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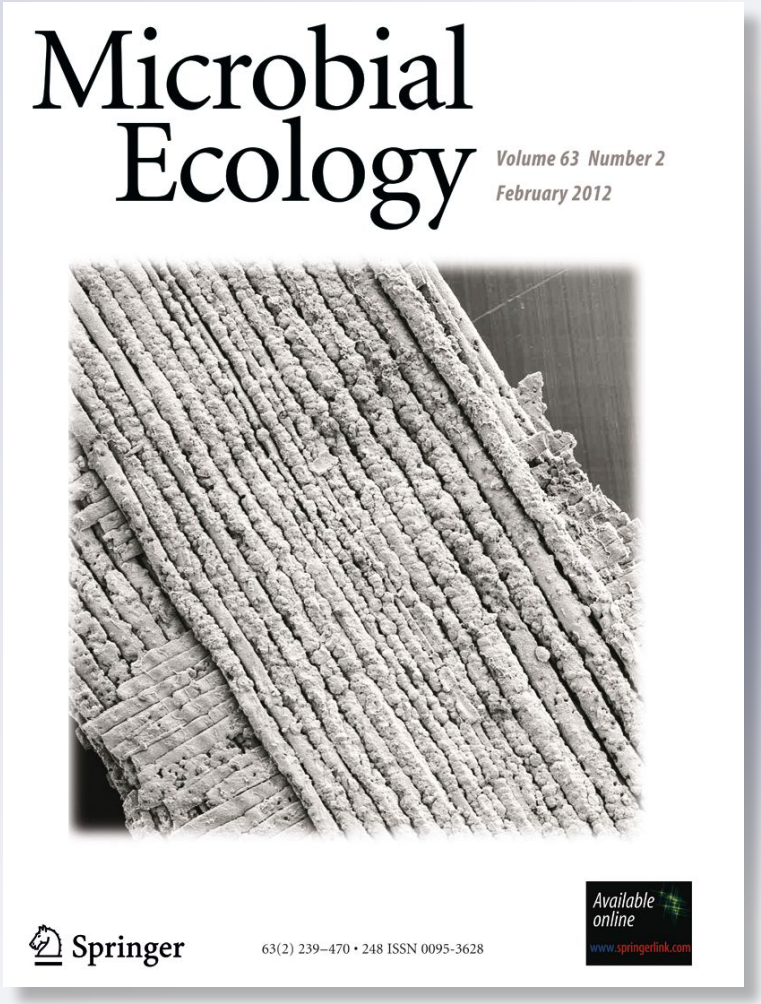
Microbial Ecology

ISSN 0095-3628

Microb Ecol
DOI 10.1007/s00248-012-0051-9



Springer

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MICROBIOLOGY OF AQUATIC SYSTEMS

Alkane Biodegradation Genes from Chronically Polluted Subantarctic Coastal Sediments and Their Shifts in Response to Oil Exposure

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Received: 3 February 2012 / Accepted: 27 March 2012

Abstract Although sediments are the natural hydrocarbon sink in the marine environment, the ecology of hydrocarbon-degrading bacteria in sediments is poorly understood, especially in cold regions. We studied the diversity of alkane-degrading bacterial populations and their response to oil exposure in sediments of a chronically polluted Subantarctic coastal environment, by analyzing alkane monooxygenase (*alkB*) gene libraries. Sequences from the sediment clone libraries were affiliated with genes described in *Proteobacteria* and *Actinobacteria*, with 67 % amino acid identity in average to sequences from isolated microorganisms. The majority of the sequences were most closely related to uncultured microorganisms from cold marine sediments or soils from high latitude regions, highlighting the role of temperature in the structuring of this bacterial guild. The distribution of *alkB* sequences among samples of different sites and years, and selection after experimental oil exposure allowed us to identify ecologically relevant *alkB* genes in Subantarctic sediments, which could be used as biomarkers for alkane biodegradation in this environment. 16 S rRNA amplicon pyrosequencing indicated the abundance of several genera for which no *alkB* genes have yet been described (*Oleispira*, *Thalassospira*) or that have not been previously associated with oil biodegradation (*Spongiibacter*—formerly *Melitea*—, *Maribius*, *Robiginitomaculum*, *Bizionia* and *Gillisia*). These genera constitute candidates for future work involving identification of hydrocarbon biodegradation pathway genes.

Electronic supplementary material The online version of this article (doi:10.1007/s00248-012-0051-9) contains supplementary material, which is available to authorized users.

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Introduction

Petroleum crude oil and its derivatives are complex chemical mixtures, which can contain up to thousands of molecules, typically 90 % hydrocarbons [1]. In the marine environment, coastal sediments are the natural sink of hydrocarbons [2]. In cold regions, in particular, stranded oil can persist in the shoreline, producing short- and long-term undesirable ecological effects [3–6]. Increasing pressure on high latitude ecosystems due to oil exploration and exploitation as well as oil-based activities requires the development of knowledge-based bioremediation tools, specific for cold marine environments [7]. The first step to achieve this goal is the identification of ecologically relevant oil-degrading bacteria and their biodegradative capabilities. As aerobic biodegradation processes play a key role in the sediment system [8], increasing our knowledge on aerobic hydrocarbon-degrading bacterial communities from cold marine sediments is fundamental.

Aerobic biodegradation of oil is a complex process involving many different microorganisms [9]. In sediments, the intrinsic biodiversity and heterogeneity of indigenous microbial communities adds a level of complexity to the identification of hydrocarbon-degrading bacterial populations and their biodegradation pathways. This hampers our understanding of the ecological mechanisms underlying the biodegradation process. Important advances have been made in this direction in temperate [10–14] and tropical [15–22] sediments. In contrast, oil-degrading bacterial

Published online: 12 May 2012

populations from cold sediments have only started to be described [23–26].

Since saturated hydrocarbons (*n*-alkanes, branched alkanes and cycloalkanes) constitute the largest fraction of crude oil by mass, their biodegradation is a major process during hydrocarbon removal from the environment [27]. Furthermore, there is evidence that alkanes could serve as cosubstrates for the cometabolic removal of more recalcitrant oil compounds like resins and asphaltenes [28, 29], as well as of other pollutants such as methyl tert-butyl ethers from gasoline [30]. Several microorganisms are known to use alkanes as carbon sources. In the marine environment, obligate hydrocarbonoclastic bacteria (OHCB), namely, *Alcanivorax* [31], *Thalassolituus* [32], *Oleiphilus* [33] and *Oleispira* [34], have been proposed as being ecologically important for the aerobic removal of alkanes [35]. Alkane-activating enzymes belong to different families, being integral membrane non-heme iron monooxygenases (AlkB) the best characterized [36–38]. The *alkB* genes have been used as functional biomarkers for the characterization of aerobic alkane-degrading bacterial populations in environmental samples [24, 25, 39–41] and in bioremediation experiments [42–44].

Ushuaia Bay, located within the Beagle Channel in the southernmost tip of South America, has been chronically exposed to various pollutants, among which anthropogenic hydrocarbons are of major relevance [26, 45–49]. Our previous work focused on microbiological and ecological aspects of polycyclic aromatic hydrocarbon biodegradation in sediments of this Subantarctic environment [26, 47, 50, 51]. The goal of this study was to identify key bacterial populations associated with aerobic alkane biodegradation processes in these Subantarctic coastal sediments. To achieve this goal, we analysed *alkB* genes from sediment samples obtained in various years, as well as shifts in this gene produced as a result of experimental oil exposure. To analyse these experiments from a phylogenetic perspective, we also performed 16 S rRNA gene amplicon pyrosequencing.

Methods

Sample Collection and Processing

Surficial sediments (0–3 cm) were collected using acrylic cores with an inner diameter of 4.4 cm. For intertidal sediment samples, sampling was performed along the low tide line at seven to ten random points in each sampling location, and the collected sediments were mixed thoroughly. The subtidal sample was collected at 11 m depth by scuba diving using the same acrylic cores, and in this case, the 0–5-cm fraction was used. All samples were stored at 4°C during transport to the laboratory and further stored at −80°C for molecular analyses and at −20°C for hydrocarbon content analysis.

Construction of Sediment-in-Seawater Slurries

The sample used for the experiment (OR08) was retrieved on 18 December 2008, which corresponds to late spring. The sediment temperature at the time of sampling was 8.2° C. Sediment-in-seawater slurries were built by mixing approximately one part of fresh sediment with two parts of 0.45-μm filtered natural seawater, as follows. A sediment: water mix was prepared, decanted and further divided into three 500-ml flasks containing 40 g of sediment and 80 ml of seawater, with the following additions: (1) 0.46-ml light petroleum crude oil from Austral basin (Comodoro Rivadavia, Argentina) in 115 ml of sediment:seawater mix (0.4 % v/v, expOR08-O), (2) 0.4 % (v/v) light petroleum crude oil with the addition of 5-mM ammonium chloride and 0.25-mM sodium phosphate (expOR08-ON) and (3) a control with neither crude oil nor nutrient addition (expOR08-c). The three systems were held at 15°C with constant agitation (150 rpm) for 20 days in the dark. Samples were obtained for molecular and hydrocarbon content analyses and stored as mentioned previously.

