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Short-term temporal variability of ammonium and urea uptake by *alexandrium catenella* (dinophyta) in cultures

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Abstract:

In batch cultures of four Mediterranean strains (from France, Italy, and Spain) of Alexandrium catenella (Whedon et Kof.) Balech growing on a daily light cycle, ammonium and urea uptake were estimated by the ^{15}N tracer technique. Ammonium uptake could be described by Michaelis–Menten kinetics along a substrate gradient of 0.1–10 µgat $N \cdot L^{-1}$ for the four strains, while two different patterns were observed for urea uptake with Michaelis–Menten kinetics for one strain and linear kinetics for the others. In all cases, an increase in uptake rates with time was noted over the daylight period. This trend led to a net increase in the maximum uptake rate (V_{max}; for saturable kinetics) and in the initial slope α. For ammonium, V_{max} increased by a factor of 2–10 depending on the strain, and, for urea, the maximal uptake rates measured increased by a factor of 2–18. Temporal variations of half-saturation constants (K_s) for both nutrients did not show a clear trend. Increases in V_{max} and α showed an acclimation of the cells' uptake system over time to a N pulse, which may be explained by the light periodicity. For two strains, extensive ammonium release was observed during urea assimilation. This mechanism removes urea from the medium, so it is no longer available to other potential competitors, but supplies N back to the medium in the form of ammonium. From a methodological point of view, the phenomenon leads to considerable underestimates of the contribution of urea to phytoplankton growth.

Keywords: Alexandrium catenella • ammonium • ammonium release • temporal variability • uptake kinetics • urea

I- INTRODUCTION

In coastal areas of the Mediterranean Sea, blooms of the toxic dinoflagellate *Alexandrium catenella* have been observed since the 1990s (Penna et al. 2005). *A. catenella* is able to use several nitrogen (N) sources for its vegetative growth in laboratory cultures (Matsuda et al. 1999; Dyhrman & Anderson 2003; Collos et al. 2004) or during bloom formation in the field (Collos et al. 2004; 2007). Ammonium (NH_4^+) and urea appear to be the main N sources for growth during bloom periods, contributing from 30 to 100 % and from 2 to 59 % respectively to N requirements (Collos et al. 2007). However, Collos et al. (2007) reported a high temporal variability in NH_4^+ and urea uptake kinetics for natural populations of *A. catenella* in Thau lagoon (France). For example, half-saturation constants varied between 0.2 and 20 μgatN.L^{-1} for NH_4^+ and between 0.1 and 44 μgatN.L^{-1} for urea over four bloom episodes. Corresponding V_{max} values on a cell basis ranged ten fold for NH_4^+ and eight fold for urea over the same period of four years. On a shorter time scale, the half-saturation constants for NH_4^+ (K_S-NH_4^+) increased from 0.5 to 6.2 μgatN.L^{-1} over a period of three days during a bloom development. This variability in kinetics parameters precludes precise estimates of the NH_4^+ and urea contributions to growth, and requires additional studies.

Over the same period of three days, the cell-based growth rate of *A. catenella* decreased by a factor of two while the K_S-NH_4^+ increased. Previous studies have shown that the variability in kinetics parameters could be related to growth rate or N-limitation. For example, a direct relationship between V_{max} of NH_4^+ and growth rate was observed for *Alexandrium minutum* by Maguer et al. (2007) and were related to N-limitation. However, for other phytoplankton species such as the diatom *Thalassiosira pseudonana,* conflicting trends have been reported for specific V_{max} for NH_4^+ as a function of growth rate/N-limitation (Caperon & Meyer 1972; Eppley & Renger 1974; McCarthy & Goldman 1979). V_{max} for NH_4^+ and urea is also known to vary during the light period (Eppley et al. 1971; Caperon & Ziemann 1976; MacIsaac 1978; Tamminen & Irmisch 1996).

Here we examine the short-term variability during the light period of NH_4^+ and urea uptake kinetics of *A. catenella* in batch cultures growing on a light-dark cycle. Results are discussed with regards to N-limitation and daily periodicity.

II- MATERIALS AND METHODS

1- Culture conditions and kinetics experiments

Four clonal strains of *Alexandrium catenella* originating from three different geographical areas were used: two strains from France (ACT03 and TL01 isolated from the Thau lagoon), one from Spain (VGO 565 from the Tarragona harbor) and one from Italy (ACATA4 from Olbia, Sardinia). Non axenic cultures of each strain were grown at 20°C on enriched seawater with nitrate (NO_3^-) as N-source. The medium used for the maintenance of the cultures and during the experiments was f/2 medium (Guillard & Ryther 1962) prepared in 0.2 µm filtered seawater for the Spanish and the Italian strains and ESAW artificial seawater medium (Andersen et al. 2005) with S = 38 for the French strains. Illumination was provided by fluorescence tubes (Grolux, Sylvania, Germany), providing an irradiance of 100 µmol photons.m^{-2}.s^{-1} using a light:dark cycle of 12h:12 h.

At the beginning of each experiment (around 10:00), cells were collected on an 11 or 20 µm mesh size net and resuspended in N-free culture medium in less than an hour. This step also allowed the removal of most of the bacteria and limited their contribution in the resuspended culture (Rausch de Traubenberg & Soyer-Gobillard 1990; Doucette & Powell 1998). Three 1 hour incubations were then performed during the light period, respectively just after resuspension and 3 h and 6 h later. Each incubation started with the addition of ^{13}C-labeled bicarbonate at a constant concentration and of $^{15}NH_4^+$ or ^{15}N-urea at 8 concentrations (0.1, 0.2, 0.5, 1, 2, 3, 5 and 10 µgatN.L^{-1}) in two different series of 50-mL samples. This range of substrate concentrations was chosen because it corresponds to the NH_4^+ or urea concentrations measured in the field areas where *A. catenella* blooms occur (Garcés et al. 2005; Collos et al. 2007). For the ACATA4 strain, the third incubation was only done with the highest substrate concentration of 10 µgatN.L^{-1}. For one strain (VGO 565), an additional incubation was also carried out 24 h after resuspension only at 10 µgat N.L^{-1}. Incubations were ended after 1h with filtration through Gelman A/E (equivalent pore size of 1 µm) 13 mm pre-combusted glass fiber filters (Gelman Sciences, Ann Arbor, MI, USA). Filters were dried at 60°C for 24 h and then stored at room temperature until analysis. Measurements of particulate nitrogen (PN), particulate carbon (PC), $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotopic ratios were done on the filters with an Integra CN elemental analysis-mass spectrometry system (PDZ Europa, UK).

