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Oyster calcifying fluid harbors persistent and dynamic autochthonous bacterial populations that may aid in shell formation

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ABSTRACT: The eastern oyster *Crassostrea virginica* is a keystone species in estuarine environments but faces threats to shell formation associated with warming temperatures and ocean acidification. Extrapallial fluid (EF), which is responsible for shell formation, harbors diverse and abundant microbial communities. Commensal microbial communities are vital to host health and fitness, yet long-term studies investigating temporal responses of the EF microbiome and its function in oyster fitness are lacking. In this study, bacterial communities of oyster EF and the water column were characterized monthly from October 2010 to September 2011. We investigated the selection, composition, and dynamics of resident and transient community members, evaluated the impact of temperature on EF microbial communities, and examined the functional role of the EF microbiome. Oyster EF communities were significantly different from those of the water column and were enriched for several taxa, including the *Deltaproteobacteria*, *Epsilonproteobacteria*, and *Gammaproteobacteria*. Overall, 94 resident members were identified in oyster EF. These members were persistent and abundant, comprising on average 33% of EF communities. Resident EF communities formed high-temperature and low-temperature groups and were more abundant overall at colder temperatures. Oyster EF resident communities were predicted to be enriched for dissimilatory nitrate reduction, nitrogen fixation, nitrification, and sulfite reductase genes. Sulfate and nitrate reduction may have a synergistic effect on calcium carbonate precipitation and indirectly aid in shell formation. Therefore, the potential role of the oyster EF microbiome in shell formation warrants further investigation as oysters and other shellfish face the future impacts of ocean warming and acidification.

KEY WORDS: *Crassostrea* · Oyster · Microbiome · Calcification · Microbial diversity · Extrapallial fluid

1. INTRODUCTION

The eastern, or American, oyster *Crassostrea virginica* is a keystone species in estuarine environments along the North American east coast but faces numerous threats from disease and habitat alteration brought on by climate change (Powell et al. 1992, Beniash et al. 2010). The protozoan parasites *Haplosporidium nelsoni* and *Perkinsus marinus*, the causative agents of the oyster diseases MSX and Dermo, respectively, are responsible for substantial annual mortalities (Andrews 1996, Burreson & Calvo 1996, Albright et al. 2007, Powell et al. 2011, Soniat et al. 2012), and warming temperatures contribute to the

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increased range, prevalence, and severity of *H. nelsoni* and *P. marinus* infections (Burreson & Calvo 1996), as well as the spread of bacterial pathogens like *Vibrio* spp. (Garnier et al. 2007, Elston et al. 2008, Vezzulli et al. 2012). In addition, ocean warming and acidification negatively impact growth rate, survival, and the shell and immunological integrity of oysters and other calcifying bivalves (Dickinson et al. 2012, Waldbusser et al. 2013, Gobler et al. 2014, Mackenzie et al. 2014a,b). Understanding factors that impact oyster fitness and their interactions with these threats will be important for improving conservation efforts and restoring the ecosystem services that oysters provide, including reducing turbidity and improving water quality (Grizzle et al. 2008), increasing local diversity by providing habitat and hard substrate (Stunz et al. 2010), protecting the shoreline from erosion (Coen et al. 2007), and playing an important role in nutrient cycling, particularly nitrogen (Kellogg et al. 2013). One potential factor that may impact oyster fitness and their interactions with the environment is the oyster microbiome.

Commensal microbial communities perform a number of functions vital to their hosts and can impact host health and fitness. Commensal communities can influence the acquisition of nutrients (Turnbaugh et al. 2006), produce essential compounds (Hill 1997), and provide protection against pathogens (Bachère 2003). The importance of commensal bacterial communities has been of particular interest in aquaculture, and probiotic administration of bacteria has increased the survival of marine invertebrate species challenged with known pathogens (Riquelme et al. 1996, Gibson et al. 1998, Nakamura et al. 1999, Lim et al. 2011, Kesarcodi-Watson et al. 2012). However, such work in *C. virginica* has been hampered by limited knowledge regarding its normal commensal microbiome (Bachère 2003).

Like other filter-feeding bivalves, oysters are exposed to a multitude of microbes from their environment. Historically, studies of oyster-associated bacteria have focused on cultivable bacteria, particularly those relevant to human health (e.g. *Vibrio* spp.), and bacteria cultivated from bivalves have been shown to differ from those cultivable bacterial populations found in surrounding water samples (Lovelace et al. 1968, Kueh & Chan 1985, Pujalte et al. 1999). However, it is well known that most marine bacteria are not readily cultivable (Ward et al. 1990), including 99.99% of bacterial cells from oysters (Romero & Espejo 2001). Furthermore, cultivated bacteria do not represent the most abundant members within the oyster bacterial flora (La Valley et al. 2009). More recently, cultivation-independent analyses of bacterial communities within oysters have reported distinct communities in the hemolymph, mantle, stomach, gut, and gills (King et al. 2012, Wegner et al. 2013, Chauhan et al. 2014, Lokmer et al. 2016a). Intraspecies and interspecies variations in commensal bacterial community composition have been observed and may be attributed to various host factors (e.g. genotype) (Lokmer et al. 2016b) and filtration rates (Banker & Vermeij 2018), respectively. Cultivation-independent reports of seasonal impacts on oyster-associated commensal communities are rare; nonetheless, water temperature appears to influence community structure and richness in wild oysters (Pierce et al. 2016), while oysters incubated under heat stress display decreased bacterial diversity (Lokmer & Wegner 2015).

One area that remains poorly studied by cultivation-independent approaches is the selection, composition, and dynamics of bacterial communities in oyster extrapallial fluid (EF). The EF is a mixture of organic and inorganic compounds secreted by the mantle into the pallial cavity and is responsible for shell formation. Models of oyster shell formation posit that Ca^{2+} precipitation occurs in granulocytes that are transported to the mineralization front. There, the crystals are released, where they interact with the organic matrix (Mount et al. 2004, Zhang et al. 2012, Wang et al. 2013). However, warming and/or acidification may result in decreased hemocyte abundance due to reallocation of resources during stress (Mackenzie et al. 2014b), an important consideration based on the role of granulocytes in shell formation (Mount et al. 2004, Wang et al. 2013). Furthermore, elevated pCO_2 and low salinities reduced hardness and fracture resistance in *C. virginica* juveniles (Dickinson et al. 2012), while warmer temperatures and acidification reduce shell strength and shell flex in the blue mussel *Mytilus edulis* (Mackenzie et al. 2014b). In these models, the role of the EF bacterial community in shell formation has not been considered (Vermeij 2014), nor have the implications of ocean warming and/or acidification on the community composition and function. However, the potential role of bacteria in the formation of other marine carbonate structures has been reported (Chafetz 1986, Uriz et al. 2012, Guido et al. 2014, Garate et al. 2017). In light of changing environmental conditions, addressing the potential role of EF-associated communities in oyster shell mineralization is particularly important.

The impact of temperature on *C. virginica* EF communities has been previously observed (Pierce et al. 2016); however, to date no long-term studies leverag-

ing next-generation sequencing have been reported. Additionally, to our knowledge no oyster microbiome study has examined commensal communities at sub-operational taxonomic unit (sub-OTU) resolution (single nucleotide variants, i.e. oligotypes of the 16S rRNA gene). The limitations of OTU-based approaches for characterizing oyster communities have been previously noted, as OTUs may be comprised of different ecotypes that could differentially impact communities and hosts (Lokmer et al. 2016b). Furthermore, clustering related organisms together hampers efforts to distinguish transient (allochthonous) community members from resident (autochthonous) members. In this study, bacterial communities of the oyster EF and surrounding water were characterized monthly by next-generation sequencing at sub-OTU (oligotype) resolution. Oligotypes were observed over an annual cycle to differentiate allochthonous from autochthonous bacteria within oysters, investigate the impact of physicochemical parameters on autochthonous and allochthonous community members, and explore the potential role the oyster bacterial microbiome plays in oyster health and fitness.

2. MATERIALS AND METHODS

2.1. Annual survey sample collection

Adult (3 yr old) cultured *Crassostrea virginica* (n = 105) were obtained from Marinetics (Cambridge, Maryland) and split between 3 wire cages on 16 September 2010. Cages contained 35 oysters each and were suspended from a pier so that they hung approximately 1 m from the bottom of the Rhode River at the Smithsonian Environmental Research Center (SERC) in Edgewater, Maryland. Oysters were allowed to acclimate to the Rhode River natural environment for 39 d prior to sampling and were subsequently maintained in the natural environment for the duration of the experiment so that they were subjected to the same environmental microbiota as wild oysters. Five oysters across the 3 cages were randomly harvested monthly from October 2010 to September 2011. Some mortality was observed over the course of the experiment, particularly during periods of low salinity; however, only live oysters were harvested for microbial analyses.

