EFFECTS OF COMMONLY THERAPEUTIC DRUG (ACETYLSALICYLIC ACID) TOXICITY IN TERMS OF BIOMARKERS DETERMINED AND HISTOLOGICAL ALTERATIONS IN *PERINEREIS CULTRIFERA* (ANNELIDA: POLYCHAETA)

MOUMENI, N.[1] – SEBBIH, A.[1] – MAAMCHA, O.[1] – BAROUR, C.[2] – DAAS, T.[1*]

[1]*Laboratory of Applied Animal Biology, Department of Biology, Faculty of Sciences, Badji Mokhtar University, Annaba 23000, Algeria*

[2]*Laboratory of Aquatic and Terrestrial Ecosystems (LEAT), Department of Biology, Faculty of Natural and Life Sciences, Mohamed Cherif Messaâdia University, BP1553, Annaba Road, Souk Ahras, Algeria*

**Corresponding author*
e-mail: tarek63daas@yahoo.fr; phone: +213-55-007-0781

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Abstract. The Mediterranean Sea is one of the busiest areas worldwide in terms of considerable anthropogenic disturbances, such as pollution by pharmaceuticals. Acetylsalicylic acid (ASA) is one of the most persistent non-steroidal anti-inflammatory drugs affecting aquatic organisms in seawater. This study was therefore designed to elucidate the biological effects of acetylsalicylic acid through short-term and long-term exposures (Acute Toxicity: AT and Chronic Toxicity: CT) to polychaetes *Perinereis cultrifera* as a non-target organism in the Algerian east coast. During an acute (96 h) and chronic period (30 days), Polychaetes were exposed to three concentrations of Acetylsalicylic acid for each treatment. The present work also aimed to assess the acute and chronic effects of acetylsalicylic acid on selected oxidative stress biomarkers [catalase: CAT, glutathione peroxidase: GPx and glutathione S-transferase: GST), as well as histological alterations in the oocyte structure and the digestive epithelium. Overall, the obtained results showed that acetylsalicylic acid significantly changed the activity of catalase and biomarkers related to the redox status of the organisms in each toxicity. In response to acetylsalicylic acid exposure, we observed a thinner and resorbed digestive epithelium with a reduction in the number and the diameters of oocytes which manifests by a weakness in the amount of yolk-grains.

Keywords: *therapeutic drug, Perinereis cultrifera, toxicity, histological study, oxidative stress biomarkers*

Introduction

The Mediterranean Sea is bordered by 21 countries, located on three continents with a total population of around 500 million in 2016 (Karadirek et al., 2019). Marine ecosystems exhibit a high susceptibility to pollution due to increased anthropogenic activities (Chandurvelan et al., 2015). Thereby, various complex mixtures of contaminants found in these zones are often the principal factors causing degradation of water quality (Blaise et al., 2016).

The Mediterranean basin includes high income and lower to upper-middle income countries having key differences covering factors such as population and demographics economic growth and industrialization, pharmaceutical manufacture and prescriptions, treatment, disposal and reuse of waste and wastewater (Gürluk, 2009; Kookana et al., 2014). Seventy percent of wastewater is generated in high income countries, such as France, Italy, followed by upper-middle income countries 38% such as Algeria, Croatia, Turkey and lower-middle income countries 30% such as Morocco, Syria and Tunisia

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(Sato et al., 2013; Madikizela et al., 2017; World Bank List of Economies, 2017). Chronic exposure to trace levels of pharmaceutical mixtures can be substantial and may cause possible threats to the aquatic environment (Fent et al., 2006a) and also to human health (de Jesus Gaffney et al., 2015), with adverse effects such as antibiotic resistance (Qiao et al., 2018; Sabri et al., 2018), and endocrine disruptive conditions (Fent et al., 2006b).

Pharmaceutical active compounds (PhACs) play a major role as an essential medical treatment for humans and other animals. It is estimated that hundreds of tons of PhACs are consumed annually and the numbers are increasing year by year (Fekadu et al., 2019). PhACs are complex, organic molecules presenting different physico-chemical and biological properties. They are classified according to therapeutic aim, including analgesics and anti-inflammatory drugs, antibiotics, beta-blockers, lipid regulators, contraceptives, neuractive compounds, and many others (Alvarez-Muñoz et al., 2016). Hundreds of PhACs have already been found in the marine environment, ranging from low ng/L to a few µg/L (Fent et al., 2006a; Aspin-Pont et al., 2016). Mezzelani et al. (2018) presented data from over 20 countries regarding various PhACs concentrations detected in seawater samples, irregular high concentrations have been detected in marine waters on several occasions (Togola and Budzinski, 2008).

The ASA is a non-steroidal anti-inflammatory, antipyretic, and analgesic drug (Pascœ et al., 2003). These non-steroidal anti-inflammatory drugs (NSAIDs) stand out to be the group of drugs with the highest worldwide consumption (Fent et al., 2006a). This drug was the first identified pharmaceutical sewage influent/effluent (Daughton and Ternes, 1999). Therefore, there are reports of the presence of various concentrations of this drug in aquatic media around the globe (Richardson and Bowron, 1985; Daughton and Ternes, 1999; Rabiet et al., 2006; Tambosi et al., 2010; Lolić et al., 2015). The mechanism of is toxic action has not been fully elucidated (Gómez-Oliván et al., 2014; Freitas et al., 2019; Nunes, 2019; Szabelak and Bownik, 2021; Afsa et al., 2023) but it is known that this compound may induce toxicity in organisms like, for example, *Daphnia magna* (Cleuvers, 2004). In addition, ASA has been shown to induce oxidative stress in fish (Nunes et al., 2015b).

The presence of different contaminants is a possible source of oxidative stress and could induce variations in antioxidant enzyme activities (Santovito et al., 2005). Differences in antioxidant enzyme activities have been observed among populations of invertebrate marine organisms from polluted and unpolluted areas (Regoli and Principato, 1995; Livingstone, 2001; Box et al., 2007; Natalotto et al., 2015). The use of biomarkers to analyze the effects of exposure to chemical contaminants in the aquatic environment is more extended in the actuality (Cossu et al., 2000; Regoli et al., 2002; Ferreira et al., 2007; Sureda et al., 2011; Semedo et al., 2012; Natalotto et al., 2015).

The aim of this study is to evaluate the acute and chronic toxicity in the laboratory on the Polychaete *P. cultrifera* by analyzing the weight, the oocyte diameter and the response of the antioxidant enzyme activities (CAT, GST and GPx). In addition, a histological study was also carried out to examine the oocyte structure and the digestive epithelium thickness.

Material and methods

Study site

Sampling operation was carried out on a beach called la Montagne (36° 53’53.33” N, 08°27’3.28” W) located in a town called El-Kala in El-Tarf City, eastern Algerian coast

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(*Fig. 1*). El-Kala is bordered on the North by the Mediterranean Sea, on the East by the Algerian-Tunisian border, on the West by the plains of Annaba City and on the South by the mountains of the Medjerda. The sampling site was classified as a biosphere reserve by UNESCO in 1990, it is also considered as a reference site (Belfetmi et al., 2020; Ramdani et al., 2021).

