Inter- and intra-specific carbon and nitrogen assimilation by dinoflagellate and diatom species

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Phytoplankton are key components in primary production and their growth is determined mainly by the availability of nitrogen-nutrients in the ocean. The paradigm that phytoplankton prefer ammonium over nitrate when both substrates are present has been tested using ^{15}N-labelled tracers on cultured diatoms and dinoflagellates isolated from two widespread geographical areas – the Baltic Sea and the Arabian Sea. We found contrary to the paradigm that both taxa preferred nitrate over ammonium and a significant within-species variation in N assimilation. Carbon uptake rates in the same experiments were estimated using ^{13}C-labelled tracer.

Keywords: Carbon, dinoflagellate, diatom species, nitrogen, stable isotopes.

OCEAN primary productivity (rate of carbon fixation, measured in mg C m^{-2} d^{-1}) is one of the major sinks of atmospheric carbon dioxide and thus an important regulator of the Earth's climate[1]. Primary productivity is mainly determined by the availability of reactive nitrogen in the ocean surface waters[2]. Reactive nitrogen substrates could either be new (NO_3^-) or recycled (NH_4^+, urea) nutrients, depending upon the dominant process in the surface ocean. The type of preferred nitrogen substrate uptake by phytoplankton determines the efficiency of the marine biological pump. Generally, NH_4^+ is believed to be preferred over NO_3^- by phytoplankton, when both substrates are present[3]. However, this paradigm has been challenged and it is found that diatoms prefer NO_3^-, whereas NH_4^+ is preferred by dinoflagellates[4]. Understanding of preferred substrate is critical to estimate oceanic nitrogen and carbon budgets. The main problem for such estimates arises because the species do not behave exactly the same way around the globe and there might even exist variability on the intra-specific uptake level, as has previously been demonstrated with other phenotypic traits[5,6]. Therefore, if we do not take such variations into account, the models for prediction will not be accurate.

Marine phytoplankton species have previously been considered to have unlimited dispersal ability due to their small size and high numbers[7]. As a consequence, phytoplankton species would have no biogeographic boundaries. Conversely, studies during the past decade have revealed high genetic and phenotypic diversity within and among geographically isolated populations of various phytoplankton taxa[5,6,8]. To understand such diversity in uptake rates, we have estimated nitrogen and carbon uptake by genotypes of different species of dinoflagellates and diatoms.

Dinoflagellates and diatoms are two dominant phytoplankton phyla present in the coastal marine waters. Here, we have used two different species of dinoflagellates – *Prorocentrum micans* and *Alexendrium ostenfeldii* (three AFLP genotyped monoclonal strains AO24, AO30, AO36)[9], and one diatom species – *Skeletonema tropicum* (three microsatellite-based genotyped monoclonal strains S3, S7, S9)[10] in two sets of incubation experiments. Two of the phytoplankton species (one monoclonal strains of *P. micans* and three *S. tropicum* strains) were isolated from the subtropical waters, i.e. the Arabian Sea. Additionally, we used three monoclonal strains of *A. ostenfeldii* isolated from the Baltic Sea in order to assess phenotypic variability of microalgae from different geographical areas.

The dinoflagellates were maintained in f/2 medium, and the diatoms were maintained in (f/2 + Si) medium in salinity of their origin (6 PSU for the Baltic Sea strains, 35 PSU for the Arabian Sea strains)[11]. Phytoplankton cultures were grown at 25°C with cycles consisting of 14 h of light and 10 h of darkness; the light intensity was 50 µE s^{-1}. Two different sets of isotopic enrichment experiments in triplicates (except for dark incubation) were performed (Tables 1 and 2).

Prior to incubation, tracers containing 99 atom% ^{13}C ($NaH^{13}CO_3$, Cambridgee Isotope Laboratories, Inc., USA) and ^{15}N ($Na^{15}NO_3$, $^{15}NH_4Cl$ and $^{15}NH_2$-CO-$^{15}NH_2$, Sigma-Aldrich, USA) were added to the bottles. A constant amount of (100 µl of 0.2 mmol ml^{-1} concentration) $NaH^{13}CO_3$ and (1 ml of 2 µmol ml^{-1} concentration) $Na^{15}NO_3$ were added together to each sample bottle containing 40 ml f/2 (or f/2 + Si) medium and 1 ml culture volume in the experiment 1 (exp#1). Next, 1 ml of 2 µmol ml^{-1} concentration of NH_4^+ and urea were added to different sets of bottles containing the same amount of medium and culture in exp#1. In experiment 2 (exp#2), a constant amount of (1 ml of 0.2 mmol ml^{-1} concentration) $NaH^{13}CO_3$ and (2 ml of 0.01 µmol ml^{-1} concentration) $Na^{15}NO_3$ was added together to each sample bottle containing 12 ml culture volume. Then 2 ml of 0.01 µmol ml^{-1} concentration of NH_4^+ was added to different sets of bottles containing the same amount of culture in exp#2. No urea incubation experiments were performed in exp#2. After the addition of tracers, incubation was performed for 4 h. Immediately after incubation, samples were filtered in dark, sequentially through pre-combusted (4 h at 400°C) 47 mm diameter and 0.7 µm pore size Whatmann GF/F filters.

