



# Environmental DNA metabarcoding reveals estuarine benthic community response to nutrient enrichment – Evidence from an *in-situ* experiment<sup>☆</sup>

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## ABSTRACT

Nutrient loading is a major threat to estuaries and coastal environments worldwide, therefore, it is critical that we have good monitoring tools to detect early signs of degradation in these ecologically important and vulnerable ecosystems. Traditionally, bottom-dwelling macroinvertebrates have been used for ecological health assessment but recent advances in environmental genomics mean we can now characterize less visible forms of biodiversity, offering a more holistic view of the ecosystem and potentially providing early warning signals of disturbance. We carried out a manipulative nutrient enrichment experiment (0, 150 and 600 g N fertilizer m<sup>-2</sup>) in two estuaries in New Zealand to assess the effects of nutrient loading on benthic communities. After seven months of enrichment, environmental DNA (eDNA) metabarcoding was used to examine the response of eukaryotic (18S rRNA), diatom only (rbcL) and bacterial (16S rRNA) communities. Multivariate analyses demonstrated changes in eukaryotic, diatom and bacterial communities in response to nutrient enrichment at both sites, despite differing environmental conditions. These patterns aligned with changes in macrofaunal communities identified using traditional morphological techniques, confirming concordance between disturbance indicators detected by eDNA and current monitoring approaches. Clear shifts in eukaryotic and bacterial indicator taxa were seen in response to nutrient loading while changes in diatom only communities were more subtle. Community changes were discernible between 0 and 150 g N m<sup>-2</sup> treatments, suggesting that estuary health assessment tools could be developed to detect early signs of degradation. Increasing variation in community structure associated with nutrient loading could also be used as an indicator of stress or approaching tipping points. This work represents a first step towards the development of molecular-based estuary monitoring tools, which could provide a more holistic and standardized approach to ecosystem health assessment with faster turn-around times and lower costs.

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## 1. Introduction

Cumulative impacts from anthropogenic activities occurring on land and in the ocean are resulting in a global loss of biodiversity, ecosystem resilience and the ecosystem services upon which people rely (Barbier et al., 2011; IPBES, 2019; Lotze et al., 2006; Worm

et al., 2006). Due to their proximity to multiple human pressures, coastal zones are among the most impacted parts of the ocean (Agardy et al., 2005). It is critical that we have good monitoring tools to detect degradation in these ecologically important and vulnerable ecosystems before a tipping point is reached. In an attempt to halt degradation of our coastal and marine environments, several national and regional initiatives have been developed (e.g. Australia's Oceans Policy, Canada's Oceans Act and Oceans Strategy, the USA's Oceans Act and Europe's Water Framework Directive and Marine Strategy Framework Directive, and South Africa's National Water Act; Borja et al., 2008). These policies generally require an assessment of ecological integrity or status

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carried out at the ecosystem level, rather than relying on single species or physical-chemical parameters alone.

Bottom-dwelling macroinvertebrate communities have long been used for ecological health assessment because they respond relatively rapidly to stress and integrate the effects of multiple stressors over time. These attributes arise because macroinvertebrate communities are diverse, span multiple trophic levels, are predominantly sedentary as adults and have species specific sensitivities to stressors (Borja et al., 2000; Dauer, 1993; Gray et al., 1979; Pearson and Rosenberg, 1978). While macrofaunal communities are a valuable indicator of ecosystem health, traditional visual morphological identification of these animals is time consuming, relatively expensive and requires taxonomic expertise that is in decline worldwide (Jones, 2008; Keeley et al., 2018). In addition, inferring ecosystem health solely from the larger, visible portion of communities neglects the contribution of meio- and microbial taxa (e.g. bacteria, protists, microalgae, nematodes), which have been shown to be extremely diverse and often more responsive to environmental change (Bianchelli et al., 2016; Eiler et al., 2013; Kemp and Aller, 2004; Kennedy and Jacoby, 1999; Li et al., 2018). Communities of bacteria, microalgae, micro- and meio-eukaryotes play an essential role in ecosystem structure and functioning (e.g. carbon and nitrogen cycling, energy transfer to higher trophic levels, sediment stabilization; Azam and Malfatti, 2007; Schratzberger, 2018; Tolhurst et al., 2008). Inclusion of these frequently overlooked communities could offer a more comprehensive view of the ecosystem, in keeping with requirements for integrated assessments of health, provide early warning signals of disturbance (because of their higher turnover) and help us to better understand connections between biodiversity and ecosystem functioning (Strong et al., 2015).

Recent advances in environmental genomics and the emergence of high-throughput sequencing (HTS) technologies are changing our ability to evaluate community composition, including characterization of invisible biodiversity. Using a technique known as environmental DNA (eDNA) metabarcoding, species diversity can be assessed at low taxonomic resolution from genetic fragments contained in small amounts of sediment (Baird and Hajibabaei, 2012; Bourlat et al., 2013; Pawlowski et al., 2018). Organisms are identified without taxonomic expertise by matching short, HTS-derived gene fragments to a reference sequence library. Although eDNA metabarcoding is rapidly expanding as a new approach to biodiversity assessment and biomonitoring, much of our understanding of the suitability of eDNA metabarcoding for environmental monitoring has relied on correlative studies (e.g. Aylagas et al., 2017; Keeley et al., 2018; Laroche et al., 2018; Montenegro et al., 2020) or experimental research conducted in laboratory settings (e.g. Chariton et al., 2014; Santi et al., 2019). Manipulative field studies are rarer (although see Birrer et al., 2019; Lawes et al., 2017) but are required to prove cause and effect, characterize the response of specific taxonomic groups to selected stressors, identify potential indicator taxa for ecological status assessment and demonstrate that these effects can be consistently detected over and above natural environmental variability. Estuaries present a particular challenge to using eDNA metabarcoding due to high environmental variability and lack of genomic studies in these habitats (Ruppert et al., 2019).

In this study, we carried out a manipulative field experiment and used eDNA metabarcoding to characterize changes in benthic community structure in response to nutrient enrichment in two estuaries. Nutrient loading is a major threat to estuaries worldwide (CENR, 2000; NRC, 2000), with two-thirds of estuaries in the US assessed to have moderate-high levels of eutrophication (Bricker et al., 2008) and one-third of European estuaries affected by nutrient enrichment (EEA, 2012). Using eDNA metabarcoding, we

characterized how eukaryotic, diatom and bacterial community structure changed in response to nutrient loading to explore whether these communities could be a sensitive indicator of nutrient enrichment. Our experiment extends previous empirical research on the response of eDNA-derived estuarine communities to sediment nutrient enrichment (Birrer et al., 2018, 2019) by moving into a new habitat (intertidal sandflats), exploring the response of diatom communities in more detail and broadening the scope of the research to test these responses under differing natural conditions. To our knowledge, this study is the first field experiment providing empirical evidence that eDNA metabarcoding can detect responses to nutrient enrichment across different trophic levels of intertidal benthic biodiversity (bacteria and eukaryotes, including diatoms) in two environmentally distinct estuarine systems and is thus an important contribution toward the development of molecular tools for ecosystem health assessment.