Separate 50-mL aliquots with 10 µgatN.L^{-1} addition were filtered every 10-15 min to follow the short-term evolution of uptake over the 1-h incubation.

2- Cell counts and nutrient analysis

To estimate growth rates at the beginning and during each experiment, cells of *A.catenella* were counted the day before, just after resuspension and 24 h later. Growth rates were calculated according to Guillard (1973).

For each experiment, concentrations of urea, NH_4^+ and NO_3^- were determined just after resuspension. Urea concentrations measurements were done using the method of Goeyens et al. (1998). NH_4^+ and NO_3^- concentrations were measured with a continuous flow analyzer following Grasshoff et al. (1983) for the Spanish and the Italian strains and using respectively the method of Koroleff (1976) and Collos et al. (1999) for the French strains. For the Spanish strains, nitrite (NO_2^-) concentrations were also measured using the method of Bendschneider & Robinson (1952) and additional measurements of nutrients concentrations were done at the end of each incubation. For the French strains, only NH_4^+ concentrations were measured at the end of the ^{15}N-urea incubations to assess NH_4^+ release during N-urea assimilation.

3- N uptake measurements and kinetic parameters

Uptake is defined as the sum of processes resulting in ^{15}N incorporation in cells. It includes adsorption and absorption. For urea, assimilation is defined as the transformation of intracellular urea to NH_4^+ and its incorporation into carbon skeletons.

Net uptake rates of NH_4^+ and urea (V in h^{-1}) were calculated from the ^{15}N-enrichment of the samples according to Collos (1987). If the relationship between net uptake rates and concentrations appeared to conform to saturable kinetics, a nonlinear regression was computed according to the Michaelis-Menten model and allowed to estimate the affinity constant (K_S in µgatN.L^{-1}), the maximum uptake rate (V_{max} in h^{-1}) and the initial slope (α in L.h^{-1}.µgatN^{-1}). The initial slope α was calculated from the uptake rate at the concentration of 0.5 µgatN.L^{-1} estimated by the model equation as recommended by Hurd & Dring (1990) and was used as an indicator of the cells competitive ability at low substrate concentrations. The other data showing unsaturable uptake were fitted to linear regressions.

The estimation of net uptake rates neglects any loss of ^{15}N during incubation. The losses under the form of NH_4^+ may be estimated from NH_4^+ concentrations and allowed to calculate gross uptake rates using the following equation:

$$\rho = V + \Delta [NH4] / (\Delta t \cdot PN)$$

Where ρ is the gross uptake rate (in h^{-1}), V is the net uptake rate (in h^{-1}), Δ [NH4] (in $\mu gatN.L^{-1}$) is the difference in NH_4^+ concentrations during the incubation time (Δ t, in h) and PN is the particulate nitrogen (in $\mu gatN.L^{-1}$).

III- RESULTS

1- Cellular growth, C/N composition ratio and nutrient conditions

At resuspension time, growth rates estimated from cell counts were respectively 0.23 d^{-1}, 0.50 d^{-1}, 0.32 d^{-1}, 0.25 d^{-1} for strains ACT03, TL01, VGO 565 and ACATA4. Additional measurements of NH_4^+ uptake were done with an ACT03 culture growing at 0.08 d^{-1}. These complementary data are only discussed with regards to the variability due to growth rates.

Variations of C/N composition ratios are available only for two strains (ACT03 and TL01) due to problems with the C measurements for the other strains. These values show increases over the light period: from 7.8 ± 0.16 (mean ± SD) molC/molN immediately after resuspension to 10.0 ± 0.20 three h later and to 11.6 ± 0.87 six h later for ACT03 and from 6.2 ± 0.27, to 7.0 ± 1.33 and to 9.8 ± 0.46 molC/molN for TL01.

Nutrients conditions during each experiment in terms of N-sources are summarized in Table 1. They showed that cells were N sufficient due to significant NO_3^- concentrations. Initial NO_3^- concentrations ranged between 3.9 and 18.5 $\mu gatN.L^{-1}$ in resuspended cultures and the monitoring of these values along the day of experiment for VGO 565 and ACATA4 indicated that only a low consumption of this N-source occurred, during the first hour after resuspension.

Strain	Medium	Nutrient	Initial concentration	Concentration at the end of incubation			
				To	To + 3h	To + 6h	To + 24h
VGO 565	Filtered seawater	NO_3^- NO_2^- NH_4^+ Urea	5.6 ± 0.5 0.1 ± 0.0 0.8 ± 0.0 2.9	4.2 ± 0.2 0.1 ± 0.0 1.2 ± 0.1	4.3 ± 0.3 0.1 ± 0.0 1.1 ± 0.3	4.4 ± 1.1 0.1 ± 0.0 1.3	4.6 ± 0.4 0.2 ± 0.0 4.7 ± 0.0
ACATA4	Filtered seawater	NO_3^- NO_2^- NH_4^+ Urea	3.9 ± 0.1 0.2 ± 0.0 0.1 2.5	2.8 ± 0.2 0.2 ± 0.0 0.9 ± 0.3	3.1 ± 0.4 0.2 ± 0.0 0.3 ± 0.5	3.0 ± 0.2 0.2 ± 0.0 0.4 ± 0.3	
ACT03	ESAW	NO_3^- NH_4^+ Urea	17.7 3.0 ± 0.3 0.9 ± 0.8	3.1 ± 0.1	2.4 ± 0.3	1.2 - 13.5 *	
TL01	ESAW	NO_3^- NH_4^+ Urea	18.5 1.4 ± 0.0 1.0 ± 0.9	1.5 ± 0.2	0.7 ± 0.2	0.1 ± 0.1	

Table 1: Nutrients concentrations (in µgatN.L^{-1}) measured in the culture medium during each experiment. The initial concentration corresponds to the nutrient concentration in the resuspended culture. Concentrations at the end of each incubation (starting just after (To), 3h after (To+3h), 6h after (To+6h) or 24h after (To+24h) the cells resuspension) correspond to the mean value of 16 samples of the two series (with addition of $^{15}NH_4^+$ or ^{15}N-urea) for NO_3^- and NO_2^- and to the mean value of 8 samples of the ^{15}N-urea incubations for NH_4^+. Data presented for the To+6h and To+24h incubations with the VGO 565 strain correspond only to measurements after addition of 10 µgatN.L^{-1} of NH_4^+ or urea. * Range of values for 8 measurements reflecting increasing NH_4^+ release upon increasing urea concentration.

