Linkages between bacterioplankton community composition, heterotrophic carbon cycling and environmental conditions in a highly dynamic coastal ecosystem.

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Summary

We used mesocosm experiments to study the bacterioplankton community in a highly dynamic coastal ecosystem during four contrasting periods of the seasonal cycle: winter mixing, spring phytoplankton bloom, summer stratification and autumn upwelling. A correlation approach was used in order to measure the degree of coupling between the dynamics of major bacterial groups, heterotrophic carbon cycling and environmental factors. We used CARD-FISH to follow changes in the relative abundance of the most abundant groups of bacteria (Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes). Bacterial carbon flux-related variables included bacterial standing stock, bacterial production and microbial respiration. The environmental factors included both, biotic variables such as chlorophyll-a concentration, primary production, phytoplankton extracellular release, and abiotic variables such as the concentration of dissolved inorganic and organic nutrients. Rapid shifts in the dominant bacterial groups occurred associated to environmental changes and bacterial bulk functions. An alternation between Alphaproteobacteria and Bacteroidetes was observed associated to different phytoplankton growth phases. The dominance of the group Bacteroidetes was related to high bacterial biomass and production. We found a significant, non-spurious, linkage between the relative abundances of major bacterial groups and bacterial carbon cycling. Our results suggest that bacteria belonging to these major groups could actually share a function in planktonic ecosystems.

Introduction

Bacterioplankton play an important role in the flow of energy and nutrients through plankton food webs, as a consequence of their great abundance, efficient nutrient uptake and large growth potential (Azam *et al.,* 1983). A number of studies have shown changes in bulk bacterial properties, as a response to biological or environmental factors (see e.g. reviews by Ducklow, 2000; Williams, 2000; del Giorgio and Cole, 2000). However, much less is known about how such factors may influence bacterial community composition, and how potential shifts in bacterial community structure may in turn influence microbial carbon cycling. Despite several studies have shown temporal and spatial changes in bacterial community composition in freshwater and marine environments (e.g. Rappé *et al.,* 2000; Dolan, 2005 and references therein), even the distribution of the major phylogenetic groups of bacteria is still not well understood. Particular attention has received the study of changes in bacterial assemblage structure associated to phytoplankton blooms (Riemann *et al.,* 2000; Fandino *et al.,* 2001; Larsen *et al.,* 2001; Pinhassi *et al.,* 2004). These studies suggest that the phylum Bacteroidetes, also known as CFB (Cytophaga-Flavobacteria-Bacteroides) might be especially relevant in the cycling of organic matter during algal blooms. Unfortunately, most of these analyses provide qualitative information, as they are mainly based on fingerprinting descriptions of the bacterioplankton assemblages. Although the same number of phylotypes can be detected in two distinct samples (samples that have the same richness), the relative abundance of each phylogenetic group can be very different (thus showing different diversity), implying that not only qualitative, but also quantitative information is needed in order to evaluate the links between bacterial assemblage structure and ecosystem functioning. One of the major advantages of fluorescence *in situ* hybridisation (FISH) is that it allows for

quantification of the actual abundance of a given phylogenetic group. This technique has now been widely used for monitoring dynamics of different phylogenetic groups in contrasting aquatic environments (e.g. Pernthaler *et al.,* 1998; Glöckner *et al.,* 1999; Brinkmeyer *et al.,* 2003; Kirchman *et al.,* 2005; Comte *et al.,* 2006; Mary *et al.,* 2006). A major limitation of FISH is the number and phylogenetic resolution of the probes (Alonso-Sáez *et al.*, 2007a). Most of the studies work at a high phylogenetic level due to the limited number of probes that can be used at a time, to rationalize counting effort. The accuracy of hybridization depends on the specificity and coverage of the probes, which must be re-evaluated frequently due to the rapidly increasing sequence information in public databases. Most of the available probes targeting broad phylogenetic groups, designed several years ago, show a far from 100% coverage, and therefore it would be more appropriate to refer not to phylogenetic groups but to operational groups or populations detected by particular probes.

Growing evidence indicates that some major bacterial groups, even at a high phylogenetic level might operate as functional units (Kirchman *et al.,* 2005). The identification of such ecological units would allow incorporating bacterial community structure into quantitative models of carbon cycling. In support of this hypothesis are several interesting recent findings: a) the very distinct uptake patterns of low molecular weight dissolved organic matter (DOM) by different bacterial groups (Alonso-Sáez and Gasol, 2007), b) the differential sensitivity of major bacterial groups to UVR (Alonso-Sáez *et al.,* 2006), and c) the differential susceptibility of specific bacterial groups to lytic viral infection (Bouvier and del Giorgio, 2007). On the other hand, some studies detecting little temporal or spatial variability at broad phylogenetic levels, such as subclass level, claimed the heterogeneity of these groups and the lack of a common functionality within the group (Pernthaler *et al.,* 1998; Longnecker *et al.,* 2006; Mary *et*

al., 2006; Pommier *et al.,* 2007). Therefore, the debate about the existence of functional bacterial groups, and particularly at which phylogenetic level should we look for, is still open.

In this context, the objective of our study was to analyse the linkages between bacterial community composition at the level of major groups, environmental factors and bacterially-driven ecosystem function, approaching this goal by statistically correlating bacterial composition with both environmental conditions and bacterial carbon cycling using a multivariate approach

Results.

We carried out four mesocosm experiments in a highly dynamic inshore ecosystem during the most relevant periods of the seasonal cycle: winter mixing, spring phytoplankton bloom, summer stratification, and autumn upwelling (Fig. 1). We analysed changes in bacterial composition using CARD-FISH with several probes targeting the most abundant groups of bacteria (Alphaproteobacteria, Gammaproteobacteria and Bacteroidetes).

