Microbial Ecology (2019) 77:343–357
https://doi.org/10.1007/s00248-018-01312-4

MICROBIOLOGY OF AQUATIC SYSTEMS

Bioreactivity and Microbiome of Biodeposits from Filter-Feeding Bivalves

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Received: 27 August 2018 / Accepted: 20 December 2018 / Published online: 5 January 2019

Abstract

Bivalves serve an important ecosystem function in delivering organic matter from pelagic to benthic zones and are important in mediating eutrophication. However, the fate of this organic matter (i.e., biodeposits) is an important consideration when assessing the ecological roles of these organisms in coastal ecosystems. In addition to environmental conditions, the processing of biodeposits is dependent on its composition and the metabolic capacity of the associated microbial community. The objectives of this study were to compare the biological reactivity, potential denitrification rates, and microbial communities of biodeposits sourced from different bivalve species: hard clam (*Mercenaria mercenaria*), eastern oyster (*Crassostrea virginica*), and ribbed mussel (*Geukensia demissa*). To our knowledge, this is the first study to investigate and compare the microbiome of bivalve biodeposits using high-throughput sequencing and provide important insight into the mechanisms by which bivalves may alter sediment microbial communities and benthic biogeochemical cycles. We show that clam biodeposits had significantly higher bioreactivity compared to mussel and oyster biodeposits, as reflected in higher dissolved inorganic carbon and ammonium production rates in controlled incubations. Potential denitrification rates were also significantly higher for clam biodeposits compared to oyster and mussel biodeposits. The microbial communities associated with the biodeposits were significantly different across bivalve species, with significantly greater abundances of *Alteromonadales*, *Chitinophagales*, *Rhodobacterales*, and *Thiotrichales* associated with the clam biodeposits. These bioreactivity and microbial differences across bivalve species are likely due to differences in bivalve physiology and feeding behavior and should be considered when evaluating the effects of bivalves on water quality and ecosystem function.

Keywords Microbiome · Biodeposits · Denitrification · Bivalves · Bioreactivity · Lability

Introduction

Filter-feeding bivalves serve an important function in connecting the pelagic and benthic compartments of aquatic ecosystems. Bivalves improve water quality by filtering particulates from the water column and may also promote nitrogen (N) removal [9, 42]. However, the fate of bivalve biodeposits (e.g., mineralization, burial) requires thorough consideration as rapid biodeposit mineralization can resupply the water column with recycled nutrients, which may fuel additional primary production and thus limit the alleviating effect of bivalves on eutrophication [38]. The fate of bivalve biodeposits is strongly influenced by local environmental conditions such as hydrologic residence time and redox gradients [9]. However, the intrinsic lability or bioreactivity of the biodeposits and the associated microbial community are likely important drivers dictating the rate that organic matter is mineralized and the degree to which biodeposits promote microbial N removal pathways.

By actively delivering organic matter to the sediments through biodeposition, bivalves can significantly affect benthic biogeochemical processes such as organic matter mineralization. Numerous studies have investigated the effect of bivalves on benthic metabolism and nutrient cycling (e.g., [21, 39, 43]). In general, bivalves enhance heterotrophic microbial metabolism in sediments, increasing benthic

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respiration and biogeochemical cycling. In particular, bivalves may fuel denitrification, the step-wise reduction of nitrate (NO_3^-) to di-nitrogen gas (N_2) [22, 42]. Denitrification is ecologically important as it mitigates excess nitrogen enrichment in aquatic systems that may contribute to eutrophication and hypoxia. The potential for bivalves to enhance denitrification has motivated the use of bivalve cultivation and restoration practices as a means to improve water quality [6]. However, the effect of bivalves on denitrification appears to be strongly dependent on environmental conditions and the specific bivalve species [40, 51].

In addition to causing shifts in biogeochemical cycling rates, high densities of bivalves can significantly alter sediment microbial community composition. For example, the invasive dreissenid mussel significantly altered the bacterial communities of lake sediments compared to nearby, uncolonized sediments and specifically promoted microorganisms important in nutrient cycling [29, 33]. Similarly, freshwater sediments associated with an invasive clam, *Corbicula* sp., harbored a higher diversity of bacteria compared to control sediments with no clams [44]. These shifts in sediment microbial community structure and diversity attributed to the presence of bivalves are likely due to a combination of factors. For example, changes in biogeochemical conditions, such as redox gradients, nutrient supply, or organic carbon availability, may result from the bivalve biodeposition, which may subsequently favor specific microbial functional groups (e.g., [13, 14]). However, the microbial community directly associated with bivalve biodeposits may also play a significant role in how biodeposition alters ambient sediment microbial communities.

Biodeposition not only delivers particulate organic matter to the sediments but may also directly inoculate sediments with the microbial community derived from the biodeposits themselves [11]. Researchers are increasingly exploring the microbial communities associated with the tissue and shell of bivalve species (e.g., [2, 34]). However, the microbiome of bivalve biodeposits has not been fully characterized, despite its likely importance in dictating the bioreactivity of biodeposits and determining the degree to which biodeposits fuel denitrification. Although, the rates that biodeposits are decomposed and fuel denitrification is highly dependent on the environmental context, the intrinsic bioreactivity of the organic matter as well as the metabolic capacity of the associated microbial community may also be important.

The objectives of this study were to compare the bioreactivity, potential denitrification, and microbiome of biodeposits sourced from hard clams, eastern oysters, and ribbed mussels. Although clams, oysters, and mussels serve similar ecological functions as filter feeders, their effects on benthic biogeochemical cycling of nutrients and carbon are quite different (e.g., [3, 43, 51]). Although differences in environmental factors likely play a role, it is also possible that differences in feeding behavior and physiology result in different bivalve species producing biodeposits with varying degrees of bioreactivity and associated microbial communities. For example, compared to the hard clam, the eastern oyster and ribbed mussel are known to produce more pseudofeces, or undigested biodeposits, with lower nutrient content [49], particularly when water column total suspended solid concentrations are high [57]. Additionally, due to differences in the size of the cirri in the gills, ribbed mussels and hard clams efficiently retain smaller particles during feeding compare to the eastern oyster [46]. We hypothesized that due to these physiological and behavioral differences among the bivalves the bioreactivity of the biodeposits and the associated microbiomes would be dependent on bivalve species.

Methods

Bivalve populations are ecologically and economically important to the eastern shore of Virginia [18]. In this region, hard clams, *Mercenaria mercenaria*, are naturally found in tidal mudflats and are also commercially produced on aquaculture leases located in shallow, generally protected, environments. Similarly, the eastern oyster, *Crassostrea virginica*, not only is extensively cultured in Virginia but also occurs naturally in subtidal and intertidal reefs as well as fringing along salt marshes. The ribbed mussel, *Guekensia demissa*, is generally found in salt marsh habitats along the eastern shore of Virginia. All three bivalve species can occur in high densities, naturally or in cultivated contexts.

Biodeposit Collection

In July 2014, live adult bivalves, including hard clams (*M. mercenaria*), eastern oysters (*C. virginica*), and ribbed mussels (*G. demissa*), were collected from nearby field locations and brought to the Virginia Institute of Marine Science's (VIMS) Eastern Shore Lab (ESL), Wachapreague, VA. The organisms were gently scrubbed to remove epifauna and separated by species in tanks with continuously flowing unfiltered water (approximately 1 l min^{-1}) sourced from Wachapreague Inlet, which provided a supply of natural phytoplankton to the organisms. Whole water samples flowing into the tanks were collected and analyzed for salinity, chlorophyll, and dissolved nutrients. Biodeposits were allowed to accumulate in the tanks over 4 to 6 h and were subsequently collected using flexible tubes attached to 60-ml syringes. The contents of the syringes were transferred to 50-ml Falcon tubes and centrifuged (3500 RPM for 10 min). Excess water was decanted and the biodeposits were transferred to aluminum dishes, homogenized, and subsampled for organic matter characterization, reactivity incubations, and microbial community analysis.

