2023

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

Miguel Semedo
Virginia Institute of Marine Science

Bongkeun Song
Virginia Institute of Marine Science

Recommended Citation

Semedo, Miguel and Song, Bongkeun, Sediment metagenomics reveals the impacts of poultry industry wastewater on antibiotic resistance and nitrogen cycling genes in tidal creek ecosystems (2023). *Science of The Total Environment*, 857(Part 2), 159496.
doi: 101016/j.scitotenv.2022.159496

Science of the Total Environment 857 (2023) 159496

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

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

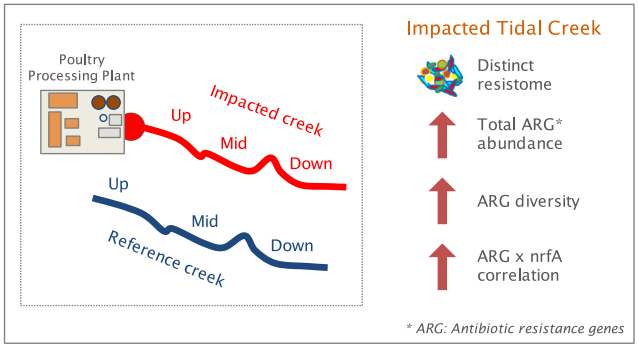
Miguel Semedo [a,b,*], Bongkeun Song [a]

[a] Department of Biological Sciences, Virginia Institute of Marine Science, College of William & Mary, Gloucester Point, VA 23062, USA
[b] Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), University of Porto, Matosinhos, Portugal

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.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Abasiofiok Mark Ibekwe

Keywords:
Wastewater
Poultry processing plant
Antibiotic resistance genes
Nitrogen cycle
Metagenomics
Nutrient pollution

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

* Corresponding author at: Department of Biological Sciences, Virginia Institute of Marine Science, College of William & Mary, Gloucester Point, VA 23062, USA.
E-mail address: msemedo@ciimar.up.pt (M. Semedo).

http://dx.doi.org/10.1016/j.scitotenv.2022.159496
Received 7 July 2022; Received in revised form 11 October 2022; Accepted 12 October 2022
Available online xxxx

ecosystems. Currently, around 50 % of surface waters in the United States (US) have detectable levels of selected antibiotics at ppb concentrations (Kolpin et al., 2002; Scott et al., 2016). Increased concentrations of antibiotics in the aquatic environment may have negative consequences, such as growth and activity inhibition of non-target beneficial bacteria and the spreading of antibiotic resistance (Danner et al., 2019).

Antibiotic usage varies between countries and regions. In the US and the European Union (EU), antibiotic usage is evenly split between human and veterinary uses (Kümmerer, 2009; Scott et al., 2016). Antibiotics are used in the livestock and poultry industry to prevent and treat disease or, in some instances, as feed supplements to promote growth (Kümmerer, 2009; USEPA, 2013). Increased antibiotic concentrations can be found in aquatic environments surrounding animal housing facilities (Campagnolo et al., 2002; Kim and Carlson, 2007; Mackie et al., 2006). Landfills and wastewater treatment plants (WWTP) are another major source of antibiotics to the surrounding environment and increased concentrations are usually found near these facilities (Danner et al., 2019; Michael et al., 2013; Scott et al., 2016; Wu et al., 2015). The sharp increase in poultry production over the last decades has led to an increase in poultry processing plants that directly discharge treated wastewater to surface waters (Semedo and Song, 2020). Due to the historical use of antibiotics in the poultry industry, these direct discharges may result in an increase in antibiotic concentrations in the aquatic environment (Kümmerer, 2009). However, the relative contribution of these discharges to antibiotic contamination or antibiotic resistance is relatively unknown.

Detecting and quantifying antibiotics in environmental matrices is not a trivial task. It is labor-intensive, costly, and time-consuming, and might not be sensitive enough for the detection of the low, sub-therapeutic doses found in the environment. The wide array of different compounds administered in livestock and poultry industries further obstructs the detection and quantification of antibiotics based on targeted approaches toward small groups of compounds. Quantification of antibiotic resistance genes (ARGs) in environmental samples can serve as a proxy for antibiotic contamination, since antibiotic exposure is a major selective pressure for the acquisition of ARGs by microorganisms, even at low environmental concentrations (Aminov and Mackie, 2007; Bengtsson-Palme et al., 2018). The collection of all the ARGs in microorganisms is known as the antibiotic resistome (Wright, 2007). Previous studies have identified and quantified ARGs in aquatic ecosystems impacted by different anthropogenic activities, such as antibiotic production waste (Bengtsson-Palme et al., 2014; Kristiansson et al., 2011), WWTPs (Chu et al., 2018; Czekalski et al., 2014; Rowe et al., 2016), and animal production facilities (Chee-Sanford et al., 2001; Mackie et al., 2006; Rowe et al., 2016). Two main approaches can be used to detect ARGs in the environment, targeted and untargeted. Targeted approaches quantify specific ARGs or associated mobile genetic elements (MGEs), such as integrons and transposons, through polymerase chain reaction (PCR) or quantitative PCR (qPCR) (Czekalski et al., 2014; Mackie et al., 2006; Tennstedt et al., 2003). These studies have considerably advanced our knowledge on the environmental contamination with antibiotics and the spreading of antibiotic resistance. However, targeted techniques may limit our ability to assess the diverse array of antibiotics potentially used in the animal industry and to understand the differential responses of different ARGs in the environment. Additionally, targeted quantitative approaches such as qPCR may be biased due to the use of selected primers for gene detection. In order to overcome these limitations, shotgun metagenomics has recently been used to detect and quantify the abundance of diverse ARGs responding to various types of antibiotic contamination (Bengtsson-Palme et al., 2014; Chu et al., 2018; Kristiansson et al., 2011; Liu et al., 2018; Rowe et al., 2016; Wu et al., 2017).

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 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 N_2 is reduced to ammonia (NH_3) through the activity of nitrogenases in the N-fixation process, which is particularly important in oligotrophic sites (Andersson et al., 2014). Non-assimilated NH_3 can then be aerobically or anaerobically oxidized through nitrification (oxidation of NH_3 to NO_2^- and NO_3^-) or anammox (the reduction of NO_2^- with NH_4^+ with the production of N_2), respectively. Nitrate (NO_3^-) or nitrite (NO_2^-) can then be step-wise reduced to N_2O and N_2 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 NH_3), denitrification leads to N loss/removal from the environment (via N_2O or N_2). It is thus expected that the DNRA/denitrification ratio is important for ecosystem health, being frequently investigated in the literature (Burgin 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 (M) (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 the 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).

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 (Danecek et al., 2021).

