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A deep dive into the epibiotic communities on aquacultured sugar kelp *Saccharina latissima* in Southern New England

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ABSTRACT

Sugar kelp cultivation at the southern end of its range on the east coast of North America is being pursued commercially for human consumption, which demands a high-quality product. Blade quality can be compromised by attached organisms – epibionts. Biweekly examination of epibionts on sugar kelp was conducted April–May 2018, on a kelp farm in eastern Long Island Sound, CT, USA. Culturable *Vibrio* spp. were not present on kelp blades until May and were limited to only old sections. No *Vibrio* colonies were human pathogens *V. parahaemolyticus* or *V. vulnificus*, based upon *ToxR*-specific multiplex PCR assays. Neither epibenthic cyanobacteria *Lyngbya* spp. nor the dinoflagellate *Prorocentrum lima,* microbes of concern because of toxigenicity, were detected on kelp by microscopy or metabarcoding of partial rRNA genes. The lacy bryozoan was the only epibiotic animal observed that could cause damage to kelp, but its abundance was low.

Summarizing most-common sequence reads, Gammaproteobacteria was the most abundant bacterial group on kelp blades (49%) and Alphaproteobacteria were the most abundant in seawater (39%). Bacillariophyta were the most abundant eukaryotes on kelp blades (36%) and Dinoflagellata were the most abundant eukaryotes in seawater (43%). Molecular operational taxonomic unit matrices were used for non-metric multidimensional scaling; the most prominent structure for both prokaryotic and eukaryotic communities was the separation between blade and seawater samples. This spatial separation explained 81% and 76% of the variation among prokaryotic and eukaryotic samples, respectively. Indicator Species Analysis identified Gammaproteobacteria (55%) and Bacillariophyta (56%) to be the most important blade indicator prokaryotes and eukaryotes, respectively. A closer examination of indicator species temporal patterns and their ecophysiology suggested that *Aquimarina*, Parcubacteria, and Peronosporomycetes are potential pathogens to sugar kelp. Ciliates may be the most important grazers that keep epiphytes (Bacillariophyta, Rhodophyta, and Phaeophyta) and Peronosporomycetes on kelp in check.

1. Introduction

Global seaweed production has grown rapidly during the 21st Century to US $6 billion annually and 30 million tons, dry weight, in 2015 [1]. Aquacultured seaweed accounted for 97% of the total production in weight. During the past decade, aquacultured seaweed grew over 27%, while the wild harvest remained relatively constant [1]. Seaweeds and seaweed products have many economic applications as sources of protein, fiber, and other nutrients for human food and animal feed (e.g., [2,3]), and they also can be used in medicine, cosmetics and food additives (e.g., [4]). In addition to intrinsic commercial value, seaweed growth provides an ecosystem service by removing inorganic nutrients from aquatic ecosystems. This has practical applications in coastal areas where nutrient inputs from human activities lead to eutrophication. Bioextraction, the practice of using bivalve shellfish and seaweed cultivation to remove excessive nutrients, has been successful in restoring ecosystem balance [5–7]. Another area where excessive inorganic nutrients can be removed by seaweed growth is in the vicinity of finfish aquaculture settings [8–12]. In those cases, ecosystem services provided by seaweed are comparable to the economic value of the seaweed products themselves [8,11].

Sugar kelp (*Saccharina latissima*) is a cold-water, brown algal species.

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It is widely distributed throughout the coastal North Atlantic and North Pacific US [13,14]. Cultivation techniques for kelp and other seaweeds are well developed in some parts of the world. For example, Japanese kelp (*Saccharina japonica*) has been cultivated widely in China, Korea, and Japan, accounting for 50% of total seaweed production in China with an annual production of approximately 1 million tons dry weight [1]. In the US, the market for sugar kelp has been met by wild harvests, but this is changing with increasing demand [15]. As a result, sugar kelp has become an aquacultured species in the US [6,16] and has been cultivated as far south as Connecticut along the US Atlantic coast.

Epibionts, both single-celled and multi-cellular attached organisms, are common on kelp and other seaweeds, with the most abundant groups being bacteria, algae (such as diatoms, chlorophytes, rhodophytes), hydroids, amphipods, gastropods, copepods, annelids, and bryozoans [17,18]. Epibionts have been documented to affect the growth and product quality of host seaweeds [19–21]. Presence of epifauna on sugar kelp, such as hydroids, reportedly degrades the taste and quality of the kelp and makes it unsuitable for human consumption [22]. The cost of seaweed preparation for market also increases when epibiotic organisms need to be removed from the kelp surface.

Epibiotic microorganisms form close relationships with seaweed hosts and affect both the safety and quality of seaweed products in significant ways. For example, if pathogenic *Vibrio* are present in seawater where sugar kelp is farmed, it is possible that kelp blades could concentrate these microbes and render kelp unsafe for human consumption. Other microorganisms of safety concern to human consumption are epibenthic, toxigenic microalgae such as the cyanobacterium *Lyngbya* found in salt marshes in Long Island Sound [23–25] and the dinoflagellate *Prorocentrum lima* found in epiphytic communities of seaweed in New England waters [26–28]. Even though these pathogenic and toxigenic microorganisms are reasonable candidates for monitoring purposes, there remains a lack of evidence for association between these microorganisms and sugar kelp, wild or aquacultured, in coastal New England waters [29]. Moreover, with human pathogens taking the center stage as microorganisms of concern [30], there is little known about microorganisms that could cause disease to sugar kelp. Reports on the temporal progression of the epibiotic microbial community, which should include both prokaryotes and eukaryotes in addition to human pathogens and kelp pathogens, remain largely unavailable. Consequently, kelp farm siting follows the same guidelines used for shellfish, which may be considered over restrictive. The burgeoning kelp farming industry and these knowledge gaps require that we better understand the sugar kelp epibionts, especially the microbial component.

To obtain a comprehensive list of epibionts on cultured sugar kelp during the main growing season at the southern end of its range, we employed both culture-independent and culture-dependent techniques. The range of methods was intended to examine epibiotic micro- and macro-organisms thoroughly, with a focus on the under-studied microbial communities. Specifically, conventional light microscopy was used to visualize microalgae; 16S (bacteria) and 18S (microalgae, protozoa, and fungi) rDNA metabarcoding (hereafter NGS for Next Generation Sequencing) was applied to describe kelp-associated (epibiotic) and ambient (planktonic) microorganisms throughout the main kelp growth season in Long Island Sound; presumptive culturing of *Vibrio* spp. on Thiosulfate-Citrate-Bile-Sucrose Agar (TCBS) and confirmatory polymerase chain reactions (PCR) were used to detect pathogenic *Vibrio* strains. Lastly, visual examination of kelp blades was conducted to identify encrusting macroorganisms that may not have been included in sub-samples subjected to NGS or TCBS/PCR.

Building upon the comprehensive methodologies used to capture different components of the kelp epibiotic communities, another highlight of our study is the application of indicator species analysis (ISA) on NGS-generated molecular taxonomic operational unit (MOTUs) matrices. ISA looks for the most consistently abundant MOTUs on blades and in seawater (a perfect indicator species will only occur in one group). Therefore, blade indicator species (IS) and seawater IS are microorganisms most enriched on blades and in seawater, respectively. Contrasts between blade IS and seawater IS could shed light on the ecophysical benefits blades and seawater provide for microorganisms to succeed (replicate) in respective habitats. Moreover, blade IS, rather than the entire suite of blade MOTUs identified through NGS, are the subset of microorganisms most strongly associated with sugar kelp, and thus serve as a pool from which the most probable sugar kelp pathogen candidates can be identified. In summary, this report will: 1) document the presence and absence of epibiotic microbes that are of a priori concern to human health if aquacultured sugar kelp is to be consumed directly (i.e., potentially pathogenic *Vibrio*, toxigenic cyanobacteria *Lyngbya,* and the dinoflagllate *Prorocentrum lima*); 2) provide a timeline of the relative abundances of important epibiotic organisms on aquacultured sugar kelp, and 3) recommend a list of microorganisms of kelp health concerns for further monitoring and research.

2. Material and methods

2.1. Sugar kelp seedstring preparation and field cultivation

Sugar kelp seedstrings were prepared in the GreenWave hatchery following established protocols [16]. Seed-string spools (strings wrapped around PVC spools) were briefly immersed in a container with seawater and a proper density of kelp spores. After allowing spores to settle on the string, spools were transferred to culture medium with recommended lighting and temperature. Juvenile sporophytes were ready to be planted in the field when they were about 1 mm in length. The specific batch of sugar kelp blades with sorus tissue was collected from Long Island Sound, at Black Ledge and Pine Island near Avery Point, Connecticut, U.S.A. in 2017 for the 2017–2018 growing season.

Open water field cultivation took place in Groton, Connecticut, USA, Groton CT (41°18′44.4″ N, 72°2′23.7″W, Fig. 1). Outplanting occurred on December 18, 2017 for the 2017–2018 growing season. The grow-out systems consisted of six longlines of 152 m length with 15 m distance between each line. The depth of the lines ranged from 5.2–7.0 m at MLW (mean low water) and 6.1–7.9 m at MHW (mean high water). Seeding of longlines was a simple procedure wherein the line was threaded through the PVC spool, and seedstring was 'spooled off' in a spiral fashion onto the line.

2.2. Sample collection

Kelp blades and seawater samples were collected on July 26, 2017 as practice samples to establish protocols. In the study year of 2018, samples were collected roughly biweekly (4/7, 4/19, 5/4, 5/17, and 5/30, corresponding to T1, T2, T3, T4, and T5, respectively). Whole kelp blades were picked randomly and kept in individual Ziploc plastic bags. Ambient seawater was collected in multiple, sterile, 50-ml Falcon tubes. All samples were placed in a cooler with ice packs and transported to the Milford Laboratory immediately after collection or stored in a refrigerator for less than 24 h before processing.

2.3. Visual inspection of encrusting macroorganisms

Three kelp blades were observed and photographed. Samples were either refrigerated and processed within 48 h or stored at −20 °C and processed within two weeks of collection. Macroalgae and invertebrates found on both sides of the blades were counted and identified to the lowest possible taxon based upon methods described by Gosner and Sears [14,31,32]. Total area of each blade, as well as the area covered by epibionts, was measured with software Image J using AREA function (https://imagej.net/Welcome). Area covered by visible epibionts in each 10-cm quadrant was also obtained to calculate the percentage of each blade covered by encrusting macroorganisms.

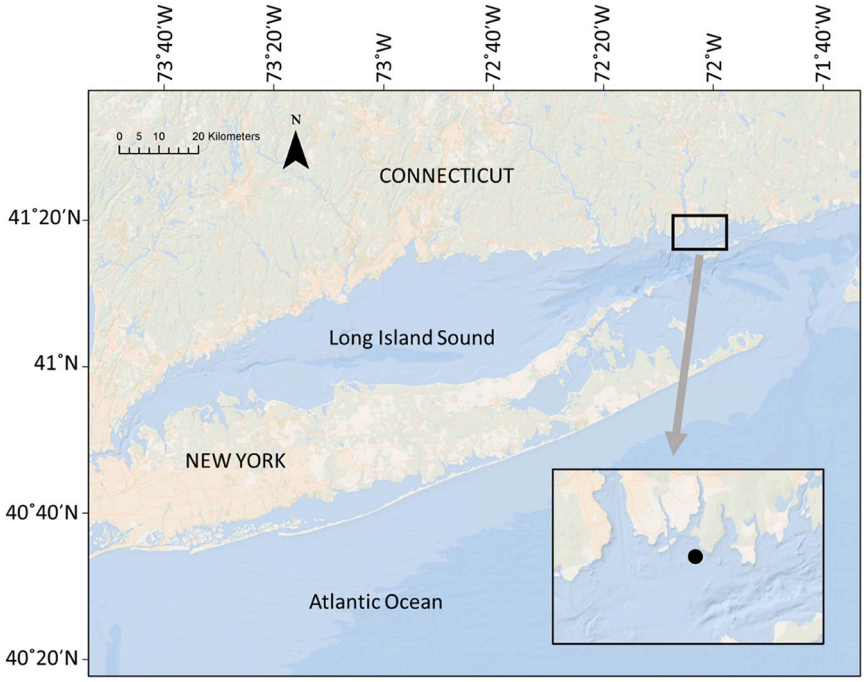

Fig. 1. Location of kelp farm at Groton, Long Island Sound, NY.

2.4. Microscopic examination of microalgae

Three individual kelp blades were scraped gently with a soft brush. Scraped materials from each blade were collected using ~200 ml 0.2-μm-filtered sea water and fixed with Lugol's solution [33] at a final concentration of 3%. Upon analysis, a known quantity (usually 50 ml) from each sample was settled with the standard Utermöhl settling chamber method [34]. Counts and identification were performed using a Zeiss Observer inverted light microscope at 200× or 400× magnification, with additional higher magnification available if necessary. Microalgal taxa were determined based upon Carmelo [35,36], supplemented by internet resource sites: World Register of Marine Science (http://www.marinespecies.org) and Algaebase – Listing the World's Algae (https://www.algaebase.org/).

2.5. Vibrio isolation on TCBS and PCR screening for V. vulnificus and V. parahaemolyticus

Three kelp blades collected on each sampling day were prepared for *Vibrio* culturing on TCBS agar plates. Each kelp blade was divided into 3 regions lengthwise, with "Tip" corresponding to the oldest 1/3 of the blade furthest from the stipe, "Middle" corresponding to the middle 1/3 of the blade, and "Bottom" being the youngest 1/3 of the blade near the stipe. A 25 cm^2 piece of tissue was removed aseptically from each of the 3 regions of the 3 blades. The 3 pieces from the same region of the 3 blades were then washed gently twice in sterile sea water (SSW) to remove loosely attached organisms and dirt, and pooled and homogenized in 100 ml SSW for 5 min. The kelp samples and surrounding water were plated in triplicate on Thiosulfate-Citrate-Bile-Sucrose Agar (TCBS) (BD-Difco), a selective medium for *Vibrio* spp. [37]. Development of bacterial colonies was recorded after 3 days of incubation at 23 °C.

