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Assessment of multimarker responses in *Perna perna*, *Mytilus galloprovincialis* and *Donax trunculus* bivalves exposed to malathion and 2,4-dichlorophenoxyacetic acid pesticides

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Abstract
The early biological effects of pesticides was assessed in three species of bivalves: *Perna perna*, *Mytilus galloprovincialis* and *Donax trunculus*, exposed to 500 µg/L of malathion **Mal** (organophosphorous pesticide; **OP**) and 2,4-Dichlorophenoxyacetic acid **2,4-D** (organochlorinated pesticide; **OC**). The responses of acetylcholinesterase (AChE, biomarker of neurotoxicity), glutathione S-transferase (GST, biomarker of detoxification) and catalase activities; and malonedialdhyde levels (CAT and MDA; biomarkers of oxidative stress and lipid peroxidation) were characterized. The mortality rate of bivalves was determined as biomarker of general stress. The results revealed critical alterations of the measured sub-cellular parameters. The alterations of the biomarkers responses depend on the pesticide and time of exposure. AChE showed a significant inhibition in all species along the experiment; which could be explained by neurotoxic effect due to the high sensitivity of AChE to pesticides, especially organophosphorous compounds. While, GST activity was significantly increased ($P< 0.01$) after 24h of exposure to 2,4-D (85 % of induction in *P. perna* at 48h). CAT activity and lipid peroxydation were induced after 12h of exposure to pesticides indicting a high generation of free oxyradicals. Meanwhile, mortality increased progressively according the exposure time (more than 30%). The multimarker responses used in these experiments demonstrated clearly the early biological effects (biochemical dysfunctions) and mortality rate increase of *Perna perna, Mytillus galloprovincialis, Donax trunculus* exposed to the sub-lethal concentration of malathion and 2,4-dichlorophenoxyacetic acid pesticides.

Key words : Perna perna, Mytillus galloprovincialis, Donax trunculus, multimarker approch, Malathion, 2,4-D.

1. Introduction
Pesticide contaminants constitute one of the major environmental concerns. The increasing application of pesticides in agriculture causes several environmental problems, leading to harmful effects at different levels of the biological organization. These contaminants can reach aquatic ecosystems (marine and fresh waters) *via* the phenomena of agricultural soils scrubbing and material haulage containing potentially toxic residues of pesticides which threat species inhabiting these ecosystems [1]. In addition to their variable environmental behavior (OC more stable than OP) their persistent proprieties in non-target organisms and ecosystems are not excluded [2].
Many works on biomonitoring of environmental contamination, especially in marine ecosystems using biological indicators in molluscs have been developed. These techniques applied in the international biomonitoring programs were proposed by several authors for screening, monitoring and identifying environmental risks on aquatic organisms [3-4-5-6] and are of a great interest for developing countries because of their simplicity, sensitivity and precocity detection of pollution and their cost effectiveness.
Some of *in situ* and *in vivo* studies carried out in our laboratory have used the biomarkers for the evaluation of the contamination by various pollutants [7-8-9-10-11]. This work, aimed to assess the responses of multiple biomarkers in three bivalves; *Perna perna*, *Mytilus galloprovincialis* and *Donax trunculus* exposed to malathion and 2,4-Dichlorophenoxyacetic acid pesticides. The selected pesticides are extensively used in Morocco (**Mal** as an insecticide and **2,4-D** as a herbicide) according to Moroccan phytosanitary index published in 2014 [12]. This multiple biomarkers approach could provide more accurate and interpretable data on the complex effects of pesticide pollutants. The results may be useful to compare them to other works having developed a similar approach to evaluate effects of various contaminants both under field and laboratory conditions [13-14-15].

2. Material and methods

2.1 Animal sampling and acclimatization

Three species of bivalves were used in this work; *Perna perna* and *Mytillus galloprovincialis* which characterise rocky areas of the littoral (mediolittoral zone) and *Donax trunculus* living in sandy beaches. 40 animals per specie (about 40 mm for *P. perna* and *M. galloprovincialis* and 20 mm for *D. trunculus*) were sampled from the reference site (site of Cap Ghir, located at 50 Km in the north of Agadir city) and transported in sea water. Once in the laboratory, the animals were kept in aquariums with seawater and at air-conditioned room (Salinity = 36 µs/cm^2, Temperature = 16 ± 2 and Light regime : 12h/12h) during 48h.

