

Bacterial biofilms on the kelp *Laminaria hyperborea*

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Dissertation for the degree philosophiae doctor (PhD)
at the University of Bergen

2011

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Scientific environment

The work presented in this thesis was carried out in the Marine Biodiversity and Geomicrobiology research groups at the Department of Biology and at the Centre for Geobiology, University of Bergen. The project was funded through a PhD grant from the University of Bergen. Additional funding for laboratory and field expenses were provided by the scholarships: Joachim Frieles legat, Bergen Myrdrkningsforenings fond and Egil og Sunniva Baardseths legat. The project was also financially supported by the company FMC Biopolymer.



Acknowledgements

Most importantly, I am deeply grateful for the amazing guidance and support that my two supervisors, Lise Øvreås and Kjersti Sjøtun, have provided during these years. You showed great courage in giving me the opportunity to work on this unusual topic when I suddenly appeared from out of the blue in 2006. Throughout the work, you have complemented each other perfectly in your expertise and supervision, and your encouragement and positive attitudes have made it such an enjoyable journey.

I want to thank all past and present members of the Geomicrobiology group, who have made everyday life at work productive and fun. Special thanks to Jørn Einen, Bjørn Olav Steinsbu and Antonio García Moyano for spontaneous discussions and for tolerating my messy side in the office. Anders Lanzén has my endless gratitude for indispensable bioinformatics assistance, fruitful collaborations, many great discussions and the best friendship and support imaginable. Thanks to Julia E. Storesund for sharing my passion for planctomycetes and for valuable help and collaboration. Thanks to Marianne Norheim, Katrine Lekang, Grete Havik, Sofia Fortunato, Alexander Tøsdal Tveit, Torill Vik Johannessen, Baye Sitotaw and Addis Simachew for great company in the labs at Jahnebakken 5. I'd also like to thank everybody in the Marine Biodiversity group for being so diverse and resourceful. It's been great to belong to the group. The Centre for Geobiology has been an exciting environment to work in. Thanks to everybody at the CGB, in particular Frida-Lise Daae for sharing her great knowledge in the laboratory and Steffen Jørgensen for help with amplicons and for always creating a good atmosphere around him.

I've had the privilege to visit and interact with some excellent research environments outside Bergen. Stein Fredriksen at the University of Oslo inspired the idea to work with kelp-associated bacteria in the first place, and has since then been an invaluable source of knowledge and support. Similarly, Kjell-Magnus Norderhaug and Hartvig Christie at NIVA in Oslo have taught me a lot about kelp forests and have provided much encouragement. Friederike Hoffmann hosted me at the Max Planck Institute for

Marine Microbiology in Bremen, where I learned FISH. She has been a good friend and somewhat of a mentor since then, and I thank her for all her wonderful help and support. Janet Jansson hosted me at the Lawrence Berkeley National Laboratory in Berkeley, a visit that was a highlight in my work. I have spent some time in Vienna during these years: Thanks to Christa Schleper and her group for great hospitality and to Tom Battin for giving me the inspiration to see beyond the kelp forest.

The financial support by FMC Biopolymer has increased the quality of the research carried out in this PhD project. I would also like to thank Jostein Veaa, Trine Stumbo and many others at FMC for the interesting discussions we had about kelp, alginate and bacteria during my visits to Haugesund.

My friends in Bergen, Oslo, and Stockholm have been extremely important during this time, I can't list you all, but you know who you are! A few of you have even contributed to the work in various ways: Thanks to Klara Eriksson, Frank Priebitz, Carol Issalene and Sam Penglase for assistance and great company during fieldwork. Marie-Lise Schläppy has provided excellent help in reviewing this thesis, thanks!

My closest family; My mother Suzanne Bengtsson, my brother Niklas Norberg, my sister Anna Bengtsson Strandqvist and her family have all been very supportive and have helped me recharge during my visits to Stockholm and their visits to Bergen. In addition to this, my father Thomas Norberg has helped with chemistry issues, given useful and encouraging comments on my writing and a lot of valuable scientific advice and inspiration. I also want to thank my grandmother, Birgitta Norberg, for her wisdom and support.

Finally, Tim Urich has been a very special positive influence in my life and work during these years. Your work-related help has been substantial and your tremendous personal support has meant everything to me. Nothing could have provided a better motivation to finish this work than the prospect of a close-distance life with you.

Abstract

This thesis deals with bacterial communities in biofilms on the surfaces of the kelp *Laminaria hyperborea* and their roles in carbon cycling. Kelp forests are characterized by high biodiversity and productivity, and cycling of kelp-produced carbon is a vital yet poorly understood process in this ecosystem. Although bacteria are assumed to play a major role in kelp forest carbon cycling, the knowledge of the composition and diversity of the bacterial communities associated to kelp is limited. *L. hyperborea* and the bacterial biofilms on its surfaces were sampled at several kelp forest localities in the archipelago near Bergen, Norway at different times of the year. The biology of *L. hyperborea* is governed by seasonal factors. Its growth, chemical composition and exudation of dissolved organic carbon changes substantially during the course of the year. The seasonal dynamics of the biofilms were addressed in **Paper I**, using genetic fingerprinting and microscopic cell counting. There was a clear seasonal cycle of the bacterial community composition. The cell density of the biofilms on the lamina was the lowest during the active growth period of the kelp in spring. The diversity of the bacterial communities was also influenced by the seasonal growth cycle of the kelp. This was shown using pyrosequencing of the 16S rRNA gene in **Paper IV**, revealing that bacterial species richness and evenness increased with kelp surface age. Bacteria belonging to *Planctomycetes* were frequently detected on kelp surface biofilms throughout the year. In **Paper II**, this bacterial group was found to be numerically dominant on kelp surfaces using fluorescence *in situ* hybridization (FISH). Several different lineages within *Planctomycetes* were detected using 16S rRNA gene clone libraries, and the most abundant clones belonged to yet uncultivated lineages. The biomass and exudates of kelp contain large quantities of organic material, such as polysaccharides, which may provide rich carbon sources for heterotrophic bacteria on kelp surfaces. By utilizing such kelp-derived carbon, these bacteria can play an important role in the ecosystem by contributing to carbon cycling. However, it is not known which bacteria are important in these processes and which carbon sources they utilize. The ability of kelp surface bacteria to utilize different kelp substances was investigated in **Paper III**

using an enrichment cultivation approach. Several bacterial strains were recovered by cultivation using a variety of kelp substances. The most abundant of these were generalists, utilizing all kelp substances tested. The same 16S rRNA gene phylotypes as some of the cultured bacterial strains were also detected in kelp surface biofilms using culture-independent methods. This suggests that the cultured bacteria may also play significant roles in kelp forests. The secondary production rate of kelp surface bacterial communities was investigated in **Paper IV** using incorporation of tritiated thymidine. Higher bacterial production was correlated with higher diversity of the bacterial communities, measured as evenness of bacterial phylotypes using pyrosequencing of the 16S rRNA gene. This shows that the composition and diversity of the bacterial community can influence bacterial production rates, and thereby have implications for kelp forest carbon cycling. The work presented in this thesis is the first to describe the bacterial communities associated to an important kelp forest forming species. The results provide a foundation for understanding the microbial ecology of kelp forests and raise several new questions about the symbiotic roles and ecosystem impacts of bacteria on kelps and other seaweeds.

List of publications

Paper I

Bengtsson, M. M., Sjøtun K., Øvreås, L. (2010) Seasonal dynamics of bacterial biofilms on the kelp *Laminaria hyperborea*. Aquatic Microbial Ecology 60(1):71-83

Paper II

Bengtsson, M. M. and Øvreås, L. (2010): Planctomycetes dominate biofilms on surfaces of the kelp *Laminaria hyperborea*. BMC Microbiology 10:#261

Paper III

Bengtsson, M. M., Sjøtun K., Storesund J. E., Øvreås, L. (2010): Utilization of kelp-derived carbon sources by kelp surface-associated bacteria. Accepted for publication in Aquatic Microbial Ecology doi: 10.3354/ame01477

Paper IV

Bengtsson, M. M., Sjøtun K., Lanzén, A., Øvreås, L.(Manuscript): Bacterial diversity and production on the surfaces of the kelp *Laminaria hyperborea*. Submitted to Environmental Microbiology

List of abbreviations

AHL = Acylhomoserine lactone

DGGE = Denaturing gradient gel electrophoresis

DOC = Dissolved organic carbon

DAPI = 4',6-diamidino-2-phenylindole

DNA = Deoxyribonucleic acid

FISH = Fluorescence *in situ* hybridization

OTU = Operational taxonomical unit

PCR = Polymerase chain reaction

POC = Particulate organic carbon

RNA = Ribonucleic acid

rRNA = Ribosomal RNA

SEM = Scanning electron microscopy

1. Introduction

A substantial part of the Norwegian coast, including the area around Bergen, is lined with underwater forests of the kelp *Laminaria hyperborea*. This kelp is an important natural resource, and provides shelter or growth substrate for many species of fish, invertebrates and seaweeds. Knowledge of the biology of *L. hyperborea* and of the ecology of the rich kelp forests it creates is therefore essential for understanding coastal productivity and biodiversity in Norway. Similarly, kelp forests consisting of other species of kelp are of economical and ecological importance in many other coastal regions of the world.

Marine heterotrophic bacteria drive carbon cycling in the worlds oceans. Recent advances in molecular microbiological methods have revealed surprisingly diverse microbial communities in seawater and have led to the discovery of many previously unknown organisms. In kelp forests, heterotrophic bacteria are also important in carbon cycling. By utilizing kelp-derived substances, they form a basis in the food web and make kelp primary production available to larger organisms. Thereby, bacteria may be critical for sustaining the diverse animal communities found in kelp forests. Yet, bacteria and other microorganisms associated with kelp have been little investigated, resulting in a limited understanding of the microbial ecology of kelp forests.

The work presented in this thesis was initiated to bridge this gap of knowledge by investigating bacterial communities associated to kelp in the context of the kelp forest ecosystem. The aim was to reveal the identity, abundance and diversity of bacteria on the surfaces of *L. hyperborea* in time and space and to address their contribution to kelp forest carbon cycling.

2. Background

2.1 Marine biofilms

A clean surface that is submerged in seawater is quickly colonized by bacteria. After 24 hours, hundreds of thousands of bacterial cells per square centimeter are typically attached (ZoBell and Allen 1935). All marine surfaces, including for example intertidal rocks, the hulls of ships, suspended particles and marine organisms are exposed to such bacterial colonization and are usually covered by bacteria as a result. Bacteria on surfaces tend to form biofilms (Fig. 1). The term biofilm is used to describe microorganisms adhering to surfaces, interfaces, and/or each other, embedded in an extracellular matrix (Costerton et al. 1995, Fig. 1A). This matrix consists of polysaccharides, proteins and other components, which are collectively referred to as exopolymers (EPS). These substances are secreted by the bacteria and their function has been linked to water retention, resistance to antibiotics and predation protection (reviewed in Flemming and Wingender 2010). Biofilms can have distinct architectures, featuring localized cell aggregations known as microcolonies (Fig. 1B) as well as channels that transport solutes and particles (see DeBeer and Stoodley 2006 and references therein). The biofilm mode of life has been likened to multicellularity (Webb et al. 2003a), as complex interactions between bacterial cells in biofilms have been discovered. These include cell to cell signaling, so called quorum sensing, that allow bacteria to "communicate" with each other using chemical cues like acylhomoserine lactone (AHL, Bassler and Losick 2006). In addition, localized cell death, which is an important component of the developmental biology of multicellular organisms, has also been indicated to play a role in biofilm architecture (Webb et al. 2003b).

Biofilm formation on marine surfaces is often the first step towards more extensive growth of microscopic and macroscopic organisms, known as biofouling. Biofouling can be a costly problem when it occurs on ship hulls, oilrigs and on other man-made structures in the marine environment, because the growth of macroscopic organisms

on surfaces causes increased hydrodynamic drag and can lead to accelerated corrosion of surface materials. For this reason, much research has been dedicated to understanding bacterial colonization and biofilm formation on marine surfaces, as to find ways to limit or prevent subsequent macroscopic biofouling. Successful strategies for biofouling control may be found among marine eukaryotes, which have always coexisted with microorganisms and have evolved various adaptations to resist excessive biofouling.

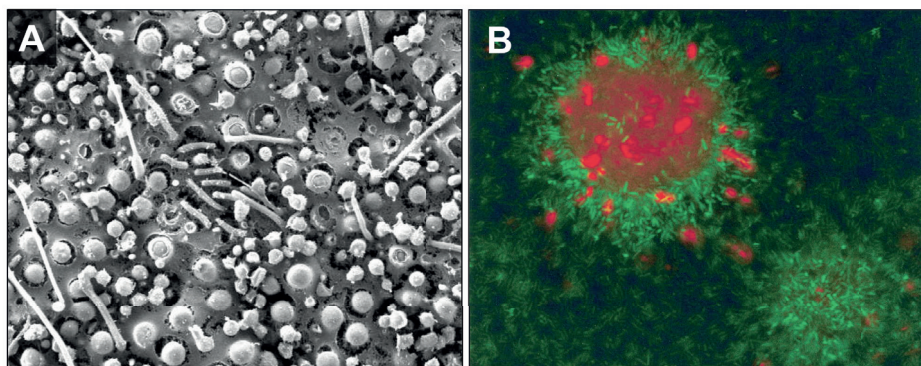


Figure 1. Biofilms: (A) The scanning electron microscopy (SEM) image shows a mature multispecies biofilm growing on a kelp surface. The cells are surrounded by EPS, visible as a smooth sheet between cells. (B) Some biofilms have distinct architectures. The fluorescence microscopy image shows two microcolonies in a single species biofilm of the biofilm model organism *Pseudomonas aeruginosa*. The green areas are made up of living cells while the red areas display localized cell death, a mechanism that may facilitate dispersal of biofilm cells. Image reproduced with permission from Jeremy Webb and ASM press (from Webb et al. 2003).

2.2 Biofilms on living surfaces

The growth of one organism on the surface of another organism is referred to as epibiosis. Many examples of strategies that limit epibiosis can be found among marine organisms (reviewed in Harder 2008, Goecke et al. 2010). Some of these are mechanical, such as periodical sloughing of surface layers including attached epibionts (Sieburth and Tootle 1981, Littler and Littler 1999, Nylund and Pavia 2005). In other cases, production and release of chemical substances deter or kill colonizers. The best studied of these chemical defense mechanisms is perhaps the production of halogenated furanones by the red seaweed *Delisea pulchra*. These

substances are believed to limit bacterial colonization and biofilm formation on the surfaces of this seaweed by inhibiting AHL mediated quorum sensing (i.e. Givskov et al. 1996, Steinberg et al. 1997, Maximilien et al. 1998, Manefield et al. 2002).

However, host-microbe interactions are not limited to chemical defense and other antifouling mechanisms. There are also positive interactions such as release of substances by the host that can be utilized as food by its microbial associates. In addition, the microorganisms themselves can influence the host negatively by acting as pathogens or parasites and positively for example by providing nutrients and protection against harmful organisms. In many cases, host-microbe interactions lead to stable and specific associations between host- and microbial species. This is referred to as symbiosis according to the broad definition of the term given by Anton de Bary (1831-1888).

Symbiosis is arguably a major force in evolution, as illustrated by the generally accepted endosymbiont theory, which states that the mitochondrial and chloroplast organelles of eukaryote cells are derived from symbiotic bacteria (Goksøyr 1967, Sagan 1967). Most research on symbiotic relationships has focused on individual host-symbiont pairs, such as legumes and *Rhizobium* bacteria, corals and zooxantellae protists and *Azolla* ferns and *Anabaena* cyanobacteria. These are all examples of mutualistic symbiotic associations, which bring a clear benefit to both the host and the symbiont. However, more than one type of microorganism is often found in association with a host and it is not always obvious what kind of interactions underlie the co-existence. Recent studies have highlighted the importance of the composition of the microbial community as a whole to host health and fitness. For example, human gut microbiota has been linked to obesity (Ley et al. 2006), and the composition of coral-associated microbial communities appears to play a role in resistance to environmental stress and disease (Reshef et al. 2006). Furthermore, genome sequencing projects have made it obvious that associated bacterial communities can provide far more genetic potential and variability than the host genome itself (Turnbaugh et al. 2007, Rosenberg et al. 2010). This has led to a renewed appreciation of the role of host-bacterial associations in evolution. It has

been suggested that a host and its collected microbial associates should be viewed as a "supra-organism" or "holobiont" rather than as discrete organisms (Turnbaugh et al. 2007, Rohwer et al. 2002). Adaptive changes in the microbial community can lead to advantages for the host, and these changes can even be inherited by coming generations, providing an alternative mode of evolution to host genome modification (Zilber-Rosenberg 2008).

Marine macroscopic organisms can be expected to display the whole spectrum of host-bacterial associations on their surfaces, ranging from passive and random epibiosis to highly specific and obligate symbioses. There is presently little knowledge of the symbiotic nature of bacterial communities of most marine living surfaces. This may change as more studies shed light on the variability and specificity of microbial communities associated to different hosts under various conditions. The potential for progress in this field is great, as new methods are enabling microbial communities to be analyzed at levels of accuracy and resolution that were previously impossible. The choice of method is an important consideration in microbial ecology, as different methods give very different types of information about microbial communities.

2.3 Microbial community analysis

The progress in microbiological research has always been limited by methodology that allows observation of life, directly or indirectly, at microscopic scales. A particular challenge for early microbiologists was to describe, with any detail, complex microbial communities in the environment. Instead, much knowledge was gained by studying microorganisms in laboratory culture.

Microorganisms that carry out specific functions in the environment are often of interest. Such functions may be for example fixation of nitrogen, oxidation of methane or degradation of specific organic substances. In enrichment cultivation, pioneered by Martinus Beijerinck (1851-1931) and Sergei Winogradsky (1856-1953), selective culture media and methods are used to enrich for microorganisms with certain characteristics (see **Paper III**). This approach offers a way to study and

manipulate microbial processes of interest in mixed cultures (Fig. 2A) and can often facilitate subsequent isolation of the organisms in pure culture (Fig. 2B).

Pure culture allows detailed investigation of the phenotypic characteristics of microorganisms that is presently not possible by studying them in their natural habitats. Techniques for cultivating microorganisms were developed by Louis Pasteur (1822-1895) and Robert Koch (1843-1919). To this day, isolating and studying bacteria in pure culture remains the only valid approach to describe bacterial species (Stackebrandt et al. 2002). Unlike macroscopic organisms and some eukaryotic microorganisms, prokaryotes (bacteria and archaea) rarely display sufficiently informative traits to allow morphological identification. Instead, a combination of phenotypic and genotypic characterization of cultured strains forms the basis of taxonomical classification (Rossello-Mora and Amann 2001, Stackebrandt et al. 2002). When pure culture is not feasible, genetic similarity alone is often used to approximate microbial species and higher-level taxa for classification. However, there is currently no universal genetic species concept for prokaryotes (Staley 2006). The existence of species as natural units of classification in prokaryotes and the eventual mechanisms of their formation (speciation) is a topic of much debate (Doolittle and Papke 2006, Spratt et al. 2006, Achtman and Wagner 2008). In this text, the term bacterial species is used to refer to genetically and ecologically coherent clusters of organisms, assuming that such clusters do exist.

Unfortunately, most environmental bacteria are not readily cultured and only a minor fraction of all bacteria is typically retrieved by culturing methods from most environments (Staley and Kanopka 1985). Therefore, cultivation-based approaches give an unrepresentative view of microbial communities. However, until the late 20th century, cultivation and microscopy were the only methods available to investigate microorganisms. Consequently, there was only very limited knowledge about microbial communities in the environment, and it was biased towards the culturable minority of bacteria.

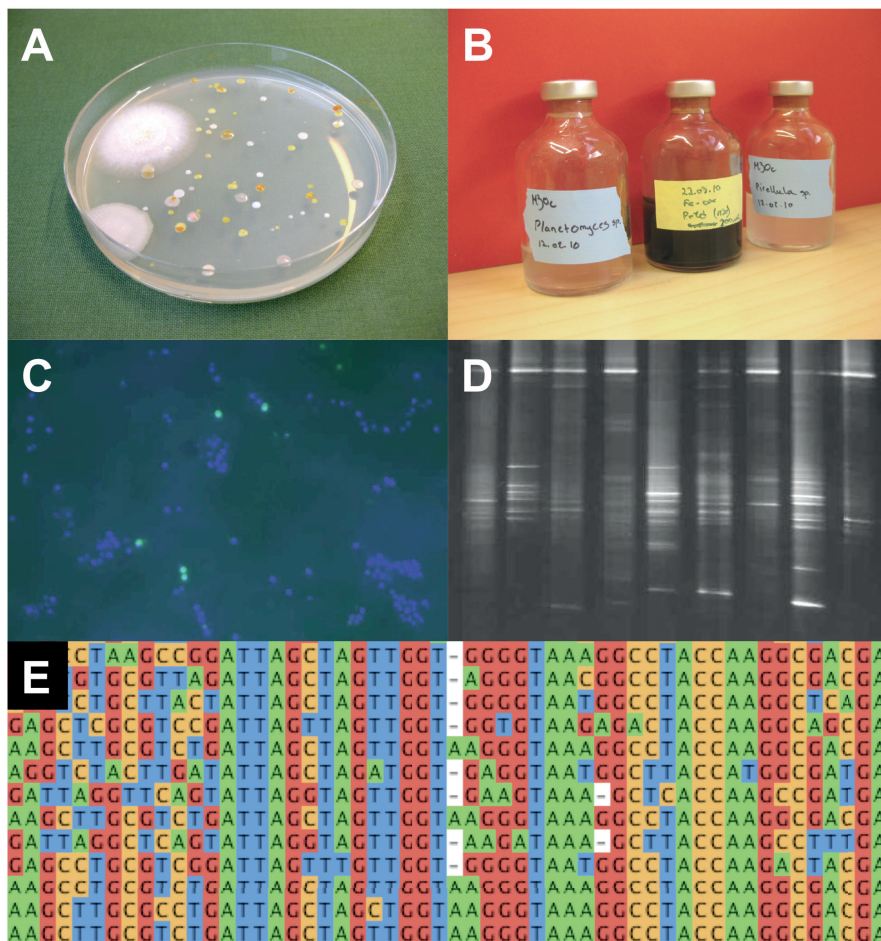


Figure 2. Methods for analyzing microbial communities: (A) Microorganisms can be enriched from the environment using selective cultivation media, exemplified by this mixed culture of bacteria and fungi growing on an agar plate. (B) Isolation of individual strains of bacteria in pure culture allows detailed investigation of their biology. (C) FISH is a cultivation-independent method that allows phylogenetic identification of bacteria in the microscope. The green cells are hybridized with a specific probe which shows that they belong to *Planctomycetes*. The blue cells are other bacteria that are stained with the unspecific stain DAPI. (D) The fingerprinting method DGGE separates PCR-amplified DNA from different organisms as bands on a gel and allows visual comparison of microbial community composition without the need for cultivation. Every vertical row of bands (lane) represents a sample with a different community. (E) The image shows a part of a multiple sequence alignment of bacterial 16S rRNA gene sequences. Such alignments are used in analysis of DNA sequences of environmental microorganisms from for example clone libraries and pyrosequencing.

A new era in microbial ecology was initiated when ribosomal RNA (rRNA) was introduced as a phylogenetic marker (Sogin et al. 1972). Analysis of rRNA enabled reconstruction of phylogenetic relationships between different cultured strains of microorganisms, resulting in the division of life into three lines of descent (Woese and Fox 1977), presently referred to as *Eukarya*, *Bacteria* and *Archaea*. A similar approach was subsequently applied directly to environmental microbial communities, without prior cultivation (Stahl et al. 1984, Stahl et al. 1985, Olsen et al. 1986). This led to the detection of many sequences that were only distantly related to known organisms, suggesting that most environmental bacteria were unknown to science (e.g. Giovannoni et al. 1990, Ward et al. 1990). These new culture-independent methods were subsequently applied to many different environments to reveal their previously hidden microbial inhabitants. This age of exploration is still going on today and new 16S rRNA gene sequences representing uncultured microorganisms are routinely detected. This suggests that the diversity of the microbial world is substantial and so far underexplored (Pedros-Alio 2006). Describing this diversity in different environments as well as understanding its functional implications is a focus of microbial ecology today, and several conceptually separate aspects of biological diversity are being considered.

One central aspect of biological diversity is the number of taxa (such as species) present in an environment, referred to as richness. The abundances of these taxa relative to each other form another component of diversity, called evenness. Further, phylogenetic diversity is concerned with how closely or distantly related the members of a community are evolutionarily. All of the components of diversity mentioned above are in theory measurable, and several diversity indexes and estimation methods have been developed to quantify them (e.g. Shannon and Weaver 1949, Pielou 1977, Faith 1992). Functional diversity refers to functional variation, for example different types of metabolic strategies represented within a community. Sometimes, the term "microbial diversity" is also used when describing the phylogenetic identities of bacteria that comprise a community. This qualitative component of diversity is in this text referred to as community composition, while the

term "diversity" is used exclusively to refer to richness and/or evenness of the bacterial communities.

During the past three decades, several methods involving the use of rRNA for microbial community analysis have been developed. In fluorescence *in situ* hybridization (FISH, DeLong et al. 1989, Amann et al. 1990), fluorescently labeled oligonucleotide probes complementary to microbial rRNA are hybridized against the ribosomes, causing the cells to shine when viewed in an epifluorescence microscope (Fig. 2C, **Paper II**). This allows both visualization and microscopic counting of cells of different phylogenetic lineages. While FISH is an excellent method to quantify cells of selected bacterial lineages, it is laborious to carry out for all taxa in an environmental sample. Consequently, other methods are often instead used to describe overall microbial community composition of a sample, for example for comparing it with other samples.

Fingerprinting methods targeting rRNA genes, such as denaturing gradient gel electrophoresis (DGGE, Muyzer et al. 1993) make rapid comparison of microbial communities possible (Fig. 2D, **Paper I**). A big advantage of these methods is that more samples can be processed at once, allowing for wide comparisons and statistical replication. A disadvantage is that the resolution of the methods often only allows detection of the most abundant members of a community. Consequently, the total number of different bacterial taxa, the richness, of the communities cannot be determined. In addition, retrieval of sequence information from fingerprinting methods is often not straightforward, making determination of the phylogenetic identity of community members difficult.

Instead, clone library analysis is often the method of choice for microbial diversity studies (Giovannoni et al. 1990, Rappé et al. 2003). Clone libraries are constructed by amplifying a target gene (usually 16S rRNA) by PCR and cloning the different fragments from a community into a host (usually *Escherichia coli*). By analyzing the sequences of the cloned fragments, it is possible to assess the richness, evenness and phylogenetic diversity of a community (Fig. 2E, **Paper II**). However, microbial

populations usually comprise millions or billions of individuals, while a typical clone library rarely consists of more than a few hundred clones. This small sample size can make accurate richness estimation difficult. Estimates from independent methods, such as DNA reassociation kinetics (Torsvik et al. 1990, Torsvik et al. 2002), and metagenomic shotgun sequencing (Venter et al. 2004) as well as from large clone libraries (Curtis et al. 2002) indicate that the richness of microbial communities in many environments is very high. Because clone library analysis is relatively expensive and time consuming, obtaining sufficient sample sizes to make accurate richness estimations may not be feasible in many environments using this method (Quince et al. 2008).

New sequencing technologies, such as pyrosequencing (Ronaghi et al. 1998), have recently enabled much deeper sampling efforts of rRNA gene sequences than previously obtained with clone libraries (e.g. Sogin et al. 2006, Roesch et al. 2007, **Paper IV**). Initial studies utilizing such technology have reported very high richness estimates in seawater and hydrothermal vent systems, corresponding to nearly 40 000 bacterial species in two hydrothermal vent samples, most of which were rare (Huber et al. 2007). The new methods and discoveries have led to a renewed interest in the potentially vast richness of microbial communities and the implications of this "rare biosphere" may have on biodiversity and biogeography (Pedros-Alio 2006, Sogin et al. 2006). It has been argued that because rare microbes are unlikely to go extinct and experience few barriers to dispersal, they may persist in the ecosystem (e.g. in a dormant stage) and represent a kind of seed bank that can respond to new environmental conditions (Pedros-Alio 2006). This would ultimately lead to a situation where "everything is everywhere" and the richness of any ecosystem would be vast, approaching the richness of the entire planet. However, the high estimates of richness obtained in early pyrosequencing studies (i.e. Sogin et al. 2006, Huber et al. 2007, Roesch et al. 2007) have recently been questioned, as they may in part have been caused by sequencing artifacts (Quince et al. 2009). In addition, the use of PCR amplification of the rRNA genes introduces biases that can skew the perceived community composition and structure, making extrapolations from such datasets

risky. Presently, estimations of the richness of microbial communities in different habitats vary by several orders of magnitude (Quince et al. 2008) and we are far away from knowing the actual number of different taxa that the $>10^{30}$ prokaryotic cells on the planet (Whitman et al. 1998) belong to.

In summary, the progress made in microbial ecology the last few decades has been enormous, largely due to the development of molecular methods enabling insight into microbial communities in the environment. However, while microbial community analysis can inform about composition and diversity, assigning functions to community members by such methods alone is rarely possible. Other methods, including different cultivation approaches and cultivation-independent methods such as metagenomics give functional information but all suffer from limitations that make it difficult to link the function of important organisms with their phylogenetic identity (Handelsman 2004). While there is broad consensus over the essential importance of microorganisms of all of the worlds ecosystems (e.g. Staley et al. 1997) it often remains unclear which community members are responsible for carrying out critical functions. Integration of microbial community analysis with existing knowledge on large-scale processes and dynamics is required to gain a deeper understanding of the structure and function of ecosystems (e.g. Fuhrman 2009). This will place microbial communities in the context of the large-scale ecosystems they inhabit, for example tropical rainforests, human guts, the open ocean, hydrothermal vents, coral reefs and kelp forests.

2.4 Kelp forest ecosystems

Kelp forests are dense stands of large brown seaweeds of the order *Laminariales*. They are three-dimensional habitats that provide shelter, food or growth substrate for a multitude of other organisms. The most visible of these include many species of seaweeds, invertebrates, fish, seabirds and mammals. The rich assemblages of associated life make kelp forests biodiversity hotspots. Kelp forests are widely distributed along rocky shores in temperate climates worldwide, yet kelps can also be

important locally in tropical latitudes (Graham et al. 2007) and in the arctic (Dunton et al. 1982).

Many human cultures have been dependent on kelp forest ecosystem services since early history. For example, the rapid migration of hunter and gatherer tribes along the pacific coastline of the American continents around 15000 years ago was likely enabled by a diet of kelp forest animals and seaweeds as summarized in the "kelp highway hypothesis" (Erlandson et al. 2007). Today, natural and cultivated kelp is harvested for food production and for extraction of valuable natural products on all of the worlds inhabited continents. In addition, kelp forests serve as feeding and nursing grounds for fish that are important for commercial and recreational fisheries (e.g. Norderhaug et al. 2005).

Kelps make important contributions to coastal primary production (Mann 1973). They fuel food webs in and near kelp forests, indicated by the isotope signals of many animals that indicate a diet of kelp-derived carbon (Duggins et al. 1989, Fredriksen 2003, Page et al. 2008). The high primary production and the rich animal life within kelp forests also have impacts that reach far beyond the relatively narrow coastal strip that they occupy. Export of kelp material to offshore areas can benefit benthic organisms in the deep through kelp falls (Vetter 1995, Harrold et al. 1998, Bernardino et al. 2010) and pelagic organisms through dissolved organic material from kelp (Koop et al. 1982, Abdullah et al. 2005) and floating kelp rafts (Smith 2002). Even terrestrial ecosystems can be affected as kelp washes up on beaches (Griffiths and Stenton-Dozey 1981, Bradley and Bradley 1993). Several species of fish, seabirds and marine mammals spend part of their lives in or around kelp forests, yet also interact with other marine or terrestrial ecosystems during their lifetime (Bradley and Bradley 1993, Estes et al. 1998).

The persistence of kelp forests globally and locally is determined by a complex interplay of biotic and physical factors. In many areas, grazing by sea urchins has led to large-scale kelp deforestation. The mechanisms that trigger these grazing events are not always understood, but removal of key predator species by human fishing or

can be an important factor (Estes and Duggins 1995). Competition with invasive introduced seaweed species has also caused declines in kelp forest cover in some areas (Chapman and Johnson 1990). Global warming may be another threat to kelp forest ecosystems, as kelps are physiologically constrained to tolerate only relatively cool seawater temperatures (Lüning 1990). Increased temperatures can also be detrimental to kelps indirectly, by contributing to overgrowth by epiphytic organisms (Scheibling and Gagnon 2009).

2.5 The kelp *Laminaria hyperborea* in Norway

Laminaria hyperborea (Gunnerus) Foslie (Fig. 3) is the main kelp forest forming species along the Atlantic coasts of Europe. It has a distribution from Portugal in the south to the Murman coast of Russia (Schoschina 1997), and has also been reported as far north as Spitsbergen, Svalbard (Olsen et al. 2004). In Norway, a considerable part of the coastline is lined with *L. hyperborea* kelp forests. The area covered is around 5000 km² according to a conservative estimate (Fosså 1995). The continuity of kelp forest distribution is disrupted in the north of Norway, where extensive areas of former kelp forests were converted to barren grounds by sea urchin grazing in the early 1970-ies (Norderhaug and Christie 2009).

One important factor influencing the local distribution and density of *L. hyperborea* kelp forests is wave exposure. Besides availability of suitable rocky substrate, light conditions and depth, wave exposure determines kelp forest density and kelp production in Norway (Sjötun and Fredriksen 1995, Sjötun et al. 1998, Bekkby et al. 2009). The optimum growth conditions for *L. hyperborea* are found on relatively exposed shores while kelp individuals growing in sheltered localities usually have a dwarfed morphology with short stipes (Sjötun and Fredriksen 1995).

