Environmental Microbiology (2007) doi:10.1111/j.1462-2920.2007.01373.x

Dynamics of the hydrocarbon-degrading *Cycloclasticus* bacteria during mesocosm-simulated oil spills

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Summary

We used catalysed reported deposition – fluorescence *in situ* hybridization (CARD-FISH) to analyse changes in the abundance of the bacterial groups *Alphaproteobacteria*, *Gammaproteobacteria* and Bacteroidetes, and of hydrocarbon-degrading *Cycloclasticus* bacteria in mesocosms that had received polycyclic aromatic hydrocarbons (PAHs) additions. The effects of PAHs were assessed under four contrasting hydrographic conditions in the coastal upwelling system of the Rías Baixas: winter mixing, spring bloom, summer stratification and autumn upwelling. We used realistic additions of water soluble PAHs (approximately 20–30 μg l^{-1} equivalent of chrysene), but during the winter period we also investigated the effect of higher PAHs concentrations (10–80 μg l^{-1} chrysene) on the bacterial community using microcosms. The most significant change observed was a significant reduction (68 ± 5%) in the relative abundance of *Alphaproteobacteria*. The magnitude of the response of *Cycloclasticus* bacteria (positive with probe CYPU829) to PAHs additions varied depending on the initial environmental conditions, and on the initial concentration of added PAHs. Our results clearly show that bacteria of the *Cycloclasticus* group play a major role in low molecular weight PAHs biodegradation in this planktonic ecosystem. Their response was stronger in colder waters, when their background abundance was also higher. During the warm periods, the response of *Cycloclasticus* was limited, possibly due to both, a lower bioavailability of PAHs caused by abiotic factors (solar radiation, temperature), and by inorganic nutrient limitation of bacterial growth.

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Introduction

Organic pollutant contamination is a constant problem in many coastal waters adjacent to urban areas. In addition to occasional oil tanker accidents, there are many recurrent sources of marine oil pollution that introduce organic pollutants, particularly polycyclic aromatic hydrocarbons (PAHs): uncontrolled releases from crude oil plants, contaminated freshwater and terrestrial run-off, etc. (Head and Swannell, 1999). Although the toxic effect of these contaminants on higher organisms, such as fish, molluscs and other invertebrates are well known (e.g. Preston, 2002), the effects on natural microbial communities are less clear (Castle *et al*., 2006). A heavy oil spill drifting over the water surface, prevents gas exchange and eliminates light and may as well directly leach toxins into the water. Immediately after an oil spill, the soluble fraction of PAHs is released into the water column. This fraction is highly toxic and remains dissolved in seawater even after the insoluble fraction has been removed. Low molecular weight (LMW) PAHs with less than three benzene rings disappear rapidly, mostly within 2–3 days. By contrast, high molecular weight (HMW) PAHs with more than four benzene rings remain in the water column for at least 9 days (Yamada *et al*., 2003).

Bacteria represent the predominant agents of hydrocarbon degradation in the marine environment and might be both, stimulated or negatively affected, by the hydrocarbons. A remarkable decrease in bacterial diversity has been frequently reported following exposure to hydrocarbons, as a consequence of a strong selection for hydrocarbon-degrading bacteria (e.g. Nyman, 1999; Röling *et al*., 2002; Castle *et al*., 2006). Many hydrocarbon-degrading marine bacteria, mostly belong-

Table 1. Mean ± standard error initial values for environmental and biological variables.

Sampling period	T	Sal	DIN	DIP	SiO_4	POC	PON	PA	Chla
March	10.5 ± 0.0	35.48 ± 0.02	4.40 ± 0.08	0.52 ± 0.00	3.17 ± 0.04	16.1 ± 1.1	2.7 ± 0.2	7.2 ± 0.3	3.2 ± 0.2
July	20.8 ± 0.0	35.02 ± 0.01	0.58 ± 0.11	0.15 ± 0.01	0.59 ± 0.05	23.4 ± 0.3	3.4 ± 0.2	11.7 ± 0.5	1.9 ± 0.1
September	15.4 ± 0.0	35.73 ± 0.00	5.66 ± 0.73	0.51 ± 0.08	0.41 ± 0.02	33.9 ± 1.6	6.2 ± 0.4	17.4 ± 0.2	10.6 ± 0.7
January	12.4 ± 0.0	35.60 ± 0.00	7.70 ± 0.44	0.48 ± 0.02	3.72 ± 0.15	9.2 ± 0.6	1.3 ± 0.1	5.8 ± 0.3	0.5 ± 0.0

Temperature (T) in °C, salinity (Sal), dissolved inorganic nitrogen (DIN) in μM, dissolved inorganic phosphorous (DIP) in μM, silicate (SiO_4) in μM, particulate organic carbon (POC) in μM, particulate organic nitrogen (PON) in μM, prokaryotic abundance (PA) in $\times 10^5$ cell ml^{-1} and chlorophyll-a concentration (Chla) in mg m^{-3}. $N = 6$.

ing to genus within the *Gammaproteobacteria* subclass, have been isolated in recent years (see review by Head *et al*., 2006). A recent study by McKew and colleagues (2007a) showed that different petroleum hydrocarbons are degraded by different bacterial taxa. Particularly, they found that PAH-degrading bacterial communities, dominated by the genus *Cycloclasticus*, were distinct from those degrading alkanes. The genus *Cycloclasticus*, a component of the *Gammaproteobacteria* subclass, had been previously identified as a key player in the degradation of petroleum aromatic hydrocarbons (Geiselbrecht *et al*., 1998; Kasai *et al*., 2002), accounting for up to 25% of the total bacterial population in severely oil-polluted waters (Maruyama *et al*., 2003; Harayama *et al*., 2004).

