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Cosmopolitan heterotrophic microeukaryotes are active bacterial grazers in experimental oil-polluted systems

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Summary

We investigated the population dynamics and prevailing 18S rDNA phylotypes of microeukaryotes (≤ 10 μm) in microcosms containing seawater from either an unpolluted oligotrophic site or a chronically oil-polluted mesotrophic site of the Aegean Sea, amended with crude oil (100 p.p.m. final concentration) and crude oil plus emulsifier (10 p.p.m. final concentration). The addition of oil alone did not result in an important increase of bacteria or their predators, while the addition of oil and emulsifiers caused an important increase in bacteria followed by nanoflagellate predator response. We observed an important shift in the microeukaryotic community structure, which was characterized by the dominance of the same heterotrophic nanoflagellates in all oil-polluted treatments. Thus, the resulting 18S rDNA phylotypes were dominated (48.1–82.4%) by *Paraphysomonas foraminifera* in all treatments containing crude oil and crude oil plus emulsifier. The origin of the seawater, i.e. unpolluted versus chronically oil-polluted, had no effect on the dominant eukaryote, suggesting that the ubiquitous *P. foraminifera* is an effective opportunist in oil-polluted aquatic systems. The next dominant phylotypes were *Monosiga brevicollis* (≤ 27.0%) and *Pseudobodo tremulans* (≤ 23.1%). However, the addition of the emulsifier increased the dominance of *P. foraminifera* but decreased that of *M. brevicollis* and *P. tremulans*. Our study revealed that these dominant oil-tolerant eukaryotes, which are commonly found in the marine environments, are important grazers of bacteria and as such their dynamics should be taken into account in bioremediation practices *in situ*.

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Introduction

What we know today is that heterotrophic nanoflagellate (HNF) protists (2–20 μm in size) play an important ecological role in freshwater and marine microbial communities, both as major consumers of picoplankton and ensuring the recycling of limiting nutrients. What we do not know a great deal about, however, are the biogeography of most of these protists (especially in the food webs of disturbed or polluted systems), or the evolutionary relationships and taxonomy for several of the protistan taxa (Adl *et al.*, 2005). This lack of important information is largely due to the fact that small protists possess few identifiable morphological features, on which taxonomic and phylogenetic schemes are based, with which they can be identified in environmental samples (Corliss, 2002; Caron *et al.*, 2004; Massana *et al.*, 2006). The advent of molecular-based approaches in the 1990s, although not unlimited, has provided some invaluable tools in studying natural assemblages of prokaryotes and eukaryotes (Amann *et al.*, 1995; Caron *et al.*, 2004).

Today, petroleum represents one of the world's most important energy sources with vast quantities being shipped by oil tankers over great distances. Unfortunately this activity leads to two major environmental problems: – accidental oil spill, and more significantly, the illegal process of cleaning oil tanks at sea. The novel molecular approaches used in this study have brought a wealth of knowledge to that of microbial diversity (in particular bacteria), and have also contributed to substantially advancing our knowledge of transformation of hydrocarbons. Today, 79 bacterial genera are known that can use hydrocarbons as a sole source of carbon and energy, as well as nine cyanobacterial, 103 fungal and 14 algal genera that degrade or transform hydrocarbons (Prince, 2005). Hydrocarbonoclastic bacteria (HCB) are considered the most important microorganisms for the bioremediation of marine crude oil pollution. However, the process of oil

Table 1. Bottle and microcosm treatments used in this study.

Preliminary bottle experiments	
OLIGbottle1control	< 10 μm oligotrophic seawater concentrated ×10
OLIGbottle1+oil	< 10 μm oligotrophic seawater concentrated ×10 + crude oil (100 p.p.m.)
OLIGbottle2control	Unaltered oligotrophic seawater < 10 μm
OLIGbottle2+oil	Unaltered oligotrophic seawater < 10 μm + crude oil (100 p.p.m.)
Microcosm experiment	
OLIGcontrol[a,b]	< 10 μm unaltered oligotrophic seawater
OLIGoil[b]	< 10 μm unaltered oligotrophic seawater + crude oil (100 p.p.m.)
OLIGoil+emu[b]	< 10 μm unaltered oligotrophic seawater + crude oil (100 p.p.m.) + emulsifier (10 p.p.m.)
POLLcontrol[a]	< 10 μm chronically polluted seawater
POLLoil[b]	< 10 μm chronically polluted seawater + crude oil (100 p.p.m.)
POLLoil+emu[b]	< 10 μm chronically polluted seawater + crude oil (100 p.p.m.) + emulsifier (10 p.p.m.)

a. 18S rRNA gene analysis was pre-formed at 0 h (before oil or emulsifier addition).
b. Treatments where 18S rRNA gene analysis was pre-formed at the HNF peak abundance.

biodegradation is a biological network that includes, apart from HCB, other prokaryotes, protistan grazers and viruses (Head *et al.*, 2006). These organisms interact with each other and the biotic environment causing negative and/or positive feedbacks in oil degradation. Primary oil degraders need to compete with other microorganisms for limiting nutrients, i.e. mostly inorganic nitrogen and phosphorus, and additionally non-oil-degrading microorganisms, can be affected by metabolites and other compounds that are released by oil-degrading bacteria and vice versa. It is now believed that degradation of pollutants is a multistep procedure where each step is performed via distinct processes of functional groups of microorganisms. This network of processes needs to be clarified if efficient bioremediation practices are desired for oil-polluted sites.

