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Nitrogen-cycling genes in oyster reefs and surrounding sediments: Relationships with environmental factors and respective nitrogen rates

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ABSTRACT

To investigate nitrogen (N) cycling in oyster reef habitats along the East coast of Australia, we assessed N-cycling gene abundances in oyster shell biofilms and surrounding sediments, and explored their correlation with environmental factors and respective N rates. We found higher abundances of the denitrification gene *nosZ*II in oyster shell biofilms, while there were not significant differences in the denitrification genes *nirS* and *nirK* between oyster biofilms and sediments. Additionally, oyster shell biofilms had a lower (*nirS* + *nirK*)/*nosZ*II ratio, indicating a greater capacity for N removal and limited nitrous oxide release compared to sediments. Abundance of *nirS*, *nirK*, and dissimilatory nitrate reduction to ammonium (*nrfA*) genes in sediments decreased with increasing content of organic material, suggesting the influence of large-scale environmental conditions. N-cycling gene abundances did not relate to N rates, emphasising the importance of investigating microbial genes to enhance our understanding of the N cycle in oyster reef habitats.

1. Introduction

Microbes such as bacteria and archaea mediate ecosystem processes that drive key ecosystem services, such as nutrient cycling via primary production, mineralisation and decomposition of organic material, and climate regulation through the release of greenhouse gases including carbon dioxide (CO_2) and nitrous oxide (N_2O) into the atmosphere (Ducklow, 2008; Falkowski et al., 2008; Gupta et al., 2017; Tiedje et al., 2022). Conversely, microbial communities and their contributions to ecosystem processes are influenced by a multitude of abiotic factors including temperature, pH, substrate and oxygen availability (Louca et al., 2016; Hou et al., 2017; Abdul Rahman et al., 2021). Microbial communities and their functions are also known to be influenced by biotic factors, such as host type and host physiological conditions (Penn et al., 2006; Zilber-Rosenberg and Rosenberg, 2008; Marzinelli et al., 2015; Woodhams et al., 2020), and relationships can change depending on abiotic factors, such as the interaction between host species and geography or depth (Olson and Gao, 2013; Woo et al., 2017; Griffiths et al., 2019; Wang et al., 2022). As such, distinguishing how abiotic and biotic factors, as well as their interactions, affect microbial communities and their functions is crucial to our understanding of the delivery of ecosystem services.

In coastal marine ecosystems, microbes experience a range of ecological conditions, driven by great environmental variation as well as the presence of biogenic habitats such as seagrass, mangroves, and shellfish reefs. For example, salinity fluctuations that can occur daily (e.g. tides) or at longer time scales (e.g. seasonality in rainfall patterns; Fortunato et al., 2012; Orvain et al., 2014; Lisa et al., 2015; Franklin et al., 2017). Human activities can also modify environmental conditions through the release of organic materials and nutrients (Kennish, 2005). Moreover, the presence of biogenic habitats can interact with microbial communities by serving as hosts as well as modifying the

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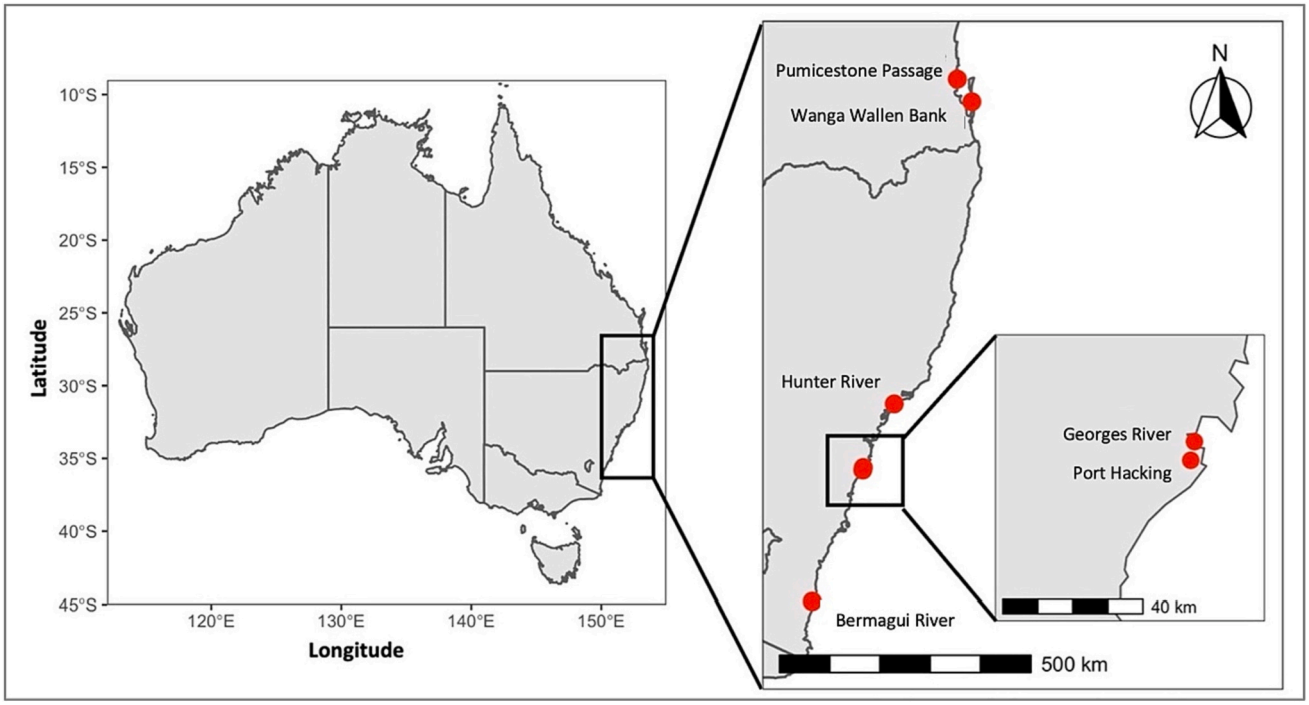

Fig. 1. Map of Australia and the six sampling sites (indicated by the red dots) on the East coast of Queensland and New South Wales. From North to South: Pumicestone Passage (−27.050544, 153.120249); Wanga Wallen Bank (−27.456107, 153.424952); Hunter River (−32.88243, 151.789136); Georges River (−34.024092, 151.135189); Port Hacking (−34.071942, 151.123659); Bermagui River (−36.421546, 150.056463). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

surrounding environments (e.g. sediments), affecting microbial functions (Ikenaga et al., 2010; Bourque et al., 2015; Feinman et al., 2018). For example, oyster reefs can host a diverse range of microbes involved in biogeochemical cycling on their shells while at the same time stimulate microbial activities in surrounding sediments through the addition of faeces and pseudofaeces, enhancing nitrogen (N) cycling (Filippini et al., 2022).