Biodeposit Characterization

Organic matter content was determined in triplicate for each biodeposit type (i.e., bivalve species) as loss on ignition of dried biodeposits after combustion at 500 °C for 5 h. Prior to combustion, dried subsamples were collected and acidified for analysis on a Carlo Erba (Thermo Electron Flash EA 1112 Series) elemental analyzer for particulate organic carbon and total nitrogen content.

Biodeposit Bioreactivity Incubation

To determine the bioreactivity of the biodeposits, which we define as the rate that the biodeposits are mineralized, we conducted slurry incubations. These bioreactivity incubations were conducted in triplicate for each species and included triplicate control water blanks, which contained no biodeposits. One gram wet weight of biodeposit material from each bivalve species was placed in separate 160-ml serum bottles filled with filtered site water (0.7 μm) and sealed with no headspace. The bottles were incubated in the dark under ambient temperature for 6 days. The water in the incubation bottles was sampled for dissolved inorganic carbon (DIC), dissolved organic carbon (DOC), ammonium (NH_4^+), nitrate plus nitrite (NO_x^-), phosphate (PO_4^{3-}), and dissolved organic nitrogen (DON) on the first and final days of the incubations. On day 5, water samples were collected from the serum vials for DIC and NH_4^+ and sampled water was replaced with additional filtered site water. Not all analytes were measured on day 5 to avoid diluting the bottles with a large volume of replacement water. Rates of production or consumption of each analyte were calculated as

$$\text{Rate} = (m \times V)/W$$

where m is equal to the slope of the linear regression of concentration (μM or mM) versus time since start of the incubation (hours); V is equal to the volume of the incubation bottles (liters); and W is the total biodeposit wet weight in the bottle at the start of the incubation (grams).

Samples collected for NH_4^+, NO_x^-, PO_4^{3-}, DON, and DOC were immediately filtered (0.45 μM Whatman polyethersulfone (PES)) and frozen until analysis. NH_4^+, NO_x^- [31, 50], PO_4^{3-} [24], and DON [26] were analyzed on a Lachat QuikChem 8000 automated ion analyzer (Lachat Instruments Milwaukee, WI, USA). DIC samples, stored in 8-ml hungate tubes (Bellco Glass), were preserved with 15 μl saturated mercuric chloride and kept cold under water until analyzed within 2 weeks of collection using a Li-Cor 6252 infrared gas analyzer [41].

Potential Denitrification Rates

Rates of potential denitrification (i.e., under optimal denitrifying conditions) associated with the biodeposits were measured using a time-series slurry method modified from Thamdrup and Dalsgaard [54] and Koop-Jakobsen and Giblin [25]. One gram wet weight of biodeposits from each bivalve species, in triplicate, was placed in 12 ml exetainer vials filled with 0.7 μm filtered anoxic site water that was N_2 saturated. Each replicate consisted of three vials, one for each time point, to allow for sequential sacrificing over time. The vials were pre-incubated overnight in the dark under ambient temperature to allow for consumption of any remaining $^{14}NO_x^-$ and to ensure anoxic conditions. In the following day, N_2-purged $^{15}NO_3^-$ (98.9 atom%) was added through the septum using a syringe to a concentration of 150 μM. One slurry from each replicate was sacrificed, by adding 200 μl saturated zinc chloride directly to the exetainer at each time point: time zero, 4 h, and 8 h after the $^{15}NO_3^-$ addition. $^{30}N_2$ was measured using a membrane inlet mass spectrometer (MIMS) [20]. The rates were calculated using a linear regression of $^{30}N_2$ over time and normalized for the weight of the biodeposits in the vials (1 g wet weight), as described above.

Microbial Community Composition

Biodeposit subsamples were collected in triplicate for each bivalve species prior to the bioreactivity incubation for characterization of the microbial communities; these samples were stored at − 80 C until amplicon library prep. DNA was extracted using the Qiagen DNeasy PowerSoil DNA extraction kit following the manufacturer's protocol. DNA extractions were quantified fluorometrically using a Qubit 3.0 (ThermoFisher, Waltham, MA) and were normalized to 3 ng μl^{-1} prior to PCR. General bacterial primers, 515F and 806R [8], were used to amplify the 16S rRNA gene; the primers had appropriate Illumina adaptors and individual 12-bp GoLay barcodes were attached to the reverse primers for demultiplexing. Each sample was amplified in triplicate using PCR conditions described in Caporaso et al. [8] and included a negative control. Libraries were confirmed with gel electrophoresis and size selection was completed by gel cutting and purification using the Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). Libraries were quantified with the Qubit 3.0 and pooled in equal molar concentrations for sequencing on the Illumina MiSeq platform using the paired-end 500-cyle kit and V2 chemistry. Sequencing was conducted at the University of Massachusetts Boston. All microbial sequences have been deposited in the Sequence Read Archive under accession number PRJNA504404.

The raw reads were demultiplexed using Illumina-Utils [10]. The DADA2 (v1.7.0) workflow, implemented in R Studio (version 3.4.1), was used to quality filter, cluster, merge

paired-end reads, remove chimeric sequences, and assign taxonomy using the Silva database [7]. To investigate specifically the bacterial communities, archaea, mitochondria, and chloroplasts were removed from the dataset. Given that between 32 and 52% of the total sequences in the original dataset were classified as *Chloroplast* and the fact that different bivalve species are known to feed on select size classes of phytoplankton, we investigated these *Chloroplast* sequences in more detail in a separate analysis from the bacterial community analysis. Since it was not possible to obtain detailed taxonomic information on these *Chloroplast* ASVs using the 16S Silva database, we blasted the representative sequences against NCBI to obtain more informative eukaryotic taxonomic classifications. Downstream data processing and statistical analyses were conducted using the Phyloseq package in R Studio (version 1.23.1) [37].

The genetic potential for denitrification within the bacterial communities was explored with a metabolic inference approach, PAPRICA (pathway prediction by phylogenetic placement), which uses phylogenetic placement onto a reference tree to assign genomic features, such as the presence of denitrification genes. We constructed a customized denitrification gene and genome database as described by Arfken et al. [2] and Semedo et al. [47]. Briefly, 8513 complete and 785 draft bacterial genomes downloaded from GenBank were curated for the presence of *nirS*, *nirK*, *nosZI*, and *nosZII* genes using the KEGG database or gene annotations for completed and draft genomes, respectively. The 4497 reads rarified from each sample in our study were aligned and placed on the phylogenetic reference tree with pplacer [36] to infer potential denitrifying taxa carrying *nirS*, *nirK*, *nosZI*, and *nosZII* genes. The estimated abundances of taxa carrying denitrification genes were calculated based on the normalized number of 16S rRNA gene copies in each sample.

Statistical Analysis

Flux measurements and potential denitrification rates were checked for homogeneity of variance using Levene's test. A one-way analysis of variance (ANOVA) was used to test for differences in rate measurements across species (clam, mussel, oyster). Tukey post hoc tests were conducted to determine significant differences when the ANOVA was significant.

To assess the overall bacterial community and chloroplast community compositions across the bivalve species, Bray-Curtis distance was used to calculate the beta diversity on the filtered, normalized amplicon sequence variants (ASVs) and was visualized using a principal coordinate analysis (PCoA) ordination. Statistical differences between microbial species composition across biodeposits sourced from the different bivalve species were assessed using permutational multivariate analysis of variance (PERMANOVA) tests implemented with the adonis2 function in the vegan package in R (v2.4.6) [1]. Pairwise comparisons across the bivalve species were conducted using DESeq2 to determine the ASVs that were differentially abundant across the bivalve species [35].