Numerous studies have been conducted in order to better understand the crude oil-HCB dynamic and the isolation of new and important bacterial species (e.g. Yakimov *et al.*, 1998; 2004; 2005; Kasai *et al.*, 2001; Golyshin *et al.*, 2002; reviews by Van Hamme *et al.*, 2003 and Head *et al.*, 2006). The presence and the potentially important role of bacterial predators during bioremediation processes in heavily polluted environments have been implied in the past (Ratsak *et al.*, 1996; Kota *et al.*, 1999; Kinner *et al.*, 2002). The scarce available studies on nanoflagellates associated with oil pollution concern their occurrence either in oil-polluted experimental systems (Elmhirst, 1922) or in crude oil (Andrews and Floodgate, 1974). Atlas and colleagues (1976) reported that one of the major changes in microbial communities after oil pollution was the dominance of flagellate protozoa over other heterotrophs. To the best of our knowledge, no study exists on the population dynamics along with the identification of eukaryotic predators of bacteria in oil-polluted marine water.

The present study was conducted in the framework of the COMMODE project (Communities of Marine Microorganisms for Oil Degradation, CE VK-CT2002-00077) that aimed to increase our understanding of the dynamics of natural and anthropogenic remediation of crude oil-polluted systems. Towards this direction, this study aimed at (i) the calculation of growth and grazing parameters of protists in oligotrophic and chronically polluted sea water contaminated with crude oil in experimental microcosms and (ii) the investigation of the dominant protistan grazers of bacteria in these systems, by using the diversity of the 18S rRNA genes.

Results

In the first bottle experiment with concentrated < 10 μm planktonic fraction from the oligotrophic area (OLIGbottle1+oil, Table 1) the initial concentration of HNF at 0 h was 15×10^3 cells ml^{-1} and it decreased dramatically after oil addition to a minimum of 0.37×10^3 cells ml^{-1} after 114 h (Fig. 1); then HNF increased 10-fold within the next 48 h with doubling time of 4.3 h (Table 2). Heterotrophic nanoflagellates were apparently feeding on bacteria which were also growing from 0 h to 192 h in the oil-amended bottle. In the control bottle, bacteria slightly increased while HNF decreased during the incubation.

In the second bottle experiment (OLIGbottle2) with < 10 μm seawater from the oligotrophic area the decrease

Table 2. Heterotrophic nanoflagellate (HNF) parameters in the different oil treatments.

Treatment	GR (h^{-1})	DT (h)	Time (h)
OLIGbottle1+oil	0.16	4.3	120–144
OLIGbottle2+oil	0.06	11.1	119–142
OLIGoil	0.02	37.5	110–135
POLLoil	0.02	34.1	90–114
OLIGoil+emu	0.14	5.0	62–86
POLLoil+emu	0.13	5.5	40–65

GR, growth rate (h^{-1}); DT, doubling time (h); Time, time interval of HNF increase. Description of different treatments in Table 1.

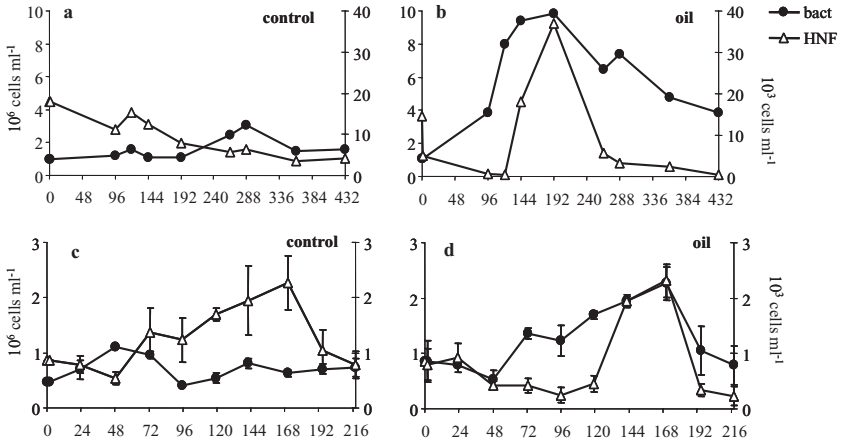

Fig. 1. Changes in cell density for bacteria (left axis) and nanoflagellate predators (HNF: right axis).
A. Bottle experiment with ×10 concentrated < 10 μm planktonic fraction (OLIGbottle1control).
B. Bottle experiment with ×10 concentrated < 10 μm planktonic fraction (OLIGbottle1+oil).
C. Bottle experiment with seawater < 10 μm control (OLIGbottle2control).
D. Bottle experiment with seawater < 10 μm+oil (OLIGbottle2+oil).
Error bars are SD of triplicates.

phase in HNF numbers was longer in the oil-amended bottle (120 h relative to 48 h in the control). In this experiment bacteria and nanoflagellate abundances showed a similar pattern and were within the same ranges in the control and oil-amended bottles. The ingestion rate on bacteria during nanoflagellate growth in the oil-amended bottle was low (3.3 bacteria HNF^{-1} h^{-1}) and the HNF doubling time was relatively long (11 h, Table 2).

In the microcosm experiments the initial concentration of bacteria was higher in the chronically polluted than in the oligotrophic seawater. The initial increase of bacterial numbers in the controls (OLIGcontrol and POLLcontrol) was followed by an increase of their HNF predators (Fig. 2) and at final bacterial numbers lower than the initial ones; this was more pronounced in the polluted microcosm. The addition of oil alone did not result in an important increase of bacteria and their predators relative to the control (OLIGoil, POLLoil, Fig. 2). The difference between the two treatments was that the oil had a dramatic effect on HNF in the oligotrophic seawater where HNF decreased from 2×10^3 to 0.2×10^3 ml^{-1} within 72 h, while it seemed to have no effect on the HNF numbers of the chronically polluted water. In both treatments HNF had relatively long doubling times (approximately 35 h).

