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BACTERIAL COMMUNITY STRUCTURE AND PETROLEUM HYDROCARBON DEGRADATION IN THE BALTIC SEA

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TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
Sarja - ser. A II osa - tom. 303 | Biologica - Geographica - Geologica | Turku 2015

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ISBN 978-951-29-6112-2 (PRINT)
ISBN 978-951-29-6113-9 (PDF)
ISSN 0082-6979
Painosalama Oy - Turku, Finland 2015

"Ymmärrättehän, tahdon päästä selville, noudattaako meri jotain
j ä r j e s t e l m ä ä vai tekeekö se aivan miten sattuu... se on tärkeätä."

Muumipappa
Tove Jansson: *Muumipappa ja meri (1965)*

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ABSTRACT

The Baltic Sea is unique by its biological, geochemical and physical features. The number of species of larger organisms is small and the species composition is distinctive. On the contrary microbial communities are diverse. Because of the low salinity levels, bacterial communities differ from the ones in the oceans. Knowing the structure of these communities better and how they response to different environmental conditions helps us to estimate how different factors affect the balance and function of the Baltic Sea ecosystem. Bacteria are the key players when it comes to natural biogeochemical processes and human-induced phenomena like eutrophication, oil spills or disposal of other harmful substances to the sea ecosystem.

In this thesis, bacterial community structure in the sea surface microlayer and subsurface water of the Archipelago Sea were compared. In addition, the effect of diatom derived polyunsaturated aldehydes on bacterial community structure was studied by a mesocosm experiment. Diesel, crude oil and polycyclic aromatic hydrocarbon degradation capacity of the Baltic Sea bacteria was studied in smaller scale microcosm experiments. In diesel oil experiments bacteria from water phase of the Archipelago Sea was studied. Sediment and iron manganese concretions collected from the Gulf of Finland were used in the crude oil and polycyclic aromatic hydrocarbon experiments. The amount of polycyclic aromatic hydrocarbon degradation genes was measured in all of the oil degradation experiments.

The results show how differences in bacterial community structure can be seen in the sea surface when compared to the subsurface waters. The mesocosm experiment demonstrated how diatom-bacteria interactions depend on other factors than diatom derived polyunsaturated aldehydes, which do not seem to have an effect on the bacterial community structure as has been suggested in earlier studies. The dominant bacterial groups in the diesel microcosms differed in samples taken from a pristine site when compared to a site with previous oil exposure in the Archipelago Sea area. Results of the study with sediment and iron-manganese concretions indicate that there are diverse bacterial communities, typical to each bottom type, inhabiting the bottoms of the Gulf of Finland capable to degrade oil and polycyclic aromatic hydrocarbon compounds.

TIIVISTELMÄ

Itämeri on ainutlaatuinen biologisilta, geokemiallisilta ja fysikaalisilta ominaisuuksiltaan. Suurempien eliöiden suhteen lajisto on Itämerelle tunnusomainen ja harvalukuinen, mutta mikrobilajisto on monimuotoinen. Alhaisemman suolapitoisuuden vuoksi bakteeriyhteisöt poikkeavat valtamerten yhteisöistä. Tutkimustieto Itämeren bakteeriyhteisöistä ja niiden vasteista erilaisille ympäristötekijöille auttaa arvioimaan myös laajemmin erilaisten tekijöiden vaikutusta Itämeren ekosysteemin tasapainoon ja toimintaan. Bakteerit ovat keskeisessä roolissa luonnon biogeokemiallisissa prosesseissa ja ihmisen aiheuttamissa ilmiöissä kuten rehevöityminen, öljyonnettomuudet tai haitallisten aineiden päätyminen mereen.

Tässä väitöskirjatyössä verrattiin Saaristomeren pinnan mikrokerroksen bakteeriyhteisöjä niiden alapuolisen veden bakteeriyhteisöihin. Lisäksi tutkittiin piilevien tuottamien monityydyttymättömien aldehydiyhdisteiden vaikutusta Itämeren veden bakteeriyhteisöihin mesokosmoskokeen avulla. Diesel- ja raakaöljyn sekä polysyklisten aromaattisten hiilivetyjen hajottamispotentiaalia seurattiin pienempien mikrokosmoskokeiden avulla. Dieselöljykokeissa tutkittiin Saaristomeren vesifaasin bakteerien kykyä hajottaa dieselöljyä. Suomenlahden sedimentin ja rauta-mangaanisaostumien bakteerien kykyä hajottaa raakaöljyä ja polysyklisiä aromaattisia hiilivetyjä tutkittiin niitä sisältävissä kokeissa. Polysyklisten aromaattisten hiilivetyjen hajoamisprosessiin liittyvien geenien määrää mitattiin kaikissa öljykokeissa.

Tulokset osoittavat kuinka Saaristomeren pinnan mikrokerroksen bakteeriyhteisöt poikkeavat alusveden bakteeriyhteisöistä. Mesokosmoskokeiden perusteella selvisi, että piilevien tuottamat monityydyttymättömät aldehydiyhdisteet eivät ole piileväbakteeri-vuorovaikutukseen vaikuttava tekijä, toisin kuin aiemmissa laboratoriotutkimuksissa on esitetty. Dieselöljykokeissa hallitsevat bakteeriryhmät riippuivat aiemmasta öljyaltistuksesta; bakteeriryhmät poikkesivat aiemmin öljylle altistuneessa vesinäytteessä puhtaan alueen näytteen yhteisöistä. Raakaöljyn- ja polysyklisten aromaattisten hiilivetyjen hajotusta tutkittaessa kokeiden tulokset osoittivat, että Suomenlahden sedimentissä ja rauta-mangaanisaostumissa on niille tyypilliset monimuotoiset bakteeriyhteisöt, joilla on kyky hajottaa näitä yhdisteitä.

ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
CFU	Colony forming unit
DGGE	Denaturing gradient gel electrophoresis
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
MS	Metal screen
OTU	Operational taxonomic unit
PAH	Polycyclic aromatic hydrocarbon
PAH-RHDα	Polycyclic aromatic hydrocarbon ring-hydroxylating dioxygenase
PCM	Polycarbonate membrane
PCR	Polymerase chain reaction
PSU	Practical salinity unit
PUA	Polyunsaturated aldehyde
qPCR	Quantitative polymerase chain reaction
SML	Sea surface microlayer
SSW	Subsurface water
T-RFLP	Terminal restriction fragment length polymorphism
TEP	Transparent exopolymer particles

LIST OF ORIGINAL PAPERS

This thesis is based on the following publications and manuscripts referred to in the text by their Roman numerals:

I Lindroos A, Szabo H M, Nikinmaa M, Leskinen P (2010). Comparison of sea surface microlayer and subsurface water bacterial communities in the Baltic Sea. Aquatic Microbial Ecology, 65:29-42.

II Paul C, Reunamo A, Lindehoff E, Bergkvist J, Mausz M A, Larsson H, Rubach A, Richter H, Wängberg S-Å, Leskinen P, Båmstedt U, Pohnert G (2012). Diatom derived polyunsaturated aldehydes do not structure the planktonic microbial community in a mesocosm study. Marine Drugs 10:775-792.

III Reunamo A, Riemann L, Leskinen P, Jørgensen K S (2013). Dominant petroleum hydrocarbon-degrading bacteria in the Archipelago Sea in South-West Finland (Baltic Sea) belong to different taxonomic groups than hydrocarbon degraders in the oceans. Marine Pollution Bulletin Vol. 72, 1:174-180.

IV Reunamo A, Yli-Hemminki P, Nuutinen J, Jørgensen K S, Lehtoranta J (2014). Degradation of crude oil and PAHs in the iron–manganese concretion and sediment bottoms in the northern Baltic Sea. Submitted manuscript.

1. INTRODUCTION

1.1 The Baltic Sea

The Baltic Sea is a relatively young inland sea with average depth being only 54 meters. The salinity ranges from 20 PSU at the Danish Straits to less than 3 PSU in the Bothnian Bay in the north. The water column is layered as a result of salinity and temperature gradients. A permanent halocline layer separates less salty surface waters from bottom water at 50-80 m depth, where the salinity starts to increase towards the bottom (Ojaveer et al. 2010). Eutrophication has caused large bottom areas to become anoxic, which increases the precipitation of harmful substances into the sediment. In addition, anoxia causes phosphorus release from the sediment, which induces algal blooms and further eutrophication. Annually temperatures vary greatly in the Baltic Sea surface water. Approximately 218, 000 square kilometers of the Baltic Sea can be covered by ice during the winter season and the ice cover is at its greatest between January and March. During summer the warming of surface water causes formation of a thermocline layer into the 10-20 m depth, where the temperature changes drastically. However, under the halocline layer, temperature changes are only small, because salinity has greater effect on the water density than temperature. Temperature in the bottom of the Baltic Sea varies between 4-10°C (Leppäranta and Myrberg 2009).

The number of macroscopic species developed in the Baltic is small, because of its unique brackish conditions and short geological history. Despite of the small diversity, the number of individuals of species found can be abundant. When it comes to plants, phytoplankton algae living in the open water are without doubt the most abundant group. Microbes, mostly bacteria along with the picoplankton, are playing an important role in the Baltic Sea ecosystem. A microbial loop (Azam et al. 1983) binds dissolved organic matter back to the food chain, available for larger organisms. Bacteria can utilize up to 50 % of carbon fixed by photosynthesis and released as dissolved organic matter (DOM) (Azam et al. 1983), however allochtonous DOM originating from anthropogenic input seems to be an important source of carbon for the pelagic bacteria as well (Tranvik 1992).

The Baltic Sea is surrounded by nine industrialized countries and it is heavily trafficked; approximately 15% of world's maritime transport is carried out in the Baltic Sea. There are number of large oil harbors in the coasts of the Baltic Sea, busiest of them being the port of St. Petersburg, Russia. The number of oil transport is steadily growing; in year 2000 the amount of oil transported in the Baltic Sea was 43.7 Mt and by the year 2010 it had increased to 157.9 Mt (Helcom 2012). The Baltic Sea has many features which make it difficult to navigate: narrow straits, shallow depths, ice cover in the winter, crossing shipping lanes and a increasing maritime traffic. Especially the Finnish Archipelago Sea in the north eastern Baltic Sea consists of numerous unique and ecologically sensitive bay and beach areas. In case of an oil spill, the spilled oil

would disperse slower in these areas causing the degradation to be less efficient than in the open sea. As a result the ecosystem might never return to the original state (Ikavalko et al. 2005). In addition to ecological damage, a major oil spill in the Finnish Archipelago would have a significant effect on its economical and recreational value.

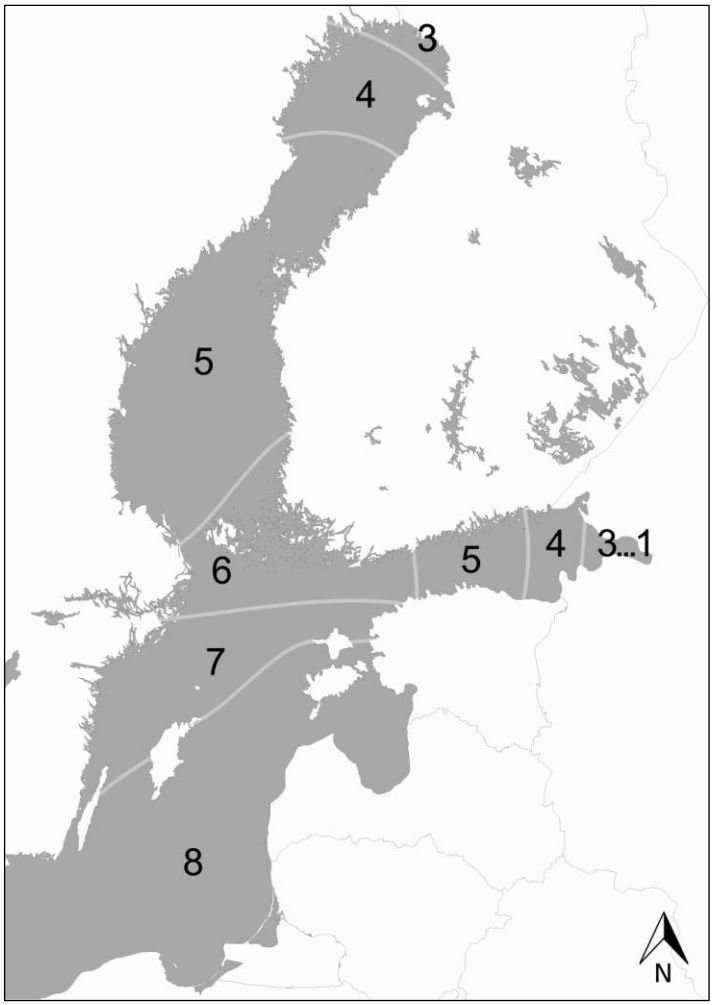

Figure 1. Salinity gradients in the Baltic Sea. Average surface salinities are shown in the map in PSU units. Oceanic average salinity is 35 PSU. The map is based on the information in Itämeriportaali.fi and constructed on a map template by Liikennevirasto.

