RESEARCH ARTICLE

Both *Cycloclasticus* spp. and *Pseudomonas* spp. as PAH-degrading bacteria in the Seine estuary (France)

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Abstract

Like other highly urbanized and industrialized estuaries, the Seine estuary (France) has, for decades, received high inputs of polycyclic aromatic hydrocarbons (PAHs). In order to estimate the bioremediation potentials and to identify the bacterial species involved in hydrocarbon degradation, we used microcosms containing seawater from the Seine estuary supplemented with either naphthalene, phenanthrene, fluorene or pyrene. In the microcosms enriched with naphthalene or phenanthrene, hydrocarbon biodegradation was significant within 9 weeks (43% or 46%, respectively), as shown by analyses in GC-MS. In similar microcosms incubated also with naphthalene or phenanthrene, analysis of the 16S rRNA gene sequences (DNA and cDNA) with denaturing gradient gel electrophoresis and clone libraries indicated that the PAH-degrading communities were dominated by *Cycloclasticus* spp., confirming their universal key role in degradation of low-molecular-weight PAHs in marine environments. However, in contrast to previous studies, we found that *Pseudomonas* spp. also degraded naphthalene and phenanthrene in seawater; this occurred only after 21 days, as was confirmed by real-time PCR. Although this genus has been abundantly described in the literature as a good PAH-degrading bacterial group in soil or in sediment, to our knowledge, this is the first evidence of a significant fitness in PAH degradation in seawater.

Introduction

The Seine basin drains the fourth largest watershed area of France (78 650 km^2) and flows into the English Channel in the North-West of France. Because it accounts for 40% of the French economic activity, including 33% of oil refining and 50% of river traffic, it is highly urbanized and industrialized. Consequently, the Seine River has, for decades, received high inputs of a large variety of micro-pollutants including polycyclic aromatic hydrocarbons (PAHs) (Fernandes *et al.*, 1997; Motelay-Massei *et al.*, 2004; Cailleaud *et al.*, 2007). PAHs are hydrophobic organic compounds consisting of two or more combined benzene rings and are important components of crude oil, creosote, asphalt and coal tar. In the Seine estuary, PAHs are almost exclusively of anthropogenic origin (automobile traffic, domestic heating or industrial emissions) (Blanchard *et al.*, 2004; Motelay-Massei *et al.*, 2007). Although major oil spills are relatively rare incidents, refining, uncontrolled releases, natural seepage from reservoirs, disposal by end users and freshwater and terrestrial run-off are all constant sources of estuary oil pollution that result in large amounts of petroleum hydrocarbons entering the estuary environment (Motelay-Massei *et al.*, 2006; Cailleaud *et al.*, 2007).

PAHs are of particular concern because of their toxic, mutagenic and carcinogenic properties, which present a significant risk to human health and the environment (Cerniglia & Heitkamp, 1989; Menzie *et al.*, 1992; Cachot *et al.*, 2006). Fortunately, natural biodegradation processes remove oil from the marine and estuary environments, with bacteria playing the dominant role (Leahy & Colwell, 1990). Following an addition of oil, the diversity of the bacterial community can be dramatically reduced (Röling *et al.*, 2002), with a strong selection of hydrocarbon-degrading species. Because organic pollutants are possibly carbon sources in an oligotrophic environment, PAH-degrading

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microorganism can be enriched at contaminated sites to at least several orders of magnitude more than in comparable noncontaminated sites (Geiselbrecht *et al.*, 1996, 1998).

Although a large number of bacterial species are able to degrade PAHs, including genera such as *Pseudomonas*, *Mycobacterium*, *Sphingomonas*, *Burkholderia, Ralstonia* and *Cycloclasticus* (Cerniglia & Heitkamp, 1989; Whyte *et al.*, 1997; Habe & Omori, 2003; Peng *et al.*, 2008), biodegradation of PAHs in the environment is often limited. This lack of success is probably due to the highly hydrophobic nature of PAHs, which reduces their availability (Bosma & Middeldorp, 1997; Peng *et al.*, 2008). Temperature, lack of inorganic nutrients or the variable fitness of PAH-degrading microorganisms could also explain this unsuccessful biodegradation (Coulon *et al.*, 2007; McKew *et al.*, 2007b; Peng *et al.*, 2008). A better understanding of the ecology of PAH-degrading microorganisms is thus essential to prevent ecological damage caused by oil spills, but also to enhance the natural attenuation of diffuse and chronic pollutions (Daane *et al.*, 2001; McKew *et al.*, 2007b).

Here, in order to estimate the bioremediation potentials in the Seine estuary and to identify the bacterial species involved in hydrocarbon degradation, we used microcosms containing seawater supplemented with either naphthalene, phenanthrene, fluorene or pyrene. Although the Seine estuary is strongly industrialized and has been highly contaminated for a long period (Fernandes *et al.*, 1997; Motelay-Massei *et al.*, 2004; Cailleaud *et al.*, 2007), it is the first time, to our knowledge, that such a study has been carried out on this estuary. While we found the kinetics of degradation and dominant PAH-degrading bacteria to be comparable to previous observations from other estuaries, we have shown that members of the genus *Pseudomonas* appeared after 21 days in the microcosms containing naphthalene and phenanthrene.

Materials and methods

Sample site and sample collection

Seawater samples were collected in February 2008 and in April 2009 at the mouth of the Seine estuary (49°29′N, 0°04′E) in the neighbourhood of a total oil refinery and the city of Le Havre, Haute-Normandie, France.

