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Continental-scale variation in seaweed host-associated bacterial communities is a function of host condition, not geography

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Summary

Interactions between hosts and associated microbial communities can fundamentally shape the development and ecology of 'holobionts', from humans to marine habitat-forming organisms such as seaweeds. In marine systems, planktonic microbial community structure is mainly driven by geography and related environmental factors, but the large-scale drivers of host-associated microbial communities are largely unknown. Using 16S-rRNA gene sequencing, we characterized 260 seaweed-associated bacterial and archaeal communities on the kelp *Ecklonia radiata* from three biogeographical provinces spanning 10° of latitude and 35° of longitude across the Australian continent. These phylogenetically and taxonomically diverse communities were more strongly and consistently associated with host condition than geographical location or environmental variables, and a 'core' microbial community characteristic of healthy kelps appears to be lost when hosts become stressed. Microbial communities on stressed individuals were more similar to each other among locations than those on healthy hosts. In contrast to biogeographical patterns of planktonic marine microbial communities, host traits emerge as critical determinants of associated microbial community structure of these holobionts, even at a continental scale.

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Introduction

Much of our understanding of the diversity, distribution and abundance of marine microorganisms and their functional importance comes from the water column, where free-living (planktonic) bacteria, archaea and viruses drive global biogeochemical cycles (Paerl and Pinckney, 1996; Arrigo, 2005) and interact with planktonic eukaryotes (Tang *et al.*, 2010; Steele *et al.*, 2011; Amin *et al.*, 2012; Eckert and Pernthaler, 2014). Large-scale sampling of planktonic microbes has revealed robust geographical patterns of microbial community structure and distribution, which are strongly coupled to variation in physical environmental factors, such as temperature and light (Rusch *et al.*, 2007; Brown *et al.*, 2012; Ghiglione *et al.*, 2012; Gilbert *et al.*, 2012). However, extensive marine microbial diversity is also found within microbial communities that grow on the surfaces or within marine macrophytes and animals (Egan *et al.*, 2008; Wahl *et al.*, 2012). Host-associated communities often have critically important roles in the normal development (Marshall *et al.*, 2006), functioning (Ruby and Nealson, 1976; Dubilier *et al.*, 2008) and defence (Engel *et al.*, 2002; Sharp *et al.*, 2007) of their hosts, and it has been suggested that in order to understand the functioning of communities, these biological associations (host and microbes) –'holobionts'– should be studied as a whole (Margulis, 1991; Rosenberg *et al.*, 2007). Such studies supporting

the interdependence of macro- and microbiota within holobionts are now emerging from diverse environments, such as corals (Rosenberg *et al*., 2007) and sponges (Fan *et al*., 2012), as well as non-marine systems including the human gut (Singh *et al*., 2013). An emergent message from this work is that disruptions to macro–micro interactions have significant, and often negative, outcomes for the host (Rosenberg *et al*., 2007; Fan *et al*., 2013).

Despite the potentially fundamental role played by marine host-associated microorganisms on the lives of their hosts, there is little (but see e.g. Taylor *et al*., 2005; Morrow *et al*., 2012 for sponge and coral hosts) or no information (particularly for key marine habitat-forming seaweed hosts) on how these communities vary at large spatial scales, nor what the main drivers of this variation are (Egan *et al*., 2008; Wahl *et al*., 2012; Goecke *et al*., 2013). Such an understanding is critical for marine systems, in particular for holobionts such as seaweeds and corals, which are major marine habitat formers supporting diverse communities of macro-organisms via the provision of food and shelter. Thus, any impacts on the interaction between these hosts and their associated microbial community may cascade throughout an entire ecosystem.

Seaweeds (macroalgae) are the dominant habitat-forming organisms in temperate systems on rocky shores (Steneck *et al*., 2002). Large canopy-forming seaweeds – kelps – are showing signs of decline from many temperate reefs around the world, and such declines are typically associated with anthropogenic environmental change (Steneck *et al*., 2002; Airoldi and Beck, 2007; Connell *et al*., 2008; Wernberg *et al*., 2011a). Seaweeds, like other living surfaces in the ocean, are covered in biofilms (Egan *et al*., 2013), but in contrast to planktonic microbial communities very little is known about how the composition of seaweed-associated communities varies across large spatial scales, as a function of seaweed condition or key environmental parameters (Lachnit *et al*., 2011).

While planktonic bacterial community composition is most strongly influenced by environmental conditions, we hypothesized that host-associated bacteria would be most strongly related to the condition of the host, i.e. stressed versus non-stressed or 'healthy' hosts, due to the intimate relationship that exists between them. To begin to understand what drives the structure and composition of microbial communities of the dominant holobionts in temperate marine systems, we sampled microbial communities on populations of the dominant kelp *Ecklonia radiata* at a total of 27 sites around temperate Australia, encompassing three distinct biogeographical provinces (Wernberg *et al*., 2013): two on the east coast (the states of New South Wales, NSW; and Tasmania, TAS), spanning 10° of latitude (*c.* 1200 km) and separated by 35° of longitude (> 4000 km), from the third province on the west coast (the state of Western Australia, WA), spanning 4° of latitude (*c.* 600 km). We assessed the frequency and severity of stressed kelp phenotypes at replicate sites in northern, central and southern regions within each province (Table 1) and took samples of host-associated microbial communities from stressed or 'bleached' individuals and 'healthy' individuals. We used high-throughput 16S rRNA gene-tag sequencing to compare the structure of bacterial and archaeal biofilms among places and algal conditions, and related these patterns to environmental variables measured *in situ*. Thus, we quantified patterns of microbial communities and measured potentially important environmental drivers on relevant spatial scales to determine how (i) geography and (ii) host condition influenced host-associated community structure.

