Chemosphere 309 (2022) 136736

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Gone with sunscreens: Responses of blue mussels (*Mytilus edulis*) to a wide concentration range of a UV filter ensulizole

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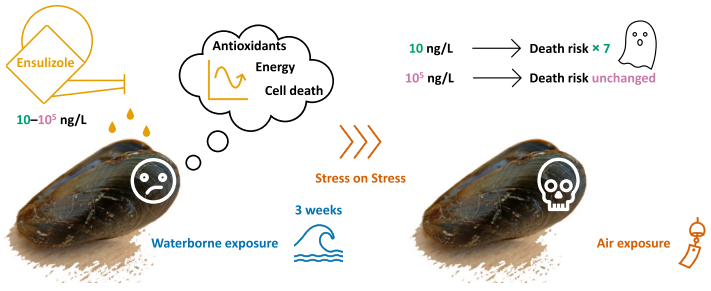
HIGHLIGHTS

- Ecotoxicological evaluation of sunscreen agent ensulizole in mussels.
- Ensulizole reduces survival in air at environmentally relevant concentrations.
- Ensulizole causes subcellular effects in the gills and digestive gland.
- Ensulizole induces non-monotonic responses.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
PBSA
SoS response
Oxidative stress
Bioenergetics
Cell signaling
Non-monotonic response

ABSTRACT

Organic UV filters have emerged as a new threat to marine organisms, but ecotoxicological studies have so far focused on only a few substances despite the chemical diversity of these synthetic sunscreen agents. Here we examined the responses of blue mussels *Mytilus edulis* to ensulizole, a non-lipophilic UV filter commonly found in the Baltic Sea. Mussels were exposed for three weeks to five ensulizole concentrations of 10, 10^2, 10^3, 10^4, and 10^5 ng/L. Stress on stress response was evaluated by subjecting mussels to air exposure. A battery of biomarkers related to detoxification and antioxidant defense, oxidative stress damage, energy reserves and metabolism, autophagy, apoptosis, inflammation, and DNA damage was measured in the gills and the digestive gland. In general, ensulizole affected the antioxidant response, energy storage, and cell death-related processes in mussel tissues. Mussels exposed to low, environmentally relevant concentrations of ensulizole had a shorter air survival time than the control. Ensulizole often showed the non-monotonic concentration-response curves, suggesting the complex effects of this UV filter at molecular, biochemical, and organismal levels.

1. Introduction

Following increased public awareness of the risk of sun overexposure, the use of sunscreen has risen steadily in recent decades (Devos et al., 2012; Ghiasvand et al., 2015; Heerfordt et al., 2017; Peacey et al., 2006). The sunscreen protection against harmful

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https://doi.org/10.1016/j.chemosphere.2022.136736
Received 2 August 2022; Received in revised form 27 September 2022; Accepted 1 October 2022
Available online 6 October 2022

ultraviolet (UV) radiation relies on its constituent UV filters including inorganic TiO_2 and ZnO nanoparticles and various organic molecules (Osterwalder et al., 2014). These UV filters are directly introduced to recreational waters due to their external application to the skin as well as released into the aquatic environment from wastewater treatment plants (WWTPs) (Daughton and Ternes, 1999; Ternes et al., 2004). Consequently, UV filters have been detected in estuarine and coastal areas including surface water, sediment, and marine biota worldwide (Cadena-Aizaga et al., 2020; Gago-Ferrero et al., 2012; Pintado-Herrera and Lara Martín, 2020; Ramos et al., 2015). The occurrence of these emerging contaminants in the marine environment has raised concerns over their adverse impacts on organisms and ecosystems (Boxall et al., 2012; Prichard and Granek, 2016).

While toxicity of inorganic UV filters to marine species has been extensively studied (Luo et al., 2020; Ma et al., 2013), the ecotoxicological effects of organic UV filters have received less attention. Furthermore, the bulk of the studies on organic UV filters is biased towards lipophilic substances including oxybenzone, enzacamene, and octocrylene, and towards sensitive model species such as corals and algae (Lozano et al., 2020). Little is known about hydrophilic UV filters with lower bioaccumulation capacity (Ramos et al., 2015) and their toxicity to other marine taxa. The ecotoxicological data of different UV filters, however, are important for evaluating the total impacts of existing sunscreen formulas as well as designing new, eco-friendly sunscreen products (Kunze et al., 2021). In this study, we evaluated the effects of a common UV filter ensulizole (2-phenylbenzimidazole-5-sulfonic acid or PBSA) on blue mussels *Mytilus edulis* from the German Baltic Sea. Ensulizole is a water-soluble UV filter approved in sunscreens in the European Union (Pawlowski et al., 2021). The reported concentrations of ensulizole in surface waters vary from thousands of ng/L in effluent-dominated German streams to dozens of ng/L in the Baltic coast and estuaries, and a few ng/L in the open Baltic Sea (Fisch et al., 2017; Orlikowska et al., 2015; Wick et al., 2010). Blue mussels are common sentinel species for coastal pollution monitoring (Farrington et al., 2016), and the abundant information on their biomarker responses provides valuable resources for ecological risk assessment (Beyer et al., 2017). *M. edulis* were selected for this study because of their sessile lifestyle, filter-feeding habit, vital ecological roles, and easy collection and maintenance (Beyer et al., 2017). The ability to absorb both dissolved and particle-adsorbed contaminants (Fabbri and Franzellitti, 2016) makes mussels an ideal model to study the toxicity of hydrophilic substances such as ensulizole (Freitas et al., 2019; Stara et al., 2020).

The assessment of ensulizole ecotoxicity is still at its early stage. Previous experiments on rainbow trout *Oncorhynchus mykiss*, zebrafish *Danio rerio*, and blue mussels *M. edulis* suggested that exposure to ensulizole led to some molecular and biochemical effects such as enhanced oxidative stress and altered activity of biotransformation enzymes without evidence of bioaccumulation (Cahova et al., 2021; Falfushynska et al., 2021; Grabicova et al., 2013; Huang et al., 2020). However, no ensulizole-induced tissue or organismal effects (such as histopathology, embryotoxicity, or mortality) were found in those studies. Therefore, in our present study we aimed to conduct a comprehensive assessment of the potential impacts of ensulizole at different levels of biological organization (molecular, cellular, and organismal) in a sentinel marine bivalve, the blue mussels *M. edulis*, and to assess the concentration-dependence of these effects within the environmentally relevant range of concentrations encompassing five orders of magnitude from 10 to 10^5 ng/L. Given the lack of mechanistic information on ensulizole toxicity, we used a wide battery of biomarkers related to stress protection including detoxification and antioxidant defense (NADPH-cytochrome P450 reductase, carboxylesterase, glutathione-*S*-transferase, and glutathione reductase activities, reduced glutathione level, and total antioxidant capacity), and potential cytotoxic mechanisms including oxidative stress damage (malondialdehyde and protein carbonyl levels), autophagy (cathepsin D activity), apoptosis (mRNA expression levels of *Bcl-2*, *Bax*, *Cas2*, *Cas3*, and *Cas8*), inflammation (*NF-κB*, *CHUK*, *TBK1*, *TGF-β*, and *TNF* mRNA expression levels), and DNA damage (*GADD45* mRNA expression level). To assess the possible metabolic disruption by ensulizole, tissue levels of energy reserves (carbohydrates and proteins) and concentrations of a reactive glycolysis intermediate, methylglyoxal, were measured. The organismal effects of ensulizole on blue mussels were assessed using stress on stress (SoS) response as a sensitive indicator of general fitness and health status (Viarengo et al., 1995). Our study revealed complex concentration-dependent toxicity mechanisms of ensulizole at the molecular and cellular levels and found that ensulizole often elicited non-monotonic responses in the mussels, particularly compromising organismal fitness at low, environmentally relevant concentrations.

2. Materials and methods

2.1. Chemicals

Ensulizole 96% (2-phenylbenzimidazole-5-sulfonic acid, CAS 27503-81-7) was obtained from Sigma Aldrich (Darmstadt, Germany). Other analytical grade chemicals were purchased from Sigma Aldrich (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), or Thermo Fisher Scientific (Schwerte, Germany) unless mentioned otherwise.

