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Biochemical and uptake responses of the macroalga *Gracilaria lemaneiformis* under urea enrichment conditions

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ABSTRACT

Thallus growth, nutrient uptake characteristics, and biochemical composition of *Gracilaria lemaneiformis* cultured at different urea concentrations of 5, 10, 25, 50, 100, 200, and 400 μM nitrogen (N) were analyzed. Urea uptake revealed two distinct patterns: a rate-unsaturated uptake kinetic for N-limited thalli and a rate-saturating uptake kinetic (V_{max} = 27.46 ± 1.24 μmol N g^{-1} DW h^{-1} and K_s = 95.81 ± 11.65 μM N) for N-replete thalli. Urea uptake rate in both N-limited and N-replete *G. lemaneiformis* increased with elevated urea concentration, and was largely enhanced in N-limited thalli relative to N-replete thalli. The relationship between tissue total nitrogen (TN) content and growth rate was not always linear for *G. lemaneiformis*. There was a significant increase in tissue TN content from the control value of 0.93%–>2.5% dry weight at high (≥100 μM N) urea concentrations, and the highest growth rate was detected at 100 μM N. The content of photosynthetic pigments Chlorophyll *a* and Phycoerythrin increased with tissue TN up to 100 μM N but decreased at higher urea concentrations. However, only minor differences in the content of soluble protein as a percentage of tissue TN were observed. The results of the present study indicate that urea uptake by *G. lemaneiformis* in the field (where urea concentration is <25 μM N) is directed towards increased growth and N storage.

1. Introduction

In recent years, the rapid expansion of worldwide urbanization and industrialization, together with marine aquaculture development, has accounted for the increase in the release of urea into coastal waters. Urea is utilized as soil nitrogen fertilizer, and about 43% of the present available commercial fertilizers can be found in the urea fertilizer (Mohammadi-Khoo et al., 2016). The effects of urea extend beyond agricultural systems; its saturation in soil prevents further absorption by crops, resulting in its transportation to sensitive coastal waters (Glibert et al., 2006). In Chesapeake Bay, the largest and most productive estuary in the USA, the urea level of the surface water increases 50-fold after urea fertilization (Lomas et al., 2002). Another pathway is the release of urea into water in the form of feed residue, feces, and excreta during the intensive farming of aquatic animals. Although ammonia excretion is predominant by most marine fish, bivalves, and shrimp (Allen and Garrett, 1971; Koshio et al., 1993; Walsh et al., 2001), urea may also contribute a substantial portion of nitrogenous waste, with some species excreting 2%–34% of the total nitrogen (TN) (Boyce, 1999; Horn, 1986; Walsh et al., 2001), while others can excrete over 90% (Gilmour et al., 1998; Wood et al., 1998). Urban sewage systems are also a main source of urea inputs to coastal waters (Cozzi et al., 2014). Urea concentration reaches 20–25 μM N during the dry season in the sewage outlet area of Daya Bay (Northern South China Sea, Lai et al., 2011) and in the aquaculture area of Jiaozhou Bay (Western Yellow Sea, Jiang et al., 2014). All of these factors can directly or indirectly result in increased concentrations of urea. Budget estimates on the gulf-wide scale of the Gulf of Trieste, northern Adriatic Sea, indicate that urea of 177–530 t (mt) N is not negligible compared with dissolved inorganic nitrogen of 409–919 mt N (Cozzi et al., 2014).

Urea is a readily available nitrogen source for microalgae (Cochlan et al., 2008; Cochlan and Harrison, 1991; Lindehoff et al., 2011) and macrophytes (Phillips and Hurd, 2004; Walker et al., 1993). The urea molecule is small, uncharged, and can easily cross the membrane by simple diffusion. A positive linear relationship between increasing external concentration and uptake rate in microalgae and macroalgae has been established (Lindehoff et al., 2011; Phillips and Hurd, 2004). In contrast, some researchers have proposed an active transport mechanism for urea uptake, usu-

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ally described as a Michaelis-Menten hyperbola (Killberg-Thoreson et al., 2014; Phillips and Hurd, 2004; Sinclair et al., 2009).

The effect of elevated urea concentration has received an increasing amount of attention in the field of phytoplankton physiology and ecology (Fan et al., 2003; Glibert et al., 2006; Lindehoff et al., 2011; Kudo et al., 2015). With concentrations often exceeding 1 μM N, urea can be utilized as a significant nitrogen source by phytoplankton (Glibert et al., 2006). Urea has been shown to contribute more than 50% of the nitrogen utilized by planktonic communities in many coastal regions (Berman and Bronk, 2003; Glibert et al., 1991; Solomon et al., 2010). And elevated concentrations of urea precede some harmful algal bloom development (Kudela and Cochlan, 2000; Li et al., 2011; Wazniak and Glibert, 2004). The identification of solutions to control urea eutrophication in coastal waters is imperative.

Macroalgae can effectively assimilate large amounts of nutrients into their tissues during normal growth and development. Therefore, culturing macroalgae has long been recognized as the most promising approach for minimizing the negative effects of animal mariculture and anthropogenic activities on the eutrophication and harmful algal blooms. Many studies have shown the potential of various species of macroalgae to accumulate inorganic nitrogen such as *Ulva* spp. (Gao et al., 2014; Rees et al., 2007), *Gracilaria* spp. (Abreu et al., 2011; Skriptsova and Miroshnikova, 2011; Wang et al., 2014; Yang et al., 2006), and *Porphyra* spp. (Kraemer et al., 2004).

