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 Co-ordinated 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 MgCl₂·6H₂O, 1.2 g CaCl₂ 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 × 10³ 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 956g 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 20,000g 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).

The amount of enzyme required to consume 1 μmol glutathione (GSH) in 1 min was defined as one unit of GPx. This activity was also expressed as units per milligram protein. The protein level was determined by the Bradford method (Bradford, 1976). Briefly, bovine serum albumin was used as a standard and absorbance was recorded at 595 nm. All measurements were performed using a model 680 microplate reader (Bio-Rad, USA).

2.5. Quantitative analyses of antioxidant enzyme gene expression

The methods for total RNA and gene expression analyses were described in detail by Hong et al. (2015b). To test the relative gene expression level of antioxidant enzymes, total RNA from the ciliates was extracted, further treated with RQ1 RNase-Free DNase (Promega, Madison, USA), and then reverse-transcribed into complementary DNA (cDNA) using the M-MLV RTase cDNA Synthesis Kit (TaKaRa, Dalian, Japan). The purity of RNA in the samples was determined by measuring the ratio of absorbance at 260, 280 and 230 nm (i.e., A260: A280 and A260: A230), using a Nano Drop ND-1000 spectrophotometer. In addition, the integrity of 18S and 28S rRNA bands was tested by 1% agarose gel electrophoresis. Manganese superoxide dismutase (MnSOD) (GenBank accession no. KJ619484) and GPx (GenBank accession no. KF049698) of *E. vannus* were used as target genes. The α -tubulin gene of *E. vannus* (GenBank accession no. Z11769) was used as an internal control. Based on preliminary findings that the amplicon efficiencies of the target and internal control genes were generally equal (data not shown), the α -tubulin gene of *E. vannus* (GenBank accession no. Z11769) was used as a reference gene.

Two MnSOD-specific primers (SOD-1 and SOD-2) were used to amplify a 104-bp fragment of MnSOD (Hong et al., 2015b). Primers GPx-1 and GPx-2 were used to amplify a 111-bp fragment of GPx (Hong et al., 2015b). Primers tubulin-1 and tubulin-2 were used to amplify a 122-bp fragment of α -tubulin as the internal control for quantitative RT-PCR (Li et al., 2014). To study mRNA relative expression of MnSOD and GPx in ciliates treated with nitrofurazone at different doses and durations of exposure, real-time PCR was performed on an ABI 7500 (Applied Biosystems, USA). The PCR reaction was performed as previously described (Hong et al., 2015b). A dissolution curve was examined to ensure that each PCR product was unique. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.6. Statistical analyses

Dose–response models were established by SigmaPlot software (Version 9) to characterize the dose–response dynamics (for details of methods, see Li et al., 2014). The Akaike information criterion (AIC) was used to measure the relative quality of statistical models for a given set of data (Akaike, 1987). Adjusted R^2 -values for the responses, standard errors of the estimates were determined (SIGMAPLOT), to indicate their goodness of fit. Paired *t*-tests were used to determine significant differences in biological responses between discrete exposure durations by using SPSS software (version 19.0 for Windows). To analyze the relationships between biological responses and nitrofurazone concentrations, as well as possible correlations between enzyme activities and relative gene expression levels, Pearson's correlation analysis was performed using SPSS software (version 19.0 for Windows). All tests used a statistical significance level of $P < 0.05$.

3. Results

3.1. SOD activity and dose–response dynamics

During each of the four periods of exposure, SOD activity rose to a peak and then declined with increasing concentration of nitrofurazone (Fig. 1). In each case, the dose–response graph comprised two roughly

Fig. 1. Dose–response dynamics for enzyme activity of superoxide dismutase (SOD) in *Euplotes vannus* exposed to different concentrations of nitrofurazone for exposure durations ranging from 6 h to 24 h. A, B, C, D: exposure durations of 6 h, 12 h, 18 h, and 24 h, respectively. E: the slopes of straight lines from A, B, C, D. Continuous lines are the best-fit to the data in the figures following equations in Table S1, respectively.

straight lines, one pre-peak and one post-peak (Fig. 1, A, B, C, D). When data of SOD activity were plotted against exposure time, the slopes of the pre-peak (K_1) and post-peak (K_2) lines were U-shaped and inverted U-shaped curves, respectively (Fig. 1, E).

