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The significance of organic nutrients in the nutrition of *Pseudo-nitzschia delicatissima* (Bacillariophyceae)

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Abstract:

The influence of organic nutrients on the evolution of Pseudo-nitzschia delicatissima cultures was investigated in an enrichment experiment with high-molecular-weight dissolved organic matter (HMWDOM) and in an uptake assay with ^{15}N-ammonium and ^{15}N-urea. HMWDOM was extracted from seawater collected at a nearby shore station during the decline of a diatom bloom. Four incubations were prepared: L1/5+DOM (*P. delicatissima* grown in L1 growth medium with 1/5 of the nitrate concentration of standard L1), (L1-N)+DOM (L1 without nitrate, i.e. nitrogen-deficient treatment), L1-DOM (control culture without added DOM) and BV+DOM (bacterial and viral control, free of microalgae). Incubations were carried out for 10 days. Chlorophyll *a* concentrations differed after day 4 and reached higher levels in the L1-DOM incubation by the end of the experiment; however, similar growth rates were observed in all incubations (1.64 ± 0.05 divisions day^{-1}). The persistently lower cellular chlorophyll content in (L1-N)+DOM during the experiment was consistent with N limitation conditions. The data suggested that the nitrogen needed for the growth of (L1-N)+DOM cells might have originated from the DOM. Based on the results of ^{15}N uptake assays, it was concluded that *P. delicatissima* more readily acquires ammonium than urea. Nevertheless, under low N conditions, *P. delicatissima* may use urea as an alternative N source, and comparable photosynthetic rates are attained on either substrate. Taken together, our results suggest a positive effect of organic nutrients on the growth of *P. delicatissima*.

Keywords: Dissolved Organic Matter (DOM) · Urea · Nitrogen Uptake · Harmful Algal Blooms (HABs)

INTRODUCTION

The role of dissolved organic matter (DOM) in aquatic food webs has been widely explored in recent years, in contrast to earlier investigations where methodological limitations restricted chemical analyses. Nevertheless, comparisons of those earlier methods with recent analytical results have shown that the former were free of serious errors (Williams, 1995). While the importance of this active organic pool remains the focus of current investigations, much remains to be understood regarding the complex mechanisms and dynamics underlying the production and decay processes inherent in multitrophic levels (Hansell and Carlson, 2002). Interactions with the microbial loop involving processes of excretion, lysis, remineralisation and predation are but a few examples (Sondergaard *et al.*, 2004). Within DOM, the dissolved organic nitrogen (DON) is an important component of the total nitrogen present in marine systems (Berman and Bronk, 2003). The increasing amounts of urea being deposited in coastal waters account for the fact that it is a significant component of the total DON pool, motivating therefore the existent research of the dynamics of this organic nitrogen resource in order to better understand its effects on the structure of the food web (Glibert *et al.*, 2006).

Several algal species forming harmful algal blooms (HABs) are able to use mixotrophic mechanisms, rather than strict autotrophic ones, to dominate phytoplankton blooms (Glibert and Legrand, 2006). Knowledge of the nutritional preferences and acquisition mechanisms employed by potentially harmful species is therefore critical to better comprehend the development and maintenance of HABs. The ability of flagellate life forms to overcome low nutrient affinity by complementing their diet of inorganic nutrients with organic ones is a well-known survival strategy of this microalgae group (Collos *et al.*, 2007; Smayda, 1997). Although inorganic nutrients are regarded as the main source of diatom sustenance, past (Lewin and Hellebust, 1976) and present (Berman and Bronk, 2003; Seitzinger and Sanders, 1999) research has focused on the availability and uptake of organic substrates by diatoms as a means of diversifying from conventional trophic pathways.

Toxic blooms of *Pseudo-nitzschia* spp. are associated with the production of domoic acid, a toxin that may induce a form of neurological damage known as amnesic shellfish poisoning (ASP) in consumers of contaminated vectors such as shellfish and sardines (Bates *et al.*, 1998; Fryxell *et al.*, 1997). The danger posed by ASP has economic consequences as well arising from the precautionary closure of shellfish harvesting when high levels of toxin and/or toxin-producing diatom species are detected (Addison and Stewart, 1989). The 11 potentially toxic species that comprise *Pseudo-nitzschia* spp. include some reported as poisonous in some areas but harmless in others. For example, *Pseudo-nitzschia delicatissima* (Cleve) Heiden is a cryptic species complex

containing both toxic strains (Fryxell *et al.*, 1997) as well as non-toxic ones (Fehling *et al.*, 2005). Genotype and environmental conditions, including nutrient status, have been suggested as triggers of the variability in toxin production (GEOHAB, 2005). *P. delicatissima* is a ubiquitous and thus physiologically versatile microalgae that forms blooms in diverse environments, from upwelling regions (Kudela *et al.*, 2005) to areas subject to anthropogenic influences (Quijano-Scheggia *et al.*, 2008a). It is considered a r-selected diatom, owing to its fast growth rate and cell yield (Quijano-Scheggia *et al.*, 2008b). Research on the *Pseudo-nitzschia* spp. trophic preferences has evidenced the ability of this genus to use organic elements as a complementary source of nutrition (Hillebrand and Sommer, 1996; Howard *et al.*, 2007) and as a dark survival strategy (Mengelt and Prézelin, 2002).

This investigation was composed of two experimental set-ups aimed at evaluating the influence of organic elements on the growth of *Pseudo-nitzschia delicatissima*. The first involved the addition of concentrated high-molecular-weight dissolved organic matter (HMWDOM) to a culture of *P. delicatissima*, while in the second ^{15}N-labeling was used to determine the *P. delicatissima* uptake kinetics of ammonium and urea.

MATERIAL AND METHODS

Experiment I. *Pseudo-nitzschia delicatissima* and DOM

Pseudo-nitzschia delicatissima culture. A strain of *P. delicatissima* (ICMB-F2B2), isolated from Arenys de Mar (Catalonia Coast, Spain), was grown in L1 medium (Guillard and Hargraves, 1993) as a unialgal culture. A second culture of this strain was acclimatized to a low-nitrogen growth medium, L1/5 (1/5 of the original L1 concentration of nitrate), three weeks before the experiment by a total of three transfers to the new medium. Both cultures were grown at a salinity of 30 and at a temperature of 20°C, with a 12:12 light:dark cycle. Illumination was provided by fluorescence tubes (Gyrolux, Sylvania, Germany) with a photon irradiance of 100 μmol photons m^{-2} s^{-1}.

DOM extraction and concentration. Seawater was collected from the oligotrophic Blanes coastal station (41º40'19"N, 2º47'11"E) in 28 May (2007), 60 km north of Barcelona, Spain, during the decline of a diatom bloom (phytoplankton base data of the Microbial Observatory of Blanes Bay, MOBB). Sampling during a period of low tourism and dry spring conditions (monthly mean rain < 1 mm; meteorological data from the area, Meteocat) additionally minimized allochthonous DOM sources from inland flow, thus favoring the presence of autochthonous DOM of autotrophic origin. HMWDOM (>1000 Da) was obtained by filtration of the seawater through a 0.2-μM cartridge (Pall

Corporation) and further concentration (50×) by tangential-flow filtration (Prep/Scale-TFF cartridge, Millipore).

Experimental design. Exponentially growing L1 and L1/5 cultures were diluted to ≈ 1×10^3 cells L^{-1} and distributed into 2.0 L polycarbonate containers (Nalgene) to a total volume of 1.8 L. Two treatments and two controls were prepared in duplicate: L1/5+DOM, (L1-N)+DOM (N-deficient with DOM addition), L1-DOM (control without added DOM), and BV+DOM (bacterial+viral control, obtained by removing *Pseudo-nitzschia delicatissima* from L1/5 medium by filtration through a 5-µm pore size filter). The starting concentrations (prior to filtration) in *P. delicatissima* culture were 8×10^3 bacteria ml^{-1} and 2×10^6 viruses ml^{-1}. Incubations were enriched with DOM in the order of 129 ± 50 µM. The experiment was carried out in semi-continuous mode by replacing 13% of the volume every two days (dilution rate = 0.07 d^{-1}). The outflow was used to estimate chemical and biological parameters. Incubation containers were mounted on a plankton wheel and rotated throughout the experiment (0.2 rpm) to promote homogeneous conditions and active growth.

