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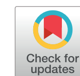
First insights into oxidative stress and theoretical environmental risk of Bronopol and Detarox® AP, two biocides claimed to be ecofriendly for a sustainable aquaculture



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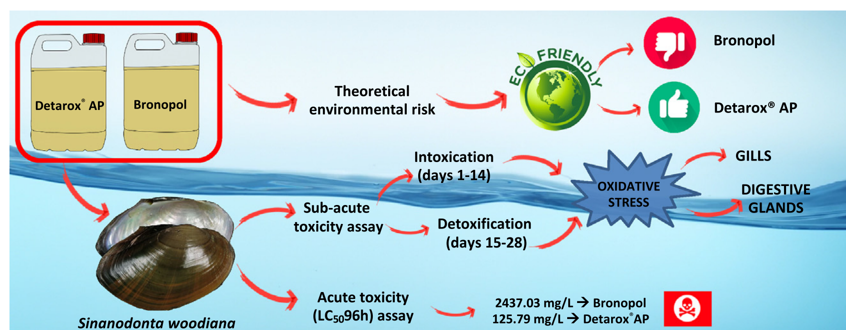
HIGHLIGHTS

- Acute and sublethal toxicity of Bronopol and Detarox®AP were assessed in *S. woodiana*.
- 96 h LC_{50} of Bronopol is higher than Detarox® AP.
- Both biocides exert weak oxidative pressure in digestive gland and gills.
- Environmental risk assessment shows a more muted risk for Detarox® AP than Bronopol.
- A greater eco-sustainability of Detarox® AP compared to Bronopol is suggested.

GRAPHICAL ABSTRACT



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ABSTRACT

Bronopol and Detarox® AP are broad spectrum antimicrobial biocides of growing interest for the aquaculture sector. While their effectiveness against aquatic pathogens has been demonstrated, toxicity data on wild or farmed species are still lacking, as is information on their potential environmental risk for aquatic ecosystems. With this study, we assessed the acute and sublethal toxicity of Bronopol and Detarox® AP in the freshwater bivalve *Sinanodonta woodiana* and their theoretical risk for aquatic ecosystem. The 96-h median lethal concentration (LC_{50}) was determined using the acute toxicity test, while for the sublethal toxicity test the bivalves were exposed to two concentrations for 14 days of Bronopol (2.5 and 50 mg/L) and Detarox® AP (1.11 and 22.26 mg/L) followed by a 14-day withdrawal period. Biocide-mediated oxidative processes were investigated via a panel of oxidative stress biomarkers (malondialdehyde, superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase). Theoretical environmental risk assessment of both biocides, with predicted concentration of no effect (PNEC), expected theoretical concentration (TEC) in the environment, and risk quotient (RQ) was performed. TEC was calculated using a model based on the size of the aquaculture facility and the receiving basin, the estimated quantity of biocide dissolved in water, and published data on biocide stability in water. Although the LC_{50} was higher for Bronopol (2440 mg/L) than for Detarox® AP (126 mg/L), fluctuations

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in oxidative stress biomarkers levels indicated that both biocides exert a slight oxidative pressure on *S. woodiana*. Theoretical environmental risk assessment suggested a muted risk with Detarox® AP and greater eco-sustainability compared to Bronopol.

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1. Introduction

There is a mounting interest by multiple food industries in the use of biocides for water treatment. Water disinfection in aquaculture - the fastest growing agri-food sector – has become increasingly important (FAO, 2020). Furthermore, it has been identified as a potential solution to meeting the food demand by the human population (60% estimated global growth by 2050) (Engle et al., 2017). Keeping breeding pathologies under control holds the primary economic importance to avert losses due to the high fish density and narrow spaces typical of intensive aquaculture that are predisposed to the development of disease and the transmission of pathogens (Marchand et al., 2012). To date, no official European guidelines on the use of disinfectants in aquaculture have been issued. Widely used by virtue of their effectiveness, biocides such as chlorine dioxide (ClO_2), sodium hypochlorite (NaClO), malachite green, and formalin have been found toxic for farmed and wild fish (Elia et al., 2006, 2008; Hu et al., 2019; Marchand et al., 2012; Pedersen et al., 2012; Souza et al., 2020; Villarini et al., 2011). The search for alternative biocides that are both effective and ecofriendly is a key focus of scientific research. A broad-spectrum antimicrobial biocide currently holding great interest is Bronopol (2-bromo-2-nitro-1,3-propanediol), an organic compound soluble in water used in industrial processes (Cui et al., 2011). Its efficacy against bacterial and fungal infections, *Saprolegnia* spp. in particular, was demonstrated in rainbow trout (*Oncorhynchus mykiss*) and salmonid eggs (Branson, 2002; Sudova et al., 2007). However, it was found toxic for phyto- and zooplankton (Tedesco et al., 2018) in the water due to rapid degradation into toxic persistent by-products (e.g., 2-bromo-2-nitroethanol, bromonitromethane, tri (hydroxymethyl) nitromethane, nitromethane, 2-bromoethanol, formaldehyde) (Cui et al., 2011). Though no data on the oxidative stress driven by Bronopol on aquatic organisms are available.

Other biocides of great interest are the peroxides, in particular hydrogen peroxide (H_2O_2), peracetic acid (CH_3COOOH), and their mixtures. Due to their chemical composition, H_2O_2 and CH_3COOOH can be considered environmentally non-toxic substances in theory. Many formulations are available; however, their effectiveness and stability depend closely on the formulation ratio (Straus and Meinelt, 2009; Marchand et al., 2011; Pedersen et al., 2013; Liu et al., 2015). Growth inhibition of the gram-negative bacterium *Flavobacterium columnare*, the fungus *Saprolegnia parasitica*, and the protozoan *Ichthyophthirius multifiliis* occurs at a lower concentration of biocides with a lower molar CH3COOOH:H2O2 ratio (Straus and Meinelt, 2009; Marchand et al., 2011), whereas a higher molar ratio reduces mortality in *Daphnia magna* (Liu et al., 2015). Within this scenario, Detarox® AP has one of the best-balanced formulations (20% hydrogen peroxide, 5% peracetic acid, and 10% acetic acid) (Perdomini-ioc.com, 2017) and so may offer a theoretical compromise between efficacy and safety. Although Detarox® AP inhibited a growth of the fungi *S. parasitica* and *S. delica* (Tedesco et al., 2018) and proved to be efficacious in the treatment of European eels (*Anguilla anguilla*) infested with the protozoan *Trichodina jadranica* (Madsen et al., 2000), its toxicity for aquatic invertebrates has not yet been investigated.

Characterizing the toxicity for non-target organisms and the potential risk of Bronopol and Detarox® AP in aquatic environments is of pivotal importance to preserve the ecological balance of aquatic ecosystems. Oxidative stress biomarkers, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase (GST) enzymes constitute a key defense pathway against the oxidative pressure driven by reactive oxygen species (ROS) in aquatic models (Semedo et al., 2012; Magara et al., 2019; Cui et al., 2020; Dörr et al., 2020; Elia et al., 2019, 2020; Pastorino et al., 2020; Stara et al., 2020). Moreover, the potential risk in aquatic environments is a crucial factor in determining the eco-sustainability of any substance, especially when information on their presence in the environment is lack, as for Bronopol and Detarox® AP.

