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Occurrence of diverse alkane hydroxylase *alkB* genes in indigenous oil-degrading bacteria of Baltic Sea surface water

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ABSTRACT

Formation of specific oil degrading bacterial communities in diesel fuel, crude oil, heptane and hexadecane supplemented microcosms of the Baltic Sea surface water samples was revealed. The 475 sequences from constructed alkane hydroxylase *alkB* gene clone libraries were grouped into 30 OPFs. The two largest groups were most similar to *Pedobacter sp.* (245 from 475) and *Limnobacter sp.* (112 from 475) *alkB* gene sequences. From 56 alkane-degrading bacterial strains 41 belonged to the *Pseudomonas* spp. and 8 to the *Rhodococcus* spp. having redundant *alkB* genes. Together 68 *alkB* gene sequences were identified. These genes grouped into 20 OPFs, half of them being specific only to the isolated strains. Altogether 543 diverse *alkB* genes were characterized in the brackish Baltic Sea water; some of them representing novel lineages having very low sequence identities with corresponding genes of the reference strains.

1. Introduction

The intercontinental brackish Baltic Sea, characterized by strong stratification, high nutrient concentration and continuous oxygen deficiency (Koskinen et al., 2011), is a habitat for a mixture of marine, freshwater and brackish water organisms (Höllfors et al., 1981). The coastline of the sea is shared by nine industrial countries that results in intensive marine traffic on the sea. Both the intensive shipping and complications in navigation (narrow traits, shallow depths, crossing shipping lanes) enhance the risk of oil pollution to the marine environment, including bays and beaches (HELCOM (Helsinki Commission. Baltic Marine Environment Protection Commission), 2013). Clean up of oil spills primarily depends on the indigenous microbes present in the environment (Leahy and Colwell, 1990) and therefore it is particularly important to investigate the catabolic capabilities of these organisms. Due to the development of the second generation sequencing techniques a number of studies, dealing with the total microbial community composition of the Baltic Sea, have been performed (Andersson et al., 2010; Koskinen et al., 2011; Tiirik et al., 2014; Dupont et al., 2014) and recently some reports analyzing the communities or/and genes involved in oil-derived hydrocarbons degradation by Baltic Sea bacterioplankton have been published (Jutkina et al., 2011; Reunamo et al., 2013; Vedler et al., 2013; Viggor et al., 2013). Alkanes originating from natural or anthropogenic sources are degraded by several different enzyme systems expressed by phylogenetically diverse bacteria (van Beilen and Funhoff, 2007). The integral-membrane alkane hydroxylases (AlkB; EC 1.14.15.3), which are responsible for hydroxylation of medium-chain-length alkanes (C10 to C16) are the most common and widely distributed genes that have been used for description of the composition of alkane-degrading communities and calculation of the abundance of oil degrading bacteria in various environments (Smith et al., 2013; Nie et al., 2014). The alkane-degradation gene clusters may be plasmid encoded, as for example the well-studied OCT plasmid of *Pseudomonas putida* GPo1 (Kok et al., 1989), but generally they are located in the chromosome (Smits et al., 1999). Usually only one *alkB* gene is found in the genome, but there are several Gram-positive and Gram-negative genera whose representatives have two or more redundant *alkB* genes, for example genus *Rhodococcus* (Whyte et al., 2002) and *Acinetobacter* (Tani et al., 2001), respectively. Although the AlkB sequences may be quite divergent they share a conserved moiety that allows the design of primers applicable for amplification of phylogenetically distant alkane hydroxylases (Smits et al., 1999; Kloos et al., 2006; Viggor et al., 2013).

This study was conducted to evaluate the reactions of indigenous microbial communities in response to a simulated oil spill (heptane, hexadecane, diesel fuel and crude oil were used as model substrates in microcosm experiments), to isolate oil-degrading bacteria, and to analyze the genetic diversity of alkane hydroxylase-encoding *alkB* genes in four different surface water samples of the Baltic Sea.

2. Materials and methods

2.1. Study area and sample collection

A total of eight surface water samples (at depths of approximately 1 m) were collected from four different Baltic Sea regions from August to September in 2008 and 2009 (Fig. 1) using 12-L sterile canisters.

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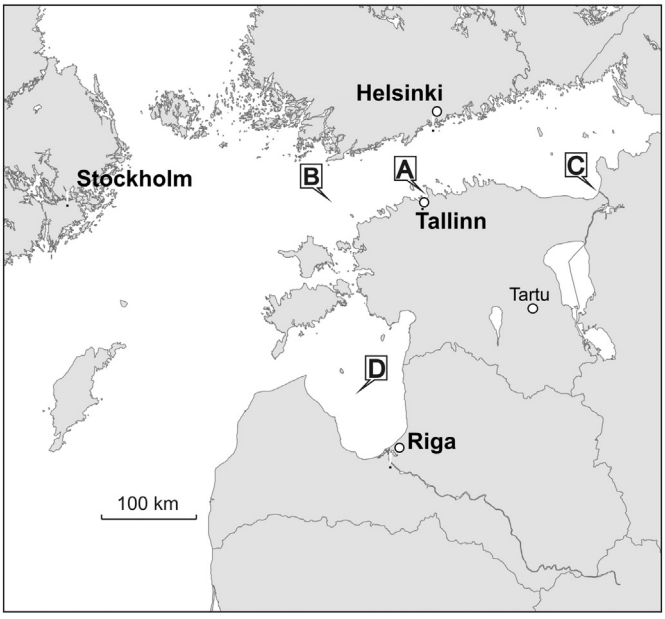

Fig. 1. The location of sampling sites (A — Tallinn Bay, B — Gulf of Finland, C — Narva Bay and D — Gulf of Riga) in Baltic Sea.

The sample code in this study consists from capital letter (A — Tallinn Bay, B — Gulf of Finland, C — Narva Bay and D — Gulf of Riga) and samples from the year 2009 have the number 2 before the letter. The sample taken ca 6 km from the port of Tallinn, A (59°32′12″ N, 24°41′18″ E), is in high sea traffic area. Sampling point C (59°28′30″ N, 28°00′30″ E) is affected by fresh water more than other sites because it is taken ca 2 km from the coast, near the estuary of the river Narva. Distances of offshore sampling sites B (59°28′60″ N, 22°57′00″ E) and D (57°37′00″ N, 23°37′00″ E) from coast were ca 37 km and 41 km, respectively. The salinity values of the sampling points were as follows A/2A — 5.8/6.0‰, B/2B — 6.3/6.1‰, C/2C — 3.7/4.1‰ and D/2D — 5.4/5.3‰. Samples were stored at 4 °C until analysis (within 24 h after collection).

