Title: Composition and functionality of bacterioplankton communities in marine coastal zones adjacent to finfish aquaculture

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Abstract:

Finfish aquaculture is one of the fastest-growing primary industries globally and is increasingly common in coastal ecosystems demanding effective impact monitoring tools. Bacterioplankton is ubiquitous in marine environment and its rapid response to environmental change makes them a potential bioindicator for environmental impacts. This study aims to verify the use of the taxonomic and functional profiles of bacterioplankton using high-throughput amplicon sequencing. Water was sampled along a distance gradient in three different depths from two lease areas and three control sites in a low-oxygen, highly stratified marine embayment. Our results revealed a vertical variation in bacterioplankton community strongly associated to NOx, conductivity, salinity, temperature and PO_4. The overall shift in the taxonomic profile (ASVs) were more noticeable than in the functional profile. Despite depth stratification, ASVs and functional annotations were detected as potential bioindicators for aquaculture activity. Differentially abundant bacteria found in

lease areas were associated with nutrient enrichment (e.g., *Desulfovibrionaceae, Psychromonadaceae,* and *Fusibacter*) or are known as potential pathogens (e.g., *Pseudomonas, Vibrio* and *Aliivibrio*) and members of the fish gut microbiome (e.g., *Leptotrichiaceae*, *Enterobacterales* and *Pseudomonadales*). Differences in the predicted functional profile were strongest in the bottom waters near leases, where pathways assigned to organic compounds and vitamin degradation, fermentation, methanogenesis and antibiotic resistance were detected. Overall, our findings indicated that the use of 16S rRNA sequencing of bacterioplankton communities are a promising and alternative method for detecting variations along an aquaculture gradient in the water column.

KEY WORDS: bioindicator, bacterioplankton, 16S rRNA gene sequencing, machine learning, aquaculture, coastal environment

1. Introduction

Aquaculture is one of the fastest-growing primary industries worldwide and is increasingly common in coastal ecosystems. Aquaculture of carnivorous fish species, such as Atlantic salmon (*Salmo salar*) is feed-additive, with a significant portion of feed flowing to the environment as metabolic waste products, faeces and waste feed (Navarro et al., 2008; Burridge et al., 2010; Zhang et al., 2015). As industries expand there is increasing concern regarding potential effects that this waste can cause to surrounding environments. Key to sustainable aquaculture operations in marine coastal systems is a better understanding around how pelagic environments assimilate and mitigate additional nutrient loadings.(Belias et al., 2003; Asami et al., 2005; á Norði et al., 2011; Elizondo-Patrone et al., 2015). There are two main pathways of enrichment in pelagic environments, being the solid and dissolved waste streams (Tsukamoto et al., 2008; Amirkolaie, 2011; Wang et al., 2012; Bouwman et al.,

2013). The solid waste stream consists of waste feed and faecal material and is available to pelagic primary consumers as the solid debris settles out of the water column through sedimentation (Macleod et al. 2004). In contrast, metabolic waste products, in particular bioactive inorganic nitrogen compounds such as ammonia and nitrate, are released into the water column as dissolved nutrients and available for assimilation by planktonic organisms (Wang et al. 2012).

Bacterial communities are known to be ubiquitous in the marine environment making up a large fraction of the planktonic ecosystem. Bacterioplankton communities (i.e. bacterial communities of the water column) play an important role in nutrient cycling and biogeochemical processes (Azam et al., 1983; Pomeroy et al., 2007; Fuhrman and Steele, 2008). Bacterioplankton are highly dynamic responding to natural or anthropogenic changes (Newell et al., 2013; Yu et al., 2014; Bowman and Ducklow, 2015; Reji et al., 2020). Previous studies on sediments beneath aquaculture operations have identified changes in the bacterial community due to nutrient enrichment (McCaig et al., 1999; Bissett et al., 2006; Dowle et al., 2015; Rubio-Portillo et al., 2019). However, bacterioplankton community change across enrichment gradients caused by aquaculture activities have yet to be fully defined . Olsen et al. (2017) studying the response of the bacterial community to nutrient input in surface waters adjacent to fish cages in southern Chile found an impact on community structure, however changes in diversity were not observed suggesting stability and functional resilience (Girvan et al., 2005). However, there have been very few other studies examining bacterioplankton and little is known about how the response may vary between different receiving environments (Yoshikawa and Eguchi, 2013; Elizondo-Patrone et al., 2015). Aquatic ecosystems are complex, with cumulative or synergetic effects frequently acting in a multifactorial way. Natural variation of the system and management of

aquaculture waste are key factors to consider when assessing the impact of this sector on the pelagic environment (Wu, 1995; Buschmann et al., 2006). Therefore, it is important to study the responses of bacterial community to environmental variations to enhance our ability to understand changes caused by both natural and anthropogenic environmental changes.

The advancement of molecular-based techniques has greatly advanced the field of microbial ecology (DeLong et al., 1993; Pace, 1997; Gilbert et al., 2010; Caporaso et al., 2011; Yarza et al., 2014; Martínez-Porchas and Vargas-Albores, 2017). The availability of genomic data now allows inference of functional capabilities based on taxon identification and relative abundances (Langille et al., 2013; Asshauer et al., 2015; Nagpal et al., 2016; Ortiz-Estrada et al., 2019; Sevigny et al., 2019). Bacterial communities can be a useful bioindicator as they are present in all marine environments (Ortiz-Estrada et al., 2019; Haro-Moreno et al., 2020; Harrison et al., 2021). The use of 16S rRNA gene amplicon sequencing and functional inference has been used to assess aquaculture impact in various aquatic systems (Keeley et al., 2018; Martins et al., 2018; Moncada et al., 2019; Ortiz-Estrada et al., 2019; Kolda et al., 2020; Frühe et al., 2021). However, natural local characteristics such as water column stratification and natural variation in environmental factors have major effects on the bacterial community structure and function (Martiny et al., 2006; Labbate et al., 2016; Zorz et al., 2019; Da Silva et al., 2021). The strong influence of the environment can make comparisons between studies undertaken in different locations difficult. However, the more we understand the natural variability across different marine systems, the better placed we are to understand common responses in bacteria community different perturbations, both natural and anthropogenic.

Macquarie Harbour is highly stratified fjord-like system with restricted water exchange with the ocean. The water column is characterized by a brackish tannin-rich water layer on top of more saline marine waters. The major freshwater source flows from the southern end carrying high amounts of organic matter with mean monthly flows varying according to the season (< 100 m^3 sec^{-1} in summer and early autumn, and 500 m^3 sec^{-1} late autumn, winter, and spring) (Carpenter et al., 1991; Teasdale et al., 2003; Hartstein et al., 2019). Marine water enters through a narrow and shallow inlet in the north, allowing the renewal of the dissolved oxygen (DO) in the deeper waters. Marine incursions into the harbour are likely driven by forces such as tide, wind, atmospheric pressure and freshwater inputs (Cresswell et al., 1989; Hartstein et al., 2019). The harbour has a history of anthropogenic impacts including mining, damming and aquaculture (King and Tyler, 1982; Crawford et al., 2003; Koehnken, 2005; Kirkpatrick et al., 2019). Pelagic DO concentrations naturally drop with increasing depth reaching around 4 to 6 mg O_2 L^{-1} at bottom layers. However, a recent report observed a considerable decline, with levels now 2 to 4 mg O_2 L^{-1} or lower below 25 m in depth (Ross et al., 2021). This decline is thought to be related to a reduction in the frequency and scalability of DO recharge along with a high biological oxygen demand due to input of organic matter from fish farms. Additionally, previous studies have suggested that pelagic microbial oxygen is likely to be an important determinant of the decline in pelagic oxygen within the Harbour (Revill et al., 2016; Da Silva et al., 2021; Ross et al., 2021). However, the impact of fish farm inputs on the bacterioplankton community remain unclear.

For this reason, the main goal of this study is to characterize the bacterioplankton community in aquaculture influenced zones within Macquarie Harbour. This will provide valuable insights into the use of these microorganisms as bioindicators of environmental change related to fish farming activity, especially in a region with restricted water exchange. The

study aims to (i) define the composition and distribution of bacterioplankton community as well as its predicted functional composition; (ii) assess the influence of aquaculture activity on microbial community structure and distribution; and (iii) evaluate the use of the taxonomic and predicted functions as potential bioindicators of localized eutrophication impacts.

2. Material and methods

Location and sample collection

In December of 2019, sampling was conducted at two fish farm leases ("Lease 1" and "Lease 2") and control stations ("Control") in Macquarie Harbour (42°18'13.8"S; 145°22'22.7"E), Tasmania, Australia (Fig. 1). At each of the leases, samples were collected at sites on replicate transects radiating from a fully stocked Atlantic salmon cage (*Salmo salar*) fed a pellet diet using 10 L Niskin bottles (Tables S1 and S2). According to the Environment Protection Authority of Tasmania, the average maximum permissible stocking density of finfish held within any fish cage is 15 kg/m^3. Seawater was collected from the surface (0-4 m), middle waters (10 – 15 m) and bottom waters (20 – 37 m) at each site except for the site inside the cage. Each transect (Lease 1: n = 2; Lease 2: n=3) included 4 distances (inside the cage + 3 distances) at the surface, and 3 distances at middle and bottom layers. Sampling over distance was carried out by letting the boat drift along the direction of the current. Three control stations (>1500 m away from cages) were sampled at the three depths mentioned above. Genetic material was collected on 0.22 µM polyethersulfone Sterivex^{TM} filter cartridges (Millipore, Darmstadt, Germany) using a multichannel peristaltic pump. Each sample was stored at -80°C until DNA extraction. Haversine's formula was used to calculate the distance between sample points by use of latitude and longitude data (Sinnott, 1984). Additional information regarding finfish farming in the Macquarie Harbour region is

available at https://epa.tas.gov.au/business-industry/regulation/salmon-aquaculture/marine-based-finfish-farms/macquarie-harbour.