Experiment II. *Pseudo-nitzschia delicatissima*: ammonium and urea uptake

Experimental design. *Pseudo-nitzschia delicatissima* cells growing exponentially in L1 were collected by 5-µm gravity filtration, which allowed the simultaneous exclusion of most bacteria from the original culture (Doucette and Powell, 1998). Cells were further resuspended in N-depleted growth medium for 6 h to ensure minimum ammonium concentrations at the beginnings of the experiments. Two series of 1-h incubations were prepared by adding ^{13}C-labeled bicarbonate to 50 ml of N-starved cultures at a fixed concentration, followed by the addition of either ^{15}N-ammonium or ^{15}N-urea in a range of nitrogen concentrations (0.1, 0.2, 0.5, 1, 2, 3, 5, and 10 µM-N), with a total of eight experimental sets per series. Concurrently, separate, short-time, 50-ml incubations with a fixed N source (^{15}N-ammonium or ^{15}N-urea) were established every 15 min to follow the short-time evolution of N-uptake. After incubation, all cultures were filtered through pre-combusted (4 h at 450°C) Gelman A/E filters (1-µm equivalent pore size). The filters were subsequently dried (60°C for 24 h) and then stored at room temperature until analysis.

N-uptake calculations. ^{15}N-enrichments were converted to N-specific uptake ratesof N-ammonium ($V_{N\text{-}ammonium}$) and N-urea ($V_{N\text{-}urea}$), as described in Collos et al. (2005). Since N-specific uptake rates vs. substrate concentrations followed saturation kinetics, the Michaelis-Menten nonlinear regression was applied to estimate the half-saturation constant (K_s, µM-N), the maximum uptake rate (V_{max}, h^{-1}), and the initial slope (α, L h^{-1} µgat N^{-1}). The latter was obtained from the uptake rate at 0.5 µM-N

concentration (Hurd and Dring, 1990). The results reflect the competitive ability of cells at low substrate concentrations.

Chemical and biological analysis. Inorganic nutrients (ammonium, nitrite, nitrate, phosphate, and silicate) samples were stored at -20ºC and subsequently analyzed by means of an auto-analyzer (Alliance Evolution II) using standard colorimetric techniques (Grasshoff *et al.*, 1983). Urea concentrations were determined according to the method of Goeyens *et al.* (1998). Samples for the analysis of organic elements were filtered through 0.7 μm filters (pre-combusted at 450ºC for 6 h and washed with Milli-Q water before sample collection). DOM was estimated by the distribution of dissolved organic carbon, dissolved organic nitrogen, and dissolved organic phosphorus (DOC, DON, and DOP, respectively). DOC was determined by high-temperature catalytic oxidation using a Shimadzu TOC-V (Álvarez-Salgado and Miller, 1998) from samples fixed with H_3PO_4 and stored at 4ºC in 10 ml flame-sealed glass ampoules (pre-combustion: 450ºC, 24h). Total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP) were determined using a Bran+Luebbe AA3·auto-analyzer, after persulfate oxidation (Grasshoff *et al.*, 1999). DON was derived by subtracting dissolved inorganic nitrogen (DIN= nitrate + nitrite + ammonium) from TDN, and DOP by subtracting dissolved inorganic phosphate from TDP. Chlorophyll *a* (chl *a*) samples were filter-extracted in acetone for 48 h and then analyzed in a Turner 10 AU fluorometer according to the method of Yentsh & Menzel (1963). Particulate nitrogen (PN) and particulate carbon (PC) as well as $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotopic ratios were estimated by means of an Integra CN elemental analysis–mass spectrometry system (PDZ Europa, UK). *Pseudo-nitzschia delicatissima* cells were maintained with Lugol-iodine solution, allowed to sediment (24 h), and quantified in a representative area with a Leica-Leitz DM-IL inverted microscope (Andersen and Throndsen, 2003). Bacterial cells were fixed with 1% paraformaldehyde, frozen in liquid N, stored at -80ºC and quantified in a Becton-Dickinson FACScalibur flow cytometer emitting at 488 nm (Gasol and delGiorgio, 2000). Further unfrozen samples were stained with 2.5 mM Syto 13 (Molecular Probes), mixed with yellow-green latex beads (Polyscience) as an internal standard, and run at low speed until 10,000 events had been registered. Bacteria were identified by their signature in a plot of side scatter (SSC) versus green fluorescence (FL1). Virus-like particles were glutaraldehyde-fixed (0.5% final concentration) and stored as described for the bacterial samples. Unfrozen samples were further diluted in TE buffer, stained with SYBR Green I for 10 min at 80ºC in the dark., cooled, and estimated by cytometry (Brussaard, 2004).

RESULTS

Experiment I: *Pseudo-nitzschia delicatissima* and DOM

A general decreasing trend in ammonium concentration was observed in all incubations (Fig. 1a), reaching a minimum value in (L1-N)+DOM (1.05 ± 0.65 µM). Nitrate consumption was accelerated at the end of the experiment in L1/5+DOM, in the DOM control (L1-DOM), and in the N-deficient incubation [(L1-N)+DOM], whereas in the BV+DOM control nitrate concentrations oscillated, with no clearly defined tendency (Fig. 1b, c). Phosphate slowly accumulated in (L1-N)+DOM (Fig. 1d), whereas the opposite pattern was observed in L1/5+DOM; in L1-DOM, phosphate peaked at day 4 and decreased thereafter. In the BV+DOM incubation, phosphate decreased at the beginning and end of the experiment, while in mid-experiment (days 4 – 8) it accumulated. In most incubations, there was an initial and final decrease in silicate concentrations (Fig. 1e) (days 0 – 2, and 8 – 10), with an increase during the middle stage of the experiment (days 2 – 8). The only exception was BV+DOM, in which no specific trend was observed. The extracted HMWDOM used for enrichment had an organic molar C:N ratio of 19. DOC concentrations were mostly stable throughout the course of the experiment (Fig. 2a). DON accumulated by the end of the experiment in L1/5+DOM and L1-DOM, with a marked increment by day 8 in the latter (Fig. 2b). In (L1-N)+DOM, DON remained constant throughout the incubation while in BV+DOM a peak was registered by day 2. DOP also peaked at day 4 in BV+DOM (Fig. 2c). In L1/5+DOM, DOP gradually increased after day 4, while in L1-DOM and (L1-N)+DOM an initial uptake period occurred, lasting until day 4 after which the DOP concentration did not vary greatly.

The growth of *Pseudo-nitzschia delicatissima* cells was similar in all experimental sets (1.64 ± 0.05 division day^{-1}) (Fig. 3). However, chl *a* concentrations markedly differed after day 4, reaching higher levels for L1-DOM and lower levels for (L1-N)+DOM by day 10 (32.6 ± 0.7 and 7.1 ± 0.1 µg L^{-1}, respectively), thus accounting for a difference in chl *a* content (320 and 207 fg chl *a* per cell on day 10, respectively). This 1.5-fold difference was already present at the beginning of the incubation period. Total bacteria (Fig. 4) rose steadily until day 6 (or day 8 in L1/5+DOM treatment), after which the number of cells declined. An exception was noted in the L1-DOM control, in which the initial increment until day 4 was followed by a second increase (days 6 – 10). Bacterial growth remained low during the experiment. Viral abundance (Fig. 5) had small variations in L1/5+DOM and (L1-N)+DOM, although in BV+DOM a peak was observed by day 4. In L1-DOM, low levels of virus were maintained until day 10, when a small increase was noted. Although the seawater sample used to extract HMWDOM was pre-filtered through a 0.2-µm filter and the concentrated HMWDOM immediately used for the enrichment experiment, the numbers of bacteria and virus in the DOM control (L1-DOM) vs. the remaining incubations at the beginning of the experiment differed, testifying to the presence of bacteria and viruses in the DOM extract.

Experiment II: *Pseudo-nitzschia delicatissima*: ammonium and urea uptake

C:N particulate molar ratios from *Pseudo-nitzschia delicatissima* cells were mainly stable during this experiment (19.4 ± 4.8 and 22.3 ± 5.0 for incubations enriched with ^{15}N-ammonium and ^{15}N-urea, respectively) due to the short incubation time (1 h). Resuspension of the cells for 6 h resulted in the development of a growth medium with very low ammonium levels (< 0.1 µM), as expected. Nitrate concentrations were low and varied only slightly (3.8 ± 0.3 µM) in all of the experimental sets, indicating low uptake of this N source and either non-interference or a constant rate of interference of nitrate to either ammonium or urea uptake. The detected nitrate levels also excluded possible modifications to N metabolism by N-limiting conditions.

The linear increase in cellular ^{15}N-ammonium and ^{15}N-urea incorporation along a temporal axis (Fig. 6) reflected the constant uptake rate of both N sources during the time elapsed, with higher uptake values for ^{15}N-ammonium. The increase in cell ^{15}N for the ammonium experiment presents a slightly concave shape, with a pronounced decrease in uptake after 70 minutes. However, those trends should not lead to significant deformation of the kinetics curve shown in Fig. 7a, for which data were obtained with an incubation time of 1 h.