Environmental risk assessment protocols are based on a set of short-term ecotoxicological studies on three main trophic levels and assessment factors to correct inherent uncertainties (European Commission, 2003). With this study we investigated the acute and sublethal toxicity of Bronopol and Detarox® AP for the freshwater bivalve *Sinanodonta woodiana* by assessing the median lethal concentration and the changes in oxidative stress biomarker levels. Also, the theoretical risk for aquatic ecosystems was determined for both compounds to determine whether they are eco-friendly biocides that ensure environmental safety as claimed.

2. Materials and methods

2.1. Animals

A single lot of adult *S. woodiana* ($N = 450$; average weight [mean ± SD] 99.31 ± 41.80 g, width 33.29 ± 6.07 cm, length 101.23 ± 13.52 cm, height 54.98 ± 9.14 cm) was purchased from a local aquarium dealer, brought to the Experimental Station of the Department of Agricultural, Forest, and Food Sciences (DISAFA), University of Turin (Italy), and acclimated for 2 weeks in nineteen 18-L tanks (equipped with compact circulation pumps) filled with artesian well dechlorinated water (pH 7.5 ± 0.3, dissolved oxygen 8.1 ± 1 mg/L, temperature 18 ± 1 °C) under a regime of 12 h light/12 h dark. After acclimatation, the main water physicochemical parameters (pH, dissolved oxygen, and temperature) were monitored daily. Vessels were randomly distributed during the experiments.

2.2. Chemical preparation and acute toxicity (96 h LC_{50}) assay

Primary stock solutions of Bronopol (32 g/L and 0.5 g/L; purity, 99.81%) and Detarox® AP (20.5 mL/L and 0.6 mL/L) were prepared in the same water for the acute (96 h LC_{50}) and the sublethal toxicity assay. The main safety criteria reported in data sheets of both products include the use of suitable side by side protection glasses, wear clothing that guarantees total protection for the skin, handling with gloves and, if the risk assessment foresees the need of air-purified respirators, use a full-face ventilated system. For the acute toxicity assay, the mussels were exposed to the same acclimation conditions reported above: 160 were placed in eight tanks (20 per tank) and exposed to Bronopol (50, 100, 200, 400, 800, 1600, 3200 mg/L); 120 were placed in six tanks (20 specimens per tank) and exposed to Detarox® AP (47.37, 94.75, 194.24, 388.47, 776.95 mg/L, corresponding to 0.12, 0.25, 0.51, 1.02, 2.05 mL/L, respectively). An unchallenged group was set aside for each biocide. Mortality was checked daily and dead specimens were immediately removed from the tanks. Assays met the test acceptability requirements of ≥90% control survival.

2.3. Sublethal toxicity assay

Sublethal toxicity assay was carried out in order to evaluate for the first time the effects of oxidative stress on mussels following a longer

Bronopol and Detarox® AP exposure rather than the ordinary very short bath treatment, that is the current method of disinfecting farmed fish.

For the sublethal toxicity test, five groups were formed: unchallenged; Bronopol (2.50 and 50 mg/L) and Detarox® AP (1.11 mg/L and 22.26 mg/L, corresponding to 3 and 60 μL/L, respectively). The lower Bronopol concentration was based on prior studies (Branson, 2002; Sudova et al., 2007; Piamsomboon et al., 2013). Bronopol was employed as a fungicidal treatment of freshwater salmonid eggs at 50 mg/L (30 min) for 15 consecutive days (Branson, 2002), and at 20 mg/L for 30 min and 14 consecutive days for treatment against anemic syndrome and bacterial gill disease (Sudova et al., 2007). The concentration of 10 mg/L (2 h, 5 consecutive days) has proven effective against *Pangasianodon hypophthalmus* (Piamsomboon et al., 2013). Since concentration can vary depending on pathogen and disease, and given the very short exposure time per day, we set the lower Bronopol concentration at 2.5 mg/L based on the average biocide concentration (≃ 26.6 mg/L), with a safety factor of 10. Differently, because there is no official information about the use of Detarox® AP in aquaculture, we set the lower concentration according to Wofasteril E400 (2.5 μL/L) for water sanitation in the presence of fish (24 h) (http://svfa-asgp.ch/textes/Kursteil_2_Desinfektionsmittel_IT.pdf). The higher biocide concentration was set 20-fold higher than the lower one and both were lower than 96 h LC_{50} measured in *S. woodiana*. The higher concentration obtained for Bronopol (50 mg/L) is close to the 96 h LC_{50} value for fish, and much higher than that of crustaceans (www.dguv.de/ifa/gestis-database), therefore it could be a challenging concentration for fish and crustacean survival when exposure is prolonged and continuous. However, in *S. woodiana* we obtained much higher 96 h LC_{50} value and no mortality was recorded during the experiment.

A total of 120 specimens of *S. woodiana* were equally distributed in 18-L tanks under the same acclimation conditions. The experiment was conducted with 3 replicates for each experimental group (3 for control and 3 for each biocide concentration) under semistatic conditions for 28 days. Mussels were exposed to the two concentrations of Bronopol (2.50 and 50 mg/L) or Detarox® AP (1.11 mg/L and 22.26 mg/L) for 14 days. After 7 and 14 days of biocides exposure, 2 specimens of control (C) and 2 for each treated group (Bronopol or Detarox® AP) and their replicates were processed, for a total number of 60 specimens. Biocides and tank water were renewed four times during the exposure period corresponding to the two experimental endpoints (days 7 and 14) and halfway between the start of experiment and day 7 and from day 7 to day 14. This experimental approach allow us to mimic a scenario of prolonged biocides exposure thus assessing their suitability in aquaculture and to limit the stressful interferences for mussels following the handling of the tanks. A withdrawal period of 14 days without biocides administration was considered for Bronopol or Detarox® AP-treated mussel and tank water was renewed under same conditions of treatment period. At 7 and 14 withdrawal days, 2 specimens of control (C) and 2 for each treated group and their replicates were sampled from each experimental group carrying out the same biochemical analyses for a total number of 60 specimens. The digestive gland and the gills were collected and stored at −80 °C until biochemical analysis.

2.4. Oxidative stress biomarkers

Levels of lipid peroxidation products (*malondialdehyde*, MDA) were measured in digestive gland and gills tissue, as reported in Elia et al. (2020). Samples were homogenized (1:5 p/v) in TRIS/HCl 20 mM buffer, pH 7.4, and 0.5 M butylated hydroxytoluene (BHT), and then centrifuged at 3000 ×g for 20 min (4 °C). The supernatant was derivatized in 1-methyl-2-phenylindole (10.32 mM in acetonitrile/methanol diluted 3:1), HCl, and dilution buffer (TRIS/HCl, pH 7.4), sample or MDA standard (0–4 μM of 1,1,3,3-tetramethoxypropane). All samples were incubated for 60 min at 45 °C and then centrifuged at 15,000 ×g. MDA content was measured spectrophotometrically at 586 nm. The results are expressed as nanomoles per gram of tissue.

Enzyme analysis of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase (GST) was performed on the cytosolic fractions of the tissues, as described elsewhere (Magara et al., 2018): the tissues (0.2 g) were homogenized (1:10 weight/volume for digestive glands and 1:5 weight/volume for gills) in 100 mM KP buffer, 0.008 TIU mL^{-1} and 0.1 mg mL^{-1}, and then centrifuged at 30,000 ×g for 30 min. Enzyme analysis was performed in triplicate with blanks (buffer and reagents only) on each sample by means of a Varian spectrophotometer (Cary 50 Thermostat Cell Holder) at 25 °C. Total protein concentration in cytosol was determined according to Lowry et al. (1951) and used to normalize enzyme activity. The SOD concentration was measured following reduction of cytochrome *c* by the xanthine/hypoxanthine system at 550 nm using a standard curve of SOD units. One unit of SOD is defined as the amount of enzyme that inhibits 50% of cytochrome *c* reduction. CAT activity was assayed in 100 mM sodium phosphate buffer (pH 7) and 12 mM H_2O_2. Consumption of H_2O_2 was measured as the decrease in absorbance at 240 nm. The activity of GPx was determined in 100 mM of sodium phosphate buffer, pH 7.5, 1 mM of EDTA, 0.12 mM of NADPH, 1 mM of NaN_3, 2 mM of GSH, 0.6 mM of H_2O_2, 1 U of GR, and sample. Oxidation of NADPH was measured at 340 nm. GST activity was measured at 340 nm using 100 mM sodium phosphate buffer, pH 6.5, 1 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and sample.