Hydrocarbon Analysis

The procedure was followed according to [49, 52]. Briefly, samples were extracted with dichloromethane in a Soxhlet device. The extract was treated with activated copper and evaporated at 40°C with highly pure N_2 until a volume of 0.2 ml was reached. The extract was loaded into an activated alumina column in order to recover the aliphatic and aromatic fractions. The aliphatic fraction was then subjected to high-resolution gas chromatography on a Thermo Trace gas chromatograph with a TriPlus Autosampler (Thermo Electron Corporation, Whaltman, MA, USA), equipped with a J & W DB5 fused silica column (30×0.25×25), split/splitless capillary injection system, and a flame ionization detector (FID). For *n*-alkanes from *n*C20 to *n*C28 range, recovery assays of spiked pristine samples resulted in 95±12 %. The percent of relative deviation (RDP) for individual aliphatic hydrocarbons varied from 0.4 % to 9 %, and the detection limit (LOD) was between 5 and 10 ng g^{-1}. The identification of resolved aliphatic hydrocarbons was achieved by comparing retention times with the corresponding standards (Chem Service Inc, West Chester, PA, USA). Total resolved aliphatics (TRA) and unresolved complex mixture (UCM) were calculated using the mean response factors of *n*-alkanes.

Individual *n*-alkane concentrations from *n*-C10 to *n*-C35, Pristane (Pr) and Phytane (Ph) isoprenoid levels, total resolved *n*-alkanes (∑*n*alk), TRA, UCM and total aliphatic

hydrocarbons (TAH=TRA+UCM) were calculated for each sample. The UCM involves cycloalkanes, branched alkanes and other compounds unresolved by the capillary column which show as a hump shape below the resolved compounds. Hydrocarbon concentration values were expressed as μg/g dry weight, after correcting for sediment moisture content.

Evaluation indices selected for this study were *n*-C17/Pristane (*n*C17/Pr), *n*-C18/Phytane (*n*C18/Ph) and TRA/UCM, in order to evaluate the relative biodegradation of *n*-alkanes [52, 53]. In addition, the ratio between low molecular weight and high molecular weight hydrocarbons (∑LMW/∑HMW, the sum of alkanes<*n*C20 / the sum of alkanes≥*n*C21) was calculated as an estimation of the relative biodegradation of lighter compounds in the experiments.

DNA Extraction

DNA was purified from 0.5 to 0.8 g wet weight sediment using the FastDNA® SPIN kit for soil (MP Biochemicals, Solon, OH, USA), according to the manufacturer's instructions with the following modifications: Samples were homogenized three times for 50 s at approximately 5,000 rpm (speed at high setting) with 1-min intervals using a mini bead-beater Biospec (Bartlesville, OK, USA). Two DNA extractions per sample were combined before further analysis.

PCR Amplification of *alkB* Genes

The *alkB* gene fragments were amplified using the *AlkB*484F (5′ GGKCAYTTCTWCRTYGARCA 3′)/*AlkB*824R (5′ CCGTAGTGYTCRABRTARTT 3′) primer set [54]. PCR reactions were performed in 25-μl volume containing 1× PCR buffer, 1.5-mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer and 1 U of T-PLUS DNA polymerase (Inbio-Highway, Tandil, Argentina). Template concentration was optimized for each sample (1–5 ng/reaction). Cycling conditions involved an initial 5-min denaturing step at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 45°C and 30 s at 72°C, and a final 15-min elongation step at 72°C. Four separate reactions were run for each sample, pooled and purified from 1.5 % (w/v) agarose gels using Wizard SV Gel and PCR clean-up System kit (Promega, Madison, WI, USA).

Clone Library Construction, Screening and Sequencing

Prior to the cloning step, 3′ A-overhangs were added to the purified *alkB* gene fragments by incubating 30 min at 72°C in the presence of PCR buffer, dATP, $MgCl_2$ and T-PLUS DNA polymerase in the same concentrations used in previous amplification reactions. Cloning was carried out using the TOPO-TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA, USA). Plasmids were purified with QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) and sequenced at Macrogen Inc. (Seoul, South Korea) or at INTA (Castelar, Buenos Aires, Argentina), using M13 primers from the cloning vector.

Pyrosequencing of 16S rRNA Gene Amplicons

The V4 hypervariable region of the 16 S rRNA gene was amplified from the same template DNA used for the construction of *alkB* gene libraries. The conserved bacterial primers 520 F (5′-AYTGGGYDTAAAGNG-3′) and 802R (5′-TACCRGGGTHTCTAATCC-3′, 5′TACCAGAGTATC TAATTC-3′, 5′-CTACDSRGGTMTCTAATC-3′, and 5′-TACNVGGGTATCTAATCC-3′) were obtained from the RDP Pyrosequencing Pipeline (http://pyro.cme.msu.edu). Each primer contained the Roche 454 sequencing A (forward) and B (reverse) adaptors, and the forward primer also contained a ten nucleotide "multiple identifier" sequence (MID) to sort the samples.

Four independent PCR reactions were performed for each sample in a 25-μl volume containing 2.5 μl of FastStart High Fidelity 10× Reaction Buffer (Roche Applied Science, Mannheim, Germany), 20 ng of template DNA, 0.4 μM of each primer, 1.25-U FastStart High Fidelity Enzyme Blend (Roche Applied Science) and 0.2-mM dNTPs. Cycling conditions involved an initial 5-min denaturing step at 95°C, followed by 30 cycles of 45 s at 95°C, 45 s at 57°C and 60 s at 72°C, and a final 4-min elongation step at 72°C. Purified PCR products were pooled in an equimolar ratio, immobilized onto DNA capture beads, amplified through emulsion-based clonal amplification and sequenced together in one PicoTiterPlate device on a Genome Sequencer FLX (Roche Applied Science) using Titanium Chemistry at INDEAR Genome Sequencing Facility (Rosario, Argentina).

Bioinformatic and Statistical Analyses

Retrieved *alkB* gene nucleotide sequences were initially visualized and truncated to exclude primer and vector sequences using FinchTV 1.4.0 program (Geospiza Inc.). Nucleotide sequences were then translated with BioEdit software [55] and screened against the GenBank database using BLAST program (*tblastn* option) [56]. Deduced protein sequences (100 amino acid positions) were grouped into operational taxonomic units (OTUs) using mothur software v.1.22.2 [57]. Diversity estimators *Chao1* [58], ACE [59] and Good's coverage [60] were also calculated with mothur. For each OTU, one representative sequence from each library was kept for phylogenetic analysis. The sequences were aligned using ClustalX [61] tool within MEGA software version 5 [62]. In MEGA 5, a pairwise distance matrix was calculated based on Dayhoff substitution model with pairwise deletion of gaps

and missing data and was used to generate a phylogenetic tree by the neighbour-joining method with 1,000 bootstrap replicates.

Analyses of 16 S rRNA amplicons generated by 454 pyrosequencing were performed with mothur software v.1.22.2 [57] following the pipeline described by Schloss and Westcott [63], with minor modifications. Briefly, sequences were trimmed when the average quality score dropped below 25 within a 50-bp window, and sequences with at least one mismatch with the MID and/or two mismatches with the primer were eliminated. Trimmed sequences were aligned to the aligned SILVA reference database [64, 65]; the alignment was filtered to conserve 85 % of the sequences that overlapped in the same alignment space and used to perform OTU clustering. Before clustering, sequences were preclustered at 99 % to account for sequencing errors [66], and chimeras were removed by Chimera Slayer with mothur. OTUs were defined at a distance threshold of 0.03 (97 % identity cutoff). Alpha diversity estimators were calculated from OTU-based analyses using mothur. Alpha diversity metrics calculated included *Chao1* [58], Good's estimated coverage [60] and Simpson's Dominance index *D* [67]. Bray–Curtis similarity index [68] among samples was calculated based on shared OTUs at 97 % identity. Sequences were taxonomically identified up to the deepest possible phylogenetic level using the Ribosomal Database Project (RDP) [69] as reference with mothur, using a bootstrap cutoff of 50 % confidence. Sequences assigned to chloroplasts were excluded from the analysis. A table was built with relative frequencies of sequences at each taxonomic level for each sample.

Sequence Accession Numbers

The partial *alkB* sequences generated in the present study have been submitted to the GenBank database under accession numbers JQ437615–JQ437743. Partial 16 S rRNA sequences were deposited in the SRA database of NCBI under accession number SRA049611.

Results

Sampling Sites and Sample Hydrocarbon Content

Sampling sites are located in Ushuaia Bay, Argentina, in an area with high maritime traffic on the coast of Ushuaia city (Fig. 1). Intertidal sediment samples were retrieved at two sites 0.3 km apart: EM (54 °48′19″S, 68 °17′39″W) and OR (54 °48′16″S, 68 °17′23″W). The OR site is located next to a jetty used for loading and offloading of refined petroleum products. For details on sampling sites, see [47, 51].