Samples collected in February 2008 were used for community analysis and for PAH measurements. The salinity was 32, temperature 7.2 °C, pH 7.5, 6.84 mg L^{-1} NO_2^{-}+NO_3^{-}, <0.01 mg L^{-1} NH_4^{+} and 0.15 mg L^{-1} PO_4^{2-}. Five litres of seawater was collected in sterile bottles at high tide for microcosm experiments. A further 450 mL of seawater was filtered on site through 0.22 μm pore size, 47-mm-diameter Millipore filters and placed immediately on dry ice throughout the sample day, and was then kept at − 80 °C for subsequent 16S rRNA gene community profile analysis.

Samples collected in April 2009 were used only for PAH measurements. The salinity was 32.5, temperature 8.2 °C, pH 7.5, 15.75 mg L^{-1} NO_2^{-}+NO_3^{-}, <0.01 mg L^{-1} NH_4^{+} and 0.59 mg L^{-1} PO_4^{2-}. Five litres of seawater was collected in sterile bottles at high tide for microcosm experiments.

Microcosm design

Two groups of microcosms were established independently from samples collected in February 2008 and in April 2009. For each experiment, microcosms were established in 250-mL sterile flasks containing 20 mL of seawater amended with nutrients (20 mg L^{-1} NH_4NO_3 and 10 mg L^{-1} KH_2PO_4) for each time point to be sampled. The microcosms were enriched separately with the following PAHs at a final concentration of 0.1% w/v: naphthalene, fluorene, phenanthrene or pyrene (Sigma, 97–99% purity). The PAHs were added directly to the microcosms without organic solvents. The following controls were also established: (1) a killed control for each PAH (seawater+nutrients and a hydrocarbon substrate and mercuric chloride 300 mg L^{-1}) to determine whether any abiotic degradation was occurring after 63 days and (2) a biotic control for each sample time (seawater+nutrients) to confirm that differences in the microbial community were caused mainly by the presence of hydrocarbons. All microcosms were incubated at 20 °C in the dark on an orbital shaker (130 r.p.m.).

A first group of microcosms was established in duplicate from samples collected in February 2008 and was used for PAH measurements and for community analysis. The duplicated microcosms were analysed after 7, 21, 42 and 63 days, except for the microcosms enriched with naphthalene, where two additional time points were sampled: after 1 and 3 days (see Table 1). Only one duplicated microcosm was chemically analysed (PAH measurements), after 21 and 63 days. For all the sample times, microbiological analyses were carried out from each duplicated microcosm, except for the 21- and 63-day samples, where a complete 20-mL microcosm was chemically analysed, leaving only one of the duplicates for the microbiological analyses for these sample times (see Table 1).

A second group of microcosms was established in triplicate from samples collected in April 2009 and was used only for the statistical analysis of the PAH degradation. Chemical analyses were performed from triplicated microcosms only after 63 days. In addition to a killed control and a biotic control, a control without nutrient (NH_4NO_3 and KH_2PO_4) was carried out, also in triplicate. For this group of microcosms, complete 20-mL microcosms were used for each chemical analysis.

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Table 1. Quantification of DNA extracted (ng μL^{-1}) from initial seawater (113 mL) or the February 2008 set of microcosms (2.5 mL)

		Biotic controls*		Naphthalene		Phenanthrene		Fluorene		Pyrene	
Time points (in days)	Initial seawater	A[†]	B[†]	A	B	A	B	A	B	A	B
	15										
T0		< 0.5[‡]	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
T1		< 0.5	< 0.5	< 0.5	< 0.5						
T3		< 0.5	< 0.5	**1.6**	**18**						
T7		< 0.5	< 0.5	**34**	**45**	**34**	**29**	< 0.5	< 0.5	1.3	0.9
T21		< 0.5	< 0.5	**60**		**49**		< 0.5		< 0.5	
T42		< 0.5	< 0.5	**61**	**51**	**54**	**68**	< 0.5	1	< 0.5	< 0.5
T63		< 0.5	< 0.5	**41**		**33**		**0.5**		< 0.5	

*Biotic controls were microcosms without PAHs.

[†]A and B were microcosm duplicates.

[‡]The threshold value of detection was 0.5 ng μL^{-1} of extracted DNA.

Positive PCR amplifications of the 16S rRNA gene sequences from extracted DNA and cDNA are highlighted in bold.

Hydrocarbon analyses

PAHs were extracted from 20 mL of aqueous samples using solid-phase extraction (SPE) tubes containing 200 mg of a hydrophobic polymeric sorbent Strata-X (Phenomenex, Torrance). The flasks containing the microcosms were rinsed three times with mQ water and then with acetone/water 20/80% (v/v) in order to also extract the attached PAHs absorbed on the flask walls. SPE tubes were first conditioned with methylene chloride, methanol and pure water. Then, after transfer of the aqueous microcosm into the SPE tubes, extracts were eluted with a mixture of 2×4 mL hexane : dichloromethane (1 : 1). Extracts were diluted 1000 times for GC analysis. A deuterated PAH (phenanthrene D10) was finally added to diluted extracts (at 1 mg L^{-1}) as an internal standard for quantification. Naphthalene, fluorene, phenanthrene and pyrene were identified and quantified by GC-MS using an HP 5890 GC gas chromatograph hyphenated to an HP 5972 mass spectrometer (Agilent technologies, Santa Clara) operating at 70 eV in the positive ion mode. Quantification was based on selected ion monitoring for better sensitivity. Chromatography was performed with a 40-m capillary column (0.15 mm internal diameter, 0.15 μm film thickness) coated with a Factor four VF-5MS phase from Varian (Les Ulis, France), with helium as the carrier gas (0.5 mL min^{-1}). Splitless injections (250 °C) with a sample volume of 0.5 μL were applied. The oven temperature was programmed at 60 °C for 2 min, then increased by 40 °C per minute up to 180 °C, and then by 3.7 °C per minute up to 280 °C. Percentages of degradation were calculated according to the initial quantity of supplemented PAH (i.e. 0.1% w/v).

