Benthic Community Composition – Lake Macquarie

Final Report June 2024

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Glossary of acronyms and common terms

Acronyms								
ANZG	Australian and New Zealand Governments							
ASV	mplicon Sequence Variant							
DCCEEW	epartment of Climate Change, Energy, the Environment and Water							
dbRDA	Distance-based Redundancy Analysis							
DISTLM	Distance-based Linear Model							
eDNA	Environmental DNA							
GLMM	Generalised Linear Mixed Model							
LOR	Limit of reporting							
PERMANOVA	Permutational Analysis of Variance							
PERMDISP	Permutational Analysis of Dispersion							
rRNA	Ribosomal ribonucleic acid							
SIMPER	Similarity Percentage							
SQGV	Sediment quality guideline value							
TOC	Total organic carbon							

Units

°C	Degree Celsius
μm	Micrometre
mg/kg	Milligrams/kilogram

Definitions

Benthic	Referring to processes that occur on or within the sediment at the botto of a water body						
Bioavailable /bioavailable fraction	Able to be taken up by an organism / a relative measure of the proportion of a chemical that an organism is exposed to through water, sediment, soil, suspended particles, organic carbon and/or food						
Catchment	The area of land that rain lands in and drains into waterways						
Community structure	The number of taxa in a community and their relative abundance						
Community composition	The identity of taxa in a community						
Contaminants	Biological or chemical substances or entities, not normally present in a system, capable of producing an adverse effect in a biological system, seriously injuring structure or function						
Ecosystem	A community of plants, animals, bacteria and the interrelated chemical and physical environment						

Environmental DNA	Nuclear or mitochondrial DNA that is released from an organism into the environment						
Guideline value	Measurable quantity (threshold) or condition of an indicator for a specific community value below or, for some stressors, above which we consider to be a low risk of unacceptable effects occurring						
Organism	Any living animal or plant; anything capable of carrying on life processes						
Sediment	Unconsolidated mineral and organic particulate material that is deposited at the bottom of a water body						

Executive Summary

The School of Natural Sciences at Macquarie University was engaged to undertake an assessment of the benthic community composition in Lake Macquarie, NSW, Australia. More specifically, the project investigated patterns in sediment infaunal and microbial abundances, richness and community composition and assessed relationships to sediment properties, including environmental contaminants. Samples were collected from the southern section of Lake Macquarie between January-February 2023 and February 2024. Ten sites were sampled, including five sites of interest due to the potential influence of contaminants from nearby power stations and ash dam repositories and five paired reference sites. Sites were selected to target similar grain sizes and total organic carbon content in the benthic sediments.

Four replicate samples were collected from each site using a top-opening Van Veen grab deployed from a boat. Samples for eDNA were collected from the top 1 cm and samples for benthic invertebrates were collected from the top 10 cm. Benthic invertebrate samples were sieved and preserved before being sorted, and individuals were identified to family/morphospecies. DNA was extracted and sequenced to investigate eukaryotic (18S rRNA gene), bacterial (16S rRNA gene) and archaeal (A16S gene) communities. Raw sequence data was processed following the DADA2 pipeline, and a final ASV table was constructed.

Thirty-four benthic invertebrate taxa from 9 phyla were identified from the sediment samples and communities were dominated by molluscs, annelids, arthropods and nematodes. After bioinformatic processing, 8,959 eukaryotic ASVs (137 eukaryotic phyla), 6,101 bacterial ASVs (107 bacterial classes) and 2,438 archaeal ASVs (21 archaeal classes) remained. The eukaryotic communities were dominated by unidentified organisms highlighting the gaps in taxonomic classification that still exist in microbial databases. Annelids and arthropods were the dominant invertebrate taxa in the eukaryotic eDNA samples. Bacterial communities were dominated by alpha- and gammaproteobacteria, phycisphaerae, planctomycetes, spirochaetia and thermodesulfovibrionia while archaeal communities were dominated by bathyarchaeia, undinarchaeia and heimdallarchaeia.

Community structure, taxon richness, total abundance and functional group analyses were used to investigate potential impacts to the ecological communities. Community structure quantifies the compositional differences between communities based on the presence and abundance of species. Community structure is particularly useful to assess in ecological studies because the metric is sensitive to changes in both the presence/absence and abundance of species that can signal environmental changes.

Taxon richness, total abundances and functional group assessments are also common metrics in ecological studies. Taxon richness is important to assess because high diversity typically indicates a healthy environment. Differences in abundances among samples are important to quantify because changes in the number of individuals can also signal environmental changes with increases potentially indicative of increased food availability. Assessing different functional groups provides insights into ecosystem functioning and health with changes in infaunal ratios e.g. between polychaetes (less sensitive, opportunistic species) and amphipods (more sensitive species) often indicative of contaminant impacts.

Analyses of community structure in the current study found no significant differences between site categories for the benthic invertebrate communities investigated here and few instances of difference between site categories within pairings. In contrast there were clear differences in microbial community structure with gradients in dilute acid extractable metals (particularly lead/zinc) often explaining separation between sites of interest and reference sites within a

pairing. This was significant for pairing 2 (i.e. site 2 and 7) and 4 (i.e. site 4 and 9) across all microbial communities, but less frequently significant for other pairings. Although we tried to control for particle size and organic carbon content in the sediments through site selection, these were still important predictors explaining some of the variation between sites. SIMPER analysis for each of the communities identified that the most abundant taxa were generally explaining patterns of difference, however none of these individual taxa were potential indicators or were directly linked to contaminants in the sediments (i.e. they didn't show consistent trends between sites of interest and reference sites).

In contrast, analyses of richness and abundance found fairly consistent significant differences between site categories within pairings for the benthic invertebrate communities, while ASV richness and abundance in the microbial communities were spatially and temporally variable (and difficult to model statistically). Interestingly, invertebrate richness and abundances tended to be greater at sites of interest than at reference sites. Possible explanations include higher organic matter at sites of interest, which can serve as a food source, or a reduction in predation pressure due to the presence of contaminants that deter predators. Based on past studies of infaunal communities, these trends may also indicate an intermediate disturbance that is selecting for more tolerant and opportunistic species, allowing them to thrive in environments where competition is reduced.

This was investigated further by analysing infaunal ratios of polychaetes/amphipods with the expectation that a higher proportion of opportunistic polychaetes than more sensitive amphipods at sites of interest might indicate that an impact is occurring. Polychaetes dominated benthic samples at sites of interest and reference sites with amphipods only found as individuals in a couple of replicates. This suggests that dominance of infaunal communities by annelids (polychaetes) is the natural state in the lake and matches previous surveys.

Recent eDNA surveys done in the lake highlighted four sensitive eukaryotic taxa; diatoms (Bacillariophyceae), dinoflagellates (Dinophyceae and Dinoflagellata) and nematodes. Therefore, these were also investigated in the current study in separate functional group analyses. None consistently followed patterns expected from a contaminant impact at sites of interest although dinoflagellates were more abundant at the site of interest than reference site for pairing 4.

Overall, the results from each of these ecological communities suggest that concentrations of metals, particularly the dilute acid extractable contaminants (Pb/Zn, Ni and Cr), were the best predictors of spatial variation, but they did not explain all of the variation suggesting other environmental drivers were also important. Benthic invertebrate richness and abundance and microbial community structure were the best differentiators between site categories within pairings, and the differences observed between sites of interest and reference sites may indicate an intermediate disturbance from metal contamination, particularly at Sites 2 and 4. There was significant temporal and spatial variability in other indicators assessed with patterns in tolerant and sensitive species not indicative of a significant impact from metal contamination at sites of interest.

1 Introduction

1.1 Project overview

The School of Natural Sciences at Macquarie University was engaged by the Science and Insights Division (S&I) of the New South Wales (NSW) Department of Climate Change, Energy, the Environment and Water (DCCEEW) to undertake an assessment of the benthic community composition within the southern section of Lake Macquarie, NSW, Australia. The information gathered provides a present-day assessment of the benthic community status of Lake Macquarie and relationships to measured contaminants and environmental variables.

The scope of works has been developed to determine patterns in sediment infaunal and microbial abundances, richness, and community composition in the southern section of Lake Macquarie and assess the potential risks to ecological receptors in the vicinity of the power stations, ash repositories, and more broadly within the southern section of Lake Macquarie. The works includes environmental DNA (eDNA) analysis, which is used to detect the species present in an area from environmental samples. Organisms present in an environment shed DNA (e.g., through skin, faeces, or spawning), which then accumulates in their surroundings. It is important to note that a positive signal does not necessarily mean that the species was present, because the DNA could originate from a dead specimen or the organism has moved on.

We used eDNA analysis in a previous study to investigate sediment health and found that these tools are sufficiently advanced to provide not only the information collected using traditional macroinvertebrate approaches, but also an order of magnitude bigger, better, faster data to differentiate locations, and to investigate cause in complex stressor scenarios (Dafforn et al., 2014). Communities analysed through metagenomics analysis were more clearly characterised in terms of their taxonomic complement, yielding clearer responses to environmental stressors and better spatial discrimination at a scale critical for biomonitoring. However, direct comparisons of traditional infaunal surveys with eDNA surveys remain few and are needed to further assess eDNA as an appropriate tool for biomonitoring in different scenarios.

The current report describes the benthic infaunal and microbial community sampled during two high-resolution sampling events carried out within Lake Macquarie between January-February 2023 and February 2024.

1.2 Aims and objectives

The primary aim of this investigation was to determine the invertebrate and microbial community composition in surface sediments at sites of interest and paired reference sites within Lake Macquarie and relationships to potential environmental drivers such as metal contaminants.

The specific objectives to be addressed were to:

- Characterise benthic invertebrate abundance and richness as well as community composition within surface sediments adjacent to ash dam repositories and nearby reference locations within Lake Macquarie and identify any relationships to contaminants and environmental variables.
- Characterise benthic microbial abundance and richness as well as community composition by collecting environmental DNA (eDNA) within surface sediments adjacent

to ash dam repositories and nearby reference locations within Lake Macquarie and identify any relationships to contaminants and environmental variables.

- Compare traditional invertebrate and eDNA community information and sensitivity to potential stressors
- Provide an ecological line of evidence to assist in assessing potential impacts from contaminants in Lake Macquarie sediments

2 Methodology

2.1 Site selection

All sites in this study were estuarine sites located within the southern section of Lake Macquarie (Figure 1).

A total of 10 sites were sampled in quadruplicate and processed individually. Site selection was guided by the results of the Surface Sediment Quality – Chemical Assessment (Table 1, DCCEEW, 2024a) and the inclusion of key areas of interest with respect to the proximity to power stations and ash dam repositories to understand any potential influence on surface sediment quality within the southern lake. Sites 1 and 2 were selected for their elevated copper and selenium concentrations; Site 3 for its elevated mercury, copper, lead, and zinc; and Sites 4 and 5 for their elevated copper, lead, and zinc. Sites 6 to 10 were selected as paired reference sites as they were in close proximity to Sites 1-5 but had lower metal concentrations then those measured in Sites 1-5. A paired design was used to accommodate the spatial variability previously observed in the lake that might otherwise have obscured potential impacts occurring at the sites of interest. Paired sites in this study had similar environmental conditions, but differed with respect to metal concentrations.

To minimise confounding effects from differences in the physical properties of the sediment, site selection was restrained to locations within the lake with similar total organic carbon (TOC) content (1.5 - 3.0%) and grain size (<0.63 μ m fraction > 80%) based on the results reported in DCCEEW (2024a) (Table 1). This range of TOC content and grain size corresponded with where elevated metal concentrations were occurring within the lake sediments.

2.2 Sediment collection

The sediment samples were collected between 31 January and 1 February in 2023, and February 2024. All sediments were sampled using a top-opening Van Veen grab sampler operated by winch from a boat. Before sub-sampling the overlying water was removed by slow siphoning using a clean tube near one corner of the grab sampler.

For the benthic eDNA assessment, the surficial (top 1 cm) sediment was collected using sterile gloves and a sterile spoon and placed into a sterile 50 mL tube. Three field blanks were collected to investigate potential contamination from the sampling methodology. This involved opening and closing three centrifuge tubes randomly during the field sampling. For the benthic invertebrate assessment, the top 10 cm of the sediment grab was homogenised and split into two 500 mL plastic containers for sieving. The top 10 cm of the sediment was selected for this assessment as this is generally recognised as the depth to which most infauna organisms will occupy (Simpson and Batley, 2016).

Four separate sediment samples (replicates) were collected at each site (8 plastic containers). Each replicate sediment sample was sieved through a 500 μ m sieve bag and combined in a single plastic container. Sieved samples were preserved by adding ~100 mL of 10%-formalin solution with Rose Bengal to the plastic container and mixing thoroughly.

Additional sediment sub-samples were collected and analysed for particle size determination, total organic carbon, dilute acid-extractable metals (1 M HCI) and porewater metals following the methods described in DCCEEW (2024b).

All samples were kept on ice during collection. The samples for eDNA analysis were placed in a freezer upon returning to land and stored at -20° C until DNA extraction. All other samples were refrigerated at 4° C until analysis.



Figure 1. Surface sediment sampling locations (n=10) in Lake Macquarie. Sites 1-5 were selected for their elevated concentrations of metals (i.e., sites of interest) and sites 6 to 10 were selected as paired reference sites (colour indicates site pairings).

Table 1. Total recoverable metal concentrations of sediment samples (top 5 cm of the sediment) collected from Lake Macquarie in 2022 (DCCEEW, 2024a). ANZG (2018) sediment quality guideline values (SQGVs) have been applied as screening criteria unless otherwise indicated.

	Scree criteria	ening (mg/kg)	Pairing 1		Pairing 2		Pairing 3		Pairing 4		Pairing 5	
2022	SQGV	GV-high	Site 1	Site 6	Site 2	Site 7	Site 3	Site 8	Site 4	Site 9	Site 5	Site 10
Total recoverable metalsª (mg/kg)ª												
As	20	70	13	15	14	14	12	12	13	15	13	14
Cd	1.5	10	0.3	0.2	0.4	0.3	0.2	0.2	0.1	0.5	0.2	0.3
Cr	80	370	22	15	27	20	29	26	27	22	28	23
Cu	65	270	80	45	70	42	62	48	71	57	66	43
Hg	0.15	1.0	0.063	0.051	0.078	0.045	0.12	0.082	0.081	0.057	0.073	0.064
Ni	21	52	12	9	12	9	15	13	14	11	14	11
Pb	50	220	29	21	28	20	65	48	49	32	44	33
Se	1 ^b	1 ⁶	0.8	0.5	1.0	0.8	0.4	0.4	0.4	0.9	0.4	0.5
Zn	200	410	190	130	180	120	300	250	260	190	260	190
TOC (%)	-	-	2.5	1.6	2.9	2.1	2.5	2.4	2.4	1.9	2.2	2.4
<63µm⁰ (%)	-	-	90	89	98	93	99	100	100	96	98	97

TOC = total organic carbon

a Site selection for the current study (Sites 1 to 10) was guided by the results and metal contour maps presented in DCCEEW (2024a). These maps indicated the areas in the southern section of Lake Macquarie where concentrations of metals were highest. Total recoverable metals for the closest sampling location in the previous study (or mean of the two closest sampling locations where the site fell between sampling locations) are presented here (Site 1: M07 and M10, Site 2: M21 and M22; Site 3: M58 and M64, Site 4: M42 and M43, Site 5: M40 and M47, Site 6: M04 and M05, Site 7: M34 and M35; Site 8: M67, Site 9: M16, and Site 10: A05). b Adopted screening criteria (SQuiRT, 2008), ^c particle size fraction

2.3 Benthic invertebrate census

Sediment samples were rinsed thoroughly to remove formalin and any sediment not removed during the in-situ field sieving and transferred to 70% ethanol within 1 week of sampling. Benthic invertebrates were counted and identified to the lowest possible level under a dissecting microscope with the aid of sorting trays and forceps. Polychaetes were identified to family or morphospecies where possible, Bivalves were identified to morphospecies and we also enumerated Nassariidae (gastropods), Copepods, Amphipods, Decapods, Ostracods, Poriferans, Chaetagnaths, Nemerteans and Nematodes, and Sipunculids.

2.4 Environmental DNA analyses

Each sediment sample (50 mL tube) was first homogenised and then DNA was extracted from 0.25g using the Powersoil DNA extraction kit (Qiagen, Carlsbad, CA, USA) according to the manufacturer's protocol. Three lab blanks were also extracted by first opening and closing

bead tubes from the extraction kit and then following the protocol. DNA concentrations were quantified using a NanoDrop ND-2000 (NanoDrop Technologies, Wilmington, DE, USA). Sequencing was done on a MiSeq Sequencing Platform (Illumina) following the manufacturer's guidelines. Briefly, amplicons targeting the V1-V3 hypervariable region of the bacterial 16S rRNA gene (27F-519R, Lane et al., 1991), the archaeal A16S gene (A2F-519R, Lane et al. 1991) and the V9 region of the eukaryotic 18S rRNA gene (1391F-EukBR, Amaral-Zettler et al. 2009) were sequenced by Ramaciotti Centre for Genomics (UNSW, Australia).

Raw sequence data for eukaryotes, bacteria and archaea were quality trimmed and adjusted to a maximum amplicon truncation length of 145bps, 290bps and 287bps for the forward reads, and 145bps, 270bps and 240bps for the reverse reads, respectively, using the dada2 package (Callahan et al., 2016). Maximum truncation lengths were determined using quality error profiles and sequences where the median Q score fell below 20 were removed. Using the learned error rates, unique sequence inferences were made using the DADA2 core denoising algorithm and subsequently sequences were merged into unique amplicon sequence variants (ASVs). Chimeric sequences and sequences only detected in the blanks were removed and a final ASV table was constructed (Callahan et al. 2016).

For bacteria and archaea, taxonomic assignment was performed using a taxonomic Naive Bayes classifier (package dada2, assignment threshold, minboot=50) trained from two taxonomic databases, SILVA v.138 database (Quast et al., 2012) and GTDB v.120 (Parks et al., 2021). The former taxonomy database was used to detect non-bacterial or archaeal sequences (i.e. chloroplast and mitochondrial ASVs), while GTDB was chosen as the final taxonomic assignment due to its higher accuracy based on whole genome microbial identification. For the eukaryote dataset, two independent taxonomic assignments were performed, using the same Naïve Bayes classifier as above (package dada2) and a second using the USEARCH algorithm (usearch_global command, usearch v11, best hit > 97%, Edgar 2018). For both methods, the taxonomic repository PR2 (v5.0 Guillaume et al., 2013) was used and a consensus taxonomic table was created with both approaches.

Raw abundance ASV tables were processed prior to statistical analysis by removing sequences that corresponded to chloroplasts, mitochondria, contaminant sequences (package decontam, prevalence method, Davis et al., 2018), singletons and ASVs with low relative abundance across all samples (i.e., <0.001%). ASV abundance tables were normalized using a scaling method to account for heterogenous library sizes (DESeq2, package *phyloseq*; Love et al., 2014).

All steps of this pipeline were done using R studio v.3.6. After bioinformatic processing, 8,959 eukaryotic ASVs (46 eukaryotic phyla), 6,101 bacterial ASVs (46 bacterial phyla) and 2,438 archaeal ASVs (11 archaeal phyla) remained.

2.5 Statistical analyses of benthic invertebrate and eDNA data

Analyses were performed separately for each year.

Community structure quantifies the compositional differences between communities based on the presence and abundance of species. Community structure is particularly useful to assess in ecological studies because the metric is sensitive to changes in both the presence/absence and abundance of species that can signal environmental changes.

Taxon richness, total abundances and functional group assessments are also common metrics in ecological studies. Taxon richness is important to assess because high diversity typically indicates a healthy environment. Differences in abundances among samples are important to quantify because changes in the number of individuals can signal environmental changes.

Assessing different functional groups provides insights into ecosystem functioning and health. Annelids, molluscs, diatoms, dinoflagellates and nematodes were assessed in this study with the expectation that tolerant groups such as annelids and nematodes would increase at sites of interest while more sensitive groups such as molluscs, diatoms and dinoflagellates would decrease if contaminants were having an impact on communities (Dafforn et al., 2013, Suzzi et al., 2023).