2- Short-term time series of uptake rates

The existence of a preconditioning effect due to growth on NO_3^-, as described by Dortch et al. (1991), or a surge uptake just after substrate addition has been tested through high frequency measurements (Fig. 1). Ammonium and urea uptake rates appeared to be constant over one hour, even for cells maintained several hours in the resuspension culture medium. Results of urea uptake by *A. catenella* cells maintained six hours in the resuspension

medium are shown in Fig. 1a for one Spanish strain (VGO 565) and one French strain (ACT03). The linearity of NH_4^+ and N-urea uptake rates over time just after substrate addition was also verified in field conditions, during the *A. catenella* bloom episode of 2001 in Thau lagoon (Fig. 1b).

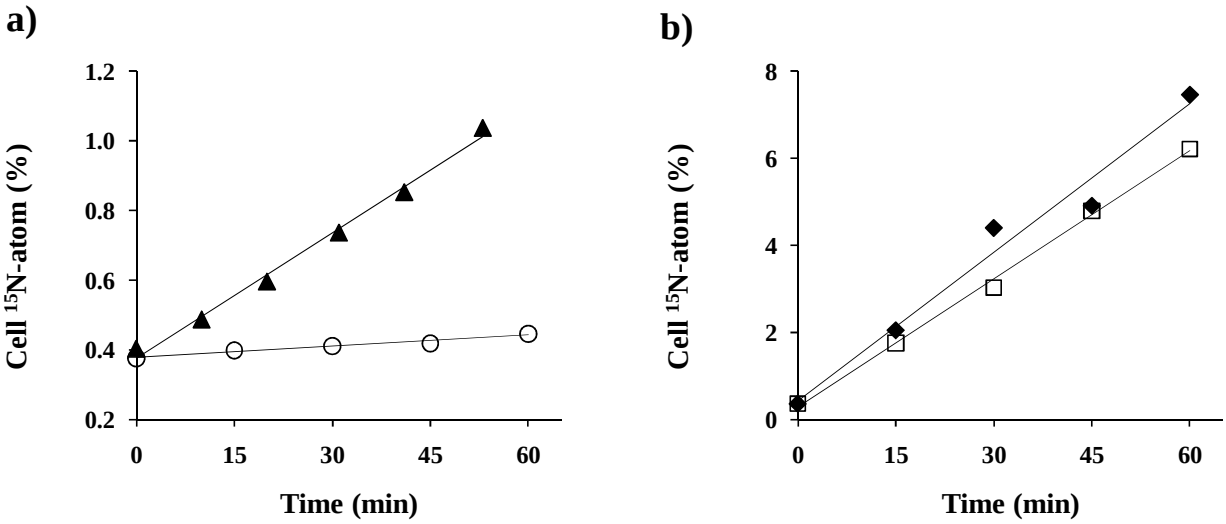

Figure 1: ^{15}N incorporation (in % of ^{15}N-atom in cells contents) over one hour just after substrate addition in cultures (a) and field conditions (*A. catenella* bloom of 2001 in Thau lagoon) (b). Measurements in cultures corresponded to ^{15}N-urea uptake by cells maintained 6h in the resuspension medium for VGO 565 (○) and ACT03 (▲). Respective r^2 values of linear regressions are 0.96 and 0.99. Linear regressions associated with field measurements of $^{15}NH_4^+$ incorporation (◆) and ^{15}N-urea incorporation (□) were characterized by respective r^2 values of 0.97 and 1.00.

3- Ammonium uptake

For the four strains tested, relations between NH_4^+ uptake rates and added NH_4^+ concentrations followed Michaelis-Menten kinetics. Uptake data obtained are presented in Fig. 2 and values of kinetics parameters (V_{max}, K_S, α) generated by the model are summarized in Table 2. Repeat values obtained a year apart for ACT03 at two different growth rates (0.23 d^{-1} and 0.08 d^{-1}) were similar for the first incubation (at To) (Fig. 2b). Three h after resuspension, results showed identical uptake rates at 10 µgat $N.L^{-1}$ but small differences at intermediate substrate concentrations.

