environmental microbiology

Environmental Microbiology (2012) **14**(9), 2348–2360 doi:10.1111/j.1462-2920.2012.02787.x

Use of phytoplankton-derived dissolved organic carbon by different types of bacterioplankton

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Summary

Phytoplankton and heterotrophic prokaryotes are major components of the microbial food web and interact continuously: heterotrophic prokaryotes utilize the dissolved organic carbon derived from phytoplankton exudation or cell lysis (DOCp), and mineralization by heterotrophic prokaryotes provides inorganic nutrients for phytoplankton. For this reason, these communities are expected to be closely linked, although the study of the interactions between them is still a major challenge. Recent studies have presented interactions between phytoplankton and heterotrophic prokaryotes based on coexistence or covariation throughout a time-series. However, a real quantification of the carbon flow within these networks (defined as the interaction strength, IS) has not been achieved yet. This is critical to understand the selectivity degree of bacteria responding to specific algal DOCp. Here we used microautoradiography to quantify the preferences of the major heterotrophic prokaryote phylogenetic groups on DOC derived from several representative phytoplankton species, and expressed these preferences as an IS value. The distribution of the ISs was not random but rather skewed towards weak interactions, in a similar way as the distributions described for stable complex non-microbial ecosystems, indicating that there are some cases of high specificity on the use of specific algal DOCp by some bacterial groups, but weak interactions are more common and may be relevant as well. The variety of IS patterns observed supports the view that the vast range of different resources (different types of organic molecules) available in the sea selects and maintains the high levels of diversity described for marine bacterioplankton.

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Introduction

Life in the ocean is microbe-based. Large amounts of carbon are constantly being processed in the global ocean and this is mainly done by phytoplankton and heterotrophic prokaryotes. Primary production (~40 Gt C $year^{-1}$) and heterotrophic respiration (~37 Gt C $year^{-1}$) alone account for more than half of global open ocean carbon processing, only within the euphotic (illuminated) layer (del Giorgio and Duarte, 2002). In this highly active layer, the dissolved organic carbon derived from phytoplankton exudation or cell lysis (DOCp) is a major energy source driving heterotrophic prokaryote respiration and growth (Hellebust, 1965; Mague *et al.*, 1980; Karl *et al.*, 1998). While heterotrophic prokaryote and phytoplankton communities are known to interact in very complex ways (Azam and Malfatti, 2007), these compartments are expected to be closely interrelated in the planktonic environment. In fact, heterotrophic prokaryote abundance and production usually covariates with phytoplankton biomass (as chlorophyll *a*) and primary production (e.g. Bird and Kalff, 1984; Cole *et al.*, 1988; Simon *et al.*, 1992; Gasol and Duarte, 2000). Although processes other than extracellular release are needed to balance bacterial demand (zooplankton excretion, sloppy feeding, etc.), it is generally accepted that phytoplankton is the principal source of organic carbon to bacteria (Baines and Pace, 1991). Recently, Fouilland and Mostajir (2010) challenged this concept, suggesting that bacterial growth depended mostly on other non-phytoplanktonic sources of carbon. However, these observations are far from being consensual (Morán and Alonso-Sáez, 2011).

The nature of phytoplankton–heterotrophic prokaryotes interactions is multifaceted; it ranges from mutualism (Aota and Nakajima, 2001) to antagonism (e.g. Mayali and Azam, 2004; Ribalet *et al.*, 2008) and competition for inorganic nutrients (Joint *et al.*, 2002; see also Van Mooy *et al.*, 2009). Another relevant interaction is the grazing on bacteria by mixotrophic phytoplankton (Unrein *et al.*, 2007; Zubkov and Tarran, 2008). The current view of marine microbial food webs (Gasol *et al.*, 2008) highlights two major carbon fluxes: the uptake of algal DOCp by heterotrophic prokaryotes, and the release of inorganic nutrients by heterotrophic prokaryotes as a result of bacterial mineralization of the organic matter, benefiting primary producers. Under this perspective, these

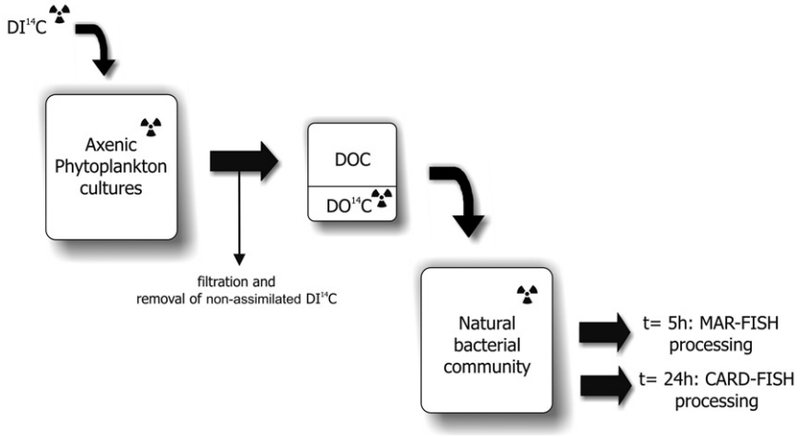

Fig. 1. Schematic representation of the experimental design. The procedure was carried out in duplicated incubation flasks.

interactions could be considered as a typical example of mutualism. The fact that these organisms persist and coexist in extremely high abundances in all the oceans on Earth supports the hypothesis that mutualism must overcome other processes, as also indicated by mathematical models (Aota and Nakajima, 2001).

The visualization of ecological interactions as a network allows the quantification of a number of parameters that are helpful to understand the relationship between network complexity and ecological stability (Montoya *et al*., 2006). This approach is particularly useful at capturing the strength of the multiple interactions within a food web, which is an imperative step in understanding the structure of natural communities and predicting how they will respond to environmental changes (Wootton and Emmerson, 2005). The carbon (or other chemical element) exchanges between two or more species or groups of organisms can be expressed in numerical values, defined as the interaction strength (IS), a concept that has been used largely in the literature for larger organisms (Sala and Graham, 2002; Berlow *et al*., 2004; Bascompte *et al*., 2005; Wootton and Emmerson, 2005), but has rarely been applied to the marine microbial food web. Nevertheless, growing evidence suggests that bacterial communities are structured by the environment in the same way as are many animal and plant communities (e.g. Fuhrman *et al*., 2006).

Most studies dealing with IS have focused on consumer–resource interactions in food webs, where IS is usually defined as the absolute or proportional effect of one species over another as a function of time (e.g. Paine, 1980). However, the concept can be extended to non-trophic interactions such as mutualism and interference competition (Wootton and Emmerson, 2005). In the context of this study, the IS concept was used to capture the mutualistic interaction of heterotrophic bacteria and six different phytoplankton species mediated by DOCp, by developing a method to estimate IS between these two compartments of the microbial food web. The use of the network approach in aquatic microbial ecology is recent and the interactions are usually defined by coexistence in a water sample or by covariation throughout time-series (Barberan *et al*., 2011; Eiler *et al*., 2011; Gilbert *et al*., 2011; Steele *et al*., 2011). However, a real quantification of the IS in terms of, for example carbon flow within these networks, has not been achieved yet.

We hypothesized that the type of dominant phytoplankton in a certain oceanic region plays a role in determining the phylogenetic structure of the heterotrophic prokaryote community. This influence would be exerted through variability in the identity and characteristics of the exuded primary production. Although not extensively characterized, evidence suggests that the nature of the molecules liberated in the water differs among phytoplankton species (Aluwihare and Repeta, 1999; Romera-Castillo *et al*., 2010). To test the hypothesis that DOC produced by different phytoplankton species selects for different bacterial community structures, we adapted the use of the MAR-FISH method (microautoradiography combined with fluorescent *in situ* hybridization) so that instead of using standard labelled molecules (such as leucine, ATP, glucose), we could use the mixed and uncharacterized substrates obtained from growing axenic algal cultures (Fig. 1). We quantified the IS between phytoplankton and heterotrophic prokaryotes through carbon extracellular release and re-assimilation, in order to evaluate the distribution patterns of the IS and capture the degree of selectivity of bacterial groups using specific algal DOCp. In a network perspective, the distribution of the IS has major ecological implications and provides insight on the extremely high level of prokaryotic diversity found in marine ecosystems.