A polychaete/amphipod ratio was also calculated for the benthic invertebrates (Gesteira and Dauvin, 2000), but since amphipods only occurred as single individuals in a couple of replicates this was not conclusive.

Multivariate analyses were done in PRIMER-E 7 and Permanova + add-on (PRIMER-E, Plymouth Marine Laboratory, UK) (Anderson, 2001). Permutational analysis of variance (PERMANOVA) was used to test for differences in community structure between site categories (levels: site of interest, reference), and also for an interaction between site categories and site pairings.

PERMANOVA is a non-parametric, distance-based approach that uses random permutations to test for significance between groups of interest. This is a robust statistic, as it does not make any assumptions regarding the distribution of variables and is acting primarily on the ranks of dissimilarities. It measures the sum-of-squares within and between groups and draws tests for significance by comparing the actual F test result to that gained from random permutations of the objects between the groups.

Site category and pairing (i.e., Pairing 1-5) were used as fixed factors. All analyses used Bray-Curtis similarities (for community structure) and Jaccard similarity (for community composition) from square root transformed data, with significance determined from 9999 permutations of the data. Permutation of residuals was done under a reduced model and using partial (type III) sum of squares for analyses of site categories for each pairing.

A test for homogeneity of dispersions (PERMDISP) for an interaction between site category and pairing was performed using centroids of 9999 permutations. No significant dispersion was detected in any of the analyses from invertebrate samples, but significant dispersion was found for in the 2024 data for eukaryotes and archaea (Table S1-S4). This means that it cannot be determined whether significant differences found in analyses are caused by differences between the factors analysed, or due to the dispersion of the data.

When significant differences occurred, similarity percentages analysis (SIMPER) of square-root transformed data was used to determine which species best explained the differences in communities between site categories within each pairing, using Bray-Curtis dissimilarities.

Distance-based linear models (DISTLM) were used to test which contaminant and environmental variables best explained variation in community structure. For values below the limit of reporting (LOR), the lowest value of reporting was used. Correlations between variables were explored and where variables were strongly correlated (r > 0.9), a single variable was selected as representative for further analyses (e.g., Pb was chosen as representative for Zn). Contaminants and environmental variables were normalised prior to analysis to adjust for different scales/units. Analyses of these relationships used AIC and a stepwise selection procedure to select the best explanatory model and marginal tests. Results were visualised with distance-based redundancy analysis (dbRDA).

All univariate models were generalized linear mixed models (GLMMs), run in the packages glmmTMB (Magnusson et al., 2017) and associated plots made in R version 4.2.2. Univariate

analyses were used to test for differences in taxon richness, the total abundance of infauna or ASVs, and the total abundance of the two most abundant phyla or class present at all sites as well as select functional groups. The model included an interaction of site category and pairing. Assumptions of homogeneity and normality of all tests were checked by plots of the residuals versus fitted values. A Poisson distribution was initially assumed in all models, but negative binomial distributions (nbinom1 or nbinom2) or tweedie distributions were used when necessary to improve residual plots. Dispersion tests in normality plots for species richness within sediment samples were significant, but different distributions did not improve the plot. Where different residual plots did not improve the models, this was noted in text. P-values were obtained using the Anova function in the R package 'car' (Fox et al. 2012). The R package 'emmeans' was used to test for pairwise comparisons for each pairing (Lenth et al.. 2019).

3 Results and discussion

3.1 Surface sediment contaminant and physico-chemical summary

All sediment contaminant data and supporting physico-chemical data for 2023 are provided in DCCEEW (2024b) and summarised here. The physico-chemical properties of the sediments collected across the lake in 2023 and 2024 are summarised in Table 1. Generally, the sediment across all sites was predominantly silty (<63um) and total organic carbon was consistently \geq 20500 mg/kg, except at site 6, where it was slightly lower (15500 and 18500 in 2023 and 2024, respectively). Moisture content varied between 54-76%.

The dilute-acid extractable metal concentrations analysed from the <2 mm fraction were averaged across sites and compared against their respective screening criteria in Table 2 and Table 3. The sediment porewater dissolved metal concentrations were also averaged across sites and summarised in Tables 4 and 5.

Table 2. Summary statistics (mean) of key physico-chemical properties of the sediments collected from each site within Lake Macquarie (n=4 per site) in 2023 and 2024.

	Pairing 1		Pairing 2		Pairing 3		Pairing 4		Pairing 5	
2023	Site 1	Site 6	Site 2	Site 7	Site 3	Site 8	Site 4	Site 9	Site 5	Site 10
Total organic carbon (mg/kg) ¤	28300	15500	30300	29500	30000	26300	27000	26300	26800	26300
Particle size (%)										
<2 µ m (clay fraction)	12	12	9	9	11	18	17	16	20	8
2-63 µm (fine silt fraction)	85	67	85	85	87	80	81	81	78	89
63 µm-2 mm (sand fraction)	3	22	6	6	2	2	2	3	2	4
<63 µm (clay and silt)	97	78	94	94	98	98	98	97	98	96
Moisture content ^c (%)	54	57	66	71	74	75	67	69	76	75
	Pairing 1		Pairing 2		Pairi	ing 3	Pair	ing 4	Pairi	ng 5
2024	Site 1	Site 6	Site 2	Site 7	Site 3	Site 8	Site 4	Site 9	Site 5	Site 10
		1								1
Total organic carbon (mg/kg)	25000	18500	27800	25300	26800	24800	25500	22500	24000	20500
Total organic carbon (mg/kg) Particle size (%)	25000	18500	27800	25300	26800	24800	25500	22500	24000	20500
Total organic carbon (mg/kg) Particle size (%) <2 µm (clay fraction)	25000 4	18500	27800 10	25300 7	26800 5	24800 3	25500 5	22500 4	24000 7	20500 7
Total organic carbon (mg/kg) Particle size (%) <2 µm (clay fraction) 2-63 µm (fine silt fraction)	25000 4 92	18500 11 80	27800 10 88	25300 7 90	26800 5 92	24800 3 94	25500 5 92	22500 4 94	24000 7 90	20500 7 91
Total organic carbon (mg/kg) Particle size (%) <2 μm (clay fraction) 2-63 μm (fine silt fraction) 63 μm-2 mm (sand fraction)	25000 4 92 4	18500 11 80 10	27800 10 88 1	25300 7 90 4	26800 5 92 3	24800 3 94 3	25500 5 92 3	22500 4 94 3	24000 7 90 3	20500 7 91 2
Total organic carbon (mg/kg) Particle size (%) <2 μm (clay fraction) 2-63 μm (fine silt fraction) 63 μm-2 mm (sand fraction) <63 μm (clay and silt)	25000 4 92 4 96	18500 111 80 10 91	27800 10 88 1 99	25300 7 90 4 96	26800 5 92 3 97	24800 3 94 3 97	25500 5 92 3 97	22500 4 94 3 97	24000 7 90 3 97	20500 7 91 2 98

^α Combustion method .^b The < 63 μm sediment particle size fraction (clay and silt) is considered a suitable representation of the sediment materials that are most readily resuspended or potentially ingested by organisms. ^c at 105°C.

	Concerning of	Screening criteria (ma /ka)		Mean dilute-acid extractable metalsª (mg/kg, n=4 per siteʰ)									
	Screening cri	feria (mg/kg)	Pair	ing 1	Pairi	Pairing 2		Pairing 3		Pairing 4		Pairing 5	
2023	SQGV	GV-high	Site 1	Site 6	Site 2	Site 7	Site 3	Site 8	Site 4	Site 9	Site 5	Site 10	
As	20	70	2.5	2.8	2.8	3.3	2.5	2.3	2.3	3.0	2.8	3.0	
Cd	1.5	10	0.18	0.25	0.23	0.48	0.33	0.28	0.28	0.18	0.20	0.23	
Cr	80	370	4.8	5.3	7.6	9.0	9.3	8.6	7.5	7.7	8.9	9.6	
Cu	65	270	27	24	37	33	33	32	30	35	36	36	
Hg	0.15	1.0					<0	.05					
Ni	21	52	2.5	2.8	2.8	3.3	4.3	3.5	3.3	3.5	4.0	3.5	
Pb	50	220	14	14	15	21	43	32	32	23	29	23	
Se] c] ¢	0.16	0.19	0.08	0.13	0.08	0.14	0.11	0.05	0.10	0.06	
Zn	200	410	85	81	99	120	190	173	150	137	168	138	

Table 3. Summary statistics for surface sediment dilute-acid extractable metal concentrations collected within Lake Macquarie in 2023. ANZG (2018) sediment quality guideline values (SQGVs) have been applied as screening criteria unless otherwise indicated.

^a 1 M HCl. ^b Values below the limit of reporting (LOR) were substituted as the LOR for calculating summary statistics. ^c Adopted screening criteria (SQuiRT, 2008).

	C		Mean dilute-acid extractable metals ^a (mg/kg, n=4 per site ^b)									
	Screening cri	Pairing 1		Pairing 2		Pairing 3		Pairing 4		Pairing 5		
2024	SQGV	GV-high	Site 1	Site 6	Site 2	Site 7	Site 3	Site 8	Site 4	Site 9	Site 5	Site 10
As	20	70	4.25	4.25	4.5	4	4	<4	<4	4	<4	4
Cd	1.5	10		<1								
Cr	80	370	5	4.5	7	4.25	6.5	6.5	6.75	6	5.25	6.25
Cu	65	270	27.5	31.25	39	20.3	33	32.8	34.8	30	28.5	30.8
Hg	0.15	1.0					•	<0.1				
Ni	21	52	3	2.75	3.5	2	3.25	3.5	3.75	3	2.75	3
Pb	50	220	21	16.5	20	14	42	35	39.8	24.5	25.8	24
Se	٦٩] c		<4								
Zn	200	410	125	94.3	143	77.3	187.5	183	185	148	140	140

Table 4. Summary statistics for surface sediment dilute-acid extractable metal concentrations collected within Lake Macquarie in 2024. ANZG (2018) sediment quality guideline values (SQGVs) have been applied as screening criteria unless otherwise indicated.

^o 1 M HCl.^b Values below the limit of reporting (LOR) were substituted as the LOR for calculating summary statistics. c Adopted screening criteria (SQuiRT, 2008).

		Mean dissolved metals (mg/kg, n=4 per site°)									
	Pairi	ng 1	Pair	ing 2	Pairi	ing 3	Pairi	ng 4	Pairing 5		
2023	Site 1	Site 6	Site 2	Site 7	Site 3	Site 8	Site 4	Site 9	Site 5	Site 10	
Ag	<0.0002										
AI	<0.01										
As	0.0095 0.0095 0.0115 0.00925 0.00825 0.0045 0.01 0.01025 0.00925 0.00775								0.00775		
Ba	0.02125	0.016	0.015	0.0155	0.017	0.0185	0.01525	0.0155	0.019	0.0168	
Be					<	0.0002					
В	3.7	3.45	3.95	3.975	3.6	3.5	3.375	4.025	3.875	3.8	
Cd					<	0.0002					
Cr					<	0.0005					
Co	0.000267	0.000275	0.0002	0.0002	0.000225	0.0002	0.0002	0.0002	0.00025	0.000275	
Cu					<	0.001					
Fe	0.228	0.295	0.13	0.193	0.145	0.475	0.233	0.12	0.26	0.273	
Hg					<	0.0004					

Table 5. Summary statistics for sediment porewater dissolved metal concentrations collected within Lake Macquarie in 2023.

	Mean dissolved metals (mg/kg, n=4 per siteª)										
	Pairing 1				Pairing	1			Pairing 1		
2023	Site 1	Site 6	Site 2	Site 7	Site 3	Site 8	Site 4	Site 9	Site 5	Site 10	
Mn	2.2	2.2 4.675 3.38 3.65 4 1.07 2.88 3.53							2.93	3.25	
Mo	0.0158 0.0165 0.0138 0.0148 0.018 0.017 0.0243 0.0158								0.0173	0.0193	
Ni	<0.001										
Pb					<0.000	5					
Se					<0.002	2					
TI					<0.000	5					
V	0.0008687	0.00165	0.00288	0.00248	0.00278	0.0008	0.00133	0.00288	0.000925	0.00108	
Zn					<0.003	3					

Table 5 cont. Summary statistics for sediment porewater dissolved metal concentrations collected within Lake Macquarie in 2023.

		Mean dissolved metals (mg/kg, n=4 per siteª)									
	Pairi	ng 1	Pairi	ing 2	Pair	ing 3	Pairi	ng 4	Pair	ing 5	
2024	Site 1	Site 6	Site 2	Site 7	Site 3	Site 8	Site 4	Site 9	Site 5	Site 10	
Ag	<0.0002										
Al		<0.01									
As	0.012	0.0075	0.00525	0.00925	0.0065	0.00575	0.00925	0.0045	0.0055	0.0045	
Ba	0.0158	0.0205	0.0185	0.0158	0.0158	0.0115	0.0165	0.0128	0.016	0.0158	
Be			^		<	0.0002					
В	3.525	3.475	3.3	3.3	3.325	3.1	3.175	3.275	3.55	3.55	
Cd					<	0.0002					
Cr					<	0.0005					
Co	0.0002 0.00015 0.000125 0.000275 0.00015 0.00025 0.0002 0.000133 0.00025 0.000175										
Cu					<	<0.001					
Hg					(0.0004					

Table 6. Summary statistics for sediment porewater dissolved metal concentrations collected within Lake Macquarie in 2024.

		Mean dissolved metals (mg/kg, n=4 per siteª)										
	Pairir	ng 1			Pair	ing 1			Pairi	ng 1		
2024	Site 1	Site 6	Site 2	Site 7	Site 3	Site 8	Site 4	Site 9	Site 5	Site 10		
Mn	2.6	4.025	3.75	3.75 3.25 1.8 1.525 1.75 2.275								
Mo	0.0165	0.0168	0.0208	0.0193	0.0263	0.0168	0.0295	0.02	0.02	0.022		
Ni					<	<0.001						
Pb					<	0.0005						
Se					<	<0.003						
TI					<	0.0005						
V	0.00103 0.0024 0.0029 0.00233 0.00518 0.0013 0.004325 0.00273 0.00128 3											
Zn					<	<0.003						

Table 6 cont. Summary statistics for sediment porewater dissolved metal concentrations collected within Lake Macquarie in 2024.

3.2 Benthic invertebrate community

3.2.1 Individual taxa

A total of 34 taxa from nine different phyla were identified across all sites and sampling years (Figures S1 and S2). Benthic invertebrate communities sampled from sites of interest and paired reference sites consisted mainly of Molluscs, Annelids, Arthropods, and Nematodes, with other phyla found in lower abundances (Figure 2). Nematode abundances increased between 2023 and 2024 at Sites 3 and 8, the pairing in the south-eastern section of Lake Macquarie, representing > 50 % of the invertebrates found in the samples.



Figure 2. Mean abundance of individuals within each phylum in sediment samples collected in 2023 and 2024 across sites in the southern section of Lake Macquarie, NSW, Australia. Bars to the left of each pair are sites of interest, and bars on the right of each pairing are paired reference sites.

3.2.2 Community structure

Analyses found a significant effect of the interaction between site category and pairing for community structure in 2023 (Table 6). Pairwise comparisons found that community structure only differed for pairing 1 (i.e., Site 1 vs Site 6), but not for any of the other pairings (Table S 5). SIMPER analysis found that differences were explained by Spionidae sp. 3, bivalve sp. 1 and Cirratulidae, which accounted for 50% of the differences between the sites (Table 7). Specifically, Spionidae sp. 3 was present at site 1 and absent at site 6 and bivalve sp. 1 was more abundant at site 1 than 6, while cirratulids were present at site 6 and absent from site 1.

In 2024, community structure did not vary between site categories, but differed between the different pairings, indicating spatial variation across Lake Macquarie (Table 6).

Table 7. Full PERMANOVA results for benthic invertebrate community across site pairings in the southern section of Lake Macquarie, NSW, Australia in 2023 and 2024. P(perm) values <0.05 indicate significant differences. Sources of variation in the model ('Source'), the degrees of freedom ('df'), sums of squares ('SS'), mean squares ('MS'), pseudo-F ratio ('Pseudo-F') and permutation P-value ('P(perm)').

Source					
Invertebrates, 2023	df	SS	MS	Pseudo-F	P(perm)
Site category	1	6934	6934	4.717	0.0002
Pairing	4	14548	3637	2.4741	0.0005
Site category x Pairing	4	11735	2934	1.9958	0.0030
Res	30	44101	1470		
Total	39	77318			
Invertebrates, 2024	df	SS	MS	Pseudo-F	P(perm)
Site category	1	4479	4779	1.7881	0.0600
Pairing	4	31732	7933	2.9683	0.0001
Site category x Pairing	4	9466	2367	0.8855	0.6770
Res	30	80175	2673		
Total	39	126150			

Table 8. Full SIMPER results for benthic invertebrate community structure sampled from pairing 1 (site 1, site 6) in 2023. A 90% cut-off was applied. Average abundance ('Av. Abund'), average dissimilarity ('Av. Diss'), dissimilarity/standard deviation ('Diss/SD'), individual contribution ('Contrib. %'), and cumulative percentage explained ('Cum %).

	Site 1 Av.	Site 6 Av.				
Taxa	Abund	Abund	Av. Diss	Diss/SD	Con %	Cum %
Spionidae sp. 3	3.17	0.00	22.5	1.46	27.9	27.9
Bivalve sp. 1	1.99	0.85	9.80	1.42	12.1	40.0
Cirratulidae	0.00	1.04	8.58	1.45	10.6	50.6
Copepoda	0.71	0.00	6.56	0.89	8.12	58.7
Spionidae sp. 2	0.25	0.71	6.25	0.95	7.73	66.5
Nereididae	0.60	0.00	5.76	0.96	7.13	73.6
Spionidae sp. 1	0.68	0.50	5.63	1.09	6.97	80.5
Nemertea	0.00	0.35	3.09	0.54	3.83	84.4
Sipunculidae	0.35	0.00	2.69	0.56	3.33	87.7
Nassariidae	0.00	0.25	2.07	0.54	2.57	90.3

3.2.3 Relationship between environmental variables and community structure

Of the environmental variables collected, total organic carbon, dilute acid extractable lead, dissolved vanadium and molybdenum in porewaters (all in 2023 and 2024), dissolved iron, manganese and arsenic in pore waters, and dilute extractable nickel and copper and moisture content (in 2023 only) and the particle size fractions 0.002 mm - 0.063 mm and < 0.002 mm (in 2024 only) explained a significant amount of variation in the benthic invertebrate community structure (Tables S6 and S7).

When visualised with a dbRDA plot, dissolved iron and molybdenum in porewaters showed a clear positive correlation with some of the reference sites (Sites 6 and 7) in 2023, whereas total organic carbon, dissolved arsenic, manganese and vanadium in porewaters, dilute acid extractable copper, lead and nickel, and moisture content were mostly correlated with sites of interest (Figure 3). In 2024, the particle size fraction <0.002 mm and dissolved vanadium and molybdenum in porewaters were positively correlated with the reference sites, while dilute extractable lead, total organic carbon and the particle size fraction 0.002 mm – 0.063 mm were positively correlated with sites of interest (Figure 3).



Figure 3. Distance-based redundancy analysis of benthic invertebrate community structure in 2023 and 2024, with contaminant and environmental variables that explained a significant amount of variation in the community overlaid as vectors. Pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.

3.2.4 Species richness and abundance

The mean, minimum, and maximum species richness and total abundance of organisms collected at each site in 2023 and 2024 are summarised in Table 8.

	<u>Richn</u>	iess	<u>Abundance</u>			
	Mean (mi	in, max)	Mean (m	iin, max)		
Site	2023	2024	2023	2024		
Sites of interest						
Site 1	4.25 (3,5)	4.25 (3,5)	23.50 (6,47)	9.50 (7,12)		
Site 2	3.25 (2,7)	3.50 (2,5)	12.20 (5,24)	5.50 (4,7)		
Site 3	4.00 (2,7)	5.25 (5,6)	24.00 (16,40)	37.3 (31,43)		
Site 4	4.50 (3,6)	5.25 (4,6)	18.20 (11,27)	17.00 (10,27)		
Site 5	5.75 (4,7)	2.00 (0,4)	24.00 (20,26)	5.50 (0,13)		
Paired reference sites						
Site 6	3.50 (3,4)	2.00 (1,4)	5.25 (4,7)	4.25 (1,12)		
Site 7	3.25 (2,5)	1.75 (1,3)	11.00 (6,19)	3.50 (1,5)		
Site 8	6.25 (5,10)	4.50 (2,7)	16.00 (13,19)	16.3 (4,45)		
Site 9	5.00 (3,6)	3.50 (3,5)	14.50 (11,18)	7.75 (5,13)		
Site 10	4.00 (3,5)	2.00 (1,4)	9.75 (5,22)	3.00 (1,6)		

Table 9. Mean (minimum, maximum) taxon richness and abundance of benthic invertebrates at each site of interest and paired reference site in the southern section of Lake Macquarie, NSW, Australia.