The $V_{N\text{-ammonium}}$ and $V_{N\text{-urea}}$ along the concentration gradient were fitted to the Michaelis-Menten model (Fig. 7a, b). There are some indications of multiphasic uptake with an initial phase transition between 3 and 5 µM-N for both N sources. In particular, for ammonium, a single Michaelis-Menten model clearly tended to overestimate uptake between 1 and 5 µM-N and to underestimate it at higher substrate concentrations. The existence of multiphasic uptake systems was confirmed by the discrepancy noted in the kinetic parameters values obtained from the two substrate concentration ranges, 0.1 – 3 and 0.1 – 10 µM-N (Table I). For both V_{max} and K_s, values for ammonium were higher than those for urea. Still, similar inorganic carbon uptake rates were attained for cells grown in either ^{15}N source, implying comparable photosynthetic rates (Fig. 8). The C:N uptake ratios were nearly 8 for the ammonium series, indicating balanced growth, and nearly 100 for the urea series, clearly revealing a deficit in nitrogen supply in those samples.

DISCUSSION

Methodological aspects

Bioassays consist of manipulated systems in which a limited number of variables are controlled in order to clarify specific aspects of an entire ecosystem that are otherwise difficult to

address. It should be noted, however, that this approach is not without disadvantages. The confinement of microorganisms and thus their disruption from natural physical (e.g., mixing, advection, sedimentation), chemical (e.g., nutrient sources and feedback mechanisms), and biological (e.g., grazing, competition, allelopathy) processes limits extrapolation of the outcome to nature (Howarth, 1988). However, organic nutrient dynamics are too complex to study in multispecific natural assemblages, where interactions between organisms and environmental parameters constantly modify the availability and nature of organic matter (Hansell and Carlson, 2002). In this investigation, the use of unialgal cultures allowed us to follow the evolution of *Pseudo-nitzschia delicatissima* cells in the presence of DOM. This approach also proved useful to explore the organic nutritional traits of several microalgae species (Doblin *et al.*, 1999; Stolte *et al.*, 2006).

Bacteria and viruses are recognized as important elements of the microbial food web, influencing nutrient biogeochemical cycles and availability to higher trophic levels (Azam *et al.*, 1983; Fuhrman, 1999). The fact that 0.2 µm filtration and DOM extraction techniques failed to efficiently remove 100% of the bacterial and viral assemblages from concentrated seawater highlights the need to follow up these variables in similar experimental sets in order to understand both holistic population interactions and systemic evolution.

DOM enrichment: experiment I

Although the pulsed nitrate was readily consumed, resulting in a generally stable concentration of this nutrient, an increase in uptake was apparent by the end of the experiment, coincident with a decrease in the ammonium concentration to below 1.4 – 2.5 µM. This result can be explained by ammonium-metabolite inactivation of nitrate uptake (Flynn, 1991) at ammonium levels higher than this threshold value, as has been previously observed in several phytoplankton species (Dortch, 1990). Inorganic N-sources may also have been used by the bacterial assemblage, leading to variations in the N concentrations measured in the BV+DOM control, in which no microalgae cells were present. Indeed, in agreement with C:N stoichiometric balance, organic substrates with high C:N ratios indicate that bacteria are forced to use inorganic nutrients as a nitrogen source (Goldman and Dennett, 2000). The uptake of dissolved inorganic nutrients (DIN) to promote bacterial growth and the consequent competition between phytoplankton and bacteria for DIN were demonstrated in previous experiments and often lead to low bacterial growth (Davidson *et al.*, 2007; Joint *et al.*, 2002). In bacteria, nitrate is considered to serve only as a secondary N source because most species lack nitrate reductase, implying a high energetic cost (extra organic material) to assimilate this inorganic nutrient (Joint *et al.*, 2002). Phosphate and silicate were in

276 excess throughout the experiment, reasonably precluding nutrient-limiting conditions for these
277 nutrients.
278 The C:N molar ratio of the extracted HMWDOM (C:N = 19) was within the range of
279 autotrophic DOM (produced by phytoplankton) (Biddanda and Benner, 1997) and reflected the
280 production of carbon-rich organic matter associated with actively growing diatoms or diatoms at the
281 decline of a bloom (Nagata, 2000; Wetz and Wheeler, 2007). The ratio was also very similar to the
282 mean DOC/DON (17) measured just outside of Thau lagoon (Souchu *et al.*, 1997), where *Pseudo-*
283 *nitzschia* species also grow to bloom proportions. The constant level of organic resources observed
284 for most incubations provides evidence for a rapid coupling between the production and uptake of
285 labile (turnover times of days) organic matter. Nonetheless, there were also episodes in which
286 organic compounds accumulated. DOM accumulation generally occurs when production and
287 consumption mechanisms are decoupled (Biddanda & Benner 1997). Various conditions may arise,
288 simultaneously or exclusively, that lead to decoupling: (1) impaired increase of organic matter
289 release and microbial uptake, (2) increase in the presence of semi-labile (turnover times of months)
290 or refractory (turnover times of years) DOM, (3) inhibition of biological decomposition processes
291 by nutrient limitation, (4) bacterial biomass control through grazing or viral lysis (Williams 1995;
292 Søndergaard et al. 2004). In the present investigation, organic matter accumulated in distinct
293 contexts, - e.g., DON and DOP peaks in BV+DOM were concurrent with an increase in the
294 concentration of virus; the DON increase in L1/5+DOM on day 10 was coincident with a decrease
295 in bacterial abundance - thereby implying the occurrence of conditions (1), (2), (3), and (4) on
296 different occasions throughout the course of the experiment. However, the estimation of DON by
297 subtracting DIN from TDN produces the accumulation of analytical errors (with respect to TDN,
298 ammonium, nitrite and nitrate) that may lead to scattering of the data (Berman & Bronk 2003).
299 Indeed, the DON accumulation detected in the L1-DOM incubation from days 6 to 8 (Fig. 2) gave
300 rise to a low DOC:DON, which, according to published literature, is not feasible (Bronk, 2002). The
301 fact that, in L1-DOM, nitrate uptake per unit chl *a* during days 6 – 8 was above the maximum
302 possible (Collos *et al.*, 2005) points to an incorrect estimation of this parameter on this occasion.
303 Although the chl *a* concentrations in (L1-N)+DOM were lower than in the other treatments,
304 *Pseudo-nitzschia delicatissima* abundance was similar in every incubation. Throughout the
305 experiment, the chl *a* cell content in (L1-N)+DOM was lower than in L1-DOM, which is consistent
306 with DIN limitation (Caperon and Meyer, 1972; Thomas and Dodson, 1972). This may also indicate
307 mixotrophic growth, whereby some or most of the nitrogen (and carbon) would have come from
308 DOM, thus reducing the need for photosynthetic pigments while maintaining comparable cell
309 concentrations (Fig. 3). Similar trends were observed in Chrysophycea (Lewitus and Caron, 1991),
310 in which glycerol addition led to a two-fold reduction in cellular chl *a* content while the growth rate

311 increased. Moreover, the fact that DON from (L1-N)+DOM was constant during the entire
312 experiment implies a fast uptake of organic nitrogen inputs. Overall, although unialgal cultures do
313 not allow the inference of straight-forward utilization of organic nutrient sources by microalgae, one
314 is tempted to suggest that organic nitrogen provides, directly or indirectly, a nutrient source to
315 maintain *P. delicatissima* at high concentrations.
316 In the DOM control (L1-DOM), virus-like particles remained at a minimum until bacteria
317 reached their maximum abundance, by the end of the experiment, implying that viruses need a
318 minimum host level to trigger an infection cycle (Weinbauer, 2004). The decrease in bacterial
319 numbers in DOM-enriched treatments over the last days (days 6 – 10) of this study likely reflected
320 the interplay between viral lysis and (as already discussed) nutrient limitation (Fuhrman, 1999;
321 Williams, 1995).
322
323 **Ammonium and urea uptake: experiment II**
324
325 Although cells were subject to a 6-h starvation period, no pre-conditioning (Dortch *et al.*,
326 1991) or surge uptake (Conway *et al.*, 1976) effects were visible in temporal incubations of ^{15}N-
327 ammonium or ^{15}N-urea (Fig. 6). The V_{max} for ammonium (about 1.4 d^{-1}) was similar to the growth
328 rate (about 1.2 d^{-1}), confirming the absence of a surge uptake at the time scale of measurement used
329 here. A C:N uptake ratio near the Redfield ratio provided further evidence of balanced growth.
330 Indeed, the trend in *P. delicatissima* may be towards uptake rather than storage, which, in turn,
331 allows a rapid assimilation of nutrients into the build-up of biomass (Collos, 1986; Tilman *et al.*,
332 1982). These data concur with the classification of *Pseudo-nitzschia delicatissima* as an r-strategist
333 (Quijano-Scheggia *et al.*, 2008b).
334 The release of ammonium during urea assimilation was observed previously in microalgae
335 and often implies the presence of interacting mechanisms to regulate the uptake of these N
336 substrates (Jauzein *et al.*, 2008). Nevertheless, in this experiment, ammonium excretion was not
337 apparent during the assimilation of urea, likely excluding interactions between ammonium and urea
338 upon their uptake. The kinetic parameters were within the range determined for coastal
339 phytoplankton assemblages and cultured neritic diatoms (Eppley *et al.*, 1969; Kudela and Cochlan,
340 2000 ref. therein). More specifically, for ammonium, the K_s value was similar to that of *P. australis*

