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2 Linkages between bacterioplankton community composition, heterotrophic

3 carbon cycling and environmental conditions in a highly dynamic coastal

4 ecosystem.

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1 **Summary**

2 We used mesocosm experiments to study the bacterioplankton community in a highly

3 dynamic coastal ecosystem during four contrasting periods of the seasonal cycle: winter

4 mixing, spring phytoplankton bloom, summer stratification and autumn upwelling. A

5 correlation approach was used in order to measure the degree of coupling between the

6 dynamics of major bacterial groups, heterotrophic carbon cycling and environmental

7 factors. We used CARD-FISH to follow changes in the relative abundance of the most

8 abundant groups of bacteria (Alphaproteobacteria, Gammaproteobacteria, and

9 Bacteroidetes). Bacterial carbon flux-related variables included bacterial standing stock,

10 bacterial production and microbial respiration. The environmental factors included both,

11 biotic variables such as chlorophyll-a concentration, primary production, phytoplankton

12 extracellular release, and abiotic variables such as the concentration of dissolved

13 inorganic and organic nutrients. Rapid shifts in the dominant bacterial groups occurred

14 associated to environmental changes and bacterial bulk functions. An alternation

15 between Alphaproteobacteria and Bacteroidetes was observed associated to different

16 phytoplankton growth phases. The dominance of the group Bacteroidetes was related to

17 high bacterial biomass and production. We found a significant, non-spurious, linkage

18 between the relative abundances of major bacterial groups and bacterial carbon cycling.

19 Our results suggest that bacteria belonging to these major groups could actually share a

20 function in planktonic ecosystems.

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1 **Introduction**

2   Bacterioplankton play an important role in the flow of energy and nutrients

3 through plankton food webs, as a consequence of their great abundance, efficient

4 nutrient uptake and large growth potential (Azam *et al.,* 1983). A number of studies

5 have shown changes in bulk bacterial properties, as a response to biological or

6 environmental factors (see e.g. reviews by Ducklow, 2000; Williams, 2000; del Giorgio

7 and Cole, 2000). However, much less is known about how such factors may influence

8 bacterial community composition, and how potential shifts in bacterial community

9 structure may in turn influence microbial carbon cycling. Despite several studies have

10 shown temporal and spatial changes in bacterial community composition in freshwater

11 and marine environments (e.g. Rappé *et al.,* 2000; Dolan, 2005 and references therein),

12 even the distribution of the major phylogenetic groups of bacteria is still not well

13 understood. Particular attention has received the study of changes in bacterial

14 assemblage structure associated to phytoplankton blooms (Riemann *et al.,* 2000;

15 Fandino *et al.,* 2001; Larsen *et al.,* 2001; Pinhassi *et al.,* 2004). These studies suggest

16 that the phylum Bacteroidetes, also known as CFB (Cytophaga-Flavobacteria-

17 Bacteroides) might be especially relevant in the cycling of organic matter during algal

18 blooms. Unfortunately, most of these analyses provide qualitative information, as they

19 are mainly based on fingerprinting descriptions of the bacterioplankton assemblages.

20 Although the same number of phylotypes can be detected in two distinct samples

21 (samples that have the same richness), the relative abundance of each phylogenetic

22 group can be very different (thus showing different diversity), implying that not only

23 qualitative, but also quantitative information is needed in order to evaluate the links

24 between bacterial assemblage structure and ecosystem functioning. One of the major

25 advantages of fluorescence *in situ* hybridisation (FISH) is that it allows for

1   quantification of the actual abundance of a given phylogenetic group. This technique

2   has now been widely used for monitoring dynamics of different phylogenetic groups in

3   contrasting aquatic environments (e.g. Pernthaler *et al.,* 1998; Glöckner *et al.,* 1999;

4   Brinkmeyer *et al.,* 2003; Kirchman *et al.,* 2005; Comte *et al.,* 2006; Mary *et al.,* 2006).

5   A major limitation of FISH is the number and phylogenetic resolution of the probes

6   (Alonso-Sáez *et al.*, 2007a). Most of the studies work at a high phylogenetic level due

7   to the limited number of probes that can be used at a time, to rationalize counting effort.

8   The accuracy of hybridization depends on the specificity and coverage of the probes,

9   which must be re-evaluated frequently due to the rapidly increasing sequence

10   information in public databases. Most of the available probes targeting broad

11   phylogenetic groups, designed several years ago, show a far from 100% coverage, and

12   therefore it would be more appropriate to refer not to phylogenetic groups but to

13   operational groups or populations detected by particular probes.

14   Growing evidence indicates that some major bacterial groups, even at a high

15   phylogenetic level might operate as functional units (Kirchman *et al.,* 2005). The

16   identification of such ecological units would allow incorporating bacterial community

17   structure into quantitative models of carbon cycling. In support of this hypothesis are

18   several interesting recent findings: a) the very distinct uptake patterns of low molecular

19   weight dissolved organic matter (DOM) by different bacterial groups (Alonso-Sáez and

20   Gasol, 2007), b) the differential sensitivity of major bacterial groups to UVR (Alonso-

21   Sáez *et al.,* 2006), and c) the differential susceptibility of specific bacterial groups to

22   lytic viral infection (Bouvier and del Giorgio, 2007). On the other hand, some studies

23   detecting little temporal or spatial variability at broad phylogenetic levels, such as

24   subclass level, claimed the heterogeneity of these groups and the lack of a common

25   functionality within the group (Pernthaler *et al.,* 1998; Longnecker *et al.,* 2006; Mary *et*

1   *al.,* 2006; Pommier *et al.,* 2007). Therefore, the debate about the existence of functional

2   bacterial groups, and particularly at which phylogenetic level should we look for, is still

3   open.

