Dynamic changes in the structure of microbial communities in Baltic Sea coastal seawater microcosms modified by crude oil, shale oil or diesel fuel

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ABSTRACT

The coastal waters of the Baltic Sea are constantly threatened by oil spills, due to the extensive transportation of oil products across the sea. To characterise the hydrocarbon-degrading bacterial community of this marine area, microcosm experiments on diesel fuel, crude oil and shale oil were performed. Analysis of these microcosms, using alkane monooxygenase \((alkB)\) and 16S rRNA marker genes in PCR-DGGE experiments, demonstrated that substrate type and concentration strongly influence species composition and the occurrence of \(alkB\) genes in respective oil degrading bacterial communities. Gammaproteobacteria (particularly the genus \textit{Pseudomonas}) and Alphaproteobacteria were dominant in all microcosms treated with oils. All \(alkB\) genes carried by bacterial isolates (40 strains), and of the 11 major DGGE bands from the microcosms, had more than 95% sequence identity with the \(alkB\) genes of \textit{Pseudomonas fluorescens}. However, the closest relatives of the majority of sequences (54 sequences from 79) of the \(alkB\) gene library from initially collected seawater DNA were \textit{Actinobacteria}. \(alkB\) gene expression, induced by hexadecane, was recorded in isolated bacterial strains. Thus, complementary culture dependent and independent methods provided a more accurate picture about the complex seawater microbial communities of the Baltic Sea.

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1. Introduction

Crude oil is a complex mixture of thousands of organic compounds. In general, crude oil contains alkanes (30%), cycloalkanes (49%), aromatic compounds (15%) and asphaltethenes (6%). Organic compounds containing O, N, S and organometallic compounds are also found in smaller amounts (Hyne 2001). The hydrocarbon molecules in crude oil range from 5 to 40 carbons in length. During the refining process, various components of crude oil are separated based on individual boiling points. For example, the fraction that boils at temperatures of 240–340 °C is called diesel. It contains hydrocarbons of 9–16 carbons in length, and is a widespread fuel used in engines. After natural crude oil, shale oil is the cheapest source for producing large amounts of liquid fuels. Shale oil is produced when shale is heated in the absence of oxygen (termed retorting; Rudzinski and Aminabhavi 2000). The obtained oil contains saturated (18%), unsaturated (7%) and aromatic hydrocarbons (total 25%; monocyclic 8%, bi- and polycyclic 17%), as well as heteroatomic compounds (total 57%; acidic 23%) (Uro and Sunberg 1999). Compared to crude oil, shale oil contains large quantities of unsaturated alkanes, with alkane molecules in shale oil ranging from 7 to 28 carbons in length.

Accidental or operational oil pollution may arise during intensive drilling, production, storage and transportation of oils at sea or on land. Although accidental spills account for only a small percentage of the oil released into the marine environment, large oil spills may cause extensive ecological damage to marine shorelines, and may have an enormous impact on local economic activities, due to the associated risk to public health (Vila et al. 2010). Bacteria play a dominant role in the natural biodegradation process of different types of oil in marine environments (Leahy and Colwell 1990; Head et al. 2006). Marine hydrocarbon-degrading bacterial isolates have been shown to belong to more than 20 genera across several groups (\textit{Alphaoa-}, \textit{Beta-} and \textit{Gammaproteobacteria}; gram-positives; \textit{Flexibacter-Cytophaga-Bacteroides}) (Floodgate 1995; Head and Swanell 1999; Head et al. 2006; Yakimov et al. 2007).

Depending on the chain-length of the alkane substrate, different enzyme systems are required to introduce oxygen to the substrate and initiate biodegradation under aerobic conditions (van Beilen and Funhoff 2007). The terminal oxidation of medium-length alkanes (from 5 to 16 carbons in length) in prokaryotes is initiated by integral membrane non-heme diiron alkane hydroxylases (van Beilen et al. 2006; van Beilen and Funhoff 2007; Rojo 2009). For example, AlkB (termed AlkM in \textit{Acinetobacter sp.}, Ratajczak et al. 1998), which was first described in the OCT plasmid of \textit{Pseudomonas putida} GPo1 (formerly \textit{P. oleovorans} GPo1; Kok et al. 1989; van Beilen et al. 1994), is the most important enzyme, because it
is prevalent in aerobic oil-degrading bacteria. More than 250 AlkB homologues have been discovered in at least 45 bacterial species of multiple genera from a wide range of environments (Wang et al. 2010b). As a result, the alkb gene has been used as a molecular marker to detect the bioremediation capacities of oil-polluted environments (Heiss-Blanquet et al. 2005; Kuhn et al. 2009; Wang et al. 2010b). Therefore, knowledge about the diversity of the alkb gene, as well as information about their host bacteria in a certain marine habitats, may help evaluate the potential of the Baltic Sea and other ocean areas to recover from an accidental oil spills (Wang et al. 2010b).

The Baltic Sea is the second largest brackish water basin in the world. It is characterised by strong stratification, high nutrient concentration, continuous oxygen deficiency (a thermocline typically occurs at 10–20 m of depth), low salinity (4.967–6.808‰) and an average depth of 55 m (Koskinen et al. 2011). The environmental conditions of the Baltic Sea arise as a result of freshwater input from rivers and precipitation, as well as by the limited inflow of saline water from the North Sea (Cabaj et al. 2006). Such environmental conditions create the unique habitat of the Baltic Sea, in which a mixture of marine, freshwater and some brackish water organisms reside (Höllfors et al. 1981). It is estimated that the Baltic Sea supports approximately 15% of the world’s total marine traffic, including the transport of different types of oil. Because so much oil is used, transported and stored in this region, oil and oil spills are considered a major threat to the Baltic Sea ecosystem (HELCOM 2009). Hence, a number of studies about the total microbial community composition and dynamics of the Baltic Sea and sediments have been conducted (Pinhassi et al. 1997; Hagström et al. 2000; Kisand and Wikner 2003; Kisand et al. 2005; Edlund et al. 2006; Riemann et al. 2008; Edlund and Jansson 2008; Andersson et al. 2010; Koskinen et al. 2011). However, data about the presence and diversity of the alkb genes in the Baltic Sea bacterioplankton have not been published and also little research has been performed regarding microbial community dynamics in response to hydrocarbons, especially in the water column.