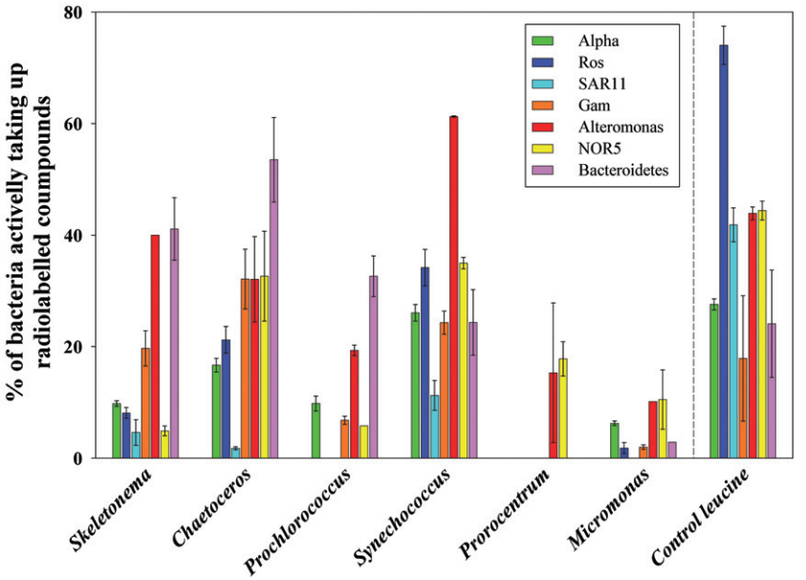

Fig. 2. Percentage of MAR-FISH positives (cells actively taking up the radiolabelled compounds) within each heterotrophic bacterial phylogenetic group ($MAR+_g$/CARD-$FISH+_g$, see text), for the different treatments. Bars indicate standard deviation for duplicated incubation flasks. *Roseobacter* and SAR11 are both subgroups of *Alphaproteobacteria*; *Alteromonas* and NOR5 are both subgroups of *Gammaproteobacteria*.

Results and discussion

MAR-FISH results (from the short-term incubations) can be presented in different ways depending on which parameter we chose to relate to the number of cells taking up the radiolabelled compounds (see Table S1). Therefore, the number of MAR-positive cells belonging to certain bacterial phylogenetic group ($MAR+_g$) can be divided by the number of cells from that phylogenetic group ($MAR+_g$/CARD-$FISH+_g$, Fig. 2), by the sum of the active cells from the different groups ($MAR+_g$/$MAR+_{total}$, Fig. 3) or by the total bacterial abundance ($MAR+_g$/DAPI counts, Table 1).

In Fig. 2 (and only here) the results are expressed as a percentage of active cells within each heterotrophic bacterial phylogenetic group ($MAR+_g$/CARD-$FISH+_g$) in order to highlight the variety of responses in processing the DOCp originated from different phytoplankton species.

The same results expressed as a percentage of total active cells ($MAR+_g$/$MAR+_{total}$) help to visualize the distribution of the different bacterial groups within the active fraction of the bacterial community in the uptake of DOCp.

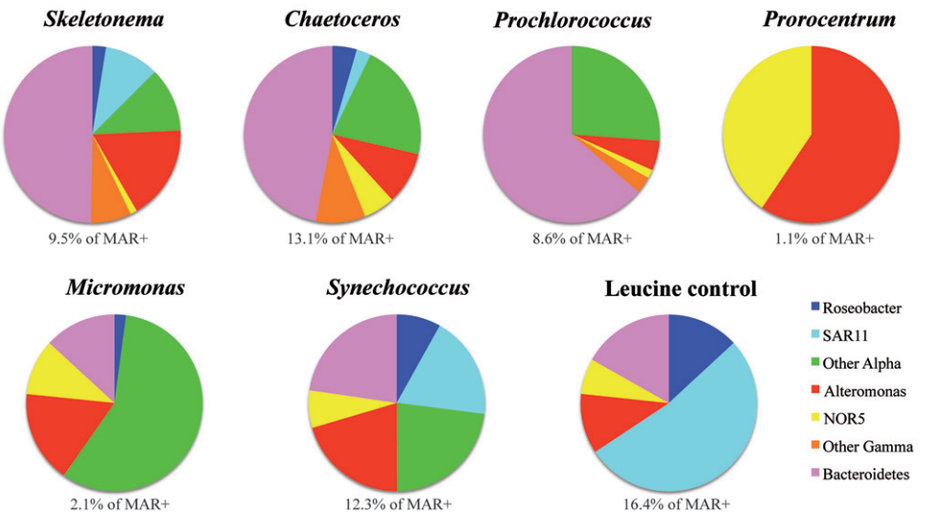

Fig. 3. Percentage of MAR-FISH positives (cells actively taking up the radiolabelled compounds) related to the sum of the active cells from the different groups ($MAR+_g$/$MAR+_{total}$, see text). Indication under each pie chart of the total percentage of active cells in each treatment ($MAR+_{total}$).

Table 1. Contribution of MAR-positive cells (percentage of cells actively taking up the radiolabelled exudates) to the total bacterial community ($MAR+_g$/DAPI counts, see text) after 5 h incubation for each of the duplicate independent incubations.

		Alphaproteobacteria				*Gammaproteobacteria*				*Bacteroidetes*
		Roseobacter	SAR11	Other Alpha	Total	*Alteromonas*	NOR5	Other Gamma	Total	Total
Skeletonema costatum	*Rep. A*	0.22	1.29	0.72	2.22	1.65	0.13	0.05	1.83	4.27
	Rep. B	0.26	0.62	1.52	2.39	n.a.	0.10	1.36	1.46	5.18
Chaetoceros sp.	*Rep. A*	0.67	0.41	3.06	4.14	1.54	0.92	< 0.05	2.47	5.11
	Rep. B	0.57	0.32	2.83	3.73	1.10	0.65	1.25	3.00	7.80
Prochlorococcus marinus pastoris	*Rep. A*	< 0.05	< 0.05	2.53	2.53	0.20	0.14	0.27	0.61	6.01
	Rep. B	< 0.05	< 0.05	2.09	2.09	0.77	n.a.	< 0.05	0.77	5.25
Prorocentrum minimum	*Rep. A*	< 0.05	< 0.05	< 0.05	< 0.05	0.99	0.38	< 0.05	1.37	< 0.05
	Rep. B	< 0.05	< 0.05	< 0.05	< 0.05	0.27	0.48	< 0.05	0.75	< 0.05
Micromonas pusilla	*Rep. A*	0.03	< 0.05	1.38	1.41	n.a.	0.16	< 0.05	0.16	0.33
	Rep. B	0.07	< 0.05	1.48	1.55	0.42	0.34	< 0.05	0.76	n.a.
Synechococcus sp.	*Rep. A*	0.93	2.72	2.74	6.39	2.52	0.86	< 0.05	3.38	3.27
	Rep. B	1.06	1.93	2.90	5.89	2.52	0.83	< 0.05	3.34	2.32
Leucine control	*Rep. A*	2.09	9.09	< 0.05	11.18	1.84	1.10	< 0.05	2.94	3.55
	Rep. B	2.23	8.20	< 0.05	10.43	1.77	1.04	< 0.05	2.81	1.99

Roseobacter and SAR11 are both subgroups of *Alphaproteobacteria*; *Alteromonas* and NOR5 are both subgroups of *Gammaproteobacteria*; n.a. stands for not available; < 0.05 stands for value below the detection limit.

