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Water Impact Statement

Stormwater runoff may be a key conduit for propagating and disseminating antibiotic resistance in the urban water cycle; however, it is unclear what impact common stormwater pollutants have on antibiotic resistance. This study demonstrates that metals in stormwater runoff significantly increase ARGs and ARB, nutrients facilitated bacterial growth but did not contribute to resistance proliferation, and salts exhibited no impact.

Elucidating the impact of common stormwater pollutants on antibiotic resistance: the role of heavy metals, nutrients, and salts

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Abstract

Antibiotic resistance poses a significant global health threat, and the urban water cycle presents an opportunity to augment or limit the spread of antibiotic resistance. In particular, stormwater runoff has recently been revealed as a key conduit for antibiotic-resistant bacteria (ARB). The specific role of stormwater pollutants, however, on antibiotic resistance has not been isolated. Understanding the impact of specific pollutants common to stormwater could help optimize design and operation of stormwater systems for management of antibiotic resistance. The objective of this research was to establish the potential contributions of common stormwater pollutants to antibiotic resistance proliferation. Lab-scale stormwater microcosms were amended with either heavy metals, nutrients, or salts, and then ARB and antibiotic resistance gene (ARG) concentrations were quantified over a seven-day period. The microcosm experiments revealed that heavy metals (5-1000 μg/L) on average significantly increased ($p<0.05$) ARGs and ARB concentrations 0.30-log and 0.96-log, respectively, and the effects were dependent on the

concentration of the metal. Total bacterial counts increased by 174% in nutrient-amended microcosms, while ARG levels remained statistically unchanged ($p>0.05$). Salts, a common pollutant in colder climates, exhibited no impact. Moving forward, targeted interventions focusing on heavy metal removal, alongside careful stormwater treatment design, could offer promising avenues for addressing antibiotic resistance dissemination in urban environments.

1. Introduction

Stormwater runoff is a critical component of the urban water cycle by conveying excess precipitation and land surface pollutants to downstream water bodies. As such, management of stormwater through conveyance networks, detention structures, and green stormwater infrastructure (GSI) is essential to reducing pollution in receiving waters. However, mitigating pollutants in stormwater runoff is a significant challenge, as stormwater runoff harbors a complex array of pollutants that render it a unique hazard to environmental and public health. Stormwater accumulates contaminants from various human activities, such as transportation, agriculture, and industrial processes, all of which contribute to the degradation of water quality. The repercussions of untreated stormwater discharge are profound, encompassing adverse effects on aquatic ecosystems, drinking water sources, and human well-being. For instance, excessive nutrient runoff can trigger harmful algal blooms and the presence of pathogens in stormwater poses significant risks to human health, causing waterborne illnesses and contaminating drinking water supplies [1,2]. As such, understanding the unique composition and potential consequences of stormwater pollution on environmental and human health is crucial for effective management and mitigation strategies.

Stormwater runoff has recently been identified as a hotspot for antibiotic resistance [3–6]. Antibiotic resistance, whereby bacteria can withstand the bactericidal or bacteriostatic effects of antibiotics, is a significant and growing public health crisis [7]. Over time, antibiotics have become less effective against bacterial infections, leading to a rise in hospitalizations and deaths [8]. The environment plays a crucial role in exacerbating antibiotic resistance by serving as a reservoir for antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) [6]. Stormwater specifically has been documented to harbor abundant ARGs and ARB at concentrations comparable to wastewater effluent, a known hotspot for antibiotic resistance [9,10]. For instance, the *sul*1 ARG, one of the most widely reported resistance genes, has been detected in stormwater at concentration ranging from 1.66 x 10^3 to 4.68 x 10^9 copies/L [10]. Similarly, ARB, such as ampicillin-resistant *E. coli,* have been measured at concentrations as high as 5-log CFU/L [11]. Stormwater runoff plays a further role in this public health crisis by facilitating the dissemination of ARGs throughout a watershed [12]. ARG dissemination could also be occurring within the bacterial community of stormwater through horizontal gene transfer (HGT) mechanisms [13]. HGT refers to the transfer of genetic material between bacteria that enables a bacterium to acquire resistance genotypes. HGT is crucial in the spread of antibiotic resistance because it enables bacteria to rapidly evolve and adapt in response to environmental pressures, including the presence of pollutants [14]. Environmental pollutants can increase the rate of HGT by inducing stress on bacterial populations, triggering mechanisms that promote genetic exchange [15]. Consequently, pollutants not only directly select for ARB but also facilitate the dissemination of ARGs within bacterial communities, exacerbating the public health threat posed by antibiotic resistance [16,17].

Various chemicals found in stormwater have been shown to propagate antibiotic resistance, including heavy metals, nutrients, and salts [18–21]. The presence of these pollutants in stormwater varies widely depending on land use, seasonal influences, and local pollution sources, leading to significant ranges reported in literature 22. Concentrations of heavy metals such as copper and zinc ranged from 10 to 500 μg/L, while nitrate and phosphate, key nutrients, have been measured between 0.1 and 30 mg/L [19,20]. Salts, particularly sodium chloride, can reach concentrations as high as 1,000 mg/L in stormwater from regions affected by road de-icing practices [21]. High concentrations of heavy metals can be toxic to bacteria, leading to the selection of a metal resistant bacterial community [22–24]. The mechanisms bacteria use to resist heavy metals, such as efflux pumps, enzymatic detoxification, and metal sequestration, can also contribute to antibiotic resistance. For example, multidrug efflux pumps such as the CusCFBA and CzcCBA systems confer resistance to both heavy metals (e.g., copper, zinc, and cadmium) and antibiotics by actively exporting toxic compounds out of bacterial cells [25,26]. Thus, the presence of heavy metals can co-select for ARB through these overlapping resistance mechanisms [27]. Nutrients, while not a selective pressure for antibiotic resistance, are a growth promoter. Bacteria rely on nutrients for growth and replication, and in environments with high nutrient concentrations, their growth can become exceptionally rapid. This accelerated growth can promote the vertical and horizontal transfer of ARGs, ultimately contributing to the spread of antibiotic resistance among bacterial populations in environments with elevated nutrient levels [28,29]. Lastly, salts can significantly influence bacterial communities by acting as a selective pressure, favoring the survival of certain species adapted to saline conditions [30,31]. This selective pressure exerted by salts can also lead to the enrichment of ARB within saline environments, due to the cross-resistance mechanisms that some bacteria may develop to cope with osmotic stress

[32,33]. The increase in antibiotic resistance by salts, though, has been found to be concentration dependent; high salinity has been reported to reduce the abundance of ARGs and limit horizontal gene transfer (HGT) [21,34].

