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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*

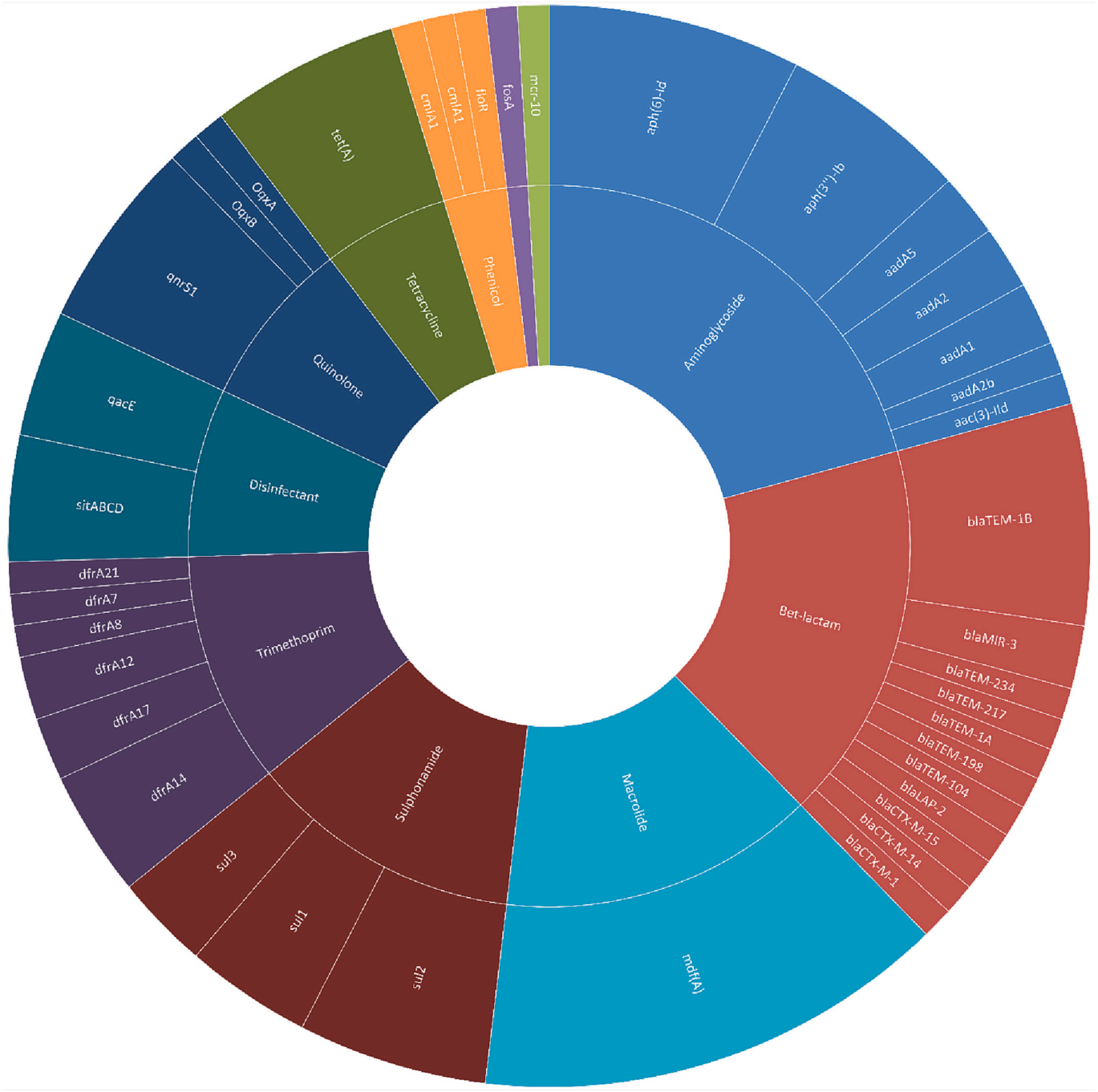

Fig. 1. Distribution of resistance genes identified in environmental isolates using WGS.

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

Professor Sabiha Y. Essack is the chairperson of the Global Respiratory Infection Partnership and a member of the Global Hygiene Council, both sponsored by unconditional educational grants from Reckitt, UK. All other authors declare that they have no conflicts of interest.

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.

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