4   In this context, the objective of our study was to analyse the linkages between

5   bacterial community composition at the level of major groups, environmental factors

6   and bacterially-driven ecosystem function, approaching this goal by statistically

7   correlating bacterial composition with both environmental conditions and bacterial

8   carbon cycling using a multivariate approach

9

10 **Results.**

11   We carried out four mesocosm experiments in a highly dynamic inshore

12 ecosystem during the most relevant periods of the seasonal cycle: winter mixing, spring

13 phytoplankton bloom, summer stratification, and autumn upwelling (Fig. 1). We

14 analysed changes in bacterial composition using CARD-FISH with several probes

15 targeting the most abundant groups of bacteria (Alphaproteobacteria,

16 Gammaproteobacteria and Bacteroidetes).

17

18 **Predominant environmental conditions.**

19   The initial environmental conditions are summarized in Teira *et al.* (2007). In

20 March, confinement in the mesocosms produced a phytoplankton bloom composed of

21 the diatoms *Lauderia annulata* and *Chaetoceros socialis* (M. Varela, pers. comm.)

22 grown at the expenses of the high initial nutrient levels. The summer (July)

23 phytoplankton community, however, was dominated by senescent diatoms (M. Varela,

24 pers. comm.) and dissolved inorganic nutrients were very low during summer

25 stratification In September, we sampled the decay of a diatom bloom, mainly composed

1 of several species of *Chaetoceros*, (M. Varela, pers. comm.), as a consequence of

2 nutrient exhaustion after the second day of the experiment. During winter mixing

3 (January), maximum concentrations of inorganic nutrients were recorded, accompanied

4 by extremely low levels of particulate matter (Teira *et al.,* 2007). The winter

5 phytoplankton community was dominated by flagellates (M. Varela, pers. comm.).

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7 Bacterial community dynamics.

8   In Figure 2 we have represented the temporal changes along a 9 day period in

9 the relative abundance of Alphaproteobacteria (positive with ALF968 probe),

10 Gammaproteobacteria (positive with GAM42a probe) and Bacteroidetes (positive with

11 CF319a probe) together with those in nitrate and chlorophyll-a concentration, for the 4

12 experiments. We have represented the mean value obtained from duplicate (in March

13 and July) and triplicate (September and January) mesocosms. The percentage of DAPI-

14 stained cells (referred to as total prokaryotic abundance) detected as Eubacteria (probe

15 EUB338) averaged 85% and was rather stable during the 4 experiments, ranging from

16 70 to 98%.

17   The relative contribution of the group Alphaproteobacteria to total prokaryotic

18 abundance (Fig. 2) ranged from values as low as 10% in September to 34% in March.

19 Repeated measures ANOVA results (RMANOVA test, $p < 0.01$) showed significant

20 differences between experiments. The relative contribution of this bacterial group was

21 significantly higher in March than in the other 3 periods (Bonferroni test, $p < 0.05$).

22 Taking into account the complete data set, Alphaproteobacteria contribution to

23 prokaryotic abundance significantly changed with time (RMANOVA test, $p < 0.05$).

24 However, there was a significant interaction Time x Experiment (RMANOVA test, p <

1   0.02), which means that temporal dynamics differed between experiments. Significant

2   temporal variability was observed only in March (Bonferroni test, $p < 0.05$, Fig. 2A).

3   The relative abundance of Gammaproteobacteria ranged between 5 %, and 58 %

4   at day 4 in January (Fig. 2). There were also significant differences between

5   experiments (RMANOVA test, $p = 0.001$), with significantly higher contribution of this

6   group to prokaryotic abundance in January than in the other 3 experiments (Bonferroni

7   test, $p < 0.005$). The relative abundance of this group was rather stable in March, July

8   and September, but significant temporal variability was observed in January (Bonferroni

9   test, $p = 0.005$), when a burst occurred between days 3 and 4 (Fig. 2D).

10   The Bacteroidetes group showed a great degree of temporal variability, clearly

11   associated with changes in the concentration of chlorophyll-a in March and September

12   (Fig. 2A & 2C). Its relative abundance varied from 10% to 61%, and was, on average,

13   the most abundant group in this planktonic system. There were significant differences

14   between experiments (RMANOVA test, $p<0.001$). These bacteria were significantly

15   more abundant in March, July and September than in January (Bonferroni test, $p <$

16   0.05), and showed significant temporal variability in March and September (Bonferroni

17   tests, $p < 0.05$, Fig. 2A & 2 C).

18

19   **Relationship between major bacterial groups, environmental factors, and bacterial**

20   **carbon cycling.**

21   Using the entire dataset, we conducted a correlation analysis in order to identify

22   any relevant physicochemical or biological variable that could explain the observed

23   variability in bacterial community composition (Table 1). The three bacterial groups

24   showed different correlations to environmental and carbon-flux related variables. None

25   of them correlated with either temperature or salinity. Regarding inorganic nutrients,

1 none of the groups correlated with either ammonium or phosphate.

2 Gammaproteobacteria and Bacteroidetes showed opposing correlations with both nitrite

3 and nitrate concentrations. The first correlated positively, whereas the later correlated

4 negatively. The correlations with dissolved and particulate organic matter also differed

5 between these three groups of bacteria. Whereas Alphaproteobacteria did not show any

6 significant correlation, Bacteroidetes correlated positively with dissolved and particulate

7 organic carbon (DOC and POC). Gammaproteobacteria correlated negatively with

8 particulate organic carbon and nitrogen (POC and PON).