A quite distinct pattern of bacteria and HNF number was observed in the 'oil+emulsifiers' microcosms. An important growth of bacteria in the beginning of the incubation was followed by a 38- and 42-fold increase in HNF numbers in the oligotrophic and chronically polluted microcosms respectively. The lag time for the appearance of a prey–predator relationship was shorter in the chronically polluted seawater (48 and 72 h in the polluted and unpolluted seawater, respectively, Fig. 2). The doubling time of HNF was similar in the two cases (approximately 5 h). The HNF present in our samples after amendments were ovoid to subovoid and between 3 and 6 μm. The dominant size of flagellates in our samples was about 3–4 mm and larger organisms were rare.

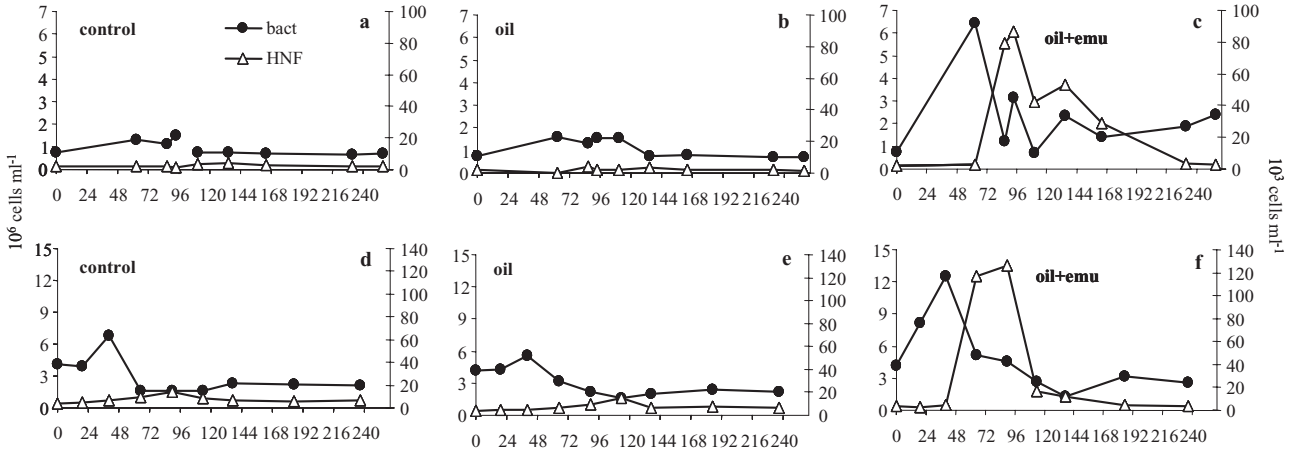

Fig. 2. Changes in cell density for bacteria (left axis) and nanoflagellate predators (HNF: right axis) in microcosm experiments.
A–C. Oligotrophic site, control (OLIGcontrol) (A), oil (OLIGoil) (B), oil+emulsifier (OLIGoil+emu) (C).
D–F. Chronically polluted site, control (POLLcontrol) (D), oil (POLLoil) (E), oil+emulsifier (POLLoil+emu) (F), and cf. Table 1.

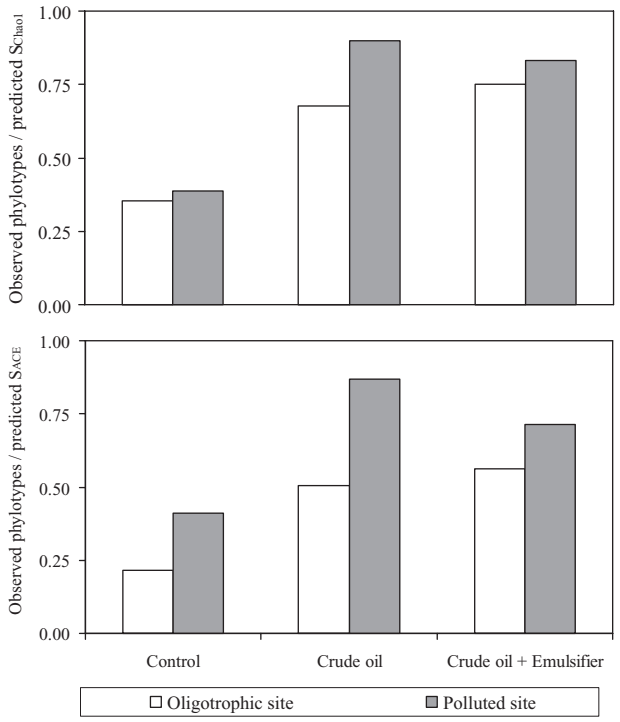

Fig. 3. 18S rRNA gene clone coverage of 18S rRNA gene libraries based on the S_{Chao1} and S_{ACE} estimators, from oil-polluted microcosms.

In total, 284 18S rDNA clones were sequenced from the seven microcosm treatments (Table 1), which belonged to 82 unique (< 98% similarity) phylotypes. In this study, we report only on the dominant phylotypes for each treatment analysed (53.1–96.1% of all phylotypes per treatment). Based on the S_{ACE} and S_{Chao1} (Kemp and Aller, 2004) clone coverage estimators (Fig. 3), our clone libraries for the control unaltered treatments was 0.216 (OLIGcontrol, 0 h) and 0.411 (POLLcontrol, 0 h) while for the polluted ones was 0.506 (OLIGoil, 86 h), 0.899 (POLLoil, 114 h), 0.561 (OLIGoil+emu, 94 h) and 0.833 (POLLoil+emu, 90 h).

