

Effects of methamidophos on antioxidants and metabolic enzymes in *Styela clava*

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The impacts of the organophosphate pesticide methamidophos on antioxidants and metabolic enzymes in *Styela clava* were investigated using five concentrations of methamidophos (5, 10, 15, 20, and 25 µg/L) with the exposure time from 0 to 96 h. The low concentration of methamidophos (5 µg/L) increased the activity of super oxide dismutase (SOD) and alkaline phosphoesterase compared to that in control animals by 34% and 20%, respectively, at 96 h. Increasing the pesticide concentration decreased the activities of peroxidase and Na⁺/K⁺-ATPase, but increased the polyphenol oxidase activity. The activity of glutamic-pyruvic transaminase increased from 0–36 h, and then decreased to different degrees depending upon the concentration. These results showed that SOD, peroxidase, polyphenol oxidase, glutamic-pyruvic transaminase, alkaline phosphatase, and Na⁺/K⁺-ATPase may be the useful biomarkers for determining the sub-lethal effects of organophosphate pesticide.

Keywords: super oxide dismutase (SOD); peroxidase; polyphenol oxidase; glutamic-pyruvic transaminase (GPT); alkaline phosphatase (AKP); Na⁺/K⁺-ATPase.

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Introduction

Organophosphate pesticides are acute neurotoxins with inhibitory effects on the activity of acetylcholinesterase located in neuromuscular junctions. Organophosphate pesticide toxicity also produces reactive oxygen free radicals and reactive nitrogen free radicals, which generate oxidative stress on the body and lipid peroxidation. Cell membrane lipid peroxidation damages the integrity of the cell membrane and causes mitochondrial dysfunction, thus negatively impacts cell survival [1]. The degree of oxidative stress is based on the balance between the generation of reactive oxygen species and antioxidant defense [2]. Many organisms have evolved protective mechanisms to minimize such damage, and one

of the most important protection mechanisms in the body is the glutathione (GSH) oxidation reduction (Redox) cycle [3, 4]. In addition to the GSH resistance of organophosphate pesticide toxicity in organisms, some antioxidant enzymes such as super oxide dismutase (SOD) and catalase (CAT) and glutathione peroxidase (GPX) [5] also significantly contribute to the removal of the detrimental peroxidation effects of organophosphate pesticides.

Organophosphate pesticides were widely used in agriculture as insecticides. They were detected in groundwater, surface water, and rainwater. The residues of organophosphate pesticides make paralic environmental pollution [6]. Total 12 organophosphate pesticides include methamidophos had been detected in California

surface water [7]. Organophosphate pesticide residues were also detected in the arctic marine as early as 2000 [8]. Methamidophos has been used in large amounts in many parts of the world. Pesticides change the structure and function of aquatic ecosystem. Invertebrates are the part of aquatic ecosystem, so they become the respondents of sublethal effect of pollutants. As a sessile, filter feeders, and low metabolism of organism, *Styela clava* may absorb and concentrate pollutants in sediment and in water environment. Therefore, it can provide useful information about the pollution of local. The effects of organophosphate pesticides on SOD in vertebrates have been reported [9]. However, few studies have examined the effects of organophosphate pesticides, especially methamidophos, on SOD and peroxide enzymes in ocean filter feeders. There are no reports describing the effects of organophosphate pesticides on polyphenol oxidase (PPO).

The liver and kidney, organs vital for pesticide degradation and metabolic waste discharge, are targets of pesticides. The activity of glutamic-pyruvic transaminase (GPT) was affected by physiological state of vital organs especially liver [10]. The activity decrease of GPT indicates the injury of liver tissue and the disturbance of protein metabolism [11, 12].

Lipid peroxidation is an important factor for reactive oxygen species to promote bodily injury. Peroxidation of membrane phospholipids not only alters the functional integrity and structure of the lipid bilayer, but also inhibits the activity of enzymes in the membrane such as Na^+/K^+ -ATPase [1]. ATPase activity is an indicator of cell vitality and toxicological effects and this enzyme is a target for organochlorine pesticides. Alkaline phosphatase (AKP) is a membrane binding protein, which is directly involved in the physiological process of phosphate group transfer and metabolism, so its activity is correlated with cell damage [13]. Few studies have reported the effects of organophosphate pesticides on ATPase, alanine aminotransferase, and alkaline phosphatase. Therefore, the goal of

the present study was to determine the effects of organophosphate pesticide methamidophos on the antioxidant enzymes including SOD, GPX, PPO, and on the metabolic enzymes including GPT and AKP, as well as on ATPase in *Styela clava*.