Because of its unique features, the Baltic Sea is sensitive for environmental perturbation. Eutrophication caused by human activity is one of the major problems for the Baltic Sea. The nutrient load from agriculture, forestry, habitation and industry interrupts the balance of this fragile inland sea and the amount of algal blooms have

increased (Elmgren 1989, Brettar and Rheinheimer 1992). Along with invasive species and pollution load, eutrophication is harmful for the vulnerable species of the Baltic Sea, effecting e.g. on primary production (Lyngsgaard et al. 2014) and behaviour of fish larvae (Engstrom-Ost and Mattila 2008).

1.2 **Bacterial diversity in the Baltic Sea**

In comparison to the low diversity of multicellular organisms, the microbial diversity in the eutrophic and brackish conditions of the Baltic Sea is abundant. The short history (< 10 000 years) of the Baltic Sea has limited the development of eukaryotic species specialized to its unique conditions. However, bacteria have been capable to quick evolutionary adaptations in this changing environment. (Riemann et al. 2008) Microorganisms are the key players in the biogeochemical cycling of materials in the oceans, as well as in the Baltic Sea. Density gradients preventing annual mixing of water in most parts of the Baltic Sea and rare inflow of salt water through Danish straits modify the structure and functions of bacterial communities (Hagström et al. 2001). Salinity in the Baltic Sea differs from approximately 3 PSU in the Northern parts and river outlets to 20 PSU near the Danish outlet (Figure 1). This modifies the bacterial community structure in different parts of the Baltic Sea. Especially in the North and near river outflows the bacterial communities are influenced by freshwater species (Holmfeldt et al. 2009, Vaatanen 1982).

Bacterial community analyses also performed in our studies are based on bacterial 16S ribosomal RNA gene (16S rRNA). It is a part of the 30S small subunit of prokaryotic ribosomes and because of the slow rate of evolution in this region of the gene, it is generally used in prokaryotic phylogeny. Carl Woese and George E. Fox (1977) were the first to study the prokaryotic domain based on 16S ribosomal RNA sequences, an approach which has since become essential in the identification and classification of organisms. They suggested that the prokaryotic domain actually includes two different lineages, bacteria and archaea, which are no more similar to each other than with the eukarya. Since 1990 the *Eukarya*, *Bacteria*, and *Archaea* have been generally accepted as the three primary divisions of organisms by Woese's classification scheme.

Not only the genetic diversity of prokaryotes, but the functional diversity among them is enormous. The source energy can originate from the sun light (phototrophs) or they can gain energy from chemical reactions (chemotrophs). Chemotrophs are divided into chemoheterotrophs, which get their energy and carbon from organic compounds e.g. carbohydrates, lipids or proteins and chemoautotrophs who can synthesize all needed compounds by fixing carbon from CO_2 and by gaining energy by inorganic energy sources (ammonia, ferrous iron, elemental sulphur, hydrogen sulfide etc.). In oxic conditions the final electron acceptor for chemoheterotrophs is O_2, as in all animals and most protozoa and fungi. In anoxic conditions, the final electron acceptor can be either organic (fermentation) or inorganic compound (electron

transport chain). The electron acceptors can be various organic compounds or when it comes to inorganic compounds, e.g. chlorate (ClO_3^-) as well as ferric iron (Fe^{3+}), manganese (IV) (Mn^{4+}), NO_3^- and S^0 (Fig. 2). Furthermore many prokaryotes harbor spesific genes for the degradation of different xenobiotics.

Temperature, salinity and the presence of oxygen shape both the taxonomical and functional diversity of bacteria in the Baltic Sea (Thureborn et al. 2013). Functional diversity can be studied by a detection of functional genes, for example the abundance of *nahAc* genes, related to hydroxylation of the aromatic ring of naphthalene (one of the GN PAH-RHDα genes), have been shown to correlate with ^{14}C-naphtalene mineralization potential in oil contaminated soil (Tuomi et al. 2004, Salminen et al. 2008). Other functional genes commonly studied are genes related to procecces like nitrogen fixation (*nifH*), denitrification (*napA*, *narG*, *nirK*, *nirS*, *nosZ*), ammonia (*amoA*), methane (*pmoA*, *mmoX*, *mxaF*) or sulfide oxidation (*sqr*).

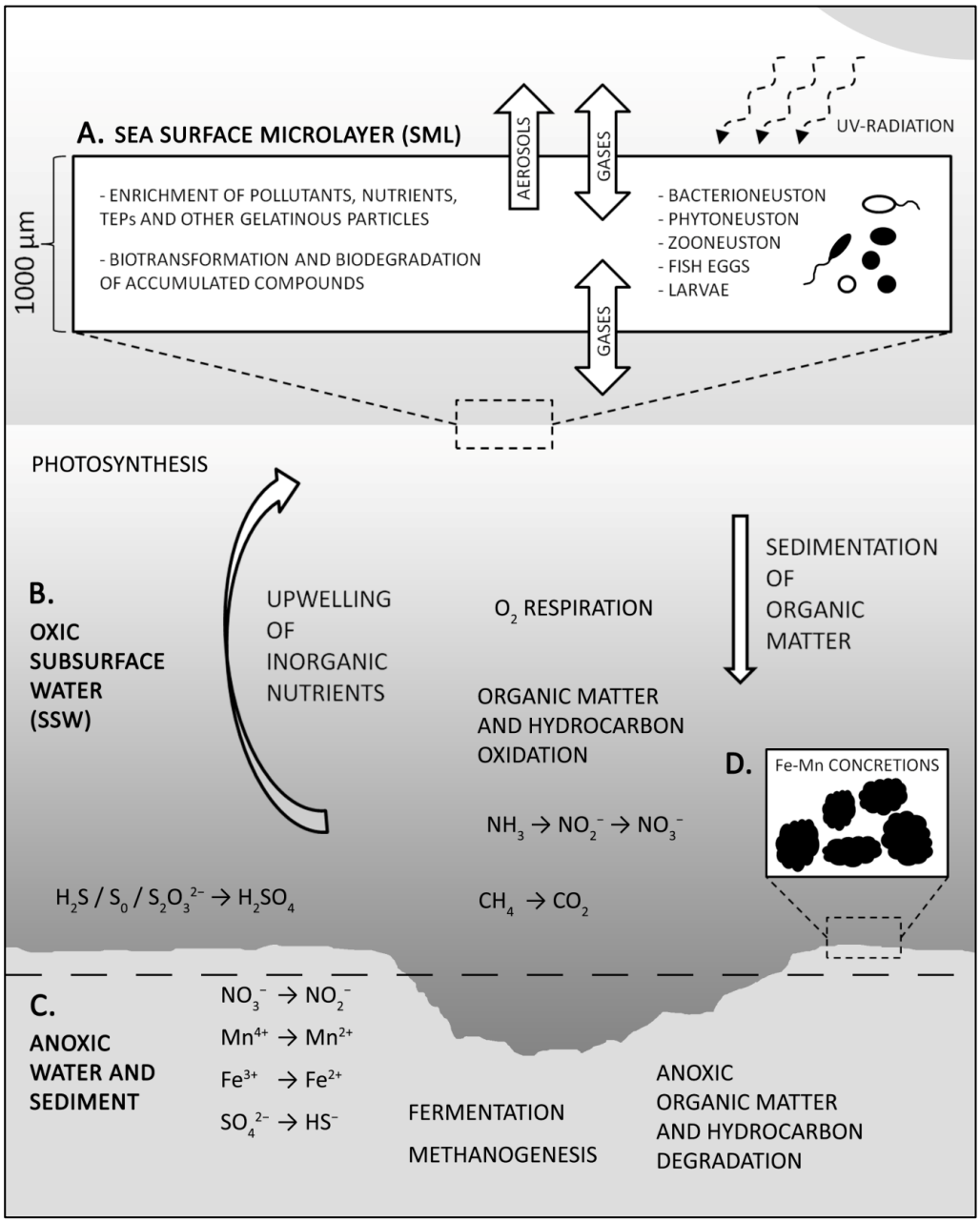

Figure 2. Bacterial processes in the sea. Examples of important microbial processes in the sea. a. Sea surface microlayer (SML) is a unique habitat for microorganisms. b. Oxic subsurface water: Microbial processes can cause anoxia in the lower parts of the subsurface water near the bottom sediment. c. Hydrocarbon degradation occurs also in the anoxic sediments. Dashed line describes a border between the oxic and anoxic zones. d. Diverse microbial communities inhabit iron-manganese concretions.

1.2.1 Sea surface microlayer

The 1 mm upper layer of the sea is considered to form the sea surface microlayer (SML) (Liss and Duce 1997, Wurl and Obbard 2004) (Fig. 2). Bacterial communities inhabiting the sea surface microlayer are considered to differ from subsurface water communities, because the SML differs from SSW in many chemical and physical properties (Lion and Leckie 1981, Hardy 1982), such as strong UV radiation and enrichment of pollutants and nutrients (Norkrans 1980, Hermansson et al. 1987). Bacteria inhabiting the SML are called bacterioneuston (Naumann 1917) in comparison to bacterioplankton in the subsurface water. Many different natural factors shape the microbial community structure of the SML, for example UV radiation and meteorological conditions (Stolle et al. 2011). The functional role of the SML bacteria is not well known. Probably they are important in the gas exchange between water and air, but it is not known how bacteria respond to organic matter enrichment in the SML (Cunliffe et al. 2008, Stolle et al. 2010). The potential of the SML bacterial communities to polycyclic aromatic hydrocarbon-degradation has been also pointed out in a temperate estuarine areas (Coelho et al. 2011).

There is a broad range of different sampling devices for the sampling of the SML (Wurl et al. 2014). These sampling devices and the thickness of the layer they collect are presented in table 1. The choice of a sampling device affects the results, because of the thickness of the SML they collect (Agogue et al. 2004).

Table 1. Sampling devices for the sea surface microlayer sampling

Sampler	Sample thickness (mm)	References
Glass plate	20–100	Harvey and Burzell, 1972
Mesh screen (Garrett screen)	150–400	Sieburth, 1965, Garrett, 1967
Rotating drum	60–100	Harvey, 1966
Polycarbonate membrane	4-40	Franklin et al. 2005, Crow et al. 1975

Only few studies have been conducted on the bacterioneuston in the Baltic Sea. Stolle et al. (2010) studied the effect of wind conditions on bacterioneston in mesocosm experiments. Wind conditions were naturally or artificially calmed and the experiment was carried out in a marina in the south-west Baltic Sea. Results showed how particle-attached bacterial assemblages increased in the SML in calm wind

conditions. This induced uncoupling of the SML and SSW bacterial community structures. Overall Stolle et al. (2010) showed that the difference between particle attached bacterial communities is greater than in non-particle attached communities.

1.2.2 Subsurface water

The Baltic sea has been shown to host a unique brackish bacterial community composition. However, because of its salinity gradients and other features, like subbasins and sills, it cannot be considered to have consistent bacterial communities throughout the whole Baltic basin (Riemann et al. 2008). Extensive sampling and bacterial community analysis has been carried out in one sampling site in the central Baltic Sea throughout one full year (Riemann et al. 2008), in which *Bacteroidetes* showed up to be the dominant phylogenetic group, while there was lack of typical marine taxa. *Bacteroidetes* is a highly diverse group inhabiting various different environments. Based on some fluorescent in situ hybridization (FISH) studies it is suggested to account for as much as half of all bacterioplankton cells in the sea (Cottrell and Kirchmann, 2000). *Bacteroidetes* is not well represented in 16S rRNA gene libraries, however, because of the bias against this cluster when common 16S rDNA primers are used (Weaver et al. 2013). In addition to *Bacteroidetes*, typical freshwater groups belonging to *Actinobacteria*, *Verrumicrobia* and *Betaproteobacteria* were strongly present. Temporal changes were observed in the community composition (Riemann et al. 2008). Clear seasonal succession was also detected in bacterioplankton of Ladsort Deep, central Baltic Sea (Andersson et al. 2010). In addition to the seasonal variation, bacterial community change was highly correlated with temperature and phosphorus concentration.

In the study of Hagström et al. (2000) the phylogenetic diversity of bacteria was studied in the Baltic Sea along with five other sea areas. This study showed how most of the observed genera were found to exist in Skagerrak, Southern California Bight and northwest Mediterranean, but the species composition in the different areas was different. Instead, in the Northern Baltic Sea the bacterial groups seemed to be more fresh water emphasized; *Pseudomonas*, *Sphingomonas* and *Shewanella*, which are common in the Baltic Sea instead of *Roseobacter*, *Alteromonas* and *Vibrio*, which all have a salt requirement for growth.

A comprehensive study with samplings performed at 60 stations in the Baltic Sea, along both vertical and horizontal salinity gradients, revealed an autochtonous microbiome existing at the Baltic Sea (Herlemann et al. 2011). In general, in the surface water the salinity was most important factor shaping the bacterial community structure. Mostly dominated by one OTU, Verrucomicrobia was shown to be abundant in most of the samples, in different depths and salinities. In the oxygen sulfide transition zone notable changes in bacterial community composition were observed: first a shift towards *Sulfurimonas* sp. and in deeper anoxic conditions sulfate-reducing *Gammaproteobacteria*.

The clear change in oxygen-sulfide transition zone has been shown in previous studies as well (Labrenz et al. 2007). In more recent study (Bergen et al. 2014) class *Spartobacteria* belonging to *Verrumicrobia* has been shown to constitute as much as 12 % of total bacteria in the brackish surface waters of the Baltic Sea. *Spartobacteria* are assumed to utilize phytoplankton-derived polysaccharides, as this group of bacteria is associated with phytoplankton cells and particles.

