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Tree of life metabarcoding can serve as a biotic benchmark for shifting baselines in urbanized estuaries

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ABSTRACT

Urbanization of estuaries drastically changed existing shorelines and bathymetric contours, in turn modifying habitat for marine foundational species that host critical biodiversity. And yet we lack approaches to characterize a significant fraction of the biota that inhabit these ecosystems on time scales that align with rates of urbanization. Environmental DNA (or eDNA) metabarcoding that combines multiple assays targeting a broad range of taxonomic groups can provide a solution, but we need to determine whether the biological communities it detects ally with different habitats in these changing aquatic environments. In this study, we tested whether tree of life metabarcoding (ToL-metabarcoding) data extracted from filtered seawater samples correlated with four known geomorphic habitat zones across a heavily urbanized estuary (Sydney Harbour, Australia). Using this method, we substantially expanded our knowledge on the composition and spatial distribution of marine biodiversity across the tree of life in Sydney Harbour, particularly for organisms where existing records are sparse. Excluding terrestrial DNA inputs, we identified significant effects of both distance from the mouth of Sydney Harbour and geomorphic zone on biological community structure in the ToL-metabarcoding dataset (entire community), as well as in each of the taxonomic subgroups that we considered (fish, macroinvertebrates, algae and aquatic plants, bacteria). This effect appeared to be driven by taxa as a collective versus a few individual taxa, with each taxon explaining no more than 0.62% of the variation between geomorphic zones. Similarly, taxonomic richness was significantly higher within geomorphic zones with large sample sizes, but also decreased by 1% with each additional kilometer from the estuary mouth, a result consistent with a reduction in tidal inputs and available habitat in upper catchments. Based on these results, we suggest that ToL-metabarcoding can be used to benchmark biological monitoring in other urbanized estuaries globally, and in Sydney Harbour at future time points based on detection of bioindicators across the tree of life. We also suggest that robust biotic snapshots can be archived following extensive curation of taxonomic assignments that incorporates ecological affinities, supported by records from relevant and regional biodiversity repositories.

1. Introduction

Sheltered coastal ecosystems provide essential ecological functions and services that support recreation, tourism, and commercial enterprises (Bennett et al., 2015; Pecl et al., 2017; Blythe et al., 2020; Gaylard et al., 2020). Estuarine habitat sustains a cosmopolitan mix of species occupying different trophic positions that collectively serve a range of important roles (see Mouillot et al., 2007; Villéger et al., 2010), and yet these environments are under considerable threat from human activity (Barbier et al., 2011). Salt marsh plants, mangroves, and seagrasses are examples of estuarine foundation species experiencing some of the greatest reductions on a global scale due to pollution, climate change,

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and urbanization (Wernberg et al., 2023). A greater understanding of the diversity of estuarine ecosystems is needed as it can provide a buffer against ongoing and future human impacts (Duffy et al., 2016), and can often be indicative of a healthy state.

Sydney Harbour is an estuary whose resident biodiversity is under threat. Extensive urbanization in the form of habitat modification (e.g., dredging, land reclamation) and construction of artificial structures (e.g., seawalls, boating and transport infrastructure) has drastically changed the shoreline (reviewed in Johnston et al., 2015; Mayer-Pinto et al., 2015). Sediment-sequestered pollution, in the form of dioxins, hastened the end of large-scale commercial fishing in the Harbour in the early 2000s (Anon, 2006), though the removal of commercial fishing pressure may have had a positive effect on biodiversity. Bacterial blooms resulting from excessive stormwater runoff regularly raise red flags with the public (Birch, 1996). In short, Sydney Harbour has been extensively modified following the arrival of the first colonists in 1788, and now serves as a recreational attraction for boaters, fishers, and tourists. And yet, few biotic baselines exist to benchmark shifts in biodiversity through space and time.

The underlying habitat of Sydney Harbour can be characterised by four geomorphic zones including the tide delta at the entrance, the central mud basin, fluvial delta, and riverine channel following in sequence when proceeding upstream (Roy, 1984; Roy et al., 2001; Mesley, 2003, Fig. 1 and Appendix S1). The tidal delta has an ebb component external to the entrance and an inner component, the flood tide delta (FTD) that explains shallower depths at the entrance of most New South Wales (NSW) estuaries when compared to further upstream in the central mud basin (CMB). The CMB, on the other hand, is a function of lowered sea levels during the Pleistocene epoch, meaning that its sedimentary signatures were integrated over millennia and only recently partially overwritten by sedimentation from urban expansion. The fluvial delta (FD) denotes a zone where terrestrial sediments are deposited, and the riverine channel (RC) is the most upstream zone of an estuary. While these structural geomorphic zones have been recognised by others (e.g., West et al., 2004), they have not been investigated in depth to determine whether they play any role in influencing the distribution of biological assemblages that inhabit the estuary. Understanding the relative spread of biota among geomorphic zones will assist the interpretation of future biodiversity monitoring in Sydney Harbour, and urban estuaries more broadly.

We would expect that a specific fraction of aquatic flora and fauna is associated with each of these geomorphic zones based on their habitat characteristics. The upper reaches of Sydney Harbour also have lower salinity levels and less habitat complexity compared to areas closer to the estuary mouth, suggesting that more taxa may inhabit the lower reaches. Water temperature and salinity shifts including increased runoff related to short- and long-term climate change, as well as urban modification, might alter the distribution or scope of geomorphic zones, and hence their biota. Further modification of the foreshore (e.g., in Sydney Harbour, a new fish market and highly rugose tiles adhered to existing seawalls; Clifton et al., 2022) will continue to enhance or decrease microhabitats, and so it will be important to provide a time-stamp for biological communities within each of these geomorphic zones to enable future monitoring.

Environmental DNA (or eDNA) metabarcoding is a tool that allows us to rapidly survey biodiversity at scale based on genetic material shed by flora and fauna into the surrounding environment (Thomsen et al., 2012; Taberlet et al., 2018; Takahashi et al., 2023). Snapshots of biodiversity generated from a single source of eDNA are often localized and reflect contemporary patterns allied with habitat characteristics (Monuki et al., 2021; Polanco F. et al., 2021). Combining multiple metabarcoding assays that target a broad range of taxonomic groups (hereafter Tree of Life or ToL-metabarcoding; Stat et al., 2017; Macé et al., 2024) extends monitoring programs that are reliant on a single or suite of proxy indicator species. ToL-metabarcoding therefore allows us to assess whether biological communities across the tree of life are bound by geomorphic zones in aquatic environments.

The aims of this study were therefore to 1) provide the first broad

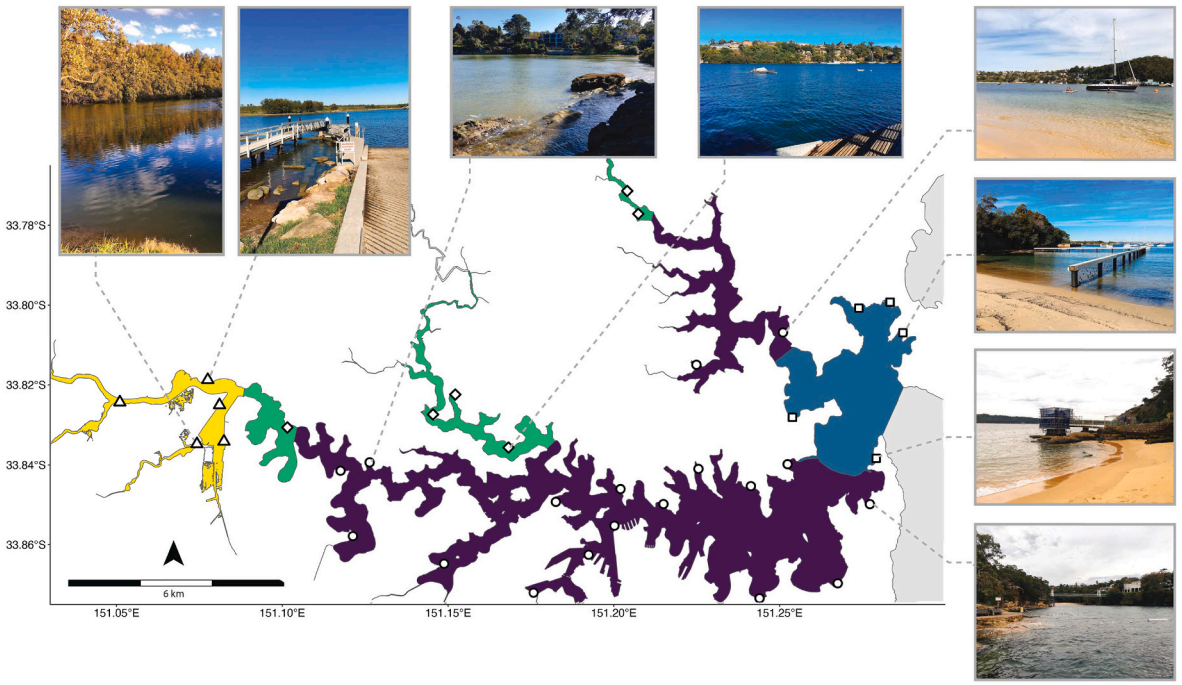

Fig. 1. Spatial distribution of the 34 water collection sites around Sydney Harbour (red symbols) with representative habitat photos for eight of the locations (clockwise: Haslams Creek Junction in Sydney Olympic Park, Riverine Channel; Ermington Boat Ramp in Melrose Park, Riverine Channel; Banjo Paterson Park in Gladesville, Central Mud Basin; Lane Cove 12 ft Sailing Skiff Club in Longueville, Fluvial Delta; Clontarf Beach in Clontarf, Flood Tide Delta; Little Manly Beach in Manly, Flood Tide Delta; Camp Cove in Vaucluse, Flood Tide Delta; Parsley Bay in Vaucluse, Central Mud Basin). Geomorphic zones are indicated by symbols (Flood Tide Delta [FTD], squares, $N = 5$; Central Mud Basin [CMB], circles, $N = 18$; Fluvial Delta [FD], diamonds, $N = 6$; Riverine Channel [RC], triangles, $N = 5$) and coloured shading on the map defines the boundaries of each geomorphic zone.