2.2. Animal collection

Adult mussels were collected in March 2020 in Warnemünde, Germany (54.1810, 12.0890). The *Mytilus* from Warnemünde are hybrids of *M. edulis* and *M. trossulus* with a genetic background similar to that of pure *M. edulis* from the North Sea (Stuckas et al., 2017); the experimental animals, therefore, were considered *M. edulis*. Within 2 h after collection, the mussels were transported in insulated containers to the University of Rostock. Byssal threads of mussels were cut and epibionts were removed. The mussels were kept in aerated, recirculating 50-L aquaria (~100 mussels per aquarium) equipped with a moving bed biofilter, a protein skimmer, and UV treatment. The mussels were acclimated at 15 ± 1 °C and salinity 15 ± 1 for one week prior to the chemical exposures.

2.3. Chemical exposures

Two single-factor experiments were conducted contemporaneously to examine the organismal effects (Experiment A) and the molecular and biochemical effects (Experiment B) of ensulizole. In each experiment, adult mussels were randomly allocated to one of the six groups, namely CT (control, no ensulizole addition), and E1, E2, E3, E4, and E5 corresponding to 10 ng/L, 10^2 ng/L, 10^3 ng/L, 10^4 ng/L, and 10^5 ng/L of ensulizole. For each exposure concentration, a 10-L experimental tank was used. Experiment A had 55 mussels per tank (shell length 3.5–4.5 cm) and Experiment B had 35 mussels per tank (shell length 4.5–5.5 cm).

Each experimental tank contained aerated artificial seawater (Instant Ocean, Aquarium Systems, Sarrebourg, France) at 15 ± 1 °C and salinity 15 ± 1. Stock solutions of ensulizole were prepared in dimethyl sulfoxide (DMSO) and diluted to the desired exposure concentrations in the seawater. The final concentration of DMSO in all groups, including the control group, was 0.0083% v/v considered safe as a vehicle in chemical testing (OECD, 2021). The exposures were performed under a semi-static condition where three-quarters of the water was replaced three times a week with new ensulizole-supplemented, temperature-adjusted water to reduce nitrate buildup without making sudden changes in temperature. Mussels were exposed to ensulizole for 21 days considered sufficient time for physiological acclimation in bivalves (Khlebovich, 2017). During the one-week maintenance and the three-week exposure, mussels were fed on alternate days with a live culture of *Nannochloropsis oculata*, *Phaeodactylum tricornutum*, and *Chlorella* sp. (Live Marine Phytoplankton–Premium Reef Blend,

Sustainable Aquatics, Jefferson City, USA). The mussels found dead during the chemical exposures were recorded and promptly removed from the aquaria.

After the three-week exposure, mussels from Experiment A were exposed to air to assess the stress on stress (SoS) response ($n = 44$–50 per group). Mussels from Experiment B were dissected on ice to collect the gills and the digestive gland. The mussel gills and digestive gland are the main sites of uptake and accumulation of xenobiotics, respectively, and thus appropriate tissues to study the toxicity of environmental contaminants (Faggio et al., 2018; Gómez-Mendikute et al., 2005). Mussel tissues were shock-frozen in liquid nitrogen and stored at −80 °C for measurement of molecular and biochemical traits ($n = 6$–10 per group). Due to logistical constraints, we could not keep the mussels individually in separate experimental tanks. By sharing the same medium (Tincani et al., 2017), the mussels from the same exposure concentration might not be truly independent experimental units, which could weaken our inferences (Colegrave and Ruxton, 2018). It is worth noting that the blue mussels naturally form dense populations (the mussel beds) on hard substrates in the shallow coastal and intertidal zone, and single housing in the laboratory could be considered an unnatural and potentially stressful condition for this gregarious species. Therefore, we maintained mussels from each experimental treatment in groups within the same tank and used individual mussels as biological replicates.

2.4. Air exposure

The tolerance of mussels to air exposure was assessed following the protocol of Thain et al. (2019) with slight modifications. Briefly, mussels were placed in plastic containers with a bottom layer of plastic filter media and kept in an environmental room at 15 ± 1 °C. Mussels were checked once every 24 h; mussels with open valves not responding to mechanical stimuli were considered dead and removed from the containers. The air exposure continued until no mussel was alive and the survival time was recorded as the response variable.

2.5. Colorimetric assays

The gills and the digestive gland were homogenized using motor-driven Potter-Elvehjem glass homogenizers. The levels of reduced glutathione (GSH), malondialdehyde (MDA), protein carbonyls (PC), soluble proteins, carbohydrates, and methylglyoxal (MGO) were measured in the homogenates via end-point colorimetric methods. The activities of NADPH-cytochrome P450 reductase (CPR), carboxylesterase (CES), glutathione-*S*-transferase (GST), glutathione reductase (GR), cathepsin D, and total antioxidant capacity (TAC) were measured via kinetic assays at the room temperature (25–27 °C). All assays were performed in 96-well plates using a microplate reader (SpectraMax iD3, Molecular Devices, Biberach, Germany) and followed protocols described elsewhere (Falfushynska et al., 2019a, 2019b; Falfushynska et al., 2021).

Briefly, reduced glutathione (GSH) level in the cytoplasmic fraction of tissue homogenates was quantified by the reaction with Ellman's reagent that forms 5-thio-2-nitrobenzoic acid measurable at 412 nm using the extinction coefficient of 14.15 mM^{-1} cm^{-1} (Eyer et al., 2003). The level of malondialdehyde (MDA) in the supernatant of trichloroacetic acid-treated tissue homogenates was determined by the reaction with thiobarbituric acid forming a product measurable at 532 nm using the extinction coefficient of 156 mM^{-1} cm^{-1} (Buege and Aust, 1978). Protein carbonyl (PC) level in the pellet of trichloroacetic acid-treated tissue homogenates was measured by the reaction with 2,4-dinitrophenylhydrazine (DNPH) that produces protein-dinitrophenylhydrazone measurable at 370 nm using the extinction coefficient of 22 mM^{-1} cm^{-1} (Levine et al., 1990). Soluble protein concentration expressed as bovine serum albumin equivalents was measured by the reaction with Bradford's reagent producing a 595 nm absorbing protein-dye complex (Bradford, 1976). The level of carbohydrates expressed as glucose equivalents in the cytoplasmic fraction was assessed by the reaction with phenol–sulfuric acid forming a 492 nm absorbing chromophore (Masuko et al., 2005). Methylglyoxal (MGO) level in the cytoplasmic fraction was evaluated by the reaction with Girard's reagent T that produces a disubstituted compound measurable at 325 nm using the extinction coefficient of 8 mM^{-1} cm^{-1} (Mitchel and Birnboim, 1977).

In the cytoplasmic fraction of tissue homogenates, the activity of NADPH-cytochrome P450 reductase (CPR) was measured by the NADPH-dependent reduction of cytochrome *c* monitored at 550 nm using 21 mM^{-1} cm^{-1} as the extinction coefficient for the difference between the oxidized and reduced forms (Guengerich et al., 2009). Carboxylesterase (CES) activity was determined by the hydrolysis of *p*-nitrophenyl acetate that forms *p*-nitrophenol traceable at 405 nm using the extinction coefficient of 18 mM^{-1} cm^{-1} (Hosokawa and Satoh, 2001). Glutathione-*S*-transferase (GST) activity was quantified by the reaction between GSH and 1-chloro-2,4-dinitrobenzene (CDNB) monitored at 340 nm using the extinction coefficient of 9.6 mM^{-1} cm^{-1} (Habig et al., 1974). The activity of glutathione reductase (GR) was assessed by the NADPH-dependent reduction of glutathione disulfide to GSH monitorable at 340 nm using the extinction coefficient of 6.2 mM^{-1} cm^{-1} (Mannervik, 1999). The free and total activities of cathepsin D in the supernatant of untreated and Triton X-100-treated tissue homogenates, respectively, were assessed by the rate of hydrolysis of denatured hemoglobin that releases tyrosine measured at 280 nm using the extinction coefficient of 1.28 mM^{-1} cm^{-1} (Dingle et al., 1971). The lysosomal activity of cathepsin D was calculated as the difference between the free and total activities. The total antioxidant capacity (TAC) expressed as Trolox equivalents was evaluated by the reduction of 2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) chromophore measurable at 734 nm (Erel, 2004).

