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Acetylcholinesterase Activity and Oxidative Stress Biomarkers in Fiddler Crabs (*Austruca occidentalis*) along the Coastal Mangrove Areas of Dar es Salaam, Tanzania

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Abstract

The aquatic organisms such as crustaceans serve as bioindicators of the environmental stressors which affect the aquatic ecosystems. The present study assessed environmental health status through fiddler crabs (*Austruca occidentalis*) towards oxidative stress biomarkers and AChE activity in mangroves with different levels of salinity and environmental contamination. Fiddler crabs were collected from Mtoni Kijichi, Kunduchi and Mbweni, which experience different levels of contamination from high to low along the Dar es Salaam coastline. Malondialdehyde the biomarker of oxidative stress was analysed by lipid peroxidation (LPO) assay and AChE activity was tested using Ellman assay in the crab tissues (muscles, hepatopancreas and gills). To this end, the results showed a significant variation in the LPO levels in analysed tissues ($p < 0.05$) with relatively higher level observed in hepatopancreas, 1.799 µM MDA/mg w.wt than in muscles and gills 0.924 and 1.378 µM MDA/mg w.wt, respectively. Although there was variation of LPO levels in *A. occidentalis* among the studied sites as well as between salinity levels, the variation was not statistically significant ($p > 0.05$). The AChE activities in *A. occidentalis* were significantly different among the study sites ($p < 0.05$), with Mbweni having lowest (4.791 nmol/min/mg protein) activity followed by Kunduchi (4.965 nmol/min/mg protein) and lastly Mtoni Kijichi (5.321 nmol/min/mg protein). The AChE activity also varied significantly by tissue ($p < 0.05$), whereas hepatopancreas and gills had lowest enzyme activity (4.254 and 4.534 nmol/min/mg protein, respectively) as compared to muscles (6.290 nmol/min/mg protein). This field study provides information on the usefulness of *A. occidentalis* in assessing the health status of marine costal ecosystems through their responses against toxicants.

Keywords*:* Fiddler crabs, Oxidative stress, Bioindicators, Acetylcholinesterase.

Introduction

The aquatic environment, particularly intertidal zone, is constantly subjected to chemical contamination from anthropogenic sources and maritime activities, thereby affecting all levels of biological organizations from the individual to the entire ecosystem (Schwarzenbach et al. 2006, Ademuyiwa et al. 2007). Tanzania mangroves are affected by anthropogenic activities mainly due to their capacity of trapping chemicals and the position in the river mouths, estuaries, and sheltered bays (Kruitwagen et al. 2008). Along the Dar es Salaam coast, the Msimbazi River has been identified as heavily polluted followed by Mtoni Kijichi estuary located south of Dar es

Salaam harbour (Machiwa 1992, Kruitwagen et al. 2008). De Wolf et al. (2001) also reported the existence of heavy metals in sediments and periwinkles, *Littoraria scabra* from mangroves along the coast of Dar es Salaam.

Aquatic organisms (e.g. fish, mussels, mudskippers, crabs and shrimps), serve as bioindicators of environmental stressors by changing their biochemical and physiological mechanisms that can precisely be detected by analysis of the biomarkers (Van der Oost et al. 2003). Fiddler crabs (*Austruca occidentalis*) are one of the aquatic species, which play important roles in nutrient cycling and energy flow in marine coastal ecosystems (Naderloo et al. 2016). These fiddler crabs are also very sensitive to environmental changes, thus may be used as bioindicators of marine pollution (Arya et al. 2014). They do occur exclusively in the upper intertidal zones of the mangrove fringe, thereby being frequently subjected to variations in the ambient salinity (Mangale and Kulkarni 2014). The mangrove forests act as sinks for chemicals and other contaminants flowing from inland to the ocean. Hence, the fauna inhabiting the mangrove forests are exposed to the toxic contaminants trapped in mangrove sediments (Kruitwagen et al. 2008). In general, aquatic organisms including fiddler crabs that respond to changing environment are preceded by alterations in biochemical and physiological mechanisms which can efficiently be detected by the analysis of the biomarkers (Borges et al. 2018).