Materials and Methods

Exposure to pesticides

The experiments were conducted in 18 basins ($40 \times 30 \times 11 \text{ cm}^3$) with each basin containing 10 L of seawater and the organophosphate pesticide methamidophos at a safe concentration. Based on the results of acute toxicity test, the LC_{50} of *S. clava* exposed to methamidophos for 96 h was 2.4 mg/L. Five nominal concentrations (5, 10, 15, 20, and 25 $\mu\text{g/L}$) were selected in this study with each giving three duplicate groups. Three control groups were set. *S. clava* individuals ($n = 140$) were placed in each basin with continuous aeration to maintain the dissolved oxygen levels above 5.0 mg/L. The animals were not fed during the experiments. The water was changed each morning and again each night for the health of the animals and to maintain relatively stable concentrations of methamidophos. During the experiment, samples were taken every 12 h with 4 animals were randomly removed from each basin each time. The whole-body weights were recorded, and the whole animals were frozen at -80°C for later determination of enzyme activities. The removed animals were replaced by additional *S. clava* that were marked for exclusion from analysis to maintain constant experimental conditions.

Enzymatic assay

The tissues were homogenized with a 1:10 ratio (w/v) of pre-cooled 0.01 M Tris-HCl buffer. The homogenate was refrigerated at 4°C for 3 h before extraction, and then centrifuged at 9,500 g for 30 min at 4°C . The supernatant fraction was retained for subsequent biochemical analysis.

The activities of PPO, SOD, GPX, AKP, GPT, and ATPase were determined by using the assay kits (Nanjing Jiancheng Technology Co., Ltd, Nanjing, Jiangsu, China) following manufacturer's instructions. One unit of PPO activity was defined as the amount of enzyme causing the increase of absorbance for 0.01 in one minute. One unit of SOD activity was defined as the amount of enzyme causing a 50% decrease of the SOD-inhibitable nitroblue tetrazolium (NBT). One unit of GPX activity was defined as the amount of enzyme oxidizing 1 μmol of NADPH per minute. One unit of AKP activity was defined as the amount of enzyme hydrolyzing para-nitrophenyl to produce 1 μmol p-nitrophenol per minute in diethanolamine (DEA) buffer (pH 9.8) at 37°C. One unit of GTP activity was defined as the amount of enzyme consuming 1 μmol sodium pyruvate at 38°C in 10 minutes. One unit of ATPase activity was defined as the amount of enzyme degrading ATP to produce 1 μmol inorganic phosphorus per hour.

All assays were performed using at least three homogenate samples. The enzymatic activity was corrected in all cases for spontaneous substrate hydrolysis. The specific activity was defined as units of activity per milligram of protein. The total protein was quantified using the procedure of Lowry et al. [14] with Folin's reagent and bovine serum albumin as the standard. The protein concentration was calculated by equation (1):

$$y = 7.7502x + 0.0458 \quad (R^2=0.9994) \quad (1)$$

where y is the absorbance of the sample to be tested, and x is the concentration of bovine serum albumin.

Data analysis

The results were calculated as mean \pm standard deviation (SD). The statistical software program SPSS for Windows (version 11.5) was employed. The probability of $P < 0.05$ was considered an acceptable level of significance. The data were analyzed by using the analysis of variance

(ANOVA) to determine the differences among all means.

Results

Effect of methamidophos on antioxidant enzyme activities in *S. clava*

Both concentration and time of exposure of methamidophos significantly affected the activity of SOD in *S. clava* ($P < 0.001$; Figure 1). The activity of SOD in tissues of *S. clava* exposed to 5 and 10 $\mu\text{g/L}$ of methamidophos increased slowly, peaked at 48 h, and then gradually decreased but still maintained an increase at 96 h of exposure of 34% and 26% respectively comparing to that in the control group. Methamidophos exposure at 15 $\mu\text{g/L}$ initially mildly decreased SOD activity comparing to that in controls. Then, after 24 h, the activity of SOD rapidly increased and reached the maximum at 48 h before decreasing to control levels after 60 h. In contrast, higher concentrations of methamidophos (20 and 25 $\mu\text{g/L}$) inhibited SOD activity with peak decreases in SOD activity reaching 25% and 35%, respectively, below that in controls at 24 h of exposure. After 24 h, the SOD activity increased slightly and remained virtually unchanged for the rest of the experiment.

