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Exploring hydrocarbonoclastic bacterial communities in the estuarine surface microlayer

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ABSTRACT: Bacteria that degrade polycyclic aromatic hydrocarbons (PAHs) in the estuarine surface microlayer (SML) of the Ria de Aveiro, Portugal—which is chronically polluted with oil hydrocarbons (OH)—were isolated and characterized; *Pseudomonas* was dominant among the PAH-degrading bacteria. Screening for PAH dioxygenase genes detected almost identical *nahAc* genes (encoding the alpha subunits of naphthalene dioxygenase) in 2 phylogenetically distinct isolates: *Pseudomonas* sp. and an unknown species of the family *Enterobacteriaceae*; this suggested that horizontal transfer of *nah* genes might be involved in PAH degradation in the SML. We also investigated the effect of PAH contamination on the spatial variability of the bacterioneuston along a gradient of pollution in the estuarine system of the Ria de Aveiro. Culture-independent techniques—fluorescence *in situ* hybridization (FISH) and denaturing-gradient gel electrophoresis (DGGE)—revealed a similar structure among the bacterioneuston communities along the estuary. In contrast, we detected differences in the relative abundance and diversity of organisms of the *Gammaproteobacteria*, including those of the genus *Pseudomonas* (which belongs to the *Gammaproteobacteria*). This is the first insight into the hydrocarbonoclastic bacterial communities in the SML of an estuarine area polluted with hydrocarbons. Our findings highlight the importance of SML-adapted hydrocarbonoclastic bacterioneuston as a potential source of new PAH-degrading bacteria (including new pseudomonads) with potential use in the bioremediation of hydrocarbon-polluted ecosystems.

KEY WORDS: Sea surface microlayer · Bacterioneuston · Polycyclic aromatic hydrocarbons · PAH degradation

INTRODUCTION

The surface microlayer (SML) is the interface between the atmosphere and the hydrosphere. This layer is roughly defined as the uppermost millimetre of the water column, and is characterized as a unique environment with distinct chemical and physical processes when compared with the underlying water (UW) (Liss & Duce 1997, Cunliffe & Murrell 2009, Cunliffe et al. 2011).

The formation of the SML results from the accumulation of particles at the air–water interface, establishing a film that extends into the UW (Cunliffe et al. 2011). Recently, the classical model of the SML as a stratified structure with an upper lipid and a lower protein–polysaccharide layer (Hardy 1982) has been reviewed. Wurl & Holmes (2008) reported that the enrichment of transparent exopolymer particles (TEPs) in the SML at several locations around Singapore appears to support the model of the SML first advanced by Sieburth (1983), i.e. a hydrated gelatinous film (Sieburth 1983, Wurl & Holmes 2008, Cunliffe & Murrell 2009, Cunliffe et al. 2011).

The organisms within the SML are known collectively as neuston; the bacterial fraction of the neuston is the bacterioneuston (Franklin et al. 2005). The importance of the SML in the control of environmental processes, and in the exchange of chemicals between the water and the atmosphere (Wurl & Obbard 2004, Obernosterer et al. 2005, Cunliffe & Murrell 2009), has

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raised interest in the effects of the activities of the bacterioneuston in this ecosystem. Cunliffe et al. (2008) found evidence that the diversity of functional genes encoding subunits of methane monoxygenase (mmox) and carbon monoxide dehydrogenase (coxL) is different in the SML when compared with the UW (Cunliffe et al. 2008). There is an emerging consensus that the SML harbours microbial communities distinct from those in the UW (for a full review see Cunliffe et al. 2011); however, there is still a lack of knowledge on both the structure and function of the SML community. For example, due to the chemical nature of the SML, natural and anthropogenic aromatic compounds—e.g. polycyclic aromatic hydrocarbons (PAHs)—can be concentrated up to 500× in this layer in relation to the rest of the water column (Wurl & Obbard 2004). High concentrations of PAHs have been found in the SML at locations chronically polluted with oil hydrocarbons (OH), such as shipping harbours (Cincinelli et al. 2001, Wurl & Obbard 2004). However, so far, no studies have exploited the potential of microorganisms in the SML to degrade PAHs, or have investigated the effect of PAHs on the bacterioneuston. It is reasonable to hypothesize that the increased levels of PAHs in the SML may stimulate the development of hydrocarbonoclastic bacterial populations in this layer. However, studies on the affect of PAH pollution on bacterioneuston are scarce, and none has specifically addressed the hydrocarbonoclastic bacterial populations of the SML.

In order to obtain more information about the bacterioneuston, and the potential of these organisms to degrade PAHs, we: (1) isolated and identified representatives of the hydrocarbonoclastic population in the SML, and (2) screened the isolates for genes encoding enzymes involved in the degradation of PAHs. We also evaluated the effect of OH pollution on the spatial variability of the bacterioneuston along a gradient of contamination in the estuarine system of the Ria de Aveiro in Portugal.

MATERIALS AND METHODS

Study site and sampling. Ria de Aveiro is a branched estuarine ecosystem, also described as a coastal lagoon, located in the northwest coast of Portugal. Samples for the selective isolation of hydrocarbonoclastic bacterioneuston were obtained from the SML in the middle section of the estuary, near a shipping harbour.