To date, quite a number of studies have investigated changes in bacterial composition associated to PAHs pollution using molecular techniques such as DGGE (denaturing gradient gel electrophoresis) of polymerase chain reaction (PCR)-amplified 16S rRNA genes. However, no consistent pattern of variability emerged from the application of these molecular tools (Macnaughton *et al*., 1999; Kasai *et al*., 2001; Ogino *et al*., 2001; Castle *et al*., 2006). Polymerase chain reaction-based techniques allow for a reasonably good characterization of the phylogenetic composition of a sample, but they give limited information on the proportions of distinct bacterial groups. In addition, PCR techniques are time-consuming and expensive and do not allow for an exhaustive study of the temporal dynamics of a given bacterial group. One of the major advantages of fluorescence *in situ* hybridization (FISH) techniques is that they allow for quantification of the actual abundance of a given phylogenetic group. Some authors have compared the results emerging from PCR techniques (clone libraries, DGGE) and FISH (Castle and Kirchman, 2004; Alonso-Sáez *et al*., 2007), and concluded that both techniques give different information and are, thus, complimentary. The number of studies assessing the effect of PAHs on the bacterial composition using FISH techniques are rather limited (Syutsubo *et al*., 2001; Yakimov *et al*., 2004; Castle *et al*., 2006).

The research project IMPRESIÓN (Impact of the oil spill from the Prestige on the planktonic microbial food web) was designed to assess the effects of the soluble fraction of PAHs derived from the Prestige oil spill on the planktonic microbial food web of the coastal Atlantic waters under four contrasting hydrographic conditions in the coastal upwelling system of the Rías Baixas: winter mixing, spring bloom, summer stratification and autumn upwelling (i.e. Cermeño *et al*., 2006). Within this project we analysed the changes in the abundance of three major phylogenetic groups of bacteria, and particularly of the hydrocarbon-degrading bacteria belonging to the genus *Cycloclasticus* using CARD-FISH (Pernthaler *et al*., 2002). We hypothesized that the dynamics of the bacterial groups and, particularly, that of *Cycloclasticus* following PAHs addition would vary depending on the experimental and environmental conditions such as the concentration of added PAHs, microbial assemblage composition, seawater temperature and seawater nutrient concentrations.

Results

For each of the four experiments we filled six mesocosms with seawater from the Ría de Vigo. Two (March and July) or three (September and January) replicates were used as controls (no PAHs addition) and two or three were amended with PAHs. The soluble fraction of PAHs was obtained from Prestige-like heavy fuel oil. Polycyclic aromatic hydrocarbons addition was done after the first sampling (day 0). The experiments lasted 9 days and were sampled every 24 h during the first 5 days and thereafter, every 48 h. At each sampling point we determined the abundance of three major bacterial groups (*Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes*) and of the hydrocarbon-degrading bacteria *Cycloclasticus* using CARD-FISH and specific oligonucleotide probes.

Initial environmental conditions

In Table 1 we have summarized the initial environmental conditions for each of the experiments. The lowest seawater temperature corresponded to early March 2005 due to strong winter mixing. Confinement in the mesocosms produced a spring phytoplankton bloom composed of the

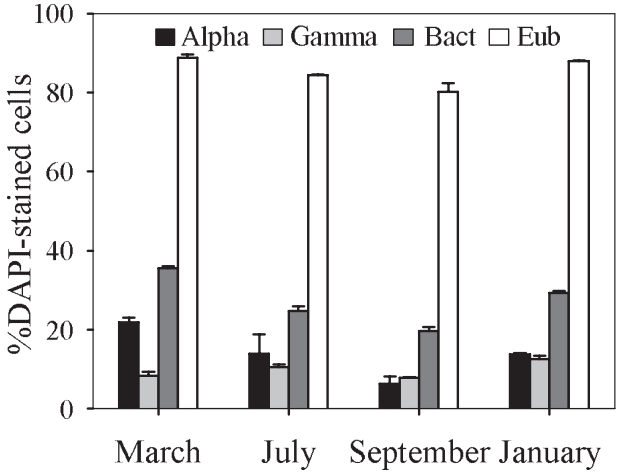

Fig. 1. Initial composition of the bacterial assemblage during the different sampling periods. The relative abundance of each group (Alpha, *Alphaproteobacteria*; Gamma, *Gammaproteobacteria*; Bact, *Bacteroidetes* and Eub, *Eubacteria*) is expressed as percentage of total DAPI-stained cells.

diatoms *Lauderia annulata* and *Chaetoceros socialis* during this experiment (M. Varela, pers. comm.) at the expenses of the high initial nutrient levels. Dissolved inorganic nitrogen (DIN) and phosphorous (DIP) concentrations were the lowest during summer stratification (July), coinciding with low chlorophyll-a (chla) levels. During winter mixing (January), maximum concentrations of DIN and silicate were recorded, accompanied by extremely low levels of particulate matter, prokaryotic abundance and chlorophyll-a. The highest initial chlorophyll-a concentration was observed in September, but, these values quickly decreased after day 1, associated to a decaying diatom bloom (M. Varela, pers. comm.), to levels as low as 1.6 mg chla m^{-3} at day 3. The levels of DIN in the mesocosms also decreased dramatically from day 0 to day 1 (from 5.9 to 0.7 μM) in September.

Initial bacterial community composition

The mean contribution of Bacteria to total prokaryotic abundance (PA) in the initial samples for each experiment ranged from 80%, in September, to 89% in March, and did not show significant differences between the four experiments (Fig. 1). The Bacteroidetes group always dominated the initial bacterial community, contributing from 20 to 36% to total prokaryotic abundance. The initial relative abundances of *Alpha-*, *Gammaproteobacteria* and *Bacteroidetes* were significantly different between experiments (ANOVA test, $P < 0.03$, $n = 20$). The *Alphaproteobacteria* and Bacteroidetes groups were relatively more abundant in January, March and July than in September (Bonferroni test, $P < 0.03$, $n = 20$). *Gammaproteobacteria* were significantly more abundant in January than in September (Bonferroni test, $P = 0.025$, $n = 20$). *Cycloclasticus* initial abundance was very close to the detection limit (10^3 cell ml^{-1}), except in January, when their initial abundance was approximately 3×10^3 cell ml^{-1}).