(Cochlan *et al.*, in press) when the entire concentration range was taken into account. There is also some evidence for multiphasic uptake in data from Cochlan *et al.* (in press) describing ammonium uptake by *Pseudo-nitzschia australis*, with phase transitions between 2 and 5 µM. Those values are similar to the ones reported here, but the authors did not attempt to estimate K_s at the lower concentration range. Concerning urea, the saturation kinetics observed here for *P. delicatissima* were very different from the linear response reported by Cochlan *et al.* (in press) for *P. australis.* The latter is more similar to that seen in some strains of *Alexandrium catenella* (Jauzein *et al.* 2008). At 10 µM-N, there was a 30-fold difference between ammonium and urea uptake in our study vs. the five-fold difference reported by Cochlan *et al*. In both species of *Pseudo- nitzschia*, ammonium is clearly preferred to urea.

The preference for ammonium is often related to transport mechanisms and the biological energetic costs involved in the synthesis of urease, needed for the assimilation of urea (Berman and Bronk, 2003). However, the K_s values suggested that during periods of low nitrogen concentration *P. delicatissima* may turn to urea as an alternative N source, as already observed for other diatoms (Rees and Syrett, 1979). In our case (Fig. 1a), the rather high K_s for ammonium indicated that *Pseudo-nitzschia* spp. could not compete with bacteria for this N source (Fuhrman *et al.*, 1988) and was thus probably forced to use organic N compounds such as urea for growth. Similar carbon-uptake levels point to comparable photosynthetic rates in *P. delicatissima* growing on either substrate, possibly due to the fact that urea provides both nitrogen and carbon to the cells (Berg *et al.*, 1997). Previous work evidenced the ability of *Pseudo-nitzschia australis* to grow equally well on urea as on other inorganic N sources (Howard *et al.*, 2007).

Summary and possible implications for the natural environment

The results of experiments I and II suggest a potential use of organic sources for the growth of *Pseudo-nitzschia delicatissima.* The presence of autotrophic DOM likely favors the development of *Pseudo-nitzschia delicatissima* during shortage of mineral nutrients. In natural environments, such conditions may be met after blooming species have depleted inorganic resources and a high content of exudated DOM is available (Sondergaard *et al.*, 2004). Indeed, in situ proliferations of *P. delicatissima* are often observed in the sequence of blooms of other diatoms, such as *Chaetoceros* spp. and *Leptocylindrus* spp. (Casas *et al.*, 1999; Quijano-Scheggia *et al.*, 2008a), or they may be monospecific, with blooms of *P. delicatissima* following those of *P. calliantha*, such as occurs in

Mediterranean areas (Caroppo *et al.*, 2005; Quijano-Scheggia *et al.*, 2008a), whereas in Korean waters *P. delicatissima* blooms occur after *P. pungens* events (Cho *et al.*, 2002).

It has been reported that natural assemblages of *Pseudo-nitzschia* spp. growing on predominantly urea-based regimens produce more domoic acid than when growing on inorganic nitrogen compounds (Howard *et al.*, 2007). The increasing loads of urea deposited into coastal waters (Glibert *et al.*, 2006) worldwide could therefore imply an increase in the toxicity of *Pseudo-nitzschia delicatissima* blooms. Conclusive evidence is nevertheless only possible by direct tests on toxic blooming strains, due to the diverse nutritional mechanisms of *P. delicatissima* cryptic complexes.

The wide temporal blooming season of *Pseudo-nitzschia delicatissima* has been explained by the succession of multiple genotypes of this species (Orsini *et al.*, 2004). It can therefore be hypothesized that strains able to profit from the presence of organic substrates thrive during periods of low inorganic nutrient concentrations, thus sustaining *P. delicatissima* blooms over prolonged periods. Furthermore, the overwhelming presence of *P. delicatissima* in mucilaginous macroaggregates (Totti *et al.*, 2005) together with the colonization by this diatom of large *Phaeocystis* spp. colonies (Sazhin *et al.*, 2007) also suggests an opportunistic ability of *P. delicatissima* to explore environments with a high organic nutrient content.

Overall, present and past research points to the ability of certain *Pseudo-nitzschia delicatissima* strains to benefit from the presence of organic substrates. Organic sources could as such represent the "missing link" to explain the growth of *P. delicatissima* when other factors fail to do so (Fehling *et al.*, 2005; Quijano-Scheggia *et al.*, 2008b). In a broader context, increasing evidence of the influence of organic substrates on the growth of several microalgae, including potentially harmful species, calls for this parameter to be included in monitoring programs and taken into account in the establishment of management and policy initiatives.

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593
594

Table I. Kinetic parameters of ammonium and urea uptake by *Pseudo-nitzschia delicatissima* cultures. Range: substrate concentrations, µM-N; K_s: half-saturation constant, µM-N; V_{max}: maximum uptake rate, h^{-1}; r^2: coefficient of determination; p: probability value; α initial slope, L h^{-1} µgat N^{-1}.

	Range	K_s	V_{max}	r^2	p	α
Ammonium	0.1 - 3	0.38	0.030	0.978	<0.01	0.034
	0.1 - 10	2.2	0.058	0.915	<0.01	0.021
Urea	0.1 – 3	0.28	0.0017	0.978	<0.01	0.002
	0.1 – 10	0.54	0.0021	0.922	<0.01	0.002

Fig. 1. Dissolved ammonium (a), nitrate (b, c), phosphate (d), and silicate (e) concentrations during the experiment. Due to the different scales of nitrate concentration, this nutrient is displayed in two distinct plots (b and c). Error bars correspond to standard deviations. L1 and L1/5 are growth media; BV+DOM is the bacteria+virus control.

Fig. 2. DOC (a), DON (b), and DOP (c) concentrations. (b) Left y-axis represents DOM-enriched incubations (L1/5+DOM; [(L1-N)+DOM]; BV+DOM); right y-axis represents DOM control incubation (L1-DOM). Error bars correspond to standard deviations; DOC = dissolved organic carbon; DON = dissolved organic nitrogen; DOP = dissolved organic phosphorus. BV+DOM: DON concentration at day 10 was not available.

Fig. 3. *Pseudo-nitzschia delicatissima* abundance (a) and chlorophyll *a* (b) concentrations. Error bars correspond to standard deviations.