Despite stormwater being a known hotspot for antibiotic resistance, the specific contribution of individual pollutants towards an increase in ARG and ARB concentrations in a stormwater matrix is unknown. The specific pollutants of interest in this research are heavy metals, nutrients, and salts because they are ubiquitous in urban stormwater runoff and are often present in high concentrations [35–39]. Furthermore, in managing stormwater runoff, green stormwater infrastructure (GSI) systems, including bioswales, bioretention cells, and constructed wetlands, have emerged as an option for removing chemical contaminants from runoff, showing promise for the removal of heavy metals, nutrients, and salts [40–42]. GSI have also been found to remove and accumulate ARGs in the soil media of GSI systems [43–45]. As such, knowing the effect of stormwater pollutants on ARG and ARB concentrations can help inform the design of GSI and its impact on ARG propagation. Following, the presence of such pollutants has strong implications for short-term propagation of antibiotic resistance in downstream receiving environments. Stormwater typically disseminates accumulated pollutants to surface water environments and has been documented to immediately and significantly impact receiving water quality [9]. Moreover, previous studies have shown that the influx of ARB and ARGs from stormwater can persist in downstream water bodies for up to 7 days [3]. However, it remains unknown how stormwater pollutants affect ARB and ARG concentrations over this 7-day period. It is therefore crucial to investigate stormwater pollutants for their ability to cause an immediate and rapid proliferation of ARB in stormwater that can pose a heightened risk to the environment

and human health, especially in urban areas where stormwater runoff is a major source of pollution.

The objective of this research was to determine the impact of stormwater pollutants on antibiotic resistance. The individual role of three stormwater pollutants, (1) heavy metals, (2) nutrients, and (3) salts, on proliferation of ARG and ARB concentrations in urban stormwater was assessed. It was hypothesized that all three pollutants would contribute to increased antibiotic resistance due to the stress they impose on the bacterial community, with heavy metals having the greatest impact due to co-selective mechanisms that promote resistance to both metals and antibiotics. To achieve this objective, microcosm experiments were conducted, and the impact of stormwater pollutants on phenotypic resistance was quantified by spread plate methods and genotypic resistance was determined via qPCR. Lastly, this work established the short-term impact of stormwater pollutants on the abundance of ARGs and ARB within stormwater by carrying out microcosm experiments over a 7-day period.

2. Methods

2.1. Sampling Locations and Events

To obtain a representative stormwater microbial community for microcosm experiments, stormwater was collected from an outfall in Wauwatosa, WI, USA (**Supplemental Material Fig. A1**). This outfall drains approximately 1.10 km^2 of mixed land use and discharges to the Menomonee River. Microcosm experiments were conducted separately for each pollutant type, with stormwater collected on different dates. The heavy metal microcosm used stormwater collected on November 5, 2022, the nutrient microcosm used stormwater from February 10, 2023, and the salt microcosm used stormwater from June 11, 2023. This approach allowed for

direct evaluation of each pollutant's impact on antibiotic resistance under controlled conditions while incorporating natural variability in stormwater microbial communities (**Supplemental Materials Fig. A16**). Streamflow data were gathered from the U.S. Geological Survey stream gage 04087120 Menomonee River at Wauwatosa, WI (**Supplemental Material Fig. A2**).

2.2. Sample Collection and Microcosm Processing

For evaluating the impact of stormwater pollutants on antibiotic resistance, three different microcosms were conducted: heavy metals, nutrients, and salts. The concentrations of the pollutants to be added to the microcosms were selected based on concentrations published stormwater literature (**Table 1**). The median and high concentration for each pollutant was calculated from the literature data set and are summarized in

Table 1. The concentrations added to the lab-scale microcosms therefore represented stormwater pollutants across a global scale and from multiple sources (e.g., outfalls, roofs, and roads). In setting up the microcosms, stormwater samples were collected from the site and transported back to the lab. All microcosms were started within 12 hours of sample collection. Each microcosm had three conditions: (1) a stormwater sample that received a median concentration of one set of pollutants (either metals, nutrients, or salts), (2) a stormwater sample that received a high concentration of the pollutants, and (3) a stormwater sample that received no pollutants, i.e., the control group used to assess temporal changes in antibiotic resistance in the microcosm without additional pollutants added. The specific type and concentration of pollutant added for each microcosm set are listed in Table 1. All microcosm conditions were tested in triplicate. For each microcosm reactor, stormwater was added into 1 L autoclaved bottles with minimal headspace. The bottles were mixed at 150 rpm on an orbital shake at room temperature [46,47]. Samples from the microcosms were taken for antibiotic resistance analysis on day 0 (i.e.,

initial conditions), day 3, and day 7 [46,47]. For each sample taken, water quality analyses were performed along with ARB and ARG analysis, all described below in the following sections.

Table 1 Stormwater Pollutant Concentrations Added to Microcosms Based on Reported Concentrations in Literature

		Added Concentrations		
		Median Concentration	**High Concentration**	**Source**
Metal Microcosm	Chromium (µg/L)	5	25	[10,35–37,48–50]
	Nickel (µg/L)	20	50	
	Copper (µg/L)	25	150	
	Cadmium (µg/L)	5	15	
	Zinc (µg/L)	250	1000	
Salt Microcosm	Sodium (mg/L) [Added as NaCl]	98.34 [250]	393.38 [1000]	[35,36,48,49,51–54]
	Magnesium (mg/L) [Added as MgCl]	63.82 [250]	255.28 [1000]	
Nutrients Microcosm	Ammonium (mg/L)	0.5	5	[35,48,49,53–55]
	Nitrate (mg/L)	2	10	
	Phosphate (mg/L)	0.5	5	

