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Evaluation of phenol-induced ecotoxicity in two model ciliate species: Population growth dynamics and antioxidant enzyme activity

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ABSTRACT

The application of identical exposure dosages in different species generally leads to a limited understanding of dose-response patterns because of species-specific factors. To evaluate phenol-induced ecotoxicity, antioxidant enzyme activity and population growth dynamics were compared in two model ciliates, the marine species *Euplotes vannus* and the freshwater species *Paramecium multimicronucleatum*. Dosage ranges of phenol exposure were based on tolerance limits of test ciliates as determined by their carrying capacity (*K*) and growth rate (*r*). When the exposure duration of phenol increased from 48 h to 96 h, the median effective dose (ED_{50}) for *P. multimicronucleatum* decreased faster than that for *E. vannus*, and the ratio of the former to the latter declined from 2.75 to 0.30. When *E. vannus* was exposed to increasing concentrations of phenol (0–140 mg l^{-1}), *r* rose initially and then dropped significantly at concentrations higher than 40 mg l^{-1}, whereas *K* decreased linearly over the entire range. For *P. multimicronucleatum*, both *r* and *K* declined gradually over the range 0–200 mg l^{-1} phenol. Dose-response patterns of activities of three individual antioxidant enzymes, and the integrative index of the three enzymes, presented a biphasic (inverse U-shaped) curve at each of four durations of exposure, i.e. 12 h, 24 h, 36 h and 48 h. Cluster analyses and multidimensional scaling analyses of antioxidant enzyme activities revealed differences in the temporal succession of physiological states between the two model ciliates. In brief, combining ED_{50} with growth dynamic parameters is helpful for designing exposure dosages of toxicants in ecotoxicity tests.

1. Introduction

Phenol and its related products are used in a wide range of industrial production processes including pharmaceuticals, plastics and disinfectants (Dieguez-Santana et al., 2016). The ecological toxicity of phenol is of growing concern due to its accumulation in the environment. Water bodies are sinks for numerous chemical compounds released into to the environment through industrial, domestic and agricultural activities (Ololade and Oginni, 2010). It has reported that phenol concentrations vary in water environments with up to 17,500 mg/l recorded in wastewater effluents (Najafpoor et al., 2015). Many of these contaminants adversely affect aquatic organisms due to their toxicity (Duan et al., 2017). Phenol has been listed as a priority pollutant by the US Environmental Protection Agency (USEPA) (Du et al., 2009). It is noteworthy that phenol is one of the most common chemicals involved in accidental marine spills (Cunha et al., 2015). For example, large amounts of phenol were spilled into the Tianjin Port Basin during the explosion accident in 12 August 2015 (Duan et al., 2017). Once phenol is spilled in port areas or at sea in significant quantities, it presents a severe threat to aquatic life. In view of these considerations, there is a growing need to develop methods for ecological toxicity assessments of phenol in aquatic ecosystems.

Ciliated protozoa (ciliates) are widely recognized as early warning bioindicators for environmental risk because of their ecological and biological properties, such as high diversity, short life cycles, cosmopolitan distribution, ease of collection, large population sizes making them amenable to statistical analyses, and rapid responses to environmental disturbance (Montagnes et al., 2012; Payne, 2013). Furthermore, evidence is accumulating for their potential as model organisms for assessing the toxicological effects of pollutants (Zhou et al., 2011; Li et al., 2014; Hong et al., 2015a, 2015b, 2017). There are, however, relatively few data on the toxicity effects of phenol on protozoa in general or on ciliates in particular (Brito-Sánchez et al., 2013; Dieguez-Santana et al., 2016; Abbasitabar and Zare-Shahabadi, 2017). There are also potential limitations to the utility of organisms in cytotoxicity testing. For example, it has been reported that organisms from different

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habitats often differ in the way they respond to certain pollutants (Fonseca et al., 2015). Furthermore, there is a risk that species-specific factors may result in the possibility that experimental data obtained in one species are not applicable to other species (Lee et al., 2012; Hong et al., 2015a). Considering the importance of species-specific factors, two species of ciliated protozoa, namely *Euplotes vannus* and *Paramecium multimicronucleatum,* were chosen as model organisms for the present study. They are widespread in marine and limnetic environments respectively, but with differing morphological features, taxonomic affiliations, behaviors, and ecological niches (Jiang et al., 2009; Chen and Song, 2002).

In recent decades, there has been increasing interest in the use of various biomarkers, both at the sub-cellular and at population/community levels, for ecotoxicity assessments of environmental pollutants in aquatic ecosystems (Hagger et al., 2006). Commonly used sub-cellular biomarkers include free radical-scavenging antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Li et al., 2014; Hong et al., 2015b, 2017). Commonly used biomarkers for ecotoxicity assessment at the population /community level include growth rate, mortality rate, and survival rate (Smolders et al., 2004). Ciliates offer biomarkers at both levels in order to advance understanding of the effects of stressors on functional ecology and on individual organisms (Pörtner et al., 2010). A major challenge, therefore, is to extrapolate responses of sub-cellular biomarkers to those observed at higher levels of biological organization (Tankoua et al., 2013). A commonly used approach is to generate integrative indices based on multiple biomarkers from which the ecological effects at higher levels of biological organization can be deduced (Luna-Acosta et al., 2017). An example of such an index is the Integrative Biological Response (IBR) index which is a frequently used tool for ecosystem health risk assessments (Panzarino et al., 2016).

