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PAH Degrading Bacteria in an Estuarine System

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Abstract—Microorganisms play an essential role in the transformation of polycyclic aromatic hydrocarbons (PAH), and biological degradation is the main process of natural decontamination in ecosystems. In this study we focus on the potential for PAH degradation of the bacterial communities present in the surface microlayer. For this purpose we compared bacterioneuston community from the surface microlayer (SML) and bacterioplankton community in underlying water (UW), in order to gain information on the spatial variability along a gradient of PAH contamination in the estuarine system Ria de Aveiro (Portugal). Fluorescence *in situ* hybridization was used to study total microorganism abundance and relative abundance of *Bacteria* and *gamma-Proteobacteria*. Enrichment cultures were used to isolate hydrocarbon-degrading microorganisms in the SML with naphthalene as sole carbon source. Prokaryotes abundance was similar in the SML and in UW. The relative abundance of the *Bacteria* domain was also similar in SML and UW. However, the *gamma-Proteobacteria* subclass, was more abundant in the estuarine sections. *Pseudomonas* and *Klebsiella* were well represented (34.5% and 31.0%, respectively) among PAH hydrocarbon-degrading isolates cultivated from SML samples.

Keywords: sea surface microlayer, bacterioneuston, polycyclic aromatic hydrocarbons, PAH degradation

INTRODUCTION

The sea surface microlayer (SML) represents the interface between the atmosphere and the hydrosphere. Organisms within the SML are known as neuston, and the community of bacteria present within this neuston layer is named bacterioneuston (Franklin *et al*., 2005). Due to the unique chemical composition of the SML, hydrophobic organic compounds of natural and anthropogenic origin, such as polycyclic aromatic hydrocarbons (PAH) are enriched in this layer when compared with the underlying water (UW) (Wurl and Obbard, 2004). High concentrations of PAH have been found in the SML at locations associated with anthropogenic coastal activities, particularly shipping harbours (Cincinelli *et al*., 2001; Wurl

and Obbard, 2004). Microcosm experiments have reported a decrease in bacterial diversity following exposure to oil hydrocarbons, in result of a selection for hydrocarbon degrading bacteria (Roling *et al.*, 2002; Castle *et al.*, 2006). It is possible that the PAH contamination in the SML may activate the bacterial communities that are able to metabolize PAH compounds. Thus, the bacterioneuston might play a significant role in the PAH decontamination of aquatic environments.

The main objective of this work was the characterization of the estuarine bacterioneuston and bacterioplankton communities along an estuarine gradient of eutrophication and contamination of hydrocarbons.

MATERIALS AND METHODS

Water sampling and salinity measurement

Ria de Aveiro is a branched estuarine ecosystem, also described as a coastal lagoon, located in the northwest coast of Portugal. Sampling was conducted along a longitudinal profile, extending from the outer section of the lagoon to the inner section of Canal de Ílhavo, one of the main branches of the estuary. The sites were designated as S1 (N 40°40′01, W 08°49′24) in the outer section, S2 (N 40°39′29, W 08°42′12) and S3 (N 40°38′20, W 08°41′32), in the mid section, located near a shipping harbour, S4 (N 40°37′21, W 08°41′01) and S5 (N 40°35′41, W 08°41′21) in the inner section of canal de Ílhavo (Fig. 1).

SML samples were collected with a Plexiglas and a glass plate according to the method described by Agogué *et al.* (2004). Samples from the UW were collected by submerging a glass bottle and opening it at a depth of 0.2 m. PAH in the SML samples were analysed 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. The result was calculated as the average of two sub-samples and expressed in ng L^{-1}.

Salinity was determined with a WTW (Wissenschaftlich TechnischeWerkstätten, Germany) Cond330i/SET conductivimeter.

