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Legacy metal contamination is reflected in the fish gut microbiome in an urbanised estuary☆

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ABSTRACT

Estuaries are critical habitats subject to a range of stressors requiring effective management. Microbes are gaining recognition as effective environmental indicators, however, the response of host associated communities to stressors remains poorly understood. We examined microbial communities from seawater, sediments and the estuarine fish *Pelates sexlineatus*, in Australia's largest urbanised estuary, and hypothesised that anthropogenic contamination would be reflected in the microbiology of these sample types. The human faecal markers Lachno3 and HF183 were not detected, indicating negligible influence of sewage, but a gradient in copy numbers of the class 1 integron (*intI-1*), which is often used as a marker for anthropogenic contamination, was observed in sediments and positively correlated with metal concentrations. While seawater communities were not strongly driven by metal contamination, shifts in the diversity and composition of the fish gut microbiome were observed, with statistical links to levels of metal contamination ($F_{2,\ 21} = 1.536$, $p < 0.01$). Within the fish gut microbiome, we further report increased relative abundance of amplicon sequence variants (ASVs; single inferred DNA sequences obtained in sequencing) identified as metal resistant and potentially pathogenic genera, as well as those that may have roles in inflammation. These results demonstrate that microbial communities from distinct habitats within estuarine systems have unique response to stressors, and alterations of the fish gut microbiome may have implications for the adaptation of estuarine fish to legacy metal contamination.

1. Introduction

Estuaries are highly diverse and productive environments, providing key ecosystem services such as nutrient cycling, water filtration and acting as nursery grounds for fish (Morrisey et al., 1997), yet they are often threatened by anthropogenic activity, resulting in high levels of disturbance from multiple stressors (Dafforn et al., 2012). As the interface between terrestrial and marine ecosystems, urbanised estuarine sediments are often sinks for a wide range of contaminants (Barletta et al., 2019), most commonly metal(loid)s, organic chemical contaminants and nutrients (Jiang et al., 2001). Metal contamination is a global problem in estuaries, often due to the legacy impacts of historical industrialisation (Förster & Wittmann, 1979). Metal(loids) are persistent in the environment and potentially bioavailable and toxic to biota even at low concentrations (Machado et al., 2014; Prabhakaran et al., 2016). Given the often highly urbanised nature of estuarine systems, understanding the impacts of anthropogenic contaminants is crucial for managing their health and stability, which requires the availability of suitable indicators to assess these impacts.

Given their significance in ecosystem stability and function and capacity to rapidly respond to environmental perturbations, microbial communities have recognised potential for monitoring anthropogenic stressors in the environment (Aylagas et al., 2017; Glasl et al., 2019; Suzzi et al., 2021). Free-living and host-associated microbial communities display high habitat specificity (Glasl et al., 2019), and may display different responses when subjected to pulse vs. press stressors. While recent work has focused on bacterial communities in coastal sediments and their responses toward anthropogenic stressors (Liu et al.,

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2014; Birrer et al., 2018; Suzzi et al., 2021), less is known about how key host associated communities compare to free living communities when impacted. Fish represent almost half of all vertebrate species (Ghanbari et al., 2015) and influence ecosystem stability, resilience and food web dynamics in estuarine systems (Holmlund & Hammer, 1999). Most of the microorganisms associated with vertebrates are found within the gastrointestinal tract, where they influence physiology, immunity and development of the host (Colston & Jackson, 2016), yet knowledge of fish gut microbiomes in temperate estuaries is only recently emerging. Host-associated communities are thought to be relatively more stable than free-living communities when exposed to changes in environmental conditions, with tighter links to host condition (Roeselers et al., 2011; Marzinelli et al., 2015; Phelps et al., 2021; Weinstein et al., 2021), but little is known about the resistance and resilience of the fish gut microbiota (Chen et al., 2021). There is contrasting evidence that the fish gut microbiome can both remain stable over the long-term and alternatively be vulnerable to disruptions (Narrowe et al., 2015). Fish are unique among vertebrates in that they have two routes of metal acquisition: aqueous uptake of water-borne chemicals (from pore waters or overlying waters) via the gills and dietary uptake of contaminated food and sediment via the gut (Sauliutė & Svecevičius, 2015). Previous research indicates that diet is the predominant route of metal uptake in a range of estuarine fish (Creighton & Twining, 2010; Pan & Wang, 2016; McDonald et al., 2021), however pore water is also important in determining bioavailability and bioaccumulation of sedimentary metals in demersal fish (Guo et al., 2019). While the toxic effects of metals in different fish organs have been well addressed (Olmedo et al., 2013; Morgano et al., 2014), effects on the gut microbiome have received less attention despite key roles in mediating biotransformation of chemicals and contaminants, physiological function and host health (Wang et al., 2017; Adamovsky et al., 2018). The gut microbiome also represents an important avenue for understanding the effects of contaminants on hosts given that it may influence individual dosages and availability *in situ*, with possible long-term implications for host adaptations in contaminated environments (Adamovsky et al., 2018).

There are several approaches currently applied to understanding impacts to microbial communities. Stressed microbiomes may display a shift to an alternative stable state (Chen et al., 2021) or greater inter-individual variability (i.e. dispersion) (Zaneveld et al., 2017) which may compromise the resilience of the community to subsequent stress events (Zaneveld et al., 2016). This can be reflected by measures of alpha and beta diversity, with low species richness, stability and dysbiosis closely associated with host stress or disease (Li et al., 2017; Nie et al., 2017). Shifts in the abundance of particular taxonomic groups may also be indicative of impacts, for example the *Lachnospiraceae* and *Bacteroidales* are commonly used as indicators of faecal material (Feng & McLellan, 2019; Feng et al., 2018), and the *Arcobacter* genus may be associated with stormwater infrastructure (Fisher et al., 2014). Specific genes have further been proposed as indicative of anthropogenic stress, such as the class 1 integron (*intI-1*) gene which has been linked to environmental contaminants such as heavy metals (Gillings et al., 2015).

Therefore, examination of whole microbial communities and quantification of microbial taxa and/or genes indicative of stress are likely to be useful approaches for investigating microbial community responses to anthropogenic stressors in estuaries. Hence, the aims of this study were to 1) investigate how the community composition and diversity of free-living and host-associated microbial communities change in response to legacy metal contamination in an urbanised NSW estuary, and 2) examine the presence and abundance of specific groups of potential indicator taxa (*Lachnospiraceae, Bacterodales, Arcobacter*) and the *intI-1* gene, which have all been linked to anthropogenic stressors. We hypothesised that the concentration of metals and indicator taxa and genes will be enriched in sites experiencing greatest anthropogenic influence, alpha and beta diversity will be negatively affected by increased anthropogenic impact, and chronic, long-term contamination of estuarine sediments will have a greater influence on the fish hindgut microbiome than the water column.

2. Methods

Lake Macquarie is the largest coastal lake in Australia. The surrounding environment is highly developed with a number of land uses along the shoreline and catchment area (Roach, 2005; Potts et al., 2011). Sample sites were selected here to represent different anthropogenic disturbances (Fig. 1a). Industrial development was previously extensive in the northern part of the estuary, resulting in a metal gradient within the lake (Roach, 2005; Batley, 1987; Roy & Crawford, 1984). The most significant operation was a lead-zinc smelter on Cockle Creek that ceased operations in 2003 (Schneider et al., 2014), with other operations including a fertiliser plant, a steel foundry, collieries and sewage treatment works (Batley, 1987). The lake is also subject to urban stormwater discharges from the fringing catchment as well as diffuse source runoff, with these identified as the largest on-going threats to the ecological health of Lake Macquarie (Department of PlanningIndustry and Environment, 2020). During large rainfall events, urban stormwater discharge from major sub-catchments drains to the northern bays (e.g. Cockle and Fennell Bays), resulting in localised impacts such as phytoplankton blooms (Department of PlanningIndustry and Environment, 2020). Compared to the northern part of the lake, water quality in the south-east part of the lake is generally classified as very good, due to low turbidity and chlorophyll *a* (Department of PlanningIndustry and Environment, 2020) and low anthropogenic influence. Therefore, Murrays Beach and Gwandalan were selected as control sites for the study here. There are coal-fired power stations including ash dams and cooling waters in the south-western reaches of the Lake contributing to elevated metals and thermal discharges, but impacts are localised and do not extend to the south-eastern part of the lake.