Nucleic acid extraction

For the first group of microcosms (seawater collected in February 2008), 10 mL (from 20 mL) of microcosms were filtered through 0.22 μm pore size, 47-mm-diameter Millipore filters and placed at − 80 °C for subsequent 16S rRNA gene community profile analysis. The flasks were shaken vigorously to detach the bacteria, but the 10-mL samples were taken with caution to avoid visible PAH crystals. In addition, 450 mL of seawater was filtered on site, as described previously.

DNA was extracted from a quarter of the filter (corresponding to 2.5 mL of each microcosm and to 113 mL of the initial seawater) using the Fast DNA Spin kit for soil (MP Biochemicals) in combination with the FastPrep FP120 bead beating system (Bio-101 Inc., CA), according to the manufacturer's instructions. The concentration of the resulting DNA was estimated by measuring $A_{260\,nm}$ with the capillary spectrophotometer GeneQuant *pro* (Amersham Biosciences, France) and by comparison with known DNA quantities in agar gel stained with ethidium bromide (0.5 μg mL^{-1}). In addition, to test the presence of PCR inhibitors, part of the extracted DNA (25 from 50 μL) was purified by elution through Elutip-D columns (Schleicher and Schuell, Dassel, Germany) according to the manufacturer's instructions.

RNA was extracted from a quarter of the filter (corresponding to 2.5 mL of each microcosm and to 113 mL of the initial seawater) using the Fast RNA Pro Soil-Direct Kit (MP Biochemicals) in combination with the FastPrep FP120 bead beating system (Bio-101 Inc.), according to the manufacturer's instructions. DNA was removed from 16 μL of the total nucleic acid extraction by DNase digestion, using the deoxyribonuclease I Amplification Grade kit (Sigma). A control PCR was performed to ensure complete removal of DNA using the primers and PCR conditions as detailed in the next section.

Reverse transcription (RT)-PCR and PCR

cDNA was produced by reverse transcription of RNA using the Enhanced Avian HS RT-PCR Kit (Sigma) as recommended by the manufacturer and using the reverse primer

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described in the next paragraph. One microlitre of the reverse transcription product was subsequently used in PCR reactions.

PCR is used to amplify the variable V3 region of the 16S rRNA gene (*Escherichia coli* positions 341–534) using the following primers: forward primer with GC clamp CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG; reverse primer ATT ACC GCG GCT GCT GG (Muyzer *et al*., 1993). The PCR mixtures (50 μL) contained 250 pM of each primer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, *Taq* PCR buffer (Gold star, Eurogentec, Belgium), 1 U of *Taq* polymerase (Gold star, Eurogentec) and 1 μL of DNA or cDNA template. Amplification was performed in a GeneAmp PCR system 9700 Thermocycler (Applied Biosystems) as follows: 95 °C for 3 min, 30 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s, with a final elongation of 72 °C for 10 min. Amplification of the target rDNA was confirmed by agar gel electrophoresis (2% w/v in 0.5 × TAE buffer at 100 V) and ethidium bromide staining.

Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE analysis of PCR and RT-PCR products was performed using the Bio-Rad D Code system, on gels consisting of 8% (w/v) polyacrylamide (acrylamide : bisacrylamide, 37 : 1) and a denaturing gradient from 40% to 60% [100% denaturant is 7 M electrophoresis-grade urea (Sigma) and 40% v/v formamide (Fluka)], in 1 × TAE (40 mM Tris-acetate, 1 mM di-sodium-EDTA, pH 8.0), at a constant 80 V and at 60 °C for 5 h. Gels were visualized under UV at 302 nm using the Alphamager System EU after staining in 500 mL of 0.25 $\mu g\,mL^{-1}$ BET in 1 × TAE for 15 min.

Relevant bands were excised using a scalpel and transferred to a 1.5-mL tube. After washing in 70% EtOH, then in TE buffer (10 mM Tris, 1 mM EDTA, pH 8), acrylamide bands were crushed in 50-μL TE buffer and incubated overnight at 4 °C. One microlitre of the supernatant was then analysed again by PCR and DGGE, and bands were extracted once more in order to eliminate any residual contamination by parasite bands. A final reamplification was performed by PCR as described previously, except that a forward primer without the GC clamp (CC TAC GGG AGG CAG CAG) was used. Double-stranded DNA sequencing was carried out by Cogenics Genome Express (Meylan, France). Nucleotide sequence data are available in the GenBank database.