Predominant environmental conditions.

The initial environmental conditions are summarized in Teira *et al.* (2007). In March, confinement in the mesocosms produced a phytoplankton bloom composed of the diatoms *Lauderia annulata* and *Chaetoceros socialis* (M. Varela, pers. comm.) grown at the expenses of the high initial nutrient levels. The summer (July) phytoplankton community, however, was dominated by senescent diatoms (M. Varela, pers. comm.) and dissolved inorganic nutrients were very low during summer stratification In September, we sampled the decay of a diatom bloom, mainly composed

of several species of *Chaetoceros*, (M. Varela, pers. comm.), as a consequence of nutrient exhaustion after the second day of the experiment. During winter mixing (January), maximum concentrations of inorganic nutrients were recorded, accompanied by extremely low levels of particulate matter (Teira *et al.,* 2007). The winter phytoplankton community was dominated by flagellates (M. Varela, pers. comm.).

Bacterial community dynamics.

In Figure 2 we have represented the temporal changes along a 9 day period in the relative abundance of Alphaproteobacteria (positive with ALF968 probe), Gammaproteobacteria (positive with GAM42a probe) and Bacteroidetes (positive with CF319a probe) together with those in nitrate and chlorophyll-a concentration, for the 4 experiments. We have represented the mean value obtained from duplicate (in March and July) and triplicate (September and January) mesocosms. The percentage of DAPI-stained cells (referred to as total prokaryotic abundance) detected as Eubacteria (probe EUB338) averaged 85% and was rather stable during the 4 experiments, ranging from 70 to 98%.

The relative contribution of the group Alphaproteobacteria to total prokaryotic abundance (Fig. 2) ranged from values as low as 10% in September to 34% in March. Repeated measures ANOVA results (RMANOVA test, $p < 0.01$) showed significant differences between experiments. The relative contribution of this bacterial group was significantly higher in March than in the other 3 periods (Bonferroni test, $p < 0.05$). Taking into account the complete data set, Alphaproteobacteria contribution to prokaryotic abundance significantly changed with time (RMANOVA test, $p < 0.05$). However, there was a significant interaction Time x Experiment (RMANOVA test, $p <$

0.02), which means that temporal dynamics differed between experiments. Significant temporal variability was observed only in March (Bonferroni test, $p < 0.05$, Fig. 2A).

The relative abundance of Gammaproteobacteria ranged between 5 %, and 58 % at day 4 in January (Fig. 2). There were also significant differences between experiments (RMANOVA test, $p = 0.001$), with significantly higher contribution of this group to prokaryotic abundance in January than in the other 3 experiments (Bonferroni test, $p < 0.005$). The relative abundance of this group was rather stable in March, July and September, but significant temporal variability was observed in January (Bonferroni test, $p = 0.005$), when a burst occurred between days 3 and 4 (Fig. 2D).

The Bacteroidetes group showed a great degree of temporal variability, clearly associated with changes in the concentration of chlorophyll-a in March and September (Fig. 2A & 2C). Its relative abundance varied from 10% to 61%, and was, on average, the most abundant group in this planktonic system. There were significant differences between experiments (RMANOVA test, $p<0.001$). These bacteria were significantly more abundant in March, July and September than in January (Bonferroni test, $p < 0.05$), and showed significant temporal variability in March and September (Bonferroni tests, $p < 0.05$, Fig. 2A & 2 C).

Relationship between major bacterial groups, environmental factors, and bacterial carbon cycling.

Using the entire dataset, we conducted a correlation analysis in order to identify any relevant physicochemical or biological variable that could explain the observed variability in bacterial community composition (Table 1). The three bacterial groups showed different correlations to environmental and carbon-flux related variables. None of them correlated with either temperature or salinity. Regarding inorganic nutrients,

none of the groups correlated with either ammonium or phosphate. Gammaproteobacteria and Bacteroidetes showed opposing correlations with both nitrite and nitrate concentrations. The first correlated positively, whereas the later correlated negatively. The correlations with dissolved and particulate organic matter also differed between these three groups of bacteria. Whereas Alphaproteobacteria did not show any significant correlation, Bacteroidetes correlated positively with dissolved and particulate organic carbon (DOC and POC). Gammaproteobacteria correlated negatively with particulate organic carbon and nitrogen (POC and PON).

The correlation between bacterial groups and biological variables revealed interesting relationships. The most striking trait is the predominance of negative correlations between Gammaproteobacteria and autotrophic variables (chlorophyll-a concentration and primary production) (Table 1), and positive correlations between Bacteroidetes and heterotrophic variables (bacterial biomass, bacterial production, and extracellular release, a process that can be considered to be the link between phytoplankton and bacteria). The Alphaproteobacteria did not correlate with any biological variable. Between 18 and 45% of the observed variability in the different bacterial components was related to each of the significant variables.

We further conducted a principal component analysis in order to reduce the amount of studied variables to a few components explaining most of the observed variability (Fig. 3). The three first principal components explained 69% of the variability in the original data. The first principal component, which explained 39% of the variability, described an environment characterized by low inorganic nutrient concentrations and high dissolved and particulate organic carbon concentrations, which sustained high levels of bacterial biomass and microbial community respiration. In this situation, the abundance of Gammaproteobacteria was very low. The second principal

component, which explained 19% of the variability, refer to a situation where temperature was high, phytoplankton biomass and primary production were very low, and the percentage of extracellular release and bacterial production, were very high. In such scenario, the group Bacteroidetes dominated the bacterial assemblage.

