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Environmental drivers impact the accumulation and diversity of antibiotic resistance in green stormwater infrastructure

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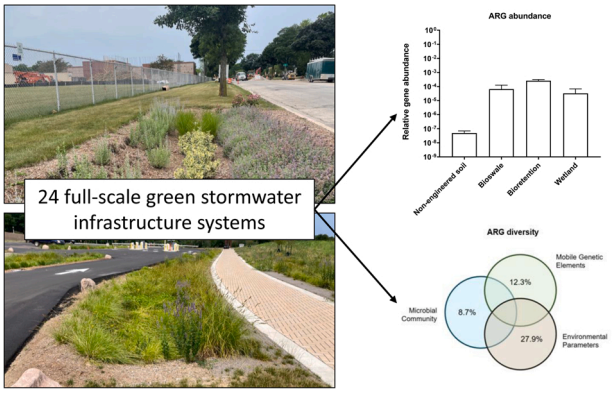
HIGHLIGHTS

- Green stormwater infrastructure soils were hotspots for ARG accumulation.
- The type of green infrastructure influenced ARG concentrations but not diversity.
- Microbial community changes were distinct from resistome changes.
- 3.27–8.83% of ARGs in GSI soils were co-occurring with a mobile genetic element.
- Environmental factors explained 27.9% of the variance in ARG diversity in GSI soils.

GRAPHICAL ABSTRACT



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ABSTRACT

Antibiotic resistance poses an urgent public health concern, with the environment playing a crucial role in the development and dissemination of resistant bacteria. There is a growing body of research indicating that stormwater is a significant source and transport vector of resistance elements. This research sought to characterize the role of green stormwater infrastructure (GSI), designed for stormwater infiltration, in accumulating and propagating antibiotic resistance in the urban water cycle. Sampling included 24 full-scale GSI systems representing three distinct types of GSI - bioswales, bioretention cells, and constructed wetlands. The results indicated that GSI soils accumulate antibiotic resistance genes (ARGs) at elevated concentrations compared to nonengineered soils. Bioretention cells specifically harbored higher abundances of ARGs, suggesting that the type of GSI influences ARG accumulation. Interestingly, ARG diversity in GSI soils was not impacted by the type of GSI design or the diversity of the microbial community and mobile genetic elements. Instead, environmental factors (catchment imperviousness, metals, nutrients, and salts) were identified as significant drivers of ARG diversity. These findings highlight how environmental selective pressures in GSI promote ARG persistence and

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proliferation independently of the microbial community. Therefore, GSI systems have the potential to be a substantial contributor of abundant and diverse ARGs to the urban water cycle.

1. Introduction

Antibiotic resistance is threatening the core of modern medicine and public health [89]. In fact, antibiotic resistant-related infections have been classified as one of the most urgent global health threats by the World Health Organization [13,69]. Moreover, antibiotic resistance has been coined "the silent pandemic" as it is expected to result in 10 million deaths per year and have an economic impact of over 100 trillion US dollars by 2050 if no actions are taken to combat this threat [67,70]. Antibiotic resistance, i.e., the ability of bacteria to resist the toxic effects of antibiotics, is spread by the movement of antibiotic resistant bacteria (ARB) as well as antibiotic resistance genes (ARGs) that bacteria can acquire. Though ARGs occur naturally, their presence has become widespread in nature due to anthropogenic activities, such as the overuse of antibiotics in the medical and agrarian sectors. In tackling environmental-based problems, the "one-water" approach has emerged as a method for viewing the connectivity between drinking water, wastewater, and the environment [68]. For instance, environmental hotspots, such as wastewater treatment effluent, could be targeted as points to mitigate the spread of antibiotic resistance into downstream water bodies [45]. Recent research has identified stormwater as an additional area of concern for ARGs entering the aquatic environment due to its ability to collect and transport ARGs from numerous sources, including impervious pavements, urban green spaces, and stormwater sewers, which can be contaminated with ARGs from soil, animal waste, human sewage, and atmospheric deposition [1,27,33,62–64]. The concentrations of ARGs in stormwater were found to be comparable to that of wastewater effluent [61], beckoning a greater understanding for how stormwater engineering systems can be employed to help mitigate the spread of ARGs [11].

In general, stormwater runoff is a significant pollution source because it is comprised of pollutants from the aquatic phase (e.g., rainwater) in combination with soil and sediment particles from surfaces. Across urban environments specifically, stormwater runoff facilitates the transport of physical, chemical, and biological contaminants from impervious surfaces into aquatic ecosystems. The focus of traditional stormwater management systems (i.e., gray infrastructure such as gutters, drains, pipes, and storage) is to mediate the physical effects of stormwater (e.g., flooding) [74,75]. Green stormwater infrastructure (GSI) has more recently been installed to work alongside traditional gray infrastructure to reduce stormwater volumes using soils and plants, while also providing additional ecological services, including pollutant reduction [55]. GSI systems and other nature-based solutions have gained significant traction in various regions globally, such as sponge cities in China and water sensitive urban design in Australia [50]. This reflects a growing recognition of their multifaceted benefits in enhancing urban resilience and promoting sustainable water management practices [39,57]. Soil-based GSI systems such as bioswales, bioretention cells, and constructed wetlands, vary in design and operational modes that could impact how they accumulate or remove stormwater pollutants [76]. Physical contaminants in stormwater, such as total suspended solids and sediments, have been found to be removed via settling, filtration, and infiltration into the GSI soils [8,18,24,76,81,90]. Moreover, GSI systems have the potential to reduce chemical contaminants in stormwater, such as nutrients, metals, ions, minerals, and organic carbon [3,18,48,76,90,92]. The ability of GSI to remove stormwater contaminants has propelled an increase in installation of GSI in many countries across the world as well as research into design considerations that could enhance their functioning [57]. The use and functional capabilities of GSI systems to manage biological contaminants in stormwater, however, has remained relatively unexplored. Research has focused primarily on fecal indictor bacteria and has indicated that removal through GSI is variable [19]. Various design factors, including system age and filter media have been identified as contributing factors to the differences exemplified in performance [72]. Despite the evidence that GSI soils can impact the fate of biological contaminants in stormwater, there has been very limited research on the occurrence of ARGs in GSI. Given that stormwater is a significant source of ARGs, it is crucial to understand the implications of stormwater dissemination within the urban water cycle. One key concern is the potential accumulation of stormwater ARGs in GSI soils, leading to GSI serving as a point source for ARG dissemination into the environment.

