Biomarkers in Rock Oysters (*Saccostrea mordax*) in Response to Organophosphate Pesticides

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Abstract

Chlorpyrifos is a xenobiotics contaminants that threats the marine environment and the living organism within the habitat. Although several marine bivalve species have been used as the indicator of marine pollution, the used of Saccostrea mordax is remaining unknown. This study aimed at investigating the suitability of lysosome membrane integrity, carboxylesterase activity, 8-oxo-2′-deoxyguanosine and condition index as biomarkers in adult S. mordax following their exposure to 0.0, 5.0 and 500 µg.L^{-1} of Chlorpyrifos for 21 days under laboratory conditions. Results indicated that the lysosome membrane integrity showed a dose-dependent response with a significant statistical number of destabilized cells between all the treatment groups. Carboxylesterase activity was significantly inhibited in 500 µg.L^{-1} chlorpyrifos treated group, while the environmentally relevant concentration (5 µg.L^{-1}) did not induce a significant inhibition with reference to the control. Similarly, the condition index showed a dose-dependent response with the oysters exposed to 500 µg.L^{-1} chlorpyrifos exhibiting a significantly reduced growth rate. There was no statistical significance in the means of both 8-oxo-2′-deoxyguanosine in all treatment groups. The reaction of S. mordax to chlorpyrifos contamination demonstrates that the species can potentially be used as sentinel organisms in environmental monitoring programs. Lysosome membrane integrity was a single out as a sensitive biomarker for exposure to chlorpyrifos and is therefore suitable for environmental monitoring for sublethal concentrations of chlorpyrifos contaminations. Additionally, the use of multiple biomarkers was found to be robust in this study and can be extrapolated to other ecotoxicological studies

Keywords: *biomarkers, chlorpyrifos, oyster, S. mordax, pesticides*

Introduction

Aquatic environments are becoming more stressed from xenobiotic contaminants. These contaminants from point and diffuse agricultural, residential and Industrial sources, are deposited into littoral areas through spray drifts and run-offs (Tedoldi *et al.*, 2016). The expansion of modern agriculture has resulted to the use of high concentration of agrochemicals such as pesticides (Insecticides, fungicides, herbicides, fumigants and rodenticides) and fertilizers (Lappharat *et al.*, 2014; Patetsini *et al.*, 2013) which coupled with natural stressors from climate change have increased vulnerability of aquatic ecosystems to pollution. Although more stringent regulations on formulations and advocacy on moderated use of pesticides are in (Osteen and Fernandez-Cornejo, 2013), trends indicate increased application of pesticides (Liu *et al.*, 2015). Consequently, causing poisoning, death, carcinogenesis, stress, endocrine disruption and upset of population size and distribution of fish. Thus raises concerns on the health integrity of the aquatic ecosystems (Patetsini *et al.*, 2013). One of the commonly used pesticides albeit associated with ecological effects is chlorpyrifos. Chlorpyrifos is effectively used as a broad-spectrum chlorinated organophosphate pesticide to control insects, termites, acaroid and nematodes in homes, agriculture and golf courses. Its use has been extensively documented following its introduction to the market in 1965 (Eaton *et al.*, 2008). Although the exposure and contamination of chlorpyrifos from households have reduced following its restriction, particularly in the US and EU, its widespread application in agriculture has escalated to unprecedented levels (Eaton *et al.*, 2008). For instance, in 1990, about 635.000 kg of chlorpyrifos was used in Central Valley of California (Bailey *et al.*, 1997) while in 1997, about 6 million kg were used in the US (Gilliom *et al.*, 2006). Chlorpyrifos remains the main pesticide applied in agriculture in Argentina; approximately 3 million liters of chlorpyrifos was used in 2012 (Martinez *et al.*, 2015).