The relationship between bacterial assemblage structure, bacterial carbon cycling (a bacterially-driven ecosystem function) and the environmental factors was assessed by means of correlation of the three similarity matrices (for details see 'experimental procedures' section on Mantel test). We found a significant correlation between bacterial assemblage structure and both carbon cycling ($r = 0.371$, $p = 0.031$) and environmental factors ($r = 0.373$, $p = 0.017$), and also between ecosystem function and environmental factors ($r = 0.333$, $p = 0.018$). We further explored if the significant correlation obtained between bacterial composition and bacterial carbon cycling was spurious, just as a result of the significant correlations between environment and both bacterial composition and carbon cycling. The partial correlation between bacterial assemblage and carbon cycling, controlling for the effects of the environment, was also significant ($r = 0.2815$, $p = 0.046$).

Bacterial community dynamics in microcosm versus mesocosms experiments.

During the mesocosm experiment conducted in January 2006 we also followed changes in bacterial composition in a parallel microcosm experiment (for details see 'experimental procedures' section) in order to test for potential enclosure effects on bacterial assemblage structure. While the mesocosms investigated held ~3000 L, the microcosms were of 5 L. If enclosure effects are significantly altering our results, we should see significant differences in parallel experiments done in 5 vs. 3000 L containers. The relative abundances of Alpha-, Gammaproteobacteria and Bacteroidetes

in the mesocosm vs. the microcosm experiment were strikingly similar (Pearson's r = 0.91, 0.98, and 0.92 respectively, with N = 6, $p < 0.02$). The relative contribution of each of the major bacterial groups did follow the same trends in both types of reservoirs (see supplementary figure on the web appendix). The dramatic increase in the relative contribution of Gammaproteobacteria and the decrease in the contribution of Bacteroidetes, did occur at the same time in the meso- and the microcosms.

Discussion.

Temporal variability.

The Ría de Vigo is a highly hydrodynamic system, as reflected in the contrasting hydrographic conditions typically found over an annual cycle (Nogueira *et al.*, 1997). This dynamism has been shown to affect biogeochemical cycling, as well as phytoplankton size-structure and composition (Tilstone *et al.,* 1999; Álvarez-Salgado *et al.*, 2001; Cermeño *et al.,* 2006). From the evident annual pattern of chlorophyll-a concentration (Fig. 1), as well as from the well-established succession pattern of phytoplankton species (Nogueira and Figueiras, 2005) we would expect to find important bacterial community composition changes. Against these expectations, a recent study by Mary *et al.* (2006), using FISH, showed that community structure at a high taxonomic level did not change dramatically over an annual cycle in the western English Channel. Schauer *et al.* (2003), using DGGE, also found a relative stability of broad phylogenetic groups in Blanes Bay, in the NW Mediterranean. Most of these studies, however, did find variability at lower phylogenetic levels. By contrast, other recent studies did find both temporal and spatial variability in the distribution of major phylogenetic groups of bacteria using either FISH (Kirchman *et al.*, 2005, Alderkamp *et*

al., 2006; Alonso-Sáez *et al.*, 2007a; 2007b; Garcés *et al.*, 2007) or PCR-based techniques (Morris *et al.,* 2005; Fuhrman *et al.,* 2006). We only analyzed 4 different sampling periods, and we also checked only for three major groups of bacteria, and even considering these limitations, we did find significant differences in bacterial community composition. The three major groups showed different dynamics, and also different correlations with environmental variables and carbon-flux related variables.

Major bacterial groups and environmental variables.

In a recent study, Frette *et al.* (2004) determined the functional characteristics of a considerable number of bacterial isolates from one freshwater and 3 marine locations. They found that 95% of the isolates were able to utilize dissolved free amino acids (DFAAs) and protein as their sole N source, and that only a few were capable to utilize ammonium, which had long been considered to be an important source of N for bacterioplankton (Kroer *et al.,* 1994). We did not find any significant correlation between any of the bacterial groups and ammonium concentration (Table 1), whereas we found significant positive correlations between Gammaproteobacteria and nitrite, nitrate and silicate, and negative correlations between Bacteroidetes and the concentrations of nitrite and nitrate. Wells and Deming (2003) also found negative correlations between Bacteroidetes and inorganic nutrient concentrations.

In our study, Bacteroidetes appear to be relatively more abundant when the concentration of inorganic nutrients is low and the concentration of dissolved organic carbon is high (Table 1, Fig. 3). The relative abundance of this group also shows a positive correlation with the percentage of extracellular release (PER), something that supports the hypothesis of the important role that this phylogenetic group is considered to play in the decomposition of recently produced phytoplankton-derived dissolved