It is imperative to characterize the amount and types of ARGs in GSI to understand the role of GSI as a sink or source of ARGs in the urban water cycle as well as if GSI could be employed to mitigate ARGs from stormwater runoff. To date, only one study investigated ARGs in full-scale GSI systems [35]. This investigation employed qPCR to quantify ARGs in three biofilters and three bioswale systems and found that ARG concentrations had a significant positive correlation with metal concentrations, suggesting that the retention of ARGs and other contaminants could be enhancing resistance via selective pressures in GSI soils [35]. This result highlights a potential fate of ARGs in GSI: proliferation. Proliferation can be driven by selective pressures as well as the presence of mobile genetic elements (MGEs) that transfer resistance genes between bacteria through horizontal gene transfer (HGT). In addition to the limited research in this field, there is a critical gap in previous work providing essential GSI-related data that may offer insights into the concentrations and diversity of ARGs in GSI soils. This gap encompasses key factors like soil properties, system design parameters, and catchment area characteristics. Moreover, no studies have investigated the diversity of ARGs in full-scale GSI systems, which is crucially needed to understand links to the soil microbial community and MGEs.

The main goal of this research was to characterize the resistome of a wide array of full-scale GSI to establish if they could act as potential hotspots for ARGs within the urban water cycle. The specific objectives of this work were to (1) characterize the abundance, occurrence, and diversity of ARGs in 24 GSI systems and a reference site, (2) investigate the co-occurrence of ARGs with MGEs, and (3) determine the contribution of the microbial community, MGEs, and environmental factors to the variation in the abundance and diversity of ARGs in GSI. Soil samples from 24 full-scale GSI systems, including bioswales, bioretention cells, and constructed wetlands, of varying system, catchment, and soil characteristics were collected for this study. Metagenomic sequencing and qPCR were performed to determine the diversity and abundance of ARGs in the soil samples. Metagenomics was also utilized to characterize the microbial community and MGEs in GSI. This dataset along with the soil quality and site information, was used to determine environmental drivers of ARG diversity through a distance-based redundancy analysis.

2. Methods

2.1. Sampling locations and collection

In total, 24 GSI sites and one reference site were selected for sampling. The GSI sites were located in the metropolitan area of Milwaukee, Wisconsin, USA (**Fig. S.1**). Four wetlands (W1 – W4), six bioretention cells (R1 – R6), and fourteen bioswales (S1 – S14) were included in this study. Bioretention, bioswale, and constructed wetland were chosen as the types of GSI to be sampled due to their widespread installation, relevance in microbiology research, and distinct design characteristics [10,37,51,52]. Specifically, bioretention cells employ engineered soils and vegetation within shallow depressions or basins to capture and treat

stormwater [36,52,73]. In contrast, bioswales utilize vegetated channels to convey and filter runoff [28,90]. Constructed wetlands emulate natural wetland environments, employing aquatic plants and soils to facilitate comprehensive stormwater treatment through a combination of physical, chemical, and biological processes [5,77]. The surface area of the GSI sites ranged between 20 and 4861 m^2 and the drainage area ranged between 248 – 15,782 m^2 (**Table S.1, Fig. S.2**). The catchments were primarily comprised of impervious surfaces, such as streets, parking lots, and roofs, with an average imperviousness of 91.6%. In addition, the GSI sites ranged in age from 3 to 18 years since installation. Site specific characteristics and further information can be found in **Table S.1**. The reference site, Scuppernong Prairie, is a Wisconsin Department of Natural Resources natural protected area located in Eagle, Wisconsin, USA [71] (**Fig. S.1**). This site allowed for comparison between natural soil communities and those of engineered GSI. Moreover, it does not receive runoff from impervious surfaces, thus providing a comparison to a site not subjected to urban stormwater dynamics.

The 25 study locations were sampled in triplicate on one day over a four-week period in July and August of 2021 (**Table S.2**). At each site, triplicate surface soil samples (0 – 10 cm) were collected using a soil core; if necessary, plants, rocks, and other material covering the soil were removed prior to soil sampling. Approximately 60 g of soil were collected from each site and transported in Whirl-Pak® sample bags (Sigma Aldrich, St. Louis, MO., USA). All field equipment was cleaned in between sites according to the US EPA's Laboratory Services and Applied Science Division operating procedure for field equipment cleaning and decontamination [84]. This study took place in the summer months for this region, with average daily temperatures ranging between 21 – 29 °C (**Table S.2, Fig. S.3**). Rainfall events were recorded in between sampling days, but a minimum antecedent dry period of 4 days was maintained for all days that were sampled to ensure that the soils were in a similar dry condition (moisture content standard deviation = 11.5%) (**Table S.2, Fig. S.3**).

2.2. Soil quality analysis

Upon transport of the soil samples back to the lab, the soil was divided into two shares. Approximately 50 g of soil were weighed for a wet weight and then were dried overnight at 105 °C and weighed again. The dry soil was then ground through a 2-mm sieve with a mortar and pestle to homogenize the sample. Soil in between analyses was stored in the dark. 20 g of the dried soil was utilized to measure organic matter [34]. 10 g of the dried soil was utilized to measure pH and electrical conductivity [83] with Thermo Scientific Orion probes (Thermo Fisher Scientific, Waltham, MA) using a 1:5 soil to water ratio. 2 g of dried soil was further sieved through an 850-μm sieve for metal extraction via nitric acid (10%). Sorbed and exchangeable metals and cations were subsequently quantified by inductively coupled plasma mass spectrometry (ICP-MS) analysis [56,82]. Finally, 15 g of dried soil were used for soil texture analysis [40].

The remaining share of the original soil sample was utilized for nutrient and DNA extraction. To maintain the integrity of the DNA and nutrients, this portion of the soil was not dried [46]. Upon division of this soil from the first share, the soil was weighed, sieved through a UV sterilized 2-mm sieve, weighed again, and stored in a − 20 °C freezer until the analyses were to be completed. Phosphorus was measured according to the Mehlich III extractable elements method [23] and potassium chloride extraction with colorimetric measurement was used to measure ammonium concentrations in the sampled soils [21]. Three samples were collected from each GSI site to generate triplicate data for each site. The three samples were analyzed each one time for all soil quality properties; therefore, n = 3. All methods used for soil quality analysis are further detailed in the Supplemental Materials.

