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Sympatric kelp species share a large portion of their surface bacterial communities

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Summary

Kelp forest ecosystems are biodiversity hotspots, providing habitat for dense assemblages of marine organisms and nutrients for marine and terrestrial food webs. The surfaces of kelps support diverse microbial communities that facilitate the transfer of carbon from algal primary production to higher trophic levels. We quantified the diversity of bacteria on the surfaces of eight sympatric kelp species from four sites in British Columbia. Kelp-associated bacterial communities are significantly different from their environment, even though 86% of their bacterial taxa are shared with seawater and 97% are shared with rocky substrate. This differentiation is driven by differences in relative abundance of the bacterial taxa present. Similarly, a large portion of bacterial taxa (37%) is shared among all eight kelp species, yet differential abundance of bacterial taxa underlies differences in community structure among species. Kelp-associated bacterial diversity does not track host phylogeny; instead bacterial community composition is correlated with the life-history strategy of the host, with annual and perennial kelps supporting divergent bacterial communities. These data provide the first community-scale investigation of kelp forest-associated bacterial diversity. More broadly, this study provides insight into mechanisms that may structure bacterial communities among closely related sympatric host species.

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Introduction

Multicellular organisms have evolved in tandem with vast communities of microbes, resulting in complex mutualisms that are essential for each other's survival (Russell *et al.*, 2014). Understanding how microbial communities are structured among closely related host species provides insight into the mechanisms by which microbial associations are assembled and maintained. For example, differences in bacterial assemblages among closely related host taxa have been identified in a diversity of systems including mammals (Ley *et al.*, 2008; Ochman *et al.*, 2010), amphibians (McKenzie *et al.*, 2012; Kueneman *et al.*, 2014; Walke *et al.*, 2014), sponges (Lee *et al.*, 2011) and termites (Mikaelyan *et al.*, 2015; Tai *et al.*, 2015), among others. Yet, data from wild populations of great apes show that the magnitude of species-specific differences in microbial structure is reduced when hosts occur in sympatry (Moeller *et al.*, 2013), suggesting that the environment plays an important role in determining the composition of host-associated communities. The homogenizing effect of colonization by environmental bacteria is especially strong for microbial communities associated with the external surfaces of their hosts (Song *et al.*, 2013; Lemieux-Labonte *et al.*, 2016), including marine organisms, whose surfaces are constantly in contact with the rich diversity of ambient free-living marine microbes (Fahimipour *et al.*, 2017).

The surfaces of marine macroalgae (i.e., seaweed) support dense communities of bacterial symbionts that are essential for the proper development, metabolic functioning and defence of their hosts (Egan *et al.*, 2013). For example many macroalgal species obtain essential morphogenetic compounds directly from their surface bacteria, rather than producing these chemicals themselves (reviewed by Wichard, 2015). The role of bacteria-mediated morphogenisis has been especially well

characterized for macroalgae in the class Ulvophyceae, which do not develop normally in the absence of surface bacteria; instead, axenically cultured Ulvophyceae are slow growing with atypical morphologies (Wichard, 2015). Similarly, both the release (Weinberger *et al*., 2007) and settlement (Joint *et al*., 2007) of marine algal spores depend on the presence of bacteria-derived signalling molecules, suggesting that microbial symbionts are necessary for even the most basic aspects of algal life history. Beyond the direct benefits to algal hosts, heterotrophic marine bacteria metabolize algae-derived polysaccharides (Michel *et al*., 2006; Bengtsson *et al*., 2011; Hehemann *et al*., 2014), providing a major pathway for the flow of carbon from algal primary production to higher trophic levels (Norderhaug *et al*., 2003; Azam and Malfatti, 2007). Thus, heterotrophic marine bacteria are responsible for metabolizing a large proportion of primary production that would otherwise be inaccessible to the marine food web (Azam, 1998).

Kelp are a morphologically diverse clade of large canopy-forming marine macroalgae (order: Laminariales) that form dense communities – kelp forests – along rocky coastlines. These kelp forest ecosystems are biodiversity hotspots, providing essential habitat for large communities of marine organisms (Dayton, 1985; Steneck *et al*., 2002) as well as an important source of energy and nutrients for both marine (Duarte and Cebrian, 1996; Harrold *et al*., 1998; Krumhansl and Scheibling, 2012; Dethier *et al*., 2014) and terrestrial food webs (Polis and Hurd, 1996; Orr *et al*., 2005; Lastra *et al*., 2008). Yet kelp forest ecosystems are easily perturbed (Steneck *et al*., 2002; Krumhansl *et al*., 2016), with reductions in kelp abundance having direct consequences for coastal biodiversity (Steneck *et al*., 2002; Marzinelli *et al*., 2016) and productivity (Wilmers *et al*., 2012; Clasen and Shurin, 2015).

Given the critical importance of surface bacteria for macroalgal health and development (Egan *et al*., 2013), describing the diversity of bacteria associated with kelp forest ecosystems is an essential step towards a more complete understanding of coastal marine ecosystem dynamics. Recent studies focusing on single kelp species have described the taxonomic diversity of kelp-associated bacteria (Michelou *et al*., 2013), identified seasonal and successional changes in bacterial diversity (Bengtsson *et al*., 2010; Bengtsson *et al*., 2012), and demonstrated that kelp-associated bacterial communities are generally stable over large spatial scales (Marzinelli *et al*., 2015). Cross species comparisons have shown that distantly related macroalgal lineages host distinct bacterial communities (Lachnit *et al*., 2009; Lachnit *et al*., 2011). However, comparisons of bacterial diversity among closely related, sympatric host species within kelp forests are lacking.

In this study, we quantified the diversity of host-associated bacterial taxa present on the surfaces of eight sympatric kelp species to test the hypothesis that kelp-associated bacterial communities are distinct from those of their environment and also distinct among species. To disentangle factors that may drive species-specific patterns of bacterial community assemblage, we also tested the hypothesis that kelp-associated bacterial community structure is correlated with the phylogenetic relatedness of host species.

Results

We sampled bacterial communities from 91 individual kelps comprising eight species and three families within the Laminariales (Table 1). These eight species included four annual and four perennial kelps. For each species, we sampled the meristematic region at the base of the blade, adjacent to the stipe, which corresponds to the newest

Table 1. Spatial and taxonomic distribution of marine macroalgae sampled in this study.