2.6. Quantitative reverse transcription PCR

The gills and the digestive gland were lysed in TRI Reagent (Zymo Research, Irvine, USA) using a bead-beating homogenizer (FastPrep-24, MP Biomedicals, Eschwege, Germany) for total RNA extraction (Chomczynski, 1993). DNA was removed (TURBO DNA-free Kit, Thermo Fisher Scientific, Schwerte, Germany) and complementary DNA (cDNA) was synthesized from 2 μg of total RNA (RevertAid RT Reverse Transcription Kit, Thermo Fisher Scientific, Schwerte, Germany) in a thermal cycler (peqSTAR 96 Universal Gradient, PEQLAB Biotechnologie, Erlangen, Germany).

Using the cDNA and gene-specific primers (Table 1), mRNA expression levels of the target genes and the reference gene *eEF1* were measured by quantitative PCR (Biozym Blue S'Green qPCR Kit Separate ROX, Biozym Scientific, Hessisch Oldendorf, Germany) in a StepOnePlus System (Thermo Fisher Scientific, Schwerte, Germany) following a protocol described earlier (Falfushynska et al., 2019a, 2019b; Falfushynska et al., 2021). Post-amplification melting curve analyses were performed to ensure the specificity of the PCR product. Gene-specific amplification efficiencies (E) were calculated using a cDNA standard dilution series (Pfaffl, 2001). The expression levels of the target genes were normalized against the expression level of the reference gene (Matz et al., 2013; Pfaffl, 2001).

2.7. Data analyses

Experimental results were analyzed using mussels as the biological replicates. For the stress on stress (SoS) response, the survival time in air exposure was used to compute the Kaplan-Meier estimates of the survival probability (Kaplan and Meier, 1958). From the Kaplan-Meier curves, the median survival time (LT50) of each exposure group was calculated. A Cox proportional hazards model (Cox, 1972) was fitted to evaluate the effects of ensulizole exposures on the death risk, represented by the hazard ratio (HR). LT50 and HR were reported with 95% confidence intervals (CI) and *p*-values.

Table 1
Primer sequences used for the amplification of the target and reference genes in *Mytilus edulis*.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	NCBI accession number
Bcl-2	CGGTGGTTGGCAAGGATTTG	CGCCATTGCGCCTATTACAC	KC545829.1
Bax	TAACTGGGGACGTGTAGGCA	CCAGGGGGCGACATAATCTG	KC545830.1
Cas2	ACAAGTGCAGATGCTGTGTTG	ACACCTCTCACATTGTCGGC	HQ424449.1
Cas3	ACGACAGCTAGTTCACCAGG	CCACCAGAAGAGGAGTTCCG	HQ424453.1
Cas8	AATGTCGGTACCCCACGATG	CGTGTATGAACCATGCCCCT	HQ424450.1
NF-κB	TGGATGATGAGGCCAAACCC	TGAAGTCCACCATGTGACGG	KF051275.1
CHUK	GTGGCCACCAGTCAAGTGAT	TAAGGCTGCAGCTTGCTGAT	KF015301.1
TBK1	TGCAGGAGCCGATAAAGCAA	CCGCCGGAACAAAATTCCAT	KF015302.1
TGF-β	TGCGGGTAAAACCAAGACCA	TCCCTGGCGGCTTCAATTAC	–
TNF	ATGTGCCAATTCCCTGTCCT	TCTGCTGTCACCTGTTCACC	KC994893.1
GADD45	TCTGTTTCGGCCATCTCTGGT	GCACAGGAAGACGGCAGAATT	AJ623737.1
eEF1	GACAGCAAAAACGACCCACC	TTCTCCAGGGTGGTTCAGGA	AF063420.1

Gene symbols: *Bcl-2* – Bcl-2 apoptosis regulator; *Bax* – Bcl-2 associated X, apoptosis regulator; *Cas2* – caspase 2; *Cas3* – caspase 3; *Cas8* – caspase 8; *NF-κB* – nuclear factor kappa B subunit 1; *CHUK* – component of inhibitor of nuclear factor kappa B kinase complex; *TBK1* – TANK binding kinase 1; *TGF-β* – transforming growth factor beta 1; *TNF* – tumor necrosis factor; *GADD45* – growth arrest and DNA damage inducible alpha; *eEF1* – eukaryotic translation elongation factor 1 alpha 1. In case of unavailable sequence information in *M. edulis*, close homologues from *M. unguiculatus* (*TGF-β*; Qi et al., 2019) and *M. galloprovincialis* were used for primer design.

Molecular/biochemical responses and mortality during chemical exposures were analyzed by permutation tests for the one-way layout and multiple comparisons with the control (Ernst, 2004). Permutation methods are more appropriate for inference in randomized experiments, particularly in the cases of small sample sizes (Ludbrook and Dudley, 1998). Briefly, the reference distributions of the test statistics were generated from 9999 permutations of the observed data and were used to compute the *p*-value of the global test and the critical value in the multiple comparisons. The family-wise error rate in these multiple comparisons was set at $\alpha = 0.05$.

We also calculated the effect size for the effects of ensulizole exposures on biomarker responses by Cliff's delta (Cliff, 1993). Cliff's delta (*d*) measures the distributional non-overlap between the responses of an ensulizole exposure group and the responses of the control group. Cliff's delta ranges from −1 (all values from the ensulizole exposure group are lower than the control) to 1 (all values from the ensulizole exposure group are higher than the control). Cliff's delta of 0 indicates identical responses between the two groups.

In this study, we adopted the language of evidence approach to reporting results based on the *p*-value (Muff et al., 2022). Thus, $p > 0.05$ implies little or no evidence against the null hypothesis (H_0: ensulizole exposures do not affect the responses of blue mussels) while the evidence was considered very strong, strong, or moderate when *p* is less than or equal to 0.001, 0.01, or 0.05, respectively.

All analyses were implemented in R (R Core Team, 2022) using the *survival* package for survival analysis (Therneau and Grambsch, 2000), the *mbRes* package for effect size calculation (Pham and Sokolova, 2022), and the *peramo* package for permutation tests, developed specifically for this study.

3. Results

3.1. Mortality in chemical exposures

Mussel mortality in six exposure groups at the end of the three-week exposure was 4.63% ($SD = 3.18\%$) (Table S1). There was little evidence that ensulizole exposures affected mortality during immersion ($p = 0.06$ and none of the multiple comparisons was significant).

3.2. SoS response

Control mussels had a median survival time of 10 days in air exposure while mussels exposed to ensulizole survived for a shorter time (LT50 = 7–9 days) (Fig. 1a, Table S2). Ensulizole-exposed mussels had the risk of death increased by 1.16–7.04 times compared with that of the control group (Fig. 1b, Table S2). There was no evidence that exposure to the highest ensulizole concentration (10^5 ng/L) compromised the survival of mussels during air exposure ($p = 0.46$). In contrast, exposures to the lower ensulizole concentrations (10–10^4 ng/L) reduced the mussel survival in the air ($p < 0.01$). Notably, the death risk was negatively associated with the test concentrations of ensulizole where exposure to the lowest concentration (10 ng/L) imposed the highest risk (Fig. 1b).

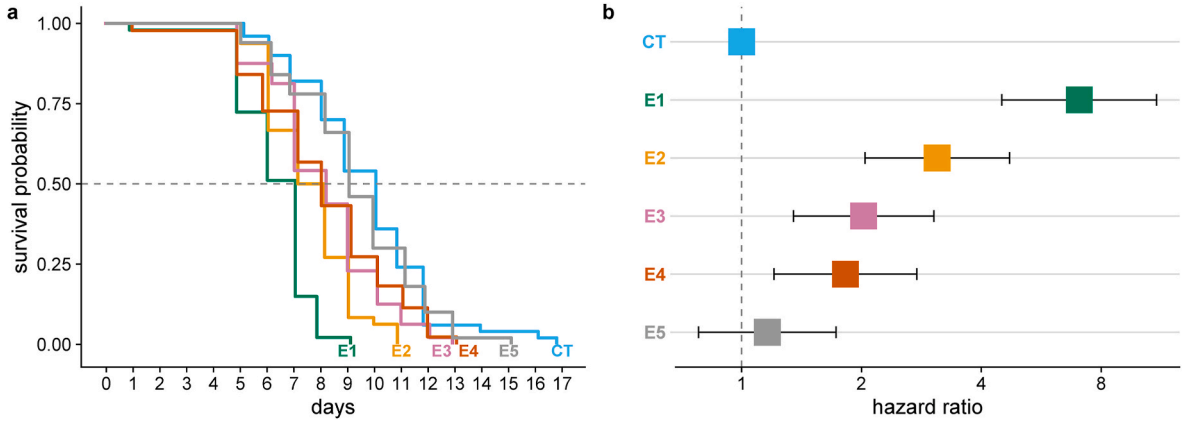

Fig. 1. Effects of ensulizole exposures on the stress on stress (SoS) response in *Mytilus edulis*. Kaplan-Meier curves represent the survival probability over time (a) and hazard ratios (HR) with 95% confidence intervals from Cox proportional hazards model represent the relative death risk in air exposure (b). Exposure groups: CT – control, E1 – 10 ng/L, E2 – 10^2 ng/L, E3 – 10^3 ng/L, E4 – 10^4 ng/L, and E5 – 10^5 ng/L of ensulizole.