2.5. Determination of theoretical environmental risk

To determine the theoretical environmental risk of Bronopol and Detarox® AP, the expected theoretical concentration (TEC) of the biocides in the environment and the predicted no-effect concentration (PNEC) were calculated. To calculate the TEC, we considered: 1) the size of the aquaculture facility; 2) the expected quantity of biocides dissolved in water; 3) the amount of water drained every day or every 2, 5, and 10 days; 4) the capacity of the receiving basin; 5) the biocide exposure every day or for a short time once a week; and 6) the stability of the biocides in water. Three increasing scales of aquaculture production at tonnages of 25, 50 and 150, corresponding to small, medium, and large-scale production, were also considered (Rawlinson and Forster, 2000). The expected quantity of biocide dispersed in water was calculated according to the equation:

$$Q_b = t \times 16.9 \times C_b \times 10^6$$

where Q_b is the mg of biocide dispersed in water, t the tonnage, 16.9 the average m^3 of freshwater withdrawal in inland aquaculture per kg production (Bosma and Verdegem, 2011), C_b the concentration of biocide (mg/L), and 10^6 the conversion factor from tonnage to kg and from volume in m^3 to liter.

Since Bronopol and Detarox rapidly dissociate in water, the TEC was calculated based on 2-bromo-2-nitroethanol (BNE) and hydrogen peroxide, the first most toxic degradation products. Three basins measuring 1500, 65,000, and 200,000 km^2, based on Lakew et al. (2019), were entered in the analysis; depths of 200, 700, and 2000 m were arbitrarily assumed for small, medium, and large basins, respectively. The average yearly TEC (TEC_y) was calculated for the daily regimen as follows:

$$TEC_1 = \left(Q_b / \left(A \times h \times 10^6\right)\right)/d$$

$$TEC_y = (TEC_1 - Cdeg_1) + \Sigma\left(\left(TEC_{(n-1)} - Cdeg_{(n-1)} + TEC_1\right) - Cdeg_{(n)}\right)/365$$

where A is the area in km^2 of the three basins, h the basin depth, 10^6 the conversion factor from km^2 to m^2, and d the estimated number of days needed to completely drain and renew the water of aquaculture facilities. Cdeg indicates the estimated concentration of biocide degraded

per day, 1 denotes day one, n every day from two to 365, n-1 the day before, and 365 the number of days in a year.

For the once-a-week regimen, two different formulas were used to calculate the average yearly TEC (TEC_{yw}) based on the estimated persistence of the biocides in the environment (Watts et al., 2007; Cui et al., 2011), which was higher than one week for Bronopol (a) and lower for Detarox (b):

a) $TEC_{yw} = (TEC_1 - Cdeg_1) + \Sigma(((TEC_{(n-1)} - Cdeg_{(n-1)}) \times 7 + TEC_1) - Cdeg_{(n)})/52$
b) $TEC_{yw} = (TEC_1 \times 52) / 365$

where n represents each week from 2 to 52 and n-1 the week before.

PNEC is the concentration beyond which a compound exerts harmful effects on an aquatic environment. For each biocide, the PNEC was calculated starting from the lower median concentration of effects (EC_{50}) on trophic levels after applying an adequate assessment factor, as reported in the Technical Guidance Document of the European Commission (EC) (2003). The most sensitive species of the three main trophic levels (primary producers and primary and secondary consumers) was chosen, assuming that if the most sensitive trophic level is protected, all other aquatic organisms will be protected as well. Since Bronopol rapidly dissociates in field waters to form the more persistent 2-bromo-2-nitroethanol (BNE), the related 24 h EC_{50} on *Chlorella pyrenoidosa* (Cui et al., 2011) was used for risk assessment. The EC_{50} of hydrogen peroxide on algae was selected from the GESTIS Substance Database (http://gestis-en.itrust.de). The risk quotient (RQ) was calculated as the TEC_y (or TEC_{yw})/PNEC ratio. Results of TEC > PNEC indicate an unacceptable environmental risk, TEC = PNEC a potential environmental risk, and TEC < PNEC a low potential environmental risk.

2.6. Statistical analysis

Probit analysis was carried out to determine 96 h LC_{50}. In this statistical approach, a sigmoid concentration-response curve is transformed into a straight line that can be analyzed by regression analysis (Finney, 1952). A formula reported by Singh and Zahra (2017), slightly modified, was applied before determination of probit (see Supplementary Material). Biochemical data are reported as the mean ± standard deviation (SD). Data analysis was performed using GraphPad Prism software. Prior to statistical analysis, the data were evaluated with Bartlett's test for normality of distribution. No statistical significant differences were found for oxidative stress biomarkers; therefore, statistical analysis was performed considering the three replicates as one group. The results are expressed as the mean ± SD. Two-way ANOVA, with concentration, time and concentration and time interaction as the independent variables, followed by Tukey's test, was used to investigate differences between the treated and the unchallenged groups. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Acute toxicity (96 h LC_{50}) assay

During the 96-h period, no mortality was recorded in the unchallenged group for Bronopol and Detarox® AP. Table 1 presents the results of median lethal concentrations following Probit analysis. The $\chi 2$ test ($p < 0.05$) showed that mortality was dependent on the concentration of both biocides. The 96 h LC_{50} of Bronopol and Detarox® AP was 2440 mg/L and 126 mg/L (corresponding to 0.33 mL/L), respectively. Lower and upper levels were 1320 and 4490 mg/L for Bronopol and 103 and 153 mg/L for Detarox® AP (corresponding to 0.27 and 0.40 mL/L). Variance of the mean was 0.019 and 0.002, respectively.

Table 1
Results of Probit analysis based on mortality of Anodonta spp. exposed to Bronopol and Detarox® AP. df = degrees of freedom; LL = lower level; UL = upper level; σ^2 = variance of mean. 95% confidence level.

Compound	df	$\chi 2$	96 h LC_{50}	LL	UL	σ^2
Bronopol (mg/L)	5	2.25	2440	1320	4490	0.019
Detarox® AP (mg/L)	3	5.67	126	103	153	0.002

3.2. Sub-acute toxicity assay and oxidative stress biomarkers

Table S1 (A, B) presents the results of two-way ANOVA of Bronopol and Detarox® AP concentrations, time, and interaction (concentrations × time) (see Supplementary Material).

The water physicochemical parameters were within the optimum range values for *S. woodiana* (Spyra et al., 2012) throughout the duration of the study.

3.2.1. Bronopol

MDA levels in the digestive gland were increased (80%) at 14 treatment days after exposure to the higher Bronopol concentration (Fig. 1A). During treatment, SOD activity was decreased after exposure to both biocide concentrations (30%), whereas at one week after biocide withdrawal enzyme activity was still reduced in the 50 mg/L group (Fig. 1C). Hepatic CAT activity was increased (1.2-fold) after exposure to the higher Bronopol concentration during the experimental period (Fig. 1E), whereas GPx activity was decreased (40%) during biocide withdrawal (Fig. 1G). GST levels were generally reduced (60%) during the experimental period (Fig. 1I).