The aim of the current study was to describe the potential impact of an oil spill (crude oil, shale oil and diesel fuel were used as model substrates) on species composition and the occurrence of alkb genes in the oil degrading bacterial community of Baltic Sea coastal waters. A polyphasic approach, based on different cultivation strategies complemented with culture-independent methods, such as PCR and DGGE analysis of the 16S rRNA and alkb genes in total community DNA, was used in this study.

2. Materials and methods

2.1. Study area and sample collection

The surface water sample was collected during March 2008 from the western part of the coastal area of Vilsandi Island (58°23′012″N, 21°48′358″E) at a depth of approximately 1 m using a sterile 12-L canister (Fig. 1). The sampling site was situated at a distance of ca. 1 km from the shoreline. Water sample salinity was 6.3‰, the pH was 8.1, the water temperature was 6 °C and the oxygen content was 6.1 mg L⁻¹. The sampling location was open to sea fluxes, and no accidental oil contamination has been recently reported in this area. Vilsandi Island is also absent of intensive agricultural activity, rivers or permanent inhabitants. The sample was stored at 4 °C in a sterilised glass bottle until analysis (within 24 h after collection).

2.2. Isolation of bacterial strains: direct selection, microcosm experiments and cell enumeration

Two approaches were used to isolate hydrocarbon-degrading bacteria from seawater: direct selection and enrichment. In the first approach, bacteria were isolated from plated-out cultures of the collected seawater on selective M9 mineral agar plates (Adams 1959; Bauchop and Eldsen 1960), containing crude oil (CO; Lukoil Oil Company OAO), diesel fuel (DF; Neste Oil), shale oil (OO; VKG Oil AS), hexane (Hex; Fluka; ≥97% purity), hexadecane (Hed; Fluka; 99% purity) or heptane (Hep; Fluka; ≥96% purity) as the only growth substrate (vapour phase). In addition, bacteria were isolated from plated-out cultures of the collected seawater on low-nutrient solid R2A plates (Difco), without any additional substrates. Plates were sealed and incubated at 15 °C for 14 days.

The microcosm (enrichment) experiments were performed in triplicate in 250-mL Erlenmeyer flasks (gas-tight), which contained 50 mL of seawater and one of the following substrates at final concentrations: crude oil (CO, 0.1% or 1%, v/v), diesel fuel (DF, 0.1% or
1%, v/v), shale oil (OO, 0.06% or 0.6%, v/v). The flasks were placed at 15 °C on a rotary shaker (100 rpm) for 9 weeks. The control microcosm contained only seawater. Isolation of hydrocarbon-degrading bacteria from different microcosms after 3, 6 and 9 weeks of cultivation was performed after serial dilution and plating on R2A agar or selective media (containing the same carbon source that was used in microcosm), followed by a 2 week incubation period at 15 °C.

Morphologically different colonies were isolated from the plates, purified and studied for BOX-PCR fingerprint patterns (according to the protocol described in Heinaru et al. 2000) to remove redundant strains, which were eliminated from further investigation. Pure bacterial cultures were stored in 20% glycerol at −80 °C.

Bacterial populations were enumerated on R2A plates (cultivable conventional heterotrophic bacteria) or on selective media containing the specified carbon sources (cultivable hydrocarbon-utilising bacteria) using the spread-plate technique. After incubation at 15 °C for 14 days, total colony numbers were counted, and the number of colony-forming units (CFUs) per ml was calculated for each sample. The plates were prepared in triplicate, from which the mean values and standard deviations were calculated.

2.3. Extraction of total community DNA

One litre of initial seawater was run through a sterile filter with a pore size of 0.2 μm (Sartorius AG) and stored at −80 °C. Bacteria for total DNA extraction, from the enrichment experiments after 3, 6 and 9 weeks of cultivation, were collected by centrifugation (15 mL of culture, 13,000 x g for 3 min) and stored at −20 °C. Total microbial community DNA was extracted from the filter and the cells collected from microcosms using a PowerSoil DNA Kit (MoBio Laboratories, Inc., USA), according to the protocol provided by the manufacturer. The extracted DNA was stored in MilliQ water at −20 °C.

2.4. PCR amplification of different target molecules

Fragments of the alkB genes encoding membrane-bound alkane hydroxylase were amplified from the community DNA and the isolated bacterial strains using the manually designed degenerate primer pair alkBF and alkBR (Table 1). It is believed that all AlkB proteins are conserved in six hydrophobic stretches, which span the cytoplasmic membrane (Shanklin et al. 1994), and eight to nine histidines, which are essential for alkane-hydroxylating activity (van Beilen et al. 2003). Based on these conserved moieties, degenerate primers for PCR detection of alkB genes were designed in earlier studies (Smits et al. 1999; Kloos et al. 2006). As the product amplified with primer pair alkB-1f and alkB-1r, designed by Kloos et al. (2006), is 550 bp long and thus not suitable for further DGGE analysis, we had to make new primer pair to obtain a shorter product. We designed our alkB primers using the conserved moieties of alkane hydroxylase amino acid sequences of phylogenetically distant genera Pseudomonas, Rhodococcus, Burkholderia, Mycobacterium, Amycolicoccus, Nocardioideas, Prauserella, Micromonaspora, Frankia, Alcanivorax, Marinobacter and Caulobacter, which were available in 2007 in the GenBank database (Supplementary Fig. S1). In design of the reverse primer alkBR we guided from the conserved region used by Kloos et al. (2006) for design of alkB-5f (that was used for hybridisation). Our forward primer alkBF was designed from an internal conserved region. As a result, we obtained 344 bp long products while using the primers designed by us. Nucleotide alignment of the same sequences was also done to verify the designed primers. DNA sourced from Rhodococcus sp. strain 4_C16_20 of the Collection of Environmental and Laboratory Microbial Strains of University of Tartu, Estonia, was used for testing PCR primers. For denaturing gradient gel electrophoresis (DGGE) analysis, a 40-bp GC-clamp was added to the 5’ end of the alkBF primer.

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The 16S rRNA gene fragments of the isolated bacterial strains were amplified using the primer pair PCRI and PCRII, adapted from the primers fD2 and rP1 (Weisburg et al. 1991). For DGGE analysis, the 16S rRNA gene fragments of bacterial communities were amplified with the primer pair PRBA338f/Lane (1991) and PRUN18r (designated as primer 2 in Muyzer et al. 1993) (Table 1).