A redoxcline is a layer of water, which is strongly stratified because of its redox gradient caused by anoxic water in the bottom and oxic water above it. This redoxcline layer can be several meters in the deep basins of the Baltic Sea and harbor elevated bacterial activity and abundance (Berg et al. 2013). In the anoxic areas alternative electron acceptors and donors are prevalent. For example Mn oxides have been suggested to be used as electron acceptors for organic carbon degradation in the redoxcline (Neretin et al. 2003), e.g., *Arcobacter* sp. making them important in the manganese cycling between the anoxic and oxic zones of the central Baltic Sea (Berg et al. 2013).

1.2.3 Sediment

The Baltic Sea bottoms consist of different kind of areas; approximately a third of the bottom areas are sites where organic matter and different pollutants are enriched by sedimentation and the rest of the areas are erosion bottoms. Large amounts of phosphorus are stored in the sediment (Lukkari et al. 2009, Emeis et al. 2000). Oxygen depletion and anoxia are common in vast areas even in the shallow coast of the Gulf of Finland (Bonsdorff et al. 1997). Anoxia causes these areas to be unsuitable for higher organisms but microbes are inhabiting them. Microbial degradation of organic matter consumes O_2 which increases Fe-bound phosphorus release into water column (Lukkari et al. 2009). In addition to phosphorus, many pollutants are accumulated in the sediments. (Fig. 2)

Bacterial communities seem to differ in pristine and polluted sediments (Edlund et al. 2006). In addition, other environmental factors such as depth, carbon content and oxygen depletion affect the bacterial community structure (Edlund et al. 2006). Bacterial communities have been shown to change along the gradients of organic carbon, nitrogen and phosphorus in the Gulf of Finland. In the most organic material-rich oxic coastal surface sediments *Flavobacteria*, *Sphingobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria* are most abundant whereas *Desulfobacula* is dominant in the same conditions in the open sea. Sulphate reducers are present up to the depths of 20 cm where they start to dimininsh and the members of family *Anaerolineaceae* (phylum *Chloroflexi*) increase (Sinkko et al. 2013). Along with other factors, eutrophication has been suggested to be an important selective factor modifying bacterial community structure in the coastal areas of the Baltic Sea (Funkey et al. 2014, Steenbergh et al. 2014).

1.2.4 Iron-manganese concretions

Iron and manganese (Fe-Mn)-rich concretions are formed by biogeochemical processes on the surface of the sediments and they can be found in many areas in world's oceans as well as in the Baltic Sea. For example in the Gulf of Finland they cover about 10 % of the bottom (Winterhalter 1966). The concretions are often spherical and their structure is porous. Bacteria are able to oxidize reduced forms of Fe and Mn which leads to precipitation and accumulation of metal oxides in the sediment (Villalobos and Tebo 2005). In addition to Fe and Mn, arsenic (As) and phosphorus (P) are coprecipitated in the concretions (Baturin and Dubinchuk 2009). (Fig. 2)

The iron-manganese concretions are a unique habitat and bacteria inhabiting them in the Baltic Sea are shown to be diverse (Yli-Hemminki et al. 2013) comprising of many unknown Fe- and Mn-oxidizing species. Approximately half of the bacteria in the concretions are shown to be proteobacteria belonging to subclasses *Alpha-* (11%), *Gamma-* (11%), *Delta-* (10%), *Beta-* (9%) and *Epsilonproteobacteria* (1%). In laboratory experiments Fe2+-oxygen gradient was shown to favor the enrichment of *Shewanella baltica*, known to reduce Fe, and *Thalassolituus oleivorans*, a known petroleum hydrocarbon-degrading bacterium.

1.3 Factors affecting the bacterial community structure in the Baltic Sea

Bacteria are highly flexible towards environmental pressures. This flexibility is based on both physiology and metabolism. When conditions are turning unfavorable towards the dominant species, the rare biosphere members of the community increase. These changes in bacterial community composition can also have effects on ecosystem processes. (Sjostedt et al. 2012, Pedros-Alio 2006, Pommier et al. 2007)

Although the oceans cover more than 70% of the earth's surface, there is still plenty of research to be carried on the marine microbial communities. Microbial populations in the environment are widespread and complex, including many uncultured and unidentified members. Major factors influencing the community structure are seasonal and spatial effects (Ghiglione et al. 2012, Du et al. 2011, Treusch et al. 2009). For example, coastal bacterial communities often differ from the communities of the open ocean. More similarity has been observed between different depths, seasons, and coastal and open waters even in the bacterial communities in the Antarctic and Arctic, regions geographically far from each other, but with a similar selection pressure (Ghiglione et al. 2012). Only surface waters showed larger difference between the poles, probably due to more variable and short-term conditions.

1.3.1 Natural effects

1.3.1.1 Salinity

It has been suggested that salinity is the most important factor modifying bacterial communities in marine environment (Wu et al. 2006). There are several studies on the influence of salinity on bacterial community composition (Bouvier and del Giorgio 2002, Cottrell and Kirchman 2003, Zhang et al. 2006, Herlemann et al 2011, Dupont et al. 2013). Dupont et al. (2013) showed the most abundant bacterial phyla and classes to change through the salinity gradient in the Baltic sea, while phytoplankton and viruses showed more stochastic pattern. In general, the amount of *Alphaproteobacteria* and *Gammaproteobacteria* has been shown to increase with increased salinity, while the proportion of *Betaproteobacteria* decreases. On the other hand, the *Cytophaga-Flavobacterium* (CF) -cluster has no clear relationship with salinity changes (Bouvier and del Giorgio 2002).

In the Baltic Sea the study of Herlemann et al. (2011) showed salinity to be the major factor affecting the surface water bacterial community composition; the relative abundance of *Actinobacteria* and *Betaproteobacteria* increased in lower salinity areas and *Alpha-* and *Gammaproteobacteria* were more abundant in higher salinity. Results of studies conducted in estuaries also show the dominance of *Betaproteobacteria* in lower salinities and *Alphaproteobacteria* in higher salinities (Bouvier and del Giorgio 2002, Cottrell and Kirchman 2003, Zhang et al. 2006). However, estuarine studies are not directly comparable to the brackish Baltic Sea, which is much more stable environment because of the long retention time of the water. The bacterial community composition can therefore not simply be a result of mixing of saline and fresh water communities, but rather gives a possibility for an autochthonous brackish microbial community to establish (Herlemann et al. 2011).

1.3.1.2 Polyunsaturated aldehydes (PUAs) produced by diatoms and bacterial communities

Some species of diatoms, phototrophic microalgae, are known to produce various fatty acid derived secondary metabolites such as polyunsaturated aldehydes (PUAs). One of these species is *Skeletonema marinoi* (Vidoudez et al. 2008, Vidoudez et al. 2011), which is an important primary producer in the North Atlantic where it is especially abundant during the spring bloom, when densities of millions of cells per liter is reported. *Skeletonema* spp. belong to the phytoplankton community in the Northern Baltic Sea as well.

The effect of PUAs on bacterioplankton has been studied in laboratory experiments (Adolph et al. 2004, Ribalet et al. 2008, Balestra et al. 2011). However, in most experiments the concentrations of PUAs have been much higher than those occurring in the environment. Balestra et al. (2011) carried out experiments with environmentally

relevant concentration (7.5 nM) and they observed PUAs to affect metabolic activity of certain bacterial groups. According to their results the bacterial groups resistant to the diatom derived polyunsaturated aldehydes might get an advantage in natural bacterial communities.

1.3.2 Anthropogenic influences

1.3.2.1 Eutrophication

Eutrophication of the Baltic Sea caused by excessive nutrient input is one of the biggest threats to its biodiversity. Eutrophication-caused anoxia makes nearly half of the Baltic Sea bottom area unsuitable for higher organisms to live in. Bacteria and archaea inhabiting these anoxic bottoms are influenced by eutrophication and thus also their activities like nutrient cycling are affected (Edlund et al. 2006).

Phosphorus release from marine sediment, which is highly dependent of redox conditions at the sediment-water interface, enhances eutrophication. Bacterial activity is in the main role when it comes to biological processes releasing and retaining phosphorus in the sediments. However, the redox conditions have been shown to explain only a small amount of the variance observed in the bacterial community structure (Steenbergh et al. 2014). Benthic bacterial communities in the Baltic Sea do not seem to be typical for either oxic or anoxic conditions and the bacterial community structure seems to have only a small influence on phosphate fluxes (Funkey et al. 2014, Steenbergh et al. 2014).

1.3.2.2 Crude oil and polycyclic aromatic hydrocarbon compounds

Crude oil and PAHs enter to the sea through various routes like shipping activity; accidental oil spills from recreational boating, tankers, oil refineries, exploration sites and terminals as well as natural seepage. In addition, municipal and industrial water and runoff from land are common contamination sources (Kostianoy et al. 2005). PAH-compounds are one of the most widespread group of organic pollutants and they are considered to be the most toxic fraction of crude oil. Seasonal and regional concentration differences of PAHs have been observed; usually the highest concentrations are found in November and in coastal areas (Witt 1995). Bioavailability of PAH-compounds is an important factor when conducting a risk assessment on petroleum-polluted sediment. The sediment type influences PAH bioavailability and in sandy or organic sediments the bioavailability can be much higher than in muddy sediments (Lindgren et al. 2014).

Hydrocarbon-degrading bacteria have been shown to increase in petroleum polluted sea environments. The pattern of bacterial group change depends on the type of petroleum hydrocarbon in question. E.g. *Alcanivorax* spp. are able to use brached chain alkanes, *Cycloclasticus* spp. aromatic hydrocarbons and *Oleiphilus* spp. and

Thalassolituus spp. both branched- and/or straight-chain saturated hydrocarbons. Succession of these different bacterial groups has been observed when analyzing samples after oil spills (reviewed in Head et al. 2006). In addition to PAHs and crude oil, metal contamination modifies bacterial community structure. For example, bacterial community structure of a certain site has been shown to be significantly different after a remediation process by dredging the sediment in the Baltic Sea (Edlund and Jansson 2006). Global climate change causing ocean acidification and increased UV-radiation might seriously affect ecosystem functioning - among other processes microbial degradation of pollutants (Coelho et al. 2013).

1.4 Petroleum hydrocarbon-degrading bacteria

Indigenous bacteria capable to degrade petroleum hydrocarbons are widespread in terrestrial and oceanic environments (Foght 2008, Head et al. 2006, Leahy and Colwell 1990, Yakimov et al. 2007). Many oil-degrading moulds, yeasts, unicellular algae and protozoa has been isolated, however, in marine environment bacteria seem to be the most important organisms responsible of oil degradation (Leahy and Colwell 1990). Petroleum hydrocarbon degrading bacteria in the oceans differ from the bacteria in soil, as the former are usually specialized to use hydrocarbons as their sole source of carbon (Yakimov et al. 2007). Petroleum hydrocarbon degradation in the oceans usually requires a succession, where one species of bacteria metabolizes only certain types of hydrocarbons and other species continue the process. In addition, members of the bacterial community which do not directly take part in the degradation process might play an important role in effective breakdown, e.g., by producing surfactants which increase the bioavailability of oil for the degraders (Head et al. 2006). Hydrocarbon-degrading bacteria usually constitute less than 1 % of total microbial community, however, in case of hydrocarbon pollution, the percentage increases approximately to 10% of the total community (Atlas 1995).

1.4.1 Degradation processes

Crude oil is a complex mixture which consist of a large number of different hydrocarbons. Most common are alkanes, cycloalkanes, aromatic hydrocarbons (including PAHs) and more complex molecules like asphaltenes. Aliphatic hydrocarbons can more readily be degraded by the natural bacterial community than the more complex chemical structures of the polycyclic aromatic hydrocarbons (PAHs), (Bamforth and Singleton 2005) which are one of the most toxic compounds found in the environment. *Alcanivorax* spp., *Thalassolituus* spp., *Cycloclasticus* spp., *Oleiphilus* spp. and *Oleispira* spp. have been shown to be most common petroleum hydrocarbon-degrading bacteria in the marine environment, each of them using hydrocarbons as a sole carbon source (reviewed in Head et al. 2006,Yakimov et al. 2007). In soil hydrocarbons are degraded by variety of bacteria, yeast and fungi (Das and Chandran 2011). Known bacterial genera responsible of petroleum hydrocarbon

degradation in soil are e.g. *Burkholderia*, *Ralstonia*, *Nocardia*, *Acinetobacter* and *Pseudomonas* (Hamamura et al. 2006).

1.4.1.1 Aliphatic hydrocarbons

Aliphatic hydrocarbons consist of alkanes (saturated hydrocarbons), alkenes and alkynes. They can be linear (e.g., n-alkanes), cyclic or branched. Alkanes are the main fraction of crude oil and many microorganisms can use them as a source of carbon and energy in both oxic and anoxic conditions. Alkanes are highly insoluble to water and it is not known how bacteria transfer them into the cell. However, many bacteria have been shown to excrete surfactants, which facilitate the process (Hommel 1990, Ron and Rosenberg 2002, Rojo et al. 2009). Surfactants contain various molecular sized compounds belonging to diverse chemical groups, e.g. glycolipids, proteins, lipoproteins, lipopolysaccharides or complex mixtures of these. They help bacteria to disperse oil and get a direct contact with the hydrocarbon substrate (Ron and Rosenberg 2002).