Presumptive *Vibrio* colonies were picked from the kelp and water plates. DNA was isolated from the strains using phenol-chloroform-isoamyl alcohol [38]. To verify the taxonomy, PCR with a *Vibrio*-specific primer set was performed following an established protocol [39]. To test for the presence of *V. parahaemolyticus* and *V. vulnificus*, multiplex PCR targeting the *toxR* gene was performed following Bauer and Rorvik [40]. Amplicon sizes for *V. parahaemolyticus* and *V. vulnificus* are 297 bp and 435 bp, respectively, readily distinguishable by gel electrophoresis.

2.6. Next generation sequencing of microorganisms on blades and in seawater

2.6.1. DNA sample collection

Triplicate seawater samples of 100 ml were filtered through Millipore polycarbonate filters (47-mm diameter, 0.2-μm pore size), which were subsequently stored in Qiagen PowerWater bead tubes in a freezer until DNA extraction. For epibiotic samples, we used kelp collected in 2017 to compare 3 methods involving different collection and extraction details. Method 1 combined blade brushing and DNA extraction using the Qiagen PowerWater kit. A clean tooth brush with gentle bristles was used to remove the epibiotic materials while a stream of pre-filtered (0.2-μm) and autoclaved seawater was applied to the blade to collect the wash-off into a sterilized glass container. The epibiotic content then was screened through a 200-μm mesh to remove macroorganisms before being collected on a Millipore polycarbonate filter (47-mm diameter, 0.2-μm pore size) using vacuum suction. These preparations were stored frozen in Qiagen PowerWater bead tubes until DNA extraction. In Method 2 and Method 3, the entire blade surface was scraped using a sterile cotton swab instead of a toothbrush. The difference between the two methods was that the Qiagen PowerBiofilm kit was used for Method 3. In all cases, triplicate epibiotic samples were taken, and DNA was extracted within 1 month of sample collection.

2.6.2. DNA extraction, PCR, & next generation sequencing

Manufacturer protocols generally were followed when extracting DNA using the two kits. Minor changes were made to the Qiagen PowerBiofilm protocol to facilitate sample resuspension at the beginning: 350 μl MBL solution was added to the thawed bead tubes which contained the cotton swabs, and gently shaken for 2 min. Upon completion of DNA extraction, a Qubit Fluorometer 2 was used for DNA quantification, and the amount of DNA retrieved using the 3 methods was normalized against the surface area sampled. Method 1 had the highest DNA retrieved per area (ng/cm^2) of the 3 tested methods (Suppl. Table 1), and was used in 2018.

Partial regions of SSU 16S and 18S rRNA genes were amplified using two universal markers [41,42]. Primer sequences including the overhang adapters (in bold) were:

18SF: 5′- **TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG** CCA GCA SCY GCG GTA ATT CC

16SF: 5′- **TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG** CCT ACG GGN GGC WGC AG

16SR: 5′- **GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**GA CTA CHV GGG TAT CTA ATC C

18SR: 5′- **GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**AC TTT CGT TCT TGA TYR A

Triplicate amplifications were run and pooled together to dilute any stochastic errors that may have occurred during PCR. PCR was run in a 25-μl system using GE Healthcare Illustra PuRe Taq Ready-To-Go PCR beads, wherein 5 μl of DNA (5 μl H_2O for negative controls) was mixed with each primer at a final concentration of 200 nM for 16S reactions and 500 nM for 18S reactions. The thermal conditions for the 16S PCR included 3 min at 95 °C, 25 cycles of 30 s at 95 °C, 30 s at 50 °C, and 30 s at 72 °C, concluded by 5 min of final extension at 72 °C. A two-step PCR was employed amplifying 18S amplicons: after the initial 5 min at 95 °C, 10 cycles of 30 s at 94 °C, 45 s at 57 °C, and 60 s at 72 °C were followed by an additional 25 cycles of 30 s at 94 °C, 45 s at 48 °C, and 60 s at 72 °C, concluding with a final extension at 72 °C for 2 min.

PCR products were checked visually using gel electrophoresis, cleaned up with AMPure XP, and re-suspended in 40 μl 1× TE (pH = 8). To add Illumina sequencing adapters and dual-index barcodes, 10 μl pure PCR product was mixed with 2.5 μl each of the Nextera primers in a 25 μl GE PCR system for 8 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by 5 min at 72 °C. Indexed PCR was cleaned up using AMPure XP, and checked for the presence of the target DNA amplicon on an Agilent 2100 Bioanalyzer. The KAPA qPCR Library Quantification Kit was used to ensure that approximately 10 nM of each library flanked by the i5 and i7 index adapter sequences was pooled and spiked with 10% phi-X for Illumina MiSeq sequencing (MiSeq Reagent Kit v3, 2 × 300 bp) at Cold Spring Harbor Laboratory Genome Center.

2.6.3. Bioinformatics & data analysis

Paired FASTQ files (NCBI BioProject ID PRJNA641861) containing paired-end sequencing reads were processed through the Mothur pipeline (v. 1.42.2, 9/30/2019) [43,44]. Sequences were classified first against the Silva reference database (v132) using the Bayesian classifier [45]. Classified sequences then were clustered into molecular operational taxonomic units (MOTUs) using the cluster.split command, in which the taxonomic information was referred to when splitting sequences into MOTUs and a 97% similarity threshold was applied. Lastly, MOTUs were re-classified using the Silva reference database (v132), and the resulted taxonomy table with MOTU read numbers was used for statistical analysis. For the 16S dataset, autotrophic MOTUs were checked for *Lyngbya* presence but were later removed from further analysis because the majority of autotrophic MOTUs were chloroplast sequences from eukaryotic microalgae [44].

Extremely rare MOTUs contributing to less than 0.1% of the total number of reads per taxon and per library were removed. Inverse Simpson Index (InvSimpson) is the inversion of Simpson Diversity Index D, where $D = \frac{\sum_{i=1}^{S_{obs}} n_i(n_i-1)}{N\,(N-1)}$, S_{obs} is the number of observed MOTUs, n_i is the read number of the i^{th} MOTU, and N is the total number of reads. A greater InvSimpson value means higher biodiversity. Two-sample *t*-tests assuming unequal variance were conducted to compare InvSimpson indices between blade and seawater samples. With the existing inconsistency in classification, e.g., diatoms have been ranked both as a class [46] and as a phylum [47], we followed classifications accepted in work using similar molecular methods (e.g., [48]). Prokaryotic and eukaryotic MOTUs were re-organized into classes or phyla when possible, depending upon the group (e.g., [48]), to characterize higher-level community composition. MOTU matrices were fed into PC-Ord (v7.08) for non-metric, multidimensional scaling (NMS), where Bray-Curtis distances were calculated between samples on slow & thorough mode [49,50]. Indicator species analysis (ISA) then was conducted in PC-Ord (v7.08) using the Dufrêne & Legendre method [51] to identify the most consistently abundant MOTUs on blades and in seawater. Lastly, Mantel test, a non-parametric test that computes the significance of correlations between distance matrices, was used to assess associations of all possible pairs among the four groups: blade prokaryotes, blade eukaryotes, seawater prokaryotes, and seawater eukaryotes. A p value <0.01 indicates a significant result for all hypothesis testing except ISA, where 0.001 was used to focus on the most significant results. Lists of all indicator species using a p value <0.05 are provided in supplementary material (Suppl. Tables 2–5).

3. Results

3.1. Encrusting macroorganisms

Four animal species (rows 1–4, Table 1) and three macroalgal species (rows 5–7, Table 1) were observed on kelp surfaces, but only in the month of May (T3 - T5) and with low or sporadic counts. NGS did not detect DNA from any encrusting macroorganisms except *Ulva* sp. (sea lettuce). The singular sea lettuce MOTU was considered extremely rare (see Section 2.6.3 for definition), and was consequently removed from statistical analysis. This shows that our sampling procedure to remove macroorganisms from NGS analysis was effective. All 7 observed epibiotic macroorganisms are considered common in New England bays and waters [52,53], with Entoprocta (*Barentsia* spp.) possibly having been introduced to Long Island Sound [54]. Lacy bryozoan, the only animal species that has been reported to form encrustation that could reduce kelp blade strength [55], appeared at T4 with a single colony and remained rare at T5 with 6 colonies. The average percentage area covered by encrusting macroorganisms generally was low, with a slight increase from 0.01% at T3 to 0.57% at T5.

3.2. Microalgae observed using light microscopic techniques

Neither the cyanobacterium *Lyngbya* spp. nor the dinoflagellate *Prorocentrum lima* was found on kelp blades throughout the growth season. Most (>50% in abundance & > 80% in number of taxon) of the epiphytic microalgae were pennate diatoms common in benthic communities, including genera *Licmophora, Navicula,* and *Nitzschia.* Detection status and total cell counts of microalgae are provided in supplementary material (Suppl. Tables 6 and 7).

3.3. Vibrio, pathogenic Vibrio, and toxigenic microalgae

Presumptive *Vibrio* colonies developed on TCBS plates that had been inoculated with seawater and tip sections of blade samples that were collected on 5/4/2018 and thereafter (Table 2). All 16 presumptive

Table 1
Counts of encrusting macroorganisms on aquacultured sugar kelp in May and the average percentage area covered by encrusting macroorganisms.

Name	T3 (5/4)	T4 (5/17)	T5 (5/30)
Entoprocta (*Barentsia* spp.)	0	100	0
Amphipod (*Leptocheirus pinguis*)	0	0	3
Common slipper shell (*Crepidula fornicate*)	0	100	0
Lacy bryozoan (*Membranipora membranacea*)	0	1	6
Ectocarpus (*Ectocarpus siliculosus*)	18	26	15
Sea lettuce (*Ulva lactuca*)[a]	0	4	0
Red algae (*Champia parvula*)	25	0	5
Percent area covered by encrusting macroorganisms (± standard error)	0.01 ± 0.006	0.09 ± 0.09	0.57 ± 0.33

[a] Detected using NGS.

Table 2
Number of colony forming units (cfu) *Vibrio* on three sections of kelp blade (cfu cm^{-2}) and in the ambient seawater (cfu ml^{-1}). NS = no sample.

Sampling date	Tip (cfu cm^{-2})	Middle (cfu cm^{-2})	Bottom (cfu cm^{-2})	Water (cfu ml^{-1})
4/7/2018	0	0	0	NS
4/19/2018	0	0	0	NS
5/4/2018	0.332	0	0	6.66
5/17/2018	0.126	0	0	36.77
5/31/2018	0.094	0	0	2.20

Vibrio colonies were later confirmed to belong to Genus *Vibrio*, but none tested positive for either *V. parahaemolyticus* or *V. vulnificus* using the multiplex PCR targeting the *toxR* gene. Similar to TCBS isolation, no *Vibrio* MOTU was detected in any samples collected prior to 5/4 (Suppl. Table 8). A singular *Vibrio* MOTU was detected in 5/4 and 5/31 blade samples, but was absent in the 5/18 blade sample and all seawater samples. This likely reflected the extremely low abundance of *Vibrio* bacteria, both on blades and in seawater. In fact, the total read number of the *Vibrio* MOTU accounted for only 0.03% of the total read number of all MOTUs.

Among autotrophic MOTUs removed from the prokaryotic dataset, ~84% represented diatom chloroplast sequences, and the rest were chloroplast sequences from other microalgae and cyanobacteria sequences. Although it was possible that the 16S primer set could have been biased against cyanobacteria, no *Lyngbya* sequence was detected. One extremely rare eukaryotic MOTU (0.0004% of the total read number) representing a *Prorocentrum* sequence was detected only in 2 of the 3 seawater samples collected on 5/31 (data not shown), which subsequently was removed from further analysis.

3.4. Prokaryotic and eukaryotic microorganisms identified by next generation sequencing

A total of 239 prokaryotic MOTUs was categorized into 13 class (phylum)-level and 3 un-classified groups (Fig. 2). The relative abundance of the 16 groups, defined as the percentage of the read number of a class to the total prokaryotic read number averaged over triplicate samples, showed some site (blade vs. seawater) and temporal differences. This difference was most visible for Alphaproteobacteria, Gammaproteobacteria, Flavobacteriia, and Actinobacteria (Fig. 2). Among these, Gammaproteobacteria was the only taxon that had greater relative abundance on blades than in seawater throughout the sampling season (49% vs. 15%), although this group's dominance on blades generally decreased from T1 (82%) to T5 (27%). Alphaproteobacteria had the highest relative abundance in seawater (39%), but relative abundance in seawater and on blades became indistinguishable from T3 (early May) onward. Similarly, the difference in the relative abundance of Flavobacteriia in seawater and on blades decreased from T1 (20% vs. 4%) to T5 (20% vs. 19%). Actinobacteria was the only taxon that was consistently more abundant in seawater than on blades throughout the sampling season (5.6% vs. 0.2%).