3.3. Detoxification and antioxidant defense

There was little evidence that ensulizole exposures altered CPR activity in the gills and digestive gland (Figs. S1a and b). Also, no evidence was found for the effect of ensulizole on CES activity in both studied tissues (Figs. S1c and d).

In the gills, no evidence for the alteration of the GST activity by ensulizole was found (Fig. 2a). In the digestive gland, exposure to 10^4 ng/L of ensulizole significantly suppressed GST activity ($d = -0.8$) while the suppression of GST activity observed at other studied concentrations was not significant (Figs. 2b and 6b).

Mussels exposed to ensulizole often had lower GR activity in the gills (Fig. 2c). Particularly, exposure to 10^2 ng/L of ensulizole significantly suppressed GR activity ($d = -0.94$). Data on GR activity in the digestive gland also showed decreases in all ensulizole exposure groups although none of the responses was significantly different from the control (Figs. 2d and 6b). The highest suppression of GR activity ($d = -0.64$) occurred in the exposure to 10^5 ng/L of ensulizole.

Mussels exposed to ensulizole often had lower GSH levels in the gills although only the decrease observed at 10^2 ng/L ($d = -0.78$) was significant (Figs. 2e and 6a). GSH level in the digestive gland, in contrast, was often higher in ensulizole-exposed mussels where exposures at 10^3–10^5 ng/L led to the increase of GSH ($ds = 0.89$–0.94) (Figs. 2f and 6b).

In the gills, exposure to 10^3 ng/L of ensulizole significantly decreased TAC ($d = -1$) (Fig. 2g). TAC of the digestive gland of ensulizole-exposed mussels was not significantly different from the control (Fig. 2h).

3.4. Oxidative stress damage

There was no evidence that exposure to ensulizole affected MDA or PC levels in the gills and digestive gland (Fig. S2).

3.5. Energy reserves and metabolism

Protein levels in the gills of mussels exposed to 10^3–10^5 ng/L of ensulizole were lower than those in the control mussels but only the decrease observed at the highest tested concentration of 10^5 ng/L ($d = -0.86$) was significant (Figs. 3a and 6a). No evidence was found for the alteration of the protein content by ensulizole in the digestive gland (Fig. 3b).

Ensulizole-exposed mussels generally had lower carbohydrate levels in the gills. Exposures to 10^4 and 10^5 ng/L of ensulizole led to the significant decreases in carbohydrate levels ($ds = -0.94$ and -1) while the declines observed at the other concentrations were not significant (Figs. 3c and 6a). Mussels exposed to 10^3 ng/L of ensulizole had a significant increase ($d = 1$) in carbohydrate level in the digestive gland (Fig. 3d).

Ensulizole-exposed mussels often had lower MGO levels in the gills. Mussels exposed to 10^4 and 10^5 ng/L of ensulizole had a significantly lower MGO level ($ds = -0.56$) while the decreases observed at the other studied concentrations were not significant (Figs. 3e and 6a). In the digestive gland, exposure to the lowest concentration of ensulizole (10 ng/L) led to elevated MGO levels ($d = 0.94$) while the changes observed at the other concentrations were not significant (Figs. 3f and 6b).

3.6. Autophagy

Exposure to the lowest ensulizole concentration (10 ng/L) led to a significant increase of total activity of cathepsin D in the gills ($d = 0.61$) (Fig. 4a). Significant elevations of the total activity of cathepsin D ($ds = 0.83$ and 1) were also observed at the lowest and highest (10^5 ng/L) ensulizole concentrations, respectively (Fig. 4b).

Mussels in ensulizole exposure groups generally had lower free activity of cathepsin D in the gills although only the reduction ($d = -0.75$) observed at 10^4 ng/L of ensulizole was significant (Fig. S3a). The changes in the free activity of cathepsin D in the digestive glands were not significant (Fig. S3b). Lysosomal activities of cathepsin D were significantly higher in the gills of mussels exposed to the lowest ensulizole concentration ($d = 0.67$) and in the digestive gland of mussels exposed to the highest concentration ($d = 1$) (Figs. S3c and d).

3.7. Apoptotic markers

The expression level of *Bcl-2* was higher in both studied tissues of ensulizole-exposed mussels compared with the controls (Fig. 4c and d). Mussels exposed to the lowest ensulizole concentration (10 ng/L) had a significant increase ($d = 0.74$) in the *Bcl-2* transcript levels in the gills while mussels exposed to the highest ensulizole concentration (10^5 ng/L) had an increase ($d = 0.9$) in the expression level in the digestive gland. The elevations of the expression level at the other concentrations were not significant (Fig. 6).

Ensulizole-exposed mussels often had higher expression levels of *Cas3* and *Cas8* in the gills although only the exposure to 10^3 ng/L of ensulizole led to significant elevations ($ds = 0.76$ and 0.78) (Fig. 4e, g, Fig. 6). No evidence was found for the effect of ensulizole on the expression level of *Cas3* or *Cas8* in the digestive gland (Fig. 4f, h).

There was no evidence that exposure to ensulizole changed the expression levels of *Bax* and *Cas2* in the gills and digestive gland (Fig. S4).

3.8. Inflammatory markers

There was no evidence that exposure to ensulizole affected the expression levels of *NF-κB* in the gills (Fig. S5a). While moderate evidence was found for the overall effect of ensulizole on the expression levels of *NF-κB* in the digestive gland (Fig. S5b), none of the multiple comparisons was significant.

The expression level of *TBK1* was higher in the gills of ensulizole-exposed mussels but only the elevation ($d = 0.76$) observed at 10^3 ng/L of ensulizole was significant (Figs. 5a and 6a). Although the expression level of *TBK1* in the digestive gland of ensulizole-exposed mussels was higher than the control ($ds = 0.42$–0.78), none of the changes was significant (Figs. 5b and 6b).

There was no evidence that exposure to ensulizole altered the expression levels of *CHUK*, *TGF-β*, and *TNF* in the gills and digestive gland (Figs. S5c–h).

3.9. DNA damage marker

No evidence was found for the effect of ensulizole on the expression level of *GADD45* in the gills (Fig. 5c). In the digestive glands, only the exposure to 10^4 ng/L of ensulizole significantly increased the expression level ($d = 0.5$) (Fig. 5d).

