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Sediment metagenomics reveals the impacts of poultry industry wastewater on antibiotic resistance and nitrogen cycling genes in tidal creek ecosystems

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HIGHLIGHTS

• Poultry processing plant wastewater may spread antibiotic resistance genes (ARGs).
• Sediment metagenomes from an impacted and a reference tidal creek were compared.
• Higher diversity and abundance of ARGs in the impacted creek was detected.
• A link between ARGs and N cycle genes was also investigated in creek sediments.
• A nitrogen retention gene (nrfA) was strongly correlated to the abundance of ARGs.

ABSTRACT

The intensification of the poultry industry may lead to the increased spread of antibiotic resistance genes (ARGs) in the environment. However, the impacts of wastewater discharge from poultry processing plants on the sediment resistome are relatively unexplored. Furthermore, its relationships with important biogeochemical pathways, such as the N cycle, are virtually unknown. The overall objective of this study was to examine the abundance and diversity of antibiotic resistance and N cycling genes in sediment microbial communities impacted by poultry industry wastewater. We performed a metagenomic investigation of sediments in an impacted and a reference tidal creek. We also quantified the abundance of the clinical class 1 integron-integrase gene (intI1) through qPCR as a secondary marker of anthropogenic contamination. Abundance and diversity of ARGs were substantially higher in the impacted tidal creek, especially near the wastewater discharge. Abundances of ARGs conferring resistance to macrolides, tetracyclines, and streptogramins were also higher in the impacted creek than the reference creek. From the N cycling genes detected in the metagenomes, nrfA, the genetic marker for dissimilatory nitrate reduction to ammonia (DNRA), had the strongest positive relationship with the total abundance of ARGs, which may indicate an increased potential of eutrophication in ARG-impacted ecosystems due to nitrogen retention. This study demonstrates that wastewater discharge from a poultry processing plant can increase the spread of ARGs, which may result in negative impacts on ecosystem health.

1. Introduction

Antibiotics are important pharmaceutical drugs used to protect humans and other animals against infectious diseases caused by microbial organisms. Their increased production and consumption in the past decades, however, have led to increased environmental concentrations in aquatic
et al., 2011; Liu et al., 2018; Rowe et al., 2016; Wu et al., 2017). The nitrogen (N) cycle is of great relevance in the environment as it plays major roles. The nitrogen (N) cycle is of great relevance in the environment ecosystem functions and metabolic cycles that play major environmental roles. Since N is an essential element of all biological molecules and has a crucial role in regulating ecosystem productivity. Through an intricate network of oxidation-reduction reactions (Kuypers et al., 2018), microbial communities transform inorganic N in both assimilatory and dissimilatory pathways. Atmospheric N2 is reduced to ammonia (NH3) through the activity of nitrogenases in the N-fixation process, which is particularly important in oligotrophic sites (Andersson et al., 2014). Non-assimilated NH3 can then be aerobically or anaerobically oxidized through nitrification (oxidation of NH4+ to NO2− and NO3−) or ammonox (the reduction of NO2− with NH4+ with the production of N2H5+ respectively. Nitrate (NO3−) or nitrite (NO2−) can then be step-wise reduced to N2O and N2 through denitrification, a crucial nitrogen removal pathway in coastal environments. Dissimilatory nitrate reduction to ammonia (DNRA) competes with denitrification for nitrate/nitrite reduction but its end-products are radically different. While DNRA leads to fixed-N retention (via NH4+), denitrification leads to N loss/removal from the environment (via N2O or N2). It is thus expected that the DNRA/denitrification ratio is important for ecosystem health, being frequently investigated in the literature (Burglin and Hamilton, 2007). Due to the importance of N cycling reactions and, in the context of ARGs contamination, it is important to investigate the potential relationships between ARG and N cycling genes and evaluate whether certain pathways are favored by high or low levels of ARG detection as a proxy of antibiotic contamination. For instance, if the presence of ARG correlates negatively with denitrification, this could present a 2-fold environmental health risk of both ARG spreading and low N removal. Metagenomic approaches are particularly suitable to investigate these relationships since the whole genomic content of all microorganisms in the sample is analyzed with the same technology. Despite their importance, the abundance relationships between ARG and N cycling genes remain largely unknown, especially in tidal estuarine ecosystems.

