

DR. BIANCA TREVIZAN SEGOVIA (Orcid ID : 0000-0002-7667-6344)

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Segovia et al.--- Diversity of Seagrass Epibiotic Microeukaryotes

Microeukaryotic Communities Associated with the Seagrass *Zostera marina* are Spatially Structured

Bianca Trevizan Segovia^{a,c*}, Rhea Sanders-Smith^{a,c}, Emily M. Adamczyk^b, Coreen Forbes^b, Margot Hessing-Lewis^c, Mary I. O'Connor^{b,c}, Laura Wegener Parfrey^{a,b,c}

^a Botany and Biodiversity Research Centre, University of British Columbia, Unceded xʷməθkʷəy̓əm (Musqueam) Territory, 3529-6270 University Blvd., Vancouver, V6T 1Z4, British Columbia, Canada

^b Zoology and Biodiversity Research Centre, University of British Columbia, Unceded xʷməθkʷəy̓əm (Musqueam) Territory, 3529-6270 University Blvd., Vancouver, V6T 1Z4, British Columbia, Canada

^c Hakai Institute, PO BOX 309 Heriot Bay, V0P 1H0, British Columbia, Canada

Correspondence

B. Segovia, Botany and Biodiversity Research Centre, University of British Columbia, Unceded xʷməθkʷəy̓əm (Musqueam) Territory, 3529-6270 University Blvd., Vancouver, V6T 1Z4, British Columbia, Canada

Telephone number: +1 778-680-1427; e-mail: biatsegovia@gmail.com

ABSTRACT

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Epibiotic microorganisms link seagrass productivity to higher trophic levels, but little is known about the processes structuring these communities, and which taxa consistently associate with seagrass. We investigated epibiotic microeukaryotes on seagrass (*Zostera marina*) leaves, substrates and planktonic microeukaryotes in ten meadows in the Northeast Pacific. Seagrass epibiotic communities are distinct from planktonic and substrate communities. We found sixteen core microeukaryotes, including dinoflagellates, diatoms, and saprotrophic stramenopiles. Some likely use seagrass leaves as a substrate, others for grazing, or they may be saprotrophic organisms involved in seagrass decomposition or parasites; their relatives have been previously reported from marine sediments and in association with other hosts such as seaweeds. Core microeukaryotes were spatially structured and none were ubiquitous across meadows. Seagrass epibota were more spatially structured than planktonic communities, mostly due to spatial distance and changes in abiotic conditions across space. Seawater communities were relatively more similar in composition across sites and more influenced by the environmental component, but more variable over time. Core and transient taxa were both mostly structured by spatial distance and the abiotic environment, with little effect of host attributes, further indicating that those core taxa would not show a strong specific association with *Z. marina*.

Keywords

Protists; community assembly; core microbiota; 18S rRNA gene; biogeography; eelgrass; biodiversity.

SEAGRASS meadows generate multiple high-value ecosystem services including carbon sequestration, coastal protection from erosion, and improvement in water quality (Duarte et al. 2013; Lamb et al. 2017). Primary productivity of seagrass meadows is amongst the highest of aquatic ecosystems (Duarte and Chiscano 1999). Much of the productivity and functions supporting coastal food webs is associated with the microbial communities living on the surface of seagrass blades (Moncreiff et al. 1992; Duffy et al. 2015); we refer to these microbes as the epibota throughout. The epibota are the primary food source for associated invertebrate epifauna – mainly isopods, amphipods

(Mittermayr et al. 2014) and gastropods (Peduzzi 1987) – that link seagrass productivity to higher trophic levels (Orth et al. 1984). These epibionts consist of prokaryotes and eukaryotes with diverse trophic strategies (Bengtsson et al. 2017), including some that can provide an additional source of nitrogen to seagrass leaves by mineralizing amino acids (Tarquinio et al. 2018).

The diversity patterns of only a few groups of microbial eukaryotes have been studied in detail, mostly microalgae such as diatoms and dinoflagellates (Chung and Lee 2008; Mabrouk et al. 2011). The protist *Labyrinthula zosterae* has also been extensively investigated because it is the pathogenic agent for “seagrass wasting disease”, which has been linked to declines in *Zostera marina* meadows on both coasts of the Atlantic Ocean in the 1930s and remains a concern today (Short et al. 1987). Studies on other microbial eukaryotes and full communities remain scarce, though two recent studies using 18S rRNA amplicon sequencing report different communities of eukaryotic epibiota across sites with contrasting environmental conditions. Hassenrück et al. show that epibiota on tape seagrass (*Enhalus acroides*) in Papua New Guinea are different near a natural CO₂ vent compared to control sites (Hassenrück et al. 2015). Similarly, eukaryotic epibiota associated with *Z. marina* in the German Baltic Sea differ between open coast and lagoon sites that are geographically close (Bengtsson et al. 2017).

Among the thousands of microbes associated with hosts, most are transient taxa that occur in low frequency and abundance (Van Der Gast et al. 2011). There are usually only a few taxa that are consistently found across host populations (Hernandez-Agreda et al. 2018). These core taxa of the host-associated community are thought to be strongly related to host functioning (Shade and Handelsman 2012). Seagrass susceptibility to infections that can lead to die-off events is an example of a function that might have a microbial basis (Ugarelli et al. 2017). Therefore, identifying core taxa may shed light on microbes that are functionally important and enable microbiome manipulation to benefit the host (Rosado et al. 2018).

Several factors affect microeukaryote community assembly on seagrass leaves. Differences in epibiota composition across sites can be due to the physical environment or due to host characteristics that select for some taxa. Environmental filtering based on niche differences between species is important in determining the structure of the regional species pool that will colonize seagrass leaves (Miller et al. 2018). Abiotic conditions of the surrounding seawater, such as water depth, salinity,

nutrient concentrations and CO₂ (Spivak et al. 2009; Hassenrück et al. 2015; Bengtsson et al. 2017; Nelson 2017), and host-related factors such as dry weight and leaf area (Mabrouk et al. 2011; Bengtsson et al. 2017) have been shown to influence the microeukaryotic community structure on seagrass leaves. In addition to local environmental conditions, regional processes such as dispersal should be important for structuring host-associated microbial communities. The strength of environmental filtering is dependent on the dispersal rates between local communities (Winegardner et al. 2012). When dispersal is sufficient environmental filtering occurs because species are able to reach suitable habitats, whereas very high dispersal rates homogenize communities by allowing species to be present in habitats where environmental conditions are outside their optima (sink populations; Mouquet and Loreau 2003). Alternatively, low dispersal can limit species from colonizing suitable habitats and can also weaken the match between local communities and environmental conditions (Heino et al. 2015).

Microbes in general are considered less affected by geographic distance than larger organisms (De Bie et al. 2012) due to their high abundance and dispersal rates (Fenchel and Finlay 2006). Environmental variation was found to be the main driver of the distribution patterns of bacteria and archaea in the ocean (Louca et al. 2016). For microeukaryotes evidence for spatial structuring has been found over large spatial scales (Foissner 2006; Martiny et al. 2006), and the relative importance of dispersal and environmental filtering may depend on body size (Villarino et al. 2018). Planktonic taxa are thought to have higher dispersal rates than epibionts, as planktonic microbes are highly connected by water flow and can be readily homogenized (Fodelianakis et al. 2019); indeed Wetzel et al. (2012) showed steeper distance decay in community similarity for epiphytic freshwater diatoms compared to planktonic diatoms. Macroalgal epiphytes also show patchy distributions and communities decrease in similarity with distance (Vanderklift and Lavery 2000; Saunders et al. 2003). Spatial patterns have seldom been investigated for epibiotic microbial communities, but spatial structuring was attributed to concomitant changes in other abiotic variables over distance (Frankovich et al. 2006).