organic matter (DOM) (Kirchman, 2002). It is important to mention that the FISH probe generally used for Bacteroidetes (CF319a) targets mainly the class Flavobacteria (formely known as order Cytophagales), which comprises the most abundant class of planktic marine Bacteroidetes (Alonso *et al.,* 2007). Several studies, based on DGGE, cloning or other PCR analyses, have shown that Bacteroidetes species dominate on marine aggregates (DeLong *et al.,* 1993; Rath *et al.,* 1998), and are involved in the degradation of complex macromolecules (Shewan and McMeekin, 1983). Riemann *et al.* (2000) observed rapid colonization of particulate matter after a mesocosm-simulated diatom bloom by specialized Alphaproteobacteria and Cytophagales-related phylotypes. Other studies also showed that members of the Flavobacteria class are exceptionally responsive to induced phytoplankton blooms (Pinhassi *et al.,* 2004), and that *Flavobacteria* are involved in the initial colonization of diatom detritus (Abell *et al.,* 2005). A recent analysis of the whole genome of a marine Bacteroidetes representative affiliated to the class Flavobacteria revealed adaptations to the degradation of polymeric organic matter (Bauer *et al.*, 2006). Our FISH data revealed that the Bacteroidetes group becomes highly dominant during the decay of diatom blooms (March and September experiments, Fig. 2A & C). Alderkamp *et al.* (2006) also showed that Bacteroidetes comprised up to 63% of the prokaryotic community at the senescent stage of a *Phaeocysitis globosa* spring bloom. Despite the lack of an overall significant correlation with chlorophyll-a concentration, there is a clear inverse pattern between Bacteroidetes and the chlorophyll-a concentration if data from January (the only experiment not dominated by diatoms) are excluded ($r = -0.65$, $p = 0.002$). Neither Alpha- nor Gammaproteobacteria responded in this straight manner to the decay of the diatom blooms.

Alphaproteobacteria also showed considerable temporal dynamics in the 4 experiments. This bacterial group has been suggested to be active colonizers of particles and good competitors under low nutrient conditions (Riemann *et al.*, 2000; Pinhassi and Berman, 2003). However, the observed lack of correlation between this group and any of the considered variables (Table 1) likely reflects complex interactions between different environmental and biological factors or the ecological heterogeneity within the group. It has been shown that two lineages of Alphaproteobacteria, Roseobacter and SAR11, present opposing seasonal patterns in Blanes Bay (Alonso-Sáez *et al.*, 2007a), with SAR11 dominating throughout the year, and Roseobacter being almost undetectable during the period of severe nutrient limitation. We have estimates of the proportion of each of these two groups of Alphaproteobacteria at the beginning of the 4 experiments (Fig. 4) and, as expected from the eutrophic nature of this planktonic system, we found that Roseobacter clearly dominate over SAR11 during the 4 sampling periods.

In our experiments, Alphaproteobacteria seemed to profit from the active growth of phytoplankton towards the end of the July and January experiments, likely as a result of nutrient regeneration. Allers *et al.* (2007) observed that the relative abundance of Roseobacter-like bacteria increased progressively during a mesocosm experiment after phosphate addition. They suggested that these bacteria benefit from phytoplankton growth. This observation agrees with microautorradiography studies showing that Roseobacter efficiently grow on, and take-up, glucose and amino acids (Alonso and Pernthaler, 2006; Alonso-Sáez and Gasol, 2007) or the algal osmolyite dimethylsulfoniopropionate (Vila *et al.*, 2004). Interestingly, Bacteroidetes and Alphaproteobacteria globally showed opposing short-time scale evolutions in our experiments (Fig. 2) which suggest an alternation of the two groups presumably linked

to the phytoplankton physiological state. We hypothesize that whereas Alphaproteobacteria appear to efficiently metabolise exudates from healthy growing phytoplankton, mainly composed of carbohydrates and/or aminoacids (Granum *et al.*, 2002), Bacteroidetes would essentially utilize more complex DOM (polymeric compounds, combined amino acids) derived from senescent phytoplankton (Kirchman, 2002).

The Gammaproteobacteria group only became considerably abundant at the end of the experiment conducted in January (Fig. 2), when phytoplankton biomass was lowest. Members of this phylogenetic group have been found to take clear advantage in dilution cultures (Fuchs *et al.,* 2000) and to be highly responsive to high concentrations of nutrients (Horňák *et al.*, 2006), or to manipulation (seawater handling or stirring, confinement, nutrient enrichment, Eilers *et al.,* 2000; Beardsley *et al.,* 2003). Allers *et al.* (2007) also showed a rapid increase in the relative abundance of Alteromonadaceae, an important member of the Gammaproteobacteria, after water enclosure in 200-L mesocosms. In contrast to all these studies, we did not observe an immediate response in Gammaproteobacteria to water confinement, neither in the mesocosms nor in the microcosms. The large volume of our mesocosms (approx. 3 m^3), together with the careful fill-up of the bags (see 'experimental procedures' section), could explain the absence of Gammaproteobacteria stimulation. However, even in the parallel microcosms of January we did not observe an immediate response of this group, which suggest that water manipulation (dilution, filtration, stirring) rather that confinement itself, could be the cause for Gammaproteobacteria selection in some of the published studies. The fact that the bloom of Gammaproteobacteria occurs only in January and exactly at the same time in micro- and mesocosms (after 3 days of enclosure), suggests a response to an external input of matter coming from the atmosphere. For example,

precipitation has been seen to be an efficient removal mechanism for water-soluble chemical substances in the atmosphere (Seitzinger *et al.,* 2003). This hypothesis is supported by meteorological data: whereas precipitation was nil from 18-28 January 2006, a total of 3.05 mm were measured during day 3 of the micro- and mecoscosm experiments (29 January 2006) (data from the station of the 'Instituto Nacional de Meteorología' in the Airport of Vigo). This precipitation would have been enough to wash out all the atmospheric material accumulated during the previous dry period. In fact, the sharp increase of Gammaproteobacteria at day 4 in January coincided with an increase in DOC concentration (from 70 μM at day 2 to 78 μM at day 4). Similarly, Alonso-Sáez *et al.* (2007a) also observed a punctual burst (50% of DAPI counts) of Gammaproteobacteria during an annual cycle in Blanes Bay. This agrees with the reported opportunistic behaviour of this phylogenetic group, and supports the use of this group's abundance, normally contributing < 10% to the bacterial community in coastal areas, as an indicator of punctual alterations in coastal waters, such as allochthonous inputs of matter (Allers *et al.,* 2007).