In order to characterize the spatial variation of the bacterioneuston, 5 collecting points were chosen for sampling. Extending from the outer segment of the lagoon to the inner section of Canal de Ílhavo, the sites were designated: S1 (40° 40′ 01″ N, 08° 49′ 24″ W) in the outer section, S2 (40°39 29 N, 08°42 12 W) and S3 (40° 38′ 20″ N, 08° 41′ 32″ W) in the middle section (near the shipping harbour), and S4 (40° 37′ 21″ N, 08° 41′ 01″ W) and S5 (40° 35′ 41″ N, 08° 41′ 21″ W) in the inner section of the Canal de Ílhavo (Fig. 1).

Samples were collected from the SML with plexiglass and glass plates. Both plates were 0.25 m wide, 0.35 m long, and 4 mm thick. Prior to sample collection, the plates were rinsed with ethanol and sterile distilled water. The plates were introduced vertically through the SML and withdrawn in the same position. Excess water was allowed to drain for about 5 s. Approximately 5 ml of water were collected each time the plates were introduced into the SML. The water adher-

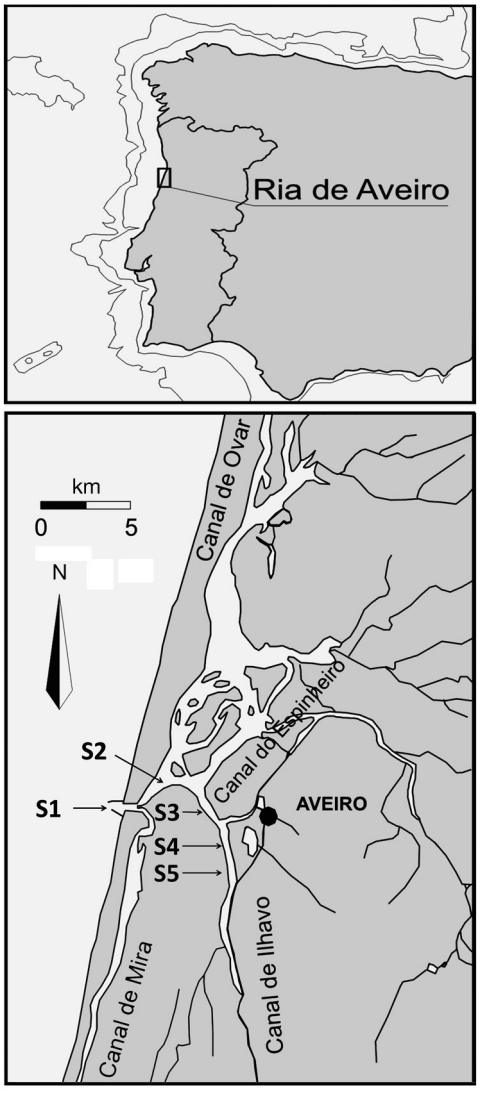

Fig. 1. Ria de Aveiro (Portugal). Sampling sites are indicated with arrows: Stn S1 in the Canal de Navegação, Stns S2 and S3 near the harbour facilities, and Stns S4 and S5 in the Canal de Ílhavo

ing to the plate was subsequently removed from both sides of the plate with a wiper blade system (Harvey & Burzell 1972). The estimated thickness of the collected SML, determined from the volume of collected sample and the area of both sides of the plate, was approximately 60 µm, which is in the range reported by Harvey & Burzell (1972) for glass plate samplers.

Salinity was determined immediately after the sampling with a WTW (Wissenschaftlich Technische Werkstätten) Cond330i/SET.

Quantification of PAHs was performed by gas chromatography–mass spectrometry (gas chromatograph Varian CP-3800 with split/splitless injection and mass spectrometry detector—Ion Trap Saturn 2200) with detection limits between 20 and 40 ng l^{-1}. The result was calculated as the average of 2 sub-samples and expressed in ng l^{-1}.

Enrichment and isolation of PAH-degrading bacteria. A PAH, 2-methylnaphthalene, was used as the sole source of carbon and energy for the enrichment and isolation of PAH-degrading bacteria in a liquid mineral medium (MM). Selective plates were prepared by adding 2% (wt/vol) agarose to MM. The 2-methylnaphthalene (Fluka) was added by spreading ethanol solutions (50 mg ml^{-1}) onto the surface of the solid medium. Inoculation was carried out only when the solvent had evaporated fully, producing a film of PAH on the surface of the medium (Ma et al. 2006).

Bacterioneuston samples for enrichment cultures were collected near the shipping harbour. A volume of 200 ml of SML sample was added to 800 ml of sterile MM with 200 mg of 2-methylnaphthalene (Fluka) in a 1 l sterilized Erlenmeyer flask. The liquid cultures were incubated at room temperature for 2 wk, in the absence of light, on a rotary shaker (90 rpm). After the enrichment, 20 ml of culture were transferred to fresh medium and incubated for the same period of time. After 2 similar subcultures, a serial dilution of the culture was spread onto the MM selective plates. These cultures were incubated at 25°C for up to 14 d, protected from light. Individual isolated colonies were further purified by re-streaking onto new MM selective plates. Control plates without 2-methylnaphthalene were also incubated.

Extraction of DNA. Genomic DNA was extracted from all the strains recovered from the isolation procedure, as previously described by Henriques et al. (2004). Environmental samples were filtered (pore size: 0.2 µm; Poretics Products). Total DNA was extracted in 3 replicates and purified using the Genomic DNA Purification Kit (MBI Fermentas) (Henriques et al. 2004).