We investigated the diversity patterns of microeukaryote epibiota associated with *Z. marina* to answer the following questions: 1) are *Z. marina* epibiota distinct from other biofilm epibiota and planktonic communities in the surrounding seawater? 2) what are the core microeukaryote taxa on *Z.*

marina? 3) which factors influence epibiota and plankton community structure? Are they similar for core and transient epibiotic taxa? We predict that *Z. marina* leaves and seawater samples harbour distinct microeukaryote communities. We expect epibiotic communities to be more spatially structured than seawater communities because planktonic microbes are highly connected by water currents. Moreover, we expect the core epibiotic taxa to be more influenced by host attributes than the abiotic environment of the surrounding seawater.

MATERIALS AND METHODS

Sample collection

During the summer of 2015 and 2016, we sampled microbial communities across five regions along four islands on the coast of British Columbia, Canada: Calvert Island (Choked, GPS: 51.66825, -128.11855 and Pruth, GPS: 51.395, -128.069), Goose (GPS: 51.92652, -128.45317), McMullin (GPS: 52.06178, -128.41239) and Triquet (GPS: 51.80869, -128.24746) islands (Fig. S1), which are located within the The Hakai Lúxvbálís Conservancy. In each region we sampled two subtidal *Z. marina* meadows approximately 1 km apart (1 meadow = 1 site), except for Choked where three sites were sampled. At each site, on SCUBA, we established three parallel transects 15 m apart, and placed three 0.25m² quadrats along each transect 15m apart to create a 3x3 array. We collected one shoot just outside each quadrat for microbial sampling at six-eight quadrats per site. This shoot was sealed into a sterile Ziploc bag underwater at the location of collection. To distinguish seagrass-associated microbial taxa from those coming from the surrounding seawater, we sampled water above each quadrat before the seagrass sampling (within ~ 1 meter of the eelgrass canopy) by filling a 500ml sterile Nalgene bottle underwater and closing in situ. We also sampled artificial seagrass deployed in the Choked region in 2015 from another experiment. Artificial seagrass consisted of 4 polyethylene seagrass ‘shoots’, each consisting of two 50 centimeter long ‘blades’, which are similar to the metrics of real seagrass shoots from this region. They were anchored to the substrate within and adjacent to the eelgrass meadow and sampled one week after deployment. We further sampled nearby substrates (i. e. rocks and shells) in 2016 from Choked, Goose and Triquet regions. Water, seagrass shoots, substrates and artificial seagrass were brought to the surface and kept cool and in the dark until further processing.

At each quadrat we harvested above ground seagrass biomass to measure seagrass density (number of shoots) and dry weight. Before drying, we also randomly selected five shoots for biometric measurements (number of seagrass blades, length and width of longest blade). We used leaf area index as a measure both the structural complexity of seagrass beds, and also the amount of habitat available for colonization by epiphytes and invertebrates (Green and Short 2003; Enríquez et al. 2019). Leaf area index (LAI) was calculated by multiplying mean blade length, width and number of blades per shoot, and then multiplying that surface area by the number of shoots in the quadrat. An additional shoot was selected for microepiphyte biomass, which was measured by scraping the biofilm with a glass slide filtering onto GF/C filters and weighting the dried filters. There is a high variation in seagrass morphology across regions. For example, seagrass shoots from Pruth, the calm and protected inland channel on Calvert Island, are short and thin, while shoots from Choked Passage on the outer, exposed coast of Calvert Island are long and wide (Fig. S2 and Table S6), so we included these measurements to investigate the effects of host attributes on microbial communities. We measured abiotic conditions to investigate how the abiotic environment influence seagrass epibionts. We measured dissolved oxygen, temperature, salinity and water depth in each site using a YSI Pro Plus. There are differences among sites; Choked sites are colder than other regions; Fig. S2 and Table S6).

We sampled microbial epibionts from shoots on the boat within two hours of collection using sterile conditions. We selected a 10cm length of the second oldest leaf close to the sheath, avoiding large epiphytes, and rinsed for 10 seconds with filtered seawater (0.22 μ m) to remove unattached organisms. We then sampled the microeukaryotes from this region using Puritan® sterile swabs by swabbing for 10 seconds. Swabs were then placed in sterile cryovials and kept cool and dark on the boat, then transferred to -80°C within 6 hours of sampling until DNA extraction. Water samples were filtered (500 ml) in the lab within 6 hours of collection through 0.22 μ m Durapore membrane filters using a peristaltic pump, and then placed in sterile Whirl-pak bags using sterile forceps. Filters were stored in -80°C until DNA extraction.

Molecular methods

We extracted DNA from swabs and filters using the Qiagen Powersoil® –htp 96 Well DNA Extraction Kit (Carlsbad, CA). The V4 region of 18S rRNA gene was targeted for amplification using

primers E572F: 5'-CYGCGGTATTCCAGCTC-3' and E1009R: 5'-AYGGTATCTRATCRTCTTYG-3' (Comeau et al. 2011). PCR, library construction, and Illumina MiSeq amplicon sequencing were carried out at the Integrated Microbiome Resource facility at the Centre for Comparative Genomics and Evolutionary Bioinformatics at Dalhousie University (Halifax, Canada) according to standard protocols (Comeau et al. 2017).

Sequence data analysis

Raw sequencing reads were demultiplexed using the Split Libraries function from the Quantitative Insights into Microbial Ecology (QIIME v.1.9) analysis pipeline (Caporaso, Kuczynski, et al. 2010). Demultiplexed reads were then trimmed to a uniform length of 250 bp using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and processed into operational taxonomic units (OTUs) using the MED method (Eren et al. 2015) as implemented in the Oligotyping microbial analysis software package (Eren et al. 2013). MED uses Shannon entropy to separate out meaningful patterns of nucleotide diversity from sequencing noise and partition the data into MED nodes, which in practice are analogous to $\geq 99\%$ OTUs. We set the minimum substantive abundance parameter (-M) at 250 reads and used default settings for all other parameters.

Taxonomy was then assigned to the representative sequence for each MED node using UCLUST V1.2.22q (Edgar 2010) and the SILVA 128 (Quast et al. 2013) database clustered at 99% similarity. OTUs that matched exactly a reference sequence in SILVA inherited the reference accession, taxonomy, and sequence. The taxonomy of the remaining representative sequences was assigned with UCLUST V1.2.22q by consensus of the top three hits; the taxonomic level annotated differs across OTUs and reflects the confidence in assignment. Taxonomy of core OTUs was refined using phylogenetic trees (see below). Chimeras, sequences belonging to *Zostera*, sequences unassigned at the domain level, and prokaryotic sequences were filtered out. We removed macroalgal sequences because their origin is generally unclear; most reflect propagules from nearby intertidal and subtidal rocky substrates (e.g. kelp forest species). We detected a few known seagrass epiphytes such as the red alga *Smithora naiadum*, but we avoided seagrass tissue with visible epiphytes during sampling, and epiphyte sequences were detected even in meadows where epiphytes were not observed. Thus, sequences from seagrass epiphytes are probably from propagules or environmental DNA; working out the distribution of macroalgal epiphytes on seagrass in this region requires a

dedicated study. We also removed the many Metazoans unlikely to be strongly interacting with *Zostera* (e.g. sequences from intertidal mussels). Metazoans belonging to Rotifera and the microcrustaceans Poecilostomatoida, Harpacticoida, Cyclopoida and Calanoida (copepods) were retained in the analysis as these are small eukaryotes that strongly interact with other communities in the biofilm matrix (Hall and Bell 1993). In our study, microbial eukaryotes include all the taxa except those clades excluded as described above. Additionally, within each sample, we removed all OTUs with fewer than two reads for that sample to minimize the impact of barcode switching. We also removed OTUs with fewer than 250 total reads and samples with low read count (fewer than 1000 reads). Alpha and beta diversity analyses were performed after rarefying the data to 1,000 reads/sample. The final OTU table across all samples consisted of 1052 unique sequences (655 OTUs in seagrass, 743 OTUs in seawater, 491 OTUs in substrates and 142 OUTs in artificial seagrass samples) and 3285 480 total reads, with a mean of 19 213 reads per sample.