Taxon richness did not differ significantly between site categories within each pairing in 2023, but was generally higher at sites of interest than reference sites in 2024 (Figure 4, Table 9). Total abundances were also generally higher at sites of interest than paired reference sites. This difference was significant for pairing 1 (Site 1 vs Site 6) and 5 (Site 5 vs Site 10) in 2023, and all pairings in 2024 (Figure 5, Table 9 and Table S8).

Analyses of the two most abundant phyla (annelids and molluscs) showed differences between years (Figure 6 and Figure 7, Table 10). In 2023, the abundance of annelids did not vary between site categories within each pairing, or between pairings or site categories individually (Figure 6). In 2024, annelids were significantly more abundant at sites of interest in each pairing although the magnitude of difference was greatest within Pairings 3 and 4 (Figure 6, Table 10). Mollusc abundances also differed between years. In 2023, molluscs were significantly more abundant at sites of interest than reference sites for Pairings 1, 3 and 5, but not for Pairings 2 and 4 (Figure 7, Table S9). In 2024, however, there were no significant differences between categories within each pairing, though overall differences between pairings were found, suggesting that spatial differences in abundance exist across the different sections of Lake Macquarie (Figure 7).


Figure 4. Mean (\pm SE) taxon richness across sites in 2023 and 2024. Site pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.



Figure 5. Mean (\pm SE) total abundance across sites in 2023 and 2024. Site pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.

Table	10. Si	tatistical	result	's from	univariate	analyses	for tax	on ri	ichness o	and to	otal al	bundance	in 2	2023	and
2024	. Pr(>	Chisq) v	alues	< 0.05	indicate si	gnificant	differer	ices.	Chi-squ	are s	tatisti	c ('Chisq')	, de	egrees	s of
freed	om ('D	F'), and	P-val	ue 'Pr(>Chisq)').										

Parameter, Year			
Invertebrate richness, 2023	Chisq	Df	Pr(>Chisq)
Site category	4.8335	4	0.9467
Pairing	0.0045	1	0.3048
Site category x Pairing	3.5760	4	0.4664
Invertebrate richness, 2024	Chisq	Df	Pr(>Chisq)
Site category	12.5975	4	0.0311
Pairing	4.6492	1	0.0134
Site category x Pairing	2.2950	4	0.6817
Invertebrate abundance, 2023	Chisq	Df	Pr(>Chisq)
Site category	7.5969	4	<0.0001
Pairing	19.3167	1	0.1075
Site category x Pairing	12.9609	4	0.0115
Invertebrate abundance, 2024	Chisq	Df	Pr(>Chisq)
Site category	59.2710	4	<0.0001
Pairing	15.2990	1	<0.0001
Site category x Pairing	0.5330	4	0.9681



Figure 6. Mean (\pm SE) abundance of annelids in 2023 and 2024. Site pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.



Figure 7. Mean (\pm SE) abundance of molluscs in 2023 and 2024. Site pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.

Table 11. Statistical results from univariate analyses for the total abundance of the two major phyla occurring across all sites in 2023 and 2024. Pr(>Chisq) values <0.05 indicate significant differences. Chi-square statistic ('Chisq'), degrees of freedom ('DF'), and P-value 'Pr(>Chisq)').

Phylum, Year			
Annelida, 2023	Chisq	Df	Pr(>Chisq)
Site category	0.9441	1	0.3312
Pairing	6.3749	4	0.1729
Site category x Pairing	8.9946	4	0.0612
Annelida, 2024	Chisq	Df	Pr(>Chisq)
Site category	14.5358	1	<0.0001
Pairing	39.0591	4	<0.0001
Site category x Pairing	1.5135	4	0.8242
Mollusca, 2023	Chisq	Df	Pr(>Chisq)
Site category	26.6800	1	<0.0001
Pairing	35.1980	4	<0.0001
Site category x Pairing	19.7080	4	0.0006
Mollusca, 2024	Chisq	Df	Pr(>Chisq)
Site category	0.5037	1	0.4779
Pairing	9.4977	4	0.0498
Site category x Pairing	8.9418	4	0.0626

3.3 Eukaryotic microbial community (eDNA)

3.3.1 Individual taxa

A total of 8,959 eukaryotic Amplicon Sequence Variants (ASVs) were found across all sites and sampling years, of which 3,108 were assigned to 137 phyla, and 5,851 could not be assigned to a phylum. Communities were dominated by ASVs that did not belong to any of the phyla identified in the benthic invertebrate samples or to unknown phyla (Figure S3), highlighting that the eDNA sampling captured taxa that were not sampled using traditional infaunal methods. When considering only the phyla that were identified in the benthic invertebrate samples, ASVs assigned to annelids and arthropods were most abundant in both years (Figure 8).



Figure 8. Mean abundance of Amplicon Sequence Variants (ASVs) in eukaryotic phyla in samples collected in 2023 and 2024 across sites in the southern section of Lake Macquarie, NSW, Australia. Only the phyla also sampled in the benthic invertebrate surveys have been included for comparison. Bars to the left of each pair are sites of interest, and bars on the right of each pairing are paired reference sites.

3.3.2 Community structure

Analyses found a significant effect of the interaction between site category and pairing for community structure in 2023 and 2024 (Table 11). Pairwise comparisons found that community structure differed for Pairings 1 (i.e., Site 1 vs Site 6), 2 (i.e., Site 2 vs Site 7), 3 (i.e., Site 3 vs Site 8) and 5 (i.e., Site 5 vs Site 10) in 2023 and Pairings 1, 2, 3, 4 in 2024 (Table S10).

SIMPER analysis found that the differences were primarily explained by organisms that could not be identified to phylum (NA) followed by a mix of diatoms (Dinophyceae), arthropods, euglenozoans, nematodes, annelids, cilophorans, apicomplexans, brachiopods, spirotricheans and gastrotrichians (Table S11). Patterns of difference were varied depending on the pairing with no consistent trends between sites of interest and reference sites suggesting differences were driven by natural spatial variation.

Table 12. Full PERMANOVA results for eukaryotic ASV community structure across site pairings in the southern section of Lake Macquarie, NSW, Australia. P(perm) values <0.05 indicate significant differences. Sources of variation in the model ('Source'), the degrees of freedom ('df'), sums of squares ('SS'), mean squares ('MS'), pseudo-F ratio ('Pseudo-F') and permutation P-value ('P(perm)').

Source					
Eukaryotes, 2023	df	SS	MS	Pseudo-F	P(perm)
Site category	1	3417	3471	1.5993	0.0069
Pairing	4	27071	6768	3.1675	0.0001
Site category x Pairing	4	15389	3847	1.8006	0.0001
Res	30	64099	2137		
Total	39	109980			
Eukaryotes, 2024	df	SS	MS	Pseudo-F	P(perm)
Site category	1	3339	3339	1.6586	0.0047
Pairing	4	22447	5612	2.7878	0.0001
Site category x Pairing	4	15389	3847	1.9113	0.0001
Res	30	60389	2013		
Total	39	101560			

3.3.3 Relationship between environmental variables and community structure

Of the environmental variables collected, total organic carbon, moisture content, dissolved manganese, iron, vanadium, arsenic and molybdenum in pore waters, and dilute acid extractable lead, nickel and copper explained a significant amount of variation in the eukaryotic community structure in 2023. Dilute acid extractable lead, dissolved vanadium and molybdenum in pore waters, and total organic carbon were also important predictors in 2024 along with the particle size fractions 0.002 mm – 0.063 mm and <0.002 mm (Table S12 and Table S13).

When visualised with a dbRDA plot, there weren't clear correlations between environmental variables and site categories with differences mainly between site pairings. For example, in 2023, Site 3 and Sites 8, 4 and 9, and 5 and 10 separated along a gradient of increasing dilute extractable lead while other site pairings separated along gradients of particle size fraction (Figure 9). Sites 1 and 6 separated along a gradient of total organic carbon with increasing TOC associated with the site of interest. In 2024, Sites 4 and 9 also separated along a gradient of increasing dilute extractable lead and to a lesser extent gradients of particle size fraction.



Figure 9. Distance-based redundancy analysis of eukaryotic ASV community structure in 2023 and 2024, with contaminant and environmental variables that explained a significant amount over variation in the community overlaid as vectors. Pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.

3.3.4 Species richness and abundance

Eukaryotic ASV richness did not differ significantly between site categories within each pairing in 2023 or 2024 (Figure 10, Table 12). Total ASV abundances varied between sites of interest and paired reference sites across years. In 2023, ASV abundances were significantly lower at the sites of interest than paired reference sites for pairing 1 (Site 1 < Site 6), whereas sites of interest had significantly higher ASV abundances than paired reference sites for pairing 4 (Site 4 > Site 9) and pairing 5 (Site 5 vs > Site 10). No significant differences were found for pairings 2 and 3.

In 2024, paired reference sites had significantly higher ASV abundances than sites of interest for Pairing 2 (Site 2 < Site 7) and pairing 3 (Site 3 < Site 8), whereas no significant differences were found between sites for Pairings 1, 4, and 5 (Figure 11, Table 12 and Table S14).



Figure 10. Mean (\pm SE) eukaryotic Amplicon Sequence Variants (ASVs) richness across sites in 2023 and 2024. Site pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.



Figure 11. Mean (\pm SE) eukaryotic Amplicon Sequence Variants (ASVs) abundance across sites in 2023 and 2024. Site pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.

Table 13. Statistical results from univariate analyses of eukaryotic Amplicon Sequence Variants (ASVs). Pr(>Chisq) values <0.05 indicate significant differences. Chi-square statistic ('Chisq'), degrees of freedom ('DF'), and P-value 'Pr(>Chisq)').

Parameter, Year			
Eukaryote richness, 2023	Chisq	Df	Pr(>Chisq)
Site category	0.3297	1	0.5658
Pairing	7.1486	4	0.1282
Site category x Pairing	2.1830	4	0.7021
Eukaryote richness, 2024	Chisq	Df	Pr(>Chisq)
Site category	0.5786	1	0.4469
Pairing	1.2408	4	0.8713
Site category x Pairing	3.2534	4	0.5163
Eukaryote abundance, 2023	Chisq	Df	Pr(>Chisq)
Site category	0.1500	1	0.6986
Pairing	87.9630	4	<0.0001
Site category x Pairing	17.385	4	0.0016
Eukaryote abundance, 2024	Chisq	Df	Pr(>Chisq)
Site category	15.2990	1	0.0737
Pairing	59.2710	4	0.0001
Site category x Pairing	0.5330	4	0.0020

Analyses of annelids and molluscs showed differences in patterns between years (Figure 12 and Figure 13, Table 13). Annelid ASV abundance did not vary significantly between site categories or pairings in 2023. Annelid ASV abundance in 2024 and mollusc ASV abundance in 2023 and 2024 varied between site categories within each pairing (Figure 12 and Figure 13, Table 13). In 2024, annelid ASVs were significantly more abundant at the site of interest than its paired reference site for pairing 3 (Site 3 > Site 8), whereas the reference site had higher annelid ASV abundances than the site of interest for Pairing 4 (Site 4 < Site 9) (Table S15).

In 2023, mollusc ASV abundance was greater at sites of interests than paired reference sites for Pairings 2 and 3. In 2024, mollusc ASV abundance was greater at sites of interests than paired reference sites for Pairing 4 only (Site 4 > Site 9), whereas the opposite was true for pairing 1 (Site 1 < Site 6) (Table S15).



Figure 12. Mean (\pm SE) annelid Amplicon Sequence Variants (ASVs) abundance in 2023 and 2024. Site pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.



Figure 13. Mean (\pm SE) mollusc Amplicon Sequence Variants (ASVs) abundance in 2023 and 2024. Site pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.

Table 14. Statistical results from univariate analyses of Amplicon Sequence Variants ASVs for annelids and molluscs. Pr(>Chisq) values <0.05 indicate significant differences. Chi-square statistic ('Chisq'), degrees of freedom ('DF'), and P-value 'Pr(>Chisq)').

Phylum, Year			
Annelida ASV, 2023	Chisq	Df	Pr(>Chisq)
Site category	0.6313	1	0.4269
Pairing	19.7012	4	0.0006
Site category x Pairing	5.3447	4	0.2537
Annelida ASV, 2024	Chisq	Df	Pr(>Chisq)
Site category	0.0014	1	0.9705
Pairing	23.9151	4	<0.0001
Site category x Pairing	19.9951	4	0.0005
Mollusca ASV, 2023	Chisq	Df	Pr(>Chisq)
Site category	7.8313	1	0.0051
Pairing	34.8164	4	<0.0001
Site category x Pairing	10.6190	4	0.0031
Mollusca ASV, 2024	Chisq	Df	Pr(>Chisq)
Site category	0.1335	1	0.7148
Pairing	106.2412	4	<0.0001
Site category x Pairing	29.5638	4	<0.0001

Analyses of diatoms (Bacillariophyceae), dinoflagellates (Dinophyceae and Dinoflagellata) and nematodes showed differences between years (Figures 14 -17, and Table 14). The abundance of diatoms differed between site categories within each pairing with diatoms more abundant at sites of interest than reference sites for Pairings 3 and 5 in 2023 and for Pairing 5 in 2024 while the opposite was observed for pairing 3 in 2024 (Figure 14). The abundance of dinoflagellates also differed between site categories within each pairing with Dinophyceae more abundant at reference sites than sites of interest for Pairing 3 and more abundant at sites of interest than reference sites for Pairing 4 in both 2023 and 2024 (Figure 15). Dinoflagellata were more abundant at sites of interest than reference sites for Pairing 4, but more abundant at reference sites than sites of interest for Pairings 1 and 3 in 2023 (Figure 16). There were no differences between site categories in 2024. Nematode abundances also differed between years. In 2023, nematodes were significantly more abundant at reference sites than sites of interest (Figure 17). In 2024, there were differences between site categories within pairings with nematodes more abundant at the site of interest than reference site for Pairing 1 and more abundant at the reference site than site of interest for Pairing 3 (Figure 17).



Figure 14. Mean (\pm SE) Bacillariophyceae Amplicon Sequence Variants (ASVs) abundance in 2023 and 2024. Site pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.



Figure 15. Mean (\pm SE) Dinophyceae Amplicon Sequence Variants (ASVs) abundance in 2023 and 2024. Site pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.



Figure 16. Mean (\pm SE) Dinoflagellata Amplicon Sequence Variants (ASVs) abundance in 2023 and 2024. Site pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.



Figure 17 Mean (\pm SE) Nematoda Amplicon Sequence Variants (ASVs) abundance in 2023 and 2024. Site pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.

Table 15. Statistical results from univariate analyses of Amplicon Sequence Variants ASVs for diatoms (Bacillarophyceae), dinoflagellates (Dinophycaea and Dinoflagellata) and nematodes. Pr(>Chisq) values <0.05 indicate significant differences. Chi-square statistic ('Chisq'), degrees of freedom ('DF'), and P-value 'Pr(>Chisq)').

Functional group, Year			
Bacillariophyceae ASV, 2023	Chisq	Df	Pr(>Chisq)
Site category	0.6313	1	<0.0001
Pairing	19.7012	4	<0.0001
Site category x Pairing	5.3447	4	<0.0001
Bacillariophyceae ASV, 2024	Chisq	Df	Pr(>Chisq)
Site category	0.0014	1	<0.0001
Pairing	23.9151	4	0.5216
Site category x Pairing	19.9951	4	0.0006
Dinophyceae ASV, 2023	Chisq	Df	Pr(>Chisq)
Site category	7.8313	1	<0.0001
Pairing	34.8164	4	0.2762
Site category x Pairing	10.6190	4	<0.0001
Dinophyceae ASV, 2024	Chisq	Df	Pr(>Chisq)
Site category	0.1335	1	0.0002
Pairing	106.2412	4	0.0174
Site category x Pairing	29.5638	4	<0.0001
Dinoflagellata ASV, 2023	Chisq	Df	Pr(>Chisq)
Site category	0.1335	1	<0.0001
Pairing	106.2412	4	0.7443
Site category x Pairing	29.5638	4	<0.0001
Dinoflagellata ASV, 2024	Chisq	Df	Pr(>Chisq)
Site category	0.1335	1	0.0005
Pairing	106.2412	4	0.0810
Site category x Pairing	29.5638	4	0.0541
Nematoda ASV, 2023	Chisq	Df	Pr(>Chisq)
Site category	0.1335	1	0.5442
Pairing	106.2412	4	0.0019
Site category x Pairing	29.5638	4	0.1266
Nematoda ASV, 2024	Chisq	Df	Pr(>Chisq)
Site category	0.1335	1	<0.0001
Pairing	106.2412	4	0.0041
Site category x Pairing	29.5638	4	<0.0001

3.4 Bacterial community (eDNA)

3.4.1 Individual taxa

Across both years, a total of 6,101 bacterial ASVs were found, of which 6,089 were assigned to 107 known classes. Communities were dominated by Alpha- and Gammaproteobacteria, Phycisphaerae, Planctomycetes, Spirochaetia, Thermodesulfovibrionia and 'unidentified Bacteria' (Figure 14).



Figure 18. Mean abundance of Amplicon Sequence Variants (ASVs) within each bacterial class in samples collected in 2023 and 2024 across sites in the southern section of Lake Macquarie, NSW, Australia. Bars to the left of each pair are sites of interest, and bars on the right of each pairing are paired reference sites.

3.4.2 Community structure

Analyses found a significant effect of the interaction between site category and pairing for bacterial community structure in 2023 and 2024 (Table 14). Pairwise comparisons found that community structure differed for Pairing 2 (i.e., Site 2 vs Site 7), Pairing 4 (i.e., Site 4 vs Site 9) and Pairing 5 (i.e., Site 5 vs Site 10) in 2023 and for Pairing 2 and Pairing 4 in 2024 but not for the other pairings (Table S 16). SIMPER analysis found that differences were explained by Alphaproteobacteria which were more abundant at reference sites than sites of interest for Pairing 2, 4 and 5 in 2023 and Pairing 2 in 2024, although the opposite was true for Pairing 4 in 2024. Gammaproteobacteria also explained a significant amount of variation in most pairings and followed a similar pattern with greater abundance at reference sites than sites than sites of interest for Pairings 2, 4 and 5 in 2023 and Pairing 4 in 2024, but differences were generally smaller (Table S17).

Table 16. Full PERMANOVA results for bacterial ASV community structure across site pairings in the southern section of Lake Macquarie, NSW, Australia. P(perm) values <0.05 indicate significant differences. Sources of variation in the model ('Source'), the degrees of freedom ('df'), sums of squares ('SS'), mean squares ('MS'), pseudo-F ratio ('Pseudo-F') and permutation P-value ('P(perm)').

Source					
Bacteria, 2023	df	SS	MS	Pseudo-F	P(perm)
Site category	1	2140	2140	1.7085	0.0023
Pairing	4	17048	4262	3.4032	0.0001
Site category x Pairing	4	6813	1703	1.3601	0.0003
Res	30	37571	1252		
Total	39	63572			
Bacteria, 2024	df	SS	MS	Pseudo-F	P(perm)
Site category	1	1840	1840	1.5149	0.0231
Pairing	4	17819	4455	3.6671	0.0001
Site category x Pairing	4	6524	1631	1.3427	0.0011
Res	30	36445	1215		
Total	39	62629			

3.4.3 Relationship between environmental variables and community structure

Of the environmental variables collected, moisture content, dissolved boron, vanadium, iron and molybdenum in porewaters, dilute acid extractable lead and nickel, and the particle size fractions 0.063 mm – 2.0 mm and <0.063 mm explained a significant amount of variation in the bacterial community structure in 2023. Dilute acid extractable lead and chromium, dissolved boron in pore waters, and moisture content and total organic carbon were important predictors in 2024 along with the particle size fractions 0.002 mm – 0.063 mm, 0.063 mm – 2.0 mm and <0.002 mm (Table S18 and Table S19).

