

# *Betaproteobacteria* growth and nitrification rates during long-term natural dissolved organic matter decomposition experiments

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**ABSTRACT:** As a first attempt to relate net nitrification rates with bacterial community structure in the coastal embayment of the Ría de Vigo (NW Spain), a set of 6 long-term (lasting 53 to 74 d) dissolved organic matter decomposition experiments were conducted in the dark and at a constant temperature of 15°C with surface seawater collected in January, February, April, and June 2008. Net nitrification rates were estimated from nitrate concentration changes. Evolution of bacterial community composition was followed using catalyzed reporter deposition-fluorescent *in situ* hybridization (CARD-FISH) and specific probes for 6 relevant bacterial groups, including *Beta*- and *Gammaproteobacteria*. The growth rates of *Betaproteobacteria* showed a significant linear positive relationship with nitrification rates that explained 82 % of the observed variability, which strongly suggests that the main nitrifying microorganisms during the incubations belonged to the *Beta* subclass of *Proteobacteria*. A positive relationship was found between net nitrification rates and both ammonium ( $r^2 = 0.92$ ) and phosphate ( $r^2 = 0.97$ ) concentrations, which suggests a tight link among the nitrogen and phosphorus cycles, likely as a result of the role of phosphate as the bacterial growth-limiting nutrient.

**KEY WORDS:** *Betaproteobacteria* · Nitrification · CARD-FISH

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## INTRODUCTION

One of the most challenging goals in ecosystem ecology is to unravel the links between biogeochemical processes and biological community composition. In the sea, the nitrogen cycle depends on the metabolic activities of selected prokaryotes (Karl & Michaels 2001). Nitrification is the microbial mediated process by which ammonium is oxidized to nitrate. This process influences the partition of N among various pools and may lead to loss of fixed N from the system. Coupled nitrification-denitrification removes up to 50 % of external dissolved inorganic N inputs to estuaries (Seitzinger et al. 2006), which reduces the risk of eutrophication in these sensitive areas. Bacteria con-

tributing to ammonia oxidation, the rate-limiting step of nitrification, belong to the subclass *Betaproteobacteria* (genera *Nitrosomonas* and *Nitrospira*) and *Gammaproteobacteria* (genus *Nitrosococcus*). The second step of nitrification, nitrite-oxidation, is carried out by a variety of bacteria belonging to different subclasses of *Proteobacteria* (*Alpha*-, *Beta*-, and *Gamma*-) and by the genus *Nitrospira* (Zehr & Ward 2002).

Several groups of *Betaproteobacteria* able to oxidize ammonia are commonly found in marine and estuarine systems (O'Mullan & Ward 2005). The potential for significant ammonia oxidation by mesophilic *Crenarchaea* has been also demonstrated in terrestrial, marine, and wastewater treatment environments (see review by Prosser & Nicol 2008). Growing evidence

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suggests that *Archaea* would prevail over bacteria under limiting ammonium and phosphate concentrations, in low pH sulfidic environments, and in low salinity estuarine sediments with high C:N ratios (Mosier & Francis 2008, Erguder et al. 2009, Martens-Habbenha et al. 2009).

A dependence of nitrification rates upon ammonium concentration is usually not observed in natural assemblages (Ward 1987, 2002). This lack of correlation has been attributed to very high affinities of ammonia-oxidizers for ammonium or to changes in the ammonia-oxidizing bacterial community composition (Ward 2005).

Rates of nitrification in the Ría de Vigo (NW Spain) obtained from nutrient budgets have been estimated to be remarkably high compared with other coastal upwelling systems, providing about 50% of the nitrate consumed in the photic layer in this upwelling ecosystem (Álvarez-Salgado & Gilcoto 2004). However, the microorganisms involved in nitrification processes have never been studied in this highly productive coastal system. As a first attempt to relate nitrification rates with bacterial community structure in this area, we conducted a set of 6 long-term (53 to 74 d) dissolved organic matter decomposition experiments with surface seawater collected from the central segment of the Ría de Vigo under contrasting hydrographic conditions in January, February, April, and June 2008. We focused on bacteria, as we hypothesized that *Archaea* likely play a minor role in this marine eutrophic system.

Net nitrification rates were estimated from the net increase of nitrate concentrations during the course of the experiments. Changes in bacterial community composition were followed using catalyzed reporter deposition-fluorescent *in situ* hybridization (CARD-FISH) and specific probes for 6 relevant groups, including *Beta*- and *Gammaproteobacteria*. Despite the limitations (reduced turbulence, darkness, and altered grazer communities) of these *in vitro* field experiments, nitrification rates have been directly linked to group-specific bacterial growth rates.

## MATERIALS AND METHODS

**Sample collection.** Water for the laboratory incubation experiments was collected from a fixed station in the middle segment of the Ría de Vigo (42° 14.09' N, 8° 47.18' W) in winter (31 January, 7 and 14 February 2008), spring (17 and 24 April 2008), and summer (26 June 2008) with an acid-washed 25 l Niskin bottle at 5 m depth. Salinity and temperature profiles were recorded prior to water collection with an SBE 9/11 CTD probe.