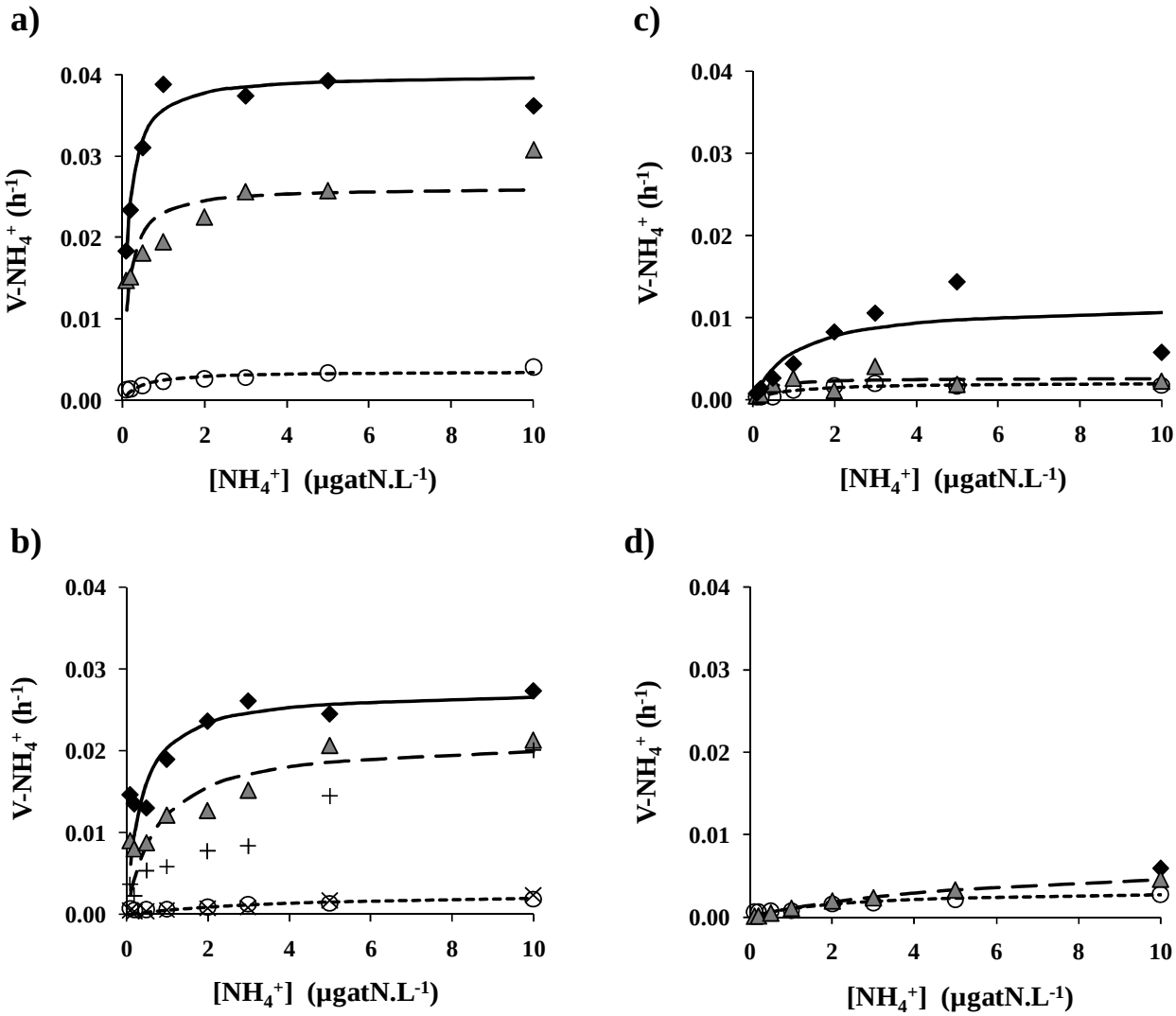

Figure 2 : Kinetic curves of NH_4^+ uptake for the TL01 strain from France (a), the ACATA4 strain from Italy (b) and the VGO 565 strain from Spain (c). Data from the three experiments, are represented by (○) for the incubation just after the resuspension (To), (▲) for the second incubation (To+3h) and (◆) for the third incubation (To+6h). The respective modeled curves by the Michaelis-Menten model correspond to a dot line (To), a dashed line (To+3h) and a solid line (To+6h).

The values of V_{max} and α showed an increase over the three successive incubations for the four strains of *A. catenella*. This trend was particularly strong for the ACT03 strain, the TL01 strain and the ACATA4 strain. For those strains, V_{max} increased, respectively, by a factor of 9, 10 and 6 over the course of the experiment, and alpha by a factor of 47, 16 and 5 (Table 2). Thus, even if the values of V_{max} and α estimated just after resuspension were very similar, differences between strains for these parameters increased with time. The maximal value of V_{max} obtained for each strain was 0.040 h^{-1} for TL01, 0.029 h^{-1} for ACT03, 0.012 h^{-1} for ACATA4 and 0.007 h^{-1} for VGO 565.

Incubation time	Linear regressions						Michaelis-Menten model			
	TL01 (France)		ACATA4 (Italia)		VGO 565 (Spain)		ACT03 (France)			
	α (× 10^{-3})	R^2	α (× 10^{-3})	R^2	α (× 10^{-3})	R^2	V_{max}	K_S	R^2	α (× 10^{-3})
To	0.1	0.96	0.3	0.99	3.0E-05	0.22	0.0004	2.3	0.90	0.1
To + 3h	0.8	0.98	0.7	0.97	2.0E-05	0.92	0.001	0.6	0.86	0.8
To + 6h	1.8	1.00	0.5	0.98	9.0E-05		0.023	2.2	0.96	0.9

Table 3: Parameters (V_{max} (h^{-1}), K_S ($\mu gatN.L^{-1}$), α ($L.h^{-1}.\mu gatN^{-1}$)) obtained for ^{15}N-urea uptake rates of A. *catenella* cells, just after the cells resuspension (To), 3h after (To+3h) and 6h after (To+6h).

Incubation time	TL01 (France)			ACT03 (France)			ACATA4 (Olbia, Italia)			VGO 565 (Spain)			VGO 565 (Spain)
	V_{max}	K_S	R^2	α (× 10^{-3})	V_{max}	K_S	R^2	α (× 10^{-3})	V_{max}	K_S	R^2	α (× 10^{-3})	α (× 10^{-3})
To	0.004	0.4	0.85	3.9	0.003	4.1	0.77	0.7	0.002	0.9	0.87	1.5	0.003	2.3	0.93	1.1
To + 3h	0.026	0.1	0.71	40.6	0.021	0.8	0.78	16.8	0.003	0.3	-	3.6	0.007	6.2	1.00	1.1
To + 6h	0.040	0.1	0.92	64.5	0.028	0.4	0.77	32.9	0.012	1.0	0.49	7.7	0.006			

Table 2: Kinetics parameters (V_{max} (h^{-1}), K_S ($\mu gatN.L^{-1}$), α ($L.h^{-1}.\mu gatN^{-1}$)) of NH_4^+uptake by the four strains of *A. catenella*, just after the cells resuspension (To), 3h after (To+3h) and 6h after (To+6h). For VGO 565, V_{max} at To+6h corresponds to the uptake rate measured after an addition of 10 $\mu atgN.L^{-1}$ of NH_4^+.

