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Incubation with macroalgae induces large shifts in water column microbiota, but minor changes to the epibiota of co-occurring macroalgae

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Abstract

Macroalgae variably promote and deter microbial growth through release of organic carbon and antimicrobial compounds into the water column. Consequently, macroalgae influence the microbial composition of the surrounding water column and biofilms on nearby surfaces. Here, we use manipulative experiments to test the hypotheses that (i) *Nereocystis luetkeana* and *Mastocarpus* sp. macroalgae alter the water column microbiota in species-specific manner, that (ii) neighbouring macroalgae alter the bacterial communities on the surface (epibiota) of actively growing *Nereocystis luetkeana* meristem fragments (NMFs), and that (iii) neighbours alter NMF growth rate. We also assess the impact of laboratory incubation on macroalgal epibiota by comparing each species to wild counterparts. We find strong differences between the *Nereocystis* and *Mastocarpus* epibiota that are maintained in the laboratory. *Nereocystis* and *Mastocarpus* alter water column bacterial community composition and richness in a species specific manner, but cause only small compositional shifts on NMF surfaces that do not differ by species, and do not change richness. Co-incubation with macroalgae results in significant change in abundance of fivefold more genera in the water column compared to NMF surfaces, although the direction (i.e., enrichment or reduction) of shift is generally consistent between the water and NMF surfaces. Finally, NMFs grew during the experiment, but growth did not depend on the presence or identity of neighbouring macroalgae. Thus, macroalgae exhibit a strong and species-specific influence on the water column microbiota, but a much weaker influence on the epibiota of neighbouring macroalgae. Overall, these results support the idea that macroalgae surfaces are highly selective and demonstrate that modulations of macroalgal microbiota operate within an overarching paradigm of host species specificity.

KEYWORDS
16S rRNA, community assembly, *Mastocarpus*, microbial ecology, *Nereocystis*, seaweed

1 | INTRODUCTION

Macroalgae (seaweeds) have an intimate relationship with their microbial symbionts. Some microbes provide benefits for their macroalgal hosts by improving nutrient acquisition (Chisholm, Douga, Ageron, Grimont, & Jaubert, 1996; Croft, Lawrence, Raux-Deery, Warren, & Smith, 2005; Ilead & Carpenter, 1975; Rosenberg & Paerl, 1981), promoting settlement and growth (Joint et al., 2002) and priming immune responses against potential pathogens (Armstrong, Yan, Boyd, Wright, & Burgess, 2001; Dobretsov & Qian, 2002; Küpper, Müller, Peters, Kloareg, & Potin, 2002; Maximilian et al., 1998; Steinberg, Schneider, & Kjelleberg, 1997; Weinberger, 2007).

Other microbes, however, cause tissue bleaching (Case et al., 2011; Zozaya-Valdes, Egan, & Thomas, 2015) and initiate or exasperate tissue degradation (Egan et al., 2013; Küpper et al., 2002). It is important to understand the assembly of macroalgal microbiota to gain insight into the factors that promote establishment and growth of beneficial or pathogenic microbes.

Macroalgae live in a rich microbial "soup" within the ocean and constantly contact a variety of microbes. Macroalgal microbiota are assembled from this microbial milieu, yet these assemblages are generally species-specific (Bondoso, Balague, Gasol, & Lage, 2014; Hollants et al., 2011; Lachnit, Meske, Wahl, Harder, & Schmitz, 2011; Staufenberger, Thiel, Wiese, & Imhoff, 2008) because they are regulated, both specifically and generally, through a variety of macroalgal exudates. The polysaccharides (alginate, carrageenan, cellulose, etc.) that compose the bulk of macroalgal biomass are rich sources of energy and carbon that can promote epibiont settlement and growth (Lachnit et al., 2011; Steinberg, 2002). Conversely, macroalgae produce antimicrobial metabolites such as hydrogen peroxide (Küpper et al., 2002) and furanones (Maximilian et al., 1998) that inhibit microbial settlement and growth. Macroalgal exudates collectively impose selection on colonizing microbes and result in diverse microbial assemblages that vary across macroalgal species.

Macroalgae modify their surrounding environment, releasing large amounts of carbon as mucilage and other exudates (Newell, Lucas, Velimirov, & Seiderer, 1980; Wada & Hama, 2013) as well as detritus (Krumhansl & Scheibling, 2012; Stuart, Lucas, & Newell, 1981). Bacteria utilize these carbon and energy inputs to fuel growth and, in turn, modify the microbial community in the water column (Clasen & Shurin, 2014; Egan et al., 2013; Lam, Stang, & Harder, 2008; Linley, Newell, & Bosma, 1981; Stuart et al., 1981) and on nearby biofilms (Fischer, Friedrichs, & Lachnit, 2014; Vega Thurber et al., 2012; Zaneveld et al., 2016). Macroalgae also release a variety of antimicrobial compounds into the water that can inhibit growth of particular bacteria, fungi and algae (Dahms & Dobretsov, 2017; Inaba et al., 2017; Lam & Harder, 2007; Lam et al., 2008). Finally, macroalgae modify the microbiota in their surroundings through the dispersal of epibiotic microbes directly into the water column or on particles of degrading algal tissue. These changes are density-dependent; greater canopy cover is associated with larger changes in the microbiota on nearby corals (Zaneveld et al., 2016) and more extensive kelp forests are associated with larger changes in water column microbial communities (Clasen & Shurin, 2014). Such microbiota alteration can have cascading effects on the health of neighbouring organisms and ecosystems. For example, increasing macroalgal canopy cover is associated with higher pathogen load in neighbouring corals (Zaneveld et al., 2016), while *Ulva* and sea grass beds enrich the concentration of bacteria that kill or inhibit harmful microalgae (Inaba et al., 2017).

Despite the well-documented changes induced by macroalgae in the structure and composition of microbial communities of nearby biofilms and water, we know little about how, or even whether, macroalgae influence the microbiota of their neighbours. Here, we use manipulative experiments to (i) determine how *Nereocystis luetkeana* (hereafter *Nereocystis*) and *Mastocarpus* sp. (hereafter *Mastocarpus*) alter the water column microbial pool, (ii) determine whether the epibiota of *Nereocystis* meristem fragments (NMFs) is altered by neighbouring macroalgae and (iii) assess growth rates of NMFs across treatments as a gross measure of macroalgal health and to ensure epibiota changes are not due to tissue death and decay. Neighbouring macroalgae could increase transmission of core microbes that are more likely to positively influence host biology and health (Hopkins, Boyle, Belden, & Wojdak, 2015; Shade & Handelsman, 2011), or may alter disease transmission and susceptibility (Hawley & Altizer, 2011), resulting in lower growth rates. Finally, we compare the epibiota of *Nereocystis* and *Mastocarpus* in the field and after laboratory incubation as a measure of the selectivity of macroalgal tissue and degree to which microbiota are retained in the laboratory. Previous studies have found that while macroalgae generally harbour species-specific communities (Lachnit, Blümel, Imhoff, & Wahl, 2009; Lemay et al., 2018), assemblages on some macroalgae differ almost completely across locations (Burke, Steinberg, Rusch, Kjelleberg, & Thomas, 2011) or after manipulation (Campbell, Marzinelli, Gelber, & Steinberg, 2015). We predict fewer microbiota changes on NMF compared to the water column because these surfaces are likely selective. We also expect con-specific neighbours to cause larger changes than hetero-specific neighbours if *Nereocystis* surfaces select for a specific microbiota assemblage.

2 | METHODS AND MATERIALS

2.1 | Sampling methods

Samples of *Nereocystis* and *Mastocarpus* for incubation experiments were collected on 5 September 2016 at Brockton Point, Vancouver, British Columbia, from the intertidal and shallow subtidal at low tide. *Nereocystis* is a rapidly growing and bed-forming kelp, and detached meristematic regions grow rapidly. We measured growth over the experiment to ensure microbiota changes are not due to tissue degradation. *Mastocarpus* is common red alga that occurs in close proximity (several metres) to *Nereocystis* at Brockton Point, albeit in different tidal zones. *Nereocystis* and *Mastocarpus* were sampled just below and above the tide line, respectively. Blades from individual *Nereocystis* and *Mastocarpus* thalli that were far enough apart so that they were not touching when submerged were collected and brought back to UBC in a cooler lined with wet paper towels (species were separated) and then transferred to overnight holding tanks with 30ppt salinity and temperature maintained at 16°C. The *Nereocystis* and *Mastocarpus* were kept in separate tanks. The next day, all samples were distributed into clean experimental tanks.