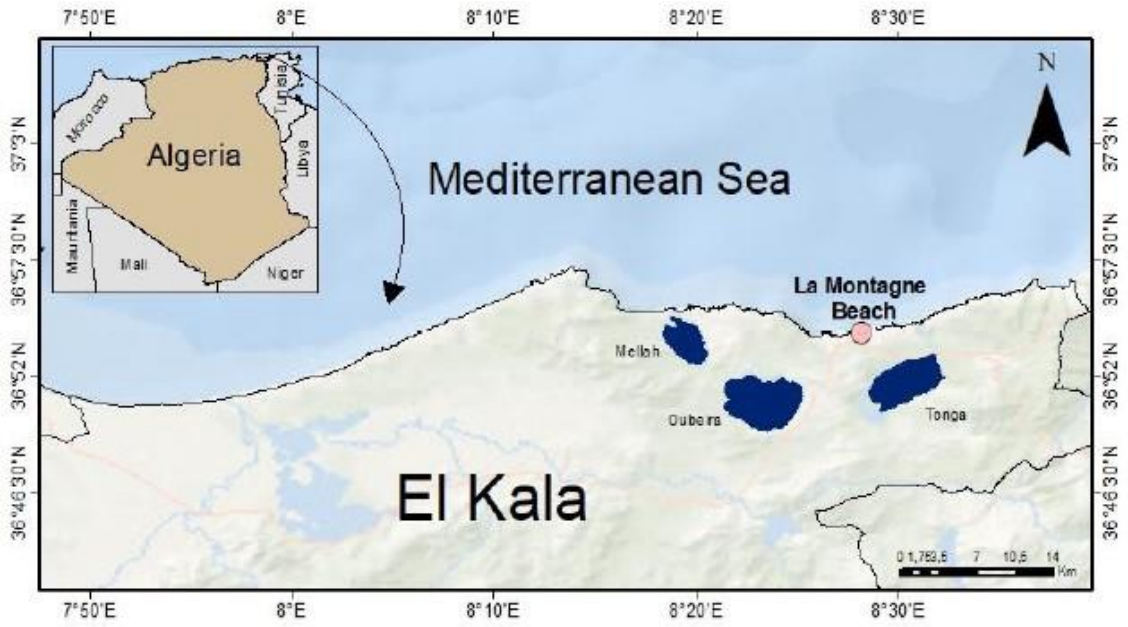

Figure 1. *The sampling geographic location in the north-east of Algeria*

Worms maintenance and experimental plan

The *P. cultrifera* individuals were chosen as an assay organism due to their wide geographical distribution but also for their abundance and availability.

The sampling method is a simple random technic; it consists of taking random samples on an area of 1 m^2 quadrate. The superficial layer of the rock which shelters Polychaete annelids as well as a very rich fauna and flora in terms of diversity was harvested by hand using a scraper at low tide (0.1 m) in the intertidal zone. The hole sample was placed in a glass container including seawater from the study site, then stored and transported in a cooler to the laboratory. Worms were rinsed with seawater of the original site then dried and identified under a binocular magnifying glass based on the shape and distribution of the paragnaths which are key elements in the description of the genus *Perinereis* (Fauvel, 1923). The females worms were placed under laboratory water-controlled conditions using a multiparameter oximeter (Multi 340i/SET), maintaining temperature at 18°C, salinity 30 PPT, pH 8, dissolved oxygen 6 mg/L.

The selected individuals for the ASA toxicity testing were placed in aquariums filled with water (6 L per aquarium) in the laboratory for 48 h before experimentation (the acclimatization period) and a 12-h light/dark cycle was also maintained. Organisms were not fed during the acclimatization period as well as for acute toxicity; in contrast to chronic toxicity, individuals were fed every 48 hours with a commercial food in flakes of TetraMin. The oxygen supply was provided by oxygen pumps, in order to simulate the natural conditions of the Polychaete annelid environment. Artificial

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galleries were placed within the aquariums; the latter was covered in order to avoid the risk of evaporation of the tested molecule.

After the acclimatization period, the treatment of *P. cultrifera* adults with ASA was conducted in aquariums (31× 16× 20 cm) as follows: distribution of 120 individuals over 8 aquariums at the rate of 15 individuals per aquarium. The first one served as a control for acute toxicity and the second one served as a control for CT, in these two aquariums there was no exposure to the tested molecule. Individuals in the remaining aquariums were exposed to ascending concentrations (C) of ASA in order to study the effect of AT (Aquarium1: 2.5 mg/L, Aquarium 2: 25 mg/L, Aquarium 3: 250 mg/L, and that for 96 h for the different concentrations) and CT (Aquarium 4: 0.005 mg/L, Aquarium 5: 0.1 mg/L, Aquarium 6: 2 mg/L, for 30 days for the different concentrations) (Gomes et al., 2019; Gumbi et al., 2017).

After exposure, 12 of the 15 individuals in each aquarium were intended for biomarkers determination while the rest were stored in formalin solution (9%) until histological study. The weight (W: the fresh weight wiped), oocyte diameter (OD) and digestive epithelium thickness (DET) were calculated for each female and its correlation was assessed by using the Spearman coefficient.

Biochemical markers

Catalase (CAT)

The activity of catalase (CAT) (200 µL of tissue homogenate, in a total volume of 1000 µL) was determined according to (Caliborne,1985), method that monitors the decomposition of H_2O_2 (final concentration of 500 mM) at λ equal to 240 nm and using a spectrophotometric ("Agilent Technologies" type CARY 60 UV-Vis.). This activity was expressed in µM of hydrolyzed substrate per minute per milligram of protein.

Glutathione S-transferase (GST)

Glutathione S-transferase (GST) activity was measured according to Habig et al. (1974) using 1-chloro 2, 4 - dinitrobenzene (CDNB) as the substrate in a final reaction mixture containing 1 mmol CDNB and 5 µM reduced glutathione activity was measured at 340 nm in a visible/UV spectrophotometer ("Agilent Technologies" type CARY 60 UV-Vis.).

Glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) activity was assayed according to the method of Flohé and Günzler (1984). This method is based on the reduction of hydrogen peroxide in the presence of reduced glutathione (GSH) which is transformed into oxidized glutathione (GSSG) under the action of GPx. The enzymatic activity was expressed in µM of GSH per minute per milligram of protein. The protein content was measured according to the Bradford method using the BSA as a standard.

Histological processing

Seaworms from each concentration were taken and sacrificed by cutting off the head. A body portion was fixed for 48 h in formalin solution (9%). The experimental tissue was dehydrated, using a series of increasing ethanol concentrations (70%, 80%, 90% and 95%), embedded in paraffin wax (72°C), and manually sectioned with a microtome

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(Thermo Scientific) to obtain histological slices of 5 μm. After rehydration in decreasing series of ethanol, the sections were stained with hematoxylin-eosin then mounted on glass slides and examined under a "Leica DM500" microscope at×60, ×150 and ×600 magnifications to examine our tissues specimens.

Data analysis

All our statistical analyses were performed using R, version 4.2.2 (R Core Team, 2022; Ihaka and Gentleman, 1996) for MacOS (http://cran.r-project.org). Shapiro-Wilk test was used to test variables normality. The influence of both acute and chronic toxicity on the biomarkers, morphometric and weight parameters was assessed by the nonparametric Kruskal-Wallis (KW) rank sum test. The KW test was followed by the nonparametric pairwise Dunn's test (with Bonferroni adjusted *p-value*) to find post-hoc statistical differences at $\alpha = 0.05$ as significant level. Correlations between variables were also calculated by using Spearman's nonparametric correlation (with Bonferroni adjusted *p-value*). Data were expressed as mean ± standard error (se). Finally, we carried out also a multivariate analysis by applying a principal component analysis (PCA as ordination technique) to characterize our toxicity experimental apparatus by the observed-effects on the explanatory variables. Several R packages were also used in our statistical analysis and to plot data results such as '*ggplot2*' (Wickham, 2016), '*ggcorrplot*' (Kassambara, 2019), '*FactoMineR*' (Le et al., 2008), '*dunn.test*' (Dinno, 2017), '*Hmisc*' R package (Harrell, 2023), '*ggpubr*' (Kassambara, 2023), '*psych*' (Revelle, 2023), and '*PMCMRplus*' (Pohlert, 2023).

