



Changes in bacterial activity and community composition caused by exposure to a simulated oil spill in microcosm and mesocosm experiments

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ABSTRACT: We studied the effects of the Prestige oil spill on Ría de Vigo bacterial abundance, production and community structure by using mesocosms (ca. 3500 l) filled with water from the center of the Ría, to which we added a realistic concentration of polycyclic aromatic hydrocarbons (PAHs; initial concentrations of approximately 20 to 30 $\mu\text{g l}^{-1}$ chrysene equivalents) at each of the 4 periods of the seasonal cycle: spring bloom, summer stratification, autumn upwelling and winter. We followed the changes in bacterial activity by leucine and thymidine incorporation, and the changes in bacterial assemblage structure by 16S rDNA DGGE. In addition, simultaneously with the winter mesocosm experiment, we ran microcosms with fuel additions equivalent to 0.5, 1, 2 and 4 \times the treatment imposed on the mesocosms in the seasonal experiments. Bacterial community structure was also analyzed by CARD-FISH. We detected significant effects of the PAHs on bacterial community structure (increased number of bands) and production only in the summer experiment. In the microcosm experiments, we found similar effects to those in the mesocosms at PAH concentrations of ca. 20 to 40 $\mu\text{g l}^{-1}$, and clear detrimental effects on phytoplankton at concentrations of ca. 80 $\mu\text{g l}^{-1}$, with large development of *Gammaproteobacteria*. Our results indicate that an oil spill of the Prestige's magnitude will have effects on the microbial resident community only at certain times of the year, while at higher PAH concentrations the effects might be more evident. For most of the year, the resident Ría de Vigo microbial communities appear to be accustomed to PAH concentrations such as those used in these experiments.

KEY WORDS: PAHs · Bacterioplankton · Production · Bacterial community structure · Mesocosms · Prestige oil

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INTRODUCTION

Bacterioplankton communities inhabiting the plankton of coastal regions use multiple sources of C and nutrients (e.g. Moran et al. 1999, Mou et al. 2008). These sources have different origins: autochthonous phytoplankton production, carbon and nutrients com-

ing from the watersheds, or allochthonous nutrient and C sources that have originated because of human activities. Petroleum hydrocarbons are some of these organic C sources, and are some of the most widespread contaminants in the environment (Santas et al. 1999). Polycyclic aromatic hydrocarbons (PAHs) are particularly influential because of their continuous

release into the water, persistence and well-known detrimental effects on marine invertebrates (Preston 2002) which make them a subject of public concern.

While bacteria are able to decompose these C molecules (e.g. Harayama et al. 1999, Kasai et al. 2002) and chronic PAH inputs to seawater may be considered just one allochthonous source of C and energy (Castle et al. 2006), immediately after an oil spill, the soluble fraction of PAHs is released into the water column and may represent point concentrations of PAHs much higher than the values measured in chronic exposures and thus affect bacterioplankton communities in ways different to just a regular C source. The effects of oil spills on the natural bacterioplankton communities of coastal ecosystems have been studied using *in situ* measurements (e.g. Bode et al. 2006), laboratory experiments (Maruyama et al. 2003, Castle et al. 2006, Cappello et al. 2007a) and experimental systems such as mesocosms (Siron et al. 1993, Ohwada et al. 2003, Cappello et al. 2007b). This last type of approach avoids the effects caused by advection, diffusion and mixing under natural conditions, and it also offers the possibility of comparison with natural populations to which PAHs have not been added, thus allowing statistical testing of hypotheses. The different types of analysis have reported contradictory results about the effects of oil spills on plankton communities. Often, no effect on primary producers has been found (e.g. Varela et al. 2006), while simultaneous significant effects on bacterial variables have been reported (Bode et al. 2006). In several experiments, bacteria have been shown to be stimulated (e.g. Delille & Siron 1993, Ohwada et al. 2003, Bode et al. 2006, Castle et al. 2006, Cappello et al. 2007b, Dalby et al. 2008), neutrally affected (Maruyama et al. 2003) or repressed (Garcia et al. 1998, Sargian et al. 2005) by high concentrations of PAHs. In most cases, a shift in bacterial community structure has been observed (Gerdtts et al. 2004, Castle et al. 2006, McKew et al. 2007), resulting in decreasing richness (Röling et al. 2002) and elevated contributions of *Gammaproteobacteria* (Gerdtts et al. 2004, Yakimov et al. 2004, Cappello et al. 2007b), particularly of groups such as *Alcalinovorax* (Cappello et al. 2007b) or *Cycloclasticus* (Kasai et al. 2002, Teira et al. 2007).

The concentration of PAHs used to experimentally simulate oil spills have ranged from 900 mg crude oil l⁻¹ (Cappello et al. 2007b) to 4.5 µg l⁻¹ chrysene equivalents (equiv.) (Ohwada et al. 2003), with all possible intermediate values. It might be possible that some of the reported effects of oil are concentration-dependent, so that they are significant at concentrations which are never experienced by natural bacteria assemblages. Accordingly, it can be hypothesized that if PAHs do not cause effects on microbial community structure and function, it is because the communities

are adapted to a certain level of these substances in the water as part of their normal C pools used for growth.

To explore this hypothesis, we used micro- and mesocosms to test the effect of PAHs on plankton communities of the Ría de Vigo. We tried to use realistic concentrations, i.e. those that were likely to be in the environment during the Prestige oil tanker accident (Bode et al. 2006, González et al. 2006). We used PAH concentrations (20 to 30 µg l⁻¹ chrysene equiv.) that were relatively low compared to other experiments (e.g. Cappello et al. 2007b), but were likely experienced by the plankton right after the Prestige accident (González et al. 2009).

The experiments were part of the project IMPRESSION, designed to test the effect of the Prestige oil spill on plankton communities of the Ría de Vigo. This coastal embayment is an area affected by a marked seasonal cycle of coastal winds (Nogueira et al. 1997). The annual cycle is divided into an upwelling season (March to September), with short relaxation intervals that enhance productivity (Álvarez-Salgado et al. 2002), and a downwelling season (October to March) characterized by low phytoplankton biomass and primary production. Microbial plankton composition is different in the contrasting situations (Nogueira et al. 1997, Figueiras et al. 2002, Teira et al. 2008). Thus, we explored the effects of oil on bacterial community structure and activity at each of the 4 relevant parts of the seasonal cycle: spring bloom, summer stratification, autumn upwelling and winter mixing.

MATERIALS AND METHODS

Experimental setup and sampling. The experimental setup was established in a small harbor of a protected bay, 4 times during a year. As detailed in Teira et al. (2007), 6 mesocosms (1.5 m in diameter, 2 m deep) were filled with seawater at the center of the Ría and transported to the harbor. The metal structure that held the mesocosms was rope-tied to a pontoon of the harbor. The mesocosm bags were filled through a 200 µm mesh in order to exclude mesozooplankton and facilitate good replication. Two of the mesocosms were used as controls, received no additions and were not treated in any way except for the 200 µm pre-filtration; 2 received a low concentration of soluble PAHs (5 to 10 µg l⁻¹ chrysene equiv.) and 2 a high concentration of soluble PAHs (approx. 20 to 30 µg l⁻¹ chrysene equiv.). The experiments lasted 9 d after the addition. An integrated tube sample was obtained from each mesocosm and brought to the lab. Sampling was done every day during the first 5 d, and thereafter every 2 d. As in the controls, there was no response in the low-concentration mesocosms; in the

subsequent experiments this treatment was eliminated and instead we triplicated the control and the high-PAH concentration treatments.