The objectives of this study were to (1) investigate the antibiotic resistome structure of sediment microbial communities impacted by poultry industry wastewater, and (2) evaluate the relationships between nitrogen cycling potential and the resistome of sediment microbial communities. To achieve these goals, we performed a metagenomic investigation of estuarine sediments collected during a field survey on two tidal creeks differently impacted by the poultry industry. We also quantified the abundance of the clinical class 1 integron-integrase gene (intI1) through qPCR as a secondary genetic marker of anthropogenic contamination and antibiotic exposure (Gillings et al., 2015).

2. Material and methods

2.1. Field survey and sediment sampling

The tidal creeks selected for this study are located in the Virginia Eastern Shore (VAES) of the Chesapeake Bay, USA, and were characterized in our previous study (Semedo and Song, 2020). Briefly, a field comparison was performed between two tidal creeks. Parker Creek, here referred to as the impacted creek, was selected because it receives the direct discharge of treated wastewater from a poultry processing plant. Nickawampus Creek, here referred to as the reference creek, does not receive the direct discharge from any processing plant. Three stations were sampled along a sediment transect of the two creeks: Upstream (U), Midstream (M), and Downstream (D) (Fig. S1). The upstream station in the impacted creek is the closest to the processing plant discharge (between 1 and 3 km) and the downstream furthest (around 5 km). In the reference creek, the three stations sampled targeted a similar salinity and distance gradient as in the impacted creek. A seasonal sampling was performed and a subset of two seasons was selected for this metagenome study: May and September 2017. At each station, three replicate sediment samples were collected with a push core device attached to core tubes (8 dia. × 30 cm length). The top 3 cm of each sediment core were homogenized and stored at −80 °C before molecular analysis. The bottom water nutrient concentrations and physicochemical characteristics of sampled tidal creeks were previously reported and can be seen in Semedo and Song (2020).

A better understanding of ARGs potential spreading from WWTP, animal processing facilities, or agricultural activities, is important for human health due to the potential risk of reaching pathogenic bacteria. Besides that, in the context of global increase in ARG environmental contamination, it is important for environmental health to understand potential links to ecosystem functions and metabolic cycles that play major environmental roles. The nitrogen (N) cycle is of great relevance in the environment.
2.2. Metagenome sequencing and functional annotation

Two replicate cores from each station in each season were used for metagenome analysis through shotgun sequencing. Genomic DNA was extracted from 0.5 to 0.75 g of sediment using the PowerLyzer PowerSoil DNA Isolation kit (MoBio), as previously described. (Semedo and Song, 2020) Purified genomic DNA was sequenced by Novogene Corporation (CA, USA). Libraries were prepared using the Illumina Nextera XT Kit with an input of 1 ng of DNA per library. The average DNA insert was approximately 350 bp. Nextera adapters were ligated on to the libraries and sequenced on an Illumina HiSeq platform (2 × 150 bp). Approximately 4 Gb (Giga base pairs) of data were generated for each sample.

The raw metagenome sequences were initially trimmed with Trimmomatic V0.33 to remove adapter sequences, short reads (<36 bp), and reads with an average quality score below 15 within 4-base windows (Bolger et al., 2014). De novo assemblies of each sample metagenome were carried out using MEGAHIT V1.1 (Li et al., 2016, 2015), with the meta-large preset and minimum contig length of 500 bp. The resulting assembled contigs were assigned to gene calls and protein-coding genes with PROKKA (Seemann, 2014), using the metagenome preset settings. To estimate gene abundance, the quality-filtered reads were mapped back to the contigs with bowtie2 (Langmead and Salzberg, 2012), using the local alignment mode while allowing for 1 base mismatch. The number of reads mapped to the target genes of this study (antibiotic resistance and nitrogen cycling genes) was counted for each metagenome using SAMtools and reads with an average quality score below 15 within 4-base windows.

