Short-term changes in the composition of active marine bacterial assemblages in response to diesel oil pollution

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Summary

The changes caused by diesel oil pollution in the metabolically active bacterioplankton from an oligotrophic coastal location were analysed in laboratory microcosms (44 l) using 16S ribosomal RNA (16S rRNA) as molecular marker. The aim was to simulate typical hydrocarbon pollution events in a coastal area exploited for seasonal touristic activities. The experiment consisted in addition of low amounts of diesel oil without nutrients to seawater collected at different times (winter and summer). Bacterial diversity was analysed by terminal-restriction fragment length polymorphism (T-RFLP) profiling of 16S rRNAs after reverse transcription polymerase chain reaction (RT-PCR), and by generation of 16S rRNA clone libraries in control and diesel-polluted microcosms. Diesel addition caused a twofold increase in prokaryotic numbers in comparison with controls at the end of the experiment, both in winter and summer microcosms. Bacterioplankton composition, determined by 16S rRNA T-RFLP data, changed rapidly (within 17 h) in response to treatment. The resulting communities were different in microcosms with water collected in summer and winter. A reduction in diversity (Shannon index, calculated on the basis of T-RFLP data) was observed only in summer microcosms. This was due to the rapid increase of phylotypes affiliated to the *Oceanospirillaceae*, not observed in winter microcosms. After diesel treatment there was a reduction in the number of phylotypes related to SAR11, SAR86 and picocyanobacteria, while phylotypes of the *Roseobacter* clade, and the OMG group seemed to be favoured. Our results show that diesel pollution alone caused profound effects on the bacterioplankton of oligotrophic seawater, and explained many of the dif-

ferences in diversity reported previously in pristine and polluted sites in this coastal area.

Introduction

Phylogenetic composition of the bacterial assemblages in different pristine coastal locations has been largely studied (Schauer *et al.*, 2003; Alonso-Sáez *et al.*, 2007; Pommier *et al.*, 2007; Lami *et al.*, 2009). However, despite the fact that coastal areas are strongly subjected to anthropogenic stress, there are only few studies describing the bacterial communities in human-exploited environments such as harbours (Schauer *et al.*, 2000; Denaro *et al.*, 2005; Kan *et al.*, 2006; Nogales *et al.*, 2007; Zhang *et al.*, 2007; Aguiló-Ferretjans *et al.*, 2008; Ma *et al.*, 2009). Harbours are particularly suitable as models for sites receiving strong anthropogenic impact because they inevitably constitute point sources for chronic pollution with hydrocarbons, heavy metals, antifouling compounds, surfactants, nutrients and sometimes fecal material, even if they have facilities for the collection of waste and for pollution control. The presence of contaminants in these waters exerts a selective pressure towards favouring microorganisms, which are either able to use these pollutants or to withstand their presence in the water. Thus, bacterial communities in harbours are different from those in adjacent areas (Nogales *et al.*, 2007; Zhang *et al.*, 2007), and there are gradual changes in community composition with phylotypes predominating in the harbour being gradually replaced by those present in pristine waters (Nogales *et al.*, 2007).

Some of the bacterial populations detected in harbours can be directly related with parameters of water pollution. In Victoria Harbor, the bacterial community structure was correlated with suspended solids, turbidity, pH, and nitrogen compounds (Zhang *et al.*, 2007), which suggested an effect of eutrophication. This was also the case of Xiamen Port (Ma *et al.*, 2009), which, as the former, receives sewage discharges. In Messina harbour, a relationship between the presence of putative hydrocarbon degrading bacteria and the increase in aliphatic hydrocarbons was reported (Denaro *et al.*, 2005). Similarly, we reported that the gradual changes in bacterial communities observed near a recreational marina in the island of Mallorca could be related with a loss of oligotrophic characteristics of the

water, as shown by correlations with chlorophyll content and the disappearance of typical oligotrophs (Nogales *et al.*, 2007; Aguiló-Ferretjans *et al.*, 2008). However, relationships between these changes and hydrocarbon pollution, *a priori* the main factor affecting this system, were difficult to demonstrate (Nogales *et al.*, 2007).

Small-scale laboratory microcosms or mesocosms constitute useful experimental approaches for the study of the effect of pollution on bacterial communities because they reduce the influence of uncontrollable environmental variables. This approach has been widely used for analysing hydrocarbon degradation by marine bacteria in experiments simulating crude oil spills and bioremediation strategies (see the reviews of Head *et al.*, 2006 and Yakimov *et al.*, 2007 and references therein). Thus, most of these experiments have been done using complex hydrocarbon mixtures (crude oils), in high amounts (in the order of several hundreds milligrams to one gram per litre), and with nutrient addition to stimulate degradation (Yakimov *et al.*, 2005; Cappello *et al.*, 2007; Coulon *et al.*, 2007; McKew *et al.*, 2007). However, the prevalent pollution events in the marine environment are not due to accidental oil spills from tankers but to the constant consumption of petroleum-derived products. Thus, it has been estimated that around 70% of the petroleum introduced in the sea from anthropogenic sources is derived from the use of hydrocarbons and involves direct accidental or intentional spills from non-tank vessels, operational discharges, illegal discharges, maritime traffic, land-based run-off and atmospheric deposition (Committee on Oil in the Sea, 2003; Ferraro *et al.*, 2009). These are the situations that are typically encountered in coastal environments with anthropogenic pressure, and occur constantly in the proximity of harbours. In this study, we have analysed the effect of this type of pollution on the composition of oligotrophic coastal marine bacterial communities by adding refined hydrocarbons (diesel oil) at much lower concentration (30 mg l^{-1}) than previously used in other studies. This concentration is the value of the improved standard of the OSPAR Recommendation 2006/4 for the management of produced water from offshore installations (http://www.ospar.org) and double the oil content allowed in effluent discharges in special areas according to the MARPOL 73/78 convention for prevention of pollution from ships (http://www.imo.org). Nutrients were not added because we did not intend to stimulate hydrocarbon bioremediation as in other studies done so far.

The aim of this study was to analyse the changes in bacterioplankton from a pristine area in response to the addition of relevant environmental concentrations of diesel oil in laboratory microcosms, as a way to understand possible factors determining the differences in community composition observed previously in the area surrounding a recreational marina (Nogales *et al.*, 2007; Aguiló-Ferretjans *et al.*, 2008). In order to obtain a view of the metabolically active bacteria responding to the treatment we used ribosomal RNA (rRNA) instead of DNA as molecular marker for diversity studies.

Results and discussion

The experiments presented here tried to simulate the most likely event of hydrocarbon pollution that occurs in an oligotrophic coastal area in the West Mediterranean, which is exploited for tourist purposes. They were done using small-scale laboratory microcosms filled with oligotrophic seawater collected at approximately 1 km from the mouth of a recreational marina, a pristine unaffected area. Microcosms were polluted with low concentrations of diesel oil (Fig. 1). Previous data on bacterial communities at this site showed that there was seasonal variation in community composition, which seemed to correlate well with changes in water temperature (Nogales *et al.*, 2007). Therefore, we analysed the bacterial response to diesel addition at two time points, when we expected different community composition. These corresponded to periods of: (i) end of winter, characterized by lower temperature, irradiance and nautical activity, as well as surface water mixing (i.e. higher nutrient concentration in surface waters); and (ii) summer, characterized by high temperature, irradiance and nautical activity, as well as thermal stratification of surface water (i.e. lower nutrient concentration in surface water). Thus, as we expected, the surface water used for preparation of the microcosms seemed to be more oligotrophic in summer than in winter, according to chlorophyll content (0.830 μg l^{-1} in winter versus 0.073 μg l^{-1} in summer). These chlorophyll concentrations were similar to those observed previously at the same location (Aguiló-Ferretjans *et al.*, 2008).

