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Influence of cetyltrimethyl ammonium bromide on nutrient uptake and cell responses of *Chlorella vulgaris*☆

Zhijie Liang[a], Fei Ge[a,*], Hui Zeng[a], Yin Xu[a], Fang Peng[a], Minghung Wong[b,**]

[a] Department of Environmental Science and Engineering, Xiangtan University, Xiangtan 411105, PR China
[b] Croucher Institute for Environmental Sciences, and Department of Biology, Hong Kong Baptist University, Hong Kong, PR China

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ABSTRACT

The removal of nutrients by algae is regarded as a vital process in wastewater treatment, however algal cell activity can be inhibited by some toxic chemicals during the biological process. This study investigated the uptake of ammonia nitrogen (NH_4^+) and total phosphorus (TP) by a green alga (*Chlorella vulgaris*) and algal cell responses under the stress of cetyltrimethyl ammonium bromide (CTAB), a representative for quaternary ammonium compounds (QACs, cationic surfactants). When the concentration of CTAB increased from 0 to 0.6 mg/L, the uptake efficiencies of NH_4^+ and TP decreased from 88% to 18% and from 96% to 15%, respectively. Algal cell responses showed a decline in photosynthesis activity as indicated by the increase of chlorophyll autofluorescence from 2.9 a.u. to 25.3 a.u.; and a decrease of cell viability from 88% to 51%; and also a drop in esterase activity as indicated by the decrease in fluorescence of fluorescein diacetate stained cells from 71.5 a.u. to 4.7 a.u. Additionally, a transcription and translation response was confirmed by an enhancement of P=O peak and amide II peak in algal cellular macromolecular composition stimulated by CTAB. The results suggest that QACs in wastewater may inhibit nutrient uptake by algae significantly through declining algal cell activities.

1. Introduction

The removal of
nutrients is becoming an important priority for wastewater treatment plants due to eutrophication caused by excessive discharge into the environment (Singh and Thomas, 2012). Algae can take up nutrients for growth and assimilate them into cellular constituents, thus achieving significant reduction in a more environmentally friendly way. Therefore, the removal of nutrients by algal growth is regarded as a vital process in wastewater treatment (Dueñas et al., 2003; Wang et al., 2010).

Some species of non-toxic algae have been extensively studied due to their high efficiencies in the removal of nutrients, e.g., a green alga (*Neochloris oleoabundans*) was reported to be able to achieve a complete removal of nitrogen and phosphorus from cultures containing up to 218 mg/L and 47 mg/L, with rates of 43.7 mg/L/day and 9.4 mg/L/day, respectively (Wang and Lan, 2011). Moreover, with nitrate or urea servicing as nitrogen source, another green alga, *Scenedesmus* sp. LX1, was able to grow well and remove 90% of nitrogen and 100% of phosphorus (Li et al., 2010).

However, algal nutrient uptake may be inhibited during wastewater treatment by potentially toxic chemicals, such as surfactants, heavy metals, and aromatic hydrocarbons. Quaternary ammonium compounds (QACs) are typical cationic surfactants with at least one hydrophobic hydrocarbon chain and the other alkyl or aryl groups (mostly short-chain substituents such as methyl or benzyl groups) linked to a positively charged nitrogen atom. QACs are extensively used as disinfectants, pesticides, personal care products, fabric softeners, and corrosion inhibitors (Garcia et al., 2001). The world annual consumption of QACs was reported as 5×10^5 tons in 2004 (CESIO, 2004) with an increasing trend. Residual QACs that are discharged into sewage treatment plants are of great concern due to their biocidal activity when dispersed into different environmental compartments (Ying, 2006).

It is known that algal growth is very sensitive to the presence of QACs. Jing et al. (2011) reported that the 96-h EC_{50} of QACs (alkyl carbon number: 12–18) on the growth of *Scenedesmus quadricauda* ranged from 0.14 mg/L to 0.58 mg/L. In addition, our previous study showed that the 96-h EC_{50} of QACs (alkyl carbon number: 8–16) on the growth of *C. vulgaris* ranged from 0.108 mg/L to 0.197 mg/L, which according to the increase of the alkyl chain

* Corresponding author. Tel.: +86 731 58298290; fax: +86 731 58298172.
** Corresponding author. Tel.: +86 852 3411 7746; fax: +86 852 3411 7743.
E-mail addresses: liangzhijie1987@126.com (Z. Liang), gefei@xtu.edu.cn, gefeixtu@sina.com.cn (F. Ge), zenghuioo@163.com (H. Zeng), xuyin123@hotmail.com (Y. Xu), pengfliping0612@126.com (F. Peng), mhwong@hkbu.ed.hk (M. Wong).

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length (Zhu et al., 2010). Xu et al. (2011) reported a concentration of 0.3 mg/L quarternary alkylammonium chloride (alkyl carbon number: 16) induced a 71% inhibition on the yield of *C. vulgaris*, and the ultra-structure of algal cells was destroyed in extreme cases. Furthermore, once entering the cell, surfactants may affect thylakoid organization and chlorophyll synthesis (with subsequent impairment of photosynthetic capacity), and may interact with bioactive macromolecules (Vonlanthen et al., 2011). It was reported that superoxide dismutase and catalase activities of *Dunaliella bardawil* were significantly promoted when exposed to 0.70–1.30 mg/L of cetyl trimethyl ammonium chloride (Qv and Jiang, 2013). However, there is currently a lack of information on the extent of QACs inhibition on the uptake of nutrients, in particular algal cellular responses under the stress of QACs, which would pose limitations to the actual application of algae in biological wastewater treatment.