K_S values decreased over time for TL01 and ACT03 (factor of 4 and 12 respectively) but did not show a clear trend for the other strains.

4- Urea uptake – saturable kinetics for ACT03

Two different patterns were observed in the relation between ^{15}N-urea uptake rates and added urea concentrations, depending on the strain tested.

For the ACT03 strain, variations of ^{15}N-urea uptake rates along the urea gradient followed the Michaelis-Menten model (Fig. 3 a). As for NH_4^+ uptake, an increase in V_{max} and α was measured over the course of the experiment (Table 3) leading to a maximal value of V_{max} of 0.023 h^{-1}. No trend could be defined in the temporal variations of Ks which varied around a mean of 1.7 µgatN.L^{-1}.

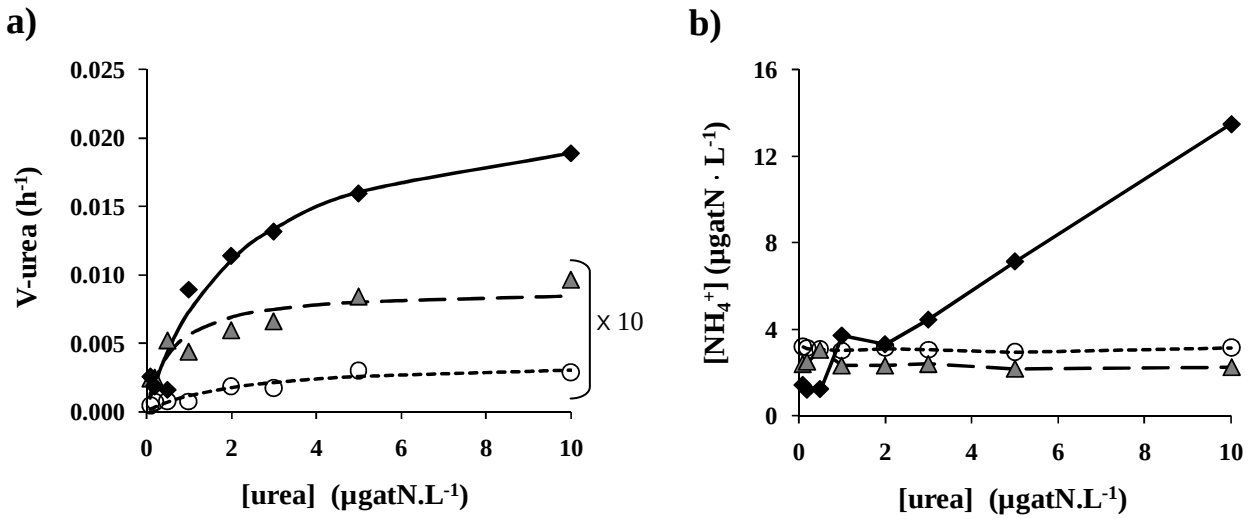

Figure 3: Kinetic curves of N-urea uptake (a) and ammonium concentration in the medium culture (b) for the ACT03 strain from France. Data from the three experiments, are represented by (○) for the incubation just after the resuspension (To), (▲) for the second incubation (To+3h) and (◆) for the third incubation (To+6h). The respective modeled curves by the Michaelis-Menten model correspond to a dot line (To), a dashed line (To+3h) and a solid line (To+6h).

Measurements of NH_4^+ concentrations in the culture medium permitted the detection of a large NH_4^+ release in cultures 6 h after resuspension (Fig. 3 b). This NH_4^+ production during urea assimilation resulted in a significant discrepancy between net and gross ^{15}N-urea uptake rates. For example, at the higher urea concentration (10 µgatN.L^{-1}), a gross uptake rate of 0.749 h^{-1} was computed from a net uptake rate of 0.019 h^{-1}, a PN value of 13.8 µgatN.L^{-1}, a

difference in NH_4^+ concentrations of 12.3 µgatN.L^{-1} and an incubation time of 1.22 h. When comparing the rate of NH_4^+ release during this third incubation period with the product of gross uptake rate and PN (ρ·PN, in µgatN.L^{-1}.h^{-1}), ^{15}N losses under the form of NH_4^+ accounted for 91-97 % of gross ^{15}N-urea uptake rate of *A. catenella* cells. Thus, the pattern of gross uptake rate as a function of the urea concentration became similar to the one of NH_4^+ release and may be modeled by a linear regression with a global slope of 0.074 L.h^{-1}.µgatN^{-1}.

5- Urea uptake – unsaturated kinetics for TL01, ACATA4 and VGO 565

For the three strains TL01, ACATA4 and VGO 565, linear relations were observed between 15N-urea uptake rates and added urea concentrations along the substrate gradient tested (Fig. 4). Estimations of the slope and r^2 values for each linear regression are summarized in Table 3. For the three data series of the TL01 strain and the first incubation of the VGO 565 strain, parameters values were computed from linear regressions without zero Y intercept.

The range of temporal variations in ^{15}N-urea uptake rates appeared to be very different between the three strains. A regular increase in ^{15}N-urea uptake rates all along the urea gradient for the TL01 strain (Fig. 4 a) led to a net increase in the regression slope during the course of the day (Table 3) and to a maximal uptake rate of 0.024 h^{-1}. For the ACATA4 strain, this trend was not clear. Even if an increase in urea uptake rates was visible between the first and the second incubation, these rates were not all maintained 3 h after (Fig. 4 b), leading to a maximal urea uptake rate of 0.008 h^{-1}. No trend was visible for VGO 565 for which very low ^{15}N-urea uptake rates (lower than 0.001 h^{-1}) were measured during the three incubations (Fig. 4 c).

