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Uptake of dissolved inorganic and organic nitrogen by the benthic toxic dinoflagellate *Ostreopsis* cf. *ovata*

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Abstract :

Environmental factors that shape dynamics of benthic toxic blooms are largely unknown. In particular, for the toxic dinoflagellate *Ostreopsis* cf. *ovata*, the importance of the availability of nutrients and the contribution of the inorganic and organic pools to growth need to be quantified in marine coastal environments. The present study aimed at characterizing N-uptake of dissolved inorganic and organic sources by *O*. cf. *ovata* cells, using the ^{15}N-labelling technique. Experiments were conducted taking into account potential interactions between nutrient uptake systems as well as variations with the diel cycle. Uptake abilities of *O.* cf. *ovata* were parameterized for ammonium (NH_4^+), nitrate (NO_3^-) and N-urea, from the estimation of kinetic and inhibition parameters. In the range of 0 to 10 µmol N L^{-1}, kinetic curves showed a clear preference pattern following the ranking $NH_4^+ > NO_3^- >$ N-urea, where the preferential uptake of NH_4^+ relative to NO_3^- was accentuated by an inhibitory effect of NH_4^+ concentration on NO_3^- uptake capabilities. Conversely, under high nutrient concentrations, the preference for NH_4^+ relative to NO_3^- was largely reduced, probably because of the existence of a low-affinity high capacity inducible NO_3^- uptake system. Ability to take up nutrients in darkness could not be defined as a competitive advantage for *O*. cf. *ovata*. Species competitiveness can also be defined from nutrient uptake kinetic parameters. A strong affinity for NH_4^+ was observed for *O*. cf. *ovata* cells that may partly explain the success of this toxic species during the summer season in the Bay of Villefranche-sur-mer (France).

Abbreviations

- EA – IRMS, Elemental Analysis – Isotope Ratio Mass Spectrometry;
- PC, particulate carbon;
- PN, particulate nitrogen

Keywords : Uptake, Nitrogen, Dinoflagellate, *Ostreopsis*, Kinetics, Interactions

1. Introduction

Benthic harmful algal blooms represent an increasing threat to human and environmental health worldwide (Parsons et al., 2012; Rhodes, 2011). Toxic dinoflagellates belonging to the genus *Ostreopsis* Schmidt are common components of tropical epibenthic microalgae communities and have also been reported in several temperate waters, including coastal waters of the Mediterranean Sea (Vila et al., 2001), New Zealand (Rhodes et al., 2000) or Japan (Taniyama et al., 2003). Along the Mediterranean coasts, massive *Ostreopsis* cf. *ovata* blooms regularly occurred during the summer season and early fall (e.g. Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2011). Some of them were associated with serious cases of human health disorders (Brescianini et al., 2006; Vila et al., 2016). Symptoms of human illnesses include skin irritations, fever or broncho-constriction, partly due to exposure to toxic marine aerosols (Ciminiello et al., 2014). Blooms of *O*. cf *ovata* can also have deleterious effects on benthic marine invertebrates (Accoroni et al., 2011; Guidi-Guilvard et al., 2012; Pagliara and Caroppo, 2012; Gorbi et al., 2013). The toxicity of *O*. cf *ovata* is associated with the presence of palytoxin-like compounds that include putative palytoxin and ovatoxins-a, b, c, d, e and f (Uchida et al., 2013; Brissard et al., 2014), and mascarenotoxins-a and c (Rossi et al., 2010; Scalco et al., 2012). Palytoxin-like compounds have already been found in Mediterranean fauna (Biré et al. 2015) but no related food poisoning has been reported.

The processes that shape dynamics of benthic dinoflagellate populations and facilitate the development of specific toxic species are still poorly understood, mainly because benthic dinoflagellates have received considerably less attention than their planktonic counterparts (Parsons et al. 2012). Among potential controlling factors, temperature may represent a key factor in the seasonality of *O*. cf *ovata* blooms in temperate areas (Mangialajo et al., 2008; Accoroni et al., 2014; Accoroni and Totti, 2016). The control of bloom dynamics by water temperate has still to be clarified, however, as its appeared to vary with geographical areas

(Accoroni and Totti, 2016). Concerning other physical parameters, several studies reported higher abundances of *O.* cf *ovata* in sheltered sites compared to the ones exposed to wave action (e.g. Totti et al., 2010; Selina et al., 2014). This suggests that hydrodynamic conditions can have strong effects on bloom development and maintenance; according to Accoroni and Totti (2016), this influence of hydrodynamics on *O.* cf *ovata* bloom may be particularly pronounced under high levels of abundance (Accoroni and Totti, 2016).

The growth and maintenance of microalgae populations are also directly dependent on nutritive sources that are fueling the blooms. The regulation of *O.* cf. *ovata* bloom dynamics by the nutrient resource is largely unknown. Cells of *Ostreopsis* are expected to be mixotrophic, able to complete their autotrophic growth (based on photosynthesis and uptake of inorganic sources) by the use of organic matter (Burkholder et al., 2008). Among potential organic sources, the phagotrophy of preys by *Ostreopsis* cells was investigated (Faust et al., 1996; Barone, 2007) but is still a matter of debate (Escalera et al., 2014). The potential use of dissolved organic phosphorus by *O.* cf *ovata* cells was tested by Pistocchi et al. (2014), when the uptake of dissolved organic nitrogen sources has not been analyzed yet. Concerning the inorganic sources of nutrients, previous studies reported conflicting results regarding relationships between nutrient availability and occurrence of *Ostreopsis* blooms (Accoroni and Totti, 2016). Several field studies conducted in the NW Mediterranean Sea did not show any relationship between epiphytic *O.* cf. *ovata* abundances and concentrations of dissolved inorganic nutrients (dissolved inorganic nitrogen, DIN, and phosphate) (Vila et al., 2001; Accoroni et al., 2011). Conversely, Parsons and Preskitt (2007) found that *Ostreopsis* sp.1 abundance was positively correlated with nutrient concentrations in the waters surrounding Hawai'i. A positive correlation between phosphate concentration and *O.* cf. *ovata* abundance was also reported by Cohu et al. (2013) in the NW Mediterranean Sea. In the Northern

Adriatic Sea, phosphate pulses in the bloom onset period may possibly stimulate *O* cf. *ovata* growth in these coastal waters (Accoroni et al., 2015).

The importance of the availability of nutrient sources and their contribution to *O* cf. *ovata* growth during bloom development and maintenance need to be quantified in marine coastal environments. In the present study, the control of *O* cf. *ovata* growth by several nitrogen (N) sources was investigated under controlled conditions, using cultures. The main goal of the present work was to characterize N-uptake of dissolved inorganic and organic sources, using the ^{15}N-labelling technique and taking into account potential interactions between nutrient uptake systems as well as variations with the diel cycle.

