.A.; Späth, J.; Fick, J.; Jansson, S.; Buckley, C.; Stark, A.; Pietruschka, B.; Martincigh, B.S. LC-MS/MS Determination of Antiretroviral Drugs in Influents and Effluents from Wastewater Treatment Plants in KwaZulu-Natal, South Africa. *Chemosphere* **2018**, *200*, 660–670. [CrossRef] [PubMed]

CHAPTER 3

ENVIRONMENTAL CONCENTRATIONS OF ANTIBIOTICS, BIOCIDES, AND HEAVY METAL FAIL TO INDUCE PHENOTYPIC ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI*

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Author contributions

Kelechi B. Chukwu – As principal investigator, co-conceptualised the study, conducted the laboratory work, analysed the data, and drafted the manuscript.

Professor A.L.K. Abia – As the main supervisor, co-conceptualised the study, supervised the laboratory work and critically revised the manuscript.

Dr Ovokeroye A. Abafe –Co-conceptualised the study, guided laboratory works, and critically revised the manuscript.

Daniel G. Amoako – Co-conceptualised the study, guided laboratory works, and critically revised the manuscript

Professor S.Y. Essack – As the Co-supervisor, co-conceptualized the study, guided the literature review and ethical clearance application, enabled data collection and analysis and undertook the critical revision of the manuscript.

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Environmental concentrations of antibiotics, biocides, and heavy metals fail to induce phenotypic antimicrobial resistance in *Escherichia coli*

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ABSTRACT

Most anthropogenically affected environments contain mixtures of pollutants from different sources. The impact of these pollutants is usually the combined effect of the individual polluting constituents. However, how these stressors contribute to the development of antimicrobial resistance in environmental microorganisms is poorly understood. Thus, a 30-day exposure experiment to environmental and sub-inhibitory concentrations of oxytetracycline, amoxicillin, zinc, copper, BAC (benzalkonium chloride) 10 and DADMAC (diallyldimethylammonium chloride) 12, was conducted using fully susceptible *E. coli* ATCC 25922 to ascertain any development of phenotypic or genotypic resistance. Furthermore, wild-type isolates were collected from the same aquatic environment as the stressors, analysed for phenotypic resistance using the disk diffusion method and genotypically through whole genome sequencing. Exposure to the various concentrations and combinations of the stressors did not trigger phenotypic resistance in the experimental bacteria. Furthermore, genotypic analysis of the WGS on the exposed isolates only found the macrolide resistance *mdf*(A) gene (also present in the control strain) and the disinfectant resistance gene *sit*ABCD. With further analysis for single nucleotide variants (SNV), mutations were detected for 19 genes that encoded for oxidative stress, DNA repair, membrane proteins efflux systems, growth and persister formations except for the *robA*, a transcription protein subset of the *ArcC/XylS* family of proteins, which confer multidrug resistance in *E. coli*. This indicates that exposure to sub-inhibitory concentrations of antibiotics, heavy metals and biocide residues in the aquatic environmental concentrations of the stressors identified in the current study could not induce phenotypic or genotypic resistance but encoded for genes responsible for the development of persistence and tolerance in bacteria, which could be a precursor to the development of resistance in environmental bacteria.

1. Introduction

Antimicrobial resistance (AMR) is a global phenomenon leading to the loss of millions of lives and trillions of dollars worth of human, animal and environmental health resources (Bengtsson-Palme and Larsson, 2015). Resistance genes have always been found in the environment long before the discovery of antibiotics (Allen et al., 2010; Bhullar et al., 2012; Costa et al., 2011; Lau et al., 2017). However, synthesising modern antimicrobials and their subsequent discharge into the environment through diverse routes have favoured the development and exponential increase of resistance in environmental bacteria, making the environment a favourable milieu for antibiotic resistance dissemination. Some studies have successfully correlated significant changes in ARGs developments to environmental stressors, thus the need for increased research in this area (Chait et al., 2012).

Stressors that have been reported include antibiotics and their residues (Azanu et al., 2018; Ebele et al., 2017; Kandie et al., 2020; Lee et al., 2017), biocides (Gao et al., 2020; Gautam et al., 2014; Zhu et al.,

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2019) and heavy metals (Chetty and Pillay, 2019; Ebenebe et al., 2017; Edokpayi et al., 2016; Eliku and Leta, 2018; Naggar, 2018). It is believed that these pollutants can remain active in the environment, exerting selective pressure on the associated microbial community, leading to the development of antibiotic resistance. Increased antibiotic resistance and metal tolerance have been observed with increased environmental metal concentrations worldwide (Manegabe et al., 2017). Limits (such as MIC and PNEC) below which no selection pressure is expected have been recommended for the environmental concentrations of these pollutants (AMR Industry Alliance, 2021; Bengtsson-Palme and Larsson, 2016).

Several studies have suggested that at sub-inhibitory concentrations of some of these stressors, bacteria can bio-accumulate and develop chronic responses, like antibiotics, heavy metal and biocide resistance through the induction of antimicrobial resistance genes (ARGs) (Andersson and Hughes, 2012; Gu et al., 2020; Holmes et al., 2016; Li et al., 2019). For example, Murray et al. (2019) exposed bacteria to sub-inhibitory concentrations of benzalkonium chloride (BAC), ciprofloxacin (CIP), chromium and trimethoprim for seven days. They observed that CIP-exposed isolates had the greatest selective capacity, developing multidrug resistance compared with the others. In another study, Christopher et al. (2014) assessed metal tolerance and its association with multidrug resistance among bacteria (*E. coli*, *Enterococcus*, *Salmonella*, *Shigella* and *Vibrio* spp.). The authors observed significant association between chromium (Cr) and nickel (Ni) tolerance and cefuroxime resistance and between Hg tolerance and ampicillin resistance. The authors further reported a strong relationship between zinc (Zn), vanadium (Va) and Ni, tolerance and ampicillin resistance, copper (Cu) tolerance and penicillin-resistant, Cd tolerance and erythromycin resistance and Hg tolerance and bacitracin resistance.

A study in Iran investigated biocide resistance in *P. aeruginosa* and attributed such resistance to the *qacEΔ1*, *qacE*, *qacG* and *cepA* (all efflux-pump gene) as well as the, *fabV* and *fabI* triclosan-sensitive enoyl-acyl-carrier reductase (ENR) (Namaki et al., 2022). Similarly, a review reported co-resistance between DDAC and cefotaxime, chloramphenicol and florfenicol in *E. coli* and between triclosan and chloramphenicol, erythromycin, imipenem, and tetracycline in *Salmonella*, which they inferred was due to enhanced efflux pump mechanisms, while in *S. aureus* they observed co-resistance between triclosan and ciprofloxacin, through the alteration of the cell membrane structure and function (Zhou et al., 2017a, 2017b). Similarly, another study conducted in a slaughterhouse, which extensively used biocides for cleaning, reported a high percentage of resistance in *Pseudomonas* species to triclosan and co-resistance to multiple antibiotics (Lavilla Lerma et al., 2015). They observed cross-resistance between biocides and antibiotics, which was linked to the MexJK-opmH, MexAB-oprM, MexCD-OprJ, MexEF-OprN, triABC-OpmH and AcrABZ-TolC systems.

Most environmental settings, like aquatic ecosystems, are not pristine and consist of numerous physical and chemical stressors that can trigger genetic changes in microorganisms. For example, the bulk of antibiotics consumed by humans and animals are not fully metabolised, and a large proportion of these antibiotics are discharged into the aquatic environment, often through wastewater treatment plants (WWTPS), most of which were not designed to remove antibiotics and their residues. Some of the antibiotics could also get into the environment through direct deposition from informal and rural settlements where sanitary facilities are usually limited, and people use nearby water bodies as dumping grounds for human wastes (Abia et al., 2015a).

According to Bengtsson-Palme et al. (2016), resistance gene development in environmental bacteria could be attributed to a combination of the concentration of the chemical stressors (antibiotics, heavy metals, biocides) and the environmental factors present. Such factors include bioaccumulation of the antibiotics (due to the ubiquitous and biodegradability nature of antibiotics), degree of exposure. Furthermore, physicochemical and environmental parameters like temperature, heavy metals, disinfectants, and poly aromatic hydrocarbons (PAHs) also impact this process. These factors could trigger the development of physiological mechanisms such as genetic mutations. The authors suggested further studies on the impact of these chemical stressors on environmental bacteria, as such information could strengthen the legislation on the amounts of antibiotics and pollutants discharged into the environment (Bengtsson-Palme and Larsson, 2015).

Studies have investigated the mechanism of bacterial resistance induced by environmental stressors, but not many have been done on the African environment. This study thus investigated the dynamics of different concentrations of antibiotics, heavy metals, and biocides on antibiotic resistance development in a previously susceptible strain of *E. coli* over a 30-day exposure period, as studies have been done on 15 and 45 days exposure periods.

2. Materials and methods

2.1. Determination of environmental concentrations of chemical stressors

Samples used to determine environmental concentrations were collected at a WWTP and upstream and downstream from the WWTP in February and March of 2020. Triplicate grab water samples were collected hourly between 8 am and 2 pm on each sampling day into 0.5 L sample bottles and later combined to form daily composite samples. The samples were transported on ice to the laboratory and frozen immediately for shipping to reference laboratories for analysis. Antibiotic and biocide analysis samples were sent to the South African Agricultural Research Council (ARC), while heavy metals were analysed at the WaterLabs (Pty) Ltd., Pretoria.

2.2. Isolation and phenotypic characterisation of environmental isolates from the same polluted environment

Escherichia coli was isolated from water samples using the Colilert-18® Quanti-tray/2000 (IDEXX Laboratories, Inc., Johannesburg, South Africa) following the manufacturer's instructions. Briefly, 100 mL of water sample was mixed with the Colilert-18® reagent, sealed in a Quanti-Tray 2000 and incubated for 24 h at 37 °C. After incubation, the Quanti-trays were viewed under an ultraviolet (UV) light for fluorescent wells, indicating *E. coli* presence. Presumptive *E. coli* isolates were harvested from fluorescent Quanti-trays wells and purified on eosin methylene blue agar as previously described (Abia et al., 2015a). Where applicable, up to five colonies were selected per sample and confirmed targeting the malate dehydrogenase (*mdh*) gene (Abia et al., 2015a) on an Applied Biosystems Quant-Studio 5 Real-time PCR system (Thermo Fisher Scientific, Waltman, Massachusetts, USA) (Abia et al., 2015c; Chukwu et al., 2019). Pure *E. coli* isolates were then tested against a panel of 19 antibiotics using the disk diffusion method (EUCAST, 2017).

2.3. Exposure experiment

2.3.1. Determination of experimental concentrations

Based on the results obtained from the chemical analysis, two heavy metals, two antibiotics and two biocides were selected for the exposure experiment (Table 1). The minimum inhibitory concentration of each chemical was determined using the 96-well microdilution method, as previously described (Wiegand et al., 2008), using their respective standards obtained from the reference laboratories. Once determined, the sub-MIC was estimated (as the next serial dilution concentration), and three other concentrations were added to obtain the four experimental concentrations. The concentrations, thus, included the sub-MIC, the maximum environmental concentration, the minimum environmental concentration, and the predicted no-effect concentration (PNEC) (AMR Industry Alliance, 2021; Bengtsson-Palme and Larsson, 2016), or the minimum allowable concentration (MAC) for antibiotics and biocides (Commission, 2003) as shown in Table 1.

Table 1
Antibiotics, heavy metals, and biocides concentrations used in the exposure experiment.

Stressor		Sub-MIC (μg/L)	Environmental concentrations (μg/L)		Limits (μg/L)
			Maximum	Minimum	
Antibiotics	Oxytetracycline	250	37.84	27.92	18[a]
	Amoxicillin	2000	136.38	73.41	0.25[a]
Heavy metals	Zinc	512,000	78	14	0.005[b]
	Copper	512,000	7	1	2[b]
Biocides	BAC 12[c]	4000	2.42	1.24	0.01[b]
	DADMAC 10[d]	16,000	0.83	0.42	0.01[b]

[a] PNEC (μg/L).
[b] MAEC (μg/L).
[c] Benzalkonium chloride.
[d] Diallyldimethylammonium chloride.

2.3.2. Experimental setup and procedure

Each of the chemicals to be tested was prepared and transferred into 1 mL Eppendorf tubes containing single-strength Muller-Hinton broth (Oxoid, Hampshire UK) to obtain the final determined experimental concentration. The tubes were then inoculated with an overnight fresh *E. coli* (ATCC 25922) culture to a final concentration of approximately 10^6 colony forming units (CFU)/mL. Each concentration was set out in triplicates. Then, a set of three tubes was made by combining all the maximum concentrations to mimic an environmental scenario. Another set of three tubes containing only the bacteria in broth was used as a control. Once the experimental setup was completed, each tube was sampled and plated unto nutrient agar (Thermo Fisher Scientific Waltham, MA, USA) to obtain Day Zero results. The tubes were then incubated at 37 °C for 24 h with agitation using a shaking incubator set at 180 rpm.

After incubation, 100 μL was transferred from each tube into a set of fresh tubes containing the initial concentrations and incubated as previously. Samples were also collected from the 24 h plates and plated unto nutrient agar (Day 1). Single colonies were collected from Day Zero plates and stored at −20 °C in Tryptic Soy Broth (TSB) (Oxoid, Hampshire, UK) supplemented with 30 % sterile glycerol and stored for further analysis. Similarly, samples were plated every day after each serial passage. The experiment was run for 30 days. The initial concentrations were maintained constant throughout the experiments.

2.3.3. Determination of phenotypic resistance to antibiotics

Following the 30 days of exposure, pure isolates from each exposure were subcultured on fresh nutrient agar and subjected to antimicrobial susceptibility testing (AST) against a panel of 19 antibiotics (Table S1, Supplementary materials), using the disk diffusion method as previously reported (EUCAST, 2017). Isolates from the control tubes were also included in the test. A fresh *E. coli* (ATCC 25922) isolate that was not included in the experiment was used as a control.

2.3.4. Determination of the presence of resistance genes using whole-genome sequencing

Genomic DNA was extracted from the 30 days exposed isolates previously subjected to antibiotic susceptibility testing, using the GenElute Bacterial Genomic DNA Kit (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. In addition to the experimental samples, DNA was extracted from 22 multidrug *E. coli* environmental isolates from our sample collection sites. This was to compare their gene profile to those of the experimental isolates. The DNA quality and quantity were checked using a Nanodrop 8000 (Thermo Fisher Scientific Waltham, MA, USA) at the 260/280 nm wavelength. The extracted DNA was shipped to the South African National Institute for Communicable Diseases (NICD) for whole genome sequencing (WGS) on an Illumina MiSeq Machine (Illumina, San Diego CA, USA). All resultant sequences were analysed using bioinformatic pipelines previously described (Amoako et al., 2019) to determine the presence of any resistance genes.

To elucidate the pathway for the emergence of resistance genes and de novo resistance, we employed single nucleotide variant (SNV) calling using the PATRIC database (https://www.bv-brc.org/app/MSA) and ANVIO analysis pipeline (https://anvio.org/help/main/workflows/sra-download/). All contiguous sequences have been deposited in GenBank with accession numbers (Table S2; Supplementary materials) under BioProject PRJNA836107.

3. Results

3.1. Phenotypic characterisation

A total of 48 isolates, one from each concentration per chemical in duplicates, were subjected to antibiotic susceptibility against 19 antibiotics, gentamycin (GEN), LAZ, FEP, CTX, ampicillin (AMP), TZP, IMP, MEM, azithromycin (AZM) SXT, nalidixic acid (NAL), chloramphenicol (CHL), ciprofloxacin (CIP), FOX, LEX, AMC, AMK, tetracycline (TET), and CRO belonging to penicillin, sulfonamide, macrolides, cephalosporin, quinolones, aminoglycosides, and carbapenem classes. All the isolates tested were susceptible to all the antibiotics tested.

3.2. Genotypic characterisation

All the laboratory isolates were successfully sequenced. However, one environmental isolate was unidentified, while five were not *E. coli*. All the experimental isolates did not carry any resistance genes, except for the *mdf*(A) gene for macrolide resistance and the disinfectant resistance gene *sit*ABCD. The control also harboured the *mdf*(A) gene but not the *sit*ABCD.

On the other hand, 15 environmental isolates harboured numerous antibiotic resistance genes (Table S3; Supplementary materials). The most detected genes were the beta-lactam genes ($bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{LAP-2}}$, $bla_{\text{MIR-3}}$, $bla_{\text{TEM-104}}$, $bla_{\text{TEM-198}}$, $bla_{\text{TEM-1A}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{TEM-217}}$, $bla_{\text{TEM-234}}$), while the least detected were those conferring resistance to colistin (*mcr*-10) and fosfomycin (*fos*A) (Fig. 1).

Unlike the experimental isolates that all carried the disinfectant resistance gene (*sit*ABCD), only four environmental isolates carried this gene, while four others carried the *qac*E gene. However, all the environmental isolates also carried the macrolide resistance gene *mdf*(A).

3.3. Single nucleotide polymorphisms

Single nucleotide variant (SNV) calling, using the PATRIC database and ANVIO analysis pipeline, showed significant mutation. A total of 2580 variants were identified, and 1197 of these were identified in all reads. Nineteen genes were identified by name in the output, viz., *yqhH* (a DNA recombination lipoprotein), *degQ* (stress protein), *purH* (a bifunctional AICAR transformarase), *epmA* (aminoacylates the protein chain elongation factor EF-P), *queG* (cellular differentiation gene), *robA* (a member of the *Xyl5/AraC* sub-family of the multi-resistance *marA/soxS/rob* regulon), *acnB* (catabolic enzyme for oxidative stress), *cusA* (part of the cusCFBA efflux system), *yddG* (a member of the aromatic amino acid/paraquet exporter (ArAA/P-E) family in the DMT super family), *hsmP* (EAL domain protein for biofilm formation) *mlc* (regulator protein controlling glucose utilisation), *tsaB* (protease that degrades tsaD), *ompD* (a porin protein), *nudK* (a nudix hydrolase), *murP* (member of the *N*-acetylmuranic acid PTS transport system), *srlE* (part of the srlABE/gutABE gene PEP-dependent sugar PTS family), *fliL* (a flagellar associated flaA locus for swarming), *mutM* (DNA glycolase) and *ptsG* (glucose hydrolase modulating the c-AMP).

