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ORIGINAL ARTICLE

Bacterial diversity in relation to secondary production and succession on surfaces of the kelp *Laminaria hyperborea*

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Kelp forests worldwide are known as hotspots for macroscopic biodiversity and primary production, yet very little is known about the biodiversity and roles of microorganisms in these ecosystems. Secondary production by heterotrophic bacteria associated to kelp is important in the food web as a link between kelp primary production and kelp forest consumers. The aim of this study was to investigate the relationship between bacterial diversity and two important processes in this ecosystem; bacterial secondary production and primary succession on kelp surfaces. To address this, kelp, *Laminaria hyperborea*, from southwestern Norway was sampled at different geographical locations and during an annual cycle. Pyrosequencing (454-sequencing) of amplicons of the 16S rRNA gene of bacteria was used to study bacterial diversity. Incorporation of tritiated thymidine was used as a measure of bacterial production. Our data show that bacterial diversity (richness and evenness) increases with the age of the kelp surface, which corresponds to the primary succession of its bacterial communities. Higher evenness of bacterial operational taxonomical units (OTUs) is linked to higher bacterial production. Owing to the dominance of a few abundant OTUs, kelp surface biofilm communities may be characterized as low-diversity habitats. This is the first detailed study of kelp-associated bacterial communities using high-throughput sequencing and it extends current knowledge on microbial community assembly and dynamics on living surfaces.
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Introduction

Kelp forests are complex three-dimensional habitats structured by large brown seaweeds of the order *Laminariales* (kelps). They dominate most subtidal rocky shores in temperate latitudes but also occur in upwelling zones in the tropics (Graham *et al.*, 2007) and in the arctic (for example Dunton *et al.* (1982)). Kelp forest ecosystems are characterized by extraordinary biodiversity and productivity (Mann, 1973; Duggins *et al.*, 1989) and provide important ecosystem services, for example as feeding grounds for commercially important fish (Norderhaug *et al.*, 2005). They sustain rich communities of invertebrates (Coyer, 1984; Christie *et al.*, 2003) and epiphytic seaweeds (Tokida, 1960). The factors regulating biodiversity of macroscopic organisms in kelp forests, and the consequences of biodiversity on ecosystem function is a central topic in marine ecology (reviewed by Steneck *et al.* (2002)). However, although the macrofauna and -flora of kelp forests have been relatively well investigated, there is still very little knowledge on the biodiversity of microorganisms associated to kelp. Recent studies using culture-independent methods have provided important information on the community dynamics and phylogenetic identity of the most abundant bacteria on the surfaces of a few kelp species (Bengtsson *et al.*, 2010). However, the diversity of these bacterial communities has most likely been underestimated so far, as many natural bacterial communities appear to possess an enormous richness of different bacterial taxa equivalent to species that are present (Torsvik *et al.*, 1990).

Secondary production by heterotrophic bacteria is of crucial importance in kelp forest ecosystems. Through full and partial degradation of dissolved and particulate organic carbon, bacteria make kelp primary production available to animal consumers (Newell and Field, 1983; Norderhaug *et al.*, 2003). Dissolved organic carbon is continuously exudated from living kelp cells (Abdullah and Fredriksen, 2004) and is also released upon fragmentation of kelp material (Newell *et al.*, 1980). Kelp dissolved organic carbon can support secondary production by

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bacteria living on the surfaces of kelp and in the surrounding water masses. Particulate organic carbon from kelp comprises everything from suspended aggregations of kelp mucosa to microscopic eroded kelp fragments and detached whole kelp thalli and may support secondary production by attached bacteria and filter feeders in or near the kelp forest (Newell and Lucas, 1981; Duggins *et al.*, 1989; Fredriksen, 2003). In addition, kelp material can be exported to shores (Bradley and Bradley, 1993), to the open ocean (Koop *et al.*, 1982) and to the deep (Harrold *et al.*, 1998; Bernardino *et al.*, 2010) and contribute to secondary production in these ecosystems. Bacteria in biofilms growing on the surfaces of living kelp and detached kelp fragments may contribute to the food web when grazed upon by other microscopic and macroscopic organisms. It is not known what controls the rate of bacterial production in these biofilms. Possible influencing factors may include temperature, defense strategies of the kelp that inhibit bacterial growth (Küpper *et al.*, 2002), but also the composition and diversity of the bacterial community itself.

L. hyperborea is the dominant kelp forest forming species along the Atlantic coasts of Europe. It has a pronounced seasonal growth cycle, in which a substantial part of its biomass, the blade-like lamina, is renewed every year. The lamina grows out from a meristematic growth region from February to early June, after which it stops growing until a new lamina replaces it the next year. This creates a gradient of kelp surface age during the year (Supplementary Figure S1), and a corresponding successional gradient of its bacterial communities.

The link between ecosystem productivity and biodiversity is an important topic in ecology (Hooper *et al.*, 2005). However, microbial diversity has rarely been studied in the context of productivity of microbial communities (Naeem *et al.*, 2000; Langenheder *et al.*, 2010; Peter *et al.*, 2011). Similarly, the process of primary succession, where freshly formed habitats become colonized by a sequence of organisms, has a long-standing tradition of study in the ecology of macroscopic organisms, yet is only since recently emerging as a topic in microbial ecology (Fierer *et al.*, 2010). We consider kelp surfaces an excellent model system to study the relationship between bacterial production and bacterial diversity, as well as the effect of succession on bacterial diversity, because of the importance of these two processes in kelp forest ecosystems.