Molecular characterization and identification of the PAH-degrading bacterial isolates. For molecular typing of the isolates, a repetitive-sequence PCR using a BOX A1R primer (BOX-PCR fingerprinting) was followed (Rademaker et al. 1998). BOX-PCR profiles were visualized after separation of PCR amplicons by electrophoresis in 2% agarose gel using 1× TAE (Tris-acetate-EDTA) at 100 V for 3 h. The gels were stained with ethidium bromide and digitalized in a Molecular Imager FXTM system (Bio-Rad Laboratories). The band positions were normalized with the GeneRuler™ 1 kb Plus DNA ladder (75–20 000 bp) and analysed with GelCompar I software (Applied Maths) using the pairwise Pearson's product–moment correlation coefficient (r-value). Cluster analysis of the similarity matrices was performed by the unweighted pair-group method (UPGMA) using arithmetic averages.

For isolates displaying distinct BOX-PCR profiles, the 16S rRNA gene was amplified by PCR using the universal bacterial primers U27 and 1492R (Weisburg et al. 1991). PCR products from the amplified 16S rRNA were used as templates in the sequencing reactions—which were carried out using an ABI PRISM_ BigDye_Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Sequences were subjected to BLAST (Basic Local Alignment Search Tool) analysis.

These isolates were also screened for the *Pseudomonas*-specific *gacA* gene using primers GACA-1F and GACA-2 (De Souza et al. 2003). All PCR reagents were purchased from MBI Fermentas unless otherwise indicated.

Detection of the dioxygenase gene. PCR was used for detecting genes that encode enzymes involved in the aerobic degradation of PAHs in the isolated strains. Primers specific for the alpha subunit of the PAH-ring hydroxylating dioxygenases, common to Gram-negative PAH degraders (PAH-RHDα GNF and PAH-RHDα GNR primers), were used to obtain amplicons of ca. 306 bp (Cébron et al. 2008). The PCR products were used as templates in the sequencing reactions. Sequencing was carried out as described in the previous section, and a comparison was made with sequences available in the GenBank database by using the BLAST service to determine their closest relative. All PCR reagents were purchased from MBI Fermentas unless otherwise indicated.

Microbial community structure. *Denaturing-gradient gel electrophoresis* (*DGGE*): DGGE was performed on the *Pseudomonas*-specific *gacA* gene sequence amplified from bacterial isolates and from environmental samples. This technique was also applied to the 16S rRNA gene sequence amplified from environmental samples.

A nested PCR approach was use to amplify the 16S rRNA gene fragments for DGGE analyses. For the first PCR, the universal bacterial primers U27 and 1492R were used to amplify ca. 1450 bp of the 16S rRNA gene

(Weisburg et al. 1991). The amount of template DNA used per reaction was ca. 10 ng. For the second PCR, 1 µl of the product of the first PCR was used as template with bacterial DGGE primers 968F-GC and 1401R (ca. 433 bp) (Nubel et al. 1996).

A nested-PCR approach was also applied to the amplification of *Pseudomonas*-specific *gacA* gene fragments for DGGE analysis (Costa et al. 2007). For the first PCR, primers GACA-1F and GACA-2 were used to amplify ca. 425 bp of the *gacA* gene (De Souza et al. 2003). The amount of template DNA used per reaction was ca. 10 ng. An aliquot of 1 µl of the product of the first PCR was used as the template for a second PCR with primers *gacA*1F-GC and *gacA*2R (Costa et al. 2007). All PCR reagents were purchased from MBI Fermentas unless otherwise indicated.

DGGE analysis was performed with a CBS-DGGE 2401 system (CBS Scientific). The GC-clamped amplicons were applied to a double-gradient polyacrylamide gel containing 6 to 9% acrylamide (Rotiphorese) with a gradient of 30 to 58% of denaturant. The run was performed in Tris-acetate-EDTA buffer (0.5 M Tris-Base, Sigma, 0.05 M EDTA, Sigma; 0.1 M CH3CO2Na, Sigma, pH 8.0) at 60°C at a constant voltage of 220 V for 16 h. The DGGE gels were silver stained (Heuer et al. 2001).

The Shannon index of diversity (H) was use to compare the complexity of the DGGE profiles. The band position and relative intensity (abundance) of each lane (community) were used as parameters to indicate categories (Costa et al. 2006). Parametric analysis of variance (ANOVA) was applied to assess significant differences between samples, provided that data were normally distributed.

Fluorescence in situ hybridization (FISH): The relative abundance of members of the domain *Bacteria* and the *Gammaproteobacteria* in the SML was assessed by FISH (Pernthaler et al. 2001) using Cy3-labeled oligonucleotide probes (MWG Biotech). Triplicate samples (1 ml) were filtered through polycarbonate filters (pore size: 0.2 µm; GE Osmonics), fixed with 4% paraformaldehyde for 30 min and rinsed with 1× PBS and MilliQ water. The filters were stored at room temperature until hybridization. The probes used in this study were EUB338 for the domain *Bacteria* (Amann et al. 1990) and EUB338-II and EUB338-III to cover the phyla Planctomycetes and Verrucomicrobia (Daims et al. 1999). For the *Gammaproteobacteria* we used the probes GAM42a (Manz et al. 1992) and an unlabelled competitor probe specific for the *Betaproteobacteria*. A non-binding probe was also used as a control for non-specific binding (Karner & Fuhrman 1997). Samples were examined with a Leitz Laborlux K microscope equipped with the appropriate filter sets for fluorescence from 4,6-diamidino-2-phenylindole (DAPI) and CY3. At least 10 fields were counted per replicate of sample. In addition to the relative abundance of organisms of the domain *Bacteria* and of the *Gammaproteobacteria*, it was also possible to obtain a measure of total microorganisms with the DAPI counts. All reagents were purchased from Fluka, except when otherwise indicated.