In oxic conditions the initial step of aliphatic hydrocarbon degradation requires molecular oxygen (O_2) as a reactant. There are several different monooxygenase enzymes, which incorporate one of the atoms of oxygen in to the oxidized hydrocarbon. Bacterial strains degrading medium-chain-length alkenes (C_5-C_{11}) and longer alkenes (C_{12}-) usually have a monooxygenase enzyme related to the well-studied *Pseudomonas putida* GPo1 *AlkB* alkane hydroxylase (Vanbeilen et al. 1994, Rojo et al. 2009). *AlkB* gene encodes a membrane-bound catalytic monooxygenase component which can be used when monitoring catabolic genes related to alkane degradation by bacteria. A gene related to short- and medium-chain-length alkane degradation in aerobic conditions is the *CYP153* (Nie et al. 2014). *almA* gene is related to long-chain alkane degradation (Throne-Holst et al. 2007) and detected to be especially common in the genera *Alcanivorax* and *Marinobacter* (Wang and Shao 2012). Shorter alkanes (C11–C12) have been shown to be degraded first, following with degradation of the the longer chain alkanes, as small hydrocarbon molecules are more readily biodegraded than larger ones (Harayama et al. 1999, Sei et al. 2003).

Although alkenes are chemically relatively inert, activating them with molecular oxygen allows much faster degradation rates than in anoxic conditions (Kloos et al. 2006). Anaerobic degradation of alkenes is less studied. However, increasing number of strictly anaerobic hydrocarbon-degrading microbes have been found since late 1980s (Widdel and Rabus 2001). They have been shown to posses very different degradation pathways from those functioning in aerobic conditions.

1.4.1.2 PAHs

The structure of polycyclic aromatic hydrocarbons (PAHs) makes them relatively persistent to biodegradation. The aqueous solubility and thus the bioavailability of the

PAHs decreases with their molecular mass, which makes the PAHs ranging in size from two-ringed naphthalene to seven ringed coronene of primary environmental concern (Johnsen et al. 2005). For degradation bacteria must take PAHs into the cell, because the initial degradation step occurs by intracellular dioxygenases (Johnsen et al. 2005).

Naphthalene is often used as a model PAH-compound in laboratory experiments, although it is the smallest PAH with its two benzene rings. The degradation of naphthalene in oxic conditions is well characterized (Goyal and Zylstra 1997). In strictly anaerobic conditions napthalene is first activated with an unknown activation mechanism and then carboxylated to form 2-naphthoic acid. (Mouttaki et al. 2012).

Bacteria possess different genes for alkane- and PAH-degradation pathways. Genes often studied include for example *nahAc* related to naphthalene degradation, *xylE* encoding catechol 2, 3 dioxygenase, an important enzyme in the degradation of toluenes, benzoates and their methyl derivatives and *PAH-RHDα* which encodes PAH ring hydroxylating dioxygenase. When oxygen is present, both gram- negative (GN) and gram-positive (GP) bacteria may express PAH-RHDα genes (Habe and Omori 2003). Cebron et al. (2008) developed two robust primer sets that amplify all known PAH-RHDα genes in the databases, i.e. 4 different in gram positive bacteria and 13 different in gram negative bacteria. The number of different bacterial petroleum hydrocarbon- and PAH-degradation genes can be used to estimate the contamination level and natural degradation capacity of the site studied (Cebron et al. 2008, Tuomi et al. 2004).

1.4.2 Petroleum hydrocarbon degradation in the Baltic Sea

Several studies in the ocean environments have shown *Gammaproteobacteria* to be most often responsible of the petroleum hydrocarbon-degradation: in the Gulf of Mexico (Hazen et al. 2010, Gutierrez et al. 2013), the North Sea (Brakstad and Lodeng 2005) and in the Mediterranean (Cappello et al. 2007).

Only few studies are available on biodegradation of oil in the brackish Baltic Sea, although there is a considerable risk for a major oil accident due to increasing maritime traffic. Suni et al. (2007) studied the the efficiency of cotton grass fibers in removing diesel oil in a mesocosm experiment constructed with water from the Baltic Sea. Part of the mesocosms were inoculated with diesel enriched microorganisms, and this enhanced the diesel removal significantly. However, in their experiments the diesel additions increased bacterial density also in the non-inoculated mesocoms, suggesting that there would also be indigenous hydrocarbon degraders in the Baltic Sea water.

Crude oil-degrading bacteria have been successfully isolated from the Baltic Sea (Bruns et al. 1993, Linden et al.1987). In more recent studies also culture-independent methods have been used; the effect of crude oil, shale oil and diesel fuel on bacterial

community composition was studied by microcosm experiments by *alkB* and 16S rRNA marker genes in PCR-DGGE in the coastal areas of eastern Baltic Sea (Viggor et al. 2013). In addition, the occurrence of PAH-degrading bacteria have been studied in contaminated sediments of Swedish coast of the Northern Baltic Sea (Edlund and Jansson 2006) using a combination of molecular methods like bromodeoxyuridine incorporation, T-RFLP and immunocapture. Their research showed the class *Deltaproteobacteria* and genus *Spirochaeta* to dominate before dredging of the polluted site and members of classes *Gammaproteobacteria* and *Flavobacteria* after the treatment.

2. AIMS OF THE STUDY

Responses of bacterial communities to different environmental factors, both anthropogenic and natural, are important when for example climate change or pollution effects are considered. There has been extensive research on biological hydrocarbon-degradation in soil and ocean environment. However, the Baltic Sea has had little attention so far. The aim of this study was to examine following questions:

I) Does the bacterial community structure of the sea surface microlayer (SML) differ from the subsurface water (SSW) communities in the Baltic Sea?

II) Are the bacterial communities affected by polyunsaturated aldehydes (PUAs) produced by *Skeletonema marinoi* -diatom?

III) What is the natural diesel degradation capacity of the bacterial communities inhabiting Baltic Sea water? Which are the dominant bacterial groups responsible for diesel degradation?

IV) What is the oil and naphthalene degradation capacity of sediment and iron-manganese concretion bacteria of the Baltic Sea? Which are the dominant bacterial groups responsible for the degradation process?

3. MATERIALS AND METHODS

Table 1. Methods

Method		**Aim**	**Article**
Sampling	Metal screen	Sampling of the SML	I
	Polycarbonate filters	Sampling of the SML	I
	Glass bottle	Sampling of the SSW	I
	Niskin bottle	Sampling of the SSW	III
	Van Veen	Sampling of sediment and iron-manganese concretions	IV
Isolation of total environmental DNA		Performing DNA-based analyses	I, II, III
T-RFLP		Bacterial community profiling	II, III
Cloning and sequencing		Detection of the main bacterial groups	IV
DGGE		Bacterial community profiling	III, IV
Quantitative PCR		Detection of PAH-degradation genes	
Flow cytometry / plate culturing		Enumeration of total / culturable bacteria	I
Mineralization analysis		Measuring of biological mineralization rate of ^{14}C-labeled naphthalene	IV
Gas chromatography		Analysing PAH and hydrocarbon content. Performed by Nova Lab and SYKE Lab	III, IV

3.1 **Study sites**

Sampling sites for our experiments were located in the Northern Baltic Sea (Fig. 3), and SML studies were performed in the Finnish archipelago Sea in SW Finland. Sites included both pristine (Stenskär) sites and harbors (Nauvo and Raisio). For the diesel experiments water samples were collected from a pristine site (Askainen) and sites with different degrees of previous pollution: a boat harbor (Raisio) and an oil harbor (Pansio). Mesocosm studies were carried out in Umeå Marine Sciences Center. The water used in the experiment was collected from the sea outside the station. Most of the concretions and sediment for the crude oil degradation experiments were collected from the Gulf of Finland (LL4A and 4 sites of A) and one of them (A) was in the Bothnian Sea.

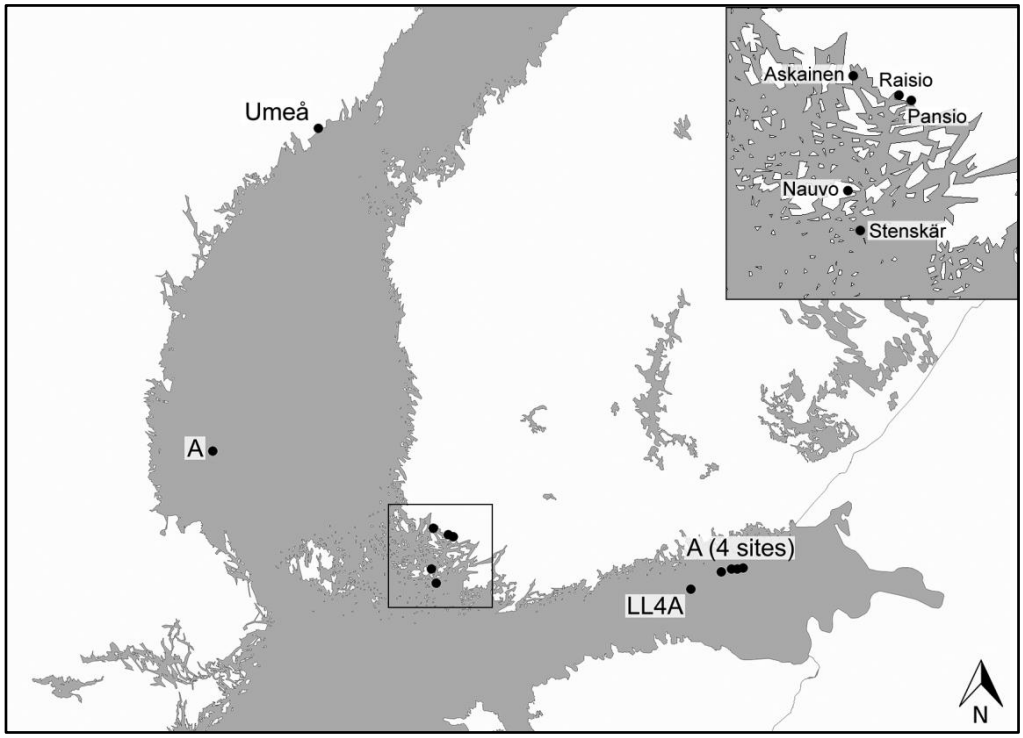

Figure 3. Map of sampling sites. Water samples for sea surface microlayer (Nauvo, Stenskär, Raisio) and diesel experiments (Askainen, Pansio, Raisio) were collected from the Archipelago Sea. Stenskär includes two pristine sites relatively closely located to each other, consisting of a small boat harbor and a pristine site. Concretions and sediment for the crude oil degradation experiments were collected from the Gulf of Finland, site LL4A and four sites marked with A (four of them located in the Gulf of Finland and one in the Bothnian Sea). Water for the mesocosm experiment was collected at the Umeå marine Sciences Center. Map template by Liikennevirasto.

3.2 Sampling

3.2.1 Sea surface microlayer and subsurface water

Samples were collected in May, June and July 2007 and June-July 2008. Sampling sites were located in the Finnish Archipelago in the harbor bays of Nauvo (N), Stenskär (H) and Raisio (R) and in a pristine bay in Stenskär (C) (Figure 2.) Site R is located close to the mainland, near a river inlet, whereas the other three sampling sites are located fairly close to each other and further out in the archipelago. R is a busy harbor with place for 700 boats, N is a medium sized harbor with place for 120 boats and H is a small harbor with place for 15 boats. SML samples were collected using a Garrett metal screen (MS) (Garrett 1965) and samples obtained from several dips of the MS were pooled. SSW samples from approximately 40 cm depth were taken with a sterile bottle. The sampling device allowed to estimate the depth of the SML sampled to be approximately 400 µm. 2.5 liters of water was sampled in total from both SML and SSW. The final volume was achieved by approximately 25 dips with the MS. Sampling days were chosen based on the weather conditions, to avoid interference of hard wind or rain. Samples were kept on ice in a cooler box until further processing in the laboratory within 8 h of collection.

Additional SML and SSW samples were collected to assess the effect of the sampling technique in October 2009 from sampling site R using both MS and a polycarbonate membrane (Cunliffe et al. 2009). In the polycarbonate sampling method, the membrane is placed on the SML for 10 seconds using sterile forceps. For the comparison of samplers water was not pre-filtered, so both free living and particle-attached bacteria were included in the analysis.

3.2.2 Subsurface water for diesel experiments

Seawater samples for the diesel experiments were collected in September 2008 from a pristine site (Askainen), a recreational harbour (Raisio), and an oil-polluted harbour (Pansio) located in the Archipelago Sea in SW Finland (Figure 2.). Ten-liter water samples were obtained from one meter depth using a Limnos water sampler (Turku, Finland). In situ water temperature was 15ºC. The samples were stored in the dark at 4°C and the experiment was started within 2 h.

3.2.3 Sediment and iron-manganese concretions

Samples were collected from the Gulf of Finland (Figure 2), the Baltic Sea, by a van Veen sampler and a box corer on board R/V Aranda. Concretions were stored in air-penetrating plastic containers or in aerated aquariums with near-bottom water at 5 °C in darkness. Sediment was stored in aerated aquariums with concretions and near-bottom water.

