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Toxicological effects of phenol on four marine microalgae

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ABSTRACT

The toxic effects of phenol on four marine microalgae (*Dunaliella salina*, *Platymonas subcordiformis*, *Phaeodactylum tricornutum* Bohlin, and *Skeletonema costatum*) were evaluated. The 96 h EC_{50} values were 72.29, 92.97, 27.32, and 27.32 mg L^{-1}, respectively, which were lower than those values of freshwater microalgae reported in the literature. During a 96-h exposure to a sub-lethal concentration of phenol (1/2 96 h EC_{50}) with green alga (*D. salina*) and diatom (*S. costatum*), reactive oxygen species (ROS) accumulation, and chlorophyll *a* (Chl *a*) content decrease were simultaneously observed in diatom cells after 48 h treatment. On the contrary, other chlorophylls in both algae were unaffected. Under transmission electron microscopy (TEM), the phenol-induced ultrastructure alterations included disappearance, or shrinkage, of nucleolus and enlargement of vacuoles, which may result in programmed cell death (PCD). The increase in number of lipid droplets may be related to phenol detoxification. These results indicate that the sensitivity of marine microalgae to phenol was dependent on some biotic factors such as cell size, ROS production, and phenol degradation ability in algal cells.

1. Introduction

Phenol, a typical compound of volatile phenols possessing biotoxicity, has a variety of adverse effects on aquatic organisms, such as inhibition of growth and reproduction (Buikema et al., 1981), changes of intermediary metabolism (Hori et al., 2006), cell damage (Abdel-Hameid, 2007), tissue damage (Hori et al., 2006), and oxidative stress (Avilez et al., 2008). Currently, phenol has been named on the list of priority pollutants (129 species) held by the US Environmental Protection Agency (USEPA) (Du et al., 2009). Generally, phenol, at a lower concentration, such as is discharged in industrial wastewater and domestic sewage, can be removed to a greater extent in secondary sewage treatment plants, producing a lower aquatic environment risk (Kueh and Lam, 2008). However, when phenol is spilled in port areas or at sea, its concentration in seawater will increase significantly, posing a severe threat to aquatic ecosystems. It has been reported that phenol is one of the most common chemicals involved in accidental marine spills (Cunha et al., 2015). Based on a risk assessment for the shipping of hazardous substances, using the method proposed by Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP) (GESAMP, 2002), among the 100 shipping chemicals transported in larger quantities between the major ports of Europe and across the English Channel, phenol was considered as one of the chemicals having a greater possibility of accidental release (HASREP, 2005). Recently, volatile phenols were spilled into the Tianjin Port Basin and surrounding marine waters during the Tianjin Port 8.12 explosion accident in 2015. Once again, the toxic effects of phenol and other volatile phenols on marine organisms attract wide concern.

Microalgae are the commonest representatives of primary producers in an ecosystem. Harmful effects accruing in these organisms may affect related food webs and indeed entire aquatic ecosystems (Gerofke et al., 2005). Their sensitivity to pollutant chemicals is usually equal to, or greater than, that of the representatives of primary and secondary consumers (such as daphnia and fish) (Weyers and Vollmer, 2000). Due to these characteristics, microalgae are especially suited to being used test organisms for toxicology research and environmental risk assessment of chemicals.

In this study, four marine microalgae: the green algae *Dunaliella salina*, *Platymonas subcordiformis*, the diatom *Phaeodactylum tricornutum* Bohlin, and *Skeletonema costatum*, were used to investigate toxic effects induced by phenol. Their sensitivity to phenol was compared based on the median effective concentration (EC_{50}). Cellular ultrastructure alterations, reactive oxygen species (ROS) production, and chlorophyll contents, induced by phenol at sub-lethal concentrations, were determined to analyse the mechanism underpinning the toxicity. The results will be conducive to an understanding of the potential ecological risks of phenol when spilled in the marine environment.

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2. Materials and methods

2.1. Chemicals

Phenol (purity ≥99%) was obtained from Bodi Chemical Industry Co., Ltd. (Tianjin, China) with a water solubility of 8.28 g per 100 mL. Other chemicals were analytical or biochemical grade, and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Test species and acclimatisation

Green algae (*P. subcordiformis* and *D. salina*) and diatom (*P. tricornutum*) were obtained from the algal culture collection at Ocean University of China (OUC). Another diatom (*S. costatum*) was supplied by the Institute of Oceanology, Chinese Academy of Sciences. Among them, two diatoms are the test species designated in the Ecological Effects Test Guidelines OCSPP 850.4500: Algal Toxicity formulated by USEPA (USEPA, 2012), and the two green algae are often used as maricultural baits for fish and oysters due to their high cellular protein, lipid, and nucleic acid contents in China. Diatom and green algae strains were acclimatised in MAA or MAA-Si (*i.e.*, MAA lacking sodium metasilicate) medium (USEPA, 2012) for more than two weeks respectively, at 20 °C under a 14/10 h illumination/dark regime at 60 μmol photons $m^{-2} s^{-1}$ irradiance.