Total microorganisms (DAPI counts) were tested for normality (Kolmogorov–Smirnov test) before the comparison of means. Parametric ANOVA was performed, provided that data were normally distributed.

RESULTS AND DISCUSSION

Physical and chemical parameters

Salinity in the analysed SML samples ranged between 31.4% (Stn S5) and 36.0% (Stn S1). Salinity is an important factor that influences the structure and composition of prokaryotic assemblages in estuarine environments (Bouvier & del Giorgio 2002, Henriques et al. 2004). However, in this study, the variation of salinity along the estuarine profile was very small, and thus does not seem to influence the distribution of bacterial communities.

The PAH analyses revealed that the composition and concentration of PAHs varied along the estuary (Table 1). Concentrations ranged from below the limit of detection of the method to 14 ± 5 ng l^{-1} of naphthalene. Stn S1 showed the lowest concentration of PAH (anthracene: 7.3 ± 0.2 ng l^{-1}). In Stns S2 and S3, located near the shipping harbour, naphthalene reached the highest concentrations (S2: 14 ± 5 ng l^{-1}; S3: 9.4 ± 1.5 ng l^{-1}). Naphthalene is the simplest of the oil PAH compounds; it is the most easily degradable and is highly volatile (Sporstol et al. 1983). The 3-ring PAH compounds anthracene (6.9 ± 0.2 ng l^{-1}) and phenanthrene (8.1 ± 1.7 ng l^{-1}) were detected at the Stns S2 and S3, respectively. At Stn S4, fluorene (9.4 ± 1.5 ng l^{-1}) was the only PAH detected. Stn S5 showed the largest spectrum of PAH compounds (phenanthrene 5.2 ± 0.1 ng l^{-1}, fluoranthrene 8.5 ± 1.1 ng l^{-1}, benzo[a]anthracene 5.1 ± 0.8 ng l^{-1} and benzo[k]fluoranthene 8.1 ± 0.6 ng l^{-1}).

PAH-degrading isolates: characterization of isolates

To isolate organisms from hydrocarbonoclastic bacterioneuston populations, in the first phase of this study, our sampling efforts were focused on the region of the Ria de Aveiro near the harbour facilities (see Fig. 1, Stns S2 and S3). This area showed the highest levels of contamination by low-molecular-weight

Table 1. Concentrations of individual polycyclic aromatic hydrocarbons (PAHs) in the sea surface microlayer at Stns S1, S2, S3, S4 and S5. LD = limit of detection, BDL = below limit of quantification. Values are means ± SD

PAH	PAH concentrations (ng l^{-1}) at site				
	LD	S1	S2	S3	S4	S5
Naphthalene	8.8	BDL	14 ± 5	9.4 ± 1.5	BDL	BDL
Acenaphthylene	5.0	BDL	BDL	BDL	BDL	BDL
Acenaphthene	1.7	BDL	BDL	BDL	BDL	BDL
Fluorene	4.1	BDL	BDL	BDL	9.4±1.5	BDL
Phenanthrene	1.9	BDL	BDL	8.1 ± 1.7	BDL	5.2 ± 0.1
Anthracene	6.2	7.3 ± 0.2	6.9 ± 0.2	BDL	BDL	BDL
Fluoranthrene	5.7	BDL	BDL	BDL	BDL	8.5 ± 1.1
Pyrene	3.9	BDL	BDL	BDL	BDL	BDL
Chrysene	7.5	BDL	BDL	BDL	BDL	BDL
Benz[a]anthracene	3.7	BDL	BDL	BDL	BDL	5.1 ± 0.8
Benzo[k]fluoranthene	8.0	BDL	BDL	BDL	BDL	8.1 ± 0.6
Benzo[b]fluoranthene	9.5	BDL	BDL	BDL	BDL	BDL
Benzo[a]pyrene	5.2	BDL	BDL	BDL	BDL	BDL
Indeno[1.2.3-cd]pyrene	28	BDL	BDL	BDL	BDL	BDL
Dibenzo[a.h]anthracene	38	BDL	BDL	BDL	BDL	BDL
Benzo(ghi)perylene	33	BDL	BDL	BDL	BDL	BDL

PAHs. These PAHs constitute a significant fraction of crude oil and petroleum products and are often used as indicators of recent or chronic contamination with petroleum hydrocarbons (Sporstol et al. 1983).

The PAH 2-methylnaphthalene, a common constituent of crude oil and fossil fuels, was used in this study as the sole source of carbon and energy for the enrichment and isolation of strains of the hydrocarbonoclastic bacterioneuston (Kasai et al. 2002). The isolation procedure recovered 42 bacterial isolates that were able to use 2-methylnaphthalene as the sole source of carbon. Genotypic diversity of the isolates, assessed by the whole-genome BOX-PCR fingerprint method (Rademaker et al. 1998), assigned the isolates to 29 different genotypes. The 16S gene sequence analysis showed that all hydrocarbonoclastic isolates were Gram-negative and had high homology with 6 different taxa: *Pseudomonas, Klebsiella*, *Serratia*, *Acinetobacter*, *Rhizobium* and *Vibrio* (Table 2).