The trend towards global urea use is expected to continue, with the potential for increasing pollution of sensitive coastal waters. *Gracilaria lemaneiformis* is an economically important red seaweed that is cultivated on a large scale in China due to the quantity and quality of industrial agar and bioactive products in its tissue. There is ample evidence of the favorable effects of its biofiltration capacity for inorganic nitrogen in waste waters (Xu and Gao, 2012; Yang et al., 2006). However, studies on the physiological responses of macroalgae following urea enrichment, specifically *Gracilaria* spp. are limited. A profound knowledge of urea uptake and assimilation in *G. lemaneiformis* is important since it can help explain the adaptability of the species to elevated urea concentration, in order to determine its potential as a biofilter in urea enriched systems. In this study, available models of urea uptake kinetics of N-limited and N-replete *G. lemaneiformis* were interpreted to assess the role of algal tissue-nutrient status on affinity for urea and its membrane transport systems to urea enrichment. After a 16-d culture, the growth rate and the biochemical composition of thalli grown under various urea concentrations were compared to determine differences in nitrogen accumulation, therefore providing fundamental information on the bioremediation potential of *G. lemaneiformis*.

2. Materials and methods

2.1. Collection and pretreatment of algae

Thalli of *G. lemaneiformis* were collected from a cultivation field at Nanao Island (116.6°E, 23.3°N), Shantou, southern China, in March 2014. The healthy thalli were transported to the laboratory in temperature preservation cases with ice. The thalli were cleaned of any visible fouling organisms, disinfected with 1% sodium hypochlorite for 2 min, and rinsed with sterilized seawater (Gao et al., 2014). Low-nutrient experimental seawater was prepared through cultivation of sufficient green seaweed *Ulva pertusa* over 4 d in filtered natural seawater and then was sterilized by autoclaving. The dissolved inorganic nitrogen (DIN) and phosphorus (DIP) in the experimental seawater were markedly reduced (DIN <1 μM, DIP undetectable). During acclimation, seaweed density was approximately 10 g fresh weight (FW) per L in 60-L laboratory tanks. N-limited *G. lemaneiformis* were obtained by maintaining thalli in low-nutrient experimental seawater and replacing the culture medium once a week over 2 weeks, whereas the N-replete thalli were cultured in low-nutrient seawater to which urea was added to achieve a concentration of 50 μM N and the medium was replaced daily over a period of 2 weeks (Smit, 2002). The temperature was maintained at 20 ± 1 °C. Illumination was provided by cool-white fluorescent lamps at 100 μmol photons m^{-2} s^{-1}, sufficient to saturate growth (Engledow and Bolton, 1992), on a photoperiod of 12 h light:12 h dark. Aeration was provided by bubbling with an air pump to ensure adequate water motion.

Table 1
Expected and measured values of urea concentrations at beginning of each kinetic uptake experiment (μM N). Data for each treatment are given as the means ± standard deviation (SD) (n = 3).

Expected value	Measured value	
	N-limited	N-replete
5	6.73 ± 1.12	5.18 ± 0.52
10	11.20 ± 2.44	10.92 ± 2.89
25	28.25 ± 7.48	24.37 ± 3.32
50	47.81 ± 7.36	58.34 ± 4.45
100	97.25 ± 20.30	105.08 ± 10.55
200	204.40 ± 24.87	210.83 ± 28.79
400	394.53 ± 25.41	404.64 ± 20.15

2.2. Uptake experiment

The uptake experiments were conducted in batches using 500-mL spherical flasks with an aeration nozzle to keep thalli movement constant, and followed-up with the N-limited and N-replete thalli. Approximately 1 g FW thalli was re-suspended in 500 mL of sterilized low-nutrient seawater, with trace elements and vitamins added according to the f/2 medium (Guillard, 1975). A total of 30 μM phosphate (KH_2PO_4) was added to all treatments to avoid phosphate limitation (Wang et al., 2014). Urea was added to the cultivation seawater as N source to give initial concentrations of 5, 10, 25, 50, 100, 200, and 400 μM N. All flasks were labeled and then randomly distributed, to avoid position artifacts, under the same pre-culture conditions as described above. A recipient containing only culture medium served as the control for each corresponding treatment. Uptake experiments for the N-limited and N-replete thalli began with the light period at 10:00 h and lasted for 24 h (12 h light + 12 h dark), with water samples collected at 0, 1, 3, 6, 12, and 24 h to monitor urea concentration. A summary of the expected and observed urea concentrations at the beginning of the experiments is presented in Table 1.

Water samples (4 mL) for urea concentration measurements were immediately filtered through combusted (450 °C, 2 h) GF/F filters and frozen in polyethylene flasks for storage until analysis. Urea was analyzed using the direct method described by Revilla et al. (2005). After the uptake experiment, thalli samples were dried to a constant weight in an oven at 60 °C for determination of dry weight (DW). The uptake rate (V) of urea was determined by the disappearance of urea from culture medium over a given time interval, calculated as follows:

$$V = [(C_0 \times V_0) - (C_t \times V_t)]/(t \times DW),$$

where C_0 and C_t are urea concentrations (μM N), and V_0 and V_t are the volumes of water (l) at the start and end of time interval, respectively; DW is the thalli dry weight (g); and t is the duration of the time interval (h).