Species- and biotype-specific grouping of the Pseudomonas strains was completed by comparing the carA gene (encodes the small subunit of the carbamoyl-phosphate synthase) sequences (Merima et al. 2006), which were amplified using the primer pair carA-F and carA-R (Hilario et al. 2004, Table 1).

BOX-PCR fingerprint patterns of the isolated bacterial strains were obtained according to the protocol described by Heinaru et al. (2000) using the primer BOXA1R (Louws et al. 1994, Table 1).

Target molecules were amplified from the samples by conventional PCR. The PCR mixture included 1 × PCR buffer (with [NH4]2SO4), 0.2 mM dNTP, 2.5 mM MgCl2, 0.8 μM of both primers.

<table>
<thead>
<tr>
<th>Primer target</th>
<th>Primers</th>
<th>Nucleotide sequence (5’ → 3’)</th>
<th>Annealing temperature (°C)</th>
<th>Extension time (min)</th>
<th>Product size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>PCRI</td>
<td>AGATTTGATATGCTGTCGAC</td>
<td>53</td>
<td>2</td>
<td>~1500</td>
<td>Weisburg et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>PCRII</td>
<td>TACGGTACCTTGAGTACAGCTT</td>
<td>53</td>
<td>2</td>
<td>~1500</td>
<td>Weisburg et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>PRBA338f</td>
<td>ATACCTATGCGGCGCACGAC</td>
<td>57</td>
<td>1</td>
<td>~180</td>
<td>Lane (1991)</td>
</tr>
<tr>
<td></td>
<td>PRUN18r</td>
<td>TTCAACACCGCATGACGGG</td>
<td>55</td>
<td>0.5</td>
<td>657</td>
<td>Muyzer et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>carA-F</td>
<td>TGATCGCCAGCCACATCC</td>
<td>53</td>
<td>0.5</td>
<td>223</td>
<td>Hilario et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>carA-R</td>
<td>GACGAATGCGCCTACAC</td>
<td>53</td>
<td>0.5</td>
<td>223</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>carARseet</td>
<td>TCAGGACCATTTGCGACG</td>
<td>50</td>
<td>1</td>
<td>344</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>alkBF1</td>
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<td>81</td>
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<td>53</td>
<td>0.5</td>
<td>81</td>
<td>This study</td>
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<td></td>
<td>alkBRseet</td>
<td>CCGCAGGACCTACCC</td>
<td>53</td>
<td>0.5</td>
<td>81</td>
<td>This study</td>
</tr>
<tr>
<td>Repeated regions in chromosome</td>
<td>BOXA1R</td>
<td>CTACGCGAACGCGAGTGAGC</td>
<td>53 → 68</td>
<td>1 → 8</td>
<td>Various</td>
<td>Louws et al. (1994)</td>
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<tr>
<td>GC clamp</td>
<td></td>
<td>CCAGGCGGCGGCGGCGGCGGGGCC</td>
<td>53</td>
<td>0.5</td>
<td>81</td>
<td>Muyzer et al. (1993)</td>
</tr>
</tbody>
</table>

a For the DGGE analysis, an additional GC clamp was added to the 5’ end of the primer.

b Primers were used for qRT-PCR.

c Primers were designed based on the amino acid sequence YGHF/Y/F/VE.

d Primers were designed based on the amino acid sequence YGHF/Y/F/VE.
2.5. Preparation and analysis of the alkB clone library

PCR products of the alkB gene amplified from the total community DNA of the initially collected seawater were purified from agarose gel using a Quick Gel Extraction Kit (Qiagen), and were ligated into a pTZY57R/T vector using an InstAClone Product Cloning Kit (Fermentas), following the manufacturer’s instructions. The constructs were transformed into Escherichia coli DH5α competent cells (Inoue et al. 1990). Cells were spread on a Luria Bertani (LB) agar plate containing 150 μg·mL⁻¹ ampicillin, 48 μg·mL⁻¹ isopropyl β-D-thiogalactopyranoside and 80 μg·mL⁻¹ 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. The screening of inserts from the white colonies of transformants was performed by direct PCR amplification using the primer pair alkBR and alkBF (suitable for subsequent DGGE analysis, Table 1). Correctly amplified inserts were identified by gel electrophoresis, and were further analysed by DGGE. Clones were grouped based on their electrophoretic mobility, with representative clones of each group being selected and sequenced.

2.6. DGGE

The alkB and 16S rRNA genes were amplified from the isolated strains, the community DNAs of the microcosms and the initial seawater with primer pairs suitable for subsequent DGGE analysis (alkBF and alkBR, and PRBA338f and PRUN518r, Table 1) using the same PCR conditions. Approximately 500 ng of the amplified samples were used for DGGE analysis following the method of Muyszer et al. (1993). Either the DCode Universal Detection System (Bio-Rad, Hercules, CA, USA) or the INGENYphorU-2 × 2 (Ingény International, Netherlands) DGGE system were used for electrophoresis, following the recommendations of the manufacturer. Either 10% or 8% (v/v) polyacrylamide gel (acylamide–bisacrylamide 37.5:1 in 1 × TAE buffer) with a linear DNA denaturing gradient was used for 16S rRNA and alkB gene fragments, respectively. The denaturing gradient was formed with deionised formamide and urea; the 100% denaturing agent is 7 M urea and 40% (v/v) deionised formamide. Linear denaturing gradients of 35–60% and 40–65% were used for the 16S rRNA and alkB gene fragments, respectively. Gels were electrophoresed in a 1 × TAE buffer for 13 h at a constant temperature (60 °C) and voltage (100 V). Gels were stained in MilliQ water containing 0.5 mg·L⁻¹ ethidium bromide, and destained twice in MilliQ water before UV transillumination. The DGGE gels were digitised and the banding pattern was analysed using cluster analysis based on the Pearson’s correlation coefficient using GelCompar II ver 4.0 (Applied Maths, Kortrijk, Belgium). The number of bands was calculated by using the programme available at http://www.changbioscience.com/genetics/shannon.html. In addition, the dominant DGGE bands, which demonstrated differences in electrophoretic mobility, were excised using a razor blade, and soaked overnight (at 37 °C) in 3 volumes of elution buffer containing 0.5 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA and 0.1% sodium dodecyl sulphate. Then, the DNA was precipitated with two volumes of absolute ethanol, pelleted and washed with 70% v/v ethanol, air dried, and dissolved in 15 μL of MilliQ water. One microlitre of the dissolved DNA was used as a template in the PCR reaction, with the same primers and conditions. Amplified products were cloned, as previously described, and then sequenced.