ToL-metabarcoding characterisation of Sydney Harbour's aquatic diversity, 2) determine whether ToL-metabarcoding detections correlate with known geomorphic habitat zones within an urbanized estuary, and 3) assess whether these detections can be used as a benchmark for future monitoring. Our null hypothesis was that there are no differences in aquatic species composition or taxonomic richness along the main axes of Sydney Harbour.

2. Material and methods

2.1. Sample collection

Water samples were collected at 34 sites across the shoreline of Sydney Harbour in the winter season from June to August 2022 to assess the efficacy of ToL-metabarcoding to delineate biodiversity based on geomorphic zone (Flood Tide Delta, $N = 5$; Central Mud Basin, $N = 18$; Fluvial Delta, $N = 6$; Riverine Channel, $N = 5$; Fig. 1 and Appendix S1).

We defined the northern limit of Sydney Harbour as the junction of Middle Harbour Creek and Rocky Creek in East Killara (33°44′52.27″S, 151°11′30.87″E), the southern limit as Blackwattle Bay (33°52′33.14″S, 151°11′21.72″E), the western limit as the weir at Parramatta (Charles Street) (33°48′47.55″S, 151°00′36.23″E), and the eastern limit as a line from the northern headland (33°49′26.58″S, 151°18′00.75″E) to the southern headland (33°49′57.67″S, 151°16′50.16″E). For further details on geographic boundaries and tributary exclusions, see DiBattista et al. (2022). The boundaries of geomorphic zones were defined according to Mesley (2003), based on sediment characteristics. As distinct from regional scale factors such as latitude, rainfall, and temperature variation, the local scale geomorphology of an estuary provides boundary conditions for physical features (tidal prism, topography, sediment deposition, bathymetry, salinity, temperature) that influence system chemistry (pH, chemical oxygen demand, total nitrogen, total phosphorous) that mediates habitat creation (mud basins, mud flats, macrophytes) that in turn control species distributions.

For each water replicate, a sterile eDNA syringe mini kit containing a 30 mm × 1.2 μm cellulose acetate syringe filter with luer-lock inlet and outlet fitting was used (Wilderlab NZ Ltd, Wellington, New Zealand). Each kit additionally included sterile nitrile gloves, a 60 cc luer-lock syringe for aspirating and pumping water over the filter membrane, and 350 μl of DNA/RNA Shield preservative (Zymo Research Cat. No. R1200-125) pre-loaded into a 3 cc luer-lock syringe.

Samples were not collected within three days of major rain events to avoid capturing stormwater runoff and allochthonous inputs (Beck and Birch, 2014). The target water filtration volume was 1 L, but in some cases, the filter clogged prior to the target volume and so only 500 mL was filtered at four sites Appendix S1. That said, recent work metabarcoding water from turbid inshore estuaries shows that even 500 mL water volume returns consistently high numbers of eDNA detections across replicates (Kumar et al., 2022). Additional metadata recorded in the field included GPS coordinates, collector, tide, moon phase, and general biotic observations at each collection site. Syringe filters were kept at ambient temperature following preservation and shipped to Wilderlab in Wellington, New Zealand within six weeks of collection for further processing. For more details on sampling using the eDNA syringe mini kits see Appendix S2.

2.2. Sample processing

The DNA/RNA Shield containing the filtrate was extracted from each filter at Wilderlab NZ Ltd using the attached preservation syringe, transferred to a DNA Lo-Bind tube (Eppendorf, Germany), and stored at −20 °C prior to DNA extraction. For DNA extraction and purification, 200 μl of each sample lysate were loaded into a Nextractor GD141 cartridge (Genolution, South Korea) and run on the Genolution Nextractor NX-48S system using default settings. DNA quality/quantity analysis, adapter-fusion, indexing, and amplification were carried out in single-step quantitative PCR (qPCR) reactions on an Applied Biosystems QuantStudio 1 qPCR instrument (CA, USA; CI assays below) or Applied Biosystems ProFlex PCR System (all other assays) enabling the detection of vertebrate, invertebrate, plant, microeukaryote and microbial DNA (Appendix S2). Also see Ling et al. (2023), Urban et al. (2023), and Wilkinson et al. (2024) for a detailed description and application of these methods in other systems.

In brief, our laboratory protocol involved the use of duplicate PCR reactions, DNA quality and quantity checks, and the inclusion of a negative PCR control reaction in the sequencing run, which consisted of deionised water in place of template DNA. DNA libraries for all assays were loaded onto iSeq V2 reagent cartridges with a 300-cycle flow cell (Illumina), which included 5% PhiX (the minimum PhiX aligned percentage recommended by Illumina; also see Appendix S3 for sequence filtration data) and was run for 290 cycles in a single direction on an Illumina iSeq 100. We used 16 metabarcoding assays that targeted the following taxonomic groups: eukaryotes (BE assay), eukaryotes and bacteria (BU assay), Chinese Mitten Crab *Eriocheir sinensis* (CE assay), aquatic insects (CI assay), Asian Paddle Crab *Charybdis japonica* (CK assay), Asian Basket Clam *Potamocorbula amurensis* (CP assay), fish (DG assay), fish (LG assay), fish (LV assay), vascular plants (MZ assay), vertebrates (RV assay), *Caulerpa* spp. (TC assay), vascular plants (TP assay), bacteria (UM assay), fish (WV assay), and decapods (ZC assay) (Appendix S2). These 16 metabarcoding assays were divided across three sequencing runs (Run 1 assays: BU, DG, LV, MZ, TP, UM, WV, ZC; Run 2 assays: BE, CI, LG, RV; Run 3 assays: CE, CK, CP, TC), with amplicon pooling only taking place after the PCR steps outlined above.

2.3. ASV generation and taxonomic assignment

The sequence fastq files were de-multiplexed in R (R Core Team, 2024) using the insect package (v 1.4.0; Wilkinson et al., 2024) and trimmed sequences were filtered to produce a table of exact amplicon sequence variants (ASVs) using the DADA2 package (Callahan et al., 2016). ASVs were identified to the lowest possible taxonomic rank using a curated database of previously detected eDNA sequences, as well as trimmed reference sequences downloaded from GenBank (Benson et al., 2010), BOLD (Ratnasingham and Hebert, 2007), and RDP (for the UM assay only; v18, Cole et al., 2014). See Appendix S2 for more information on ASV generation and taxonomic assignment.

2.4. Data curation and validation

ASV taxonomic assignments were examined within individual assays and then aggregated among all 16 metabarcoding assays (Appendix S1and Appendix S2). ASVs designated "root" were removed as these were unassignable even at the superkingdom level. We also removed ASVs assigned to "cellular organisms" and "environmental samples" as their taxonomic identity could not be inferred. The World Register of Marine Species (WORMS, https://www.marinespecies.org/) was used to source the accepted taxonomic nomenclature for each ASV (accessed June–July 2023), as well as determine whether it was associated with primarily marine, brackish, freshwater, or terrestrial habitat. All strictly terrestrial taxa were excluded from downstream statistical analysis unless otherwise noted. For the aquatic taxa (i.e., marine, brackish, and freshwater), we used the Atlas of Living Australia (ALA) online repository of Australian plants, animals, and fungi (ALA Accessed August–September 2023) to assess whether it had previously been recorded in Australia as well as the number of records, and whether it had previously been recorded in Sydney Harbour following our defined boundaries. ALA assembles biodiversity data from several sources including natural history collections at museums and herbaria, government monitoring programs, independent research projects, Indigenous knowledge, and citizen science platforms (Belbin et al., 2021). The Shiny Wheels app was used to visualise the phylogenetic tree for all datasets (https://wilderlab.shinyapps.io/ShinyWheel/). Spatial data

visualisation was curated in R v4.0.3 using the 'tidyverse', 'ggspatial' and 'sf' packages (Pebesma, 2018; Wickham et al., 2019; Dunnington, 2023).

2.5. Statistical analysis

The amount of eDNA present in the environment can be influenced by biotic and abiotic factors that dictate the amount of DNA released from each organism, rates of DNA degradation, and the removal of DNA from that environment (Stewart, 2019). Similarly, laboratory methods at multiple points in the workflow can introduce biases related to the state and quality of DNA available for PCR amplification in each metabarcoding assay. We have therefore taken a conservative approach and restricted our statistical inferences to the presence or absence of taxa that we detected based on sequence data in each sample, versus their relative abundance as a function of the number of reads.

Separate analyses were run for the entire community and each of the following major taxonomic subgroups: fish, macroinvertebrates (here defined as crustaceans, echinoderms, flatworms, insects, mites and ticks, molluscs, and worms), a combination of algae (green, heterokont, and red) and aquatic plants, and bacteria. Permutational multivariate ANOVA (PERMANOVA; PERMANOVA+, PRIMER-E Ltd) was used to test for differences in eDNA community structure between the four geomorphic zones based on the presence/absence (detections) of ASVs.