4. Discussion

4.1. Subcellular effects of ensulizole

Previous studies with ensulizole show that this hydrophilic UV filter is stable in both the fresh and sea water, and the measured water concentrations are close to the nominal concentrations added to the medium (Falfushynska et al., 2021; Grabicova et al., 2013; Huang et al., 2020). Therefore, even though the seawater concentrations of ensulizole were not measured in the our present study due to the budgetary limitations, we are confident that given the stability of the compound and the static renewal design with frequent water changes, the nominal concentrations closely correspond to the actual exposure concentrations. Earlier studies also showed that ensulizole did not bioaccumulate in the tissues of the exposed animals including mussels and fish (Falfushynska et al., 2021; Grabicova et al., 2013). The lack of bioaccumulation has been attributed to the effective elimination of

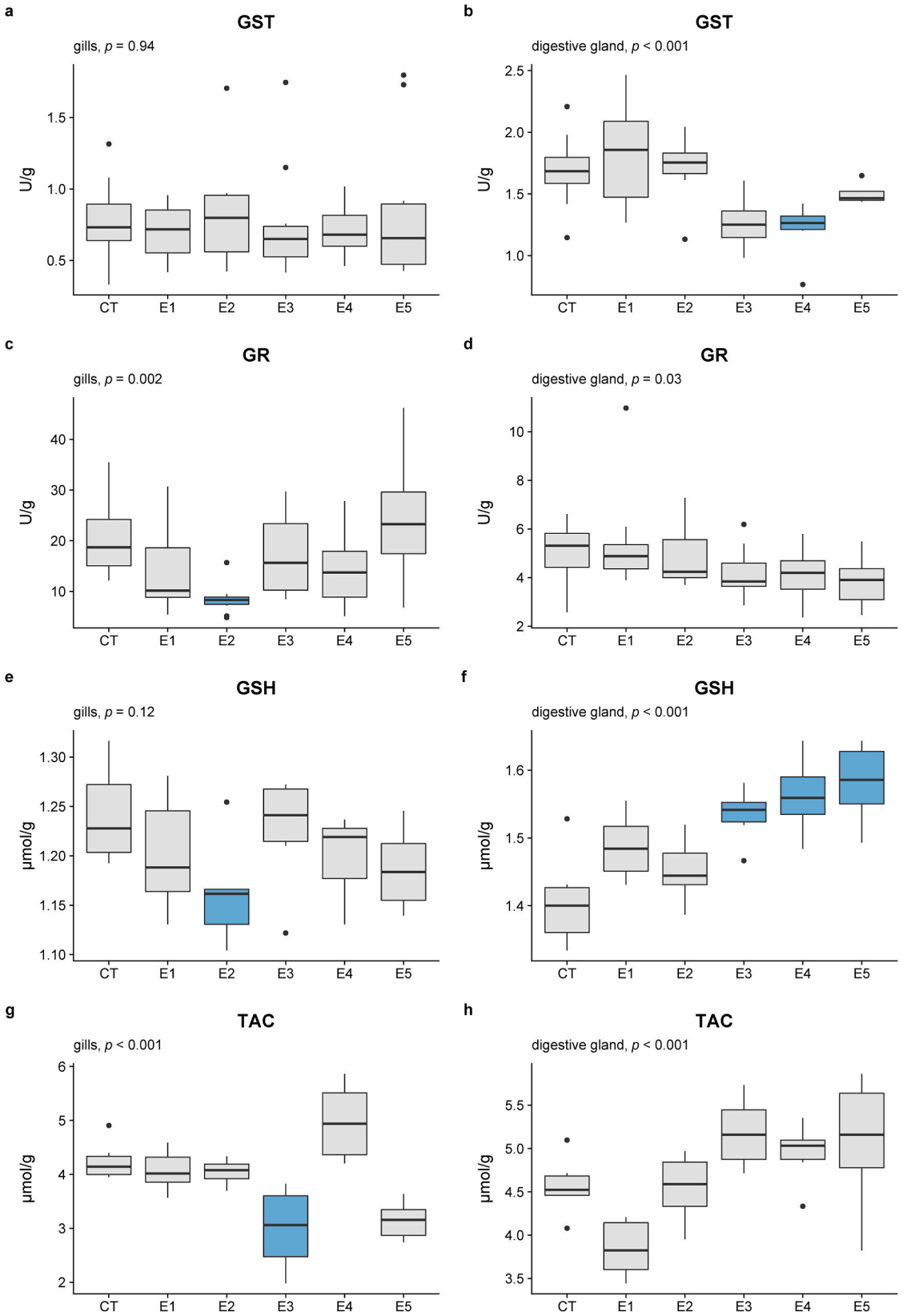

Fig. 2. Effects of ensulizole exposures on glutathione-*S*-transferase (GST) activity, glutathione reductase (GR) activity, reduced glutathione level (GSH), and total antioxidant capacity (TAC) in the gills and digestive gland of *Mytilus edulis*. Exposure groups: CT – control, E1 – 10 ng/L, E2 – 10^2 ng/L, E3 – 10^3 ng/L, E4 – 10^4 ng/L, and E5 – 10^5 ng/L of ensulizole. The *p*-value is given for the global test and responses significantly different from the control ($\alpha = 0.05$) in the multiple comparisons are indicated by boxplots of a different color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

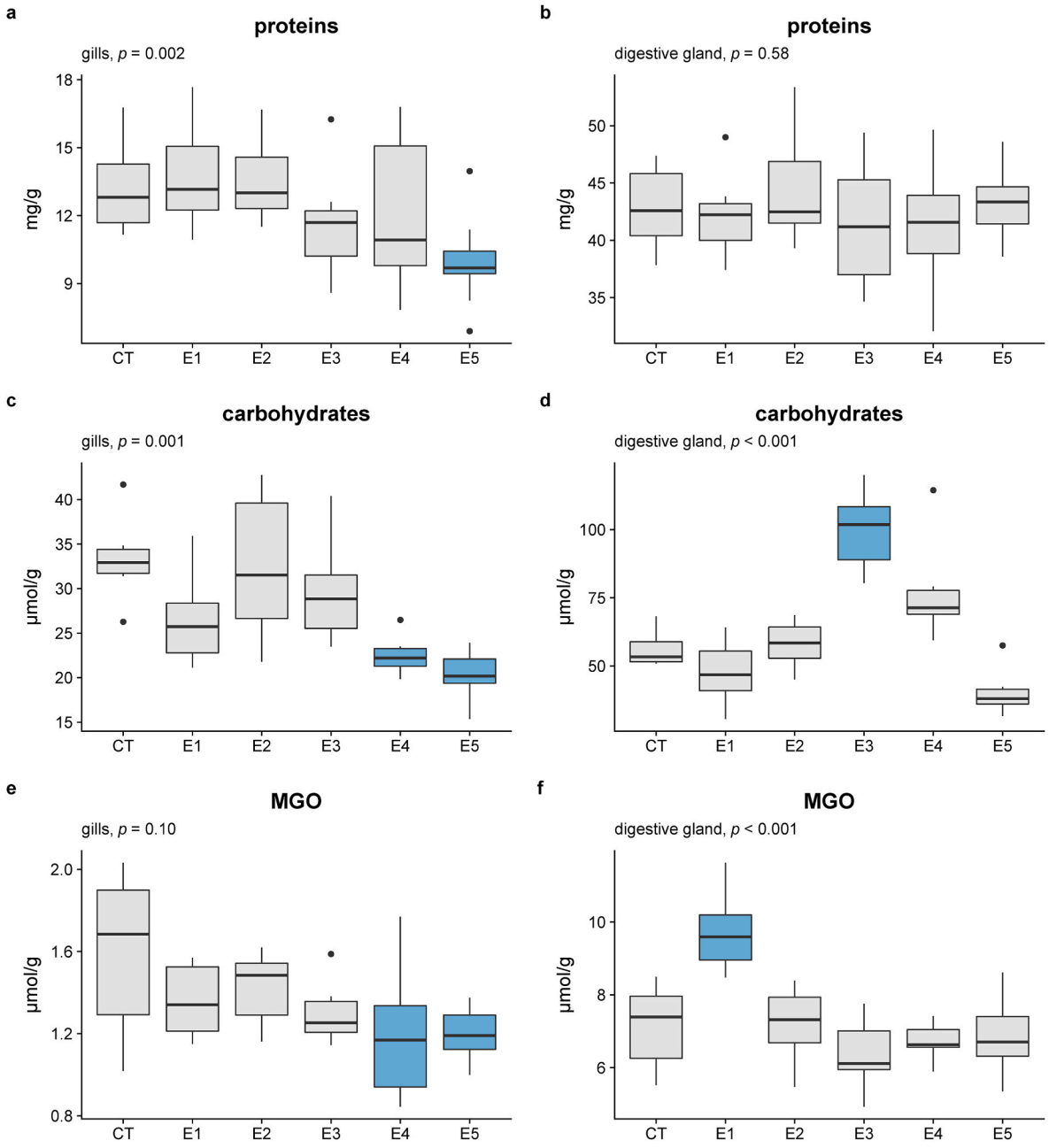

Fig. 3. Effects of ensulizole exposures on the levels of soluble proteins, carbohydrates, and methylglyoxal (MGO) in the gills and digestive gland of *Mytilus edulis*. Exposure groups: CT – control, E1 – 10 ng/L, E2 – 10^2 ng/L, E3 – 10^3 ng/L, E4 – 10^4 ng/L, and E5 – 10^5 ng/L of ensulizole. The *p*-value is given for the global test and responses significantly different from the control ($\alpha = 0.05$) in the multiple comparisons are indicated by boxplots of a different color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

ensulizole or the biotransformation into metabolites not detectable by the methods used to assess the parent compound (Falfushynska et al., 2021). However, our present study as well as earlier published research (Falfushynska et al., 2021) indicate that despite the lack of bio-accumulation, ensulizole or its metabolites can interact with cellular targets causing subcellular effects in the gills and digestive gland of blue mussels *M. edulis*.