Oysters act as benthic-pelagic couplers, taking in pelagic food particles and releasing faeces and pseudofaeces (biodeposits) rich in high labile organic carbon (C) and N. These biodeposits accumulate on the surrounding sediments, thereby increasing the availability of organic material, which in turn facilitates microbial metabolism and N-cycling (Filippini et al., 2022). Additionally, oysters' excretion of ammonium (NH_4^+) provides substrates for N-cycling microbial communities on their shells (Welsh and Castadelli, 2004; Caffrey et al., 2016). Previous studies on N-cycling in oyster reefs have mainly focused on quantifying the amount of dinitrogen gas (N_2) released by the system (e.g. sediments surrounding reefs; Ray and Fulweiler, 2021a; Filippini et al., 2022). Specifically, N_2 fluxes were found to vary significantly across different components of the oyster reefs, such as the oysters themselves and the sediments surrounding these reefs (Filippini et al., 2023). Conversely, very little is known about how microbial communities and their functions differ between oysters and surrounding sediments.

In-situ or *ex-situ* incubation chambers/cores have been utilised to measure biogeochemical fluxes (e.g. exchange of N across the sediment-water interface) in different systems (e.g. Eyre and Ferguson, 2005; Roth et al., 2019). However, these ecosystem processes are fundamentally regulated by the genes found in microbial genomes. These genes undergo transcription and translation processes, ultimately resulting in protein synthesis and enabling enzyme-catalysed chemical reactions (Crick, 1958). Therefore, the identification and quantification of genes can be used to understand changes in microbial ecosystem processes and the mechanisms behind such changes. These techniques include metagenomics (coupled with bioinformatic tools like PICRUST or Tax4Fun; Langille et al., 2013; Aßhauer et al., 2015), microarrays (He et al., 2007; Yergeau et al., 2007), and quantitative polymerase chain reaction (qPCR; Ligi et al., 2014; Lammel et al., 2015). By quantitatively linking microbial genes to biogeochemical fluxes there is the potential to improve the predictive capacity of N-cycling, and better understand the influence of environmental factors on microbial N functions associated with oyster reefs. Moreover, it can potentially help disentangle the role of microbial communities on N-cycling from that of environmental factors.

N-cycling is a complex process consisting of several microbial pathways including those that fix N_2 (i.e. N fixation), those that retain N in the system, e.g. dissimilatory nitrate reduction to ammonium (DNRA), and those that remove N from the system, e.g. denitrification. Each of these pathways are mediated by at least one microbial gene (Damashek and Francis, 2018). N fixation is a one step process where N_2 is converted into NH_4^+ via the nitrogenase enzyme (e.g. gene marker *nifH*); and DNRA has two steps whereby nitrate (NO_3^-) is firstly reduced to nitrite (NO_2^-; e.g. gene marker *narG*) and successively into NH_4^+ via the ammonia-forming nitrite reductase (i.e. gene marker *nrfA*). Denitrification is a four-step metabolic process that converts NO_3^- to N_2, and its associated genes encode functions for nitrate (e.g. gene markers *narG* or *napA*), nitrite (i.e. gene markers *nirS* and *nirK*), nitric oxide (e.g. gene marker *norB*), and nitrous oxide (i.e. gene marker *nosZ*) reduction (Zumft, 1997). This reaction is a modular process in that different microbes can harbour different subsets of genes, allowing them to engage in distinct stages of the pathway (Zumft, 1997; Graf et al., 2014). Each of these microbial pathways in the N cycle can be influenced by a wide range of interactions between habitat types and environmental conditions. For example, in reduced conditions, N removal via denitrification is enhanced by NO_3^- availability and organic matter supply (Cornwell et al., 2014; Bartoli et al., 2021). In anoxic/eutrophic environments, N retention via DNRA may be upregulated compared to denitrification under conditions with low NO_3^- availability (Burgin and Hamilton, 2007). While NO_3^- availability is observed to affect the rates of these processes, this has not always resulted in a change in microbial communities contributing to these processes (Wallenstein et al., 2006).

Table 1
Primer pair sequences and amplicon size of the genes investigated in qPCR.

Gene	Primers	Sequences	Amplicon size	References
nirS	Cd3aF	GTSAACGTSAAGGARACSGG	409 bp	Throbäck et al., 2004
	Rc3d	GASTTCGGRTGSGTCTTGA		
nirK	F1aCu	ATCATGGTSCTGCCGCG	472 bp	Throbäck et al., 2004
	R3Cu	GCCTCGATCAGRTTGTGGTT		
nosZ	nosZ2F	CGCRACGGCAASAAGGTSMSSGT	267 bp	Henry et al., 2006
	nosZ2R	CAKRTGCAKSGCRTGGCAGAA		
nrfA	nrfA6F	GAYTGCCAYATGCCRAAAGT	220 bp	Takeuchi, 2006
	nrfA6R	GCBKCTTTYGCTTCRAAGTG		
nifH	nifH-F	AAAGGYGGWATCGGYAARTCCACCAC	460 bp	Rösch et al., 2002
	nifH-R	TTGTTSGCSGCRTACATSGCCATCAT		

Understanding factors that facilitate one microbial pathway over another is fundamental for estimating N budgets. This knowledge is particularly important for managing N in coastal marine systems subjected to excessive N inputs derived from anthropogenic activities such as the use of fertiliser and burning of fossil fuels.

In this study, we used gene marker analysis (via qPCR) to study how microbial functional capacity can affect N-cycling in oyster reefs present on the East coast of Australia, and whether those explain the previously observed differences in N-cycling between oyster reef components (oysters vs surrounding sediments). Specifically, we aimed to (i) compare the abundances of N-cycling genes between oyster shell biofilms (hereafter, oyster biofilms) and sediments surrounding oyster reefs (hereafter, sediments); and (ii) examine relationships among N-cycling gene abundances, environmental factors (e.g. water column inorganic N concentrations and content of sediment organic material), and N fluxes (e.g. N_2 and NH_4^+ fluxes). We hypothesised that the abundance of N-cycling genes would be higher in oyster biofilms compared to sediments as suggested by Arfken et al. (2017), and would reflect previously observed patterns in N fluxes (Filippini et al., 2023). While we did not expect a strong correlation between the abundance of N-cycling genes and water column inorganic N concentrations in both oyster biofilms and sediments, we predicted that the abundance of these genes would be positively correlated with organic matter content (in sediments only). Furthermore, as observed in previous studies (e.g. Bowen et al., 2014; Lindemann et al., 2016), we postulated that the abundances of N-cycling genes would not necessarily be correlated with their respective N fluxes such as denitrification genes with N_2 fluxes and DNRA genes with NH_4^+ fluxes.

2. Materials and methods

2.1. Study sites

During January and May 2021, we sampled oyster reefs in six sites spanning a latitudinal gradient of 10 degrees along the East coast of Australia. Two sites (Pumicestone Passage and Wanga Wallen Bank) were in South Queensland and four sites (Hunter River, Georges River, Port Hacking, and Bermagui River) were in New South Wales. Of these, two sites (Georges River and Port Hacking) were in the Sydney Metropolitan Region (Fig. 1) and were the closest in proximity, separated by 5 km. Pumicestone Passage was the northernmost site, while Bermagui River was the southernmost, resulting in a span of 1080 km.

