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Dark metabolism and carbonï nitrogen uncoupling in the toxic dinoflagellate *Alexandrium catenella* (Dinophyceae)

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Abstract :

Uptake rates of three potential N-sources (ammonium, nitrate and N-urea) and two potential C-sources (HCO_3^- and C-urea) were estimated during growth of *Alexandrium catenella* in both light and dark phases. According to the variations observed in ^{13}C-isotopic ratio, *A. catenella* cells were not able to use C-urea. Furthermore, decreases in ^{13}C cell content during darkness revealed a probably high involvement of C recently fixed in dark respiration. Dark N-uptake capacities of *A. catenella* were characterized by dark/light uptake ratio of 27%, 43% and 65% for NO_3^-, NH_4^+ and N-urea, respectively. An accumulation of C-rich compounds during the light period was highlighted through strong diel variations in C:N ratio and would provide C and energy for these dark uptake processes indicating an uncoupling between N and C metabolism. Total costs in terms of C associated with dark N-uptake and assimilation were estimated and revealed that the main part of those costs may be associated with maintenance metabolism in *A. catenella* cells. The relatively low C-costs of biosynthesis in darkness suggest that dark uptake and C-storage strategies correspond to a benefit in terms of competitiveness for *A. catenella*, optimized by the migrating abilities of this species.

Highlights

3 Dark nitrogen uptake and carbon storage of *Alexandrium catenella* are reported. 3 Strong diel variations in C:N ratio show uncoupling between C and N metabolism. 3 Dark/light uptake ratios are 27%, 43%, and 65% for nitrate, ammonium, and urea. 3 Estimations of total C costs associated with dark N metabolism are 10ï 14 gC gN^{-1}. 3 Dark N uptake represents a low additional cost in terms of C for this species.

Abbreviations :
- D/L, dark:light
- PC, particulate carbon
- PN, particulate nitrogen

Keywords : Diel cycle ; Dark uptake ; Nitrogen ; Carbon ; Uncoupling ; *Alexandrium catenella*

1. Introduction

Biological processes of phytoplankton cell metabolism may show a diel periodicity, initiated by extracellular oscillations in environmental growth conditions, internal variability associated with cell division cycle or circadian rhythms driven by a biological clock (*cf*. Sweeney, 1987; Edmunds, 1988). For phytoplankton cells, irradiance oscillation throughout the light:dark cycle corresponds to the most significant cyclic environmental variable: it can regulate metabolic processes through the influence of day or night length, maximum irradiance or spectral composition, which can act individually or in combination (Prézelin, 1992). This regulation may not only affect photosynthesis activity, but also metabolic processes dependent on end products of photosynthesis. For nutrition metabolism, nitrogen (N) uptake and assimilation may be linked with photosynthesis through different metabolic pathways (Turpin, 1991) as they require production of ATP, reducing power (NAD(P)H) and carbon (C) fixation. A strong coupling between N and C metabolism may lead to diel periodicity of N uptake and assimilation, provided that cells are in N-sufficient conditions; N-limitation conditions may induce the storage of C-rich compounds which facilitates dark nutrition processes and the decline of diel periodicity (Turpin, 1991).
In the previous study of Jauzein et al. (2008), diurnal patterns (*i.e*. during the light period) were highlighted for *Alexandrium catenella* cells and a short-term (6h) increase in C:N composition ratios was interpreted as a consequence of uncoupling between C and N uptake despite N-sufficient conditions. The present work was conducted to carry out additional analyses of N-uptake and C-fixation by *A. catenella* cells throughout a complete light:dark cycle, in particular to test the potential for dark N-uptake which would be sustained by C accumulation during the day as reflected by the diurnal increase in C:N ratio. Three potential N-sources that are known to yield similar growth rates for *A. catenella* (Collos et al., 2004) were tested: nitrate (NO_3^-), ammonium (NH_4^+) and N-urea. Some phytoplankton species are capable of utilizing urea not only as a N-source, but also as a C-source (Lomas, 2004). This study gave the opportunity to test the potential use of C-urea by *A. catenella* cells.

2. Materials and methods

2.1. Culture conditions

The *A. catenella* strain (ACT03) used in this study was isolated from the Thau lagoon in 2003. Cultures were maintained at 20°C on ESAW artificial seawater (Andersen et al., 2005) with nitrate (NO_3^-) as N-source. Cells were grown on a 12:12 h light:dark cycle using a photon flux density of 100 µmol photons m^{-2} s^{-1}.

2.2. Experimental procedures

To investigate the diel variations of N and C-uptake by *A. catenella* cells, two experiments were conducted in batch cultures, based on the same experimental design but different ranges of N-addition: *(1)* in the first one, N-additions were performed to ensure N-sufficient conditions all along the experiment duration, *(2)* in the second one, lower N-additions were made to allow a depletion of N-sources before the end of the experiment. The last experiment was also performed to test the potential uptake of C-urea. As N-uptake and assimilation vary with the growth rate (Maguer et al., 2007), one 3 L-stock culture in exponential growth phase was used for each experiment to ensure that cells were in similar growth conditions.

Each experiment started at the beginning of the light period with the resuspension of *A. catenella* cells in 3 L of ESAW medium without N-source using an 11 µm mesh size net. To avoid modifications of metabolic processes due to agitation during repeated sampling, the resuspended culture was immediately split in 40ml-aliquots and different ^{15}N and ^{13}C-additions were performed.

In the first experiment *(1)* four series of samples were run for testing three different N-sources (NO_3^-, NH_4^+ and urea) in N-sufficient conditions, one series without N-addition being used as a control. N-sources were added as ^{15}N-labeled nutrient at an initial concentration between 66 and 140 µgatN L^{-1}.^{13}C-labeled bicarbonate ($NaH^{13}CO_3^-$) was added simultaneously at 78 µM-C in each sample.

The second experiment *(2)* tested three N-conditions, applying lower pulses of urea (15 µgatN L^{-1}) or NH_4^+ (15 µgatN L^{-1}) or no N-addition leading to N exhaustion over the short term. Four series of samples were performed as follow: addition of ^{15}N-^{13}C-urea only, addition of ^{15}N-^{13}C-urea and $NaH^{13}CO_3^-$, addition of $^{15}NH_4^+$ and $NaH^{13}CO_3^-$, and neither ^{15}N nor ^{13}C-addition. Pulses of $H^{13}CO_3^-$ corresponded to 78 µM-C additions.

Incubations started with the addition of ^{15}N and ^{13}C-sources and samples were immediately replaced in the culture chamber under initial conditions. During each experiment, at least one aliquot was used every hour during light periods and every 2 h (except for one missing incubation time) during dark periods for parameters measurements. Experiments *(1)* and *(2)* lasted 26 and 29 h to ensure a complete diel cycle.