In this study, we investigate the diversity (richness, evenness and community composition) of bacterial communities on surfaces of the kelp *L. hyperborea* in relation to bacterial secondary production and successional dynamics. More specifically, we ask the following questions: (i) Is there a relationship between the bacterial diversity and the secondary production rate of the kelp surface biofilms? (ii) How does bacterial diversity change during the natural successional process caused by the growth and aging of the kelp lamina? We used pyrosequencing of amplicons of the 16S rRNA gene of bacteria to study the diversity of kelp surface bacterial communities, analyzed as richness, evenness and phylogenetic affiliation of bacterial operational taxonomical units (OTUs). To explore the relationship between bacterial diversity and bacterial production, incorporation of tritiated thymidine was measured in kelp surface biofilms.

Materials and methods

Sampling

Kelp material was collected for bacterial production measurements and diversity analyses in July 2009 at three different sampling sites. These sites were chosen to capture different wave exposure regimes representative to the study area, as wave exposure is an important factor influencing the growth of *L. hyperborea* (Kain, 1971) and may also influence its associated bacterial communities. The sites were; Tekslo (60° 09.706′ N, 5° 02.371′ E), Landro (60° 25.338′ N, 4° 57.472′ E) and Flatevossen (60° 16.119′ N, 5° 12.432′ E). Tekslo is the most wave-exposed site, followed by Landro and Flatevossen (see Supplementary Figure S2). Wave exposure level was determined according to a geographical information system (GIS)-based wave exposure model (Isaeus, 2004). Kelp material was collected by dredging (Tekslo) and free diving (Landro and Flatevossen) at a depth of 3–6 m. Four (Tekslo) or six (Landro and Flatevossen) replicate kelp individuals were collected from each site. Bacterial production measurements, DNA extraction and subsequent diversity analyses were carried out as described in the following sections.

For bacterial diversity analyses along a successional gradient, kelp was collected at one sampling site (Tekslo) during an annual cycle (March–November 2007). The kelp sampling during an annual cycle was performed as described in detail in Bengtsson *et al.* (2010). In summary, kelp was collected by dredging from a boat at 2-monthly intervals during the year 2007 (see Supplementary Table S1). One sampling occasion (February 2007) was excluded from this study because the sampling procedures differed slightly compared with the other occasions (macroscopic epiphytes such as bryozoans and ascidians were avoided during biofilm sampling at all sampling occasions except in February 2007). Six replicate kelp individuals were sampled at each time point and two distinct thallus regions (meristem and lamina) of each kelp individual were sampled. The samples from the different time points and kelp thallus regions represent a gradient in kelp surface age, ranging from freshly formed ($T=0$ months) surface to aged ($T=12$ months) surface (see Supplementary Figure S1). Biofilm was removed from the kelp surfaces by means of scraping with a sterile scalpel. The scraped off biofilm material was used for DNA extraction

and subsequent diversity analyses as described in the following sections. A detailed overview of the different samples collected is given in Supplementary Table S1.

Bacterial production measurements

To account for any heterogeneity in the biofilm density and diversity within each individual, three spatially separate but morphologically equivalent areas of the kelp laminae were sampled. Pieces (4×4 cm) were cut out from each area using a sterile scalpel and were thoroughly rinsed with sterile seawater. The kelp pieces were further subdivided into 16 1×1 cm pieces. Before the bacterial production measurements, the kelp pieces were preincubated in sterile seawater at 10 °C for 24 h under constant agitation in order for the kelp tissue to acclimatize.

Bacterial production was measured as incorporation of ^{3}H-labeled thymidine (Fuhrman and Azam, 1980) in bacterial cells attached to kelp surfaces. Three 1×1 cm kelp pieces, one from each separate area of the same kelp individual, were incubated in 10 ml of sterile seawater in glass scintillation vials at a concentration of 12.5 nM (methyl)-^{3}H-Thymidine (specific activity 48 Ci mmol^{-1}, PerkinElmer, Waltham, MA, USA) for 1 h at 10 °C under constant agitation (200 r.p.m.). The temperature was near the ambient seawater temperature at the time of sampling. Different ^{3}H-Thymidine concentrations and incubation times were evaluated in pilot experiments to ensure a linear uptake rate. Incubations were terminated by adding formaldehyde to a final concentration of 1% to the incubation vials. The biofilm was separated from both sides of the kelp pieces by scraping with a scalpel. The scraped off biofilm material was suspended in 1.5 ml ice-cold trichloroacetic acid and pelleted by centrifugation. The pellet was washed twice in ice-cold trichloroacetic acid and twice in cold 70% ethanol. The pellet was subsequently air-dried and suspended in scintillation cocktail (Ultima Gold, PerkinElmer), transferred to 5 ml scintillation vials filled with the same liquid and incubated overnight before scintillation counting. Bacterial biofilm uptake of ^{3}H-Thymidine was measured as decompositions per minute (DPM) in a TRI-CARB 1900 CA liquid scintillation counter (Packard Instruments, PerkinElmer). DPM values were corrected by subtracting the values from a control incubation where formaldehyde was added before ^{3}H-Thymidine addition. Triplicate incubations of each kelp individual were carried out. Two sets of parallel incubations without the addition of ^{3}H-Thymidine and formaldehyde were carried out using kelp pieces from the same areas of the same kelp individual. Biofilm material from the first set of parallel incubations was scraped off and processed as described in Bengtsson *et al.* (2010) and used for DNA extraction and subsequent bacterial diversity analyses. The kelp pieces from the second set were fixed, stained with 4', 6-diamidino-2-phenylindole (DAPI) and used for biofilm cell counting as described in Bengtsson *et al.* (2010). Average cell division times were calculated from ^{3}H-Thymidine incorporation (DPM) and cell density data according to Fuhrman and Azam (1982) using a conversion factor of 2.0E18 cells dividing per mol of thymidine incorporated (Pollard and Moriarty, 1984).