Results

Morphometric parameters

In this research paper, morphometric parameters related to weight (W), oocyte diameter (OD) and digestive epithelium thickness (DET) were used as biomarkers to demonstrate the possible effects of ASA concentrations on the reproduction and the digestion of *P. cultrifera*. The Morphometric averages of the parameters studied by concentrations of ASA are summarized in *Table 1*.

Table 1. *Variations in weight (W), oocyte diameter (OD) and digestive epithelium thickness (DET) of P. cultrifera exposed to different concentrations of acetylsalicylic acid ASA (acute toxicity: AT and chronic toxicity: CT) (m ± SE, n = 8)*

		Type of toxicity							
		AT				**CT**			
		C	**2.5 mg/L**	**25 mg/L**	**250 mg/L**	**C**	**0.005 mg/L**	**0.1 mg/L**	**2 mg/L**
Morphometric parameters	W (g)	0.24± 0.02 **a**	0.16± 0.01**ab**	0.15± 0.01 **b**	0.16± 0.01**b**	0.27± 0.01**a**	0.20± 0.02**ab**	0.19± 0.02 **b**	0.15± 0.01 **b**
	OD (μm)	253.00± 8.23**a**	224.75± 5.98**a**	221.50± 9.96 **ab**	169.20± 7.08**b**	254.50± 6.52**a**	246.88± 10.06**a**	217.25± 7.32 **ab**	193.75± 5.71**b**
	DET (μm)	163.62± 4.80**a**	152.62± 4.69 **ab**	144.75± 3.94 **ab**	140.12± 3.90**b**	151.00± 7.44 **a**	134.62± 3.10 **ab**	125.75± 4.20 **ab**	119.12± 3.87**b**

C: Control Group (0 mg/L); C1(AT: 2.5 mg/L; CT: 0.005 mg/L); C2 (AT: 25 mg/L; CT: 0.1 mg/L); C3 (AT: 250 mg/L; CT: 2 mg/L). For each parameter, the different letters indicate significant differences (*Dunn's test*) between concentrations

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Results revealed that the highest values of practically all the morphometric parameters, for both acute and chronic toxicity, were observed in the control groups and decreased progressively with each exposure concentration. In addition, W, OD and DET were significantly higher (KW test, p = 0.00) in the treated groups compared to the control groups. While, a highly significant difference was highlighted for the DET by chronic and acute toxicity (KW test, p = 0.01) (*Table 1*). This result is clearly visible on the 1st axis of the PCA (*Fig. 2*).

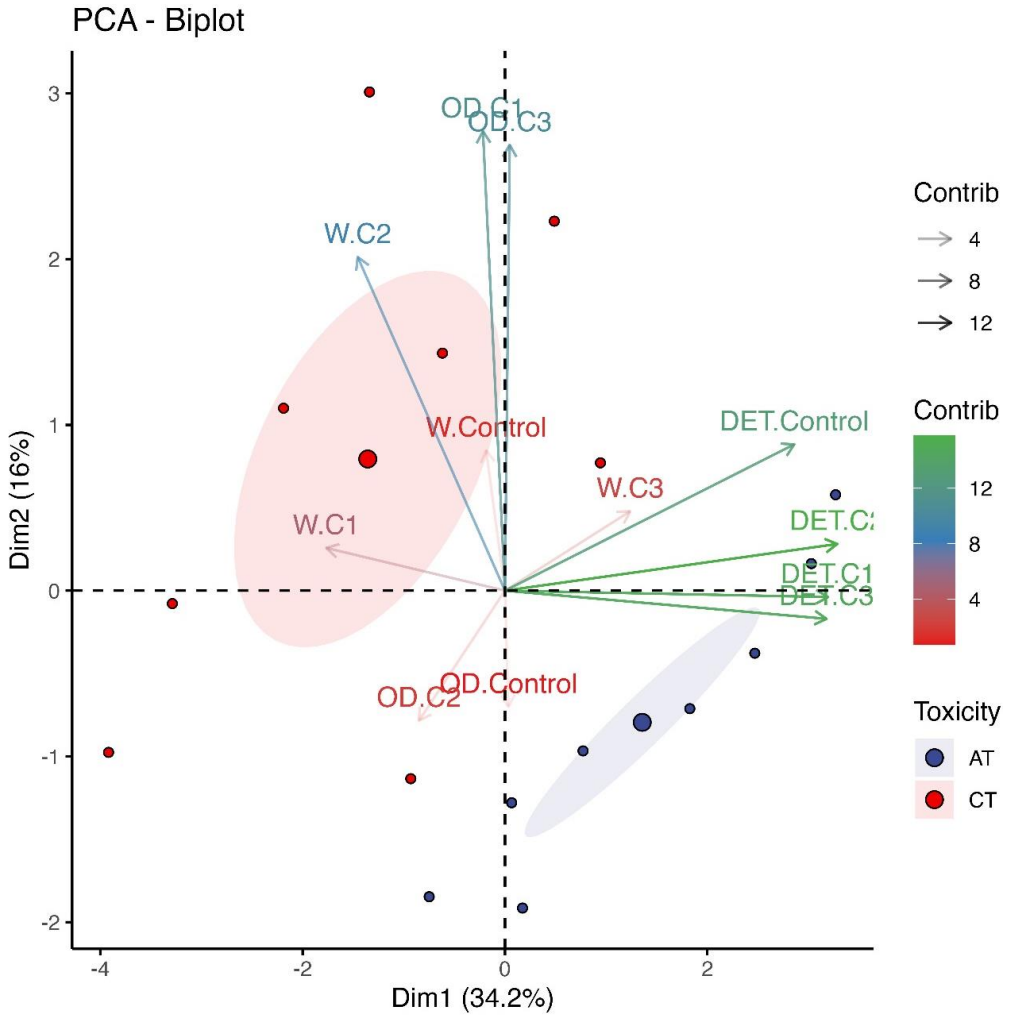

Figure 2. *Principal component analysis (PCA) biplot of acute and chronic toxicity and explanatory morphometric variables (n = 4). The biplot shows the PCA scores of the explanatory variables as vectors (in colors) and the toxicity experimental apparatus as points. Points on the same side as a given variable should be interpreted as having a high contribution on it. The magnitude of the vectors shows the strength of their contribution to each axis. Colored concentration ellipses (0.95 probability level) show the observation related to the toxicity type grouped by mark class. (Dim 1: 34.2% and Dim 2 = 16.0%)*

In a synthetic way, PCA (ordination technique) carried out on the toxicity data and the morphometric and weight measurements showed different patterns of correlations between DET, OD and W variables in relation to AT and CT (*Fig. 2*). In addition, the PCA resulted in two relevant components that accounted for 50.2% of data variance

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(total inertia). On the one hand, Dim1 alone represented by 34.2% of the variance and showed strongly positive correlation especially with DET:DET.control (r = + 0.84; cos^2 = 0.72), DET.C1 (r = + 0.94; cos^2 = 0.89), DET.C2 (r = + 0.97; cos^2 = 0.94) and DET.C3 (r = + 0.94; cos^2 = 0.88). On the other hand, PC2 just explained 16% of the total data variance and it was also positively correlated with OD.C1 (r = + 0.82; cos^2 = 0.67), OD.C3 (r = + 0.79; cos^2 = 0.63) and W.C2 (r = + 0.59; cos^2 = 0.35).