2.2 Animal contamination

After 2 days of acclimatization period, mussels (about 60 specimens per set) were transferred to the exposure aquariums containing 500 µg/L of each pesticide (Mal or 2,4-D used as chemicals with 99% purity from Sigma-Aldrich ®) in 5L of natural seawater originating from the reference site. Controls were separately grouped in non-contaminated sea water. A permanent bubbling allows for saturating the medium with oxygen and water was renewed every 2 days for both controls and exposed animals. 6 to 8 specimens per species per each tested pesticide and of controls were taken after 0.5 day (12h), 1 day (24h), 2 days (48h) and 7 days (168h) of exposure and used for analysis.

2.3 Biochemical analysis

Mussels were dissected and the whole soft tissues of each animal were homogenized with an ultra-turrax (at 5000 rpm), in 100 mM, pH 7.4 Tris buffer (tris [hydroxymethyl] aminomethane) with ratio of 3ml of Tris solution per 1 gram of fresh tissue. The homogenates were centrifuged at 9000 g for 30 min. All procedures were carried out at 4°C. The supernatants (S_9) were collected and analysed.

2.3.1. Acethylcholinesterase (AChE) determination

AChE activity was measured according to the method of Ellman et al. [16] by kinetic measurement at 412 nm. Reaction mixture contained 5,5'-dithiobis-[2-nitrobenzoic acid] (DTNB) as Ellman's reagent, stock cytosolic containing acetylcholinesterase fractions and acetylthiocholine (AsCh) as substrate.

2.3.2. Catalase (CAT) determination

CAT activity was determined by the method of Aebi [17], using the enzymatic decomposition rate of hydrogen peroxide (H_2O_2) which is determined by absorbance decrements at 240 nm.

2.3.3. Malondialdehyde content (MDA) determination

Lipid peroxidation was estimated in terms of malonedialdhyde levels using 1,1,3,3-tetramethoxypropane (TMP) as a standard. The reaction was determined at 532 nm using thiobarbituric acid reagent as per the method of Sunderman [18].

2.3.4. Glutathione S-transferase (GST) determination

GST activity was assayed by the method described by Habig et al. [19], using glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrate.

The total proteins concentration in the S_9 fraction was determined according to the method of Lowry [20] with bovine serum albumin as a reference.

2.4 Mortality rates calculation

The mortality assessment is performed simultaneously in exposed and non-exposed animals depending on exposure times and type of pesticide. Mortality rates in exposed molluscs are calculated compared to non-exposed ones by the following formula:

$\mathbf{X_e}$: number of dead in exposed mussels
$\mathbf{X_n}$: number of dead in non-exposed mussels
N : Total exposed mussels

$$\textbf{Mortality \%} = \frac{\textbf{Xe - Xn}}{\textbf{N}}$$

2.5 Expression of enzymatic activities and statistical analyses

Enzymatic activities were expressed as nmol of transformed substrat per minute per milligram protein in S_9. While MDA content was expressed as nmol equivalent MDA per milligram protein in S_9. For each measurement, means and standard deviation (m ± SD) were calculated from the 6 to 8 replicas (n= 6 to 8). The comparison of the means was carried out by the variance analysis (ANOVA) and the Low Significant Difference test (LSD) using Statistica software (release 10, Edition StatSoft, 2010). The significance levels are set at $p>0.05$ (*) and $p>0.01$ (**).