To investigate the impacts of poultry industry wastewater on the resistome and to exclude the quantification of very low specificity resistance genes (e.g. multidrug efflux pumps), we performed a string-based word search on the PROKKA annotated metagenomes for protein names associated with antibiotics approved for use in the poultry industry (USEPA, 2013): bacitracin, chlortetracycline, erythromycin, gentamicin, lasalocid, lincomycin, monensin, neomycin, nystatin, oxytetracycline, penicillin, spectinomycin, streptomycin, sulfadimethoxine, tetracycline, tylosin, and virginiamycin. To quantify the abundance of nitrogen cycling genes (NCGs), we performed a string-based word search for the following protein names in each metabolic reaction (gene name in parenthesis): Nitrate reduction – respiratory nitrate reductase alpha subunit (*narG*), periplasmic nitrate reductase (*napA*); DNRA - cytochrome *c*-552 (*nrfA*); denitrification – nitrite reductase (*nirS*), copper-containing nitrite reductase (*nirK*), nitric oxide reductase subunit B (*norB*), and nitrous-oxide reductase (*nosZ*); nitrification – ammonia monooxygenase alpha subunit (*amoA*) and hydroxylamine oxidoreductase (*hao*); anammox – hydrazine synthase subunit alpha (*hzo*) and hydrazine dehydrogenase (*hdh*); nitrogen fixation – nitrogenase iron protein (*nifH*). All protein annotations were verified in the reviewed (Swiss-Prot) manually annotated UniProtKB database. (Consortium, 2021).

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 obtain the coverage for each gene (Chu et al., 2018). To account for differences in sequencing depth between samples, the number of mapped reads to each target gene was normalized against the average count of three single-copy genes found in nearly all free-living bacteria (Dupont et al., 2012): RecA protein (*RecA*), DNA gyrase subunit B (*gyrB*), and DNA-directed RNA polymerase subunit beta (*rpoB*). The normalized abundance of a target gene (ARG or NCG) was then expressed as an average genomic copy number, similar to that proposed by Nayfach and Pollard (2016) (Nayfach and Pollard, 2016), and was obtained using the formula below:

$$Average\ Genomic\ Copy\ Number = \frac{\left\{\frac{\Sigma counts\ of\ target\ gene}{gene\ length\ (bp)}\right\}_{ARG\ or\ NCG}}{mean\left\{\frac{\Sigma counts\ of\ reference\ gene}{gene\ length\ (bp)}\right\}_{RecA,gyrB,rpoB}}$$

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-assembles 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 (Chaumeil 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 were prepared 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 intIF165 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 H_2O. 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 R^2 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 quantitative 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 685R 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. *IntI1* 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 and being marginally non-significant ($R^2_{partial} = 0.07, p = 0.081$), probably due to the high variability observed and the limited replication. The two creeks, however, presented a different spatial trend across the three locations. While the samples from the reference creek do not present a clear separation between upstream, midstream, and downstream stations, the samples from the impacted creek present some separation from upstream to downstream stations. This spatial variation in the impacted creek also corresponds to a dissimilarity trend with the samples from the reference creek. 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 ($n_{ARG} = 11$) was around 6 times higher than the corresponding number in the reference creek ($n_{ARG} = 2$) and even exceeded the number of genes present in both creeks ($n_{ARG} = 10$) (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

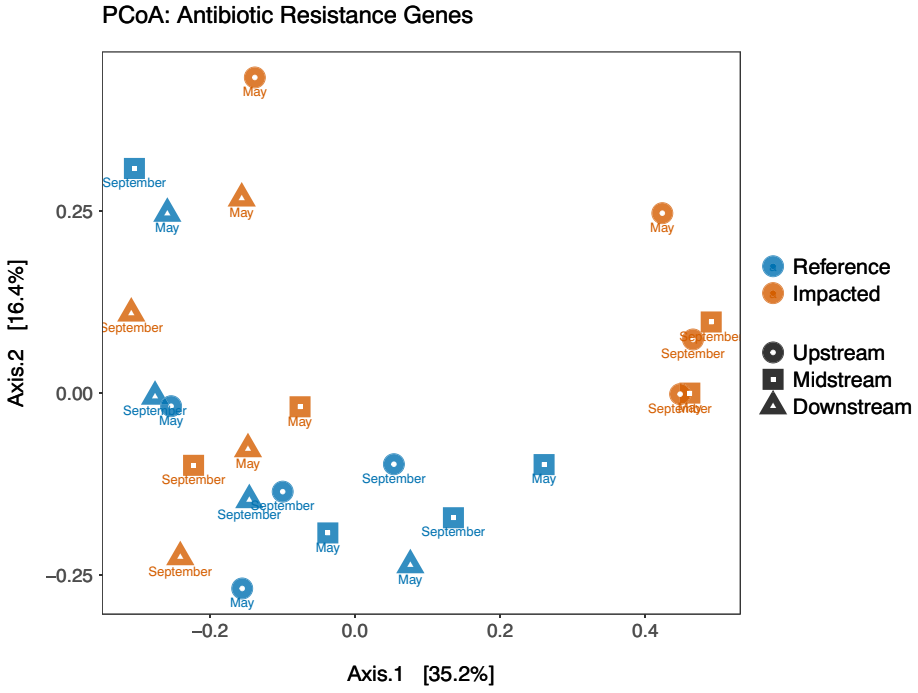


Fig. 1. Principal Coordinate Analysis (PCoA) plot representing the dissimilarity in the normalized abundance of antibiotic resistance genes in the sediments collected from the reference (blue) and the impacted (red) creeks. Upstream (circles), midstream (squares), and downstream (triangles) samples from May and September are shown. Sample dissimilarity and distance analysis was calculated using the Bray-Curtis dissimilarity index.