2.3. ARB Quantification

Samples collected on days 0, 3, and 7 were plated using the spread plate method on media containing seven different antibiotics to measure phenotypic resistance to antibiotics as well as R2A media (no antibiotic) to measure heterotrophic bacteria. Plates were prepared under aseptic conditions for the following antibiotics: ampicillin (AMP) at 32 µg/mL, sulfamethoxazole (SULF) at 100 µg/mL, rifampicin (RIF) at 4 µg/mL, ciprofloxacin (CIP) at 4 µg/mL, trimethoprim (TRIM) at 16 µg/mL, tetracycline (TET) at 16 µg/mL, and vancomycin (VAN) at 32 µg/mL. These antibiotics were selected based on their frequent detection in stormwater environments and their relevance to both clinical and environmental antibiotic resistance concerns. These concentrations are based on diagnostic concentrations inferring

clinical resistance from the Clinical and Laboratory Standards Institute (CLSI) dilution method (CLSI, 2018) as done previously [56]. While actual antibiotic concentrations in stormwater can vary widely, often in the ng/L to low µg/L range, the selected concentrations were chosen to better understand the potential for resistance under conditions of higher contamination exposure and to allow for easier detection and quantification of resistance phenotypes. All plates had a 100 µg/mL of cycloheximide addition for fungal growth prevention. After plating, the individual plates were stored in an incubator (set to 30℃ following growth plate protocol), and colony forming units were manually counted after five days of incubation. Any colony forming unit grown on antibiotic plates after incubation were presumed to be ARB.

2.4. DNA Extraction and ARG Quantification

Samples from the microcosms were vacuum filtered through a 0.22 µm Merck Millipore Express Plus® membrane filter. DNA was extracted from the filters via FastDNA Spin Kit (MP Biomedicals, Santa Ana, CA) manufacturer's protocol with the addition of three liquid nitrogen freeze-thaw cycles for cell lysis [57]. Three ARGs, *sul1, tet*W, and *erm*F, the integrase gene of the class 1 integrons, *intI*1, and the 16S rRNA gene were quantified from the DNA extracts via qPCR (**Supplemental Materials Table A1**). The genes chosen were selected because of their frequency of detection in stormwater [3]. To quantify the genes, a previously published qPCR protocol was followed [58–60].

2.5. Water Quality Analysis

Following the transport of the water samples back to the lab, pH of each sample was measured with a Thermo Scientific Orion probe (Thermo Fisher Scientific, Waltham, MA). US EPA Method 415.3 was utilized to quantify the dissolved organic carbon (DOC) with a TOC-

196 V_{CSN} analyzer (Shimadzu, Kyoto, JP). Metals and ions were quantified via inductively coupled
197 plasma mass spectrometry (ICP-MS) as described previously [61]. Lastly, total phosphate was
198 quantified with the Hach Phosphate Color Disc Test Kit (0-40 mg/L PO_4), nitrate was quantified
199 with the Hach Nitrate Color Disc Test Kit (0-10 mg/L NO_3^--N), and ammonia was quantified
200 with the Hach TNTplus Vial Test (0.015-2.000 mg/L NH_3 -N).

201 **2.6. Statistical Analyses**

202 All genes quantified *via* qPCR and water quality parameters were measured in triplicate.
203 Error between triplicate values was calculated through the standard deviation of the mean. In
204 addition, statistically significant relationships across sampling locations were evaluated with
205 one-way analysis of variance (ANOVA) with the *post hoc* Tukey's multiple comparisons test.
206 Significant relationships were assessed at a p-value$\leq$0.05. Gene relative abundances were
207 determined by dividing the gene's absolute concentration by the 16S rRNA gene absolute
208 concentration.
209

210 **3. Results and Discussion**

211 **3.1. Heavy Metals in Stormwater Cause Growth with Selection for Resistance**

212 Heavy metals at the high concentration caused ARB concentrations to increase. Relative
213 to the control, ARB concentrations increased between 0.30 and 0.88-log by day 3 and between
214 0.67 and 1.24-log by day 7 (**Fig. 1** and **Supplemental Material Fig. A3**). Ampicillin and
215 ciprofloxacin resistant bacteria exhibited the greatest increase in concentration across time.
216 Along with the increase in ARB concentrations, the total heterotrophic bacterial count (as
217 measured by the R2A plates) increased across time in comparison to the control (**Supplemental**
218 **Material Fig. A4**). The median concentration dosage of metals exemplified a similar result as

219 the high metal concentration dosage on day 3, with heterotrophic bacteria and most ARB
220 increasing in concentration relative to the control, except for tetracycline (**Fig. 1** and
221 **Supplemental Material Fig. A3**). However, on day 7, the ARB concentrations generally
222 decreased or showed no change from day 3, leading to variable results.