3. Results and Discussion

The effect of Mal and 2,4-D pesticides on AChE activity is shown in the Fig. 1 (**a**). The results revealed a significant decrease ($p<0.01$) in AChE activity in the three species exposed to the two pesticides. The AChE activity diminished gradually from 12 h to 168h of exposure. Indeed, the lowest activities were recorded after

168h of exposure with the values of 0.28 ± 1.42, 1.98 ± 0.37 and 7.10 ± 2.93 nmol/min/mg proteins compared to the controls (0.88 ± 3.73; 3.39 ± 0.15 and 35.03 ± 2.63 nmol/min/mg proteins) respectively in *P. perna*, *M. galloprovincialis* and *D. trunculus*. The AChE inhibition was more pronounced in the molluscs exposed to organophosphorous pesticide (Mal) than those exposed to organochlorinated one. Indeed, 2,4-D did not have significant effect on *P. perna* and *M. galloprovincialis*, however an inhibitory effect of this compound on *D. trunculus* was observed ($p<0.05$) after 24 hours of exposure (0% against 39% inhibition, respectively, 12 and 48)

Exposure of molluscs to pesticides provoked a very significant ($p<0.01$) increase of GST activity levels (Figure 1 **b**) after 24h of exposure compared to the controls. This biomarker was more induced by 2,4-D in *P. perna* and *M. galloprovincialis* than *D. trunculus* (78% ; 65% and 28% of induction respectively at 48h). On the other hand, the induction of GST was more highlighted in the case of 2,4-D than in that of Mal for *P. perna* and *M. galloprovincialis* (respectively 78% and 65% for 2,4-D against 40% and 4% for Mal at 48h of exposure).