2.3. Chemical and biological parameters

Each incubation was ended with sample filtration through precombusted (4h at 450 °C) A/E filters (Gelman Sciences, Ann Arbor, MI, USA). Measurements of nutrient concentrations were performed from the filtrate using the methods of Collos et al. (1999), Koroleff (1976) and Goeyens et al. (1998) respectively for NO_3^-, NH^+ and urea. Filters were dried at 60 °C overnight and kept at room temperature until analysis. Estimations of particulate nitrogen (PN), particulate carbon (PC) and $^{15}N/^{14}N$ and $^{13}C/^{12}C$ isotopic ratios were obtained from filters analysis on an Integra CN elemental analysis-mass spectrometry system (PDZ Europa, UK). Uptake rates were calculated from the regressions of isotopic ratios vs. time using equations in Collos (1987). Total costs in terms of carbon loss associated with N uptake and assimilation into *A. catenella* cells during darkness were estimated from variations in PC relatively to changes in PN (æPC / æPN) using the following equations:

$$\frac{\Delta PC}{\Delta PN} = \frac{a}{b} \times C{:}N_1 \qquad (1)$$

$$\text{with} \quad a = \frac{\Delta PC}{PC_1} = \frac{C{:}N_2}{C{:}N_1} \times (b+1) - 1 \qquad (2)$$

$$\text{and} \quad b = \frac{\Delta PN}{PN_1} = \frac{1 - {}^{14}\text{N-atom\%}_{-1}}{1 - {}^{15}\text{N-atom\%}_{-2}} - 1 \qquad (3)$$

where, for each variable X (particulate carbon, PC; particulate nitrogen, PN; ratio between PC and PN, C:N; $^{15}N/^{14}N$ isotopic ratio, ^{15}N-atom%), X_1 and X_2 represent the values obtained at the beginning and at the end of the dark period, respectively.

Those total costs estimates are based on C:N values and $^{15}N/^{14}N$ isotopic ratios ($C{:}N_1$, $C{:}N_2$, $^{15}N\text{-atom\%}_{-1}$, $^{15}N\text{-atom\%}_{-2}$) obtained from data regressions during the dark period. They include costs of N uptake and assimilation as well as all other processes taking place during

the dark period, such as uptake of other nutrients or maintenance metabolism. The equation *(3)* relies on the hypothesis defining the uptake of ^{14}N as negligible. This postulate leads to the relation æ$PN = P^{15}N_2$ ï $P^{15}N_1 = PN_2 \times {}^{15}N\text{-atom}\%_{-2}$ ï $PN_1 \times {}^{15}N\text{-atom}\%_{-1}$ that can be easily modified to obtain equation *(3)*.

Comparisons between groups used either ANOVAs followed by Tukeyôs multiple comparison tests or unpaired t tests depending on the number of groups compared. Statistical analyses were done with Prism software (GraphPad Software Inc.). All statistics are based upon the multiple time points collected during each light/dark phase.

3. Results

Considering variations of NH_4^+ concentrations in the series of samples where an initial ^{15}N-urea addition was performed, no extensive NH_4^+ release, associated with the urea assimilation process, was observed during both experiments (Fig. 1). Thus, no N-losses have to be taken into account in the analysis of ^{15}N-isotopic ratios.

3.1. Experiment in N-replete conditions

For the first experiment *(1)*, a linear decrease in nutrient concentrations was observed with time for the three series of samples performed with an initial ^{15}N-addition. Despite this consumption, nutrient concentrations remained above 12 µgatN L^{-1} until the end of the experiment: final concentrations were 81, 69 and 12 µgatN L^{-1} for NO_3^-, NH_4^+ and urea respectively.

A. catenella cells were capable of using the three N-sources, NO_3^-, NH_4^+ and urea, during the light and the dark periods (Fig. 2). Considering data of each consecutive light/dark period, most appeared to increase linearly with time, indicating a constant uptake rate. To compare N-uptake capacities of *A. catenella* cells under the different nutrient and light conditions, linear regressions of the ^{15}N-ratios as a function of time were computed for each light/dark period. Mean uptake rates estimated from regression slopes are presented in Table 1 with corresponding standard deviations and R^2 values. For the first light period, all N uptake rates were different from each other ($p < 0.001$), with NH_4^+ uptake being the highest and N-urea the lowest. For the dark period, NH_4^+ uptake was significantly higher than NO_3^- ($p < 0.001$) or N-urea ($p < 0.001$) uptake. Dark uptake rates corresponded to 27%, 43% and 65% of light uptake rates for NO_3^-, NH_4^+ and N-urea respectively.

^{13}C cell contents showed increases in the light and decreases in the dark for all series of samples (Fig. 3). The first linear rise, identified between 0 and 6 h, allowed the definition of maximal ^{13}C-uptake rates ($V_{max\text{-}C}$) from regression slopes (Table 1). Uptake rates obtained were very close between series (0.028 ± 0.003 h^{-1}), but the $V_{max\text{-}C}$ estimated for the $^{15}NH_4^+$ series was significantly lower than values of both ^{15}N-urea and control series ($p < 0.001$). After 17:00, ^{13}C-uptake rates appeared to slow down during the 3 h preceding the dark period (20:00). Linear decreases in ^{13}C isotopic ratios were then observed during darkness, characterized by similar negative slopes for the four series of samples with a mean of -0.0037 ± 0.0004 h^{-1} (NS).

C:N composition ratios revealed a clear diel cycle showing a general increase during the light period followed by a decrease during the dark period (Fig. 4). During the first light period, the largest increase in C:N ratios was noted for the series without initial N-pulse, reaching 12.1 molC $molN^{-1}$. All slopes were significantly different (p ranging from < 0.001 to < 0.05) with the ranking: control > urea > NO_3^- > NH_4^+. Data obtained between 9h and 21h of incubation were

used to estimate regression slopes associated with decreases in darkness (Table 1): slope values between -0.07 and -0.20 molC $molN^{-1}$ h^{-1} were obtained, with the lowest calculated for the ^{15}N-urea series (significantly lower than values estimated for the $^{15}NH_4^+$and control series, $p < 0.001$) and the highest for the $^{15}NH_4^+$ series.

Integrating C:N variations along the first 24h (limit indicated by a dashed line on Fig. 4), C:N ratios measured at the end of the complete diel cycle ($C:N_{24h}$) were 9.0, 7.3, 9.3 and 11.1 for the $^{15}NO_3^-$, $^{15}NH_4^+$, ^{15}N-urea and control series respectively. For the $^{15}NH_4^+$ series, the $C:N_{24h}$ value was close to the initial value of 6.8, indicating that the increase during the light period was entirely compensated by the decrease in darkness. Such compensation was only partial for the other series. Similar $C:N_{24h}$ values were obtained for the series based on $^{15}NO_3^-$ and ^{15}N-urea and corresponded to the initial ratio increased by 33 % and 37 % respectively. The series without N-addition showed the greatest increase (64%) between initial C:N and $C:N_{24h}$. C:N values and ^{15}N isotopic ratios were used to estimate total costs associated with N-uptake and assimilation into *A. catenella* cells during darkness. Respective costs of 14.2, 13.7 and 10.3 gC gN^{-1} were calculated for $^{15}NO_3^-$, $^{15}NH_4^+$ and ^{15}N-urea series.