The DOCp from *Synechococcus* and *Chaetoceros* was the substrates that enhanced more bacterial activity during the short-term incubations, as inferred from the addition of the active cells (MAR+) of all bacterial groups, per phytoplankton species (Fig. 3).

The active fraction of the total community (indicated on the bottom of each pie chart in Fig. 3) was relatively low (7.8% on average for algal exudates, and 16.4% for leucine) compared with the values found in literature, which are usually in the range of 20% to 40% (reviewed by del Giorgio and Gasol, 2008). However, these studies used as tracers small very labile molecules such as leucine, thymidine, glucose, ATP or other aminoacids, with the exception of Elifantz and colleagues (2005), who used exopolymers from a heterotrophic diatom culture growing in glucose. The phytoplankton exudates are probably more complex substrates, made of higher molecular weight molecules, harder to assimilate in such short-time incubations. Additionally, our labelled compounds were diluted with non-labelled and equally labile molecules (as deduced from our experimental procedure, Fig. 1), which decreased the sensitivity of the technique, at least at this range of incubation times.

For the calculations of the IS in the short-term incubations we used the percentage of MAR positives related to the total heterotrophic bacterial community ($MAR+_g$/DAPI counts) as it allows a more accurate comparison between treatments at the community level (Table 1). The same results expressed in other ways are available in Table S1. In this study, a weaker IS corresponds to a lower percentage of active cells, while a stronger IS corresponds to a higher percentage of active cells.

Duplicated flasks yielded remarkably similar results (Tables 1 and S1). All bacterial groups used leucine in relatively high numbers, but the uptake of the radiolabelled exudates was more selective (Figs 2 and 3): no uptake of DOCp from *Prorocentrum* could be observed by members of *Bacteroidetes* or *Alphaproteobacteria* (*Roseobacter* and SAR11). Perhaps surprisingly, these two subgroups of *Alphaproteobacteria* also did not use exudates from *Prochlorococcus*.

Based on the sum of the percentage of MAR-FISH positive in all treatments, it appears that *Bacteroidetes* was the group with most active cells, with particularly high affinity towards the DOC produced by diatoms (*Chaetoceros* and *Skeletonema*) and cyanobacteria (*Prochlorococcus* and *Synechococcus*). *Alphaproteobacteria* were moderately active using diatom (*Chaetoceros* and *Skeletonema*) and *Synechococcus* exudates, and were the highest fraction of the active cells in the uptake of DOCp from *Micromonas* (Fig. 3). *Gammaproteobacteria* had less specific and weaker interactions during the short-term incubations with the exception of the *Synechococcus* and *Chaetoceros* treatments where the percentage of active cells was higher. However, in the incubation with DOCp from *Prorocentrum*, the extremely low number of active cells was split between *Alteromonas* and NOR5 (Fig. 3).

The results from the long-term incubations were very different from those of the short-term incubations: SAR11 bacteria, which contributed 20% of the initial community, were present in very low numbers after 24 h in all treatments except in the 'leucine addition' and the 'no addition control' samples (Fig. 4). Other rare groups (< 5% of the initial counts) as *Alteromonas* or *Roseobacter* increased their abundance in all the enrichment treatments. The clade NOR5 was an exception as it was present in the initial sample (2.4% of the DAPI counts) and decreased in

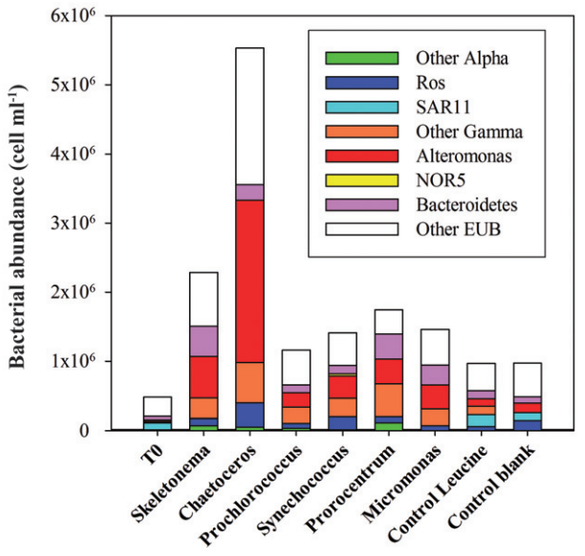

Fig. 4. Abundance of the different heterotrophic bacterial groups in the initial community (T0) and after 24 h incubation in presence of the compounds of different origin (algal exudates and leucine). *Roseobacter* and SAR11 are both subgroups of *Alphaproteobacteria*; *Alteromonas* and NOR5 are both subgroups of *Gammaproteobacteria*; in white, unidentified *Eubacteria*.

all treatments except in the *Synechococcus* exudates, where NOR5 cell abundance actually increased after 24 h. The abundance of *Bacteroidetes* cells increased in all enriched treatments, although their contribution to the overall bacterial community (which was 11.5% in the initial sample) decreased. *Alteromonas* increased the most in cell abundance after 24 h, especially in the incubations containing DOCp from *Chaetoceros* sp., which were also the incubations that showed the greatest increase in total bacterial numbers, indicating that this carbon source was highly usable (Fig. 4).

Most diatoms produce polysaccharide-rich exudates (Myklestad, 2000) which are highly labile, and this probably explains the higher bacterial growth on this substrate in the long-term incubations (Fig. 4), in agreement with the high percentage of active cells in the short-term incubations (Table 1 and Fig. 4). The same comparison between short term (active cells) and long term (bacterial growth) for the non-diatom cultures revealed more intriguing results: *Prorocentrum* and *Micromonas* produced low percentages of active cells in the short term (Figs 3 and 4), resulting in relatively low bacterial growth in the long term (Fig. 4). However, cyanobacteria (*Synechococcus* and *Prochlorococcus*) produced high percentages of active cells in the short term (Table 1 and Fig. 4) but low bacterial growth in the long term (Fig. 4). This indicates that the molecules excreted by prokaryotes are substantially different from those excreted by eukaryotes as shown by the analysis of excitation/emission matrices of the excreted fluorescent dissolved organic matter (Romera-Castillo *et al.*, 2011). We can hypothesize that the DOCp from prokaryotic phytoplankton enhances more bacterial respiration than biomass production, but further studies are needed to confirm this hypothesis.

Interaction patterns in the short-term versus long-term incubations

While the MAR-FISH short-term incubations results (Table 1) reveal the most active groups of heterotrophic prokaryotes in the uptake of the different organic carbon sources, the CARD-FISH analyses after 24 h incubations (Fig. 4) show the increases in biomass (cell abundance) of each bacterial group, integrating several factors that affect net growth, such as the different growth rates inherent to each bacterial type, and mortality by grazing or viral lysis (the samples had not been filtered nor processed in any way).

This experimental approach allowed the quantification of the IS between several phytoplankton species (representative of the major functional groups that occur in coastal Mediterranean waters) and the main phylogenetic groups of bacterioplankton through the utilization of the DOCp. Plotting the IS of each bacterial group from both experiments (long-term versus short-term incubations), we can infer the contribution of each bacterial group to the DOCp uptake at the beginning of the experiment, and how the different groups contributed to the total community after 24 h of incubation (Fig. 5). In absence of grazing or any other source of mortality, and if all heterotrophic prokaryote groups were able to express similar growth rates, all the points on the plot should lay on the 1:1 line. Our results demonstrate that this was not the case and in which side of the 1:1 line the points are provides valuable information about the ecological strategy of each bacterial group.

In all the enriched conditions with exception of the leucine control, *Gammaproteobacteria* (particularly *Alteromonas*) and *Roseobacter* (*Alphaproteobacteria*) appear above the 1:1 line, indicating a high contribution to total community in terms of biomass, probably related to high specific growth rates, in accordance to what has been observed in growth rate measurements of different phylogenetic bacterioplankton groups in the field (Yokokawa *et al.*, 2004; Teira *et al.*, 2009; Ferrera *et al.*, 2011). This can also indicate that these bacterial groups were able to overcome mortality losses caused by grazing and viral lysis. On the other hand, *Bacteroidetes* and *Alphaproteobacteria* appeared under the 1:1 line, indicating a very active uptake of radiolabelled compounds in the first 5 h that was not converted into biomass in the same proportion after 24 h.

