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Antimicrobial effects, and selection for AMR by non-antibiotic drugs in a wastewater bacterial community

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ABSTRACT

Antimicrobial resistance (AMR) is a major threat to human, animal, and crop health. AMR can be directly selected for by antibiotics, and indirectly co-selected for by biocides and metals, at environmentally relevant concentrations. Some evidence suggests that non-antibiotic drugs (NADs) can co-select for AMR, but previous work focused on exposing single model bacterial species to predominately high concentrations of NADs. There is a significant knowledge gap in understanding a range of NAD concentrations, (including lower μg/L concentrations found in the environment) on mixed bacterial communities containing a diverse mobile resistome. Here, we determined the antimicrobial effect and selective potential of diclofenac, metformin, and 17-β-estradiol, NADs that are commonly found environmental pollutants, in a complex bacterial community using a combination of culture based, metagenome, and metratranscriptome approaches. We found that diclofenac, metformin, and 17-β-estradiol at 50 μg/L, 26 μg/L, and 24 μg/L respectively, significantly reduced growth of a bacterial community although only 17-β-estradiol selected for an AMR marker using qPCR (from 7 μg/L to 5400 μg/L). Whole metagenome sequencing indicated that there was no clear selection by NADs for antibiotic resistance genes, or effects on community composition. Additionally, increases in relative abundance of some specific metal resistance genes (such as *arsB*) were observed after exposure to diclofenac, metformin, and 17-β-estradiol. These results indicate that environmentally relevant concentrations of NADs are likely to affect community growth, function, and potentially selection for specific metal resistance genes.

1. Introduction

Antimicrobial resistance (AMR) is a significant threat to human health, with a predicted 1.27 million deaths already attributable to resistant bacterial infections in 2019 (Murray et al., 2022) and 10 million deaths estimated to occur annually by 2050 (O'Neill, 2014). Antibiotic resistance (occurring when bacteria become unable to be killed or inhibited by antibiotics), is commonly selected for by antibiotics, even at low concentrations, but can also co-selected for by metals and biocides (Baker-Austin et al., 2006; Chapman, 2003; Murray et al., 2024). There is increasing evidence that this also occurs with NADs.

The evidence for co-selective properties of NADs is increasing. NADs can decrease the growth of human gut strains (Maier et al., 2018), pathogens (Younis et al., 2015), and clinical and lab strains of bacteria (Campbell et al., 2019; Dastidar et al., 2000; Harbut et al., 2015). Additionally, some NADs can also increase horizontal gene transfer (HGT) rates between bacterial species through transformation (Lu et al., 2022; Wang et al., 2020), and conjugation (Ding et al., 2022; Jia et al., 2021; Wang et al., 2019). NADs have been shown to select for antibiotic resistance through increasing mutation rates (Jin et al., 2018; Guðmundsdóttir et al., 2021; Riordan et al., 2011; Meunier et al., 2019). Therefore, there are concerns that NADs may be selecting for AMR in polluted environments worldwide.

NAD concentrations are generally found at in the mg/L range in the human gut, (Schulz et al., 2012) and in the ng/L – μg/L range in the aquatic environment (aus der Beek et al., 2016; Wilkinson et al., 2022). For example, diclofenac has been detected at 8 g/L in sewage sludge, 2 mg/L in urban wastewater, and 100μg/L in surface water (Graumnitz,

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2021). Low concentrations of antibiotics and metals can select or co-select for antibiotic resistance genes (ARGs) (Gullberg et al., 2014; Gullberg et al., 2011; Murray et al., 2018; Stanton et al., 2020; Hayes et al., 2022), and these concentrations can be environmentally relevant. Therefore, co-selection for AMR by NADs could potentially occur across large geographic scales.

However, most current studies investigating NADs use single, model species of bacteria and high concentrations of NADs, e.g. g/mL or mg/mL concentrations, although there are some recent exceptions (e.g. (Hall et al., 2024)). It is unlikely that bacteria would be exposed to such high concentrations of NADs. Furthermore, single species experiments are unrealistic when considering that most bacteria naturally exist in mixed species communities with a diverse reservoir of AMR genes. In addition, previous work has shown that the presence of a community increases the selective concentration of gentamicin and kanamycin for a focal species (Klümper et al., 2019) suggesting that single species experiments may overestimate selective effects.

Here, we tested the effects of diclofenac, ibuprofen, haloperidol, metformin, and 17-β-estradiol on bacterial growth and selection for AMR using a complex bacterial community. Effects on growth and selection for AMR were experimentally determined. For the former, a simple growth-based assay (Murray et al., 2020) was used to identify which NADs cause significant reductions in community growth. For the latter, phenotypic resistance was tested by plating NAD-evolved communities onto antibiotic amended agar plates. Further, DNA was extracted from the evolved communities and was used in qPCR assays for AMR marker genes (for *intI1* and *cintI1*) and metagenome sequencing for diverse AMR endpoints. Metatranscriptome analyses were carried out on communities evolved for seven days with a high concentration of diclofenac, metformin, and 17-β-estradiol to identify changes in gene expression.

2. Materials and Methods

2.1. Wastewater collection

Raw untreated wastewater influent was collected from the wastewater treatment plant in Falmouth, UK, on the 2/2/2020, and frozen at 1:1 with 40 % glycerol and stored at −70 °C until use. This wastewater was used as the inoculum in all experiments in this study. This community includes species associated with the human gut microbiome, and include opportunistic human pathogens, providing an indication of how species of clinical interest respond to NADs. Although freezing these communities inevitably alters community structure, we did so to standardise the inoculum, reducing variability between experiments.

2.2. Non-antibiotic drugs

Diclofenac (Sigma Aldritch), ibuprofen (Sigma Aldritch), haloperidol (Sigma Aldritch), metformin (Enzo Life Sciences), and 17-β-estradiol (Sigma Aldritch) were dissolved in water, water, acetone, water, and ethanol respectively to produce 1 mg/mL stocks. These were kept at −20 °C before use and used within nine days.

These compounds were chosen since they have previously been demonstrated to reduce bacterial growth in bacterial pathogens such as *Escherichia coli* (Gümüş et al., 2019) and *Staphylococcus aureus* (Abbas et al., 2020) and commensal or lab strains (Maier et al., 2018; Liu et al., 2020; Laudy et al., 2016), reduce biofilm formation (Abbas et al., 2020; Abbas, 2015), increase HGT rates (Wang et al., 2020), be substrates of efflux pumps (Laudy et al., 2016; Elkins and Mullis, 2006) and in some cases, increase antibiotic resistance (Riordan et al., 2011; Wei et al., 2022; Verma et al., 2018). Additionally, these NADs can be detected in the aquatic environment (aus der Beek et al., 2016; Graumnitz, 2021), and are commonly used in clinical medicine.