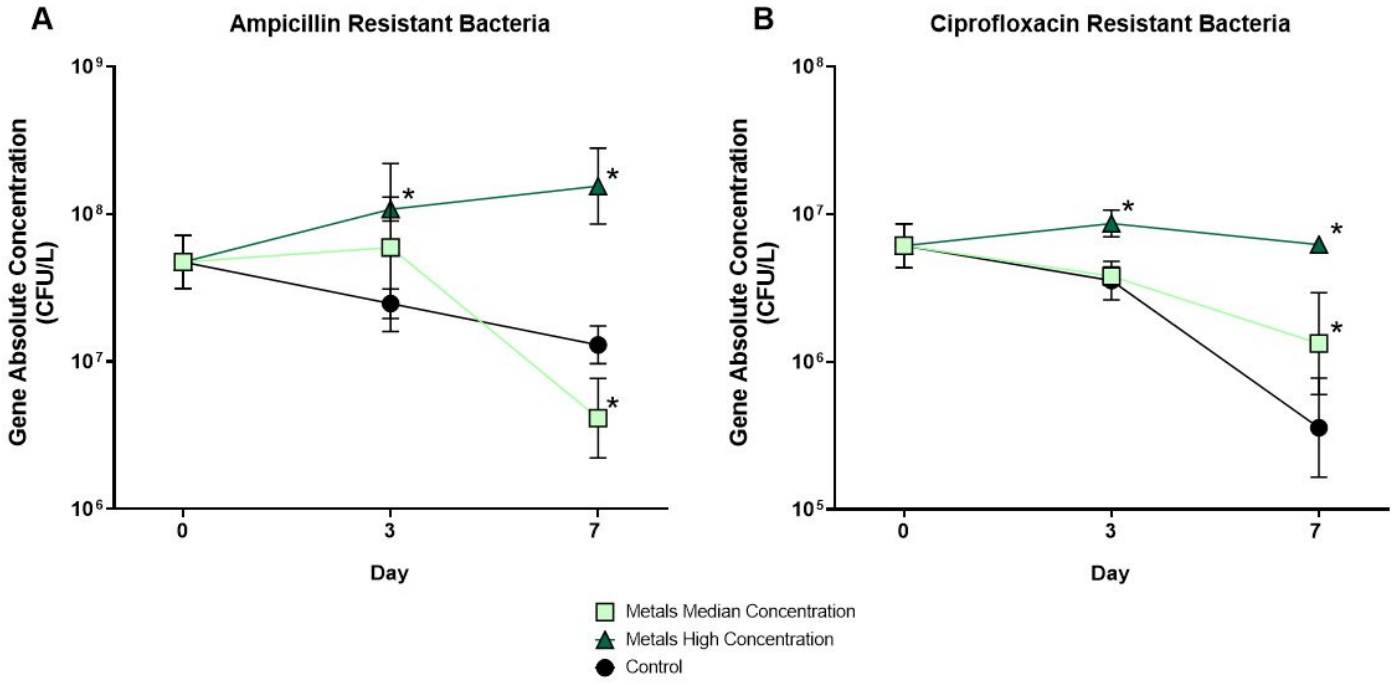

Fig. 1. Results of the ARB analysis from the heavy metal microcosm. Absolute concentration of bacteria resistant to ampicillin (A) and ciprofloxacin (B) over time when exposed to median and high metal concentrations. Error bars represent standard deviation from the mean ($n \geq 6$). * indicates a statistical difference ($p < 0.05$) of the microcosm dosed condition from the control at the respective timepoint.

223 The observed increase in abundance of ARB following the addition of heavy metals at a
224 high concentration prompts consideration of several potential mechanisms. It's plausible that the
225 elevated metal concentrations exerted a selective pressure on the microbial community, favoring
226 the proliferation of ARB capable of withstanding these stressors. Heavy metals are known to
227 interact with bacterial cells in ways that can induce stress responses, such as the activation of
228 efflux pumps, alteration of membrane permeability, and enzymatic detoxification. These

mechanisms help bacteria cope with metal toxicity, but they may also confer resistance to antibiotics that share similar modes of action or mechanisms of resistance. Additionally, the co-selection of resistance traits could play a critical role in this process. Genes conferring resistance to heavy metals and antibiotics are often co-located on the same mobile genetic elements, such as plasmids, transposons, or integrons [62,63]. This linkage facilitates the simultaneous transfer of resistance to both types of stressors, accelerating the spread of resistance genes in environments where heavy metals are present. Moreover, heavy metals are essential micronutrients for microbial growth, and their increased availability due to supplementation could have stimulated bacterial proliferation, including ARB. In environments where metals are typically limiting, their addition could provide a nutritional advantage to bacteria capable of tolerating high concentrations. This metabolic boost may further enhance bacterial fitness, contributing to the persistence and spread of ARB. The increase in total bacterial load alongside the rise in ARB concentrations suggests that metal exposure might also induce shifts in microbial community composition or adaptations within the bacterial population. These shifts could be driven by changes in metabolic pathways, such as the activation of stress response systems, or competitive interactions among bacterial species.

ARGs, similar to ARB, in the high concentration heavy metal microcosms, primarily increased in concentration relative to the control (**Fig. 2**). The 16S rRNA gene, a marker for bacterial load, also increased by 0.58-log on day 3 and 0.46-log on day 7. Where the ARG results varied from the ARB, however, is for the *sul*1 and *intI*1 genes on day 7 as both were less concentrated than the control (**Supplemental Material Fig. A5**). This result is due to a significant increase in the concentration of *sul*1 and *intI*1 in the control sample. *sul*1 is a sulfonamide ARG, while *intI*1 is a class 1 integron integrase mobile genetic element (MGE).

252 *intI*1 thus indicates mobility in the resistome, while it also has been found to be a marker for
253 anthropogenic pollution in the environment [64]. As an MGE, integrons can carry ARGs in their
254 gene cassettes and regularly *sul*1 concentrations are correlated with *intI*1 concentrations,
255 implying that the *sul*1 gene has been mobilized by the integron [64,65]. Therefore, it is likely that
256 *sul*1 is co-located on the integron in the stormwater samples collected in this study as these two
257 genes are showing the same relationship. At the median metal concentration dosage, ARG
258 concentrations primarily did not increase relative to the control. This result, paired with that of
259 ARB at the median concentration dosage, exemplifies that the promotion of antibiotic resistance
260 in stormwater via heavy metals is concentration dependent [26]. It is recognized that these
261 experiments stem from collection of stormwater on a single day. While the results demonstrate a
262 proof-of-concept that metals indeed can select for antibiotic resistance in stormwater, future
263 research could investigate the role of various stormwater microbial communities and how they
264 respond to metals. Overall, these results indicate GSI that reduces metal concentrations in
265 stormwater could help mitigate impacts that stormwater has on antibiotic resistance in
266 downstream aquatic surface waters.