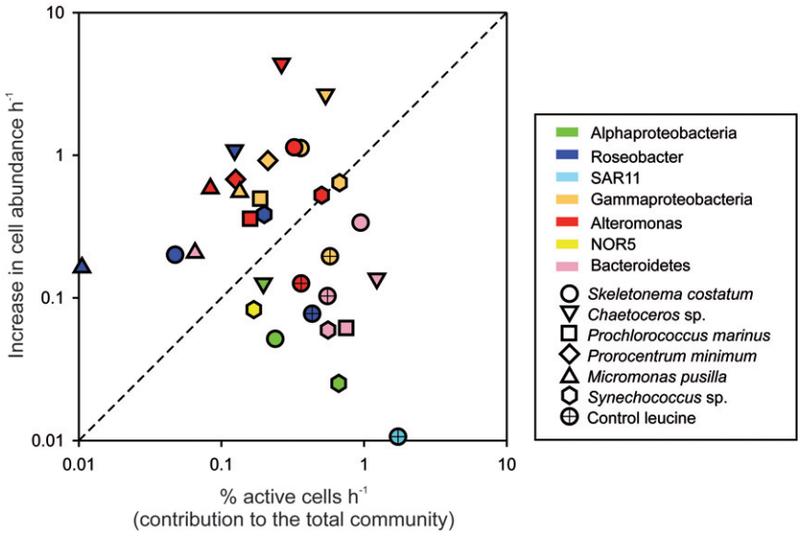

Fig. 5. Contribution of MAR-FISH positive cells to the total community (after a 5 h incubation) compared with the net growth in cell abundance 24 h after the enrichment with ~50 μM DOC produced by different phytoplankton species. Data from short-term incubations were obtained as contribution of each heterotrophic prokaryote group to the total community ($MAR+_g$/DAPI counts, Table 1) divided by incubation time (in hours). Bacterial groups featuring a contribution to the total community < 1% at the end of the experiment are not represented. The dotted line indicates the 1:1 line.

Selectivity of bacterial groups using DOCp

The early studies on this recurrent topic of marine microbial ecology considered only bulk measurements that integrate the positive and the negative feedbacks between specific phytoplankton and heterotrophic prokaryotes, ignoring the phylogenetic affiliation of the microorganisms involved. At that time technology limited the access to the taxonomical affiliation of bacterial communities, and there was no other choice than assuming that all prokaryotes should use all the DOCp in an indiscriminated way (e.g. Bell and Mitchell, 1972; Bell *et al*., 1974; Iturriaga and Hoppe, 1977; Cole, 1982; Cole *et al*., 1982; Bell, 1983; Berman and Kaplan, 1984). Lately, with the upraise of molecular biology tools, a series of reports of co-occurrence of specific algal and prokaryotic species or groups suggested the existence of particular associations between specific types of phytoplankton and bacterioplankton (e.g. González *et al*., 2000; Schafer *et al*., 2002; Pinhassi *et al*., 2004; Grossart *et al*., 2005; Rooney-Varga *et al*., 2005; Sapp *et al*., 2007a). This somehow generated the idea of an extreme specialization in the bacterioplankton utilization of the DOCp. However, most of these later studies provide few quantitative data, and generally report circumstantial observations of phytoplankton and heterotrophic prokaryote communities coexisting in a specific environmental context. Co-occurrence does not necessarily imply interaction, nor that the observed bacterial groups are the only ones using the products produced by the algae. Our experimental procedure allowed tracking the production and the use of DOCp from different algal sources in order to resolve this issue.

The patterns of interactions revealed specific linkages and a large range of intensities between the bacterial phylogenetic groups and the phytoplankton species (Fig. 6). The ISs were not randomly distributed but clearly skewed towards the weaker interactions (Fig. 7), in a similar way as the distributions described for stable complex non-microbial ecosystems (McCann, 2000). Although there were some cases of high specificity in the use of specific algal DOCp by some bacterial groups, the numerous weak interactions were more common, as it happens in most mutualistic networks (Montoya *et al*., 2006).

Despite the low taxonomic resolution that can be achieved with the used CARD-FISH probes, the method is highly effective for quantifying large phylogenetic groups of heterotrophic prokaryotes (reviewed by Amann and Fuchs, 2008). The responses observed in our MAR-FISH results corroborate the hypothesis that specific groups of heterotrophic prokaryotes have developed selective capabilities of processing the DOCp derived from certain species of phytoplankton, as suggested by several previous circumstantial observations (e.g. Rooney-Varga *et al*., 2005; Sapp *et al*., 2007b). Using molecular fingerprinting techniques, most of these studies suggest that the bacterial groups responding to phytoplankton are generally members of *Roseobacter* or *Bacteroidetes*, while *Alteromonas* have been described as having a typical particle-attached lifestyle (Acinas *et al*., 1999; Ivars-Martinez *et al*., 2008; Thomas *et al*., 2008). Pinhassi and colleagues (2004) reported a remarkable response of members of the *Flavobacteriaceae* (*Bacteroidetes*) to a diatom bloom (*Chaetoceros socialis*) induced in laboratory mesocosms, and Riemann and colleagues (2000) detected *Roseobacter* and *Bacteroidetes* phylotypes responding to an induced diatom bloom (*Thalassiosira* sp.), generated by nutrient enrichment. In algal cultures, two closely related diatom species had