2.3. Optical density growth experiments

Reduction in the overall growth of the community during exponential phase was used to identify if NADs could significantly affect growth. Resistance to antibiotics commonly incurs a fitness cost, resulting in growth reduction of the resistant bacterial population compared to the sensitive population (Murray et al., 2020; Ahmad et al., 2023; Andersson and Hughes, 2010; Dahlberg and Chao, 2003; Gifford et al., 2016). Previous work has shown that reduction in growth of a bacterial community is a good proxy for selection for AMR markers, including *intI1* (Murray et al., 2020). Furthermore, modelling approaches have predicted that a reduction in growth rate is the most significant factor when attempting to estimate the minimal selective concentration (Greenfield et al., 2018).

For all growth curve experiments, a 5 mL aliquot of frozen wastewater influent was thawed, and centrifuged at 14.8 rpm for 2 min, before the supernatant was removed, and pellet resuspended with 0.85 % NaCl (Sigma Aldrich). This centrifugation and re-suspension step was repeated to minimise nutrient and glycerol carry over. The re-suspended pellet was used to inoculate Iso-Sensitest broth (Oxoid) at 10 % vol/vol.

1.5 mL of the bacteria-spiked broth was taken and NAD added to this to result in twice the highest concentration required. 200 μL of the bacteria-spiked broth was added to all wells of a 96 well plate, with the last six wells left as a negative control. 200 μL of the NAD- and bacteria-spiked broth was added to the first six wells. This was serially diluted down the entire plate, leaving six wells as a no-NAD control and six as the non-inoculated control, resulting in six replicates per treatment. Concentration ranges started at 20 μM, since this has previously been shown to reduce gut bacterial growth (Maier et al., 2018), and then descended in a two-fold dilution until tested concentrations did not have any significant difference to the no-NAD control. The highest concentrations tested were: diclofenac 6400 μg/L, ibuprofen 4100 μg/L, haloperidol 7500 μg/L, metformin 3300 μg/L, and 17-β-estradiol 5400 μg/L.

The plates were sealed with a transparent optical seal (MicroAmp) and incubated at 37 °C in Varioskan Flash (Thermo Fisher) plate reader for 11 h, with continual shaking at 180 rpm. Optical density at 600 nm (OD600) was taken every hour. We used 37 °C as our incubating temperature to select towards human associated, and potentially pathogenic species, which could be of clinical interest. Additionally, previous work has indicated that identifying growth based effect concentrations is not significantly affected by temperature, and high nutrient media (Murray et al., 2020).

2.4. Growth-based effect concentration analyses

The Lowest Observed Effect Concentrations (LOECs) were identified as previously described (Murray et al., 2020). In summary, the time point during exponential phase with the greatest dose response was determined using either a Spearman's or Pearson's correlation test. Then, OD600 at all concentrations at that time point were tested to determine the concentrations that differ significantly from the control growth using a Dunn's test in R (*dunn.test* v1.3.5). The LOEC was recorded as the lowest tested concentration at this time point that was significantly different to the no-NAD control.

2.5. Selection experiments

Week-long selection experiments were conducted to determine how the populations changed after exposure for a longer period. These experiments were used to provide data for the plating experiment, the qPCR analyses, and the metagenomic sequencing.

Firstly, a 40 mL aliquot of wastewater influent was thawed, centrifuged at 3,500 rpm for 10 min, the supernatant removed, and the pellet resuspended with 0.85 % NaCl. This was repeated to remove contaminants and glycerol carryover. The washed bacterial community was added to Iso-Sensitest broth (Oxoid) at 10 % vol/vol. This spiked broth

was then separated into 30 mL aliquots, and NAD added to each 30 mL to result in different NAD concentrations. Diclofenac concentrations used: 6400μg/L, 2100μg/L, 710μg/L, 240μg/L, 79μg/L, 26μg/L, 8μg/L, 0μg/L. Metformin concentrations used: 3300μg/L, 1100μg/L, 367μg/L, 122μg/L 41μg/L, 14μg/L, 5μg/L, 0μg/L. 17-β-estradiol concentrations used: 5440μg/L, 1813μg/L, 604μg/L, 201μg/L, 67μg/L, 22μg/L, 7μg/L, 2μg/L, 0μg/L. These 30 mL aliquots were then separated into five 5 mL replicate microcosms.

At the beginning of the experiment, two 1 mL aliquots of each microcosm were taken, and centrifuged at 14.8G for 2 min, the supernatant removed, and the pellet resuspended in 20 % glycerol and frozen at −70 °C.

The microcosms were then incubated at 37 °C with shaking at 180 rpm. Every day, a 1 % transfer occurred into fresh medium with fresh NAD. On day seven, four 0.5 mL aliquots were taken and mixed 1:1 with 40 % glycerol and frozen at −70 °C.

2.6. Growth on antibiotic agar experiment

We tested whether communities grown in NAD at 20 μM had a greater ability to grow on antibiotic amended agar plates. Communities were exposed to either 6400 μg/L diclofenac, 3300 μg/L metformin, or 5400 μg/L 17-β-estradiol for seven days in a selection experiment as described above.

After seven days, 20 μL of a dilution series of each of the five biological replicates for each treatment was plated onto Chromocult agar (Millipore) containing the EUCAST resistance endpoints for six antibiotics (Supplementary Table 1) and spread across the plate using glass beads. A no-antibiotic control plate was also used to determine the proportion of resistant colonies. Antibiotics were obtained: ampicillin (Melford), azithromycin (Merck), cefotaxime (Fisher), ciprofloxacin (Sigma Aldritch), gentamicin (Melford) and tetracycline (Formedium), and dissolved in water, ethanol, water, 0.5 M hydrochloridic acid, water, and water respectively.

We used Chromocult agar to focus this experiment on Gram-negative coliforms. Coliforms include species that are common human pathogens that can often be highly antibiotic resistant, such as *E. coli*. They are also used as indicator species in environmental surveillance.

2.7. QPCR assays

QPCR was carried out on selection experiments with diclofenac, metformin, and 17-β-estradiol to determine changes in gene abundance after exposure to these NADs over a seven-day period. Day zero samples were also included in the assays to enable identification of positive selection, or persistence (relative changes in negative selection), since without day zero data, there is no ability to determine whether any increase with treatment at day seven is above the relative gene abundances at day zero (Stanton et al., 2020).