3.2. GPx activity and dose–response dynamics

GPx activity increased markedly with increasing nitrofurazone

Fig. 2. Dose-response dynamics for enzyme activity of glutathione peroxidase (GPx) in *Euplotes vannus* exposed to different concentrations of nitrofurazone for exposure durations ranging from 6 h to 24 h. A, B, C, D: exposure durations of 6 h, 12 h, 18 h, and 24 h, respectively. E: the slopes of straight lines from A, B, C, D. Continuous lines are the best-fit to the data in the figures following equations in Table S2, respectively.

concentration (Fig. 2). At exposure duration of 6 h, GPx activity increased linearly at lower nitrofurazone concentrations and exponentially at higher nitrofurazone concentrations (Fig. 2, A). At exposure durations of 12 h, 18 h, and 24 h, GPx activity increased linearly with increasing nitrofurazone concentration ($P < 0.001$; Fig. 2, B, C, D). Furthermore, the slopes of the lines (K) increased almost linearly with increasing durations of exposure from 6 h to 24 h (Fig. 2, E).

Fig. 3. Dose-response dynamics for mRNA relative expression levels of superoxide dismutase (SOD) in *Euplotes vannus* exposed to different concentrations of nitrofurazone for exposure durations ranging from 6 h to 24 h. A, B, C, D: exposure durations of 6 h, 12 h, 18 h, and 24 h, respectively. E: the slopes of straight lines from A, B, C, D. Continuous lines are the best-fit to the data in the figures following equations in Table S3, respectively.

3.3. Relative levels of expression of SOD mRNA and dose-response dynamics

The relative levels of expression of SOD mRNA increased at lower nitrofurazone concentrations to a peak and then decreased at higher concentrations (Fig. 3). At exposure durations of 6 h, 12 h, and 18 h, each dose-response graph comprised two roughly straight lines, one pre-peak and one post-peak (Fig. 3, A, B, C). With increasing exposure durations, the slopes of the dose-response lines (K_1) increased pre-peak whereas the absolute values of slopes decreased post-peak (Fig. 3, E). At

Fig. 4. Dose–response dynamics for mRNA relative expression levels of glutathione peroxidase (GPx) in *Euplotes vannus* exposed to different concentrations of nitrofurazone for exposure durations ranging from 6 h to 24 h. A, B, C, D: exposure durations of 6 h, 12 h, 18 h, and 24 h, respectively. E: the Max and N_c in the dose–response curves from A, B, C, D. Max is the maximum values of mRNA relative expression levels of GPx and N_c is the nitrofurazone concentration for Max. Continuous lines are the best-fit to the data in the figures following equations in Table S4, respectively.

the 24 h exposure, relative expression levels of SOD mRNA increased exponentially to a peak and then decreased linearly with increasing nitrofurazone concentration (Fig. 3, D).

3.4. Relative expression of GPx mRNA and dose–response dynamics

GPx mRNA expression relative to nitrofurazone concentration presented a typical bell-shaped curve with gene expression increasing to a peak before declining at higher concentrations of nitrofurazone (Fig. 4, A, B, C, D). Both the peak value (Max) of relative expression of mRNA, and the nitrofurazone concentration (N_c) at which the peak occurred, decreased with increasing exposure time (Fig. 4, E).

Fig. 5. Box-chart analyses of antioxidant enzyme activity and mRNA relative expression level. A: Activities of superoxide dismutase (SOD); B: Activities of glutathione peroxidase (GPx); C: mRNA relative expression levels of SOD; D: mRNA relative expression levels of GPx.

3.5. Box-chart analyses of antioxidant enzyme activity and mRNA expression

The statistical distribution patterns of between-group data, and the variations of within-group data, are illustrated by box-chart analyses (Fig. 5). SOD activities at each duration of exposure had a relatively large box size compared to the whiskers extending vertically from the boxes (Fig. 5, A). T-test analyses showed no significant differences between the groups at different durations of exposure ($P > 0.05$). By contrast, significant differences in GPx activities were found at different durations of exposure (Fig. 5, B; $P < 0.05$). Compared to antioxidant enzyme activities, mRNA gene expression presented significantly different distribution patterns at different durations of exposure. SOD mRNA expression presented no distinct variation, however, the box sizes first increased and then decreased after peaking at an exposure duration of 18 h (Fig. 5, C). The box sizes and whiskers in GPx mRNA expression generally decreased with longer durations of exposure (Fig. 5, D; $P < 0.05$). In brief, these results indicated that dose-responses were influenced by a combination of nitrofurazone concentration and duration of exposure.