The PAHs were added as a soluble fraction which was prepared from 15 kg of Prestige-like heavy oil provided by the 'Oficina Técnica de Coordinación del Programa de Intervención Científica en la Catástrofe del Prestige' mixed with 300 l of 0.2 μm -filtered seawater. The mixture was vigorously stirred during 4 h and the resulting extract (approx. 700 $\mu\text{g l}^{-1}$ soluble PAHs) was separated from the insoluble fuel oil by decantation, collected in 25 l polyethylene barrels and added to the mesocosms. PAH concentrations were measured following the MARPOLMON protocol (IOC 1984), with modified volumes, and referred to a chrysene standard (see details in González et al. 2006).

The Prestige oil spill was found to consist of a complex mixture of hydrocarbons, where the aromatic fraction (mainly naphthalene, phenanthrene and alkyl derivatives) comprised ca. 53% (Alzaga et al. 2004). PAHs represented 99.7% of the water-soluble fraction of the Prestige oil and alkanes were almost undetectable in that fraction (J. Albaigés pers. comm.). Although it was not possible to use the Prestige oil, we used oil with a very similar composition.

Addition of oil was done after the Time 0 sample was taken. Sampling of the mesocosms was then done daily at sunset with an integrating tube that collected water down to 1 m above the bottom of the tanks.

The experiments were carried out during the 4 periods of the seasonal cycle in the coastal NE Iberian Atlantic (Nogueira et al. 1997): spring bloom (March 2005), summer stratification (July 2005), autumn upwelling (September 2005) and winter mixing (January 2006).

Additional microcosm experiments. As described by Teira et al. (2007), a microcosm experiment was run in parallel to the winter mesocosms using the same initial seawater in order to determine the response of the community to a larger gradient of PAH concentrations. For the microcosm experiment, ten 5 l PET bottles were kept opened and temperature was controlled by circulating surface seawater. A gradient of 4 concentrations (10, 20, 40 and 80 $\mu\text{g l}^{-1}$ chrysene equiv.) plus the control were prepared in duplicate. This is equivalent to 0.5, 1, 2 and 4 \times what was added in the simultaneous mesocosms. The microcosms were kept for 8 d, sampling every 24 h for bacterial abundances, bulk heterotrophic production and bacterial community structure analyses.

Organism abundances. Chlorophyll *a* (chl *a*) concentration was used as an estimator of phytoplankton biomass. Seawater (250 ml) was sequentially filtered through 20, 2 and 0.2 μm polycarbonate filters. The filters were frozen and chl *a* was extracted in 90%

acetone for 24 h at 4°C. The fluorescence of the extracted chl *a* was measured with a Turner-TD-700 fluorometer previously calibrated with pure chl *a*. Total chl *a* concentration was obtained by addition of the concentration in each of the 3 filter size classes.

Prokaryote abundance was measured by flow cytometry as described in Gasol & del Giorgio (2000). The subsamples were run in a Becton-Dickinson FACS-Calibur cytometer after staining with SYBR Green (10 \times final concentration, Molecular Probes), and bacteria were detected by their signature in a plot of side scatter (SSC) versus green fluorescence (FL1). Calibration of the cytometer, and of the SSC-size relationship, was done as described in Calvo-Díaz & Morán (2006). Since *Archaea* are not abundant in most temperate coastal marine sites (e.g. Alderkamp et al. 2006), prokaryote abundance and heterotrophic production will hereafter be referred to as bacterial abundance and production.

Samples for nanoflagellate abundance were collected 4 or 5 times in every experiment. Subsamples of 60 to 80 ml were fixed with glutaraldehyde (1%, final concentration). The samples were filtered through 0.6 μm black polycarbonate filters (Millipore), and stained with DAPI (Porter & Feig 1980) at a final concentration of 5 $\mu\text{g ml}^{-1}$ (Sieracki et al. 1995). Abundance of these microorganisms was determined at 1000 \times magnification with an Olympus BX40-102/E epifluorescence microscope. The heterotrophic and phototrophic nanoflagellates were counted under both UV (blue fluorescence) and blue excitation (B2 filter). Under blue light we discriminated phototrophic nanoflagellates (PNF, showing red-orange autofluorescence and/or plastidic structures) from colorless nanoflagellates classified as heterotrophic (HNF). With this method we could not distinguish mixotrophic nanoflagellates. Random 10 mm transects (100 μm width) were examined and between 20 and 100 HNF and between 10 and 50 PNF per filter were counted.

Bacterial activity and production. Bacterial heterotrophic production was estimated using both the ^3H -leucine and ^3H -thymidine (TdR) incorporation methods (Kirchman et al. 1985). Triplicate or quadruplicate aliquots of 1.2 ml were taken for each sample and 1 or 2 trichloroacetic acid (TCA)-killed controls. The Leu tracer was used at 40 nM and the TdR tracer at 20 nM final concentrations in incubations lasting approximately 2 h. The incorporation was stopped with the addition of 120 μl of cold 50% TCA to the samples and, after mixing, they were kept frozen at -20°C until processing, which was carried out by the centrifugation method of Smith & Azam (1992). The samples were counted on a Beckman scintillation counter, 24 h after addition of 1 ml of scintillation cocktail.

We used the standard conversion factors of 3.1 kg C mol Leu⁻¹ and 40 kg C mol TdR⁻¹, since we had previously determined that conversion factors (CFs) in the Ría were on average close to these theoretical ones (Morán et al. 2002). The final value of heterotrophic bacterial production was computed as the average of the Leu and TdR-based determinations.

Collection of community DNA. Microbial biomass was collected by sequentially filtering around 5 l of seawater through a 3 µm pore size polycarbonate filter (Millipore, 46 mm) and 0.2 µm Sterivex filter (Millipore). The Sterivex units were filled with 1.8 ml of lysis buffer (50 mM Tris-HCl pH 8.3, 40 mM EDTA pH 8.0, 0.75 M sucrose) and stored at -80°C. We used the Sterivex units for the analyses. Microbial biomass was treated with lysozyme, proteinase K and sodium dodecyl sulphate, and the nucleic acids were extracted with phenol and concentrated in a Centricon-100 filter (Millipore). Nucleic acids were extracted by a standard protocol using phenol/chloroform (see details in Schauer et al. 2003).

Fingerprinting analysis. DGGE and gel analysis were performed essentially as described previously (Schauer et al. 2000, Sánchez et al. 2007). Briefly, 16S rRNA gene fragments (around 550 bp in length) were amplified by PCR using the universal primer 907rm and the bacterial specific primer 358f, with a GC-clamp. PCR products were loaded on a 6% polyacrylamide gel with a DNA-denaturant gradient ranging from 40 to 80%. The gel was run at 100 V for 16 h at 60°C in 1× TAE running buffer. DGGE gel images were analyzed using the Diversity Database software (BIO-RAD). A matrix was constructed for all lanes taking into account the relative contribution of each band (%) to the total intensity of the lane. Based on this matrix, we obtained a dendrogram by the UPGMA clustering method (Euclidean distances, Statistica 6.0).

Bacterial community composition. We used CARD-FISH to study the composition of the bacterial communities at the start and end of the microcosm experiment. We followed the method as in Pernthaler et al.

(2002): 5 ml were fixed with paraformaldehyde (2% final concentration) and, after 12 to 18 h storage at 4°C in the dark, were filtered through a 0.2 µm polycarbonate filter (Millipore, GTTP, 25 mm filter diameter) supported by a cellulose nitrate filter (Millipore, HAWP, 0.45 mm), washed twice with Milli-Q water, dried and stored in a microfuge vial at -20°C until further processing. We used oligonucleotide probes specific for the domain *Eubacteria* (EUB338) (Amann et al. 1990), the *Alpha-* (ALF968) (Glöckner et al. 1999) and *Gammaproteobacteria* (GAM42a) (Manz et al. 1992) subclasses and the class *Flavobacteria* of phylum *Bacteroidetes* (CF319a) (Manz et al. 1996). We also used probe CYU829 for *Cycloclasticus* (Maruyama et al. 2003, Teira et al. 2007). The Eub antisense probe Non 338 was used as negative control. Further details are presented in Teira et al. (2008).