Links between bacterial assemblage structure, ecosystem function and environment.

The prokaryotic community involved in DOM decomposition in the ocean includes different groups of organisms with potentially different biogeochemical roles (Kirchman, 2004). In contrast to a number of ecosystem models which discriminate several phytoplankton functional groups with differential key roles in biogeochemical cycles, such as coccolithophores, diatoms, prasinophytes, or cyanobacteria (e.g. Le Queré *et al.*, 2005; Hood *et al.*, 2006; Litchman *et al.,* 2006), current ocean carbon cycle models either ignore bacteria or include them as a single homogeneous compartment.

The main difficulty for bacteria is the limited knowledge about the distribution patterns and the metabolic diversity of even the major phylogenetic groups (Dolan, 2005). Recent studies, however, have revealed temporal patterns in the distribution of different bacterial groups, which suggests the existence of low functional redundancy (Furhman *et al.,* 2006). These authors show that many bacterial groups are highly predictable from both abiotic and biotic variables. In our study we found a significant, non-spurious correlation between the relative abundances of major bacterial groups and bacterial carbon cycling. There are only few studies which have tried to link bacterial assemblage structure not only to environmental factors, but also to the rates of microbial carbon cycling. Some of these studies have failed to detect a significant effect of bacterial assemblage structure in bulk bacterial functions (Arrieta *et al.,* 2004; Langenheder *et al.,* 2006), although some of them did find a relation to more specific functions, such as some enzymatic activities (Langenheder *et al.,* 2006). In contrast, a similar significant correlation between bacterial composition and bacterial carbon cycling was reported by Alonso-Sáez *et al.* (2007b) along a productivity gradient in the NE Atlantic Ocean. It is important to note that some of these studies described qualitative changes in bacterial assemblage structure. Even if the number/type of bacterial groups does not change, a change in the proportion/abundance of each may have important consequences for the carbon cycling mediated by the bacterial assemblage and thus, for ecosystem functioning. We are aware that the proportions of three operational populations detected by FISH probes provide only a rather simplistic picture of bacterial community structure. However, the fact that changes in the relative abundances of each of these 3 major bacterial groups we considered are related to changes in environmental conditions and in bacterial carbon cycling, supports the emerging notion that members of these

major bacterial groups could actually share a function in the pelagic ecosystem and thus operate as meaningful ecological units (Kirchman *et al.,* 2005).

In summary, the bacterial assemblage in this coastal ecosystem displayed considerable short-time scale variability at a broad phylogenetic level, showing significant correlations both with environmental factors and with variables related to bacterial carbon cycling. The dominance of the group Bacteroidetes was related to high bacterial biomass and production. We observed a clear alternation between Alphaproteobacteria (which peaked during active phytoplankton growth) and Bacteroidetes (which increased their relative abundance during the decay phase of diatom blooms), likely reflecting contrasting phytoplankton derived-DOM preferences. The group Gammaproteobacteria appeared to rapidly respond to an episodic event, suggesting an opportunistic behaviour of members of this bacterial subclass.

Experimental procedures

Mesocosms of 1.5 m in diameter and 2 m deep were filled with seawater from the Ría de Vigo. The bags were filled from their bottom through a 200 μm mesh, in order to exclude mesozooplankton. Once filled, the bags were closed with a stopper. A total of 4 experiments were run under contrasting initial conditions: one in spring (March 2005), one in summer (July 2005), one in early autumn (September 2005) and one during winter (January 2006). Two (in March and July) or three (September and January) mesocosms were used for each experiment. The experiments covered 4 relevant periods of the seasonal cycle: spring bloom, summer stratification, autumn upwelling and winter mixing (Fig. 1). Each experiment lasted for 9 days and samples were taken every day during the first 5 days, and thereafter every 2 days. Samples from the mesocosms were collected with an integrated 1.5 m tube minimizing stirring, to

avoid resuspension from the bottom of the bags, and deposited into polycarbonate carboys that were brought back to the laboratory, less than 30' later. No nutrients were added to the mesocosms.

Dissolved inorganic nutrients. Water samples were collected in polyethylene bottles, directly from the carboys, and kept frozen (-20 ºC) until determination using standard segmented flow analysis with colorimetric procedures (Grashoff *et al.*, 1983).

Dissolved organic carbon and nitrogen (DOC). Samples were filtered under low N_2-flow pressure through pre-combusted GF/F filters (using an acid-clean filtration system), dispensed into glass pre-combusted ampoules, acidified to pH < 2 with phosphoric acid, heat-sealed and stored at 4 ºC until analysis. DOC was measured with a Shimadzu TOC-5000 organic carbon analyser (Álvarez-Salgado and Miller, 1998).

Particulate organic matter. Suspended organic matter (0.5-1.5 L of seawater filtered) was collected under low vacuum on pre-combusted GF/F filters. Filters were dried overnight and frozen (-20ºC) until analysis. Measurements of particulate organic carbon and nitrogen (POC and PON) were carried out with a Perkin Elmer 2400CHN analyser.

Chlorophyll-a concentration. 250 mL samples were sequentially filtered through 20, 2 and 0.2 μm polycarbonate filters. The filters were frozen and chlorophyll-a extracted in 90% acetone for 24 h at 4ºC. The fluorescence of the extracted chlorophyll-a was assessed with a Turner-TD-700 fluorometer previously calibrated with pure chlorophyll-a. The total concentration of chlorophyll-a was obtained by addition of the concentration in each of the three size classes.

