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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

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ABSTRACT

The natural petroleum hydrocarbon degrading capacity of the Archipelago Sea water in S-W Finland was studied in a microcosm experiment. Pristine and previously oil exposed sites were examined. Bacterial community fingerprinting was performed using terminal restriction fragment length polymorphism (T-RFLP) and samples from selected microcosms were sequenced. The abundance of PAH degradation genes was measured by quantitative PCR. Bacterial communities in diesel exposed microcosms diverged from control microcosms during the experiment. Gram positive PAH degradation genes dominated at both sites in situ, whereas gram negative PAH degrading genes became enriched in diesel microcosms. The dominant bacterial groups after a 14 days of diesel exposure were different depending on the sampling site, belonging to the class *Actinobacteria* (32%) at a pristine site and *Betaproteobacteria* (52%) at a previously oil exposed site. The hydrocarbon degrading bacteria in the Baltic Sea differ from those in the oceans, where most hydrocarbon degraders belong to *Gammaproteobacteria*.

1. Introduction

The Baltic Sea is one of the largest brackish water bodies in the world with a coastline shared by nine industrialized countries. Therefore, many busy shipping routes traverse the Baltic Sea and as much as 166 million tonnes of oil was transported in 2009. There is no information on the actual oil pollution load to the Baltic Sea, but the yearly ~300 deliberate illegal oil discharges from ships and ~13 tanker accidents, as well as numerous minor leakages from ships and recreational boats, represent a serious environmental concern (Helcom, 2012). Moreover, there is a considerable risk of a single major oil spill since the Baltic Sea is difficult to navigate, with its narrow straits, shallow depths, crossing shipping lanes and a continuously increasing maritime traffic.

The Finnish Archipelago Sea in the north eastern Baltic Sea represents a particularly vulnerable ecosystem. In case of a major accident, oil spilled here would contaminate numerous ecologically sensitive and unique bays and beaches. In particular, numerous islands and shallow depths hamper oil dispersal. Such features make oil degradation slower than in the open sea, and in some cases, the ecosystem never returns to the original state (Ikavalko et al., 2005). In addition to ecological losses, a major oil spill in the Finnish Archipelago would significantly affect its recreational and economic value.

The capacity of indigenous bacteria to degrade oil appears widespread in oceanic and terrestrial environments (Foght, 2008; Head et al., 2006; Leahy and Colwell, 1990; Yakimov et al., 2007). In particular, *Gammaproteobacteria* has been shown to degrade oil in the oceans (Al-Awadhi et al., 2012; Baelum et al., 2012; Hazen et al., 2010; Head et al., 2006; Wang et al., 2010). In contrast, few studies are available on biodegradation of oil in the brackish Baltic Sea. In early studies, crude oil degrading bacteria were successfully isolated from the Baltic Sea (Bruns et al., 1993; Linden et al., 1987) and more recently addition of diesel oil was shown to stimulate bacterioplankton growth in a mesocosm experiment with water from the Baltic Sea (Suni et al., 2007). However, detailed information on the identity of oil degrading Baltic bacteria and their potential responsiveness to oil pollution is currently lacking. With the aim of understanding and ultimately predicting the ability of the marine biota to cope with an extensive oil pollution knowledge of the response of the bacterioplankton is essential. Since indigenous Baltic bacterioplankton assemblages are influenced by freshwater bacterial species (Riemann et al., 2008), especially in the northern Baltic Sea and nearby river outlets (Holmfeldt et al., 2009; Vaatanen, 1982), and since they differ in composition

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relative to oceanic bacterial communities (Herlemann et al., 2011; Riemann et al., 2008), the ability and capacity of the bacterioplankton to degrade oil is probably different in the Baltic Sea than in the oceans. Moreover, this will likely also be affected by the unique conditions (e.g., nutrient and carbon load, climate, and hydrology) prevailing in the Baltic Sea.