Statistical analysis. We used ANOVA to test for the differences between treatments. A repeated measures ANOVA with 1 between-subjects factor (Treatment) was conducted to discriminate the treatment from the time effects and all possible interactions. Time was a within-subject factor because the same mesocosm was sampled at sequential time periods (every 24 to 48 h). A 1-way ANOVA was performed to determine differences in the microcosms experiment.

RESULTS

The initial environmental conditions were different in each season (Table 1). In winter and spring, inorganic nutrients were present in high concentrations. In autumn, chl *a* was very high and dissolved inorganic nitrogen (DIN), but not silicate, was also high, since our sampling coincided with the decline of an algal bloom (Fig. 1C). Bacterial abundance and heterotrophic production were high in summer and autumn.

The added PAHs disappeared from the water in a similar exponential way during the 4 experiments (Fig. 1). On Day 2 of the experiments, only ca. 35% of

Table 1. Initial conditions of the mesocosm experiments with respect to temperature (T, °C), salinity (Sal), dissolved inorganic nitrogen (DIN, µM), dissolved inorganic phosphorus (DIP, µM), silicate (SiO₄, µM), particulate organic carbon (POC, µM), particulate organic nitrogen (PON, µM), chlorophyll *a* concentration (chl *a*, mg m⁻³), total bacterial abundance (BA, ×10⁵ cell ml⁻¹) and bacterial heterotrophic production (BHP, µg C l⁻¹ d⁻¹; average of Leu and TdR with standard conversion factors, 3.1 kg C mol Leu⁻¹ and 40 kg C mol TdR⁻¹). Data are presented as means ± SE. N (number of replicates) = 6

Expt	Date (dd/mm/yy)	T	Sal	DIN	DIP	SiO ₄	Chl <i>a</i>	BA	BHP
Spring	02/03/05	10.5 ± 0.0	35.48 ± 0.02	4.40 ± 0.08	0.52 ± 0.00	3.17 ± 0.04	5.50 ± 0.55	6.13 ± 0.18	3.05 ± 0.4
Summer	02/07/05	20.8 ± 0.0	35.02 ± 0.01	0.58 ± 0.11	0.15 ± 0.01	0.59 ± 0.05	2.60 ± 0.12	9.92 ± 0.72	69.94 ± 3.4
Autumn	22/09/05	15.4 ± 0.0	35.73 ± 0.01	5.66 ± 0.73	0.51 ± 0.08	0.41 ± 0.02	6.21 ± 0.71	13.82 ± 0.40	26.25 ± 8.11
Winter	24/01/06	12.4 ± 0.0	35.60 ± 0.00	7.70 ± 0.44	0.48 ± 0.02	3.72 ± 0.15	0.95 ± 0.02	5.29 ± 0.06	16.88 ± 0.31

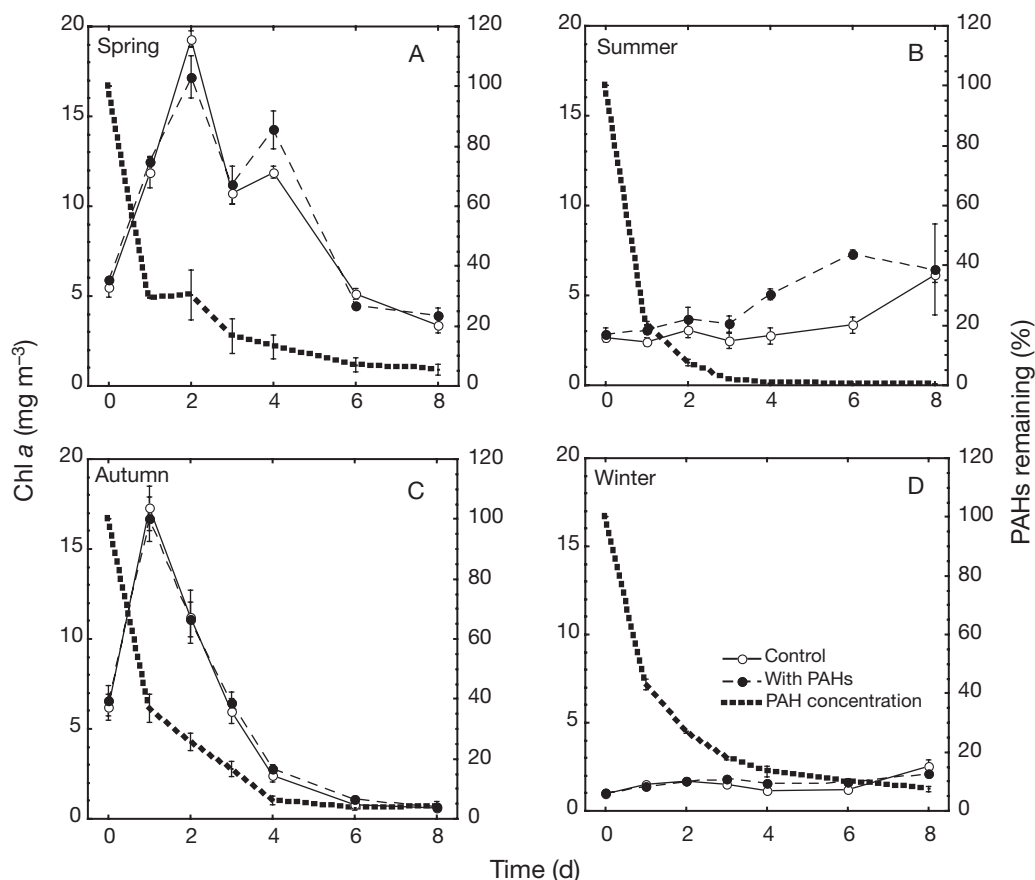


Fig. 1. Changes in dissolved polycyclic aromatic hydrocarbon (PAH) concentration and in chlorophyll *a* (chl *a*) concentrations in the control (open circles) and treatment (filled circles) mesocosms for (A) spring, (B) summer, (C) autumn and (D) winter. Data presented are means \pm SD of 2 (spring) or 3 (other seasons) replicates

the added PAHs were found in solution except in summer, where the high ambient temperature caused faster volatilization of the added compounds and only 10% remained in the water (Fig. 1). In summer, no PAHs were detected after Day 4, while in the other seasons between 5 and 15% of the added substrates remained in the mesocosms at Day 4. The PAHs exponential decay rate was similar in all the experiments (around -0.35 d^{-1}), except in the summer experiment when it was higher (-0.7 d^{-1} , Table 2).

Chl *a*

The pattern of chl *a* change in the mesocosms differed between experiments and no obvious general effect of oil could be discerned. In spring (Fig. 1A), water confinement caused a bloom (up to $10 \text{ mg chl } a \text{ m}^{-3}$), probably because we had sampled growing healthy algae. In autumn, in contrast, the starting concentration of chl *a* was 9 mg m^{-3} ; this decreased drastically during the 4 subsequent days. In summer and winter there were few changes in chl *a* during the experiments, just small, slow

increases. The oil effects were only significant ($p < 0.01$) in the summer experiment (Fig. 1B, Table 3): the increase of chl *a* observed in the oil-added mesocosms after Day 4 and towards the end of the experiment was higher (ca. 2 \times) than in the control mesocosms (Fig. 1B).