Table 1. Location of and *in situ* water temperature (8–12 m depth) at the 27 sites sampled in three biogeographical provinces (BP) along the east (NSW, TAS) and west (WA) coasts of Australia during April–May 2011.

BP	Region	Site	Latitude	Longitude	Temperature (°C)
NSW	Crowdy Head	Me	S 31° 46.08′	E 152° 48.53′	23
		Di	S 31° 42.79′	E 152° 48.15′	23
		Cr	S 31° 50.28′	E 152° 45.23′	23
	Sydney	CB	S 33° 59.90′	E 151° 14.80′	22
		Ku	S 34° 01.16′	E 151° 13.89′	22
		LB	S 33° 58.00′	E 151° 15.42′	22
	Batemans Bay	To	S 35° 44.88′	E 150° 15.27′	21
		Pr	S 35° 48.00′	E 150° 13.99′	21
		Mo	S 35° 46.85′	E 150° 14.23′	21
TAS	Bicheno	B7	S 41° 54.53′	E 148° 19.28′	15
		B8	S 41° 50.79′	E 148° 16.71′	15
		B9	S 41° 52.16′	E 148° 18.18′	15
	Fortescue Bay	F4	S 43° 08.39′	E 148° 00.10′	15
		F5	S 43° 07.40′	E 147° 58.55′	15
		F6	S 43° 08.21′	E 147° 58.19′	15
	Southport	S1	S 43° 27.53′	E 146° 59.49′	14
		S2	S 43° 31.78′	E 146° 57.40′	14
		S3	S 43° 25.54′	E 147° 01.39′	14
WA	Jurien Bay	J1	S 30° 15.96′	E 114° 58.53′	24
		J2	S 30° 18.63′	E 114° 58.42′	24
		J3	S 30° 18.21′	E 114° 59.35′	24
	Marmion	M1	S 31° 51.87′	E 115° 42.48′	24
		M2	S 31° 47.33′	E 115° 40.73′	24
		M3	S 31° 46.67′	E 115° 40.57′	24
	Hamelin Bay	H1	S 34° 13.80′	E 115° 00.86′	23
		H2	S 34° 16.07′	E 115° 01.13′	23
		H3	S 34° 15.39′	E 115° 00.48′	23

Results

Kelp condition and associated microbial communities

Over half of all kelp individuals sampled had some visible signs of thallus bleaching (Fig. 1A), but the proportion of bleached individuals in each population or the severity of bleaching (% cover of bleaching per thallus) across the three biogeographical provinces did not differ [analysis of

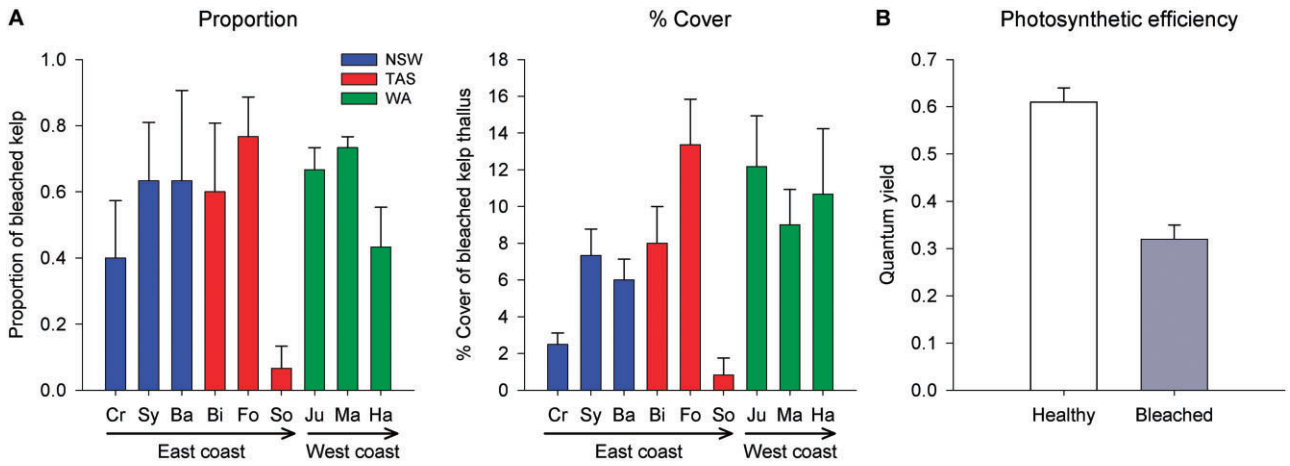

Fig. 1. Host bleaching was common across the temperate coastlines of eastern and western Australia (A, proportion of kelp individuals with bleaching in each population and percentage cover of bleached tissue on the thallus of each individual; mean + SE, $n = 30$ per region) and negatively affected kelp photosynthetic efficiency (B, quantum yield describes the probability that an absorbed light quanta are utilized to push electrons through photosystem II; mean + SE, $n = 15$). NSW, TAS and WA: biogeographical provinces along the east and west coast of Australia (Table 1). Arrows point towards increasing latitude.

variance (ANOVA) $F_{2,18} = 0.56$ and 3.45, respectively, $P > 0.05$, Fig. 1A]. Bleaching was rare only at the southernmost (coldest) site in TAS (~ 10%; Fig. 1A). Bleached individuals exhibited signs of physiological stress, with significantly lower photosynthetic efficiency than unbleached kelp (ANOVA $F_{1,28} = 47$, $P < 0.001$, Fig. 1B).