9   The correlation between bacterial groups and biological variables revealed

10 interesting relationships. The most striking trait is the predominance of negative

11 correlations between Gammaproteobacteria and autotrophic variables (chlorophyll-a

12 concentration and primary production) (Table 1), and positive correlations between

13 Bacteroidetes and heterotrophic variables (bacterial biomass, bacterial production, and

14 extracellular release, a process that can be considered to be the link between

15 phytoplankton and bacteria). The Alphaproteobacteria did not correlate with any

16 biological variable. Between 18 and 45% of the observed variability in the different

17 bacterial components was related to each of the significant variables.

18   We further conducted a principal component analysis in order to reduce the

19 amount of studied variables to a few components explaining most of the observed

20 variability (Fig. 3). The three first principal components explained 69% of the

21 variability in the original data. The first principal component, which explained 39% of

22 the variability, described an environment characterized by low inorganic nutrient

23 concentrations and high dissolved and particulate organic carbon concentrations, which

24 sustained high levels of bacterial biomass and microbial community respiration. In this

25 situation, the abundance of Gammaproteobacteria was very low. The second principal

1   component, which explained 19% of the variability, refer to a situation where

2   temperature was high, phytoplankton biomass and primary production were very low,

3   and the percentage of extracellular release and bacterial production, were very high. In

4   such scenario, the group Bacteroidetes dominated the bacterial assemblage.

5   The relationship between bacterial assemblage structure, bacterial carbon

6   cycling (a bacterially-driven ecosystem function) and the environmental factors was

7   assessed by means of correlation of the three similarity matrices (for details see

8   'experimental procedures' section on Mantel test). We found a significant correlation

9   between bacterial assemblage structure and both carbon cycling (r = 0.371, p = 0.031)

10   and environmental factors (r = 0.373, p = 0.017), and also between ecosystem function

11   and environmental factors (r = 0.333, p = 0.018). We further explored if the significant

12   correlation obtained between bacterial composition and bacterial carbon cycling was

13   spurious, just as a result of the significant correlations between environment and both

14   bacterial composition and carbon cycling. The partial correlation between bacterial

15   assemblage and carbon cycling, controlling for the effects of the environment, was also

16   significant (r = 0.2815, p = 0.046).

17

18   **Bacterial community dynamics in microcosm versus mesocosms experiments.**

19   During the mesocosm experiment conducted in January 2006 we also followed

20   changes in bacterial composition in a parallel microcosm experiment (for details see

21   'experimental procedures' section) in order to test for potential enclosure effects on

22   bacterial assemblage structure. While the mesocosms investigated held ~3000 L, the

23   microcosms were of 5 L. If enclosure effects are significantly altering our results, we

24   should see significant differences in parallel experiments done in 5 vs. 3000 L

25   containers. The relative abundances of Alpha-, Gammaproteobacteria and Bacteroidetes

1   in the mesocosm vs. the microcosm experiment were strikingly similar (Pearson's r =

2   0.91, 0.98, and 0.92 respectively, with N = 6, p < 0.02). The relative contribution of

3   each of the major bacterial groups did follow the same trends in both types of reservoirs

4   (see supplementary figure on the web appendix). The dramatic increase in the relative

5   contribution of Gammaproteobacteria and the decrease in the contribution of

6   Bacteroidetes, did occur at the same time in the meso- and the microcosms.

7

8   **Discussion.**

9

10   **Temporal variability.**

11   The Ría de Vigo is a highly hydrodynamic system, as reflected in the contrasting

12   hydrographic conditions typically found over an annual cycle (Nogueira *et al.*, 1997).

13   This dynamism has been shown to affect biogeochemical cycling, as well as

14   phytoplankton size-structure and composition (Tilstone *et al.,* 1999; Álvarez-Salgado *et*

15   *al.*, 2001; Cermeño *et al.,* 2006). From the evident annual pattern of chlorophyll-a

16   concentration (Fig. 1), as well as from the well-established succession pattern of

17   phytoplankton species (Nogueira and Figueiras, 2005) we would expect to find

18   important bacterial community composition changes. Against these expectations, a

19   recent study by Mary *et al.* (2006), using FISH, showed that community structure at a

20   high taxonomic level did not change dramatically over an annual cycle in the western

21   English Channel. Schauer *et al.* (2003), using DGGE, also found a relative stability of

22   broad phylogenetic groups in Blanes Bay, in the NW Mediterranean. Most of these

23   studies, however, did find variability at lower phylogenetic levels. By contrast, other

24   recent studies did find both temporal and spatial variability in the distribution of major

25   phylogenetic groups of bacteria using either FISH (Kirchman *et al.,* 2005, Alderkamp *et*

1   *al.*, 2006; Alonso-Sáez *et al.*, 2007a; 2007b; Garcés *et al.*, 2007) or PCR-based

2   techniques (Morris *et al.*, 2005; Fuhrman *et al.*, 2006). We only analyzed 4 different

3   sampling periods, and we also checked only for three major groups of bacteria, and even

4   considering these limitations, we did find significant differences in bacterial community

5   composition. The three major groups showed different dynamics, and also different

6   correlations with environmental variables and carbon-flux related variables.

7

8   **Major bacterial groups and environmental variables.**

9   In a recent study, Frette *et al.* (2004) determined the functional characteristics of

10   a considerable number of bacterial isolates from one freshwater and 3 marine locations.

11   They found that 95% of the isolates were able to utilize dissolved free amino acids

12   (DFAAs) and protein as their sole N source, and that only a few were capable to utilize

13   ammonium, which had long been considered to be an important source of N for

14   bacterioplankton (Kroer *et al.,* 1994). We did not find any significant correlation

15   between any of the bacterial groups and ammonium concentration (Table 1), whereas

16   we found significant positive correlations between Gammaproteobacteria and nitrite,

17   nitrate and silicate, and negative correlations between Bacteroidetes and the

18   concentrations of nitrite and nitrate. Wells and Deming (2003) also found negative

19   correlations between Bacteroidetes and inorganic nutrient concentrations.