Of the 19 detected genes, 12 genes, *acnB*, *cusA*, *degQ*, *epmA*, *hsmP*, *mlc*, *purH*, *queG*, *srlE*, *tsaB*, *yddh* and *yqhH* genes (encoding for oxidative stress maintenance, efflux activities, biofilm formation and growth), were detected in all the exposed isolates. The swarming and motility *filA*

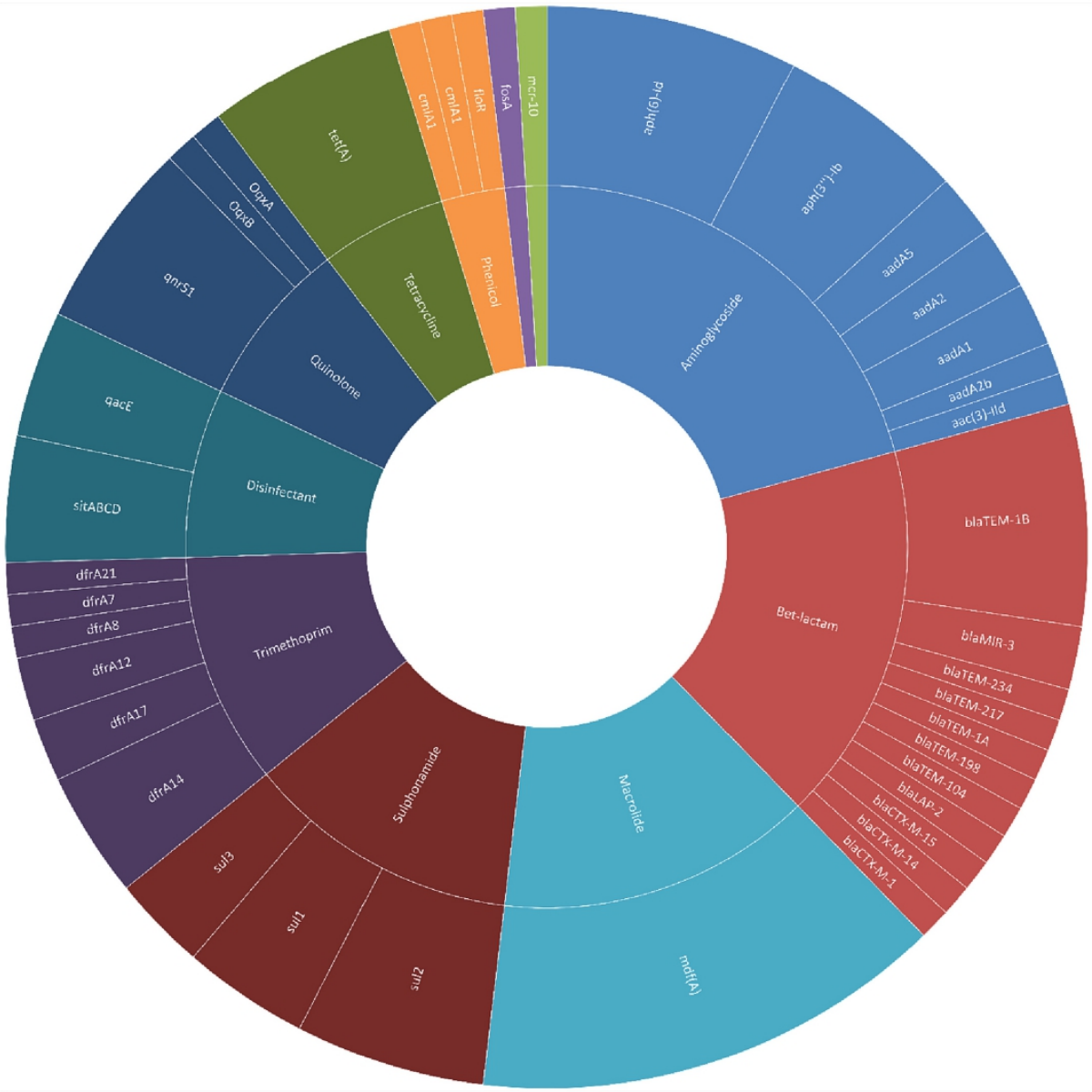

Fig. 1. Distribution of resistance genes identified in environmental isolates using WGS.

genes were detected in only the oxytetracycline and BAC12-exposed isolates, and the *mutM* gene was detected in zinc-exposed isolates only. The biofilm formation *nudK* gene was detected in all the exposed isolates except the DADMAC12 exposed isolates, and the growth and phosphotransferase *ptsG* was detected in only the oxytetracycline exposed isolates. In contrast, the porin protein *ompD* was detected in only DADMAC10 exposed isolates.

The environmental samples were also subjected to SNV calling using the PATRIC database and ANVIO analysis pipeline, and a total of 180,573 variants were detected, with 50 of the variants identified in all read libraries. All the genes detected in the exposed isolates (except the *robA*) were also detected in the environmental isolates.

A large proportion of the total exposed isolates (82.05 %) had mutations (Table 2) compared to the control. Of these, the heavy metal exposure was associated with the least number of mutations at 58.3 % of the Zn-exposed isolates had mutations for new genes, and 66.7 % of the Cu-exposed isolates, had mutations when compared with the control., while 91.6 % of the biocide-exposed isolates exhibited mutations. The antibiotics-exposed isolates also had more mutations than the metal-exposed isolates; 91.6 % of all the oxytetracycline-exposed isolates had mutations, and 83.3 % of all the AMX-exposed isolates had mutations when compared with the control, respectively. Interestingly there was 100 % mutation in isolates exposed to the antimicrobial combinations and in the environmental isolates.

4. Discussion

This study investigated the effect of 30 days of laboratory exposure of

Table 2
SNV gene calling report.

SN	Gene	OXYTET	AMX	ZIN	COP	DAD	BAC	ALL	ENV
1	*acnB*	Y	Y	Y	Y	Y	Y	Y	Y
2	*cusA*	Y	Y	Y	Y	Y	Y	Y	Y
3	*degQ*	Y	Y	Y	Y	Y	Y	Y	Y
4	*epmA*	Y	Y	Y	Y	Y	Y	Y	Y
5	*filL*	Y	N	N	N	N	Y	Y	Y
6	*hsmp*	Y	Y	Y	Y	Y	Y	Y	Y
7	*mlc*	Y	Y	Y	Y	Y	Y	Y	Y
8	*murP*	Y	Y	Y	Y	Y	Y	Y	Y
9	*mutM*	N	N	Y	N	N	N	Y	Y
10	*nudK*	Y	Y	Y	Y	N	Y	Y	Y
11	*ompD*	N	N	N	N	Y	N	Y	Y
12	*ptsG*	Y	N	N	N	N	N	Y	Y
13	*purH*	Y	Y	Y	Y	Y	Y	Y	Y
14	*queG*	Y	Y	Y	Y	Y	Y	Y	Y
15	*robA*	Y	Y	Y	Y	Y	Y	Y	N
16	*srlE*	Y	Y	Y	Y	Y	Y	Y	Y
17	*tsaB*	Y	Y	Y	Y	Y	Y	Y	Y
18	*yddG*	Y	Y	Y	Y	Y	Y	Y	Y
19	*yqhH*	Y	Y	Y	Y	Y	Y	Y	Y
No of genes detected	19	17	15	16	15	15	16	19	18
No of variants/all read library	2580/1197	433/212	435/212	409/219	415/211	434/216	405/214	262/219	180,573/50
No of isolates affected/total	64/78 (82.05 %)	11/12 (91.67 %)	10/12 (83.33 %)	7/12 (58.33 %)	8/12 (66.67 %)	11/12 (91.67 %)	11/12 (91.67 %)	6/6 (100 %)	20/20 (100 %)

Y = positive and N = negative. GENE = gene detected, OXT = 30 days oxytetracycline-exposed isolates, AMX = 30 days amoxicillin-exposed isolates, ZIN = 30 days zinc-exposed isolates, COP = 30 days copper exposed isolates, DAD = 30 days DADMAC10-exposed isolates, BAC = 30 days bac12 exposed isolates, ALL = 30 days combinations-exposed isolates, ENV = environmental isolates, NS = non-synonymous, INS = insertion, SYN = synonymous, NA = not applicable.

E. coli to six environmentally identified stressors, including antibiotics, heavy metals and biocides. The study further assessed the isolates for phenotypic antibiotic resistance against 19 antibiotics to assess the co-selection and/or cross-resistance potential of the biocides and heavy metals. In addition, the genotypic profile of the isolates was evaluated post-exposure using WGS and the results were compared to environmental isolates collected from the same milieu. No phenotypic resistance was observed in the experimental isolates. Also, no antibiotic resistance genes were observed except for the disinfectant tolerance *sitABCD* gene. On the other hand, the environmental isolates carried resistance genes against the antibiotics tested.

Several studies have indicated that environmental concentrations of pollutants such as antibiotics, heavy metals, and biocides could trigger the development of resistance in environmental bacteria (Imran et al., 2019; River et al., 2020; Silva et al., 2021; Squadrone, 2020; Vos et al., 2020; Zhou et al., 2017a, 2017b). However, these studies have inferred such resistance and coresistance through correlation analysis or co-occurrence instead of direct laboratory investigations. For example, a US study observed a correlation between tetracycline and sulphonamide residues and resistance genes in a wastewater treatment plant (Gao et al., 2012). Regarding heavy metals, it was demonstrated through an experimental investigation that tetracycline resistance was associated with exposure to arsenic, copper and zinc (Chen et al., 2015). Similarly, the *mer* gene responsible for mercury resistance has been associated with numerous antibiotic resistance genes, including the *tet*(A) gene conferring resistance to tetracycline (Mcintosh et al., 2008). Apart from heavy metals and antibiotics, the presence of biocides has also been shown to be associated with the presence of antibiotic resistance in the environment, probably due to the location of biocide-associated genes and antibiotic resistance genes on the same mobile genetic elements (Bengtsson-Palme et al., 2018; Liu et al., 2017; Perron et al., 2004).

In this study, *E. coli* was exposed to six compounds, including two antibiotics (amoxicillin and oxytetracycline), two heavy metals (copper and zinc), and two biocides (BAC 10 and DADMAC 12) chosen due to their relatively high concentrations in water environments based on previous sampling. Following exposure to these environmental concentrations individually and in combinations for 30 days, no phenotypic resistance nor the presence of resistance genes was detected. However, the disinfectant tolerance gene, *sitABCD*, involved in Mn^{2+} and Fe^{2+} transport and conferring resistance to disinfecting agents like hydrogen peroxide (Lozica et al., 2022) was detected in all the isolates, including the control. This gene was first discovered in *Salmonella* as a homologue to a metal transporter in *Yersenia pestis* (Sabri et al., 2008). However, most homologues of this gene may not induce disinfectant resistance in the absence of other ion transport systems (Sabri et al., 2006). Therefore, the lack of other supporting systems and the difference in the mode of action between different antimicrobials could have led to the presence of the *sitABCD* gene not translating to any phenotypic resistance. Furthermore, discordances have been reported regarding resistance, with lack of phenotypic resistance to antimicrobials occurring even in the presence of resistance genes (Roedel et al., 2021).

Environmental isolates obtained from the same environment as the chemicals harboured numerous resistance genes. For example, the environmental isolates harboured the *tet*(A) and the *bla* genes responsible for resistance to tetracycline and amoxicillin, respectively, to which the isolates were exposed experimentally. The experimental observations in the current study could be due to several reasons. First, only a few chemical stressors were selected in the current study. Second, the concentrations used were constant throughout the experiment. Third, other environmental parameters, such as pH, nutrients, and temperature, were not considered. However, environmental conditions are more complex than the ones considered in the current study. Microorganisms are exposed to multiple stressors at any time in the aquatic environment (Patel et al., 2019). These parameters could also change with high and low values fluctuations due to the flowing waters. Therefore, the current study's static conditions could have influenced the outcome observed.

Ntabugi et al. (2021) argued that the interaction between bacteria and heavy metals pollution in the environment confers metal tolerance and prompts antibiotic resistance, as the expression of genes is closely linked to the two. Since antibiotic concentration decreases due to rapid degradation, sorption and sequestration, the authors observed that bacteria in heavy metal-polluted environments tend to develop heavy metal tolerance instead of antimicrobial resistance genes. This is because heavy metal tolerance gave them greater survival chances and indirectly conferred antibiotic resistance by co-selection. Nevertheless,

no heavy metal and Biocide resistance genes were recorded.

Considering that these isolates were subjected to the same environmental concentrations identified before the start of the study, it can be inferred that the resistance observed in the environmental isolates was not elicited ab initio due to the concentration of these stressors in the environment, but these isolates could have been introduced into that ecosystem from another hotspot with very high chemical concentrations (Martinez, 2009; Karkman et al., 2019). Moreover, some authors have suggested that antibiotic-resistant bacteria in the environment could be from faecal pollution and not selective pressure due to exposure to environmental pollutants (Larsson and Flach, 2021).

Our results were at variance with other studies that observed resistance in bacteria exposed to sub-MIC concentrations of antibiotics (Sanz-García et al., 2022), heavy metals (Xu et al., 2022; Zhang et al., 2018) and biocides (Lu et al., 2018). A probable reason may be the duration of the exposure (Gu et al., 2020) observed that bacteria needed a long period of exposure to sub-MIC concentration, up to 60 days, to enable moderate resistance and tolerance gene developments, although some studies observed at 15 days of exposure (Bernardi et al., 2021; Lu et al., 2018).

Subjecting the sequences to single nucleotide Variant (SNV) calling, using the PATRIC database and ANVIO analysis pipeline, allowed us to observe a significant amount of mutation. A total of 2580 variants were identified and 1197 of these were identified in all reads. Nineteen genes were identified by name in the output, and they are *yqhH*, *degQ*, *purH*, *epmA*, *queG*, *robA*, *acnB*, *cusA*, *yddG*, *mlc*, *tsaB*, *ompD*, *nudK*, *murP*, *srlE*, *fliL*, *mutM* and *ptsG*.

These results agree with Bernardi et al. (2021) and Gu et al. (2020), as most of these genes are responsible for biofilm (*hsmP*), DNA repairs (*mlc*), triggering SOS responses (*degQ*) and formation of persisters (*acnB*), which could lead to drug tolerance and eventual resistance gene development in bacteria (Levin-Reisman et al., 2017a, 2017b). Most of these genes were like those detected in a similar study by Gu et al. (2020), and this suggests that even though outright resistance was undetected in the exposed isolates, mutation towards biofilm and persisters formations had started, and the exposed isolates may be on the pathway towards resistance (Levin-Reisman et al., 2017a, 2017b).

All the variants' genes detected in the exposed isolates (except the *robA*) were also detected in the environmental isolates (Table 2), suggesting that environmental isolates might have also used the same route to eventual AMR development. Interestingly, although *robA* was detected in all the isolates, there was still no phenotypic expression of antimicrobial resistance. This could be attributed to a couple of factors. *RobA*, *soxR*, and *marA* are transcription protein subsets of the *ArcC/XylS* family of proteins that confer multidrug resistance in *E. coli* (Chetri et al., 2020). However, unlike *soxR* and *marA*, which are actively expressed in the presence of stressors, *robA* is constitutively expressed but remains inactive in cells due to its sequestration in the intercellular loci. This could be attributed to the presence of the C-terminal domain, as its absence, as seen in rob133, makes them active (Griffith et al., 2009). Secondly, because of the absence of *soxR* and *marA*, which represses the promoter salicylate, there will be no up-regulatory expression of the *ArcAB-TolC* multidrug efflux pump, and the inhibitory RNA, *micF* that regulates the outer membrane porin *ompF*, that contributes to drug resistance (Wipt and George, 2008). Instead, the *robA* could be contributing towards persistence. *n*-Hexanes increase the expression of *robA*, leading to increased tolerance to organic solvents, as *robA* encodes a 33-kDa Rob protein that binds the oriC border region and expression of the Plasmid POST4034, which confers decreased susceptibility to antibiotics and leads to multidrug drug resistance in bacteria (Chetri et al., 2020; Nakajima et al., 1995). So even though there was an SNP mutation for *rob*A, it could not lead to phenotypic antibiotic resistance in the isolates, but perhaps towards organic solvent tolerance and persistence in the isolates.

From the percentage of the isolates exhibiting mutation, from the SNV calling, the fact that the heavy metals exhibited the least percentage of mutation could be attributed to the low toxicity of Cu and Zn at very low concentrations, which instead of being irritants to the bacteria, may have been beneficial to it. And this may be probably why, in the combined (ALL) exposed isolates, there was 100 % of exposed isolates and 19/19 of the genes were also detected, including the *mutM* gene, which was only seen among the heavy metals (Zn). This agrees with previous studies on metal resistance and the development of resistance genes in which the authors stated that metal pollution was concentration-dependent, which increases mutation and, as such, was associated with increases in metal resistance genes and ARG abundance (Gupta et al., 2022; Souza et al., 2018; Wang et al., 2021; Zou et al., 2021).

5. Conclusion

Laboratory exposure of *E. coli* to environmental concentrations of Cu, Zn, BAC 10, DADMAC 12, oxytetracycline and amoxicillin did not result in the organisms developing phenotypic or genotypic resistance to antibiotics. Rather, SNV calling revealed that the isolates instead underwent mutations, resulting in variants and the development of genes responsible for SOS responses, biofilm formation, DNA repairs and the development of persister cells. This suggests that the pathway to resistance for bacteria exposed to environmental stressors is not direct but through a stepwise process beginning with tolerance. Furthermore, the current study, although limited by the number of stressors evaluated compared to those observed in the environment, indicating that the development of antibiotic resistance by environmental isolates requires further research.

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CRediT authorship contribution statement

Conceptualization, K.B.C., S.Y.E. and A.L.K.A.; methodology, K.B.C., O.A.A., and A.L.K.A.; software, K.B.C., O.A.A., and A.L.K.A.; validation, S.Y.E., D.G.A. and A.L.K.A.; formal analysis, K.B.C., O.A.A., D.G.A.; investigation, K.B.C.; resources, S.Y.E.; data curation, K.B.C. and D.G.A.; writing-original draft preparation, K.B.C.; writing-review and editing, all authors; supervision, S.Y.E. and A.L.K.A.; project administration; funding acquisition, S.Y.E. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

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Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.165721.

References

Abia, A.L.K., Ubomba-Jaswa, E., Momba, M.N.B., 2015a. Impact of seasonal variation on Escherichia coli concentrations in the riverbed sediments in the Apies River, South Africa. Sci. Total Environ. 537, 462–469. https://doi.org/10.1016/j.scitotenv.2015.07.132.

Abia, A.L.K., Ubomba-Jaswa, E., Momba, M.N.B., 2015b. High prevalence of multiple-antibiotic-resistant (MAR) Escherichia coli in river bed sediments of the Apies River, South Africa. Environ. Monit. Assess. 187, 652. https://doi.org/10.1007/s10661-015-4879-6.

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J., Handelsman, J., 2010. Call of the wild: antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. 8, 251–259. https://doi.org/10.1038/nrmicro2312.

Amoako, D.G., Somboro, A.M., Abia, A.L.K., Allam, M., Ismail, A., Bester, L.A., Essack, S. Y., 2019. Whole-genome Shotgun Sequence of Drug-resistant Staphylococcus aureus Strain SA9, Isolated From a Slaughterhouse Chicken Carcass in South Africa. https://doi.org/10.1128/MRA.

AMR Industry Alliance, 2021. List of predicted no-effect concentrations (PNECs). In: AMR Ind. Alliance Antibiot. Disch. Targets, pp. 1–5.

Andersson, D.I., Hughes, D., 2012. Evolution of antibiotic resistance at non-lethal drug concentrations. Drug Resist. Updat. 15, 162–172. https://doi.org/10.1016/j.drup.2012.03.005.

Azanu, D., Styrishave, B., Darko, G., Weisser, J.J., Abaidoo, R.C., 2018. Occurrence and risk assessment of antibiotics in water and lettuce in Ghana. Sci. Total Environ. 622–623, 293–305. https://doi.org/10.1016/j.scitotenv.2017.11.287.

Bengtsson-Palme, J., Larsson, D.G.J., 2015. Antibiotic resistance genes in the environment: prioritizing risks. Nat. Rev. Microbiol. 13, 396. https://doi.org/10.1038/nrmicro3399-c1.

Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: proposed limits for environmental regulation. Environ. Int. 86, 140–149. https://doi.org/10.1016/j.envint.2015.10.015.

Bengtsson-Palme, J., Hammarén, R., Pal, C., Östman, M., Björlenius, B., Flach, C.F., Fick, J., Kristiansson, E., Tysklind, M., Larsson, D.G.J., 2016. Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. Sci. Total Environ. 572, 697–712. https://doi.org/10.1016/j.scitotenv.2016.06.228.

Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J.J., 2018. Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiol. Rev. 42, 68–80. https://doi.org/10.1093/femsre/fux053.

Bernardi, S., Anderson, A., Macchiarelli, G., Hellwig, E., Cieplik, F., Vach, K., Al-Ahmad, A., 2021. Subinhibitory antibiotic concentrations enhance biofilm formation of clinical *Enterococcus faecalis* isolates. Antibiotics. 10 (7), 874. https://doi.org/10.3390/antibiotics10070874.

Bhullar, K., Waglechner, N., Pawlowski, A., Koteva, K., Banks, E.D., Johnston, M.D., Barton, H.A., Wright, G.D., 2012. Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS One 7, 1–11. https://doi.org/10.1371/journal.pone.0034953.