Results

Environmental and Biodeposit Characteristics

General environmental conditions including ambient water column nutrient concentrations are provided in Table 1. Biodeposit characteristics prior to and post incubations are provided in Table 2. Prior to the incubation, oyster biodeposits had the highest organic matter content compared to the clam and mussel biodeposits; however, clam biodeposits had a lower C/N compared to oyster and mussel biodeposits. After the bioreactivity incubations, the percent organic C content and total N content decreased across all biodeposit bivalve treatments. The C/N ratio decreased post-incubation indicating that (1) more carbon was mineralized relative to nitrogen, (2) there was an increase in protein content as a result of microbial production, and/or (3), potentially, non-labile humic nitrogen accumulated as a result of humification [45].

Biodeposit Reactivity Experiments

There was significant production of DIC, NH_4^+, and PO_4^{3-} over the course of the incubation from all bivalve species' biodeposits (Fig. 1). Oyster biodeposits were generally less bioreactive than clam and mussel biodeposits as revealed by significantly lower DIC and nutrient production rates (Fig. 1, Table 3). DIC production from clam biodeposits, which averaged 2494.8 (± 118.9) nmol gDW^{-1} h^{-1}, was significantly higher than from oyster biodeposits (1630.0 ± 87.0 nmol gDW^{-1} h^{-1}) but similar to mussel biodeposits, which averaged 2130.0 (± 95.8) nmol gDW^{-1} h^{-1}. PO_4^{3-} production mirrored the trends observed in DIC production. NH_4^+ production associated with the clam biodeposits averaged 281.5 (± 11.9) nmol gDW^{-1} h^{-1} and was significantly greater than that of mussel (198.9 ± 10.7 nmol gDW^{-1} h^{-1}) and oyster biodeposits (123.7 ± 4.8 nmol gDW^{-1} h^{-1}). Rates of NO_x^- production were generally

Table 1 Water column environmental characteristics of Wachapreague Inlet at the time of biodeposit collection (July 2014)

Parameter	Wachapreague Inlet
Chlorophyll (μg l^{-1})	1.51
Temp. (°C)	29
Salinity	31
PO_4^{-3} (μM)	0.13
NO_x (μM)	0.30
NH_4^+ (μM)	1.12

Table 2 Average percent carbon, percent nitrogen, C/N, and percent organic matter of biodeposits collected from Wachapreague, before and after bioreactivity incubations. Standard errors are provided in parentheses; due to limited sample volume, replicate samples for clam biodeposits were not available

		Pre-incubation	Post-incubation
% total N	Clam	0.64	0.42 (0.01)
	Mussel	0.58 (0.02)	0.39 (0.02)
	Oyster	0.62 (0.01)	0.34 (0.02)
% organic C	Clam	3.35	1.81 (0.19)
	Mussel	3.11 (0.06)	1.98 (0.27)
	Oyster	3.38 (0.09)	1.62 (0.22)
C/N	Clam	6.07	5.00 (0.60)
	Mussel	6.24 (0.09)	5.88 (0.66)
	Oyster	6.41 (0.10)	5.63 (0.56)
% organic matter	Clam	13.35	n.d.
	Mussel	14.80 (0.21)	n.d.
	Oyster	15.05 (0.27)	n.d.

n.d. no data available

Table 3 Statistical results of the one-way ANOVA and Tukey post hoc tests determining the effect of bivalve species on flux and potential denitrification (DNF) rates of biodeposits

Response	*F*	df	*p*	Post hoc summary
DIC	21.24	2	*0.0019*	O < M; O < C; M = C
NH_4^+	71.46	2	6.54E−05	O < M < C
NO_x^-	1.584	2	0.28	n.s.
PO_4^{3+}	7.545	2	*0.023*	O < C; O = M; C = M
DON	0.782	2	0.499	n.s.
DOC	0.621	2	0.569	n.s.
DNF	10.79	2	*0.010*	O < C; M < C; O = M

Italic values, $p < 0.05$

O oyster, *C* clam, *M* mussel

low compared to the other nutrient rates, with no significant difference across biodeposit treatments. DON fluxes were also generally low and similar across bivalve treatments, with a small net production observed for all biodeposit types. DOC consumption was observed for all biodeposits with similar magnitude across species. The consumption of DOC was smaller than the production of DIC, suggesting DOC production concurrent with consumption was occurring.

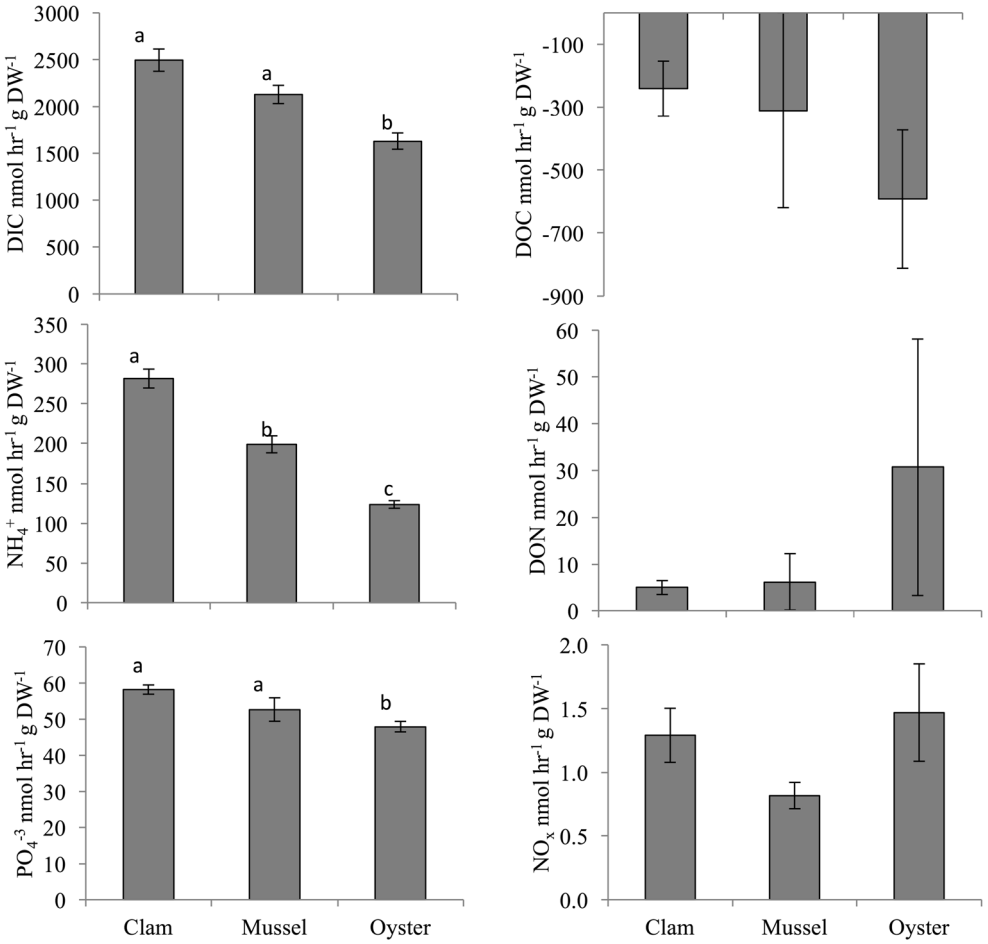

Fig. 1 Rates of dissolved inorganic carbon (DIC), dissolved organic carbon (DOC), ammonium (NH_4^+), phosphate (PO_4^{3+}), dissolved organic nitrogen (DON), and nitrate plus nitrite (NO_x^-) during the closed chamber incubations of biodeposits sourced from clams, mussels, and oysters. Lower case letters above the bars represent significant differences across bivalve species (Tukey post hoc statistical results)

Biodeposit Potential Denitrification Rates

Clam biodeposits had significantly higher potential denitrification rates, which averaged 20.8 (± 2.1) nmol gWW^{-1} h^{-1} compared to oyster and mussel biodeposits (Fig. 2, Table 3). Oyster and mussel biodeposits had similar rates, averaging 12.5 (± 0.8) nmol gWW^{-1} h^{-1} and 11.8 (± 1.4) nmol gWW^{-1} h^{-1}, respectively.