Because we were working with oligotrophic seawater in microcosms, we expected changes due to water confinement that could interfere with the effect of the treatment as previously reported (Lee and Fuhrman, 1991; Schäfer *et al.*, 2000). For this reason, we prepared microcosms with a water volume of 44 l (Fig. 1). In addition, we performed a preliminary control experiment, with seawater without any amendment, to determine the maximum incubation time of the microcosms. This time was set to 5 days (120 h), according to viable and total (DAPI-stained) cell counts. Fluorescent *in situ* hybridization (FISH) counts with probes EUBI-III were also done in order to detect the expected proliferation of fast-growing bacteria with high ribosome content, in response to confinement, which would interfere with our rRNA-based approach. This was observed after 136 h (Fig. S1).
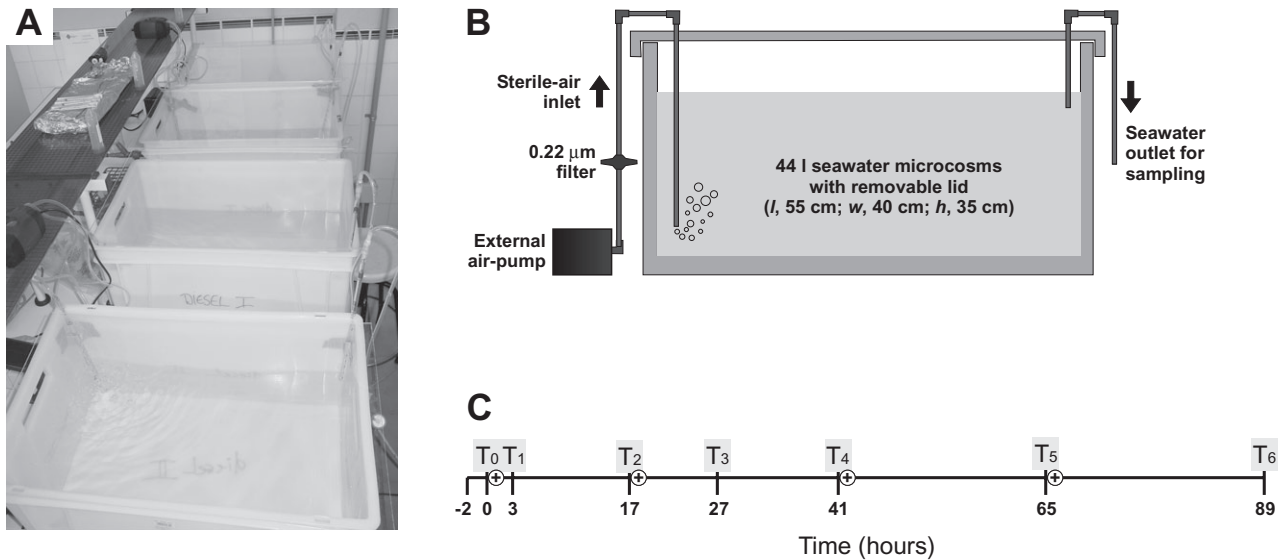
Fig. 1. Microcosm set-up.
A. Photograph of the microcosms.
B. Schematic view of the microcosm set-up
C. Scheme of the microcosms sampling times. Plus symbols indicate diesel additions.

Diesel treatment stimulated rapid bacterial growth

Two sets of four microcosms (with and without diesel addition, with duplicates) were prepared with seawater collected in winter and summer (hereafter denominated as winter and summer microcosms). Incubations were done at room temperature: 20°C and 26°C for winter and summer microcosms respectively. Total cell counts revealed a low effect of confinement and laboratory incubation with average increases of 3×10^5 cells ml^{-1} and 4.8×10^5 cells ml^{-1} by the end of the experiment in summer and winter microcosms respectively (Fig. 2). We attribute the increase of cell counts in winter control microcosms observed between 27 and 65 h to the difference in water temperature in the laboratory with respect to environmental conditions (14.5°C). The increase in cell numbers in the diesel-treated winter microcosms up to 41 h of incubation might be attributed to an effect of temperature as well. However, at longer incubation times (41–89 h) there was an increment in cell numbers in the diesel-treated microcosms, not observed in the controls (Fig. 2).

In comparison with control microcosms, cell counts were approximately twofold higher in diesel-treated microcosms at the end of the experiment (increases of 9×10^5 and 1.8×10^6 cells ml^{-1} in winter and summer respectively). The stimulatory effect of diesel was observed earlier (after 17 h) in summer microcosms. On average, $24 \pm 2\%$ (winter microcosms) and $28 \pm 1\%$ (summer microcosms) of DAPI-stained cells were detected using the mixture EUBI-III probes at the start of the experiment.

As expected from the stimulation of fast-growing bacteria in response to the treatment, this value increased in diesel-treated but not in control microcosms, reaching $41 \pm 6.2\%$ in winter and $69 \pm 3.5\%$ in summer microcosms by the end of the experiment (Fig. S2). Viable counts followed the same dynamics and were always higher in diesel-treated microcosms (particularly summer ones), irrespective of the carbon source added to the plates (data not shown).

The increase in cell numbers obtained after diesel addition in our experiments was comparable to those obtained

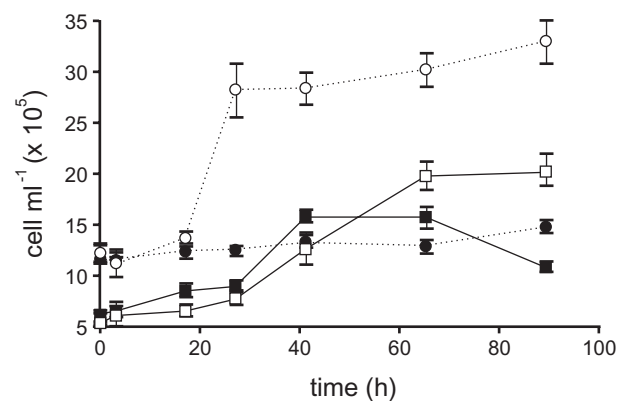**Fig. 2.** Variation in the average values of total number of prokaryotic cells (DAPI counts) in the different microcosms during the experiments. Square symbols with solid lines correspond to winter microcosms. Circle symbols and dashed lines correspond to summer microcosms. Closed symbols correspond to controls and open symbols to diesel treatments. Standard errors of the two replicate microcosms for each condition are shown.

Table 1. Shannon diversity indices calculated for the different microcosms according to the size and relative abundance of T-RFs generated with the enzyme AluI.

Time (h)	Winter microcosms		Summer microcosms	
	Control	Diesel	Control	Diesel
0	3.73	4.12	4.32	4.25
3	3.83	3.68	4.73	4.08
17	3.41	3.78	4.17	3.57
27	3.58	3.38	4.19	2.71
41	3.30	3.48	3.93	2.79
65	3.53	3.44	4.28	2.57
89	3.68	3.25	3.83	3.23

in a study of nutrient limitation of bacterioplankton growth in a close location in the Western Mediterranean Sea, in which pulses of nitrate (4 μM), phosphate (0.13 μM) and carbon (40 μM) were added (Sala *et al.*, 2002), and much lower than those reported in bioremediation experiments with oil and nutrient addition at concentrations between 73–560 μM phosphate and 0.5–5.0 mM nitrogen (Yakimov *et al.*, 2005; Cappello *et al.*, 2007; Coulon *et al.*, 2007). Therefore, our results indicate that pulses of low amounts of hydrocarbons, such as those used in this study, can stimulate bacterioplankton growth in the same manner as other nutrient sources shown to be limiting microbial growth.

Response of bacterial communities to diesel was rapid and differed depending on the sampling season

In order to evaluate the changes in diversity of presumably metabolically active bacteria after diesel addition we used terminal-restriction fragment length polymorphism (T-RFLP) profiling of microcosm seawater in both sets of experiments (winter and summer) at all sampling times, by targeting community 16S rRNAs after reverse transcription polymerase chain reaction (RT-PCR). The analysis revealed different responses in the two sets of experiments done. Thus, the calculated values of Shannon diversity index (H), based on 5′ terminal restriction fragments (T-RFs) obtained with the enzyme AluI (Table 1), showed no differences between winter control and diesel-treated microcosms except at the end of the incubation after 89 h. In contrast, there was a decrease in diversity in diesel-treated summer microcosms, which was evident already 3 h after initiating the experiment (Table 1). Oil treatment has been shown to decrease bacterial diversity, and this has been explained by a selection of specialist hydrocarbon-degrading bacteria, particularly when nutrients were added (Kasai *et al.*, 2002; Coulon *et al.*, 2007). In contrast, our results show that under conditions of lower hydrocarbon concentration and without biostimulation, a decrease in diversity and the proliferation of putative hydrocarbon degraders (see

below) might or might not occur. This might be dependent on the initial composition of the communities (which varies in different seasons as shown below), their nutritional status and other environmental factors.