2.2. Isolation of bacteria: direct selection and microcosm experiments

Hydrocarbon-degrading bacterial strains were isolated from plated-out cultures of collected seawater on selective M9 mineral agar plates (Adams, 1959; Bauchop and Elsden, 1960) containing crude oil (CO; Lukoil Oil Company OAO), diesel fuel (DF; Neste Oil), heptane (Hp; Fluka; ≥96% purity) or hexadecane (Hd; Fluka; 99% purity) as the only growth substrate (vapor phase). Plates were sealed and incubated at 15 °C for 14 days.

The microcosms (enrichment and control) experiments were performed as described in Jutkina et al. (2011) using crude oil (CO), diesel fuel (DF), heptane (Hp) or hexadecane (Hd) at final concentration 1% (*v*/*v*) as the only growth substrate in enrichments. The experiments were done with each seawater sample in 250-mL sterile flasks containing 50 mL of liquid media at 15 °C on rotary shaker (130 rpm) for three weeks. Isolation of hydrocarbon-degrading bacteria from different microcosms was performed after serial dilution and plating on selective media (containing the same carbon source that was used in microcosm), followed by a 2 week incubation period at 15 °C.

Purified, morphologically different bacterial cultures having unique BOX-PCR fingerprint pattern (Heinaru et al., 2000) and *alkB* gene were stored in 20% glycerol at −80 °C.

The carbon source utilization tests of the bacteria were performed on the minimal media supplemented with octane (C8; AlfaAesar; ≥98% purity), hexadecane (C16/Hd; Fluka; 99% purity), octadecane (C18; AlfaAesar; 99% purity) or docosane (C22; Sigma-Aldrich; 99% purity) as the only growth substrate (vapor phase).

2.3. Extraction of total community DNA

Total community DNA was extracted from initial seawater and from the first enrichment step of microcosm experiments as described by Viggor et al. (2013) using a PowerSoil DNA Kit (MoBio Laboratories, Inc., USA), according to the protocol provided by the manufacturer. The extracted DNA was quantified with spectrophotometer NanoDrop ND-1000 (NanoDrop Technologies, USA) and stored in MilliQ water at −20 °C.

2.4. PCR amplification of target molecules

The 16S rRNA genes and membrane-bound alkane hydroxylase encoding *alkB* gene fragments of the isolated bacterial strains, and the 16S rRNA gene fragments of bacterial communities (eight samples of the initial water and samples obtained from microcosm enrichment experiments with all seawater samples each supplemented with heptane, hexadecane, diesel fuel or crude oil) for denaturing gradient gel electrophoresis (DGGE) analysis were amplified as described in our previous work (Viggor et al., 2013). The specific primer pair HydroAlkBF (5′-CCTACGGCCATTTCTTCATTGA-3′) and HydroAlkBR (5′-GGCTGTAATG CTCGAGATAATT-3′) was designed based on *Hydrocarboniphaga effusa* AP103 *alkB* gene sequence (EIT68388). The PCR conditions were identical to those of the alkBF and alkBR primers (Viggor et al., 2013).

2.5. Clone libraries

Gel purified (Qiaquick Gel Extraction Kit, Qiagen) PCR products of the *alkB* genes amplified from the total community DNAs of the initially collected seawater samples were cloned into a pTZ57R/T vector and transformed into competent *Escherichia coli* DH5α cells, following the manufacturer's protocol of InsT/Aclone product Cloning Kit (Thermo Fisher Scientific Inc). Screening and thereafter grouping of correctly amplified inserts obtained from community DNAs were done using DGGE analysis (Viggor et al., 2013). The PCR products of the *alkB* genes amplified from the isolated strains that resulted in ambiguous bases during direct sequencing, suggesting overlapping sequences referring to intra-species gene redundancy, were cloned similarly. In the case of *alkB* clone libraries of the strains the screening of inserts from white colonies of transformants was performed using the vector specific primer pair (M13-R 5′-AACAGCTATGACCATG-3′ and M13-F 5′-CATTTTGCTTGC CGG-3′; Thermo Fisher Scientific Inc.). At least ten correctly amplified inserts were randomly picked and sequenced. One representative of each group of different sequences was chosen for further phylogenetic analyses.

2.6. DGGE

The INGENYphorU-2 × 2 (Ingeny International, Netherlands) DGGE system was used for analysis of the *alkB* gene clone libraries of initially collected seawater and 16S rRNA genes amplified from the community DNAs of initially collected seawater and enrichment experiments, following the recommendations of the manufacturer. Detailed description of used DGGE conditions and subsequent analysis of DGGE gels are described earlier in Viggor et al. (2013). The cluster analysis of PCR-DGGE fingerprints of the partial 16S rRNA genes of the control sample (initial seawater without additional substrate) communities and enrichment communities revealed that after three week incubation the community profiles of the Baltic Sea water without substrate formed separate cluster from enrichment communities (data not shown) as was reported in Viggor et al., 2013.