3.6. Correlations between antioxidant enzyme activity, mRNA expression, and nitrofurazone dose

Correlations between antioxidant enzyme activity, mRNA

Table 1

Correlation between antioxidant enzymes (activity, mRNA expression level), nitrofurazone concentration and duration of exposure using the Pearson's correlation coefficient.

Variables	Exposure Duration			
	6 h	12 h	18 h	24 h
SOD activity × Nitrofurazone Con.	−0.004	0.215	0.277	−0.458
SOD mRNA expression × Nitrofurazone Con.	0.513	0.442	0.766**	0.881**
SOD activity × mRNA expression	0.477	0.102	0.678**	−0.544*
GPx activity × Nitrofurazone Con.	0.816**	0.896**	0.954**	0.968**
GPx mRNA expression × Nitrofurazone Con.	0.828**	0.760**	0.721**	0.344
GPx activity × mRNA expression	0.391	0.547	0.806**	0.317

expression, and nitrofurazone concentration varied according to the durations of exposure to nitrofurazone (Table 1). SOD activity had no significant dose relationship ($P > 0.05$) whereas mRNA expression was significantly correlated with nitrofurazone concentration at 18 h and 24 h of exposure ($P < 0.01$). SOD enzyme activity was, however, positively correlated with mRNA expression at 18 h exposure to nitrofurazone ($P < 0.01$) but negatively correlated at 24 h exposure ($P < 0.01$). GPx enzyme activity was positively correlated with nitrofurazone concentration for all four durations of exposure ($P < 0.01$). GPx mRNA expression was positively correlated with nitrofurazone concentration from 6 h to 18 h exposure ($P < 0.01$), but was not significantly correlated at 24 h exposure ($P > 0.05$). GPx mRNA expression was significantly correlated with enzyme activity at 18 h exposure only. Results of correlation analyses indicated that the correlation depended on enzyme properties, biological endpoint levels, and duration of exposure.

4. Discussion

The broad-spectrum antibiotic nitrofurazone has been widely used in human and veterinary medicine (Reynolds, 1982). The mechanism by which it inactivates bacteria is based on nitrofurazone metabolites causing oxidative DNA damage by generating reactive oxygen species (ROS) through their interaction with copper (Hiraku et al., 2004). As mentioned above, nitrofurazone is still widely used in aquaculture in many countries because of its low cost and efficiency (Du et al., 2014; Vlastos et al., 2010). Furthermore, with the drastic development of intensive aquaculture, the amount of antibiotics accumulating in aquaculture environments is increasing (Puckowski et al., 2016). Although the concentrations of nitrofurazone used in the present study exceeded those typically found in aquaculture waters (Yanong, 2003), the dose-response patterns will nevertheless provide insights for verifying the existing model as well as predicting future ecotoxicity trends.

In aerobic organisms, antioxidant enzymes are crucial components of defense systems that prevent the uncontrolled formation of free radicals and activated oxygen species (Doyen et al., 2006). Among the central antioxidant enzymes, SOD catalyzes the conversion of superoxide anions, generated in peroxisomes and mitochondria, to water and hydrogen peroxide (Velisek et al., 2011; Stara et al., 2012). Furthermore, its activity reflects levels of oxidative stress in cells (Hu et al., 2016). GPx is another important endogenous antioxidant enzyme and catalyzes the conversion of peroxides to water and oxygen with GSH as the electron donor (Kutlu and Susuz, 2004; Drevet, 2006; Liu et al., 2010). Antioxidant enzyme activity and gene expression have been used as sensitive biomarkers for monitoring environmental stress (Cossu et al., 1997; Hagger et al., 2006; Kerambrun et al., 2016). It has also been reported that superoxide anion radicals and hydrogen peroxide are formed during the reductive metabolism of nitrofurazone in rat brain tissue (Gherzi-Egea et al., 1998). The present study shows that levels of both activity and mRNA expression of the antioxidant enzymes SOD and GPx varied with nitrofurazone dose, confirming their potential

as biomarkers for ecotoxicity as suggested previously (Li et al., 2014; Hong et al., 2015b).