To compare the bacterial community structure in all the samples, we summarized the generated 16S rRNA T-RFLP profiles in datasets including T-RFs originated with two restriction enzymes (AluI and HhaI). Cluster analysis using UPGMA method and Bray–Curtis similarity coefficient was then performed (Fig. 3). Profiles for control microcosms along the experiment were on average 75% and 80% similar (winter and summer microcosms respectively) to the profiles at the beginning of the experiment (T0). Therefore, confinement caused only small differences in bacterial composition, mainly at the end of the experiment (T5–T6). In contrast, similarity values to pro-
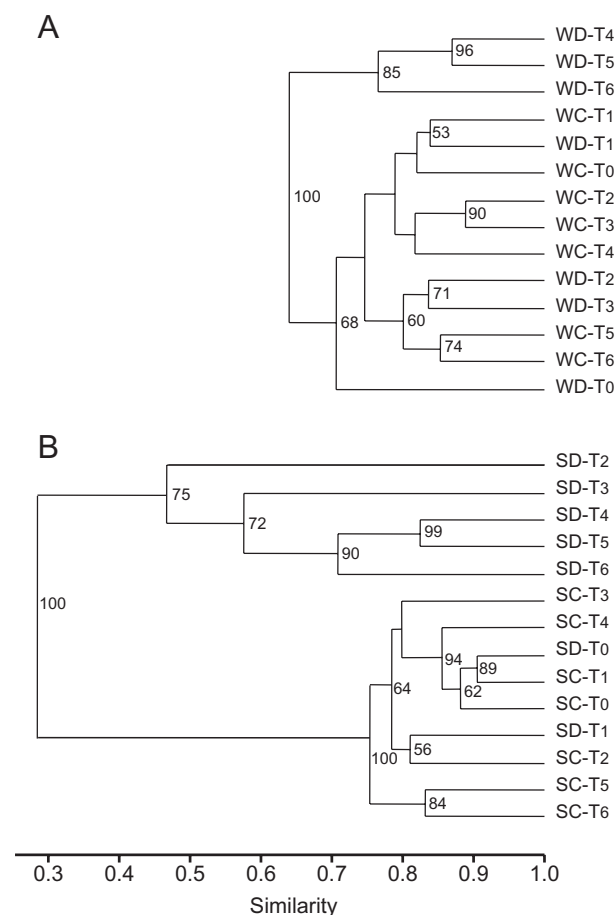
Fig. 3. UPGMA dendrogram showing the clustering of T-RFLP profiles of amplified 16S rRNAs obtained from microcosm samples. Samples are designated with letters 'W' and 'S' for winter and summer microcosms respectively, and with letters 'C' and 'D' for control and diesel treatment respectively. The sampling time is indicated in each case. Bootstrap values higher than 50% (1000 resamplings) are shown.
A. Winter microcosms.
B. Summer microcosms.

files at T0 in diesel-treated microcosms were lower (60% and 35% on average in winter and summer respectively), which showed the influence of the treatment on bacterial communities. These values are similar or even lower than those obtained in an experiment of crude oil degradation in seawater from the North Sea after 7 days of incubation (Brakstad and Lødeng, 2005). Significant differences in community composition with respect to the controls were observed in just few hours (17 h, T2) in summer microcosms (Fig. 3B) and at later time in winter microcosms (41 h, T4, Fig. 3A). Moreover, profiles from control and treated microcosms from corresponding sampling times were less dissimilar (75% on average) in winter microcosms than in summer microcosms (43%). These results indicated that the changes caused by diesel were stronger in the summer microcosms. The significance of the changes in T-RFLP profiles obtained from treated versus untreated microcosms was confirmed by ANOSIM ($P < 0.01$). Our results agree with those of Cappello and colleagues (2007), who reported shifts in community composition within two days in nutrient supplemented microcosm experiments simulating an oil spill in harbour seawater. This rapid response of bacterial communities to hydrocarbon addition might have been neglected in most of the studies done with longer time scales of days or weeks (Brakstad and Lødeng, 2005; Coulon *et al.*, 2007). When the profiles of the two experiments (winter and summer microcosms) were compared together (data not shown), we observed that there was no convergence in the profiles of bacterial communities in the presence of diesel. This result indicated that different communities developed in response to the treatment in summer and winter microcosms. However, it has to be taken into account that the communities at the start of the experiment in winter and summer microcosms were also different (similarity of 40%).

Shifts in the relative abundance of main components of the bacterioplankton of pristine oligotrophic water after diesel addition

In order to determine which bacterial populations, presumably metabolically active, were affected by diesel treatment, six 16S rRNA clone libraries were constructed with samples from winter (W) and summer (S) microcosms: two at the beginning of the experiment (0 h, T0) just before adding diesel (libraries designated WC0 and SC0 respectively), and the other four after 65 h of incubation (T5) for control (libraries WC5 and SC5) and diesel microcosms (WD5 and SD5). This time was selected because the T-RFLP profiles of untreated samples remained similar (approx. 80%) to the initial conditions (Fig. 3). Clones were initially screened by RFLP with the enzyme HhaI (see supplementary Table S1). Represen-

tatives of all groups as well as unique clones were sequenced. After discarding chimeras we obtained a total of 274 sequences of approximately 800 nt on average. We also used the clone libraries to generate a collection of T-RFs for enzymes AluI and HhaI that we then compared with the fragments obtained in profiles from the whole communities. The aim was to infer the identification of the bacterial populations generating the T-RFs, which had higher relative abundances and discriminated better the communities in control and diesel-treated microcosms [determined by their contribution to the ordination of the T-RFLP profiles by Principal Component Analysis, PCA (data not shown)]. This also allowed us to analyse their temporal dynamics during the experiments.

The overall phylogenetic composition found in the libraries is shown in Fig. 4, in which all the clones belonging to the same RFLP group were affiliated according to the sequenced representative clones. All the sequences affiliated with groups commonly found in surface coastal seawater, such as *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes* and *Cyanobacteria*. In addition, we have detected sequences affiliated with *Verrucomicrobia*, *Betaproteobacteria* and a sequence of picoeukaryote chloroplast in winter microcosms (see supplementary Table S2). The composition of the libraries was statistically compared using LIBSHUFF analysis. In agreement with the results of the T-RFLP analysis, the two libraries corresponding to T0 (winter and summer microcosms) were significantly different ($P = 0.001$), confirming the temporal variability of the bacterial communities in this area reported previously (Nogales *et al.*, 2007; Aguiló-Ferretjans *et al.*, 2008). Libraries from control microcosms at T0 and T5 were not significantly different ($P = 0.014$ and $P = 0.019$ in winter and summer microcosms respectively), confirming a low effect of confinement. Besides, there was no apparent proliferation of fast-growing cultivable bacteria, since most of the clones were related to sequences of uncultured marine bacteria. Libraries from diesel-treated microcosms were significantly different from those at T0, as well as to the corresponding controls at T5 ($P = 0.001$). Sequence analysis confirmed that the composition of the libraries from the two diesel treatments at T5 (winter and summer microcosms) was significantly different ($P = 0.001$), in agreement with the results of the T-RFLP profiling. Rarefaction curves based on the sequence types defined with 0.03 distance cut-off showed that the diversity of the bacterial communities was reasonably well explored (data not shown), and coverage values ranged between 55% and 75%.

The composition of the 16S rRNA libraries at the beginning of microcosm experiments resembled that commonly found in pristine surface coastal waters in the Mediterranean (Alonso-Sáez *et al.*, 2007; Aguiló-Ferretjans *et al.*, 2008) and different locations distributed around the world
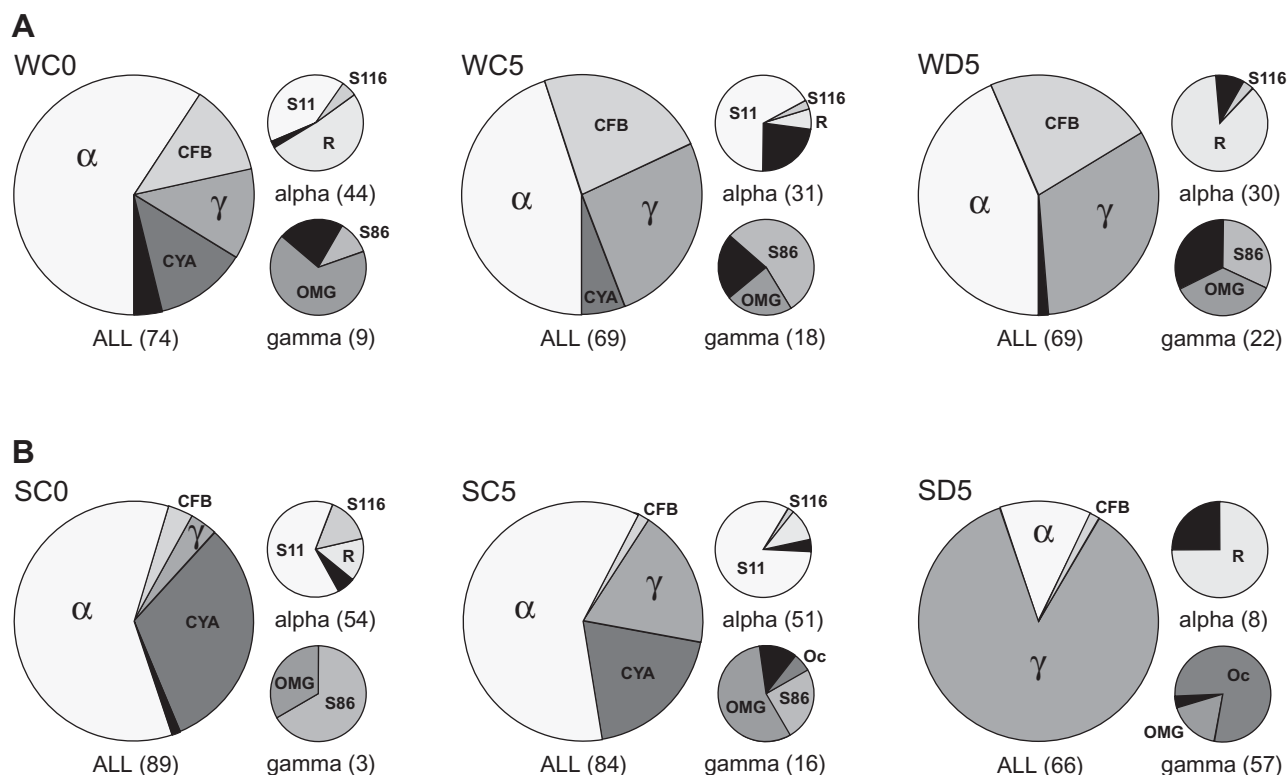
A