At all sites, oyster reefs (mainly formed by populations of the Sydney rock oyster; *Saccostrea glomerata*) were consolidated forming three-dimensional structures (McAfee et al., 2020), except Wanga Wallen Bank, where numerous small clumps of oysters formed unconsolidated reefs over sediments (i.e. oyster bed). The oyster reefs sampled were mainly located in estuary channels (Pumicestone Passage, Hunter River, Georges River, Port Hacking, and Bermagui River), and only the Wanga Wallen Bank bed was situated in a shallow sloping sand area outside the main channel.

2.2. Experimental design and sampling

To study microbial capacity for N-cycling in oyster biofilms and sediments, and link these to environmental factors and respective N fluxes, we randomly collected five oyster clumps of similar sizes from the reefs and selected five unvegetated sediment plots located within 2 m of the reefs at each site (Fig. S1). Using dark and clear *in-situ* incubation chambers (surface area 0.18 m^2, volume ~ 29 l) as described in Filippini et al. (2023), we measured water column environmental parameters and N fluxes for each oyster clump and sediments surrounding oyster reefs (Fig. S1). Briefly, each oyster clump was placed on a circular acrylic plate attached to the chamber base to exclude sediment effects during incubation, whereases circular metal chamber bases were inserted ~10 cm into the selected sediment plots. Incubations were run over two days, with dark and light incubations conducted the same day for approximately 1.15 and 2 h each - depending on tidal period. At the start and end of each incubation, water samples were collected and subsequently poisoned with 20 μl of 7 % $HgCl_2$ to measure dissolved N_2-N, or filtered through 45 μm cellulose acetate filters to measure inorganic N concentrations: NH_4^+, NO_3^-, and nitrite (NO_2^-). During incubations, temperature parameters were measured with a PME miniDOT® (PME, Inc.) logger located in the top port of the chambers. Salinity was collected between dark and light incubations at each site using a calibrated multiparameter water quality sonde (YSI, Inc., Yellow Springs, OH, United States).

After incubations and during low tide (<0.80 m water depth), oyster biofilms and sediments were sampled for microbial marker gene analysis. Specifically, oyster biofilms were sampled by swiping two sterile cotton swabs over the shell of three random Sydney rock oysters from each oyster clump. Cotton swabs were then placed into 2 ml cryogenic vials. Cryogenic vials (5 ml) were used to collect surface sediments from each unvegetated sediment plot surrounding oyster reefs. Oyster biofilm and sediment samples were stored on ice in the field and at −80 °C in the lab. The top 2 cm of sediment was collected using a glass jar (600 ml) for grain size, organic material content, and total nitrogen analyses. These samples were kept cool in the field and frozen at −20 °C in the lab.

2.3. DNA extraction

From each sample type (oyster biofilms and sediments) collected at each site, we randomly selected three subsamples out of five for molecular analyses. Specifically, DNA was extracted from three oyster biofilm (two swabs) and three sediment (0.25 g) samples from each site using the QIAGEN DNeasy PowerSoil Pro Kit (catalog: 47016), following the manufacturer's protocols. Overall, we obtained 36 samples (18 from each sample type) of extracted DNA. Extracted DNA was stored at −80 °C until further analyses.

2.4. Primers selections, product specification, and qPCR assays

Absolute qPCR was used to determine the abundance of N-cycling genes in oyster biofilms and sediments. For qPCR analysis, we firstly

Table 2
Primer concentrations and qPCR conditions used for each gene.

Gene	Primer concentration (nM)	Annealing temperature and time	Extension temperature and time	Holding temperature and time (data collection)	Number of cycles
nirS	200	54 × 30 s	72 × 5 s	80 × 10 s	40
nirK	300	60 × 30 s	72 × 5 s	80 × 10 s	40
nosZ	300	60 × 30 s	72 × 1 s	NA	40
nrfA	200	53 × 30 s	72 × 1 s	76 × 10 s	50
nifH	200	61 × 30 s	72 × 5 s	81 × 10 s	40

selected several existing primer sets targeting N-cycling genes of bacterial and archaeal DNA (Table S1). Then to ensure the specificity of the primers with our samples, we conducted validation using melting curve analysis and gel electrophoresis, followed by Sanger sequencing analysis that was completed at the Ramaciotti Centre for Genomics (University of New South Wales, Australia). It was not possible to perform further qPCR analysis for the *norB* (denitrification pathway), *nosZ* clade I (denitrification pathway), *amoA* (bacteria and archaea; nitrification pathway), *hzo* and *hzsA* (anammox pathway) genes due to the lack of product specificity in our samples (e.g. multiple peaks and/or bands following melt curve and gel electrophoresis analysis respectively). In summary, we were able to quantify the abundance of the following genes: *nirS*, *nirK*, and *nosZ* clade II (denitrification pathway), *nrfA* (DNRA), and *nifH* (nitrogen fixation) in oyster biofilms and sediments via qPCR using primers summarised in Table 1. It is important to note that the *nosZ* clade II (*nosZ*II) gene is also carried by non-denitrifying microbes (Hallin et al., 2018). Therefore, by targeting this gene, we were able to capture measurements of both denitrifying and non-denitrifying populations with the ability to reduce N_2O to N_2.

The qPCR reactions were carried out in a final volume of 10 μl which contained 5 μl of KAPA SYBR® FAST qPCR Master Mix (Roche), 0.2 or 0.3 μl of each forward and reverse primer (200 or 300 nM respectively; Table 1), 1 μl of template DNA (<20 ng), and nuclease free water (to volume). Thermocycling was conducted with a LightCycler® 480 (Roche), and the conditions used included an initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s, with gene-specific temperatures and times for annealing, extension and holding steps as indicated in Table 2. The specificity of target DNA amplification was checked by including controls lacking DNA template (no-template control) and by a melting analysis (95 °C for 5 s, 65 °C for 1 min and a continuous fluorescence detection at 97 °C). Each 384-well plate contained triplicates of no-template controls, standards, and samples. Quantification of each gene in oyster biofilm and sediment samples were estimated from at least four-point serial curve generated from artificially synthesised gBlock Gene Fragments (Integrated DNA Technologies) used as standards. Each gBlock fragment was designed to contain the amplicon of interest (for specific features of each gBlock fragment, see Table S2). Obtained copy numbers were normalised to DNA extraction yield as DNA concentration varied between oyster biofilm and sediment samples. A coefficient of variation (CV) was also calculated for the technical replicates and samples, where samples with CV > 2 % had a replicate removed from the analysis. Amplification efficiencies ranged from 82 to 87 % for *nirK*, *nosZ*II and *nrfA*; and from 92 to 99 % for *nirS* and *nifH* reactions. All standard curves had an R^2 of 0.99 except for *nirS*, which was 0.98. The abundances of denitrification genes (*nirS*, *nirK*, and *nosZ*II) were also used to determine the ratios of *nir* to *nosZ* genes in the microbial community present in oyster biofilm and sediments. These ratios were used as indicators of the microbial community's ability to facilitate N removal, with higher ratios (e.g. >1) potentially indicating N_2O accumulation and subsequent emission (Pereira et al., 2015). We reported the ratios of *nirS*/*nosZ*II, *nirK*/*nosZ*II, and (*nirS* + *nirK*)/*nosZ*II abundances because individual microbial species typically possess either the copper-based nitrite reductase (*nirK*) or the cytochrome-based nitrite reductase (*nirS*), but not both (Throbäck et al., 2004). Our ratios included the *nosZ*II and not the *nosZ*I because of the lack of product specificity found for this gene.