Bacterial diversity analyses

16S rRNA gene amplicon generation and pyrosequencing. DNA extraction of the biofilm material from all samples was carried out as described in Bengtsson *et al.* (2010). For the seasonal cycle samples, the extracted DNA from the six replicate kelp individuals was pooled (in equal volumes) into one sample per sampling occasion and kelp region (meristem and lamina). This was done to get a representative kelp forest-scale view of the bacterial diversity of each seasonal sampling occasion. An analysis of most (MarL and SepL were not analyzed by denaturing gradient gel electrophoresis (DGGE)) of the replicated samples that were used to make the pooled samples was carried out on DGGE data gathered in a previous study (Bengtsson *et al.*, 2010). The extracted DNA from the production experiment samples was not pooled, and each sample represents DNA from the biofilm of a single kelp individual.

PCR amplification of a part of the 16S rRNA gene of bacteria was carried out using the primers 787f (5′-ATTAGATACCCNGGTAG-3′) and 1492r (5′-GNT ACCTTGTTACGACTT-3′) (Roesch *et al.*, 2007) at a concentration of 1 μM each in HotStarTaq mastermix (Qiagen, Hilden, Germany). The DNA template concentration was adjusted to achieve equal concentrations of the final PCR product. The PCR conditions were: 15 min 95 °C hot start followed by 25 cycles of 45 s 95 °C, 45 s 50 °C annealing, 60 s 72 °C extension and a final extension of 72 °C for 7 min. Triplicate PCR reactions were pooled. The PCR amplicons were then used as template for a second 5-cycle PCR using the same conditions above. The primers for the second PCR were modified as follows. Unique multiplex identification tags (error corrected MIDs or 'barcodes') sequences for each sample was added to the forward primer (787f) in addition to a GS-FLX 'A' adaptor sequence. The reverse primer (1492r) was modified with a GS-FLX 'B' adaptor sequence. The resulting amplicons were pooled in equimolar amounts and kept at −80 °C until pyrosequencing, which was carried out at The Norwegian High-Throughput Sequencing Centre using Lib-L chemistry and GS-FLX Titanium technology (454 Life Sciences, Roche, Branford, CT, USA).

Sequence analysis. Quality filtering and noise removal of pyrosequencing reads of 16S rRNA gene

amplicons was carried out using AmpliconNoise (version 1.1) (Quince *et al.*, 2011), which corrects and compensates for PCR- and pyrosequencing-generated errors, thereby preventing an overestimation of bacterial diversity. The sequences were clustered into OTUs using complete linkage clustering as described in Quince *et al.* (2011) using OTU definitions of 97% and 99% similarity. The sequences were classified to Phylum-Class level using Ribosomal database project (RDP) classifier (Wang *et al.*, 2007). On the basis of this classification, sequences of chloroplast origin were identified and subsequently removed from the data set.

Statistical analyses. Statistical analyses were carried out using the R software (version 2.13.0) (R Development Core Team, 2011), including functions from the vegan package (version 1.17–10) (Oksanen *et al.*, 2011). Two aspects of bacterial diversity were evaluated: OTU richness and OTU evenness. To obtain comparable OTU richness estimates despite varying sequencing depth between samples, rarefaction analysis was used. The samples from the seasonal and production experiment data sets were rarefied at the number of sequences of the sample with the least sequencing depth in the respective data sets. One sample (sample 'T1', see Supplementary Table S1) in the production experiment data set was excluded from comparative analyses because of poor sequencing depth. In addition to rarefied richness, non-parametric (Chao, 1987) and parametric (Quince *et al.*, 2008) richness estimates were calculated. Evenness of the community was estimated using Pielou's evenness index $E = D/\log S$ (Pielou, 1977), where S is rarefied OTU richness and D is Shannons diversity index (Shannon and Weaver, 1949). All statistical analyses were carried out using OTU definitions of both 97% and 99% sequence similarity. Bacterial production numbers, measured as DPM cm^{-2}, were normalized to biofilm density and were log transformed. For community composition analysis, rare OTUs (<10 occurrences) were removed and the data set was subjected to Hellinger transformation (Legendre and Gallagher, 2001) to account for differences in sequencing depth among samples. Bray–Curtis distance was used for non-metric multidimensional scaling (nMDS). Regression analysis was carried out using linear regression followed by ANOVA to test for significance. Spearman correlation was used for non-parametric correlation analysis.

Results

The entire pyrosequencing data set consisted of 228 517 sequence reads with an average sequence length of 317 bp after quality filtering and chloroplast sequence removal. The average number of reads per sample ($n = 23$) was 9936 (max = 17 435, min = 1379, SD = 3985). The sequence reads cover a portion of the 16S rRNA gene of bacteria corresponding to *Escherischia coli* positions 804 to approximately 1170. The total OTU richness was 1108 at the 97% OTU definition and 1819 at 99% OTU definition. Only results from the 97% OTU clustering are hereafter presented, as all analyses yielded very similar results with both definitions. In the parametric diversity estimation, the inverse gaussian model gave the best fit for most samples and estimations based on this model are given in Supplementary Table S1. The sequencing effort required for capturing 90% of all OTUs ranged from 2.4 to 82 times the achieved sequencing depths (Supplementary Table S1), indicating that the communities were not exhaustingly sampled.