B

Fig. 4. Phylogenetic composition of the clone libraries retrieved from control and diesel treated microcosms. Clones belonging to the same RFLP group were affiliated according to the sequence of the representative clones. At the level of bacterial division or class, sectors represent percentages of each group to the total number of clones in each library (shown in parenthesis). Smaller circles represent the abundance of clones belonging to different groups within the classes *Alpha-* and *Gammaproteobacteria*, with respect to the total of clones from these two classes (shown in parenthesis). Sectors in black colour represent the sum of clones belonging to other, not specified, phylogenetic groups. Labels are as follows: α, *Alphaproteobacteria*; γ, *Gammaproteobacteria*; CFB, *Bacteroidetes*; CYA, *Cyanobacteria*; S11, SAR11 group; S116, SAR116 group; R, *Roseobacter* clade; S86, SAR86; OMG, oligotrophic marine *Gammaproteobacteria*; Oc, *Oceanospirillaceae*.
A. Winter microcosms: WC0 (control T0); WC5 (control T5) and WD5 (diesel T5).
B. Summer microcosms: SC0 (control T0); SC5 (control T5) and SD5 (diesel T5).

(Pommier *et al.*, 2007). Libraries from control microcosms (winter and summer) were dominated by sequences belonging to *Alphaproteobacteria*, and particularly to the SAR11 group (Fig. 4), which is the most abundant heterotrophic microbe in the sampled site and in oligotrophic oceans worldwide (Morris *et al.*, 2002; Aguiló-Ferretjans *et al.*, 2008). However, we did not detect sequences from SAR11 in any of the libraries from diesel-treated microcosms (Fig. 4). These results agree with previous environmental data, which showed the absence of SAR11-like sequences in the polluted waters of a recreational marina near the location sampled for the microcosms (Nogales *et al.*, 2007; Aguiló-Ferretjans *et al.*, 2008).

To explore the apparent disappearance of SAR11 phylotypes in the presence of diesel we interrogated the T-RLP profiles for the presence of T-RFs characteristic of clones of this group. We found that a fragment exclusive to all SAR11 clones (obtained with AluI and sized as 171 nt, hereafter designated as A171) was one of the most important fragments differentiating the T-RFLP profiles of the communities in diesel-treated, both in summer and winter microcosms. Figure 5A shows that there was a fast decrease in the relative abundance of this fragment in both, summer and winter, diesel-treated microcosms. Within 27 h there was a 50% decrease in the relative abundance of T-RF A171 in diesel winter microcosms (total reduction of 92% at the end of the experiment), and this percentage was much higher in summer microcosms (94% decrease at 27 h, 97% at the end of the experiment). This would explain why SAR11 sequences were not detected in libraries from diesel-treated microcosms at T5. The reduction in the relative abundance of fragment A171 could be due to a detrimental effect of diesel over SAR11 populations or simply to a lack of detection of their rRNAs due, for example, to the proliferation of bacteria with ribosome contents higher than these oligotrophic organisms with diluted cytoplasm (Giovannoni *et al.*, 2005). The significant increase in hybridization percentages with probes EUB I-III (Fig. S2), observed in diesel-treated summer microcosms at 27 h, would support the
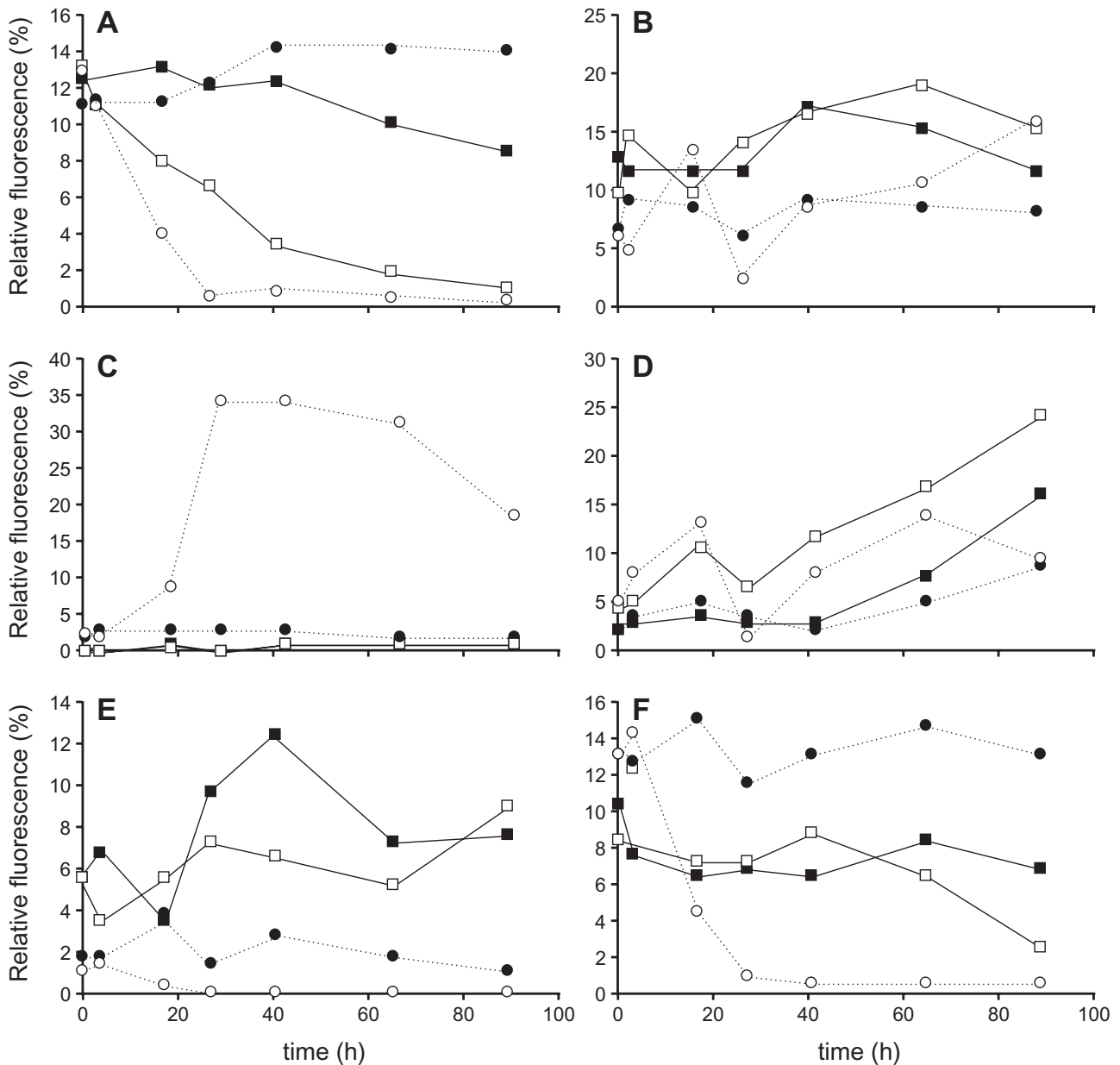
Fig. 5. Variation in the relative abundance of selected T-RFs, diagnostic for different bacterial groups, during the microcosm experiments. Square symbols with solid lines correspond to winter microcosms. Circle symbols and dashed lines correspond to summer microcosms. Closed symbols correspond to controls and open symbols to diesel treatments.
A. SAR11 group (T-RF AluI size 171 nt).
B. *Roseobacter* group (sum of relative abundances of T-RFs AluI sizes 245 and 248 nt).
C. *Oceanospirillaceae* (sum of relative abundances of T-RFs HhaI sizes 209, 361 and 369 nt).
D. OMG group (sum of relative abundances of T-RFs AluI 229 and HhaI 354 nt).
E. SAR 86 group (sum of T-RFs HhaI 365 and 366 nt).
F. *Synechococcus* (sum of relative abundances of T-RFs AluI sizes 125, 189 and 204 nt).