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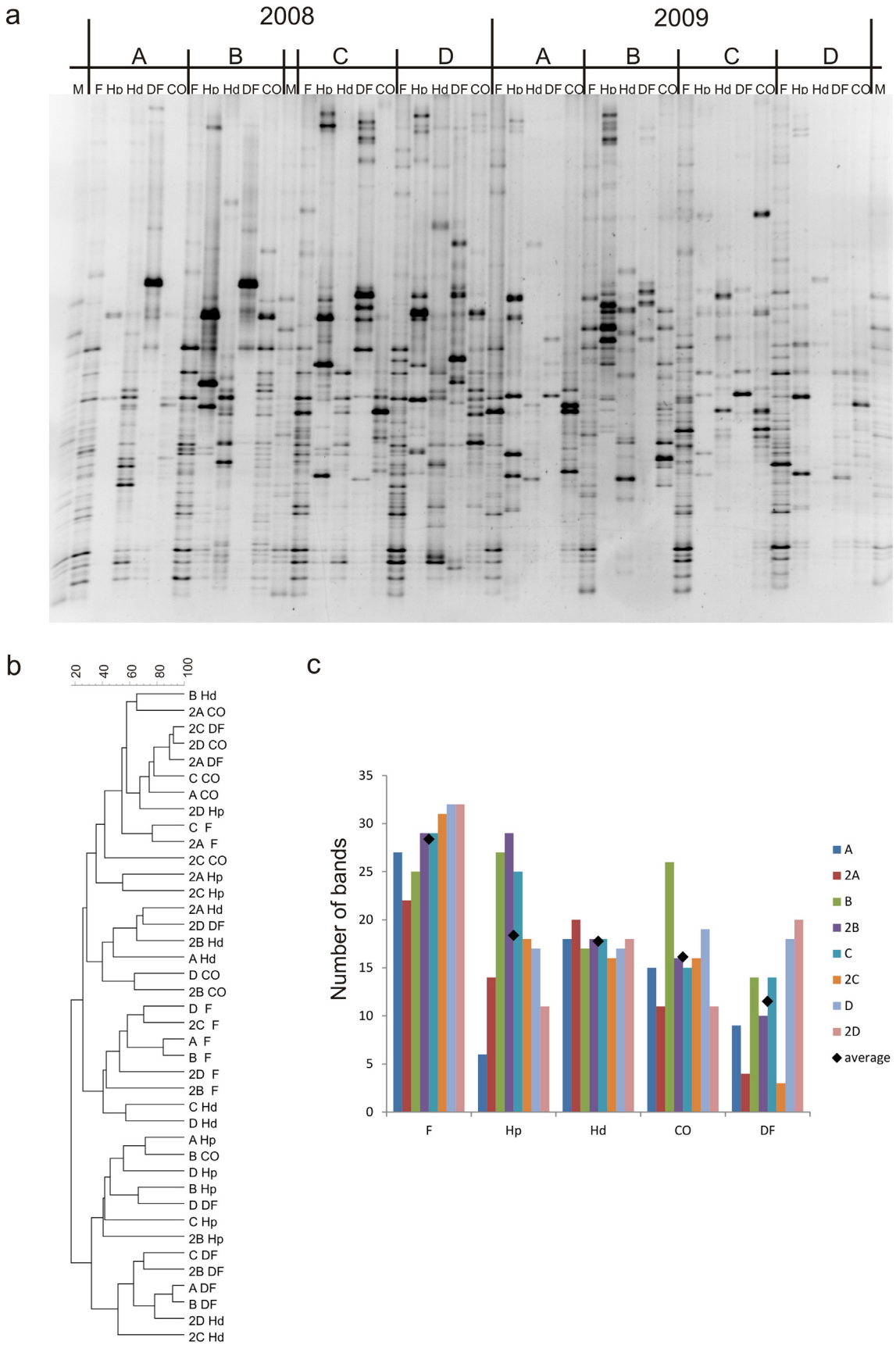

Fig. 2. Original DGGE profiles of the partial 16S rRNA genes of microbial communities (a), cluster analysis of PCR-DGGE fingerprints (b), and number of bands (c) calculated from DGGE analysis of the enrichment experiments. Abbreviations: A — Tallinn Bay, B — Gulf of Finland, C — Narva Bay and D — Gulf of Riga, samples taken in 2009 have number 2 before the legend; F — initially collected seawater; M — marker line (2009 B F); CO — crude oil; DF — diesel fuel; Hp — heptane; and Hd — hexadecane. Data of a single experiment are presented; variability among different experiments was minimal.

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2.7. Sequencing and data analysis

All the *alkB* and 16S rRNA gene fragments were nucleotide sequenced using a BigDye®Terminator v3.1 Cycle Sequencing Kit for Applied Biosystems 3730χ/DNA Analyser following the manufacturer's instructions. Reference sequences were obtained from the GenBank/NCBI based on the alignments performed with the programs BLASTN and BLASTP.

The *alkB* nucleotide sequences were translated in silico, and phylogenetic trees from ClustalW aligned sequences were constructed by neighbor-joining (NJ) analysis using MEGA6 software (Tamura et al., 2013) with 1000 bootstrap replicates. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.79). All positions containing gaps and missing data were eliminated.

The evolutionary history of the nucleotide sequences of the 16S rRNA genes was inferred using the Neighbor-Joining method. Phylogenetic trees from ClustalW aligned sequences were constructed by using MEGA6 software (Tamura et al., 2013) with 1000 bootstrap replicates. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated.

The AlkB sequences with >82% identity (the cut-off value) were assigned to the same operational protein families (OPF) using Fungene software available at http://fungene.cme.msu.edu/FunGenePipeline/ (Fish et al., 2013). Same software was used for chimera check and for calculation of the coverage estimates of the *alkB* gene library. 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). Good's coverage estimator was calculated using the following formula C = 1-(s/N), where s is the number of singletons and N is the total number of clones in the library (Good, 1953). The nucleotide sequences reported in this study were deposited in the GenBank database under accession numbers KT229361–KT229447 and KT229449–KT229515.

2.8. Growth measurements

The growth of *Rhodococcus* sp. strains D1RHp1 and 2D43 (possessing redundant *alkB* genes) on R2A and mineral medium supplemented with 0.1% *v/v* liquid (C16 and C18) or solid (0.05% wt/v) alkanes (C22 and C26 (Sigma-Aldrich; 99% purity)) was measured spectrophotometrically at 580 nm. All growth experiments were performed at 30 °C in triplicate.

2.9. Statistical analysis

Excel Data Analyses Toolbox was used for statistical analyses.