				Sample location				
Sample type	Order	Family	Species	WB	SI	GI	TB	TOTAL
Macroalgae	Laminariales	Agaraceae	*Costaria costata*[a]	6	5		3	14
	(kelp)	Alariaceae	*Alaria marginata*[a]	5				5
			Pterygophora californica[b]	6	4	5	5	20
		Laminariaceae	*Cymathaere triplicata*[a]	4	5			9
			Laminaria setchellii[b]	4			3	7
			Nereocystis luetkeana[a]	5	5			10
			Saccharina groenlandica[b]		4	5	6	15
			Saccharina latissima[b]		6		5	11
	Desmarestiales	Desmarestiaceae	*Desmarestia ligulata*	4	4	5	4	17
Rock	–	–	–	5	5	5	5	20
Seawater	–	–	–	2	3	4	5	14

a. Annual kelp.
b. Perennial kelp.
Numbers are sample sizes from each site on the central coast of British Columbia: West Beach (WB), Starfish Island (SI), Golden Island (GI) and Triquet Bay (TB). The absence of samples indicates that the species was not present at a particular site.

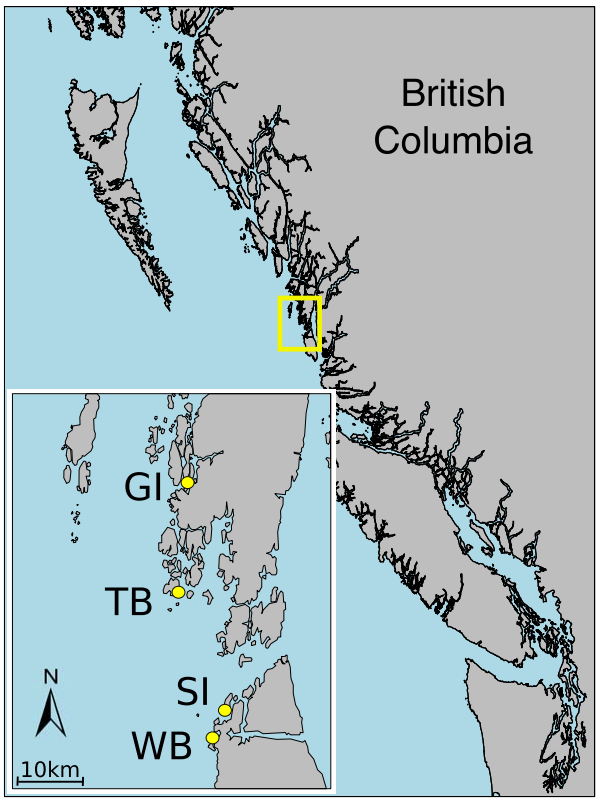

Fig. 1. Map of sample locations at four sites on the central coast of British Columbia: West Beach, Calvert Island (WB), Starfish Island (SI), Golden Island (GI), Triquet Bay (TB). See Table 1 for a list of macroalgal species and Table 2 for oceanographic conditions at each site. [Colour figure can be viewed at wileyonlinelibrary.com]

region of blade growth. While there is some variability on the timing of blade growth among these species, in general peak blade growth occurs in the spring for all species sampled. Sampling was carried out on June 26 and June 28, 2015 at four subtidal sites of similar depth ($\sim$ 5 m) near that Hakai Institute field station on the central coast of British Columbia, Canada (Fig. 1). Sampling locations were selected based on their high diversity of kelp species and the similarity of their oceanographic properties (Table 2).

Additional bacterial samples were collected from rocky substrate ($n = 20$), seawater ($n = 14$), and from a sympatric species of brown macroalgae, *Desmarestia ligulata* ($n = 17$). Sequencing the V4 region of the 16S rRNA gene produced 6.5 million paired-end reads, with an average coverage of 45 550 quality-filtered reads per sample. Minimum entropy decomposition (MED) and subsequent filtering of these data resulted in a total of 1535 bacterial operational taxonomic units (OTUs) for downstream analyses. For all subsequent results, the term 'macroalgae' will be used to refer to the eight kelp species plus *D. Ligulata*, whereas 'kelp' will be used to refer specifically to the eight species within the Laminariales.

The surfaces of macroalgal blades shared a large proportion of OTUs with their environment; of the 1535 OTUs identified across all samples in this study, 1256 (82%) were detected in all three environments (macroalgal surfaces, rocky substrate and seawater). Only 25 OTUs were unique to macroalgal surfaces. These 25 OTUs were present at low abundance among macroalgal species; we recovered between 386 and 4785 reads ($<$ 0.1% of all sequence reads) for these OTUs across all macroalgal samples. These OTUs are distributed across the commonly detected bacterial phyla and belong to Saprospirae (5 OTUs), Flavobacteria (5 OTUs), Alphaproteobacteria (3 OTUs), Gammaproteobacteria (8 OTUs), Verrucomicrobiae (3 OTUs) and Planctomycetes (1 OTU). Given the low abundance of these OTUs on macroalgal surfaces, these OTUs may occur in the environment but at abundances below our detection threshold. 24 OTUs were unique to rocky substrate; we did not identify any bacterial OTUs unique to seawater.

A large proportion of OTUs were shared among sympatric species of macroalagae; of the 1506 OTUs present on macroalgal surfaces, 544 OTUs (36%) were observed in at least one individual of all nine macroalgal species (eight kelps plus *D. ligulata*). When only kelps were examined there were 1497 bacterial OTUs present, of which 1449 OTUs (97%) were detected on at least two species and 549 OTUs (37%) were shared across at least one individual of all eight species. The most abundant bacteria identified on macroalgal surfaces were alpha- and gamma-Proteobacteria (17% and 38% of kelp sequences respectively), Bacteriodetes (Flavobacteria 13% and Saprospirae 11% of sequences) and Verrucomicrobiae (9% of sequences; Fig. 2). Only 36 OTUs were unique to a single macroalgal species, all at low abundance ($<$ 1200 reads/OTU). Nine of these OTUs were unique to *D. ligulata*.

Table 2. Oceanographic properties of each sample location used in this study.

Sample location	Date	Depth (m)	Dissolved O_2 (mg/l)	Temperature (°C)	Salinity (ppt)
Golden Island	June 28, 2015	4	10.5	15.1	30.0
Starfish Island	June 26, 2015	4	10.8	14.6	29.7
Triquet Bay	June 28, 2015	4	10.7	15.1	30.2
West Beach	June 26, 2015	4	10.8	15.0	29.0

Measurements were obtained on the same date as microbial sampling.