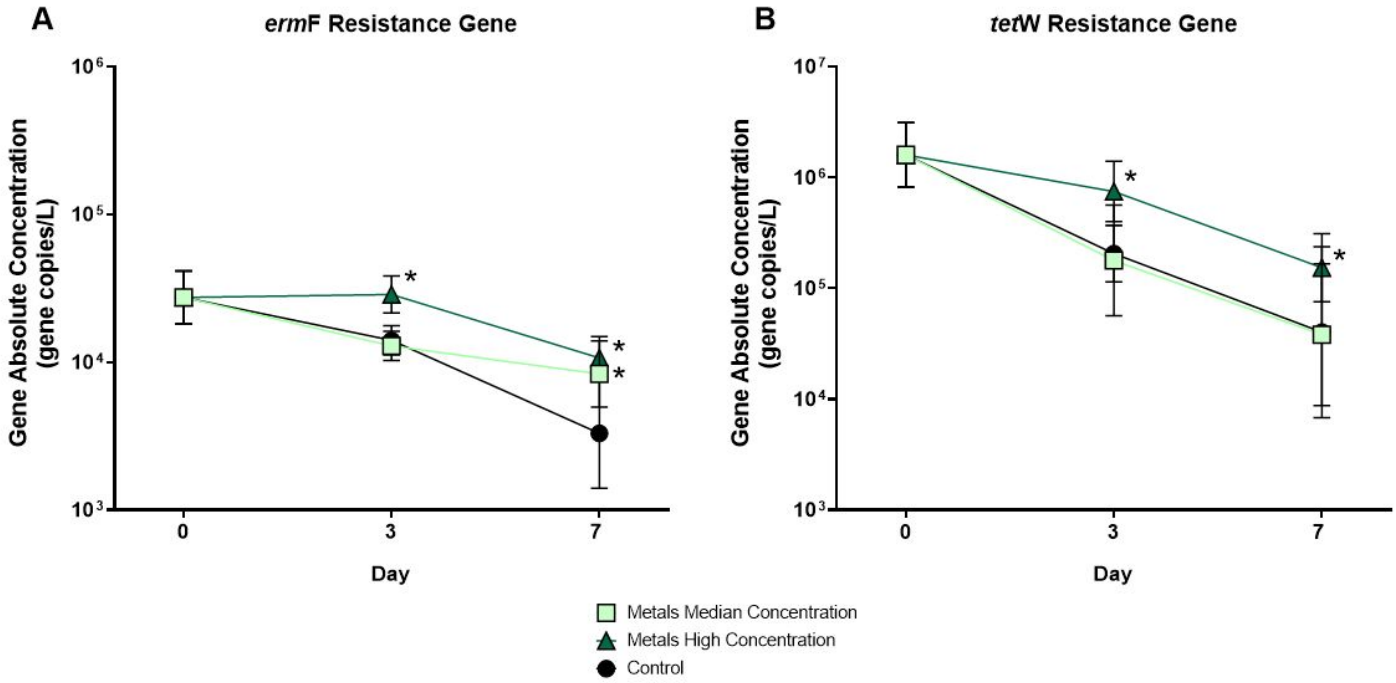

Fig. 2. Results of the ARG analysis from the heavy metal microcosm. Absolute concentration of the *erm*F resistance gene (A) and *tet*W resistance gene (B) over time when exposed to median and high metal concentrations. Error bars represent standard deviation from the mean (n≥6). * indicates a statistical difference ($p<0.05$) of the microcosm dosed condition from the control at the respective timepoint.

3.2. Nutrients in Stormwater Cause Growth without Selection for Resistance

Nutrients added to the stormwater led to growth of total bacteria (**Fig. 3**). The 16S rRNA gene was quantified by qPCR as a representative measurement of genotypic bacterial biomass. Across the microcosms, under both the median and high concentration conditions, the 16S rRNA was statistically greater ($p<0.05$) than the control (**Fig. 3B**). Similarly, total heterotrophic biomass (measured on R2A) increased relative to the control ($p<0.05$) with the addition of a high nutrient concentrations, however, there was no statistical difference ($p>0.05$) from the median concentration condition from the control (**Fig. 3A**). The increase in the 16S rRNA gene and total heterotrophic biomass was expected because nitrogen and phosphorus are essential for microbial

activity and growth [66]. Nitrate concentrations tended to decrease over the experiment, while ammonia increased (**Supplemental Material Fig. A7**). Total phosphate concentrations exhibited an initial increase by day 3 but were subsequently reduced by the end of the experiment conclusion (**Supplemental Material Fig. A7**). It is necessary to clarify that these measurements represent dissolved concentrations of nitrogen and phosphorus. While the observed fluctuations in nutrient concentrations align with expectations of microbial utilization, the persistence of certain nutrients, particularly phosphate, suggests potential limitations or complexities in nutrient cycling dynamics within the microcosm environment. Notably, an increase in phosphate concentration could indicate cellular death and insufficient nutrient uptake, which warrants further investigation. Moreover, it is worth considering the sensitivity of our measurements and their ability to capture changes relevant to microbial growth dynamics. Future work should focus on refining experimental methods to comprehensively understand the intricate interplay between nutrient availability and microbial responses in the stormwater microcosm.