For qPCR assays, DNA was extracted from all day zero and day seven replicates using a Qiagen DNeasy UltraClean Microbial Kit, carried out to manufacturer's instructions, with the initial centrifugation step increased to one minute. Extracted DNA was diluted 5X with 10 mM Tris-HCl (Sigma Aldritch), and kept at 4 °C until use in qPCR assays, or frozen at −20 °C. If samples were thawed, they were kept at 4 °C.

The gene targets used were *intI1* and *cintI1*, and gene abundances were normalised to the 16S rRNA gene. Both a general class I integrase gene (*intI1*) and a clinical class I integrase gene (*cintI1*) were used as targets since *intI1* is often recommended as a gene target for AMR surveillance (Abramova et al., 2023; Gillings et al., 2015), and because both can be associated with various antibiotic resistance gene cassettes (Gillings et al., 2015) that can be selected for by antibiotics (Stanton et al., 2020). *CintI1* is more often associated with clinical pathogens and was used to understand the responses of pathogens after NAD treatment, and the general *intI1* gene was used to identify changes to this important genetic platform in a wider population.

QPCR was carried out on all experiments with either *intI1* or *cintI1* quantity normalised to the 16S rRNA gene. The reaction mix contained 10 μL Master Mix (PrecisionPlus) with ROX and SYBR, 1 μL forward primer, 1 μL reverse primer, 0.2 μL bovine serum albumin (Fisher), and 2.8 μL nuclease free water (Ambion). Primers were used at 9 mM. The reaction cycle was as follows: 120 s hold at 95 °C, 40 rounds of cycling DNA amplification, with 10 s at 95 °C and 60 s at 60 °C for data collection. Only qPCR runs that had an $R2 > 0.9$ and an efficiency of between 90–110 % were analysed. For *cintI1* assays, the annealing temperature was increased to 70 °C. Primer sequences can be found in Supplementary Table 2. Standard curves were carried out with 10-fold concentrations of the target gene, with five concentrations of DNA used as standards, from $5e^6$ to $5e^2$ copy number. DNA standards (gBlocks) were purchased from IDT Technologies and sequences are presented in Supplementary Table 2. Target gene molecular prevalence was determined by dividing the gene target quantity by the 16S rRNA quantity (i.e., *intI1* quantity/16S rRNA quantity).

2.8. Metagenome sequencing

Metagenome sequencing was carried out to determine changes to the overall resistome and community composition after exposure to the NADs after seven days.

DNA was extracted from day seven samples using the Qiagen DNeasy UltraClean Microbial Kit as detailed above. DNA was extracted from three replicates for each treatment. DNA was purified before being sent for sequencing using an Ampure XP protocol. DNA was sequenced by the Exeter Sequencing Centre using a NovaSeq 6000 to a depth of ~ 5 GB. The diclofenac and 17-β-estradiol experiments were sequenced using NEB Ultra ll FS library prep, and the metformin experiment used a PCR free library prep. This difference was due to changes in protocol at the Exeter Sequencing Centre. The Exeter Sequencing Centre trimmed the adaptors from the reads, and only trimmed reads passing quality checks were used in all downstream analyses.

2.8.1. Taxonomic pipeline

MetaPhlAn v2.0 (Truong et al., 2015) was used to determine community taxonomy. Firstly, forward and reverse reads were paired using FLASH2 (Magoč and Salzberg, 2011) before being piped into MetaPhlAn. MetaPhlAn produces relative abundances of bacterial and archaeal taxa by mapping input reads using BLAST (Altschul et al., 1990) against clade-specific marker sequences.

2.8.2. Antibiotic resistance gene profiling

ARGs-OAP v2.0 (Yin et al., 2018) was used to identify relative abundance of antibiotic resistance genes (ARGs). Reads were mapped to the SARG database which was manually curated using data from CARD (Alcock et al., 2023) and ARDB (Liu and Pop, 2008). Default parameters were used for ARGs-OAP (a percent identity of 95 % or greater, matching 25 bp or higher, with a $1e^{-7}$ evalue cutoff). ARG hits were normalised to 16S reads generated during the ARGs-OAP pipeline process.

2.8.3. Metal and biocide resistance gene profiling

Biocide and metal resistance genes (BMRG) were identified using BacMet (Pal et al., 2014). We used the experimentally determined BacMet database to ensure no spurious hits to genes that had not been identified as BMRGs. Sample reads were mapped to the BacMet database using BLAST, and the pipeline outputs contained counts of each gene per sample.

2.9. Metatranscriptome sequencing

For metatranscriptome analysis, a separate selection experiment was carried out, using the highest tested concentration of diclofenac, metformin, 17-β-estradiol (6400 μg/L, 3300 μg/L, and 5400 μg/L

respectively), and a no-NAD control. The experiment was set up as outlined earlier. On day seven, after the initial transfer, communities were incubated for six hours to allow communities to enter exponential phase. At this time point, 1 mL samples were taken, pelleted, and the supernatant removed. Then, the samples were freeze-dried using 100 % ethanol and dry ice. Once frozen, samples were stored at −70 °C overnight until packaging for transport.

Samples were transported on dry ice to Novogene. All RNA extraction, sequencing, quality control, and analysis were performed by Novogene. In brief, RNA was extracted from the samples, and quality checked. The samples were then sequenced produce 1.9 GB to 3.5 GB raw data per sample.

2.10. Metatranscriptome analysis

All analyses of metatranscriptome data were conducted by Novogene. Quality of reads were checked, and only good quality reads were used in analyses. Reads were assembled using Trinity (Grabherr et al., 2011), and analysed to determine taxonomic changes, and functional gene analyses. Species annotation was carried out using DIAMOND (Buchfink et al., 2021). Functional gene annotation was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2023; Kanehisa and Goto, 2000; Kanehisa, 2019). Differential gene expression analysis (those with a > log2 fold change) was performed by mapping sample reads to the assembled transcriptome and converting read counts to fragments per kilobase of transcript sequenced per million base pairs sequenced. Read counts were normalised, and adjusted p values were produced.

The reads for each sample were also piped through the ARGs-OAP and BacMet pipelines by the authors to determine changes in expression of these genes. The data from these were analysed as for the metagenome analyses.

2.11. Data analysis

All statistical analyses were conducted in R v4.1.10 (R Core Team, 2019). All plots were made using *ggplot* v3.3.5 (Wickham, 2016) and *Metbrewer* v0.2.0 (Mills and MetBrewer, 2022). All statistical tests with multiple comparisons were corrected using false discovery rate (*fdr*). For all models, the most parsimonious model was used, determined by sequentially deleting terms, and using X^2 tests to compare the model fits. All models used for analysis had residuals that fitted the model assumptions. Fit of residuals were checked using *DHARMa* v0.4.6 (Hartig, 2020).