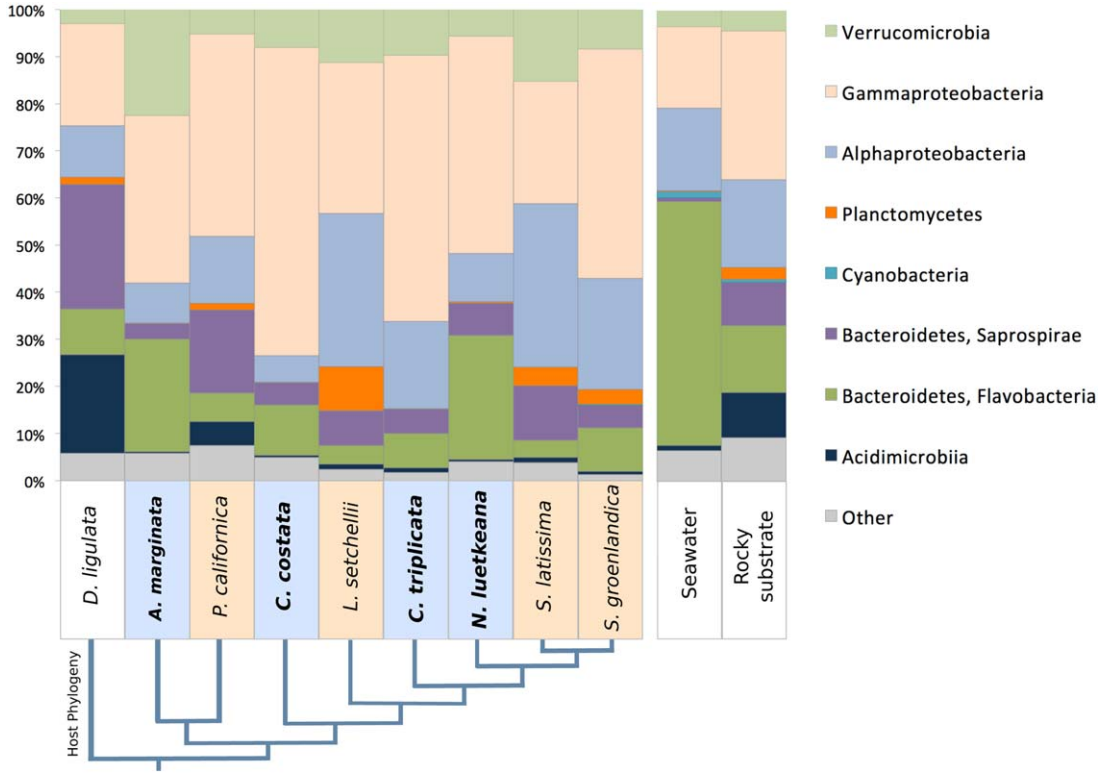

Fig. 2. The relative abundance of bacterial taxa among macroalgal species, seawater and rocky substrate. Annual kelp species are in bold font with blue shading; perennial kelp species are in plain font with orange shading. A phylogram depicting the relationship among macroalgal species is included (modified from Fig. 6 of Lane *et al.* 2006). [Colour figure can be viewed at wileyonlinelibrary.com]

Bacterial OTU richness (Chao1 index) was significantly different among sample types (nine macroalgal species, seawater and rocky substrate; $F = 25.5$, df = 10, $P < 0.0001$). Pairwise contrasts revealed that rocky substrates supported significantly greater bacterial richness than all other samples. Seawater had lower richness than rocky substrate and greater richness than four of the kelp species. Bacterial richness was generally consistent and lowest among kelp species (Fig. 3A). This statistical model included sample location as a fixed factor; both location ($F = 7.5$, df = 3, $P = 0.0001$) and the interaction term (sample type × sample location) had a significant effect on bacterial richness ($F = 3.0$, df = 17, $P = 0.0003$). However, investigating differences at the site-level revealed that the overall patterns observed across sample types were generally robust despite the significant interaction (see Supporting Information File 1). Rocky substrate always has the greatest OTU richness, but there is some variation in kelp-associated OTU richness across sample locations (Supporting Information File 1).

Bacterial community structure differs significantly across sample types (nine macroalgal species, seawater and rocky substrate; unweighted UniFrac distance permutational multivariate analysis of variance (PERMANOVA): pseudo-$F = 9.1$, df = 10, $P = 0.0001$; Figs. 3B and 4). The PERMANOVA model accounted for among-site variability, which was significant (sample location: pseudo-$F = 3.7$, df = 3, $P = 0.0001$), as was the interaction term (sample type × sample location; pseudo-$F = 2.1$, df = 17, $P = 0.0001$). These results were consistent among the three distance metrics tested (unweighted and weighted UniFrac and Bray Curtis; Supporting Information File 2).

To further investigate the effect of sample locations, we used PERMANOVAs to test for differences in unweighted UniFrac distance for each sample type (rocky substrate, seawater and nine macroalgal species) among sites. This analysis revealed significant structure among sample locations for all macroalgal species except *L. setchellii* (Supporting Information File 3). Bacterial communities on rocky substrate also differed among sample locations, while seawater communities did not significantly differ among sites.

Host phylogenetic distance and bacterial diversity are not correlated when samples from all locations were combined (Mantel test with unweighted UniFrac distance: $R = 0.12$, $P = 0.3$), or when sites are analysed separately (West beach: $R = 0.35$, $P = 0.09$; Triquet Bay: $R = 0.05$, $P = 0.5$, Starfish Island: $R = 0.23$, $P = 0.24$; Golden Island: Not tested – only two species were present at this site). These results are consistent across all dissimilarity metrics. In support of this conclusion, the topology of the UPGMA dendrogram