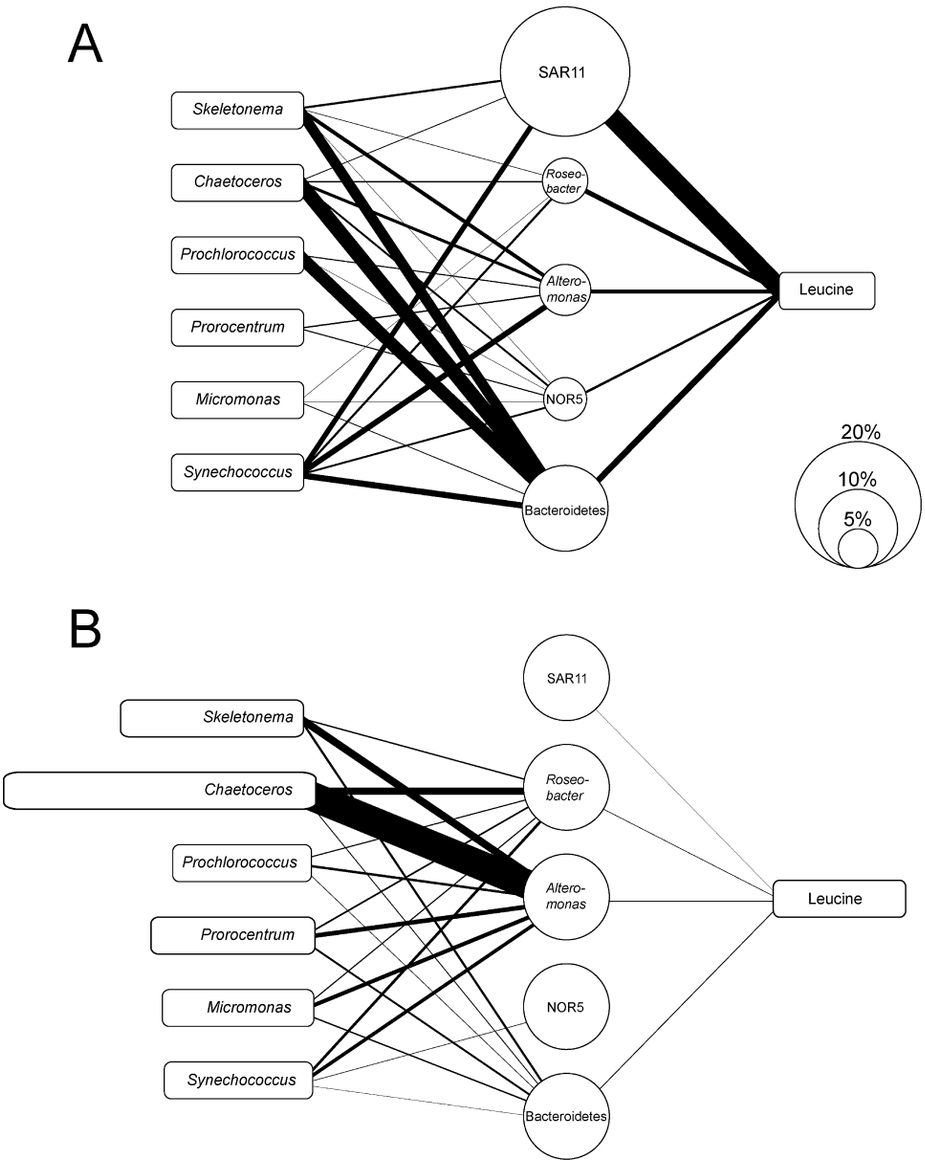

Fig. 6. IS between phytoplankton and the major phylogenetic groups of marine heterotrophic bacteria in short-term (A) and long-term (B) incubations. In short-term incubations (A), the thickness of the lines represents the IS, which is proportional to the contribution of MAR-FISH positive cells (actively incorporating the radiolabelled exudates) to the total community (MAR_{+g}/DAPI counts, Table 1), after 5 h of incubation. The diameter of the circles is proportional to the contribution of each phylogenetic group to the total community at the beginning of the experiment. In long-term incubations (B), the thickness of the lines (the IS) is proportional to the net cell abundance changes in 24 h. The length of the boxes is proportional to the lability of the DOC as given by the total bacterial abundance reached in each incubation flask after 24 h. *Roseobacter* and SAR11 are both subgroups of *Alphaproteobacteria*; *Alteromonas* and NOR5 are both subgroups of *Gammaproteobacteria*.

distinct attached bacterial assemblages dominated by the *Flavobacteria* and *Sphingobacteria* groups of the *Bacteroidetes* phylum, whereas the free-living bacterial assemblages were mainly formed by members of the *Roseobacter* clade (Schafer *et al*., 2002; Grossart *et al*., 2005; see also Sapp *et al*., 2007a).

In addition, our results clearly indicate the existence of numerous weak interactions not observed in previous studies (Fig. 7). With the network perspective, the distribution of the IS is determinant in maintaining and promoting persistence in diverse communities. Weak interactions serve to limit energy flow in a potentially strong consumer–resource interaction and, therefore, to inhibit runaway consumption that destabilizes the dynamics of food webs. This is called the 'weak-interaction' effect (reviewed by McCann, 2000). Our results indicate that the

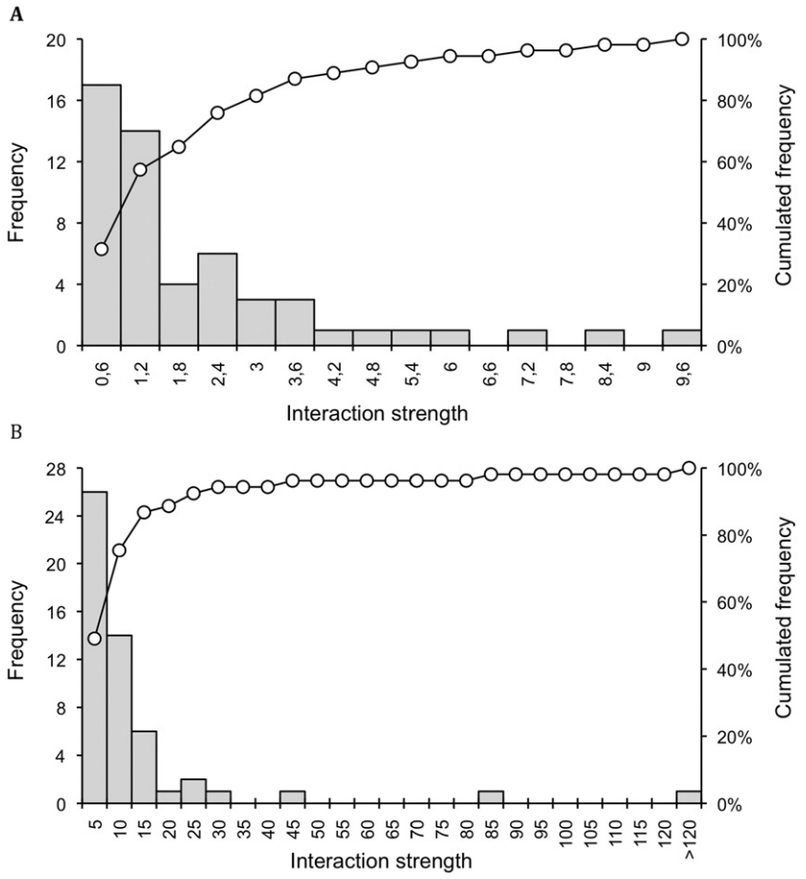

Fig. 7. Frequency distributions of the ISs between different phytoplankton species and the major phylogenetic groups of marine heterotrophic prokaryotes, as given by (A) the contribution of MAR-positive cells (actively taking up the radiolabelled exudates) to the total community after a 5 h incubation, and given by (B) the growth rate during the 24 h enrichment with DOC produced by different phytoplankton species. Results of the probes for *Alphaproteobacteria* and *Gammaproteobacteria* were not considered to avoid over-representation (presented are only the subgroups). Duplicates were considered as individual observations in this diagram.

weak-interaction effect could also operate in the microbial food web.

Our goal was to study the interaction of free-living bacteria with the dissolved organic matter, but in the samples containing exudates from *Chaetoceros* (and only in these), and in spite of the filtration of the phytoplankton cultures through 0.22 μm filters, we observed the formation of micro-aggregates after the 24 h incubation which, being larger than 0.22 μm, could be considered as particulate material (Fig. 8). Thus, there was a spontaneous particle-generating process in the *Chaetoceros* treatment flasks. These aggregates could be the result of a spontaneous complexation of the organic compounds, or a bacterially mediated process (reviewed by Simon *et al.*, 2002). The fact is that the formation of these micro-aggregates crucially affected bacterial

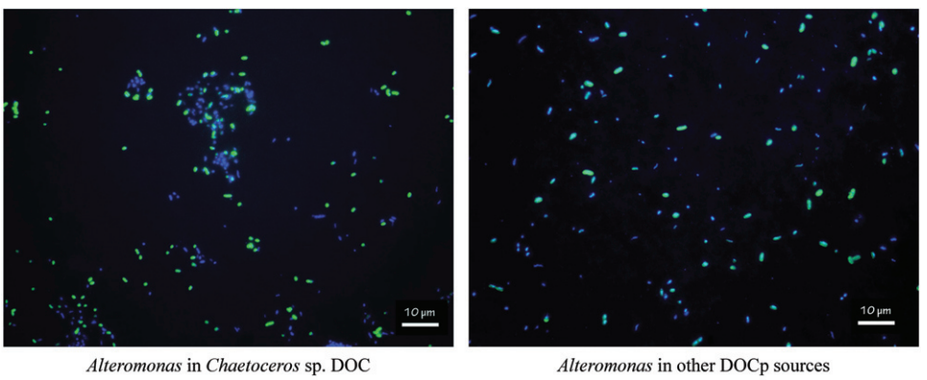

Fig. 8. Epifluorescence microscopy micrographs showing the atypical formation of micro-aggregates with the DOCp from *Chaetoceros* sp. as compared with other treatments. DAPI-stained cells appear in blue; cells hybridized with the probe for *Alteromonas* (Alt1413) appear in green.

growth, as most cells developed on the aggregates (Fig. 8). This is an experimental evidence for the essential role of particles in shaping heterotrophic prokaryote community structure, by generating environmental (micro) heterogeneity and providing a support for variety of lifestyles, as highlighted in previous studies (e.g. Kiørboe and Jackson, 2001; Grossart *et al*., 2007; Stocker *et al*., 2008). The interaction of bacteria with particles, for example diatom aggregates, is a relevant process in the ocean (Smith *et al*., 1992) and the bacterial communities colonizing particles show higher specificity than free-living bacteria (Grossart *et al*., 2005; 2006). Motility and potential for ecto-enzymatic activity are essential requirements for a chemosensory behaviour of particle-colonizing bacteria (Kiørboe and Jackson, 2001). The exact proportion of marine bacteria with these characteristics is still under debate (Grossart *et al*., 2001), but it is certain that not all marine bacteria have the capabilities of colonizing particles (Kiørboe *et al*., 2002), which is somehow an indication of specialization.