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The various pathways through which organophosphate pesticides become bioavailable in the aquatic systems complicate their biochemical reactions. Previous studies indicate the obliquity of biochemical pathway of organophosphate pesticides with regards to non-target species. For example, Canesi *et al.* (2011) in a study with marine mussels conclude that chlorpyrifos caused endocrine disruption of natural estrogen, subsequently impairing the uptake of Ca^{2+} and metabolic processes. Besides, the extent of influence of natural stressors on the biochemical reactions of organophosphates has never been exhaustively demystified (Relyea and Hoverman, 2006). There is no single biomarker that is robust enough to diagnose environmental contamination. The current trend in ecotoxicology is the use of a battery of biomarkers that provide early-warning signs of environmental contamination, thus, enable appropriate diagnostic, prognostic and intervention tools which are geared towards sound environmental best practices and environmental risk assessments. Although chemical analyses can be used to detect the presence of a contaminant in aquatic system, they do not assess the impacts of such contaminants on aquatic organism, therefore, the suitability of biomarkers (Sarkar *et al.*, 2006). Some studies have indicated that bioaccumulation of organophosphate pesticides in marine gastropods initiate different physio-pathological processes that include the elevation of the generation of reactive oxygen species (ROS). ROS are products from partial reduction of molecular oxygen in the cells arising from biochemical process in an organism (Lushchak, 2011). Although some ROS such as peroxides (H_2O_2), hydroxyl (OH^-) and superoxide (O_2^-) are naturally produced in the mitochondria and endoplasmic reticulum, such ROS are controlled through a counter-reaction with antioxidant enzymes such as catalases and antioxidants. However, exposure to contaminants induces overproduction of ROS which diffuse through biological membranes and cause damages in places far from their origins, resulting into oxidative stress (Oliveira *et al.*, 2010).

Oysters have been used in ecotoxicological studies to deduce both temporal and spatial contaminations in aquatic environments (Domouhtsidou *et al.*, 2004; Edge *et al.*, 2012). They feed through filtration of the water column, thereby enhancing light penetration. Oysters accumulate contaminants through body surfaces and feeding. In addition, oysters are sessile hence are ideal for site exposure studies. The contaminants are often at a higher concentration than in the surrounding medium, essentially making them ideal for ecotoxicological studies (Moreira *et al.*, 2013). The marine pollution have been evaluated using biomarkers features in some marine bivalves. However, the use of *Saccrostrea mordax* is still unknown. The aim of the present study is to investigate the use of 8-oxo-gd, carboxylesterase activity, lysosomal membrane integrity and condition factor in *S. mordax* as a biomarker of the environmental contamination with chlorpyrifos.

Materials and Methods

Chemicals

The organophosphate insecticide used was commercial-grade formulation (Chlorpyrifos 500EC insecticide; active constituent: 500 $g.L^{-1}$ chlorpyrifos; in 480 $g.L^{-1}$ in liquid hydrocarbon solvent) purchased from Nufarm Australia Limited, Western Australia. Other chemicals used were of high purity levels and unless stated, they were obtained from Sigma-Aldrich, Australia.

Animals

Since oysters have no specific size, the present study will use the average wet weight of *Saccostrea mordax.* A total number of 36 of *S. mordax* (60.775 ± 17.091 g) were harvested from oyster reef in Kalbarri, Western Australia. This site was particularly chosen due to its isolation from agricultural activities hence less likelihood of organophosphate contamination. The oysters were transported in moist eskies to Curtin Aquaculture Research Laboratory in Bentley, Western Australia. The oysters were acclimated for 10 days in 20 L water (Salinity of 35 ppt; 26°C) and were fed on a commercial product of known as shellfish diet 1800 (instant algae) composed of concentrates of six marine microalgae species (40% *Isochrysis galbana*) at a rate of $7.0x10^6$ cells mL^{-1} every 24 h, for the acclimation and the exposure periods. Half of the water in the holding tanks was changed after every 36 h.

Exposure of the oysters

A set of 36 aquaria of a capacity of 1 L were used in this experiment. The set was sub-divided into 3 subsets, each containing 12 aquaria designated for control, low (5 $\mu g.L^{-1}$) and high (500 $\mu g.L^{-1}$) concentration treatments. The aquaria were randomly placed in a large water bath whose temperature was maintained at 26°C. Each aquarium had 2 oysters that had been randomly sampled from the original batch. Air was gently bubbled through each aquarium for the entire exposure period. The oysters were exposed to 14 and 10 h of light and darkness respectively, for a period of 21 days. The ambient conditions were maintained as follows; temperature 24.799±0.729ºC, percentage dissolved oxygen 82.173±0.402%, dissolved oxygen

5.593±0.026 mgL^{-1}, salinity 33.374 ±0.094 ppt and pH of 7.156±0.026. The whole of the water in the aquaria was being change after every 36 h.