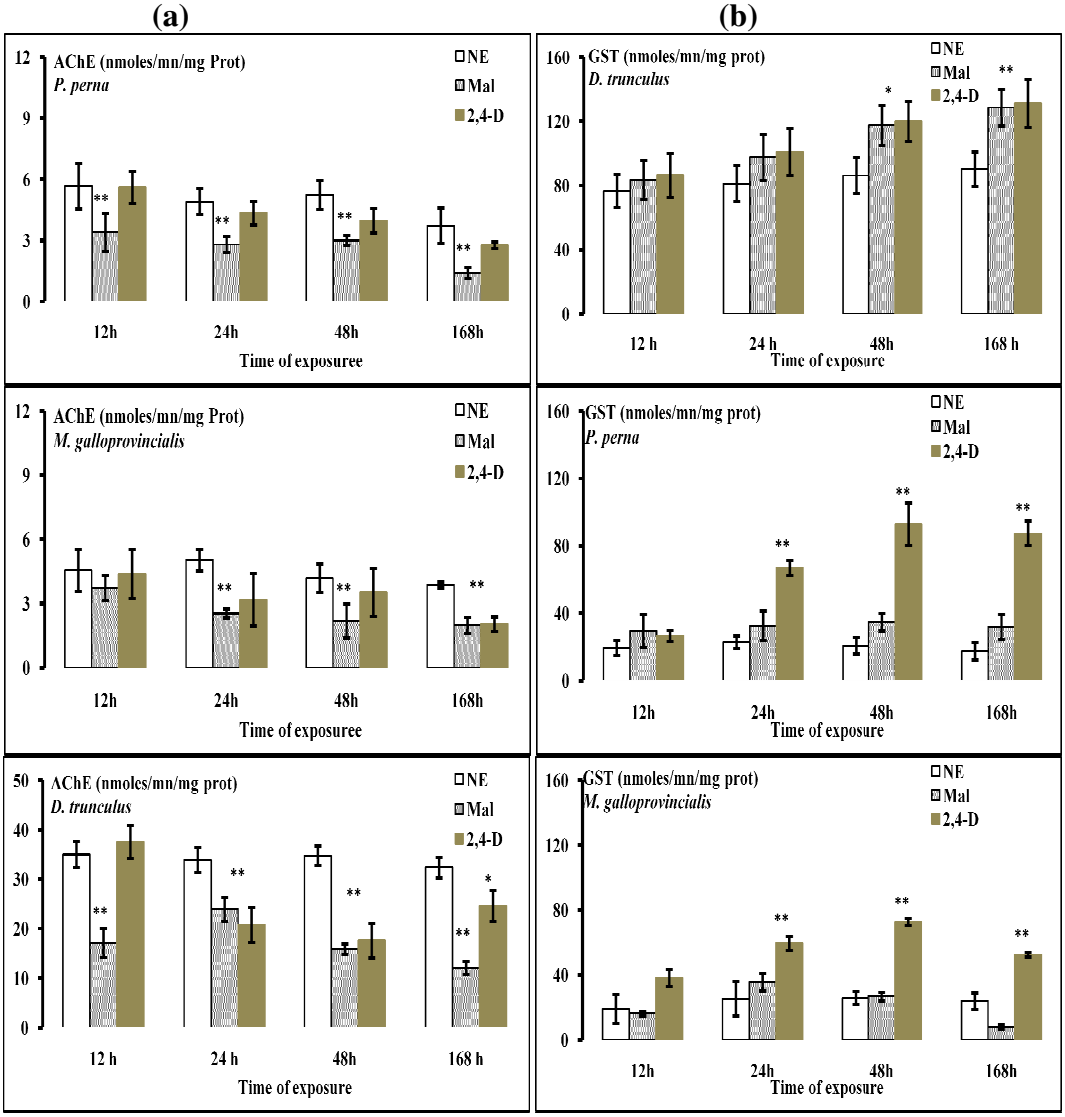

Figure 1. Evolution of acetylcholinesterase AChE (**a**) and glutathion S-transferase GST (**b**) in *P. perna*, *M. galloprovincialis and D. trunculus* in vivo exposed to 500 µg/L of Mal and 2,4-D during 7 days.

The CAT response showed a similar profile in the three species for both Mal and 2,4-D (Figure 2 **a**). This activity is induced after 12 h of exposure by the malathion ($p<0.05$) with rates of 6.12 ± 1.17 among exposed compared to controls (3.61 ± 0.61 nmol/min/mg proteins) in *P. perna* and 5.09 ± 0.55 nmol/min/mg proteins in *M. galloprovincialis* compared to the control values (3.51 ± 0.6 nmol/min/mg proteins). Concerning *D.trunculus*, catalase activity is induced significantly ($P<0.01$) after 24 hours of exposure to pesticides which is maintained all along the exposure (20.36±1,6 and 19.73 ± 2.72 nmol/min/mg proteins respectively at 24h for Mal and at 48h for 2,4-D compared to controls). As for

the lipid peroxydation (Figure 2 **b**), higher rates of MDA were observed in exposed mussels after 12h of exposure with a percentage of induction of 30% and 70% respectively for Mal and 2.4-D in *P. perna* and about 72% and 29% respectively for Mal and 2,4-D in *M. galloprovincialis*. The Induction seems to be gradually unsignificant after 24h of exposure, with rates varying depending on the exposure time and the kind of pesticide. However, in *D. trunculus* à strong production of MDA ($p< 0.01$) occurs from 12 hours of exposure and continues until the end of the experiment with the highest rates caused by 2,4-D (59% against 49% of induction respectively at 12h and 168h). It was also noted that the levels of MDA registered in controls of *M. galloprovincialis* are higher than those measured in *P. perna* and *D. trunculus* (average value of 0.8; 0.5 nmol /mg proteins in *P. perna* and *D. trunculus* against 5 nmol /mg proteins in *M. galloprovincialis*).