Phylogenetic trees

We placed core OTUs into phylogenetic trees to investigate their relationships and refine taxonomic assignments. We used SILVA 128 as a phylogenetic backbone (https://github.com/parfreylab/SILVA_128/tree/master/18s) and added additional sequences of close relatives from GenBank; these additional sequences where identified via BLASTn search. We aligned the representative sequences of OTUs to SILVA 128 with PyNAST (Caporaso, Bittinger, et al. 2010) then placed them into the SILVA128 tree with EPA algorithm (Berger et al. 2011) using RaxML version 8 (Stamatakis 2014).

Data Analysis

To investigate if *Z. marina* epibiota is distinct from other biofilm epibiota and planktonic communities in the surrounding seawater (**question 1**), we performed Analysis of Variance (ANOVA) to test for differences in alpha diversity metrics, and Permutational Analysis of Variance (PERMANOVA) to test for differences in community structure (detailed below) between all sample types. Because substrates were only sampled in three regions in 2016, we performed both analyses restricting the data set to coinciding sites and year to further confirm if *Z. marina* is distinct from substrates. Using the same reasoning, we also performed both analyses restricting the dataset to the Choked region in 2015 to further confirm if *Z. marina* is distinct from the artificial seagrass.

For alpha diversity analyses, we calculated OTU richness using the non-parametric Chao1 index (Chao 1984). We also calculated Shannon diversity (Jost 2006) and Pielou's evenness (Pielou 1966). We performed an ANOVA to test for differences in alpha diversity metrics between sample types using the “aov” function and “TukeyHSD” to correct for pairwise comparisons.

We used non-metric multidimensional scaling (NMDS) to ordinate samples based on Bray-Curtis (abundance) dissimilarity to visualize the similarity of epibiota on *Z. marina*, substrates and artificial seagrass, as well as seawater microbial eukaryotes across regions and years. PERMANOVA was used to test for differences in community structure between sample types with function “adonis” in the vegan R package (Oksanen et al. 2018). Pairwise differences were conducted using Benjamini and Hochberg correction with function “adonis.pair” in the EcolUtils package (Salazar 2019). Including comparisons with other samples from the surrounding environment resulted in an uneven number of samples between sample types. PERMANOVA is robust to differences in dispersion, but only with balanced sampling designs (Anderson and Walsh 2013), so we tested for the presence of heterogeneity of dispersion. Some of the data showed heteroscedasticity (difference in dispersion), in which cases we randomly subsampled the groups so that designs were balanced for further PERMANOVA tests to confirm initial results. Statistics were generated using 999 permutations for all permutational tests.

To identify the core microbial eukaryotes living on seagrass leaves (**question 2**), we used Indicator Species Analysis (indval; Dufrêne and Legendre 1997). Indval identifies indicator species based on specificity (i.e. OTUs that are found in higher relative abundances in seagrass compared to the surrounding seawater and substrates) and fidelity (i.e. OTUs that are present across most samples from that habitat; also called prevalence (Legendre 2013). We used the function “multipatt” in the indicSpecies R package (De Caceres and Legendre 2009) with 999 random permutations. We considered OTUs to be part of the core seagrass epibiota if they were: 1) significant ($p < 0.01$) according to the permutation tests, 2) present in at least 50% of the samples and 3) present in both years.

To investigate which factors influence seagrass epibiota and planktonic communities (**question 3**), we tested for differences in community structure between regions and years for *Zostera marina* and seawater samples using “adonis2” (for marginal effects) in the vegan R package (Oksanen

et al. 2018). We used this same function to evaluate which environmental variables explain more variation in the *Zostera marina* overall microeukaryotic communities, and in core and transient taxa separately.

To further explore microeukaryote diversity patterns across space, we used distance-decay of similarity (DDS; Nekola and White 1999). We calculated pairwise dissimilarities of Hellinger-transformed (Legendre and Gallagher 2001) microeukaryote OTU matrices between sites in each year using the Bray-Curtis index. For geographic distances, we measured paths between sites across the ocean using Google Earth Pro 2018 version 7.3.2.5495. A distance matrix was constructed with the pairwise distances in kilometers across all sites. Distance between regions varied between 10km and 56km. We performed mantel analyses (999 permutations) using the “mantel” function in the vegan R package (Oksanen et al. 2018) to test for the statistical significance of the relationship between community similarity and geographic distance for seagrass epibiota and planktonic communities in each year for the whole dataset. We then fit linear regressions with geographic distances and used the slope (standardized regression coefficient (β) as a measure of the rate of decay in similarity as a function of geographical distances, whereas the intercept was used as a measure of initial similarity, which reflects beta diversity over small spatial extents (Soininen et al. 2007). We tested for differences in the slopes of the linear regressions of seagrass epibiota and planktonic communities within each year using the function “emmeans” in the emmeans (Lenth 2020) package.

Because the distance decay of similarity can arise from different processes (i.e. an increase in environmental heterogeneity with geographic distance or a decrease in dispersal rates), we further investigated which component was responsible for most of the variation in community structure. We partitioned the variance of seawater and seagrass epibiotic communities using a partial redundancy analysis (pRDA; Borcard et al. 1992)) to identify the relative contribution of environmental variables and geographic distance on the community assembly of microeukaryotes. We further analyzed the data adding year as a component explaining variation to account for interannual changes in our data and disentangled the environmental component of *Z. marina* samples into abiotic conditions and host attributes. We also analyzed core and transient taxa to explore if the relative contribution of spatial distance, abiotic conditions, host attributes and year for these groups is distinct. Thus, three variance components were generated in the first variation partitioning: (1) Pure spatial component (SPA):

spatial patterns in the microeukaryotes data that is independent of the environmental variables included in the analysis; (2) Pure environmental component (ENV): amount of variation in microeukaryotes community structure explained by environmental variables that are not spatially structured; (3) Shared component (SPAENV): the variation in microbial data explained by spatially structured environmental variables. Further variation partitioning analyses including pure abiotic (ABIOT), host attributes (HOST) and year components (YEAR) follow the same reasoning, where the explained variation of each component is shown independently of others (pure component), whereas the intersection of components reveal their shared contribution (i.e. ABIOT and YEAR shared component indicates the importance of interannual changes in the abiotic conditions in explaining variation in a community). We performed variation partitioning using the function “varpart” in the vegan R package (Oksanen et al. 2018) with adjusted canonical R^2 (Peres-Neto et al. 2006). Lastly, we tested the significance of each component ($P < 0.05$) through 999 Monte Carlo permutations (Peres-Neto et al. 2006).

For further details on statistical analysis, see Supporting Information (Methods S1). All data analyses were performed in software R version 3.6.1 (R Core Team 2019).

RESULTS

1) Are *Z. marina* epibiota distinct from other biofilm epibiota and planktonic communities in the surrounding seawater?

The ANOVA overall test and post hoc pairwise comparisons between all sample types showed that microbial eukaryotes in the biofilms (*Z. marina*, substrates and artificial seagrass) have generally lower alpha diversity than the microbial eukaryotes in the surrounding seawater (Fig. 1 and Table S1). Further comparisons between *Z. marina* and substrates, restricting the dataset to 2016 in the three meadows where those were both sampled, showed that they had similar diversity (Shannon: $F_{1,59} = 0.089$; $P = 0.766$) and evenness (Pielou: $F_{1,59} = 0.16$; $P = 0.69$), but richness was higher in the seagrass samples (Chao1: $F_{1,59} = 7.737$; $P < 0.001$). When comparing *Z. marina* with artificial seagrass within the same area and year, all alpha diversity metrics were similar (Shannon: $F_{1,11} = 0.022$; $P = 0.886$; Pielou: $F_{1,11} = 1.938$; $P = 0.191$; Chao1: $F_{1,11} = 4.718$; $P = 0.0526$).