MDA levels in the gills were increased after 14 post treatment days to the higher Bronopol concentration (1.5-fold, Fig. 1B) and SOD content (50%) at 14 treatment days (Fig. 1D). CAT activity (Fig. 1F) was increased after 7 treatment days (50 mg/L, 80%), 7 post treatment days (both concentrations, up to 1.2-fold), and 14 post treatment days (50 mg/L, one fold). At 7 post treatment days GPx activity was increased after exposure to 50 mg/L Bronopol (Fig. 1H). GST levels (1.5-fold) were also increased (Fig. 1L).

3.2.2. Detarox® AP

The MDA levels in the digestive glands were consistently higher (1.2-fold) in the biocide-exposed mussels during the experiment (Fig. 2A). SOD (Fig. 2C) and GPx (Fig. 2G) activities were increased significantly (2-fold) in mussels exposed to the higher Detarox® AP concentration during the withdrawal period. CAT (Fig. 2E) activity was markedly decreased (40%), before rising significantly at later endpoints (1.5-fold). GST levels (Fig. 2I) were decreased after exposure to the higher biocide concentration (60%), and to both concentrations during the withdrawal period (60%).

The MDA level (1.5-fold) was increased in the gills only at 14 post treatment days in the group exposed to 22.26 mg/L (corresponding to 60 μL/L) (Fig. 2B). SOD (Fig. 2D) and CAT (Fig. 2F) levels were increased at 14 treatment days: 50% for SOD after exposure to the higher Detarox® AP concentration and 40% for CAT after exposure to both concentrations. GPx activity was increased (2-fold) after exposure to 22.26 mg/L (Fig. 2G) during the withdrawal period and at 7 post treatment days together with the lower Detarox® AP concentration (80%). GST activity was increased (80%), except at 14 treatment days, when no significant change between the unchallenged and the biocide-exposed groups was observed (Fig. 2L).

3.3. Theoretical environmental risk

The average TECs for theoretical environmental risk assessment were calculated for each combination of the estimated water drainage time and size (small, medium, large) of aquaculture facility and of the receiving basin. Tables 2A and 3A and Fig. 3 present the RQ based on the average TECs and PNEC of the Bronopol by-product (2-bromo-2-nitroethanol). In the daily regimen scenario, the RQ was consistently higher than the threshold level for the higher biocide concentration,

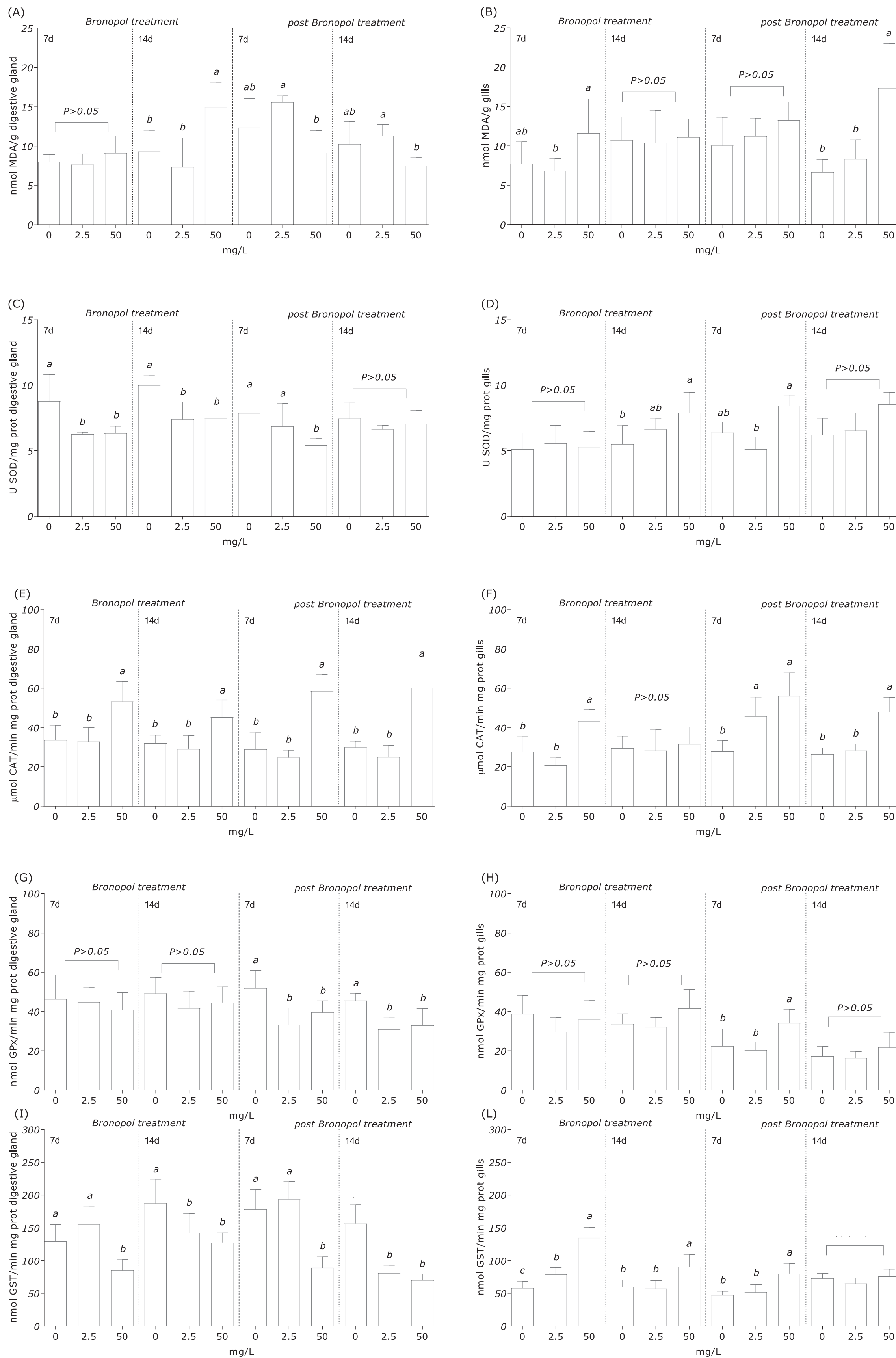

Fig. 1. Oxidative stress biomarkers in digestive gland (A, C, E, G, I) and gills (B, D, F, H, L) of *Sinanodonta woodiana* exposed to 2.5 and 50 mg/L Bronopol. Letters denote significant statistical differences between the unchallenged and the treated groups (Tukey multiple comparison test $p < 0.05$). Malondialdehyde (MDA; A, B); Superoxide dismutase (SOD; C, D); Catalase (CAT; E, F); Glutathione peroxidase (GPx; G, H); Glutathione S-transferase (GST; I, L). Bronopol treatment (7 and 14 days); post Bronopol treatment (7 and 14-days withdrawal period).