Fluorescence in situ hybridization (FISH)

The relative abundance of *Bacteria* and *gamma-Proteobacteria* in the SML and in UW was assessed by FISH using the Cy3-labeled oligonucleotide probes (MWG Biotech) as described previously by Pernthaler *et al.* (2001). Samples (1 mL) were filtered through 0.22 μm polycarbonate filters (GE Osmonics). The probes used in this study for the *Bacteria* domain were EUB338 (Amann *et al.*, 1990), EUB338-II and EUB338-III to cover the *Planctomycetes* and *Verrucomicrobia* phyla (Daims *et al.*, 1999). For *gamma-Proteobacteria* subclass, the probes used were GAM42a (Manz *et al.*, 1992) and an unlabelled competitor probe specific for *beta-Proteobacteria*. Total DAPI counts were used as an estimate of total prokaryote abundance. All reagents were purchased from Fluka,

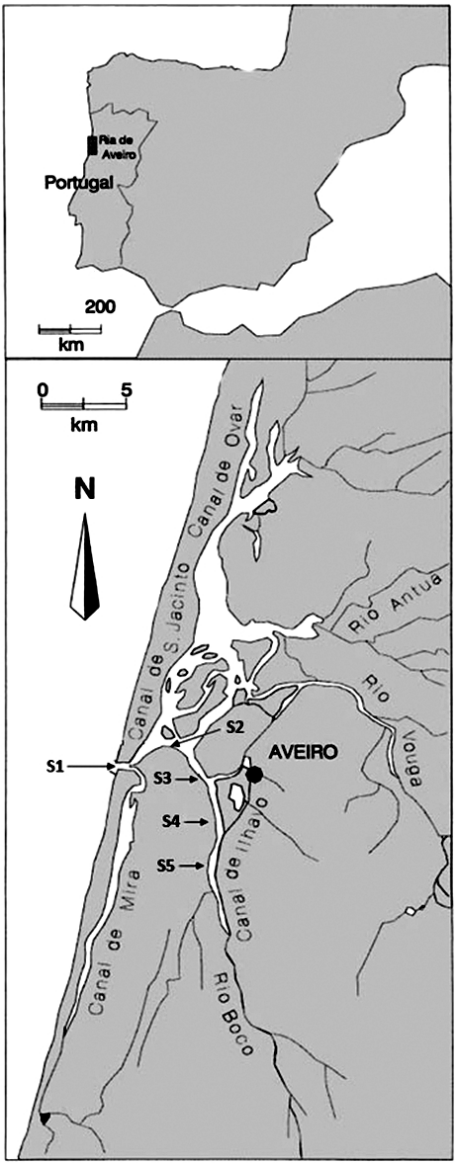

Fig. 1. Ria de Aveiro (Portugal); sampling sites indicated with arrows: S1 in Canal de Navegação, S2 and S3 near the harbour facilities, S4 and S5 in Canal de Ílhavo.

except when otherwise indicated.

Total prokaryote abundance (DAPI counts), the relative abundance of the *Bacteria* domain and of the *gamma-Proteobacteria* subclass as percentages of total DAPI counts were tested for normality (Kolmogorov-Smirnov test) before

the comparison of means. Parametric analysis of variance (ANOVA) was performed for normally distributed data.

Enrichment and isolation of the PAH-degrading bacteria

2-Methylnaphthalene was used as the sole carbon source for the enrichment and isolation of the PAH-degrading bacteria in a liquid mineral medium (MM) (Ma *et al.*, 2006).

Water from the SML collected at S3 site, next to the shipping harbour, was used as inoculum. A volume of 200 mL of SML was added to 800 mL of sterile MM medium in a 1 L sterilized Erlenmeyer with 200 mg of 2-methylnaphthalene (Fluka). The culture was incubated at room temperature for two weeks on a rotary shaker operating at 90 rpm. After the enrichment, 20 mL of the culture were transferred to fresh medium and further incubated. After two subcultures, a serial dilution of the culture was spread on plates of selective medium. Plates of selective solid medium were prepared by adding 2% (wt/vol) agarose to MM. The hydrocarbon 2-methylnaphthalene (Fluka) was added by spreading ethanol solutions on the plates. Inoculation was performed after the solvent was totally evaporated producing a film of the hydrocarbon on the plate surface (Ma *et al.*, 2006). These cultures were incubated at room temperature for fourteen days. Individual isolated colonies were purified on the same MM selective plates.

Molecular characterization and identification of the PAH-degrading bacterial isolates

DNA extraction was performed as described by Henriques *et al.* (2004). For the molecular typing of the isolates, a repetitive sequence PCR using a BOX A1R primer (BOX-PCR fingerprinting) was performed (Rademaker *et al.*, 1998). For isolates with distinct BOX-PCR profiles, the 16S rRNA gene was amplified by PCR using the universal bacterial primers U27 and 1492R (Weisburg *et al.*, 1991) and sequenced by using an ABI PRISM BigDye_Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA). Sequences were compared with sequences available in the GenBank database by using the BLAST (Basic Local Alignment Search Tool) service to determine their closest relative.