Moreover, the Dim1 (1st axis) clearly indicates a clear distinction between AT and CT where the morphometric parameter that contributes much more to this difference is DET. This latter states that the digestive epithelium is thicker in AT. Also, the Dim2 (2nd axis) could explain that CT is mainly characterized by very high values for W and OD; but unfortunately, the amount of inertia on this axis is weak (16%). In conclusion, the resultant biplot showed distinct partitioning in our toxicity experimental apparatus according to the first PCA axis and in a secondary way by the second PCA axis.

A Spearman correlation analysis was also performed to identify relationships between the different morphometric parameters, W, OD and DET of Polychaete annelids exposed to acute and chronic toxicity to ASA (*Fig. 3*).

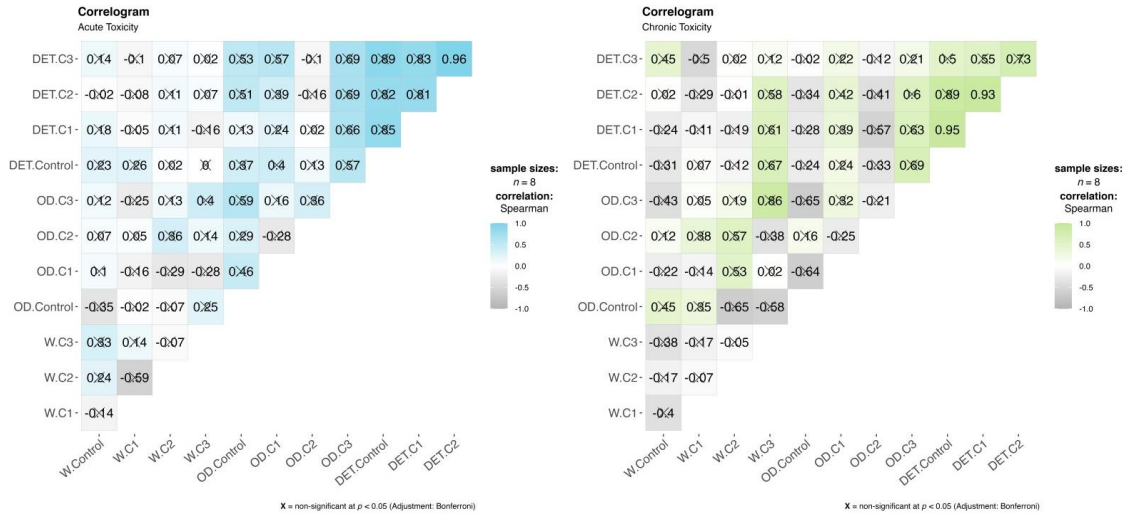

Figure 3. *Spearman correlation carried out on DET with the other variables in worms exposed to acetylsalicylic acid (in AT and CT)*

The Spearman's correlation analysis showed that in AT, DET.C3 was strongly correlated with DET.C2, DET.C1, and with DET.C with a significant correlation coefficient of r = 0.96; r = 0.83 and r = 0.8 respectively. Additionally, a strongly positive correlation was revealed between DET.C2 and DET.C1 (r = 0.81) as well as between DET.C2 and DET.C (r = 0.82). Moreover, the correlation between DET.C1 and DET.C was also very strong with a coefficient of r = 0.85.Regarding CT, a positive correlation (r = 0.93) was identified between DET.C2 and DET.C1 and also between DET.C2 and DET.C (r = 0.89), and another positive correlation (r = 0.95) was observed between DET.C1 and DET.C (*Fig. 3*). For AT, a non-significant correlation is recorded in individuals treated at different concentrations for oocyte diameters between OD.C3 and OD.C (r = 0.59) and for weight between W.C3 and W.C (r = 0.33).On the other hand, for the chronic toxicity a non-significant positive correlation was recorded between OD.C3 and W.C3 (r = 0.86).

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Biochemical markers

CAT activity

During this study, we found that CAT activity increased in contaminated worms. Indeed, the highest values were obtained at the highest ASA concentration (1.9 ± 0.04 µmol min^{-1} mg^{-1} protein and 1.52 ± 0.02 µmol min^{-1} mg^{-1} protein respectively for AT and CT). Comparison of CAT activity by the KW test between the concentrations revealed a significant differences ($p < 0.05$) and also observed within the two studied toxicities (*Figs. 4* and *5*).