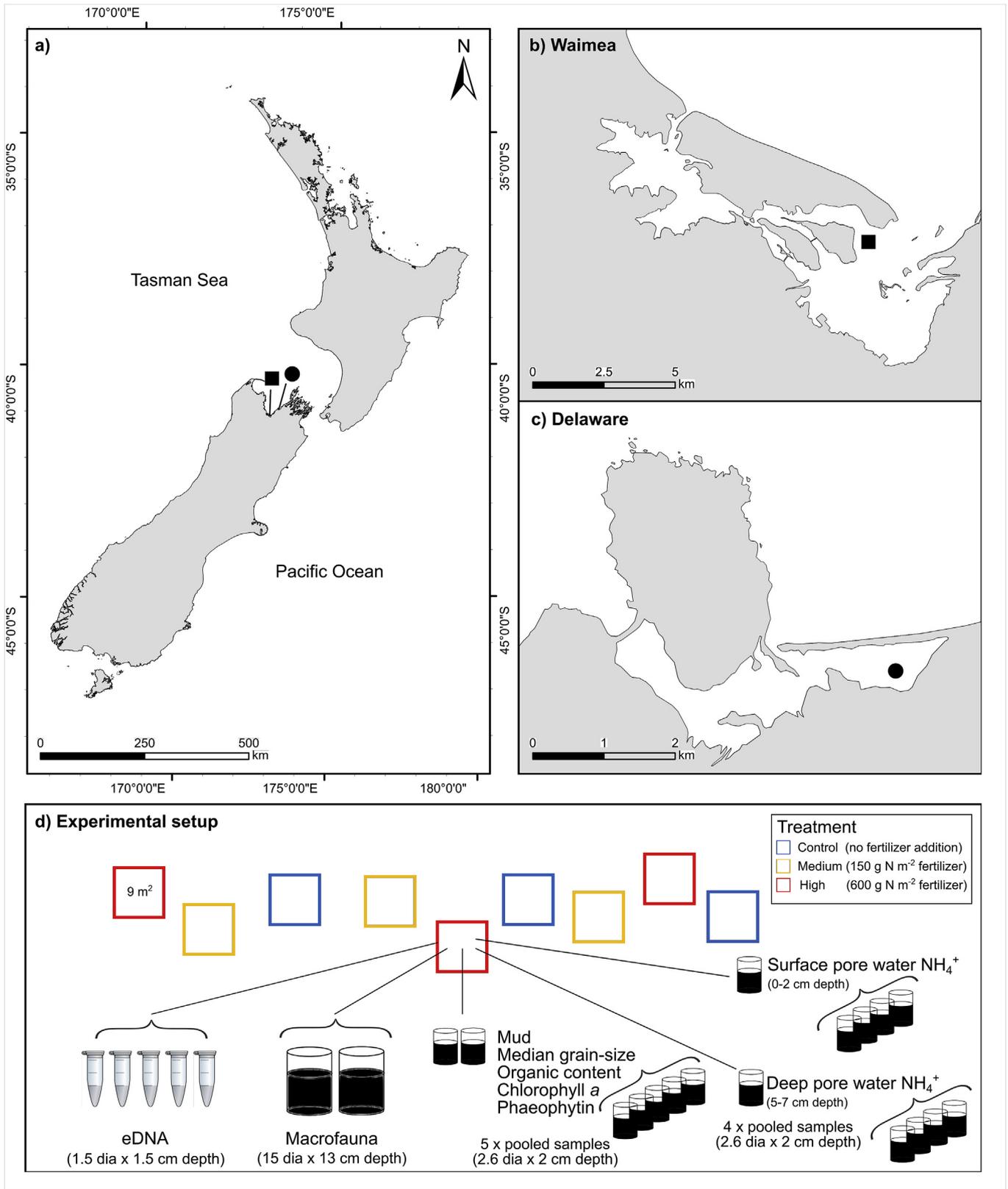
## 2. Material and methods

### 2.1. Field experiment

In April 2017, manipulative nutrient enrichment experiments were set up on unvegetated mid-tide sandflats in two estuaries located 25 km apart near Nelson, New Zealand (Fig. 1). The site in Waimea Estuary (173° 11' 06.59 E, 41° 17' 33.36 S) was located close to the estuary mouth on exposed sandflats while the site in Delaware Inlet (173° 27' 39.16 E, 41° 09' 50.42 S) was positioned in a more sheltered area of the estuary. Catchments of both estuaries were dominated by native and exotic forest but modelled mean annual nitrate concentrations (Plew et al., 2015) were higher in Waimea (49.7 mg m<sup>-3</sup>) than Delaware (27.7 mg m<sup>-3</sup>), likely a result of the larger catchment size of Waimea (903 km<sup>2</sup> vs 78 km<sup>2</sup>) and slightly more intensive land use (more horticulture).

At each site, nine treatment plots arranged parallel to the incoming tide were interspersed across the sandflat, at least 3 m apart (Fig. 1). Plots were set up by measuring a 3 × 3 m area on the surface of the sandflat and marking the corners of the plots with stakes. Plots were exposed to the elements and no attempt was made to control organism movement (e.g. with fences or cages), replicating natural conditions where organisms could respond to nutrient treatments depending on their preference and motility. The plots were randomly assigned a nutrient treatment: control (0 g N m<sup>-2</sup>), medium (150 g N m<sup>-2</sup> fertilizer) and high (600 g N m<sup>-2</sup> fertilizer) enrichment ( $n = 3$  plots per treatment). To simulate nutrient loading, we used Nutricote® slow release nitrogen (urea) fertilizer (140–200 d, 40-0-0 N:P:K) injected uniformly into the sediment at a depth of 15 cm following established methods (Douglas et al., 2016). Fertilizer granules were added to the plots by removing a sediment core (3 cm diameter x 15 cm depth), adding the fertilizer and immediately replacing the plug to maintain sediment structure. Cores were evenly spaced (20 cores m<sup>-2</sup>), with more granules added per core to achieve higher nutrient loading. This technique has been demonstrated to elevate surface (0–7 cm) sediment pore water NH<sub>4</sub><sup>+</sup> concentrations equivalent to those measured in enriched estuaries globally, with enrichment effects undetectable 0.5 m beyond the plot boundary (Douglas et al., 2016, 2017; Gladstone-Gallagher et al., 2020; Thrush et al., 2017).