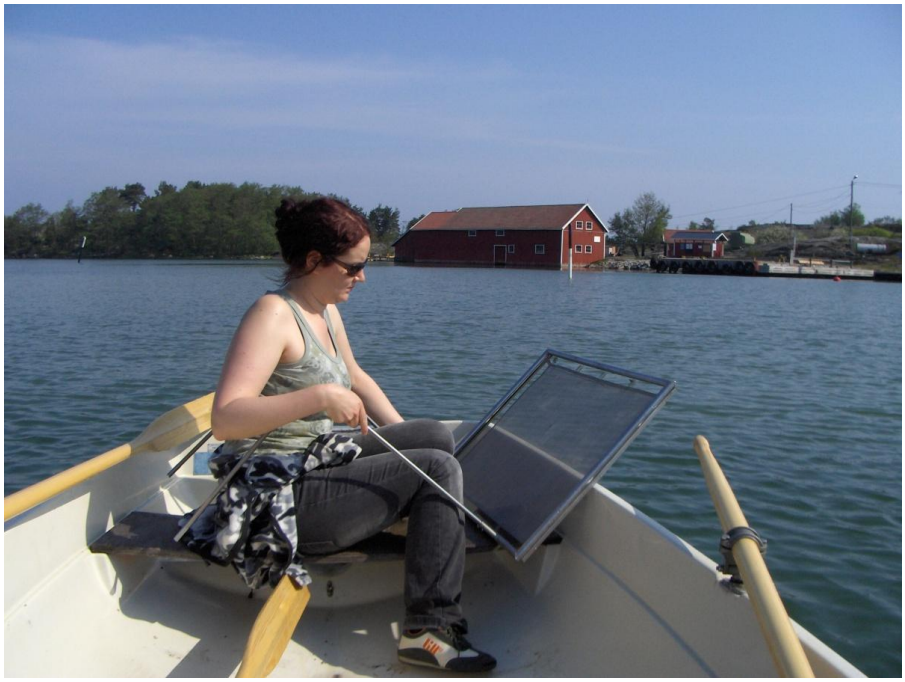

Figure 4. Sampling the SML with a Garrett metal screen in a small boat harbor in Stenskär, Nauvo.

Figure 5. Pristine sampling site in Stenskär, Nauvo.

3.3 Experimental setup

3.3.1 Impact of polyunsaturated aldehydes produced by diatoms on bacterial communities in the water phase

The experiment was performed at the mesocosm facility of the Umeå Marine Science Center (UMSC, Umeå, Sweden), between 3 May (day 0) and 22 May (day 19) 2010. The experiment was run in 12 indoor polyethylene mesocosm towers of 5 m depth and a volume of approximately 2000 L each. Thermal advection, achieved by heating the lowest section of the tanks, resulted in a slow mixing of the water column with a turnover time of approximately 24 h. All tanks were filled with unfiltered seawater (29 April) with its natural microbial community from the Bothnian Sea and were kept at 8 °C to 10 °C, corresponding to ambient field conditions outside the mesocosm. Nutrients were added to all tanks directly after filling to a concentration of 13 µM $NaNO_3$-N, 4 µM NH_4Cl-N and 4 µM NaH_2PO_4. The nutrient levels mimicked the unlimited conditions at the time of the spring bloom in the Bothnian Sea. Nutrient levels were regularly monitored on days 8 and 14 and adjusted if necessary with nitrate and phosphate and additionally, on day 17, with silicate, to initial conditions.

Two treatments were supplemented with two different *Skeletonema marinoi* strains (*S. marinoi* abbreviated SKE1 and *S. marinoi* abbreviated SKE2) having different growth and PUA-releasing characteristics. The addition of *Skeletonema* was adjusted to roughly double the concentration of chlorophyll a in the tanks after 4 days of growth, thereby leading to a *Skeletonema* dominated phytoplankton community throughout the experiment. Samples for bacterial diversity, bacterial abundance, viral abundance, chlorophyll a, phytoplankton cell abundance and inorganic nutrient determinations were taken 7 to 8 times during the experiment depending on the analysis.

3.3.2 Microcosm experiments

3.3.2.1 Diesel experiments in sea water

The samples were distributed in 2-L glass jars (1300 ml total volume). Triplicates of diesel spiked (0.2% w/v) microcosms and duplicates of controls without diesel were established with water from each of the sampling sites. Microcosms were shaken at 130 rpm at 15°C for 22 days under a 12:12 hour light-dark regime. Samples for molecular analyses were obtained at the start and then after 2, 7, 14 and 22 d. Samples for cloning and sequencing were selected based on T-RFLP results. Sequencing and qPCR were carried out with microcosm samples from pristine and oil-polluted site microcosms after 14-day incubation. Samples for hydrocarbon analysis were obtained from all diesel spiked microcosms 1 h after diesel addition and at the end of the experiment.

3.3.2.2 Crude oil and ^{14}C-naphthalene mineralization experiments with sediment and concretions

3.3.2.2.1 Mixture of concretions

A mixture of spherical concretions of different sizes was weighted (10 g ± 1 wet weight, WW) in flasks with 0.01 g (± 0.001) of Russian crude oil. Ten mL of artificial brackish sea water (Yli-Hemminki et al. 2014), was added into the flasks so that concretions were nearly covered. Crude oil formed a film on the water but the oil was still in touch with the concretions. Twelve anoxic flasks were gassed with N_2 for five minutes to gain anoxia, whereas 23 oxic flasks were aerated regularly every week. Three replicate oxic and/or anoxic flasks were emptied every sampling time by destructive sampling. For every sampling series there was an autoclaved control (autoclaved two times 60 min). Negative control contained only artificial brackish sea water and crude oil without concretions. Autoclaved and negative controls were aerated weekly. The flasks were incubated at 10 °C, in the darkness, and slowly (60 rpm) agitated.

3.3.2.2.2 Conretions LL4A and sediment LL4A

Sediment or concretions (10 g WW) from site LL4A or both (10 g WW total) were weighed into 100 mL flasks in triplicates. Ten mL of artificial brackish sea water was added into the flasks. One set of concretions as well as of sediment received nutrition addition (5 mM NH_4NO_3 and 0.35 mM K_2HPO_4) twice during the experiment to test the effects of the nutrients on naphthalene mineralization. Sediment and concretions together were autoclaved to form controls (120 °C for 60 min on three following days).

Oil and PAH analyses were performed in the beginning (after 1 h settling) and at the end of the experiment from two parallel sets. Destructive sampling emptied the whole bottle. Samples for DNA analysis and Fe and Mn measurements were taken from a third parallel set. All the experimental sets were incubated at 10°C in darkness, and slowly (70 rpm) agitated. In addition, the oxic experiment was aerated once a week by a 60 mL syringe whereas anaerobic experiment was driven anoxic by rinsing the flasks with nitrogen gas for at least five minutes.

3.3.2.2.3 Mineralization measurements

Approximately 3 mg crude oil and ^{14}C-labeled naphthalene was added to one set of LL4A flasks. ^{14}C-naphtalene mineralization to CO_2 was studied by radiorespirometry (Tuomi et al. 2004, Nyyssonen et al. 2006). An amount of 2 kBq of ring-labelled ^{14}C-naphtalene [specific activity 31.3 mCi/mmol; 99% radiochemical purity (Larodan Fine Chemicals Ab; Sweden)] was added to concretion or sediment samples in 100 mL infusion bottles. The sealed bottles were incubated for 75 days. The production of ^{14}C-labeled CO_2 in the bottles was monitored by 0.5 mL 1 M NaOH traps, which were analyzed in a Winspecial 1414 Liquid scintillation counter (Wallac Oy; Turku,

Finland) using Ultima Gold high flash point LSC- scintillation cocktail (PerkinElmer; MA, USA).

3.4 Bacterial community analysis

3.4.1 Cloning and sequencing

For cloning, the selected bacterial communities were first compared by using denaturing gradient gel electrophoresis (DGGE). Cluster analysis of DGGE bands showed that the bands varied less between the replicates than between the treatments. Triplicate DNAs were pooled before preparing the clone library. Total of 11 clone libraries were constructed and 987 sequences obtained from mixed concretion, LL4A concretion and LL4A sediment experiments. The cloned sequences were classified by means of RDP classifier with confidence threshold of 95% for classification to each phylogenetic level.

3.4.2 Terminal restriction fragment length polymorphism (T-RFLP) data analysis

For the T-RFLP data analysis (I, II) the fluorescence peaks were sized using the PeakScanner software (Applied Biosystems) with a threshold value of 50 relative fluorescence units. The peak data was further processed using the T-REX program (Culman et al., 2009) with default values for noise filtering and peak alignment and with a clustering threshold of 0.6. Data from replicate analyses of the same sample was averaged, peak heights were relativized within samples and TRFs occurring in less than 5% of samples were omitted. Data matrices containing relativized and aligned peak height data for each microcosm were exported to Primer 6 program (*PRIMER-E Ltd, United Kingdom*), which was used for non-metric multi-dimensional scaling (MDS) analysis and data clustering. Prior to these analyses, the data was log(x+1) transformed and a similarity matrix was constructed using Bray-Curtis distance measure.

3.4.3 OTU assignment and diversity analysis

In the diesel experiments (III) sequences were checked with the CHECK_CHIMERA program (Maidak et al., 2001) and putative chimeras were removed. The sequences were then compared with the GenBank nucleotide database using BLASTn. For the crude oil- and PAH-experiments (IV) Mothur program (Schloss et al. 2009) was used for sequence analysis, Venn diagram analysis, determination the operational taxonomical units (OTUs) and for the construction of rarefaction curves. The R software (version 3.1.0) was used for plotting the rarefaction graph. OTUs were assigned at 97% identity threshold and the diversity of OTUs in the initial concretion and sediment samples was compared by the rarefaction analysis (IV, Fig. S1).

3.4.4 Amplification of PAH-degradation genes

qPCR reactions were carried out in triplicate for the microcosms using a 7300 real time PCR instrument (Applied Biosystems). The primers used were: gram–positive (GP) forward 5′–CGG CGC CGA CAA YTT YGT NGG–3′ and reverse 5′–GGG GAA CAC GGT GCC RTG DAT RAA–3′ or gram–negative (GN) forward 5′–GAG ATG CAT ACC ACG TKG GTT GGA–3′ and reverse 5′–AGC TGT TGT TCG GGA AGA YWG TGC MGT T–3′ (Cebron et al. 2008), 0.3 µmol each. The 25 µL reaction contained also 12.5 µL Maxima™ SYBR Green qPCR Master Mix (Fermentas, Vilnius, Lithuania), and approximately 7.0-24.0 ng DNA sample. The DNA extracts were diluted 1:10 to reduce the effect of possible PCR inhibition. Thermal cycling conditions were 10 min at 95°C, 42 cycles of 15 s at 95°C, 30 s at 54°C, 30 s at 72°C, and 27 s at 86°C for GP reaction or 83°C for GN reaction. After the elongation step a fluorescence reading step was applied in order to exclude signals derived from primer dimer or unspecific products having melting point below 86°C (GP) or 83°C (GN). Melting curve analysis was performed after each run. Standards used in the analysis were *Escherichia coli* plasmids with PAH–RHDα inserts amplified and cloned from *Pseudomonas putida* G7 (GN) or *Mycobacterium vanbaalenii* DSM 7251 (GP). Standards with 3×10^{-1} and from 10^{0} to 10^{8} gene copies μL^{-1} were used for the calibration curve. The detection limit of the assay was 200 gene copies g^{-1} sediment or concretion. The amplification efficiency of the qPCR reactions, estimated by using the standard curve, was 100% ±10%. The average gene copy numbers of PAH–RHDα–genes of gram–positive and gram–negative bacteria were calculated per gram dry weight (DW) of concretions, using 65% dry matter, which was an average of the mixed concretions from different sites and of varying shapes and sizes.

3.5 **Statistical analysis**

To assess whether there are significant differences between the organic parameters such as DOC, high molecular weight (HMW), and low molecular weight (LMW) fractions of SML and SSW samples (I), a one-way ANOVA was performed. To compare SML and SSW at the same site, an independent t-test was used. The relation between the organic parameters and bacterial counts was assessed by the Pearson correlation coefficient r. Statistical analysis was performed with the statistical program SPSS 15.0 (IBM).

One–way ANOVA or Mann–Whitney non-parametric test analysis was performed for the crude oil and PAH experiment data (IV) using 95% confidence interval.The analyses were performed by the SPSS software (version 22, IBM Corp. Released 2013).

4. RESULTS AND DISCUSSION

4.1 Sea surface microlayer and subsurface water

T-RFLP analysis was carried out to compare SML and SSW bacterial community profiles. The aim was to determinate whether they differ from each other, because of the exposure of the SML bacteria to different environmental conditions with regard to UV-radiation, accumulation of pollutants and nutrients. The bacterial abundance was measured by colony counting and flow cytometric analysis. DOC, cytotoxicity, and metal concentrations (cadmium, copper, lead and mercury) was measured. Different time points and sites were tested and the samplings were performed between May and July in the summers of 2007 and 2008. There were four sampling sites in total.