2.3. DNA extraction, metagenomic sequencing prep, and quantification of ARGs

DNA was extracted with the QIAGEN DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) from the soil cores collected from each sampling site. The manufacturer's protocol was followed for extraction and the vortex option was utilized for bead beating. The extracted DNA was used for both quantitative polymerase chain reaction (qPCR) and metagenomic sequencing. For sequencing, a composite sample of the extracts was prepared for each site by combining the triplicate samples by an equal DNA mass that was measured on an Invitrogen™ Qubit™ Flex Fluorometer (Thermo Fisher Scientific, Waltham, MA). For qPCR, four ARGs, *sul1, sul*2, *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. These genes were selected because of the frequency of their quantification and abundances reported in soil environments [15,35,44,80,94]. Additionally, the ARGs represent different resistance mechanisms, including antibiotic target replacement (*sul*1 and *sul*2) [85], antibiotic target protection (*tet*W) [16], and antibiotic target alteration (*erm*F) [2]. Genes were quantified according to previously published qPCR protocols [41–43]. Additional primer information is provided in **Table S.3**.

2.4. Metagenomic analysis and bioinformatics

Samples were sequenced at the SeqCenter (previously Microbial Genome Sequencing Center, Pittsburgh, PA) on the Illumina NextSeq 2000 platform (151-bp paired end) at a sequencing depth of 650 Mbp. The raw metagenomic sequencing data have been deposited in the Sequence Read Archive under accession number PRJNA1024991. The sequenced reads were first quality filtered using Trimmomatic (v0.40) to remove adaptors and low-quality sequences [7]. The quality filtered reads were utilized to assign taxonomy using MetaPhlAn v4.1 [6]. Following, reads were *de novo* assembled into contigs using metaSPAdes (v3.15.5) [60]. After each sample had its own assembly created using metaSPAdes, these assemblies were individually annotated using resistance gene identifier (RGI) v6.0.3 to discover ARGs [2], geNomad v1.7.0 to annotate plasmids and viruses [31], and Integron Finder v2 to annotate integrons [59]. To estimate abundances of annotated ARGs, quality-filtered reads were mapped to FASTA sequences from RGI output files using Kallisto (v0.50.0) [9]. Following, relative gene abundance was calculated for each ARG as the mapped reads per kilobase of gene length per million total reads (RPKM). Finally, a specific subset of ARGs were isolated for all further analyses; ARGs without direct clinical relevance, such as those that confer resistance through protein overexpression or rRNA mutations, were removed. To identify co-location between ARGs and MGEs, the contig name associated with each ARG from RGI, and each MGE from geNomad and Integron Finder were isolated within each specific sample's assembly. Therefore, co-location of ARG and MGEs from the same sample was determined by matching the ARG contig names with the MGE contig names from each sample's assembly. This determined if an ARG shared a contig with an MGE, or if it was by itself on the contig. This method can only determine co-location and does not provide any insight into whether or not an MGE sharing a contig with an ARG is flanking it or not.

2.5. Statistical analysis

All genes quantified via qPCR and soil quality parameters were measured in triplicate. Statistically significant relationships across sampling locations were evaluated with one-way analysis of variance (ANOVA) with the post hoc Tukey's multiple comparisons test. Significant relationships were assessed at a p-value$<$ 0.05. Gene relative abundances were determined by dividing the gene's absolute concentration by the 16S rRNA gene absolute concentration.

Multiple linear regression analysis was conducted to determine the

influence of environmental factors, such as GSI soil properties, catchment, and site characteristics, on the absolute and relative abundance of ARGs, *intI*1, and the 16S rRNA gene. As this objective was specific to GSI sites, the reference site was not included in this analysis. The specific system and catchment parameters inputted into the model and the modelling process are detailed in the Supplemental Materials [17,53].

Statistical analyses for metagenomic results were also performed in R (v.4.2.2). The package phyloseq (v.1.42.0) was used to calculate the alpha and beta diversities of the ARG, microbial community, and MGE datasets. The metrics utilized for the alpha and beta diversity analyses were the Shannon diversity index and Bray-Curtis dissimilarity index, respectively. Non-metric multidimensional scaling (NMDS) was further used to ordinate and plot the beta diversity dissimilarity distances. To assess statistical significance between samples an ANOVA test with the Tukey's post hoc multiple comparison test was applied for alpha diversity and the permutational multivariate analysis of variance (PERMANOVA) (999 permutations) test adonis2 (vegan package v.2.6-4) was applied to the Bray-Curtis distance matrices. Both were assessed at a significance level of p-values$\leq$ 0.05 [95].

Variance partitioning was used for this study to determine the degree to which different factors – environmental, MGE, and microbial community – explained the variance of ARG diversity at GSI sites. The GSI soil quality, catchment, and site characteristics were used as one explanatory factor, described collectively as "environmental factors". The MGEs and microbial community datasets were the other two explanatory factors in this analysis. Explanatory factors were standardized prior to analysis and the ARG diversity matrix was used as the response factor. The variance partitioning analysis was completed in R using the vegan package. To further investigate the specific contribution of environmental factors to the variance of ARGs across GSI sites, the individual environmental factors were modeled through a distance-based redundancy analysis (db-RDA) (vegan package). The process is detailed in the Supplemental Materials. All plots were created in either GraphPad Prism 7® (GraphPad Software, La Jolla, CA) or R (ggplot2 package v.3.4.1).

3. Results and discussion

3.1. GSI accumulate ARGs at elevated concentrations

The qPCR results revealed that GSI sites were hotspots for ARG sequestration. The relative abundance of all ARGs quantified was higher at each of the GSI sites compared to the reference site (Fig. 1 **and Table S.5**). This result was found despite a statistically ($p < 0.05$) greater biomass concentration at the reference site in comparison to all GSI systems, except bioswales 7 (S7) and 2 (S2) (**Fig. S.8 and Table S.4**). Therefore, the higher concentrations of ARG observed in the GSI systems cannot solely be attributed to the bacterial load of the soil. These results offer further evidence that ARGs accumulate in GSI soils at higher concentrations and occur more frequently within the microbial community compared to the rural and non-engineered soil samples studied in this work. This finding suggests that GSI soils may retain ARGs from stormwater runoff, potentially serving as a long-term reservoir for ARGs, thereby heightening the potential of ARB proliferation and dissemination into the urban water environment.