To estimate gene abundance, the quality-filtered reads were mapped back to the contigs with bowtie2 (Langmead and Salzberg, 2012), using the local alignment mode while allowing for 1 base mismatch. The number of reads mapped to the target genes of this study (antibiotic resistance and nitrogen cycling genes) was counted for each metagenome using SAMtools (Danecek et al., 2020).

To allow for gene-to-gene comparisons, the number of mapped reads to each gene was normalized by gene length (obtained from the UniProtKB) to the reviewed (Swiss-Prot) manually annotated UniProtKB database. To normalize ARG richness estimates, PROKKA gene calls were also randomly subsampled to the minimum number of gene calls in a sample of this dataset.

To further explore the relationships between N cycling genes’ presence and the detection of ARGs in the metagenomes, metagenomic binning was performed in merged sequence libraries from each location. Quality-trimmed sequence libraries from each station from each creek were merged and co-assemblies with MEGAHIT V1.2.9 (Li et al., 2016, 2015) and contig binning was performed with MetaBAT v1.7 (Kang et al., 2015). The CheckM software (Parks et al., 2015) was used to keep only medium- and high-quality metagenome assembled genomes (MAGs), according to the following published criteria: >90% and 50% completeness and <5% and 10% of contamination for high- and medium-quality MAGs, respectively (Bowers et al., 2017). The obtained MAGs were functionally annotated using PROKKA, as described above, and taxonomy was inferred by using the GTDB-Tk v1.7.0 (Chausseil et al., 2020). The metagenome binning workflow was performed using the Kbase environment (Arkin et al., 2018).

2.3. qPCR of clinical class 1 integron-integrase (intI1) and total bacteria (16S)

The prevalence of anthropogenic pollution in the sediments was quantified by quantitative polymerase chain reaction (qPCR) of clinical class 1 integron-integrase gene, intI1, in the extracted DNA standards. Extraction was performed through a serial dilution of M13 PCR products from plasmids carrying the target gene and quantified using an Agilent 220 TapeStation System (Agilent Technologies). The primers intI1F and intI1R476 were used to generate 311 bp amplicons (Gillings et al., 2015). The 20 μL qPCR reactions for intI1 quantification consisted of 10 μL of SYBR green Go-Taq qPCR Master Mix (Promega), 0.05 μL of CRX dye, 0.25 μL of each primer (10 μM), 0.2 μL of bovine serum albumin (BSA), 2 ng of template DNA, and were adjusted to final volume with nuclease-free H2O. The qPCR conditions for intI1 quantification were the following: 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 62 °C, 60 s at 72 °C, and 3 s at 80 °C for fluorescence detection. Amplification efficiency was 78% and the R² value of the standard curve was 0.97. All reactions were performed in 96 well plates with two negative controls, which contained no template DNA, to exclude any potential contamination. Reaction specificity was confirmed using gel electrophoresis in comparison with standards and monitored by analysis of dissociation curves during quantification amplification. The relative abundance of the intI1 gene was normalized against total bacterial abundance, estimated by qPCR of the 16S rRNA gene. The primers used for 16S rRNA gene amplification were EU341F and 68SR and the qPCR components and thermal cycling conditions were the same as previously described (Semedo et al., 2018).