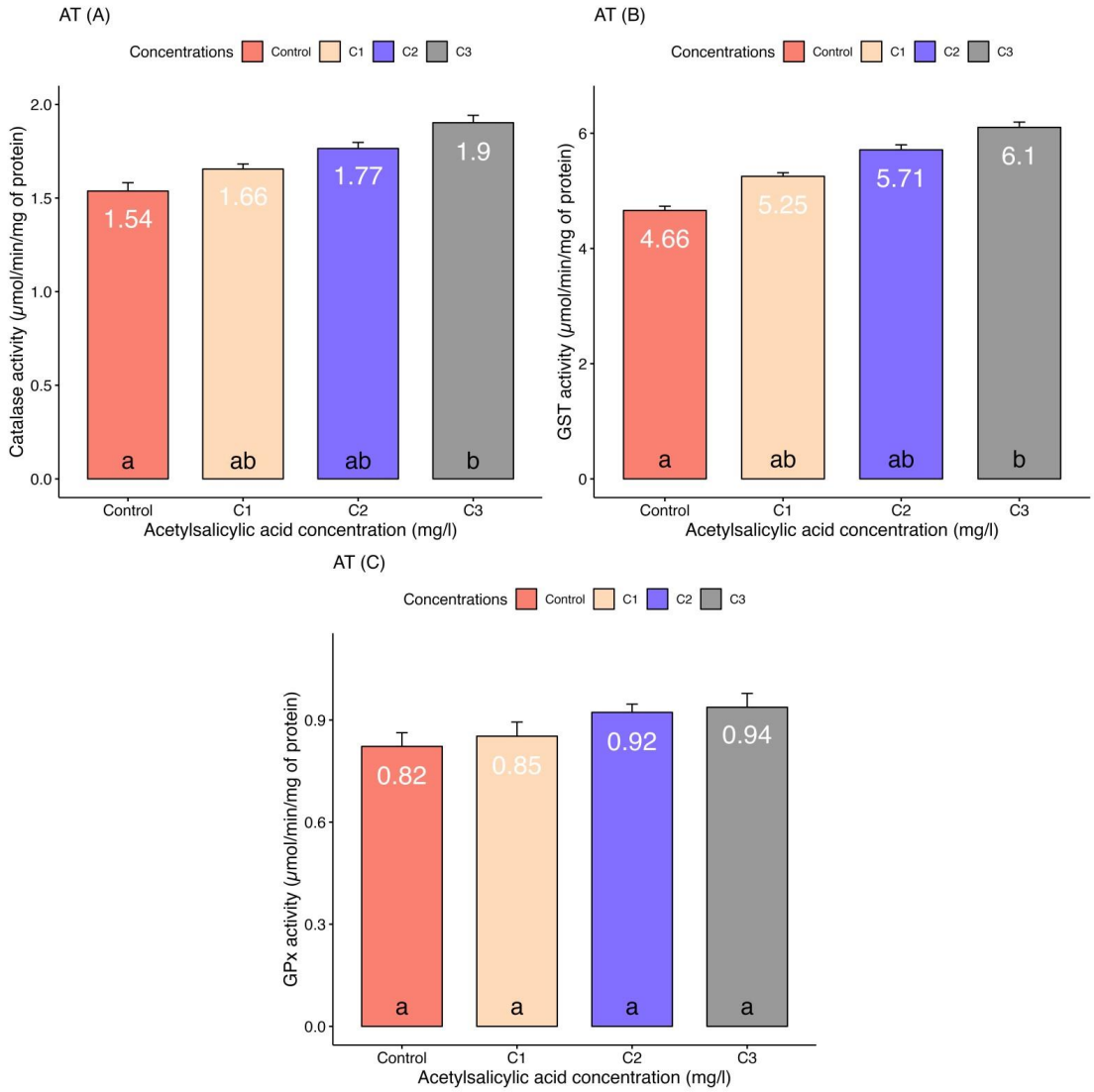

Figure 4. *Variations in biochemical markers; CAT: A (µmol^{-1} min^{-1} mg protein); GST: B (µmol^{-1} min^{-1} mg protein); and GPx: C (µmol^{-1}min^{-1} mg protein) after acute exposure to acetylsalicylic acid (m ± SE, n = 4). Data are expressed as mean ± standard error (SE)*

GST activity

The lowest values of GST activity vary respectively between acute and chronic toxicity (4.66 ± 0.07, 4.49 ± 0.08 µmol min^{-1} mg^{-1} protein) were observed in *P.*

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cultrifera exposed to the lowest ASA concentrations, then GST activity increased gradually with increasing exposure concentration to reach its maximum values (AT:6.1 ± 0.09 µmol min^{-1} mg^{-1} protein; CT: 5.81 ± 0.06 µmol min^{-1} mg^{-1} protein) in the highest concentration. The chronic and acute toxicity by ASA showed a significant differences (KW test, $p < 0.05$) between the different concentrations where the induction is observed from C2 for the CT and C3 for the AT (*Figs.4* and *5*).

GPx activity

GPx activity varied greatly among ASA concentrations, where a gradual increase in GPx activity was noticed with the increase in concentrations. No significant differences were observed between contaminated organisms by ASA during all AT period (KW test, $p > 0.05$). Moreover, in the CT, GPx was highly increased at C1 where the comparison by the KW test revealed a significant differences ($p < 0.05$) between all concentrations (*Figs. 4* and *5*).

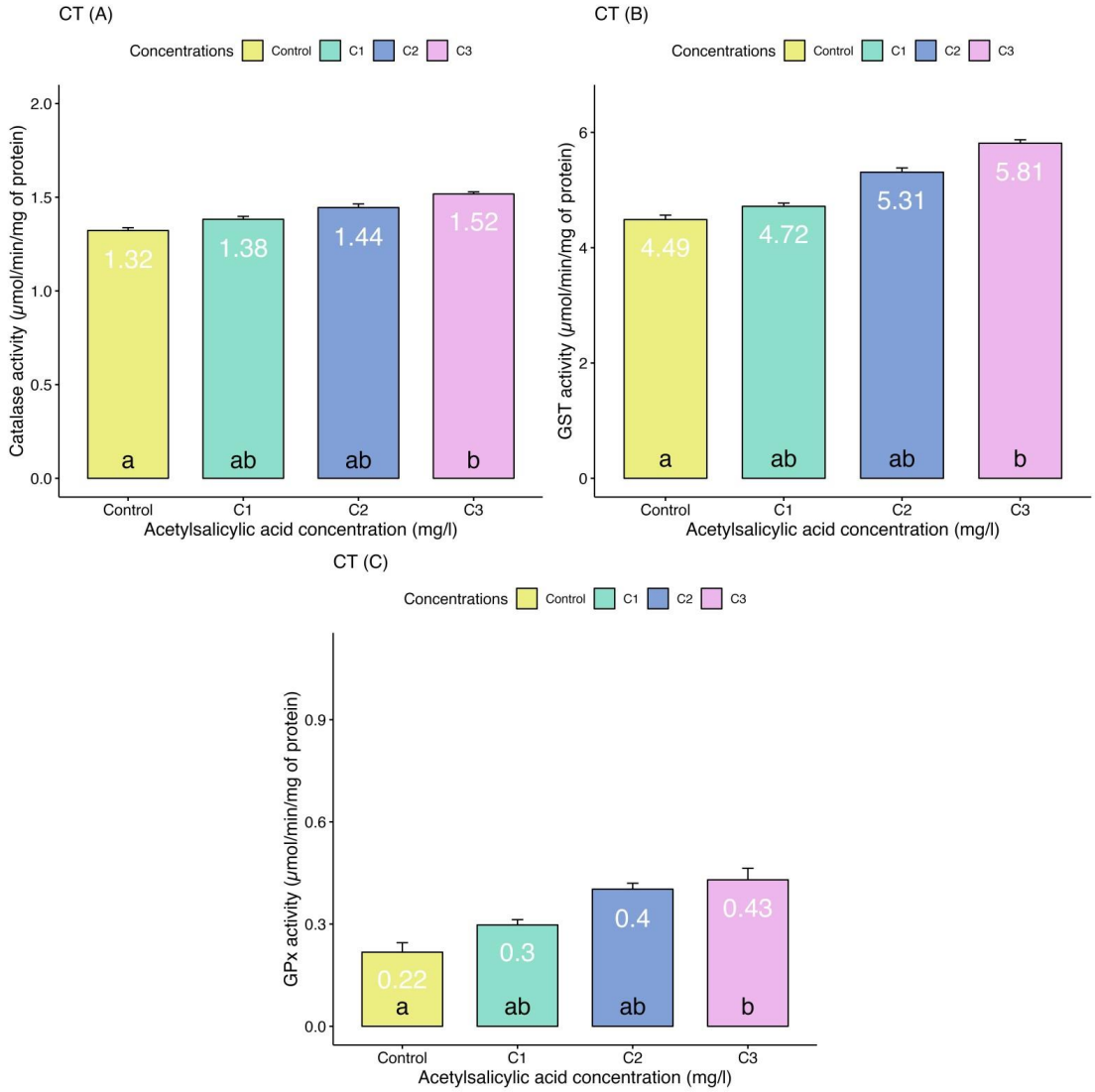

Figure 5. *Variations in biochemical markers; CAT: A (µmol^{-1} min^{-1} mg protein); GST: B (µmol^{-1} min^{-1} mg protein); and GPx: C (µmol^{-1} min^{-1} mg protein) after chronic exposure to acetylsalicylic acid (m ± SE, n = 4). Data are expressed as mean ± standard error (SE)*

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Histological examination

During the two periods of toxicity, the same trend was recorded for the W which underwent a progressive reduction during the toxicity from C1 (2.5 mg/L) in acute toxicity and from C1 (0.005 mg/L) in chronic toxicity to reach its lowest value in C3 and this jointly for the two toxicity tests. To this difference in weight is added the oocyte diameter which varies since the administration of C1 of the two toxicities, recording the lowest diameters at the end of the study periods with a diameter of 169.20 µm in C3 = 2.5 mg/L and 193.75 µm in C3 = 250 mg/L (*Fig. 6*).