Biomarkers are biochemical, physiological and/or histopathological measurements that indicate alterations in biochemical or cellular pathways of living organisms as a response to toxicants (Van der Oost et al. 2003). Various biomarkers ranging from haematological, immunological and enzymes of biotransformation to oxidative stress can be used as indicators to assess the level of contamination in aquatic ecosystems. Contamination either induces oxidative stress or increases production of free radicals such as reactive oxygen species (ROS) (Rochette et al. 2014). Oxidative stress biomarkers are categorized into two main groups as biomarkers of exposure and those of effects. Antioxidant system responses have mainly been studied as biomarkers of exposure or defense which enables aquatic organisms to survive or adapt in contaminated environment (Mansour et al. 2020). When intoxication is acute, the oxidative stress might cause DNA damage, enzyme inactivation, and protein damage as well as lipid peroxidation (LPO) (Lushchak 2011, Mansour et al. 2020). In recent years acetylcholinesterase (AChE) activity has been widely used as a biomarker of exposure to neurotoxic compounds such as pesticides, metals, detergents, and complex mixtures of persistent organic pollutants in monitoring programmes across the animal Kingdom (Tu et al. 2012, Lionetto et al. 2013 and Mansour et al. 2020). Salinity has been reported to modulate the toxicity of contaminants on the oxidative stress and AChE activity interactively in aquatic organisms (Zanette et al. 2011). Thus, the current study sought to assess the environmental health status by determining the activity of AChE and oxidative stress responses in fiddler crabs (*A. occidentalis*) as a measure of environmental changes with respect to salinity and exposure to polluted and unpolluted mangrove stands along the coast of Dar es Salaam.

Materials and Methods

Study area

The study was conducted along the Dar es Salaam coast, which has different mangrove stands with different levels of contamination. Dar es Salaam is located at 6°48' south, 39°17' east of the Western Indian Ocean. The three sampling sites were Mtoni Kijichi, Kunduchi, and Mbweni based on the level of contamination as reported by Machiwa (1992) and Kruitwagen et al. (2008). Mtoni Kijichi mangrove stand is considered as most polluted as it receives the Kizinga (major) and the Mzinga (minor) tributaries. The Kizinga tributary transports mixed wastes from household, agricultural, as well as wastes of industrial origins such as Murzah oil mills,

coastal steel industry. The mangroves of Kunduchi and Mbweni are located at 20 and 30 km north of the Dar es Salaam harbour, respectively (Figure 1). The Kunduchi mangroves are in the proximity of human settlements, several touristic hotels (such as Kunduchi Beach Hotel and Resort, Maua Beach Hotel) with heavy fishing activities marked by the presence of fish market than the other mangroves included in this study. The Mbweni mangrove stand is located North of Kunduchi, next to a small fishing village with apparent influences of the city of Dar es Salaam (De Wolf et al. 2001, Kruitwagen et al. 2008). It is located at the mouth of Mpigi River, which mainly drains agricultural areas since the main activity in the area is agriculture. All the sampling areas were inhabited with crabs such as *Neosarmatium meinertide* Mann and fiddler crab species including *Austruca occidentalis*.

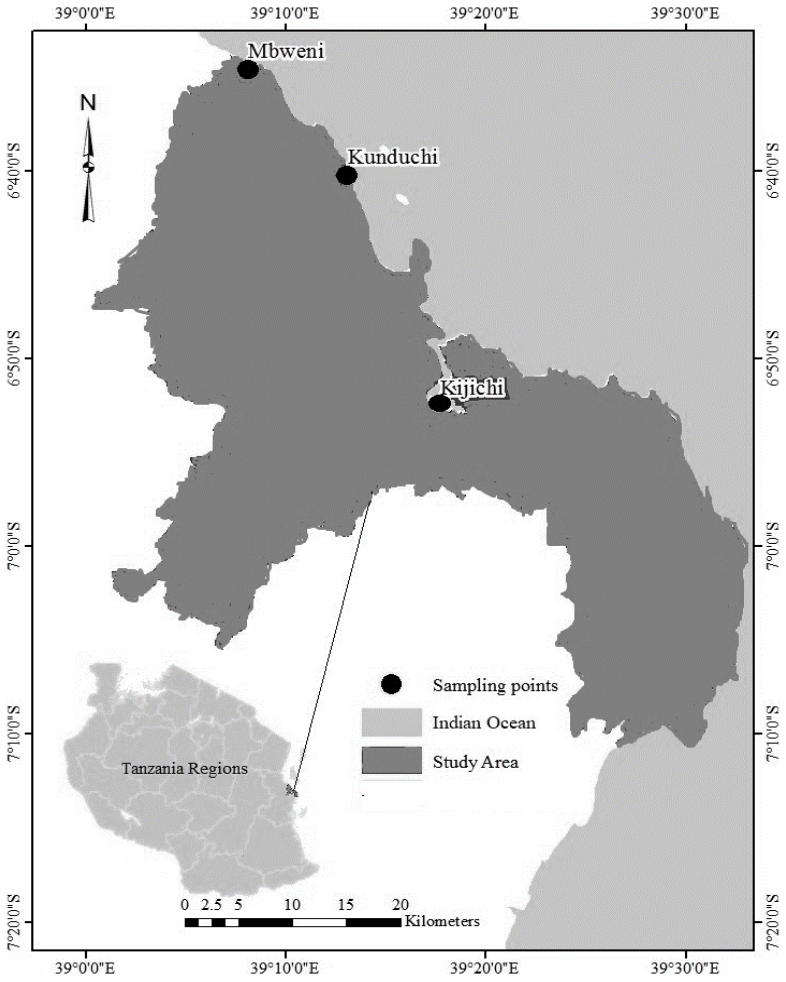

Figure 1: Map of Dar es Salaam region showing sampling points along the Dar es Salaam coast (Kijichi = Mtoni Kijichi).