Sample preparations

After the exposure period, the whole weight and the wet weight and the shell weight were measured to the nearest 0.001 using analytical weighing scale and all the measured parameters were recorded for working condition index. The shells were opened using oysters knife. The digestive glands of 12 individuals from a subset were each carefully excised and immediately analysed for lysosome membrane integrity. The digestive glands of the rest of the exposed oysters were individually excised, divided into 2 parts and immediately refrigerated at -85°C for later biochemical analyses.

Determination condition index and lysosomal membrane integrity

Condition index was determined according to Filgueira *et al.* (2013) with following equations: CI = wet weight/(whole weight-shell weight) x 100. The lysosomal membrane integrity assay was conducted according to the procedure adopted from Ringwood *et al*. (1998). Fresh tissues of the digestive gland (0.02 g) were rinsed in calcium- and magnesium-free saline (CMFS contained 20 mM HEPES, 480 mM NaCl, 12.5 mM KCl, and 5.0 mM $CaCl_2$, pH adjusted to 7.4 using 6N NaOH), minced and rinsed again, and transferred into microcentrifuge tubes kept in ice. The tissues were disintegrated and cells dissociated through the addition of CMFS followed by gentle shaking on reciprocating shaker at 120 rpm for 20 mins Further cellular dissociation was achieved through the addition of trypsin at pH 7.4, followed by shaking on a reciprocating shaker at 120 rpm for 20 mins. The mixture was sheared before filtration through a 23 µm screen. The filtrate was centrifuged at 300 g for 5 mins. The supernatant was discarded and the cells were resuspended in1 ml CMFS and again centrifuged 300 g for 5 mins. Finally, the cell resuspended in 900 µl of CMFS and mixed with freshly prepared neutral red (Secondary solution of NR of 0.08 mg/ml dissolved in CMFS) at 9:1 respectively. The dyed cell suspension was incubated for 1 h at room temperature before observed under 40X objective lens with a total magnification of 400x. The scores were recorded for 50 cells. Based on the dye retention ability, the cells with dye enclosed in the lysosomes were classified as stable while the ones in which the dye oozed into the cytosol were considered destabilised. The percentage of destabilised cells were then calculated by dividing the number of cells with the dye in the cytosol with the total number of cells counted then multiplied by 100.

Determination of Carboxylesterase Activities

The carboxylesterase activity in the digestive gland of each oyster (n=12) was analysed using 1-Naphthyl acetate as a substrate as adopted by Baker *et al.* (2016). The digestive system which had been kept at -85°C was slowly thawed in ice, weighed and homogenised in 0.02 M phosphate buffer solution (with 1% triton X-100, pH 8.0)) at a ratio of 1unit weight of the sample to 3.5unit volume of the homogenisation buffer. The homogenate was pipetted into a 5 ml glass tube and diluted with 0.2M homogenisation buffer solution (devoid of triton X-100) at volume ratio of 1:20 respectively, and the resulting solution centrifuged at 14,000 g for 4 mins at 4°C. The resulting supernatant was further diluted with 0.2M homogenisation buffer solution (devoid of triton X-100) at 1:4 respectively. Twenty mL of 0.2M homogenisation buffer solution was pipetted into blank on the microplate, 20 µl of the diluted supernatant was pipetted into microplate in triplicate in specific plate layout, followed by addition of 190 µl assay buffer (50mg of Fast Blue RR salt dissolved in 25 ml freshly prepared 0.2M homogenisation buffer, devoid of triton X-100, diluted in 500 µl 1-Naphthyl acetate stock solution) to the wells of the microplate. The increase in absorbance was carried out for each minute for 10 mins at 450 nm and 25°C using microplate reader. The proteins content was analysed by Bradford assay (Bradford, 1976) using bovine globulin solution as a standard, and read on microplate at 595 nm. The values were entered into excel and the final results were compared to the control, and expressed as nmol min^{-1} mg^{-1}.