Harvested oysters were rinsed with deionized water and scrubbed with 70% ethanol prior to EF extraction. Extrapallial fluid was extracted from each oyster with a 5 ml syringe (23G needle) through a hole drilled (3/32 inch drill bit) into the oyster posterior at the interface between valves. Following EF extraction, oysters were shucked. Oysters generally appeared to be visually healthy, although this was not empirically determined. EF samples were placed on ice and transported to Newark, Delaware, for processing. At each sampling, we also collected 10 l of water. The water sample was placed in a cooler filled with ambient water to maintain temperature during transport to Newark. Temperature, pH, salinity, chlorophyll, conductivity, and dissolved oxygen (DO) were downloaded (http://nmnhmp.riocean.com) from the on-site continuous monitoring station maintained by the SERC. Measurements were taken with an EXO2 Sonde (YSI) within 15 min of sample collection (Table 1; courtesy of Charles Gallegos, SERC). Physicochemical water conditions represent single instantaneous measurements and were recorded for all months except January and February 2011 when frozen conditions required sensor removal.

2.2. Bacterial abundance and correlations

A 200 µl aliquot of each oyster sample was combined with 37% formalin to a final concentration of 1% (v/v), snap-frozen in liquid nitrogen, and stored at −80°C prior to bacterial enumeration. A 4.5 ml aliquot of each water sample was treated similarly. Thawed samples were combined with 0.22 µm fil-

Table 1. Rhode River (Edgewater, Maryland, USA) surface water physicochemical parameters measured at the time of each oyster sample collection from October 2010 to September 2011

Month	Temp. (°C)	Salinity (ppt)	pH (total)	Specific cond. (mS cm^{-1})	Dissolved oxygen (mg l^{-1})	Chlorophyll (µg l^{-1})
Oct	15.25	12.25	8.32	20.42	9.9	13.9
Nov	10.77	12.1	8.36	20.25	11.53	15.3
Dec	3.8	9.79	8.37	16.9	13.24	17.6
Mar	8.59	6.91	8.34	12.08	12.13	14.2
Apr	14.21	6.08	8.32	10.68	10.24	21.3
May	21.8	2.85	8.94	5.28	8.41	91.8
Jun	25.22	4.22	7.91	7.65	7.02	22.8
Jul	28.21	7.54	7.78	13.16	6.16	26.5
Aug	26.47	10.1	7.64	17.2	5.61	31.4
Sep	20.12	2.87	8.69	5.31	9.37	57.7

tered 1× phosphate-buffered saline (PBS) as follows: 10 µl EF in 990 µl PBS for oyster samples; 70 µl water in 930 µl PBS for water samples. Solutions were rocked moderately at 30°C for 20 min and then vacuum filtered onto 0.02 µm Anodisc filters (Whatman). Filters were stained with 2.5× SYBR Gold (Thermo Fisher Scientific) in the dark for 15 min. Bacteria were visualized with a 100× oil-immersion objective on an Olympus BX51 upright epifluorescence microscope at a wavelength of 495 nm. Images were taken at 15 random sites per filter. Bacterial abundance was calculated as follows: Bt = Bc / Fc × At / Af / S, where Bt = bacterial abundance ml^{-1}, Bc = number of bacteria counted, Fc = number of fields counted, At = surface area of the filter ($µm^2$), Af = area of each field ($µm^2$), and S = volume of sample filtered (ml) (adapted from Suttle & Fuhrman 2010). Annual mean bacterial abundances between oyster and water samples were compared by a mixed-effects model with treatment (oyster EF vs. water), time, and treatment × time interactions. Mean bacterial abundances across all oyster and water samples were compared by a Mann-Whitney test. Individual monthly differences ($p < 0.05$) between oyster and water bacterial abundances were identified by Mann-Whitney tests.

2.3. Bacterial DNA isolation

Oyster EF samples were processed individually. EF from each oyster was combined with sterile 1× PBS buffer (1:25) and rocked moderately for 1 h at 30°C to improve filtration. The EF–PBS sample mixture of ca. 500 ml was filtered through a Millipore Sterivex 0.22 µm filter unit. The 10 l water sample was split into 2 replicates. Approximately 150 ml of water were filtered through a Millipore Sterivex 0.22 µm filter unit per replicate. DNA was extracted from each filter as previously described (Crump et al. 2003) with amendments. Briefly, Proteinase K (20 mg ml^{-1}) and lysozyme (100 mg ml^{-1}) were combined with DNA extraction buffer (DEB: 100 mM Tris buffer pH 8, 100 mM NaEDTA pH 8, 100 mM phosphate buffer pH 8, 1.5 M NaCl, 1% CTAB) and added to the filter. Filters were incubated at 37°C for 30 min and subjected to 3 freeze–thaw cycles of −80 and 37°C, followed by incubation at 37°C for 30 min. DEB was removed from the filter, combined with 10% (w/v) sodium dodecyl sulfate and incubated for 2 h at 65°C. DNA within the aqueous phase was extracted twice with buffered phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform. DNA was precipitated with 0.6 volumes of 100% isopropanol and resuspended in sterile 1× TE buffer.

2.4. 16S rRNA gene amplification, barcoding, and sequencing

Universal 16S primers with 454 adapters 27F (5′-GCC TTG CCA GCC CGC TCA GTC AGA GTT TGA TCC TGG CTC AG-3′) and 338R (5′-GCC TCC CTC GCG CCA TCA G-barcode-CAT GCT GCC TCC CGT AGG AGT-3′) with 8-mer barcodes on the reverse primer (Hamady et al. 2008) were used to amplify ~300 bp of the 16S rRNA gene from the DNA extractions of 3 randomly selected oyster EF and 2 water samples each month. Bacterial DNA (0.5–1 ng) was combined with 10× buffer (1× final concentration), dNTPs (0.25 mM each), forward primer mix (0.1 µM final concentration), reverse primer mix (0.1 µM final concentration), and TaKaRa Ex *Taq* DNA polymerase (1.25 U) to a final volume of 25 µl. PCR amplification of samples was performed using the following conditions: 95°C for 5 min; 30–34 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min; 72°C for 7 min. The entire PCR volume was run on a 1.8% agarose gel. Amplicon bands were excised and DNA purified using the Qiaquick Gel Extraction kit. Amplified DNA (100 ng per sample) was used for sequencing. Samples were sequenced on the Roche 454 Genome Sequencer with FLX Titanium technology. Sequences have been submitted to the Sequence Read Archive (SRA) with BioProject PRJNA 450640 and BioSample accessions SAMN08943017–SAMN08943076.

2.5. Denoising and taxonomic assignment

Sequence reads were dereplicated in QIIME (Caporaso et al. 2010) with the following parameters: minimum quality score of 25, minimum length of 295 bp, zero barcode errors, maximum 1 primer mismatch. Only reads passing these parameters were retained. Barcodes were removed from the sequence reads, and all sequence reads were trimmed to a final length of 295 bp. Retained sequence reads were prefiltered for 454 homopolymer indels using the optional filterIndels.py script with default parameters prior to assignment of oligotypes (single nucleotide polymorphism variants) by Cluster Free Filtering (Tikhonov et al. 2015) with a maximum of 1 expected error per sequence (Table S1 in the Supplement at www.int-res.com/articles/suppl/m653p057_supp.

pdf). Oligotypes were assigned taxonomies using QIIME with the Greengenes 13_8 reference database (DeSantis et al. 2006). Oligotypes identified as chloroplast sequences were discarded from future analyses.

2.6. Oyster EF and water bacterial community correlations with physicochemical water conditions

Oyster EF and water bacterial abundances, alpha diversity, and UniFrac distances were compared by Spearman's Rank correlation to contemporaneous physicochemical conditions in the water column, as well as physicochemical conditions after introducing a 1 mo lag. Alpha diversity was calculated as mean monthly Chao1 and Shannon index values for oyster EF and water samples in QIIME. Communities were sub-sampled 1000 times at a depth of 1000 sequences. Community similarity amongst oyster EF samples each month was calculated as the mean pairwise weighted UniFrac distance (Lozupone & Knight 2005) between samples. Community similarity amongst monthly water samples was calculated similarly. Values were compared to physicochemical water conditions for all months except January and February, when water condition data were unavailable.

2.7. Oyster EF and water bacterial community comparisons

Alpha diversity was compared between oyster EF and water samples by Chao1 and Shannon indices in QIIME. Communities were sub-sampled 1000 times at a depth of 1000 sequences, and significance between EF and water was determined by 999 Monte Carlo permutations. Oyster EF and water communities were compared by ANOSIM (Clarke 1993) with 999 permutations using distance matrices of unweighted and weighted UniFrac distances. Bacterial taxa (class level) with significantly different (false discovery rate [FDR] $p < 0.05$) relative abundances between oyster EF and water samples were identified by a Kruskal-Wallis test. Oligotypes significantly associated with oyster EF and water samples (FDR $p < 0.05$) by relative abundance were identified by a Kruskal-Wallis test in QIIME after removing oligotypes observed in fewer than 9 samples. Bacterial taxa (class level) significantly more abundant (FDR $p < 0.05$) in autochthonous or allochthonous oyster EF communities were identified by a Kruskal-Wallis test. A phylogenetic tree of oligotypes significantly (FDR $p < 0.05$) associated with oyster EF and water samples by relative abundance was made in QIIME using RAxML v.7.3.0 (Stamatakis 2006).