2.11.1. Analysis of growth on antibiotic plates

The proportion of resistant colonies were determined by dividing the resistant CFU/mL by the CFU/mL of the no-antibiotic plates. Linear mixed effects models were used to identify changes to growth on antibiotic amended plates as a function of NAD treatment, using *lme4* v1.1.31 (Bates et al., 2015). Antibiotic plate and NAD treatment were used as fixed effects, and microcosm as a random effect. Models were run on all data (*E. coli* and presumptive coliform) and both separately. Post-hoc tests were performed using *emmeans* v1.8.2 (Lenth et al., 2018) to determine which treatments had significantly altered phenotypic resistance.

2.11.2. QPCR analyses

Linear mixed effect models (*lme4* v1.1.31) were used to determine effect of treatment and time on the prevalence of *intI1* or *cintI1* in the samples. We included microcosm as a random effect. Post-hoc comparisons were performed using *emmeans* v1.8.2, which determined pairwise comparisons between day seven prevalence data to determine selective concentrations (comparing the day seven prevalence control data to the day seven treatment prevalences).

2.11.3. Metagenome analyses

All outputs from the bioinformatic pipelines were used in all downstream analyses. For both the community taxonomy and resistome, changes to genes or species as a function of NAD concentration was determined.

Firstly, all hits to the BacMet database were filtered to 80 % percent identity match of at least 25 bp to match the default parameters used in ARGs-OAP. Subsequently, hits were subsetted into 'genes associated with plasmids', and 'genes associated with efflux' using metadata extracted from the BacMet database.

Kruskal-Wallis tests were used to determine changes in any gene or species in at least one treatment. Any genes that showed a significant change in at least one treatment were plotted and modelled.

The beta diversity of the resistome and taxonomy for communities exposed to each NAD were determined using Bray-Curtis dissimilarity distance using *vegan* v2.5–7 (Dixon, 2003). This dissimilarity distance was then visualised using Non-metric MultiDimensional Scaling (NMDS), with a starting seed of 123. An Analysis of Similarity (ANOSIM) was used to determine differences in treatments using *vegan* using 9999 permutations to determine effect.

During the resistome analysis for the metformin selection experiment, *tolC* analysis was carried out using *drc* v.3.0.1 (Ritz et al., 2015). A four parameter log logistic curve was fitted.

3. Results

3.1. Three non-antibiotic drugs decrease growth of a bacterial community during exponential growth phase

Firstly, we investigated whether NADs could reduce bacterial growth and aimed to identify a lowest observed effect concentration (LOEC). We found that diclofenac, metformin, and 17-β-estradiol significantly decreased the growth of a bacterial community in exponential phase, with the LOECs of each identified as: 50 μg/L diclofenac, 26 μg/L metformin, and 24 μg/L 17-β-estradiol (Supplementary Fig. 1). We did not determine any effect of ibuprofen (from 4100μg/L to 0.25μg/L) or haloperidol (from 7500μg/L to 0.46 μg/L) on the growth of the community, and so these were not used in further experiments.

After this, longer, seven-day selection experiments were carried out to identify changes to the community after longer exposure to diclofenac, metformin, and 17-β-estradiol. We hypothesised that if these NADs did have antimicrobial effects, that they would select for antimicrobial resistance genes within the bacterial populations, and we would be able to identify these with a range of phenotypic and genotypic assays.

3.2. Diclofenac, metformin, and 17-β-estradiol did not increase bacterial ability to grow on antibiotic amended agar

To test whether exposure to NADs increased phenotypic resistance to antibiotics we plated day seven cultures of populations exposed to a high concentration of NAD (6400 μg/L diclofenac, 3300 μg/L metformin, and 5400 μg/L 17-β-estradiol) onto antibiotic amended agar plates. We tested a range of antibiotics to be able to identify any wide co-selective effects.

We found that when both *E. coli* and presumptive coliform CFU/mL were combined, NAD treatment did not significantly affect CFU/mL counts (NAD treatment main effect, ($X^2 = 6.85$, d.f. = 3, p = 0.077). There was a similar response seen in the *E. coli* only data, with NAD treatment not significantly affecting the data ($X^2 = 1.95$, d.f. = 3, p = 0.58). However, when only presumptive coliform counts were analysed (Fig. 1), we found that both NAD treatment (NAD main effect: $X^2 = 10.74$, d.f. = 3, p = 0.013), and type of antibiotic plate (antibiotic main effect: $X^2 = 84.32$, d.f. = 5, p=<0.0001) significantly affected the proportion of resistant CFU/mL. Pairwise comparisons show that 17-β-estradiol decreased resistance to ampicillin (p = 0.0306) and gentamicin (p = 0.017), and non-significantly with azithromycin treatment.