The microbiota of five *Nereocystis* was sampled in situ at Brockton Point (referred to as Brockton) on 6 September 2016 at two locations: meristem (10 cm from the stipe) and mature blade (50 cm from the stipe on a different blade). Individuals were at least 5 metres apart and just below the low tide line. The microbiota of five *Mastocarpus* individuals was sampled in situ by swabbing the mid-

blade; individuals were at least 3 m apart just above the low tide line. For all samples, the blade surface was rinsed with sterile artificial seawater (ASW, always 30 ppt unless noted otherwise) for 10 s and then swabbed with a sterile cotton swab (Puritan-Item no.: CA10805-154) for 10 s. The cotton swab tip was then snapped off into 2-ml cryotubes (VWR-Item no.: 10018-760) and kept on ice until return to the laboratory. Field (wild) samples were compared to experimental macroalgae in the laboratory to test whether laboratory incubation significantly affected microbial community composition and diversity on macroalgal surfaces.

Microbiota of ten mature *Nereocystis* blades (roughly 50 cm from the stipe) were sampled as above in August 2016 from "Starfish" site in Choked Passage at the Hakai Research Institute on Calvert Island, British Columbia. This remote location (referred to as Hakai) more than 500 km from Brockton Point enables us to assess how *Nereocystis* blade microbiota vary across large geographic distances. Five blades were located inside dense kelp beds ("inner"), whereas five blades were located on kelp bed peripheries ("outer"). Water column samples from inner and outer kelp beds (two each) were also collected. Water samples were collected in sterile 500-ml PPE bottles, prefiltered with an acid-sterilized 150 μm sieve to remove algal detritus fragments and large animals and then pumped through sterile 0.22 μm membranes (Durapore-Item no.: GVWP04700) with a peristaltic pump (Cole-Parmer-Item no.: RK-77913-70) at approximately 180 rpm (level 30) to collect microbial biomass. Filters were immediately frozen at −20°C in 2-ml cryotubes (VWR-Item no.: 10018-760). The tubing was rinsed with 500 ml of 2% HCl, followed by a rinse with 1500 ml deionized water between replicates.

2.2 | Macroalgae–Water experiment

In the first experiment, referred to hereafter as the "Macroalgae–Water (M–W) experiment," we assessed the degree to which microbes are transferred from macroalga to the surrounding water column by incubating *Nereocystis* and *Mastocarpus* alone in sea water for 6 days (see Figure 1a for experimental design). Ten 10-L tanks were placed in a two-layer water table. The temperature of all tanks was regulated by the water table and kept at 16°C. Lights were 24 hr/day. Tanks were aerated by placing small stone bubblers in each tank that were attached via tubing to one of two large air pumps. Gas valves to each stone bubbler were opened completely. Treatment positions (and thus which air pump it was attached to) were randomized in the water table. Five tanks contained only *Nereocystis* and the other five tanks only *Mastocarpus* (Figure 1a). Each tank contained approximately 100 g (wet weight) of tissue from two or three individuals. At the end of the incubation period, we sampled one random macroalga individual and took one 500 ml water sample from each tank. Macroalgae and water samples were processed as above. As above, all water samples were prefiltered with a sterile 150 μm sieve to remove algal fragments prior to collecting the microbiota on a 0.22 μm membrane.

2.3 | Macroalgae–Water–Nereocystis Meristem Fragment (NMF) experiment

We conducted a second experiment (the Macroalgae–Water—NMF [M–W–NMF] experiment) to determine how the presence of macroalga influences the surface microbial community of neighbouring

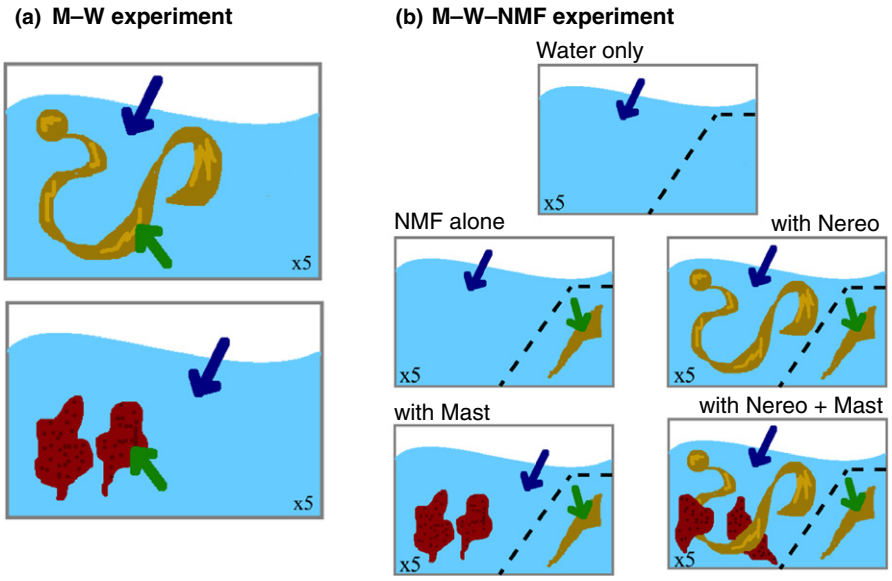

FIGURE 1 Experimental design. (a) Macroalgae–Water (M–W) experiment to assess changes in the water column following macroalgal incubation. *Mastocarpus* (n = 5) or *Nereocystis* (n = 5) were incubated in 10-L tanks. Water (dark blue arrows) and macroalgal surfaces (green arrows) were sampled on day 6. (b) Macroalgae–Water–NMF (M–W–NMF) experiment to assess impact of macroalgal co-incubation on neighbouring macroalgae. NMFs were incubated with either *Nereocystis* (n = 5), *Mastocarpus* (n = 5), or both (n = 5). A NMF alone control (n = 5) and a water only control (n = 5) were also included. The dashed line represents coarse plastic mesh that was included in the tanks to separate NMFs from other macroalgae, and we also included in controls. Water and NMF surfaces were sampled on day 5

macroalgae (see Figure 1 for experimental design). Twenty-five tanks with 5 L of 30 ppt water each were incubated in a water table held at 16°C. Tanks were divided into five treatments: (i) water only, (ii) water with one NMF, (iii) water with one NMF fragment and approximately 50 g (wet weight) of *Nereocystis* blades, (iv) water with one NMF and approximately 50 g (wet weight) of *Mastocarpus* blades and (v) water with one NMF and approximately 50 g (wet weight) combined of *Nereocystis* and *Mastocarpus* blades. Tanks received mature blades from two or more individuals. All treatments were incubated for 5 days. Dissolved oxygen, pH, temperature and salinity were measured at the beginning and end of the experiment using the Orion STAR A329 (ThermoScientific, Item no.:STARA3295) and a standard refractometer. Additionally, we measured growth rate of NMFs to assess macroalgal health and to determine whether microbiota changes altered growth. *Nereocystis* can grow up to 14 cm per day (Kain, 1987), which maximizes the potential effect size for differential growth rates between treatments. Areas of new growth have less microbial diversity (Bengtsson, Sjøtun, Lanzén, & Øvreås, 2012) and are highly defended. Thus, the surfaces of *Nereocystis* meristems are optimal areas to test for meaningful shifts in microbiota community structure because they are highly selective surfaces. NMFs were prepared by cutting 10-cm fragments of *Nereocystis* meristem from the base of each blade with scissors. Length and width were measured using a measuring tape to the nearest half millimetre, and wet weight was determined by blotting twice on a paper towel and weighing on a scale to the nearest 0.01 g. NMFs and other algal tissue were kept physically separated by coarse plastic mesh, although this approach may not have completely prevented NMFs from touching other macroalgae and thus directly transferring microbiota. At the end of the incubation period, NMFs were measured and their microbiota were sampled as above. Separate 500 ml volumes of were also collected from each tank and processed as above.