When visualised with a dbRDA plot, there weren't clear correlations between environmental variables and site categories with differences mainly between site pairings. For example, in 2023, Site 2 and Sites 7, 4 and 9 and 5 and 10 separated along a gradient of dissolved metals and particle size fractions. Sites 3 and 4 were also associated with increasing dilute extractable lead and nickel in 2023 and 2024 (Figure 15).



Figure 19. Distance-based redundancy analysis of bacterial ASV community structure in 2023 and 2024, with contaminant and environmental variables that explained a significant amount over variation in the community overlaid as vectors. Pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.

3.4.4 Species richness and abundance

Bacterial ASV richness and abundance differed significantly between site categories within each pairing in 2023 (abundance only) and 2024 (Figures 16 and 17, Table 15). In 2023, ASV abundances were significantly lower at the site of interest than the paired reference site for Pairing 5 (Site 5 < Site 10), while in 2024 ASV abundances were significantly higher at the site of interest than the paired reference site for Pairing 1 (Site 1 > Site 6). In 2024, ASV richness was also significantly higher at Site 1 than Site 6 (Figure 17, Table 15, Table S16). Overall trends across sites and times were highly variable and statistical models did not fit the data well.



Figure 20. Mean (\pm SE) bacterial Amplicon Sequence Variants (ASVs) richness across sites in 2023 and 2024. Site pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.



Figure 21. Mean (\pm SE) bacterial Amplicon Sequence Variants (ASVs) abundance across sites in 2023 and 2024. Site pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.

Table 17. Statistical results from univariate analyses of bacterial Amplicon Sequence Variants (ASVs). Pr(>Chisq) values <0.05 indicate significant differences. Chi-square statistic ('Chisq'), degrees of freedom ('DF'), and P-value 'Pr(>Chisq)').

Parameter, Year			
Bacterial richness, 2023	Chisq	Df	Pr(>Chisq)
Site category	3.2791	1	0.0702
Pairing	6.4654	4	0.1670
Site category x Pairing	5.9309	4	0.2044
Bacterial richness, 2024	Chisq	Df	Pr(>Chisq)
Site category	0.6788	1	0.4100
Pairing	13.2575	4	0.0101
Site category x Pairing	11.3476	4	0.0229
Bacterial abundance, 2023	Chisq	Df	Pr(>Chisq)
Site category	1.2825	1	0.2574
Pairing	17.0825	4	0.0019
Site category x Pairing	10.6386	4	0.0309
Bacterial abundance, 2024	Chisq	Df	Pr(>Chisq)
Site category	0.9715	1	0.32432
Pairing	50.6394	4	<0.0001
Site category x Pairing	10.6129	4	0.0313

3.5 Archaeal community (eDNA)

3.5.1 Individual taxa

Across both years, a total of 2,438 archaeal ASVs were found, of which 2,289 were assigned to 21 known classes. The most abundant classes across both years were Bathyarchaeia and Undinarchaeia, followed by Heimdallarchaeia (Figure 18).



Figure 22. Mean abundance of Amplicon Sequence Variants (ASVs) within each archaeal class in samples collected in 2023 and 2024 across sites in the southern section of Lake Macquarie, NSW, Australia. Bars to the left of each pair are sites of interest, and bars on the right of each pairing are paired reference sites.

3.5.2 Community structure

Analyses found a significant effect of the interaction between site category and pairing for archaeal community structure in 2023 and 2024 (Table 16). Pairwise comparisons found that community structure differed for all pairings in 2023 and all except for Pairing 3 in 2024 (i.e. Site 3 vs Site 8) (Table S 21). SIMPER analysis found that differences were explained by Bathyarchaeia, which in many cases explained more than 50% of differences between paired sites. Bathyarchaeia were more abundant at sites of interest than reference sites for Pairings 1 and 3 in 2023 and pairing 3 in 2024, but otherwise were most abundant at reference sites (Table S22).

Table 18. Full PERMANOVA results for archaeal ASV community structure across site pairings in the southern section of Lake Macquarie, NSW, Australia. P(perm) values <0.05 indicate significant differences. Sources of variation in the model ('Source'), the degrees of freedom ('df'), sums of squares ('SS'), mean squares ('MS'), pseudo-F ratio ('Pseudo-F') and permutation P-value ('P(perm)').

Source					
Archaea, 2023	df	SS	MS	Pseudo-F	P(perm)
Site category	1	2520	2520	3.9496	0.0001
Pairing	4	19931	4983	7.8087	0.0001
Site category x Pairing	4	5725	1431	2.2431	0.0001
Res	30	19143	638		
Total	39	47319			
Archaea, 2024	df	SS	MS	Pseudo-F	P(perm)
Site category	1	2828	2828	4.7902	0.0001
Pairing	4	21555	5389	9.127	0.0001
Site category x Pairing	4	6860	1715	2.9051	0.0001
Res	30	17712	590		
Total	39	48956			

3.5.3 Relationship between environmental variables and community structure

Of the environmental variables collected, dissolved barium, boron, iron, vanadium, molybdenum and manganese, dilute acid extractable lead, nickel and chromium, the 0.063 ount of variation in the archaeal community structure in 2023. Dissolved barium, boron and vanadium in porewater, dilute acid extractable lead, nickel and chromium, most size fractions and moisture content were also important predictors in 2024 (Table S23 and Table S24).

When visualised with a dbRDA plot, there weren't clear correlations between environmental variables and site categories with differences mainly between site pairings (Figure 19). For example, in 2023 and 2024, Sites 3 and 8, 4 and 9 and 5 and 10 separated along a gradient of increasing dilute acid extractable nickel and lead/zinc, while Sites 1 and 6 and Sites 2 and 7 separated out along a particle size gradient.



Figure 23. Distance-based redundancy analysis of archaeal ASV community structure in 2023 and 2024, with contaminant and environmental variables that explained a significant amount over variation in the community overlaid as vectors. Pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.

3.5.4 Species richness and abundance

Archaeal ASV abundance differed significantly between site categories within each pairing in 2023 and 2024, but richness did not significantly differ across categories or within pairings (Figure 20 and Figure 21, Table 17). In 2023, ASV abundances were significantly higher at the site of interest than the paired reference site for Pairing 5 (Site 5 > Site 10), while in 2024 ASV abundances were significantly lower at the site of interest than the paired reference site for Pairing 5. (Site 5 > Site 10), while in 2024 ASV abundances were significantly lower at the site of interest than the paired reference site for Pairing 2. (Site 2 < Site 7) (Figure 21, Table 17, Table 5.). Overall trends across sites and times were highly variable and statistical models did not fit the data well.



Figure 24. Mean (\pm SE) archaeal Amplicon Sequence Variants (ASVs) richness across sites in 2023 and 2024. Site pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.



Figure 25. Mean (\pm SE) archaeal Amplicon Sequence Variants (ASVs) abundance across sites in 2023 and 2024. Site pairings have the same colours. Filled bars are sites of interest and open bars are paired reference sites.

Table 19. Statistical results from univariate analyses of archaeal Amplicon Sequence Variants (ASVs). Pr(>Chisq) values <0.05 indicate significant differences. Chi-square statistic ('Chisq'), degrees of freedom ('DF'), and P-value 'Pr(>Chisq) ').

Parameter, Year			
Archaeal richness, 2023	Chisq	Df	Pr(>Chisq)
Site category	0.0143	1	0.9049
Pairing	3.0920	4	0.5425
Site category x Pairing	7.2057	4	0.1254
Archaeal richness, 2024	Chisq	Df	Pr(>Chisq)
Site category	0.7893	1	0.3743
Pairing	7.6436	4	0.1055
Site category x Pairing	4.5089	4	0.3415
Archaeal abundance, 2023	Chisq	Df	Pr(>Chisq)
Site category	1.7664	1	0.1838
Pairing	29.1212	4	<0.0001
Site category x Pairing	9.9168	4	0.0419
Archaeal abundance, 2024	Chisq	Df	Pr(>Chisq)
Site category	10.9140	1	0.0010
Pairing	18.8460	4	0.0008
Site category x Pairing	14.3000	4	0.0064

4 Conclusions

Community structure, taxon richness, total abundance and functional group analyses were used to investigate potential impacts from contaminants to the ecological communities between reference sites and sites of interest in Lake Macquarie. Community structure quantifies the compositional differences between communities based on the presence and abundance of species. Community structure is particularly useful to assess in ecological studies because the metric is sensitive to changes in both the presence/absence and abundance of species that can signal environmental changes.

Taxon richness, total abundances and functional group assessments are also common metrics in ecological studies. Taxon richness is important to assess because high diversity typically indicates a healthy environment. Differences in abundances among samples are important to quantify because changes in the number of individuals can also signal environmental changes with increases potentially indicative of increased food availability. Assessing different functional groups provides insights into ecosystem functioning and health. Annelids, molluscs, diatoms, dinoflagellates and nematodes were assessed in this study with the expectation that tolerant groups such as annelids, polychaetes and nematodes would increase at sites of interest while more sensitive groups such as molluscs, amphipods diatoms and dinoflagellates would decrease if contaminants were having an impact on communities (Dafforn et al., 2013, Suzzi et al., 2023).

Thirty-four benthic invertebrate taxa from 9 phyla were identified from the sediment samples, and communities were dominated by molluscs, annelids, arthropods and nematodes. This was comparable to the study by Chariton et al. (2011) who found 40 taxa across a larger area of the lake. After bioinformatic processing, 8,959 eukaryotic ASVs (137 eukaryotic phyla), 6,101 bacterial ASVs (107 bacterial classes) and 2,438 archaeal ASVs (21 archaeal classes) remained. The eukaryotic sampling found the same order of magnitude of ASVs as Suzzi et al. (2023) who retained 6857 ASVs in their surveys of Lake Macquarie. The eukaryotic communities were dominated by unidentified organisms highlighting the gaps in taxonomic classification that still exist in databases. Annelids and arthopods were the dominant invertebrate taxa in the eukaryotic eDNA samples. Bacterial communities were dominated by alpha- and gammaproteobacterial, phycisphaerae, planctomycetes, spirochaetia and thermodesulfovibrionia while archaeal communities were dominated by bathyarchaeia, undinarchaeia and heimdallarchaeia.

Analyses of community structure and composition found no significant differences between site categories for the benthic invertebrate communities investigated here and few instances of difference between site categories within pairings. In contrast, there were clear differences in microbial community composition with gradients in dilute acid extractable metals (particularly lead and zinc) often explaining separation between sites of interest and reference sites within a pairing (Table 18). This was significant for Pairing 4 (i.e., Sites 4 and 9) across all microbial communities, but less frequently significant for other pairings.

The different patterns observed for the benthic invertebrate communities compared to the microbial communities is likely related to the taxonomic resolution provided by these different methods. The greater taxonomic resolution provided by environmental DNA can increase the power of discrimination between samples and include additional species such as bacteria and diatoms that are more sensitive to spatial variation in environmental stressors (Dafforn et al.,2014).

While the shifts in community composition of the microbial communities do not conclusively indicate an impact from sediment contaminants (further experimental work would be necessary

to determine causality), the relationship to environmental predictors such as dilute extractable lead, zinc, nickel and chromium suggests that at some sites of interest the microbial communities may have been influenced by the presence of elevated contaminants (Sun et al., 2012). The amount of variation explained by any single environmental variable was generally less than 20%, but this may still be significant for several reasons. Firstly, ecological communities are inherently complex and influenced by a multitude of biotic and abiotic factors. It is common for ecological data to have a high degree of variability that cannot be fully captured by a single model or a small set of variables. Therefore, even explaining a small percentage of the variation can provide valuable insights into key drivers of community structure. Additional variables not measured here such as temperature, nutrients and distance from the influence of freshwater impacts may also be important. Secondly, even if the explained variation is low, identifying the variables that contribute to this variation can be crucial even if they are not the sole drivers. Finally, in some cases, subtle environmental effects may only explain a small proportion of the variation but can still be ecologically significant. For instance, small changes in nutrient levels or contamination might only slightly alter community composition, but these changes can have cascading effects on the ecosystem.

Analyses of richness and abundance found fairly consistent significant differences between site categories within pairings for the benthic invertebrate communities, while ASV richness and abundance in the microbial communities were spatially and temporally variable. Based on previous research (e.g., meta-analysis by Johnston and Roberts, 2009), we would expect any negative effects of contaminants to manifest through decreases in abundance and diversity of benthic invertebrates, but interestingly, we observed that invertebrate richness and abundances tended to be greater at sites of interest than at reference sites. This suggests that any effects of contaminants in Lake Macquarie are not significant and may reflect lower bioavailability of contaminants due to the high silt and organic content of the sediments, which also serve as a food source (Chapman and Wang, 2001).

Based on past studies of infaunal communities, greater richness and abundances at sites of interest may suggest an intermediate disturbance that is selecting for more tolerant and opportunistic species, allowing them to thrive in environments where competition is reduced (Olsgard, 1999). This was investigated further by analysing infaunal ratios of polychaetes/amphipods with the expectation that a higher proportion of opportunistic polychaetes than more sensitive amphipods at sites of interest might indicate that an impact is occurring (Gesteira and Dauvin, 2000). However, polychaetes dominated all benthic samples with amphipods only found as individuals in a couple of replicates. Thus, patterns are more likely explained by other environmental drivers such as organic enrichment rather than contamination.

Overall, the results from each of these ecological communities suggest that concentrations of metals, particularly the dilute acid extractable contaminants (Pb/Zn, Ni and Cr) were the best predictors of spatial variation, but they did not explain all of the variation, suggesting that other environmental drivers were also important. Benthic invertebrate richness and abundance, and microbial community structure were the best differentiators between site categories within pairings and the differences observed between sites of interest and reference sites may indicate an intermediate disturbance from metal contamination, with separation between sites of interest and reference sites 3 and 4. There was significant temporal and spatial variability in other indicators assessed with patterns in tolerant and sensitive species not indicative of a significant impact from metal contamination.

	Invertebrates		Eukaryotes		Bacteria		Archaea	
	2023	2024	2023	2024	2023	2024	2023	2024
Al (dissolved) ^a								
As (dissolved) ^a	Х		Х					
Ba (dissolved)ª			Х	Х			Х	Х
B (dissolved) ^a			Х	Х	Х	Х	Х	Х
Co (dissolved) ^a								
Cu (dissolved)ª								
Fe (dissolved) ^a	X		X		Х		Х	
Pb (dissolved) ^a								
Mn (dissolved) ^a	Х		Х	Х			Х	
Mo (dissolved) ^a	X	Х	X	X	Х		Х	
V (dissolved) ^a	X	Х	X	X	Х		X	Х
As1M HCl extract ^b								
Cd 1M HCl extract ^b								
Cr 1M HCl extract ^b			Х	Х	Х	Х	Х	Х
Cu 1 M HCl extract ^b	Х							
Pb 1M HCl extract ^b	Х	Х	Х	Х	Х	Х	Х	Х
Ni 1M HCl extract ^b	Х		Х		Х		Х	Х
Se 1 M HCl extract ^b								
Moisture	Х		Х	Х	Х	Х	Х	Х
<0.002 mm		Х	Х	Х		Х		Х
0.002 mm - 0.063 mm		Х		Х		Х		Х
< 0.063 mm			Х		Х		Х	
0.063 mm - 2.0 mm			Х	Х	Х	Х	Х	Х
TOC	Х	Х	Х	Х		Х		Х

Table 20. Summary of significant environmental predictors for each ecological community investigated

 $^{\rm a}$ Variables are estimated dissolved (<0.45 μm) metal concentrations in the pore waters.

^b Variables are 1 M HCl extract is the dilute-acid extractable concentration of the sediment.

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6 Appendix

Table S1. Full PERMDISP results for invertebrate community structure when comparing between the interaction of site category and pairing in 2023 and 2024. P(perm) values <0.05 indicate significant differences. Sources of variation in the model ('Source'), F-statistic ('F'), the degrees of freedom ('df'), and permutation P-value ('P(perm)').

Source				
Invertebrates, 2023	F	Df1	Df2	P (perm)
Community structure	1.665	9	30	0.6125
Invertebrates, 2024	F	Df1	Df2	P (perm)
Community structure	4.1004	9	30	0.0544

Table S2. Full PERMDISP results for eukaryotic community structure when comparing between the interaction of site category and pairing in 2023 and 2024. P(perm) values <0.05 indicate significant differences. Sources of variation in the model ('Source'), F-statistic ('F'), the degrees of freedom ('df'), and permutation P-value ('P(perm)').

Source				
Eukaryotes, 2023	F	Df1	Df2	P (perm)
Community structure	3.5951	9	30	0.0611
Eukaryotes, 2024	F	Df1	Df2	P (perm)
Community structure	5.5215	9	30	0.0103

Table S3. Full PERMDISP results for bacterial community structure when comparing between the interaction of site category and pairing in 2023 and 2024. P(perm) values <0.05 indicate significant differences. Sources of variation in the model ('Source'), F-statistic ('F'), the degrees of freedom ('df'), and permutation P-value ('P(perm)').

Source				
Bacteria, 2023	F	Df1	Df2	P (perm)
Community structure	3.0538	9	30	0.2199
Bacteria, 2024	F	Df1	Df2	P (perm)
Community structure	2.4077	9	30	0.5868

Table S4. Full PERMDISP results for archaeal community structure when comparing between the interaction of site category and pairing in 2023 and 2024. P(perm) values <0.05 indicate significant differences. Sources of variation in the model ('Source'), F-statistic ('F'), the degrees of freedom ('df'), and permutation P-value ('P(perm)').

Source				
Archaea, 2024	F	Df1	Df2	P (perm)
Community structure	3.8375	9	30	0.0848
Archaea, 2024	F	Df1	Df2	P (perm)
Community structure	8.3673	9	30	0.0006

6.1 Benthic invertebrate community

6.1.1 Individual taxa



Figure S1. Mean abundance of individual taxa collected across sites in 2023 in the southern section of Lake Macquarie, NSW, Australia.



Figure S2. Mean abundance of individual taxa collected across sites in 2024 in the southern section of Lake Macquarie, NSW, Australia.

6.1.2 Community structure

Table S5. Pairwise comparisons of invertebrate community structure among site categories within each pairing for 2023. P-values <0.05 indicate significant differences. Sources of variation in the model ('Source'), t-test ('t'), permutation P-value ('P(perm)'), and number of unique permutations ('Unique perms').

Source						
Invertebrates, 2023						
Pairwise comparisons	t	P(perm)	Unique perms			
Pairing 1 (Site 1 vs Site 6)	1.9754	0.0291	35			
Pairing 2 (Site 2 vs Site 7)	1.5523	0.0879	35			
Pairing 3 (Site 3 vs Site 8)	1.5942	0.0582	35			
Pairing 4 (Site 4 vs Site 9)	1.038	0.3987	35			
Pairing 5 (Site 5 vs Site 10)	1.4903	0.0608	35			

6.1.3 Relationship between environmental variables and community structure

Table S6. Full DISTLM results of marginal tests for invertebrate community structure in 2023. P-values <0.05 indicate significant differences. Sums of squares ('SS'), pseudo-F ratio ('Pseudo-F') and P-value ('P-value') and proportion of variation explained.