Clone libraries

Two DNA clone libraries were constructed from one of the duplicated microcosms enriched in naphthalene or phenanthrene (seawater collected in February 2008), after 42 days. In each case, at least three independent PCR products were used for cloning. Almost full-length 16S rRNA gene fragments were amplified using universal primers 8f (AGA GTT TGA TCC TGG CTC AG) and 1541r (AAG GAG GTG ATC CAG CCG CA). The PCR mixtures (50 μL) contained 250 pM of each primer, 200 μM of each dNTP, *Phusion* PCR buffer (Finnzymes), 1 U of the high-fidelity *Phusion* hot start DNA polymerase (Finnzymes) and 1 μL of DNA template. Amplification was performed in a GeneAmp PCR system 9700 Thermocycler (Applied Biosystems) as follows: 95 °C for 3 min, 30 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s, with a final elongation of 72 °C for 10 min. An additional step was used to add adenine in the 3′ extremities of the PCR products: 72 °C for 10 min with ATP and *Taq* Gold star Polymerase (Eurogentec). Modified PCR products were cloned into the pGEMT plasmid using a TA cloning kit (Invitrogen) according to the manufacturer's instructions. About 50 clones were selected randomly and checked for correct insert size via PCR and agarose gel electrophoresis and sequenced using the T7 primer of the plasmid.

Sequences (about 800 bp) were identified using the Ribosomal Database Project database (http://rdp.cme.msu.edu/index.jsp). Moreover, two clones (Nap42 and Phe42 clones) were fully sequenced, included in the phylogenetic analysis and deposited in the GenBank database with acccession no. GQ34341 and GQ34342.

Real-time PCR

Each quantitative PCR (qPCR) was performed in a 25-μL reaction mixture containing the MESA Blue qPCR Mastermix for SYBR Green (Eurogentec), 800 pM of each primer and 2 μL of DNA template. The primers used were OLIPCR8/OLIPCR5 T (Bodilis *et al*., 2006) and 63f/BU16S4 (Plassart *et al*., 2008) for the amplification of the *oprF* gene and the 16S rRNA gene, respectively. The *oprF* gene is present in the whole *Pseudomonas* genus and in no other bacterial genus. Moreover, the *oprF* gene is amplified with the OLIPCR8/OLIPCR5T primers from all *Pseudomonas* species, except from *Pseudomonas stutzeri* (Bodilis *et al*., 2006). Real-time PCR amplifications were performed in 96-well optical plates with a Chromo 4 Real Time PCR Detector (Bio-Rad) as follows: 95 °C for 10 min, 35 cycles of 95 °C for 40 s, 60 or 64 °C (for *oprF* or 16S rRNA gene amplifications, respectively) for 45 s and 72 °C for 30 s, with a final elongation of 72 °C for 5 min. A melting curve analysis was performed after amplification by slow heating from 30 to 100 °C. Finally, the same standard curve was used for the quantification of *oprF* and 16S rRNA genes, using the threshold values and the corresponding copy numbers of each gene from a serial dilution of the chromosome of the *Pseudomonas fluorescens* SBW25 strain. Because the genome of this strain was sequenced (Silby *et al*., 2009), both the

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genome size (6 722 540 bp) and the gene copy number per genome were known, i.e. one and five copies for *oprF* and 16S rRNA genes, respectively. Hence, copy numbers in the standard curve were easily deduced from the DNA quantification of the chromosomal DNA extract. Real-time PCR was carried out in duplicate for both the standards and the samples. The results presented correspond to the means of these duplicates.

Phylogenetic analysis

The reference sequences used for phylogenetic analyses were retrieved from the GenBank and Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp). The 16S rRNA gene sequences from the DGGE bands and some clones were aligned with the reference sequences using CLUSTAL X version 1.81, with default parameters (Thompson *et al.*, 1997). A second alignment was carried out with the reference sequences only (without sequences from the DGGE bands and clones and without the short reference sequence AM501652). Both the alignments were truncated to the same size as the shortest sequence: positions 359–517 (in the *E. coli* numbering system) for the alignment containing all the sequences (including our sequences) and positions 99–1414 for the alignment containing only the reference sequences. Both the alignments were optimized visually and all ambiguous positions and positions with gaps were removed.

A neighbour-joining tree was inferred using the Kimura two-parameter correction (Kimura, 1980) with MEGA v2.0 (Saitou & Nei, 1987; Kumar *et al.*, 2001). A maximum likelihood analysis was performed using fastDNAml based on the HKY model (Hasegawa *et al.*, 1985) with PHYLOWIN v2.0 (Galtier *et al.*, 1996). The degree of statistical support for the branches was determined with 1000 bootstrap replicates for the distance matrix analyses or 100 bootstrap replicates for the maximum likelihood analyses.

Statistical analysis

All tests were performed using XLSTAT™. The significance of hydrocarbon degradation relative to controls was determined using the nonparametric one-way Kruskall–Wallis test.

Results

Two independent sets of microcosms were established from seawater collected in the same area (at the mouth of the Seine estuary, see Materials and methods), in February 2008 and in April 2009. The microcosms in April 2009 were used only for PAH measurements, while the microcosms in February 2008 were used for community analysis and for PAH measurements.

Table 2. Degradation of PAHs in the April 2009 set of microcosms after 63 days

	% Degradation*		
PAHs	With additional nutrients (N and P)	Without additional nutrients (N and P)	In killed controls†
Naphthalene	58.8 (± 4.1)a	42.9 (± 4.4)b	15.1 (± 4.8)c
Phenanthrene	47.7 (± 9.3)a	24.8 (± 9.1)b	1.5 (± 0.1)c
Fluorene	11.2 (± 6.8)a	8.0 (± 3.0)a	2.8 (± 8.9)a
Pyrene	23.0 (± 13.6)a	13.5 (± 13.5)a	25.8 (± 7.1)a

*Values correspond to averages of three replicates ± SD ($n = 3$). Values with a different letter (a, b or c) within the same line are significantly different at $P < 0.05$.