In the oligotrophic seawater microcosm, there was a shift in the dominant phylotypes from autotrophic to heterotrophic phylotypes. At 0 h the oligotrophic seawater microcosm (OLIGcontrol, 0 h) was dominated by the autotrophs *Geminigera cryophila* and *Micromonas pusilla* (18.4% and 10.2% respectively) while at the HNF peak (OLIGcontrol, 134 h) it was dominated by the heterotrophs *Paraphysomonas foraminifera* and *Diaphanoeca grandis* (29.4% and 20.6% respectively) (Table 3; Fig. 4). In the chronically polluted microcosm (POLLcontrol, 0 h), although a few autotrophs were present, the dominant phylotypes were heterotrophic as well, namely an unknown *Cercozoa* (32.0%) related to *Cryothecomonas longipes* and an unknown *Alveolata* (10.0%).

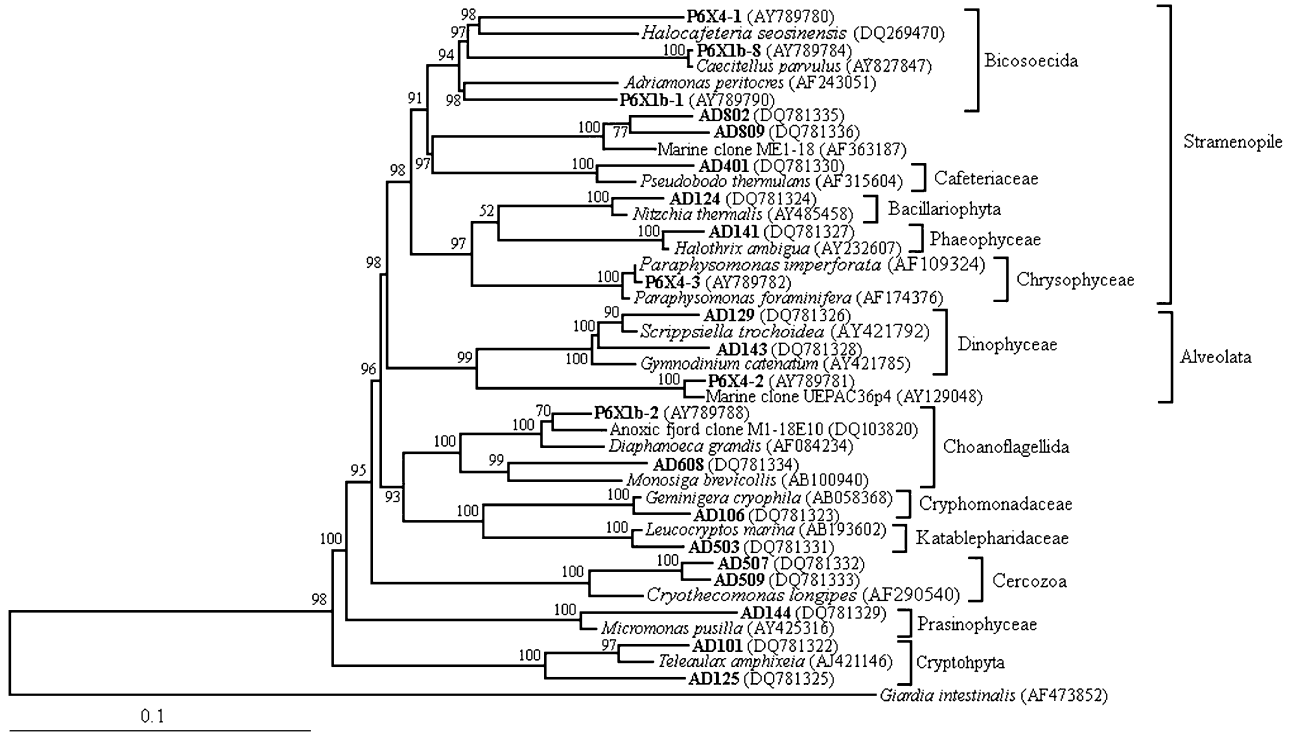

Fig. 4. Neighbour-joining phylogenetic tree of partial SSU rRNA genes obtained from oil-polluted microcosms. Numbers at nodes represent the bootstrap percentages from 1000 replicates. Values below 50% are not shown. Bar indicates the number of substitutions per site.

Table 3. Dominant (> 2%) 18S rDNA phylotypes and their closest relatives found in the oil-polluted treatments and controls.