DNA Extraction and Quantification of 8-oxo-dG

The DNA extraction was conducted according to spin-column protocol as outlined in a commercially available kit (QIAGEN, 2006). Briefly, the previously refrigerated digestive tissues were slowly thawed in ice. Tissues (0.025 g) were sliced out and placed into 1.5 ml tubes followed by addition of buffer ATL and proteinase K to lyse the tissue and digest proteins respectively. The mixture was thoroughly vortexed and incubated at 56°C with occasional vortexing. Further lysis of cells and membranes was achieved through the addition of buffer AL and the DNA precipitated through addition of absolute ethanol. The mixture was transferred into spin column and centrifuged at 6000 g. The debris in the spin column was washed using Buffers AW1 following centrifugation for 1 minute at 6000 g. Further washing was achieved through addition of buffer AW2 following centrifugation for 3 mins at 13500 g. Finally, 100 µl buffer AE was used to elute DNA from the filter in the spin column at centrifugation speed of 6000 g for 1 min. The DNA material was then quantified using thermos scientific NanoDrop 2000

spectrophotometer. The unwinding and deploy-merisation of the collected DNA materials were achieved through the addition of DNase 1 and alkaline phosphatase respectively into the samples.

The DNA materials were quantified using a commercial enzyme-like immunosorbent assay (ELISA) kit (Trevigen, 2015). Briefly, 25 µl of 8-oxo-dG standard (diluted with Assay Diluent) and another 25 µl DNA samples were added to appropriate wells on a 96 pre-coated wells plate in duplicates. Assay Diluent was added to the blanks as control, followed by addition of 25 µl Anti-OHdG monoclonal solution. The plate was covered with film sealer and incubated for 1 h at 25°C. Following incubation, plate was washed 4 times with phosphate-buffered saline tween (PBS and 1% Tween 20) using immunowash model 1575 (Bio-Rad Laboratories Pty Ltd, NSW, Australia). Twenty-five microlitres of Goat anti-mouse IgG-HRP conjugate was added to each well, with the exception of the blanks, and the mixture again incubated for 1hr at 25°C. The wells were washed using PBST as before, followed by the addition of 50 µl TACS-Sapphire colorimetric substrate which had been pre-warmed to room temperature. The plate was then incubated in the dark for 15 minute at room temperature. The reaction was stopped by with 0.2M HCl and the plate was immediately read using a Bio-Rad iMark Microplate Reader through an absorbance of 450 nm. The microplate readings were used to calculated the 8-oxo-dg concentration using the worksheet provided on Trevigen website using MS excel.

Data analysis

Normality of distribution and homogeneity of variance was analysed by Kolmogorov–Smirnoff and Levene tests respectively. The data for all the assays conformed to Normality of distribution and homogeneity of variance. The significance level was fixed at α=0.05. Significant difference between means was analysed with one-way ANOVA for all the assays and Tukey's HSD test was subsequently used to compare the variations between treatment groups. Statistical analysis was performed using SPSS v. 23.

Result and Discussion

The condition index, lysosome membrane integrity, carboxylesterase activity, 8-oxo-2′-deoxyguanosine can be used as biomarkers in adult rock oyster (*S. mordax*). The exposure of chlorpyrifos pesticide at low and high concentration affect the lysosome membrane integrity and carboxylesterase activity. Condition indices in bivalves occur at organismal level thus are considered as late signal of stress, however, they have been used in ecotoxicological experiments as markers of chronic contamination. In the present study, there was no significant difference ($F_{2,33}$= 0.458 p=0.636) in condition index of *S. mordax* between the control and the treatment groups. The condition index of low and high treatments were 37.16 ± 1.59 and 38.57 ± 1.91, respectively (Figure 1.). Generally, studies have indicated that condition index is a reliable biomarker of contamination in bivalves (Smith and Reddy, 2012). For instance, Benali *et al.* (2015) demonstrated dose-dependent response in condition index of *M. galloprovincialis* in a field study. However, the lack of significant difference in condition index between the treatment groups in the current study may be explained by the fact that condition index is a late signal of stress which occur at organismal level following the damages at cellular and sub-cellular levels due to exposure to a contaminant. additionally, oysters are known to compensate for the weight loss of energy-storage structures with water (Lucas and Beninger, 1985). This phenomenon makes it difficult to detect change in weights even in cases where such changes occur. Similar findings have been shown in previous studies with other bivalves. For instance, common mussels (*M. edulis*) shown insignificant differences in their conditions in separate field studies of contamination with tributyltin and organochloride compounds. On the contrary, a field study on *C. virginica* indicated a greater response to salinity rather that the presences and concentrations of xenobiotic contaminants (Volety, 2008). Although the condition index is potentially useful as a biomarker in *S. mordax,* it should be coupled with other biomarkers in order to ensure a robust ecotoxicological study.