Ensulizole exposures altered the antioxidant defense (i.e., GR, GSH, and TAC) without causing oxidative stress damage (i.e., accumulation of MDA or PC) in the studied tissues of mussels. The suppression of GR activity was a notable effect of ensulizole, which is consistent with the findings of Grabicova et al. (2013) and Falfushynska et al. (2021). While GR helps regenerate GSH from glutathione disulfide, the suppressed GR activity was not always associated with the depletion of GSH as shown by the increase of GSH level in the digestive gland. This might indicate a compensatory upregulation of GSH biosynthesis in ensulizole-exposed mussels.

We observed tissue- and concentration-dependent change in TAC, a marker of antioxidant status, in the gills and digestive gland at tested ensulizole concentrations. Generally, TAC was suppressed at the lowest tested ensulizole concentration (10 ng/L) in both studied tissues of the mussels. This was also the concentration where the highest increase in the risk of air mortality was detected by SoS. At the higher exposure concentrations of ensulizole (at or above 10^2 ng/L), TAC levels in the digestive gland increased indicating stimulation of antioxidant defense. In the gill, the response to higher ensulizole concentration was variable. Notably, Falfushynska et al. (2021) reported higher production of reactive oxygen species (ROS) in the hemocytes of blue mussels after

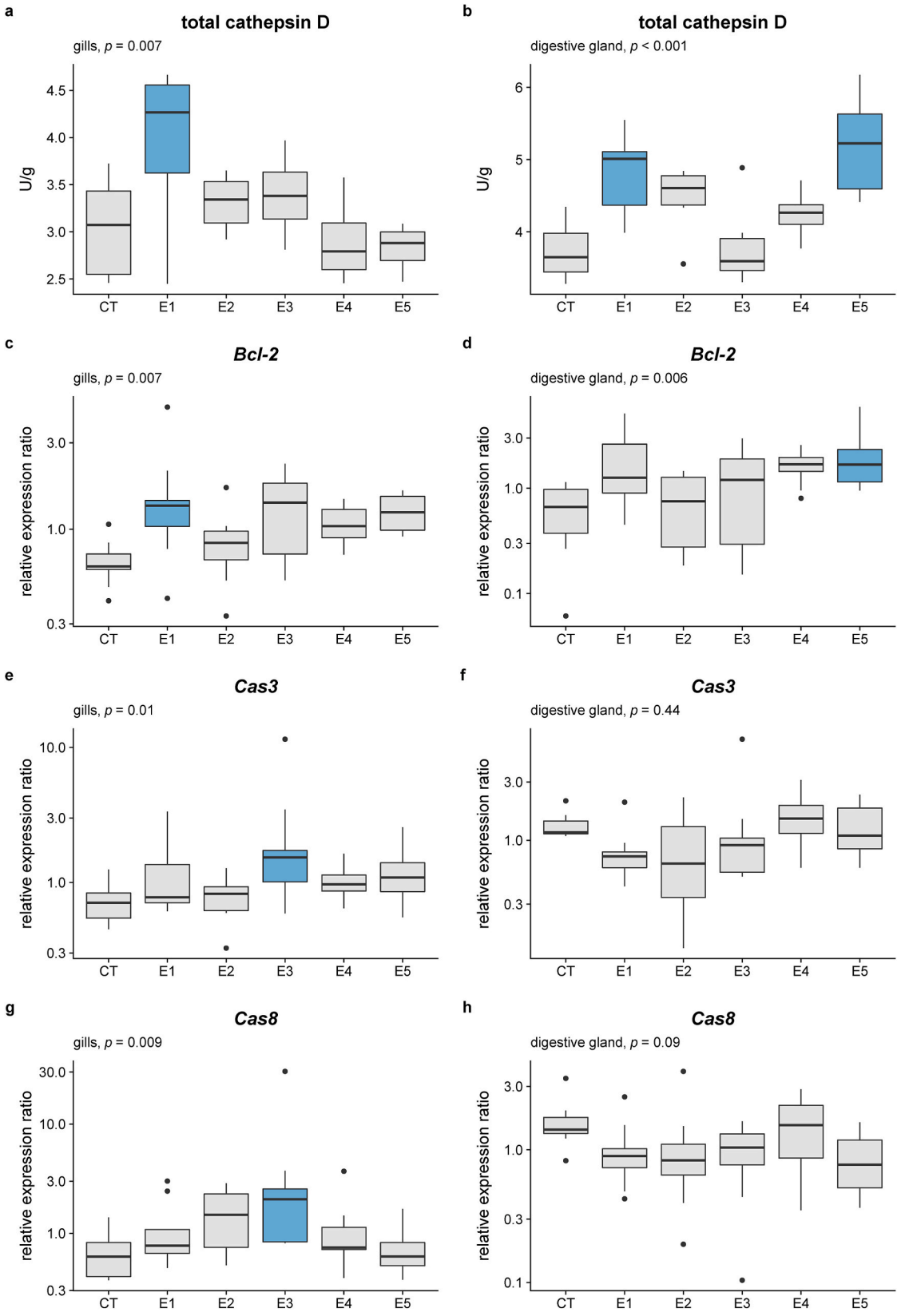

Fig. 4. Effects of ensulizole exposures on total activity of cathepsin D and mRNA expression levels of *Bcl-2*, *Cas3*, and *Cas8* in the gills and digestive gland of *Mytilus edulis*. Exposure groups: CT – control, E1 – 10 ng/L, E2 – 10^2 ng/L, E3 – 10^3 ng/L, E4 – 10^4 ng/L, and E5 – 10^5 ng/L of ensulizole. The *p*-value is given for the global test and responses significantly different from the control ($\alpha = 0.05$) in the multiple comparisons are indicated by boxplots of a different color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