Variable	SS (trace)	Pseudo-F	P-value	Proportion
Total Organic Carbon	8818.8	4.8922	0.0001	0.11406
Manganese (dissolved)ª	7197	3.9002	0.0009	0.093083
lron (dissolved)¤	6194.5	3.3096	0.0031	0.080117
Lead 1M HCI extract ^b	5485.3	2.9017	0.0078	0.070944
Vanadium (dissolved) ^a	4924.6	2.585	0.0185	0.063692
Nickel 1M HCI extract ^b	4588.5	2.3974	0.0236	0.059346
Moisture	4587.8	2.397	0.0241	0.059337
Arsenic (dissolved)ª	4519.4	2.3591	0.0269	0.058452
Molybdenum (dissolved) ^a	4214.1	2.1905	0.0402	0.054504
Copper 1M HCl extract ^b	4021.5	2.0849	0.0481	0.052013
Selenium 1M HCl extract ^b	3941.4	2.0412	0.0543	0.050977
Size fraction 0.002 mm - 0.063 mm	3828.6	1.9797	0.0616	0.049518
Boron (dissolved) ^a	3464.3	1.7825	0.0969	0.044807
Chromium 1M HCI extract ^b	3340.6	1.7159	0.11	0.043206
Cadmium 1M HCl extract ^b	3112.2	1.5937	0.1351	0.040252
Size fraction < 0.063 mm	2715.3	1.3831	0.2044	0.035119
Size fraction 0.063 mm - 2.0 mm	2546.8	1.2943	0.2471	0.03294
Cobalt (dissolved)ª	1885	0.94961	0.4642	0.02438
Size fraction <0.002 mm	1444.5	0.72347	0.662	0.018683
Lead (dissolved)ª	1421.1	0.71152	0.6627	0.01838
Arsenic 1M HCI extract ^b	1274.3	0.63677	0.7232	0.016481
Barium (dissolved) ^a	1078.2	0.53741	0.8041	0.013945
Aluminium (dissolved)ª	975.27	0.48544	0.8379	0.012614
Copper (dissolved) ^a	-9.29E-12	-2.53E-15	1	-1.20E-16

^a Variables are estimated dissolved (<0.45 um) metal concentrations in the pore waters.

^b Variables are 1M HCl extract is the dilute-acid extractable concentration of the sediment.
Variable	SS (trace)	Pseudo-F	P-value	Proportion
Lead 1M HCl extract ^b	17725	6.2122	0.0001	0.14051
Vanadium (dissolved)ª	9020	2.9263	0.0044	0.071501
Total Organic Carbon	7140	2.2798	0.0186	0.056599
Size fraction <0.002 mm	6992.8	2.23	0.0209	0.055432
Molybdenum (dissolved)¤	6382.8	2.0251	0.0367	0.050596
Size fraction 0.002 mm - 0.063 mm	6257.1	1.9831	0.0425	0.0496
Nickel 1M HCl extract ^b	5773.8	1.8226	0.0584	0.045768
Selenium (dissolved)ª	5621.6	1.7723	0.1262	0.044562
Chromium 1M HCl extract ^b	4810	1.5063	0.134	0.038129
Moisture	4760.9	1.4903	0.1374	0.037739
Mercury 1M HCl extract ^b	4787.7	1.4991	0.1395	0.037952
Copper 1M HCl extract ^b	4594.6	1.4363	0.1708	0.036422
Manganese (dissolved)ª	4387.1	1.3691	0.1923	0.034776
Selenium 1 M HCl extract ^b	4171.5	1.2995	0.2224	0.033067
Arsenic 1M HCI extract ^b	4047.1	1.2595	0.2376	0.032081
Size fraction 0.063 mm - 2.0 mm	3691.7	1.1456	0.3171	0.029264
Size fraction < 0.063 mm	3609.9	1.1194	0.3461	0.028615
Boron (dissolved) ^a	3323.9	1.0283	0.4096	0.026348
Barium (dissolved)ª	3243.3	1.0028	0.4296	0.02571
Copper (dissolved) ^a	2703.1	0.83208	0.474	0.021428
Zinc (dissolved)ª	2703.1	0.83208	0.4809	0.021428
Cobalt (dissolved) ^a	2977.9	0.91871	0.5113	0.023606
Nickel (dissolved) ^a	2566.8	0.78925	0.5969	0.020347
Chromium (dissolved) ^a	2192.4	0.67209	0.7474	0.017379
Arsenic (dissolved)ª	1108.3	0.33679	0.9663	0.008785

Table S7. Full DISTLM results of marginal tests for invertebrate community structure in 2024. P-values <0.05 indicate significant differences. Sums of squares ('SS'), pseudo-F ratio ('Pseudo-F') and P-value ('P-value') and proportion of variation explained.

^a Variables are estimated dissolved (<0.45 um) metal concentrations in the pore waters.

6.1.4 Species richness and abundance

Table S8. Pairwise comparisons of total abundance of invertebrate taxa between sites of interest and paired reference sites for each pairing in 2023. P-values <0.05 indicate significant differences. Standard error ('SE'), degrees of freedom ('df'), t-statistic ('t-ratio'). Results are given on the log (not the response) scale.

Parameter, Year					
Invertebrate abundance, 2023	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	1.499	0.33	29	4.503	0.0001
Pairing 2 (Site 2 vs Site 7)	0.108	0.31	29	0.348	0.7304
Pairing 3 (Site 3 vs Site 8)	0.405	0.28	29	1.446	0.1588
Pairing 4 (Site 4 vs Site 9)	0.230	0.29	29	0.796	0.4325
Pairing 5 (Site 5 vs Site 10)	0.901	0.30	29	3.026	0.0052

Table S9. Pairwise comparisons of total mollusc abundance between sites of interest and paired reference sites for each pairing in 2023. P-values <0.05 indicate significant differences. Standard error ('SE'), degrees of freedom ('df'), t-statistic ('t-ratio'). Results are given on the log (not the response) scale.

Phylum, Year					
Mollusca, 2023	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	1.099	0.487	29	2.258	0.0316
Pairing 2 (Site 2 vs Site 7)	-0.302	0.342	29	-0.885	0.3837
Pairing 3 (Site 3 vs Site 8)	1.070	0.265	29	4.033	0.0004
Pairing 4 (Site 4 vs Site 9)	0.365	0.287	29	1.271	0.2137
Pairing 5 (Site 5 vs Site 10)	1.609	0.338	29	4.757	<0.0001

6.2 Eukaryotic microbial community (eDNA)

6.2.1 Individual taxa



Figure S3. Mean abundance of Amplicon Sequence Variants (ASVs) within each eukaryotic phylum in samples across sites in 2023 and 2024 in the southern section of Lake Macquarie, NSW, Australia.

6.2.2 Community structure

Table S10. Pairwise comparisons of eukaryotic community structure among site categories within each pairing for 2023. P-values <0.05 indicate significant differences. Sources of variation in the model ('Source'), t-test ('t'), permutation P-value ('P(perm)'), and number of unique permutations ('Unique perms').

Source			
Eukaryotes, 2023			
Pairwise comparisons	t	P(perm)	Unique perms
Pairing 1 (Site 1 vs Site 6)	1.4028	0.0313	35
Pairing 2 (Site 2 vs Site 7)	1.2982	0.0309	35
Pairing 3 (Site 3 vs Site 8)	1.1891	0.0605	35
Pairing 4 (Site 4 vs Site 9)	1.3742	0.0277	35
Pairing 5 (Site 5 vs Site 10)	1.3791	0.0268	35
Eukaryotes, 2024			
Pairwise comparisons	t	P(perm)	Unique perms
Pairing 1 (Site 1 vs Site 6)	1.5521	0.0278	35
Pairing 2 (Site 2 vs Site 7)	1.2124	0.0301	35
Pairing 3 (Site 3 vs Site 8)	1.4431	0.0304	35
Pairing 4 (Site 4 vs Site 9)	1.4306	0.0302	35
Pairing 5 (Site 5 vs Site 10)	1.2250	0.0860	35

Table S11. Full SIMPER results for eukaryotic community structure sampled in 2023 and 2024. A 90% cut-off was applied. Average abundance ('Av. Abund'), average dissimilarity ('Av. Diss'), dissimilarity/standard deviation ('Diss/SD'), individual contribution ('Contrib. %'), and cumulative percentage explained ('Cum %). 'UI' = unidentified.

Ταχα, 2023	Site 1 Av. Abun	Site 6 Av. Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
NA	463	361	7.37	1.33	19.9	19.9
Dinophyceae	95.3	150	2.67	1.76	7.21	27.1
Nematoda	27.1	96.3	1.74	2.96	4.71	31.8
Brachiopoda	0	60.4	1.56	0.55	4.22	36.0
Spirotrichea	28.7	77.0	1.27	1.37	3.43	39.5
Euglenozoa	162	124	1.23	1.05	3.32	42.8
Syndiniales	36.0	74.2	1.17	1.25	3.15	45.9
Gastrotricha	9.07	53.2	1.1	1.39	2.98	48.9
Arthropoda	67.4	67.2	1.06	1.66	2.87	51.8
Trebouxiophyceae	31.5	69.5	1.04	1.37	2.8	54.6
Bacillariophyceae	125	162	1	1.26	2.71	57.3
Cercozoa	15.5	54.0	1	1.95	2.69	60
Apicomplexa	90.9	118	0.92	1.05	2.47	62.5
Mediophyceae	180	196	0.88	1.37	2.37	64.8
UI Archaeplastida	13.3	46.1	0.85	1.68	2.3	67.1
Mollusca	8.61	32.7	0.73	1.31	1.97	69.1
Annelida	25.5	29.9	0.69	0.88	1.87	71.0
UI TSAR	18.1	39.7	0.63	1.62	1.7	72.7

Dinoflagellata	25.3	34.1	0.62	1.73	1.68	74.3
Ciliophora	9.21	29.2	0.56	1.62	1.5	75.9
UI Gyrista	9.29	29.4	0.48	1.83	1.3	77.2
UI Alveolata	14.3	32.3	0.48	1.43	1.29	78.4
Nemertea	0	17.0	0.44	1.2	1.18	79.6
Filosa-Thecofilosea	8.92	14.3	0.33	1.23	0.9	80.5
Filosa-Imbricatea	6.05	13.9	0.33	1.44	0.89	81.4
UI Metazoa	23.1	26.8	0.29	1.29	0.79	82.2
Platyhelminthes	4.24	12.6	0.27	0.89	0.72	82.9
Cyclotrichium	7.3	6.97	0.26	0.91	0.71	83.6
Fungi	4.92	13.9	0.26	1.45	0.71	84.3
Gregarinomorphea	31.0	32.3	0.26	1.22	0.71	85.1
UI Amoebozoa	13.8	8.91	0.25	1.08	0.67	85.7
Chytridiomycota	5.58	12.7	0.22	1.31	0.6	86.3
Raphidophyceae	15.3	21.1	0.21	1.34	0.57	86.9
Diplonemea	7.25	14.0	0.2	1.99	0.54	87.4
Streptophyta_X	0	8.06	0.2	4.03	0.54	88.0
Globothalamea	8.71	9.44	0.19	1.31	0.52	88.5
Evosea_X	3.51	10.3	0.18	1.85	0.48	89.0
Foraminifera	5.65	9.59	0.16	1.4	0.43	89.4
UI Opisthokonta	9.87	4.51	0.15	1.55	0.41	89.8
Sagenista	13.4	17.6	0.14	1.43	0.38	90.2

	Site 2	Site 7				
Taxa, 2023	Abun	Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
NA	381	475	10.3	1.36	26.1	26.1
Dinophyceae	141	72.6	2.97	1.08	7.54	33.7
Arthropoda	104	39.6	2.37	1.31	6.03	39.7
Euglenozoa	68.2	129.6	2.12	2.06	5.37	45.1
Apicomplexa	59.7	123.7	2.11	1.07	5.36	50.4
Mediophyceae	107	163.3	1.63	1.95	4.14	54.6
Syndiniales	60.4	46.2	1.5	1.79	3.81	58.4
Bacillariophyceae	104	148.5	1.36	1.29	3.45	61.8
UI TSAR	16.0	44.1	1.02	1.23	2.59	64.4
Spirotrichea	35.1	25.1	0.91	1.55	2.32	66.7
Trebouxiophyceae	32.2	32.1	0.79	1.79	2	68.7
Gastrotricha	25.5	5.18	0.67	1.62	1.7	70.4
Nematoda	25.4	35.9	0.65	1.2	1.65	72.1
UI Metazoa	24.9	13.5	0.59	1.26	1.49	73.6
Platyhelminthes	19.2	2.29	0.56	1.12	1.41	75
Dinoflagellata	17.7	9.2	0.49	1.16	1.24	76.2
Annelida	13.0	2.1	0.48	0.62	1.23	77.5

UI Amoebozoa	8.78	13.7	0.39	1.47	0.99	78.5
Cercozoa	15.3	12.0	0.38	1.74	0.97	79.4
Peronosporomycetes	6.73	18.6	0.37	2.67	0.94	80.4
Filosa-Thecofilosea	12.7	7.95	0.36	1.28	0.92	81.3
Gregarinomorphea	19.3	23.8	0.36	1.32	0.92	82.2
UI Archaeplastida	11.9	9.38	0.31	1.72	0.79	83.01
Ciliophora	12.0	7.29	0.29	1.43	0.74	83.8
UI Opisthokonta	8.43	3.94	0.26	1.27	0.66	84.4
Filosa-Imbricatea	7.22	4.23	0.25	2.55	0.63	85.0
Raphidophyceae	15.6	21.7	0.25	1.13	0.63	85.7
UI Alveolata	12.8	10.3	0.24	1.5	0.6	86.3
UI Gyrista	7.48	14.9	0.23	1.53	0.59	86.9
Diplonemea	4.12	10.5	0.2	3.3	0.5	87.4
Chytridiomycota	6.15	2.86	0.2	1.73	0.5	87.8
Cyclotrichium_like	5.67	0.66	0.19	1.46	0.49	88.3
Euglenida	0.56	7.09	0.19	2.14	0.49	88.8
UI Evosea	0.96	5.76	0.19	0.86	0.47	89.3
Sagenista	7.39	13.2	0.17	1.46	0.44	89.7
Phaeophyceae	6.02	11.1	0.17	2.21	0.43	90.2

	Site 3 Av.	Site 8 Av.				
Taxa, 2023	Abun	Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
NA	1199	765	11.3	1.42	30.8	30.8
Dinophyceae	366	129	3.58	1.54	9.76	40.6
Arthropoda	182	65.8	1.87	1.31	5.1	45.7
Apicomplexa	245	154	1.59	1.59	4.35	50.0
Syndiniales	135	52.5	1.25	1.51	3.4	53.4
UI Metazoa	97.9	22.8	0.97	1.25	2.64	56.1
UI Archaeplastida	87.0	22.2	0.95	2.38	2.6	58.7
Dinoflagellata	81.5	25.8	0.82	2.32	2.23	60.9
Trebouxiophyceae	98.7	47.9	0.8	1.26	2.18	63.1
Filosa-Thecofilosea	68.2	21.1	0.79	1.57	2.16	65.3
Filosa-Imbricatea	63.7	12.8	0.75	1.85	2.03	67.3
Spirotrichea	68.2	32.0	0.74	1.67	2.02	69.3
Cercozoa	69.0	27.8	0.63	1.48	1.71	71.0
Mediophyceae	265	240	0.61	1.27	1.66	72.7
Bacillariophyceae	216	203	0.5	1.29	1.38	74.1
Annelida	42.0	17.9	0.49	1.28	1.33	75.4
UI Amoebozoa	14	41.4	0.48	0.75	1.32	76.7
Ciliophora	44.7	20.2	0.47	1.94	1.3	78.0
Nematoda	44.4	45.5	0.4	1.36	1.1	79.1
Euglenozoa	222	212	0.4	1.54	1.09	80.2

UI Alveolata	59.7	37.9	0.37	1.34	1	81.2
Gastrotricha	32.5	9.27	0.33	1.8	0.91	82.1
Fungi	29.2	9.9	0.28	1.67	0.77	82.9
Chytridiomycota	23.9	7.73	0.27	1.67	0.75	83.6
Gregarinomorphea	45.6	48.1	0.27	1.49	0.73	84.3
Prostomatea	18.7	3.61	0.25	1.03	0.68	85.0
Gyrista_X	28.9	13.5	0.23	2.35	0.61	85.6
Platyhelminthes	18.8	6.16	0.2	1.24	0.54	86.2
Sagenista	35.2	30.9	0.19	1.61	0.51	86.7
UI TSAR	53.3	48.8	0.17	1.36	0.47	87.2
Basidiomycota	11.3	3.39	0.15	1.64	0.4	87.6
Foraminifera	18.6	9.68	0.13	1.66	0.37	87.9
Ascomycota	10.8	7.43	0.13	2.21	0.35	88.3
UI Gyrista	25.8	22.7	0.13	1.15	0.35	88.6
Coscinodiscophyceae	16.9	9.04	0.13	2.09	0.35	89.0
Apusomonada_X	13.2	5.91	0.13	1.27	0.34	89.3
Evosea_X	12.5	4.49	0.12	1.51	0.34	89.7
Peronosporomycetes	35.3	28.4	0.12	0.85	0.34	90
Rhodophyta_X	13.9	7.68	0.12	1.16	0.34	90.3

	Site 5	Site 10				
Taxa, 2023	Av. Abun	Av. Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
NA	1489	723	16.4	1.46	42.6	42.6
Dinophyceae	122	214	2.19	1.56	5.67	48.3
Arthropoda	111	138	1.44	2.08	3.74	52.0
Euglenozoa	302	233	1.44	1.18	3.73	55.7
UI Amoebozoa	98.5	36.3	1.13	0.94	2.92	58.7
Mediophyceae	245	183	1.1	1.45	2.85	61.5
Syndiniales	62.4	96.8	0.94	1.56	2.45	64.0
Bacillariophyceae	239	184	0.92	1.8	2.4	66.4
Apicomplexa	164	172	0.71	1.53	1.83	68.2
Filosa-Thecofilosea	30.9	39.5	0.67	1.36	1.72	69.9
Trebouxiophyceae	49.3	61.6	0.61	1.91	1.59	71.5
Spirotrichea	33	50.7	0.57	1.6	1.49	73.0
UI Metazoa	47.0	23	0.56	0.9	1.46	74.4
Nematoda	26.8	58.7	0.49	1.25	1.27	75.7
Annelida	20.9	43.2	0.44	1.02	1.13	76.9
UI Alveolata	33.1	38.5	0.41	1.3	1.07	77.9
Cercozoa	35.0	32.1	0.37	1.28	0.96	78.9
Peronosporomycetes	45.8	22.5	0.37	2.86	0.95	79.8
Ciliophora	17.1	34.3	0.36	1.41	0.94	80.8
UI Archaeplastida	27.2	32.7	0.35	1.72	0.92	81.7
Dinoflagellata	33.1	33.8	0.35	3.06	0.91	82.6

Platyhelminthes	76	25.5	033	0.96	0.86	83.5
ridryneininnes	7.0	23.5	0.00	0.70	0.00	00.5
UI TSAR	43.8	35.8	0.33	1.34	0.86	84.3
Gastrotricha	8.1	24.5	0.32	1.66	0.84	85.2
Filosa-Imbricatea	9.97	21.5	0.32	1.09	0.83	86.0
UI Opisthokonta	19.3	3.49	0.29	0.93	0.74	86.7
Gregarinomorphea	35.2	25.9	0.24	0.9	0.62	87.4
Globothalamea	17.5	2.17	0.24	1.36	0.61	88.0
UI Gyrista	29.1	18.0	0.22	1.05	0.58	88.5
Chytridiomycota	11.6	19.3	0.21	1.66	0.54	89.1
Foraminifera	14.8	6.63	0.19	0.67	0.49	89.6
Raphidophyceae	30.7	19.9	0.18	1.51	0.48	90.0

	Site 1 Av.	Site 6 Av.				
Таха, 2024	Abun	Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
NA	662	262	10.1	1.32	27.4	27.4
Dinophyceae	98.5	259	4.17	1.47	11.4	38.8
Arthropoda	47.2	133	2.25	1.65	6.14	45.0
Euglenozoa	173	84.4	2.13	1.51	5.82	50.8
Apicomplexa	182	179	1.13	1.4	3.08	53.9
Nematoda	84.3	53.7	0.92	1.15	2.52	56.4
Gastrotricha	31.2	47.3	0.92	1.35	2.5	58.9
Bacillariophyceae	182	165	0.87	1.82	2.37	61.3
Spirotrichea	31.4	44.2	0.76	1.54	2.07	63.3
Trebouxiophyceae	20.5	50.2	0.74	1.45	2.03	65.4
Mediophyceae	281	257	0.73	1.48	1.99	67.3
Diplonemea	31.4	2.48	0.67	2.48	1.84	69.2
Syndiniales	23.8	33.7	0.56	1.66	1.53	70.7
UI Archaeplastida	22.1	37.1	0.54	1.34	1.48	72.2
Ciliophora	19.7	25.4	0.5	1.71	1.36	73.6
Filosa-Thecofilosea	9.02	21.8	0.48	1.11	1.31	74.9
Gregarinomorphea	16.8	35.8	0.48	1.33	1.31	76.2
Prostomatea	0	20.8	0.45	0.8	1.24	77.4
Annelida	17.9	27.6	0.43	2.01	1.19	78.6
UI Metazoa	9.45	26.9	0.41	1.05	1.12	79.7
UI Amoebozoa	22.8	5.39	0.41	1.62	1.12	80.8
UI Alveolata	32.7	25.6	0.4	1.88	1.09	81.9
Dinoflagellata	17.5	15.3	0.38	1.49	1.05	83.0
Cercozoa	21.1	28.6	0.33	1.11	0.9	83.9
UI TSAR	43.6	39.9	0.3	1.45	0.81	84.7
Mollusca	0.79	13.3	0.29	0.79	0.8	85.5
Monothalamids	15.0	3.66	0.26	1.92	0.71	86.2
Platyhelminthes	3.01	12.9	0.26	1.71	0.7	86.9