20   In our study, Bacteroidetes appear to be relatively more abundant when the

21   concentration of inorganic nutrients is low and the concentration of dissolved organic

22   carbon is high (Table 1, Fig. 3). The relative abundance of this group also shows a

23   positive correlation with the percentage of extracellular release (PER), something that

24   supports the hypothesis of the important role that this phylogenetic group is considered

25   to play in the decomposition of recently produced phytoplankton-derived dissolved

1   organic matter (DOM) (Kirchman, 2002). It is important to mention that the FISH probe

2   generally used for Bacteroidetes (CF319a) targets mainly the class Flavobacteria

3   (formely known as order Cytophagales), which comprises the most abundant class of

4   planktic marine Bacteroidetes (Alonso *et al.,* 2007). Several studies, based on DGGE,

5   cloning or other PCR analyses, have shown that Bacteroidetes species dominate on

6   marine aggregates (DeLong *et al.,* 1993; Rath *et al.,* 1998), and are involved in the

7   degradation of complex macromolecules (Shewan and McMeekin, 1983). Riemann *et*

8   *al.* (2000) observed rapid colonization of particulate matter after a mesocosm-simulated

9   diatom bloom by specialized Alphaproteobacteria and Cytophagales-related phylotypes.

10   Other studies also showed that members of the Flavobacteria class are exceptionally

11   responsive to induced phytoplankton blooms (Pinhassi *et al.,* 2004), and that

12   *Flavobacteria* are involved in the initial colonization of diatom detritus (Abell *et al.,*

13   2005). A recent analysis of the whole genome of a marine Bacteroidetes representative

14   affiliated to the class Flavobacteria revealed adaptations to the degradation of polymeric

15   organic matter (Bauer *et al.*, 2006). Our FISH data revealed that the Bacteroidetes group

16   becomes highly dominant during the decay of diatom blooms (March and September

17   experiments, Fig. 2A & C). Alderkamp *et al.* (2006) also showed that Bacteroidetes

18   comprised up to 63% of the prokaryotic community at the senescent stage of a

19   *Phaeocysitis globosa* spring bloom. Despite the lack of an overall significant correlation

20   with chlorophyll-a concentration, there is a clear inverse pattern between Bacteroidetes

21   and the chlorophyll-a concentration if data from January (the only experiment not

22   dominated by diatoms) are excluded ($r = -0.65$, $p = 0.002$). Neither Alpha- nor

23   Gammaproteobacteria responded in this straight manner to the decay of the diatom

24   blooms.

1   Alphaproteobacteria also showed considerable temporal dynamics in the 4

2 experiments. This bacterial group has been suggested to be active colonizers of particles

3 and good competitors under low nutrient conditions (Riemann *et al.,* 2000; Pinhassi and

4 Berman, 2003). However, the observed lack of correlation between this group and any

5 of the considered variables (Table 1) likely reflects complex interactions between

6 different environmental and biological factors or the ecological heterogeneity within the

7 group. It has been shown that two lineages of Alphaproteobacteria, Roseobacter and

8 SAR11, present opposing seasonal patterns in Blanes Bay (Alonso-Sáez *et al.,* 2007a),

9 with SAR11 dominating throughout the year, and Roseobacter being almost

10 undetectable during the period of severe nutrient limitation. We have estimates of the

11 proportion of each of these two groups of Alphaproteobacteria at the beginning of the 4

12 experiments (Fig. 4) and, as expected from the eutrophic nature of this planktonic

13 system, we found that Roseobacter clearly dominate over SAR11 during the 4 sampling

14 periods.

15   In our experiments, Alphaproteobacteria seemed to profit from the active growth

16 of phytoplankton towards the end of the July and January experiments, likely as a result

17 of nutrient regeneration. Allers *et al.* (2007) observed that the relative abundance of

18 Roseobacter-like bacteria increased progressively during a mesocosm experiment after

19 phosphate addition. They suggested that these bacteria benefit from phytoplankton

20 growth. This observation agrees with microautorradiography studies showing that

21 Roseobacter efficiently grow on, and take-up, glucose and amino acids (Alonso and

22 Pernthaler, 2006; Alonso-Sáez and Gasol, 2007) or the algal osmolyite

23 dimethylsulfoniopropionate (Vila *et al.,* 2004). Interestingly, Bacteroidetes and

24 Alphaproteobacteria globally showed opposing short-time scale evolutions in our

25 experiments (Fig. 2) which suggest an alternation of the two groups presumably linked

1 to the phytoplankton physiological state. We hypothesize that whereas

2 Alphaproteobacteria appear to efficiently metabolise exudates from healthy growing

3 phytoplankton, mainly composed of carbohydrates and/or aminoacids (Granum *et al.*,

4 2002), Bacteroidetes would essentially utilize more complex DOM (polymeric

5 compounds, combined amino acids) derived from senescent phytoplankton (Kirchman,

6 2002).