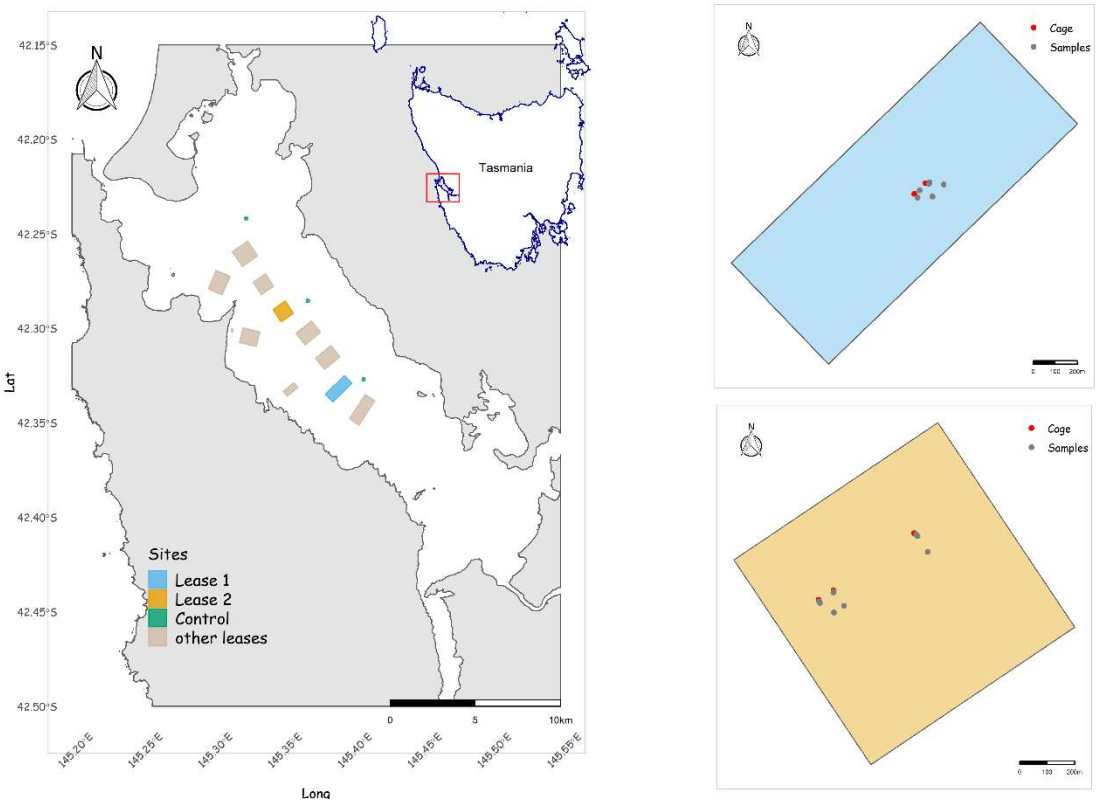


Fig. 1 – Map on the left displays the locations of sites sampled within this study. Green points are control sites and squares are leases located in the Macquarie Harbour. Sampled leases are the blue (Lease 1) and orange (Lease 2) ones. The two maps on the right show the sampled leases where the red points are the cages and the grey points are the samples collected along a distance gradient.

Environmental variables

Physiochemical parameters including temperature (°C), dissolved oxygen (DO, mg L^{-1}), turbidity (NTU) and salinity were measured *in situ* at each site using a YSI 6600 V2 Multi Parameter Water Quality Sonde with a YSI 650 MDS logger. Nutrient analyses were assayed using a Bran + Luebbe AA3 HR segmented flow analyzer following standard spectrophotometric methods (Grasshoff et al., 2009). Detection limits for NO_3^-/NO_2^- were 0.015 µmol/L, for PO_4^{-3} to 0.01 µmol/L, and for NH_4^+ to 0.004 µmol/L.

DNA extraction and sequencing

166 DNA was extracted using a modified phenol:chloroform:isoamyl based DNA extraction
167 protocol of the DNeasy PowerWater SterivexTM Kit (MoBio-Qiagen,vHilden, Germany) as
168 described in Appleyard et al. (2013). Amplicon sequencing targeted the V1 to V3 region of
169 the bacterial 16S rRNA gene (27F–519R) (Lane et al., 1985; Lane, 1991). Libraries were
170 separately generated and sequenced for each sample at the Ramaciotti Centre for Genomics
171 (University of New South Wales, Sydney) using 300 bp paired-end sequencing on the MiSeq
172 platform (Illumina Inc., USA). Raw sequence reads and metadata are publicly available at the
173 Sequence Read Archive under the BioProject accession PRJNA803985
174 (https://www.ncbi.nlm.nih.gov/sra/PRJNA803985).
175
176 **Bioinformatic Pipeline**
177 Paired end sequences of the 16S rRNA gene amplicons were analyzed through the DADA2
178 package pipeline, version 1.20.0 (Callahan et al., 2016) in R 4.1.0 environment (R Core
179 Team, 2021). Reads were filtered with the following parameters: truncLen = c(290, 225),
180 truncQ = 2, maxEE = c(2, 2). Merging of the forward and reverse reads was done with the
181 mergePairs function using the default parameters (minOverlap = 12, maxMismatch = 0).
182 Chimeras were removed using *removeBimeraDenovo* with default parameters. Taxonomic
183 classification was performed using the *assignTaxonomy* function using the parameters (tryRC
184 = FALSE, minBoot = 50) with the SILVA v138 database (Yilmaz et al., 2014) using the
185 naive Bayesian classifier method described in Wang et al. (2007). Mitochondria-derived
186 sequences were removed from the original dataset, obtaining 24575 ASVs. ASV abundance
187 tables, taxonomy and environmental data were imported into the phyloseq R package version
188 1.36.0 (McMurdie and Holmes, 2013). To reduce the data complexity (Cao et al., 2021),
189 bacterial ASVs were filtered to keep taxa observed at least 2 times in at least 2 samples
190 resulting in 3260 ASVs for further analyses (2.7 % of total reads removed).

191

192 **Functional Annotations**

193 Functional composition were predicted using PICRUSt2 version 2.4.1 (Douglas et al., 2020).
194 First, the nearest sequenced taxon index (NSTI) values are obtained as a metric to determine
195 the degree to which ASVs in a sample are related to available reference genomes and
196 environmentally sampled organisms. Then, gene content inference per ASV was performed
197 using hidden-state prediction (HSP) approaches. Before prediction, data was normalized by
198 their 16S rRNA gene copy number abundance. Finally, metagenome inference was used to
199 generate MetaCyc pathway predictions (Caspi et al., 2018). MetaCyc pathways were linked
200 to their respective secondary superclass levels using the tools available at https://metacyc.org.

201

202 **Data Analysis and Statistics**

203 All data analysis and statistical tests were conducted in R 4.1.0 environment (R Core Team,
204 2021). Environmental variables were explored using the tidyverse package v.1.3.1 (Wickham
205 et al., 2019). Bacterial and functional communities were centered log-ratio (CLR)
206 transformed to address the negative correlation bias intrinsic to compositional data (Gloor et
207 al., 2017) using CoDaSeq v.0.99.6 (Gloor, 2016) and zCompositions v.1.3.4 (Palarea-
208 Albaladejo and Martín-Fernández, 2015) packages. To visualize differences among samples
209 based on environmental and community (ASVs composition and functional annotations) data,
210 Principal Composition Analysis (PCA) followed by K-means clustering and distance-based
211 Redundancy Analysis (db-RDA) on Aitchison distance (Aitchison, 1986) were performed,
212 respectively. PCA were implemented using tidymodels v.0.1.3 (Kuhn and Wickham, 2020),
213 K-mean clustering and validation methods using factoextra v.1.0.7 (Kassambara and Mundt,
214 2020), and db-RDA using vegan v.2.5.7 (Oksanen et al., 2018) packages. Environmental
215 variables which could explain variation found on db-RDA ordinations were fitted using both

