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Biodegradation of phthalate acid esters by different marine microalgal species

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ABSTRACT

Biodegradation of diethyl phthalate (DEP) and di-n-butyl phthalate (DBP) by three marine algae was investigated. When they were coexistent, DBP was degraded more quickly than DEP. The first-order biodegradation rate constants of DBP in the algal solutions were in the order of *Cylindrotheca closterium* ($0.0169\ h^{-1}$) > *Dunaliella salina* ($0.0035\ h^{-1}$) and *Chaetoceros muelleri* ($0.0034\ h^{-1}$). When singly existed, DEP was degraded more quickly than in a mixture with DBP, indicating that DBP had inhibitory effect on the biodegradation of DEP. Moreover, the degradation trends of DEP and DBP in both extra- and intracellular crude extracts were similar to those in algal solutions. At the end, DEP was largely in water phase, whereas DBP remained in both water phase and algal phase. It can be concluded that biodegradation of DEP was mainly by algal extracellular enzymes, and both extra- and intracellular enzymes played key roles in the degradation of DBP.

1. Introduction

Phthalate acid esters (PAEs) are mainly used as additives and plasticizers to enhance the flexibility and workability of polymeric materials. They have been detected in various environment media, such as water, sediments, soils, and even in the atmosphere (Wang et al., 2008; Mahmoud et al., 2012). Most of PAEs have been classified as priority pollutants by United States Environment Protection Agency (USEPA) and its counterparts in several other countries. In addition, because of their endocrine disruption, respiratory effects, reproductive and developmental toxicity, PAEs have caused serious concerns (Matsumoto et al., 2008). The coastal ocean is among the most heavily impacted environments by human activities because it receives wastewater and runoff from inland and coastal human populations. Different levels of PAEs have been found in coastal waters (Turner and Rawling, 2000; Brossa et al., 2009).

As the primary producers of the aquatic food web, microalgae support more than half of the global primary production. The pollutants can enter aquatic food chains through microalgal bioaccumulation, so the response of algae will undoubtedly affect upper tropic levels. Moreover, it has been known that microorganism degradation is a critical process affecting the environmental fate of PAEs (Staples et al., 1997; Chang et al., 2004). Microalgae are a class of autotrophic microorganisms capable of photosynthesis. In addition to producing oxygen to meet the needs of heterotrophic bacteria, and consequently stimulate activities of the bacteria degrading organic pollutants, microalgae also have the ability to degrade organic pollutants directly, such as phenolics, polycyclic aromatic hydrocarbons, pesticides, petroleum, and PAEs (Yan et al., 1995; Subashchandrabose et al., 2013). However, to date, most studies on the removal of PAEs by microalgae via bioaccumulation and degradation have been limited to freshwater microalgae (Staples et al., 1997; Yan et al., 2002; Yan and Pan, 2004). Therefore, the investigation on the removal of PAEs by marine microalgae can facilitate the assessment of environmental fate and risk of PAEs in marine ecosystems and provide a valuable evidence for coast ecological remediation.

In this study, three marine microalgal species, including two planktonic microalgae *Dunaliella salina* Teod (Chlorophyta) and *Chaetoceros muelleri* Lemm (Bacillariophyta) and one benthic microalga *Cylindrotheca closterium* (Ehrenberg) Reimann et Lewin (Bacillariophyta), were selected. Benthic microalgae are an important component of productive shallow systems and intertidal areas. In many shallow ecosystems, the biomass of benthic microalgae often exceeds that of the planktonic microalgae in the overlying waters (Brito et al., 2009). Due to the stimulus of light, tide and wind, cells of benthic microalgae tend to vertically migrate, and resuspend into overlaying water, resulting in the mixture with phytoplankton. The three species are widely distributed

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groups and present fast growth in laboratory condition. The objective of this study is to investigate the biodegradation of di-ethyl phthalate (DEP) and di-n-butyl phthalate (DBP) by the three marine microalgae. Furthermore, the mechanism was also discussed.

2. Material and methods

2.1. Materials and chemicals

DEP and DBP were purchased from Sigma (purity 99%). Analytical grade dichloromethane (purity 99.5%) was obtained from Tianjin Chemical Reagent Factory and rectified before use.

Marine microalgae *D. salina*, *C. muelleri* and *C. closterium* were provided by Institute of Oceanology, Chinese Academy of Sciences, Qingdao City, China. The microalgae were cultivated in sterile f/2 medium (Guillard, 1973) made by artificial seawater (Araujo et al., 2010) at 25 ± 1 °C, under a 16:8 light:dark cycle provided by cool white fluorescent tubes at a light intensity of 3000 Lux. The algal cultures were maintained in mid-log exponential growth by serial transfers of subcultures to fresh medium every 7 days. Characteristics of the three marine microalgae are shown in Table S1.

Surface sediment samples were collected from Bohai Bay (38° 53′4″N, 117°43′24″E) which is a large, semi-enclosed shallow water basin located along the western region of Bohai Sea in the northeastern part of China. After air-dried, the samples were ground to pass through a 2 mm sieve. Aqueous extract of the sediment samples was prepared at a sediment/artificial seawater ratio of 1:5 (w/v) and was used in the degradation experiment. The extraction treatment included stirring with a glass rod for 30 min, settling for 30 min, and then filtrating through a 0.45 μm pore-size filter. The physicochemical characteristics of the sediment extract are listed in Table S2.

