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Oxidative stress biomarkers in the gills of the bivalve *Mactra stultorum* exposed to acrylamide

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Summary: Acrylamide (ACR) is among the most deleterious pollutants in the environment and presents a serious risk to humans and ecosystems. The purpose of this study was to assess its effects when administered at different concentrations (5, 10 and 20 mg L^{-1}) to evaluate antioxidant status in the gills of *Mactra stultorum*. Our results showed, after five days of treatment, an increase in malondialdehyde (MDA), lipid hydroperoxides (LOOH), advanced oxidation protein products (AOPP), reduced glutathione (GSH), ascorbic acid (Vit C) and metallothionein (MDA) levels in gills of treated clams compared with controls. Moreover, an increase in superoxide dismutase (SOD) and a significant decrease in glutathione peroxidase (GPx) activities were also observed. Acrylamide induced neurotoxicity, as evidenced by the inhibition of acetylcholinesterase (AChE) activity in a dose-dependent manner. Overall, our results indicated that oxidative stress may be considered one of the mechanisms behind acrylamide toxicity in bivalves, although the subject requires more research.

Keywords: acrylamide; exposure; *Mactra stultorum*; gills; antioxidant status; acetylcholinesterase.

Biomarcadores de estrés oxidativo en las branquias del bivalvo *Mactra stultorum* expuesto a acrilamida

Resumen: La acrilamida (ACR) es uno de los contaminantes más perjudiciales en el medio ambiente y presenta un grave riesgo para los seres humanos y los ecosistemas. El objetivo de este estudio es evaluar los efectos del ACR administrado a diferentes concentraciones (5, 10 y 20 mg L^{-1}) con el fin de evaluar el estado antioxidante en las branquias de *Mactra stultorum*. Nuestros resultados mostraron, después de 5 días de tratamiento, un aumento en los niveles de malondialdehído (MDA), hidroperóxidos lipídicos (LOOH), proteínas de oxidación avanzada (AOPP), glutatión reducido (GSH), ácido ascórbico (vit C) y metalotioneínas (MTs) en las branquias de las almejas tratadas en comparación con los controles. Además, también se observó un aumento en la superóxidodismutasa (SOD) y una disminución significativa en las actividades de glutatión peroxidasa (GPx). La acrilamida indujo neurotoxicidad como lo demuestra la inhibición de la actividad de la acetilcolinesterasa (AChE) de una manera dependiente de la dosis. En general, nuestros resultados indicaron que el estrés oxidativo puede considerarse como uno de los mecanismos detrás de la toxicidad por ACR en los bivalvos, aunque el tópico debería beneficiarse de más investigaciones.

Palabras clave: acrilamida; exposición; *Mactra stultorum*; branquias; estado antioxidante; acetilcolinesterasa.

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INTRODUCTION

Over the last few decades, the use and development of the biomonitoring approach in environmental studies has increased considerably. Indeed, this type of biological test provides supplementary information that may not be revealed by chemical methods. Biomonitoring programmes based on measuring hazardous pollutants in marine organisms are interesting tools from a human health viewpoint (Contardo-Jara et al. 2009). Bivalves are among the first sentinel organisms used in programmes for monitoring chemical contamination in marine and freshwater ecosystems. They have a high water-filtering activity, good accumulation ability, low excretion rates and a low metabolic capacity for contaminant degradation (Zorita et al. 2006, Sheehan and McDonagh 2008). *Mactra stultorum* (Mollusca: Bivalvia) is sedentary and easy to collect and keep alive in the laboratory (Chetoui et al. 2010, Abdallah 2013).

Acrylamide (ACR), a ubiquitous chemical, is an industrial compound with a variety of applications, including water treatment, agricultural processes, textile manufacturing and life science research (Friedman 2003, Tepe and Çebi 2017). Due to its substantial hydrophilicity, ACR has a high migration capability and may be easily transferred to the environmental soil and groundwater (Tepe 2015). ACR was first discovered in numerous fried or baked carbohydrate-rich foods such as French fries, potato chips, bread, biscuits and coffee (Mottram et al. 2002). It is considered one of the most dumped contaminants, reaching high concentrations (≈1000 mg L^{-1}) in several accident cases (Kusnin et al. 2015, Tepe 2015). High ACR levels found in the environment are especially encountered in workplaces where ACR is used or in the vicinity of industrial facilities (Kusnin et al. 2015). The stationary point sources of ARC (about 93%) are located in China, USA, Western Europe and Japan (Touzé et al. 2015).