Altogether, our observations have relevant ecological implications providing experimental evidence of what has been assumed for years, that is, that a very high number of different resources (different types of organic molecules) available in the sea selects and maintains the astonishing diversity within bacterioplankton. A major obstacle to fully test this hypothesis is that the DOC pool is composed of so many different molecules that it is practically impossible to characterize it in a comprehensive way with the techniques available today (Benner, 2002). The only realistic approach is tracing the biological responses of heterotrophic prokaryotes to the different DOC pools. However, the generalist paradigm that all bacterial groups are capable of equally using all (or most of all) available organic molecules is clearly falsified by our results.

Overall, we demonstrated that the DOCp originated from different phytoplankton species stimulated the major heterotrophic prokaryote phylogenetic groups differently. Similarly to stable complex non-microbial ecosystems, we observed many weak and few strong interactions between phytoplankton and bacterioplankton.

Experimental procedures

Phytoplankton cultures and preparation of radiolabelled exudates

We prepared radiolabelled DOCp from six axenic cultures representative of the major functional groups of phytoplankton that occur in the coastal oceans and particularly in the coastal northwestern Mediterranean (Margalef, 1978; Sournia, 1982), and used it as a tracer in the MAR-FISH technique. The strains obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) were cultured in axenic conditions: the diatoms *Chaetoceros* sp. (CCMP199) and *Skeletonema costatum* (Greville) Cleve (CCMP2092), the dinoflagellate *Prorocentrum minimum* (Pavillard) J. Schiller (CCMP1329), the prasinophyte *Micromonas pusilla* (R.W. Butcher) (CCMP1545) I. Manton & M. Parke, and the cyanobacteria *Prochlorococcus marinus pastoris* Chisholm *et al*. (CCMP2389) and *Synechococcus* sp. (CCMP1183). *Skeletonema costatum* and *Prochlorococcus marinus pastoris* are strains isolated from the Mediterranean Sea. Centric diatoms (among them *Chaetoceros* and *Skeletonema*) are the most abundant group during winter–spring and autumn in the coastal NW Mediterranean. The rest of the year cycle, picophytoplankton dominate, with cyanobacteria (*Synechococcus* and *Prochlorococcus*) and *Prasinophytes* (e.g. *Micromonas*) ranking among the most abundant groups. Dinoflagellates (as *Prorocentrum*) often dominate the summer phytoplankton community (Mura *et al*., 1996; Siokou-Frangou *et al*., 2010), particularly in near-shore bays. The cultures were grown in 50 ml tissue-culture flasks in presence of 150 μCi of $NaH^{14}CO_3$, in F/2 culture medium elaborated with filtered and autoclaved coastal Mediterranean seawater, and incubated at 20°C under artificial photosynthetic active radiation radiation of 100 $\mu mol_{photon}\ m^{-2}\ s^{-1}$, in a 16:8 h light : dark cycle, until cell density increased about one order of magnitude (exponential phase). Aliquots for phytoplankton counts were taken at the beginning and end of the incubation period: 3 days for all microalgae except for *P. minimum*, which was incubated for 7 days. Bacterial contamination was checked in the same samples by epifluorescence microscope observations under blue and UV wavelength excitation, following 4,6-diamidino-2-phenylindole (DAPI) staining (10 μg ml^{-1}, final conc.). Parallel non-radioactive cultures were run simultaneously in the exactly same conditions in order to measure the DOC in each culture. Dissolved organic carbon analysis was performed by standard methods as described in Romera-Castillo and colleagues (2010).

An overview of the experimental design is presented in a schematic way in Fig. 1. To obtain the labelled DOCp, roughly 40 ml of culture were gently filtered onto 0.2 μm Sterivex filters in order to isolate the dissolved exudates from cells. The filtrates were acidified with 3 ml of 6 M HCl and left open in an orbital shaker overnight for dissipation of unassimilated $NaH^{14}CO_3$ as described in Morán and Estrada (2002). The pH of the exudates was then adjusted to ~8 with NaOH 6 M and a 3 ml subsample was radioassayed ($DO^{14}C$) in 15 ml of Ultima Gold liquid scintillation cocktail. To determine the amount of labelled particulate organic carbon ($PO^{14}C$), 1 ml of each culture was filtered onto 0.22 μm Millipore GSWP filters which were treated with concentrated HCl fumes overnight before the addition of 4.5 ml of ReadySafe liquid scintillation cocktail.

Microautoradiography coupled with catalysed reporter deposition fluorescence in situ *hybridization*

MAR-FISH allows tracing of the carbon passage through phytoplankton and its re-assimilation by heterotrophic prokaryotes, revealing how many and which kind of heterotrophic prokaryote really incorporate each type of DOCp. Duplicates of subsurface 150 μm pre-filtered seawater samples (20 ml each) taken on the 30th September of 2008 from a NW Mediterranean coastal site (Blanes Bay Microbial

Observatory, http://www.icm.csic.es/bio/projects/icmicrobis/bbmo/) were incubated at dark controlled *in situ* temperature with addition of the different radiolabelled compounds (~50 μM DOC final conc.). The DOC concentration on that date at the Blanes Bay Microbial Observatory was 94.7 μM. Alonso-Sáez and colleagues (2008) reported an average DOC of 109 μM in the monthly sampling at the same site in 2003/2004, with a maximum of 177 mM during the spring phytoplankton bloom. The average value for the 2008–2010 period was 80.9 μM (C. Romera-Castillo, C. Marrasé, X.A. Álvarez-Salgado, M. Galí and J.M. Gasol., submitted) with maxima reaching 140 μM. In both studies, the difference between the average and the spring maxima is close to 50 μM, the concentration we used in our experiments.

In order to achieve similar amounts of DOC additions in each incubation flask, we varied the volume of exudate solution added. Control incubations with a mixture of labelled and unlabelled leucine (in order to obtain similar DOC concentration and similar disintegrations per minute as in the other treatments) were also carried out. Dead control incubations were performed by fixing the sample with formaldehyde (1.8%) before (> 5 min) the addition of the radiolabelled compounds. False positives obtained in these dead controls (always < 0.5% of DAPI-stained cells) were subtracted. Another control incubation was created by incubating the sample without DOC during the same period of time (blank control).

Subsamples were taken after 5 h (short-term) and 24 h (long-term) of incubation. After the incubation, samples were fixed overnight with formaldehyde (1.8%) at 4°C, and gently filtered on 0.2 μm polycarbonate filters (Millipore, GTTP, 25 mm diameter). The short-term incubation samples were processed with the MAR-FISH protocol (microautoradiography coupled with catalysed reporter deposition fluorescence *in situ* hybridization – CARD-FISH).