2.5. Sediment physiochemistry, water column characteristics and N fluxes

Methodologies used to estimate the content of fines (%Fines), percentage of sediment organic content (%OC), content of total nitrogen (%TN), C:N ratio, dissolved N_2-N concentrations, water column inorganic N concentrations (NH_4^+, NO_3^-, and NO_2^-), and the formula used to calculate fluxes are described in detailed in Filippini et al. (2023). Briefly, grain size distributions were assessed using a laser particle size analyser and %Fines was obtained using the package "G2sd" in R (Fournier et al., 2014). The %OC was measured by loss on ignition (LOI) method and the %TN as well as C:N ratio were determined by using a CHNS-O Elemental Analyser. Dissolved N_2-N concentrations were analysed using the N_2/Ar technique (Kana et al., 1994) and a Membrane Inlet Mass Spectrometer (MIMS). N_2/Ar values were obtained using the 'mimsy' package in R (Kelly, 2020) and subsequently multiplied by the theoretical Ar concentration of the sample given its temperature and salinity to estimate sample N_2-N concentration (Ray and Fulweiler, 2020). Inorganic N concentrations were analysed according to APHA-4500 (2017).

To provide a comprehensive understanding of the relationship between the abundance of the different N-cycling genes studied and overall concentrations in the water column and fluxes of N forms, net concentrations of NH_4^+, NO_3^-, and NO_2^- were determined by averaging measurements taken at the start of each dark and light incubation. Since NO_3^- and NO_2^- concentrations were very low and highly correlated (Pearson's correlation coefficient > 0.80), we combined them to estimate total oxidised nitrogen (NO_x). Nutrient concentrations below detection (<0.005 N mg/l) were treated as 0 for analyses. Net N_2 and NH_4^+ fluxes were calculated by averaging the respective dark and light fluxes. Net N_2 or NH_4^+ fluxes were not calculated when one of the measurements was missing. Net NO_x flux was not estimated as it did not represent a final product of any of the N-cycling genes investigated.

2.6. Statistical analyses

To compare abundances of denitrification (*nirS*, *nirK*, and *nosZ*II), nitrogen fixation (*nifH*) genes, and the *nirS*/*nosZ*II, *nirK*/*nosZ*II and (*nirS* + *nirK*)/*nosZ*II ratios between oyster reef components (oyster biofilms vs sediments), we utilised linear mixed-effects models from the "lme4" package (Bates et al., 2015). We included 'Site' (6 levels: Pumicestone Passage, Wanga Wallen Bank, Hunter River, Shell Point, Port Hacking, and Bermagui River) as a random factor to account for spatial variability. Assumptions of normality and homogeneity of variance were examined and visualised through Q-Q and residual plots, respectively. Data were log transformed to meet the assumptions of the models as well as reduce the range of the data. If deviations were detected, outliers were tested using the Rosner test (EnvStats package in Millard, 2013) and excluded from the models one by one until obtaining a good fit. After running our models, we evaluated the significance of fixed factors using the "anova" function from the package "stats" (R core Team, 2021). We did not compare the abundance of the DNRA gene (*nrfA*) between oyster biofilms and sediments as this gene was only present in one site in the oyster biofilm samples.

Linear mixed-effects models were also used to investigate relationships between the abundances of each gene and ratio, with each environmental variable and respective N transformation rates for oyster

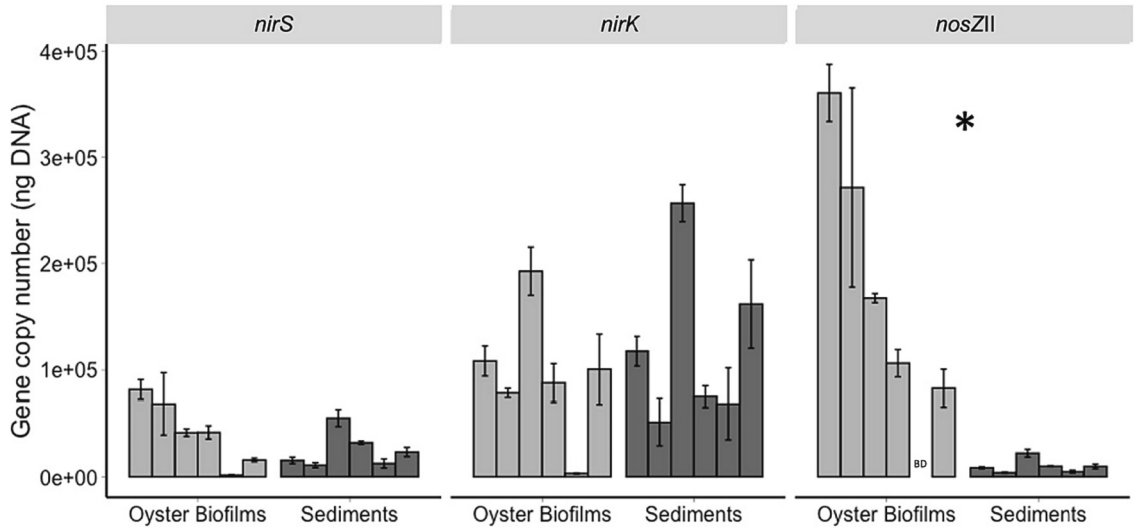

Fig. 2. Number of copies per ng of DNA of the denitrification genes (*nirS*, *nirK* and *nosZ*II) in oyster biofilms (*Light grey*) and sediments (*Dark grey*). Error bars indicate standard error of the mean. *Asterisks* indicate significant differences between the two sample types (oyster biofilms vs sediments; $p < 0.05$). Sites are ordered from North to South: Pumicestone Passage, Wanga Wallen Bank, Hunter River, Georges River, Port Hacking, and Bermagui River. 'BD' stands for below detection.