PERMANOVA is the equivalent of an ANOVA performed on similarity values and uses permutations to test the significance of differences among groups (Anderson et al., 2008). Given that distance, specifically in terms of variation in salinity proceeding upstream from the mouth of the harbour may influence community structure, this term was fitted to the data first, and then the effect of geomorphic zone was investigated. We did not consider the interaction term between distance and geomorphic zone because it is not a relevant consideration within zones. Analyses were performed on Bray-Curtis dissimilarity values calculated between sample pairs, and results were visualised using Canonical Analysis of Principle coordinates (CAP) plots. Bray-Curtis resemblance performs equally well (i.e., is equivalent) to other methods that are dedicated to presence-absence data (e.g., Jaccard Index). CAP plots are appropriate for accompanying PERMANOVA significance testing because the CAP routine determines axes through the multivariate data cloud that best discriminate among *a priori* groups (Anderson et al., 2008), in this case, geomorphic zones. Where significant differences were found, pairwise PERMANOVA tests were used to identify which geomorphic zones differed.

Significant PERMANOVA results may arise from differences in the location of data groups (usually the characteristic of interest), or differences in multivariate data spread, or both. The PERMDISP routine was used to test for potential differences in multivariate data spread among groups; PERMDISP is the multivariate equivalent of Levene's test for homogeneity of variance in ANOVA (Anderson, 2014). For the entire community, Similarity Percentages (SIMPER) analyses were conducted to examine the contribution of each taxon (i.e., ASV) to the differences between geomorphic zones.

A similar analysis was used to compare taxonomic richness among geomorphic zones while accounting for the effect of distance from the mouth of the estuary. A generalised linear model (GLM) with a Poisson distribution and a log link was used due to the positive count data. The assumption of the Poisson distribution, where variance equals the mean, was statistically verified (overdispersion test: dispersion ratio = 1.13, P = 0.2820). GLMs were conducted in R v.4.3.1, and pairwise comparisons of marginal means between geomorphic zones were conducted using the *contrast* function in the package 'emmeans'.

3. Results

3.1. ASVs and taxonomic assignments

In total, 12,189 ASVs were generated in the full dataset (i.e., all ASVs considered in each assay independently) through bioinformatic processing, with 6759 ASVs assigned to "root", 1 ASV assigned to "cellular organisms", and 5 ASVs assigned to "environmental samples" excluded from further analysis. Raw sequence reads are available in.fastq format from the NCBI GenBank Sequence Read Archive (BioProject Accession Number: PRJNA1107684). Appendix S3 sequentially reports the raw reads from sequencing, reads after quality filtering, reads after denoising, and reads after taxonomic identification based on the sequencing library ID. Note that we did not detect any animal DNA in our negative control reactions; however, we detected one plant genus (*Allium*), one yeast genus (*Cyberlindnera*), 7 bacterial genera, and 16 bacterial assignments at higher taxonomic ranks. The ASV profiles of the negative control reactions included commonly detected bacteria such as *Cupriavidus*, *Methylobacterium*, and *Novosphingobium*, all consistent with the microbial profiles previously encountered using this workflow and assay panel (Wilkinson et al., 2024).

Based on the remaining 5424 ASVs, the mean number of ASVs per assay was 387.50 ± 127.48, with the CI assay having the most ASVs (1832 ASVs), followed by the UM assay (826 ASVs), and four assays having under 100 ASVs (CK = 8 ASVs, MZ = 61 ASVs, TC = 2 ASVs, ZC = 94 ASVs). Note that two of the assays (CE, CP) only generated ASVs for the excluded ambiguous taxonomic assignments (see above). Also note that due to genetic variation in the barcoding genes associated with each assay, in some cases, multiple ASVs were assigned the same taxon.

Our dataset included 1427 unique taxa once it was aggregated across all assays, which consisted of 1118 aquatic taxa (Fig. 2): 1073 predominantly marine taxa, 45 predominantly brackish or freshwater taxa, and the remaining 309 taxa were strictly associated with terrestrial habitat (see Appendix S4 for full phylogenetic tree including all unique taxa). Of these 1427 unique taxa, 3 (<1%) were assigned at the level of kingdom (plus 1 superkingdom), 34 (2.4%) to phylum, 57 (4.0%) to class (plus 1 infraclass and 4 subclasses), 106 (7.4%) to order (plus 1 superorder and 3 suborders), 211 (14.8%) to family (plus 4 superfamilies and 34 subfamilies), 493 (34.5%) to genus (plus 3 subgenera), 394 (27.6%) to species (plus 2 subspecies), and 38 (2.7%) additional taxa assigned to alternate ranks (i.e., clade, cohort, tribe, subtribe). In addition, 38 (2.7%) were assigned "no rank", but in every case, these were identified as plants, animals, fungi, or bacteria (Fig. 3).

Based on the 1427 unique taxa in the aggregated dataset, a significant proportion were made up of fish (14.0%), macroinvertebrates (15.2%), algae and aquatic plants (9.4%), and bacteria (14.6%), thus justifying our independent statistical interrogation of these well-represented subgroups (Appendix S1 and Fig. 3). The remaining taxa were identified as amoebae (<1%), archaea (<1%), birds (1.9%), bryozoans (<1%), ciliates (4.5%), cnidarians (4.3%), cryptomonads (<1%), diatoms (3.2%), dinoflagellates (1.6%), fungi (<1%), mammals (2.6%), mosses (<1%), oomycetes (<1%), terrestrial plants (13.9%), rotifers (<1%), sponges (1.3%), springtails (<1%), and 5.5% as "other", in most cases due to its higher taxonomic assignment (Appendix S1 and Fig. 3).

Based on the 1427 unique taxa, 1135 (79.5%) of the taxa were previously identified in Australia according to records sourced from ALA (Appendix S1). Specifically for the 1118 aquatic taxa, 879 (78.6%) were previously identified in Australia and 521 (46.6%) were previously identified in Sydney Harbour. It should be noted a heterokont algae (*Hecatonema stewartense*, 9 ALA records) and a red algae (*Pyropia corniculate*, 25 ALA records) only contained records from New Zealand or the Northwest Pacific Ocean on ALA. Of the 239 (21.4%) aquatic taxa that did not have records on ALA (Appendix S1), 26 taxa had taxonomic classifications not recognised by ALA at the order, suborder, subfamily, tribe, group, or clade level, and 11 taxa did not have records at the species level that we identified (i.e., only listed at the generic level on

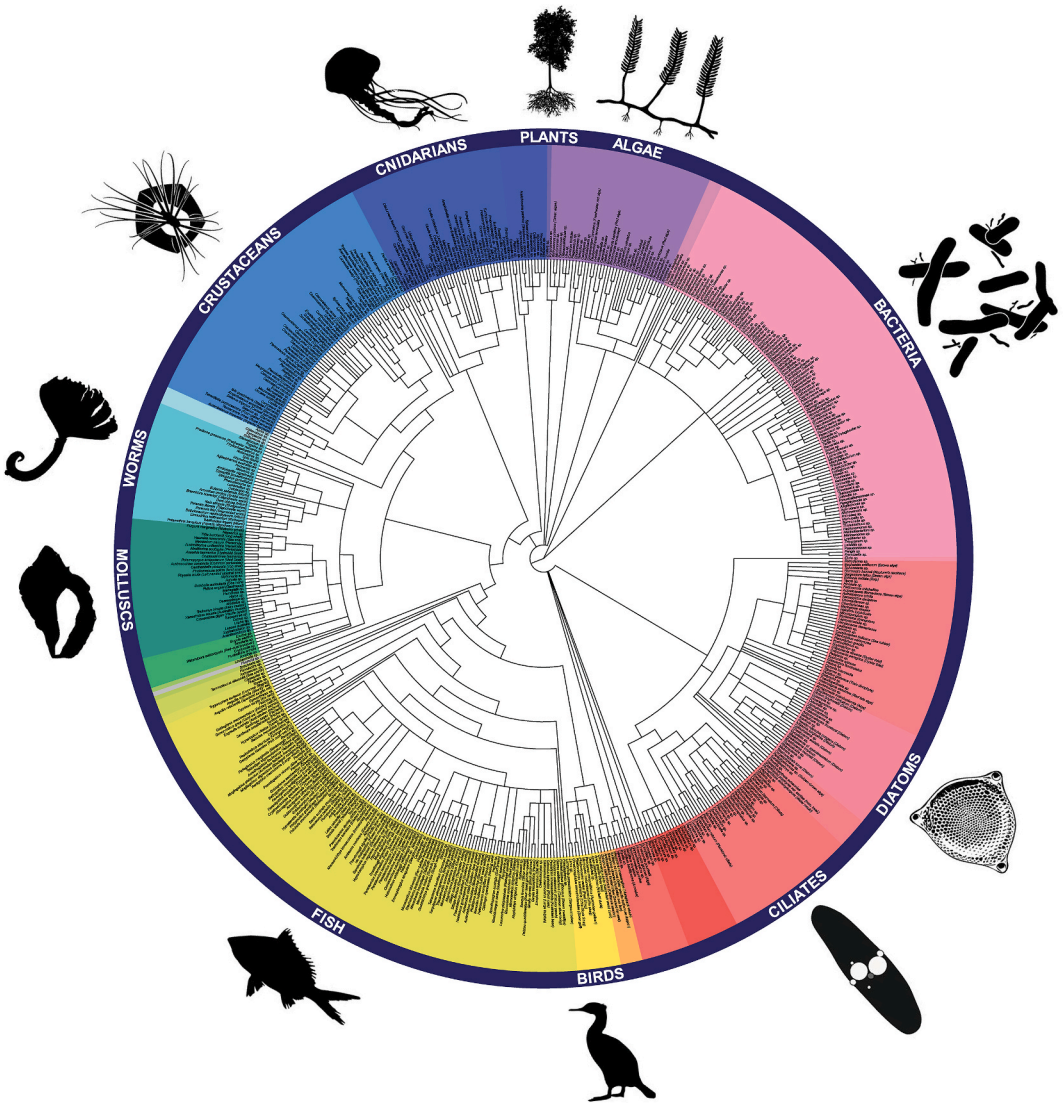

Fig. 2. Phylogenetic tree (i.e., "Wheel of Life") based on all *aquatic* tree of life (ToL) metabarcoding detections ($N = 1118$ unique taxa) as defined by WORMS (accessed June–July 2023). For a full phylogenetic tree based on all metabarcoding detections (marine, brackish, freshwater, and terrestrial taxa), see Appendix S4.