ACR has been classified as a "Group 2A carcinogen" by the International Agency for Research on Cancer (IARC) (IARC 1994). It is common knowledge that its low molecular weight and high water solubility enable this compound to easily pass through various biological membranes. A study conducted by Larguinho et al. (2014) showed that the LC50 (96 h) for ACR in water is estimated to be 411 mg L^{-1} in bivalves (*Mytilus galloprovincialis*). Indeed, the chemical structure of ACR is distinctive as a result of an α,β-unsaturated carbonyl group which constitutes its "Michael-type" reactivity towards thiol, hydroxyl or amino groups, and to a lesser extent the nucleophilic centres in DNA (Adamsa et al. 2010). However, recent reports have revealed that ACR exposure is associated with carcinogenesis, genotoxicity, neurotoxicity and reproductive toxicity (Erkekoglu and Baydar 2014, Duan et al. 2015).In toxicological studies, ACR has been observed to elicit its toxicity through oxidative stress induction associated with the over-generation of reactive oxygen species (ROS) including the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$) (Yilmaz et al. 2017, Trabelsi et al. 2019). ROS are able to damage all cellular biomolecules, including proteins, DNA and lipids (Fasulo et al. 2012, Kim et al. 2015).

Our knowledge of potential toxicity following ACR exposure in bivalves is largely based on very limited data (Larguinho et al. 2014, Trabelsi et al. 2019), suggesting that more extensive experimental studies in this field are required. In an effort to improve the mechanisms of ACR toxicity, the present work was performed to examine the effect of different concentrations of ACR on the function of *M. stultorum* gills.

MATERIALS AND METHODS

Sampling and experimental design

Clams of *Mactra stultorum* (3.5±0.63cm length and 8.03±0.47 g weight) were collected from Bizerta lagoon situated in northern Tunisia. The clams were transferred directly to the laboratory, where they were acclimated for a week in 20-L aquaria renewed daily with fresh seawater. The acclimation arrangement contained a closed-circuit system with continuous aeration, and some physical-chemical parameters such as salinity (34±1) and photoperiod (12/12h) were maintained. After acclimation, clams were randomly divided into four groups and transferred to 50-L experimental aquaria in duplicate (n=8 clams per replicate). They were exposed for five days under controlled conditions, as mentioned above, to graded acrylamide (ACR, powder 99%) concentrations as follows:

1**.** Control group
2. Group A: 5 mg L^{-1}
3. Group B: 10 mg L^{-1}
4. Group C: 20 mg L^{-1}.

The doses of ACR were chosen according to other studies involving aquatic organisms (Larguinho et al. 2014, Trabelsi et al. 2019). The doses were also selected to ensure observable effects in order to investigate the toxic effects of ACR and its mechanism of action in terms of oxidative stress and metabolic alteration. During the experimental test, the clams were not fed to avoid prandial effects. No mortality was detected after five days. Specimens were collected and dissected, and the gills were removed using sterile scissors. Some portions of the tissue were homogenized in 10% of Tris-HCl buffer (100 mM, pH: 7.4) and then centrifuged at 10000 rpm for 25 min at 4°C. The obtained supernatants were stored at –80°C in aliquots for further biomarker and protein assays.

Chemicals

All reagents used in the present study were of the highest quality available and were obtained from Sigma Aldrich (Germany) or Merck (Germany).

Biochemical analyses in *Mactra stultorum* gills

Protein quantification

Proteins contents were measured according to the method of Lowry et al. (1951) using bovine serum albumin as standard. Results were expressed as mg/g of tissue.

Malondialdehyde (MDA)

Lipid peroxidation was estimated by measuring thiobarbituric acid (TBA)–reactive substances and was expressed in terms of malondialdehyde (MDA) content by a colorimetric method according to Draper and Hadley (1990). An aliquot of 0.2 mL of the tissue extract supernatant was mixed with 1 mL of trichloroacetic acid solution and centrifuged at 2500×g for 10 min. The resulting supernatant was then incubated at 90°C for 15 min with 1 mL of a solution containing 0.67% TBA. The absorbance of TBA–MDA complex was measured spectrophotometrically at 532 nm. The MDA amounts were calculated using 1,1,3,3-tetraethoxypropane as standard and expressed as nmol/mg of protein.

