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The effect of naphthenic acids on physiological characteristics of the microalgae *Phaeodactylum tricornutum* and *Platymonas helgolandica* var. *tsingtaoensis*☆

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ABSTRACT

Naphthenic acids (NAs) account for 1–2% of crude oil and represent its main acidic component. However, the aquatoxic effects of NAs on marine phytoplankton and their ecological risks have remained largely unknown. Using the marine microalgae *Phaeodactylum tricornutum* and *Platymonas helgolandica* var. *tsingtaoensis* as the target, we studied the effects of NAs on their growth, cell morphology and physiological characteristics. The cell density decreased as the concentrations of NAs increased, indicating that they had an adverse effect on growth of the investigated algae in a concentration-dependent manner. Moreover, scanning electron microscopy revealed NAs exposure caused damage such as deformed cells, shrunken surface and ruptured cell structures. Exposure to NAs at higher concentrations for 48 h significantly increased the content of chlorophyll (Chl) a and b in *P. tricornutum*, but decreased their levels in *P. helgolandica* var. *tsingtaoensis*. NAs with concentrations no higher than 4 mg/L gradually enhanced the Chl fluorescence (ChlF) parameters and decreased the ChlF parameters at higher concentrations for the two marine microalgae. Additionally, NAs induced hormesis on photosynthetic efficiency of the two microalgae and also have the species difference in their aquatic toxicity. Overall, the results of this study provide a better understanding of the physiological responses of phytoplankton and will enable better risk assessments of NAs.

1. Introduction

Naphthenic acids (NAs) are mixtures of naturally-occurring unsaturated monocarboxylic acids that occur in cyclic or non-cyclic forms with the molecular formula $C_nH_{2n+z}O_x$, where 'n' stands for the number of carbon atoms and 'z' is related to hydrogen deficiency and generally zero or negative. Some NAs also contain aromatic acids, N, S and other heteroatoms (Swigert et al., 2015). NAs are by-products produced during petroleum refining that can also be released from bitumen during oil sands extraction. NAs account for 1–2% of crude oil and represent its main acidic component (Headley and Mcmartin, 2004a, 2004b; Yen et al., 2004). Currently, high-acid crude oil production accounts for about 5% of global annual crude oil production, and this value is growing at an average rate of 0.3%, indicating the increasing production and usage of NAs (Speight, 2015). Residues of NAs are frequently detected in petroleum refinery waste waters and oil sands process-affected water (OSPW). NAs have also been detected in coastal sediments, oil polluted seawater, ground water and aquatic organisms because of marine crude oil pollution (Ahad et al., 2018; Headley et al., 2009; Sun et al., 2017; Wan et al., 2014). The frequent detection of NAs in various environmental media has caused increasing public concern; however, current investigations of the toxicity effects of NAs and their ecological risks are based on the production and usages of NAs already in place.

NAs have been reported to have acute and subacute toxic effects on aquatic organisms such as freshwater fish, amphibians and luminescent bacteria, and petrogenic NAs were revealed as weak estrogen receptor agonists (Thomas et al., 2009). Additionally,

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OSPW containing NAs with concentrations of 12–18 mg/L can significantly inhibit cell proliferation of the RAW 264.7 mouse macrophage cell line, and exposure to whole OSPW (at NAs doses of 10–20 mg/L) induced cytotoxic and immunomodulatory changes in mouse macrophages (Fu et al., 2017). Long-term exposure of low dose NAs has also been reported to induce reproductive toxicity, endocrine disruption of zebrafish, and earthworms, as well as to block the immune response (Garcia-Garcia et al., 2011; Knag et al., 2013; Rowland et al., 2011; Wang et al., 2015).

However, the aquatic toxic effects of NAs on marine phytoplankton and their ecological risks have remained largely unknown. Phytoplankton blooms are important biological processes in coastal waters and one of the key components of marine food webs. Changes in the distribution of phytoplankton population can affect nutrient transmission, productivity levels and the action of the bio-pump in the ocean carbon cycle. The density of marine phytoplankton has been reported to increase after long periods of oil spills or oil sands tailings leaching (Abbriano et al., 2011; Leung et al., 2001, 2003; Woodworth et al., 2012). Investigations of how NAs affect growth and algal morphology and physiological characteristics of different marine phytoplankton are essential to understanding of marine eco-toxicities of NAs.

In the present study, the effects of NAs on the growth, cell morphology, chlorophyll (Chl) a and b content and chlorophyll (Chl) fluorescence (ChlF) of two marine microalgae, *Phaeodactylum tricornutum* and *Platymonas helgolandica* var. *tsingtaoensis*, were investigated. The hormesis on photosynthetic efficiency induced by NAs and the differences in the aquatic toxicity of NAs between species were also comparatively studied. To the best of our knowledge, this is the first investigation of NAs-induced aquatic toxicity toward *P. tricornutum* and *P. helgolandica* var. *tsingtaoensis*. Our results will facilitate further risk assessment of NAs and related contaminants in marine ecosystems.