216 the *envfit*, and *ordistep* functions, the latter with forward and backward stepwise selections.
217 Significant correlation between environmental variables and ordination (p-value < 0.01 in
218 both analyses) were plotted as vectors (arrows) whose direction and length indicate the
219 environmental variable gradient and the correlation, respectively. Prior to both ordination
220 methods, autocorrelated environmental variables (i.e., r > 0.7, p-value ≤ 0.05) were combined
221 into one for downstream analyses.
222
223 To verify the magnitude of variation along a gradient of environmental changes, a correlation
224 between bacterial community and functional annotation distance matrices was calculated
225 using Mantel tests (Spearman rank correlation and 9999 permutations). To evaluate the
226 difference in community composition between leases and control sites, ASVs and functional
227 annotations were subjected to two widely used statistical methods: ALDEx2 (Fernandes et
228 al., 2013) and DESeq2 (Love et al., 2014) packages. The combination of these two methods
229 aims to overcome the drawbacks found in differential abundance methods, such as low power
230 and high False Discovery Rate (FDR) (Hawinkel et al., 2019). For ALDEx2, clr-transformed
231 abundance table was used as the input for the analyses, whereas DESeq2 used variance-
232 stabilized data to perform the evaluation. ASVs and pathways were considered differentially
233 abundant if they satisfied the following assumptions: an effect size > |1.5| in ALDEx2 and an
234 adjusted P value (FDR) < 0.05 and a log2foldchange > |2| in DESeq2 analysis. The package
235 ggtern v.3.3.5 (Hamilton and Ferry, 2018) was used for data visualization. Then, the
236 differentially abundant ASVs and pathways were used to model input data (clr-transformed
237 count table) to verify the relationship with the environmental variables (NH_4, NOX, turbidity
238 and oxygen).
239

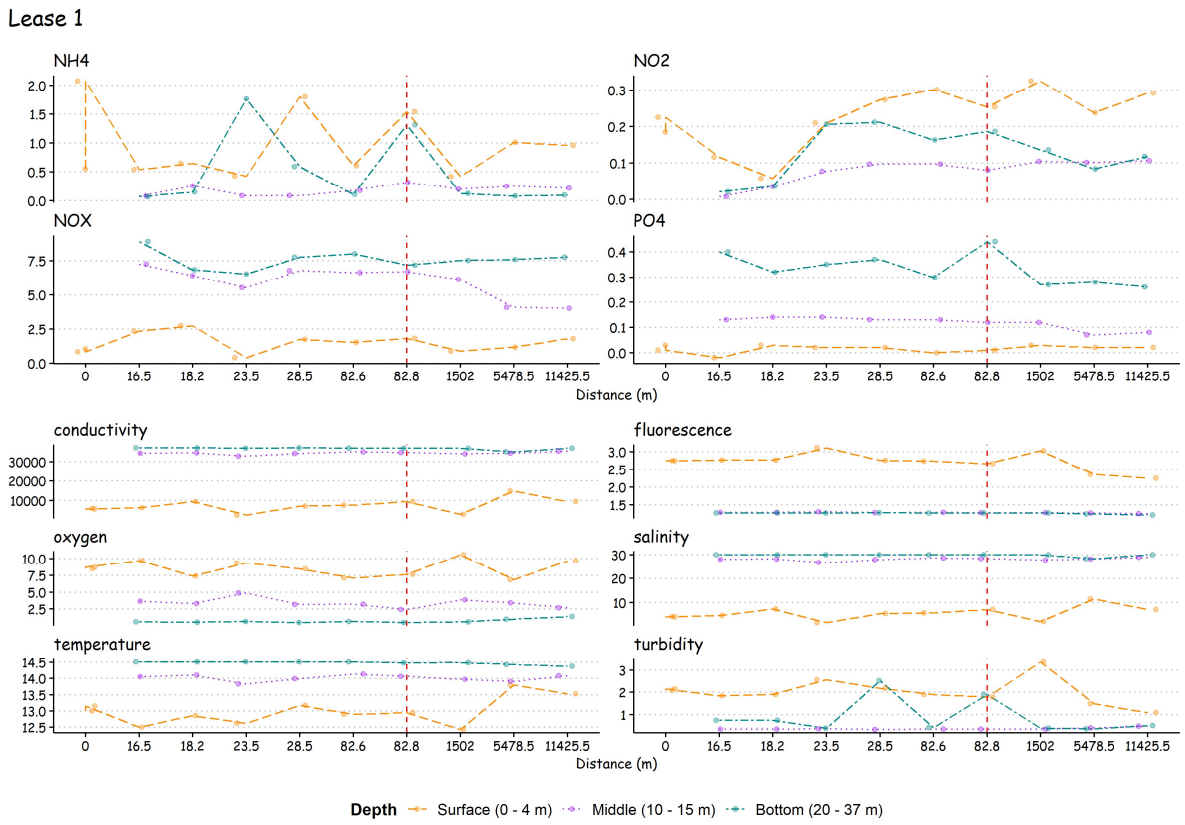
240 Random forest regression modelling was implemented through tidymodels v.0.1.3 (Kuhn and
241 Wickham, 2020) and randomForest v.4.6.14 (Liaw and Wiener, 2002) packages to test the
242 ability to predict estimates of the variability of the aforementioned environmental variables.
243 For this process normalized clr-transformed data was split into training and testing sets in a
244 proportion of ¾. To tune the hyperparameters (mtry and min_n), a k-fold cross validation
245 scheme (k = 10 with 3 repeats) with 501 trees of the training set was used to estimate the best
246 model based on root-mean-square error (RMSE) metric. The model was finalized using the
247 *finalize_workflow* and *select_best* functions. After that, the model performance was tested in
248 the training set and the metrics RMSE and R^2 were collected using the *collect_metrics*
249 function. Variable importance was measured by the residual sum of squares. Model
250 predictions and variable importance were extracted with the *collect_predictions* function and
251 vip v.0.3.2 package (Greenwell and Boehmke, 2020), respectively and visualized using
252 tidyverse package v.1.3.1 (Wickham et al., 2019). Code used for data analyses in this study
253 can be found in the associated GitHub repository
254 (https://github.com/ricrocha82/MH_2019_16s).
255

256 **3. Results**

257 **Environmental Variables**

258 The samples from leases and control sites showed a uniform trend in most environmental
259 variables when distances from cages were considered, however for NH_4, NO_2 and NOx,
260 concentrations were highly variable at both leases (Fig. 2; Tables S2, S3 and S4). Despite this
261 variation, NH_4 level was observed to decrease as distance from cage increased at the surface
262 of Lease 2 (2.84 ± 0.47 μM to 0.1 ± 0.03 μM) and NOx decreased in concentration with
263 distance in the middle waters of Lease 1 (6.38 ± 1.22 μM to 1.29 ± 0.46 μM). Also, at Lease

1, NO_2 showed an increase in concentration as distance increased after ~18 m (0.04 ± 0.05 μM to 0.28 ± 0.04 μM).



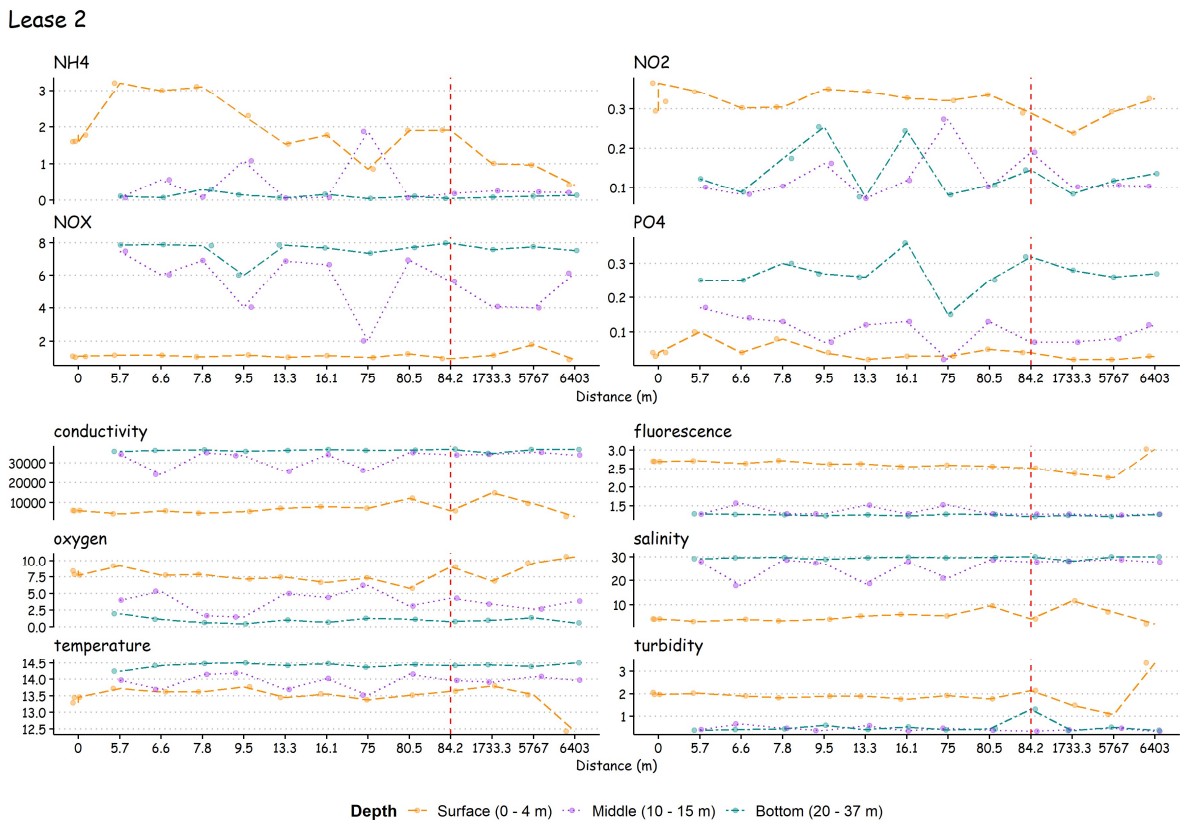


Fig. 2 – Environmental variables along a distance gradient in each lease area. Each line in the graph displays environmental variable measurements in each depth. Red dashed line shows the farthest distance from the cage. (i.e., end of cage influenced zone). After this line cage site distances are displayed. Distance 0 m indicates

sample from inside the cages (2 cages in Lease 1 and 3 cages in Lease 2). Dissolved nutrients (µmol l^{-1}), conductivity, fluorescence, oxygen (mg/l), temperature (°C), turbidity (NTU)