Dynamics of prokaryotes after PAHs addition

The temporal dynamics of the response of prokaryotes to PAHs addition relative to unamended controls, revealed only important changes for PA and the relative abundance of *Alphaproteobacteria* (Fig. 2). Repeated measures

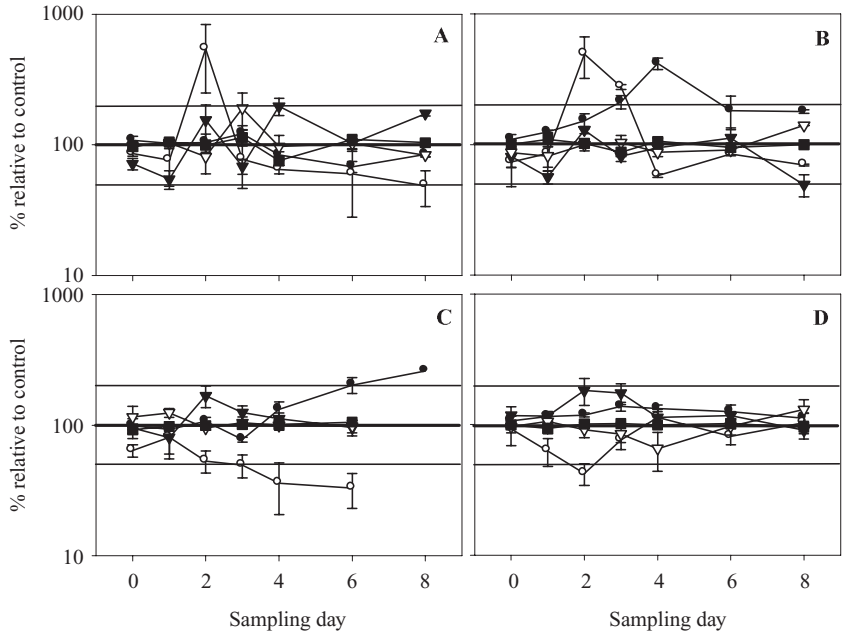

Fig. 2. Time-course of mean total prokaryotic abundance (black circles), *Alphaproteobacteria* (white circles), *Gammaproteobacteria* (black triangles) *Bacteroidetes* (white triangles) and *Eubacteria* (squares) in the PAHs-amended mesocosms expressed as a percentage relative to the values in the control mesocosms (Abundance in treat × 100/Abundance in control) in March (A), July (B), September (C) and January (D). The error bars represent the standard error from two replicates in March and July and three replicates in September and January. The horizontal lines in each graph represent the 200, 100% (no change) and 50% relative to control.

Table 2. Repeated measures ANOVA with one within-subjects factor (sampling day, time) and two between-subjects factors (experiment, exp; and treatment, treat).

Effects	PA	%EUB	%ALPHA	%GAMMA	%BACT	CYCLO
Within-subjects						
Time	**< 0.001**	**0.006**	**0.011**	**< 0.001**	**< 0.001**	**< 0.001**
	0.547	*0.360*	*0.302*	*0.555*	*0.555*	*0.543*
Time × Exp	**< 0.001**	**0.002**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
	0.775	*0.585*	*0.667*	*0.881*	*0.881*	*0.669*
Time × Treat	**< 0.001**	0.301	0.332	0.089	0.957	**< 0.001**
	0.503	*0.153*	*0.129*	*0.186*	*0.021*	*0.523*
Time × Exp × Treat	**< 0.001**	0.450	0.166	0.996	0.965	**< 0.001**
	0.671	*0.354*	*0.354*	*0.081*	*0.064*	*0.668*
Between-subjects						
Exp	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
	0.904	*0.904*	*0.938*	*0.947*	*0.947*	*0.970*
Treat	**< 0.001**	0.373	**< 0.001**	0.411	0.411	**< 0.001**
	0.965	*0.114*	*0.830*	*0.069*	*0.069*	*0.969*
Exp × Treat	**< 0.001**	0.959	**0.011**	0.271	0.271	**< 0.001**
	0.958	*0.040*	*0.732*	*0.230*	*0.230*	*0.931*

PA, prokaryotic abundance; %EUB, relative abundance of bacteria (over DAPI counts); %ALPHA, relative abundance of *Alphaproteobacteria*; %GAMMA, relative abundance of *Gammaproteobacteria*; %BACT, relative abundance of Bacteroidetes; CYCLO, abundance of *Cycloclasticus*. For each pair factor or factor combination and variable, the significance (upper value) and the partial η^2, which reflects the proportion of variance associated with each factor or factor combination (lower value, italics) are given. Significant effects are in bold.

ANOVA (RMANOVA) results showed a significant effect of PAHs addition (treatment) on PA and the relative abundance of *Alphaproteobacteria* (%ALPHA) (Table 2, 'Treat' effect). The effect of PAHs addition was stronger on PA than in %ALPHA, as reflected by the higher proportion of variance explained by the treatment (0.965 for PA, 0.830 for %ALPHA, see Table 2). There was a significant interaction between time (sampling day) and treatment (PAHs addition) for PA but not for %ALPHA (Table 2, 'Time × Treat' effect). The interaction plots (see *Experimental procedures*) and multiple tests based on estimated marginal means (Fig. 3A) indicate that PA was significantly higher from day 2 to day 8, in the treated than in the control mesocosms. A clear interaction effect between experiment (sampling period) and treatment was also detected for both PA and %ALPHA (Table 2, 'Exp × Treat' effect). This means that the effect of PAHs addition differed between experiments. Interaction plots revealed that there is no significant effect of PAHs addition on PA in March, and on %ALPHA in July (Fig. 3B and D). Whereas PAHs addition stimulated PA (positive effect), the relative abundance of *Alphaproteobacteria* was negatively affected by the treatment. Although the effects of PAHs were significant, the magnitude of the PA stimulation was rather small, being, on average, 150% relative to control (excluding data from March), and the relative abundance of *Alphaproteobacteria* was, on average, 68%, excluding data from July.

During the last experiment (January 2006) we additionally run microcosm experiments in 5-l bottles, in order to test for the effect of higher PAHs concentrations (Fig. 4). We observed a strong positive response of PA in the treatment with the highest PAHs concentration, where the per cent relative to control reached 712% at day 8. In all the other three treatments the per cent was, on average < 150%. The main phylogenetic groups did not show any response to PAHs addition, except for *Alphaproteobacteria*, that showed a considerable reduction in all but the treatment with the lowest PAHs addition (on average < 50% relative to control in days 7–8).