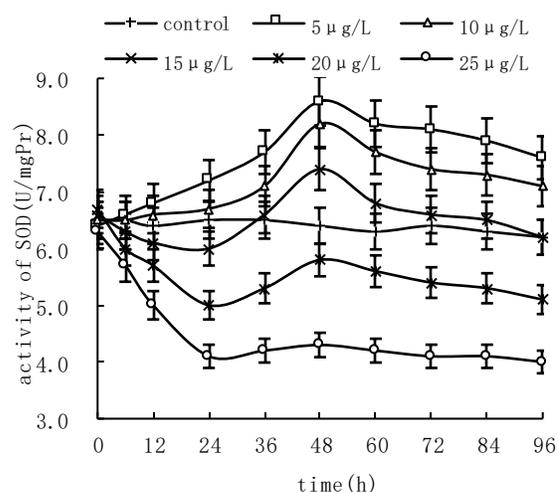


Figure 1. Effect of methamidophos on SOD activity in *S. clava*.

The effect of methamidophos on peroxidase activity in *S. clava* is shown in Figure 2. The activity of peroxidase of *S. clava* sharply increased to a maximum level at 12 h of 41%, 33%, 21%, 15%, and 7% over that in controls at methamidophos concentrations of 5, 10, 15, 20, and 25 $\mu\text{g/L}$, respectively. From 12 to 24 h, the activity of peroxidase decreased rapidly, then continued a slow decline throughout the duration of the experiment (96 h). In general, the activity of peroxidase was higher at 1, 10, and 15 $\mu\text{g/L}$ of methamidophos than that in controls, but lower than that in controls at 20 and 25 $\mu\text{g/L}$. Thus, the lower the concentration of methamidophos, the higher the activity of peroxidase was in *S. clava*.

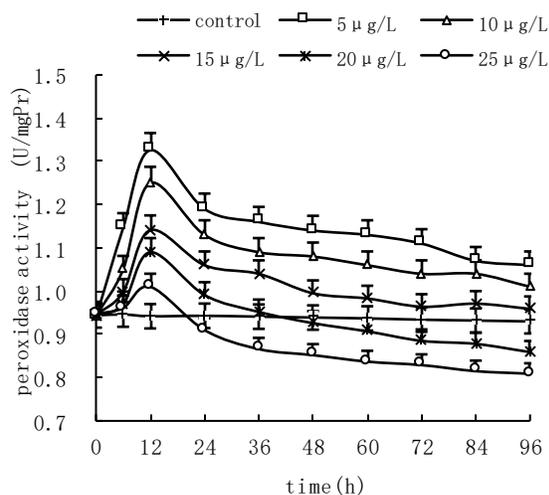


Figure 2. Effect of methamidophos on peroxidase activity in *S. clava*.

The influence of methamidophos on polyphenol oxidase in *S. clava* is presented in Figure 3. The methamidophos concentration as well as exposure time significantly affected polyphenol oxidase in *S. clava* ($P < 0.01$). Polyphenol oxidase activity increased gradually, peaked at 48 h, and then remained virtually unchanged at all tested methamidophos concentrations, and increased with the increase of the concentration of methamidophos.

Effect of methamidophos on metabolic enzyme activities in *S. clava*

Although all tested concentrations of

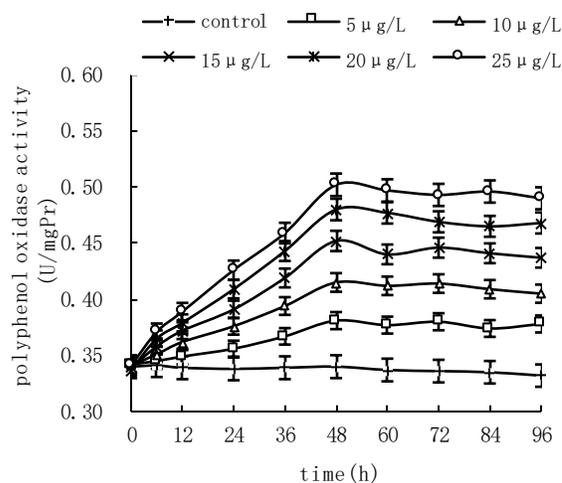


Figure 3. Effect of methamidophos on polyphenol oxidase activity in *S. clava*.

methamidophos increased the activity of GPT in *S. clava* comparing to that in control animals, the increase was inversely proportional to the concentration. The higher the methamidophos concentration, the lower the induced increase in GPT activity was observed (Figure 4). From 0 to 36 h of exposure, the activity of GPT continued to gradually increase. After 36 h, the activity of GPT in animals exposed to 5 and 10 $\mu\text{g/L}$ of methamidophos remained unchanged, whereas the GPT activity decreased to different levels with concentrations of methamidophos at 15, 20, and 25 $\mu\text{g/L}$.