Sediments contained total aliphatic hydrocarbons (TAH) in concentrations ranging from 19.7 to 347.2 μg/g dry weight sediment (dws, Online Resource 1). TAH content of OR samples from different years was fairly stable, while EM site showed the highest value (Online Resource 1). Values were in the range considered by UNEP/IOC/IAEA [70] as characteristic of polluted sediments from areas related to ports and industrial activities and in the category of moderately polluted sediments according to a previous survey carried out in the Patagonian coast [49]. All samples presented *n*-alkane homologous series, the isoprenoids pristane and phytane and unresolved complex mixture (UCM), characteristic of petrogenic origin. Biodegradation indices *n*C17/Pr and *n*C18/Ph ranged between 1.1 and 1.5 (Online Resource 1), suggesting ongoing biodegradation processes [52]. In addition, the ratio between total resolved aliphatics (TRA) and UCM (TRA/UCM) showed relatively low values (from 0.12 to 0.21, Online Resource 1) adding evidence to these results [49].

Diversity of *alkB* Gene Sequences in Subantarctic Coastal Sediments

Using a highly degenerate primer set [54], we amplified *alkB* gene fragments from five Subantarctic sediment samples and used them to build PCR clone libraries. We analysed by sequencing a total of 202 randomly chosen clones

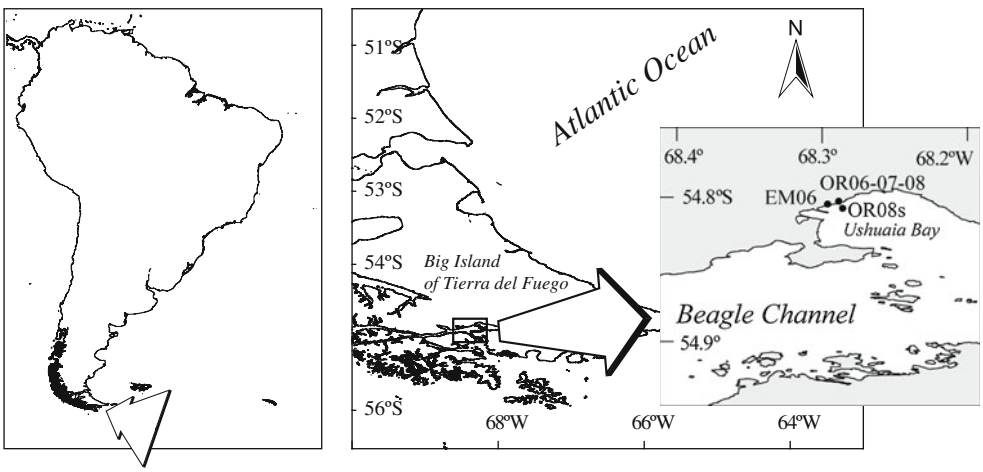

Figure 1 Sampling sites in Ushuaia Bay, Tierra del Fuego, Argentina. Samples are named as the site followed by the last two digits of the sampling year and followed by "s" for the subtidal sample. The map was constructed with Surfer version 8.02 (Golden Software, Inc.)

from the five libraries (Online Resource 1). All deduced AlkB sequences matched alkane monooxygenases from the GenBank database with significant identity and low *e* values [56, 71]. With one exception, they contained the terminal histidines of the conserved histidine box II, essential for alkane hydroxylase activity [71–73]. In addition, other residues conserved in most alkane monooxygenases [72] were also present. Deduced protein sequences were grouped into operational taxonomic units (OTUs) with a distance threshold of 0.20 (80 % sequence identity at the amino acid level), due to the high sequence variability of this gene [24, 25]. Online Resource 2 shows an alignment of a representative AlkB sequence from each OTU.

For diversity analysis, the sequences from the five clone libraries were pooled. A total of 30 OTUs were identified, and we estimated 95 % coverage of the overall *alkB* gene diversity in this environment, as far as it can be amplified with our primer set (Table 1). Richness estimated by ACE and *Chao1* indices was 39 and 48 OTUs, respectively (Table 1). OTUs contained sequences with average distances of up to 0.14, indicating that there was internal variability within each OTU. This was also evidenced by the increase in the number of OTUs when the cutoff distance was decreased (Table 1). Our estimations can thus be considered as a conservative measure of the *alkB* richness in this chronically polluted environment.

The chosen primer set was able to recover a high number of *alkB* genes with wide phylogenetic diversity (Fig. 2), with the exception of *alkB2* from *Alcanivorax borkumensis* SK2 (BAC98366) and related sequences, which were not covered by our primer set [54]. Subantarctic sediment clone libraries were mostly composed by AlkB sequences related to the ones described in *Proteobacteria* (165 sequences, 82 %). Ninety-nine of these sequences grouped into a large and diverse cluster related to AlkB1 of *A. borkumensis*, which also includes sequences from microorganisms such as *Pseudomonas*, *Thalassolituus*, *Marinobacter* and *Oleiphilus* (Fig. 2). The rest of the sequences were related to different types of genes, including sequences from *Alcanivorax dieselolei, Alcanivorax venustensis and Alcanivorax hongdengensis*, and to AlkM sequences from *Acinetobacter*. With the exception of OTU #6, which showed 85 % amino acid identity to *Marinobacter* sp. S17-4 (ACT31523), the OTUs related to AlkB from *Proteobacteria* showed identity to sequences from isolated microorganisms ranging from 51 % to 74 %. Moreover, sequences retrieved from the libraries were more closely related to AlkB sequences from uncultured microorganisms from cold marine sediments or soils from high-latitude regions (Online Resource 3).

Sequences related to AlkB from *Actinobacteria* accounted for the next most abundant group (33 sequences, Fig. 2). The most abundant OTU (#24) was, however, most closely related to an Arctic soil clone, with no near match from a cultured representative (Online Resource 3). The other three minority OTUs showed high identity values to AlkB sequences from *Rhodococcus* and *Arthrobacter* (Online Resource 3). In addition, there were four divergent sequences detected in the EM06 sample, which clustered with AlkB sequences from members of the *Bacteroidetes*, forming deep but highly supported branches in the phylogenetic tree (Fig. 2).

Oil Exposure and Biostimulation Experiment

In order to evaluate the response of the indigenous alkane-degrading bacterial populations to crude oil exposure, we set up laboratory scale experiments (sediment-in-seawater slurries) using OR08 sediment sample. The three conditions were (1) 0.4 % (v/v) crude oil (expOR08-O), (2) 0.4 % (v/v) crude oil with the addition of 5-mM ammonium chloride and 0.25-mM sodium phosphate (expOR08-ON) and (3) a control with neither crude oil nor nutrient addition (expOR08-c) (Online Resource 1).

Alkane Biodegradation

After 20 days, expOR08-O slurry evidenced a decrease in the relative concentration of *n*-alkanes lower than *n*C20, with respect to the crude oil gas chromatography profile (Online Resource 4). This reduction extended to the majority of the resolved alkanes in the expOR08-ON slurry, where the profile was composed mainly of UCM (Online Resource 4). Accordingly, the biodegradation indices *n*C17/Pr, *n*C18/Ph and TRA/UCM decreased in both oil-exposed slurries with respect to the added crude oil, being lower in expOR08-ON. The ratio ∑HMW/∑LMW followed the same trend (Online Resource 1). These results indicate that after only 20 days, a certain level of biodegradation occurred in the expOR08-O slurry and that this process was more efficient with the addition of nutrients.

Table 1 *alkB* gene sequence diversity in coastal sediments from Ushuaia Bay

Number of samples	Number of sequences	Cutoff distance[a]	S_{obs}[b]	*Chao1*	ACE	Coverage
5	202	0.20	30	48 (34–105)	39 (32–61)	0.95
		0.02	46	72 (55–119)	71 (56–109)	0.89

[a] Distance threshold at the amino acid level used to define OTUs

[b] Number of observed OTUs. For richness estimators, 95 % confidence intervals are shown in parentheses