Across the GSI soils, the concentration of ARGs were found to vary depending on the type of GSI system. Specifically, bioretention cell sites had significantly ($p < 0.05$) higher levels of *sul*1, *sul*2, and *erm*F genes compared to bioswales and constructed wetland sites (**Fig. S.12**). The increased abundances of these ARGs in bioretention cells could be linked to differences in their design or function. For instance, bioretention cells are designed to maximize stormwater infiltration, which involves the use of specialized soils and vegetation to facilitate infiltration and could thus promote the removal of ARGs through adsorption to the soil media [73]. On the other hand, in some cases, bioswales are designed to convey sheet flow, potentially limiting the degree of infiltration and retention of ARGs compared to bioretention cells [22]. Furthermore, constructed wetlands are intentionally designed to replicate the functions of natural wetlands, placing a primary focus on ecological processes [77]. This ecological emphasis may, in turn, be limiting the engineered elements that promote ARG removal. In addition, the constructed wetlands selected for this study were the largest in terms of surface area, a

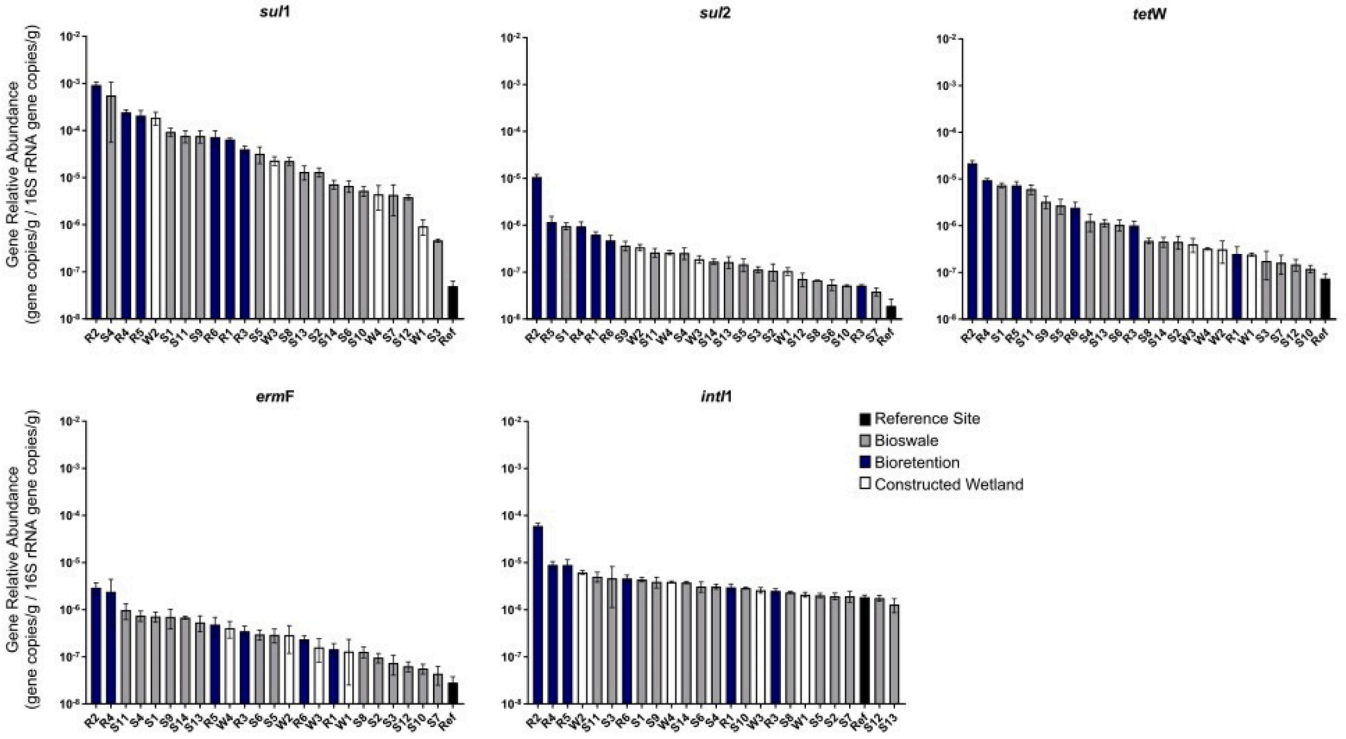

Fig. 1. Relative Abundance of the four ARGs and *intI*1 MGE quantified at 24 GSI sites and 1 reference site by qPCR.

characteristic that could have contributed to dispersed and irregular stormwater infiltration and ARG retention patterns throughout the site. This finding also contrasts with the results of a previous study by [35], which did not detect differences in ARG concentrations among various GSI locations [35]. That study though had a smaller sample size (n = 6).

From this analysis, bioretention cell 2 (R2) also emerged as a site of interest as it had the greatest relative abundance for each ARG and the *intI*1 gene. This could be attributed to selective pressures as site R2 had high concentrations of metals, specifically nickel and cobalt (**Fig. S.13**). However, this trend is not consistent across all sites; for example, R1 also had high metal concentrations but ARGs were not similarly abundant. This discrepancy suggests that, while metal concentrations may in part affect the prevalence of ARGs in GSI soils, other intricate factors are at play to contribute to the overall ARG abundance.

Environmental, site, and catchment characteristics were used as explanatory factors in a multiple linear regression analysis to predict the abundance of ARGs in GSI soils (**Table S.12**). The model indicated that the absolute and relative abundance of select ARGs in GSI can be predicted using environmental variables with the proportion of variance being explained (R^2-adjusted) falling between 0.419 and 0.862. Notably, heavy metals were not revealed as significant variables in the models, contrary to the results found in other GSI soils [66]. Rather, factors consistently identified in the models were percent catchment imperviousness of the drainage area, soil pH, and soil electrical conductivity.

Across the ARGs quantified, *sul*1 was the most abundant for every site. *intI*1, a class 1 integron-integrase gene, was the second most abundant gene (Fig. 1). *intI*1 was also strongly (Pearson r = 0.44 – 0.99) and significantly ($p < 0.05$) correlated with the relative abundances of all ARGs (**Table S.6**). The concentrations of ARGs in numerous environments have previously been found to correlate with *intI*1 concentrations and such a result has been used to suggest ARG mobility via integrons [32,35,54,87]. It is thus possible that ARGs are co-located on integrons in GSI soils. *intI*1 has also been suggested to be a marker for anthropogenic pollution in the environment [30]. In the GSI soils sampled for this study, where anthropogenic pollution is prevalent, the occurrence of *intI*1 reinforces this claim due to significant relationships with heavy metal concentrations, particularly zinc, as well as pH, calcium, and electrical conductivity (**Table S.7**). Additionally, in the multiple linear regression analysis catchment imperviousness was found to contribute to the variance of *intI*1 concentrations, implying that GSI design elements may also play a role in elevating pollution and resistance levels in the soil environments.

