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The use of selected biomarkers, fatty acid and histopathology features to detect the possible toxic effects of the penconazole-containing fungicide Topas on marine clam siphons (*Ruditapes decussatus*)

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Summary: Widespread use of pesticides in agriculture has the potential to harm non-target creatures diffusely and contaminate aquatic habitats through leaching and runoff events. Triazoles are among the fungicides used extensively worldwide due to their efficacy against fungal crop diseases and their broad spectrum of action. In this study, the impact of Topas on the antioxidant defence system, fatty acid composition and histopathological injuries was experimentally examined under three concentrations (4, 40, and 400 µg L^{-1}) over 96 hours in *Ruditapes decussatus* siphons. Our results showed that Topas exposure induced a significant decrease in the levels of saturated fatty acids. However, an increase of monounsaturated fatty acids and polyunsaturated fatty acids, mainly the eicosapentaenoic (C20:5n-3), docosahexaenoic (C22:6n-3) and arachidonic (C20:4n-6) acids. Topas exposure enhanced the levels of hydrogen peroxide, malondialdehyde and protein carbonyls and altered enzymatic and non-enzymatic antioxidant status in all treated clams. Acetylcholinesterase activity was inhibited with the increase of Topas concentrations. Eventually, histopathological changes detected in treated animals varied in a concentration-dependent manner and were herein consistent with the biochemical outcomes. Our findings shed new light on the relationship between redox state and fatty acid composition changes, allowing us to understand Topas-triggered toxicity.

Keywords: *Ruditapes decussatus*, Topas, siphons, antioxidant status, fatty acid profiling, histopathological features.

El uso de biomarcadores seleccionados, ácidos grasos y características histopatológicas para detectar los posibles efectos tóxicos del fungicida Topas, que contiene Penconazole, en los sifones de los almejas marinos (*Ruditapes decussatus*)

Resumen: El uso generalizado de pesticidas en la agricultura tiene el potencial de dañar de manera difusa a criaturas no objetivo y contaminar los hábitats acuáticos a través de eventos de lixiviación y escorrentía. Los triazoles se encuentran entre los fungicidas más utilizados en todo el mundo debido a su eficacia contra enfermedades fúngicas de los cultivos y su amplio espectro de acción. En este estudio, se examinó experimentalmente el impacto de Topas en el sistema de defensa antioxidante, la composición de ácidos grasos y las lesiones histopatológicas en tres concentraciones (4, 40 y 400 µg L^{-1}) durante 96 horas en sifones de *Ruditapes decussatus*. Nuestros resultados mostraron que la exposición a Topas indujo una disminución significativa en los niveles de ácidos grasos saturados. Sin embargo, se observó un aumento de ácidos grasos monoinsaturados y poliinsaturados, principalmente los ácidos eicosapentaenoico (C20:5n-3), docosahexaenoico (C22:6n-3) y araquidónico (C20:4n-6). La exposición a Topas mejoró los niveles de peróxido de hidrógeno, malondialdehído y carbonilos proteicos, además de alterar el estado antioxidante (enzimático y no enzimático) en todas las almejas tratadas. La actividad de la acetilcolinesterasa se inhibió con el aumento de las concentraciones de Topas. Finalmente, los cambios histopatológicos detectados en los animales tratados variaron de manera dependiente de la concentración y aquí fueron consistentes con los resultados bioquímicos. Nuestros hallazgos arrojan nueva luz sobre la relación entre el estado redox y los cambios en la composición de los ácidos grasos, lo que nos permite comprender la toxicidad provocada por Topas.

Palabras clave: *Ruditapes decussatus*, Topas, sifones, estado antioxidante, perfil de ácidos grasos, características histopatológicas.

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INTRODUCTION

The aquatic ecosystem serves as a primary sink for potentially dangerous chemicals released from industrial and domestic sources. Among these contaminants, pesticide use has skyrocketed in recent years. Approximately 2×10^9 kg are utilized annually (Kalliora et al. 2018), but only 0.1% of the applied pesticides reach the target pests raising public concerns about potential hazardous effects on non-target creatures (Hart and Pimentel 2002).

Coastal ecosystems such as lagoons are complex and dynamic, with constantly changing environmental circumstances (Kamel et al. 2014). Some Mediterranean lagoons in Tunisia have become the most extensively changed and threatened habitats, owing primarily to urbanization and agricultural and industrial releases (Barhoumi et al. 2014), which reach shorelines via precipitation, irrigation or groundwater flux (Surfrider 2020), making them an extremely useful system for ecotoxicological monitoring. At the local level, various studies have been conducted to assess the environmental influence associated with aquatic pollution in Tunisia, indicating different sources of pollutants and causes of pesticide contamination (López Martinez 2024). Indeed, in the Bizerte lagoon system, Necibi and Mzoughi (2020) reported contamination of sediments by pesticides, manifested by high concentrations of organochlorine pesticide pollutants. In addition, several authors have reported that the uncontrolled use of pesticides and fertilizers on agricultural land has increased the nitrate content in Tunisian aquatic ecosystem pollution (Grünberger et al. 2024).

Penconazole (PEN) is a typical sterol demethylation inhibitor of triazole fungicide that controls numerous pathogens in crops such as fruits, vegetables and tea plants (Husak et al. 2017). Several characteristics make it persistent in soil and water, such as high chemical stability and low biodegradability (Wang et al. 2011). Accordingly, there is concern about the extensive application of triazole fungicides and their possible detrimental effects on non-target organisms in both terrestrial and aquatic ecosystems resulting from spray drift and surface runoff (Konwick et al. 2006). However, little is known about PEN availability in surface water. For example, the study of Dalvie et al. (2003) showed that surface waters in the Western Cape, South Africa, contain less than 2 µg L^{-1} of this fungicide.

High concentrations of triazole fungicides cause various toxic outcomes, including carcinogenicity, reproductive toxicity and hepatotoxicity in mammals (Peffer et al. 2007). In addition, they are principally considered to affect lipid biosynthesis and metabolism pathways (Hermsen et al. 2011). An LC_{50} (median lethal concentration) of 20.55 mg L^{-1} for paclobutrazol has been reported for zebrafish (*Danio rerio*) (Ding et al. 2009). The acute toxicity of difenoconazole on zebrafish was 1.17 mg L^{-1} for larvae, 1.45 mg L^{-1} for adult fish, and 2.34 mg L^{-1} for embryos (Mu et al. 2013). The hepatic antioxidant enzymes, the RNA/DNA ratio and haematological and plasma biochemical parameters are affected in rainbow trout (*Oncorhynchus mykiss*) after exposure to 0.5 mg L^{-1} of propiconazole (Li et al. 2010a). A previous study also detected gene expression changes in zebrafish embryos after treatment with 4 mg/L of flusilazole (Hermsen et al. 2011). According to the harmonized classification and labelling approved by the European Union, PEN is highly toxic to aquatic organisms with long-lasting effects (hazard statements H400 and H410) (European Chemicals Agency (ECHA), 2023). However, the impact of PEN on bivalves, namely clams, is quite limited. Only the work of Yoloğlu (2019) reported the assessment of Na^+/K^+-ATPase, Mg^{2+}-ATPase, Ca^{2+}-ATPase, and total-ATPase activities in gills of freshwater mussels exposed to PEN.

In addition to their varying harmfulness, triazole fungicides are also known to induce oxidative stress, one of the main mechanisms of toxicity associated with these xenobiotics (Monserrat et al. 2007). Indeed, PEN has been displayed to increase production of reactive oxygen species (ROS), prompting alterations in the intracellular redox status and inducing oxidative damage to cellular macromolecules, as observed in invertebrates and vertebrate species (Chaâbane et al. 2016, Yoloğlu 2019). In this line, it is well documented that lipids and their most common components, fatty acids (FAs) are particularly susceptible to the oxidative reactions of ROS (Pamplona 2008). Though lipids and FAs have been demonstrated to be potent proxies for contamination-induced stress in animals (Signa et al. 2015), no scientific research has yet been released regarding PEN's impact on the composition of FAs in bivalves.

Molluscs, particularly bivalves, have assumed a major role in assessing levels of contaminants worldwide due to characteristics such as their sedentary behaviour, filter-feeding practices, high filtration rate and capacity to concentrate contaminants (Chalghmi et al. 2016). The clam *Ruditapes decussatus* (Linnaeus, 1758), one of the plentiful bivalves on the Tunisian coasts (Hamza-Chaffai et al. 2003) living in muddy sand deposits of coastal areas, is commonly used and judged as an effective sentinel species, specifically in confined coastal environments (Costa et al. 2013). Given the aforementioned factors, it is quite important to investigate the damage produced by Topas 100 EC to the antioxidant defence system, histoarchitecture, and FA profiles in the siphons of *R. decussatus*, which represent the primary routes of toxicants to enter its body at different concentrations (4, 40 and 400 µg L^{-1}). Hence, based on the current study, using the battery of cellular and biochemical markers along with histological analysis proves useful for assessing Topas 100 EC contamination in marine invertebrates.

MATERIAL AND METHODS

Chemicals and reagents

The commercial fungicide used in the present study was Topas, purchased from the Syngenta company (Bâle, Switzerland), which includes 100 g L^{-1} of penconazole, the active substance. Glutathione (GSH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA), 2,4-dinitrophenylhydrazine (DNPH), 1-chloro-2,4-dinitrobenzene (CDNB), ethylenediamine tetraacetic acid (EDTA), xylenol orange, hydrogen peroxide (H_2O_2), NaCl and nitro blue tetrazolium (NBT) were purchased from Sigma Chemical Co. (MO, USA). All other analytical-grade chemicals were obtained from different commercial suppliers.

Sampling method and experimental design

Clams were collected from a site that is far from anthropogenic activities within the Bizerte lagoon (37°11'20.4"N 9°51'16.2"E), located on Tunisia's North coast in the western Mediterranean Basin (Ghribi et al. 2020). To minimize differences in biochemical responses, organisms with similar shell length (3.86±0.39 cm) and weight (8.75±2.33 g) were collected in October 2021. *R. decussatus* specimens were carried straight to the laboratory at the Faculty of Science of Tunis in aerated tanks holding saltwater, and then acclimated for 72 hours in an aquarium containing 35 L of seawater with constant aeration (temperature 19±2°C, salinity 36±1, oxygen 6.2 mg/L and a 12 light to 12 dark photoperiod). The sampling area's properties were used to maintain salinity, temperature and pH during the acclimation and experimental periods (Ghribi et al. 2020). Following acclimation, the *R. decussatus* specimens were separated into four groups of 30 individuals each and transferred to 25 L experimental aquaria in a duplicate design (n=15) under controlled circumstances, as described in the present study.

Before exposure, the Topas 100 EC stock solution (Syngenta, Bâle, Switzerland) containing penconazole (active ingredient of 100 g L^{-1}) was prepared in seawater. We diluted the solution to obtain three concentrations (4, 40, and 400 µg L^{-1}). The Topas-exposed and non-exposed groups were designed as follows (Fig. 1):

- Group I (control): unexposed *R. decussatus* kept in Topas-free water.
- Group II (D1): *R. decussatus* exposed to Topas dose of 4 µg L^{-1} for 96 hours.
- Group III (D2): *R. decussatus* exposed to Topas dose of 40 µg L^{-1} for 96 hours.
- Group IV (D3): *R. decussatus* exposed to Topas dose of 400 µg L^{-1} for 96 hours.