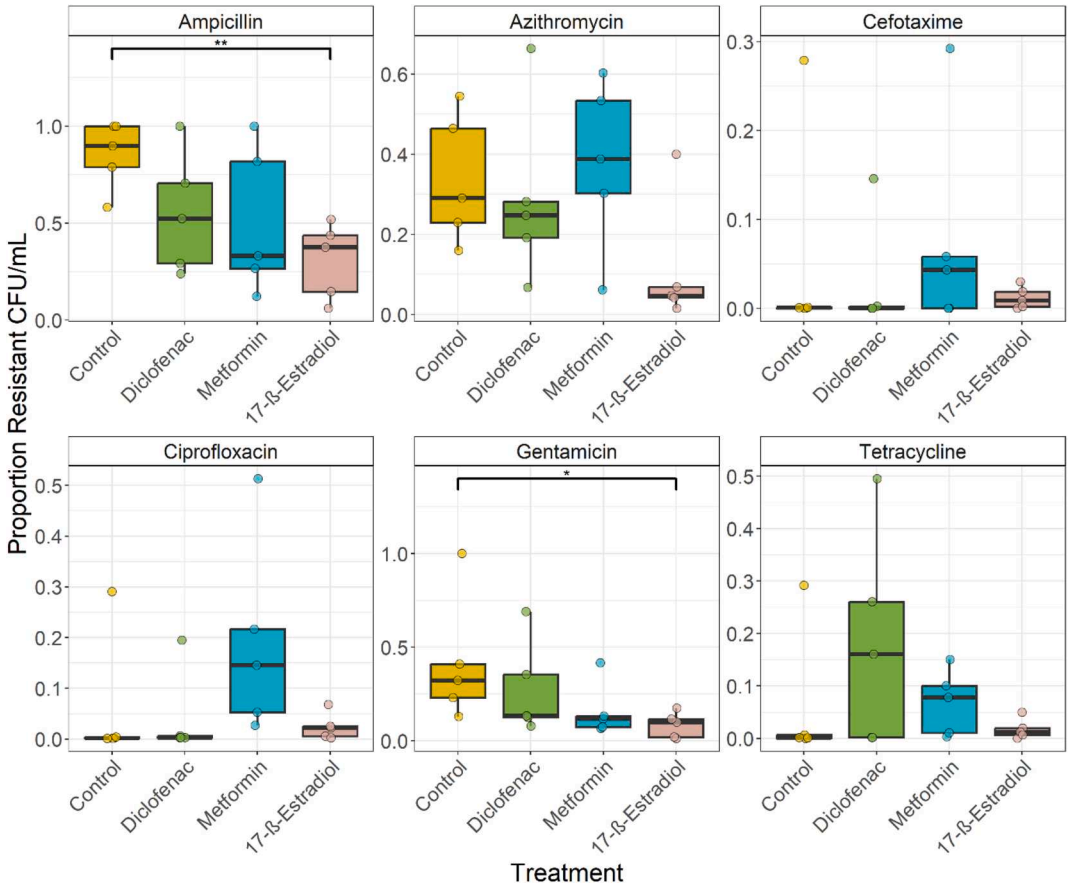

Fig. 1. Resistant proportion of colony forming units (CFU) per mL growth on different antibiotic plates, with treatment with diclofenac, metformin, and 17-β-estradiol. Graphs show presumptive coliform growth only.

We see that metformin exposure increased phenotypic resistance against cefotaxime and ciprofloxacin although these were non-significant.

3.3. 17-β-estradiol selected for intI1, diclofenac and metformin did not

Next, we used qPCR to identify changes to *intI1* and *cintI1* after exposure to NADs for seven days. Firstly, we found that *intI1* prevalence (the *intI1* quantity/16S rRNA quantity) was not changed with diclofenac or metformin exposure (Supplementary Figs. 2 and 3). However, 17-β-estradiol selected for *intI1* after exposure for seven days (Fig. 2). *IntI1* prevalence increased after the seven-day exposure (time main effect: $X^2 = 103.31$, d.f. = 1, $p < 2.2e\text{-}16$), and increased with increasing 17-β-estradiol concentration (concentration main effect: $X^2 = 29.76$, d.f. = 8, $p = 0.00023$). Pairwise comparisons show that 7 μg/L ($p = 0.0004$), 22 μg/L ($p = 0.0026$), 67 μg/L ($p = 0.0017$), 201 μg/L ($p = 0.0006$), 604 μg/L ($p = 0.0027$), 1800 μg/L ($p = 0.0050$), and 5400 μg/L ($p = 0.0008$) all had significantly increased *intI1* prevalence at day seven compared to the day seven control.

None of the three NADs selected for or against *cintI1* prevalence after seven days exposure (Supplementary Figs. 2-4). Since these qPCR assays were used as an indicator of selection, but were inconclusive, and the plating indicated reduced selection, we performed metagenome sequencing on the evolved communities to understand if these NADs were selecting for or against other AMR genes.

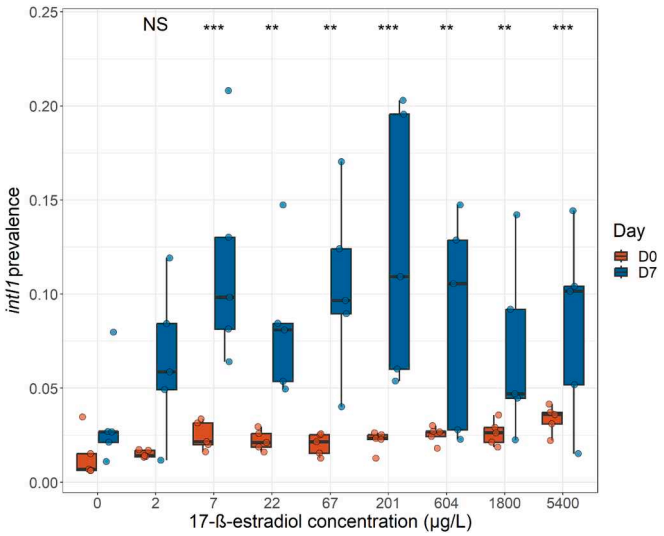

Fig. 2. *intI1* prevalence for selection experiment with 17-β estradiol. Asterisks indicate significant difference in day seven prevalence compared to day seven prevalence at 0 μg/L. Significance values as follows: NS – non-significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.4. There was limited ecological species sorting effects with NAD treatment

Overall, we did not observe large differences in the diversity of the evolved communities that had been exposed to NADs. The alpha diversity (richness) and the beta diversity did not differ between the evolved communities (details of these are found in the Supplementary File, and Supplementary Figs. 5-10). We also did not see any significant changes to the dominant genera present in each community exposed to the three NADs (Supplementary Figs. 8-10). There were also no changes

to any specific taxa after treatment with either diclofenac, metformin, or 17-β-estradiol (Kruskal-Wallis, $p > 0.05$, *fdr* adjustment).

3.5. There were some specific changes to ARGs after exposure to diclofenac, metformin, or 17-β-estradiol

We tested all resistance genes to determine if any changed with treatment with one of the three NADs Firstly, we qualitatively observed that the richness of resistance genes increased with metformin treatment (Supplementary Fig. 12) and decreased with 17-β-estradiol treatment (Supplementary Fig. 13) however, these changes were not statistically significant.

When we tested whether any specific gene had changed in response to treatment, we found that diclofenac and 17-β-estradiol did not significantly select for any ARGs in the metagenome (Kruskal-Wallis, $p > 0.05$, *fdr* adjustment), and neither significantly altered the beta diversity of the ARGs within the evolved communities (Supplementary Figs. 11 and 13, details of tests in Supplementary File Section 2).

Although metformin did not specifically change the relative abundance of any antibiotic gene class (Supplementary Fig. 12), metformin treatment of 13.6 μg/L to 3300 μg/L decreased the diversity of ARGs compared to 0 μg/L and 4.5 μg/L evolved communities (ANOSIM, statistic $R = 0.37$, $p = 0.0016$). Additionally, metformin treatment appeared to select against the efflux pump gene *TolC* (Kruskal-Wallis, $p = 0.026$) (Fig. 3). The associated efflux genes *acrA* and *acrB* also showed decreased abundance, but this was not significant (Kruskal-Wallis, $p = 0.08$, and $p = 0.051$ respectively).