Dynamics of Cycloclasticus *abundance after PAHs addition*

Cycloclasticus reached maximum abundances (15–20×10^3 cell ml^{-1}) in March and January (Fig. 5A and D). The specific growth rate of *Cycloclasticus* was also higher in March (1.28 d^{-1}) and January (0.99 d^{-1}) than in July (0.85 d^{-1}) and September (0.51 d^{-1}). In all the four experiments, these bacteria increased their abundance after PAHs addition during 2–4 days, and thereafter their numbers decreased to a constant level, usually still significantly higher than the background abundance measured in the control bags. The maximal contribution of *Cycloclasticus* bacteria to total prokaryotic abundance ranged from 0.3% in July to 6.4% in March. When taking into account the complete data set, we found a significant effect of PAHs addition on the abundance of *Cycloclasticus* (RMANOVA, Table 2). There were significant time × treatment and experiment × treatment effects, which indicate that the effect of PAHs on the abundance of *Cycloclasticus* varied in time and that the response was

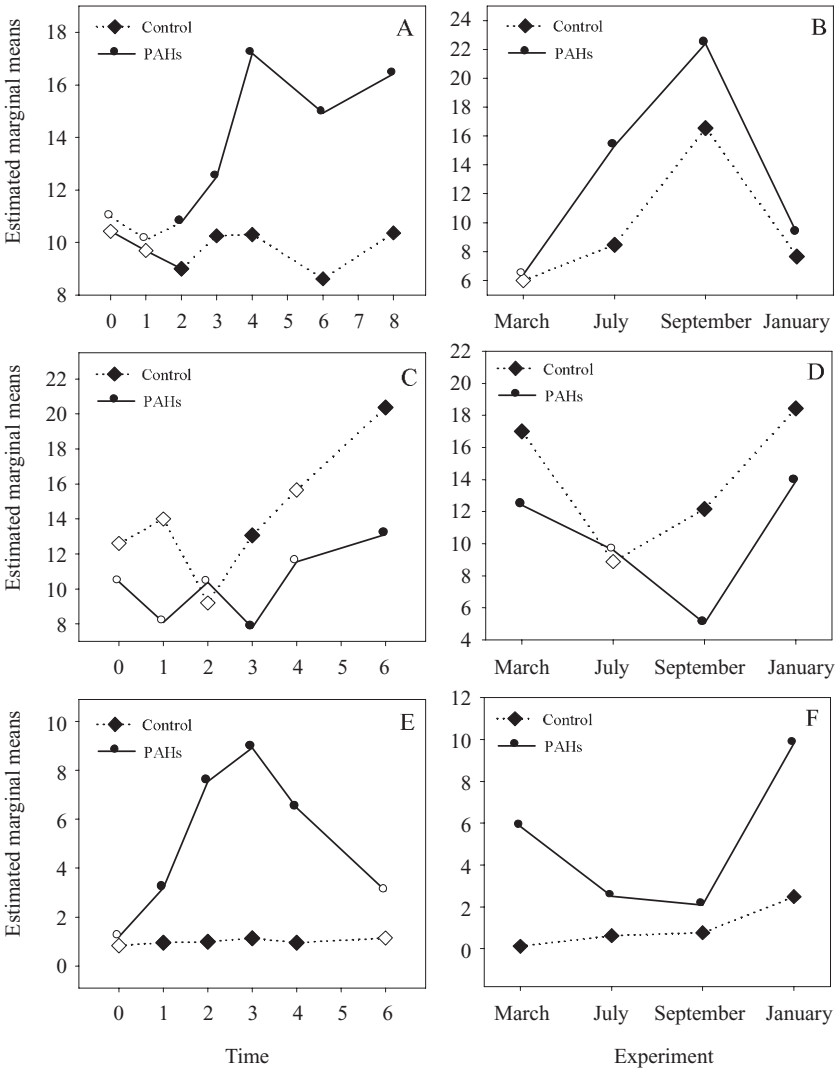

Fig. 3. Interaction plots showing estimated marginal means (means of each variable predicted by the ANOVA model) of prokaryotic abundance (A, B), % *Alphaproteobacteria* (C, D) and *Cycloclasticus* abundance (E, F) in control (dashed lines) and PAHs-amended (solid lines) mesocosms across time (A, C, E) or experiment (B, D, F). Solid symbols represent a significant effect of treatment at each level of time or experiment; open symbols, not significant effect.

different between experiments. Interaction plots show that the positive effects of PAHs addition was significant from day 1 to day 4 (Fig. 3E) and that the magnitude of the response is higher in March and January (Fig. 3F).

In the microcosm experiments run during the last experiment (January 2006), the abundance of *Cycloclasticus* followed a similar temporal dynamics as that observed in mesocosms, reaching maximum abundances at days 4–5 (Fig. 6). We observed that on average, the half-life time of PAHs was higher in the microcosms than in the mesocosms (65 and 24 h respectively), something that we attributed to a lower atmosphere contact area of the microcosms, and that would explain the relatively longer persistence of *Cycloclasticus* growth in the microcosms than in the mesocosms. The maximal abundance (approximately 2×10^5 cells ml^{-1}) was observed at the highest initial PAHs concentration, comprising 11% of the total prokaryotic community. There was a highly significant lineal relationship between the mean maximum abundance of *Cycloclasticus* and the mean initial PAHs concentration ($r^2 = 0.97$, $P = 0.015$, $n = 4$). When we did this analysis with data from the mesocosm experiments only (Fig. 7), the obtained model was not significant ($P = 0.214$, $n = 4$). The maximal abundance of *Cycloclasticus* in July and September was lower than expected by the initial concentration of PAHs.