Chait, R., Vetsigian, K., Kishony, R., 2012. What counters antibiotic resistance in nature? Nat. Chem. Biol. 8, 2–5. https://doi.org/10.1038/nchembio.745.

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, 23390–23404. https://doi.org/10.3390/ijms161023390.

Chetri, S., Das, B.J., Bhowmik, D., Chanda, D.D., Chakravarty, A., Bhattacharjee, A., 2020. Transcriptional response of mar, sox and rob regulon against concentration gradient carbapenem stress within Escherichia coli isolated from hospital acquired infection. BMC Res. Notes 13, 1–7. https://doi.org/10.1186/s13104-020-04999-2.

Chetty, S., Pillay, L., 2019. Assessing the influence of human activities on river health: a case for two South African rivers with differing pollutant sources. Environ. Monit. Assess. 191 https://doi.org/10.1007/s10661-019-7308-4.

Christopher, M., Paul, O., Hamadi, B., 2014. Association of metal tolerance with multidrug resistance among environmental bacteria from wetlands of Lake Victoria basin. ID - 20143225882. Agric. Biol. J. North Am. 5, 24–32. https://doi.org/10.5251/abjna.2014.5.1.24.32.

Chukwu, M.O., Abia, A.L.K., Ubomba-Jaswa, E., Obi, L.C., Dewar, J.B., 2019. Antibiotic resistance profile and clonality of E. coli isolated from water and paediatric stool samples in the north-west, province South Africa. J. Pure Appl. Microbiol. 13, 517–530. https://doi.org/10.22207/JPAM.13.1.58.

Commission, E, 2003. Document on risk assessment. In: Tech. Guid. Doc. Ris Assess. Part II, p. 337.

Costa, V.M.D., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N., Wright, G.D., 2011. Antibiotic resistance is ancient. Nature 477, 457–461. https://doi.org/10.1038/nature10388.

Ebele, A.J., Abou-Elwafa Abdallah, M., Harrad, S., 2017. Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment. Emerg. Contam. 3, 1–16. https://doi.org/10.1016/j.emcon.2016.12.004.

Ebenebe, P.N., Shale, K., Sedibe, M., 2017. South African mine effluents: heavy metal pollution and impact on the ecosystem. Int. J. Chem. Sci. Res. 15, 198.

Edokpayi, J., Odiyo, J., Popoola, O., Msagati, T., 2016. Assessment of trace metals contamination of surface water and sediment: a case study of Mvudi River, South Africa. Sustainability 8, 135. https://doi.org/10.3390/su8020135.

Eliku, T., Leta, S., 2018. Spatial and seasonal variation in physicochemical parameters and heavy metals in Awash River, Ethiopia. Appl Water Sci 8, 1–13. https://doi.org/10.1007/s13201-018-0803-x.

EUCAST, 2017. Antimicrobial susceptibility testing EUCAST disk diffusion method. Eucast 0, 1–22.

Gao, P., Munir, M., Xagoraraki, I., 2012. Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. Sci. Total Environ. 421–422, 173–183. https://doi.org/10.1016/j.scitotenv.2012.01.061.

Gao, X., Wang, X., Li, J., Ai, S., Fu, X., Fan, B., Li, W., Liu, Z., 2020. Aquatic life criteria derivation and ecological risk assessment of DEET in China. Ecotoxicol. Environ. Saf. 188 https://doi.org/10.1016/j.ecoenv.2019.109881.

Gautam, P., Carsella, J.S., Kinney, C.A., 2014. Presence and transport of the antimicrobials triclocarban and triclosan in a wastewater-dominated stream and freshwater environment. Water Res. 48, 247–256. https://doi.org/10.1016/j.watres.2013.09.032.

Griffith, K.L., Fitzpatrick, M.M., Keen, E.F., Wolf, R.E., 2009. Two functions of the C-terminal domain of Escherichia coli rob: mediating "sequestration-dispersal" as a novel off-on switch for regulating Rob's activity as a transcription activator and preventing degradation of rob by Lon protease. J. Mol. Biol. 388, 415–430. https://doi.org/10.1016/j.jmb.2009.03.023.

Gu, Y., Wang, S., Huang, L., Sa, W., Li, J., Huang, J., Dai, M., Cheng, G., 2020. Development of resistance in escherichia coli atcc25922 under exposure of sub-inhibitory concentration of olaquindox. Antibiotics 9, 1–22. https://doi.org/10.3390/antibiotics9110791.

Gupta, S., Graham, D.W., Sreekrishnan, T.R., Ahammad, S.Z., 2022. Effects of heavy metals pollution on the co-selection of metal and antibiotic resistance in urban rivers in UK and India. Environ. Pollut. 306, 119326 https://doi.org/10.1016/j.envpol.2022.119326.

Holmes, A.H., Moore, L.S.P., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., Guerin, P.J., Piddock, L.J.V., 2016. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet 387, 176–187. https://doi.org/10.1016/s0140-6736(15)00473-0.

Imran, M., Das, K.R., Naik, M.M., 2019. Co-selection of multi-antibiotic resistance in bacterial pathogens in metal and microplastic contaminated environments: an emerging health threat. Chemosphere 215, 846–857. https://doi.org/10.1016/j.chemosphere.2018.10.114.

Kandie, F.J., Krauss, M., Beckers, L.M., Massei, R., Fillinger, U., Becker, J., Liess, M., Torto, B., Brack, W., 2020. Occurrence and risk assessment of organic micropollutants in freshwater systems within the Lake Victoria South Basin, Kenya. Sci. Total Environ. 714, 136748 https://doi.org/10.1016/j.scitotenv.2020.136748.

Karkman, A., Pärnänen, K., Larsson, D.G.J., 2019. Fecal pollution can explain antibiotic resistance gene abundances in anthropogenically impacted environments. Nat. Commun. 10, 1–8.

Larsson, D.G.J., Flach, C.-F., 2021. Antibiotic resistance in the environment. Nat. Rev. Microbiol., 0123456789 https://doi.org/10.1038/s41579-021-00649-x.

Lau, C.H.-F., van Engelen, K., Gordon, S., Renaud, J., Topp, E., 2017. Novel antibiotic resistance determinants from agricultural soil exposed to antibiotics widely used in human medicine and animal farming. Appl. Environ. Microbiol. 83, 1–18. https://doi.org/10.1128/aem.00989-17.

Lavilla Lerma, L., Benomar, N., Casado Muñoz, M. del C., Gálvez, A., Abriouel, H., 2015. Correlation between antibiotic and biocide resistance in mesophilic and psychrotrophic Pseudomonas spp. isolated from slaughterhouse surfaces throughout meat chain production. Food Microbiol. 51, 33–44. https://doi.org/10.1016/j.fm.2015.04.010.

Lee, Jangwoo, Shin, S.G., Jang, H.M., Kim, Y.B., Lee, Joonyeob, Kim, Y.M., 2017. Characterization of antibiotic resistance genes in representative organic solid wastes: food waste-recycling wastewater, manure, and sewage sludge. Sci. Total Environ. 579, 1692–1698. https://doi.org/10.1016/j.scitotenv.2016.11.187.

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., 2017a. Antibiotic tolerance facilitates the evolution of resistance, 830, 826–830.

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N., Balaban, N.Q., 2017b. Antibiotic tolerance facilitates the evolution of resistance. Science (80-.) 355, 826–830. https://doi.org/10.1126/science.aaj2191.

Li, X., Gu, A.Z., Zhang, Y., Xie, B., Li, D., Chen, J., 2019. Sub-lethal concentrations of heavy metals induce antibiotic resistance via mutagenesis. J. Hazard. Mater. 369, 9–16. https://doi.org/10.1016/j.jhazmat.2019.02.006.

Liu, W.J., Fu, L., Huang, M., Zhang, J.P., Wu, Y., Zhou, Y.S., Zeng, J., Wang, G.X., 2017. Frequency of antiseptic resistance genes and reduced susceptibility to biocides in carbapenem-resistant Acinetobacter baumannii. J. Med. Microbiol. 66, 13–17. https://doi.org/10.1099/jmm.0.000403.

Lozica, L., Villumsen, K.R., Li, G., Hu, X., Maurić Maljković, M., Gottstein, Ž., 2022. Genomic analysis of Escherichia coli longitudinally isolated from broiler breeder flocks after the application of an autogenous vaccine. Microorganisms 10. https://doi.org/10.3390/microorganisms10020377.

Lu, J., Jin, M., Nguyen, S.H., Mao, L., Li, J., Coin, L.J.M., Yuan, Z., Guo, J., 2018. Non-antibiotic antimicrobial triclosan induces multiple antibiotic resistance through genetic mutation. Environ. Int. 118, 257–265. https://doi.org/10.1016/j.envint.2018.06.004.

Manegabe, B.J., Marie-Médiatrice, N.K., Barr Dewar, J., Christian, S.B., 2017. Antibiotic resistance and tolerance to heavy metals demonstrated by environmental pathogenic bacteria isolated from the Kahwa River, Bukavu Town, Democratic Republic of the Congo. Int. J. Environ. Stud. 74, 290–302. https://doi.org/10.1080/00207233.2017.1284461.

Martinez, J.L., 2009. The role of natural environments in the evolution of resistance traits in pathogenic bacteria. Proc. R. Soc. B Biol. Sci. https://doi.org/10.1098/rspb.2009.0320.

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, 1221–1228. https://doi.org/10.1093/jac/dkn123.
Murray, A.K., Zhang, L., Snape, J., Gaze, W.H., 2019. Comparing the selective and co-selective effects of different antimicrobials in bacterial communities. Int. J. Antimicrob. Agents 53, 767–773. https://doi.org/10.1016/j.ijantimicag.2019.03.001.
Naggar, Y. Al, 2018. Environmental Pollution by Heavy Metals in the Aquatic Ecosystems of Egypt. https://doi.org/10.19080/OAJT.2018.03.555603.
Nakajima, H., Kobayashi, K.E.I., 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*, 61, 2302–2307.
Namaki, M., Habibzadeh, S., Vaez, H., Arzanlou, M., Safarirad, S., Bazghandi, S.A., Sahebkar, A., Khademi, F., 2022. Prevalence of resistance genes to biocides in antibiotic-resistant Pseudomonas aeruginosa clinical isolates. Mol. Biol. Rep. 49, 2149–2155. https://doi.org/10.1007/s11033-021-07032-2.
Ntabugi, K.M.M., Manegabe, B.J., Dewar, J.B., Simbahan, J.F., Flavier, M.E., Sekomo, C. B., 2021. Synergistic increase in antibiotic resistance with tolerance to cadmium and lead in environmental bacteria isolated from the San Cristobal River, Laguna De Bay, Philippines. Int. J. Environ. Stud. 78, 165–183. https://doi.org/10.1080/00207233.2020.1834307.
Patel, M., Kumar, R., Kishor, K., Mlsna, T., Pittman, C.U., Mohan, D., 2019. Pharmaceuticals of emerging concern in aquatic systems : chemistry, occurrence, effects, and removal methods. Chem. Rev. 119, 3510–3673. https://doi.org/10.1021/acs.chemrev.8b00299.
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, 8761–8768. https://doi.org/10.1074/jbc.M312080200.
River, Y., Delhi, N., Siddiqui, M.T., Mondal, A.H., Gogry, F.A., 2020. Metal Co-resistance among ESBL-producing Isolates, pp. 1–17.
Roedel, A., Vincze, S., Projahn, M., Roesler, U., Robé, C., Hammerl, J.A., Noll, M., Al Dahouk, S., Dieckmann, R., 2021. Genetic but no phenotypic associations between biocide tolerance and antibiotic resistance in escherichia coli from german broiler fattening farms. Microorganisms 9, 1–17. https://doi.org/10.3390/microorganisms9030651.
Sabri, M., Léveillé, S., Dozois, C.M., 2006. A SitABCD homologue from an avian pathogenic Escherichia coli strain mediates transport of iron and manganese and resistance to hydrogen peroxide. Microbiology 152, 745–758. https://doi.org/10.1099/mic.0.28682-0.
Sabri, M., Caza, M., Proulx, J., Lymberopoulos, M.H., Brée, A., Moulin-Schouleur, M., Curtiss, R., Dozois, C.M., 2008. Contribution of the SitABCD, MntH, and FeoB metal transporters to the virulence of avian pathogenic Escherichia coli O78 strain χ7122. Infect. Immun. 76, 601–611. https://doi.org/10.1128/IAI.00789-07.
Sanz-García, F., Hernando-Amado, S., Martínez, J.L., 2022. Evolution under low antibiotic concentrations: a risk for the selection of Pseudomonas aeruginosa multidrug-resistant mutants in nature. Environ. Microbiol. 24, 1279–1293. https://doi.org/10.1111/1462-2920.15806.
Silva, I., Tacão, M., Henriques, I., 2021. Selection of antibiotic resistance by metals in a riverine bacterial community. Chemosphere 263. https://doi.org/10.1016/j.chemosphere.2020.127936.
Souza, L.R.R., da Silva, V.S., Franchi, L.P., de Souza, T.A.J., 2018. Toxic and beneficial potential of silver nanoparticles: the two sides of the same coin. Adv. Exp. Med. Biol. 1048, 251–262. https://doi.org/10.1007/978-3-319-72041-8_15.
Squadrone, S., 2020. Water environments: metal-tolerant and antibiotic-resistant bacteria. Environ. Monit. Assess. 192 https://doi.org/10.1007/s10661-020-8191-8.
Vos, M., Sibleyras, L., Lo, L.K., Hesse, E., Gaze, W., Klümper, U., 2020. Zinc can counteract selection for ciprofloxacin resistance. FEMS Microbiol. Lett. 367, 1–8. https://doi.org/10.1093/femsle/fnaa038.
Wang, X., Lan, B., Fei, H., Wang, S., Zhu, G., 2021. Heavy metal could drive co-selection of antibiotic resistance in terrestrial subsurface soils. J. Hazard. Mater. 411, 124848 https://doi.org/10.1016/j.jhazmat.2020.124848.
Wiegand, I., Hilpert, K., Hancock, R.E.W., 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Protoc. 3, 163–175. https://doi.org/10.1038/nprot.2007.521.
Wipt, P., George, K.M., 2008. 基因的改变NIH Public Access. Bone 23, 1–7.
Xu, Y., Tan, L., Li, Q., Zheng, X., Liu, W., 2022. Sublethal concentrations of heavy metals Cu2+ and Zn2+ can induce the emergence of bacterial multidrug resistance. Environ. Technol. Innov. 27, 102379 https://doi.org/10.1016/j.eti.2022.102379.
Zhang, Y., Gu, A.Z., Cen, T., Li, X., He, M., Li, D., Chen, J., 2018. Sub-inhibitory concentrations of heavy metals facilitate the horizontal transfer of plasmid-mediated antibiotic resistance genes in water environment. Environ. Pollut. 237, 74–82. https://doi.org/10.1016/j.envpol.2018.01.032.
Zhou, Q., et al., 2017a. Is the potential interconnection between antibiotics susceptibility and disinfectant resistance a threat to food safety, 0, 5–10.
Zhou, Q., Tang, M., Zhang, J., Zhang, X., Tang, X., Chen, D., Gu, R., Lu, J., Gao, Y., 2017b. Is the potential interconnection between antibiotics susceptibility and disinfectant resistance a threat to food safety. Agric. Sci. Technol. 18, 1033–1037.
Zhu, Q., Jia, J., Wang, Y., Zhang, K., Zhang, H., Liao, C., Jiang, G., 2019. Spatial distribution of parabens, triclocarban, triclosan, bisphenols, and tetrabromobisphenol A and its alternatives in municipal sewage sludges in China. Sci. Total Environ. 679, 61–69. https://doi.org/10.1016/j.scitotenv.2019.05.059.
Zou, H.Y., He, L.Y., Gao, F.Z., Zhang, M., Chen, S., Wu, D.L., Liu, Y.S., He, L.X., Bai, H., Ying, G.G., 2021. Antibiotic resistance genes in surface water and groundwater from mining affected environments. Sci. Total Environ. 772 https://doi.org/10.1016/j.scitotenv.2021.145516.

CHAPTER 4

IMPACT OF ENVIRONMENTAL SUB-INHIBITORY CONCENTRATIONS OF ANTIBIOTICS, METALS AND BIOCIDES ON THE EMERGENCE OF TOLERANCE AND EFFECTS ON THE MUTANT SUSCEPTIBILITY WINDOW IN *E. COLI*

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Dr Ovokeroye A. Abafe – Co-conceptualised the study, guided laboratory works, and critically revised the manuscript.

Daniel G. Amoako – Co-conceptualised the study, guided laboratory works, and critically revised the manuscript

Arshad Ismail – Co-conceptualised the study, guided laboratory works, and critically revised the manuscript

Professor S.Y. Essack – As the Co-supervisor, co-conceptualized the study, guided the literature review and ethical clearance application, enabled data collection and analysis and undertook the critical revision of the manuscript.

Objectives met – Paper answers objective 4 and 5

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Impact of Environmental Sub-Inhibitory Concentrations of Antibiotics, Heavy Metals, and Biocides on the Emergence of Tolerance and Effects on the Mutant Selection Window in *E. coli*

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Abstract: Bacteria's ability to withstand the detrimental effects of antimicrobials could occur as resistance or tolerance with the minimum inhibitory concentration, the mutant prevention concentration, and the mutant selection window as salient concepts. Thus, this study assessed the impact of exposure to extremely high doses of ampicillin on the level of persistence and tolerance development in isolates previously exposed to different concentrations of selected antibiotics, biocides, and heavy metals. These isolates were previously exposed to oxytetracycline (OXYTET), amoxicillin (AMX), copper (Cu), zinc (Zn), benzalkonium chloride (BAC) 10, dimethylammonium chloride (DADMAC) 12 and a combination of all the individual pollutants (ALL). The isolates were exposed to very high concentrations (25 × MIC) of ampicillin, and their tolerance was calculated as the time required to kill 99.9% of the bacterial population ($MDK_{99.9}$). The $MDK_{99.9}$ increased by 30 to 50% in test isolates (DADMAC, OXYTET, Zinc = 28 h; BAC, Copper = 30 h; amoxycillin, ALL = 26 h) compared to the untreated control. BAC-exposed isolates decreased from 2.5×10^8 CFU/mL to 2.5×10^4 CFU/mL on the second day, displaying the highest tolerance increase. The tolerance appeared to originate from two sources, i.e., stochastic persistence and genetic-induced persistence, involving multiple genes with diverse mechanisms. The mutant selection window of the isolates to ampicillin, amoxicillin, and oxytetracycline also slightly increased compared to the control, indicating the selective survival of persister cells during the 30-day exposure. These findings indicate that bacterial exposure to sub-inhibitory concentrations of environmental chemical stressors may not always result in the development of antimicrobial resistance but could initiate this process by selecting persisters that could evolve into resistant isolates.

Keywords: environmental stressors; antibiotic resistance; selection pressure; public health; tolerant bacteria; environmental pollution; mutation; single nucleotide polymorphisms

1. Introduction

Bacteria and other microorganisms have continuously adapted to adverse stressors originating from natural and anthropogenic activities in the environment. These adaptations are exhibited phenotypically through persistence, biofilm formation, resistance, and tolerance, and genotypically through the acquisition of resistance and tolerance genes.

Resistance is the ability of bacteria to survive, grow, and replicate in the presence of antimicrobials at concentrations beyond the minimum inhibitory concentrations (MIC); this usually manifests as direct drug inactivation, decreased intake, increased efflux of the drug, and the alteration of the drugs' binding sites [1–3]. Tolerance, on the other hand, is the ability of the bacteria to survive low to extremely high antimicrobial concentrations, usually above bactericidal concentrations but without growth [2]. Tolerance occurs through mechanisms such as dormancy, reduced metabolism, oxidative stress, and adenosine triphosphate (ATP) level maintenance. On their part, persisters are a naturally occurring sub-population of bacteria, making up about 0.000001% of the overall population. Their lack of growth makes them non-susceptible to antimicrobials and other environmental stressors, favouring their survival in the presence of extremely high pressure from these stressors [4]. Unlike resistance, which involves a one-gene-one-phenotype expression, persistence and tolerance are associated with multiple genes [5–8].