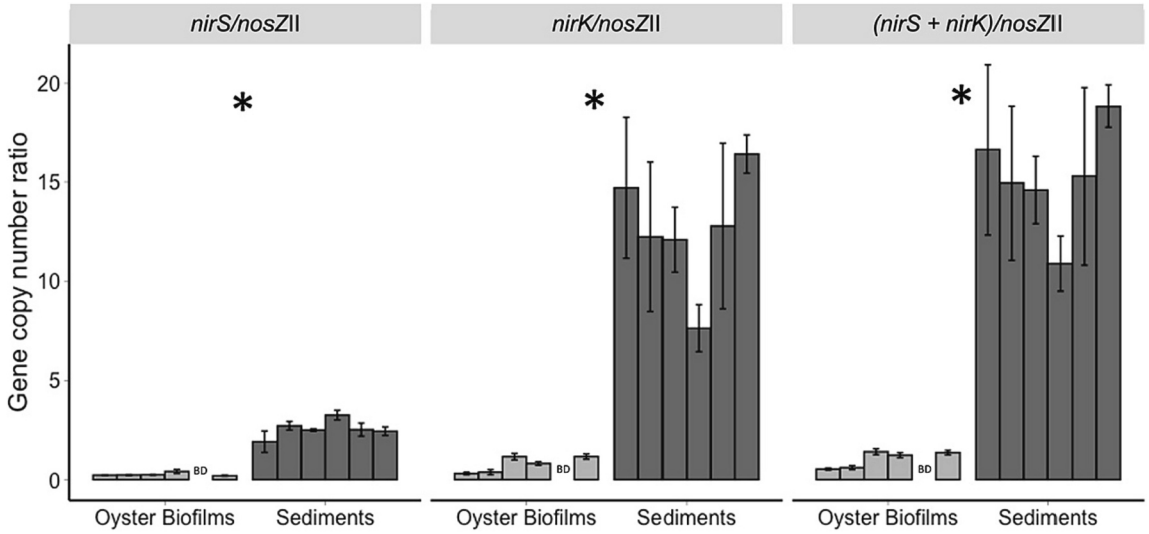

Fig. 3. Ratio of *nirS/nosZ*II, *nirK/nosZ*II, and (*nirS* + *nirK*)/*nosZ*II in oyster biofilms (*Light grey*) and sediments (*Dark grey*). Error bars indicate standard error of the mean. *Asterisks* indicate significant differences between the two sample types (oyster biofilms vs sediments; $p < 0.05$). Sites are ordered from North to South: Pumicestone Passage, Wanga Wallen Bank, Hunter River, Georges River, Port Hacking, and Bermagui River. 'BD' stands for below detection.

biofilms and sediments separately. Site was included as a random factor, and assumptions of the models were checked as described above. When models encountered issues due to a singularity problem, we first tried data transformation. If the problem persisted, we explored alternative approaches by utilising different families within the "glmer" function from the "lme4" package (Bates et al., 2015). In cases where singularity problems continued, we removed the random factor and used the linear model via the function "lm" from the package "stats" (R core Team, 2021). As such, linear models were only conducted to explore the relationships between the ratio *nirS/nosZ*II and temperature in oyster biofilm samples, and between (*nirS* + *nirK*)/*nosZ*II and net N_2 flux. We used Pearson's correlation coefficient from the "PerformanceAnalytics" package to test correlations between environmental factors (Peterson and Carl, 2020). As a result, the variable %TN was not considered in the models as it was highly correlated with %OC (Pearson's correlation coefficient > 0.80; Fig. S2). In summary, for oyster biofilms, we examined the relationships between the abundances of each gene and water column variables (temperature, NH_4^+ and NO_x concentrations). For sediments, we investigated the relationships between the abundances of each gene and both water column (same as above) and sediment (% Fines, %OC, and C:N ratio) factors. For both oyster biofilm and sediment samples, we tested the relationships between the abundance of denitrification genes, including their ratios, and net N_2 flux. Additionally, we examined the relationships between nitrogen fixation gene (*nifH*) and net NH_4^+ flux. Relationships between DNRA (*nrfA*) and net NH_4^+ flux were only tested for sediment samples. Fluxes were scaled using the package "scales" to improve model fit (Wickham and Seidel, 2022). All statistical analyses were conducted in R (version 4.1.0).

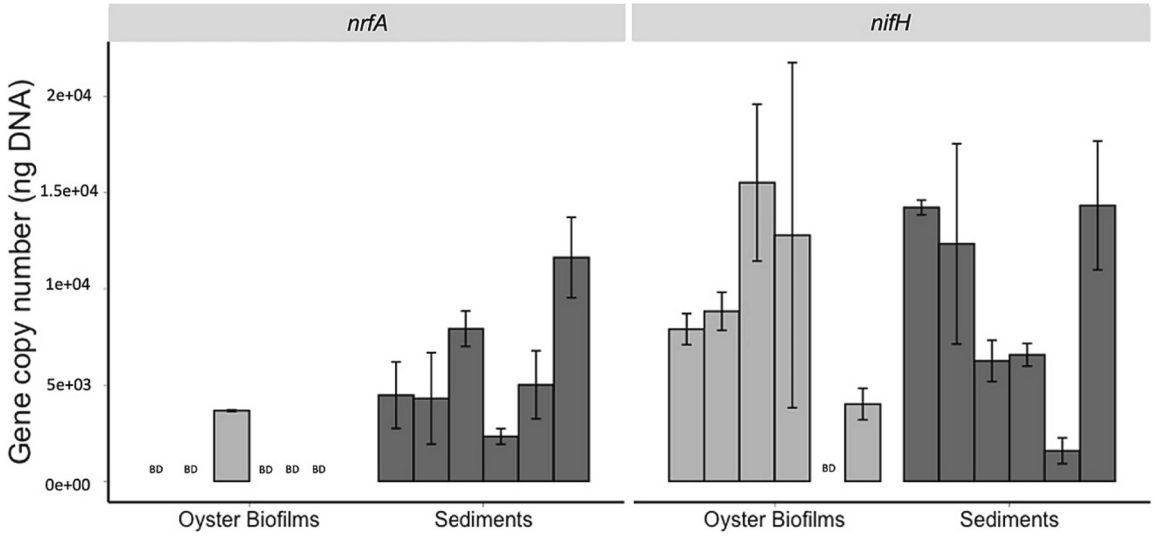

Fig. 4. Number of copies per ng of DNA of the *nrfA* (DNRA pathway) and *nifH* (N-fixation pathway) in oyster biofilms (*Light grey*) and sediments (*Dark grey*). Error bars indicate standard error of the mean. Sites are ordered from North to South: Pumicestone Passage, Wanga Wallen Bank, Hunter River, Georges River, Port Hacking, and Bermagui River. 'BD' stands for below detection.