The plots were left undisturbed for the next seven months and sampled in November 2017. From each plot we collected two sediment samples (each consisting of five 2.6 cm diameter x 0–2 cm depth samples pooled) for grain-size, organic content, chlorophyll *a* and phaeophytin analyses, one sediment sample for pore water NH<sub>4</sub><sup>+</sup> analyses (four, 2.6 cm diameter, split into 0–2 cm and 5–7 cm depth sections and pooled) and two cores (13 cm diameter x 15 cm depth) for macrofauna community composition



**Fig. 1.** a) Map of New Zealand showing the location of Waimea Estuary (square) and Delaware Inlet (circle), b) & c) location of the study site within each estuary, d) experimental layout showing samples collected from each of the plots. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 1). Chlorophyll *a* and phaeophytin concentrations were measured as a proxy for microphytobenthic biomass. Five sediment samples (1.5 cm diameter x 1.5 cm depth) were also randomly collected from each plot for eDNA metabarcoding using separate pairs of gloves and sterilized sampling vials. Field negatives for eDNA analysis were collected and consisted of three empty sampling vials handled in the same way as samples but not filled with sediment. Macrofaunal samples were sieved to 500  $\mu\text{m}$ , preserved in 70% isopropyl alcohol and later counted and identified to the lowest possible taxonomic resolution (70% to species level). All other samples were kept in the dark, transported on ice to the laboratory and frozen ( $-20^\circ\text{C}$ ) until further processing, except for pore water, which was extracted immediately.

## 2.2. Analysis of environmental variables

Sediment grain-size was measured, after digestion in 10% hydrogen peroxide, on a Malvern Mastersizer 3000 (particle size range 0.01–3500  $\mu\text{m}$ ; Singer et al., 1988). Organic content was determined by drying sediment to a constant weight ( $60^\circ\text{C}$ ) and measuring weight loss on ignition of dry sediments ( $550^\circ\text{C}$  for 4 h; Parker, 1983). Chlorophyll *a* and phaeophytin were extracted from sediment in 90% buffered acetone and measured fluorometrically before and after acidification (Arar and Collins, 1997). Pore water was extracted by centrifugation, filtered (1.1  $\mu\text{m}$  Whatman GC glass fibre filter), frozen ( $-20^\circ\text{C}$ ) and analysed for  $\text{NH}_4^+$  following the methods in Douglas et al. (2016).

## 2.3. Environmental DNA extraction, amplification, sequencing, and bioinformatics

Each step of the molecular processing (i.e. DNA extraction, polymerase chain reaction (PCR) and metabarcoding library preparation) was carried out in a separate sterile laboratory dedicated to that step with sequential workflow to ensure no cross-contamination. Each laboratory was treated with ultra-violet light for at least 15 min before use and all working surfaces wiped with 5% bleach. The PCR set-up and template addition were undertaken in laminar flow cabinets. Filter pipet tips (Axygen® and Thermo Fisher Scientific) were used throughout and gloves changed frequently.

Environmental DNA sediment samples were homogenized via bead beating (MiniG™ 1600) for 2 min. DNA was then extracted from 2 g of sediment using the Qiagen DNeasy PowerSoil Kit, following the manufacturer's protocol. The field negative controls were processed the same way and extraction controls were added at the start of each new DNeasy PowerSoil Kit ( $n = 2$ ). The quantity and quality of extracted DNA were measured using a Nano-Photometer (Implen). All extract products were stored frozen ( $-20^\circ\text{C}$ ) until further analysis.

Three gene markers were chosen to represent communities that were expected to respond to nutrient enrichment, either directly or indirectly (i.e. through changes in the macrofaunal communities that consume them). Bacterial communities were represented by short ca. 80–450 base-pair (bp) fragments of the nuclear 16S rRNA gene (V3-4 region), eukaryotic communities (including diatoms) were represented by the nuclear 18S rRNA gene (V4 region) and diatom communities were further investigated using the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcl) gene (Stoof-Leichsenring et al., 2012), which provides highly resolved, complementary information to 18S (Kerमारrec et al., 2013; Visco et al., 2015; Supplementary Table S1). In this study, diatom communities assessed using the rbcl gene will be referred to separately from the eukaryotic communities assessed using the 18S gene, despite eukaryotic communities also containing diatom taxa.

Separate PCR analyses were performed on each eDNA sample for each of the three gene markers. PCR amplifications were undertaken on an Eppendorf Mastercycler in a total reaction volume of 50  $\mu\text{l}$  using MyFi™ Mix (Bioline) according to the mastermix recipe and thermocycling conditions outlined in Supplementary Table S1. One sample containing nuclease-free water (Ambion®) in place of DNA template was used as a 'no-template' negative control. PCR products were visualized on 1.5% agarose gels stained with Red-Safe™ Nucleic Acid Staining Solution (Intron) to confirm the presence of 16S, 18S and rbcl fragments. Purification followed the Agencourt™ AMPureXP protocol (Beckman Coulter) using magnetic beads with products quantified using a Qubit™ 2.0 Fluorometer (Invitrogen). Purified amplicons were diluted to 3 ng  $\mu\text{l}^{-1}$  and sent to New Zealand Genomic Limited, University of Auckland, for library preparation following a two-step tailed PCR amplicon procedure using the Nextera XT kit and sequencing (Kozich et al., 2013). The final loading concentration of the library was 7 pM with a 15% PhiX spike and paired-end sequences ( $2 \times 250$  bp; MiSeq v2 reagents kit) were generated on a MiSeq instrument. Sequence data were automatically demultiplexed using MiSeq Reporter (v2). Raw sequence reads were deposited in the NCBI short read archive under the Project ID: PRJNA627491.

The same bioinformatics pipeline was applied for the bacterial, eukaryotic and diatom datasets, except where explicitly stated. Primers were removed using CUTADAPT (version 1.18; Martin, 2011) with a single mismatch allowed and reads were subsequently processed using the DADA2 package (version 1.16; Callahan et al., 2016) within R software (version 3.6.1). Briefly, quality control of the reads was undertaken by truncating the reads (bacteria and eukaryotes forward 230 bp, reverse 228 bp; diatoms forward and reverse 110 bp), trimmed based on quality and filtered with a maxEE (maximum number of 'expected errors' allowed) of 2 for all forward reads, 4 for bacterial reverse reads and 6 for eukaryotic and diatom reverse reads. Reads were discarded if they did not match these criteria. Sequence variants for the forward and reverse reads were inferred using pseudo-pooling based on derived error profiles (first  $10^8$  bp in the dataset) after sequence dereplications. Using a maximum mismatch of 1 bp and a required minimum overlap of 10 bp paired-end reads were merged, discarding any reads that did not merge correctly. Chimeras were removed using the consensus method in DADA2. The resulting chimera-checked, merged amplicon sequence variants (ASVs) were taxonomically assigned using the DADA2 method, based on the rdp classifier (Wang et al., 2007) with a confidence of 50, using three distinct sequencing referencing databases. For bacteria (16S), the SILVA version 132 database (Pruesse et al., 2007) was used as a reference. For eukaryotes (including diatoms, 18S), the Protist Ribosomal Reference (PR2) database (version 4.11.1; Guillou et al., 2012) was used. For diatoms (rbcl), the reference sequences were downloaded from the National Center of Biotechnology Information (NCBI; Sayers et al., 2018) and formatted for use with DADA2. The results were then parsed into a table using the phyloseq package.