Lipid hydroperoxides

Lipid hydroperoxide (LOOH) levels in gills were estimated using the ferrous oxidation in the xylenol orange assay (FOX assay) described by Jiang et al. (1992). The amount of hydroperoxides produced was calculated using the molar extinction coefficient of 4.6×9×104/M/cm and the results were expressed as nmol/mg of protein.

Advanced oxidation protein products

The advanced oxidation protein product (AOPP) levels were determined according to the method of Kayali et al. (2006). Briefly, 400 µL of digestive gland supernatant was treated with 800 µL Tris-Hcl (100 mM, pH 7.4). After 2 min, 100 µl of potassium iodide (1.16 M) was treated with the previous solution followed by 0.1 mL of acetic acid. The reaction mixture was immediately recorded at 340 nm. The concentration of AOPP was calculated using the extinction coefficient (261/cm/mM) and results were expressed as µmol/mg of protein.

Non-enzymatic antioxidants

Reduced glutathione (GSH): GSH level in *M. stultorum* digestives glands was determined according to Ellman's (1959) method as modified by Jollow et al. (1974). The method is based on the reductive cleavage of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by a sulfhydryl (-SH) group to yield a yellow colour. Briefly, a mixture of tissue homogenates in phosphate buffer and sulfosalicylic acid (4%) was centrifuged at 1600×g for 15 min. A volume of the obtained supernatant was taken and added to Ellman's reagent. Reduced GSHwas measured as the difference in the absorbance values of samples in the presence and the absence of DTNB at 412 nm. GSH value was expressed as µg/mg of protein.

Ascorbic acid (Vit C): Ascorbic acid levels were determined according to Jacques-Silva et al. (2001) in clams' digestive glands. Supernatants were precipitated in a cold trichloroacetic acid solution (5%). After incubation at 85°C for 30 min, 1 mL of sulfuric acid (H_2SO_4) solution (65%) was added to the previous mixture. The reaction product was determined using colour reagent containing dinitrophenyl hydrazine and copper sulphate and measured at 540 nm. Results were expressed as nmol/mg of protein.

Metallothioneins (MTs): MT content was determined according to the method of Viarengo et al. (1997) modified by Petrovic et al. (2001). An aliquot of the supernatant was mixed with cold absolute ethanol and chloroform and centrifuged at 6000 g for 10 min. The collected supernatant was combined with three volumes of cold ethanol, maintained at −20°C for 1 h and centrifuged at 6000 g for 10 min. The obtained MT-containing pellets were then rinsed with 87% ethanol and1% chloroform and centrifuged at 6000 g for 10 min. MT concentration was quantified by evaluating the SH residue content by a spectrophotometric method, using Ellman's reagent (DTNB). The absorbance was evaluated at 412 nm. Concentrations were expressed as µmol GSH/mg of protein.

Enzymatic antioxidant activities

Glutathione peroxidase (GPx): GPx was assayedby Flohe and Gunzler's (1984) method. GPx oxidizes reduced glutathione in the presence of peroxides. A reaction mixture containing 0.3 mL of phosphate buffer (0.1 M, pH 7.4), 0.2 mL of 2mM glutathione (GSH), 0.1 mL of sodium acid (10 mM), 0.1 mL of H_2O_2 (1 mM) and 0.3 mL of tissue homogenates was used. After 15 min incubation at 37°C, 0.5 mL 5% trichloroacetic acidwas added to the mixture. The tubes were centrifuged at 1500 g for 10 min and the collected supernatant was assayed for glutathione content using DTNB reagent (10 mM). The absorbance was recorded at 420 nm. Resultswere expressed as nmol of GSH/min/mg of protein.

Superoxide dismutase (SOD): SOD activity was estimated by the riboflavin/nitro blue tetrazolium (NBT) photochemistry analysis method, as described by Beauchamp and Fridovich (1971). The reaction mixture consisted of tissue homogenates in potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mML-methionine, 2 µM riboflavin and 75 µM NBT. The reduction of NBT by superoxide radicals to blue colour was followed at 560 nm. The SOD activity was expressed as the amount of enzyme required to inhibit the reduction of NBT by 50% and the activity was expressed as U/mg of protein.

Acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was determined in *M. stultorum* digestive glands according to the method described by Ellman et al. (1961), using acetylthiocholine iodide as a substrate. Enzyme activity was recorded over 5 min after addition of the substrate concentration. The enzymatic reaction rate was measured spectrophotometrically at 412 nm against a

Table 1. – MDA LOOH and AOPP levels in *Mactra stultorum* gills of control and specimens treated with graded doses of ACR (5, 10 and 20 mg L^{-1}) after five days of treatment.Values were expressed as means ±SD. The number of determinations was n=8. Groups A, B and C vs. control group: * p<0.05, ** p<0.01, *** p<0.001.

Parameters and Treatment	Control	A (5 mg L^{-1})	B (10 mg L^{-1})	C (20 mg L^{-1})
LOOH (nmol/mg of protein)	$6\times10^3\pm0.7\times10^3$	$15\times10^3\pm3\times10^{3***}$	$21\times10^3\pm4\times10^{3***}$	$22\times10^3\pm1\times10^{3***}$
MDA (nmol/mg of protein)	6.71±1.46	12.61±2.05***	16.80±4.07***	16.82±0.91***
AOPP (µmol/mg of protein)	0.14±0.03	0.18±0.03**	0.25±0.03***	0.24±0.01***

Table 2. – Correlation (Pearson) between various antioxidant parameters in gills from control and exposed *M. stultorum.* Significance level at p<0.05=a, p<0.01=b, p<0.001=c.

	Pr	LOOH	MDA	SOD	GPx	GSH	Vit C	MTs	AOPP	AChE
Pr	1.00									
LOOH	**–0.91** b	1.00								
MDA	**–0.90** b	**0.95** c	1.00							
SOD	–0.48	0.43	0.46	1.00						
GPx	0.27	–0.44	–0.34	–0.18	1.00					
GSH	**–0.91** b	**0.89** b	**0.85** b	0.47	–0.31	1.00				
Vit C	**–0.79** a	**0.75** a	**0.71** a	0.40	–0.14	0.66	1.00			
MTs	**–0.96** c	**0.94** c	**0.90** b	0.45	–0.32	**0.90** b	**0.80** a	1.00		
AOPP	**–0.91** b	**0.93** c	**0.91** b	0.44	–0.28	**0.81** a	**0.77** a	**0.92** b	1.00	
AChE	**0.82** a	**–0.85** b	**–0.82** a	–0.35	0.54	**–0.84** b	–0.46	**–0.79** a	**–0.75** a	1.00

blank without substrate for each activity. AChE activity was expressed as nmol of substrate/min/mg protein.

Statistical analysis

Multivariate statistical analysis was performed using Statistica software analysis (8.0, USA). The obtained results were assessed for normality and homogeneity via the Shapiro test. A one-way ANOVA (Tukey HSD test) and a non-parametric Kruskal-Wallis test (Student t-test) for analysed parameters were performed in order to determine the significant differences between control and treated groups at p<0.05. The whole data set of the non-enzymatic and enzymatic antioxidants in gills of *M. stultorum* was statistically treated by principal component analysis (PCA) using R software version 2.15.2 (R Core Team 2017). PCA is used to look for directions in space that best represent the correlations between random variables. The aim of PCA is to obtain an approximate representation of the cloud in a low-dimensional subspace "k" by projection on well-chosen axes. The main "k" axes are those that maximize the inertia of the projected cloud, that is, the weighted average of the squares of the distances from the projected points to their centre of gravity.

RESULTS

Estimation of MDA, LOOH and AOPP levels in *M. stultorum* gills

The exposure of groups A, B and C to ACR (5, 10 and 20 mg L^{-1}, respectively) caused a significant, dose-dependent increase in MDA levels (88%, 150% and 150%, respectively), LOOH levels (134%, 124% and 125%, respectively) and AOPP levels (29%, 74% and 61%, respectively) in gills of *M. stultorum* in comparison with the control group (Table 1). The AOPP levels showed a positive correlation with MDA (p<0.01) and LOOH (p<0.001) in gills of *M. stultorum* (Table 2).