2.7. Sequencing and data analysis

All the alkB, 16S rRNA and carA gene fragments were nucleotide sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit for Applied Biosystems 3730x/3DNA Analyser following the manufacturer’s instructions. Reference sequences were obtained from the GenBank/NCBI using the programmes BLASTN and TBLASTX. Nucleotide sequences were translated in silico, and phylogenetic trees were constructed by neighbour-joining (NJ) analysis using MEGAS software (Tamura et al. 2011) with 1000 bootstrap replicates. Sequences with >82% identity (the cut-off value) were assigned to the same operational protein families (OPF) using FastGroupII software available at http://phage.sdsu.edu/research/tools/fastgroup/ (Yu et al. 2006). A cut-off value of 82% was selected to compare the obtained data with published alkB information (Kuhn et al. 2009; Wasmund et al. 2009; Guibert et al. 2012). The coverage estimates of the alkB gene library were calculated using a programme available at http://www.aslo.org/lomethods/free/2004/0114a.html (Kemp and Aller 2004). The nucleotide sequences reported in this study were deposited in the GenBank database under accession numbers GU226434–GU226482, GU226485–GU226489, GU226491, GU226493–GU226494, GU226496, GU226497, GU226499, GU226503–GU226505, GU226508–GU226516, GU226518–GU226520, GU226529, GU226530, GU226533, GU226535–GU226537, GU226539, GU226540, GU226542–GU226544, GU226546, GU226548–GU226556, GU226564–GU226569, JN462459, JN462460, JN462662–JN462464, JN462668, JN462670, JN462673–JN462675 and JQ253561.

2.8. Quantitative reverse transcription-PCR of alkB mRNA in bacterial isolates

Pseudomonas fluorescens biotype A strains TN44 and Hp2, biotype B strain Hd1, biotype C strain TN24, biotype G strain TN23, biotype F strains D46 and TN43 (Supplementary Table S2, Fig. 4B), which were obtained from the direct isolation or enrichment experiments, were grown in minimal medium. The medium contained M9 salts and trace elements, which were supplemented with 0.2% (w/v) casamino acids. For induction, the medium was supplemented with 1% (v/v) hexadecane. The experiments were performed in triplicate in 100-ml Erlenmeyer flasks containing 20 ml of media at 25 °C with rotary shaking (180 rpm). Fresh media were inoculated with the stationary phase culture grown on casamino acids. After inoculation, the OD_{580 nm} of the culture was approximately 0.2. Bacterial cultures were incubated for 24 h, then 500 μL of the late logarithmic growth phase cultures were collected by centrifugation at 8000 × g at 4 °C for 5 min, and cell pellets were then rapidly frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated from the cell pellets of induced and control treatments using Trizol Reagent (Invitrogen), according to the protocol provided by the manufacturer. Additional treatment with DNase I (Fermentas) was performed according to the protocol (Fermentas). The resulting RNA was quantified with a spectrophotometer (Nanodrop ND1000, NanoDrop Technologies) and its integrity was evaluated by electrophoresis in 0.8% agarose.

Quantitative reverse transcription qRT-PCR was performed in the real-time PCR system Rotor–Gene (Qiagen), with a total reaction volume of 10 μL using a SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen). The reaction mixture contained 4 μL of total RNA and 400 nM of both primers (Table 1, primers
carAFeest and carARseest, and alkBFseest and alkBRseest were designed using the alkB and carA gene sequences of the seven strains listed in the beginning of the chapter). The primers were designed to generate products of 81 and 223 bp. For standard curve generation, and to assess the suitability of the primers, carA and alkB mRNAs were in vitro synthesised by T7 RNA polymerase (Ferments) using linearised plasmids containing the cloned carA and alkB gene sequences from TN43 as a template. Ten-fold serial dilutions of the in vitro synthesised carA and alkB mRNAs were used as a template in qRT-PCR. Highly linear standard curves (R² values > 0.99; PCR efficiency = 99%) were obtained for both carA and alkB mRNAs over a dilution range of 10⁶–10³ copies per reaction. The PCRs were run using the following programme: 50°C for 3 min, 95°C for 5 min, 40 cycles of 95°C for 15 s, 15 s at the annealing temperature (Table 1), 72°C for 30 s, followed by 40°C for 1 min. At the end of the run, melting curve analysis was performed by increasing the temperature from 76°C to 95°C, using a 3-s interval at 0.35°C with continuous fluorescence recording. Data from the qRT-PCR were analysed with Rotor-Gene Series software, version 2.0.2 (Qiagen). The carA gene was used as the housekeeping control to correct for differences in the amount of starting material. The calculated threshold cycle (Ct) of alkB gene was normalised to the Ct of carA that was amplified from the corresponding sample. Changes in alkB mRNA levels of the bacterial strains were calculated using the 2-ΔΔCt method (Livak and Schmittgen, 2001). qRT-PCR was performed in triplicate for each sample.

3. Results

3.1. Bacterial abundance in microcosm experiments and isolation of oil-degrading bacteria

The number of cultivable aerobic heterotrophic bacteria in the initial seawater sample was 6 × 10⁴ CFU mL⁻¹, with a similar range of approximately 10⁴–10⁵ CFU mL⁻¹ being recorded in the microcosm experiment. At the beginning of the crude oil, diesel fuel or shale oil microcosm experiments, the numbers of cultivable hydrocarbon-utilising bacteria were in the range of 2–4 × 10³ CFU mL⁻¹ (Fig. 2). The same carbon source as the microcosm was fed was used in the determination of these counts. After 3 weeks, the bacterial abundance in oil containing microcosms increased up to 10- to 100-fold (up to 10⁵ CFU mL⁻¹), and remained relatively stable until the end of the experiment. Higher values were obtained in microcosms with shale oil after 6 weeks of cultivation, when colony counts reached up to 2 × 10⁶ CFU mL⁻¹. In the case of the 1% concentration of crude oil, the lowest numbers of cultivable hydrocarbon-utilising bacteria were obtained (Fig. 2).

Based on the ability to grow on different oil products, a total of 191 hydrocarbon-degrading bacterial strains (redundant strains removed) were isolated from direct cultivation (55 strains) and microcosms experiments (136 strains) (Supplementary Table S1). On non-selective R2A media, 20 bacterial strains were obtained from the initial seawater by direct cultivation, and 22 strains were obtained from the control microcosm (I, Supplementary Table S1).