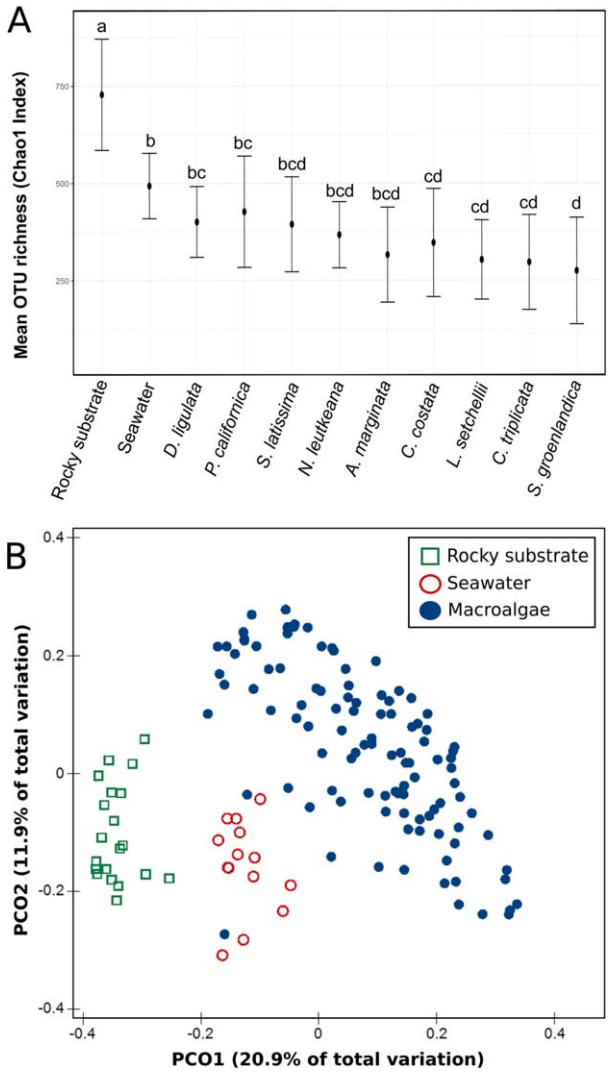

Fig. 3. (A) Bacterial OTU richness (raw mean ± standard deviation) for each sample type. Letters denote significant differences following Benjamini–Hochberg correction at a false discovery rate of 0.10; (B) Principal coordinates analysis of unweighted UniFrac distance among bacterial communities collected from seawater, rocky substrate and macroalgal hosts. For clarity, all macoalgal species are denoted with the same symbol; visualization of differences among species is presented in Figs. 4 and 5. [Colour figure can be viewed at wileyonlinelibrary.com]

constructed using bacterial community distances does not reflect the evolutionary history of kelps, but rather suggests that alternate kelp life history strategies (annual vs. perennial growth) harbour divergent bacterial communities (Fig. 5). Replicate UPGMA dendrograms based on 50 different rarefactions consistently place perennial and annual kelps into two reciprocally monophyletic groups. However, in several dendrograms (12/50), *A. marginata* (annual species) was placed either in its own clade or in a clade with *P. californica* (perennial species); these two species are both in the family Alariaceae (see Table 1), suggesting the possibility of some phylogenetic signal in this clade.

We further tested the hypothesis that bacterial community assemblages differ between annual and perennial kelp species using a three-factor PERMANOVA with 'life history type' (annual vs. perennial) coded as a fixed factor, kelp species ($n = 8$) as a random factor nested within life-history type, and sample location ($n = 4$) as a random factor. Both weighted and unweighted UniFrac metrics show a significant correlation between host life-history strategy and their microbial communities (weighted UniFrac: df = 1, pseudo-$F = 2.0$, $P = 0.038$; unweighted UniFrac: df = 1, pseudo-$F = 1.5$, $P = 0.035$), but the Bray-Curtis dissimilarity metric does not (df = 1, pseudo-$F = 1.1$, $P = 0.34$; Supporting Information File 4). Discordance among distance metrics may result from the fact that UniFrac (both weighted and unweighted) are phylogenetic methods that take into account the degree of similarity between microbial taxa as measured by the fraction of unshared branch-lengths in a phylogenetic tree (Lozupone and Knight, 2005), whereas Bray Curtis is a measure of taxonomic overlap in which values are based solely on OTU counts. It has been suggested that phylogenetic approaches may be more sensitive for revealing underlying ecological patterns (Lozupone *et al.*, 2008; Parks and Beiko, 2013).

Following the identification of significant bacterial community structure between annual and perennial samples, we used Kruskal–Wallis tests to compare OTU abundance between annual and perennial kelps. This test revealed an increase of Alphaproteobacteria (Rhodobacterales) and Planctomycetes (Pirellulaceae) on perennial kelp species relative to annuals (Supporting Information File 5).

Discussion

Bacterial assemblage among host species

We show that sympatric species of kelp share a large proportion of their microbiota. Yet, despite the high proportion of shared OTUs, variation in relative abundance of bacterial taxa contributes to differences in community structure among sympatric host species. The high degree to which OTUs are shared among kelp species likely reflects a combination of factors including their close physical proximity within the same environment, similarities in the abundance of dominant carbohydrates present in kelp blades (Schiener *et al.*, 2015), as well as their recent evolutionary diversification (Silberfeld *et al.*, 2010).

Early research into the community composition of microbes associated with marine macroalgae found significant differences in bacterial assemblage among sympatric hosts from the three algal lineages (red, green and brown algae; Lachnit *et al.*, 2009), likely reflecting the immense divergence time among host clades: these independent lineages of eukaryotes split roughly 1500 million years ago (Parfrey *et al.*, 2011). In contrast, our current study has quantified algal-associated bacterial diversity at a much

finer taxonomic scale – among kelp species within a single order (Laminariales). The Laminariales are a relatively young clade, and the species included here diverged from each other during the last 30 million years (Silberfeld *et al.*, 2010). Since their divergence, the Laminariales have diversified into ~ 30 genera and nearly 100 species (Lane *et al.*, 2006). Yet species within the Laminariales readily hybridize with each other, even across different families (Liptack and Druehl, 2000). We hypothesize that the high proportion of shared OTUs among kelps may in part reflect their relatively recent diversification and the corresponding genetic and chemical similarities among these species.

The observed similarity of kelp-associated bacterial communities is likely also influenced by their co-occurrence in sympatry, which exposes them to the same source population of bacteria. For example, Moeller *et al.* (2013) found that chimpanzees and gorillas share 53% more bacteria in sympatry than in allopatry, with local environmental factors significantly altering the distribution of OTUs across the most abundant bacterial clades.