Taxonomic composition of the most abundant taxa is strongly differentiated across sample types (Fig. 2). The most abundant microeukaryotes found on *Z. marina* are diatoms (such as Bacillariophyceae clades 1 and 2, and *Tabularia*) and dinoflagellates (*Amphidinium* and *Prorocentrum*) (Fig. 2). Artificial seagrass samples were dominated by diatoms, but there was a clear dominance of Bacillariophyceae clade 1 OTUs, in contrast to *Z. marina* sampled in the same region (Choked), which were dominated by Bacillariophyceae clade 2. The most abundant taxa on substrates were copepods (Harpacticoida), gregarines (*Lecudina polymorpha*) and rotifers (Ploimida). Seawater samples were dominated by planktonic dinoflagellates (such as *Gyrodinium* and *Heterocapsa*), microalgae (coccoid haptophytes), and symbionts of marine organisms (Syndiniales and *Lecudina polymorpha*) (Fig. 2). *Z. marina* shared similar percentages of OTUs with other sample types: 33% with artificial seagrass, 43% with substrates and 38% with seawater.

Microbial eukaryote community structure overall is also significantly different between *Z. marina* and other biofilms and seawater samples, as visualized in NMDS plots of Bray-Curtis dissimilarity, which takes abundance into account, and shown in the PERMANOVA (Table S2 and Fig. 3). Pairwise comparisons showed that microeukaryotic communities on natural *Zostera marina* leaves are more similar to those on substrates and artificial seagrass than to those in seawater (Table S2 and Fig. 3). We found similar results when using presence-absence data (Jaccard), indicating that differences were also related to community composition (Table S2). Differences between microeukaryotic community structure of *Z. marina* and substrates (PERMANOVA $F_{1,57} = 11.752$, $P < 0.001$, $R^2 = 0.13$) and of *Z. marina* and artificial seagrass (PERMANOVA $F_{1,11} = 7.702$, $P < 0.001$, $R^2 = 0.41$) were confirmed when analyses were restricted to only the year/region in which artificial seagrass and substrates were sampled.

2) What are the core taxa of microbial eukaryotes living on seagrass leaves?

We used indicator species analysis to identify the microeukaryotes that are characteristic of seagrass and enriched on surfaces compared to the water column. In order to be considered part of the seagrass core, indicator OTUs had to meet three criteria: 1) significant Indval score (<0.01), 2) present on least 50% of seagrass samples, 3) be present in both years. We identified 55 eukaryotic core OTUs on seagrass, and 12 additional OTUs that were indicators of *Zostera marina* + substrates and/or artificial seagrass leaves (Table S3). In all high-throughput sequencing studies, numerous OTUs are generated

by sequencing artifacts which expand the apparent diversity, particularly for abundant taxa. We placed all 67 indicator OTUs (55 indicators of seagrass plus 12 indicators of seagrass + other surfaces) into phylogenetic trees to identify OTUs that cluster together and have similar distribution across samples, indicating that they likely belong to the same biological entity. Finally, we collapsed them into core clades. Phylogenetic trees were also used to refine taxonomic annotations (See Supporting Information Fig. S3 – Fig. S8 for trees and additional details). The resulting 16 core taxa, 10 of which were exclusive to seagrass leaves, include dinoflagellates, diatoms, saprotrophic/parasitic stramenopiles, and heterotrophs (Fig. 4).

The core taxa show strong spatial structure, with core taxa identified as indicators of *Zostera marina* alone being much more common in the Triquet, Goose, and McMullin meadows and nearly absent from Choked (Fig. 4). Interestingly, core taxa represented in Choked were identified as indicators of *Zostera marina* + artificial seagrass, sometimes also including substrates (Fig. 4). Artificial seagrass shoots were placed only in Choked Passage in 2015. These core taxa include several diatoms and one clade of *Aplanochytrium* (Fig. 4 and Table S3).

3) Which factors influence seagrass-associated and seawater microeukaryotic community structure?

We used several analyses to determine which factors most strongly influence community structure in the water column and on seagrass. We found that microeukaryotic community structure differs by region and year for both seawater and seagrass (Fig. 3), but the regional distinction is stronger for seagrass epibiota as region explains over 10 times more variation than year (PERMANOVA region: $F_{4,88} = 16.338$, $P < 0.001$, $R^2 = 0.41$; year: $F_{1,88} = 4.874$, $P < 0.001$, $R^2 = 0.03$). In contrast, for communities in seawater interannual and regional differences explain roughly the same amount of variation (PERMANOVA region: $F_{4,42} = 3.818$, $P < 0.001$, $R^2 = 0.18$; year: $F_{1,42} = 17.621$, $P < 0.001$, $R^2 = 0.21$). Further, we found that 13.8 % of OTUs in seawater samples were found in all regions, whereas only 5 % of OTUs in seagrass samples are found in all regions. We note that two regions (McMullin and Pruth) were sampled in only one year. When restricting the dataset to only the 3 regions sampled in both years, the relative importance of region versus year is similar for seagrass (PERMANOVA region: $F_{2,63} = 18.398$, $P < 0.001$, $R^2 = 0.35$; year: $F_{1,63} = 4.780$, $P < 0.001$, $R^2 = 0.04$),

but interannual changes in seawater become much more evident (PERMANOVA region: $F_{2,32} = 5.037$, $P < 0.001$, $R^2 = 0.16$; year: $F_{1,32} = 18.468$, $P < 0.001$, $R^2 = 0.30$).

We used distance decay of similarity analysis to further compare the influence of spatial distance on community composition between seagrass and seawater. Similarity in species composition decreased with spatial distance for both seagrass and seawater samples and in both years (Fig. 5). However, the rate of decrease in community similarity is steeper for seagrass compared to seawater both in 2015 (t-ratio: 6.499; $P < 0.001$) and 2016 (t-ratio: 13.566; $P < 0.001$). Seawater samples are also more similar across small spatial scales, as indicated by their larger intercept compared with the intercepts of seagrass samples within each year (Fig. 5)

We used variation partitioning to determine the extent to which change in environmental conditions across space underlies the spatial structure in our dataset. The two years of sampling in our dataset was helpful in breaking apart the correlation between environmental conditions and space. We found that variation in seawater and epibiotic community structure was significantly explained by both the spatial and environmental components, but space plays a larger role for seagrass epibiota, whereas environmental conditions explain more community variation for seawater microeukaryotes (Fig. 6). We further partitioned the environmental variables into the abiotic environment and host attributes for seagrass-associated communities and added year as an explanatory factor to account for interannual changes in our data. For seagrass epibiota, a large contribution of the shared spatial and abiotic components indicates that spatially structured abiotic variables play an important role in community assembly, whereas host attributes have a minor influence in overall explanation. PERMANOVA analysis of the individual variables showed that temperature was the most important environmental factor explaining variation in seagrass epibiota (14%) without taking site variation into account, whereas when including site variation, nearly all of the explained variation is attributed to site differences (26%) (Table S4). For seawater microeukaryotes, the large contribution of the shared year and abiotic components suggests that interannual changes in the abiotic environment explain much of the variation in community structure (Fig. 6).

Core and transient taxa showed similar patterns on the relative contribution of the abiotic environment and host attributes (Fig. 6). The spatial component explained the most variation for both groups (Fig. 6). PERMANOVA showed that core taxa were mostly affected by both temperature and

salinity, whereas temperature was the most important factor affecting transient taxa. Host attributes, in general, explain a small portion of the variation (Table S4).