RESULTS

Physico-chemical analyses

Salinity ranged between 31.1% and 36.0%, being the lowest value registered in the UW of the site S5, and the highest value registered at site S1.

Values of PAH concentration in the SML are presented in Table 1. PAH concentration ranged between values below the limit of detection of the method and 14 ± 5 ng L^{-1} of naphthalene. Site S1 registered the lowest concentration of PAH. At this site, only anthracene was detected (7.3 ± 0.2 ng L^{-1}). At sites S2 and S3, naphthalene, a low molecular weight PAH, reached the highest concentration

Table 1. Concentrations of individual PAH in the sea surface microlayer (SML) at sites S1, S2, S3, S4 and S5. (LQ - Limit of quantification; BLQ - Below the limit of quantification).

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

(I2 - 14 ± 5 ng L^{-1}; I3 - 9.4 ± 1.5 ng L^{-1}). The only PAH detected at S4 was fluorene (9.4 ± 1.5 ng L^{-1}). Site S5 presented the largest spectrum of detected PAH, including benzo[a]anthracene (5.1 ± 0.8 ng L^{-1}) and benzo[k]fluorantene (8.1 ± 0.6 ng L^{-1}), two high molecular weight PAH.

Bacterial abundance (FISH)

Total prokaryote abundance (Fig. 2A) estimated from DAPI counts ranged from $2.3 \pm 0.3 \times 10^9$ cells L^{-1} in UW at site S1 to $5.1 \pm 0.4 \times 10^9$ cells L^{-1} in UW at site S5, defining a clear gradient of enrichment from the outer to the inner sections of the estuary. Significant differences (ANOVA < 0.05) between the SML and UW in terms of total microorganisms abundance were not found.

The relative abundance of the *Bacteria* domain (Fig. 2B) varied between 48.2 ± 6.4% in the UW at the sample site S4, and 69.9 ± 6.1% in the SML at site S1. A consistent pattern of variation along the longitudinal profile could not be defined.

The relative abundance of *gamma-Proteobacteria* (Fig. 2C) varied between 1.8 ± 1.4%, in UW at site S2, and 19.7 ± 1.0%, in the SML at site S3. The variation of the relative abundance of this group along the longitudinal profile showed an increase from the outer to the mid-inner sections of the estuary. Significant differences (ANOVA < 0.05) between the SML and UW were found in sites S3 and S5, being the relative abundance 1.3 and 1.4 times higher in the SML than in the UW, respectively.

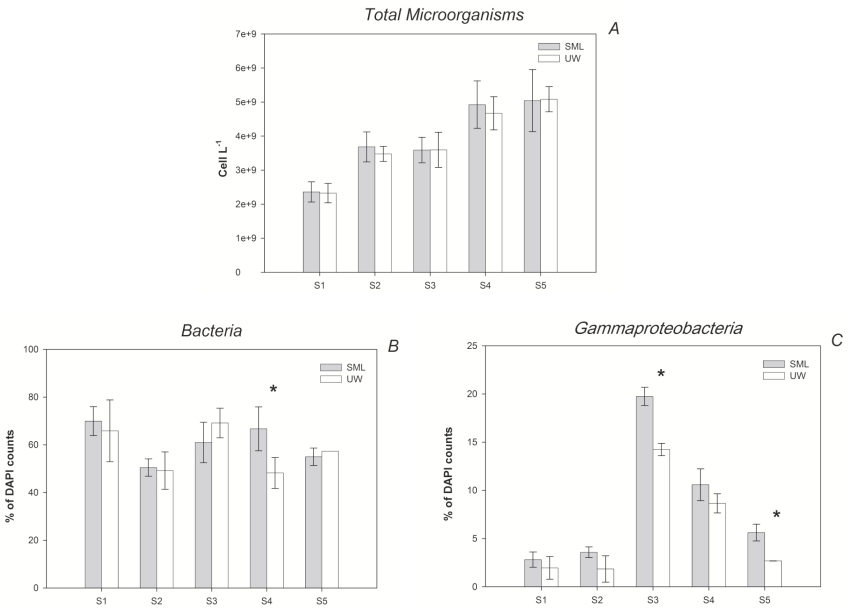

Fig. 2. Variations in total prokaryote abundance and in relative abundance of specific groups determined by FISH in bacterioneuston (SML) and bacterioplankton (UW) communities at sites S1, S2, S3, S4 and S5. Significant differences between the two communities are marked with ∗.