When depth is considered, stratification is clear for all environmental variables at all leases and control sites (Fig. 3 – A; Tables S1-S4). Despite the variation observed in nutrient concentrations, NH_4 (1.53 ± 0.85 µM to 0.3 ± 0.48 µM) and NO_2 (0.28 ± 0.08 µM to 0.14 ± 0.07 µM) decreased with depth whereas NOx (1.29 ± 0.51 µM to 7.57 ± 0.63 µM) and PO_4 (0.03 ± 0.02 µM to 0.3 ± 0.07 µM) displayed the opposite trend. Similarly to NOx and PO_4, conductivity (6914.73 ± 2781.28 to 36573.31 ± 548.15), salinity (5 ± 2.26 to 29.7 ± 0.5) and temperature (13.28 ± 0.41 °C to 14.45 ± 0.07 °C) were greater at depth, whereas fluorescence (2.68 ± 0.2 to 1.26 ± 0.022 °C), oxygen (8.10 ± 1.14 mg/l to 0.86 ± 0.4 mg/l), PAR (0.62 ± 1 to 0.003 ± 0.001) and turbidity (2 ± 0.4 NTU to 0.72 ± 0.6 NTU) were all lower at depth as seen for NH_4 and NO_2 concentrations. NOx was highly correlated to conductivity, salinity, temperature and PO_4, whereas NH_4 was correlated to NO_2 and oxygen to PAR and fluorescence (Fig.S2). Therefore, NOx, NH_4 and oxygen were used as proxies of their highly correlated peers.

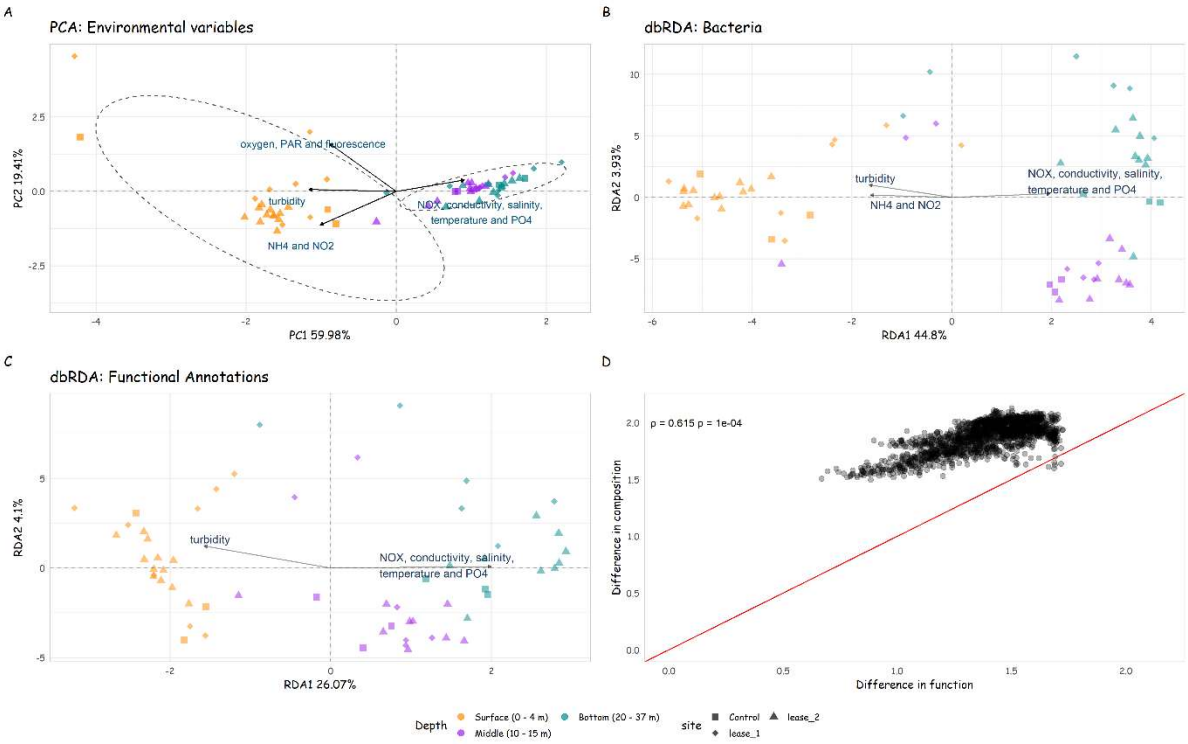


Fig. 3 – Ordination plots of the samples and vectors (arrows). (**A**) Principal Component Analysis (PCA) of environmental data. Dashed lines highlight the results of K-mean clustering analyses. (**B-C**) Distance-based Redundancy Analysis (db-RDA) of the composition differences of bacterial and functional communities. To avoid multicollinearity, NOx included conductivity, salinity, temperature and PO_4, and NH_4 included NO_2. Vectors indicate a significant correlation between environmental variables and ordination ($p < 0.01$) using *envfit* function of vegan package. (**D**) Differences in ASV bacterial and functional compositions. Equal changes are shown by the red line. Mantel test shows a significant correlation between the two distances matrices (Spearman rho = 0.6154).

Bacterial and Functional Communities Structure

At phylum and class levels, bacterial community at all sites was dominated by *Proteobacteria* (Control: 58.4 %; Lease 1: 55.3 %; Lease 2: 57.9 %) and *Gammaproteobacteria* (Control: 30.4 %; Lease 1: 30.7 %; Lease 2: 31.7 %), respectively. *Proteobacteria* was followed by *Bacteroidota* (Control: 15.6 %; Lease 1: 17 %; Lease 2: 18.1 %) at all sites, *Actinobacteriota* (5.6 %) at Control sites and *Campylobacterota* (Lease 1: 7.63 %; Lease 2: 5.45 %) at lease sites. *Gammaproteobacteria* was followed by *Alphaproteobacteria* (Control: 28.1 %; Lease 1: 24.6 %; Lease 2: 21.6 %) and *Bacteroidia* (Control: 15.2 %; Lease 1: 16.7 %; Lease 2: 17.9 %) at all sites. ASVs encompassing the class *Actinobacteria* (Control: 4.3 %; Lease 1: 1.93 %; Lease 2: 1.90 %) were more abundant at control sites whereas *Campylobacteria* ASVs were more abundant at lease sites (Control: 3.3 %; Lease 1: 7.63 %; Lease 2: 5.45 %) Fig. S3).

Functional annotations were uniformly distributed among leases and control sites. Amino-acid biosynthesis was the most abundant pathway (Control: 17 %; Lease 1: 16.8 %; Lease 2: 16.9 %), followed by nucleotide-biosynthesis (Control: 16.2 %; Lease 1: 16.3 %; Lease 2: 16.3 %) and vitamin-biosynthesis (Control: 9.93 %; Lease 1: 10.2 %; Lease 2: 10.1 %) (Fig. S4). Despite little variation in both communities among sites, depth stratification was evident when ordination analyses were performed (Fig. 3 - B and C). db-RDA explained 49.47% and 31.18% of the variation in bacterial and functional communities, respectively. The ordination of bacterial community distribution was significantly correlated to NOx ($r^2 = 0.76$; $p < 0.01$),

318 NH_4 ($r^2 = 0.49$; $p < 0.01$) and turbidity ($r^2 = 0.36$; $p < 0.01$) whereas functional profiles were
319 significantly related to NOx ($r^2 = 0.74$; $p < 0.01$) and turbidity ($r^2 = 0.41$; $p < 0.01$) (Table 1).
320 The results indicate that environmental parameters and both bacterial and functional
321 communities are more affected by depth stratification than by aquaculture activity. Moreover,
322 the correlation between the distance matrices of bacterial ASVs and functional compositions
323 suggested a significant relationship (rho: 0.62; $p < 0.05$). In other words, the differences in
324 the taxonomic profile between samples co-vary with the differences in the functional profile
325 between samples. However, bacterial community showed a more prominent overall shift than
326 the associated functional data (Fig. 3 - D).

327 Table 1 - Result of redundancy analysis (*ordistep* and *envif* functions) and random forest (rf) of bacterial and
328 functional communities in the Macquarie Harbour. Regarding the db-RDA, we considered significant, if the
329 environmental variable had a p-value < 0.01 in both analyses. To avoid multicollinearity, NOx included
330 conductivity, salinity, temperature and PO_4. NH_4 included NO_2.

