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Evaluation of biomarkers for ecotoxicity assessment by dose-response dynamic models: Effects of nitrofurazone on antioxidant enzymes in the model ciliated protozoan *Euplotes vannus*

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ABSTRACT

Understanding dose-responses is crucial for determining the utility of biomarkers in ecotoxicity assessment. Nitrofurazone is a broad-spectrum antibiotic that is widely used in the aquaculture industry in China despite its detrimental effects on ecosystems. Potential dose-response models were examined for the effect of nitrofurazone on two antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx), in the ciliated protozoan *Euplotes vannus*. This was achieved by measuring enzyme activity and gene expression profiling of SOD and GPx in ciliate cells exposed to nitrofurazone at doses ranging from 0 to 180 mg l^{-1} for 6 h, 12 h, 18 h and 24 h. Dose-response dynamics were characterized by mathematical models. Results showed that: 1) dose-response patterns differed significantly among the tested endpoints, nitrofurazone concentrations and durations of exposure; 2) GPx activity was the best candidate biomarker because of its linear dose-response relationship; 3) SOD activity and mRNA relative expression levels of GPx and SOD are also candidate biomarkers but their dose-responses were non-linear and therefore more difficult to interpret; 4) partitioning the dose-response dynamic model by piecewise function can help to clarify the relationships between biological endpoints. This study demonstrates the utility of dynamic model analysis and the potential of antioxidant enzymes, in particular GPx activity, as a candidate biomarkers for environmental monitoring and risk assessment of nitrofurazone in the aquaculture industry.

1. Introduction

Veterinary antibiotics are widely used in farmed-animal industries for prophylactic and therapeutic purposes, to promote growth, and to increase feeding efficiency (Chen et al., 2014; Puckowski et al., 2016). It is estimated, however, that 40–90% of the administered veterinary antibiotics are released into the environment in the form of parent compounds and/or their metabolites (Phillips et al., 2004; Kumar et al., 2005; Kemper, 2008). Furthermore, the abuse of antibiotics in intensive aquaculture has caused a range of environmental problems including antibiotic resistance and the appearance of ultra-virulent bacteria, which in turn necessitate the use of more antibiotics, further aggravating the deterioration of the environment (Tendencia and Peña, 2002; Qi et al., 2009; Dafale et al., 2016). Moreover, antibiotics not only affect bacteria but also can seriously impair non-target organisms, thus impacting on wider biological processes (Białk-Bielińska et al., 2011; Hassan et al., 2016). These problems increasingly affect community composition and ecosystem function in aquaculture ecosystems (Puckowski et al., 2016). There is therefore a growing need to monitor water quality and to evaluate the ecotoxicological risks of antibiotic use in the aquaculture industry (Vutukuru et al., 2007; Puckowski et al., 2016).

Biological assays have a number of advantages over chemical assays for monitoring water quality. These include the ability to reflect the bioavailability of toxicants, the effects of mixtures of toxic compounds, both known and unknown, on biota and the mechanisms by which the toxic compounds act (Hendriks et al., 1994; Van et al., 2010; He et al., 2012; Li et al., 2013; Hassan et al., 2016). Furthermore, the use of bioassays is well established at multinational (e.g. the EU OSPAR Coordinated Environmental Monitoring Program) and national (e.g. the UK National Marine Monitoring Program) levels (Hagger et al., 2006). Bioassays rely on the quantitative relationships between environmental stressors, including contaminant level and exposure time, and biomarker responses elicited in the target organisms (Mushak, 2013).

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There is, however, uncertainty over the precise nature of some of these relationships which is hampering the selection of ideal biomarkers and limiting the application of biological assays in practice (Li et al., 2014; Hong et al., 2015a, 2015b). These uncertainties are due to a combination of the complexity of biomarker responses, the properties of the contaminant and a lack of pragmatic methods for processing biomarker response patterns (Hong et al., 2015b; Vidal-Liñán et al., 2016). Recent advances in dynamic model analyses, particularly the development of dynamic models for estimating dose-responses, are addressing such methodological problems both by helping to reinterpret existing data and to generate novel predictive models (McCarty, 2013; Li et al., 2014; Hong et al., 2015a, 2015b).

Exposure of organisms to antibiotics can increase the generation of reactive oxygen species (ROS) leading to oxidative stress (Kohanski et al., 2007; Páez et al., 2011; Grant et al., 2012; Hong et al., 2015a, 2015b). Consequently, free radical-scavenging antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), are being intensively studied due to their potential as biomarkers (Velisek et al., 2011; Stara et al., 2012). Both the activity and the gene expression of antioxidant enzymes are induced by increasing production of ROS and might therefore serve as indicators of oxidative stress toxicity (Winston and Giulio, 1991; Cossu et al., 1997; Hagger et al., 2006). In some cases the detoxification processes may be completed by the entire antioxidant defense system involving complicated interactive mechanisms among these antioxidant enzymes (Li et al., 2013; Kaur and Kaur, 2015). Further complexities and uncertainties are caused by other factors such as dose-response, time-response, and interactions between the biological endpoints (Hagger et al., 2006; Li et al., 2013; Kaur and Kaur, 2015). With the development of intensive aquaculture practices the use of antibiotics, and consequently their concentration in the aquatic environment, is increasing. Thus, it is necessary to investigate the ecotoxicological effects of antibiotics in a greater concentration range than is usually present in the aquatic environment. Key questions that therefore arise include: 1) do higher concentrations of antibiotics affect the dose-response patterns of antioxidant enzymes in the same way as they do at lower concentrations? 2) do divergences exist in the response patterns between biological endpoints? 3) can a dynamic dose-response model be developed that takes account of these divergences?