3.2. Experiment with N-depletion

For the second experiment *(2)*, an initial pulse of 15 µgatN $L^{-1}$of NH_4^+ or N-urea was performed for three of the four series of samples. Nutrient depletion occurred during the first light period (5h30) for the $^{15}NH_4^+$ series and at the end of the dark period (21h) for the ^{15}N-^{13}C-urea series (Fig. 1). This difference came from the maximal N-uptake rate measured during the light period for the $^{15}NH_4^+$ series (0.026 ± 0.001 h^{-1}) that was found to be three times higher (significantly, $t = 35.5$, $p < 0.0001$) than the one measured for ^{15}N-^{13}C-urea series (0.007 ± 0.001 h^{-1}). These uptake rates were estimated from the linear part of the increase in cellular ^{15}N isotopic ratio with time (Fig. 5). Variations in ^{15}N isotopic ratio were similar for both ^{15}N-^{13}C-urea series, so associated results were considered as replicates and are reported on Fig. 5 as mean values. As in the first experiment in N-replete conditions, N-urea uptake rate was measured also during the dark period, with a ratio between dark and light uptake rates of 53 %. Increases observed in ^{15}N cell contents ended with nutrient depletion and values were then globally maintained at a constant level until the end of the experiment, 15.2 ± 0.9 ^{15}N-atom % and 10.3 ± 0.7 ^{15}N-atom % for the $^{15}NH_4^+$- and the ^{15}N-^{13}C-urea series respectively.

Assimilation of ^{13}C by *A. catenella* cells was measured only for samples where an initial addition of $H^{13}CO_3^-$ had been performed (Fig. 6). When comparing ^{13}C isotopic ratios of the series based on ^{15}N-^{13}C-urea addition (without $H^{13}CO_3^-$) and the series without initial pulses of ^{15}N and ^{13}C, results were found equivalent, suggesting that *A. catenella* cells were not able to use urea as a C-source. Variations in ^{13}C-ratios observed for the other series with $H^{13}CO_3^-$ additions ($^{15}NH_4^+$ + $H^{13}CO_3^-$ and ^{15}N-^{13}C-urea + $H^{13}CO_3^-$) showed trends similar to those highlighted under N-replete conditions: increases during the light periods and decreases during the dark period, however decreases appeared exponential rather than linear in this second experiment. As no ^{13}C-assimilation was noted after an initial addition of ^{15}N-^{13}C-urea only, variations observed for the series compiling both potential ^{13}C-sources, ^{15}N-^{13}C-urea and $H^{13}CO_3^-$, were representative of ^{13}C assimilation from $H^{13}CO_3^-$ exclusively and allowed estimations of $H^{13}CO_3^-$ uptake rates. $V_{max\text{-}C}$ from $H^{13}CO_3^-$ estimated during the first light period were 0.040 ± 0.003 h^{-1} and 0.046 ± 0.004 h^{-1} for the series based on $^{15}NH_4^+$ and ^{15}N-^{13}C-urea respectively (significant difference, $t = 3.4$, $p = 0.003$).

4. Discussion

4.1.Dark N-uptake under N-sufficient conditions

Among the three potential N sources tested (NO_3^-,NH_4^+ and N-urea) for A. catenella, NH_4^+seems to be the preferred N-source during both light and dark phases. Results from the experiment *(1)* allow the characterization of dark N-uptake capacities under N-sufficient conditions. These nutritive conditions can be justified referring to N-uptake kinetics reported for *A. catenella* cells by Collos et al. (2004) and Jauzein et al. (2008) (strains ACT2000 and ACT03 respectively): *A. catenella* cells were able to sustain maximal N-uptake rates all over the experiment *(1)* duration according to ranges ofNO_3^-, NH_4^+ and N-urea concentrations observed. During the light period, the mean N-urea uptake rate estimated in the present study under N-sufficient conditions is low compared to the maximum uptake rate (V_{max} = 0.025 ± 0.008 h^{-1}) reported by Collos et al. (2004) for cells acclimated to urea as a N-source, however. If differences between strains in terms of N-urea uptake capacities may explain such a discrepancy (Jauzein et al. 2008), potential interferences due to preconditioning effects cannot be rejected and may have limited N-urea uptake capacities of *A. catenella* cells in the present study. Only relative data of uptake rates between light and dark periods are discussed below; these ratio values might be less sensitive to potential interferences due to preconditioning.

Abilities of marine microalgae to carry out uptake at night under N-sufficient conditions are observed for various taxonomic groups, including dinoflagellates, prymnesiophytes and diatoms (Paasche et al., 1984; Clark et al., 2002; Needoba and Harrison, 2004). No global classification of phytoplankton taxonomic groups may be proposed on such a basis because reported capacities vary strongly between genera of the same class and even between species of the same genus. For dinoflagellates, a high variability inside genera can be highlighted for *Prorocentrum*, *Heterocapsa* or *Alexandrium* from previous studies (Table 2). Concerning $NO_3^-$and NH_4^+, *A. catenella* shows low capacities for N-uptake at night, with respective dark:light (D/L) uptake ratios of 27 % and 43 % when reported D/L uptake ratios for other species under N-sufficient conditions range from 1% to 75% for NO_3^- and from 21 % to 100% for NH_4^+ (Clark et al., 2002;Granumet al., 2002; Needoba and Harrison, 2004; Table 2). Dark N-urea uptake capacities of phytoplankton cells in N-sufficient conditions are much less studied than NO_3^- or NH_4^+. The estimation of D/L N-urea uptake ratio for *A. catenella* (65%; present study) is in the range of values reported for the diatom *Phaeodactylum tricornutum* (35 %; Rees and Syrett, 1979) and the dinoflagellate *Alexandrium tamarense* (98 %; Leong et al., 2010; see Table 2). According to Sinclair et al. (2009), dark uptake and assimilation abilities may also vary between strains of the same species, revealing specific adaptations to different environmental conditions. From the study of three strains of *Karenia brevis* isolated from different geographic regions, they obtained ranges of D/L uptake ratios of 55 % - 136 % for NH_4^+ and 32 % - 89 % for N-urea, but from cultures that were N-depleted and maintained under low light.