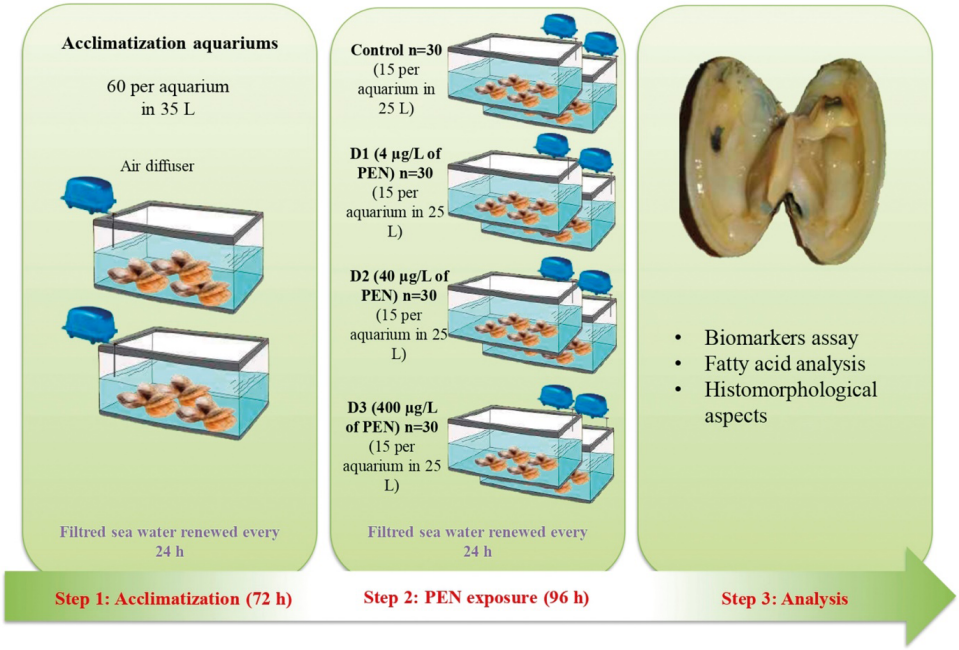

Fig. 1. - Experimental design of *Ruditapes decussatus* exposure to Topas.

The D1 concentration used in this study was based on the PEN concentration found in surface waters (2 µg L^{-1}) in the Western Cape, South Africa (Dalvie et al. 2003). To ascertain whether *R. decussatus* specimens are tolerant to elevated concentrations of this pesticide, D2 and D3 were selected to ensure a detectable impact of PEN.

The saltwater was replaced every 24 hours to ensure water quality, and Topas concentrations were restored. Throughout the experiment, there was no mortality in either the control or the Topas-treated groups. The trials were carried out following ethical standards (EC Directive 86/609/EEC) and were authorized by the Ethical Committee of the Faculty of Sciences of Tunis.

R. decussatus specimens (n=15) were dissected on ice to obtain siphon tissues, then homogenized in 10% Tris-HCl buffer (100 mM; pH=7.4) containing 1 mM EDTA and 1 mM PMSF and centrifuged at 9000 × g for 20 min at 4°C (Centrifuge UNIVERSAL 320R, Hettich, Germany). The obtained supernatants were stored in Eppendorf tubes at −80°C for biomarker assays (Fouzai et al. 2020a). Other gill specimens were homogenized using an Ultra-Turrax (T18: UGS 13396299, IKA, Germany) and then conserved at -20°C for FA analysis (n=6). For histological analysis, three specimens from each condition (n=3) were cleaned under running water, fixed in 10% buffered formalin solution and embedded in paraffin (Martoja and Martoja 1967).

Protein quantification

Siphon protein content was quantified following the method of Lowry et al. (1951), in order to normalize all oxidative stress biomarkers per mg of protein. Using bovine serum albumin as a standard, the quantity of protein in the reaction is related to its optical density at 500 nm. The protein values were expressed as mg of protein/g tissue.

Ferric-reducing antioxidant power activity

The ferric-reducing antioxidant power (FRAP), a simple and reliable colorimetric assay originally developed by Benzie and Strain (1996) was tested by adding 50 µL of the homogenate, 1500 mL of FRAP reagent containing acetate buffer (300 mM; pH 3.6), 2,3,5-triphenyltetrazolium chloride (TPTZ; 10 mM) and ferric chloride (20 mM) (10:1:1), followed by incubation for 10 min at room temperature.

Finally, the absorbance was measured at 593 nm. The values obtained were referred to a calibration curve from a 0.001 M ferrous sulphate heptahydrate ($S_2SO_47H_2O$) standard solution. The results were expressed as µmoles of FRAP/mg of protein.

Acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was evaluated in the siphon tissues according to the method of Ellman et al. (1961), using acetylthiocholine iodide as a substrate. Fifty µL of siphon supernatant was added to 850 µL of phosphate buffer (0.1 M; pH=7.5) and 50 µL of DTNB (0.01 M). After a 5 min pre-incubation at 20°C, the reaction begins with adding 50 µL of acetylthiocholine iodide (8.25 mM). The absorbance was measured at 412 nm and results were conveyed as nmoles of substrate/min/mg of protein.

Hydrogen peroxide levels

Hydrogen peroxide (H_2O_2) generation in siphon tissues was monitored by the ferrous ion oxidation xylenol orange (FOX1) according to the method of Ou and Wolff (1996). The technique consisted of adding 100 µL of the supernatant to 900 µL of the FOX1 buffer (sorbitol 0.1 M; orange xylenol 100 µM; ferric ammonium sulphate 250 µM; and sulfuric acid 25 mM) in a cuvette cell. The reagents were mixed, and absorbance was measured in a spectrophotometer at 560 nm. Residual H_2O_2 was calculated by reference to the extinction coefficient of H_2O_2 in the FOX1 reagent of 2.35×10^5 M^{-1} cm^{-1}. The results were presented as mmoles of H_2O_2 mg^{-1} protein.

Determination of lipid peroxidation product

The siphon concentrations of malondialdehyde (MDA), index of lipid peroxidation, were determined spectrophotometrically as described by Draper and Hadley (1990). An aliquot of 500 µL was mixed with 500 µL of trichloroacetic acid solution (TCA 30%). After centrifugation at 3500 g for 10 min at cold, 1 mL of a solution containing 0.67% TBA (pH: 7.4) was added to 1 mL of supernatant and then incubated for 15 min at 90°C and cooled. The absorbance of the TBA-MDA complex was quantified at 532 nm using a spectrophotometer. The MDA values were calculated using TEP (1,1,3,3 tetraethoxypropane) as standard and expressed as nmoles of MDA/mg of protein.

Determination of protein oxidation products

The siphon levels of advanced oxidation protein products (AOPP), biomarkers of protein oxidation, were determined according to the method of Kayali et al. (2006). Briefly, 400 µL of the siphon supernatant was mixed with 0.8 mL of phosphate buffer (0.1 M; pH 7.4). After 2 min, 0.1 mL of 1.16 M potassium iodide (KI) was treated with the previous solution followed by 0.2 mL of acetic acid. The absorbance of the reaction mixture was observed at 340 nm. The concentration of AOPP was calculated using the extinction coefficient of 261 mM^{-1} cm^{-1}, and the results were expressed as nmoles of AOPP/mg of protein.

Protein carbonyl (PCO) content in siphon was determined according to the method of Reznick and Packer (1994). Simply, 100 µL of the aqueous phase was added to 500 µL of DNPH (10 mM) and hatched for 1 h in darkness. Then, trichloroacetic acid (20%) was added and the mix was centrifuged at 3500×g for 10 min at 4°C after 15 min. After centrifugation, the siphon pellet was washed more than two times with ethyl acetate-ethanol (V1:V1; 1 mL) followed by centrifugation at 4000 × g for 15 min. The reaction was activated when the precipitate was dissolved in guanidine (6 M), and the absorbance was read at 370 nm. Calculation of the PCO level was based on the molar extinction coefficient of DNPH (£=2.2×10^4 M^{-1} cm^{-1}), and the results were expressed as µmoles of PCO/mg of protein.

Determination of non-enzymatic and enzymatic antioxidant activities

Total GSH concentration in the siphon tissues was quantified by the reduced glutathione recycling assay of Ellman (1959) modified by Jollow et al. (1974), which is based on the development of a yellow colour when DTNB is added to compounds containing sulfhydryl groups. An aliquot of 500 µL of siphon homogenate was deproteinized by addition of 3 mL of sulfosalicylic acid (4%) and then centrifuged at 1.600×g for 15 min. Five hundred mL of supernatant was taken and added to Ellman's reagent.

The absorbance of GSH levels was measured at 412 nm after DTNB addition (10 mM). The level of GSH was calculated by a standard concentration and conveyed as µg of GSH/mg of protein.

The metallothionein (MT) content of siphons was evaluated according to Viarengo et al. (1997) modified by Petrović et al. (2001). One mL of siphon supernatant was added to 1 mL of cold absolute ethanol and 80 µL of chloroform and centrifuged at 6000×g for 10 min. The resulting supernatant was mixed with absolute ethanol (3V) and incubated at -20°C for 1 h. After incubation, the mixture was centrifuged at 6000×g for 10 min and the pellet was cleaned with 87% ethanol and 1% chloroform. The pellet containing MTs was resuspended in 150 µL NaCl (0.25 M) and 150 µL HCl (0.5 N) containing EDTA (2 mM). Before centrifugation at 3000 g for 5 min, 4.2 mL of NaCl (2 M) containing DTNB (0.6 mM) buffered with Na-phosphate (0.2 M; pH=8) was added to each pellet at room temperature. MT absorbance was measured at 412 nm and the results were expressed as µmoles GSH/mg of protein using GSH as a standard.

Ascorbic acid (AA) content in siphon tissues was determined using the DNPH method described by Jacques-Silva et al. (2001). Protein was precipitated in a cold trichloroacetic acid solution (4%), centrifuged for 10 min and incubated at 85°C for 30 min with DNPH (4.5 mg mL^{-1}) and CuSo4 (0.075 mg mL^{-1}). The reaction product was detected at 540 nm and results were expressed as mmoles of AA/mg protein.

Catalase (CAT) activity was estimated according to the method of Aebi (1984), using H_2O_2 (0.5 M) as a substrate. The reaction was started by adding an aliquot of 20 µL of the homogenized siphon and the substrate (H_2O_2) to a concentration of 0.5 M in a medium containing 100 mM phosphate buffer (pH 7.4). The H_2O_2 decomposition level was followed by monitoring absorption at 240 nm ($\varepsilon = 40\ mM^{-1}\ cm^{-1}$). CAT activity was calculated in terms of nmoles H_2O_2 consumed/min/mg protein.

Determination of superoxide dismutase (SOD) activity was based on the ability of superoxide dismutase to inhibit the reduction of NBT by superoxide anion as described by Beauchamp and Fridovich (1971). The reaction was started by adding NBT (2.64 mM) to the mixed supernatant. One unit (U) of SOD activity corresponded to the amount of enzyme required to cause 50% inhibition of NBT reduction at 560 nm. SOD activity was expressed as international units per milligram soluble protein (U/mg protein).