Several lines of evidence from studies have indicated that lysosome membrane integrity is adversely affected following exposures to xenobiotic contaminants. However, there is no particular pathway through which contaminants affect lysosomes. Results indicated that there was a significant difference in lysosome membrane integrity between all treatment groups ($F_{2,30}$=50.319 p< 0.001) (Figure 2.). *S. mordax* exposed chlorpyrifos at high level resulted on the highest lysosomal integrity. Further both low and high chlorpyrifos exposure have also significantly higher lysosome membrane integrity than control. In the present study, the background level of destabilisation was above the 30% designated as a normal background lysosomal instability in oysters (Ringwood *et al.*, 2005). It is probable that the reproductive cycle (spawning) elevated the normal background levels of lysosome destabilisation in this study given that the study coincided with the spawning period of *S. mordax*.

This observation is consistent with previous study which indicated that lysosome membrane integrity in marine bivalves were adversely affected as a result of physiochemical changes that accompany spawning Cho and Jeong (2005). The results of this study demonstrated that chlorpyrifos, even in low concentrations, adversely affects the lysosome membrane integrity of *S. mordax*. Although our low concentration (5 µg.L^{-1} of Chlorpyrifos) was higher than that used by Patetsini *et al.* (2013) it was below agricultural concentrations of 26.6 µg.L^{-1} as reported by Otieno *et al.* (2012). Therefore, our finding justifies the regulation of the use of Chlorpyrifos in Agriculture given that a lot of agricultural activities occur in areas likely to be eroded through run–offs to aquatic system hence the possibility of affecting oysters.

In the current study, lysosomes showed high sensitivity to chlorpyrifos and the membrane destabilisation correspondingly increased with contamination gradient. This finding agrees with previous study in which chlorpyrifos induced adverse effects on the lysosome membranes of haemocytes in *Mylitus galloprovicialis* (Patetsini *et al.*, 2013). The sensitivity of lysosome membrane integrity as a broad-based biomarker of stressors is further supported by several lines of evidence from both field and laboratory studies of aquatic animals (Moore *et al.*, 2006; Webb, 2011). In a study of *S. glomerata* in Homebush Bay and Port Kembla in Australia, Edge *et al.* (2012) indicated that the lysosome membranes were destabilised following exposure to metals and poly aromatic hydrocarbons (PAH). Further evidence has been recorded in *M. galloprovincialis* exposed to

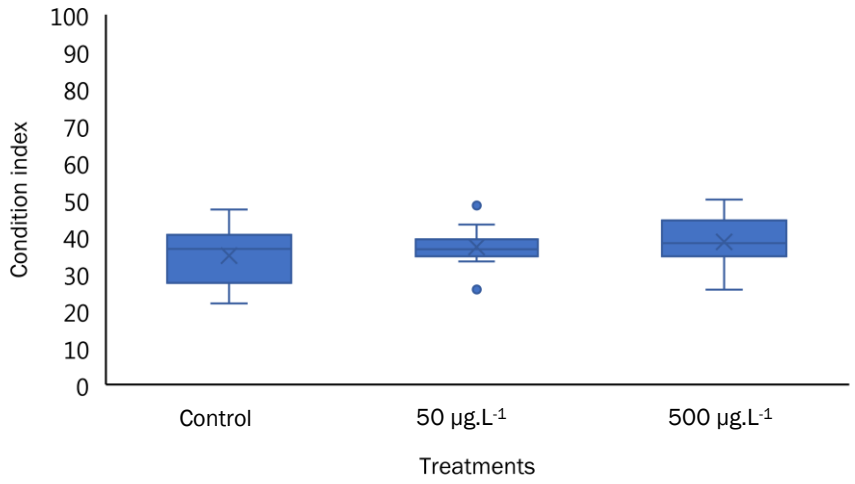

Figure 1. Condition index of *S. modax* exposed to 0, 5 and 500 µg.L^{-1} of chlorpyrifos for a 21 days. There was no significant differences in the means of condition index of oyster (P> 0.05)