ALA). Moreover, of the 239 aquatic taxa that did not have records on ALA (Appendix S1), 24 (10.0%) were identified as fish, 16 (6.7%) as macroinvertebrates, 38 (15.9%) as algae and aquatic plants, and 90 (37.7%) as bacteria (Appendix S1).

3.2. Multivariate biological community analysis

PERMANOVA indicated significant effects of distance from the mouth of the Sydney Harbour estuary and geomorphic zone on community structure in the aggregated eDNA dataset (entire community) and each of the taxonomic subgroups investigated (PERMANOVA, Table 1 and Fig. 4). However, each result for zone was accompanied by a significant PERMDISP statistic (Table 1), suggesting differences between zones may be driven by either community differences, the extent of variability among samples, or both. Examination of the pairwise results and the degree of separation among data groups in CAP plots were used to draw conclusions regarding causal effects (see below).

Pairwise PERMANOVA tests indicated that the biological community of the CMB differed significantly from all other zones, but other zones did not differ from each other. Although the spread of multivariate data also differed between CMB and each of the RC or FD (Table 2), visual inspection of the CAP plot showed a clear separation in the data groups (Fig. 4a), suggesting that differences in biological communities were driving the significant PERMANOVA results rather than merely differences in multivariate data spread.

Despite the significant global PERMANOVA result for fish, none of the pairwise comparisons among zones were significant (Table 2, Fig. 4b); this statistical difference may have therefore been driven by differences in multivariate spread (PERMDISP results, Table 2). Like the pairwise result for the entire community, CMB differed significantly from all other zones for macroinvertebrates, but again other zones did not differ from each other (Table 2, Fig. 4c). Although multivariate data spread also differed between CMB and each of the RC or FD for macroinvertebrates (PERMDISP, Table 2), visual inspection of the CAP plot showed almost no overlap among these data groups (Fig. 4c). Pairwise results for a combination of algae and aquatic plant taxa also indicated that the CMB differed from both RC and FD (Table 2, Fig. 4d); although multivariate data spread differed between CMB and RC, there was almost no overlap of these two data groups apparent in the CAP plot (Fig. 4d). Finally, pairwise results for bacteria were like those for the entire community, with marginally significant results based on comparisons between CMB and all other zones (Table 2). Despite a significant difference in multivariate data spread between CMB and RC for bacteria, data points for these two zones did not overlap (Fig. 4e). The most interesting result was that statistical differences did not appear to be dependent on whether the compared zones were geographically

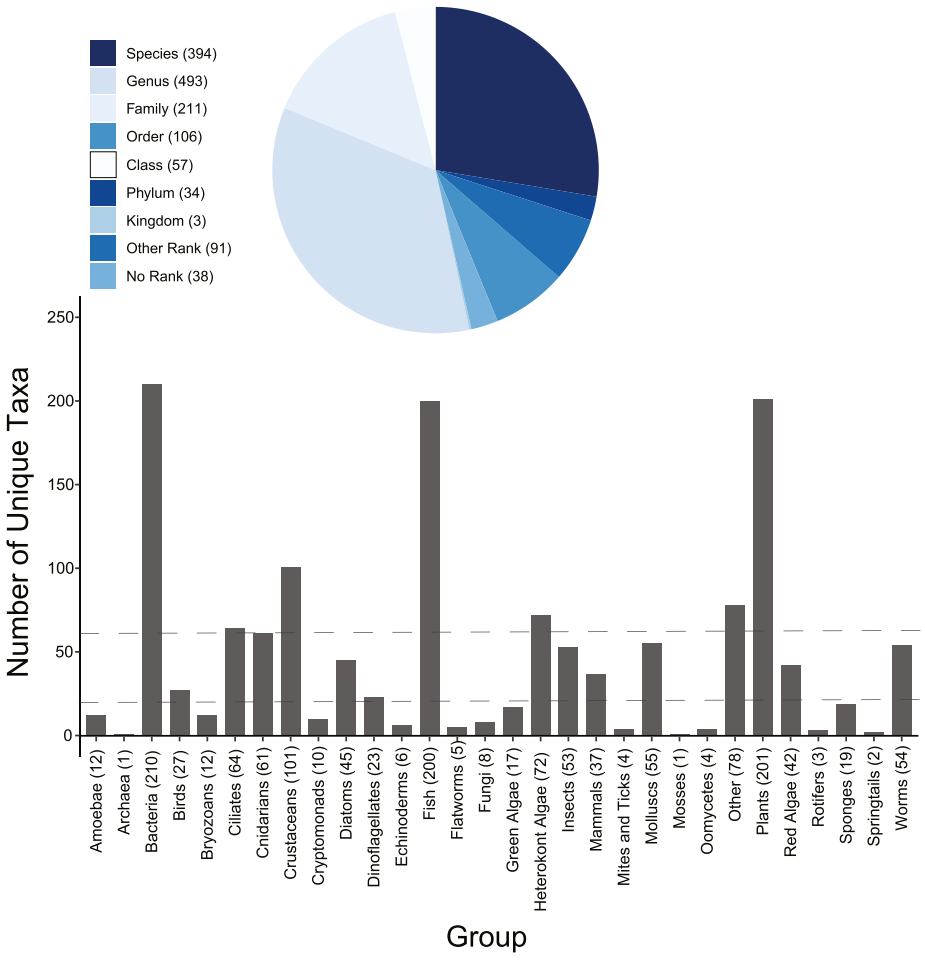

Fig. 3. Bar plot of the number of unique taxa in the aggregated dataset ($N = 1427$) as a function of taxonomic group and accompanying pie chart displaying the taxonomic rank of those assignments. Numbers in parentheses represent the number of taxa. "Other" taxonomic ranks include clade, subtribe, tribe, cohort, subspecies, subgenus, subfamily, superfamily, suborder, superorder, subclass, infraclass, and superkingdom. The dashed lines indicate 95% confidence intervals of the mean number of unique taxa across groups.

Table 1
Results of global PERMANOVA comparisons of community structure across geomorphic zones, accounting for distance from the estuary mouth. Analyses were conducted separately for the entire community and for each of the four taxonomic subgroups. PERMDISP was used to test for significant differences in multivariate data spread among zones, results that assist in the interpretation of the main PERMANOVA result. P-values in bold are significant (<0.05).

Taxonomic Group	Model Term	Pseudo-F	PERMANOVA P-value	PERMDISP P-value
Entire Community	Distance	5.77	**0.0001**	–
	Zone	1.95	**0.0001**	**0.0001**
Fish	Distance	5.36	**0.0001**	–
	Zone	1.36	0.0327	**0.0001**
Macroinvertebrates	Distance	7.57	**0.0001**	–
	Zone	1.76	**0.0001**	**0.0030**
Algae and Aquatic Plants	Distance	5.39	**0.0001**	–
	Zone	2.43	**0.0001**	**0.0001**
Bacteria	Distance	4.28	**0.0001**	–
	Zone	1.74	**0.0009**	**0.0007**

adjacent versus separated by at least on other geomorphic zone (Table 2).

SIMPER analyses indicated that a broad spectrum of taxa contributed to the zonal differences in the entire community analysis (Fig. 5). Indeed, the highest individual contribution for any single taxon was only 0.62%, and it required 495 taxa to explain 80% of the differences between the CMB and FTD, 435 taxa to explain 80% of the differences between the CMB and FD, and 387 taxa to explain 80% of the differences between the CMB and RC.

3.3. Univariate analysis and taxonomic richness

Significant differences in taxonomic richness were found among geomorphic zones, with the richness of the CMB higher than that of the FD ($P = 0.0315$) and RC ($P < 0.001$) (Fig. 6). Richness of the CMB and FTD, however, did not differ significantly ($P = 0.1627$). Taxonomic richness decreased with increasing distance from the entrance of the estuary ($P = 0.0347$, Fig. 7), decreasing by 0.8% with each additional kilometer from the estuary mouth (coefficient [95% CI]: 0.992 [0.984–0.999]).