2.4 | Library preparation

The 96-well MoBio PowerSoil DNA extraction kit was used to extract DNA from both the water filters and macroalgae swabs. Filters and swabs were transferred to the extraction kit using tweezers sterilized with 2% HCl and then with ethanol and flame. Extractions followed the MoBio Powersoil DNA extraction protocol with modifications based on recommendations in the Earth Microbiome protocol (http://www.earthmicrobiome.org/), except that plates were shaken at 20 shakes per second for 20 min. The DNA was stored at −20°C. A map of the layout of samples is provided (Figure S9).

The 16S small subunit ribosomal RNA marker gene was sequenced to profile bacteria and archaea. The amplicon library prep was carried out in laboratory using the following 16S rRNA gene primers: barcoded 515 forward primers (5′-GTGYCAGCMGCCGCG GTAA-3′) and 806 reverse primers (5′-GGACTACHVGGGTWTCTA AT-3′). Primers were used at final concentrations of 0.5 μm with 4 μl of DNA extract. DNA extracts were amplified in 20 μl reactions using Phusion Flash High-fidelity proofreading Mastermix (Thermofisher-Item no.: F548L). Reactions underwent the following thermocycler settings: 98°C for 10 s; 25 cycles of 98°C (1 s), 50°C (5 s), 72°C (24 s); and a final extension phase of 72°C for 1 min. Lastly, the successful PCR products were quantified using Pico-green (Thermofisher-Item no.: P11496) and pooled at 45 ng per sample. The pooled samples were then sent to the Centre for Comparative Genomics and Evolution Bioinformatics (CGEB) at Dalhousie University for sequencing on the Illumina MiSeq platform with 2 × 300 bp chemistry.

2.5 | Sequence processing

Raw samples were demultiplexed with split_libraries_fastq.py in Quantitative Insights into Microbial Ecology (QIIME) version 1.9 (Caporaso, Kuczynski et al., 2010), yielding 3,688,981 reads. Sequences were trimmed (fastx_trimmer), clipped (fastx_clipper), and filtered (fastq_quality_filter) using the Fastx Toolkit (Hannon Lab) to 250 bp with a minimum quality threshold of Q19. The remaining 3,661,707 raw sequences were processed into operational taxonomic units (OTUs) using minimum entropy decomposition (MED; Eren et al., 2014) with the minimum substantive abundance (−m) parameter set to 100, yielding 1,363 unique OTUs and 3,050,864 reads. MED is an OTU picking method that does not use similarity cut-offs: instead, MED clusters similar reads together to form a "node" and evaluates entropy changes before and after clustering to determine whether each node is valid. In practice, MED nodes are generally 98.5% or greater in sequence similarity. The most abundant read within each node was used as its representative sequence. Taxonomy was assigned to OTUs by matching the representative sequence to the SILVA 128 database clustered at 99% similarity with assign_taxonomy.py in QIIME using uclust V1.2.22q (Edgar, 2010). The resulting OTU matrix was transcribed into a QIIME -compatible format.

Chloroplast, mitochondrial and eukaryotic sequences were filtered out of the data set. Additionally, 70 OTUs (representing 4% of total reads) for which taxonomy was unassigned by uclust and that did not match bacteria at greater than 98% in a BLAST search of the NCBI database (NCBI Resource Coordinators, 2016) were filtered out. Each sample was also filtered to remove OTUs that had fewer than five reads to minimize any cross-contamination between wells. Three OTUs (one *Pseudomonas*, one *Achromobacter* and an *Escherichia*) were removed because they occurred in PCR controls and across most samples and were suspected laboratory contaminants. The extraction control (E.CON) and one PCR negative control (NEG.-CON) contained 1,043 and 739 raw reads, respectively, and we investigated these further. Controls were plotted with all samples in an NMDS plot; E.CON appears similar to *Nereocystis*, while NEG.-CON is distinct from other samples though closer to water. Neither control grouped closely with samples from adjacent wells in the extraction and PCR plate. This suggests localized contamination of the extraction control by a macroalgal swab and of NEG.CON with water. We assessed all OTUs in E.CON and NEG.CON by comparing to a published data set (Lemay et al., 2018) and other literature. None showed a characteristic contamination pattern of ubiquity

across samples and higher abundance in otherwise low read count samples. The majority (50 of 56 OTUs) match the Lemay et al., 2018 data set at 97% similarity, and all are commonly detected on other macroalgae or in marine water column samples, suggesting these represent bacteria found in these environments rather than laboratory contaminants. However, we cannot rule out contamination with certainty. OTU tables, mapping files which include sample metadata, and a comparison of OTUs found here and in Lemay et al. (2018), and analysis of OTUs from controls can be found at https://github.com/mechen10/NereoIncubProject. Lastly, samples with fewer than 800 reads were removed.

The final OTU table consisted of 1,220 unique OTUs and 2,689,912 reads, with a mean of 30,919 reads per sample. For alpha and beta diversity analysis, samples were rarefied to 800 reads per sample. Representative sequences were aligned with PYNAST (Caporaso, Bittinger et al., 2010) in QIIME, and a phylogenetic tree was created using FASTTREE (Price, Dehal, & Arkin, 2009) in QIIME with the make_phylogeny.py script. Custom R scripts used for graphing and analysis can be found at https://github.com/mechen10/NereoIncubProject.

2.6 | Community dissimilarity

To compare community composition across treatments, distance matrices were created with the beta_diversity.py script in QIIME with the rarefied OTU table using the weighted UniFrac metric, unweighted UniFrac metric (Lozupone & Knight, 2005) and the Bray–Curtis dissimilarity index (Bray & Curtis, 1957). Results from all three are generally consistent, and we show the Bray–Curtis dissimilarity index, which considers abundance and membership in microbial communities. In the few cases where results differ by metric, we present the results from all three. Dissimilarity matrices were imported into R, and the "isomds" command from the "MASS" package (Venables & Ripley, 2002) was used to created two-dimensional NMDS plots. Polygons were drawn around treatments using "chull" in the "GRDEVICES" package (R Core Team, 2016). Differences between macroalgal species and water samples overall were assessed statistically with a PERMANOVA implemented in "adonis" from the "VEGAN" package (Oksanen et al., 2017). The model included fixed factors for species (*Nereocystis*, *Mastocarpus*) and site (laboratory, Brockton point, Calvert Island) and their interaction, and factors were tested sequentially in this order. Pairwise PERMANOVAs were also calculated across treatments of laboratory experiments using "adonis," and *p*-values were adjusted for multiple comparisons using the Benjamini–Hochberg method (also known as the "false discovery rate (FDR)" method; Benjamini & Hochberg, 1995) with "p.adjust" in the "STATS" package (R Core Team, 2016). We tested for differences in dispersion between groups using "betadisper" in the "VEGAN" package (Oksanen et al., 2017) when PERMANOVA results were significant.

2.7 | Alpha diversity

Richness for each treatment was calculated in QIIME using the alpha_diversity pipeline. The metrics Chao1 (Chao, 1984), PD_whole_tree (Faith & Baker, 2006) and observed_otus were used. Results were similar for all three metrics, and Chao1, a richness measure that corrects for rare taxa, is shown. Overall differences in richness between host species and across laboratory and field sites were assessed using an ANOVA with host species and site as fixed factors. The richness of experimental treatments were compared using Welch's *t* tests. Pairwise comparisons between treatments was calculated using "*t* test" in the "STATS" package (R Core Team, 2016) with the method "Welch's *t* test" and Benjamini–Hochberg *p*-value adjustments. Tables were initially created using "xtable" in the package "XTABLE" (Dahl, 2016) and then edited manually in LaTex.