Biodeposit Bacterial Communities

After quality filtering, clustering, and removing chimeric sequences, a total of 217,318 high-quality sequences were obtained from the nine samples; sequencing depth ranged from a minimum of 12,351 to a maximum depth of 35,183 across the samples. After filtering out chloroplasts (31.9–51.7%), mitochondria (2.9–10.8%), and archaea (0–0.13%) and normalizing across samples to an even sampling depth (4497 reads), a total of 889 amplicon sequence variants (ASVs) were retained in the dataset. Of these ASVs, 170 were shared across all three of the bivalve species, which accounted for between 68.4 and 77.9% of the total bacterial community within each sample. The bacterial communities from the mussel and oyster biodeposits shared an additional 116 ASVs that were not found in clam biodeposits. In fact, the clam biodeposits shared much fewer taxa with both the mussel (16 ASVs) and the oyster (41 ASVs) than the mussel and oyster shared with each other (116 ASVs). Oyster biodeposits harbored more unique ASVs (274 total) than clam and mussel biodeposits.

Alpha diversity, including species richness and evenness did not differ significantly across bivalve species (Table 4). However, in general, oyster biodeposits had higher species richness followed by mussel and, finally, clam biodeposits. Microbial communities were dominated by ASVs in the Classes *Gammaproteobacteria* (29.3–37.4%), *Deltaproteobacteria* (20.6–27.9%), *Bacteroidia* (15.8–24.1%), and *Alphaproteobacteria* (7.5–18.2%) (Fig. 3). Within the *Gammaproteobacteria* was a highly diverse group of bacteria, dominated by *Cellvibrionales*, an order found in similar abundances across bivalve species. *Pseudomonadales* were not found in oyster biodeposits, but were important in both clam and mussel biodeposits. The *Deltaproteobacteria* was dominated by *Desulfobacterales* and *Desulfuromonadales*, with similar mean relative abundances across bivalve species observed within both orders. *Bacteroidia* was mainly composed of *Flavobacteriales*, with slightly more occurring in the mussel biodeposits compared to the clam and oyster. Finally, the *Alphaproteobacteria* was dominated by *Rhodobacterales*, with generally higher mean relative abundances observed in the clam biodeposits.

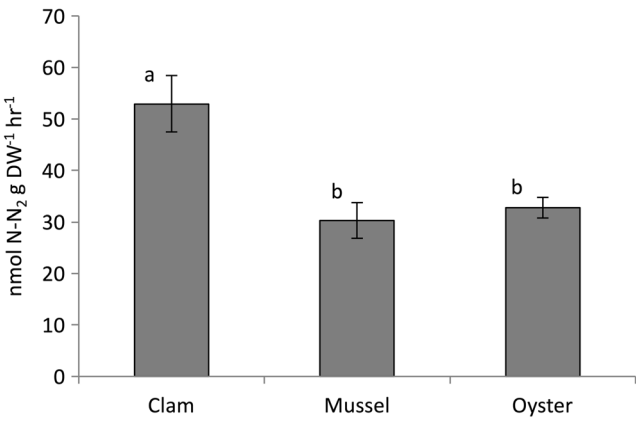

Fig. 2 Potential denitrification rates associated with clam, mussel, and oyster biodeposits, measured as $^{30}N_2$ production during anaerobic, $^{15}NO_3^-$ enriched, slurry incubations. Letters represent Tukey post hoc statistical results

Despite a general similarity in the relative abundance of the dominant ASVs across bivalve biodeposits, there was a significant effect of bivalve species on the microbial community structures as illustrated in the PCoA ordination constructed with the Bray-Curtis similarity matrix (Fig. 4; PERMANOVA $p = 0.003$). The microbial communities in the clam biodeposit replicates separated from the microbial communities associated with the oyster and mussel biodeposits along the primary axis, while the oyster and mussel appeared more similar to each other along the primary axis, but did have separation along the secondary axis.

Pairwise comparisons among the bivalve species revealed 23 differentially abundant ASVs across the bivalve species (DESeq2, adjusted p values < 0.10 [35]). These ASVs only accounted for between a total of 4.4 and 11.0% of the relative abundances within each community (Fig. 5a). The only ASV that was significantly different between the oyster and mussel biodeposits was classified at the genus level as *Pseudomonas*, which was, on average, 4.2% of the mussel biodeposit community, 1.1% of in the clam biodeposit community, and completely absent in the oyster biodeposit communities. There were 12 ASVs that were significantly different between the clam and oyster biodeposit communities and 19 ASVs that differed between the clam and mussel biodeposit communities. The clam biodeposits had significantly greater abundances of ASVs classified within the orders *Alteromonadales*, *Chitinophagales*, *Rhodobacterales*, and *Thiotrichales* and significantly lower abundances of ASVs classified as *Chromatiales*, *Bacteroidales*, and *Thiotrichales* compared to both the oyster and mussel biodeposit communities (Fig. 5a).

Of the 23 ASVs that were significantly different in relative abundance across the bivalve species, 7 of them were identified by the PAPRICA analysis to contain one or more genes associated with the denitrification pathway (i.e., *nirS*, *nirK*, *nosZI*, and/or *nosZII*) (Fig. 5b; Table 5). Specifically, PAPRICA predicted that the distinguishing denitrifying ASVs associated with the clam biodeposits were taxa that contained only *nirK* and/or *nosZII*, whereas the other bivalves

Table 4 Mean alpha diversity metrics of the bacterial dataset (i.e., no chloroplast, archaea, or mitochondria) and *Chloroplast* dataset by bivalve type including the standard error across the three replicate samples and the one-way ANOVA results

	Clam	Mussel	Oyster	*F* stat	*p* value
Bacterial taxa					
Observed ASVs	212 (± 7.9)	236 (± 67.9)	371 (± 21.7)	4.31	0.07
Shannon index	4.90 (± 0.04)	4.85 (± 0.23)	5.30 (± 0.03)	3.29	0.11
Inverse Simpson	91.1 (± 3.2)	78.6 (± 13.1)	106.3 (± 4.4)	2.89	0.13
Chloroplast taxa					
Observed ASVs	21 (± 2.0)	24 (± 1.5)	29 (± 0.9)	6.30	*0.03*
Shannon index	1.78 (± 0.08)	1.75 (± 0.06)	2.02 (± 0.06)	5.22	0.05
Inverse Simpson	3.69 (± 0.23)	3.48 (± 0.13)	4.27 (± 0.20)	4.58	0.06

Italic values, $p < 0.05$

were differentiated by taxa containing all four denitrifying genes. The denitrifying taxa in the clam biodeposit community had little overlap with denitrifying taxa in the mussel and oyster biodeposits. In contrast, the oyster and mussel biodeposits shared similar denitrifying ASVs. In the clam biodeposits, the distinguishing denitrifying taxa were generally N_2O-reducing organisms. In general, oyster biodeposits harbored a more diverse set of predicted denitrifying genes, with two ASVs having the capacity to both reduce nitrite (*nirS* or *nirK*) and nitrous oxide (*nosZI* or *nosZII*), with evidence for niche separation due to little overlap among denitrifying gene combinations (Fig. 5b).

When the entire dataset was included (i.e., not just the significantly different ASVs), PAPRICA revealed trends in the predicted relative abundances of denitrifying genes across the different bivalve biodeposit communities. The relative abundances of *nirS*, *nirK*, and *nos*ZI genes inferred by PAPRICA were not significantly different across bivalve treatments (Fig. 6a–c). The relative abundance of *nosZII* was significantly higher in the oyster biodeposit community (6.8 ± 0.3%) compared to the mussel (5.9 ± 0.01%) (ANOVA: $p = 0.04$, $F = 5.43$); while the clam biodeposit community had similar inferred relative abundances of *nosZII* (6.4 ± 0.2%) to both the oyster and mussel communities (Fig. 6d). The relative abundance of "complete denitrifiers," carrying both a nitrite reductase (either *nirS* or *nirK*) and a nitrous oxide reductase (either *nosZI* or *nosZII*), was significantly higher in the mussel biodeposit community compared to the clam, while the oyster community was similar to both the clam and mussel (Fig. 6e).