3. Results and discussion

3.1. Dynamic changes in bacterial community composition according to DGGE profiles of the 16S rRNA gene fragments in microcosms modified by oil born compounds

All eight seawater samples taken from four different parts of Baltic Sea in two year period were used in microcosm experiments supplemented with heptane, hexadecane, diesel fuel or crude oil to enrich the growth of hydrocarbon-degrading bacteria. PCR-DGGE method was used to assess the impact of different oil components on bacterial community composition (Fig. 2). Cluster analysis of the DGGE profiles of the 16S rRNA gene showed difference between the microcosm samples supplemented with different substrates after 3 week incubation and the initial seawater samples (Fig. 2b). The profiles of the bacterial communities enriched through hydrocarbon exposure were different if four samples of one year or one sample in two sampling years were compared. Considering the same sample, the used hydrocarbon was responsible for the selection of specific bacterial communities and cluster analysis of DGGE profiles showed distinction of samples supplemented with different substrates into separate clusters (Fig. 2b). The number of DGGE bands of the amplified 16S rRNA gene in initial samples was approximately the same during the two year period (average number of bands 28), but decreased after three weeks lasting microcosm experiments. The greatest changes in the number of bands were observed in the cases of microcosms containing diesel fuel (average number of bands 11), while in the cases of heptane, hexadecane and crude oil diminishing was usually smaller (average number of bands 17) (Fig. 2c). The finding is consistent with previous researches (Röling et al., 2002; McKew et al., 2007; Vila et al., 2010; Viggor et al., 2013) where it has been shown that oil contamination may reduce the diversity of a microbial community due to the selection of limited number of biodegradative species. The DGGE analysis of microcosm experiments in this study indicates that Baltic Sea is a habitat for bacteria able to degrade different oil born compounds.

3.2. Analysis of the isolates possessing alkB genes

Although we are living in the age of genomics the fact remains that the most efficient way to gather information about a microorganism is to study it in culture and relate that knowledge to field observations (Giovannoni and Stingl, 2007). Workgroups of Herlemann et al. (2011) and Dupont et al. (2014) pointed to the fact that the brackish Baltic Sea community was a combination of freshwater and marine bacterial groups, along with populations unique to this environment. Therefore there is a need for investigation of such communities to map important bacterial players and to identify their specific biogeochemical roles. A total of 175 strains were isolated in the current study by applying direct plating of seawater on minimal media with selective substrates and microcosm enrichment experiments. 56 of these strains (13 strains from initial seawater and 43 from microcosms) possessed the alkane hydroxylase-encoding *alkB* gene (Table S1).

3.2.1. Phylogenetic analysis of the 16S rRNA genes of the isolates

According to the phylogenetic analysis of the 16S rRNA genes (~419 bp) 48 bacterial strains belonged to the phylum *Proteobacteria* and 8 to the phylum *Actinobacteria* (Fig. 3). Among the class *Gammaproteobacteria* the genus *Pseudomonas* was most common (41 strains), while genera *Perlucidibaca*, *Hydrocarboniphaga* and *Solimonas* were each represented by one isolate. *Pseudomonas* strains formed four clusters in the phylogenetic tree (Fig. 3) and their partial 16S rRNA gene sequences were identical with the sequences of the strains from the *Pseudomonas* species *anguilliseptica* (I), *protegens* (II), *fluorescens* (III) and *putida* (IV), respectively. Classes *Alpha-* and *Betaproteobacteria* were represented by strains belonging to the genera *Novosphingobium* (one strain), and *Acidovorax* (one strain) and *Aquabacterium* (two strains), respectively. All Gram-positive strains

Fig. 3. Phylogenetic tree of partial 16S rRNA gene sequences (~419 bp) obtained from isolates. The colored rectangles represent *Alpha* — (blue), *Beta* — (red), *Gammaproteobacteria* (green) and *Actinobacteria* (yellow). The details of the *Pseudomonas* group are shown on the right side of the figure (the roman numerals note different genera). Reference sequences from the GenBank database are labeled with the accession numbers of 16S rRNA genes and the name of the strain. There were a total of 380 positions in the final dataset. Bootstrap values (per 1000 trials) ˃50% are indicated at the nodes. The scale bars represent 0.05 substitutions per base pair.

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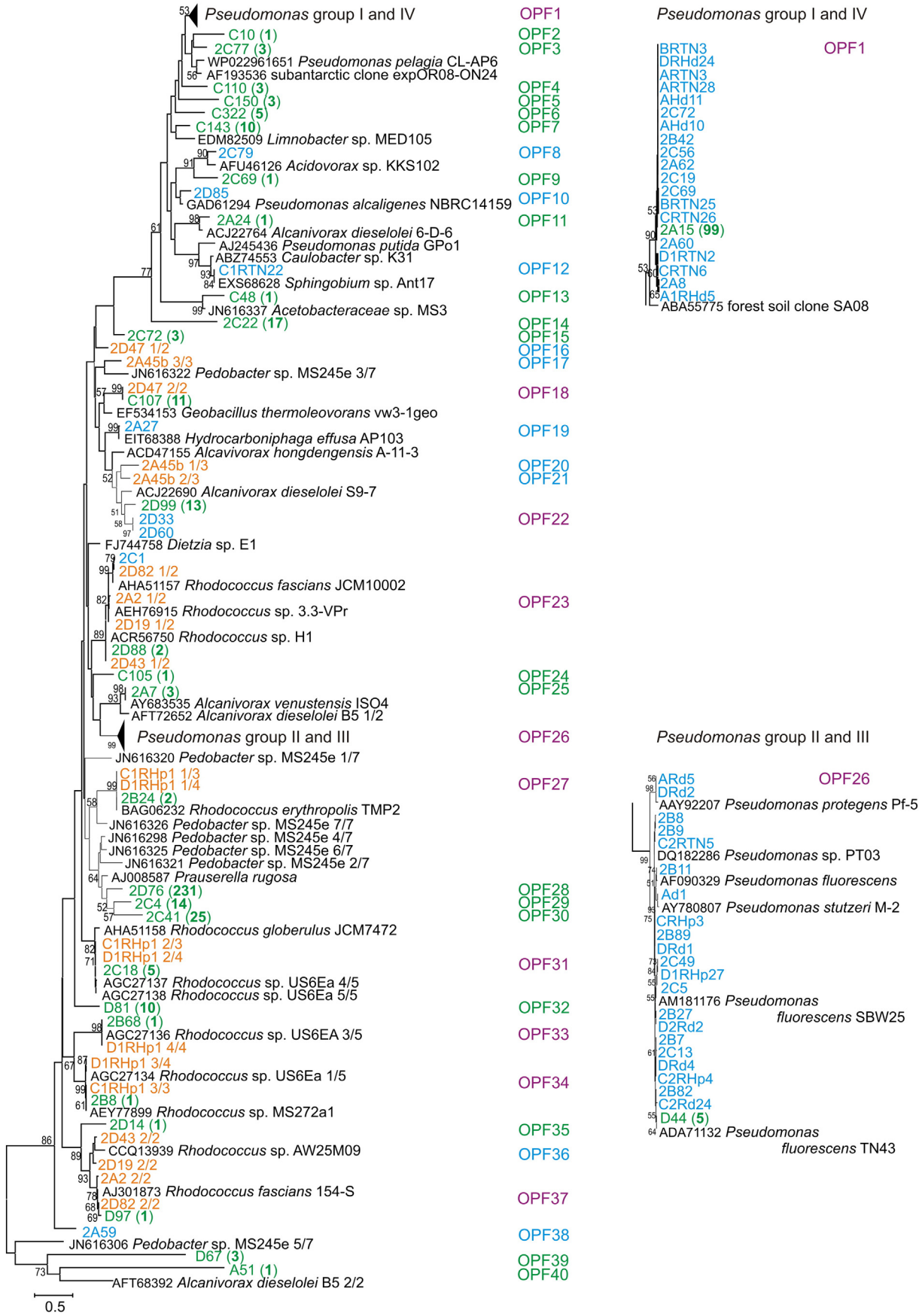
(8) belonged to the genus *Rhodococcus*. The metagenomic research of the V6 region of the 16S rRNA gene on Illumina HiSeq2000 of the same Baltic Sea samples revealed that dominant phyla in these communities were *Actinobacteria* (35–50%), *Proteobacteria* (15–40%), *Bacteroidetes* (10–20%) and *Firmicutes* (5–10%) (Tiirik et al., 2014). Domination of these phyla in Baltic Sea surface water samples are in