2.8 | OTU enrichment and Taxa summaries

Fold-change enrichment of OTUs and genera were calculated on unrarefied OTU tables using "DESeq2" in the R package "DESEQ2" (Love, Huber, & Anders, 2014) with the "Wald" test. For the genus-level tests, the unrarefied OTU table was collapsed at level 6 using summarize_taxa.py (QIIME), and only genera with more than 100 reads in at least one sample were included in the analysis. If the taxonomy was not defined at the genus level, or listed as uncultured, only family is listed. For the OTU-level analysis, different OTUs with the same taxonomy are differentiated by a ".number" following the name. We note that in several cases, SILVA identifies subclades of paraphyletic genera with "_number." Genera (or OTUs) that were significantly enriched or reduced ($p < .05$, after Benjamini–Hochberg *p*-value adjustment) and were observed at abundances >3% in at least two samples in the overall data set were plotted using "heatmap.2" in the "GPLOTS" package (Warnes et al., 2016). Taxa summary plots were generated in R and depict genera with >3% relative abundance in at least two samples overall. The remaining genera are plotted in white.

3 | RESULTS

3.1 | Macroalgae and water column communities from the field and laboratory

Macroalgal surface communities are distinct from the water column and cluster by species identity across all field and laboratory samples (Figure 2a). There is an effect of site (laboratory, Brockton, Hakai) on the composition of microbiota on macroalgae, but this effect is nested within an overarching pattern of species specificity (PERMANOVA; host species: $p = .001$, $R^2 = .202$, $df = 1,38$, site: $p = .001$, $R^2 = .070$, $df = 1,38$, site*host species: $p = .012$, $R^2 = .041$, $df = 1,38$). Thus, *Mastocarpus* and *Nereocystis* retain characteristic microbiota when incubated in the laboratory. *Mastocarpus* surface communities were significantly more diverse than *Nereocystis* surface communities (ANOVA: host species: $p < .001$, $F_{1,38} = 29.406$, Site: $p = .0397$, $F_{1,38} = 4.539$, site*host species: $p = .005$, $F_{1,38} = 8.880$ Figure 2b).

We sampled mature *Nereocystis* blades from within ("inner") and on the periphery of ("outer") *Nereocystis* beds with the aim of assessing the influence of macroalgal density on epibiotic communities in

the field. The microbiota of these groups were not significantly different from each other in composition (PERMANOVA $p = .226$, $R^2 = .197$, $df = 1,6$) or richness (Welch's t test $p = .466$, $t_{3.874} = -0.808$). Inner kelp bed *Nereocystis* samples were significantly more dispersed than outer kelp bed samples according to the unweighted UniFrac metrics ($p = .002$, $F_{1,6} = 28.441$), but not by Bray–Curtis or weighted UniFrac (PERMDISP $p = .101$, $F_{1,6} = 3.746$ and $p = .072$, $F_{1,6} = 4.752$, respectively).

3.2 | Microbiota changes following co-incubation with macroalgae

Macroalgae alter the water column microbial community in a species-specific manner in laboratory experiments. The addition of small meristem fragments of *Nereocystis* (NMF alone treatment) increases richness and alters community composition of the water column (Figure 3a and b, Table 1). The addition of larger volumes of mature macroalgae induced larger shifts in water column communities: treatments with NMF plus *Nereocystis*, *Mastocarpus* or both were significantly different in composition and had higher richness compared to those incubated with NMF alone (Figure 3a, Table 1). Pairwise comparisons among treatments that incubated NMF with mature *Nereocystis*, *Mastocarpus* or both suggest species-specific changes to the water column community. There is no difference in dispersion across treatments (PERMDISP $p = .745$, $F_{4,19} = 0.487$). The water column communities in the treatment with mature *Nereocystis* differ significantly from the treatment with *Mastocarpus* in composition (PERMANOVA, FDR corrected $p = .017$) and *Mastocarpus* harbours richer communities (Welch's t test, FDR corrected $p = .0141$) in the M–W–NMF experiment (Figure 3, Table 1). We find that *Nereocystis* has a stronger effect on water column community composition than *Mastocarpus* as the treatment with both macroalgae is significantly different from the treatment with only *Mastocarpus* and NMF (PERMANOVA $p = .0135$), but not different from the *Nereocystis* only treatment (PERMANOVA $p = .12$, Figure 3a, Table 1). Similarly, water incubated with *Nereocystis* or *Mastocarpus* in the M–W experiment harbours significantly different microbial communities (PERMANOVA for Bray–Curtis and unweighted UniFrac $p < .05$, although weighted UniFrac metric is not significant $p = .15$, Table S1, Figure S1). Water column richness does not differ in the M–W experiment (Table S1 and Figure 2b), although it is significantly greater on *Mastocarpus* tissue (Figure S1b).

Macroalgae also alter the epibiota of neighbouring fragments of actively growing *Nereocystis* meristem fragments (NMFs), although these changes do not depend on the identity of the co-incubated macroalgae. Microbial communities on NMF surfaces incubated alone were different than communities on NMFs incubated with macroalgae (Figure 4a, Table 1). However, NMF surface communities did not differ according to the identity of the co-incubated macroalga (Table 1). Richness was higher on NMF surfaces in co-incubation treatments than the NMF alone treatment, but again did not differ according to the identity of the co-incubated macroalgae (Figure 4b, Table 1).

3.3 | Taxonomic composition of communities and enrichment of select genera

We assessed changes in the water column and NMF surface communities across treatments using DESEQ2 (Love et al., 2014). Overall, the taxa we find in the water column that are consistent with previous studies and are dominated by Flavobacteria (e.g., *Dokdonia*), Alphaproteobacteria (e.g., *Sulfitobacter*, *Roseibacterium* and *Marivita*) and Gammoproteobacteria (e.g., *Pseudohongiella* and Alteromonadaceae; Figure 6). Some enriched taxa were macroalga species-specific. For example, *Marivita* (Alphaproteobacteria) was enriched in treatments with *Mastocarpus*, whereas *Dokdonia* (Flavobacteriia) and

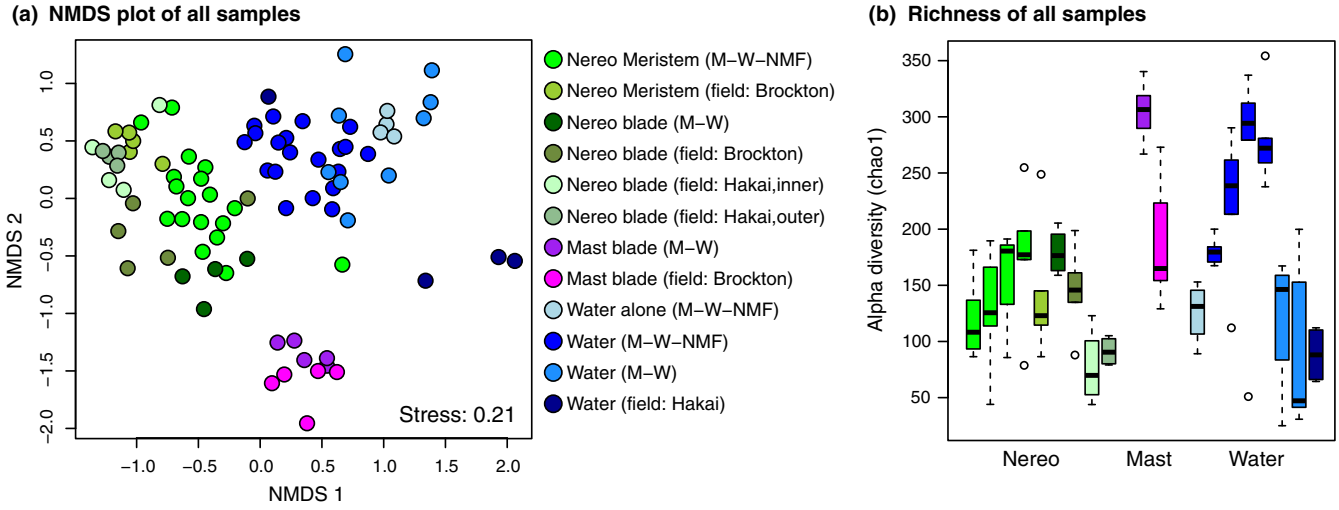

FIGURE 2 Comparison of microbial community composition and richness across macroalgal surfaces and associated water column samples in M–W–NMF experiment. (a) NMDS plot of *Nereocystis leutkeana* ($n = 40$), *Mastocarpus* sp. ($n = 10$), and water samples ($n = 37$) from created from a Bray-Curtis dissimilarity matrix. (b) Bacterial richness (Chao1 index) from same samples as in (a)