For the first incubation of VGO 565, a low r^2 value was obtained (r^2 = 0.22) even though the linear regression was not forced through the origin of axes. The Y intercept may only be representative of the standard error of measurements. For the TL01 strain (Fig. 4 a), significant Y intercepts were also necessary to yield high r^2 values for the linear regressions. Considering the significant trends observed with r^2 > 0.96, these deviations may be representative of a physiological process. They suggest that a complete description of the relation between urea uptake and concentration may involve biphasic kinetics. These mixed kinetics could be described by a Michaelis-Menten relationship under very low urea

concentration (probably less than 1 µgatN.L^{-1}.h^{-1}) and by a linear fit at higher concentrations (1–10 µgatN.L^{-1}.h^{-1}). However, more uptake rate data are required under 1 µgatN.L^{-1}.h^{-1} of urea to test and estimate parameters of these biphasic models.

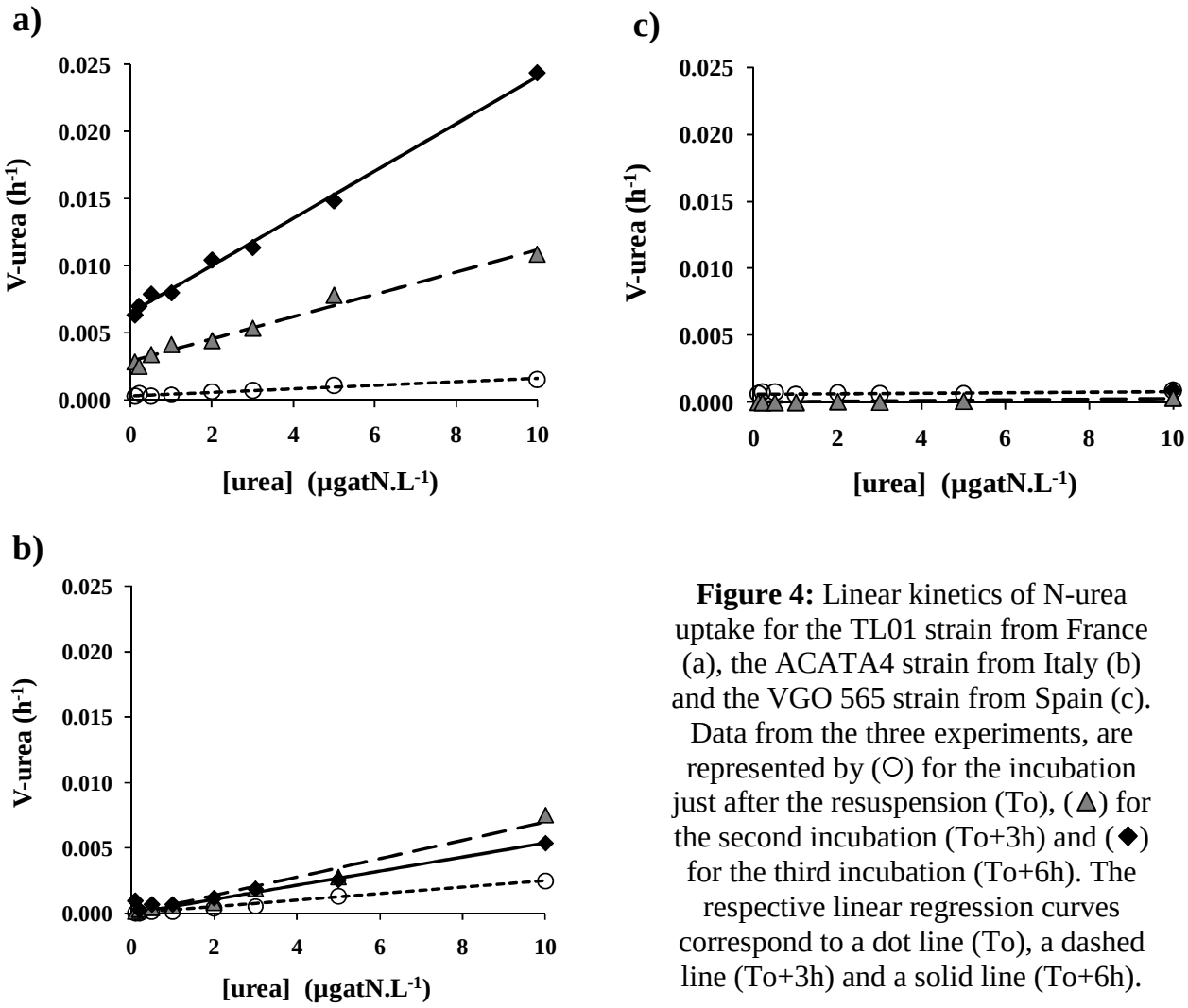

Figure 4: Linear kinetics of N-urea uptake for the TL01 strain from France (a), the ACATA4 strain from Italy (b) and the VGO 565 strain from Spain (c). Data from the three experiments, are represented by (○) for the incubation just after the resuspension (To), (▲) for the second incubation (To+3h) and (◆) for the third incubation (To+6h). The respective linear regression curves correspond to a dot line (To), a dashed line (To+3h) and a solid line (To+6h).

For those experiments, no change in NH_4^+ concentrations in the medium could be noted for these three strains over the incubation period (Table 1). So, no N losses have to be taken into account during the incubations and the net and gross N-urea uptake rates could be considered as similar for the TL01, ACATA4 and VGO 565 strains. Twenty four hours after resuspension, NH_4^+ excretion was observed for the Tarragona strain (VGO 565) (Table 1). At this point, about 45 % of the N taken up as urea (10 µgat N.L^{-1} addition) was recovered in the medium under the form of NH_4^+.