4.1.1 Bacterial community profiles

A study comparing the SML and SSW bacterial communities in the Archipelago Sea in south-west Finland was carried out. The occurrence of differences in the community profiles was expected between the SML and SSW samples, since it has been shown in previous studies in other sea areas (Franklin et al. 2005, Cunliffe et al. 2009b). Our T-RFLP results (I) support the difference between SML and SSW bacterial communities. The samplings were performed in calm wind conditions and we focused on the non-particle attached fractions. Approximately half of the SML and SSW sampling pairs compared were considered to represent diverging communities. In general, more similarity was observed between bacterial community profiles from the SML of different sampling sites than between the SML and the SSW of the same site. Most of the R samples formed a completely separate cluster (I, Fig. 4). This is probably explained by its location nearest to the mainland coast in an active recreational harbor (Fig. 3). Apart from a few individual samples, the community profiles constructed with both HhaI and RsaI clustered in a similar manner, which suggests that the clusters are based on real differences between communities instead of random differences produced by the method.

4.1.2 Bacterial counts

The number of bacteria in the SML and SSW was measured by plating on marine agar and flow cytometric analysis. Plating resulted in bacterial numbers of 10^2-10^4 cfu/ml (I, Fig.2) and flow cytometric analysis in 10^5-10^6 cells/ml sea water (I, Fig.3). The number of cfu was slightly higher in most of the SML samples, showing significantly ($p<0.05$) larger numbers of cfu in third of the samplings. It is well known that only a portion of bacteria can be grown on a agar plate (Jannasch and Jones 1959, Xu et al. 1982, Ferguson et al. 1984, Eilers et al. 2000). In our experiment the fraction of culturable bacteria was approximately 2 % based on the total number achieved from the flow cytometric analysis.

4.1.3 DOC, cytotoxicity and metals

Different parameters were measured in our experiments: DOC (I, Table 2), cytotoxicity, and metal concentrations. Results from these analysis showed no significant differences between the SML and SSW samples. The amount of pollutants in the coastal SML is usually higher than in SSW, because of the direct input from the coastal areas. It has been shown that the amount of pollutants can be up to 500 times higher in the SML than in the underlying water column (Wurl and Obbard 2004). Fish eggs and larvae are often found in the SML and the pollution load can cause mortality and abnormality in them. In addition, the pollutants in the SML cause deleterious effects on marine food webs (Wurl and Obbard 2004). In the cold sea areas with small species diversity, like in the Baltic Sea, the effects can be even more harmful to the less complex food webs than to the more complex food webs of the warmer seas (Corsolini et al. 2002, Wurl and Obbard 2004).

4.1.4 The effect of sampling device

The 1 mm upper layer of the sea is suggested to form the sea surface microlayer (Liss and Duce 1997). The SML thickness and composition sampled varies when different samplers are used. Thus we performed an additional sampling to ensure that the community structure analysis differences between SML and SSW were not caused by the metal screen (MS) sampling method. Sampling was carried out in site R in October 2009 using both a polycarbonate membrane (PCM) and metal screen (MS). Based on our results the sampling technique did not seem to have a significant effect on any of the SML and SSW findings (I, Fig. 5). However, in some of the samples taken with PCM the DNA yield was lower, and we did not succeed in amplifying the 16S RNA gene from DNA preparations of some of the replicate polycarbonate membranes. In general, the use of PC membrane limits the number of analysis that can be performed. The purpose of the sampling must be taken into consideration when choosing the sampling device for the SML sampling (Table 1) (Wurl et al. 2014).

4.2 **The effect of diatom derived polyunsaturated aldehydes (PUAs) on bacterial communities**

The possible effect of *Skeletonema marinoi*-produced PUAs on bacterial community structure was studied in a mesocosm experiment. Previous studies suggest that these compounds might have a role in shaping the bacterioplankton composition (Adolph et al. 2004, Ribalet et al. 2008, Balestra et al. 2011). In addition, several other parameters (bacterial, phytoplankton and viral abundance, chlorophyll a concentration) were measured during the four-week mesocosm experiment using 2000 L tanks mimicking natural conditions with different treatments.

Table 2. Abbreviations for different treatments in the mesocosm experiment. SKE1 and SKE2 represent *Skeletonema marinoi* strains with a different growth and PUA-releasing characteristics, SKE2 producing higher amouts of PUAs than SKE1.

Mesocosm treatment	Abbreviation
Seawater (control)	CTRL
PUA addition	PUA+
S. marinoi addition	SKE1
S. marinoi addition	SKE2

4.2.1 Bacterial community profiles

A cluster analysis performed for the T-RFLP results of the study included samples taken during the first PUA addition (day 3), samples taken directly after the second PUA addition period (day 14) and samples taken towards the end of the experiment. The results showed how on the day 3, CTRL and PUA+ group close to each other, indicating a similar (> 80% similarity) bacterial community in these treatments (II, Fig. 5).

The bacterial community profiles, presented by a cluster analysis, showed the change of the bacterial community over time most clearly. Similar results have been gained previously in mesocosm experiments with *S. costatum* (Riemann et al. 2000). Other observed patterns were the similarity of CTRL and PUA+ at day 14. In general, at day 14 all samples, with the exception of SKE2, grouped together. However, after three days SKE2 grouped again together with SKE1, possibly indicating only a temporary change in the community.

4.2.2 Bacterial counts

The bacterial abundance was analysed using an epifluorescence microscope. At the beginning of the experiment there was 1×10^6 cells mL^{-1} in all treatments. This corresponded to the natural bacterial community of the Bothnian Sea, as the tanks were filled with seawater. No differences in the bacterial abundance between the treatments were detected during the first 10 days. Later significantly enhanced bacterial growth was observed in both CTRL and PUA+ mesocosms when compared to SKE1 and SKE2 (II, Fig. 3A).

Obviously the presence of PUA was not the factor limiting bacterial growth, since PUA+ mesocosms did not differ from the CTRL during the experiment. Even though the PUA concentrations applied in the experiment were way above those reached in nature. Competition for essential nutrient might have caused the suppression of bacterial growth in the SKE1 and SKE2 mesocoms (Rhee 1972). Also, the

antimicrobial activity of *Skeletonema costatum* has been described (Naviner et al. 1999, Sarno et al. 2007). *S. costatum* has been shown to produce antimicrobial substances especially during its steady-state growth phase (Terekhova et al. 2009). This might explain the decreased bacterial abundance in our experiment. Our results suggest, however, that the antibacterial activity of PUAs detected in laboratory studies (Adolph et al. 2004, Ribalet et al. 2008) is not sufficient to inhibit bacterial growth in the plankton.

4.3 Petroleum hydrocarbon-degrading bacteria in the Baltic Sea

4.3.1 Diesel experiments

Microcosm experiments were carried out to investigate the diesel-degradation capacity and main bacterial groups responsible of it. The hypothesis was that samples from the Finnish Archipelago Sea water would host bacteria capable of oil degradation. After oil exposure these bacteria would proliferate in the experiment. Through oil-amendment experiments with water from pristine and polluted sites we sought to identify bacteria involved in oil degradation.

4.3.2 Bacterial community structure

The bacterial communities of the three studied sites differed from each other according to the multidimensional scaling plot and hierarchical clustering constructed from T-RFLP-analysis data. Communities in microcosms with diesel addition diverged from the controls and formed a separate cluster within each series of microcosms (A, P, R) (III, Fig. 2 and 3). T-RFLP profiles showed that the average T-RF richness at the pristine site, at the oil polluted site and at the recreational harbor was 60, 40 and 70 peaks, respectively. The species richness has been shown to decrease during bioremediation, because of the selection towards oil-degrading bacterial groups (Roling et al. 2002, Looper et al.). The dominant bacterial groups were determined after a 14-day diesel exposure by sequencing. The sequence analysis show how the dominant groups differed between the sampling sites (III, Fig. 4).

In the polluted site (P) microcosm was dominated by *Betaproteobacteria* after 14-day incubation. 52% of the clones belonged to the order *Burkholderiales* and in more detail family *Comamonadaceae*. Burkholderia are especially known to degrade petroleum compounds in soil (Okoh 2003, Yrjala et al. 2010). In addition, petroleum-degrading bacteria belonging to the *Comamonadaceae* family have been found for example in coking wastewater (Geng et al. 2014) and in a waste water plant of an oil refinery (Rouviere et al. 2003). Relative abundance of *Betaproteobacteria* and *Actinobacteria* has been shown to increase in lower salinity areas in the Baltic Sea as *Alpha-* and *Gammaproteobacteria* are more abundant in higher salinity areas (Herlemann et al. 2011). Studies conducted in estuaries also show the dominance of *Betaproteobacteria* in lower salinities and *Alphaproteobacteria* in higher salinities (Bouvier and del Giorgio 2002, Cottrell and Kirchman 2003, Zhang et al. 2006).

Sequence analysis revealed the pristine site (A) microcosm with diesel exposure to differ from the polluted site microcosm; *Actinobacteria* (32%) was the largest group, followed by *Alphaproteobacteria* (25%) (Fig. 8). *Actinobacteria* are known to be responsible for diesel and fuel oil degradation in soil (Chikere et al. 2009, Hamamura et al. 2006, Lin et al. 2010) and have been used for bioremediation of petroleum-contaminated soils (Alvarez et al. 2011, Baek et al. 2007). *Cyanobacteria* were also abundant (15%) in the pristine site (A) diesel-exposed microcosm. They are thought to contribute indirectly to the oil degradation process (Abed 2010, Harayama et al. 2004, Ibraheem 2010, Tang et al. 2010). Using the released CO_2 they provide O_2 to aerobic oil-degrading bacteria and cyanobacteria are also known to produce compounds (biosurfactants, extracellular substances) which increase the pollutant bioavailability (Tang et al. 2010). For example during the Gulf War in Kuwait, cyanobacteria blooms emerged along with oil release into the sea (Ibraheem 2010, Sorkhoh et al. 1992). *Alphaproteobacteria* was the dominating group in the control microcosms without diesel addition (54% in both microcosms). The second largest group was *Betaproteobacteria* (23% in pristine and 32% in polluted site microcosm without diesel addition). Since *Alphaproteobacteria* has been earlier reported to be enriched in bottle cultures in laboratory conditions (Riemann et al. 2008), it is possible that the microcosm experiment was a selection factor in our control treatments.

Several studies have shown that there are natural petroleum hydrocarbon-degrading microbes in different environments. Main bacterial genera reported to degrade oil in marine environment are *Alcanivorax* spp., *Oleiphilus* spp., *Oleispira* spp. and *Thalassolituus* spp. (Head et al. 2006) and in some areas for example *Acinetobacter* and *Pseudomonas* (Wang et al. 2010). All these genera belong to the *Gammaproteobacteria*, which are often described to be the main bacterial group responsible of oil degradation in the sea (Al-Awadhi et al. 2012, Baelum et al. 2012, Hazen et al. 2010, Head et al. 2006, McKew et al. 2007, Wang et al. 2010). However, bacterial communities of the Baltic Sea are influenced by freshwater species, especially in the northern, less saline, areas of the Baltic and near river outflows (Holmfeldt et al. 2009, Vaatanen 1982). It has been suggested, that there exists a unique brackish water bacterial community in the Baltic Sea (Riemann et al. 2008) consisting of freshwater and marine species. In addition, our sampling sites were located in harbors or near the coast, which makes it logical that the oil degrading bacterial groups in our experiment were similar to bacteria, which are previously known degraders in soil.

