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Understanding the nitrogen uptake and assimilation of the Chinese strain of *Aureococcus anophagefferens* (Pelagophyceae)

Linjian Ou[1], Yangyang Cai[1], Wenyu Jin, Zhou Wang, Songhui Lu*

Research Center of Harmful Algae and Marine Biology, and Key Laboratory of Eutrophication and Red Tide Prevention of Guangdong Higher Education Institutes, Jinan University, 510632 Guangzhou, PR China

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ABSTRACT

Brown tides caused by *Aureococcus anophagefferens* have occurred annually and severely impacted on the local ecology and economy in the coastal waters of Qinhuangdao, China since 2009. The uptake preference, pigment composition and growth responses of the Chinese strain of *A. anophagefferens* to different nitrogen substrates were studied in the laboratory to better understand its nitrogen (N) strategies and compare the findings with those from the other strains in the U.S.A. Our results indicated that *A. anophagefferens* had a high absolute uptake rate (ρ_{max}) for NH_4^+ and low half-saturation constants (K_s) for NO_3^- and urea. It grew faster on the organic substrates (urea and glutamic acid) than that on the inorganic substrates (NO_3^- and NH_4^+). *A. anophagefferens* had higher contents of cellular pigments for both photosynthesis (chlorophyll *a*, chlorophyll c_2, 19′-butanoyloxyfucoxanthin and fucoxanthin) and photoprotection (β-Carotene and diadinoxanthin) on urea than did on NO_3^- and NH_4^+, suggesting a stronger capability for photosynthesis on urea. *A. anophagefferens* had higher contents of cellular carbon, N and proteins on urea than did on NO_3^- and NH_4^+, suggesting a stronger capability for the acclimation and storage of C and N on urea. *A. anophagefferens* produced more β-carotene rather than chlorophyll *a* on glutamic acid and thus inhibited photosynthesis and carbon fixation. We found in this study that urea was the best N substrate for the growth of *A. anophagefferens*. *A. anophagefferens* might have a competitive advantage over other phytoplankton in waters enriched with DON, particularly with urea.

1. Introduction

Since the first report of brown tides in the Narragansett Bay in the summer of 1985, *Aureococcus anophagefferens* Hargraves et Sieburth (Pelagophyceae) has been one of the most well-known and destructive harmful algal species in the eastern coastal waters of the U.S.A. over the last 30 years [1–3]. Brown tides of *A. anophagefferens* have also been reported to thrive frequently in the Saldanha Bay, South Africa since 1997 [4]. The notorious brown tides not only caused the large-scale die-off of seagrass beds of *Zostera marina* but also poisoned bivalves, in particular scallops (*Argopecten irradians*) and clams (*Mercenaria mercenaria*) [3,5,6].

China is the third country threatened by brown tides [7]. The *A. anophagefferens* blooms have been recorded annually in the coastal waters of Qinhuangdao of the Bohai Sea since 2009 [7–9]. The brown tides could last from May to August with a maximum cell density of approximately 10^9 cells l^{-1} [7,10]. The affected sea area by brown tides could even be larger than 3400 km^2 [11]. The significant negative impacts on both scallop mariculture and coastal tourism in the region were also reported [10,12]. The average economic loss due to brown tides was higher than $14.5 million per year [7].

The possible mechanisms of brown tide outbreaks in the U.S.A. have been studied [3,5,13]. The results suggested that the proliferation of brown tides was the combined effect of multiple factors, such as the decrease of ingestion rates of zooplankton, the weakening of the net flows of estuaries, and suitable conditions of temperature, salinity and nutrients [3,5], among which the increase of dissolved organic nitrogen (DON) in seawater was considered as an important factor for high densities of brown tides [14–18]. The input of dissolved inorganic nitrogen (DIN) inhibited the formation of brown tides, whereas the input of DON stimulated their occurrences [17,19,20]. *A. anophagefferens* could efficiently utilize the organic nutrients released by previous diatom blooms to become dominant population in phytoplankton communities [21]. Many studies emphasized the importance of urea during brown tides [4,15,22,23]. The affinity of *A. anophagefferens* for urea was much higher than that for NO_3^- [22,24]. Mulholland et al.