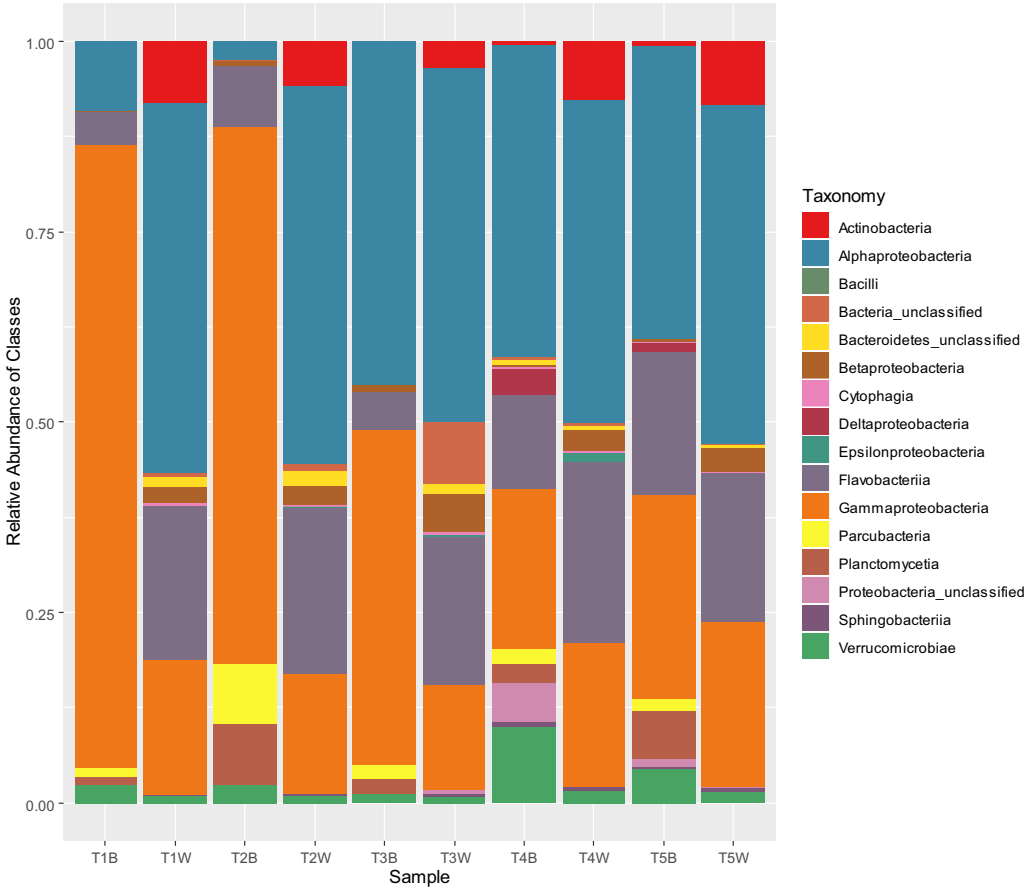

Fig. 2. Relative abundance of prokaryotic groups averaged over triplicates.

Planctomycetia, Parcubacteria, Deltaproteobacteria, and Bacilli were detected only on blades. Planctomycetia (4 MOTUs) and Deltaproteobacteria (6 MOTUs) were detected in all blade samples, with each contributing 3.9% and 0.9%, respectively, of the total read numbers averaged throughout the season. Parcubacteria and Bacilli, each represented by a singular MOTU, were detected on all and only one blade sample, respectively. Consequently, although Parcubacteria contributed 2.9% of the total read number throughout the sampling season, Bacilli contributed only 0.007% of the total read number. No bacterial taxon was detected in seawater only.

A total of 212 eukaryotic MOTUs was categorized into 26 groups generally considered as phylum-level and 2 unclassified groups (Fig. 3). Baccilliariophyta, Ciliophora, and Arthropoda had the highest relative abundance on blades (36%, 31%, and 19%); whereas, Dinoflagellata and Chlorophyta had the highest relative abundance in seawater (43% and 13%). The dominance of Bacillariophta on blades was most prominent early on (T1 - T3, 60% - 45%) when Ciliophora gradually increased in relative abundance before reaching a peak at T4 (83%). Arthropoda demonstrated the greatest difference in relative abundance between blades and seawater at T1 (29% vs. 0.2%) and T5 (46% vs. 4.7), and there was a less continuous temporal trend than for Bacillariophyta or Ciliophora. The difference in the relative abundance of Dinoflagellata in seawater and on blades was lowest at T3 (12% vs. 0.2) but was greater than 30% at other times. Relative abundance of Chlorophyta at T1 and T2 was below 3%, both in seawater and on blades. This remained true for blade samples for the rest of the season, but seawater samples showed an increase in Chlorophyta relative abundance at T3 (11%) before reaching a peak at T5 (39%).

Peronosporomycetes, Bryozoa, and Rotifera were detected on blades only. Peronosporomycetes (0.9%), also known as Oomycetes or water molds, are saprotrophs (organisms living on non-living organic matter) or parasites [56–58]. Bryozoa (0.3%) are suspension-feeding, colonial animals that live on surfaces including kelp, with tentacles that also can absorb dissolved organic compounds in the water [59]. Both bryozoan MOTUs belonged to the Order Ctenostomatida, a group with chitinous, gelatinous, or membrane-like exoskeletons [60]. Rotifera (0.08%) are effective grazers on marine microorganisms including bacteria, algae, protozoa, and particulate organic detritus. Unique seawater detections included marine phytoplankton Cryptophyta (4%), Chrysophyta (1%), Pelagophyta (0.4%), and MOCH-2 (0.008%) [61], as well as heterotrophic microorganisms such as Picozoa (0.2%), Choanoflagellida (0.04%), Katablepharid (0.04%), and Telonema (0.02%) [62–64].

3.5. Microbial communities on blades and in seawater

3.5.1. Prokaryotes – NMS ordination

The mean InvSimpson value was 13.7 for blade samples and 17.1 for seawater samples, and there was no statistically significant difference between the two ($p = 0.41$). Multivariate analysis using NMS identified a 1-dimensional solution (stress = 0.077) in which blade samples were

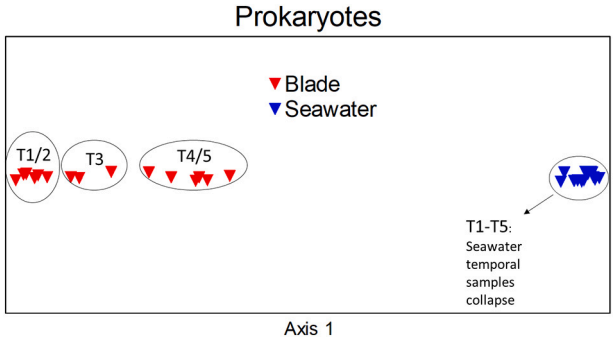

Fig. 4. Non-metric multidimensional scaling (NMS) of prokaryotes.

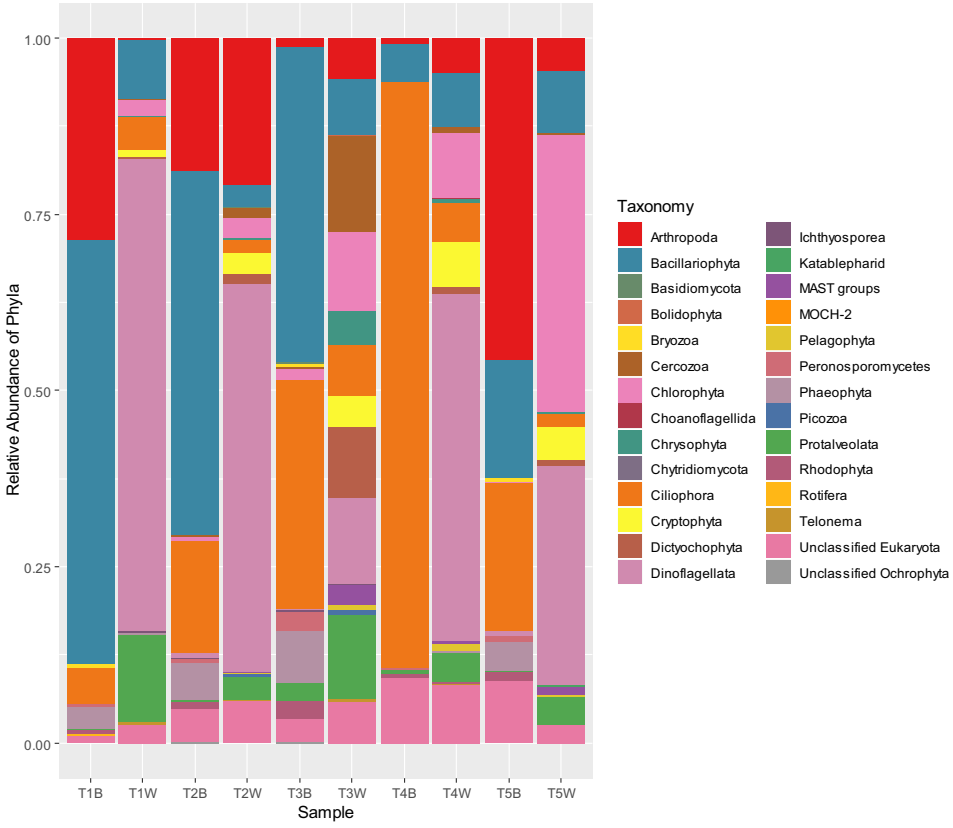

Fig. 3. Relative abundance of eukaryotic groups averaged over triplicates.

distinct from the seawater samples (Fig. 4). This ordination solution represented ~81% variation among all samples. In addition to the separation between blade and seawater samples, there was also some separation of blade samples collected at different times, but all seawater samples clustered together (Fig. 4), suggesting greater temporal dynamics of epibiotic prokaryotes than planktonic prokaryotes.

3.5.2. *Prokaryotes – indicator species analysis*

Indicator species analysis (ISA) identified 20 and 74 indicator MOTUs for blade and seawater samples, respectively, and these MOTUs were further categorized into 5 blade and 9 seawater indicator groups (Fig. 5). While more than 50% of blade indicator MOTUs were Gammaproteobacteria (Fig. 5, upper), Gammaproteobacteria, Alphaproteobacteria, and Flavobacteriia each accounted for ~25% of seawater indicator MOTUs (Fig. 5, lower). The nature of ISA dictates that blade indicator MOTUs are different from seawater indicator MOTUs, even when they belong to the same high-level taxon (a bacterium cannot be more abundant both on blades and in seawater). Blade indicator Gammaproteobacteria included *Marinobacter*, a genus with many members able to degrade hydrocarbons [65,66]; *Psychromonas*, a psychrophilic or psychrotolerant genus with some members being aerotolerant/anaerobic [67], some being agarolytic (breaking down agar, a polysaccharide) [68], some being piezophilic [69], and some biofilm-forming [70]; *Arenicella*, a genus with two species isolated from marine sediment and sea urchin tissue [71,72]; *Alcanivorax*, a group of hydrocarbon-degrading marine bacteria that was once suggested to be obligate oil-degrading bacteria [73] until recently [74]; *Saccharospirillum*, bacteria isolated from various marine surfaces such as halophyte sea purslane [75]; and unclassified Vibrionaceae. Seawater indicator Gammaproteobacteria included only a few unclassified MOTUs and *Reinekea*, a group of bacteria associated with both seawater and marine sediments [76,77]. Blade indicator Alphaproteobacteria included *Planktomarina*, a genus with only one species isolated from the Wadden Sea [78]; *Litorimonas*, a genus with one species isolated from a sandy beach [79]; and two other species isolated from green macroalga *Cladophora stimpsoni* and sponge [80,81]. Seawater indicator Alphaproteobacteria included 4 genera in the family Rhodobacteraceae and the Genus *Pelagibacter,* a group of highly abundant marine heterotrophic bacteria. Of the 4 Rhodobacteraceae genera, *Amylibacter* was first isolated from surface seawater [82], *Loktanella* has been isolated from both freshwater and marine ecosystems [83], *Octadecabacter* is a member of ubiquitous marine Roseobacter clade [84], and *Sulfitobacter* has numerous representatives isolated from both seawater [85–87] and from macroorganisms such as starfish, sea grass, corals, and the red macroalga *Pyropia yezoensis* (nori) [88–90]. Blade indicator Flavobacteriia included two genera, *Aquimarina* and *Muricauda*. *Aquimarina* is a genus with many agarolytic and chitin-degrading strains [91,92] that cause diseases in marine eukaryotes, such as bleaching of red macroalga *Pyropia yezoensis* (nori) when *Aquimarina* relative abundance reached 9.3% [93]. Association of *Aquimarina* with brown algae was only recently discovered (unspecified brown algae) [94] but their agarolytic activities towards brown algae are unknown. Species in the Genus *Muricauda* are mesophilic and neutrophilic bacteria found in both seawater and sediments that are capable of growing on a range of carbohydrates and some amino acids [95]. Seawater indicator Flavobacteriia included Genera *Crocinitomix* and *Salinirepens* from a poorly-defined Family Cryomorphaceae with few cultivated taxa [96], *Polaribacter*, a genus with representatives isolated from both seawater and macroalgae but especially red macroalgae [97–101], and *Tenacibaculum*, a genus with many opportunistic pathogens of finfish [102] and Pacific oysters [103]. Blade indicator Verrucomicrobia included an unclassified MOTU and *Rubritalea*, a genus with many species isolated from sponges and sea squirts [104,105]; whereas, seawater indicator Verrucomicrobia had only one MOTU representing *Roseibacillus*, a genus established in 2008 with three species found in marine environments [106].

Betaproteobacteria, Epsilonproteobacteria, Actinobacteria, Cytophagia, and Sphingobacteriia were indicator groups unique to seawater samples. The only indicator group unique to blade samples was Parcubacteria (candidate phylum Parcubacteria) represented by a singular MOTU. None of Parcubacteria species has been isolated in the laboratory, and almost all Parcubacteria sequences have been recovered from anoxic environments [107] except a recent study in which partial Parcubacteria genomes were reconstructed from oxic groundwater samples [108]. Evidence of Parcubacteria's ability to respire was not definitive as only 3 of the 8 reconstructed genomes contained genes associated with the ability to use O_2 as a terminal electron acceptor [108]. This study by Nelson & Stegen confirmed that the reduced genomes of Parcubacteria often were linked to a fermentation-based lifestyle and the absence of biosynthetic pathways, all pointing to very specialized life styles of

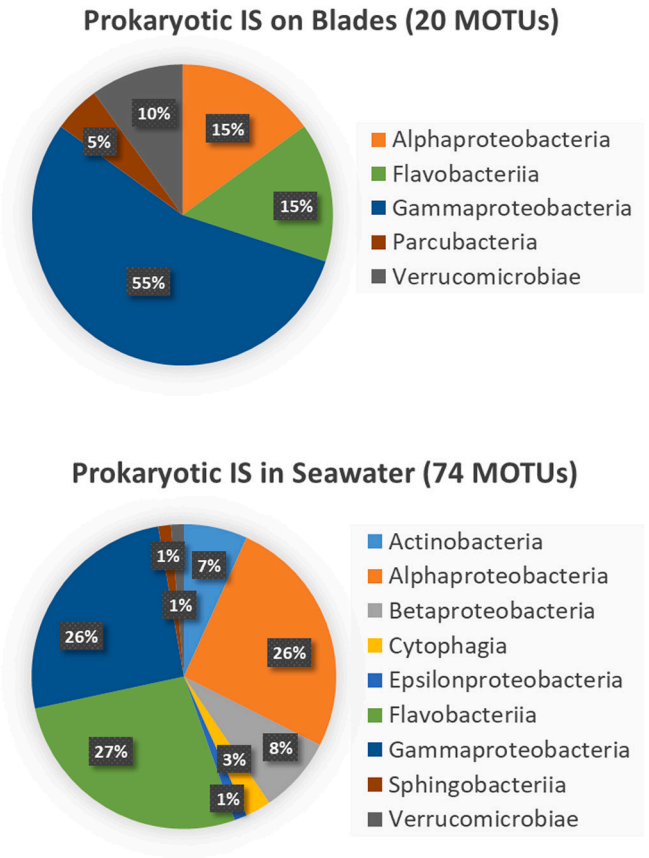

Fig. 5. Composition of indicator prokaryotes on kelp blades (upper) and in seawater (lower).