Detected contamination was negligible in negative controls, with the total number of reads in each control <350, except for two negative controls which had 1050-2410 reads for three ASVs (a Oncholaimidae nematode and two *Cylindrotheca* diatoms). The number of reads for each ASV found in negative controls was subtracted across all other samples following the method described in Bell et al. (2018). Non-target taxa were also removed from the bacterial (eukaryotes, chloroplasts, and mitochondria) and eukaryotic (mammals and Actinopterygii) samples. We retained diatom taxa in the 18S eukaryotic community dataset. ASVs with a total of <0.005% reads across all samples were removed from the dataset. Rarefaction curves and the number of reads and ASVs remaining in each sample are presented in Fig. S1 and Tables S2a

and S2b of the Supplementary Material. As we were primarily interested in community structure, we retained samples with >5000 reads and converted the number of reads to proportional abundance for the downstream statistical analyses.

#### 2.4. Data analysis and statistics

Environmental data were averaged by plot and transformed, if necessary, to meet assumptions of normality (square-root was used for mud content; log was used for organic matter and porewater concentrations). Two-way analysis of variances (ANOVAs) with 'site' and 'treatment' as fixed factors were carried out in R (v 3.6.1) to test whether sediment properties, microphytobenthic biomass and pore water  $\text{NH}_4^+$  varied significantly between sites and treatments.

Multivariate analyses were used to investigate whether eukaryotic, diatom and bacterial community structure changed across nutrient enrichment treatments. Bray-Curtis dissimilarity matrices were calculated for each dataset using fourth root transformed eDNA proportional read abundance and the results were plotted using Principal Coordinates Analysis (PCO). The PCO revealed four outliers (two from the control treatment at Delaware, one from the control treatment at Waimea and one from the high treatment at Waimea) and further investigation showed these samples had low DNA concentrations, numbers of ASVs or numbers of reads. These samples were removed from all three eDNA community datasets (and subsequent analyses) and the PCO was re-run.

Two-way permutational ANOVAs (PERMANOVAs) with 'site' and 'treatment' as fixed factors were used to test whether eDNA-derived community structure varied with nutrient enrichment and whether this response varied with site. Permutations of residuals under a reduced model was used, with 9999 permutations, type III sum of squares and pairwise *post-hoc* tests to identify significant differences between treatments. Differences between treatments were visualized using Canonical Analysis of Principal Coordinates (CAP; Anderson and Willis, 2003), with 'treatment' as a factor and 9999 permutations. CAP allows a constrained ordination to be carried out based on any dissimilarity measure and determines the PCO axes that are best at discriminating among *a priori* groups. The appropriate number of axes ( $m$ ) used in each CAP model was chosen by the software, which maximizes a leave-one-out allocation success to groups (the proportion of samples allocated into their correct group using a leave-one-out procedure). Allocation success was also used as a measure of the sensitivity of each community at detecting nutrient enrichment effects. Allocation success was chosen in preference to canonical correlation for determining model performance because canonical correlation, and the separation between treatments on the CAP plots, increases as the number of axes in the model increases, even if the predictive capability of the underlying CAP model does not improve (Anderson et al., 2008). Based on the highly significant PERMANOVA site  $\times$  treatment interaction for each community, CAP was performed on each site separately. Tests of homogeneity of dispersions (PERMDISP), which were used to quantify the variability in community structure between treatments, were also performed for each site separately, using 'treatment' as a group factor, 9999 permutations and calculating distances to centroids. All multivariate statistical analyses were carried out using PRIMER 7 (v 7.0.13) with the PERMANOVA + add-on (Anderson et al., 2008; Clarke and Gorley, 2015).

At each site, eukaryotic (including diatoms), diatom and bacterial taxa indicative of each nutrient enrichment treatment were identified using the *indicspecies* R package (version 1.7.8; De Caceres, 2019), with Indicator Values (IndVal) measuring the strength of the association between a taxon and a treatment. ASVs

present in less than three samples were discarded, taxa were then aggregated to genus-level and *indicspecies* was carried on proportional abundance read data using *multipatt* function with 9999 permutations and a significance level of 0.05.

Fourth root transformed macrofaunal community abundance data were plotted using PCO and CAP to see if patterns observed using eDNA-derived communities generally aligned with those resulting from traditional monitoring techniques. Results from other analyses (i.e. PERMANOVA, CAP, PERMDISP, *indicspecies*) carried out on macrofaunal abundance data can be found in the Supplementary Material.

### 3. Results

The two-way ANOVAs showed that sediment properties varied between sites but not between treatments, with Delaware Inlet having a smaller median grain-size ( $df = 1$ ,  $F = 234.99$ ,  $p < 0.0001$ ) and higher proportion of mud (particles  $< 63 \mu\text{m}$ ;  $df = 1$ ,  $F = 184.5$ ,  $p < 0.0001$ ), organic content ( $df = 1$ ,  $F = 1048.0$ ,  $p < 0.0001$ ), chlorophyll *a* ( $df = 1$ ,  $F = 13.1$ ,  $p = 0.0035$ ) and phaeophytin ( $df = 1$ ,  $F = 45.5$ ,  $p < 0.0001$ ) content than Waimea (Table 1 and Supplementary Table S3). The nitrogen fertilizer addition increased surface ( $df = 2$ ,  $F = 25.6$ ,  $p < 0.0001$ ) and deep ( $df = 2$ ,  $F = 58.0$ ,  $p < 0.0001$ ) pore water  $\text{NH}_4^+$  concentrations in the medium and high treatments and this did not vary with site (Table 1 and Supplementary Table S3). Pore water  $\text{NH}_4^+$  concentrations in the medium treatment were 5–76 times greater than controls while concentrations in the high treatment were 118–760 times higher than controls (Table 1).

For all four communities (eukaryotes, diatoms, bacteria and macrofauna), the greatest variance in community structure was between sites rather than across treatments, with 30–75% of the total variance explained along the first PCO axis that separated the two sites (Fig. 2). PERMANOVA tests showed there was a highly significant site  $\times$  treatment interaction for each community type (eukaryotes  $df = 2$ , pseudo- $F = 2.38$ ,  $p = 0.0001$ ; diatoms  $df = 2$ , pseudo- $F = 2.40$ ,  $p = 0.0001$ ; bacteria  $df = 2$ , pseudo- $F = 2.30$ ,  $p = 0.0001$ ; macrofauna  $df = 2$ , pseudo- $F = 2.07$ ,  $p = 0.0040$ ), which meant the response to nutrient addition varied with site (Table S4). Within-site *post-hoc* testing showed that there were significant differences ( $p < 0.05$ ) in eukaryotic, diatom and bacterial community structure between all treatments, with the exception of eukaryotic communities in the medium and high treatments at Waimea ( $t = 1.2$ ,  $p = 0.0684$ ) and bacterial communities in the control and medium treatments at Delaware ( $t = 1.1$ ,  $p = 0.1600$ ; Supplementary Table S4).

