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Abundance, Dynamics, and Biogeographic Distribution of Seven Polycyclic Aromatic Hydrocarbon Dioxygenase Gene Variants in Coastal Sediments of Patagonia

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Novel polycyclic aromatic hydrocarbon dioxygenase gene variants were present in abundances similar to or higher than those of *phnA1* from *Cycloclasticus* spp. at a chronically polluted subantarctic coastal marine environment in Patagonia. These novel gene variants were detected over a 6-year time span and were also present in sediments from temperate Patagonian sites.

Polycyclic aromatic hydrocarbons (PAHs), organic molecules with two or more fused benzene rings, are recognized as chemicals of concern due to their recalcitrance and their potentially deleterious effects on the ecosystem and human health (11). As a result of the hydrophobic nature of PAHs, sediments are the primary repository of these compounds in the marine environment (4). The identification of functionally important PAH-degrading bacterial populations in marine sediments is the primary step to advancing our understanding of the ecological mechanisms governing PAH biodegradation in this environmental matrix (9). The most common target used to study PAH-degrading bacterial populations is the gene encoding the alpha subunit of the dioxygenase that catalyzes the first step of aerobic degradation pathways (5). In previous studies (2, 9), we identified eight distinct variants of this functional gene in intertidal sediments of Patagonia using PCR clone libraries targeting PAH dioxygenase genes previously identified in Gram-negative bacteria. Three of these gene variants showed ≥95% identity at the amino acid level with PAH dioxygenases from cultured representatives: *phnA1* from *Cycloclasticus* spp. (15), *phnAc* from *Alcaligenes faecalis* AFK2 (GenBank accession number AB024945), and other *Betaproteobacteria*, as well as *nahAc* from *Pseudomonas* spp. (6). The rest of the gene variants, identified as A to E, showed <70% identity at the amino acid level with previously described sequences (9). These novel genes were detected only in subantarctic sediments, in spite of analyzing intertidal sediments covering 12° latitude on the Patagonian coast (9). Most novel genes were poorly represented in the PCR clone libraries with the exception of the C dioxygenase gene variant, whose relative abundance widely varied in sediments obtained at the same site in different years (2). In the present work, we used quantitative PCR (qPCR) to quantify each of seven of these gene variants in intertidal sediments from Patagonia, in order to evaluate the ecological significance of bacterial populations carrying these genes. We excluded dioxygenase gene variant E from the analysis, since we were able to recover only one sequence of this gene variant in our previous study (2, 9) and it was not possible to guarantee an appropriate coverage in the design of a primer set. Our working hypotheses were as follows: (i) PAH dioxygenase genes *nahAc*, *phnAc*, and *phnA1* are more abundant than novel gene variants A to D in subantarctic coastal sediments, (ii) relative abundances of these genes exhibit temporal variations when analyzed over a long time span, and (iii) novel variants of PAH dioxygenase genes present a narrow biogeographic distribution, restricted to subantarctic environments.

For this study, we selected the two most prominent hydrocarbon-polluted sites of the Argentinean coast of Patagonia: Orion Plant (OR) (54°48′S, 68°17′W) and Córdova Cove (CC) (45°45′S, 67°22′W) (Fig. 1 and references 1–3 and 9). OR is located next to an oil jetty in Ushuaia Bay, within the Beagle Channel, and is mainly polluted with refined petroleum products (2, 9). CC, on the other hand, is located on the coast of the San Jorge Gulf, in the Atlantic Ocean, and is mainly affected by crude oil extraction and transportation activities (1). A third sampling location, Fracasso Beach (PF) (42°25′S, 64°7′W), was chosen as a reference pristine site (9). OR belongs to the "Channels and Fjords of Southern Chile" marine ecoregion, while both CC and PF belong to the marine ecoregion "North Patagonian Gulfs" (14). Surficial sediments (top 3 cm) were retrieved from 7 to 10 random points in the low tide line, combined, and mixed thoroughly in order to produce a composite sample. These intertidal sediment samples were named according to the sampling site (OR, CC, or PF) and the last two digits of the sampling year. Sediment PAH concentrations were measured by gas chromatography-mass spectrometry. More information regarding sampling sites, sampling method, and chemical analysis of sediment samples can be found in the supplemental material. As expected, PAHs of anthropogenic origin were found in all the sediment samples retrieved from OR and CC sites (151 to 4,127 μg kg dry weight sediment^{-1}), while no PAHs were detected in sample PF08 (Fig. 2; see Table S1 in the supplemental material).