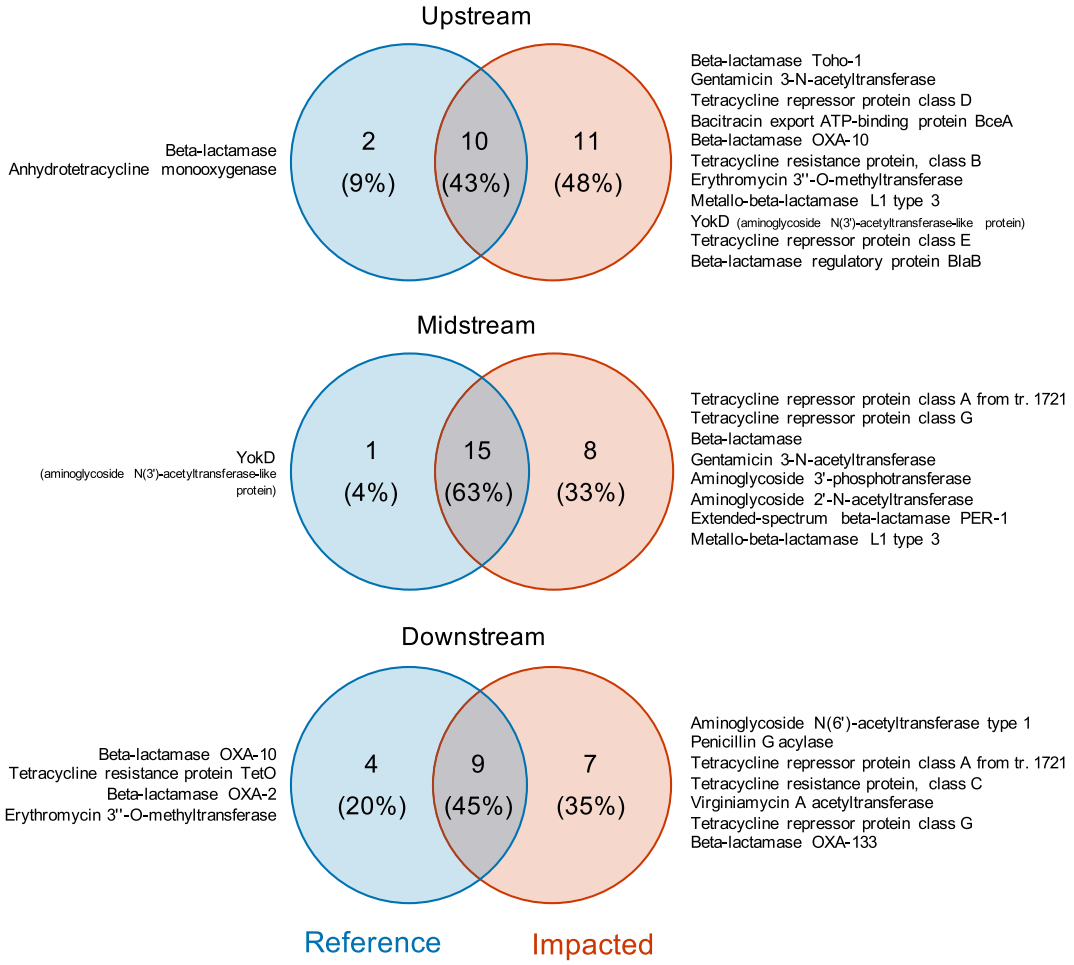


Fig. 2. Venn diagrams describing the ARGs identified exclusively in the reference (blue) or the impacted creek sediments (red). The shaded overlap represents the number of genes shared between the two creeks. The percentage (in parenthesis) represents the proportion of genes from each sub-group in relation to the total number of ARGs identified in each station.

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

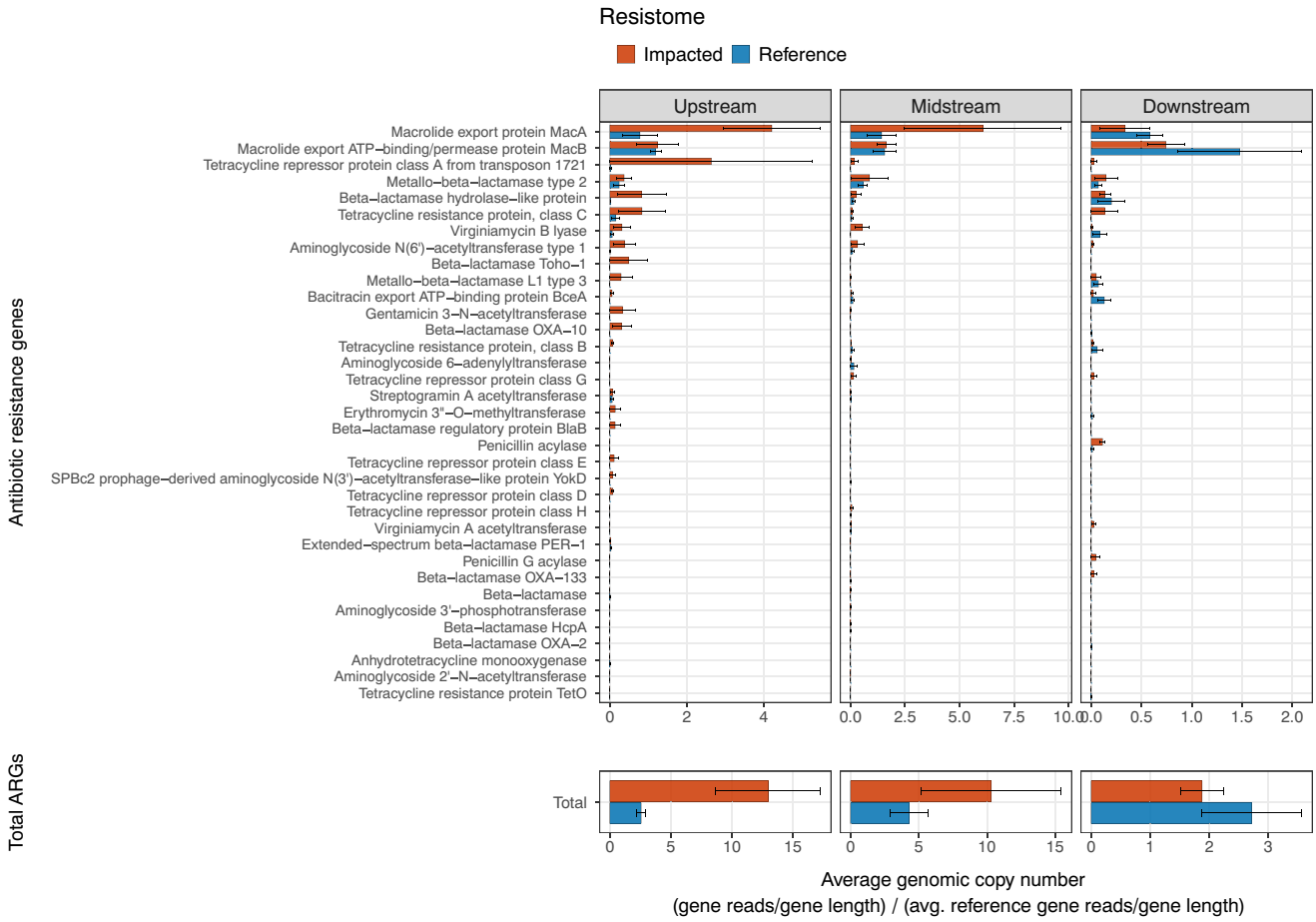


Fig. 3. Normalized abundance of antibiotic resistance genes in assembled metagenomes from reference (blue) and impacted (red) sediments. Bars represent mean ± SE of four cores (2 replicates × 2 months) in each location (upstream, midstream, or downstream). The abundance is expressed as the average genomic 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.