Filosa-Imbricatea	3.86	12.3	0.24	1.58	0.66	87.5
Apusomonada_X	12.0	3.52	0.19	1.25	0.53	88.1
Ascomycota	4.35	8.12	0.19	1.89	0.52	88.6
Symbiontida	8.09	0	0.19	1.91	0.52	89.1
UI Gyrista	13.9	16.7	0.17	1.25	0.46	89.6
Chlorodendrophyceae	0.97	8.07	0.17	2.74	0.45	90.0

	Site 2 Av.	Site 7 Av.				
Taxa, 2024	Abun	Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
NA	707	1929	23.47	1.54	54.5	54.5
Dinophyceae	187	98.8	1.61	1.81	3.75	58.2
Arthropoda	100	18.4	1.38	2.34	3.19	61.4
Euglenozoa	119	123	1.2	1.08	2.79	64.2
UI TSAR	48.7	95.1	1.15	0.92	2.67	66.9
Gastrotricha	72.78	9.6	1.12	1.83	2.6	69.5
Mediophyceae	281	284	1.09	1.93	2.54	72.0
Spirotrichea	74.7	16	1.01	2.46	2.34	74.3
Apicomplexa	105	152	0.96	1.7	2.24	76.6
Bacillariophyceae	196	181	0.74	1.58	1.72	78.3
Annelida	29.5	18.9	0.51	1.14	1.19	79.5
Syndiniales	52.5	23.2	0.51	2.47	1.18	80.7
UI Metazoa	10.4	28.3	0.45	1.36	1.04	81.7
UI Opisthokonta	6.48	32.3	0.44	0.87	1.01	82.7
Pirsoniales	0.99	25.5	0.34	0.64	0.79	83.5
UI Amoebozoa	9.78	26.5	0.31	2.1	0.73	84.2
Nematoda	29.9	21.2	0.29	1.25	0.67	84.9
Filosa-Thecofilosea	17.4	9.9	0.29	1.38	0.67	85.6
Dinoflagellata	17.6	14.7	0.28	1.67	0.64	86.2
Sagenista	10.2	22.8	0.27	0.93	0.62	86.8
UI Alveolata	25.8	13.3	0.26	1.73	0.6	87.4
Ciliophora	20.3	7.34	0.24	1.52	0.56	88.0
Cercozoa	23.9	12.6	0.22	1.56	0.5	88.5
Apusomonada_X	23.3	9.97	0.21	2.77	0.48	89.0
Globothalamea	4.54	15.5	0.21	1.56	0.48	89.4
Trebouxiophyceae	24.3	13.9	0.2	1.78	0.47	89.9
Platyhelminthes	12.1	2.02	0.2	0.88	0.47	90.4

Ταχα, 2024	Site 3 Av. Abun	Site 8 Av. Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
NA	896	1197	9.54	1.38	22.6	22.6
Dinophyceae	137	505	4.47	1.74	10.6	33.1

Nematoda	52.0	223	2.08	2.41	4.92	38.1
UI Metazoa	33.3	198	1.83	0.99	4.33	42.4
Annelida	48.6	178	1.55	1.6	3.65	46.0
Ciliophora	19.1	149	1.54	3.24	3.63	49.7
Apicomplexa	210	312	1.33	1.74	3.15	52.8
Arthropoda	99.2	161	1.2	1.39	2.84	55.7
Bacillariophyceae	225	319	1.17	2.39	2.77	58.4
Syndiniales	24.1	107	1.04	2.32	2.46	60.9
UI Archaeplastida	27.9	101	0.98	1.7	2.31	63.2
Euglenozoa	165	242	0.88	2.75	2.07	65.3
Spirotrichea	29.2	100	0.87	1.58	2.06	67.3
Mollusca	62.7	81.2	0.86	0.99	2.04	69.4
Filosa-Thecofilosea	22.2	67.1	0.75	1.48	1.77	71.1
UI Alveolata	20.7	67.8	0.64	1.64	1.5	72.6
Dinoflagellata	21.1	65.9	0.6	1.73	1.43	74.1
Gastrotricha	1.86	45.4	0.56	1.72	1.32	75.4
Trebouxiophyceae	25.3	65.6	0.51	1.56	1.22	76.6
Cercozoa	24.3	59.5	0.51	1.51	1.21	77.8
Filosa-Imbricatea	9.51	47.9	0.5	1.93	1.19	79
Brachiopoda	2.42	37.4	0.43	1.63	1.03	80.0
Mediophyceae	327	321	0.43	1.23	1.02	81.0
UI TSAR	33.2	65.6	0.41	1.72	0.97	82
Platyhelminthes	2.72	38.8	0.39	1.27	0.92	82.9
Cnidaria	16.4	44.9	0.38	1.47	0.9	83.8
Peronosporomycetes	19.9	47.8	0.34	2.33	0.8	84.6
Prostomatea	0	24.4	0.3	0.98	0.71	85.3
Sagenista	16.9	36.6	0.28	2	0.65	86.0
CONTH_3	5.88	23.7	0.23	1.93	0.54	86.5
UI Gyrista	14.5	33.2	0.23	2.16	0.53	87.1
Endomyxa	0	17.1	0.22	2.41	0.53	87.6
Raphidophyceae	11.0	29.4	0.22	1.52	0.51	88.1
UI Amoebozoa	24.4	15.9	0.22	0.98	0.51	88.6
Gregarinomorphea	11.4	26.8	0.21	1.23	0.5	89.1
Ascomycota	5.93	17.4	0.17	2.04	0.41	89.5
Foraminifera	6.13	17.8	0.16	1.7	0.38	89.9
Diplonemea	16.7	28.1	0.15	1.66	0.36	90.3

Taxa, 2024	Site 4 Av. Abun	Site 9 Av. Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
NA	488	582	7.6	1.55	23.76	23.8
Dinophyceae	221	90.9	2.91	1.42	9.08	32.8
Euglenozoa	145	232	1.92	1.31	6	38.8

Arthropoda	102	46.4	1.41	1.12	4.4	43.2
Apicomplexa	180	136	1.15	1.63	3.6	46.8
Annelida	74	17.8	1.13	1.96	3.54	50.4
Spirotrichea	59.1	19.4	0.92	1.8	2.88	53.3
Gastrotricha	55.1	24.1	0.91	1.68	2.85	56.1
Bacillariophyceae	187	215	0.72	1.59	2.26	58.4
Filosa-Thecofilosea	40.8	8.89	0.69	1.36	2.17	60.6
Mollusca	36.2	2.25	0.66	1.72	2.08	62.6
Cnidaria	41.7	11.7	0.65	1.12	2.04	64.7
Mediophyceae	288	318	0.64	1.54	2	66.7
UI Metazoa	45.9	31.9	0.62	1.95	1.95	68.6
Ciliophora	46.1	20.6	0.59	1.37	1.86	70.5
UI Archaeplastida	45.1	23.1	0.59	1.34	1.83	72.3
Syndiniales	41.1	20.7	0.55	1.36	1.71	74
Trebouxiophyceae	37.8	15	0.5	1.37	1.57	75.6
Cercozoa	41.3	23.7	0.48	1.31	1.49	77.1
Unidentified opisthokonta	7.17	29.3	0.45	0.82	1.4	78.5
UI TSAR	41.3	22.5	0.45	1.45	1.39	79.9
Dinoflagellata	29	20.1	0.44	1.61	1.38	81.3
UI Alveolata	47.9	31.2	0.39	1.06	1.21	82.5
Filosa-Imbricatea	20.6	7.15	0.35	1.63	1.09	83.6
Platyhelminthes	19	5.74	0.34	1.28	1.07	84.6
Peronosporomycetes	35.8	27.6	0.25	1.06	0.78	85.4
Gregarinomorphea	14.4	16.4	0.21	1.26	0.65	86.1
Nematoda	28.9	35.2	0.2	1.3	0.64	86.7
Endomyxa	2.23	9.76	0.16	1.73	0.49	87.2
Diplonemea	13.5	20	0.16	1.5	0.49	87.7
Gyrista_X	12.6	6.68	0.13	1.86	0.4	88.1
UI Amoebozoa	7.91	8.24	0.13	1.39	0.39	88.5
UI Gyrista	13.3	18.2	0.12	1.45	0.39	88.9
Apusomonada_X	8.28	8.72	0.12	1.3	0.36	89.2
Pyramimonadophyceae	10.3	6.1	0.11	1.09	0.35	89.6
Rozellomycota	6.33	1.84	0.11	1.89	0.35	89.9
Globothalamea	3.71	7.48	0.11	1.85	0.35	90.3

6.2.3 Relationship between environmental variables and community structure

Table S12. Full DISTLM results of marginal tests for eukaryotic community structure in 2023. P-values <0.05 indicate significant differences. Sums of squares ('SS'), pseudo-F ratio ('Pseudo-F') and P-value ('P-value') and proportion of variation explained.

Variable	SS (trace)	Pseudo-F	P-value	Proportion
Boron (dissolved) ^a	9433.9	3.5656	0.0001	0.085781
lron (dissolved)ª	6561.5	2.4111	0.0001	0.059664
Vanadium (dissolved) ^a	8502.8	3.1842	0.0001	0.077315
Lead 1M HCI extract ^b	10510	4.0152	0.0001	0.095565
Molybdenum (dissolved) ^a	5761	2.1006	0.0017	0.052384
Nickel 1M HCl extract ^b	5556.6	2.0222	0.0039	0.050526
Moisture	5387.6	1.9575	0.0046	0.048989
Total Organic Carbon	5033.6	1.8227	0.0085	0.04577
Size fraction 0.063 mm - 2.0 mm	4702.9	1.6976	0.0128	0.042763
Size fraction < 0.063 mm	4607.6	1.6617	0.015	0.041897
Manganese (dissolved) ^a	4567.5	1.6466	0.0166	0.041532
Arsenic (dissolved) ^a	4525.6	1.6308	0.0188	0.041151
Chromium 1M HCl extract ^b	4371.8	1.5731	0.0255	0.039753
Barium (dissolved) ^a	4338.1	1.5605	0.0268	0.039446
Size fraction <0.002 mm	4055.9	1.4551	0.0427	0.03688
Cobalt (dissolved) ^a	3796.4	1.3587	0.0711	0.03452
Size fraction 0.002 mm - 0.063 mm	3714.3	1.3283	0.0845	0.033774
Arsenic 1M HCl extract ^b	3630.1	1.2971	0.097	0.033008
Lead (dissolved) ^a	3363.5	1.1989	0.1658	0.030584
Copper 1M HCl extract ^b	3315.8	1.1813	0.1679	0.030151
Cadmium 1 M HCl extract ^b	3161.4	1.1247	0.2247	0.028747
Selenium 1 M HCl extract ^b	2551.5	0.90258	0.6177	0.023201
Aluminium (dissolved) ^a	2184	0.76995	0.8487	0.019859
Copper (dissolved) ^a	-2.17E-11	-8.11E-15	1	-1.97E-16

^a Variables are estimated dissolved (<0.45 um) metal concentrations in the pore waters.

Table S 13. Full DISTLM results of marginal tests for eukaryotic community structure in 2024. P-values <0.05 indicate significant differences. Sums of squares ('SS'), pseudo-F ratio ('Pseudo-F') and P-value ('P-value') and proportion of variation explained.

Variable	SS (trace)	Pseudo-F	P-value	Proportion
Lead 1M HCI extract ^b	6936.6	2.7856	0.0001	0.068298
Size fraction 0.002 mm - 0.063 mm	5.02E+03	1.97E+00	0.0009	4.94E-02
Boron (dissolved)ª	4687.9	1.8389	0.0017	0.046157
Barium (dissolved) ^a	4748.6	1.8638	0.0019	0.046754
Moisture	4440.4	1.7373	0.0062	0.04372
Size fraction 0.063 mm - 2.0 mm	4589.2	1.7983	0.0067	0.045185
Vanadium (dissolved) ^a	4567.1	1.7892	0.0086	0.044967
Molybdenum (dissolved) ^a	4814.5	1.891	0.0103	0.047404
Total Organic Carbon	4192.6	1.6362	0.0107	0.04128
Size fraction <0.002 mm	4122.6	1.6077	0.0108	0.040591
Manganese (dissolved) ^a	3926.3	1.5281	0.0199	0.038658
Chromium 1M HCI extract ^b	3840.3	1.4933	0.0241	0.037812
Nickel 1M HCl extract ^b	3373.9	1.3057	0.0805	0.033219
Cobalt (dissolved) ^a	3100.4	1.1965	0.1633	0.030526
Arsenic 1M HCl extract ^b	3026.8	1.1673	0.1877	0.029802
Size fraction < 0.063 mm	2826.9	1.088	0.2809	0.027834
Copper 1M HCl extract ^b	2758.5	1.0609	0.3189	0.02716
Selenium 1 M HCl extract ^b	2769.2	1.0651	0.3453	0.027265
Arsenic (dissolved) ^a	2618.6	1.0057	0.4122	0.025783
Selenium (dissolved) ^a	2648.5	1.0175	0.4264	0.026077
Chromium (dissolved) ^a	2524.8	0.96873	0.4552	0.024859
Mercury 1M HCl extract ^b	2304.8	0.88235	0.6228	0.022693
Nickel (dissolved) ^a	1.96E+03	7.47E-01	0.8452	1.93E-02
Zinc (dissolved) ^a	1722.8	0.65571	0.8689	0.016963

^a Variables are estimated dissolved (<0.45 um) metal concentrations in the pore waters.

6.2.4 Species richness and abundance

Table S14. Pairwise comparisons in eukaryotic Amplicon Sequence Variants (ASVs) abundance between sites of interest and paired reference sites for each pairing in 2023 and 2024. P-values <0.05 indicate significant differences. Standard error ('SE'), degrees of freedom ('df'), t-statistic ('t-ratio'). Results are given on the log (not the response) scale.

Parameter, Year					
Eukaryote abundance, 2023	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	-0.758	0.30	29	-2.527	0.0172
Pairing 2 (Site 2 vs Site 7)	-0.050	0.30	29	-0.166	0.8689
Pairing 3 (Site 3 vs Site 8)	-0.284	0.30	29	-0.948	0.3511
Pairing 4 (Site 4 vs Site 9)	0.731	0.30	29	2.436	0.0212
Pairing 5 (Site 5 vs Site 10)	0.621	0.30	29	2.071	0.0473
Eukaryote abundance, 2024	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	0.089	0.315	29	0.283	0.7794
Pairing 2 (Site 2 vs Site 7)	-1.117	0.315	29	-3.550	0.0013
Pairing 3 (Site 3 vs Site 8)	-0.767	0.315	29	-2.439	0.0211
Pairing 4 (Site 4 vs Site 9)	0.213	0.315	29	0.677	0.5040
Pairing 5 (Site 5 vs Site 10)	0.324	0.315	29	1.030	0.3115

Table S15. Pairwise comparisons in Amplicon Sequence Variants (ASVs) of annelids and molluscs between sites of interest and paired reference sites for each pairing in 2023 and 2024. P-values <0.05 indicate significant differences. Standard error ('SE'), degrees of freedom ('df'), t-statistic ('t-ratio'). Results are given on the log (not the response) scale.

Phylum, Year					
Mollusca ASV, 2023	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	-1.220	1.230	29	-0.990	0.3303
Pairing 2 (Site 2 vs Site 7)	4.170	1.590	29	2.629	0.0135
Pairing 3 (Site 3 vs Site 8)	3.550	1.230	29	2.889	0.0072
Pairing 4 (Site 4 vs Site 9)	1.050	1.240	29	0.846	0.4042
Pairing 5 (Site 5 vs Site 10)	1.500	1.230	29	1.223	0.2313
Annelida ASV, 2024	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	-0.419	0.753	29	-0.557	0.5816
Pairing 2 (Site 2 vs Site 7)	1.161	0.752	29	1.543	0.1337
Pairing 3 (Site 3 vs Site 8)	-1.867	0.752	29	-2.483	0.0191
Pairing 4 (Site 4 vs Site 9)	2.227	0.752	29	2.960	0.0061
Pairing 5 (Site 5 vs Site 10)	1.161	0.752	29	-1.543	0.1337
Mollusca ASV, 2024	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	-4.782	1.32	29	-3.3620	0.0011
Pairing 2 (Site 2 vs Site 7)	-19.650	6163.94	29	-0.003	0.9975
Pairing 3 (Site 3 vs Site 8)	-1.025	1.24	29	-0.825	0.4161
Pairing 4 (Site 4 vs Site 9)	4.981	1.26	29	3.963	0.0004
Pairing 5 (Site 5 vs Site 10)	-0.693	1.51	29	-0.458	0.6506

Table S 16. Pairwise comparisons in Amplicon Sequence Variants (ASVs) of annelids and molluscs between sites of interest and paired reference sites for each pairing in 2023 and 2024. P-values <0.05 indicate significant differences. Standard error ('SE'), degrees of freedom ('df'), t-statistic ('t-ratio'). Results are given on the log (not the response) scale.

Phylum, Year					
Bacillariophyceae ASV, 2023	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	-0.344	0.183	29	-1.884	0.0696
Pairing 2 (Site 2 vs Site 7)	0.293	0.199	29	1.473	0.1515
Pairing 3 (Site 3 vs Site 8)	0.314	0.140	29	2.239	0.0330
Pairing 4 (Site 4 vs Site 9)	0.160	0.131	29	1.217	0.2332
Pairing 5 (Site 5 vs Site 10)	0.866	0.152	29	5.692	<0.0001
Bacillariophyceae ASV, 2024	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	0.332	0.240	29	1.385	0.1766
Pairing 2 (Site 2 vs Site 7)	0.280	0.190	29	1.468	0.1530
Pairing 3 (Site 3 vs Site 8)	-0.390	0.157	29	-2.488	0.0188
Pairing 4 (Site 4 vs Site 9)	-0.192	0.192	29	-0.999	0.3262
Pairing 5 (Site 5 vs Site 10)	0.579	0.195	29	2.962	0.0061
Dinophyceae ASV, 2023	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	-0.965	0.570	29	-1.693	0.1012
Pairing 2 (Site 2 vs Site 7)	1.027	0.602	29	1.706	0.0986
Pairing 3 (Site 3 vs Site 8)	-1.702	0.429	29	-3.971	0.0004
Pairing 4 (Site 4 vs Site 9)	2.421	0.604	29	4.010	0.0004
Pairing 5 (Site 5 vs Site 10)	-0.614	0.522	29	-1.177	0.2490
Dinophyceae ASV, 2024	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	-1.086	0.542	29	-2.003	0.0550
Pairing 2 (Site 2 vs Site 7)	0.739	0.547	29	1.353	0.1869
Pairing 3 (Site 3 vs Site 8)	-1.894	0.464	29	-4.079	0.0003
Pairing 4 (Site 4 vs Site 9)	1.225	0.584	29	2.098	0.0451
Pairing 5 (Site 5 vs Site 10)	-1.016	0.527	29	-1.929	0.0640
Dinoflagellata ASV, 2023	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	-1.5151	0.556	29	-2.725	0.0108
Pairing 2 (Site 2 vs Site 7)	1.0504	0.665	29	1.580	0.1250
Pairing 3 (Site 3 vs Site 8)	-1.1635	0.369	29	-3.151	0.0038
Pairing 4 (Site 4 vs Site 9)	1.3353	0.322	29	4.150	0.0003
Pairing 5 (Site 5 vs Site 10)	-0.0241	0.434	29	-0.056	0.9560
Nematoda ASV, 2024	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	1.388	0.414	29	3.350	0.0023
Pairing 2 (Site 2 vs Site 7)	0.548	0.555	29	0.988	0.3317
Pairing 3 (Site 3 vs Site 8)	-2.260	0.340	29	-6.641	<0.0001
Pairing 4 (Site 4 vs Site 9)	-0.406	0.479	29	-0.847	0.4044
Pairing 5 (Site 5 vs Site 10)	-0.723	0.454	29	-1.594	0.1221

6.3 Bacterial community (eDNA)

6.3.1 Individual taxa

Individual taxa were not analysed for the bacterial community.