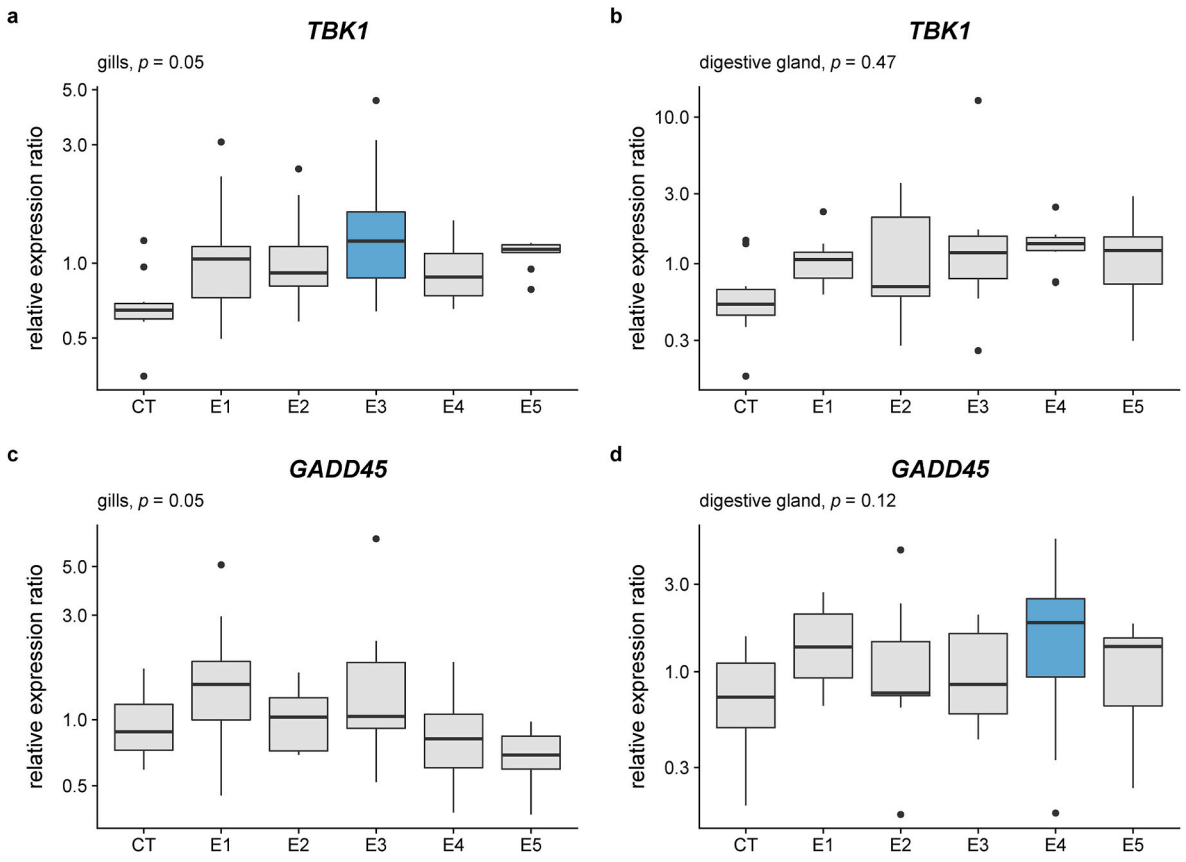

Fig. 5. Effects of ensulizole exposures on mRNA expression levels of *TBK1* and *GADD45* in the gills and digestive gland of *Mytilus edulis*. Exposure groups: CT – control, E1 – 10 ng/L, E2 – 10^2 ng/L, E3 – 10^3 ng/L, E4 – 10^4 ng/L, and E5 – 10^5 ng/L of ensulizole. The *p*-value is given for the global test and responses significantly different from the control ($\alpha = 0.05$) in the multiple comparisons are indicated by boxplots of a different color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

two-week exposure to 10^4 ng/L compared with those kept at 10^5 ng/L. Grabicova et al. (2013), however, found no change in the activities of catalase (CAT) and superoxide dismutase (SOD) in rainbow trout liver after six-week exposure to 10^6 ng/L of ensulizole. Similarly, Huang et al. (2020) reported no change in SOD activity in zebrafish liver after two-week exposure to above 10^5 ng/L of ensulizole but a decrease in SOD activity after one week. In our study, MDA and PC as markers of lipid and protein oxidation, respectively, were found unchanged while Huang et al. (2020) and Falfushynska et al. (2021) showed the increase of these biomarkers in the tissues of zebrafish and mussels exposed to ensulizole. Taken together, these findings indicate that disturbances of the redox balance (such as ROS generation and suppression of antioxidants) might occur in aquatic organisms including fish and mussels especially at low ensulizole concentrations albeit these disturbances are not always associated with accumulation of oxidative lesions to lipids and proteins.

Activities of Phase I biotransformation enzymes CPR and CES and Phase II enzyme GST were mostly unaltered in the studied tissues of ensulizole-exposed mussels except for suppression of GST in the digestive gland at high ensulizole concentrations. In rainbow trout liver, the induction of a Phase I enzyme cytochrome P450 oxidase (CYP) was observed after three-week exposure to 10^3–10^6 ng/L of ensulizole but CYP activity returned to the baseline levels after six-week exposure (Grabicova et al., 2013). Falfushynska et al. (2021) showed suppressed activity of CPR, which is critical for CYP-catalyzed oxidations, but also observed suppression and stimulation of CYP1A in the digestive gland of mussels exposed to 10^4 and 10^5 ng/L of ensulizole for two weeks. These ensulizole concentrations also had opposite effects on CES activity in the mussel digestive gland (Falfushynska et al., 2021). Huang et al. (2020) and Falfushynska et al. (2021) also reported the unchanged or stimulated activity of GST in zebrafish and mussel tissues. Based on the molecular structure of ensulizole, it is unlikely that the hydrolysis by CES and the glutathione conjugation by GST are directly involved in the biotransformation of this UV filter (Wishart et al., 2022). Instead, future studies might focus on the hydroxylation by CYPs and particularly the glucuronidation by UDP-glucuronosyltransferase which produces possibly hepatotoxic/immunotoxic metabolites (Banerjee et al., 2018; King et al., 2000).

Exposure to high ensulizole concentrations led to the depleted energy reserves as shown by the lower protein and carbohydrate levels in the mussel gills, the first exposure site of ensulizole. It is possible that ensulizole exposures imposed elevated energetic costs of detoxification, repair, and adaptation mechanisms on the gill cells (Sokolova et al., 2012) that required the breakdown of glycogen and even proteins to produce more energy. In contrast, exposure to ensulizole sometimes led to an increased carbohydrate level in the mussel digestive gland, probably due to enhanced glycogenesis or suppressed glycogenolysis. We observed a high level of MGO, a deleterious by-product of glycolysis and lipid/protein metabolisms, in the digestive gland of mussel exposed to the lowest ensulizole concentration, indicating the possible effects of ensulizole on the detoxification of MGO. Falfushynska et al. (2021) also suggested that ensulizole may disturb the lipid metabolism via its impact on acetyl-CoA carboxylase (ACC). Therefore, further investigations on the glyoxalase system (Allaman et al., 2015) and cellular energy allocation (De Coen and Janssen, 1997) might be useful to shed light on the bioenergetics implications of ensulizole toxicity in the coastal mussel populations.