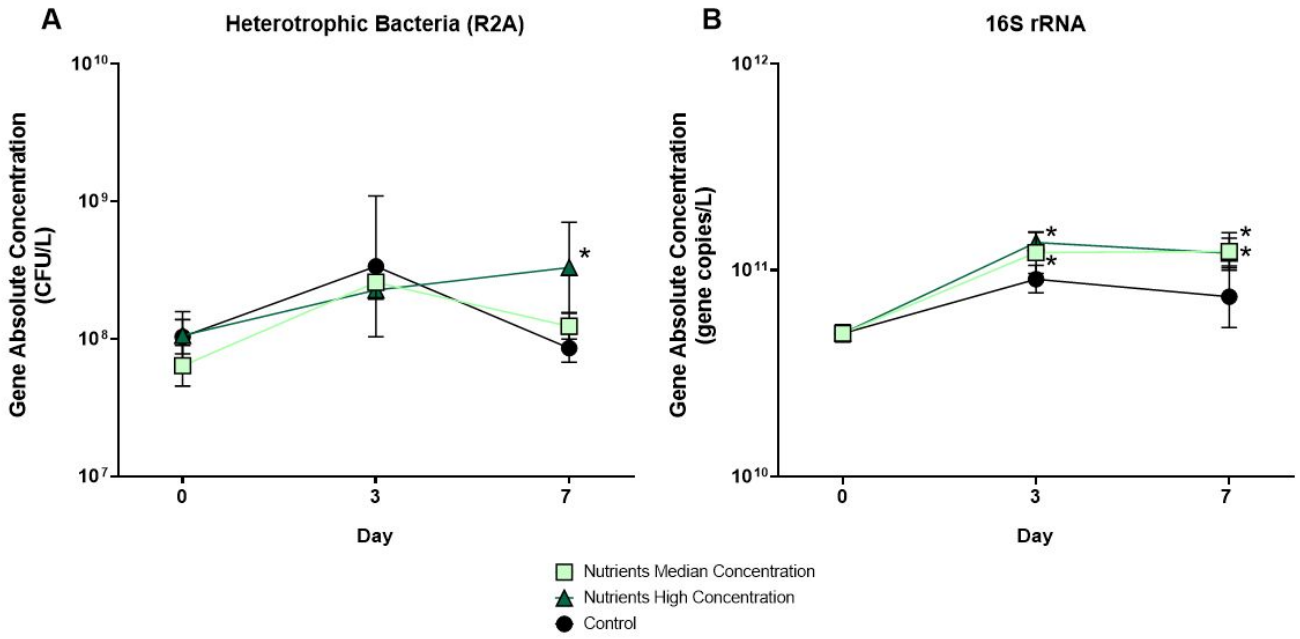

Fig. 3. Bacterial biomass concentration over the time of the nutrient microcosm experiment. Quantified as CFU growth on R2A agar (A) and copies of the 16S rRNA gene (B). Error bars represent standard deviation from the mean ($n \geq 6$). A * indicates a statistical difference ($p<0.05$) of the microcosm dosed condition from the control at the respective timepoint.

Despite the growth in the bacterial community, ARGs and ARB did not change in concentration across the nutrient microcosm experiment. **Fig. 4** depicts an example of one ARB, vancomycin resistant bacteria, and one ARG, *tet*W, while the remaining ARB and ARGs can be found in **Supplemental Material Figs. A9** and **A10**, respectively. In no instance did an ARB or ARG increase or decrease statistically relative to the control under the high or median nutrient concentration conditions on day 3 and day 7. This indicates that nutrients in stormwater do not cause an increase in ARGs or ARB. This result lies in contrast to the results of the heavy metal microcosm, as in both cases growth in the bacterial community was observed, but resistance only increased when heavy metals were added. This work indicates that, while nutrients can increase bacterial biomass, nutrients do not exert a selective pressure for antibiotic resistance in stormwater.

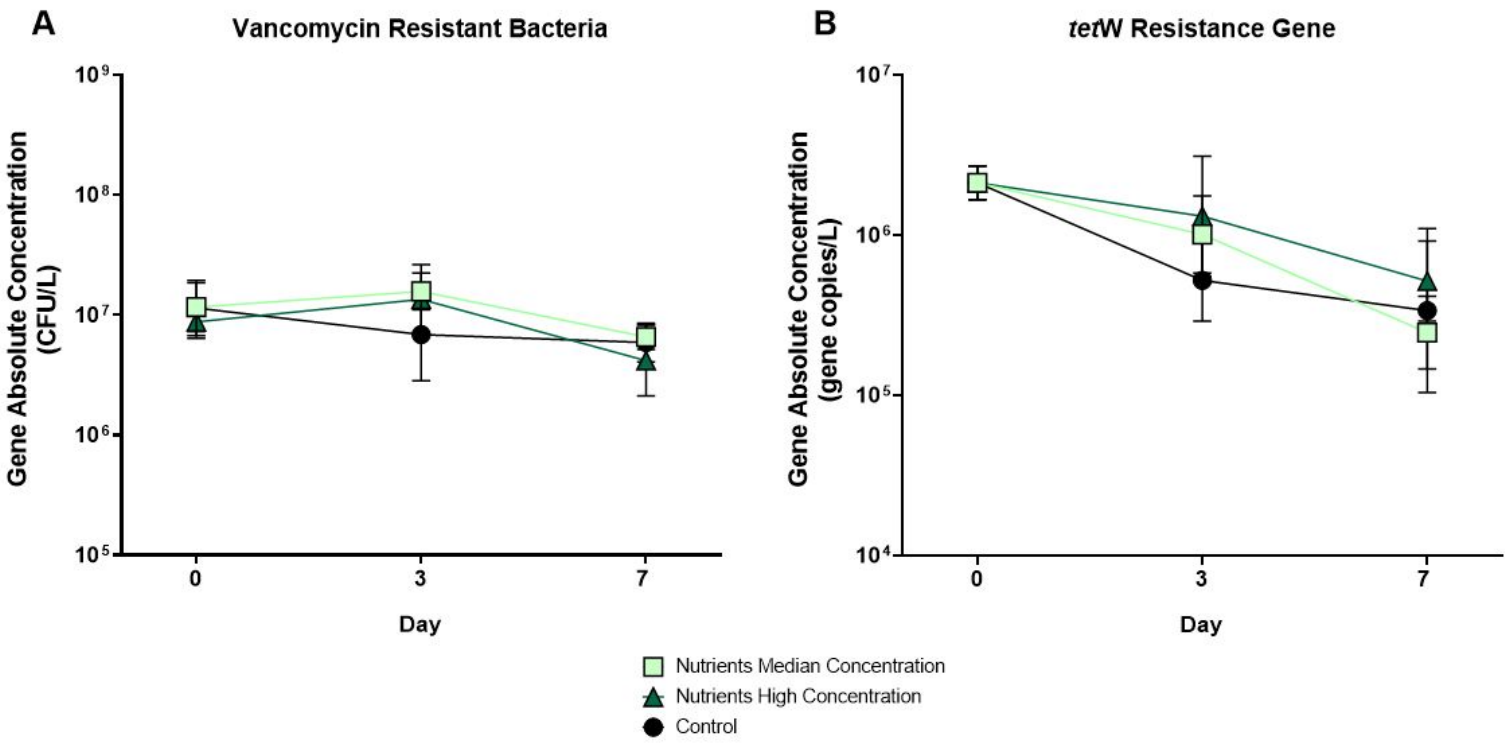

Fig. 4. Results of the ARB and ARG analyses from the nutrient microcosm. Absolute concentration of bacteria resistant to vancomycin (A) and the concentration of the *tet*W gene (B) over time when exposed to median and high nutrient concentrations. Error bars represent standard deviation from the mean (n≥6).

Previous research has documented that nutrients contribute to an enrichment of ARB and ARGs, specifically in soil-based environments [67,68]. Therefore, while nutrients may not be directly responsible for an increase in resistance in stormwater, they likely still can play a role in proliferation in receiving environments, such as green stormwater infrastructure (GSI). For instance, in downstream environments, abundant nutrient concentrations could support a diverse bacterial community, while also promoting growth rates [67]. The increased diversity paired with selective pressure from heavy metals could lead to ARGs being transferred to diverse bacterial genera [69]. Nutrients, by increasing bacterial growth, could also increase the vertical transfer of ARGs [70].