Under normal physiological conditions, SOD levels maintain a dynamic balance and meet the need of the organism to eliminate superoxide anions (Hu et al., 2016). The balance between the formation and removal of superoxide anions can, however, easily be disrupted by stress (Liu et al., 2011). In this study, SOD enzyme activity in *E. vannus* exposed to increasing concentrations of nitrofurazone rose to a peak and then declined (Fig. 1). These findings suggest that *E. vannus* suffered nitrofurazone-induced oxidative stress, the level of stress being dependent upon both the concentration and the duration of exposure to nitrofurazone. In agreement with our previous study, SOD activity rose in *E. vannus* cells exposed to lower concentrations of nitrofurazone (0–30 mg l^{−1}) for durations ranging from 6 h to 24 h (Hong et al., 2015b). The effects of higher concentrations of nitrofurazone on antioxidant enzymes in ciliated protozoa have been rarely reported. However, it has been suggested that SOD activity can be inactivated by excessive hydrogen peroxide (Modesto and Martinez, 2010). Observations that lower concentrations of contaminants stimulate SOD activity while higher concentrations suppress SOD activity have been reported in other organisms, e.g. crucian carp (*Carassius auratus*) exposed to hexavalent chromium (Li et al., 2013) and phthalates (Zheng et al., 2013), and the snail (*Cantareus apertus*) exposed to carbamate pesticide (Leomanni et al., 2015). Correlation analyses showed no significant dose-dependent relationship in SOD activity in *E. vannus* ($P > 0.05$); SOD activity presented a non-linear dose-response pattern with increasing nitrofurazone concentrations. Moreover, the results of box-chart analyses indicated that SOD activity varied slightly with either nitrofurazone concentration or duration of exposure. In particular, no significant differences were detected between cells exposed to nitrofurazone for different durations (t -test, $P > 0.05$). These findings suggest that the impact of nitrofurazone dose on SOD activity was insignificant and that SOD activity is therefore not a suitable biomarker to evaluate nitrofurazone-induced ecotoxicity in *E. vannus*.

In contrast to SOD activity, GPx activity in *E. vannus* cells presented a linear dose-response pattern, with activity increasing as the nitrofurazone concentration increased ($P < 0.001$; Fig. 2, B, C, D). This is consistent with the findings of Hong et al. (2015b) who investigated GPx activity in *E. vannus* at lower concentrations of nitrofurazone (0–30 mg l^{−1}) for the same durations of exposure. In the dose-response plots, the slopes of the straight lines (K) generally increased linearly with increasing durations of exposure from 6 h to 24 h (Fig. 2, E). Results of box-chart analyses indicated that GPx activity varied significantly with both nitrofurazone concentration and duration of exposure (t -test, $P < 0.05$). Additionally, GPx activity was significantly positively correlated with nitrofurazone concentration ($P < 0.05$). Consequently, it was concluded that GPx activity is a candidate biomarker for the evaluation of nitrofurazone-induced ecotoxicity in *E. vannus*.

Gene expression profiling based on mRNA is a useful supplement for enzyme activity detection and has been used to identify biomarkers of toxicity of potentially hazardous contaminants (Brulle et al., 2010; Chen et al., 2011; Hu et al., 2016). In the present study, t -test and box-chart analyses demonstrated that gene expression profiling of the antioxidant enzymes SOD and GPx was significantly influenced by both the concentration of nitrofurazone and the duration of exposure (Fig. 5). Relative expression levels of SOD mRNA increased to a peak and then decreased with increasing concentrations of nitrofurazone (Fig. 3). In a previous study, elevated levels of mRNA expression for SOD were also observed in *E. vannus* exposed to a lower range of concentrations of nitrofurazone (0–30 mg l^{−1}) for the same durations of exposure (Hong et al., 2015b). Similar results have been reported by Hu et al. (2016) who investigated gene expression of SOD mRNA in the earthworm (*Eisenia fetida*) after 14 days repetitive exposure to Pb and to the brominated flame retardant BDE209. There have also been reports on contaminant-induced down-regulation of antioxidant genes in