second hypothesis, and might explain the rapid decrease in the abundance of fragment A171 observed. In contrast, EUB I-III hybridization percentages were equally low in control and diesel-treated winter microcosms during most of the experiment. No apparent proliferation of high-ribosome containing bacteria occurred in these micro-

cosms at times when there was a decrease in the relative abundance of fragment A171. Therefore, this result would support the hypothesis of a negative effect of diesel treatment over SAR11 populations.

In libraries from diesel-treated microcosms, the dominant alphaproteobacterial sequences belonged to the

Roseobacter clade, particularly in winter microcosms (Fig. 4). Sequences related to groups RCA, CHAB-I-5 (Buchan *et al.*, 2005) as well as to the isolate *Marinovum algicola*, were observed in diesel-treated and control microcosms (Fig. 6). However, most of the sequences from diesel treatments formed a group related to *Roseovarius* spp. and the sequences of isolate C21, described as hydrocarbon degrader (Harwati *et al.*, 2007), and to clones BES37 and BEW120, obtained from a beach near the area sampled for microcosm preparation (Aguiló-Ferretjans *et al.*, 2008). Interestingly, a clone sequence retrieved from the winter diesel microcosm was identical to clone MAW9, related to *Loktanella* spp., retrieved from a recreational marina in the proximity of the sampling site (Aguiló-Ferretjans *et al.*, 2008). Thus, these sequences could represent bacteria adapted to pollution, which may be indigenous to the area near that marina. Some clones were only found in the absence of diesel, and therefore they might represent populations negatively affected by this pollutant. These sequences were related to group NAC11-7 and to clone BAS35, retrieved previously from the pristine location sampled in this study (Aguiló-Ferretjans *et al.*, 2008).

The increase in the abundance of T-RFs characteristic of the *Roseobacter* clade (A245 and A248), expected from the results of the libraries, was evident only at the later incubation times (Fig. 5B). Accordingly, these fragments had a minor contribution to the overall differentiation of the profiles. Although, there was a transient increase in the abundance of T-RFs for *Roseobacter* clade shortly after diesel addition, peak dynamics seemed to indicate that the positive response of the *Roseobacter* clade to diesel addition was slow. We can then hypothesize that the increase in the abundance of roseobacters might be linked to hydrocarbon utilization in agreement with previous results (Brakstad and Lødeng, 2005; Coulon *et al.*, 2007; McKew *et al.*, 2007), and the later genomic studies showing the genetic potential for hydrocarbon utilization of this bacterial group (for a revision see Buchan and González, 2010).

In contrast with the results for *Alphaproteobacteria*, there were important differences in the proportion and composition of gammaproteobacterial sequences in winter and summer diesel-treated microcosms (Figs 4 and 7). Gammaproteobacterial sequences were the second most abundant group in the winter microcosm libraries as reported previously in the area sampled (Aguiló-Ferretjans *et al.*, 2008). The sequences belonged mainly to the groups SAR86 and OMG (Cho and Giovannoni, 2004): OM60, KI89A and SAR92. Control libraries from summer microcosms had similar proportion and composition of *Gammaproteobacteria* than the libraries from winter microcosms. However, in the library from the diesel treatment in summer microcosms, clones affiliated

to the *Gammaproteobacteria* outnumbered by far those of the *Alphaproteobacteria* (Fig. 4B). The increase in *Gammaproteobacteria* was confirmed by FISH using Gam42a probe (Manz *et al.*, 1992). Percentages of cells hybridizing with this probe in control and diesel-treated summer microcosms after 65 h were 3% and 18% respectively. A group of clone sequences (7 clones) from the summer diesel microcosm affiliated with the OM60 group clustered together with MAW74 and MAS35, clones previously obtained in the nearby marina (Aguiló-Ferretjans *et al.*, 2008). Most of the rest of gammaproteobacterial sequences in this microcosm (23) were affiliated to the family *Oceanospirillaceae*, which includes some of the oligotrophic marine hydrocarbon degrading bacteria described so far (Garrity *et al.*, 2005). The clones were related to *Oceanospirillum*, *Neptuniibacter*, and to strain CAR-SF, a carbazole-degrading bacterium related to *Neptunomonas naphthovorans* (Fuse *et al.*, 2003). Sequences related to isolate CAR-SF were also detected in microcosms with contaminated sediments of Milazzo Harbor supplemented with nutrients (Yakimov *et al.*, 2005). Proliferation of hydrocarbon degraders of the genus *Alcanivorax*, *Cycloclasticus* or *Thalassolituus* was not observed in this study as was in others (Kasai *et al.*, 2002; Cappello *et al.*, 2007; Coulon *et al.*, 2007; McKew *et al.*, 2007; Teira *et al.*, 2007), probably due to the conditions of our experiment done with low concentrations of diesel and without nutrient addition.

The dynamics of T-RFs representatives of the groups OMG, SAR86 and *Oceanospirillaceae* was also analysed. The T-RFs characteristic of clones from the *Oceanospirillaceae* (represented in Fig. 5C by the sum of T-RFs H209, H361 and H369) were the most important in differentiating profiles from control and diesel treatment in summer, because they were virtually undetectable in winter (relative abundance below 1%). As can be seen in Fig. 5C, the increase in the abundance of these T-RFs in the presence of diesel was very rapid (higher than 1700% increase in 27 h) and agreed with the increase in total cell counts (Fig. 2), with the decrease in diversity in the diesel-treated microcosms (Table 1), and probably with the increase in percentages of detection with EUB I-III probes (Fig. S2). All these evidences indicated that populations of *Oceanospirillaceae*, present in low proportion at the beginning of the experiment (relative abundance around 1.7%), responded very rapidly to diesel addition in summer but not in winter microcosms. Therefore, we can hypothesize that there were primed populations of putative hydrocarbon degraders in seawater in summer, ready to react to the presence of hydrocarbons in the water.