3.6. All three NADs showed selection for metal resistance genes

We found that diclofenac changed the beta diversity of BMRGs between the evolved populations (ANOSIM statistic $R = 0.26$, $p = 0.012$). These differences may have been non-specific, since the communities did not become more dissimilar the more they varied with concentration (Mantel statistic $R = -0.012$, $p = 0.51$). Neither metformin, nor 17-β-estradiol significantly altered the beta diversity of the BMRGs present in the evolved communities (metformin: ANOSIM statistic $R = 0.17$, $p = 0.054$, 17-β-estradiol: ANOSIM statistic $R = 0.077$, $p = 0.19$).

We tested the BMRGs that were specifically plasmid-borne, since HGT of resistance genes is thought to be a major driver of AMR across and within environments (von Wintersdorff et al., 2016). Furthermore, plasmid-borne genes are more likely to be co-located with multiple different genes that could confer resistance. We found that all three NADs significantly altered plasmid-borne BMRGs.

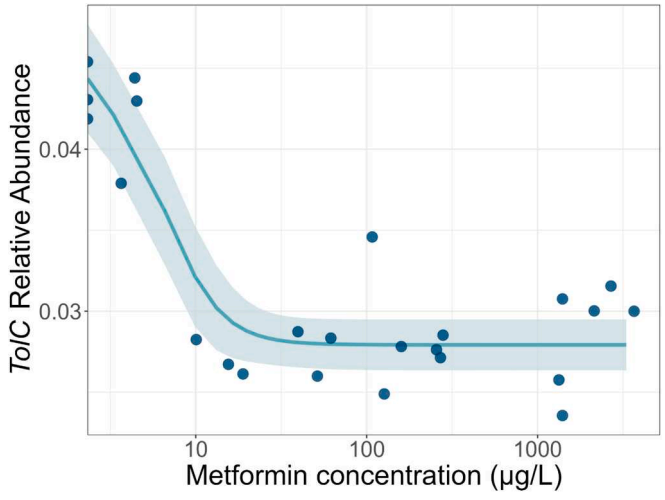

Fig. 3. Relative abundance of *tolC* as a function of metformin concentration. Four parameter log logistic curve plotted (line) with 95% confidence intervals (pale ribbon) against all data points.

Diclofenac treatment significantly altered relative abundance of 57 plasmid associated genes (including arsenic, mercury, nickel, and tellurium resistance genes, Supplementary Table 3). However, these were not altered in a dose-dependent manner (Kruskal-Wallis test, $p < 0.05$, *fdr* adjustment). Metformin treatment significantly altered relative abundance of 133 plasmid associated genes (Supplementary Table 4), again, in a non-dose-dependent manner (Kruskal-Wallis, $p < 0.05$, *fdr* adjustment). 17-β-estradiol treatment significantly altered 142 plasmid associated genes (Kruskal-Wallis, $p < 0.05$, *fdr* adjustment, Supplementary Table 5), and of these, 138 were not dose-dependent. However, the relative abundance of four genes significantly correlated with 17-β-estradiol concentration (Fig. 4). We found that 17-β-estradiol positively selected for two copies of *arsB* and for *arsR* but showed negative selection for *ncrA*.

3.7. NADs did not change ARG expression levels

Since we did not find any specific selection for heritable ARGs or BMRGs that associated with the reduction in growth, we decided to investigate antibiotic resistance gene expression changes in communities exposed to the three NADs after seven days exposure.

We did not find any ARGs that were differentially expressed when communities were grown at high concentrations of diclofenac, metformin, or 17-β-estradiol when compared to the control. Additionally, there was no change in genes associated with common functions (as annotated using KEGG) (Kruskal-Wallis, $p > 0.05$, *fdr* adjustment). However, data indicated that there were several thousand unidentified genes that were only expressed in either the diclofenac ($n = 8{,}967$), metformin, ($n = 11{,}063$), or 17-β- estradiol ($n = 9{,}494$) treatments and not the control, with a further 1,081 unidentified genes expressed in all the NAD treatments but not the control populations.

3.8. NADs can alter BMRG expression levels

NAD treatment significantly altered the expression of BMRGs associated with plasmids. Five plasmid-associated BMRGs were significantly different across the NAD treatments (Fig. 5, Kruskal-Wallis, $p < 0.05$, *fdr* adjustment). We found that diclofenac reduced the expression of *arsR* (Dunn's test, $p = 0.024$), and metformin reduced the expression of *arsB* (Dunn's test, $p = 0.014$). Additionally, metformin increased the expression of *merP* (Dunn's test, $p = 0.044$). Finally, 17-β-estradiol reduced the expression of *arsR* (Dunn's test, $p = 0.031$) and *merA* (Dunn's test, $p = 0.013$).

4. Discussion

The effects of five NADs at environmental concentrations were tested on a wastewater influent bacterial community. We found that three of these NADs reduced bacterial growth and showed significant changes to metal resistance gene abundances.

Ibuprofen and haloperidol did not significantly reduce growth of the wastewater influent bacterial community here, although they have previously been indicated to have growth inhibitory effects (Maier et al., 2018; Paes Leme and da Silva, 2021). These differences may be due to different experimental designs. For example, in this study we used a mixed bacterial community derived from wastewater, whereas much previous work has focused on single species models (e.g. (Maier et al., 2018; Younis et al., 2015)). It is known that the presence of a bacterial community can be protective against antibiotic stress (Klümper et al., 2019) and this may occur with NADs.

Diclofenac, metformin, and 17-β-estradiol significantly reduced the growth of the community down to low, μg/L, concentrations and were tested further. This reduction in exponential phase, has been suggested to indicate selection occurring on those populations (Murray et al., 2020; Greenfield et al., 2018). These NADs affected growth at