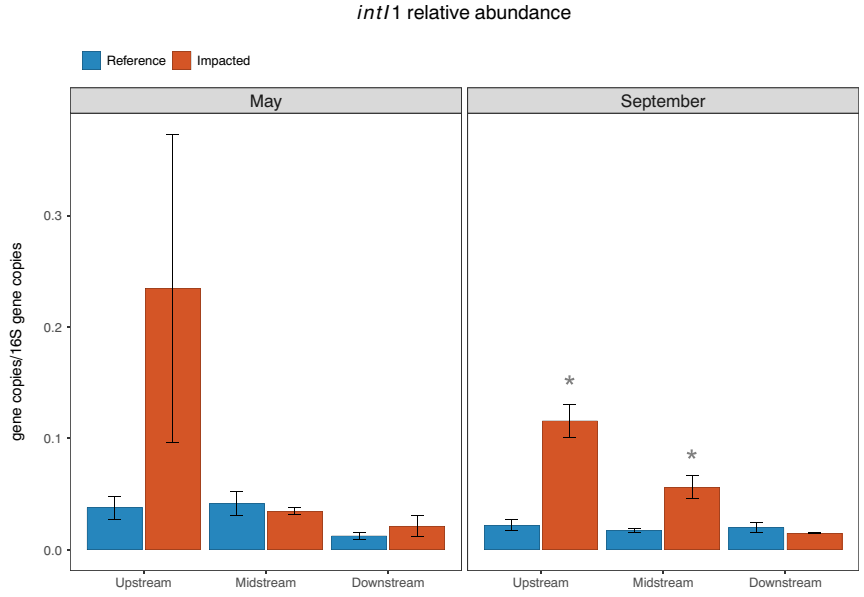


Fig. 4. Relative abundance of clinical class 1 integron-integrase (intI1) gene in the reference (blue) and the impacted (red) creek sediments. Bars represent mean ± SE of triplicate cores. Significant difference between the two creeks at each station are marked with * (2-way ANOVA, $p < 0.05$).

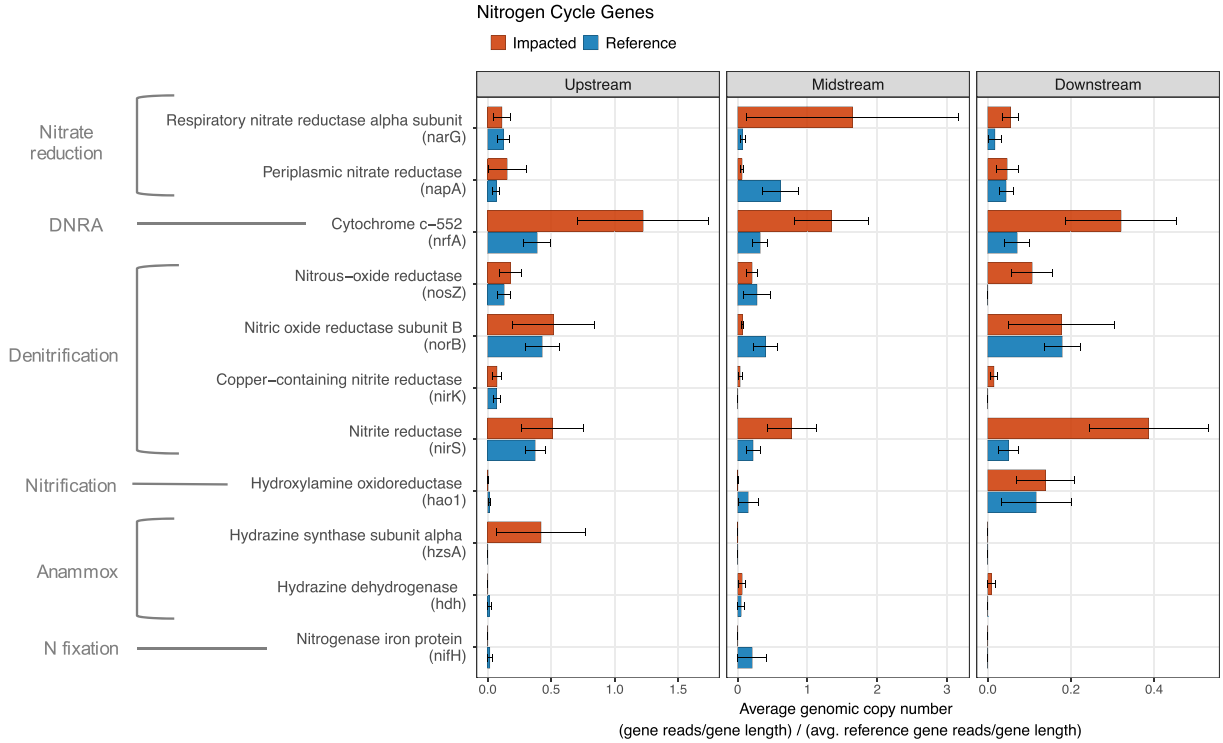


Fig. 5. Normalized abundance of N cycling genes in assembled metagenomes from the reference (blue) and the impacted (red) sediments. Bars represent mean ± SE of four cores (2 replicates × 2 months) in each location (upstream, midstream, or downstream). The abundance is expressed as the average genomic copy number to normalize for gene length and sequencing depth, as described in the Material and methods section. The genes are vertically ordered by N metabolic pathway.

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, *norB* 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 highest 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, closest 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