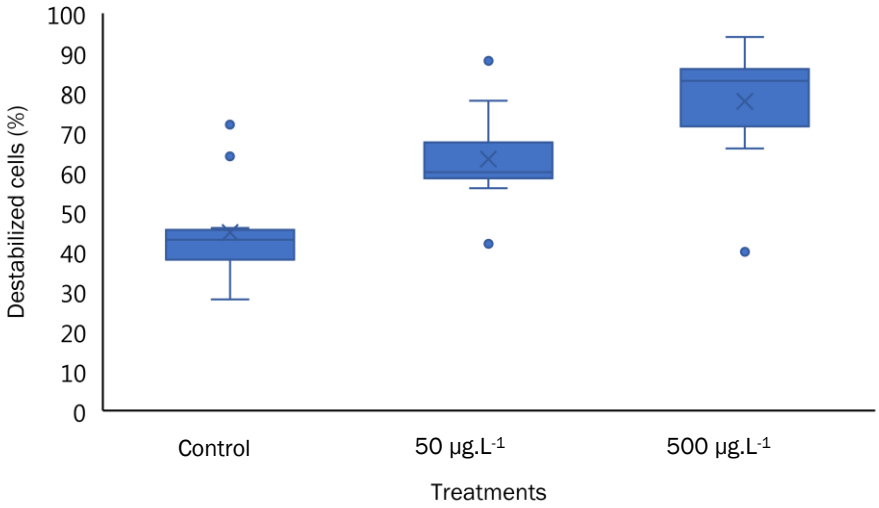

Figure 2. Percentage of cells with destabilized lysosome membranes in *S. mordax* exposed with treatment levels of chlorpyrifos. Group treated to high concentration of chlorpyrifos had a significantly higher destabilized cells (p<0.05).

Table 1. The condition index, lysosome membrane integrity, carboxylesterase activity and DNA damage of *S. mordax* after chlorpyrifos exposure at two levels. Value with different supercript (^{a,b}) in the similar column represent significant difference at $P<0.05$.

Treatments	Condition Index	Lysosome Membrane Integrity	Carboxylesterase Activity	DNA Damage
Control	34.87±2.40^{a}	44.83±3.46^{a}	86.59±10.53^{a}	27.45±3.28^{a}
5 µg.L^{-1}	37.16±1.59^{a}	63.33±3.29^{b}	73.64±7.22^{a}	20.51±1.90^{a}
500 µg.L^{-1}	38.57±1.91^{a}	77.83±4.15^{b}	34.23±6.08^{b}	21.10±3.92^{a}

trace metal contamination (Domouhtsidou *et al.*, 2004) and in *M. edulis* exposed to 3-Methylcholanthrene (Moore *et al.*, 2006). A general consensus can be drawn that lysosome membrane integrity in a powerful biomarker of anthropogenic stressors in many organisms. Based on the sensitivity of lysosome membrane integrity, this study indicates that *S. mordax* is a potential sentinel particularly in areas of Australia and New Zealand where this species is endemic.

The *in vivo* inhibition of carboxylesterase, is an indication of the general endowment of an individual organism/species against ester-containing compounds (*e.g.* organophosphates), and further reflects the sensitivity of the organism to the organophosphate compounds. A significance difference was detected in the specific activity between the treatment groups ($F_{2,32}$=12.238, P<0.001). There was a significantly lower specific activity (umol.min^{-1}.mg^{-1} protein) of carboxylesterase in oysters exposed to high concentration treatment than those exposed to a low concentration and control. Specific activity (umol.min^{-1}.mg^{-1} protein) of carboxylesterase was not significantly different in groups treated to low concentration of chlorpyrifos and the control (Figure 3.). The relative insensitivity of carboxylesterase activity in *S. mordax* to chlorpyrifos except to high (500 µg.L^{-1}) concentration in this study made it challenging to draw clear conclusion over the suitability of carboxylesterase as a biomarker of exposure to chlorpyrifos in the natural environment. Unfortunately, this study did not investigate a dose-response relationship over a wide range of chlorpyrifos contamination with regards to carboxylesterase activity, therefore, it was difficult to tell whether statistically significant specific activity of carboxylesterase could be induced by environmentally relevant chlorpyrifos concentrations above 5 µg.L^{-1} already reported by Otieno *et al.* (2012). In contrast, other studies have revealed that concentrations below or equal to 5 µg.L^{-1} induced inhibition of carboxylesterase activities in other organisms. Rivadeneira *et al.* (2013) indicated that 5 µg.L^{-1} of Chlorpyrifos significantly inhibited carboxylesterase in snail (*Planorbarius corneus*) while Wheelock *et al.* (2005) found a significant inhibition of carboxylesterase in chinook salmon (*Oncorhynchus Tshawytscha*) upon exposure to 1.2 µg.L^{-1} Chlorpyrifos. Therefore, the future of investigations should use a range of chlorpyrifos concentration to gauge the sensitivity of carboxylesterase as a potential biomarker in *S. mordax*.