Table 2. Conditions of the treatments with added polycyclic aromatic hydrocarbons (PAHs) in the different experiments. Microcosm experiments occurred simultaneously to the winter 2006 mesocosms (see text for further explanations). Data are the average of 2 or 3 replicates \pm SD

Expt	Date/ oil conc.	Initial PAH concentration ($\mu\text{g l}^{-1}$)	Exponential decay rate (d^{-1})
Spring	02/03/05	14.95 ± 5.59	-0.370 ± 0.024
Summer	02/07/05	29.03 ± 6.08	-0.714 ± 0.015
Autumn	22/09/05	23.63 ± 6.11	-0.415 ± 0.045
Winter	24/01/06	18.51 ± 4.68	-0.326 ± 0.014
Microcosms (Winter)	0.5 \times	9.60 ± 0.99	-0.231 ± 0.009
	1 \times	19.81 ± 0.42	-0.241 ± 0.001
	2 \times	38.85 ± 0.64	-0.234 ± 0.003
	4 \times	73.21 ± 2.97	-0.212 ± 0.009

Table 3. Results of the repeated measures ANOVA with 1 within-subject factor (Time) and 1 between-subjects factor (Treatment). N = 2 to 3 for the mesocosm experiments, and N = 4 for the microcosm experiment. Significant ($p < 0.05$) effects are in **bold**

	Treatment		Time (Treatment)	
	F	p	F	p
Chlorophyll a				
Spring	0.43	0.525	68.04	<0.01
Summer	9.81	<0.01	4.09	<0.01
Autumn	0.11	0.733	45.55	<0.01
Winter	3.21	0.084	15.63	<0.01
Microcosms	130.85	<0.01	79.49	<0.01
Total bacterial abundance				
Spring	16.10	<0.01	85.07	<0.01
First half	1.18	0.31		
Second half	32.91	<0.01		
Summer	35.08	<0.01	7.59	<0.01
First half	1.54	0.24		
Second half	37.29	<0.01		
Autumn	5.95	0.021	4.67	<0.01
First half	1.34	0.29		
Second half	60.89	<0.01		
Winter	20.31	<0.01	32.60	<0.01
First half	2.25	0.15		
Second half	8.88	0.02		
Microcosms	727.79	<0.01	94.81	<0.01
Bacterial production				
Spring	4.71	0.47	55.65	<0.01
Summer	43.54	<0.01	5.44	<0.01
Autumn	2.35	0.136	9.43	<0.01
Winter	0.215	0.646	2.73	0.014
Microcosms	325.44	<0.01	87.15	<0.01
Bacterial growth rate				
Spring	0.56	0.47	103.09	<0.01
Summer	0.92	0.36	6.55	<0.01
Autumn	3.98	0.06	5.21	<0.01
Winter	5.46	0.03	12.82	<0.01
Microcosms	4.39	<0.01	18.53	<0.01

Bacterial abundance

The changes in bacterial abundances also showed contrasting trends in the different seasons (Fig. 2) and no obvious general effect of oil could be discerned, except in the last days of each experiment. With the exception of the autumn experiment, there was a general initial increase in abundance (Day 1) that was soon followed by a decrease in bacterial abundance (Days 3 to 4) and by a second peak, higher in magnitude, at about Day 6 to 8. The autumn experiment showed just 1 peak. The effect of oil was clear in the summer experiment (Fig. 2b): the 2 peaks were higher in magnitude when PAHs had been added. When separating the first (Days 0 to 4) from the second (Days 5 to 8) part of the experiments, the PAH treatments affected the second

part of all the experiments (Table 3), particularly evident in the autumn experiment.

Bacterial production

Thymidine and leucine incorporation were positively correlated throughout the experiments (TdR = $51.9\text{Leu}^{0.69}$, $r^2 = 0.56$, $N = 165$), with an average Leu:TdR ratio of 30.4 ± 2.8 . This ratio was lower (average = 12.8 ± 1.2) in summer and larger in spring. It was relatively stable throughout all the experiments in summer and winter, but increased in the spring experiment (46.0 ± 8.9) and decreased in the autumn experiment. Using repeated measures ANOVA, the only significant differences in the Leu:TdR ratio in treated versus control mesocosms were observed in summer (data not shown), when it was significantly lower in the mesocosms that had received PAHs (9.7 ± 0.7) than in those that had not (13.7 ± 0.9).

Bacterial production was much higher in the autumn experiment than in the rest of the experiments (Fig. 3), in concordance with the fact that this experiment coincided with the end of a phytoplankton bloom that slowly decomposed during our mesocosm experiment (Fig. 1C). In general, bacterial production tended to reproduce the same pattern as bacterial abundances (Fig. 2), with an initial and a secondary peak after Day 4. This pattern was evident in the spring and winter experiments (Fig. 3A,D), slightly less clear in the summer experiment and did not hold in the autumn experiment (Fig. 3C). Differences between treatment and control were significant in the summer experiment (Fig. 3B, Table 3); the PAH-treated mesocosms had bacterial production values about 1.5-fold higher than the controls.

Bacterial growth rates showed a pattern similar to that of production, with initial values ranging from 0.3 (spring) to 3 d^{-1} (summer) (data not shown). There was only a slight initial effect of oil on bacterial growth rates in the winter experiment, when they were slightly faster in the oil treatments than in the controls (ca. 25% decrease in growth rate).

Heterotrophic nanoflagellates

Flagellates were enumerated in the first 3 experiments and the pattern was quite similar: they first increased up to Day 3 or 4, and then decreased until the end of the experiment (see example in Fig. 4). The peak of HNF abundance was reached at different times in the different seasons, at Day 4 in the spring experiment (Fig. 4), and at Day 2 in the summer and autumn experiments. At the peak of flagellate devel-

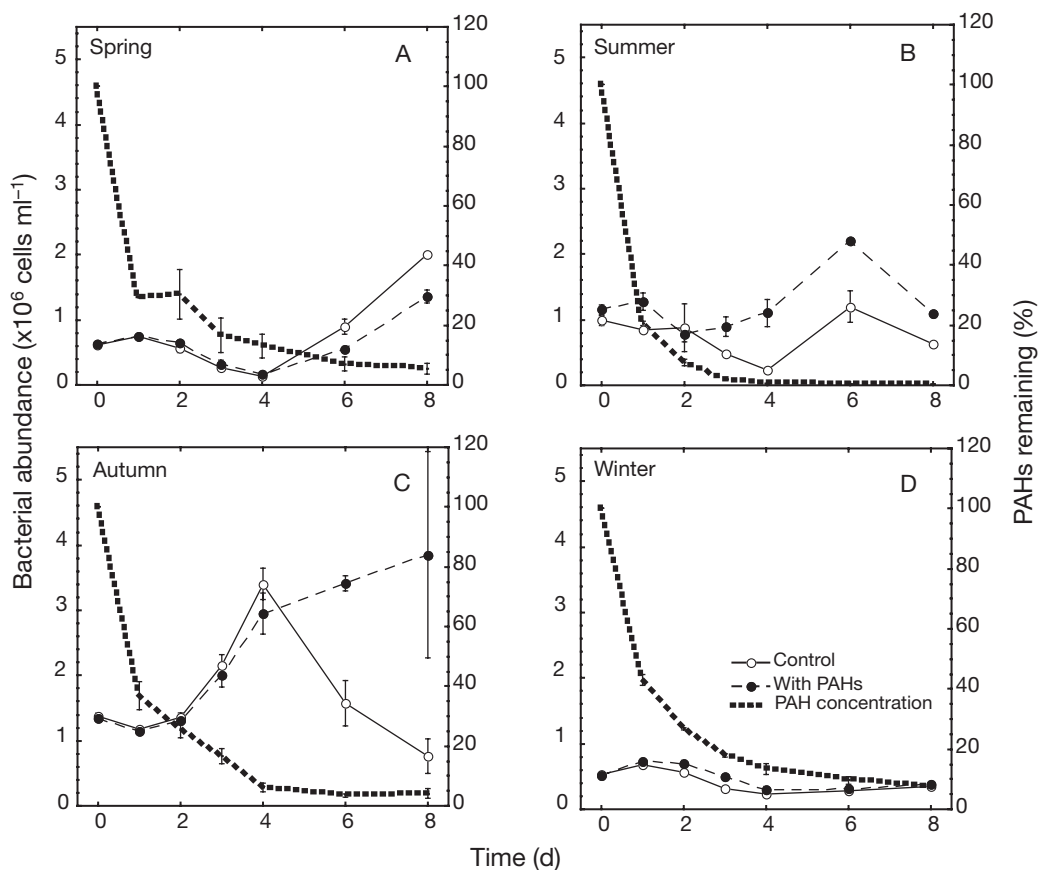


Fig. 2. Changes in dissolved polycyclic aromatic hydrocarbons (PAH) concentration and in bacterial abundance in the control (open circles) and treatment (filled circles) mesocosms for (A) spring, (B) summer, (C) autumn and (D) winter. Data presented are means \pm SD of 2 (spring) or 3 (other seasons) replicates

opment, the abundance was higher in the mesocosms (ca. double) that had received PAHs than in the controls (see example for the spring experiment in Fig. 4).