* Corresponding author at: Research Center of Harmful Algae and Marine Biology, Jinan University, Guangzhou, Guangdong 510632, PR China.
E-mail address: lusonghui1963@163.com (S. Lu).
[1] These authors contribute equally.

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[15,23] suggested that urea was the main nitrogen (N) source available during brown tides. The potential uptake rate of urea contributed 58–64% to the total N uptake during a brown tide [22]. The results from laboratories also support the advantages of *A. anophagefferens* in utilizing DON [25–28]. *A. anophagefferens* can utilize at least 8 different types of N sources (*e.g.* urea, amino acids, purines, polypeptides and proteins), and the ability to utilize different N sources makes it significantly different from those species that can utilize only a few N sources, such as *Thalassiosira pseudonana* [25]. Results from urease activities also supported that *A. anophagefferens* has higher affinity for urea [26]. Wurch et al. [27,28] found a suite of genes of *A. anophagefferens* were upregulated under N deficiency to utilize reduced N compounds and DON, which suggested the importance of these N sources in the bloom formation.

A. anophagefferens is an emerging HAB causative species in China [7,9]. Zhen et al. [10] indicated that a decrease of DIN was an important factor in the formation of *A. anophagefferens* in the area. The cell densities of *A. anophagefferens* were correlated with the increase of DON during brown tides [10,29]. High DON concentrations coupled with high extracellular enzyme activities during brown tides suggested an efficiency of microorganisms in utilizing dissolved organic nutrients [30]. All these results suggested that DON might also be an important factor in triggering brown tides in the coastal waters of Qinhuangdao, which was suggested by previous studies [16–23]. Considering that differences might exist among geographical strains, there is a need to ascertain the physiological characteristics of N utilization to the Chinese strain of *A. anophagefferens* for better understanding of nutritional strategies and to make clear whether N is also important during brown tides in China, even many studies have been done on the U.S.A strains.

In this study, we employed a physiological approach to examine the uptake and metabolism of N in *A. anophagefferens*, a strain from Qinhuangdao, China. The uptake kinetics, physiological and growth parameters were analyzed in a batch culture under treatments with different N substrates. The comparison between organic and inorganic N sources has been made to understand the potential importance of the organic N. The objective of this study is to demonstrate the N utilization strategies in the occurrences of brown tides in Qinhuangdao, China, based on evidences from field investigations in the area and the findings from the other strains in the U.S.A.

2. Materials and methods

2.1. Algal strain and culture conditions

The strain of *A. anophagefferens* (No. AA-1) was isolated from the coastal waters of Qinhuangdao in the Bohai Sea (119°37.911′E, 39°54.111′N) during a brown tide in July 2012 and was maintained in the Algal Collection, Research Center of Harmful Algae and Marine Biology, Jinan University, China.

Prior to the experiment, the culture was re-inoculated 3 times during the exponential phase in the Aquil* artificial seawater media (enriched with f/2) [31]. The concentrations of NO_3^- in the media were reduced gradually from 882 to 400, then to 100 μmol l^{-1} at each inoculation. The cultures were maintained at 21 ± 1 °C in a light dark cycle of 12:12 h with an irradiation of ~75 μmol photon m^{-2} s^{-1}. Antibiotics (Penicillin G, 3 g l^{-1}, and streptomycin sulfate, 5 g l^{-1}) were used to eliminate bacterial contamination 48 h before the next inoculation [32]. Cells were collected by centrifugation (6000 × *g*, 5 min), washed once with nitrogen-free sterile artificial seawater and then inoculated in a fresh medium without N addition for two days to get rid of the internal N pools. The cultures were checked for bacterial contamination with 4′,6-diamidino-2-phenylindole (DAPI)(Sigma) stain at regular intervals by microscopic inspection.