2. Material and methods

2.1. Culture conditions

Two strains of *Ostreopsis* cf. *ovata*, MCCV 054 and MCCV 055, were obtained from the Mediterranean Culture Collection of Villefranche (MCCV). They were both isolated in 2014 from Villefranche Bay, South of France (43°41′34.83″ N and 7°18′31.66″ E), during the same bloom event. Non-axenic stock cultures were grown in modified K/10 medium (originally defined by Keller et al. (1987)), where addition of silicate and Tris base was omitted, phosphorus was added as KH_2PO_4 (final concentration of 4 µM) and ZnSO4 was added at a final concentration of 0.08 nM. Culture medium was prepared using autoclaved old seawater filtered on 0.2 µm (FSW) at salinity 38. Cultures were maintained at 23°C, under 250 µmol photons m^{-2} s^{-1}, with a 16:8 h light:dark cycle. Stock cultures were grown in batch mode without bubbling, in 15 mL of culture medium. Culture flasks were maintained in flat culturing conditions in order to optimize the surface area for gas exchange and growth of benthic cells. Before each experiment, one stock culture in exponential phase of growth was

successively diluted in order to scale up the culture volume from 15 ml (in flask of 25 cm² surface area) to 350 ml (in flask of 300 cm² surface area). The final large volume culture was used to inoculate three or four replicated cultures of 350 ml. Experiments were run using a set of replicated cultures in exponential phase and characterized by a cell density higher than 1,500 cell ml^{-1}.

2.2. Micro-algal cell resuspension in low N medium

Experiments were conducted under controlled conditions of nitrogen (N) availability in order to help for a precise characterization of N-uptake capabilities of *O.* cf. *ovata* cells. Each experiment started with the resuspension of micro-algal cells in culture medium where no NH_4^+ or NO_3^- addition was performed (–N medium). Concentrations of NH_4^+ and NO_3^- were determined for the –N medium used for running the experiments. Full resuspension of *O.* cf. *ovata* cells was completed in about 1h. Cells were collected on an 8 or 10 µm mesh size net by gravity filtration, then rinsed with –N medium before being resuspended in –N medium. To ensure that the net was not clogged due to mucus accumulation, these collection, rinsing and resuspension steps were performed on successive aliquots of 35 or 40 mL of culture and a new piece of net was used every four aliquots. A gentle agitation of the net in –N medium did not allow for passive resuspension of *O.* cf. *ovata* cells. Thus, for each aliquot of culture, micro-algal cells concentrated on the net were collected by pipetting repeatedly and carefully ~1 ml of –N medium above the net, then this volume was finally poured in a culture flask (75 cm² surface area) filled with 40 mL of –N medium. The resuspension and rinsing steps allowed for the removal of most of the bacteria present in the growth medium and limited their contribution in the resuspended cultures (Raush de Traubenberg and Soyer-Gobillard, 1990).

The resuspended culture flasks were kept aside in the culture chamber, under initial culture conditions, during 1-2h before starting the incubations. This lag reduced the potential impact of stress associated with the resuspension step on uptake rates and also contributed to start incubations under really low N concentrations.

2.3. Kinetic experiments

Uptake kinetics of three potential N-sources, nitrate (NO_3^-), ammonium (NH_4^+) and urea, were characterized for the two *O.* cf. *ovata* strains, MCCV 054 and MCCV 055. For each strain, *O.* cf. *ovata* cells were resuspended from three replicated cultures of 350 ml in exponential phase. Each mother culture allowed for the creation of one series of eight 40 ml samples and was used to characterize the uptake kinetics of one N-source. Incubations started with the addition of ^{15}N ($^{15}NO_3^-$, $^{15}NH_4^+$ or ^{15}N-urea) at eight graded concentrations (0.1, 0.2, 0.5, 1, 2, 3, 5, and 10 µmol N L^{-1}). Samples were incubated for 1h under initial culture conditions. At the end of the incubation, samples were filtered through precombusted (4 h at 450°C) A/E filters (Gelman Sciences) and rinsed with 20 mL of FSW. Filters were dried at 60°C overnight and analyzed by EA-IRMS (Elemental Analysis – Isotope Ratio Mass Spectrometry) for measurements of particulate carbon (PC), particulate nitrogen (PN) and $^{15}N/^{14}N$ isotopic ratios.

An additional experiment was conducted in order to characterize N-urea uptake capabilities of *O.* cf. *ovata* cells taking into account the potential role of preconditioning effects. Cells of *O.* cf. *ovata* were grown on a modified K/10 medium containing three potential N-sources: NO_3^- added at 28.8 µmol N L^{-1} and NH_4^+ and N-urea added at 5 µmol N L^{-1}. These growth conditions were maintained during several culture transfers in batch mode. Then, one culture of 350 mL in exponential phase was used for running a replicated kinetic

experiment, in order to estimate N-urea uptake rates along a concentration gradient of 0 – 10 µmol N L^{-1}.

2.4. Interaction experiments

An experiment was run in order to characterize the potential interaction between NH_4^+ and NO_3^- uptake. Four replicated cultures of 350 mL (MCCV 054) were used to carry out two successive series of incubations, one testing the influence of NH_4^+ on the maximal uptake rate of NO_3^-, and the other the influence NO_3^- on the maximal uptake rate of NH_4^+. During each part of the experiment, the uptake rate of one nutrient, added at a reference concentration of 10 µmol N L^{-1}, was measured as a function of the increasing concentration of the other nutrient (0, 0.1, 0.2, 0.5, 1, 2, 3, 5, and 10 µmol N L^{-1}). For each set, two series of incubations were performed in parallel in order to simultaneously assay uptake rates of both nutrients (NH_4^+ and NO_3^-) for all nutritive conditions. These coupled incubations were based on the same nutrient regime, with only one of the two N-sources labeled with ^{15}N: for one series of samples, the nutrient added under various concentration (0-10 µmol N L^{-1}) was labeled with ^{15}N and, for the other series of samples, the nutrient added at saturating concentration (10 µmol N L^{-1}) was labeled with ^{15}N. Incubations started with the addition of $^{15}NH_4^+$ or $^{15}NO_3^-$ into 40 mL samples and lasted 1h. Incubations ended with the filtration of samples through precombusted (4 h at 450°C) A/E filters (Gelman Sciences). Filters were rinsed with 20 mL of FSW, then dried at 60°C overnight. Analyses were run using EA-IRMS in order to obtain measurements of PN, PC and $^{15}N/^{14}N$ isotopic ratios.

2.5. Diel cycle experiments

Variations of NH_4^+- and NO_3^--uptake by *O.* cf. *ovata* cells were investigated over the diel cycle. For each N-source, three replicated cultures of 350 ml (MCCV 054) were used and

allowed for the preparation of three series of ten resuspended samples, each of them containing 40 mL of –N medium. At the beginning of the incubations, all samples were spiked with a solution of ^{15}N ($^{15}NH_4^+$ or $^{15}NO_3^-$) at 100 µmol N L^{-1} final concentration and were immediately replaced in the culture chamber under initial conditions. This level of concentration was used to ensure N-sufficient conditions all along the experiment duration. Regular stops in the incubations were done during a 24h survey (every 3 h during light periods and three times during the dark phase). At each stop, three samples were taken, one originating from each of the replicated mother cultures. Cells of *O.* cf. *ovata* were collected on precombusted (4 h at 450°C) A/E filters (Gelman Sciences) and rinsed with 20 mL of FSW. Half of the samples were used to follow nutrient concentrations during the experiment, collecting 20 mL of the culture filtrate during microalgal cell collection (before the rinsing step). Filters were finally dried at 60°C overnight, then analyzed by EA-IRMS in order to obtain measurements of PN, PC and $^{15}N/^{14}N$ isotopic ratios.