Supplementary data related to this article found, in the online version, at doi:10.1016/j.micres.2013.02.006.

3.2. Molecular screening of oil-degrading strains for the presence of alkane hydroxylase genes

All 191 isolated strains were further tested for the presence of alkane hydroxylase-encoding alkB genes. The results revealed 40 alkB-positive strains. Of these, 14 alkB-positive strains were obtained from the direct cultivations of the initially collected seawater, with a further 26 strains being obtained from the microcosm experiments (Supplementary Table S1).

Phylogenetic analysis of the deduced amino acid sequences of these alkB genes (98 aa) showed high homologies only to Pseudomonas-like alkB genes (identity >95%, Fig. 3, Supplementary Table S2). The phylogenetic analysis of 16S RNA gene sequences of 11 representative alkB-positive strains (selected from different clusters of the phylogenetic tree of alkB nucleotide sequences of the isolates) also revealed high similarity of the strains with different pseudomonads (Fig. 4a, Supplementary Table S2). These strains were found to be clustered to 5 biotypes (A–G) of Pseudomonas fluorescens, based on a comparison of their carA gene sequences (Fig. 4b).

Supplementary data related to this article found, in the online version, at doi:10.1016/j.micres.2013.02.006.

3.3. Analysis of the alkB clone library

The clone library of the alkB gene was constructed to characterise the main bacterial alkB groups in the initial Vilsandi seawater sample. The sequences from the gene library were found to be more diverse compared to the alkB gene sequences of the isolated bacteria (Fig. 3). Sequence homologies to published sequences ranged from 38% to 96% (identity of amino acids) (Supplementary Table S3). The majority of the alkB clone sequences (54 out of 79) were mostly similar to those from the actinobacterial bacterium Prauserella rugosa NRRLB-22095 (identities between 72% and 78%, Fig. 3, OPF II and III). The second largest clone group (Fig. 3, OPF I) consisted of 21 sequences, which had high sequence homologies (identity over 95%) to alkane hydroxylase sequences of Pseudomonas strains. Two other clones were related to Gammaproteobacteria, with low sequence homology. One sequence (clone 9, OPF V) was similar to alkB of Alcanivorax dieselolaei (identity 68%), while the other sequence (clone 47, OPF VI) was related to alkB of Marinobacter aquaeolaei (identity 55%). The alkB clone 17s sequence shared high sequence homology (91% identity) with alkBs related to Alphaproteobacteria, namely Caulobacter sp. (Fig. 3, OPF VII). In comparison, the alkB clone 35s sequence showed low sequence homology (38% of identity) with alkBs related to Rhodobacterales sp. (Fig. 3, OPF VIII).

Supplementary data related to this article found, in the online version, at doi:10.1016/j.micres.2013.02.006.
Fig. 3. The bootstrap consensus tree based on the deduced amino acid sequences (98 aa) of alkane hydroxylases (alkB) from clones (boxed light grey), strains (letters and numbers in bold) and DGGE gel fragments (boxed dark grey) obtained in this study (130 sequences in total; GenBank protein accession numbers in parentheses). The details of the operational protein family I are shown on the right side of the figure. Reference sequences from the database are labelled with the name of the strain and the GenBank protein accession number in parentheses. The roman numerals represent different Operational Protein Families. The tree was constructed using the neighbour-joining method in the MEGAS programme with the Dayhoff model. The percentages of replicate trees in which the associated taxa are clustered together in the bootstrap test (1000 replicates) are shown next to the branches (higher than 50%). The scale bar represents 0.2 substitutions per amino acid site.
Fig. 4. Phylogenetic trees of partial 16S rRNA gene sequences (~150 bp) obtained from isolates (bold letters) or DGGE gel bands (boxed grey) (a) and the carA gene sequences (617 bp) of 11 isolates belonging to the genus Pseudomonas (b). The GenBank accession numbers of 16S rRNA and carA genes are shown in parentheses, * after parentheses indicates that a longer (~580 bp) 16S rRNA gene sequence of the isolated strain was submitted to GenBank than was used in the construction of phylogenetic tree. The coloured rectangle and ovals represent Alpha- (blue), Beta- (red), Gammaproteobacteria (yellow) and Actinobacteria (green). The trees were constructed from the evolutionary distance matrix by the neighbour-joining method in MEGA5, with the p-distance model. Bootstrap values (per 1000 trials) higher than 50% are indicated at the nodes. The scale bars represent 0.1 substitutions per base pair. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)
3.4. Dynamic changes in bacterial community composition according to DGGE profiles of the 16S rRNA and alkB gene sequences

DGGE analyses of the alkB and 16S rRNA gene fragments from microcosm samples were performed to assess the impact of different oil components on microbial community composition, as well as on the diversity of the alkB genes. Cluster analysis of the DGGE profiles of the 16S rRNA gene showed a clear difference among the microcosm samples supplemented with different oil compounds and the initial seawater samples (Fig. 5a). In addition, microcosms with different concentrations of oil products formed separate clusters in the dendrogram, with the exception of the crude oil (1%) microcosm sample from week 3, which formed a discrete branch.

The samples from the 0.06% and 0.6% shale oil (OO) microcosms were clustered together with the samples from diesel fuel (DF) and crude oil (CO) microcosms, respectively. The DGGE profile of the initial seawater was clustered together with the profile of the mixture of isolated strains; however, bands with the same electrophoretic mobility as those of the isolated strains were also observed in other microcosms. In general, the number of DGGE bands of the amplified 16S rRNA gene decreased during the microcosm experiments (Fig. 5b and c). In the microcosms with higher concentrations of OO and CO, the numbers of bands were relatively stable between week 0 and 3; however, during week 6, the numbers of bands decreased and remained stable until the end of the experiment. The greatest change in the number of bands was observed for the microcosm containing 0.06% shale oil where the number of bands decreased...
Fig. 6. Original DGGE profiles of alkB gene fragments of microbial communities in the enrichment experiments. STRAINS – alkB gene fragments of the reference hydrocarbon-degrading strains isolated from hexadecane (strain Hdl1), 1% crude oil (strain TN23) and 0.1% crude oil (strain TN43). CLONES – alkB gene fragments obtained from the respective cloning library. Alk1–Alk11 indicate the numbers of sequenced bands. Abbreviations: I – initially collected seawater; CO – crude oil; OO – shale oil; DF – diesel fuel; the number under the substrate used in the microcosm indicates the sampling week number. Data of a single experiment are presented; variability among different experiments was minimal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

significantly during week 3, and then increased to around the initial level during week 6, before majorly decreasing during week 9 (Fig. 5c). Changes in the pattern of band number were similar in the DF microcosms. For instance, band number remained relatively stable after decreasing in week 3, whereas it was lower compared to the corresponding control microcosm containing the initially collected seawater (Fig. 5c).