Microcosm experiment

In the winter season we ran a microcosm experiment simultaneous to the mesocosm experiments. As we have shown before (Teira et al. 2007), the changes of bacterial abundance were similar in the mesocosms and the microcosms that had received the same oil additions. PAHs, however, disappeared more slowly from the microcosms (Fig. 5A) than from the mesocosms (Fig. 1D), probably because the water surface in contact with the atmosphere was smaller in the case of the microcosms. PAH concentrations had an overall negative effect on chl *a* that was much stronger at $80 \mu\text{g l}^{-1}$ (Fig. 5B, Table 3). Bacterial abundance was also affected by the added PAHs. The decrease in the bacterial peak occurred later the more PAHs had been added and did not occur when the maximal concentra-

tion of $80 \mu\text{g l}^{-1}$ was added (Fig. 5C). Bacterial production did not show significant differences between treatments until Day 4, when the $80 \mu\text{g l}^{-1}$ sample increased tremendously (Fig. 5D). Bacterial growth rates were significantly affected by the oil addition (data not shown). HNF developed much more in the control of the microcosms (up to $3500 \text{ cells ml}^{-1}$) than in the PAH treatments, which were all rather similar and had few changes in HNF concentration (ca. 1500 ml^{-1} , with a slight increase towards the end of the experiment; data not shown).

Bacterial community structure and estimates of richness

Fig. 6 shows 2 examples of the fingerprints of bacterial community structure in the mesocosms. In 3 cases (spring, autumn and winter), the band patterns showed no differences between control and PAH-added mesocosms, as exemplified by the March DGGE gel and the corresponding dendrogram (Fig. 6A). Only in

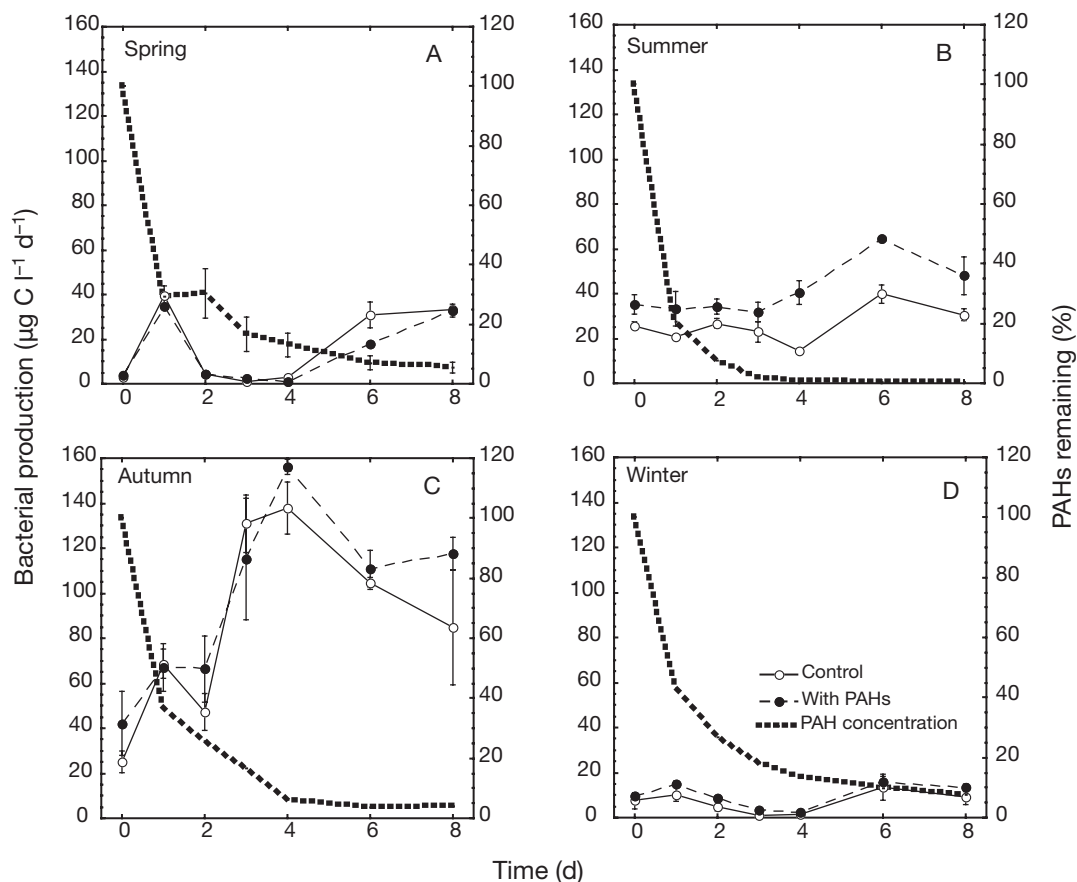


Fig. 3. Changes in dissolved polycyclic aromatic hydrocarbon (PAH) concentration and in bacterial production (leucine incorporation) in the control (open circles) and treatment (filled circles) mesocosms for (A) spring, (B) summer, (C) autumn and (D) winter. Data presented are means \pm SD of 2 (spring) or 3 (other seasons) replicates

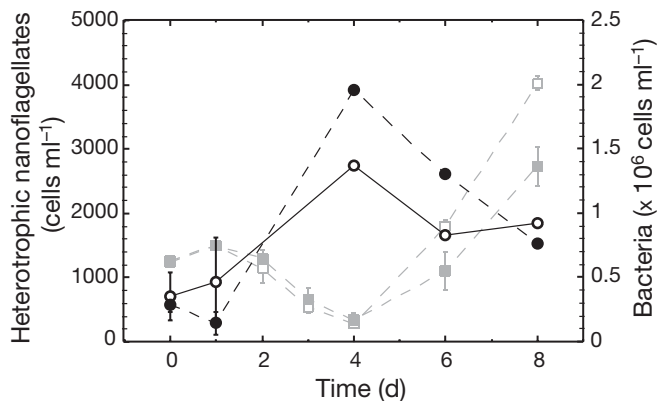


Fig. 4. Changes in heterotrophic nanoflagellate abundance in the control (open circles) and the oil-added treatments (filled circles) in the spring experiment. Data presented are means \pm SD of 2 replicated mesocosms. Bacterial abundances in the same control (open squares) and treatment (filled squares) mesocosms are shown for reference (same data as in Fig. 2A)

the case of the summer experiment was there a detectable oil effect (Fig. 6B), the dendrogram showing a cluster of samples labeled 'A' (PAH addition) after Day 4. The presence and absence of bands in the DGGE analysis was used to estimate bacterial richness of each sample. Band number varied between 7 and 17, with an overall average of 13. Differences in band richness were not significant for any of the experiments, except for the summer where we detected higher richness in the PAH-amended mesocosms, particularly in the first part of the experiment.

The DGGE analysis and the associated dendrogram of the microcosm experiment showed at the end of the experiment a clear ordering according to the added oil concentration (Fig. 6C). The samples from Day 7 all clustered together, however, indicating that the time course was probably more important than PAH addition in determining community structure. The lowest richness was detected with the highest PAH concentration and differences were significant with respect to the other times (1-way ANOVA, $p < 0.01$).