2.6. Cell counts and nutrient analysis

Growth rate was estimated for each mother culture used for running experiments, from measurements of cell density done just before the resuspension step and 24h earlier. These growth rate estimations allowed to verify that *O.* cf. *ovata* cells were growing under optimal growth conditions when incubations started. For sampling, three 2 mL-aliquots of culture were taken after a gentle mixing of the culture and pooled together before counting. Samples were fixed with acidic lugol solution at 1% (vol/vol) final concentration and stored at +4°C until analysis. Cell counts were done in triplicate using a 1 mL Sedgewick rafter counting chamber. Growth rates were calculated according to Guillard (1973), using the following formula:

$$\mu = \frac{\ln(C2) - \ln(C1)}{t2 - t1}$$

where μ is the growth rate (d^{-1}), C_1 and C_2 are the cell concentrations at time 1 (t_1, d) and time 2 (t_2, d), respectively.

Measurements of NH_4^+ concentrations were performed few hours after sampling, using the fluorometric method (Taylor et al., 2007). Samples for estimations of NO_3^- concentration were immediately frozen at -20°C and stored until analysis. Concentrations of NO_3^- were measured using an automated colourimetry system (Seal Analytical continuous flow AutoAnalyser III, AA3)) as described by Bendschneider and Robienson (1952).

2.7. N-uptake measurements and kinetic parameters

For determination of PN, PC and $^{15}N/^{14}N$ isotopic ratios, EA-IRMS experiments were done with an Elementar Vario Pyro Cube analyzer in CN mode (combustion oven 920°C, reduction oven 600°C) coupled to an Isoprime 100 IRMS (Isotope Resolved Mass Spectrometer). Calibration of measurements was performed with certified caffeine (AIEA-600) and other laboratory standards (commercially available glycine (Sigma), acetinalide (Merck)).

Uptake rates (V in h^{-1}) were calculated from the ^{15}N enrichment of the samples according to Collos (1987). For kinetic and interaction experiments, relationship between uptake rates and concentrations that showed clear saturating kinetics were modeled using the original or a modified equation of the Michaelis-Menten model. When the ^{15}N-source was added at graded concentrations, uptake data were modeled using the original Michaelis-Menten relation:

$$V_N = V_{max\text{-}N} \times [N] / (K_s + [N]) \qquad (1)$$

Where V_N (in h^{-1}) is the N-uptake rate under a nutrient concentration of $[N]$ (in μmol N L^{-1}), $V_{max\text{-}N}$ is the maximal uptake rate (in h^{-1}) and K_s is the half-saturation constant (in μmol N L^{-1}).

For these kinetics, the initial slope α was also calculated from the uptake rate at the concentration of 0.5 µmol N L^{-1} estimated by the model equation as recommended by Hurd and Dring (1990), and was used as an indicator of the competitive ability of the cells at low substrate concentrations.

For the interaction experiment, the exponential decrease in the uptake rate of one nutrient (N1) when increasing the concentration of the other (N2) was fitted to the reverse Michaelis-Menten relation (Varela and Harrison, 1999):

$$V_{N1} = V_{max\text{-}N2=0} \times (1 - (I_{max} \times [N2] / (K_I + [N2])) \quad (2)$$

Where the N-uptake rate of the nutrient N1, V_{N1} (in h^{-1}), is function of the maximum uptake rate without inhibition ($V_{max\text{-}N2=0}$, in h^{-1}), the concentration of the inhibitory nutrient *[N2]* (in µmol N L^{-1}), the maximum inhibition I_{max} (values from 0 to 1) and of the inhibition constant K_I (concentration of N2 at which $I = I_{max} / 2$, in µmol N L^{-1}).

For the diel cycle experiment, uptake rates during each light and dark period were estimated from linear regressions of isotopic ratios *vs*. time.

Values of kinetic parameters were obtained from non-linear regressions of data sets, using the Statgraphics Centurion software (Manugistics, Inc.). Statistical tests (significance and comparison of regression slopes) were performed using the same software.

3. Results

3.1. Culture medium and cellular growth

In terms of nutrient availability, low N-conditions were verified in the culture medium used for running the experiments. The medium used for resuspension and incubation of *O.* cf. *ovata* cells was characterized by an NH_4^+ concentration of 0.82 µmol N L^{-1} (SD± 0.27 µmol N L^{-1}) and NO_3^- concentration of 1.00 µmol N L^{-1} (SD± 0.01 µmol N L^{-1}).

All cultures used for running the experiments were growing exponentially, under similar growth conditions. At resuspension time, the growth rates of replicated cultures were, on average, 0.39 d^{-1}, 0.45 d^{-1} and 0.51 d^{-1} for the kinetic experiment, the interaction experiment and the diel cycle experiment, respectively. Quantities of particulate organic nitrogen (PN) and carbon (PC) allowed for estimations of C:N (atomic) ratio. Mean C:N ratios of 12.3 (SD± 1.0) and 12.5 (SD± 0.8) were estimated for the strains MCCV 054 and MCCV 055, respectively, from the compilation of data sets obtained during short-term experiments (1h-incubations). Extra-cellular mucilage might have interfered in the precision of these estimations. Trends observed in C:N ratios did not allow for a detailed characterization of the coupling between N- and C-fluxes over the diel cycle.

3.2. Uptake rates during kinetic experiments

Variations of ^{15}N-enrichment over 1h-incubations showed that *O.* cf. *ovata* cells were able to use dissolved inorganic N-sources (NH_4^+ and NO_3^-) and dissolved organic nitrogen (N-urea). Similar saturating kinetic curves were observed for the two strains of *O.* cf. *ovata* tested, when NH_4^+, NO_3^- or N-urea was added as a unique N-source along a gradient of 0 – 10 µmol N L^{-1} (Figure 1). Out of the three potential N-sources tested, *O.* cf. *ovata* cells showed a clear preference pattern following the ranking: NH_4^+ > NO_3^- > N-urea. No potential preconditioning effect influenced this ranking because *O.* cf. *ovata* cells were grown in K/10 medium with NH_4^+ and NO_3^- added as N-sources and an acclimation of cells to the presence of N-urea in the culture medium did not induce a clear modification of N-urea kinetics (Figure 1C). For both strains, on average along the whole gradient, NH_4^+ uptake rate was 4 to 5 times higher than NO_3^- uptake rate, when NO_3^- uptake rate was 8 to 9 times higher than N-urea uptake rate.

Relationships between uptake rates and nutrient concentrations were characterized by the Michaelis-Menten model. Similar estimations of kinetic parameters (V_{max}, K_s, α) were obtained for the two strains (Table 1). This similarity allowed to characterize abilities of *O.* cf. *ovata* cells isolated in the Bay of Villefranche-sur-mer by a maximal uptake rate (V_{max}) of 0.021 h^{-1} (SD± 0.001 h^{-1}), 0.008 h^{-1} (SD± 0.003 h^{-1}), 0.0005 h^{-1} (SD± 0.0001 h^{-1}) for NH_4^+, NO_3^- and N-urea, respectively. The associated K_s values were 0.5 µmol N L^{-1} (SD± 0.1 µmol N L^{-1}), 2.3 µmol N L^{-1} (SD± 2.1 µmol N L^{-1}) and 0.3 µmol N L^{-1} (SD± 0.1 µmol N L^{-1}) for NH_4^+, NO_3^- and N-urea, respectively. For the characterization of abilities under low nutrient concentrations, the initial slope of kinetic curves (α) was 0.011 L µmol N^{-1} h^{-1} (SD± 0.001 L µmol N^{-1} h^{-1}), 0.002 L µmol N^{-1} h^{-1} (SD± 0.001 L µmol N^{-1} h^{-1}) and 0.0003 L µmol N^{-1} h^{-1} (SD± 0.0001 L µmol N^{-1} h^{-1}) for NH_4^+, NO_3^- and N-urea, respectively.