3.3. Salts in Stormwater have No Observable Impact on Antibiotic Resistance

Road salts were found to have no impact on ARB and ARG concentrations in stormwater (**Fig. 5** and **Supplemental Material Figs. A11 – A13**). In no instance was there a statistical increase in ARB or ARG concentrations above the control samples when the stormwater resistome was exposed to a median or high concentration of road salts ($p>0.05$). Moreover, there was no impact to the bacterial community, as heterotrophic bacteria (R2A) and the 16S rRNA gene also displayed no statistical increase relative to the control.

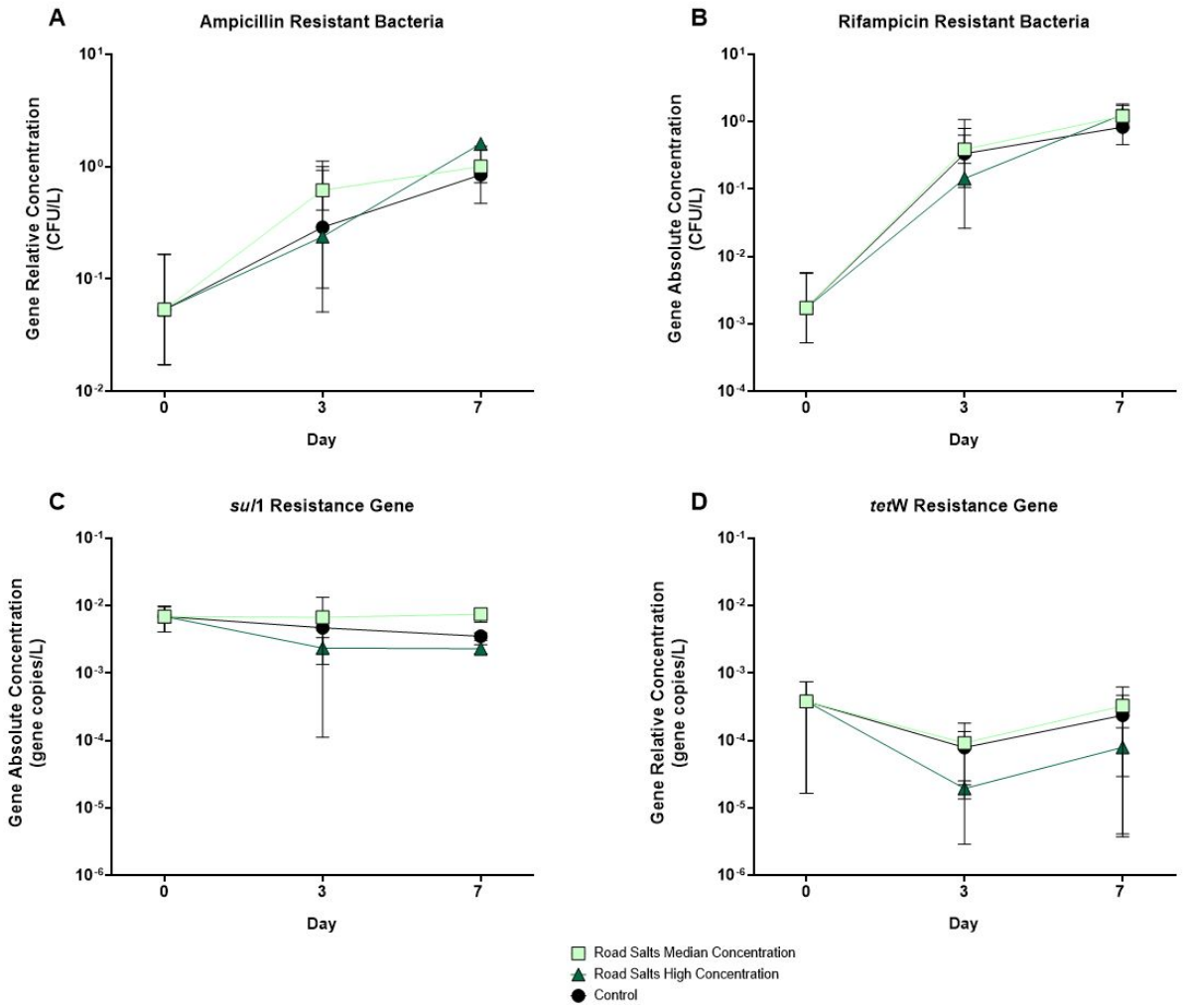

Fig. 5. Results of the ARB and ARG analyses from the road salt microcosm. Relative concentration of bacteria resistant to ampicillin (A) and rifampicin (B) as well as the concentration of the *sul*1 (C) and *tet*W genes (D) over time when exposed to median and high road salt concentrations. Error bars represent standard deviation from the mean ($n\geq6$).

316 The expectation for this work, based on previous research, was that road salts would lead
317 to an increase in resistance. Conversely, the results of this study do not support the finding that
318 salts are a selective pressure for antibiotic resistance. The lack of impact of salts on antibiotic
319 resistance, however, was observed only over a 7-day period. The influx of salt concentrations in
320 the environment, though specific to winter months, has been shown to persistent into the spring
321 and summer months [71]. Moreover, previous work that has concluded that salinity can increase
322 antibiotic resistance and the rate of HGT made these observations after 45 and 90 days [72]. In
323 addition, saline stress has also been found to significantly alter the microbial community in soil
324 and aquatic environments after a minimum of 29 days [73–75]. As such, road salts in stormwater
325 may not have an immediate and short-term impact on ARB and ARG concentrations, but still
326 may pose long term consequences.
327 Interestingly, the ARB under all conditions – high salts, median salts, and no salts –
328 displayed an increase in relative abundance (**Fig. 5**). Specifically, on average the relative
329 concentration doubled from day 0 to day 7 for all ARB. This result indicates that baseline
330 concentrations of pollutants in stormwater have an impact on antibiotic resistance. Following,
331 ARB and ARGs were evaluated in the controls for all microcosm experiments from day 0 to day
332 7, and several of the ARB and ARGs were found to increase in relative abundance across time
333 (**Supplemental Material Fig. A14** and **Fig. A15).** For example, within the control for the metal,
334 nutrient, and salt microcosms, sulfonamide ARB increased 0.15, 1.56, and 0.68-log, while *sul*1
335 increased 0.25, 0.04, and 0.43-log, respectively. Ampicillin ARB, rifampicin ARB, and
336 trimethoprim ARB also increased in relative concentration over time for at least two of the
337 sampling events. Ciprofloxacin ARB, vancomycin ARB, tetracycline ARB, *erm*F, and *tet*W,
338 however, only increased in relative concentration for one sampling event. The observed increase