2.1. Field sampling

Sampling occurred between late February and early March 2020, during dry weather conditions, whereby there had been less than 1 mm of rain day^{-1} in the 7 days prior. This allowed us to assess the impact of legacy metal contamination on microbial communities under background conditions. Environmental parameters were recorded at each site using a Horiba U-50 water quality meter to record temperature, salinity, pH, turbidity and dissolved oxygen. Seawater was collected from each site for chlorophyll *a* determination (n = 3), which was subsequently performed using acetone extraction (Parsons et al., 1984). Samples for microbial analysis were collected in sterile 1 L bottles (rinsed in 10% bleach solution) (n = 5) and approximately 800 mL from each sample was filtered onto 0.2 μm pore sized Sterivex filters, depending on the amount of particulate organic matter present, For sediment analysis, 50–60 mL was collected for sediment granulometry, determination of organic matter, mud content and metal analysis (n = 3) using a 50 mL Luer Lock syringe plunged vertically into the sediment to approximately 100 mm depth. Additional sediment samples were collected from the upper 1 cm layer using sterile 15 mL tubes for microbial analysis (n = 5). Sediments were not collected from Speers Point due to a mix of large rocky and fine silty sediment. Seagrass (*Zostera muelleri*) blades were collected at each site for stable isotope analysis of nitrogen (n = 3).

Specimens of an estuarine fish species, the eastern striped grunter (*Pelates sexlineatus*), were collected using a 10 m seine net pulled through seagrass beds for stable isotope analysis of nitrogen (n = 3) and to investigate host-associated microbial communities via 16 S rRNA gene sequencing (n = 5). *P. sexlineatus* is one of the 10 most common estuarine fish families occupying Australian seagrass meadows and is distributed along the south-east coast of New South Wales (Pollard, 1984; Trnski & Neira, 1998; Smith & Suthers, 2000). Fish could not be collected at Speers Point likely due to a lack of suitable seagrass habitat here. All samples were immediately stored on ice and processed within

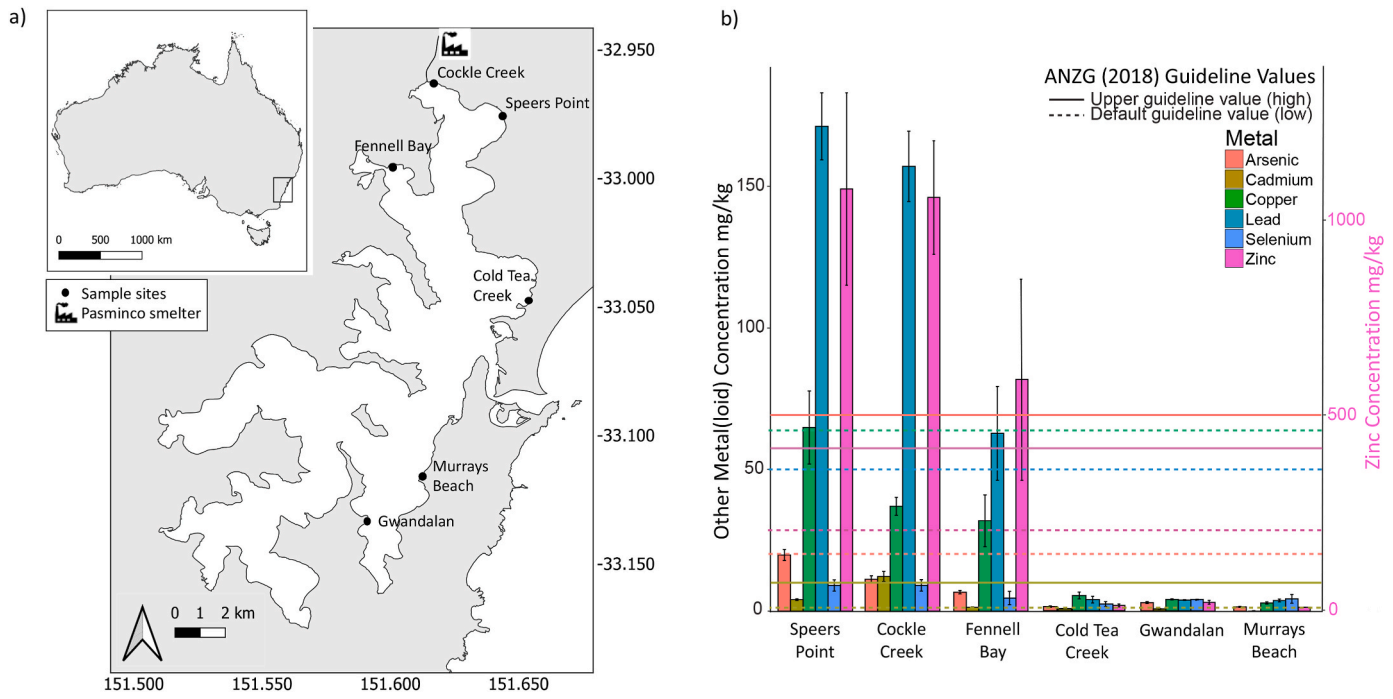

Fig. 1. Location of a) and total metal concentrations in sediments from b) sample sites in Lake Macquarie. Zinc concentration is shown on the right y axis, with arsenic, cadmium, copper, lead and selenium shown on the left y axis. Horizontal lines on the graph represent ANZG (2018) guideline values for each metal, colour coded to match the legend. Below default guideline values (low) there is a low risk of unacceptable impacts occurring. Upper guideline values (high) provide an indication of concentrations at which toxicity-related adverse effects may be observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

6 h of collection.

2.2. Metal analysis

Sediment samples were dried until they reached constant weight at 60 °C and homogenised using a Retsch MM200 mixing mill. For total recoverable metals, approximately 500 mg of homogenised sample was combined with 12 mL of aqua regia (HNO_3/HCl: 1:3, ICP-MS grade, Merck) and digested using a microwave digestor (ETHOS EASY – Advanced Microwave Digestion System, Milestone) on the soil digestion program – "EPA:3051 A (alternative)". Once digested, samples were filtered through a DigiFILTER (SCP Science) with a pore size 0.45 μm hydrophilic PTFE membrane and made up to 50 mL volume. Further, we used the weak acid partial extraction method for bioavailable metal analysis (Duodu et al., 2017; Roe et al., 2020). About 50 mg of sediment sample was weighed in a centrifuge tube and mixed with trace metal grade 1 M HNO_3 (Seastar chemicals) to a final volume of 50 mL and was then rotated for 6 h. A 0.45 μm syringe filter (Millipore cellulose acetate hydrophilic membrane) was used to filter the resulting aliquot, and 10 mL filtrate was taken in an inductively coupled plasma mass spectrometer (ICP-MS) tube (polypropylene). Selected candidate metals, copper (Cu), zinc (Zn), arsenic (As), selenium (Se), cadmium (Cd), and lead (Pb) were analysed using an Agilent 7900 (Agilent Technologies, Tokyo, Japan) ICP-MS coupled with an autosampler (SPS 4, Agilent Technologies). A multi-elemental standard was used in the calibration process (High-Purity Standards, SC, USA). Internal standards were used to prevent calibration drift, and 2% HNO_3 was used as a matrix blank. To verify recovery within the digestion process, we used standard reference materials, marine sediment (PACS-2, Canada) and Montana soil (2711) (Table S1).