Metagenomic DNA was purified from sediment samples using the FastDNA SPIN kit for soil (MP Biomedicals, Solon, OH) (for detailed information see the supplemental material). Novel PAH dioxygenase gene variants A to D (9) and the gene variants with cultured representatives *phnA1* (*Cycloclasticus* spp.), *phnAc* (*Alcaligenes faecalis* AFK2 and other *Betaproteobacteria*), and *nahAc*

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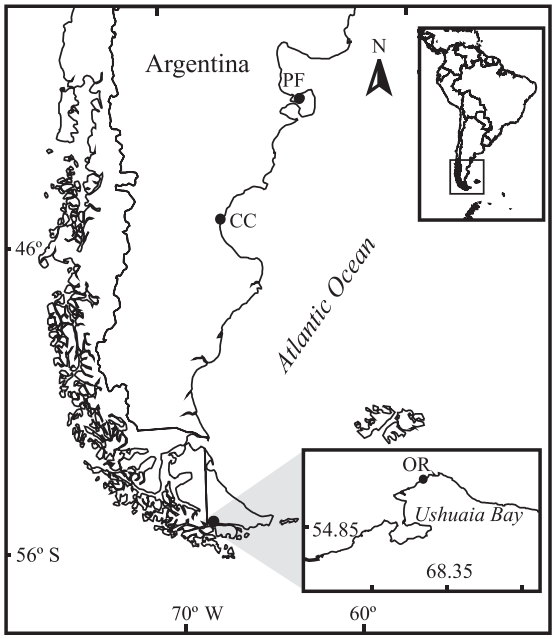

FIG 1 Location of the sampling sites Fracasso Beach (PF), Cordova Cove (CC), and Orion Plant (OR). (Modified from reference 9.)

(*Pseudomonas* spp.) were quantified using SYBR green I qPCR assays with the primer sets and conditions detailed in the supplemental material. In addition, we estimated the relative abundance of bacterial 16S rRNA genes in the sediments, to be used as a reference value (see Table S2 in the supplemental material). We quantified these targets in six intertidal sediment samples retrieved at the OR site over a 6-year time span. In most sediment samples, C and D gene variants were more abundant than *nahAc*, *phnA1*, and *phnAc* genes (Fig. 2A), with relative abundances representing 0.01 to 0.63 and 0.08 to 1.94% of the quantified bacterial 16S rRNA genes, respectively. The B gene variant was also detected in all the sediment samples, although at lower concentrations (0.01 to 0.03% of 16S rRNA genes). These results suggest that populations carrying these novel gene variants are ecologically relevant members of this microbial community. Archetypical *nahAc* genes were also detected in all the analyzed samples, although at concentrations below the quantification limit of the assay (Fig. 2A). This result is in contrast with *nahAc* relative abundances found in the PCR clone libraries, where this gene was dominant in the majority of the analyzed samples from Ushuaia Bay (2, 9). This could be due to biases often found in this approach, introduced in endpoint PCR involving multiple templates, as well as in the cloning step (13).