The original pyrosequencing output files of each sample have been submitted to the NCBI sequence read archive under the accession SRA025960 (http://www.ncbi.nlm.nih.gov/Traces/sra). In addition, representative sequences of the 100 most abundant OTUs (97% clustering) have been submitted to GenBank under the accession numbers HQ541982–HQ542081.

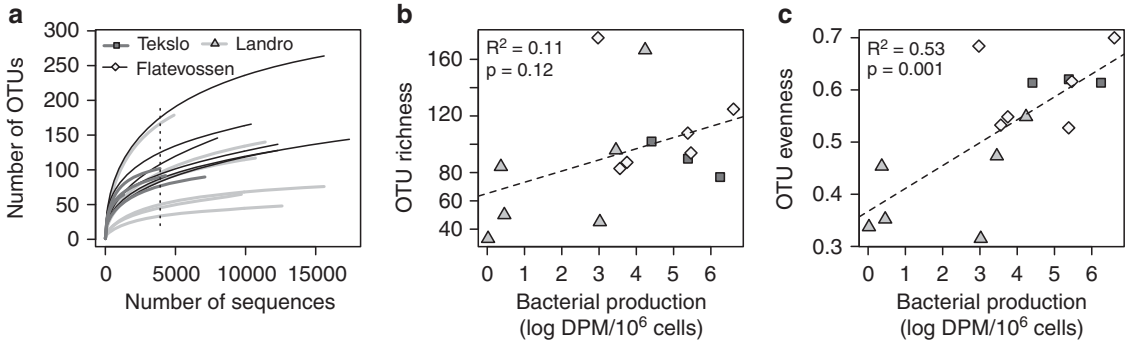

Figure 1 Bacterial production and bacterial diversity on kelp surfaces. (**a**) The rarefaction curves show the observed 97% OTU richness with increasing sequencing depth of the kelp biofilm sampled at the three different sites in July 2009 for bacterial production measurements. The dotted line shows at what sequencing depth rarefied OTU richness was calculated. (**b**) There is no clear relationship between 97% OTU richness and bacterial production as measured by incorporation of ^{3}H-Thymidine. (**c**) 97% OTU evenness is positively correlated with bacterial production.

Bacterial production and bacterial diversity

There was a relationship between bacterial production on kelp surfaces and the evenness of the bacterial communities in our experiments (Figure 1). A significant ($R^2 = 0.53$, $P = 0.001$) increase in bacterial production with increased OTU evenness of the bacterial communities (Figure 1c) was observed. No clear relationship between bacterial production and OTU richness was observed ($R^2 = 0.11$, $P = 0.12$, Figure 1b). We were not able to find a single 97% OTU or set of OTUs whose abundance correlates positively with bacterial production, implying that the OTUs responsible for high production are not the same in the different samples. However, the combined abundances of the OTUs 132 and 1322 (Planctomycetes) show a negative correlation with bacterial production (Supplementary Figure S3b).

There was no apparent correlation between bacterial production and biofilm cell density (Supplementary Figure S3a). Neither was there a correlation between biofilm cell density and bacterial OTU richness or evenness (Supplementary Figures S3c and d) in the production experiment. However, there was a clear difference between the cell densities recorded on kelp surfaces from the three sampling sites (Supplementary Figure S3f). The sampling sites were chosen to differ in wave exposure. The site with the highest wave exposure, Tekslo, had the lowest cell density in July 2009, whereas the most sheltered site, Flatevossen, had the highest cell density.

Successional changes in bacterial diversity

The bacterial communities that were sampled during an annual cycle revealed a strong positive relationship between kelp surface age and bacterial OTU diversity. A significant increase in both OTU richness ($R^2 = 0.85$, $P = 0.002$) and OTU evenness (Spearman correlation = 0.79, $P = 0.039$) was observed with increasing kelp surface age (Figures 2a–c). In March, the kelp is growing rapidly at the meristem region (age = 0 months). The bacterial community of the meristem in March was characterized by low OTU richness and evenness (Figures 2a–c). The very low evenness (Figure 2c) is due to the dominance of one 97% OTU belonging to the Gammaproteobacteria (OTU 46) that makes up nearly 84% of the sequences in the March meristem sample (Table 1). The meristem is still growing in May (surface age $\leqslant 2$ months), though at a slower pace (Kain, 1979), and the richness was still low (Figure 2b), but the dominance of a few OTUs was replaced by a more even community (Figure 2c). As the kelp surface ages from May through to November, the OTU richness continued to increase (Figure 2b), whereas the evenness was relatively unchanged (Figure 2c). The highest richness was observed on the lamina in March (Figures 2a and b), which is approximately 1 year older (age = 12 months) than the meristem. The contrasting richness and evenness on the coexisting meristem and lamina in March (Figures 2a–c) indicates that kelp surface age is more important than other seasonal factors (for example, temperature) in regulating bacterial diversity on kelp surfaces. An analysis of DGGE data from replicated samples collected at most of the same time points generally supports the trend of increased diversity as the kelp surface ages (Supplementary Figure S5a). No clear relationships were found between cell density and richness, evenness or kelp surface age (Supplementary Figure S4).