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Table 1. Cell-specific rates of carbon and nitrogen uptake by a dinoflagellate (*Prorocentrum micans*) and a diatom (*Skeletonema tropicum*) in culture experiment #1

Species	C uptake ± σ * (pM C cell^{-1} h^{-1})	N uptake ± σ * (pM N cell^{-1} h^{-1})		
		NO_3^-	NH_4^+	Urea
Light				
P. micans	55.8 ± 6.8	1.5 ± 0.5	0.22 ± 0.00	0.23 ± 0.01
S. tropicum	24.0 ± 0.2	0.1 ± 0.0	0.04 ± 0.01	0.04 ± 0.00
Dark				
P. micans	24.1	1.08	0.18	0.22
S. tropicum	5.0	0.35	0.03	0.03

*σ is standard deviation of triplicates, wherever reported.

Table 2. Cell-specific rates of carbon and nitrogen uptake by three monoclonal cultures of a dinoflagellate (*Alexendrium ostenfeldii*) and a diatom (*S. tropicum*) species in culture experiment #2

Species	Strain	C uptake ± σ * (pM C cell^{-1} h^{-1})	N uptake (pM N cell^{-1} h^{-1}) ± σ *	
			NO_3^-	NH_4^+
Light				
A. ostenfeldii	AO24	326.1 ± 83.4	31.1 ± 11.4	0.2 ± 0.0
	AO30	342.3 ± 36.0	56.7 ± 12.6	0.1 ± 0.1
	AO36	1821.4 ± 1129	81.7 ± 42.4	0.4 ± 0.2
S. tropicum	ST3	8.0 ± 2.6	1.3 ± 0.4	0.005 ± 0.002
	ST7	18.8 ± 10.9	0.6 ± 0.3	0.003 ± 0.001
	ST9	14.4 ± 9.5	5.5 ± 3.7	0.008 ± 0.005
Dark				
A. ostenfeldii	AO24	49.1 ± 6.8	51.8 ± 7.2	Not measured
	AO30	75.5 ± 6.5	46.4 ± 4.0	
	AO36	77.5 ± 34.4	94.2 ± 41.8	
S. tropicum	ST3	1.3 ± 0.3	0.8 ± 0.2	
	ST7	1.1 ± 0.5	0.6 ± 0.3	
	ST9	8.0 ± 5.2	3.6 ± 2.3	

*σ is standard deviation of triplicates.

A Finnigan Delta Plus mass spectrometer was used to measure particulate organic nitrogen (and carbon) and atom% ^{15}N (and ^{13}C) in the filters. For nitrogen, calibrated casein and international standards $(NH_4)_2SO_4$ (IAEA-N-2) and KNO_3 (IAEA-NO-3) were used. While for carbon, calibrated starch and international standard ANU sucrose were used. The external precisions of the measurements were better than 0.5‰. (For more experimental details see ref. 12.) For the calculation of nitrogen and carbon uptake rates, we used mass balance equations[13,14].

One-way ANOVA was followed to test the significance levels and associated *P* values are provided in the discussion below. Cell abundances were determined by microscopic cell counts in Sedgewick Rafter chambers. Cells were counted on the day of incubation for exp#1, and over a period of 1–9 days for exp#2. The growth curves of the three strains of *A. ostenfeldii* and *S. tropicum* respectively, are shown in Figure 1.

All the strains were in exponential growth phase on the day of the incubation experiment. The intra-specific growth rate varied: for *S. tropicum* S7 attained maximum growth (2.5 d^{-1}), whereas S3 had minimum growth rate (1.1 d^{-1}; $P = 0.18$). For *A. ostenfeldii*, AO24 displayed maximum growth rate (0.41 d^{-1}), but this was not significantly different from the observed lower value (0.26 d^{-1}) for AO36 ($P = 0.46$).