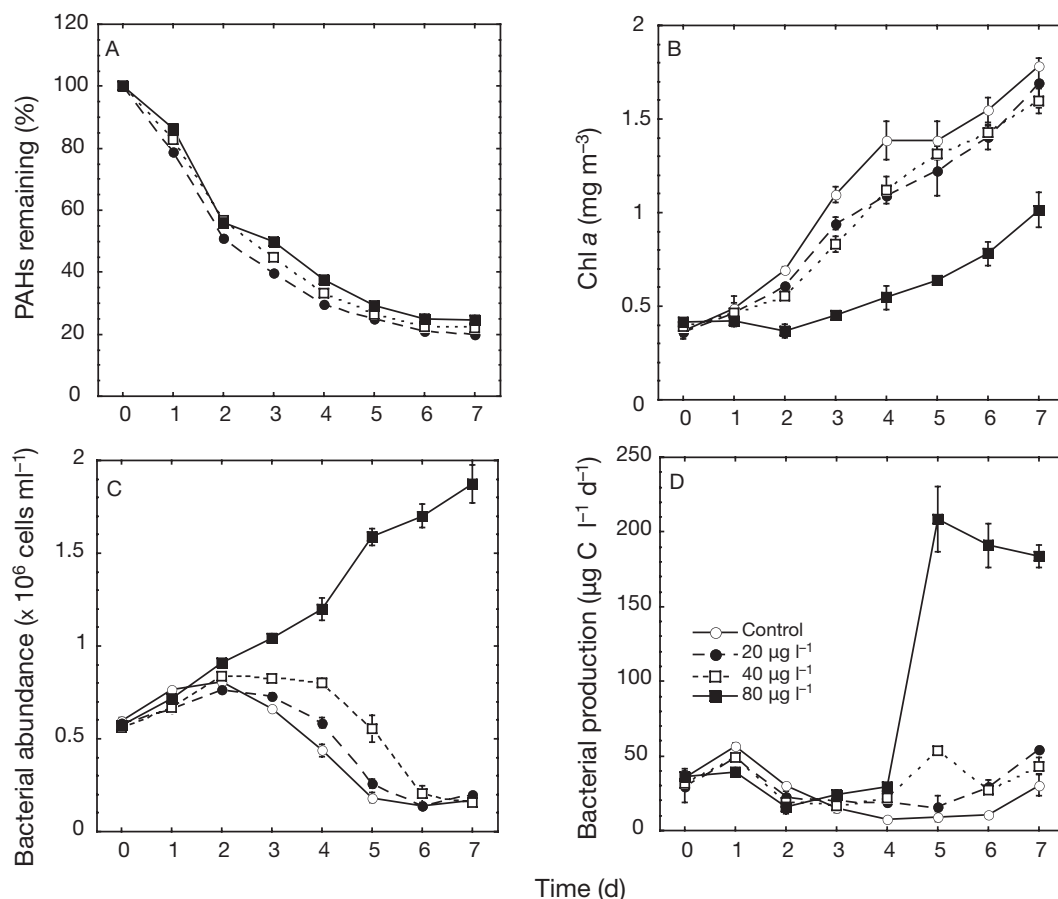


Fig. 5. Time course changes in the microcosm experiments: (A) polycyclic aromatic hydrocarbon (PAH) concentration, (B) chlorophyll a, (C) bacterial abundance and (D) bacterial production. A gradient of 4 concentrations is presented: control (○), 20 μg l⁻¹ (●), 40 μg l⁻¹ (□) and 80 μg l⁻¹ (■). For simplicity, the ca. 10 μg l⁻¹ treatment is omitted

In the microcosm experiments we also followed the changes in bacterial subgroup abundance using CARD-FISH. In Table 4 we show the initial and final values. *Alphaproteobacteria* abundances had similar decreases (ca. 1×10^5 cells ml⁻¹) in all treatments except in the 2× treatment, where it was lower (5×10^4 cells ml⁻¹). *Bacteroidetes* showed a higher decrease in the tank that was amended with the highest concentration of PAHs (Table 4). The most important changes occurred, however, in the *Gammaproteobacteria*, which slightly decreased in the treatment that received no PAHs, but increased greatly in the treatment that had received most PAHs. Thus, community structure in the microcosms that received PAHs shifted from ca. 50% *Bacteroidetes*, 35% *Alphaproteobacteria* and 15% *Gammaproteobacteria* to 20% *Bacteroidetes*, 10% *Alphaproteobacteria* and 73% *Gammaproteobacteria*. Community structure also shifted in the microcosms that did not receive PAHs, but the large decrease in *Bacteroidetes* (50 to 14%) was accompanied by a smaller dominance of *Gammaproteobacteria* (55%).

Table 4. Composition of the bacterial assemblage in the microcosm experiment determined by CARD-FISH. Concentrations (cells ml⁻¹) at the beginning (t_0) and end (t_t) of the experiment are given for a gradient of PAH concentrations: control (0×), 20 mg l⁻¹ chrysene equiv. (1×), 40 mg l⁻¹ (2×) and 80 mg l⁻¹ (4×). Change in abundance (abund.) = cells produced (or lost) during the experiment (±SD)

	t_0	t_t	Change in abund.
<i>Alphaproteobacteria</i>			
0×	1.48×10^5	3.31×10^4	-115000 ± 5300
1×	1.32×10^5	0.68×10^4	-126000 ± 6000
2×	0.76×10^5	1.70×10^4	-59000 ± 1700
4×	1.63×10^5	6.36×10^4	-99000 ± 5100
<i>Gammaproteobacteria</i>			
0×	9.25×10^4	0.76×10^5	-16000 ± 800
1×	5.09×10^4	1.97×10^5	146000 ± 13000
2×	4.07×10^4	1.29×10^5	88000 ± 6000
4×	8.14×10^4	6.87×10^5	606000 ± 44000
<i>Bacteroidetes</i>			
0×	2.50×10^5	0.18×10^5	-232000 ± 14000
1×	1.83×10^5	0.51×10^5	-132000 ± 5700
2×	1.68×10^5	0.64×10^5	-104000 ± 2800
4×	1.83×10^5	1.11×10^5	-719000 ± 2700