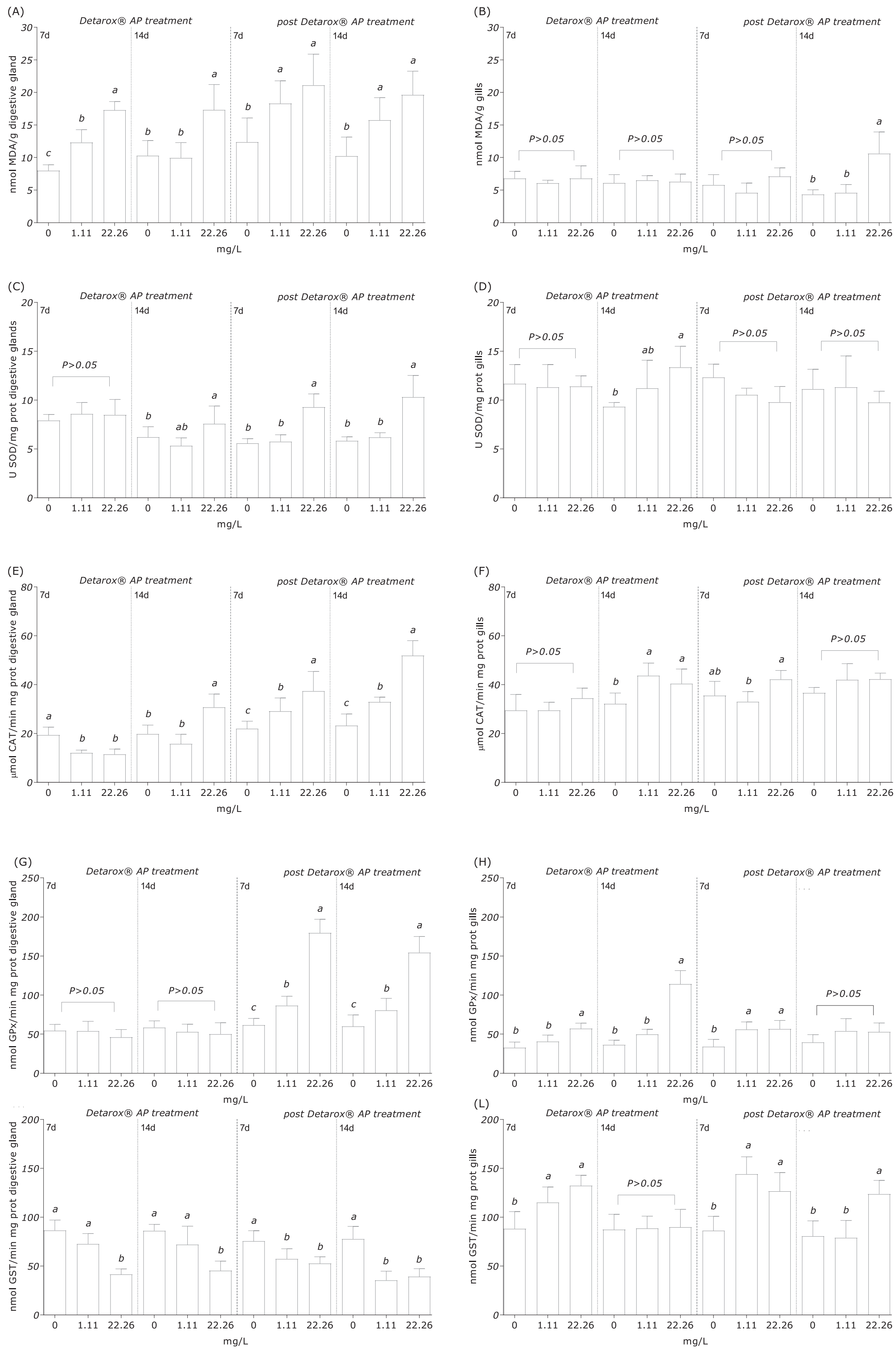

Fig. 2. Oxidative stress biomarkers in digestive gland (A, C, E, G, I) and gills (B, D, F, H, L) of *Sinanodonta woodiana* exposed to 1.11 and 22.26 mg/L (corresponding to 3 and 60 μL/L) Detarox® AP. Letters denote significant statistical differences between the unchallenged and the treated groups (Tukey multiple comparison test $p < 0.05$). Malondialdehyde (MDA; A, B); Superoxide dismutase (SOD; C, D); Catalase (CAT; E, F); Glutathione peroxidase (GPx; G, H); Glutathione S-transferase (GST; I, L). Detarox® AP treatment (7 and 14 days); post Detarox® AP treatment (7 and 14-days withdrawal period).

Table 2
Risk quotient (RQ) of (A) 2-bromo-2-nitroethanol as Bronopol *by-product* (2.5 and 50 mg/L) and (B) H_2O_2 as Detarox® AP *by-product* (1.11 and 22.26 mg/L) released daily for one year in three basins (1500, 65,000 and 200,000 km^2) by small, medium and large-size aquaculture facilities (25, 50 and 100 tonnage of production). A period of 1, 2, 5 and 10 days was considered to completely drain and renew the water of the aquaculture facilities.

A) RQ of 2-bromo-2-nitroethanol as Bronopol *by-product*

		Small-size facility (25 t)				Medium-size facility (50 t)				Large-size facility (150 t)			
	mg/L	1d	2d	5d	10d	1d	2d	5d	10d	1d	2d	5d	10d
SB	2.5	2.7×10^2	1.4×10^2	55.4460	27.7230	5.5×10^2	2.8×10^2	1.1×10^2	55.4460	1.6×10^3	8.3×10^2	3.3×10^2	1.7×10^2
	50	5.54×10^3	2.7×10^3	1.1×10^3	5.5×10^2	1.1×10^4	5.5×10^3	2.2×10^3	1.1×10^3	3.3×10^4	1.6×10^4	6.6×10^3	3.3×10^3
MB	2.5	1.8279	0.9139	0.3656	0.1828	3.6558	1.8279	0.7311	0.3656	10.9673	5.4837	2.1935	1.0967
	50	36.5578	18.2789	7.3115	3.6558	73.1156	36.5578	14.6231	7.3116	2.2×10^2	1.1×10^2	43.8693	21.9347
LB	2.5	0.2079	0.1039	0.0416	0.0208	0.4158	0.2079	0.0832	0.0416	1.2475	0.6238	0.2495	0.1247
	50	4.1584	2.0792	0.8317	0.4158	8.3169	4.1584	1.6634	0.8317	24.9507	12.4753	4.9901	2.4951

B) RQ of H_2O_2 as Detarox® AP *by-product*

		Small-size facility (25 t)				Medium-size facility (50 t)				Large-size facility (150 t)			
	mg/L (μL/L)	1d	2d	5d	10d	1d	2d	5d	10d	1d	2d	5d	10d
SB	1.11 (3)	0.4937	0.2469	0.0987	0.0494	0.9874	0.4937	0.1975	0.0987	2.9623	1.4812	0.5925	0.2962
	22.26 (60)	9.9023	4.9512	1.9805	0.9902	19.8046	9.9023	3.9609	1.9805	59.4140	29.7070	11.8828	5.9414
MB	1.11 (3)	0.0032	0.0016	0.0006	0.0003	0.0065	0.0032	0.0013	0.0006	0.0195	0.0098	0.0039	0.0019
	22.26 (60)	0.0653	0.0326	0.1306	0.0065	0.1306	0.0653	0.0261	0.0130	0.3917	0.1959	0.0783	0.0392
LB	1.11 (3)	0.0004	0.0002	7.4×10^{-5}	3.7×10^{-5}	0.0007	0.0004	0.0001	7.4×10^{-5}	0.0022	0.0011	0.0004	0.0002
	22.26 (60)	0.0074	0.0037	0.0015	0.0007	0.01485	0.0074	0.0030	0.0015	0.0445	0.0223	0.0089	0.0044

Small basin (LB); Medium Basin (MB); Large Basin (LB).

with a minimum of 1.66 (large basin × medium-size aquaculture facility) and a maximum of 3.3×10^4 (small basin × large-size facility). Acceptable RQs were sporadically obtained for the large basin and a longer time period to completely drain the water from the aquaculture systems. Alarmingly high values were still observed with the lower biocide concentration, when the small and the medium basins were analyzed. In the once-a-week regimen, RQs < 1 were observed only with the lower biocide concentration and the medium or the large basin.