Biodeposit Chloroplast Communities

A total of 44 ASVs were classified in the original dataset to be of the order *Chloroplast* and were re-classified using

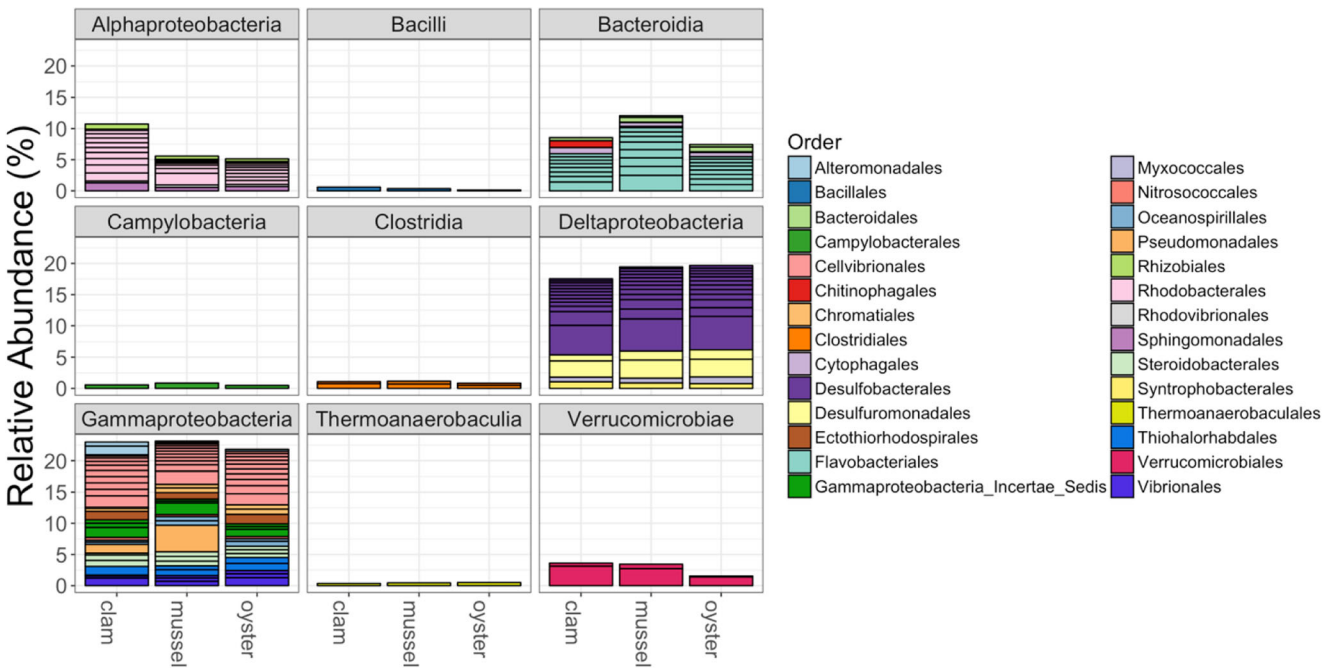

Fig. 3 Mean relative abundance of ASV's by bivalve type (includes only the ASV's with > 1% mean relative abundance in the dataset); the color depicts order classification and the data are faceted by class

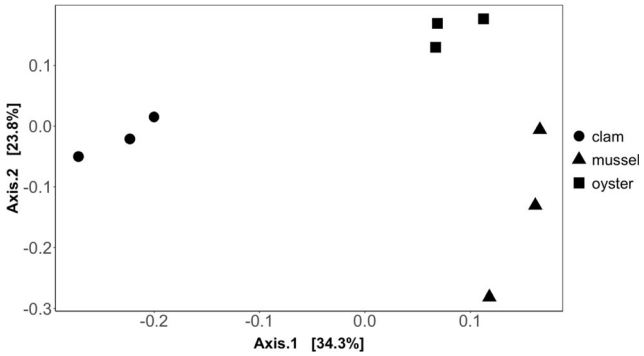

Fig. 4 Beta diversity of the normalized (filtered dataset (filtered out chloroplast, archaea, and mitochondria) depicted using a principal coordinate analysis constructed with Bray-Curtis similarity illustrating a significant effect of bivalve-source on the microbial community structure associated with the biodeposit (PERMANOVA; $F = 3.49$, $p = 0.003$)

NCBI. In general, the oyster biodeposits had higher alpha diversity compared to the clam and mussel biodeposits (Table 4). The majority of taxa were diatoms, from the phylum *Bacillariophyta*, averaging 87.1, 93.1, and 94.7% of the total chloroplast dataset in the clam, oyster, and mussel biodeposits, respectively. Of these diatoms, centric diatoms (*Coscinodiscophyceae Thalassiosirophycidae*) made up the majority of the taxa, ranging from 34.7% in clams to 47.4% in oysters. Pennate diatoms (*Fragilariophycidae*) were also abundant, ranging from 29.8% in oysters to 40.9% in clams. A significant effect of bivalve species on the community structure of *Chloroplast* ASVs was observed (Fig. 7, PERMANOVA $p = 0.002$). There were 19 ASVs that were differentially abundant across the bivalve species (Fig. 8). Generally, these differences were driven by lower abundances of particular taxa in the clam biodeposits compared to the mussel and oyster. Within these differentially abundant ASVs, eight were identified as separate ASVs, but were classified into one of four taxa (*Planoglabratella* (g), *Thalassiosiraceaea thalassiosira*, *Stephanodiscaceae cyclotella*, and *Fragilariaceae diatoma*). Further, for all four of these taxa, the clam biodeposits only harbored one of the two, while the oyster biodeposits consistently harbored both oligotypes.

Discussion

We found significant differences across bivalve species in terms of (1) the bulk biogeochemical composition of the biodeposits (i.e., percent carbon, percent nitrogen, and organic matter content), (2) the rate at which the biodeposits decomposed, (3) the capacity of the biodeposits to fuel denitrification, (4) the microbial communities associated with the biodeposits, and (5) the potential denitrifying taxa. These distinct differences likely influence the fate of the different types of biodeposits in the environment and are important to consider when assessing the ecological role of each bivalve species in coastal ecosystems. These results, however, are only based on a single sampling event at one location; additional sampling is required to draw broad conclusions regarding seasonal and spatial effects on these results.

Bioreactivity

Our study shows that different bivalve species produce biodeposits that vary in their bioavailability to the microbial community, resulting in different rates of mineralization. We found clam biodeposits to be the most bioreactive, with significantly higher rates of DIC and NH_4^+ production, followed by mussel and finally oyster biodeposits. These findings were corroborated by a lower C/N ratio of the clam biodeposits compared to the mussel and oyster biodeposits, suggesting higher organic matter quality leading to greater accessibility to heterotrophic microbial pathways. This is despite the fact that clam biodeposits had the lowest percent organic matter content.

Although dissolved oxygen (DO) was not measured during the incubations, it is likely that the DO in the vials became depleted over the course of the incubation, and thus, the mineralization pathways shifted from aerobic to predominantly anaerobic. It is also likely that given the higher rates of DIC production, the clam and mussel treatments became anoxic prior to the oyster biodeposits. If, hypothetically, the production of DIC relative to the consumption of DO was 1:1, the mussel and clam vials would be anoxic after 3.2 and 2.7 days, respectively, while the oyster treatment would be anoxic only after 6.1 days. Further, the stoichiometry of the DIC/PO_4^{-3} fluxes corroborates this; the clam and mussel treatments had much higher ratios (~ 29 and 30, respectively) compared to the oyster (~ 16.7), suggesting a greater release of PO_4^{-3} relative to DIC, potentially due to anoxic conditions that promote PO_4^{-3} release from iron precipitates. However, the likely differences in DO concentrations across the bivalve treatments does not alter the conclusion that different biodeposits had fundamentally different bioavailability when the bivalves were offered identical food sources, though whether that remineralization was aerobic, anaerobic, or a combination of the two could not be determined.