Pseudomonas and *Klebsiella* were preponderant, accounting to 34.5 and 31.0%, respectively, of the total representative isolates (10 isolates were assigned to the genus *Pseudomonas* and 9 to *Klebsiella*). These results point to a high genotypic diversity in the populations of hydrocarbonoclastic *Pseudomonas* in the SML (Table 2). Members of this genus have a remarkable ability to degrade a wide range of organic pollutants, including PAHs, halogenated derivates and recalcitrant organic residues (Johnsen et al. 1996, Bhattacharya et al. 2003). Besides their capacity to degrade toxic compounds, species such as *P. putida* can efficiently produce a range of valuable compounds with industrial uses (Wackett 2003). These abilities, together with enhanced stress resistance, amenability to genetic manipulation and suitability as a host for heterologous expression, makes strains of *Pseudomonas* particulary useful for biotechnological applications (Puchałka et al. 2008).

As well as contamination with PAHs, the estuarine system of the Ria de Aveiro is also subject to chronic contamination with domestic sewage (Cunha et al. 2000). This might explain the large number of isolates related to the family *Enterobacteriaceae*, such as *Klebsiella* and *Serratia.* Nonetheless, the catabolic capacity of *Klebsiella* strains to degrade hydrocarbons, including PAHs, has been previously described. A recent study suggested that *Klebsiella* can be an important part of the OH-degrading bacterial groups in estuarine areas exposed to sewage contamination (Rodrigues et al. 2009).

Acinetobacter and *Rhizobium* were also part of the culturable fraction of PAH degraders in the SML. Many environmental strains of *Acinetobacter* with hydrocarbon-degrading capacities have been isolated in terrestrial and marine environments (Vanbroekhoven et al. 2004, Rodrigues et al. 2009). Reports of the presence of *Rhizobium* in PAH-contaminated soils, and its hydrocarbon-degrading metabolism, are also available (Poonthrigpun et al. 2006). One of the isolates was identified as *Vibrio*, which has also been reported as a PAH degrader in marine environments (Hedlund & Staley 2001).

PAH-degrading isolates: detection of dioxygenase genes

In the present study, an attempt was made to detect genes encoding the enzyme system PAH-RHDα (involved in the initial step in bacterial metabolism of PAH) in the bacterioneuston isolates. All isolates, including those having similar BOX-PCR profiles, were screened. Although the isolates were cultivated with 2-methylnaphthalene as the sole source of carbon, and reference to the PAH-degrading capacity was obtained from the literature for all the identified genera (Hedlund & Staley 2001, Vanbroekhoven et al. 2004, Ma et al. 2006, Poonthrigpun et al. 2006, Rodrigues et al. 2009), PAH-RHDα gene sequences were detected in only 2 of the isolates. A positive

Table 2. Analysis of the 16S rRNA gene sequences from the isolated strains, and their tentative assignment to different taxonomic categories. GenBank sequence accession numbers of the respective isolate. Number of genotypes clustered in the same BOX group. Blast-N max identity classification. GenBank sequence accession number of most closely related bacterial sequence(s)

Isolate code	Sequence accession no.	BOX group	BLAST-N identity	Phylogenetic similarity	Accession no.
1	GU935753	1	*Serratia* sp.	99	EF111121.1
2	GU935754	4	Uncultured *Klebsiella* sp.	95	EU344923.1
5	GU935755	2	*Acinetobacter johnsonii*	98	FJ263917.1
6	GU935756	1	*Serratia* sp.	97	EU109729.1
7	GU935757	1	*Klebsiella* sp.	93	DQ923489.1
10	GU935758	1	*Pseudomonas* sp.	96	FJ424813.1
12	GU935759	1	Uncultured *Pseudomonas* sp.	96	EU705005.1
13	GU935760	1	*Pseudomonas* sp.	98	FN429930.1
14	GU935761	1	*Pseudomonas* sp.	97	AB088548.1
15	GU935762	1	*Pseudomonas* sp.	94	DQ839561.1
16	GU935763	2	*Klebsiella terrigena*	95	AF129442.1
19	GU935764	1	*Serratia proteamaculans*	97	AY559499.1
20	GU935765	1	*Klebsiella* sp.	97	EU545402.1
21	GU935766	1	*Acinetobacter johnsonii*	98	AM184278.1
22	GU935767	1	*Rhizobium* sp.	99	EF599760.1
23	GU935768	1	Uncultured *Klebsiella* sp.	97	EU344923.1
24	GU935769	1	*Pseudomonas* sp.	97	FJ789687.1
25	GU935770	1	Uncultured *bacterium*	99	GQ069755.1
26	GU935771	1	*Serratia* sp.	96	EF111121.1
27	GU935772	1	Uncultured *Pseudomonas* sp.	98	DQ295987.1
28	GU935773	3	*Klebsiella* sp.	95	DQ229100.1
31	GU935774	2	Uncultured *Klebsiella* sp.	97	EF679185.1
32	GU935775	1	Uncultured *Pseudomonas* sp.	95	DQ295987.1
36	GU935776	4	*Klebsiella ornithinolytica*	100	AF129441.1
37	GU935777	2	*Pseudomonas* sp.	97	FN429930.1
40	GU935778	1	*Pseudomonas* sp.	99	AB506040.1
41	GU935779	1	*Rhizobium* sp.	98	EU741078.1
42	GU935780	2	*Klebsiella* sp.	97	EU888474.1
44	GU935781	*1*	*Vibrio proteolyticus*	93	DQ995521.1

PCR amplification product was obtained only for isolates #10 (*Pseudomonas* spp.) and #25, an unknown member of the family *Enterobacteriaceae*. However, the lack of amplification does not exclude the presence of dioxigenases in the other isolates; instead, this result may indicate the presence of novel genes encoding PAH-degrading enzymes in these hydrocarbonoclastic organisms. Moreover, because we did not monitor the complete cleavage of the benzene rings in this study, it cannot be excluded that the isolated strains oxidize only the methyl group of 2-methylnaphtalene. It is possible that the *ndo*-negative isolates use the methyl group as a source of carbon and energy without cleaving the benzene ring.