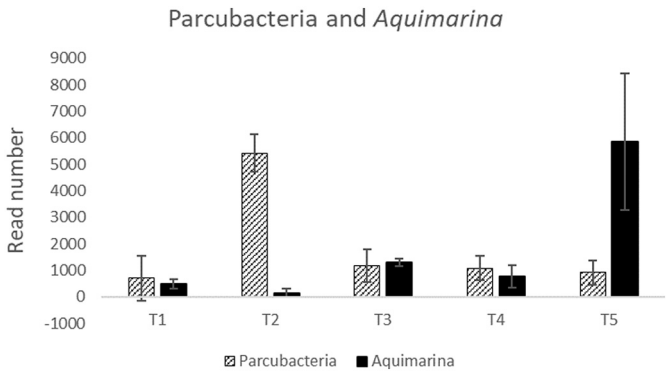

Fig. 6. Read numbers of Parcubacteria and *Aquimarina* throughout the sampling season. Error bar represents standard deviation.

symbiotic, commensal, and parasitic organisms.

3.5.3. Prokaryotes – temporal analysis of potential bacterial pathogens of sugar kelp

With little known about sugar kelp pathogens, we consider blade indicator species (enriched on blades) with demonstrated pathogenicity to other macroalgae as potential pathogens for sugar kelp. Indicator species Parcubacteria (singular MOTU) and *Aquimarina* (2 MOTUs) meet such criteria. The former is potentially pathogenic, based upon genomic information [108], and the latter has been found to be pathogenic to nori [93]. A closer examination of read numbers at different times showed different temporal patterns of the two groups (Fig. 6). Parcubacteria had highest read number of 5426 at T2, but were much less abundant at other times. *Aquimarina*, however, had lower read numbers from T1 to T4 before reaching a peak read number of 5859 at T5.

3.5.4. Eukaryotes – NMS ordination

The mean InvSimpson value of blade samples, 6.4, was significantly smaller than that of seawater samples, 15.6 ($p = 0.001$). Multivariate analysis using NMS identified a 2-D solution (stress = 0.079) wherein blade samples were distinct from seawater samples along axis 1. Similar to prokaryotes, this axis represented the majority of the variation among all samples, 76% in this case. Unlike prokaryotes, an additional axis 2 represented 15% of the variation among all samples, wherein both blade and seawater samples showed temporal changes, with blade samples showing greater variation (Fig. 7).

3.5.5. Eukaryotes – indicator species analysis

Indicator species analysis (ISA) identified 25 and 54 indicator MOTUs for blade and seawater samples, respectively, and these MOTUs were further categorized into 7 blade and 13 seawater indicator groups (Fig. 8). The most MOTU-rich blade indicator group, Bacillariophyta, accounted for 55% of blade indicator MOTUs, and the rest of the blade indicator phyla contributed between 4% and 12% of blade indicator MOTUs (Fig. 8, upper). The most MOTU-rich seawater indicator group, Dinoflagellata, accounted for ~25% of seawater indicator MOTUs, and the rest of the seawater indicator groups contributed between 2% and 10% of the seawater indicator MOTUs (Fig. 8, lower). Bacillariophyta and Ciliophora are two groups that had both blade indicator species and seawater indicator species. Of the 14 blade indicator MOTUs that were Bacillariophyta, 5 were unclassified and the remaining 9 were all benthic diatoms, with three representing *Navicula*, two representing *Nitzschia*, and one each representing *Cylindrotheca*, *Licmophora*, *Pleurosigma*, and *Tabularia*. Seawater indicator MOTUs that were Bacillariophyta included one each from the genera *Brockmanniella*, *Guinardia*, *Leptocylindrus*, *Skeletonema*, and *Thalassiosira*, all planktonic species. As for Ciliophora, two blade indicator MOTUs belonged to the group Conthreep, with one further classified as Phyllopharyngea. Three seawater indicator MOTUs belonged to the group Oligotrichia, with two further classified as *Spirotontonia* and *Strombidium*. Generally, the blade-indicator ciliates, Phyllopharyngea, include many species that are morphologically specialized benthic species [109]; whereas, seawater indicator ciliates, *Spirotontonia* and *Strombidium*, are mostly planktonic [110].

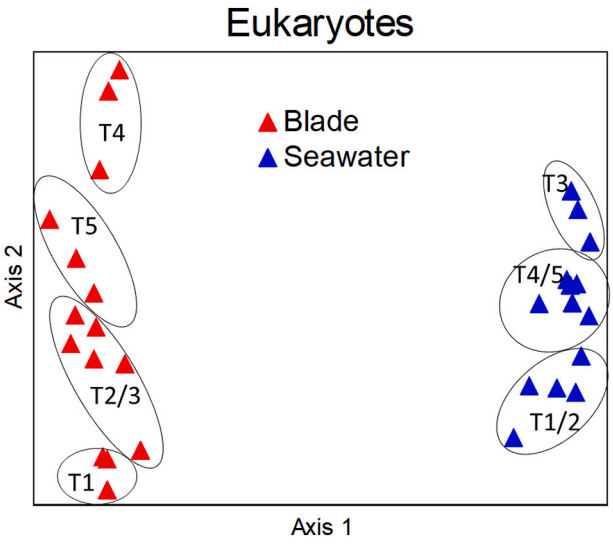

Fig. 7. Non-metric multidimensional scaling (NMS) of eukaryotes.

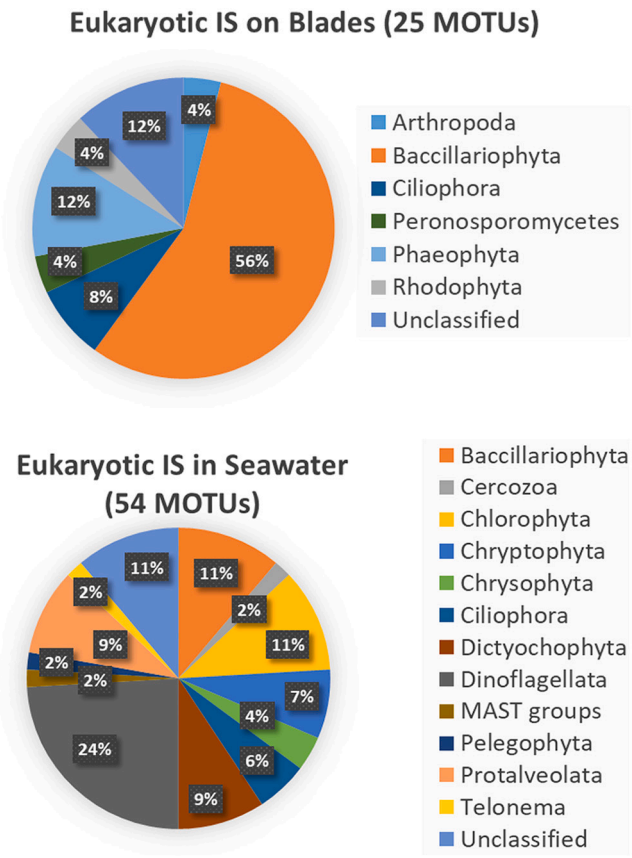

Fig. 8. Composition of indicator eukaryotes on kelp blades (upper) and in seawater (lower).

Arthropoda, Peronosporomycetes, Phaeophyta, and Rhodophyta were indicator groups unique to blade samples. Indicator Arthropoda comprised a singular MOTU representing an unclassified Maxillopoda. Indicator Peronosporomycetes (Oomycetes or water molds), also comprising a singular MOTU, are fungus-like saprotrophs, yet taxanomically Stramenopiles. Peronosporomycetes were detected only on blades, and these microorganisms are known pathogens of commercially important red macroalga *Pyropia yezoensis* (nori) and giant kelp *Macrocystis pyrifera* [56,58]. Indicator Phaeophyta (brown algae) and indicator Rhodophyta (red algae) were not classified to genus levels. Among these, two Phaeophyta MOTUs belonged to the Order Ectocarpales, which includes many filamentous brown algae. Indicator eukaryotic microorganisms unique to seawater were composed mainly of planktonic microalgae including Chlorophyta (*Micromonas* and *Mamiella*), Chryptophyta (*Rhodomonas* and *Teleaulax*), Chrysophyta (uncultured), Dictyochophyta (*Dictyocha* and Order Pedinellales), Dinoflagellata (*Gyrodinium*, *Gymnodinium*, and *Heterocapasa*), Pelagophyta (Order Sarcinochrysidales), and Protalveolata (Order Syndiniales). Lastly, Cercozoa [111], *Telonema*, and MAST (MArine STramenopiles) groups, each represented by a single MOTU, are abundant grazers feeding on

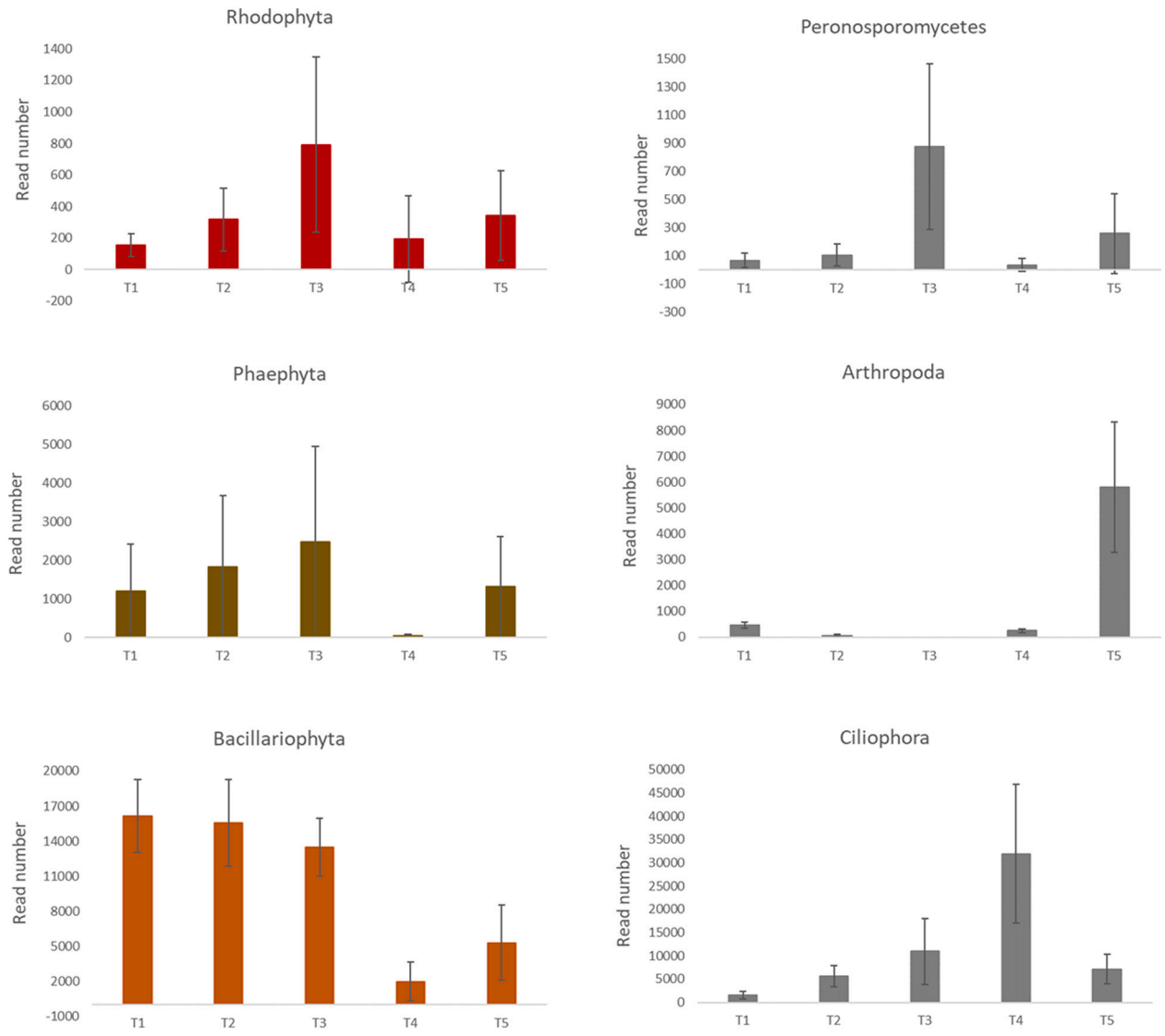

Fig. 9. Read numbers of blade indicator epiphytes (Rhodophyta, Phaeophyta, and Bacillariophyta), a potential pathogen (Peronosporomycetes), and grazers (Arthropoda and Ciliophora) throughout the sampling season. Error bar represents standard deviation.

bacteria and picophytoplankton [61,112].