**Experimental design.** Filtration of the sample water started within 10 min of collection; one part was fil-

tered through a dual-stage (0.8 and 0.2  $\mu\text{m}$ ) filter cartridge (Pall-Acropak Supor Membrane) which had been pre-washed with 10 l of Milli-Q water; the second part was filtered through pre-combusted (450°C for 4 h) GF/C filters to collect the bacterial community (<1.2  $\mu\text{m}$  size fraction). After filtration, the water was kept in the dark until arrival in the base laboratory, within 2 h of collection. The 0.2  $\mu\text{m}$  filtrate was transferred into a 20 l carboy, and the bacterial community (i.e. the 1.2  $\mu\text{m}$  filtrate) was added in a proportion corresponding to 10% of the total volume. The water was distributed into 24 glass bottles (500 ml), with 4 replicate bottles being analyzed on Days 0, 1, 2, 4, 12, and 53 or 74 (summer experiment only). The glass bottles were kept in the dark at a constant temperature of 15°C in the 6 experiments. All glassware was acid-washed and rinsed with Milli-Q water prior to use. Samples for the analysis of the dissolved phase were collected from each of the 4 replicate incubation bottles by filtration through 0.2  $\mu\text{m}$  filters (Pall, Supor Membrane Disc Filter) to follow ammonium ( $\text{NH}_4^+$ ), nitrite ( $\text{NO}_2^-$ ), nitrate ( $\text{NO}_3^-$ ), phosphate ( $\text{HPO}_4^{2-}$ ), and dissolved organic carbon (DOC) concentrations. Water samples for nutrient analyses were collected in 50 ml acid-washed polyethylene bottles and kept frozen (-20°C) until analysis. Aliquots for DOC analysis were collected in 10 ml pre-combusted (450°C, 12 h) glass ampoules and preserved by adding 50  $\mu\text{l}$  25%  $\text{H}_3\text{PO}_4$ . Subsamples for the bacterial community were collected on Days 0, 1, 2, 4, and 53 or 74.

**Chemical analyses.** DOC samples were measured using a Shimadzu TOC-CSV organic carbon analyzer (Pt-catalyst). To avoid the small error associated with day-to-day instrument variability, all samples from a given experiment were analyzed on a single day. The instrument was checked daily against the deep Sargasso Sea reference water (2600 m) provided by the laboratory of D. A. Hansell (Miami, FL, USA). We obtained a mean  $\pm$  SD concentration of  $46.0 \pm 2.0 \mu\text{mol C l}^{-1}$  while the nominal value provided by the reference laboratory was  $44.0 \pm 1.5 \mu\text{mol C l}^{-1}$ .

For the same set of experiments discussed here, Lønborg et al. (2010) adjusted the kinetics of DOC utilization to a first-order exponential decay function using the Marquardt-Levenberg algorithm and taking the refractory pool into account:

$$\text{DOC} = \text{BDOC} \times \exp(-k_{\text{DOC}} \times t) + \text{RDOC} \quad (1)$$

where  $\text{DOC}(t)$  is the concentration of DOC at incubation times 0, 4, 12, and 53–74 d, BDOC is the bioavailable DOC pool ( $\mu\text{mol l}^{-1}$ ),  $k_{\text{DOC}}$  is the degradation rate constant ( $\text{d}^{-1}$ ),  $t$  is time (in days), and RDOC is the refractory DOC pool ( $\mu\text{mol l}^{-1}$ ). RDOC was defined as the concentration at the end of the incubation time (53 or 74 d) and BDOC as the difference between the ini-

tial DOC concentration and RDOC. Since BDOC and RDOC are calculated prior to adjusting the time evolution of  $\text{DOC}(t)$ , the only parameter that is adjusted with Eq. (1) is  $k_{\text{DOC}}$ .

Nutrient salts were determined by standard colorimetric methods with an Alpkem segmented flow analyzer (SFA) (Grasshoff et al. 1983). A 4-point calibration curve of potassium nitrate with concentrations ranging from 0 to 15  $\mu\text{mol l}^{-1}$  was used to standardize the nitrate+nitrite SFA method, based on the reduction to nitrite in a Cu/Cd column followed by the determination of nitrite by the standard methods of azoic salt. Nitrite was measured simultaneously, and the concentration of nitrate was obtained by subtracting the nitrite concentration of the sample divided by the yield of the reduction column to the nitrate+nitrite concentration. The accuracy of the method is  $\pm 0.1 \mu\text{mol l}^{-1}$  and the curve is linear up to 30  $\mu\text{mol l}^{-1}$ . Net nitrification rates (in  $\mu\text{mol m}^{-3} \text{d}^{-1}$ ) were calculated from the accumulation of nitrate during the course of each experiment by subtracting the initial from the final nitrate concentration and dividing by the incubation time.