Finally, a spatial depiction of the mean number of taxa and taxonomic richness estimated from the entire biological community (Appendix S5), as well as metrics estimated from taxa with broad phylogenetic similarities (fish, macroinvertebrates, algae and aquatic plants, bacteria; Fig. 8), showed no obvious pattern in terms of their spatial clustering across and within each geomorphic zone. For fish, FD appeared to have the highest mean number of taxa (with CMB having the lowest), for macroinvertebrates FTD appeared to have the highest mean number of taxa (with RC having the lowest), whereas in algae, aquatic plants, and bacteria, CMB had the highest mean number of taxa (with RC having the lowest in all cases).

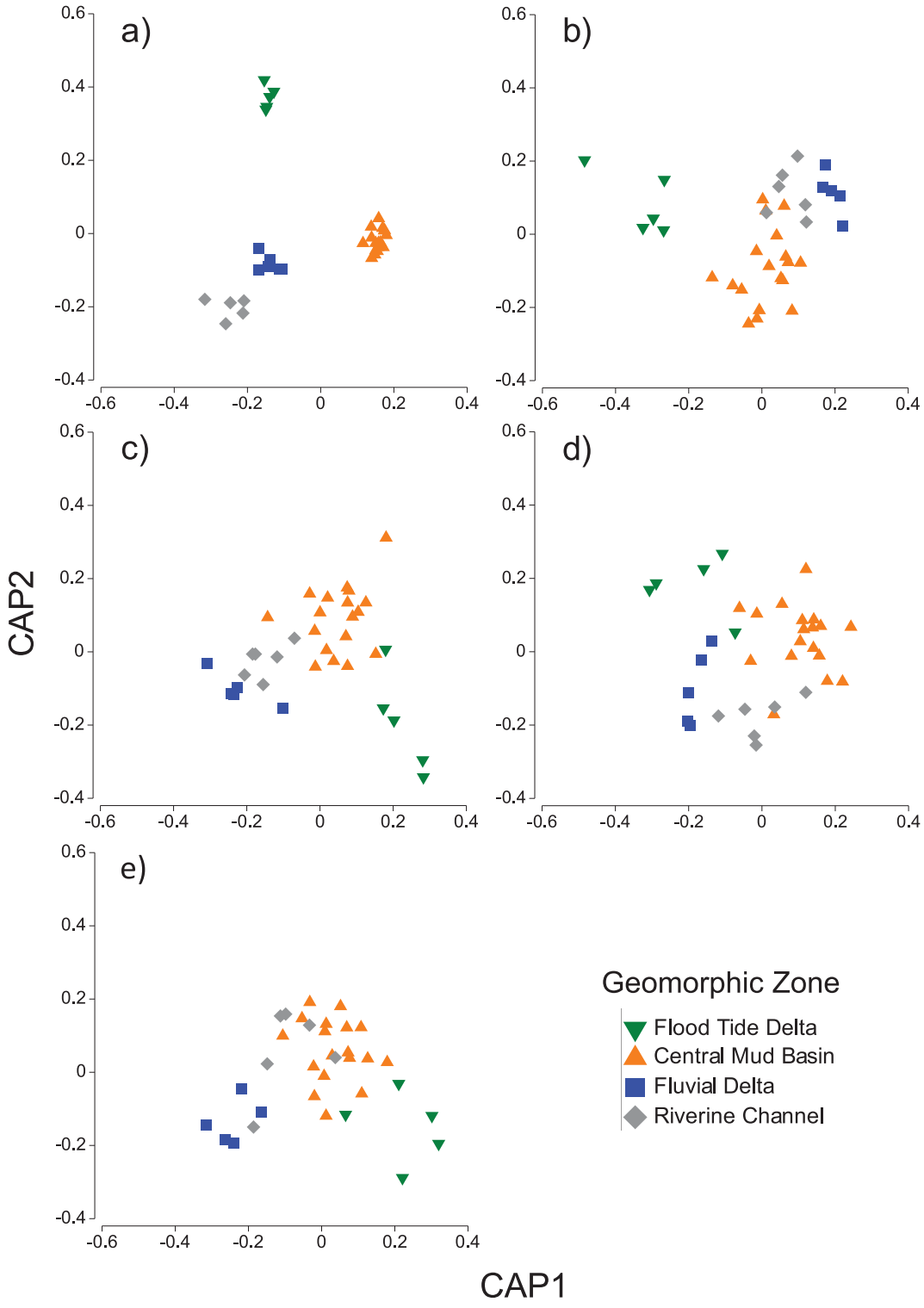

Fig. 4. Canonical Analysis of Principle Coordinates (CAP) ordination of the tree of life (ToL) metabarcoding detections based the 34 water collection sites around Sydney Harbour coded by geomorphic zone (Flood Tide Delta [FTD], $N = 5$; Central Mud Basin [CMB], $N = 18$; Fluvial Delta [FD], $N = 6$; Riverine Channel [RC], $N = 5$). Five taxonomic subgroupings of unique aquatic taxa were investigated: a) entire community ($N = 1118$), b) fish ($N = 200$), c) macroinvertebrates (crustaceans, echinoderms, flatworms, insects, mites and ticks, molluscs, and worms; $N = 217$), d) algae (green, heterokont, and red) and aquatic plants ($N = 134$), and e) bacteria ($N = 208$).

4. Discussion

Our study integrates water sampling in an urbanized estuary with 16 eDNA metabarcoding assays to generate a snapshot of biodiversity across the tree of life, previously referred to as ToL-metabarcoding, with an increased sampling effort (i.e., number of samples, total filtered water volume) compared to some recent applications (Stat et al., 2017; Macé et al., 2024). We interrogated biodiversity differences obtained by genetic isolation to assess whether the identified biological communities were constrained by geomorphic zones, and additionally discuss how this method can be used to establish baselines and scaled for biotic benchmarking in other urbanized estuaries or at more frequent

Table 2
Results of pairwise PERMANOVA (PERM) comparisons of community structure between geomorphic zones for the entire community and each of the four taxonomic subgroups. PERMDISP was used to test for significant differences in multivariate data spread between zones, results that assist in the interpretation of the PERMANOVA result. P-values in **bold** are significant (<0.05). Proceeding upstream from the estuary entrance: FTD = Flood Tide Delta, CMB = Central Mud Basin, FD = Fluvial Delta, RC = Riverine Channel.

Zones Compared		Entire Community			Fish			Macroinvertebrates			Algae and Aquatic Plants			Bacteria		
Up stream	Down stream	Pseudo-t	PERM P-value	PERM DISP P-value	Pseudo-t	PERM P-value	PERM DISP P-value	Pseudo-t	PERM P-value	PERM DISP P-value	Pseudo-t	PERM P-value	PERM DISP P-value	Pseudo-t	PERM P-value	PERM DISP P-value
CMB	FTD	1.29	**0.0103**	0.6693	1.16	0.1176	0.1906	1.38	**0.0095**	0.1573	1.21	0.1501	0.3833	1.26	**0.0429**	0.2623
FD	CMB	1.34	**0.0033**	**0.0015**	1.12	0.2025	**0.0003**	1.32	**0.0135**	**0.0011**	1.68	**0.0061**	0.2357	1.27	**0.0454**	0.3380
RC	CMB	1.40	**0.0008**	**0.0001**	1.01	0.4320	**0.0001**	1.27	**0.0273**	**0.0107**	1.50	**0.0172**	**0.0002**	1.26	**0.0496**	**0.0006**
FD	FTD	1.11	0.2062	**0.0230**	1.13	0.2051	**0.0071**	1.23	0.1000	0.4503	0.90	0.6103	**0.0275**	1.02	0.4327	0.6149
RC	FTD	0.83	0.8173	**0.0084**	0.96	0.5710	**0.0053**	0.96	0.5518	0.4990	0.39	0.9850	**0.0081**	0.74	0.9019	**0.0167**
RC	FD	1.00	0.4912	**0.0069**	1.04	0.3768	0.3770	0.88	0.7349	0.9816	1.02	0.4174	**0.0058**	0.86	0.7151	**0.0113**

Fig. 5. Similarity Percentages (SIMPER) analysis showing the contribution of each unique aquatic taxa (N = 1118) to overall dissimilarity between the geomorphic zone of Central Mud Basin (CMB) and a) Flood Tide Delta (FTD), b) Fluvial Delta (FD), and c) Riverine Channel (RC). Taxa were ordered from most to least contribution.

Fig. 6. Mean richness (number of taxa) across geomorphic zones. Bars indicate 95% confidence intervals.

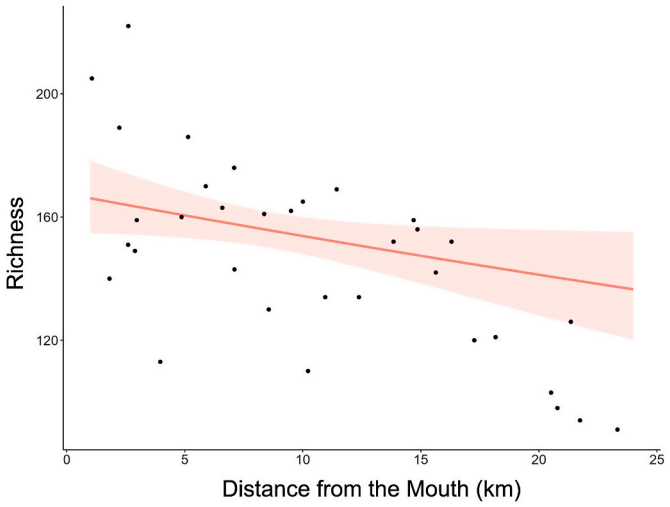

Fig. 7. Partial effect of distance from the estuary mouth on richness (number of taxa) in Sydney Harbour. The pink shaded area represents the 95% confidence interval of the modelled effect. Data points indicate raw data.

intervals.