The inclusion of the non-kelp brown algal species, *D. ligulata*, serves as an interesting point of comparison from the Laminariales; *D. ligulata*, differed from all kelp species at all sample locations with the exception of *P. californica* at Golden Island (Fig. 4). *Desmarestia ligulata* is enriched in Acidimicrobiales (see Fig. 2), a clade containing many acidophilic bacteria, as its name suggests. Several *Desmarestia* species, including *D. ligulata*, accumulate high concentrations of sulfuric acid within cell vacuoles; acid concentration in *D. ligulata* can be as high as 18% of dry mass, resulting in an internal pH between 2.08 and 2.70 (Pelletreau and Muller-Parker, 2002). The accumulation of sulfuric acid in *D. ligulata* provides chemical defence against invertebrate grazers (Pelletreau and Muller-Parker, 2002), and may also confer defence against heterotrophic bacteria. While much of the observed bacterial variation between *D. ligulata* and the kelps is likely explained by the low pH of *D. ligulata* tissues, the much greater phylogenetic distance between *D. ligulata* and kelps may also play a role. The Desmarestiales diverged from other brown algae (Phaeophyceae) 125 million years ago (Silberfeld *et al.*, 2010), providing ample time for divergence of other aspects of chemical composition and host characteristics that may influence surface microbial communities.

Kelp life history strategy

Beyond the species-level, kelp-associated bacterial assemblages vary according to life history strategy (annual versus perennial growth) rather than by host phylogeny. The observation that annual and perennial kelps have divergent bacterial communities warrants further investigation as this finding is supported by two of the three distance metrics. Importantly, we sampled new growth in the meristematic region near the stipe in both annual and perennial species, so tissue age was roughly consistent for all species.

We hypothesize that the observed differences between annual and perennial kelp-associated bacteria reflect different successional stages of bacterial colonization, with perennial species hosting more mature communities compared to annual species. Perennial species of kelp are characterized by stipe and holdfast tissues that persist year after year and new blades grow out of the stipe each summer and senesce in the fall and winter. Thus, the perennial tissue (stipe and holdfast) could act as a bacterial reservoir facilitating the colonization of newly grown blade tissue (Bengtsson *et al.*, 2010). The presence of bacterial reservoirs has previously been shown to affect the colonization and composition of host-associated bacterial communities (Zarraonaindia *et al.*, 2015; Loudon *et al.*, 2016). Conversely, in annual species, macroscopic tissues are generally absent outside of the growing season and grow anew each year. These annual species thus lack the potential for a bacterial reservoir and would be dependent either on the pool of available microbes in the water column or possibly vertical transmission. Our results are in line with those of Bengtsson *et al.* (2010; 2012) who examined the seasonal dynamics of bacteria on the surface of the kelp *Laminaria hyperborea* and observed an enrichment of Gammaproteobacteria on young tissue, and an in increase in Alphaproteobacteria and Planctomycetes on older kelp surfaces, later in the growing season (Bengtsson *et al.*, 2010; Bengtsson *et al.*, 2012). Similarly, we observed enriched Alphaproteobacteria and Planctomycetes on the surfaces of older perennial species compared with the annuals (Fig. 2 and Supporting Information File 5).

Other possible explanations for this pattern include differences in chemical ecology between annuals and perennials, which in turn could affect their microbial communities. For example, it has been shown that annual species of marine macroalgae tend to have greater caloric content than perennial species (Paine and Vadas, 1969). While the reason for these caloric differences are unknown, Paine and Vadas (1969) hypothesize that the accumulation of energy-rich compounds may be a by product of rapid growth and maturation. The distribution and concentrations of secondary metabolites such as phlorotannins, which are used as chemical defence against herbivory (Cronin, 2001), may also vary between annuals and perennials and differentially impact surface microbial communities. Allocation of defensive compounds is variable among the tissues of many kelp species, with greater concentrations of phlorotannins generally occurring in tissues that determine kelp fitness, such as meristems and reproductive regions (Steinberg, 1984; Van Alstyne *et al.*, 1999; Cronin, 2001). Similarly, Tugwell and Branch (1989)

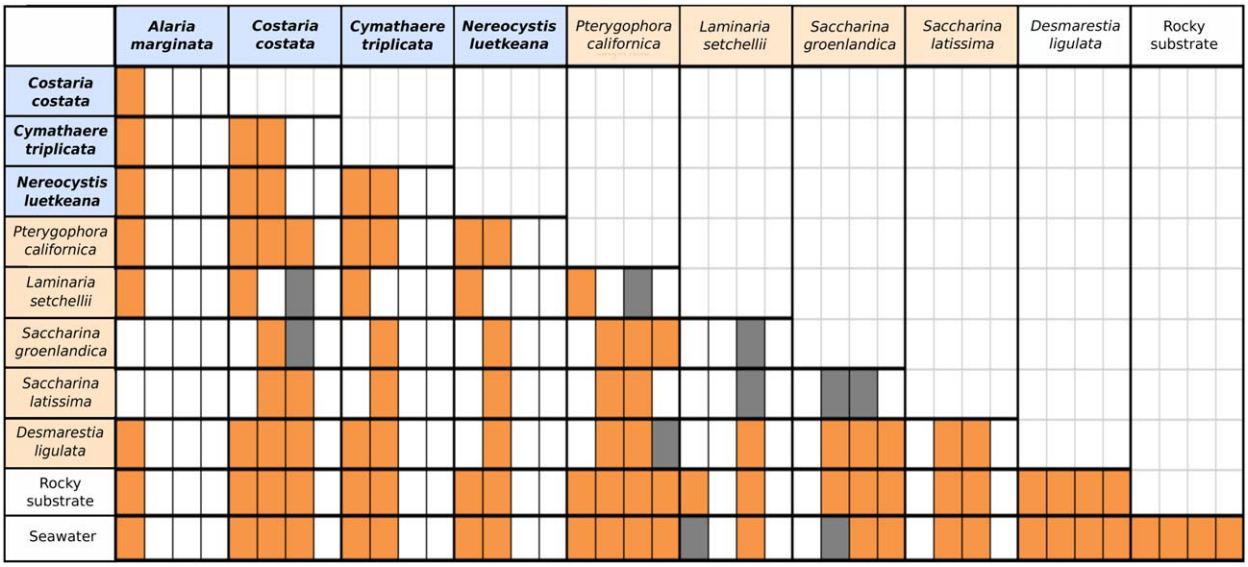

Fig. 4. Pairwise comparisons of community structure among samples. Orange boxes indicate significant differences based on PERMANOVA. Each comparison is divided into four boxes corresponding to results from each site: West Beach, Starfish Island, Triquet Bay and Golden Island (in that order). *P* values are corrected using the Benjamini–Hochberg procedure with a false discovery rate of 0.10. Annual kelp species are in bold font with blue shading; perennial kelp species are in plain font with orange shading. [Colour figure can be viewed at wileyonlinelibrary.com]

found significantly more anti-herbivory polyphenolic compounds in holdfasts, stipes and meristems compared to vegetative blades of three perennial kelp species; the preferential protection of perennial tissues potentially safeguarding the long-term survival (and fitness) of these species (Van Alstyne *et al*., 1999; Iken *et al*., 2007). Future work is needed to test these hypotheses.