These differences in bioreactivity may be due to physiological differences across the bivalve species. *G. demissa*, *C. virginica*, and *M. mercenaria* are known to have different feeding behaviors and physiological rates [46, 53]. When the concentration of total suspended solids (TSS) in the water column is high, concurrent with elevated mineral content in the TSS, mussels and oysters produce significant amounts of pseudofeces, which are biodeposits that have not passed through the digestive tract [17, 57]. On the other hand, clams typically respond to elevated TSS concentrations by reducing clearance rates and subsequently decreasing biodeposit production [5]. This difference in feeding behavior likely affects

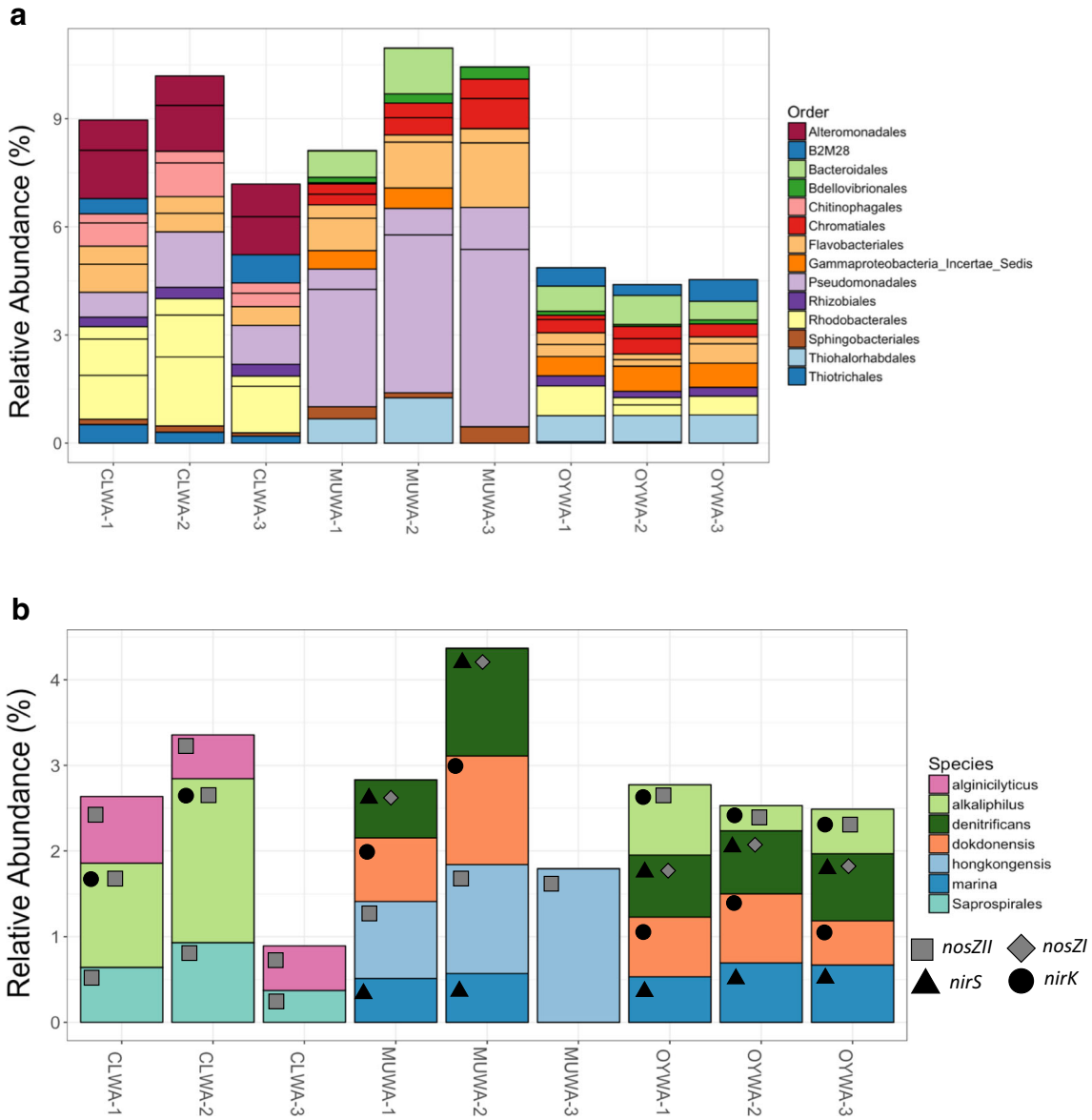

Fig. 5 **a** The relative abundances of the ASVs that were significantly different in the pairwise comparisons between bivalve species (DESeq2; adjusted p value < 0.1). Colors depict order classifications. **b** The relative abundances of the significantly different ASVs that were identified by PAPRICA to have denitrifying genes (symbols within the stacked bars). Taxonomic information for these 7 ASVs, including both the Silva database classifications and the PAPRICA identities, are provided in Table 5. CLWA, clam; MUWA, mussel; OYWA, oyster

the biogeochemical and microbiological characteristics of the biodeposits and may explain the generally greater bioreactivity and organic matter quality associated with clam biodeposits compared to mussel and oyster biodeposits. Smaal and Prins [49] found that nutrient content of bivalve feces is higher than of pseudofeces and that mineralization of pseudofeces was lower than feces. Additional data characterizing the water column during biodeposit collection, such as TSS concentrations and percent organic matter would be useful in determining the relative proportion of pseudofeces to feces that each bivalve species produced.

Aside from differences in feeding behavior, clearance rates and biodeposition rates are significantly higher in mussels followed by oysters and finally clams [53]. However, our study aimed to control for the quantity of biodeposits in the incubations (1 g wet weight) as we were interested in determining differences in bioreactivity, independent of the supply of organic matter. Incorporating the effects of bivalve physiological rates, such as biodeposition rates and pseudofeces production, on the fate of the biodeposits, thus, requires further study.

Potential Denitrification Rates

We found that clam biodeposits fueled significantly higher rates of potential denitrification compared to mussel and

Table 5 Taxonomic identification of the ASV's designated as both significantly different in abundances across bivalve species (DeSeq $p < 0.1$) and containing denitrifying genes (PAPRICA results), including both the PAPRICA identification and the taxonomy based on the Silva database

Paprica	Ref.	Silva database taxonomy					
Genome_ID (genus species names)		Kingdom	Phylum	Class	Order	Family	Genus
GCF_001270965.1_DRAFT_Sunxiuqinia_dokdonensis	[32]	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidetes_BD2-2	NA
GCF_001579945.1_Steroidobacter denitrificans_strain=DSM 18526	[12]	Bacteria	Proteobacteria	Gammaproteobacteria	Thiohalorhabdales	Thiohalorhabdaceae	NA
GCF_000152805.1_DRAFT_Maritimibacter_alkaliphilus_HTCC2654	[28]	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	NA
GCF_001187785.1_Wenzhouxiangella marina_strain=KCTC 42284	[56]	Bacteria	Proteobacteria	Gammaproteobacteria	Gammaproteobacteria_Incertae_Sedis	Unknown_Family	NA
GCF_000236705.1_Owenweeksia hongkongensis DSM 17368_strain=DSM 17368	[27]	Bacteria	Bacteroidetes	Bacteroidia	Flavobacteriales	Cryomorphaceae	NA
Saprospirales	[16]	Bacteria	Bacteroidetes	Bacteroidia	Chitinophagales	Saprospiraceae	Lewinella
GCF_001310225.1_Algibacter alginicilyticus_strain=HZ22	[52]	Bacteria	Bacteroidetes	Bacteroidia	Flavobacteriales	Flavobacteriaceae	NA