2.3. Stable isotope analysis

Eastern striped grunter and seagrass (n = 3) were processed for stable isotope analysis of nitrogen. Prior to processing, fish were weighed, total length was recorded, and Fulton's condition factor (*K*) was calculated using the following: $K = 100 \times W/L^3$ (where W and L are the recorded weight and total length, respectively) (Froese, 2006). Muscle tissue was excised from fish and seagrass blades were carefully cleared of epiphytes. Samples were dried at 60 °C for 24 h, ground to a fine powder using a Retsch MM200 mixing mill and then weighed into tin capsules and sent to the Stable Isotope Laboratory at Griffith University for nitrogen isotope analysis using a Secron Hydra 20–22 automated Isoprime Isotope Ratio Mass Spectrometer.

2.4. DNA extraction

Eastern striped grunter specimens were dissected and hindgut contents removed, and a 1 g sample was weighed from collected sediments. Prior to DNA extraction, all samples were stored at −80 °C. DNA extraction for fish hindgut and sediment samples was carried out using Qiagen PowerSoil Kits and from seawater using Qiagen PowerWater Kits, with sample quantity and quality checked using a NanoPhotometer NP80.

2.5. Quantitative PCR (qPCR)

Quantitative PCR (qPCR) was used to target the 16 S gene, using the primers BACT1369F and PROK1492R paired with the TM1389F probe (Table S2), to deliver a proxy measure of bacterial abundance in all samples. To detect human faecal material indicative of sewage, we employed two qPCR assays, including the HF183 assay, which targets the human associated HF183 *Bacteroidales* cluster (Templar et al., 2016), and Lachno3, which targets human associated *Lachnospiraceae* (Feng et al., 2018). In addition, we quantified *Arcobacter*, which is a bacterial genus commonly associated with stormwater infrastructure, using the ARCO assay targeting the 23 S rRNA *Arcobacter* gene (Bastyns et al., 1995). We also quantified the Class 1 Integron gene, *intI-1* (Mazel et al.,

2000), which has previously been shown to be an excellent marker for anthropogenic contamination (Gillings et al., 2015). See Table S2 for specific details of all assays.

All qPCR analyses were performed on a BIO-RAD CFX384 Touch™ Real-Time PCR Detection System™. In each case, gene copies were calculated for each target, using a standard curve and BIO-RAD's CFX MAESTRO™ software version 1.1. Standard curves were generated from known concentrations of the targeted section of each gene. Each qPCR was run in triplicate with SYBR assays consisting of 5 μL reaction volumes that consisted of 2.5 μL BIO-RAD iTaq Universal SYBR® Green Supermix, 1.1 μl nuclease free water, 0.2 mM of each forward and reverse primer and 1 μL of diluted (1:20) DNA template. Probe assays consisted of 2.5 μL BIO-RAD iTaq UniversalProbes® Supermix, 1 μL nuclease free water, 0.2 mM of each forward and reverse primer, 0.2 mM of probe and 1 μL of diluted (1:20) DNA. A dilution of 1:20 was made to eliminate the influence of DNA inhibitors on amplicon efficiency. The dilution was chosen after performing qPCR on a range of dilutions on both hindgut and seawater samples, and selection based on the lowest dilution that provided consistent results. Calibration curves were run with every plate. Plate preparation was conducted using an epMotion® 5075 I Automated Liquid Handling System. QPCR cycling conditions consisted of an initial denaturation step at 95 °C for 3 min, 45 cycles of: 95 °C for 15 s and a variable annealing temperature for 1 min, with the exception of *intI-1* which had an annealing time of 30 s. Melt curves were added to all SYBR assays to confirm the amplification of a single product.

2.6. 16 S rRNA sequencing

Hindgut and seawater bacterial communities were characterised using 16 S rRNA amplicon sequencing. The V3–V4 region of the 16 S rRNA gene from prokaryotes was amplified using universal primers 341 F (5′-CCTACGGGNGGCWGCAG -3′) and 805 R (5′-GACTACHVGGGTATCTAATCC -3′) attached with Illumina adaptors in PCR with the following cycling conditions: 95 °C for 3 min, then 25 cycles of: 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, then 72 °C for 5 min. PCR products were sequenced using the Illumina Miseq v3 (2 × 300bp) platform at the Ramaciotti Centre for Genomics at the University of New South Wales. Resultant amplicons were processed using the R pipeline DADA2 with default parameters (Callahan et al., 2016), chimeras removed and contigs assembled. Reads were clustered to produce amplicon sequence variants (ASVs) and sequences were aligned to the SILVA v132 database (Yilmaz et al., 2014) for taxonomic assignment. The dataset was further cleaned by removing singletons and those identified as non-bacterial or chloroplasts.

2.7. Statistical analysis

Statistical analysis was carried out using R Studio (R Core Team, 2018). To analyse differences in environmental parameters and fish length, weight and condition factor among sites, analysis of variance (ANOVA) with Tukey's HSD were employed using the 'aov' and 'TukeyHSD' functions in the 'vegan' package (Oksanen et al., 2019). Differences in metal concentrations between sites as determined by ANOVA and Tukey's HSD were used to classify metal contamination into three groups: high, mid and low (see results). Differences in the abundance of *Lachnospiraceae, Bacterodales, Arcobacter* and the *intI-1* gene between sites and sample types were also determined using ANOVA and Tukey's HSD. The abundance of these microbial markers were further tested for correlation with environmental parameters using Pearson correlation and visualised using scatterplots.

For microbial community data, seawater and hindgut samples were analysed separately using the 'phyloseq' package (McMurdie & Holmes, 2015). Data was rarefied using the 'rarefy even depth' function to the smallest sample size: 1432 reads per sample for hindguts and 159,128 for seawater. Biodiversity metrics were then calculated using rarefied sequence data. Alpha diversity measures (Richness, Shannon index and Simpson index) were compared between sites and analysed with Pairwise Wilcoxon Rank Sum Tests. Beta diversity was investigated with Bray-Curtis distance matrices and presented using principal coordinates analysis (PCoA). PERMANOVA was carried out using the 'adonis' function in the 'vegan' package (Oksanen et al., 2019), with pairwise comparisons carried out using the 'pairwise.perm.manova' function in the 'RVAideMemoire' package (Hervé, 2021). For further investigation into fish hindgut ASVs that differed significantly in abundance between high, mid and low metal contaminated sites, SIMPER was carried out using the 'simper' function in the 'vegan' package (Oksanen et al., 2019). Canonical correspondence analysis was used to assess how environmental variables and abundance of *Lachnospiraceae, Bacteroidales, Arcobacter* and the *intI-1* gene as determined by qPCR may be linked with microbial community composition, using the function 'cca' in the 'vegan' package (Oksanen et al., 2019). Environmental variables were checked for collinearity using Pearson correlations. Those that were highly correlated ($R^2 \geq 0.8$; $p < 0.05$) were removed from the analysis with only one parameter retained for each group of highly correlated variables.

3. Results

3.1. Environmental conditions

Water column temperature, salinity, dissolved oxygen and turbidity varied throughout the lake (21.16 °C–26.22 °C, 18.79–34.46 ppt, 5.95 mg^{-L} – 9.18 mg^{-L} and 0–23.9 NTU respectively) (Table S3). Water column pH did not vary greatly throughout the lake, ranging from 7.53 at Cockle Creek to 8.04 at Speers Point (Table S3). Water column chlorophyll *a* differed between sites (ANOVA, $F_{5, 12} = 10.74$, $p < 0.01$), with the concentration significantly higher at Cockle Creek and Speers Point than all other sites, except for Gwandalan ($p < 0.05$). Sediment grain size differed between sites ($F_{5, 12} = 9.769$, $p < 0.01$), ranging between medium (250–500 μm) and fine (125–250 μm) sand (Wentworth, 1922) throughout Lake Macquarie, with Gwandalan differing significantly from all other sites, dominated by medium sand ($p < 0.01$; 393.8 ± 54.35). Sediment organic matter also varied throughout the lake, ranging between 1.19 and 6.8% (Table S3).