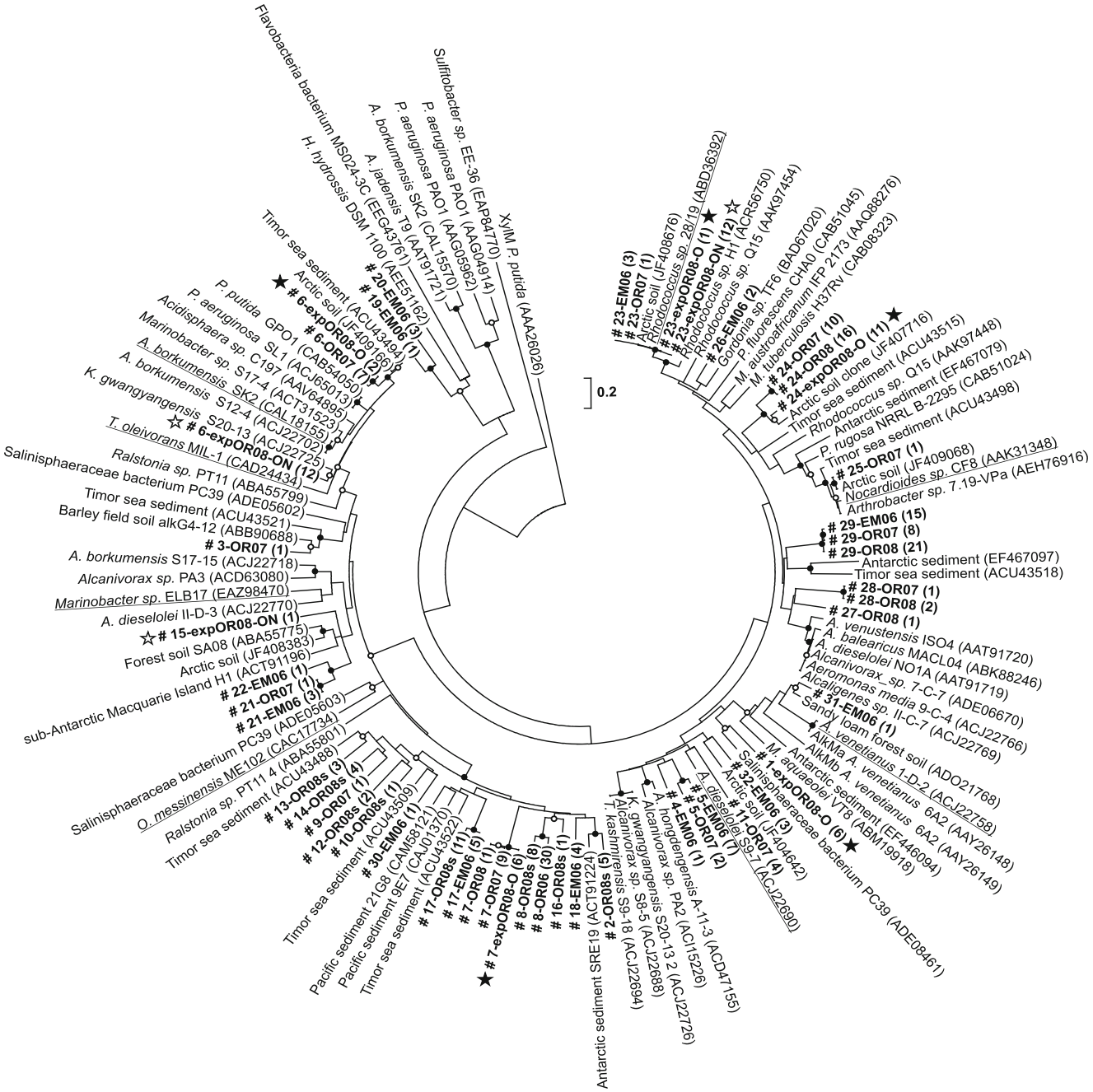

Figure 2 Phylogenetic tree of translated AlkB sequences from clones obtained in this study and representative sequences from the databases. Accession numbers from GenBank database are shown in parentheses. One representative sequence for each OTU formed at 0.20 cutoff distance from each library was used to build the tree. OTUs are shown in *bold type* as: **OTU number-library (number of sequences)**. Oil-exposed and oil-plus-nutrient experimental systems are indicated by *filled* and *open stars*, respectively. The tree was constructed from deduced amino acid sequences using the neighbour-joining algorithm with MEGA 5 [62]. Bootstrap values ≥60 % and ≥90 % (1000 repetitions) are presented at nodes as *open* and *filled circles*, respectively. The *scale bar* represents 0.20 sequence divergence. The tree was rooted with xylene monooxygenase catalytic subunit (XylM) from *Pseudomonas putida* TOL plasmid (AAA26026)

Changes in alkB Gene Diversity

Five *alkB* gene OTUs were observed in the library of expOR08-O slurry after 20 days of oil exposure. Four of them were also present in the libraries constructed from the environmental samples (Fig. 2, Online Resource 3). The most abundant OTU (#24, 42 % of the total sequences) was related to AlkB sequences mostly described in members of *Actinobacteria*, although no closely related sequence from an isolate has yet been described. OTUs #1 and #7

were the next most abundant gene sequences in the library (23 % each). OTU #1 clustered strongly with an AlkB sequence from an unclassified *Gammaproteobacteria* from the *Salinisphaerales* (Fig. 2). On the other hand, OTU #7 clustered in a highly supported branch including only sequences from uncultured microorganisms, mostly from deep sea sediments. This branch is in the clade where *A. borkumensis* AlkB1 is located (Fig. 2). The two minority OTUs were OTU #6, clearly affiliated with uncultured clones from the Arctic and deep sea, and #23 with 100 % identity to *Rhodococcus* sp. 28/19 (ABD36392) (Fig. 2).

In the *alkB* clone library from expOR08-ON slurry, only two OTUs (#6 and #23) accounted for 96 % of the sequences. They were equally represented and were also observed, although at low frequencies, in the expOR08-O slurry (Fig. 2). However, in this case, amino acid sequences belonging to OTU #6 were unequivocally placed with AlkB from *Kordiimonas gwangyangensis* (ACJ22725). OTU #23 was 100 % identical to AlkB from *Rhodococcus* sp. 28/19 (ABD36392).

PCR amplifications from the control slurry (expOR08-c) resulted in low yields and in the cloning of a high proportion of non-target DNA fragments, probably due to the low target concentration in this experimental condition.

Changes in Bacterial Community Structure

We analysed a total of 44,380 pyrosequencing reads of the V4 hypervariable region of the 16 S rRNA gene, amplified from the three slurries (expOR08-O, expOR08-ON and expOR08-c) as well as the original sediment sample (OR08) (Table 2). Coverage values were at least 94 % in all cases. The OR08 sample, as well as the expOR08-c and expOR08-O slurries, showed high bacterial richness and low dominance values. The OR08 sample was the most diverse, with approximately 1,800 and 3,000 observed and estimated OTUs, respectively (Table 2). The expOR08-c slurry experienced a decrease in richness of about 38 % with respect to the original sediment, which can be attributed to the passage to laboratory conditions (Table 2). However, this slurry was the most similar to the original sediment (Bray–Curtis similarity index based on 16 S rRNA OTUs=0.48). The expOR08-O slurry showed slightly lower richness values than expOR08-c (Table 2), with Bray–Curtis similarity indices of 0.47 with expOR08-c and 0.34 with OR08. On the other hand, expOR08-ON was the most simplified, with only 163 and 288 observed and estimated OTUs, respectively (Table 2). This was also the only sample which showed an asymptotic behaviour in its rarefaction curve (data not shown). The structure of the bacterial community of the expOR08-ON slurry was also very different from the sediment and other treatments (Bray–Curtis similarity indices from 0.03 to 0.07).

Various bacterial genera known to include hydrocarbon degraders [9, 35, 74] were relatively abundant in Ushuaia Bay sediment sample OR08 (Fig. 3). These included *Oleispira*, *Pseudoalteromonas*, *Nocardioides*, *Sulfitobacter* and *Marinobacter*. Only *Sulfitobacter* and *Nocardioides* were also present in expOR08-O in relatively high abundances, while other genera were strongly enriched (*Spongiibacter*, *Sphingopyxis*, *Maribius* and *Robiginitomaculum*). Altogether, the aforementioned genera accounted for 38.5 % of the bacterial community of the expOR08-O slurry, as estimated by 16 S rRNA gene analysis.

In the expOR08-ON slurry, sequences classified as belonging to *Alcanivorax* were highly enriched (45.9 %), followed by *Thalassospira*, *Sulfitobacter* and various Flavobacteriales (*Flavobacterium*, *Bizionia* and *Gillisia*) (Fig. 3).

Discussion

The results of this study indicate the existence of a high diversity of relatively divergent *alkB* genes in the sediments of the chronically polluted environment of Ushuaia Bay.

Table 2 Bacterial diversity in OR08 sediment sample and slurries, as estimated by pyrosequening of 16S rRNA amplicons

Sample		Number of sequences[b]	S_{obs}[c]	Coverage	*Chao1*	*D*[d]
Type	Name[a]					
Sediment	OR08	15,344	1,829	0.94	3,011 (2,817–3,243)	0.043 (0.041–0.045)
Experimental systems	expOR08-c	9,357	1,128	0.94	1,961 (1,788–2,179)	0.034 (0.032–0.036)
	expOR08-O	11,636	896	0.96	1,503 (1,366–1,680)	0.033 (0.032–0.035)
	expOR08-ON	8,043	163	0.99	288 (230–398)	0.247 (0.238–0.256)

[a] *expOR08-c* control slurry (nor oil neither nutrients added), *expOR08-O* oil-exposed slurry, *expOR08-ON* oil-plus-nutrient amended slurry

[b] Obtained after discarding low-quality reads, chimeras and sequences assigned to chloroplasts

[c] Number of observed OTUs, defined at 0.03 distance threshold

[d] Simpson's dominance index [66]. For richness and dominance estimators, 95 % confidence intervals are shown in parentheses