Hosts versus the environment

The surfaces of kelp blades are nutrient rich and chemically active substrates (Michelou *et al*., 2013), providing unique bacterial niches that are not found in the abiotic environment (Egan *et al*., 2013). While the mechanisms by which kelp surfaces obtain their bacterial communities are not well understood, our data suggest that a large proportion of surface communities on kelps are assembled from the pool of available bacteria within the environment. Of the 1497 OTUs identified on the surface of kelp, 86% of these bacterial taxa are shared with seawater and 97% are shared with rocky substrate.

The high degree of shared OTUs between kelp-associated and environmental communities is contrary to previous research on kelp bacterial ecology in which only very small proportion of OTUs were shared between kelp and seawater (Bengtsson *et al*., 2010; Burke *et al*., 2011a; Michelou *et al*., 2013). For example, a recent study on the giant kelp, *Macrocystis pyrifera*, found that only 2% of OTUs were shared between seawater and kelp samples (Michelou *et al*., 2013). The increased sharing of OTUs identified in our study may reflect advances in sequencing technology that allow for greater sequencing depth per sample, with a corresponding improvement in the ability to detect rare OTUs in seawater. These differences could also be associated with the particular species being examined (*M. pyrifera* was not included in our survey) or to methodological differences, such as the location along the thallus that was sampled, differences in water filtering protocols, and the methods used for bacterial sampling (swabbing the blade versus incubating the blade in artificial seawater).

However, we acknowledge that some overlap between water and algal bacterial communities may be due to sampling, since some water column bacteria were likely still present on the algal samples after sterile rinsing, and water samples taken adjacent to kelp blades may contain trace quantities of kelp-associated bacteria that have been dislodged. Nonetheless, the water column bacteria are in constant contact with the kelp surface, providing a continuous pool of bacteria that can potentially colonize. Further, our observation that annual and perennial kelps support distinct bacterial communities consistent with early and late successional stages suggests that the colonization of kelp blades occurs continuously throughout their life cycle.

Previous research on the bacteria associated with marine macroalgae has generally observed that bacterial community structure is consistent across large spatial scales (Lachnit *et al*., 2009). However, regional differences in community structure have been reported for the kelp species *Saccharina latissima* (published as *Laminaria*

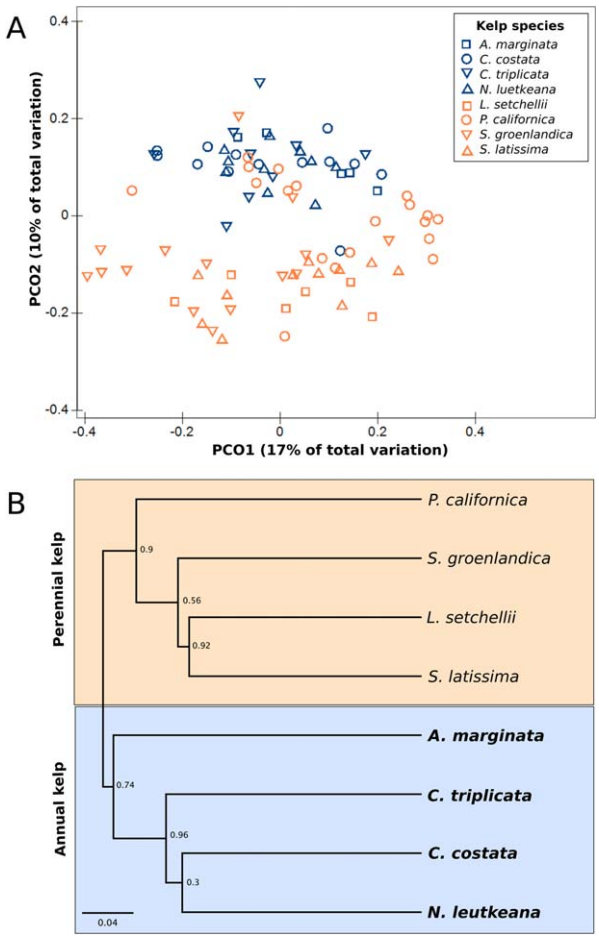

Fig. 5. (A) Principal coordinates analysis of unweighted UniFrac distance comparing bacterial communities among kelp species. Colours represent annual (blue) and perennial (orange) life history strategies. (B) An UPGMA tree constructed using bacterial distance among kelp species (unweighted UniFrac distance). For this analysis the abundance of each OTU (in sequence reads) was averaged across replicates of the same species. Jackknife support values were obtained by comparing
50 replicate trees each rarefied to 5000 sequences/sample. Annual kelp species are in bold font with blue shading; perennial kelp species are in plain font with orange shading. [Colour figure can be viewed at wileyonlinelibrary.com]

saccharina; Lachnit *et al.*, 2009), *Laminaria hyperborea* (Bengtsson *et al.*, 2012) and *Ecklonia radiata* (Marzinelli *et al.*, 2015). Our data show significant regional differences in bacterial communities on the surfaces of eight macroalgal species and rocky substrate. We collected all samples within a three-day period (June 26–28, 2015) to minimize any temporal effects on the kelp associated microbial communities and the four sites were selected because they have similar depth and oceanographic properties (Table 2); indeed, we find that bacterial communities in the ambient seawater to do not significantly differ among sample locations (Supporting Information File 3). The observed variation in host-associated microbial communities may be indicative of small differences in the colonization of macroalgae by ambient bacteria at each site. For example, it has been suggested that algal-associated bacterial strains have a high-degree of ecological equivalence, and that communities may be assembled on a functional rather than taxonomic basis (Burke *et al.*, 2011b; Bengtsson *et al.*, 2012). Such a process could generate the differences across site observed here.