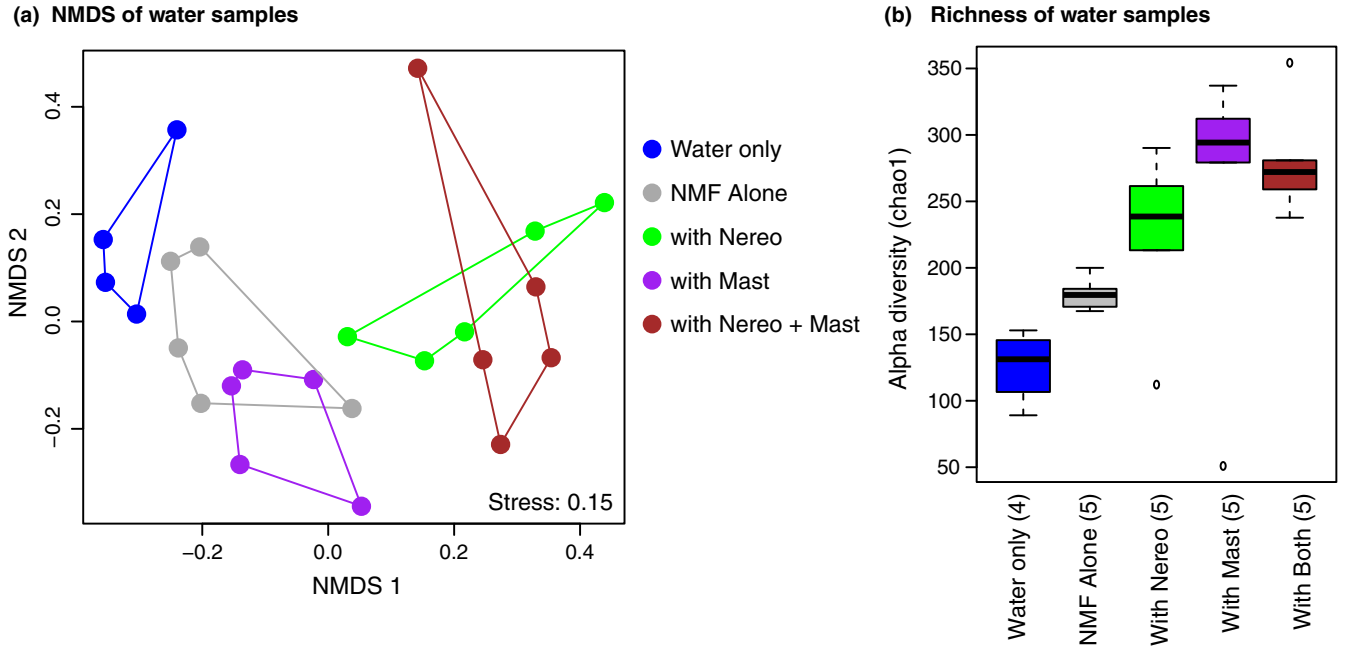

FIGURE 3 Comparison of water column communities following co-incubation with macroalgae in M–W—NMF experiment. (a) NMDS plot of water column community composition created from a Bray Curtis dissimilarity matrix. (b) Richness (Chao1 index) of water column communities across treatments. $n = 4$ for water only treatment and $n = 5$ for all other treatments. Refer to Table 1 for statistical results

TABLE 1 Comparison of microbial communities in M–W–NMF experiment

		PERMANOVA (Bray-Curtis community dissimilarity)		Welch's *t*-test (Chao1 richness)	
Group 1	**Group 2**	**Water samples**	**NMF surface**	**Water samples**	**NMF surface**
Water Only	NMF Alone	**0.018** (R^2 = .22, $F.model_{1,7}$ = 1.979)		**0.0211** ($t_{3.66}$ = 4.058)	
NMF Alone	Nereo, Mast, NereoMast	**0.004** (R^2 = .124, $F.model_{1,18}$ = 2.541)	**0.002** (R^2 = .115, $F.model_{1,16}$ = 2.088)	**<0.001** ($t_{39.849}$ = −4.052)	0.49 ($t_{36.81}$ = −0.697)
Nereo	Mast	**0.0135*** (R^2 = .300, $F.model_{1,8}$ = 3.435)	0.111* (R^2 = .245, $F.model_{1,6}$ = 1.945)	**0.0141*** ($t_{4.672}$ = −4.879)	0.519* ($t_{12.69}$ = −0.978)
Nereo	NereoMast	0.120* (R^2 = .150, $F.model_{1,8}$ = 1.415)	0.298* (R^2 = .209, $F.model_{1,6}$ = 1.589)	0.092* ($t_{10.634}$ = −2.491)	0.805* ($t_{8.067}$ = −.256)
Mast	NereoMast	**0.0135*** (R^2 = .350, $F.model_{1,8}$ = 4.308)	0.128* (R^2 = .123, $F.model_{1,8}$ = 1.118)	0.181* ($t_{16.372}$ = −1.637)	0.519* ($t_{5.753}$ = −1.052)

*FDR-adjusted *p*-values.
Bold values indicate $p < .05$. Colours indicate treatment.

Paraglaciecola (Gammaproteobacteria) were enriched in treatments with *Nereocystis* (Figure 5). Several genera (e.g., *Glaciecola*, *Polaribacter*, *Lewinella*) were significantly enriched in the water column in all treatments (Figure S3). There were no genera consistently reduced across water samples, but some genera appear to respond to a particular species: for example, an unidentified strain of Flavobacteria is reduced when co-incubated with *Mastocarpus* (Figure 5). More taxa (both OTUs and genera) are significantly enriched or reduced in the water column than on NMF surfaces when incubated with macroalgae (Figure 5 and Figures S2 and S3), although water column and NMF surface communities have similar richness in the baseline NMF alone treatment (Figures 3b and 4b). NMF surfaces are also more variable than water column communities (Figure 6). Only one genus, *Persicirhabdus* (Verrucomicrobia), declined significantly compared to the control across all treatments. *Persicirhabdus* is also at fairly high abundance in the field at both locations, but is more common on mature blades (Figure S5). The relative abundance of the genus *Rubritalea* on NMF surfaces declines when any macroalgae is