Primary production. Rates of primary production (PP) were derived from the incorporation of ^{14}C-bicarbonate into particulate (PPP) and dissolved (DPP) fractions, under *in situ* light and temperature conditions, following the protocol of Marañón *et al.* (2004). The percentage of extracellular release is defined by the formula: DPP*100/(DPP+PPP).

Microbial community respiration. Rates of respiration (RESP) were obtained from *in vitro* changes in oxygen concentration during 24 h *in situ* incubation in the dark, using the Winkler method with an automated Metrohm 721 Net Titrino, adopting a potentiometric endpoint (Serret *et al.,* 1999).

Bacterial biomass. Bacterial standing stock was obtained from prokaryotic abundance (as derived from microscopic counts of DAPI-stained cells counts) and assuming a conservative conversion factor of 20 fg C cell-1 (Fukuda *et al.*, 1998).

Bacterial production. Rates of bacterial production (BP) were estimated from ^{3}H-Leucine incorporation following the protocol by Kirchman *et al.* (1985) but in eppendorf vials which were processed by centrifugation and TCA rinsing.

Bacterial community composition. 5 mL were fixed by adding 0.2-µm filtered paraformaldehyde (2% final conc.) and subsequently, the samples were stored at 4ºC in the dark for 12-18 h. Thereafter, each sample was filtered through a 0.2 µm polycarbonate filter (Millipore, GTTP, 25 mm filter diameter) supported by a cellulose nitrate filter (Millipore, HAWP, 0.45 µm), washed twice with Milli-Q water, dried and stored in a microfuge vial at -20ºC until further treatment in the laboratory.

Bacterial community composition changes were monitored using Fluorescence *In Situ* Hybridisation techniques with oligonucleotide probes specific for the domain Eubacteria (EUB338) (Amann, *et al.*, 1990), the *Alpha-* (ALF968) (Glöckner *et al.*, 1999) and Gammaproteobacteria (GAM42a) (Manz *et al.*, 1992) subclasses and the class Flavobacteria of phylum Bacteroidetes (CF319a) (Manz *et al.*, 1996). We also used a general probe targeting Betaproteobacteria (BET42a) (Manz *et al.*, 1992), but this group was very close to the detection limit (< 0.5 %), so these data are not included. The Eub antisense probe Non338 probe was used as negative control. Additionally, the relative abundance of the SAR11 and Roseobacter clusters was also analysed at the beginning of each experiment using the specific probes SAR11-441R (Morris *et al.*, 2002) and Ros537 (Eilers *et al.*, 2001).

Filters for CARD-FISH were embedded in low-gelling-point agarose and incubated with lysozyme (Pernthaler *et al.* 2002, Teira *et al.*, 2004). Filters were cut in sections and hybridized at 35 ºC with horseradish peroxidase (HRP)-labeled oligonucleotide probes during 2-4 hours. Tyramide-Alexa488 was used for signal amplification (30-40 minutes) following the protocol described in Teira *et al.* (2004). We used 55% of formamide for all probes excepting for ALF968 and SAR11-441R (45 % formamide). Cells were counter-stained with a DAPI-mix (5.5 parts of Citifluor [Citifluor, Ltd.], 1 part of Vectashield [Vector Laboratories, Inc.] and 0.5 parts of PBS with DAPI (final concentration 1 $\mu g\ mL^{-1}$).

The slides were examined under a Leica DMBL microscope equipped with a 100-W Hg-lamp and appropriate filter sets for DAPI and Alexa488. More than 800 DAPI-stained cells were counted per sample. For each microscope field, 2 different categories were enumerated: (i) total DAPI-stained cells, (ii) cells stained with the specific probe. Negative control counts (hybridization with HRP-Non338) averaged

0.5% and were always below 1.5% of DAPI-stained cells. The counting error, expressed as the percentage of standard error between replicates, was < 2% for DAPI counts and < 9% for FISH counts.

Additional microcosm experiment. In January 2006 we conducted an additional microcosm experiment. Two microcosms were run in parallel to the mesocosms and with the same original seawater. The microcosms consisted in 5L-PET bottles, with a wide opening. The microcosms were kept open to the atmosphere and refrigerated by circulating surface seawater. The microcosms were kept for 8 days, sampling every 24h, except at day 6, for bacterial composition analysis. The goal of this additional experiment was to compare the bacterial assemblage dynamics in micro- vs. mesocosms in order to test for potential enclosure effects.

Statistical analysis. A repeated measures ANOVA (RMANOVA) was conducted to asses time and experiment (sampling period) effects. All data fitted a normal distribution, however, even after log or arcsine data transformation, the homogeneity of covariance matrices failed for some datasets. For the latter case we applied the Huynh-Feldt adjustment to correct p values (Scheiner and Gurevitch, 1993). Multiple comparisons (Bonferroni test) with the estimated marginal means were used to interpret interactions between factors. The Pearson coefficient was used to analyse correlations between bacterial community composition and both environmental and carbon cycling variables. Principal component analysis (PCA) was used to reduce the complexity of multivariate data, creating new variables that explain most of the variability in the original data. The Mantel test (Mantel, 1967) was used to analyse the association between i) bacterial assemblage structure, ii) environmental factors and iii)

heterotrophic carbon cycling similarity matrices using Passage software (v1.1). The 3 similarity matrices (Pearson coefficient, N=24) included the following variables: i) the relative abundance of Alphaproteobacteria, Gammaproteobacteria and Bacteroidetes, ii) concentration of inorganic nutrients (nitrite, nitrate and silicate), dissolved organic carbon concentration, chlorophyll-a concentration, primary production and percentage of extracellular release, and iii) bacterial biomass, bacterial production and microbial respiration. A modification of the Mantel test was applied in order to calculate the partial correlation coefficient between matrix i) and ii), controlling the effect of matrix iii).