Furthermore, while resistance increases the MIC of the mutants compared with the susceptible parental strains, the MIC of the parental and the evolved strains remain the same during tolerance. Contrarily, tolerance increases the MDK_{99}, the minimum time required to kill 99% of the bacteria in a culture [9]. Generally, persister cells are genetically similar to parental and non-persister cells in a given bacterial population and have the same MIC. However, their presence is responsible for the biphasic killing pattern observed in bacteria, which usually starts exponentially with the killing of the susceptible cells, followed by the persister cells [5,9].

In addition to resistance and tolerance, another factor influencing microbial non-susceptibility to antimicrobials is the mutant selection window (MSW). The MSW represents the concentration range which allows the emergence of resistant mutants within a bacterial population. It is the range between the minimum inhibitory concentration (MIC) and the mutant prevention concentration (MPC) [10]. The MIC is the minimum concentration of an antimicrobial that inhibits bacterial growth, while the MPC is the threshold above which it is predicted that selection pressure would rarely lead to the proliferation of resistant mutants in the bacterial population [11]. The length of the MSW plays a crucial role in the selection of resistance in bacteria. The shorter the MSW, the smaller the drug concentration range required to eliminate the bacteria, and the better the chances of preventing the development of resistant mutants [12].

Effluents from hospital and manufacturing sites may contain chemical pollutants, including pharmaceuticals, especially antibiotics and heavy metals, at concentrations usually higher than the environmental values. For example, in Africa, sulfamethoxazole (SMX) has been detected at concentrations of 20.6 μg/L in hospital effluents compared to 6.8–7.8 μg/L in wastewater treatment plants and surface waters [13]. Also, ciprofloxacin (CIP) levels detected in industrial effluents were up to 31,000 μg/L, over 100 times the toxic level of most bacteria [13–15]. Karkman et al. [14] suggested that such high concentrations, above the bactericidal levels, were responsible for the emergence of antimicrobial resistance (AMR) in the environment. Although concentrations above the MPC rarely favour the emergence of resistant mutants [16,17], environmental concentrations of these stressors are not static, usually fluctuating between very low to extremely high levels, depending on the distance from the source and prevailing weather conditions. Such fluctuations could expose the bacteria to sub-inhibitory concentrations and contribute to the emergence of persister cells and the subsequent development of tolerance in the bacterial populations. Therefore, it is essential to investigate the effect of prolonged exposure to sub-bactericidal concentrations, as seen in the environment, on the emergence of tolerance to stressors in bacteria.

The standard technique to assess tolerance is through time-kill measurements, in which bacteria are exposed to an antimicrobial and the viable colony forming units (CFUs) are determined and plotted against time [18]. When the killing is exponential, the killing rate can be used to measure tolerance, which is the minimum duration of killing (MDK) at a certain percentile of the population; the percentile is expressed as an index in the MDK value. Therefore, MDK_n is the minimum time required to kill *n*% of a bacterial population. Conversely, a high MDK suggests that killing the bacteria would require more time, corresponding to high tolerance [18]. Hence, tolerance is the ability of bacteria to stay alive even at bactericidal antimicrobial concentrations [19].

In our previous experiment, it was observed that the environmental concentrations of oxytetracycline (OXYTET), amoxicillin (AMX), copper (Cu), zinc (Zn), benzalkonium chloride (BAC) 10, dimethylammonium chloride (DADMAC) 12 and a combination of all the individual pollutants (ALL) could not elicit phenotypic or genotypic resistance in *E. coli* following exposure for 30 days [20]. Therefore, the current study assessed the impact of these exposures on the level of persistence and tolerance development in the exposed isolates, using an extremely high ampicillin concentration. The study further assessed the associations with observed mutations via whole genome sequencing (WGS) and single nucleotide polymorphisms (SNP) and investigated the impact of such exposure on the MSW of these exposed *E. coli* isolates to ampicillin, oxytetracycline, and amoxicillin. Although previous studies have demonstrated the development of resistance in bacteria following exposure to pharmaceuticals, such studies have mostly used unrealistically high concentrations, which would seldom be encountered in the environment [21,22]. Here, the concentrations used were those previously identified in the environment [23] to mimic real environmental conditions. It was hypothesised that exposure to environmental concentrations of biocides, antibiotics, and heavy metals induce tolerance in the exposed bacteria, increasing their MSW.

2. Materials and Methods

All chemical stressors used in this study were purchased from Sigma-Aldrich (Kempton Park, South Africa). The preparation of the standards for each chemical has previously been reported [23]. The concentrations used for the 30-day exposure experiments have also been published [20]. In the current study, two sets of experiments were conducted using the 30-day exposed isolates, first to determine the MSW to amoxicillin, oxytetracycline and ampicillin and then their $MDK_{99.9}$ when exposed to extremely high doses of ampicillin.

2.1. Test Organisms

The test organisms were the 30-day exposed *E. coli* (ATCC strain 25922) isolates from the earlier experiment [20], while wholly susceptible *E. coli* (ATCC strain 25922) were used as control. Bacteria inocula at concentrations of 1.5×10^8 CFU/mL (McFarland standard) were prepared using the 30-day exposed isolates and the wholly susceptible *E. coli* (ATCC strain 25922).

2.2. Determination of Bacterial Tolerance

For this experiment, the time required to kill 99.99% of the isolates ($MDK_{99.99}$) was estimated by modifying the method by Fridman et al. [24]. First, the exposed isolates were cultured overnight on nutrient agar at 37 °C to obtain fresh, viable, and concentrated isolates to monitor killing rates adequately. Then, the cultured isolates were scraped into 1 mL of Luria-Bertani (LB) broth (Merck Life Science (Pty) Ltd., Johannesburg, South Africa) and cultured overnight. After that, 1 mL of the concentrated culture was inoculated into 5 mL LB broth supplemented with 100 g/mL ampicillin (about 25 times the *E. coli* ampicillin MIC value). Each culture mixture was prepared in 2 × triplicate sets for three different time intervals (T_a = 3, 5, and 8 h), and incubated at 37 °C in a shaking incubator at 200 rpm.

After incubation and depending on the time interval (3, 5 and 8 h), the culture was washed twice in 5 mL of LBL by centrifuging at 1400 g for 10 min. Next, the pellets were resuspended in 1 mL of LBL and incubated for up to 24 h (21, 19 and 16 h, respectively) at 37 °C in a shaking incubator at 200 rpm. This gave a fresh 24 h culture for the following day's exposures. After 24 h, the plate was cell counted and recorded. The overnight culture was then subjected to the same exposure as the previous day for three consecutive days, under the same experimental conditions. To confirm tolerance and not resistance, the surviving *E. coli* cells were also subjected to antimicrobial susceptibility testing (AST) using the broth microdilution method to ascertain changes in their MICs compared to the original stock [24].

2.3. Mutant Selection Window

The MSW is an antimicrobial concentration range between the MIC and MPC. The MIC was performed using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) broth microdilution method [25–27]. Briefly, two-fold serial dilutions of the test agents (environmentally determined amoxicillin, ampicillin and oxytetracycline) were dispensed into microdilution plates and inoculated with *E. coli*. The plates were incubated at 37 °C for 24 h and read visually. The MPC was obtained by determining the MIC of a higher microbial load (≥10^9 CFU/mL), as previously described [28].

To determine gene mutations that could lead to tolerance, isolates from all the experimental rounds were subjected to whole genome sequencing. All contiguous sequences for the isolates were deposited in GenBank with accession numbers under BioProject PRJNA836107.

3. Results

3.1. Determination of Tolerance

3.1.1. Tolerance among Biocide-Exposed Isolates

The tolerance of the biocide-exposed isolates was measured as the minimum duration for killing 99.99% ($MDK_{99.99}$) of the BAC and the DADMAC-exposed isolates when treated with a very high ampicillin concentration. There was a decrease in the initial bacterial concentration of 2.5 × 10^8 CFU/mL to 2.5 × 10^4 CFU/mL on the second day, giving an $MDK_{99.99}$ of 30 h for BAC 12 (Figure 1). For DADMAC-exposed isolates, the bacterial count dropped from 1.8 × 10^8 to 1.5 × 10^4 CFU/mL, giving an $MDK_{99.99}$ value of 28 h (Figure 1). There was no reduction in the bacterial count of the controls.

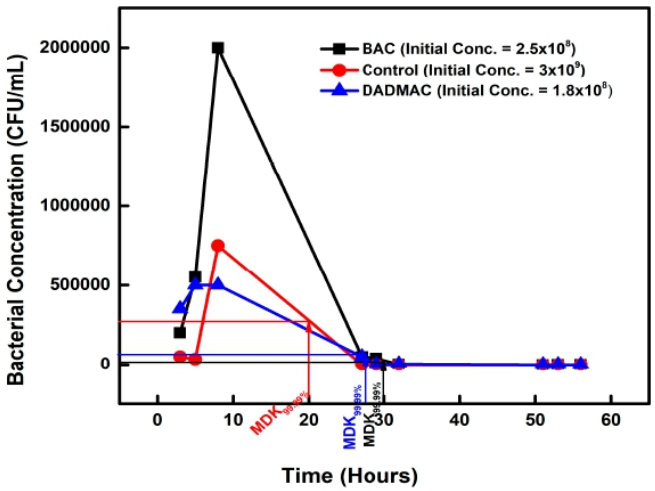

Figure 1. The minimum duration for killing 99.99% ($MDK_{99.99}$) of bacterial cells in the population for BAC (benzalkonium chloride)- and DADMAC (dimethylammonium chloride)-exposed isolates compared to the control.

3.1.2. Tolerance among Antibiotics Residue Exposed Isolates

The microbial count of the amoxicillin-exposed isolates decreased from 1×10^8 CFU/mL to 1×10^4 CFU/mL on the second day, when exposed to a very high ampicillin concentration, giving an $MDK_{99.99}$ of 26 h (Figure 2). The OXYTET-exposed isolates had an $MDK_{99.99}$ value of 28 h, with the initial concentration of 2×10^8 CFU/mL decreasing to 2×10^4 CFU/mL on the second day (Figure 2).

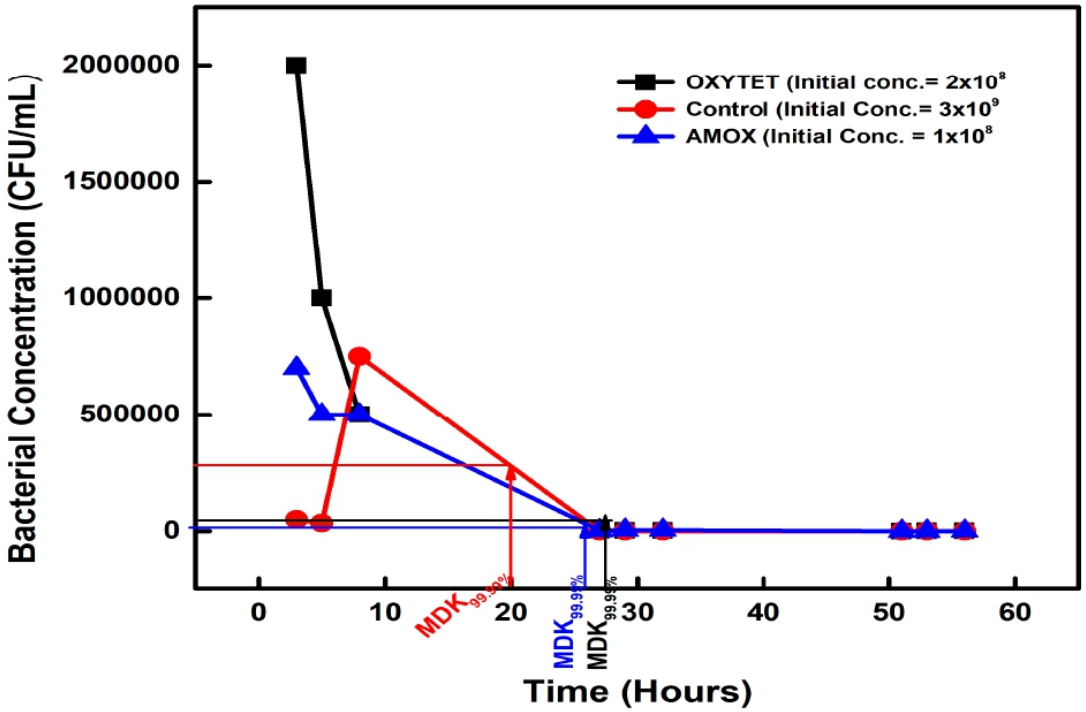

Figure 2. The minimum time required to kill 99.99% ($MDK_{99.99}$) of the AMX (amoxycillin)- and OXYTET (oxytetracycline)-exposed isolates compared to the control.

3.1.3. Tolerance among Metal-Exposed Isolates

When subjected to a high ampicillin concentration, the Zn- and Cu-exposed isolates had an $MDK_{99.99}$ of 28 h and 30 h, with bacterial counts reducing from 2×10^8 CFU/mL to 2×10^4 CFU/mL and 1.5×10^8 CFU/mL to 1.5×10^4 CFU/mL on the second day, respectively (Figure 3).

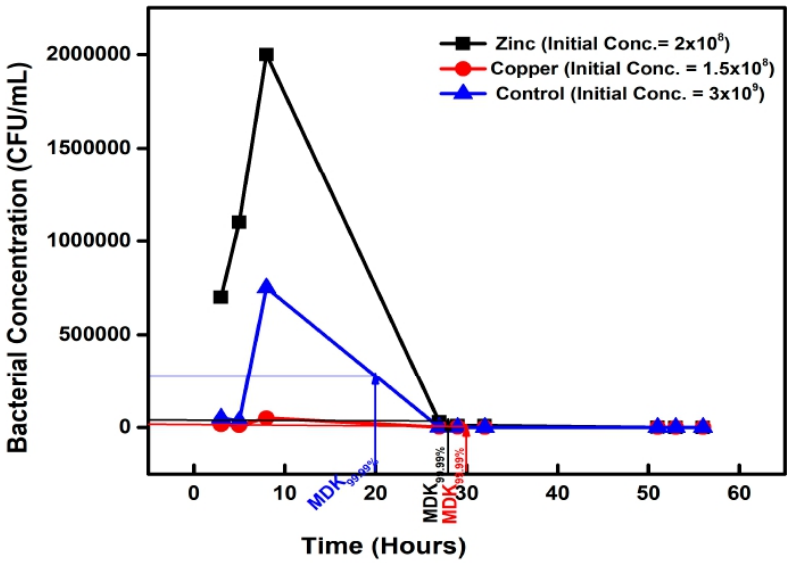

Figure 3. The minimum time required to kill 99.99% ($MDK_{99.99}$) of the zinc- and copper-exposed isolates compared to the control.

3.1.4. Tolerance for the Combined Chemical-Exposed Isolates

The combined chemical (ALL)-exposed isolates subjected to a high ampicillin concentration recorded an $MDK_{99.99}$ value of 26 h with a concentration of 2.5×10^4 CFU/mL on the second day, from an initial 2.5×10^8 CFU/mL (Figure 4).

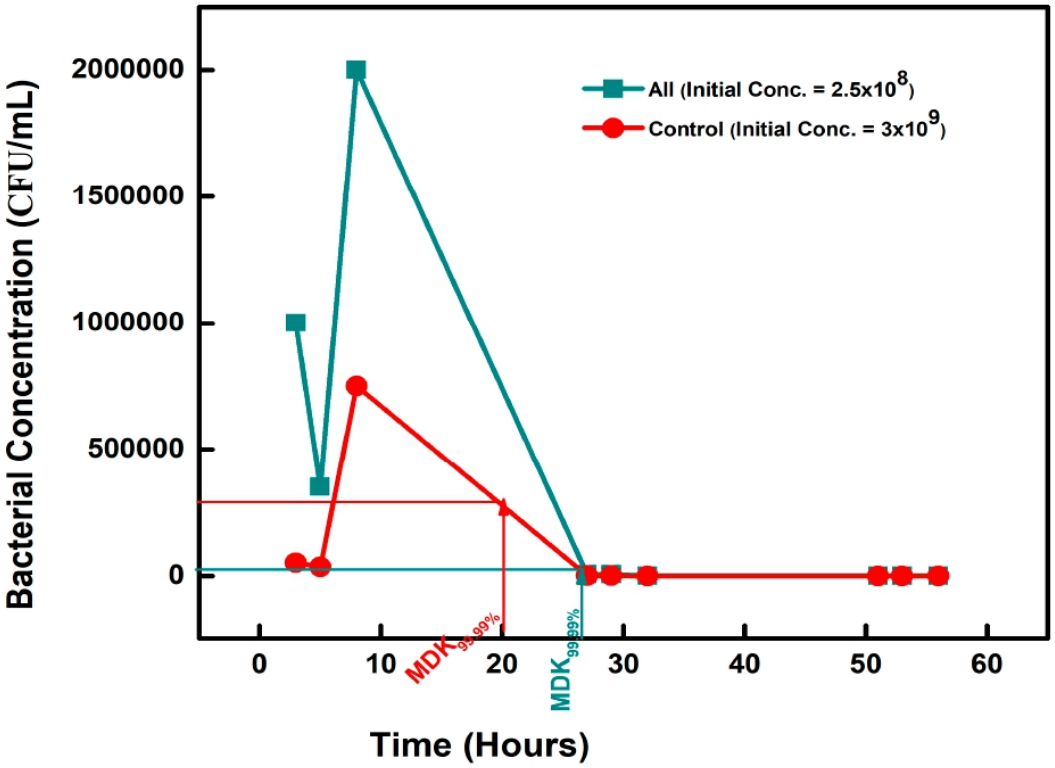

Figure 4. The minimum time required to kill 99.99% (MDK99.99) of the ALL (combined chemicals)-exposed isolates compared to the control.

3.1.5. Comparison of Pollutant-Treated Isolates to Control in the Determination of the $MDK_{99.99}$

The $MDK_{99.99}$ of all the pollutant-exposed isolates, compared to that of the control, are summarised in Table 1. Compared with the control with an initial bacterial count of 3.5×10^9 and an $MDK_{99.99}$ of 20 h, the BAC-exposed isolates showed a 50% increase in the duration of killing, while the DADMAC-exposed isolates recorded a 40% increase following treatment with a very high ampicillin concentration. Also, for the AMX and OXYTET-exposed isolates, the $MDK_{99.99}$ increased by 30% and 40%, respectively, after high ampicillin exposure. Furthermore, there was a 40% and 30% increase in the $MDK_{99.99}$ for Zn and Cu-exposed isolates, respectively, compared to the control. Finally, ALL-treated isolates displayed a 30% increase in the $MDK_{99.99}$ value compared to the control upon treatment with high antibiotic concentrations.

Table 1. Summary of the tolerance experiment results showing the initial concentrations, final concentrations, $MDK_{99.99}$, and percentage difference compared to the control.

Chemical-Exposed Isolate	Initial Concentration (CFU/mL)	Concentration (CFU/mL) on Day 2	$MDK_{99.99}$	Percentage Difference
BAC 12	2.5×10^8	2.5×10^4	30	+50%
DADMAC 10	1.5×10^8	1.5×10^4	28	+40%
AMOXICILLIN	1×10^8	1×10^4	26	+30%
OXYTETRACYCLINE	2×10^8	2×10^4	28	+40%
ZINC	2×10^8	2×10^4	28	+40%
COPPER	1.5×10^8	1.5×10^4	26	+30%
CONTROL	3×10^9	3×10^5	20	0

BAC = benzalkonium chloride; DADMAC = dimethylammonium chloride; MDK = minimum duration of killing; CFU = colony forming units.