2.4. Statistical analysis

A principal coordinate analysis (PCoA) was performed to evaluate dissimilarity of ARGs structure using Bray-Curtis distances between samples with the phyloseq package in R (McMurdie and Holmes, 2013). Significant effects of creek, location, and month in gene structure dissimilarity were tested by permutational ANOVA (PERMANOVA) using the adonis function of the vegan package in R (Oksanen et al., 2017). All PERMANOVA models, either including only creek, only location, or only month, and the different combinations of the three factors were compared with the Akaike Information Criterion (AIC) and the model with lowest corrected AIC and highest weight was used for subsequent analysis and interpretation. A one-way ANOVA was also performed to test for significant differences of samples dissimilarity distance (Bray-Curtis) between sampling location (upstream, midstream, downstream). Differences in the relative abundance of the intI1 gene between impacted and reference creeks at each month were analyzed using two-way ANOVAs. The factors included in the ANOVA were creek (categorical; reference or impacted) and location (categorical; upstream, midstream, downstream). Tukey’s-HSD test was used to perform multiple comparisons between groups. Normality and homoscedasticity
were assessed with Q-Q plots and residual plots. Int1 relative abundance was log-transformed to meet ANOVA assumptions. Significant relationships for all tests were considered at \( \alpha < 0.05 \). All statistical analyses were conducted in R (version 3.2.2. Copyright 2015 The R Foundation for Statistical Computing).

3. Results

3.1. Antibiotic resistance genes (ARGs) in sediment metagenomes

An average of 25,087,219 sequences per sample was obtained for contig assembly and functional annotation following quality screening and removal of adapter sequences (Table S1). From an average of 168,413 contigs per sample, 130,354 gene calls were detected, on average, representing an average of 5000 unique genes in each sample.

After subsampling all samples to the same number of gene calls (39,017) to allow for normalized comparisons of ARG diversity between samples, we identified 35 different genes conferring resistance to antibiotics among all samples. A PCoA, representing the dissimilarity between the samples based on the relative abundance of the antibiotic resistance genes (ARGs), is presented in Fig. 1.

The first two principal coordinates represented 51.6 % of the variation in the resistome structure of these communities. The best PERMANOVA model, selected through an AICc model comparison, included creek only as of the predictor variable (Table S2), despite the low explanatory power of the model, selected through an AICc model comparison, included creek only as of the predictor variable (Table S2), despite the low explanatory power of the model. The upstream stations from the impacted creek cluster further apart from the corresponding stations in the reference creek than the midstream or downstream stations, indicating a more distinct resistome structure at the upstream station, near the wastewater discharge of the poultry processing plant. In fact, if we calculate the Bray-Curtis distances between the two creeks, these are significantly higher between the upstream stations, when compared to the midstream and downstream stations (Fig. S2).

Among the 35 different ARGs detected in this study, 9 were found exclusively in the impacted creek, 3 exclusively in the reference creek, and 23 detected in both creeks. The difference between the creeks is more obvious at the upstream stations, where the number of ARGs found exclusively in the impacted creek (\( n_{\text{ARG}} = 11 \)) was around 6 times higher than the corresponding number in the reference creek (\( n_{\text{ARG}} = 2 \)) and even exceeded the number of genes present in both creeks (\( n_{\text{ARG}} = 10 \) (Fig. 2). These differences are dissipated in the midstream and downstream stations, where the number of shared genes between the two creeks is always higher than the genes found exclusively in the impacted creek. When considering ARGs associated with individual compounds, it is worth noticing the diversity of tetracycline resistance genes exclusively found in the impacted creek, from classes B, D, and E.

The average genomic copy number of total ARGs at the upstream station in the impacted creek was five times higher than in the reference creek (Fig. 3). Due to the large variability observed and limited sample size, however, this difference was not considered to be significant (t-test). Nevertheless, it is important to note that virtually every single ARG had their average abundance higher in the impacted creek than in the reference creek, at the upstream stations (with the only exception of extended-spectrum beta-lactamase PER-1).