**Bacterial community composition.** Fifteen ml were fixed by adding 0.2- $\mu\text{m}$  filtered formaldehyde (1 to 2% final concentration) and the samples were subsequently stored at 4°C in the dark for 12 to 18 h. Thereafter, each sample was filtered through a 0.2  $\mu\text{m}$  polycarbonate filter (Millipore, GTTP, 25 mm filter diameter) supported by a cellulose nitrate filter (Millipore, HAWP, 0.45  $\mu\text{m}$ ), washed twice with Milli-Q water, dried, and stored in a microfuge vial at -20°C until further treatment in the laboratory.

Bacterial community composition changes were monitored using CARD-FISH techniques as described by Teira et al. (2008) with a mix of oligonucleotide probes specific for the domain *Eubacteria* (EUB338, EUB338II, EUB338III; Daims et al. 1999), the *Beta*-(BET42a) (Manz et al. 1992) and *Gammaproteobacteria* (GAM42a) (Manz et al. 1992) subclasses, and the class *Flavobacteria* of phylum *Bacteroidetes* (CF319a) (Manz et al. 1996). Additionally, the relative abundance of the SAR11, *Roseobacter*, and SAR86 clusters was also analyzed using the specific probes SAR11-441R (Morris et al. 2002), Ros537 (Eilers et al. 2001), and SAR86/1245 (Zubkov et al. 2001). The Eub antisense probe Non338 probe was used as a negative control.

Filters for CARD-FISH were embedded in low-gelling-point agarose and incubated with lysozyme. Filters were cut in sections and hybridized at 35°C with horseradish peroxidase (HRP)-labeled oligonucleotide probes over 4 to 12 h. Tyramide-Alexa488 was used for signal amplification (30 to 40 min). We used 55% of formamide for all probes except SAR11-441R (45% formamide). Cells were counter-stained with a DAPI-mix (5.5 parts of Citifluor, 1 part of Vectashield, and 0.5

parts of phosphate-buffered saline with DAPI, final concentration 1  $\mu\text{g ml}^{-1}$ ).

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: (1) total DAPI-stained cells, (2) cells stained with the specific probe. Negative control counts (hybridization with HRP-Non338) averaged 0.5% of DAPI-stained cells. The counting error, expressed as the percentage of standard error between replicates, was on average <5% for DAPI counts and <10% for FISH counts. The counting error was relatively higher (33%) for the less abundant groups (*Betaproteobacteria* and SAR86).

Group-specific bacterial net growth rates over the full incubation period were estimated using the logistic equation:

$$N = Kn/[1 + \exp(a - \mu \times t)] \quad (2)$$

where  $N$  represents cell abundance (cells  $\text{ml}^{-1}$ ),  $Kn$  is the cell abundance at the end of the experiment (Day 53 or 74),  $a$  is a constant parameter,  $t$  is the time (d), and  $\mu$  is the growth rate ( $\text{d}^{-1}$ ). Student's  $t$ -test was used to assess whether the derived growth rates were significantly higher than 0.

## RESULTS AND DISCUSSION

The percentage of detected bacteria (EUB-positive cells) slightly increased during the incubation in January and February. By contrast, during the experiments conducted in April and June, the percentage of detected bacteria significantly decreased (Fig. 1). The set of 6 FISH probes used accounted on average for 85 and 80% of total detected bacteria at the beginning and at the end of the experiments, respectively.

The initial bacterial community was dominated by the group *Bacteroidetes*, except in January, when the group SAR11 was the most abundant (Fig. 1). The dominance of *Bacteroidetes* has been widely reported in productive marine pelagic systems (Riemann et al. 2000, Alderkamp et al. 2006, Teira et al. 2008). The dominance of SAR11 in January was related to the entry of DOC-poor Eastern North Atlantic Central Water into the Ría de Vigo associated with the upwelling-downwelling dynamics of this embayment (Teira et al. 2009). The relative abundance of *Beta*- and *Gammaproteobacteria*, which include several nitrifying bacteria, was low at the beginning of the experiments (on average 2 and 4% of DAPI counts, respectively). *Betaproteobacteria* significantly increased their relative abundances during the incubation time in all