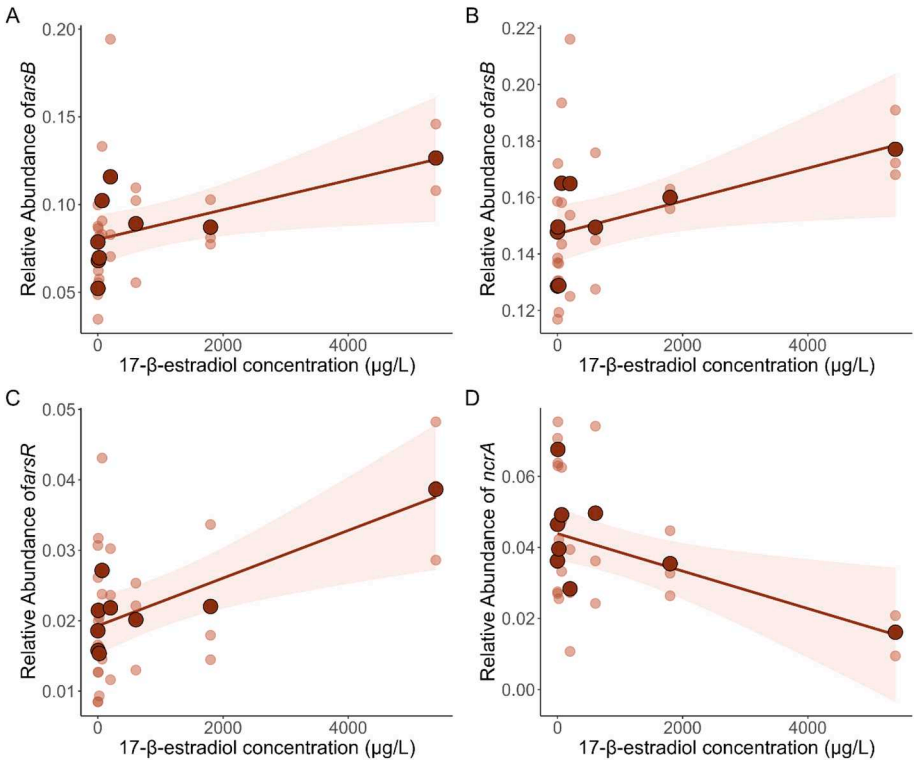

Fig. 4. Genes that are significantly positively or negatively selected for with 17-β-estradiol concentration. A – *arsB*, B – *arsB,* C – *arsR*, D – *ncrA*. Plotted points indicate raw data, larger points show the concentration mean. Line indicates model fit, and the shaded ribbon indicates the 95% confidence interval.

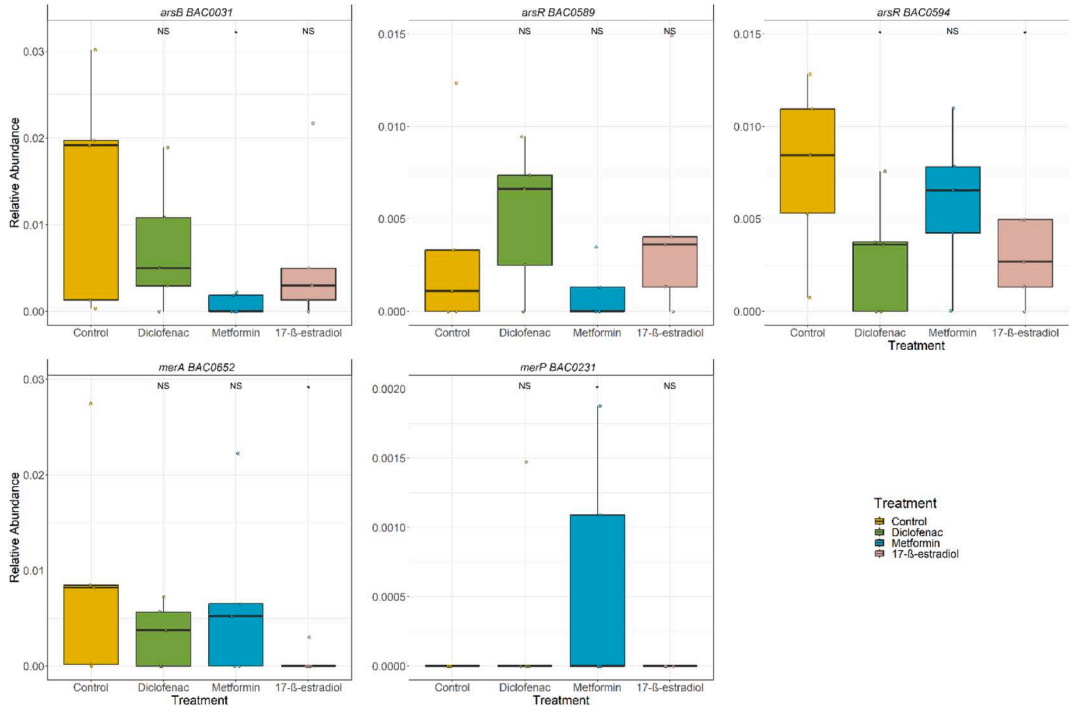

Fig. 5. Expressed BMRGs in communities evolved with diclofenac, metformin, or 17-β-estradiol, or a no-NAD control. P < 0.05 = *, p < 0.01 = **.

concentrations similar to those found in the aquatic environment, both globally (aus der Beek et al., 2016) and in England and Wales (UKWIR, 2020). This suggests that in the aquatic environment, diclofenac, metformin, and 17-β-estradiol may alter the function of bacterial populations, indicating that increased pollution via wastewater into the freshwater environment (for example through sewage overspills) may affect ecosystem functioning.

To confirm selection for AMR, we used qPCR assays and metagenome sequencing. Only 17-β-estradiol selected for a qPCR target (*intI1*), from 7 μg/L to 5400 μg/L, in a non-dose-dependent manner. We observed positive selection, which then plateaued. It may be that the gene/s under selection in the integron provided a low-level benefit to the community,

but the upper limit of benefit was quickly reached. However, we do not know which genes associated with *intI1* may be driving this response. *IntI1* is recommended as a gene to use in environmental AMR surveillance as it can be associated with a large range of ARGs (Gillings et al., 2008), biocide, and metal resistance genes (Gillings et al., 2015). Future research could sequence the gene cassettes within the class I integrons in these communities, to find those that are closest to the promoter (and therefore would be the most highly expressed). Regardless, selection for *intI1* suggests that there could be a risk of increased mobilisation of genes, which could be ARGs, and therefore increased potential for dissemination of AMR throughout 17-β-estradiol impacted bacterial communities. This selection for increased evolvability may be of concern when 17-β-estradiol is found in mixtures with antibiotics, with increased mobilisation coupled with strong selection for AMR. However, whether 17-β-estradiol can indeed increase HGT would need to be experimentally tested in future work.

Interestingly, we did not observe any selection for ARGs by diclofenac, metformin, or 17-β-estradiol using metagenome analyses, and no statistically significant increases in phenotypic resistance for the antibiotics tested. Metformin selected against *tolC* in the metagenome analysis. *TolC* is a member of the acrAB-TolC efflux pump, known to provide resistance to multiple antibiotics and disinfectants (Li et al., 2015). Metformin might have selected against carriage of this gene or inhibited the growth of *tolC* bearing bacterial species. Regardless, exposure to metformin may reduce co-selection for AMR, however, this is not captured in the other analyses presented here, such as the phenotypic assays. Metformin treatments of 13.6 μg/L and greater appeared to decrease the beta diversity of ARGs compared to the control, suggesting that metformin did select against specific ARGs, but that this could not be detected with statistical approaches used at the ARG or ARG class level. Furthermore, we hypothesised that exposure to 17-β-estradiol would select for various ARGs, however this was not the case, and phenotypically, exposure to 17-β-estradiol increased sensitivity to gentamicin and ampicillin. This suggests that 17-β-estradiol may not select for antibiotic resistance and may increase sensitivity in this community.