The 16S rRNA gene sequencing information for the microbial community of 260 algal samples was analysed at the level of operational taxonomic units (OTU) at 97% sequence identity. Overall, 1107 OTUs were obtained using a closed-reference protocol, which only considered reads that matched the Greengenes database (> 97% identity), thus representing the part of *E. radiata*'s microbial community that has been previously described in other 16S rRNA gene-based studies. These OTUs belonged to 235 families, 116 orders, 89 classes and 49 phyla, revealing a taxonomically diverse microbial community on *E. radiata*. In addition, 7426 OTUs were obtained using an open-reference protocol, which is based on *de novo* clustering of sequences and therefore describes the total OTU diversity, previously described or not. A comparison of the two protocols indicated that about 85% of the OTU diversity on *E. radiata* could not be classified to previously described bacteria and archaea. Communities on both kelp phenotypes were well represented for both protocols (Fig. S1).

Microbial community structure consistently differed between healthy and bleached kelp, despite significant variability among sites, using both the relative abundance of OTUs ('structure') and composition (only presence/absence of OTUs) obtained through the closed-reference protocol [permutational multivariate analyses of variance (PERMANOVA) pseudo-$F_{17,208} = 2.50$ and 1.70 for structure and composition, respectively, on independent bleached or healthy tissue samples at each site, $P < 0.001$, Table 2, Fig. 2A]. This difference was consistent for 89% (structure) and 77% (composition) of all sites across Australia (post-hoc pairwise tests, $P < 0.05$, Table S1). Overall, 85 OTUs (8%) were unique to healthy kelp and 60 (5%) to bleached kelp. Estimates of the components of variation in the PERMANOVA analyses showed that kelp condition (healthy versus bleached) explained a significantly greater amount of the variation in microbial communities across the continent than geographical location (Table S2 and Fig. 2B), despite differences between biogeographical provinces and regions within each province (Table 2, Fig. 2A).

These differences in microbial community structure and composition between host types were even stronger when using the open-reference protocol, with significant differences for 92% (structure) and 96% (composition) of the sites (PERMANOVA pseudo-$F_{17,208} = 2.41$ and 1.85 for structure and composition, respectively, $P < 0.001$; post-hoc pairwise tests, $P < 0.05$; Table 2 and Table S1). Kelp condition, rather than spatial differences, again explained a significantly greater amount of the variability in microbial community structure for these data (Table S2).

The extent to which geography affected patterns in microbial community structure differed between healthy and stressed thalli, with a lack of consistent latitudinal pattern for microbial communities on stressed, but not healthy hosts (PERMANOVA, interaction between kelp condition and region, pseudo-$F_{2,17} = 1.80$, $P < 0.05$, Table 2). Microbial communities on bleached individuals were more similar to each other among regions, while healthy hosts maintained region-specific biofilm structures (post-hoc pairwise tests, $P < 0.05$, Table 2).

Table 2. PERMANOVAs based on Bray–Curtis dissimilarity measure for square root-transformed relative abundance or presence/absence (P/A) of closed- or open-reference OTUs on healthy and bleached kelp across Australia.[a]

		Closed-reference						Open-reference					
		Square root			P/A			Square root			P/A		
Source	*df*	MS	pseudo-*F*	*P*	MS	pseudo-*F*	*P*	MS	pseudo-*F*	*P*	MS	pseudo-*F*	*P*
Co	1	34 941	30.55	< **0.001**	6776	8.20	< **0.001**	52 465	22.82	< **0.001**	27 164	22.72	< **0.001**
BP	2	8958	4.82	< **0.001**	4417	3.41	< **0.001**	19 484	6.65	< **0.001**	11 238	7.00	< **0.001**
Re	2	4171	2.24	**0.005**	2318	1.79	**0.044**	7460	2.55	< **0.001**	4145	2.58	< **0.001**
Co × BP	2	4350	3.80	< **0.001**	1782	2.16	**0.009**	9067	3.94	< **0.001**	3839	3.21	< **0.001**
Co × Re	2	1944	1.70	0.053	936	1.13	0.312	4132	1.80	**0.025**	2040	1.71	0.050
BP × Re	4	5281	2.84	< **0.001**	3609	2.79	< **0.001**	8336	2.85	< **0.001**	5396	3.36	< **0.001**
Si (BP × Re)	17	1859	4.06	< **0.001**	1294	2.66	< **0.001**	2928	3.07	< **0.001**	1606	2.48	< **0.001**
Co × BP × Re	4	1381	1.21	0.205	963	1.17	0.252	3205	1.39	0.058	1774	1.48	0.054
Co × Si (BP × Re)	17	1144	2.50	< **0.001**	826	1.70	< **0.001**	2299	2.41	< **0.001**	1196	1.85	< **0.001**
Residual	208	457			487			953			647		
Pairwise tests													
Co × Si [Re(BP)]: Co		He ≠ Bl in 89% of sites (Table S1)			He ≠ Bl in 77% of sites (Table S1)			He ≠ Bl in 92% of sites (Table S1)			He ≠ Bl in 96% of sites (Table S1)		
Co × BP: BP		He and Bl: NSW ≠ TAS ≠ WA			He: NSW ≠ TAS ≠ WA Bl: NSW ≠ TAS = WA			He and Bl: NSW ≠ TAS ≠ WA			He and Bl: NSW ≠ TAS ≠ WA		
BP × Re: BP		N: NSW ≠ TAS = WA C and S: NSW ≠ TAS ≠ WA			N: NSW ≠ TAS = WA C and S: NSW ≠ TAS ≠ WA			N, C, S: NSW ≠ TAS ≠ WA			N, C, S: NSW ≠ TAS ≠ WA		
BP × Re: Re		NSW: N = C; N ≠ S; C = S TAS: N = C; N = S; C ≠ S WA: N ≠ C ≠ S			NSW: N ≠ C = S TAS and WA: N ≠ C ≠ S			NSW: N ≠ C = S TAS and WA: N ≠ C ≠ S			NSW: N ≠ C = S TAS and WA: N ≠ C ≠ S		
Co × Re: Re								He: N = C ≠ S Bl: N = S ≠ C					