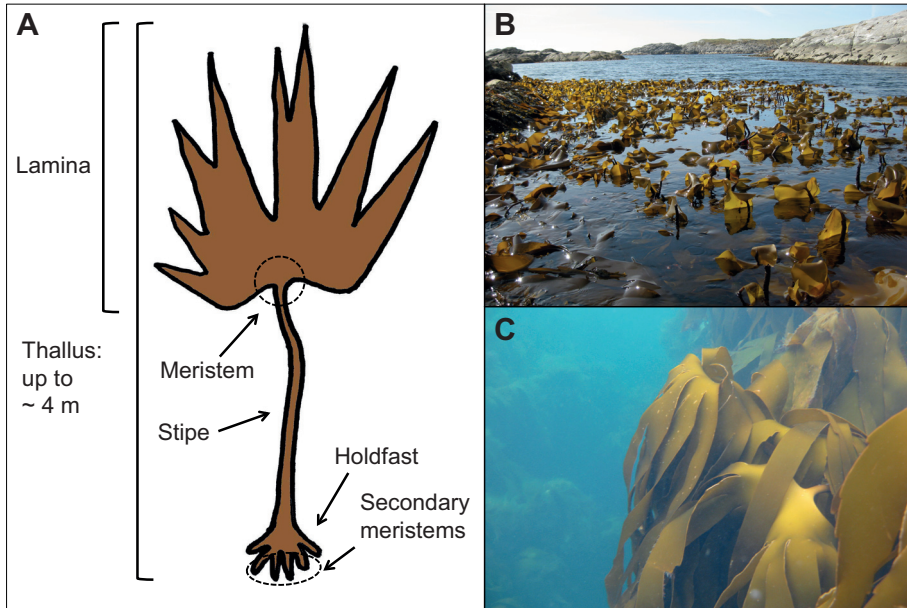


Figure 3: The kelp *Laminaria hyperborea*: (A) The schematic illustration shows the morphology of a typical *L. hyperborea* sporophyte thallus. The lamina is renewed each year through rapid growth at the meristem in winter-spring. (B) The canopy of an *L. hyperborea* kelp forest exposed at low tide near Store Kalsøy, Hordaland, Norway. (C) *L. hyperborea* laminae photographed underwater in Øygarden, Hordaland, Norway.

Laminaria hyperborea has a pronounced seasonal cycle, and several aspects of its growth and physiology vary considerably during the year. In simple terms, photosynthesis and storage of fixed carbon primarily take place during the summer months (June - September) while production of new lamina tissue is allocated to winter and spring (January - June). This allows the kelp to take advantage of the favorable light conditions during summer and the higher seawater nutrient concentrations during winter. Stored carbon fixed during summer is used for lamina regeneration the following winter and spring (Kain 1971). This seasonal growth strategy results in a gradient of kelp lamina tissue age throughout the year. The youngest tissue is found in the meristem during the most rapid growth period in March. The lamina tissue ages progressively throughout the year, until the old lamina is detached from the rest of the kelp thallus in April the following year. Figure 1 in **Paper I** illustrates the stages of this growth cycle that were sampled during the work presented in this thesis. The chemical composition of *L. hyperborea* cells is also

subject to seasonal variation. Laminaran, which is the primary storage compound of kelp cells, is a major cellular component after summer (up to 30 % of dry mass, Haug and Jensen 1954). This is later consumed for growth and the young lamina in spring is instead rich in the structural component alginate. A portion of the photosynthetically fixed carbon is lost from the kelp cells to the surrounding water masses through exudation. The exudation of dissolved organic carbon is especially high in summer, when kelp photosynthesis is the most active (Abdullah and Fredriksen 2004).

The annual kelp biomass production in Norwegian kelp forests is estimated at 50 million metric tonnes wet weight (Fredriksen 2003). A small fraction of this forms the raw material for extraction of alginate, which presently comprises a commercial industry in Norway. Yet, how the bulk of *L. hyperborea* primary production is processed and distributed in marine ecosystems is not known.

2.6 The role of bacteria in kelp forest food webs

While many kelp forest animals are sustained by kelp-produced carbon (Fredriksen 2003), most of these animals do not graze directly on kelp. This suggests that there are intermediate levels in the food web that make kelp carbon available to them. Heterotrophic bacteria utilizing kelp-derived carbon sources can function as an important link between kelp and animal consumers (Fig. 4). Several separate bacterial populations may contribute to the food web in this way. First, bacteria attached to the surface of living kelp can utilize exudates from kelp cells or kelp surface mucus. Second, pelagic bacteria in the surrounding water masses can also benefit from kelp exudates in the form of dissolved organic carbon (DOC). A third population of bacteria that may utilize kelp DOC are attached bacteria on other surfaces than kelp, for example on rock and on living organisms such as sponges in or near the kelp forest. In addition, bacteria degrade particulate organic carbon (POC) originating from fragmented kelp material. This fourth population of bacteria is likely important, because substantial part of kelp primary production is converted into POC through continuous wave mediated erosion of the kelp laminae as well as detachment of

whole kelp thalli (Newell and Lucas 1981). The secondary production of all these bacterial populations, based on kelp primary production, can form a basis for the food web in kelp forests. Bacteria can enter the food web through grazing by protists (Azam et al. 1983, Armstrong et al. 2000a) and biofilm grazers such as mollusks (Toth and Pavia 2002). Bacteria attached to kelp POC can be consumed by suspension and detritus feeding animals (Norderhaug et al. 2003, Fredriksen 2003, Kaehler et al. 2006).

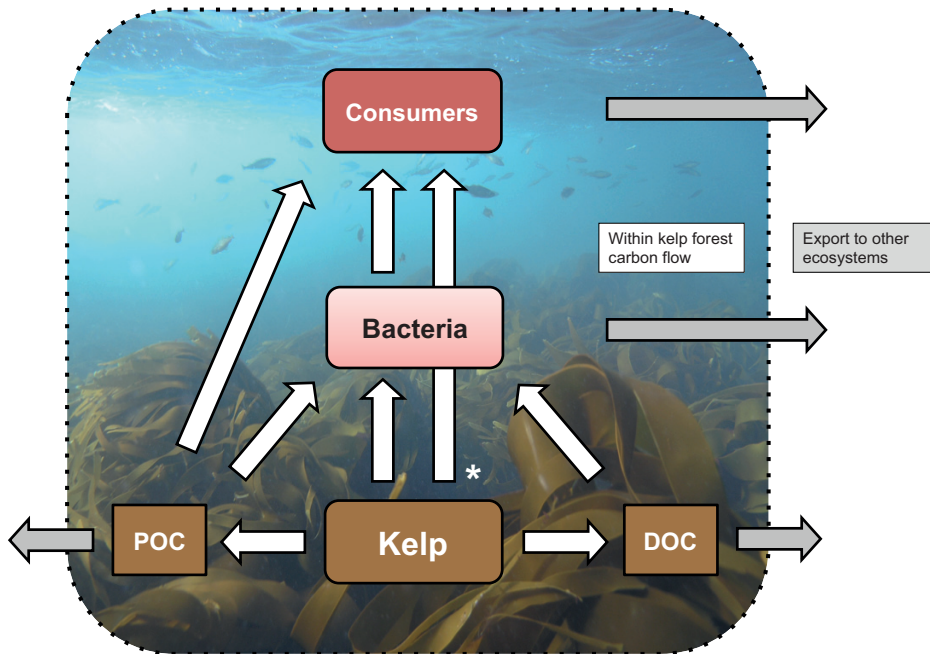


Figure 4. The flow of kelp-produced carbon in kelp forest ecosystems. Kelp carbon in the form of living kelp (Kelp) dissolved (DOC) and particulate organic carbon (POC) can fuel kelp forest food webs according to the white arrows, but can also be exported to other ecosystems (grey arrows). Direct grazing on living kelp by animals (*) is probably a minor pathway of kelp carbon under stable ecosystem conditions. Instead, bacteria may play a central role in making kelp primary production available to higher levels in the food chain.

Background photograph by Kjell-Magnus Norderhaug.

A summary of the possible ways in which kelp produced organic carbon can flow in kelp forest food webs and be exported to other ecosystems is shown in Figure 4. With few exceptions (Newell and Lucas 1981), the relative importance of the different routes has not been addressed for kelp forest ecosystems around the world, including

the roles of the different bacterial populations described above. Thus, the flow and the fate of kelp-produced carbon in and outside the kelp forest are unclear.

2.7 Kelp surface associated microorganisms

Bacteria on the surfaces of living kelp represent one of the bacterial populations that can play a role in carbon cycling in kelp forests, as discussed in the previous section. In addition, bacteria and other microorganisms on kelp surfaces interact with their host, and may thereby influence kelp health by acting as pathogens or by protecting against harmful organisms. They may also be a reservoir of bioactive substances, such as antibiotics and polymer-degrading enzymes, and therefore of interest in biotechnological applications. Despite this, relatively few studies have addressed microorganisms inhabiting kelp surfaces prior to the work presented in this thesis.

Early studies used cultivation-based methods to study kelp-surface microorganisms. For example, Laycock (1974) studied the bacteria on *Saccharina longicuris* (formerly *Laminaria longicuris*) in eastern Canada during a seasonal cycle. Psychrophilic (cold-loving) and mesophilic (moderate temperature-loving) populations of culturable bacteria were detected in winter and summer, respectively. The psychrophilic bacteria were typically utilizing laminaran, while the mesophilic bacteria showed an affinity towards mannitol, alginate and proteins. The culturable bacterial populations were quantitatively and qualitatively different on old, decaying parts of the kelp thallus than younger parts (Laycock 1974). Higher numbers of culturable bacteria were also found on the older tips than on younger parts of the thallus on two kelp species in South African kelp forests (Mazure and Field 1980).

More recent studies using cultivation-based methods include the 16S rRNA gene sequences of isolated strains making phylogenetic classification of bacteria possible. For example, Lee and co-workers (2006) isolated several strains related to the genera *Psychrobacter* sp. and *Psychromonas* sp. (*Gammaproteobacteria*) and to *Bacillus* (*Firmicutes*) from the kelp *Undaria pinnatifida* in Korea. All isolates were closely related to previously described psychrophilic bacterial strains (Lee et al. 2006). Wiese and co-workers (2009) conducted a large survey of bacterial strains isolated from the

kelp *Saccharina latissima* to investigate their antibiotic activities. Nearly half of the 210 strains investigated displayed the ability to inhibit the growth of at least one type of microorganism, suggesting that kelp bacterial communities are a promising source for the discovery of new antibiotic substances (Wiese et al. 2009). The isolates belonged to *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Firmicutes* and *Actinobacteria* and several represented previously undescribed genera and species.

Because kelps are commercially valuable in many parts of the world, pathogenic bacteria that compromise harvests have been a concern and a subject of research. Bacterial strains belonging to the genus *Pseudoalteromonas* (Gammaproteobacteria) associated to spot-wounding disease of farmed *Saccharina japonica* were isolated by Sawabe and co-workers (Sawabe et al. 1992, 1998, 2000). Many of these strains produce extracellular alginate lyase enzymes that enable them utilize alginate as a carbon source. A similar strain was also identified as the disease-causing agent of natural populations of *Saccharina religiosa* in Japan (Vairappan et al. 2001).

In addition to bacteria, other microorganisms, such as protists and microalgae live on kelp surfaces. Heterotrophic protists can also play an important role in kelp forest food webs as grazers on the bacterial biofilms. They may also be able to utilize kelp-produced carbon sources directly (Armstrong et al. 2000b). Heterotrophic protists such as amoeba and flagellates reached numbers up to 50 individuals cm⁻² on the kelp *Laminaria digitata*. Damaged (older) tissue consistently sustained higher populations than undamaged tissue (Armstrong et al. 2000a). Many types of microscopic algae colonize kelp surfaces (Ellertsdottir and Peters 1997). Brown algae of the genus *Laminariocolax* are known to live endo- and epiphytically on different species of kelp. Their phylogeny suggests that they may have co-evolved with their hosts (Burkhardt and Peters 1998).

Direct observation of kelp surfaces and their attached microbial communities using scanning electron microscopy (SEM) enabled a detailed study of cell density and morphology of bacteria in biofilms on the kelp *Laminaria digitata* on the Atlantic

coast of France (Corre and Prieur 1990). Densities ranging from 10^6 - 6×10^7 cells cm^{-2} were observed with a maximum in summer on the old, decaying tips of the laminae. The bacterial cell morphologies were also different in summer and winter and between the middle and the tips of the kelp lamina, implying differences in bacterial community composition. The total counts of bacteria using SEM were compared with counts of culturable bacteria, which were consistently between one and five orders of magnitude lower (Corre and Prieur 1990). This observation illustrates the limitations of cultivation-based methods.

Recently, cultivation-independent methods have been used to study the community composition of kelp surface-associated bacteria, enabling a less biased view of the bacterial communities. In parallel to the work presented in this thesis, Staufenger and co-workers (2008) investigated bacteria from the surfaces of another kelp, *Saccharina latissima*, using 16S rRNA gene clone library construction and DGGE. They found that different parts of the kelp thallus, corresponding to holdfast, stipe, meristem and old lamina displayed different bacterial communities. The bacteria mainly belonged to *Alphaproteobacteria*, followed by *Gammaproteobacteria* and *Bacteroidetes* (Staufenger et al 2008).

In summary, these studies have all shed light on different aspects of kelp surface-associated microorganisms. General patterns emerge, such as the importance of kelp surface region or age for determining microbial cell density and community composition (Laycock 1974, Mazure and Field 1980, Corre and Prieur 1990, Armstrong et al. 2000, Staufenger et al. 2008). Still, the knowledge is fragmentary because of the few studies that have addressed the topic and the methods that have been used. For example, early studies were limited by the bias introduced cultivation-based methods. In addition, the lack of phylogenetic classification of the cultured bacteria from early studies makes it difficult to compare their results with current findings. Cultivation-independent methods are just beginning to be applied to kelp surface biofilms and have a great potential for uncovering community composition and dynamics. This type of knowledge is vital for a holistic understanding of host-microbial interactions and the role of bacteria in kelp forest ecosystems.

3. Research questions

Against the background of the current knowledge outlined in previous sections, the following questions were formulated:

Q1. Are there certain bacterial lineages that predominate in *Laminaria hyperborea* surface biofilms? At the commencement of the work presented in this thesis, there were no published investigations of kelp surface bacterial communities using culture-independent methods. Therefore, the community composition of the biofilms was unknown. The question was addressed in **Paper I**, using DGGE fingerprinting of the 16S rRNA gene and in **Paper II** using FISH and 16S rRNA gene clone library analysis.

Q2. How do the density, composition and diversity of the biofilm communities vary in time and space? Because *Laminaria hyperborea* physiology, including growth and exudation of DOC is controlled by temporal (seasonal) and spatial factors, the bacterial communities on its surfaces may also be directly or indirectly affected by these factors. The question was addressed in **Paper I** through analysis of the composition and density of the biofilms during a seasonal cycle at two spatially separated sampling sites using DGGE fingerprinting of the 16S rRNA gene and microscopic cell counting. In **Paper IV**, the diversity of the bacterial communities was investigated during a seasonal cycle and at three sampling sites differing in wave exposure using pyrosequencing of the 16S rRNA gene and microscopic cell counting. In **Paper II**, the question was further explored by a detailed analysis of *Planctomycetes* using FISH and 16S rRNA gene clone library analysis at different times of the year.

Q3. Do heterotrophic bacteria in the biofilms utilize kelp-produced carbon?

Kelp contains and exudates large quantities of organic material such as polysaccharides. Heterotrophic bacteria living on kelp surfaces may utilize these rich carbon sources, thereby contributing to the carbon turnover in the kelp forest (Fig. 4). This question was addressed in **Paper III** using an enrichment approach to cultivate

bacteria using different kelp substances as carbon sources. The cultivated bacteria were compared with 16S rRNA gene sequences from indigenous kelp surface biofilms obtained using cultivation-independent methods to assess their significance in the kelp forest environment.

Q4. Is there a relationship between the rate of bacterial secondary production in the biofilms and the composition and diversity of the bacterial communities?

Variation in bacterial community composition and diversity may lead to a corresponding functional variation. Secondary production by heterotrophic bacteria is an aspect of bacterial community function that is highly relevant to the kelp forest ecosystem (Fig. 4). This question was addressed in **Paper IV** by analysis of bacterial community composition and diversity by pyrosequencing of the 16S rRNA gene accompanied by bacterial production measurements using incorporation of ³H-Thymidine.

4. Discussion

The purpose of this discussion is to synthesize and provide a holistic view of the major findings of this PhD. More detailed discussion on specific results can be found in the respective papers. In addition, some results and observations that were not included in the papers are presented and discussed.

4.1 Bacterial community composition

Several lineages within *Bacteria* were detected on the surfaces of *Laminaria hyperborea*. Figure 5 shows their relative proportions as revealed by the two separate molecular methods DGGE and pyrosequencing on a comparable set of samples from four seasons (**Paper I** and **Paper IV**). Both methods identify *Planctomycetes* as the most abundant group, and the numerical dominance of this group was confirmed using the more quantitative method FISH (**Paper II**). The high abundance of planctomycetes is an unusual and interesting feature of *L. hyperborea* surface biofilms that can give insights into the lifestyle habits of this poorly studied group of bacteria (**Paper II**). *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* were also abundant in both datasets (Fig. 5). This shows that in addition to *Planctomycetes*, these are the predominating bacterial lineages on *L. hyperborea*, answering **Q1**.

However, there are considerable differences in the relative proportions of some other bacterial lineages between the different methods. For example, the *Verrucomicrobia* and the *Cyanobacteria* were detected in high abundances using DGGE, but are nearly absent in the pyrosequencing dataset (Fig. 5). This discrepancy can primarily be attributed to bias introduced by the different primers used for the PCR steps of DGGE and pyrosequencing. Further, the lack of band intensity information could cause some groups to be underrepresented in the DGGE dataset. This illustrates how important it is to be aware of methodological bias when interpreting community composition results and to confirm interesting observations using more quantitative methods, such as FISH (**Paper II**) or real-time PCR, for example.

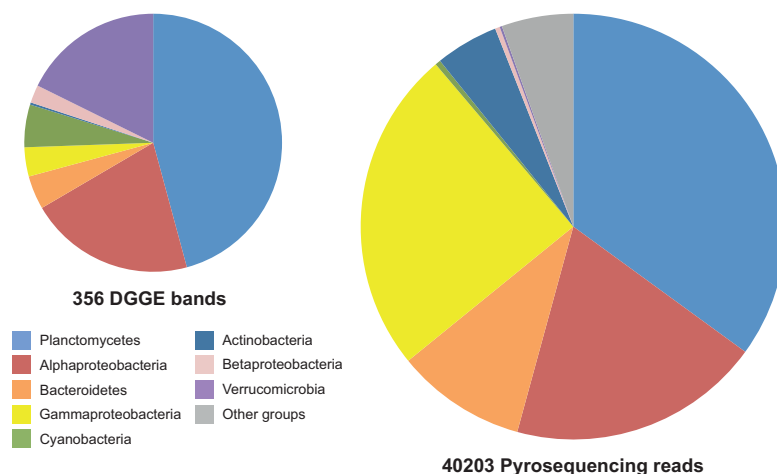


Figure 5. The major bacterial lineages on kelp surfaces: The pie charts show the relative abundances of 16S rRNA gene sequences that classify as the respective groups according to the two independent methods DGGE and pyrosequencing. Both charts are based on comparable sets of samples from February, May, July and November 2007.

In addition to *Bacteria*, sequences affiliated with *Archaea* were also sporadically detected on kelp surfaces using pyrosequencing. However, only 0.01 % of all sequences were of archaeal origin (**Paper IV**). Also, specific primers failed to amplify the 16S rRNA gene of *Archaea* on a selection of samples from different times of the year (results not shown), further indicating that archaea comprise a very small portion of the microbial communities on kelp surfaces.

Within the major bacterial lineages depicted in Figure 5, many individual bacterial species are presumably hidden. It is not possible to delineate bacterial species with the 16S rRNA approaches used in the work presented here, as cultivation and detailed phenotypic characterization are required according to current practice (Stackebrandt et al. 2002). Instead, partial 16S rRNA gene sequences were clustered into OTUs (operational taxonomical units) based on different sequence similarity values as an approximation of species (**Papers I, II, III and IV**). A few of these OTUs appear to be very common on kelp surfaces and were detected very frequently. Such OTUs include the planctomycete OTU "P44" in **Paper I** and the OTUs referred to as the

"core community" (see Fig. 5 in **Paper IV**). Unfortunately, it is not possible to directly compare the OTUs defined in **Papers I, III and IV**, because different areas of the 16S rRNA gene were targeted by the primer sets used for DGGE, colony PCR and pyrosequencing respectively. However, by analyzing the partial 16S rRNA gene sequences together with closely related full-length reference sequences it is possible to compare them indirectly, thereby consolidating the results from the different studies. This is illustrated in Figure 6, where selected sequences obtained using DGGE (**Paper I**), clone library analysis (**Paper II**), pyrosequencing (**Paper IV**) and enrichment cultivation (**Paper III**) are displayed in phylogenetic trees together with reference sequences. For example, this shows that the planctomycete OTU 1322, that was the single most abundant OTU in the pyrosequencing dataset (**Paper IV**), corresponds to the near full length clone sequence with the accession number HM369075 (**Paper II**).

4.2 Specificity of kelp surface bacterial communities

Phylogenetic analysis shows that the most closely related reference sequences of common kelp surface bacteria are often derived from the surfaces of other seaweeds (Fig. 6, **Paper II**). In Figure 6, several sequences derived from clone libraries of biofilms on the seaweeds *Delisea pulchra* and *Ulva australis* (Longford et al. 2007) appear closely related to *Laminaria hyperborea* surface bacteria (Fig. 6). A symbiont of the red seaweed *Prionitis decipiens* (Ashen and Goff 2000) is among the closely related reference sequences of the alphaproteobacterial OTUs OTU A2 (**Paper III**) and OTU 90 (**Paper IV**). One clone from the surface of the kelp (i.e. brown seaweed) *Saccharina latissima* clusters with the gammaproteobacterial OTUs OTU G96 (**Paper I**), OTU G2 (**Paper III**) and OTU 46 (**Paper IV**). These apparent similarities between bacterial communities on red, green and brown seaweeds suggest that there are bacterial lineages that are generally adapted to a life on seaweed surfaces, rather than one type of seaweed specifically. This is remarkable, because although red, green and brown seaweeds are all photosynthetic eukaryotes, they are not closely related evolutionarily and are often structurally and physiologically dissimilar. OTUs that are found on all types of seaweeds may therefore represent bacterial species that

display a high degree of phenotypic plasticity, enabling them to be successful generalists. Alternatively, they may represent closely related but separate bacterial species that have a different functional repertoire, perhaps due to horizontal gene transfer of genes involved in metabolism.

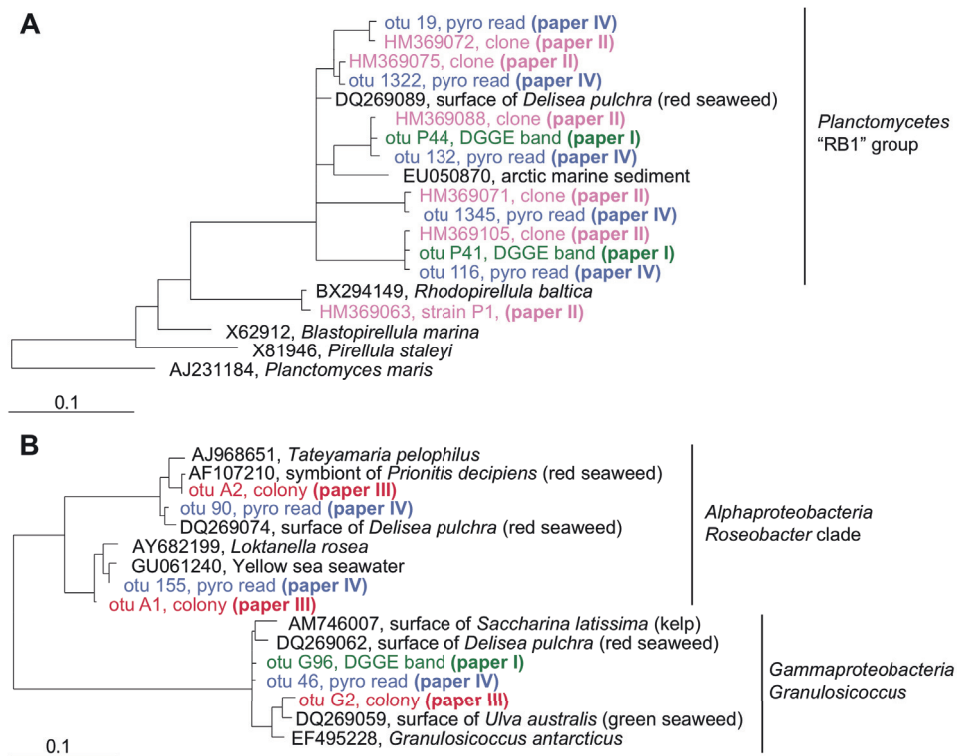


Figure 6. The "core community" and relatives: The phylogenetic trees show the relationships between 16S rRNA gene sequences of the bacterial OTUs identified as the "core community" (in blue, Paper IV) and related sequences obtained in **Papers I, II** and **III** as well as reference sequences. The planctomycetes tree (**A**) is based on the tree in **Paper II**, where partial 16S rRNA gene sequences have been added using the parsimony tool in the ARB software. The tree including alphaproteobacteria and gammaproteobacteria (**B**) was calculated using 110 near full-length reference sequences using PhyML, no positional filter and 100 bootstraps. The partial sequences from kelp surfaces (including from *S. latissima*) were added to the tree as described above. Several sequences were omitted from both trees for clarity of presentation. The scale bars indicate 10 % sequence divergence.

However, although these results suggest that closely related organisms are found on different types of seaweeds (Fig. 6), the bacterial communities as a whole may differ substantially. Indeed, Lachnit and co-workers (2009) found that host phylogeny determined bacterial community composition more than geographic location on several species of red, green and brown seaweed in the Baltic and North seas. Seaweed species was also a more important determinant than season (Lachnit et al. 2010). In addition, the bacterial community of *L. hyperborea* surfaces and that of its red seaweed epiphyte, *Palmaria palmata*, showed very little overlap when compared using DGGE, sharing only 3 of 41 OTUs (M.M. Bengtsson, unpublished results).

Seawater bacterial communities comprise a pool of potential colonizers of seaweed surfaces. Which bacteria successfully colonize newly formed seaweed surfaces may be determined both by stochastic processes influenced by the abundance of different bacteria in the seawater and by host-bacterial interactions that select certain bacteria over others. The bacterial community composition of *L. hyperborea* biofilms and seawater showed minor overlap when compared by DGGE (**Paper I**). Similar results have been reported from other seaweeds (Staufenberger et al. 2008, Burke et al. 2010), indicating that seaweed surfaces are colonized by low abundance populations in the seawater.

The core community of bacterial OTUs that was identified in **Paper IV** indicates that the colonization of kelp surfaces is a non-random process that selects for certain organisms over others. However, the high variability of the relative abundances of the core community OTUs (**Paper IV**) may be an indication that stochastic processes also play a role in community assembly. Further, the finding of closely related OTUs on *L. hyperborea* and other seaweed species raise important questions about the specificity of host-bacterial interactions on seaweed surfaces. More comparative studies of bacterial communities on different seaweed species are however necessary before any conclusive statements can be made on this subject.

4.3 Bacterial diversity

In contrast to the core community OTUs, that are frequently detected and abundant, there are several OTUs that are only sporadically detected in low abundances. These rare OTUs are in fact the majority, with 31% of OTUs represented only one pyrosequencing read, and 61% represented by less than five reads (**Paper IV**). This makes the rare OTUs responsible for the differences in OTU richness observed for example between kelp surfaces of different ages (**Paper IV**). The bacteroidetes have the most rare OTUs in relation to their abundance (**Paper IV**), indicating that kelp surfaces are a suitable habitat for a wide variety of members of this group.

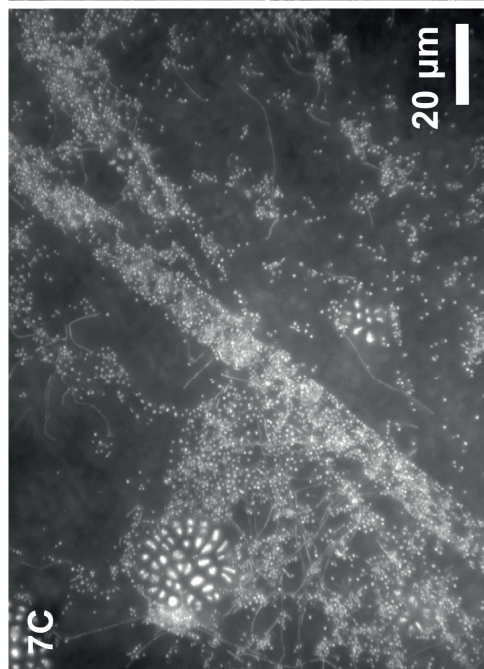
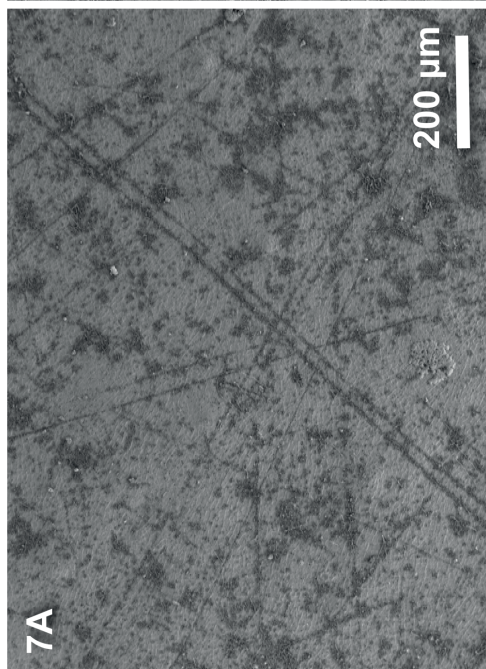
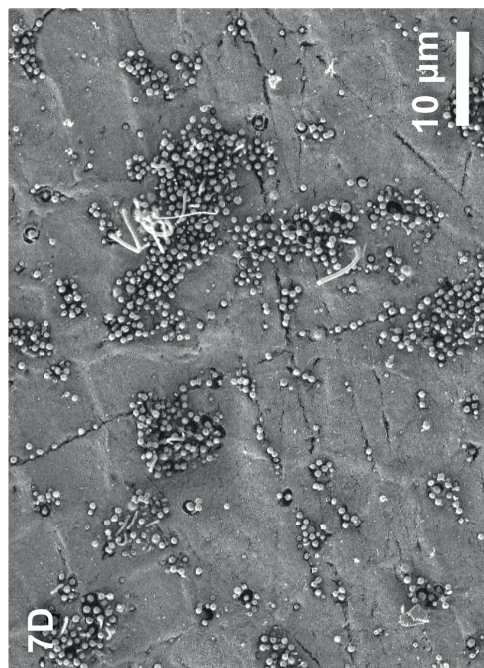
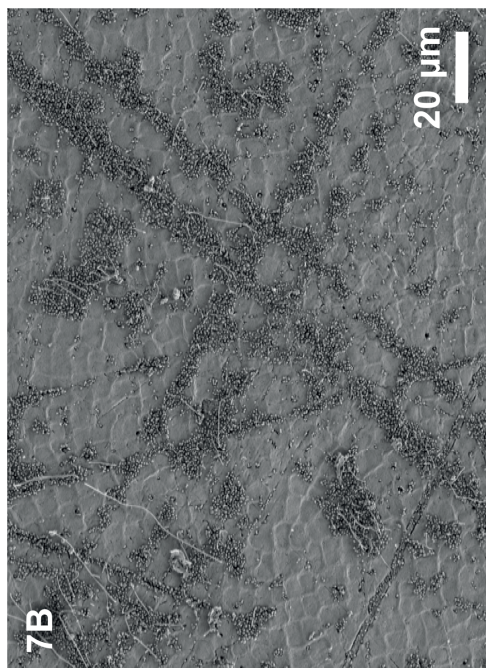
Are kelp surfaces diverse in relation to other microbial habitats? This question is difficult to answer due to extremely variable sampling and experimental procedures that make comparisons to other habitats difficult. For example, many of the existing studies that have utilized pyrosequencing of 16S rRNA gene amplicons, as in **Paper IV**, have not accounted for sequencing noise, leading to inflated richness estimates (Quince et al. 2009). However, in some situations, including the extreme dominance of a few RB1 planctomycete OTUs in summer (**Paper II, Paper IV**) and the dominance of the gammaproteobacterial OTU 46 on young kelp surface in March (**Paper IV**), kelp surfaces can certainly be characterized as low diversity microbial habitats.