6.3.2 Community structure

Table S 17. Pairwise comparisons of bacterial community structure among site categories within each pairing in 2023 and 2024. P-values <0.05 indicate significant differences. Sources of variation in the model ('Source'), t-test ('t'), permutation P-value ('P(perm)'), and number of unique permutations ('Unique perms').

Source			
Bacteria, 2023			
Pairwise comparisons	t	P(perm)	Unique perms
Pairing 1 (Site 1 vs Site 6)	1.0789	0.1731	35
Pairing 2 (Site 2 vs Site 7)	1.2415	0.0321	35
Pairing 3 (Site 3 vs Site 8)	1.0702	0.0547	35
Pairing 4 (Site 4 vs Site 9)	1.1985	0.0289	35
Pairing 5 (Site 5 vs Site 10)	1.4048	0.0318	35
Bacteria, 2024			
Pairwise comparisons	t	P(perm)	Unique perms
Pairing 1 (Site 1 vs Site 6)	1.1618	0.0585	35
Pairing 2 (Site 2 vs Site 7)	1.3294	0.0292	35
Pairing 3 (Site 3 vs Site 8)	1.1179	0.0806	35
Pairing 4 (Site 4 vs Site 9)	1.1851	0.0273	35
Pairing 5 (Site 5 vs Site 10)	1.1174	0.1766	35

Table S18. Full SIMPER results for bacterial community structure sampled in 2023 and 2024. A 90% cutoff was applied. Average abundance ('Av. Abund'), average dissimilarity ('Av. Diss'), dissimilarity/standard deviation ('Diss/SD'), individual contribution ('Contrib. %'), and cumulative percentage explained ('Cum %). 'UI' = unidentified.

	Site 2	Site 7				
Taxa, 2023	Av. Abun	Av. Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
Alphaproteobacteria	611	699	1.73	1.25	15.8	15.8
UI Bacteria	296	335	0.84	1.65	7.68	23.5
Planctomycetes	209	192	0.7	1.34	6.43	29.9
Gammaproteobacteria	175	177	0.69	1.56	6.35	36.3
Phycisphaerae	197	216	0.55	1.16	5	41.3
Spirochaetia	195	193	0.48	1.53	4.43	45.7
Thermodesulfovibrionia	92.7	114	0.4	1.86	3.63	49.3
Polyangia	47.4	38.0	0.29	1.32	2.61	51.9
UBA1135	61.9	71	0.28	1.46	2.56	54.5
Verrucomicrobiae	86.4	91.8	0.27	1.48	2.45	56.9
Anaerolineae	34.3	37.4	0.26	1.6	2.41	59.3
Hydrogenedentia	69.0	81.5	0.25	1.46	2.31	61.7
UBA8108	36.5	30.9	0.21	1.19	1.93	63.6
Nitrospiria	34.4	44.0	0.18	1.63	1.64	65.2
Lentisphaeria	34.2	30.2	0.17	1.19	1.52	66.7
Koll11	23.0	17.8	0.15	1.31	1.42	68.2
Bacteroidia	57.3	60.9	0.15	1.14	1.35	69.5
Dehalococcoidia	25.5	20.4	0.13	1.43	1.19	70.7
UI Planctomycetota	37.7	32.4	0.13	2.14	1.18	71.9
Vicinamibacteria	20.7	18.8	0.12	1.5	1.11	72.9
MSB-5A5	30.9	32.7	0.12	1.48	1.1	74.1
Acidimicrobiia	30.4	25.8	0.12	1.38	1.09	75.2
Deferrisomatia	22.7	24.4	0.11	1.34	1	76.2
Nitrospinia	15.1	10.7	0.09	1.52	0.83	77
Fibrobacteria	17.7	21.5	0.09	1.39	0.83	77.8
Babeliae	13.8	15.6	0.09	1.37	0.81	78.6
Campylobacteria	9.07	13.3	0.09	1.56	0.8	79.4
Aminicenantia	15.3	13.1	0.08	1.53	0.74	80.2
Bacilli	7.61	7.77	0.08	1.33	0.72	80.9
Desulfobulbia	7.86	12.0	0.08	2.2	0.7	81.6
Desulfobacteria	2.6	4.93	0.07	1.43	0.67	82.3
B62-G9	15.7	14.2	0.07	1.59	0.66	82.9
Calditrichia	3.85	7.37	0.07	1.51	0.63	83.6
Ignavibacteria	8.64	6.52	0.07	1.84	0.63	84.2
Sumerlaeia	6.99	4.72	0.07	1.35	0.62	84.8
Rhodothermia	6.79	9.41	0.07	1.38	0.6	85.4
UI Acidobacteriota	13.8	14.6	0.07	1.55	0.59	86.0
Clostridia	5.98	5.39	0.06	1.56	0.59	86.6

2.35	5.68	0.06	1.48	0.55	87.1
5.32	4.5	0.06	1.29	0.53	87.7
3.78	6.61	0.05	1.38	0.48	88.1
14.6	15.3	0.05	1.24	0.45	88.6
7.84	7.72	0.05	1.47	0.42	89
6.13	5.5	0.04	1.45	0.41	89.4
4.93	2.79	0.04	1.2	0.38	89.8
3.61	3.36	0.04	1.33	0.37	90.2
	2.35 5.32 3.78 14.6 7.84 6.13 4.93 3.61	2.355.685.324.53.786.6114.615.37.847.726.135.54.932.793.613.36	2.355.680.065.324.50.063.786.610.0514.615.30.057.847.720.056.135.50.044.932.790.043.613.360.04	2.355.680.061.485.324.50.061.293.786.610.051.3814.615.30.051.247.847.720.051.476.135.50.041.454.932.790.041.23.613.360.041.33	2.355.680.061.480.555.324.50.061.290.533.786.610.051.380.4814.615.30.051.240.457.847.720.051.470.426.135.50.041.450.414.932.790.041.20.383.613.360.041.330.37

Ταχα, 2023	Site 4 Av. Abun	Site 9 Av. Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
Alphaproteobacteria	660	735	4.21	1.21	16.7	16.7
Gammaproteobacteria	281	299	2.24	1.36	8.85	25.5
UI Bacteria	377	392	2.16	1.3	8.54	34.1
Planctomycetes	252	282	2.14	1.22	8.46	42.5
Phycisphaerae	290	297	2.13	1.36	8.44	50.9
Spirochaetia	229	258	1.72	1.3	6.81	57.8
Thermodesulfovibrionia	135	148	0.79	1.32	3.14	60.9
Verrucomicrobiae	78.4	104	0.78	1.09	3.1	64.0
Bacteroidia	92.5	84.3	0.71	1.41	2.82	66.8
Hydrogenedentia	68.1	86.1	0.58	1.08	2.31	69.1
Anaerolineae	69.6	46.0	0.48	1.63	1.91	71.0
UBA1135	81.5	88.7	0.43	1.22	1.71	72.7
Acidimicrobiia	40.9	34.7	0.38	1.61	1.51	74.3
Lentisphaeria	44.1	49.9	0.35	1.27	1.39	75.6
Polyangia	53.2	54.0	0.34	1.34	1.36	77
Dehalococcoidia	37.8	31.9	0.33	1.4	1.33	78.3
Nitrospiria	44.9	47.6	0.32	1.35	1.25	79.6
Vicinamibacteria	35.8	32.2	0.31	1.66	1.22	80.8
UBA8108	48.6	45.3	0.26	1.49	1.03	81.8
MSB-5A5	36.1	39.8	0.25	1.29	0.99	82.8
UI Planctomycetota	30.8	36.7	0.24	1.2	0.93	83.7
Deferrisomatia	28.3	28.2	0.21	1.35	0.84	84.6
Nitrospinia	19.1	22.4	0.21	1.22	0.83	85.4
Aminicenantia	18.7	18.4	0.17	1.39	0.66	86.1
UI Acidobacteriota	19.8	19.3	0.15	1.1	0.6	86.7
Koll11	20.1	23.9	0.14	1.16	0.57	87.3
BMS3Abin14	11.0	13.7	0.13	1.2	0.51	87.8
Fibrobacteria	26.1	20.0	0.12	1.45	0.49	88.3
UI Fibrobacterota	25.9	20.1	0.12	1.33	0.48	88.7
Babeliae	18.1	18.0	0.12	1.44	0.48	89.2
B62-G9	14.5	12.8	0.11	1.46	0.45	89.7
Acidobacteriae	8.89	16.2	0.11	1.88	0.43	90.1

	Site 5	Site 10				
Ταχα, 2023	Av. Abun	Av. Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
Alphaproteobacteria	609	895	3.88	1.82	15.8	15.8
UI Bacteria	307	473	2.26	1.93	9.22	25.0
Phycisphaerae	216	371	2.11	1.96	8.6	33.6
Planctomycetes	220	363	2.09	2.4	8.54	42.2
Gammaproteobacteria	252	392	1.93	1.5	7.87	50.1
Spirochaetia	199	318	1.61	2.39	6.58	56.6
Verrucomicrobiae	72.4	137	0.87	2.74	3.55	60.2
Thermodesulfovibrionia	115	171	0.76	1.9	3.11	63.3
Bacteroidia	65.2	120	0.76	2.04	3.1	66.4
Hydrogenedentia	63.7	104	0.55	1.86	2.25	68.7
UBA1135	73.7	111	0.51	2.46	2.07	70.7
Anaerolineae	47.9	78.0	0.41	2.14	1.68	72.4
Polyangia	44.9	72.6	0.39	1.46	1.6	74
Nitrospiria	38.0	66.4	0.39	2	1.57	75.6
UI Planctomycetota	21.5	49.6	0.38	2.81	1.57	77.1
MSB-5A5	25.6	52.1	0.36	3.24	1.47	78.6
Lentisphaeria	32.2	56.8	0.33	1.82	1.34	79.9
Deferrisomatia	22.7	42.0	0.26	1.97	1.08	81.0
UBA8108	39.4	58.5	0.26	1.89	1.07	82.1
Vicinamibacteria	31.5	50.1	0.25	1.29	1.04	83.1
Dehalococcoidia	22.0	38.1	0.22	1.54	0.91	84.0
BMS3Abin14	5.7	19.1	0.18	4.66	0.74	84.8
Desulfobulbia	6.22	18.5	0.17	2.55	0.68	85.5
Rhodothermia	4.78	16.9	0.17	2.67	0.67	86.1
UI Acidobacteriota	10.8	22.3	0.16	1.62	0.67	86.8
B62-G9	9.67	20.1	0.15	1.47	0.6	87.4
Acidimicrobiia	25.5	36.2	0.14	1.57	0.59	88.0
Aminicenantia	16.2	26.6	0.14	1.36	0.58	88.6
Koll11	18.5	28.9	0.14	1.51	0.58	89.2
Ellin6529	6.41	15.9	0.13	2.36	0.51	89.7
Acidobacteriae	7.22	16.1	0.12	2.54	0.5	90.2

Tawa 2024	Site 2 Av.	Site 7 Av.			Contrib %	C 9/
Taxa, 2024	Abun	Abun	AV.DISS	0155/30	Contrib %	Com. %
Gammaproteobacteria	294	209	1.7	1.63	12.8	12.8
Planctomycetes	277	203	1.52	1.64	11.5	24.3
Alphaproteobacteria	681	691	1.1	1.25	8.28	32.6
Phycisphaerae	254	228	0.87	1.44	6.56	39.1
UI Bacteria	349	352	0.75	1.02	5.69	44.8
Spirochaetia	230	199	0.71	1.68	5.37	50.2

Polyangia	70.9	44.0	0.45	1.68	3.39	53.6
Verrucomicrobiae	112	90.4	0.42	1.85	3.17	56.7
Bacteroidia	84.1	71.6	0.35	1.41	2.67	59.4
Thermodesulfovibrionia	111	107	0.31	1.23	2.31	61.7
MSB-5A5	46.9	28.5	0.29	1.75	2.21	63.9
UBA1135	83.8	83.0	0.25	1.35	1.91	65.8
Dehalococcoidia	33.7	29.3	0.23	1.71	1.74	67.6
Anaerolineae	52.3	39.4	0.22	1.57	1.63	69.2
Lentisphaeria	42.2	31.4	0.19	1.19	1.45	70.7
UBA8108	48.3	38.7	0.18	1.98	1.39	72.1
Vicinamibacteria	27.1	16.5	0.18	1.69	1.38	73.4
UI Planctomycetota	40.4	33.1	0.18	1.41	1.37	74.8
Hydrogenedentia	79.7	82.8	0.16	1.48	1.22	76.0
Nitrospiria	49.3	44.6	0.15	2.1	1.17	77.2
Koll11	29.6	22.0	0.15	1.4	1.12	78.3
Deferrisomatia	27.9	22.2	0.12	1.68	0.92	79.2
Acidimicrobiia	32.6	29.8	0.11	1.5	0.85	80.1
Babeliae	17.6	13.6	0.1	1.45	0.78	80.9
Aminicenantia	17.0	12.9	0.1	0.93	0.74	81.6
Sumerlaeia	9.73	3.69	0.09	1.57	0.71	82.3
UI Acidobacteriota	20.9	19.7	0.09	1.63	0.69	83.0
Fibrobacteria	18.3	24.0	0.09	1.94	0.69	83.7
Ellin6529	7.98	2.61	0.08	2.22	0.63	84.3
Gemmatimonadetes	11.9	9.90	0.08	1.3	0.61	84.9
Bacilli	10.3	9.40	0.08	1.21	0.6	85.5
Desulfobulbia	11.5	7.85	0.07	2.09	0.56	86.1
Rhodothermia	9.80	7.32	0.07	1.49	0.49	86.6
Clostridia	10.4	6.74	0.06	1.51	0.48	87.1
B62-G9	16.2	15.0	0.06	1.71	0.47	87.5
Acidobacteriae	12.9	11.5	0.06	1.37	0.44	88.0
UBA2968	6.29	4.55	0.06	1.15	0.43	88.4
Campylobacteria	8.12	9.08	0.06	1.92	0.42	88.8
Gracilibacteria	5.10	2.38	0.05	1.57	0.41	89.3
BMS3Abin14	14.9	14.5	0.05	1.5	0.39	89.7
UBA4802	5.40	3.96	0.05	1.54	0.39	90.0

Ταχα, 2024	Site 4 Av. Abun	Site 9 Av. Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
Alphaproteobacteria	908	846	1.41	1.2	13.4	13.4
Gammaproteobacteria	374	415	1.04	1.43	9.92	23.4
Planctomycetes	329	362	0.96	1.42	9.09	32.5
UI Bacteria	453	466	0.76	1.48	7.23	39.7

Phycisphaerae	361	371	0.72	1.42	6.84	46.5
Spirochaetia	292	305	0.39	1.35	3.73	50.3
Bacteroidia	115	110	0.36	1.58	3.45	53.7
Thermodesulfovibrionia	176	170	0.31	1.47	2.99	56.7
UBA1135	114	102	0.26	1.52	2.43	59.1
Polyangia	65.3	78.6	0.23	1.52	2.22	61.4
Anaerolineae	70.5	66.7	0.23	1.24	2.2	63.6
Verrucomicrobiae	104	114	0.17	1.46	1.63	65.2
Hydrogenedentia	107	101	0.16	1.15	1.5	66.7
Nitrospiria	61.5	63.0	0.15	1.5	1.39	68.1
Koll11	25.9	36.1	0.14	1.45	1.33	69.4
Acidimicrobiia	41.5	52.9	0.14	1.12	1.3	70.7
UBA8108	58.0	60.9	0.14	1.46	1.28	72.0
Dehalococcoidia	45.8	47.0	0.13	1.47	1.27	73.2
Vicinamibacteria	42.6	45.0	0.12	1.52	1.16	74.4
Lentisphaeria	57.7	62.0	0.12	1.54	1.15	75.6
UI Planctomycetota	40.1	41.6	0.12	1.47	1.11	76.7
Deferrisomatia	40.2	38.5	0.09	1.49	0.81	77.5
MSB-5A5	55.7	54.3	0.08	1.41	0.78	78.3
Campylobacteria	8.61	14.1	0.08	1.42	0.77	79.0
Aminicenantia	23.8	23.9	0.07	1.45	0.71	79.7
Gemmatimonadetes	15.6	15.9	0.07	1.52	0.71	80.4
UI Acidobacteriota	24.2	22.1	0.07	1.7	0.69	81.1
Thermoleophilia	5.21	11.6	0.07	1.14	0.67	81.8
Fibrobacteria	28.7	23.8	0.07	1.11	0.66	82.5
Nitrospinia	32.9	32.4	0.07	1.32	0.65	83.1
Rhodothermia	9.27	14.8	0.07	1.32	0.64	83.8
Acidobacteriae	12.9	16.9	0.06	1.33	0.6	84.4
Ignavibacteria	9.75	7.79	0.06	1.43	0.54	84.9
Babeliae	20.0	24.7	0.06	1.41	0.54	85.4
Bacilli	6.49	10.8	0.06	1.51	0.53	86.0
BMS3Abin14	15.9	17.0	0.06	1.45	0.53	86.5
Clostridia	13.2	15.6	0.06	1.2	0.53	87.0
Marinisomatia	4.16	5.68	0.05	1.3	0.5	87.5
Calditrichia	10.3	11.9	0.05	1.2	0.49	88.0
Desulfobulbia	17.3	16	0.05	1.48	0.48	88.5
Actinomycetia	5.45	8.07	0.05	1.75	0.48	89.0
UI Fibrobacterota	28.4	25.6	0.05	1.09	0.46	89.4
Ellin6529	9.51	7.44	0.04	2.04	0.42	89.9
Gracilibacteria	7.5	8.29	0.04	1.38	0.41	90.3

6.3.3 Relationship between environmental variables and community structure

Table S19. Full DISTLM results of marginal tests for bacterial community structure in 2023. P-values <0.05 indicate significant differences. Sums of squares ('SS'), pseudo-F ratio ('Pseudo-F') and P-value ('P-value') and proportion of variation explained.

Variable	SS (trace)	Pseudo-F	P-value	Proportion
Boron (dissolved) ^a	6766.4	4.5263	0.0001	0.10644
Vanadium (dissolved)ª	5838.6	3.843	0.0001	0.091842
Lead 1M HCI extract ^b	8340.1	5.738	0.0001	0.13119
Moisture	5419	3.541	0.0001	0.085242
Nickel 1M HCl extract ^b	4668.5	3.0117	0.0003	0.073436
lron (dissolved) ^a	3966.9	2.529	0.0009	0.062401
Molybdenum (dissolved) ^a	3981.8	2.5391	0.001	0.062634
Chromium 1M HCl extract	3938.7	2.5099	0.0013	0.061957
Size fraction 0.063 mm - 2.0 mm	2771.2	1.732	0.0338	0.043591
Size fraction < 0.063 mm	2665.9	1.6633	0.0416	0.041934
Barium (dissolved) ^a	2435.9	1.5141	0.0589	0.038317
Size fraction 0.002 mm - 0.063 mm	2428.1	1.509	0.0608	0.038195
Manganese (dissolved) ^a	2306.3	1.4305	0.0698	0.036278
Arsenic 1M HCl extract ^b	2172.1	1.3443	0.0996	0.034168
Cadmium 1M HCl extract ^b	2181.1	1.3501	0.1057	0.034309
Copper 1M HCl extract ^b	2062.6	1.2742	0.127	0.032445
Arsenic (dissolved) ^a	2060.7	1.2731	0.1397	0.032416
Total Organic Carbon	1994.1	1.2306	0.1683	0.031367
Selenium 1 M HCl extract ^b	1860.3	1.1455	0.2219	0.029263
Size fraction <0.002 mm	1660.5	1.0192	0.3595	0.026119
Cobalt (dissolved) ^a	1581.3	0.96931	0.4442	0.024874
Lead (dissolved) ^a	1156.4	0.70401	0.8871	0.01819
Aluminium (dissolved)a	942.98	0.57215	0.9745	0.014833
Copper (dissolved) ^a	-9.58E-12	-6.25E-15	1	-1.51E-16

^a Variables are estimated dissolved (<0.45 um) metal concentrations in the pore waters.