a. Condition (Co) was fixed with two levels (healthy He versus bleached Bl); biogeographical province (BP) was fixed, orthogonal with three levels (NSW, TAS, WA); region (Re) was fixed, orthogonal, with three levels (north N, centre C, south S); site (Si) was random, nested in St × Re, with three levels except for one region in WA, with two levels. The replicates were the kelp laminae ($n = 5$).

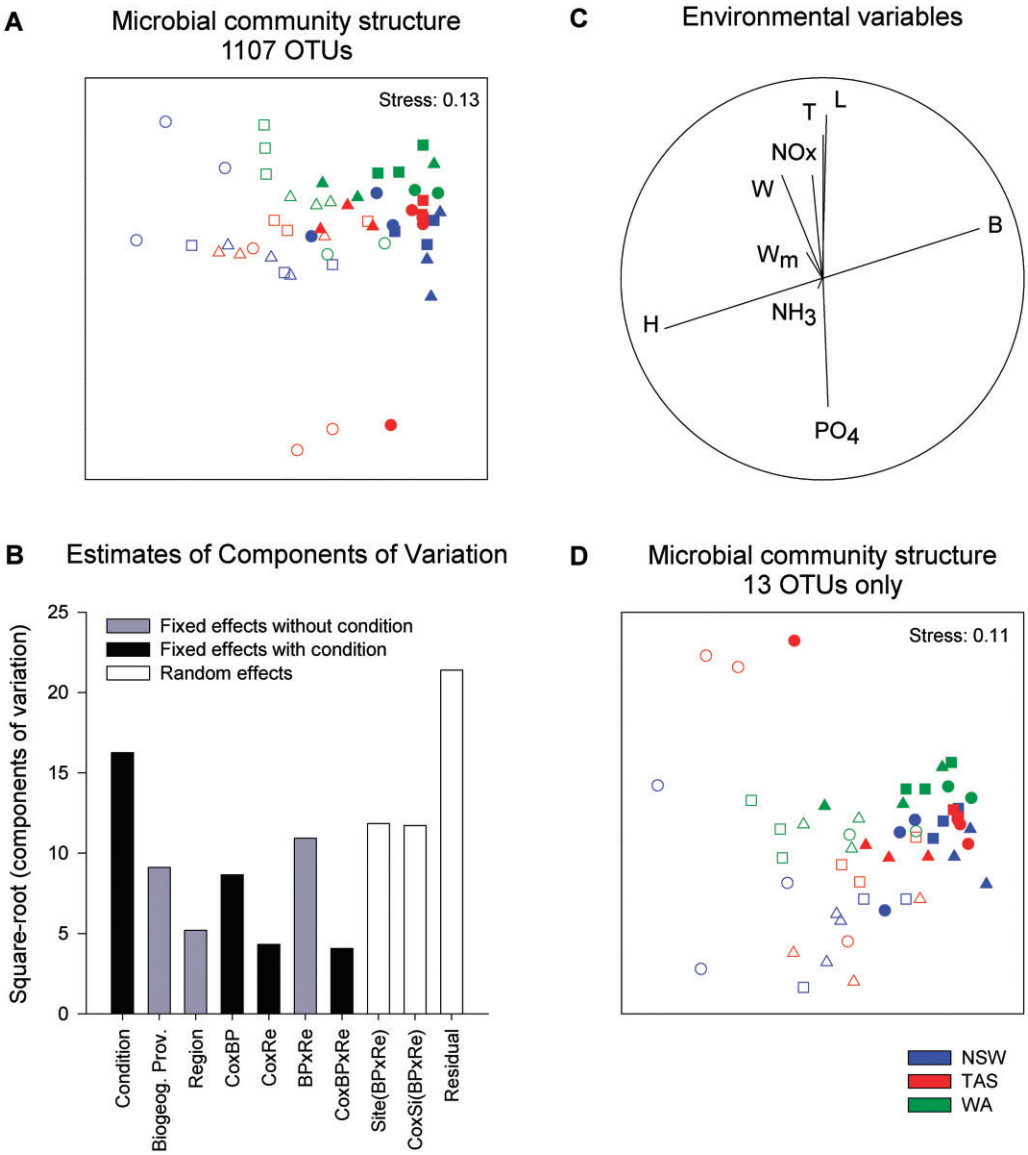

Fig. 2. Continental-scale variation in microbial communities was more strongly related to host condition than geography (A, B) or associated environmental variables (C), and was explained by small numbers of OTUs (D).
A. nMDS of distances among centroids (sites; Bray–Curtis on square-root OTU abundance, closed-reference protocol) for healthy (empty symbols) or bleached hosts (filled symbols) in each biogeographical province (NSW, TAS, WA; regions north: circles; centre: squares; south: triangles).
B. Components of variation estimates for fixed and random terms in the model (community structure, closed-reference protocol).
C. Pearson's correlations between community structure (in A) and environmental variables (T: mean temperature, L: mean light intensity, W: mean wave energy, W_m: maximum wave energy); host condition (H: healthy, B: bleached).
D. nMDS (as in A) based on a subset of 13 OTUs strongly correlated to the full OTU set (1107 OTUs).