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Table 1. Pearson correlation coefficients between the relative abundance (expressed as % of total DAPI counts) of Alphaproteobacteria (%Alpha), Gammaproteobacteria (%Gamma), Bacteroidetes (%CFB) and bacterial and environmental variables. Chla, chlorophyll-a concentration; PP, primary production; PER, percentage of extracellular release; BB, bacterial biomass; BP, bacterial production; RESP, respiration; Temp, temperature; Sal, salinity; DOC, dissolved organic carbon; POC, particulate organic carbon; and PON, particulate organic nitrogen. Positive correlations are in bold. n.s, not significant, * $p<0.05$, ** $p<0.01$.

N=24-27	% Alpha	%Gamma	%CFB
BB	n.s	-0.466*	**0.671****
BP	n.s	n.s	**0.498****
RESP	n.s	-0.452*	n.s.
Chla	n.s.	-0.427*	n.s.
PP	n.s	-0.522*	n.s
PER	n.s	n.s	**0.459***
Temp	n.s	n.s	n.s
Sal	n.s	n.s	n.s
Ammonium	n.s	n.s	n.s
Nitrite	n.s	**0.400***	-0.532**
Nitrate	n.s	**0.407***	-0.432*
Phosphate	n.s	n.s	n.s
Silicate	n.s	**0.438***	n.s
DOC	n.s	n.s	**0.560****
POC	n.s	-0.526**	**0.419***
PON	n.s	-0.624**	n.s

Figure legends.

Figure 1. Typical annual variability in nitrate (black symbols) and chlorophyll-a (white symbols) concentration in the Ría de Vigo. Data represent mean (± standard error) values for the period 1987-1996 (modified after Nogueira *et al.,* 1997). Shadow areas indicate the 4 mesocosm experiments.

Figure 2. Time course changes in the mean relative abundance of Alphaproteobacteria (ALPHA), Gammaproteobacteria (GAMMA), and Bacteroidetes (CFB), expressed as percentage of total DAPI-stained cells; nitrate concentration, expressed in μM; and chlorophyll-a concentration, expressed in mg m^{-3}, in March (A), July (B), September (C) and January (D). The error bars represent the standard error from two replicates in March and July and three replicates in September and January.

Figure 3. Principal component analysis (PCA) of all the variables determined in this study, showing how each of the three major groups of bacteria (Alphaproteobacteria, alpha; Gammaproteobacteria, gamma; Bacteroidetes, CFB) cluster with different combinations of environmental and carbon-cycling related variables. For abbreviations see Table 1.

Figure 4. Relative abundance of SAR11 (cells positive with probe SAR11-441R) and Roseobacter (positive with probe Ros537) as detected by CARD-FISH in the Ría de Vigo at the beginning of the 4 experiments. Error bars represent counting errors.