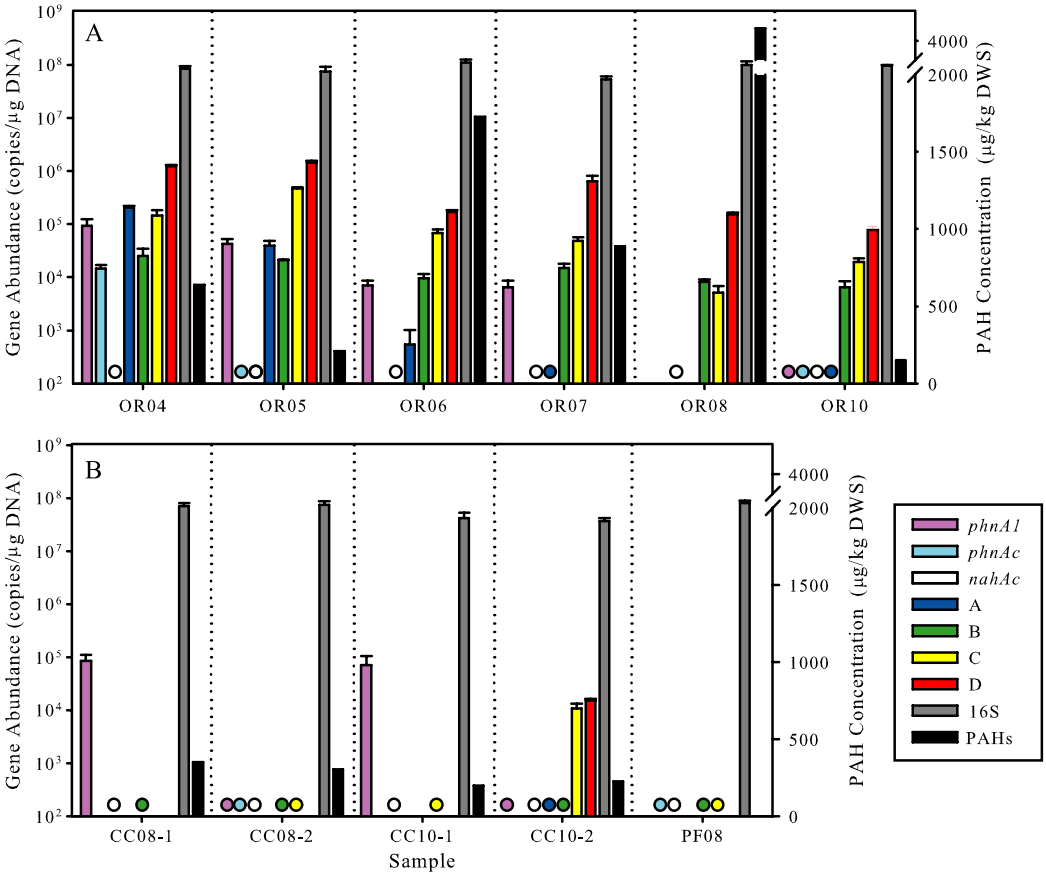

FIG 2 Relative abundance of PAH dioxygenase gene variants in intertidal sediments of OR (A), CC, and PF (B) sites. Samples were named according to sampling location (OR, Orion Plant; CC, Córdova Cove; PF, Fracasso Beach) and the last two digits of sampling year. Genes that were detected in qPCRs but could not be quantified because they were present at concentrations below the quantification limit of the technique (8×10^2 copies/μg DNA for assays *phnA1*, *nahAc*, B and D, 9×10^2 copies/μg DNA for assay A, and 1.3×10^3 copies/μg DNA for assays *phnAc* and C) are indicated in the figure with colored circles. The abundance of the 16S rRNA gene (16S) and the concentration of total PAHs (μg/kg dry weight sediment [DWS]) are also indicated. Individual PAH concentrations and sampling dates can be found in Table S1 in the supplemental material.

We observed a decrease in the overall abundance of the quantified PAH dioxygenase genes over time (Fig. 2A), with an order of magnitude difference between the first two samples and the last two samples of the time series. The two genes with the highest reduction in abundance were A and *phnA1*, with two orders of magnitude difference over this time. Nonmetric multidimensional scaling analysis based on gene variant abundances in OR sediment samples showed a temporal trend in the ordination of the samples (see Fig. S1A in the supplemental material). (For information about statistical analyses, see the supplemental material.) This result was supported by the grouping pattern obtained by cluster analysis (see Fig. S1B in the supplemental material). Overall, these results suggest the occurrence of nonrandom temporal variations in the relative abundances of the target genes in intertidal sediment samples of this time series. Additionally, we used metadata information in order to explore which factors could be affecting the abundance of these microbial populations. The BIO-ENV analysis showed that two- and four-ring PAHs, total PAHs, and time elapsed since first sampling comprised the subset of variables that, when combined, gave the highest correlation between gene abundances and metadata information ($\rho = 0.693$; see Table S3 in the supplemental material). This result suggests that changes in type and abundance of PAHs could provoke changes in the abundance of the microbial populations that carry the genes targeted in this study. In this time series, however, the overall abundance of targeted PAH dioxygenase genes decreased over time, despite the presence of various PAHs in the sediments (Fig. 2A; see Table S1 in the supplemental material). One possible explanation for the observed decline in PAH dioxygenase gene abundance could be that populations carrying these genes were outcompeted by other PAH-degrading populations not targeted in this study but present at this site, such as Gram-positive bacteria (10). Further studies should explore this possibility.

In order to explore whether the biogeographic distribution of dioxygenase gene variants A to D is restricted to Ushuaia Bay, as suggested in our previous study (9), or, alternatively, if these gene variants could also be found in other coastal environments in Patagonia, we analyzed sediment samples from CC and PF sites (Fig. 1). All novel gene variants were detected in intertidal sediments of CC, although they were often found at concentrations below the quantification limit of the assays (Fig. 2B). In addition, gene variants B and C were detected in the pristine site PF, also below quantification limits. Our work extends the known biogeographic distribution of novel PAH dioxygenase gene variants A to D to the temperate marine ecoregion North Patagonian Gulfs. It still needs to be determined if these genes are also present in microorganisms from other marine environments or in other habitats. In a recent work, Yagi and Madsen (18) identified sequences which clustered with PAH dioxygenase gene variant A in PCR clone libraries from a coal tar waste-contaminated aquifer (79.5% and 92.3% identity at nucleotide and amino acid levels, respectively). Although these sequences were only 78 bp long, this finding suggests that novel dioxygenase gene variant A could be diverse and broadly distributed.

The *phnA1* gene was detected in 9 of the 11 samples from the two biogeographic regions analyzed in this study (Fig. 2). We found a strong positive correlation between *phnA1* gene abundances and the concentrations of three-ring PAHs ($\rho = 0.789$; $P = 0.004$) and phenanthrene ($\rho = 0.781$; $P = 0.005$). In addition, a correlation was found between *phnA1* gene abundances and low-molecular-weight PAHs (two- and three-ring PAHs; $\rho = 0.708$; $P = 0.015$). On the other hand, no correlation could be found between PAHs with more than three rings and *phnA1* gene abundances, although the low bioavailability of high-molecular-weight PAHs could preclude finding meaningful correlations between gene abundances and PAH concentrations (12). The observed correlations are in agreement with substrate preferences of the recombinant dioxygenase from *Cycloclasticus* sp. A5, which is able to transform, within the PAHs tested, naphthalene, phenanthrene, and substituted naphthalenes but not anthracene, pyrene or benzo[a]pyrene (8). Our results suggest that bacterial populations carrying *phnA1* dioxygenase genes could play an important role in the biodegradation of (at least) two- and three-ring PAHs in coastal sediments of Patagonia.