Community similarity amongst oyster EF samples was calculated by weighted UniFrac distances. This same approach was used to calculate community similarity amongst water samples and between oyster EF and water samples. To examine the change in community similarities with the passage of time, pairwise UniFrac distances were sorted based on the number of months between samples (e.g. pairwise distances of oyster EF samples from October and November would be grouped into the 1 mo separation category, as would samples from June and July, etc.).

2.8. Correlations between oligotypes

The relative abundances of paired oligotypes (oligotypes present in both oyster EF and water samples) were compared over time by Spearman's rank correlation in R v. 3.5.1 (R Core Team, 2013). Paired oligotypes were tested for correlated relative abundances over time without a lag and after introducing a 1 mo lag in oyster EF relative abundances. Paired oligotypes were considered correlated if $p < 0.05$ for either no-lag or 1 mo lag analyses. Predicted absolute abundances of oligotypes in oyster EF were also compared with the following physicochemical parameters over time by Spearman's rank correlation in R: temperature, salinity, dissolved oxygen, pH, and chlorophyll. Spearman's rank R-values were used to create a correlation profile for each oligotype. Oyster-associated oligotypes were clustered in R by hierarchical clustering using the 'Heatmap.2' package (Warnes et al. 2009) with default settings. Autochthonous and allochthonous oligotypes were grouped by positive and negative R-values when correlated with each physicochemical parameter and then compared by Kruskal-Wallis tests within each group (e.g. autochthonous and allochthonous oligotypes that were positively correlated with temperature, etc.).

2.9. Predicting metabolic potential of bacterial communities

The metabolic potential of oligotypes significantly associated with oysters (autochthonous oligotypes) and oligotypes observed in oysters but not significantly

associated with them (allochthonous oligotypes) were predicted using PiCrust (Langille et al. 2013). The relative abundances of genes involved in nitrogen and sulfur metabolism were identified, and enrichment of specific metabolic processes in autochthonous communities was examined by comparing predicted gene abundances between autochthonous and allochthonous communities by Mann-Whitney tests.

3. RESULTS

3.1. Oyster EF and water column bacterial abundances

Oyster EF and water column bacterial abundances varied over time (Fig. S1A). Mean bacterial abundance in EF was approximately twice that of the ambient water over 1 annual cycle (Fig. S1B), and bacterial abundances between the 2 environments were not correlated (Fig. S1C, $R^2 = 0.15$). However, EF and water column bacterial abundances were correlated when introducing a 1 mo lag in EF bacterial abundances (Fig. S1C, $R^2 = 0.75$, $p < 0.001$). EF bacterial abundances also correlated with water temperature when a 1 mo lag was introduced (Table 2).

3.2. Oligotype distributions between oyster EF and water samples

In total, 574 oligotypes were identified in oyster EF (151 651 reads; n = 32) and water (112 585 reads; n = 24) samples (Table S1). Most oligotypes (441, i.e. 77% of all observed oligotypes) were not significantly associated with either environment (FDR $p > 0.05$) and should be considered allochthonous bacterial populations in oysters. These allochthonous oligotypes accounted for on average ~63% of oyster EF communities and ~83% of water communities (Table 3). An additional 4% of the oyster EF community consisted of oligotypes significantly (FDR $p < 0.05$) associated with the water column (Table 3). These oligotypes should also be considered allochthonous in oysters.

Oligotypes observed in water samples were also observed in oyster EF. Only 14 oligotypes were observed exclusively in water samples, and none were significantly associated with the water column (Table 3). In contrast, 107 oligotypes were observed exclusively in oyster EF samples, including 52 that were significantly (FDR $p < 0.05$) associated with oysters and 55 that were not significantly associated with either environ-

Table 2. Spearman's Rank R values of correlations between water column physicochemical parameters and bacterial communities in oyster extrapallial fluid (EF) and the water column. + Lag: results after introducing a 1 month lag in observed oyster EF and water bacterial community abundances and diversity behind measured water column physicochemical properties. Significant ($p < 0.05$) correlations are highlighted in **bold**

	Bacterial abundance		Chao1		Shannon index		UniFrac distance	
	Oyster (+ Lag)	Water (+ Lag)	Oyster (+ Lag)	Water (+ Lag)	Oyster (+ Lag)	Water (+ Lag)	Oyster (+ Lag)	Water (+ Lag)
Temperature (°C)	0.60	**0.72**	0.42	0.38	0.20	–0.22	0.15	–0.18
	(0.83)	(0.53)	(0.45)	(0.48)	(0.47)	(–0.37)	(0.43)	(0.10)
Salinity (ppt)	0.45	–0.39	–0.26	**–0.72**	–0.21	**–0.78**	0.61	0.19
	(–0.22)	(–0.50)	(–0.37)	(–0.32)	(–0.13)	(0.12)	(0.35)	(0.12)
pH (total)	**–0.69**	0.08	–0.18	0.28	0.18	**0.69**	–0.01	0.10
	(–0.32)	(–0.33)	(–0.15)	(–0.21)	(–0.23)	(0.00)	(–0.54)	(0.27)
Specific conductivity (mS cm^{-1})	0.45	–0.39	–0.26	**–0.72**	–0.21	**–0.78**	0.61	0.19
	(–0.22)	(–0.50)	(–0.37)	(–0.32)	(–0.13)	(0.12)	(0.35)	(0.12)
Dissolved oxygen (mg l^{-1})	**–0.64**	**–0.67**	–0.37	–0.37	–0.18	0.21	–0.19	0.22
	(–0.77)	(–0.57)	(–0.53)	(–0.57)	(–0.55)	(0.27)	(–0.37)	(–0.22)
Chlorophyll (µg l^{-1})	0.05	**0.70**	0.27	**0.79**	0.31	0.43	–0.02	–0.42
	(0.50)	(0.48)	**(0.68)**	**(0.70)**	(0.58)	(–0.13)	(–0.17)	(0.27)

Table 3. Distribution of bacterial small-subunit ribosomal RNA gene oligotypes in oyster extrapallial fluid (EF) and water samples. Selected community: environment with which oligotypes were significantly associated (FDR $p < 0.05$, Kruskal-Wallis test); observed environment: indicates whether oligotypes were observed in only oyster samples, water samples, or both; correlated paired oligotypes: indicates whether oligotypes displayed significantly correlated relative abundances over time in oyster and water samples (Spearman rank correlations $p < 0.05$); no. (%) of oligotypes: number of oligotypes observed in each descriptive category and their percentage of total (574) oligotypes; mean % oyster community: mean relative abundance of oligotypes in each descriptive category across oyster EF samples (n = 36); mean % water community: mean relative abundance of oligotypes in each descriptive category across water samples (n = 24)

Selected community	Observed environment	Correlated paired oligotypes?	No. of oligotypes (% of oligotypes)	Mean % oyster community	Mean % water community
Oysters	Oysters	–	52 (9.06)	7.2	0.0
	Oysters & water	No	37 (6.45)	19.7	0.5
	Oysters & water	Yes	5 (0.87)	6.2	0.2
	Total		94 (16.4)	33.0	0.6
Water	Water	–	0 (0)	0.0	0.0
	Oysters & water	No	29 (5.05)	2.3	11.2
	Oysters & water	Yes	10 (1.74)	1.9	5.1
	Total		39 (6.79)	4.2	16.3
None	Oysters	–	55 (9.58)	4.9	0.0
	Water	–	14 (2.44)	0.0	0.8
	Oysters & water	No	112 (19.51)	12.7	23.0
	Oysters & water	Yes	260 (45.30)	45.2	59.4
	Total		441 (76.89)	62.8	83.1
	Grand total		574 (100)	100	100

ment (Table 3). In all, 94 oligotypes were significantly associated with oyster EF samples, accounting on average for 33% of the bacterial community in oysters and <1% of the bacterial community in the water column (Table 3). The 94 oligotypes significantly associated with oyster EF samples should be considered autochthonous bacterial populations under environmental selection in oyster EF.

3.3. Oligotype persistence and abundance in oyster EF and water samples

Over half (56%) of all observed oligotypes were present in EF samples for ≤4 mo, as were 74% of oligotypes in water samples (Fig. S2E). Oligotypes that were more persistent (i.e. observed more frequently over time) in water samples were also more persistent in oyster EF samples (Fig. S2C). In contrast, oligotypes that were more persistent in oyster EF were not as persistent in water samples (Fig. S2A). Oligotype persistence and abundance was significantly positively correlated ($r^2 = 0.70$, $p < 0.001$) in oyster EF samples (Fig. S2B), but no correlation between persistence and abundance was observed in water samples ($r^2 = 0.29$, $p > 0.05$) (Fig. S2D). Additionally, the persistence of oligotypes in the water column had no bearing on their abundance in oyster EF.