Non-enzymatic antioxidant status of *M. stultorum* gills

Exposure to ACR of groups A, B and C increased the ascorbic acid levels (24%, 44% and 39%, respectively) in *M. stultorum* gills in comparison with the control group (Table 3). They also significantly increased GSH levels (p<0.001) and MT levels (p<0.001). In addition, MT levels showed a strong positive correlation with GSH content (p<0.001) (Table 2).

Enzymatic antioxidant status in *M. stultorum* gills

Exposure to ACR of groups B and C led to a significant increase (28% and 21%, respectively) in SOD activity in gills of *M. stultorum* in comparison with the control group (Table 3). In groups A, B and C it also led to a significant decrease in GPx activity (17%, 120% and 30%, respectively) (Table 3).

AChE activity in *M. stultorum* gills

Exposure to ACR of groups A, B and C caused a significant decrease in AChE activity (79%, 96% and 94%, respectively) in comparison with the control

Table 3. – GSH, ascorbic acid and MT levels and GPx and SOD activities in *Mactra stultorum* gills of control and specimens treated with graded doses of ACR (5, 10 and 20 mg L^{-1}) after five days of treatment. Values were expressed as means ±SD. The number of determinations was n=8. Groups A, B and C vs. control group: * p<0.05, ** p<0.01, *** p<0.001.

Parameters and treatment	Control	A (5 mg L^{-1})	B (10 mg L^{-1})	C (20 mg L^{-1})
GSH (µg/mg of protein)	0.54±0.10	1.81±0.50***	1.96±0.33***	1.85±0.22***
Ascorbic acid (nmol/g of protein)	14.89±4.47	18.51±3.54*	21.55±5.05**	20.70±1.36**
MTs (µmol GSH/mg of protein)	0.35±0.03	0.58±0.10***	0.69±0.14***	0.61±0.03***
GPx (nmol GSH/min/mg of protein)	16.25±1.46	13.48±1.44***	13.48±1.46***	11.31±3.73***
SOD (U/mg of protein)	25.99±5.95	30.32±7.80	33.17±8.69*	31.35±4.38*

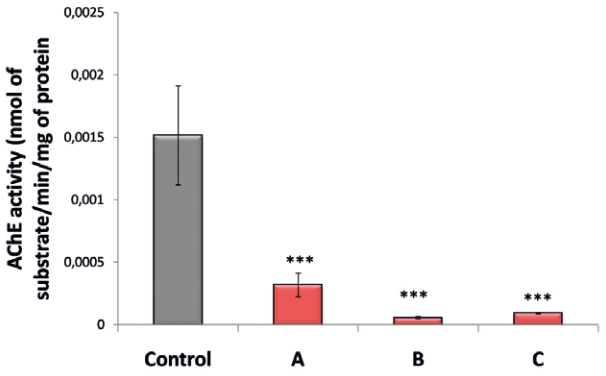

Fig. 1. – AChE activity in *Mactra stultorum* gills of control and specimens treated with graded doses of ACR (5, 10 and 20 mg L^{-1}) after fivedays of treatment. Values are expressed as means ±SD, n=8. A, B, C groups vs. Control group: *** p<0.001.

group (Fig. 1). Levels of AChE in gills were positively correlated with protein content (Table 2).

Principal component analysis

The PCA was applied to our biomarker measurements in order to have a better understanding of their variation in *M. stultorum* gills exposed to graded doses of ACR after five days of treatment. The explanation of the results is limited to the first two factorial axes, which explain most of the cloud variance of the initial variables. The two principal components (F1, F2) explained 83.4% of the total variance. The first axis, with 73.1% of variance, allowed an almost complete classification of the different experimental groups by separating the controls from those exposed to ACR-treated groups. This axis was negatively correlated with MDA, LOOH, AOPP, SOD, GSH, Vit C and MTs and showed a positive contribution to proteins and AChE. However, the second axis (10.3%) represented the high positive