DISCUSSION

Epibiotic microeukaryote communities of seagrass are unique, but more similar to other substrates than to seawater

The epibiotic microeukaryotes on seagrass leaves were distinct from those in the water column and on nearby surfaces (Fig. 2 and Fig. 3). We found that seagrass leaves harboured a lower richness, diversity and evenness than seawater, but were similar to other substrates and artificial seagrass (Fig. 1). Seagrass leaves grow in constant contact with the seawater and microbes are exchanged between habitats; water provides a pool of potential colonists, while exudates from seagrass change plankton communities (Lamb et al. 2017), as do exudates from seaweed (Lam et al. 2008; Chen and Parfrey 2018). Indeed, we found that ~38% of OTUs are shared across habitats, though often at much lower abundance in one habitat or the other. We took steps during sampling to avoid mixing, including rinsing leaves with sterile seawater prior to swabbing, and collecting water first at each quadrat to minimize detection of organisms dislodged from seagrass surfaces or sediments into the water column. Our results are consistent with other studies reporting distinct epibiotic bacterial communities on seagrass and seaweeds compared to the surrounding seawater communities (Burke et al. 2011; Crump et al. 2018; Lemay et al. 2018; Ugarelli et al. 2019), with the exception of a global study in which seagrass leaves were not rinsed prior to sampling (Fahimipour et al. 2017). The body of literature on microeukaryotic communities living on seagrass using microscopy and other methods also find that these communities are distinct from the water column, although many organisms are shared with sediments and other surfaces (Hurtado-McCormick et al. 2019). This work highlights that the widespread pattern of a strong distinction between planktonic and epibiotic communities extends to microeukaryotes.

Core microeukaryotes on seagrass are not host-specific

We sought to identify core microbes to describe the characteristic members of seagrass epibiota and determine how consistent communities are over space and time. It is a widely held expectation from

microbial ecology, which is largely focused on bacteria and archaea, that core microbes are more likely to be functionally important to their host (Shade and Handelsman 2012). Of course, the core identified based on prevalence and specificity will include commensal and pathogenic symbionts as well as those that might be beneficial.

Identifying host-specific microbiota is a challenge even for well-studied hosts such as corals and there is no agreed upon definition (Hernandez-Agreda et al. 2016). We believe that it is crucial to consider samples from the nearby environment to distinguish taxa that are widespread everywhere from taxa that are enriched on hosts, though the core is often defined only by the prevalence and/or abundance of a taxon across host individuals (Hernandez-Agreda et al. 2016). Sampling across sites and over time also enabled us to identify core taxa that are widespread and stable over time. Using seawater samples and other surfaces to differentiate seagrass epibionts from the broader species pool, we identified 55 core OTUs that were found in over 50% of seagrass samples and present in both years (Table S3). Twelve additional OTUs were enriched on seagrass as well as nearby substrates and/or artificial seagrass leaves, suggesting that they are also commonly found in other marine surfaces. According to phylogenetic trees, all 67 core taxa collapsed into 16 core clades, 10 of which were exclusive to seagrass leaves. Even amongst the core we find striking regional variation, with most of the core found in three or four of the five regions sampled (Fig 4).

Dinoflagellates and diatoms are highly represented in the core taxa and are at high relative abundance in the dataset overall. Dinoflagellates related to *Prorocentrum foraminosum* (Fig. S6) and *Amphidinium* (Fig. S3) dominate the Triquet, Goose and McMullin regions in both years, whereas diatoms that cannot be identified beyond Bacillariophyceae (Fig. S7) dominate the Choked and Pruth regions on Calvert Island (Fig. 2). These core taxa are present across regions, but at lower relative abundance. This distribution pattern suggests that some environmental conditions and perhaps dispersal limitation are influencing the distribution of those two taxa.

Prorocentrum foraminosum is a benthic marine dinoflagellate. It was initially discovered in floating detritus of Twin Cays (Faust 1993), and later observed in tropical macroalgae and seagrass species also in the Caribbean (Okolodkov et al. 2014) and in *Sargassum* spp. in Malaysia (Mohammad-Noor et al. 2007). In the Sea of Japan, *P. foraminosum* was found in association with several brown and red algae species, over a wide range of temperatures (8-22°C) (Selina 2017). They

are also able to firmly attach to substrates and are adapted to low light conditions (Hoppenrath et al. 2013). The core OTUs here are identical, or nearly identical, to sequences from the *P. foraminosum* voucher (KT203864; Fig. S6). Cyst formation occurs in *P. foraminosum* (Hoppenrath et al. 2013) potentially helping to explain its widespread distribution, considering that resting cysts allow survival under unfavourable environmental conditions and increase the likelihood of dispersal (Bravo and Figueiroa 2014). Core *Amphidinium* OTUs here form a clade with *A. steinii* but are only 92% similar (Fig. S3). *Amphidinium steinii* is also usually found in benthic marine samples (Saburova et al. 2010) and associated with macroalgae (Fukuyo 1981; Koon and Usup 2004), but they have also been recorded over a variety of other environments (i.e. Brackish pond and freshwater swamps).

Amphidinium is a diverse genus of dinoflagellates, and often found in high abundance in marine habitats. Finally, one core dinoflagellate taxon belongs to Syndiniales, which shows a broad distribution across marine environments and is likely a parasite of one of the highly prevalent animals or dinoflagellates (Guillou et al. 2008).

Benthic diatoms (Bacillariophyceae clade 1, *Navicula* spp. and Diatomea ME.Euk.FW10) were amongst the core taxa enriched in both *Z. marina*, substrates and artificial seagrass (Fig. 4). *Navicula* is a late colonizing genus that often dominates the diatom communities on *Z. marina* leaves (Chung and Lee 2008), and other seagrass species such as *Enhalus acoroides* (Yap-Dejeto and Baqueros 2015), *Posidonia oceanica* and *Cymodocea nodosa* (Mabrouk et al. 2014). Diatomea ME.Euk.FW10 has been found in high abundance in sporophytes of *Mastocarpus* spp. (Lemay et al. 2018). Seagrass-associated diatom communities usually resemble the ones inhabiting adjacent meadow sediments, suggesting that the sediment would be the major source of individuals colonizing seagrass surfaces (Sullivan 1977). No evidence for diatom-host specificity was found when different seagrass species (Sullivan 1979) or even distinct hosts such as seaweeds and seagrass (Main and McIntire 1974) were sampled within the same area. Differences in community structure of epiphytic algae across distinct species of seagrass can usually be attributed to factors such as lifespan and growth rates of the host that lead to differences in colonization stages (Chung and Lee 2008; Mabrouk et al. 2014).

Fungal-like stramenopiles (i.e. Labyrinthulomycota and Oomycota) are cosmopolitan organisms found in marine, freshwater and soil ecosystems, with many taxa known to be pathogens of

plants (Beakes et al. 2014). We found three core clades of *Aplanochytrium* in our dataset (Fig. S5). *Aplanochytrium* are typical of marine ecosystems and known to be important decomposers and common inhabitants of marine surfaces in general, such as seagrass and seaweeds (Raghukumar 2017). Indeed, *Aplanochytrium* core clade 3 is prevalent across all meadows and also common on artificial seagrass leaves (Fig 4). It was first reported to infect the seagrass *Halodule wrightii* (Quick 1974) and recently it was also associated with a seagrass wasting-type disease in *Zostera marina* (Hughes et al. 2018). The other two core taxa belonged to Class Peronosporomycetes (Oomycota), which are commonly found in coastal waters, particularly in the sediment (Massana et al. 2015). Peronosporomycetes have also been found in the epiphytic biofilm of seagrass *Enhalus acroides* in Papua New Guinea (Hassenrück et al. 2015). Some taxa are saprotrophs and others are broad host range pathogens. Specifically, the *Anisoploidium* clade were similar to sequences from intertidal marine sediments in Greenland (Stoeck et al. 2007), which were later found to be closely related to pathogens of seaweeds (Fletcher et al. 2015; Badis et al. 2019). These sequences are 99% similar to *Anisoploidium rosenvingei*, which is known to infect the brown algae *Pylaiella littoralis* (Gachon et al. 2017) (Fig. S4). The Peronosporomycetes clade (Fig. 4) are 99% similar to parasites of red algae in the order Pontismatales (Fig. S4), being closely related to *Pontisma lagenidiooides* (Buaya et al. 2020), a pathogen of green filamentous alga *Chaetomorpha media* (Raghukumar and Chandramohan 1988) and red algae such as *Ceramium rubrum* (Buaya et al. 2019).