The T-RFs for OMG and SAR86 were relevant in the differentiation of profiles from diesel treatment, particularly in winter microcosms when their relative abundance was
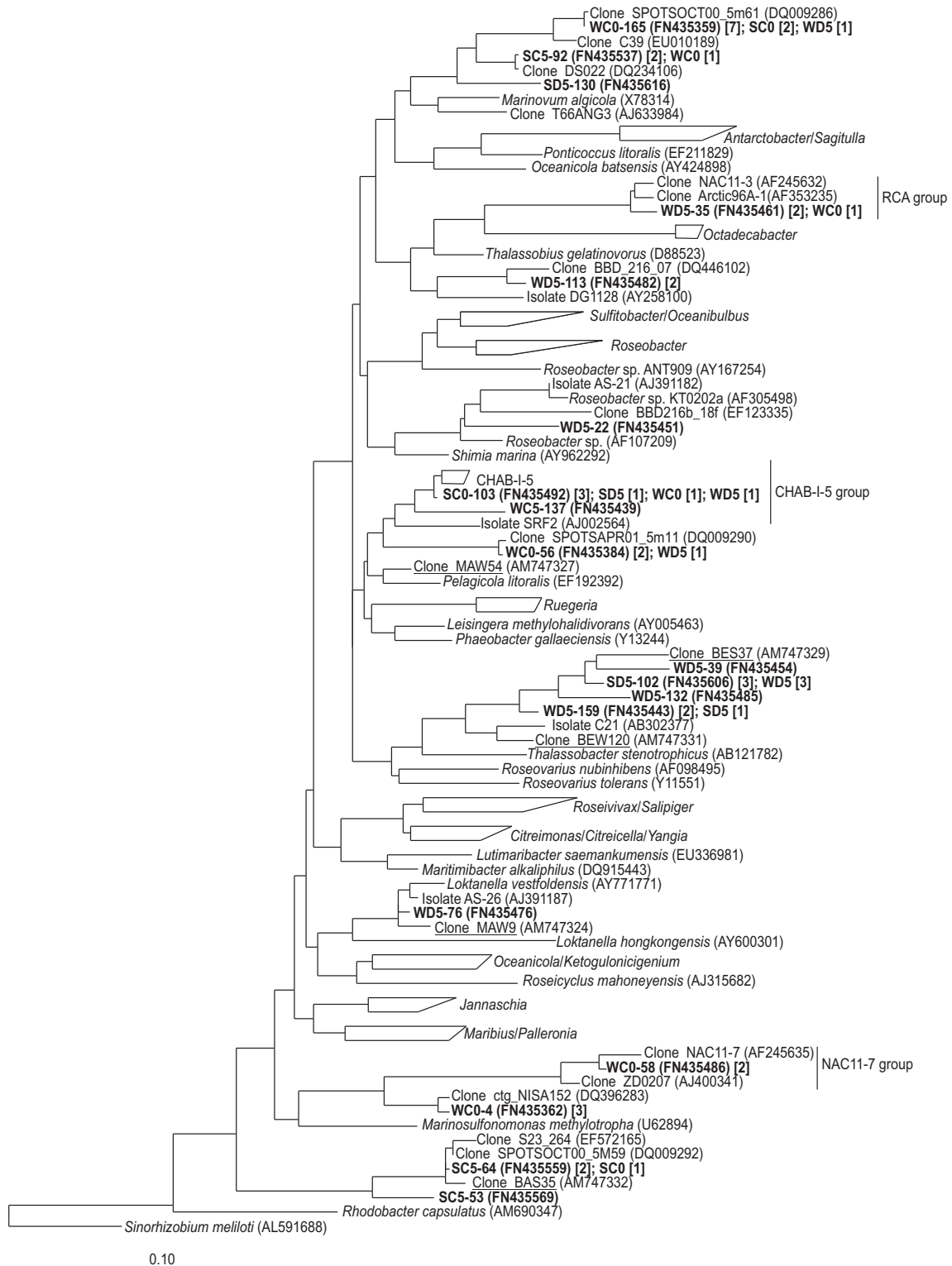
Clone SPOTSOCT00_5m61 (DQ009286)
WC0-165 (FN435359) [7]; SC0 [2]; WD5 [1]
Clone C39 (EU010189)
SC5-92 (FN435537) [2]; WC0 [1]
Clone DS022 (DQ234106)
SD5-130 (FN435616)
Marinovum algicola (X78314)
Clone T66ANG3 (AJ633984)
Antarctobacter/Sagitulla
Ponticoccus litoralis (EF211829)
Oceanicola batsensis (AY424898)
Clone NAC11-3 (AF245632)
Clone Arctic96A-1(AF353235)
WD5-35 (FN435461) [2]; WC0 [1]   RCA group
Octadecabacter
Thalassobius gelatinovorus (D88523)
Clone BBD_216_07 (DQ446102)
WD5-113 (FN435482) [2]
Isolate DG1128 (AY258100)
Sulfitobacter/Oceanibulbus
Roseobacter
Roseobacter sp. ANT909 (AY167254)
Isolate AS-21 (AJ391182)
Roseobacter sp. KT0202a (AF305498)
Clone BBD216b_18f (EF123335)
WD5-22 (FN435451)
Roseobacter sp. (AF107209)
Shimia marina (AY962292)
CHAB-I-5
SC0-103 (FN435492) [3]; SD5 [1]; WC0 [1]; WD5 [1]   CHAB-I-5 group
WC5-137 (FN435439)
Isolate SRF2 (AJ002564)
Clone SPOTSAPR01_5m11 (DQ009290)
WC0-56 (FN435384) [2]; WD5 [1]
Clone MAW54 (AM747327)
Pelagicola litoralis (EF192392)
Ruegeria
Leisingera methylohalidivorans (AY005463)
Phaeobacter gallaeciensis (Y13244)
Clone BES37 (AM747329)
WD5-39 (FN435454)
SD5-102 (FN435606) [3]; WD5 [3]
WD5-132 (FN435485)
WD5-159 (FN435443) [2]; SD5 [1]
Isolate C21 (AB302377)
Clone BEW120 (AM747331)
Thalassobacter stenotrophicus (AB121782)
Roseovarius nubinhibens (AF098495)
Roseovarius tolerans (Y11551)
Roseivivax/Salipiger
Citreimonas/Citreicella/Yangia
Lutimaribacter saemankumensis (EU336981)
Maritimibacter alkaliphilus (DQ915443)
Loktanella vestfoldensis (AY771771)
Isolate AS-26 (AJ391187)
WD5-76 (FN435476)
Clone MAW9 (AM747324)
Loktanella hongkongensis (AY600301)
Oceanicola/Ketogulonicigenium
Roseicyclus mahoneyensis (AJ315682)
Jannaschia
Maribius/Palleronia
Clone NAC11-7 (AF245635)
WC0-58 (FN435486) [2]   NAC11-7 group
Clone ZD0207 (AJ400341)
Clone ctg_NISA152 (DQ396283)
WC0-4 (FN435362) [3]
Marinosulfonomonas methylotropha (U62894)
Clone S23_264 (EF572165)
Clone SPOTSOCT00_5M59 (DQ009292)
SC5-64 (FN435559) [2]; SC0 [1]
Clone BAS35 (AM747332)
SC5-53 (FN435569)
Rhodobacter capsulatus (AM690347)
Sinorhizobium meliloti (AL591688)

0.10

Fig. 6. Dendrogram of the 16S rRNA sequences affiliated to the class *Alphaproteobacteria*. Clones are designated as 'W' and 'S' for winter and summer microcosms, followed by 'C' (for control) or 'D' (diesel treatment) and a number. The tree was calculated with nearly complete reference sequence data from databases. Clone sequences representative of each phylotype (defined at a distance cut-off of 0.03) were then added using the parsimony tool in ARB package. Numbers in brackets indicate the total number of sequences in every phylotype in each of the libraries where it was observed. Underlined clones belong to a previous study done in the area (Aguiló-Ferretjans *et al.*, 2008). The sequence of *Sinorhizobium meliloti* was used as outgroup.
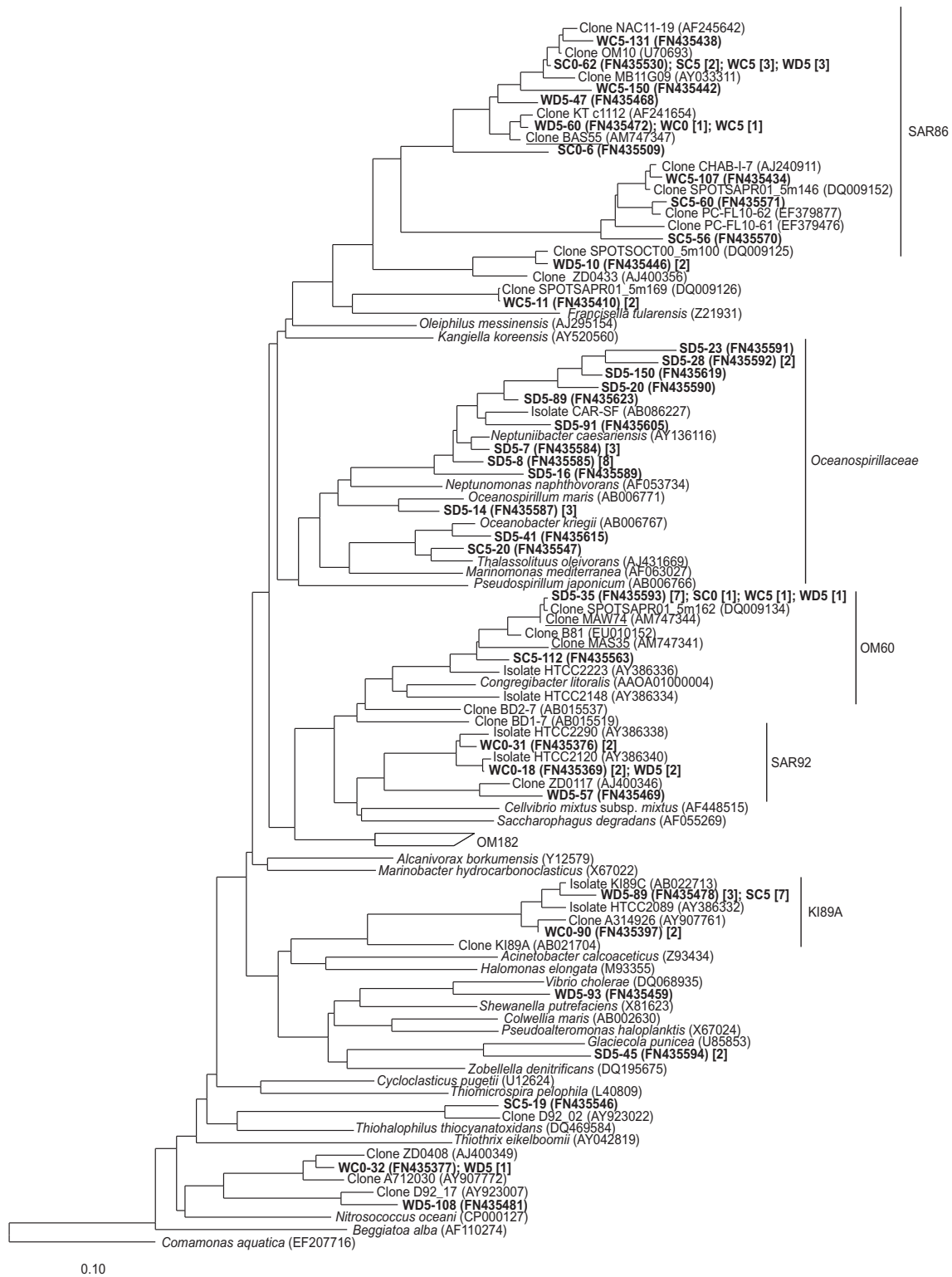
Fig. 7. Dendrogram of the 16S rRNA sequences affiliated to the class *Gammaproteobacteria*. Clones are designated as 'W' and 'S' for winter and summer microcosms, followed by 'C' (for control) or 'D' (diesel treatment) and a number. The tree was calculated with nearly complete reference sequence data from databases. Clone sequences representative of each phylotype (defined at a distance cut-off of 0.03) were then added using the parsimony tool in ARB package. Numbers in brackets indicate the number of sequences in every phylotype in each of the libraries where it was observed. Underlined clone names belong to a previous study done in the area (Aguiló-Ferretjans *et al.*, 2008). The sequence of *Comamonas aquatica* was used as outgroup.