The TECs of H_2O_2 produced by both concentrations of Detarox® AP were generally lower than the PNEC derivatized from the EC_{50} measured on algae (http://gestis-en.itrust.de), particularly after assessing the lower biocide concentration (Table 2B and Table 3B). In the daily regimen scenario, RQ was >1 mainly for 22.26 mg/L (corresponding to 60 μL/L) Detarox® AP in the small basin, with a minimum of 1.98 (small basin × small-size facility) and a maximum of 59.4 (small basin x large-size facility). RQ <1 was often found for the medium and large basins (0.0007–0.39). Differently, the RQ of Detarox® AP 1.11 mg/L (corresponding to 3 μL/L) was <1 in almost all cases, except for the small basin x large-size aquaculture facility (up to 2.96), in which water drainage was rapid. In the once-a-week regimen scenario, RQ was elevated for the small basin in medium and large-size facilities (up to 5.10).

Table 3
risk quotient (RQ) of (A) 2-bromo-2-nitroethanol as Bronopol (2.5 and 50 mg/L) *by-product* and H_2O_2 as Detarox® AP *by-product* (1.11 and 22.26 mg/L) (B) released once-a-week for one year in three basins (1500, 65,000 and 200,000 km^2) by small, medium and large-size aquaculture facilities (25, 50 and 100 tonnage of production).

A) RQ of 2-bromo-2-nitroethanol as Bronopol *by-product*

	Bronopol 2.5 mg/L			Bronopol 50 mg/L		
	25 t	50 t	150 t	25 t	50 t	150 t
SB	37.9960	75.9919	2.27×10^2	7.6×10^2	1.5×10^3	4.5×10^4
MB	0.2505	0.5010	1.5031	5.0104	10.0209	30.0627
LB	0.02850	0.0570	0.1710	0.5699	1.1399	3.4196

B) RQ of H_2O_2 as Detarox® AP *by-product*

	Detarox® AP 1.11 mg/L (3 μL/L)			Detarox® AP 22.26 mg/L (60 μL/L)		
	25 t	50 t	150 t	25 t	50 t	150 t
SB	0.0424	0.0847	0.2544	0.8503	1.7006	5.1020
MB	0.0003	0.0005	0.0017	0.0056	0.0112	0.0336
LB	3.17×10^{-5}	6.36×10^{-5}	0.0001	0.0006	0.0013	0.0038

Small basin (LB); Medium Basin (MB); Large Basin (LB).

4. Discussion

In this study we measured the toxic effects of Bronopol and Detarox® AP on *S. woodiana* and their theoretical environmental risk in order to determine the biological and environmental safety of these two novel biocides claimed to be eco-friendly. Chinese pond mussel (*Sinanodonta woodiana*) was chosen as the model for acute and sublethal assays because it is a bioindicator of water pollution due to its sedentary life, feeding by filtration, and ability to easily accumulate pollutants. Moreover, it represents an important economic resource for many non-European countries (Falfushynska et al., 2013).

Although the 96 h median lethal concentration of Bronopol has been determined for various fish species (Novartis Report, 2006; Chaturvedi et al., 2012), no data on mussels are available, neither is there information on acute toxicity for Detarox® AP. The only information for Detarox® AP is the safety data sheet, which describes each component of the mixture (https://docplayer.it/53699415-Scheda-di-sicurezza-detarox-ap.html). In this scenario, determination of 96 h LC_{50} of both biocides on *S. woodiana* is crucial to gain knowledge on their toxicity and to fill the present gap in the literature. Finney's probit analysis showed that the 96 h LC_{50} was 2440 mg/L for Bronopol and 126 mg/L (corresponding to 0.33 mL/L) for Detarox® AP. A previous study on rainbow trout (*O. mykiss*) reported a 96 h LC_{50} for Bronopol 20 mg/L (Novartis Report, 2006), whereas Chaturvedi et al. (2012) found values of 25.20 mg/L, 30.50 mg/L, and 2.10 mg/L for freshwater fish *C. carpio*, *Colisa fasciatus* and *Heteropneustes fossilis* exposed to Pyceze™ (commercial name of Bronopol). In this context, the 96 h LC_{50} of Bronopol for *S. woodiana* was at least 100-fold higher than that measured in fish (Novartis Report, 2006; Chaturvedi et al., 2012), suggesting a higher tolerance of this mussel species for the biocide. A similar outcome was observed for the peroxide-based biocide, which exerted 50% mortality at a more than 10-fold higher concentration compared to single 35% *v*/v H_2O_2 at the same time point in fish (https://docplayer.it/53699415-Scheda-di-sicurezza-detarox-ap.html).

Despite the tolerance of Chinese pond mussel to biocides suggested by the acute toxicity assay, fluctuations in oxidative stress biomarkers in the digestive gland and gills indicated that Bronopol and Detarox® AP