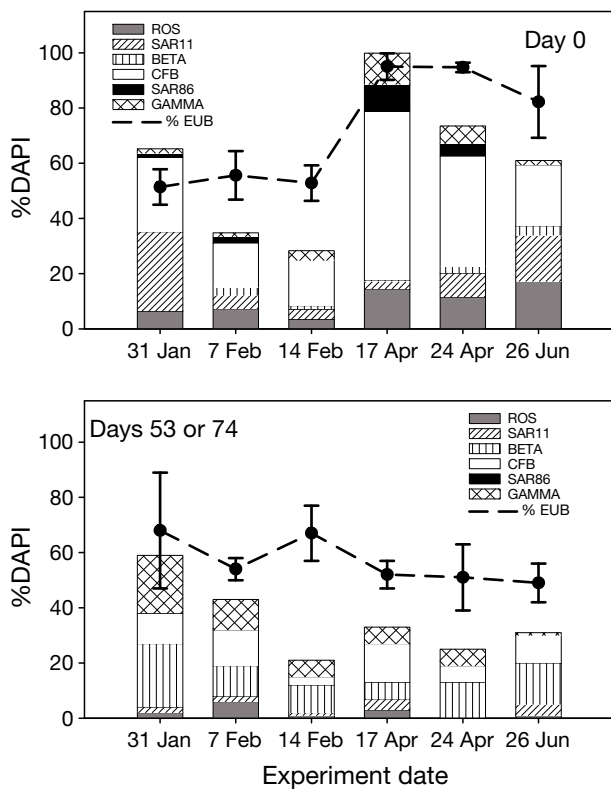


Fig. 1. Composition of the bacterial assemblage at the beginning (Day 0) and at the end (Days 53 or 74) of 6 incubation experiments conducted in 2008. The relative abundance of each group (ROS: *Roseobacter*; SAR11; BETA: *Betaproteobacteria*; CFB: *Bacteroidetes*; SAR86; GAMMA: *Gammaproteobacteria*) is expressed as a percentage of total DAPI-stained cells. The dashed black line represents the percentage of prokaryotes detected with the mix of oligonucleotide probes specific for the domain *Eubacteria* (EUB). Error bars represent  $\pm$  SE

experiments ( $t$ -test,  $p < 0.045$ ), accounting for up to 23% of DAPI-counts at the end of the experiment conducted in January. The *Gammaproteobacteria* showed a significantly higher relative abundance at the end than at the beginning of the experiment conducted in January ( $t$ -test,  $p = 0.012$ ). All other groups either remained constant ( $t$ -test,  $p > 0.05$ ) or showed a significant decrease in their relative abundances ( $t$ -test,  $p < 0.05$ ).

In most cases, the concentration of nitrate did not change much over the first 12 d of incubation; it accumulated between Days 12 and 50 (Fig. 2). Exceptions to this trend were Expts 1 and 2 (where a linear increase was observed) and 5 (where a logarithmic increase was observed). For this reason, we decided to calculate the net nitrification rate just by subtracting the initial from the final nitrate concentration and dividing by the incubation time. Net nitrification rates ranged from 6 to 103  $\mu\text{mol NO}_3 \text{ m}^{-3} \text{ d}^{-1}$ , being higher in winter than in spring and summer. Although the net

nitrification rates derived from our incubations were within the range of previous estimates by Álvarez-Salgado & Gilcoto (2004) for the same region, the highest rate was 1 order of magnitude lower than the highest nitrification rate ( $1400 \mu\text{mol NO}_3 \text{ m}^{-3} \text{ d}^{-1}$ ) calculated by these authors under natural field conditions. It is important to note the significant methodological differences between our study (10% dilution bacterial cultures, long incubations, dark conditions) and that by Álvarez-Salgado & Gilcoto (2004) (*in situ* nutrient budgets), which preclude us from directly comparing the magnitude of nitrification during both studies. Nevertheless, the measured nitrification rates agreed very well with those rates of ammonium oxidation measured by Ward (2005) over a 2 yr study in Monterey Bay, California, USA ( $<20$  to ca.  $80 \mu\text{mol m}^{-3} \text{ d}^{-1}$ ), using the  $^{15}\text{N}$  trace method during 3 to 6 h incubations under simulated *in situ* conditions.

*Betaproteobacteria* net growth rates over the whole incubation period (53 or 74 d) were calculated from the time-course of their absolute abundances in the 6 experiments using a logistic equation (Fig. 3). All net growth estimates were significantly higher than 0 except that of the 24 April experiment, and ranged from 0.4 to  $1.4 \text{ d}^{-1}$  (Table 1). The determination coefficient of the adjusted models ranged from 0.85 to 0.99. Higher net growth rates were measured in winter than in spring and summer. The net growth rate of *Gammaproteobacteria* was significantly different from 0 only in the experiment conducted in January ( $0.46 \text{ d}^{-1}$ ). As mentioned before, the abundance of all other groups remained stable or decreased during the incubations, showing a positive net growth only for 1 to 2 d.

The net growth rates of *Betaproteobacteria* showed a significant linear positive relationship with the net nitrification rates (Fig. 4A). Furthermore, the net growth of this bacterial group explained 82% of the variability observed in net nitrification rates, which strongly suggests that the main nitrifying microorganisms during our incubations belong to the *Beta* subclass of *Proteobacteria*. Although both *Beta*- and *Gammaproteobacteria* include ammonia-oxidizing bacteria (AOB), which carry out the limiting step in nitrification, it has been observed that *Gammaproteobacteria* AOB are not very abundant in seawater (O'Mullan & Ward 2005). Nonetheless, Lam et al. (2007) found that despite their low abundance, ammonia-oxidizing *Gammaproteobacteria* can significantly contribute to nitrification in the Black Sea suboxic zone.