3.5.6. Eukaryotes – temporal analysis of epiphytes, a potential kelp pathogen, and micro-grazers associated with sugar kelp

Rhodophyta and Phaeophyta (Order Ectocarpales) were unique blade indicator groups, and they were likely epiphytes with holdfasts attaching to the sugar kelp that were too small to be noticed by visual inspection. The red and brown epiphytes had similar temporal trends in that they demonstrated gradual increases in read numbers from T1 to T3, a significant dip at T4 (mid-May), and a recovery at T5 (Fig. 9). The other autotrophic blade indicator group, Bacillariophyta, had a similar dip at T4 and a recovery at T5 as Rhodophyta and Phaeophyta, but unlike Rhodophyta and Phaeophyta, diatom read numbers from T1 to T3 were already high despite a slight decreasing trend (16,173 to 13,494). Interestingly, the blade indicator Peronosporomycetes, a potential kelp pathogen, followed almost the same temporal trend as Rhodophyta and Phaeophyta. Meanwhile, the blade indicator grazer group, Ciliophora, experienced a gradual increase in its read number from T1 to T4, with a significant peak value of 31,925 at T4 when read numbers of all indicator epiphytes and Peronosporomycetes were at their lowest. At T5, while the read number of indicator ciliates dropped, the read number of blade indicator Arthropoda had a dramatic increase to 5813.

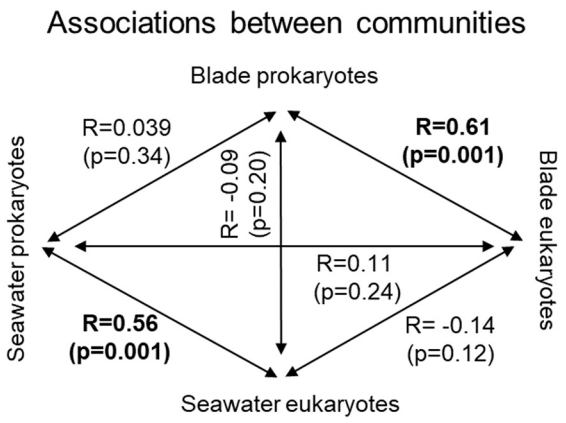

Fig. 10. Mantel test associations of all possible pairs among 4 communities – blade prokaryotes, blade eukaryotes, seawater prokaryotes, and seawater eukaryotes.

3.5.7. Prokaryote-eukaryote associations

Mantel tests using class matrices for prokaryotes and phylum matrices for eukaryotes revealed two significant positive associations, one between prokaryotes and eukaryotes on blades and the other between prokaryotes and eukaryotes in seawater (Fig. 10). No significant associations were found between blade and seawater microbial communities.

4. Discussion

Epibionts play an important role in maintaining the safety and quality of aquacultured sugar kelp as a consumer product. We used *Vibrio*-specific TCBS selection as well as PCR assays, light microscopy, and NGS to look closely for microorganisms that are of a priori concern to kelp human consumption. To gain deeper insights into interactions between microorganisms and the kelp host, ISA was applied to high-resolution datasets generated by NGS. Through ISA, we identified potential pathogens to sugar kelp, highlighted a possible new application of sugar kelp in ecosystem restoration, and outlined important epibiotic trophic interactions though temporal analysis of blade indicator species. Combining observations of both micro- and macro- epibiotic organisms, we recommend some best industrial practices and monitoring efforts. We conclude by comparing aspects of prokaryotic and eukaryotic epibiotic communities, which sheds light on the tight interactions between sugar kelp and epibiotic microorganisms.

4.1. Microorganisms of a priori concern to aquacultured sugar kelp – pathogenic Vibrio and toxigenic microalgae

Vibrio bacteria are natural inhabitants of seawater. Some strains, such as *V. vulnificus* and *V. parahaemolyticus*, however, could infect humans through open wounds or raw shellfish consumption, which leads to septicemia and death in severe cases. The initial isolation of *Vibrio* using TCBS has to be followed by confirmatory immunological or molecular tests to detect pathogenic *Vibrio,* because TCBS also enriches non-pathogenic *Vibrio* and other Gammaproteobacteria such as *Aeromonas*, *Pseudomonas*, and *Plesiomonas* [113,114]. NGS read numbers of the Genus *Vibrio* provided culture-independent, numerical insight into the onset and development of *Vibrio* abundance on aquacultured sugar kelp, and it agreed with TCBS culturing in that *Vibrio* detection started in May but abundance was low throughout May. Using a qPCR assay, Barberi and co-workers discovered the association between the highest detection of *V. alginolyticus* on kelp blades with the lowest detection of *V. alginolyticus* in seawater [30]. It is possible that water circulation conditions on farm sites and the bacterial composition in the ambient seawater, both in terms of *Vibrio* species present and other bacteria in the community that can facilitate or inhibit *Vibrio* colonization on kelp, will affect kelp-*Vibrio* interactions.

The epibenthic nature of toxigenic *Lyngbya* spp. and *Prorocentrum lima* led us to consider these as possible kelp epibionts with human health concern. We did not observe any *Lyngbya* spp. or *Prorocentrum lima* cells under microscope. No *Lyngbya* sequence was detected using NGS throughout the kelp growth season. It is possible that the relatively open and rocky environment at our study site is not conducive to growth of *Lyngbya*, which tends to inhabit salt marshes. Alternatively, microalgal composition may have significantly changed since earlier detections of *Lyngbya* in Long Island Sound [23,24]. The extremely low detection of a *Prorocentrum* sequence was from seawater samples collected on the last sampling day. This shows that not only was the detected *Prorocentrum* rare, but also it may not be the toxigenic *P. lima*. In fact, the only microalgal group positively associated with sugar kelp was the benthic diatoms, and no harmful species were among those detected. Our study suggests that toxigenic microalgae did not pose a safety problem for aquacultured sugar kelp to be used for direct consumption during our study.

4.2. Microorganisms positively associated with aquacultured sugar kelp revealed by indicator species analysis (ISA)

ISA on NGS data proved to be a powerful tool to identify important microoganisms driving the differences between blade and seawater samples. Such identification is not often straightforward from data presentation at higher taxonomic levels. Class Flavobacteriia, for example, had on average lower relative abundance on blades than in seawater (Fig. 2); however, ISA discovered two blade indicator Flavobacteriia MOTUs, both representing potential kelp pathogen *Aquimarina*. Similarly, even though Rotifera was detected only on blades while Arthropoda was detected on both blades and in seawater (Fig. 3), Arthropoda, but not Rotifera, was identified as a blade indicator eukaryote, because Rotifera was detected at only 2 of 5 sampling times, and at very low abundance. It is also noteworthy that, even though Bacillariophyta were abundant on kelp blades (Fig. 3), it was only through ISA that we achieved a clear understanding of the association between kelp blades and benthic diatoms. Not only did ISA identify ecological relationships that made sense, but also it helped us gain new insights into the ecophysiology of kelp-associated microbes that otherwise are difficult to study (see below).

Prokaryotic seawater indicator species included bacteria well established as plankters, such as *Pelagibacter*. In contrast, prokaryotic blade indicator species identified in this study were composed mainly of epibiotic bacteria. Some are specialized metabolizers, such as *Alcanivorax* and *Marinobacter* using hydrocarbons, as well as *Psychromonas* and *Aquimarina* using polysaccharides; some are commonly found on halophytes such as *Saccharospirillum* and *Litorimonas*; some could be pathogenic, such as a newly defined phylum Parcubacteria with a small genome that is compatible with a symbiotic/pathogenic lifestyle [108] and *Aquimarina*, a known pathogen to red macroalgae including *Pyropia yezoensis* (nori) [91,92,115]. The once-considered obligate oil-degrading blade indicator Gammaproteobacteria *Alcanivorax* and *Marinobacter* [73] have been found recently to survive on a variety of non-hydrocarbon substrates [74]. Both *Alcanivorax* and *Marinobacter* are considered to play important roles in oil-remediation in marine environments [116]. Our finding of these two bacteria as blade indicator species supports the notion that *Alcanivorax* and *Marinobacter* may be more versatile than obligate hydrocarbon degraders, and it also points to a new potential for aquacultured sugar kelp to be used as inoculum in oil contaminated marine environments. Follow-up bioremediation research is needed to verify the practicality. Whether or not Parcubacteria and *Aquimarina* could infect sugar kelp warrants further investigation. Laboratory experiments should be conducted to examine the potential pathogenicity of these bacteria to sugar kelp, both in the gametophyte stage (i.e., Is the increased abundance of *Aquimarina* a result of hatchery conditions?) and in the sporophyte stage (i.e., Do sugar kelp enrich *Aquimarina* from the seawater on culture ropes?).

Another unique ecophysiological feature observed only among blade indicator bacteria is tolerance of anoxic condition. Some *Psychromonas* species are aerotolerant anaerobic organisms [67], and almost all Parcubacteria sequences have been recovered from anoxic environments [107]. These findings suggest the presence of anoxic pockets hosting anaerobic bacteria on sugar kelp, and caution against simply dropping sugar kelp to the ocean floor to achieve permanent carbon burial. After reaching the sea floor, a majority of the organic carbon fixed by aquacultured sugar kelp will likely be degraded first by aerobic bacteria. Once the local dissolved oxygen is depleted, aerotolerant and anaerobic bacteria could continue degrading organic carbon that is more readily degraded under anoxic conditions. As a result, not only will CO_2 be released from oxic and anoxic organic carbon degradation, but also the native benthic communities could be compromised by locally anoxic conditions.

Macroalgae have been shown to possess antimicrobial capacity to prevent biofilm accumulation on thallus surfaces [117,118], but macroalgae are also hosts to beneficial microorganisms that help strengthen

host disease resistance [119]. ISA revealed that, even though many bacteria were enriched on sugar kelp compared to seawater, only a very small proportion of them are potentially pathogenic to sugar kelp. One possible example of evidence for sugar kelp's antimicrobial capacity for self-defense comes from our findings with *Tenacibaculum* (Class Flavobacteriia). Even though some *Tenacibaculum* are agarolytic and have been isolated from *Pyropia yezoensis* (nori) [120], this genus was found to be a seawater, rather than a blade, indicator species. This means aquacultured sugar kelp kept the abundance of *Tenacibaculum* on blades low despite high abundance in seawater throughout the kelp growing season. The strong positive and negative associations between aquacultured sugar kelp and bacteria revealed in this study (Suppl. Tables 2–5) are useful to future studies, such as developing sugar kelp probiotics and prospecting for antimicrobial chemicals.

For eukaryotes, the distinction between blade indicator species and seawater indicator species reflected strongly benthic or planktonic lifestyles. All 9 blade indicator diatoms classified to genus level were benthic taxa; whereas, 5 seawater indicator diatoms classified to genus level were characteristically planktonic. The same benthic vs. planktonic distinction also was observed for indicator ciliates. Moreover, all seawater indicator microalgae covering 7 phyla were mostly planktonic species. All blade indicator microalgae were from a single phylum, Bacillariophyta, and more than half of blade indicator MOTUs belonged to Bacillariophyta. This strong association between aquacultured sugar kelp and benthic diatoms may be caused by the dominance of pennate diatoms in the benthic microalgal community [121], which could have served as a "seeding" pool for kelp blades.

Considering potential eukaryotic kelp pathogens, it was estimated that an average of 10%, and regionally as high as 20%–60%, of annual production of nori culture, the most valuable seaweed aquaculture industry, was lost to Peronosporomycetes pathogens [122], a potential kelp pathogen identified in this study. There is little known about Peronosporomycetes, and the only effective treatment of diseased nori has been to acid-wash the crop [122]. Our study shows that aquacultured sugar kelp supports Peronosporomycetes growth, and further studies using higher resolution primers for this group such as ITS primers [123] should be conducted to characterize the strains associated with aquacultured sugar kelp. Meanwhile, lab experiments on the potential pathogenicity of *Pythium porphyrae* and *Olpidiopsis* sp. on sugar kelp may be worthwhile, because these two Peronosporomycetes are the main species causing disease in nori [56,124]. *Anisolpidium ectocarpii* is another Peronosporomycetes that may be worth follow up because it has been shown to infect giant kelp *Macrocystis pyrifera*, a brown alga [58].

4.3. Temporal patterns of epibiotic organisms

Snail grazing and lacy bryozoan encrustation have been associated with lesser ability of macroalgae to withstand wave forces and increased frond breakage [55]. No kelp-grazing snails such as *Lacuna vincta* were observed during the present study. Even though lacy bryozoan started appearing mid-May, abundance remained low throughout and did not pose a threat to frond integrity. NGS also detected bryozoan Order Ctenostomatida, but the detection level was too low to be concerning (0.3%), not to mention there hasn't been any report suggesting Ctenostomatida affects kelp growth.

Temporal changes in microorganisms related to sugar kelp quality and safety revealed highly dynamic microbial communities. For prokaryotes, the peaks in abundance for Parcubacteria and *Aquimarina*, both potential kelp pathogens, did not overlap (Fig. 6), which could be caused by different temperature preferences, different grazer groups, or varying dissolved oxygen (DO), assuming that Parcubacteria prefer low DO and there were local sub-oxic pockets on kelp blades at times. Regardless, there does not seem to be synchronization in growth of Parcubacteria and *Aquimarina*.

For eukaryotes, temporal patterns in blade indicator groups revealed interesting trophic dynamics (Fig. 9). Abundance of benthic diatoms slowly decreased throughout April, which aligns with diatoms as spring bloom species. Meanwhile, epibiotic red algae and brown algae experienced steady increases in abundance. Yet, none of these epiphytes caused appreciable biofouling on kelp blades (confirmed by visual inspection), which was likely attributable to ciliate grazing, especially by the Class Phyllopharyngea. In fact, ciliates had a consistent increase in abundance from April to mid-May, probably benefiting from the higher availability of prey, including bacteria, diatoms, and other epiphytes, as temperature and day length increased and sugar kelp fixed more organic carbon to fuel the epibiotic ecosystem. The potential pathogen Peronosporomycetes also seemed to have experienced grazing from ciliates as it followed a temporal trend similar to that of blade indicator epiphytes. The drastic increase in Arthropoda abundance at the end of May could reflect a transition of dominant grazers from ciliates to arthropods (Class Maxillopoda) on kelp blades.