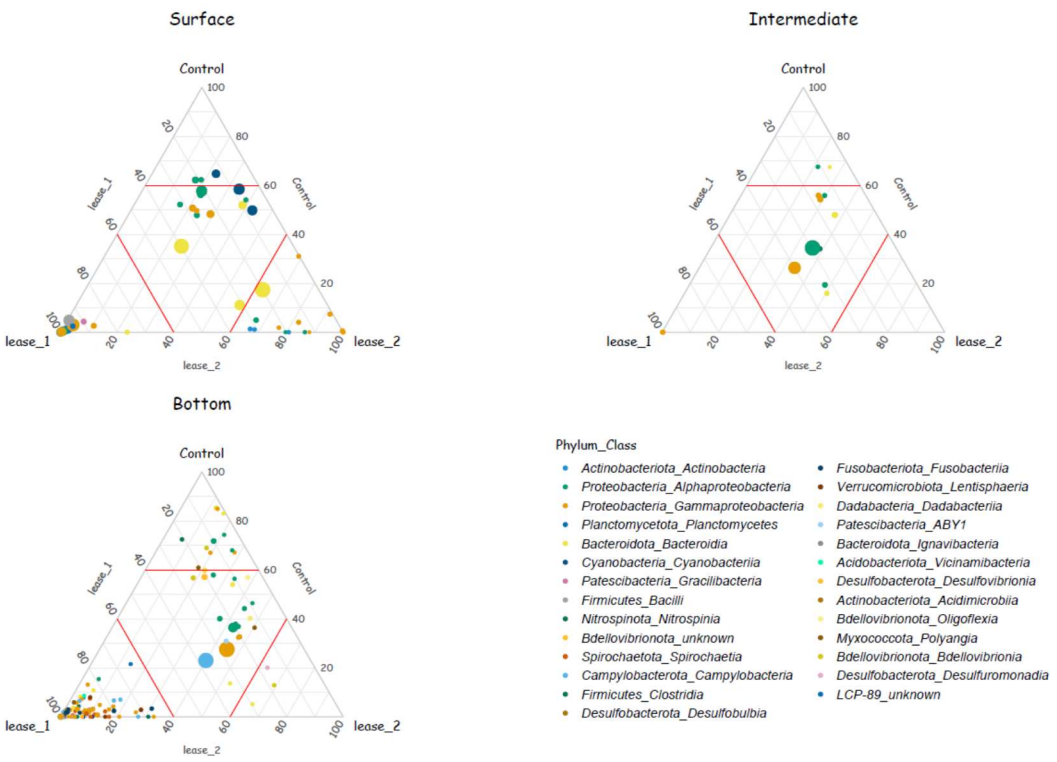
Community	env variable	p-value (ordistep)	p-value (envfit)	r2 (envfit)	RMSE (rf)	R2 (rf)
Bacteria	NOx*	0.005	0.001	0.76	1.14	0.87
	turbidity	0.005	0.001	0.36	0.66	0.44
	NH_4**	0.005	0.001	0.49	0.70	0.53
Functional annotation	NOx	0.005	0.001	0.74	1.23	0.82
	turbidity	0.005	0.001	0.41	0.53	0.59

331 *NOx included conductivity, salinity, temperature and PO_4.
332 **NH_4 included NO_2.
333

334 **Differential Abundance Among Sites by Depth**

335 To detect key members in bacterial and functional communities at lease and control sites,
336 differential abundance analyses were performed considering the effect of depth stratification
337 observed in the community structure analyses. Therefore, the analyses were conducted by
338 executing pairwise comparisons between sites nested by depth (i.e., surface, middle and
339 bottom water layers). ALDEx2 and DESeq2 found a total of 90 and 167 bacterial ASVs and
340 82 and 61 functional annotations differentially abundant, respectively. At all depths, ASVs
341 and functional annotations selected by both methods were more abundant in both lease
342 influential zones (i.e., relative abundance $\geq$ 60%) compared to control site. The difference in

bacterial community was pronounced at the bottom and surface layers (Fig. 4). Most of the enriched ASVs found in Lease 1 area inhabited the bottom layers (Fig. S5 and Table S5). The taxon with the highest number of differentially abundant ASVs were those belonging to the genus *Psychromonas* (family *Psychromonadaceae*) followed by *Spirochaeta* 2 (family *Spirochaetaceae*), unknown *Arcobacteraceae* (order *Campylobacterales*), unknown *Bacteroidales*, *Hypnocyclicus* and an unknown genus (both assigned to the family *Leptotrichiaceae*), *Draconibacterium* and three unknown genera related to the order *Bacteroidales*, *Sulfurimonas* (family *Sulfurimonadaceae*), *Desulfovibrio* (family *Desulfovibrionaceae*), *Pseudomonas* (family *Pseudomonadaceae*), and *Woeseia* (family *Steroidobacterales*). In contrast, at the surface of Lease 2, *Aliivibrio* (family: *Vibrionaceae*) had the highest number of differentially abundant ASVs (Fig. S6 and Table S6). In the control, taxa related to *Nitrospina* and *Gammaproteobacteria* were more abundant in the bottom layers (Fig. S7 and Table S7).



358 Fig. 4 – Ternary plots of per-depth relative abundances of differentially abundant ASVs. Each point represents
359 an ASV, and its position indicates the proportion of its relative abundance at each site. Points closer to the
360 ternary plot corners indicate that a greater proportion of the total relative abundance of this ASV was found in
361 this site. Point colors indicate the Phylum and Class of the ASV. The size of each point indicates the mean
362 relative abundance of the ASV in all samples. The red lines inside the ternary plot indicate the 60% level of
363 relative abundance of each of the sites.
364

365 Regarding functional community, differences were prominent at the Lease 1 influenced zone
366 (Fig. 5, S8 and S9; Tables S8 and S9). In the bottom layer at Lease 1, the analysis detected
367 pathways related with organic matter degradation such as fermentation, carbohydrates,
368 amino-acids, aromatic compounds, and alcohol degradations, as well as those associated with
369 trace metal and macro-nutrient limitations (secondary metabolites, cofactor, vitamins, and
370 amino acids biosynthesis), methanogenesis and cell metabolism. Pathways associated with
371 vitamin degradation and cell metabolism (cell-structure/cell-wall biosynthesis) and those
372 related to alcohol degradation and vitamin biosynthesis were more abundant at the surface
373 and middle layers, respectively. At the control site, lipid biosynthesis and secondary
374 metabolite biosynthesis were differentially expressed at the middle and bottom layers,
375 respectively. These results suggest that the differences observed in bacterioplankton
376 community (16S rRNA gene sequencing) and functional profile (functional annotation:
377 PICRUSt2) at each depth between the lease and control stations are likely to reflect changes
378 in the local environment due to aquaculture activities.

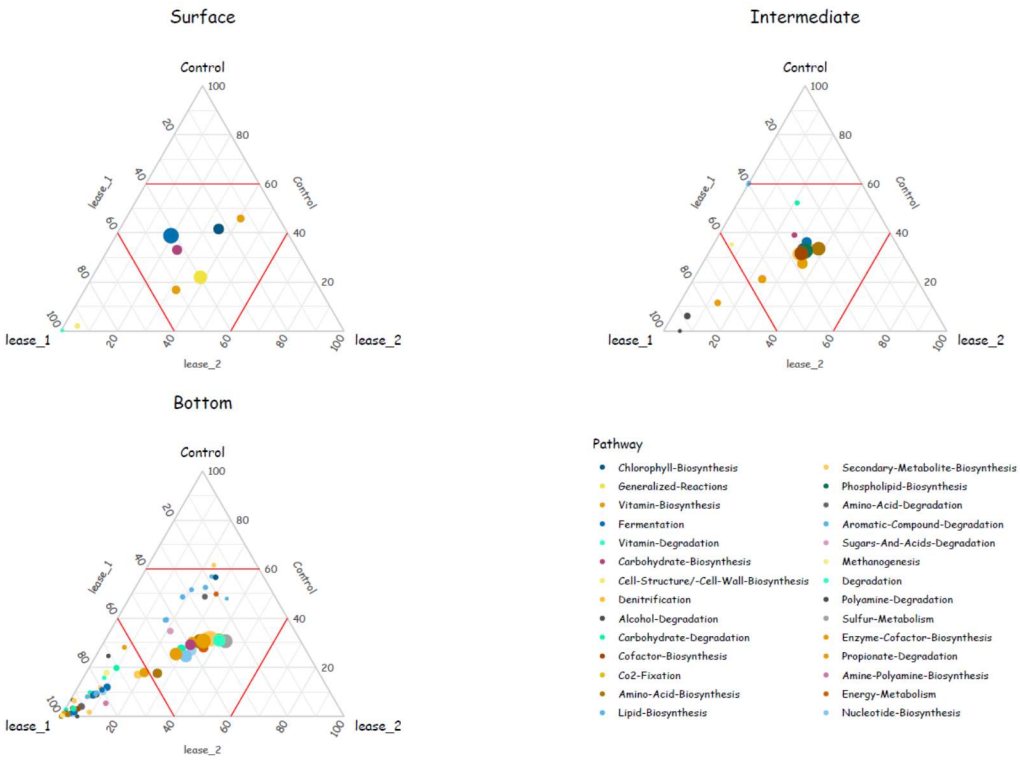


Fig. 5 – Ternary plots of per-depth relative abundances of differentially abundant functions. Point colors indicate the pathway of the function according to https://metacyc.org. The size of each point indicates the mean relative abundance of the function in all samples. The red lines inside the ternary plot indicate the 60% level of relative abundance of each of the sites.