Cell-specific carbon uptake rate for *P. micans* (55.8 pM C cell^{-1} h^{-1}) was more than double of that *S. tropicum* (24.0 pM C cell^{-1} h^{-1}) for light incubations in exp#1 (Table 1). Furthermore, in the dark incubations in the same experiment, carbon assimilation by *P. micans* (24.1 pM C cell^{-1} h^{-1}) was approximately five times that of *S. tropicum* (5.0 pM C cell^{-1} h^{-1}). In addition, both light and dark nitrogen (all forms) assimilation rates were an order of magnitude higher for *P. micans* than that of *S. tropicum*. Both *P. micans* and *S. tropicum* consumed more NO_3^- than NH_4^+ and urea together, highlighting the preferential uptake of oxidized form of nitrogen by these two dinoflagellate and diatom species. Earlier studies have also reported higher NO_3^- uptake rates of dinoflagellates (~1 pM N cell^{-1} h^{-1}) than that of diatoms

(~0.03 pM N cell^{-1} h^{-1}), but such uptake rates were smaller than those observed here[15].

Light incubation rates for all tested strains were always higher than dark incubation rates (Tables 1 and 2). When we did similar experiments with three different strains of each species, carbon and nitrogen uptake rates were up to 100 times higher for the dinoflagellate strains compared to the diatom strains, for both the light and dark incubations (Table 2; $P < 0.05$). AO36 had the highest carbon and nitrogen assimilation rates among the *A. ostenfeldii* strains for both light and dark incubations ($P < 0.05$). However, for *S. tropicum*, strain ST7 had the highest carbon uptake rate for the light incubation ($P < 0.05$), whereas ST9 had it for the NO_3^- and NH_4^+ uptake in light ($P < 0.001$), and carbon and NO_3^- uptake in dark. It is believed that NO_3^- assimilation by phytoplankton is generally inhibited exponentially with increasing NH_4^+ concentration, but it is never curbed completely[15–17]. Later it was discovered that diatoms and dinoflagellates prefer nitrate and ammonium as nitrogen source respectively[4]. However, our analysis suggests that NO_3^- is always preferred over NH_4^+. C : N uptake ratios were generally higher than the Redfield ratio (C : N = 16), except in the dark incubations of *S. tropicum* in exp#2.

Overall, our study suggests a significant difference in the carbon and nitrogen assimilation rates for light and dark incubations: monoclonal strains of dinoflagellates and diatoms display intra-specific variable phenotypic traits with respect to nitrogen and carbon uptake rates. The ability of these species to utilize different forms of nitrogen and thus to exploit a temporal and spatial variable environment is the key to survive and maintain growth in competitive coastal regions. However, NO_3^- seems to be the preferred nitrogen substrate for both the dinoflagellate and diatom species tested. Some earlier studies have also suggested that NO_3^- is the preferred substrate for diatoms at low temperatures[18], but our results are contrary to the generally accepted paradigm that NH_4^+ is the preferred nitrogen substrate for dinoflagellates. NO_3^- uptake is the key component in modelling new production; understanding strain-specific rates will improve the predictive nature of such models. These findings will add to our understanding of strain-specific nitrogen and carbon uptake rates by dinoflagellate and diatom species.

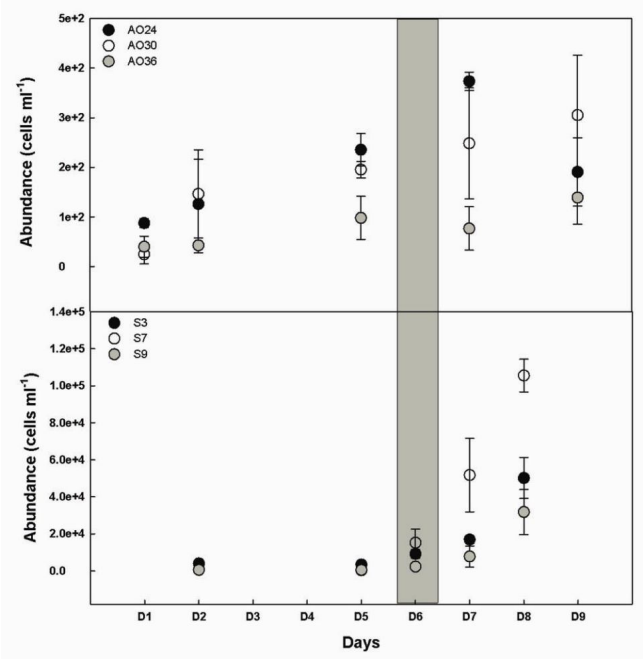

Figure 1. Growth of three monoclonal strains of *Alexendrium ostenfeldii* (AO24, AO30, AO36) and *Skeletonema tropicum* (S3, S7, S9) species in culture experiment #2. Vertical grey bar indicates the day experiment was performed.