The CAP ordinations (Fig. 2) provide a visual representation of site-specific differences in community structure among nutrient enrichment treatments. Correlations from CAP plots based on eukaryotic, diatom and bacterial communities offer strong support for significant differences in community structure between treatments (canonical correlation = 0.73–0.99,  $p = 0.0001$ –0.0315; Table 2). CAP models derived from these communities were able to correctly allocate observations into the appropriate nutrient enrichment treatment 61–83% of the time, which is considerably better than the 33% success expected by chance if samples were randomly allocated into three groups. Models derived from diatom and bacterial communities performed best at Waimea (83% and 81% allocation success, respectively) followed by eukaryotic communities (71% allocation success). At Delaware, models based on eukaryotic communities performed the best (81% allocation success) followed by diatoms and bacteria (67% and 61% allocation success, respectively). The poorer performance of CAP models for eukaryotic communities at Waimea and diatom and bacterial communities at Delaware agree with the *post-hoc* PERMANOVA test

**Table 1**  
Sediment properties (average  $\pm$  1 standard deviation,  $n = 6$  except for pore water where  $n = 3$ ) in experimental plots at Waimea and Delaware seven months after addition of slow-release nitrogen fertilizer (control:  $0 \text{ g N m}^{-2}$ ; medium:  $150 \text{ g N m}^{-2}$ ; high  $600 \text{ g N m}^{-2}$ ). Full statistical results are presented in Table S3 of the Supplementary Material.

Variable	Waimea			Delaware		
	Control	Medium	High	Control	Medium	High
<b>Sediment properties</b>						
Mud ( $\% < 63 \mu\text{m}$ ) <sup>a</sup>	0.4 (0.7)	0.9 (0.5)	1.4 (0.9)	16.1 (4.7)	14.4 (2.4)	17.4 (5.2)
Median grain-size ( $\mu\text{m}$ ) <sup>a</sup>	151.0 (1.7)	151.0 (1.6)	149.0 (2.3)	103.0 (8.8)	106.0 (5.9)	103.0 (9.3)
Organic content ( $\%$ ) <sup>a</sup>	1.3 (0.0)	1.3 (0.1)	1.3 (0.1)	2.7 (0.3)	2.7 (0.2)	2.8 (0.3)
Microphytobenthic biomass ( $\mu\text{g g}^{-1}$ sediment)						
Chlorophyll <i>a</i> <sup>a</sup>	2.7 (0.6)	3.7 (0.9)	2.5 (0.4)	4.2 (0.6)	4.3 (0.5)	4.4 (1.2)
Phaeophytin <sup>a</sup>	1.2 (0.2)	1.9 (1.0)	1.7 (0.2)	3.4 (0.8)	3.1 (0.3)	3.7 (0.6)
Pore water $\text{NH}_4^+$ ( $\mu\text{mol N L}^{-1}$ )						
Surface sediments (0–2 cm) <sup>b</sup>	29 (28)	2190 (2439)	15,500 (14,240)	68.0 (16)	351 (188)	7980 (7104)
Deeper sediments (5–7 cm) <sup>b</sup>	40 (14)	2900 (3816)	28,500 (19,918)	114 (44)	2080 (1137)	32,500 (21,982)

<sup>a</sup> Differed significantly ( $p < 0.004$ ) between sites (see Table S3).

<sup>b</sup> Differed significantly ( $p < 0.02$ ) between treatments (see Table S3).

results described above and the marginally significant differences between bacterial communities in the medium and high treatments at Delaware ( $t = 1.1$ ,  $p = 0.0448$ ; Supplementary Table S4). For example, the PERMANOVA test showed no significant difference between bacterial communities in the control and medium treatments at Delaware ( $t = 1.1$ ,  $p = 0.1600$ ) and the CAP model was only able to correctly allocate samples into the control treatment 46% of the time. CAP ordinations based on macrofaunal communities showed the same patterns as those observed from ordinations of eDNA-derived communities (Fig. 2).

PERMDISP results showed that at Delaware, variability in bacterial and diatom community structure was similar across all treatments (bacteria  $df = 2$ ,  $F = 1.07$ ,  $p = 0.4388$ ; diatom  $df = 2$ ,  $F = 1.86$ ,  $p = 0.1968$ , respectively) but greater variation in eukaryotic community structure was observed in the high treatment compared to the control ( $t = 3.0$ ,  $p = 0.0085$ ) and medium ( $t = 2.6$ ,  $p = 0.0193$ ) treatments, with the average Bray-Curtis distance-to-centroid 3% greater in the high treatments (Supplementary Table S6). At Waimea, the high nutrient treatment was associated with greater variation in community structure (eukaryotes  $df = 2$ ,  $F = 23.69$ ,  $p = 0.0001$ ; diatoms  $df = 2$ ,  $F = 20.54$ ,  $p = 0.0001$ ; bacteria  $df = 2$ ,  $F = 13.62$ ,  $p = 0.0001$ ) across all three eDNA-derived communities, with the average Bray-Curtis distance-to-centroid 8–16% greater than the controls and 3–8% greater than the medium treatment.