2.2. N uptake kinetics and N preference of A. anophagefferens

2.2.1. Experiment 1 design

The initial cell densities of *A. anophagefferens* in the media were ~3.5 × 10^5 cells ml^{-1}. The uptake kinetics were determined for three N substrates (NO_3^-, NH_4^+ and urea) (Cambridge Isotope Laboratories, all 99 at.% of ^{15}N). The ^{15}N was diluted with ^{14}N to obtain a constant 20 at.% enrichment. Considering that urea consists of 2 N atoms, each substrate was added at the final N concentrations of 0, 0.5, 1.0, 5.0, 15.0, 40.0 and 80.0 μmol N l^{-1}, with two replicates at each concentration. Incubation was terminated after 0.5 h by gentle filtration of 50 ml of seawater onto pre-combusted GF/F filters (450 °C, 2 h) and rinsed twice with sterile artificial seawater. The filters were dried at 60 °C for 48 h prior to isotope analysis. Samples for particulate N (PN) were also filtered onto pre-combusted GF/F filters and dried at 60 °C overnight.

2.2.2. Experiment 2 design

The initial cell densities were ~5.3 × 10^6 cells l^{-1}. Three treatments were set up in triplicate. All three N substrates (NO_3^-, NH_4^+ and urea) were added simultaneously in each treatment, and each substrate had a final concentration of 2.0 μmol N l^{-1}. In each treatment, only one type of N substrate was labeled with ^{15}N; for example, in treatment 1, only NO_3^- was labeled with ^{15}N and in treatment 2, only NH_4^+ was labeled with ^{15}N. The treatments were incubated at 21 ± 1 °C for 0.5 h with an irradiation of ~75 μmol photon m^{-2} s^{-1}. Incubations were terminated by gentle filtration with 50 ml of seawater onto pre-combusted GF/F filters (450 °C, 2 h) and rinsed twice with sterile artificial seawater. The filters were used for isotope analysis.

2.2.3. Calculations

N specific uptake rates (V, h^{-1}) were calculated according to the equation of Dugdale and Wilkerson [33]. The cellular absolute uptake rates (ρ, μmol N cell $^{-1}$ h^{-1}) were calculated by multiplying V by the PN, and then dividing by the cell densities. The parameters of N uptake kinetics were based on the Michaelis-Menten equation:

$$\rho = \frac{\rho_{\max} S}{K_s + S}$$

where ρ is the specific uptake rate in cellular units (μmol N cell $^{-1}$ h^{-1}), ρ_{max} is the maximum specific uptake rate (μmol N cell $^{-1}$ h^{-1}), S is the substrate concentration (μmol N l^{-1}) and K_s is the half-saturation constant for the N substrate (μmol N l^{-1}). The affinity coefficient α was calculated as the ratio of ρ_{max}/K_s [34].

The relative preference index (*RPI*) which assesses the degree to which a specific N substrate is selected when different N substrates coexist was calculated according to the method of McCarthy et al. [35]. For example, NO_3^- was calculated by

$$RPI_{NO_3^-} = \frac{\frac{V_{NO_3^-}}{V_{NO_3^-} + V_{NH_4^+} + V_{urea}}}{\frac{[NO_3^-]}{[NO_3^-] + [NH_4^+] + [urea]}}$$

where $V_{NO_3^-}$, $V_{NH_4^+}$ and V_{urea} are the specific uptake rates for NO_3^-, NH_4^+ and urea (h^{-1}), respectively, and [NO_3^-], [NH_4^+] and [*urea*] are the respective substrate concentrations in the media (μmol N l^{-1}). A value of unity for a specific N substrate reflects equitable utilization availability and one in excess of unity indicates preference.

2.3. Physiological growth of A. anophagefferens under different N substrates

2.3.1. Experiment 3 design

The initial cell densities were ~3.5 × 10^5 cells ml^{-1}. Four N substrates (NO_3^-, NH_4^+, urea and glutamic acid) were added separately with the final concentrations of ~30 μmol N l^{-1}. The treatments were