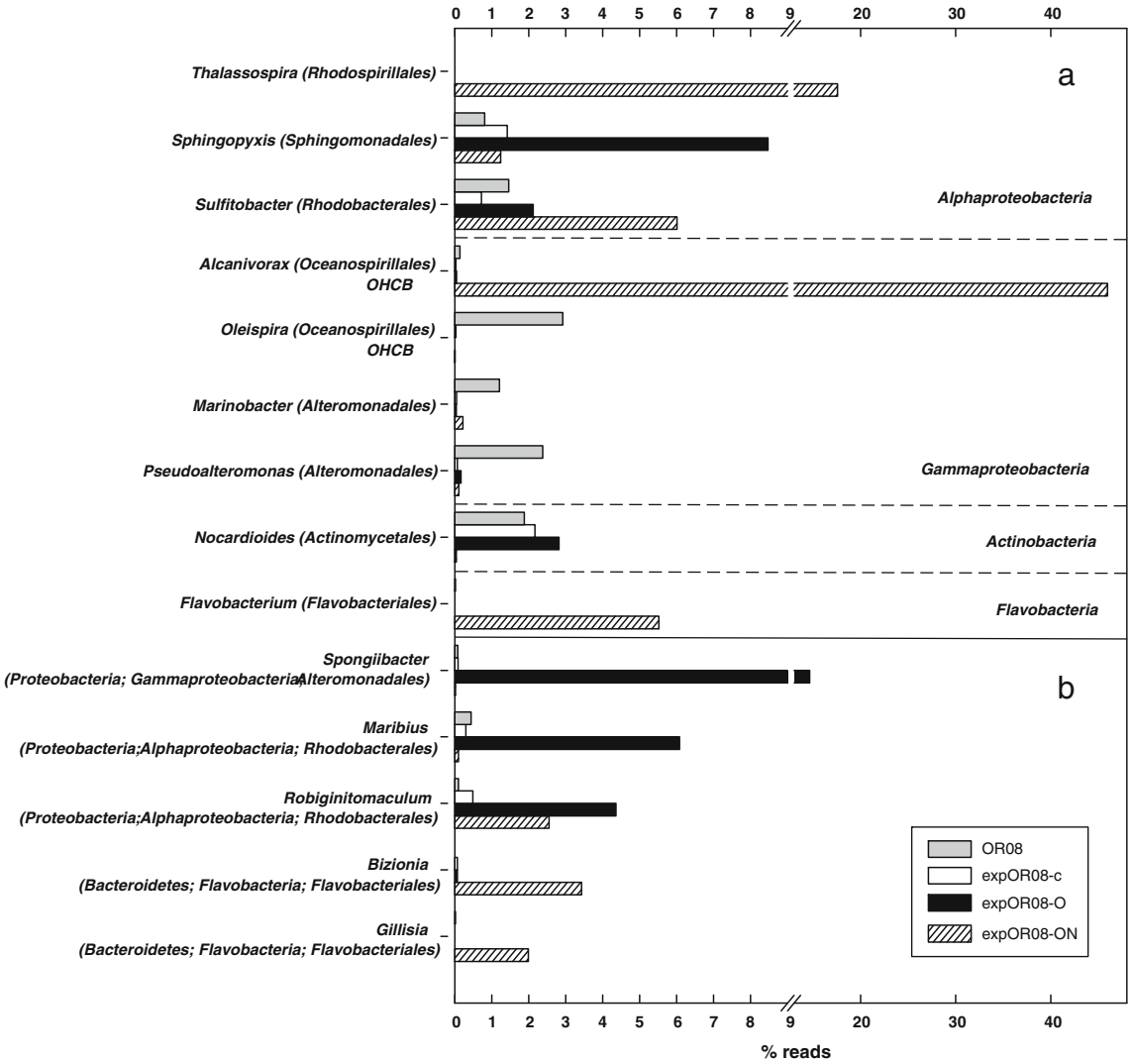

Figure 3 Relative frequency of selected bacterial genera from OR08 sediment sample and experimental systems, as estimated by 16 S rRNA amplicon pyrosequencing analysis. **a** Genera linked to hydrocarbon biodegradation in marine environments [9, 35, 74]. **b** Genera not previously linked to hydrocarbon biodegradation, but showing important shifts in frequency among treatments. Only genera with frequency values above 1 % in at least one sample are shown. *expOR08-c* control slurry (nor oil neither nutrients added), *expOR08-O* oil-exposed slurry, *expOR08-ON* oil-plus-nutrient amended slurry, *OHCB* obligate hydrocarbonoclastic bacteria. Analyses were performed with mothur v.1.22.2

Moreover, the most abundant *alkB* gene variants found in our study were detected across sampling sites and years, and various were further selected in oil enrichment experiments, suggesting the ecological relevance of bacterial populations carrying these genes in Subantarctic sediments. Chronic hydrocarbon pollution at this site [46, 49] has been key for the establishment of an active and diverse bacterial alkane-degrading guild in these sediments. In general, the closest matches of AlkB sequences from this study were sequences from uncultured microorganisms, in accordance with reports from other underexplored coastal and deep sea environments [24, 25]. The matches most often found were *alkB* genes detected in diesel-contaminated Antarctic marine sediments (GenBank acc. no. ACT91224 and related sequences), Arctic soils contaminated with high-alkane content fuel [75] and natural seepages in deep-sea sediments [24]. Various components including type of pollution and environmental factors such as temperature seem to be shaping the bacterial guilds associated with aerobic alkane biodegradation. Studies are needed in this direction in order to quantitatively assess the relative contribution of the different environmental factors to the structuring of the hydrocarbon-degrading bacterial guilds in the marine environment.

Ushuaia Bay sediments receive small and frequent inputs of refined petroleum products [46, 49]. The addition of a different type of oil (crude oil) resulted in a rapid disappearance of *n*-alkanes and a concomitant shift in bacterial community structure that was different in each nutritional

condition. Other studies have linked preexposure to hydrocarbons to a fast structural and functional response of the microbial community in soils [76, 77] and temperate sediments [14]. As regards to the suitability of the *alkB* gene to reflect the response of the bacterial community to oil exposure, results are still controversial. Hamamura and collaborators were able to detect key alkane-degrading populations in soil with various group-specific *alkB* primers and proposed their use as functional biomarkers [44]. In a study involving quantitative PCR, the proportion of *alkB*-carrying microorganisms was positively correlated to the concentration of *n*-alkanes in soil [42]. On the other hand, in experiments involving temperate coastal sediments, the expression of this gene could not be linked to the community response to oil pollution probably due to the transient nature of the transcripts, although oiled microcosms showed higher diversity of *alkB* genes than controls [78]. We assessed the selection of bacterial populations carrying *alkB* genes after oil exposure, as measured by DNA-based analysis and a time scale of 20 days. The presence of oil resulted in strong amplification and selection of specific *alkB* gene sequences in both conditions (oil and oil-plus-nutrients). In contrast, poor amplification and cloning of unspecific products were observed in the control, suggesting lack of selection of *alkB*-carrying bacterial populations after the dilution of the alkanes present in OR08 sample due to the addition of non-polluted seawater in this slurry. The identification of *alkB* gene sequences from key populations achieved in this study is the first step towards their targeted use as functional biomarkers using molecular techniques with quantitative power, in both basic and applied contexts.

In accordance with the detection of *alkB* gene sequences affiliated with the *Proteobacteria* in the oil-exposed slurry, members of the *Gammaproteobacteria* and *Alphaproteobacteria* were identified as highly abundant by 16 S rRNA gene amplicon pyrosequencing. Of these populations, the ones classified as belonging to the *Spongiibacter* genus (*Gammaproteobacteria*, *Alteromonadales*, formerly *Melitea* [79, 80]) were the most prominent. Bacteria belonging to this genus have been isolated from seawater and described as truly marine as they require salt for growth [81]. Although to the best of our knowledge members of this genus have not been previously linked to hydrocarbon biodegradation, strains from the closely related genus *Marinobacter* [82, 83] are well known to participate in hydrocarbon biodegradation in sediments [19, 20]. The *alkB* genes related to the ones described in *Proteobacteria* that were identified in this slurry could be carried by *Spongiibacter* populations, although this possibility has to be confirmed. Another *alkB* gene strongly selected in this experimental condition, as well as abundant in the sediments obtained at the OR site in two consecutive years, was closely related to an Arctic soil clone. Its deduced protein sequence clustered, among others, with AlkB from *Nocardioides*. Coincidentally, *Nocardioides* 16 S rRNA sequences were relatively abundant in the oil-exposed slurries and also in the sediment sample, suggesting that this *alkB* gene (OTU #24) could be carried by members of this genus.