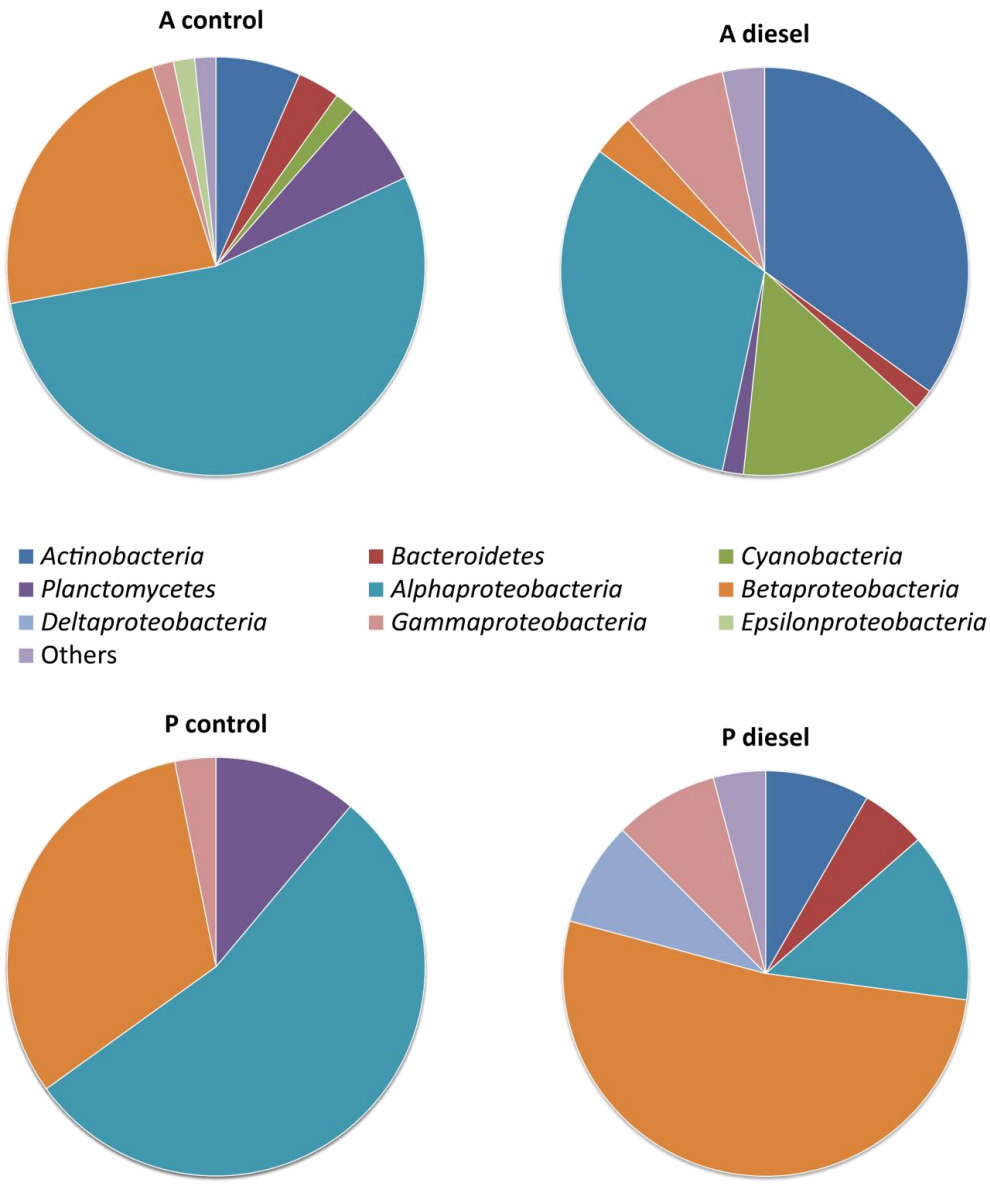

Figure 8. Bacterial phyla in the microcosms after 14 days of incubation. The distribution is based on clone libraries of 16S rRNA genes. A: pristine site, P: previously exposed site. Number of clones sequenced: A control 61, A diesel 60, P control 63, P diesel 96. *Actinobacteria*, followed with *Alphaproteobacteria* and *Cyanobacteria*, became dominant in the pristine site (A) microcosm with diesel exposure and *Betaproteobacteria* in the polluted site (P) microcosm with diesel exposure.

4.3.2.1 PAH-degradation genes

PAH degradation potential was observed at both pristine (A) and previously polluted (P) sites. The number of gram-positive PAH-degradation genes was high in both in situ samples (III, Fig. 5). Gram-negative genes were observed in situ only at the polluted site. GN PAH-degradation genes were enriched in all microcosms during our experiment. The highest copy numbers were observed in the diesel microcosms from the previously polluted site. The gram negative order *Burkholderiales* was predominant in these microcosms. The number of PAH-RHD GP genes was notably lower than GN gene copy number after the diesel exposure. Similar pattern of large GN PAH-degradation gene enrichment was observed in the study of Wang et al. (2014) at the Yellow Sea after crude oil exposure.

Since certain oil- and PAH-degradation genes have been shown to correlate with the bacterial degradation potential, the functional gene abundances can be used to assess bioremediation efficacy. For example the abundance of *nahAc* genes, related to hydroxylation of the aromatic ring of naphthalene (one of the GN PAH-RHDα genes), have been shown to correlate with ^{14}C-naphtalene mineralization potential in oil contaminated soil (Tuomi et al. 2004, Salminen et al. 2008). However, the use of alkane hydroxylase gene *alkB* for direct monitoring of degradation rates was considered not to be suitable, as the expression was observed both in control and oil spiked microcosms by Paisse et al. (2011). The abundance and expression of different degradation genes vary in different phases of the process, since many different bacterial groups are usually involved in the degradation process (Roling et al. 2002). This should be considered when estimating the bacterial degradation potential of petroleum hydrocarbons in the environment.

4.4 Crude oil and PAH -experiments

In these experiments, the difference in crude oil and naphthalene degradation efficiency between Fe-Mn concretions and sediment was tested in microcosm experiments. We wanted to investigate how crude oil and PAHs are biodegraded under oxic and anoxic conditions in these different bottoms of the Baltic Sea. The aim was to study natural recovery of these bottom types after oil contamination and gain information of the dominant bacterial groups responsible of the crude oil and PAH-degradation.

4.4.1 Disappearance of crude oil and PAHs by concretions and sediment in oxic and anoxic conditions

4.4.1.1 Mixed concretions

In the experiment with mixed concretions 59% (oxic) and 51% (anoxic) of the added oil disappeared after seventeen weeks of incubation. In the beginning of the experiment the oil disappearance rate was observed to be faster in the oxic than in the anoxic

conditions (IV, Fig. 1), but later the disappearance rates were approximately at the same level. No crude oil disappearance was observed in the autoclaved controls containing only artificial sea water and crude oil. However, the oil amount dropped 46% after seven weeks in the controls with autoclaved concretions. This suggests that the actual biological degradation would be approximately 13% in oxic and 5-10% in anoxic incubations. On the other hand the disappearance of the added crude oil from the autoclaved microcosms may partly be explained by the fact that autoclaving changed the chemical structure of the concretions and sediment and thus altered their reactivity or sorption properties (Tuominen et al 1994). This is supported by the stable concentrations of oil in the aerated control incubations containing only water and crude oil (IV, Table 2). In addition, the oil disappearance rates in the mixed concretion microcosms were fast (IV, Fig. 1), whereas in the autoclaved controls the decrease in the crude–oil amount happened drastically once after seven weeks and then stayed at constant level. It is possible that a long incubation time caused recovery of bacteria in the autoclaved samples. However, no sign of recovery was observed based on the naphthalene mineralization (IV, Table 2) or the disappearance pattern of total PAH fractions (IV, Fig. 2).

4.4.1.2 LL4A concretions and sediment

In the microcosms with LL4A concretions 70–-81% of the crude oil disappeared in the oxic conditions after 11 weeks incubation (IV, Table 2). In the sediment microcosms the amount was 54–80%. In anoxic conditions the amounts of crude oil diminished by 35–61% and 41–47% in the LL4A concretions and sediment, respectively. Crude oil diminished also in the autoclaved controls, including both LL4A sediment and concretions, by 10% in the anoxic and 80% in the oxic experiment. This unexpected result is difficult to explain. One explanation may be that the autoclaving causes a release of metals from the concretions and sediment and thus the chemical structure of the autoclaved control is different than that of the non-autoclaved control. This may lead to unpredictable chemical reactions. Therefore autoclaving is perhaps not the best way of producing controls for studying abiotic reactions. Autoclaving was meant to sterilize the concretions, and for that it seems to work. In the experiment with ^{14}C-naphthalene, its mineralization occurred only in the non-autoclaved microcosms, showing biological degradation.

The concentration of 18 different PAH compounds was analysed in the LL4A experiment (IV, Table S1 and S2). The analysis included 1–methylnaphthalene and 2–methylnaphthalene, which are not among the 16 commonly analysed EPA PAHs. These two compounds constituted >95% of the total PAHs in the crude oil in this study. The amount of total PAHs diminished (96%) in all incubations. Even high molecular weight PAHs diminished during the experiment. The disappearance of PAHs was up to 79% also in the autoclaved oxic controls. However, the pattern of PAHs differed between the autoclaved and the non-autoclaved microcosms (IV, Fig. 2), indicating that mineralization had an important role in the disappearance of the

PAHs. In the non-autoclaved microcosms the PAHs consisted mainly of 4-, 5-, and 6-ringed PAHs, whereas in the autoclaved controls the 2-,3-, and 4-ringed PAHs were most abundant in the end of the experiment (IV, Fig. 2) Less PAHs (35%) disappeared in the autoclaved controls (IV, Table 2), otherwise results were similar in the anoxic experiment.

It has been shown in laboratory experiments, that crude oil degradation rates vary depending on experiment conditions, e.g. temperature. In a ten-week experiment by McKew et al. (2007) > 99% of low molecular weight alkanes, 41-84% of high molecular-weight alkanes and 32-88% of PAHs were degraded in a temperature of 20°C. Temperature in the bottom of the Baltic Sea varies between 4–10°C. In these temperatures the degradation of complex compounds might be slower compared to 20°C. Less efficient petroleum hydrocarbon degradation rate in low temperatures has been showed in many studies (Michaud et al. 2004, Brakstad and Bonaunet 2006, Coulon et al. 2007). However, no significant difference was observed by Delille et al. (2009) in 4°C than in 20°C and crude degradation has been shown to occur efficiently even in -1°C seawater (McFarlin et al. 2014).

Our results suggest that, if taking into consideration the amount of crude oil diminished in the autoclaved control units, the proportion of biological degradation was at least 13 % and 5 % for mixed concretions, in the oxic and anoxic conditions, respectively. For the LL4A concretions it was at least 25-50% and around 30% in the sediment in the anoxic experiment. However, as the abiotic controls may have been altered during the autoclaving, this overestimates the abiotic disappearance, thus affecting these values.

4.4.2 Mineralization of ^{14}C-naphthalene

4.4.2.1 LL4A concretions and sediment

The amount of ^{14}C–naphthalene mineralized during the 17-week experiment remained rather low in both oxic and anoxic experiments (IV, Fig. 3). The highest amount mineralized was 14% in the oxic sediment flasks. The nutrient addition did not enhance the mineralization; only 5% of ^{14}C–naphthalene was mineralized in the sediment flasks with nutrient addition. In the anoxic experiment only 1–4% of the ^{14}C–naphthalene was mineralized (IV, Table 2, Fig. 3). In the controls without crude oil the mineralization of ^{14}C–naphthalene started rapidly. Only 0.2% of ^{14}C–naphthalene was mineralized in the autoclaved microcosms (IV, Table 2, Fig. 3) showing the mineralization in other microcosms to be due to biological activity.

4.4.3 Quantification of PAH-degradation genes

4.4.3.1 Mixed concretions

PAH ring–hydroxylating dioxygenase (PAH–RHDα) is an enzyme acting in the initial step of aerobic metabolism of PAH. We carried out quantitative PCR to detect the number gene copies encoding this enzyme. In the experiments with mixed concretions and 5–6 mg of crude oil (no naphthalene addition) the copy number of the gene encoding gram positive PAH ring–hydroxylating dioxygenase (GP PAH–RHDα) increased in both oxic (1.8×10^5 $\pm9.4\times10^4$) and anoxic (1.3×10^5 $\pm1.3\times10^5$) incubations during the first four weeks, but there was no significant difference. In the end of the experiment the gram-negative (GN) and GP PAH-RHDα gene copy levels were approximately at the same level as in the initial samples (IV, Table 2). Traces of oxygen in the beginning of the experiment may have increased the gene copy numbers also in anoxic conditions. It is beneficial for bacteria to keep the gene also in the anoxic conditions as the oxygen levels in the sediment vary. These aerobic PAH degradation-genes have been for example observed in anoxic petroleum–hydrocarbon contaminated subsurface soil (Tuomi et al. 2004).

4.4.3.2 LL4A concretions and sediment

In the experiments with LL4A concretions and sediment the increase of GN PAH-RHDα gene was notable; especially in the oxic LL4A concretion flasks the number of GN PAH–RHDα gene copies increased 1000-fold (IV, Table 2). In the oxic LL4A sediment experiment the number of GN PAH-RHDα gene copies increased during the experiment significantly ($p < 0.05$) in the flasks with nutrient additions. Instead, the gene copy number of GP PAH–RHDα stayed relatively stable during both the oxic and anoxic experiments.