3.2. Determination of the Mutant Selection Window

The MSW of the isolates exposed for 30 days to the different environmental stressors was obtained by determining the MIC and MPC of these isolates. The average MSWs for the various isolates, compared to the control, are presented in Table 2.

Table 2. Mean mutant selection window values for the ampicillin-, oxytetracycline-, and amoxicillin-treated isolates.

MSW Treatment	Mean MSW	Standard Deviation
Ampicillin	266.86	27.67
Ampicillin control	144.766	8.76
Oxytetracycline	254.76	0.12
Oxytetracycline control	255.45	0.17
Amoxycillin	452.16	42.84
Amoxycillin control	348.57	59.02

No significant increase in the MSW was observed for the OXYTET treatment compared to the control. For the AMX treatment, no significant differences were observed in the MSW of the AMX, OXYTET, and Cu-exposed isolates compared with the control. However, there was a 41.67% increase in the MSW of Zn-exposed isolates and a 100% increase in the MSW for the DADMAC, BAC, and ALL-exposed isolates. Finally, ampicillin treatment revealed a 100% (Zn-exposed isolates) and 300% (ALL-exposed isolates) increase in the MSW of the test isolates compared to the control (Table 3).

Table 3. Mutant selection window of the 30-day-exposed isolates following oxytetracycline, amoxicillin and ampicillin treatment.

MSW Treatment	Exposure Pollutant	Test Isolates			Controls		
		MIC	MPC	MSW	MIC	MPC	MSW
OXYTETRACYCLINE	AMX	2	256	0.5–256	2	256	2–256
	OXYTET	2	256	2–256	2	256	2–256
	COPPER	2	256	2–256	2	256	2–256
	ZINC	2	256	2–256	2	256	2–256
	BAC	2	256	2–256	2	256	2–256
	DADMAC	0.5	256	0.5–256	0.5	256	0.5–256
	ALL	2	256	2–256	2	256	2–256
AMOXICILLIN	AMX	8	512	8–512	8	512	8–512
	OXYTET	8	512	8–512	8	512	8–512
	COPPER	8	512	8–512	8	512	8–512
	ZINC	8	512	8–512	8	256	8–256
	BAC	8	512	8–512	8	256	8–256
	DADMAC	8	256	8–256	8	128	8–128
	ALL	8	512	8–512	8	256	8–256
AMPICILLIN	AMX	8	256	8–256	8	256	8–256
	OXYTET	8	256	8–256	8	256	8–256
	COPPER	8	256	8–256	8	256	8–256
	ZINC	8	256	8–256	8	128	8–128
	BAC	8	512	8–512	8	128	8–128
	DADMAC	8	256	8–256	8	256	8–256
	ALL	8	258	8–258	8	128	8–128

MSW = mutant selection window; MPC = mutant prevention concentration; MIC = minimum inhibitory concentration; BAC = benzalkonium chloride; DADMAC = dimethylammonium chloride; OXYTET = oxytetracycline; AMX = amoxycillin.

4. Discussion

The present study investigated the impact of exposure to different environmental pollutants on the development of resistance in bacteria, using *E. coli* as a model organism. Previously exposed isolates were treated with extremely high ampicillin concentrations, and the time to reduce their population by 99.99% was determined. It was observed that exposure to environmental concentrations of biocides, antibiotics, and heavy metals induced tolerance in the test organism. This was demonstrated by an increase in the time required to kill 99.99% of the initial count of the exposed bacterial population and a broadening of the mutant selection window.

4.1. Tolerance in Exposed Isolates

Tolerance develops when the number of persister cells in a bacterial population increases depending on prevailing conditions [2,4,5,9]. This means that the increase in the number of persister cells should translate to an increase in tolerance, indicated by an increase in the $MDK_{99.99}$ of the isolates. This is triggered by the expression of genes, as seen in the 30-day-exposed fully susceptible *E. coli* in the current study. Such gene expression is a first step towards resistance, as further exposure may lead to the development of resistance genes. Furthermore, this shows that tolerance enables the bacteria to survive stress, which if not eliminated, lowers the bacterial fitness cost for selecting and expressing resistance genes [24,29,30].

The current experiment reveals that exposing *E. coli* to sub-inhibitory concentrations of different chemicals can increase the time required to kill the exposed isolates ($MDK_{99.99}$). This observation indicates that the exposed isolates may survive longer in the

environment compared to unexposed cells (in this case, the control). From the results, BAC 12 had the highest $MDK_{99.99}$, 50% higher than the control, while amoxicillin and copper had the lowest $MDK_{99.99}$ (30% higher than the control). In an earlier experiment on these isolates, whole genome sequencing showed mutations in the isolates after 30 days of exposure, with no phenotypic resistance [20]. However, this study identified more survivors in the exposed isolates compared to the control isolates, despite the exposure to a very high ampicillin concentration (MIC × 25). This indicates a probable adaptation to antimicrobials through tolerance, as there was an increase in MDK instead of the MIC [24].

Most of the genes detected through WGS/SNP (*acnB, cusA, degQ, epmA, queG, hsmp, mlc, murP, nudK, ptsG, purH, queG, robA, srlE, tsaB, yddG* and *yqhH*) [20] are involved in the repression of oxidative stress, SOS-dependent gene repairs, toxin/antitoxin efflux actions, skin permeability, biofilm formation, or cellular physiological processes. These are factors mostly employed by bacteria for tolerance and persister cell production. [31,32].

Isolates exposed to BAC 12 had the highest $MDK_{99.99}$. In addition to the genes above, these isolates also harboured the *fli*L *gene*. The *fli*L gene was only detected in BAC 12 and oxytetracycline isolates, which may have contributed to the high $MDK_{99.99}$ observed in these two isolates. *fliL* is one of the seven genes within the flagellar-associated *flaA* locus that works with specific proteins to increase bacteria motility [33,34]. Cell motility contributes to bacteria survival and virulence, and survival due to motility does not increase the MIC of the survivors [35]. This result agrees with previous studies indicating that the exposure of *E. coli* to sub-MIC concentrations of BAC resulted in the expression of genes associated with efflux, outer membrane porins and motility, increasing tolerance to BAC [36–39]. Therefore, the detected *filL* gene likely contributed to increased tolerance to stressors in the BAC 12 and OXYTET-exposed isolates, demonstrated by the increased MDK when compared to control.

Oxidative stress, which results from over-accumulating reactive oxygen species (ROS) (produced by normal metabolism and essential for cell signalling and homeostasis), leads to DNA damage and cell death. For example, the *mut*M and *Fpg* (formamidopyrimidine glycolase) genes were only detected in zinc-exposed isolates (with a 40% increase in tolerance compared to the control). *Fpg* is a bifunctional DNA glycosylase that cleaves the N-glycoside bond of redox-damaged purines and incises the phosphodiester backbone to yield single-strand breaks with 3′ and 5′phosphoryl ends [40]. In repairing oxidative-damaged DNA, *mut*M is the primary DNA glycosylase that removes the oxidised purines and some pyrimidines [40]. As such, it is actively involved in the repair of lesions in the transcription of intermediates [41–43]. The repair of genetic materials is part of the SOS response, which in *E. coli*, contributes to the transcription of genes involved in DNA repair, the production of persister cells, biofilm formation, and tolerance mechanisms [31,44,45].

Another gene only detected in DADMAC isolates was *omp*D, a major porin protein in the outer membrane of cells, involved in the efflux of toxins/antitoxins through the cell membrane, which is very important in tolerance [46–48]. Furthermore, the *nud*K gene, also known as GDP-mannose hydrolase (which was expressed by other isolates except for DADMAC-exposed isolates), is a member of the ADP-ribose pyrophosphate sub-family of the *Nudix* hydrolases, and promotes biofilm formation, contributing to persister production and tolerance [31,49]. In addition, the *hsmP* gene, also detected in all the isolates, encodes for biofilm formation [50]. Biofilm formation is very important for tolerance as it encourages the production of persister cells within the population.

Another gene detected in all the isolates was the *mur*P gene. This gene contributes to tolerance by encoding the permease component of the N-acetylmuramic acid PTS transport system, facilitating the uptake and transportation of anhydrous acetylmuramic (*anyMurNAc*) acid. In addition, it encodes *anm*K (anhydro-N-acetyl muramic acid kinase), which is needed to convert imported *anhMurNA*c to *MurNAc-P*, a carbon and energy source for *E. coli*. The cAMP and catabolic response genes in *E. coli* negatively relay *rpoS*, so the over-expression of *rpoS* induces stationary phase cells and persister production and increases tolerance to antimicrobials [31,51–54]. *acnB* is also similar [55–57].

During exposure to antimicrobials, tolerant bacteria can withstand antimicrobial exposure and resume growth and virulence once the stressor is removed; hence their ability to stay alive in fluctuating exposure to antimicrobials, especially above the MIC [19]. This is facilitated by *degQ,* a serine endoprotease and a homologous member of the *HtrA* (high-temperature requirement A) protein family with *degP* and *degS*, which is involved in the degradation of transient proteins, stress sensing, regulation, and protection during unfolded protein responses, especially in isolates exposed to high temperature and nutrient deprivation [31,54,58].

Mutations that lead to tolerance are essential for the continued survival of the bacterial population, as the increased number of persisters creates a reservoir of non-susceptible bacteria, enabling these bacteria to survive antimicrobials at bactericidal concentrations [24]. Therefore, tolerance allows bacteria to adapt to adverse conditions for extended periods; if conditions continue, they select resistance genes with a decreased fitness cost. This can be attributed to the fact that persister cells naturally develop resistance genes, although these genes are usually lost due to high fitness cost. However, tolerance could lead to an increase in persister cells that can easily spread these genes within the population, leading to pseudo resistance. Therefore, tolerance has been observed as the first step towards resistance in most bacteria, especially in environments polluted with very high antimicrobial concentrations [29,59,60].

4.2. Effect of Exposure on the Mutant Selection Window

The MIC and MPC results showed that of the 30-day Cu and Zn-exposed isolates, only Zn-exposed isolates had an increase in their MSW to the tested antibiotics, compared to the unexposed control. On the other hand, there was a significant increase in the MSW of amoxicillin and ampicillin for the Zn-exposed isolates, while the MSW of OXYTET remained the same. This signifies that the exposure of susceptible bacteria to sub-MIC concentrations of heavy metals like Zn in the environment may contribute to the development of more persister cells within the population. These non-susceptible cells could outgrow susceptible ones in the population, increasing the chances of antimicrobial resistance development and spread in bacterial population [28,61,62]. This also agrees with Fridman et al. [24] and Levin-Reisman et al. [60], who stated that tolerant strains enhance the population's survival and extend their survival window beyond the MPC, thereby increasing the MSW.

For the biocides, BAC-exposed isolates exhibited the most significant increase in the MSW compared to the control, especially when tested against amoxicillin and ampicillin but not with oxytetracycline. This shows that biocides in the environment, like heavy metals, can also trigger selection pressures for tolerant strains, contributing to the further resistance of the isolates against known antimicrobials and facilitating mutation [28,61,62].

Unlike the heavy metals and biocides tested in the current study, the antibiotic-exposed isolates all had similar results, indicating only a slight increase in the MSW following ampicillin treatment. This suggests that antibiotics in the environment may not be major contributory factors for increased antimicrobial resistance [12]. This agrees with a previous study which stated that antibiotics in the environment exert less selection pressure for antimicrobial resistance than other stressors, such as heavy metals [62].

5. Conclusions

Although several studies have reported that the exposure of environmental bacteria to different antimicrobials would lead to the development of resistance, this has mostly been undertaken using unrealistically high concentrations of the stressors. The present study revealed that exposing *E. coli* to different chemicals at environmental concentrations for 30 days triggered increased tolerance in the bacterial population when exposed to a high ampicillin concentration. This affected the MSW of the isolates against amoxicillin and ampicillin treatment. Survival in the presence of antimicrobials, even above the MIC value, can be attributed to the emergence of more persister cells, hence increased

tolerance, and this contributed to the observed differences in the MSW of the exposed isolates compared to controls that were not exposed to any chemical. Given the complexity of the environmental dimension of AMR, these results call for a deeper analysis of the mechanisms influencing antimicrobial resistance in the environment. It should, however, be noted that the current study was conducted under static physicochemical conditions and that other factors like temperature, pH, and organic substances present in the natural environment were not investigated. Nevertheless, the results call for greater attention to the release of antimicrobials in the environment; these could have severe negative ecological and public health consequences, especially in resource limited areas without access to basic sanitation facilities and potable water supplies.

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References

1. Costa, V.M.D.; King, C.E.; Kalan, L.; Morar, M.; Sung, W.W.L.; Schwarz, C.; Froese, D.; Zazula, G.; Calmels, F.; Debruyne, R.; et al. Antibiotic Resistance Is Ancient. *Nature* **2011**, *477*, 457–461. https://doi.org/10.1038/nature10388.
2. Sulaiman, J.E.; Lam, H. Evolution of Bacterial Tolerance Under Antibiotic Treatment and Its Implications on the Development of Resistance. *Front. Microbiol.* **2021**, *12*, 617412. https://doi.org/10.3389/fmicb.2021.617412.
3. Wright, M.S.; Peltier, G.L.; Stepanauskas, R.; McArthur, J.V. Bacterial Tolerances to Metals and Antibiotics in Metal-Contaminated and Reference Streams. *FEMS Microbiol. Ecol.* **2006**, *58*, 293–302. https://doi.org/10.1111/j.1574-6941.2006.00154.x.
4. Bergh, B. Van Den; Michiels, J.E.; Wenseleers, T.; Windels, E.M.; Boer, V.; Kestemont, D.; Meester, L. De; Verstrepen, K.J.; Verstraeten, N.; Fauvart, M.; et al. Frequency of Antibiotic Application Drives Rapid Evolutionary Adaptation of *Escherichia coli* Persistence. *Nat. Microbiol.* **2016**, *1*, 16020. https://doi.org/10.1038/nmicrobiol.2016.20.
5. Sulaiman, J.E.; Lam, H. Application of Proteomics in Studying Bacterial Persistence. *Expert Rev. Proteom.* **2019**, *16*, 227–239. https://doi.org/10.1080/14789450.2019.1575207.
6. Song, S.; Wood, T.K. PpGpp Ribosome Dimerization Model for Bacterial Persister Formation and Resuscitation. *Biochem. Biophys. Res. Commun.* **2020**, *523*, 281–286. https://doi.org/10.1016/j.bbrc.2020.01.102.
7. Harms, A.; Maisonneuve, E.; Gerdes, K. Mechanisms of Bacterial Persistence during Stress and Antibiotic Exposure. *Science* **2016**, *354*, aaf4268. https://doi.org/10.1126/science.aaf4268.
8. Wood, T.K.; Knabel, S.J.; Kwan, B.W. Bacterial Persister Cell Formation and Dormancy. *Appl. Environ. Microbiol.* **2013**, *79*, 7116–7121. https://doi.org/10.1128/AEM.02636-13.
9. Balaban, N.Q.; Helaine, S.; Lewis, K.; Ackermann, M.; Aldridge, B.; Andersson, D.I.; Brynildsen, M.P.; Bumann, D.; Camilli, A.; Collins, J.J.; et al. Definitions and Guidelines for Research on Antibiotic Persistence. *Nat. Rev. Microbiol.* **2019**, *17*, 441–448. https://doi.org/10.1038/s41579-019-0196-3.

10. Zhang, L.; Wang, H.; Bai, Y.; Wang, L.; Bai, Y.; Hu, J. Evaluation of the Mutant Selection Window of Danofloxacin against Actinobacillus Pleuropneumoniae in an in Vitro Dynamic Model. *Front. Vet. Sci.* **2023**, *10*, 1107608. https://doi.org/10.3389/fvets.2023.1107608.
11. Krajewska, J.; Tyski, S.; Laudy, A.E. Mutant Prevention Concentration, Frequency of Spontaneous Mutant Selection, and Mutant Selection Window—A New Approach to the In Vitro Determination of the Antimicrobial Potency of Compounds. *Antimicrob. Agents Chemother.* **2023**, *67*, e01373-22. https://doi.org/10.1128/aac.01373-22.
12. Drlica, K.; Zhao, X. Mutant Selection Window Hypothesis Updated. *Clin. Infect. Dis.* **2007**, *44*, 681–688. https://doi.org/10.1086/511642.
13. Ngigi, A.N.; Magu, M.M.; Muendo, B.M. Occurrence of Antibiotics Residues in Hospital Wastewater, Wastewater Treatment Plant, and in Surface Water in Nairobi County, Kenya. *Environ. Monit. Assess.* **2020**, *192*, 18. https://doi.org/10.1007/s10661-019-7952-8.
14. Karkman, A.; Pärnänen, K.; Larsson, D.G.J. Fecal Pollution Can Explain Antibiotic Resistance Gene Abundances in Anthropogenically Impacted Environments. *Nat. Commun.* **2019**, *10*, 80. https://doi.org/10.1038/s41467-018-07992-3.
15. Larsson, D.G.J.; Andremont, A.; Bengtsson-Palme, J.; Brandt, K.K.; de Roda Husman, A.M.; Fagerstedt, P.; Fick, J.; Flach, C.F.; Gaze, W.H.; Kuroda, M.; et al. Critical Knowledge Gaps and Research Needs Related to the Environmental Dimensions of Antibiotic Resistance. *Environ. Int.* **2018**, *117*, 132–138. https://doi.org/10.1016/j.envint.2018.04.041.
16. Dong, Y.; Zhao, X.; Kreiswlrth, B.N.; Drlica, K. Mutant Prevention Concentration as a Measure of Antibiotic Potency: Studies with Clinical Isolates of Mycobacterium Tuberculosis. *Antimicrob. Agents Chemother.* **2000**, *44*, 2581–2584. https://doi.org/10.1128/AAC.44.9.2581-2584.2000.
17. Blondeau, J.M.; Zhao, X.; Hansen, G.; Drlica, K. Mutant Prevention Concentrations of Fluoroquinolones for Clinical Isolates of *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* **2001**, *45*, 433–438. https://doi.org/10.1128/AAC.45.2.433.
18. Brauner, A.; Shoresh, N.; Fridman, O.; Balaban, N.Q. An Experimental Framework for Quantifying Bacterial Tolerance. *Biophys. J.* **2017**, *112*, 2664–2671. https://doi.org/10.1016/j.bpj.2017.05.014.
19. Kester, J.C.; Fortune, S.M. Persisters and beyond: Mechanisms of Phenotypic Drug Resistance and Drug Tolerance in Bacteria. *Crit. Rev. Biochem. Mol. Biol.* **2014**, *49*, 91–101. https://doi.org/10.3109/10409238.2013.869543.
20. Chukwu, K.B.; Abafe, O.A.; Amoako, D.G.; Essack, S.Y.; Abia, A.L.K. Environmental Concentrations of Antibiotics, Biocides, and Heavy Metals Fail to Induce Phenotypic Antimicrobial Resistance in *Escherichia coli*. *Sci. Total Environ.* **2023**, *899*, 165721. https://doi.org/10.1016/j.scitotenv.2023.165721.
21. Lau, C.H.F.; Tien, Y.C.; Stedtfeld, R.D.; Topp, E. Impacts of Multi-Year Field Exposure of Agricultural Soil to Macrolide Antibiotics on the Abundance of Antibiotic Resistance Genes and Selected Mobile Genetic Elements. *Sci. Total Environ.* **2020**, *727*, 138520. https://doi.org/10.1016/j.scitotenv.2020.138520.
22. Brown, L.P.; Murray, R.; Scott, A.; Tien, Y.C.; Lau, C.H.F.; Tai, V.; Topp, E. Responses of the Soil Bacterial Community, Resistome, and Mobilome to a Decade of Annual Exposure to Macrolide Antibiotics. *Appl. Environ. Microbiol.* **2022**, *88*, e00316-22. https://doi.org/10.1128/aem.00316-22.
23. Fridman, O.; Goldberg, A.; Ronin, I.; Shoresh, N.; Balaban, N.Q. Optimization of Lag Time Underlies Antibiotic Tolerance in Evolved Bacterial Populations. *Nature* **2014**, *513*, 418–421. https://doi.org/10.1038/nature13469.
24. Wiegand, I.; Hilpert, K.; Hancock, R.E.W. Agar and Broth Dilution Methods to Determine the Minimal Inhibitory Concentration (MIC) of Antimicrobial Substances. *Nat. Protoc.* **2008**, *3*, 163–175. https://doi.org/10.1038/nprot.2007.521.
25. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing*; Twenty-Seventh Informational Supplement. CLSI Document M100-S27; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2017; ISBN 1562387855.
26. EUCAST. *Testing Breakpoint Tables for Interpretation of MICs and Zone Diameters; version 10.02020*. European Committee on Antimicrobial Susceptibility Testing: Växjö , Sweden.
27. Blondeau, J.M. New Concepts in Antimicrobial Susceptibility Testing: The Mutant Prevention Concentration and Mutant Selection Window Approach. *Vet. Dermatol.* **2009**, *20*, 383–396. https://doi.org/10.1111/j.1365-3164.2009.00856.x.
28. Balcázar, J.L.; Subirats, J.; Borrego, C.M. The Role of Biofilms as Environmental Reservoirs of Antibiotic Resistance. *Front. Microbiol.* **2015**, *6*, 1216. https://doi.org/10.3389/fmicb.2015.01216.
29. Levin-Reisman, I.; Ronin, I.; Gefen, O.; Braniss, I.; Shoresh, N.; Balaban, N.Q. Antibiotic Tolerance Facilitates the Evolution of Resistance. *Science* **2017**, *355*, 826–830.
30. Trastoy R, Manso T, Fernández-García L, Blasco L, Ambroa A, Pérez Del Molino ML, Bou G, García-Contreras R, Wood TK, Tomás M. Mechanisms of Bacterial Tolerance and Persistence in the Gastrointestinal and Respiratory Environments. Clin Microbiol Rev. 2018 Aug 1;31(4):e00023-18. doi: 10.1128/CMR.00023-18. PMID: 30068737; PMCID: PMC6148185.
31. Abdallah, M.; Benoliel, C.; Drider, D.; Dhulster, P.; Chihib, N.E. Biofilm Formation and Persistence on Abiotic Surfaces in the Context of Food and Medical Environments. *Arch. Microbiol.* **2014**, *196*, 453–472. https://doi.org/10.1007/s00203-014-0983-1.
32. Tachiyama, S.; Chan, K.L.; Liu, X.; Hathroubi, S.; Li, W.; Peterson, B. The Fl Agellar Motor Protein FliL Forms a Scaffold of Circumferentially Positioned Rings Required for Stator Activation. *Proc. Natl. Acad. Sci. USA* **2022**, *119*, e2118401119. https://doi.org/10.1073/pnas.2118401119/-/DCSupplemental.Published.
33. Malakooti, J.; Komeda, Y.; Matsumura, P. DNA Sequence Analysis, Gene Product Identification , and Localization of Flagellar Motor Components of *Escherichia coli*. *J. Bacteriol.* **1989**, *171*, 2728–2734.