We expected that the ToL-metabarcoding data expressed either as whole biological communities or taxonomic richness would correlate with known geomorphic habitat zones within an urbanized estuary, and this was indeed the case in Sydney Harbour. Each zone possesses distinct habitat characteristics that host a unique suite of flora and fauna across the tree of life and occupy different sections of the harbour. The RC is found at the upper (western) reaches of the Parramatta River. The FD is found just east of this in the Parramatta River but also at the upper (northwestern) reaches of the Lane Cove River and Middle Harbour. The FTD is found at the estuary mouth, and the CMB is the largest of the four zones that occupies a transitional territory. As examples of habitat differences between the zones, the broad leaf seagrass *Posidonia australis* exists almost exclusively in the FTD, with rare instances along the margins of the adjacent CMB (West et al., 2004), though its distribution has reduced significantly even in the last two decades (Evans et al., 2018). Fishes that associate predominantly with *P. australis* (such as many Syngnathidae species) will therefore nominally be restricted to the lower part of the estuary where FTD characteristics are present. That said, we did not detect either *P. australis* or Syngnathidae species in this study. *Posidonia australis* meadows have been reported to be declining at a rate of 10% per year in Sydney Harbour based on a loss of 1.12 ha between March 2010 and September 2014, a loss that is the greatest among the many estuaries of the Manning-Hawkesbury ecoregion and far exceeds the global rate of seagrass decline (Evans et al., 2018). This

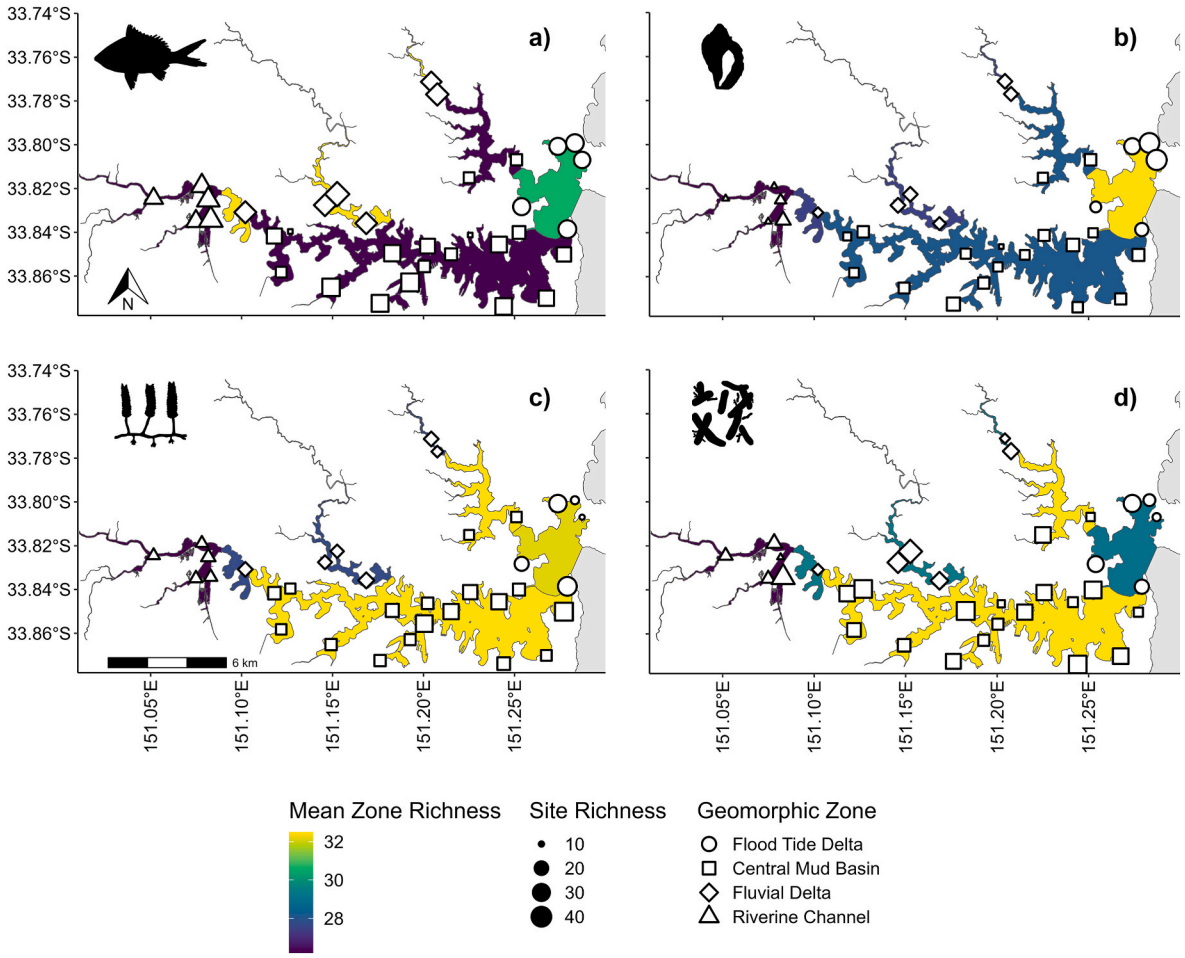

Fig. 8. Map of richness (number of taxa) from 34 water collection sites around Sydney Harbour based on aquatic tree of life metabarcoding detections for a) fish (N = 200), b) macroinvertebrates (crustaceans, echinoderms, flatworms, insects, mites and ticks, molluscs, and worms; N = 217), c) algae (green, heterokont, and red) and aquatic plants (N = 134), and d) bacteria (N = 208). Geomorphic zones are indicated by symbols (Flood Tide Delta [FTD], N = 5; Central Mud Basin [CMB], N = 18; Fluvial Delta [FD], N = 6; Riverine Channel [RC], N = 5) and the size of each symbol indicates the number of taxa at each water collection site. Shading on the map represents the mean taxa across each geomorphic zone.

reduction may have influenced our ability to detect remnant populations of *P. australis* using eDNA in our study.

Seagrasses in the family Zosteraceae are more widespread in Sydney Harbour than *P. australis*, found in the near-shore shallows of the FTD and CMB, as well as the downstream portion of the FD. Reflecting this, we detected *Zostera* spp. in 7 of the 34 samples, 6 from the CMB and one from the FTD. With respect to the lack of Syngnathidae detections across all geomorphic zones, this could indicate a primer bias in our fish metabarcoding assays, which have previously necessitated the re-design of general fish primers to also detect fishes in the Syngnathidae family (Nester et al., 2020). That said, more extensive temporal sampling at two sites in Sydney Harbour (Camp Cove in the FTD and Parsley Bay in the CMB) as part of a companion study detected 3 Syngnathidae with the same suite of 16 metabarcoding assays (*Hippocampus* sp. in one sample, *Stigmatopora nigra* in 5 samples, and *Vanacampus margaritifer* in one sample; J.D. DiBattista, unpublished data). Moreover, the re-designed primers in Nester et al. (2020) are used in 1 of our 16 eDNA metabarcoding assays (assay WV; Appendix S2).

The consistent multivariate differences between geomorphic zones detected across broad taxonomic subgroupings with vastly different ecological properties (fish, macroinvertebrates, algae and aquatic plants, and bacteria) suggest that these zones have a considerable impact on community composition in Sydney Harbour. A partitioning of bacterial communities (phylogenetic diversity, relative abundance, and gene function) by rainfall and nutrient regimes has previously been tested in Sydney Harbour (Jeffries et al., 2016), but associations with geomorphic zones for these and other taxonomic groups had not yet been considered. Bracewell et al. (2023) identified similarities in benthic bacterial and eukaryotic communities across the upper catchments of temperate estuaries in eastern Australia, though bacteria appeared to be the most susceptible to immediate environmental changes. Previous research in highly urbanized estuaries like Sydney Harbour suggests that particularly species of bacteria may serve as useful indicators for localised anthropogenic stressors (Birrer et al., 2021).

Suspended and sequestered bacteria generally drive high heterotrophic activity and biogeochemical cycling in estuaries (Crump and Bowen, 2024). Due to abiotic gradients and anthropogenic discharges, the composition of bacterial communities can shift between aerobic, chemoheterotrophic, and anaerobic, down to groups adapted to more extreme environmental conditions (e.g., thermophilic, halophilic; Yi et al., 2020; Santoferrara et al., 2022). Though inferring shifts in phylogenetic diversity and gene function of bacteria is beyond the scope of our study in Sydney Harbour owing to our lack of relative abundance data for bacteria and more broadly across the tree of life (but see Jeffries et al., 2016), we note that bacteria were one of most represented groups in our dataset (14.6% of the total taxa), but also one of the least reported on groups owing to a lack of formal biodiversity records on ALA. There was however agreement in the apparent differences among geomorphic zones for bacterial communities when compared to higher order taxonomic groups, which suggests an interrelationship between prokaryotic and eukaryotic taxa. Given that bacteria form the foundation of food webs as prey for many planktonic organisms (e.g., copepods, rotifers) and infauna (e.g., nematodes, polychaetes), play key ecological functions, and are closely associated with plants (i.e., nitrogen fixation) and animals (i.e., microbiomes) that inhabit estuaries, we suggest further interrogation of the interactions among ToL-metabarcoding detections using network analyses (DiBattista et al., 2020; Codello et al., 2023), with the added requirement of increased replication within and among catchments.