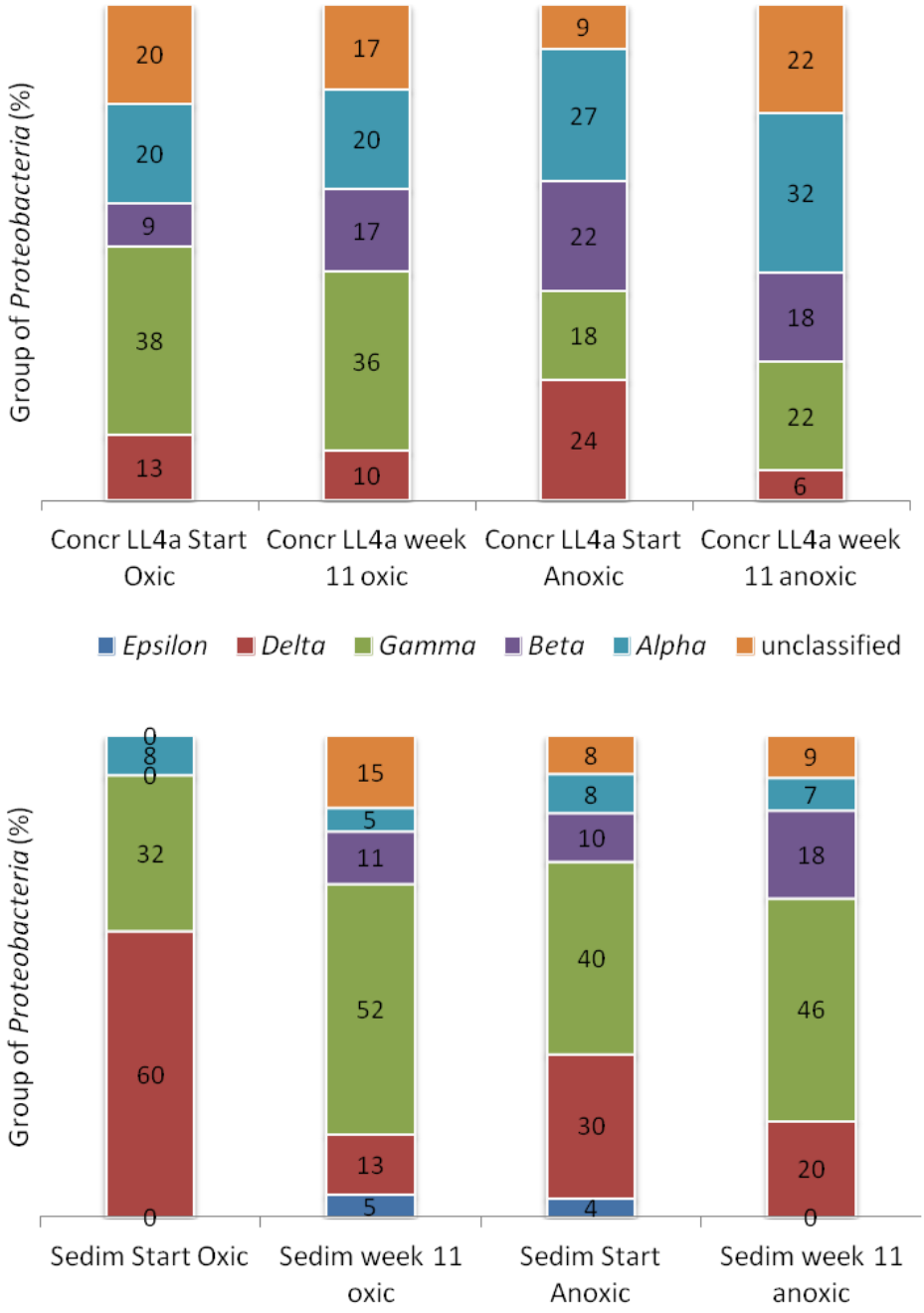

Figure 10. The proportions (%) of different classes of *Proteobacteria* in the microcosms. Cloned sequences classified as *Proteobacteria* from the LL4A concretions (upper figure) and sediments (lower figure) were classified to different classes of *Proteobacteria*.

4.4.4 Bacterial community structure in the concretion and sediment microcosms

Not the oil addition, but rather the concretion as a special habitat, seemed to be the selective factor for bacterial community; after the crude oil exposure clones from the concretion microcosms affiliated to clones previously found from concretions (Yli-Hemminki et al. 2014) and other metal-rich environments (IV, Table 3). In the sediment microcosms the bacterial communities changed more towards clones previously found from oil polluted sediment. PAH and metal pollution has been shown to shape bacterial communities in estuarine areas, metals being even stronger factor modifying the communities (Sun et al. 2012, Sun et al. 2013).

Approximately 50% of initial bacterial sequences from concretion and sediment samples could be assigned to to *Proteobacteria* (Fig. 10, IV, Fig. 4). The proportion of bacterial sequences remaining unclassified were 35-40% in the concretions and 20–29% in the sediment samples. The largest group of *Proteobacteria* in the initial concretion samples was *Gammaproteobacteria* (30–38%) whereas in the sediment it was *Deltaproteobacteria* (30–60%) (IV, Fig.5). *Epsilonproteobacteria* were observed only in the sediment samples (4% of the *Proteobacteria*). Mixed concretion samples had the highest proportion of unclassified *Proteobacteria* (32%). After the oil exposure mainly *Alpha-* and *Betaproteobacteria* were enriched in the concretions and *Beta-* and *Gammaproteobacteria* in the sediment (Fig. 10, IV, Fig. 5).

In many previous studies (Röling et al. 2002, McKew et al. 2007, reviewed in Head et al. 2006 and Yakimov et al. 2007) bacterial diversity has been shown to decrease during oil exposure favouring the enrichment of known oil–degrading species such as *Thalassolituus oleivorans*, *Cycloclasticus* and *Alcanivorax*. This was not observed in the study of Dell'Anno et al. (2012) which showed biodegradation efficiency to increase along with the bacterial richness and evenness. In our study the number of OTUs was approximately the same after different treatments when compared to the initial samples. Rarefaction curves (IV, Fig. S1) showed that the number of OTUs were slightly higher in the initial sediment than in the initial concretions. We obtained total number of 515 OTUs in the 11 clone libraries sequenced (at 0.03 distance). The proportion of shared OTUs between the initial LL4A concretions and sediment was only 8.9% and the value was even lower (2.3%) between the initial samples of mixed concretions and LL4A sediment.

In general, a large fraction (99%) of the cloned sequences matched to uncultured clones which could not be classified to bacterial order or family. The cloned sequences were compared to their closest matches in the GenBank and categorized into ecotypes based on the environment they were from (IV, Fig. 6). We observed the largest proportion of the clones (30 of 90 sequences) from the initial mixed concretions being similar (90–100%) to the sequences earlier found from the Baltic concretions by Yli–Hemminki et al. (2014). These 30 clones consisted of only 8 different closest matches (classified as *Gammaproteobacteria* and *Betaproteobacteria*) (IV, Fig. 6, Table 3).

This was also observed within all of the initial LL4A concretions, where *Gamma-* and *Deltaproteobacteria* were classified as the main groups (IV, Table S3). The sediment samples showed a different pattern and only few clones from sediment samples matched to the sequences from the Fe–Mn concretions of the Baltic Sea (Yli–Hemminki et al. 2014) (IV, Fig. 6). After the oxic incubation of sediment the proportion of clones matching to sequences originating from sediment and oil–contaminated sites, as well as from water or waste water origin, increased. Clones classified as *Gammaproteobacteria*, similar to sequences originating from oil–contaminated sediment (Paisse et al. 2010) and Baltic Sea sediment (Edlund et al. 2008) enriched. After anoxic incubation of sediment there was also increase in clones which matched to sequences originating from metal–containing environments such as Mn–rich sediment (Vandieken et al. 2012).

5. CONCLUSIONS

- Difference between the sea surface microlayer and subsurface bacterial communities in the Baltic Sea was observed: bacterial community profiles from the sea surface microlayer of different sampling sites were more similar with each other than between the sea surface microlayer and the subsurface water of the same site. The number of bacteria was somewhat higher in most of the sea surface microlayer samples than in the subsurface water.

- In this study the diatom-derived polyunsaturated aldehydes did not have an effect on bacterial community structure or abundance. The sampling time was the factor which seemed to modify the community structure more strongly. Bacterial community structure changed over time in the experiment. The mesocosms with two different *Skeletonema marinoi* -strains seemed to develop unique and dynamic bacterial communities related to these algal blooms. The results show that factors other than PUA are of significance for interactions between diatoms and bacteria.

- The dominant bacterial groups enriched in the presence of oil were shown to be variable, but seemed to possess similar capacity to degrade oil compounds. Bacterial groups were different depending on the sampling location, as well as the target habitat (water, sediment, iron-manganese concretions). The number of different bacterial groups decreased in the water microcosms with diesel additions. However, this was not detected in the sediment and concretion experiments. The oil and polycyclic aromatic hydrocarbon degradation processes seemed to be as efficient in the Fe-Mn concretions as in the surrounding sediment, although bacteria responsible of it were different.

6. FUTURE PERSPECTIVE

Information of the sea surface microlayer and bacteria inhabiting it is crucial, as it is an interface between the sea and atmosphere having an important role in climate processes, gas exchange and in the fate of pollutants and other compounds (Wurl et al. 2014). In addition to bacteria, diatoms play an important role of carbon cycle and biochemical cycles of other important elements in the oceans. Studying the diatom-bacteria interactions helps us to interpret these processes and how e.g. climate change affects them (Amin et al. 2012). The Baltic Sea differs from the oceans, and when it comes to oil spill response, species conservation and maritime planning this has to be taken into consideration. Knowledge of bacterial community structure and function is important for applying proper methods for management of this vulnerable sea area.

The amount of oil transport is constantly increasing and because of new oil terminals opened in Russia, especially the Gulf of Finland has become one of the risk areas (Nikula and Tynkkynen 2007). In addition to development of preventing measures and mechanical oil spill response, it is important to plan methods to improve the recovery of contaminated sites. Petroleum hydrocarbon degradation capacity of indigenous bacteria is previously not comprehensively studied in the Baltic Sea. This study provided valuable information on diesel, crude oil and PAH degradation processes in the Baltic Sea. Depending on the sampling location, as well as the target habitat, dominant bacterial groups enriched in the presence of oil are variable, but seem to possess similar capacity to degrade oil compounds. In our experiments the Fe-Mn concretion bottoms of the Baltic Sea were for the first time shown to have potential for crude oil and PAH degradation.

In future more extensive sequencing with modern high-throughput sequencing methods would be interesting to carry out. Our clone libraries were very small and the real benefit of using next generation sequencing would be to get a much more covering picture of the diversity of the community. In addition, studying the active members of the bacterial community by using e.g. RNA-based methods would be important. Also, methods such as single cell genomics could create a link between the taxonomy and function of single species of bacteria. Other challenges could be on the study of the anaerobic petroleum hydrocarbon degradation using molecular tools. In general, also the study of a larger suite of genes involved in petroleum hydrocarbon degradation. The limitations of laboratory experiments should be carefully taken into account when interpreting the results and applying them to the natural conditions. However, the results of this thesis give a great foundation for more studies on the topic. For efficient bioremediation it is important to understand the function of microbial populations responsible of crude oil degradation in different habitats of the Baltic Sea.

ACKNOWLEDGEMENTS

This study was carried out in the Laboratory of Animal Physiology in the University of Turku and in the Marine Research Centre in the Finnish Environment Institute SYKE in Helsinki. The work was funded by the Nessling Foundation, Turku University Foundation, Finnish Cultural Foundation, Maa- ja vesitekniikan tuki ry and Rotary Club Finlandia Hall.

First I want to thank my supervisors, Piia Leskinen who supervised my thesis work for the first years and Kirsten Jørgensen who gave me an opportunity to finalize my thesis in SYKE. Kirsten, I have really learned a lot from you. You are always so helpful, motivating and on a good mood! I also want to thank professor Mikko Nikinmaa, it has been an honour to do my PhD thesis under your guidance. In addition, I am grateful to everyone in the Laboratory of Animal Physiology and in the Laboratory of Genetics in the University of Turku. I especially want to thank Nina Vuori for helping me in the laboratory and Mirella Kanerva and Kristiina Vuori, who have been great friends and "supervisors" for me during my work. I also want to thank many other PhD students and post docs who I got to know while I was in Turku.

I sincerely thank professor Marja Tiirola and Dr. Matthias Labrenz for reviewing this thesis and giving valuable comments.

I have worked in many different places and I want to thank all my great co-authors, in Finland and abroad. I am very grateful for having a possibility to author also other than PhD project-related articles during the last few years. I especially thank Sigitas Sulcius, researchers in the Centre for Maritime Studies of the University of Turku and members of the SCOR Working Group on Sea-Surface Microlayers. I have learned a lot from you all.

In addition to my supervisor Kirsten, I want to thank other people in Marine Research Centre in the Finnish Environment Institute. Pirjo, Aura, Noora, Raisa, Anna S., Eeva and other PhD students, I want to thank you for your support, friendship and interesting discussions. Many thanks to my co-authors Jouni Lehtoranta and Jari Nuutinen too. I also want to thank the technical personnel in Hakuninmaa, especially Kaisa Heinonen and Ilse Heiskanen, and Anne Markkanen, who performed the oil analysis and Timo Sara-Aho for metal analytics.

During my PhD work I got an opportunity to work in the laboratory of professor Lasse Riemann in Kalmar. I am really grateful for that and I want to thank Lasse and his research group and the laboratory staff in Kalmar. I also want to thank Lasse for co-authoring one of my articles and giving me really good comments for improving it. I also spent time in the beautiful Umeå Marine Sciences Center and I want to thank all the researchers working there with me as well as the technical personnel.

I spent the year 2011 in Pori which was one of the most wonderful years of my life. I want to thank Sari Repka from the Centre for Maritime Studies of the University of Turku for giving me an interesting position while I was looking for a new supervisor for my PhD thesis. I also want to thank the whole staff in the Centre for Maritime Studies.

I have a large number of friends to thank. First of all my friends from the University of Helsinki: Leena, Hanna, Aura, Stiina, Vesa, Päivi, Tuomas and many other microbiologists - thank you, you are valuable for me both as friends and colleagues! And especially thank you Leena Pitkänen for being such a helpful when I needed guidance from a microbiologist while working in Turku. Secondly I want to thank all the amazing members of TYRMY and other friends in Turku, you really made my time in Turku fun! Heidi, Ville, Tuomo, Lilli, Riina, Eeva, Toni and so many others: thank you and let's keep on rocking! In addition, I have many many wonderful friends to thank in Helsinki, Tampere and Pori. You are all dear to me and help me to forget work from time to time, thank you for that.

Finally, I want to thank my parents for their support, which I experienced through words, food and funding. During my thesis work my sisters have helped me through the fields of their expertise, I thank you for that Jenni and Emma. My dear husband Antti, I want to thank you for all the love, support and technical help with my thesis, you are the best! Finally I want to thank my cats Edvin, Usva and Bertsa for all the supportive purring and my miniature schnauzer Elli for relaxing outdoor activities.

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