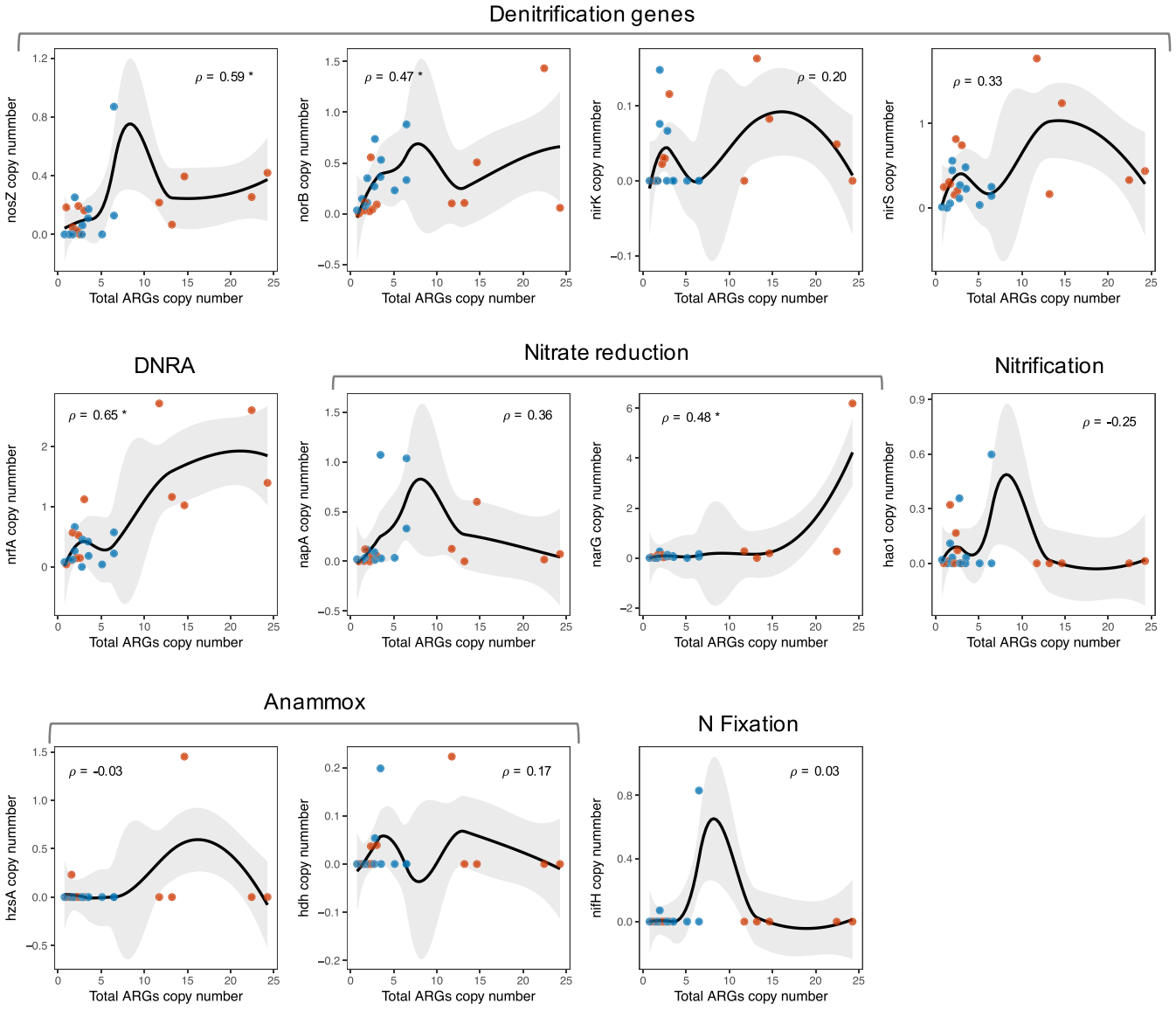


Fig. 6. Relationship between N cycle and antibiotic resistance genes. The abundance of each N cycle gene is expressed as the average genomic copy number and is compared with the total average genomic copy number of antibiotic resistance genes per sample. Each point represents a sample from the reference (blue) and the impacted (red) creek sediments. The spearman correlation coefficient is also shown for each relationship. An asterisk indicates a significant spearman correlation. The shaded area represents the 95 % confidence interval of the locally estimated scatterplot smoothing predictions (solid black line).

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-loose" 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

Station	Creek	MAG ID	Genus (class/phylum)	# ARG	Nitrate red.		DNRA	Denitrification				Nitrif.	Anammox		N fix.	% comp.	% cont.
					narG	napA	nrfA	nosZ	norB	nirK	nirS	hao1	hzsA	hdh	nifH		
Upstream	Impacted	bin.010	Piscinibacter (Gamma-proteob.)	6		yes	yes				yes					95,6	5,7
		bin.013	UBA3961 (Bacteroidia)	6				yes								87,1	2,0
		bin.057	unclass. (Verrucomicrobiae)	6		yes										99,3	7,4
		bin.017	PHOS-HE28 (Bacteroidia)	5				yes								95,1	3,6
		bin.018	Aestuariivirga (Alpha-proteobacteria)	5	yes	yes	yes			yes						93,2	4,2
		bin.025	unclass. (Acidimicrobiia)	5												95,9	1,8
		bin.037	JAAZBK01 (Acidimicrobiia)	5		yes										94,0	6,3
		bin.042	JJ008 (Bacteroidia)	4				yes								77,6	1,1
		bin.055	JACADZ01 (Alpha-proteobacteria)	4	yes		yes									94,4	2,1
		bin.061	unclass. (Thermoanaerobaculia)	4	yes	yes										66,9	8,4
		bin.016	Methylomirabilis (Methylomirabilia)	3	yes	yes					yes			yes		90,2	3,5
		bin.038	UBA7227 (Anaerolineae)	3	yes	yes	yes				yes					70,4	4,2
		bin.046	JAAUPO01 (Cyanobacteriia)	3												84,1	1,0
		bin.047	unclass. (Kapabacteria)	3		yes		yes								86,9	0,1
		bin.050	Terricaulis (Alpha-proteobacteria)	3	yes											81,6	4,9
		bin.051	unclass. (Ignavibacteria)	3		yes	yes									86,5	1,9
		bin.056	unclass. (Myxococcota)	3	yes	yes					yes					60,7	4,4
		bin.006	unclass. (Gamma-proteob.)	2		yes		yes			yes					95,7	1,9
		bin.019	UBA8403 (Bacteoidia)	2												68,9	1,0
	Ref.	bin.009	Sulfuricella (Gamma-proteob.)	2		yes	yes	yes	yes		yes					85,7	6,9
		bin.004	SM1-50 (Thermoplasmatota)	0												93,2	3,2
		bin.013	PALSA-986 (Thermoproteota)	0												55,5	1,9
					narG	napA	nrfA	nosZ	norB	nirK	nirS	hao1	hzsA	hdh	nifH		
Midstream	Impacted	bin.003	unclass. (Actinobacteriota)	5											yes	97,4	2,3
		bin.040	Rubrivivax (Gamma-proteob.)	5		yes		yes			yes					75,9	6,0
		bin.020	UBA11591 (Bacteroidia)	4		yes		yes								72,7	1,3
		bin.014	Methylomirabilis (Methylomirabilia)	3		yes					yes					80,5	3,2
		bin.031	Robiginitalea (Bacteroidia)	3				yes								66,0	0,3
		bin.034	9FT-COMBO-42-15 (Nitrospiria)	3	yes		yes	yes		yes						90,9	5,9
		bin.008	CAADGV01 (Bacteroidota)	2	yes	yes	yes									78,6	5,2
		bin.022	unclass. (Bacteroidia)	2	yes			yes								97,4	0,0
		bin.032	unclass. (Desulfobacterota)	2		yes	yes									76,9	3,6
		bin.038	Gallionella (Gamma-proteob.)	2						yes	yes					86,3	3,3
		bin.012	Nitrospira (Nitrospiria)	1								yes				52,1	8,6
		bin.011	unclass. (Thermodesulfovibrionia)	0		yes		yes	yes			yes				92,5	0,9
	Reference	bin.028	unclass. (Gamma-proteob.)	4	yes	yes		yes	yes						yes	94,5	5,4
		bin.029	unclass. (Anaerolineae)	3		yes									yes	57,3	2,0
		bin.031	Hydrogenophaga (Gamma-proteob.)	3		yes	yes									88,4	3,4
		bin.032	Desulfuromusa (Desulfuromonadia)	3								yes				74,5	3,1
		bin.037	Yeosuana (Bacteroidia)	3				yes								80,1	1,2
		bin.040	Sulfurovum (Campylobacteria)	1		yes		yes	yes							78,2	6,3
		bin.010	Sulfurovum (Campylobacteria)	0		yes			yes							87,0	8,8
					narG	napA	nrfA	nosZ	norB	nirK	nirS	hao1	hzsA	hdh	nifH		
Downstream	Impacted	bin.009	Robiginitalea (Bacteroidia)	3				yes								51,0	1,6
		bin.003	SLDE01 (Gamma-proteob.)	2							yes					53,5	0,0
		bin.008	SG8-38 (Polyangia)	2		yes	yes	yes							yes	73,9	3,9
		bin.016	M0040 (Desulfuromonadia)	2	yes	yes										53,9	1,0
		bin.019	GCA-2746365 (Gamma-proteob.)	2												50,3	0,0
		bin.022	BM004 (Desulfobulbia)	2		yes	yes									98,8	4,4
		bin.025	unclass. (Desulfuromonadia)	0												60,8	1,5
	Reference	bin.014	JABDQZ01 (Gamma-proteob.)	4												74,8	3,2
		bin.001	UBA9214 (Gamma-proteob.)	3					yes		yes					94,6	4,7
		bin.015	M0040 (Desulfuromonadia)	2		yes									yes	96,7	4,0
		bin.016	BM004 (Desulfobulbia)	2			yes									87,1	2,2
		bin.029	Robiginitalea (Bacteroidia)	2												67,4	2,2
		bin.006	Draconibacterium (Bacteroidia)	1												55,1	2,6
		bin.008	Sulfurovum (Campylobacteria)	1		yes										92,1	5,7
		bin.020	GCA-2746365 (Gamma-proteob.)	1		yes										89,5	3,8