3.4. Community composition of oyster EF and water samples

Oyster EF and water bacterial communities displayed similar richness (Chao1) and evenness (Shannon index). *Alphaproteobacteria* were the most abundant bacterial taxa (class level) in both oyster EF (30% of community) and water (31% of community) communities over the annual survey but were not significantly more abundant in either environment (Fig. 1A). In total, 12 taxa (class level) were significantly more abundant (FDR $p < 0.05$) in oyster EF over the annual survey, including *Deltaproteobacteria*, *Epsilonproteobacteria*, *Gammaproteobacteria*, *Nitrospira*, and *Spirochaetes* (Table 4). Nine bacterial taxa (class level) were significantly associated with the water column over this period and included *Flavobacteriia*, *Actinobacteria*, and *Betaproteobacteria* (Table 4).

Oyster EF and water communities were significantly different by both unweighted ($p < 0.01$) and weighted ($p < 0.01$) ANOSIM analyses. Community similarity between oyster EF and water samples as measured by weighted UniFrac distance fluctuated throughout the year and was highest in June (greatest similarity between samples, i.e. lowest UniFrac distances) and lowest in February and March (least similarity between samples, i.e. highest UniFrac distances) (Fig. 1B).

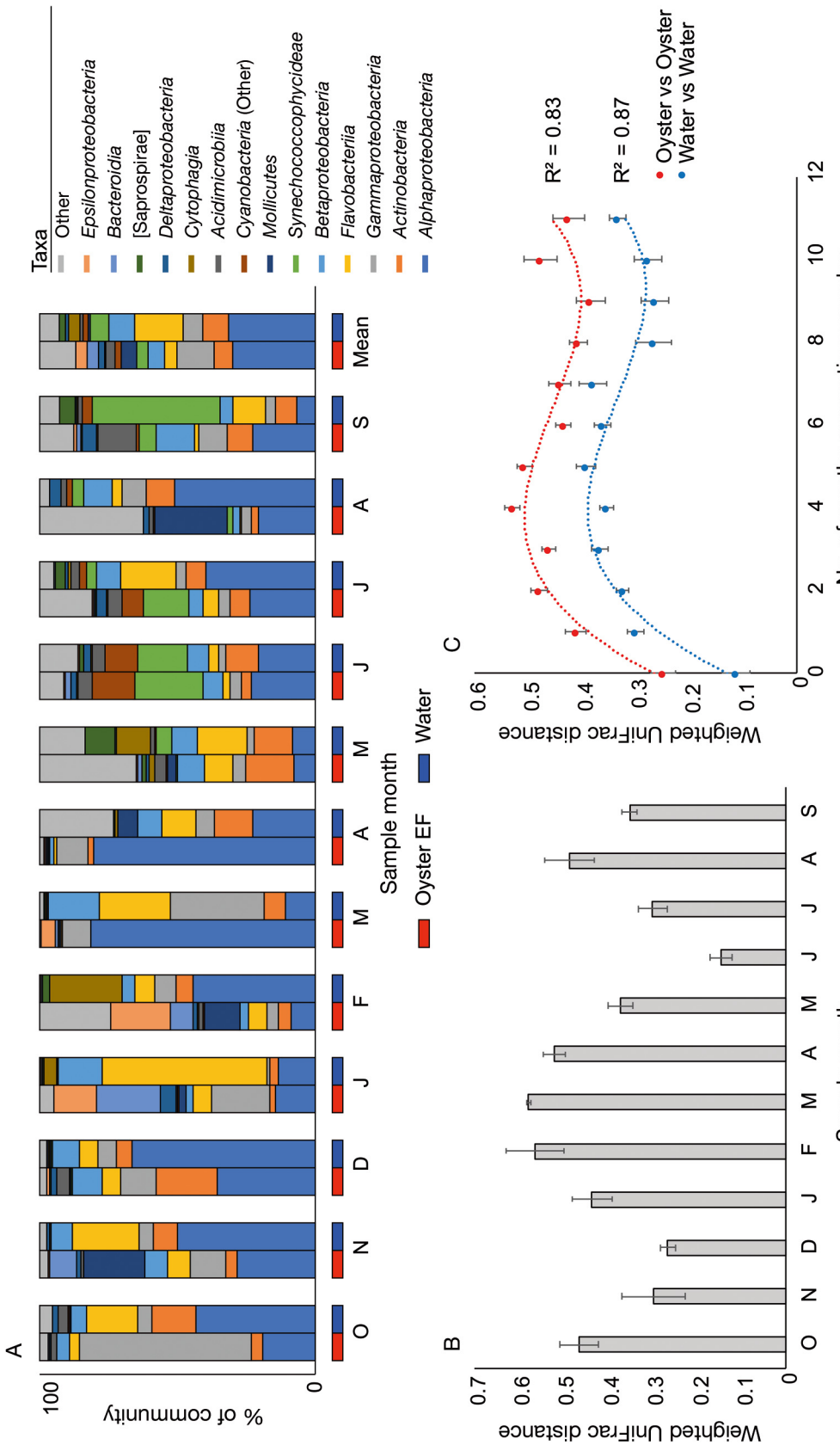

Fig. 1. Community composition and dynamics of oyster extrapallial fluid (EF) and water samples. (A) Monthly and 12 mo average bacterial community compositions (class level) of oyster EF (red) and water (blue) samples. Each month is the average community composition of 3 oyster or 2 water samples. (B) Mean community similarity between oyster EF and water samples each month as determined by weighted UniFrac distance. Lower values indicate greater similarity between communities. (C) Mean similarity of oyster EF communities (red) or water communities (blue) as a function of the amount of time between samples. Community similarities were calculated by weighted UniFrac distance and grouped by the time between samples (e.g. January vs. March and July vs. September communities both belong to the '2 mo between samples' comparison). UniFrac distances were significantly lower ($p < 0.05$) for water column communities as compared to oyster EF communities at all time intervals except for 7 mo between samples. Error bars are SE

Table 4. Bacterial taxa (class level) showing significantly (FDR $p < 0.05$) greater association with either oyster extrapallial fluid (EF) or water over 1 annual cycle

Phylum	Class	Oyster EF community mean %	Water community mean %
Acidobacteria	*Acidobacteria-6*	0.03	0.00
	Holophagae	0.03	0.00
Actinobacteria	*Actinobacteria*	6.61	9.37
Bacteroidetes	[*Saprospirae*]	0.24	2.23
	Bacteroidia	4.06	0.06
	Cytophagia	0.34	4.01
	Flavobacteriia	4.55	17.63
	Sphingobacteriia	0.19	0.83
	Other	0.03	2.17
Chloroflexi	*Anaerolineae*	0.01	0.05
Cyanobacteria	*Nostocophycideae*	0.00	0.14
Firmicutes	*Bacilli*	0.55	0.00
	Clostridia	0.26	0.00
Nitrospirae	*Nitrospira*	0.29	0.00
Proteobacteria	*Betaproteobacteria*	5.74	9.29
	Deltaproteobacteria	2.20	1.01
	Epsilonproteobacteria	3.91	0.06
	Gammaproteobacteria	13.48	7.40
Spirochaetes	[*Brachyspirae*]	1.40	0.01
	Spirochaetes	0.29	0.02
Unassigned	Other	8.20	1.49

Oyster EF communities were more variable than water column communities (i.e. higher UniFrac distances), but the communities in both environments displayed similar temporal trends (Fig. 1C). EF communities were most similar between oysters sampled in the same month and became less similar as time passed between samples. Peak dissimilarity between samples was observed when 4 mo separated oyster EF samples. Oyster EF communities then became gradually more similar when separated by greater than 4 mo (Fig. 1C). This trend was also observed between water column communities (Fig. 1C), although water column communities were significantly ($p < 0.05$) less variable than oyster EF communities at every time interval except at 7 mo of separation between samples (Fig. 1C).

3.5. Oyster-associated community composition and dynamics

Autochthonous oligotypes (i.e. oligotypes significantly associated with oyster EF samples) spanned a broad range of taxonomic diversity. Certain taxonomic groups, like the *Flavobacteriia* and *Alphaproteobacteria*, were more commonly associated with water samples than oysters (Fig. 2); nevertheless, specific oligotypes significantly associated with oysters were present within each taxonomic lineage (Fig. 2). In contrast, oligotypes from several taxonomic groups were associated predominantly or entirely with oyster EF samples, including the *Bacteroidia*, *Mollicutes*, *Deltaproteobacteria*, *Epsilonproteobacteria*, and *Gammaproteobacteria* (Fig. 2).