However, we found that diclofenac, metformin, and 17-β-estradiol altered relative abundances of some metal resistance genes. After treatment with diclofenac, metformin, or 17-β-estradiol, the relative abundances of arsenic resistance genes (e.g. *arsA*), and mercury resistance genes (e.g. *merT*) were significantly altered across at least one concentration. This suggests that there could be a generalisable response to NADs, using metal resistance pathways to manage any toxic effects of these NADs.

More interestingly, exposure to 17-β-estradiol dose-dependently increased the relative abundance of two arsenic resistance genes (*arsB,* and *arsR*) and decreased the relative abundance of *ncrA*. The arsenic resistance genes are involved in the production of an arsenite efflux pump (Escobar-Niño et al., 2021; Yang et al., 2012). These genes were isolated from three different bacterial species – *Yersinia enterocolitica, Staphylococcus aureus*, and *Acidiphilium multivorum* (Pal et al., 2014). This suggests that the increase in these genes occurs across phyla and is not driven by a dominant species. This is supported by the lack of a significant alteration in the community composition following treatment with 17-β-estradiol. This arsenite efflux pump may confer cross-resistance to 17-β-estradiol and should be a target for future studies. The gene *ncrA* is involved in the ncrABCY efflux system (Marrero et al., 2007) and its negative selection in this study suggests it is not beneficial to the community, potentially to offset the cost of positive selection for the arsenite efflux pump genes.

We did not observe any changes in ARG expression after exposure to diclofenac, metformin, or 17-β-estradiol. However, expression of various metal resistance genes decreased in expression, in contrast to increased relative abundances of these genes. For example, 17-β-estradiol reduced *arsR* expression at the highest concentration, but had increased *arsR* gene relative abundance in the metagenome. This indicates that expression of genes is not always coupled to heritable selection.

Additionally, as mentioned, there were various gene transcripts that were only expressed in response to one of the three NADs that were not present in the control populations. These are uncharacterised, and it would be prudent for future work to identify these genes to understand if these are related to AMR in some way, or are unknown genes involved in adaptation to pharmaceutical pollution. However, since *arsR* appeared to be decreased in expression, but increased in absolute gene abundance, alongside an increase in *intI1* prevalence, this suggests that these genes are co-located with other unidentified resistance mechanisms. These mechanisms may be 17-β-estradiol specific, but further work would be required to identify these.

There are limitations associated with this work. Firstly, we used a mixed bacterial community derived from sewage and incubated at high temperature in high nutrient media. Therefore, in natural environments any effects due to exposure to NADs may differ, since different species may not be able to survive in our laboratory conditions that would survive in those environments (or vice versa). To address this, future work should aim to use environment-specific conditions (e.g. anaerobic conditions to mimic the human gut).

Secondly, genes may have been selected for that confer resistance to antibiotics but are unannotated in the ARGs-OAP database. For example, metabolic genes can confer antibiotic and biocide resistance (Murray et al., 2023) but would not be identified as ARGs. This is a common issue regarding environmental AMR work since databases used to identify ARGs are predominantly based on clinically derived data and are therefore biased towards clinical pathogens and human associated bacteria.

Thirdly, due to the nature of the experimental design, it was difficult to identify low effect size changes in the metagenome data. Community experiments are noisy due to the large variation in strains and species present. Therefore, subtle changes in responses are difficult to identify. Further work should aim to include increased replicative power to try to be able to identify effects that may have been lost in this study.

Finally, we propose that these experimental conditions are not fully replicative of how bacterial communities would encounter environmental pollutants. In the environment, pollutants are present as a complex mixture, including NADs (Wilkinson et al., 2022), antibiotics (Danner et al., 2019), and other pollutants, including chemicals used in agriculture (Tang et al., 2021), biocides (Paun et al., 2022), and chemicals from road run-off (Müller et al., 2020). Therefore, the effects of mixtures of these compounds might be more important in understanding the real-world effects of NADs. Even when prescribed clinically, NADs are often combined with other medications, whether transiently (e.g. with ad-hoc usage of painkillers) or long-term (e.g. multiple pharmaceuticals prescribed for long-term conditions). Therefore, although the NADs tested here do not appear to strongly co-select for antibiotic resistance when tested alone, what occurs when these NADs are in combination with one or multiple other compounds should also be investigated.

5. Conclusion

This study suggests that diclofenac, metformin, and 17-β-estradiol do not select for antibiotic resistance in wastewater derived bacterial communities but are associated with significant increases in metal resistance genes. These data add to existing single species work investigating the selective effects of NADs but expand this to complex real-world mixed species communities. This research indicates that environmental pollutants such as NADs select for adaptive traits such as metal resistance in anthropogenically impacted environments worldwide. Future work should aim to further understand these effects in a larger range of environmental microbial communities, and in specific environmental laboratory conditions (e.g. low temperature and nutrient limited media). Overall, this research expands our knowledge of selective effects of NADs and indicates that environments polluted with NADs

are likely to be selecting for AMR in bacterial communities.

CRediT authorship contribution statement

April Hayes: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lihong Zhang:** Writing – review & editing, Supervision. **Edward Feil:** Writing – review & editing, Supervision. **Barbara Kasprzyk-Hordern:** Writing – review & editing, Supervision. **Jason Snape:** Writing – review & editing, Supervision. **William H Gaze:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Aimee K Murray:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

JS was formerly employed by AstraZenceca. All remaining authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2025.109490.

Data availability

The datasets generated and analysis code used in this study are available at Zenodo https://doi.org/10.5281/zenodo.11109355. The raw metagenome and metatranscriptome sequence files generated as part of this study are available at ENA with Accession PRJEB74464.

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Glossary

Antimicrobial Resistance (AMR): a phenomenon when microorganisms become unable to be killed by antimicrobials. For example, antibiotic resistant bacteria are bacteria that are unable to be treated by antibiotics.
Co-selection: occurs when one compound (e.g. antibiotic) selects for resistance to itself and another compound (such as a metal)
Diclofenac: Non-steroidal anti-inflammatory drug, commonly used as a pain reliever
Metformin: pharmaceutical used in front-line diabetes treatment
Non-antibiotic drugs: pharmaceuticals that are not antibiotics, for example, painkillers, and drugs used for long term conditions such as statins
17-β-estradiol: a hormone naturally produced in mammals, also used pharmaceutically in hormone replacement therapy