Phylogenetic analysis, by BLAST, of our PAH-RHDα gene sequences and their closest relative showed that both PAH-RHDα genes clustered together with the archetypal *nahAc* gene carried by the plasmid pNAH20 (Yen & Gunsalus 1982, Heinaru et al. 2000, Sota et al. 2006) (Fig. 2). As identical PAH-RHDα genes with high homology to those in naphthalene-degrading plasmids occur in different bacterial families, it is reasonable to speculate that horizontal gene transfer may also play a role in the occurrence and spreading of hydrocarbonoclastic capacity in the SML. Horizontal exchange of genes encoding the degradation of xenobiotic compounds is a common phenomenon in biofilms (Singh et al. 2006). A preponderance of biofilm-growing cells in the SML could favour ecological processes that occur in communities with cells in close proximity, such as horizontal gene transfer and quorum sensing (Cunliffe & Murrell 2009).

The effect of sampling site on the community structure of the bacterioneuston

Whole-community and *Pseudomonas* structural diversity

Because most microorganisms are refractory to cultivation, culture-independent methods are fundamental in the characterization of the structure of microbial communities in the environment (Amann et al. 1995).

DGGE was used to assess the structural diversity of the overall bacterial community. The banding pattern

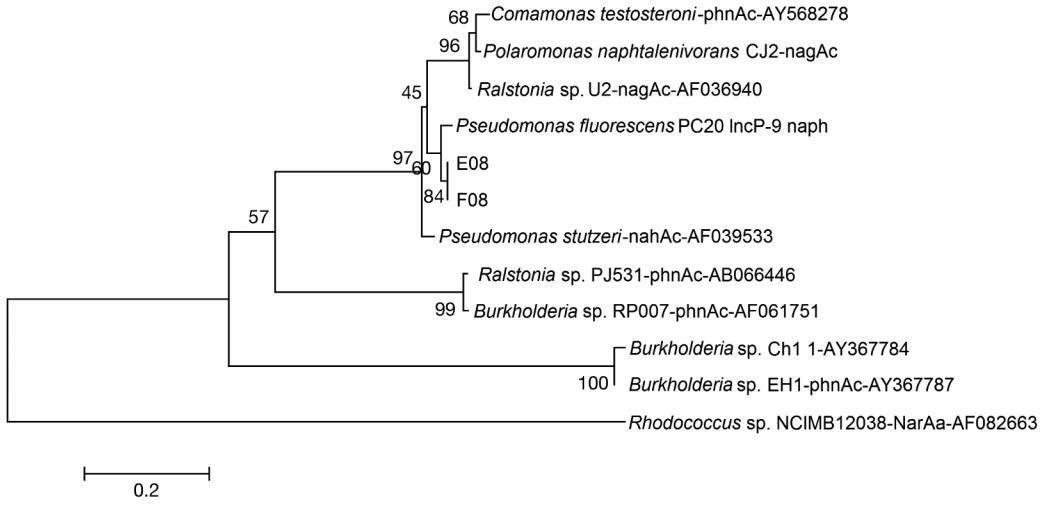

Fig. 2. Phylogenetic relationships between dioxygenase-encoding genes amplified from the isolated strains (E08 was obtained from Clone 25, F08 from Clone 10). The tree was constructed by using the neighbor-joining method and bootstrapping analysis (1000 repetitions). Numbers on the branches indicate percentages of bootstrap values. Scale bar represents the percentage of amino acid divergence

analysis of the PCR-amplified 16S rRNA gene showed a large number of equally abundant bands in bacterioneuston communities from all the sampling sites (Fig. 3). The Shannon diversity index revealed no significant differences between sites (Table 3). This analysis suggests that the dominant bacterial communities are very similar in the SML of different sampling sites. Earlier studies, using DGGE, found that the dominant bacterioneuston communities were similar in 2 different areas of the Blyth estuary (Cunliffe et al. 2008). Recently, this author also reported that bacterioneuston communities from 2 different areas, close to the Hawaiian Island of Oahu, were more similar to each other than to their subjacent bacterioplankton communities (Cunliffe et al. 2009).

Due to the preponderance of *Pseudomonas* among the bacteria isolated from the PAH-enrichment culture, and because of the recognized ability of the organisms of this genus to degrade PAHs, we paid particular attention to the diversity of a *Pseudomonas* genetic marker in the SML at all sites. The *Pseudomonas*-specific *gacA* gene was used as a genetic marker for this genus. This gene influences the production of several secondary metabolites in *Pseudomonas* sp., and it can be used as a complementary genetic marker for detecting bacteria of this genus in environmental samples (De Souza et al. 2003).