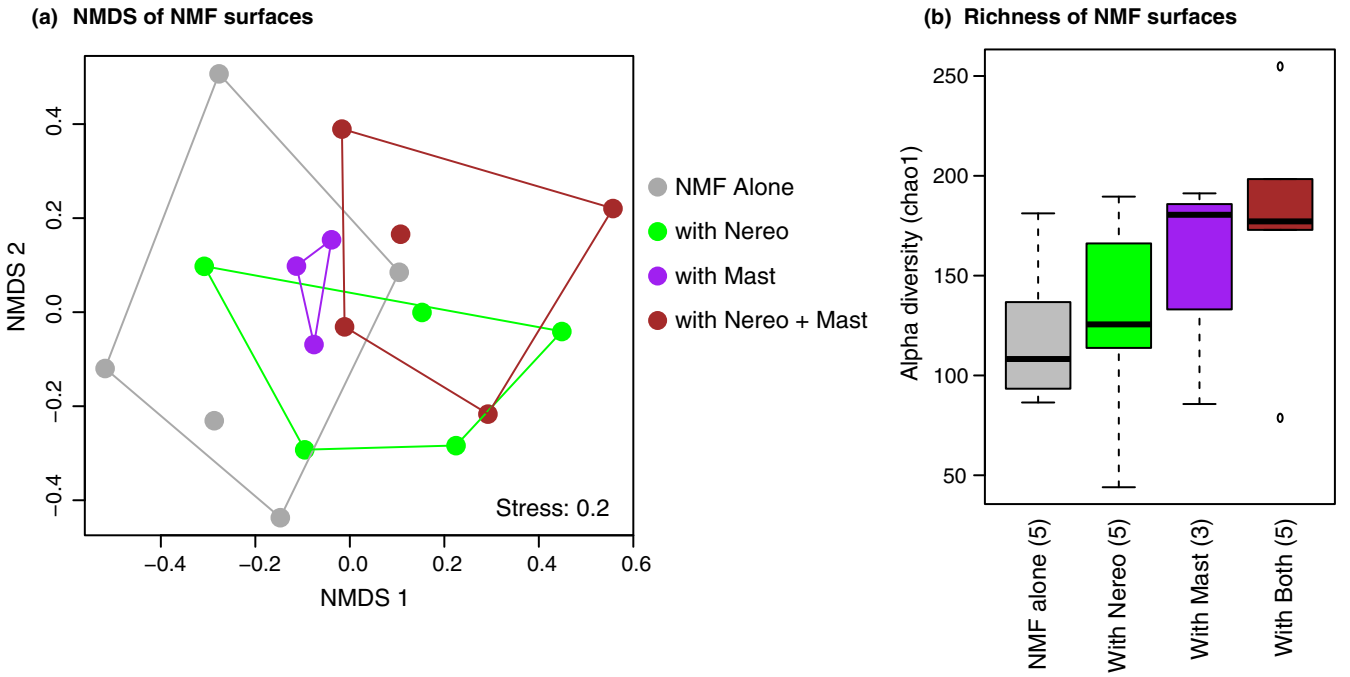

FIGURE 4 Comparison of NMF surface microbial communities following co-incubation with macroalgae in M–W–NMF experiment. (a) NMDS plot of NMF surface communities created from a Bray-Curtis dissimilarity matrix. (b) Box plots of richness (Chao1 index) of NMF surface communities. $n = 3$ for with *Mastocarpus* treatment and $n = 5$ for all other treatments. For statistical results, refer to Table 1

added, and declines are significant in the presence of mature *Nereocystis* (Figures 5 and 6). *Rubritalea* is rare in the water-only control, and it is more abundant on *Nereocystis* meristems compared to mature blades sampled in situ from Brockton Point (Figure S5). *Rubritalea* is also found in high abundances on *Nereocystis* blade samples from our remote location (Hakai) (Figure S5). Together, these observations suggest *Rubritalea* is naturally associated with *Nereocystis* and *Nereocystis* meristems.

3.4 | NMF growth and water quality during experiment

All NMFs grew during the experiment, and growth was proportional to their original surface area (regression of original area to area after incubation: $p < .001$, $R^2 = .97$; Figure S7). Growth indicates that meristem fragments were alive and productive. We calculated an ANOVA on the residuals of this regression to test for differential growth across treatments and find that NMF growth rates do not differ (ANOVA: growth residuals $p = .83$, $F = 0.288$; Figure S7). We measured temperature, salinity, dissolved oxygen and pH of the water in both the M–W and M–W–NMF experiments (Figure S8). Dissolved oxygen and pH increased in treatments with macroalgae across the M–W–NMF experiment, likely as a result of photosynthesis from greater macroalgal biomass (one-way ANOVA: dissolved oxygen $p < .001$, $F_{4,58} = 15.429$; pH $p < .001$, $F_{4,58} = 11.556$). They did not differ between treatments in the M–W experiment (one-way ANOVA: dissolved oxygen $p = .316$, $F_{1,18} = 1.0621$; pH $p = .058$, $F_{1,18} = 4.115$). Salinity and temperature sometimes differ by treatment (Figure S7).

4 | DISCUSSION

We investigated the extent to which macroalgae alter the microbial communities in the surrounding water column and on neighbouring macroalgal surfaces. We find that the distantly related macroalgae *Nereocystis* (kelp; Phaeophyceae) and *Mastocarpus* (Rhodophyceae) alter the microbial community composition of the water column and these shifts are species specific (Figure 3), consistent with previous results (Lam & Harder, 2007; Lam et al., 2008). In general, *Nereocystis* had a stronger effect on community composition than *Mastocarpus*, while treatments with *Mastocarpus* showed a trend towards increased richness. Treatments with both species yield water column communities that are similar in composition to *Nereocystis* treatments, but with the higher richness (Figure 3). *Nereocystis* produced qualitatively more mucilage than *Mastocarpus*. Kelp mucilage is known to contain various polysaccharides and antimicrobial compounds; this in combination with the larger volume of exudates may explain its larger effect on community composition. Suppression by *Nereocystis* antimicrobial exudates may play a role. Comparing water column communities across experiments suggests that *Mastocarpus* induces larger shifts in the water column microbiota when incubated alone in the M—W experiment (Figure S6). These samples have high relative abundance of NS3a (Flavobacteria) and *Glaciecola* (Gammaproteobacteria) (Figure S4), which are present at low abundance in other samples and are commonly associated with algal blooms in other systems (Teeling et al., 2016). In any case, the effects of macroalgal species are not strictly additive: the treatment with both *Nereocystis* and *Mastocarpus* is less rich than *Mastocarpus* alone (Figure 3b), although this trend is not significant ($p = .181$;

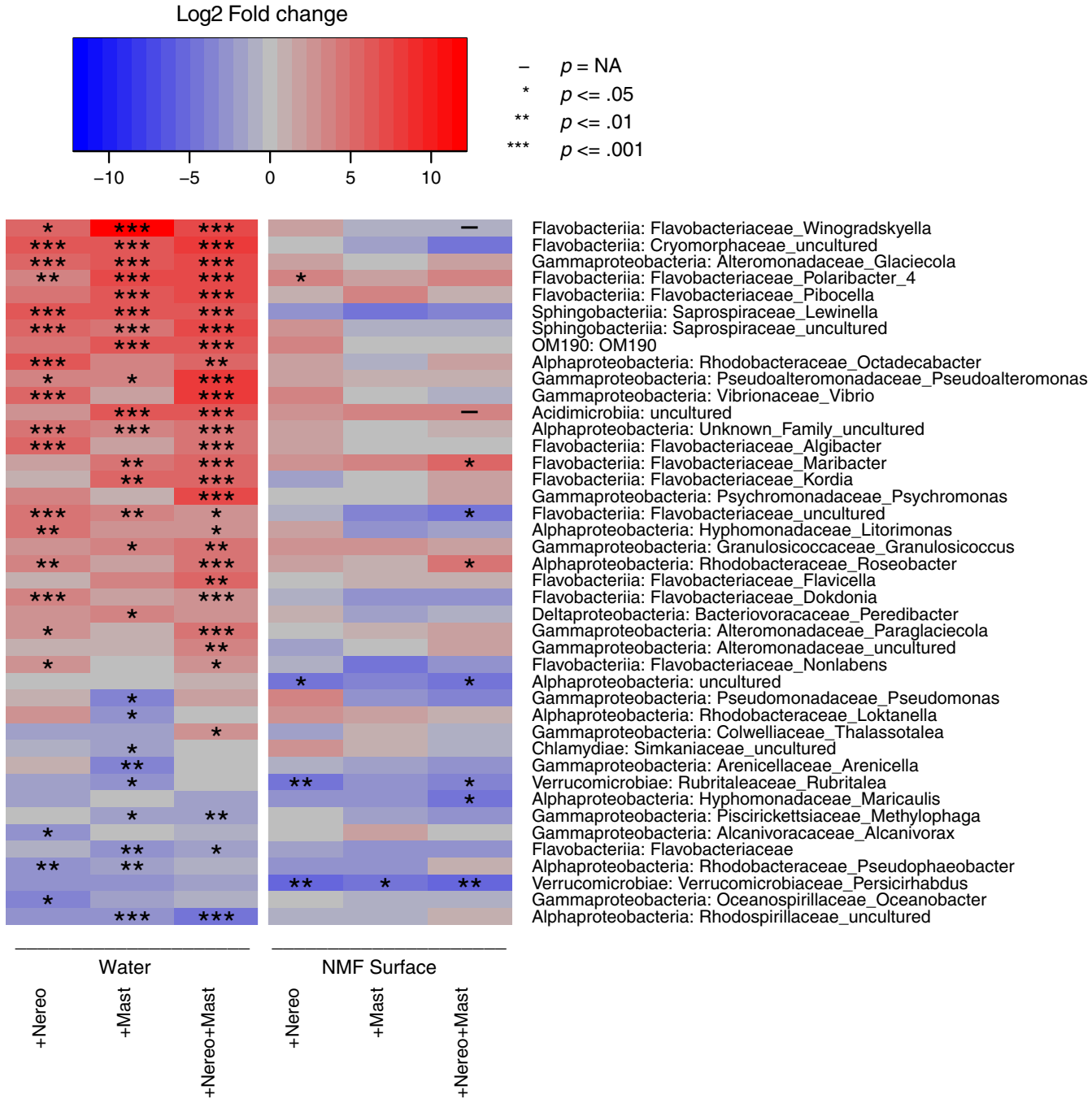

FIGURE 5 Enriched and reduced genera in response to co-incubation with macroalgae in the M–W–NMF experiment. Changes in relative abundance of bacterial genera compared to the NMF alone treatment was calculated in the "DESEQ2" package in R. When OTUs could not be assigned a genera, the next highest level of taxonomy was used. The genera shown are those with *p*-values below .05 after Benjamini-Hochberg correction and which occur at >3% relative abundance in at least two samples; see Figure S3 for all genera. Asterisks indicate significance level and "–" refers to genera whose *p*-value could not be calculated in DESeq2 due to outliers or filter cut-offs. Colours indicate fold change: red is enriched in macroalgal addition treatments, while blue is suppressed. Relative abundance plot of taxa shown in Figure 6. OTU-level analysis in Figure S2