Glutathione peroxidase (GPx) activity in siphon was measured using reduced GSH as a substrate according to the method of Flohé and Günzler (1984). A 200 µL aliquot of siphon extract was mixed with 100 µL of phosphate buffer (0.1 Mm; pH=7.4) and 200 µL of glutathione (4 mM). This mixture was incubated for 10 min at 37°C and then 500 µL of H_2O_2 (5 mM) and 1 mL of TCA (5%) were added. The reaction was detected after addition of DTNB (10 mM) to the 100 µL of the mixture using spectrophotometric absorbance at 420 nm. GPx amounts were extrapolated using the extinction coefficient of 6.22 $mM^{-1}\ cm^{-1}$ and expressed as nmoles of GSH oxidized/min/mg protein.

Glutathione S-transferase (GST) activity in siphon tissues was determined according to the method of Habig et al. (1974), using CDNB (60 mM) as a substrate. A 10 µL aliquot of siphon extract was combined with 390 µL of phosphate buffer (100 mM; pH=6.5). This mixture was vortexed. Then, 100 µL was obtained and incorporated within 200 µL of the reaction solution (containing 4.95 mL of phosphate buffer (100 mM; pH=6.5), 0.9 mL of GSH (10 mM) and 0.15 mL of CDNB (60 mM)). The absorbance was measured at 340 nm for 2 min, and the results were expressed as mmoles of GST/min/mg of protein.

Determination of FA composition

Total lipids were extracted from the control and the treated *R. decussatus* siphons using a chloroform-methanol (2v/1v) solution as a mixture solvent with 0.01% butylated hydroxyl toluene, as described by Floch et al. (1957). The lipid extract fraction was trans-esterified to methyl esters by the addition of sodium methylate ($NaOCH_3$) and sulfuric acid (H_2SO_4) following the procedure of Cecchi et al. (1985). The nonadecanoic acid (C19:0) (Sigma) was utilized as an internal standard. Fatty acid methyl esters were recovered by centrifugation at 3000 tr for 10 min and evaluated using gas chromatography employing an Agilent Technologies HP 6890 chromatogram equipped with an INNO-WAX capillary column (30 m × 0.25 µm) and supplied by a carrier gas: nitrogen. FA peaks were integrated using the Agilent G2070BA GC Hewlett-Packard Chemstation ssoftware and identified by comparing their durations of retention to the reference methyl esters (Supelco 47,085 U PUFA No 3 and Supelco 37 component FAME mix 47,885-U) and marine oil (Mehaden oil by Supelco). The FA composition of siphons was expressed as a percentage.

Histopathological analysis

For histological examination, the technique described by Martoja and Martoja (1967) was used. The siphons were extracted from living animals, taking care not to harm them and avoiding the overabundance of mucus and fine sand that frequently stick to their epithelia. Siphon sections were promptly fixed for 48 hours in buffered formalin (10%). Then they were moved into a series of graded ethanol solutions (70%), cleared in toluene and embedded in paraffin wax. Sections were cut (thickness ≈ 0.5 µm) using a rotative microtome (Thermo Scientific; Shandon Finesse 325) and stained with haematoxylin-eosin. The histological sections were examined in detail under light microscopy (Leica DM 750 equipped with an ICC50 HD camera and LAS EZ software; Leica, Germany).

Statistical analysis

Results were expressed as means±standard error. The R package version 4.2.2 was used for statistical analysis. The normality of data was first checked using the Shapiro-Wilk W test. Then, the homogeneity of variance was tested using the Levene test. Significant differences between each exposed and control group were evaluated by one-way analysis of variance followed by Tukey's post hoc test. Differences were deemed significant when the p value was lower than 5%. Principal component analysis (PCA) and THE Spearman correlation matrix were performed using the FactoMineR R package and the corrplot R library to assess the significant differences between the biochemical parameters of PEN-treated and untreated clam siphons. Furthermore, we used the Heatmaply R package to produce a heatmap and a hierarchical clustering dendrogram analysis that elucidated the behaviour of FAs at every PEN concentration.

RESULTS

Estimation of FRAP capacity and AChE activity

As shown in Figure 2A, the antioxidant capacity measured by FRAP was increased significantly in all treated Topas groups ($p<0.01$). However, AChE activity decreased significantly by 74% and 65% in groups D2 and D3 during the treatment period (Fig. 2B).

Estimation of H_2O_2 levels and lipid peroxidation (MDA) index

The effect of Topas on H_2O_2 amounts and lipid peroxidation index (MDA) levels in siphons is summarized in Table 1. Our results showed an increase in H_2O_2 levels (+31%) at dose D3 compared with the untreated group (control). Moreover, our data revealed a significant increase (+37%) of MDA levels at dose D1 when compared with the corresponding control values.

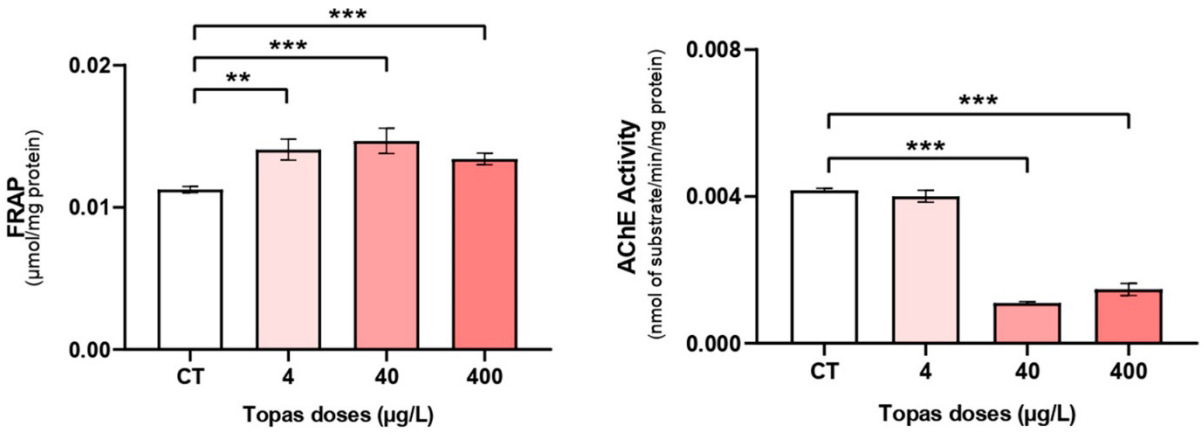

Fig. 2. - (A) FRAP capacity and (B) acetylcholinesterase activity (AChE) in the siphons of untreated (control) and treated *Ruditapes decussatus* with different concentrations of Topas (4, 40 and 400 µg L^{-1}) for 96 hours. Values are expressed as means±SE (n=8). D1, D2 and D3 groups vs control group (CT): *p<0.05, **p<0.01, ***p<0.001.

Table 1. - Hydrogen peroxide (H_2O_2), malondialdehyde (MDA), advanced oxidation protein product (AOPP) and protein carbonyl (PCO) levels in siphons of *Ruditapes decussatus* under exposure to graded Topas doses (4, 40 and 400 µg L^{-1}) for 96 hours.

Parameters and treatment	Control	4 µg L^{-1}	40 µg L^{-1}	400 µg L^{-1}	F¥/H£	P
H_2O_2 α	9.88±0.16[a]	9.5±0.47[a]	8.75±0.22[a]	12.9±0.53[b]	21.65	<0.001
MDA β	15.64±1.14[a]	21.43±1.74[b]	17.72±1.32[ab]	19.88±0.99[ab]	3.66	0.0242
AOPP β	8.36±0.14[a]	8.18±0.21[a]	11.42±0.86[b]	16.26±0.7[c]	25.22	<0.001
PCO λ	23.62±0.51[a]	40.78±2.57[b]	39±1.37[b]	48.66±2.19[c]	32.42	<0.001

Values are means±standard error for eight clams in each group.
Superscript letters a, b, and c indicate significant differences ($p<0.05$) between exposure concentrations.
α mmol/mg of protein
β nmol/mg of protein
λ µmol/mg of protein
¥ ANOVA F Test
£ Kruscal-Wallis H test

Estimation of protein oxidation: AOPP and PCO levels

Total protein damage was determined by measuring both the AOPP and the PCO derivatives (Table 1). Topas exposure was found to increase the amount of AOPP and PCO in the groups treated with doses D2 and D3, respectively, when compared with the control.

Enzymatic antioxidant levels

Table 2 and Figure 3 show the levels of enzymatic antioxidative responses in the siphons of *R. decussatus* clams. SOD activities were significantly incremented by the Topas treatment (+20 and +59%) at doses D2 and D3 when compared with the control group, while no significant change was observed at dose D1. For CAT activity, a significant enhancement was observed in siphon tissues of *R. decussatus* ($p<0.001$) exposed to all three concentrations of Topas when compared with the negative control group (Table 2). Additionally, Topas exposure led to a significant 44% increase in GPx activity in siphons of clams treated with dose D3 when compared with the control. For GST activity ($p<0.001$), a significant increase was recorded for all treated groups (Table 2).

Non-enzymatic antioxidants levels

The effect of Topas on non-enzymatic biomarkers, including MTs, GSH and AA in siphon tissues of *R. decussatus*, is summarized in Table 2 and Figure 3. As shown, a significant 140% increase in MT levels at dose D3 was observed when compared with the controls. Moreover, GSH levels at the two tested concentrations (D2 and D3) were significantly increased by 24% and 28%, respectively, compared with the control values, and AA levels in Topas-treated groups tended to increase significantly ($p < 0.001$) by 133% at dose D3.

Fatty acid composition

The FA profiles of the siphons are illustrated in Table 3. Twenty-four different FAs were identified for all specimens. The major FA class in the untreated (control) group was saturated fatty acids (SFAs) (up to 47.11% of total FAs), followed by polyunsaturated fatty acids (PUFAs) and then monounsaturated fatty acids (MUFAs) (with 43.03% and 9.87%, respectively, of total FAs) in siphons. Overall, compared with the control group, PUFAs increased significantly, whereas SFAs decreased significantly. Following the treatment of *R. decussatus* with Topas, the amount of n-6 PUFAs (16.73% of total PUFAs in the control group in siphons) increased significantly ($p<0.001$) in siphons. In addition, a similar trend was observed for those of n-3 PUFAs in doses D1 and D2 (24.35% of total PUFAs in the control group) but showed a significant depletion ($p<0.05$) at the highest dose exposure, D3.

Table 2. - Enzymatic and non-enzymatic parameters: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), metallothionein (MTs), glutathione reduced (GSH) and ascorbic acid levels in siphons of *Ruditapes decussatus* under exposure to graded doses of Topas (4, 40 and 400 µg L^{-1}) for 96 hours.