higher. The T-RFs from the OMG group (A229 and H354) increased steadily in all microcosms but mainly in the presence of diesel (Fig. 5D). In contrast, the relative abundance of T-RFs characteristic of the SAR86 group (H365 and H366) was higher in control microcosms than in the diesel-treated microcosms, particularly in summer, when T-RFs for SAR86 were not detected after 27 h of incubation (Fig. 5E). These results agree again with environmental data available on the area sampled, which shows a decrease of SAR86-like bacteria and an increase of OM60-like bacteria in the more polluted waters (Nogales *et al.*, 2007; Aguiló-Ferretjans *et al.*, 2008).

Apparently, diesel treatment did not cause any effect on the abundance of sequences from the group *Bacteroidetes* in libraries from the control and diesel-treated microcosms at T5 (Fig. 4). There were pronounced seasonal differences in the detection of *Bacteroidetes* sequences in the libraries, and their proportion in libraries from summer microcosms was very low. These observations were also supported by the dynamics and relative abundance of the T-RFs representative for this group (sum of H92 and H94) in the microcosms (Fig. S3). These results contrast with environmental data which showed a higher abundance of *Bacteroidetes* in water from the marina (Nogales *et al.*, 2007; Aguiló-Ferretjans *et al.*, 2008).

The last group of sequences observed in the libraries, which were more abundant in summer microcosms, affiliated to the *Cyanobacteria* (genus *Synechococcus*). These sequences were only obtained in libraries from control microcosms (Fig. 4). The dynamics of characteristic T-RFs of these picocyanobacteria in summer microcosms (sum of A125, A189 and A204) followed the same pattern than that of T-RF A171 from SAR11, and opposite to the increase of the T-RFs from the *Oceanospirillaceae*. In winter, no clear decrease in their abundance of these T-RFs was observed in the diesel-treated microcosms according to the sum of signature T-RFs (Fig. 5F).

The results presented in this study show that diesel addition cause significant and rapid changes in the composition of the metabolically active bacterioplankton from coastal oligotrophic seawater. The effect on communities sampled at the same location but at different times was different, presumably because community composition and water physico-chemical conditions also differed. The most important changes in diversity observed in the experiments presented here agree with previous data comparing the composition of bacterioplankton communities in environments with high and low pollution levels. Therefore, diesel pollution alone, without considering other factors such as nutrient enrichment, might explain why certain oligotrophic marine bacterial groups are excluded from polluted environments such as harbours.

Experimental procedures

Seawater sampling

Surface water (top 2–3 centimetres) from a coastal area in the SW of Mallorca Island, Spain (39°29′05″N, 2°28′16″E) was collected to prepare the microcosms. Water at this location has been shown to hold typical oligotrophic bacterioplankton and considered to be undisturbed despite its proximity, approximately 1 km, to a recreational marina (Nogales *et al.*, 2007; Aguiló-Ferretjans *et al.*, 2008). The experiment was done with water collected at two time points, on a late winter day (March) and a summer day (July) of year 2007. Water was taken directly in acid-washed plastic containers and immediately transported to the laboratory. Different parameters, such as *in situ* water temperature, pH and chlorophyll *a* concentration in seawater, were analysed as described before (Nogales *et al.*, 2007).

Microcosms setup

Four microcosms, control and treatment with duplicates, were prepared in plastic tanks filled with 44 l of surface seawater at the two different sampling times. They were covered with loose methacrylate lids and incubated under natural light at room temperature in the laboratory. Water was continuously oxygenated and mixed using aquarium pumps (Eheim Air pump 400) expelling filtered air (0.2 μm, pore diameter syringe filters, Nalgene) to the bottom of each tank. Once set, the microcosms were let to stabilize for 2 h before the first sample was taken (T0). Then, two microcosms were supplemented with diesel oil at an initial concentration of 30 mg l⁻¹. Subsequent diesel additions (10 mg l⁻¹) were performed daily, 24 h after the last addition to compensate evaporation losses and adhesion to the walls of the tanks (3 additions in total). The other two microcosms, without any treatment, were used as control. Samples were taken 3, 17, 27, 41, 65 and 89 h after initiating the experiment to carry out the analysis. A scheme of the microcosms and sampling procedure is shown in Fig. 1. The length of the incubation was optimized in a previous experiment run for 15 days with untreated seawater taken from the same sampling site in which we determined that bacterial communities were strongly affected by confinement after six days (Fig. S1).

Total cell counts, fluorescent in situ *hybridization and plate counts*

At each sampling time, samples were taken from each microcosm (control and treatment with duplicates) and fixed overnight at 4°C with formaldehyde (4% v/v, final concentration). After fixation, cells were collected onto 0.2 μm polycarbonate filters (Millipore) and washed twice with phosphate buffered saline. Hybridizations and microscopy counts of hybridized and DAPI (4′,6-diamidino-2-phenylindole)-stained cells were done as described previously (Glöckner *et al.*, 1996). Probes EUB338 I-III (Amann *et al.*, 1990; Daims *et al.*, 1999) for the domain *Bacteria* and Gam42a with competitor (Manz *et al.*, 1992) for *Gammaproteobacteria* were used. Probes were synthesized with Cy3 fluorochrome at the 5′-end (Interactiva

Biotechnologie). For each sample, between 1300 and 2600 DAPI-positive cells were counted with an Axiophot microscope (Zeiss).

All samples were plated by triplicate in minimal marine medium (MMM) solidified with washed agar (15 g l^{-1}). MMM is based in the composition of medium ONR7a (Dyksterhouse *et al.*, 1995) with slight modifications: it was prepared with artificial seawater (Sigma-Aldrich), 0.1 M Tris-HCl pH 7.4 as buffer, 0.63 mM Na_2HPO_4, and 5 μM of $Fe(NH_4)_2(SO_4)_2 \times 6H_2O$ as iron source. Plate counts for oligotrophic heterotrophic bacteria were made on MMM and supplemented with glucose (10 mg l^{-1}, 55.6 μM) and casaminoacids (5 mg l^{-1}). For counts in the presence of hydrocarbons, 25 μl of diesel oil was spread on the lid of plates prepared with MMM without added carbon source. Plates were incubated at same temperatures than microcosms (20°C in winter and 26°C in summer), under natural daylight conditions. Colony counts were made after 7 days of incubation.