4.2. Uncoupling between photosynthesis and nutrient uptake

Dark N-uptake capacities in photosynthetic cells are dependent on particular metabolic adaptations. Nutrient uptake and assimilation in darkness require an additional expense of previously fixed C to be supplied with energy (ATP), reductant (NAD(P)H) and C-skeletons, creating an uncoupling between photosynthesis and nutrition processes (Cosper, 1982). Photosynthetic C fixed in excess during the light period may be stored into C-rich and N-free macromolecules, such as carbohydrates (Cuhel et al. 1984; Clark and Flynn, 2002; Granum et al. 2002) or neutral lipids (Fábregas et al. 2002) which can account for 10-50% of the dry

weight of the cell (Geider and La Roche, 2002). Accumulation of large pools of C-rich storage compounds may modify significantly C:N and C:P ratios (Geider and La Roche, 2002).

In the present study, strong diel variations in C:N ratio were observed for *A. catenella* cells growing on NO_3^-, NH_4^+ and N-urea, but also for the control series without N-addition. C-uptake from urea did not interfere in these trends as *A. catenella* cells were not able to use urea as a C-source (Fig. 6) under the light levels used in the present study (100 ε mol photons.m^{-2}.s^{-1}). Similar detailed patterns of C:N diel variations were described for species maintained under N-replete conditions, such as *Skeletonema costatum* (Burkhardt et al.1999b), *Thalassiosira weissflogii* (Clark et al. 2002) and *Emiliana huxleyi* (Bucciarelli et al., 2007), and N-limited conditions, as for *Dunaliella tertiolecta* (Sciandra et al., 1997). For dinoflagellates, the only study reporting variations of C:N during the light:dark cycle to our knowledge is the one of MacIntyre et al. (1997) on *A. tamarense*. Even if samples were taken only twice daily on a 14h:10h light:dark cycle in this study, C:N oscillations were visible for *A. tamarense* cells maintained under N-replete conditions when measurements were done around the middle of successive light and dark periods.

Under N-sufficient conditions, an increase in C:N during the light period suggests that photosynthetic C is stored in excess relatively to requirements for N-uptake and assimilation. In darkness, a decrease results from C losses, through respiration and DOC excretion and N-uptake at night. In the present study, the large C:N decrease observed for the control series (without N-addition) suggests that C:N variations in darkness are mainly governed by C-losses and the main part of these losses are independent of dark N-uptake and assimilation. For *A. catenella*, storage of C-rich compounds from photosynthesis may mostly balance the high C-requirement for maintenance respiration in darkness.

4.3. Variations of ^{13}C-isotopic ratio during the light:dark cycle

Analysis of ^{13}C-isotopic ratios sustains the hypothesis that most of C-fluxes in *A. catenella* cells during the light:dark cycle are not dependent on dark N-uptake and assimilation. During the light period, ^{13}C-uptake rates reveal that *A. catenella* cells maintain an optimized photosynthetic activity during the main part of the light period, with a drop in C-uptake rate noted during the 3h preceding the dark phase. $V_{max\text{-}C}$ values obtained during the light period of the experiment *(1)* do not mirror the differences in terms of C-requirements associated with dark N-uptake and assimilation: close $V_{max\text{-}C}$ values were estimated from the four series of samples (growth on NO_3^-, NH_4^+, N-urea or without N-addition) and the lowest value is not associated with the control.

During the dark phase, similar variations in ^{13}C-isotopic ratio *vs*. time were also observed from the different nutritive conditions of growth. These decreases in ^{13}C-isotopic ratio could result either from an unbalanced loss of carbon (loss of products enriched in ^{13}C relatively to natural abundance) or from dilution of the PC labeling by uptake of unlabelled organic products. The second hypothesis was previously proposed by Collos et al. (2006) to explain a concomitant decrease in both ^{13}C- and ^{15}N-isotopic ratios of *A. catenella* cells in cultures once the extracellular concentration of inorganic nitrogen was exhausted. However, results obtained in experiment *(2)* (Fig. 5) did not reveal a decrease in ^{15}N cell content after exhaustion of $^{15}NH_4^+$ (during the first light period) or ^{15}N-urea (at the end of the dark phase). Thus, if uptake of unlabelled organic compounds has occurred during experiments *(1)* and *(2)*, this process would not be the only one to contribute to the decrease in ^{13}C cell contents noted during the dark phase.
Concerning the first hypothesis and according to Burkhardt et al. (1999a), an unbalanced loss of carbon may be the result of either a loss of ^{13}C-enriched dissolved organic carbon from the cell, or fractionation processes associated with carboxylation/decarboxylation reactions or finally complete decarboxylation of ^{13}C-rich organic compounds. The latter

process is likely to have contributed the most to the trends observed. According to Geider and Osborne (1989), dinoflagellates may be characterized by high ratios between dark respiration and maximum net photosynthesis rates, generally higher than 25% and reaching up to 59%. In the present results, ^{13}C-decreases during darkness represented 12-15% of the maximal C-uptake ($V_{max\text{-}C}$) estimated during the previous photophase. Based on the ratios of dark respiration *vs.*net photosynthesis proposed by Geider and Osborne (1989), a contribution of 20-60% of ^{13}C in the pool of C respired during the dark phase may explain slopes of ^{13}C-decrease observed for *A. catenella* cells. Thus, the decrease in ^{13}C cell content in darkness may proceed from the complete decarboxylation of ^{13}C-rich organic compounds synthesized during the previous photophase, reflecting a high involvement in dark respiration of C recently fixed. Furthermore, the similar decreases obtained for the series with and without N-addition may suggest a high involvement of maintenance metabolism in dark respiration.

4.4. Metabolic costs associated with dark metabolism

If a part of the energy requirements of phototrophic cells during the light phase can be provided by photosynthesis, dark respiration corresponds to the exclusive source of ATP and reductant during darkness at the expense of C-storage reserves (carbohydrates or neutral lipids) used as substrates (Geider and Osborne 1989). Assuming a coupling in respiratory energy conversion, dark respiration rates can be used to estimate energy requirements associated with maintenance metabolism and biosynthesis during darkness. In the present study, variations in C:N ratio and cell content in terms of ^{13}C and ^{15}N allow the analysis of the respective energy requirements for dark N-uptake and assimilation compared to basal metabolism in *A. catenella* cells.