In our study, the CMB exhibited the greatest differences compared to other geomorphic zones based on multivariate (biological communities) and univariate (taxonomic richness) statistical tests, and this was reasonably consistent across the subset of taxonomic groups examined. This statistical difference can be attributed to the ecological attributes of each geomorphic zone (see above), but also a potential bias in statistical power. With respect to the latter, our sampling favoured the much larger and more accessible CMB ($N = 18$) compared to the other geomorphic zones (FTD, $N = 5$; FD, $N = 6$; RC, $N = 5$). The greater replication for CMB may have provided additional power for pairwise comparisons that included this zone, increasing the likelihood of detecting a statistical difference if one existed. A corollary of this is the reduced power for pairwise comparisons that did not include CMB, which could in turn increase the likelihood of a Type II error. Therefore, some community differences may exist between other zones in the present study, as indicated by the considerable separation observed between data clusters for all zones within the CAP plot of the entire community (Fig. 4a).

Our observed decrease in taxonomic richness with increasing distance from the estuary mouth is consistent with environmental changes that are known to affect biodiversity, including decreases in salinity and habitat complexity (also see Saenz-Agudelo et al., 2022). Given that ecological characteristics might change over time in terms of mean values or error terms (or both) within and between geomorphic zones, this observation is important when calibrating ToL-metabarcoding in future applications. Our results also indicate that monitoring must standardise for distance from the mouth of the estuary, in that samples need be taken at consistent distances to avoid the confounding of temporal trends.

The upper reaches of the estuary are more brackish and presumably have less habitat diversity compared to areas closer to the estuary mouth, it is therefore likely that species compositions will differ from downstream. Moreover, organisms with an oceanic life-stage, including numerous fishes, might also be expected to decrease in abundance with increasing distance from the mouth given the additional dispersal capability required (e.g., tidal currents or directional swimming) (Fortier and Leggett, 1982; Loneragan et al., 1986; Ford et al., 2010; Franca et al., 2012). As one example, the Yellowtail Kingfish *Seriola lalandi*, an active pelagic/benthopelagic fish, is generally thought to shoal, feed, and spawn in offshore marine environments, but younger fish of this species sometimes enter estuaries (Clarke et al., 2023). In our study, we detected this species 16.3 km from the estuary mouth (Cabarita Beach), but the mean distance from the mouth was 7.5 ± 1.8 SEM based on 8 total detections. In contrast, the Goldspot Mullet *Gracilimugil argentea*, an endemic fish species well adapted to estuarine and even freshwater habitat as juveniles, and one that spawns in the open ocean, was detected at 24 of 34 (71%) of the sampling locations, with a mean distance from the estuary mouth of 10.9 ± 1.3 SEM. In fact, of the 13 fish detected at more locations than *S. lalandi* and identified to the species level (range = 9 to 31 detections), only one other species was considered pelagic with pronounced offshore migrations (Tailor *Pomatomus saltatrix*, $N = 15$ detections), though juvenile *P. saltatrix* are known to inhabit estuaries (Schilling et al., 2018). An inability to discriminate between DNA material shed from adults, juveniles, transitional phases, or larvae across all contexts remains a shortcoming of the ToL-metabarcoding approach.

4.1. ToL-metabarcoding for biotic benchmarking

Advantages of the ToL-metabarcoding approach is that it is non-destructive in comparison to some traditional sampling techniques (e.g., fishing nets, traps, plankton tows), and it does not require as much taxonomic expertise as traditional studies (Thomsen and Willerslev, 2015; Deiner et al., 2017), though the latter remains relevant for effective DNA sequence assignment and curation. It can also be cost-effective when compared to video-based survey methods (e.g., Baited Remote Underwater Videos or BRUVs) that require extensive manual post-processing (Connolly et al., 2021). Cost effectiveness represents an advantage in a research climate that is investing less in our taxonomic capabilities (Engel et al., 2021). A further advantage of ToL-metabarcoding in urbanized environments is that it expands the utility and scope of biological indicators, traditionally a combination of biotic and abiotic metrics used to identify and, in some cases, predict

negative or positive changes to ecosystems (Holt and Miller, 2011). This is often manifested as microbial indicators (though these are usually expressed as bacterial counts or relative read abundance), algal indicators, planktonic indicators, and macroinvertebrate indicators, which often include insects (Hosmani, 2013; Jeffries et al., 2016; Sutcliffe et al., 2019; Pajunen et al., 2020; Shackleton et al., 2021). No single species could possibly be diagnostic of the effects of anthropogenic pressures across an entire ecosystem however, and so novel methods, like ToL-metabarcoding, capable of rapidly detecting biota across the tree of life, need to be developed further in this capacity (Wilkinson et al., 2024).

Some species may respond rapidly to a particular disturbance (e.g., land reclamation or a pollutant), but perhaps not to another disturbance, or at least not to the same degree. This lack of sensitivity is particularly true for low-level, chronic stressors when compared to extreme and/or acute stressors. To support this idea, no single taxon explained more than 0.62% of the variation in biological community as a function of geomorphic zone in our study, a proxy for different habitat types. It was therefore the presence/absence of biota in their entirety that provided the most information, not a particular species. Based on this, we recommend a shift from established biological indicator species with rapid life cycles to developing "biotic signatures" across the tree of life in urbanized estuaries using ToL-metabarcoding, though this will require further refinement (e.g., Schulte et al., 2023). This does not replace the biological indicator concept but simply provides a means to upscale the data available for interpretation, increase the speed at which it can be generated, and decrease the cost of doing so.

Another advantage to the ToL-metabarcoding approach is that it allows the detection of invasive species, often representing threats to human health, with or without targeting these species *a priori*. For example, in Australia, there are four toxic marine dinoflagellates designated as pests that can survive as cysts in the sediment, causing harmful algal blooms when conditions support their proliferation, and pose a threat to human health through the consumption of intoxicated shellfish (Hallegraeff, 2010). These toxic marine dinoflagellate species include *Alexandrium catenella*, *A. minutum*, *A. tamarense*, and *Gymnodinium catenatum* (Furlani, 1996), which are all included on the Australian Priority Marine Pest List (https://www.marinepests.gov.au/pests/identify). Though not classified to species, *Alexandrium* sp. was detected at Camp Cove and Fairlight Rocks in our study, two beaches popular with ocean swimmers and SCUBA divers, and both in the FTD. This genus was also detected at CMB sites: Taronga Zoo Beach in Mosman, Pirrama Park in Pyrmont, and Kirribilli in North Sydney. Similarly, although not identified to species or even genus in our study, we had detections of ASVs assigned to the Gymnodiniaceae family or the Gymnodiniales order at multiple locations in Sydney Harbour, higher taxonomic divisions where *Gymnodinium catenatum* would be nested.

Our panel of 16 genetic metabarcoding assays did include those optimized to detect the following animals or plants from the Australian Priority Marine Pest List: Chinese Mitten Crab *Eriocheir sinensis* (CE assay), Asian Paddle Crab *Charybdis japonica* (CK assay), Asian Basket Clam *Potamocorbula amurensis* (CP assay), and *Caulerpa* spp. (TC assay). Of these four, only the non-toxic *Caulerpa taxifolia* was detected within the FTD exclusively (Manly Cove), which historically represents the most significant population of this invasive algal species in Sydney Harbour (Creese et al., 2004). Although this species is native to northern Australia, its extension to temperate regions brings the risk of it outcompeting native species, degrading habitat, and impacting swimming nets and anchorages (Creese et al., 2004).

4.2. Methodological caveats

Despite the apparent utility of ToL-metabarcoding to broad applications in urbanized estuaries, including biotic benchmarking and biosecurity, there are some limitations to this approach and how it was applied in our study. For example, given that our study aimed to provide a snapshot of an urban ecosystem and parse biological communities among recognised geomorphic zones, single replicates were collected at 34 locations that spanned these habitat divisions. With more time and financial resources, we instead would have doubled or tripled our sampling efforts at each of these sites to address potential biases introduced by the stochasticity of sampling and filtering a finite volume of seawater. To this end, collecting more replicates at fewer locations in Sydney Harbour is the focus of future works aimed at quantifying eDNA sampling bias and how it affects the power to detect temporal shifts in biological communities revealed by ToL-metabarcoding.

Tide and moon phases can influence the origin of DNA particles and the amount of DNA shed into the water column (Harrison et al., 2019), particularly when fish (Tillotson et al., 2018; Wu et al., 2022) and macroinvertebrate spawning is triggered (Ip et al., 2023). Previous work in much larger Ramsar protected wetlands (i.e., Rio Cruces Wetland, Chile, a non-urbanized estuary) however, showed that the distribution of eDNA from different animal species was spatially structured despite a more complex hydrodynamic system and greater tidal range than Sydney Harbour (Saenz-Agudelo et al., 2022). Another study conducted in a nearshore marine habitat (Juneau, Alaska, USA) subject to large tides, strong currents, and significant freshwater input found that these hydrological influences on eDNA detection of fish communities were minimal (Larson et al., 2022). Finally, Jeunen et al. (2019) and Kelly et al. (2018) inferred localized signals of nearshore eukaryotic diversity based on eDNA sampling in temperate marine habitats (Otago Harbour, New Zealand and Hood Canal, Washington, USA, respectively) even though sample sites were subject to significant tides and directional water flow. A lack of tidal influence on eDNA signatures and their association with distinct habitat types appears to be a consistent result from studies conducted in tropical marine systems (Palmyra Atoll, USA; Lafferty et al., 2021). This consistency of eDNA signatures when subjected to variable tides or currents may reflect the rapid rates of DNA degradation suspended in estuarine environments (Harrison et al., 2019).