34. Stabryla, L.M.; Johnston, K.A.; Diemler, N.A.; Cooper, V.S.; Millstone, J.E.; Haig, S.J.; Gilbertson, L.M. Role of Bacterial Motility in Differential Resistance Mechanisms of Silver Nanoparticles and Silver Ions. *Nat. Nanotechnol.* **2021**, *16*, 996–1003. https://doi.org/10.1038/s41565-021-00929-w.
35. Rodríguez-López, P.; Cabo, M.L. Tolerance Development in Listeria Monocytogenes-*Escherichia Coli* Dual-Species Biofilms after Sublethal Exposures to Pronase-Benzalkonium Chloride Combined Treatments. *Food Microbiol.* **2017**, *67*, 58–66. https://doi.org/10.1016/j.fm.2017.06.002.
36. Bore, E.; Hébraud, M.; Chafsey, I.; Chambon, C.; Skjæret, C.; Moen, B.; Møretrø, T.; Langsrud, Ø.; Rudi, K.; Langsrud, S. Adapted Tolerance to Benzalkonium Chloride in *Escherichia Coli* K-12 Studied by Transcriptome and Proteome Analyses. *Microbiology* **2007**, *153*, 935–946. https://doi.org/10.1099/mic.0.29288-0.
37. Moen, B.; Rudi, K.; Bore, E.; Langsrud, S. Subminimal Inhibitory Concentrations of the Disinfectant Benzalkonium Chloride Select for a Tolerant Subpopulation of *Escherichia Coli* with Inheritable Characteristics. *Int. J. Mol. Sci.* **2012**, *13*, 4101–4123. https://doi.org/10.3390/ijms13044101.
38. Forbes, S.; Morgan, N.; Humphreys, G.J.; Amézquita, A.; Mistry, H.; McBain, A.J. Loss of Function in *Escherichia Coli* Exposed to Environmentally Relevant Concentrations of Benzalkonium Chloride. *Appl. Environ. Microbiol.* **2019**, *85*, e02417-18. https://doi.org/10.1128/AEM.02417-18.
39. Morland, I.; Rolseth, V.; Luna, L.; Rognes, T.; Bjørås, M.; Seeberg, E. Human DNA Glycosylases of the Bacterial Fpg/MutM Superfamily: An Alternative Pathway for the Repair of 8-Oxoguanine and Other Oxidation Products in DNA. *Nucleic Acids Res.* **2002**, *30*, 4926–4936. https://doi.org/10.1093/nar/gkf618.
40. Makasheva, K.A.; Endutkin, A.V.; Zharkov, D.O. Requirements for DNA Bubble Structure for Efficient Cleavage by Helix–Two-Turn–Helix DNA Glycosylases. *Mutagenesis* **2019**, *35*, 119–128. https://doi.org/10.1093/mutage/gez047.
41. Fromme, J.C.; Banerjee, A.; Verdine, G.L. DNA Glycosylase Recognition and Catalysis. *Curr. Opin. Struct. Biol.* **2004**, *14*, 43–49. https://doi.org/10.1016/j.sbi.2004.01.003.
42. Schalow, B.J.; Courcelle, C.T.; Courcelle, J. *Escherichia Coli* Fpg Glycosylase Is Nonrendundant and Required for the Rapid Global Repair of Oxidized Purine and Pyrimidine Damage In Vivo. *J. Mol. Biol.* **2011**, *410*, 183–193. https://doi.org/10.1016/j.jmb.2011.05.004.
43. Pourahmad Jaktaji, R.; Pasand, S. Overexpression of SOS Genes in Ciprofloxacin Resistant *Escherichia Coli* Mutants. *Gene* **2016**, *576*, 115–118. https://doi.org/10.1016/j.gene.2015.09.069.
44. Valat, C.; Hirchaud, E.; Drapeau, A.; Touzain, F.; de Boisseson, C.; Haenni, M.; Blanchard, Y.; Madec, J.Y. Overall Changes in the Transcriptome of *Escherichia coli* O26:H11 Induced by a Subinhibitory Concentration of Ciprofloxacin. *J. Appl. Microbiol.* **2020**, *129*, 1577–1588. https://doi.org/10.1111/jam.14741.
45. Porin, M.; Santiviago, C.A.; Toro, C.S.; Hidalgo, A.A.; Youderian, P.; Mora, G.C. Global Regulation of the Salmonella Enterica Serovar Typhimurium. *J. Bacteriol.* **2003**, *185*, 5901–5905. https://doi.org/10.1128/JB.185.19.5901.
46. Li, M.; Liu, Q.; Teng, Y.; Ou, L.; Xi, Y.; Chen, S.; Duan, G. The Resistance Mechanism of *Escherichia Coli* Induced by Ampicillin in Laboratory. *Infect. Drug Resist.* **2019**, *12*, 2853–2863. https://doi.org/10.2147/IDR.S221212.
47. Gogol, E.B.; Rhodius, V.A.; Papenfort, K.; Vogel, J.; Gross, C.A. Small RNAs Endow a Transcriptional Activator with Essential Repressor Functions for Single-Tier Control of a Global Stress Regulon. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 12875–12880. https://doi.org/10.1073/pnas.1109379108.
48. Boto, A.N.; Xu, W.; Jakoncic, J.; Pannuri, A.; Romeo, T.; Bessman, M.J.; Gabelli, S.B.; Amzel, L.M. Structural Studies of the Nudix GDP-Mannose Hydrolase from E. Coli Reveals a New Motif for Mannose Recognition. *Proteins Struct. Funct. Bioinform.* **2011**, *79*, 2455–2466. https://doi.org/10.1002/prot.23069.
49. Bobrov, A.G.; Kirillina, O.; Perry, R.D. The Phosphodiesterase Activity of the HmsP EAL Domain Is Required for Negative Regulation of Biofilm Formation in Yersinia Pestis. *FEMS Microbiol. Lett.* **2005**, *247*, 123–130. https://doi.org/10.1016/j.femsle.2005.04.036.
50. Dahl, U.; Jaeger, T.; Tra, B.; Sattler, J.M.; Mayer, C. Identification of a Phosphotransferase System of *Escherichia Coli* Required for Growth on N-Acetylmuramic Acid. *J. Bacteriol.* **2004**, *186*, 2385–2392. https://doi.org/10.1128/JB.186.8.2385.
51. Heravi, K.M.; Altenbuchner, J. Cross-Talk among Transporters of the Phosphoenolpyruvate- Dependent Phosphotransferase System in Bacillus Subtilis. *J. Bacteriol.* **2018**, *200*, 10–1128. https://doi.org/10.1128/JB.00213-18.
52. Ruscitto, A.; Hottmann, I.; Stafford, G.P.; Schäffer, C. Identification of a Novel N-Acetylmuramic Acid (MurNAc) Transporter in Tannerella Forsythia. *J. Bacteriol.* **2016**, *198*, 3119–3125. https://doi.org/10.1128/JB.00473-16.
53. van den Bergh, B.; Fauvart, M.; Michiels, J. Formation, Physiology, Ecology, Evolution and Clinical Importance of Bacterial Persisters. *FEMS Microbiol. Rev.* **2017**, *41*, 219–251. https://doi.org/10.1093/femsre/fux001.
54. Araujo, J.; Ottinger, S.; Venkat, S.; Gan, Q.; Fan, C.; Werner, C. Studying Acetylation of Aconitase Isozymes by Genetic Code Expansion. *Front. Chem.* **2022**, *10*, 862483. https://doi.org/10.3389/fchem.2022.862483.
55. Brock, M.; Maerker, C.; Schu, A.; Vo, U. Oxidation of Propionate to Pyruvate in *Escherichia Coli* Involvement of Methylcitrate Dehydratase and Aconitase. *Eur. J. Biochem.* **2002**, *6194*, 6184–6194. https://doi.org/10.1046/j.1432-1033.2002.03336.x.
56. Cunningham, L.; Gruer, M.J.; Guest, J.R. Transcriptional Regulation of the Aconitase Genes (AcnA and AcnB) of *Escherichia coli*. *Microbiology* **1997**, *143*, 3795–3805.
57. Wilmaerts, D.; Dewachter, L.; De Loose, P.J.; Bollen, C.; Verstraeten, N.; Michiels, J. HokB Monomerization and Membrane Repolarization Control Persister Awakening. *Mol. Cell* **2019**, *75*, 1031–1042.e4. https://doi.org/10.1016/j.molcel.2019.06.015.

58. Kim, J.S.; Cho, D.H.; Heo, P.; Jung, S.C.; Park, M.; Oh, E.J.; Sung, J.; Kim, P.J.; Lee, S.C.; Lee, D.H.; et al. Fumarate-Mediated Persistence of *Escherichia Coli* against Antibiotics. *Antimicrob. Agents Chemother.* **2016**, *60*, 2232–2240. https://doi.org/10.1128/AAC.01794-15.
59. Cohen, N.R.; Lobritz, M.A.; Collins, J.J. Microbial Persistence and the Road to Drug Resistance. *Cell Host Microbe* **2013**, *13*, 632–642. https://doi.org/10.1016/j.chom.2013.05.009.
60. Liu, Y.; Cui, J.; Wang, R.; Wang, X.; Drlica, K.; Zhao, X. Selection of Rifampicin-Resistant *Staphylococcus Aureus* during Tuberculosis Therapy: Concurrent Bacterial Eradication and Acquisition of Resistance. *J. Antimicrob. Chemother.* **2005**, *56*, 1172–1175. https://doi.org/10.1093/jac/dki364.
61. Pasquali, F.; Manfreda, G. Mutant Prevention Concentration of Ciprofloxacin and Enrofloxacin against *Escherichia Coli*, *Salmonella Typhimurium* and *Pseudomonas Aeruginosa*. *Vet. Microbiol.* **2007**, *119*, 304–310. https://doi.org/10.1016/j.vetmic.2006.08.018.
62. Xu, Y.; Xu, J.; Mao, D.; Luo, Y. 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.* **2017**, *220*, 900–908. https://doi.org/10.1016/j.envpol.2016.10.074.

CHAPTER 5

CONCLUSION, LIMITATIONS, AND RECOMMENDATIONS

5.1. Conclusion

This study describes the effect of detected environmental concentrations, as well as sub-inhibitory concentrations of antimicrobials, on the development of antibiotic resistance in *E. coli.* The findings of the study in relation to our five objectives are:

1. Determine the presence of selected environmental stressors, viz., antibiotics, biocides and heavy metals, and their respective concentrations from water samples collected from a WWTP effluent and upstream and downstream from its receiving river.

    i. A total of 69 heavy metals were screened, of which 26 heavy metals were detected, with seven having concentrations above those recommended by the World Health Organization (WHO) and South African National Standard (SANS). Of these, the highest mean metal concentration was observed for sodium (27.734 mg/L), while the least was for iron (0.001 mg/L).
    ii. Although aluminium 3.43 mg/L) and zinc (0.078 mg/L) were higher than the SANS limits (aluminium - 0.3 mg/L; zinc - 0.005 mg/L), other heavy metals (arsenic, silver, gold, barium, beryllium, cadmium, cobalt, chromiun, mercury, molybdenum, nickel, lead, antimony, selenium, uranium, vanadium V) were detected at very low concentrations.
    iii. A total of 24 antibiotics were targeted, and we detected 14 across all samples, with the WWTP effluent samples recording the overall highest total antibiotic concentration compared to the other sites. Sulfamethoxazole was the most detected at the highest concentration (286.180 µg/L) and penicillin at the lowest (2.2 µg/L). Most importantly, some of these concentrations were higher than the recommended PNEC values, as observed for sulfamethoxazole tetracycline, oxytetracycline, lincomycin, and amoxicillin.
    iv. Eight of the 10 targeted biocides detected in this study had quantifiable values. Benzalkonium chloride (BAC) 12 had the highest detected concentration of 29,58 µg/L, while BenthEZ was the most frequently detected in 75% of the samples.
    v. The overall highest mean antibiotic, metal and biocide concentrations were observed for sulfamethoxazole (286.180 µg/L), neodymium (Nd; 27.734 mg/L), and benzalkonium chloride (BAC 12) (7.805 µg/L), respectively. In decreasing order per sampling site, the pollutant concentrations were effluent > downstream > upstream.
2. Determine the antibiotic resistance profiles of *E. coli* from the WWTP and its receiving river.

i. *E. coli* was also detected from the samples, with a total of 142 confirmed *E. coli* isolates tested against 19 antibiotics to which they exhibited resistance s.

ii. Of these, 109 (76.76%) were resistant to at least one of the antibiotics tested. The highest percentage resistance was observed against amoxicillin-clavulanic acid (104/109; 95.41%), while the least was against azithromycin (Table S2.7, Chapter 3). All the isolates were susceptible to ceftazidime, cefotaxime and meropenem (Table S2.7, Chapter 3).

iii. Furthermore, 57/109 (52.29%) were resistant to three or more antibiotics, with some isolates being resistant to eight antibiotics.

3. Determine, via 30-days exposure experiments, the effect of environmental concentration of the selected antibiotics, biocides, and heavy metals, on the development of resistance in previously susceptible *E. coli* (ATCC 25922) strain phenotypically by AST and genotypically using WGS and single nucleotide variants (SNV).

i. EXposure to the various concentrations and combinations of the stressors did not trigger phenotypic resistance in the experimental bacteria. When subjected to antibiotic susceptibility against 19 antibiotics, all the isolates tested were susceptible to all the antibiotics tested.

ii. Genotypic analysis through WGS on the exposed isolates only found the macrolide resistance *mdf*(A) gene (also present in the control strain) and the disinfectant resistance gene *sit*ABCD.

iii. With further analysis for SNVs, mutations were detected for 19 genes that encoded for oXidative stress, DNA repair, membrane proteins effluX systems, growth and persister formations except for the *robA*, a transcription protein subset of the *ArcC/ XylS* family of proteins, which confer multidrug resistance in *E. coli*.

iv. A total of 2580 variants were identified and 1197 of these were identified in all reads. Nineteen genes were identified by name in the output. They are *yqhH*, *degQ*, *purH*, *epmA*, *queG*, *robA*, *acnB*, *cusA*, *yddG*, *mlc*, *tsaB*, *ompD*, *nudK*, *murP*, *srlE*, *fliL*, *hsmp, mutM* and *ptsG*.

4. Determine the effect of 30 days exposure to selected stressors, on the development of persistence and tolerance in exposed isolates, compared to previously susceptible *E. coli* (ATCC 25922) strain.

i. Re-exposure of our 30-day isolates to very high (25 x MIC) concentrations of ampicillin showed an increase in the $MDK_{99.99}$ values by 30 to 50%, when compared to untreated exposed control.

ii. This indication of increased persister population and a probable increase in tolerance, was confirmed by the MIC, which indicated no increase in the MIC of the isolates, thereby confirming tolerance.

5. Determine the effect of 30-days exposure to selected stressors, on mutant selection window of exposed isolates, compared to previously susceptible *E. coli* (ATCC 25922) strain.

i. Exposing *E. coli* to different chemicals at environmental concentrations for 30 days triggered increased tolerance in the bacterial population when exposed to a high ampicillin concentration.

ii. Survival in the presence of antimicrobials, even above the MIC value, can be attributed to the emergence of more persister cells, hence increased tolerance, and this contributed to the observed increased MSW of the exposed isolates compared to controls that were not exposed to any chemical.

iii. There was a slight increase in the MSW of the zinc and BAC-exposed isolates to ampicillin and amoxicillin. For the DADMAC-exposed isolates, only the MSW for amoxicillin was slightly increased, when compared to the unexposed control.

5.2. Limitations

1. The study focused on selected stressors and not all the stressors identified during the field sampling. However, within the environment, bacterial are exposed to more complex systems than those investigated in this study, and this could lead to different outcomes compared to those reported here.
2. The study would have benefitted from sequencing of more isolates besides the 30 days isolates, like the 15^{th} and 7^{th} day isolates, to determine exact time for mutation. This was however not possible due to financial constraints.

5.3. Recommendations

1. It was observed that although low concentrations reduced their antimicrobial effect, it does not eliminate the risk of inducing selection pressure for antimicrobial resistance in environmental bacteria. Therefore, there is need for stringent measures to prevent the discharge of these chemicals into the environment. Where such pollution has occurred, there is an urgent need for improved remediation strategies to remove these chemicals from the environment.

2. Wastewater treatment plants must treat wastewater longer, varying the treatment time and chemicals to eliminate persister cells that have resumed growth after tolerance.

3. More studies need to be conducted to ascertain the role of other chemicals and stressors in the environment on the development of antibiotic resistance. This is because the environment constitutes a mixture of diverse stressors and many factors come into play, besides the concentration of the chemicals studied in this study.

4. Also, there is need for more studies to ascertain the effect of the chemical concentrations and exposure times on rate of persister generation and resistance development.