3.2. Legacy metal contamination

Analysis of variance (ANOVA) was used to determine significant site differences according to overall combined total metal contamination ($F_{5, 12} = 10.74$, $p < 0.01$), and to group sites by level of metal contamination, resulting in high (Cockle Creek and Speers Point), mid (Fennell Bay) and low (Cold Tea Creek, Gwandalan and Murrays Beach) groups (Fig. 1b; Table S4). ANOVA comparing each metal individually further revealed significant differences for all total and bioavailable sediment metal concentrations between sample sites, except for selenium (selenium total: $F_{5, 12} = 3.382$, $p = 0.06$; selenium bioavailable: $F_{5, 12} = 2.073$, $p = 0.14$). Total and bioavailable concentrations of zinc ($F_{5, 12} = 10.95$, $p = 0.00$; $F_{5, 12} = 12.42$, $p < 0.01$) and lead ($F_{5, 12} = 65.36$, $p < 0.01$; $F_{5, 12} = 62.75$, $p < 0.01$) were significantly elevated in northern sites Cockle Creek and Speers Point, followed by Fennell Bay, in comparison to southern sites (Table S4). Concentrations of total and bioavailable cadmium ($F_{5, 12} = 39.45$, $p < 0.01$; $F_{5, 12} = 41.24$, $p < 0.01$) were most elevated at Cockle Creek followed by Speers Point and Fennell Bay, and copper ($F_{5, 12} = 14.22$, $p < 0.01$; $F_{5, 12} = 17.82$, $p < 0.01$) and arsenic ($F_{5, 12} = 53.57$, $p < 0.01$; $F_{5, 12} = 88.59$, $p < 0.01$) were most elevated at Speers Point, followed by Cockle Creek and Fennell Bay (Table S4).

Bioavailable concentrations of zinc, lead, copper and cadmium comprised 70–95% of total metal concentrations in northernmost sites Cockle Creek and Speers Point, 65–100% at Fennell Bay and 0–100% across Cold Tea Creek, Gwandalan and Murrays Beach (Table S4). Bioavailable concentrations of arsenic ranged from 60 to 77% across all

sample sites within Lake Macquarie, and selenium displayed the lowest bioavailability with bioavailable concentrations 2–12% of total metal concentrations across the study (Table S4).

3.3. *Pelates sexlineatus* condition factor and site fidelity

Pelates sexlineatus length (mm) varied between sample sites ($F_{4, 20} = 4.104$, $p < 0.05$) with significantly larger fish recorded at Murrays Beach (78.8 ± 5.5 mm) compared to northern sites Cockle Creek (49.2 ± 6.7 mm) and Fennell Bay (53.4 ± 4.1 mm) (Table S5). Fulton's condition factor (*K*) did not differ significantly between sites ($F_{4, 20} = 1.314$, $p = 0.29$) (Table S5). Pearson correlations revealed significant relationships between bioavailable zinc, lead, copper, cadmium and arsenic concentrations and total length of *Pelates sexlineatus* (Table S6).

Both seagrass blade and *Pelates sexlineatus* muscle tissue $\delta^{15}N$ differed significantly between sites ($F_{5, 12} = 78.88$, $p < 0.01$ and $F_{4, 10} = 10.82$, $p < 0.01$ respectively), ranging from 1.6 ± 0.2–4.7 ± 0.2‰ for seagrass and 8.5 ± 0.2–10.2 ± 0.4‰ for fish. Pearson correlation revealed a significant positive relationship between fish and seagrass $\delta^{15}N$ ($R = 0.66$, $p < 0.05$) throughout the lake (Fig. S1).

3.4. Quantification of microbial indicators

Copy numbers of the 16 S gene, used here as a proxy for total bacterial abundance, did not differ significantly between sample type ($F_{2, 71} = 1.067$, $p = 0.34$), and within sample types differed by site for seawater samples ($F_{4, 20} = 4.709$, $p = 0.00$) but not sediment ($F_{4, 20} = 2.544$, $p = 0.07$) or hindgut ($F_{4, 19} = 0.958$, $p = 0.45$). Within seawater samples, copy numbers were significantly greater at Gwandalan compared to all other sites except for Fennell Bay ($p < 0.05$) (Table S7). For seawater samples, an average of $4.28 \times 10^6 \pm 6.63 \times 10^5$ gene copies mL^{-1} were detected, compared to $9.18 \times 10^8 \pm 1.10 \times 10^8$ copies g^{-1} for sediment and $3.23 \times 10^{11} \pm 3.16 \times 10^{11}$ g^{-1} in hindguts. Copy numbers from hindgut samples were variable, with one sample from Cockle Creek reporting a count of 9.2×10^{12}, 3 orders of magnitude greater than mean counts recorded from other sites (Table S7). The human faecal markers Lachno3 and HF183 were not detected in any samples across Lake Macquarie.

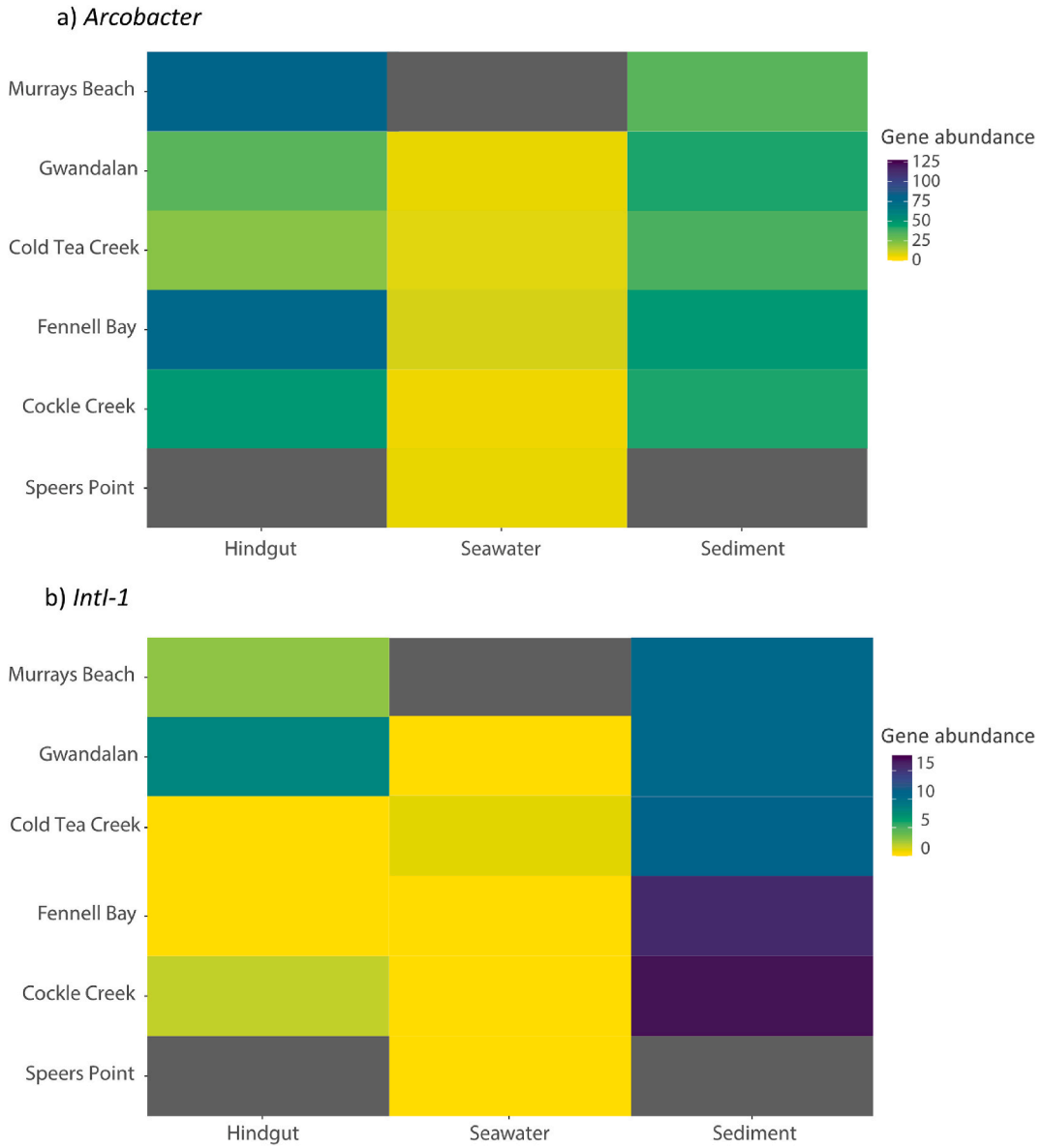

Fig. 2. Fourth root transformed gene abundances of a) *Arcobacter* and b) *intI-1* between hindgut, seawater and sediment samples. Abundances are in number of gene copies g^{-1} for sediment and fish hindgut samples, and gene copies mL^{-1} for seawater.