It is possible that some of the NGS-detected microorganisms were from macroscopic epiphytes. Considering the lack of detection of macroscopic epiphytes by visual inspection, this fraction should be considered small.

4.4. Suggestions for best industrial practices and monitoring efforts

Encrusting colonies feed more quickly and grow faster in gentle than in strong currents [125,126]. Therefore, kelp grown at sites with intense water circulation and steady currents will less likely suffer from severe encrustation in May when kelp accelerates biomass accumulation. Some growth, both in terms of rate and C/N ratio, however, may be compromised at high wave exposure levels [126]. Hence, growth rates, nutrient content, and encrustation of aquacultured sugar kelp should all be considered when deciding the suitability of a location as a farm site. Our study in a relatively open area at the eastern end of Long Island Sound demonstrates that aquacultured sugar kelp could reach an average length of 1.3 m (4 ft) without concerning levels of encrustation or kelp grazers. Beyond maintaining a pristine look for marketability, aquacultured sugar kelp also remained free from epiphytic biofouling and potential microbial pathogens throughout May, likely at least partially attributable to persistent grazing from ciliates April – May, and probably from arthropods starting early in June. In summary, the physical and biological conditions at the eastern end of Long Island Sound are conducive to aquacultured sugar kelp maintaining self-cleaning capacity. Harvesting at different times for different usage of kelp could further guarantee product quality. Harvesting by June could assure the quality and safety of the crop, if the intended use is direct human consumption.

Using NGS and ISA, we identified microorganisms enriched on aquacultured sugar kelp and make suggestions on what microorganisms to monitor, considering safety concerns both for human consumption and for kelp health. In CT, where the study was conducted, Gammaproteobacteria *Vibrio*, *Salmonella*, *E.coli* O157:H7 and *Shigella* [29] are considered bacterial pathogens of concern. ISA, indeed, discovered more than 50% of blade indicator MOTUs to be Gammaproteobacteria. None of the blade indicator Gammaproteobacteria, however, were pathogenic to humans or sugar kelp. Furthermore, all CT bacterial pathogens of concern were absent from the NGS dataset except *Vibrio*, but this genus was present only at extremely low abundance. Although not human pathogens, *Aquimarina* and Parcubacteria (prokaryotes), as well as Peronosporomycetes (eukaryotes), had significantly higher growth on kelp blades than in seawater and could potentially cause disease in sugar kelp, based on putative pathogenicity [108], established pathogenicity towards other macroalgae [58,92,93], and recent detection on brown algae [94]. Therefore, natural occurrence, seasonal variation, and effects of these microorganisms on sugar kelp should be further investigated.

NGS did not capture sequences from toxigenic microalgae with read numbers high enough to be considered non-noise, in seawater or on blades. Furthermore, ISA identified benthic diatoms to be the only kind

of microalgae enriched on kelp blades. Accordingly, future farm site selection and monitoring efforts could benefit most from a better understanding of the benthic communities, especially diatoms. Additionally, Cyanobacteria- and Dinoflagellata-specific molecular assays could be employed to better focus on *Lyngbya* and *Prorocentrum* detection if there is a history of outbreak events caused by these species. In the context of climate change, tropical and sub-tropical benthic toxigenic species may expand beyond their current geographic range; therefore, it would be beneficial for aquaculture practitioners to perform benthic and planktonic microbial community surveys with a frequency that is practical and also relevant to climate change.

4.5. Aquacultured sugar kelp hosts distinct, dynamic, and interactive microbial communities

'Everything is everywhere: but the environment selects' [127] is a well-accepted notion in microbial biogeography. Multiple discoveries made in this study suggest that sugar kelp was more selective than seawater for microorganisms, possibly more so for prokaryotes than for eukaryotes. The clearest evidence was from ISA, wherein the ratio of blade/seawater indicator MOTUs for prokaryotes and eukaryotes was 0.27 and 0.46, respectively (see 4.2). This did not mean, however, that the prokaryotic community on kelp blades was less diverse or less dynamic, which brings us to the second and third lines of evidence to appreciate the more nuanced detail of how sugar kelp shaped its epibiotic microbial communities.

Judging by a general biodiversity index such as InvSimpson, it may seem that blades and seawater were equally selective towards prokaryotes (see Section 3.5.1). The ratios of blade/seawater indicator MOTUs for prokaryotes and eukaryotes (0.27 vs. 0.46), however, suggested that blades may have shaped the prokaryotic community more strongly in the sense that only a smaller proportion of prokaryotes (compared to eukaryotes) established high abundance on blades than in seawater. In other words, the blade habitat was suitable for a relatively small subset of bacteria to thrive and become indicator species while still allowing diverse bacteria to be present so that the InvSimpson was comparable to that in seawater.

The third line of evidence that sheds light on how kelp blades shaped the epibiotic microbial community is from the temporal structure revealed by NMS (Figs. 4 & 7). For both prokaryotes and eukaryotes, but more so for prokaryotes, the temporal variation on blades was greater than that in seawater. This means that kelp blades supported microorganisms, and especially prokaryotes, whose abundances changed more with time than those in seawater. The greater temporal dynamics of bacterial communities could be driven by temporal changes in "food" (various organic materials provided by kelp) availability and in chemicals released from kelp that may affect bacterial growth.

Lastly, Mantel tests revealed two significant associations between prokaryotes and eukaryotes, one on blades and the other in seawater, but none between any groups across blades and seawater. This shows that not only were epibiotic and planktonic microbial communities distinct from each other throughout the kelp growth season (Figs. 4 & 7), but also strong microbial associations were limited to within each habitat (blade vs. seawater). This reinforces that aquacultured sugar kelp develops unique epibiotic communities and hosts various microorganisms closely interacting with each other.

5. Conclusions

There was clear separation between epibiotic and planktonic microbial communities, for both prokaryotes and eukaryotes. Aquacultured sugar kelp hosted distinct, diverse, and dynamic epibiotic communities, more so for prokaryotes than for eukaryotes, which may be a reflection of attachment surfaces and various resources made available by sugar kelp for bacterial growth.

Neither *Vibrio* nor microalgae detected in this study (through microscopy, TCBS isolation, PCR confirmatory assay, and microbial DNA metabarcoding) are pathogenic or toxigenic based on what is known about them so far. It would be beneficial for the kelp industry to continue monitoring these microorganisms and other potential tropical and subtropical epibiotic species, in the context of climate change and possible spatial and temporal expansions of potential human pathogens.

No kelp grazers and only very low counts of encrusting lacy bryozoan were observed starting in late May. Therefore, aquacultured sugar kelp did not show any sign of safety or quality compromise caused by macroscopic epibionts in eastern Long Island Sound as late as the end of May.

Indicator species analysis (ISA) revealed that Gammaproteobacteria were enriched on aquacultured sugar kelp, but there were no human or kelp pathogens among them. Instead, two Gammaproteobacterial indicator genera, *Alcanivorax* and *Marinobacter,* known to play important roles in oil-remediation in marine environments, dominated. *Aquimarina*, Parcubacteria, and Peronosporomycetes had significantly enhanced presence on sugar kelp compared to seawater. Because these taxa can be pathogenic to other macroalgae, we suggest that these microorganisms be investigated as candidate pathogens of sugar kelp. Ciliates may be the most important contributor to maintaining sugar kelp self-cleaning capacity by grazing down epibiotic bacteria and algae.

CRediT authorship contribution statement

Yuan Liu: Conceptualization, Formal analysis, Investigation, Data curation, Writing - Original Draft, Review & Editing, Visualization, Funding acquisition.

Gary H. Wikfors: Conceptualization, Writing - Review & Editing, Funding acquisition.

Paul Clark: Investigation.

Stephen Pitchford: Investigation.

Melissa Krisak: Investigation.

Mark S. Dixon: Investigation.

Yaqin Li: Conceptualization, Validation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2022.102654.

References

[1] FAO, The Global Status of Seaweed Production, Trade and Utilization, Globalfish Research Program, Rome, Italy, 2016, 120pp.
[2] K. Nisizawa, Preparation and Marketing of Seaweeds as Foods, 1987.
[3] M.H. Norziah, C.Y. Ching, Nutritional composition of edible seaweed gracilaria changgi, Food Chem. 68 (2000) 69–76.
[4] S.L. Holdt, S. Kraan, Bioactive compounds in seaweed: functional food applications and legislation, J. Appl. Phycol. 23 (2011) 543–597.

[5] S.B. Bricker, R.E. Grizzle, P. Trowbridge, J.M. Rose, J.G. Ferreira, K. Wellman, C. Zhu, E. Galimany, G.H. Wikfors, C. Saurel, R.Landeck Miller, J. Wands, R. Rheault, J. Steinberg, A.P. Jacob, E.D. Davenport, S. Ayvazian, M. Chintala, M. A. Tedesco, Bioextractive removal of nitrogen by oysters in Great Bay Piscataqua river estuary, New Hampshire, USA, Estuaries and Coasts 43 (2020) 23–38.

[6] J.K. Kim, G.P. Kraemer, C. Yarish, Use of sugar kelp aquaculture in Long Island sound and the Bronx River estuary for nutrient extraction, Mar. Ecol. Prog. Ser. 531 (2015) 155–166.

[7] J.M. Rose, S.B. Bricker, M.A. Tedesco, G.H. Wikfors, A role for shellfish aquaculture in coastal nitrogen management, Environmental Science & Technology 48 (2014) 2519–2525.

[8] M.A. Carras, D. Knowler, C.M. Pearce, A. Hamer, T. Chopin, T. Weaire, A discounted cash-flow analysis of salmon monoculture and integrated multi-trophic aquaculture in eastern Canada, Aquac. Econ. Manag. 24 (2020) 43–63.

[9] M.D. Chambers, in: Research and development of steelhead trout Oncorhynchus mykiss aquaculture in sea cages, 2013, pp. 1–158.

[10] J. Fossberg, S. Forbord, O.J. Broch, A.M. Malzahn, H. Jansen, A. Handå, H. Førde, M. Bergvik, A.L. Fleddum, J. Skjermo, Y. Olsen, The potential for upscaling kelp (Saccharina latissima) cultivation in Salmon-driven integrated multi-trophic aquaculture (IMTA), Frontiers in Marine Science 5 (2018), https://doi.org/10.3389/fmars.2018.00418.

[11] J.R.C. Freitas, J.M. Salinas Morrondo, J. Cremades Ugarte, Saccharina latissima (Laminariales, Ochrophyta) farming in an industrial IMTA system in Galicia (Spain), J. Appl. Phycol. 28 (2016) 377–385.

[12] A. Handå, S. Forbord, X. Wang, O.J. Broch, S.W. Dahle, T.R. Størseth, K.I. Reitan, Y. Olsen, J. Skjermo, Seasonal- and depth-dependent growth of cultivated kelp (Saccharina latissima) in close proximity to salmon (Salmo salar) aquaculture in Norway, Aquaculture 414–415 (2013) 191–201.

[13] C. Paulino, J. Neiva, N.C. Coelho, T. Aires, N. Marba, D. Krause-Jensen, E. A. Serrao, Characterization of 12 polymorphic microsatellite markers in the sugar kelp saccharina latissima, J. Appl. Phycol. 28 (2016) 3071–3074.

[14] J.R. Sears, NEAS Keys to the Benthic Marine Algae of the Northeastern Coast of North America from Long Island Sound to the Strait of Belle Isle, 2nd ed., Northeast Algal Society, UMASS Dartmouth, Dartmouth, MA, USA, 2002.

[15] T.S. Breton, J.C. Nettleton, B. O'Connell, M. Bertocci, Fine-scale population genetic structure of sugar kelp, saccharina latissima (Laminariales, Phaeophyceae), in eastern Maine, USA, Phycologia 57 (2018) 32–40.

[16] S. Redmond, L. Green, C. Yarish, J. Kim, C. Neefus, in: New England Seaweed Culture Handbook-Nursery Systems, Connecticut Sea Grant CTSG-14-01, 2014, p. 92. http://seagrant.uconn.edu/publications/aquaculture/handbook.pdf.

[17] C. Peteiro, Ó. Freire, Epiphytism on blades of the edible kelps undaria pinnatifida and saccharina latissima farmed under different abiotic conditions, J. World Aquacult. Soc. 44 (2013) 706–715.

[18] A.L. Schmidt, R.E. Scheibling, A comparison of epifauna and epiphytes on native kelps (Laminaria spp.) and an invasive alga (Codium fragile ssp. Tomentosoides) in Nova Scotia, Canada, Botanica Marina 49 (2006) 315–330.

[19] S. Egan, N.D. Fernandes, V. Kumar, M. Gardiner, T. Thomas, Bacterial pathogens, virulence mechanism and host defence in marine macroalgae, Environ. Microbiol. 16 (2014) 925–938.

[20] E.M. Marzinelli, A.H. Campbell, E.Z. Valdes, A. Vergés, S. Nielsen, T. Wernberg, S. Bennett, J.G. Caporaso, T. Thomas, P.D. Steinberg, T.D. Bettignies, Continental-scale variation in seaweed host-associated bacterial communities is a function of host condition, not geography, Environmental microbiology 17 (10) (2015) 4078–4088.

[21] J.-C. Simon, J.R. Marchesi, C. Mougel, M.-A. Selosse, Host-microbiota interactions: from holobiont theory to analysis, Microbiome 7 (2019) 5.

[22] C.S. Park, E.K. Hwang, Seasonality of epiphytic development of the hydroid Obelia geniculata on cultivated saccharina japonica (Laminariaceae, Phaeophyta) in Korea, J. Appl. Phycol. 24 (2012) 433–439.

[23] V.J. Chapman, Salt-marsh ecology: sections VI and VII. Comparison with marshes on the East Coast of North America, J. Ecol. 28 (1940) 118–152.

[24] D.S. Johnson, H.H. York, The Relation of Plants to Tidal Levels, Washington D. C, Carnegie Institution of Washington, 1915.

[25] K. Baker, Systematics and ecology of lyngbya spp. And associated species (Cyanophyta) in a New England salt marsh, J. Phycol. 23 (2004) 201–208.