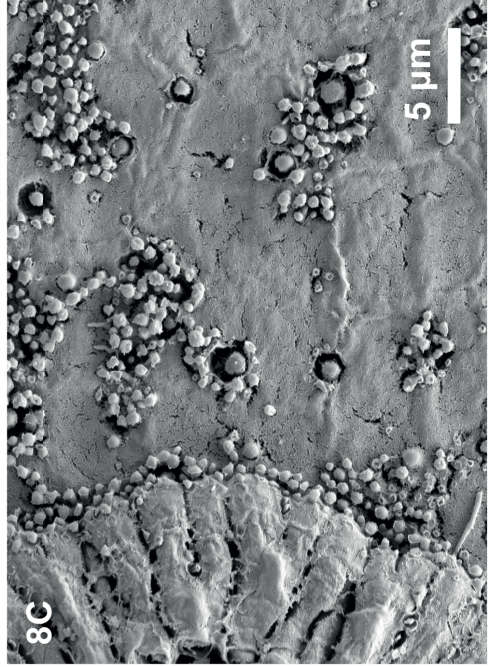
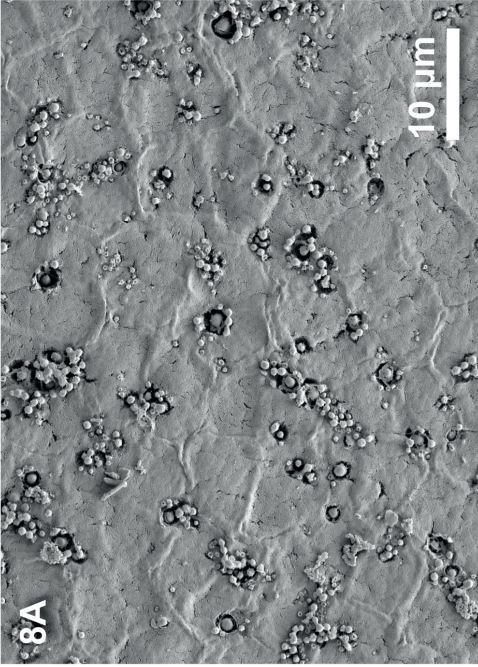
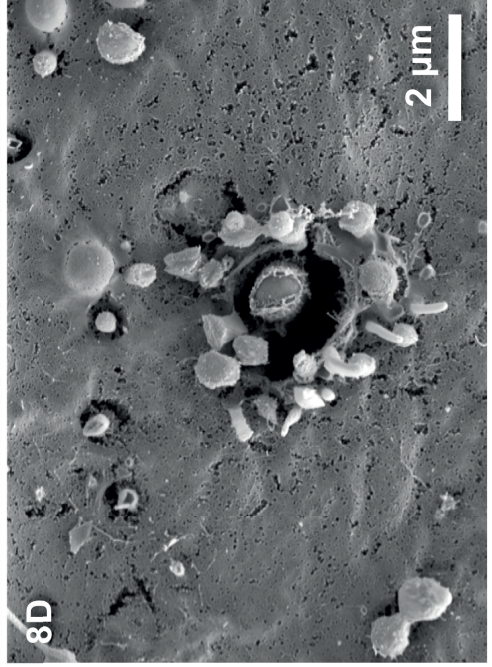
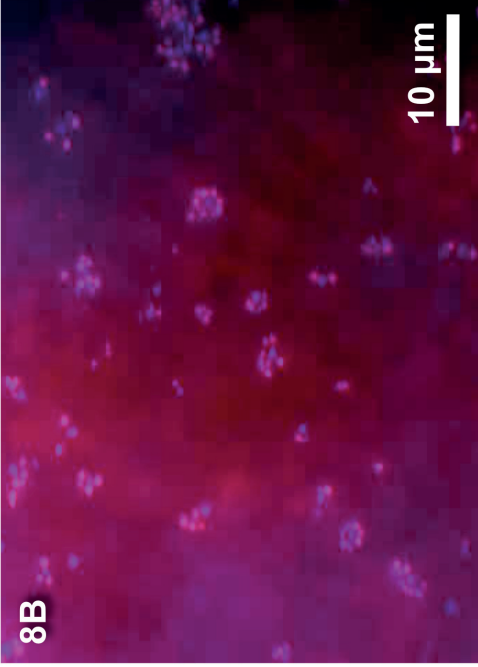
4.4 Biofilm cell density and architecture

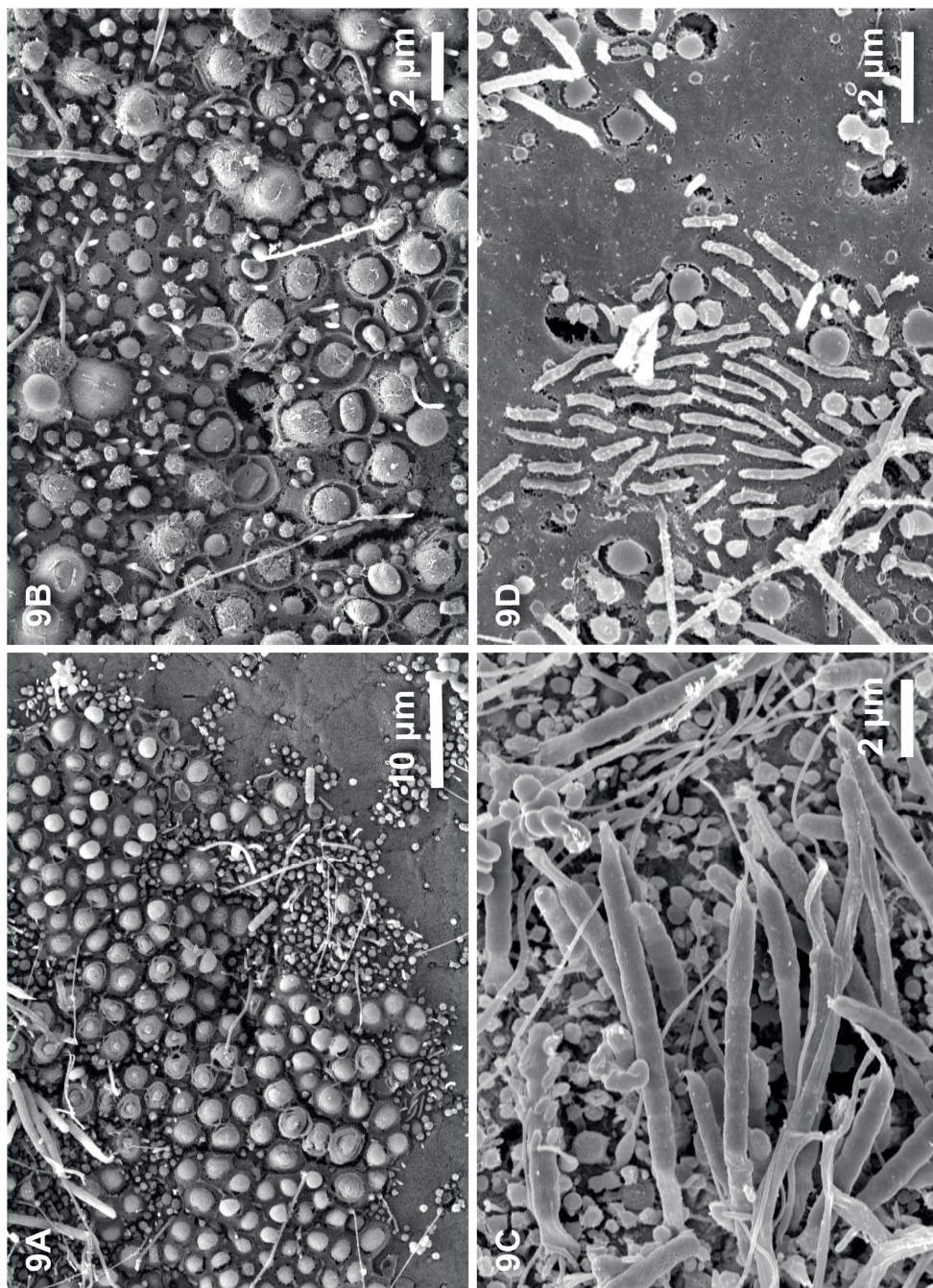
The overall average biofilm cell density on *Laminaria hyperborea* surfaces was around 10^7 cells cm^{-2} , although the variation between kelp individuals is large and displays marked seasonal and environmental trends (**Paper I, Paper IV**). These numbers are comparable to cell densities recorded on the closely related kelp *Laminaria digitata*, which also followed a seasonal pattern with low densities on the lamina in March (Corre and Prieur 1990). Considering the inevitable process of biofouling that leads to rapid overgrowth of inert marine surfaces (ZoBell and Allen 1935), the cell densities on kelps are relatively low. This implies the presence of mechanisms that inhibit biofilm formation.

Biofilms on *Laminaria hyperborea* were sometimes dense, resembling lawns of tightly packed cells (Figures 9B, 9C and 10A). However, biofilms did not appear to be more than one cell layer thick. In contrast, biofilms on inert surfaces typically develop complex multilayered structures, known as microcolonies (DeBeer and Stoodley 2006). These microcolonies form in an organized matter, involving quorum sensing by AHL (Davies et al. 1998). The absence of microcolonies on kelp surfaces may indicate that mechanisms that interfere with biofilm organization prevent them from forming. Borchardt and colleagues (2001) demonstrated that haloperoxides produced by the kelp *Laminaria digitata* deactivate AHL in the presence of H₂O₂, which is normally formed and released during photosynthesis in seaweeds (Pedersen et al. 1996). Disruption of biofilm organization by deactivation of quorum sensing may thus represent an important antifouling mechanism in kelps. Similar mechanisms have been observed in red algae, involving halogenated furanones that act as specific antagonists of AHL, thereby inhibiting biofilm quorum sensing and subsequent biofilm development (Givskov et al. 1996). Other antifouling strategies, for example direct toxicity of oxygen radicals exuded by the kelp (Küpper et al. 2001, 2002) as well as antimicrobial activity of some biofilm bacteria (Wiese et al. 2009, Rao et al. 2007) probably also contribute to the low cell densities typically observed on *L. hyperborea*.

Scanning electron microscopy and fluorescence microscopy reveal complex and beautiful microbial landscapes on kelp surfaces. Many different bacterial cell morphologies, epiphytic microalgae as well as other cell types of unknown identity are to be seen. Curious assemblages of cells and non-random patterns of cell distribution hint at complex biotic interactions and evolutionary adaptations. Unfortunately, very little can be interpreted and less can be concluded about the biology of the biofilm inhabitants by these observations. A collection of microscopic images illustrating some of the commonly observed features of cell distribution and morphology is found in Figures 7-10.







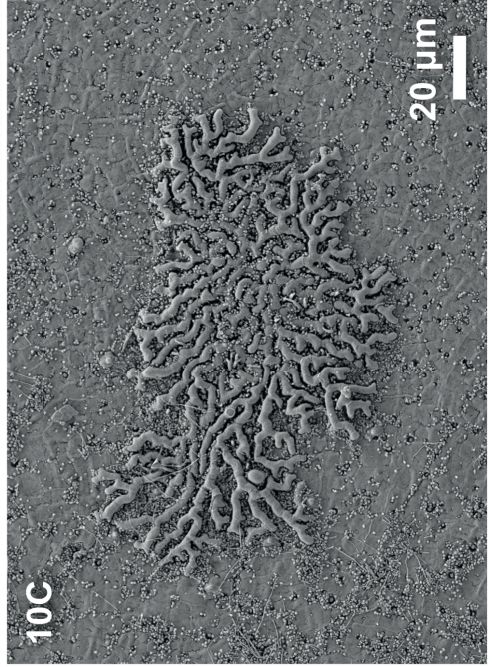
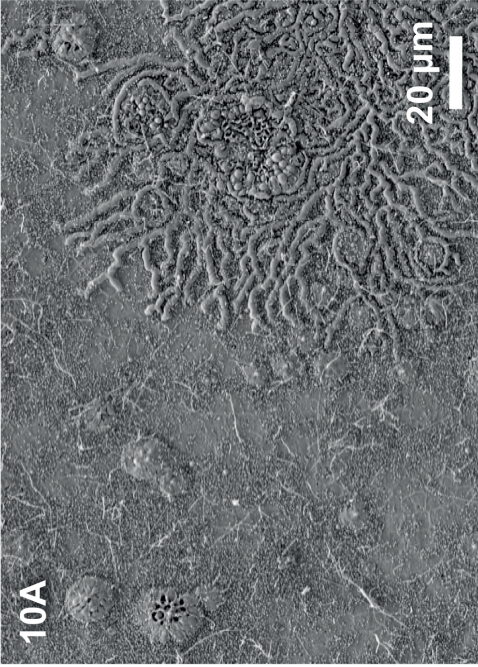
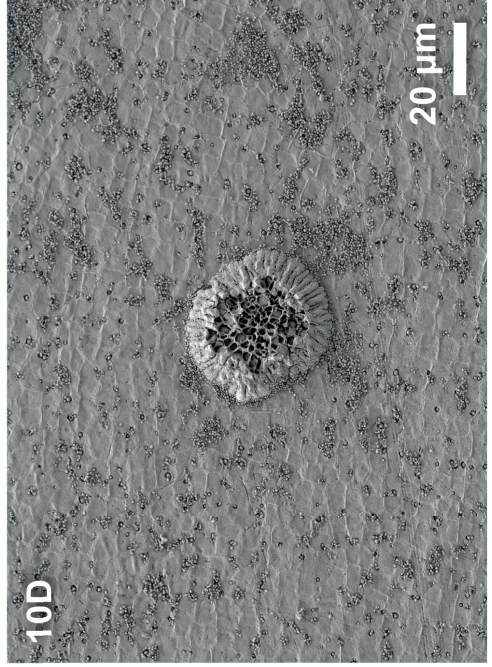
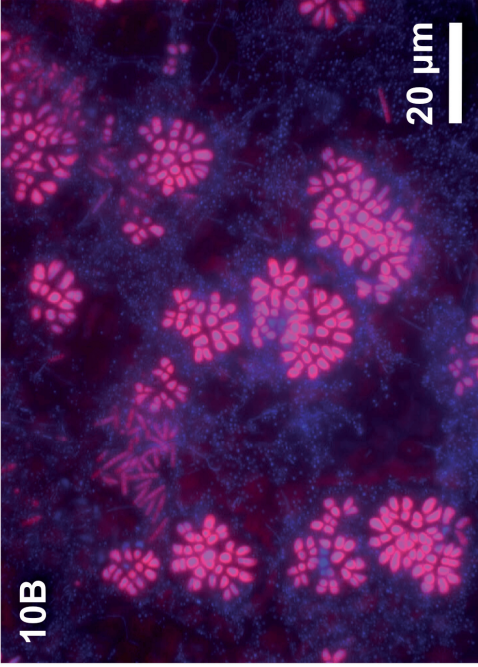


Figure 7. Colonization patterns (page 39).

Bacterial cells attached along straight lines on the kelp surface were frequently observed. These lines were often long (7A) and were not aligned with the underlying kelp epithelial cells (7B), visible as a grid pattern on the surface. These line structures were observed both in SEM (7A, 7B, 7D) and in the fluorescence microscope (7C). Upon closer inspection, small scratches or tears on the surface layer were visible, and bacterial cells appeared preferentially associated with these structures (7D). The colonization of bacterial cells along straight lines may be due to scratches in the kelp surface mucus layer caused by mechanical forces associated to wave action and contact with sharp objects. This disturbance of the mucus layer may facilitate attachment of bacterial cells.

Figure 8. Bacterial associations (page 40).

In biofilms of relatively low cell density, cell assemblages consisting of one central cell surrounded by a ring of cells were frequently observed. In SEM, the central cell appeared to be sitting in a pit in the surface mucus layer, while the surrounding cells were attached on top of the mucus layer (8A, 8C, 8D). FISH revealed that the surrounding cells were often planctomycetes, visible as red signals (8B), while the central cell hybridized with eubacterial probes, but not planctomycetes-specific probes. The unidentified central cells may have created the depressions they are sitting in by releasing extracellular enzymes that degrade the kelp surface mucus layer. The surrounding planctomycetes (and other) cells may be benefiting from metabolites produced by the central cell. Alternatively, their attachment may have been facilitated by the disturbance in the mucus layer.

Figure 9. Different cell morphologies (page 41).

SEM revealed diverse morphologies of bacterial cells on kelp surfaces. Large cocci (9A, 9B), narrow rods (9D) and thick filaments (9C) displaying typical cyanobacterial autofluorescence were common features, in addition to the smaller irregular cocci that presumably represent the dominant planctomycetes.

Figure 10. Microscopic algae (page 42).

Several unidentified algal cells were observed on kelp surfaces. Round multicellular thalli (10A, 10B, 10D) displayed strong autofluorescence indicative of red algal pigments (10B). Branched filamentous thalli were also frequently observed (10A, 10C), and typically displayed weaker autofluorescence. The branched filamentous forms sometimes displayed an endophytic habit, with filaments extending into the kelp epithelial tissue. These algae may represent habitually microscopic species or microscopic stages of some of the common epiphytic seaweeds found on *L. hyperborea*.

4.5 Variation in time and space

4.5.1 Seasonal variation

Temporal variation was addressed through the investigation of kelp surface biofilms during a seasonal cycle. Seasonal factors were expected to influence the biofilms, both due to the highly seasonal growth habit and physiology of *Laminaria hyperborea* and due to seasonal fluctuations in physical conditions such as seawater temperature. The results in **Paper I** demonstrated that the biofilm cell density and bacterial community composition are indeed subject to seasonal variation. The planctomycetes subset of the bacterial communities also displayed differences in relative abundance, community composition and diversity at different times of the year (**Paper II**). The diversity of the total bacterial communities varied throughout the year as well (**Paper IV**). All these observations show that seasonal factors influence kelp surface biofilms substantially. However, it is difficult to say what specific factors are responsible for these trends, as seasonal factors include both biotic factors mainly relating to kelp physiology and abiotic factors like temperature, nutrient levels and light conditions. One important component of the observed seasonal patterns is apparently kelp surface age (**Paper I, Paper IV**). The rapid expansion of the kelp surface tissue during the growth period in spring clearly influenced the density of the biofilms (**Paper I**). In addition, the strong relationship between bacterial diversity (richness and evenness) and kelp surface age in **Paper IV** implies that it also plays a role in structuring the communities. Several other studies on surface microorganisms on different species of kelp have also reported quantitative and qualitative differences in microbial populations that can be attributed to age of the kelp tissue (Laycock 1974, Mazure and Field 1980, Corre and Prieur 1990, Armstrong et al. 2000, Staufenberger et al. 2008). However, the seasonal community composition resembled a cycle (Fig. 11A), where the aging kelp lamina in February and the fresh kelp surface in May had many OTUs in common (**Paper I**). This indicates that factors other than those that relate to the kelp also influence the bacterial communities to some extent.

Season was the only scale of temporal variation investigated in this work and thus year-to-year variation was not explicitly addressed. However, the relative abundances of the "core-community" OTUs between samples from July 2007 and July 2009 (**Paper IV**) suggest that there may be some differences between the two years. The degree of year-to-year variation can show the reproducibility of biofilm community assembly, as every year represents a new colonization event. This would be an interesting aspect to address in future investigations.

4.5.2 Variation between sampling sites

Laminaria hyperborea was sampled at different sites in the Bergen area, including outer fjord areas facing the open ocean and in the inner archipelago, closer to the mainland. These sites differ primarily in the exposure to wave action experienced by the kelp forests (see map in Fig. 2 in **Paper IV**). The two sites sampled in **Paper I** were chosen to be spatially separated yet similar with respect to wave exposure while the three sites sampled in **Paper IV** represented a gradient in wave exposure, ranging from "moderately exposed" (Tekslo) to "very sheltered" (Landro) to "extremely sheltered" (Flatevossen) according to definitions by Isaeus (2004). A clear difference in biofilm cell density was observed between these three sites (**Paper IV**), and less wave exposure appeared to be associated with higher cell density. The sites also differed in bacterial community composition as shown in Fig. 11B. In contrast, the two similarly wave exposed yet spatially separated sites did not display significant differences in cell density, and only occasional differences in community composition (**Paper I**). At this local spatial scale (Bergen area), wave exposure thus appears to be an important influence on the biofilms, directly or indirectly. Again, as with seasonal factors, it is difficult to separate biotic factors relating to the kelp from physical factors such as wave exposure *per se* when it comes to the influence they may have on the biofilm communities. Wave exposure acts on the kelp itself influencing productivity and growth (Sjötun and Fredriksen 1995, Sjötun et al. 1998), which may correlate with differences in kelp physiology that in turn influence the bacterial communities. Alternatively, lower wave exposure could also contribute to

increased sedimentation of particles on kelp surfaces, promoting attachment of bacteria leading to denser biofilms in sheltered areas.

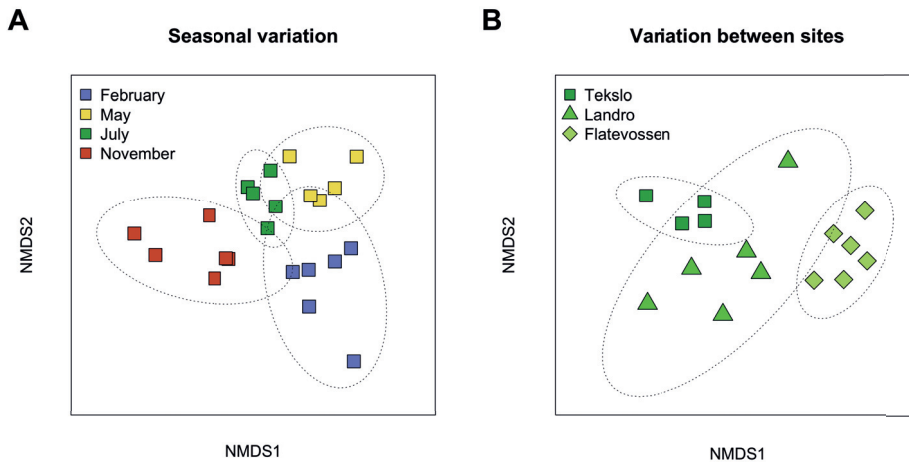


Figure 11. Variation of bacterial community composition in time and space: The non-metric multidimensional scaling plots illustrate the similarity of OTU composition between samples. Seasonal variation (**A**) is based on DGGE data (**Paper I**) from kelp collected throughout the year 2007 at the sampling site Tekslo. Variation between sites (**B**) is based on pyrosequencing data (**Paper IV**) from kelp collected in July 2009. The sites differ in wave exposure; Tekslo is the most exposed, Landro intermediate and Flatevossen the most sheltered.

In conclusion, *Laminaria hyperborea* surface biofilms vary in time according to seasonal factors and appear to vary in space in relation to wave exposure (**Q2**). Both biotic and abiotic factors linked to season and wave exposure can be expected to act upon and influence bacterial communities on kelp surfaces. However, the observed seasonal patterns can largely be explained by biology of the kelp, through kelp surface age (**Paper I** and **Paper IV**). Wave exposure also has a strong influence on the biology of the kelp. This suggests that most of the variation of kelp surface biofilms in time and space (at the scales investigated) is driven by the biology of the kelp host.

4.6 Culturability of kelp surface bacteria

Seaweed surfaces have previously been identified as environments where recovery of bacteria using cultivation-based approaches is relatively high (Jensen et al. 1996,

Bouvy et al. 1986). The most dominant bacteria on *Laminaria hyperborea* surfaces, the "RB1" planctomycetes (**Paper II**), defied culture with the approaches employed in **Paper II** and **Paper III**. Instead, a planctomycetes strain closely related to *Rhodopirellula baltica* was isolated (**Paper II**). Partial 16S rRNA gene sequences corresponding to this strain make up only 0.1 % of pyrosequencing reads from kelp lamina in September 2008, which is the same habitat that it was isolated from (results not shown). This planctomycete is thus only a minor-abundance member of the community. The most abundant planctomycetes, for example groups RB1 and OM190 (**Paper II**), have no close cultured relatives which makes them interesting candidates for further cultivation efforts.

Several OTUs from the enrichment cultivation experiment were also abundant enough to be detected on kelp surfaces using cultivation-independent methods (**Paper III**). Three of these OTUs (OTU A1, OTU A2 and OTU G2) are displayed in Fig. 6B together with close relatives recovered by other methods and reference sequences. Interestingly, they appear closely related to OTUs that belong to the "core community" of frequent and abundant OTUs (**Paper IV**). Two are alphaproteobacteria within the *Roseobacter* clade (OTU 90 and OTU 155). This marine group has received considerable attention in later years, because they are both readily culturable and frequently detected in the environment (e.g. Wagner-Doebler and Biebl 2006, Brinkhoff et al. 2008, Tang et al. 2010, Newton et al. 2010). Gammaproteobacteria closely related to *Granulosicoccus antarcticus* (Lee et al. 2007) were also detected by both enrichment cultivation and other methods (Fig. 6b). OTUs belonging to this cluster were abundant in samples from the young meristem in March (**Paper IV**) and may represent a pioneer colonizer on fresh kelp surface. Related sequences were also found on the kelp *Saccharina latissima* (Staufenberger et al. 2008), the brown seaweed *Fucus vesiculosus* (Lachnit et al. 2010), the green seaweed *Ulva australis* and the red seaweed *Delisea pulchra* (Longford et al. 2007) suggesting that organisms within this cluster are seaweed-associated yet ecologically versatile.

The recovery in enrichment culture of bacteria closely related to many of the most abundant bacteria detected with culture-independent methods (**Paper III**, Fig 6B) is remarkable. It is contrary to the experiences made in many other marine habitats, where cultured bacteria are usually very rare members of the community (e.g. Pedros-Alio 2007) and abundant members are isolated only with comparably large efforts (Rappé et al. 2002). However, a word of caution must be said, because although the cultured strains are closely related to environmental sequences from kelp, they may represent different and ecologically distinct species. Nonetheless, the results suggest that kelp surfaces could make an excellent model habitat, providing a unique possibility to study abundant community members both in their natural environment and in pure culture.

4.7 The role of kelp surface bacteria in kelp forest carbon cycling

In order for kelp surface bacteria to play a role in the turnover of kelp primary production, they must utilize kelp substances as carbon sources (see **Q3**). The alternative is that they merely use the kelp as a surface to attach to, while they utilize seawater DOC of non-kelp origin (i.e. from phytoplankton) as a carbon source. They may also be autotrophic, fixing carbon themselves through photosynthesis. In **Paper III**, the ability of bacteria from kelp surfaces to grow on substances that are major constituents of *Laminaria hyperborea* was tested using a cultivation approach. Several alphaproteobacteria and gammaproteobacteria and one *Firmicutes* strain that grew on one or more of these substances were enriched. Some of the same, or closely related, bacteria were abundant enough on kelp surfaces to be detected by DGGE (**Paper III**). This shows that some kelp surface bacteria indeed have the ability to utilize kelp-produced carbon and answers **Q3** partially. However, the majority of the bacteria on kelp surfaces are planctomycetes (**Paper II**), which were not captured in the cultivation approach in **Paper III**. Their dominance in numbers makes their secondary production, based on kelp primary production or not, of significance for addressing **Q3**. Genomic analysis of other cultured and uncultured marine planctomycetes suggests that they are specialized in degrading sulfated polysaccharides (Woebken et al. 2007). The high content of fucoidan and other

sulfated polysaccharides in *L. hyperborea* invites to speculation that planctomycetes on kelp surfaces utilize these substances as carbon sources. Yet, this hypothesis demands further investigation before conclusions about the carbon sources of kelp surface planctomycetes can be made.

Total heterotrophic bacterial production of biofilm communities on kelp pieces appeared to increase with OTU diversity. More specifically, evenness of the communities correlated with ^3H -Thymidine incorporation (**Paper IV**). This answers **Q4**, by showing that bacterial diversity can influence bacterial production. This effect is to some extent related to the relative abundance of the dominant planctomycetes, as low evenness is the same as high dominance of a few OTUs. This suggests that community composition as well as diversity affects bacterial production (**Q4**). Due to the experimental setup of the production measurements (**Paper IV**), involving the use of cut out kelp pieces, it is difficult to say if the observed trends directly apply to surfaces of living kelp plants. However, the kelp pieces may resemble fragmented kelp material (POC, see Fig. 4), which are highly relevant to kelp forest food webs (e.g. Duggins et al. 1989, Fredriksen 2003, Kaehler et al. 2006). In any case, the results imply that there is a potential for the bacterial community composition and diversity to influence bacterial secondary production and ultimately kelp forest carbon cycling. This shows that the microbial ecology of kelp forests should be considered in order to understand large-scale fluxes of carbon in the ecosystem.

Finally, how important are kelp surface bacterial communities for kelp forest food webs? While the aim of work presented here was not to quantify the contribution of kelp surface biofilms to the carbon flow in kelp forests (Fig. 4), the results allow some general comments. Biofilms on the surfaces of living kelp are dominated by planctomycetes, which are generally slow-growing bacteria (Ward et al. 2006). In addition, the cell density of the biofilm is often relatively low (**Paper I, Paper IV**), probably as a result of antifouling mechanisms of the kelp. Bacterial production estimates were in general also relatively low, even if it is unclear if they reflect the conditions on living kelp surfaces (**Paper IV**). Based on all these observations, bacterial secondary production on the surfaces of living kelp can be expected to make

a minor contribution to kelp forest food webs in comparison to that of bacterial communities that degrade fragmented and detached kelp (POC). This dead kelp material is probably under less influence of kelp antifouling mechanisms and may thereby support denser and more productive bacterial communities. However, the relative contribution of kelp surface bacteria to bacterial secondary production likely varies in time and space with factors that influence biofilm community composition (for example the abundance of planctomycetes), diversity and cell density. The work presented here clearly shows that season (**Papers I, II and IV**) and wave exposure (**Paper IV**) are among these factors.

4.8 Main conclusions

- *Planctomycetes* is the most abundant of the major bacterial lineages on kelp surfaces (**Q1, Paper II**).
- Temporal and spatial variation in kelp surface biofilms (**Q2**) is tightly coupled to factors that influence the biology of the kelp host, such as season (**Paper I**), and wave exposure (**Paper IV**).
- The diversity of bacterial OTUs in the biofilm increases with kelp surface age (**Q2, Paper IV**).
- Some bacteria from kelp surface biofilms utilize kelp produced carbon sources (**Q3, Paper III**).
- The diversity of the bacterial communities can influence the bacterial production on kelp surfaces (**Q4, Paper IV**).

5. Future perspectives

The results obtained during this PhD have uncovered fundamental information on the bacterial communities on the important kelp forest forming species *Laminaria hyperborea*. This knowledge forms a foundation from which new hypotheses about the roles of bacteria in kelp forest ecosystems as well as host-bacterial interactions can be formulated. The following paragraphs describe a few of the topics that should be explored further.

The finding of a core community that is stable across seasonal and other environmental factors (**Paper IV**) raises the question to what extent this community is specific for *L. hyperborea*, or if the same bacteria are also found on other kelp and seaweed species. Preliminary comparisons suggest that core community OTUs are closely related to bacteria detected on different seaweeds (Fig. 4). On the other hand, no planctomycetes were detected on the kelp *Saccharina latissima* (Staufenberger et al. 2008). Kelps share a common evolutionary descent, but the species also differ in several distinct ways. A systematic comparison of bacterial communities on geographically and phylogenetically separated kelps in combination with existing contextual data on the kelps, such as biogeography, phylogeny, chemical constituents and cellular and morphological characteristics could reveal factors that determine the presence or absence of certain lineages of bacteria. This would provide insight into the specificity of host-bacterial interactions on kelp surfaces, as well as into the functional roles of specific bacterial lineages.

The biofilms on detached kelp material (POC, Fig. 4) may be at least as important for kelp forest carbon turnover as biofilms on the surfaces of living kelp. However, their bacterial communities are likely to be substantially different because of the lack of active antimicrobial defense by the kelp. Controlled kelp decomposition experiments, where the bacterial community composition is monitored over time could reveal successional patterns that suggest the roles of different bacteria in the degradation process.

The initial cultivation attempts presented in this work (**Paper II** and **Paper III**) suggest that several of the abundant kelp surface bacteria can be isolated in culture. Further cultivation efforts could potentially bring some of the planctomycetes, many alphaproteobacteria (for example roseobacters) and gammaproteobacteria (for example *Granulosicoccus* spp.) into culture. Cultured strains of representative bacteria would be an invaluable resource to test hypotheses of the roles of these bacteria on kelp surfaces. For example, carbon source affinities and production of bioactive compounds could be studied extensively in pure culture.

If cultivation of the most abundant kelp surface bacteria should prove unsuccessful, the natural enrichment of RB1 planctomycetes in the biofilms offers other alternatives to investigating their function using cultivation-independent methods such as metagenomics, metatranscriptomics and metaproteomics. The identification of genes or proteins involved in metabolism could reveal what carbon sources these planctomycetes utilize on kelp surfaces as well as many other aspects of their physiology and interactions with the kelp host.

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Seasonal dynamics of bacterial biofilms on the kelp *Laminaria hyperborea*

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ABSTRACT: Seasonal variations of the cell density and bacterial community composition in biofilms growing on the surface of the kelp *Laminaria hyperborea* from 2 sites on the southwestern coast of Norway were investigated using total cell enumeration and denaturing gradient gel electrophoresis (DGGE) fingerprinting. The major taxonomical groups of bacteria inhabiting the biofilms were identified by DGGE band sequence classification. The microbial cell density of the biofilm appeared to be highly affected by the seasonal growth cycle of the kelp and was found to be lowest on growing kelp in March (minimum 8.3×10^2 cells cm^{-2}), while on non-growing kelp in July to February, it was around 1.0×10^7 cells cm^{-2} with large fluctuations. The composition of the bacterial community of the biofilm followed a continuous seasonal succession that may be explained by the influence of both biotic factors such as seasonal changes in the kelp substrate and abiotic factors such as seawater temperature. *Planctomycetes* and *Alphaproteobacteria* were frequently detected throughout the year, while *Verrucomicrobia*, *Cyanobacteria*, *Gammaproteobacteria*, *Betaproteobacteria*, and *Bacteroidetes* were more sporadically detected. The bacterial communities of the biofilm on kelp showed little overlap with that of the surrounding seawater.

KEY WORDS: Kelp · Bacteria · Biofilm · Marine · Seasonal dynamics · Seaweed · DGGE

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INTRODUCTION

Kelps (large brown algae of the order Laminariales) form the basis of extensive underwater ecosystems known as kelp forests, which are found on rocky coasts both in temperate seas and in upwelling zones in the tropics (Graham et al. 2007). Kelps are large contributors to primary production in these areas (Mann 2000), and the unique 3-dimensional habitats that kelp forests constitute make them biodiversity hotspots (Christie et al. 2003, Graham 2004, Coleman et al. 2007). In many countries, kelp is harvested for the extraction of industrially valuable chemicals such as alginate as well as for food production.

Bacteria associated with kelp are believed to be critically important in many processes in kelp forest ecosystems. Several studies have addressed the flow of matter in kelp forests in South Africa (Lucas et al. 1981, Stuart et al. 1981, Newell & Field 1983). Their many findings highlight the important role of heterotrophic

bacteria as secondary producers utilizing kelp-produced carbon. Norderhaug et al. (2003) showed experimentally that kelp material that had been partially degraded by naturally associated bacteria was a better food source than undegraded kelp for amphipods living in kelp forests. These studies have focused on the bacteria as a group and have not been able to identify the bacteria responsible for these processes. Other studies have aimed to describe the composition and spatial and temporal dynamics of the bacterial communities associated with kelp. Early studies (Chesters et al. 1956, Laycock 1974) focused on culturable bacteria associated with kelp and their degradation of kelp substances. Laycock (1974) also found a seasonal difference in the culturable bacteria from kelp manifested as a predominance of psychrophilic and mesophilic bacterial strains isolated in winter and summer, respectively. The application of scanning electron microscopy (SEM) and fluorescent stains enabled *in situ* enumeration of bacteria growing on kelp without the need of

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culturing. Corre & Prieur (1990) studied the density and morphology of bacteria on the kelp *Laminaria digitata* using SEM. They found a change of the bacterial communities both in terms of the density of bacterial coverage and the morphology of the bacteria in response to the aging of the kelp tissue. In a recent study, Staufenberger et al. (2008) used molecular methods to investigate the bacteria associated with the kelp *Saccharina latissima* (as *Laminaria saccharina*). They could identify several different bacterial lineages based on 16S ribosomal RNA gene sequences and found differences in the composition of the bacterial communities present on different morphological parts of the kelp. They also found indications of seasonal and geographical differences.