†Microcosms containing additional nutrients (N and P) and mercuric chloride at 300 mg L^{-1}.

A yellow colouration was indicative of naphthalene and phenanthrene degradation in the two sets of microcosms, appearing after 3 and 7 days, respectively. By contrast, the microcosms enriched with pyrene or fluorene, as well as all the control microcosms (biotic and killed controls), presented no change of colour after 63 days.

Analyses by GC-MS from the April 2009 set of microcosms showed, by comparison with chemical analysis on killed controls, a significant biodegradation ($P < 0.05$) only for naphthalene and phenanthrene (43% and 46%, respectively), while biodegradation of fluorene was inconclusive (8%, $P = 0.275$), and no biotic degradation was observed for the pyrene (Table 2). It is important to note that for the killed controls enriched with naphthalene and pyrene, substantial decreases of PAH were observed (15% and 25%, respectively). Comparison with chemical analysis from microcosms just after adding the PAH (data not shown) revealed that this decrease of naphthalene corresponded to an abiotic degradation, while, in microcosms enriched with pyrene, high extraction losses occurred.

Finally, microcosms enriched with PAH alone (without nutrients) were used to test the effects of an additional source of nitrogen and phosphate. The results showed that biotic degradation did occur (at least for naphthalene and phenanthrene) in microcosms without nutrients, but was significantly less ($P < 0.05$) than when nutrients were added (Table 2). Because our main objective was to identify the bacterial species involved in hydrocarbon degradation, we decided to ensure that we had sufficient production of bacterial biomass.

Analyses by GC-MS from the February 2008 set of microcosms (used for microbiological analysis) were also carried out, but only from a single and complete microcosm (after 21 and 63 days), leaving only one of the duplicates for microbiological analyses for these sample times (data not shown). The absence of replicates prevented us from performing a statistical test. Nevertheless, the coherence

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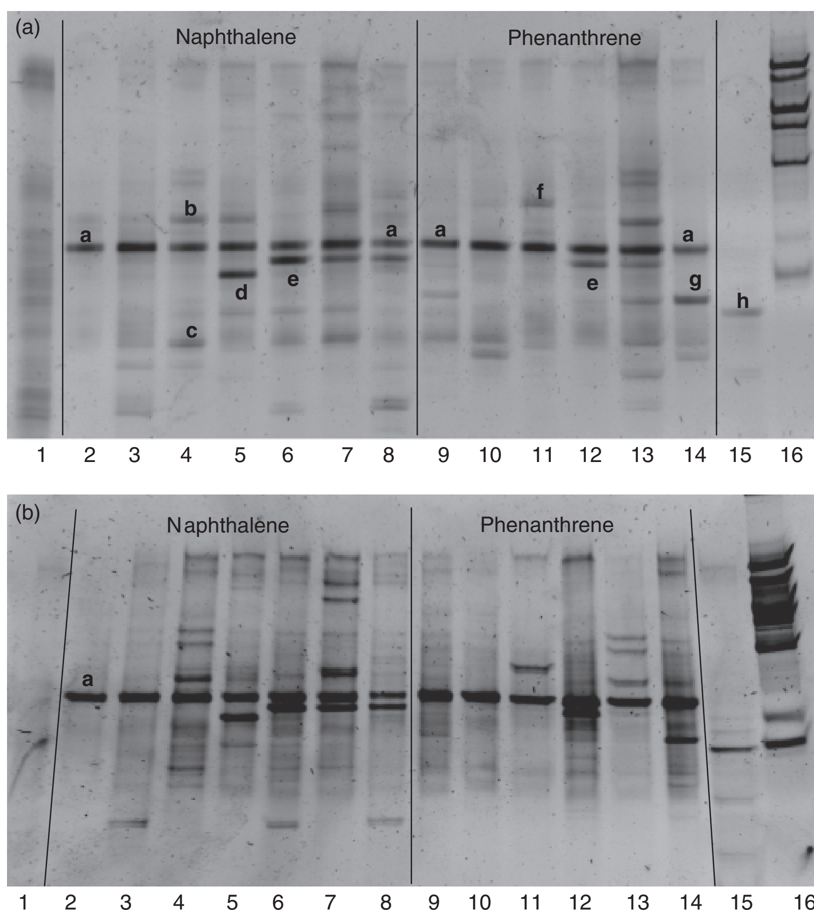

Fig. 1. Dynamics analysis of the bacterial community in the February 2008 set of microcosms revealed by DGGE profiles of PCR-amplified V3 region fragments of the 16S rRNA genes from DNA (a) and cDNA (b). Lane 1, initial bacterial community. Lanes 2–8, supplemented with naphthalene (lane 2, after 3 days; lanes 3–4, after 7 days; lane 5, after 21 days; lanes 6–7, after 42 days; lane 8, after 63 days). Lanes 9–14, supplemented with phenanthrene (lanes 9–10, after 7 days; lane 11, after 21 days; lanes 12–13, after 42 days; lane 14, after 63 days). Lane 15, supplemented with fluorene, after 63 days. Lane 16, SmartLadder (Eurogentec). About 160 bp of the 16S rRNA gene sequences were determined from dominant bands denoted by a letter.

observed between the measurements after 21 and 63 days (data not shown), taken together with almost the same degradation observed in the April 2009 set of microcosms, strongly suggested to us that it is indeed the seawater microorganisms that played a major role in degrading naphthalene and phenanthrene.