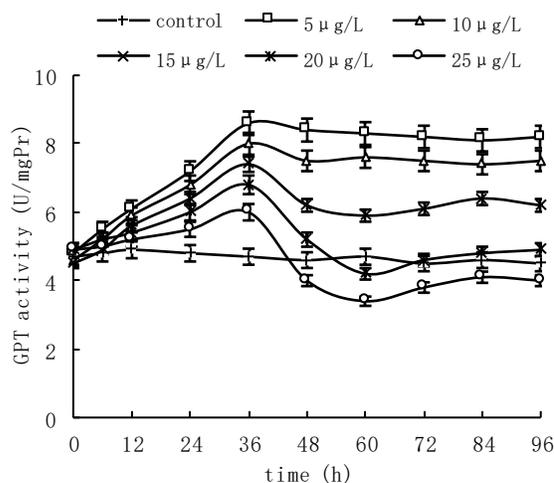


Figure 4. Effect of methamidophos on GPT activity in *S. clava*.

As shown in Figure 5, with increasing time of exposure and concentration of methamidophos up to and including 15 $\mu\text{g/L}$, AKP activity in *S. clava* increased comparing to that in control animals. The increase in AKP activity induced by methamidophos at concentrations above 15 $\mu\text{g/L}$ was not as marked as that induced at the lower concentrations.

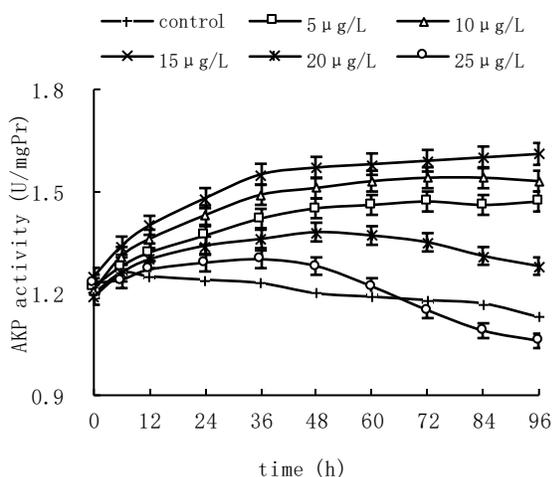


Figure 5. Effect of methamidophos on AKP activity in *S. clava*.

Effect of methamidophos on ATPase in *S. clava*

The activity of ATPase in *S. clava* was significantly altered by methamidophos ($P < 0.01$; Figure 6). The ATPase activity at the two higher methamidophos concentrations of 20 and 25 $\mu\text{g/L}$ decreased throughout the duration of the experiment with peak reductions of 15% and 26%, respectively, at 48 h. In contrast, ATPase activity was increased at the lower methamidophos concentrations of 5, 10, and 15 $\mu\text{g/L}$ from 0 to 24 h, and then gradually decreased.

Discussion

The toxicity of organophosphate pesticides is related to the effects of the induced oxidative stress. Oxidative stress is generated by the imbalance in free radicals and the antioxidant defense system [15]. During sub-chronic exposure to organophosphate pesticides,

organisms protect themselves by producing excessive amounts of antioxidants [4, 16], which prevent or inhibit oxidative damage to biomacromolecule targets. Antioxidants are relatively stable molecules that neutralize free radicals by donating electrons. Many compounds with antioxidant activity are presently known to exist in the human body. Their main defense is mediated through enzyme systems including SOD, hydrogen peroxide, and enzymes containing sulfur. All antioxidants possess specific types of activity, and they frequently work together to enhance the body's antioxidant capacity.

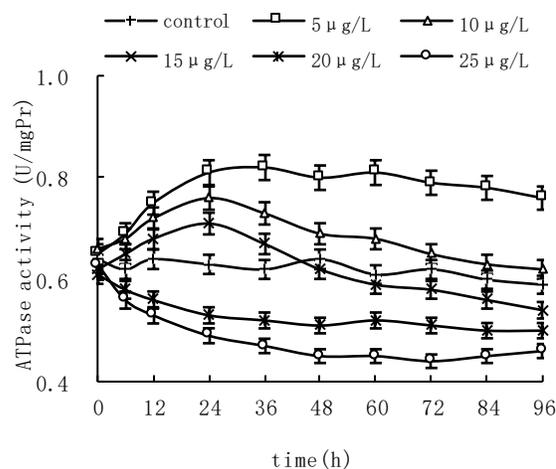


Figure 6. Effect of methamidophos on ATPase activity in *S. clava*.