3.3. Uptake rates during interaction experiments

The influence of NH_4^+ on the maximal uptake rate of NO_3^- ($V_{max\text{-}NO3\text{-}}$) was analyzed from estimations of $^{15}NH_4^+$ and $^{15}NO_3^-$-uptake rates after an addition of 10 µmol N L^{-1} of NO_3^- and an NH_4^+ concentration varying from 0 to 10 µmol N L^{-1} (Figure 2A). In the presence of 10 µmol N L^{-1} of NO_3^-, the relationship between NH_4^+ uptake rate and NH_4^+ concentration displayed a saturating kinetic (Figure 2A) with a high V_{max} of 0.034 h^{-1} and a K_s value (0.7 µmol N L^{-1}) close to estimations done when NH_4^+ was added as the only N-source (Table 1). Variations of $V_{max\text{-}NO3\text{-}}$ along the NH_4^+ gradient showed an exponential decrease that could be characterized by fitting the data set to the reverse Michaelis-Menten model (2) (Figure 2A). The inhibition parameters generated by the model (Table 1) showed a strong NH_4^+ inhibition of NO_3^- uptake rate, with a maximum inhibition value of 67% and a K_I value of 6.2 µmol N L^{-1}.

Under reverse nutrient conditions (addition of 10 µmol N L^{-1} of NH_4^+ along a NO_3^- gradient of 0-10 µmol N L^{-1}), variations of NO_3^- uptake rates were characterized by kinetic parameters (V_{max} = 0.008 h^{-1}, K_s = 2.8 µmol N L^{-1}) after fitting the data set to the Michaelis-Menten model (1) (Figure 2B, Table 1). Concerning variations of $V_{max\text{-}NH4+}$ with increasing NO_3^- concentration, the reverse Michaelis-Menten model did not converge, however, even if the NH_4^+ uptake rate obtained after addition of 10 µmol N L^{-1} of NH_4^+ and 10 µmol N L^{-1} of NO_3^- appeared to be slightly low Figure 2B). Globally, NH_4^+ uptake rate was stable along the NO_3^- gradient, with $V_{max\text{-}NH4+}$ = 0.026 h^{-1} (SD± 0.002 h^{-1}).

3.4. Diel cycle experiment

After the addition of 100 µmol N L^{-1} of ^{15}N ($^{15}NH_4^+$ or $^{15}NO_3^-$), linear decreases in nutrient concentrations were observed with time for both series of incubations (data not shown). Despite the consumption of nitrogen sources, final concentrations of NH_4^+ and NO_3^- in replicated flasks were 84 µmol N L^{-1} (SD± 9 µmol N L^{-1}) and 82 µmol N L^{-1} (SD± 4 µmol N L^{-1}), respectively, after 24h of incubation. These estimations showed the maintenance of N-replete conditions over the whole experiment duration (24h).

According to estimations of ^{15}N-enrichment of *O.* cf. *ovata* cells over the diel cycle, microalgal cells were capable of using both NH_4^+ and NO_3^- during the light and the dark periods (Figure 3). For both NH_4^+ and NO_3^-, linear increases in ^{15}N-atom (%) of *O.* cf. *ovata* cells were observed with time during each consecutive light and dark periods. During the first light period, trends were highly significant for both NH_4^+ and NO_3^- (linear regressions with r^2 = 0.99, $p < 0.001$, Table 2 and Figure 3). Slopes of linear regressions allowed for precise estimations of N-uptake rates and indicated that NH_4^+-uptake rate was higher (0.032 h^{-1}) than but also close to NO_3^--uptake rate (0.030 h^{-1}) during the light period. During the subsequent dark phase, values of ^{15}N-atom (%) were coherent between replicated flasks and showed an

increasing trend with time for both NH_4^+ and NO_3^- (Figure 3). Slopes of linear regressions that modelled dark processes were not significant ($r^2 \geq 0.78$, $p \geq 0.06$, Table 2), however, suggesting a lack of precision in dark N-uptake rate estimations. From present results, dark uptake rates corresponded to 19% and 10% of light uptake rates for NH_4^+ and NO_3^-, respectively.

4. Discussion

Nutrient uptake capabilities of phytoplankton cells are known to vary as a function of cell size (Litchman et al., 2007), nutritional history and physiological status of the cells (Mulholland and Lomas, 2008), growth rates (Maguer et al., 2007), N substrate interactions (Maguer et al., 2007; Jauzein et al., 2008a) and environmental factors such as irradiance and temperature (Lomas and Glibert, 1999; Kudela and Cochlan, 2000). Consequently, it is often difficult to determine taxa-specific differences in uptake capabilities and environmental control on uptake from field studies. Characterization of uptake capabilities under controlled conditions from culture studies gives the opportunity to better understand uptake regulation. Relatively few studies have determined N-uptake kinetics from cells that are nitrogen replete. In previous culture works, kinetic parameters were often characterized for cells under N-limited conditions or after several days of N-starvation (e.g. Nishikawa et al., 2009; Kwon et al., 2013). Nutrient depletion can lead to transient or surge uptake, however, when an uncoupling between nutrient uptake and growth occurs (Dortch et al., 1982; Mulholland and Lomas, 2008). In the present study, efforts were made at characterizing N-uptake of cells growing under optimal conditions. Experiments started by the resuspension of exponentially growing cells in –N medium, when physical conditions were optimized to ensure no limitation of uptake capabilities. Ammonium concentrations used to monitor uptake rates

were also lower than concentrations known to potentially inhibit growth of dinoflagellate cells (Collos and Harrison, 2014; Siu et al., 1997). Thus, patterns and non-linear regressions of data sets obtained allow for the characterization and parameterization of functional responses of *O.* cf. *ov*ata cells to N-sources availability. In particular, values of parameters, such as half-saturation constants and inhibition parameters, are crucial for ecological modeling and understanding of forcing functions (Tian, 2006). In the present study, estimations were done when uptake of microalgal cells was tightly coupled with growth; values of parameters obtained can be used for the definition of mechanistic formulations that simulate the function for nutrient limitation of *O.* cf. *ov*ata growth.

Nutrient uptake by microalgal cells is an active process, whose response to extracellular nutrient concentration can generally be modelled as that of an enzyme, using Michaelis-Menten kinetics. In the Michaelis-Menten model, the maximum uptake rate (V_{max}) and half-saturation constant (K_s) are often said to be biological parameters, dependent on the number of carrier sites on cell membrane and specific efficiency of each transporter (Aksnes and Egge, 1991; Litchman et al., 2007). Estimations of these parameters, along with the affinity coefficient α, have been used to assess the relative preference for different N-substrates or competitive abilities between species under various nutritive conditions (Cochlan et al., 2008; Mulholland and Lomas, 2008). In the present study, kinetic curves were characterized in the range of few µmol N L^{-1}, for two strains isolated during the same bloom event. They show that *O.* cf. *ovata* cells are able to use dissolved inorganic N-sources (NO_3^- and NH_4^+) and dissolved organic sources (N-urea) with a clear preference pattern: this pattern follows the ranking NH_4^+ > NO_3^- > N-urea and was well defined all over the gradient tested (0.1 – 10 µmol N L^{-1}). Preconditioning effects did not interfere in these trends for neither of the N-sources tested. Results also show that the preferential uptake of NH_4^+ relative to NO_3^- is accentuated for *O.* cf. *ovata* cells by an inhibitory effect of NH_4^+ concentration on NO_3^-

uptake capabilities. Conversely, no influence of NO_3^- availability on NH_4^+ uptake was observed for this species. Repression of NO_3^- uptake by NH_4^+ has been well studied for many decades for several phytoplankton species (Glibert et al., 2016), but never for benthic dinoflagellates. The maximal inhibition estimated for *O*. cf. *ovata* cells (I_{max} = 67%) is similar to values reported for other dinoflagellates (*Alexandrium minutum*, *Prorocentrum minimum*, *Gyrodinium uncatenum*) (Lomas and Glibert, 1999; Maguer et al., 2007). The half-inhibition constant (K_I) estimated for *O*. cf. *ovata* (6.2 µmol N L^{-1}) appears really high compared to values reported in previous studies, however, in particular for N-sufficient microalgal cells (e.g., Lomas and Glibert, 1999; Maguer et al., 2007); this suggests a low sensitivity of NO_3^- uptake of *O*. cf. *ovata* cells to NH_4^+ concentration, in particular under low NH_4^+ availability.