We then extracted DNA and RNA from the initial seawater sample as well as from all the February 2008 microcosms. The quantities of extracted DNA are presented in Table 1. It is important to note that for some microcosms, the quantity of extracted DNA was under our quantification threshold (0.5 ng μL^{-1} of extracted DNA). This was expected because the DNA extracted from the initial sample of seawater (i.e. from a volume about 50-fold higher than for the microcosms) had a concentration of only 15 ng μL^{-1} (Table 1). Moreover, amplification of the V3 region of the 16S rRNA genes from extracted DNA and from cDNA was not possible for all the microcosms, as shown in Table 1: amplification failed for biotic controls, as well as for all the microcosms enriched with pyrene. For the microcosms enriched with fluorene, amplification of the 16S rRNA genes was possible only after 63 days.

As expected, we observed a very good correlation between the quantity of extracted DNA, and whether or not amplification of the 16S rRNA genes was possible, all the negative samples having a quantity of DNA close to or lower than our quantification threshold (about 0.5 ng μL^{-1} of extracted DNA). Interestingly, we also observed a perfect correlation between the amplification from DNA and the amplification from cDNA (Table 1).

The diversity of the amplified 16S rRNA genes was then investigated by DGGE (Fig. 1). As expected, compared with the initial seawater, we observed fewer bands for microcosms enriched with PAHs. Furthermore, the number of bands increased gradually in time for the microcosms enriched with naphthalene or phenanthrene. For these microcosms, the fingerprints were very similar to each other. The same band (band a) was observed for all the microcosms enriched with naphthalene or phenanthrene (Fig. 1). This band was almost single at the start of incubation and continued to be dominant even after 63 days. Sequencing of 159 bp showed 100% of identity with the 16S rRNA gene of *Cycloclasticus spirillensus* and *Cycloclasticus pugetii* type strains (AY026915 and L34955, respectively) as well as with

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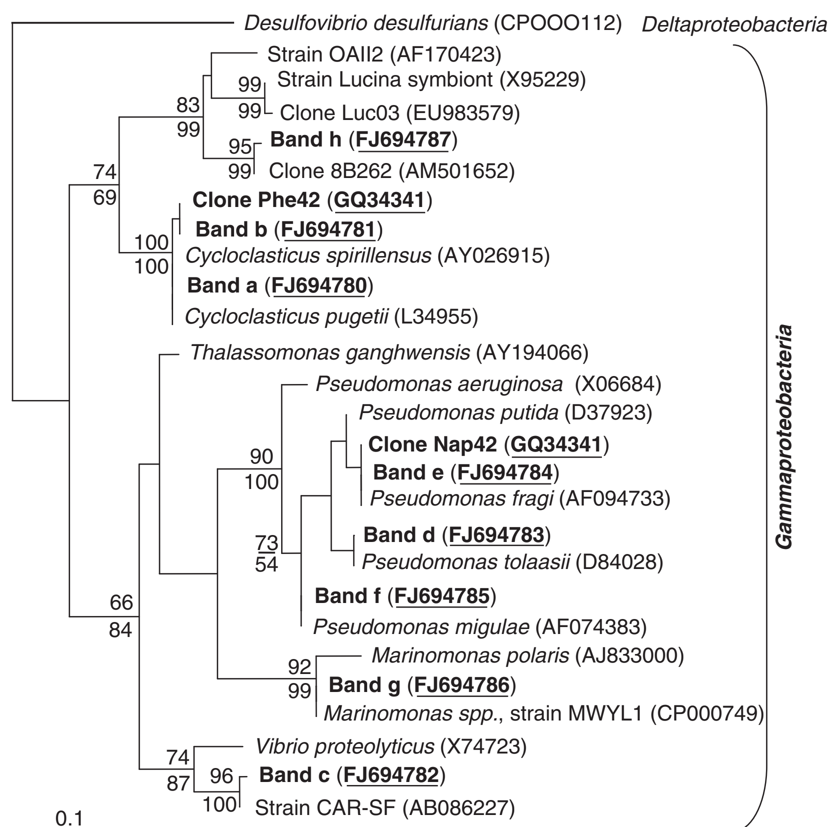

Fig. 2. 16S rRNA gene phylogeny of dominant bacteria from seawater microcosms enriched with different PAHs. Sequences of closest relatives and representative PAH-degrading bacteria are included in the analysis. The unrooted dendrogram was generated using maximum likelihood analysis. The scale bar corresponds to 0.1 substitutions per nucleotide position. Numbers on tree branches indicate bootstrap results from maximum likelihood analysis (above branch) and distance matrix (below branch) analyses for those branches having ≥50% support. Sequences provided in this study are in bold.