Table S20. Full DISTLM results of marginal tests for bacterial community structure in 2024. P-values <0.05 indicate significant differences. Sums of squares ('SS'), pseudo-F ratio ('Pseudo-F') and P-value ('P-value') and proportion of variation explained.

Variable	SS (trace)	Pseudo-F	P-valu <u>e</u>	Proportion
Lead 1M HCI extract ^b	6853	4.669	0.0001	0.10942
Size fraction 0.002 mm - 0.063 mm	4585.4	3.002	0.0002	0.073216
Moisture	4052.4	2.6289	0.0005	0.064705
Size fraction 0.063 mm - 2.0 mm	4708.1	3.0888	0.0006	0.075174
Boron (dissolved)ª	3507.3	2.2543	0.0029	0.056001
Chromium 1M HCI extract ^b	3220.8	2.0602	0.0058	0.051427
Size fraction <0.002 mm	2855	1.815	0.0163	0.045587
Total Organic Carbon	2580.6	1.6331	0.0481	0.041204
Barium (dissolved)ª	2441.4	1.5414	0.0557	0.038982
Nickel 1M HCl extract ^b	2394.9	1.5109	0.0584	0.038239
Manganese (dissolved) ^a	2373.3	1.4967	0.0732	0.037894
Mercury 1M HCl extract ^b	2326.4	1.466	0.0807	0.037146
Arsenic 1M HCl extract ^b	2579.6	1.6324	0.0962	0.041188
Vanadium (dissolved)ª	2234.9	1.4062	0.112	0.035684
Selenium (dissolved)ª	1939.2	1.2142	0.1309	0.030964
Molybdenum (dissolved)a	1892.2	1.1839	0.1729	0.030213
Arsenic (dissolved) ^a	1802.7	1.1262	0.2285	0.028784
Size fraction < 0.063 mm	1737.8	1.0845	0.2903	0.027748
Selenium 1M HCl extract ^b	1630.8	1.0159	0.324	0.026039
Cobalt (dissolved) ^a	1668.5	1.0401	0.3316	0.026641
Nickel (dissolved) ^a	1315.4	0.81526	0.5566	0.021003
Copper 1M HCl extract ^b	1424.2	0.88423	0.5928	0.02274
Copper (dissolved) ^a	1198.4	0.7413	0.6698	0.019135
Zinc (dissolved) ^a	1198.4	0.7413	0.677	0.019135
Chromium (dissolved) ^a	994.72	0.61328	0.9011	0.015883

^a Variables are estimated dissolved (<0.45 um) metal concentrations in the pore waters.

6.3.4 Species richness and abundance

Table S21. Pairwise comparisons in bacterial Amplicon Sequence Variants (ASVs) abundance between sites of interest and paired reference sites for each pairing. P-values <0.05 indicate significant differences. Standard error ('SE'), degrees of freedom ('df'), t-statistic ('t-ratio'). Results are given on the log (not the response) scale.

Parameter, Year					
Bacteria abundance, 2023	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	-0.250	0.261	29	-0.957	0.3465
Pairing 2 (Site 2 vs Site 7)	-0.366	0.234	29	-1.564	0.1287
Pairing 3 (Site 3 vs Site 8)	0.231	0.195	29	1.186	0.2453
Pairing 4 (Site 4 vs Site 9)	0.206	0.217	29	0.947	0.3514
Pairing 5 (Site 5 vs Site 10)	-0.543	0.217	29	-2.504	0.0182
Bacteria richness, 2024	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	1.1860	0.387	29	3.065	0.0047
Pairing 2 (Site 2 vs Site 7)	0.3184	0.262	29	1.214	0.2347
Pairing 3 (Site 3 vs Site 8)	-0.1855	0.248	29	-0.749	0.4597
Pairing 4 (Site 4 vs Site 9)	-0.1767	0.232	29	-0.762	0.4522
Pairing 5 (Site 5 vs Site 10)	0.0476	0.269	29	0.177	0.8607
Bacteria abundance, 2024	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	0.854	0.345	29	2.474	0.0194
Pairing 2 (Site 2 vs Site 7)	-0.112	0.236	29	-0.473	0.6398
Pairing 3 (Site 3 vs Site 8)	-0.307	0.205	29	-1.498	0.1448
Pairing 4 (Site 4 vs Site 9)	0.138	0.200	29	0.688	0.4971
Pairing 5 (Site 5 vs Site 10)	0.285	0.179	29	1.593	0.1220

6.4 Archaeal community (eDNA)

6.4.1 Individual taxa

Individual taxa were not analysed for the archaeal community.

6.4.2 Community structure

Table S22. Pairwise comparisons of community structure among site categories within each pairing for 2023. P-values <0.05 indicate significant differences. Sources of variation in the model ('Source'), t-test ('t'), permutation P-value ('P(perm)'), and number of unique permutations ('Unique perms').

Source			
Archaea, 2023			
Pairwise comparisons	t	P(perm)	Unique perms
Pairing 1 (Site 1 vs Site 6)	1.7405	0.0320	35
Pairing 2 (Site 2 vs Site 7)	1.7816	0.0259	35
Pairing 3 (Site 3 vs Site 8)	1.3311	0.0313	35
Pairing 4 (Site 4 vs Site 9)	1.5504	0.0289	35
Pairing 5 (Site 5 vs Site 10)	1.6248	0.0278	35
Archaea, 2024			
Pairwise comparisons	t	P(perm)	Unique perms
Pairing 1 (Site 1 vs Site 6)	2.0898	0.0284	35
Pairing 2 (Site 2 vs Site 7)	1.9119	0.0283	35
Pairing 3 (Site 3 vs Site 8)	1.3402	0.0540	35
Pairing 4 (Site 4 vs Site 9)	1.7122	0.0281	35
Pairing 5 (Site 5 vs Site 10)	1.8445	0.0299	35

Table S23. Full SIMPER results for archaeal community structure sampled in 2023 and 2024. A 90% cutoff was applied. Average abundance ('Av. Abund'), average dissimilarity ('Av. Diss'), dissimilarity/standard deviation ('Diss/SD'), individual contribution ('Contrib. %'), and cumulative percentage explained ('Cum %). 'UI' = unidentified.

	Site 1 Av.	Site 6 Av.				
Taxa, 2023	Abun	Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
Bathyarchaeia	1901	1358	11.1	1.1	61.8	61.8
Nitrososphaeria	190	112	1.56	1.17	8.68	70.5
Aenigmatarchaeia	93.2	72.8	0.88	1.26	4.91	75.4
Lokiarchaeia	95.9	55.9	0.79	1.32	4.4	79.8
E2	77.8	71.8	0.73	1.36	4.05	83.8
Undinarchaeia	521	493	0.67	1.34	3.76	87.6
UI Thermoproteota	114	91.6	0.56	1.6	3.1	90.7

	Site 2 Av.	Site 7 Av.				
Taxa, 2023	Abun	Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
Bathyarchaeia	2061	2134	4.66	1.67	47.7	47.7
E2	124	162	1.12	2.04	11.5	59.1
Aenigmatarchaeia	84.3	131	0.89	1.28	9.11	68.2
Nitrososphaeria	193	195	0.57	1.58	5.79	74.0
Lokiarchaeia	108	144	0.54	1.56	5.54	79.6
UI Thermoproteota	135	153	0.46	1.42	4.66	84.2
Thermoplasmata	47.3	52.8	0.31	1.5	3.18	87.4
UI Archaea	71.4	62.4	0.31	1.65	3.17	90.6

	Site 3 Av.	Site 8 Av.				
Ταχα, 2023	Abun	Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
Bathyarchaeia	1634	1428	10	0.94	64.4	64.4
E2	83.9	95.9	1.16	1.67	7.47	71.9
Nitrososphaeria	116	92.0	0.99	1.07	6.4	78.3
UI Thermoproteota	93.5	77.7	0.65	1.4	4.17	82.5
Aenigmatarchaeia	58.5	47.0	0.52	1.22	3.37	85.8
UI Archaea	75.5	76.8	0.44	1.38	2.81	88.6
Undinarchaeia	472	483	0.41	1.87	2.61	91.3

Ταχα, 2023	Site 4 Av. Abun	Site 9 Av. Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
Bathyarchaeia	1677	1746	7.46	1.46	59.2	59.2
Nitrososphaeria	136	169	0.93	1.39	7.36	66.5
E2	96.0	82.8	0.66	1.37	5.26	71.8
Aenigmatarchaeia	56.5	66.9	0.6	1.27	4.79	76.6
UI Thermoproteota	85.7	95.2	0.47	1.48	3.75	80.3
UI Archaea	62.3	61.3	0.42	1.23	3.37	83.7
Lokiarchaeia	56.2	77.1	0.41	1.42	3.25	86.9
Thermoplasmata	18.3	31.3	0.36	1.98	2.87	89.8
Undinarchaeia	511	529	0.35	1.52	2.81	92.7

Taxa, 2023	Site 5 Av. Abun	Site 10 Av. Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
Bathyarchaeia	1515	1794	8.94	1.44	58.9	58.9
Nitrososphaeria	117	161	1.27	1.77	8.34	67.3
UI Thermoproteota	72.3	101	0.71	1.65	4.68	72.0
Aenigmatarchaeia	30.4	65	0.7	1.89	4.59	76.5
Lokiarchaeia	51.1	89.3	0.7	1.78	4.59	81.1
E2	54.0	86.7	0.67	1.4	4.38	85.5
Undinarchaeia	527	521	0.57	1.27	3.77	89.3
UI Archaea	57.1	70.5	0.38	1.26	2.53	91.8

	Site 1	Site 6				
Taxa, 2024	Abun	Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
Bathyarchaeia	2120	2242	3.42	1.62	39.3	39.3
E2	93.1	209	1.52	1.31	17.5	56.8
Aenigmatarchaeia	118	150	0.63	1.51	7.19	63.9
Nitrososphaeria	197	231	0.52	1.29	5.99	69.9
Undinarchaeia	539	507	0.47	1.51	5.4	75.3
UI Archaea	75.1	97.1	0.37	1.46	4.27	79.6
UI Thermoproteota	118	133	0.3	1.3	3.48	83.1
Hydrothermarchaeia	13.2	35.5	0.29	1.62	3.38	86.5
Heimdallarchaeia	72.7	55.7	0.23	3.9	2.62	89.1
UI Thermoplasmatota	12.1	27.5	0.22	1.93	2.5	91.6

Taxa, 2024	Site 2 Av. Abun	Site 7 Av. Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
Bathyarchaeia	2086	3390	13.0	1.42	54.4	54.4
E2	118	369	2.37	1.29	9.87	64.2
Aenigmatarchaeia	84.1	260	1.66	1.35	6.93	71.2
UI Thermoproteota	145	297	1.51	1.49	6.31	77.5
Nitrososphaeria	212	322	1.11	1.58	4.62	82.1
Thermoplasmata	33.4	138	0.99	1.23	4.11	86.2
UI Archaea	68.1	145	0.75	1.23	3.15	89.3
Lokiarchaeia	115	181	0.66	1.26	2.75	92.1

Taxa, 2024	Site 3 Av. Abun	Site 8 Av. Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
Bathyarchaeia	1841	1654	6.66	1.35	57.5	57.5
E2	96.2	81.3	0.81	1.39	7.02	64.5
Nitrososphaeria	152	129	0.81	1.37	7	71.5
Undinarchaeia	477	492	0.55	1.3	4.74	76.3
Aenigmatarchaeia	76.1	52.1	0.54	1.32	4.64	80.9
UI Archaea	86.7	72.7	0.45	1.48	3.86	84.8
Lokiarchaeia	87.8	81.3	0.42	1.38	3.63	88.4
UI Thermoproteota	105	90.1	0.37	1.11	3.22	91.6

Taxa 2024	Site 4 Av. Abun	Site 9 Av. Abun	Av Diss	Diss/SD	Contrib%	Cum %
	7.0011	Abon	AV. B133	B133/5B	comino /0	CO 111. /0
Bathyarchaeia	1982	2074	3.83	1.48	46.7	46.7
Undinarchaeia	511	556	0.83	1.36	10.1	56.8
E2	112	120	0.71	1.54	8.62	65.4
Nitrososphaeria	187	194	0.51	1.4	6.2	71.6
Aenigmatarchaeia	66.8	85.9	0.45	1.11	5.49	77.1
Lokiarchaeia	85.1	102	0.34	1.27	4.17	81.3
UI Archaea	89.0	74.4	0.28	2.16	3.45	84.7
UI Thermoproteota	87.6	102	0.24	1.24	2.95	87.7
Thermoplasmata	30.8	32.2	0.24	1.26	2.92	90.6

Ταχα. 2024	Site 5 Av. Abun	Site 10 Av. Abun	Av.Diss	Diss/SD	Contrib%	Cum.%
Bathyarchaeia	1851	2092	4.95	2.53	52.1	52.1
Nitrososphaeria	145	217	1.11	2.05	11.6	63.7
UI Thermoproteota	81.2	126	0.68	2.27	7.17	70.9
Undinarchaeia	518	538	0.61	0.98	6.45	77.3
E2	75.9	113	0.56	1.44	5.87	83.2
Aenigmatarchaeia	58.2	76.3	0.3	1.35	3.12	86.3
Lokiarchaeia	89.7	99.2	0.27	1.59	2.82	89.1
UI Archaea	66.7	67.4	0.18	1.24	1.95	91.1

6.4.3 Relationship between environmental variables and community structure

Table S- 24. Full DISTLM results of marginal tests for archaeal community structure in 2023. P-values <0.05 indicate significant differences. Sums of squares ('SS'), pseudo-F ratio ('Pseudo-F') and P-value ('P-value') and proportion of variation explained.

Variable	SS (trace)	Pseudo-F	P-value	Proportion
Boron (dissolved)ª	6938.3	6.5291	0.0001	0.14663
Iron (dissolved)ª	4772.9	4.2629	0.0001	0.10087
Vanadium (dissolved)ª	6924	6.5135	0.0001	0.14633
Lead 1M HCI extract ^b	8476.1	8.2921	0.0001	0.17913
Moisture	5246.9	4.739	0.0001	0.11088
Molybdenum (dissolved)ª	5158.5	4.6494	0.0002	0.10901
Nickel 1M HCl extract ^b	4415.6	3.9109	0.0003	0.093315
Size fraction 0.063 mm - 2.0 mm	4032.9	3.5404	0.0004	0.085228
Chromium 1M HCl extract ^b	4168.6	3.671	0.0009	0.088094
Size fraction < 0.063 mm	3873.8	3.3883	0.0009	0.081865
Manganese (dissolved) ^a	3644.2	3.1706	0.0023	0.077012
Barium (dissolved)ª	3194.2	2.7508	0.0047	0.067503
Total Organic Carbon	3170.1	2.7285	0.0053	0.066993
Cadmium 1M HCl extract ^b	2786	2.3773	0.0093	0.058876
Size fraction 0.002 mm - 0.063 mm	2772.1	2.3647	0.0134	0.058583
Selenium 1 M HCl extract ^b	2618.5	2.226	0.0175	0.055337
Arsenic (dissolved)ª	2619.1	2.2265	0.0202	0.055349
Copper 1M HCl extract ^b	2110.8	1.7742	0.055	0.044608
Size fraction <0.002 mm	1773.5	1.4796	0.1146	0.037478
Arsenic 1M HCI extract ^b	1682.8	1.4012	0.1511	0.035563
Cobalt (dissolved)ª	1502.9	1.2465	0.2152	0.03176
Lead (dissolved)ª	1032.7	0.84778	0.5616	0.021823
Aluminium (dissolved)a	777.01	0.63439	0.8298	0.01642
Copper (dissolved) ^a	7.28E-12	5.39E-15	1	1.54E-16

 $^{\rm a}$ Variables are estimated dissolved (<0.45 um) metal concentrations in the pore waters.

Variable	SS (trace)	Pseudo-F	P-value	Proportion
Lead 1M HCI extract ^b	9243.9	8.8454	0.0001	0.18882
Size fraction 0.002 mm - 0.063 mm	5027.3	4.3488	0.0002	0.10269
Size fraction 0.063 mm - 2.0 mm	5607.9	4.9161	0.0002	0.11455
Moisture	5358.5	4.6705	0.0003	0.10946
Chromium 1M HCl extract ^b	4309.9	3.6683	0.0008	0.088037
Total Organic Carbon	3676.6	3.0855	0.0025	0.0751
Nickel 1M HCl extract ^b	3574.1	2.9927	0.0037	0.073006
Size fraction <0.002 mm	3070.6	2.5429	0.009	0.062721
Boron (dissolved)ª	3148.7	2.6121	0.0109	0.064318
Barium (dissolved)a	2644	2.1694	0.0207	0.054007
Vanadium (dissolved)ª	2523.7	2.0654	0.0328	0.05155
Arsenic 1 M HCl extract ^b	2219	1.8042	0.051	0.045326
Manganese (dissolved) ^a	2213.4	1.7994	0.0556	0.045212
Molybdenum (dissolved) ^a	2237.2	1.8197	0.0628	0.045698
Copper 1M HCl extract ^b	2106.3	1.7085	0.067	0.043026
Cobalt (dissolved) ^a	2098.5	1.7018	0.0714	0.042866
Selenium (dissolved)ª	2217.6	1.803	0.0735	0.045298
Mercury 1M HCI extract ^b	2016.6	1.6325	0.0869	0.041192
Arsenic (dissolved)ª	1269.2	1.0114	0.3661	0.025926
Size fraction < 0.063 mm	1236.5	0.98468	0.4126	0.025258
Zinc (dissolved)ª	1060.5	0.84143	0.4495	0.021663
Copper (dissolved) ^a	1060.5	0.84143	0.4555	0.021663
Nickel (dissolved)ª	1139.1	0.90522	0.4572	0.023267
Selenium 1 M HCl extract ^b	908.13	0.71822	0.745	0.01855
Chromium (dissolved) ^a	773.04	0.60967	0.8966	0.015791

Table S25. Full DISTLM results of marginal tests for archaeal community structure in 2024. P-values <0.05 indicate significant differences. Sums of squares ('SS'), pseudo-F ratio ('Pseudo-F') and P-value ('P-value') and proportion of variation explained.

^a Variables are estimated dissolved (<0.45 um) metal concentrations in the pore waters.

6.4.4 Species richness and abundance

Table S 26. Pairwise comparisons in archaeal Amplicon Sequence Variants (ASVs) abundance between sites of interest and paired reference sites for each pairing. P-values <0.05 indicate significant differences. Standard error ('SE'), degrees of freedom ('df'), t-statistic ('t-ratio'). Results are given on the log (not the response) scale.

Parameter, Year					
Archaeal abundance, 2023	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	0.0361	0.0524	29	0.689	0.4966
Pairing 2 (Site 2 vs Site 7)	-0.0929	0.0524	29	-1.774	0.0865
Pairing 3 (Site 3 vs Site 8)	0.0029	0.0524	29	0.055	0.9568
Pairing 4 (Site 4 vs Site 9)	0.0970	0.0524	29	1.851	0.0743
Pairing 5 (Site 5 vs Site 10)	0.1127	0.0524	29	2.152	0.0399
Archaeal abundance, 2024	Estimate	SE	df	t-ratio	P-value
Pairwise comparisons					
Pairing 1 (Site 1 vs Site 6)	-0.1243	0.1400	29	-0.889	0.3812
Pairing 2 (Site 2 vs Site 7)	-0.6702	0.1400	29	-4.796	<0.0001
Pairing 3 (Site 3 vs Site 8)	-0.0030	0.1400	29	-0.022	0.9829
Pairing 4 (Site 4 vs Site 9)	-0.1077	0.1400	29	-0.770	0.4473
Pairing 5 (Site 5 vs Site 10)	-0.1272	0.1400	29	-0.910	0.3704