The potential major role of *Archaea* as ammonia-oxidizing microorganisms has recently been reported in several environments. From the available information on the potential niches of ammonia-oxidizing *Archaea* (AOA) and AOB, we presumed that *Archaea* do not

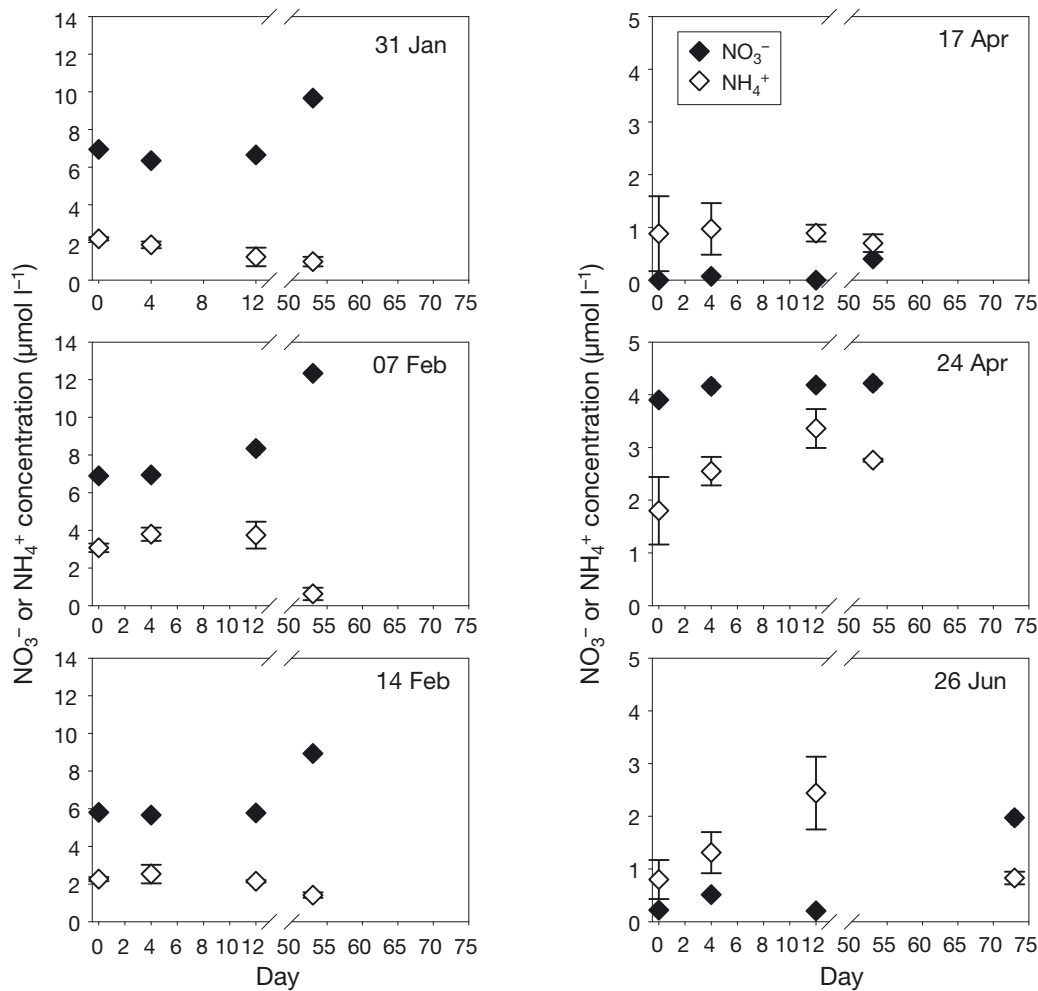


Fig. 2. Time course of nitrate and ammonium concentration during 6 dilution experiments conducted in 2008. Error bars represent  $\pm$ SD from 4 replicates. Note the different scales for the 17 April, 24 April, and 26 June experiments

play a relevant role in nitrification in this eutrophic pelagic ecosystem (Mosier & Francis 2008, Di et al. 2009, Erguder et al. 2009, Martens-Habbenha et al. 2009). Assuming that CARD-FISH detection rates were

Table 1. *Betaproteobacteria* (*Beta*) growth rates, nitrification rates, and dissolved organic carbon degradation rate ( $k_{\text{DOC}}$ ) ( $\pm$ SE) estimated for 6 experiments conducted in 2008.  $k_{\text{DOC}}$  values were taken from Lønborg et al. (2010). NS: not significantly different from 0

Date (2008)	<i>Beta</i> growth ( $\text{d}^{-1}$ )	Nitrification ( $\mu\text{mol NO}_3 \text{ m}^{-3} \text{ d}^{-1}$ )	$k_{\text{DOC}}$ ( $\text{d}^{-1}$ )
31 Jan	$1.1 \pm 0.2$	$51 \pm 7$	$0.11 \pm 0.03$
7 Feb	$1.2 \pm 0.1$	$103 \pm 5$	$0.11 \pm 0.01$
14 Feb	$0.9 \pm 0.2$	$59 \pm 4$	$0.20 \pm 0.01$
17 Apr	$0.4 \pm 0.1$	$8 \pm 1$	$0.20 \pm 0.02$
24 Apr	$0.5 \pm 0.4$ (NS)	$6 \pm 4$	$0.20 \pm 0.02$
26 Jun	$0.5 \pm 0.1$	$24 \pm 2$	$0.30 \pm 0.08$