7   The Gammaproteobacteria group only became considerably abundant at the end

8 of the experiment conducted in January (Fig. 2), when phytoplankton biomass was

9 lowest. Members of this phylogenetic group have been found to take clear advantage in

10 dilution cultures (Fuchs *et al.*, 2000) and to be highly responsive to high concentrations

11 of nutrients (Horňák *et al.*, 2006), or to manipulation (seawater handling or stirring,

12 confinement, nutrient enrichment, Eilers *et al.*, 2000; Beardsley *et al.*, 2003). Allers *et*

13 *al.* (2007) also showed a rapid increase in the relative abundance of Alteromonadaceae,

14 an important member of the Gammaproteobacteria, after water enclosure in 200-L

15 mesocosms. In contrast to all these studies, we did not observe an immediate response

16 in Gammaproteobacteria to water confinement, neither in the mesocosms nor in the

17 microcosms. The large volume of our mesocosms (approx. 3 m^3), together with the

18 careful fill-up of the bags (see 'experimental procedures' section), could explain the

19 absence of Gammaproteobacteria stimulation. However, even in the parallel

20 microcosms of January we did not observe an immediate response of this group, which

21 suggest that water manipulation (dilution, filtration, stirring) rather that confinement

22 itself, could be the cause for Gammaproteobacteria selection in some of the published

23 studies. The fact that the bloom of Gammaproteobacteria occurs only in January and

24 exactly at the same time in micro- and mesocosms (after 3 days of enclosure), suggests

25 a response to an external input of matter coming from the atmosphere. For example,

1 precipitation has been seen to be an efficient removal mechanism for water-soluble

2 chemical substances in the atmosphere (Seitzinger *et al.,* 2003). This hypothesis is

3 supported by meteorological data: whereas precipitation was nil from 18-28 January

4 2006, a total of 3.05 mm were measured during day 3 of the micro- and mecoscosm

5 experiments (29 January 2006) (data from the station of the 'Instituto Nacional de

6 Meteorología' in the Airport of Vigo). This precipitation would have been enough to

7 wash out all the atmospheric material accumulated during the previous dry period. In

8 fact, the sharp increase of Gammaproteobacteria at day 4 in January coincided with an

9 increase in DOC concentration (from 70 µM at day 2 to 78 µM at day 4). Similarly,

10 Alonso-Sáez *et al.* (2007a) also observed a punctual burst (50% of DAPI counts) of

11 Gammaproteobacteria during an annual cycle in Blanes Bay. This agrees with the

12 reported opportunistic behaviour of this phylogenetic group, and supports the use of this

13 group's abundance, normally contributing < 10% to the bacterial community in coastal

14 areas, as an indicator of punctual alterations in coastal waters, such as allochthonous

15 inputs of matter (Allers *et al.,* 2007).

16

17 **Links between bacterial assemblage structure, ecosystem function and**

18 **environment.**

19   The prokaryotic community involved in DOM decomposition in the ocean

20 includes different groups of organisms with potentially different biogeochemical roles

21 (Kirchman, 2004). In contrast to a number of ecosystem models which discriminate

22 several phytoplankton functional groups with differential key roles in biogeochemical

23 cycles, such as coccolithophores, diatoms, prasinophytes, or cyanobacteria (e.g. Le

24 Queré *et al.*, 2005; Hood *et al.*, 2006; Litchman *et al.,* 2006), current ocean carbon cycle

25 models either ignore bacteria or include them as a single homogeneous compartment.

1   The main difficulty for bacteria is the limited knowledge about the distribution patterns

2   and the metabolic diversity of even the major phylogenetic groups (Dolan, 2005).

3   Recent studies, however, have revealed temporal patterns in the distribution of different

4   bacterial groups, which suggests the existence of low functional redundancy (Furhman

5   *et al.,* 2006). These authors show that many bacterial groups are highly predictable from

6   both abiotic and biotic variables. In our study we found a significant, non-spurious

7   correlation between the relative abundances of major bacterial groups and bacterial

8   carbon cycling. There are only few studies which have tried to link bacterial assemblage

9   structure not only to environmental factors, but also to the rates of microbial carbon

10   cycling. Some of these studies have failed to detect a significant effect of bacterial

11   assemblage structure in bulk bacterial functions (Arrieta *et al.,* 2004; Langenheder *et*

12   *al.,* 2006), although some of them did find a relation to more specific functions, such as

13   some enzymatic activities (Langenheder *et al.,* 2006). In contrast, a similar significant

14   correlation between bacterial composition and bacterial carbon cycling was reported by

15   Alonso-Sáez *et al.* (2007b) along a productivity gradient in the NE Atlantic Ocean. It is

16   important to note that some of these studies described qualitative changes in bacterial

17   assemblage structure. Even if the number/type of bacterial groups does not change, a

18   change in the proportion/abundance of each may have important consequences for the

19   carbon cycling mediated by the bacterial assemblage and thus, for ecosystem

20   functioning. We are aware that the proportions of three operational populations detected

21   by FISH probes provide only a rather simplistic picture of bacterial community

22   structure. However, the fact that changes in the relative abundances of each of these 3

23   major bacterial groups we considered are related to changes in environmental conditions

24   and in bacterial carbon cycling, supports the emerging notion that members of these

1   major bacterial groups could actually share a function in the pelagic ecosystem and thus

2   operate as meaningful ecological units (Kirchman *et al.,* 2005).

3   In summary, the bacterial assemblage in this coastal ecosystem displayed

4   considerable short-time scale variability at a broad phylogenetic level, showing

5   significant correlations both with environmental factors and with variables related to

6   bacterial carbon cycling. The dominance of the group Bacteroidetes was related to high

7   bacterial biomass and production. We observed a clear alternation between

8   Alphaproteobacteria (which peaked during active phytoplankton growth) and

9   Bacteroidetes (which increased their relative abundance during the decay phase of

10   diatom blooms), likely reflecting contrasting phytoplankton derived-DOM preferences.

11   The group Gammaproteobacteria appeared to rapidly respond to an episodic event,

12   suggesting an opportunistic behaviour of members of this bacterial subclass.