Molecular techniques can provide timely information for choosing a bioremediation strategy, as well as for monitoring its efficiency and reliability (16). However, insufficient knowledge regarding key biomarkers is still hindering the application of molecular biological tools in this field (17). Our study shows that yet-unidentified bacteria carrying novel dioxygenase gene variants, in concert with known obligate hydrocarbonoclastic bacteria, might play an important role in the biodegradation of aromatic compounds in coastal sediments of Patagonia. As detecting a sequence is not necessarily an indication that the gene will be expressed and the enzyme will carry out the assumed function in the environment (7), further studies are needed to link these candidate biomarkers to their function within the microbial community.

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Supplemental Material Chemical Analysis

Sediment sampling: Two chronically-polluted and one pristine coastal marine environment located on the coast of Patagonia, Argentina, were chosen for this study (Figure 1). The first polluted sampling site, Orion Plant, is located in Ushuaia Bay, within the Beagle Channel, on the South coast of the Big Island of Tierra del Fuego (OR, 54º 48' S, 68º 17' W). Loading and offloading of refined petroleum products at this jetty and the intense maritime traffic and port operations taking place within Ushuaia Bay have exposed this site to chronic oil pollution (2-4). The main environmental features of this area have been recently reviewed (2). The second polluted site is Córdova Cove, located in the San Jorge Gulf over the Atlantic Ocean (CC, 45° 45' S, 67° 22' W). This site is affected by hydrocarbon inputs due to nearby crude oil extraction activities, buoys used for loading this oil for maritime transportation (5.5 million m^3 a year), and activities of a small fishing port. In December 2007, an accidental crude oil spill from a tanker affected at least two kilometers of coast at this cove, and intertidal sediment samples were obtained at the affected area approximately 100 (CC08 samples) and 900 days (CC10 samples) after this oil spill. OR site has a humid and cold-temperate climate, while CC has an arid and temperate climate. Average air temperatures are, respectively, 2.4ºC and 6.6°C on the coldest months and 9.6°C and 19.6°C on the warmest months (Servicio Meteorológico Nacional, www.smn.gov.ar). The unpolluted site, Fracasso Beach (PF, 42º 25' S, 64º 07' W), is located on the coast of the San José Gulf at the Valdes Peninsula (Figure 1). This Peninsula is a protected area, which has been listed as an UNESCO natural world heritage site since 1999. Like the CC sampling site, PF has an arid and temperate climate.

Surficial intertidal sediment samples (top 3 cm) were retrieved using acrylic cores with an inner diameter of 4.4 cm, at seven to ten random points along the low tide line. Samples were placed in sterile glass flasks and stored at 4°C during transport to the laboratory, where they were mixed thoroughly and stored at -80°C for molecular analyses and at -20°C for chemical analyses. All samples were named according to their sampling site (PF, CC or OR) and the last two digits of the sampling year.

Chemical Analysis: PAH concentrations were determined using gas chromatography - mass spectrometry techniques according to US EPA 8100 (6), Dean (1) and Sloan et al. (5). Sediments were homogenized, and anhydrous Na_2SO_4 was added to absorb the water from the sample. The sediments were mixed immediately to avoid clumping and hardening of the sodium sulfate, and mixing was continued until they appeared dry. Dichloromethane was then added and PAHs were extracted by sonication for 12 h. Extracts were filtered through a 0.45 μm fiberglass filter. A gravity-flow silica/alumina chromatography column was used to remove extraneous polar compounds from the extract. The eluant was collected and concentrated with a rotary evaporator to a final volume of 1 ml. Two μl were directly injected into a GC injection port operating in splitless mode. A Shimadzu gas chromatograph 17A V 1.3 model with mass spectrometer QP 5050A and an MS Workstation Class 5000 (Shimadzu Corp., 1999) was used to analyze the PAHs. Experimental conditions included: (a) a PTE-5 fused-silica capillary column (30 m x 0.25 mm i.d. x 0.25 μm film thickness, Supelco, Bellafonte, PA), (b) a linear velocity of carrier Helio of 36.2 cm/s, splitless, with sampling time of 2 min and total flow of 11.7 ml/min, (c) a temperature program of 60°C for 2 min heated to a final temperature of 300 °C at 10°C/min, held at this temperature for 10 min, (d) an injector temperature of 250 °C, and (e) a capillary interface temperature of 300 °C. Samples were analyzed using SIM mode for optimal

50 sensitivity, scanning only the quantification ions for each PAH. Quality assurance criteria included the percent recoveries from spiked sediments, which was between 70 –
52 130 %. In addition, the relative standard deviation (RSD) of the concentrations of each replicated analyte was considered acceptable if the RSD was <15% for at least 85% of
54 the analytes. Quantification was performed by the use of external standards (Restek, Bellafonte, PA). Quantification limit of the technique was 10 µg/kg of dry weight
56 sediment.