3.2. GSI harbor a diverse resistome that is not influenced by the type of GSI

The relative abundance of all ARGs annotated in the sample resistomes varied significantly across the GSI sites (Fig. 2**A**). In comparison to the reference site, all GSI sites – except for R6 – had a greater relative ARG abundance. Specifically, the RPKM of ARGs in the genomes of 15 of the 24 sites was 1.5 times or higher than the reference genome. Across the sites, however, only two ARGs were at all 25 sites: *van*W and *APH* (2″)-Ig. Moreover, there were no ARGs exclusively found in the GSI soil environments. The two ARGs that were at the most GSI sites (n = 20) and not the reference site were *van*Y and *bah*A. ARGs in GSI soils were generally unique to the individual systems. This could be in part the result of isolating specific ARG following annotation for analysis. Specifically, 54% of the genes retained were only found at one or two sites (**Fig. S.4 and S.5**). Therefore, the ARG removal step implemented in this work, which aimed to extract ARGs without direct clinical relevance, also allowed for unique distinctions in the ARG composition across the samples to be isolated.

ARGs were diverse in the GSI soils with the Shannon diversity index ranging from 2.68 to 5.06 (Fig. 2**B**). The Shannon diversity index is a function of the number of different ARGs and their relative abundance in a sample. The high diversity in GSI could be the result of more heterogeneous inputs to GSI as stormwater runoff could be modifying and introducing foreign resistance elements into the soil. Moreover, elevated diversity observed at certain sites may suggest an influence of individual site designs. In certain instances, like at wetland site 4 (W4) and bioretention site 5 (R5), the locations exhibited comparatively low Shannon diversity but high relative abundance (RPKM) values. This finding indicates the presence of a high number of ARGs, with fewer distinct types of ARGs. It's feasible that the difference in ARG diversity across the sites could be due to the adsorptive capacity of the soil media to capture and retain ARGs from influent stormwater [12], nutrient content to sustain microbial functioning [76], and presence of heavy metals at concentrations to promote antibiotic resistance [26]. In exploring this hypothesis, Pearson correlation analysis revealed significant correlations ($p < 0.05$) between ARG alpha diversity and clay content (r = 0.423), which supports the theory of ARG accumulation due to soil composition, as well as pH (0.404), and system age (−0.433) (**Table S.8**).

No difference in ARG beta diversity was found based on the type of GSI system (Fig. 3**A** PERMANOVA $p > 0.05$). This result falls contrary to the qPCR relative abundance data, suggesting that the treatment processes and design factors specific to each GSI type do not exert a substantial influence on the overall diversity of ARGs. Following, hierarchical clustering was utilized to determine which sites were similar in terms of ARG composition (**Fig. S.7**). Except for S10 and S12, all sites formed distinct clusters of mixed GSI types apart from the reference site. This underscores the likelihood that factors beyond GSI type, such as engineered soil composition, urban location, or exposure to stormwater, contribute to the divergence in GSI resistomes compared to those of native soil environments.

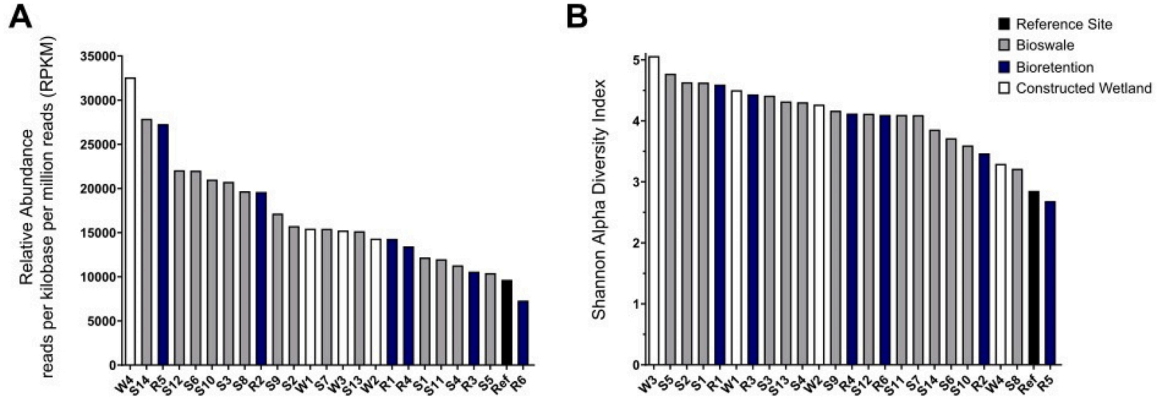

Fig. 2. Alpha Diversity; **A:** The number of ARG-like reads per kilobase million (RPKM) bacterial genome number; **B:** ARG Shannon alpha diversity index.

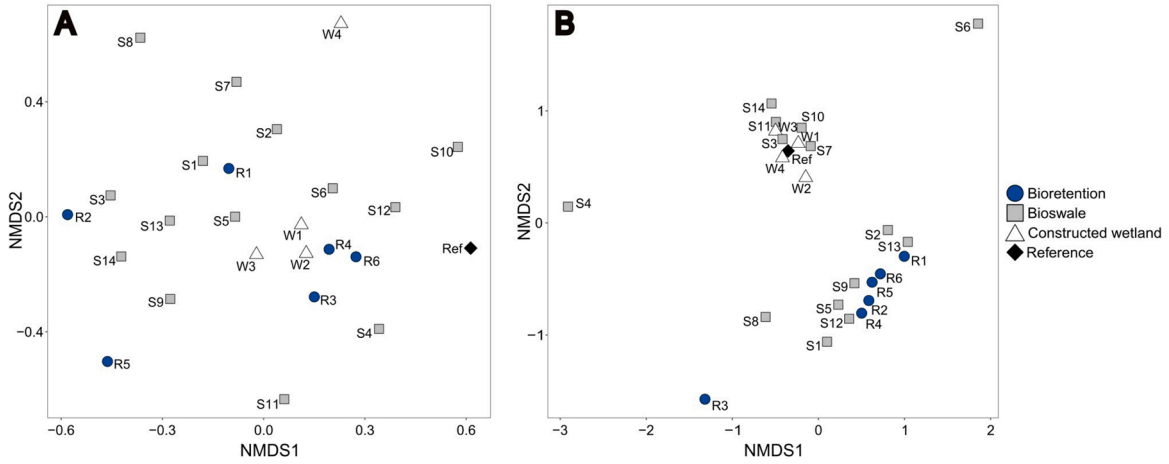

Fig. 3. Beta Diversity; **A:** Beta Diversity of ARGs at GSI sites and reference site. PERMANOVA indicates no statistical difference in ARG composition by type of GSI system ($p > 0.05$); **B:** Beta Diversity of the microbial community at the GSI sites and reference site. PERMANOVA indicates a statistical difference in community composition by type of GSI system ($p < 0.05$).