oyster biodeposits. This is likely related to the greater quality and bioreactivity of organic matter associated with the clam biodeposits that we observed in the bioreactivity incubations. As denitrification is typically a heterotrophic metabolism, denitrifiers require labile organic carbon. Interestingly, field studies report highest rates of denitrification associated with oyster reefs [21] and a study comparing the effects of oysters and clams on denitrification found significantly higher rates associated with oyster sediments [51]. This divergence between our controlled laboratory experiment and sediment flux studies emphasizes the tremendous importance of environmental factors such as local hydrodynamics, redox conditions, and the presence of other ecologically important fauna, which were omitted in our study. Additionally, high variability in oyster sediment-associated denitrification was reported in a synthesis paper that compared findings across study systems [22], highlighting the importance of local environmental conditions in influencing the fate of bivalve biodeposits and the potential to stimulate denitrification. For example, an important variable omitted in our study is the effect of nitrate supply on denitrification rates, as our experiment provided ample nitrate during the incubation and, therefore, measured potential rates of denitrification. In situ, the supply of nitrate may be as important as the supply of organic matter. When the supply of nitrate is sourced from nitrification, it can be drastically depleted by high inputs of labile organic matter (e.g., clam biodeposits), which decreases oxygen availability and increases sulfide accumulation in sediments. These complex factors (i.e., sulfide accumulation and nitrate supply) were not addressed in our study and should be considered further under field conditions across different locations and seasons. Our study was a laboratory incubation which sought to control for most environmental variables, including nitrate supply, to mechanistically target the intrinsic differences across biodeposits sourced from different bivalve species. Therefore, although this study provides valuable information about the biogeochemical properties of bivalve biodeposits, scaling-up the measured rates to a natural ecosystem or even on a per square meter basis is not realistic and would require additional experiments addressing the influence of environmental conditions across different locations and seasons.

Microbial Community Differences

Aside from the biogeochemical composition and bioreactivity of the bivalve biodeposits, the microbial community associated with the biodeposits was also significantly different across bivalve species. Our study, which is the first to compare bivalve biodeposit microbiomes with high-throughput sequencing, highlights the importance of bivalve biodeposition on not only delivering organic matter to benthic habitats but also potentially shuttling specific microbial taxa to the sediments. We hypothesize that through direct inoculation, bivalve biodeposition may influence the resident sediment microbial community structure and function. Although numerous studies have determined bivalve populations can increase bacterial respiration and microbial diversity (e.g., [13, 44]), very few studies have characterized the influence of bivalve populations on the

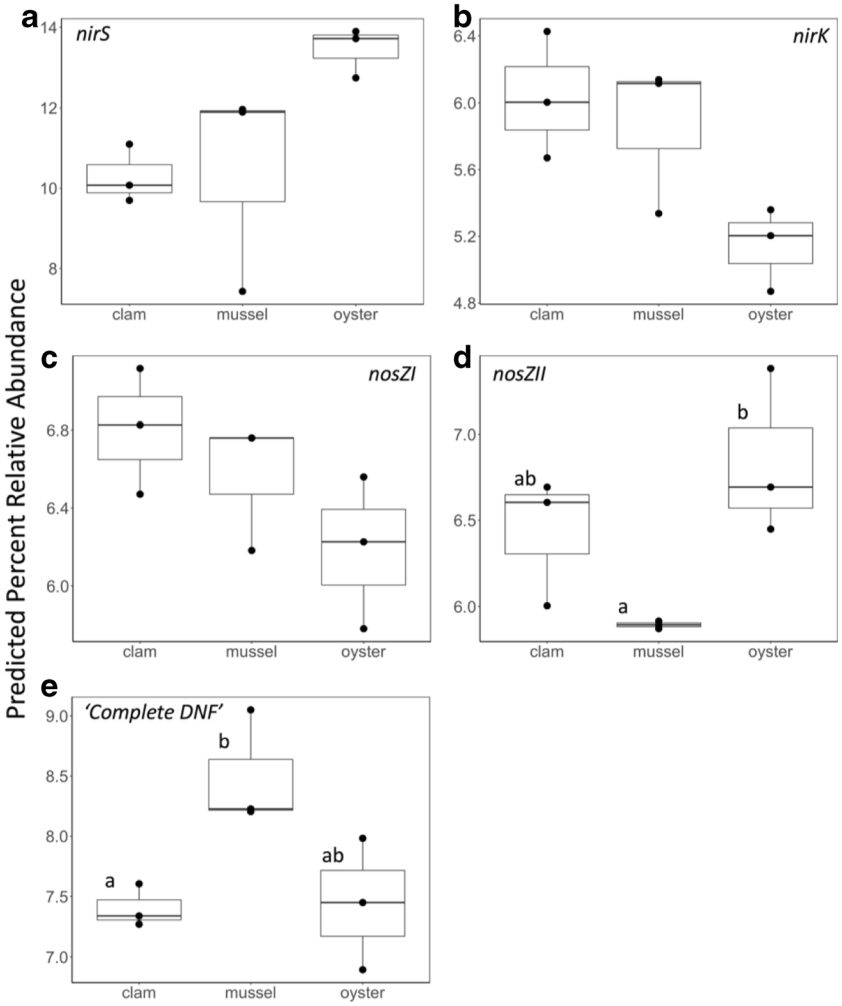

Fig. 6 Percent relative abundance of gene copies of **a** *nirS*, **b** *nirK*, **c** *nosZI*, **d** *nosZII*, and **e** both a nitrite reductase (*nirS* or *nirK*) and a nitrous oxide reductase (*nosZI* or *nosZII*), obtained from the metabolic inference with PAPRICA. Lower case letters indicate significant differences across bivalve species (ANOVA $p < 0.05$)

taxonomic composition of sediment microbial communities using high-throughput sequencing. One study showed freshwater mussels caused an overall decrease in *Proteobacteria* and increase in *Nitrospirae* in sediments, but attributed these taxonomic shifts to changes in biogeochemical conditions in the sediments from biodeposition, as opposed to biodeposit inoculation [4]. However, using enzymatic activity measurements Grenz et al. [15] suggested a large portion of mussel biodeposits supplied to sediments are degraded by bacteria sourced from the digestive tract of the bivalves and shed upon egestion. Further studies comparing the microbial communities associated with both the bivalve biodeposits and the proximal sediments are required to fully understand the influence bivalves have on benthic microbial community structure and composition.

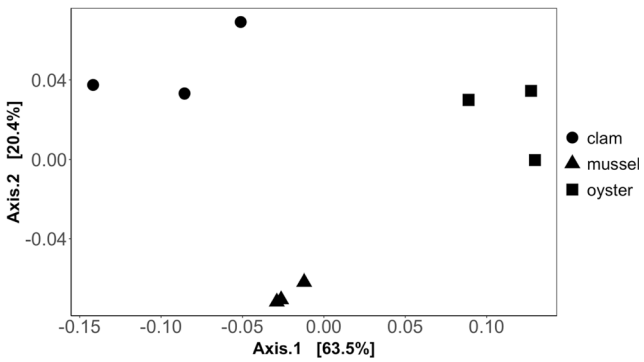

Fig. 7 Beta diversity of the normalized *Chloroplast* dataset depicted using a principal coordinate analysis constructed with Bray-Curtis distance illustrating a significant effect of bivalve-source on the *Chloroplast* community structure associated with the biodeposit (PERMANOVA; $F = 10.93$, $p = 0.002$)

Both the bivalve food source and the tissue microbiome may contribute to structuring the microbial community of bivalve biodeposits [48]. In our study, characterizing the microbial community of the water column could provide further information about the origin of the microorganisms in the biodeposits. However, as the bivalves in our experiment fed on the same source water during biodeposition collection, differences in the microbiomes are likely due to bivalve species-specific factors that can influence both diet and specific tissue microbiomes. For example, the size ranges of particles that are efficiently retained during feeding vary across