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concordance with the results obtained independently by workgroups of Herlemann et al. (2011) and Dupont et al. (2014) who analyzed metagenomes of the Baltic Sea water samples taken in the same period as in the current work (June/July 2008 and July 2009, respectively).

3.2.2. alkB diversity in the isolated strains

The membrane bound alkane hydroxylase encoding *alkB* genes of strains analyzed in the current study were amplified by PCR using degenerate primer pair alkBF and alkBR (Viggor et al., 2013), except the strain *Hydrocarboniphaga effusa* 2A27 for which specific primer pair was designed. Together 68 *alkB* gene sequences (330 bp) were obtained from strains, whereas eight strains were found to have redundant *alkB* genes (Table S1, Fig. 4). Pooling of all AlkB sequences obtained from isolated strains resulted in 20 operational protein families (OPFs) (cut-off value of 82% sequence identity at the amino acid level (Fig. 4; Table S1). Pseudomonads that clustered into four groups on the 16S rRNA phylogenetic tree (Fig. 3) were placed into two separate groups on the phylogenetic tree of the deduced amino acid sequences of *alkB* gene (100 aa). The AlkB sequences of the strains from species *P. fluorescens* were >90% identical with those described in species *P. fluorescens* or *P. gessardii* (OPF1), while strains from species *P. anqulliseptica* had the highest identity (71%) with AlkB sequence of *Limnobacter* sp. from the class *Betaproteobacteria* (OPF26). AlkB sequences of the four strains, belonging to *P. putida* by 16S rRNA gene sequence, were 61–63% identical with AlkB sequence of *Alcanivorax dieselolei* 6-D-6. The AlkB sequence of strain 2D85 that was 86% identical with respective sequence of *P. alcaligenes* clustered separately from other pseudomonads (Table S1, Fig. 4). Strains belonging to *P. fluorescence* and *P. protegens* group gave weak growth on hexadecane and octadecane, while *P. angulliseptica* and *P. putida* strains gave vigorous growth (Table S1). Phylogenetic groupings of strains (Fig. 3) did not coincide well with groupings based on their AlkB sequences (Fig. 4), especially in the case of the genus *Pseudomonas*. Such difference in clustering has been described by other workgroups (Nie et al., 2014), and also in the case of other catabolic genes, for example multicomponent phenol hydroxylase and catechol 2,3-dioxygenase (Vedler et al., 2013). This phenomenon has been explained by horizontal gene transfer of *alkB* genes (van Beilen et al., 2003), evolution of duplicated genes or gene fusions (Nie et al., 2014), although detailed mechanisms are needed to be further investigated. The phylogenetic analyses revealed that the AlkB sequences of the *Pseudomonas* strains clustered into two separate clusters (OPF1 and OPF26, Fig. 4). The sequences of the cluster OPF1 had >70% identity with AlkB sequences of the betaproteobacterial *Limnobacter* sp. MED105 (Table S1). Such a low sequence identity with sequences found in GenBank database may indicate that we have described a new, so far not determined AlkB lineage. An interesting fact is also that Vedler et al. (2013) were the first who isolated four *Limnobacter* sp. strains from Baltic Sea surface water samples and showed their ability to degrade phenol. The whole genome sequencing of these strains revealed the possessing of AlkB sequences highly identical (~99%) with AlkB sequence of *Limnobacter* sp. MED105 (Vedler, personal communication). The role of the genus *Limnobacter* in degradation of *n*-alkanes still needs further clarification. AlkB sequences from OPF26 were identical to sequences determined in different pseudomonads, including those isolated from Baltic Sea coastal seawater (Viggor et al., 2013).

Strains belonging to the genus *Hydrocarboniphaga* have been shown to grow on aliphatic hydrocarbons from C6 to C19 (Palleroni et al., 2004; Liu et al., 2011) and genome analysis of the *Hydrocarboniphaga effusa* AP103T (ATCC BAA-332T) revealed the presence of genes encoding AlkB-like enzymes that could possibly be involved in alkane degradation (Chang et al., 2012). *Hydrocarboniphaga effusa* strain 2A27 isolated from the Baltic Sea surface water grows well on solid agar plates containing alkanes from C8 to C22 as the only growth substrate (Table S1). From the same water sample Vedler et al. (2013) identified a phenol, benzoate, *m*-toluate and salicylate-degrading *Hydrocarboniphaga effusa* strain 2Atol2 indicating that strains belonging to this genus are versatile degraders of oil-derived hydrocarbons.