Predictive Performance of the Selected ASVs

To measure the importance of these differentially expressed features in predicting the environmental drivers found in the db-RDA analysis (Fig. 3 and Table 1), supervised machine learning was used to estimate the relationship between feature distribution and environmental variable measurements. Then, RF regression models were trained with 257 ASVs and 143 functional annotations. Only NOx (included conductivity, salinity, temperature and PO_4) concentration prediction models yielded desirable performance for both communities (Table 1). The most important ASV and functional annotation in reducing variance and uncertainty to NOx values were assigned to *Oligoflexaceae*

(

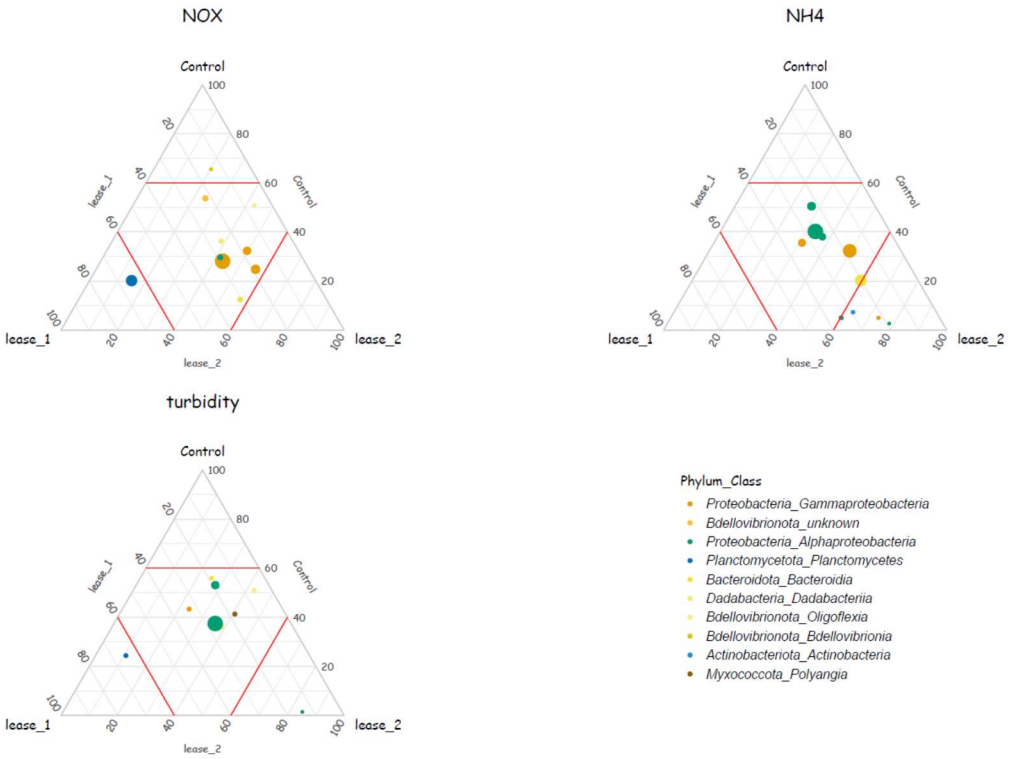


Fig. 6 and S10; Table S8**Error! Reference source not found.**) and to vitamin biosynthesis, respectively (Fig. 7 and S11; Table S9). Additionally, the most important features in both communities were uniformly distributed among sites, except for some ASVs and functional annotations. For instance, ASVs that could predict NOx concentration (i.e., OM27 clade (*Bdellovibrionaceae*) and *Planctomycetes*) were found more abundant (> 60% relative abundance) at the control and Lease 1 samples, respectively. And two functional annotations assigned to methanogenesis and vitamin biosynthesis, both important in NOx prediction, were more enriched in Lease 1 samples.

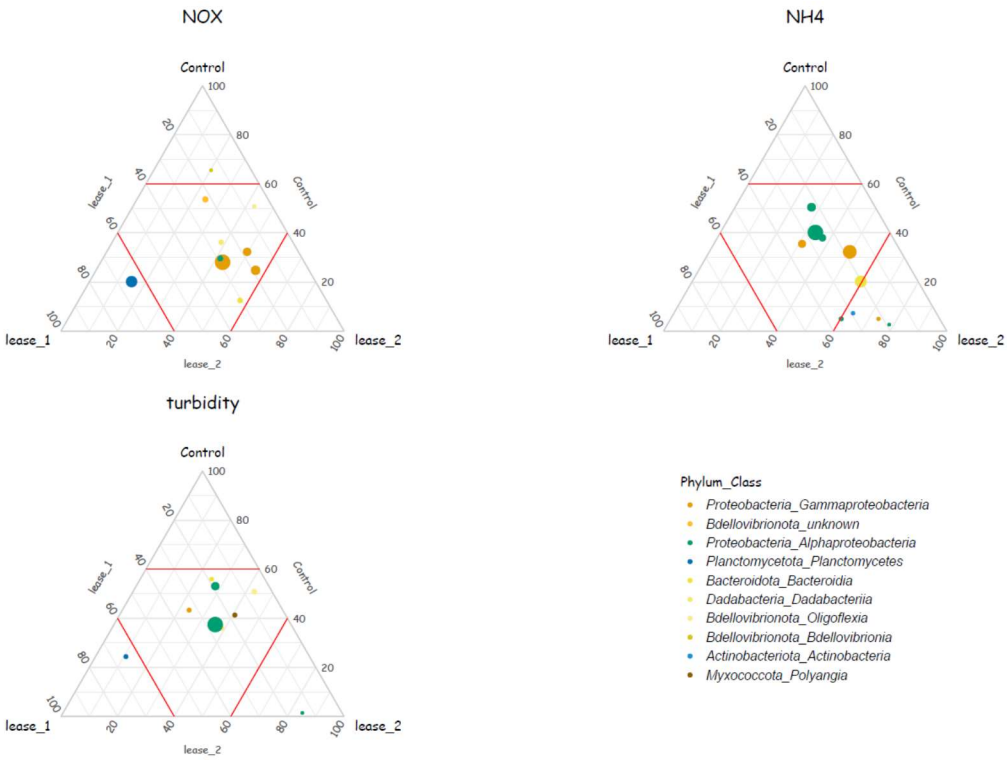


Fig. 6 – Ternary plot showing the distribution of the 10 most important ASVs based on the residuals sum of square in regression-based prediction model of the environmental drivers of the bacterial community (NH_4, NOx and turbidity). The size of each point indicates the mean relative abundance of the ASV in all samples. The red lines inside the ternary plot indicate the 60% level of relative abundance of each of the sites. To avoid multicollinearity, NOx included conductivity, salinity, temperature and PO_4; and NH_4 included NO_2.

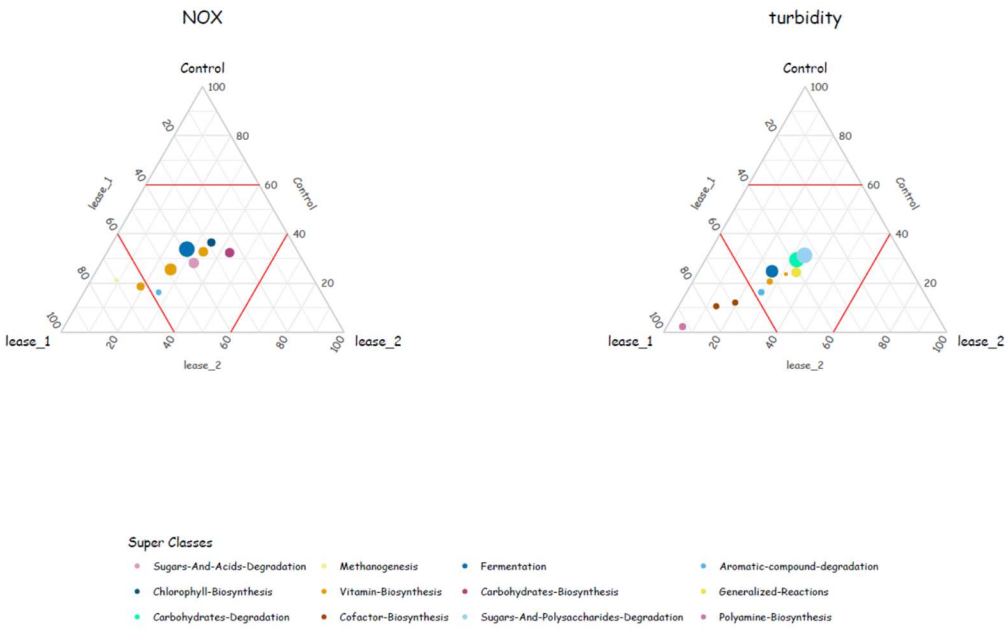


Fig. 7 – Ternary plot showing the distribution of the 10 most important pathways based on the residuals sum of square in regression-based prediction model of the environmental drivers of the functional community (NOx and turbidity). The size of each point indicates the mean relative abundance of the function in all samples. To avoid multicollinearity, NOx included conductivity, salinity, temperature and PO_4.