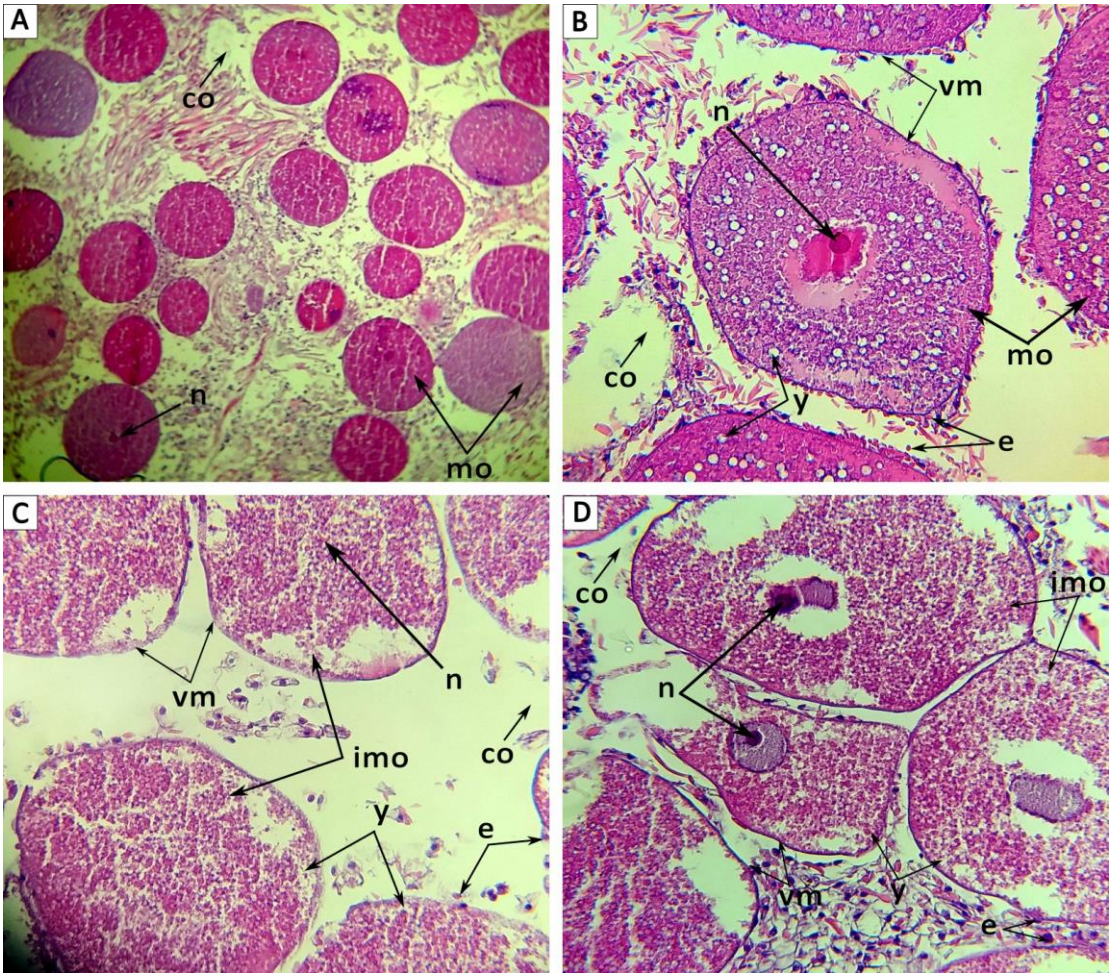

Figure 6. *Representative histological organization of coelomic fluid of Perinereis cultrifera from control group to different exposure tests at ASA. (A) Control group (Asynchronous vitellogenesis), X150. (B) Mature oocyte with yolk-grains, X600. (C) Immatures oocytes after C3 exposure of ASA at AT, X600. (D) Immature oocytes after C3 exposure of ASA at CT, X600. co, coelomic fluid; mo, mature oocytes*; *imo, immatures oocytes; vm, vitelline membrane; n, nucleus; e, eleocytes; y, yolk-grains*

Histological observations show in controls an asynchronization of oocyte growth where the cells are surrounded by a thin membrane. In the oocytes, we see the presence of very dense yolk-grains surrounding the nucleus in the center of oocyte (*Fig. 6A, B*).

After exposure of females to ASA at different concentrations during two toxicities tests (acute and chronic), a deterioration of the oocytes was showed results in a

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weakness in the quantity of yolk-grains and their absence at the periphery of the cells in females treated with C3 (250 mg/L) in acute toxicity at 96 h of treatment (*Fig. 6C*).

Furthermore, this reduction in yolk-grains is even greater in females treated with C3 (2 mg/L) after 30 days of treatment where resorption occurs at the periphery and in the center of the oocytes (*Fig. 6D*).

The histological sections carried out in the controls allowed the recognition of a large central gut surrounded by the longitudinal dorsal muscle located at the base of the paprapods and the presence of oocytes in the coelomic fluid (*Fig. 7*).

In response to ASA exposure, we observe a thinner and resorbed digestive epithelium (*Fig. 7B*) with a reduction in the number of oocytes which have small oocyte diameters (*Fig. 6C*). On the other hand, and at the level of the histological sections of females exposed to C3 (2 mg/L) of ASA, a chronic alteration affects the thinner and irregular digestive epithelium (119.12 µm) surrounded by much fewer immature oocytes (*Fig. 7C*).

It was observed a statistically significant difference only for the DET, particularly when exposed to AT. However, for the other morphometric parameters such as W and OD, no significant differences were observed.