Oxidative DNA damage from ROS in the cells often results into generation of 8-oxo-dG in the cells. The ROS are often induced by the presence of xenobiotic stressors such as chlorpyrifos. The DNA damage manifested as 8-oxo-dG following the exposure of organisms to chlorpyrifos, often results from the induction of ROS by Chlorpyrifos as evidenced in some studies (Rosen, 1997; Cacciatore *et al.*, 2015; Chauhan *et al.*, 2016). Analysis of variance test did not detect any significant differences ($F_{2,10}$=0.095, p=0.910) in the levels of 8-Oxo-dG between the treatment groups in *S. mordax* (Figure 4.). Previous study have shown that chlorpyrifos causes DNA damage in a concentration-dependent fashion (Ali *et al.*, 2009). However, in the present study, there was no significance difference in the amount of 8-oxo-dG between the treatment groups. On the contrary, DNA damage as a result of exposure to low concentrations of chlorpyrifos has been recorded in freshwater fish (*Channa punctatus*) (Ali *et al.*, 2009). The findings of (Ali *et al.*, 2009) has been reaffirmed in a recent study of genotoxicity of chlorpyrifos to zebrafish (*Danio rerio*) (Wang *et al.*, 2014). Generally, the insignificance damage of the DNA illustrated in this experiment may be an indicator that oysters have better mechanisms of either neutralising chlorpyrifos or fast-tracking the repair of the damaged DNA. Our observation supports earlier observations that oysters are less responsive to organophosphate compounds (Ha Park *et al.*, 2004). Although 8-oxo-dG has been well studied in human medicine, its use in ecotoxicology with reference to exposure to bivalves is limited. Based on the available literatures, it is the first time that 8-oxo-dG is studied as a potential biomarker of exposure to chlorpyrifos in oysters. Furthermore, the concentrations of chlorpyrifos used in this study are within ranges that have been recorded in the natural environment therefore, this study offers a base and a reference for future studies of genotoxicity of chlorpyrifos in oysters.

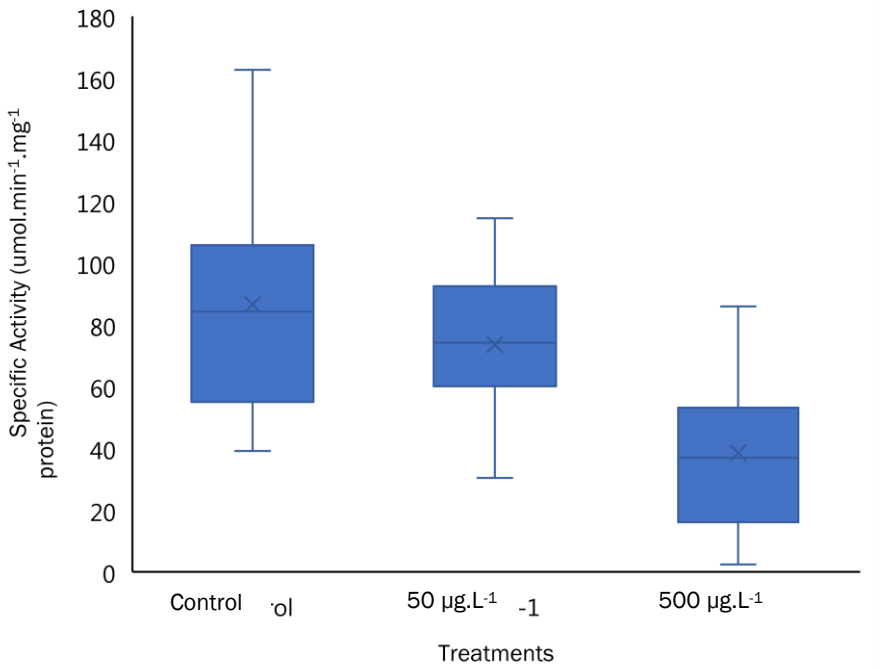

Figure 3. Specific activity (umol.min^{-1}.mg^{-1} protein) of carboxylesterase in the digestive glands of adult *S. mordax* following exposure to 0, 5 and 500 µg.L^{-1} concentrations of Chlorpyrifos. Group treated to high concentration of chlorpyrifos had a significantly lower specific activity (P<0.05).