Relative *Arcobacter* gene abundances differed between sample type ($F_{2, 71} = 4.867$, $p < 0.01$), with counts from hindgut samples higher than both seawater and sediment (Fig. 2b) (Table S7). Gene abundances also differed significantly between site for both sediment ($F_{4, 20} = 3.297$, $p < 0.05$) and seawater samples ($F_{4, 20} = 5.151$, $p < 0.01$), but not hindgut ($F_{4, 18} = 1.359$, $p = 0.287$) (Table S7). In sediment samples, *Arcobacter* concentrations were greatest at Fennell Bay ($6.7 \times 10^6 \pm 1.8 \times 10^6$ gene abundances g^{-1}) and lowest at Murrays Beach ($1.6 \times 10^6 \pm 3.3 \times 10^5$ gene abundances mL^{-1}) (Table S7). In seawater samples, *Arcobacter* was found at comparatively lower concentrations, ranging from 59.02 ± 3.0 gene abundances mL^{-1} at Gwandalan to $5.5 \times 10^3 \pm 1.7 \times 10^3$ gene abundances mL^{-1} at Fennell Bay (Table S7).

Relative *IntI-1* gene abundances differed between sample type ($F_{2, 71} = 12.03$, $p < 0.01$), with copy numbers in sediment differing from both hindgut and seawater (Fig. 2a). *IntI-1* was highest in sediment samples, and within sediments differed significantly between sites ($F_{4, 20} = 4.609$, $p < 0.01$), significantly elevated at the northern highly metal contaminated site Cockle Creek ($4.7 \times 10^4 \pm 1.1 \times 10^4$ gene abundances g^{-1}) and decreasing in southern sites characterised by low metal contamination, Murrays Beach and Gwandalan (Table S7). Further, Pearson correlations comparing environmental variables and sediment *intI-1* abundances revealed significant positive correlations between the concentration of bioavailable metals in sediments and the number of gene abundances g^{-1} of *intI-1* in sediments (Fig. 3). *IntI-1* gene abundances did not differ significantly between sample sites for hindgut ($F_{4, 19} = 2.281$, $p = 0.09$) or seawater samples ($F_{4, 16} = 1.499$, $p = 0.24$).

3.5. Bacterial community diversity and composition

A total of 1,175,570 sequences were obtained for fish hindgut

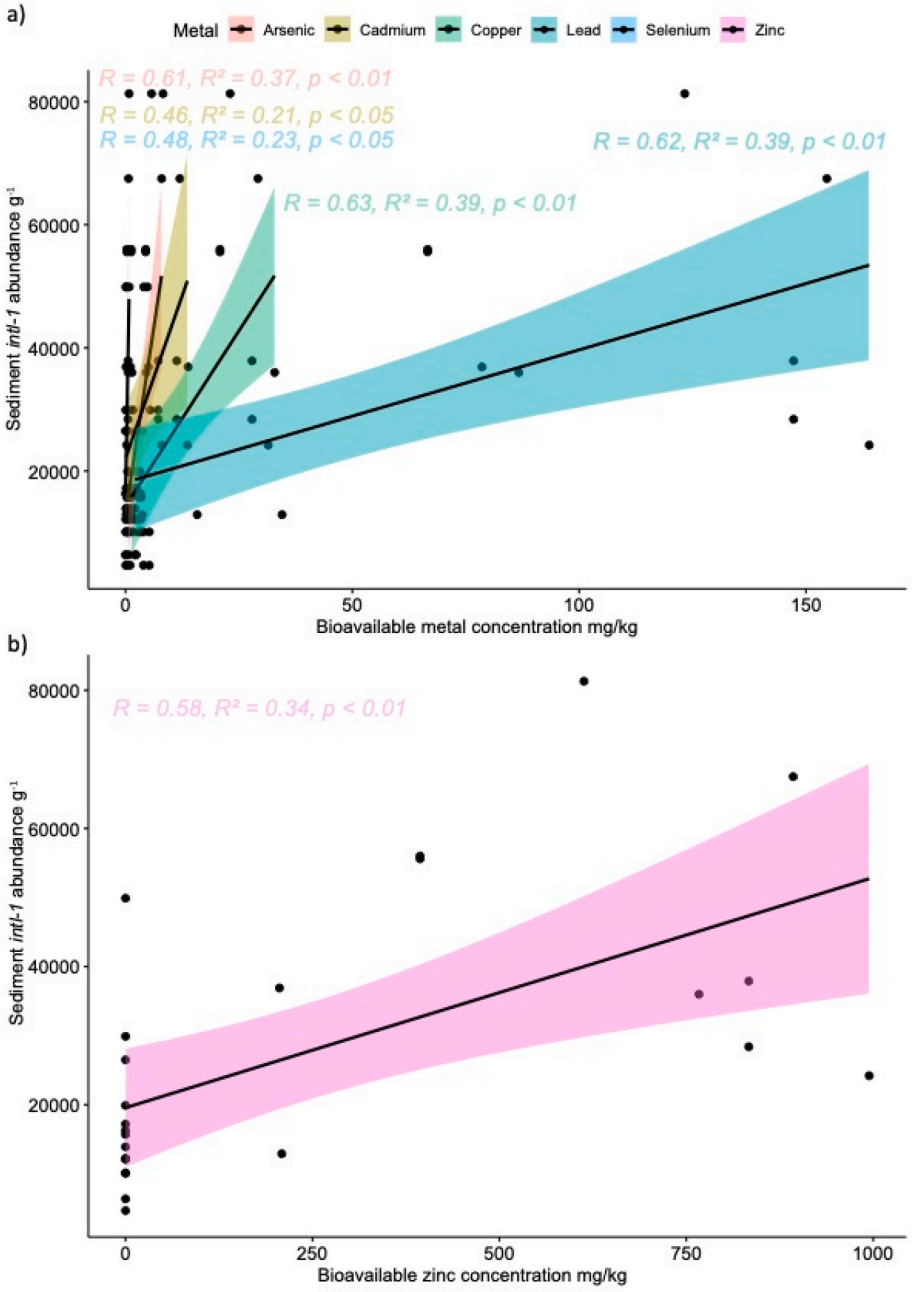

Fig. 3. Pearson correlations of sediment *intI-1* gene abundance g^{-1} and bioavailable sediment metal concentrations (mg/kg): a) arsenic, cadmium, copper, lead and selenium; and b) zinc. Zinc is plotted separately due to higher concentrations than other metals.

samples (mean 48,982.08 ± 14,963.14) and 5,657,766 for seawater (226,310.64 ± 6621.33) after merging, quality filtering, denoising and removal of chimeric and non-bacterial sequences. After rarefying, sequences were assigned to 530 bacterial ASVs for hindgut samples and 5143 ASVs for seawater samples. Alpha diversity measures did not differ significantly between sites for hindgut or seawater samples (Fig. 4a; Fig. S2a), however some trends towards differences between level of metal contamination were evident. More specifically, observed species richness and Chao1 differed significantly between sites characterised by high and low metal contamination for hindgut communities ($p < 0.05$ and $p < 0.05$ respectively) (Fig. 4a). Simpson diversity differed between all levels of metal contamination for seawater communities ($p < 0.05$) (Fig. S2a). PERMANOVA revealed significant variation for both hindgut and seawater communities between sites ($F_{4,\ 19} = 1.386$, $p < 0.01$ and $F_{4,\ 20} = 14.416$, $p < 0.01$ respectively) and level of metal contamination ($F_{2,\ 21} = 1.536$, $p < 0.01$ and $F_{2,\ 22} = 4.915$, $p < 0.01$ respectively). Pairwise comparisons revealed that sites with low metal contamination differed from those with mid and high metal contamination for hindgut communities (Fig. 4b), while seawater communities displayed clear clustering by site, with all sites and levels of metal contamination differing significantly ($p < 0.05$) (Fig. S2b).