MAR-FISH was performed with the protocol initially described by Alonso and Pernthaler (2005) with some modifications. After hybridization following the CARD-FISH protocol (described hereafter) the filters were glued onto glass slides with an epoxy adhesive (UHU plus; UHU GmbH, Bühl, Germany). The slides were embedded in 46°C tempered photographic emulsion (KODAK NTB-2) containing 0.1% agarose (gel strength 1%, > 1 kg cm^{-2}) in a dark room and placed on an ice-cold metal bar for about 5 min to allow the emulsion to solidify. They were subsequently placed inside black boxes at 4°C until development. We found that the optimal exposure time was of 9 days (data not presented). For development, we submerged the exposed slides for 3 min in the developer (KODAK D19), 30 s rinsing with distilled water, and 3 min in fixer (KODAK Tmax) followed by 5 min of washing with tap water. The slides were then dried in a dessicator overnight, stained with DAPI (1 μg ml^{-1}), and inspected in an Olympus BX61 epifluorescence microscope. CARD-FISH positive cells (hybridized with the specific probe) appear in bright green under blue light excitation. MAR-FISH positives contain, additionally, dark silver grains accumulated above the bacterial cells on the photographic emulsion, resulting from radioactive decay of labelled DOCp, and are clearly visible under white light in the same microscope.

The long-term incubation samples were used to determine the changes in community structure in the long run (with CARD-FISH) derived from the use of the labelled compounds in the presence of the entire microbial food web. CARD-FISH was carried out following the protocol described previously (Pernthaler *et al.*, 2004). Several horseradish peroxidase probes were used to characterize the composition of the bacterial community in the original water samples, using the same procedure as described in Alonso-Sáez and Gasol (2007). The horseradish peroxidase-labelled probes used were: EUB 338-II and -III (target most *Eubacteria*) (Amann *et al.*, 1990; Daims *et al.*, 1999); GAM42a (targets most *Gammaproteobacteria*) (Manz *et al.*, 1992); ALF968 (targets most *Alphaproteobacteria*) (Neef, 1997); CF319 (targets many groups belonging to the *Bacteroidetes* group) (Manz *et al.*, 1996); ROS537 (targets members of the *Roseobacter–Sulfitobacter–Silicibacter* group) (Eilers *et al.*, 2001); NOR5-730 (targets members of the NOR5 cluster) (Eilers *et al.*, 2001); Alt1413 (targets *Alteromonas* and *Colwellia*) (Eilers *et al.*, 2000); SAR11-441R (targets the SAR11 cluster) (Morris *et al.*, 2002); EUB antisense probe NON338 (Wallner, 1993) was used as a negative control. All probes were purchased from biomers.net (Ulm, Germany). Specific hybridization conditions were established by addition of formamide to the hybridization buffers (20% formamide for the NON338 probe, 45% formamide for the ALF968 and SAR11-441R probes, 50% for NOR5-730, 60% for Alt1413 and 55% for the other probes).

Counterstaining of CARD-FISH preparations was performed with DAPI (1 μg ml^{-1}). Between 500 and 1000 DAPI-positive cells of each phylogenetic were counted in a minimum of 10 fields.

Inevitably, the exudates added to the seawater samples contained large amounts of inorganic nutrients (as the algae were growing in the relatively nutrient-rich F/2 culture medium). We assumed that during the 24 h incubations all the bacterial communities were in inorganic nutrient-replete conditions and responded primarily to the different organic compounds added in the form of DOCp. We also assumed that there were no major changes in bacterial community structure during the first 5 h of incubation. This was actually tested previously using the quantitative CARD-FISH technique in an independent preliminary experiment performed to optimize the incubation conditions.

Calculating IS and network building

The networks were built aggregating the results of the different treatments in one single network. The IS between a phytoplankton strain and the different heterotrophic prokaryote groups mediated through the use of DOCp was calculated in two different ways, depending on the duration of the incubation. For the short-term incubations, the IS was the number of MAR-FISH positives (cells actively taking up the radiolabelled compounds) as a proportion of the total bacterial community (MAR_{+g}/DAPI counts). If all cells of a group used a particular DOCp, then that group and the corresponding algae would have a strong interaction, while when no cells used the substrate, this interaction would be zero (no interaction). In the long-term incubations, net growth (in cell abundance) was calculated as: $(n_{final} - n_{initial})\ n_{initial}^{-1}$, where n is the cell abundance of each heterotrophic prokaryote group (in cell ml^{-1}).

Acknowledgements

We thank Clara Ruiz-González, Irene Forn, Eléonore Toussaint and Sophie Gusbin for laboratory assistance at different stages of the experiments. We are thankful to José M. Montoya, David Kirchman, Silvia Acinas and Ramon Massana and two anonymous reviewers for their valuable comments to the manuscript and their constructive suggestions. H. S. benefited from grants from the Spanish MEC (SB2006-0060) and MCyI (Juan de la Cierva Fellowship JCI-2008-2727), and Portuguese MCTES (FCT grant SFRH/BPD/34041/2006). The work was supported by Spanish MCyT projects MICROOCEAN PANGENOMES (CGL2011-26848), MICRODIVERSITY (CGL2008-00762/BOS), SUMMER (CTM2008-03309/MAR) and STORM (CTM2009-09352/MAR).

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Supporting information

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

Table S1. Detailed results of the MAR-FISH incubations presented in different ways depending on which parameter we chose to relate to the number of cells taking up the radiolabelled compounds.

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MAR+g/CARD-FISH+		Alpha-proteobacteria				Gamma-proteobacteria				Bacteroidetes
		Roseobacter	SAR11	Other Alpha	Total	Alteromonas	NOR5	Other Gamma	Total	Total
Skeletonema	Dupl. A	7.43	6.23	<0.05	**13.66**	40.00	5.49	<0.05	**45.49**	**37.14**
Skeletonema	Dupl. B	8.78	2.99	<0.05	**11.77**	*n.a.*	4.26	13.21	**17.47**	**45.07**
Chaetoceros	Dupl. A	22.91	1.96	<0.05	**24.87**	37.50	38.33	<0.05	**75.83**	**44.53**
Chaetoceros	Dupl. B	19.53	1.57	<0.05	**21.10**	26.67	26.92	<0.05	**53.59**	**67.95**
Prochlorococcus	Dupl. A	<0.05	<0.05	10.73	**10.73**	4.86	5.80	<0.05	**10.66**	**52.31**
Prochlorococcus	Dupl. B	<0.05	<0.05	8.86	**8.86**	18.67	*n.a.*	<0.05	**18.67**	**45.69**
Prorocentrum	Dupl. A	<0.05	<0.05	<0.05	**<0.05**	24.15	15.63	<0.05	**39.78**	**<0.05**
Prorocentrum	Dupl. B	<0.05	<0.05	<0.05	**<0.05**	6.45	20.00	<0.05	**26.45**	**<0.05**
Micromonas	Dupl. A	1.11	<0.05	4.87	**5.98**	*n.a.*	6.76	<0.05	**6.76**	**2.84**
Micromonas	Dupl. B	2.49	<0.05	4.08	**6.57**	10.17	14.29	<0.05	**24.46**	*n.a.*
Synechococcus	Dupl. A	31.88	13.15	<0.05	**45.02**	61.36	35.71	<0.05	**97.08**	**28.49**
Synechococcus	Dupl. B	36.49	9.35	<0.05	**45.83**	61.18	34.25	<0.05	**95.42**	**20.16**
Control Leucine	Dupl. A	71.60	44.00	<0.05	**115.60**	44.72	45.61	<0.05	**90.33**	**30.91**
Control Leucine	Dupl. B	76.47	39.69	<0.05	**116.17**	43.10	43.22	<0.05	**86.32**	**17.29**