Parameters and treatment	Control	4 µg L^{-1}	40 µg L^{-1}	400 µg L^{-1}	F^{α} /H^{∂}	P
SOD$^{\alpha}$	229.48±4.54^{a}	246.37±9.01^{ab}	275.11±10.73^{b}	364.72±14.33^{c}	22,39	<0.001
CAT$^{\beta}$	10.12±0.47^{a}	66.6±1.26^{b}	24.25±0.73^{c}	54.76±0.76^{d}	29,09	<0.001
GPx$^{\lambda}$	233.582±0.93^{a}	257.11±5.05^{a}	235.6±8.7^{a}	336.28±17.38^{b}	7,83	<0.001
GST$^{\varepsilon}$	0.002±0^{a}	0.007±0^{b}	0.01±0.001^{c}	0.01±0^{c}	25,35	<0.001
MTs$^{\gamma}$	0.99±0.06^{a}	1.21±0.1^{a}	1.13±0.04^{a}	2.38±0.11^{b}	20,08	<0.001
GSH$^{£}$	12.55±0.66^{a}	14.42±0.76^{a}	15.59±1.4^{b}	16.01±0.78^{b}	2,7	0.0654
Ascorbic acid $^{¥}$	11.16±0.53^{a}	13.61±1.26^{a}	12.74±1.06^{a}	26±2.3^{b}	19,59	<0.001

Values are means ± standard error for eight clams in each group.
Superscript letters a, b, c and d indicate significant differences ($p<0.05$) between exposure concentrations.
$^{\alpha}$ unit/mg of protein
$^{\beta}$ nmol of H_2O_2/min/mg of protein
$^{\lambda}$ nmol of GSH/min/mg of protein
$^{\varepsilon}$ mmol/min/mg of protein
$^{\gamma}$ µmol of GSH/mg of protein
$^{£}$ µg/mg of protein
$^{¥}$ mmol/mg of protein
$^{\alpha}$ ANOVA F Test
$^{\partial}$ Kruscal-Wallis H test

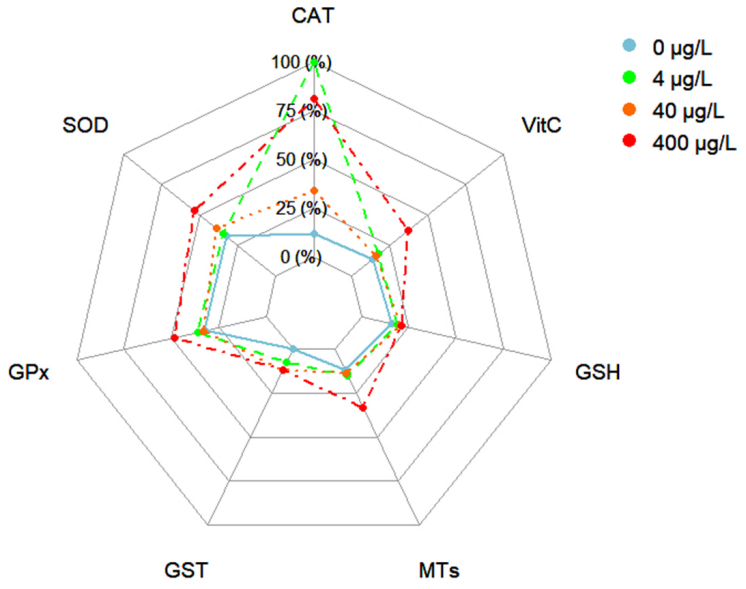

Fig. 3. - The star plots of biomarker responses (CAT, SOD, GPx, GST, MTs, GSH and AA) for the clam *R. decussatus* exposed to different concentrations of Topas (4, 40 and 400 µg L^{-1}) for 96 hours.

Table 3. - The fatty acid composition (%) in the siphon tissue of *Ruditapes decussatus* control (CT) and groups exposed to different doses of Topas (4, 40 and 400 µg L^{-1}) for 96 hours.

Fatty acids	Control	D1 (4 µg L^{-1})	D2 (40 µg L^{-1})	D3 (400 µg L^{-1})
C14: 0	24.15±0.88	21.21±1.85	5.51±0.48***	21.69±0.47*
C15: 0	1.26±0.12	1.13±0.17	0.81±0.09*	1.91±0.19*
C16: 0	11.57±0.6	7.58±0.68**	4.82±0.3***	7.79±0.22**
C18: 0	4.64±0.12	5.86±0.63	3.72±0.18**	5.74±0.27**
C20: 0	2.07±0.22	0.22±0.05***	2.09±0.16	0.85±0.09**
C22: 0	3.42±0.53	0.37±0.02**	5.56±0.25**	0.41±0.02**
ΣSFA	**47.11±0.31**	**36.38±0.46***	**22.51±0.85*****	**38.39±0.47*****
C15: 1	0.42±0.03	0.41±0.05	0.5±0.002*	1.2±0.09***
C16: 1	2.1±0.05	2.1±0.36	1.28±0.17**	1.09±0.11***
C18: 1	3.25±0.6	10.45±1.27**	2.85±0.29	6.52±0.18**
C20: 1	2.76±0.51	3.19±0.44	4.17±0.28*	4.1±0.33*
C22: 1	1.34±0.11	0.62±0.08**	2.59±0.12***	0.58±0.06**
ΣMUFA	**9.87±0.85**	**16.77±0.76***	**11.39±0.26**	**13.49±0.27****
C18: 2n6 (LA)	6.95±0.68	3.95±0.36**	2.4±0.09**	2.6±0.41**
C18: 3n6	0.85±0.11	1.07±0.13	0.77±0.07	2.4±0.37**
C18: 3n3 (ALA)	3.17±0.16	2.34±0.22*	2.38±0.22*	1.09±0.11***
C18: 4n3	2.93±0.33	5.12±0.45**	3.79±0.09*	2.17±0.23
C20: 2n6	2.86±0.19	13.07±0.81***	3.89±0.18**	11.15±1.19**
C20: 3n6	0.76±0.07	2.41±0.11***	3.2±0.15***	1.1±0.16
C20: 4n6 (ARA)	2.22±0.27	3.38±0.38*	5.79±0.18***	2.56±0.34
C20: 5n3 (EPA)	3.68±0.23	4.41±0.34	5.11±0.23**	2.86±0.16*
2i/2j (NMID)	1.94±0.08	3.55±0.47*	8.33±0.09***	2.97±0.22**
C22: 2n6	2.22±0.23	1.88±0.17	7.15±0.47***	1.39±0.25*
C22: 5n6	0.87±0.1	0.34±0.05**	3.74±0.51**	0.51±0.06*
C22: 5n3 (DPA)	2.43±0.09	3.23±0.41	6.16±0.46***	2.56±0.35
C22: 6n3 (DHA)	12.14±0.51	10.36±0.85	13.39±0.06*	14.76±0.28**
ΣPUFA	**43.03±0.54**	**55.11±0.31****	**66.09±0.87*****	**48.12±0.28*****
n-3 PUFA	24.35±0.28	25.47±1.74	30.83±0.42***	23.45±0.18*
n-6 PUFA	16.73±0.6	26.1±0.88***	26.93±0.44***	21.7±0.5**
n-6/n-3 PUFA	0.69±0.03	1.03±0.04**	0.87±0.01**	0.93±0.03**

Values are expressed as means±standard error (n=6).
*p < 0.05; **p < 0.01; ***p < 0.001.
SFA, saturated fatty acid(s); MUFA, monounsaturated fatty acid(s); PUFA, polyunsaturated fatty acid(s); NMID, non-methylene-Interrupted dionic fatty acids (C22:2i + C22:2j); n-3 PUFA, omega 3 fatty acids; n-6 PUFA, omega 6 fatty acids; ARA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; SA, stearic acid; GLA, γ-linolenic acid.; LA, linoleic acid; ALA, α-linolenic acid. The bold values in Table 3 represent the sum of SFA, MUFA, and PUFA. This bold formatting is used to highlight these totals.

Our results revealed a significant increase in arachidonic acid (ARA, C20:4n-6) in the siphons treated with the doses D1 and D2 when compared with the control. For the levels of eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) in siphons, similar responses were observed, with a significant increase ($p<0.01$) of EPA at dose D2 and a significant increase of DHA ($p<0.01$) at doses D2 and D3. Consequently, there was a significant positive correlation between EPA and DHA levels in siphons. A non-methylene-interrupted dienoic (NMID) FA (C22:2i/2j) was found in our specimens and its level was greatly elevated in all treatment groups. Following the identification of all FAs, the data were utilized for a hierarchical cluster analysis using a heatmap and a dendrogram. This produced a group of samples of the control group that had been isolated from the three Topas doses (Fig. 4).

Histopathological analysis

Histopathological observations showed significant lesions of the tissue analysed compared with specimens collected from the control tanks (Fig. 5). The siphons of the control group showed no morphological abnormalities and well-defined epithelial cells (Fig. 5A). In contrast, compared with the control group, exposure to Topas at different concentrations led to an increase in damage severity in a concentration-dependent manner. Contaminated siphons exposed to doses D1 and D2 of Topas showed changes such as rupture of epithelial cells (Fig. 5B and C). Dose D3 caused further and more severe histological damage ranging from lipofuscin granules to rupture, haemocyte infiltration, vacuolization and deformation of epithelial cells when compared with the control group (Fig. 5D).

Multivariate analysis

The PCA, applied to better elucidate the differential effects of the series of Topas concentrations, produced a two-dimensional pattern explaining 65.9% of the total variance, including factor 1 (43.7%) and factor 2 (22.2%), as shown in Figure 6. The PCA biplot of all the biochemical data depicted a clear separation between control and Topas-exposed clams. In this line, a strong correlation was noted between the oxidative stress biomarkers and doses D1, D2, and D3, as evidenced by an increase in FRAP, MDA, H_2O_2, AOPP, PCO, and GSH levels, as well as SOD, CAT, GPx, GST, AA and MT levels (Fig. 7). Overall, the PCA performed on the whole dataset highlighted the clear separation between the experimental groups, indicating activation of detoxification mechanisms. The relationships observed between the studied parameters were statistically confirmed using Pearson's linear correlation analysis, as represented in Figure 7.

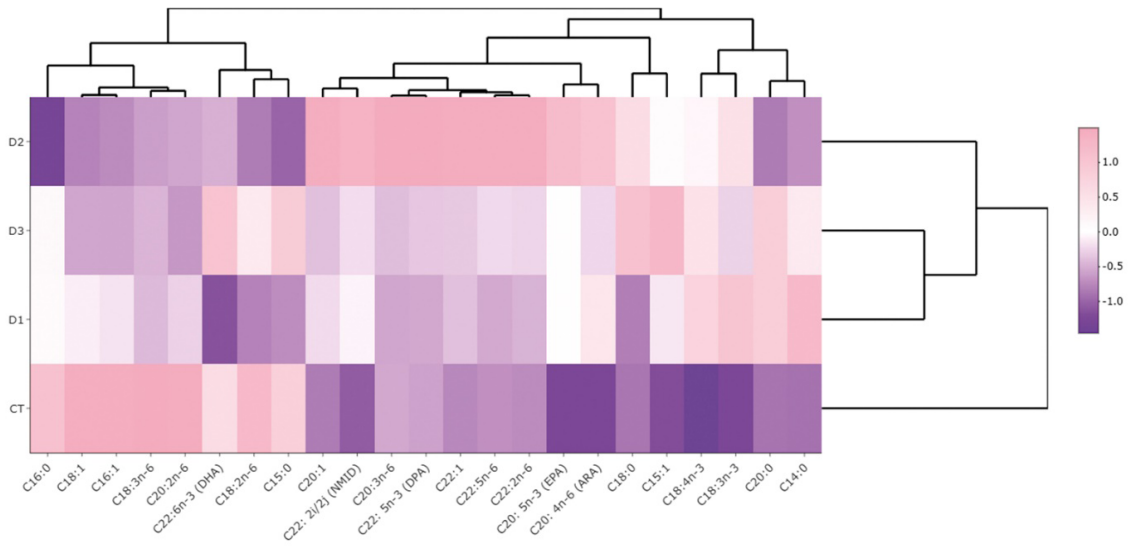

Fig. 4. - Heatmap depicting an overall comparison of the whole fatty acids data set in *Ruditapes decussatus* siphons against different concentrations of Topas (µg L^{-1}). The pink square border shows the fatty acids with the highest sensitivity when compared with the respective ones in the control. CT, control; D1, 4 µg L^{-1}; D2, 40 µg L^{-1}; D3, 400 µg L^{-1}.