Bacterial community composition on kelp surfaces

The bacterial community composition showed no apparent relationship with bacterial production as indicated by the lack of a visible trend between size and position of the bubble points in Figure 3a. Rather, the samples from the production experiment cluster according to sampling site with respect to community composition (Figure 3a).

The successional development of the bacterial communities appeared to follow a kelp surface age gradient, although other unknown factors also must

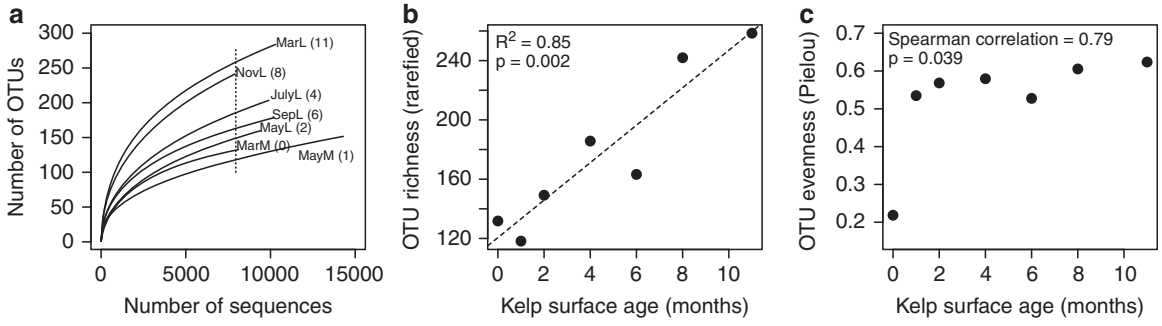

Figure 2 Successional changes in bacterial diversity. (**a**) The rarefaction curves show the observed 97% OTU richness with increasing sequencing depth of the kelp biofilm sampled across a gradient of kelp surface age (March 2007–November 2007, see Supplementary Figure S1). Sample id is indicated by abbreviated month names and 'L' and 'M' for the kelp lamina and meristem, respectively. Numbers in parentheses indicate the approximate age of the kelp surface in months. The dotted line shows at what sequencing depth rarefied OTU richness was calculated. (**b**) Rarefied 97% OTU richness increases with kelp surface age. (**c**) 97% OTU evenness, measured by Pielou's evenness index, increases with kelp surface age.

Table 1 The relative abundances of the 15 most abundant OTUs

OTU #	*Genbank accession #*	*RDP classification*	*Succession experiment (Tekslo, 2007)*							*Production experiment (July 2008)*					
			March meristem	*May meristem*	*May lamina*	*July lamina*	*September lamina*	*November lamina*	*March lamina*	*Tekslo (n = 4)*		*Landro (n = 6)*		*Flatevossen (n = 6)*	
										Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*
1322	HQ541982	Planctomycetes	0.89	4.99	5.78	7.35	6.21	0.00	2.56	27.25	10.88	51.02	9.41	24.86	12.62
132	HQ541983	Planctomycetes	0.45	26.14	11.95	13.53	2.21	4.28	0.90	2.53	2.24	18.50	9.47	5.30	2.12
19	HQ541984	Planctomycetes	0.01	0.08	2.89	12.14	11.36	20.43	7.18	0.67	0.92	0.64	1.55	4.11	2.18
46	HQ541985	Gammaproteobacteria	83.65	0.77	2.19	12.83	9.16	10.71	18.88	0.18	0.14	0.14	0.15	0.05	0.05
116	HQ541986	Planctomycetes	0.00	0.35	1.13	0.70	6.00	2.70	0.67	1.12	0.86	2.34	2.03	8.43	8.72
1345	HQ541987	Planctomycetes	0.18	2.10	7.29	9.73	6.50	0.40	6.52	6.20	7.16	5.00	3.56	2.81	1.23
76	HQ541988	Alphaproteobacteria	0.00	0.08	0.89	2.03	0.17	0.90	0.05	0.06	0.11	0.03	0.06	12.27	11.30
323	HQ541989	Alphaproteobacteria	0.66	6.23	6.10	4.77	0.96	4.41	3.74	0.00	0.00	0.03	0.07	0.05	0.12
154	HQ541990	Planctomycetes	0.00	0.00	0.00	0.41	3.96	4.60	0.00	0.00	0.00	0.30	0.34	0.73	0.49
502	HQ541991	Alphaproteobacteria	0.00	6.16	11.10	0.03	0.01	0.05	0.00	0.00	0.00	0.28	0.31	4.43	3.31
485	HQ541992	Actinobacteria	0.12	0.00	0.17	0.22	0.51	2.60	6.67	0.00	0.00	0.01	0.02	0.01	0.01
146	HQ541993	Alphaproteobacteria	0.05	0.00	0.01	0.03	0.01	0.00	0.01	2.16	2.66	2.49	4.79	3.72	2.84
473	HQ541994	Gammaproteobacteria	0.10	0.00	0.06	7.41	33.64	9.29	0.01	0.02	0.03	0.01	0.01	0.01	0.01
324	HQ541995	Alphaproteobacteria	0.00	0.16	0.00	0.27	0.02	0.00	0.00	4.28	5.66	2.86	4.52	3.29	6.01
90	HQ541996	Alphaproteobacteria	0.00	0.03	0.03	0.02	0.03	0.04	0.01	1.96	2.11	1.91	2.70	3.26	2.50

The relative abundances are displayed as a percentage of the total sequence reads in each sample. For the succession experiment, the numbers correspond to individual samples derived from pooled DNA from six kelp individuals. For the production experiment, mean and SD of relative abundances of samples deriving from replicate kelp individuals (four or six, only the lamina region was sampled) are shown. 'RDP classification' indicates the taxonomical affiliation of the OTU sequences.