We also found heterotrophic grazers in the core taxonomic group that likely use seagrass leaves as a substrate, and a symbiont. The grazers belong to Ploimida (rotifer), a Cercozoa Novel Clade Gran-1. Most periphytic rotifers do not show host selectivity (Pejler B. 1995). Cercozoa consist of ameboid and flagellated protists that live in the soil, freshwater and marine environments (Bass et al. 2009), but little is known about the ecology of Novel Clade Gran-1 found here.

Therefore, the core seagrass microeukaryotes are likely not host-specific, and most of the highly persistent taxa are probably using the seagrass leaves as a substrate (i.e. diatoms, dinoflagellates), for grazing (i.e. rotifers and Cercozoa) or may be saprotrophic organisms associated with seagrass decomposition, including some potentially pathogenic taxa (i.e. *Anisoploidium* sp. and other Peronosporomycetes). Additional research is necessary to understand the impact of particular

microbes on host health and physiology. Further, including samples from nearby sediments and other marine surfaces will be necessary to identify the true habitat range of seagrass epibionts.

Seagrass-associated microeukaryotes are more spatially structured than seawater communities

Regional differences were much more important for seagrass epibiota than interannual differences, whereas differences between years were slightly more important for seawater microeukaryotes (PERMANOVA results). The distance decay of community similarity was steeper for epibionts than for plankton (Fig 5). Epibiotic communities were spatially structured at the regional level, but also showed high variability among seagrass plants at small spatial scales. Only 5% of OTUs were shared among seagrass epibiotic communities from different regions. This is a much lower fraction than observed in seawater samples (14%), reinforcing a conclusion that there was higher spatial variability in the epibiotic communities. A higher overlap across sites between seawater than between seagrass associated bacterial communities was also found in seagrass meadows in Florida, USA, although their numbers were much higher (seagrass: 47% and seawater: 75%; Ugarelli et al. 2019), likely due to the smaller spatial scale sampled. The higher initial similarity found for seawater samples is indicative of a lower beta diversity over small spatial scales. This, together with the shallower distance-decay slope, corroborates our prediction that seawater microeukaryotes would be highly connected (Fodelianakis et al. 2019) and thus more similar in composition than seagrass epibionts at the same spatial scale (Ugarelli et al. 2019). The higher variability of seagrass epibiota over short distances could be the result of microspatial gradients of environmental variables such as light and temperature (Perkins et al. 2016), exposure to ocean currents, which could select for species with different adhesive strengths (Tanaka 1986; Costa et al. 2014), or differences in the microenvironment of leaf surfaces (Pinckney and Micheli 1998; Papazian et al. 2019).

The spatial structuring of seagrass communities seems to be driven mostly by spatial distance and spatial structuring of the measured abiotic variables, whereas interannual changes in the abiotic environment seem relatively more important for seawater microeukaryotes (Fig. 6). A similar pattern was found in phytoplankton and periphytic diatoms along the Negro River in the Amazon, where periphytic communities showed higher spatial dependency than planktonic ones, likely due to lower dispersal abilities (Wetzel et al. 2012). However, other unmeasured environmental variables, or the indirect effect of spatial processes through a direct effect of space on different organisms that they

interact with (Peres-Neto and Legendre 2010), could also be responsible for the spatial structuring of seagrass microeukaryotes. Here, we provide additional evidence that the relative strength of environmental and spatial processes in structuring associated and free-living microeukaryotic communities within the same area may differ (Chen et al. 2017).

We then asked which of the measured environmental variables explained most of the variation in seagrass epibiota. When taking into account the effects of site variation, nearly all of the variation was explained by the spatial differences (26%), and other variables were not significant. In the analysis without including site as a factor temperature explained around 14% of the variation (Table S4). This suggests that temperature, together with other unmeasured spatially structured variables, are important factors affecting seagrass epibiota distribution. Indeed, temperature is one of the main environmental variables determining community composition of aquatic microbial communities (Sunagawa et al. 2015). However, further comparative studies encompassing a broader range of environmental variation across sites or nearby sites with contrasting environmental characteristics, along with experimental manipulations, are needed to tease apart and confirm the importance of these variables on seagrass epibiota.

Core and transient taxa are more influenced by the abiotic environment

Core and transient taxa both showed similar patterns to the overall community, with spatial distance being the most important structuring factor (Fig. 6). Our results point to abiotic variables of the surrounding seawater being more important in affecting both core and transient taxa than the variation in host attributes (Fig. 6 and Table S4). This is contrary to the expected for core taxa, further highlighting a lack of strong, specific association between microeukaryotes and seagrass. Other studies had previously identified biofilm chlorophyll-*a* and leaf area to influence the microeukaryote community structure of seagrass leaves (Mabrouk et al. 2011, 2014; Bengtsson et al. 2017), but they included fewer samples and fewer regions. Other unmeasured variables related to the host such as carbon release and metabolites (Papazian et al. 2019) could also be important drivers of microeukaryote distribution. However, a strong environmental filtering on the species pool of colonists can surpass the effects of host selection (Miller et al. 2018).

CONCLUSION

Seagrasses are foundation species in nearshore ecosystems, providing habitat for a wide range of animals, seaweeds, and microbes. Microbial eukaryotes are a key part of this ecosystem, both contributing to primary production and linking seagrass productivity to higher trophic levels. By sampling 10 seagrass meadows over two years, we found that epibiotic and planktonic communities are distinct, although we found a relatively high degree of OTU overlap in both communities. We also identified a handful of core taxa present across space and time; however, all core taxa found on seagrass leaves can also be found in a variety of other marine surfaces, including other hosts and benthic habitats. Therefore, the core seagrass microeukaryotes are likely not host-specific, with some using the seagrass leaves as a substrate, others for grazing, or they may be saprotrophic organisms related to decomposition or parasites. Seagrass epibiota were more spatially structured than planktonic communities, and this seem to be driven by both spatial distance and changes in abiotic conditions across space. Seawater communities were relatively more similar in composition across sites and more influenced by the environmental component, but more variable over time. Core and transient taxa showed similar patterns and were mostly structured by spatial distance and the abiotic environment, with little effect of host attributes, further indicating that those core taxa would not show a strong specific association with *Z. marina*.

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CONFLICT OF INTEREST

The authors declare no competing interests.

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FIGURE LEGENDS

Fig. 1 Alpha diversity metrics of microbial eukaryotes for *Z. marina*, substrates, artificial seagrass and seawater samples. Boxplot shows interquartile range (IQR: first to the third quartiles), median = vertical bar in the center of the box, whiskers = minimum and maximum numbers ($\pm 1.5 \times \text{IQR}$).

Fig. 2 Relative abundance of the 15 most abundant taxa of microbial eukaryotes in *Z. marina*, substrates, artificial seagrass and seawater samples from four regions along the BC Coast. We included the most abundant taxa of each sample type in all plots for comparison.

Fig. 3 Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities of microbial eukaryotes for *Z. marina*, substrates, artificial seagrass and seawater samples across different regions. Substrates consisted of shells, rocks and wood sampled nearby the quadrats.