Mollusks, especially bivalves, have a wide range of resistance at the cellular level to harmful compounds. Their antidotal antioxidant system allows for the neutralization and elimination of harmful parent compounds, metabolites, and by-products. The process of detoxification is mostly controlled by inducible proteins with their expression or activation related to the content of specific toxic substances in the cells [17].

Following exposure to low concentrations of methamidophos, the enzyme activity of SOD in *S. clava* in the present study initially slowly increased and then gradually declined. For the duration of the experiment, the SOD activity was

higher than that in control animals, which indicated that SOD resisted the reactive oxygen free radicals produced by methamidophos and many SOD enzymes were induced. Eventually, the activity of SOD was higher than that in controls indicating that *S. clava* activated Redox cycling compounds and displayed antioxidant stress [18]. In the *S. clava* exposed to relatively high concentrations of methamidophos in the present study, SOD activity rapidly decreased at the beginning of the exposure to the lowest level, then increased slightly and remained virtually unchanged for at least 96 h. The higher the concentration of methamidophos, the lower the SOD enzyme activity was, which suggested that the large amount of reactive oxygen free radicals produced by methamidophos rapidly consumed the SOD and that the SOD generated was not enough to neutralize the reactive oxygen free radicals. Thus, the activity of SOD in the methamidophos-exposed animals was lower than that in the controls, which suggested that *S. clava* had certain amount of tolerance and resistance to organophosphate pesticide stress. Peroxidase is an important enzyme for the degradation of organophosphate pesticides in organisms. Following exposure to methamidophos, the activity of peroxidase in *S. clava* was reduced after an initial increase suggesting that the reactive oxygen species produced by the organophosphate pesticide further induced the peroxidase. With the degradation of the pesticide, much of the peroxide produced was consumed by the peroxidase, a scenario consistent with the results of Orus and Usta [19].

In invertebrate animals, polyphenol oxidase exists in the form of pro-polyphenoloxidase in granular cells. Because of the existence of small molecule compounds within the organism such as the sugar lipopolysaccharide, pro-polyphenoloxidase is active after hydrolyzed by serine protease and is important in the function of the invertebrate immune system. Polyphenol oxidase is a metabolic oxidase [20] and plays an important role in the resistance to disease in animals and is a potential target of pesticides. In

invertebrate animals, polyphenol oxidase is associated with cytotoxicity [21].

During the exposure to methamidophos in the present study, the activity of polyphenol oxidase in *S. clava* gradually increased with time, reaching a maximum at 48 h and remaining virtually unchanged thereafter. The vast majority of cytotoxicity is caused by hydroxyl radicals. Thus, this increase in polyphenol oxidase activity indicates that *S. clava* is in oxidative stress [20]. Previous research showed that polyphenol oxidase activity in cells increased significantly when cell toxicity led to tissue necrosis in *S. clava* [21]. Oxidation is the first step in the biological degradation of pesticides, and polyphenol oxidase is involved in the degradation of organophosphate pesticides in organisms. Thus, the increase in the polyphenol oxidase activity observed for *S. clava* in the present study indicated that methamidophos was in the first stage of *in vivo* metabolism [22].

GPT is involved in the decomposition of metabolic amino acids and catalyzes transamination between amino acids. It is a bridge between sugar metabolism and protein metabolism [23]. Both an increase and decrease in GPT reflect damage in liver, kidney, and gill tissues [24] as well as liver dysfunction [25]. Normally, GPT exists mainly in liver cells, and its activity in serum is low and relatively stable. When liver function is damaged, GPT is released from the liver into the blood stream causing a significant increase in blood GPT levels. In addition, GPT is an important transaminase and plays a key intermediation role in the synthesis of the non-essential amino acids and the metabolism of protein. Therefore, an increase in hepatic transaminase activity indicates marked metabolism of amino acid.