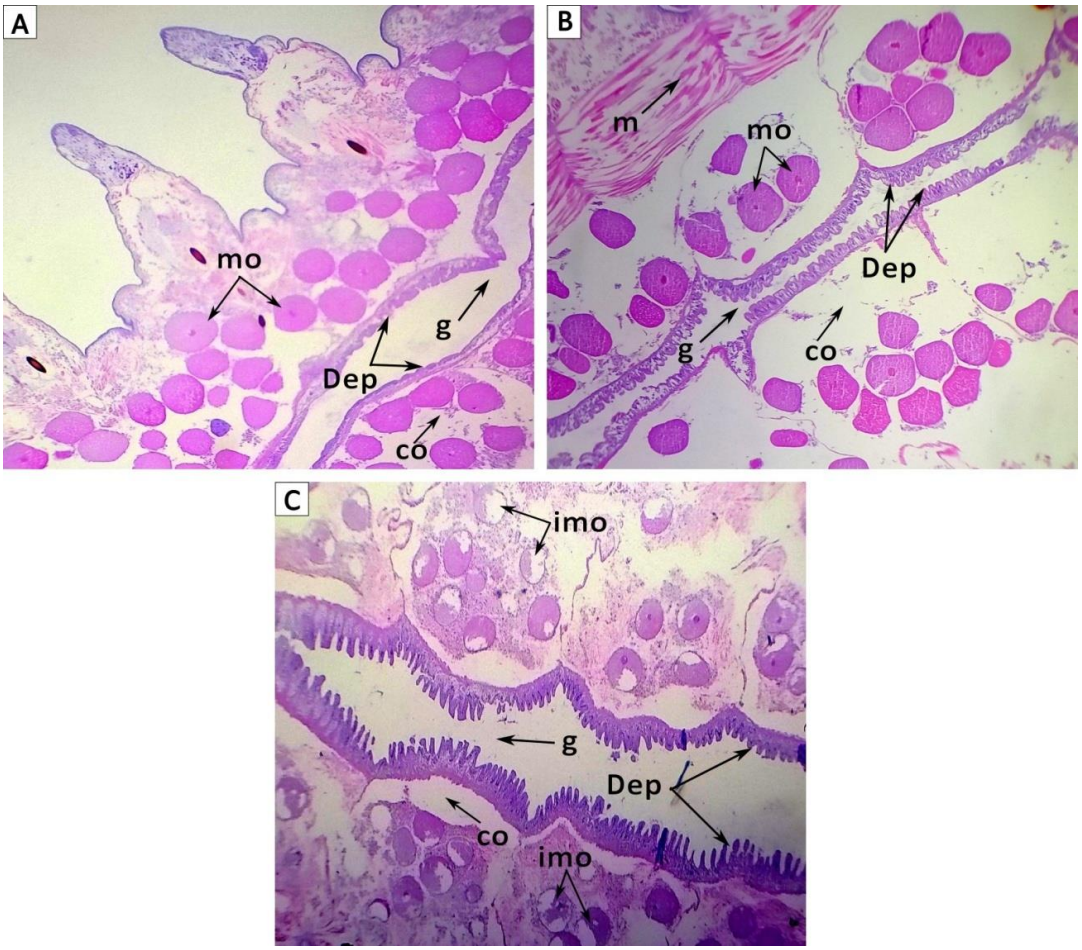

Figure 7. *Digestive epithelium histological sections of Perinereis cultrifera after exposure to ASA. (A) Control group, X60. (B) Digestive epithelium and matures oocytes after C3 exposure of ASA at AT, X60. (C) irregular digestive epithelium and immatures oocytes after C3 exposure of ASA at CT, X150. co, coelomic fluid; mo, mature oocytes; imo, immature oocytes; g, gut; m, muscle; Dep, digestive epithelium*

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Discussion

Relationship between morphometric parameters

The study of morphometric parameters; W, OD and DET thickness were carried out for the females individuals of *P. cultrifera* in order to detect a possible effect of exposure of ASA at acute and chronic toxicity on reproduction and digestion functions of the target species. The toxicological effects of ASA on *P. cultrifera*, after exposure to different concentrations, where the highest values for both acute and chronic toxicity, were observed in the control groups and decreased progressively with increasing exposure concentration. However, the two physiological functions targeted in our experiment are reproduction and digestion. Organisms can increase their survival process in an environment polluted by development of resistance with an evolutionary response to pollutants.

Throughout the experimental exposure, a progressive decrease response was recorded on W, OD and DET for the different concentrations of ASA during AT where it is showing a positive correlation between them. This is in agreement with a study carried out showing the effect of environmental stress on Polychaete annelids in Skikda and El Kala cities (Guemouda et al., 2014), by revealing the existence of a pollution effect on weight and length in females of *P. cultrifera*. A significant decrease in weight and oocyte diameter was observed after exposure to the first concentration and which becomes more significant for individuals at the end of the ASA exposure in CT. This corresponds, obviously, with the sexual differentiation period. The females chosen for our experiment are characterized by OD varying from 220 to 250 μm. Furthermore, according to Ramdani et al. (2020), the oocyte growth process is asynchronous in *P. cultrifera* and its reproduction function is more intense from March to May; however, the oocytes take 9 to 12 months to mature and their diameter at maturity is between 250 and 350 μm.

In general, the worms that colonize a disturbed environment have a reduced weight gain stage (Durou et al., 2007, 2008; Gillet et al., 2008; Mouneyrac et al., 2009). However, the evolution of this resistance is usually accompanied by an expression physical form, such as reduction of reproduction, growth (Wirgin and Waldman, 2004) or reduced tolerance to various stressors (Meyer and De Giulio, 2003).

In addition to the effect of ASA, these disruptions could be due to the hormones and the abiotic factors (Temperature, Salinity, Food, etc.). They also confirmed that worms living in disturbed environments have a reduced weight gain stage (Durou et al., 2007, 2008; Gillet et al., 2008; Mouneyrac et al., 2009).

To this aim, mussel digestive gland was selected as target organ because it plays vital functions in the accumulation, biotransformation and detoxification processes of xenobiotics (Cappello et al., 2018). Also, these females were treated with ASA during the start of the reproductive period of this species which overlaps the March month. As stated by Costa et al. (1998), the existence of a noticed continuous reproductive activity all year long, in which the gut was reabsorbed and feeding ceased, as observed for this species and other Nereididae during its sexual maturity period (Golding and Yuwono, 1994; Last and Olive, 1999; Belfetmi et al., 2021).

Biomarkers responses

The Algerian coasts like several Mediterranean wetlands are subject to an increasing pressure in the anthropogenic activities (urbanization, industry, pollution, aquaculture,

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tourism and overfishing). The littoral is highly vulnerable to a wide assortment of contaminants and micropollutants directly released into the seas and oceans, to which are added those released into the air and drained by soils and rivers (Bensouda and Soltani-Mazouni, 2014). Invertebrates, in particular worms, are essential vectors of contaminants from environmental compartments (water, food or sediment) to higher trophic levels (Cattaneo et al., 2009). Polychaetes such as *P. cultrifera* (Grübe, 1840) are commonly used in ecotoxicological studies because of their high sensitivity towards organic contamination (Bouraoui et al., 2009). Many authors have observed that different species of bivalves exposed to environmental pollution may have slow growth (Peteiro et al., 2006). Changes in environmental factors imposed on these aquatic lives are usually gradual, rhythmic and predictable (Ahamefula, 2014).

Biomarkers are relatively effective in revealing overall toxicity of complex mixtures, particularly those that are at a high level of a biological organization such as physiological biomarkers relating to the growth or reproduction of organisms.

In this work, the evaluation of the biological effect of ASA in Polychaetes during an acute and chronic exposure allowed us to collect valuable information about a pharmaceutical effect on aquatic organisms. Polychaetes as the mussels were reconfirmed as valid bioindicators in the ecotoxicology field; owing to their ability to elaborate a wide range of biological responses following exposure to chemical compounds (Dean, 2008; Vassalli et al., 2015; Piscopo et al., 2018; Świacka et al., 2019; Cappello et al., 2021; Caliani et al.,2022).

The Algerian coasts, which are akin to numerous other wetlands in the Mediterranean regions, are experiencing a surge in anthropogenic activities that are exerting pressure on them. This pressure is a result of various factors such as industrialization, urbanization, aquaculture, tourism, pollution, and overfishing. The vulnerability of the coast to a variety of contaminants and micropollutants that are frequently released into the oceans and seas, is heightened by the additional pollutants that are discharged into the atmosphere and carried through the soil and rivers (Bensouda and Soltani-Mazouni, 2014).

The results of our study indicate that organisms which were exposed to high concentrations of ASA for a short period of time (acute toxicity 2.5 mg/L, 25 mg/Land 250 mg/L) showed an increased level of CAT activity. This response may be considered a defense mechanism against an excess of H_2O_2 and to prevent damage caused by lipid peroxidation. Similarly, significantly increase of CAT activity was shown in the Oligochaete *Lumbriculus variegatus* exposed to contaminated sediment even at one day exposure (Contardo-Jara and Wiegand, 2008). On the other hand, in the case of organisms which were exposed to ASA over a long period of time for 30 days (chronic toxicity 0.005 mg/L, 0.1 mg/L and 2 mg/L), an increase in CAT activity was reported. This increase was aimed at eliminating excessive ROS to prevent further damage. CAT also gives information on the oxidative stress response status of organisms, as this is protecting the cell against oxyradicals induced by numerous factors (Winston and Di Giulio, 1991).