13

14   **Experimental procedures**

15   Mesocosms of 1.5 m in diameter and 2 m deep were filled with seawater from

16   the Ría de Vigo. The bags were filled from their bottom through a 200 μm mesh, in

17   order to exclude mesozooplankton. Once filled, the bags were closed with a stopper. A

18   total of 4 experiments were run under contrasting initial conditions: one in spring

19   (March 2005), one in summer (July 2005), one in early autumn (September 2005) and

20   one during winter (January 2006). Two (in March and July) or three (September and

21   January) mesocosms were used for each experiment. The experiments covered 4

22   relevant periods of the seasonal cycle: spring bloom, summer stratification, autumn

23   upwelling and winter mixing (Fig. 1). Each experiment lasted for 9 days and samples

24   were taken every day during the first 5 days, and thereafter every 2 days. Samples from

25   the mesocosms were collected with an integrated 1.5 m tube minimizing stirring, to

1 avoid resuspension from the bottom of the bags, and deposited into polycarbonate

2 carboys that were brought back to the laboratory, less than 30' later. No nutrients were

3 added to the mesocosms.

4

5 **Dissolved inorganic nutrients.** Water samples were collected in polyethylene bottles,

6 directly from the carboys, and kept frozen (-20 ºC) until determination using standard

7 segmented flow analysis with colorimetric procedures (Grashoff *et al.*, 1983).

8

9 **Dissolved organic carbon and nitrogen (DOC).** Samples were filtered under low N_2-

10 flow pressure through pre-combusted GF/F filters (using an acid-clean filtration

11 system), dispensed into glass pre-combusted ampoules, acidified to pH < 2 with

12 phosphoric acid, heat-sealed and stored at 4 ºC until analysis. DOC was measured with

13 a Shimadzu TOC-5000 organic carbon analyser (Álvarez-Salgado and Miller, 1998).

14

15 **Particulate organic matter.** Suspended organic matter (0.5-1.5 L of seawater filtered)

16 was collected under low vacuum on pre-combusted GF/F filters. Filters were dried

17 overnight and frozen (-20ºC) until analysis. Measurements of particulate organic carbon

18 and nitrogen (POC and PON) were carried out with a Perkin Elmer 2400CHN analyser.

19

20 **Chlorophyll-a concentration.** 250 mL samples were sequentially filtered through 20, 2

21 and 0.2 μm polycarbonate filters. The filters were frozen and chlorophyll-a extracted in

22 90% acetone for 24 h at 4ºC. The fluorescence of the extracted chlorophyll-a was

23 assessed with a Turner-TD-700 fluorometer previously calibrated with pure

24 chlorophyll-a. The total concentration of chlorophyll-a was obtained by addition of the

25 concentration in each of the three size classes.

1

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.

1   Bacterial community composition changes were monitored using Fluorescence

2   *In Situ* Hybridisation techniques with oligonucleotide probes specific for the domain

3   Eubacteria (EUB338) (Amann, *et al.*, 1990), the *Alpha-* (ALF968) (Glöckner *et al.*,

4   1999) and Gammaproteobacteria (GAM42a) (Manz *et al.*, 1992) subclasses and the

5   class Flavobacteria of phylum Bacteroidetes (CF319a) (Manz *et al.*, 1996). We also

6   used a general probe targeting Betaproteobacteria (BET42a) (Manz *et al.*, 1992), but

7   this group was very close to the detection limit (< 0.5 %), so these data are not included.

8   The Eub antisense probe Non338 probe was used as negative control. Additionally, the

9   relative abundance of the SAR11 and Roseobacter clusters was also analysed at the

10   beginning of each experiment using the specific probes SAR11-441R (Morris *et al.*,

11   2002) and Ros537 (Eilers *et al.*, 2001).

12   Filters for CARD-FISH were embedded in low-gelling-point agarose and

13   incubated with lysozyme (Pernthaler *et al.* 2002, Teira *et al.*, 2004). Filters were cut in

14   sections and hybridized at 35 ºC with horseradish peroxidase (HRP)-labeled

15   oligonucleotide probes during 2-4 hours. Tyramide-Alexa488 was used for signal

16   amplification (30-40 minutes) following the protocol described in Teira *et al.* (2004).

17   We used 55% of formamide for all probes excepting for ALF968 and SAR11-441R (45

18   % formamide). Cells were counter-stained with a DAPI-mix (5.5 parts of Citifluor

19   [Citifluor, Ltd.], 1 part of Vectashield [Vector Laboratories, Inc.] and 0.5 parts of PBS

20   with DAPI (final concentration 1 µg mL^{-1}).

21   The slides were examined under a Leica DMBL microscope equipped with a

22   100-W Hg-lamp and appropriate filter sets for DAPI and Alexa488. More than 800

23   DAPI-stained cells were counted per sample. For each microscope field, 2 different

24   categories were enumerated: (i) total DAPI-stained cells, (ii) cells stained with the

25   specific probe. Negative control counts (hybridization with HRP-Non338) averaged

1 0.5% and were always below 1.5% of DAPI-stained cells. The counting error, expressed

2 as the percentage of standard error between replicates, was < 2% for DAPI counts and <

3 9% for FISH counts.

4

5 **Additional microcosm experiment.** In January 2006 we conducted an additional

6 microcosm experiment. Two microcosms were run in parallel to the mesocosms and

7 with the same original seawater. The microcosms consisted in 5L-PET bottles, with a

8 wide opening. The microcosms were kept open to the atmosphere and refrigerated by

9 circulating surface seawater. The microcosms were kept for 8 days, sampling every 24h,

10 except at day 6, for bacterial composition analysis. The goal of this additional

11 experiment was to compare the bacterial assemblage dynamics in micro- vs. mesocosms

12 in order to test for potential enclosure effects.