in antibiotic resistance across the various stormwater samples suggests that multiple pollutants present in stormwater may collectively influence antibiotic resistance dynamics. This underscores the need for future research to explore the potential synergistic effects of different stormwater pollutants on the proliferation of antibiotic resistance, highlighting a critical avenue for understanding and mitigating environmental factors contributing to antibiotic resistance dissemination.

3.4. Study Implications

The findings of this study highlight the critical role that metal contamination plays in the proliferation of ARB and ARGs in stormwater. Given the significant contribution of metals to the emergence of resistance, it is imperative for stormwater management strategies to prioritize the reduction of metal concentrations in urban runoff. GSI systems present an opportunity to address this issue. Vegetated swales, bioretention basins, and constructed wetlands, known for their ability to filter stormwater through natural processes such as adsorption and filtration, are particularly effective at removing metals [76]. Enhancing these systems with plant species that are capable of metal uptake or materials like biochar with high sorptive capacities could further increase their efficiency in mitigating antibiotic resistance. Monitoring metal concentrations regularly would be essential to ensure that stormwater treatment systems continue to provide effective management of metal pollution.

While metals were shown to be the primary drivers of antibiotic resistance in this study, the role of nutrients in promoting bacterial growth warrants attention. Although nutrients alone were not directly linked to resistance proliferation, their combined effects with metals may exacerbate the concentration of ARB and ARGs. Therefore, a comprehensive stormwater

treatment strategy should address nutrient management as well. Techniques like denitrification, phosphorus precipitation, and biofiltration could be used to mitigate nutrient pollution, preventing the amplification of antibiotic resistance under the combined effects of metals and nutrients.

3.5. Future Research Needs

A limitation of this study is the relatively short duration (7 days) of the microcosm experiments. This time frame was selected as it is reflective of both the time scale of stormwater processes that have a time of concentration of hours to days, and the observed persistence of ARGs in stormwater within downstream water bodies that have been shown to remain above baseline conditions for up to a week [3]. As such, this study provides a temporally relevant understanding of the immediate, short-term impacts of stormwater pollutants on ARB and ARG dynamics, which is critical for evaluating how rapidly resistance can proliferate in response to contamination. The 7-day period allowed us to capture early changes in the bacterial community and provided valuable insights into how quickly resistance might emerge under high contamination exposure.

While 7 days is valuable for assessing initial impacts, it does not fully capture the long-term dynamics of ARB and ARG concentrations in stormwater. Therefore, future research should undertake longer studies, such as those extending 30 days or an entire season, to provide a broader understanding of how resistance evolves over time and the persistence of pollutants like metals and nutrients in stormwater systems. Long-term studies would also give us a better picture of how microbial communities adapt to chronic exposure to stormwater pollutants, allowing for the identification of longer-term ecological and public health implications.

In terms of real-world relevance, it is important to assess the long-term efficacy of stormwater treatment systems, including GSI, in mitigating antibiotic resistance. Ongoing monitoring of GSI systems after pollutant introduction would help evaluate how effectively these systems reduce antibiotic resistance over time and under varying conditions. Furthermore, future studies should examine the synergistic effects of multiple stormwater pollutants, such as the combined influence of metals, nutrients, and salts, to understand how these pollutants may amplify the effects on ARB and ARG concentrations. In addition, other emerging contaminants, such as nanoparticles, should be investigated for their role in ARG proliferation in stormwater. Recently research has shown that nanoparticles, like ZnO and PMMA, can promote the horizontal gene transfer of ARGs in aquatic environments, potentially amplifying the spread of resistance [77]. These nanoparticles can also impose selective pressure on microbial communities and influence the dissemination of ARGs, highlighting the need for further study on their ecological impact and the risks they pose in stormwater management systems [78].

4. Conclusions

In addressing the public health challenge of antibiotic resistance, adopting a One Health approach that considers the interconnectedness of human health and the environment is crucial. Stormwater, with its rich diversity of ARGs, ARB, and MGEs, is an environment of concern for the emergence and spread of novel resistance elements. While this study is limited to stormwater samples collected on a single day and at a single location, it indicates that within the unique composition of stormwater, metals are the significant factor influencing the abundance of ARB and ARGs in stormwater, suggesting that managing metal levels in stormwater runoff could help mitigate the exacerbation of antibiotic resistance. Stormwater commonly contains high levels of salts, nutrients, and metals; however, these results emphasize that metals are the primary driver

for antibiotic resistance. Therefore, targeting locations with elevated metal pollution, such as rooftops and roads, for intervention, along with using stormwater treatment systems such as GSI that target the removal of metals could substantially mitigate the effects of stormwater on antibiotic resistance proliferation in downstream aquatic systems. Future research is still needed though to fully understand the relationship between metal concentrations in stormwater and ARG proliferation through selective pressures and horizontal gene transfer. Additionally, while nutrients alone were observed to affect bacterial growth in stormwater, their combined effect with other selective pressures, such as metals, could potentially amplify the concentrations of ARB and ARGs. Careful consideration in GSI design is needed to prevent unintended consequences on antibiotic resistance proliferation. Considering these findings, targeted interventions focusing on metal removal in stormwater runoff, alongside the implementation of stormwater treatment systems like GSI, could substantially alleviate the impact of stormwater on antibiotic resistance proliferation in downstream aquatic systems, marking a positive step forward in safeguarding public health and environmental integrity.

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Data Availability Statement

The data supporting this article have been included as part of the Supplementary Information.