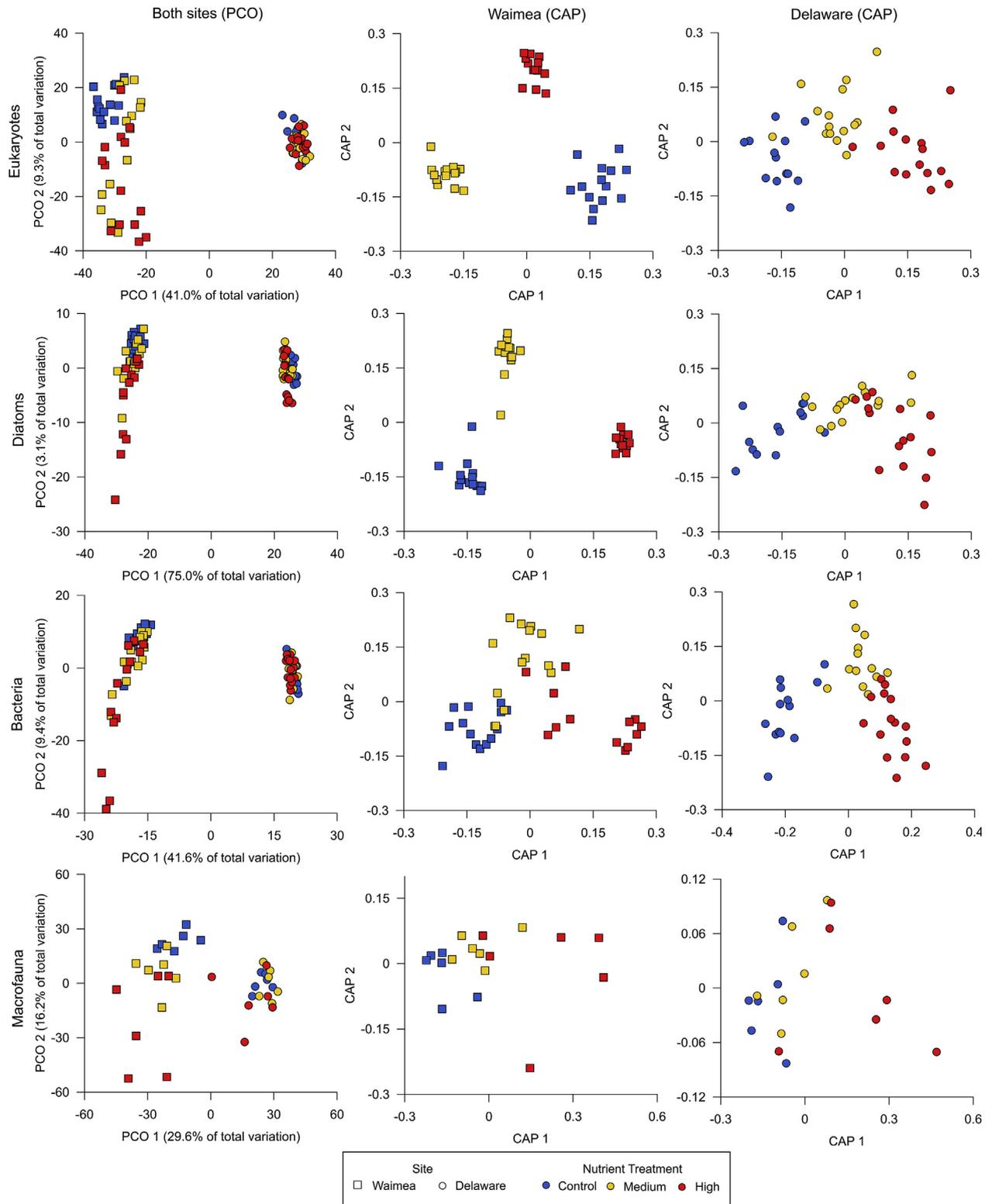
Indicator species analysis identified eukaryotic ( $n = 31$ ), diatom ( $n = 4$ ) and bacterial ( $n = 52$ ) taxa significantly ( $p < 0.05$ ) associated with different nutrient enrichment treatments. None of the eukaryotic community indicator taxa were diatoms. Eukaryotic and bacterial communities showed a clear shift in response to nutrient loading with several indicator species only present in the medium and high nutrient treatments or present in higher abundances than the control treatments (Fig. 3). Eukaryotic and diatom indicator taxa were site-specific for all treatments and no diatom taxa were associated with high nutrient enrichment. Most bacterial indicator taxa were also site-specific, except for *Fusibacter* and *Soehngenia*, which were indicative of the medium-high treatment at Waimea and the high treatment at Delaware. Twelve bacterial indicator taxa associated with high nutrient enrichment were also shared between sites. Indicator values and abundances per treatment for each of the indicator taxa are provided in the Supplementary Material (Table S7).

#### 4. Discussion

In our manipulative experimental study, the nutrient addition

elevated sediment pore water  $\text{NH}_4^+$  to levels found within eutrophic estuaries globally (Douglas et al., 2016), with clear differences in sediment pore water  $\text{NH}_4^+$  observed as nutrient loading increased across treatments, but not between sites. Sediment properties (i.e. granulometry and organic content) were not altered by the nutrient addition, therefore, responses in benthic communities can be confidently attributed to nutrient enrichment. Nutrient enrichment is known to modify sediment and water chemistry leading to changes in the composition, biomass and diversity of benthic communities (NRC, 2000). Benthic communities (eukaryotes, diatoms, bacteria and macrofauna) at the two studied estuaries were distinct from each other, most likely reflecting differing environmental conditions at each site. Regardless of the underlying differences in community structure, changes in these communities were observed at both sites in response to nutrient enrichment, demonstrating their potential use for ecosystem health assessment in response to eutrophication pressure.

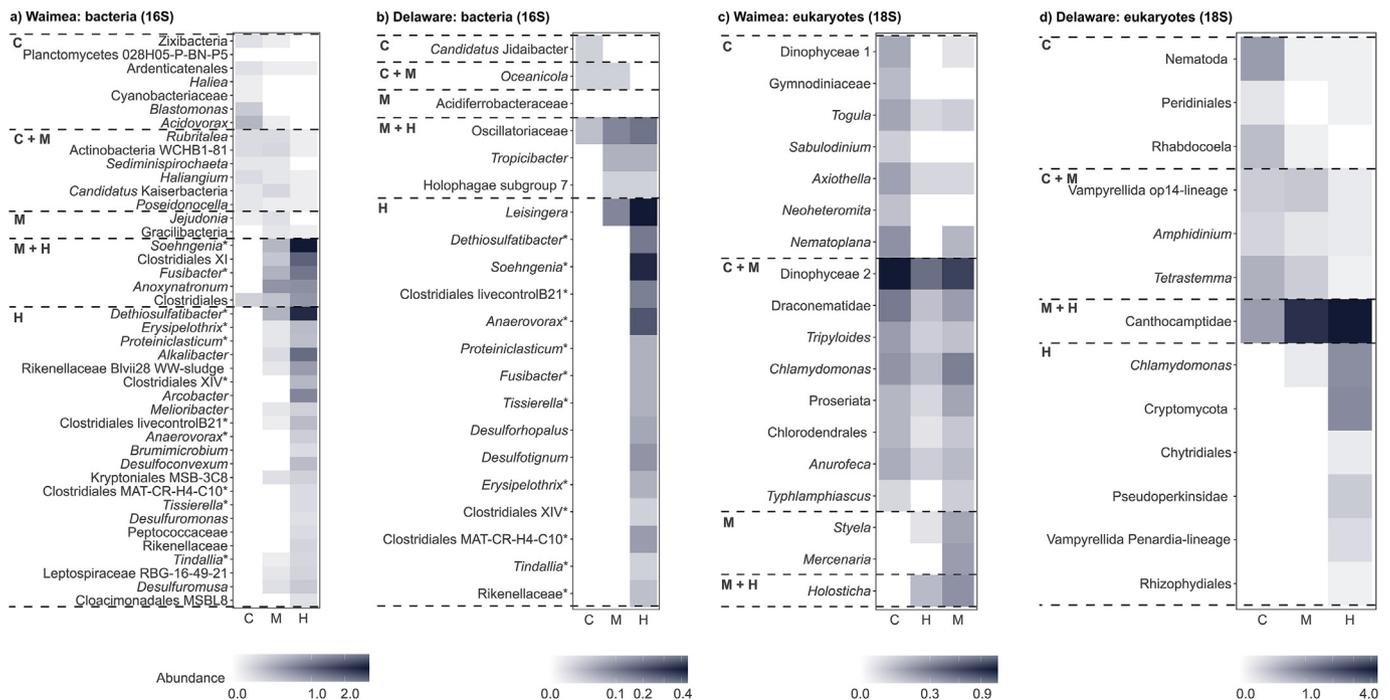
Before noticeable eutrophication-related structural changes occur in macroinvertebrate assemblages, considerable shifts in the composition of microbenthos are expected in affected habitats, providing early signals of functional disturbance (Keeley et al., 2018). Diatom only and bacterial communities showed the strongest response to nutrient enrichment at Waimea while eukaryotic communities (including diatoms) were most sensitive to changes in nutrient loads at Delaware. The differing sensitivities of these communities to nutrient enrichment may reflect the differing environmental conditions at each site and suggests the development of indicators may be context dependent. For example, as nutrient loading at Waimea increased there was a reduction in diazotrophic cyanobacteria (Cyanobacteriaceae in the Order Nostocales; Blais et al., 2012) and increase in *Proteiniclasticum*, which has been found to be abundant in situations where nitrate reduction is high (Li et al., 2016). These changes may indicate that the addition of fertilizer to the sandy sediments at Waimea altered nitrogen acquisition pathways in bacterial communities (e.g. by switching from nitrogen fixation to assimilation) resulting in shifts in bacterial community composition and a strong response to enrichment. Conversely, bacterial communities in the naturally enriched muddy sediments at Delaware may already possess the ability to undertake these functional pathways, therefore, the nutrient addition may have only adjusted their rates, without major shifts in community composition. Further studies across a wider range of sites is required to understand drivers of site-specific responses. Other studies have also shown diatom (Agatz et al., 1999; Kafouris et al., 2019; Tsikopoulou et al., 2020; Weckström and Juggins, 2006), bacterial (Dowle et al., 2015; Keeley et al., 2018;