Discussion

The mesocosms experiments were designed in order to describe the effect of PAHs derived from the *Prestige* oil spill on the planktonic microbial food web of the coastal Atlantic waters under four contrasting hydrographic conditions. Although we tested relatively low PAHs concentrations (20–30 $\mu g\ l^{-1}$ chrysene), they were three- to sixfold higher than the 90% percentile of the concentrations found along the Galician coast affected by the *Prestige* oil spill just after the accident, which rarely exceeded

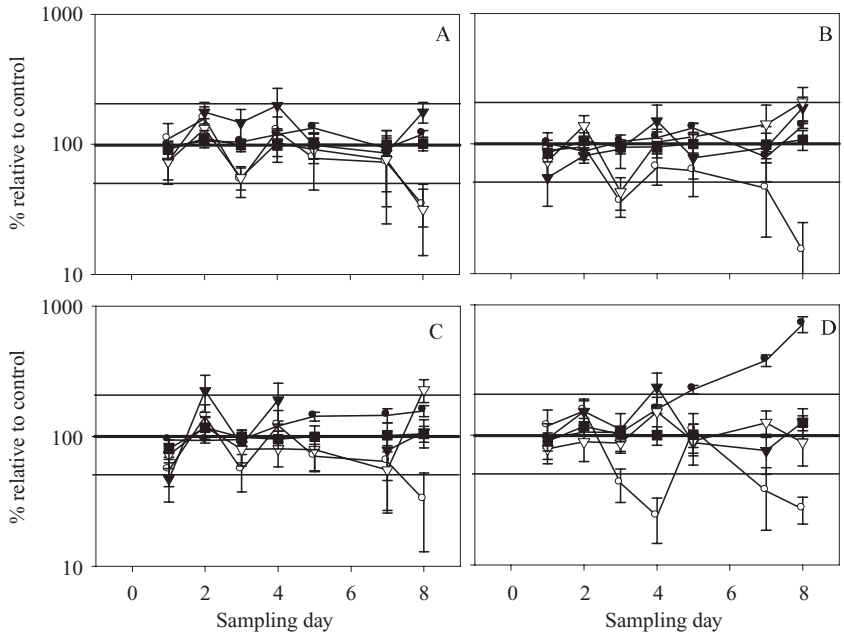

Fig. 4. Time-course of total prokaryotic abundance (black circles), *Alphaproteobacteria* (white circles), *Gammaproteobacteria* (black triangles) *Bacteroidetes* (white triangles) and *Eubacteria* (squares) in microcosms amended with 10 (A), 20 (B), 40 (C) and 80 (D) μg l^{-1} of PAHs, expressed as a percentage relative to the values in the control mesocosms. The error bars represent the standard error. The horizontal lines in each graph represent the 200, 100% (no change) and 50% relative to control.

5 μg l^{-1} chrysene (González *et al*., 2006). Experiments performed with higher PAH additions might have given more contrasted results, but we were interested in the effects that had possibly been created by that oil spill. 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 approximately 53% (Alzaga *et al*., 2004). Polycyclic aromatic hydrocarbons represented 99.7% of the water soluble fraction of the Prestige oil and alkanes were almost undetectable (J. Albaigés, pers. comm.). Although it was not possible to use exactly the Prestige oil, we used an oil with a very similar composition (see *Experimental procedures*).

After fuel addition bacterial abundances usually tend to increase according to both experimental and field observations (Ohwada *et al*., 2003; Nayar *et al*., 2005; Sargian *et al*., 2005; Bode *et al*., 2006). Our results also show an overall increase of PA after PAHs treatment as compared with the control. However, the effect of fuel additions on PA

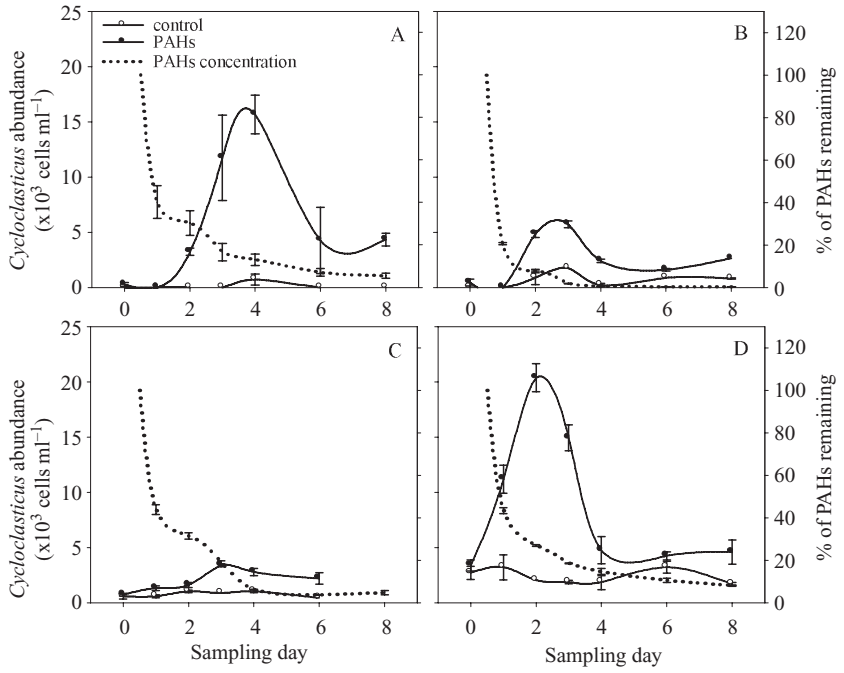

Fig. 5. PAHs concentrations (expressed as percentage of initial concentration, in μg l^{-1}), and changes in *Cycloclasticus* abundance in control and PAHs-amended mesocosms in March (A), July (B), September (C) and January (D).

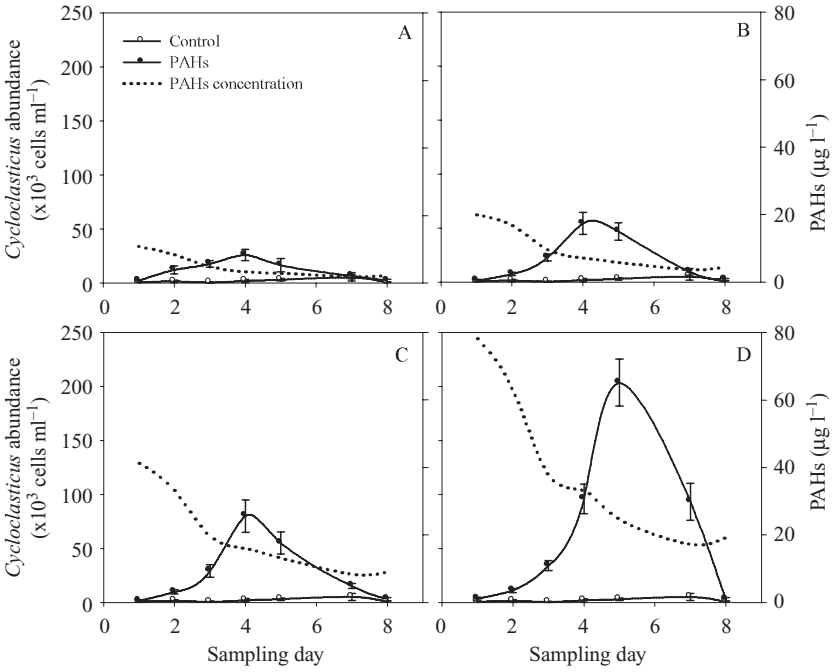

Fig. 6. PAHs concentration and changes in *Cycloclasticus* abundance in microcosms amended with 10 (A), 20 (B), 40 (C) and 80 (D) µg l^{-1} of PAHs.