13

14 **Statistical analysis.** A repeated measures ANOVA (RMANOVA) was conducted to

15 asses time and experiment (sampling period) effects. All data fitted a normal

16 distribution, however, even after log or arcsine data transformation, the homogeneity of

17 covariance matrices failed for some datasets. For the latter case we applied the Huynh-

18 Feldt adjustment to correct p values (Scheiner and Gurevitch, 1993). Multiple

19 comparisons (Bonferroni test) with the estimated marginal means were used to interpret

20 interactions between factors. The Pearson coefficient was used to analyse correlations

21 between bacterial community composition and both environmental and carbon cycling

22 variables. Principal component analysis (PCA) was used to reduce the complexity of

23 multivariate data, creating new variables that explain most of the variability in the

24 original data. The Mantel test (Mantel, 1967) was used to analyse the association

25 between i) bacterial assemblage structure, ii) environmental factors and iii)

1   heterotrophic carbon cycling similarity matrices using Passage software (v1.1). The 3

2   similarity matrices (Pearson coefficient, N=24) included the following variables: i) the

3   relative abundance of Alphaproteobacteria, Gammaproteobacteria and Bacteroidetes, ii)

4   concentration of inorganic nutrients (nitrite, nitrate and silicate), dissolved organic

5   carbon concentration, chlorophyll-a concentration, primary production and percentage

6   of extracellular release, and iii) bacterial biomass, bacterial production and microbial

7   respiration. A modification of the Mantel test was applied in order to calculate the

8   partial correlation coefficient between matrix i) and ii), controlling the effect of matrix

9   iii).

10

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1   Table 1. Pearson correlation coefficients between the relative abundance (expressed as

2   % of total DAPI counts) of Alphaproteobacteria (%Alpha), Gammaproteobacteria

3   (%Gamma), Bacteroidetes (%CFB) and bacterial and environmental variables. Chla,

4   chlorophyll-a concentration; PP, primary production; PER, percentage of extracellular

5   release; BB, bacterial biomass; BP, bacterial production; RESP, respiration; Temp,

6   temperature; Sal, salinity; DOC, dissolved organic carbon; POC, particulate organic

7   carbon; and PON, particulate organic nitrogen. Positive correlations are in bold. n.s, not

8   significant, * p<0.05, ** p<0.01.

9

10

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

1   **Figure legends.**

2   Figure 1. Typical annual variability in nitrate (black symbols) and chlorophyll-a (white

3   symbols) concentration in the Ría de Vigo. Data represent mean (± standard error)

4   values for the period 1987-1996 (modified after Nogueira *et al.,* 1997). Shadow areas

5   indicate the 4 mesocosm experiments.

6   Figure 2. Time course changes in the mean relative abundance of Alphaproteobacteria

7   (ALPHA), Gammaproteobacteria (GAMMA), and Bacteroidetes (CFB), expressed as

8   percentage of total DAPI-stained cells; nitrate concentration, expressed in μM; and

9   chlorophyll-a concentration, expressed in mg m^{-3}, in March (A), July (B), September

10   (C) and January (D). The error bars represent the standard error from two replicates in

11   March and July and three replicates in September and January.

12   Figure 3. Principal component analysis (PCA) of all the variables determined in this

13   study, showing how each of the three major groups of bacteria (Alphaproteobacteria,

14   alpha; Gammaproteobacteria, gamma; Bacteroidetes, CFB) cluster with different

15   combinations of environmental and carbon-cycling related variables. For abbreviations

16   see Table 1.