2.3. The 96 h algal growth inhibition test

MAA and MAA-Si media supplemented with different phenol concentrations (Table 1) were freshly prepared. Generally, these dosing levels were designed to be a geometric progression of twofold according to OCSPP 850.4500 (USEPA, 2012) after finishing a range-finding test. However, for *S. costatum*, the exposure levels were set up in arithmetic progression, because it presented a narrow response range in the range-finding test, i.e. its growth inhibition (0–100%) was only observed when phenol concentration varied in the range of 15–40 mg L^{-1}. The bioassays were carried out in 500 mL flasks containing 250 mL of medium according to the OCSPP 850.4500 (USEPA, 2012). For each algal species, an initial inoculum (1×10^4 cells mL^{-1}) in the exponential growth phase was added to each medium. Microalgae incubated in the same culture medium, but without added phenol, were used as a control. All assays were carried out in quadruplicate for 96 h without medium replacement. Suspension cultures were maintained under the same conditions as were used for algal acclimatisation in an incubator, shaken by hand twice daily. To avoid loss of phenol due to volatilisation, the cultures were always maintained in a closed state except for sampling for cell density measurement. The cell density (10^4 cells mL^{-1}) were measured every 24 h by direct counting, using a light microscope (YS2-H, Nikon, Japan) at 400× magnification with a 0.1 mm deep hemocytometer during the assay.

Specific growth rates (r) were calculated for each species during the exponential growth phase according to the following equation (Fogg, 1975):

$$r = \frac{\ln N_2 - \ln N_1}{t}$$

where N_2 and N_1 are cell densities (cells mL^{-1}) at times t_2 and t_1, respectively and t is the time interval from t_1 to t_2 in hours.

The generation time (T_d), *i.e.*, the doubling time of cell density, was calculated based on the following equation (USEPA, 2012):

$$T_d = \frac{\ln 2}{r}$$

Microalgal growth curves under different initial phenol concentrations were plotted against time, and the area (A) under the growth curve for each treatment based on cell density was calculated using the following equation (USEPA, 2012):

$$A = \left(\frac{b_1 - b_0}{2}\right)t_1 + \left(\frac{b_1 + b_1 - 2b_0}{2}\right)(t_2 - t_1) + \left(\frac{b_{n-1} + b_n - 2b_0}{2}\right)(t_n - t_{n-1})$$

where b_0, b_1, b_2, and b_n are the observed cell densities at test initiation (t_0), time (day) of the first measurement (t_1), the second measurement (t_2) and the n^{th} measurement (t_n) after test initiation.

The percent inhibition (%I) at each treatment level with respect to controls at 96 h was calculated using the following equation (USEPA, 2012):

$$\%I = \frac{A_C - A_T}{A_C} \times 100$$

where A_C is the area under the growth curve in the control group, and A_T is the area under the growth curve in the phenol treatment group.

The median effective concentration values for 96 h (96 h EC_{50}) were calculated by means of a probit analysis (Finney, 1971) of the percent inhibition (%I).

2.4. Transmission electron microscopy (TEM) observation

To study the cytological changes induced by phenol, *D. saliva* and *S. costatum* were exposed to phenol at concentrations of 36.15 mg L^{-1} and 13.66 mg L^{-1} (*i.e.*, half of their own 96 h EC_{50}) respectively, under the same conditions as described in Section 2.2. The control was MAA-Si and MAA media, respectively, with algal inoculation but without phenol addition. After 96 h, the algal cells were collected by centrifugation for 15 min at 5000*g* and 4 °C. According to the method described by Kadar et al. (2012), the resulting pellet was fixed for 1 h at 4 °C with 2.5% (w/v) glutaraldehyde in 0.2 mol L^{-1} phosphate buffer (pH 7.2), followed by post-fixation for 2 h with 1% OsO_4 in the same buffer. The material was then dehydrated in an acetone graded series from 10% to absolute acetone for 10 min each. After embedding in Spurr's resin and sectioning with a Reichert Ultracut E ultramicrotome, the thin slices were stained for 15 min in a saturated solution of uranyl acetate followed with lead citrate. Then, the slices were examined under a transmission electron microscope (JEM 1200EX, JEOL, Japan).

2.5. Effects on photosynthesis and ROS formation

D. saliva and *S. costatum* were cultivated under the conditions described in Section 2.3, with triplicate experiments for each control and treatment. Samples were taken for determination of the following parameters after 48 h and 96 h exposure, respectively.

2.5.1. Chlorophyll content determination

After 5 mL of algal culture was centrifuged at 4000*g* for 15 min, the supernatants were removed and 5 mL 90% acetone was added to the pellets and extracted in the dark for 24 h. The lysates were clarified by centrifugation at 4000*g* for 15 min, and the absorbance was measured at 664 and 647 nm (for *D. saliva*) and 630 and 664 nm (for *S. costatum*), respectively. To calculate chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and Chlorophyll *c* (Chl *c*), the following equations were used (Jeffrey and Humphrey, 1975). Formulae (1) and (2) were used in the calculation of Chl *a* and Chl *b* of *D. saliva*, and (3) and (4) were used for Chl *a* and Chl *c* of *S. costatum*. The results were described as quality of a certain number of algal cells (μg/10^6 cells).

Table 1
Concentration gradients of phenol in the acute toxicity test.

Species	Phenol concentration (mg L^{-1})				
D. salina	0	10	20	40	80
P. subcordiformis	0	20	40	80	160
P. tricornutum	0	10	20	40	80
S. costatum	0	20	25	30	35