Laminaria hyperborea is the largest canopy-forming kelp in the Northern Atlantic. *L. hyperborea* is estimated to produce approximately 50 million tonnes of biomass (wet weight) annually along the Norwegian coast (Fredriksen 2003). It forms the raw material for industrial algininate extraction presently carried out in Norway, and the kelp forests are an invaluable resource for fisheries as they serve as nursing grounds for several commercially important species of fish (Norderhaug 2003). *L. hyperborea* consists of a smooth blade-like structure called the lamina that is attached to an upright and flexible stipe, which can be up to 3 m long. A holdfast structure at the bottom of the stipe attaches the kelp to the substrate. *L. hyperborea* has a pronounced seasonal growth pattern, as the lamina is renewed every year during a growth period lasting from January until June (Fig. 1). The most rapid growth takes place from March until May (Kain 1979). This generates fresh surface area for marine microbes to colonize and makes an excellent system to study natural biofilm succession and dynamics. The old lamina is shed in April and is replaced by a new lamina that has grown out since January. There is also a seasonal pattern of the carbon and nitrogen content of *L.*

hyperborea cells (Sjötun et al. 1996) as well as exudation of dissolved organic matter in the form of carbohydrates and phenolic compounds (Abdullah & Fredriksen 2004). Because the contents and exudates of the kelp cells may be sources of nutrition for kelp-associated bacteria, seasonal variations in these are likely to be important factors controlling the bacterial communities on *L. hyperborea*.

In this study, biofilms growing on the surfaces of the kelp *Laminaria hyperborea* at different times of the year are described using total microbial cell enumeration as well as molecular fingerprinting and classification based on the 16S rRNA gene sequences of bacteria. We aimed to reveal some of the fundamental factors that control the composition and dynamics of bacterial communities in kelp forests by studying them in a seasonal context. This may lay a foundation for increased understanding of the roles of bacteria in kelp forests, which can ultimately shed more light on both the ecology of kelp forests and the dynamics of natural biofilms on living surfaces.

MATERIALS AND METHODS

Sampling of kelp biofilms and seawater bacteria.

Kelp *Laminaria hyperborea* was collected by dredging from a boat at 2 sites outside Bergen, Norway (Stn 1: 60° 30.505' N, 4° 49.776' E, Stn 2: 60° 09.706' N, 5° 02.371' E) at a depth of 5 to 9 m. Sampling was performed at approximately 2 mo intervals evenly spread out throughout 2007, starting in early February and ending in late November. At each site and sampling occasion, 6 intact, full-grown (stipe length >40 cm) kelp individuals were selected from the kelp gathered by the rake-shaped specialized kelp dredge. The kelp individuals were transported to the laboratory in an insulated box and were processed within a few hours after sampling. Two different morphological regions

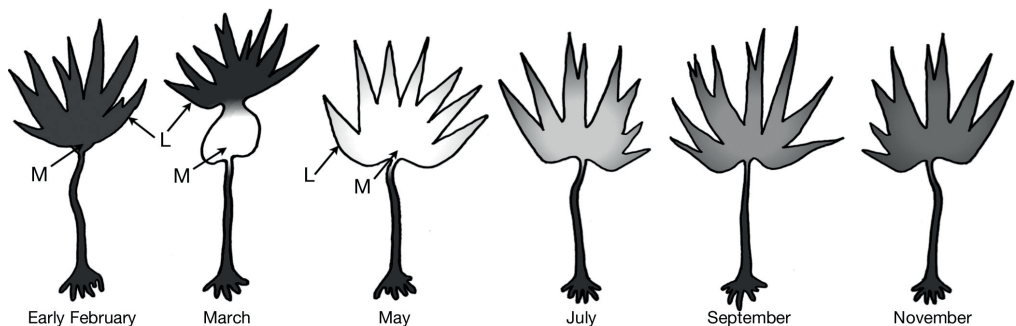


Fig. 1. *Laminaria hyperborea*. Morphology and different growth cycle stages of sampled kelp. Shading of the lamina (L) and meristem (M) indicates the age of the surface tissue (light = younger, dark = older)

from each kelp individual were sampled; the lower part of the lamina (meristematic region) and the middle part of the lamina (Fig. 1). These 2 regions are hereafter referred to as meristem and lamina. Areas that were heavily epiphytized, for example, by bryozoans and seaweeds, were avoided. A surface of approximately 50 cm² from each region of every individual kelp plant (the area included both sides of the flattened thallus) was cut out and thoroughly rinsed with sterile seawater to remove any loosely attached contaminating bacteria originating from the sampling process. The kelp surface was scraped using a sterile scalpel, as microscopic inspection proved this method to be more effective in removing all attached bacteria than wiping with a cotton swab (results not shown). The scraped off material was suspended in 10 ml of sterile phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4) and mixed by vortexing. A volume of 1.5 ml was immediately taken out from the suspension and centrifuged in order to pellet the bacterial cells. Pellets were kept at -20°C until DNA extraction. One 0.5 l water sample was also collected from a depth of 10 m at each site directly adjacent to where the kelp was collected. Water samples were filtered first onto 2.0 µm and then onto 0.2 µm polycarbonate membrane filters (GE Osmotics). Both filters were kept at -20°C until DNA extraction.

Enumeration of microbial cells in the biofilm. Areas directly adjacent to the scraped areas of the meristem and lamina regions were used for cell enumeration. One small piece of kelp surface per kelp individual and region was cut out and fixed in a buffered 2% paraformaldehyde solution at 4°C overnight. The pieces were washed twice in ethanol:PBS (1:1) and kept in the same solution at -20°C until DAPI staining. The kelp biofilms were stained by immersing approximately 0.25 cm² kelp pieces in 2% DAPI solution for 10 min. The pieces were then rinsed in several volumes of sterile distilled water. Mounting on microscope slides was achieved by placing a piece of adhesive poster tack at the 4 corners of 25 × 25 mm coverslips to create the correct distance between slide and coverslip to accommodate the thick (up to 3 mm) kelp pieces. The kelp pieces were mounted in a few drops of Citi-fluor:Vectashield (4:1). Pictures of the stained biofilms were captured on a Zeiss AxioImager.Z1 fluorescent microscope with an AxioCam MRm digital camera (Zeiss Microimaging). Ten randomly selected but evenly distributed microscope fields were captured per sample kelp piece. Because of the 3-dimensional structure of the kelp surface and of the attached biofilm, it was necessary to capture several focal planes in order to visualize all biofilm cells in 1 microscope field. This was achieved by using the 'Z-stack' and 'extended

focus' functions of the Zeiss AxioVision software. One 'extended focus' image was created for each microscope field consisting of information from several images captured at different focal planes with 1 µm intervals through the entire depth of the biofilm of the sample. The number of images used to create the extended focus image varied between 5 and about 50, depending on the topography of the kelp surface and/or the biofilm thickness. The DAPI-stained cells were manually counted in each extended focus image. When an image contained more than approximately 200 cells, a grid with known dimensions was digitally overlaid on the image, and the cells within 10 predefined squares of the grid were counted. The cell density of each sample was estimated by calculating the average density of the 10 captured microscope fields.

DNA extraction. DNA from the pellets of scraped off material from the kelp surface layers was extracted using a modification of the method described by Øvreås et al. (2003b). Briefly, the pellet was suspended in a solution of lysozyme (1 mg ml⁻¹) and RNase (0.5 mg ml⁻¹) in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and incubated for 15 min at 37°C. Proteinase K was added to a final concentration of 40 µg ml⁻¹, and samples were incubated for another 15 min at 37°C. SDS was added to a concentration of 0.5 mg ml⁻¹, and samples were incubated at 55°C for 15 min. Next, NaCl (5M) was added to a concentration of 1 M followed by a CTAB buffer (10% w/v cetyl trimethylammonium bromide in 0.7% NaCl) to a final concentration of 1.6% CTAB. Samples were incubated at 65°C for 10 min. An equal volume of chloroform: isoamylalcohol (24:1) was then added, and the samples were shaken on a vortexer for 10 min. After a brief centrifugation, the aqueous phase was collected and precipitated in 0.6 volumes of isopropanol. The DNA pellet was washed in 70% ethanol, dried, and resuspended in PCR-grade water. The same method was used to extract DNA from whole membrane filters with filtered pelagic water samples.

PCR amplification of 16S rDNA. A part of the 16S ribosomal RNA gene of bacteria including the V7 and V8 variable regions was amplified using the primers 1070f (5'-ATG GCT GTC GTC AGC T-3') (Ferris et al. 1996) and 1392r (Lane et al. 1985) with an attached GC-clamp (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CAC GGG CGG TGT GTR C-3'). Each 50 µl PCR reaction contained nuclease-free water, F511 buffer (Finnzymes), 0.1 mM of each dNTP (F506L, Finnzymes), 0.02% BSA, 0.5 µM of each primer, 0.02 U Dynazyme II F501-L (Finnzymes), and approximately 30 ng template DNA. PCR conditions were: 94°C for 5 min, 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s, and final elongation at 72°C for 7 min.

Denaturing gradient gel electrophoresis (DGGE) profiling of biofilm and seawater bacterial communities. PCR products were separated on 30 to 70% denaturant (7 M urea and 40% deionized formamide) DGGE gels as described by Øvreås et al. (2003a). Gel images were captured with a ChemiGenius Bio Imaging System (Synoptics). All visible unique bands were picked for sequencing, and bands recurring several times on the same gel were picked at least twice. Band material was cut out from the gel and processed according to Øvreås et al. (1997), and reamplification of the bands was carried out using the same PCR conditions described in the previous section. A subset of the reamplified bands was run again on a second DGGE to check their purity. Reamplified bands were diluted 20 times with sterile water prior to sequencing.

Sequencing of DGGE bands. Sequencing was carried out using the BigDye Terminator v3.1 sequencing kit (Applied Biosystems). The 1070f primer was used in the sequencing reaction. The sequencing was carried out on an ABI 3700 sequencing system at the Bergen sequencing facility (www.seqlab.uib.no).

Sequence processing and analysis. Base calling of the sequence chromatogram files was done by the Phred software (Ewing et al. 1998), which also assigns a quality value to each base of the sequence. A strict quality filtering of sequences was carried out based on the Phred quality data. Bad quality ends of the sequences were cropped until the average quality of the distal 10 base pairs was above 25. Sequences that were shorter than 200 base pairs after cropping were discarded, as were sequences with an overall average quality below 33. A few additional sequences were subsequently removed from the analysis after manual inspection in the chromatogram viewer 4peaks (www.mekentosj.com). The filtered sequences were aligned with MUSCLE (Edgar 2004), and the JalView alignment editor (Waterhouse et al. 2009) was used to trim the sequences to a similar length and to remove the GC-clamp at the end of each sequence. The resulting sequences were submitted to the NAST aligner (DeSantis et al. 2006a) of the Greengenes database project (DeSantis et al. 2006b). The NAST alignment was then used to assign a phylum to class level taxonomic classification to each sequence using the classify tool of Greengenes with the G2-chip as the chosen taxonomy. The NAST-alignment of the sequences was also used to create a distance matrix using the Greengenes distance matrix tool. The distance matrix was analyzed with DOTUR (Schloss & Handelsman 2005), which assigns operational taxonomical units (OTUs) defined by different similarity percentages based on the distance matrix. An OTU definition of 95% similarity was adopted for this study, as it appeared to correspond well with the DGGE band positions. When using

a 97% similarity definition, DGGE band sequences from the same position on a gel sometimes belonged to different OTUs. The OTUs were named by using the first letter(s) of the taxonomical group to which they belonged, followed by the number assigned to them by DOTUR. An OTU does not represent a bacterial species or any other natural taxonomical entity; it is merely a tool used for managing the sequence information obtained in the study in a convenient way.

DGGE image analysis. High-resolution printouts of the DGGE images were analyzed visually. To compare the banding patterns between many separate DGGE gels, the sequence information of the selected bands was used as an internal standard. This allowed the OTU composition of each sample to be deduced based on both the banding pattern and the OTU assignments of the sequenced bands on the same gel. Bands that were not chosen for sequencing but were located at the same position as the sequenced bands were assigned to the same OTU as the sequenced bands within each gel. Using this approach, most (80%) of the visible bands on the DGGE gels could be assigned to an OTU. OTUs were recorded as either present or absent in each sample.

Statistical analyses. Statistical analyses were performed using the R-project software (R Development Core Team 2009) including functions from the vegan package (Oksanen et al. 2009). The cell density dataset was square root transformed prior to analysis. Differences in biofilm cell densities were tested using 1-way analysis of variance (ANOVA; R function `aov`) and Tukey's honestly significant difference (HSD; R function `TukeyHSD`). The DGGE results were analyzed with non-metric multidimensional scaling (nMDS, R function `metaMDS`) using the Jaccard similarity metric to visualize the similarities between samples. Anosim (R function `anosim`) was used to measure the separation between samples from the different regions and seasons. For comparison between seasons, the presence/absence tables of the meristem and lamina region samples from the same kelp individual were pooled into 1 presence/absence table per kelp individual prior to the analyses. Samples containing less than 3 detected OTUs were removed from the analyses.

RESULTS

Density of microbial cells in kelp surface biofilms

The density and distribution of DAPI-stained microbial cells in the kelp surface biofilms was highly variable. This variation was seen between seasons (Figs. 2 & 3), between replicate kelp individuals (Table 1 and Fig. 3), and between different microscope fields in

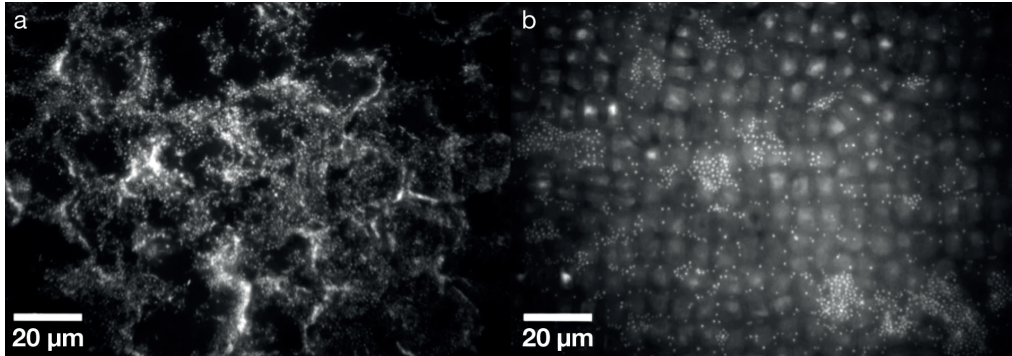


Fig. 2. Two examples of DAPI-stained biofilm from kelp. (a) Meristem region sampled in February (cell density: 3.8×10^7); (b) lamina region sampled in July (cell density: 1.3×10^7). Images are cropped and thus cover a smaller area than the whole microscope field images used for cell enumeration

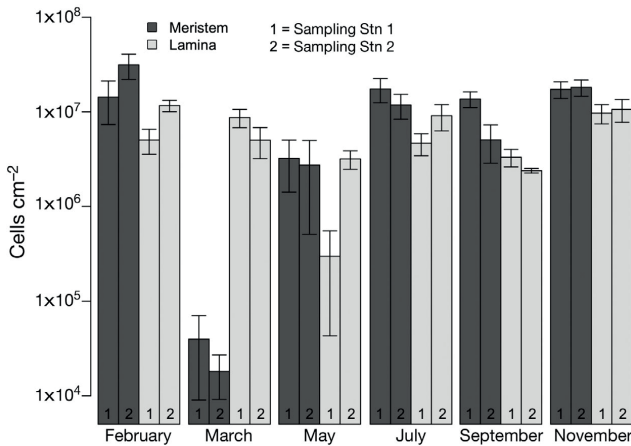


Fig. 3. Densities of DAPI-stained biofilm cells cm^{-2} on the meristem and middle lamina regions of kelp at different seasons represented as the mean of 6 replicate samples (except Stn 2 in March with only 2 replicates). Error bars represent SE

each sample (results not shown). Fig. 2 shows 2 examples of images captured of DAPI-stained biofilm cells, illustrating different biofilm architectures. The average cell densities on the meristem and lamina of kelp individuals from the 2 sampling stations throughout the year are summarized in Table 1 and visualized in Fig. 3. The large SDs of the average values in Table 1 and the large error bars (representing standard error, SE) in Fig. 3 highlight the considerable variation of the cell density between replicate kelp individuals. The highest cell densities were seen in samples from the meristem region in February with sample averages

close to 6.5×10^7 cells cm^{-2} . The lowest densities were recorded in the rapidly growing meristem region in March with average cell numbers down to 8.3×10^2 cells cm^{-2} in some samples. The overall average cell density across all seasons and kelp plant regions was 9.0×10^6 cells cm^{-2} and the median density was 5.4×10^6 cells cm^{-2} . Differences in cell densities between seasons, stations, and between meristem and lamina were tested with 1-way ANOVA, followed by Tukey's HSD test (see Appendix 1 for a complete list of p-values). Although highly variable, the overall cell densities of both meristem and lamina during February, July, and November and of the lamina in March were not significantly different and hence appear not to be affected by season. However, the growing meristem in March at Stn 1 had significantly lower cell densities ($p < 0.05$) than the meristem region in all other months except May. The meristem in March at Stn 1 also had significantly ($p < 0.01$) lower cell density than the lamina in March. The meristem region in March at Stn 2 appeared to have lower cell density than both the meristem of other months and the lamina in March, yet p-values are not consistently significant due to the low number of replicate samples (due to technical problems during sample processing, only 2 samples were included in the analysis) in March at Stn 2. In May, both the lamina and the meristem displayed cell densities that were significantly lower than in February, July, and November

Table 1. Summary of the results, showing the numbers of denaturing gradient gel electrophoresis (DGGE) bands and operational taxonomical units (OTUs) within each taxonomical group and season. Mean (± 1 SD) cell densities (cells cm^{-2}) on the meristem and lamina of kelp individuals from the 2 sampling stations throughout the year are also shown. Seawater temperature measured at 10 m depth at times close to the sampling occasions in a nearby fjord is also included (J. Egge unpubl. data). N/A: not applicable

Season	No. of DGGE bands	No. of OTUs	Cell density, meristem	Cell density, lamina	Seawater temperature ($^{\circ}\text{C}$)
February	88	22	2.3×10^7 ($\pm 2.1 \times 10^7$)	8.3×10^6 ($\pm 5.0 \times 10^6$)	6.3
March	N/A	N/A	3.4×10^4 ($\pm 6.4 \times 10^4$)	7.8×10^6 ($\pm 4.4 \times 10^6$)	6.2
May	65	11	3.0×10^6 ($\pm 4.8 \times 10^6$)	1.7×10^6 ($\pm 1.9 \times 10^6$)	8.3
July	94	10	1.5×10^7 ($\pm 1.0 \times 10^7$)	6.9×10^6 ($\pm 5.6 \times 10^6$)	12.3
September	N/A	N/A	9.4×10^6 ($\pm 7.2 \times 10^6$)	2.9×10^6 ($\pm 1.3 \times 10^6$)	10.3
November	113	16	1.8×10^7 ($\pm 8.2 \times 10^6$)	1.0×10^7 ($\pm 6.0 \times 10^6$)	8.4
Taxonomical group					
<i>Planctomycetes</i>	167 (46.4%)	8			
<i>Verrucomicrobia</i>	63 (17.5%)	5			
<i>Cyanobacteria</i>	19 (5.3%)	1			
<i>Bacteroidetes</i>	15 (4.2%)	3			
<i>Alphaproteobacteria</i>	74 (20.6%)	5			
<i>Betaproteobacteria</i>	8 (2.2%)	2			
<i>Gammaproteobacteria</i>	13 (3.6%)	6			
<i>Actinobacteria</i>	1 (0.3%)	1			

with few exceptions. The cell density of the lamina region in September was also significantly lower than in February and November for Stn 2. The lamina region appears to have a lower average cell density than the meristem when the lamina is not growing at the meristem, i.e. in February, July, September, and November (see Fig. 1). However, these differences were not significant ($p > 0.18$). There were also no significant ($p > 0.13$) differences in biofilm cell densities between the different sampling stations.

DGGE profiling of kelp biofilm and seawater bacterial communities

DGGE profiles from 112 individual samples from 4 seasons were included in the analyses. One example of a DGGE gel is shown in Fig. 4. Out of a subset of the picked and reamplified bands, a large majority (85%) was represented as 1 clear band in the correct position when run on a second DGGE. Of the 426 bands that were sequenced, 141 were filtered out because of

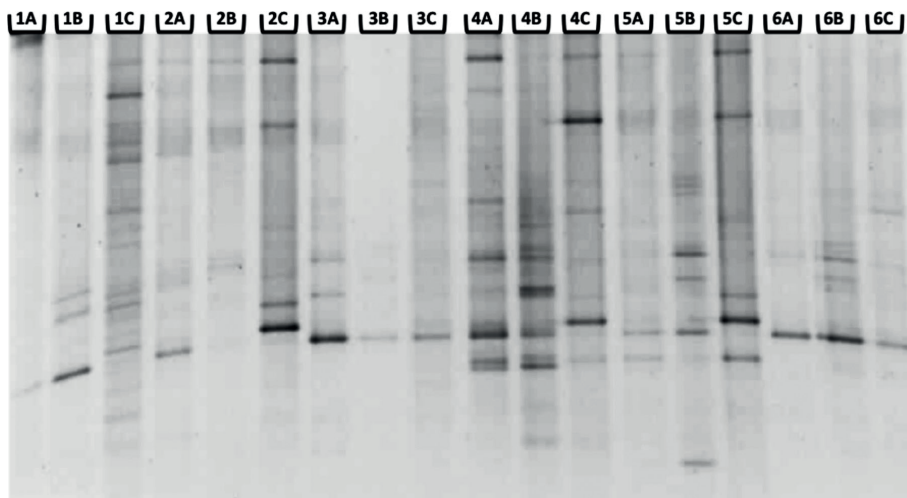


Fig. 4. Representative denaturing gradient gel electrophoresis (DGGE) gel used in the analysis of the seasonal dynamics of the bacterial communities on kelp. Numbers 1 to 6 represent different individual kelp plants, A to C designate the area of the kelp plant sampled. A: stipe (not included in the analyses), B: meristem, C: lamina. The gel shows samples from February

insufficient quality, and 12 represented chloroplast and mitochondrial sequences and were removed from the analysis. The remaining 273 good quality, bacterial 16S rRNA gene sequences spread among 38 different OTUs using the 95% similarity OTU definition. When analyzing the DGGE images, 401 DGGE bands could be assigned to an OTU, which corresponds to 80% of the visible bands. Of these bands, 360 derived from kelp biofilm and 41 from seawater.

Taxonomical classification of the DGGE band sequences

The 360 DGGE bands deriving from kelp fell into 8 broad taxonomical groups (phylum and class) based on the Greengenes classification tool. These were *Planctomycetes*, *Verrucomicrobia*, *Cyanobacteria*, *Bacteroidetes*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Actinobacteria*. The number of DGGE bands belonging to each group in the different seasons is illustrated in Fig. 5. The most frequently detected group was the *Planctomycetes*, with a total number of 167 bands. A majority of the planctomycete sequences classified as *Pirellulae* (G2-chip taxonomy). The *Alphaproteobacteria* were also frequently detected (74 bands in total), where all sequences classified to the order *Rhodobacterales*. The number of OTUs that belonged to each group varied from only a single OTU within the *Cyanobacteria* to 8 different OTUs within the *Planctomycetes*. The numbers of

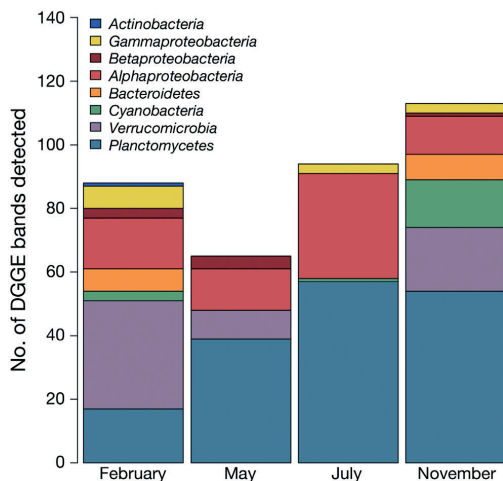


Fig. 5. Major groups of bacteria detected on kelp, shown as the number of denaturing gradient gel electrophoresis (DGGE) bands belonging to each group detected in the different seasons

DGGE bands and OTUs within each taxonomical group and season are summarized in Table 1. One representative sequence of each OTU was submitted to GenBank under accession numbers GU292011 to GU292048.

Seasonal variation of bacterial community composition in kelp surface biofilms

Analysis of the OTU composition of the 4 different seasons revealed seasonal changes in bacterial community composition in kelp surface biofilms. The nMDS ordination in Fig. 6a illustrates the seasonal pattern of biofilm community composition. Samples that are plotted closer to each other in the ordination have a more similar OTU composition than samples plotted farther away from each other. The samples from the respective seasons cluster together with varying degrees of overlap between the different seasons.

The degree of separation between the seasons was tested with Anosim. Table 2 shows the values of the Anosim statistic R when seasons were compared pairwise. R is scaled to have a value between -1 and +1, where 1 corresponds to complete separation of the compared groups and 0 indicates no separation. According to a conventional interpretation of R, values above 0.75 indicate completely separated groups, while R = 0.5 indicates that groups are clearly separated but overlapping and R = 0.25 indicates barely separable groups (Clarke & Warwick 2001). The validity of R is assessed by a permutation test that gives a probability value 'p' indicating whether R is meaningful for the tested dataset (Clarke 1993). In the pairwise comparisons between seasons, R values ranged between 0.35 and 0.77, where the greatest separation (R = 0.77, p < 0.001) occurred between May and November followed by July and November (R = 0.70, p < 0.001). The lowest separation was between February and May (R = 0.35, p = 0.007).

Fig. 6b shows the positions of the common OTUs (>4 occurrences) in the nMDS ordination. Their position in relation to the seasonal 95% confidence ellipses indicates their influence on the seasonal clustering of the samples. For example, the *Cyanobacteria* OTU C17 that is positioned within the November ellipse, adjacent to the February and July ellipses (see Fig. 6b) was detected in February, July, and November, but was most frequently detected in November. The *Planctomycetes* OTU P40 was only detected in November, while P41 was detected in May and February. The *Planctomycetes* OTU P44 that is positioned centrally in the plot was common in all seasons and was detected in 78% of the samples.

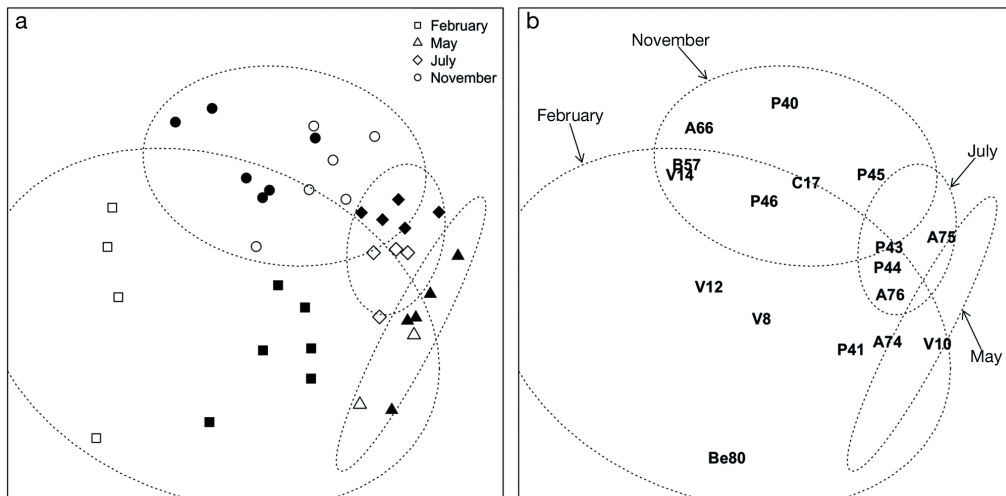


Fig. 6. (a) Non-metric multidimensional scaling (nMDS) ordination illustrating the similarity of samples from the 4 different seasons based on operational taxonomic unit (OTU) composition. Open (filled) symbols represent samples from Stn 1 (Stn 2). The dashed ellipses outline 95% confidence intervals of sample point position. (b) The same nMDS ordination where, instead of samples, common OTUs are displayed. Letter(s) of the OTU label indicate to which of the major taxonomic groups the OTU belongs. P: *Planctomycetes*, V: *Verrucomicrobia*, A: *Alphaproteobacteria*, C: *Cyanobacteria*, B: *Bacteroidetes*, Be: *Betaproteobacteria*. One OTU (P32) was omitted from the figure because it was in the same position as P44

Table 2. Values of the analysis of similarity (Anosim) statistic R from pairwise Anosim comparisons of the operational taxonomic unit compositions of the different seasons. All R values $p < 0.001$

Season	February	May	July	November
February		0.35	0.59	0.41
May			0.49	0.77
July				0.70
November				

Fig. 5 also suggests a seasonal pattern at a higher taxonomical level. Bands belonging to the *Verrucomicrobia* are absent in July, and the *Bacteroidetes* are represented only in February and November. Bands from the different taxonomical groups are more evenly distributed in February and November, while a few abundant groups dominate in May and July.

Spatial variation of bacterial community composition in kelp surface biofilms

No clear difference in the biofilm community composition between the 2 sampled regions of the kelp thallus, the meristem and the lamina was seen.

Within-season comparisons of the 2 regions yielded Anosim R values for February ($R = 0.15$, $p = 0.10$), May ($R = 0.41$, $p = 0.06$), July ($R = 0.10$, $p = 0.08$), and November ($R = 0.35$, $p = 0.002$). The p-values for February, May, and July indicate that the R statistic may not be reliable for these datasets, while the R statistic in November shows a slight difference between the 2 sampled regions.

A difference in the kelp biofilm community composition between sampling stations within seasons is indicated in Fig. 6a and was confirmed by Anosim in February ($R = 0.64$, $p < 0.001$) and in July ($R = 0.46$, $p < 0.001$), while no significant difference could be demonstrated in May ($R = -0.2455$, $p = 0.757$) and November ($R = 0.09$, $p = 0.102$).

Bacterial community composition in kelp surface biofilms compared to seawater

The 2 seawater samples collected during each season (1 from each station) showed little OTU overlap with kelp biofilm communities. In total, 3 OTUs (V10, P32, and A66) were found in both kelp biofilms and seawater. Only 1 of these (P32) was encountered in both kelp biofilms and seawater during the same season (July). V10 was detected in seawater in July, but

was only detected in kelp biofilm in May and November. A66 was detected in seawater in May and July and in kelp biofilm in February and November.

DISCUSSION

The investigation of cell density and bacterial community composition of the microbial biofilms on the surfaces of the kelp *Laminaria hyperborea* at different times of the year revealed that seasonality has an important influence on the biofilms. However, seasonal changes include several different factors that can affect microbial biofilm communities. These include biotic factors such as kelp growth, exudation of substances by the kelp that are either antimicrobial or act as a food source, the composition of the microbial communities in the surrounding seawater, and the activity of grazers of kelp biofilms. Abiotic factors such as temperature, wave action, light conditions, and seawater nutrient levels may also be important. The seasonal context of this study allows evaluation and discussion of the influence of some of these different factors on kelp biofilms.

Seasonal dynamics of cell density and community composition in kelp surface biofilms

March to May: establishment of the biofilm on new kelp substrate

During spring, a rapid expansion of the kelp meristem tissue takes place and provides fresh substrate for colonizing bacteria (see Fig. 1). In March to April, this expansion corresponds to a lamina elongation of nearly 1 cm d^{-1} (Kain 1979, Sjøtun et al. 1996). The drastically reduced cell densities found on the meristem in March in this study (minimum 8.3×10^2 , average $3.4 \times 10^4 \text{ cells cm}^{-2}$) are probably limited by the rate at which new kelp surface is produced. If this is the case, bacterial attachment and division does not happen at a rate sufficient to achieve higher cell densities on the rapidly expanding kelp surface. In addition, bacterial attachment and division may be suppressed by exudation of antibacterial substances such as phenols, which are high in March compared to other months (Abdullah & Fredriksen 2004). The lamina region in March still consisted of old kelp tissue grown out the previous year and displayed cell densities that were comparable to other months, yet significantly higher than the growing meristem in March (see Fig. 3).

Two months later, in May, both the meristem and lamina regions consist of fresh tissue, and growth is still taking place at the meristem, but at a slower rate

than in March (Kain 1979, Sjøtun et al. 1996). The cell densities of both meristem (average $3.0 \times 10^6 \text{ cells cm}^{-2}$) and lamina (average $1.7 \times 10^6 \text{ cells cm}^{-2}$) were still significantly lower in May (with few exceptions) compared to all other months except March, indicating that the biofilm was not yet fully established. The OTUs detected in May, which primarily belong to *Planctomycetes*, *Verrucomicrobia*, and *Alphaproteobacteria*, can be regarded as early colonizers of the fresh kelp substrate.

July to February: maturation and succession of the biofilm

The elongation of the kelp lamina stops in June (Kain 1979, Sjøtun et al. 1996), and the surface tissue is available for bacterial colonization and biofilm maturation until the lamina is shed in the next spring (see Fig. 1). From July to February, the cell densities of meristem and lamina appeared to stabilize at values close to $1.0 \times 10^7 \text{ cells cm}^{-2}$ (average 1.16×10^7), although fluctuations were large (Fig. 3, Table 1). Yet the biofilm community composition was constantly changing. This was evident both in the different biofilm architectures found in the microscopic images (Fig. 2) and in the DGGE results (Figs. 5 & 6).