Sequence analysis of the 41 dominant 16S rRNA gene fragments (Fig. 5b) revealed the dominance of *Gammaproteobacteria* (23 bands), particularly the genus *Pseudomonas* (12 bands) in all microcosms including controls. In addition, bands in the DGGE profiles of original seawater and control microcosms contained a sequence highly similar to that of *Marinomonas* sp., *Nevskia* sp. and *Shewanella* sp. (Fig. 4a; Supplementary Table S2). The sequences of the bands in profiles of OO and CO microcosms were highly similar with *Glaciecola* sp. (3 bands) and *Halomonas* sp. (Fig. 4a, Supplementary Table S2). *Alphaproteobacteria* were identified in 10 bands. The sequenced bands 11 and 12 had 100% identity with the genus *Thalassospira*, and were present in all treatments at variable band intensities. One of the dominant bands (band 16) in the DGGE profiles of original seawater, DF and OO microcosms contained a sequence highly similar to that of a *Roseobacter* sp. (Fig. 5b).

Two other prevailing bands were common to the communities of CO microcosms. These bands were identified as *Phaeobacter* sp. (band 22) and *Parvibacterium* sp. (band 19). *Betaproteobacteria* (2 bands) were identified in CO and DF microcosms, while the dominant band 21 sequence shared high identity with the family *Rhodocyclaceae*, and band 32 sequence shared high identity to *Achromobacter xylosidans*. The actinobacterial genus *Propionibacterium* (bands 2, 25 and 27) was dominant in almost all microcosms. The four remaining sequenced bands had high similarities with representatives of uncultured *Actinobacteria* (bands 15 and 17), *Gammaproteobacteria* (band 18) and *Rhodospirillales* clones (band 20), and were present in all treatments at variable band intensities.

The DGGE profiles of the alkB gene fragments from the microcosm experiments confirmed that different oil compounds have impact on microbial community as was obtained with the cluster analysis of DGGE profiles of the 16S rRNA gene fragments (although due to unidentified technical problems, smeared lines were obtained in the alkB DGGE profiles of some samples). Generally, the greatest changes in microbial community composition and the diversity of the alkB gene, occurred within 3 weeks of the beginning of the experiment, and remained relatively stable until the end of the experiment (Fig. 6). The alkB gene profiles showed that the alkB gene fragments obtained from the clone library and the isolated strains are common in DGGE profiles of the microcosms and the initial seawater samples (Fig. 6).

Sequence analysis of the 11 dominant alkB gene fragments revealed the prevalence of *P. fluorescens*-like alkB genes in microcosms (bands Alk2–Alk6, Alk9–Alk11; identities > 92%). The *Prauserella*-like band Alk1 (identity 77%) was also dominant in all types of enrichment substrates, while *Alcanivorax*-like alkB genes (bands Alk7 and Alk8; identity 67%) only became dominant at the end of the CO and DF microcosm experiments (Figs. 3 and 6).

### 3.5. Comparative analysis of all alkB gene sequences

When all obtained alkB gene sequences (from clones, isolated strains and DGGE gel bands; 98 aa) were pooled, 8 Operational Protein Families (OPF) were distinguished (Fig. 3) using FastGroupII software (cut-off value 82% sequence identity at the amino acid level). The rarefaction curve did not reach an asymptote for the obtained alkB gene sequences (Supplementary Fig. S2a), indicating that greater diversity was present than it was revealed by the sequencing effort. *SACE* and *SChao1* estimators also indicated that more OPFs were present, 22 and 13 respectively, than were detected in our alkB gene pool (Supplementary Fig. S2b).

Supplementary data related to this article found, in the online version, at doi:10.1016/j.micres.2013.02.006.

### 3.6. Quantitative expression analysis of the alkB gene in bacterial isolates

To assess the possible effect of hexadecane on the alkB mRNA level in bacterial strains, growth experiments were performed with and without 1% (v/v) hexadecane as an inducer in the growth
medium, followed by quantification of the corresponding mRNA by quantitative reverse transcription-PCR. One AlkB-positive bacterial strain was selected from each P. fluorescens carA type (Fig. 4b). The expression levels of the alkB gene relative to the housekeeping reference gene (carA) were compared in induced and uninduced conditions. The obtained fold changes are presented in Table 2. The results revealed the expression of the alkB gene in selected bacterial strains in the presence and absence of hexadecane in the growth medium. However, according to differences in the fold changes of the alkB mRNA level, alkB gene expression in P. fluorescens strains Hd1, D46, TN23, TN43 and TN44 was enhanced in the presence of hexadecane (Table 2). The results also revealed that the levels of induction of alkB gene expression in bacterial strains belonging to different P. fluorescens biotypes were similar.

### Table 2

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Pseudomonas fluorescens biotypea</th>
<th>Fold changeb</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN44</td>
<td>A</td>
<td>1.67 ± 0.82</td>
</tr>
<tr>
<td>Hr2</td>
<td>A</td>
<td>1.18 ± 0.55a</td>
</tr>
<tr>
<td>Hd1</td>
<td>B</td>
<td>1.62 ± 0.85</td>
</tr>
<tr>
<td>TN24</td>
<td>C</td>
<td>1.01 ± 0.39a</td>
</tr>
<tr>
<td>D46</td>
<td>F</td>
<td>3.72 ± 0.32</td>
</tr>
<tr>
<td>TN43</td>
<td>F</td>
<td>1.49 ± 0.36</td>
</tr>
<tr>
<td>TN23</td>
<td>G</td>
<td>2.13 ± 0.85</td>
</tr>
</tbody>
</table>

* Means and standard deviations of the three independent experiments are presented, p<0.05.
* Not significant, p>0.1.
* According to carA gene phylogeny.