Although *Solimonas* sp. strain 2A45b had three redundant *Alcanivorax*-like *alkB* genes (identities 70–77%), its growth on tested *n*-alkanes was moderate (Table S1). Workgroup of Wang et al. (2010) isolated diesel oil degrading *Solimonas* sp. S10-1 from the Atlantic Ocean surface water sample and identified the presence of *alkB* and two quite divergent P450 CYP153A sequences. The AlkB sequences identities of strains 2A45b and S10-1 were between 68 and 77%, reflecting the phylogenetic divergence of these strains.

In the case of the *Betaproteobacteria* the identified *alkB* gene phylogeny in *Acidovorax* sp. strain 2C79 was consistent with 16S rRNA gene phylogeny, while in *Aquabacterium* sp. strains 2D60 and 2D47 they were 68–79% identical with the respective genes of *Alcanivorax* sp. Strains from these genera have been shown to be involved in degradation of hydrocarbons in various environments (Yang et al., 2014; Jechalke et al., 2013). Lately, from the genome of a soil bacterium, *Aquabacterium* sp. strain NJ1, capable of utilizing both liquid and solid alkanes, two copies of cytochrome P450-type, five copies of AlkB-type, and eight copies of AlmA-type oxygenases were identified and further research will explore the physiological roles of each gene in the degradation of *n*-alkanes (Masuda et al., 2014).

Isolates from the genus *Rhodococcus* had vigorous biomass production on minimal agar plates containing alkanes from 8 to 22 carbon atoms, and six strains from eight had more than one AlkB homolog with high identity to sequences from *Rhodococcus* species (Table S1). The amino acid sequences of the two redundant AlkBs share 50% to 71% identity, depending on the strain. Previously it has been shown that *Actinobacteria* represent an abundant, active and diverse component of bacterioplankton in the northern Baltic Sea (Holmfeldt et al., 2009); the genus *Rhodococcus* together with other actinobacterial genera *Nocardia*, *Dietzia*, *Mycobacterium* and *Gordonia* are recognized as ideal candidates for the biodegradation of hydrocarbons because of their ability to degrade a wide range of organic compounds, hydrophobic cell surfaces, production of biosurfactants, and robustness and ubiquity in the environment (Larkin et al., 2005). It is supposed that the number of AlkB homologs in a strain correlates with the range of *n*-alkanes that can be metabolized by that strain. In many cases strains with multiple alkane hydroxylases are able to oxidase alkanes up to C32 to C36 (Whyte et al., 2002; van Beilen et al., 2002), compared for example with *Pseudomonas putida* GPo1 that has one AlkB and degrades alkanes up to C12 (Smits et al., 2002). Some strains from the family *Rhodococcus* may contain in addition to *alkB* gene(s) also other type of alkane hydroxylases in their genomes, for example the cytochrome P450 enzyme(s) of the CYP153 family (Amouric et al., 2010; Nie et al., 2014). *alkB* genes found in a single rhodococcal strain are usually quite divergent (Whyte et al., 2002), the current study confirmed this

Fig. 4. Neighbor-Joining tree based on the deduced amino acid sequences of the alkane hydroxylases (*alkB*; 100 aa) of strains and clones obtained in this study. The details of the *Pseudomonas* groups are shown on the right side of the figure. Names of the strains having one or multiple *alkB* gene are colored blue and yellow, respectively. In the case of the multiple *alkB* homologs the homolog number is shown after the strain name and total number of homologs is given after slash. The bold numbers in parentheses after clone numbers (green) represent the number of identical clones obtained from clone libraries of initially collected seawater samples. The numbers of the operational protein families (OPF; cut-off value 82% sequence identity at the amino acid level) are shown next to the phylogenetic tree; green, blue and purple color of the OPF denotes that OPF contains AlkB sequences obtained only from clone libraries, only from strains, and both from clones and strains, respectively. Reference strains obtained from GenBank are labeled with accession numbers and the name of the strain. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branch (˜50%). The analysis involved 143 amino acid sequences. The scale bars represent 0.5 substitutions per amino acid.

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finding. An intriguing result was obtained in case of strains D1RHp1 and C1RHp1. Their AlkB sequences were found to be 97–100% identical (100 aa between histidine cluster 2 and HYG-motif) to the AlkB sequences of three rhodococci originating from geographically distinct areas: *Rhodococcus* sp. Q15 from Lake Ontario sediment, Canada (Whyte et al., 1998), *R. erythropolis* NRRL B-16531 (ATCC15960) from petroleum–contaminated soil, Japan (Iizuka and Komagata, 1964), and *R. erythropolis* 23-D from soil near a car repair shop in Bremen, Germany (van Beilen et al., 2002). These strains degrade a broad range of alkanes — growth of strains D1RHp1 and C1RHp1 was tested on alkanes up to 26 carbons in length (Fig. S1, Table S1), strain Q15 is able to oxidase *n*-alkanes (C8 to C32), branched alkanes and substituted cyclohexane (Whyte et al., 1998) while strains NRRL B-16531 and 23-D degrade C6 to C36 *n*-alkanes (van Beilen et al., 2002). It is also important to mention that the organization of *alk* gene cluster may be different within and between the strains (Whyte et al., 2002) and as we saw in the growth experiments the strains with two (2D43) and four (D1RHp1) redundant *alkB* genes have different growth patterns on alkanes (Fig. S1). Therefore further work is needed to specify the role of each of the rhodococcal *alkB* gene in the degradation of alkanes and clarify how the gene fusions (for example AlkB-rubredoxin) may affect degradation spectrum of the long-chain *n*-alkanes.