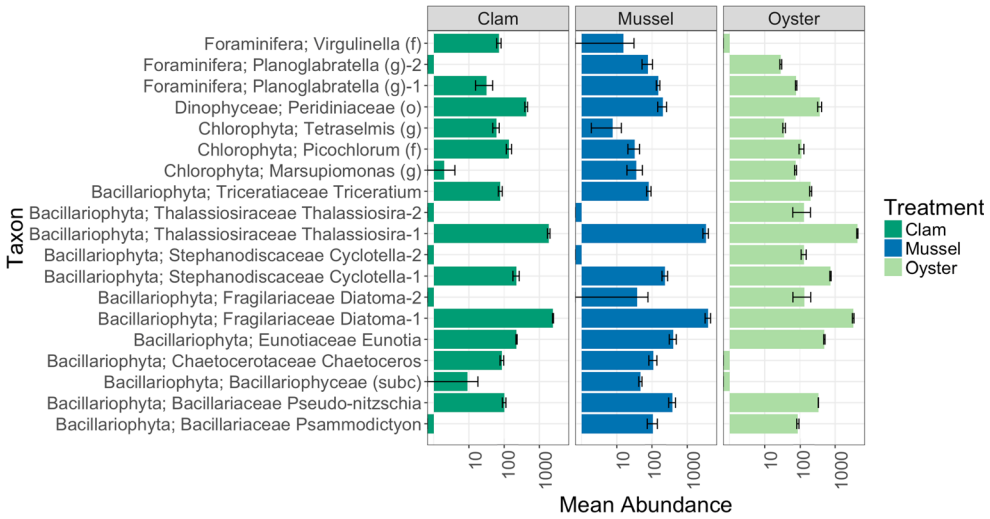

Fig. 8 The mean abundances and standard errors of the 19 ASVs initially identified against Silva database as belonging to the order *Chloroplast* and subsequently determined to have significantly different abundances across bivalve species (DESeq2, adjusted p value < 0.05); axis is on a log(10) scale. Further taxonomic identification is based on BLAST against the NCBI database and denoted along the vertical axis as class and the lowest possible taxonomic rank

these bivalve species due to physiological differences. Due to larger latero-frontal cirri in the gills, ribbed mussels and hard clams have the capacity to access smaller particles (> 4 μm) more efficiently compared to the eastern oyster, which has a smaller latero-frontal cirri allowing them to entirely retain particles above 5–6 μm [46]. Additionally, direct comparisons across these three species revealed significantly higher filtration rates by *C. virginica* and *G. demissa* compared to *M. mercenaria* [46]. Not surprisingly, we found very high abundances of *Chloroplasts* across the biodeposit communities, ranging from ~ 32 to 52% of the sequences. We found significant differences in the *Chloroplast* taxonomic composition across the bivalve species, which suggests strong control of bivalve physiology on biodeposit microbiomes. Whether it be through selective feeding, relative pseudofeces production, and/or differences in digestion rates and efficiencies, the bivalve biodeposits harbored significantly different microorganisms identified as *Chloroplast*, most of which were diatoms, a preferred food source for these filter feeders.

In addition to differences in feeding and digestion, the microbiota associated with the bivalve tissues, particularly those associated with feeding and digestion, such as the gill and gut, likely differ across bivalve species and may explain the observed differences in the microbial communities associated with biodeposits. The majority of studies investigating the microbiome associated with bivalve tissue focus on oysters, with no comparative studies across other bivalve species. In general, the microbial communities we found associated with bivalve biodeposits are similar to those reported for oyster gut-associated microbiota, with the most common phyla being *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* [30, 55]. Although other studies showed *Mollicutes* (*Mycoplasma*) and *Planctomycetes* to be important members of the upper gastrointestinal tract of oysters [23, 34], we found very low abundances of these groups in the biodeposits (typically < 1%).

Interestingly, the clam biodeposits, which had the highest rates of potential denitrification, harbored a very different denitrifying community, as predicted by PAPRICA, compared to the mussel and oyster biodeposits. The differentially abundant denitrifying taxa associated with the clam biodeposits included taxa predicted to have the capacity to reduce N_2O to N_2 (*nosZII*) (i.e., classes *Chitinophagales*, *Rhodobacterales*, and *Flavobacterales*) (Fig. 6b, Table 5). Clam biodeposits had higher abundances of complete denitrifying taxa in class *Rhodobacterales*, and thus, this group may be responsible for the higher rates of denitrification associated with clam biodeposits. In contrast to the clam biodeposits, the distinguishing taxa associated with the oyster and mussel biodeposits were similar to each other and consisted of a metabolically diverse group of denitrifying bacteria, resulting in more complete assemblages of both nitrite reducers (*nirS* and *nirK*) and nitrous oxide reducers (*nosZI* and *nosZII*) (Fig. 6b). PAPRICA provides the ability to predict the co-occurrence of denitrifying genes within single taxa or ASVs, which is not possible with quantitative PCR approaches. However, without genomic context, for example through metagenome-assembled genomes, it is not possible to confirm which specific bacterial taxa across the bivalve biodeposits are canonical denitrifiers.

In general, the PAPRICA analysis revealed no clear trends of the relative abundances of denitrifying genes across bivalve species. The discrepancy between the PAPRICA results and the direct potential rate measurements suggests that organic carbon quality may be more important in dictating potential rates of denitrification than the predicted microbial metabolic capacity or relative abundances of denitrifying functional genes. Additionally, numerous caveats and assumptions are associated with the phylogenetic inference approach we used to predict denitrification capacity. Since denitrification is not phylogenetically constrained [19], making inferences about the potential for an organism to conduct denitrification based

on phylogeny is challenging. Despite this, PAPRICA provided additional insight into the taxonomy of the potential denitrifiers in the biodeposit communities, contributing both greater taxonomic resolution and the specific denitrifying genes associated with the differentially abundant taxa across the bivalve species.

Conclusions

Bivalves serve an important ecological function in filtering particulates from the water column and removing nutrients in coastal environments; restoration of bivalve populations is often proposed to alleviate symptoms of eutrophication (e.g., Bricker et al. [6]). However, the bioreactivity or degradability of bivalve biodeposits is a critical consideration as high mineralization rates may lead to recycled nutrients supplied back to the water column promoting additional primary production. The biological reactivity of bivalve biodeposits is the direct result of a combination of the organic matter quality and the microbial community composition and its metabolic capabilities. Our results indicate that oyster biodeposits have lower bioreactivity and a different bacterial community structure than biodeposits from clams and mussels; however, we are unable to determine which factor (i.e., organic carbon quality or the bacterial community) is more important in determining remineralization rates. Although our study did not include environmental variables such as oxygen availability or sulfide concentrations, it is important to recognize that the environmental context is critical in determining the fate of bivalve biodeposits (e.g., decomposition rates and their potential to fuel denitrification). The question of whether the observed intrinsic differences in bioreactivity and microbiomes across biodeposit types are reflected in differences in the processing of these biodeposits under ambient environmental conditions remains unanswered and requires further directed work. Our study provides important information about the intrinsic biodegradability of biodeposits sourced from different bivalve species and highlights the potential importance of bivalves in inoculating sediments with particular microbial communities that differ across bivalve species.

Acknowledgements Many thanks to Jennifer Stanhope and Hunter Walker at VIMS for the logistical and laboratory support. Discussions with Ashley Smyth and Lisa Kellogg greatly improved this research effort. This work was supported by the VIMS Research Experiences for Undergraduates program through grants awarded from the National Science Foundation (grant # NSF OCE 1062882) to Linda Schaffner and Rochelle Seitz, Virginia Sea Grant (NA10OAR4170085, #R/71515W) awarded to A. Murphy, grant #NSF OCE 1233801 awarded to BK Song, and NSF Virginia Coast Reserve Long Term Ecological Research (LTER 0080381, 0621014).

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