equal or even lower on Days 53 or 74 than on Day 0, the fact that in the winter experiments (31 January, 7 and 14 February) the initial contribution of bacteria to total prokaryotic abundance (52, 55, and 53%, respectively) did not significantly decrease from its contribution at the end of the incubations (68, 54, and 67%, respectively;  $t$ -test,  $p > 0.16$ ) suggests that *Archaea* did not grow considerably in our experimental bottles. By contrast, the relative abundance of bacteria significantly decreased ( $t$ -test,  $p < 0.05$ ) at the end of the incubation in the experiments conducted in spring and summer (from 95, 95, and 82% at the beginning to 52, 51, and 49% at the end on 17 and 24 April and 26 June, respectively), which might indicate an increase in archaeal abundance. Unfortunately, we do not have information about the abundance and the community composition of *Archaea* in our experiments, which precludes us from speculating about their potential role as nitrifiers in the Ría de Vigo.

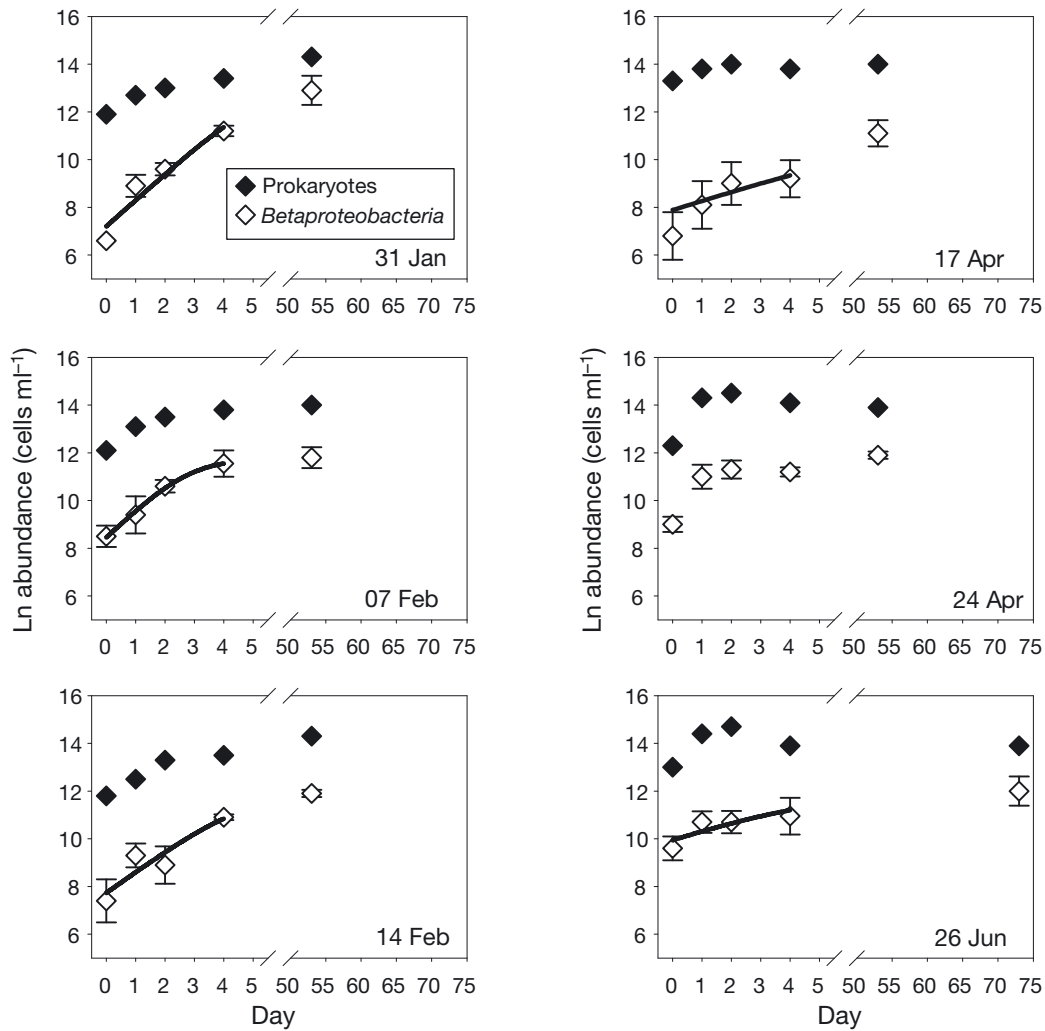


Fig. 3. Time course of prokaryotic abundance (DAPI-stained cells) and *Betaproteobacteria* abundance during 6 dilution experiments conducted in 2008. Error bars represent  $\pm$ SE from 2 replicates. For clarity, the adjusted logistic growth curve (black line) for *Betaproteobacteria* has been drawn only between Days 0 and 4