2.2. Experiment design

2.2.1. Biodegradation experiment of PAEs in algal solutions

After pre-cultivation, algal cells in the exponential phase of growth were collected by centrifugation (4000 rpm, 5 min). The harvested cells were washed twice with sterile artificial seawater, and were used as inoculums for experimental studies. The mixture stock solution of DEP and DBP (each of 2000 mg L^{-1}) were prepared in N,N-dimethyl formamide (DMF). The stock solution was added into sterilized sediment extract to achieve a mixture of DEP and DBP of about (0.4 + 0.4) mg L^{-1}. The final concentration of DMF was controlled below 0.4% (v/v), which was found to have no significant effects on the growth of the three marine microalgae. Each algal species was inoculated into the sediment extract at an initial cell density of 2.5×10^5 cells mL^{-1} and cultured under the same conditions as the pre-cultivation. The flasks were shaken every 8 h or before sampling. At time intervals of 0, 24, 48, 72, 120 and 168 h, triplicate flasks of each algal culture were removed for the measurement of residual PAEs in the algal solutions. The concentrations of PAEs in algae were measured at 168 h for *D. salina* and *C. muelleri* and at 120 h for *C. closterium*. Esterase activity of each algal species was measured at 0, 48, 120 and 168 h. At the same time, the flasks without microalgae were used as the controls to evaluate the abiotic effect on PAEs removal. The control experiment was performed under the same conditions as the experiments with algae.

In order to clarify the effect of DBP on the biodegradation of DEP, degradation of single DEP was also performed under the same conditions as DEP–DBP mixture. The residual DEP in the algal solutions was sampled after 0-, 24-, 72-, 120- and 168-h incubation.

2.2.2. Degradation experiment of PAEs in extra- and intracellular crude extracts of algae

The microalgae were inoculated at an initial cell density of 2.5×10^5 cells mL^{-1} under the same conditions as the pre-cultivation, and then were harvested by centrifuging (4000 rpm, 5 min) after 168-h incubation. Two hundred mL of the cell-free supernatant was collected and spiked with stock solution of DEP or DEP–DBP mixture to achieve each PAE concentration of about 0.4 mg L^{-1}, which was used for the extracellular degradation experiment. The pellets were washed twice with sterile f/2 medium and disintegrated by grinding for 15 min under ice-cold condition. After centrifugation (12,000 rpm, 20 min, room temperature), the supernatant was pipetted into 200 mL of sterile f/2 medium with DEP (about 0.4 mg L^{-1}) or mixture of DEP and DBP (each of about 0.4 mg L^{-1}) for the intracellular degradation experiment. Both extra- and intracellular degradation experiments were carried out under the same condition as the pre-cultivation. Triplicate flasks of each experiment were retrieved to determine residual PAEs at 0, 24, 72 and 120 h.

2.3. Analysis

For the analysis of residual PAEs in the algal solution, ten mL of algal solution was extracted with dichloromethane for three times and each for 10 min. The dichloromethane layer was concentrated to a final volume of 0.5 mL for GC-FID analysis. For PAEs concentrations in microalgae, the cells were harvested from 50 mL of culture medium by centrifugation at 4000 rpm for 5 min. Then the pellet was extracted in a histoid grinding tube with 4 mL dichloromethane at 500 rpm for 10 min. The solution was centrifuged at 4000 rpm for 5 min. After removal of the water phase, the dichloromethane layer was reduced to a final volume of 0.5 mL.

Samples were analyzed by an Agilent 6890N gas chromatograph fitted with a splitless injector, a fused-silica capillary column (HP-5, 0.32 × 30 m) and a flame ionization detector. The temperatures of injector and detector were both set at 250 °C. Nitrogen was used as a carrier gas at a flow rate of 50 mL min^{-1}, while the flow rates of hydrogen and air were 37 and 550 mL min^{-1}. Injection volume: 1 μL. The retention times of DEP and DBP were 5.62 and 6.88 min. The detection limits ($n = 6$) for DEP and DBP were 20 and 17.5 μg L^{-1} for water, 0.03 and 0.026 mg kg^{-1} for microalgae, respectively. Average recoveries ($n = 3$) of DEP and DBP were 93.8% and 101% in algal solutions, 85% and 95% in algae. The relative standard deviation (RSD) was 7.7–8.9% for algal solutions and 4.5–15.0% for algae.

Esterase activity was determined according to the method of Brar et al. (2009). In brief, esterase activity was measured using 4-nitrophenyl acetate as a substrate. The assay mixture consisted of 1 mL of a 100 mmol L^{-1} Tris–HCl buffer at pH 7 ± 0.1, 100 mL of culture supernatant and 10 mL of the substrate solution. This mixture was allowed to incubate for 3 min at room temperature before the increase of the absorbance at 405 nm was measured at room temperature using a UV–vis spectrophotometer. A blank was measured using 100 mL buffer instead of sample. The increase of the absorbance at 405 nm indicates an increase of 4-nitrophenolate due to hydrolysis of the substrate. The activity was calculated in U mL^{-1}, where 1 U mL^{-1} is the amount of enzyme required to hydrolyze 1 μmol of substrate per second under the given pH and temperature conditions.

2.4. Statistical analysis

Statistical analysis for the data in this study was conducted by a one-way analysis of variance using the software called SPSS (SPSS 18.0 for windows, SPSS Inc., USA)