In the oil-plus-nutrient amended slurry, the high abundance of *alkB* sequences related to genes from *Proteobacteria* was evident. In accordance, *Alcanivorax* (*Gammaproteobacteria*) and *Thalassospira* (*Alphaproteobacteria*) were detected as very abundant by our phylogenetic-based analysis. The capability of *Alcanivorax* for scavenging nutrients is well known [35, 84]. On the other hand, *Thalassospira*, originally associated with polycyclic aromatic hydrocarbon biodegradation [85, 86], has been recently linked to alkane biodegradation [87]. Therefore, we cannot exclude the possibility that *alkB* gene OTU #6 belongs to this genus. However, the analysis based on the *alkB* gene precludes us from drawing more definite conclusions, due to the possibility of horizontal gene transfer [88], the presence of multiple and divergent *alkB* genes in members of the same genus, characteristic of *Alcanivorax* [89] and lack of catabolic gene information from *Thalassospira*. The other *alkB* gene found in a very high abundance in this slurry was identical at the amino acid level to AlkB from *Rhodococcus* (OTU #23). This result was not in accordance with the 16 S rRNA gene pyrosequencing data, where only 0.25 % of sequences were classified as *Rhodococcus*. This lack of agreement may be due to a bias introduced by the *alkB* primer set, which was designed using a majority of *Rhodococcus* sequences [54]. Alternatively, it is possible that bacteria other than *Rhodococcus* could be carrying this gene, acquired by horizontal gene transfer [90]. Interestingly, this same discrepancy has been recently observed for *Rhodococcus* genes in a metagenomic study of diesel-contaminated Arctic soils [91]. The complementation of functional gene-based information with a phylogenetic approach allowed us to build a more complete picture of the bacterial communities inhabiting Subantarctic sediments and their response to an oil input. For example, sequences classified as belonging to *Oleispira* [34, 92] were detected in Ushuaia Bay sediments in relatively high abundance by 16 S rRNA gene amplicon pyrosequencing. These microorganisms were not selected in any of the slurries, which were performed at 15° C resembling Ushuaia summer temperatures [48]. Both the latitude of Ushuaia and our experimental results suggest that *Oleispira* populations present in these sediments could be more closely related to the psychrophilic bacterium *Oleispira antarctica* [34] than to the recently described *Oleispira lenta*, whose optimum growth temperature is 28°C [92]. Although its role in hydrocarbon biodegradation has been recognized [35, 93, 94], the pathway genes of *Oleispira* have not yet been described. The high-throughput information provided by pyrosequencing was fundamental for the detection of members of this genus in the complex microbial community of

Ushuaia Bay sediments. Other microorganisms for which no *alkB* information is known, or even no biodegradation capabilities have been reported, could also be identified as relevant in our study when using the phylogenetic approach. In the slurries, genera such as *Spongiibacter*, *Maribius*, *Robiginitomaculum*, *Thalassospira*, *Flavobacterium*, *Bizionia* and *Gillisia* were detected in relatively high abundances with respect to the control slurry. These genera constitute candidates for future work involving identification of hydrocarbon biodegradation pathway genes.

Insufficient information is still hampering our understanding of hydrocarbon biodegradation processes in cold marine environments. The results from this study contribute to the knowledge on the identity and behaviour of key alkane-degrading populations from Subantarctic regions.

Acknowledgments LMG, CLL and MSM are recipients of graduate student fellowships from the National Research Council of Argentina (CONICET). ML, HMD and MGC are staff members from CONICET. Grants from CONICET, National Agency for the Promotion of Science and Technology (ANPCyT, Argentina) and Secretary of Science, Technology and Innovation of the Chubut Province (Argentina) supported this research.

We would like to thank M. Gil, J. L. Esteves, H. Ocariz, A. Torres and R. Vera for their help during sample collection.

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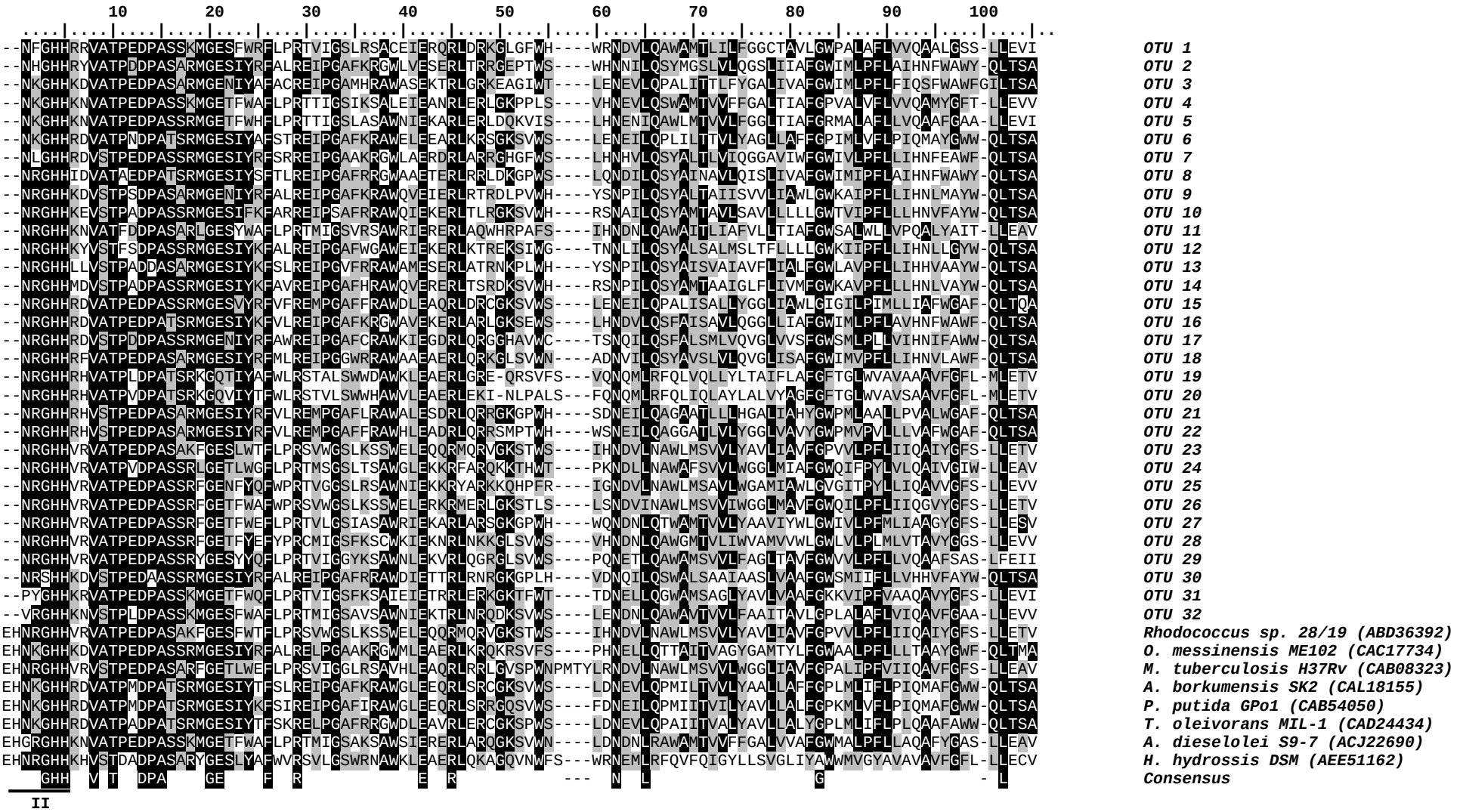
Online Resource 1 Hydrocarbon content and *alkB* PCR clone library information of coastal sediment samples from Ushuaia Bay

Sample		*Hydrocarbon content (μg/g dws)* [b]			*Aliphatic diagnostic indices*				*alkB PCR clone libraries*		
type	*name* [a]	TRA [c]	UCM [d]	TAH [e]	*n*C17/Pr [f]	*n*C18/Ph [g]	∑LMW/∑HMW [h]	TRA/UCM	*N* [i]	S_{obs} [j]	*Coverage*
Ushuaia Bay sediments	EM06	38.78	308.4	347.2	1.32	1.13	*na*	0.13	50	14	0.90
	OR06	5.09	28.03	33.12	1.27	1.48	*na*	0.18	30	1	1
	OR07	8.74	68.26	77.00	1.27	1.34	*na*	0.13	46	12	0.87
	OR08	4.06	19.44	23.50	1.19	1.43	*na*	0.21	41	5	0.95
	OR08s	2.06	17.64	19.70	1.35	1.34	*na*	0.12	35	8	0.94
Crude oil	O	*na*	*na*	*na*	2.17	3.18	4.09	0.76	-	-	-
Experimental systems	expOR08-c	*na*	*na*	*na*	1.00	1.20	*na*	0.16	-	-	-
	expOR08-O	*na*	*na*	*na*	1.99	2.82	2.34	0.41	26	5	0.96
	expOR08-ON	*na*	*na*	*na*	0.02	0.20	0.78	0.12	25	3	0.96

na: not applicable; [a] sediment samples are named as site followed by the last two digits of the sampling year. Experimental systems are named as expOR08-c: control slurry (nor oil neither nutrients added), expOR08-O: oil-exposed slurry, expOR08-ON: oil-plus-nutrient amended slurry; [b] hydrocarbon content values are expressed as μg/g dry weight sediment; [c] total resolved aliphatics; [d] aliphatic unresolved complex mixture; [e] total aliphatic hydrocarbons (TRA + UCM); [f] ratio between *n*-C17 alkane and pristane; [g] ratio between *n*-C18 alkane and phytane; [h] ratio between low and high molecular weight hydrocarbons; [i] number of sequences; [j] number of OTUs defined at 0.20 cutoff distance at the amino acid level.