At the midstream and downstream stations, the difference between the two creeks weakens and the distribution of ARGs becomes more similar between reference and impacted creeks. The most abundant ARGs in both creeks and across all stations were the macrolide export ATP-binding permease protein (MacB) and macrolide export protein (MacA), with combined average abundances of 2.37 and 1.17 average genomic copy numbers in the impacted and reference creek, respectively. At the upstream stations, the difference between the two creeks was larger, with a combined average

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**Fig. 1.** Principal Coordinate Analysis (PCoA) plot representing the dissimilarity in the normalized abundance of antibiotic resistance genes in the sediments collected from the reference (blue) and the impacted (red) creeks. Upstream (circles), midstream (squares), and downstream (triangles) samples from May and September are shown. Sample dissimilarity and distance analysis was calculated using the Bray-Curtis dissimilarity index.
abundance of 2.72 and 0.98 in the impacted and reference creek, respectively. When considering the resistance to tetracycline, one of the most commonly used antibiotics in the animal industry, we found an average genomic copy number of 0.184 in the impacted creek and 0.016 in the reference creek. The tetracycline genes identified belong to classes A, B, C, D, E, G, H, and O of tetracycline resistance determinants. The most abundant was class C, with an average genomic copy number of 0.347 in the impacted creek and 0.069 in the reference creek. We also found three genes conferring resistance to virginiamycin, a streptogramin commonly used in the animal industry as a growth promoter and therapeutic drug: virginiamycin A acetyltransferase, virginiamycin B lyase, and streptogramin A acetyltransferase. The average genomic copy number of these genes was 0.109 in the impacted creek and 0.027 in the reference creek.

3.2. Abundance of clinical class 1 integron-integrase gene (intI1)

Relative abundance of the clinical class 1 integron-integrase gene (intI1) determined by qPCR assays is shown in Fig. 4. The intI1 relative abundance in the reference creek ranged from 0.013 to 0.041 gene copies/16S gene copies without any monthly or spatial trend. In the impacted creek, the intI1 relative abundance ranged from 0.015 to 0.235 gene copies/16S gene copies with a clear spatial trend. In both sampling months, the highest abundances in the impacted creek were observed at the upstream station, closest to the wastewater discharge from the processing plant. The relative abundance of this gene at the upstream station in the impacted creek was on average 6 times higher than in the reference creek. In September, the difference between the two creeks was also observed in the midstream stations.

3.3. Abundance of N cycling genes in sediment metagenomes

The average genomic copy number of all N cycling genes found in the studied sediments is shown in Fig. 5. The estimated abundance of nitrate reduction genes, narG and napA, was not different, in general, between the two creeks. The normalized abundance of nrfA, the marker gene for dissimilatory nitrate reduction to ammonium (DNRA), was approximately four times higher in the impacted creek than in the reference creek across all stations sampled. Regarding denitrification, which competes with DNRA for dissimilatory nitrate/nitrite reduction, the trend is less clear. Despite occasionally higher mean abundances in the impacted creek (e.g. nirS and nosZ at the downstream location), the variability is high and no substantial differences were observed for nirS, nirK, norB, or nosZ genes. The combined observations of increased DNRA genetic potential and no consistent changes in denitrification potential indicates that the impacted creek is more prone to reactive N retention than removal when compared to the reference creek.

When considering ammonium oxidation pathways, we detected genes from both aerobic (nitrification) and anaerobic (anammox) pathways. The hydroxylamine oxidoreductase gene (hao) was the only gene found for nitrification and its abundance was five and 50 times lower in the impacted creek, when compared to the reference creek, at the upstream and midstream stations, respectively. In the downstream stations, there was...
Fig. 3. Normalized abundance of antibiotic resistance genes in assembled metagenomes from reference (blue) and impacted (red) sediments. Bars represent mean ± SE of four cores (2 replicates × 2 months) in each location (upstream, midstream, or downstream). The abundance is expressed as the average genomic copy number to normalize for gene length and sequencing depth, as described in the Material and methods section. The genes are vertically ordered by decreasing abundances. The lower panel (total ARGs) represents the sum of all genes normalized abundance in each sample.