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 *MacA* 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 clinical class 1 integron-integrase (*intI*1) 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 integrases and transposases, near wastewater discharges or antibiotic-polluted environments has been reported in previous studies (Bengtsson-Palme et al., 2014; Kristiansson et al., 2011). The *intI*1 gene was proposed as a genetic marker of anthropogenic pollution due to its rapid response to various human pollutants, such as antibiotics, disinfectants, and heavy metals (Gillings et al., 2015). Livestock waste products are characterized by increased levels of diverse antibiotic resistance genes and enriched concentrations of heavy metals (Zhu et al., 2013), so the increased abundance in the impacted creek is not surprising. Indeed, the presence of class 1 integrons was previously reported to increase in poultry house litter, CAFOs aerosols, and groundwater impacted by animal farming (Hong et al., 2013; Ling et al., 2013; Nandi et al., 2004). This result underscores that this creek is highly impacted by the chicken industry, when compared to the reference creek.

In this study, we also aimed to explore the metagenomic profile of N cycling genes in the sediments of impacted and reference tidal creeks as well as the potential relationships with ARGs distribution. Our results suggest that the DNRA pathway was favored at the upstream station of the impacted creek, compared with the same station of the reference creek, while denitrification gene differences were not detectable in the metagenomes. The denitrification gene results contrast with our previous observations of inhibited denitrification activity in the same station of the impacted creek, when compared to the reference creek (Semedo and Song, 2020). A disconnect between metagenomic gene abundance and activity measurements is not unique to this study (Frostegård et al., 2022) and, in this case, was possibly explained by an inhibition at the expression or even at the enzymatic activity level. Another possible explanation for the inconsistency is that metagenomic sequencing coverage for target genes (e.g. denitrification) is expected to be relatively low, in comparison to targeted approaches, such as qPCR.

The increased DNRA potential observed in the impacted creek may lead to increased N retention, which is supported by the significantly higher levels of bottom DIN (both NH_4^+ and NO_3^-) previously found in this creek, especially at the upstream and midstream stations (Semedo and Song, 2020). The lower abundance of nitrifying *hao* also contributes to explaining the higher NH_4^+ found in this creek. Despite higher DNRA and lower nitrification potential in the impacted creek, however, the NO_3^- was still substantially higher at the upstream station of the impacted creek (Semedo and Song, 2020), which is probably explained by the continuous supply of external NO_3^- to the creek, commonly observed in waterways draining WWTP and/or animal facilities (Carey and Migliaccio, 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 NO_3^-/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 NO_x 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 NO_3^- 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 should not be ignored in future estimates of antibiotic resistance levels in the Anthropocene. Our results also suggest that microbial communities highly impacted by antibiotic resistance genes, a N retention pathway (DNRA) may be favored in relation to a N removal pathway (denitrification), which presents a two-fold concern for ecosystem health related to aquatic eutrophication.

CRediT authorship contribution statement

Miguel Semedo: Experimental plan, data collection, analysis, and curation. Writing first draft, review, and editing manuscript.

Bongkeun Song: Experimental plan, data curation, supervision, project administration. Review and editing manuscript.

Data availability

Data will be made available on request.

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.

Acknowledgements

The authors are grateful to Marcos Krull for the assistance provided with the multivariate analysis and AIC model selection. We also thank all members of the Microbial Ecology Lab that provided invaluable assistance during sampling campaigns and laboratory work. This study was performed using computing facilities at the College of William and Mary which were provided by contributions from the National Science Foundation, the Commonwealth of Virginia Equipment Trust Fund and the Office of Naval Research. This study was supported by funding from National Science Foundation (OCE1737258) and through fellowships from the Virginia Sea Grant and the Fulbright Program.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159496.

References

Agga, G.E., Cook, K.L., Netthisinghe, A.M.P., Gilfillen, R.A., Woosley, P.B., Sistani, K.R., 2019. Persistence of antibiotic resistance genes in beef cattle backgrounding environment over two years after cessation of operation. PLoS One 14. https://doi.org/10.1371/journal.pone.0212510.

Aminov, R.I., Mackie, R.I., 2007. Evolution and ecology of antibiotic resistance genes. FEMS Microbiol. Lett. 271, 147–161. https://doi.org/10.1111/j.1574-6968.2007.00757.x.

Andersson, B., Sundbäck, K., Hellman, M., Hallin, S., Alsterberg, C., 2014. Nitrogen fixation in shallow-water sediments: spatial distribution and controlling factors. Limnol. Oceanogr. 59, 1932–1944. https://doi.org/10.4319/lo.2014.59.6.1932.

Arkin, A., Cottingham, R., Henry, C., et al., 2018. KBase: the United States department of energy systems biology knowledgebase. Nat. Biotechnol. 36, 566–569.

Beate, K., E, T.H., Ritin, S., G, K.M., G, F.T., L, H.R., S, G.J., Marc, S., 2014. The environmental controls that govern the end product of bacterial nitrate respiration. Science (1979) 345, 676–679. https://doi.org/10.1126/science.1254070.

Bengtsson-Palme, J., Boulund, F., Fick, J., Kristiansson, E., Larsson, D.G.J., 2014. Shotgun metagenomics reveals a wide array of antibiotic resistance genes and mobile elements in a polluted lake in India. Frontiers in Microbiology 5, 1–14. https://doi.org/10.3389/fmicb.2014.00648.

Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J., 2018. Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiol. Rev. 42, 68–80. https://doi.org/10.1093/femsre/fux053.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for illumina sequence data. Bioinformatics 30, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

Bowers, R.M., Kyrpides, N.C., Stepanauskas, R., Harmon-Smith, M., Doud, D., Reddy, T.B.K., Schulz, F., Jarett, J., Rivers, A.R., Eloe-Fadrosh, E.A., Tringe, S.G., Ivanova, N.N., Copeland, A., Clum, A., Becraft, E.D., Malmstrom, R.R., Birren, B., Podar, M., Bork, P., Weinstock, G.M., Garrity, G.M., Dodsworth, J.A., Yooseph, S., Sutton, G., Glöckner, F.O., Gilbert, J.A., Nelson, W.C., Hallam, S.J., Jungbluth, S.P., Ettema, T.J.G., Tighe, S., Konstantinidis, K.T., Liu, W.-T., Baker, B.J., Rattei, T., Eisen, J.A., Hedlund, B., McMahon, K.D., Fierer, N., Knight, R., Finn, R., Cochrane, G., Karsch-Mizrachi, I., Tyson, G.W., Rinke, C., Kyrpides, N.C., Schriml, L., Garrity, G.M., Hugenholtz, P., Sutton, G., Yilmaz, P., Meyer, F., Glöckner, F.O., Gilbert, J.A., Knight, R., Finn, R., Cochrane, G., Karsch-Mizrachi, I., Lapidus, A., Meyer, F., Yilmaz, P., Parks, D.H., Murat Eren, A., Schriml, L., Banfield, J.F., Hugenholtz, P., Woyke, T., Consortium, T.G.S., 2017. Minimum information about a single amplified genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria and archaea. Nat. Biotechnol. 35, 725–731. https://doi.org/10.1038/nbt.3893.

Bu, C., Wang, Y., Ge, C., Ahmad, H.A., Gao, B., Ni, S.-Q., 2017. Dissimilatory nitrate reduction to ammonium in the Yellow River estuary: rates, abundance, and community diversity. Sci. Rep. 7, 6830. https://doi.org/10.1038/s41598-017-06404-8.

Burgin, A., Hamilton, S., 2007. Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. Front. Ecol. Environ. 5, 89–96. https://doi.org/10.1890/1540-9295(2007)5[89:HWOTRO]2.0.CO;2.

Campagnolo, E.R., Johnson, K.R., Karpati, A., Rubin, C.S., Kolpin, D.W., Meyer, M.T., Esteban, J.E., Currier, R.W., Smith, K., Thu, K.M., McGeehin, M., 2002. Antimicrobial residues in animal waste and water resources proximal to large-scale swine and poultry feeding operations. Sci. Total Environ. 299, 89–95. https://doi.org/10.1016/S0048-9697(02)00233-4.

Carey, R.O., Migliaccio, K.W., 2009. Contribution of wastewater treatment plant effluents to nutrient dynamics in aquatic systems: a review. Environ. Manag. 44, 205–217. https://doi.org/10.1007/s00267-009-9309-5.

Chaumeil, P.-A., Mussig, A.J., Hugenholtz, P., Parks, D.H., 2020. GTDB-tk: a toolkit to classify genomes with the genome taxonomy database. Bioinformatics 36, 1925–1927. https://doi.org/10.1093/bioinformatics/btz848.

Chee-Sanford, J.C., Aminov, R.I., Krapac, I.J., Garrigues-Jeanjean, N., Mackie, R.I., 2001. Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. Appl. Environ. Microbiol. 67, 1494–1502. https://doi.org/10.1128/AEM.67.4.1494-1502.2001.

Chu, B.T.T., Petrovich, M.L., Chaudhary, A., Wright, D., Murphy, B., Wells, G., Poretsky, R., 2018. Metagenomics reveals the impact of wastewater treatment plants on the dispersal of microorganisms and genes in aquatic sediments. Appl. Environ. Microbiol. 84, 1–15. https://doi.org/10.1128/AEM.02168-17.

Consortium, T.U., 2021. UniProt: the universal protein knowledgebase in 2021. Nucleic Acids Res 49, D480–D489. https://doi.org/10.1093/nar/gkaa1100.

Czekalski, N., Gascón Díez, E., Bürgmann, H., 2014. Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. ISME J. 8, 1381–1390. https://doi.org/10.1038/ismej.2014.8.

Danecek, P., Bonfield, J.K., Liddle, J., Marshall, J., Ohan, V., Pollard, M.O., Whitwham, A., Keane, T., McCarthy, S.A., Davies, R.M., Li, H., 2021. Twelve years of SAMtools and BCFtools. Gigascience 10, giab008. https://doi.org/10.1093/gigascience/giab008.

Danner, M.-C., Robertson, A., Behrends, V., Reiss, J., 2019. Antibiotic pollution in surface fresh waters: occurrence and effects. Sci. Total Environ. 664, 793–804. https://doi.org/10.1016/j.scitotenv.2019.01.406.

Dupont, C.L., Rusch, D.B., Yooseph, S., Lombardo, M.-J., Richter, R.A., Valas, R., Novotny, M., Yee-Greenbaum, J., Selengut, J.D., Haft, D.H., Halpern, A.L., Lasken, R.S., Nealson, K., Friedman, R., Venter, J.C., 2012. Genomic insights to SAR86, an abundant and uncultivated marine bacterial lineage. ISME J. 6, 1186–1199. https://doi.org/10.1038/ismej.2011.189.

Frostegård, Å., Vick, S.H.W., Lim, N.Y.N., Bakken, L.R., Shapleigh, J.P., 2022. Linking meta-omics to the kinetics of denitrification intermediates reveals pH-dependent causes of N2O emissions and nitrite accumulation in soil. ISME J. 16, 26–37. https://doi.org/10.1038/s41396-021-01045-2.

Giblin, A., Tobias, C., Song, B., Weston, N., Banta, G., Rivera-Monroy, V., 2013. The importance of dissimilatory nitrate reduction to ammonium (DNRA) in the nitrogen cycle of coastal ecosystems. Oceanography 26, 124–131. https://doi.org/10.5670/oceanog.2013.54.

Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.-G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 9, 1269–1279.

Hong, P.Y., Yannarell, A.C., Dai, Q., Ekizoglu, M., Mackie, R.I., 2013. Monitoring the perturbation of soil and groundwater microbial communities due to pig production activities. Appl. Environ. Microbiol. 79, 2620–2629. https://doi.org/10.1128/AEM.03760-12.

Kang, D.D., Froula, J., Egan, R., Wang, Z., 2015. MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. PeerJ 3, e1165. https://doi.org/10.7717/peerj.1165.

Kim, S.C., Carlson, K., 2007. Temporal and spatial trends in the occurrence of human and veterinary antibiotics in aqueous and river sediment matrices. Environ. Sci. Technol 41, 50–57. https://doi.org/10.1021/es060737+.

Kobayashi, N., Nishino, K., Yamaguchi, A., 2001. Novel macrolide-specific ABC-type efflux transporter in Escherichia coli. J. Bacteriol. 183, 5639–5644. https://doi.org/10.1128/JB.183.19.5639-5644.2001.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. Streams, 1999–2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–1211. https://doi.org/10.1021/es011055j.

Kristiansson, E., Fick, J., Janzon, A., Grabic, R., Rutgersson, C., Weijdegård, B., Söderström, H., Joakim Larsson, D.G., 2011. Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. PLoS One 6. https://doi.org/10.1371/journal.pone.0017038.

Kümmerer, K., 2009. Antibiotics in the aquatic environment–a review–part I. Chemosphere 75, 417–434. https://doi.org/10.1016/j.chemosphere.2008.12.006.