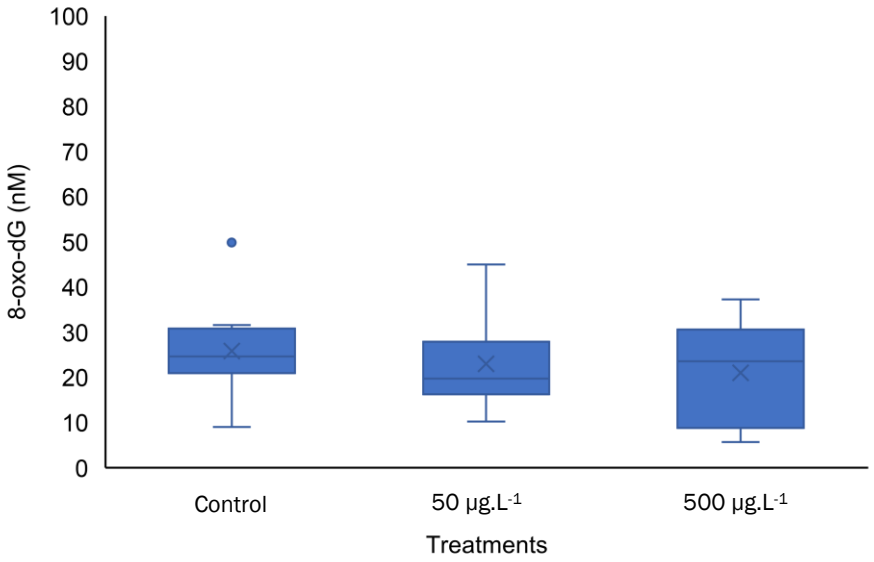

Figure 4. Amount of 8-oxo-dG in the cells of the digestive glands of *S. mordax* following exposure to Chlorpyrifos treatments. There was no significant difference between the treatment groups (P<0.05).

Biomarkers in chlorpyrifos regulation and monitoring

There is no single biomarker can authoritatively be applied in environmental monitoring. As argued by Gagnon and Rawson (2016) individual biomarker does not reliably portray the true health status of an organism following contamination. Their argument supports previous assertions that although sub-organismal biomarkers particularly at molecular and cellular levels are important as early signal of contamination, they are poorly linked to effects at high biological organisations (Sarkar *et al.*, 2006) hence they need to be looked at holistically in order to draw consistent and useful information for environmental management. In this study, lysosome membrane integrity was identified as sensitive biomarker in *S. mordax* to chlorpyrifos contamination hence can be

applied to regulations of the pesticide as well as environmental monitoring. However, the fact that condition index and DNA materials were not significantly affected in this study is an indication of unreliable assumptions of the general health status of the oysters in case lysosome membrane integrity was looked at in isolation. In addition, following the trend in the present study that carboxylesterase activity is a potential biomarker in *S. mordax* with regards to high chlorpyrifos contaminations

Conclusion

This study indicated that the environmentally relevant concentration (5 $\mu g.L^{-1}$) chlorpyrifos is able to adversely affect the lysosome membrane integrity of *S. mordax* in marine environment. Likewise, chlorpyrifos is able to inhibit specific activity of carboxylesterase but only in concentrations higher that 5 $\mu g.L^{-1}$. Furthermore, the condition index of *S. mordax* would probably be affected by chronic exposure to chlorpyrifos. However, it is difficult to predict whether or not chlorpyrifos would induce DNA damage in *S. mordax* in marine environment following the exposure to chlorpyrifos. A recommendation that future investigators of 8-oxo-dG as a biomarker of DNA damage in *S. mordax* exposed chlorpyrifos should periodically measure 8-oxo-dG during and at the end of exposure in order to ascertain recovery or (lack thereof) of the NA damage. This study has equally demonstrated the necessity to utilise multiple biomarkers in ecotoxicological studies due to uniqueness in sensitivity biomarkers to a contaminant. In our case, a combination of lysosome membrane integrity, carboxylesterase activity, 8-oxo-dG and condition index provided ecologically relevant information that can be useful in regulation and monitoring with reference to the coastlines that are inhabited by oysters.

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