The bacterial community composition of the biofilm in July overlapped with that in May (Anosim $R = 0.49$) and was characterized by a stable community dominated by only a few OTUs within *Planctomycetes* and *Alphaproteobacteria* (see Fig. 5 and Table 2). The summer is a period of relatively high water temperatures, high photosynthetic activity of the kelp, and also high exudation rates of carbohydrates (Abdullah & Fredriksen 2004). Such conditions are likely to be beneficial for heterotrophic bacteria that live on kelp exudates. The dominating OTUs in July may represent a highly specialized community adapted to life in kelp biofilms that is able to outcompete other bacteria. The frequently detected *Planctomycetes* OTU P44 is probably an example of one of these specialized bacteria.

In November, the community was supplemented with different OTUs within *Planctomycetes*, *Alphaproteobacteria*, *Cyanobacteria*, and *Bacteroidetes*. Many of these OTUs were also present in February. The biofilm in February was characterized by a diverse community including OTUs from all major taxonomical groups found in the study. The big spread of sample points from February in the nMDS ordination in Fig. 6a illustrates that there are big variations in the community composition between different kelp individuals. This diverse and unspecific community contrasted with the stable and specialized community in July and may be explained by the wide range of niches available for

different bacteria involved in breaking down the aging kelp tissue in February. The result agrees well with the study of Staufenberg et al. (2008), who found that old laminas (phyloids) of the kelp *Saccharina latissima* displayed less specific bacterial communities than the younger meristem and stipe (cauloid) regions.

The Anosim R values showed a large degree of overlap between the bacterial communities in May and February ($R = 0.35$), indicating that some of the OTUs found on aging tissue in February also acted as early colonizers on the fresh tissue in May. This closes the apparent 'seasonal cycle' of bacterial community composition in kelp surface biofilms. It also shows that aging of the kelp substrate and the resulting maturation of the biofilm is not the only factor explaining the succession of the bacterial communities. Instead, other factors such as seawater temperature, which was similarly cold in February and May at the sampling sites (Table 1; J. Egge unpubl. data), may have contributed to shaping the bacterial communities. Laycock (1974) showed that seawater temperature selects for either psychrophilic or mesophilic culturable bacterial communities on the kelp *Laminaria longicruris*. Seawater temperature may be an important factor besides the biotic factors relating to the growth cycle of the kelp in controlling the bacterial community composition of kelp biofilms.

The similarity of the bacterial communities found between the aging lamina in February and the freshly grown out lamina in May also implies transmission of bacteria from old kelp tissue to young kelp tissue, either via the seawater or by direct contact. The stipe and holdfast regions of *Laminaria hyperborea* (not investigated in this study) are not renewed every year, and the biofilms residing on these older surfaces could serve as stock populations for biofilm bacteria colonizing fresh kelp surface tissue.

Spatial dynamics of cell density and community composition in kelp surface biofilms

The meristem has a lower content of phenolic substances than the lamina in *Laminaria hyperborea* (Connan et al. 2006). This could potentially influence biofilms by causing differential exudation of substances in the different regions of the kelp that either have an antibacterial effect or act as a food source for the bacteria. No significant differences in cell density between the meristem and lamina regions could be demonstrated in this study, although the lamina appears to have a lower average cell density than the meristem (see Fig. 3). However, the large variations in cell density between kelp individuals and even different microscope fields shows that kelp biofilms are

extremely heterogeneous, and stochastic variation could easily obscure general trends. A lower cell density in the lamina region could reflect its higher phenolic content, given that the phenolic substances have antimicrobial activity.

The meristem and lamina regions of the kelp sampled in this study showed slight differences in biofilm community composition only in November. These results support the findings of Staufenberg et al. (2008), who detected specific associations of bacterial communities with different parts of the kelp *Saccharina latissima* including the equivalents of the meristem and lamina.

The 2 sampling stations for kelp were chosen to be similar with respect to kelp size and morphology. Yet differences in the cover and composition of macroscopic epiphytes (red seaweeds for example) indicated that other environmental factors such as wave exposure may differ between the stations. Differences in bacterial community composition between kelps from the 2 different sampling stations in this study were evident only in February and July. The lack of difference in May and November may indicate that there are large stochastic variations between the sampled kelp plants that obscure differences between kelp plants from the different stations. The kelp forest is a heterogeneous environment, and local scale variations in depth, wave exposure, and currents, all factors that may influence bacterial biofilms growing on kelp, make it difficult to sample a representative selection of kelp plants from 1 station, especially with the dredging technique used in this study. The differences observed between the stations in July and February as well as the large variations within the stations are likely to be caused by local environmental factors rather than geographical distance.

Regulation of the microbial cell density of kelp surface biofilms

The overall cell density on kelp tissue in this study was around 1×10^7 cells cm^{-2} . This number is comparable to cell densities recorded by Corre et al. (1990) on the closely related kelp species *Laminaria digitata*. This number is surprisingly low considering the rapid overgrowth by bacteria and other fouling organisms of most surfaces exposed to colonization in natural seawater (Zobell et al. 1935). The cell densities of both meristem and lamina are rather constant on the non-growing kelp tissue from July to February. The exception to this rule is the lower cell density on the lamina in September, which is not easily explained by the seasonal aspects of kelp growth and exudation, and may reflect unknown environmental factors or unrepresentative

tative sampling. The constant and low cell densities imply a regulation of the biofilm, preventing high cell densities. This may represent anti-fouling properties of the kelp, such as exudation of phenolic compounds (Arnold & Targett 2002) or activated oxygen species (Küpper et al. 2001) to prevent settlement or growth of biofilm bacteria. The regulation may also be attributed to the biofilm bacteria themselves, which can control cell division and attachment within the biofilm by quorum sensing and production of inhibitory compounds. A recent study by Wiese et al. (2008) isolated and identified several bacterial strains producing antibiotics from the kelp *Saccharina latissima* (*Laminaria saccharina*). In addition, Rao et al. (2007) showed that biofilms grown from strains of epiphytic bacteria isolated from the marine seaweed *Ulva australis* inhibit settlement of bacteria and other fouling organisms at biofilm densities down to 100 cells cm⁻².

Bacterial community composition in kelp surface biofilms compared to the surrounding seawater

Another factor influencing kelp biofilms may be the composition of the bacterial community of the surrounding seawater. As the results from the total microbial cell numbers in March and May showed, the new surface area formed during growth of the *Laminaria hyperborea* meristem was rapidly colonized by biofilm bacteria. One likely source of recruitment of bacteria is the surrounding seawater. Little overlap was found between the kelp biofilm and the seawater bacterial communities that were sampled in this study. Similar results have been reported in earlier studies on microbial biofilms on seaweed (Bolinches et al. 1988, Staufenberger et al. 2008). This shows that the dominant bacterial types are not the same in the kelp biofilm and the surrounding seawater. Therefore, biofilm bacteria must be recruited from less abundant populations in the seawater through selective processes or via transmission of bacteria by direct contact with surfaces. Due to limitations in the resolution of the DGGE technique, rare populations in the seawater are not easily detected. This makes it difficult to correlate fluctuations in the seawater bacterial communities with the composition of the bacterial communities in biofilms on kelp. Of the few OTUs that were detected in both seawater and kelp biofilm, most were not detected in both habitats during the same season. These OTUs may represent bacterial types alternating between a biofilm and free-living lifestyle on a seasonal basis. Alternatively, the OTUs may represent phylogenetically closely related but ecologically different bacterial types.

Bacterial groups that inhabit kelp biofilms

The most frequently detected groups were *Planctomycetes*, *Alphaproteobacteria*, *Verrucomicrobia*, and *Cyanobacteria*. These groups have relatively few OTUs in relation to the number of detected DGGE bands (Table 1). In contrast, *Gammaproteobacteria* have 6 OTUs among only 13 DGGE bands. This may indicate that *Planctomycetes*, *Alphaproteobacteria*, *Verrucomicrobia*, and *Cyanobacteria* make up a community consisting of a few specialized 'strains' adapted to life in kelp biofilms and are therefore consistently abundant. The different *Gammaproteobacteria* OTUs that occasionally reach high enough abundances to be detected in kelp biofilms may either be generalists that grow rapidly when competition is low or they may represent highly specialized strains that exploit rare niches on the kelp. *Betaproteobacteria* and *Verrucomicrobia* were only detected during February, May, and November, when the seawater temperature was below 10°C; this may indicate a preference for cold seawater temperatures among these groups.

Interestingly, *Planctomycetes*, and *Verrucomicrobia*, which were frequently detected in this study, were not detected on the related kelp species *Saccharina latissima* in the recent study by Staufenberger et al. (2008). This may reflect a real difference in the bacterial community composition between the 2 different kelp species, but it may also result from a PCR primer bias, as Staufenberger et al. (2008) used a different 16S rRNA gene primer combination for clone library construction.

In summary, the seasonal fluctuations in cell density appear to be connected to the growth cycle of *Laminaria hyperborea*, or more specifically, to the age of the kelp tissue. The cell densities in growing or young kelp tissue may be suppressed by rapid growth of the kelp substrate. The overall cell density is low and implies a strong regulation of kelp biofilms. On the OTU level, the biofilm community composition reflects a seasonal cycle, where the succession of bacterial OTUs is a continuous process during the year. May and November display the least similar biofilm communities, while February and May are highly overlapping. This successional pattern may only be explained by the combined influence of biotic factors relating to the growth cycle of the kelp and of abiotic factors such as seawater temperature. The community composition of the biofilm is characterized by a stable 'basal community' consisting of *Planctomycetes* and *Alphaproteobacteria* that has already established in the fresh kelp tissue in May. This community is supplemented with bacteria belonging to *Gammaproteobacteria*, *Bacteroidetes*, and *Cyanobacteria* as the kelp tissue is aging and the biofilm

matures. *Verrucomicrobia* and *Betaproteobacteria* may be favored by colder seawater temperatures, as they are detected in February, May, and November but not in July. Seawater and kelp biofilm bacterial communities show little overlap.

Acknowledgements. We thank T. Sørli, T. Urich, and D. Kohlmeier for assistance during sampling, J. Egge for the seawater temperature data, A. Lanzén for bioinformatics assistance, R. Telford for advice on the statistical analyses, and 3 anonymous reviewers for constructive comments on the manuscript. The study was funded by 2 scholarships from The Bergen University Fund and a donation from FMC BioPolymer (Philadelphia, PA).

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Appendix 1. P-values from 1-way ANOVA followed by Tukey's HSD tests of differences in cell density between seasons, between meristem and lamina regions, and between sampling stations. Shaded cells represent p-values <0.05. M: meristem, L: lamina, 1: Stn 1, 2: Stn 2

	February		March		May		July		September		November	
	M	L	M	L	M	L	M	L	M	L	M	L
	1	2	1	2	1	2	1	2	1	2	1	2
February	M 1	-										
	2	0.35	-									
	L 1	0.91	0.20	-								
	2		0.24									
March	M 1	0.01		-								
	2	<0.01		1.00	-							
	L 1		0.53	<0.01	1.00	-						
	2		0.52	0.83		-						
May	M 1	0.27		0.67		-						
	2	<0.01		0.95		1.00	-					
	L 1		<0.01		<0.01	0.93	0.13	-				
	2		0.02		0.98	1.00		-				
July	M 1	0.94		<0.01		0.04		-				
	2	0.16		0.14		0.19		1.00	-			
	L 1		1.00	0.40		<0.01		0.18	0.87	-		
	2		0.80	0.96		0.27		1.00	1.0		-	
September	M 1	0.99		<0.01		0.10		1.00		-		
	2	<0.01		0.57		0.88		0.78		0.79	-	
	L 1		0.97	0.15		<0.01		0.99	0.22	1.00		-
	2		<0.01	0.92		1.00		0.14	1.00			-
November	M 1	0.90		<0.01		0.03		1.00		0.99		-
	2	0.79		0.02		0.01		0.82		0.16		1.00
	L 1		0.28	1.00		<0.01		0.19		0.06		0.93
	2		0.99	0.77		0.07		0.98		0.03		0.91

Editorial responsibility: Staffan Kjelleberg, Sydney, Australia

Submitted: December 14, 2009; Accepted: January 28, 2010
 Proofs received from author(s): April 19, 2010

RESEARCH ARTICLE

Open Access

Planctomycetes dominate biofilms on surfaces of the kelp *Laminaria hyperborea*

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Abstract

Background: Bacteria belonging to *Planctomycetes* display several unique morphological and genetic features and are found in a wide variety of habitats on earth. Their ecological roles in these habitats are still poorly understood. Planctomycetes have previously been detected throughout the year on surfaces of the kelp *Laminaria hyperborea* from southwestern Norway. We aimed to make a detailed investigation of the abundance and phylogenetic diversity of planctomycetes inhabiting these kelp surfaces.

Results: Planctomycetes accounted for 51-53% of the bacterial biofilm cells in July and September and 24% in February according to fluorescence *in situ* hybridization (FISH) results. Several separate planctomycetes lineages within *Pirellulaceae*, *Planctomyces* and OM190 were represented in 16S rRNA gene clone libraries and the most abundant clones belonged to yet uncultured lineages. In contrast to the abundance, the diversity of the planctomycete populations increased from July to February and was probably influenced by the aging of the kelp tissue. One planctomycete strain that was closely related to *Rhodopirellula baltica* was isolated using selective cultivation techniques.

Conclusions: Biofilms on surfaces of *L. hyperborea* display an even higher proportion of planctomycetes compared to other investigated planctomycete-rich habitats such as open water, sandy sediments and peat bogs. The findings agree well with the hypothesis of the role of planctomycetes as degraders of sulfated polymeric carbon in the marine environment as kelps produce such substances. In addition, the abundant planctomycete populations on kelp surfaces and in association with other eukaryotes suggest that coexistence with eukaryotes may be a key feature of many planctomycete lifestyles.

Background

Bacteria belonging to the phylum *Planctomycetes* have revealed several remarkable features that set them apart from other bacteria. Their cryptic morphology led early microbiologists to mistake them for fungi, and the discovery of their cell compartmentalization, featuring membrane bounded organelles, raised fundamental questions about the evolution of eukaryotes [1,2]. Further, the unique anammox metabolism found in some planctomycetes has revolutionized the view of microbial nitrogen cycling [3]. The planctomycetes also possess cell walls without peptidoglycan, a characteristic that they share only with the obligate intracellular bacteria within *Chlamydiae*. In addition to the interest sparked by these unusual and fascinating features,

planctomycetes have in later years attracted considerable attention because of their presence in a wide variety of environments on earth. By investigating bacterial communities using molecular methods (sequences coding for 16S rRNA), planctomycetes have been repeatedly detected in soil, sediments, marine and freshwater systems and in terrestrial hot springs to mention just a few (for a detailed review see [4]). However, their metabolic potential and function in these ecosystems is often unclear, as 16S rRNA gene sequence investigations only rarely give clues to ecological roles.

In the marine environment, DeLong and co-workers [5] found that planctomycetes were more abundant in clone libraries from marine aggregate (marine snow) attached bacteria than from free-living bacteria. Since then, results from several studies suggest that planctomycetes favor a biofilm lifestyle, adhering to surfaces in aquatic environments including marine sediments [6]

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(among others), diatom cells [7], seaweeds and other aquatic macrophytes [8,9]. *Rhodopirellula baltica* is an extensively studied marine particle-attached planctomycete. Its genome sequence reveals a large number of genes involved in the breakdown of sulfated polysaccharides [10], a carbon source found in marine photosynthetic organisms such as microalgae and seaweeds, whose detrital material is thought to generate marine snow. Such genes are also encountered in other planctomycete genomes and planctomycete-derived metagenomic fosmid libraries from seawater collected in upwelling zones [11]. The overrepresentation of such genes, and the association of *R. baltica* and other planctomycetes with marine snow has led to the hypothesis that heterotrophic planctomycetes are specialized degraders of sulfated polymeric carbon, for example in marine snow [10,11]. Given the significance of marine snow as part of the so-called "biological pump" of carbon in the oceans [12,13], planctomycetes may thereby be playing a crucial role in global carbon turnover [11]. Still, quantitative data on the distribution of planctomycetes in the marine environment and elsewhere is still scarce, and very little is known about the yet uncultured planctomycete lineages that are assumed to carry out the bulk of these globally critical processes.

Kelps are large brown seaweeds of the order *Laminariales*. They often form dense stands along rocky coastlines that are referred to as kelp forests. Kelp forest ecosystems are some of the most productive ecosystems in the world [14]. Their immense importance for coastal biodiversity, productivity and human economy has long been recognized in temperate regions of the world and is only beginning to be understood in the tropics [15]. Kelp forests along the Atlantic coasts of Europe are dominated by the large kelp *Laminaria hyperborea*. Bacteria associated to kelp are believed to be important in the carbon and nitrogen turnover in kelp forest food webs [16,17], but it is still not known what types of bacteria are involved in these processes. Recently, the seasonal dynamics of the cell density and bacterial community composition in biofilms on *L. hyperborea* were addressed. In this study, planctomycetes were frequently detected throughout the year but their abundance and phylogenetic relationships were not considered [18]. In order to address the importance of this group of bacteria in kelp forests, we therefore aimed to take an in-depth look at the abundance and phylogenetic diversity of planctomycete communities inhabiting *L. hyperborea* surface biofilms. This was achieved by using fluorescence *in situ* hybridization (FISH) to quantify their abundance and visualize their distribution in the biofilm as well as 16S rRNA gene clone library construction to elucidate their phylogenetic relationships, community

composition and diversity at different times of the year. In addition, a selective cultivation approach was used to assess the culturability of planctomycetes from kelp surfaces.

Results

Abundance of planctomycetes in kelp surface biofilms

Quantification of planctomycetes in samples from July 2007, February 2007 and September 2008 using FISH showed that they make up a large part of the kelp surface biofilm community in all three sampling occasions. In July and September they dominated the community, with cells hybridizing with the *Planctomycetes*-specific probe Pla46 [19] accounting for over 50% of the total DAPI stained cells on average (Table 1 and Figure 1). In February, the planctomycetes were less abundant; with Pla46 hybridized cells corresponding to an average of 24% of total DAPI stained cells. Samples that were also subjected to hybridization with the Pir1223 [20] probe showed similar percentages ($\pm 1\%$) of hybridized cells as the with Pla46 probe (results not shown). Inspection of the cloned 16S rRNA gene sequences revealed that the Pir1223 target sequence was present in all clones except those belonging to the OM190 lineage (see the following sections) suggesting that the specificity of this probe needs to be reevaluated. The different formamide concentrations (20-40%) used in hybridization with the Pla46 probe did not change the proportion of Pla46 hybridized cells significantly (results not shown). The average proportion of the DAPI stained cells that hybridized with the Eub338 probes was 79% in July, 74% in September and 52% in February (Table 1 and Figure 1).

Cell distribution of planctomycetes in the biofilms

Fluorescence microscopy images of DAPI and FISH stained biofilm cells revealed a complex and variable microscopic landscape. The microbial cells appeared to be unevenly distributed on the kelp surface, often occurring in clusters (Figure 2a, c and 2g) or growing along straight lines (Figure 2c). Planctomycetes cells were found within all these structures, and appeared to grow evenly intermingled with other cells (Figure 2b, d and 2f). Fluorescence microscope images showed DAPI and FISH signals corresponding to different cell morphologies in the biofilm, ranging from long filaments, cocci of different sizes and small rods (Figure 2). The planctomycete FISH signals were always in the shape of small and medium sized cocci (Figure 2b, d and 2f) and displayed the "ring" shape typical of planctomycete cell organization [19] (Figure 2b inset). The Eub338 FISH signals included the whole range of morphologies (Figure 2h) and were both ring-shaped and solid.

Table 1 A summary of the results

Sampling time	Avg. cells/cm ² (DAPI) ± 1SD	Avg.% Eub338 I-III of DAPI ± 1SD	Avg.% Pla46 of DAPI ± 1SD	% Pla46 of Eub338 I-III	No. of clones	No. of OTUs (98%)	Shannon diversity index	Chao1 OTU richness estimate ± SE
February 2007	8.2e+06 ± 1.9e+06	51.6 ± 18.5	23.7 ± 9.3	45.9	73	20	2.56	29 ± 12.5
July 2007	7.4e+06 ± 4.8e+06	78.7 ± 5.2	52.5 ± 9.3	66.7	89	9	1.85	9 ± 0.73
September 2008	1.7e+07 ± 6.4e+06	73.6 ± 4.7	50.8 ± 7.2	69.0	89	15	2.32	16 ± 3.4

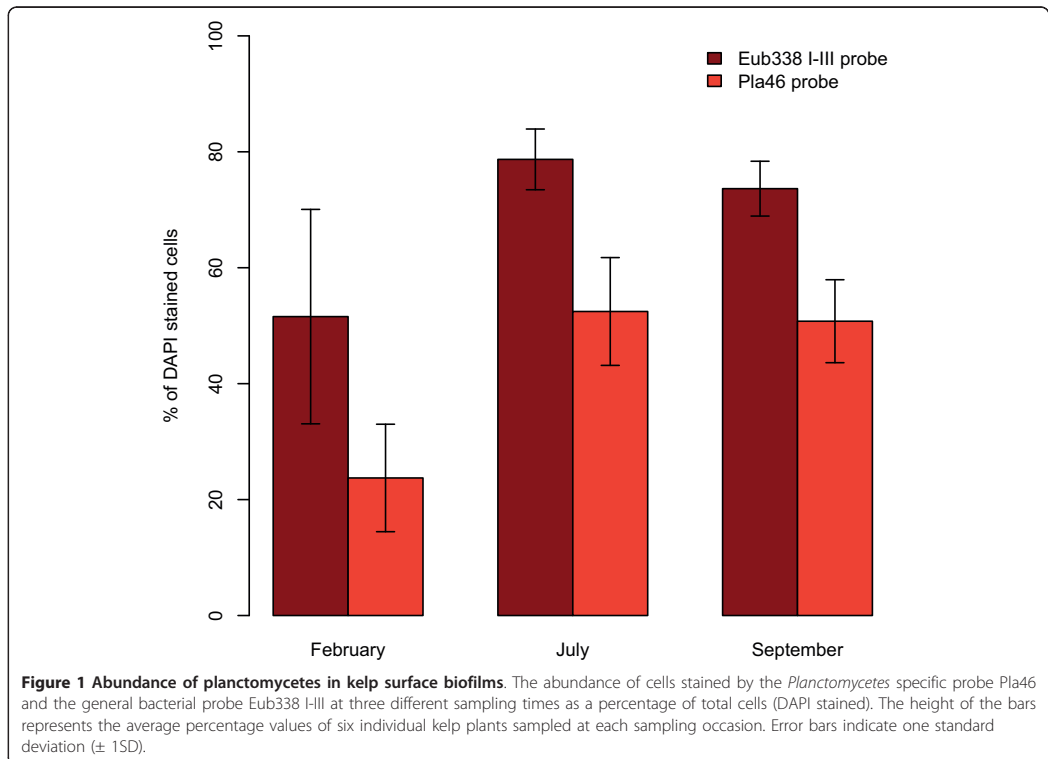
Isolation and cultivation of planctomycetes from kelp surfaces

One strain, named “P1”, belonging to *Planctomycetes* was isolated from kelp surface biofilm material from September 2008. It displayed morphological features typical for *Rhodopirellula baltica*, with ovoid cells and rosette formation (Figure 3). It formed pink colonies on M30 solid media that were visible after approximately seven days of incubation in room temperature after inoculation. It was closely related to the type strain of *Rhodopirellula baltica* (Figure 4, 99.5% 16S rRNA gene sequence similarity) and to *Rhodopirellula* strain K833

isolated from seawater in Iceland [21] (Figure 4, 99.9% sequence similarity). However, it was not closely related to any of the clone library sequences from kelp surface biofilms (Figure 4).

Phylogenetic diversity of planctomycetes from kelp surface biofilms

Three clone libraries, from February 2007, July 2007 and September 2008, constructed with the *Planctomycetes*-specific primer Pla46f and the general bacterial primer 1542r were analyzed to gain insight into the phylogenetic diversity of the planctomycetes growing in kelp



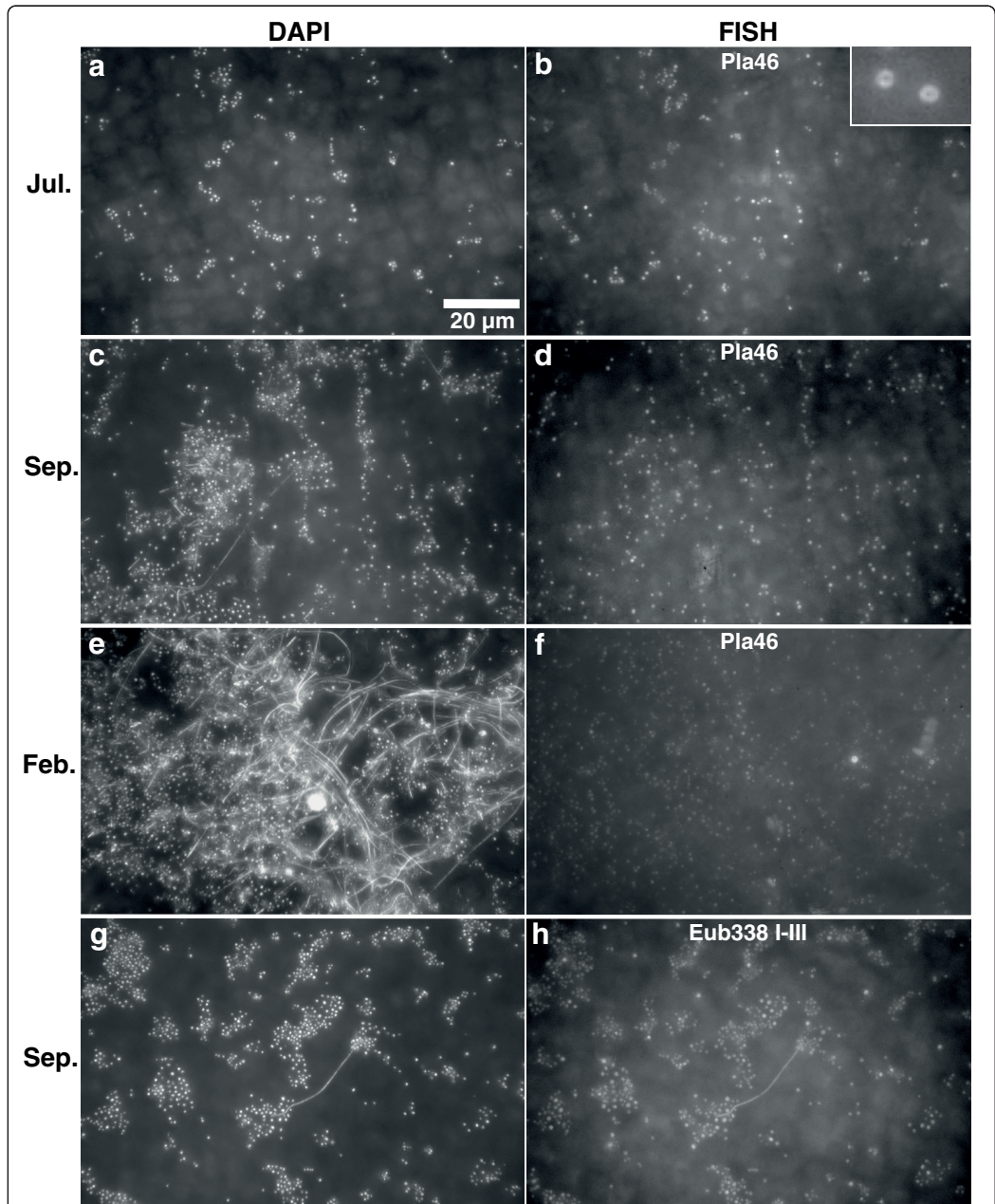
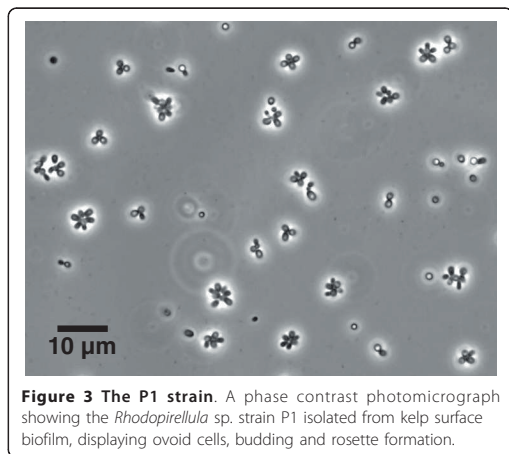


Figure 2 Distribution of planctomycete cells in the biofilm. Fluorescence microscopy images of *Laminaria hyperborea* surface biofilm. Images a, c, e and g show DAPI stained biofilm while b, d, f and h show FISH signals in the same microscope fields from hybridizations with either the Pla46 probe (b, d and f) or the Eub 338 I-III probe mix (h). Images show representative microscope fields of samples from July 2007 (a-b), September 2008 (c-d, g-h) and February 2007 (e-f). The enlarged inset image in b shows the typical ring shaped FISH signals of planctomycetes.



surface biofilms. In total, 266 clones were sequenced in the forward direction from the three clone libraries, resulting in partial 16S rRNA gene sequences of approximately 850 basepairs. Of these, only 9 sequences (3.4%) did not classify as belonging to *Planctomycetes* and were discarded from the further analyses. These unspecific sequences classified as *Deltaproteobacteria* (three), *Gammaproteobacteria* (two), *Actinobacteria* (two) and *Verrucomicrobia* (one) while one remained unclassified using the Greengenes G2Chip classifier [22]. The remaining 257 partial planctomycete 16S rRNA gene sequences clustered into 23 OTUs at 98% sequence similarity. Other OTU definitions (95-99%) gave different numbers of OTUs, but the general trends observed in the dataset were the same. One to six representative clones of each OTU were selected for sequencing in the reverse direction in order to assemble near full-length 16S rRNA gene sequences. Of the assembled sequences, three were removed from the analyses because of poor sequence quality and two because of indications of chimeric origin. The remaining 46 near full-length planctomycete 16S rRNA gene sequences have been deposited to GenBank under the accession numbers HM369064 to HM369109, and the sequence of the P1 isolate under HM369063.

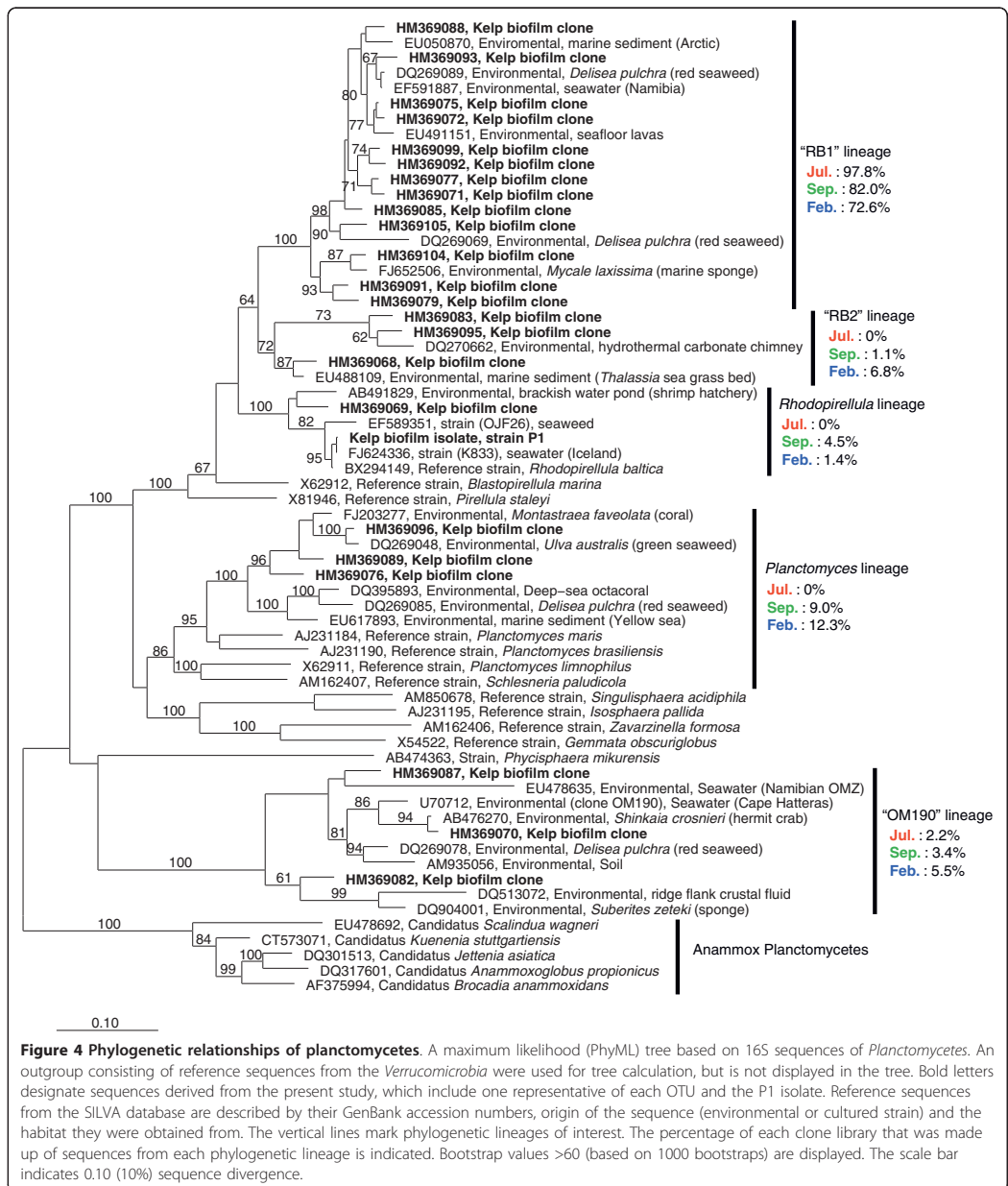
The clone libraries from February, July and September showed considerable overlap in OTU composition (Figure 5). The July library had the lowest OTU richness and consisted of a subset of the OTUs detected in the other two libraries. The highest OTU richness and the most unique OTUs (seven) were found in February. September was intermediate in OTU richness and the number of unique OTUs (Figs. 5 and 6). The diversity of the three clone libraries is illustrated in Figure 6 using rarefaction curves showing the expected number

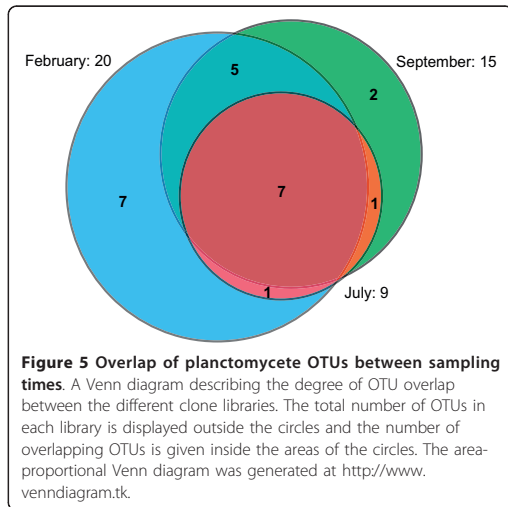
of OTUs encountered with clone sampling effort. July displays a near asymptotic curve, indicating low diversity, while September is intermediate and February displays the highest diversity. The Shannon diversity index and the Chao1 richness estimates for the clone libraries (Table 1) show the same relative diversity pattern.