We further investigated whether there were fundamental differences in the properties of communities on healthy versus stressed hosts using network analysis. These analyses showed significantly higher connectedness among OTUs in communities on healthy than on bleached kelp [average clustering coefficient (CC): 0.35 ± SE 0.01 versus 0.20 ± SE 0.01, ANOVA $F_{1,4} = 140$, $P < 0.001$; Fig. S2].

Relationships between geographical differences in microbial communities and environmental variables for healthy and bleached hosts

Because environmental variables were measured on a per site basis (which included both healthy and stressed kelps), it was not possible to do an analysis examining the interactive effect of these variables and host condition on

microbial communities. Thus, analyses of relationships between environmental variables and microbial communities were done separately for healthy or bleached hosts (*Experimental procedures*). Differences in environmental variables explained two to three times the amount of the variability in microbial communities on healthy than on bleached kelp among biogeographical provinces and regions (24–33% and 10–13%, respectively, distance-based linear modelling (DISTLM) analyses, Table S3 and Fig. 2C). This is consistent with the idea that healthy *E. radiata* maintain microbial communities more specific to their environment, but when algae become stressed and bleach this specificity breaks down. *In situ* mean water temperature and mean light intensity were strongly and consistently related to microbial communities on healthy and bleached kelp, with changes in community structure and composition following a gradient from lowest (TAS) to highest (WA) mean temperature and light values. Mean and maximum wave motion were also strongly related to microbial communities, but only on healthy hosts, while closed-reference community composition was strongly related to phosphate concentration (Fig. 2C, Table S3 and Fig. S3).

Structural redundancy in host-associated microbial communities

In order to determine whether there was a 'core' set of microbial taxa in the kelp holobiont, we identified the smallest subset(s) of OTUs with a multivariate pattern of community structure that closely matched that of the whole community using a stepwise procedure (*Experimental procedures*). A smallest subset of 13 OTUs (1.2% of the total pool) was found whose similarity matrix across all samples correlated strongly with that of the full OTU set obtained through the closed-reference protocol (1107 variables; BVSTEP analyses $\rho > 0.9$, Fig. 2D). This was not the only subset of OTUs that could explain the observed pattern: three other independent sets of 17 (1.5%), 23 (2.1%) and 32 (2.9%) OTUs were also found to correlate strongly ($\rho > 0.9$) with the multivariate pattern of the entire microbial community (all OTUs belonged to Bacteria). In combination, these subsets represented < 8% of the total number of OTUs and ~ 33% of the total sequences obtained. In subsequent subsets ($\rho < 0.9$), the numbers of OTUs increased abruptly as ρ decreased (Fig. S2).

We further used SIMPER analysis to determine the OTUs contributing specifically to the differences in the communities on healthy versus bleached hosts. Forty-two (4%) and 286 (26%) OTUs contributed to 50% and 90% of the difference in community structure between healthy and bleached kelp respectively (Fig. 3). The OTUs that were more abundant in bleached samples belonged at the family-level, mostly to the taxa *Rhodobacteraceae* and *Flavobacteriaceae* (16 out 29 OTUs that were enriched in bleached samples). Within those families, some OTUs could be assigned to genera whose bacterial members have previously been shown or implied in causing disease