Sequence	No. of similar (≥ 98%) clones (% abundance)	Closest relative (GenBank accession No.) [similarity]	Putative affiliation
OLIGcontrol, 0 h			
AD106	9 (18.4)	*Gemingera cryophila* (AB058368) [99%]	*Cryptomonadaceae*
AD144	5 (10.2)	*Micromonas pusilla* (AY425316) [99%]	*Prasinophyceae*
AD101	3 (6.1)	*Teleaulax amphioxeia* (AJ421146) [97%]	*Cryptohpyta*
AD143	2 (4.1)	*Gymnodinium catenatum* (AY421785) [96%]	*Dinophyceae*
AD125	2 (4.1)	Clone RD010517.7, coastal sea (AY295757) [99%] *Teleaulax amphioxeia* (AJ421146) [96%]	*Cryptohpyta*
AD124	2 (4.1)	*Nitzschia thermalis* (AY485458) [98%]	*Bacillariophyta*
AD141	2 (4.1)	*Halothrix ambigua* (AY232607) [99%]	*Phaeophyceae*
AD129	1 (2.0)	*Scrippsiella trochoidea* (AY421792) [98%]	*Dinophyceae*
OLIGcontrol, 134 h			
P6X4-3	10 (29.4)	*Paraphysomonas foraminifera* (AF174376) [99%]	*Paraphysomonadaceae*
P6X1b-2	7 (20.6)	*Diaphanoeca grandis* (AF084234) [96%]	*Acanthoecidae*
P6X1b-1	3 (8.8)	*Bicosoecida* sp. (AY665992) [100%]	*Bicosoecida*
P6X1b-8	2 (5.9)	*Caecitellus parvulus* (AY827847) [99%]	*Bicosoecida*
P6X4-1	1 (2.9)	*Bicosoecida* sp. (AY665994) [100%]	*Bicosoecida*
OLIGoil, 86 h			
P6X4-3	18 (69.2)	*Paraphysomonas foraminifera* (AF174376) [99%]	*Paraphysomonadaceae*
AD401	6 (23.1)	*Pseudobodo tremulans* (AF315604) [97%]	*Cafeteriaceae*
P6X4-1	1 (3.8)	*Bicosoecida* sp. (AY665994) [100%]	*Bicosoecida*
OLIGoil+emu, 94 h			
P6X4-3	42 (82.4)	*Paraphysomonas foraminifera* (AF174376) [99%]	*Paraphysomonadaceae*
AD401	4 (7.8)	*Pseudobodo tremulans* (AF315604) [97%]	*Cafeteriaceae*
POLLcontrol, 0 h			
AD507	16 (32.0)	Clone BL010 625.27, oligotrophic coastal (AY426931) [99%] *Cryothecomonas longipes* (AF290540) [96%]	*Cercozoa*
P6X4-2	5 (10.0)	Clone UEPAC36p4, Pacific Ocean (AY129048) [98%]	*Alveolata*
AD509	4 (8.0)	Clone BL010 625.27, oligotrophic coastal (AY426931) [99%] *Cryothecomonas longipes* (AF290540) [96%]	*Cercozoa*
AD143	3 (6.0)	*Gymnodinium catenatum* (AY421785) [96%]	*Dinophyceae*
P6X1b-2	3 (6.0)	*Diaphanoeca grandis* (AF084234) [96%]	*Acanthoecidae*
AD129	2 (4.0)	*Scrippsiella trochoidea* (AY421792) [98%]	*Dinophyceae*
AD503	2 (4.0)	*Leucocryptos marina* (AB193602) [99%]	*Katablepharidaceae*
P6X4-3	1 (2.0)	*Paraphysomonas foraminifera* (AF174376) [99%]	*Paraphysomonadaceae*
POLLoil, 114 h			
P6X4-3	25 (48.1)	*Paraphysomonas foraminifera* (AF174376) [99%]	*Paraphysomonadaceae*
AD851	7 (13.5)	*Monosiga brevicollis* (AF100940) [100%]	*Codonosigidae*
AD608	7 (13.5)	*Monosiga brevicollis* (AF100940) [94%]	*Codonosigidae*
P6X4-1	5 (9.6)	*Bicosoecida* sp. (AY665994) [100%]	*Bicosoecida*
P6X1b-1	2 (8.8)	*Bicosoecida* sp. (AY665992) [100%]	*Bicosoecida*
P6X1b-2	1 (1.9)	*Diaphanoeca grandis* (AF084234) [96%]	*Acanthoecidae*
POLLoil+emu, 90 h			
P6X4-3	32 (58.2)	*Paraphysomonas foraminifera* (AF174376) [99%]	*Paraphysomonadaceae*
AD809	7 (12.7)	Clone ME1-18, open ocean (AF363187) [95%]	Unidentified
AD802	4 (7.3)	Clone ME1-18, open ocean (AF363187) [95%]	Unidentified
P6X1b-8	4 (7.3)	*Caecitellus parvulus* (AY827847) [99%]	*Bicosoecida*
AD401	1 (1.8)	*Pseudobodo tremulans* (AF315604) [97%]	*Cafeteriaceae*
P6X4-1	1 (1.8)	*Bicosoecida* sp. (AY665994) [100%]	*Bicosoecida*
AD851	1 (1.8)	*Monosiga brevicollis* (AF100940) [100%]	*Codonosigidae*

The addition of crude oil resulted in the dominance of *P. foraminifera* at the peak of the HNF abundance in both the oligotrophic (OLIGoil, 86 h) and the chronically polluted (POLLoil, 114 h), with 69.2% and 48.1% of the observed phylotypes respectively. The same organism was also dominant at the peak of the HNF abundance of both treatments containing crude oil+emulsifier, comprising 82.4% (OLIGoil+emu, 94 h) and 58.2% (POLLoil+emu, 90 h) of the observed phylotypes in each treatment. Other species with considerable representation were *Pseudobodo tremulans* (23.1%, OLIGoil, 86 h) *Monosiga brevicollis* (27.0%, POLLoil, 114 h).

Discussion

In all our experiments bacterial growth was followed by an HNF response in terms of biomass and enhanced grazing on bacteria indicating that at least a fraction of HNF could survive in oil-polluted seawater and that they could tightly control bacterial growth keeping it always $\leq 10^7$ bacteria

ml^{-1}. For both bacteria and HNF, the addition of oil alone in the microcosms containing < 10 μm natural seawater did not cause important increase of their abundance. It is known that hydrocarbon degradation is typically limited by the bioavailability of nutrients such as nitrogen and phosphorous (Head *et al.*, 2006, and references therein). Only in one bottle experiment (OLIGbottle1) with concentrated < 10 μm planktonic fraction the increase of bacteria and the prey–predator relation between bacteria and HNF in the oil-amended sample could be attributed to the enrichment of the seawater in organic substrate (Fig. 1; Table 2).

The addition of 'oil+emulsifiers' resulted in significant increase of bacteria (comparison of slopes $P < 0.001$; Zahr, 1984) followed by a 40-fold nanoflagellate predator response (Fig. 2; Table 2). The bio-emulsifier Alasan used in our experiments is produced from *Acinetobacter radioresistens* and is a high-molecular-weight complex of polysaccharides and proteins (Navon-Venezia *et al.*, 1995; Toren *et al.*, 2001). It is known that emulsifiers promote oil bioavailability and degradation by bacteria through enhancement of cell motility, substrate accession, avoidance of toxic elements and may also be used as carbon and energy sources (Van Hamme *et al.*, 2006), leading, thus, to increase in bacterial cells and subsequent higher protistan numbers.