Autochthonous oligotypes accounted for on average 8–67% of oyster EF bacterial communities any given month (Fig. 3) and were dynamic in oyster EF over time. Autochthonous oligotypes formed 2 general groups according to correlations with water temperature and DO (Fig. 4A). High-temperature and low-temperature groups were comprised of similar taxa at the class level (Fig. 4B). Warm water-associated (i.e. positively associated with water temperature) autochthonous and allochthonous oligotypes displayed similar strengths of correlation with water temperature. In contrast, cold water-associated autochthonous oligotypes were significantly ($p < 0.01$) more strongly correlated with water temperature than their allochthonous counterparts (Fig. 5A). The strength of correlation between cold water-associated autochthonous oligotypes and water temperature increased further when introducing a 1 mo lag ($p = 0.06$), but no such increase was observed in cold water-associated allochthonous oligotypes (Fig. 5A).

Allochthonous oligotype communities in oyster EF samples mirrored the temporal dynamics of the entire EF community and the water column (Figs. 1C & 5B) and became increasingly different over a period of 4–5 mo before gradually becoming more similar again (Fig. 5B). In contrast, while autochthonous communities in oyster EF samples also became increasingly different over 4–5 mo, they did not become more similar again as time passed (Fig. 5B).

3.6. Predicted metabolic potential of oyster EF autochthonous and allochthonous oligotypes

The potential range of metabolic capabilities among autochthonous and allochthonous oligotypes was predicted by taxonomic composition using PiCrust (Lan-

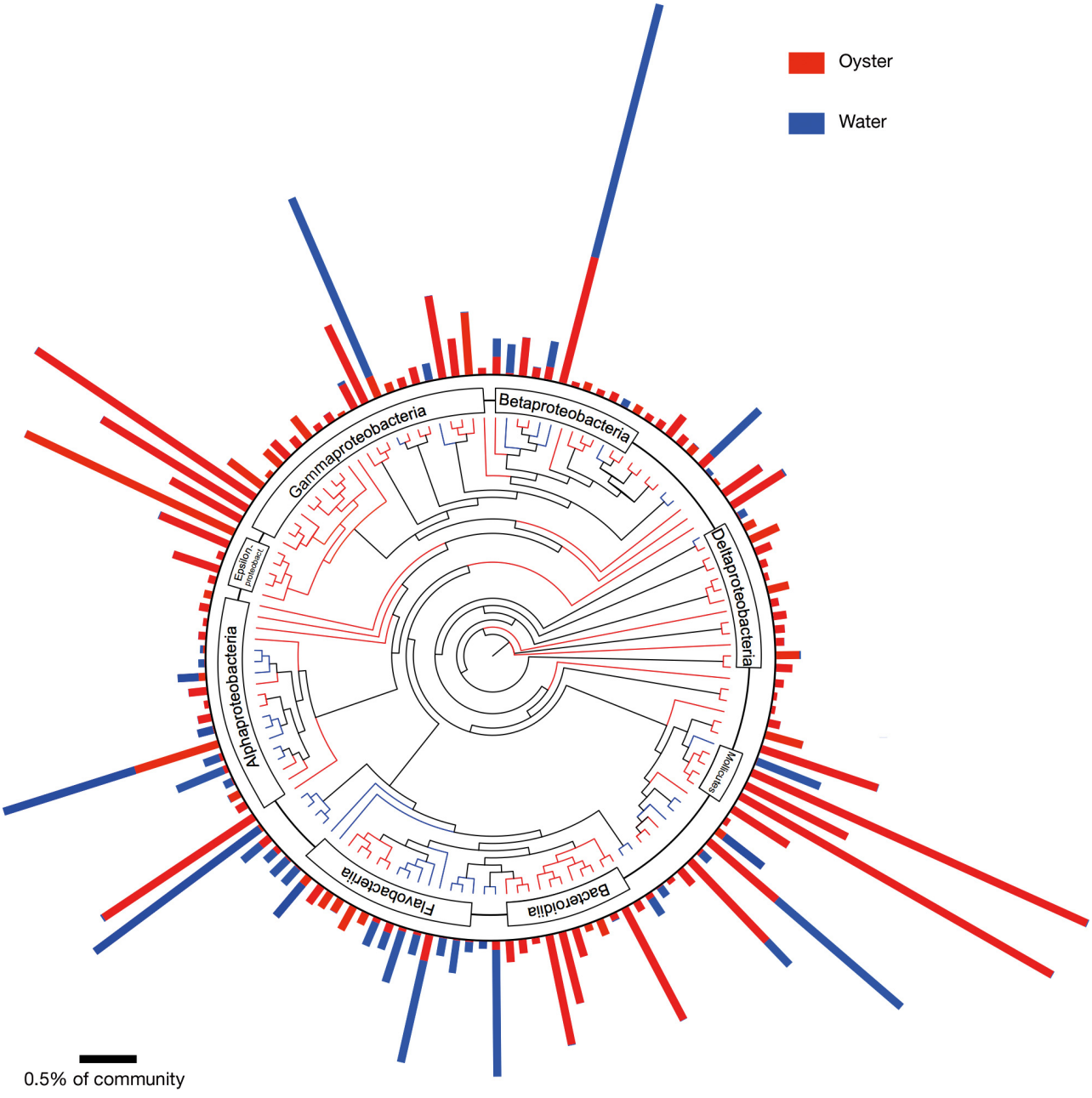

Fig. 2. Oligotypes that were significantly (FDR $p < 0.05$) associated with oyster and water samples from October 2010 to September 2011 in the Rhode River. The environment with which they were primarily associated is indicated by the color of the cladogram branches. Bars represent the mean relative abundance of oyster-associated (autochthonous) oligotypes (n = 94) and water-associated oligotypes (n = 39) over the course of the study

gille et al. 2013). In total, 933 Kyoto Encyclopedia of Genes and Genomes (KEGG) identifiers (Kanehisa & Goto 2000) were significantly ($p < 0.05$) enriched among autochthonous oligotypes and 700 among allochthonous oligotypes. Notably, autochthonous oligotypes were predicted to be significantly enriched for genes involved in dissimilatory nitrate reduction, nitrogen fixation, and nitrification pathways compared to allochthonous oligotypes (Fig. 6A). Autochthonous and allochthonous oligotypes were also predicted to be enriched for different assimilatory sulfate reduction genes for the conversion of sulfite to sulfide (*cysJ* vs. *sir*),