Interestingly, contrary to what was observed for the bacterial fingerprints, analysis of the *Pseudomonas* community showed a significant ($p_{ANOVA} < 0.05$) increase in diversity (Table 3) from the outer (Stn S1) to the middle (Stn S3) and inner (Stn S5) sections of the estuary, following the concentrations of PAH and organic matter. This pattern may explain the high abundance of *Pseudomonas* strains recovered from the SML-PAH enrichments obtained in the area with higher levels of lighter (low-molecular-weight) PAHs. As previously mentioned, members of this genus are known for their ability to metabolize light PAH compounds and other monoaromatic hydrocarbons.

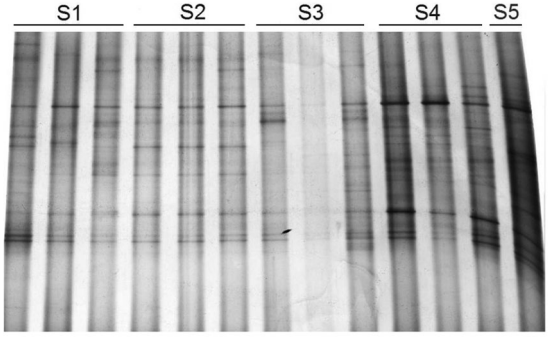

Fig. 3. Denaturing-gradient gel electrophoresis fingerprints of 16S rRNA gene fragments amplified from bacterioneuston obtained from the surface microlayer at Stns S1, S2, S3, S4 and S5

The *gacA* DGGE pattern of the isolated strains, and the *gacA* profile of whole bacterioneuston, show a common band with the mobility exhibited by isolate #27 (Fig. 4). This band is present in the bacterioneuston from Stns S1, S2 and S3, but is absent in samples from Stns S4 and S5, at the inner section of the estuary. Interestingly, isolate #27 showed a high

Table 3. Mean ± SD of the Shannon diversity indices calculated from denaturing-gradient gel electrophoresis (DGGE) profiles of bacterial 16S rRNA and the *Pseudomonas*-specific *gacA* gene in bacterioneuston from the surface microlayer at Stns S1, S2, S3, S4 and S5

DGGE profile	Shannon diversity indices at Stn				
	S1	S2	S3	S4	S5
Pseudomonas-specific *gacA*	0.9 ± 0.2	1.3 ± 0.5	2.0 ± 0.2	2.0 ± 0.2	2.2 ± 0.2
Bacterial 16S rRNA	2.36 ± 0.29	2.24 ± 0.24	2.44 ± 0.29	2.22 ± 0.37	2.58[a]

[a]It was possible to obtain only 1 replicate of bacterial 16S rRNA at Stn S5

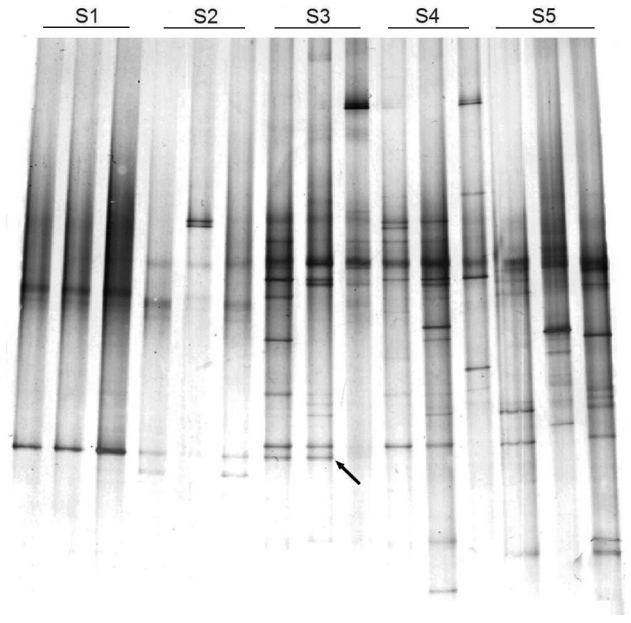

Fig. 4. Denaturing-gradient gel electrophoresis (DGGE) fingerprints of *Pseudomonas*-specific *gacA* gene fragments amplified from bacterioneuston in the surface microlayer collected at Stns S1, S2, S3, S4 and S5. The position of the arrow corresponds to the melting behaviour of isolate #27, classified as an uncultured *Pseudomonas* sp.

homology with an uncultured *Pseudomonas* which has been linked to the transformation of organic compounds in coastal seawater (Mou et al. 2007). These results suggest that the distribution of *Pseudomonas* populations may also be related to the quality and composition of lipophilic organic compounds in the SML.

Microbial abundance overall and in the *Gammaproteobacteria*

FISH was applied in this study to assess the spatial variability in the total abundance of microorganisms and also for quantifying members of the domain *Bacteria* and the *Gammaproteobacteria* in the SML (Amann et al. 1990, Manz et al. 1992, Karner & Fuhrman 1997, Daims et al. 1999, Pernthaler et al. 2001).

The overall abundance of microorganisms estimated from DAPI counts ranged from $2.3 \pm 0.2 \times 10^6$ cells ml^{-1} at Stn S1, to $5.0 \pm 0.9 \times 10^6$ cells ml^{-1} at Stn S5, defining a significant ($p_{ANOVA} < 0.05$) gradient of enrichment from the outer to the inner sections of the estuary (Fig. 5). The total cell number is within the range (4.9×10^5 cells ml^{-1} to 6.2×10^6 cells ml^{-1}) observed in the Ria de Vigo estuary (Zdanowski & Figueiras 1997). The pattern of variation that we detected corresponds, in general, to the structure of an estuary in which bacterial abundance and phytoplankton biomass are maximal at the intermediate sections (Wright & Coffin 1983, Fuks et al. 1991, Cunha et al. 2003). This distribution is probably related to the quality (lability) of the available organic matter along the estuary.