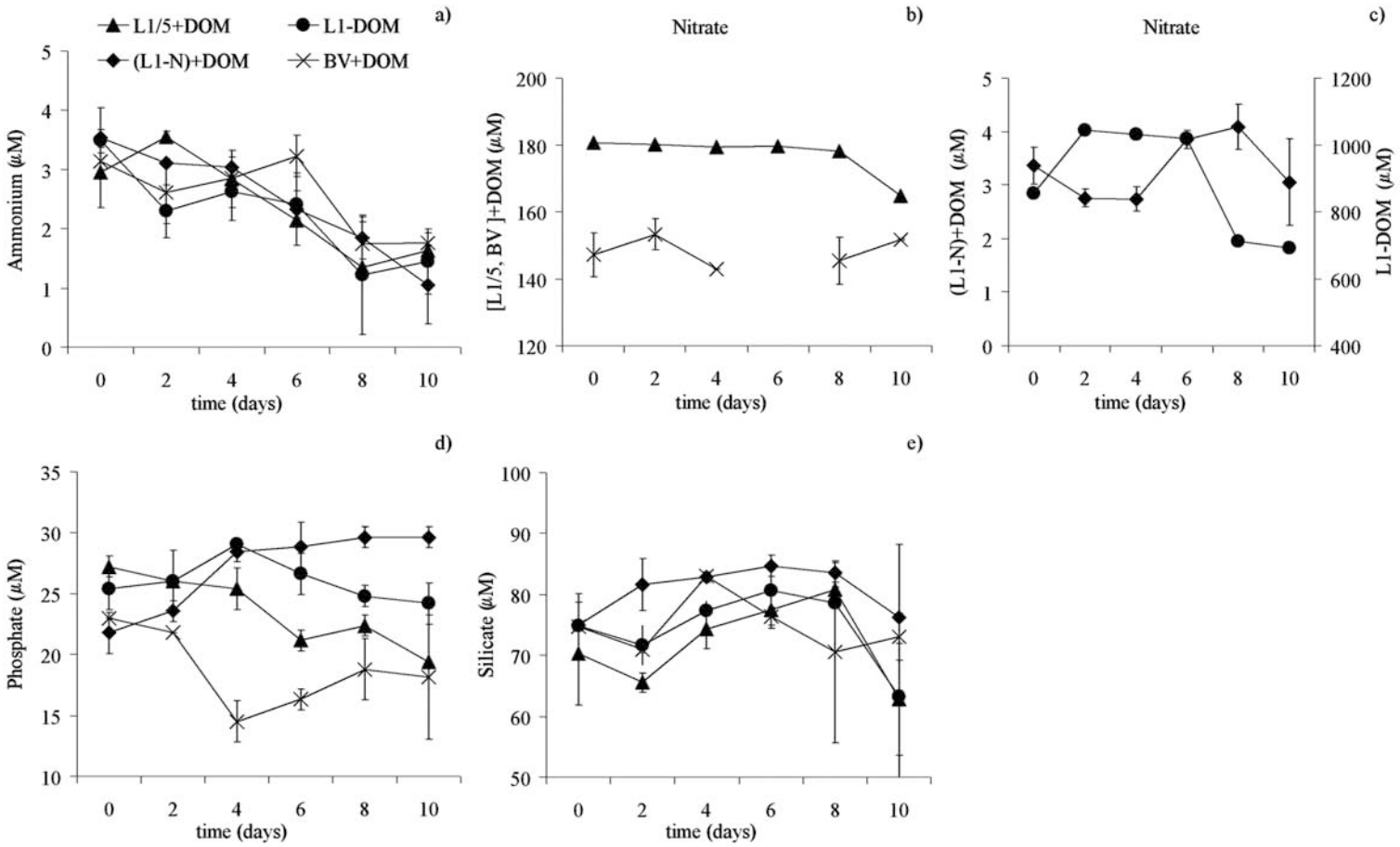
Fig. 4. Bacteria concentrations. Error bars correspond to standard deviations.

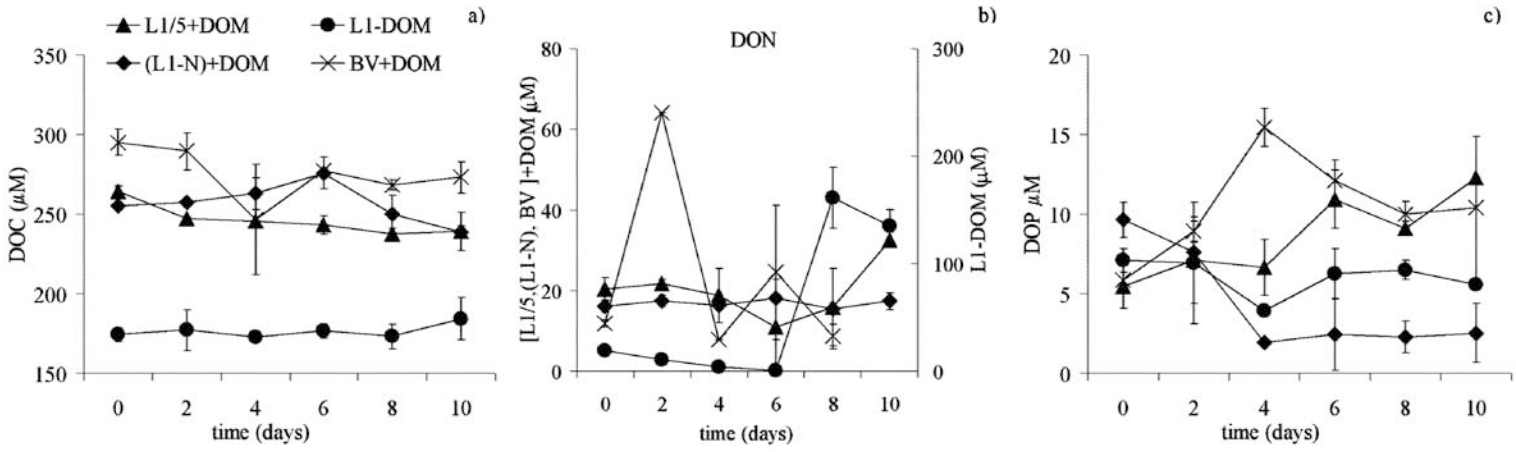
Fig. 5. Virus concentrations. Error bars correspond to standard deviations.

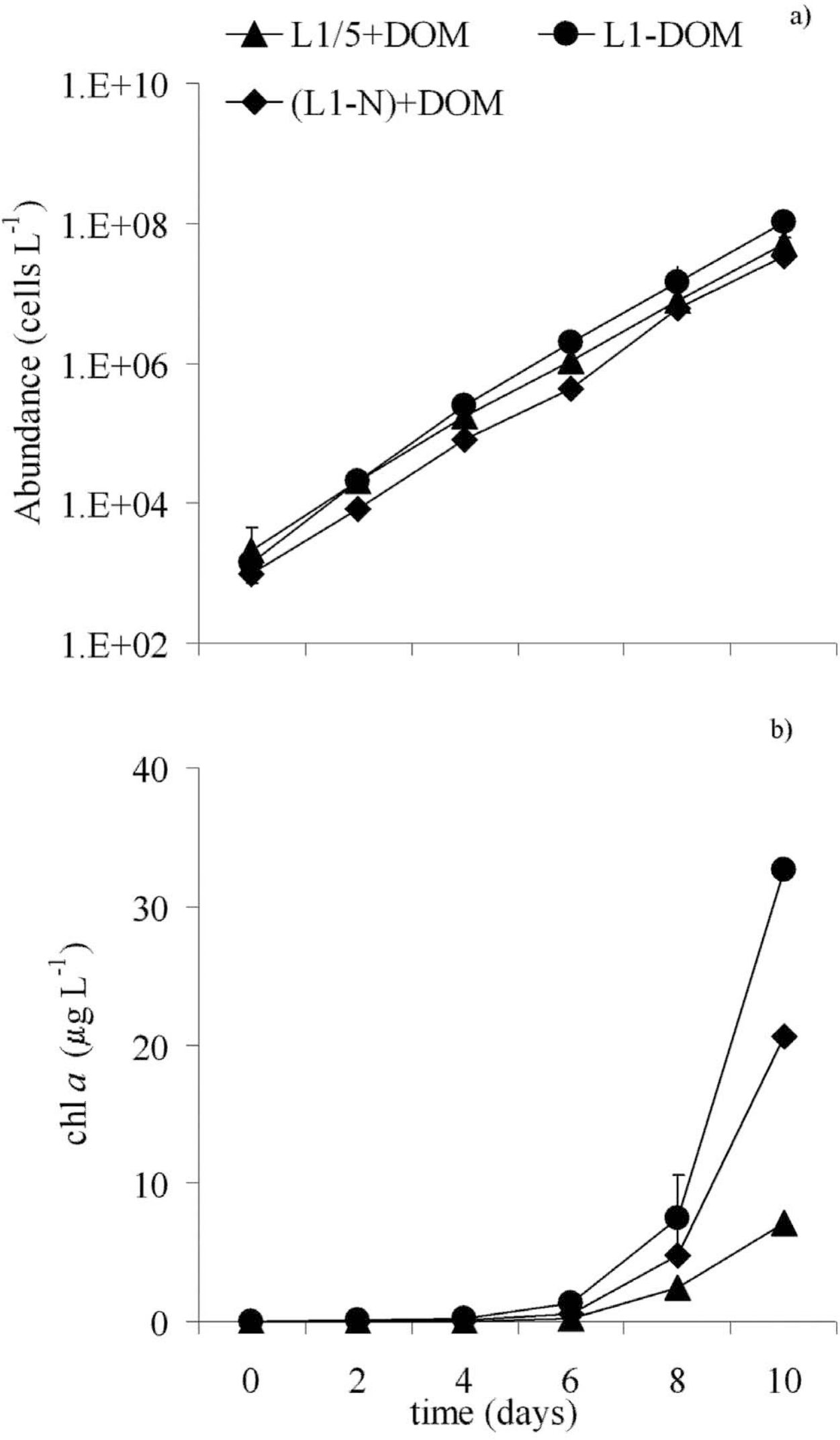
Fig. 6. ^{15}N-incorporation by *Pseudo-nitzschia delicatissima* cells during short-term incubations over a temporal axis ($p<0.001$).