APPENDICES

APPENDIX I:

Ethical Approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

17 March 2017

Prof SY Essack
Department of Pharmaceutical Sciences
School of Health Sciences
essacks@ukzn.ac.za

Dear Prof Essack

Title: One Health approach to the containment of antibiotic resistance.
Degree: Non-degree
BREC Ref No: BCA444/16

CLASS APPROVAL

The Biomedical Research Ethics Committee (BREC) has considered the abovementioned application at a meeting held on 13 September 2016.

The study was provisionally approved by BREC pending appropriate responses to queries raised. Your responses dated 28 February 2017 to queries raised on 19 September 2016 have been noted and approved by the Biomedical Research Committee at a meeting held on 14 March 2017.

This approval is valid for one year from **17 March 2017.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

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Biomedical Research Ethics Committee
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Table S2.1 Overall mean metal concentration.

Metal (mg/L)	F_Up	F_Down	F_Eff	M_Up	M_Down	M_Eff	Mean	SD
Nd	13.068	20.687	67.791	0.088	28.022	36.749	27.734	21.27198
Ca	6.637	8.017	17.358	0.083	16.386	20.375	11.476	7.107189
Mn	4.138	4.671	6.266	0.194	6.956	6.521	4.791	2.288649
La	1.847	2.906	10.051	0.006	3.137	4.413	3.726	3.132304
Sm	4.092	5.328	2.768	0.125	2.18	2.633	2.854	1.613021
Ga	2.198	3.197	0.167	0	0.084	0.097	0.957	1.264778
Al	2.05	2.901	0.269	0	0.1	0.1	0.903	1.141096
Pb	0.072	0.076	2.326	0.086209	0.052	0.155	0.461	0.834439
Na	0.16	0.254	0.122	0	0.025	0.054	0.102	0.086941
Ba	0.074	0.109	0.015	0.002123	0.019	0.026	0.041	0.037834
Ti	0.037	0.05	0.054	0.003637	0.043	0.054	0.04	0.017381
B	0.006	0.006	0.024	0.002582	0.01	0.023	0.012	0.008565
V	0.017	0.017	0.012	0.000593	0.001	0	0.008	0.00754
Co	0.011	0.018	0	0	0	0	0.005	0.007064
Gd	0.01	0.013	0.001	7.48E-05	0.002	0.002	0.005	0.005194
Y	0.01	0.014	0	0.000338	0.001	0	0.004	0.005633
Se	0.002	0.003	0.009	0.000355	0.002	0.004	0.003	0.002787
Mg	0.001	0.001	0.009	0.000265	0.001	0.003	0.002	0.002923
Cu	0.003	0.004	0.007	0	0	0.001	0.002	0.002552
Ni	0.006	0.009	0	0	0	0	0.002	0.003457
Li	0.005	0.008	0	0	0	0	0.002	0.00321
Dy	0.005	0.007	0.001	0	0.001	0	0.002	0.0027
Pd	0.004	0.006	0	0	0	0	0.002	0.00244
Zn	0.004	0.006	0	0	0	0	0.002	0.002287
Os	0.002	0.002	0.002	0.00166	0.001	0	0.001	0.000659

Cr	0.003	0.004	0	0	0	0	0.001	0.001625
Ag	0.002	0	0	0.00172	0.001	0	0.001	0.000695
Si	0.001	0.002	0	0.000436	0.001	0	0.001	0.000572
P	0.002	0.001	0.001	0	0	0	0.001	0.000745
Pr	0.001	0.002	0.001	0	0	0	0.001	0.000639
K	0.001	0.001	0.002	0	0	0.001	0.001	0.000466
Au	0.001	0	0.002	0	0.001	0.001	0.001	0.000539
Rb	0.001	0.002	0	0	0	0	0.001	0.000845
Hf	0.001	0.002	0	0	0	0	0.001	0.000763
Sr	0.001	0.002	0	0	0	0	0.001	0.000745
Hg	0.001	0	0.002	0	0	0.001	0.001	0.000517
Fe	0.001	0.002	0	0	0	0	0	0.000657

Table S2.2 Metal comparison across sampling sites

	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Ag	1.907	2	0.197	0.000804127	-0.001010293	0.002618547
Al	2.373	2	0.141	0.919916667	-0.748043806	2.58787714
Au	2.068	2	0.175	0.000638875	-0.000690511	0.00196826
B	2.019	2	0.181	0.012028704	-0.013601121	0.037658528
Ba	3.397	2	0.077	0.042983961	-0.011463695	0.097431617
Ca	4.971	2	0.038	13.59083333	1.826391044	25.35527562
Ce	1.858	2	0.204	0.004808589	-0.006324875	0.015942052
Co	1.943	2	0.191	0.001132621	-0.001375248	0.003640489
Cr	3.152	2	0.088	0.002381375	-0.000869771	0.00563252
Cu	2.211	2	0.158	0.0021793	-0.002061443	0.006420043
Dy	1.885	2	0.2	0.000450526	-0.000577794	0.001478845
Fe	2.189	2	0.16	0.97116667	-0.9379916	2.8803249
Ga	2.656	2	0.117	0.004920678	-0.003050123	0.01289148
Gd	1.88	2	0.201	0.000522582	-0.000673639	0.001718803
Hf	1.626	2	0.245	0.000502463	-0.000827013	0.001831939
Hg	2.964	2	0.097	0.000642807	-0.000290222	0.001575835
K	2.461	2	0.133	4.02925	-3.0162263	11.0747263
La	1.887	2	0.2	0.002202377	-0.002820644	0.007225398
Li	1.4	2	0.296	0.002441537	-0.005059768	0.009942841
Mg	23.64	2	0.002	5.91725	4.8402823	6.9942177
Mn	3.489	2	0.073	0.1671667	-0.038973	0.373306
Na	2.829	2	0.106	30.92416667	-16.11520028	77.96353361
Nd	1.876	2	0.202	0.002365057	-0.003060187	0.007790302
Ni	6.229	2	0.025	0.001457572	0.000450794	0.00246435
Os	5.155	2	0.036	0.00069499	0.0001149	0.001275079

P	1.183	2	0.358	0.461038451	-1.215902598	2.1379795
Pb	1.934	2	0.193	0.001696133	-0.002078214	0.00547048
Pd	3.471	2	0.074	0.000660052	-0.000158214	0.001478319
Pr	1.925	2	0.194	0.000585663	-0.000723255	0.001894582
Rb	2.222	2	0.156	0.003420491	-0.003202251	0.010043234
Se	2.337	2	0.145	0.000788324	-0.000663364	0.002240012
Si	10.583	2	0.009	3.22	1.9108357	4.5291643
Sm	1.874	2	0.202	0.000509622	-0.000660593	0.001679838
Sr	6.579	2	0.022	0.043718999	0.015125577	0.072312421
Ti	7.615	2	0.017	0.007875948	0.003425813	0.012326083
V	2.031	2	0.179	0.004272177	-0.00477834	0.013322694
Y	1.922	2	0.194	0.001584638	-0.001962135	0.005131412
Zn	3.138	2	0.088	0.032610162	-0.012103403	0.077323727

Table S2.3 Antibiotics distribution across all sampling points

ANTIBIOTICS	US am 1	US am 2	US pm1	USpm2	Upstream	EFam1	EFam2	EF pm1	EF pm 2	Effluent	DS am 1	DS am 2	DS pm1	DS pm 2	Downstream	Highest Conc
Sulfamethoxazole	18.64	2.8	19.74	5.32	11.625	280.26	11.2	286.18	0	144.41	54.18	0.62	59.78	14.72	32.325	286.18
Amoxycillin	0	120.37	0	128.88	62.3125	0	125.94	0	112.99	59.7325	0	136.38	0	73.41	52.4475	136.38
Tetracycline	0	0	0	0	0	40.21	0	0	0	10.0525	39.6	0	39.32	0	19.73	40.21
Oxytetracycline	37.42	0	37.72	0	18.785	36.94	0	37.84	0	18.695	37.13	0	37.72	0	18.7125	37.84
Lincomycin	0	0	15.65	0	3.9125	31.8	0	0	0	7.95	8.96	0	22.28	0	7.81	31.8
Doxycycline	10.76	0	12.25	0	5.7525	13.38	0	13.34	0	6.68	10.8	0	11.61	0	5.6025	13.38
Sulfapyridine	0	0	0	0	0	13.31	0	7.35	0	5.165	0	0	1.26	0	0.315	13.31
Sulfamonomethoxine	0	0	0	0	0	0	0	0	0	0	0	0	13	0	3.25	13
Albendazole	0	10.88	0	5.8	4.17	0	9.37	0	9.6	4.7425	0	8.85	0	7.8	4.1625	10.88
Sulfadimidine	0	0	3.79	0	0.9475	9.78	0	5.89	0	3.9175	0	0	4.03	0	1.0075	9.78
Lasal ºCid A	6.84	0	6.84	0	3.42	0	0	6.74	0	1.685	6.66	0	6.69	0	3.3375	6.84
Monensin	0	0	3.21	0	0.8025	3.74	0	3.5	0	1.81	1.75	0	3.37	0	1.28	3.74
Sulfamethazine	0	0	3.16	0	0.79	3.63	0	3.31	0	1.735	0	0	2.93	0	0.7325	3.63
Penicillin	0	0	0	0	0	0	0	0	0	0	0	0	2.2	0	0.55	2.2

Overall Concentration	Total
Upstream	450.07
Downstream	605.05
Effluent	1066.3

Table S2.4 One-Sample Test

Test Value = 0

	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Sulfamethoxazole	2.67	21	0.014	54.80136364	12.12352537	97.4792019
Amoxycillin	6.894	21	0	80.22621212	56.02486014	104.4275641
Tetracycline	2.45	21	0.023	8.145227273	1.231492556	15.05896199
Oxytetracycline	3.715	21	0.001	13.63962121	6.004321586	21.27492084
Lincomycin	2.568	21	0.018	5.618409091	1.068348859	10.16846932
Doxycycline	3.684	21	0.001	4.433787879	1.930760707	6.93681505
Sulfapyridine	2.022	21	0.056	1.767424242	-0.0506446	3.585493085
Sulfamonomethoxine	1.572	21	0.131	1.28030303	-0.41392108	2.974527141
Albendazole	6.85	21	0	6.041515152	4.207439056	7.875591248
Sulfadimidine	2.56	21	0.018	1.690227273	0.317114333	3.063340212
Lasal ºCid A	3.285	21	0.004	2.101742424	0.771173864	3.432310985
Monensin	3.169	21	0.005	0.995681818	0.342271881	1.649091756
Sulfamethazine	2.852	21	0.01	0.855984848	0.231841895	1.480127802
Penicillin	1.572	21	0.131	0.216666667	-0.070048183	0.503381516

Table S2.5 Biocide IN THE ENVIRONMENT

Biocide	US am 1	US am 2	US pm 1	US pm 2	Upstream	EF am 1	EF am 2	EF pm 1	EF pm 2	Effluent	DS am 1	DS am 2	DS pm 1	DS pm 2	Downstream	Highest conc
BAC 10	0.97	0	0.76	0.95	0.67	0.72	0	0.61	1.05	0.595	0	1.08	1.14	0	0.555	1.08
BAC 12	0	1.64	0	29.58	7.805	0	0	0	9.01	2.2525	0	0	1.24	6.19	1.8575	29.58
BAC 14	0	0	0	0	0	0	0	0	0	0	0	0	2.42	0	0.605	2.42
BAC 16	0	0	1.37	0	0.3425	0	0	0.5	0.5	0.25	0	0	0.39	0	0.0975	1.37
BenthEZ	0.06	0	0.09	0.11	0.065	0.07	0.07	0	0.12	0.065	0	0.04	0.04	0.06	0.035	0.12
DDAC	0	0	0	0	0	0	0	0.77	0	0.1925	0	0.83	0	0.83	0.415	0.83
DADMAC 10	0	14.53	0	0	3.6325	0	14.65	4.53	0	4.795	0	0	0	10.26	2.565	14.65
DADMAC 12	0	0	0	0.22	0.055	0	0	0	0	0	0	1.2	0.01	0.02	0.3075	1.2

Table S2.6 AST OF ENVIRONMENTAL ISOLATES

Isolate 1	GEN	CAZ	FEP	CTX	AMP	TZP	IMP	MEM	AZM	SXT	NAL	CHL	CIP	FOX	LEX	AMC	AMK	TET	CRO
EF24	19	24	29	28	16	22	24	27	20	22	19	25	30	22	15	18	17		25
US12A6	18	25	30	30	6	27	20	28	20	20	17	25	33	6	6	6	19		26
EF11A3	22	29	33	31	17	25	25	30	25	24	19	25	35	22	11	18	19		25
DS33	20	25	28	27	13	22	22	27	20	6	16	22	30	21	15	17	17		23
U11A5	19	27	28	25	18	23	27	28	20	24	20	25	30	22	15	19	15		24
UA13	20	27	31	30	6	25	19	28	20	24	20	25	40	6	6	6	19		26
EF412	20	25	30	25	18	23	24	28	20	22	19	25	30	22	16	19	18		25
D11A6	20	27	30	29	18	25	25	30	25	25	12	25	28	24	16	20	18		28
D11A4	20	25	34	28	26	25	22	30	18	6	19	25	40	6	6	10	20		27
D1P2	20	30	35	35	20	27	30	35	20	24	6	27	14	24	19	20	20		30
US14	22	28	30	30	20	25	25	30	25	25	22	25	40	24	19	22	20		28
U1P8	22	33	33	35	22	25	30	30	25	28	23	30	40	27	20	23	20		32
US16	17	23	25	24	17	20	23	26	20	22	18	22	30	23	17	19	17		25
EF42	22	30	33	33	20	26	28	34	25	26	20	25	37	25	18	20	17		26
DS45	15	23	25	24	10	22	16	25	20	15	18	25	30	6	6	6	15		22
U2P6	20	23	26	23	10	20	18	25	20	20	18	22	40	6	6	6	19		23
U11A7	17	23	24	24	6	20	15	25	20	6	19	21	30	6	6	6	16		25
DS26	24	28	30	30	18	25	28	30	20	25	20	25	35	24	16	19	18		30
EF43	20	29	29	30	18	25	27	30	25	27	20	25	32	23	17	19	17		28
U11A4	19	25	24	25	6	24	24	25	26	25	20	24	28	25	15	20	16		25
CONTROL	24	28	33	30	18	25	27	30	24	25	24	28	35	27	17	19	22		29
US11	22	30	34	34	19	31	32	32	22	27	23	26	39	23	19	20	21		30
DS36	22	30	34	34	22	25	30	32	20	24	22	25	36	25	20	22	20		30
D10A5	22	26	30	30	6	25	25	27	20	25	18	26	30	22	19	23	21		28
U10A8	24	30	35	35	23	28	30	30	20	25	25	26	40	25	20	24	21		30
DS11	20	27	33	30	6	25	20	30	20	20	6	24	26	6	14	18	21		30
D1P6	21	28	30	30	18	25	25	25	20	24	21	25	34	25	19	20	19		29
UF41	20	25	30	28	18	25	25	30	20	25	20	25	33	22	18	20	17		25
EF48	22	28	30	30	19	25	27	30	20	27	20	25	30	24	17	19	19		27
US11A3	20	25	30	27	6	25	21	30	23	6	18	23	30	6	6	15	19		29
UF23	22	30	33	33	20	30	30	33	20	28	22	25	35	27	18	20	19		30
U2P7	20	27	30	30	6	25	18	26	20	24	20	25	35	6	6	6	18		30
EF46	20	28	30	32	19	25	29	30	20	27	22	27	35	25	18	20	18		28
U11A6	21	26	30	28	6	24	26	30	20	24	16	25	30	22	20	20	20		25
D1P7	22	28	30	30	6	25	27	30	22	25	14	26	26	23	18	18	18		28
D1P4	20	28	30	30	19	26	26	30	24	26	19	27	25	23	18	20	19		28
U11A10	20	28	28	28	8	20	25	30	20	6	20	25	26	27	17	22	18		22
DS12	18	21	24	20	14	20	15	22	17	22	20	24	30	6	6	6	15		20
US47	22	27	32	30	19	25	26	30	26	22	6	25	37	23	20	22	20		29
EF418	20	30	33	32	19	27	29	30	20	25	20	26	34	24	17	20	18		27
DS41	22	30	32	33	22	29	22	30	20	27	24	27	34	18	16	15	18		30
D10A9	17	20	25	20	6	20	16	22	20	20	17	25	30	6	6	6	15		20
D11A9	16	22	20	17	6	15	20	25	15	20	18	26	30	20	12	20	15		12
DS17	18	23	23	20	15	20	22	25	20	22	18	22	30	25	14	18	15		16
U2P8	20	25	25	25	13	20	20	25	20	24	20	25	30	25	14	22	17		20
U1P10	19	25	25	26	20	25	27	28	20	22	19	25	30	22	16	20	17		25

CONTROL	24	28	33	30	18	25	27	30	24	25	24	28	35	27	17	19	22		29
D2P1	24	30	34	32	10	27	20	28	22	29	23	25	40	6	12	10	20	18	25
EF410	21	31	34	36	20	29	30	34	20	30	22	28	37	25	18	20	19	18	27
US43		28	30	30	12	25	20	28	22	21	19	24	35	6	14	10	15	15	24
U1P4	25	33	38	40	20	30	32	34	27	32	26	32	40	32	22	28	24	24	35
D11A10	23	25	32	33	18	24	25	28	24	25	17	27	30	23	15	17	16	15	24
EF411	23	30	34	30	20	26	27	30	20	29	21	26	40	23	19	22	17	17	40
US12A6	20	27	30	28	16	25	27	30	25	25	15	22	30	18	15	17	16	14	22
D2P3	20	28	32	30	6	28	22	29	20	26	22	26	35	6	12	10	18	20	28
DS410	22	34	35	25	20	30	21	30	24	30	26	30	40	6	10	10	19	22	30
US17	22	30	32	32	6	25	28	30	20	25	19	25	30	25	20	20	18	18	29
D11A10	24	28	32	32	10	25	30	32	20	27	20	24	30	22	19	19	20	15	26
EF44	20	30	37	35	20	29	30	35	22	30	23	27	36	26	19	20	20	18	30
DS13	25	30	34	34	20	28	27	30	20	26	23	28	35	25	21	20	20	19	30
EF28	22	32	34	34	20	28	32	32	20	27	20	25	35	24	20	20	20	18	30
D1P8	20	30	33	40	20	30	27	30	20	29	25	30	36	28	22	25	19	21	30
EF414	20	29	30	30	20	26	25	30	20	27	22	25	33	23	19	18	19	15	27
US41	22	25	30	30	20	23	25	28	23	23	17	25	30	21	15	17	18	15	25
US34	22	28	30	32	6	25	28	29	21	26	20	26	30	23	20	22	20	18	28
EF47	23	33	35	34	20	29	30	32	22	30	22	26	36	25	18	20	20	18	29
US18	24	30	32	32	20	25	29	30	20	26	20	22	32	22	16	20	18	17	25
D1P1	20	25	39	29	13	24	20	25	20	6	19	24	30	22	16	22	12	17	20
EF419	25	30	30	32	18	25	27	30	20	27	18	26	33	22	15	19	18	10	27
US35	22	30	35	35	20	28	30	33	20	30	24	26	37	24	19	21	20	19	33
D1P9	24	28	30	33	16	29	22	36	23	28	25	28	35	25	18	25	18	20	27
EF210	24	30	33	34	22	30	30	34	20	30	22	25	35	25	18	20	20	18	29
CONTROL	25	33	35	35	20	30	30	30	24	25	26	28	35	27	19	20	22	20	32
DS46	21	26	28	26	10	22	24	25	20	22	15	20	25	20	18	16	17	14	23
EF21	20	30	33	33	20	25	25	28	17	25	20	25	23	23	16	19	18	16	25
EF49	23	26	33	30	19	25	25	30	20	26	20	25	22	22	16	18	19	14	26
U10A10	20	26	31	27	6	25	24	33	20	6	15	22	20	20	18	18	18	6	28
EF417	20	26	31	30	19	24	25	28	20	24	18	24	22	22	19	19	17	15	23
D10A2	21	28	30	30	6	25	25	30	19	25	17	24	20	20	20	20	20	15	24
D2P9	23	25	28	26	6	22	22	27	20	20	15	24	20	20	18	18	20	14	24
EF420	21	30	32	31	20	26	28	30	20	27	19	25	22	22	17	17	19	15	25
D2P8	20	25	28	28	6	22	28	25	20	20	16	24	21	21	18	18	20	16	24
DS21	20	29	30	32	14	25	18	25	22	25	20	26	10	10	8	8	20	20	26
U10A1	22	28	30	30	19	25	25	30	18	26	20	25	24	24	14	14	20	15	25
EF45	22	28	32	31	18	26	29	30	23	26	20	25	22	22	18	18	19	16	26
DS32	22	30	33	32	20	25	25	30	15	27	20	25	24	24	20	20	18	18	29
U1P9	19	22	29	25	6	22	20	30	18	6	17	20	10	10	15	15	17	8	22
D10A8	23	28	31	30	6	25	25	27	18	23	20	25	25	25	22	22	20	15	29
CONTROL	25	33	35	35	20	30	30	30	24	25	26	28	27	27	22	20	22	20	32
DS411	22	30	35	32	10	30	27	30	22	6	23	20	26	34	22	23	20	16	30
DS24	22	28	33	32	6	28	20	29	20	28	25	28	37	6	6	6	17	19	26