With the above background information, the objectives of this study were to investigate: (1) nitrogen and phosphorus uptake; and (2) cell response (variance of chlorophyll fluorescence, cell viability, esterase activity, and cellular macromolecular composition) of *C. vulgaris* under the stress of cetyltrimethyl ammonium bromide (CTAB), as a representative of QACs.

2. Materials and methods

2.1. Algal culture and chemicals

C. vulgaris was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. The culture medium method was formed based on the Organization for Economic Co-operation and Development (OECD, 2006). The pH of the medium was adjusted to 8.0 with NaOH or HCl. Erlenmeyer flasks (250 mL) containing the culture medium (100 mL) were incubated in the shaking incubator (HZ-200LG, Wuhan Ruihua Instrument and Experiment Co. Ltd., Wuhan, China) with a rate of 120 rpm at 25 ± 0.5 °C under a light intensity of 2500 lx with a daily light:dark cycle of 12 h:12 h by fluorescent lamp.

CTAB (analytical grade) was purchased from Robiot Co., Ltd. (Nanjing, China) and the stock solution was prepared with deionized water to 40 mg/L and stored at 4 °C. Fluorescein diacetate (FDA) and propidium iodide (PI) were purchased from Sigma–Aldrich (Milan, Italy). Stock solutions of FDA and PI were prepared with acetone and deionized water to 60 mg/L and 30 mg/L and stored at −20 °C and 4 °C, respectively.

2.2. Experimental setup

For the uptake of NH_4^+ and TP by *C. vulgaris*, algal cells in the late log growth phase (96 h, Fig. S1 in Supplementary Material) were collected by centrifugation (4000 rpm, 10 min). After being washed with deionized water twice, algal cells were incubated into 100 mL of modified OECD medium (without NH_4^+ and TP) in Erlenmeyer flasks (250 mL) with an initial cell density of 1×10^6 cells/mL. Initial concentrations of NH_4^+ and TP were settled at 10 mg/L and 2 mg/L according to our previous study (Fig. S2 in Supplementary Material), respectively. Appropriate volumes of CTAB stock solution were added into the tested culture to give serial concentrations of 0.05, 0.1, 0.2, 0.4, 0.6 mg/L for six days, which were enough to achieve stable removal efficiencies (Fig. S3 in Supplementary Material). The tested cultures were incubated in the shaking incubator under the same culture condition. Cell density was measured daily with a spectrophotometer (722S, Shanghai Precision and Scientific Instrument Co. Ltd., Shanghai, China) at 680 nm and calculated as the method described by Xu et al. (2011). 10 mL of algae suspension withdrawn from the flasks was centrifuged at 4000 rpm for 10 min at the end of the six-day test. The supernatant was used for NH_4^+ and TP measurement with standard methods established by the National Environment Protection Agency of China (2002).

2.3. Chlorophyll autofluorescence

For groups treated and untreated with CTAB, the algal biomass was harvested by centrifugation (4000 rpm, 15 min) at the end of the six-day test, and washed with PBS (pH 7.2) twice. The cells were re-suspended in PBS (pH 7.2) with an initial cell density of 1×10^6 cells/mL, and 3 mL of the algal re-suspension was taken for Chlorophyll autofluorescence measurement. Fluorescence emission spectrum of the algae was recorded with a spectrofluorimeter (LS-50B, Perkin Elmer, USA) excited at 480 nm. Suspension of algae cultured in OECD medium was also measured.

2.4. Cell viability

Since fluorescence of FDA stained algal cells requires esterase activity and an intact membrane and PI can get into cells only through the membrane of dead or dying cells, FDA/PI double staining method can provide information about cell viability (Hurst et al., 2007). 1 mL of the algal re-suspension (mentioned in Section 2.4) was taken for staining with FDA and PI solutions at the concentrations of 20 mg/L and 10 mg/L in the final volume of 3 mL, respectively. After stained with FDA and PI, the algal cells were analyzed under a confocal laser scanner microscope (CLSM) (Fluoview FV1000, Olympus, Tokyo, Japan). Argon (488 nm) and PI (536 nm) lasers were used for excitation and images were acquired for calculation of cell viability by setting the detection bandwidth between 500 and 530 nm for FDA fluorescence, 610–630 nm for PI. Digital image analysis was performed using the freeware Image J 1.29× downloadable from the website http://rsb.info.nih.gov/ij (Nancharaiah et al., 2007).

2.5. Esterase activity

FDA can enter the cells easily and intracellular cleavage of FDA by esterase results in free fluorescence, hence quantification of the fluorescence could reflect metabolic activity of cells (Regel et al., 2002). 1 mL of the algal re-suspension (mentioned in Section 2.4) was withdrawn for staining with FDA at the concentration of 20 mg/L for 15 min in the final volume of 3 mL. The stained algal re-suspension was excited at 513 nm and the emission fluorescence was recorded with a spectrofluorimeter (LS-50B, Perkin Elmer, USA) at 615 nm. Suspension of unstained algae was used as control for each sample, including the untreated group.

2.6. Macromolecular composition analyses

After dehydration by freeze-drying, algal cell powder was mixed with KBr and molded with 769YP-24B hot-press (Keqi High Technology Co., Tianjin, China). The infrared measurements were performed using a Nicolet 380 Fourier transform infrared spectrophotometer (Thermo Scientific Brand, America) in 4000–400 cm^{-1} range of wave numbers.

2.7. Data analysis

The removal efficiencies of NH_4^+ and TP were calculated based on mass balance (Maltais-Landrya et al., 2009). The average growth rate of algae was calculated as the daily increase of cell density during the six-day test period. Cell viability was defined as the percentage of viable cells within the whole population, in which non-viable cells are the supplementary percentage (Argüello-García et al., 2004).