Conclusion

Kelp forest ecosystems provide structural habitat for diverse assemblages of marine organisms and are major contributors to coastal primary productivity. Yet, these ecosystems are subject to periods of collapse and recovery (Steneck *et al.*, 2002; Filbee-Dexter and Scheibling, 2014; Krumhansl *et al.*, 2016), which results in associated losses or gains to biodiversity, changes in carbon and nutrient cycling, and other ecosystem services. The structure, function and abundance of kelp-associated bacterial communities are affected by reductions in kelp biomass (Clasen and Shurin, 2015), highlighting the need for research into the ecological implications of altered bacterial community structure during periods of kelp forest decline. Our study is a step towards this goal, providing the first community-scale investigation of kelp forest-associated bacterial diversity and foundational knowledge for future studies in this productive ecosystem. Beyond kelp, this study deepens our understanding of the mechanisms that may drive bacterial community structure among closely related sympatric species. At this taxonomic scale (sympatric species within the same family), host phylogeny had no significant impact on bacterial community structure. Instead, our data suggest that ecological differences (in this case life history strategy) can be a predictor of host-associated bacterial community structure.

Experimental procedures

Sample collection

At each site 3–6 individuals from each available kelp species were collected by SCUBA within 1 m of the substrate and placed in individual sterile Ziploc® bags for transport to the surface. At the surface each blade was rinsed with sterile seawater for 10 s to remove transient (non-host-associated) bacteria. Samples were collected from each specimen using a Puritan® sterile swab, which was stored in an individual sterile cryovial (VWR). Samples were placed on ice for transport back to the lab where they were transferred to −80°C for storage. All bacterial communities were sampled by swabbing for 10 s the newest growth on kelp blades close to, but not touching the stipe, which contains meristem tissue. Not all species were present at all sites, however, given our aim of quantifying kelp-associated bacterial diversity, we prioritized sampling a greater number of host species over having each species replicated at all sites. This approach provides more data on the

breadth of kelp-associated bacterial diversity at the expense of reduced statistical power to detect site-specific differences (unbalanced design).

In terms of the relative age of sampled blade tissue, recruitment of the four annual kelp species occurs in early spring with peak blade elongation occurring in spring and summer (Maxell and Miller, 1996; McConnico and Foster, 2005). For two of the perennial species (*Pterygophora californica* and *Laminaria setchellii*) stipes and holdfasts persist for several years while the blades erode completely and regrow anew each spring (De Wreede, 1984; Bartsch *et al*., 2008). The other two perennial species (*Saccharina groenlandica* and *Saccharina latissima*) have perennial stipes and holdfasts, and experience meristematic blade growth with distal blade erosion year-round; however, the rate of blade growth fluctuates throughout the year with peak blade growth occurring in spring (Druehl *et al*., 1987; Nielsen *et al*., 2014).

At each site we also collected bacterial communities from environmental samples (ambient seawater and rocky substrates) and from *D. ligulata* (order Desmarestiales). Bacterial samples from rocky substrates (small cobbles with size < 1 kg) and from *D. ligulata* were collected using sterile swabs as described above. Seawater from within the kelp forest was collected from the same depth as algal samples (within 1 m of substrate) using sterile 500 ml plastic containers. Water samples were pre-filtered through a 150μm mesh. Bacteria were filtered from seawater in the lab using a Cole-Parmer Master-Flex L/S peristaltic pump with a 0.22 μm Durapore® membrane filter (Merk Millipore). Filters from each seawater sample were stored at −80°C in individual Whirl-Pak® bags.

Molecular methods

DNA was extracted from swabs and water filters using the MoBio PowerSoil®-htp 96 well DNA extraction kit (Carlsbad, CA) following the manufacturers recommended protocol. The V4 region of 16S rRNA gene in Bacteria and Archaea was targeted for amplification using redesigned versions of the primers *515f/806r* (Caporaso *et al*., 2012): *515f*: 5'–GTG YCAGCMGCCGCGGTAA–3', *806r*: 5'–GGACTACNVGGGTW TCTAAT–3'. Forward primers were tagged with a 12 bp Golay barcode to facilitate sample pooling. Each PCR contained 10 μl of 5-Prime Master Mix, 1 μl of each primer (final concentration = 0.2 μM each), 0.5 μl of peptide nucleic acid (PNA) chloroplast blocking primer (Lundberg *et al*., 2013; 0.2 μM final concentration, purchased from PNA Bio, Thousand Oaks, CA), 2 μl of DNA, and PCR grade water to a final volume of 25 μl. PCR was carried out with an initial denaturation step at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 45 s, PNA clamping at 75°C for 60 s, primer annealing at 50°C for 60 s, and extension at 72°C for 90 s, with a final extension step of 72°C for 10 min. PCR products were quantified using Quant-IT Pico Green® ds DNA Assay Kit (Life Technologies). Equal amounts (25 ng) of each sample were pooled and then purified using the MoBio UltaClean® PCR clean-up kit. Pooled library quantitation and paired-end Illumina MiSeq sequencing (2×300 bp) was carried out at the Integrated Microbiome Resource facility in the Centre for Genomics and Evolutionary Bioinformatics at Dalhousie University (Halifax, Canada).

Sequence data

Raw sequencing reads were demultiplexed using the Split Libraries function from the Quantitative Insights into Microbial Ecology (QIIME v.1.9) analysis pipeline (Caporaso *et al*., 2010b). Demultiplexed reads were then trimmed to a uniform length of 250 bp using FastX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), and processed into operational taxonomic units using the MED method (Eren *et al*., 2015) as implemented in the Oligotyping microbial analysis software package (Eren *et al*., 2013). MEDs perform *de novo* taxonomic clustering using Shannon Entropy to separate biologically meaningful patterns of nucleotide diversity from sequencing noise; the processed data are partitioned into phylogenetically homogeneous units (MED-nodes) for downstream bacterial diversity analyses. This analysis was carried out with the minimum substantive abundance parameter (–M) set at 250 reads. All other parameters were run with default settings; the maximum variation allowed per node (–V) was automatically set at three nucleotides. In practice, the MED-nodes identified in this study are analogous to $\geq 99\%$ OTUs.