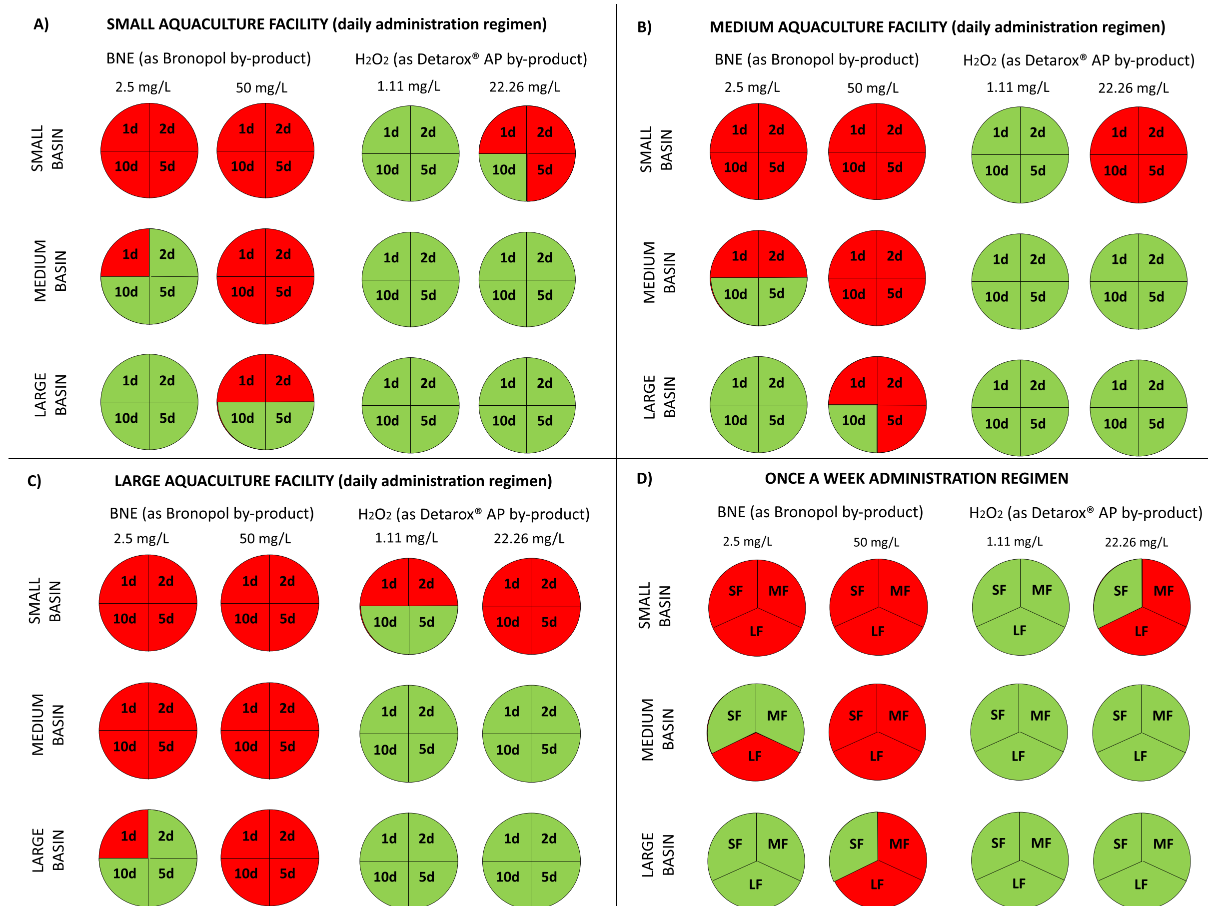

Fig. 3. Graphical representation of risk quotient (RQ) of BNE as Bronopol by-product (2.5 and 50 mg/L) and H2O2 as Detarox® AP by-product (1.11 and 22.26 mg/L) released daily (A, B, C) or once a week (D) for one year in three basins (1500, 65,000 and 200,000 km2) by small, medium and large-size aquaculture facilities (25, 50 and 100 tonnage of production). A period of 1, 2, 5 and 10 days was considered to completely drain and renew the water of the aquaculture facilities. SF = small-size aquaculture facility; MF = medium-size aquaculture facility; LF = large-size aquaculture facility. Green color indicates values of RQ ≤ 1. Red color indicated values of RQ > 1.

can exert a slight oxidative pressure on this freshwater species. Respiratory and hepatic tissues have a high potential for ROS production, which is offset by protective mechanisms in aquatic organisms (Livingstone et al., 1992; Elia et al., 2006, 2010; Pastorino et al., 2020). Severe response was observed mainly in the digestive gland following exposure to both biocides and even during the 14 withdrawal period. Bronopol quickly degrades in water, where it forms toxic, persistent by-products (2-bromo-2-nitroethanol, bromonitromethane, tri(hydroxymethyl)nitromethane, nitromethane, 2-bromoethanol, formaldehyde) (Cui et al., 2011), which can cause early and late impairment of the antioxidant pathway. Previous studies showed higher hepatic MDA levels in *Mytilus galloprovincialis* exposed for 10 days to 1 mg/L quaternium-15, a strong formaldehyde releaser preservative (Faggio et al., 2016; Pagano et al., 2016), and elevated ROS and MDA production in mouse liver treated with formaldehyde (Liu et al., 2014a, Ye et al., 2013).

Moreover, levels of thiobarbituric acid reactive substances (TBARS) generated during oxidative stress were increased after a formalin bath at 0.2 mL/L for 20 min in rainbow trout *O. mykiss* (Tkachenko and Grudniewska, 2016). The results we obtained in the digestive gland of *S. woodiana* were similar to those reported above, suggesting a strong correlation between lipid peroxidation and the formaldehyde by-products. However, the antioxidant biomarker response differed from those reported in mussels and fish treated with formaldehyde releaser compounds (Faggio et al., 2016; Hodkovicova et al., 2019). Bronopol at both concentrations led to decreased SOD activity in the digestive gland, contrasting with published data on *M. galloprovincialis* (Faggio et al., 2016). Unfortunately, it is difficult to delineate a specific mechanism of action of Bronopol because of the lack of studies on oxidative stress the biocide or its by-products exert. Nonetheless, based on the severe effects on SOD activity we observed compared to the milder ones previously reported in aquatic organisms exposed only to formaldehyde, synergistic effects between the by-products cannot be excluded. CAT activity was consistently higher than for the unchallenged group in both tissues, especially in the digestive gland. This result was unexpected, since the SOD levels were generally lower or unchanged in the digestive gland and the gills, respectively. This outcome suggests a SOD-independent route of hydrogen peroxide production, the substrate of catalase. A mechanistic study showed that formaldehyde is involved in the inhibition of complex I of the mitochondrial respiratory chain (Teng et al., 2001) and thus in the regeneration of ATP coupled to O_2 reduction to water (Ott et al., 2007). The defective reduction in O_2 may have increased ROS generation, resulting in an overproduction of H_2O_2. Moreover, formaldehyde can reduce GSH levels (Teng et al., 2001), one of the most potent antioxidant molecules in living organisms. This may explain the drop in hepatic GPx activity during the withdrawal period or the consistently lower GST levels in digestive gland during the experimental period. While the results of GPx were in line with those reported for rainbow trout exposed to formalin (Ispir et al., 2017), those obtained for GST were in contrast with the previous study and showed no changes in activity for common carp *C. carpio* (Hodkovicova et al., 2019) or increased enzyme levels in silver pomfret *Pampus argenteus* (Hu et al., 2019). The higher GST activity in the gills recorded during biocide treatment and until 7 post treatment days suggests and efficient protective role against lipid peroxidation. However,

the return to the levels noted for the unchallenged group during the last experimental week may have contributed to the significantly higher MDA levels measured in the gills at the end of the experiment (14 post treatment days, 50 mg/L).

MDA levels were consistently higher in the Detarox® AP-exposed mussels than in unchallenged group, indicating a high concentration of hydroxyl anion (-OH•) delivered by an excess of hydrogen peroxide via the Fenton reaction. The enzyme levels of were raised during the experimental period. At day 7, however, a transient decrease in CAT activity was measured in the mussels exposed to both biocide concentrations. This observation is shared by previous results for SOD and CAT levels in the cardiac tissue of rainbow trout *O. mykiss* after peracetic acid treatment (Tkachenko et al., 2013). The reduction in this pivotal enzymatic shield against oxidative pressure could have been due to the peracetic acid in the Detarox® AP formulation. Mechanistic studies showed that peracetic acid and H_2O_2 decay following first order kinetics (Pedersen et al., 2009), despite the different routes involved. Chemical oxidation is bindweed in the degradation of peracetic acid, in contrast to the enzymatic removal of H_2O_2 by catalase, as reported for bacteria (Block, 1991). Moreover, the amount of organic matter can play a key role in the degradation of peracetic acid, as reported by Pedersen et al. (2009). In their study, decay was significantly reduced with the increase in fish stocking density, following half-life constants of 4.6 and 1.7 h at 12 and 63 kg/m^3, respectively (Pedersen et al., 2009). This was a crucial point when the treatment with peracetic acid and hydrogen peroxide was combined, showing that, under conditions of a low organic component, the degradation of H_2O_2 was significantly reduced. This outcome suggests that peracetic acid is able to inactivate microbial catalase activity (Pedersen et al., 2009). In the present study, because the biomass of *S. woodiana* per liter was far below that reported by Pedersen et al. (2009), we may assume that the half-life of peracetic acid was not affected by the organic matter of mussels. It is likely that peracetic acid can inactivate CAT activity also in *S. woodiana*, ensuring the longer stability of Detarox® AP. This hypothesis is also supported by the results noted for hepatic GST. Although the enzyme levels in the digestive gland were generally raised, GST activity in the Detarox®-treated groups was consistently lower than in the unchallenged group. This outcome was unexpected, since increased GST activity was previously reported in fish exposed to peracetic acid (Elia et al., 2006; Maurício et al., 2020). To our best knowledge, no information is available on the effect of H_2O_2 on enzyme activity in mussels or fish; however, a mechanistic study in the rat found that hydrogen peroxide can boost GST activity (Aniya and Anders, 1992). In the present study, it is possible that the lower GST activity in the digestive gland may have been due to a synergistic effect between H_2O_2 and peracetic acid. The impaired enzyme functionality can be considered both a positive and a negative event. As a phase II enzyme, the lower levels measured in the treated groups compared to the unchallenged group indicate that GST, together with CAT, may control the fate of Detarox® AP and so prolong its stability. GST is also involved in the modulation of the innate immune system against bacterial infection in mussels (Wang et al., 2013). The immune response can be weakened and the defensive shield against pathogens maintained only by virtue of the effectiveness of Detarox® AP. From 7 post treatment days onwards, antioxidant enzyme activity was significantly increased in a time-dose dependent manner. This can be related to the delay of peracetic acid degradation in the tissues during biocide treatment. Following the cleavage of peracetic acid, acetic acid and H_2O_2 are produced, maintaining the elevated concentration of hydrogen peroxide longer. Furthermore, when peracetic acid cleavage is delayed, its ability to slow CAT activity fails and the enzyme may be induced, together with GPx, to metabolize the peroxide. According to findings by Elia et al. (2006, 2008) for chlorine dioxide, sodium hypochlorite, and peracetic acid, it is possible that the rise in the antioxidant enzyme levels following exposure to Detarox® AP protect the animal from the oxidative damage induced by the disinfectant. Furthermore, it could also provide a support pathway for immune defense still weakened by the prolonged low GST levels.