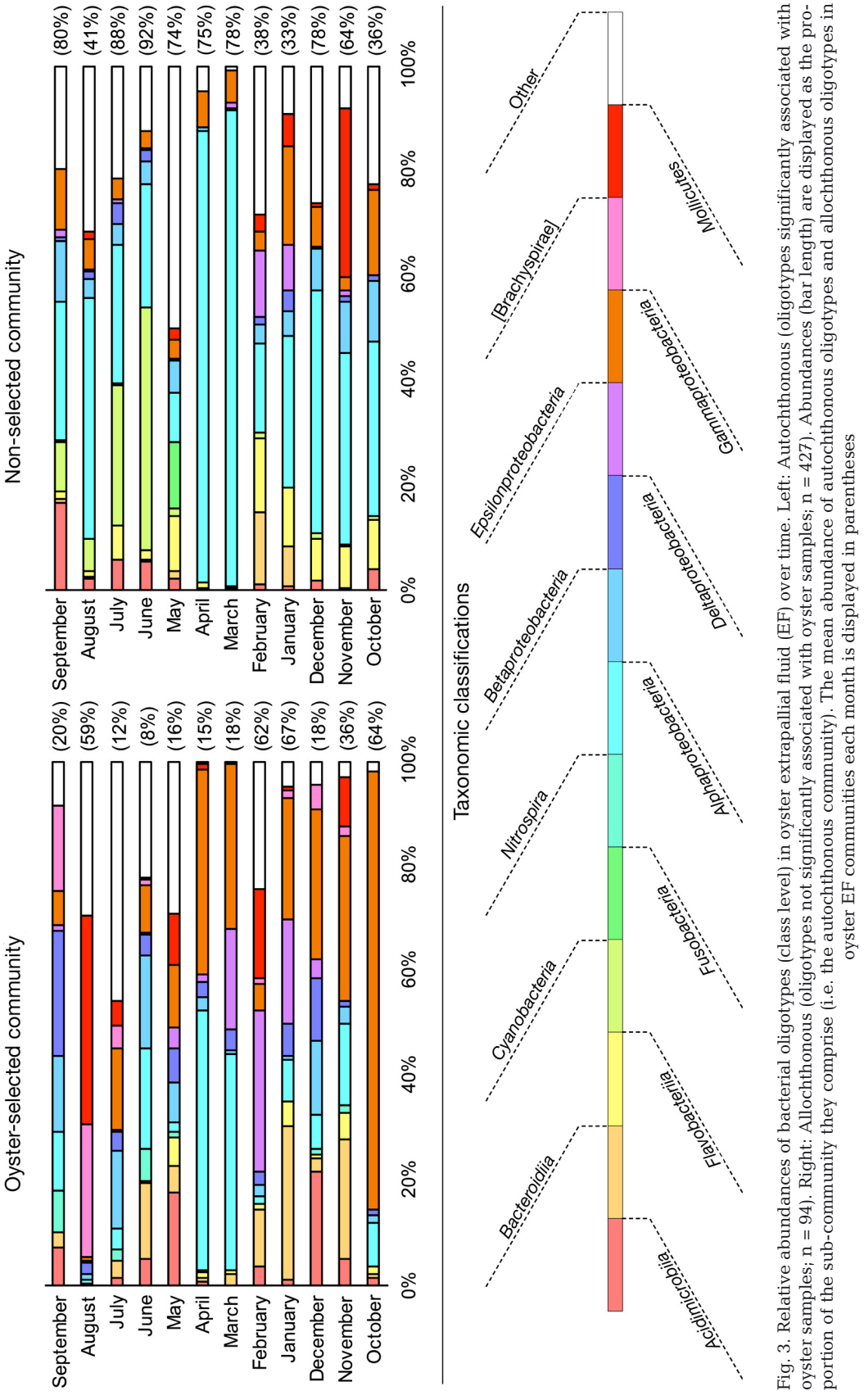

Fig. 3. Relative abundances of bacterial oligotypes (class level) in oyster extrapallial fluid (EF) over time. Left: Autochthonous (oligotypes significantly associated with oyster samples; n = 94). Right: Allochthonous (oligotypes not significantly associated with oyster samples; n = 427). Abundances (bar length) are displayed as the proportion of the sub-community they comprise (i.e. the autochthonous community). The mean abundance of autochthonous oligotypes and allochthonous oligotypes in oyster EF communities each month is displayed in parentheses

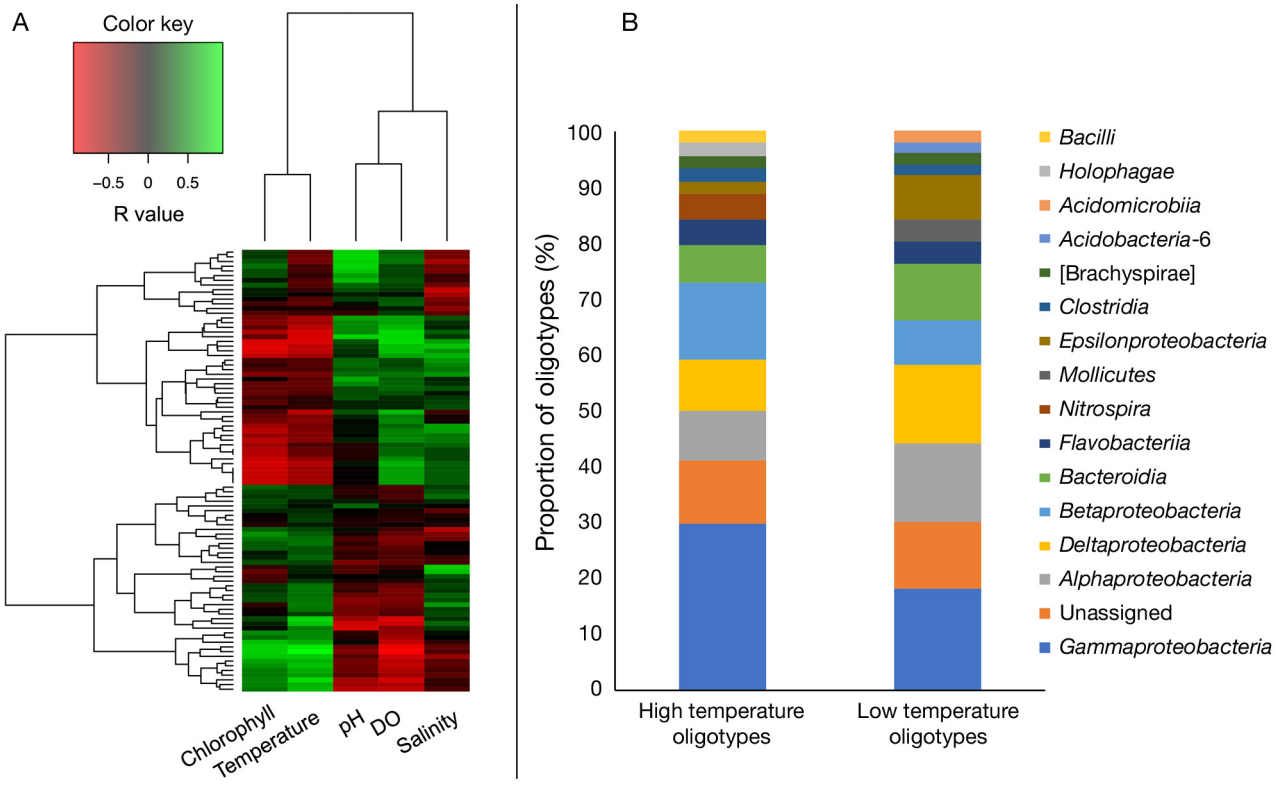

Fig. 4. Impact of environmental conditions on oyster extrapallial fluid autochthonous oligotypes. (A) Hierarchical clustering of autochthonous oligotypes (vertical cladogram) based strength of correlation between oligotype absolute abundance and measured environmental parameters (temperature, chlorophyll, salinity, dissolved oxygen [DO], and pH). (B) Taxonomic distribution of autochthonous oligotypes in high temperature and low temperature-associated groups as determined by hierarchical clustering

while dissimilatory sulfite reductase (*dsrA*) was predicted to be enriched in autochthonous oligotypes (Fig. 6B).

4. DISCUSSION

4.1. Selection for unique bacterial communities in oyster EF

Bacterial communities in oyster EF are largely a reflection of the water column diversity (Banker & Vermeij 2018). This background of diversity from the water column hinders efforts to distinguish resident from transient community members, particularly when related but distinct organisms are grouped together as OTUs. We used the higher resolution provided by Cluster Free Filtering (Tikhonov et al. 2015) to identify bacterial oligotypes and differentiate between closely related organisms. By tracking oligotypes over time, we were able to discern autochthonous oligotypes from allochthonous background diversity introduced by the water column. In this study, most bacterial oligotypes observed in water samples were also observed in oyster EF samples (Table 3). In fact, 77% of bacterial oligotypes (441 oligotypes) were not significantly associated with oysters or water samples (Table 3), and 260 of these oligotypes displayed significantly correlated temporal dynamics (Table 3) between environments. They also comprised on average 63% of oyster EF communities (Table 3). This agrees with a report of bacterial communities in *Crassostrea gigas* hemolymph, where the most abundant OTUs in water samples were identified in 85% of oyster samples and accounted for up to 43% of the hemolymph bacterial community (Lokmer et al. 2016a).

Yet, in agreement with previous reports (Kueh & Chan 1985, Pujalte et al. 1999, La Valley et al. 2009, Thomas et al. 2014), the taxonomic composition of bacterial communities in oyster EF was significantly different from that of the water column (ANOSIM, $p < 0.01$). This was attributable to a fraction (<20%) of bacterial oligotypes that were more persistent and abundant in oyster EF, comprising on average one-