The phylogenetic analysis of the near full-length sequences obtained in this study and other planctomycete sequences obtained from the Silva reference database [23] revealed that highly divergent lineages of the *Planctomycetes* phylum are represented in kelp surface biofilms (Figure 4). The kelp surface biofilm clone sequences appear to cluster within five major lineages that have been labeled as: "RB1" and "RB2" (defined in this study), *Rhodopirellula*, *Planctomyces* and "OM190". The "RB1" and "RB2" lineages appear more closely related to the *Rhodopirellula* and *Blastopirellula* genera than to the *Pirellula* genus and were given their labels based on that (RB = *Rhodopirellula*/*Blastopirellula*). Yet the phylogenetic analyses do not place them consistently with either of the genera. Sequence similarities of 86-90% to *Rhodopirellula baltica* and *Blastopirellula marina* indicate that they probably represent distinct phylogenetic lineages that could correspond to new genera according to conventional taxonomical practice. The "RB1" lineage was by far the most represented in all three clone libraries (Figure 4). Sequences that cluster within the "RB2", *Rhodopirellula* and *Planctomyces* lineages were only represented in September and February, indicating a seasonal difference, while OM190 representatives were present at low numbers in all three clone libraries (Figure 4).

Discussion

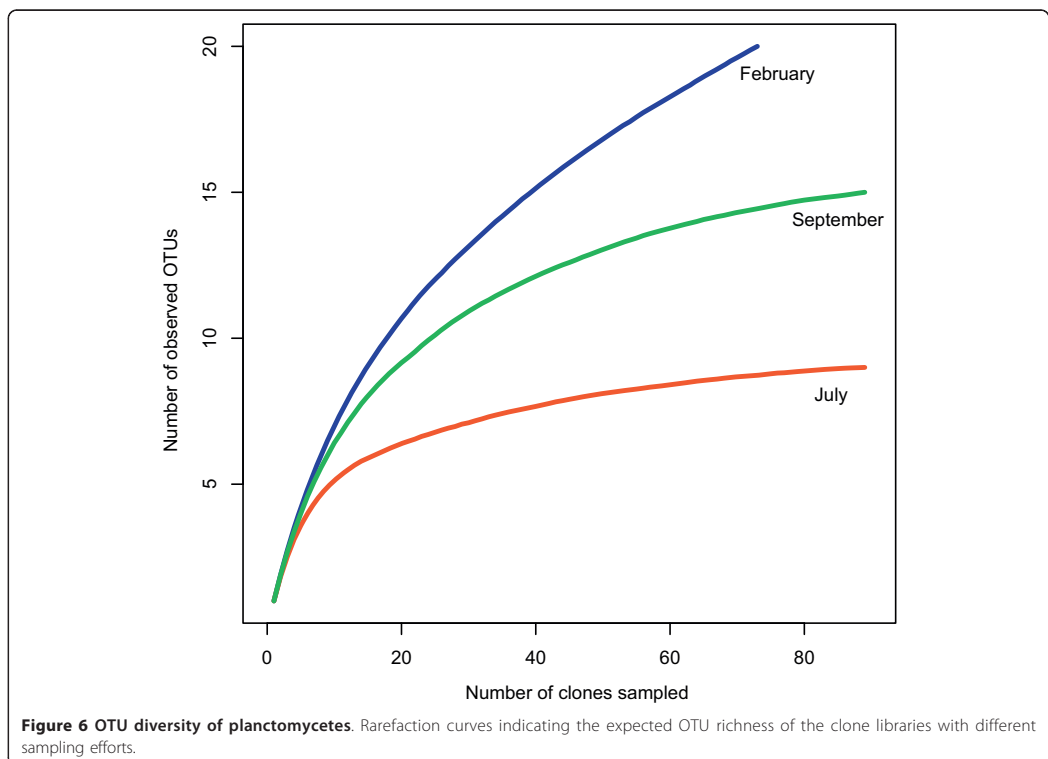
To our knowledge, the kelp surface biofilms investigated in this study display the highest proportion of bacteria belonging to *Planctomycetes* reported in a natural bacterial community so far. This observation is consistent with earlier results from a DGGE based study on seasonal variation of *Laminaria hyperborea* (kelp) surface biofilm communities [18]. Other habitats where a high abundance of planctomycetes has been reported include seawater during a diatom bloom where planctomycetes related to *Pirellula* were detected attached to diatom cells and were among the dominant lineages in the bloom samples [7]. In investigations of sandy sediments containing algal cells [24,25], planctomycetes were also abundant, accounting for up to 20% of total cells, accompanied by *Cytophaga/Flavobacteria*. Gade and co-workers [20] used order-, genus- and strain specific FISH probes to detect planctomycetes in a range of aquatic habitats and recorded abundances up to 11% of total cells in some lakes. Peat bogs with *Sphagnum* moss have also been reported to harbor abundant (up to





13% of total bacterial numbers) planctomycete populations [26]. Similarly to kelp surfaces, these environments are all highly influenced by photosynthetic eukaryotes.

The studies mentioned above have all quantified planctomycetes using specific FISH probes. Several other studies have detected planctomycetes using PCR based cloning and fingerprinting methods followed by sequencing. Two of the most frequently used general bacterial PCR primers, targeting the 16S rRNA gene around *E. coli* positions 8-27 and 338-355, contain mismatches against planctomycete sequences [27,28]. This may have caused planctomycete abundances to be underestimated in many habitats, leading investigators to turn their attention towards bacterial groups that appear more abundant. Despite awareness of this problem, the literature and the sequence databases probably reflect a tradition of neglect towards the planctomycetes. In the light of this, it is difficult to say whether the dominance of planctomycetes on *Laminaria hyperborea* surface biofilms represents a unique feature of this



habitat, or if other planctomycete-dominated bacterial communities have been overlooked until now. For example, Staufenger and co-workers [29] did not detect planctomyces in surface biofilms of another species of kelp (*Saccharina latissima*) using general bacterial primers for cloning and DGGE analysis. Yet, use of different primers has led to the detection of planctomyces on both the kelps *S. latissima* and *Laminaria digitata* (Bengtsson, unpublished results).

A possible explanation for the suitability of kelp as a habitat for planctomyces is its content of sulfated polysaccharides, a class of molecules that some marine planctomyces are known for being able to degrade [10]. For example, *Laminaria hyperborea* contains fucoidan, a class of complex brown algal sulfated polysaccharides. These substances are secreted to the surface of *L. hyperborea* via mucilage channels [30]. It is reasonable to assume that planctomyces living on kelp surfaces utilize substances produced by the kelp, for example fucoidan, as carbon sources. However, the presence of suitable carbon sources appears insufficient to explain the observed dominance of planctomyces, as they must not only be able to grow and divide, but also outcompete other bacteria to be successful. Another contributing factor to the success of planctomyces on kelp surfaces may be resistance to chemical antimicrobial defense compounds produced by the kelp. Antibacterial activity has been detected in extracts from many species of kelp, yet the substances responsible for the activity have often not been identified [31]. The lack of peptidoglycan in planctomycete cell walls makes them resistant to conventional cell wall targeting antibiotics like ampicillin. Resistance to other antibiotics, targeting for example protein synthesis (streptomycin) has also been reported in some marine planctomyces [32,33].

In many cases the reference sequences that are the most closely related to kelp surface planctomyces are obtained from other marine eukaryotes such as for example red and green seaweeds, corals, crustaceans and sponges (Figure 4). The frequent association of planctomyces to eukaryotes has previously been noted [34]. This could point to a general lifestyle pattern of heterotrophic planctomyces. They may combine an affinity for sulfated polysaccharides and other polymeric carbon molecules [10,11] produced by their eukaryote hosts with a resistance to eukaryote chemical defense molecules. The resulting competitive advantage over other bacterial groups that are utilizing the same kind of substrates, for example the *Bacteroidetes* [35] might be one of the keys to the success of planctomyces in a wide variety of environments on earth.

Our results show differences between the different sampling times (February, July and September), in planctomycete abundance, OTU composition and diversity.

For example, in February there is a relatively low abundance of planctomyces (Figure 1) compared to July and September. This may be linked to the age of the kelp tissue, as the kelp lamina is older in February compared to in July and September due to the seasonal growth cycle of the kelp. Aging of the kelp tissue could be associated with lowered antibacterial chemical defense by the kelp, as the old kelp lamina is to be shed soon after February, and does therefore not need to be defended against microbial colonization. Without the presence of chemical defense substances, the planctomyces could lose their competitive advantage over other bacterial groups, explaining their lower abundance in February. The senescence of the kelp tissue as it ages could also cause the appearance of new niches involved in degradation of different kelp constituents, thereby enabling the more diverse planctomycete communities that are observed in February compared to July and September (Table 1, Figure 6).

Among the different planctomycete lineages that are represented on the kelp, the lineage defined as "RB1" in this study appears to be the most abundant, accounting for a majority of the clones at all sampling times (Figure 4). The high abundance of RB1 planctomyces may thus be the cause of the observed dominance of planctomyces on kelp surfaces (Figs. 1 and 2). Their high abundance implies a lifestyle that makes them particularly successful on kelp surfaces. Yet the lineage also includes reference sequences from a variety of other marine habitats, indicating that RB1 is not a kelp-specific lineage. The RB1 and RB2 lineages, defined in this study, are clearly related to the "*Pirellulaceae*", a lineage including the genera *Pirellula*, *Rhodopirellula* and *Blastopirellula* (formerly all included in the genus *Pirellula*). Yet our phylogenetic analyses did not place them reliably with any of the described genera, indicated by the bootstrap support for the relevant branches in Figure 4. There are no sequences of cultured strains within the RB1 and RB2 lineages available in the databases. Another uncultured lineage, the so-called OM190 planctomyces (Silva taxonomy) is also represented by clones from kelp surfaces at all sampling times, yet in low numbers. This is a lineage that appears to branch off deeply in the planctomycete tree and representatives have been detected in a variety of environments including seawater [36], soil [37] and marine eukaryotes [8,38]. These lineages have yet to be cultured and described and will reveal valuable information on planctomycete metabolism and evolution if cultivation is successful.

Using conventional approaches, the *Rhodopirellula sp.* strain P1 could easily be isolated. Several closely related strains have been brought into culture earlier [21]. However, the 16S rRNA gene sequence of P1 does not correspond to any of the abundant OTUs detected on the

kelp surfaces, for example within the RB1 lineage. Kelp surfaces are nevertheless a promising source for isolation of novel planctomycete strains, using more ambitious and creative approaches that take into account the environmental factors experienced by bacteria on kelp surfaces. The rewards awaiting such attempts can be substantial, given the representation of highly divergent lineages of the planctomycete tree in kelp surface biofilms.

Conclusions

Kelp (*Laminaria hyperborea*) surface biofilms have a uniquely high relative abundance of planctomyces. Several distinct lineages are represented, and the diversity and composition of the planctomyces change during the year, probably influenced by aging of the kelp tissue. The finding of abundant planctomycete populations in kelp surface biofilms agrees well with the view of heterotrophic planctomyces as surface attached, specialized degraders of sulfated polysaccharides in the marine environment, as kelps are known to produce such substances. Furthermore, we wish to extend this view by hypothesizing that many heterotrophic planctomyces share a preference of intimate coexistence with eukaryotes, which may be linked to antibiotic resistance. The study addresses the urgent need for more detailed, quantitative knowledge on the diverse marine planctomyces.

Methods

Sample collection and preparation

Kelp (*Laminaria hyperborea*) was collected at one site near Bergen, Norway (60° 09.706' N, 5° 02.371' E) in February 2007 and in July 2007. These sampling times were selected based on a previous study that detected low (February) and high proportions (July) of planctomyces at these times [18]. In addition, kelp was sampled at the same site in September 2008 to obtain fresh biofilm material for cultivation of planctomyces. Six replicate kelp individuals were collected from a depth of 5 to 9 m by dredging from a boat at each sampling occasion and were kept cool until further processing (a few hours). Biofilm samples were obtained from the middle part of the kelp lamina (blade) of each kelp individual. The lamina areas used for biofilm sampling were thoroughly washed with sterile seawater. Biofilm for DNA extraction was sampled by scraping off material from the kelp surface with a sterile scalpel as described previously [18]. Biofilm for FISH was sampled by cutting out whole pieces of the kelp lamina in order for the attached biofilm to remain intact. Sample collection and preparation procedures are described in greater detail in [18].

FISH

Kelp lamina pieces (1 × 0.5 cm) were fixed in 2% buffered paraformaldehyde overnight, washed twice in 50% EtOH in PBS and stored in the same solution at -20°C. Prior to FISH, the kelp pieces were dehydrated in 96% EtOH and air-dried. Each sample kelp piece was further divided into 0.5 × 0.5 cm pieces, that were used for hybridization either with the general Bacterial probe mix Eub338 I-III [28] or the planctomycete specific probe Pla46 [19]. In addition, a subset of samples were hybridized with the probe Pir1223 [20] that is reported to be specific for the genera *Pirellula*, *Blastopirellula* and *Rhodopirellula* (formerly all included in *Pirellula*). Several samples were also hybridized with the Non338 probe to check for signals caused by unspecific hybridization or autofluorescence of bacterial cells. All probes were bound to the fluorochrome Cy3, as previous investigations have shown that it gives superior fluorescence signals over the otherwise troublesome autofluorescence of the kelp cells compared to other fluorochromes such as fluorescein (Bengtsson, unpublished data). The formamide concentrations in the hybridization solution for the respective probes were 35% for the Eub338 I-III mix, 30% for Pla46 and 30% for Pir1223. Formamide concentrations of 20, 25, 30, 35 and 40% were evaluated on a subset of the September samples for the Pla46 probe. FISH was carried out according to [39] with some modifications. In summary, the dry kelp pieces were soaked in hybridization solution and hybridized at 46°C for 3 hours inside capped 0.5 ml plastic tubes. After stringent washing and subsequent washing with dH₂O, the kelp pieces were counter-stained with DAPI and mounted on glass slides as described in [18].

Fluorescence microscopy

Digital images of randomly selected microscopic fields were captured for counting of DAPI stained cells and FISH hybridized cells. Image capture and counting were carried out as previously described [18]. The percentage FISH hybridized cells of the total cell count (DAPI stained cells) was calculated for every individual microscope field captured, and an average percentage was calculated for each sample.

Isolation and cultivation of planctomyces from kelp surfaces

Freshly scraped off biofilm material from September 2008 suspended in sterile seawater was used to inoculate M30 medium [4] diluted in 3/4 parts sterile seawater supplemented with ampicillin (0.2 mg/ml). After growth was detected, the liquid culture was plated out on M30 medium solidified with gellan gum (Gelzan, Sigma-Aldrich), and individual colonies were picked and replated several times to obtain pure cultures.

DNA extraction

Scraped off biofilm was suspended in sterile filtered and autoclaved seawater and the cells were pelleted by centrifugation. DNA was extracted from the pellets as previously described [18]. Equal volumes of the DNA extracts from the 6 replicate kelp plants from each sampling time were pooled into one sample per sampling time. The purpose of this was to obtain an overall picture of the planctomycete populations at each sampling time. Variation in OTU composition between individual kelp laminae is not captured by this approach, but has been addressed previously for the whole bacterial communities [18]. The pooled DNA extracts (from February 2007, July 2007 and September 2008) were used for the subsequent PCR amplification and clone library construction.

PCR amplification and clone library construction

The *Planctomycetes* specific forward primer Pla46f (5'-GGA TTA GGC ATG CAA GTC-3') complementary to the Pla46 FISH probe [19] and the general bacterial reverse primer 1542r (5'-AAG GAG GTG ATC CAG CCG CA-3') [40] were used to amplify a near full length fragment of the 16S rRNA gene of *Planctomycetes*. PCR conditions were: 94°C for 5 min, 25 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, and final elongation at 72°C for 10 min. Each 25 µl PCR reaction contained nuclease-free water, F511 buffer (Finnzymes), 0.1 mM of each dNTP (F506L, Finnzymes), 0.02% BSA, 0.5 µM of each primer, 0.02 U Dynazyme II F501-L (Finnzymes), and approximately 30 ng template DNA. Three clone libraries, one from each sampling occasion, were constructed using the TOPO TA cloning kit (Invitrogen). Ninety-six clones were picked from each clone library. Cloned fragments were reamplified using the supplied M13 primer pair according to the manufacturers instructions.

Sequencing and sequence processing

All cloned fragments were sequenced in one direction using the Pla46f primer. Sequencing was carried out with the BigDye Terminator v3.1 kit (Applied Biosystems) at the Bergen Sequencing Facility <http://www.seqlab.uib.no> using an ABI 3700 sequencing system. Base calling from the chromatogram files was done using the Phred software [41] (version 0.020425.c). The resulting sequences representing partial fragments of the 16S rRNA gene were used to select a subset of clones to sequence in the reverse direction in order to obtain near complete length 16S rRNA gene fragments. The sequences were trimmed to approximately 750 bp of good quality sequence and aligned against the Silva seed alignment (release 102) using the SINA web aligner [23]. The alignment was imported into the ARB software package [42] (version

5.0) and was manually edited to improve alignment quality. The resulting alignment was used to create a distance matrix in ARB, which was used to cluster the sequences into OTUs using the furthest neighbor algorithm in the Mothur software [43] (version 1.9.0). Rarefaction and overlap analysis were carried out in Mothur. The Shannon diversity index and the Chao1 richness estimate was calculated in the R statistical environment ([44], functions: diversity and estimateR, package: vegan). Based on the OTU clustering, one to six representatives of each OTU were sequenced in reverse using the 1542r primer. The resulting sequences were assembled using CAP3 [45] with the corresponding forward sequences to build contigs spanning nearly the full length of the 16S rRNA gene. The contigs were manually cropped to roughly the same length using the Phred base quality scores of the ends of the contigs as a guide. The resulting same-length (about 1250 bp), good quality contiguous sequences were checked for chimeras using Bellerophon [46] through the online Greengenes interface [22]. The *Rhodospirillum* sp. strain P1 was sequenced in forward and reverse direction several times with different 16S rRNA gene primers. The individual sequence reads were manually assembled into one full-length consensus sequence.

Phylogenetic tree reconstruction

The near full-length sequences were aligned using the SINA web aligner, imported into ARB and edited as described in the previous section. Reference sequences that were closely related to the clone sequences from this study and sequences from cultured planctomycetes were selected from the SILVA database and were included in the tree calculations. Several tree calculation methods including neighbor joining (NJ), maximum likelihood (ML) and maximum parsimony (MP) were used in combination with different conservatory filters in ARB and the tree topologies compared to ensure a reliable result. The final ML tree was calculated in ARB with 175 sequences using PhyML [47] applying bootstrap analysis (1000 bootstraps) and no filter. Four *Verrucomicrobia* sequences (accession numbers: AY271254, DQ302104, AB297805, AB297806) were used as an outgroup in the tree calculation. The tree was edited by removing some of the reference sequences for clarity of presentation and the final result is shown in Figure 4.

Acknowledgements

The authors wish to thank Tomas Sørli and Julia Storesund for sampling assistance, Friederike Hoffmann for valuable advice on FISH and Anders Lanzén for bioinformatics assistance. Kjersti Sjøtun, Antonio García Moyano, Jeffrey Keen, Tim Ulrich and three anonymous reviewers provided constructive comments that considerably improved the manuscript. Funding for sampling and laboratory expenses was provided by FMC Biopolymer. The authors are funded by the University of Bergen.

Authors' contributions

MMB carried out the sampling, laboratory work and data analysis and wrote the manuscript. LØ conceived the study, supervised the laboratory work and data analysis and participated in editing the manuscript.

Received: 4 June 2010 Accepted: 15 October 2010

Published: 15 October 2010

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doi:10.1186/1471-2180-10-261

Cite this article as: Bengtsson and Øvreås: Planctomycetes dominate biofilms on surfaces of the kelp *Laminaria hyperborea*. *BMC Microbiology* 2010 **10**:261.

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Utilization of kelp-derived carbon sources by kelp surface-associated bacteria

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ABSTRACT: The surfaces of kelp are covered with bacteria that may utilize kelp-produced carbon and thereby contribute significantly to the carbon flux in kelp forest ecosystems. There is scant knowledge about the identity of these bacteria and about which kelp-derived carbon sources they utilize. An enrichment approach, using kelp constituent carbon sources for bacterial cultivation, was used to identify bacterial populations associated with the kelp *Laminaria hyperborea* that degrade kelp components. In order to assess whether the cultured bacteria are significant under natural conditions, partial 16 rRNA gene sequences from the cultured bacteria were compared to sequences obtained from the indigenous bacterial communities inhabiting natural kelp surface biofilms. The results identify different members of the Roseobacter clade of *Alphaproteobacteria* in addition to members of *Gammaproteobacteria* that are involved in kelp constituent degradation. These bacteria are observed sporadically on natural kelp surfaces and may represent opportunistic bacteria important in degradation of dead kelp material. Many of the cultured bacteria appear to be generalists that are able to utilize different kelp carbon sources. This study is the first to link culturable kelp-associated bacteria with their occurrence and possible roles in the natural environment.

KEY WORDS:

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INTRODUCTION

The kelp *Laminaria hyperborea* forms extensive underwater forests along the Atlantic coasts of Europe with large abundances in Norway. These forests provide food, shelter or growth substrate for a great biodiversity of macroscopic organisms, such as invertebrates, fish and epiphytic seaweeds (Tokida 1960, Christie et al. 2003). Kelp forests also sustain an invisible, but even more diverse, world of microbes that are for example found growing on the surfaces of the kelp. These biofilms, consisting mainly of bacteria (M. M. Bengtsson unpubl. data), coat all surfaces of the kelp and may play vital roles in the kelp forest ecosystem. Some heterotrophic marine bacteria are known to have the ability to utilize complex polysaccharides, for example, alginate and fucoidan that are produced by kelp (Wong et al. 2000, Sakai et al. 2002, Sakai et al. 2003). Kelp-associated bacteria with such abilities can serve as food for micrograzers and filter feeding animals in the kelp forest, and contribute to the nutritional

value of kelp material for these consumers (Norderhaug et al. 2003). In these ways, bacteria play a key role in making kelp primary production available to higher levels in the food chain (Newell & Field 1983, Norderhaug et al. 2003).

Several strains of bacteria that are capable of degrading different kelp substances have been isolated from marine environments and investigated (e.g. Chesters & Bull 1963, Uchida 1995, Sakai et al. 2002, Sakai et al. 2003, Descamps et al. 2006). In these studies, the individual strains and their physiology were the focus rather than the ecological importance of the bacteria on kelp surfaces or in kelp forest ecosystems. Other studies have adopted a more ecological perspective, by studying the seasonal (Laycock 1974), spatial (Koop et al. 1982, Davis et al. 1983) or successional (Hollohan et al. 1986) dynamics of bacteria that grow on kelp substances. In these studies, the bacteria involved were not identified with any taxonomical resolution and it was not possible to compare the cultured bacteria with indigenous bacterial communities on

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kelp due to the methodological limitations at the time when the studies were carried out. Consequently, there is little knowledge of which types of kelp-carbon utilizing bacteria are captured by cultivation efforts and of their significance in indigenous kelp-associated bacterial communities.

The bacterial communities inhabiting the surfaces of kelps have recently been investigated using culture-independent, molecular tools based on 16S rRNA (Staufenberger et al. 2008, Bengtsson et al. 2010). On surfaces of *Laminaria hyperborea*, the most frequently detected lineages were *Planctomycetes*, *Alphaproteobacteria*, *Verrucomicrobia*, *Bacteroidetes* and *Gamma-proteobacteria* (Bengtsson et al. 2010). However, the ecological roles of the different types of bacteria have not been investigated. For example, it is not known which of the bacteria are sustained by kelp-produced substances and which substances they utilize.

The aim of this study was to pinpoint and identify bacterial populations that utilize different kelp constituents in order to gain insight into the ecological roles of the bacteria that inhabit kelp surface biofilms. This was achieved by an enrichment-based approach, using kelp constituent substances as carbon sources for cultivation of bacteria derived from kelp surface biofilms. Comparisons of sequences obtained from the cultured bacteria with sequences from the indigenous bacterial communities on kelp surfaces, were made to assess the ecological significance of the cultured bacterial lineages on kelp surfaces.

MATERIALS AND METHODS

Sampling and sample processing. *Laminaria hyperborea* was sampled in September 2008 at 1 site (60° 09.706' N, 5° 02.371' E) close to Bergen, Norway. Six kelp individuals were sampled by dredging from a boat and were brought back to the laboratory for processing within a few hours. Three 5 × 5 cm pieces were cut out from different places (evenly distributed) on the middle lamina of each kelp individual and were rinsed thoroughly in sterile (autoclaved and sterile filtered) seawater to remove loosely attached (non-biofilm) bacteria. Biofilm material was scraped off the surfaces of both sides of the kelp pieces using a sterile scalpel. The biofilm material from all 3 pieces from each kelp individual was pooled by suspending it in 10 ml of sterile seawater. Thus, each suspension contained 150 cm² of biofilm from the same kelp individual. The biofilm suspensions were mixed thoroughly by vortexing and were kept on ice for a few hours until inoculation of the culture media (described in the next section).

Three smaller (0.5 × 0.5 cm) pieces were cut out from each kelp individual and used for enumeration of

biofilm cells. They were fixed, stained with DAPI (4',6-diamidino-2-phenylindole) and photographed under a fluorescence microscope. Biofilm cells were counted as described in Bengtsson et al. (2010).

Cultivation media. Six different media were prepared for cultivation of kelp biofilm bacteria. Of these, 4 contained a minimal base medium supplemented with different kelp constituents as carbon sources, and 1 was a control containing the minimal base medium without a carbon source. A commercial marine broth (Difco Marine Broth 2216) medium with complex carbon sources, such as peptone and yeast extract, was included in the experiments. All media were solidified with agar (Bacto Agar, grade A). The minimal base medium consisted of 1.25 mM NH₄NO₃ and 1.8 % agar in 90 % natural seawater (v/v), as well as 0.5 mM Na₂HPO₄, trace elements (Whitman et al. 2006) and vitamins (Wolin et al. 1963) that were added after autoclaving. The chemical structures of the kelp constituent carbon sources that were used in this study are given in Fig. 1.

Alginate is an anionic polysaccharide (Fig. 1) that is a structural component of brown seaweed cell walls, and is also found intercellularly. It makes up approximately 15 to 35 % of the dry weight of *Laminaria hyperborea* laminae (Haug & Jensen 1954). Fucoidan is a sulphated polysaccharide (Fig. 1) that is produced in specialized secretory cells in *L. hyperborea* and secreted to the surface of the thallus via mucilage channels (Evans et al. 1973). The exact structure of *L. hyperborea* fucoidan has not been determined. Fucoidan appears to be a minor constituent of *Laminaria* kelps, making up less than 4 % of the dry weight (Black 1954). Laminaran is a storage polysaccharide of the cells of *L. hyperborea* and other brown seaweeds; It constitutes up to 30 % of the dry weight of *L. hyperborea* laminae, and concentrations are highest between August and February, as a result of storage of carbon fixed by photosynthesis during the summer (Haug & Jensen 1954). D-mannitol is a relatively simple sugar alcohol (Fig. 1) that, like laminaran, is an intracellular storage compound; however, it may also be released from kelp cells into the surrounding seawater by exudation (Wada et al. 2007). D-mannitol constitutes up to 20 % of the dry weight of *L. hyperborea* laminae, and concentrations are highest from July to September (Haug & Jensen 1954).

The polymers alginate (Protanal HF, FMC Biopolymer), fucoidan and laminaran were extracted from Norwegian *Laminaria hyperborea* and provided by FMC Biopolymer while D-mannitol was obtained commercially (Pearlitol 160 C, Roquette). The kelp constituents were added to the minimal base medium at 1 g l⁻¹ medium before autoclaving. The marine broth was diluted according to the manufacturers instructions. The media were poured onto petri dishes (90 mm diameter).

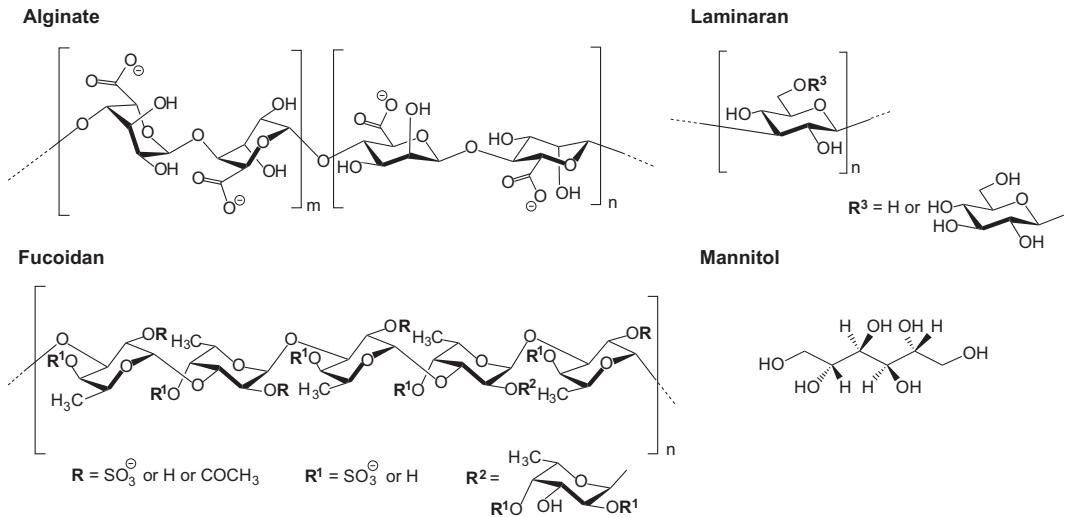


Fig. 1. The structures of the kelp constituent carbon sources used for enrichment cultivation of bacteria in the present study. The letters 'n' and 'm' indicate repeating units in the polymeric substances

Enrichment cultivation. The biofilm suspensions from the 6 kelp individuals were plated on the 6 different media described in the previous section in 3 different dilutions, corresponding to 5, 10 and 20 μl per plate. Plates were incubated at 15°C in darkness for 4 wk. Colony counts were recorded for all plates and the sums of the counts of the 3 different dilutions per biofilm suspension and media type were used in further calculations. The colony diameter was measured for 30 randomly selected colonies on each plate and the average colony diameter of the 3 different dilutions per biofilm suspension and media type was calculated. Sixteen randomly selected colonies from each biofilm suspension and medium type were picked for colony PCR and sequencing.

Colony PCR and sequencing. Colony material was transferred with sterile pipette tips to 50 μl PCR grade water in individual wells of a 96 well PCR microtiter plate. The colony suspensions were incubated at 99.9°C in a PCR thermocycler for 10 minutes to disrupt the cells and inactivate nucleases. The colony suspensions were immediately frozen and stored at -20°C until further analyses. PCR amplification of a part of the 16S rRNA gene of bacteria was carried out according to the PCR conditions described in Bengtsson et al. 2010, using 1 μl of the colony suspensions as template for 25 μl PCR reactions. The primers 1070f (5'-ATG-GCTGTCGTCAGCT-3') (Ferris et al. 1996) and 1392r (5'-ACGGGCGGTGTGTRC-3') (Lane et al. 1985) that were used cover approximately 300 bp of the 16S rRNA gene. Positive PCR products were sequenced in

1 direction using the 1070f primer and BigDye Terminator v3.1 technology on an ABI 3700 sequencer (Applied Biosystems) at the Bergen sequencing facility (www.seqlab.uib.no).

Sequence analysis. Base calling of sequence chromatogram files was done using Phred (Ewing et al. 1998) and quality filtering and sequence end trimming was carried out via the Greengenes online interface (DeSantis et al. 2006). The quality-trimmed sequences were aligned against the Silva seed alignment, version 102 (Pruesse et al. 2007). The resulting alignment was imported into the software package ARB (version 5.0) (Ludwig et al. 2004) together with the 40 nearest neighbor reference sequences to each colony sequence. The alignment was manually edited in ARB to improve alignment quality. A distance matrix was constructed in ARB, using all alignment positions and Jukes-Cantor correction. The distance matrix was used to generate OTUs of different similarity thresholds in mothur (version 1.8.0) (Schloss et al. 2009), using the furthest neighbor clustering algorithm.

The sequences obtained from the enrichment cultivation experiment described in this study were compared with sequences from indigenous kelp-associated bacterial communities obtained with cultivation-independent methods. These sequences comprised of 587 bands obtained with denaturing gradient gel electrophoresis (DGGE) from bacterial communities collected during a seasonal cycle from kelp biofilms, the surrounding seawater and biofilms from the surfaces of the red seaweed *Palmaria palmata* that is an epiphyte on kelp. A

part of this dataset, representing bands from *Laminaria hyperborea* meristem and lamina and seawater from 4 seasons, has been published in Bengtsson et al. (2010). The DGGE sequences were generated in that study using the same primers as described above. Sample collection and DGGE procedures are described in Bengtsson et al. (2010). All sequence analyses in this study, including distance matrix construction and OTU clustering were carried out concomitantly using sequences from both the enrichment cultivation and cultivation-independent sources. Representative sequences (the longest) of each 97 % OTU from the cultivation experiment have been deposited in GenBank under the accession numbers HQ331511 to HQ331524.