2. Materials and methods

2.1. Chemical reagents and materials

NAs (purity > 97%, CAS NO. 1338-24-5, Sigma-Aldrich Catalogue: 70340) were purchased from Sigma-Aldrich. Algal chl a and b levels were determined using 80% acetone (CAS NO. 67-64-1). Sodium dihydrogen phosphate (CAS NO. 13472-35-0), disodium hydrogen phosphate, glutaraldehyde (CAS NO. 10039-32-4), ethanol (CAS NO. 64-17-5) and T-butanol (CAS NO. 75-65-0) were used to prepare algae electron microscopy samples. All chemicals were of analytical grade. *P. tricornutum* and *P. helgolandica* var. *tsingtaoensis* were obtained from the Algal Center of the Institute of Oceanography, Chinese Academy of Sciences, Qingdao, China.

2.2. Observation of algal growth

NAs and filtered seawater were mixed at a volume ratio of 1:9 and then stirred at room temperature for 24 h. The mixture was subsequently transferred to a separatory funnel to separate the bottom aqueous phase, after which the separated stock solutions were kept at 4 °C for 4 h without light.

P. tricornutum and *P. helgolandica* var. *tsingtaoensis* were pre-cultured in a 3000 mL conical flask using f/2 seawater nutrients (Guillard, 1975).

The surface salinity of the f/2 seawater nutrients was 30 and the pH was 8.2. During pre-culture, the light intensity was set at 70 μmol photons m^{-2}. s^{-1}. Both algae in the logarithmic growth phase were transferred to 250 mL Erlenmeyer flasks. The starting densities of *P. tricornutum* and *P. helgolandica* var. *tsingtaoensis* were 199.133×10^6 ml^{-1} and 15.303×10^6 ml^{-1}, respectively. Stock solutions containing NAs at 0, 0.5, 2, 4, 8 and 16 mg/L were added to the Erlenmeyer flasks, after which the algae were cultured for an additional 24, 48, 72 and 96 h. Next, 0.5 mL aliquots of the cultures were collected and preserved in Lugol's solution, then enumerated using a hemocytometer under an optical microscope (MoticSFC-18, Motic China, Xiamen, China).

2.3. Electron microscopy analysis

The effects of the exposure to NAs at 16 mg/L on cell morphology of the two algae were investigated by scanning electron microscopy (SEM). Cells from the control and treatment groups were collected on day 4 of the culture and then incubated in the presence of 2.5% glutaraldehyde at 4 °C overnight. Cells were subsequently washed with PBS (PH = 7.2), dehydrated with ethanol and finally dried in a critical point dryer. For cell morphology analysis, cells of two algae were first mounted on copper stubs, then sputter-coated using gold palladium and finally scanned by field emission scanning electron microscopy at 10 kV using a Hitachi S4800 FE-SEM (Hitachi, Japan).

2.4. Determination of chlorophyll a and b

Algal cultures (5 mL) were centrifuged at 4000 rcf for 15 min, after which the supernatant was collected and 5 mL of 80% acetone were added. Following incubation in the dark for 24 h, the mixture was centrifuged at 4000 rcf for 15 min, after which the supernatant was collected to determine the chlorophyll contents using a TU-1800 (PERSEE, China) ultraviolet visible spectrophotometer. The optical density (OD) of the supernatants at 663 nm and 645 nm was then measured and the content of Chl a and b was calculated using the following equations (Sestak, 1971).

$$\text{Chl a} = 12.7OD_{663} - 2.69OD_{645}$$

$$\text{Chl b} = 20.13OD_{645} - 5.03OD_{663}$$

2.5. Determination of chlorophyll fluorescence parameters

The ChlF parameters for Photosystem (PS), Fv/Fm (maximal photochemical efficiency of PSII), energy electrons transfer rate of PS II (ETR), Yield (the potential activity of PSII) and qP (the PSII antenna pigment captured by the light energy used for photochemical electron transfer) were measured using a Water-PAM Water chlorophyll fluorescence spectrometer (Walz, Effeltrich, Germany). Aliquots of cultured algae (5 mL) were incubated at dark for 15 min under different light intensities. The initial fluorescence Fo at open PS II centers was measured at 0.12 μmol m^{-2} s^{-1} and the maximum fluorescence Fm at closed PSII centers was determined at 4000 μmol $m^{-2} \cdot S^{-1}$ with a pulse time of 0.8 s. The Fv/Fm, Yield, ETR and qP were calculated as previously described (Lichtenthaler et al., 1992).

2.6. Statistical analysis

All experiments were performed in triplicate and the data were presented as the means ± standard deviation (SD). Multiple comparisons of data were performed using the LSD method and treatments were analyzed by one-way analysis of variance (ANOVA) using the IBM SPSS 22.0 statistical software. A $p < 0.05$ was considered to indicate significance.