[26] J.E. Lawrence, J. Grant, M. Quilliam, A.G. Bauder, A. Cembella, Colonization and growth of the toxic dinoflagellate Prorocentrum lima and associated fouling macroalgae on mussels in suspended culture, Mar. Ecol. Prog. Ser. 201 (2000) 147–154.

[27] L. Maranda, S. Corwin, P. Hargraves, Prorocentrum lima (Dinophyceae) in northeastern USA coastal waters: IAbundance and distribution, Harmful Algae 6 (2007) 623–631.

[28] L. Maranda, S. Corwin, S. Dover, S.L. Morton, Prorocentrum lima (Dinophyceae) in northeastern USA coastal waters: II: toxin load in the epibiota and in shellfish, Harmful Algae 6 (2007) 632–641.

[29] A. Concepcion, K. DeRosia-Banick, N. Balcom, Seaweed Production and Processing in Connecticut: A Guide to Understanding and Controlling Potential Food Safety Hazards, T.C. Connecticut Sea Grant, Department of Agriculture Bureau of Aquaculture, 2020.

[30] O.N. Barberi, C.J. Byron, K.M. Burkholder, A.T. St, A.K.Williams Gelais, Assessment of bacterial pathogens on edible macroalgae in coastal waters, J. Appl. Phycol. 32 (2020) 683–696.

[31] K.L. Gosner, Guide to identification of marine and estuarine invertebrates, in: Cape Hatteras to the Bay of Fundy, John Wiley and Sons, Inc, New York, 1971.

[32] K.L. Gosner, A Field Guide to the Atlantic Seashore from the Bay of Fundy to Cape Hatteras, Houghton Mifflin Company, New York, 1978.

[33] J. Throndsen, Preservation and storage, in: A. Sournia (Ed.), Phytoplankton Manual, UNESCO, Paris, 1978, pp. 69–74.

[34] G.R. Hasle, The inverted microscope method, in: A. Sournia (Ed.), Phytoplankton Manual, UNESCO, Paris, 1978, pp. 88–96.

[35] R.T. Carmelo, in: Marine Phytoplankton A Guide to Naked Flagellates and Coccolithophorids, Adademic Press, Inc, Diego, New York, Boston, London, Sydney, Tokyo, Toronto, 1993, p. 263.

[36] R.T. Carmelo, Identifying Marine Diatoms and Dinoflagellates, Academic Press Inc, San Diego, New York, Boston, London, Sydney, Tokyo, Toronto, 1996 pp.

[37] C. Pfeffer, J.D. Oliver, A comparison of thiosulphate-citrate-bile salts-sucrose (TCBS) agar and thiosulphate-chloride-iodide (TCI) agar for the isolation of vibrio species from estuarine environments, Lett. Appl. Microbiol. 36 (2003) 150–151.

[38] K. Wilson, Preparation of genomic DNA from bacteria, Curr. Protoc. Mol. Biol. 56 (2001) 241–245.

[39] G. Panicker, D.R. Call, M.J. Krug, A.K. Bej, Detection of pathogenic vibrio spp. In shellfish by using multiplex PCR and DNA microarrays, Appl. Environ. Microbiol. 70 (2004) 7436–7444.

[40] A. Bauer, L.M. Rorvik, A novel multiplex PCR for the identification of Vibrio parahaemolyticus, vibrio cholerae and Vibrio vulnificus, Lett. Appl. Microbiol. 45 (2007) 371–375.

[41] A. Klindworth, E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, F. O. Glöckner, Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies, Nucleic Acids Res. 41 (1) (2013) e1, https://doi.org/10.1093/nar/gks808.

[42] T. Stoeck, D. Bass, M. Nebel, R. Christen, M.D. Jones, H.W. Breiner, T.A. Richards, Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water, Mol. Ecol. 19 (Suppl 1) (2010) 21–31.

[43] P.D. Schloss, S.L. Westcott, T. Ryabin, J.R. Hall, M. Hartmann, E.B. Hollister, R. A. Lesniewski, B.B. Oakley, D.H. Parks, C.J. Robinson, J.W. Sahl, B. Stres, G. G. Thallinger, D.J. Van Horn, C.F. Weber, Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities, Appl. Environ. Microbiol. 75 (2009) 7537–7541.

[44] J.J. Kozich, S.L. Westcott, N.T. Baxter, S.K. Highlander, P.D. Schloss, Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq illumina sequencing platform, Appl. Environ. Microbiol. 79 (2013) 5112–5120.

[45] Q. Wang, G.M. Garrity, J.M. Tiedje, J.R. Cole, Naïve bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy, Appl. Environ. Microbiol. 73 (2007) 5261–5267.

[46] L.K. Medlin, I. Kaczmarska, Evolution of the diatoms: VMorphological and cytological support for the major clades and a taxonomic revision, Phycologia 43 (2004) 245–270.

[47] F.E. Round, R.M. Crawford, D.G. Mann, The Diatoms: Biology & Morphology of the Genera, Cambridge University Press, 1990.

[48] N. Somboonna, A. Wilantho, A. Assawamakin, S. Monanunsap, D. Sangsrakru, S. Tangphatsornruang, S. Tongsima, Structural and functional diversity of free-living microorganisms in reef surface, Kra island, Thailand, BMC Genomics 15 (2014) 607, https://doi.org/10.1186/1471-2164-15-607.

[49] J.E. Peck, Multivariate Analysis for Community Ecologists: Step-by-Step using PC-ORD, MjM Software Design, Gleneden Beach, OR, 2016.

[50] B. McCune, J.B. Grace, Analysis of Ecological Communities, Gleneden Beach, Oregon, 2002.

[51] M. Dufrêne, P. Legendre, Species assemblages and indicator species: the need for a flexible asymmetrical approach, Ecol. Monogr. 67 (1997) 345–366.

[52] C.D. Wells, A.L. Pappal, Y. Cao, J.T. Carlton, Z. Currimjee, J.A. Dijkstra, S. K. Edquist, A. Gittenberger, S. Goodnight, S.P. Grady, L.A. Green, L.G. Harris, L. H. Harris, N.-V. Hobbs, G. Lambert, A. Marques, A.C. Mathieson, M.I. McCuller, K. Osborne, J.A. Pederson, M. Ros, J.P. Smith, L.M. Stefaniak, A. Stevens, in: Rapid Assessment Survey of Marine Species at New England Bays and Harbors, Massachusetts Office of Coastal Management (CZM) pursuant to the National Oceanic and Atmospheric Administration (NOAA), 2014, p. 26.

[53] N.W. Jeffery, S.G. Heaslip, L.A. Stevens, R.R.E. Stanley, in: S.B. Coastal Ecosystem Science Division, Oceans Canada Fisheries (Eds.), Biophysical and Ecological Overview of the Eastern Shore Islands Area of Interest (AOI), Fisheries and Oceans Canada, Canadian Science Advisory Secretariat, 200 Kent Street, Ottawa ON K1A 0E6, 2020.

[54] N. Balcom, W. Hyatt, N. Murray, P. Bresnahan, in: Connecticut aquatic nuisance species management plan, 2006.

[55] K.A. Krumhansl, J.M. Lee, R.E. Scheibling, Grazing damage and encrustation by an invasive bryozoan reduce the ability of kelps to withstand breakage by waves, J. Exp. Mar. Biol. Ecol. 407 (2011) 12–18.

[56] A. Schwelm, J. Badstöber, S. Bulman, N. Desoignies, M. Etemadi, R.E. Falloon, C. M.M. Gachon, A. Legreve, J. Lukeš, U. Merz, A. Nenarokova, M. Strittmatter, B. K. Sullivan, S. Neuhauser, Not in your usual Top 10: protists that infect plants and algae, Mol. Plant Pathol. 19 (2018) 1029–1044.

[57] B.J. Slater, S. McLoughlin, J. Hilton, Peronosporomycetes (Oomycota) from a Middle Permian permineralised peat within the Bainmedart Coal Measures, Prince Charles Mountains, Antarctica, PLOS ONE 8 (2013) e70707-e70707.

[58] P. Murúa, D.G. Müller, M. Etemadi, P. van West, C.M.M. Gachon, Host and pathogen autophagy are central to the inducible local defences and systemic response of the giant kelp Macrocystis pyrifera against the oomycete pathogen Anisolpidium ectocarpii, New Phytol. 226 (2020) 1445–1460.

[59] E.E. Ruppert, R.S. Fox, R.D. Barnes, Invertebrate Zoology: A Functional Evolutionary Approach, 7 ed., Brooks/Cole, 2004.