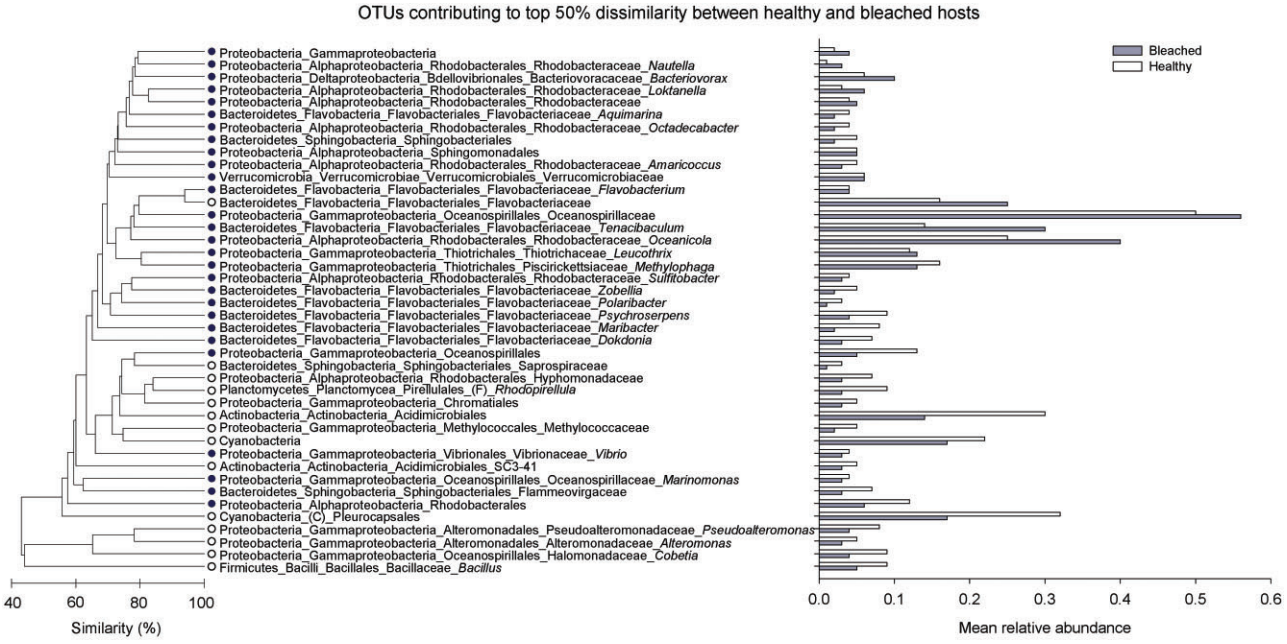

Fig. 3. Dendrogram of Bray–Curtis similarities for OTUs' standardized abundance showing the 4% of known OTUs that contributed 50% to the dissimilarity in community structure on healthy versus bleached hosts. Mean abundance (square root-transformed) on healthy (white bars) and bleached (grey bars) hosts is shown next to each OTU.

or damage to macroalgae. These include, for example, the genus *Nautella*, which contains a pathogen that caused a bleaching disease in the red algae *Delisea pulchra* (Case *et al*., 2011), and the genus *Aquamarina*, whose members have been shown to possess diverse agarolytic and algicidal activities (Chen *et al*., 2012; Lin *et al*., 2012).

Discussion

Our study assessed community structure and distribution of marine microorganisms associated with sessile seaweed hosts at a continental scale across three distinct biogeographical provinces. These microbial communities were most strongly and consistently associated with host condition, i.e. whether the host exhibited signs of stress or not, rather than with geographical location or related differences in environmental variables. While there was geographical variation in these communities as well, particularly where hosts were healthy, the strong geographical pattern observed in large-scale studies of marine planktonic microbial communities was not evident. This suggests that host traits are potentially more important in influencing the structure of host-associated microbial communities than large-scale spatial and environmental factors (Rosenberg *et al*., 2007; Singh *et al*., 2013).

Recent theoretical work has shown that planktonic microorganisms can evolve at a faster rate than their rate of dispersal by oceanographic processes, leading to strong biogeographical differences in community composition (Hellweger *et al*., 2014). This model is supported by data from large-scale sampling of planktonic microbial communities, which also show that gradients of environmental variables, such as water temperature and light, strongly influence such communities (Rusch *et al*., 2007; Brown *et al*., 2012; Gilbert *et al*., 2012). In contrast, our data showed strong consistency in the structure of host-associated microbial communities at similar spatial scales. This could suggest that the oceans may act as sources of microbial diversity, where microbes evolve rapidly (potentially as a result of shifts in oceanographic conditions), whereas hosts may act as reservoirs, colonized by a subset of bacteria available in the planktonic species pool that persist in the microenvironment provided by the host.

Where relationships between latitude or environmental variables and host-associated microbial communities did exist, these were stronger for communities associated with healthy hosts than those on bleached hosts. This suggests that environmental effects and consequent spatial variation in microbial communities on healthy hosts may break down when hosts are stressed, with the relationship between hosts and their associated microbial communities becoming disrupted and less stringent.

While microbial communities associated with stressed versus healthy hosts differed significantly, these differences could be attributed to a relatively small subset of OTUs. Less than 8% of the OTUs explained most of the variability in community structure, and within these, several independent subsets of OTUs showed the same pattern of overall community structure, indicating ecological redundancy among microbial taxa. That is, there are likely different subsets of bacteria that have the same or closely related affinity to host traits or the microenvironment provided by the host, or that are correlated as a result of ecological interactions. This is consistent with the competitive lottery model derived from macroecology (Sale, 1976), where several functionally redundant microbial 'species' can colonize a particular habitat, but the taxonomic outcome for the final community composition depends on the randomness of the initial colonization (Burke *et al*., 2011). Neutral ecological processes may result in different bacteria colonizing surfaces of hosts in different locations, but host traits may subsequently determine a 'core' community assembly through selective processes.

While we do not yet know if the differences in microbial communities are causally linked to host condition for these kelp, disruptions to the microbial consortium of holobionts via host stress can have strong detrimental effects on the host because bacteria within surface biofilms can provide key nutrients, can be integral to the hosts' normal development and may contribute to host defences against natural enemies (Nasrolahi *et al*., 2012; Egan *et al*., 2013). Several studies on other organisms, such as corals and sponges, and even humans, have shown that these disruptions can result in disease (Rosenberg *et al*., 2007; Fan *et al*., 2013; Singh *et al*., 2013), and some of the bacteria found here to be enriched in bleached kelp fall into taxonomic clades that contain known or putative seaweed pathogens (e.g. *Nautella*). In marine systems, e.g. in tropical coral reefs (Rosenberg *et al*., 2007), disease is emerging as a significant ecological factor, and stressed organisms are generally more susceptible to diseases. However, with some rare exceptions (Campbell *et al*., 2011; Case *et al*., 2011), an understanding of the impact of disease on temperate algal forests is almost completely lacking. Algal bleaching can be a sign of stress (Scrosati and DeWreede, 1998; Martone *et al*., 2010) or disease (Campbell *et al*., 2011). This phenomenon appears to be widespread for kelp, with similar proportion of individuals and severity across several biogeographical provinces along the continent. Moreover, observed bleaching was similar to the bacterially induced bleaching described for the red seaweed *D. pulchra* in SE Australia (Campbell *et al*., 2011; Case *et al*., 2011). Although a causal link between bleaching and surface bacteria has not been established for *E. radiata*, the parallels with *D. pulchra* are notable.