Heterotrophic nanoflagellate and bacterial numbers show in natural environments prey–predator cyclical behaviour (Fenchel, 1982). The HNF growth rate in this study ranged from 0.02 h^{-1} in the oil treatments where bacteria showed a weak increase up to 0.15 h^{-1} in the oil+emulsifier treatments where bacteria were very abundant (in the order of 10^7 bacteria ml^{-1}). In cultures *P. foraminifera*, *P. tremulans* and *M. evicollis* are about 3.8 μm (range 3.1–4.4 μm), 4.5–6 μm and 4–6 μm long respectively (Collection of Roscoff Biological Station). The dominant size of flagellates in our samples was about 3–4 μm larger organisms were rare. However, larger HNF can also graze on smaller HNF, thus the growth rates calculated during exponential growth phase may be slightly underestimated.

Previous culture experiments with different HNF species inoculated in the presence of bacterial food at concentrations of the order of 10^7–10^8 cells ml^{-1} have shown growth rates between 0.15 and 0.25 h^{-1}, while HNF maintained a steady-state growth at least as 0.028 h^{-1} at bacterial densities of around 2×10^6 bacteria ml^{-1} (Fenchel, 1982). These same experiments (Fenchel, 1982) indicated that HNF half saturation constants in cultures fell within 1.3×10^6 to 3.8×10^7 bacteria ml^{-1}. The highest bacterial concentration in this study was recorded in the chronically polluted seawater mesocosm (POLLoil+emu) and was of 1.25×10^7 bacteria ml^{-1}, which falls within the range set by Fenchel (1982). In our experiments HNF were always present and responded quickly to bacterial increase (Figs 1 and 2; Table 2). Previous studies focusing on bioremediation have reported that oil-degrading bacteria like *Alcanivorax* spp., are early colonizers after nutrient amendments of oil spill but decline to much lower numbers within a few weeks (Röling *et al.*, 2001; Syutsubo *et al.*, 2001). Bacterial decrease due to predation is often undesired to bioremediation processes because it decreases the number of oil-degrading bacteria (Kota *et al.*, 1999). Considering a growth rate of 5 h (Table 2), and assuming that protists fed exclusively on bacteria, then a protist of 4 μm needed to ingest 160 bacteria $flag^{-1}$ h^{-1}. This value is 16-fold higher than the value obtained based on the change of bacterial numbers in the microcosm with oil+emulsifiers (which would be about 10 bacteria $flag^{-1}$ h^{-1}) which strongly indicates that the presence of bacterial predators can also stimulate bacterial production. Protozoan grazers constitute a potentially significant source of surface-active material (e.g. Iwabuchi *et al.*, 2002) in areas where protists are abundant, such as the sediment–water interface and microbial loop-dominated oligotrophic regimes (Kujawinski *et al.*, 2002). In addition, Safonova and colleagues (1999) have reported more efficient black oil degradation by associations of algae with alcanotrophic bacteria than by pure culture of HCB. Finally, grazing of bacteria by protists causes remineralization of inorganic nutrients, prolonging thus the activity of bacteria. The exact role of each mechanism and the quantification of their rates remains an open issue.

Following the above, the question was now set to: (i) was there an important shift in the HNF population after oil and oil+emulsifier amendments? and (ii) which species do these abundant HNF belong to? As the morphology-based identification of marine protists solely down to the species level is often impossible (Corliss, 2002; Caron *et al.*, 2004; Massana *et al.*, 2006), we addressed these questions by investigating the 18S rRNA gene diversity of the dominant microscopic eukaryotes in the oil-polluted microcosms. As such, the aim was not to unravel the full extent of the existing diversity in the clone libraries but rather to recognize the dominant species. Indeed, in the control microcosms, the S_{Chao1} and S_{ACE} clone coverage estimators were low, ranging between 21.6% and 41.1% (Fig. 3). On the contrary, in the polluted treatments, these values ranged between 50.6% and 89.9%, reflecting the dominance of only a few phylotypes and suggesting satisfactory clone coverage. Possible reasons that limit the coverage of full diversity could be attributed to general polymerase chain reaction (PCR) innate problems (Qiu *et al.*, 2001) and to a recent suggestion that single PCR primer sets do not amplify well the 18S rRNA gene from all protistan taxa (Caron *et al.*, 2004).

The microcosms containing chronically polluted seawater always had higher clone coverage (Fig. 3), probably due to the lower eukaryotic diversity compared with the oligotrophic seawater containing microcosms. Apart from the dominant *P. foraminifera* (see below) the next dominant phylotypes, in most cases, were closely related to known representatives of the *Stramenopile*, *Alveolata*, *Choanoflagellida*, *Cryphomonadaceae*, *Katablepharidaceae*, *Cercozoa*, *Prasinophyceae* and *Cryptophyta* (Fig. 4).

Paraphysomonas foraminifera dominated at the peak abundance of all treatments, which contained crude oil and crude 'oil+emulsifier'. This shift was particularly obvious in the oligotrophic seawater treatments, where at the beginning of the experiment it was undetectable but after 134 h it dominated the eukaryotic assemblage with nearly 30% of all phylotypes, while in the respective treatments containing crude oil it reached almost 70% and when emulsifier was added the percentage was even higher, reaching more than 80%. Similar increasing trend, but with lower intensity, was observed for the chronically polluted treatments. After oil amendments the chronically oil-polluted water showed a higher diversity and lower percentage of *P. foraminifera* contribution (48.1% and 58.2% in the oil and 'oil+emulsifier' treatments respectively) than the oligotrophic seawater (69.2% and 82.4% in the oil and 'oil+emulsifier' treatments respectively). It is possible that the planktic community of the chronically oil-polluted site is a more stable one, less susceptible to major shifts, hosting more species that can tolerate the harmful effects of oil spill, as has been suggested for benthic populations in polluted sediments (e.g. Klerks and Levinton, 1992). The above results potentially render *P. foraminifera* an advantageous competitor over other HNF in dominating coastal systems subject to oil spills, with or without previous oil pollution history.