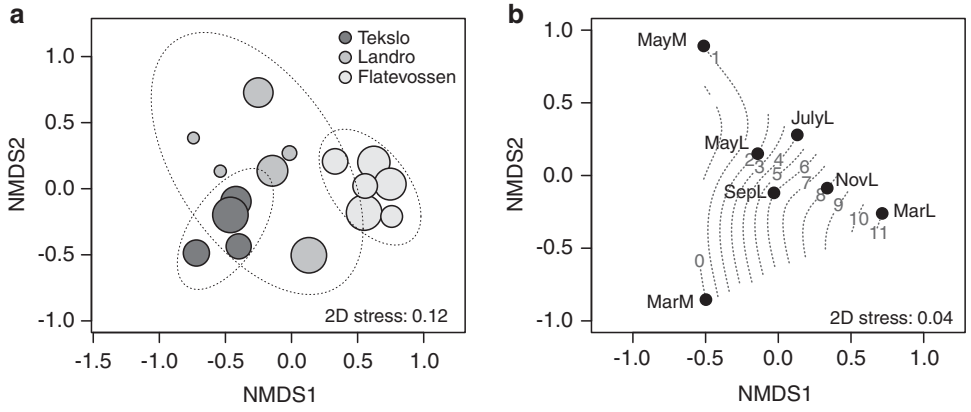

Figure 3 Bacterial community composition in relation to bacterial production and succession. (**a**) The nMDS ordination illustrates similarity of OTU composition between samples in the production experiment. The diameter of the bubbles is proportional to the bacterial production measured in each sample. The ellipses represent 95% confidence intervals of sample ordination grouped by sampling site. (**b**) The nMDS ordination illustrates similarity of OTU composition between samples of different kelp surface age. The contours show the gradient of kelp surface age (see Supplementary Figure S1), fitted to the ordination plot using function ordisurf in R.

be influential, as indicated by the large distance between the meristem in March (age = 0 months) and the meristem in May (age = 1 month) in the nMDS plot in Figure 3. An nMDS analysis of replicated samples using DGGE showed a similar trend (Supplementary Figure S5b).

The relative abundances of the major bacterial lineages largely confirm the results from previous studies on *L. hyperborea* biofilms (Bengtsson and Øvreås, 2010; Bengtsson *et al.*, 2010). For example, the Planctomycetes was again found to be the most abundant lineage (55.7% of sequence reads). However, the in-depth pyrosequencing approach employed in this study for the first time allows comparison of the richness between bacterial lineages. Bacteroidetes and Alphaproteobacteria displayed a higher OTU richness compared with the Planctomycetes and Gammaproteobacteria, although sequences from the latter lineages were more abundant in the data set (Figure 4a). Most samples were dominated by a few abundant OTUs, mostly belonging to the Planctomycetes (Table 1). There was a positive relationship between the relative abundance and the frequency of detection of OTUs, as shown in Figure 4a. Overall, the rare OTUs were numerous, with 61% of OTUs represented by below five sequences. The most rare OTUs, the singletons (detected only once in the data set) made up 31% of the entire data set. Archaea were also detected on kelp surfaces at low abundances, with 0.015% of all sequences classifying as Archaea.