some clones and strains isolated worldwide (Fig. 2). The other bands were of variable intensities, but some were either found frequently or presented considerable intensity. Band b differed from band a by only one nucleotide (identity 99.4%, 158 out of 159 bp) and likely also corresponded to a strain of the *Cycloclasticus* genus. Band c had 99.4% of identity (158 out of 159 bp) with an unidentified carbazole-degrading strain (AB086227) isolated from seawater in Japan (Fuse *et al*., 2003) (Fig. 2). Bands d, e and f appeared after 21 days and presented 100% identity with some type strains of the *Pseudomonas* genus (*Pseudomonas tolaasii*, D84028; *Pseudomonas fragi*, AF094733; *Pseudomonas migulae*, AF074383; respectively). Interestingly, after 42-day incubation, band e was found in all the microcosms enriched with naphthalene or phenanthrene. Band g appeared specifically in the microcosms enriched with phenanthrene and became codominant after 63 days. Sequencing of 161 bp showed 100% of identity with the 16S rRNA gene of the MWYL1 strain identified as *Marinomonas* spp. (complete genome, CP000749), known to be involved in phenanthrene degradation (Melcher *et al*., 2002). Finally, the microcosm enriched with fluorene revealed, after 63 days, only one band (band h), which did not appear in the microcosms enriched with naphthalene or phenanthrene. Sequencing of 159 bp showed only 92.5% (147 out of 159 bp) identity with the OAII2 strain, an unidentified sulphur-oxidizing bacterium isolated from a hydrothermal vent in shallow water (AF170423, S.M. Sievert *et al*., unpublished data). This band also had 99.4% (158 out of 159 bp) identity with a clone found in anoxic sediments from Venice (AM501652, S. Borin *et al*., unpublished data) (Fig. 2). However, no cultivable strain was close, highlighting the interest in studying the total bacterial community and not only the cultivable bacteria.

The diversity of the 16S rRNA gene (from cDNA amplifications) was also investigated by DGGE (Fig. 1b). Except from the initial seawater (from which we failed to amplify cDNA), the fingerprints obtained were almost identical to those obtained from DNA, with only some minority bands presenting slightly different intensities.

To further investigate the proportion of the dominant PAH-degrading bacteria, two DNA clone libraries were constructed from one of the duplicated microcosms enriched in naphthalene or phenanthrene (seawater collected in February 2008), after 42 days (Fig. 3a). In both the clone libraries, *Gammaproteobacteria* were dominant (91% and 89%, for naphthalene and phenanthrene, respectively), with a majority of *Pseudomonas* (57% and 5%, respectively) and *Cycloclasticus* (34% and 84%, respectively).

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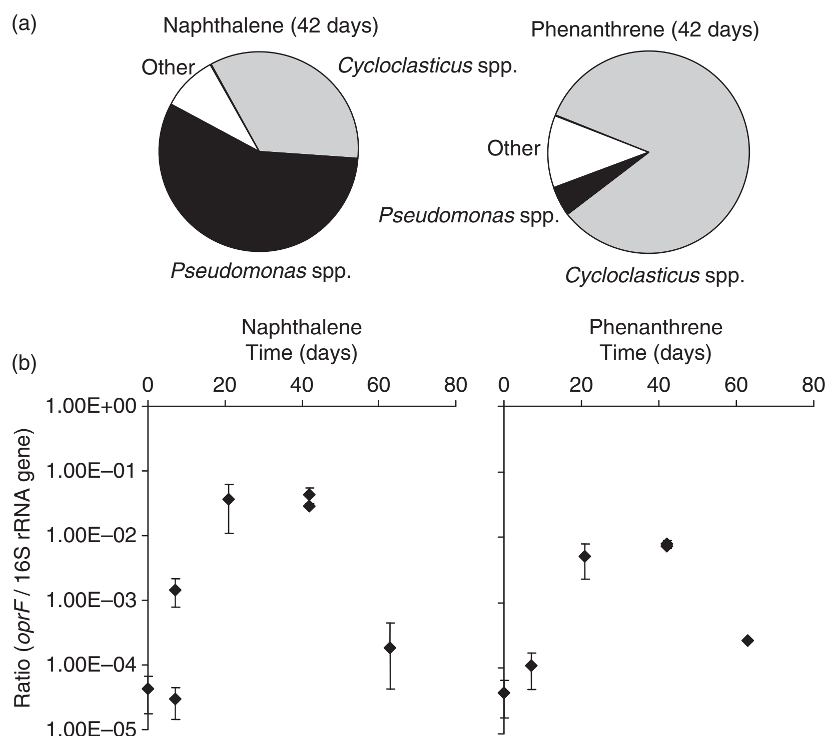

Fig. 3. (a) Relative abundance (%) of clones in 16S rRNA gene clone libraries from the February 2008 set of microcosms after 42 days. For each library, 44 (for naphthalene) and 43 (for phenanthrene) sequences (about 800 bp) were identified using the Ribosomal Database Project database (http://rdp.cme.msu.edu/index.jsp). Sectors titled 'other' were comprised of some bacterial genera present only as single clones. (b) Proportion of *Pseudomonas* in the total bacterial community (number of *oprF* gene copies/number of 16S rRNA gene copies) from microcosms enriched with naphthalene and phenanthrene, as determined by real-time PCR ($n = 2$).