IV- DISCUSSION

1- Temporal variations in nutrients kinetics

There is a strain-dependent acclimation over time of the cells uptake system to a N pulse (either NH_4^+ or urea) along the concentration gradient. Except for N-urea uptake by VGO 565, this acclimation is expressed through an increase in V_{max} and α for saturated uptake kinetics and by an increase in the slope α for linear kinetics (Fig. 2, 3 and 4, and Tables 2 and 3). According to the results of short time-series experiments (Fig. 1), constant uptake rates were observed over the incubation period. Although in contrast with previous studies showing preconditioning effect (Dortch et al. 1991) or surge uptake (Conway et al. 1976; Glibert & Goldman 1981; Goldman & Glibert 1982; Horrigan & McCarthy 1982), these trends are in accordance with the results of Cochlan & Harrison (1991a) in cultures of *Micromonas pusilla* for both NH_4^+ and urea, Wheeler et al. (1982) for Chesapeake Bay phytoplankton at high nutrient stations and Tamminen & Irmisch (1996) in the Baltic Sea. Therefore, no such effect has interfered in the trends observed here, even for incubations six hours after resuspension. Stress due to resuspension does not seem to be involved here as inorganic carbon uptake did not differ with time at low N additions. For example, values of 0.030, 0.033 and 0.026 h^{-1} were observed for inorganic C uptake by TL01 at To, three h and six h respectively following NH_4^+ addition. For the series with urea additions, corresponding values were 0.036, 0.031 and 0.027 h^{-1}. Therefore, those trends could be due to two major causes: daily periodicity in uptake due to the light/dark cycle or change in the cells nutrient state following resuspension in low nitrogen medium.

Concerning the first cause, very few studies exist on changes in kinetic parameters over the light-dark cycle. Eppley et al. (1971) noted a peak in NH_4^+ uptake by *Skeletonema costatum* around noon in continuous cultures on a day-night cycle (factor of 3 increase over 6 h). Ammonium uptake by *Pavlova lutheri* increased by a factor of 4 over 6 hours in the light period (Caperon & Ziemann 1976). In natural populations of marine phytoplankton dominated by *Gonyaulax polyedra*, V_{max} for NH_4^+ increased two fold over 4 to 8 hours depending on stations (McIsaac 1978). For urea uptake, Tamminen & Irmisch (1996) reported a five fold increase in uptake rate over 12 h under natural irradiance, with the maximum uptake taking place in the late afternoon (around 18:00). The only study on changes in both K_S and V_{max} for a major limiting nutrient over a day/night cycle that we are aware of is that of

Chisholm & Stross (1976 a,b) on phosphate uptake by *Euglena gracilis*. For P-sufficient cells, they observed an increase in V_{max} (factor of 2.5 272 over 5 h) and an increase in K_S (2 fold over 5 h, 3.5 fold over 10 h) during the light phase (Chisholm & Stross 1976 a). They related those changes to the daily oscillation in energy source (L-D cycle) or to an endogenous rhythm.

The alternative explanation to temporal variations in V_{max} and α is a change in the nutrient status of cells (Caperon & Meyer 1972; Conway et al. 1976; Collos 1980). Thus, Harrison (1976) managed to separate effects of daily periodicity from those of N deficiency by comparing N sufficient and N starved (24 h without N) cells. Such coupled experiments have revealed that nutrient limitation may modify the daily rhythm of nutrient uptake. Chisholm and Stross (1976 b) have observed a temporal shift in the maximal P-uptake rate from noon for P-sufficient cells to the end of the light period for P-limited cells. A small increase in V_{max} for P-limited cells (1.5 fold over 12 h) was also observed, as well as no variation in K_S which contrasts with the increase noted for P-sufficient cells (Chisholm & Stross 1976 a,b). In the present study, the presence of significant NO_3^- concentrations indicate that *A. catenella* cells were N-sufficient during experiments. Therefore, increases in C/N ratios measured for ACT03 and TL01 over 6 h must be due to the daily irradiance (uncoupling between carbon fixation and N assimilation) and not to N deficiency.

Thus, the changes in kinetic parameters with time as observed here are due to the daily irradiance cycle.

2- Variability among strains

Large differences in uptake kinetics are seen between strains. The clearest one concerns urea uptake and contrasts ACT03 to the other strains. This strain is the only one for which a large NH_4^+ release linked with urea assimilation has been observed during the incubations and for which net N-urea uptake rate can be modeled by Michaelis-Menten kinetics over the concentration range tested. The existence of a release process during urea assimilation may not be limited to this strain. A strong NH_4^+ release was also noted for the Tarragona strain (VGO 565) but only 24 h after resuspension. This illustrates the need for a better knowledge of the time scale of this process. A number of unicellular algae have been reported to release NH_4^+ during urea assimilation (Rees & Bekheet 1982; Price & Harrison 1988; Uchida 1976). In particular, *Prorocentrum micans* released large amounts of NH_4^+

during urea assimilation, with a mean of 60 % of urea uptake, and individual values ranged between 23 and 81 % of urea taken up (Uchida 1976). For *A. catenella* cells, no NH_4^+ release was observed in a previous work (Collos et al. 2004) using another strain (ACT 2000). The strong NH_4^+ release measured for the ACT03 strain (more than 90 % of gross N-urea uptake) is accompanied by very high gross N-urea uptake rates, which are nevertheless within physiological bounds (McCarthy & Goldman 1979). Comparing these gross N-urea uptake rates with NH_4^+ uptake rates, the preferred N source for ACT03 appears to be urea 6 h after resuspension, whereas NH_4^+ clearly remains the preferred N source for the other strains. The NH_4^+ release process by ACT03 is consistent with the observed lack of intracellular NH_4^+ accumulation in that strain in contrast with TL01 (Collos et al. 2006). Such a mechanism removes urea from the medium, thereby making it unavailable to other potential competitors, but it supplies N back to the medium under the form of NH_4^+. The net result is a rather limited incorporation of N, so the ecological advantage of such a limiting nutrient acquisition strategy is not obvious. Ammonium release could also be a mechanism for reducing intracellular NH_4^+ toxicity during uncoupling between urease activity and the maximum rate of NH_4^+ assimilation following exposure to a urea pulse.

The second characteristic of the urea uptake system of ACT03 was represented by saturable kinetics. Values of K_S ranged from 0.6 to 2.3 μgatN.L^{-1} (Table 3) in our study and agree with the range of 0.4-1.7 μgatN.L^{-1} generally observed for urea uptake by marine phytoplankton in laboratory cultures (McCarthy 1972; Rees & Syrett 1979; Cochlan & Harrison 1991b). In particular for dinoflagellates, (Fan et al. 2003a) reported values of 0.9-1.8 μgatN.L^{-1} for *Prorocentrum minutum*. No data exist in the literature for other *Alexandrium* species.