Artificial seagrass shoots were made with polyethylene cut to a similar size of the natural seagrass in the Choked region.

Fig. 4 Relative abundance of the core taxa of microbial eukaryotes for each sample type and region. White boxes indicate core category: core taxa identified on *Zostera marina* alone, and in combination with artificial seagrass and/or substrate (shells, rocks and wood found within seagrass meadows).

Fig. 5 Distance decay relationship for microbial eukaryotes of *Z. marina* and seawater samples in each year, showing Bray-Curtis similarity between pairs of communities against geographic distance between sampling sites.

Fig. 6 Venn diagram showing proportion of variation in community structure explained by each component and shared effects using variation partitioning analysis (varpart) for microbial eukaryotes of *Z. marina* and seawater samples. The first varpart shows the contribution of the spatial (SPA) and environmental (ENV) components. We further considered interannual variation (YEAR), and disentangled the environmental component for *Z. marina* overall, core and transient taxa into abiotic conditions (ABIOTIC) and host attributes (HOST). Asterisks represent $p < 0.001$. Shared effects (overlapping circles) are not testable.

SUPPORTING INFORMATION

Methods S1. Detailed description of statistical analyses

Table S1 Results of Analysis of Variance (ANOVA) showing pairwise comparisons in alpha diversity metrics between sample types. Asterisks represent significant differences ($p < 0.05$) after Tukey HSD correction for multiple comparisons.

Table S2 Results of Permutational Analysis of Variance (PERMANOVA) showing pairwise comparisons in microeukaryotic community structure between sample types for abundance (Bray Curtis) and presence-absence (Jaccard) data. Asterisks represent significant differences ($p < 0.05$) after

Benjamini and Hochberg correction for multiple comparisons.

Table S3 Indval results showing the core taxa of microbial eukaryotes on *Zostera marina* leaves and *Z. marina* + substrates and/or artificial seagrass. OTUs considered core met three criteria 1) significant Indval score (<0.01), 2) present on least 50% of seagrass samples, and 3) present in both years. Taxon = most specific taxonomy level reliably assigned. Preval.*Z.marina* = prevalence of taxa in seagrass samples (A = specificity). Rel.abun.*Z.marina* = relative abundance of taxa in seagrass samples (B = fidelity). Functional group categories: ‡ = autotrophic or mixotrophic; † = heterotrophic; ϕ = saprotrophic or parasitic.

Table S4 Results of PERMANOVA showing the proportion of variation explained by each environmental variable in *Zostera marina* microeukaryotic community structure. Asterisks represent significant differences ($p<0.05$).

Table S5 Results of PERMANOVA showing the proportion of variation explained by each environmental variable in the core and transient portions of *Zostera marina* microeukaryotic community structure. Asterisks represent significant differences ($p<0.05$).

Table S6 Mean values of seagrass metrics (measured at the quadrat level) and abiotic variables (measured at the site level) on each of the sites studied.

Fig. S1 Map showing the five regions sampled in the coast of British Columbia: Pruth, Choked, Triquet, Goose, and McMullin.

Fig. S2 Principal component analysis (PCA) showing variation in abiotic (A) and host attributes variables (B) across regions and year.

Fig. S3 Maximum likelihood phylogenetic tree constructed with RAxML using GTRCAT. Operational taxonomic units from this study are in blue, and core OTUs are bold. Core OTUs of *Amphidinium* fall into one clade that is similar to *A. steinii*.

Fig. S4 Maximum likelihood phylogenetic tree constructed with RAxML using GTRCAT. Operational taxonomic units from this study are in blue, and core OTUs are bold. Core OTUs of Peronosporomycetes fall into two clades.

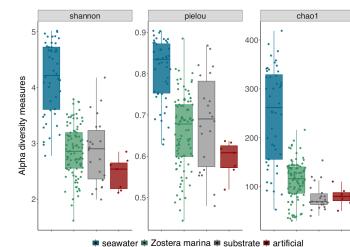
Fig. S5 Maximum likelihood phylogenetic tree constructed with RAxML using GTRCAT. Operational taxonomic units from this study are in blue, and core OTUs are bold. Core OTUs of *Aplanochytrium* fall into three clades.

Fig. S6 Maximum likelihood phylogenetic tree constructed with RAxML using GTRCAT. Operational taxonomic units from this study are in blue, and core OTUs are bold. Core OTUs of *Prorocentrum* fall into one clade nearly identical to the voucher for *Prorocentrum foraminosum*, and we find one additional clade of core Dinophyceae. SILVA sequences belonging to the same genus, or all uncultured, are collapsed for clarity.

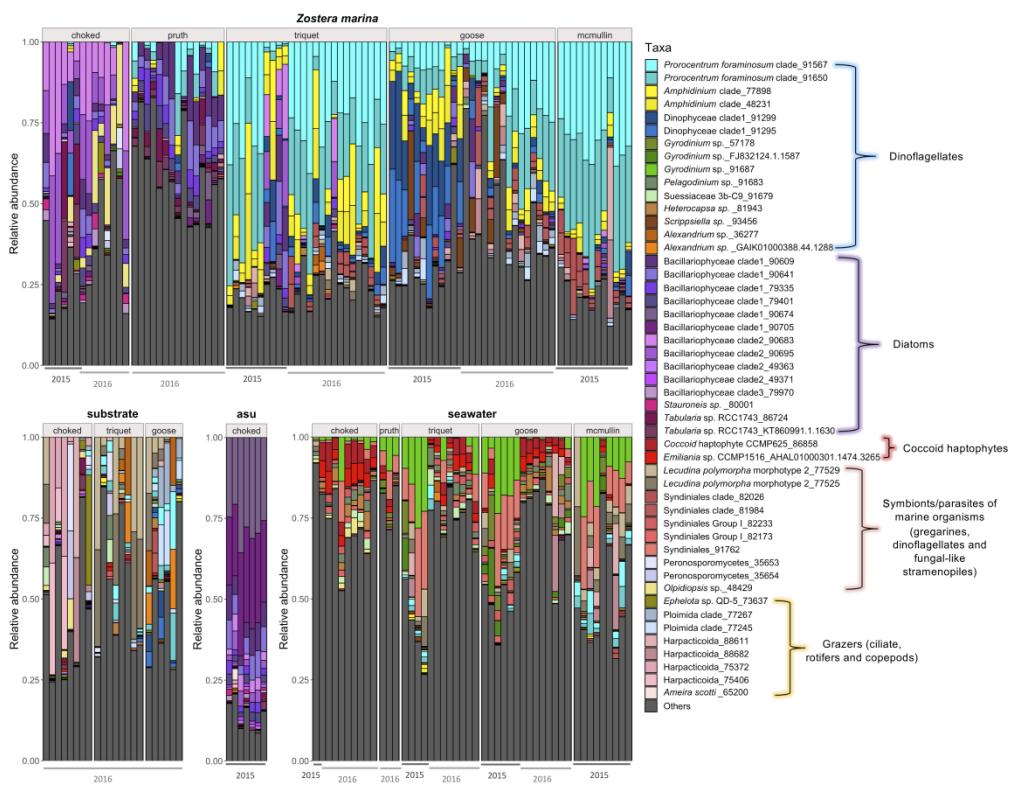
Fig. S7 Maximum likelihood phylogenetic tree constructed with RAxML using GTRCAT. Operational taxonomic units from this study are in blue, and core OTUs are bold. Core OTUs of Bacillariophyceae fall into one clade. 35 additional OTUs form a clade with *Planothidium* (collapsed here for clarity). SILVA sequences belonging to the same genus, or all uncultured, are collapsed for clarity.

Fig. S8 Maximum likelihood phylogenetic tree constructed with RAxML using GTRCAT. Operational taxonomic units from this study are in blue, and core OTUs are bold. Core OTUs assigned to Dinophyceae fall into one clade, but with low similarity to neighboring clades. SILVA sequences belonging to the same genus, or all uncultured, are collapsed for clarity.