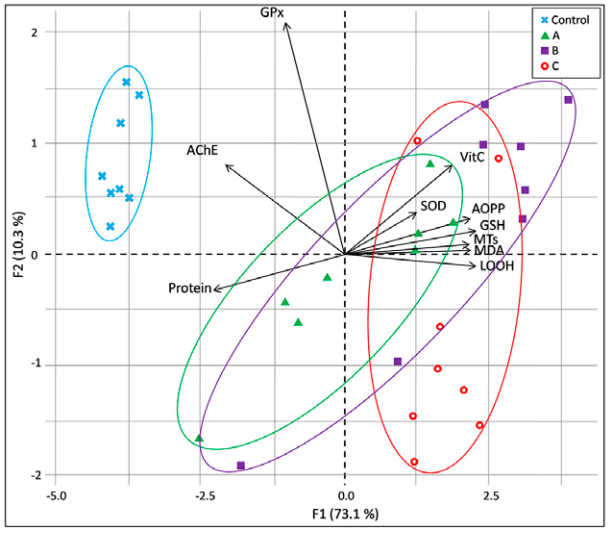

Fig. 2. – Principal component analysis (PCA) represented by two factors (F1=73.1% and F2=10.3%) and produced by biochemical variables (MDA, LOOH, AOPP, GPx, SOD, GSH, vit C, MT and AChE) in *Mactra stultorum* gills of control and specimens treated with graded doses (5, 10 and 20 mg L^{-1}) of ACR for five days.

loads with GPx. The projection of individuals (each specimen from each dose) on the same factorial plan 1-2 (Fig. 2) showed that *M. stultorum* of control and treated groups were clustered into four groups. The group belonging to the controls showed a positive contribution tothe second component (10.3%). This correlation coincided with the high AChE and GPx activities and protein concentration, the low MDA, LOOH, AOPP, GSH, vit C and MT levels, and the low SOD activity. Clams exposed to 5 and 10 mg L^{-1} overlap in a group that is explained by the similarity in response to oxidative stress. The group of specimens exposed to 20 mg L^{-1} contribute positively tothe axis 1 and negatively to axis 2. MDA, LOOH, AOPP, GSH, vit C, MTs levels and SOD activity increased when those of AChE, GPx and proteins decreased.

DISCUSSION

Acrylamide is considered one of a variety of dumped contaminants that reach high concentrations in marine ecosystems. It is an organic molecule with very high water solubility, which facilitates its rapid absorption by and distribution through aquatic organisms (Andersen et al. 2005, Kusnin et al. 2015). Bivalves' gill are widely used in monitoring programmes, because that organ perform a multitude of functions in organisms' body and represent the first point of pollutant access from the surrounding aquatic environment (Della Torre et al. 2015). Therefore, the present study was designed to examine the impact of ACR on redox status in *M. stultorum* gills.

Gills are sensitive sensory organs and the first barrier against natural impacts (pathogens and natural toxins) and anthropogenic pollution. This tissue may therefore be the main organ involved in prooxidant and antioxidant processes, resulting in both increased production and accumulation of ROS (Della Torre et al. 2015, Bejaoui et al. 2018). Lipids and protein, the mainfunctional and structural components of cell membranes, are the target of free radical stress (Favier 2003). In cell membranes, polyunsaturated fatty acids (PUFAs), fatty acids with two or more double bonds, are the main target of ROS, leading to their peroxidation and to functional and structural disorders (Stanicka et al. 2015). The result of our present study showed an increase in levels of MDA and LOOH, the products of lipid peroxidation, in the gills of ACR-exposed clams. A conclusive and positive correlation (p<0.001) was found between MDA and LOOH levels in the gill tissues. The results of the current study are corroborated by the findings of Trabelsi et al. (2019), who found a rise in the MDA content in *M. stultorum*'s digestive gland when it was exposed to ACR.

However, increased formation of ROS also leads to enhanced oxidative damage to proteins and polypeptides. Protein oxidation can lead to ROS-induced modifications of amino acid residues/prosthetic groups of enzymes, as well as fragmentation/aggregation of proteins in the cell (Adams 2001). The most frequently evaluated biomarkers of oxidative protein damage are AOPPs, which are dityrosine-containing cross-linked

protein products (Alderman et al. 2002). In this study, the assessed AOPP was significantly higher in the gills of ACR-treated species than in the control group. Hence, it may reflect the prooxidant effect of the ACR active compound, which can easily react with free sulfhydryl groups in proteins (LoPachin and Gavin 2012). Furthermore, a positive correlation ($p<0.01$) and ($p<0.001$) was found between the AOPP levels and the lipid peroxydation products (MDA and LOOH, respectively) in the gills of treated groups compared with controls.