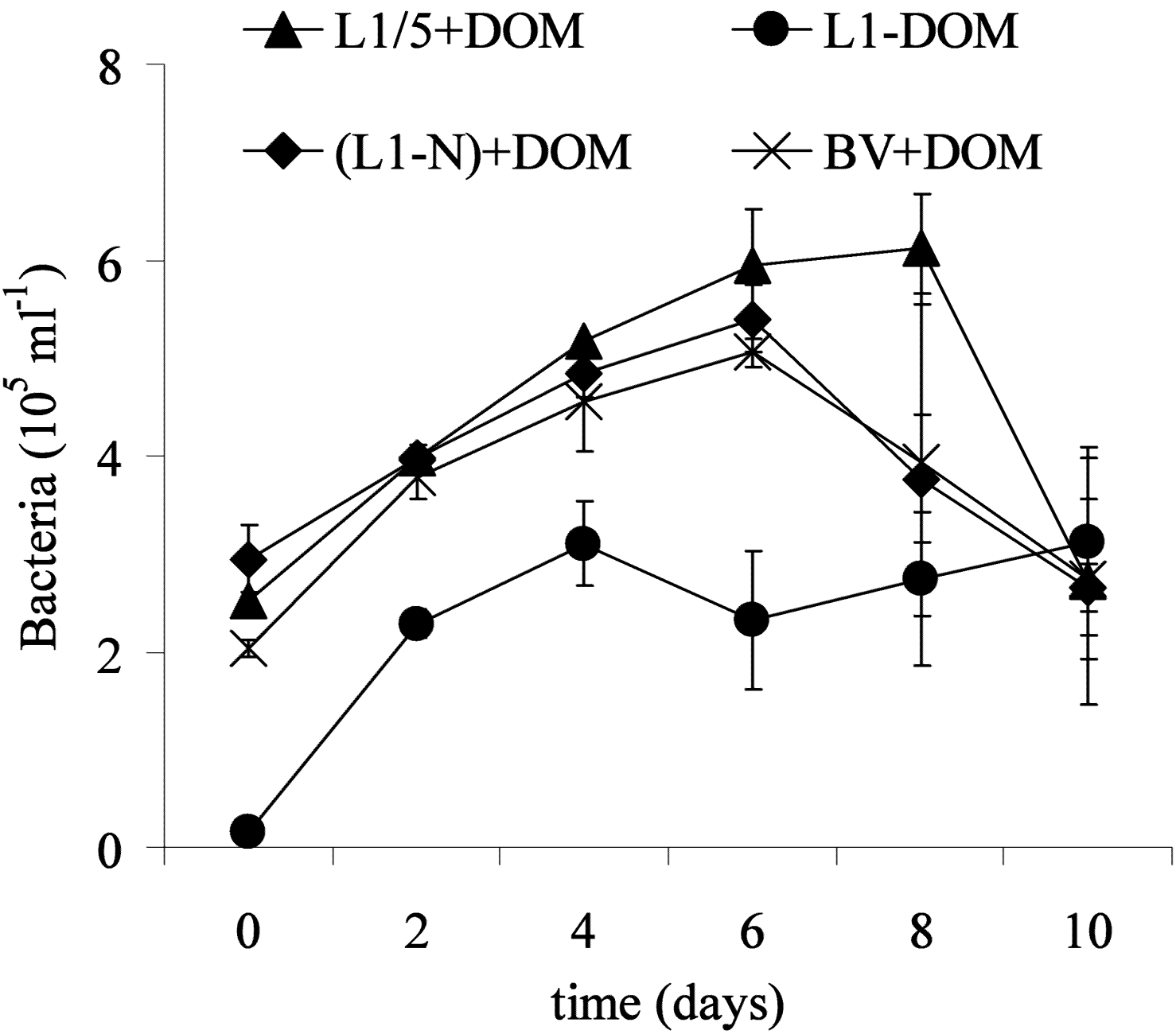
Fig. 7. Michaelis-Menten kinetic curves of $V_{N\text{-ammonium}}$ (a) and $V_{N\text{-urea}}$ (b) in *Pseudo-nitzschia delicatissima* cultures. Note the difference of scales between plots.

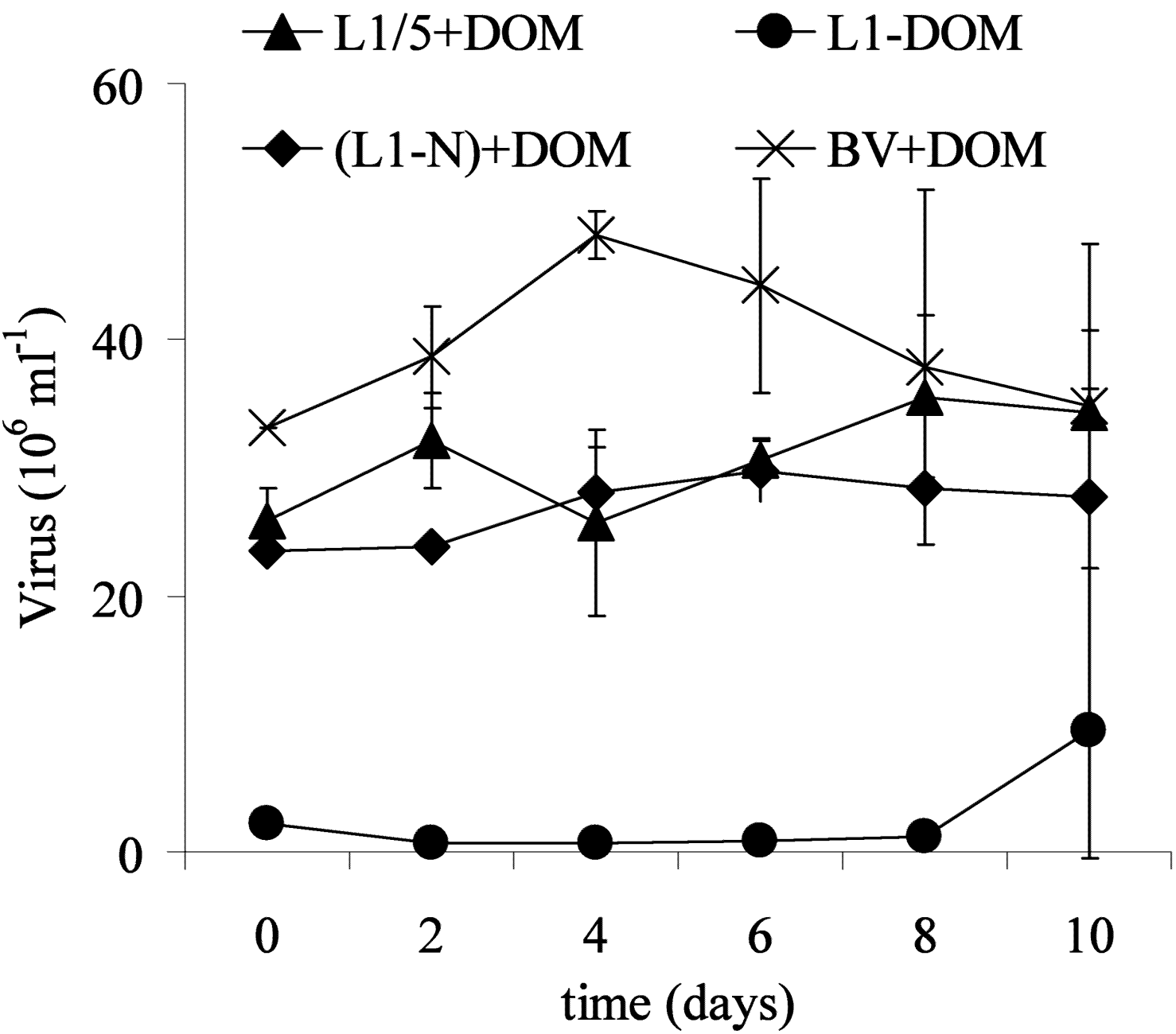
Fig. 8. ^{13}C assimilation in cells as a function of elapsed time.