Given that habitat-forming seaweeds fundamentally define temperate coastal rocky shores, the potential for algal disease to dramatically affect temperate marine ecosystems is no less significant than that of diseases of coral reefs in the tropics (Steneck *et al*., 2002; Wernberg *et al*., 2011b). Holistic studies linking specific, identified pathogens to their ecological impact, or testing specific hypotheses about microbial disease and habitat or environmental change, are almost entirely lacking for kelp forests. Understanding how hosts and microorganisms interact and how the holobiont adapts to environmental stresses is critical.

Experimental procedures

Sampling design and kelp condition

A total of 27 *Ecklonia radiata* populations were surveyed along the east and west coast of Australia from 5 April to 5 May 2011 (Table 1), encompassing most of the distribution range of this kelp (South Queensland to mid-WA). We surveyed kelp at 8–12 m depth in three sites separated by 3–4 km within three regions (north, centre, south), 100–270 km apart in each of three states, New South Wales (NSW; eastern Australia), Tasmania (TAS; south-eastern Australia) and Western Australia (WA), each in a different biogeographical province (Wernberg *et al*., 2013).

In order to compare the influence of host condition with that of geographical variation, microbial communities of 'stressed' versus healthy kelp were compared at each site. The marker of stress was bleaching, visible as a whitening of algal tissue, often associated with loss of surface integrity and known to be associated with microbial disease in other macroalgae (Campbell *et al*., 2011; Case *et al*., 2011). We distinguished bleaching as defined here from discolouration and decay associated with natural senescence by excluding the older tissue (distal tips) of the primary and secondary laminae from our sampling. To characterize the incidence of bleaching, 10 kelp were haphazardly selected while scuba diving, and the presence and percentage cover of thallus bleaching were quantified.

In addition, to characterize the impact of bleaching and to have an objective measure of stress of these hosts, laminae of five healthy and five bleached individuals were collected at three Sydney sites (NSW; Table 1) to quantify photosynthetic efficiency *in situ* (quantum yield, i.e. the probability that an absorbed light quanta are utilized to push electrons through photosystem II, using a Diving-PAM fluorometer), as well as reflectance (colour) at 600 nm in the laboratory (using an OceanOptics spectrometer) to obtain a quantitative measure of the bleaching phenotype. Visibly bleached tissue was clearly distinguishable, with a significantly higher reflectance than 'healthy' tissue (ANOVA, $F_{1,28} = 80$, $P < 0.001$).

Measurement of environmental variables

At each site, a rope with a hemispherical buoy was attached to a flat section of the seabed and Hobo® pendant data loggers, and waveline magnets (Onset HOBO #UA-002-64 and Onset HOBO G-logger #UA-004-64) were cable-tied to the upward facing flat side of the buoy to measure *in situ* water temperature (°C), illuminance (Lux) as a measure of light intensity, and wave motion ($m\ s^{-2}$) daily at ~ 50 cm above the seabed (approximate kelp laminae height). Loggers were collected after 1 month, and the mean and maximum values were calculated for each variable. In addition, water samples (50 ml) were collected *in situ* using a syringe. Samples were then taken to the surface, filtered using a 0.45 μm polypropylene GMF syringe filter and stored at –20°C until processing. NH_3, NO_x and PO_4 concentrations ($mg\ l^{-1}$) were quantified using a Lachat Quickchem 8500 Flow Injection Analyser (Hach, CO, USA).

Analysis of associated microbial communities

To characterize microbial communities associated with surfaces of healthy and bleached kelp tissues, we consistently sampled the middle section of a secondary lamina located at approximately the same distance from the meristem of five healthy and five bleached individuals per site. Samples were enclosed individually inside press-seal bags *in situ* and brought to the surface where 20 cm^2 of each algal surface was rinsed with filtered seawater, then wiped gently with a sterile cotton swab for 30 s. The swabs were aseptically transferred into sterile cryo-tubes and immediately stored in a dry-shipper with liquid nitrogen onsite. Samples were then transferred to a –80°C freezer in the laboratory until processing.

Microbial DNA was extracted from each cotton swab using a 96-well PowerSoil DNA Isolation Kit (MO Bio Laboratories) and stored at –20°C. The v4 region of the bacterial and archaeal 16S rRNA gene was amplified and sequenced on a HiSeq2000 platform as part of the Earth Microbiome Project (EMP) (Caporaso *et al*., 2012). Sequences were processed using the QIIME software (Caporaso *et al*., 2010) following the standard protocols of the EMP (http://www.earthmicrobiome.org), which consists of the following steps. The sequencing reads were quality-filtered by first trimming the 3′ end of each read until the first base with a quality score of 3 or less. If the truncated read was shorter than 75 bp or its barcode did not match the expected collection of barcodes, it was discarded. Quality-filtered reads were then assigned to OTUs using both a closed-reference and a subsampled open-reference OTU picking protocol (Caporaso *et al*., 2010). In the closed-reference protocol, only the reads that matched with a minimum of 97% identity to the Greengenes database (DeSantis *et al*., 2006) were assigned to OTUs and the remaining reads were ignored. For the subsampled open-reference protocol, first reads that failed to match the Greengenes database with a minimum of 60% sequence identity were filtered out. The remaining reads were clustered against a reference database, and those that fail to hit the reference were randomly subsampled and clustered *de novo*. A representative sequence of each of these new OTU clusters was used to make a new reference. In a second round of closed reference clustering, more sequences were clustered against the reference obtained in the last step. Reads that failed to hit the reference were clustered *de novo* (Caporaso *et al*., 2010). Quality-filtered reads from the HiSeq Illumina platform may still produce spurious OTUs (Reeder and