Fig. 4. Relative abundance of clinical class 1 integron-integrase (intI1) gene in the reference (blue) and the impacted (red) creek sediments. Bars represent mean ± SE of triplicate cores. Significant difference between the two creeks at each station are marked with * (2-way ANOVA, p < 0.05).
no detected difference between the two creeks. Regarding anammox, no apparent differences between the two creeks were detected.

The genetic potential for nitrogen fixation, quantified by the normalized abundance of the nitrogenase iron protein gene (nifH), was apparently lower in the impacted creek than in the reference creek. In fact, this gene was exclusively detected in the reference reek, at the upstream and midstream stations.

### 3.4. Relationship between the resistome and N cycling genes

The relationship between the normalized abundance of the N cycle and antibiotic resistance genes is shown in Fig. 6. Between the two genes encoding for nitrate reduction to nitrite, narG genes showed a positive and significant correlation with total ARG abundance, while napA showed no significant correlation. However, it is worth saying that the observed positive correlation between narG and total ARGs may be biased due to the presence of a single sample with extremely high ARG and narG normalized abundances (Fig. 6). Among the denitrification genes quantified, narB and nosZ were significantly and positively correlated with total ARG abundance while the nitrite reduction genes (nirS and nirK) did not present a significant correlation. On the other hand, nrfA, responsible for nitrite reduction in DNRA was significantly and positively correlated with the total ARGs abundance in the studied sediments.

Regarding ammonia oxidation (aerobic and anaerobic) and N fixation genes, no significant correlations with total ARGs were observed. However, it is worth noticing a negative spearman correlation coefficient of hao (nitrification) with total ARGs, indicating that lower genetic potential for nitrification was observed in samples where total ARGs were highest, which occurred mostly at the upstream station of the impacted creek.

To further explore whether the significant correlations observed between N cycling genes and ARGs were driven by genes occurring in the same genomes and eventual co-selection, we performed contig binning from the metagenomic reads (Fig. 7). Similarly to the normalized abundances shown above, the metagenome assembled genomes (MAGs) with the highest number of ARGs were found at the upstream station of the impacted creek. At this location, the mean ARG count per MAG is 3.95 in the impacted creek and 0.67 in the reference creek. At the midstream station is 2.67 and 2.42 in the impacted and reference creeks, respectively, while at the downstream stations is 1.86 and 2.00. Regarding the taxonomic classification of high-ARG containing MAGs (with 3 or more ARGs), we observed a wide range of taxa, but Gamma-proteobacteria consistently had a representative MAG with the higher number of ARGs at each station from both creeks.

The significant correlation observed between normalized abundance of ARGs and nrfA gene (Fig. 6) did not translate into a clear co-occurrence pattern in MAGs, i.e. high-ARG containing MAGs were not always carrying the nrfA gene and this gene was also present in low-ARG containing MAGs (Fig. 7). In total, we found 31 high-ARG containing MAGs (with 3 or more ARGs) and 25 low-ARG containing MAGs (with <3 ARGs). The nrfA gene was present in 23 % of the high-ARG containing MAGs as well as in 24 % of the low-ARG containing MAGs. The other N cycling genes were also evenly distributed among high- and low-ARG containing MAGs and only spatial patterns already described above could be observed after the MAG analysis.

### 4. Discussion

The diversity and abundance of antibiotic resistance genes (ARGs) in the impacted creek were considerably higher than in the reference creek. This result was particularly clear at the upstream stations, close to the discharge of the poultry processing plant in the impacted creek. At these stations, nearly half (48 %) of all ARGs found were observed exclusively in the impacted creek. On the other hand, at the downstream stations, farther from the processing plant, the difference in the resistome abundance and diversity between impacted and reference creeks is minimal. This decreasing gradient of dissimilarity and the higher abundances detected at the upstream stations suggest that the wastewater discharge, occurring upstream of the three analyzed stations, is the main source of most ARGs or
releases the chemicals enhancing the ARG abundance. These results are comparable to previous studies which found higher abundances of ARGs near wastewater treatment plants or animal feeding operations, when compared to reference or more distant sites (Chu et al., 2018; Czekalski et al., 2014; Lu et al., 2022; Mackie et al., 2006; Pruden et al., 2012). Others have found even larger differences, for example, by comparing lakes directly affected by antibiotic manufacturing to remote pristine lakes where differences of more than three orders of magnitude can be found (Bengtsson-Palme et al., 2014). In the terrestrial environment, previous studies have also shown spatial and temporal trends, with higher abundances in soils collected near livestock feeding areas and lower abundances of ARGs years after termination of farming operation (Agga et al., 2019).