Variations observed in C:N and ^{15}N-isotopic ratios of *A. catenella* cells during the dark phase were used to estimate total costs (combining biosynthesis and basal metabolism) in terms of C loss associated with dark N-uptake and assimilation of NO_3^-, NH_4^+ and N-urea. These costs based on material budgets are comparable with the values reported for the diatom *Thalassiosira weissflogii* by Clark et al. (2002). Uptake and assimilation of $NO_3^-$and N-urea in darkness are associated with higher energy requirement and C-reserve mobilization than NH_4^+. For NO_3^-, the additional energetic expense comes from the requirement of ATP, reductant and enzyme synthesis (NO_3^- and NO_2^-reductases) for the reduction of NO_3^- successively into nitrite (NO_2^-) and NH_4^+ (Syrett,1981). This may lead to a cost of N-assimilation into protein (derived from cell C budget) four times higher for NO_3^-than NH_4^+ (Syrett, 1956; Penning de Vries et al., 1974) and may explain the stronger dependence on light noted for NO_3^- uptake for several marine phytoplankton species (Clark and Flynn, 2002; Clark et al., 2002; Table 2). For *A. catenella*, the limitation of NO_3^- dark uptake by the energetic demand is confirmed from estimations of costs in terms of C: a similar value was obtained (around 14 g C g N^{-1}) for both NO_3^- andNH_4^+, while dark uptake rate of NO_3^- represents less than half the rate of NH_4^+.

With a D/L uptake ratio of 65%associated with a total C-cost of 10 g C g N^{-1}, N-urea uptake appears less dependent on light than NO_3^- (27 %) and NH_4^+(43 %) even though N-urea assimilation includes a transformation step of urea into NH_4^+involvingureaseactivity. A similar ranking of D/L uptake ratios as a function of oxidation state (< 10 %, 66 %, 98 % respectively for NO_3^-, NH_4^+ and N-urea) was reported by Leong et al. (2010) for *A. tamarense* cells maintained semi-continuously at 100 µM-N. An explanation of this lowest dependence on light noted for N-urea uptake could lie in the potential use of urea as a C-source which would facilitate dark nutrition processes; present results show that *A. catenella* is not able to use C-urea, however.

The total C-costs associated with dark uptake and assimilation of NO_3^- and $NH_4^+$by *A. catenella* cells appear to be much higher than values (< 3 g C g N^{-1}) reported for the diatom *T. weissflogii* in N-replete conditions (Clark et al., 2002). If higher costs can arise from inefficiency in respiratory energy conversion leading to increased C-reserve consumption, this cannot explain the discrepancy observed. According to variations in C:N ratio and ^{13}C cell content, *A. catenella* can be characterized by high maintenance costs that contribute mainly to C-losses in darkness. Thus, differences in total C-costs noted between *A. catenella* and the diatom may rely on maintenance metabolic energy requirements more than on costs associated with dark N-uptake and assimilation.

Geider and Osborne (1989) reported a high involvement of maintenance metabolism in dark respiration for the close species *A. tamarense*. *A. tamarense* is characterized by a high maintenance respiration rate (0.30 d^{-1}) compared to other species (Ó0.2 d^{-1} for 4 out of 32 cases), and in particular to *T. weissflogii* (average of 0.04 d^{-1} under the light:dark cycle, Table 3 in Geider and Osborne, 1989). Furthermore, even if *A. tamarense* shows a strong dependence of dark respiration on growth, its low growth rate probably limits a lot the contribution of biosynthesis in dark respiration: the maintenance respiration rate r_0= 0.30 d^{-1}represents 55 % to 70 % of the total dark respiration rate r_d (d^{-1}) when a maximum growth rate (ε, d^{-1}) of 0.3 d^{-1} - 0.6 d^{-1} (Laabir et al., 2011) is used in the linear relationship r_d (d^{-1}) = r_0 (d^{-1}) + 0.40 × ε (d^{-1}) defined by Geider and Osborne (1989) for *A. tamarense*.

Difference in maintenance costs between flagellates and diatoms cannot be explained by motility as synthesis and use of flagella are likely to be of minor metabolic costs (Raven and Richardson, 1984). We suggest that a significant part of the difference observed between *Alexandrium* spp. and *T. weissflogii* may come from costs associated with protein turnover. Protein turnover is considered to be one of the most significant components of maintenance processes (Penning de Vries, 1975; Raven et al. 2000). When *T. weissflogii* is characterized by a protein content of 40-60 g prot.L^{-1} cell volume (Price, 2005), a two-times higher protein content (90-130 g prot.L^{-1} cell volume) can be estimated for *A. tamarense* from estimations of Murata et al (2006) standardized by cell volumes reported by Sullivan and Swift (2003). The higher total C-costs associated with dark uptake of NO_3^- and NH_4^+ estimated for *A. catenella* (present study) compared to *T. weissflogii* (Clark et al., 2002) could be due to higher maintenance respiration rate and more specifically protein turnover in dinoflagellates.

4.5. Ecological implications

According to Litchman et al. (2004), the impact of the dark N-uptake strategy on species competitiveness depends on the duration of the light period in the diel cycle (dependent on the day length and the mixing depth *in situ*) and on the energetic cost of the nutrient uptake and assimilation. For *A. catenella*, additional costs associated with dark uptake and assimilation may not reduce the competitiveness of this species as they appear to be low compared to maintenance costs. Furthermore, the higher D/L uptake ratio obtained for N-urea uptake of *Alexandrium* cells compared to NH_4^+ (Leong et al. 2010; present study) suggests that costs of uptake and assimilation may not be the only metabolic parameters interfering in costs-benefits of dark uptake strategy.

For flagellate species such as *A. catenella*, benefits from dark uptake strategy are also linked with their ability to migrate actively in the water column. Migration behavior during the diel cycle allows the improvement of dark N-uptake at night optimizing nutrient conditions, as well as C storage capacities during the daylight optimizing irradiance conditions. In a reverse and complementary way, accumulation of C-rich compounds over the daylight may increase the specific density of cells and favor the passive sinking to the deeper layers at night. Such a role of carbohydrates as ballast may have an ecological interest also for non-flagellate phytoplankton species, such as diatoms: intracellular accumulation of carbohydrates in

response to nutritive stress may trigger cell sinking into deeper layers where more nutrient sufficient conditions may be encountered (Richardson and Cullen, 1995). However, this buoyancy regulation process operates over longer time scales than diel variations. Thus, even if some diatoms species show higher D/L uptake ratios than *A. catenella* in N-replete cultures (Clark et al., 2002; Needoba and Harrison, 2004), the potential benefits of their dark uptake strategy *in situ* can be higher for the latter species, in particular in water columns where nutrients distribution is not homogenous.

Flynn et al. (2002) consider that dinoflagellates may have a competitive disadvantage for dark uptake and assimilation due to their relatively N-rich status, which may reduce C availability for supporting dark metabolism processes. Under N-replete conditions, detailed diel variations of C:N ratio do not support this hypothesis: differences between the minimum and maximum value of C:N over the diel cycle were reported to be 1.7 and 4.7 for two diatoms (Bukhardt et al., 1999b; Clark et al. 2002), 1.0 for a prymnesiophyte (Bucciarelli et al., 2007) and 2.9 for the dinoflagellate *A. catenella* (present study). According to these values, potential C-storage availability does not appear to be a competitive disadvantage for *A. catenella*.