Characterization of putative PAH-degrading isolates

From the isolation procedure, 42 bacterial isolates able to use methylnaphthalene as sole carbon source were retrieved. BOX-PCR analysis assigned all the isolates to 29 different clusters (Table 2).

The 29 representatative isolates were assigned with high confidence to six genera: *Pseudomonas*, *Klebsiella*, *Serratia*, *Acinetobacter*, *Rhizobium* and *Vibrio*. *Pseudomonas* and *Klebsiella* were dominant accounting for 34.5% and 31.0% of the total representative isolates (10 isolates assigned to *Pseudomonas* and 9 assigned to *Klebsiella*). *Serratia* accounted for 13.8% of the total representative isolates (4 isolates) and *Acinetobacter* and *Rhizobium* accounted 6.9% (2 isolates each) of the isolates. One isolate showed similarity (93%) with *Vibrio proteolyticus*. One was related to uncultured bacteria with 99% similarity with strains of *Enterobacteriaceae* family.

DISCUSSION

Bacterioneuston and bacterioplankton in contaminated estuarine waters

This study reveals a gradient of enrichment of total number of prokaryotic cells from the mouth to the inner sections of the estuary. This pattern corresponds

Table 2. Analysis of the 16S rRNA gene sequences from the isolated strains, and their tentative assignment into different taxonomical categories.

[a]Box representative	[b]Box group	Closest phylogenetic relative		
		BLAST-N identity	%	[c]Accession No.
1	1	*Serratia* sp.	99	EF111121.1
2	4	Uncultured *Klebsiella* sp.	95	EU344923.1
5	2	*Acinetobacter johnsonii*	98	FJ263917.1
6	1	*Serratia* sp	97	EU109729.1
7	1	*Klebsiella* sp.	93	DQ923489.1
10	1	*Pseudomonas* sp.	96	FJ424813.1
12	1	Uncultured *Pseudomonas* sp.	96	EU705005.1
13	1	*Pseudomonas* sp.	98	FN429930.1
14	1	*Pseudomonas* sp.	97	AB088548.1
15	1	*Pseudomonas* sp.	94	DQ839561.1
16	2	*Klebsiella terrigena*	95	AF129442.1
19	1	*Serratia proteamaculans*	97	AY559499.1
20	1	*Klebsiella* sp.	97	EU545402.1
21	1	*Acinetobacter johnsonii*	98	AM184278.1
22	1	*Rhizobium* sp.	99	EF599760.1
23	1	Uncultured *Klebsiella* sp.	97	EU344923.1
24	1	*Pseudomonas* sp.	97	FJ789687.1
25	1	Uncultured bacterium	99	GQ069755.1
26	1	*Serratia* sp.	96	EF111121.1
27	1	Uncultured *Pseudomonas* sp.	98	DQ295987.1
28	3	*Klebsiella* sp.	95	DQ229100.1
31	2	Uncultured *Klebsiella* sp.	97	EF679185.1
32	1	Uncultured *Pseudomonas* sp	95	DQ295987.1
36	4	*Klebsiella ornithinolytica*	100	AF129441.1
37	2	*Pseudomonas* sp.	97	FN429930.1
40	1	*Pseudomonas* sp.	99	AB506040.1
41	1	*Rhizobium* sp	98	EU741078.1
42	2	*Klebsiella* sp.	97	EU888474.1
44	1	*Vibrio proteolyticus*	93	DQ995521.1

[a]Isolate representative of the BOX cluster analyses.
[b]Number of isolates clustered in the BOX group.
[c]GenBank sequence accession number of most closely related bacterial sequence(s).

in general, to the structure of an estuary, where bacterial abundance and phytoplankton biomass reach the highest values at the intermediate estuarine sections (Wright and Coffin, 1983; Fuks *et al*., 1991; Cunha *et al*., 2003). This distribution is probably related with the quality (lability) of the available organic matter, along the estuary.