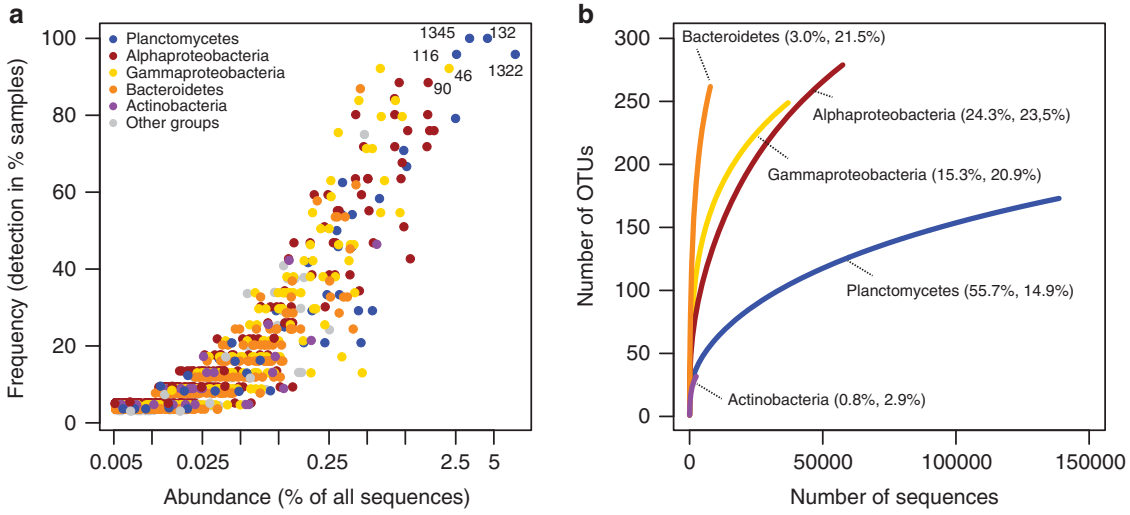

Figure 4 General characteristics of bacterial communities on kelp surfaces. (**a**) Every dot represents a bacterial 97% OTU that is plotted by its frequency of detection (percentage of samples it is present in) against its relative abundance in the entire data set ($n = 1108$). OTUs are color coded according to which of the major phylogenetic groups they belong to. Individual OTUs mentioned in the text are indicated by their number. (**b**) The rarefaction curves show the number of 97% OTUs that belong to the five most represented bacterial groups. Numbers in parentheses give the percentage of sequences represented by that group, and the percentage of OTUs, respectively. Most of the sequences belong to Planctomycetes, but Alphaproteobacteria and Bacteroidetes are more diverse (have a higher OTU richness).

Discussion

In this study, we have used a pyrosequencing approach to make a detailed study of the bacterial diversity of kelp surfaces. We have chosen to relate bacterial diversity to two processes that are fundamental in this environment, primary succession and bacterial secondary production. In addition, because this is the first study using a high-throughput sequencing approach in a kelp surface environment, special attention is given to differences in OTU richness observed between bacterial lineages and successional dynamics of individual OTUs as this may indicate their ecological roles in this, and other similar microbial habitats.

Relationship between bacterial diversity and bacterial production

To explain the intriguing result that higher OTU evenness is linked to higher bacterial production (Figure 1c), we must consider what OTU evenness reflects in terms of the composition of the bacterial community. Low evenness is equivalent to the dominance of a few OTUs in the community, which belong to the Planctomycetes in our case (Table 1). The observed negative correlation between bacterial production and the relative abundance of the dominant planctomycete OTUs 132 and 1322 is therefore not surprising and may suggest that bacterial cells belonging to these OTUs were not contributing significantly to the bacterial production measured during the experiment. This is also indicated by the very low production estimates recorded on some samples (see Supplementary Table S1). This may, in part, be due to the generally slow-growing nature of Planctomycetes (Ward *et al.*, 2006). However, the use of small, excised kelp pieces in the production experiment may also have caused a shift in bacterial growth conditions as they probably offer a different environment for bacteria compared with the surfaces of whole living kelp individuals. However, even if our experimental conditions may differ from the natural conditions of living kelp surfaces, they do not represent a completely unnatural situation. Kelp pieces, and whole kelp thalli, also detach in the kelp forest, and the bacterial communities on their surfaces likely undergo shifts as a response to the change in environment also in these cases. Our results therefore indicate that the Planctomycetes that dominate on living kelp surfaces (Bengtsson and Øvreås, 2010) are less important in the degradation of fragmented kelp material.

An alternative explanation for the observed trends is that inability by some types of bacteria to incorporate thymidine (Jeffrey and Paul, 1990) could cause community composition to influence measured bacterial production. Though this effect cannot be ruled out entirely, there was no indication that the relative abundance of any specific group of bacteria (for example Planctomycetes) correlated with bacterial production (Supplementary Figure S3).

Primary succession on kelp surfaces

The renewal of the *L. hyperborea* lamina each year results a temporal gradient of surface age throughout the year. This is accompanied by a spatial gradient along the kelp lamina, where the meristem region is younger than the distal lamina at all times (illustrated in Supplementary Figure S1). Primary

succession on kelp surfaces is an example on endogenous heterotrophic succession as classified by Fierer *et al.* (2010). The predicted development in such a successional process is an initial dominance of a copiotrophic subpopulation, followed by a gradual increase in a more specialized, oligotrophic subpopulation. This model of succession is consistent with the observed dominance of one bacterial OTU (OTU 46, Gammaproteobacteria, Table 1) on the rapidly growing meristem in March, which could represent a copiotrophic, primary colonizer. The planctomycete OTUs that dominate during the rest of the successional process likely represent specialized, slow-growing oligotrophs. Interestingly, the increase in richness as the kelp surface ages appears nearly linear (Figure 2b). There is no indication of a climax or plateau of richness during the lifespan of the kelp lamina. New OTUs are continuously being added to the community, whereas old OTUs persist. A likely explanation for this observation is the more varied surface environment on the older kelp lamina, resulting from the accumulation of small injuries on the kelp surface caused by mechanical forces or grazing. This may lead to more niches for bacteria becoming available over time, due to the more structurally varied habitat and access to different carbon sources leaching out of lysed kelp cells, for example. The analysis of replicate samples by DGGE (Supplementary Figure S5a) also showed a general increase in richness over time, despite large variations between replicates. Because this analysis has a much lower resolution (maximum eight OTUs were detected instead of >200 OTUs), the similar trend indicates that the increase in diversity with kelp surface age applies to both very abundant members of the community and to more rare ones. The community composition, as detected by DGGE, also showed a gradual change as the kelp surface ages. However, the variation between replicate samples causes considerable overlap between sampling points (Supplementary Figure S5b). The similar results obtained by these two different analysis techniques (pyrosequencing and DGGE) demonstrates the usefulness of 'classical' fingerprinting methods such as DGGE in revealing ecological responses of microbial communities.