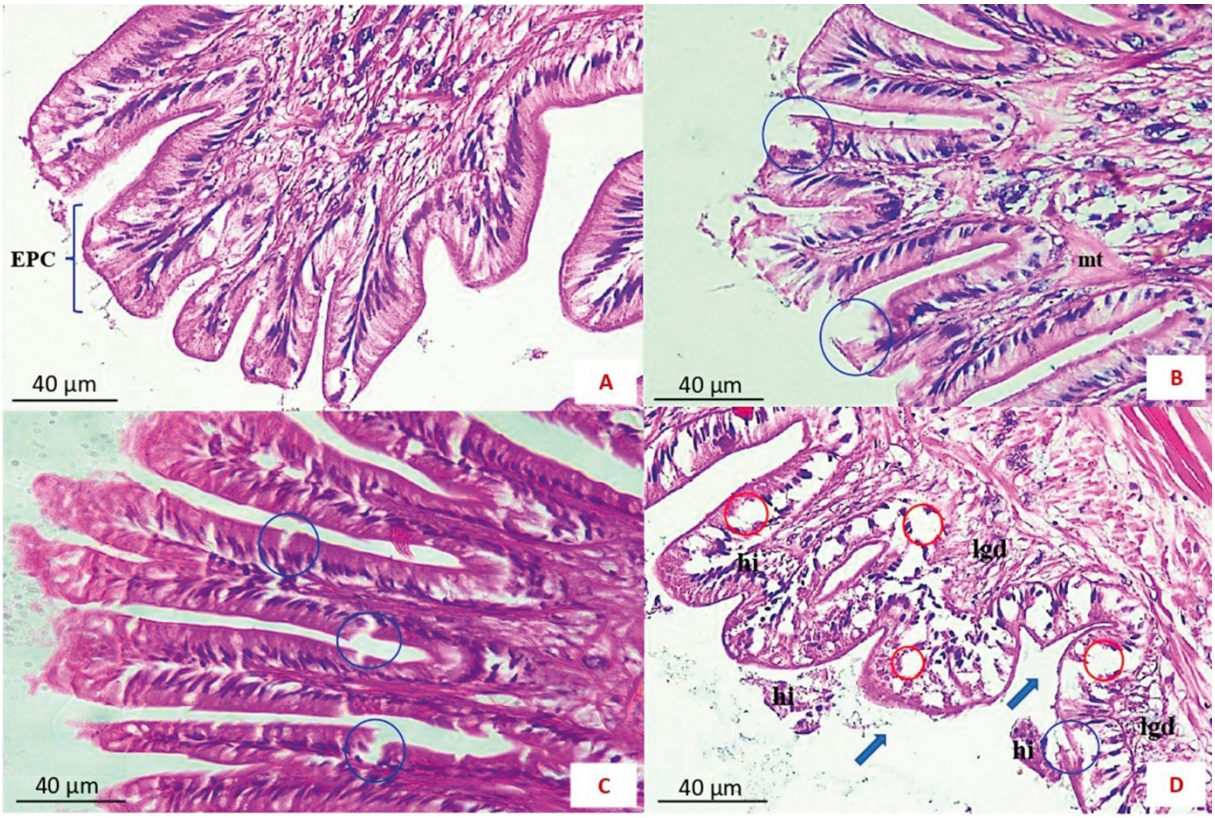

Fig. 5. - Representative histological sections of the siphons of *Ruditapes decussatus* control (A) and exposed to different Topas concentrations (B, 4 µg L^{-1}; C, 40 µg L^{-1}; D, 400 µg L^{-1}) after 96 hours of exposure, stained with haematoxylin-eosin. EPC, epithelial cells; mt, muscular tissue; hi, haemocyte infiltration; lgd, lipofuscin granule degradation. Blue circles indicate rupture; red circles indicate vacuolization; arrowheads indicate deformation of epithelial cells. Scale bar=40 µm

DISCUSSION

Health risks posed by emerging chemicals, including pesticides such as penconazole, are a growing global concern due to their widespread presence, increased exposure and substantial toxic effects (Chaâbane et al. 2018, Yoloğlu 2019). However, few studies have examined PEN's distribution in non-target organisms such as mussels, crayfish and fish (Icoglu Aksakal and Ciltas 2018, Yoloğlu 2019, Alkan Uçkun and Barım Öz 2020). The current report offers valuable insights into the potential oxidative damage in the siphons and the FA composition of the bivalve *Ruditapes decussatus* exposed to the triazole fungicide Topas, which contains PEN. This study provides new tools for evaluating the sensitivity of clams to environmental pollution, grounded in scientific understanding of metabolic pathways linked to adaptation under stress from varying doses of Topas.

Following Husak et al. (2017), Topas enhanced generation of ROS such as superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$) and induced oxidative stress in fish. This finding was supported herein by a significant rise in H_2O_2 levels in the D3 group, possibly implying an aberration of the mitochondrial respiration chain. Using the Fenton/Haber-Weiss route, H_2O_2 reacts with free iron (Fe^{2+}) to produce a more reactive and combative radical species, $HO^{\bullet}$ (Krumova and Cosa 2016). This chemical is involved in the lipid peroxidation (LPO) process, resulting in loss of membrane integrity (Ayala et al. 2014). In agreement with this, the current study found that all Topas-treated clams developed LPO, showing that the accumulation of Topas in the organism resulted in oxidative toxicity that exceeded the antioxidant defence capacity of the clams and caused oxidative damage. In accordance with our results, it has been reported in various studies that MDA levels rise significantly in fish tissues due to pesticide use (Hatami et al. 2019).

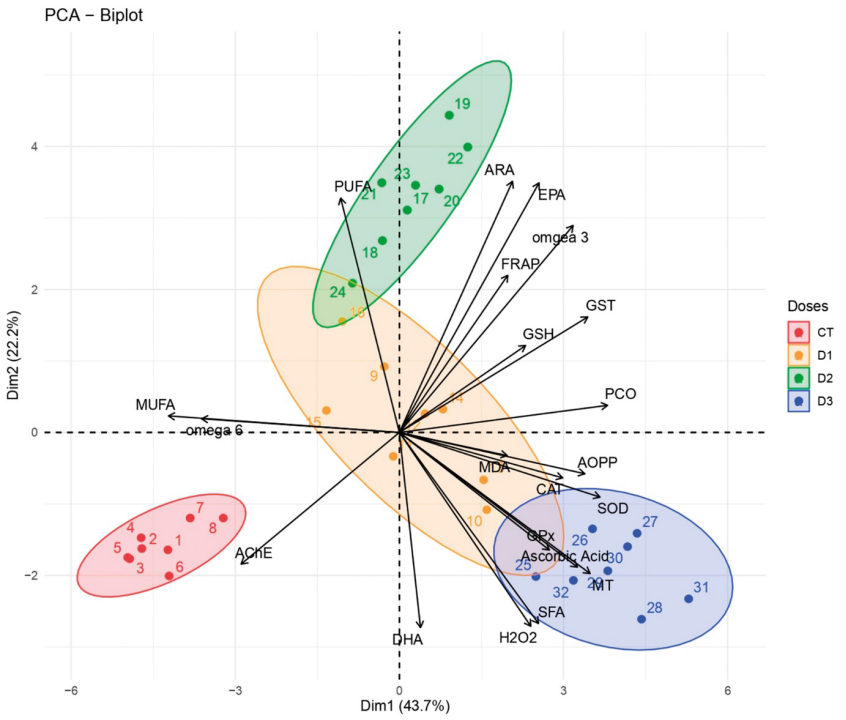

Fig. 6. - Principal component analysis (PCA) represented by two factors (F1=43.7% and F2=22.2%) and produced by biochemical variables (MDA, H_2O_2, PCO, CAT, GPx, SOD, GSH, AA, MT, AChE, PUFA, MUFA, SFA, ω6 (n-6 PUFA), ω3 (n-3 PUFA), ARA, EPA, DHA) in *Ruditapes decussatus* siphons of control (CT) and specimens treated with a series of Topas concentrations (D1, 4 µg L^{-1}; D2, 40 µg L^{-1}; D3, 400 µg L^{-1}) for 96 hours.

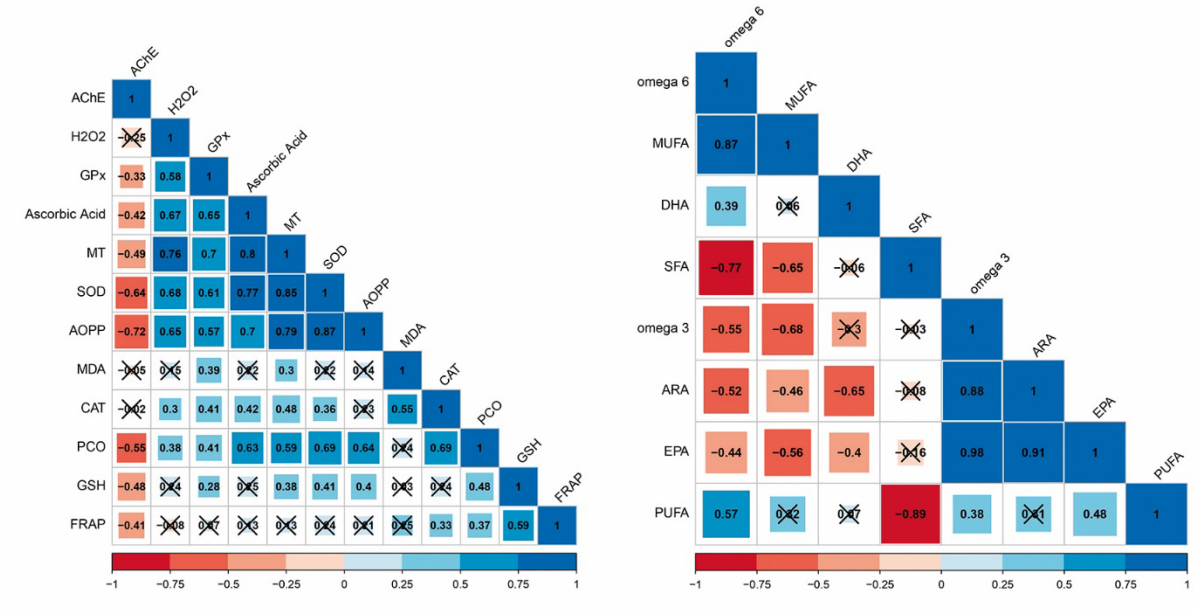

Fig. 7. - Correlation matrix for redox state biomarkers and fatty acid profiles. Each cell indicates Pearson correlation coefficient value, r, for a pair of biomarkers. Positive correlations (0<r<1.0) are displayed in blue and negative correlations (–1<r<0) in red. Colour intensity is proportional to the correlation coefficients.