We did not explicitly account for the potential effects of tide and moon phase in our analyses because there was insufficient power in the statistical design to do so while still providing a sufficient test for the effect of geomorphic zones. Importantly, sampling of eDNA was conducted across a range of tide and moon phases, with no pattern across geomorphic zones that could potentially bias comparisons among them. As such, the significant results obtained are highly unlikely to be the result of potential biases introduced by tide or moon effects. However, the increased variance in eDNA introduced by sampling at different tide and moon phases may have reduced our ability to detect differences among some geomorphic zones. Increased replication in future studies may allow these potential effects, and others, to be appropriately accounted for within statistical models, or least be overcome for the purposes of spatial comparison.

We mitigated stochasticity associated with filtration volume (1 L) and filter pore size (1.2 μm) by standardizing these across all samples, bar four samples where only half the volume could be filtered due to turbid conditions, though 500 mL has proven to be sufficient for consistency in eDNA detections within turbid inshore estuaries (Kumar et al., 2022). A filtration volume of 1 L also nests well within the range used by most nearshore and coastal aquatic eDNA studies aimed at detecting bacteria, phytoplankton, macroinvertebrates, and fish (but not protists; Bunholi et al., 2023). We additionally mitigated stochasticity in the PCR process by running metabarcoding duplicates and pooling them for sequencing, which increased our likelihood of detecting low abundance taxa (Shirazi et al., 2021), though no single taxon greatly contributed to our statistical inferences and how the abundance of different species might influence these results should be considered in future studies. It should be noted that the four sites where only 500 mL of water were processed were not biased towards any one geomorphic zone (Central Mud Basin, $N = 2$; Fluvial Delta, $N = 1$; Riverine Channel, $N = 1$), had greater (Rozelle Bay) or equivalent levels of estimated

richness (i.e., between 0 and 2 standard deviations lower than the mean; Echo Point Park, Haslams Creek Junction, Willoughby Bay) across the taxonomic subgroups, and clustered with samples that achieved the standardized volume in ordination space (see Fig. 4a).

Biases associated with water sampling, filtration, and particularly PCR may be magnified by using many metabarcoding assays in ToL-metabarcoding (Stat et al., 2017; Macé et al., 2024) versus fewer metabarcoding assays characteristic of other approaches (e.g., West et al., 2020; Saenz-Agudelo et al., 2022). That said, 6.8% of our ASVs were detected by multiple genetic assays (280 ASVs in two assays, 51 ASVs in three assays, 21 ASVs in four assays, 10 ASVs in five assays, 3 ASVs in six assays, 1 ASV in seven assays, 1 ASV in eight assays, and 1 ASV in nine assays) across all taxonomic groups except fungi, mites and ticks, mosses, oomycetes, and springtails. This represents a "balancing advantage" when using multiple metabarcoding assays, which in our study increased confidence in the detection and assignment of taxa, consistent with findings in other systems (Jeunen et al., 2019; Alexander et al., 2020; McElroy et al., 2020; Berry et al., 2023; Stepien et al., 2023). In our case, the use of four assays capable of independently amplifying fish DNA for example, including one assay modified to additionally detect fishes from the Syngnathidae family, increased the confidence and resolution of our taxonomic assignments (or lack thereof) for that subgroup.

The obvious trade-off with multiplicity is that by maximizing taxonomic coverage, in this case by applying 16 separate metabarcoding assays, the associated costs of staff and reagents to optimize each of the assays will be accessible to only a small group of commercial laboratories. In our case, the ToL-metabarcoding panel was part of a standard package currently available with Wilderlab in New Zealand for $300 NZD per sample. This transferable service enables other researchers outside of our group to replicate our study in the same estuary, or to test new hypotheses in other estuaries with comparable sampling effort. Moreover, as sequencing technologies improve, become more portable, and the costs associated with using them fall, primers than can recover longer eDNA fragments (i.e., entire genes or mitochondrial genomes) might soon replace the need for the optimization of independent metabarcoding assays as we have done here (Krehenwinkel et al., 2019). That said, long-read metabarcoding is still in the early stages of development (Egeter et al., 2022; but see Buetas et al., 2024 for microbiome applications), it is associated with higher error rates of sequencing (Krehenwinkel et al., 2019), it requires more complex restriction digests or ligation laboratory workflows (Krehenwinkel et al., 2019), and it may be limited by the fragmented and degraded state of DNA isolated from our aquatic environments (Jo, 2023)

The final limitation of our approach was primer bias as well as equivocal taxonomic assignment related to putative gaps in DNA reference databases (Schenekar et al., 2020; Bunholi et al., 2023; Stepien et al., 2023). The first issue of primer bias is mitigated by our use of multiple metabarcoding assays versus a single assay as described above, but this remains an area of active improvement. The disproportionate matching of DNA sequences to a DNA reference database on the other hand is of particular concern for regional studies or under-sampled taxonomic groups (Marques et al., 2021; Blackman et al., 2023; Bunholi et al., 2023), both of which would apply to the flora and fauna of Sydney Harbour. Interrogation of our taxonomic assignments indicates that 53.4% (597 of 1118 taxa) were not previously recorded from Sydney Harbour. For one of the most taxonomically resolved groups, we note that of the 28 fish taxa not previously recorded from Sydney Harbour, 20 of the names were not recognised due to taxonomic uncertainty in classification between WORMs and ALA, 5 were known only from outside of Australia (e.g., New Zealand, Northwest Pacific Ocean, Southwest Atlantic Ocean), and 3 were known from Australia but not included on the most recent checklist of Sydney Harbour fishes (DiBattista et al., 2022). The three fish species absent from the existing checklist included Pink Ling (*Genypterus blacodes*, detected only at Wentworth Point in the RC), Orange Roughy (*Hoplostethus atlanticus,* detected only at Pirrama Park in the CMB), and a lanternfish (*Lampanyctus* sp., detected only at Manly Cove in the FTD), which may represent incursions of free-floating DNA material from outside of the harbour, fish processing at the fish market in the CMB, or fish waste from human consumption along the shoreline. One other unexpected detection worth highlighting, which may not represent taxonomic misassignment, is the detection of a California Sealion (*Zalophus californianus* and *Zalophus* sp.) from a single sampling site in front of Taronga Zoo Beach in the CMB. Indeed, this site is a known discharge point for UV sterilized water from their aquaria that houses two California Sealions.

The task of assigning recognised names to ASVs extracted from environmental samples remains a challenge due to: (1) limited knowledge of the genetic diversity of the targeted amplicon across the tree of life and the gaps in DNA sequence databases (Kvist, 2013) and (2) poor taxonomic resolution associated with short amplicons characteristic of eDNA assays (Waugh, 2007). Taxonomic-independent tests are viable alternatives to estimate composition and richness based on DNA sequence diversity to explore patterns in space (as we have done here) and time, provided the sequences are not artifacts of the laboratory workflow. Extensive curation of taxonomic assignments based on their ecological affinities and records from regional biodiversity repositories however unlocks the full potential of biotic benchmarking.

5. Conclusions

Globally, urbanization is putting estuarine species at risk and so new approaches to effectively monitor, manage, and maintain their habitats are critical. Our study has rapidly broadened knowledge of marine biodiversity and community composition in Sydney Harbour and across a broader spectrum of taxa, relative to more traditional collection methods that have slowly accumulated biodiversity detections over many decades (Hutchings et al., 2013; DiBattista et al., 2022). We suggest that ToL-metabarcoding data can serve as a benchmark for biological monitoring, particularly if the data are extensively curated, the records (alongside comprehensive metadata) are deposited in public databases, and the samples are archived in perpetuity and can be re-examined (Jarman et al., 2018; Berry et al., 2021), recognizing that the cost and effort required to optimize this approach may be prohibitive for some laboratories and study systems. At present, ToL-metabarcoding, as applied here, enables relative comparisons between samples, but not necessarily comparisons between other approaches that apply different genetic assays or divergent field methodologies.

To make this biotic benchmark most effective, we recommend that future research should target the separation of natural variability in heterogenous environments from that due to anthropogenic stressors. As complementary technologies develop, particularly in the field of artificial intelligence, ToL-metabarcoding can be further improved as these data are incorporated into biotic indices that reflect ecosystem health (Cordier et al., 2017; Wilkinson et al., 2024). Similarly, ToL-metabarcoding built on multiple genetic assays can be expanded to alternative substrata (i.e., sediment) in estuaries to further develop predictive biotic signatures of disturbance (Chariton et al., 2010, 2015) as library preparation practices and sequencing technologies improve.

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CRediT authorship contribution statement

Joseph D. DiBattista: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ashley M. Fowler:**

Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Tanika C. Shalders:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Robert J. Williams:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shaun Wilkinson:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Joseph DiBattista reports financial support was provided by Australian Museum. Joseph DiBattista reports a relationship with Australian Museum that includes: employment. Shaun P. Wilkinson is a director and shareholder of Wilderlab NZ Ltd., a commercial eDNA processing laboratory. All other co-authors declare no known competing financial interests or personal relationships that influenced the work reported in this paper. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All raw data needed to replicate the study and statistical analyses are available as supplementary material. Raw sequence data are also available as .fastq files at NCBI BioProject PRJNA1107684.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2024.119454.

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