Individual PAH concentrations of intertidal sediment samples of OR, CC and PF
58 is shown in Table S1. Between 1 and 11 different PAHs were detected in samples retrieved at the two polluted sites, OR and CC, while no PAH contamination was found
60 in the pristine site PF. Sixty three percent of the detected PAHs were found to be above interim marine sediment quality guidelines, while 8.7% of them exceeded probable
62 effect level thresholds, where adverse biological effects are expected to be observed (Table S1).

Table S1. PAH concentrations (µg/kg dry weight sediment) and sampling dates of the intertidal sediment samples used in this study.

	Sample										
PAHs	**OR04**	**OR05**	**OR06**	**OR07**	**OR08**	**OR10**	**CC08-1**	**CC08-2**	**CC10-1**	**CC10-2**	**PF08**
Naphthalene	-	-	35[a]	67[a]	52[a]	86[a]	-	-	172[a]	172[a]	-
Acenaphthylene	-	-	42[a]	-	-	-	-	-	61[a]	30[a]	-
Acenaphthene	-	-	44[a]	480[b]	-	-	78[a]	71[a]	-	-	-
Fluorene	-	-	88[a]	-	-	30[a]	-	-	-	bql	-
Phenanthrene	640[b]	-	110[a]	-	-	-	170[a]	-	23	-	-
Anthracene	-	14	323[b]	71[a]	-	-	110[a]	91[a]	bql	-	-
Fluoranthene	-	14	149[a]	107	-	-	240[a]	167[a]	40	-	-
Pyrene	-	-	59	158[a]	-	bql	210[a]	207[a]	82	bql	-
Chrysene	-	-	-	-	140[a]	bql	70	-	-	-	-
Benzophenanthrene	-	-	-	-	-	19	-	-	-	-	-
Perylene	-	-	-	-	-	bql	-	-	-	-	-
Dibenz(*a*,*h*)anthracene	-	-	141[b]	-	-	-	70[a]	89[a]	-	-	-

Benzo(*a*)pyrene	-	-	-	-	-	-	-	-	-	252[a]	-
Indeno(1,2,3-*cd*)pyrene	-	86	402	-	208	-	106	133	-	-	-
Indeno(1,2,3-*cd*)fluoranthene	-	-	-	-	412	16	-	-	-	-	-
Benzo(*g,h,i*)perylene	-	95	333	-	3,315	bql	-	-	-	-	-
TOTAL PAHs	**640**	**209**	**1726**	**883**	**4,127**	**151**	**1,054**	**758**	**378**	**454**	**-**
Sampling date (month/year)	10/04	09/05	04/06	06/07	12/08	09/10	04/08	04/08	06/10	06/10	05/08

Samples were named according to sampling location (OR: Orion Plant, CC: Córdova Cove, PF: Fracasso Beach) and the last two digits of sampling year.

-: not detected; bql: detected below the quantification limit of the technique (10 µg/kg dry weight sediment).

[a]PAH concentrations exceeding interim marine sediment quality guidelines, although below probable effect level thresholds; [b]Values exceeding probable effect level thresholds (Canadian Environmental Quality Guidelines, http://st-ts.ccme.ca/). Benzophenanthrene, Perylene, Indeno(1,2,3-*cd*)pyrene, Indeno(1,2,3-*cd*)fluoranthene and Benzo(*g,h,i*)perylene are not included in this report.

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Supplemental Material qPCR Analysis

Metagenomic DNA extraction and quantification: Metagenomic DNA was purified from 0.8 to 1 g wet sediments using FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH), following the manufacturer's instructions. Samples were homogenized three times for 50 s at approximately 5,000 rpm (speed at high setting) with 1 min intervals using a mini-beadbeater Biospec (Bartlesville, OK). Two extractions per sample were combined before further analysis. DNA concentrations were measured using the DNA binding-fluorophore EvaGreen® (Biotium, Inc., Hayward, CA) in a Chromo4 thermal cycler (Bio-Rad, Hercules, CA), according to Wang and collaborators (15), using λ bacteriophage DNA (Promega, Madison WI) as standard.