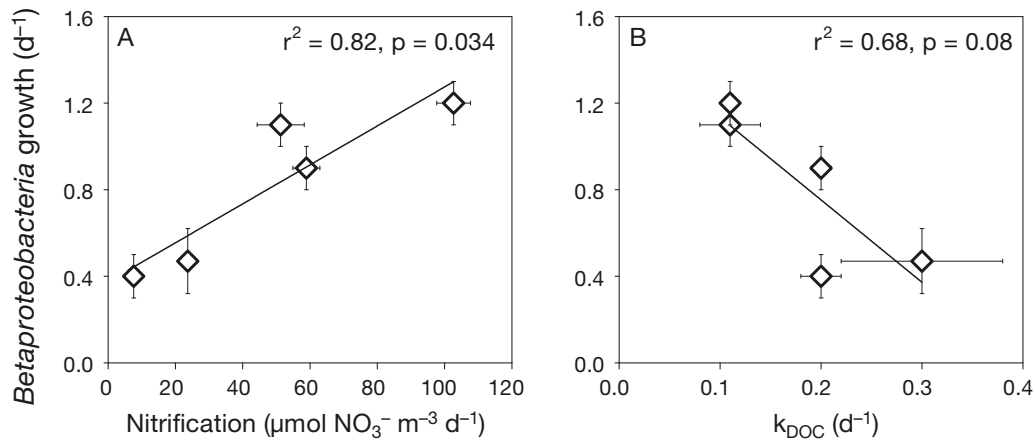


Fig. 4. Relationship between *Betaproteobacteria* growth and (A) nitrification rates ( $y = 0.009x + 0.37$ ) or (B) dissolved organic carbon (DOC) degradation rates ( $k_{\text{DOC}}$ ;  $y = -3.8x + 1.51$ ). Errors bars represent  $\pm$ SE from 2 (*Betaproteobacteria* growth) or 4 (nitrification and DOC degradation rates) replicates. Data point from 24 April was excluded due to no significant growth rate (see Table 1)

Table 2. Environmental conditions at the sampling site (5 m depth) in the Ría de Vigo on the water collection dates in 2008. Temp: temperature, Chl *a*: chlorophyll *a*, DOC: dissolved organic carbon

Date (2008)	Salinity	Temp (°C)	Chl <i>a</i> ( $\mu\text{g l}^{-1}$ )	$\text{HPO}_4^{2-}$ ( $\mu\text{mol l}^{-1}$ )	$\text{NO}_3^-$ ( $\mu\text{mol l}^{-1}$ )	$\text{NH}_4^+$ ( $\mu\text{mol l}^{-1}$ )	DOC ( $\mu\text{mol l}^{-1}$ )
31 Jan	35.0	13.0	1.52	$0.46 \pm 0.01$	$6.9 \pm 0.1$	$2.2 \pm 0.1$	$75 \pm 1$
7 Feb	34.5	13.1	0.81	$0.56 \pm 0.01$	$6.9 \pm 0.1$	$3.2 \pm 0.1$	$77 \pm 1$
14 Feb	35.2	13.4	1.13	$0.42 \pm 0.01$	$5.8 \pm 0.1$	$2.3 \pm 0.1$	$73 \pm 1$
17 Apr	34.8	14.3	3.04	$0.09 \pm 0.02$	$0.1 \pm 0.1$	$0.9 \pm 0.7$	$81 \pm 1$
24 Apr	25.0	15.5	8.42	$0.02 \pm 0.01$	$3.9 \pm 0.1$	$1.8 \pm 0.6$	$85 \pm 1$
26 Jun	35.1	17.4	4.32	$0.27 \pm 0.01$	$0.2 \pm 0.1$	$0.8 \pm 0.1$	$88 \pm 1$

The derived maximum net growth rate for *Betaproteobacteria* was within the range of reported rates for ammonia ( $0.48$  to  $2.11 \text{ d}^{-1}$ ) and nitrite-oxidizing bacteria ( $0.43$  to  $1.39 \text{ d}^{-1}$ ; Prosser 2007), and was also similar to maximum growth rates of the AOA '*Candidatus Nitrosopumilus maritimus*' strain SCM1 (Martens-Habbena et al. 2009). AOB are autotrophic bacteria that use ammonium as their sole source of energy, and carbon dioxide as a carbon source, which results in rather inefficient growth. Therefore, it is generally accepted that the growth rate of nitrifying bacteria is significantly lower than that of heterotrophic bacteria. This can certainly be true under culture conditions. However, the growth rates of nitrifying bacteria reported in natural conditions are not remarkably lower than the range of growth rates reported for heterotrophic bacteria in marine waters (Yokokawa et al. 2004, Teira et al. 2009).

It is possible that not all of the *Betaproteobacteria* detected with CARD-FISH are nitrifiers and, thus, autotrophic; however, the high positive correlation with nitrification and the negative marginally significant ( $p = 0.08$ ) correlation with DOC degradation rates (Fig. 4B) strongly suggest that a significant fraction of the *Betaproteobacteria* detected during the incuba-

tions were autotrophic. The fact that *Betaproteobacteria* growth is lower as the DOC degradation rates are higher could reflect a direct competition of autotrophic and heterotrophic bacteria for the available ammonium. It has been reported that autotrophic ammonia oxidation can be either depressed (Verhagen et al. 1992) or enhanced (Clark & Schmidt 1966) by heterotrophic bacteria. A recent study by Taylor & Townsend (2010) suggests that heterotrophic bacteria would out-compete nitrifying bacteria for ammonium uptake under a situation of nitrogen limitation (high DOC:nitrate ratios). The highest DOC:nitrate ratios were found in the spring and summer experiments (Table 2). Thus, the low nitrification rates measured in spring and summer could result from heterotrophic bacteria outcompeting nitrifying bacteria.