Although higher abundances of microorganisms can be found in the SML, when compared to the UW (Carlucci *et al*., 1991). Statistically significant differences between these two layers were not confirmed in this study. This result suggest that bacterioneuston of the Ílhavo Channel is not different, in terms of

abundance, of bacterioplankton.

At the inner and outer sections of the estuary the subclass *gamma-Proteobacteria* was relatively more abundant in bacterioneuston (SML) than in bacterioplankton (UW) (Fig. 2). The *gamma-Proteobacteria* subclass includes major PAH-degrading genera, such as *Alcanivorax*, *Cycloclasticus*, *Pseudomonas*, *Oleiphilus*, *Oleispira*, and *Thalassolituus* (Watanabe, 2001; Head *et al.*, 2006). The variation in the relative abundance of this group observed in this study might be related with the variation of PAH and organic matter concentration along the estuary. The relative abundance of this class reached its maximum value at site S3 where naphthalene and phenanthrene were detected.

Although the concentration of PAH can influence the relative abundance of *gamma-Proteobacteria* along the estuary, other sources of organic carbon are also determinant in the distribution of this group. In estuarine environments, bacterial abundance depends in part of the quantity and quality of the pool of organic matter used as substrate for growth. The middle and inner sections of the estuarine system Ria de Aveiro are exposed to contamination associated with harbour activities, industries, aquaculture plants, diffuse domestic sewage drains and run-off from agriculture fields. The preference of *gamma-Proteobacteria* for high nutrient concentration has been previously reported, and peaks of abundance appeared to be related to nutrient point sources (Bouvier and del Giorgio, 2002; Henriques *et al.*, 2004).

The differences between the SML and UW in the relative abundance of the subclass *gamma-Proteobacteria* were more pronounced at the sites were PAH concentration was higher. All the significant differences between these two layers corresponded to an enrichment at the SML. However, this effect may result from the accumulation of other contaminants at the more eutrophic mid and inner sections of the estuary.

Culturable PAH degrading bacteria in the SML

Because high concentrations of PAH are frequently reported in the SML at sampling locations associated with anthropogenic coastal activities (Wurl and Obbard, 2004), the characterization of the culturable fraction was specifically directed to the SML community. *Pseudomonas* and *Klebsiella* emerged as abundant genera in the culturable PAH-degrading bacterial community in the SML.

Pseudomonas species have a remarkable capacity for the degradation of a broad range of organic pollutants, including PAH, halogenated derivates and recalcitrant organic residues (Bhattacharya *et al.*, 2003). *Pseudomonas putida* (Caldini *et al.*, 1995) and *Pseudomonas fluorescens* (Serebriiskaya *et al.*, 1999), are two examples of well known species capable of PAH degradation frequently reported in PAH-impacted environments. In Antarctic soils, twenty-two PAH-degrading bacterial strains were isolated, and all except one, were *Pseudomonas* (Ma *et al.*, 2006).

In addition to PAH contamination, the estuarine system Ria de Aveiro is

subject to chronic contamination with domestic sewage from point sources. This might explain the high number of isolates belonging to the *Enterobacteriaceae* family such as *Klebsiella* and *Serratia* genera. Nonetheless, the catabolic capacity of *Klebsiella* strains to degrade hydrocarbons, including PAH, has been previously described (Bhattacharya *et al*., 2003). A recent study suggests that this genus could be an important part of the oil-degrading microbial community in estuarine areas exposed to sewage (Rodrigues *et al*., 2009).

Acinetobacter and *Rhizobium* were also found among the PAH degrading isolates retrieved from the SML. Many environmental strains of *Acinetobacter* with hydrocarbon degrading capacities have been isolated in terrestrial and marine environments (Karolien *et al*., 2004; Rodrigues *et al*., 2009). There are also reports on the presence of *Rhizobium* in PAH impacted soils and on hydrocarbon degradation metabolism (Poonthrigpun *et al*., 2006).

CONCLUSIONS

Although the overall abundance of *gamma-Proteobacteria* was similar in bacterioneuston and bacterioplankton communities, this study suggests that the structure of these two communities may be different as to the occurrence of genera related with PAH and organic matter degradation. *Pseudomonas* and *Klebsiella* were well represented in the culturable fraction of bacterioneuston. The results point to the relevant role of bacterioneuston in organic matter recycling in human-impacted estuaries.

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