3.3. Analysis of the alkB clone libraries

3.3.1. Description of libraries

The analysis of the *alkB* gene (300 bp) clone libraries constructed to characterize the main bacterial *alkB* groups in the initial Baltic Sea seawater samples cover 475 *alkB* gene sequences (Table S2). The rarefraction curves of all constructed libraries expressed asymptotic behavior (Fig. S2), indicating that the sampling effort was satisfactory. Good's coverage estimator values of libraries were > 0.90, also indicating that almost complete coverage was obtained (Table 1). The number of operational protein families (OPF) (cut-off value of 82% sequence identity at the amino acid level) and the richness and diversity estimates were calculated for each constructed library and annually (Table 1). The number of observed OPFs (Sobs) varied from 4 to 16, whereas the lowest and the highest value was obtained for libraries from samples of Gulf of Finland (sample B) in 2008 and Narva Bay (sample C) in 2008, respectively. The number of OPFs in summarized annual samples in 2008 and 2009 was 19 and 17, respectively and all together 30 unique OPFs were detected. As the salinity of water in four sampling points was different and it has been shown previously that salinity is a major factor controlling the distribution of the biota in aquatic systems (Herlemann et al., 2011; Dupont et al., 2014) we decided to test whether there is a correlation between diversity estimates and salinity. As a result, a statistically ($p < 0.05$) important negative linear correlation was obtained both between number of observed OPFs (Sobs) and estimated Chao1 richness (SChao1) (correlation coefficients −0.849 and −0817, respectively) with the salinity of the sample. Statistical measures of diversity (Shannon diversity indices, H', and eveness indeces, E) did not vary consistently with salinity of sample. So far, there have been only few reports on the diversity of *n*-alkane genes and environmental factors in marine or freshwater ecosystems. Smith et al. (2013) concluded after studying the water samples taken from the Gulf of Mexico that community structure (composition, richness and diversity) of alkane-degrading bacteria varied among sites independently of depth and location. While King et al. (2013) analyzing the phylogenetic composition and diversity of communities of the same samples as the previously sited article deduced that communities' compositions (16S-rRNA-gene-based method) varied substantially with depth, but diversity indices did not, which indicated that the structure of bacterioplankton communities was relatively stable across large gradients in physical–chemical and biological variables. An important factor in determining the composition of oil-degrading consortia at specific sites is oil type and concentration (Röling et al., 2002; McKew et al., 2007; Viggor et al., 2013). Nutrient amendment over a wide range of concentrations in sea sediment microcosms significantly improved oil degradation, and a marked effect on the composition and diversity of the developing bacterial community was also observed (Röling et al., 2002; Singh et al., 2014).

Table 1
Observed bacterial *alkB* gene richness and diversity estimates of the clone libraries of analyzed samples (distance cut-off 0.18).

Sample[a]	N	Sobs	SChao1	H'	E	C
A	62	6	10	1.377	0.708	0.95
B	22	4	4	0.945	0.682	0.95
C	78	16	21	2.329	0.840	0.94
D	56	6	6	1.059	0.591	0.98
2008	218	19	30	2.219	0.741	0.98
2A	77	6	6	1.120	0.625	0.99
2B	39	8	11	1.636	0.745	0.90
2C	74	11	14	1.700	0.709	0.96
2D	67	6	6	0.641	0.358	0.97
2009	257	17	23	1.739	0.601	0.98

[a] The capital letter in samples's code denotes the isolation site (A - Tallinn Bay, B – Gulf of Finland, C – Narva Bay and D – Gulf of Riga) and samples from the year 2009 have number 2 before the letter. Abbreviations: N - number of clones; Sobs - observed richness; SChao1 - Chao1 richness estimate; H' - Shannon index of diversity; E - evenness; C - Good's coverage. Because each OPF may be present at multiple sites the sum of Sobs at all sites is not equal to the pooled (annual) value for Sobs.

3.3.2. Phylogenetic analysis of alkB clone libraries

The deduced amino acid sequences of the 30 representative OPFs had 56% to 100% identity to published sequences (Table S2). More than half of the *alkB* clone sequences (248 from 475) were most similar to those of the bacterium *Pedobacter* sp. MS245e from the phylum *Bacteroidetes* (identities between 73% and 76%; Table S2). The sequences of the second largest clone group (169 from 475) were most similar (identities between 63% and 100%) to those obtained from the bacteria belonging to the phylum *Proteobacteria*, specifically to the classes *Alphaproteobacteria* (19), *Betaproteobacteria* (116) and *Gammaproteobacteria* (32). Remaining sequences (60 from 475) were similar to *alkB* genes described in Gram-positive bacteria from the phyla *Actinobacteria* (identities between 67% and 100%) and *Firmicutes* (identity 77%). This result is consistent with the result of sequencing of communities' 16S rRNA genes (Tiirik et al., 2014) where the same phyla were found to be dominant, although not even a single strain from the phylum of *Bacteroidetes* was isolated in the current work. In the recent research Nie et al. (2014) investigated the distribution of alkane hydroxylase *alkB* genes among the 137 metagenomes deposited in GenBank and reported that the terrestrial, freshwater and marine metagenomes had different distributional patterns. However, in case of the current study the pattern of phylogenetic distribution of AlkB sequences of the brackish Baltic Sea bacterioplankton among phyla is not identical to none of them and it supports the hypothesis that the Baltic Sea is a unique habitat for a mixture of terrestrial, marine and freshwater organisms.

4. Comparison of the *alkB* genes acquired by using cultivation-based and culture-independent methods

The brackish Baltic Sea is characterized by high level of nutrients, stratification and contrasting oxygen concentrations; its microbial community is specific. Therefore it is not reliable to compare AlkB diversity indices of communities, for example the number of observed OPFs, obtained in the current work with those found in other studies dealing with oceans or other seas. However, detailed information on the oil-degrading bacteria and distribution of degradative genes in the Baltic Sea bacterioplankton is currently lacking. In the nonpolluted coastal waters (salinity 6.3‰) of the Baltic Sea eight OPFs of *alkB* gene (82% sequence identity at amino acid level) were observed (Viggor et al., 2013). Characteristics of AlkB clone libraries (Table 1) indicate that the observed richness of samples are similar to the coastal water sample