5. Conclusions

Experiments performed have allowed the test of two potential nutritive strategies of *A. catenella* cells: dark N-uptake and C-urea uptake capacities. *A. catenella* cells show no ability to use C-urea, but they are characterized by dark N-uptake capacities at the expense of previously fixed carbon with a high efficiency for the dissolved organic source N-urea. *In situ*, the efficiency of these dark N-uptake and C-storage strategies may be optimized by the ability of the cells to migrate actively. The analysis of costs in terms of C-losses associated with biosynthesis processes and basal metabolism in darkness revealed that specific C-costs of dark N-uptake and assimilation may be negligible compared to cell maintenance in *A. catenella*. This suggests that the dark N-uptake strategy corresponds to a benefit in terms of competitiveness for this species.

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Tables

Table 1
Estimations of ^{15}N-uptake rates (V_N in h^{-1}), ^{13}C-uptake rates ($V_{max\text{-}C}$ in h^{-1}) and slopes from regressions of C:N ratio *vs.* time for *A. catenella* cells during the light and dark periods, for the four series of samples (controls without N-addition).

	^{15}N-uptake		^{13}C-uptake		C:N composition ratio	
	V_N	R^2	$V_{max\text{-}C}$	R^2	Slope	R^2
First light period						
$+^{15}NO_3^-$	0.012 (±0.001)	0.96	0.028 (±0.003)	0.94	–	–
$+^{15}NH_4^+$	0.018 (±0.001)	0.99	0.025 (±0.002)	0.97	–	–
$+^{15}N$-urea	0.005 (±0.001)	0.85	0.031 (±0.001)	0.99	–	–
Control	–	–	0.030 (±0.003)	0.96	–	–
Dark period						
$+^{15}NO_3^-$	0.003 (±0.001)	0.98	–	–	0.139 (±0.032)	0.79
$+^{15}NH_4^+$	0.008 (±0.001)	0.92	–	–	0.196 (±0.054)	0.73
$+^{15}N$-urea	0.003 (±0.001)	0.95	–	–	0.071 (±0.020)	0.71
Control	–	–	–	–	0.156 (±0.024)	0.89

R^2 values correspond to each data regression. ^{13}C-uptake rates ($V_{max\text{-}C}$) were estimated from the linear part of variations in ^{13}C-isotopic ratio. Standard deviations in parentheses.

Table 2
Dark/light uptake ratios (in %) reported for dinoflagellates species from culture experiments in N-sufficient conditions.

Genera	Species	Nutrient			Reference
		NO_3^-	NH_4^+	N-urea	
Amphidinium	*carterae*	NA	65	NA	Paasche et al. (1984)
Gymnodinium	*galatheanum*	12	NA	NA	Paasche et al. (1984)
Gyrodinium	*aureolum*	1	21	NA	Paasche et al. (1984)
Scrippsiella	*trochoidea*	29	44	NA	Paasche et al. (1984)
Prorocentrum	*micans*	15	36	NA	Paasche et al. (1984)
	minimum	60	86	NA	Paasche et al. (1984)
Heterocapsa	*triquetra*	75	108		Paasche et al. (1984)
	illdefina	<20	NA	NA	Clark et al. (2002)
Alexandrium	*catenella*	27	43	65	Present study
	tamarense	<10	66	98	Leong et al. (2010)

Among the different N-conditions tested in the study of Leong et al. (2010), data presented here were obtained from cultures maintained semi-continuously on 100 μM-NO_3^-, under the culture mode called "continuous-N supply".

Fig. 1. Variations of urea and ammonium concentrations in *Alexandrium catenella* cultures where an initial ^{15}N-urea addition was performed to ensure N-replete conditions over 24h (a) or short-term depletion of urea (b). Series of samples with supplementary addition of $H^{13}CO_3^-$ are represented by black symbols (urea, black diamonds; ammonium, black triangles) and series without by white symbols (urea, white circles; ammonium, white squares).