Provided that ammonia oxidation is the limiting step in nitrification, both AOB growth rates and nitrification rates are expected to be directly linked to the availability of ammonium. We actually found a strong positive relationship ( $r^2 = 0.92$ ) among the initial ammonium concentration and the nitrification rates (Fig. 5A). However, the relationship was not linear, and fitted better to a positive exponential model. Fig. 4A suggests that

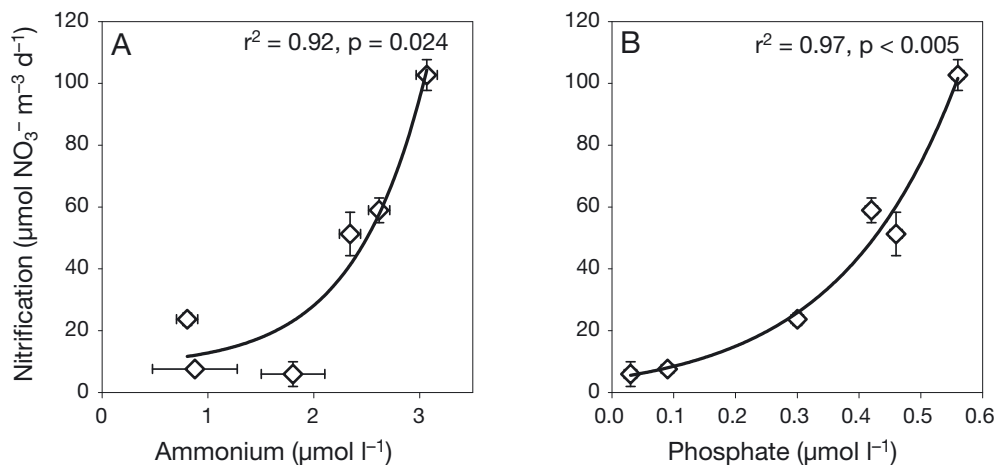


Fig. 5. Relationship between nitrification rates and (A) ammonium or (B) phosphate concentration at the beginning of the experiments. Error bars represent  $\pm$ SE from 4 replicates

at low ammonium concentration ( $<2 \mu\text{mol l}^{-1}$ ), other factors were ultimately controlling nitrification rates. Most studies on environmental factors controlling nitrification in marine pelagic systems point to temperature, light, and oxygen concentrations, besides ammonium availability, as the main regulatory factors (de Bie et al. 2002, Andersson et al. 2006, Sugimoto et al. 2009). Regardless of the very limited range of variability in water temperature and initial oxygen concentration (Table 2) covered during our sampling period, the estimated nitrification rates varied by more than 1 order of magnitude (Table 1). In addition, all incubations were conducted in the dark and at a constant temperature ( $15^\circ\text{C}$ ), suggesting that the aforementioned factors must play a minor role in explaining the variability in our dataset.

We explored the relationship of nitrification rates and other environmental variables such as salinity, DOC concentration, or phosphate concentration. We found that phosphate concentration explained 97% of the variability in nitrification rates (Fig. 5B). In fact, nitrification rates appear to be better explained by phosphate concentration when nutrients are in short supply. There is not much information about the effect of phosphate deficiency on nitrification. Herfort et al. (2007) also demonstrated a positive correlation between crenarchaeotal *amoA* gene copies and phosphate concentration in the southern North Sea. A positive correlation between nitrification or *amoA* gene copies and phosphate can merely reflect the role of phosphate as a bacterial growth-limiting nutrient (see review by Church 2008). The fact that during spring and summer, when the phosphate concentration was lower than in winter, the nitrification rates were low and the DOC degradation rates were high, suggests that a severe P deficiency may limit even more the ability of nitrifying bacteria to compete for N with heterotrophic bacteria. Purchase (1974) demonstrated the influence of phosphate deficiency on nitrification and concluded that nitrite oxidizers are more sensitive to P deficiency than ammonia oxidizers, which can severely affect their ability to compete for nitrogen, ultimately limiting nitrification. Our results thus demonstrate that nitrification depends not only on the availability of ammonia but also on the availability of phosphate.

## CONCLUSIONS

*Betaproteobacteria* growth explained 82% of the observed variability in nitrification rates in the coastal eutrophic system of the Ría de Vigo, which highlights the role of members of this group as nitrifiers in this system. Nevertheless, further research is needed to simultaneously assess the potential role of both bacteria and *Archaea*.

Ammonium concentration explained 92% of the variability observed in nitrification rates in the Ría de Vigo, although phosphate also appears to be an important controlling factor, which implies a tight link between the nitrogen and phosphorus cycles.

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