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Frontier Article

Cellular responses and bioremoval of nonylphenol and octylphenol in the freshwater green microalga *Scenedesmus obliquus*

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ABSTRACT

The removal of nonylphenol (NP) and octylphenol (OP) by the freshwater green microalga *Scenedesmus obliquus* was studied in cultures exposed to different concentrations of NP and OP for 5 days. In most cases, low NP and OP concentrations (<1 mg/L) did not affect the growth, fluorescence transient (F_v/F_m), photosynthetic pigments and cell ultrastructure of *S. obliquus*, whereas high NP and OP concentrations (>1 mg/L) suppressed algal growth, decreased F_v/F_m and photosynthetic pigments, and destroyed algal ultrastructure. *S. obliquus* had a rapid and high ability to remove NP and OP. After 5 days of culturing, >89 percent NP and >58 percent OP were removed by this alga, with the highest removal efficiency being near 100 percent. The removal of NP and OP was mainly caused by algal degradation. Extracellular NP contents of *S. obliquus* were lower than intracellular NP contents, with the ratios changing from 0 to 0.74. However, most of extracellular OP contents of *S. obliquus* were higher than intracellular OP contents, with the ratios changing from 0.74 to 2.15. The two alkylphenols exhibited a high bioconcentration potential, with one-day bioconcentration factors (BCF) of NP and OP varying between 3393 to 13262 and 949 to 3227, respectively. After 5 days of culturing, high BCF values were still recorded when NP and OP initial concentrations were higher than 0.5 mg/L. These results demonstrated potential application of this algal species in the removal of organic contaminants including alkylphenols in addition to nutrients and metals.

1. Introduction

Nonionic surfactants, alkylphenol ethoxylates (APEs), are widely used in various domestic and industrial detergents, and pesticide formulations (Ying et al., 2002). APEs could be converted into alkylphenols such as nonylphenol (NP) and octylphenol (OP) in wastewater treatment plants and finally reach the environment via discharge of effluents and disposal of sludge. In the last decade, NP and OP were frequently detected in various environmental compartments such as rivers, lakes, oceans, groundwater, sediments and soils (Soares et al., 2008; Ying et al., 2002). For example, NP and OP have been found in US sewage effluents at concentrations up to 37 and 0.673 μg/L, respectively (Snyder et al., 1999). NP and OP were also detected in five German rivers at the concentrations up to 458 and 189 ng/L, respectively (Bolz et al., 2001). Although the parent surfactants APEs are less toxic, NP and OP have been found to be more persistent and toxic (Vazquez-Duhalt et al., 2005; Ying et al., 2002).

Alkylphenols NP and OP are known as endocrine disruptors, which possess the ability to mimic natural estrogens and disrupt the endocrine systems of higher organisms by interacting with the estrogen receptor (Soares et al., 2008; Vazquez-Duhalt et al., 2005), thereby potentially producing adverse effects on the reproductive system. For example, when exposed to NP or OP, male rainbow trout developed synthesis of vitellogenin and concomitant inhibition of testicular growth (Jobling et al., 1996). In addition, they can affect plankton community structure when released into aquatic environments. For example, in response to exposure of NP, the abundances of cladocera and copepoda were reduced, and rotifer abundance was increased, thereby further indirectly influencing phytoplankton populations though the changes of zooplankton grazing pressure (Hense et al., 2005). Considering the negative effects of NP and OP to aquatic organisms, it is necessary to understand their environmental fate in aquatic ecosystems.

Due to their substantial biomass and extensive range of habitat and diversity, microalgae constitute the largest and most widely distributed group of photosynthetic organisms in aquatic ecosystems. As primary producer in aquatic ecosystems, microalgae play an important role in generating primary organic substances and it is the base of aquatic food-chain. Algae can interact with aquatic contaminants, which include negative effects on algal growth and

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function and algal degradation of the contaminants (Semple et al., 1999; Wang et al., 2007; Yang et al., 2002). Algae may also take up aquatic contaminants, thereby acting as a medium for bioconcentration and subsequent biomagnification in higher trophic levels (Sijm et al., 1998; Soares et al., 2008; Vazquez-Duhalt et al., 2005). Algae have also been suggested to be used in wastewater treatment processes (Park et al., 2011). Thus it is important to understand the mechanism involved with the algal degradation process of contaminants in wastewater. So far, little information is available about accumulation and biodegradation processes of emerging organic contaminants such as alkylphenols by algae, and physiological and internal ultrastructure changes of the algal cells during the processes.