Phylogenetic tree reconstruction. Full-length nearest neighbor 16S rRNA gene reference sequences obtained from the Silva 102 database (as described in the previous section) were used for tree calculations. Tree topologies were compared between Neighbor Joining and Maximum Likelihood (ML) tree calculation methods to ensure stable branching. The final ML tree was calculated with 601 full-length sequences in ARB using all alignment positions and an archaeal sequence (GenBank accession CP000866) as an outgroup. The partial sequences obtained in this study were added to the tree afterwards using the ARB parsimony tool. The tree was edited by removing all but one representative sequence of each OTU and all but the most closely related reference sequences.

Statistical analysis. Fishers exact test for count data (Fisher 1935) was used to test for independence of counts of 'growth' and 'no growth' of colonies of individual culturing media and individual OTUs. The significance of the test result was used to indicate whether or not the different culturing media influenced the OTU composition of the sequenced bacterial colonies. The statistical analyses were carried out in the R software for statistical computing (R Core Development Team 2010).

RESULTS

Colony number and size

The total numbers of colonies recorded on the different cultivation media are displayed in Fig 2. The colony numbers give a range of 15 to 10485 CFUs (colony forming units) per cm² of kelp surface if the sampled surface area that the inoculum volume represents is taken into account. Assuming that 1 CFU corresponds to 1 bacterial cell, this equates to a cultivation success in the range of 0.0002 to 0.063 %, based on the counts of DAPI stained biofilm cells. The addition of kelp substances as carbon sources to the growth media

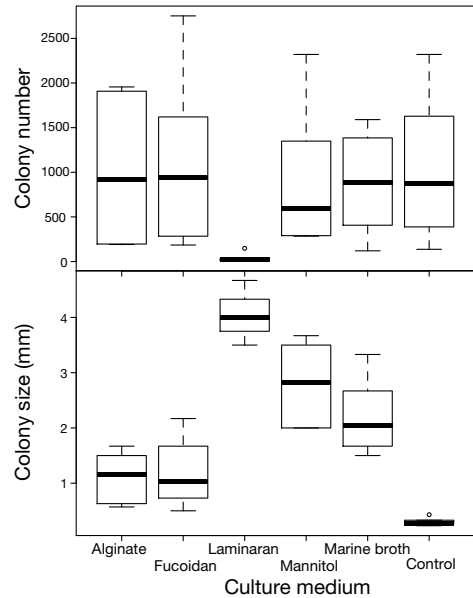


Fig. 2. Box plots illustrating the number (sum of 3 inoculum dilutions) and the average size (diameter) of colonies recorded for the different culture media. The diagrams depict the lower and upper quartiles (boxes), minimum and maximum (bars) and median (solid line) values of 6 different kelp individuals. Outliers (3) are also shown

did not (with the exception of laminaran) result in any detectable difference in the number of bacterial colonies observed compared to the control medium that contained no kelp carbon source (Fig. 2). However, there was a clear increase in colony size in all kelp-carbon-source media compared to the control (Fig. 2). The bacterial colonies that grew on the control medium were all below 0.5 mm in diameter and barely visible to the naked eye. The larger colonies on all kelp-carbon-source media show that the addition of kelp substances indeed had an effect on bacterial growth, indicating that they are utilized as carbon sources by the bacteria. The most striking effect was observed for the medium containing laminaran, where a dramatic increase in colony size was accompanied by a decrease in colony number compared to the control medium and the other media (Fig. 2).

Influence of culture media on bacterial OTU composition

In total, 19 different OTUs at 97 % sequence similarity were detected among the 282 colonies that were

Table 1. The number of sequenced colonies belonging to each OTU in the different culturing media. The actual number of colonies is given and the table cells are shaded according to the percentage of the total number of sequenced colonies for each medium. Significant p-values ($p < 0.05$) derived from Fishers exact test indicate that the occurrence of an OTU is influenced by medium (rows) and that a medium influences the composition of OTUs (columns). The number of samples in which the OTU was detected by DGGE in natural kelp surface biofilms is given. Cultured OTUs that occurred only once in the dataset are not displayed.

Carbon source type: Medium OTU	Kelp constituent				Complex	No	p-value	Detection by DGGE (# of samples)
	Alginate	Fucoidan	Laminaran	Mannitol	Marine broth	Control		
A1	4	3	0	4	4	3	0.89	-
A2	6	12	17	10	6	9	<0.001*	-
A3	22	22	2	29	26	20	0.006*	9
A4	2	6	0	1	0	4	0.04*	-
A5	0	3	0	3	0	0	0.08	-
A6	9	1	0	1	0	4	<0.001*	-
A7	0	0	2	1	2	1	0.09	-
A8	0	0	0	4	0	0	0.01*	-
A9	1	1	0	0	0	3	0.19	-
A10	4	0	1	1	5	1	0.07	-
A11	1	0	0	0	3	0	0.07	1
G1	3	0	0	0	0	0	0.05*	3
G2	0	0	0	1	1	0	0.08	6
F1	0	4	0	0	0	0	0.008*	-
p-value	0.001*	0.004*	<0.001*	0.02*	0.005*	0.5		

sequenced. The OTUs occurring only once were not included in the following analyses to reduce the influence of stochastic variation. The occurrence of the remaining 14 OTUs on the different cultivation media is displayed in Table 1. All media except the control had a significant influence on the bacterial OTU composition, and several of the OTUs were significantly influenced by the medium type according to the statistical analyses (using Fisher's exact test on individual growth media and OTUs). The OTUs that show a significant preference for one or more of the different culture media are indicated by highlighted p-values in **Table 1**. The overall OTU composition was dominated by 2 OTUs, A2 and A3, that were present on all culturing media, including the control (Table 1). Both of these OTUs were significantly influenced by medium type, and closer inspection revealed that A2 was overrepresented, while A3 was underrepresented on the laminaran medium. This indicates that A2 preferably utilizes laminaran while A3 shows no such preference. Both A2 and A3 appeared to be evenly represented on the other medium types. Of the less abundant OTUs, A8 was exclusively detected on the mannitol medium, G1 on the alginate medium and F1 on the fucoidan medium (Table 1). This may indicate that these OTUs are specialized in utilizing these substances. As all culturing media contained agar as a solidifying agent, some of the bacterial OTUs may be utilizing this as a carbon source. However, no depres-

sions in the agar surface were observed associated with colonies, which is a common indication of agarolytic properties of bacteria. The OTUs that were also detected on the control medium, that contained no added carbon source, must be able to utilize agar or other trace carbon sources present in the medium or in the inoculum.

Phylogenetic identity of the cultured bacteria

The bacterial OTUs detected in this study belonged to the major bacterial lineages *Alphaproteobacteria*, *Gammaproteobacteria* and *Firmicutes*. A phylogenetic tree illustrating the relation of the OTUs with their closest relatives is displayed in **Fig. 3**. The OTUs A1 to A5 clustered among cultured and uncultured representatives of the Roseobacter clade (Fig 3), a relatively well-studied group within the *Alphaproteobacteria*. Remaining alphaproteobacterial OTUs (A9 to A11), cluster with other cultured strains, many of which contain bacteriochlorophyll and have a photoheterotrophic lifestyle. The gammaproteobacterial OTU G1 has no close cultured relatives, and clusters with a cloned sequence from arctic marine sediment (Fig 3), while G2 is related to *Granulosicoccus antarcticus* isolated from seawater (Lee et al. 2007). One OTU (F1) belonged to *Firmicutes* and clustered with a cultured *Bacillus* strain isolated from the sediment of an estuary.

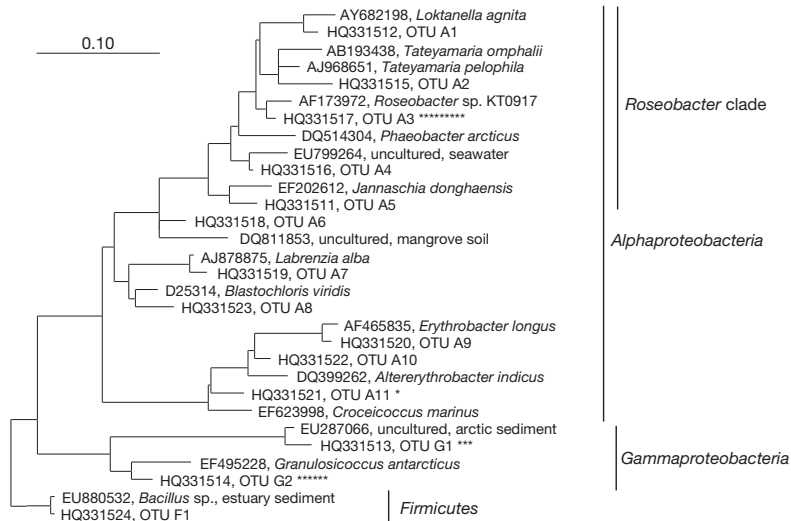


Fig. 3. A maximum likelihood tree of 16S rRNA gene sequences displaying the OTUs that were detected more than once in the enrichment cultivation experiment (in bold), as well as closely related reference sequences (represented with Genbank accession number and name/description). Asterisks indicate cultivation-independent detection of OTUs in natural kelp surface biofilms. The tree was calculated with 600 full length 16S rRNA gene sequences from bacteria and one archaeal outgroup sequence. All but the reference sequences that were the most closely related to the cultivation OTUs have been removed for clarity of presentation. The scale bar indicates 10% sequence divergence

Similarity between cultivated bacteria and bacteria detected on indigenous kelp surface biofilms

The OTU composition of the cultured bacteria differed substantially from that of natural kelp biofilms as revealed by comparison with sequences obtained with the cultivation-independent method DGGE, comprising 119 97% OTUs. For example, 2 of the most frequently detected major bacterial lineages on natural kelp biofilms, *Planctomycetes* and *Verrucomicrobia*, were not detected among the cultured bacteria in this study. However, several of the cultured OTUs (97%) were also detected in kelp surface biofilms (marked with asterisks in Fig. 3). As DGGE has a limited sensitivity, it can only detect relatively abundant members of a community. Therefore, the detection of cultured OTUs by DGGE on kelp surfaces indicates that identical or closely related bacterial species indeed reach high abundances also under natural conditions. The most abundant cultured OTU, A3, was detected by DGGE on kelp surfaces in February, May, September and November (in a total of 9 samples) as well as on the surface of the red alga *Palmaria palmata* in September (in 1 sample) and in seawater in May (in 2 samples). The OTU A11 was detected on kelp surfaces in July (in 51 sample). Both gammaproteobacterial OTUs

were detected on natural kelp surfaces, G1 in February, May and July (in 1 sample in each season) and G2 in February (in 1 sample), March (in 3 samples) and May (in 2 samples).

DISCUSSION

The kelp surface is a habitat rich in potential carbon sources for heterotrophic bacteria. The kelp fixes carbon through photosynthesis. Some of this carbon is exuded from the cells (Abdullah & Fredriksen 2004) and some is stored inside the cells, primarily in the form of mannitol and laminaran. The stored carbon is mobilized when it is used for biosynthesis of new kelp tissue, which to a large extent consists of the cell wall material alginate (Haug & Jensen 1954). The kelp also excretes mucus on its surface, which in part consists of the sulfated polysaccharide fucoidan (Evans et al. 1973). Knowledge of how these carbon sources are utilized by bacteria is essential for understanding the flux of carbon in kelp forest ecosystems. In the present study, enrichment cultivation of bacteria from kelp surfaces using kelp constituent carbon sources was used to identify bacteria able to degrade these kelp-derived substances. The results highlight a few bacterial OTUs

that may be important in these processes, some of which are also detected in high abundances in indigenous kelp biofilms.

It must be pointed out that bacteria belonging to the same OTU at 97% partial 16S rRNA gene sequence similarity are not necessarily derived from the same bacterial species sharing the same phenotypic traits and ecological roles as even strains showing 100% 16S rRNA gene similarity can vary considerably in their phenotypic features (see Hanage et al. 2006 and references therein). However, we consider the clustering of sequences into OTUs a useful tool to study trends in community composition as a first step towards linking community structure to function. More detailed physiological analyses are required to definitively link phylogenetic identity to function and are beyond the scope of the present study.

The majority of the OTUs that were captured by cultivation in the present study belong to the Roseobacter clade of *Alphaproteobacteria* (Fig. 3). Roseobacters are frequently recovered by cultivation-based and cultivation-independent methods in a wide variety of marine habitats and are often dominant community members (Buchan et al. 2005). Several genome sequences of roseobacters already exist and genomic and physiological data describe a bacterial lineage with considerable metabolic versatility (Newton et al. 2010). The most abundant OTU detected in this study, A3, clusters within the Roseobacter clade (Fig. 3) and is closely related to a strain isolated from the North Sea (Eilers et al. 2000). A3 was able to grow on all of the culturing media tested in the present study (Table 1), which suggests that it is a generalist that can utilize many different carbon sources. The detection of A3 on kelp surfaces in different seasons, on the surfaces of a red alga as well as in seawater further supports the notion of a generalist lifestyle. Its ability to reach high abundances—both in culture and occasionally under natural conditions—suggests that A3 is an opportunist, combining a wide substrate affinity with a fast growth rate in order to reach high abundances when the conditions are temporarily suitable. The roseobacterial out, A2, was also detected on all culturing media (Table 1). However, A2 was clearly overrepresented on the medium containing laminaran (Table 1), indicating that it has a special affinity for this carbon source. The few but on average large colonies that were observed on the laminaran medium (Fig. 2) consisted of bacteria of this OTU. A2 is closely related to bacterial strains of the newly described genus *Tateyamaria* (Fig. 3). *T. pelophila*, that was isolated from tidal flat sediments (Sass et al. 2010), like A2 has the ability to utilize laminaran, while *T. omphalii* (Kurahashi & Yokota 2007), isolated from the shell of a mollusc, does not (Sass et al. 2010). Both described strains are otherwise versatile in

their carbon source utilization, as A2 also appears to be. A2 was not detected with DGGE in indigenous kelp surface biofilms, indicating that it is a relatively low abundance community member.

Both of the 2 gammaproteobacterial OTUs, G1 and G2, were also detected in indigenous kelp surface biofilms. G1 may be an alginate degrader on kelp surfaces based on its preference for the culture medium containing alginate (Table 1). G2 is closely related to the bacterial strain *Granulosicoccus antarcticus* isolated from seawater close to Antarctica (Lee et al. 2007). It was detected on kelp surfaces predominantly in March and May, when the kelp surface tissue is growing rapidly, which may indicate that it is one of the early colonizers of newly formed kelp surfaces.

As in virtually all cultivation efforts of environmental bacteria, the bacteria that were captured by cultivation in the present study were not the same as those which dominate kelp surface biofilm communities under natural conditions. The most frequently detected OTUs in kelp surface biofilms belong to *Planctomycetes*, *Alphaproteobacteria* and *Verrucomicrobia* (Bengtsson et al. 2010) and were not detected by cultivation in the present study. The maximum culturability of 0.06% of kelp biofilm cells in this experiment makes the cultured OTUs relatively rare community members on the kelp surfaces that were sampled. However, the detection of some of the same OTUs by cultivation-independent methods in other samples indicates that these organisms (or close relatives) do occasionally reach high abundances also under natural conditions. This confirms that these OTUs are not rare and random guests on the kelp surfaces, but can play an important role when conditions allow them to multiply.

Culture media containing kelp constituent carbon sources represent an environment that is distinctly different from living kelp surfaces in many ways. One factor that is likely to influence kelp surface biofilm communities strongly, but not laboratory cultures, is biotic interactions between different bacteria and, importantly, between the kelp itself and the bacteria. While the kelp is alive and healthy, not all of its carbon sources are available for microbial utilization, due to microbial defense strategies of the kelp. These strategies are not well understood (Goecke et al. 2010), but there are indications that the kelp can select for a non-destructive microbial community on its surface. For example, the kelp *Laminaria digitata* reacts with an oxidative burst, exuding hydrogen peroxide that can kill attached bacteria when it is exposed to oligoguluronates—building blocks of alginate that can be released during bacterial breakdown of kelp cell walls (Küpfer et al. 2001). The composition of kelp surface bacterial communities on living kelp surfaces is there-

fore likely to reflect such defense strategies, and a predominance of bacteria that do not harm the kelp by degrading structural or intracellular kelp constituents such as alginate and laminaran may be expected. Instead, the most abundant kelp surface bacteria may utilize dissolved carbon that is exudated from kelp cells as well as fucoidan or other components of the extracellular mucus found on kelp surfaces. An example of this strategy may be found within the *Planctomycetes* lineage, which often dominates the indigenous communities on kelp surface biofilms (Bengtsson & Øvreås 2010). Some marine *Planctomycetes* are hypothesized to be specialized degraders of sulphated polysaccharides (Woebken et al. 2007), such as fucoidan, which might enable them to utilize kelp-produced carbon without harming the kelp and thereby eliciting microbial defense responses. Culture media containing kelp constituent carbon sources are devoid of kelp microbial defenses and may rather resemble dead kelp material, where all constituents of the kelp cells are available for microbial degradation. Thus, the bacterial OTUs detected with this enrichment cultivation approach, may be more important in degradation of dead kelp material than utilization of the exudates and excretions of living kelp cells. For example, the combination of a wide carbon source affinity with a fast growth rate, such as displayed by the *Roseobacter* OTUs, A2 and A3, would make them successful opportunists, taking advantage of the rich carbon sources present in kelp material that become available upon the death of kelp cells.

CONCLUSIONS

The most abundant bacteria captured by the enrichment cultivation approach using kelp constituent carbon sources belonged to the *Roseobacter* clade of *Alphaproteobacteria*. Other *Alphaproteobacteria* and *Gammaproteobacteria* were also among the cultured bacteria. Several of the cultured OTUs also occasionally reached high abundances in natural kelp surface biofilms at different times of the year. The sporadic nature of their detection on kelp surfaces and their apparent generalist lifestyles suggests that they are opportunists that may be important in degradation of dead kelp material. The present study provides a fresh look on the culturable fraction of bacteria associated with kelp, and links their identity to possible ecological roles in the environment. This bridges the gap between cultivation-based approaches that have been traditionally used to study kelp-associated bacteria and the cultivation independent methods that are the cornerstones of microbial ecology research today.

Acknowledgements. The authors thank Tomas Sørli for assistance during sampling, Knut Helge Jensen for advice on statistics, Thomas Norberg for providing the chemical structures in Fig. 1 and FMC Biopolymer for providing the kelp constituents used in the cultivation experiments as well as funding for the study. Additional funding was provided through a scholarship awarded to M.M.B. from Egil and Sunniva Baardseth's legat. Two anonymous reviewers made constructive and encouraging comments on the manuscript.

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Editorial responsibility: Paul del Giorgio, Montreal, Canada

Submitted: October 4, 2010; Accepted: November 24, 2010
Proofs received from author(s):

Bacterial diversity and production on the surfaces of the kelp

2 *Laminaria hyperborea*

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8

Running title: Bacterial diversity and production on kelp

10

SUMMARY

2 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.

4 Secondary production by heterotrophic bacteria on the surfaces of kelp may be important in the food
web as a link between kelp primary production and kelp forest consumers. The aim of this study was
6 to reveal factors that regulate the bacterial diversity on kelp surfaces and to investigate its impact on
bacterial secondary production. Kelp, *Laminaria hyperborea*, from southwestern Norway was
8 sampled during an annual cycle to determine the influence of seasonal factors, in particular kelp
surface age, on bacterial diversity. The relationship between bacterial diversity and bacterial
10 production was investigated on kelps collected at sites differing in wave exposure. Pyrosequencing
(454-sequencing) of amplicons of the 16S rRNA gene of bacteria was used to study bacterial
12 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.

14 There is a core community of a few bacterial OTUs (operational taxonomical units), mainly
belonging to *Planctomycetes*, that is present across different seasons and environmental conditions.

16 Higher bacterial OTU evenness is linked to higher bacterial production, confirming that bacterial
diversity impacts ecosystem function. Due to the dominance of a few abundant OTUs, kelp surface
18 biofilm communities may be characterized as low diversity habitats.

INTRODUCTION

2 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
4 in upwelling zones in the tropics (Graham et al., 2007) and in the arctic (e.g. (Dunton et al., 1982)).
Kelp forest ecosystems are characterized by extraordinary biodiversity and productivity (Mann,
6 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
8 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
10 biodiversity on ecosystem function is a central topic in kelp forest ecology (reviewed by (Steneck et
al., 2002)). However, while the macrofauna and -flora of kelp forests have been relatively well
12 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
14 community dynamics and phylogenetic identity of the most abundant bacteria on the surfaces of a
few kelp species (Staufenberger et al., 2008; Bengtsson and Øvreås, 2010; Bengtsson et al., 2010).
16 However, the bacterial diversity on kelp surfaces has most likely been underestimated, since many
natural bacterial communities appear to possess an enormous diversity in terms of the number of
18 different bacterial taxa equivalent to species that are present (Torsvik et al., 1990; Curtis et al., 2002;
Torsvik et al., 2002; Venter et al., 2004; Sogin et al., 2006). Ambitious sequencing strategies may be
20 required to adequately describe this diversity (Curtis, 2006; Quince et al., 2008).

Bacteria play a crucial role in kelp forest ecosystems, and therefore a deeper understanding of
22 bacterial diversity and its regulation is needed. Through full and partial degradation of dissolved-
(DOC) and particulate- (POC) organic carbon, bacteria make kelp primary production available to
24 animal consumers (Newell and Field, 1983; Norderhaug et al., 2003). DOC is continuously exudated

from living kelp cells (Abdullah and Fredriksen, 2004) and is also released upon fragmentation of
2 kelp material (Newell et al., 1980). Kelp DOC can support secondary production by bacteria living
on the surfaces of kelp and in the surrounding water masses. POC from kelp comprises everything
4 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
6 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.,
8 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 plants
10 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
12 production in these biofilms. Possible influencing factors may include temperature, defense
strategies of the kelp that inhibit bacterial growth, but also the composition and diversity of the
14 bacterial community itself.

Laminaria hyperborea (Fig. 1) is the dominant kelp forest forming species along the Atlantic coasts
16 of Europe. An important factor controlling its local distribution and growth is wave exposure (Sjötun
and Fredriksen, 1995; Sjötun et al., 1998; Bekkby et al., 2009). *L. hyperborea* has a pronounced
18 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,
20 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, and a corresponding successional gradient of its bacterial
22 communities. Seasonal factors have been shown to influence the composition of bacterial
communities inhabiting the *L. hyperborea* lamina surface (Bengtsson et al., 2010). However, only
24 the few most abundant bacterial phylotypes could be observed and identified. Therefore, it was not
determined how seasonal factors influence the diversity of the bacterial communities.

In this study, we aim to uncover seasonal and environmental factors controlling the diversity of
2 bacteria on kelp surfaces. In addition, we investigate if bacterial diversity influences bacterial
secondary production, which is highly relevant to the function of kelp forest ecosystems. We used
4 pyrosequencing of amplicons of the 16S rRNA gene of bacteria to study the diversity of kelp surface
bacterial communities, measured as richness and evenness of bacterial operational taxonomical units
6 (OTUs). Bacterial OTU richness and evenness were compared between kelp surface biofilm samples
collected during a seasonal cycle (March to November) to evaluate the importance of seasonal
8 factors, in particular kelp surface age, on bacterial diversity. To explore the relationship between
bacterial diversity and bacterial production, incorporation of tritiated thymidine was measured on
10 biofilm samples collected in summer (July) from three different sampling sites differing in wave
exposure.

12

RESULTS

2 Seasonal variation in bacterial diversity

The bacterial communities that were sampled during the seasonal cycle revealed a strong
4 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 ($R^2 = 0.53$, $p = 0.04$) was observed with
6 increasing kelp surface age (Fig. 2 a-c, Table 1). These results were identical using both 97% and
99% OTU clustering. In March, the kelp is growing at the meristem region and new kelp surface is
8 being formed rapidly (surface age ≤ 1 month). The bacterial community of the meristem in March
was characterized by low OTU richness and evenness (Fig. 2a-c). The very low evenness (Fig. 2c) is
10 due to the dominance of one 97% OTU (two 99% OTUs) belonging to the *Gammaproteobacteria*
(OTU46) that makes up nearly 84 % of the sequences in the March meristem sample (Table 3). The
12 meristem is still growing in May (surface age ≤ 2 months), though at a slower pace (Kain, 1979;
Sjötun et al., 1996), and the richness was still low (Fig. 2b), but the dominance of a few OTUs was
14 replaced by a more even community (Fig. 2c). As the kelp surface ages from May through to
November, the OTU richness continued to increase (Fig. 2b), while the evenness was relatively
16 unchanged (Fig. 2c). The highest richness was observed on the lamina in March (Fig. 2a-b), which is
approximately one year older (12 months) than the fresh meristem surface in March. The contrasting
18 richness and evenness on the coexisting meristem and lamina in March (Fig. 2a-c) shows that kelp
surface age is more important than other seasonal factors (e.g. temperature) in regulating bacterial
20 diversity on kelp surfaces.

Bacterial production and bacterial diversity

22 There was a relationship between bacterial production on kelp surfaces and the evenness of the
bacterial communities in our experiments (Fig. 3, Table 2). A significant ($R^2 = 0.53$, $p = 0.001$)

1 increase in bacterial production with increased OTU evenness of the bacterial communities (Fig. 3c)
2 was observed. No clear relationship between bacterial production and OTU richness was observed
($R^2 = 0.11$, $p = 0.12$, Fig. 3b). Again, the correlation results were identical between 97% and 99%
4 OTU clustering. 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
6 production are not the same in the different samples. However, the abundance of the single most
dominant 97% OTU 1322 (*Planctomycetes*) shows a weak negative correlation with bacterial
8 production ($R^2 = 0.33$, $p = 0.01$, results not shown).

Biofilm cell density did not appear to correlate with bacterial OTU richness ($R^2 = -0.03$, $p = 0.44$) or
10 evenness ($R^2 = -0.06$, $p = 0.64$, results not shown) in the production experiment. However, there was
a clear difference between the cell densities recorded on kelp surfaces from the three sampling sites
12 (Figure 4b). 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 while the most sheltered site,
14 Flatevossen, had the highest cell density (Fig. 4b).

The bacterial communities on kelp surfaces

16 The bacterial communities on kelp surfaces were characterized by a few OTUs that were frequently
detected in samples across different seasons and environmental conditions and that were also
18 abundant overall in the dataset. These OTUs can be regarded as a "core community" that is inherent
to kelp surfaces (Fig. 5a, loosely defined as OTUs detected in >85% of samples and that make up
20 >1% of the entire dataset). This core community includes four OTUs belonging to *Planctomycetes*.
One of them, OTU 1322, makes up nearly 25 % of the sequences in the entire dataset (Table 3, Fig.
22 5a). Other members of the putative core community include the gammaproteobacterial OTU 46 and
the alphaproteobacterial OTU 90. The relative abundances of the six OTUs that have been defined as
24 the putative core community are highly variable between samples (Table 3). *Bacteroidetes* and

Alphaproteobacteria displayed a higher OTU richness compared to the *Planctomycetes* and *Gammaproteobacteria*, although the latter lineages were more abundant in the dataset (Fig. 5b). 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 dataset) made up 31% of the entire dataset. Of these, 26% belonged to *Alphaproteobacteria*, 18% to *Bacteroidetes*, 17% to *Gammaproteobacteria* and 16% to *Planctomycetes* (97% OTU clustering). *Archaea* were also detected on kelp surfaces at low abundances, with 0.015% of all sequences classifying as *Archaea*.

The entire dataset consisted of 228517 sequence reads with an average sequence length of 317 bp. The total OTU richness was 1108 OTUs at the 97% OTU definition and 1819 OTUs at 99% OTU definition. Parametric and non-parametric diversity estimation indicated that the bacterial communities were not exhaustively sampled (Tables 1 and 2). Of the different taxa-abundance distributions tested in the parametric diversity estimation (Quince et al., 2008), inverse gaussian gave the best fit for most samples and estimations based on this model are given in Tables 1 and 2. The sequencing effort required for capturing 90% of all OTUs ranged between 2.4 to 82 times the achieved sequencing depths (Tables 1 and 2).

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.

20

DISCUSSION

2 Regulation of bacterial diversity on kelp surfaces

Kelp surfaces provide a habitat rich in potential carbon sources for heterotrophic bacteria. Kelp is also a living surface. This central fact has consequences for kelp surface biofilms in many ways, including the regulation of the density, community composition and diversity of bacteria. For example, kelp must defend itself against pathogenic bacteria and excessive fouling of macroscopic organisms that may follow bacterial colonization. Active antifouling strategies are probably the cause of the relatively low density of biofilm cells typically observed on kelp surfaces (Fig. 1, Fig. 4b, (Corre and Prieur, 1990; Bengtsson et al., 2010). These defense mechanisms are not completely understood in kelps, yet they include production of reactive oxygen species (Küpper et al., 2001; Küpper et al., 2002) and oxidized halogens that interfere with biofilm cell signaling (Borchardt et al., 2001). The lower cell density that was observed in the more wave-exposed sites (Fig. 4b) may suggest a higher activity of antimicrobial defense mechanisms of the kelp at these sites. *L. hyperborea* individuals growing under high wave exposure are typically more productive (Sjötun and Fredriksen, 1995; Sjötun et al., 1998), which may imply that they have more resources to spend on defense.

The renewal of the lamina each year is likely also a strategy that ensures that fouling is kept at a minimum on surfaces that are needed for photosynthesis. Freshly formed kelp surfaces are immediately colonized by bacteria, as is evident from the bacterial community already present on the rapidly growing meristem in March (Tables 1 and 3). The gammaproteobacterial OTU 46 appears to be one of the pioneer colonizers of new kelp surfaces (Table 3). Its dominance on the meristem in March suggests a strategy of rapid attachment and growth at the early stages of biofilm formation. However, already in May the dominance of OTU 46 has been replaced by a more even community characterized by a high proportion of planctomycetes (Table 3). A gradual diversification of the

bacterial community follows as the kelp surface ages (Fig. 2). This successional increase in bacterial diversity could be a result of more niches for bacteria becoming available over time, for example as a result of the accumulation of small injuries on the kelp surface caused by mechanical forces or grazing (see Fig. 1b-c). Such damage to the kelp could provide a more structurally varied habitat and access to different carbon sources leaching out of lysed kelp cells.

6 **Relationship between bacterial diversity and bacterial production**

³H-Thymidine incorporation by bacterial cells during DNA synthesis is a measure of overall community production, which may include high production of some community members and low or nonexistent production of others. To explain the intriguing result that higher OTU evenness is linked to higher bacterial production, or conversely low evenness to low bacterial production (Fig 3c), 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. The observed negative correlation between bacterial production and the relative abundance of the dominant planctomycetes OTU 1322 is therefore not surprising and may suggest that bacterial cells belonging to this OTU were not growing much during the experiment. However, the correlation between bacterial production and OTU 1322 abundance ($R^2 = 0.33$, $p = 0.01$) is weaker than between bacterial production and evenness ($R^2 = 0.53$, $p = 0.001$), suggesting that other mechanisms are probably also contributing to the effect. In ecological studies, higher ecosystem production with higher species diversity is often attributed to niche complementarity of different species, resulting in more efficient overall resource utilization (e.g. Hooper (Hooper et al., 2005; Thebault and Loreau, 2006)). Assuming that kelp biomass and exudates comprise the resource that sustains heterotrophic bacterial communities on kelp surfaces, many different types of bacteria utilizing separate carbon sources could thus account for higher overall community production.

While resource use (niche) complementarity is a mechanism that could explain a positive correlation
2 between bacterial production and diversity, the low production estimates recorded on some samples
(Table 2) suggest that other explanations may be more likely. The lowest ³H-thymidine
4 incorporation rates measured in our experiments correspond to average cell division times of thousands
of days (see Table 2), suggesting that most of the bacteria in these samples were not growing during
6 the incubations. Such extremely low bacterial production on kelp surfaces that harbor up to 1.5E07
bacterial cells/cm² appear unrealistic, and may indicate that the experimental conditions caused a
8 shift of the bacterial communities, causing the abundant bacteria (for example the OTU 1322, Table
3) to stop growing. The use of small, excised kelp pieces in the production experiment may have
10 caused this shift as they probably offer a different environment for bacteria compared to the surfaces
of whole living kelp individuals in several ways. Upon detachment, exudation of potential carbon
12 sources from the kelp cells may be altered and antimicrobial defense may cease, for example. Any of
these changes may favor or disfavor different bacterial populations causing increases or decreases in
14 their growth rates as a result. However, even if our experimental conditions probably differ from the
natural conditions of living kelp surfaces, they may not represent a completely unnatural situation.
16 Kelp pieces, and whole kelp thalli, also get detached in the kelp forest, and the bacterial
communities on their surfaces likely undergo shifts as a response to the change in environment also
18 in these cases. What we are witnessing in our experiments may therefore be the early steps of the
successional process starting from a bacterial community adapted to life on living kelp surfaces
20 leading to a bacterial community adapted to the degradation of detached kelp material. The rate of
bacterial production in this early successional stage could then be seen as an indication of how
22 rapidly the community responds to the change in the environment. A more even community, co-
dominated by several different types of bacteria, may be more likely to respond rapidly, because the
24 initial abundances of potential opportunist bacteria are higher. This may be a plausible explanation
for the observed relationship between OTU evenness and bacterial production in this study. The

ability of diverse bacterial communities to respond rapidly to changing conditions is a general
2 mechanism by which bacterial diversity can influence the productivity of a bacterial community, and
may also apply for other bacterial communities undergoing environmental change.

4 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
6 measured bacterial production. Though such artifacts cannot be ruled out entirely, there was no
indication that the relative abundance of any specific group of bacteria (for example *Planctomycetes*)
8 correlated with bacterial production. It should also be pointed out that because bacterial production
measurements and bacterial diversity analyses were carried out on parallel samples, corresponding to
10 kelp pieces cut out directly adjacent to each other, they might represent bacterial communities that
differ to some extent due to the patchy nature of biofilms. This may cause noise in the data, making
12 it difficult to detect biological trends, but should not introduce systematic artifacts.