**Fig. 2.** Principal coordinates analysis (PCO) plots of data from both sites (left-hand column) and canonical analysis of principal coordinates (CAP) plots of data from Waimea (middle column) and Delaware (right-hand column). Each row displays plots from different communities; eukaryotes (including diatoms), diatom only, bacteria and macrofauna. Analyses were based on Bray-Curtis dissimilarities of fourth root transformed eDNA proportional read abundance data or macrofaunal abundance data with nutrient enrichment treatment used as a grouping factor for the CAP analyses. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Table 2**  
Summary of canonical analysis of principal coordinates (CAP) analyses carried out on fourth root transformed eDNA proportional read abundance data for eukaryotic (includes diatoms), diatom only and bacterial communities at two sites. \*Model performance is assessed using the allocation success, with higher values indicating better performance. Details from the CAP analysis based on macrofaunal communities are presented in Table S5 of the Supplementary Material because a direct comparison with eDNA-derived communities is not possible due to differing numbers of replicates.

Site	Waimea			Delaware		
	Eukaryotes	Diatoms	Bacteria	Eukaryotes	Diatoms	Bacteria
Correlation	0.98	0.99	0.85	0.92	0.87	0.95
Canonical correlation	0.97	0.99	0.73	0.85	0.75	0.89
Total variation explained	99%	96%	70%	63%	63%	78%
Number of PCO axes ( <i>m</i> )	34	30	12	11	11	23
<i>p</i>	0.0040	0.0001	0.0001	0.0001	0.0006	0.0315
Allocation success* (%)	71.4	83.3	81.0	81.4	67.4	61.0
Control	85.7	100	100	76.9	61.5	46.2
Medium	64.3	73.3	66.7	86.7	73.3	69.2
High	64.3	76.3	76.9	80.0	66.7	66.7



**Fig. 3.** Average abundance of indicator taxa for bacterial (a, b) and eukaryotic (c, d) communities (including diatoms) across three nutrient enrichment treatments (control, medium, high) at sites in Waimea and Delaware estuaries. The nutrient enrichment treatment (or groups of treatments) that each taxon is associated with is indicated for each group (C = control, M = medium, H = high). An asterisk beside the name denotes indicator taxa shared by both sites. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Lawes et al., 2016, 2017; Santi et al., 2019; Stoeck et al., 2018) and eukaryotic (Chariton et al., 2015; Santi et al., 2019) communities to be sensitive indicators of enrichment, with diatom and bacterial communities often responding more strongly than general eukaryotes (Birrer et al., 2018; Minerovic et al., 2020; Pochon et al., 2019).

Clear shifts in eukaryotic and bacterial indicator taxa were seen in response to nutrient loading but indicator taxa common to both sites were restricted to bacterial communities. These shared bacterial taxa were almost completely absent from control treatments, and often the medium treatments as well, suggesting that they were favored once nutrients reached a certain level. Most shared indicator taxa were from the Clostridiales group, which includes a diverse range of species representing a variety of degradation pathways (Wiegel et al., 2006). Some of these taxa, such as ammonifying bacteria in the genus *Tindallia* (Kevbrin et al., 1998), identified as an indicator species in this study, can be linked to the

degradation of organic matter, which is expected to increase with nutrient addition due to the stimulation of primary and secondary production. Accumulation of organic matter can lead to the formation of anaerobic sediments, which favor bacteria adapted to these environments, such as the anaerobic sulfur-reducing bacteria *Fusibacter* (Fadhlaoui et al., 2015), which was also associated with nutrient enrichment at both sites.

Many of the site-specific bacterial taxa associated with the high nutrient treatment are known to play roles in the sulfur cycle, including the anaerobic sulphate-reducing bacteria *Desulfuromonas*, *Desulfoconvexum*, *Desulfotignum*, *Desulfuromusa* and members of *Arcobacter* and the Peptococcaceae and Rhodobacteraceae families (Pujalte et al., 2014; Schink et al., 2002; Stackebrandt, 2014; Widdel and Pfennig, 1992). Consistent with our study, sulphate-reducing bacteria have been found to respond positively to organic carbon and nitrogen in seagrass and mangrove sediments (Sun et al., 2015; Zhu et al., 2018). Increased abundances of sulphate-reducing

bacteria have also been reported from enriched sediments near fish farms (Dowle et al., 2015; Kawahara et al., 2009; Keeley et al., 2018) and changes in the gene expression of microbial nitrogen and sulfur metabolisms were observed in response to excess organic enrichment in a manipulative field experiment (Birrer et al., 2019).

For eukaryotes, unique indicator taxa were identified at each of the sites and included taxa commonly associated with estuarine sediments (e.g. dinoflagellates, nematodes, platyhelminths). Eukaryotic taxa indicative of the high nutrient treatment were only identified at Delaware, corresponding with the finding that eukaryotic communities were the most responsive to changes in nutrient loads at this site. These taxa included green algae (*Chlamydomonas* sp.) and aquatic fungi (Cryptomycota, Chytridiales, Rhizophydiales) known to infect algae (e.g. diatoms, dinoflagellates, green algae). The increase in green algae in the high nutrient treatment likely arises from nutrient loading fueling algal metabolism (Stevenson, 2014) while the increase in parasitic fungi may be indirectly linked to changes in the abundance of the aquatic algae with whom they associate.