MAR+g/CARD-FISH+		Alpha-proteobacteria				Gamma-proteobacteria				Bacteroidetes
		Roseobacter	SAR11	Other Alpha	Total	Alteromonas	NOR5	Other Gamma	Total	Total
Skeletonema	Avg	8.10	4.61	<0.05	**12.71**	40.00	4.87	13.21	**31.48**	**41.11**
Chaetoceros	Avg	21.22	1.77	<0.05	**22.99**	32.08	32.63	<0.05	**64.71**	**56.24**
Prochlorococcus	Avg	<0.05	<0.05	9.80	**9.80**	11.76	5.80	<0.05	**14.66**	**49.00**
Prorocentrum	Avg	<0.05	<0.05	<0.05	**<0.05**	15.30	17.81	<0.05	**33.12**	**<0.05**
Micromonas	Avg	1.80	<0.05	4.48	**6.28**	10.17	10.52	<0.05	**15.61**	**2.84**
Synechococcus	Avg	34.18	11.25	<0.05	**45.43**	61.27	34.98	<0.05	**96.25**	**24.33**
Control Leucine	Avg	74.04	41.85	<0.05	**115.89**	43.91	44.42	<0.05	**88.33**	**24.10**

MAR+g/MAR+total		Alpha-proteobacteria				Gamma-proteobacteria				Bacteroidetes
		Roseobacter	SAR11	Other Alpha	Total	Alteromonas	NOR5	Other Gamma	Total	Total
Skeletonema	Dupl. A	10.64	15.60	<0.05	**26.24**	4.26	3.55	29.08	**36.88**	**36.88**
Skeletonema	Dupl. B	20.00	5.81	<0.05	**25.81**	*n.a.*	2.58	30.32	**32.90**	**41.29**
Chaetoceros	Dupl. A	31.33	3.61	<0.05	**34.94**	9.04	13.86	7.83	**30.72**	**34.34**
Chaetoceros	Dupl. B	31.45	3.14	<0.05	**34.59**	2.52	17.61	11.95	**32.08**	**33.33**
Prochlorococcus	Dupl. A	<0.05	<0.05	28.79	**28.79**	5.30	3.03	11.36	**19.70**	**51.52**
Prochlorococcus	Dupl. B	<0.05	<0.05	29.25	**29.25**	13.21	*n.a.*	7.55	**20.75**	**50.00**
Prorocentrum	Dupl. A	<0.05	<0.05	<0.05	**<0.05**	83.33	16.67	<0.05	**100.00**	**<0.05**
Prorocentrum	Dupl. B	<0.05	<0.05	<0.05	**<0.05**	50.00	50.00	<0.05	**100.00**	**<0.05**
Micromonas	Dupl. A	5.41	<0.05	51.35	**56.76**	*n.a.*	13.51	2.70	**16.22**	**27.03**
Micromonas	Dupl. B	16.36	<0.05	25.45	**41.82**	43.64	14.55	<0.05	**58.18**	*n.a.*
Synechococcus	Dupl. A	20.48	17.27	<0.05	**37.75**	21.69	20.08	<0.05	**41.77**	**20.48**
Synechococcus	Dupl. B	22.88	12.71	<0.05	**35.59**	22.03	21.19	<0.05	**43.22**	**21.19**
Control Leucine	Dupl. A	21.40	20.30	<0.05	**41.70**	20.30	19.19	<0.05	**39.48**	**18.82**
Control Leucine	Dupl. B	20.31	20.31	<0.05	**40.63**	19.53	19.92	<0.05	**39.45**	**19.92**

MAR+g/MAR+total		Alpha-proteobacteria				Gamma-proteobacteria				Bacteroidetes
		Roseobacter	SAR11	Other Alpha	Total	Alteromonas	NOR5	Other Gamma	Total	Total
Skeletonema	Avg	15.32	10.70	<0.05	**26.02**	4.26	3.06	29.70	**34.89**	**39.08**
Chaetoceros	Avg	31.39	3.38	<0.05	**34.77**	5.78	15.73	9.89	**31.40**	**33.84**
Prochlorococcus	Avg	<0.05	<0.05	29.02	**29.02**	9.26	3.03	9.46	**20.23**	**50.76**
Prorocentrum	Avg	<0.05	<0.05	<0.05	**<0.05**	66.67	33.33	<0.05	**100.00**	**<0.05**
Micromonas	Avg	10.88	<0.05	38.40	**49.29**	43.64	14.03	2.70	**37.20**	**27.03**
Synechococcus	Avg	21.68	14.99	<0.05	**36.67**	21.86	20.63	<0.05	**42.49**	**20.83**
Control Leucine	Avg	20.86	20.30	<0.05	**41.16**	19.91	19.56	<0.05	**39.47**	**19.37**

MAR+g/DAPI counts		Alpha-proteobacteria				Gamma-proteobacteria				Bacteroidetes
		Roseobacter	SAR11	Other Alpha	Total	Alteromonas	NOR5	Other Gamma	Total	Total
Skeletonema	Dupl. A	0.22	1.29	0.72	**2.22**	1.65	0.13	0.05	**1.83**	**4.27**
Skeletonema	Dupl. B	0.26	0.62	1.52	**2.39**	*n.a.*	0.10	1.36	**1.46**	**5.18**
Chaetoceros	Dupl. A	0.67	0.41	3.06	**4.14**	1.54	0.92	<0.05	**2.47**	**5.11**
Chaetoceros	Dupl. B	0.57	0.32	2.83	**3.73**	1.10	0.65	1.25	**3.00**	**7.80**
Prochlorococcus	Dupl. A	<0.05	<0.05	2.53	**2.53**	0.20	0.14	0.27	**0.61**	**6.01**
Prochlorococcus	Dupl. B	<0.05	<0.05	2.09	**2.09**	0.77	*n.a.*	<0.05	**0.77**	**5.25**
Prorocentrum	Dupl. A	<0.05	<0.05	<0.05	**<0.05**	0.99	0.38	<0.05	**1.37**	**<0.05**
Prorocentrum	Dupl. B	<0.05	<0.05	<0.05	**<0.05**	0.27	0.48	<0.05	**0.75**	**<0.05**
Micromonas	Dupl. A	0.03	<0.05	1.38	**1.41**	*n.a.*	0.16	<0.05	**0.16**	**0.33**
Micromonas	Dupl. B	0.07	<0.05	1.48	**1.55**	0.42	0.34	<0.05	**0.76**	*n.a.*
Synechococcus	Dupl. A	0.93	2.72	2.74	**6.39**	2.52	0.86	<0.05	**3.38**	**3.27**
Synechococcus	Dupl. B	1.06	1.93	2.90	**5.89**	2.52	0.83	<0.05	**3.34**	**2.32**
Control Leucine	Dupl. A	2.09	9.09	<0.05	**11.18**	1.84	1.10	<0.05	**2.94**	**3.55**
Control Leucine	Dupl. B	2.23	8.20	<0.05	**10.43**	1.77	1.04	<0.05	**2.81**	**1.99**

MAR+g/DAPI counts		Alpha-proteobacteria				Gamma-proteobacteria				Bacteroidetes
		Roseobacter	SAR11	Other Alpha	Total	Alteromonas	NOR5	Other Gamma	Total	Total
Skeletonema	Avg	0.24	0.95	1.12	**2.31**	1.65	0.12	0.71	**1.65**	**4.72**
Chaetoceros	Avg	0.62	0.36	2.95	**3.93**	1.32	0.79	1.25	**2.73**	**6.46**
Prochlorococcus	Avg	<0.05	<0.05	2.31	**2.31**	0.48	0.14	0.27	**0.69**	**5.63**
Prorocentrum	Avg	<0.05	<0.05	<0.05	**<0.05**	0.63	0.43	<0.05	**1.06**	**<0.05**
Micromonas	Avg	0.05	<0.05	1.43	**1.48**	0.42	0.25	<0.05	**0.46**	**0.33**
Synechococcus	Avg	1.00	2.32	2.82	**6.14**	2.52	0.84	<0.05	**3.36**	**2.79**
Control Leucine	Avg	2.16	8.65	<0.05	**10.81**	1.81	1.07	<0.05	**2.88**	**2.77**