Design of qPCR primers: Primer sets for qPCR analysis targeting the PAH dioxygenase gene variant *phnA1* from *Cycloclasticus* spp., as well as novel gene groups B and C have been previously reported [(3), Table S2]. Primers for the quantification of gene variants *nahAc* from *Pseudomonas* spp., *phnAc* from *Alcaligenes faecalis* AFK2 and related sequences, as well as novel dioxygenase gene variants A, D and E were designed for this study (Table S2). To design the primer sets, we aligned the nucleotide sequences of each gene variant using ClustalX 2.0 (13). Sequences included in each alignment correspond to those previously amplified from coastal sediments of Patagonia (9), as well as sequences of genes *phnA1*, *phnAc* and *nahAc* available at the GenBank database by July 2007. The consensus sequence for each gene variant was obtained using the GeneFisher software (5), and primers were manually designed on conserved regions of the consensus sequences following general guidelines for qPCR primer design (1). The specificity of the different potential primers was evaluated by comparing their sequences with the GenBank database, using the BLASTN tool

adjusted for short nearly exact matches (10). The stability of secondary structures and the potential formation of primer-dimers were tested using Oligonucleotide Properties Calculator (www.basic.northwestern.edu/biotools/oligocalc.html) and AutoDimer softwares (14). In addition, we selected primers 1055f and 1392r (Table S2) to quantify bacterial 16S rRNA genes in the intertidal sediment samples.

qPCR conditions: All qPCR reactions were optimized and carried out in a Chromo4 thermal cycler (Bio-Rad, Hercules, CA) using the primer sets detailed in Table S2. Reactions were performed in 20 µl final volume containing 1× PerfeCTa® SYBR® Green Supermix (Quanta BioSciences, Inc., Gaithersburg, MD), primers at concentrations indicated in Table S2, and template DNA. Control reactions where template DNA was replaced with bidistilled water were included in all runs. The amplification program consisted of an initial denaturation step at 95°C during 5 min and 45 cycles of: 30 s at 95°C, 30 s at the temperature indicated in Table S2, and a final elongation step of 30 s at 72°C. The amplification protocol for the *nahAc* assay included, in addition, an incubation step at 79°C for 20 s to avoid the detection of primer-dimers (11). After the amplification protocol, melting curves were performed in order to analyze the specificity of the amplified fragments, by measuring fluorescence signal at incrementing temperatures of 0.2°C, from 70°C to 95°C.

The most abundant clones from the PCR clone libraries constructed from intertidal sediments were chosen as standards of each assay [A: Ac-OR04-B5; B: Ac-OR05-71; C: Ac-OR06-180; D: Ac-OR05-93; E: Ac-OR06-11; *phnA1*: Cycp-OR05-30; *phnAc*: Ac-SC04-5; *nahAc*: Ac-OR06-41 (9)]. The 16S rRNA gene cloned from *Escherichia coli* DH5-α was used as standard for the bacterial 16S rRNA gene quantification assay. Plasmids were purified using the QIAprep® Spin Miniprep Kit (QIAGEN Inc., Valencia, CA), linearized using the *Not*I restriction enzyme (Promega,

Madison, WI) and subsequently purified using Wizard SV gel and PCR clean-up system
52 (Promega, Madison, WI) to eliminate the restriction enzyme and buffer. The concentration of each standard DNA was measured using the Hoechst 33258 fluorescent
54 dye (Amersham Biosciences, Piscataway, NJ) in a DyNA Quant 200 fluorometer (Hoefer Scientific Instruments, San Francisco, CA), and the measured concentration
56 was confirmed by agarose gel electrophoresis using High DNA Mass™ Ladder (Invitrogen, Carlsbad, CA). The number of gene copies/µl standard DNA was
58 calculated considering plasmids and inserts lengths, and assuming a molecular mass of 660 Da per base pair. Standard curves were constructed for each assay using serial
60 dilutions of the stock solutions, and the linearity ($r^2 > 0.99$) extended over more than 6 orders of magnitude. Amplification efficiencies, measured as previously described (11),
62 were higher than 88% for the assays targeting gene variants A to E, *phnA1* and bacterial 16S rRNA, 78% for *phnAc* and 70% for *nahAc*, based on standard DNA curves.

64 Due to the widespread inhibition by metagenomic DNA observed in these samples, optimum DNA concentrations were determined for each qPCR assay and each
66 sample. Samples were analyzed in triplicate at concentrations between 1 and 7.5 ng of DNA per reaction, where the least PCR inhibition was detected. A fourth amplification
68 reaction containing the optimal environmental DNA concentration was spiked with 10^3 copies of standard DNA per reaction in order to calculate PCR inhibition as previously
70 described (2), which was <10% in the majority (80%) of the quantified samples. The number of quantified gene copies in each DNA sample was corrected accordingly. We
72 chose to report the qPCR data as copies of the target gene per µg of metagenomic DNA, because although qPCR assays can provide relatively accurate measurements of gene
74 abundance expressed in this manner, the conversion of this information to copies per

gram of sediment or cells per gram of sediment will introduce several sources of error, as recently reviewed (12).