On a broader point of view, NH_4^+ is commonly found to be the preferred N-source over NO_3^- and N-urea for phytoplankton uptake (Mulholland and Lomas, 2008; Glibert et al., 2016, and references therein). The preferential use of NH_4^+ is attributed largely to the low energetic demand for its uptake and assimilation (Syrett, 1981). Exceptions have been documented, however, such as the preference of *Pseudo-nitzschia australis* for NO_3^- over NH_4^+ and N-urea reported by Cochlan et al. (2008). Most importantly, the preference for NH_4^+ over NO_3^- may strongly depend on the range of nutrient considered. As well explained and conceptualized by Glibert et al. (2016), the preference for NH_4^+/NO_3^- can be inverted under high nutrient conditions, due to either *(i)* the toxicity and growth inhibition of high NH_4^+ concentrations and/or *(ii)* the potential acceleration of NO_3^- uptake in the presence of NO_3^- that can lead to biphasic kinetics. In the present study, results suggest that such an acceleration on NO_3^- uptake occurs for *O* cf. *ovata* cells exposed to high NO_3^- concentrations. Indeed, NH_4^+ uptake rates were 4 to 5 times higher than NO_3^- uptake rates during kinetic experiments that were conducted in the range 0.1 – 10 µmol N L^{-1}. During the light period of the diel-cycle experiment, estimations of mean NO_3^- uptake rate (0.030 h^{-1}) were close to

estimations of mean NH_4^+ uptake rate (0.032 h^{-1}) after addition of 100 µmol N L^{-1} of NH_4^+ or NO_3^-, however. This mean NO_3^- uptake rate obtained under high nitrate concentrations was more than 3 times higher than the V_{max} value of 0.008 h^{-1} estimated in the range 0.1 – 10 µmol N L^{-1}. Such variations in N-uptake capabilities are consistent with the existence of a two-component NO_3^- uptake system, involving a high-affinity low-capacity constitutive component and a low-affinity high capacity inducible uptake component (Glibert et al., 2016). Results obtained in the present study are consistent with the threshold of about 60 µmol N L^{-1} that was reported for the transition between biphasic kinetics of NO_3^- uptake for several phytoplankton species (Collos et al., 1992; Lomas and Glibert, 2000).

The monitoring of N-uptake rates done over the diel cycle allows for the characterization of dark N-uptake capability of *O* cf. *ovata* under N-sufficient conditions. Dark uptake of N compounds is commonly observed in marine waters (e.g. Cochlan et al., 1991; Fan and Glibert, 2005; Maguer et al., 2015). For photosynthetic cells, nutrient uptake and assimilation in darkness occurs at the expense of previously accumulated carbon that will supply dark processes with energy (ATP), reductant (NAD(P)H) and C-skeletons (Turpin, 1991). Photosynthetic carbon can be stored in excess during the light period into C-rich and N-free macromolecules, such as carbohydrates (Clark and Flynn, 2002; Granum et al., 2002) or neutral lipids (Fabrégas et al., 2002). Detailed observations of morphological and metabolic features *O*. cf. *ovata* cells revealed that their cytoplasm is often full of neutral lipid droplets, in all stages of growth under N-sufficient conditions (Honsell et al., 2013). This suggests a potential for C-storage strategies that could support dark processes. Various taxonomic groups of phytoplankton, including dinoflagellates, prymnesiophytes and diatoms, carry out uptake at night under N-sufficient conditions (e.g. Paasche et al., 1984; Clark et al., 2002; Needoba and Harrison, 2004), with reported dark:light (D/L) uptake ratios ranging from 1% to 75% for NO_3^- and from 21% to 100% for NH_4^+ (Jauzein et al., 2011, and references therein). With D/L

uptake ratios of 10% and 19% measured for NO_3^- and NH_4^+ in the present study, *O*. cf. *ovata* shows low capabilities for N-uptake in darkness, at least under N-sufficient conditions. Thus, the lipid storage strategy noted by Honsell et al. (2013) has to be explored further through additional experiments to define its implications in the species competitiveness. As dark N-uptake processes have been shown to be enhanced under N-limited conditions (Paasche et al., 1984; Turpin, 1991), it could also be interesting to complete the characterization of dark N-uptake capabilities of *O*. cf. *ovata* testing N-limited conditions and/or *in situ* measurements.

According to present results, ability to take up nutrients in darkness cannot be seen as a competitive advantage for *O*. cf. *ovata*. Additional information about species competitiveness can be defined from nutrient uptake kinetic parameters (e.g. Smayda, 1997; Litchman et al., 2007). On a broad point of view, reviews of parameter values reported in both field studies and experimental works show that dinoflagellates have generally higher K_s for DIN than diatoms (Smayda, 1997; Kudela et al., 2010). Diatoms were also characterized by higher carbon-specific V_{max} (values standardized by C units in order to diminish the effect of cell size) for NO_3^- than other taxa, including dinoflagellates (Litchman et al., 2007). Thus, a general trend of low competitive abilities for acquisition of DIN can be defined for dinoflagellates that puts emphasis on the contribution of organic matter in fueling dinoflagellate blooms in oligotrophic or mesotrophic coastal waters (e.g. Collos et al., 2004).

For the benthic compartment in particular, studies reporting uptake parameters are rare. Estimations of NO_3^- uptake kinetics were done for several species of benthic diatoms by Kwon et al. (2013) and for *O*. cf. *ovata* by Pistocchi et al. (2014). These studies report surprisingly high K_s values (ranging from 6.75 µmol N L^{-1} to 9.29 µmol N L^{-1}) compared to literature on planktonic species (Kudela et al., 2010). For *O*. cf. *ovata*, K_s value reported by Pistocchi et al. (2014) (8.4 µmol N L^{-1}) is strongly higher than values (< 4 µmol N L^{-1}) obtained in the present study for strains isolated from French coastal waters. Strong

differences in N-uptake abilities can be observed between strains of the same species (Jauzein et al., 2008b). It has also to be noted that some methodological choices can partly explain the high level of K_s reported in Kwon et al. (2013) and Pistocchi et al. (2014) for NO_3^- uptake of benthic species. These studies were conducted on a large NO_3^- concentration gradient, ranging from 1 µmol N L^{-1} to 100 µmol N L^{-1}, when kinetic experiments of the present study were run on 0.1-10 µmol N L^{-1}. Collos et al. (2005) pooled data from 20 different studies dealing with phytoplankton uptake and showed a direct increase in K_s values with maximal NO_3^- concentration used in the respective studies. This correlation can be explained by an acclimation of microalgal cells to high NO_3^- concentrations coming from the existence of multiphasic uptake systems (Collos et al., 2005; Glibert et al., 2016); as explained above, such a biphasic system is suspected for *O.* cf. *ovata*. Thus, kinetic parameters estimated by Kwon et al. (2013) and Pistocchi et al. (2014) are probably representative of microalgal cell responses to high nutrient availability, but might not provide an appropriate representation of uptake capabilities under low N-conditions, making these results hardly comparable to the present study.