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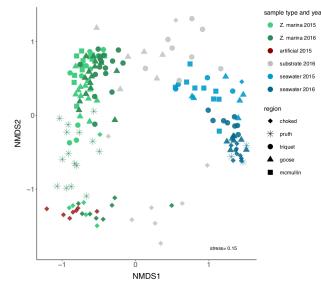


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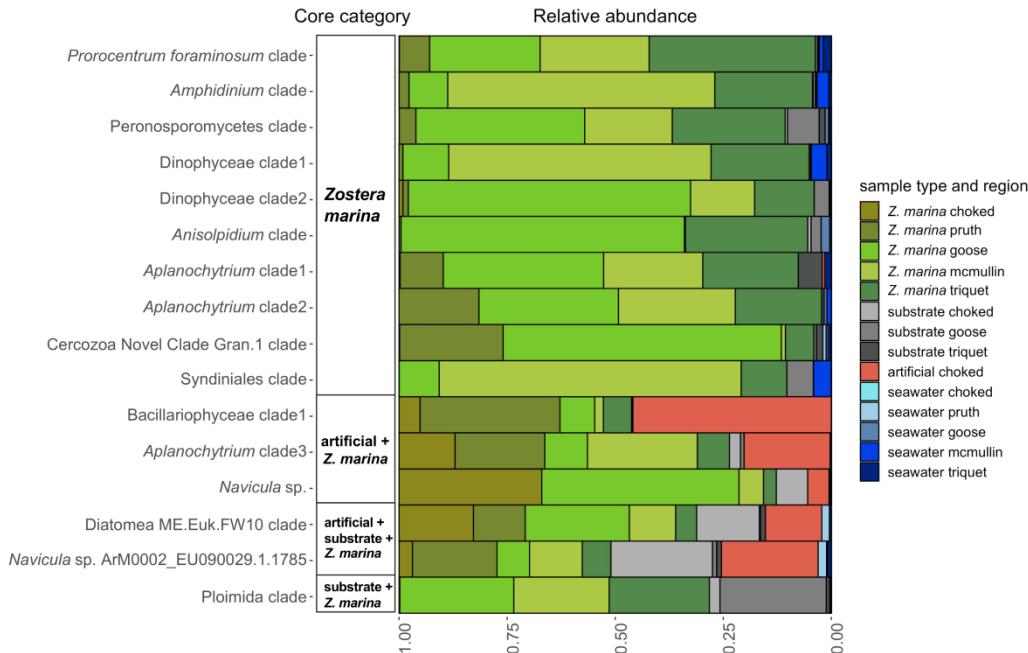


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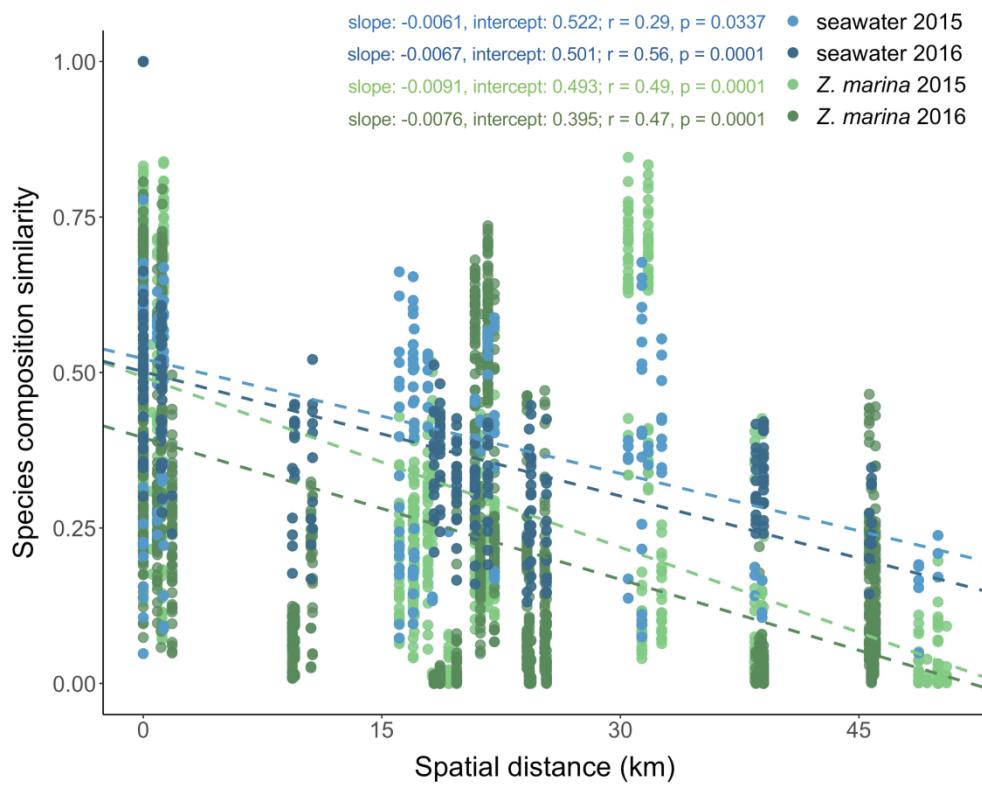
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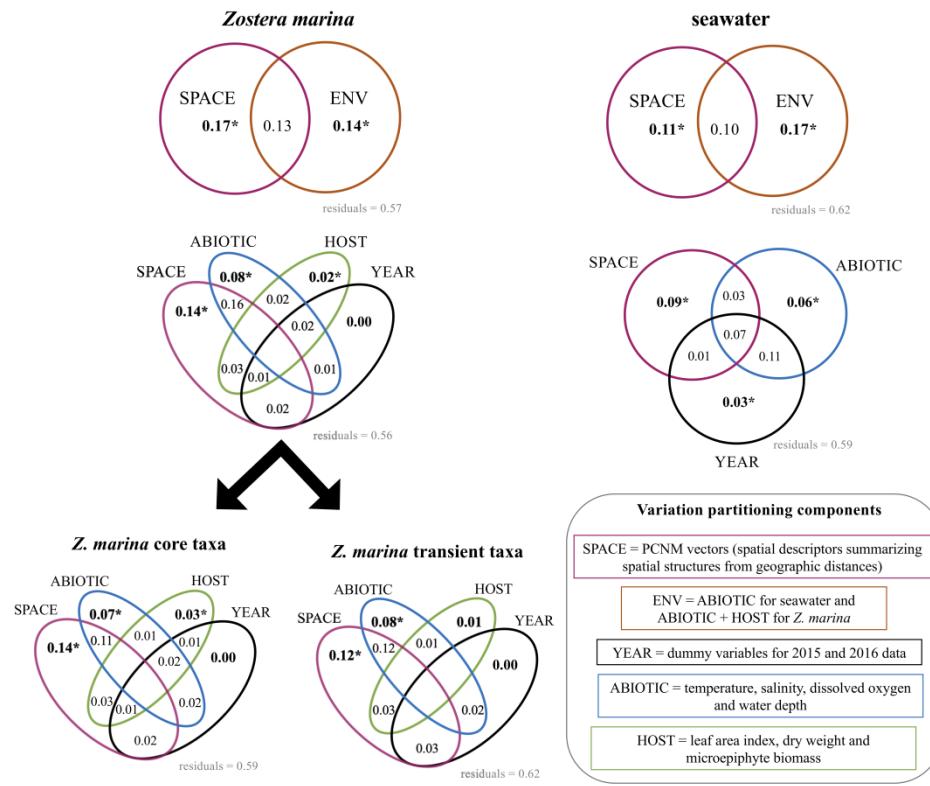
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