Determination of RQ, i.e., the ratio between the expected concentration of a xenobiotic in environment and its PNEC for biota, is one of the most useful and informative approaches to assess the potential risk in aquatic ecosystems (Papageorgiou et al., 2016). In the present study, TEC values were calculated taking several factors into account, among which biocide stability played a crucial role. Bronopol is unstable in field water and can dissociate in just a few hours (Cui et al., 2011). The first by-product is 2-bromo-2-nitroethanol (BNE), a much more toxic compound than Bronopol. The 24 h EC_{50} of BNE on algae *C. pyrenoidosa* was 4.76 μmol/L, 6-fold higher than the 28.80 μmol/L of the precursor biocide. Furthermore, it is able to persist for up to 100 days in aquatic environments (Cui et al., 2011). For this reason, BNE has been used as a model to assess the theoretical environmental risk due to Bronopol environmental exposure. Information on the stability of Detarox® AP in water is lacking. Liu et al. (2014b) reported that decomposition of commercial products based on peracetic acid and hydrogen peroxide, such as Wofasteril® E400, E250, and Lspez, depends on the ratio of peracetic acid and hydrogen peroxide. Wofasteril® Lspez, which contains 3% peracetic acid, resulted the least stable though the general degradation period of peracetic acid of all three compounds was only a few hours. Accordingly, we assumed that also Detarox® AP can degrade within a few hours and that peracetic acid releases H_2O_2. In aquatic environments, hydrogen peroxide can be quickly transformed into H_2O, while in the presence of other contaminants it can remain stable for up to 32 h (Watts et al., 2007). The environmental risk assessment of Detarox® AP was thus based on the H_2O_2 produced after degradation. According to the Technical Guidance Document of the European Commission (EC) (2003), the PNEC can be derivatized from both acute or chronic toxicity tests, considering that the data obtained from longer-period assays, like the no observed effect concentration (NOEC), require a lower assessment factor. And although long-term parameters may seem preferable for obtaining a more realistic environmental risk assessment, previous studies have pointed out that this kind of data are often scanty and prone to high uncertainty (Moore and Caux, 1997; Musee, 2018; Riva et al., 2019). For this study, PNEC was derivatized from EC_{50}, in order to build a solid dataset that could ensure reliable evaluation of the potential aquatic risk for Bronopol and Detarox® AP. According to the GESTIS Substance Dataset, the EC_{50} of hydrogen peroxide is 24.4 mg/L (96 h) in fish, 13.2 mg/L (48 h) in crustaceans and 3.36 mg/L (72 h) in algae (http://gestis-en.itrust.de).

Acute toxicity data of BNE for aquatic species have been reported only by Cui et al. (2011) on algae. Although evaluation of environmental risk requires the EC_{50} of at least one species for all the three main trophic levels and bases the assessment on the lower acute toxicity value, we used this data and applied an assessment factor of 1000. Algae have ecological attributes and are at the base of the trophic chain in aquatic ecosystems (Stevenson and Rollins, 2017). Assessment of the potential toxic effects driven by xenobiotics and the related ecological risk on algal populations is of pivotal importance, since an imbalance in the community of producers can dramatically disrupt the entire aquatic food-web. The RQ calculated for both Bronopol and Detarox® AP indicates that the environmental risk of the two biocides is closely related to the geomorphological characteristics of the receiving basin and the size of the aquaculture facility. Neither compound seems to have environmental issues when used in large-size basins. Problems arise for medium and small basins. In this context, Detarox® AP, especially at the lower concentration, may be the safer biocide for aquatic environments than Bronopo, due to the lower and less frequent higher RQ. Furthermore, its use may be more advisable in small and medium-sized aquaculture plants, which are the most common worldwide due to the economically advantageous cost/production ratio (Rawlinson and Forster, 2000). Our results suggest that biocide choice and appropriate concentrations should match both hygiene and eco-sustainability criteria according to the frequency and the duration of administration and the size of the aquaculture facility and receiving basin. This is especially crucial for Bronopol, which is used at a concentration of 50 mg/L as

a fungicidal for freshwater salmonid eggs (Branson, 2002). The RQ calculated for the same concentration indicates that Bronopol could have a potential for environmental risk, even with once-a-week regimens instead of the recommended 15 consecutive days (Branson, 2002).

5. Conclusions

Acute toxicity testing showed a higher median lethal concentration for Bronopol than Detarox® AP in *S. woodiana*. Sublethal exposure to both biocides resulted in changes in oxidative stress biomarker levels, suggesting a slight oxidative pressure. Environmental risk assessment based on primary producer algae showed a muted risk following the predicted occurrence in water of Detarox® AP. Detarox® AP could provide a more ecofriendly alternative compared to Bronopol and other biocides currently used in aquaculture. The study findings give initial insights into oxidative stress and the aquatic risk of two biocides claimed to be eco-friendly. Further studies investigating the toxicity and environmental sustainability of the compounds are needed.

CRediT authorship contribution statement

Gabriele Magara: Investigation, Methodology, Writing – original draft. **Akkarasiri Sangsawang:** Investigation, Methodology, Writing – review & editing. **Paolo Pastorino:** Data curation, Writing – review & editing. **Sara Bellezza Oddon:** Methodology. **Barbara Caldaroni:** Data curation, Methodology. **Vasco Menconi:** Methodology. **Uthaiwan Kovitvadhi:** Funding acquisition, Writing – review & editing. **Laura Gasco:** Methodology, Supervision, Writing – review & editing. **Daniela Meloni:** Funding acquisition, Writing – review & editing. **Ambrosius Josef Martin Dörr:** Methodology, Writing – review & editing. **Marino Prearo:** Conceptualization, Writing – review & editing. **Ermanno Federici:** Funding acquisition, Writing – review & editing. **Antonia Concetta Elia:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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