The results from our study support the abundant literature reporting dissemination of ARGs in environments impacted by anthropogenic activities generating human or animal-related wastewater (Bengtsson-Palme et al., 2014; Chu et al., 2018; Pruden et al., 2012; Scott et al., 2016).

As discussed in previous works, the higher abundance of ARGs in metagenomes from the impacted creek can be explained by a selective pressure from historical exposure to high concentrations of antibiotics in the creek or due to migration of resistant bacteria directly from the processing plant (Bengtsson-Palme et al., 2014). The presence of antibiotics in the environment (creek or processing plant) favors an increase in the frequency of ARGs and acts as a major selection force of antibiotic resistant bacteria (Wu et al., 2017). It is true that other contaminants, such as metals or disinfectants, may co-select or be major drivers for the presence of some ARGs (Danner et al., 2019; Lu et al., 2022). However, our approach of searching for a subset of ARGs that confer resistance to a particular group of antibiotics (authorized for usage in the poultry industry), aimed to eliminate the weight of the co-selection confounding factor by pollutants other than antibiotics. It is thus reasonable to expect that the main selective force of the examined ARGs is antibiotics, rather than other co-selectors. Since ARGs can remain in bacterial communities even after the selective pressure is removed, the “easy-to-get, hard-to-lose” phenomenon (Aminov and Mackie, 2007), we cannot determine if the antibiotic pressure is due to a recent or past exposure; this determination is out of the scope of this work.

When considering the individual ARGs detected in this study, the two most abundant ARGs, MacA and MacB confer resistance to macrolides. Macrolide resistance genes were also abundant ARGs in the sediment.
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**Fig. 7.** Nitrogen cycling genes and number of ARGs detected in mid- and high-quality MAGs from creek sediments. N cycling genes presence ("yes") is highlighted with yellow background. For each location, MAGs are ordered by decreasing number of ARGs detected. Percent completeness and contamination is shown for each MAG. Archaeal MAGs are shown in purple font.
metagenomes near WWTP outfalls (Chu et al., 2018). Macrolide antibiotics, such as erythromycin and tylosin, are commonly used in the poultry industry and up to 67% of tylosin may be excreted by livestock or poultry when orally administered, favoring environmental dissemination (USEPA, 2013). The average normalized abundance of the two genes was higher in the impacted creek, especially MacB at the upstream station, where its average genomic copy number was four times higher than in the reference creek. The proteins encoded by MacB and MacA are part of a transmembrane complex (MacAB) that provides macrolide-specific resistance through active efflux (Kobayashi et al., 2001). The relatively high abundance of these genes in the impacted creek suggests the exposure to macrolide compounds, which is possibly due to their high usage in the poultry industry.