Validation of qPCR assays: Due to the growth of the GenBank database since the date of the primer design, we repeated the analysis of primer coverage, this time considering the sequences deposited on the database by September 2011. It is important to notice, however, that these sequences probably only represent a part of the true diversity of PAH dioxygenase genes and as a consequence, this analysis can only partially estimate their actual coverage (6). Concerning the assay targeting *phnA1* gene of *Cycloclasticus* spp., the 16 *phnA1* gene sequences available in GenBank by that time perfectly matched the primer pair (8 sequences from *Cycloclasticus* spp. and 8 from uncultured bacteria). Similarly, 92 out of 94 gene sequences of the database related to the *phnAc* gene variant identified in *A. faecalis* AFK2 had a perfect match with the primer set, and the other two sequences presented only one mismatch at the 5' end of the reverse primer. Seven of these sequences were retrieved from bacterial strains belonging to the genera *Burkholderia* (4 sequences), *Alcaligenes* (1 sequence), *Acidovorax* (1 sequence) or *Delftia* (1 sequence), while 87 were obtained in culture-independent studies. Concerning the assay targeting the *nahAc* gene, we found 119 sequences of this archetypical gene available in GenBank whose sequence included both primers. Sixty three sequences were retrieved from pure cultures of *Pseudomonas* spp. and 56 from uncultured microorganisms. The *nahAcf* - *nahAcr* primers have a perfect match with 115 of these sequences. Two sequences present one mismatch with the forward primer within the last 4 bases at their 3' end and 2 present one mismatch with the reverse primer within the last 7 bases at their 5' end. None of the primers matched sequences that did not belong to their specific variant. In addition, we used TestProbe 2.2 (www.arb-silva.de/search/testprobe) to test for the specificity of the

100 selected bacterial 16S rRNA gene primer set (Supplemental Table S2) against a database of more than 500,000 high quality sequences of this gene. The primers
102 presented a perfect match with the 51.3% and 54.7% of the sequences of the database (SSU r108) for the forward and reverse primers, respectively. Although unable to detect
104 every bacterium present in the sediments as any primer set targeting this gene, the quantification of this target is still able to provide useful reference values.

106 After the optimization of each qPCR assay, we evaluated their specificity by agarose gel electrophoresis, cloning and sequencing of representative PCR products. In
108 addition, the specificity was tested in all reactions by analyzing their melting curve. All assays were found to be highly specific, with the exception of the *nahAc* gene assay,
110 which produced specific as well as nonspecific amplicons in some of the environmental samples (data not shown). The *nahAc* gene was not very abundant in the sediments, and
112 under these conditions primers can randomly missanneal leading to the formation of nonspecific products (7). In fact, when this assay was tested using a sample from a
114 bioreactor that presented a high abundance of *nahAc* genes, the assay presented a high specificity (data not shown).

Supplemental Table S2. Primer sets used for qPCR analysis of PAH dioxygenase gene variants and bacterial 16S rRNA gene in intertidal sediment samples, as well as optimal primer concentrations and annealing temperatures for these assays.

Target gene variant	Primers	Sequences (5' – 3')	Product length (bp)	Primer concentrations	Annealing temperature	References
A	Agroup-f	CAGAAAGATGGTTCGGTCAAAG	83	600 nM	58°C	This study
	Agroup-r	GTATTCCCTGCTTCAACTGATG		400 nM		
B	Bgroup-f	GGATTTGTCTACGGTTGTTTCG	75	600 nM	61°C	(3)
	Bgroup-r	GAGGTACCACGCAAATTCTC		600 nM		
C	Cgroup-f	CTTCGTRTTCGGATGCATG	86	600 nM	59°C	(3)
	Cgroup-r	CATGAAGCTATYCAGATACCAG		600 nM		
D	Dgroup-f	AACCCTCACCTGCTCCTAC	86	600 nM	59°C	This study
	Dgroup-r	TAAAGTAGGCGCTYTGCTCC		600 nM		

phnA1 *Cycloclasticus* spp.	*phnA1f*	GGGTGGACTAGCTGGAA	120	600 nM	60°C	(3)
	phnA1r	TTCGCATGAATAGCGATGG		600 nM		
phnAc *Alcaligenes faecalis* AFK2	*phnAcf*	CCYAGCTTGAATGACTATCTTG	109	600 nM	56°C	This study
	phnAcr	AGTTYAAYAATGATCGACTTGG		600 nM		
nahAc *Pseudomonas* spp.	*nahAcf*	TATCACGGCTGGGRCTTC	138	400 nM	63°C	This study
	nahAcr	GAASCCATGGAAGCTCTC		400 nM		
Bacterial 16S rRNA	16S-1055f	ATGGCTGTCGTCAGCT	352	500 nM	50°C	(4, 8)
	16S-1392r	ACGGGCGGTGTGTAC		500 nM		

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Supplemental Material Statistical Analyses

Multivariate analyses were performed using the PRIMER v6 software package [Plymouth Marine Laboratory, (4)]. The Bray-Curtis similarity index (2) was calculated between sediment samples from OR site based on the information of PAH dioxygenase gene abundances. This index takes into account both the number of shared species and their relative abundances to calculate the percent similarity between pairs of community samples (1). Before computing similarities, relative gene abundance data were square-root transformed (7), since very abundant genes could mask the influence of low abundance genes on samples ordination. In addition, gene values that were detected below the quantification limit of the qPCR assay were replaced by the quantification limit for each assay (*phnA1*: 800 copies/µg DNA; A: 900 copies/µg DNA; *phnAc*: 1,300 copies/µg DNA). The *nahAc* gene abundances were excluded from the analysis, as this gene was detected below quantification limit of the technique in all samples. The Bray-Curtis similarity matrix was used to perform a non-metric multidimensional scaling (MDS) ordination (6). The similarity matrix was further used to perform a hierarchical clustering analysis, using a group-average linking method. The result of this analysis was superimposed on the MDS plot at two arbitrary similarity levels (75% and 65% similarity), in order to best reflect the formed groups.