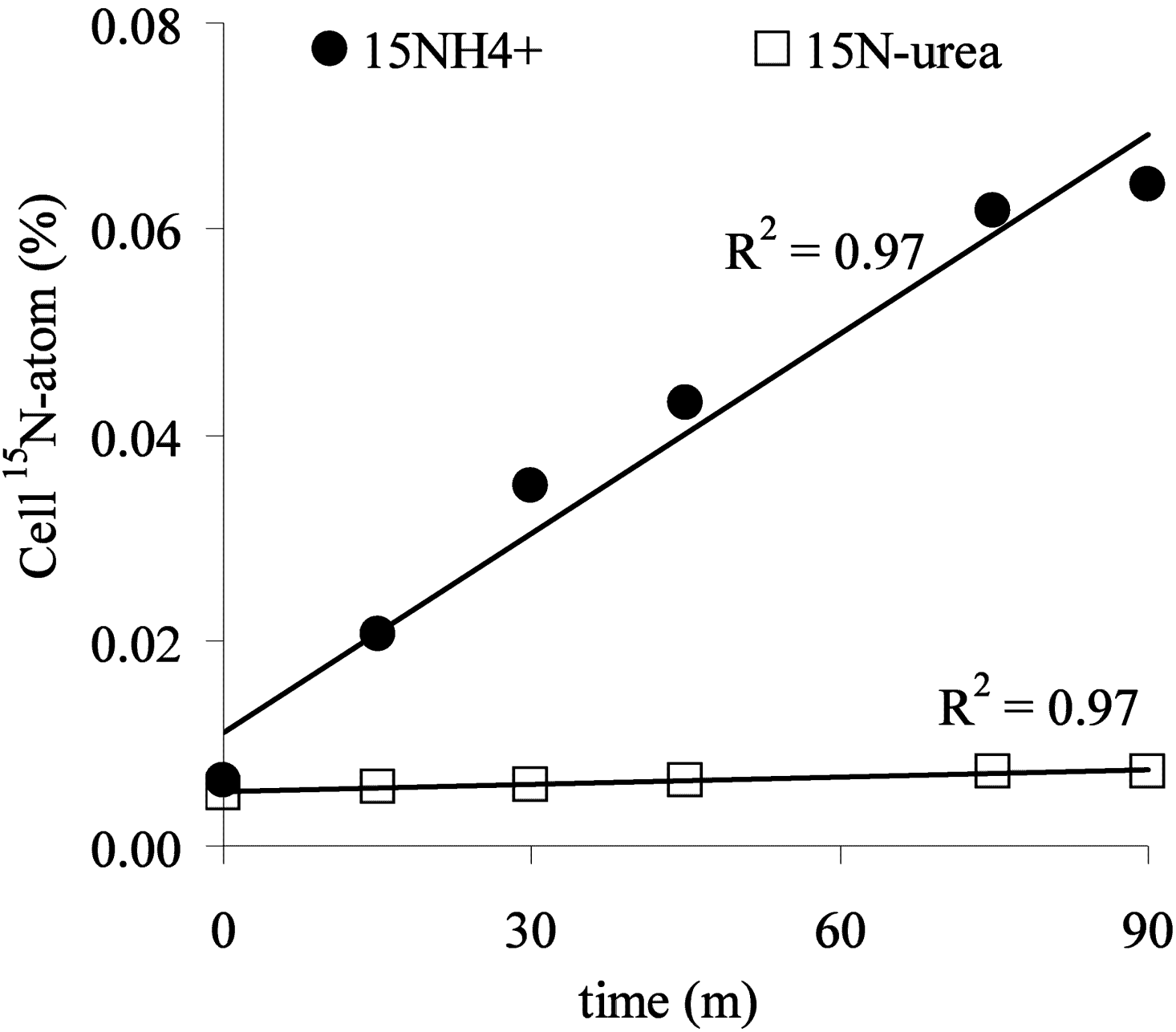

a)
L1/5+DOM
L1-DOM
(L1-N)+DOM
BV+DOM
Ammonium (µM)
time (days)
b)
Nitrate
[L1/5, BV]+DOM (µM)
time (days)
c)
Nitrate
(L1-N)+DOM (µM)
L1-DOM (µM)
time (days)
d)
Phosphate (µM)
time (days)
e)
Silicate (µM)
time (days)

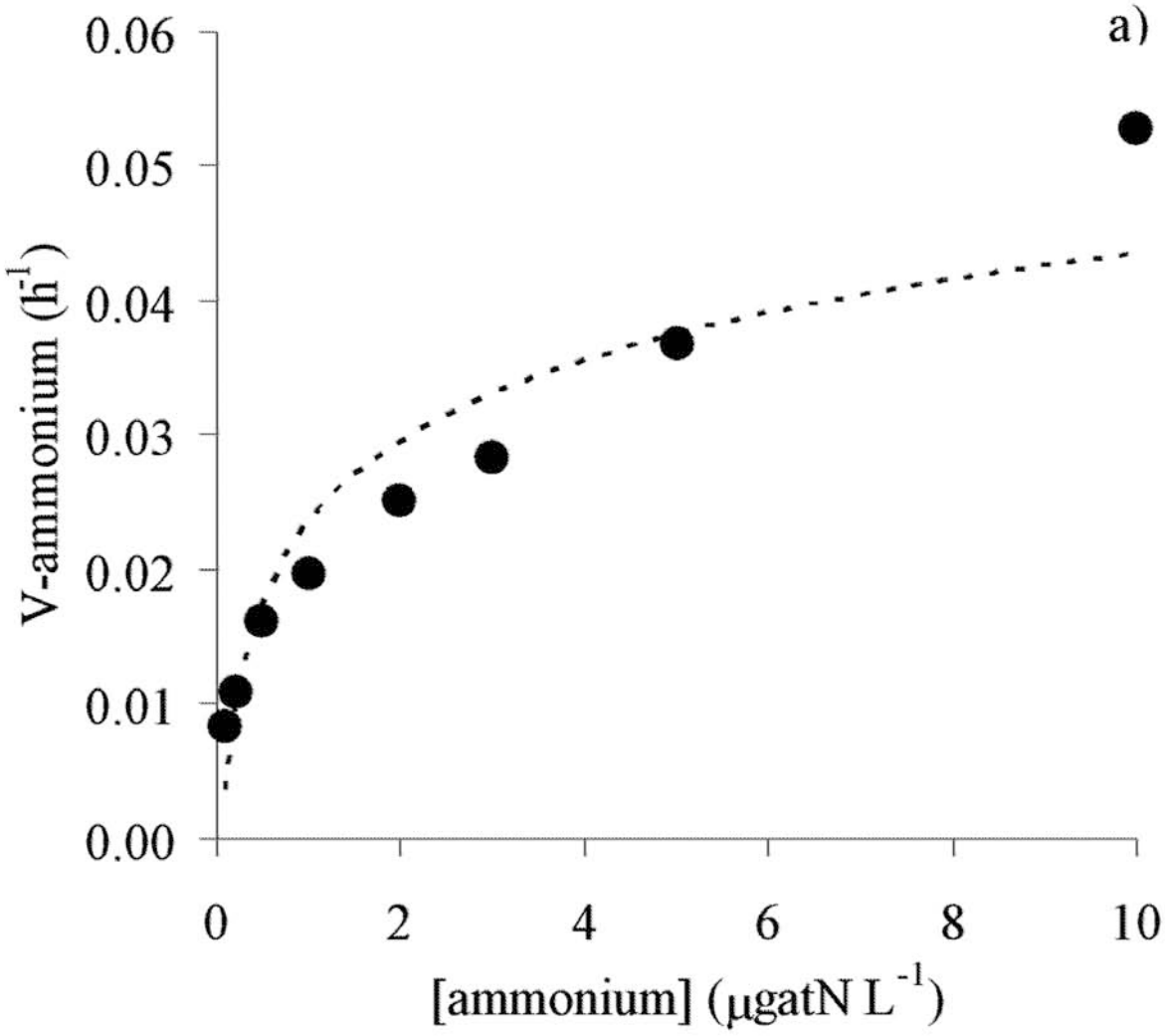

a)
L1/5+DOM
L1-DOM
(L1-N)+DOM
BV+DOM
DOC (µM)
350
300
250
200
150
0
2
4
6
8
10
time (days)
b)
DON
[L1/5,(L1-N), BV]+DOM (µM)
L1-DOM (µM)
80
60
40
20
0
300
200
100
0
0
2
4
6
8
10
time (days)
c)
DOP µM
20
15
10
5
0
0
2
4
6
8
10
time (days)