We also found substantially higher diversity and abundance of tetracycline resistance genes in the impacted creek than in the reference creek. The two most abundant tetracycline resistance genes found in our study belong to class A and class C that were mostly found in the impacted creek. A previous study identified classes C, E, and O as associated with WWTPs and classes H, Q, S, and T to be associated with animal feeding operations (Storteboom et al., 2010). We found genes from all classes of the first group (CEO) with a cumulative genomic copy number of 0.84 in the reference creek and 4.61 in the impacted creek, but very low to zero abundances of genes in classes H, Q, S, or T. The distribution of the tetracycline resistance classes in our study suggests that the molecular signature of these sediment metagenomes is representative of a WWTP more so than an animal feeding operation, which is not surprising since the processing plant contains its own WWTP to treat the wastewater before discharging to the creek. This finding is also in agreement with the microbial community results described in our previous work (Semedo and Song, 2020), which showed a community composition characteristic of WWTP effluent. However, we also found an increased abundance of the genes conferring resistance to streptogramin, such as virginiamycin, which is not expected near urban or municipal WWTP outfalls (Chu et al., 2018). Two genes conferring resistance to virginiamycin were found (virginiamycin B lyase and virginiamycin A acetyltransferase), with a cumulative average genomic copy number of 0.64 in the reference creek and 3.61 in the impacted creek. This result indicates that these ARGs may be specific to wastewater from the poultry industry.

The relative abundance of the classical class 1 integron-integrase (int1) gene in this study was also higher in the impacted creek than the reference creek, especially at the upstream and midstream stations. Higher abundance of mobile genetic elements (MGEs), including integrase and transposases, near wastewater discharges or antibiotic-polluted environments has been reported in previous studies (Bengtsson-Palme et al., 2009; Lofton et al., 2007). Despite these observations, and through a biogeochemical point of view, it was still surprising to find a higher DNRA/DNF ratio potential in these stations, since this ratio is expected to be lower in high NO3−/organics sites (Beate et al., 2014; Yoon et al., 2015), such as the upstream station of the impacted creek. With the observation of the significant relationship between nrfA and total ARGs (Fig. 6), one could speculate that the DNRA pathway may have been more co-selected with ARGs than denitrification genes in the impacted creek. However, after metagenomic binning, it does not appear that any particular N cycling pathway is directly linked to the presence of ARGs in individual genomes or that any specific gene is being co-selected with ARGs in the same genomes (Fig. 7). The correlation may thus be driven by a parallel response to factors co-occurring in the same location, the upstream station of the impacted creek in this case. Antibiotic resistance genes are probably responding to the wastewater input and the associated contaminants (Bengtsson-Palme et al., 2014; Chu et al., 2018; Pruden et al., 2012; Scott et al., 2016), while nrfA gene abundance could be simply responding to the high NO3 levels detected since it is a required electron acceptor, despite the abundant literature describing DNRA as being electron donor limited (Beate et al., 2014; Bu et al., 2017; Giblin et al., 2013; Yoon et al., 2015). Alternatively, both genes could be responding to the environmental disturbance caused by wastewater input. The clarification of these mechanisms, however, could only be achieved by experimental work with wastewater exposure and assessment of ARGs and nrfA gene co-evolution in different conditions of NO3− supply, which future studies may address. Nevertheless, it is important to underscore that finding significant or non-significant correlations in field observations such as the ones in this study must be cautiously considered since a causal relationship is not certain and requires further validation through targeted experiments and theoretical support.

In summary, the results from this study show that wastewater discharge from a poultry processing plant may lead to an increase in ARGs and integron-integrase genes in receiving waterbodies. Simultaneous high abundances of ARGs and mobile genetic elements in impacted creeks are a cause for concern due to an increased risk of antibiotic resistance propagation among bacterial communities in the aquatic environment, which may have negative consequences on environmental and human health. Due to the increasing trend of global poultry production, this source of antibiotic resistance genes could be responding to environmental disturbance caused by wastewater input. The clarification of these mechanisms, however, could only be achieved by experimental work with wastewater exposure and assessment of ARGs and nrfA gene co-evolution in different conditions of NO3− supply, which future studies may address. Nevertheless, it is important to underscore that finding significant or non-significant correlations in field observations such as the ones in this study must be cautiously considered since a causal relationship is not certain and requires further validation through targeted experiments and theoretical support.

CRediT authorship contribution statement

Miguel Semedo: Experimental plan, data collection, analysis, and curation. Writing first draft, review, and editing manuscript.
Bongleun Song: Experimental plan, data curation, supervision, project administration. Review and editing manuscript.