varied between experiments (Table 2). The magnitude of PA increment was considerably higher in July and September than in January, and not significant in March (Fig. 3), thus confirming the hypothesis of a variable response of natural bacterial assemblages to oil additions, depending on the initial environmental (temperature, nutrient concentration) and biological (planktonic assemblage composition, trophic relationships) conditions.

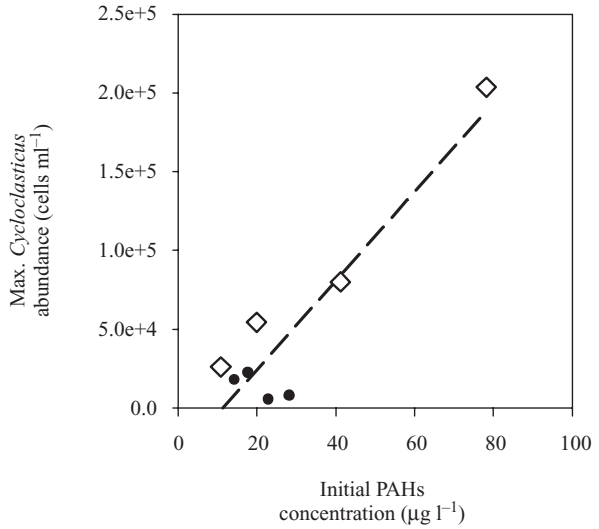

Fig. 7. Relationship between the average maximal abundance of *Cycloclasticus* and the average initial concentration of PAHs comparing data from mesocosms (black symbols) and microcosms (white symbols). Dashed line represent the regression line obtained with microcosm data.

Effects of PAHs addition on major phylogenetic bacterial groups

Only a few studies in the past years have focused on the structural changes that occur in natural marine planktonic bacteria after oil pollution. They have used either qualitative (e.g. fingerprinting methods, Yakimov *et al*., 2004; Denaro *et al*., 2005; Castle *et al*., 2006; Coulon *et al*., 2007; McKew *et al*., 2007a) or quantitative methods (e.g. Fluorescence *In Situ* Hybridization, Castle *et al*., 2006 or Q-PCR analysis, McKew *et al*., 2007b). Castle and colleagues (2006) found that naphthalene (an LMW-PAH) caused a reduction in the number of detectable phylogenetic groups: 3 days after the addition, both *Alphaproteobacteria* and *Bacteroidetes* groups became undetectable with FISH. In contrast, we did not observe such losses of entire groups at any time in the four mesocosm experiments, although the concentration of PAHs in our study was one order of magnitude lower that the concentration used by these authors. However, we did find a significant reduction in the relative abundance of *Alphaproteobacteria* in the PAHs-amended mesocosms (Table 2 and Fig. 3D). There are at least two plausible explanations for the reduction of these bacteria after PAHs addition: they could be outcompeted by other groups, or their growth could be inhibited by the chemicals added. We did not observe a parallel increment in the relative abundance of either *Gammaproteobacteria* or *Bacteroidetes* groups, which would support the first explanation. Two recent studies provide evidence for the inhibitory effects hypothesis. McKew and colleagues (2007a) suggested that *Roseobacter*-related bacteria, an important group of

Alphaproteobacteria which may play a key role in the degradation of *n*-alkanes, could be inhibited by PAHs. Labbé and colleagues (2007) also found that the relative abundance of *Alphaproteobacteria* was about twice higher in pristine than in hydrocarbon-contaminated Alpine soils. The fact that a reduction in *Alphaproteobacteria* was not observed in July, even a transient increment of *Alphaproteobacteria* occurs between day 1 and 4, could be related to a faster degradation of PAHs due to abiotic factors, such as temperature or solar radiation which were higher in that experiment (see next section).

Gammaproteobacteria have been found to become predominant after petroleum-derived hydrocarbon additions (Yakimov *et al*., 2004; Castle *et al*., 2006; McKew *et al*., 2007a). Surprisingly, in our study we did not observe any significant effect of PAHs addition over the relative abundance of *Gammaproteobacteria*. This contrasting finding could be related to the concentration of PAHs added in our mesocosm experiments as compared with the levels of addition in the aforementioned studies. Our highest concentration was approximately 30 $\mu g\ l^{-1}$ chrysene, while the initial hydrocarbon concentration was, e.g. 640 μg naphthlene l^{-1} in the Castle and colleagues (2006) study. Other possible explanation is related to the specificity of the probe used to detect *Gammaproteobacteria*. We did find a significant increase in *Cycloclasticus* abundance, which actually belong to *Gammaproteobacteria*, however, it is very likely that the *Gammaproteobacteria* probe did not target *Cycloclasticus* (see *Experimental procedures*). Finally, It could also be related to the use of crude oil rather than soluble PAHs additions (Yakimov *et al*., 2004; McKew *et al*., 2007a). Both these studies showed a dominance of the *Gammaproteobacteria* subclass related to alkane-degrading bacteria *Thalassolituus* or *Oleispira,* that grows on aliphatic hydrocarbons, alkanoles and alkanoates.