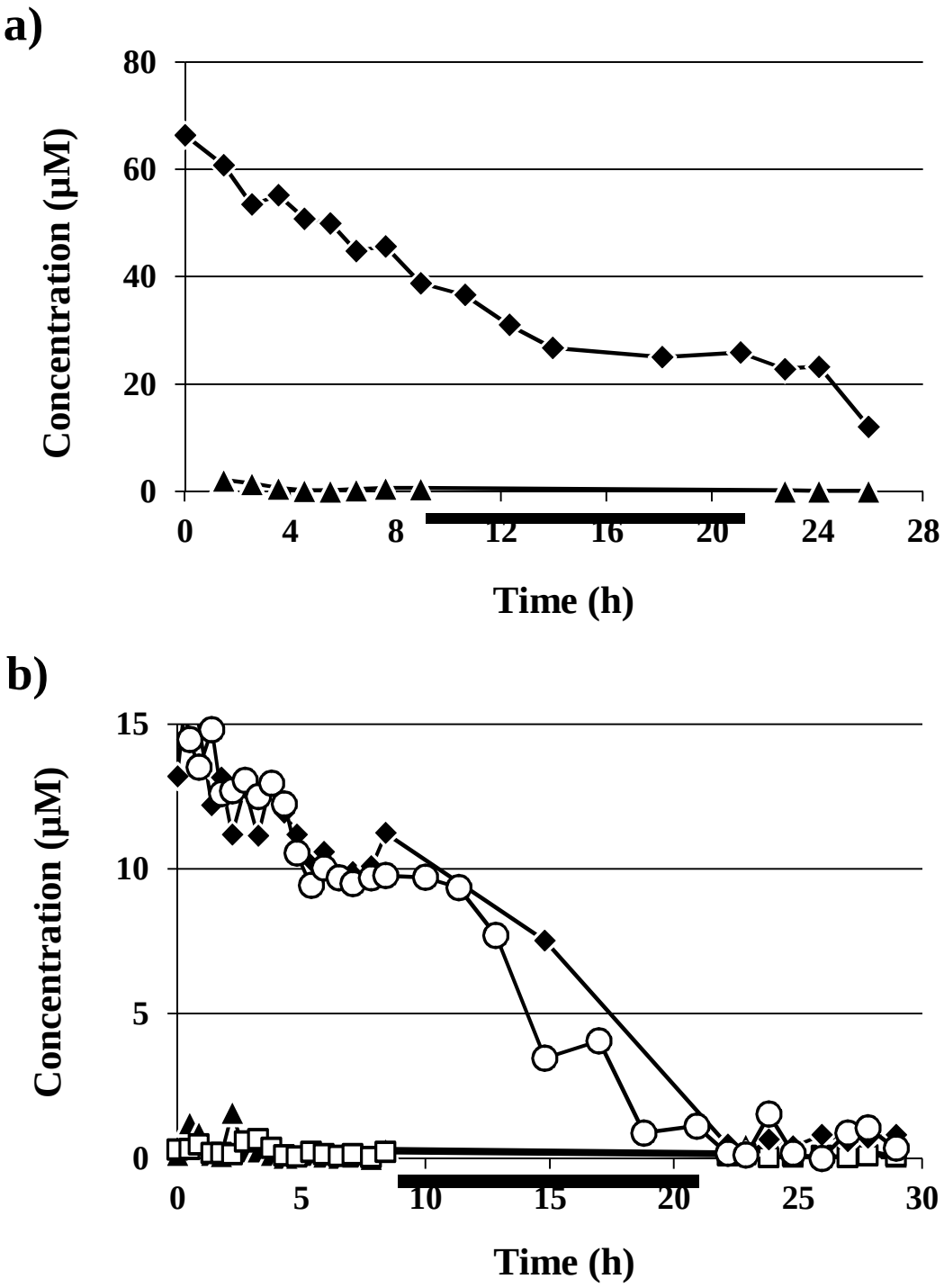

Fig. 2. Variations in ^{15}N isotopic ratios of *Alexandrium catenella* cells in N-sufficient conditions, with $^{15}NH_4^+$,$^{15}NO_3^-$or ^{15}N-urea additions, and without N-addition. The dark period is indicated by the horizontal solid line and the end of the complete light:dark cycle (24h) by the vertical dotted line.

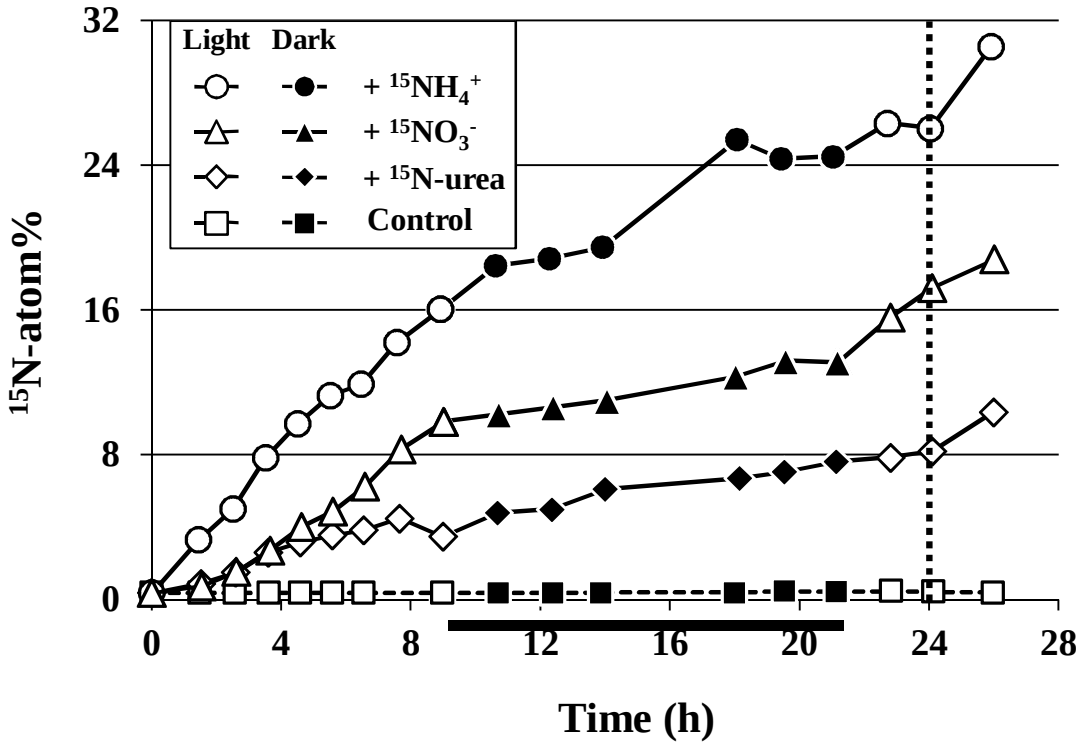

Fig. 3.Variations in ^{13}C isotopic ratios of *Alexandrium catenella* cells in N-sufficient conditions, with $^{15}NH_4^+$, $^{15}NO_3^-$ or ^{15}N-urea additions, and without N-addition. The dark period is indicated by the horizontal solid line and the end of the complete light:dark cycle (24h) by the vertical dotted line.

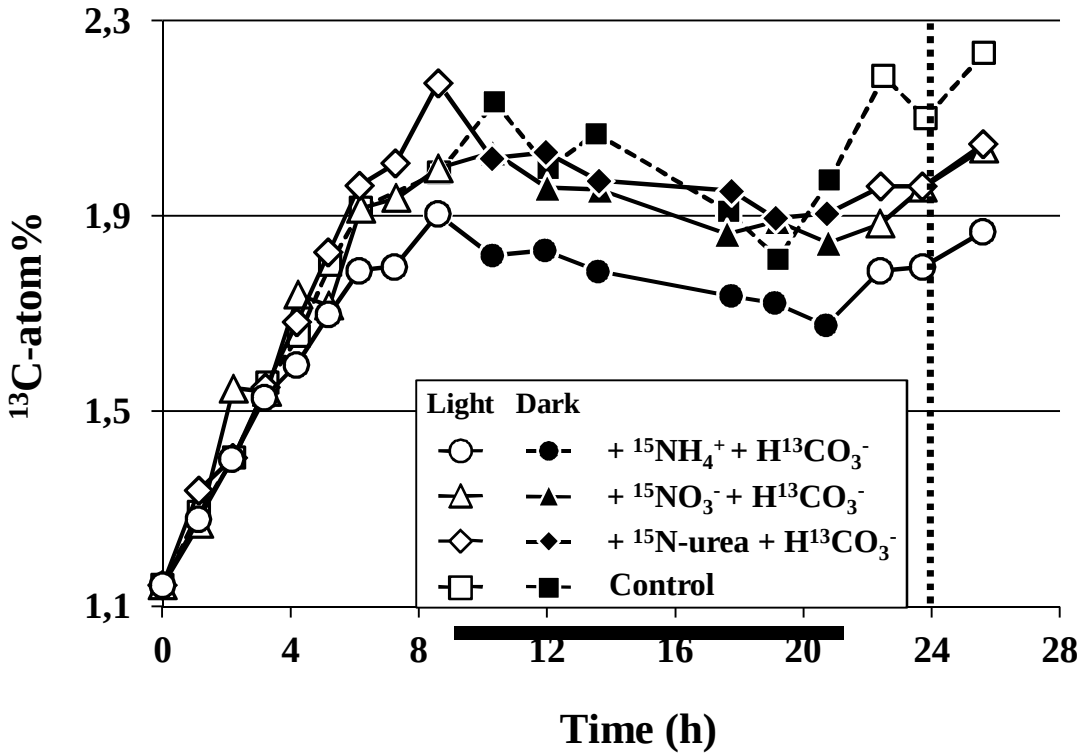

Fig. 4. Variations in C:N composition ratios of *Alexandrium catenella*cells in N-sufficient conditions, with $^{15}NH_4^+$, $^{15}NO_3^-$ or ^{15}N-urea additions, and without N-addition. The dark period is indicated by the horizontal solid line and the end of the complete light:dark cycle (24h) by the vertical dotted line.