Table 1). It is unclear whether this lower richness in this treatment is due to antagonistic effects between *Mastocarpus* and *Nereocystis* exudates, or a result of less total *Mastocarpus* tissue (which drives high richness) in the combined treatment. Our results demonstrate that the surrounding water column community differs in both composition and richness when incubated with macroalgae and that species of macroalgae differentially affect the composition and richness of microbial communities.

We note that the degree to which macroalgae impact the water column and neighbouring macroalgal microbiota is likely lower in

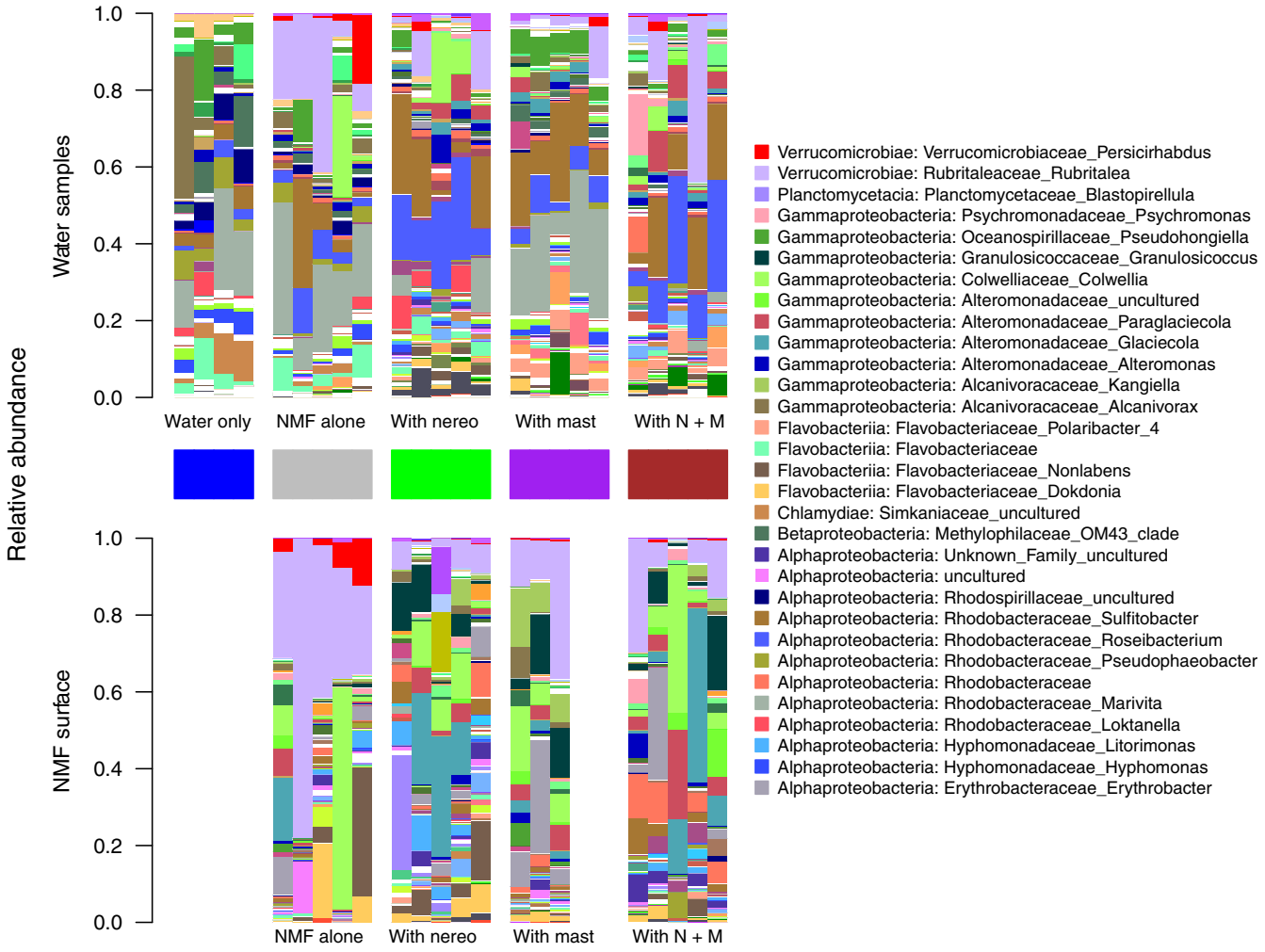

FIGURE 6 Relative abundance of genera found on NMF surfaces and water column following co-incubation with macroalgae in the M–W–NMF experiment. The legend lists the class, family, and genus of the most abundant taxa, and colours are consistent across all taxa summaries

nature than in our experiments, where these species would be farther apart and their exudates diluted by large-scale water exchange due to currents and tides. *Nereocystis* and *Mastocarpus* were chosen for this experiment for several reasons. They are distantly related (red algae and kelp diverged more than 1 billion years ago; Parfrey, Lahr, Knoll, & Katz, 2011) and previous work has shown that red and brown algae have different epibiota (Lachnit et al., 2009), and Lam and Harder have shown that *Mastocarpus stellatus* and *Laminaria digitata* (another kelp) have large impacts on the water column community (Lam & Harder, 2007), enabling us to assess the effect of species identity on the water column microbiota and the epibiota of neighbouring macroalgae. *Nereocystis* is a rapidly growing and bed-forming kelp, which enables us to link our results into the larger body of work on the impact of kelp on environmental microbiota (Clasen & Shurin, 2014; Linley et al., 1981; Newell et al., 1980; Stuart et al., 1981). The rapid growth from detached meristematic regions in *Nereocystis* enabled us to track growth rates over the experiment and ensure that microbiota changes observed are not due to tissue degradation. Further, *Nereocystis* and *Mastocarpus* are common species and occur in close proximity (several metres) of each other. However, they do not directly interact in situ as *Mastocarpus* grows on and within rocks of the low intertidal, whereas *Nereocystis* grows in the shallow subtidal and blades float near the water surface.

Macroalgae alter surrounding microbiota through diverse mechanisms, including enrichment due to dissolved or particulate organic carbon inputs (Stuart et al., 1981), release of antimicrobial compounds (Dahms & Dobretsov, 2017; Egan et al., 2013; Lam et al., 2008) and direct dispersal from macroalgal tissue (Lam & Harder, 2007). We assessed overall changes in the water column microbiota and in the epibiota of NMF in response to neighbouring macroalgae, but did not distinguished among these possible mechanisms. We find that more microbes are enriched in the water column than are suppressed in the presence of mature macroalgae (treatments with co-incubates added to NMF fragments; Figure 5). The taxa that bloom (such as *Algibacter* [Martin et al., 2016] and Saprospiraceae [McIlroy & Nielsen, 2014]) include known degraders of algal polysaccharides, suggesting that response to additional carbon sources drives part of

the community shifts. Many of the water column microbiota that change in relative abundance are not common on macroalgal surfaces, such as *Roseibacterium*, *Pseudohongiella* and *Sulfitobacter* (Figure 5 and Figures S5), which likely reflects the widespread ability of coastal water column microbes to bloom in response to the presence of macroalgal carbon. This is an apparent contrast to microbes in artificial indoor environments, for example, in which most detected microbes appear to be derived from their inhabitants (Lax et al., 2014). We used a large mesh prefilter (150 μm) to remove only large detritus particles in order to study overall changes that result from co-incubation with macroalgae. Future studies that distinguish between dissolved organic carbon (e.g., mucilage) and particulate algal debris would be enlightening as these have differential impacts on bacterial productivity (Linley et al., 1981; Stuart et al., 1981) in kelp forests, and more generally, marine particle-associated bacteria differ from those that are free living (Rieck, Herlemann, Jrgens, & Grossart, 2015).