Kuypers, M.M.M., Marchant, H.K., Kartal, B., 2018. The microbial nitrogen-cycling network. Nat. Rev. Microbiol. 16, 263.

Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with bowtie 2. Nat. Methods 9, 357.

Li, D., Liu, C.-M., Luo, R., Sadakane, K., Lam, T.-W., 2015. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de bruijn graph. Bioinformatics 31, 1674–1676. https://doi.org/10.1093/bioinformatics/btv033.

Li, D., Luo, R., Liu, C.-M., Leung, C.-M., Ting, H.-F., Sadakane, K., Yamashita, H., Lam, T.-W., 2016. MEGAHIT v1.0: a fast and scalable metagenome assembler driven by advanced methodologies and community practices. Methods 102, 3–11. https://doi.org/10.1016/j.ymeth.2016.02.020.

Ling, A.L., Pace, N.R., Hernandez, M.T., LaPara, T.M., 2013. Tetracycline resistance and class 1 integron genes associated with indoor and outdoor aerosols. Environ. Sci. Technol. 47, 4046–4052. https://doi.org/10.1021/es400238g.

Liu, X., Yang, S., Wang, Y., Zhao, H.P., Song, L., 2018. Metagenomic analysis of antibiotic resistance genes (ARGs) during refuse decomposition. Sci. Total Environ. 634, 1231–1237. https://doi.org/10.1016/j.scitotenv.2018.04.048.

Lofton, D.D., Hershey, A.E., Whalen, S.C., 2007. Evaluation of denitrification in an urban stream receiving wastewater effluent. Biogeochemistry 86, 77–90. https://doi.org/10.1007/s10533-007-9146-7.

Lu, L., He, Y., Peng, C., Wen, X., Ye, Y., Ren, D., Tang, Y., Zhu, D., 2022. Dispersal of antibiotic resistance genes in an agricultural influenced multi-branch river network. Sci. Total Environ. 830, 154739. https://doi.org/10.1016/j.scitotenv.2022.154739.

Mackie, R.I., Koike, S., Krapac, I., Chee-Sanford, J., Maxwell, S., Aminov, R.I., 2006. Tetracycline residues and tetracycline resistance genes in groundwater impacted by swine production facilities. Anim. Biotechnol. 17, 157–176. https://doi.org/10.1080/10495390600956953.

McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, 1–11. https://doi.org/10.1371/journal.pone.0061217.

Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., Dagot, C., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: a review. Water Res. 47, 957–995. https://doi.org/10.1016/j.watres.2012.11.027.

Nandi, S., Maurer, J.J., Hofacre, C., Summers, A.O., 2004. Gram-positive bacteria are a major reservoir of Class 1 antibiotic resistance integrons in poultry litter. Proc. Natl. Acad. Sci. U. S. A. 101, 7118–7122. https://doi.org/10.1073/pnas.0306466101.

Nayfach, S., Pollard, K.S., 2016. Toward accurate and quantitative comparative metagenomics. Cell 166, 1103–1116. https://doi.org/10.1016/j.cell.2016.08.007.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, B.R., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2017. vegan: Community Ecology Package.

Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., Tyson, G.W., 2015. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 25. https://doi.org/10.1101/gr.186072.114.

Pruden, A., Arabi, M., Storteboom, H.N., 2012. Correlation between upstream human activities and riverine antibiotic resistance genes. Environ Sci Technol 46, 11541–11549. https://doi.org/10.1021/es302657r.

Rowe, W., Verner-Jeffreys, D.W., Baker-Austin, C., Ryan, J.J., Maskell, D.J., Pearce, G.P., 2016. Comparative metagenomics reveals a diverse range of antimicrobial resistance genes in effluents entering a river catchment. Water Sci. Technol. 73, 1541–1549. https://doi.org/10.2166/wst.2015.634.

Scott, G.I., Porter, D.E., Norman, R.S., Scott, C.H., Uyaguari-Diaz, M.I., Maruya, K.A., Weisberg, S.B., Fulton, M.H., Wirth, E.F., Moore, J., Pennington, P.L., Schlenk, D., Cobb, G.P., Denslow, N.D., 2016. Antibiotics as CECs: an overview of the hazards posed by antibiotics and antibiotic resistance. Front Mar Sci. 3, 1–15. https://doi.org/10.3389/fmars.2016.00024.

Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30, 2068–2069. https://doi.org/10.1093/bioinformatics/btu153.

Semedo, M., Song, B., 2020. From genes to nitrogen removal: determining the impacts of poultry industry wastewater on Tidal Creek denitrification. Environ. Sci. Technol. 54, 146–157. https://doi.org/10.1021/acs.est.9b03560.

Semedo, M., Song, B., Sparrer, T., Phillips, R.L., 2018. Antibiotic effects on microbial communities responsible for denitrification and N2O production in grassland soils. Front. Microbiol. 9. https://doi.org/10.3389/fmicb.2018.02121.

Storteboom, H., Arabi, M., Davis, J.G., Crimi, B., Pruden, A., 2010. Identification of antibiotic-resistance-gene molecular signatures suitable as tracers of pristine river, urban, and agricultural sources. Environ. Sci. Technol 44, 1947–1953. https://doi.org/10.1021/es902893f.

Tennstedt, T., Szczepanowski, R., Braun, S., Pühler, A., Schlüter, A., 2003. Occurrence of integron-associated resistance gene cassettes located on antibiotic resistance plasmids isolated from a wastewater treatment plant. FEMS Microbiol. Ecol. 45, 239–252. https://doi.org/10.1016/S0168-6496(03)00164-8.

USEPA, 2013. Literature Review of Contaminants in Livestock and Poultry Manure and Implications for Water Quality. Washington, DC.

Wright, G.D., 2007. The antibiotic resistome: the nexus of chemical and genetic diversity. Nat Rev Microbiol 5, 175.

Wu, D., Chen, G., Zhang, X., Yang, K., Xie, B., 2017. Change in microbial community in landfill refuse contaminated with antibiotics facilitates denitrification more than the increase in ARG over long-term. Sci. Rep. 7, 41230.

Wu, D., Huang, Z., Yang, K., Graham, D., Xie, B., 2015. Relationships between antibiotics and antibiotic resistance gene levels in municipal solid waste leachates in Shanghai, China. Environ. Sci. Technol 49, 4122–4128. https://doi.org/10.1021/es506081z.

Yoon, S., Cruz-García, C., Sanford, R., Ritalahti, K.M., Löffler, F.E., 2015. Denitrification versus respiratory ammonification: environmental controls of two competing dissimilatory NO3(-)/NO2(-) reduction pathways in shewanella loihica strain PV-4. ISME J. 9, 1093–1104. https://doi.org/10.1038/ismej.2014.201.

Zhu, Y.-G., Johnson, T.a., Su, J.-Q., Qiao, M., Guo, G.-X., Stedtfeld, R.D., Hashsham, S.a., Tiedje, J.M., 2013. Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc. Natl. Acad. Sci. U. S. A. 110, 3435–3440. https://doi.org/10.1073/pnas.1222743110.