Although a variety of oil-degrading molds, yeasts, unicellular algae and protozoa have been isolated, bacteria seem to assume the greatest importance in oil degradation in marine environments (Leahy and Colwell, 1990). While terrestrial petroleum hydrocarbon degrading bacteria commonly utilize a range of substrates, most of their marine counterparts are highly specialized, using hydrocarbons as the sole carbon source (Yakimov et al., 2007). Crude oil consists of a large variety of petroleum hydrocarbons that have different chemical properties. Typically, one species of bacteria can metabolize only certain hydrocarbons and thus a succession of different bacteria is needed for the complete break up of oil. Further, petroleum hydrocarbon-degrading communities also need members responsible for other functions, such as production of surfactants, which makes the oil more accessible to the degraders (Head et al., 2006). In fact, only 10–20% of microorganisms extracted from an oil-polluted environment can effectively degrade petroleum hydrocarbons (Dai et al., 2004; Sugiura et al., 1997). The most toxic components of crude oil are the polycyclic aromatic hydrocarbons (PAHs). Although their structures make them relatively persistent for biodegradation, they can for instance be degraded through hydroxylation by dioxygenases encoded by bacterial PAH ring hydroxylating dioxygenase (PAH-RHDα) genes. Under aerobic conditions, both gram-negative (GN) and gram-positive (GP) bacteria may express these genes (Habe and Omori, 2003); consequently, enrichment of bacterial PAH-RHDα genes has been used as an indicator of PAH contamination level in environmental samples by Cebron et al. (2008). These authors developed two robust primer sets that amplify all known PAH-RHDα genes in the databases, i.e. four different in gram positive bacteria and 13 different in gram negative bacteria.

We hypothesized that samples from the Finnish Archipelago Sea in the Baltic Sea would host bacteria capable of oil degradation that would proliferate once exposed to oil. Through oil-amendment experiments with water from pristine and polluted sites we sought to identify bacteria actively involved in oil degradation.

2. Materials and methods

2.1. Sampling

Seawater samples were collected in September 2008 from a pristine site (Askainen), a recreational harbor (Raisio), and an oil polluted harbor (Pansio) located in the Archipelago Sea SW Finland (Table 1). Ten-L water samples were obtained from 1-m depth using a Limnos water sampler (Turku, Finland). In situ water temperature was 15 °C. The samples were stored in the dark at 4 °C and processed within 2 h.

2.2. Microcosms

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 h light–dark regime. Samples for molecular analyses were obtained at the start and then after 2, 7, 14 and 22 days. Samples for cloning and sequencing were selected based on T-RFLP results. Sequencing and qPCR was 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.

2.3. Quantification of petroleum hydrocarbon content

The determination of hydrocarbon content in the range C10–C40 was done by Novalab Oy, Finland. Briefly, the hydrocarbons were extracted from samples using heptane according to the standard CEN/TC 292/WG 5N 148E. The greases were separated by aluminum oxide treatment and the hydrocarbon contents were analyzed and quantified according to the standard SFS-EN ISO 9377-2 using a gas chromatograph equipped with a flame ionization detector.

2.4. DNA extraction and PCR

For DNA extraction, 100 mL of water was filtered onto 47 mm diameter, 0.22 μm pore size polycarbonate filters (GE Water & Process Technologies). DNA extraction was carried out as described by Ghiglione et al. (2005). For terminal restriction fragment length polymorphism (T-RFLP) analysis, partial 16S rRNA genes were PCR amplified using bacterial primers 27F_FAM (AGA GTT TGA TCC TGG CTC AG) and 926R_HEX (CCG TCA ATT CCT TTG AGT) (Pandey et al., 2007). The reaction mixture (25 μL) consisted of 0.1 μL template DNA, 25 mM dNTPs, 10 μM of each primer, buffer and 0.5 units of KAPA Taq polymerase (KAPA Biosystems). Three replicate reactions were carried out from each DNA sample.

2.5. Terminal restriction fragment length polymorphism (T-RFLP)

Restriction enzymes *Rsa*I and *Hha*I were used in parallel for each sample. The fluorescence peaks were sized using the PeakScanner software (Applied Biosystems) and a threshold value of 50 relative fluorescence units. The peak data were further processed using the T-REX program (Culman et al., 2009) with default values for noise filtering and peak alignment 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

Table 1
Characteristics of the three sampling sites in the Finnish Archipelago Sea.

Site	Location	Background information	Concentration in seawater (μg/L)	
			Total P	Total N
Pansio (P)	60°26′32″N 22°9′ 26″E	Oil harbor, long term constant exposure to petroleum contaminants	64	700
Raisio (R)	60°27′47″N 22°7′10″E	Recreational harbor, close to a ship yard and municipal wastewater outlet	160	970
Askainen (A)	60°31′17″N 21°52′45″E	No previous pollution	36	410

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