Our research showed that GPT activity in *S. clava* increased at low concentrations of methamidophos (5 and 10 $\mu\text{g/L}$) indicating that transamination was enhanced to raise the levels of oxaloacetic acid, pyruvic acid, and alpha-ketoglutaric acid [3] or to help the

transamination of glucogenic amino acids to reinforce gluconeogenesis [6] to meet the growing energy demand of the stress environment. The change in GPT activity is affected by vital organs, especially by the physiology of the liver. A reduction in GPT activity manifests the damage of liver tissue and protein metabolism disorders [26]. After 36 h of methamidophos exposure (15 µg/L) in the present study, the GPT activity was markedly reduced, which indicated hepatopancreas degeneration in *S. clava*. The GPT activity recovered gradually after 72 h, demonstrating the regeneration of hepatic cells or an enzyme release from mitochondria or other organs [27].

AKP is a membrane-binding protein directly involved in the transfer of phosphate groups *in vivo* and metabolic processes associated with maintaining an appropriate calcium-phosphorus ratio in the body. AKP is also a hydrolytic metabolic enzyme. It plays an important role in antioxidative defense in mollusks and in their oxygen-carrying systems. It is a phosphomonoesterase that plays a key physiological role in catalyzing the transfer of phosphate groups. AKP is also an important detoxification enzyme *in vivo*, having a significant role in ossification of the organism and the digestion, absorption, and transport of phosphide and other nutrients [13]. Thus, its activity is related to cellular damage [27].

Exposure to low concentrations of methamidophos increased AKP activity in *S. clava* in the present study indicating that the liver was involved in the detoxification of this organophosphate pesticide [28]. After a transient increase, AKP activity rapidly decreased at the high methamidophos concentrations indicating that the liver cells were mildly injured by higher pesticide concentrations. However, the decrease in AKP activity at high pesticide concentrations was indicative of serious hepatopancreas injury in *S. clava*.

Because AKP is a membrane-binding protein, damage to the cell membrane affects the activity of AKP. The large number of free radicals produced by organophosphate pesticides in an organism induces membrane lipid peroxidation, inevitably affecting the biological function of the membrane, and thus affecting the enzyme activity associated with the membrane. When the degree of membrane lipid peroxidation is low, low concentrations of organophosphate pesticides mildly disturb the active site of AKP, loosening its highly organized and tightly regulated structure to increase AKP activity. A high concentration of pesticide perturbs the overall conformation of the enzyme, further damages the structure of active site and leads to loss of enzyme activity.

Na⁺/K⁺-ATPase is a membrane-bound enzyme that plays an important role in maintaining *in vivo* K⁺ and Na⁺ transmembrane homeostasis. Na⁺/K⁺-dependent ATPase is responsible for the transport of these ions across the plasma membrane and is thus believed to regulate critical physiological activities [29]. The most basic function of ATPase is to catalyze the hydrolysis of the end phosphate group of ATP using the energy emitted by the reaction to counter the ionic electrochemical gradient to produce the Na⁺ and K⁺ active transport. Low concentrations of methamidophos markedly increased the activity of ATPase in *S. clava* in the present study. Exposed to the stress environment, ATPase activity *in vivo* increased, which might be a compensation for ionic regulation damage such as a superficial osmotic pressure increase [30]. At higher concentrations of methamidophos, the ATPase activity in *S. clava* markedly decreased and remained reduced with time in the present study. Na⁺/K⁺-ATPase possesses a large number of lysine and cysteine residues, as well as disulfide bridges. Thus, the decrease in the enzyme activity may be due to an interference of sulfhydryl groups [31]. The maintenance of the tertiary structures in cellular membrane proteins including ATPase is highly dependent on the associated lipids. The destruction of these lipids may result in

structural alterations followed by changes in protein or enzyme functions. Changes in composition and structure of membrane can cause the loss of ATPase or a decrease of its activity [32]. Organophosphate pesticides are lipophilic and, when located in a conducive area, can interfere with the fluid lipids to affect the activity of the ATP enzyme [33]. Organophosphate pesticides *in vivo* produce large numbers of free radicals leading to lipid peroxidation of the bilayer, destroying the biological function of the membrane, which inevitably affects many physiological functions associated with the membrane including membrane-bound enzyme activity. The Na⁺/K⁺-ATPase is a membrane cation pump regulating the active transport of Na⁺ and K⁺. Therefore, its decrease following exposure to organophosphate pesticides in the present study shows that osmotic regulation based on ions is negatively impacted in *S. clava*.

Conclusion

The exposure to the organophosphate pesticide methamidophos affected the activities of SOD, peroxidase, polyphenol oxidase, glutamic-pyruvic transaminase, alkaline phosphatase, and Na⁺/K⁺-ATPase in *S. clava* suggesting that these enzymes might be the useful biomarkers for determining the sub-lethal effects of organophosphate pesticides in marine animals.

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