3.3. (Lack of) correlation between ARG and microbial community diversity in GSI

ARG beta diversity did not correlate to microbial community diversity (Fig. 3; Mantel test, $p > 0.05$). Moreover, correlation analysis conducted between ARG alpha diversity and microbial alpha diversity (Fig. 2**B** and **Fig. S.6**) also revealed no relationship within the GSI soil samples (**Table S.9**). This finding suggests that, unlike many other environments such as soil, water, urban sewage, sediments, biofilms, sludge, and fecal samples, where a strong and significant correlation between microbial community diversity and ARG diversity has been consistently observed [25,49,78], the diversity of the bacterial communities in GSI may not exert a substantial influence on the diversity of ARGs.

Within GSI soils, factors beyond the microbial community must be driving the composition of ARGs. Potential factors include MGEs and environmental conditions. Variations in environmental selection pressures, for example, could be forcing the microbial community in GSI to adapt to the prevailing environmental conditions, thereby favoring the proliferation of distinct ARGs. The lack of correlation between microbial and ARG diversity may also, to some extent, be attributed to the ARG removed prior to this analysis. The genes that were excluded had no direct clinical relevance, such as genes associated with global gene regulators, two component system proteins, and signaling mediators as well as general efflux pumps and genes encoding subunits that are part of multiple efflux pumps. Such genes may have had a stronger association with particular bacterial strains, making the remaining genes less a consequence of phylogenetic distinctions and more a reflection of the different environmental conditions prevalent at each site.

The diversity of microbial community was influenced by GSI type (Fig. 3**B**, PERMANOVA $p = 0.026$). This result is supported by other studies of GSI [10,28,29,51] wherein many different factors were found to contribute to the differences across the types of systems, including the plant species, soil composition, and hydrology. Plants, for instance, can influence the types of microbes present in GSI through altering the infiltration rates, promoting uptake of specific pollutants, and providing resources in root extrudates [58]. Furthermore, engineered soils used in GSI differ according to the specific system. Bioretention cells, for instance, typically have a layered soil profile consisting of sandy loam or loamy soils to support infiltration. In contrast, constructed wetlands often have more clayey soils to support wetland vegetation. This soil design can significantly enhance GSI performance, modulating the extent of water infiltration, nutrient retention, and pollutant removal [37]. These soil variations, in turn, can shape microbial communities, as certain bacterial species are better adapted to specific soil conditions, such as limited nutrient availability [20]. GSI design also largely impacts hydrologic functioning, specifically water retention and drainage. These functional traits can influence the composition and activity of microbial communities by exposing them to fluctuating moisture conditions and affecting the availability of oxygen. Bioswales and bioretention cells for instance, experience intermittent wetting and drying cycles, and, depending on the intensity and duration of the storm event, will temporarily retain water. Constructed wetlands though are designed to have submerged zones, leading to anaerobic and aerobic zones across the system. The varying bacterial communities across GSI system types therefore indicates that these differing design factors force the bacterial community to adapt to the specific environmental conditions, effectively creating distinct niche environments within the GSI soils.

3.4. ARGs Co-located with MGEs in GSI systems are driven by environmental factors

The percentage of ARGs at each GSI site that were identified as co-occurring with a MGE ranged from 3.27 – 8.83%, with the reference site having the second highest proportion of potentially mobilized ARGs (Fig. 4**A**). MGEs were further explored in this work to elucidate their role in shaping the composition of ARGs within GSI soils as the diversity analysis alluded to factors beyond the microbial community influencing the diversity of ARGs in GSI soils, and qPCR analysis revealed significant correlations between ARGs and the MGE *intI*1. The results, however, fall contrary to the expectation that there would be an increased occurrence of ARGs co-located on MGEs in GSI soils comparatively to the reference site [30].

It was also expected though that elevated concentrations of stormwater pollutants, including selective pressures, would increase ARG mobility [30]. This hypothesis is supported by correlation analysis (Pearson $r > 0.5$, $p < 0.05$), which indicated that sites with higher concentrations of arsenic, phosphorus, magnesium, and calcium also had a high ratio of mobilized ARGs (**Table S.10**). Correlation analysis was also conducted by GSI type, and different results were found. A higher ratio of mobilized ARGs in bioswales was correlated with soil texture ($r = 0.76$, $p < 0.05$), suggesting that the presence of mobilized ARGs is primarily on account of the site's adsorption capacity. Conversely, in bioretention cells and constructed wetlands a higher ratio was strongly ($r > 0.85$) and significantly ($p < 0.05$) correlated to soil properties (i.e., soil moisture, phosphorus, and arsenic) and pH, respectively. Therefore, in bioretention cells and constructed wetlands, mobilization of ARGs is more the result of soil condition and selective

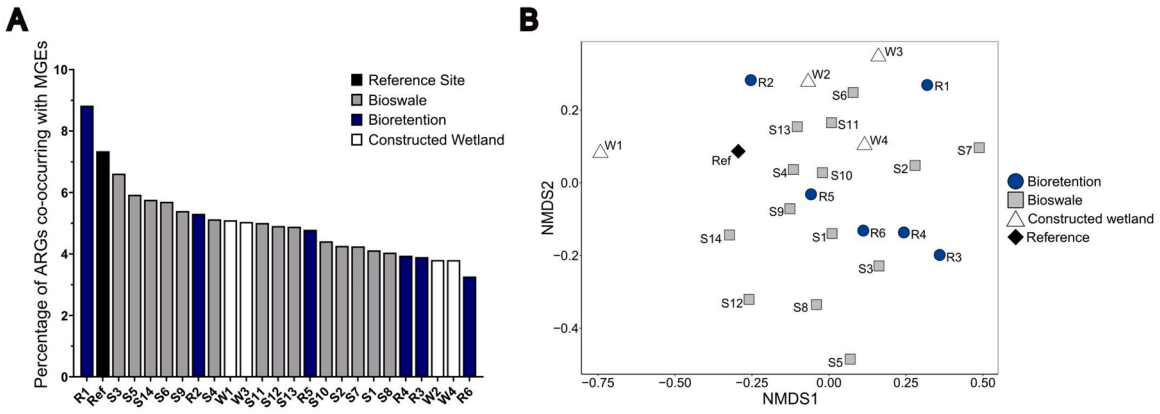

Fig. 4. Mobile Genetic Elements; **A**: From the total number of ARG annotated, the percentage of those ARGs co-occurring with MGE at each site; **B**: The beta diversity of the mobilized ARGs. PERMANOVA indicates no statistical difference in ARG composition by type of GSI system ($p > 0.05$).

pressures. The presence of MGEs carrying ARGs, moreover, have been demonstrated to provide a selective advantage upon bacteria harboring these elements [4]. As a result, it is possible that, in bioretention cells and constructed wetland environments, the accumulation of potentially toxic ions selects for ARGs and the dissemination of ARGs through MGEs. The diversity of the mobilized ARGs (Fig. 4**B**), however, was not significantly correlated (Mantel test, $p > 0.05$) to the overall ARGs diversity (Fig. 3**A**). Paired together, these findings suggest that, while mobilized ARGs can contribute to proliferation in GSI soils, they were not the primary diver contributing to the overall ARG variance observed in the GSI soils in this study.