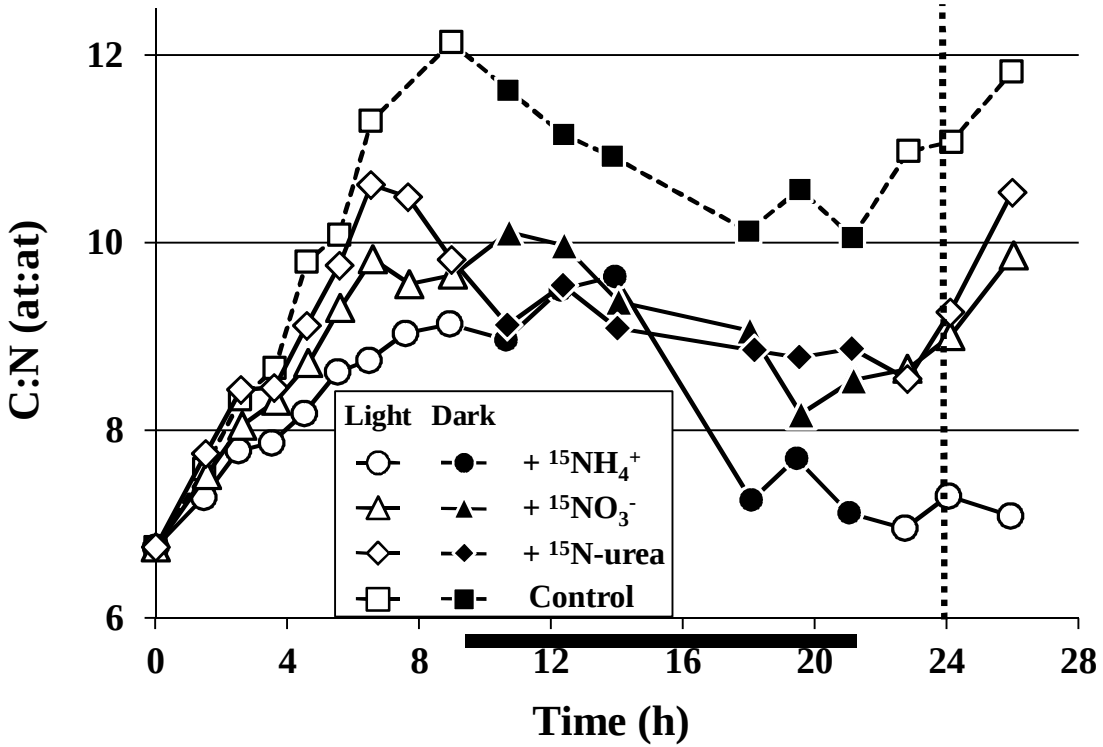

Fig. 5. Variations in ^{15}N isotopic ratios of *Alexandrium catenella* cells after an initial pulse (15 µgatN L^{-1}) of $^{15}NH_4^+$, ^{15}N-^{13}C-urea and without ^{15}N-addition. The ^{15}N-^{13}C-urea data include the results obtained from both series, with and without supplementary addition of $H^{13}CO_3^-$. The vertical lines indicate standard deviations and the horizontal solid line the dark period.

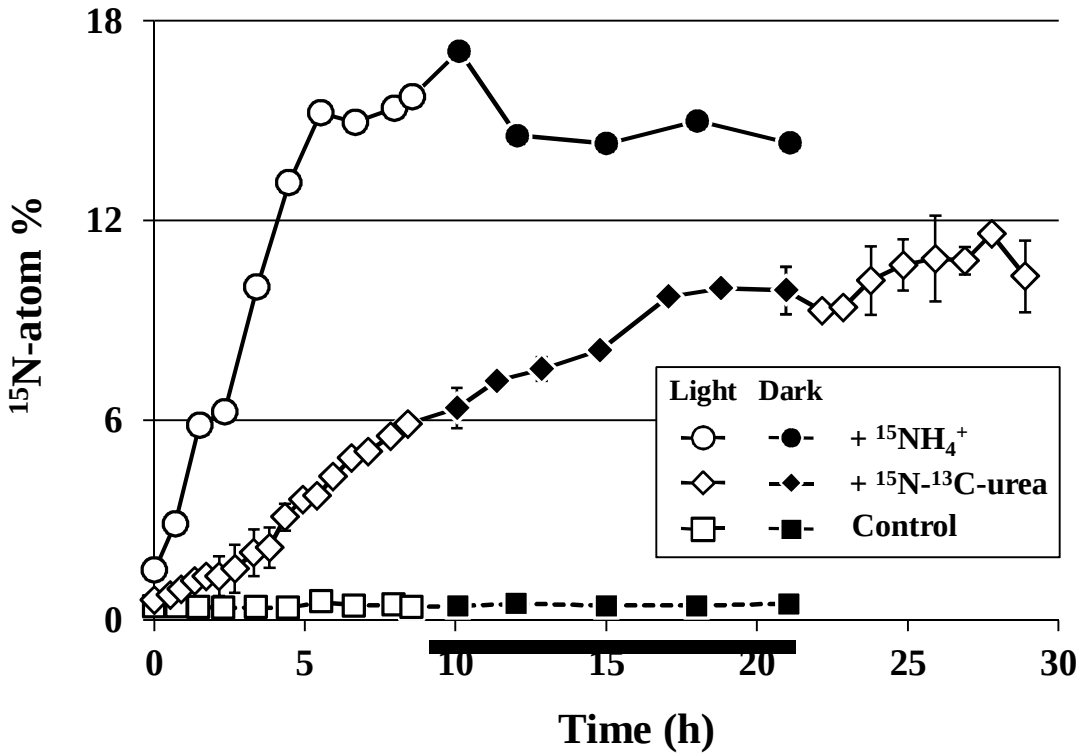

Fig. 6.Variations in ^{13}C isotopic ratio of *Alexandrium catenella* cells with or without initial pulses of ^{15}N and ^{13}C, in the form of $^{15}NH_4^+$, ^{15}N- ^{13}C-ureaand $H^{13}CO_3^-$.^{15}N-additions correspond to 15 µgatN L^{-1} and $H^{13}CO_3^-$ additions to 78 µM-C. The solid line indicates the dark period.