Cycloclasticus *dynamics after PAHs addition*

Diverse petroleum-degrading bacteria inhabit marine environments, including hydrocarbonoclastic bacteria, which use hydrocarbons almost exclusively as carbon source (see reviews by Head *et al*., 2006; McKew *et al*., 2007a). These specialists are usually present in very low numbers, and given the appropriate conditions can grow and multiply rapidly (Head *et al*., 2006). We observed a quick response of bacteria belonging to the genus *Cycloclasticus* after PAHs addition, reaching maximum abundances in about 3 days. In a very recent paper, McKew and colleagues (2007a) identified bacteria belonging to the *Cycloclasticus* genus dominating the community of bacteria degrading naphthalene, phenanthrene and pyrene. These LMW-PAHs have been shown to degrade within 2–3 days (Yamada *et al*., 2003). This would perfectly explain the rapid response of *Cycloclasticus* observed both in mesocosms and microcosms. The significant lineal relationship obtained between initial PAHs concentration and the maximal abundance of *Cycloclasticus* in the microcosm experiments suggests that this genus was largely responsible for the degradation of the LMW fraction of the added PAHs. Kasai and colleagues (2002) also showed that PAHs degradation occurs in parallel with the growth of *Cycloclasticus* cells on the surface of oil-polluted grains of gravel. The explanation for the quick decline of *Cycloclasticus* abundance after days 4–5 is, however, not clear. Their abundance could drop off by grazing, or, they could become inactive and die off, once the substrate they are specialized on is depleted. The contribution of *Cycloclasticus* to total prokaryotic abundance was relatively low (from 0.3 to 6.4%) compared with the abundance detected after the Nakhodka oil spill (Maruyama *et al*., 2003), likely reflecting a lower concentration of PAHs in our experiments. In July and September, the maximal relative abundance of the genus *Cycloclasticus* was the lowest (0.3% and 0.5%), which could be related to a predominance of other groups of hydrocarbon-degrading bacteria.

These variability in the oil-degrading microbial community could be related to environmental differences, such as solar radiation, seawater temperature or nutrient concentrations. Dutta and Harayama (2000), for example, observed that sunlight promotes a decrease in the oil aromatic fraction. Significant faster photodegradation rates have been observed specially for LMW-PAHs (Nadal *et al*., 2006). The high solar radiation in July could have favoured photooxidation of PAHs, leading to low maximal abundances of *Cycloclasticus*.

Coulon and colleagues (2007) recently showed that seawater temperature can lead to the selection of different hydrocarbon-degrading bacterial groups, and concluded that a change in temperature may have a much more profound effect on the oil-degrading microbial communities than nutrient additions. However, *Cycloclasticus* is a cold-tolerant and versatile group of bacteria that has been shown to grow in the temperature range 4–20°C (Coulon *et al*., 2007). Additional experiments conducted in our lab showed that when exposing a natural seawater assemblage amended with naphthalene (500 $\mu g\ l^{-1}$) to different temperatures (from 8 to 25°C) *Cycloclasticus* growth increased with increasing temperature (details not shown). On the other hand, high temperatures can directly modify the bioavailability of the PAHs by increasing volatilization and solubility of some hydrocarbons (Coulon *et al*., 2007), and act synergistically with UVB radiation enhancing photodegradation rates (Nadal *et al*., 2006). A faster volatilization of the LMW-PAHs during July and September, due to the higher ambient temperatures, could explain the relatively low abundance of *Cycloclas-*

Table 3. Regression model relating maximum *Cycloclasticus* abundance (cells ml^{-1}) to initial PAHs concentration (PAHs, in $\mu g\ l^{-1}$) and dissolved inorganic nitrogen concentration at day 1 (DIN, in μM).

Independent variable	*N*	R^2	Adjusted R^2	*F*	*P*	Coefficient ± SE	*B*	Coefficient *P*
PAHs	8	0.945	0.923	43.2	0.001	2 583 ± 332	0.842	0.010
DIN						5 526 ± 1 897	0.315	0.033
Constant						–5 756 ± 14 403		0.100

ticus after the simulated oil spill. The very short half-life of PAHs in July compared with the other three experiments (11 h, vs. 18–24 h in March, September and January) could be related to a greater importance of abiotic degradation processes (volatilization, photodegradation) in July than in the other three periods.

Finally, the inorganic nutrient concentration was also lower in July (day 1 dissolved inorganic nitrogen, DIN, 1.16 μM DIN) and September (DIN, 0.72 μM) than in March (DIN, 2.14 μM) and January (DIN, 8.66 μM). The availability of limiting resources is a key factor controlling hydrocarbon degradation, and some studies point out that nutrient concentrations might directly influence the relative degradation of polycyclic aromatic and saturated hydrocarbons, through a change in bacterial composition. Laboratory experiments using beach-simulation tanks, demonstrated that *Cycloclasticus* cells grow up to two orders of magnitude more after fertilization with nitrogen and phosphorus compared with unamended tanks (Smith *et al*., 1998). A stepwise multiple regression analysis including as independent variables temperature, DIN concentration at day 1 and initial PAHs concentration, excluded temperature as a significant variable but resulted in a model which explained 95% of the variability observed in the maximum abundance reached by *Cycloclasticus* (Table 3).

In conclusion, we clearly show that bacteria belonging to the genus *Cycloclasticus* play a major role in LMW-PAHs biodegradation in a planktonic ecosystem. Their response is stronger in cold waters, where their background abundance is also higher. During the warm periods, the response of *Cycloclasticus* is limited, possibly due to both, a higher removal of PAHs by abiotic factors (solar radiation, temperature), and because of inorganic nutrient limitation.

Experimental procedures

Experimental set-up and sampling

Six mesocosms of 1.5 m in diameter and 2 m deep were filled with seawater from the middle Ría de Vigo. The bags were filled from their bottom through a 200-μm mesh, in order to exclude mesozooplankton. Once filled, the bags were closed with a bottom stopper and gently transported to shore, where they were attached to a harbour in a protected bay. Two of the mesocosms were used as controls, two were treated by adding a low concentration of soluble PAHs (approximately 5–10 $\mu g\ l^{-1}$ chrysene) and two with a high concentration of soluble PAHs (approximately 20–30 $\mu g\ l^{-1}$ chrysene). Due to logistic problems during the first two experiments, which affected the bags with low PAH concentration, in the other two experiments we eliminated the low concentration treatment in order to triplicate the control and the high PAHs concentration treatments. Polycyclic aromatic hydrocarbons addition took place after the first sampling (named as day 0). The water soluble fraction of PAHs was prepared by addition of 15 kg of Prestige-like heavy fuel oil provided by the Oficina 'Técnica de Coordinación del Programa de Intervención Científica en la Catástrofe del Prestige' in 300 l of 0.2 μm-filtered seawater, taken from the cultivation plant facilities of the Instituto de Investigaciones Marinas. The mixture was vigorously stirred with a mechanical stirrer during 4 h to allow extraction of the soluble fraction into seawater. The resulting extract, with approximately 700 $\mu g\ l^{-1}$ of soluble PAHs, was separated from the insoluble fuel oil by decantation and collected on 25 l polyethylene barrels. Finally, the content of the barrels were added to the mesocosms to get the desired initial soluble PAHs concentrations. Polycyclic aromatic hydrocarbons were measured following the MARPOLMON protocol (UNESCO, 1984), with modified volumes, and referred to a chrysene standard.