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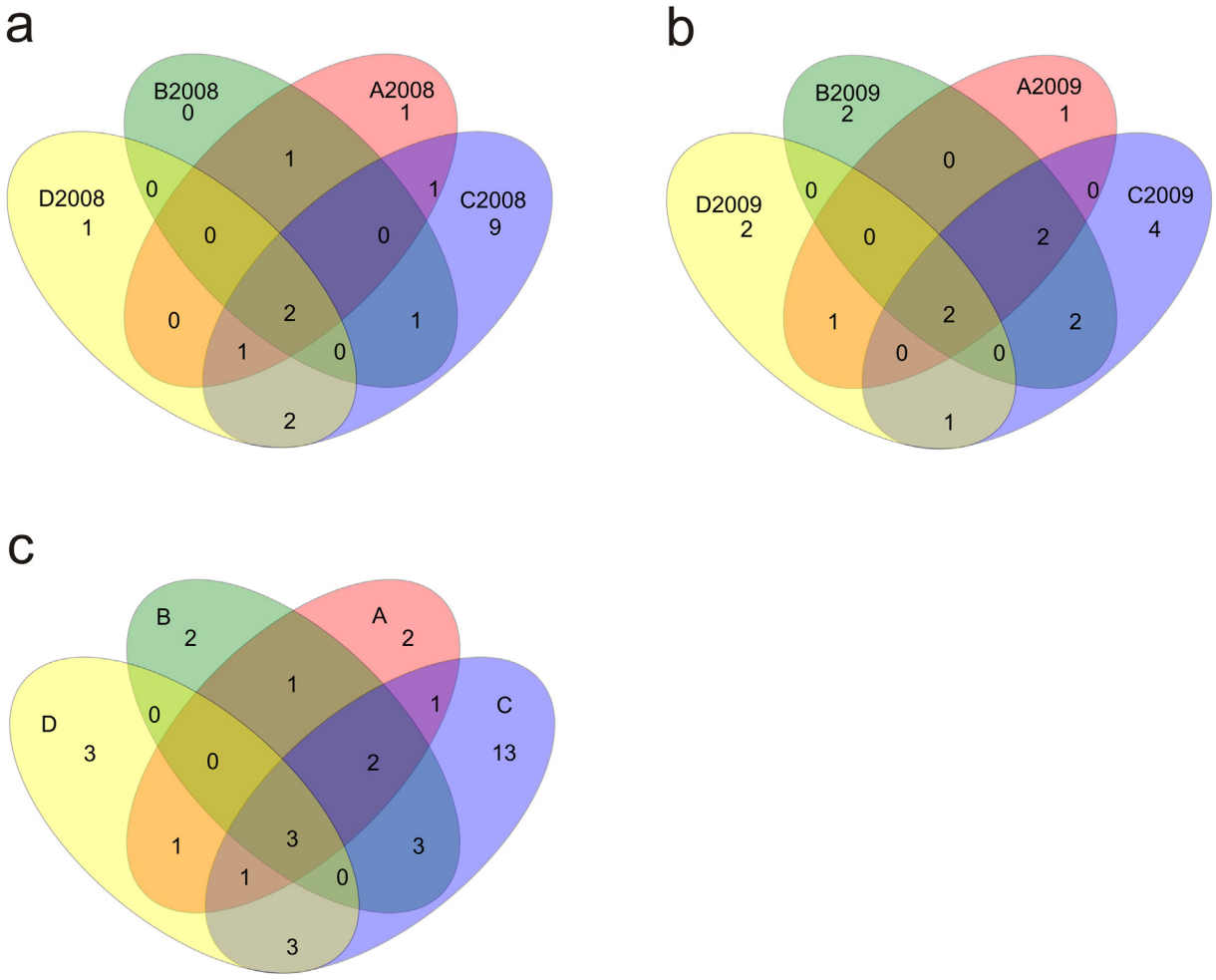

Fig. 5. Venn diagrams of *alkB* clone libraries of samples taken in 2008 (a), 2009 (b) and in pooled samples (c) using an 82% sequence similarity cutoff. The numbers represent the observed richness and the shared richness of each library. Abbreviations: A — Tallinn Bay, B — Gulf of Finland, C — Narva Bay and D — Gulf of Riga.

analyzed previously (Viggor et al., 2013), whereas in samples taken near the Narva Bay, where there is a strong influence of inland freshwater, the highest richness was detected. Analysis of the Venn diagrams of annual samples (Fig. 5) reveals that samples taken from Narva Bay have more unique AlkB sequences than other samples and all constructed libraries have three common OPFs and several OPFs are shared between two or three libraries. This observation shows that even distantly separated (ca 500 km) samples of the Baltic Sea surface water share common AlkB phylotypes.

Analysis of complex communities with different methods usually gives a more proper overview of it. When all AlkB sequences obtained in this work were pooled, 40 OPFs were distinguished (82% sequence similarity cut-off), from which half were from clone libraries, quarter hold sequences from strains and quarter contained sequences both from clone libraries and strains (Fig. 4; Tables S1 and S2). Using such approach shortcomings arising from cultivation and incubation or DNA separation and PCR methods will be balanced. Isolation of strains possessing phylogenetically different *alkB* genes is requisite to resolve inconsistency between *alkB* and 16S rRNA gene phylogeny. For example, *Limnobacter*-like (69–75% identity) sequences from the second largest OPF (Table S2) clustered on phylogenetic tree with *alkB* genes obtained from *P. putida* and *P. anguilliseptica* strains (Fig. 4) revealing the need for careful interpretation of *alkB* phylogeny in the case of low identities with reference sequences.

In summary, the results from PCR-DGGE analysis of the Baltic Sea bacterioplankton using 16S rRNA as a marker gene showed the formation of specific communities with reduced diversity after three week incubation of seawater with heptane, hexadecane, diesel fuel or crude oil. The isolates obtained from direct cultivation of seawater and after performing microcosm experiments belong to well-known oil-degrading strains from the phyla *Proteobacteria* and *Actinobacteria*, whereas the genera *Pseudomonas* and *Rhodococcus* were represented with the biggest number of strains. Rhodococcal strains grew well on alkanes up to 26 carbons in length and two-thirds of isolates from this genus had two to four redundant *alkB* sequences. Analysis of *alkB* gene clone libraries revealed the domination of *Bacteroidetes*-like sequences, but as the identities with genes of reference strains were low (56–76%) and *alkB* and 16S rRNA genes phylogeny was not always consistent, confident conclusions about phylogeny of catabolic genes cannot be made. The communities' diversity indices' values were found to correlate with the salinity of the sample. Further study will be focused to close examination of the strains expressing redundant *alkB* genes, as well as to characterization of distribution and role of other bacterial alkane hydroxylases in the Baltic Sea.

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