Discussion

In this study we surveyed changes in microbial community structures of Baltic Sea coastal seawater microcosms that were modified with crude oil, shale oil or diesel fuel, using both culture dependent and culture independent methods. The occurrence and diversity of the alkB gene, which is widely used as a marker gene for the detection of alkane biodegradation potential, was also evaluated in the bacterioplankton of the Baltic Sea and in oil-enriched microcosms. As expected, the alkB gene sequences obtained from culture independent methods (gene library and DGGE) were more diverse compared to sequences obtained from isolated bacteria. AlkB phylogenetic analysis revealed the dominance of sequences similar to alkB genes of pseudomonads and Actinobacteria among isolated strains (all 40 strains) and clones (54 clones from 79), respectively. Kloos et al. (2006) also observed significantly different AlkB populations in soil when using different screening methods (culture independent and culture dependent).

The AlkB sequences analysed in this work contained terminal histidines of the conserved histidine box II, which are essential for alkane hydroxylase activity (Smits et al. 1999; Smits et al. 2002; Guibert et al. 2012). The deduced protein sequences were grouped into Operational Protein Families (OPFs), with a distance threshold of 0.18 (82% sequence identity at amino acid level). Due to the high sequence variability of this gene (Kuhn et al. 2009; Wasmund et al. 2009), this low threshold level was used to obtain values that were comparable with those found in literature. Seven out of eight AlkB OPFs (Fig. 3) were divergent from previously characterised AlkB sequences, with amino acid sequence identities being in the range of 38–91% (Supplementary Tables S2 and S3). In addition, the AlkB sequences of OPF II and III seemed more closely related to each other than to reference sequences, and formed a relatively concentrated cluster that was distinct from previously reported AlkB sequences (Fig. 3). This result may reflect the particular properties of Baltic Sea water microbial communities in general, which could be considered as autochthonous estuarine communities adapted to primarily brackish environmental conditions (Riemann et al. 2008). AlkB sequence diversity (evaluated by the high number of OPFs) in the nonpolluted coastal waters of the Baltic Sea appeared to be at the same level as that described for pristine or slightly polluted sea sediments. For example, Wasmund et al. (2009) observed eight to 15 OPFs of alkB gene in Timor Sea sediment samples, with total hydrocarbon concentration (THC) ranging from 0.01 to 8.4 µg g⁻¹. In comparison, Kuhn et al. (2009) found four and 15 OPFs of the alkB gene in contaminated (THC 5 µg g⁻¹) and pristine Antarctic marine sediments, respectively. The highest AlkB diversity (30 OPFs) was detected by Guibert et al. (2012) in chronically polluted sub-Antarctic coastal sediments, which had a THC ranging from 19.7 to 347 µg g⁻¹. Up to 85% of n-alkanes are degraded after they have settled (Wasmund et al. 2009) and that may result in higher number of OPFs in (continuously) polluted sediments than in pristine sediments or surface water.

Oil type and composition is considered an important factor in determining the composition of oil-degrading consortia at specific experimental sites (Röling et al. 2002; McKew et al. 2007). We also found that different oil products, and their respective concentrations, had a substantial impact on microbial community composition and the diversity of alkB genes (Figs. 5 and 6). Substrates used in microcosm experiments were a mixture of various hydrocarbons containing different amounts of n-alkanes of varying chain length, in addition to other organic compounds. For example, when crude oil and shale oil are characterised by a wide range of hydrocarbon molecules (CO 5–40 carbons and OO 9–29 carbons in length) with high concentrations of aromatics (CO 10–20%; OO ∼25%), the size of hydrocarbon molecules in diesel fuel ranges from 9 to 16 carbons in length, with the concentration of aromatic compounds usually being less than 10%. Shale oil is discerned from other oils by its alkene content (7%) and high-polar compounds (>10%). Microbial attack has been shown to occur primarily towards n- and iso-alkanes, then towards cycloalkanes and 1- to 3-ring aromatics, and finally towards polyaromatics (Sugiura et al. 1996; Mana Capelli et al. 2001). Sei et al. (2003) reported that shorter alkanes (up to 12 carbons in length) were first degraded in the seawater microcosm containing crude oil, with bacteria containing alkB genes quickly proliferating after substrate addition. In our study, a major rearrangement in microbial community composition was observed during the early stage of the experiments (after 3 weeks). In microcosms with shale oil (that may contain substances that may be probably more recalcitrant than those found in other used substrates), the communities became dominated by a lesser number of phytypes as compared to other microcosms. Also, cluster analysis of the DGGE profiles of the 16S rRNA gene revealed that the shale oil microcosms that had low concentrations of shale oil clustered near to crude oil microcosms, while the microcosm that had high concentrations formed a separate cluster in the dendrogram (Fig. 5a). These observations are in accordance with previous research where it has been shown that microbial community diversity in marine environments contaminated by oil spills, or microcosms that mimic such environments, may be substantially reduced, due to strong selection for a limited number of hydrocarbon-degrading species (Röling et al. 2002; McKew et al. 2007; Vila et al. 2010). Sei et al. (2004) observed also concentration dependent change in the microbial community structure of seawater microcosms during salicylate degradation.

The natural AlkB community of Baltic Sea coastal waters contained sequences similar to the alkB genes of Alphaproteobacteria (e.g. Caulobacter and Rhodobacterales), Gammaproteobacteria (e.g. Pseudomonas, Marinobacter and Alcanivorax) and Actinobacteria (Prauserella) (Fig. 3). The present study supports existing research, where by Gammaproteobacteria, and to a lesser extent Alphaproteobacteria, become dominant in the bacterial communities of marine
ecosystems following exposure to oil hydrocarbons (Head et al. 2006; Yakimov et al. 2007; Greer 2010; Kostka et al. 2011). Most previous studies have shown that Gammaproteobacteria, namely Alteromonas, is ubiquitous; however, while it usually occurs in small quantities in unpolluted marine waters, it only becomes dominant in marine communities affected by oil or its components (McKew et al. 2007; Röling et al. 2002; Vila et al. 2010; Gertler et al. 2012). The sequencing of DGGE bands showed that AlkB sequences similar to those found in Pseudomonas and Prauserella strains were dominant throughout the experiment, while the Alcanivorax-like sequences were detected at the end of the microcosm experiments. This observation may be explained by a number of adaptations exhibited by Alcanivorax. For instance, it is susceptible to low temperatures (Yakimov et al. 2007), it requires the addition of mineral nutrients (phosphorus and nitrogen; Head et al. 2006) and it is moderately halophilic (optimum salt concentrations 3–10%). In our experiments, nutrients were not added, temperature was kept at 15 °C and seawater salinity was only 6.3‰, which only facilitated the proliferation of strains that tolerate such growth conditions. Other hydrocarbon-degrading members of Gammaproteobacteria, Pseudomonas and Marinobacter are metabolically more versatile than Alcanivorax, and have been shown to degrade poly cyclic aromatic hydrocarbons, as well as alkanes (Kostka et al. 2011). The dominance of gram-positive Actinobacteria (including the genus Prauserella) in oil contaminated environments has been demonstrated in several studies (Kloos et al. 2006; Quarini et al. 2008; Alonso-Gutierrez et al. 2009; Kuhn et al. 2009). Such dominance has been associated with the presence of more than one alkB gene homologue in these strains, the degradation of n-alkanes of up to 36 carbon molecules and branched alkanes, and the ability to produce biosurfactants (van Beilen et al. 2003). The presence of the genus Propionibacterium in microcosms was an unexpected finding. Yamane et al. (2008) have previously shown that these bacteria are also common in petroleum crude oils produced in Asia. 