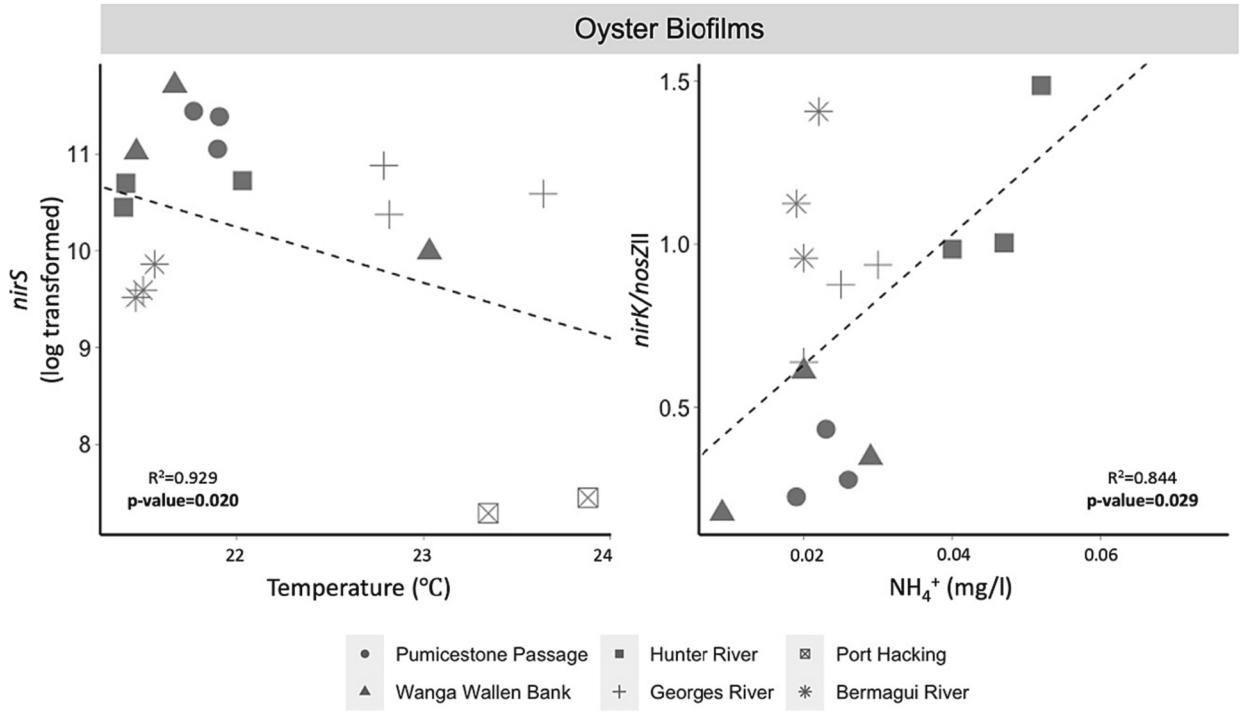

Fig. 5. Significant relationships between gene abundances of *nirS* and ratio of *nirK/nosZ*II and water column environmental parameters in oyster biofilms. Water column variables: temperature and ammonium (NH_4^+). R^2 correspond to the conditional r-squared values for mixed models calculated based on Nakagawa et al. (2017). Gene abundance were 'log transformed' in the models.

3. Results

3.1. Comparing abundances of N-cycling genes between oyster biofilms and sediments

Overall, results showed that the abundances of the N-cycling genes varied between oyster biofilms and sediments (Figs. 2–4; Table S3). Specifically, the *nosZ*II gene had approximately 20-fold higher copies on average in oyster biofilms compared to sediments (Fig. 2, Table S3), and the abundance of the *nirS* gene was almost double in oyster biofilms than in sediments (Fig. 2). Conversely, the abundance of the *nirK* gene was approximately 1.5 times lower in oyster biofilms than sediments (Fig. 2). Despite these patterns, differences in the abundances of the *nirS* and *nirK* genes between oyster biofilms and sediments were not significant (Table S3).

All the *nirS/nosZ*II, *nirK/nosZ*II, and (*nirS* + *nirK*)/*nosZ*II ratios were significantly higher in sediments than in oyster biofilms (Fig. 3, Table S3). Specifically, in sediments, ratios ranged from approximately 2.6 ± 0.14 (mean ± standard error) in *nirS/nosZ*II to 16.94 ± 2.16 in (*nirS* + *nirK*)/*nosZ*II; while, in oyster biofilms, they varied from about 0.26 ± 0.03 in *nirS/nosZ*II to 1.02 ± 0.10 in (*nirS* + *nirK*)/*nosZ*II (Fig. 3).

In oyster biofilms, the abundance of the *nrfA* gene was below

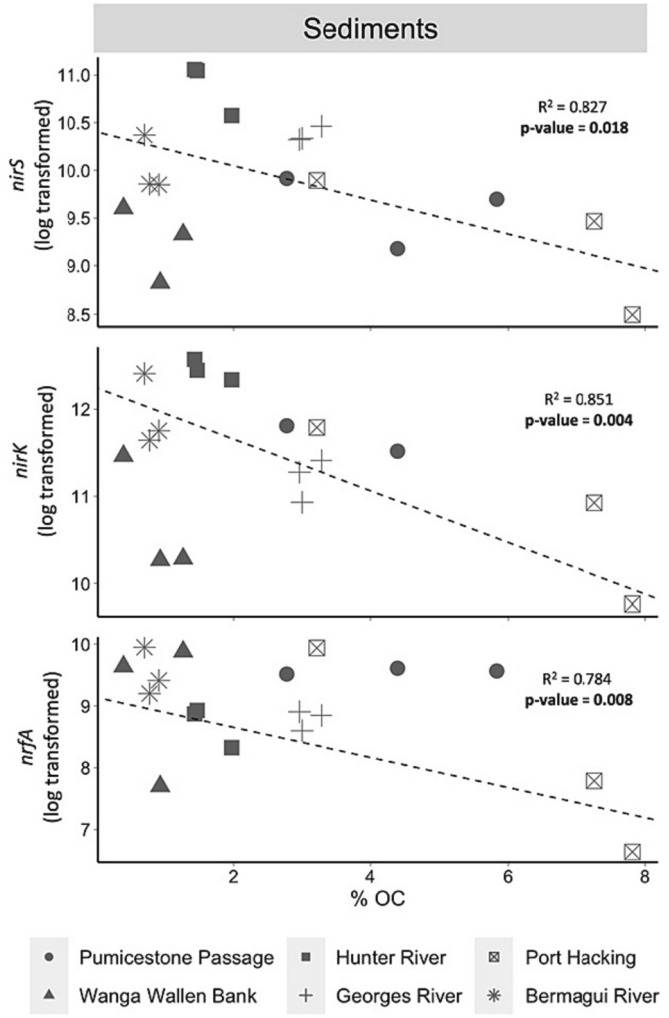

Fig. 6. Significant relationships among gene abundances of *nirS*, *nirK*, and *nrfA* and percentage of organic content (%OC) in sediments. R^2 correspond to the conditional r-squared values for mixed models calculated based on Nakagawa et al. (2017). Genes abundance were 'log transformed' in the models.

detection in all sites except for one (Hunter River), where there was $3.67 \times 10^3 \pm 1.36 \times 10^1$ copies per ng of DNA (Fig. 4). By contrast, *nrfA* was found in all sediment samples with an overall mean of $5.95 \times 10^3 \pm 9.36 \times 10^2$ copies per ng of DNA (Fig. 4).