A total of four experiments were run under contrasting initial conditions: one in spring (March 2005), one in summer (July 2005), one in early autumn (September 2005) and one during winter (January 2006). The experiments were carried out during the four most relevant periods of the seasonal cycle in the coastal NE Iberian Atlantic waters: winter mixing, spring bloom, summer stratification and autumn upwelling. In this article we present only data from the control and high concentration treatment for the four experiments. The experiments run for 8 days after the PAH additions. Samples were taken every day during the first 5 days, and thereafter every 2 days.

Additional microcosm experiments

In January 2006 we conducted additional microcosm experiments in order to test the response of the community to a gradient of PAHs concentrations. The microcosms were run in parallel to the mesocosms and with the same initial seawater, although the PAHs were already added at day 0. We prepared a total of 10 microcosms, consisting in 5-l PET bottles, with a wide opening. The microcosms were kept opened and refrigerated by circulating surface seawater and were incubated outside the Institute. Two microcosms were used as controls (no PAHs addition), the other eight were spiked, in duplicate, with final PAHs concentration of

approximately 10, 20, 40 and 80 μg l^{-1}. These concentrations were, thus, 0.5 ×, 1 ×, 2 × and 4 × the 'High' treatment in the mesocosms. The microcosms were kept for 8 days, sampling every 24 h, except at day 6. Samples were taken for analysis of bacterial community composition, as described below.

Bacterial community composition

Samples from the mesocosms were collected with an integrated 1.5 m tube minimizing stirring, to avoid resuspension from the bottom of the bags, and deposited into polycarbonate carboys that were brought back to the laboratory. Less than 30 min later, 5 ml of water samples were fixed by adding to them 0.2-μm filtered paraformaldehyde (2% final conc.) and subsequently, the samples were stored at 4°C in the dark for 12–18 h. Thereafter, the samples 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 μm), washed twice with Milli-Q water, dried and stored in a microfuge vial at −20°C until further processing.

Bacterial assemblage composition changes were monitored using FISH techniques with 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, the *Bacteroidetes* group (CF319a) (Manz *et al*., 1996) and the genus *Cycloclasticus* (CYPU829) (Maruyama *et al*., 2003). We also tried a general probe targeting *Betaproteobacteria* (BET42a) (Manz *et al*., 1992), but this group was very close to the detection limit (< 0.4%), so these data are not included. We checked the specificity of the GAM42a probe using the BLAST program (Altschul *et al*., 1997). The probe sequence did not produce significant alignments with known sequences of many hydrocarbon-degrading bacteria, such as *Cycloclasticus*, *Alcanivorax*, *Thalassolituus*, but it aligned to sequences from other bacteria able to degrade hydrocarbons, such as *Pseudomonas* or *Marinobacter.*

Filters for CARD-FISH were embedded in low-gelling-point agarose and incubated with lysozyme. Filters were cut in sections and hybridized with horseradish peroxidase (HRP)-labelled oligonucleotide probes and tyramide-Alexa488 for signal amplification following the protocol described in Pernthaler and colleagues (2002) and Teira and colleagues (2004). Cells were counter-stained with a DAPI-mix [5.5 parts of Citifluor (Citifluor), 1 part of Vectashield (Vector Laboratories) and 0.5 parts of PBS with DAPI (final concentration 1 μg ml^{-1})].

The slides were examined under a Zeiss Axioplan 2 microscope equipped with a 100-W Hg-lamp and appropriate filter sets for DAPI and Alexa488. More than 800 DAPI-stained cells were counted per sample. For each microscope field, two different categories were enumerated: (i) total DAPI-stained cells and (ii) cells stained with the specific probe. Negative control counts (hybridization with HRP-Non338) averaged 0.5% and were always below 1.0% of DAPI-stained cells. The counting error, expressed as the percentage of standard error between replicates, was < 2% for DAPI counts and < 9% for FISH counts.

Statistical analysis

In order to test for differences at day 0 between the four experiments, we used ANOVA after log or arcsine data transformation. For post hoc multiple comparisons we used the Bonferroni test in order to control for type I errors.

A repeated measures ANOVA (RMANOVA) with one within-subjects factor (time) and two between-subjects factors (experiment and treatment) was conducted to asses time effects and all possible interactions. Time is a within-subjects factor because the same mesocosm is sampled at sequential time periods (every 24–48 h). All data fitted a normal distribution (Kolmogorov-Smirnoff test), however, even after log or arcsine data transformation, the homogeneity of covariance matrices failed for some variables. For the latter case we applied the Huynh-Feldt adjustment to correct *P*-values (Scheiner and Gurevitch, 1993). Profile plots and multiple comparison tests with the estimated marginal means were used to interpret interactions between factors. The marginal means are the means of each variable across levels of each factor predicted by the model. Profile plots are the line plots of marginal means of a response variable across levels of a factor. When two factors are involved these are called interaction plots. We constructed the interaction plots representing time or experiment factors along the *X*-axis and the treatment factor as different lines in the same plot. Parallel or roughly parallel lines indicate little or no interaction.

Acknowledgements

We thank all the people involved in the project IMPRESION who helped with the preparation and sampling of the mesocosms during the four experiments. We also would like to thank the captain and crew on board the RV *Mytilus* and RV *Arao*. This research was supported by the MEC contract IMPRESION (VEM2003-20021). E.T. was funded by an European Community Marie Curie Reintegration Fellowship (MERG-CT-2004-511937) and a Juan de la Cierva-MEC contract.

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