Dominant phyla across hindgut samples (>1% of all sequences across all samples) were *Proteobacteria* (mean relative abundance of 76.9% ± 5.1), *Firmicutes* (13.0% ± 3.9), *Tenericutes* (3.6% ± 3.5), *Actinobacteria* (1.3 ± 0.4), *Cyanobacteria* (1.3 ± 0.3), *Bacteroidetes* (1.2% ± 0.4), *Chlamydiae* (0.9% ± 0.6), *Chloroflexi* (0.8 ± 0.4) *Planctomycetes* (0.2% ± 0.4), *Epsilonbacteraeota* (0.2% ± 0.1) and *Fusobacteria* (0.2% ± 0.1) (Fig. 4c). SIMPER identified those that differed significantly in abundance between high, mid and low metal contamination sites (Table S8). Several ASVs belonging to the Rhodobacteraceae family were found to be more abundant at sites characterised by a high and mid metal contamination in comparison to low impact, as well as ASVs identified as the genera *Turicibacter, Stenotrophomonas* and those from the family Burkholderiaceae (*Ralstonia* and *Burkholderia*) (Table S8).

Seven phyla dominated seawater samples (>1% of all sequences across all samples): *Proteobacteria* (62.9 ± 1.4), *Bacteoridetes* (13.0% ± 2.1), *Cyanobacteria* (10.3% ± 0.9), *Actinobacteria* (6.1% ± 0.7), *Marinimicrobia* (3.8% ± 0.6), *Planctomycetes* (1.5% ± 0.2), and *Verrucomicrobia* (0.9% ± 0.1) (Figure S2c). While the PCoA plot based on Bray-Curtis dissimilarity shows that sites with a high and mid metal contamination cluster together (Cockle Creek, Speers Point and Fennell Bay), sites with a low metal contamination (Cold Tea Creek and Gwandalan) are quite distinct from one another and from all other sites (Figure S2b). Cold Tea Creek is characterised by an increased relative abundance of *Bacteroidetes,* with decreased *Cyanobacteria* and *Proteobacteria* relative to other sites (Figure S2c).

3.6. Relationship with environmental variables

Bioavailable sediment metal concentrations (lead, copper, cadmium and arsenic were removed from CCA analysis due to collinearity with zinc; Table S9), *intI-1* abundance, grain size and organic matter as well as water column pH, temperature, salinity and chlorophyll *a* were identified as drivers of variation in fish hindgut microbial community

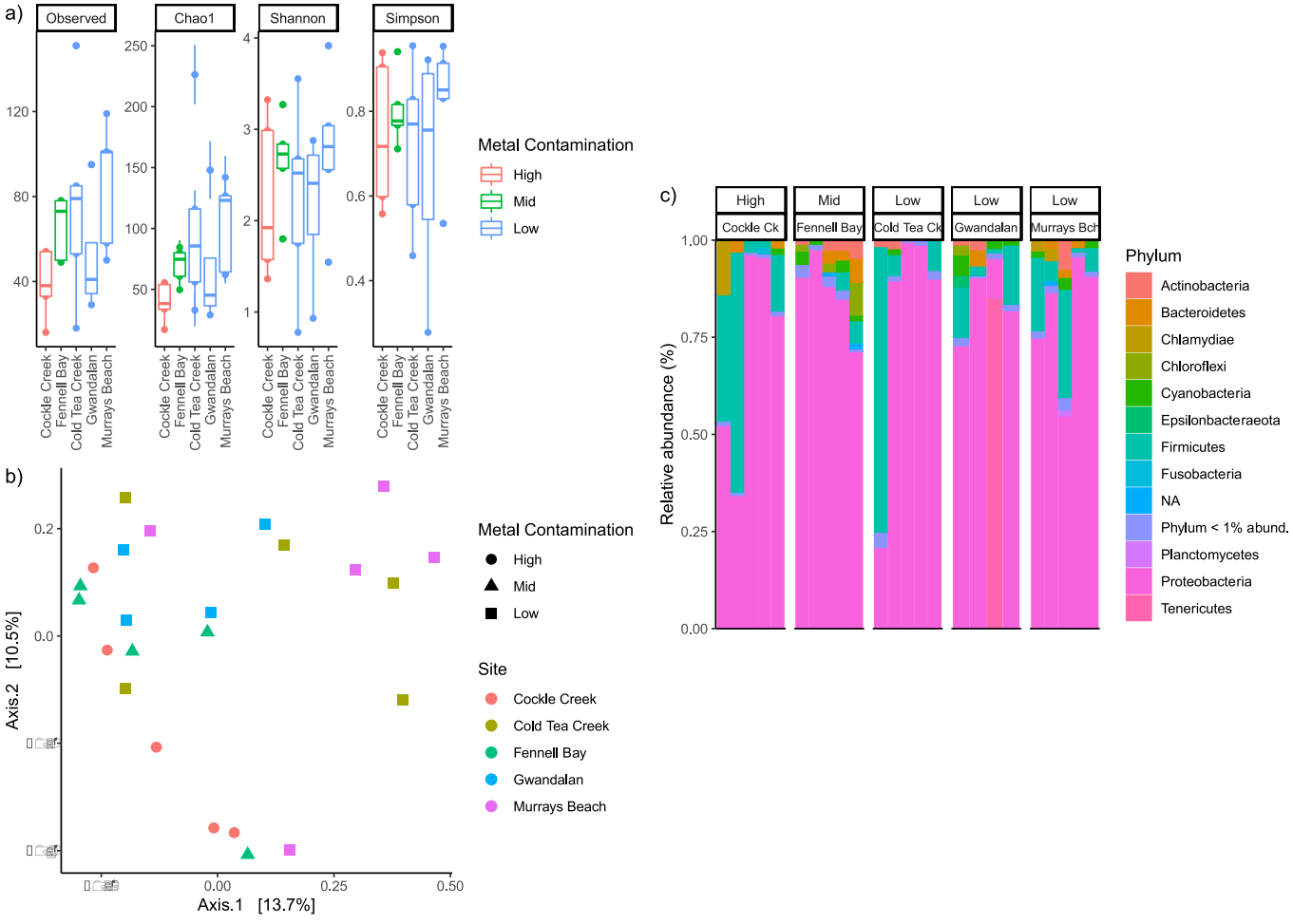

Fig. 4. Microbial community composition of *Pelates sexlineatus* hindgut samples across sample sites and levels of metal contamination: a) PCoA plot based on Bray-Curtis dissimilarity and barplot of eigenvalues for each axis b) alpha diversity and c) relative abundance of bacterial phyla.

composition (Fig. 5a). Increased bioavailable metal concentrations, sediment organic matter and temperature were linked to distinct communities from Cockle Creek and Fennell Bay, sites with high and mid metal contamination (Fig. 5a). Samples from low metal contamination sites were more closely linked to increased chlorophyll *a*, sediment grain size and *intI-1* abundance (Fig. 5a). Water column pH, salinity and temperature were identified as drivers of variation in seawater microbial communities, as well as sediment grain size, organic matter and metal concentrations (Fig. 5b). Sediment organic matter was linked to communities from Fennell Bay and Gwandalan, while bioavailable metals and temperature were linked with communities from Cockle Creek (Fig. 5b).