3.5. *Environmental factors drive the diversity of ARGs in GSI*

Partitioning analysis indicated that environmental factors significantly influence ARG diversity, specifically accounting for 27.9% of the observed variance in ARGs (Fig. 5**A**). The analysis also confirmed that the microbial community minimally impacted ARG diversity as it was the factor that explained the least proportion of variance, accounting for only 8.7% of the observed variability. Following, MGEs explained 12.3% of the observed variation. Such results reveal that in GSI there is an interplay between environmental factors, the microbial community, and resistance elements that is creating a unique dynamic at each site leading to varying antibiotic resistance diversity. Moreover, this finding highlights the critical role that abiotic and design factors play in shaping the prevalence and distribution of ARGs within GSI soil environments. From this analysis, important insights into the dynamics shaping ARGs in GSI were confirmed, however a notable portion of the variance—-specifically, 51.1%—remains unexplained. This unexplained variance suggests the presence of additional factors or interactions that have yet to be accounted for in our current model and highlights the need for further research.

The environmental factors were individually explored to elucidate their impact on ARG diversity; copper, catchment imperviousness, phosphorus, magnesium, and calcium were identified as significant factors contributing to the model. A db-RDA was utilized for this analysis, but prior to modelling all factors were summarized into PCA axes to reduce collinearity in the model (**Fig. S.9 and Table S.11**). The db-RDA modelling indicated that four of the PCAs contributed to a significant model ($p < 0.05$) (Fig. 5**B**). To interpret this result, correlation analyses

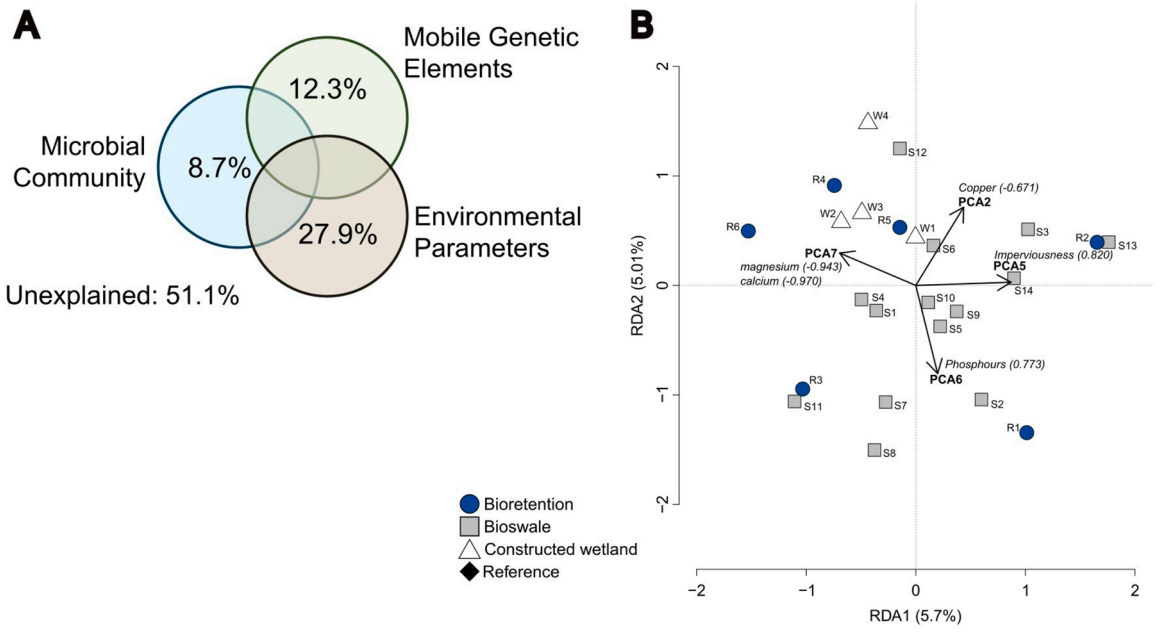

Fig. 5. Variance Partitioning; **A**: The results of the variance partitioning analysis, values in Venn diagram are adjusted R^2; **B**: RDA results; model p-value = 0.039 and $R^2 = 0.189$.

were completed between the PCA axes identified and the environmental factors summarized by each PCA (shown on Fig. 5**B**). The correlation analyses revealed the factors that primarily drove the diversity of ARGs in GSI soils: copper (PCA2, $r = -0.671$), catchment imperviousness (PCA5, $r = 0.820$), phosphorus (PCA6, $r = 0.773$), magnesium (PCA7, $r = -0.943$) and calcium (PCA7, $r = -0.970$).

Two of the factors identified, metals and catchment imperviousness, were initially hypothesized to influence ARG diversity. The rationale for this hypothesis was based on the understanding that metals are a known selective pressure for the maintenance of ARGs and are prevalent in urban runoff (Q. [86]). Copper concentrations were found to be negatively correlated with ARG diversity (Fig. 5). This relationship can be explained by high copper concentrations having a bactericidal effect on the community, leading to a reduction in overall diversity, while lower copper concentrations can induce stress and HGT among the bacterial population [86,93]. This heightened HGT activity under lower copper stress can contribute to an increase in ARG diversity. Catchment imperviousness on the other hand, was positively correlated to ARG diversity. This finding reveals that GSI located in proximity to impervious surfaces such as parking lots and roofs tend to accumulate a more diverse resistome. This outcome can be attributed to the fact that impervious surfaces accumulate more dynamic and varied pollutants as well as yield increased runoff volumes [14,88]. Consequently, GSI located in such areas may be exposed to a wider array of environmental stressors, potentially fostering a greater diversity of antibiotic resistance mechanisms.