The green microalga *S. obliquus* is commonly found in most types of freshwater, and plays an important part in the function of aquatic ecosystem. The objective of this study was to investigate the removal mechanisms of *S. obliquus* for alkylphenols NP and OP in aqueous systems. The algal responses to NP and OP were also evaluated by measuring algal growth, algal ultrastructure and photosynthetic parameters such as photosynthetic pigment and chlorophyll *a* fluorescence.

2. Materials and methods

2.1. Algal strain and culture medium

S. obliquus was isolated from a hypereutrophic environment (the East Lake, Wuhan, China). BG11 medium was used as the growth medium for the algal species. The growth media contained the following chemicals: $NaNO_3$, 1.5 g/L; $K_2HPO_4 \cdot 3H_2O$, 0.04 g/L; $MgSO_4 \cdot 7H_2O$, 0.075 g/L; $CaCl_2 \cdot 2H_2O$, 0.036 g/L; Na_2CO_3, 0.02 g/L; citric acid, 0.006 g/L; ferric ammonium citrate, 0.006 g/L; EDTA, 0.001 g/L; and A_5+Co solution (1 mL/L) that consists of H_3BO_3, 2.86 g/L, $MnCl_2 \cdot 4H_2O$, 1.81 g/L; $ZnSO_4 \cdot 7H_2O$, 0.222 g/L; $CuSO_4 \cdot 5H_2O$, 0.079 g/L; $Na_2MoO_4 \cdot 2H_2O$, 0.390 g/L; and $Co(NO_3)_2 \cdot 6H_2O$, 0.0494 g/L (Rippka et al., 1979).

2.2. Experimental set up

Nonylphenol (NP) and 4-*t*-octylphenol (OP) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, German). The stock solutions of the two compounds were prepared in methanol at a concentration of 2000 mg/L. The stock solution of each compound was spiked into the growth medium to obtain different working concentrations of 0, 0.25, 0.5, 1.0, 2.0 and 4.0 mg/L. Relatively high concentrations for NP and OP were selected in order to study the interaction between algae and target contaminant. The toxicity of methanol to algal cells was checked and no observed effect concentration of methanol was determined to be 0.50 percent (v/v) from the preliminary experiment. The final concentration of methanol in the experiment was controlled at 0.40 percent (v/v) for all experimental media.

Algal cells at the exponential growth phase were inoculated into the medium spiked with different concentrations of NP or OP, with the initial algal densities of *S. obliquus* being 3.2×10^5 cells/mL. Culturing was performed in 250-mL Erlenmeyer flasks containing 150 mL culture volume at 150 rpm and 25 ± 1 °C. Light was provided by continuous cool white fluorescent lamps at 3000 lx with a dark/light cycle of 12 h:12 h. The experiments were conducted for 5 days, and all tests were carried out in triplicate. All treatments had corresponding controls without algae, which were used for measuring the abiotic loss of target chemicals from the culture medium.

2.3. Analysis

2.3.1. Determination of cell density and dry weight of S. obliquus

Optical density (OD) of the algae cultures was measured daily at 650 nm as the cell density and dry weight indicator using a BMG microplate reader (BMG Lab technologies, Offenburg, Germany). The cell density was determined using a haemocytometer under a light microscope. For cell dry weight measurement, a 20 mL aliquot of culture was filtered through pre-weighed 0.45 μm pore-size Whatman GF/F glass-fiber filter paper. The filter paper with algal cells was dried overnight in an oven at 60 °C till a constant weight was reached. The difference between the final weight and the weight before filtration was the dry weight of algal cells. The linear relationship between algal density (N, cells/mL), dry weight and OD_{650} was shown in Eqs. (1),(2).

$$\text{Cell density}\left(10^5 \text{cells/mL}\right) = 78.953 \times OD_{650} - 1.9331R = 0.995 \quad (1)$$

$$\text{Dry weight}(\text{mg/mL}) = 0.7244 \times OD_{650} - 0.0176R = 0.9998 \quad (2)$$

Growth rate (μ,/*d*) of *S. obliquus* was calculated using the following Eq. (3):

$$\mu = (\ln N_t - \ln N_0)/(t_t - t_0) \quad (3)$$

where N_t is the cell number at time t_t, N_0 is the initial cell number at the beginning of the test.