4. Discussion

We demonstrated that in highly urbanised estuaries, sediments act as sinks for metal contaminants from historical industrial activity and long-term exposure to this contamination can lead to impacts that affect the gut microbiome of a common estuarine fish. Hindgut microbial communities of the abundant estuarine fish *P. sexlineatus* displayed shifts in composition and diversity at sites characterised by legacy metal contamination, with an increased abundance of metal tolerant and potentially pathogenic bacterial taxa relative to low impact sites.

4.1. Anthropogenic contaminants

Our analysis revealed a trace metal gradient in Lake Macquarie consistent with previous reports (Batley, 1987; Roy & Crawford, 1984). Both total and bioavailable metal concentrations were significantly greater at the northernmost sites and declined in southern sites. Since the 1980s, concentrations of lead, zinc and cadmium in sediments have decreased throughout Lake Macquarie (Batley, 1987; Roach, 2005; Lopez et al., 2014). Our results indicate trends of contemporary decreases in metals, with all recorded metals at Cockle Creek found in lower concentrations than those reported in Roach (2005), except for zinc and selenium. Interestingly, both Lopez et al. (2014) and the present study recorded increases in the concentration of metals at Speers Point, whereby levels exceeded those recorded by Roach (2005) by up to tenfold. Elevated loads of metal(loid)s in Cockle Creek are likely attributable to resuspension of legacy loads from inputs from the decommissioned Pb/Zn Smelter and/or spatial variability in sampling sediment. There are two operational coal-fired power stations on the southern shores of Lake Macquarie, Eraring and Vales Point, with sediments near these power stations contaminated with selenium-contaminated fly ash (Batley, 1987; Kirby et al., 2001). Selenium contamination in the southern basin of the lake has been attributed to ongoing activities of these coal-fired power stations and may explain elevated selenium at these sites (Kirby et al., 2001).

Selenium, copper and zinc are essential for fish metabolism and occur naturally in aquatic systems (Bury et al., 2003), while cadmium and lead have no known biological role (Canli & Atli, 2003). When additional metals are introduced from polluting industries, aquatic organisms may be exposed to concentrations that induce sublethal or lethal toxic effects (Biddinger & Gloss, 1984). Despite an overall decreasing trend in metal contamination since the 1980s, most total and bioavailable metal concentrations recorded in the present study at northern sites Cockle Creek, Speers Point and Fennell Bay exceeded ANZG (2018) lower guideline values. Zinc and cadmium further exceed upper guideline values at some northern sites. Default guideline values indicate a risk of adverse biological and ecological impacts, with upper guideline values providing an indication at which toxicity-related adverse effects may be observed (ANZG, 2018). Guideline values are not currently available for selenium due to limited data availability, however Kirby et al. (2001) indicated background concentrations of selenium in the lake at 0.3 mg/kg. Total selenium concentrations at all sites exceeded this background concentration indicating widespread contamination of selenium, however bioavailable selenium made up a low percentage of total concentrations throughout the lake indicating little biological risk (ANZG, 2018). Exceedances of guideline values do not guarantee ecological impacts, with effects varying from increased bioaccumulation in the tissues of organisms and changes in reproduction and growth to dramatic reductions in biodiversity and functioning of benthic communities (Roach, 2005; Chapman et al., 1989; Lamberson et al., 2018; ReynoldsonThomas et al., 1987; Scott, 1989). The total length of *Pelates sexlineatus* individuals collected in Lake Macquarie showed a significant negative relationship with increasing concentrations of bioavailable cadmium, copper, lead, zinc and arsenic in sediments in this study, indicating that growth may have been affected by available metals. Metals have been shown to directly transfer from contaminated sediments to benthic organisms (Selck et al., 1998; Wang et al., 1999; Griscom et al., 2002; Wang et al., 2002), and can then transfer from prey items to fish (trophic uptake route) (Dallinger et al.,

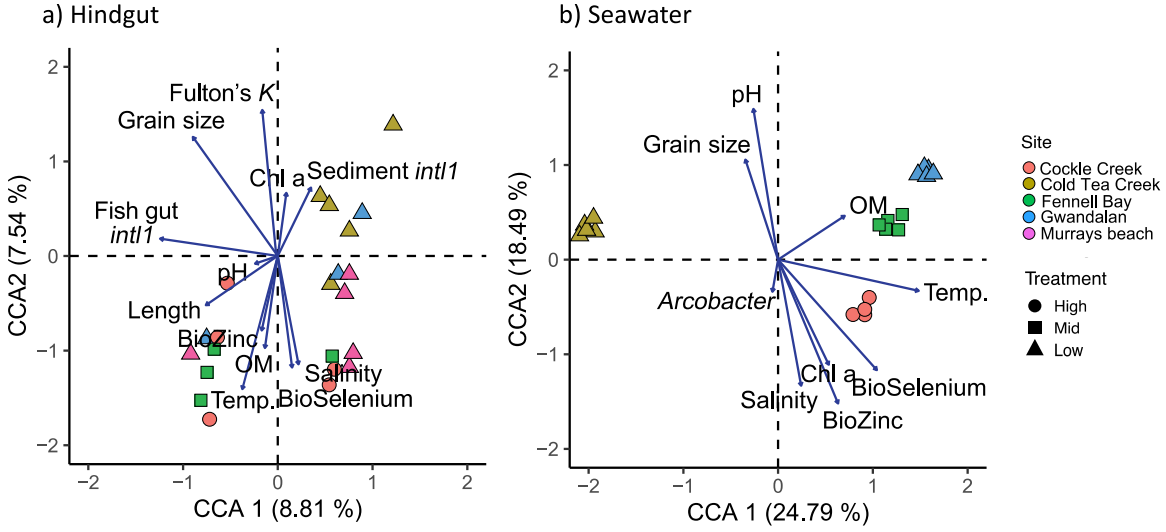

Fig. 5. Canonical correspondence analysis (CCA) plots comparing environmental parameters and qPCR assays and a) *P. sexlineatus* hindgut and b) seawater microbial communities. DO stands for water column dissolved oxygen and temp. Stands for seawater temperature. Grain size and OM refer to sediment grain size and organic matter. Pearson correlation was employed to test bioavailable metal concentrations for collinearity, with lead, copper, cadmium and arsenic all highly correlated with zinc. Zinc was therefore retained in the analyses with lead, copper, cadmium and arsenic removed.

1987; Sauliutė & Svecevičius, 2015), meaning even mobile species may be affected. Like many estuarine species within seagrass beds, *P. sexlineatus* is a generalist feeder, consuming a wide variety of benthic invertebrates depending on prey availability (Sanchez-Jerez et al., 2002). Pore and overlying waters are important uptake pathways for demersal fish and benthic invertebrates that suspension feed or irrigate burrows with overlying water, while deposit feeders likely uptake most of their metals from ingested sediments (Simpson & Batley, 2009). Given their foraging behaviours, the most likely uptake route of metals in *P. sexlineatus* is dietary from contaminated food and sediment by the gut (Sauliutė & Svecevičius, 2015). At low concentrations, selenium, copper and zinc may be regulated in fish due to their essential nature for metabolic processes (Denton and Burdon-Jones, 1986), however given elevated concentrations and long-term exposure, metals in Lake Macquarie are at risk of harmful accumulation in fish tissues (Roach et al., 2008; UseroIzquierdo et al., 2003). Zinc, arsenic, lead, cadmium and selenium have been shown to bioaccumulate in fish tissues in heavily contaminated estuaries (UseroIzquierdo et al., 2003; Roach et al., 2008; Kirby et al., 2001) with growth and reproductive success commonly affected as well as increases in larval abnormalities (Kingsford & Gray, 1996; Van Derveer & Canton, 1997).