Knight, 2009); however, this can be dealt with by applying additional abundance filters to the OTU table (Bokulich *et al*., 2013). Therefore, the OTUs obtained from the subsampled open-reference protocol were removed whenever they had an average abundance across all samples below one. Ten samples, all from a site in north WA (J3 in Table 1), were lost resulting in a final total of 260 sequenced microbial communities.

Statistical analyses

The proportion of bleached kelp at each site and the percentage cover of bleached tissue per individual were compared across biogeographical provinces (fixed, three levels: WA, NSW, TAS), among regions (fixed, three levels: North, Centre, South) and sites (random, nested in state × region, three levels) using ANOVA in GMAV 5 (University of Sydney, Australia). Analysis of variance was also used to compare photosynthetic efficiency between healthy and bleached tissues. Microbial community data (closed- and open-reference) were compared between kelp condition (fixed, two levels: healthy versus bleached) and biogeographical provinces, regions and sites (as above) using PERMANOVA (Anderson, 2001) in the PERMANOVA+ add-on for PRIMER v6 (PRIMER-E, UK) (Anderson *et al*., 2007). Similarity matrices based on Bray–Curtis distances of square root-transformed relative abundance ('community structure', which takes into account identity and abundance of OTUs) or presence/absence ('community composition', which only takes into account identity) were generated for the analyses, which used 9999 permutations of residuals under a reduced model. Estimates of components of variation for fixed and random effects in the model were also obtained from PERMANOVA to compare the relative importance of different terms in the model towards explaining overall variation (Underwood and Petraitis, 1993; Anderson *et al*., 2007). To visualize multivariate patterns in microbial assemblages, distances among centroids for each site were calculated in PERMANOVA+, and non-metric multidimensional scaling (nMDS) was used as an ordination method using PRIMER v6.

Multivariate multiple regression (DISTLM) (McArdle and Anderson, 2001) was used to determine relationships between microbial community structure and composition with environmental variables. Environmental variables that were strongly inter-correlated ($|r| > 0.9$) were excluded from the model, leaving one of these to represent the mutually correlated set (detailed in *Supporting information*). Analyses used Akaike's AIC (An Information Criterion) selection criterion and a best selection procedure to select the best explanatory model, and were done in DISTLM within the PERMANOVA+ add-on (Anderson *et al*., 2007). *P*-values for marginal tests (testing relationships with each environmental variable independently of the others) were calculated using 9999 permutations.

For community structure data obtained using the closed-reference protocol, SIMPER analysis (Clarke, 1993) was used to determine the OTUs contributing most to the difference between bleached versus healthy kelp. A Bray–Curtis OTU similarity matrix from standardized relative abundance was constructed for the subset of OTUs contributing to the top 50% dissimilarity between both conditions to determine patterns of relationships among them using CLUSTER analysis (Clarke and Warwick, 1998). The OTUs selected by SIMPER with < 0.5% relative abundance difference between healthy and bleached hosts were not included in the CLUSTER analysis (< 2%). In addition, BVSTEP analysis was used to find the smallest subset(s) of OTUs with a multivariate pattern of community structure that closely matched that of the whole community, i.e. a between-samples similarity matrix using the minimum number of OTUs with a Spearman rank correlation $\rho > 0.9$ to the total OTU similarity matrix. A stepwise procedure was used to randomly select subsets of OTUs, with five random starts each (Clarke and Warwick, 1998). The selected OTU subset was removed from the matrix, and the procedure was repeated 10 times. These multivariate analyses were done in PRIMER v6 (PRIMER-E, UK).

Network analysis

Networks were generated to compare connectivity between OTUs in communities on healthy and bleached hosts. Data were separated by biogeographical province providing three replicate networks for both healthy and bleached kelp. Correlations between OTUs were calculated using the maximal information coefficient (MIC) (Reshef *et al*., 2011). The MIC between OTU pairs were only considered if present in > 80% of the samples and with $P < 0.001$. Network average CC, which is a measure of connectedness, was calculated for each network using NetworkAnalyser (Assenov *et al*., 2008). The CCs were compared between healthy and bleached communities using a one-factor ANOVA ($n = 3$). Networks were visualized using CYTOSCAPE v2.8.3 by uniting the three healthy and, separately, the three bleached networks (Lopes *et al*., 2010).

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Supporting information

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

Fig. S1. Rarefaction curves for the closed- and open-reference protocols.

Fig. S2. Connectedness and structural redundancy in microbial communities on kelp.

Fig. S3. Distance-based redundancy analyses of relationships between environmental variables and microbial communities.

Table S1. Outcome of pairwise comparisons of analyses of microbial communities at each site.

Table S2. Estimates of components of variation in the microbial community analysis.

Table S3. Distance-based linear models of relationships between environmental variables and microbial communities.