The activity of total cathepsin D increased in the tissues of mussels

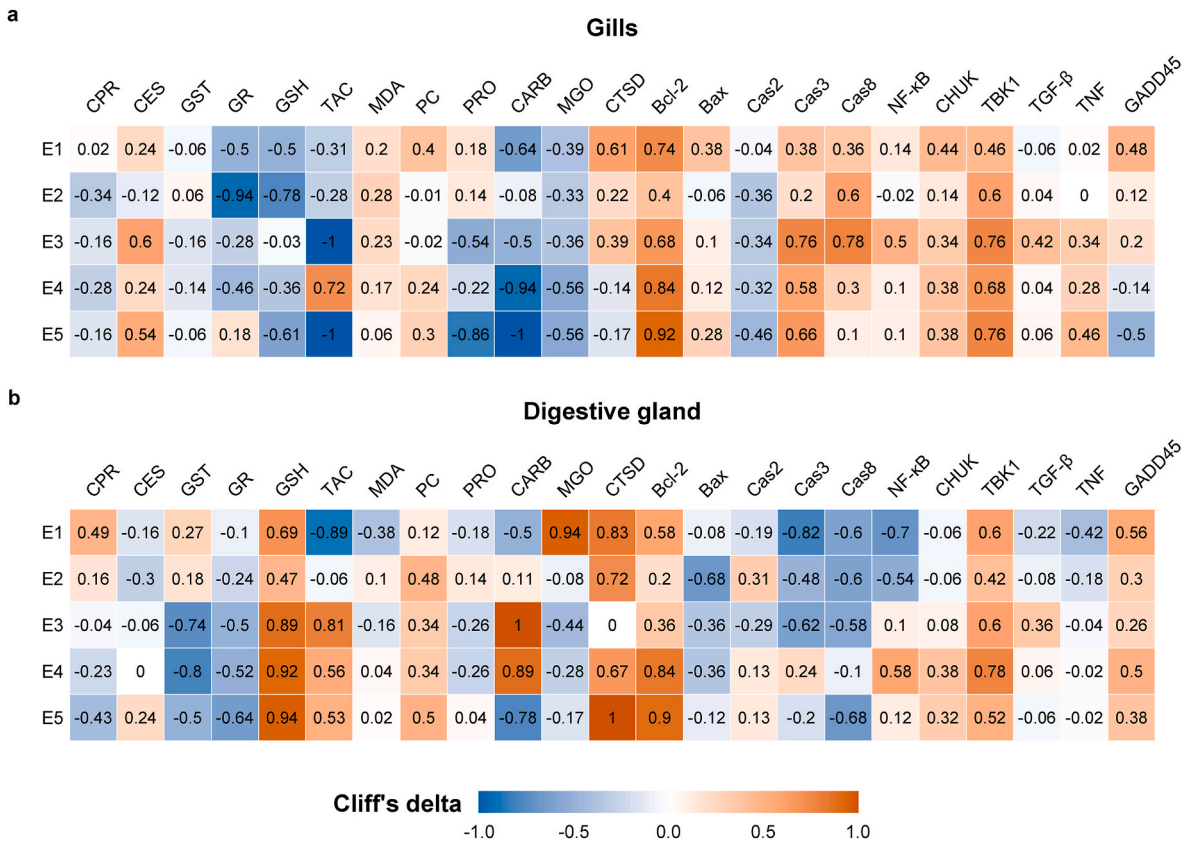

Fig. 6. Cliff's delta effect size for the effects of ensulizole exposures on molecular/biochemical biomarkers in the gills (a) and digestive gland (b) of *Mytilus edulis*. Exposure groups: E1 – 10 ng/L, E2 – 10^2 ng/L, E3 – 10^3 ng/L, E4 – 10^4 ng/L, and E5 – 10^5 ng/L of ensulizole. Biomarkers: NADPH-cytochrome P450 reductase (CPR), carboxylesterase (CES), glutathione-*S*-transferase (GST), and glutathione reductase (GR) activities, reduced glutathione level (GSH), total antioxidant capacity (TAC), malondialdehyde (MDA) and protein carbonyl (PC) levels, protein (PRO), carbohydrate (CARB), and methylglyoxal (MGO) levels, total cathepsin D activity (CTSD), and mRNA expression levels of *Bcl-2*, *Bax*, *Cas2*, *Cas3*, *Cas8*, *NF-κB*, *CHUK*, *TBK1*, *TGF-β*, *TNF*, and *GADD45*.

exposed to the lowest and highest ensulizole concentrations, possibly indicating the elevated autophagic removal of damaged proteins in lysosomes or increased breakdown of cellular constituents for energy supply (Rabinowitz and White, 2010). The upregulation of gene encoding anti-apoptotic protein Bcl-2 in both tissues of ensulizole-exposed mussels and the unchanged expression of gene encoding pro-apoptotic effector Bax, as also observed by Falfushynska et al. (2021), implied an adaptive response to prevent the mitochondrial-mediated apoptosis (Orrenius et al., 2011). The transcriptional induction of genes encoding initiator caspase 8 and effector caspase 3 in the gills suggested the enhancement of the receptor-mediated apoptotic pathway (Tummers and Green, 2017). In contrast to Falfushynska et al. (2021), we found little alteration in the expression levels of genes encoding initiator caspase 2 and protein GADD45 which often respond to oxidative stress and DNA damage (Puccini et al., 2013; Zhan, 2005). This discrepancy might reflect differences in the degree of the oxidative stress experienced by the test organisms in these two studies as shown by the lack of MDA and PC accumulation in our present study contrasted with elevated levels of MDA and PC in the exposures reported by Falfushynska et al. (2021). Among studied inflammatory markers, only the expression of *TBK1* encoding TANK-binding kinase 1 showed the potential induction, which might lead to NF-κB-dependent inflammatory responses (Yu et al., 2012). Overall, these findings indicate that ensulizole does not cause inflammation but might be implicated in the initiation of cell death mechanisms in the mussels.

4.2. Whole-organism responses to ensulizole

Our study showed that three-week exposure to 10–10^5 ng/L of ensulizole did not increase the mortality of blue mussels *M. edulis* in the water environment. This result is consistent with the report of Falfushynska et al. (2021) in which no mortality of *M. edulis* was recorded during two-week exposure to 10^4 and 10^5 ng/L of ensulizole. Huang et al. (2020) reported that the median lethal concentration (LC50, unknown duration) of ensulizole in zebrafish *Danio rerio* was greater than 10^8 ng/L. Cahova et al. (2021) also found that exposure to 10^2–10^6 ng/L of ensulizole in 24–96 h did not affect the mortality and hatching rate of zebrafish embryos. These findings suggest that ensulizole at environmental concentrations (<10^4 ng/L; Fisch et al., 2017; Orlikowska et al., 2015; Wick et al., 2010) probably has no acute lethal effects on aquatic organisms.

Our results on the SoS response of *M. edulis*, nevertheless, indicated that ensulizole could reduce the individual fitness with potentially lethal consequences. The use of SoS response as a general stress indicator was based on the assumption that mussels under the effect of anthropogenic stressors (i.e., contaminants) have a lower tolerance for natural stressors such as air exposure (Bayne, 1986; Viarengo et al., 1995). Accordingly, LT50 of 10 days or more in the air suggests a healthy condition (Vethaak et al., 2017), which was the case for control mussels in our experiment, while LT50 of 5 to less than 10 days implies moderate stress, as happened in the ensulizole exposed mussels. This indicates that cumulative effects of multiple mild cellular disturbances caused by ensulizole such as those found in glutathione, energy metabolism, and cell death pathways might lead to decline in the whole-organism fitness despite the lack of strong inflammatory or oxidative stress responses.

The observed disturbance of energy metabolism in ensulizole-exposed mussels might be particularly relevant to the reduced survival in air. Unlike intertidal mussels that may maintain a valve gap for oxygen intake during emersion (Eertman et al., 1993; Gagné et al., 2015), subtidal mussels, including the ones from the non-tidal Baltic Sea used in our study, must close their valves completely to avoid desiccation during air exposure. The ability to sustain adductor muscles keeping the valves closed is thought to depend on the amount of ATP produced by glycogen-fueled anaerobic respiration (Thain et al., 2019). Although the exposure to contaminants often costs energy for subcellular stress responses such as detoxification processes, Eertman et al. (1993) found that mussels living in contaminated areas and showing shorter survival time in air had similar glycogen content to those in cleaner sites. Therefore, the lower air tolerance caused by contaminant exposures could be better explained by the disturbances of anaerobic catabolism which reduce the ATP yield (Eertman and de Zwaan, 1994; Martínez-Gómez et al., 2017). Future studies might explore the impacts of ensulizole on the succinate-propionate fermentation pathway (Wang et al., 1992) to explain the organismal effects of this UV filter.

4.3. Concentration-response relationships and risk assessment

In general, observed responses of blue mussels at molecular, biochemical, and organismal levels were non-monotonic functions of ensulizole concentration. For example, mussels exposed to the lowest ensulizole concentration had the lowest tolerance to air exposure while mussels exposed to the highest concentration had similar tolerance to the control. Similarly, an earlier study in the blue mussels using two ensulizole concentrations (10^4 and 10^5 ng/L) showed a stronger evidence of toxicity at a lower of the two tested concentrations (Falfushynska et al., 2021). Although non-monotonic concentration-response relationships have been shown in the cases of essential nutrients (Hayes, 2007) and some toxicants (Calabrese et al., 2007), particularly endocrine disruptors (Vandenberg et al., 2012), the exact mechanisms by which ensulizole induced non-monotonic responses in mussels remain unknown. Regardless of the mechanisms, our study suggests that the theory of monotonic concentration-response relationship might not be ideal in the ecological risk assessment of cosmetics and personal care products including UV filters. The risk of these emerging contaminants should not be neglected simply due to their low environmental concentrations as our study showed that ensulizole at the ng/L range was capable of compromising mussel fitness, making the organisms more vulnerable to environmental stressors.

CRediT author statement

Duy Nghia Pham: Methodology, Software, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. **Eugene P. Sokolov**: Methodology, Validation, Investigation, Data Curation, Writing - Review & Editing. **Halina Falfushynska**: Methodology, Validation, Investigation, Data Curation, Writing - Review & Editing. **Inna M. Sokolova**: Conceptualization, Methodology, Validation, Resources, Data Curation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data, associated metadata, and calculation tools are publicly available on CRAN at https://cran.r-project.org/package=peramo and on GitHub at https://github.com/phamdn/peramo.

Acknowledgements

This work was in part supported by the project BluEs - "Blue_-Estuaries - Developing estuaries as habitable sustainable ecosystem despite climate change and stress" funded by the Federal Ministry of Education and Research of Germany (project BluEs-Uni-HRO #03F0864B) to I.M. Sokolova, by Alexander von Humboldt Fellowship to H. Falfushynska, and by Erasmus Mundus Scholarship via MER Consortium to D.N. Pham.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.136736.

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