Uptake kinetics are also known to potentially vary with N-history and physiological status of microalgal cells (Mulholland and Lomas, 2008). When compiling exclusively results obtained from actively growing cultures, kinetic parameter values determined for NH_4^+, NO_3^- and N-urea uptake by *O.* cf. *ovata* in the present study are in accordance with values reported for other planktonic dinoflagellates (Table 3). For NO_3^-, the compilation of these data sets also highlights higher V_{max} for diatoms compared to dinoflagellates, when no pattern can be defined for K_s (Table 3).

In the Bay of Villefranche-sur-mer (South of France), nutritive conditions can be seen as oligotrophic to mesotrophic (Selmer et al., 1993). Recent blooms of *O.* cf. *ovata* in this bay were observed when NH_4^+, NO_3^- and N-urea concentrations ranged between 0.04 – 0.27 µmol

N L^{-1}, 0.20 – 2.12 µmol N L^{-1} and 0.3 – 2.25 µmol N L^{-1}, respectively (data not shown). Under such low availability in N-sources, affinity (K_s) more than velocity (V_{max}) should control species competitiveness. In the present study, values of K_s estimated for NH_4^+, NO_3^- and N-urea uptake by *O.* cf. *ovata* are in the upper part of these ranges of *in situ* concentrations. This suggests a good adaptation of the cells to field conditions but not a strong affinity strategy. To go further on the definition of specific competitive abilities, affinity for N-sources can also be compared to other taxa taking into account field studies and other culture works. Indeed, characterization of N-uptake kinetics of microalgal cells as a function of N-limitation did not always show variations of K_s with N-status or cell nutritional history (Hu et al., 2014; Maguer et al., 2007). According to the review done by Kudela et al. (2010), *O.* cf. *ovata* shows relatively high K_s (low affinity) for NO_3^-, as most of the dinoflagellates do, but a low K_s for NH_4^+ compared to both dinoflagellates and diatoms. This defines strong competitive abilities of *O.* cf. *ovata* for NH_4^+ uptake under low N-conditions, like the ones encountered during bloom seasons in the Bay of Villefranche-sur-mer. Mixotrophic abilities of *O.* cf. *ovata* have not been fully characterized yet and are still a matter of debate (Escalera et al., 2014). Current results characterize the potential use of N-urea as a source of labile DON for *O.* cf. *ovata* cells. According to uptake capabilities and ranges of *in situ* conditions, contribution of N-urea to growth of *O.* cf. *ovata* in Villefranche-sur-mer Bay is probably low compared to DIN sources, even if this source can be rapidly regenerated in the water column (Lomas et al., 2002). Out of the three N-sources tested in the present study, the main N-source fueling blooms *O.* cf. *ovata* in the Bay of Villefranche-sur-mer is probably NH_4^+, a recycled N-source for which *O.* cf. *ovata* showed highest uptake rates and good competitive abilities.

5. Conclusions

The present study provides a detailed parameterization of N-uptake by *O.* cf. *ovata*. Kinetic and inhibition parameters can be used for the definition of mechanistic formulations in order to simulate growth limitation by nutrient availability. Ability to take up nutrients in darkness could not be defined as a competitive advantage for *O.* cf. *ovata* during exponential growth. Conversely, a strong affinity for NH_4^+ was observed for *O.* cf. *ovata* cells and may partly explain the success of this species during the summer season in the Bay of Villefranche-sur-mer (France). Further studies will be necessary to clarify the role of organic matter in growth of *O.* cf. *ovata* cells during bloom development and maintenance. It could also be interesting to better characterize links between C fluxes and other metabolic processes in *O.* cf. *ovata* cells, trying to define for example the potential role of the lipid storage strategy in terms of species competitiveness.

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Figures

Figure 1. Kinetic curves of NH_4^+, NO_3^- and N-urea uptake for *Ostreopsis* cf. *ovata*. Data points show variations of NH_4^+, NO_3^- and N-urea uptake rates as a function of each respective nutrient concentration for the French strains MCCV 054 (A) and MCCV 055 (B). Detailed representations of variations of N-urea uptake rate, with or without acclimation to N-urea as a N-source, are shown for MCCV 054 (C) and MCCV 055 (D). Respective modeled curves (straight lines for NH_4^+, dotted lines for NO_3^- and dashed lines for N-urea) correspond to Michaelis-Menten model and values of parameters (V_{max}, K_s, α), as well as associated r^2, are listed in Table 1.