US37	25	30	33	32	10	25	28	30	20	25	20	26	33	24	22	20	20	17	30
D11A4	20	33	34	34	22	30	30	34	25	28	23	26	35	25	22	22	20	18	30
DS22	20	29	34	30	6	29	22	28	20		19	25	34	6	6	6	17	18	25
DS18	25	28	34	32	6	28	25	30	22	6	21	25	40	6	6	6	20	18	28
U1P9	24	32	34	35	6	28	30	30	20	6	22	24	38	25	22	20	20	18	30
US32	22	26	24	17	6	26	28	32	20	6	14	25	27	23	12	18	20	18	10
U1P3	22	30	34	35	20	28	28	30	22	26	22	30	35	25	19	20	19	20	30
US2P1	25	30	34	35	12	26	28	30	20	26	20	30	33	25	21	24	20	20	30
US45	24	28	30	31	10	27	25	30	21	26	20	27	34	25	20	25	22	20	27
U2P3	23	30	34	35	22	29	28	32	25	6	15	25	27	24	18	20	20	6	30
DS31	20	30	34	37	16	28	20	30	20	29	23	26	38	6	10	6	20	20	34
DS23	21	35	35	37	6	30	30	34	22	29	12	22	27	25	20	20	20	19	33
D1P3	23	31	34	35	11	29	28	32	20	6	22	26	37	25	18	20	20	20	30
D2P6	22	30	33	34	18	27	28	30	20	28	20	25	35	24	19	19	19	17	30
U10A2	20	29	34	34	19	30	30	35	25	19	14	24	40	25	17	20	20	17	30
US10A5	22	29	34	35	20	28	27	30	20	25	20	26	30	24	20	24	20	18	27
DS412	20	29	32	30	20	27	25	32	20	25	21	25	35	24	20	20	20	17	30
CONTROL	25	30	33	30	19	26	26	30	24	24	24	28	40	26	18	20	25	20	30
DS49	24	32	29	33	12	32	28	35	22	32	25	32	36	30	24	26	22	23	33
D11A7	24	33	33	33	6	26	26	28	12	25	20	26	33	25	21	22	22	17	30
D10A7	25	33	34	34	16	30	26	28	22	12	26	30	40	28	21	26	22	24	27
DS28	23	30	34	32	6	30	30	34	30	6	23	30	38	25	18	19	21	6	30
D2P7	23	28	34	33	6	27	24	30	22	6	23	25	40	12	12	18	20	20	6
DS16	22	34	34	33	11	33	28	30	20	30	24	30	40	28	22	30	22	24	26
D11A5	22	30	35	36	22	26	28	32	22	23	22	26	35	22	18	21	18	20	33
DS15	23	30	32	34	19	24	26	28	20	26	20	26	32	24	20	22	21	19	28
D10A1	22	30	36	35	6	29	25	33	22	30	22	26	36	25	12	10	22	6	30
US26	22	32	36	34	20	29	30	34	20	29	20	26	34	25	19	22	20	17	32
U10A4	22	30	34	33	22	22	30	34	22	6	14	25	26	24	20	20	19	6	30
US42	20	29	30	30	17	26	19	26	20	20	17	24	30	6	10	8	18	18	26
U1P10	22	30	37	35	14	30	30	35	20	28	21	26	34	25	14	20	23	19	35
U10A7	20	30	32	33	6	25	26	30	25	25	15	26	27	25	18	18	19	6	27
U1P2	22	34	38	38	20	34	30	33	22	30	29	34	36	30	23	26	21	8	31
US45	22	29	34	32	20	28	26	30	22	25	21	25	24	25	19	20	19	17	30
US31	22	32	34	35	10	30	22	28	20	28	22	26	35	6	10	10	19	18	28
EF413	22	30	34	34	20	25	27	30	17	30	23	27	34	23	19	21	20	18	30
CONTROL	26	30	35	35	20	29	26	30	25	25	27	32	40	29	21	22	24	22	34
D11A8	24	29	34	34	25	31	34	35	24	23	27	28	40	29	19	24	23	21	32
DS35	25	29	34	34	6	26	25	30	20	6	21	24	38	25	21	20	23	20	32
US316	22	30	34	34	21	28	30	32	22	26	22	26	38	25	20	21	20	18	32
U10A3	6	32	35	34	6	29	30	32	6	6	24	25	40	25	20	20	21	6	32
DS25	24	30	34	33	6	28	25	30	22	6	16	25	32	6	6	10	20	20	30
EF212	20	29	34	32	20	26	30	30	20	27	21	28	34	25	19	20	20	18	30
US46	20	25	32	30	6	24	22	30	20	25	16	24	30	10	12	15	19	18	25
US36	25	30	32	33	16	28	24	26	20	27	20	25	34	25	19	25	20	20	28

U2P4	22	28	30	30	15	28	22	26	18	24	21	26	32	25	19	22	19	20	24
D2P5	20	29	32	32	14	26	22	28	22	28	21	27	35	6	10	10	19	19	26
U11A2	22	27	30	29	6	24	21	30	25	6	20	24	30	6	10	18	20	6	30
CONTROL	25	30	34	34	19	27	28	30	25	25	24	27	37	27	19	21	23	20	32
US38	21	28	32	32	16	25	28	30	25	26	22	28	37	25	19	20	19	17	29
US2P2	23	28	32	31	16	26	28	32	25	6	21	26	37	23	17	17	20	6	29
D10A3	23	30	30	28	6	25	24	28	21	6	20	26	34	26	20	23	23	20	26
U11A9	25	34	35	33	14	30	25	29	20	28	22	30	37	27	20	25	22	21	25
D10A6	20	29	32	30	11	27	20	26	24	23	21	26	30	6	12	6	19	19	25
D10A10	24	28	33	30	16	29	25	30	22	6	20	24	34	24	22	25	20	19	27
DS43	20	30	33	33	10	27	20	27	20	25	22	25	36	6	18	8	19	20	28
UP25	22	28	32	30	14	25	22	27	20	28	20	26	33	25	18	24	20	20	25

Table S2.7 Antibiograms of environmental isolates with resistance to three or more antibiotics

Antibiogram	Number of antibiotics	Number of isolates
AMC-AMK-TET	3	5
AMP-FOX-AMC	3	1
AMP-SXT-TET	3	3
FOX-AMC-TET	3	1
FOX-LEX-AMC	3	5
LEX-AMC-TET	3	1
NAL-CIP-TET	3	1
SXT-AMC-TET	3	1
TZP-CIP-TET	3	1
AMP-AMK-TET-CRO	4	1
AMP-FOX-LEX-AMC	4	5
AMP-LEX-AMC-TET	4	1
AMP-SXT-AMK-CRO	4	1
FOX-AMC-AMK-TET	4	1
AMP-FOX-LEX-AMC-AMK	5	1
AMP-FOX-LEX-AMC-TET	5	4
AMP-NAL-FOX-AMC-TET	5	1
AMP-SXT-AMC-AMK-TET	5	1
AMP-SXT-FOX-LEX-AMC	5	2
AMP-SXT-LEX-AMC-CRO	5	1
FEP-AMC-AMK-TET-CRO	5	1
GEN-AMP-AZM-SXT-TET	5	1

SXT-FOX-LEX-AMC-TET	5	1
TZP-CHL-AMC-AMK-TET	5	1
TZP-CHL-CIP-AMC-TET	5	2
TZP-CIP-FOX-LEX-AMC	5	1
AMP-SXT-FOX-LEX-AMC-AMK	6	1
AMP-SXT-FOX-LEX-AMC-CRO	6	1
AMP-SXT-FOX-LEX-AMC-TET	6	2
GEN-AMP-FOX-AMC-AMK-TET	6	1
TZP-NAL-CHL-CIP-AMC-TET	6	1
IMP-FOX-LEX-AMC-AMK-TET-CRO	7	1
AMP-IMP-FOX-LEX-AMC-AMK-TET-CRO	8	1
AMP-IMP-SXT-FOX-LEX-AMC-AMK-TET	8	1
FEP-TZP-NAL-CIP-FOX-AMC-AMK-TET	8	1
GEN-AMP-IMP-FOX-LEX-AMC-AMK-TET	8	1
GEN-FEP-AMP-TZP-LEX-AMK-TET-CRO	8	1

Table S3.1 WGS GENE ANALYSIS.

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sampleID	Aminoglycoside	Beta.lactam	Colistin	Fosfomycin	Macrolide	Phenicol	Quinolone	Sulphonamide	Tetracycline	Trimethoprim
D11A-3	aph(6)-Id	blaCTX-M-1			mdf(A)			sul2	tet(A)	
D11A-3	aph(3")-Ib	blaTEM-1B								
D1P-4					mdf(A)					
D2P-10		blaCTX-M-15			mdf(A)		qnrS1			
D2P-4	aadA2				mdf(A)	cmlA1		sul3		dfrA12
D2P-4	aadA1									
DS2-9	aph(6)-Id	blaTEM-1B			mdf(A)		qnrS1	sul2		dfrA12
DS2-9	aadA2						OqxB	sul1		dfrA8
DS2-9							OqxA			
DS2-9	aph(3")-Ib									
DSI-4	aph(6)-Id	blaMIR-3			mdf(A)		qnrS1	sul3	tet(A)	dfrA14
DSI-4		blaTEM-1B								
DSI-4		blaTEM-104								
DSI-4		blaTEM-198								
DSI-4		blaTEM-217								
DSI-4		blaTEM-234								
EF1P-1					mdf(A)					
EF2-7					mdf(A)					
EF2P-1	aadA1	blaTEM-1B			mdf(A)	cmlA1		sul3	tet(A)	dfrA17
EF2P-1	aadA5							sul2		
EF2P-1	aadA2b									
U10A-3	aph(6)-Id	blaTEM-1B			mdf(A)			sul2	tet(A)	dfrA17
U10A-3	aac(3)-IId				mph(A)			sul1		
U10A-3	aadA5									
U10A-3	aph(3")-Ib									
UIP-9	aph(6)-Id	blaTEM-1B			mdf(A)			sul1		dfrA7
UIP-9	aph(3")-Ib							sul2		
US2-5		blaMIR-3	mcr-10	fosA				sul1		dfrA21
US3-2	aph(6)-Id	blaLAP-2			mdf(A)		qnrS1	sul2		dfrA14

US3-2		**blaCTX-M-14**						
US3-2	**aph(3'')-Ib**							
US3-3	**aph(6)-Id**	**blaTEM-1B**	**mdf(A)**		**qnrS1**	**sul3**	**tet(A)**	**dfrA14**
US3-9	**aph(6)-Id**	**blaTEM-1A**	**mdf(A)**	**floR**	**qnrS1**	**sul2**	**tet(A)**	**dfrA14**
US3-9	**aph(3'')-Ib**							

Table S4.1 Nineteen genes identified by name in the output: Single nucleotide Variant (SNV) calling, using the PATRIC database and ANVIO analysis pipeline, showing a significant mutation.

S/NO	GENE	DESCRIPTION	EFFECT
1	*acnB* aconitase gene B	*acnB* (pairing with *acnA*) are involved in catalysing the reversible isomerisation of the citrate and is ºCitrate. They are catabolic enzymes expressed during nutritive and oxidative stress periods and are expressed more during the exponential growth phase decreasing during the persister /stationary phase.	PERSISTENCE. (Araujo et al., 2022; Brock et al. 2002, Cunningham et al., 1997)
2	*cusA*	*cusA* is part of the resistance-nodulation-cell division (RND) transporter superfamily, and part of the *cusCFBA* efflux system responsible for the detoxification of the copper/silver ions.	Metal Tolerance (Chacón et al., 2014; Franke et al., 2003; Mealman et al., 2012)
3	*degQ*	*degQ* is a serine endoprotease and a homologous member of the *HtrA* (high temperature requirement A) protein family with *degP* and *degS*. It is involved in the degradation of transient proteins, and stress sensing, regulation, and protection during unfolded protein responses especially in high temperature and nutrient deprived isolates.	Protein synthesis regulation. (Abfalter et al., 2016; Sawa et al., 2011; Waller and Sauer, 1996)
4	*epmA*	*epmA,* EF-A post elongation factor A, has a sequence that is like the class11lsyRS-tRNA synthetases, only lacking the anti-codon binding site.	Pays a role in growth. (Bergh et al., 2016; Roy et al., 2011; Vivijs et al., 2016; Yanagisawa et al., 2010)
5	*fliL*	*fliL* is one of the seven genes with the flagellar associated *flaA* 1 ºCus. It interacts with the rotor and stator proteins, involved in the swimming and swarming mobility of bacteria by increasing the motor output through stabilising and increasing the efficiency of the stators, this enhances the torque generation, which is very essential in swarming over surface.	Motility. (Malakooti et al., 1989; Partridge et al., 2015; Tachiyama et al., 2022)
6	*Hsmp*	This is an EAL domain protein that inhibits biofilm formation. Their products in Yesinia pestis are quite like those of *pgaABCD(ycdSRQP)* products in *E. coli*, which are required for biofilm formation and biosynthesis of glucosamine.	Biofilm formation. (Bobrov et al., 2005)
7	*Mlc*	Also known as *dgsA* (makes large colonies) or DNA-binding transcriptional repressor *mlc*.	Growth. (Pan et al., 2021; Schiefner et al., 2005)

		It is a transcriptional repressor that controls the expression of gene encoding enzymes of the PTS and PEP system, responsible for glucose and mannose transportation. A regulator protein controlling glucose utilisation in *E. coli* and a member of the ROK (repression, open reading frames and kinases) family of transcriptional regulators, which include xylose regulators (xylR) and a series of glucose/fructose kinases. Its repression activity is mediated by Zinc.		
8	*murP*	*murP* encodes the permease component of the N-acetylmuramic acid PTS transport system. It facilitates the uptake and transportation of anhydrous acetylmuramic (anyMurNAc) acid but not its phosphorylation. It encodes anmK (anyhydro-N-acetyl muramic acid kinase) which is needed to convert imported *anhMurNA*c to *MurNAc-P*, which is the source of carbon and energy for *E. coli.*	Growth.	(Dahl et al., 2004; Heravi and Altenbuchner, 2018).
9	*mutM*	Also known as Fpg (formamidopyrimidine glycolase) which is a bifunctional DNA glycosylase that cleaves the N-glycoside bond of redox-damaged purines and incises the phosphodiester backbone to yield single strand breaks with 3'and 5'phosphoryl ends. In the repair of oxidative damaged DNA, *mutM* is the primary DNA glycosylase that removes the oxidised purines and some oxidised pyrimidines and is actively involved in the repair of lesions in the transcription intermediates. Its involvement has been noted in the molecular mechanism of lesion recognition, formation of productive complex and lesion excision.	DNA repair.	(Fromme et al., 2004; Makasheva et al., 2019; Schalow et al., 2011)
10	*nudK*	*nudK* also known as GDP-mannose hydrolase, is a member of the ADP-ribose pyrophosphate sub-family of the Nudix hydrolases. Over expression of the *nudK* suppresses the *lapB* essentiality which encodes the protein involved in lipopolysaccharide (LPS) assembly.	Biofilm formation.	(Boto et al., 2011).
11	ompD	*ompD* is a major porin protein in the outer membrane of cells. It constitutes about 50% of the cell wall porins during favourable conditions and increases with increased anaerobolism and stress in the environment. It is involved in the efflux of toxins out of the cell membrane.	Stress porin and efflux activities.	(Santiviago et al., 2003)
12	*ptsG*	*ptsG* is a member of the glucose specific phosphotransferase system (PTS^{GLC}), which is the most effective glucose transportation system in bacteria. It plays a huge role in the carbon carbolite repression (CCR) signalling system and modulates the cyclic AMP (cAMP) in cells.	Growth, glycolysis, tricarboxylic acid (TCA) cycle, acetate metabolism.	(Green et al., 2014; Jung et al., 2019; Liang et al., 2015)
13	*purH*	*purH*, also known as Bifunctional AICAR transformylase / IMP cyclohyydrolase encodes 5'-phosphoribosyl 5-aminoimidazole- 4-	Signalling pathways, carbohydrate metabolism.	

		carboxamide transformylase (EC2.1.2.3) which are enzymes involved in de novo purine nucleotide synthesis. Of the 10 steps in the purine biosynthesis pathway (PBP), *purH* is involved in the last 2 steps.	(Aiba and Mizobuchi, 1989; Cruz et al., 2019)	
14	*queG*	This gene is responsible for the last step in the step formation of Queuosine (Q), a very highly modified tRNA nucleoside from epoxyqueuosine (oQ)	Virulence and cellular differentiation. (Frey et al., 1988; Payne et al., 2015)	
15	*robA*	*robA* is a member of the *Xyl5/AraC* sub-family of DNA, which shares about 49% identity with *marA* and *soxS* on the N-terminal domain. They make up the *marA/soxS/rob* regulon, which have over 50 genes, that can be independently or together activated. The over-expression of *robA* induces multiple antimicrobial resistance in *E. coli*.	Antibiotic resistance, metal, and organic solvent tolerance. (Chubiz et al., 2012; Gu et al., 2020; Horii et al., 1997; Saini, 2016; Nakajima et al., 1995)	
16	*srlE*	Part of the *srlABE* also known as the *gutABE* genes, are the sorbitol PTS permease which belong to the phosphoenolpyruvate (PEP)-dependent sugar transporting phosphotransferase system (PTS^{SUGAR}).	Sorbitol transport. (Boyd et al., 2000; Ng et al., 2018)	
17	*tsaB*	tsaB is involved in the biosynthesis and decoding tRNA. *tsaB* role is possibly the regulation of the enzymatic activity of *tsaD*, so *tsaB* acts as a protease that specifically degrades *tsaD*	Growth. (Handford et al., 2009; Lauhon, 2012; Missoury et al., 2018; Thiaville et al., 2016)	
18	*yddG*	yddG is a member of the aromatic amino acid/paraquet exporter (ArAA/P-E) family within the drug/metabolite transporter (DMT) super family and is an amino acid exporter. Expression of yddG results leads to increased resistance on *E. coli* to aromatic amino acids.	Amino acid transport and resistance. (Doroshenko et al., 2007; Tsuchiya et al., 2016; Wang et al., 2013)	
19	*yqhH*	*yqhH* is a verified lipoprotein with a leucine zipper region that is envisaged to play a part in the protein-protein interaction and DNA recombination.	Cell viability. (Rao et al., 2020)	