The relative abundance of organisms of the domain *Bacteria* varied between 50.4 ± 3.6% at Stn S2 and 66.7 ± 9.2% at Stn S4 (Fig. 5). The proportion of cells hybridized by the *Bacteria* probes is within the 60% average reported for the Delaware estuary (Kirchman et al. 2005). The missing percentage could be related to other groups not covered by this study. By applying CARD-FISH, one study found that up to 37% of the total cell numbers recovered from the SML in high mountain lakes hybridized with *Archaea*-specific probes (Auguet & Casamayor 2008). The relative abundance of organisms of the domain *Bacteria* did not show a defined trend of spatial variation along the estuary.

On the contrary, the highest value for the relative abundance of organisms of the *Gammaproteobacteria* (19.7 ± 1.0%) was found in the SML at Stn S3 (Fig. 5), where naphthalene and phenantrene were detected. *Gammaproteobacteria* were selected as a target for FISH analysis because this group includes many of the PAH-degrading genera, such as *Alcanivorax*, *Cycloclasticus*, *Pseudomonas*, *Oleiphilus*, *Oleispira* and *Thalassolituus* (Watanabe 2001, Head et al. 2006). Although the abundance and distribution of microorganisms in marine and estuarine waters is influenced by complex biotic and abiotic interactions

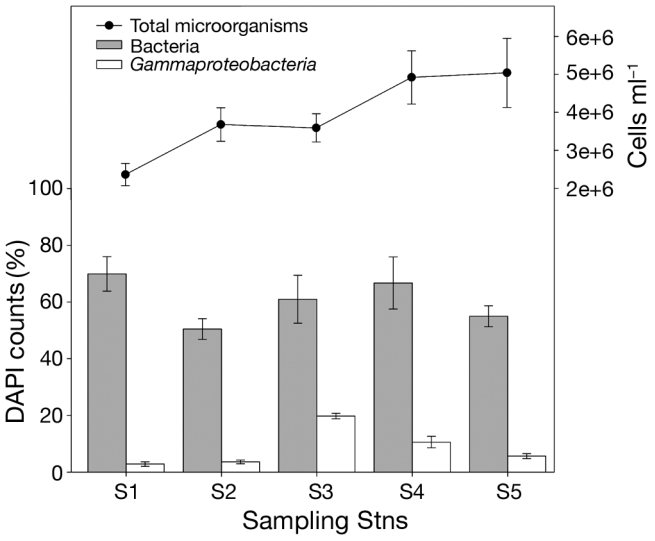

Fig. 5. Variations in total prokaryote abundance (as counted by 4 ,6-diamidino-2-phenylindole [DAPI] staining) and relative abundance of Bacteria and *Gammproteobacteria* (% of DAPI counts) determined by fluorescence *in situ* hybridization in bacterioneuston in the surface microlayer collected at Stns S1, S2, S3, S4 and S5. (In the scale: 2e+6 = 2 × 10^6 etc.)

(Shiah & Ducklow 1995), the high concentration of low-molecular-weight PAHs, generally easily degraded (Yamada et al. 2003), at sampling Stn S3 can be related to the high relative abundance of *Gammaproteobacteria* at this site. Exposure to hydrocarbons has been shown to decrease bacterial diversity, as a result of selection in favour of PAH-degrading bacteria (Röling et al. 2002, Castle et al. 2006). Nonetheless, other sources of organic carbon might also have influenced the distribution of this group. The preference of *Gammaproteobacteria* for high concentrations of nutrients has been previously reported, and peaks of abundance appeared to be related to particular point sources of nutrients (Bouvier & del Giorgio 2002, Henriques et al. 2004).

CONCLUSIONS

To our knowledge, we are the first to isolate and characterize hydrocarbonoclastic strains of bacterioneuston from estuarine SML exposed to chronic OH contamination; the most dominant isolates belonged to the genus *Pseudomonas* (members of the *Gammaproteobacteria*).

The SML is the first marine habitat to be in contact with hydrophobic OH pollutants after an oil spill accident. The possibility of using hydrocarbonoclastic bacterioneuston isolates in a bioremediation strategy for the rapid degradation of oil slicks should be examined in future studies. The SML has the potential to be a natural 'seed bank' that can be useful for isolating and 'domesticating' novel bacteria for PAH degradation. The ability of the hydrocarbonoclastic bacterioneuston to withstand and degrade toxic compounds, together with its capacity to survive in this environment, make the isolates recovered in this study interesting candidates for future research on *in situ* SML bioremediation.

The cultivation-independent analyses (FISH counts and *Pseudomonas*-DGGE) agreed with the isolation results. These findings indicate that the abundance of *Gammaproteobacteria* and the diversity of *Pseudomonas* in the bacterioneuston have undergone significant changes in areas contaminated with elevated levels of OH in the Ria de Aveiro. However, the ecological role of the bacterioneuston in the degradation of hydrophobic OH pollutants is still unclear. Future work should focus on more in-depth functional analyses of genes whose expression/repression are most likely related to SML contamination by OH compounds.

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