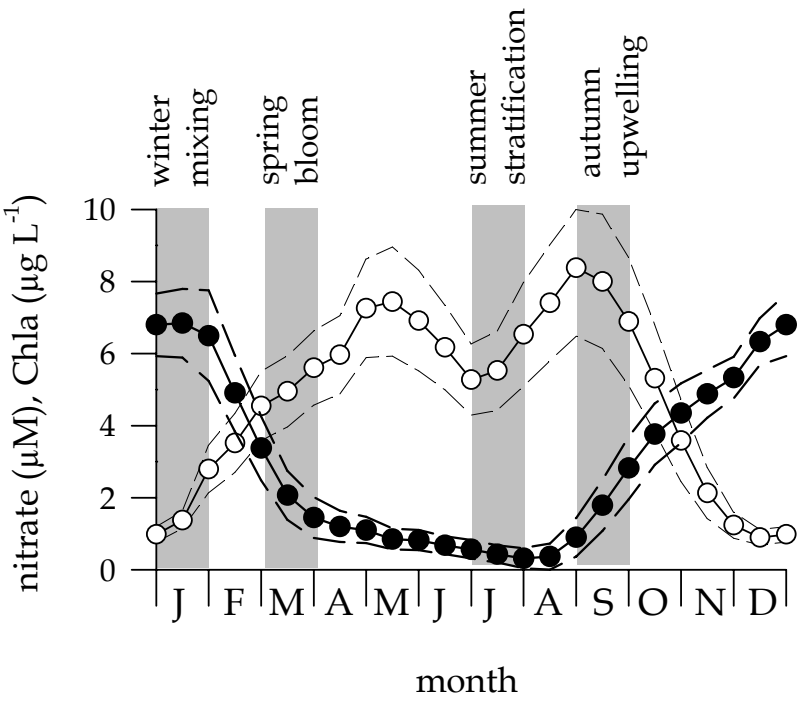
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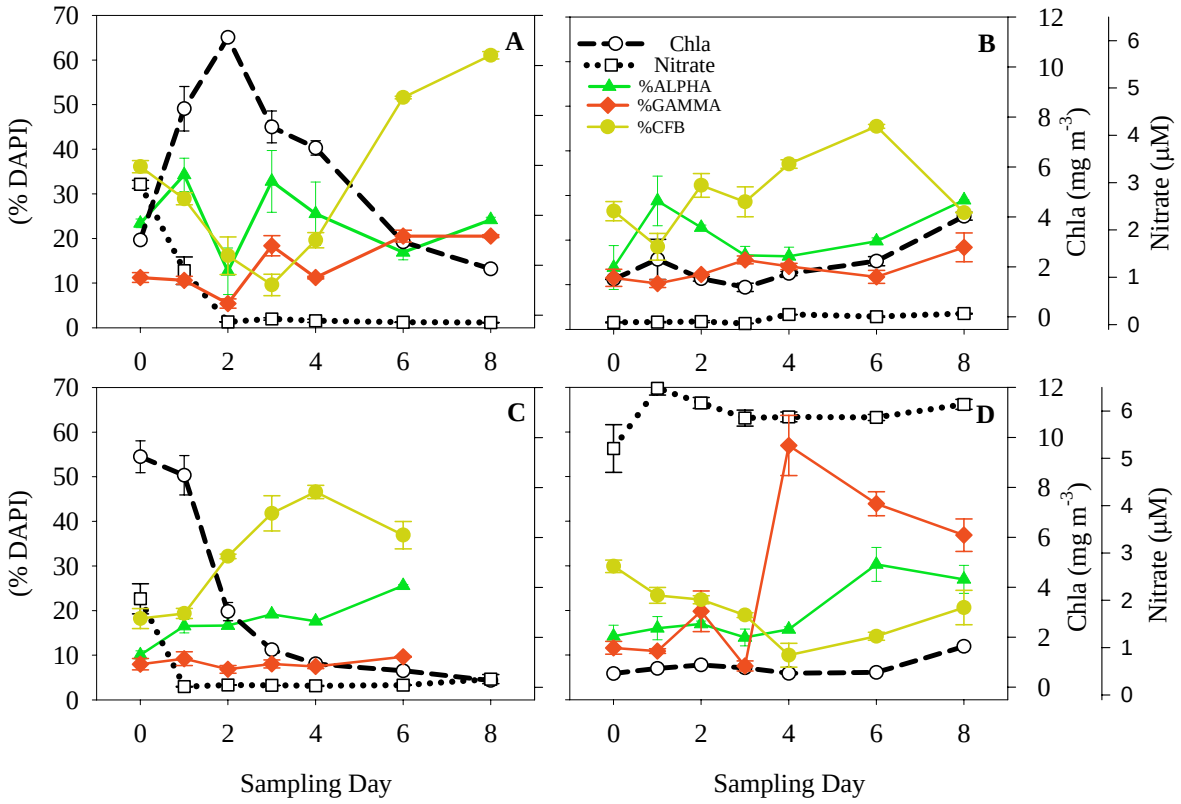
Figure 1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Sampling Day

Figure 2

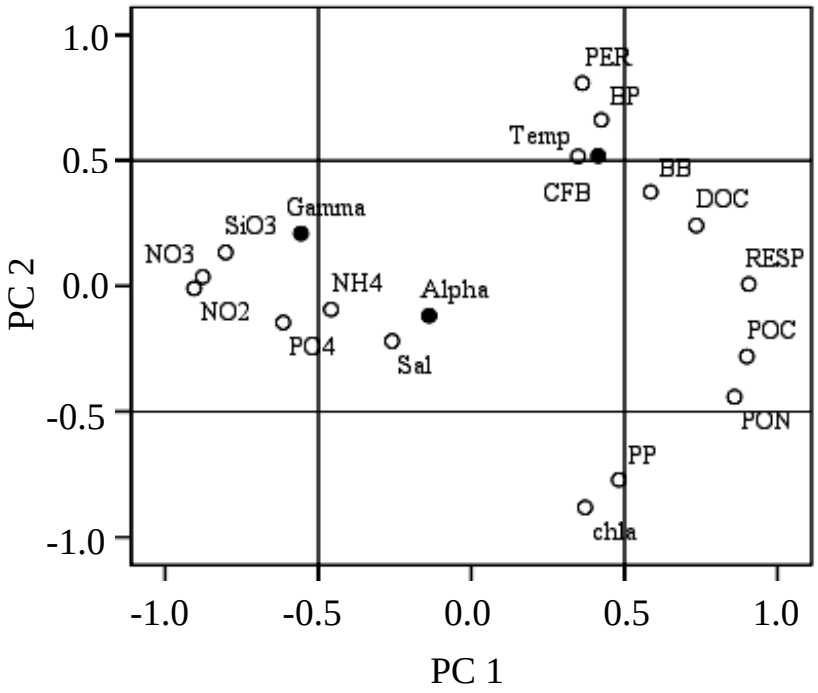
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Figure 3

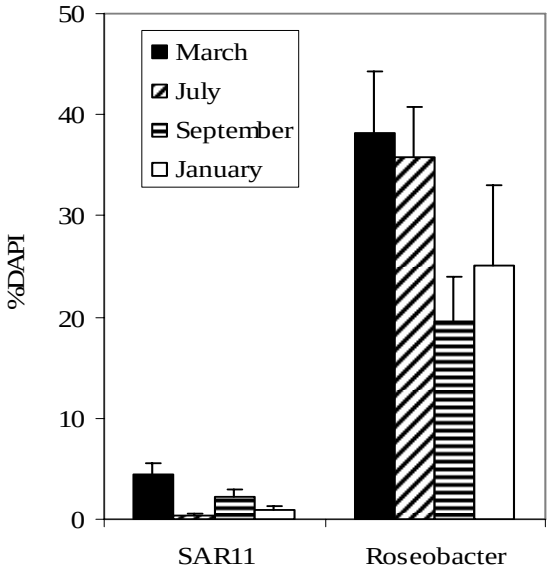
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Figure 4