The addition of emulsifier had a somewhat different effect on the dominant phylotypes. As mentioned already, in the case of *P. foraminifera*, the addition of emulsifier increased its relative abundance in both the oligotrophic (from 69.25% to 82.4% without and with emulsifier respectively) and chronically oil-polluted waters (from 48.1% to 58.2% without and with emulsifier respectively). On the other hand, a negative effect was observed after the addition of the emulsifier on the relative abundance of both the *P. tremulans* and *M. brevicollis* phylotypes in the oligotrophic and chronically oil-polluted waters, the second dominant eukaryotes in the microcosms (7.85% and 27.0% respectively). This could be due to the increased *P. foraminifera* dominance, which reduces the available bacterial prey for other species.

Bacterial communities that develop in oil-polluted systems are highly specialized (Head *et al.*, 2006, and references therein). Thus, one could expect that the HNF, which grazed efficiently on these bacteria, could be also characterized as oil-tolerant specialists of unique ecophysiology. Our study revealed that *P. foraminifera* and the other HNF dominant in the oil and oil+emulsifier treatments belonging in their great majority to *Stramenopile* and *Choanoflagellida* are cosmopolitan organisms in the world's oceans (Massana *et al.*, 2006). It has been shown that easily enriched organisms are present in low abundance in natural assemblages. For example, Lim and colleagues (1999) have enriched coastal seawater samples from different seasons and locations and have shown that *Paraphysomonas imperforata* – which phylogenetically is closely related to *P. foraminifera* – was found at low percentages in natural plankton assemblages, and became always dominant in enriched cultures. Based on these findings they concluded that *P. imperforata* is an opportunistic species growing rapidly to high abundances when prey concentrations are high.

Overall this work has shown that heterotrophic, cosmopolitan non-specialists can grow efficiently in oil-polluted seawater and control bacteria. Future work is needed to quantify the role of these eukaryotes in bioremediation practices *in situ*. Apart from oil bioremediation, these opportunistic eukaryotes exhibit a broader ecological interest in better assessment of their ecophysiology and their potential role in disturbed and/or changing marine ecosystems.

Experimental procedures

Preliminary experiments

Prior to the microcosm experiment, in order to determine the effect of oil addition on eukaryotes, the lag time between oil addition and bacterial and nanoflagellate response, and the replication of treatments, two preliminary experiments were performed in June 2004. The water used for these experiments was from the oligotrophic (OLIG) Anavissos Bay, Aegean Sea.

First experiment (June 2004, OLIGbottle1, Table 1*)*

For this experiment we concentrated 6 l of 10 μm screened seawater down to 600 ml by filtering on 1 μm filters (the filter was changed three times and each time 200 ml was recovered). The purpose we did this was to obtain a seawater sample rich in nanoflagellates in order to have a more pronounced result of the effect of oil on these organisms. This concentrated seawater was also rich in organic material (phytoplankton and detritus), which was a good substrate for bacterial growth providing nutrients. Two plastic 250 ml Sarstedt tissue culture flasks were each filled with 150 ml of filtered concentrated seawater. One bottle served as control and another was amended with 600 p.p.m. of Arabian light crude oil (the same Arabian light crude oil was used in all experiments, provided by M. Yakimov, Istituto Sperimentale Talassografico – CNR, Messina, Italy). Incubations lasted 18 days, and the bottles were maintained under low agitation

(45 r.p.m.) at 21°C in the dark to promote the growth of the heterotrophic compartment of the food web, and sampled 10 times for bacteria and nanoflagellate abundance.

Second experiment (October 2004, OLIGbottle2)

For this experiment, 600 ml plastic Sarstedt tissue culture flasks were each filled with 200 ml of 10 μm screened seawater. Three bottles served as control and three other were amended with 600 p.p.m. of Arabian light sweet crude. Incubations lasted 13 days, and the bottles were maintained under low agitation (45 r.p.m.) at 21°C in the dark and sampled 10 times for bacteria and nanoflagellate abundance.

Microcosm experiments

Seawater from an unpolluted oligotrophic site (OLIG – Anavissos Bay) or a chronically oil-polluted mesotrophic site (POLL – Elefsina Bay, Aegean Sea) was collected in April and June 2005 respectively. The water was passed through a 10 μm Polycarbonate Nuclepore filter (147 mm) in a gravity filtration device (Bailey's Plastic Fabrication, Dartmouth, NS, Canada) to screen out larger organisms. The seawater was then distributed evenly into three 100 l capacity glass aquaria, each one holding approximately 75 l of water. After filling to 75 l, the following was added to each tank: (i) control, nothing added, (ii) crude oil (100 p.p.m. final concentration) and (iii) crude oil and Alasan emulsifier (100 and 10 p.p.m. final concentrations respectively). Alasan (Navon-Venezia *et al*., 1995) is a bioemulsifier complex of an anionic polysaccharide and proteins (provided by M. Yakimov, Istituto Sperimentale Talassografico – CNR, Messina, Italy). Gentle continuous water mixing in each microcosm was accomplished by using a commercial fish pump (Tunze, Italy) with 35 l h^{-1} circulating capacity. Each pump was affixed to the bottom at one corner of the tank for water intake by way of a small 6 mm plastic fan and was circulated through 6 mm silicon tube over the top of the tank where it re-entered the water at the other corner 2 cm above the surface. Incubations took place in 21°C in the dark. Microcosm incubations lasted 10 days and sampled nine times for bacteria and nanoflagellate abundance. DNA samples were taken for each experiment at 0 h and during the exponential HNF growth peak in the microcosms.