Furthermore, to protect themselves against stressful environmental conditions, a considerable change in the FA composition was noticed in all Topas-treated clams, following LPO (Trabelsi et al. 2019, Fouzai et al. 2020b). The PUFAs (such as ARA, EPA and DHA) have been identified as essential components of all cell membranes and tissues. They are crucial components that not only determine the nutritional value of clams but also guarantee normal growth and development, and recently they have been used as a bio marker to determine xenobiotic impacts (Monroig and Kabeya 2018). In addition, they influence membrane permeability, cell signals and several physiological pathways, as well as providing energy (Liu et al. 2015). The current findings revealed a clear alteration in the lipid fraction of treated *R. decussatus* siphons compared with the control values. There was a tendency for an increase in PUFAs, ARA, EPA, and DHA in Topas-treated groups compared with the control. This result could be interpreted as an adaptive response of clams against environmental stressors, where they expend effort and energy to scavenge the ROS overproduction and attempt to maintain the stability and fluidity of lipid membranes. Conversely, this investigation showed a highly significant decline in SFA amounts in all treated groups. These FAs may be used as an energy source due to their high caloric content. As a result, SFA levels may decrease following Topas exposure. Consistent with this, the findings described above showed that the PUFA levels showed an opposite tendency to the SFA levels. In this line, the increase in PUFAs may occur mainly at the expense of SFA levels. Indeed, the decrease in SFA amount may be due to its metabolization when the organism completes the elongation and the desaturation processes to synthesize the PUFAs, which showed a higher concentration in the treated groups. According to Yin et al. (2017), these essential FAs improve the stress resistance of aquatic organisms. It is well known that ARA is naturally stored within lipid bodies in immune cells (Tallima and El Ridi 2018). There are many biological activities involved in its metabolism, including the regulation of innate immunity and the resolution of inflammation (Calder 2010). Indeed, from the significant increase in the n-6 PUFA/n-3 PUFA ratio it can be inferred that this pollutant has a pro-inflammatory effect. Consequently, the significant rise in ARA levels observed in Topas-treated siphons may reflect its involvement in the inflammatory cell response (Calder 2010) and highlight one of the defensive mechanisms of the molluscs to mitigate the harmfulness of Topas.

In addition, NMID FA (C22:2i/2j) can be synthesized de novo by bivalves and used to recover the more sensitive PUFAs such as DHA (Zhukova 1991). Their isolated double bonds can protect membrane phospholipids by slow auto-oxidation, contrary to the normal structure of PUFAs (Fokina et al. 2013, Signa et al. 2015). To the best of our knowledge, this is the first study to evaluate Topas's effect on NMID FAs. In the siphons of Topas-treated groups, NMID FAs were detected at higher levels than the control values, indicating that increased LPO had resulted in a loss of membrane fluidity. Taken together, these results indicate that Topas has toxic effects likely via affecting lipid metabolism. Our results were in line with those recorded in bivalves exposed to lead and acrylamide, respectively (Chetoui et al. 2019, Trabelsi et al. 2019). Overall, the hierarchical cluster analysis confirmed the sensitivity of the FA composition of siphons to Topas exposure.

It has been reported in the present study that some PUFAS (ARA, EPA and NMID) involve a biphasic response characterized by enhancement at dose D2 followed by diminishment at dose D3 in siphon tissues of *R. decussatus.*

This pattern may reflect a general adaptive response to cope with stress or as part of the organism's reaction to the stressor. The significant increase observed at dose D2 could result from changes in lipid metabolism or activation of enzymes involved in FA synthesis. However, at dose D3, the fungicide may disrupt normal enzyme function in FA synthesis and metabolism, causing a decrease in their levels. Thus, responses to chemical stress may vary with exposure time, dose and the vulnerability of the species (Cheung et al. 2001).

As with lipids, other major functional components such as proteins, mainly those of the membrane, may be the target of ROS attack (Fokina et al. 2013). The generation of free radicals can cause structural and functional damage to proteins (Alderman et al. 2002), as evidenced in our study by the significant increase in protein oxidation indicators, primarily AOPP and PCOs, in all treated clam siphons. According to our Pearson correlation, these findings are correlated with the H_2O_2 levels, thereby reflecting an excess of ROS production and protein oxidative damage in clam siphons.

Exposure of *R. decussatus* siphons to Topas may lead to irreversible and adverse changes at the cellular level in this soft body part. Consequently, 96 hours of treatment at higher Topas concentrations significantly improved FRAP capacity in siphon tissue. According to Llesuy et al. (2001), the FRAP assay has been established as a reliable measure of the system's ability to control ROS-induced damage.

Following this, our results clearly reflected the significant enhancement of FRAP to neutralize ROS damage (Fig. 2A). Based on the above results and to further prevent cellular oxidative damage, antioxidant enzymes such as SOD, CAT, GPx and GST play a crucial role in helping organisms adapt to stressful conditions by protecting against ROS overproduction and lipid peroxidation, thereby preventing cellular oxidative damage (Ullah et al. 2014). Accordingly, the overgeneration of H_2O_2 in exposed clams, especially at dose D3, may be due to the action of SOD, which was significantly higher under this dose. SOD can convert superoxide radical (O_2^{-}) to H_2O_2 and molecular oxygen (O_2), consequently rendering the potentially harmful (O_2^{-}) less hazardous (Rodríguez-Fuentes et al. 2015). Similar findings were reported in goldfish that were exposed to PEN (Husak et al. 2017). The activity of the primary antioxidant enzyme CAT also increased in Topas-treated siphons, similar to findings in the liver of *G. aculeatus* under prochloraz contamination (Sanchez et al. 2008) and in rainbow trout liver after long-term exposure to propiconazole (Li et al. 2010b). To break down H_2O_2 into water (H_2O) and mitigate oxidative stress, the rise in GPx activity in all exposed specimens indicates a collective response from other enzymatic activities to protect cells from damage. The data of the present study are in accordance with those of Fouzai et al. (2020b), who demonstrated a significant increase in GPx activities in bivalves after exposure to lambda-cyhalothrin. Indeed, GST is best known for its ability to catalyse the conjugation of the reduced form of GSH to xenobiotic substrates for the purpose of detoxification (Jifa et al. 2006).

The increased trend of GST in all groups treated in the present study confirms the activation of the cellular detoxification process to address oxidative injuries, a finding that aligns with similar results observed in the gills of PEN-exposed goldfish (Husak et al. 2017).

Defence against damage can also be facilitated by non-enzymatic antioxidants, which form a primary system to limit free radical toxicity. As the most abundant low-molecular-weight thiol in cells, GSH plays a key role in preventing ROS-induced damage. In our study, the notable increase in GSH levels in Topas-treated *R. decussatus* likely reflects its active role in detoxification through thiol (-SH) groups in response to free radical accumulation. Additionally, the chemical composition of penconazole promotes direct conjugation with GSH, suggesting that the detoxification of Topas in siphons occurs via direct GSH conjugation, enhancing the hydrophilicity and excretion of xenobiotics (Sanchez et al. 2008). Furthermore, the considerable rise in AA could be related to the increased amount of GSH in the siphons of Topas-treated clams. Following Krishnan et al. (2009), the recycling process of AA is aided by GSH, and the increase of this molecule and in particular AA counteracts oxidative damage. Our results were in line with those of Telahigue et al. (2020) describing the increase in the AA antioxidant in the sea cucumber *Holothuria forskali* exposed to glyphosate and its commercial formulation Roundup. Furthermore, the present findings corroborate with those recorded by Sayeed el al. (2003) in the fish *Channa punctatus* exposed to deltamethrin.

Given their molecular properties and their role in metal uptake, transport, storage and excretion, MTs play a major role in detoxification, homeostatic regulation of metals and protection against oxidative stress by acting as a metal-chelating agent for the excess of metals in the cells (Mao et al. 2012). Our results showed that exposure to Topas-induced MT synthesis in *R. decussatus* siphons reflected a high binding affinity between Topas and MTs. To explore data, the star plots served as a helpful visual tool that was applied in this study to combine multi-biomarker responses in the clams (CAT, SOD, GPx, GST, GSH, AA and MTs).

In addition to the non-enzymatic antioxidant discussed above, AChE activity is widely used as a neurotoxicity biomarker in bivalves (Smii et al. 2021), as it terminates nerve impulses by catalysing acetylcholine hydrolysis. AChE is a primary inhibition target for pesticides, particularly biocides, organophosphorus pesticides and carbamates (Uluturhan et al. 2019). In our case, the results showed that Topas had destructive effects on esterase mechanisms by inhibiting AChE activities (Alkan Uçkun and Barım Öz 2020). Topas concentrations showed a positive correlation with AChE inhibition in siphon tissues at doses D1 and D2. However, even at dose D3 AChE's inhibitory impact decreased. This outcome may be explained by the findings of Ahammad Sahib et al. (1980), who discovered that malathion's inhibitory effect decreases over prolonged exposure due to acetylcholine accumulation in fish tissues from AChE inhibition caused by pesticide stress. Our results also align with those of Cravo et al. (2012), who reported AChE activity inhibition in *R. decussatus* from the Ria Formosa lagoon, Portugal, where various contaminants, including metals, PAHs and tributyltin, are present.

Collectively, the multivariate analysis, including the PCA and Pearson correlation, performed on the biochemical data matrix has further highlighted the clear distinction between the control and Topas-treated groups. In addition, this analysis confirms the differential sensitivity/defensive response of *R. decussatus* clams to Topas exposure.

Histopathology is considered another effective method for keeping an eye on anthropogenic contamination. This study is the first to demonstrate the possible histological effects of Topas on *R. decussatus* siphons at gradual levels. The morphological state of *R. decussatus* siphons revealed the rupture of epithelial cells at doses D1 and D2. However, marked lipofuscin granules and vacuolization associated with haemocytes infiltration, rupture and deformation of epithelial cells were recorded at dose D3. The current histopathological findings could be attributed to ROS formation and the subsequent lipid peroxidation consequences, which can induce cell membrane rupture and disrupt membrane permeability and fluidity. The presence of abundant lipofuscin deposits and haemocyte infiltrations in the analysed tissues unequivocally indicate infiltrative inflammation in response to fungicide exposure (De Vico and Carella 2012). Indeed, as mentioned above, lipofuscin aggregates confirm the course of inflammation of tissues as a result of the pro-phenoloxidase (PO) activating systems, a chain of immune mechanisms involved in phenomena such as recognition and encapsulation of foreign matter (Stara et al. 2021). Haemocyte infiltration is certainly related to an increase in haemolymph flow, facilitating the migration of defensive cells to the site of inflammation and penetrating the epithelium through the diapedesis process (Pagano et al. 2016). These two reaction patterns were mainly documented in the siphon tissues of the specimens analysed, showing a dose-dependent exposure effect compared with the controls (see current results). Therefore, because of the lack of research targeting Topas toxicity and histopathological features in molluscs, our study makes an original contribution.

CONCLUSION

Our results suggest that Topas exposure could induce significant changes in the physiology of *R. decussatus*. It induces oxidative damage in clam siphons, as evidenced by an increase in lipid peroxidation and protein oxidation, in addition to perturbations in the enzymatic and non-enzymatic antioxidant status. Exposure to this fungicide also affected the cholinergic system, FA profiles and siphon histoarchitecture. The knowledge gained from our findings indicates for the first time that Topas is a potential neurotoxicant pesticide that exerts its neurotoxic effects via the generation of oxidative stress. Consequently, exposure to Topas should be carefully monitored.

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DATA AVAILABILITY

The authors declare that the data supporting the findings of this study are available within the paper. Should any raw data files be needed in another format, they are available from the corresponding author upon reasonable request. Source data are provided in this paper.

CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

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ETHICAL STATEMENT

All experiments were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

AUTHORS' CONTRIBUTION STATEMENT

Boutheina Ben Abdallah: Investigation, Methodology, Software, Formal analysis, Writing - original draft, Data curation, Visualization. **Safa Bejaoui:** Investigation, Conceptualization, Methodology, Resources, Data curation, Review and editing. **Wafa Trabelsi:** Methodology. **Dalya Belhassen:** Methodology, Software. **Zeineb Khila:** Methodology. **Samir Boubaker:** Methodology, Resources. **Chayma Ben Fayala:** Methodology. **Nejla Soudani:** Conceptualization, Supervision, Writing - review and editing, Validation.

REFERENCES

Aebi H. 1984. Catalase in Vitro. Methods Enzymol. 105: 121-126. https://doi.org/10.1016/S0076-6879(84)05016-3

Ahammad Sahib I.K., Sailatha D., Ramana Rao K.V. 1980. Impact of malathion on acetylcholinesterase in the tissues of the fish *Tilapia mossambica* (Peters)—a time course study. J. Biosci. 2(1): 37-41. https://doi.org/10.1007/BF02703131

Alderman C.J.J., Shah S., Foreman J.C., et al. 2002. The role of advanced oxidation protein products in regulation of dendritic cell function. Free Radical. Biol. Med. 32(5): 377-385. https://doi.org/10.1016/j.clinbiochem.2011.03.134

Alkan Uçkun A., Barım Öz Ö. 2020. Acute exposure to the fungicide penconazole affects some biochemical parameters in the crayfish (*Astacus leptodactylus* Eschscholtz, 1823). Environ. Sci. Pollut. Res. 27(28): 35626-35637. https://doi.org/10.1007/s11356-020-09595-2

Ayala A., Muñoz M.F., Argüelles S. 2014. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4- hydroxy-2-nonenal. Oxidative Med. Cell. Longev. 2014: 360438. https://doi.org/10.1155/2014/360438

Barhoumi B., Clérandeau C., Le Menach K., et al. 2014. Pollution biomonitoring in the Bizerte lagoon (Tunisia), using grass goby, *Zosterisessor ophiocephalus* (Teleostei, Gobiidae) as a bioindicator species. Mar. Environ. Res. 101: 184-195. https://doi.org/10.1016/j.marenvres.2014.07.002

Beauchamp C., Fridovich I. 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44(1): 276-287. https://doi.org/10.1016/0003-2697(71)90370-8

Benzie I.F.F., Strain J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. Anal. Biochem. 239(1): 70-76. https://doi.org/10.1006/abio.1996.0292

Calder P.C. 2010. Omega-3 fatty acids and inflammatory processes. Nutrients 2(3): 355-374. https://doi.org/10.3390/nu2030355

Cecchi G., Biasini S.C.J. 1985. Methanolyse rapide des huiles en solvants. Note de Laboratoire. Rev. Franc. Corps. Gras. 4: 163-164.

Chaâbane M., Ghorbel I., Elwej A., et al. 2016. Penconazole alters redox status, cholinergic function, and membrane-bound ATPases in the cerebrum and cerebellum of adult rats. Hum. Exp. Toxicol. 36(8): 854-866. https://doi.org/10.1177/0960327116672911

Chaâbane M., Elwej A., Ghorbel I., Chelly S., Mnif H., Boudawara T., Ellouze chaabouni S., Zeghal N., Soudani, N. 2018. Penconazole alters redox status, cholinergic function and lung's histoarchitecture of adult rats: Reversal effect of vitamin E. Biomed. Pharmacother. 102: 645-652.

Chalghmi H., Bourdineaud J.P., Haouas Z., et al. 2016. Transcriptomic, Biochemical, and Histopathological Responses of the Clam *Ruditapes decussatus* from a Metal-Contaminated Tunis Lagoon. Arch. Environ. Contam. Toxicol. 70(2), 241-256. https://doi.org/10.1007/s00244-015-0185-0

Chetoui I., Bejaoui S., Trabelsi W., et al. 2019. Exposure of *Mactra corallina* to acute doses of lead: effects on redox status, fatty acid composition and histomorphological aspect. Drug Chem. Toxicol. 45(1): 311-323. https://doi.org/10.1080/01480545.2019.1693590

Cheung C.C.C., Zheng G.J., Li A.M.Y., et al. 2001. Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, *Perna viridis*. Aquat. Toxicol. 52: 189-203. https://doi.org/10.1016/S0166-445X(00)00145-4

Costa P.M., Carreira S., Costa M.H., et al. 2013. Development of histopathological indices in a commercial marine bivalve (*Ruditapes decussatus*) to determine environmental quality. Aquat. Toxicol. 126(2013): 442-454. https://doi.org/10.1016/j.aquatox.2012.08.013

Cravo A., Pereira C., Gomes T., et al. 2012. A multibiomarker approach in the clam *Ruditapes decussatus* to assess the impact of pollution in the Ria Formosa lagoon, South Coast of Portugal. Mar. Environ. Res. 75: 23-34. https://doi.org/10.1016/j.marenvres.2011.09.012

Dalvie M.A., Cairncross E., Solomon A., et al. 2003. Contamination of rural surface and ground water by endosulfan in farming areas of the Western Cape, South Africa. Environ Health. 2(1): 1. https://doi.org/10.1186/1476-069X-2-1

De Vico G., Carella F. 2012. Morphological features of the inflammatory response in molluscs. Res. Vet. Sci. 93(3): 1109-1115. https://doi.org/10.1016/j.rvsc.2012.03.014

Ding F., Song W.H., Guo J., et al. 2009. Oxidative stress and structure-activity relationship in the zebrafish (*Danio rerio*) under exposure to paclobutrazol. J. Environ. Sci. Health. - Part B Pesticides, Food Contaminants, and Agricultural Wastes. 44(1): 44-50. https://doi.org/10.1080/03601230802519652

Draper H.H., Hadley M. 1990. Malondialdehyde determination as index of lipid Peroxidation. Methods. Enzymol. 186(C): 421-431. https://doi.org/10.1016/0076-6879(90)86135-I

Ellman G.L. 1959. Tissue Sulfhydryl Groups. Arch. Biochem. Biophys. 82: 70-77. https://doi.org/10.1016/0003-9861(59)90090-6

Ellman G.L., Courtney K.D., Andres V. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7: 88-95. https://doi.org/10.1016/0006-2952(61)90145-9

European chemicals agency (ECHA) 2023. Retrieved 23 May 2024 from https://echa.europa.eu/substance-information/-/substanceinfo/100.060.231

Floch J., Lees M., Sloane Stanley G.H. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226(1): 497-509. https://doi.org/10.1016/S0021-9258(18)64849-5

Flohé L., Günzler W.A. 1984. Assays of Gluthathione Peroxidase. Methods. Enzymol. 105: 114-120. https://doi.org/10.1016/S0076-6879(84)05015-1

Fokina N.N., Ruokolainen T.R., Nemova N.N., et al. 2013. Changes of blue mussels *Mytilus edulis* L. lipid composition under cadmium and copper toxic effect. Biol. Trace. Elem. Res. 154(2): 217-225. https://doi.org/10.1007/s12011-013-9727-3

Fouzai C., Trabelsi W., Bejaoui S., et al. 2020a. Cellular toxicity mechanisms of lambda-cyhalothrin in *Venus verrucosa* as revealed by fatty acid composition, redox status and histopathological changes. Ecol. Indic. 108. https://doi.org/10.1016/j.ecolind.2019.105690

Fouzai C., Trabelsi W., Rabeh I., et al. 2020b. Fatty acid profile and antioxidant status in *Venus verrucosa* gills as λ-cyhalothrin toxic effects. Toxicol. Ind. Health. 36(11): 898-907. https://doi.org/10.1177/0748233720961219

Ghribi F., Richir J., Bejaoui S., et al. 2020. Trace elements and oxidative stress in the Ark shell *Arca noae* from a Mediterranean coastal lagoon (Bizerte lagoon, Tunisia): are there health risks associated with their consumption? Environ. Sci. Pollut. Res. 27(13): 15607-15623. https://doi.org/10.1007/s11356-020-07967-2

Grünberger O., Hamdi R., Lagacherie M., et al. 2024. Pesticide contamination pattern of surface water in an urban-agricultural mediterranean watershed (Wadi Guenniche, Bizerte Lagoon, Northern Tunisia). J. Environ. Sci. Health. Part B. 59(8): 521-539. https://doi.org/10.1080/03601234.2024.2375905

Habig W.H., Pabst M.J., Fleischner G., et al. 1974. The identity of glutathione S-transferase B with ligandin, a major binding protein of liver. Proc. Natl. Acad. Sci. 71(10): 3879-3882. https://doi.org/10.1073/pnas.71.10.3879

Hamza-Chaffai A., Pellerin J., Amiard J.C. 2003. Health assessment of a marine bivalve *Ruditapes decussatus* from the Gulf of Gabès (Tunisia). Environ. Int. 28(7): 609-617. https://doi.org/10.1016/S0160-4120(02)00102-2

Hatami M., Banaee M., Nematdoost Haghi B. 2019. Sub-lethal toxicity of chlorpyrifos alone and in combination with polyethylene glycol to common carp (*Cyprinus carpio*). Chemosphere 219: 981-988. https://doi.org/10.1016/j.chemosphere.2018.12.077

Hart K.A., Pimentel D. 2002. Environmental and economic costs of pesticide use. In: Pimentel D, editor. Encyclopedia of pest management. New York: Marcel Dekker; p. 237-239. https://doi.org/10.1201/NOE0824706326.ch99

Hermsen S.A.B., Pronk T.E., van den Brandhof E.J., et al. 2011. Chemical class-specific gene expression changes in the zebrafish embryo after exposure to glycol ether alkoxy acids and 1,2,4-triazole antifungals. Reprod. Toxicol. 32(2): 245-252. https://doi.org/10.1016/j.reprotox.2011.05.010

Husak V.V., Mosiichuk N.M., Storey J.M., et al. 2017. Acute exposure to the penconazole-containing fungicide Topas partially augments antioxidant potential in goldfish tissues. Comp. Biochem. Physiol. Part - C. 193: 1-8. https://doi.org/10.1016/j.cbpc.2016.12.003

Icoglu Aksakal F., Ciltas A. 2018. Developmental toxicity of penconazole in Zebrfish (*Danio rerio*) embryos. Chemosphere. 200: 8-15. https://doi.org/10.1016/j.chemosphere.2018.02.094

Jacques-Silva M.C., Nogueira C.W., Broch L.C. 2001. Diphenyl diselenide and ascorbic acid changes deposition of selenium and ascorbic acid in liver and brain of mice. Pharmacol. Toxicol. 88: 119-125. https://doi.org/10.1034/j.1600-0773.2001.d01-92.x

Jifa W., Yu Z., Xiuxian S., et al.2006. Response of integrated biomarkers of fish (*Lateolabrax japonicus*) exposed to benzo[a]pyrene and sodium dodecylbenzene sulfonate. Ecotoxicol. Environ. Saf. 65(2): 230-236. https://doi.org/10.1016/j.ecoenv.2005.08.002

Jollow D.J., Mitchell J., Zampaglione N., et al. 1974. Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3,4-bromobenzene oxide as the hepatotoxic metabolite. Pharmacology. (11): 151-169. https://doi.org/10.1159/000136485

Kalliora C., Mamoulakis C., Vasilopoulos E., et al. 2018. Association of pesticide exposure with human congenital abnormalities. Toxicol. Appl. Pharmacol. 346(March): 58-75. https://doi.org/10.1016/j.taap.2018.03.025

Kamel N., Burgeot T., Banni M., et al. 2014. Effects of increasing temperatures on biomarker responses and accumulation of hazardous substances in rope mussels (*Mytilus galloprovincialis*) from Bizerte lagoon. Environ. Sci. Pollut. Res. 21: 6108-6123 https://doi.org/10.1007/s11356-014-2540-5

Kayali R., Çakatay U., Akçay T., et al. 2006. Effect of alpha-lipoic acid supplementation on markers of protein oxidation in post-mitotic tissues of ageing rat. Cell Biochem. Funct: Cellular biochemistry and its modulation by active agents or disease. 24(1): 79-85.