Ciliated protozoa are important components of microbial food webs and play a crucial role in controlling the flux of materials and energy within ecosystems (Eccleston-Parry and Leadbeater, 1994). Due to their short life cycles, cosmopolitan distribution, and rapid response to environmental disturbance, ciliated protozoa are increasingly recognized as ideal indicator organisms for environmental impact assessment (Nilsson, 1989). Montagnes et al. (2012) reviewed the utility of protozoa as model organisms in a wide range of fields of study including ecology, ecotoxicology and physiology, and concluded that they have numerous advantages. Ciliated protozoa in particular are increasingly used as model organisms in bioassays. The freshwater ciliate *Tetrahymena thermophila*, for example, has been used as a model organism to investigate the ecotoxicity of nanoparticles of CuO and ZnO in natural water (Blinova et al., 2010) and ecotoxicological assessments of biochar, a type of charcoal commonly used for soil amendment (Oleszczuk et al., 2013). There have also been several recent studies using marine ciliates to evaluate the ecotoxicity of various aquatic pollutants (Zhou et al., 2011; Gomiero et al., 2012; Li et al., 2014; Hong et al., 2015a, 2015b).

Nitrofurazone is a broad-spectrum antibiotic whose use in aquaculture is prohibited in many countries. Nevertheless, it is still widely used because of its low cost and efficacy (Vlastos et al., 2010; Du et al., 2014). In two recent studies, we have demonstrated the potential of the marine ciliate *Euplotes vannus* as a model organism for evaluating antioxidant enzymes as biomarkers of nitrofurazone-induced ecotoxicity at low (0–24 mg l^{-1}) concentrations of exposure (Li et al., 2014; Hong et al., 2015a, 2015b). In the present study, *E. vannus* was used to investigate the ecotoxicological effects of exposure to nitrofurazone in a greater range of concentrations (0–180 mg l^{-1}). Specifically, activity and gene expression of the antioxidant enzymes SOD and GPx were used as biomarkers of nitrofurazone ecotoxicity. The main aims were to develop a dose-response dynamic model for evaluating the potential of these biomarkers for risk assessment of nitrofurazone in aquatic ecosystems and to improve their utility for inclusion in routine monitoring protocols.

2. Materials and methods

2.1. Nitrofurazone

Nitrofurazone (5-nitro-2-furfural semicarbazone) was obtained from Sigma-Aldrich Shanghai Trading Co., Ltd., Shanghai, China (CAS No.: 59870). A stock solution (200 mg l^{-1}) of nitrofurazone was prepared with sterile artificial marine water (AMW) consisting 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‰). The stock solution was diluted with sterile AMW to give appropriate concentrations of nitrofurazone in toxicity exposure experiments (Li et al., 2014; Hong et al., 2015b).

2.2. Organisms and media

The marine ciliate *Euplotes vannus*, identified by a combination of morphological and molecular information (Chen and Song, 2002), was obtained from the Laboratory of Protozoology Ocean University of China, Qingdao, China. Clonal cultures of *E. vannus* were established and maintained in Petri dishes with sterilized AMW (prepared as described above) at 25 °C. Rice grains were added to each Petri dish to enrich the natural bacterial populations as a food source for *E. vannus*.

2.3. Toxicity exposure of nitrofurazone

Cells of *E. vannus* in logarithmic growth phase were exposed to nitrofurazone at concentrations of 0, 0.5, 1.5, 3, 6, 12, 24, 36, 42, 48, 54, 66, 90, 138, and 180 mg l^{-1}. In each case, durations of exposure were 6 h, 12 h, 18 h, and 24 h. Treatments were performed in 50 ml sterilized conical flasks, each containing 20 ml of solution, sealed with air-permeable film and incubated at 25 °C in the dark. The density of ciliate cells in each solution was approximately 4×10^3 ind. ml^{-1}. The ciliates were not fed throughout the experiment. At the end of the exposure period, ciliate cells were washed gently with sterilized AMW and incubated for 12 h in the presence of 10 mg ml^{-1} lysozyme. They were then centrifuged at 956*g* at 4 °C for 3 min and washed gently with sterilized AMW. This purification procedure was repeated three times. After purification, cells were lysed for protein and RNA extraction.

2.4. Determination of antioxidant enzyme activity

Cells were lysed in lysis buffer, 1% 100 mM Phenylmethanesulfonyl fluoride (PMSF, purity > 99%; Beyotime Co., China) for Western Blot and the immunoprecipitation of proteins (IP). The samples were kept on ice during the pre-processing procedure. Cells in lysed buffer were homogenized on ice using a homogenizer (Sonifier cell disrupter, UH-100A, Tianjin). Total cell homogenates were centrifuged at 20,000*g* for 10 min at 4 °C and the supernatant was used to determine the antioxidant enzyme activity. SOD activity was measured by the ferricytochrome c method (McCord and Fridovich, 1969). Briefly, xanthine: xanthine oxidase was used as a source of superoxide radicals and absorption values were monitored at 550 nm. One unit of SOD activity is defined as the amount of enzyme required to inhibit cytochrome c reduction by 50%. Enzyme activity is expressed as units per milligram protein. GPx activity was determined by the rate of NADPH oxidation in a coupled reaction with glutathione reductase and measured by absorbance in a micro-plate reader at 412 nm (Lawrence and Burk, 1976).