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Compensatory effects of medicinal plants of Pakistan upon prolongation of coagulation assays induced by *Naja naja karachiensis* bite

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The present study was carried out to evaluate 28 medicinal plants of Pakistan having folklore claims to neutralize coagulopathy induced by *Naja naja karachiensis* bite in comparison with standard antidote. Venom was tested on citrated human plasma to determine its effect on prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT). Snake venom (200 µg/ml) was found to delay PT (13 ± 0.57 to 23 ± 0.57 sec), aPTT (35 ± 1.52 to 48 ± 2.0 sec) and TT (13 ± 0.57 to 33 ± 0.57 sec) within 4.5% coefficient of variance. Prolongation of PT and TT suggested the presence of thrombin-like or plasminogen activating enzymes. Methanolic plant extracts (5 µg/ml) were considered as effective standard antidote. *Enicostemma hyssopifolium* (Willd.) Verdoorn (PT = 22 ± 0.57 sec, aPTT = 36 ± 1.00 sec, TT = 19 ± 0.57 sec) and *Stenolobium stans* (L) D. Don (PT = 16 ± 0.57 sec, aPTT = 36 ± 0.57 sec, TT = 29 ± 0.57 sec) were considered the most protective (≥70%, but ≤ 92%) from the rest of the listed medicinal plants. Nevertheless, further studies are required for identification and segregation of bioactive constituent(s) as an alternate and cheap source to treat anticoagulation.

Keywords: Antidote, coagulopathy, medicinal plants, *Naja naja karachiensis*.

INCIDENCES of snake-bite poisoning are particularly frequent in tropical and subtropical areas of the world, resulting in high rate of mortality and morbidity. Hence it has received the attention of several researchers to find out the root cause of snake-bite poisoning and to pave the way for possible treatment[1]. Like other countries of the world, snake-bite envenomation is common in Pakistan, where 20,000 deaths are reported annually[2]. Among various species of Asiatic *Naja* (complex Asiatic cobras), *Naja naja karachiensis* causes serious disorders in the victims of snake bite. Severe pain, necrosis, bleeding from wounds, blood in urine, inflammation, gum bleeding and coagulopathies are some of the complications arising due to *N. n. karachiensis* bite[3–5].

Coagulopathy is one of the key after-effects of *N. n. karachiensis* envenomation. It is the clotting defect in which blood is unable to congeal contrary to normal blood. Clotting disorders have been monitored by coagulation assays in diagnostic laboratories[5]. These include PT (prothrombin time), aPTT (activated partial thromboplastin time) and TT (thrombin time). Coagulation assays are surrogate markers for various blood-clotting factors. PT has presumptive evidences about II, V, VII and X clotting factors, while aPTT possesses information about VIII, IX and XI factors. TT is linked with fibrinogen (factor I) along with its measurements[5]. Administration of antisera is an appropriate therapy to combat snake-bite envenomation. However, due to their limited supply and high cost, the rural population is unable to afford them[2].

Consequently victims have to rely on medicinal plants to treat snake bite as they have been reported in the literature to neutralize various snake venoms[6]. In the present study, various medicinal plants of Pakistan (widespread in different locations) were collected to facilitate the victims against *N. n. karachiensis* venom-induced coagulopathies. These included *Albizia lebbeck* (L.) Benth, *Allium cepa* L., *Allium sativum* L., *Althaea officinalis* L., *Bauhinia variegate* L., *Brassica nigra* (L.) W. D. J. Koch, *Calotropis procera* (Aiton) W. T. Aiton, *Cedrus deodara* (Roxb. ex D. Don) G. Don, *Citrullus colocynthis* (L.) Schrad, *Citrus limon* (L.). Burm. f, *Cuminum cyminum* L., *Enicostemma hyssopifolium* (Willd.) I. Verd, *Fogonia cretica* L., *Leucas capitata* Desf, *Matthiloa incana* (L.)

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