418 **4. Discussion**

419 In this study, we used taxonomic and functional profiles to characterize the bacterioplankton
420 community of a coastal embayment containing finfish aquaculture operations in Tasmania
421 (Australia). Using unsupervised and supervised analysis techniques of 16S rRNA bacterial
422 community composition, we observed that the effects of depth stratification on
423 bacterioplankton dominates over aquaculture effects. Despite this natural influence, we also
424 detected differentially abundant ASVs and functional annotations between both marine farm
425 leases (< 85 m) and control sites (> 1500 m), that could be used as good bioindicators for
426 aquaculture activity. According to the random forest regression model, these features could
427 only predict NOx concentrations (along with conductivity, salinity, temperature and PO_4).
428

429 **4.1. Community variation and depth stratification**

430 The main driver of variation in both the environmental variables and communities was water
431 column stratification. Depth stratification was most notable in bacterioplankton community
432 structure with each of the three sampled depths (i.e., surface, middle and bottom waters)
433 being quite distinct. While taxonomic distribution changed, functional profiles were more
434 stable in comparison, suggesting functional redundancy (Fernández et al., 1999; Allison and
435 Martiny, 2008). The stratified distribution of both communities could be explained by the
436 variation in the environmental variables. Macquarie Harbour is considered a highly stratified
437 embayment with distinct communities inhabiting each water layer (Da Silva et al., 2021). The
438 higher turbidity and NH_4 concentration at the surface could be related to the Gordon River
439 inputs carrying labile and recalcitrant organic matter as well as high amounts of tannin
440 affecting light penetration (Terry, 2001; Hartstein et al., 2019). Rivers are commonly
441 described as nutrient sources in coastal environments affecting biogeochemical cycling and
442 the microbial community, particularly in semi-closed coastal systems (Bartl et al., 2018; Hou

et al., 2018). Low light penetration and high amounts of NH_4 and organic matter in the surface can favor nitrification and bacterial respiration. This could explain the increasing NOx and decreasing DO and NH_4 concentrations with increasing depth. These mineralized nutrients are then used as source of energy by the bacterial community in the water column (Berman and Bronk, 2003). Thus, it is unsurprising that the differences in bacterial communities observed in this study are related to physical parameters observed within the water column (Bowman and Ducklow, 2015; Langenheder and Lindstrom, 2019; Zorz et al., 2019).

4.2. Effects of aquaculture on the bacterial community composition

In this study, differential abundance analysis revealed an effect in the taxonomic and functional profiles at both the surface and the near-bottom water layers in lease areas. Most previous aquaculture-environmental impact studies focus on sediments and associated effects to the benthic microbial community (Borja et al., 2009; Fodelianakis et al., 2014; Dowle et al., 2015; Aylagas et al., 2017; Cordier et al., 2018; Stoeck et al., 2018; Keeley et al., 2021). The pelagic environment is less studied and as such the information regarding bacterioplankton response to aquaculture operation is limited, despite their known sensitivity to environmental changes (Wei et al., 2016; Lindh and Pinhassi, 2018; Kolda et al., 2020). The observed differences in the water column were similar to previous studies, where the heterotrophic community degrading organic matter has been enriched near fish cages (La Rosa et al., 2002; Navarro et al., 2008; Šestanović et al., 2016; Kolda et al., 2020).

Aquaculture activities are known to increase nutrient loading and change trophic status near the fish cages from waste, by-products, fish feces or feed products inputs (Belias et al., 2003; Buschmann et al., 2006; Amirkolaie, 2011). Several differentially abundant ASVs were found in both lease areas that could be related to higher levels of organic matter,

469 eutrophication or pollution. These include members of the taxa *Psychromonadaceae*
470 (*Psychromonas*)*, Fusobacteriales (Fusobacteriaceae), Vibrionaceae, Desulfovibrionaceae*
471 *(Desulfovibrio), Sulfurimonadaceae (Sulfurimonas), Sulfurovaceae (Sulfurovum),*
472 *Fusibacteraceae (Fusibacter), Rhizobiales,* and *Sphingomonadaceae.* Various microbial
473 groups have been associated with marine environments receiving high level of nutrients and
474 some have been reported as potential aquaculture impact biomarkers in the past (Blancheton
475 et al., 2013; Kandel et al., 2014; Rasigraf et al., 2017; Verhoeven et al., 2018; Yang et al.,
476 2019; Haro-Moreno et al., 2020; Zhang et al., 2020; Aalto et al., 2021; Dully et al., 2021;
477 Frühe et al., 2021; Mahmoud and Magdy, 2021; Pan et al., 2021). According to these studies,
478 such taxa are usually found in high abundance in near-cages sites, including *Sulfurovum,*
479 *Vibrionaceae, Desulfovibrionaceae, Methylotenera, Hyphomonadaceae*, *Spirosomaceae* and
480 *Fusibacter, Flavobacteriaceae* (NS3a marine group) and *Bacteriovoracaceae*. Moreover,
481 *Psychromonadaceae* (*Psychromonas*), *Fusobacteriales (Fusobacteriaceae)* and *Vibrionaceae*
482 have been also reported as members of the fish gut microbiome(Verhoeven et al., 2018;
483 Frühe et al., 2021; Pan et al., 2021).
484

485 **4.3. Bacterioplankton community associated with aquaculture activity**

486 **4.3.1. ASVs important to nutrient cycling and biogeochemical processes**

487 Interestingly, ASVs that play a role in biogeochemical cycling (e.g., sulphur, nitrogen,
488 methane, carbon) were enriched at lease sites. These bacteria have been reported as microbes
489 related to aquaculture operations. For instance, sulphate-reducing bacteria such as members
490 of the family *Desulfovibrionaceae* have been found to be enriched in sediments by
491 aquaculture activity (Blancheton et al., 2013; Aranda et al., 2015; Frühe et al., 2021).
492 Sulphate reducing bacteria can be enriched by high levels of organic matter relying on, for
493 instance, fermented to acidic end products (i.e., by *Psychromonadaceae*, *Vibrionaceae*, and

494 other marine (facultatively) anaerobic chemoorganotrophs), leading to the increased
495 production of H_2S (Barton and Hamilton, 2007; Kwon et al., 2016). *Fusibacter* also
496 contributes to organic matter and sulphur compounds cycling since it is able to respire using
497 sulphur and thiosulfate and use a wide range of organic donor compounds (Ravot et al., 1999;
498 Fadhlaoui et al., 2015). This genus has been found in a range of impacted aquatic systems
499 including aquaculture and mining sites (Aranda et al., 2015; Won et al., 2017; Verhoeven et
500 al., 2018).
501
502 *Thioglobaceae* (SUP05 cluster) and *Beijerinckiaceae* (*Methylocystis*) were abundant in the
503 surface water samples at Lease 1 and have been previously reported in aquaculture sites (Fan
504 et al., 2019; Haro-Moreno et al., 2020). The former is capable of nitrate reduction and dark
505 sulphide oxidation processes found below the photic zone in marine environments (Reji et al.,
506 2020; Chun et al., 2021) while the latter is methanotrophic (i.e., methane oxidizing bacteria)
507 (Fan et al., 2019). Methanotrophic bacteria participate in the nitrogen cycle by linking
508 methane oxidation when under hypoxic conditions and performing denitrification when
509 exposed to nitrate (Kits et al., 2015). Different methylotrophic bacteria were also found at the
510 surface of Lease 2. *Methylophilaceae* (*Methylotenera*) are able to consume C_1 compounds
511 (i.e., methanol, methylamine) linked to denitrification (Mustakhimov et al., 2013; Hernandez
512 et al., 2015) and can be found enriched in salmon hatcheries (Lokesh et al., 2019; Minich et
513 al., 2020) and associated to "red-operculum" disease in crucian carps aquaculture ponds (Li
514 et al., 2017). The presence of these microbes could be related to oxygen concentration and
515 feed inputs (Hernandez et al., 2015; Fan et al., 2019).
516
517 ASVs assigned to the chemoheterotrophs *Hyphomonadaceae* and *Spirosomaceae* were
518 enriched at the surface waters of the Lease 2 sample area, similar to a previous study