Bacterial and nanoflagellate cell counts

To enumerate HNF and bacteria, 5–15 ml and 1–5 ml of samples, respectively, were preserved with formaldehyde at a final concentration of 2%. Samples were kept at 4°C in the dark, filtered on black Nuclepore filters (pore size: 0.8 μm and 0.2 μm for nanoflagellates and bacteria respectively) and stained with DAPI (Porter and Feig, 1980) within a few hours of sampling and stored at –20°C until counting. Heterotrophic nanoflagellates and bacteria were enumerated using an Olympus AX-70 PROVIS epifluorescence microscope at 1000×. Growth (μ, h^{-1}) of nanoflagellates was calculated according to Frost (1972).

$$\mu = [\ln(n_t/n_0)]/t \quad (1)$$

where n_t and n_0 are the number of flagellates and t is time (h).

The doubling time or generation time (GT) of flagellates was calculated as

$$GT = \ln 2/\mu \quad (2)$$

Molecular characterization and phylogenetic analysis

18S rRNA gene analysis was performed in a total of seven samples in the microcosm experiment. Sampling was performed at 0 h in unaltered seawater and during the growing phase of the eukaryotes as follows: at 135, 86 and 94 h for the treatments 'oligotrophic control' (OLIGcontrol), 'oligotrophic oil' (OLIGoil); 'oligotrophic oil+emulsifier' (OLIGoil+emu), and at 114 and 90 h 'polluted oil' (POLLoil), 'polluted oil+emulsifiers' (POLLoil+emu) respectively (cf. Table 1, Figs 1 and 2). The sample for DNA extraction of the 'polluted control' (POLLcontrol) at the nanoflagellate peak (90 h) was lost and thus was not analysed.

One litre of microcosm water was filtered on 0.2 mm pore size polycarbonate filters and the filters were stored at –80°C. DNA was extracted using the UltraClean Soil DNA isolation kit (MoBio Laboratories, USA) according to the manufacturer's protocol after slicing the filters with a sterile scalpel. For PCR amplification, 0.5 ml of the DNA template was used. Using nested PCR, a *c*. 1200 bp portion of the 18S rDNA gene was amplified on a MyCycler thermocycler (Bio-Rad, USA). For the first amplification, the EukF (5′-AACCTGGTTGATCCTGCCAGT-3′) and EukR (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) primers were used and the PCR consisted of a 1 min pre-PCR hold at 94°C, followed by 15 cycles consisting of a 45 s denaturation step at 94°C, a 45 s annealing step at 50°C and a 2 min elongation step at 72°C. These PCR products (0.5 ml) were re-amplified in the second PCR using the EukF and 1179r (EUK) (5′-CCCGTGTTGAGTCAAATT-3′) primers. This PCR consisted of a 1 min pre-PCR hold at 94°C, followed by 26–28 cycles consisting of a 45 s denaturation step at 94°C, a 45 s annealing step at 47°C, a 2 min elongation step at 72°C, and at the end of the 26–28 cycles, a 7 min finishing step at 72°C. The specificity of primers was checked against bacterial and archaeal DNA; no amplification occurred in either case.

Polymerase chain reaction products were gel-purified with the Wizard SV Gel and PCR Clean-Up System kit (Promega, USA). The purified products were A-tailed to improve cloning efficiency by mixing approximately 50 ng of purified PCR product with 5 ml of 10× PCR buffer [200 mM Tris (pH 8.55), 100 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 300 mM KCl, 0.1% (w/v PCR buffer) gelatin, 0.5% (v/v PCR buffer) NP-40], 5 μl deoxynucleoside triphosphate (2 mM) and 1 U of *Taq* DNA polymerase (Promega, USA). After incubating the mixture at 72°C for 10 min, 4 ml was immediately used for cloning using the TOPO XL PCR cloning kit (Invitrogen Corporation, USA) according to the manufacturer's specifications. The PCR products were cloned using the TOPO XL PCR cloning kit (Invitrogen Corporation, USA) using chemically competent cells according to the manufacturer's specifications. For each sample and each gene, 26–55 clones (in total 284) were analysed. These clones were screened for unique restriction fragment length polymorphism (RFLP) patterns after digestion with the RsaI, AluI and HinfIII (Fermentas UAB, Lithua-

nia) enzymes. Clones with unique RFLP patterns were grown in liquid LB medium with kanamycin and their plasmids were purified using the Nucleospin Plasmid QuickPure kit (Macherey-Nagel GmbH and Co. KG, Germany) for DNA sequencing.

Purified plasmids were sent to Macrogen (Korea) for sequencing their 18S rDNA inserts. Sequencing data were obtained by capillary sequencing with the M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) primers. For each individual clone, forward and reverse reads were assembled using Sequencher 3.0 (Gene Codes Corporation, USA). Sequences were checked for chimeras (Hugenholtz and Huber, 2003) using the CHIMERA_CHECK function of the Ribosomal Database Project II (Maidak *et al*., 2001).

Sequence data were compiled using the ARB software (http://www.arb-home.de) and aligned with sequences obtained from the ARB and GenBank databases, using the ARB FastAligner utility, and followed by manual aligning according to secondary structure. Analyses were performed using minimum evolution and parsimony methods implemented in PAUP (Swofford, 2000). Bootstrapping under minimum evolution and parsimony criteria was performed with 1000 replicates. Sequences from the present study have GenBank No. AY789780–AY789790 and DQ781322–DQ781337. For each clone library, the coverage was estimated according to Kemp and Aller (2004).

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