Only four indicator taxa (all site-specific) were identified for the diatom only community at the genus level, suggesting that taxon-specific response of these communities to nutrient enrichment was more subtle than that of eukaryotic and bacterial communities. Diatom species within a genus may differ in their sensitivity to nutrient enrichment (Hillebrand and Sommer, 1997) and other environmental variables (An et al., 2018), therefore, indicator taxa may not be revealed at the genus level. For example, the diatom *Melosira moniliformis* was shown respond positively to *in situ* nitrogen enrichment in the Baltic Sea, but no response was observed for the closely related species *M. nummuloides* (Hillebrand and Sommer, 1997).

Our results suggest that bacterial communities, which had indicator taxa common to both sites, show the most promise for the development of benthic health assessment tools. Other studies have also shown bacterial communities to be relatively non-specific to differences in water flow regime, site and geographic region (Frühe et al., 2020; Keeley et al., 2018), suggesting changes in these communities may be temporally consistent and regionally transferable. For index development and validation, the scale of the study will need to be expanded to ensure any patterns hold true across wider spatial and temporal scales and identify drivers of inconsistent responses. Despite the fact that nutrient enrichment did not consistently select for particular indicator diatom and eukaryotic taxa across study sites, their strong community-level structural response to nutrient enrichment shows potential for use in multivariate and multitrophic ecosystem health metrics.

Besides structural and compositional changes, response to disturbance can be manifested through other benthic community characteristics (e.g. species diversity, variation, or turnover rates). In this study, for example, benthic community variation increased with nutrient loading, supporting the idea that increased variability can act as an indicator of stress in marine communities and proximity to tipping points (Brock and Carpenter, 2006; Guttal and Jayaprakash, 2009; Litzow et al., 2008; Warwick and Clarke, 1993). At Delaware, this pattern was only detected in eukaryotic communities while at Waimea, the trend was stronger and was observed in all eDNA-derived communities (eukaryotes, diatoms, and bacteria). Community metrics (species abundance, richness, diversity, taxonomic distinctness) and ecosystem function responses (sediment oxygen consumption, ammonium flux and gross primary production) show greater variability in sandy sediments than muddy sediments, with mud acting as a ceiling factor that limits variability, possibly explaining the weaker response at Delaware (Pratt et al., 2013; Thrush et al., 2003).

The response of eDNA-derived communities to enrichment

aligned with results from traditional morphological identification of macrofauna, confirming that eDNA can provide concordant, and potentially better (Dafforn et al., 2014), information than that collected using current monitoring approaches. Macrofaunal communities appeared to be less responsive to enrichment effects than eDNA-derived communities, however, due to differences in the scale of sampling (number of replicates, area sampled), comparisons of quantitative results between eDNA-derived and macrofaunal communities should be undertaken with caution. The poorer response of macrofaunal communities could be a result of the fewer replicates collected in this study, the lower taxonomic resolution and limited range of taxa often associated with morphological identification, or the slower turnover rates of macrofauna compared with bacteria and eukaryotes (e.g. bacteria turnover rate is minutes to days; Luna et al., 2002). eDNA sample processing has been estimated to be three times quicker and half the cost of traditional monitoring (Aylagas et al., 2018), with effort and cost decreasing as the number of samples increases. This allows more samples to be collected for an equivalent cost, while providing unprecedented volumes of biodiversity information, which can increase the power to detect change (as in this study) or expand the spatial or temporal scope of monitoring programs. Furthermore, the wide range of taxa captured by metabarcoding allows for greater discrimination between ecosystem responses integrated across different temporal scales, than a dataset constrained to only macrofaunal responses.

Most ecological assessment methods can easily distinguish between unimpacted and impacted sites, however, it is more difficult to discriminate smaller relative differences between pristine reference sites and moderately impacted sites (Chariton et al., 2010). In our study, eDNA metabarcoding enabled eukaryotic, diatom and bacterial communities to differentiate relatively subtle changes between medium and high levels of nutrient enrichment. The community shifts and identification of eukaryotic and bacterial indicator taxa associated with medium or medium-high levels of nutrient enrichment suggests that eDNA-based biodiversity assessments could detect low-level nutrient enrichment before estuaries become too degraded. In this regard, bacteria show more potential than eukaryotes because more indicator taxa indicative of medium and medium-high nutrient enrichment were found for this group. The ability for eDNA-derived communities to distinguish between two levels of nutrient enrichment has also been demonstrated for bacterial biofilms (Lawes et al., 2017) and eukaryotic and bacterial plankton communities (Santi et al., 2019). Detecting community change in response to low levels of impact is a crucial step in the advancement of modern biomonitoring as it would allow for implementation of management or remediation strategies at an early stage, increasingly the effectiveness of these actions (Birrer et al., 2017). The detectable response of eDNA-derived communities to low levels of nutrient enrichment in the field demonstrated in this study, is an important step towards developing genomic tools for ecosystem health assessment, but further work across a wider range of conditions is required to identify consistent patterns in community responses and indicator taxa.

## 5. Conclusion

With rapid advancement of molecular technologies and constantly reducing costs of genomic sample processing, there are intensifying calls for applying omics information in environmental risk assessment and management (Leung, 2018; Pawlowski et al., 2018). However, despite efforts to integrate genomic tools into monitoring programs (Aylagas et al., 2018; Bourlat et al., 2013; Valentini et al., 2016) and the development of metabarcoding-

based indices (e.g. Aylagas et al., 2017; Borja, 2018; Keeley et al., 2018), genomics-based monitoring of ecosystem health has yet to be implemented by regulatory frameworks (Cordier et al., 2020). In order to increase the pace of uptake and utilization of these powerful technologies, coordinated efforts to stimulate the use of omics and build up evidence from relevant case studies are imperative. In this context, the current study provides valuable insights into the applicability of eDNA-based biodiversity information for a more holistic and standardized approach to monitoring estuary health. eDNA-derived communities showed great promise for the development of monitoring tools at our two study sites but before such tools could be practically applied for ecosystem health assessment, the scale of the study needs to be expanded across wider and spatial and temporal scales to identify consistent responses. In addition, these tools would need to be tested in naturally enriched sediments to ensure responses are reliable under true conditions. Future research could also examine functional genes associated with nutrient processing (e.g. Birrer et al., 2019; Fasching et al., 2019) and the structure of biotic interactions within ecological networks (Faust and Raes, 2012) to better understand the processes shaping community responses.

## Summary

An *in-situ* experiment demonstrated that eukaryotic, diatom and bacterial communities show great promise for the development of molecular monitoring tools for estuary health assessment.

## CRedit authorship contribution statement

**D.E. Clark:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Project administration, Funding acquisition. **C.A. Pilditch:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **J.K. Pearman:** Software, Data curation, Writing - review & editing. **J.I. Ellis:** Writing - review & editing, Supervision. **A. Zaiko:** Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision.

## Declaration of competing interest

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

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## Appendix A. Supplementary data

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