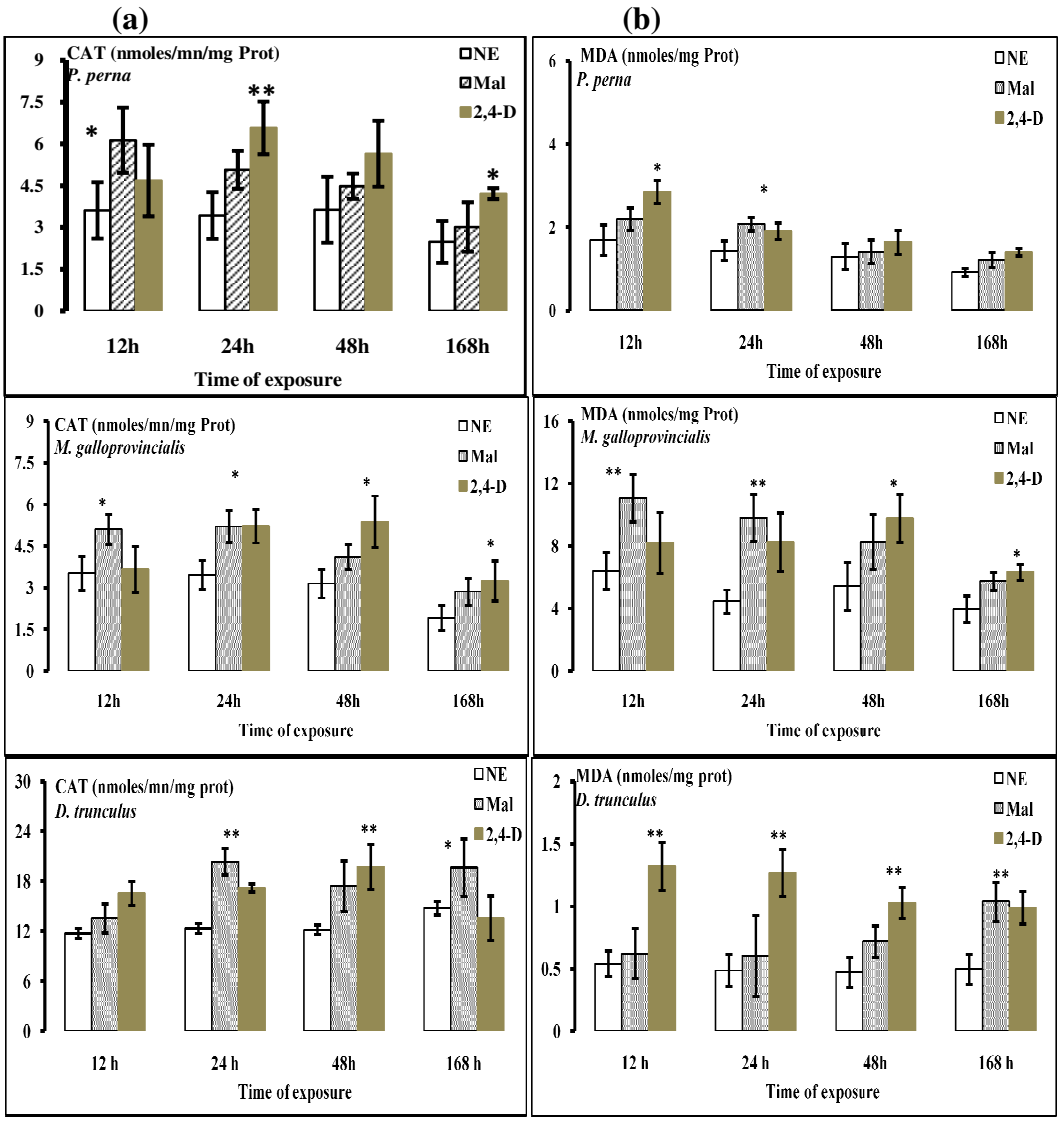

Figure 2. Evolution of catalase CAT (**a**) and malonedihadehyde MDA (**b**) in *P. perna, M. galloprovincialis and D. trunculus* in vivo exposed to 500 µg/L of Mal and 2,4-D during 7 days.

The mortality rates of exposed mussels to pesticides are represented in Fig.3. The results show a gradually increase in rate mortality of the treated mussels with pesticides from 12h of exposure to achieve the highest rate at the end of the experiment (12% versus 38% and 9% versus 37% respectively at 12h and 168h in *D. trunculus* and *P. perna*). On the other hand, Mal appears to induce more mortalities that 2,4-D (37% against 24% in *P. perna* to 168h). Such results were described in mussels exposed to organophosphorous pesticide [21] or to sediments originating from contaminated areas by hydrocarbons and pesticides [9], showing mortality rates exceeding 50%.