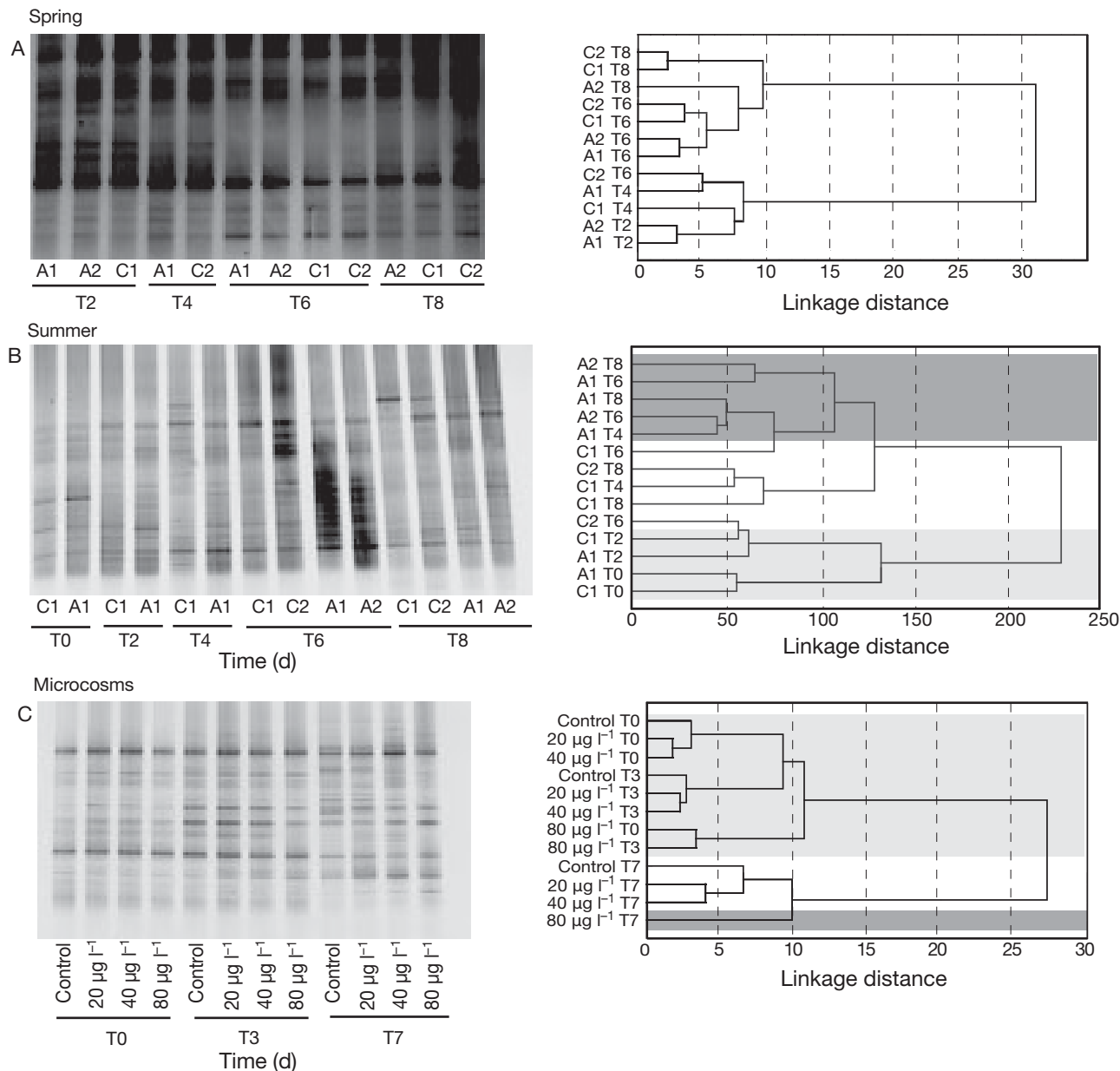


Fig. 6. Examples of DGGE gels of bacterial 16S rRNA gene fragments from (A) spring, (B) summer and (C) microcosm experiments and dendrogram classification (Ward's method, Euclidean distances according to the band pattern). DGGE lanes represent the different treatments: control mesocosms (C1 and C2) and polycyclic aromatic hydrocarbon (PAH)-amended mesocosms (A1 and A2). Sampling time is given in days (T0, T2, T4, T6 and T8). In the microcosm experiment, the different PAH concentrations are control, 20, 40 and 80 $\mu\text{g l}^{-1}$, and the sampling time is given by T0, T3 and T7. Light-grey bands represent the initial times (T0–T3) in the different treatments; dark grey bands represent the PAH-amended treatments (Panels B) and the 80 $\mu\text{g l}^{-1}$ microcosm (>T4 in Panels C)

DISCUSSION

We used a mesocosm approach to test for the effects of realistic additions of oil on bacterial function and assemblage structure. We repeated the experiment 4 times at different phases of the seasonal cycle of Ría de Vigo to discern whether the assayed levels of PAHs affected microbial communities at all times or only de-

pending on the initial microbial communities or environmental settings. Furthermore, we also ran a variable-concentration experiment to find the threshold generating significant effects. The 4 experiments were run at 4 different conditions: spring with a starting phytoplankton bloom, summer with little development of algae, autumn with a declining algal bloom and winter with low chlorophyll values. The fact that the

algal communities maintained constant populations in the mesocosms in summer and winter indicates that our sampling and filling protocols were accurate and did not disturb the communities too much.

Our aim in the present study was to determine the effects of an oil spill on the resident bacterial community of Ría de Vigo following the accident of the Prestige oil tanker on the Galician coast in November 2002. Our objective was to test to what extent the bacterial assemblages of Ría de Vigo were pre-adapted to the oil concentrations likely to be found in an oil spill accident. We hypothesized that if the assemblages did not change in diversity nor in function after point additions of PAHs, it would indicate that the assemblages were accustomed to the presence of similar PAHs concentrations in the waters, and that they could use and metabolize this type of allochthonous C.

The concentrations of PAHs measured after similar oil spill accidents are very variable (as are in fact the methodologies used to measure the concentration). After the oil spill in Paraíso Bay, Antarctica, values of 50 to 100 $\mu\text{g l}^{-1}$ were measured, while the values measured after the Exxon Valdez spill were much lower, ca. 10 to 30 ng l^{-1} (González et al. 2006). The values measured in the area of the Prestige oil spill were similar to our additions (20 to 30 $\mu\text{g l}^{-1}$, González et al. 2009). One month after the accident, however, they were ca. 20% the initial values (González et al. 2006); the same pattern was found in our meso- and microcosm experiments (at Day 3 they were about 20% the values that we had added initially).

It is difficult to compare different units of PAH concentrations that have been previously reported because not all the units are interchangeable. In any case, the values used in our experiment were generally much lower than those used in the past in experimental studies. Siron et al. (1993), for example, used a range of oil concentrations of 0.7 to 4.4 mg l^{-1} in mesocosms, and Kasai et al. (2001) measured values of 0.04 to 0.17 $\text{mg total petroleum hydrocarbons l}^{-1}$. Some authors have used large crude oil concentrations (900 mg l^{-1} , Cappello et al. 2007b; 100 mg l^{-1} , Cappello et al. 2007a) and Castle et al. (2006) used extremely high concentrations of one PAH molecule (naphthalene, 6.4 g l^{-1}). When crude oil is added, the real concentration of PAHs that microorganisms experience will be much smaller, as not everything in crude oil are PAHs. In our experiments, we chose values (Table 2) similar to what was detected *in situ* after the Prestige oil spill, and below what had been used in previous similar studies; these concentrations should be a more realistic representation of what the Ría de Vigo bacterial community experienced.

Our results indicate that the concentrations of PAHs tested in the mesocosms appeared to affect bacterial

abundance in all experiments while, however, chlorophyll and bacterial production were only affected significantly during the summer experiment, and growth rates were not affected significantly (Table 3). This contrasting result (i.e. effects on abundance and no effects on the rest of the variables) can perhaps be explained by the fact that the effects on bacterial abundance were only significant in the second part of the experiment (Table 3) after flagellates had grazed down the community and had, presumably, affected its composition (see below). It is possible that the organisms growing during this secondary peak are affected by the oil while those growing initially are not.

That the effects were stronger in summer, a period of less available inorganic nutrients (Table 1), may be due to increased mineralization activity by bacteria stimulated by the introduced PAHs, which could generate more inorganic nutrients, in turn stimulating algal growth. In contrast, the excess of nutrients in the water in winter would mask the increase caused by increased bacterioplankton mineralization. It is interesting to note that we found the highest effect in the season in which PAHs disappeared faster from the water (0.70 d^{-1} in summer and 0.35 d^{-1} in the other seasons), probably due to the higher temperatures (Table 1). Temperature also plays a significant role in controlling the nature and extent of microbial hydrocarbon metabolism (Nedwell 1999), and directly affects both the rate of biodegradation (Brakstad & Bonaumet 2006) and the physicochemical behaviour of oil hydrocarbons, such as viscosity, diffusion and volatilization, which changes oil composition and bioavailability of the water-soluble components (Northcott & Jones 2000, Rowland et al. 2000). Coulon et al. (2007) showed that a change in temperature had a much more pronounced effect on the oil-degrading microbial communities than nutrient additions.