4.2. Pelates sexlineatus site fidelity

Fish are thought to be good indicators of long term and broad-scale effects given their relatively long life spans and well known distributions and life histories in comparison to frequently sampled macro-invertebrate groups (Barbour et al., 1999; Namba et al., 2020). Small-bodied, sedentary fish may be more sensitive to local contamination impacts than large-bodied, mobile species (Namba et al., 2020). Isotope signatures provide a tool to trace fish site fidelity and movement within estuaries (Haas et al., 2009; Weinstein et al., 2010; Muller & Strydom, 2017). Highly mobile species will have more isotopically homogenous signatures as they assimilate resources from various locations (Hansson et al., 1997), while species exhibiting high site residency will show site specific signatures (Harrod et al., 2005). Primary producers such as seagrass readily assimilate available nitrogen, with nitrogen stable isotopes capable of providing an indication of local nitrogen sources (Lepoint et al., 2004; Pitt et al., 2009; Suzzi et al., 2021). *P. sexlineatus* is resident to seagrass beds where it is an opportunistic carnivore (Sanchez-Jerez et al., 2002). The significant positive correlation of nitrogen isotope values between fish and seagrass recorded here indicates that *P. sexlineatus* displays high site fidelity and is therefore likely able to provide a reliable indication of localised impacts.

4.3. Abundance of arcobacter and intI-1 in sediments and fish hindguts

The absence of Lachno3 and HF183 gene copies throughout the study suggests that contamination by sewage was negligible during the time of sampling. The *intI-1* gene has been used as a marker for anthropogenic contamination in a range of environments, providing an indication of sewage, heavy metals and disinfectant contamination as well as a proxy for antibiotic resistant gene occurrence (Gillings et al., 2015; Koczura et al., 2016). We demonstrate that legacy metal concentrations are positively correlated with abundance of *intI-1* within Lake Macquarie sediments, consistent with evidence that this marker gene is a good proxy for anthropogenic impact (Gillings et al., 2015). Levels of *intI-1* found in sediments in Lake Macquarie are also consistent with levels reported in contaminated sediments elsewhere (e.g. Wright et al., 2008; Koczura et al., 2016). Our results further indicate that the presence of *intI-1* and *Arcobacter* in the surrounding environment may influence their abundance in fish hindguts. While the genus *Arcobacter* naturally occurs in diverse environments, it has been recently recognised as a potential pathogen and for its association with urban waste infrastructure (Collado & Figueras, 2011; Fisher et al., 2014). Interestingly, both *intI-1* and *Arcobacter* were found elevated in hindgut samples from southern sites despite sediment concentrations displaying a strong north south gradient. Fish are in constant contact with surrounding seawater, so it is possible that these sites are receiving pulse inputs of urban wastewater resulting in spikes of *Arcobacter* and *intI*-1 that are ingested by fish and may accumulate in the gut microbiome (Xue et al., 2021; Marti et al., 2018; Fu et al., 2017), persisting here as a legacy microbial signature of previous anthropogenic impacts which are less evident in the water column at the time of sampling. Increased abundance of *intI-1* in fish guts also suggests that anthropogenic impact not only poses a contamination risk for estuarine environments but may also contribute to the emergence and spread of antibiotic resistance in fish.

4.4. Bacterial community response to anthropogenic contaminants

The gut microbiota plays crucial roles in nutrition, development, metabolism and immunity of the host (Wang et al., 2017). Shifts in fish hindgut microbial community composition and diversity in response to metal exposure have been demonstrated previously (Meng et al., 2018; Xia et al., 2018; Kakade et al., 2020). These shifts can result in dysbiosis of the fish gut (Kakade et al., 2020) and subsequent loss of metabolic function (Degregori et al., 2021; Xia et al., 2018). While we did not see a dramatic shift in hindgut bacterial phyla across the study, we found that beta diversity (determined by the Bray-Curtis metric) differed between sites characterised by increased metal contamination and abundance of *Arcobacter* and *intI-1* (Cockle Creek and Fennell Bay), when compared to those with lower levels of these contaminants. In addition to this, we found a loss of bacterial alpha diversity (as measured by observed richness and Chao1 index) in samples from the most heavily contaminated site, Cockle Creek when compared to other sites. A loss in gut microbial community diversity has previously been reported in metal exposed fish (Zhai et al., 2017; Chang et al., 2019) and has significance in that the stability and diversity of the gut microbiome plays an important role in the maintenance of host health (Jones & Lennon, 2010).

Metal tolerant bacteria can influence the bioavailability of metals and promote plant growth in soils (Jiang et al., 2008; Pages et al., 2008; González Henao et al., 2021), however, the abundance and role of these bacteria in the gut microbiome of metal exposed fish has received less attention. We report a significant increase in the relative abundance of ASVs identified as metal tolerant taxa in sites characterised by high and mid metal contamination compared to those with low metal contamination. An ASV identified as *Stenotrophomonas maltophila* was found in greatest abundance at the mid metal contaminated site. Strains of this species are known to tolerate high levels of various metals (e.g. Cd, Pb, Co, Zn, As and Hg) (Pages et al., 2008; Ryan et al., 2009; Mukherjee & Roy, 2016). This species has also been identified as an emerging pathogen in animals including fish as well as humans (Brooke, 2012; Abraham & Adikesavalu, 2016). ASVs from the *Burkholderiaceae* family (genera *Burkholderia* and *Ralstonia*) were also found to be significantly more abundant in fish from metal affected sites compared to those with low metal impact. Strains of these genera have been identified as heavy metal resistant (Goris et al., 2001; Huang et al., 2021) with some *Burkholderia* also capable of aiding plant growth in contaminated sediments (Jiang et al., 2008). It has been proposed that the *Burkholderia* may present characteristics that aid fish in resisting environmental contaminants (Nolorbe-Payahua et al., 2020). Other ASVs identified belong to the family *Erysipelotrichaceae*, particularly the genus *Turicibacter*. This genus has been found to occur in elevated levels within the gut microbiota of mice and chickens following exposure to Cd, Pb and Cr (Breton et al., 2013; Li et al., 2021), and is also thought to increase under inflammatory conditions (Munyaka et al., 2016; Bretin et al., 2018). Significant increases of these taxa in fish exposed to high and mid-levels of metal contamination relative to those from low impact sites demonstrates that long-term exposure to legacy metal contamination results in an increase of metal tolerant and potentially pathogenic bacteria in the hindgut of fish. These findings may have significance for the ability of

fish to resist environmental contaminants, as well as the maintenance of development and health, however the underlying mechanisms of these alterations to the gut microbiome and the consequences of these require further investigation.

5. Conclusion

While contaminants may be rapidly diluted in seawater, sediments act as reservoirs for contaminants in urbanised estuaries. Within sediments, we identified a metal contamination gradient and correlations between legacy metal concentrations and the *intI-1* gene, a proxy for anthropogenic pollution and antibiotic resistance, of importance in estuaries with a history of industrial pollution as well as increasing anthropogenic activity. At the time of sampling, seawater microbial communities did not appear to be strongly driven by these impacts and instead reflected local environmental conditions, however the hindgut microbiome of *P. sexlineatus* from metal contaminated sites demonstrated shifts in community composition and diversity, with an increase in ASVs identified as metal resistant and potentially pathogenic bacterial taxa. These results indicate that the fish gut microbiome is influenced by long-term exposure to chronic metal contamination and may contribute to the ability of fish to resist contaminated environments. Historical industrial activity and increasing urbanisation place estuarine systems at risk from multiple stressors that threaten the function and stability of these ecosystems. Given the importance of the fish gut microbiota in maintaining host fitness and its demonstrated sensitivity to a range of stressors, urbanisation poses a threat to the health of fish communities within estuaries.

Author statement

Alessandra L. Suzzi: Conceptualisation; Formal analysis; Funding acquisition; Investigation; Methodology; Writing – original draft. **Michael Stat**: Conceptualisation; Methodology; Resources; Writing – review & editing; Supervision; Funding acquisition. **Troy F. Gaston**: Conceptualisation; Methodology; Supervision; Writing – review & editing; Funding acquisition.**Geoff R. MacFarlane**: Resources; Writing – review & editing.**Justin R. Seymour**: Resources; Writing – review & editing.**Nathan L. R. Williams**: Investigation; Writing – review & editing.**Md Rushna Alam**: Investigation; Writing – review & editing. **Megan J. Huggett**: Conceptualisation; Methodology; Resources; Writing – review & editing; Supervision; Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.120222.

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