Considering gross N-urea uptake rates, kinetics of uptake for the ACT03 strain appeared to be linear, as for the three other strains of *A. catenella* (Fig. 4). Such linear kinetics are surprising because urea assimilation by *A. catenella* cells involves the enzyme urease (Dyhrman & Anderson 2003) whose activity has always been related to Michaelis-Menten kinetics (Mobley et al. 1995; Fan et al. 2003b). This discrepancy can be explained by the existence of separated regulation systems between urease activity and urea uptake or by a saturation kinetics model involving a very high K_S. Results obtained in previous works supported the second hypothesis. Collos et al. (2004) reported K_S-urea values of 28 and 44 μgatN.L^{-1} respectively for cultures and natural populations of *A. catenella*. For the strain ACT 2000 (Collos et al. 2004), saturation of urea uptake occurred only above 20 μgatN.L^{-1}. Linear

kinetics are usually interpreted as diffusion of substrate (Glibert et al. 2006) or non-saturable kinetics which maximize the uptake rates at high concentrations (Fan et al. 2003a). In the present study, the linearity between uptake rates and substrate concentration may reflect a different process: very low capacities of *A. catenella* cells to take up urea at low concentrations through saturable kinetics with very high K_S.

For NH_4^+ kinetics, K_S-NH_4^+ values obtained for *A. catenella* cells ranged from 0.1 to 6.2 µgatN.L^{-1} in our study (from 0.1 to 0.4 µgatN.L^{-1} for TL01 and from 2.3 to 6.2 µgatN.L^{-1} for VGO 565). Concerning other *Alexandrium* species, values between 0.25 and 0.38 µgatN.L^{-1} were observed in *A. minutum* by Maguer et al. (2007) and a value of 2 µgatN.L^{-1} was reported by MacIsaac (1978) for *A. tamarense*. So the variability between strains of *A. catenella* observed here is as large as that between species of the same genus.

From a temporal point of view, marked differences between strains were also noted in acclimation capacities of uptake kinetics to N pulses. The range of acclimation potential observed widens the differences in N-uptake between strains with time. Thus, a large range of maximal values of uptake rates (close to the V_{max} value for saturable kinetics) were measured 6 h after resuspension: 0.007 to 0.040 h^{-1} for NH_4^+, and 0.001 to 0.024 h^{-1} for urea (or 0.001 to 0.749 h^{-1} for gross uptake rates), depending on the strain. These differences in maximal uptake rates could not be explained by differences in growth rates. Additional measurements done with the ACT03 strain (Fig. 2b) showed that NH_4^+ uptake rates measured at the higher concentration (10 µgatN.L^{-1}) were similar for two different growth rates (0.08 d^{-1} and 0.23 d^{-1}) just after and three h after resuspension. The small differences in utptake rates observed at lower concentrations suggest that the affinity to NH_4^+ may be reduced when the growth rate decreased, as observed by Collos et al. (2007) during an *A. catenella* bloom in Thau lagoon. However, the uptake rates at 0.08 d^{-1} (Fig. 2b) do not change intra- and inter strain trends and ranking of uptake capacities. Moreover, the Spanish strain VGO 565 presented a high growth rate but the lowest uptake rates of NH_4^+ and urea 6 h after resuspension. Thus, the range of uptake rates achieved during each experiment represents differences in uptake capacities between strains. Values obtained for both French strains (TL01 and ACT03) are in accordance with V_{max} estimated in cultures by Collos et al. (2004) with the strain ACT 2000 and confirm previous results on such differences in V_{max}-NH_4^+ between TL01 and ACT03 (Collos et al. 2006).

The comparison of kinetics parameters allows the ranking of those strains as a function of their acclimation potential and N acquisition capacities. For NH_4^+, the coupled

increases in V_{max} and alpha (Table 2) allow to define the following ranking (from lowest to highest acquisition capacities): VGO 565 < ACATA4 < ACT03 < TL01. For urea (Table 3), net uptake capacities may only be compared with the maximal uptake rate measured (after addition of 10 $\mu gatN.L^{-1}$) because of the difference in kinetics patterns between strains and lead to the same ranking between strains. The comparison of gross N-urea fluxes changes this ranking, giving ACT03 the best N-urea influx capacity 6 h after resuspension. So, for ACT03, NH_4^+ losses by excretion clearly decrease the competitiveness of this strain when urea is the limiting resource. The fact that similar rankings were observed between strains from net uptake rates and that both French strains remain close in these rankings may indicate specific adaptations of *A. catenella* cells to environmental conditions characterizing the three originating areas, the Tarragona harbor in Spain (for VGO 565), the Olbia Bay in Italy (for ACATA4) and the Thau lagoon in France (for ACT03 and TL01).

V- CONCLUSION

In conclusion, differences observed in N-uptake characteristics between strains of *A. catenella* point out difficulties to extrapolate results obtained in culture to the field. However, the analysis of several strains has shown a range of daily variations in N-uptake kinetics. This range encompasses the variations in V_{max} observed in the field over a few days by Collos et al. (2007), but not those of K_S. The latter could be due to an interaction between light and N limitation. Studies of N limited *A. catenella* in cultures are presently seriously hampered by methodological problems (Collos et al. 2006). Nevertheless, the relationship between N-uptake kinetics and the daily light cycle highlights the importance of assessing daily uptake rates from successive short-term measurements rather than a sole one during the day. Two of the four strains tested, the ACT03 and the VGO 565 strain, seem to have complex regulation systems of N-urea fluxes, including extensive N losses during N-urea assimilation through NH_4^+ excretion. Moreover, this excretion process points out potential interactions between NH_4^+ and urea uptake. Such nutrient interactions may be part of the regulation system of N urea uptake and require further investigations. A wider implication of our results concerns the estimation of urea uptake by the ^{15}N tracer technique. If N losses due to NH_4^+ release during urea assimilation are not taken into account, it is likely that the importance of urea in the N nutrition of phytoplankton has been widely underestimated (up to 90 %).

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