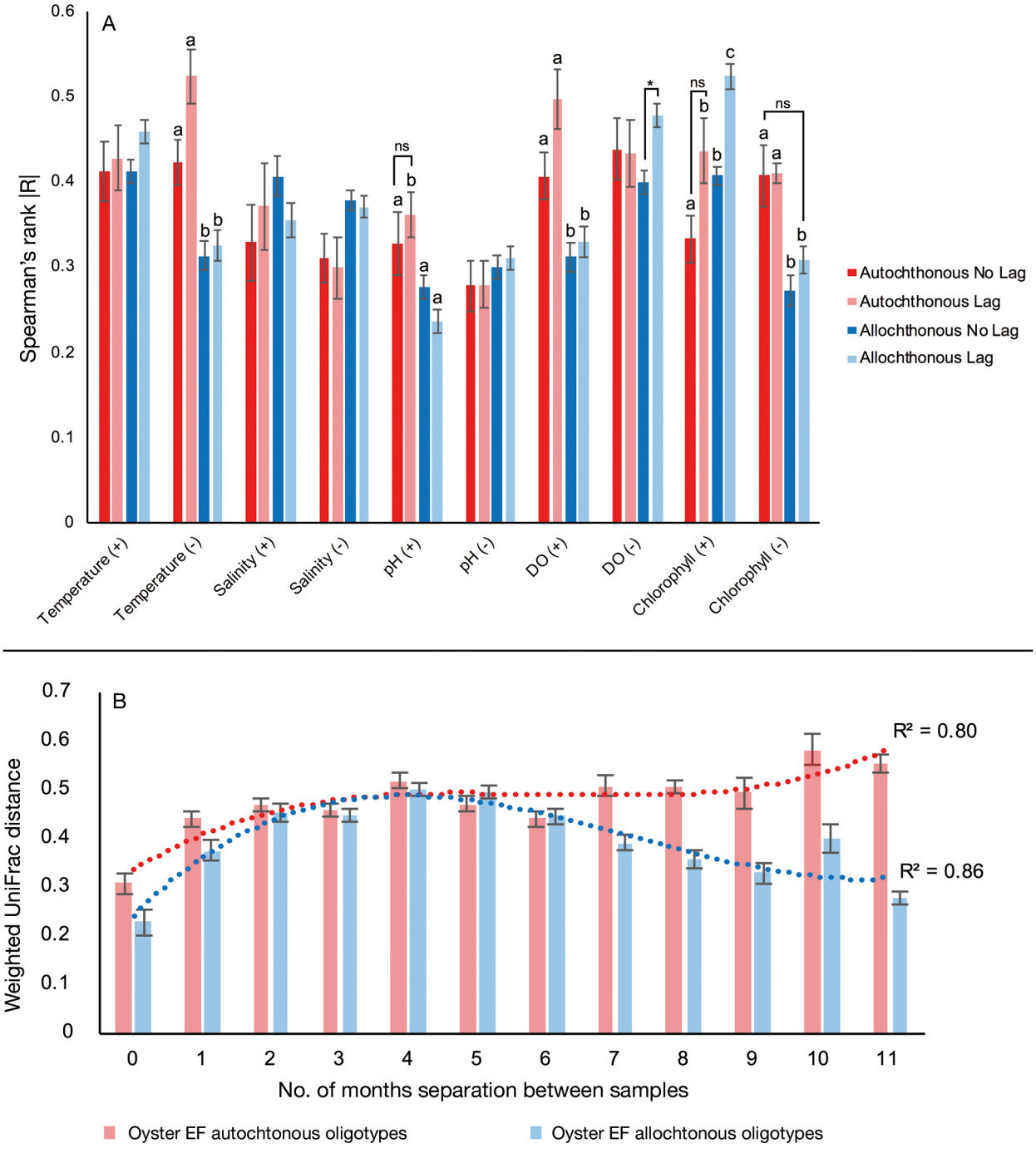

Fig. 5. Impacts of time and environmental conditions on autochthonous and allochthonous communities. (A) Strength of correlation of oligotype absolute abundance with measured environmental conditions. Oligotypes were split into positive and negative R-value groups for each comparison as noted after each environmental condition. Oligotypes were also correlated with environmental conditions after introducing a 1 mo lag behind measured environmental parameters. Letters denote statistically significant groups (Kruskal Wallis, $p < 0.05$) except where noted (ns: not significant). Only 1 comparison was significantly different for the dissolved oxygen (DO) (–) strength of correlations (noted by *). (B) Similarity of autochthonous and allochthonous communities in oyster extrapallial fluid (EF) over time. Community similarities were calculated by weighted UniFrac distance and grouped by the amount of time that passed between samples (e.g. January vs. March and July vs. September communities both belong to the '2 mo between samples' comparison). Error bars are SE

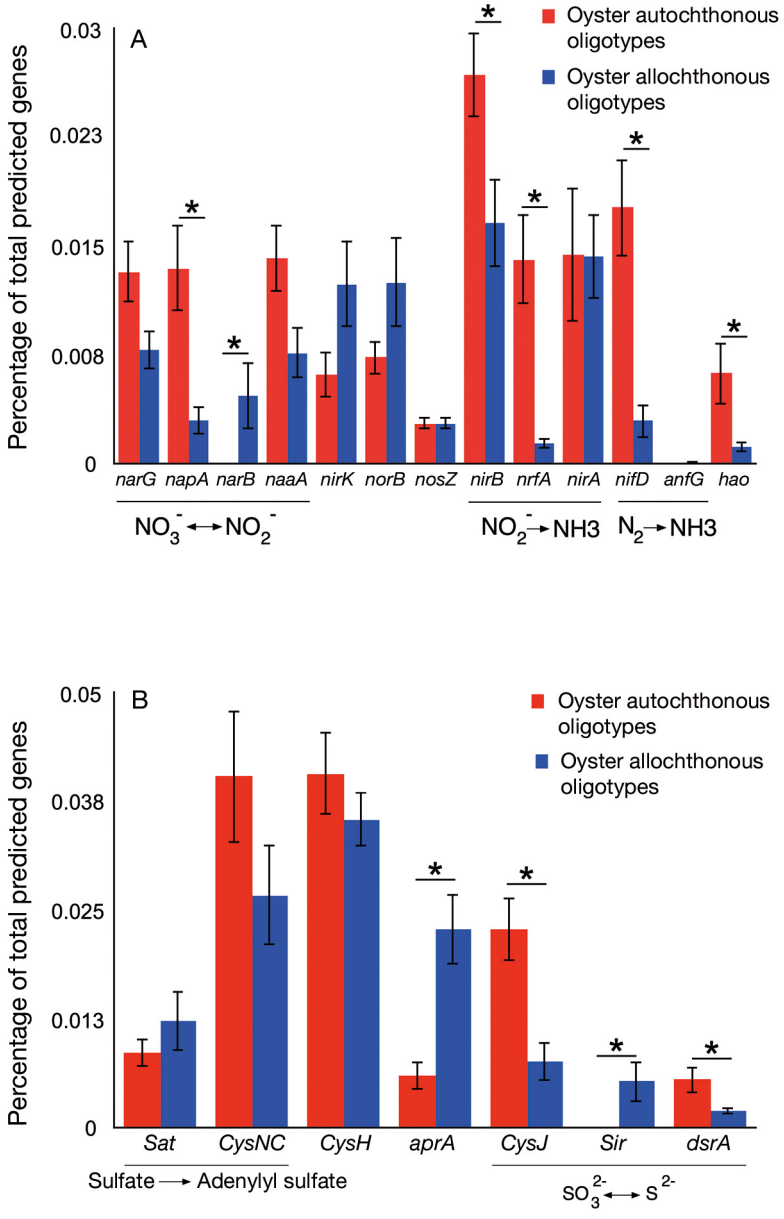

Fig. 6. Relative proportion of genes predicted to be involved in (A) nitrogen and (B) sulfur redox pathways in the oyster extrapallial fluid (EF) autochthonous and allochthonous communities. The metabolic potential was predicted by the taxonomies of autochthonous and allochthonous oligotypes by PiCrust. An asterisk indicates significantly different (Mann-Whitney, $p < 0.05$) predicted gene abundances between EF and water. Error bars are SE

third of the oyster EF community but <1% of the water community (Table 3). Oligotype persistence and abundance were positively correlated in oysters (Fig. S2B), a phenomenon that was not observed in water column communities (Fig. S2D), suggesting establishment and active growth of autochthonous bacterial populations within the oyster. Previous studies have noted that bacteria within an exogenous inoculum are rapidly removed from oysters via depuration as compared to autochthonous bacteria (reviewed by Froelich & Noble 2014). It is hypothesized that this phenomenon may be due to the competitive exclusion of exogenous bacteria by the established bacterial community within the oyster (Froelich & Noble 2014). Therefore, it is likely that the persistence and abundance of autochthonous oligotypes in EF was attributable to their establishment within the oyster and successful exclusion of other populations.

Early colonization may play a key role in determining which populations become established within the oyster EF. In this study, 26 autochthonous oligotypes shared ≥97% 16S rRNA gene sequence identity with allochthonous oligotypes. Exclusion of closely related bacteria has been observed in natural *C. virginica* populations where high salinities resulted in the loss of estuarine *Vibrio* species; subsequently, oysters were colonized by halo-tolerant *Vibrio* species that prevented recolonization by estuarine *Vibrio* even after their detection in the water column (Froelich et al. 2012). A similar mechanism may occur in the EF whereby early colonizers prevent the later establishment of other closely related bacterial populations. Additionally, the oyster immune system may impact the establishment of certain specific oligotypes but not others in oyster EF, especially since the immune system is known to be active in the pallial cavity and respond to pathogens like *Roseovarius crassostreae*, the causative agent of *Roseovarius* oyster disease (ROD, formerly known as juvenile oyster disease) (Boardman et al. 2008). Oysters do not possess an adaptive immune system, but agglutinins promote phagocytosis of bacterial cells by oyster hemocytes (Olafsen et al. 1993). Interestingly, *C. virginica* was found to agglutinate *Vibrio cholerae* but not 79 other environmental isolates (Tamplin & Fisher 1989). Similarly, *C. gigas* contained agglutinins for *V. anguillarum* but not *V. salmonicida* (Olafsen et al. 1993). This may also help explain previous reports of the impact of host factors like genotype on bacterial community composition (Lokmer et al. 2016b).