[60] R.A. Pohowsky, The boring ctenostomate bryozoa: taxonomy and paleobiology based on cavities in calcareous substrata, Bull. Am. Paleontol. 73 (1978).
[61] R. Massana, J. del Campo, M.E. Sieracki, S. Audic, R. Logares, Exploring the uncultured microeukaryote majority in the oceans: reevaluation of ribogroups within stramenopiles, ISME J 8 (2014) 854–866.
[62] A.Z. Worden, C. Dupont, A.E. Allen, Genomes of uncultured eukaryotes: sorting FACS from fiction, Genome Biol. 12 (2011) 117.
[63] N. Okamoto, C. Chantangsi, A. Horák, B.S. Leander, P.J. Keeling, Molecular phylogeny and description of the novel Katablepharid Roombia truncata gen. et sp. nov., and establishment of the Hacrobia Taxon nov, PLOS ONE 4 (2009), e7080.
[64] D. Klaveness, K. Shalchian-Tabrizi, H. Thomsen, W. Eikrem, K. Jakobsen, Telonema antarcticum sp. nov., a common marine phagotrophic flagellate, Int. J. Syst. Evol. Microbiol. 55 (2005) 2595–2604.
[65] E.M.S. Brito, R. Guyoneaud, M. Goñi-Urriza, A. Ranchou-Peyruse, A. Verbaere, M. A.C. Crapez, J.C.A. Wasserman, R. Duran, Characterization of hydrocarbonoclastic bacterial communities from mangrove sediments in Guanabara Bay, Brazil, Res. Microbiol. 157 (2006) 752–762.
[66] M.J. Gauthier, B. Lafay, R. Christen, L. Fernandez, M. Acquaviva, P. Bonin, J.-C. Bertrand, Marinobacter hydrocarbonoclasticus gen. nov., sp. nov., a New, Extremely Halotolerant, Hydrocarbon-Degrading Marine Bacterium, Int. J. Syst. Evol. Microbiol. 42 (1992) 568–576.
[67] D.O. Mountfort, F.A. Rainey, J. Burghardt, H.F. Kaspar, E. Stackebrandt, Psychromonas antarcticus gen. nov., sp. nov., A new aerotolerant anaerobic, halophilic psychrophile isolated from pond sediment of the McMurdo ice shelf, antarctica, Arch. Microbiol. 169 (1998) 231–238.
[68] S. Hosoya, J.-H. Jang, M. Yasumoto-Hirose, S. Matsuda, H. Kasai, Psychromonas agarivorans sp. nov., a novel agarolytic bacterium, Int. J. Syst. Evol. Microbiol. 59 (2009) 1262–1266.
[69] Y. Xu, Y. Nogi, C. Kato, Z. Liang, H.J. Rüger, D. De Kegel, N. Glansdorff, Psychromonas profunda sp. nov., a psychropiezophilic bacterium from deep Atlantic sediments, Int. J. Syst. Evol. Microbiol. 53 (2003) 527–532.
[70] T. Groudieva, R. Grote, G. Antranikian, Psychromonas arctica sp. nov., a novel psychrotolerant, biofilm-forming bacterium isolated from Spitzbergen, Int. J. Syst. Evol. Microbiol. 53 (2003) 539–545.
[71] L.A. Romanenko, N. Tanaka, G.M. Frolova, V.V. Mikhailov, Arenicella xantha gen. nov., sp. nov., a gammaproteobacterium isolated from a marine sandy sediment, Int. J. Syst. Evol. Microbiol. 60 (2010) 1832–1836.
[72] O.I. Nedashkovskaya, I. Cleenwerck, N.V. Zhukova, S.B. Kim, P. de Vos, Arenicella chitinivorans sp. nov., a gammaproteobacterium isolated from the sea urchin Strongylocentrotus intermedius, Int. J. Syst. Evol. Microbiol. 63 (2013) 4124–4129.
[73] M.M. Yakimov, K.N. Timmis, P.N. Golyshin, Obligate oil-degrading marine bacteria, Curr. Opin. Microbiol. 18 (2007) 257–266.
[74] S.S. Radwan, M.M. Khanafer, H.A. Al-Awadhi, Ability of the so-called obligate hydrocarbonoclastic bacteria to utilize nonhydrocarbon substrates thus enhancing their activities despite their misleading name, BMC Microbiol. 19 (2019) 41.
[75] C. Fidalgo, J. Rocha, D.N. Proença, P.V. Morais, A. Alves, I. Henriques, Saccharospirillum correiae sp. nov., an endophytic bacterium isolated from the halophyte Halimione portulacoides, Int. J. Syst. Evol. Microbiol. 67 (2017) 2026–2030.
[76] H. Kang, H. Kim, Y. Joung, K. Joh, Reinekea marina sp. nov., isolated from seawater, and emended description of the genus Reinekea, Int. J. Syst. Evol. Microbiol. 66 (2016) 360–364.
[77] A. Choi, J.C. Cho, Reinekea aestuarii sp. nov., isolated from tidal flat sediment, Int. J. Syst. Evol. Microbiol. 60 (2010) 2813–2817.
[78] H.A. Giebel, D. Kalhoefer, R. Gahl-Janssen, Y.J. Choo, K. Lee, J.C. Cho, B. J. Tindall, E. Rhiel, C. Beardsley, S. Voget, R. Daniel, M. Simon, T. Brinkhoff, O. Aydogmus, Planktomarina temperata gen. nov., sp. nov., belonging to the globally distributed RCA cluster of the marine Roseobacter clade, isolated from the German Wadden Sea, Int J Syst Evol Microbiol 63 (2013) 4207–4217.
[79] J.Y. Jung, J.M. Kim, H.M. Jin, S.Y. Kim, W. Park, C.O. Jeon, Litorimonas taeanensis gen. nov., sp. nov., isolated from a sandy beach, Int. J. Syst. Evol. Microbiol. 61 (2011) 1534–1538.
[80] O.I. Nedashkovskaya, A.D. Kukhlevskiy, N.V. Zhukova, S.J. Kim, S.K. Rhee, Litorimonas cladophorae sp. nov., a new alphaproteobacterium isolated from the Pacific green alga Cladophora stimpsoni, and emended descriptions of the genus Litorimonas and Litorimonas taeaensis, Antonie Van Leeuwenhoek 103 (2013) 1263–1269.
[81] J. Schellenberg, H.J. Busse, M. Hardt, P. Schubert, T. Wilke, P. Kämpfer, S. P. Glaeser, Proposal of Litorimonas haliclonae sp. nov., isolated from a marine sponge of the genus Haliclona, Int. J. Syst. Evol. Microbiol. 68 (2018) 835–843.
[82] M. Teramoto, M. Nishijima, Amylibacter marinus gen. nov., sp. nov., isolated from surface seawater, Int. J. Syst. Evol. Microbiol. 64 (2014) 4016–4020.
[83] R. Ma, J. Wang, Q. Wang, Z. Ma, J. Li, L. Chen, Draft genome sequence of Loktanella cinnabarina strain XM1 isolated from coastal surface water, Genome Announc. 5 (2017) e01310–e01317.
[84] J. Vollmers, S. Voget, S. Dietrich, K. Gollnow, M. Smits, K. Meyer, T. Brinkhoff, M. Simon, R. Daniel, Poles Apart: Arctic and Antarctic Octadecabacter strains Share High Genome Plasticity and a New Type of Xanthorhodopsin, PLOS ONE 8 (2013), e63422.
[85] J.-H. Yoon, S.-J. Kang, M.-H. Lee, T.-K. Oh, Description of Sulfitobacter donghicola sp. nov., isolated from seawater of the East Sea in Korea, transfer of Staleya guttiformis Labrenz et al. 2000 to the genus Sulfitobacter as Sulfitobacter guttiformis comb. nov. and emended description of the genus Sulfitobacter, International Journal of Systematic and Evolutionary Microbiology 57 (2007) 1788–1792.
[86] M.J. Kwak, J.S. Lee, K.C. Lee, K.K. Kim, M.K. Eom, B.K. Kim, J.F. Kim, Sulfitobacter geojensis sp. nov., Sulfitobacter noctilucae sp. nov., and Sulfitobacter noctilucicola sp. nov., isolated from coastal seawater, Int. J. Syst. Evol. Microbiol. 64 (2014) 3760–3767.
[87] R. Pukall, D. Buntefuss, A. Frühling, M. Rohde, R.M. Kroppenstedt, J. Burghardt, P. Lebaron, L. Bernard, E. Stackebrandt, Sulfitobacter mediterraneus sp. nov., a new sulfite-oxidizing member of the alpha-proteobacteria, Int. J. Syst. Bacteriol. 49 (Pt 2) (1999) 513–519.
[88] E.P. Ivanova, N.M. Gorshkova, T. Sawabe, N.V. Zhukova, K. Hayashi, V. V. Kurilenko, Y. Alexeeva, V. Buljan, D.V. Nicolau, V.V. Mikhailov, R. Christen, Sulfitobacter delicatus sp. nov. and Sulfitobacter dubius sp. nov., respectively from a starfish (Stellaster equestris) and sea grass (Zostera marina), Int. J. Syst. Evol. Microbiol. 54 (2004) 475–480.
[89] P. Kumari, S. Bhattacharjee, A. Poddar, S.K. Das, Sulfitobacter faviae sp. nov., isolated from the coral Faviaveroni, Int. J. Syst. Evol. Microbiol. 66 (2016) 3786–3792.
[90] Y. Fukui, M. Abe, M. Kobayashi, M. Satomi, Sulfitobacter pacificus sp. nov., isolated from the red alga Pyropia yezoensis, Antonie Van Leeuwenhoek 107 (2015) 1155–1163.
[91] O.I. Nedashkovskaya, S.G. Kim, A.M. Stenkova, A.D. Kukhlevskiy, N.V. Zhukova, V.V. Mikhailov, Aquimarina algiphila sp. nov., a chitin degrading bacterium isolated from the red alga Tichocarpus crinitus, Int. J. Syst. Evol. Microbiol. 68 (2018) 892–898.
[92] B. Lin, G. Lu, Y. Zheng, W. Xie, S. Li, Z. Hu, Aquimarina agarilytica sp. nov., an agarolytic species isolated from a red alga, Int. J. Syst. Evol. Microbiol. 62 (2012) 869–873.
[93] E. Zozaya-Valdes, S. Egan, T. Thomas, A comprehensive analysis of the microbial communities of healthy and diseased marine macroalgae and the detection of known and potential bacterial pathogens, Front. Microbiol. 6 (2015).
[94] R. Selvarajan, T. Sibanda, S. Venkatachalam, H.J.O. Ogola, C. Christopher Obieze, T.A. Msagati, Distribution, Interaction and Functional Profiles of Epiphytic Bacterial Communities from the Rocky Intertidal SeaweedsSouth Africa, Scientific Reports 9 (2019) 19835.
[95] J.P. Bowman, Muricauda, in: M.E. Trujillo, S. Dedysh, P. DeVos, B. Hedlund, P. Kämpfer, F.A. Rainey, W.B. Whitman (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria, 2018, pp. 1–12.
[96] J.P. Bowman, Out From the Shadows – Resolution of the Taxonomy of the Family Cryomorphaceae, Front. Microbiol. 11 (2020).
[97] S. Park, J.S. Lee, K.C. Lee, J.H. Yoon, Algibacter undariae sp. nov., isolated from a brown algae reservoir, Int. J. Syst. Evol. Microbiol. 63 (2013) 3704–3709.
[98] W. Xu, X.Y. Chen, X.T. Wei, D.C. Lu, Z.J. Du, Polaribacter aquimarinus sp. nov., isolated from the surface of a marine red alga, Antonie Van Leeuwenhoek 113 (2020) 407–415.
[99] Y. Fukui, M. Abe, M. Kobayashi, H. Saito, H. Oikawa, Y. Yano, M. Satomi, Polaribacter porphyrae sp. nov., isolated from the red alga Porphyra yezoensis, and emended descriptions of the genus Polaribacter and two Polaribacter species, Int. J. Syst. Evol. Microbiol. 63 (2013) 1665–1672.
[100] O.I. Nedashkovskaya, A.D. Kukhlevskiy, N.V. Zhukova, Polaribacter reichenbachii sp. nov.: a new marine bacterium associated with the green alga Ulva fenestrata, Curr. Microbiol. 66 (2013) 16–21.
[101] O.I. Nedashkovskaya, S.G. Kim, L.A. Balabanova, N.V. Zhukova, I.Y. Bakunina, V. V. Mikhailov, Polaribacter staleyi sp. nov., a polysaccharide-degrading marine bacterium isolated from the red alga Ahnfeltia tobuchiensis, Int. J. Syst. Evol. Microbiol. 68 (2018) 623–629.
[102] C. Fernández-Álvarez, Y. Santos, Identification and typing of fish pathogenic species of the genus Tenacibaculum, Appl. Microbiol. Biotechnol. 102 (2018) 9973–9989.
[103] E.A.V. Burioli, K. Varello, S. Trancart, E. Bozzetta, A. Gorla, M. Prearo, M. Houssin, First description of a mortality event in adult Pacific oysters in Italy associated with infection by a Tenacibaculum soleae strain, J. Fish Dis. 41 (2018) 215–221.
[104] M. Scheuermayer, T.A.M. Gulder, G. Bringmann, U. Hentschel, Rubritalea marina gen. nov., sp. nov., a marine representative of the phylum 'Verrucomicrobia', isolated from a sponge (Porifera), Int. J. Syst. Evol. Microbiol. 56 (2006) 2119–2124.
[105] J. Yoon, S. Matsuda, K. Adachi, H. Kasai, A. Yokota, Rubritalea halochordaticola sp. nov., a carotenoid-producing verrucomicrobial species isolated from a marine chordate, Int. J. Syst. Evol. Microbiol. 61 (2011) 1515–1520.
[106] J. Yoon, Y. Matsuo, K. Adachi, M. Nozawa, S. Matsuda, H. Kasai, A. Yokota, Description of Persicirhabdus sediminis gen. nov., sp. nov., Roseibacillus ishigakijimensis gen. nov., sp. nov., Roseibacillus ponti sp. nov., Roseibacillus persicicus sp. nov., Luteolibacter pohnpeiensis gen. nov., sp. nov. and Luteolibacter algae sp. nov., six marine members of the phylum 'Verrucomicrobia', and emended descriptions of the class Verrucomicrobiae, the order Verrucomicrobiales and the family Verrucomicrobiaceae, Int J Syst Evol Microbiol 58 (2008) 998–1007.
[107] J.K. Harris, S.T. Kelley, N.R. Pace, New perspective on uncultured bacterial phylogenetic division OP11, Appl. Environ. Microbiol. 70 (2004) 845–849.
[108] W. Nelson, J. Stegen, The reduced genomes of Parcubacteria (OD1) contain signatures of a symbiotic lifestyle, Front. Microbiol. 6 (2015).
[109] M.-H. Park, G.-S. Min, A New Marine Cyrtophorid Ciliate, Dysteria nabia nov. spec. (Ciliophora: Phyllopharyngea: Cyrtophorida: Dysteriidae), from South Korea, Acta Protozool. 53 (2014) 257–268.

[110] P. Sun, L. Huang, D. Xu, A. Warren, B. Huang, Y. Wang, L. Wang, W. Xiao, J. Kong, Integrated space-time dataset reveals high diversity and distinct community structure of ciliates in mesopelagic waters of the northern south China Sea, Front. Microbiol. 10 (2019).
[111] N.A.T. Irwin, D.V. Tikhonenkov, E. Hehenberger, A.P. Mylnikov, F. Burki, P. J. Keeling, Phylogenomics supports the monophyly of the Cercozoa, Mol. Phylogenet. Evol. 130 (2019) 416–423.
[112] K. Shalchian-Tabrizi, W. Eikrem, D. Klaveness, D. Vaulot, M.A. Minge, F. Le Gall, K. Romari, J. Throndsen, A. Botnen, R. Massana, H.A. Thomsen, K.S. Jakobsen, Telonemia, a new protist phylum with affinity to chromist lineages, Proc. Biol. Sci. 273 (2006) 1833–1842.
[113] V.J. Harwood, J.P. Gandhi, A.C. Wright, Methods for isolation and confirmation of Vibrio vulnificus from oysters and environmental sources: a review, J. Microbiol. Methods 59 (2004) 301–316.
[114] M. Yeung, T. Thorsen, Development of a more sensitive and specific chromogenic agar medium for the detection of Vibrio parahaemolyticus and other vibrio species, J. Vis. Exp. (2016) 54493.
[115] W.-M. Chen, F.-S. Sheu, S.-Y. Sheu, Aquimarina salinaria sp. nov., a novel algicidal bacterium isolated from a saltpan, Arch. Microbiol. 194 (2012) 103–112.
[116] X. Xu, W. Liu, S. Tian, W. Wang, Q. Qi, P. Jiang, X. Gao, F. Li, H. Li, H. Yu, Petroleum hydrocarbon-degrading bacteria for the remediation of oil pollution under aerobic conditions: a perspective analysis, Front. Microbiol. 9 (2018).
[117] T. Staufenberger, V. Thiel, J. Wiese, J.F. Imhoff, Phylogenetic analysis of bacteria associated with Laminaria saccharina, FEMS Microbiol. Ecol. 64 (2008) 65–77.
[118] V.K. Michelou, J.G. Caporaso, R. Knight, S.R. Palumbi, The ecology of microbial communities associated with macrocystis pyrifera, PLOS ONE 8 (2013), e67480.
[119] M. Saha, F. Weinberger, Microbial “gardening” by a seaweed holobiont: Surface metabolites attract protective and deter pathogenic epibacterial settlement, J. Ecol. 107 (2019) 2255–2265.
[120] Z.X. Xu, P. Yu, D.S. Mu, Y. Liu, Z.J. Du, Tenacibaculum agarivorans sp. nov., an agar-degrading bacterium isolated from marine alga Porphyra yezoensis Ueda, Int. J. Syst. Evol. Microbiol. 67 (2017) 5139–5143.
[121] J.L. Pinckney, A mini-review of the contribution of benthic microalgae to the ecology of the continental shelf in the South Atlantic Bight, Estuar. Coasts 41 (2018) 2070–2078.
[122] C.M.M. Gachon, T. Sime-Ngando, M. Strittmatter, A. Chambouvet, G.H. Kim, Algal diseases: spotlight on a black box, Trends Plant Sci. 15 (2010) 633–640.
[123] T. Riit, L. Tedersoo, R. Drenkhan, E. Runno-Paurson, H. Kokko, S. Anslan, Oomycete-specific ITS primers for identification and metabarcoding, MycoKeys 14 (2016) 17–30.
[124] G.M. Ward, J.P. Faisan Jr., E.J. Cottier-Cook, C. Gachon, A.Q. Hurtado, P.E. Lim, I. Matoju, F.E. Msuya, D. Bass, J. Brodie, A review of reported seaweed diseases and pests in aquaculture in Asia, J. World Aquacult. Soc. 51 (2020) 815–828.
[125] M.C. Pratt, Living where the flow is right: how flow affects feeding in bryozoans, Integr. Comp. Biol. 48 (2008) 808–822.
[126] W. Visch, G.M. Nylund, H. Pavia, Growth and biofouling in kelp aquaculture (Saccharina latissima): the effect of location and wave exposure, J. Appl. Phycol. 32 (2020) 3199–3209.
[127] L.G.M.B. Becking, Geobiologie of inleiding tot de milieukunde, in: WP Van Stockum & Zoon, 1934.