The factors identified in the model that were not anticipated to be influential were phosphorus as well as magnesium and calcium. Phosphorus is an abundant pollutant in stormwater runoff, and GSI soils are regularly designed to capture and retain nutrients, including phosphorus, to mitigate downstream environmental impacts [65]. Consequently, GSI soils tend to have elevated nutrient concentrations [81]. Phosphorus also plays a pivotal role as an essential nutrient supporting a healthy microbial community. Increased phosphorus concentrations, in combination with selective pressures like metals, could thus facilitate the selective growth of ARB, increasing the overall diversity of the resistome in GSI. Magnesium and calcium are also widespread stormwater pollutants, typically associated with road salts. Road salts, in this case magnesium chloride and calcium chloride, are a significant environmental concern in regions where they are applied to impervious surfaces during the winter months to combat ice buildup caused by winter storms and cold temperatures [38,47]. Even though these samples were collected in the summer months, salts have been shown to persist in water bodies, even being documented in urban streams at concentrations exceeding regulatory chloride thresholds in summer months [47,79]. In microbial communities, salinity, associated with magnesium and calcium ions, is a known selective pressure as elevated salt concentrations can induce intracellular osmotic stress and trigger the SOS responses [91]. As observed with heavy metals, genes conferring tolerance to salt stress have been found to co-occur with ARGs, and consequently, salinity can promote the propagation of ARGs through co-resistance mechanisms [91]. Consequently, while the diversity of the microbial community was found to not significantly impact ARG diversity, this analysis suggests that ARG diversity is impacted by the response of a microbial community to environmental stressors. Furthermore, the impact of salinity on ARGs within GSI is supported by the results of the ARG abundance regression analysis (**Table S.12**). In this analysis, pH, conductivity, and calcium were all correlated with ARG abundances. Notably, these correlations displayed a negative trend, implying that high salt concentrations are toxic to the microbial community, limiting ARG abundance and diversity. Lower salt concentrations though can induce stress within the microbial community, leading to both an increase in ARG abundance and diversity.

3.6. Implications and future directions

In assessing the risk associated with the environmental presence of antibiotic resistance, there is a need to examine the role played by GSI in potentially exacerbating this public health concern. This study specifically investigated two critical aspects: GSI's role in accumulating diverse and abundant ARGs from stormwater and its potential to accelerate the dissemination of resistance within the microbial community inhabiting GSI soils. This work concludes that GSI soils harbor a diverse and abundant resistome, above what is found in native, non-engineered soils, and that heavy metals, nutrients, and road salts, are the factors influencing this diversity in GSI environments.

The overarching implications of GSI being a hotspot for antibiotic resistance involves a potential cycle wherein ARGs from stormwater are temporarily retained within GSI soils, environmental stressors trigger HGT, and following, a diverse and abundant community of ARB are mobilized back into the environment during subsequent rainfall events. This phenomenon could make GSI soils a significant source of antibiotic resistance within the urban water cycle. Future research will be required to confirm these potential consequences, including monitoring GSIs across a longer time scale. This research though does highlight key factors to be considered in GSI design when assessing the potential accumulation of ARGs in GSI soils. Factors such as the type of GSI, for instance, can increase the retention of ARGs from stormwater through design that prioritizes stormwater infiltration mechanisms, such as soil composition and plant diversity. Site characteristics were also identified as influencing factors, including catchment area imperviousness, which indicates that the placement of GSI in an urban landscape can impact the ARG diversity in runoff. Furthermore, this research revealed that heavy metals, nutrients, and road salt concentrations in GSI can be used as indicative markers for the stress being placed on the microbial community, potentially driving selective HGT. Minimum selective concentration experiments will need to be conducted to determine the levels at which metals and road salts induce stress. These data would assist in identifying high risk GSI environments for ARG dissemination and mobilization and offers guidance for optimizing GSI design to mitigate antibiotic resistance proliferation and protect the surrounding environment.

4. Conclusions

This was the first research to investigate the resistome of GSI. The main objective was to characterize the resistome of GSI while also determining if GSI is a hotspot for antibiotic resistance. This research led to the following conclusions:

1. GSI soils are hotspots for ARGs accumulation as the concentrations of ARGs were greater in GSI than a nonengineered soil environment
2. ARG concentrations are influenced by GSI type as ARG concentrations differed based on the type of system (i.e., bioswale, bioretention cells, and constructed wetland)
3. GSI soils harbor a diverse resistome that is not influenced by GSI type
4. No relationship was observed between the diversity of the microbial community and ARGs, indicating that the diversity of the microbial community had minimal influence over the diversity of ARGs in GSI
5. ARGs were not co-located on MGEs in GSI above what was observed in native, un-engineered soil
6. Environmental factors, including copper, catchment imperviousness, phosphorus, magnesium, and calcium, significantly shaped the diversity of ARGs in GSI

This research demonstrates that GSI soils act as significant reservoirs for ARGs. For this work only one non-GSI site was sampled, and therefore, future work should seek to compare GSI soils to further non-engineered soil environments. It was further determined that the type of GSI is driving ARG accumulation, indicating the possibility of

optimizing the engineered aspects of GSI to effectively control ARGs in stormwater runoff. The study also reveals that environmental factors play crucial roles in shaping the resistome diversity within these systems. While catchment imperviousness, metals, nutrients, and salts were identified as influential factors, the complex interplay among these elements, microbial communities, and MGEs contributes to the observed variation in ARG diversity in GSI soils. This work highlights the importance of considering GSI systems as potential hotspots for ARGs and emphasizes the need for further research to develop strategies for managing antibiotic resistance in urban environments.

Environmental implication

Green Stormwater Infrastructure (GSI) soils are environmentally relevant because of their potential role as reservoirs for hazardous materials, specifically antibiotic resistance genes (ARGs). In this research, GSI sites were identified as hotspots for ARG sequestration. Moreover, the diversity in antibiotic resistomes was influenced by environmental parameters including heavy metal, nutrients, and salt concentrations. The accumulation of ARGs in GSI soils raises concerns about the environmental dissemination and proliferation of antibiotic resistance throughout the urban water cycle. Understanding ARG dynamics in GSI is crucial for informing design decisions to leverage GSI as a tool to mitigate the spread of antibiotic resistance.

CRediT authorship contribution statement

Walter McDonald: Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Conceptualization. **Numair Ali:** Methodology, Formal analysis, Data curation. **Christopher Marshall:** Writing – review & editing, Visualization, Methodology, Formal analysis, Data curation. **Patrick McNamara:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Conceptualization. **Thuy "Duyen" Lam:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Emily Lou LaMartina:** Methodology, Formal analysis, Data curation. **Kassidy O'Malley:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.133923.

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