Online Resource 2 Alignment of deduced AlkB sequences from clones obtained in this study and related sequences from public databases. Only one representative sequence per OTU is shown. The conserved histidine box II [1, 2] is indicated. The beginning of histidine box II in the sequences from this study is not included, as it was included in the forward primer [3]. Residues ≥50% identical and similar are shaded in black and gray, respectively. CLUSTAL, MEGA5 and BioEdit softwares were used to build and shade the alignment.

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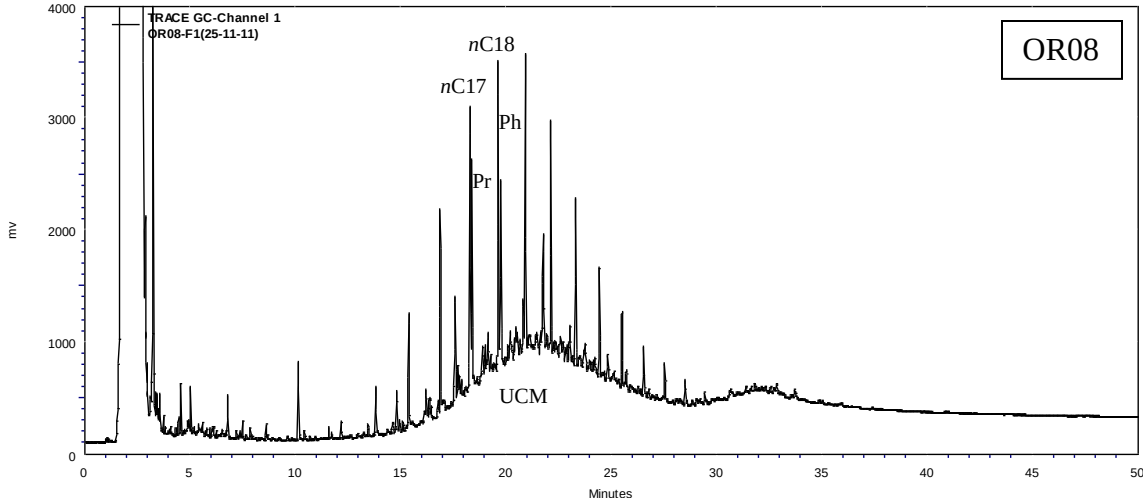
Online Resource 3 BLAST results of the *alkB* gene OTUs identified in this study. OTUs are ordered by relative abundances. For each OTU, the first BLAST match and the sequence from the closest isolated microorganism is indicated, with GenBank accession numbers in parentheses. Percent identity at the amino acid level is shown. *N*: number of sequences in the OTU; *n:* number of samples where the OTU was detected; samples correspond to: S (sediment), O (oil-exposed slurry), ON (oil-plus-nutrient amended slurry).

OTU	*N*	*n*	*sample*	*BLAST match*	*% Identity*	*e-value*
29	44	3	S	*alkB* Forest soil clone alkB22CB (HM441106)	67	3.00E-41
				alkB Alcanivorax dieselolei NO1A (AAT91719)	64	4.00E-41
8	38	2	S	*alkB* Antarctic sediment clone SRE-19 (ACT91224)	73	8.00E-45
				alkB Ralstonia sp. PT11 isolate 4 (ABA55801)	57	6.00E-35
24	26	2	S	*alkB* Arctic soil clone GO0VNXF07IJMIO (JF407716)	80	8.00E-55
				alkB Gordonia DSM 44995 (ACZ17528)	66	7.00E-41
	11	1	O	*alkB* Arctic soil clone GO0VNXF07IJMIO (JF407716)	89	4.00E-61
				alkB Nocardia sp. 3.2-VPr (AEH76914)	70	9.00E-43
6	7	1	S	*alkB* Soil clone OTU28 (ACB11552)	93	2.00E-62
				alkB Marinobacter sp. S17-4 (ACT31523)	85	3.00E-57
	2	1	O	*alkB* Soil clone OTU28 (ACB11552)	93	9.00E-63
				alkB Marinobacter sp. S17-4 (ACT31523)	85	2.00E-57
	12	1	ON	*alkB* France sediment clone cl3 (CCA29153)	96	6.00E-66
				alkB Kordiimonas gwangyangensis S20-13 clone 1 (ACJ22725)	91	6.00E-63
23	5	3	S; O	*alkB Rhodococcus* sp. 28/19 (ABD36392)	100	3.00E-67
	12	1	ON	*alkB Rhodococcus* sp. 28/19 (ABD36392)	99	2.00E-66
7	16	3	S;O	*alkB* Antarctic sediment clone SRE-19 (ACT91224)	67	1.00E-41
				alkB Ralstonia sp. PT11 isolate 4 (ABA55801)	62	2.00E-35
17	16	2	S	*alkB* Timor deep-sea sediment clone G17 (ACU43522)	70	1.00E-48
				alkB Kordiimonas gwangyangensis S20-13 clone 1 (ACJ22725)	58	1.00E-35

OTU	*N*	*n*	*sample*	*BLAST match*	*% Identity*	*e-value*
5	9	2	S	*alkB Alcanivorax hongdengensis* A-11-3 (ACD47155)	74	3.00E-47
1	6	1	O	*alkB* Salinisphaeraceae bacterium PC39 (ADE08461)	73	1.00E-47
2	5	1	S	*alkB* Antarctic sediment clone SRE 19 (ACT91224)	84	3.00E-55
				alkB Alcanivorax borkumensis s17-15 (ACJ22718)	62	8.00E-35
11	4	1	S	*alkB Alcanivorax dieselolei* S9-7 (ACJ22690)	62	5.00E-37
14	4	1	S	*alkB* Antarctic sediment clone SRE_F11 (ACT91241)	68	7.00E-44
				alkB Ralstonia sp. PT11 isolate 1 (ABA55798)	55	1.00E-32
18	4	1	S	*alkB* Timor deep-sea sediment clone G17 (ACU43522)	72	4.00E-41
				alkB2 Salinisphaeraceae bacterium PC39 (ADE05603)	62	6.00E-30
21	4	2	S	*alkB* Forest soil clone SA08 (ABA55775)	67	6.00E-35
				alkB Alcanivorax dieselolei 6-D-6 (ACJ22764)	59	3.00E-29
13	3	1	S	*alkB* Timor deep-sea sediment clone F33 (ACU43488)	73	7.00E-47
				alkB Ralstonia sp. PT11 isolate 5 (ABA55802)	58	3.00E-33
20	3	1	S	*alkB Haliscomenobacter hydrossis* DSM 1100 (AEE51162)	50	2.00E-22
28	3	2	S	*alkB2 Alcanivorax dieselolei* Qtet3 (ADN21388)	66	1.00E-45
32	3	1	S	*alkB* Soil clone alkB36mpn (ACZ64725)	73	6.00E-46
				alkB Alcanivorax dieselolei S9-7 (ACJ22690)	71	8.00E-45
12	2	1	S	*alkB* Pacific sediment metagenomic clone 9E7 (CAJ01370)	64	2.00E-37
				alkB Ralstonia sp. PT11 isolate 1 (ABA55798)	51	2.00E-26
26	2	1	S	*alkB Rhodococcus* sp. H1 (ACR56750)	82	2.00E-53
3	1	1	S	*alkB* Barley field soil clone alkG4-12 (ABB90688)	83	8.00E-56
				alkB Ralstonia sp. PT11 isolate 2 (ABA55799)	65	3.00E-36
4	1	1	S	*alkB Alcanivorax hongdengensis* A-11-3 (ACD47155)	71	6.00E-44
9	1	1	S	*alkB* Timor deep-sea sediment clone F33 (ACU43488)	70	3.00E-46
				alkB Alcanivorax sp. PA3 (ACD63080)	55	2.00E-31