Taxonomy was assigned to the resulting MED-nodes (hereafter, referred to as operational taxonomic units; OTUs) using the RDP classifier v.2.2 (Wang *et al*., 2007) as implemented in the Assign Taxonomy function of QIIME v.1.9 retrained on the GreenGenes (gg_13_8) database (DeSantis *et al*., 2006). OTUs annotated to either chloroplast or mitochondrial sequences were removed as putative host contamination. Additional OTUs were removed if they had fewer than 100 reads or if they occurred in only a single sample (regardless of read abundance). Representative sequences for the remaining OTUs ($n = 1535$) were aligned with PyNAST v.1.2.2 (Caporaso *et al*., 2010a) using the GreenGenes 13_8 alignment as a template, and a tree was constructed using FastTree (Price *et al*., 2010) as implemented in QIIME v.1.9.

Bacterial OTU richness was calculated for each sample using the non-parametric Chao1 index (Chao, 1984) after rarefying to 5000 sequences/sample. Chao1 estimates species abundance for each sample by adding a correction factor to the number of observed species to account for rare unsampled taxa. These attributes make Chao1 well suited for estimating diversity in microbial communities where the abundance of rare taxa means that samples are likely not representative of the entire community (Hughes *et al*., 2001; Haegeman *et al*., 2013). For these calculations, the bias-corrected version of Chao1 was used as implemented in QIIME v.1.9.

To quantify patterns of bacterial community assemblage among samples, we constructed distance matrices based on three different metrics (weighted and unweighted UniFrac distance (Lozupone and Knight, 2005) and Bray Curtis dissimilarity) using data rarefied to 5000 sequences/sample in QIIME v.1.9. Distances matrices were visualized with Principal Coordinates plots created in PRIMER E v. 6 (Clarke and Gorley, 2006).

Statistical analyses

We compared bacterial OTU richness (Chao1 index) among samples from each species of marine macroalgae (eight kelps plus *D. ligulata*), rocky substrate and seawater using a two-

factor analysis of variance (ANOVA) in which 'sample type' (11 levels: nine macroalgal species, seawater and rocky substrate) and sample location were each coded as a fixed factors. This analysis was carried out in R v.3.2.3 (R Development Core Team, 2015). *P* values for pairwise comparisons were corrected using the Benjamini-Hochberg procedure with a false discovery rate of 0.10.

Differences in bacterial community assemblage among samples collected from macroalgal species, rocky substrate and seawater were statistically assessed using a PERMANOVA on all three distance metrics as implemented in PRIMER v. 6 (Clarke and Gorley, 2006). The statistical model was constructed as described above, with 'sample type' (11 levels) and sample location coded as fixed factors. Each PERMANOVA described in this study was carried out using 9999 permutations. *P* values for pairwise comparisons were corrected using the Benjamini-Hochberg procedure with a false discovery rate of 0.10.

In many systems, bacterial diversity tracks the phylogenetic diversity of host species (Ochman *et al.*, 2010; Jones *et al.*, 2013). To test for co-diversification patterns of kelp species and their associated bacterial communities, a Mantel test (ade4 package; Dray and Dufour, 2007) was used to test for a correlation between phylogenetic distance and bacterial diversity. For this analysis, phylogenetic distance was based on the kelp phylogeny derived by Lane and colleagues (2006). For bacterial distance we used three different dissimilarity matrices (weighted and unweighted UniFrac and Bray Curtis distance) in which OTU abundance was averaged across replicate individuals of each species, and then rarefied to 5000 sequences. Bacterial OTU distance matrices were also visualized using UPGMA dendrograms, with bootstrap support estimated from 50 replicate rarefied distance matrices. For each analysis that focused on kelp hosts (i.e., where environmental samples were removed), the OTU table was re-filtered to remove OTUs that had fewer than 100 reads or occurred in only a single sample (regardless of read abundance).

Given the observed differences in bacterial communities between annual and perennial kelps, we tested for specific OTUs that are differentially abundant on the surfaces of annual and perennial kelp species. Unlike the community-level analysis, this objective of this test was to identify bacterial OTUs that are diagnostic of each growth strategy. To facilitate this analysis we first filtered the data to retain only OTUs that are enriched on kelp relative to their environment using the Sloan neutral model (Sloan *et al.*, 2006) with scripts and methods described by Venkataraman *et al.* (2015). For this analysis, environmental samples (rocky substrate and seawater) were coded as the 'source' of OTUs, and we retained only those OTUs with abundance on kelp greater than expected given the environmental abundance. We then used the Group Significance function in Qiime v.1.9 to compare the frequency of each OTU between annual and perennial kelps. This analysis uses Kruskal–Wallis tests on each OTU to test for significant differences in the frequency of each OTU between groups (in this case annual and perennial kelp species).

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Data accessibility

Raw Illumina MiSeq reads and associated MiMARKS compliant metadata have been accessioned in the European Bioinformatics Institute (www.ebi.ac.uk; Accession Number PRJEB15100).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

File S1. Bacterial OTU richness (raw mean ± standard error) for each sample type at each sample location.
File S2. PERMANOVA output testing for bacterial community differences among sample type (fixed factor with 11 levels). Sample location is included as a fixed factor in the model. This analysis was repeated with three distance metrics (unweighted UniFrac, weighted UniFrac and Bray Curtis).
File S3. Significant differences in bacterial community structure were observed among study sites for the majority of macroalgal species and environmental samples. This table presents the results of statistical tests (PERMANOVA) carried out on unweighted UniFrac distance among sample locations for each species of kelp and environmental samples.
File S4. PERMANOVA output testing for bacterial community differences between annual and perennial kelps (fixed factor). Kelp species and sample location are both included as random factors in the model. This analysis was repeated with three distance metrics (unweighted UniFrac, weighted UniFrac and Bray Curtis).
File S5. Heatmap showing the abundance of significantly differentiated OTUs from annual and perennial kelp species. This analysis is based on Kruskal–Wallis tests of OTU abundance between life history types, and presents all those that are significant following Bonferroni correction. Numbers on the left of each cell are the mean number of sequence reads for that OTU across replicate individuals; numbers in parentheses are the proportion of individuals that had that OTU. Only those OTUs that were significantly enriched on kelps relative to the environment were included in this analysis ($n = 725$ OTUs).