17   Figure 4. Relative abundance of SAR11 (cells positive with probe SAR11-441R) and

18   Roseobacter (positive with probe Ros537) as detected by CARD-FISH in the Ría de

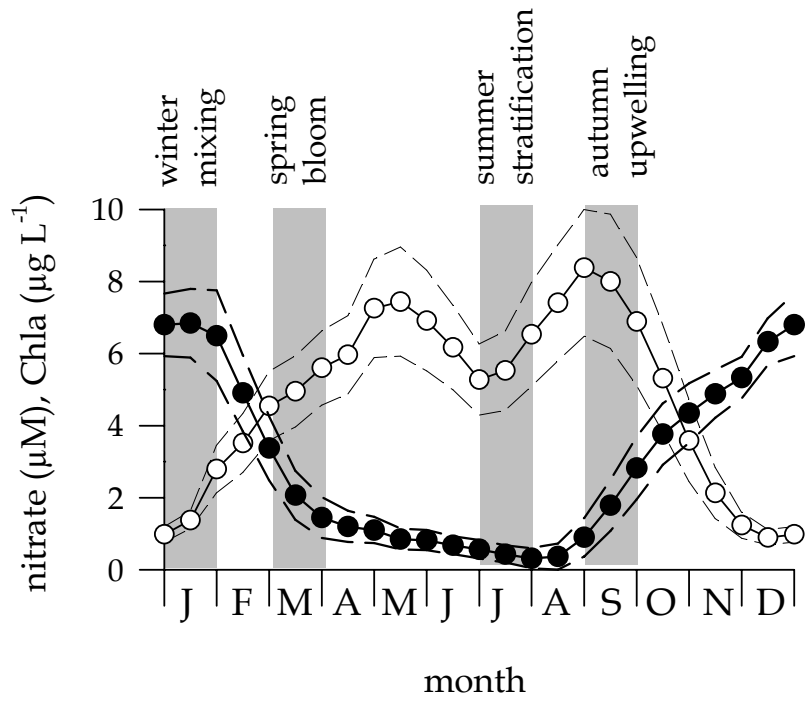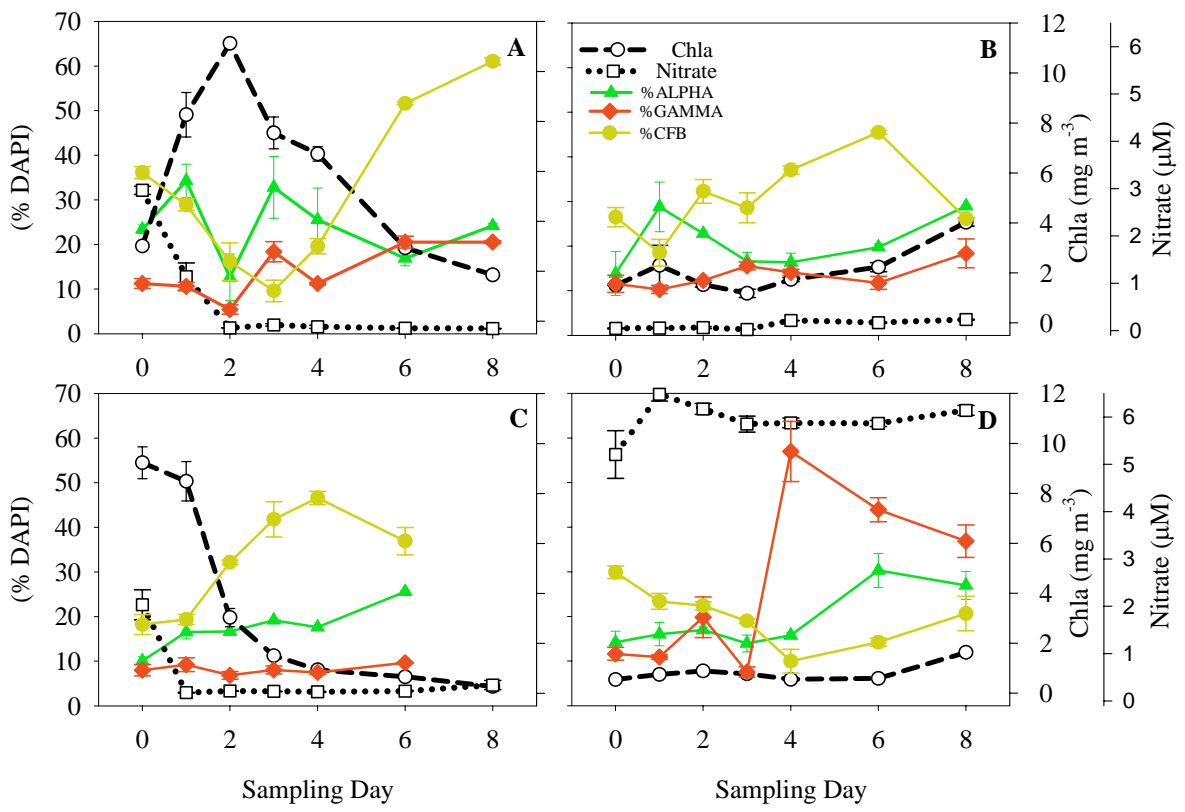19   Vigo at the beginning of the 4 experiments. Error bars represent counting errors.
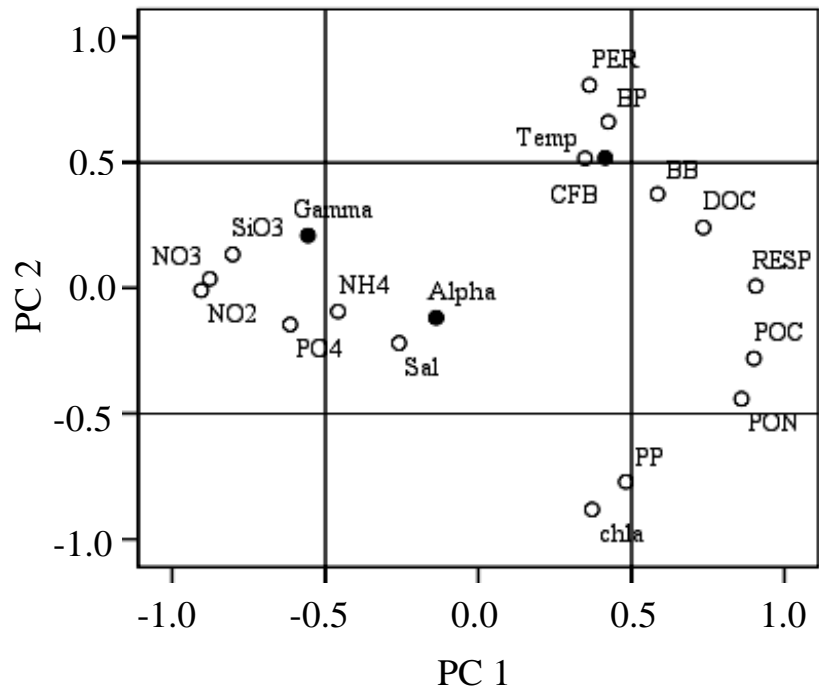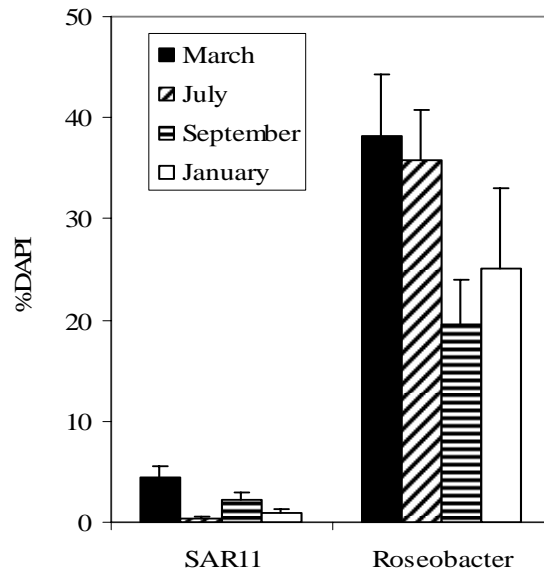
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Figure 1

Figure 2

Figure 3

Figure 4