OTU	*N*	*n*	*sample*	*BLAST match*	*% Identity*	*e-value*
10	1	1	S	*alkB* Antarctic sediment clone SRE-19 (ACT91224)	65	8.00E-40
				Pseudomonas aeruginosa SL1 (ACJ65013)	57	5.00E-28
15	1	1	ON	*alkB* Arctic soil clone GO0VNXF07IG6EW (JF408383)	75	2.00E-39
				alkB Alcanivorax dieselolei II-D-3 (ACJ22770)	69	1.00E-34
16	1	1	S	*alkB* Antarctic sediment clone SRE-19 (ACT91224)	73	2.00E-49
				alkB Marinobacter sp. S17-4 (ACT31523)	66	6.00E-39
19	1	1	S	*alkB Haliscomenobacter hydrossis* DSM 1100 (AEE51162)	54	1.00E-25
22	1	1	S	*alkB* Arctic soil clone GO0VNXF07IG6EW (JF408383)	69	2.00E-44
				alkB Alcanivorax borkumensis s17-15 (ACJ22718)	59	2.00E-35
25	1	1	S	*alkB Arthrobacter* sp. 7.19-Vpa (AEH76916)	96	4.00E-65
27	1	1	S	*alkB Alcanivorax venustensis* ISO4 (AAT91720)	71	9.00E-48
30	1	1	S	*alkB* Timor deep-sea sediment clone H6 (ACU43509)	72	1.00E-34
				alkB1 Salinisphaeraceae bacterium PC39 (ADE05602)	59	3.00E-28
31	1	1	S	*alkB* Forest soil clone alkB15XB2 (ADO21745)	87	2.00E-59
				alkB Acinetobacter venetianus 1-D-2 (ACJ22758)	73	1.00E-49

a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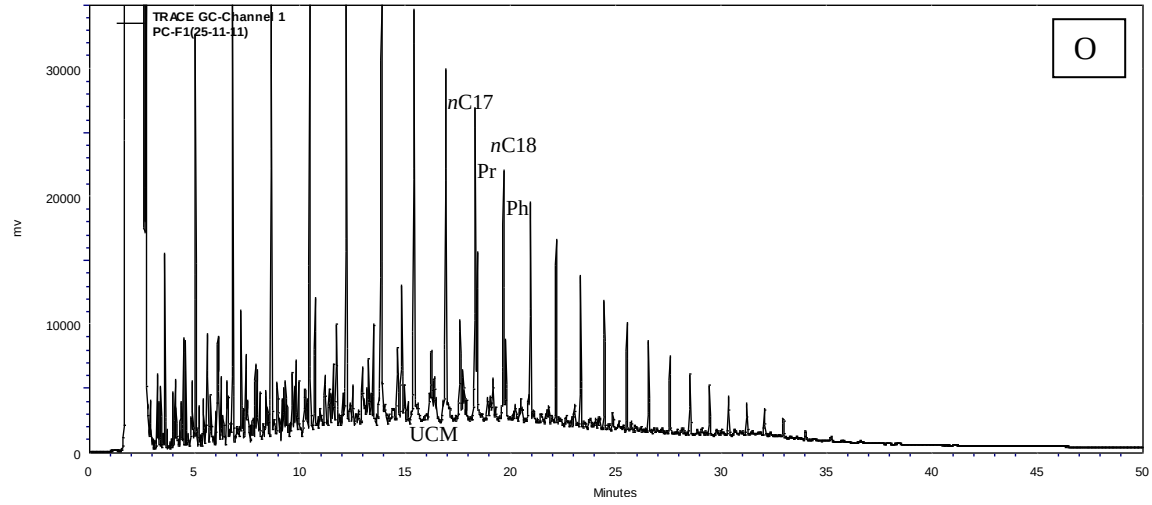
nC17
Ph
Pr
UCM
mv
Minutes

b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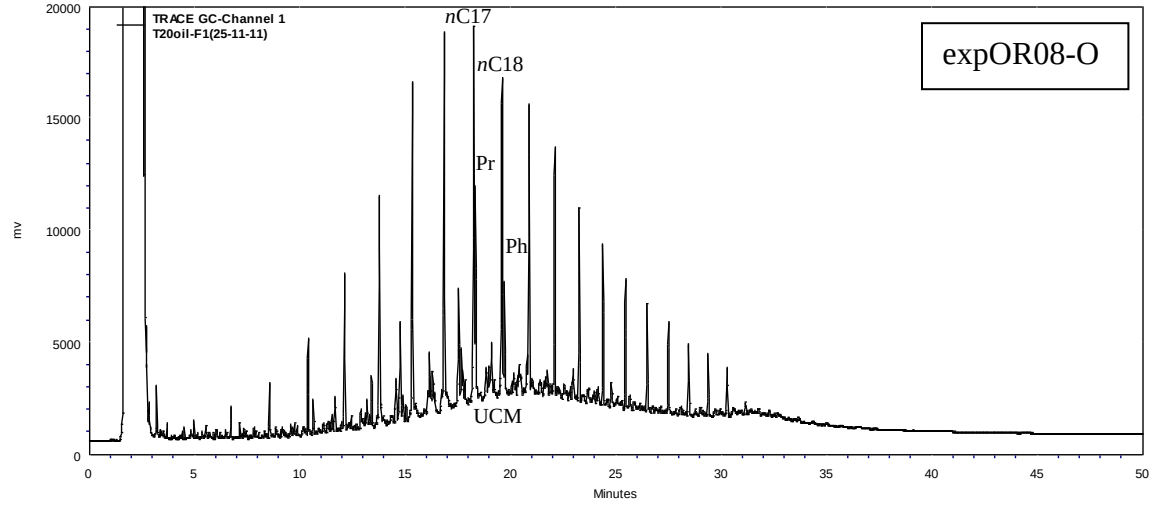
PC-F1(25-11-11)
O
nC17
nC18
Pr
Ph
UCM
mv
Minutes

c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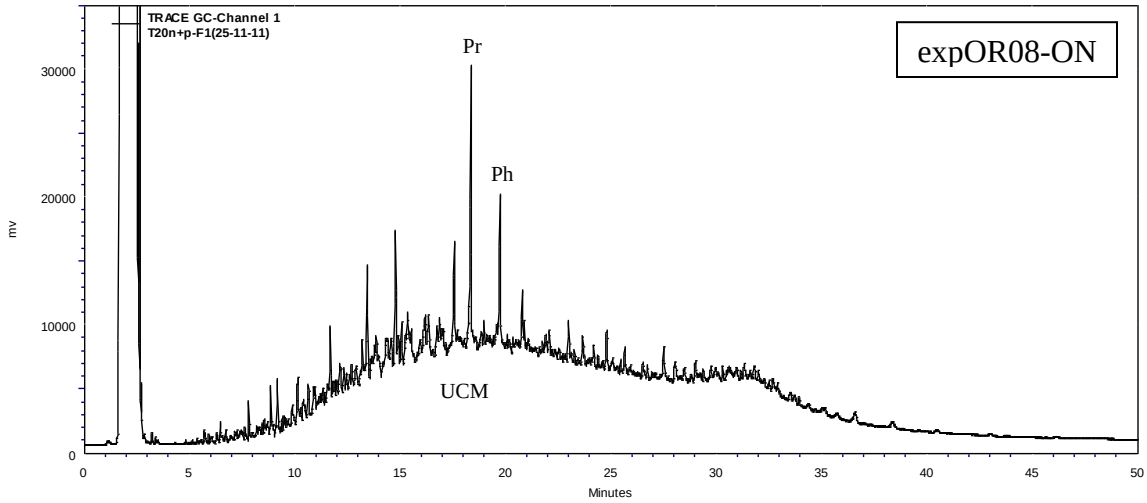
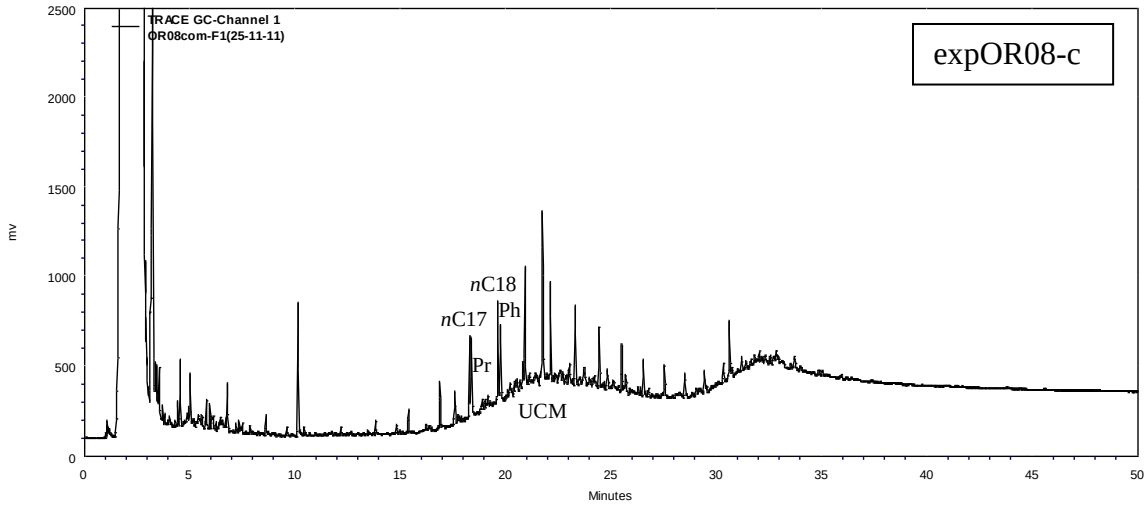
nC18
Pr
Ph
UCM
mv
Minutes

d



e



Online Resource 4 Profiles obtained by high resolution gas chromatography of the aliphatic hydrocarbon fraction of sediments and experimental systems. **a.** OR08 sediment sample from Ushuaia Bay. **b.** crude oil added to the experimental systems. **c.** oil-exposed slurry (expOR08-O) after twenty days of exposure. **d.** oil-plus-nutrient amended slurry (expOR08-ON) after twenty days of exposure. **e.** control slurry (expOR08-c, neither oil nor nutrients added) after twenty days. Representative compound abbreviations are indicated above the corresponding peaks. UCM: unresolved complex mixture