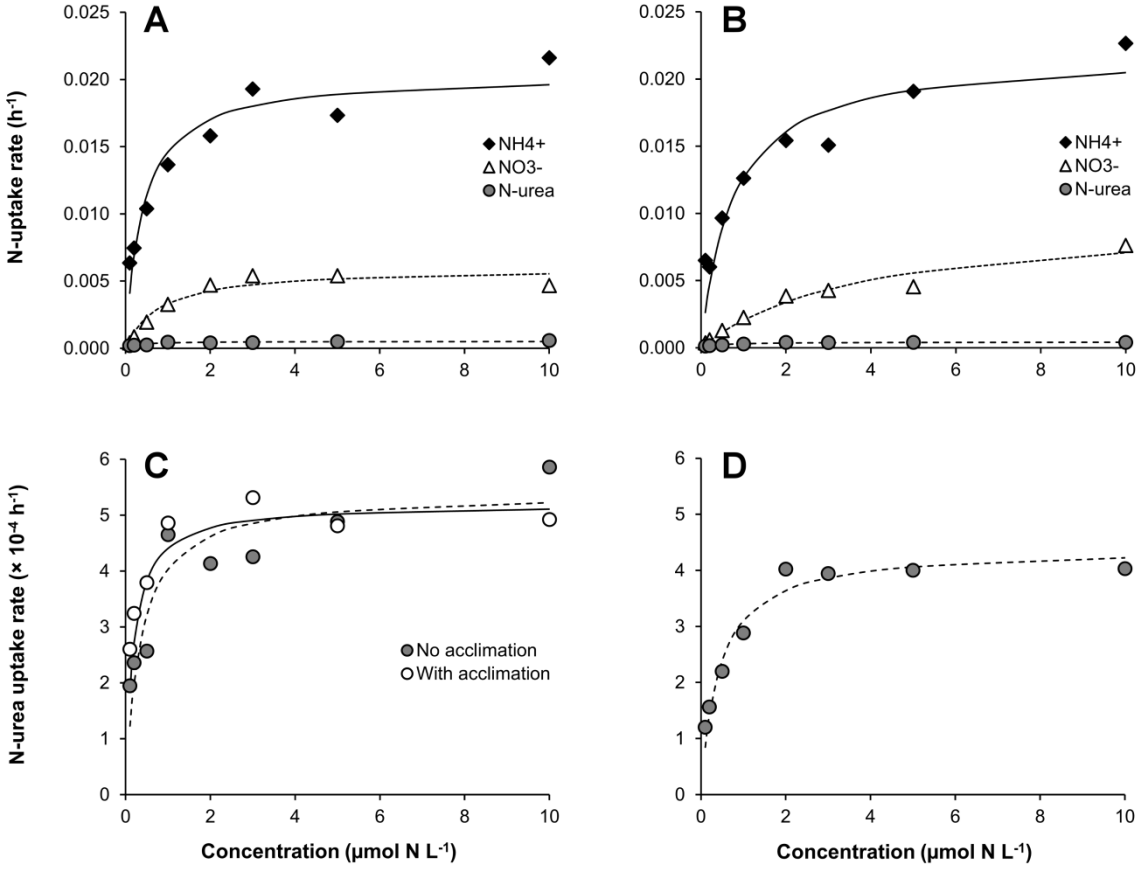

Figure 2. Variations in NH_4^+ and NO_3^- uptake rates for the strain MCCV 054 of *Ostreopsis* cf. *ovata* after an addition of 10 µmol N L^{-1} of NO_3^- and along a graded NH_4^+ concentration of 0 to 10 µmol N L^{-1} (A), or after an addition of 10 µmol N L^{-1} of NH_4^+ and along graded NO_3^- concentration of 0 to 10 µmol N L^{-1}. The modeled curves of NH_4^+ and NO_3^- uptake data correspond to solid and dotted lines, respectively. Values of parameters used for modelling these data sets are listed in Table 1 with associated r^2 values.

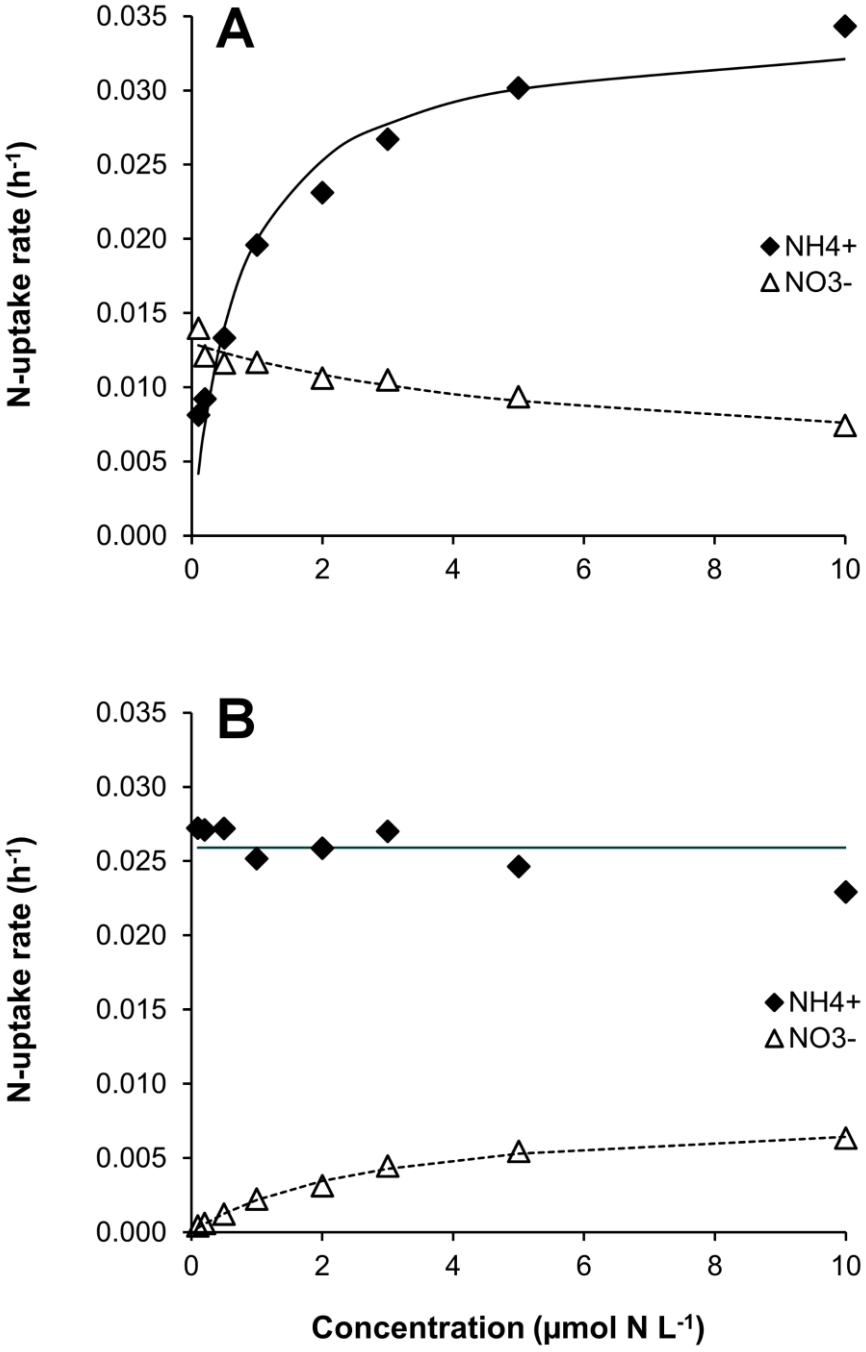

Figure 3. Variations in ^{15}N isotopic ratios of *Ostreopsis* cf. *ovata* cells over 24h, after addition of 100 µmol N L^{-1} of $^{15}NH_4^+$ or $^{15}NO_3^-$. The dark period is indicated by the horizontal solid line. Vertical lines indicate standard deviations from three replicate cultures. Linear regressions of data sets are represented by solid lines and dashed lines for incubations with $^{15}NH_4^+$ and $^{15}NO_3^-$, respectively. For the first light period, equations of linear regressions are Y = 0.0318 X – 0.3111 (r^2 = 0.99) for $^{15}NH_4^+$ incubations and Y = 0.0300 X – 0.3415 (r^2 = 0.99) for $^{15}NO_3^-$ incubations. For the dark period, equations of linear regressions are Y = 0.0059 X + 0.2558 (r^2 = 0.99) for $^{15}NH_4^+$ incubations and Y = 0.0029 X + 0.2421 (r^2 = 0.78) for $^{15}NO_3^-$ incubations.