We used software package PRIMER v6 (4) to find the genes that most influenced the observed ordination pattern. From all possible combinations of genes, this software selected the smallest subset whose Bray-Curtis similarity matrix best correlated with the complete dataset matrix. In this analysis, a Spearman coefficient (ρ) of at least 0.95 is considered a robust correlation (4). The subset of genes that produced a Bray-Curtis similarity matrix that highly correlated with the similarity matrix produced by the complete data set (ρ = 0.95) was *phnA1*, *phnAc*, A and D, indicating

that the observed ordination was product of the variation in various gene abundances rather than a change in the relative abundance of a particular gene variant.

The BIO-ENV tool of the PRIMER v6 software (3) was used in order to link samples metadata to dioxygenase gene abundances. The BIO-ENV analysis computes (non-parametric) Spearman rank correlations between two distance matrices: the Bray-Curtis dissimilarity matrix based on the complete gene abundance data and a Euclidean distance matrix among all samples based on metadata information. The metadata used to calculate the Euclidean distance matrix were: concentration of 2 to 6-ring PAHs, total PAHs, average temperature of the month of sampling and the time elapsed (in months) since the first sample collection, October 2004. PAH concentrations that were detected below quantification limit (10 μg/kg dry weight sediment, dws) were replaced by half the quantification limit of the technique, as recommended when substitutions represent less than 30% of the data points (5). Each value was normalized by subtracting the mean and dividing by the standard deviation across all samples to remove units from the data (4).

Univariate analyses of correlation between individual PAH dioxygenase gene abundances (square-root transformed) and normalized PAH concentrations were performed using the Spearman rank correlation test in SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Figure S1. Similarities among sediment samples of OR site, based on square root-transformed PAH dioxygenase gene abundances. **A-** Non-metric multi-dimensional scaling (MDS) ordination plot. **B-** Cluster analysis. Two arbitrary similarity levels were superimposed on the figures: 75% similarity (solid line) and 65% similarity (dotted line). Similarity index: Bray-Curtis.

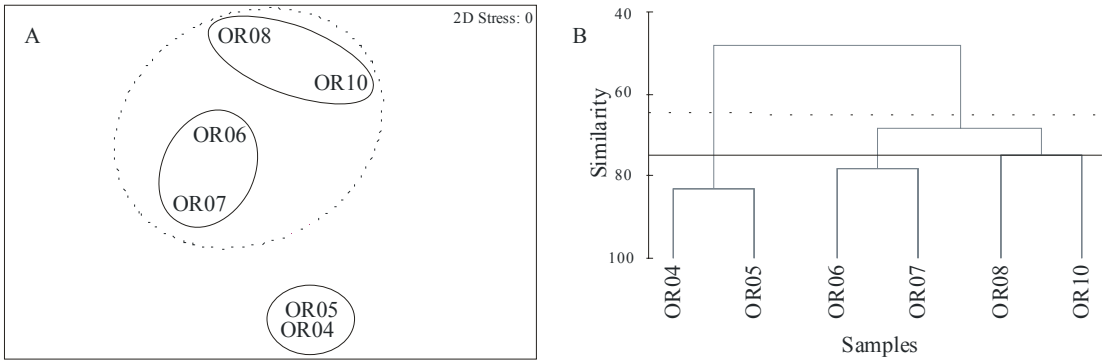

Table S3. Combination of environmental factors (taken *k* at a time) and their correlations to PAH dioxygenase gene abundance data of Subantarctic intertidal sediment samples. (BIO-ENV analysis, Primer v6 software)

Spearman rank correlation (ρ_s)

k	Best variable combinations				
1	2-ring	Tem	4-ring	T-PAHs	…
	(0.602)	(0.564)	(0.146)	(0.096)	
2	4-ring, Tem	2-ring, Tem	T-PAHs, Tem	2-ring, T-PAHs	…
	(0.593)	(0.561)	(0.557)	(0.539)	
3	2-ring, T-PAHs, Tem	2-ring, 6-ring, Tem	2-ring, 4-ring, Tem	…	
	(0.629)	(0.604)	(0.593)		
4	**2-ring, 4-ring, T-PAHs, Tem**	2-ring, 4-ring, 6-ring, Tem	...		

64		**(0.693)**	(0.650)
	5	2-ring, 3-ring, 4-ring, T-PAHs, Tem	…
66		(0.611)	

Tem: temporal variable (months); 2-ring: 2-ring PAHs; 3-ring: 3-ring PAHs; 4-ring: 4-ring PAHs; 6-ring: 6-ring PAHs; T-PAHs: total PAHs

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