Supporting the detection of alkB gene sequences affiliated with the Proteobacteria and Actinobacteria, members of these phyla were also identified as dominant by sequencing bands excised from 16S rRNA gene DGGE gels (Fig. 5b). Several studies have investigated total microbial community composition of Baltic Sea waters and sediments (Hagström et al. 2000; Edlund and Jansson 2008; Riemann et al. 2008; Koskinen et al. 2011). For example, Hagström et al. (2000) described the presence of members of the Alphaproteobacterial genus Sphingomonas and the gammaproteobacterial genera Pseudomonas, Acinetobacter and Shewanella, in parallel to the absence of typical marine genera of Gammaproteobacteria (Vibrio, Pseudoalteromonas and Alteromonas) and Alphaproteobacteria (Roseobacter) in 38 plate isolates. Recently, Koskinen et al. (2011) used pyrosequencing to show that Pseudomonas is the most dominant genus among 169 identified genera, comprising 45% of all sequences. The class Gammaproteobacteria, particularly the genus Pseudomonas, was also dominant in our study. For instance, all isolates containing alkB genes, which are the second largest clone cluster of the alkB gene library, as well as about one third of dominant bands in DGGE profiles of 16S rRNA gene and alkB gene fragments, belonged to this genus. The genera Shewanella and Marinomonas, which were detected in the initial seawater profiles, and genera Halomonas and Glaciecola, which were represented in the oil microcosm profiles, have been associated with hydrocarbon degradation in marine environments (Martín-Gil et al., 2004; Brakstad et al. 2008). Sequences of the excised DGGE bands showed high identity to sequences of the isolates, and in the case of alkB gene, also with sequences obtained from the clone library. For example, P. fluorescens strain Hd1 (which was isolated from the initially collected seawater by spreading on hexadecane containing plates) was a representative strain of redundant strains isolated in all of the enrichment experiments. On DGGE profiles of 16S rRNA and alkB gene fragments, the band with the same electrophoretic mobility as that of strain Hd1 was present in all lanes; hence, we suggest that this strain is important in degrading the oil products used in our experiments. The potential importance of alpha- and betaproteobacterial genera as marine oil degraders have been shown (Coulon et al. 2007; McKew et al. 2007; Wang et al. 2010a; Minif et al. 2011; Gertler et al. 2012) and they have also been associated with the degradation of polyaromatic compounds (Alonso-Gutierrez et al. 2009). Experiments performed in this study showed that Alphaproteobacteria are common in initial seawater and they were present in all microcosms at variable band intensities. However, Betaproteobacteria became abundant only in diesel fuel spiked microcosms and did not represent the dominant organisms in the original seawater community (Fig. 5b). The obtained results reveal the importance of these bacterial groups in biodegradation of oil compounds and more effort should be made to improve the isolation methods that allow the proper description of biodegradation potential of bacteria belonging to these groups.

In addition to confirming the presence of catabolic genes in microbial communities, their expression must be addressed to better estimate the biodegradable potential of the microbial community. The alkB gene expression was obtained in case of all seven studied bacterial strains belonging to different P. fluorescens bio-types both in the presence and absence of hexadecane, as an inducer in the growth media. It has also been previously reported that the presence and/or the expression of alkB genes cannot be directly related to the presence of oil (pollution) in the environment, or with the capacity of aliphatic hydrocarbon removal (Païsse et al. 2011). alkB gene expression is induced by many different natural aliphatic compounds, including those produced by living organisms (Widdel and Rabus 2001). Nevertheless, in our study five bacterial strains isolated from the waters of the Baltic Sea demonstrated moderate alkB gene inducibility. However, it is important to mention that other genes may also be involved in alkane degradation, such as cytochrome P450 (van Beilen et al. 2006), which has been found in a broad range of aquatic environments (Wang et al. 2010a,b), or the long chain alkane hydroxylase encoded by alma (Throne-Holst et al. 2007; Liu et al. 2011; Wang and Shao 2012). Hence, further study of these genes, including analysis of gene expression levels in different strains induced by a wider range of hydrocarbon molecules, in bacterioplankton of the Baltic Sea may provide more information about the ability of alkane degradation in this environment.

In conclusion, this study showed that the formation of distinct microbial communities depends on the concentration and the type of oil in Baltic Sea coastal water microcosms that were supplemented with crude oil, shale oil and diesel fuel. In total, 130 alkB gene sequences were studied, and phylogenetic analysis showed that most sequences were linked to three groups: the F. fluorescens- (69 sequences), the Prauserella- (55 sequences) and the Alcanivorax-like alkB group (3 sequences). Some novel alkB gene sequences, which were distant from all other respective sequences, were also present in this unique brackish environment. 16S rRNA-based analysis also suggested that Gammaproteobacteria as well as Actinobacteria are the key players in alkane biodegradation in Baltic Sea coastal water. However, the representatives of Alphaproteobacteria might also become important alkane biodegraders in case of accidental oil spills as they became abundant in all microcosm experiments, but obviously they contain more distant alkB genes as they were found only in minor scale in AlkB clone library. In addition, expression of alkB genes was proved in the representative bacterial strains of the studied hydrocarbon-growing bacterial community. Further research is in progress to assess the presence and diversity of alkane hydroxylase genes in bacteria at different open sea sampling sites and at different time points.