4.2. Physicochemical impacts on oyster EF communities

Bacterial communities in the water column and allochthonous communities in oysters displayed temporal patterns (Figs. 1C & 5B) similar to the stable patterns observed in the surface waters of the San Pedro Channel long-term time series (Cram et al. 2015), while autochthonous communities displayed no such pattern (Fig. 5B). Instead, autochthonous communities in oyster EF became increasingly dissimilar over time. Nevertheless, autochthonous oligotypes were split into low-temperature and high-temperature groups (Fig. 4A), suggesting some influence of season on autochthonous community composition. Distinct low-temperature and high-temperature autochthonous communities may be replaced or exist below the level of detection when less favorable seasonal conditions arise. As ecological niches within the oyster microenvironment open during seasonal transition periods, these niches could be filled by the early colonizers better adapted to the current environmental conditions and contribute to the increasingly dissimilar autochthonous communities observed over time (Fig. 5B).

Pierce et al. (2016) also observed high-temperature and low-temperature communities within *C. virginica* pallial fluid and noted that pallial fluid communities between oysters were more similar to each other during colder months. We saw a similar, albeit insignificant, trend whereby smaller UniFrac distances (i.e. more similar communities) correlated with lower water temperatures ($R = 0.15$, $p = 0.68$). However, smaller UniFrac distances were even more strongly correlated ($R = 0.43$, $p = 0.24$) with lower water temperatures when a 1 mo lag between measured water temperature and community composition was introduced (Table 2). Greater community similarity and lower alpha diversity (Table 2) in oyster EF at colder temperatures may be a function of decreased valve opening, feeding, and respiration rates by *C. virginica* in colder and lower light conditions (Loosanoff 1958, Shumway & Koehn 1982, Comeau et al. 2008, Comeau et al. 2012), which would limit the input of exogenous bacteria from the water column. A comparison of bacterial communities in *C. gigas* and *Ostrea lurida* revealed the communities of *C. gigas* to be more similar to the water column than those of *O. lurida*. The authors concluded that this may have been influenced by differences in filtration rates between the 2 species, with the higher filtration rate of *C. gigas* contributing to a more similar bacterial community to the water (Banker & Vermeij 2018). Longer periods of valve closure and lower feeding and respiration rates would also limit exogenous sources of nutrients and alter the oxygen concentration and pH of the oyster microenvironment (Crenshaw & Neff 1969). Therefore, during colder months it would be expected that the oyster microenvironment would exert a greater selective pressure on bacterial communities and enrich specific taxa within the oyster. Supporting this hypothesis, low temperature autochthonous oligotypes were significantly ($p < 0.01$) more strongly correlated with lower temperatures than their allochthonous counterparts (Fig. 5A).

4.3. Bacterial community composition of *C. virginica* EF

Oyster EF communities were enriched for several taxa (Table 4) and shared similarities with communities reported for other oyster tissues using cultivation-independent approaches. For example, *Mycoplasma* dominated the gill microbiome of *Crassostrea gigas* (Wegner et al. 2013), while *C. virginica* stomach communities were also dominated by *Mollicutes* (King et al. 2012). In this study, 2 oligotypes most closely related to *Mycoplasma gypis* strain B1/T1 (85%) and *M. moatsii* strain MK405 (83%) were the 4th and 36th most abundant oligotypes on average in oyster EF and were among the most abundant members of autochthonous EF communities (Fig. 2). Small sub-unit ribosomal RNA RFLP banding patterns from Chilean oyster *Tiostrea chilensis* homogenates indicated that *Arcobacter* (*Epsilonproteobacteria*) were common and abundant (Romero et al. 2002), and the oyster-selected EF community contained 5 *Arcobacter* oligotypes (Fig. 2). It is also notable that *Arcobacter* were enriched in *C. gigas* hemolymph and may be indicative of healthy oysters (Lokmer et al. 2016a), suggesting that these organisms may play an important but poorly understood role in the microbial communities of multiple oyster species.

Several taxonomic groups, including the *Flavobacteriia* and *Betaproteobacteria*, were enriched in water samples (Table 4), but specific oligotypes of these taxa were also part of autochthonous EF communities (Fig. 2). The juxtaposition of seawater-associated bacteria and oyster-specific bacteria in oyster autochthonous communities may reflect community responses to changes in oxygen, pH, and substrate availability with valve opening and closing (Crenshaw & Neff 1969, Lokmer et al. 2016b). Identifying the composition and metabolic functions of possible

alternative stable communities in oyster EF will be critical to evaluating the impact of changing environmental conditions on the oyster microbiome and subsequent implications for oyster health and fitness.

4.4. Metabolic potential of autochthonous communities and a possible role in shell formation

Oyster EF autochthonous communities were predicted to be significantly enriched for genes involved in dissimilatory nitrate reduction, nitrogen fixation, and nitrification pathways (Fig. 6A). The sulfite reductase genes *cysJ* and *dsrA* were also enriched in autochthonous communities (Fig. 6B), suggesting that these communities play an important role in biogeochemical cycling in the oyster microenvironment. It is noteworthy that high temperature and low temperature oligotypes were composed of similar taxa (Fig. 4B), but autochthonous oligotypes comprised on average 44% of EF communities in October–March and just 22% of EF communities in April–September (Fig. 3). Therefore, high-temperature and low-temperature autochthonous communities would be predicted to have similar functional roles in oyster EF but may have a lesser impact in warmer conditions.

The extrapallial cavity where EF is located is the site of calcium precipitation and shell formation, and one intriguing potential functional role of autochthonous EF communities is indirectly aiding shell formation. Although seawater is supersaturated with calcium carbonate, precipitation does not spontaneously occur (Braissant et al. 2007). In contrast, rates of bivalve shell mineralization are much faster than abiotic precipitation (Waldbusser et al. 2013). Interestingly, EF communities shared similarities with the microbial communities of rhodoliths (Cavalcanti et al. 2014), coralline algae that form calcareous structures. Both organisms had communities enriched in *Gamma*- and *Deltaproteobacteria* populations. The observance of *Deltaproteobacteria* in both of these organisms and functional groups related to organomineralization in rhodoliths suggests a potential role for commensal bacterial communities in biomineralization and shell formation (Cavalcanti et al. 2014).

Many *Deltaproteobacteria* are capable of sulfate reduction and are known as sulfate-reducing bacteria (SRB). However, SRB have also been implicated in a number of calcification processes. They are key members of lithifying microbial communities (Braissant et al. 2007) and play a role in the formation of pool fingers, stalactites, and stalagmites in caves (Cacchio et al. 2012). In fact, hypogean environments such as caves appear to select for calcifying microbes (Cacchio et al. 2012), and our data indicate the same for the EF. The role of SRB in remote calcification of oyster shells has been proposed, but experimental evidence is lacking (Vermeij 2014). Banker & Vermeij (2018) specifically sought to correlate SRB with calcifying fluid in 2 oyster species (*C. gigas* and *O. lurida*) but observed no enrichment of SRB in oysters as compared to water samples. However, only 3 oysters of each species were sampled, and samples were collected at a single time point in the summer. In contrast, we observed *Deltaproteobacteria* to be significantly more abundant in EF than in water (2.20 vs. 1.01%; Table 4), and 11 *Deltaproteobacteria* oligotypes were autochthonous members of EF communities (Fig. 2). While autochthonous *Deltaproteobacteria* oligotypes comprised just 1.4% of oyster EF communities on average (versus 0.02% of water communities), low relative abundances may not indicate low community impact. Indeed, Banker & Vermeij (2018) noted that SRB could impact shell formation despite comprising a small fraction of the bacterial community, as was observed in rhodoliths (Cavalcanti et al. 2014).

Furthermore, interactions between SRB and nitrate-reducing autochthonous community members may have a synergistic effect on indirect shell formation. SRB produce substantial amounts of exopolymeric substances (EPS), which can act as nucleation sites for the precipitation of $CaCO_3$(Braissant et al. 2007). Many heterotrophic bacteria can degrade EPS, which may serve to release calcium (Braissant et al. 2007). Additionally, denitrification and ammonification increase alkalinity and may aid indirectly in $CaCO_3$ precipitation (Cacchio et al. 2012). It is also noteworthy that dissimilatory nitrate reduction in anaerobic aquatic sediments is enhanced when S^{2-} is available as an electron donor (Bonin 1996). It is possible that S^{2-} from SRB metabolism in oyster EF also enhances nitrate reduction, providing a potential mechanism whereby sulfate reduction and nitrate reduction play a coordinated role in $CaCO_3$ precipitation and shell formation. Understanding the potential role of the oyster microbiome in shell formation will be essential to better evaluating the impacts that future ocean warming and acidification may have on oyster populations, especially since autochthonous EF oligotypes comprised a larger fraction of EF communities in colder conditions.

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