RNA extraction

At each sampling time 2 l water samples were taken from the different microcosms and they were filtered sequentially through 5 and 0.22 μm pore diameter polyvinylidene fluoride membrane filters (Millipore) for microbial biomass collection. Filters were frozen immediately in liquid nitrogen and kept at −80°C until processed. Since total and FISH counts indicated a similar response in the duplicate microcosms we selected only one replicate of the control and the treatment for molecular analyses. Total nucleic acids from 0.22 μm filters were extracted by using the protocol of Nogales and colleagues (2002) with slight modifications to simplify the procedure for water samples. Briefly, extraction buffer containing proteinase K, lysozyme and SDS was added to each filter and incubated at 37°C for 20 min with shaking. After adding NaCl and hexadecylmethylammonium bromide (CTAB), the samples were incubated at 65°C for 10 min and then subjected to a freeze-thaw cycle by submerging them in liquid nitrogen and subsequently in a water bath at 65°C. This step was repeated three times. The lysate was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v). The aqueous phase was recovered and nucleic acids were precipitated by adding 0.6 volume of isopropanol with 0.3 M sodium acetate (pH 4.8) and 10 mM $MgCl_2$. The pellet containing nucleic acids obtained after centrifugation at 4°C at 16 000 *g* for 30 min was washed with 70% ethanol, air dried and resuspended in DEPC-treated deionized water. All reagents were prepared using diethyl pyrocarbonate (DEPC)-treated Milli-Q water (Millipore) and autoclaved. Aliquots of extracted nucleic acid were treated with 0.6 U μl^{-1} of RNase-free DNase I (Roche Applied Science) at 37°C for 90 min in 10 mM sodium acetate and 0.5 mM $MgSO_4$ (pH 5.0) followed by heat incubation at 75°C for 5 min to inactivate DNase activity. The quality and concentration of the extracted RNA was determined with a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies) according to manufacturer's instructions.

RT-PCR amplification of 16S rRNA

To discard the possibility of contaminating DNA in the RNA extracts, control PCR reactions targeting 16S rRNA in samples, which contained RNA and were not previously subjected to reverse transcription (RT), were prepared. When no amplification product was obtained, reverse transcription reactions were carried out by using the enzyme Superscript III reverse transcriptase (Invitrogen) as recommended by the manufacturer. The RT reaction mixtures (20 μl) contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, each deoxynucleoside triphosphate at a concentration of 500 μM, 250 ng of random primers (Life Technologies), 2–6 μg total RNA and 200 U of Superscript III reverse transcriptase. RT products (cDNA) were kept frozen at −20°C until they were used for PCR amplification. Partial 16S rRNAs were amplified from cDNA using universal primers 27f and 907rm (Lane, 1991 and Muyzer *et al.*, 1998). For T-RFLP the forward primer was labelled at the 5′-end with the phosphoramidite dye 6-FAM (Applied Biosystems). PCR reactions were carried out in 50 μl reaction volumes containing reaction buffer [10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$], each of the four deoxyribonucleoside triphosphates (Roche Applied Science) at 100 μM concentration, 0.4 μM concentration of primers (Invitrogen), 1 μl template cDNA and 1.5 U of *Taq* DNA polymerase (GE Healthcare). Reactions were done with a Mastercycler personal thermal cycler (Eppendorf). PCR cycling conditions were 94°C for 5 min followed by 30 cycles of 94°C for 60 s, 50°C for 60 s, 72°C for 90 s, with a final extension step of 10 min at 72°C.

Terminal-restriction fragment length electrophoresis of 16S rRNA

Amplified products were purified using the PureLink PCR Purification Kit (Invitrogen) according to the manufacturer's instructions. Ten microlitres of the purified products was digested separately with 20 U of restriction endonucleases AluI and HhaI (Roche Applied Science and New England Biolabs respectively) at 37°C during 3.5 h. Restriction fragments were mixed with a ROX-labelled size standard GeneScan-500 (Applied Biosystems), denatured by heating at 96°C for 5 min in formamide and loaded in an ABI 3130 Genetic Analyzer (Applied Biosystems) for electrophoretic separation, using 36 cm length capillaries filled with POP-7 polymer (Applied Biosystems). Samples were electrophoresed at 15 kV, 35–45 mA, for 30 min at 60°C; the injection time was 10 s, the injection voltage 7 kV and laser power 15 mW. Replicate profiles were run for each sample. Electrophoretic patterns were analysed with PeakScanner Software version 1.0 (Applied Biosystems), using a threshold value of 75 relative fluorescence units. Profiles were checked for possible artefacts and incorrect peak determination. Standardization of peak fluorescence data was performed as described previously (Fahy *et al.*, 2005). Homologous peaks in replicate profiles were aligned using the program T-Align to generate a consensus profile for each sample in which peaks appearing in a single replicate were excluded (Smith *et al.*, 2005). Sample consensus profiles were then compared in order to generate a comparison profile among all the samples (Smith *et al.*, 2005). A file with the results containing the two types of T-RFs generated for AluI and HhaI for each sample was obtained and used for further analyses. Comparison of sample profiles and generation of dendrograms was carried

out by hierarchical clustering analysis using the Unweighted Pair-Group Moving Average (UPGMA) method and the Bray–Curtis coefficient for distance measure, using the software PAST-Paleontological Statistics version 1.29 (Hammer *et al.*, 2001). Statistic comparison of the profiles and diversity indexes was also calculated with this package.

Clone library construction

Six clone libraries from cDNA were generated in total from the winter and summer microcosms. In each case, the first sample for cloning corresponded to the start of the experiment (0 h, T0). The other four libraries were prepared from samples taken after 65 h (T5) from the control and the diesel-treated microcosms. Amplified PCR products were cloned using the TOPO TA cloning kit (Invitrogen) and the clones selected according to the manufacturer's recommendations. Approximately 100 clones from each library were grouped after restriction fragment length polymorphism (RFLP) analysis with HhaI restriction endonuclease (New England Biolabs). Clones exhibiting the same RFLP patterns were grouped and representatives from every pattern, including those with only one representative, were sequenced. Besides, those groups with four or more representatives were selected for T-RFLP analysis. The sizes of T-RFs corresponding to the AluI and HhaI 5′-end (6-FAM-labelled fragments) for each clone were recorded and compared with the collection of T-RFs obtained with the environmental water samples.

Sequencing and phylogenetic analysis

The nucleotide sequences of the selected clones were obtained as described in Aguiló-Ferretjans and colleagues (2008). Cloned 16S rRNA sequences were compared with reference sequences in databases using the BLAST program (Altschul *et al.*, 1990). The programs Mallard and Pintail (Ashelford *et al.*, 2005; 2006) were used to screen all sequences for potential chimeras. Sequences judged to be potentially chimeric by these software packages were manually confirmed using BLAST and the ARB package (Ludwig *et al.*, 2004) and excluded from the analysis. Sequences were aligned using the ARB package. Evolutionary distances, derived from sequence pair dissimilarities, were calculated using the correction of Jukes and Cantor (Jukes and Cantor, 1969). Phylogenetic trees were calculated using the neighbour-joining distance method (Saitou and Nei, 1987) with nearly complete reference 16S rDNA sequences. The partial sequences generated in this study were added to the calculated trees using the parsimony tool in the ARB package. Statistical comparison of library composition was done with the program LIBSHUFF (Singleton *et al.*, 2001). Assignation of sequences to phylotypes was done with the program Mothur (Schloss *et al.*, 2009) using sequence dissimilarities of 0.03.

Nucleotide sequence data

The sequence data generated in this study has been deposited in the EBI sequence database under the accession numbers FN435351 to FN435624.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Results of the preliminary experiment for determining the maximum time for microcosm incubation. Solid squares represent DAPI counts; open squares represent FISH counts with probes EUB I-III; grey circles represent plate counts in marine agar. Standard errors of two replicate microcosms are shown.

Fig. S2. Variation in the percentage of prokaryotic cells hybridizing with probes EUBI-III (with respect to total DAPI counts) during microcosm incubations. Square symbols with solid lines correspond to winter microcosms. Circle symbols and dashed lines correspond to summer microcosms. Closed symbols correspond to controls and open symbols to diesel treatment.

Fig. S3. Variation in the relative abundance of T-RFs representative of *Bacteroidetes*. The sum of the relative abundances of two T-RFs obtained with the enzyme HhaI corresponding to sizes 92 and 94 nt are shown. Square symbols with solid lines correspond to winter microcosms. Circle symbols with dashed lines correspond to summer microcosms. Closed symbols correspond to controls and open symbols to diesel treatment.

Table S1. Detailed information on the results of the screening of clone libraries by RFLP analysis with enzyme HhaI.

Table S2. Detailed information on the accession numbers and affiliation of the clone sequences generated in this study.

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