The core community of kelp surface biofilms

14 A few OTUs were almost always detected (frequent) and were also some of the most abundant in the
dataset. They can be regarded as a "core community" of kelp surface biofilms (Fig. 5a). Their
16 presence under varying seasonal and environmental conditions suggests that they represent bacterial
species that exploit niches that are always available on kelp surfaces. They may also be well adapted
18 to kelp antimicrobial defense mechanisms, giving them a competitive advantage over other bacterial
species. It is possible that they are even indirectly selected by the kelp by not triggering specific
20 defense mechanisms, such as the oxidative burst presumably elicited by alginate-degrading bacteria
in the closely related kelp *Laminaria digitata* (Küpper et al., 2001).

22 Four of the most abundant OTUs on *L. hyperborea* detected in this study belong to *Planctomycetes*
(Fig. 5a, Table 3). This is consistent with the recent finding that planctomycetes are numerically
24 dominant on kelp surfaces (Bengtsson and Øvreås, 2010). They are all related to planctomycetes of

the *Rhodopirellula* and *Blastopirellula* genera ("RB1" lineage in (Bengtsson and Øvreås, 2010)).

2 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
4 (Woebken et al., 2007). Such substances are produced by kelps (Evans et al., 1973), and may
possibly be utilized by kelp surface planctomycetes. One gammaproteobacterial OTU, (OTU 46, the
6 pioneer colonizer of fresh kelp surfaces in March), is also part of the core community (Fig. 5a, Table
3). It classifies as a member of the genus *Granulosicoccus* (Lee et al., 2007). Related sequences have
8 been detected on the red seaweed *Delisea pulchra*, the green seaweed *Ulva australis* (Longford et
al., 2007), the brown seaweed *Fucus vesiculosus* (Lachnit et al., 2010) and on the kelp (brown
10 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
12 (maximum 7.65%), yet frequently detected on *L. hyperborea* (Fig. 5a, Table 3). It is closely related
to the roseobacter *Tateyamaria pelophila* (Sass et al., 2010), an isolate from tidal flat sediment that
14 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*
16 surfaces (Bengtsson et al., *in press*). Thus, the OTU 90 possibly represents a bacterial species of the
genus *Tateyamaria* that utilizes laminaran as a carbon source on kelp surfaces.

18 A stable sub-population of bacteria was also observed on the surface of the green seaweed *Ulva*
australis (Tujula et al., 2010), consisting of members of the *Alphaproteobacteria* and *Bacteroidetes*.
20 However, a following study of the same seaweed concluded that the relative abundances of the
OTUs that were always detected were too variable to be considered a core community (Burke et al.,
22 2010). The variation of the relative abundances of the core OTUs was substantial also in our dataset
(Table 3). This variation may reflect unknown environmental or host-related gradients that affect the
24 biofilms, selecting for different bacteria. However, it may also suggest an element of stochasticity in
the colonization of kelp surfaces, as suggested for *U. australis* (Burke et al., 2010). In such a case,

several functionally equivalent bacterial species may be recruited from adjacent bacterial
2 populations (for example seawater), when fresh kelp tissue is formed. Their relative abundances
would be determined by stochastic events during colonization, rather than actual ecological
4 advantages determined by their phenotypes. The four planctomycete core OTUs (Fig. 5, Table 3)
may represent such ecologically equivalent organisms. This model of community assembly is
6 assumed in the neutral theory (Hubbell, 2001), which has successfully predicted species distribution
curves for communities of macroscopic organisms (e.g. (Hubbell, 2005)) as well as microbial
8 communities (Sloan et al., 2006).

A random recruitment of functionally equivalent bacterial species does not exclude symbiotic roles
10 of bacterial communities on kelp surfaces. This functional redundancy leads to a genetic diversity
that may have implications in situations of intensive selection pressure, for example when the kelp
12 host is exposed to stress or disease. Mounting evidence suggests that bacterial communities are
important components of adaptation and fitness of many host-microbial consortia, or "holobionts"
14 (Zilber-Rosenberg and Rosenberg, 2008; Rosenberg et al., 2010). In coral reefs, the resistance to
disease of the coral animal appears to be connected with the composition of the coral-associated
16 bacterial communities (e.g. (Reshef et al., 2006; Garren et al., 2009)). Kelps are also susceptible to
disease, for example brought on by pathogenic bacteria (Sawabe et al., 2000; Vairappan et al., 2001)
18 and endophytic algae (Ellertsdottir and Peters, 1997). Their surface bacterial communities could play
a role in resistance to such diseases, for example by inhibiting settlement of pathogenic organisms
20 (Rao et al., 2007). At this point, relatively little is known about the symbiotic roles of bacterial
communities of kelps and other seaweeds. Detailed investigations comparing communities of hosts
22 in different states, for example diseased and healthy, could shed light on the symbiotic roles of
bacterial communities on kelps and other seaweeds, and their possible impacts on ecosystems such
24 as kelp forests.

How diverse are kelp surface biofilms?

2 The pyrosequencing approach employed in this study and others allows a much more detailed view
on the diversity of microbial communities compared to traditional fingerprinting and cloning
4 methods. An important consideration to make when designing experiments and analyzing
sequencing data is whether the sequencing depth adequately captures the diversity of the community
6 of study (Quince et al., 2008). Although the bacterial communities were not exhaustively sampled in
this study, the moderate estimated sequencing efforts required to capture 90% of the OTUs in each
8 sample (Tables 1 and 2) suggest that a near complete analysis of kelp biofilm bacterial communities
is within reach using current technology.

10 The total number of 97% OTUs detected in this study, 1108 in 228517 sequence reads from samples
spanning wide seasonal and environmental gradients, appears modest in comparison to other recent
12 studies using pyrosequencing of 16S rRNA gene amplicons in marine environments (Sogin et al.,
2006; Huber et al., 2007; Kirchman et al., 2010). However, comparisons of the observed bacterial
14 richness numbers between separate studies may be inappropriate due to differing sampling and
sample processing strategies. Importantly, quality filtering and noise removal of sequence reads can
16 have large impacts on observed richness (Quince et al., 2009; Kunin et al., 2010). The rigorous
filtering approach employed in this study yields lower, but more accurate richness values. This may
18 make comparisons with most existing studies difficult, and conclusions about whether kelp surface
biofilms harbor relatively high or low bacterial richness are therefore difficult to draw. However, by
20 considering the other component of diversity, evenness, it may be possible to make more general
statements. The dominance of one or a few OTUs (e.g. 1322 and 46) that causes the low evenness
22 observed in many samples (Figures 2c and 3c) suggests that kelp surface biofilms may indeed be
characterized as low bacterial diversity habitats in some instances.

While the abundant organisms in the core community (Fig. 5a) 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 (Fig. 5b). 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; Pedros-Alio, 2007). On kelp surfaces, substantial environmental change takes place when the kelp is fragmented or detached, a continuous and natural process in kelp forests. Detached kelp is rich in organic substrates and active antimicrobial defense mechanisms may be reduced. Rare inhabitants of the biofilm communities could take advantage of the new conditions upon fragmentation, and outcompete the initially more abundant core community of living kelp surfaces.

Conclusions

Kelp surface biofilms feature a core community of a few abundant OTUs, which belong primarily to *Planctomycetes*. Beyond these core OTUs, kelp surfaces harbor considerable bacterial diversity that increases as the kelp surface ages. 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. This degradation may be carried out by substantially different bacterial communities than those found on living kelp surfaces, possibly recruited from rare bacteria in the biofilms.

EXPERIMENTAL PROCEDURES

2 **Sampling**

For bacterial diversity analyses during a seasonal cycle, kelp was collected at one sampling site
4 (Tekslo, 60° 09.706' N, 5° 02.371' E) in March - November 2007. In addition, kelp was collected
for bacterial production measurements and diversity analyses in July 2009 at three different
6 sampling sites differing in wave exposure. The sites were; Tekslo ("moderately exposed"), Landro
(60° 25.338' N, 4° 57.472' E, , "very sheltered") and Flatevossen (60° 16.119' N, 5° 12.432' E,
8 , "extremely sheltered") (see Fig. 4a). Wave exposure was determined according to a GIS-based
wave exposure model (Isaeus, 2004).

10 The kelp sampling during a seasonal cycle was performed as described in detail in (Bengtsson et al.,
2010). In summary, kelp was collected by dredging from a boat at two-monthly intervals during the
12 year 2007 (see Table 4). One sampling occasion (February 2007) was excluded from this study
because the sampling procedures differed slightly compared to the other occasions (macroscopic
14 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
16 and two distinct regions (meristem and lamina) of each kelp individual were sampled. Biofilm was
removed from the kelp surfaces by means of scraping with a sterile scalpel. The scraped off biofilm
18 material was used for DNA extraction and subsequent diversity analyses as described in the
following sections.

20 The kelp sampled in July 2009 was collected by dredging (Tekslo) and free diving (Landro and
Flatevossen) at a depth of 3 - 6 metres. Four (Tekslo) or six (Landro and Flatevossen) replicate kelp
22 individuals were collected from each site. The sampled kelp from July 2009 was used for bacterial

production measurements, DNA extraction and subsequent diversity analyses as described in the following sections. An overview of the different samples collected is given in Table 4.

Bacterial production measurements

To account for any heterogeneity in the biofilm density and diversity within each individual, three separate but morphologically equivalent areas of the kelp laminae were sampled. Pieces (4 x 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 x 1 cm pieces. Prior to the bacterial production measurements, the kelp pieces were pre-incubated in sterile seawater at 10°C for 24 hours under constant agitation in order for the kelp tissue to acclimatize as kelp may produce substances that inhibit bacterial growth upon stress (Küpper et al., 2001), which may be experienced when the tissue is cut.

Bacterial production was measured as incorporation of ^3H -labelled thymidine (Fuhrman and Azam, 1980) in bacterial cells attached to kelp surfaces during growth. Three 1 x 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]- ^3H -Thymidine (Specific activity 48 Ci/mmol, PerkinElmer) for 1 hour at 10°C under constant agitation (200 RPM). The temperature was near the ambient seawater temperature at the time of sampling. Different ^3H -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 (TCA) and pelleted by centrifugation. The pellet was washed twice in ice-cold TCA 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 ^3H -Thymidine was
2 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
4 control incubation where formaldehyde was added prior to ^3H -Thymidine addition. Triplicate
incubations of each kelp individual were carried out. Two sets of parallel incubations without the
6 addition of ^3H -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
8 off and processed as described previously (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
10 DAPI and used for biofilm cell counting as described in (Bengtsson et al., 2010). Average cell
division times were calculated from ^3H -Thymidine incorporation (DPM) and cell density data
12 according to (Fuhrman and Azam, 1982) using a conversion factor of 2×10^{18} cells dividing per mol of
Thymidine incorporated (Pollard and Moriarty, 1984).

14 **Bacterial diversity analyses**

16S rRNA gene amplicon generation and 454-sequencing: DNA extraction of the biofilm
16 material from all samples (see Table 4) was carried out as described previously (Bengtsson et al.,
2010). For the seasonal cycle samples, the extracted DNA from the six replicate kelp individuals was
18 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
20 each seasonal sampling occasion, not accounting for heterogeneity between kelp individuals, which
was previously addressed (Bengtsson et al., 2010). The extracted DNA from the production
22 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
2 (5'-ATTAGATACCCNGGTAG-3') and 1492r (5'-GNTACCTTGTTACGACTT-3')(Roesch et al.,
3 2007) at a concentration of 1µM each in HotStarTaq mastermix (Qiagen). The DNA template
4 concentration was adjusted to achieve equal concentrations of the final PCR product. The PCR
5 conditions were; 15 min 95°C hot start followed by 25 cycles of 45 sec 95°C, 45 sec 50°C annealing,
6 60 sec 72°C extension and a final extension of 72°C for 7 min. Triplicate PCR reactions were
7 pooled. The PCR amplicons were then used as template for a second 5-cycle PCR using the same
8 conditions above. The primers for the second PCR were modified as follows. Unique multiplex
9 identification tags (error corrected MIDs or "barcodes") sequences for each sample was added to the
10 forward primer (787f) in addition to a GS FLX "A" adaptor sequence. The reverse primer (1492r)
11 was modified with a GS FLX "B" adaptor sequence. The resulting amplicons were pooled in
12 equimolar amounts and kept at -80°C until pyrosequencing, which was carried out at The Norwegian
13 High-Throughput Sequencing Centre using Lib-L chemistry and GS-FLX Titanium technology (454
14 Life Sciences, Roche).

Sequence analysis: Quality filtering and noise removal of pyrosequencing reads of 16S rRNA gene
15 amplicons was carried out using AmpliconNoise (version 1.1, Quince et al. *in press*), which corrects
16 and compensates for PCR- and pyrosequencing-generated errors, thereby preventing an
17 overestimation of bacterial diversity. The sequences were clustered into OTUs using complete
18 linkage clustering as described in Quince et al. (*in press*). OTU definitions of 97% similarity and
19 99% similarity were used in the following statistical analyses. All OTU numbers referred to in the
20 text and figures are from the 97% clustering. The sequences were classified to Phylum-Class level
21 using RDP classifier (Wang et al., 2007). Based on this classification, sequences of chloroplast
22 origin were identified and subsequently removed from the dataset.

Statistical analyses: Statistical analyses were carried out using the R software (version 2.10.1, R Development Core Team, 2010) including functions from the vegan package (version 1.5-4, (Oksanen et al., 2010)). 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 datasets were rarefied at the number of sequences of the sample with the least sequencing depth (indicated by a dotted line in Figures 2a and 3a) in the respective datasets. One sample in the production experiment dataset was excluded from comparative analyses because of poor sequencing depth (see Table 4). In addition to rarefied richness, non-parametric (Chao, 1987) and parametric (Quince et al., 2008) richness estimates were calculated (Tables 1 and 2). 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 / cm², were normalized to biofilm density and were log transformed.

16

ACKNOWLEDGEMENTS

18 The authors thank Tomas Sørli for superb assistance during sampling, Professor Janet Jansson for valuable discussions, Morten Skage and others at The Norwegian High-Throughput Sequencing Facility for excellent pyrosequencing service and advice and Kjell-Magnus Norderhaug and Trine Bekkby for providing the wave exposure map. This study was funded by a scholarship awarded to M.M.B. by Egil and Sunniva Baardseths legat and by a donation from FMC Biopolymer.

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Table 1: A summary of the results of the seasonal experiment.

Parametric OTU richness estimates and estimated sequencing depth required to capture 90% of OTUs are given with 95% confidence intervals representing minimum, median (**bold**), and maximum values. OTU evenness refers to Pielou's evenness index.

Sample id	Avg. cells/cm ²	Sequencing depth	Observed 97% OTUs	Chao1 97% OTU richness	Parametric 97% OTU richness	Sequencing depth for 90% of OTUs	97% OTU evenness
MarM	1.25E4	8195	133	166	160 210 359	43000 99600 390000	0.22
MarL	4.33E6	10024	284	427	371 467 677	47800 96300 251000	0.62
MayM	3.27E6	14634	152	251	315 614 1945	242000 1200000 13800000	0.53
MayL	2.73E6	9434	160	250	237 380 1088	83900 290000 3110000	0.57
JulL	8.76E6	9969	204	324	306 462 1070	80400 234000 1590000	0.58
SepL	2.43E6	10288	179	251	213 277 436	48900 96300 312000	0.53
NovL	1.20E7	7952	242	369	319 444 809	48900 125000 539000	0.61

Table 2: A summary of the production experiment results.

Parametric OTU richness estimates and estimated sequencing depth required to capture 90% of OTUs are given with 95% confidence intervals representing minimum, median (bold), and maximum values. OTU evenness refers to Pielou's evenness index. The "*" indicates that the sample was not included in correlation analyses (Fig. 3b and c) because of poor sequencing depth.

Sample id	Avg. cells/cm ²	³ H-Thymidine uptake (DPM/cm ²)	Avg. cell division time (days)	Sequencing depth	Observed 97% OTUs	Chao1 97% OTU richness	Parametric 97% OTU richness	Sequencing depth for 90% of OTUs	97% OTU evenness
T1	1.06E6	349.3	11.29	1379*	65	82	64 75 105	6600 14100 43300	0.62
T2	4.0E6	872.4	17.00	4354	92	105	88 99 125	8780 15100 37000	0.62
T3	3.37E6	276.8	45.03	3909	102	126	88 95 108	9000 9470 17600	0.61
T4	5.49E6	2838.7	7.17	7190	90	107	91 106 137	12600 23500 56300	0.61
F1	1.00E7	7398.3	5.03	10453	166	217	154 175 214	15200 26300 52900	0.70
F2	1.52E7	3303.8	17.06	8024	146	251	188 285 676	42400 138000 1040000	0.53
F3	1.25E7	2955.9	15.67	12354	137	235	148 199 397	40300 101000 621000	0.62
F4	1.40E7	594.0	87.58	12316	128	185	122 150 230	27100 55500 191000	0.55
F5	1.19E7	232.5	189.64	15636	264	305	259	23400	0.68

							285 326	35000 57200	
F6	1.36E7	478.0	105.4	17435	144	191	161 206 330	55000 121000 191000	0.53
L1	7.51E6	519.7	53.54	4917	179	238	188 218 274	30800 53200 109000	0.55
L2	8.73E6	179.2	180.43	9791	65	128	97 224 1042	85000 671000 17400000	0.31
L3	7.12E6	225.0	117.19	11242	140	183	157 211 371	44500 109000 438000	0.47
L4	9.05E6	13.1	2568.2	10748	117	131	133 174 279	35500 84800 290000	0.45
L5	5.31E6	8.4	2341.35	15658	76	86	76 95 145	30400 68700 220000	0.35
L6	7.64E6	7.8	3621.22	12615	48	83	51 85 857	32600 160000 22500000	0.34

Table 3: The core community.

The table shows the relative abundances (in %) of the most abundant and frequently detected OTUs, the putative core community of *L. hyperborea* biofilms (Fig. 5a).

OTU classification	Planctomycetes <i>Rhodopirellula/Blastopirellula</i> spp. ("RB1 lineage")				Gammaproteobacteria <i>Granulosicoccus</i> sp.	Alphaproteobacteria <i>Tateyamaria</i> sp.
	1322	132	1345	116	46	90
MarM	0.89	0.45	0.18	0	83.65	0
MarL	2.56	0.90	6.52	0.67	18.88	0.01
MayM	4.99	26.14	2.10	0.35	0.77	0.03
MayL	5.78	11.95	7.29	1.13	2.19	0.03
JulyL	7.35	13.53	9.73	0.70	12.83	0.02
SepL	6.21	2.21	6.50	6.00	9.16	0.03
NovL	0	4.28	0.40	2.70	10.71	0.04
T1	34.47	2.32	16.80	1.01	0.29	0
T2	34.67	5.71	2.82	1.17	0.30	4.36
T3	11.54	0.59	3.98	0.10	0.08	0.38
T4	28.31	1.49	1.19	2.21	0.04	3.10
F1	13.16	7.92	3.60	15.28	0.08	2.12
F2	38.49	4.77	3.95	0.91	0.12	1.18
F3	15.19	5.18	2.42	3.39	0	3.12
F4	35.96	5.07	3.52	5.47	0.06	4.44
F5	11.96	1.80	0.57	2.64	0.01	7.65
F6	34.41	7.05	2.83	22.89	0.03	1.05
L1	36.29	9.26	2.38	0.08	0.26	0.67
L2	64.81	20.83	5.78	3.74	0.02	0.74
L3	48.59	13.05	2.85	1.16	0.37	6.90
L4	47.95	9.98	1.91	0.41	0.02	3.14
L5	54.31	24.58	11.44	4.90	0.15	0.02
L6	54.16	33.29	5.64	3.74	0	0

Table 4: An overview of the kelp surface biofilm samples.

The table summarizes the properties of the different samples analyzed in the seasonal and production experiments. M = meristem part of kelp, L = lamina part of kelp.

Sample id	Experiment	Site	Month	Year	Kelp part	Surface age	Area sampled	No. kelp ind.
MarM	Seasonal	Tekslo	Mar.	2007	M	1	300	6
MarL	Seasonal	Tekslo	Mar.	2007	L	12	300	6
MayM	Seasonal	Tekslo	May	2007	M	2	300	6
MayL	Seasonal	Tekslo	May	2007	L	3	300	6
JulyL	Seasonal	Tekslo	Jul.	2007	L	5	300	6
SepL	Seasonal	Tekslo	Sep.	2007	L	7	300	6
NovL	Seasonal	Tekslo	Nov.	2007	L	9	300	6
T1	Production	Tekslo	Jul.	2009	L	5	6	1
T2	Production	Tekslo	Jul.	2009	L	5	6	1
T3	Production	Tekslo	Jul.	2009	L	5	6	1
T4	Production	Tekslo	Jul.	2009	L	5	6	1
F1	Production	Flatevossen	Jul.	2009	L	5	6	1
F2	Production	Flatevossen	Jul.	2009	L	5	6	1
F3	Production	Flatevossen	Jul.	2009	L	5	6	1
F4	Production	Flatevossen	Jul.	2009	L	5	6	1
F5	Production	Flatevossen	Jul.	2009	L	5	6	1
F6	Production	Flatevossen	Jul.	2009	L	5	6	1
L1	Production	Landro	Jul.	2009	L	5	6	1
L2	Production	Landro	Jul.	2009	L	5	6	1
L3	Production	Landro	Jul.	2009	L	5	6	1
L4	Production	Landro	Jul.	2009	L	5	6	1
L5	Production	Landro	Jul.	2009	L	5	6	1
L6	Production	Landro	Jul.	2009	L	5	6	1

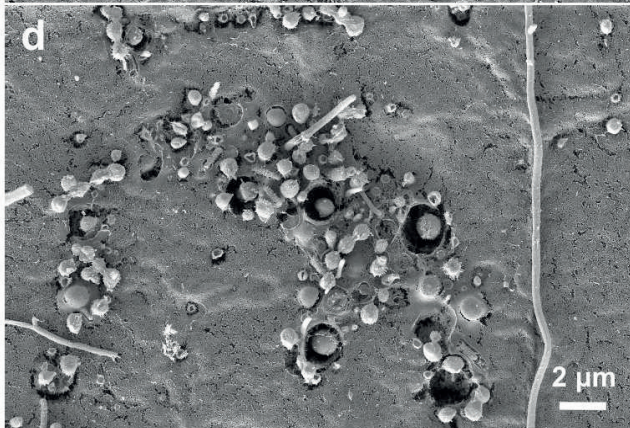
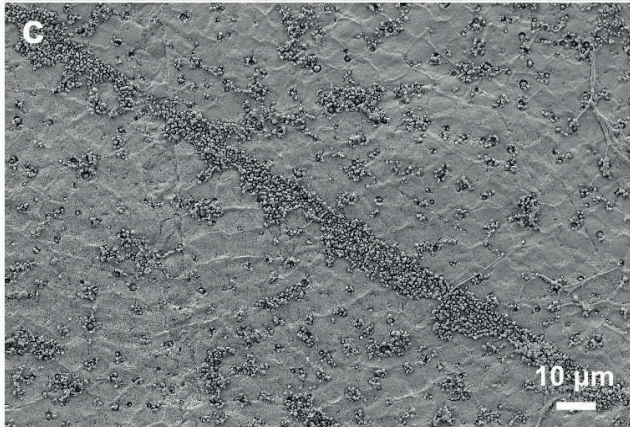
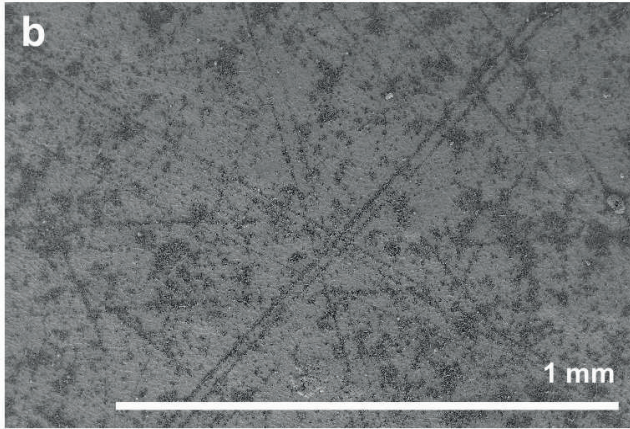


Figure 1: The ecosystem of study.

a) The photograph shows the canopy of a *Laminaria hyperborea* kelp forest, displaying numerous kelp laminae and fish (photographer: Kjell-Magnus Norderhaug).

b) A close up of the surface of a kelp lamina is visualized with scanning electron microscopy (SEM). The darker lines and patches consist of bacteria. They appear to colonize the kelp surface along straight lines that may represent tears or scratches in the kelp surface mucus layer. **c)** A

further close up makes individual bacterial cells and aggregations visible. Also note the outlines of the kelp epithelial cells, visible as grid-like elevations in the mucus layer that covers the kelp surface. **d)** A

bacterial assemblage with several different cell morphologies is portrayed. Some bacterial cells appear to grow in pit-like depressions, while others grow embedded in or on top of the mucus layer. All SEM images are from kelp collected in September 2008.

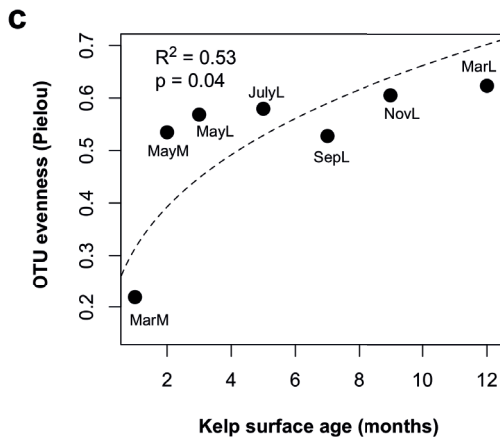
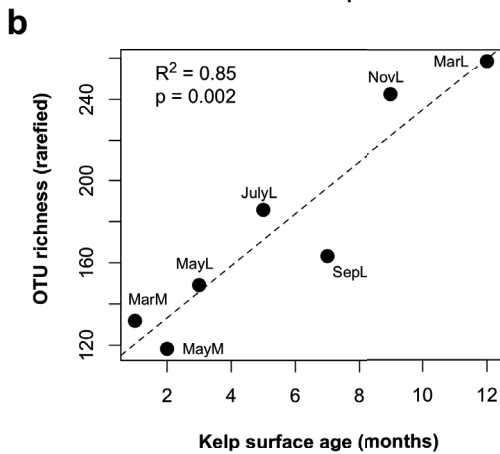
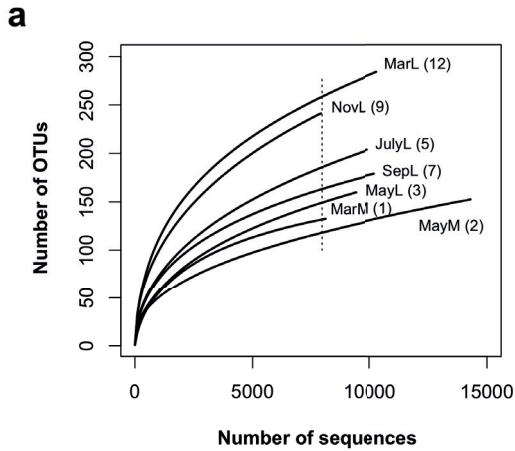


Figure 2: The influence of kelp surface age on bacterial diversity.

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). 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.

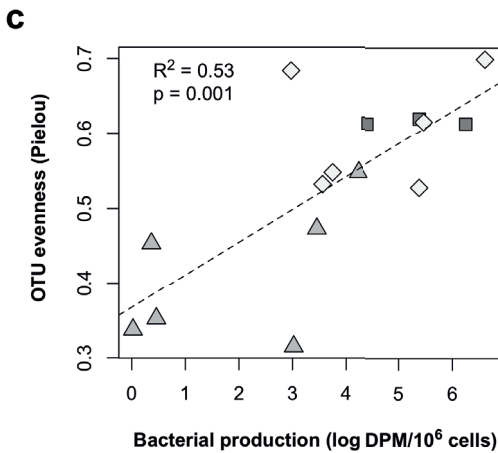
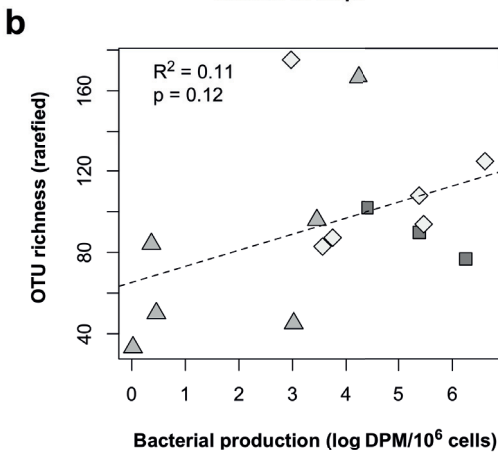
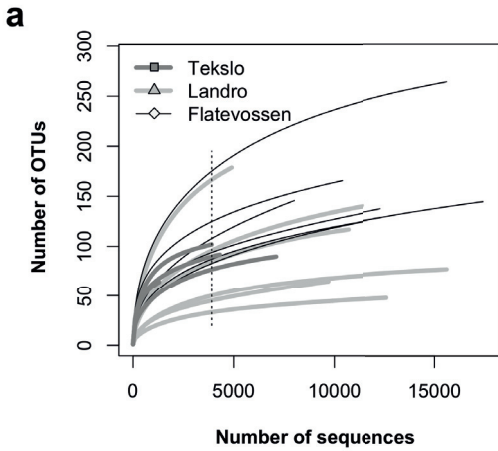


Figure 3: Kelp surface bacterial diversity and bacterial production.

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 ³H-Thymidine. **c)** 97% OTU evenness is positively correlated with bacterial production.

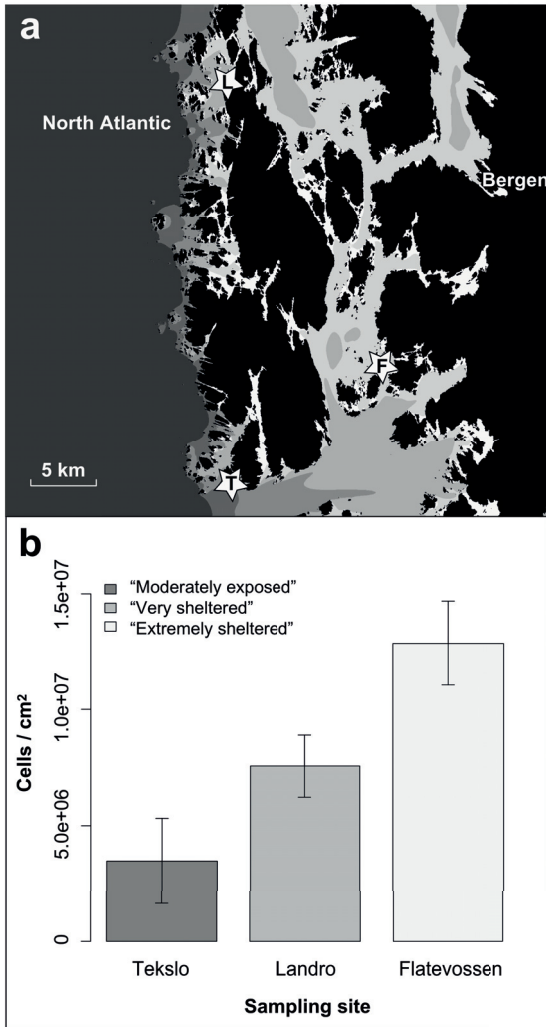


Figure 4: Wave exposure and kelp surface biofilm cell density.

a) The location of the three different sampling sites close to Bergen, Norway where kelp was collected for this study are marked by stars and letters abbreviating the names of the sites. The shading of the water on the map indicates modeled wave exposure. **b)** The biofilm cell densities that were recorded for the kelp collected in July 2009 at the different sampling sites are given as total counts of DAPI-stained bacterial-sized cells. The error bars indicate one standard deviation based on 6 (Flatevossen and Landro) or 4 (Tekslo) replicate kelp individuals.

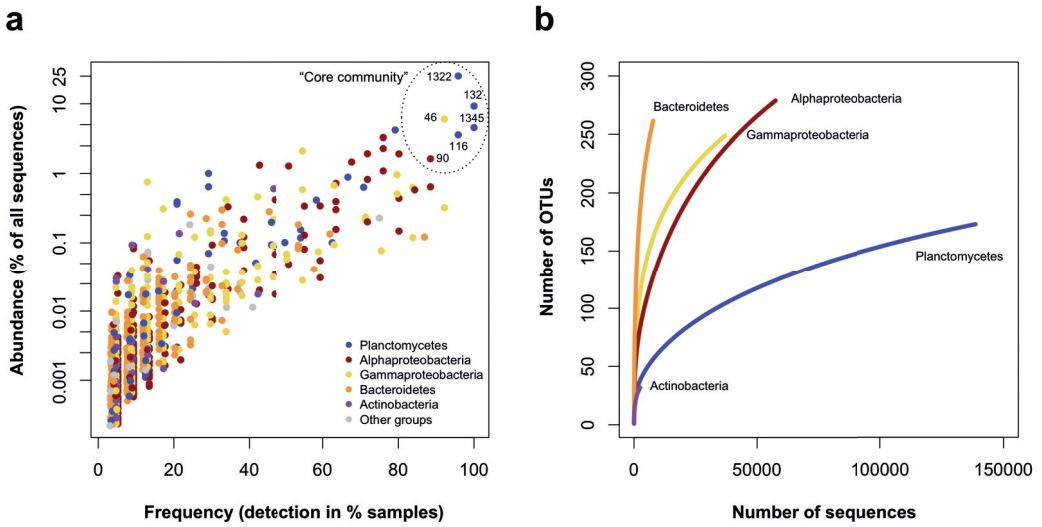


Figure 5: The bacterial communities of kelp surfaces.

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 dataset ($n = 1108$). OTUs are color coded according to which of the major phylogenetic groups they belong to. There is a "core community" of OTUs that is present across seasons and varying environmental conditions. **b)** The rarefaction curves show the number of 97% OTUs that belong to the five most represented bacterial groups. Most of the sequences belong to *Planctomycetes*, but *Alphaproteobacteria* and *Bacteroidetes* contain many more OTUs.