The abundance of *nifH* gene was similar between oyster biofilms and sediments, with approximately $9.65 \times 10^3 \pm 1.50 \times 10^3$ copies per ng of DNA in both (Fig. 4, Table S3).

3.2. Relationships between abundances of N-cycling genes and environmental factors

Overall, results indicate that some water column environmental parameters (temperature, NH_4^+ and NO_x concentrations) were significantly related to N-cycling genes in oyster biofilms, but not in sediments (Figs. S3–S4, Tables S4–S5). Specifically, in oyster biofilms the abundance of *nirS* denitrification gene showed a negative correlation with temperature, but no significant relationships were found between the denitrification genes (*nirS*, *nirK*, and *nosZII*) and inorganic N concentrations (Fig. 5, Fig. S3, Table S4). Furthermore, in those samples, the ratio of *nirK*/*nosZ*II increased with increasing concentration of NH_4^+ in the water column (Fig. 5, Table S4). On the other hand, the ratios *nirS*/*nosZ*II and (*nirS* + *nirK*)/*nosZ*II were not related to water column environmental parameters (Fig. S3, Table S4). Similarly, the abundance of the *nifH* gene did not show a significant relationship with either temperature, NH_4^+ or NO_x concentrations (Fig. S3, Table S4).

In sediments, the abundances of *nirS*, *nirK* and *nrfA* genes decreased with increasing %OC (Fig. 6, Table S6). However, there were no significant relationships between %OC and either abundance of *nosZ*II and *nrfA* genes or *nirS*/*nosZ*II, *nirK*/*nosZ*II, *nirS* + *nirK*/*nosZ*II ratios (Fig. S5, Table S6). In those samples, no significant relationships were found among the abundance/ratio of the N-cycling genes and %Fines or C:N ratio (Fig. S5, Table S6).

3.3. Relationships between abundances of N-cycling genes and N fluxes

There were no significant relationships between the abundances or ratios of denitrification genes (*nirS*, *nirK* and *nosZ*II) and net N_2 fluxes, or the abundance of *nifH* and net NH_4^+ fluxes in both oyster biofilms and sediments (Figs. S6–S7, Tables S7–S8). Likewise, the abundances of *nrfA* did not show significant associations with net NH_4^+ fluxes in sediment samples (Fig. S7, Table S8).

4. Discussion

N-cycling is underpinned by highly complex microbial reactions, and our understanding of the biotic and abiotic drivers of these processes remains limited. Our study aimed to assess microbial potential to drive N-cycling in oyster reef habitats. In this study, we found a higher abundance of the gene linked to the final step of denitrification (*nosZ*II) in oyster biofilms compared to sediments surrounding oyster reefs, with no significant differences found in the abundances of the other denitrification genes investigated (*nirS* and *nirK*). On the other hand, gene associated with DNRA (*nrfA*) was less abundant (and often absent) in oyster biofilms than sediments (Fig. 7). Furthermore, our findings revealed distinct patterns in N dynamics between oyster biofilms and sediments surrounding reefs, as evidenced by the different ratios between N_2O producers (*nirS* and *nirK*) and reducers (*nosZ*II). While in oyster biofilms a ratio < 1 (observed for *nirS*/*nosZ*II and for *nirK*/*nosZ*II) may suggest the presence of a large community of non-denitrifying microbes carrying the *nosZ*II gene in these systems, in sediments a ratio > 1 may be due to truncated denitrification pathways lacking the *nosZ*II gene (Zumft, 1997; Wood et al., 2001; Jones et al., 2008). Findings align with our previous results showing a higher release of N_2 by oysters compared to sediments surrounding their reefs (Filippini et al., 2023), and suggest that these differences are largely driven by microbial functional capacity.

Our results suggest that oyster biofilms may have greater capacity to reduce N_2O to N_2 (*nir*/*nosZ*II ratio ≤ 1) than sediments surrounding reefs, which may have a higher likelihood of accumulating, and therefore releasing, greenhouse gases due to a *nir*/*nosZ*II ratio > 1. Interestingly, García-Lledó et al. (2011) also showed a higher (*nirS* + *nirK*)/*nosZ* ratio in vegetated than in unvegetated sediments, due to higher availability of organic material and N. Similarly, the accumulation of oyster biodeposits in nearby sediments may select for the higher abundance of *nir* genes. Thus, while the role of oyster reefs as carbon sinks remains uncertain (Fodrie et al., 2017), sediments next to them might have the potential to be a source of the greenhouse gas N_2O, thus having a negative climate impact. However, other studies measuring N_2O fluxes have found that emissions of this greenhouse gas are generally low and sometimes negative (i.e. sink) in sediments under farmed oysters (Erler et al., 2017; Ray et al., 2019; Ray and Fulweiler, 2021b). Therefore, future research is needed to better understand the role of sediments surrounding oyster reefs in the release of N_2O.

We observed a higher abundance of *nosZ*II in oyster biofilms than sediments (Figs. 2 and 7). Similarly, Arfken et al. (2017) reported higher abundance of the *nosZ*I gene in oysters and their shells compared to sediments. Greater abundance of the *nosZ* gene (clade II in our case) in oyster biofilms may suggest a specific interaction between microbes carrying this gene and the host (oyster). One possible explanation for

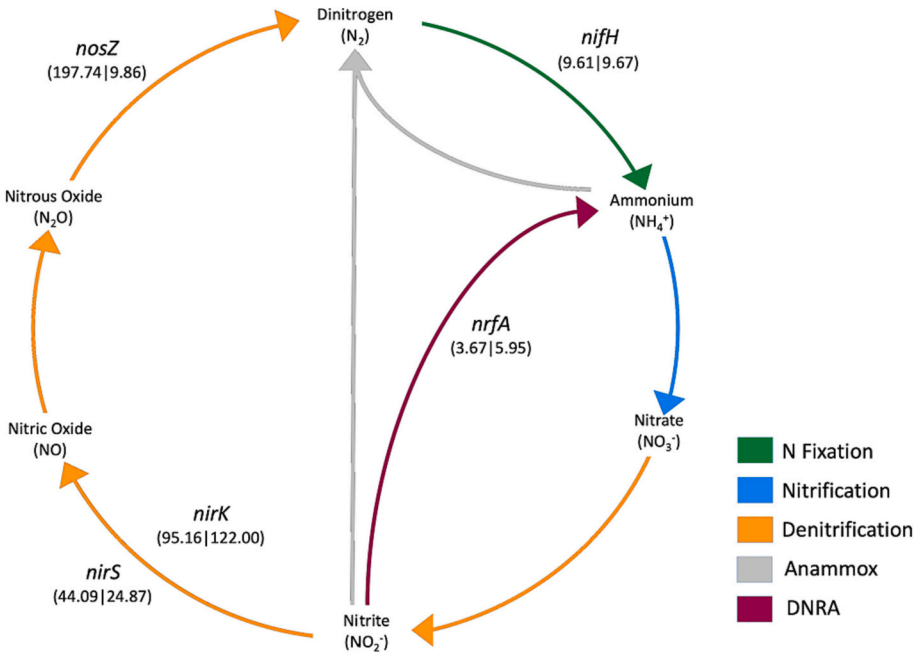

Fig. 7. Conceptual diagram showing the nitrogen (N) cycling processes with the genes examined in this study (*nirS*, *nirK*, *nosZ*II, *nrfA* and *nifH*) and respective products. The two values beneath each gene represent the mean of the gene abundance measured in oyster biofilms and in sediments surrounding oyster reefs (i.e. oyster biofilms | sediments). Values were normalised by dividing them by 1000. Colours represent different processes: nitrogen (N) fixation in green; nitrification in blue, denitrification in orange, anammox in grey, and dissimilatory nitrate reduction to ammonium (DNRA) in purple. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