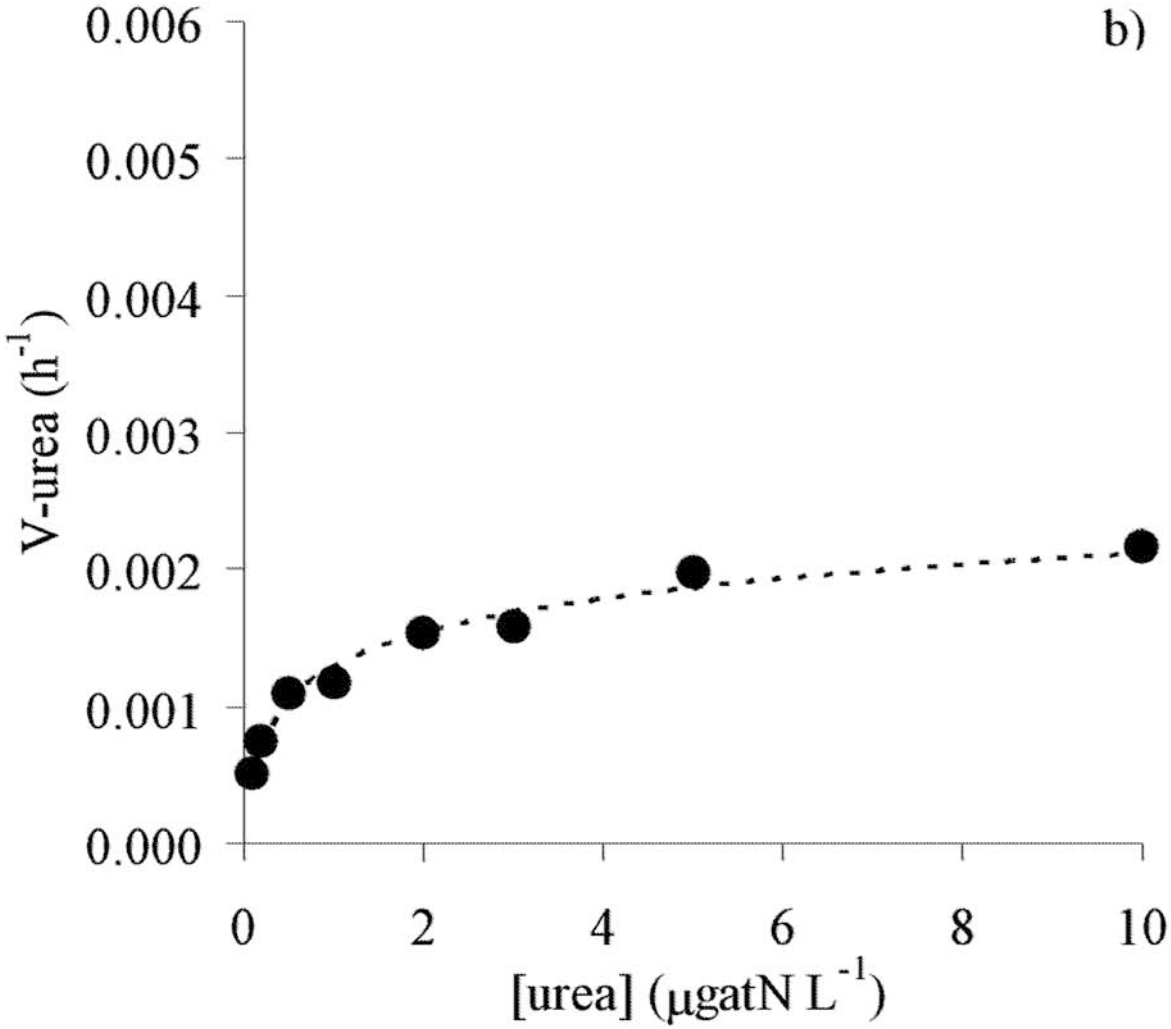

a)
L1/5+DOM
L1-DOM
(L1-N)+DOM
Abundance (cells L-1)
1.E+10
1.E+08
1.E+06
1.E+04
1.E+02
b)
chl a (µg L-1)
40
30
20
10
0
0
2
4
6
8
10
time (days)

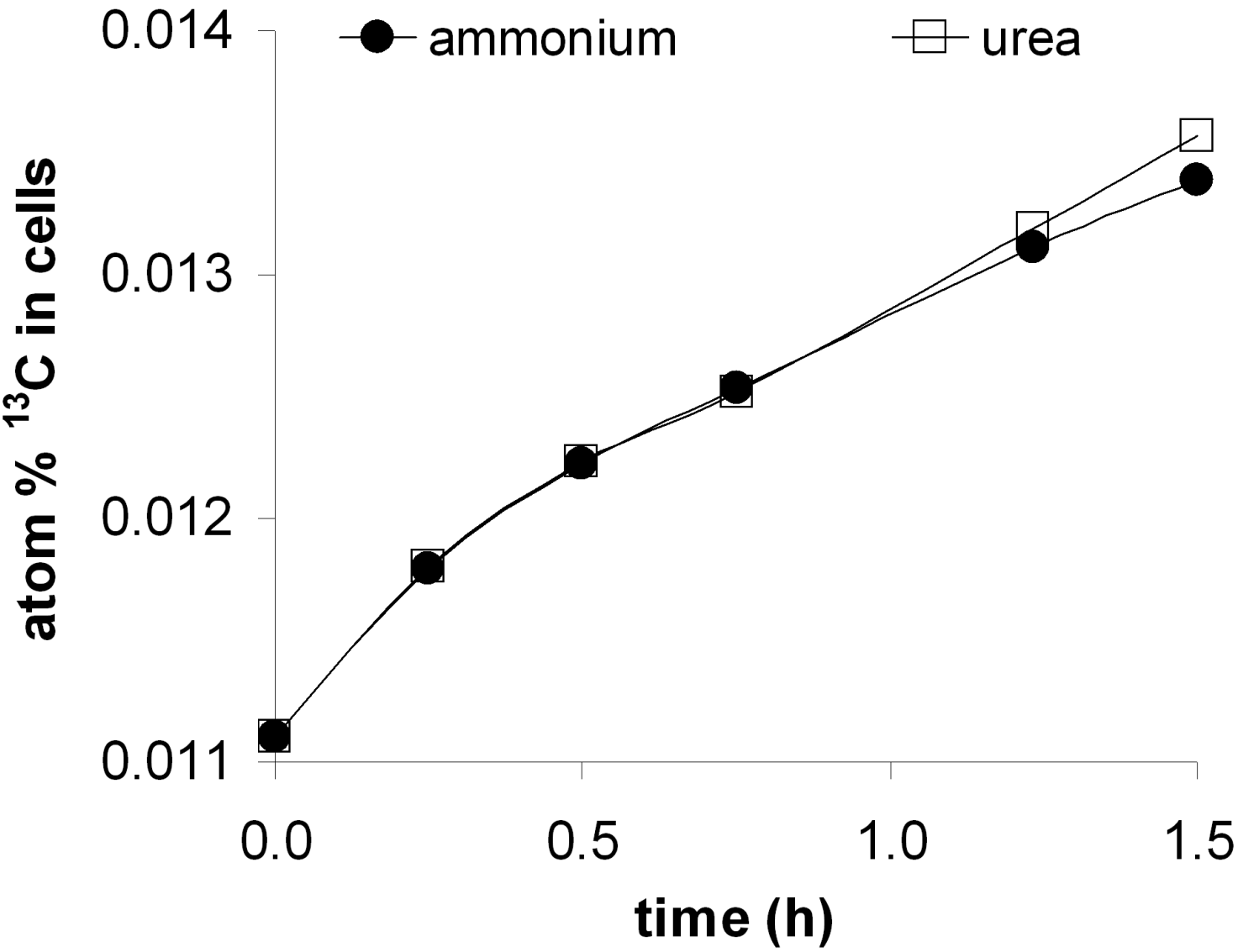

L1/5+DOM
L1-DOM
(L1-N)+DOM
BV+DOM
Bacteria (10^5 ml^{-1})
0
2
4
6
8
0
2
4
6
8
10
time (days)


L1/5+DOM
L1-DOM
(L1-N)+DOM
BV+DOM
60
40
20
0
Virus (10^6 ml^{-1})
0
2
4
6
8
10
time (days)


0.08
0.06
0.04
0.02
0.00
Cell ^{15}N-atom (%)
15NH4+
15N-urea
$R^2 = 0.97$
$R^2 = 0.97$
0
30
60
90
time (m)


a)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
V-ammonium (h-1)
0
2
4
6
8
10
[ammonium] (μgatN L-1)


b)
0.006
0.005
0.004
0.003
0.002
0.001
0.000
V-urea (h-1)
0
2
4
6
8
10
[urea] (μgatN L-1)


ammonium
urea
0.014
0.013
0.012
0.011
atom % 13C in cells
0.0
0.5
1.0
1.5
time (h)