The rise in GST at C1 at acute and chronic exposure for ASA respectively at 2.5 mg/L for 96 h and 0.001 mg/L for 30 days, similar to what was documented by Freitas et al. (2019) in Mussels exposed to higher SA concentrations for 28 days. The presence of effects concerning the activity of GSTs, after both acute and chronic exposures, suggests that following exposure to ASA, the detoxifying system mechanism of *H. diversicolor*, namely through GSH conjugation, was activated. As shown in

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Figures 4 and *5*, GST activities of *P. cultrifera* exposed to ASA were time dependently upregulated. The highest level of GST activity was observed after 24 h of exposure, and maintained a high level of activity over 96 h after exposure. By contrast, Praskova et al. (2012) investigated the acute (96 h) effects of ASA in *Danio rerio*, at concentrations of 340 mg/L, 380 mg/L, and 420 mg/L, and obtained higher GST activity. Nunes et al. (2015) reported that the activity of GSTs in fish may not be influenced by salicylic acid (SA).

De Luca-Abbott et al. (2005) demonstrated species-dependent biomarker responses by showing that GST and CAT had great responses than other antioxidant biomarkers in the Manila clam *Ruditapes philippinarum* when compared to the green-lipped mussel *Pernaviridis*.

The GPx activities of *P. cultrifera* was highly increased after 96 h of chronic exposure chronically exposed to the concentrations 0.1 mg/L and 2 mg/L for 30 days. Nunes et al. (2015) showing an increase in the activity of GPx in the liver and gills of *Salmo truttafario*. Also, Zivna et al. (2013) obtained an increase in the activity of GPx in the fish *Danio rerio* exposed to ASA. Herein, Data on GPx activity revealed a significant difference in Polychaetes by exposure to ASA at concentrations above 0.005 mg/L (C1) for 30 days. This could be related to the detoxifying activity of GST, the most important phase II biotransformation enzyme (Manduzio et al., 2004). The reactions catalyzed by GPx involve the reduction of a peroxide to its corresponding alcohol, by simultaneously converting reduced glutathione to glutathione disulfide, by oxidation (Moreira et al., 2006).

In this study, no significant differences were observed in terms of GPx after acute exposure. In *P. cultrifera*, GPx was slightly increased at chronic exposure by ASA.A further hypothesis advanced by Zivna et al. (2016) is related to the possibility of certain concentrations of ASA to lead to damage at the mitochondrial level.

Oocytes and gut histology

Histological analysis provides a better presentation of the morpho-anatomical reactions of the organs affected by contamination. In this study, alterations were recorded in certain tissues exposed to ASA at different concentrations.

The weight and the oocyte diameter decreased drastically after the first concentration administered in the two toxicity studies. Apart from the effect of ASA, this decrease may be related to other factors such as adaptation, temperature and photo period.

Similarly, the studies examining the effects of contaminants on the growth and reproduction of *P. cultrifera* have shown that individuals collected in polluted environments have reduced morphometric parameters compared with those collected in healthier environments (Ramdani et al., 2020; Belfetmi et al., 2021; Rezzag Mahcene et al., 2022; Sebbih et al., 2023). Also, other work has demonstrated that change in timing of spawning and life cycle of Polychaetes could be a consequence of temperature and to climate factors (Ait Alla et al., 2006).

The biometric measurements show a similar evolution and that oocyte growth is asynchronous. Moreover, in related species such as *Platynereis dumerilli* (Fisher et Donesteijer, 2004) and *Nereis virens* (Hoeger et al., 1999), oocyte growth is synchronous.

The effect of ASA during the two toxicity tests on the OD presents the lowest averages of this parameter at 96 h and 30 days with respectively 169.20 µm and 193.75 µm as well as a low distribution of yolk-grains. These values reach

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approximately 200 µm, the diameter marking the maturity phase, the last stage before reproduction which coincides with our study period (February-March). This result corroborates that individuals of *P. cultrifera* reproduced exclusively by epitok from March (Daas et al., 2011) and that *Nereis diversicolor* living in disturbed environments have a reduced weight gain stage (Durou et al., 2008; Mouneyrac et al., 2009).

As for the DET, it was found that the most significantly low average was that obtained from C2 to C3 during chronic toxicity (125.75 ± 4.20 µm to119.12 µm), unlike AT where the reduction in thickness was gradual and significant.

On the other hand, in mussel gills tissues during the 12 days chronic exposure, SA did not cause any morphological alterations (Giuseppe di Marco et al., 2022).

To this aim, DET of *P. cultrifera* was damaged after the C3 of ASA by maintaining a decrease in the thickness of the digestive epithelium and especially in chronic toxicity which could also be due to its diet by flakes of TetraMin.

It is well known that *P. cultrifera* as an omnivore species (Scaps, 2002) prefers algae, diatoms and detritus (Goerke, 1971). Exposure to these concentrations of ASA caused degeneration of oocytes (CT/C2-C3: 217.25 ± 7.32 µm and 193.75 ± 5.71 µm) and resorption of the digestive epithelium. The existence of a noticed continuous reproductive activity throughout the year (Costa et al., 1998), in which the gut was reabsorbed and feeding ceased, as observed for this species and other Nereididae during its sexual maturity period (Golding and Yumono, 1994; Last and Olive, 1999).

The thickness of the digestive epithelium could suggest its degeneration, which can be translated into the induction of cellular degeneration, a process that could be linked to epitoky in reproduction. A related study revealed that the intestinal changes associated with epitoky illustrate an example of programmed cellular degeneration (Dakhamaet al., 1985).

Conclusion

The impact of PhAcs on non-target marine organisms was clarified through our study on adult female individuals of *P. cultrifera* (Nereidae). The present work made it possible to assess the risk of environmental contamination by determining the toxicological effects after exposure of individuals to ASA. Two forms of toxicity tests were applied: Acute (2.5 mg/L, 25 mg/L and 250 mg/L) and Chronic (0.005 mg/L, 0.1 mg/L and 2 mg/L). Data was obtained from a multi-biomarker approach (CAT, GST and GPx) following the effect of ASA exposure on tissue damage levels.

The results showed increased CAT activity after acute and chronic exposures. This response may be considered a defense mechanism against an excess of H_2O_2 preventing damage caused by lipid peroxidation. The GST activities of *P. cultrifera* exposed to ASA were dose-dependently upregulated. The highest level of GST activity was observed and maintained during the study. Also, GPx was slightly increased during chronic exposure to ASA but no significant differences were observed after acute exposure.

The weight and the oocyte diameter decreased drastically after the first concentration was administered in the two toxicity studies. The biometric measurements showed a similar evolution and that oocyte growth was asynchronous. The effect of ASA during the two toxicity tests on the oocyte diameter presented the lowest averages of this parameter at 96 h and 30 days. For the DET, it was found that the most significantly low average was obtained at C3 during chronic toxicity, unlike acute toxicity where the

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reduction in thickness was gradual and significant. The thickness of the digestive epithelium could suggest its degeneration, which can be translated into the induction of cellular degeneration, a process that could be linked to epitoky in reproduction.

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