519 conducted in a trout aquaculture farm, where these two aerobic bacteria were abundant in the
520 pond-water (Mahmoud and Magdy, 2021). Finally, oligotrophic chemoheterotroph
521 *Flavobacteriaceae* (NS3a marine group) and the predatory *Bacteriovoracaceae* were already
522 observed in other microbial aquaculture studies in the sediments (Frühe et al., 2021) and in a
523 zero-discharge system (Kandel et al., 2014), respectively.
524
525 Our study shows that these enriched bacterial communities are most likely related to organic
526 enrichment caused by aquaculture operations. These distinctive groups of microorganisms
527 represent potential biomarkers that could be used to trace and monitor environment changes
528 caused by the effects of aquaculture.
529
530 **4.3.2. ASVs as potential pathogens or related to the fish gut**
531 Interestingly, several ASVs enriched at the lease sites are known as potential pathogens
532 and/or can be part of the gut microbiome of fish. *Spirochaetaceae* ASVs are capable of
533 anaerobic carbohydrate metabolism commonly found in the salmon gut microbiome (Fogarty
534 et al., 2019). Members of this genus are often found in aquaculture impacted sites
535 (Verhoeven et al., 2016; Kolda et al., 2020; Frühe et al., 2021). Another potential pathogen
536 potentially present in the gut of fish (and with aquaculture systems in general) are the
537 members of *Arcobacteraceae.* These bacteria can be pathogens, opportunistic pathogens and
538 commensals being detected globally (Ho et al., 2008). Species from this group are connected
539 to sewage, marine contamination and zoonotic intestinal disease (Figueras et al., 2014;
540 Kristensen et al., 2020). The anaerobic family *Leptotrichiaceae* can be found in the fecal
541 microbiome of farmed fish (Moncada et al., 2019; Kopprio et al., 2021). They contribute to
542 organic matter degradation and have been described as indicators of eutrophic conditions and
543 mariculture activities (Moncada et al., 2019; Kopprio et al., 2021). *Enterobacterales* and

544 *Pseudomonadales* species were enriched in all depths at fish cage vicinities and commonly
545 associated to the presence of fish production (Blancheton et al., 2013; Martins et al., 2013;
546 Aylagas et al., 2017; Algammal et al., 2020; Haro-Moreno et al., 2020; Pan et al., 2021). For
547 instance, *Pseudomonas* and *Vibrionaceae* species (*Vibrio* and *Aliivibrio*) were abundant at the
548 bottom and middle waters of the Lease 1, and at the surface of Lease 2, possibly due to their
549 metabolic diversity being capable of inhabiting different aquatic habitats (Aylagas et al.,
550 2017). They are also potential opportunistic pathogens of salmonids (Wakabayashi, 1980;
551 Loch et al., 2012). *Aliivibrio* bacteria were found in the gut microbiome of salmon grown in
552 Tasmania (Zarkasi et al., 2016) and have been correlated to a poor growth performance of
553 Atlantic salmon (Bozzi et al., 2021).
554
555 The ASVs found in our study are of particular interest for the aquaculture industry since they
556 can cause serious losses to the sector affecting fish and human health (Novotny et al., 2004).
557 It is important to highlight the absence or low abundance of these pathogens in the control
558 sites, indicating a localised impact due the presence of the fish cages. Focusing on these
559 bacteria may allow prediction of outbreaks, design accurate diagnosis approaches, and aid
560 prophylactic measures in aquaculture management to ensure its sustainability (Mustafa,
561 2015). Therefore, the use molecular-based methods of these emerging pathogens could assist
562 efforts to ensure the quality and control of the aquaculture operation.
563
564 **4.4. Functional structure in the context of aquaculture activity**
565 Taxonomic composition of bacterial communities through the use of 16S rRNA gene
566 profiling is useful in showing differences in community composition between sites, as well as
567 providing understanding regarding taxonomic response to the presence of fish cages in the
568 harbour. However, evidence on potential functional composition can provide important

569 insights into the interaction between metabolic pathways and environmental stress
570 (Mukherjee et al., 2017; Wu et al., 2019; Laroche et al., 2021; Raes et al., 2021). PICRUSt2
571 is a computational approach to predict the functional composition of a metagenome from the
572 16S rRNA sequence (Douglas et al., 2020), and has been used widely in previous studies on
573 aquaculture environmental impact (Hornick and Buschmann, 2017; Ortiz-Estrada et al., 2019;
574 Vargas-Albores et al., 2019; Laroche et al., 2021; Qu et al., 2021). By applying PICRUSt2,
575 we are able to explore how the functional community changes with the presence of
576 aquaculture activities.
577
578 Overall, we found that the relative stability of the functional community was greater than the
579 taxonomic community between lease and control sites. The stability detected in this study
580 may be related to the concept of functional redundancy among prokaryotes (Bissett et al.,
581 2006; Bissett et al., 2007; Louca et al., 2016; Hornick and Buschmann, 2017; Louca et al.,
582 2018; Cordier, 2020). On the other hand, Galand et al. (2018) argued that functional
583 redundancy in the marine ecosystem is low and the investigation of microbial functional
584 profiles should be performed using all functional aspects despite partial redundancy not being
585 discarded by the authors. However, deep metagenomic sequencing across many samples is
586 economically prohibitive in many studies. Additionally, metagenome prediction tools can
587 lead to incorrect predictions as a result of gene transfer rate, phylogenetic variability, or genes
588 related to the uncultured microorganisms. Also, the performance of these tools for inference
589 on genes associated to “housekeeping” function drops significantly in environmental samples
590 (Langille et al., 2013; Sun et al., 2020).
591
592 In this study, PICRUSt2 inferred a methanogenesis pathway from bacterial 16S rRNA
593 amplicon sequencing data. It is well known that methanogenesis is only seen in the archaeal

kingdom. Although only bacterial sequences were used as input, this inference could be related to an incorrect clustering with reference archaeal sequences. For these reasons, results from functional prediction tools are useful and insightful but should be interpreted with caution (Douglas et al., 2020). The differences were more pronounced in the near-bottom water layers of leases areas where enriched pathways were associated to organic compounds and vitamin degradation, fermentation, methanogenesis as well as antibiotic resistance. These pathways could be related to the altered surrounding environment caused by the presence of fish which is consistent with studies on functional alteration along a pollution gradient (Galand et al., 2016; Cordier et al., 2019). The use of taxonomic and predicted functional profiles allowed us to explore both taxonomic and function of bacterioplankton communities. Despite associated weaknesses and limitations, both are valuable methods for evaluating the changes in the environment caused by aquaculture farms in Macquarie Harbour.

4.5. Natural physicochemical variation as an important driver of the bacterioplankton community

It is important to note a greater number of differentially abundant ASVs associated with the Lease 1 local area, particularly in the near-bottom waters, compared to Lease 2 and control locations. The difference could be related to the production (e.g., stock density, faeces), management practices (e.g., feed input rate), hydrodynamics and location (e.g., shallow or deeper waters) of that specific lease (Belias et al., 2003; Sarà et al., 2007; Sanderson et al., 2008; Tsagaraki et al., 2011; Jansen et al., 2016). Hydrodynamics and location of both leases could be important factors explaining this difference. While Lease 1 is located near the harbour's southern end, Lease 2 is further north, near the deepest basin with a depth of approximately 52 m. Macquarie Harbour is a fjord-like system with periods of deep-water renewal entering by a narrow and shallow entrance located at the harbour's northern end. Due

619 to its geological formation, and freshwater, seasonal or weather-related influences, the
620 distribution of the marine water intrusions are heterogeneously distributed leading to a less
621 frequent and less pronounced deep water renewal events in the southern harbour (Hartstein et
622 al., 2019; Ross et al., 2021). Also, Teasdale et al. (2003) revealed increasing organic carbon
623 and sulphide concentrations from north to south which was influenced by riverine inputs.
624 This north-south environmental gradient may affect the oxygen and organic matter
625 concentration in the water column in the Lease 1 area, causing changes in the environmental
626 conditions as we observed in our study. Although our findings suggest bacterioplankton as a
627 potential indicator for aquaculture activities, more work is needed to confirm its reliability
628 over time in the context of natural variation. Understanding seasonal change in the bacterial
629 community across the harbour will be particularly important to improve our capacity to detect
630 disturbances caused by both natural and anthropogenic environmental changes (Allison and
631 Martiny, 2008; Magurran et al., 2010; Lindh et al., 2015).
632

633 **5. Conclusions**

634 In summary, investigation of bacterioplankton communities and predicted functions in water
635 column revealed localised changes in the presence of salmon cages. Local enriched taxa were
636 related to biochemical processes (i.e., sulphur, nitrogen, methane, and carbon), potential
637 pathogens or members of the fish gut microbiome, while the relative abundance of function
638 annotations was associated with organic compounds and vitamin degradation, fermentation,
639 methanogenesis and antibiotic resistance. Our study demonstrates that molecular-based
640 methods could be a valuable tool in monitoring aquaculture effects. Trends in nutrient
641 concentrations were likely be masked by the natural environmental variation found in the
642 water column which also has a strong influence on the distribution of the bacterial taxonomic
643 and functional communities. The robustness of the indicators suggested by this study could

be further validated through sampling across both season and farm production cycle to examine temporal variation in taxonomic and functional bacterioplankton communities. Overall, the study showed that the 16S rRNA sequencing and functional annotation of the bacterioplankton communities have the potential to becoming effective tools in understanding the interaction between finfish aquaculture and the pelagic environment.

6. Author contributions

RRPS, CW and JR: Conceptualization. **RRPS**: DNA extraction, Formal analysis, Writing- Original draft preparation. **CW, JR and JB**: Supervision, Writing- Reviewing and Editing**.**

7. Declaration of competing interest

We declare that the submitted work was conducted in the absence of any commercial or financial relationships that could potentially be construed as a conflict of interest.

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