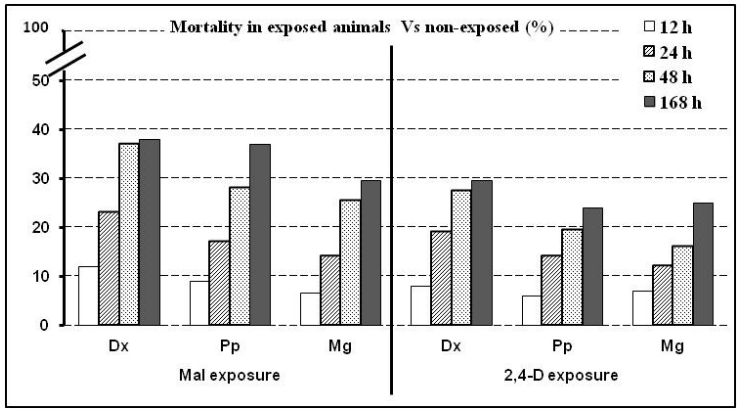

Figure 3. Evolution of mortality rates in exposed compared to non-exposed mussels during 7 days exposure to pesticides (Mal and 2,4-D) (in %).

Our results showed that tested pesticides exert a notable inhibitory effect on AChE activity in exposed mussels especially at the end of the exposure (over than 50% reduced AChE activity in all species at 168h of exposure for OP pesticide). Such inhibitory effect of organophosphorous and carbamate insecticides on the cholinesterase enzymes is well known [22-23] but its intensity depends on several factors such as the nature of pesticide, levels of exposure and studied species [24-25]. Suggestions reported by these authors agreed with our results, relating variable response in the species and depending on the type of pesticide. It was also demonstrated, that the inhibition of AChE is correlated, whether *in vivo* or *in situ*, with increase levels of pesticide in the environment [13-5-26-27]. Indeed, the inhibitory effect of pesticides, particularly organophosphates, manifested by the irreversible reaction of biotransformation products of pesticides with the hydroxyls groupings of the serine of the AChE active site [28-29-30]. A significant reduction (up than 40%) of the brain AChE in fish from polluted sites of Italy was reported by Lionetto et al. [31] related with presence in the sediment of great variety of compounds, especially pesticide residues. Similar results were affirmed by the study on brain AChE activity assessed in fish *Seriola dumerilli* which significantly inhibited after 7 days of Mal exposure [32]. Cantry et al. [33] reported that exposure of the blue mussel, *M. edulis*, to organophosphorous pesticide for periods of up to 24h caused a significant reduction in acetylcholinesterase activity.
Several studies have shown that lipoperoxidation is one of the molecular mechanisms that reflect an early toxicity [34] particularly due to contamination by pesticides [35]. They act by inducing oxidative stress and the production of free radicals and by the alteration of enzymatic and non-enzymatic defence systems [36]. In the same way, some works conducted on the effects of organic substances (pesticides, hydrocarbons …) suggested different effects depending on the nature of pollutants and the species. Therefore, Narbonne *et al*., [5] observed that many organic substances including pesticides induce lipid peroxydation in *M. galloprovincialis*. Thus, our results clearly demonstrated the induction of lipid peroxidation in exposed mussels resulting in increasing concentrations of MDA, along with a significant induction of CAT activity (antioxidant enzyme) that might be due to the effect of Mal and 2,4- D.
GST isoforms are involved in the metabolism of organochlorinated pesticides [37] and consequently were used as biomarkers for these substances and other organic compounds (HAPs and PCBs) in shellfish [38]. This affirmation is in perfect agreement with our results revealing high induction of GST in exposed molluscs to 2,4-D. These compounds seem to exert similar effects as those caused by organic compounds recognized by their inductive effect on GST.

Conclusion

The biochemical effects of sub-lethal concentrations to 500 µg/L of malathion and 2,4-dichlorophenoxyacetic acid pesticides was assessed in three species of bivalves: *Perna perna*, *Mytilus galloprovincialis* and *Donax trunculus*, under laboratory conditions. The malathion and 2,4-dichlorophenoxyacetic acid pesticides exposures caused a significant inductions of glutathion S-transferase, catalase and lipid peroxidation. The increase of oxidative stress biomarkers was negatively associated with the AChE inhibition in the three tested species. In addition, the present study provides additional evidences for the usefulness of a set of biomarkers in assessing the health of bivalves exposed to the models of pesticides.

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