2.3.2. Measurement of fluorescence transient

Due to its very sensitive and rapid response to xenobiotics, the ratio of variable and maximal fluorescence, F_v/F_m, is often used to evaluate the toxic effect of chemicals on microalgae in recent years (Gao and Tam, 2011; Perron and Juneau, 2011; Gattullo et al., 2012). Chlorophyll fluorescence transient was measured using a plant efficiency analyzer (PEA, Hansatech Instruments Ltd., UK) at time intervals of 0.5, 2, 5 and 10 h (Perron and Juneau, 2011). Algal samples (2 mL each) were put into 1-cm diameter vials (2.5 mL each) and placed in darkness to adapt for 15 min at the room temperature. Then measurements were performed using the aqueous-phase attachment of the PEA. The transients were induced by a red light (peak at 650 nm) of 3500 μmol photons $m^{-2}\,s^{-1}$ provided by an array of six high-intensity light-emitting diodes, and measured on a time scale from 10 μs to 1 s. The fluorescence intensity at 50 μs was designated as the initial fluorescence (F_0) and the maximum level of fluorescence as F_m.

The parameter F_v/F_m was calculated using the following Eq. (4):

$$F_v/F_m = (F_m - F_0)/F_m \quad (4)$$

where F_0, F_m and F_v are the initial fluorescence, maximal fluorescence and variable fluorescence, respectively.

2.3.3. Determination of photosynthetic pigment content

Photosynthetic pigment measurement was carried out on the last day of each test according to the procedure described by Zhou et al. (2012). Photosynthetic pigment contents were calculated from the absorbances at 665, 649 and 470 nm according to the following Eqs. (5)–(8):

$$C_A = 13.95A_{665} - 6.88A_{649} \quad (5)$$

$$C_B = 24.96A_{649} - 7.32A_{665} \quad (6)$$

$$C_T = C_A + C_B = 6.63A_{665} + 18.08A_{64} \quad (7)$$

$$C_K = (1000A_{470} - 2.05C_A - 114.8C_B)/245 \quad (8)$$

where C_A (mg/L) is the content of chlorophyll *a*, C_B (mg/L) is the content of chlorophyll *b*, C_T (mg/L) is the content of total chlorophyll, and C_K (mg/L) is the content of carotenoids.

2.3.4. Electron microscopy (TEM)

On the last day of experiment, algal cells were harvested by centrifugation (9168 g, 10 min), and then fixed by 2.5 percent glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.2) for 24 h at the room temperature. After centrifugation, the centrifuged algae were subsequently post-fixed overnight in the same buffer containing 1 percent OsO_4. The centrifuged material was dehydrated in a graded series of acetone solution, and embedded in Spurr resin. They were cut with a glass knife on an ultramicrotome, and then ultrathin sections were stained with uranyl acetate and lead citrate. Observations were carried out with a transmission electron microscope (HITACHI H-7000FA) (Zhou et al., 2012).

2.3.5. Determination of NP and OP concentrations

The concentrations of NP and OP in the medium samples were measured at time intervals of 0.5, 2, 5, 10, 24, 72 and 120 h. Culture medium samples (1.5 mL) were centrifuged at 9168 *g* for 5 min to remove the algae cells, and then NP or OP was extracted three times from 1 mL of supernatant solutions with dichloromethane. The extracts were pooled together for the determination of NP and OP concentrations in the water. The recovery rates of NP and OP from the water were 99.5 and 97.6 percent, respectively.

The NP and OP adsorbed/absorbed in algal cells were determined at time intervals of 24, 72 and 120 h. The algal culture fluid (1.5 mL) was centrifuged at 9168 *g* for 5 min. After the supernatant was discarded, the concentrated algal cells were re-suspended in 1.5 mL of 10 percent methanol and shaken for approximately 30 s to wash the NP and OP adsorbed on the cell walls. The sample solution was then centrifuged for a further 5 min at 9168 *g*, and the NP and OP contained in supernatant solutions was used for the determination of NP and OP contents adsorbed on the algal cells (Correa-Reyes et al., 2007). The algal cells after 10 percent methanol wash were frozen (−20 °C) overnight until the cell walls were broken. The processed algae were then extracted three times with dichloromethane-methanol (1:2 v/v) to obtain the NP and OP absorbed in algal cells (Correa-Reyes et al., 2007).

The target compounds (NP and OP) were analyzed by using an Agilent 1200 series high performance liquid chromatograph (HPLC) coupled to a fluorescence detector. A XDB-C18 RS column (4.6 × 150 mm, 5 μm) was used for the separation of NP or OP. The elution was carried out under isocratic conditions with