Finally, the ratio of the *oprF* gene to the 16S rRNA gene was determined from qPCR assays and reflected the proportion of the *Pseudomonas* in the bacterial community, because the *oprF* gene is specific to the *Pseudomonas* genus (Bodilis & Barray, 2006). It is important to note that the ratio of the *oprF* gene to the 16S rRNA gene does not correspond exactly to the proportion of *Pseudomonas* because there is one copy of *oprF* per genome, while the copy number of the 16S rRNA gene varies from 1 to 15 in bacteria, and 4 to 7 in *Pseudomonas* (Acinas *et al*., 2004). Nevertheless, we demonstrated that the genus *Pseudomonas* was strongly selected during the degradation of naphthalene and phenanthrene, with an increase of two or three orders of magnitude (for naphthalene and phenanthrene, respectively) by comparison with the initial seawater (Fig. 3b). Moreover, a decrease of the proportion of the *Pseudomonas* was observed after 63 days for both the microcosms enriched in naphthalene or phenanthrene.

Discussion

Because we added a large quantity of PAH to the microcosms ($1\,g\,L^{-1}$), we simulated an oil spill and not a diffuse pollution event. We thus studied only a potential PAH degradation. However, we could suppose that the bacteria highlighted here also participate in the natural attenuation of PAH contaminations in the Seine estuary.

As in various other studies, we observed a rate of degradation that decreased according to the number of carbon rings (Bidaud & Tran-Minh, 1998; McKew *et al*., 2007a). However, fluorene showed a greatly reduced rate of biodegradation in comparison with phenanthrene (Table 2), a PAH of similar size. Other authors have already reported this observation (Wammer & Peters, 2005; McKew *et al*., 2007a); Wammer & Peters (2005) have suggested that the slightly planar five-carbon ring of fluorene may restrict the access into narrow enzyme-binding pockets. Overall, we observed lower rates of degradation than those found in the study of McKew *et al*. (2007a), which was carried out on Thames estuary water, using a similar protocol. Moreover, unlike the Thames estuary water study, our study showed neither biodegradation of pyrene (Table 2) nor production of bacterial biomass in microcosms enriched with this PAH (Table 1), thus highlighting a lower potential of PAH degradation in the Seine estuary.

Because our microcosms had small volumes and because of high extraction losses (about 80% for DNA extraction, data not shown), the quantities of extracted DNA and RNA were low, i.e. under our detection threshold (about $0.5\,ng\,\mu L^{-1}$) for biotic controls and for most of the microcosms enriched with fluorene and pyrene (Table 1). Although we were aware that it would have been possible to increase the extraction yield, to optimize the PCR amplification or to carry out the extractions from larger volumes of microcosms, our detection (and amplification) threshold

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presented the advantage that only the amplification of the 16S rRNA genes from bacteria that could significantly develop was observed. These are the bacteria that use, directly or indirectly, PAHs as sources of carbon and energy.

Clone libraries and DGGE showed that *Cycloclasticus* spp. were dominant or codominant at every sample time in the microcosms enriched with naphthalene or phenanthrene, and so are key bacteria in the degradation of these PAHs in the Seine estuary. Interestingly, the same DGGE band (i.e. 100% of identity) was dominant in microcosms produced under similar conditions from the water of the Thames estuary (Mckew *et al*., 2007a), but also in various studies around the world. *Cycloclasticus* spp. were also dominant in other studies using enriched microcosms with seawater or sediments from the Atlantic or Pacific Oceans (Kasai *et al*., 2002; McKew *et al*., 2007a; Teira *et al*., 2007; Cui *et al*., 2008; Wang *et al*., 2008). Although we were not able to highlight a pyrene-degrading bacterial community, it was previously found that some *Cycloclasticus* spp. also play a role in the degradation of this PAH, even when it is the sole source of carbon (Wang *et al*., 2008). Finally, Maruyama *et al.* (2003) have observed a considerable *in situ* enrichment by this cosmopolitan bacterial genus after an oil spill in Japan, demonstrating its universal key role in the degradation of low-molecular-weight PAHs in a marine environment.

Finally, we observed a late role (after 21 days) in the degradation of naphthalene and phenanthrene of at least three different strains of *Pseudomonas*. *Pseudomonas* spp. are ubiquitous and are abundantly described in the literature as competitive PAH-degrading bacteria in polluted soils and sediments (Weissenfels *et al*., 1990; Daane *et al*., 2001; Cebron *et al*., 2008). Moreover, because the estuarine water is not a typical marine environment, the detection of *Pseudomonas* at the mouth of the Seine estuary (Fig. 3b) was expected. Nevertheless, although McKew *et al.* (2007a) also identified a sequence from a minor DGGE band with 100% identity to *Pseudomonas*, members of this genus have not previously been described as good PAH-degrading bacteria in seawater (i.e. with a salinity of 32). Hence, most studies focused on the first days of PAH degradation in a marine environment because it is especially these first days that are critical in improving remediation after oil spills (McKew *et al*., 2007b). However, in order to better understand natural attenuation of PAHs during chronic contamination along the continuum of a watershed, it would be interesting to study, in particular, the diversity of *Pseudomonas* spp. associated with this degradation. Strains of this genus being easily cultivable, further investigation of the mechanisms of competition and adaptation associated with the cleanup function of bacteria would be facilitated.

In this context, we were surprised that *Pseudomonas* spp. played a competitive role in the degradation of naphthalene and phenanthrene in seawater. These bacteria are generally described as having an r-strategy, i.e. they grow fast as soon as a new carbon source is added to the medium (De Leij *et al*., 1993), and so it was even more surprising that the degradation by *Pseudomonas* spp. only appeared after 21 days. To further investigate this last point, it could be interesting to study the potential of PAH degradation of *Pseudomonas* alone or in competition with *Cycloclasticus*.

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