The summer season in the NW Iberian zone is characterized by upwelling due to the dominance of north winds over the adjacent shelf. Surface water leaves the Ría moving offshore and this water is replaced by subsurface oceanic Eastern North Atlantic Central Water, which enters the Ría and has a profound effect on its hydrography (e.g. Álvarez-Salgado et al. 1993). The bacterial assemblages at this time of the year, previously surviving in deep oceanic waters, are probably less adapted to the amounts of allochthonous organic matter present in the Ría. In fact, using CARD-FISH we detected the presence of some *Betaproteobacteria* (typically considered freshwater groups, Methé et al. 1998) during all seasons except summer, and the *Gamma*proteobacteria PAH-degrading genus *Cycloclasticus* was detected in September and January, but not in summer (Alonso-Gutiérrez et al. 2009). In addition, the SAR11 subgroup of the *Alphaproteobacteria*

was not detected in summer, consistent with the deep-water origin of the bacterial assemblage (SAR11 decreases at mesopelagic depths, e.g. Baltar et al. 2007).

The microcosm experiments ran in parallel to the January mesocosm demonstrate that the lack of effect of the added PAH concentrations in the spring, autumn and winter experiments was most probably due to PAH concentrations that were too low. In those microcosms, ca. 20 $\mu\text{g l}^{-1}$ of PAHs had no effect, while twice this amount was detectable and 4 \times this amount had a pronounced effect on the microbial communities, decreasing algal development, increasing bacterial abundance and production and decreasing bacterial richness. PAH addition stimulated bacterial abundance and production both in the summer experiment and in the microcosm experiments (Figs. 2 & 5), but had contrasting effects on chlorophyll development, which was stimulated in summer and depressed in the microcosms. A previous study reported a stimulatory effect of oil addition to autotrophs but a toxic effect at higher concentrations; however, the bacterial response was positive at all concentrations (Koshikawa et al. 2007).

Inspection of the DGGE gels indicated that there were significant changes in bacterial community structure caused by the PAH additions only in the summer experiment, when an increase in the number of bands was observed (estimate of bacterial richness). This result differs from the previous idea that hydrocarbon-polluted areas should have low diversity and a high dominance of a few bacterial groups (Harayama et al. 1999, MacNaughton et al. 1999, Kasai et al. 2001, R ling et al. 2002), and from the results of Sch fer et al. (2000), who reported that experimental manipulations should reduce the diversity of microbial communities. Since the experimentally added PAHs can be considered as a C source, in addition to other C sources present in the water, it could be speculated that increased richness would indicate the appearance of more niches for bacterial development when the PAHs were added. Interestingly, in the microcosm experiments we observed an increase in richness with the additions of 20 and 40 $\mu\text{g l}^{-1}$ as compared to the control, but a decrease in richness with the highest concentration (80 $\mu\text{g l}^{-1}$). The overall structure of the community changed little, however, as detected by DGGE (Fig. 6C), and it seemed to do so more at the end of the experiment, when the 80 $\mu\text{g l}^{-1}$ treatment was more distinct from the control treatment.

This is in contrast with the CARD-FISH results (Table 4), which showed a clear increase in abundance of *Gammaproteobacteria* in the 4 \times (80 $\mu\text{g l}^{-1}$) treatment. This pattern was also apparent, but much lower in magnitude, in the 1 and 2 \times treatments. This discrepancy is maybe due to the fact that the high addition treatment presented much higher total bacterial abun-

dances compared to the other treatments. The abundances of *Bacteroidetes* and *Alphaproteobacteria* decreased in all treatments, but with contrasting patterns: *Alphaproteobacteria* decreased more at low than at high oil additions, while *Bacteroidetes* were replaced by *Gammaproteobacteria* particularly at the highest PAH addition. These results at times seem to contradict a previous analysis (Teira et al. 2008); however, that analysis was based on the percent contribution of each group to total bacterial abundance (DAPI counts), whereas the present study used absolute abundance.

Gammaproteobacteria are generally dominant after oil additions to seawater (e.g. Kasai et al. 2001, R ling et al. 2002, Cappello et al. 2007b, McKew et al. 2007), 2 types in particular: *Alcanivorax*, which were shown to dominate in oil-contaminated seawater when nutrients were adequately supplied (Kasai et al. 2002), and the aromatic hydrocarbon decomposer *Cycloclasticus pugetii*, which dominated after the Nakhoda oil spill (Maruyama et al. 2003). Since the type of oil we used was rich in PAHs and poor in aliphatics, we expected to see the development of *Cycloclasticus* spp. Indeed, an increase of *Cycloclasticus* spp. bacteria in the 4 mesocosm experiments, reaching different levels depending on the season, was reported by Teira et al. (2007), and in the microcosm experiments in the present study this organism showed a proportional increase depending on the amount of added PAHs. The *Cycloclasticus* genus is well known for serving a key role in PAH degradation (Kasai et al. 2002, Yakimov et al. 2004).

For the analyses of bacterial community structure, we used the DGGE approach, as other studies have done in oil-polluted marine environments (Macnaughton et al. 1999, Kasai et al. 2001, Ogino et al. 2001, Castle et al. 2006). Bacterial richness (as estimated by DGGE) or community structure was only affected in the summer experiment. Observation of the DGGE band patterns (Fig. 6) and the statistical analyses reported in Table 3 indicates that Time was the most important factor determining the changes in bacterial community structure in the experiments, and was also one of the main factors determining the changes in chl *a*, bacterial abundance and production (Table 3). Bacterial community structure changed in our mesocosm experiments as they did in many other published mesocosm experiments (e.g. Lebaron et al. 1999, Pinhassi et al. 2006, Allers et al. 2007). Bacteria had a small initial positive abundance response to the mesocosm startup (Days 1 to 2), then decreased in abundance (Days 3 to 4) and increased again towards the end of the experiments. It is worth noting that it was this second bacterial peak (the one after Day 4) that was most affected by oil (see Table 3).

There is still disagreement as to whether the abundance, productivity and diversity of pelagic bacteria are determined mainly by predators or nutrients (Pernthaler 2005). Grazing has been shown to influence bacterial community composition in laboratory and field experiments (van Hannen et al. 1999, Gasol et al. 2002, Simek et al. 2002). Allers et al. (2007), in a mesocosm experiment with Mediterranean waters, found a succession of bacterial communities. The initial abundance peak consisted of predominantly *Alteromonadaceae*, with very similar phylotypes in the different treatments. These organisms decreased concomitantly with an increase in HNF numbers and afterwards different *Rhodobacteraceae* phylotypes developed in the various treatments, particularly those that had received P additions. If the bacterial assemblages of coastal waters are composed of rather stable populations with peaks of fast-growing organisms showing rapid temporal fluctuations, manipulation of the ecosystem might destabilize it and promote the growth of these fast-growing populations, which will be responsible for changes in bacterial production and community structure (Pernthaler 2005).

CONCLUSIONS

PAHs, which are known to have toxic effects on organisms are also, however, a significant allochthonous C source for bacteria. Our microcosm study showed that the addition of PAHs in concentrations higher than those that were present in the seawater after the Prestige oil tanker accident resulted in a change in bacterial community structure. However, the mesocosm experiments showed that, in 3 of the 4 seasons assayed, the natural assemblage seldom reacted to the PAHs added, perhaps because the added oil concentration was a negligible C source as compared to the total bioavailable dissolved organic C. In these instances, the oil did not produce significant changes in community structure, probably indicating that for a large part of the year the resident communities are pre-adapted to the presence of oil in the water. Furthermore, Teira et al. (2007) observed that PAH additions triggered short-lived peaks of *Cycloclasticus* in the Ría de Vigo; it is obvious that the organisms with the potential to use the allochthonous added substances (e.g. *Cycloclasticus*) were present, albeit at very low abundances. The response of communities in micro- or mesocosm studies is more likely to shed light on the true behaviour of natural microbial communities when realistic oil or PAH concentrations are added in experiments. The addition of larger concentrations, like the ones tested here in the microcosms, might however, be useful to describe the toxic effects of the allochthonous substances.

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