Konwick B.J., Garrison A.W., Avants J.K., et al. 2006. Bioaccumulation and biotransformation of chiral triazole fungicides in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 80: 372-381. https://doi.org/10.1016/j.aquatox.2006.10.003

Krishnan N., Kodrík D., Kłudkiewicz B., et al. 2009. Glutathione-ascorbic acid redox cycle and thioredoxin reductase activity in the digestive tract of *Leptinotarsa decemlineata* (say). Insect. Biochem. Mol. Biol. 39: 180-188. https://doi.org/10.1016/j.ibmb.2008.11.001

Krumova K., Cosa G. 2016. Chapter 1: Overview of reactive oxygen species, in Singlet oxygen: applications in biosciences and nanosciences, Volume 1, pp. 1-21. https://doi.org/10.1039/9781782622208-00001

Li Z.H., Zlabek V., Grabic R., et al. 2010a. Effects of exposure to sublethal propiconazole on the antioxidant defense system and Na+-K+-ATPase activity in brain of rainbow trout, *Oncorhynchus mykiss*. Aquat. Toxicol. 98(3): 297-303. https://doi.org/10.1016/j.aquatox.2010.02.017

Li Z.H., Zlabek V., Li P., et al. 2010b. Biochemical and physiological responses in liver and muscle of rainbow trout after long-term exposure to propiconazole. Ecotoxicol. Environ. Saf. 73: 1391-1396. https://doi.org/10.1016/j.ecoenv.2010.05.017

Liu J.J., Green P., John-Mann J., et al. 2015. Pathways of polyunsaturated fatty acid utilization: Implications for brain function in neuropsychiatric health and disease. Brain. Res. 1597: 220-246. https://doi.org/10.1016/j.brainres.2014.11.059

Llesuy S., Evelson P., Campos A.M., et al. 2001. Methodologies for evaluation of total antioxidant activities in complex mixtures. A critical review. Biol. Res. 34(2): 51-73. https://doi.org/10.4067/S0716-97602001000200009

López Martínez R. 2024. Study of the establishment of an allocated zone for aquaculture (AZA) in the Lagoon of Bizerte, Tunisia. 112 pp.

Lowry O.H., Rosebrough N.J., Farr A.L., et al. 1951. Protein measurement with the Folin phenol reagent. J. Biolo. Chem. 193(1): 265-275. https://doi.org/10.1016/S0021-9258(19)52451-6

Mao H., Wang D.H., Yang W.X. 2012. The involvement of metallothionein in the development of aquatic invertebrate. Aquat. Toxicol. 110-111: 208-213. https://doi.org/10.1016/j.aquatox.2012.01.018

Martoja R., Martoja-Pierson M. 1967. Initiation aux techniques de l'histologie animale, Masson: Paris, 345.

Monroig Ó., Kabeya N. 2018. Desaturases and elongases involved in polyunsaturated fatty acid biosynthesis in aquatic invertebrates: a comprehensive review. Fisheries. Sci. 84(6): 911-928. https://doi.org/10.1007/s12562-018-1254-x

Monserrat J.M., Martinez P.E., Geracitano L.A., et al. 2007. Pollution biomarkers in estuarine animals: critical review and new perspectives. Comp. Biochem. Physiol. - Part C 146: 221-234. https://doi.org/10.1016/j.cbpc.2006.08.012

Mu X., Pang S., Sun X., et al. 2013. Evaluation of acute and developmental effects of difenoconazole via multiple stage zebrafish assays. Environ. Pollut. 175: 147-157. https://doi.org/10.1016/j.envpol.2012.12.029

Necibi M., Mzoughi N. 2020. Distribution of organochlorine pesticides in sediment cores from the Bizerte Lagoon (Tunisia). Int. J. of Environ. Anal. Chem. 100(10): 1118-1132. https://doi.org/10.1080/03067319.2019.1650173

Ou P., Wolff S.P. 1996. A discontinuous method for catalase determination at "near physiological" concentrations of H_2O_2 and its application to the study of H_2O_2 fluxes within cells. J. Biochem. Biophys. Methods. 31(1-2): 59-67. https://doi.org/10.1016/0165-022X(95)00039-T

Pagano M., Capillo G., Sanfilippo M., et al. 2016. Evaluation of Functionality and Biological Responses of *Mytilus galloprovincialis* after Exposure to Quaternium-15 (Methenamine 3-Chloroallylochloride). Molecules. 21(2): 1-12. https://doi.org/10.3390/molecules21020144

Pamplona R. 2008. Membrane phospholipids, lipoxidative damage and molecular integrity: a causal role in aging and longevity. Biochim. Biophys. Acta. 1777(10): 1249-1262. https://doi.org/10.1016/j.bbabio.2008.07.003

Peffer R.C., Moggs J.G., Pastoor T., et al. 2007. Mouse liver effects of cyproconazole, a triazole fungicide: role of the constitutive androstane receptor. Toxicol. Sci. 99: 315-325. https://doi.org/10.1093/toxsci/kfm154

Petrović S., Ozretić B., Krajnović-Ozretić M., et al. 2001. Lysosomal membrane stability and metallothioneins in digestive gland of mussels (*Mytilus galloprovincialis* Lam.) as biomarkers in a field study. Mar. Pollut. Bull. 42(12): 1373-1378. https://doi.org/10.1016/S0025-326X(01)00167-9

Reznick A.Z., Packer L. 1994. Oxidative damage to proteins: Spectrophotometric method for carbonyl assay. Methods. Enzymol. 233: 357-363. https://doi.org/10.1016/S0076-6879(94)33041-7

Rodríguez-Fuentes G., Rubio-Escalante F.J., Noreña-Barroso E., et al. 2015. Impacts of oxidative stress on acetylcholinesterase transcription, and activity in embryos of zebrafish (*Danio rerio*) following Chlorpyrifos exposure. Comp. Biochem. Physiol Part - C: Toxicology and Pharmacology. 172-173: 19-25. https://doi.org/10.1016/j.cbpc.2015.04.003

Sanchez W., Piccini B., Porcher J.M. 2008. Effect of prochloraz fungicide on biotransformation enzymes and oxidative stress parameters in three-spined stickleback (*Gasterosteus aculeatus* L.). J. Environ. Sci. Health. B 43 (1): 65-70. https://doi.org/10.1080/03601230701735151

Sayeed I., Parvez S., Pandey S., et al. 2003. Oxidative stress biomarkers of exposure to deltamethrin in freshwater fish, *Channa punctatus* Bloch. Ecotoxicol. Environ. Saf. 56(2): 295-301. https://doi.org/10.1016/S0147-6513(03)00009-5

Signa G., Di Leonardo R., Vaccaro A., et al. 2015. Lipid and fatty acid biomarkers as proxies for environmental contamination in caged mussels *Mytilus galloprovincialis*. Ecol. Indic. 57: 384-394. https://doi.org/10.1016/j.ecolind.2015.05.002

Smii H., Khazri A., Ali M., et al. 2021. Titanium dioxide nanoparticles are toxic for the freshwater mussel *Unio ravoisieri*: Evidence from a multimarker approach. Diversity. 13(12): 1-17. https://doi.org/10.3390/d13120679

Stara A., Pagano M., Albano M., et al. 2021. Effects of long-term exposure of *Mytilus galloprovincialis* to thiacloprid: A multibiomarker approach. Environ. Pollut. 289(August): 117892. https://doi.org/10.1016/j.envpol.2021.117892

Surfrider. 2020. Chemical pollution of the ocean: the pesticide issue. Retrieved October 3, 2024, from https://www.surfrider.eu/learn/news/pollution-chimique-ocean-question-pesticides

Tallima H., El Ridi R. 2018. Arachidonic acid: Physiological roles and potential health benefits - A review. J. Adv. Res. 11: 33-41. https://doi.org/10.1016/j.jare.2017.11.004

Telahigue K., Rabeh I., Hajji T., et al. 2020. Assessment of the impacts of glyphosate and its commercial formulation Roundup® on the respiratory tree of the sea cucumber *Holothuria forskali* using a multivariate biomarker approach. Chemosphere. https://doi.org/10.1016/j.chemosphere.2020.129376.

Trabelsi W., Chetoui I., Fouzai C., et al. 2019. Redox status and fatty acid composition of *Mactra corallina* digestive gland following exposure to acrylamide. Environ. Sci. Pollut. Res. 26: 22197-22208. https://doi.org/10.1007/s11356-019-05492-5.

Ullah R., Zuberi A., Ullah S., et al. 2014. Cypermethrin induced behavioral and biochemical changes in mahseer, Tor putitora. J. Toxicol. Sci. 39: 829-836. https://doi.org/10.2131/jts.39.829

Uluturhan E., Darılmaz E., Kontas A., et al. 2019. Seasonal variations of multi-biomarker responses to metals and pesticides pollution in *M. galloprovincialis* and *T. decussatus* from Homa Lagoon, Eastern Aegean Sea. Mar. Pollut. Bull. 141: 176-186. https://doi.org/10.1016/j.marpolbul.2019.02.035

Viarengo A., Ponzano E., Dondero F., et al. 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: An application to Mediterranean and Antarctic molluscs. Mar. Environ. Res. 44(1): 69-84. https://doi.org/10.1016/S0141-1136(96)00103-1

Wang C., Wu Q., Wu C., et al. 2011. Application of dispersion-solidification liquid-liquid microextraction for the determination of triazole fungicides in environmental water samples by high-performance liquid chromatography. J. Hazard. Mater. 185(1): 71-76. https://doi.org/10.1016/j.jhazmat.2010.08.124

Yin X., Chen P., Chen H., Jin W. Yan X. 2017. Physiological performance of the intertidal Manila clam (*Ruditapes philippinarum*) to long-term daily rhythms of air exposure. Sci. rep. 7: 1-12. https://doi.org/10.1038/srep41648.

Yoloğlu E. 2019. Assessment of Na^{+}/K^{+} -ATPase, Mg^{2}+-ATPase, Ca^{2}+-ATPase, and Total-ATPase Activities in Gills of Freshwater Mussels Exposed to Penconazole. Comm. J. Biol. 3: 88-92. https://doi.org/10.31594/commagene.632082

Zhukova N.V. 1991. The pathway of the biosynthesis of non-methylene-interrupted dienoic fatty acids in molluscs. Comp. Biochem. Physiol. 100(4): 801-804. https://doi.org/10.1016/0305-0491(91)90293-M