We find that the microbiota on actively growing *Nereocystis* meristem surfaces are resistant to change compared to the water column, consistent with them being highly selective surfaces. NMF microbiota shift in the presence of neighbouring macroalgae, but the shift is much less pronounced than in the water column. Community composition on NMF surfaces was sensitive to the presence or absence of a co-incubated macroalgae, but the changes do not depend on macroalgal species (Figure 4a and Table 1). Richness did not change appreciably in any of the treatments (Figure 4b and Table 1). Underlying these diversity patterns, many fewer taxa on NMF surfaces compared to the water column change significantly in relative abundance when incubated with neighbouring macroalgae (Figures 5 and 6). Differences between control (NMF alone) and treatment samples were largely driven by the reduction in a few genera, particularly *Rubritalea* and *Persicirhabdus* (Verrucomicrobia) (Figure 5). We also find that these relatively small microbiota differences do not impact growth; all NMF fragments grew over the course of the 5-day experiment but growth was proportional to starting size and independent of treatment (Figure S7).

The genus *Rubritalea* was found on all NMF surfaces, while other genera were highly variable across samples. *Rubritalea* are also found in higher abundances on *Nereocystis* meristems compared to mature *Nereocystis* blades in the field (Figures S4 and S5). *Rubritalea* are proportionally less represented in treatments with macroalgae in the M–W–NMF experiment (Figures 5 and 6), which generally have higher microbial richness, and thus may be outcompeted by other members of the microbiota when near mature blades of macroalgae. *Rubritalea* and *Persicirhabdus* are also common at our remote field site (Hakai; Figure S5). Representatives of the genus *Rubritalea* produce pink-orange pigments and squalene (Kasai et al., 2007; Scheuermayer, Gulder, Bringmann, & Hentschel, 2006; Yoon et al., 2007; Yoon,Matsuo, Matsuda et al., 2008), the latter of which is a precursor to steroids and D-vitamins (Bloch, 1983). Interestingly, both steroids and D-vitamins are known to promote growth in some species of macroalgae (Fries, 1983). *Rubritalea* was previously isolated from sponges (Scheuermayer et al., 2006) and is a close relative to *Akkermansia*, which is a commensal in humans. Two other genera of Verrucomicrobia are common on *Nereocystis*: *Persicirhabdus* and *Roseibacillus* (Figure S5, Figure 6); these have been isolated from brown algae and marine sediments and can also produce red pigments (Yoon, Matsuo, Adachi et al., 2008). Further work must be done to clarify the functional role *Rubritalea* and other Verrucomicrobia play, if any, in the *Nereocystis* microbiome.

Comparing our results to other studies of kelp microbiota suggests the presence of a common suite of taxa. At the broad level, seaweed and kelp microbial communities are typically dominated by Gammaproteobacteria, Alphaproteobacteria and Bacteroidetes, with Verrucomicrobia and Planctomycetes found consistently but at lower abundances (Bengtsson et al., 2012; Burke, Thomas, Lewis, Steinberg, & Kjelleberg, 2011; Lemay et al., 2018; Michelou, Caporaso, Knight, & Palumbi, 2013). At more detailed taxonomic resolution, similarity in composition across kelp is more striking. For example, Verrucomicrobia and Planctomycetes are predominately represented by characteristic genera, including *Rubritalea* and *Persicirhabdus* (Verrucomicrobia) and *Blastopirellula* (Planctomycetes; Bengtsson & Øvreås, 2010; Bengtsson et al., 2012; Lemay et al., 2018; Vollmers, Frentrup, Rast, Jogler, & Kaster, 2017; Figure S5 and Figure 6; Table S2). Within the Gammaproteobacteria, *Granulosicoccus* is highly abundant and is indicative of annual kelp (Lemay et al., 2018), and dominant in early season samples of *Laminaria* (Bengtsson et al., 2012). *Granulosicoccus* is also common in this study (Figure 6). To better assess commonality, we compared our OTUs to those reported by Lemay et al. (2018) at 97% similarity and find that approximately two-thirds match, and account for ~80% of total reads (Table S2). Another common feature of macroalgal-associated microbiota is a high degree of overlap in the composition of the epibiota and surrounding seawater (Lemay et al., 2018). Across all samples from laboratory experiments, 68% of OTUs are shared (found in at least one macroalgal and one water sample), while 16% are shared between water and epibiota in the Hakai field data set. This is lower than the 86% of shared OTUs reported by Lemay et al. (2018) because we have fewer samples overall (61 in the laboratory and 12 at Hakai, versus 124) and more stringent filtering to remove low abundance OTUs.

We note that despite efforts to keep conditions the same across tanks and treatments, including randomizing the location of treatment tanks within the experimental array of tanks, the treatments do differ in abiotic parameters (Figure S8). Differences in dissolved oxygen and pH likely reflect differential rates of photosynthesis across treatments, with the NMF alone treatment having the smallest biomass of algal tissue and the NMF + *Nereocystis* + *Mastocarpus* having the most algal biomass. This variation in abiotic conditions may underlie some of the microbial community differences we observe.

We find a strong signal of host specificity in our data. Comparisons between all samples show that the strongest driving factor of microbial community composition is macroalgal species (*Nereocystis* vs *Mastocarpus*) and that these differences are maintained on individuals incubated in the laboratory (Figure 2). While both our study

and previous studies show high variation in microbial community membership within a single species of macroalgae (Burke, Steinberg et al., 2011; Burke, Thomas et al., 2011) our data suggest that differences in microbial community composition are even greater between species of macroalgae. Other studies that compare within-species with between-species variation in microbiota structure have also found that species is a stronger predictor of microbial community composition than location (Lachnit et al., 2009; Lemay et al., 2018). A fuller understanding of the relative importance of host identity, geographic location and other factors in driving community assembly and maintenance on macroalgae await studies that simultaneously test these variables. These results emphasize that the effects of treatments on microbial community structure are subtle modulations on a more general pattern of species specificity and provide a framework for interpreting future results in a broader ecological context.

5 | CONCLUSION

In conclusion, we find that neighbouring macroalgae influence the microbial communities on actively growing macroalgal tissue. However, the changes they induce in surface communities are muted compared to changes in the surrounding water column. Many fewer genera are differentially enriched on NMF surfaces compared to the water column, and changes do not depend on the co-incubated macroalgal species. In contrast, macroalgae induce species-specific changes in column communities. Further, while water column communities are significantly more diverse when co-incubated macroalgae are added, NMF surfaces are not. This suggests that macroalgal surfaces are more resistant to change than the surrounding water column. Further supporting the selectivity of macroalgae, we find that *Nereocystis* and *Mastocarpus* each retain a characteristic microbiota in the laboratory that resembles epibiota in the field and that *Nereocystis*-associated communities retain host specificity despite variation across treatments. Whether the subtle changes in microbiota observed on NMFs translate to biologically important functional differences is unknown, but the bacteria identified here are candidates for symbionts that might enhance disease resistance or promote health.

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AUTHOR CONTRIBUTIONS

M.C. conceived of the research idea, designed and performed the research, analysed the data and wrote the first draft. L.P. supervised the project, provided feedback on design of experiments and on analysis and made major contributions to writing.

DATA ACCESSIBILITY

Sequence data and MiMARKS compliant metadata are accessioned at the European Bioinformatics Institute (PRJEB23525). OTU tables and mapping files which include sample metadata can be found at https://github.com/mechen10/NereoIncubProject.

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