The consequences of the generation of free radicals can bring further changes to antioxidant enzyme activities (Schwarz 1996). GPx is a glycoprotein comprised of a single seleno-cysteine residue at the active site of each sub-unit. Hence, it participates in the detoxification of LOOHs through its reduction to water and the related alcohols (Jomova and Valko 2011). In the present study, we found that the activity of GPx decreased in the gills of *M. stultorum* under ACR exposure, which may imply that GPx was degraded by ROS or over utilized to overcome the excess of lipid hydroperoxides. Moreover, SOD is considered to be the first line of defence against the superoxide anion radical ($O_2^{\bullet-}$) by catalysing its conversion to H_2O_2 (Haleng et al. 2007). Our findings show an increase in SOD activity in the treated animals. The increased SOD activity suggests the protective behaviour of gill cells against $O_2^{\bullet-}$ radicals generated under a plausible ACR toxicity. Trabelsi et al. (2019) reported a disruption of the enzymatic antioxidant status in digestive glands of the ACR-treated *M. stultorum*. Meanwhile, other indicators of oxidative stress, GSH, Vitamin C and MTs were measured to evaluate the potential oxidative damage induced by ACR exposure. During the test, the gills of ACR-treated organisms had a high content of GSH. It is the abundant non-protein thiol (–SH) that is implicated in cell protection by suppressing toxicants and quenching unstable species such as free radicals (Birben et al. 2012). Furthermore, the detoxification pathway of ACR is driven by its conjugation with a GSH molecule (Greim and Snyde 2018). The latter is also directly implicated in recycling ascorbic acid, an important water soluble antioxidant, which protects the cell membrane from oxidative stress (Krishnan et al. 2009). Under experimental conditions, ascorbic acid levels increased significantly in the gills of ACR-treated *M. stultorum*. MTs, cysteine-rich proteins with low molecular weight, are believed to protect DNA, lipids and proteins from ROS production and oxidative injury (Cai et al. 1999). In fact, MTs have the potential to directly inactivate the hydroxyl, ($OH^{\bullet}$) and superoxide radicals ($O_2^{\bullet-}$) (Zhou et al. 2002). In our study, the levels of MTs increased in all the treated groups. Indeed, the active compound (α,β-unsaturated carbonyl) in an ACR molecule is likely to be added to the thiol group in MT molecules and forms cysteine-acrylamide adducts (Zamora et al. 2010). Recently, high MT levels were found in *M. stultorum* digestive glands treated with graded concentrations of ACR (Trabelsi et al. 2019). This finding is also supported by the Pearson's correlation results, in which GSH was positively correlated with MT levels ($p<0.01$). This increase in thiol-rich molecules may be a compensatory adaptive mechanism to neutralize increased levels of ACR molecules binding with the –SH group of GSH and MTs.

Acetylcholine is an important neurotransmitter. Its action is dependent on it being able to metabolize the AChE enzyme, which is essential for the correct transmission of the nerve impulse (Schmatz et al. 2009). In the present study, the AChE activity of *M. stultorum* gills exposed to graded doses of ACR was significantly inhibited. This enzyme activity can be inhibited by free radicals generated by ACR and its inhibition could disturb the metabolic and nervous activities and induce differential membrane permeability and ionic refluxes. As noted above, the neurotoxic effect of ACR must be related to the reaction of α,β-unsaturated carbonyl with the thiol molecules, either in the presynaptic membranes, with the subsequent reduction of the neurotransmitter releasing AChE, or in the enzyme itself. Recent studies have demonstrated the cholinergic damage of ACR through the inhibition of the AChE activity in the digestive glands of *M. stultorum* bivalves (Trabelsi et al. 2019).

CONCLUSION

Although the ACR concentrations used in our study are unlikely to occur in coastal areas, this study shows that ACR might have a hazardous impact on *M. stultorum* gills following the installation of oxidative stress. Additionally, our results are particularly important because the observed alterations might induce several types of physiological damage depending on the doses used. We can therefore consider the biomarker responses to be useful tools for evaluating ACR toxicity. Further research using lower concentrations is required to validate the exact signalling pathways targeted by ACR and mediating its toxicity action in cell metabolism.

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Conflict of Interest The authors declare that they have no competing interests.

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