The exposure dosage of a toxicant is a key factor for the evaluation of its ecotoxicological effects. Different species often exhibit different response patterns to the same dosage exposure due to variations in their tolerance to the toxicant in question. The application of inappropriate ranges of dosage exposure is a potentially important cause of failure in elucidating the relationship between biomarkers at different biological levels (Hong et al., 2015a, 2015b). Exposure dosage can be set in terms of the growth dynamics of the test organism based on the growth rate (r) and carrying capacity (K) of a population during a defined period of exposure. According to Dynamic Energy Budget theory, the maximum tolerance limit of an organism is achieved when it is in a physiological state of 'maintenance' wherein all available energy is allotted to survival rather than population growth, i.e., r = 0 (Kooijman, 2010). This exposure dosage is, however, specific for the test organism used. We therefore hypothesize that dose-response patterns will differ in the two test ciliates when exposed to identical dose range of phenol because of species-specific factors, whereas dose-response patterns will be similar when the exposure dose range extends to the maximum tolerance limits of the test organisms. In order to test this hypothesis, it would be preferable to design the maximum exposure dosage based the population dynamic parameters K and r such that the test organisms attain the equivalent physiological state, specifically the maximum exposure dosage of toxicant that results in the same parameters among the test organisms, i.e., K = the initial inoculum abundance and $r = 0$. Furthermore, determining the tolerance limits of the test organisms based on population dynamic parameters would also be relevant to the effects of the toxicant on ecosystem function. Thus, population growth dynamics could provide both an objective method for determining the maximum exposure dosage of a toxicant and a novel strategy to investigate the relationship between biomarkers at different biological levels.

The main aim of the present study is to evaluate the ecotoxicological effects of phenol exposure to two species of ciliates, i.e., *Euplotes vannus* and *Paramecium multimicronucleatum,* using biomarkers at two levels of biological organization, i.e., antioxidant enzyme activity and parameters of population growth dynamics. The sensitivity /tolerance of each species to phenol exposure was assessed and maximum exposure dosages were determined using population growth dynamics. Dose-response patterns of antioxidant enzyme activity were investigated in each species by exposing them to a range of doses of phenol for different durations of exposure. Finally, to reveal the temporal succession of physiological states in the test ciliates, correlations between the profiles of antioxidant enzyme activities at each exposure duration were analyzed by multivariate statistical analyses.

2. Materials and methods

2.1. Ciliate cultivation

Two model ciliates, i.e. the marine species *Euplotes vannus* and the freshwater species *Paramecium multimicronucleatum,* provided by Laboratory of Protozoology, South China Normal University, Guangzhou, China, were used as the test organisms. Clonal cultures were established for both species at 25 ± 1 °C. *Euplotes vannus* was cultivated in Artificial Marine Water (AMW) which consisted of 28 g NaCl, 0.8 g KCl, 5 g $MgCl_2{\cdot}6H_2O$, 1.2 g $CaCl_2$ and 1000 ml distilled water, pH 8.2, salinity 30‰. *Paramecium multimicronucleatum* was cultivated in Watson's distilled water with added minerals (Watson's, Guangzhou). In both cases, rice grains were added in order to enrich the growth of natural bacteria which are the food source for the ciliates.

2.2. Phenol

Laboratory grade phenol was obtained from China Sigma-Aldrich Shanghai Trading Co Ltd Shanghai, China (CAS No.: 108-95-2). Stock solutions (2000 mg l^{-1}) for *E. vannus* were prepared by dissolving phenol in AMW. Stock solutions (2000 mg l^{-1}) for *P. multimicronucleatum* were prepared by dissolving phenol in Watson's distilled water. Test solutions of phenol at different concentrations were prepared by diluting the stock solutions with AMW or Watson's distilled water, respectively. All solutions were stored at 4 °C until used.

2.3. Acute toxicity test for determining ED_{50}

The median effective dose (ED_{50}) for inhibition of population growth for each species was determined by acute toxicity tests according to Hong et al. (2015a). All tests were carried out in flat-bottomed culture plates (Φ = 30.0 mm, h = 15.0 mm) at 25 °C. After an acclimation period of 48 h, ciliates were inoculated into a series of phenol solutions of different concentrations. Phenol concentrations were 0, 20, 40, 60, 80, 100, 120, and 140 mg l^{-1} for *E. vannus* and 0, 20, 60, 100, and 200 mg l^{-1} for *P. multimicronucleatum*. Each solution was randomly assigned to triplicate wells and each well was stocked with 30 ciliate cells in a 3 ml solution. Measures taken in order to maintain the concentration of phenol included conducting experiments in the dark, sealing the culture plate, and transferring the test ciliates to newly prepared solution of phenol every 24 h. Cells that were unable to swim or creep along the bottom of the well, together with disappeared cells, were regarded as dead (Madoni and Romeo, 2006). Mortality was measured at 24 h intervals under a dissecting microscope (20–30 ×). Results were analyzed and the median effective dose (ED_{50}) was determined by probit analysis (Finney, 1971).

2.4. Chronic toxicity test for determining population growth dynamics

Population growth dynamics of ciliates were investigated in flat-bottomed culture plates (Φ = 30.0 mm, h = 15.0 mm). The media and culture conditions were the same as those for the ED_{50} assay. After an acclimation period of 48 h, ciliate cells were transferred into a series of phenol concentrations, i.e., for *E. vannus* 0, 20, 40, 60, 80, 100, 120 and 140 mg l^{-1} phenol in AMW, and for *P. multimicronucleatum* 0, 20, 60,