Mechanisms of community assembly

The variation in the relative abundances of the dominant OTUs between samples was substantial in our data set (Table 1). This variation may reflect unknown environmental or host-related gradients that affect the biofilms, selecting for different bacteria (that is, species sorting). However, it may also suggest an element of stochasticity in the community assembly of kelp surfaces. In such a case, several functionally equivalent bacterial species may be recruited from adjacent bacterial populations (for example seawater), when fresh kelp tissue is formed. Their relative abundances would be determined by stochastic events during colonization, rather than ecological advantages determined by their phenotypes. The most abundant planctomycete OTUs (Table 1, Figure 4b) may represent such ecologically equivalent organisms. This model of community assembly is assumed in the neutral theory (Hubbell, 2001), which has successfully predicted species distribution curves for communities of macroscopic organisms (Hubbell, 2005) as well as microbial communities (Sloan *et al.*, 2006). Similar observations indicating a high variability of bacterial OTUs between host individuals have also been reported for the green seaweed *Ulva australis* (Burke *et al.*, 2011b), suggesting that seaweeds (including both kelps and green algae such as *Ulva*) display common patterns of surface bacterial community assembly. In the case of *U. australis*, it was recently shown that this lack of phylogenetic similarity (inferred by 16S rRNA gene sequences) is contrasted by a high similarity of functional genes (Burke *et al.*, 2011a). This makes a convincing case for stochastic recruitment of functionally equivalent bacterial species as an important mechanism of community assembly. The abundant OTUs in our study were also generally widespread in time and space, as illustrated by the positive relationship between relative abundance and frequency of detection (Figure 4a). This type of relationship is indeed predicted by neutral theory, further indicating that stochastic processes may be important in bacterial community assembly on kelp surfaces. However, several other explanations for such relationships have also been proposed (Östman *et al.*, 2010). For example, widespread and abundant OTUs could conceal several distinct ecotypes that occupy different environmental niches.

In addition to the variation between kelp individuals, there is of also variation in community composition between sampling sites (Figure 3a), which is accompanied by a difference in cell density (Supplementary Figure S3f). These differences indicate that wave exposure may have a role in regulating the bacterial communities on kelp surfaces, as this is the only factor that is known to differ between the sites.

Community composition and proposed functions of abundant OTUs

Four of the most abundant OTUs on *L. hyperborea* detected in this study belong to Planctomycetes (Table 1, Figure 4a). They are all related to Planctomycetes of the *Rhodopirellula* and *Blastopirellula* genera ('RB1' lineage in Bengtsson and Øvreås (2010)). There are no close cultured relatives of these Planctomycetes, and their roles on kelp surfaces are yet unknown. Other marine Planctomycetes are presumably degraders of sulfated polysaccharides (Woebken *et al.*, 2007). Such substances are produced by kelps (Evans *et al.*, 1973), and may

possibly be utilized by kelp surface Planctomycetes. The gammaproteobacterial OTU 46 is also frequently detected and periodically dominant (Table 1, Figure 4a). Its dominance on the meristem in March (Table 1) suggests that it is a pioneer colonizer of fresh kelp surfaces and implies a strategy of rapid attachment and growth at the early stages of biofilm development. It classifies as a member of the genus *Granulosicoccus* (Lee *et al.*, 2007). Related sequences have been detected on the red seaweed *Delisea pulchra*, the green seaweed *U. australis* (Longford *et al.*, 2007), the brown seaweed *Fucus vesiculosus* (Lachnit *et al.*, 2010) and on the kelp (brown seaweed) *Saccharina latissima* (Staufenberger *et al.*, 2008), suggesting that organisms within this genus share a preference for seaweeds. The alphaproteobacterial OTU 90 is less abundant (maximum 7.65%), yet frequently detected on *L. hyperborea* (Table 1, Figure 4a). It is closely related to the roseobacter *Tateyamaria pelophila* (Sass *et al.*, 2010), an isolate from tidal flat sediment that is capable of degradation of laminaran, a brown algal storage polysaccharide. Cultivation experiments using laminaran as a carbon source yielded highly similar strains from *L. hyperborea* surfaces (Bengtsson *et al.*, 2011). Thus, OTU 90 possibly represents a bacterial species of the genus *Tateyamaria* that utilizes laminaran as a carbon source on kelp surfaces.

Although abundant organisms such as the dominant Planctomycetes (Table 1, Figure 4a) can be expected to have lifestyles that make them well adapted to kelp surfaces, the roles of the many rare inhabitants detected in this study are not easily predicted. Bacteroidetes are represented by the most rare OTUs in relation to the abundance of sequences belonging to this group (Figure 4b). Some of these rare OTUs could represent transient 'visitors' from other marine habitats where they are more abundant. They may also function as a seed bank, whose members can be recruited when environmental conditions change (Pedros-Alio, 2006). The dominance of one or a few OTUs (for example 1322 and 46, Table 1) that causes the low evenness observed in many samples (Figures 1c and 2c) suggests that kelp surface biofilms may indeed be characterized as low bacterial diversity habitats in these instances.

Conclusions

(i) High evenness of the bacterial community is linked to higher bacterial production rates, which may be relevant especially during initiation of degradation of fragmented kelp. (ii) The successional changes in diversity on kelp surfaces are characterized by a steady increase in richness as the kelp surface ages. The bacterial community detected during initial colonization during the rapid growth period in March is substantially different from the Planctomycetes-dominated community found during the rest of the year. Although a small number of planctomycete OTUs are nearly always detected and abundant, Bacteroidetes are overrepresented among the rare members of kelp surface biofilms. A high variability in OTU relative abundance between individuals suggests that stochastic processes have a role in community assembly, consistent with observations made on other seaweed surfaces.

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