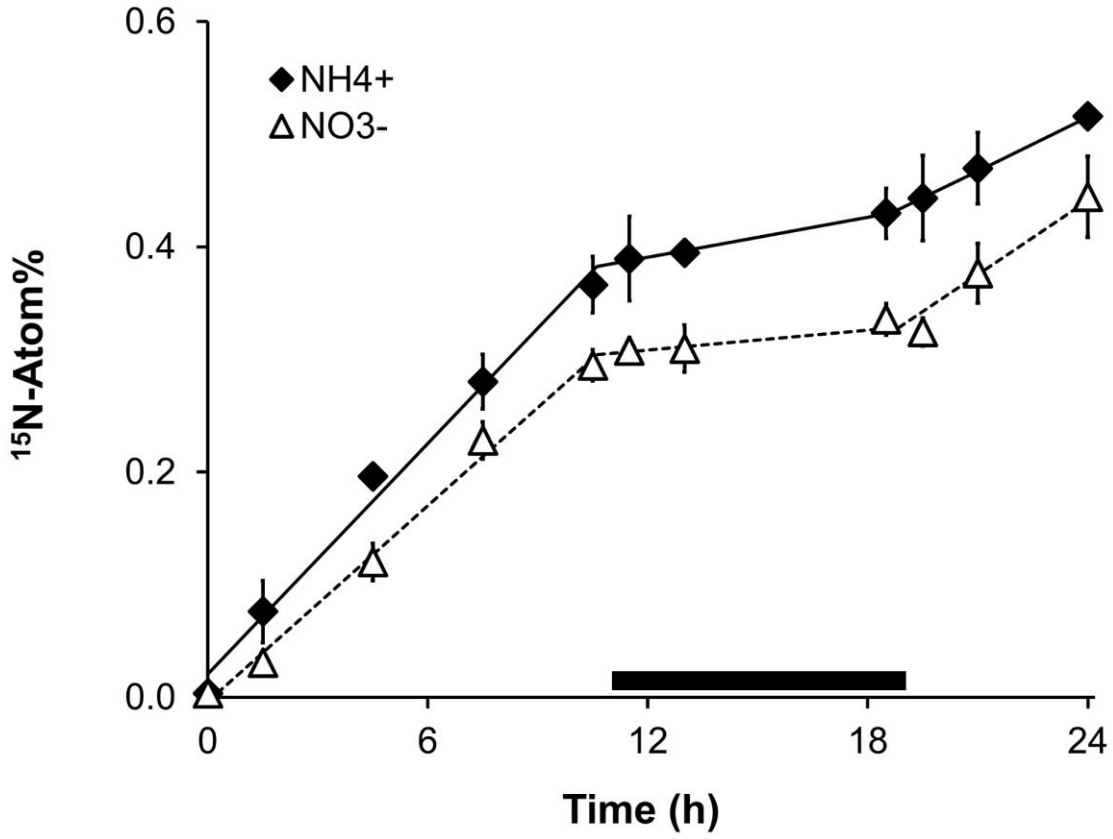

Table 1. Values of kinetic parameters (V_{max} in h^{-1}, K_s in µmol N L^{-1}, α in L µmol N^{-1} h^{-1}) and inhibition parameters ($V_{max\text{-}N=0}$ in h^{-1}, K_I in µmol N L^{-1}, I_{max}) obtained for *Ostreopsis* cf. *ovata* under various culture conditions. Uptake abilities were characterized for three potential N-sources (NH_4^+, NO_3^- and N-urea), added under graded nutrient concentrations (listed in µmol N L^{-1}), as a unique N-source or in combination with another one. Non-linear regressions were based on the Michaelis-Menten model or the reverse Michaelis-Menten relation.

Experiment (Strain)	^{15}N-addition		Kinetic parameters			r^2
	N-source	Concentration	V_{max}	K_s	α	
Kinetics (MCCV 054)	NH_4^+	0.1 - 10	0.020	0.40	0.0114	0.92
	NO_3^-	0.1 - 10	0.006	0.80	0.0023	0.94
	N-urea	0.1 - 10	0.0005	0.28	0.0003	0.82
Acclimation to N-urea	N-urea	0.1 - 10	0.0005	0.11	0.0004	0.93
Kinetics (MCCV 055)	NH_4^+	0.1 - 10	0.021	0.58	0.0098	0.89
	NO_3^-	0.1 - 10	0.010	3.73	0.0011	0.96
	N-urea	0.1 - 10	0.0004	0.38	0.0002	0.96
Interaction NH_4^+/NO_3^- (MCCV 054)						
NH_4^+ gradient, 10 µM NO_3^-	NH_4^+	0.1 - 10	0.034	0.72	0.0139	0.95
NO_3^- gradient, 10 µM NH_4^+	NO_3^-	0.1 - 10	0.008	2.77	0.0012	0.99

	Inhibition parameters		
	$V_{max\text{-}N=0}$	K_I	I_{max}

NH_4^+ gradient, 10 µM NO_3^-	NO_3^-	10	0.013	6.24	0.67	0.91

Table 2. Estimations of ^{15}N-uptake rates (in h^{-1}) of *Ostreopsis* cf. *ovata* cells in cultures under both light and dark phases of the diel cycle.

Uptake abilities were characterized for NH_4^+ and NO_3^- under N-sufficient conditions.

Experiment (Strain)	^{15}N-addition		Mean uptake rate	r^2 (p value)
	N-source	Concentration		
Diel cycle (MCCV 054)				
First light period	NH_4^+	100	0.032	0.99 ($p < 0.001$)
	NO_3^-	100	0.030	0.99 ($p < 0.001$)
Dark period	NH_4^+	100	0.006	0.99 ($p = 0.06$)
	NO_3^-	100	0.003	0.78 ($p = 0.12$)

Table 3. Values of kinetic parameters (maximal uptake rate V_{max} in h^{-1} and half-saturation constant K_s in µmol N L^{-1}) reported for phytoplankton species from culture experiments with N-sufficient cells. Ranges of nutrient concentration used for kinetics are indicated in µ mol N L^{-1}. Most of the references listed reported results obtained for actively growing N-replete cells. Some experiments were conducted from recently N-depleted cultures at the end of the growth phase and are indicated with a footnote.

Species	Substrate	NH_4^+		NO_3^-		N-urea		Reference
	concentration	V_{max}	K_s	V_{max}	K_s	V_{max}	K_s	
Diatoms								
Chaetoceros sp.	0.01 - 40			0.110	3.10			Lomas and Glibert (2000)
Pseudo-Nitzschia australis	0.1 - 40	0.071	5.37	0.105	2.82	0.0300		Cochlan et al. (2008)[ab]
Skeletonema costatum	0.01 - 40			0.100	0.40			Lomas and Glibert (2000)
Thalassiosira weissflogii	0.01 - 40			0.170	2.80			Lomas and Glibert (2000)
Dinoflagellates								
Alexandrium catenella	0.1 - 10	0.002 - 0.026	0.1 - 6.2			0.0004 - 0.001	0.6 - 2.3	Jauzein et al. (2008b)[c]
Alexandirum minutum	0.1 - 30		0.33		0.28			Maguer et al. (2007)[d]
Ostreopsis cf. *ovata*	0.01 - 10	0.021	0.49	0.008	2.27	0.0005	0.33	Present study
Prorocentrum minimum	0.01 - 40			0.050	5.00			Lomas and Glibert (2000)
	0.4 - 30		2.48		5.18		1.82	Fan et al. (2003)
	0.2 - 20	0.046	1.25			0.0004	0.05	Li et al. (2011)
Prorocentrum donghaiense	0.1 - 50	0.075	7.10			0.0400	0.12	Hu et al. (2014)
Haptophyte								

Pavelova lutheri	0.01 - 40			0.120	22.70			Lomas and Glibert (2000)
Chlorophyte								
Dunaliella tertiolecta	0.01 - 40			0.030	11.10			Lomas and Glibert (2000)
Raphydophyte								
Heterosigma akashiwo	0.1- 12	0.028	1.44	0.018	1.47	0.0029	0.42	Herndon and Cochlan (2007)[b]

Footnotes

[a] Non-saturating kinetics were observed for N-urea uptake; the V_{max} value indicated for N-urea uptake of *P. asutralis* in this table corresponds to the uptake rate estimated at 36 µmol N L^{-1}.

[b] Experiments were conducted on recently N-depleted cultures, in late growth phase.

[c] Reported values correspond to incubations done just after and 3h after resuspension of N-replete cells in -N medium.

[d] Reported values correspond to results obtained for the higest growth rate, when µ/µmax = 0.42.