various organisms including Javanese medaka (*Oryzias javanicus*) exposed to heavy metals (Woo et al., 2009) and *Eisenia fetida* exposed to BDE209 and Pb (Hu et al., 2016). Nitrofurazone in high concentrations has thus been linked to oxidative stress, probably as a result of decreased expression of antioxidant mRNA genes (Gherzi-Egea et al., 1998). In the case of SOD mRNA, the slopes of the linear dose-response patterns at exposure durations of 6 h, 12 h, and 18 h significantly increased at lower concentrations and decreased at higher concentrations of nitrofurazone. However, the dose-response pattern at 24 h exposure deviated from the above trend. This suggests that SOD mRNA expression can be used as a biomarker under defined conditions of nitrofurazone concentration and duration of exposure. For example, when nitrofurazone concentration is less than 50 mg l^{-1} and exposure duration is less than 18 h, toxicological effects of nitrofurazone can be assessed by SOD mRNA expression in *E. vannus*.

GPx mRNA expression presented a typical bell-shaped curve with increasing concentrations of nitrofurazone (Fig. 4). Both the peak value (Max) of mRNA expression and the equivalent nitrofurazone concentrations (N_c) decreased with increasing durations of exposure (Fig. 4, E). Deng et al. (2012) proposed that the piecewise function can be used for fitting the biphasic dose-response curves obtained from chronic toxicity assays. Therefore, based on Max and N_c , each biphasic dose-response curve of GPx mRNA expression can be divided into two monomial curves by piecewise function, which will be more operable in practice. In agreement with previous observations (Calabrese and Baldwin, 2001), both biphasic and linear dose-response relationships are found in the test biological responses to nitrofurazone. Moreover, whether the biological responses are non-monotonic or monotonic partly depends on the concentration range of the test contaminant (Calabrese and Baldwin, 2001). Therefore, the problem that arises in biomarker use is that identical biological responses can be detected in test organisms at different concentrations of toxin. Such problems can largely be resolved by using piecewise function where the binomial curves are split into two monomial lines (Deng et al., 2012). A key challenge is how to evaluate the biological responses near the specific nitrofurazone dose where these responses peaked. In order to do this, biological responses for a narrower dose range of nitrofurazone that covers the above mentioned dose point should be investigated.

These findings demonstrate the potential of antioxidant enzymes, in particular GPx activity, as candidate biomarkers of nitrofurazone-induced toxicity. This does not mean, however, that GPx activity should be adopted uniquely. On the contrary, a battery of biomarkers coupled with chemical analyses should be used since this approach has been shown to provide the most comprehensive indication of ecosystem health (Solé, 2002; Hagger et al., 2006). By using a suite of biomarkers, a weight-of-evidence approach can be adopted thereby minimizing the influence of natural variation and allowing the accurate assessment of environmental quality (Hagger et al., 2006). Furthermore, a suite of biomarkers at different levels of biological organization facilitates a better understanding both of the biological processes affected and of the mechanism of toxicity, allowing bio-monitoring programs to be optimized. We therefore suggest that, within a battery of biomarkers, those presenting linear dose-response patterns should be given the greatest weight while others should be weighted less heavily.

Inconsistency of response patterns commonly occurs between biomarkers from different biological levels or components in identical systems, thus leading to equivocality in the correlations (Zheng et al., 2013; Hong et al., 2015b; Vidal-Liñán et al., 2016). Such phenomena occurred in the present study making the application of biomarkers more complicated. However, biological response patterns, particularly those that are non-linear, can be divided into several linear patterns by piecewise function based on specific parameters (e.g. Max and N_c of GPx mRNA expression). This allows significant correlations, and dose-response trends of the biomarkers, to be determined.

5. Conclusions

The results of the present study support the potential of the antioxidant enzymes SOD and GPx, and in particular levels of activity and mRNA gene expression, as candidate biomarkers of nitrofurazone-induced toxicity in aquaculture systems. Dynamic model analyses showed that GPx activity was the most reliable biomarker because of its linear dose-response. Other measures of antioxidant enzyme response, i.e. GPx mRNA expression and SOD activity, are useful but should be less heavily weighted as their responses are more complex. Furthermore, in order to optimize the utility of these biomarkers, we suggest that non-linear dose-response data (e.g. Max and N_c of GPx mRNA expression) should be divided by piecewise function based on the specific dose of nitrofurazone. However, the interactive effects between different antioxidant enzymes need to be further investigated during the biological process of detoxification in order to gain a better understanding of dose-response dynamics.

Conflict of interest

None declared.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ecoenv.2017.06.069>.

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