this pattern might be the characteristic of the substratum, which can significantly influence microbial attachment rates (Wahl et al., 2012). Shellfish shell surfaces offer a complex structure with crevices and pores that create heterogenous oxygen gradients (Heisterkamp et al., 2013), providing a suitable habitat for the colonisation of diverse aerobic and anaerobic microbes (Pfister et al., 2014; King et al., 2021). Sediments exhibit a different oxygen profile, usually with higher oxygen availability in the uppermost layers that quickly decreases with depth (depending on sediment composition or presence of infaunal communities). Furthermore, a previous study revealed the *nosZ*II to be more prevalent and active in more oxygenated environments (Sun et al., 2017). Thus, the variation in oxygen gradients between oyster biofilms and sediments may help to explain the higher abundance of the *nosZ*II gene found in the oyster biofilms. While our sampling of oyster biofilms likely captured both aerobic and anaerobic microbes, the sediment sampling targeted the top 2 cm which may have contained mostly anaerobic microbes, and hence less *nosZ*II. It is important to note that our findings specifically apply to clade II of the *nosZ* gene, and may not be applicable to clade I. Clade I and II of the *nosZ* genes are different in protein physiology and responses to different environmental factors such as oxygen availability (Wittorf et al., 2016), which may result in distinct occurrences among habitat types (Sanford et al., 2012; Jones et al., 2013; Graf et al., 2014).

Although we hypothesised that in sediments an increase in organic material content due to the addition of oysters' biodeposits would positively correlate with N-cycling genes by providing substance and energy for microbes such as denitrifiers, our findings did not support this. Instead, we found that the abundance of these genes decreased with increasing organic content, as opposed to previous studies conducted across a wide range of ecosystem types such as estuaries and soil (e.g. Kandeler et al., 2006; Lindemann et al., 2016; Fan et al., 2019; Tao et al., 2021). This could be because microbial communities and their functions are known to be influenced by both small- and large- scale environmental conditions (Orland et al., 2020). Thus, while results from previous studies (mainly undertaken within one or a few sites) might have reflected an increased influence of small-scale environmental conditions (e.g. difference in sediment composition, chemistry and depth from within an estuary), our study covered a larger spatial scale. Hence, it is possible that our results might have been additionally driven by large-scale environmental conditions (e.g. differences in climate, hydrodynamics or sedimentation rates among estuaries), as well as ecological processes occurring at larger scales, such as species pool and connectivity.

Interestingly, we found that in oyster biofilms, the *nirK*/*nosZ*II ratio significantly increased with the concentration of NH_4^+ in the water column. A similar trend (although not significant) was found when considering the (*nirS* + *nirK*)/*nosZ*II ratio and this nutrient, but not with *nirS*/*nosZ*II. Thus, it might be possible that higher concentration of NH_4^+ in the water column affects the composition of the microbial communities and therefore their functions present on oyster shells, resulting in a greater proportion of microbes carrying the *nirK* within the community (Clark et al., 2020; Nordström et al., 2021). Additionally, previous studies have shown that NH_4^+ present in the system can influence the composition of the microbial communities existent in the environment (Lage et al., 2010; Tee et al., 2021; Gao et al., 2022). Thus, while the abundance of individual N-cycling genes may not correlate with inorganic N concentrations in the water column, their abundances relative to other genes may change, as reflected in the *nirK*/*nosZ*II ratio. Our study highlights the importance of exploring the correlations between gene ratios and environmental conditions to get a more comprehensive understanding of the microbial dynamics in the environments.

Findings supported our hypothesis that the abundances of the N-cycling genes in both oyster biofilms and sediments would not correlate with respective N_2 or NH_4^+ fluxes. Our results provide evidence that these relationships are rarely observed and emphasise the importance of avoiding assumptions in this regard (Rocca et al., 2015). Factors that can obscure the relationship between genes and their corresponding processes include: (i) the complexity of biogeochemical processes (e.g. coexistence of multiple, sometimes competing pathways); (ii) the influence of other biotic factors which may interfere with fluxes but not necessarily gene abundance (e.g. primary producers as competitors for remineralised N, infaunal/epifauna communities driving solute

variation); (iii) temporal variation in the presence of genes versus gene expression versus enzymes versus products; and (iii) the potential variability in methodological/molecular techniques (e.g. measurement of fluxes; use of specific primers). For example, N_2 is the final product of denitrification and anammox pathways, and considering genes from just one pathway (e.g. denitrification) may not provide a comprehensive picture. However, we suggest that both gene abundances and flux analyses should be included when studying N processes as they may reflect the status of the ecosystem over a longer and shorter timeframe respectively, and thus provides a more holistic understanding of the functions in the system. Moreover, assessing gene abundances is essential to untangle the role of microbial communities on N-cycling from that of environmental factors. This information is essential to advance our understanding of the drivers of these complex processes.

5. Conclusion

Here, we provide evidence that microbial communities in oyster reef habitats (including their surrounding sediments) have the potential to remove N, but oyster biofilms seem to play a bigger role in denitrification than sediments, supporting findings in our previous study. Our results also suggest that oyster biofilms could enable the reduction of greenhouse gas N_2O to N_2, while sediments surrounding the reefs may contribute to the release of this harmful gas. On the other hand, N fluxes were not explained by microbial functional capacity. Therefore, further studies are needed to disentangle the balance between these processes and the function of microbial communities associated with oyster reefs. These results highlight the complexity of N-cycling processes within these systems, as they reveal the influence of various biotic and abiotic factors on N-cycling.

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CRediT authorship contribution statement

GF, ABB, AF, PEG and KAD conceived the ideas and designed methodologies; GF, ABB, KE and JP collected the data; GF, JP, DV, and NS conducted molecular analysis; GF, ABB, DV, NS, KE, and KAD analysed the data; ABB, DV, AF and KAD supervised GF; GF wrote the first draft of the manuscript; ABB, DV, AF, PEG, NS, KE, JP and KAD revised and edited the manuscript with contributions and inputs from all authors.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2023.115710.

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