Sample collection

Fiddler crabs for oxidative stress determination were collected from March to September 2018 for LPO assay and AChE activity analysis, from the three sites. At each site, two sampling points were established based on the availability of the *A. occidentalis* crabs, where salinity was measured. The fiddler crabs were collected from Mbweni, Kunduchi and Mtoni Kijichi based on exposure to the two categories of salinity, low 22.5‰ and high 33.8‰. These salinity categories were established at each site. The distance between the two sampling points was approximately 100 m apart. Salinity was measured by salinometer during sampling at different sites along the coast. These sites included, in the decreasing order of pollution level: Mtoni Kijichi, Kunduchi and Mbweni. Fiddler crabs were collected by hand or by excavating their holes and then rinsed with seawater to remove sediment particles, placed in specimen jars, labelled, and kept in plastic containers for transportation to the laboratory for further analysis.

Sample preparation

Crabs were anaesthetised in 2% of 2-phenoxyethanol and briefly blot dried. The hepatopancreas, gill and muscle tissues were extracted on ice for assays. Tissue preparation for LPO and AChE assays were pooled from three individuals to make one sample of 100 mg wet weight. The glass douncer was employed for homogenization, where 1 ml ice-cold phosphate buffer (50 mM KH_2PO_4/K_2HPO_4, pH 7.5) and 100 mg tissue were added. Then using a pestle, the sample was homogenized with 20 strokes in a douncer and the homogenate was transferred to microtubes chilled on ice.

Biochemical investigations

Oxidative stress biomarker was investigated by measuring the level of malondialdehyde (MDA) as it is used for estimation of damage by reactive oxygen species (ROS). In this study, 1-methyl-2-phenylindole was used as chromogenic agent to assess the production of MDA at 45 °C and 1,1,3,3-tetramethoxypropane was used to prepare MDA standard according to Siddique et al. (2012). The AChE activity was determined using the fixed time endpoint calorimetric method (Benabent et al. 2014). Total protein content in the fraction was estimated as described by Bradford (1976) using bovine serum albumin as a standard.

Data analysis

Data were expressed as mean ± standard error of the means (SEM). All variables were checked for normality using Kolmogorov-Smirnov test ($p > 0.05$) using InStat™ software, v. 2.01/93 (GraphPad) to meet statistical demands. Differences in LPO and in AChE activity from the three sites with respect to salinity and tissues were confirmed using analysis of variance (ANOVA), followed by a post hoc test to investigate the pair-wise difference of the study area. IBM SPSS Statistics 2.0 software was used for statistical analysis with significant level set at $p < 0.05$. Data were not normally distributed ($p < 0.05$), therefore, preceding to the analysis, LPO data were square root transformed, whereas AChE data were natural logarithm transformed to attain the normal distribution and homogeneity of variance to be analysed by ANOVA.

Results

Oxidative stress responses by *A. occidentalis* crabs in mangroves with different levels of environmental contamination

The oxidative stress was determined for samples of *A. occidentalis* crabs collected from Mbweni, Kunduchi and Mtoni Kijichi. The results showed a decreasing trend of LPO from Mbweni to Mtoni Kijichi in low salinity and the pattern was vice versa in high salinity. However, the LPO levels per site were different as compared between low and high salinity and their trend was not clear for example, crabs from low salinity in Mbweni

had high LPO levels, which was vice versa for crabs from Kunduchi and Mtoni Kijichi in low salinity (Figure 2A). Two-way ANOVA revealed that there was no significant difference in LPO levels for both, salinity and site $p > 0.05$; however, there was significant interaction of site and salinity on LPO of *A. occidentalis* $p < 0.05$.

The levels of LPO examined in three tissues of *A. occidentalis* collected from three study sites and in two categories of salinities showed that LPO significantly varied between tissues ($p < 0.05$). The muscles had the lowest LPO levels ranging from 0.4 to 1.98 µM MDA/mg w.wt followed by the gills (0.72–2.07 µM MDA/mg w.wt) and the hepatopancreas with 0.57–2.56 µM MDA/mg w.wt). The finding revealed that, generally the level of oxidative stress did not show much variations as the minimum and maximum values ranged from 0.6 to 2.25, 0.4 to 2.48 and 0.45 to 2.56 µM MDA/mg w.wt for Mbweni, Mtoni Kijichi and Kunduchi, respectively. However, Mtoni Kijichi crabs had relatively lower average of LPO levels than the other sites though it was not significantly different ($p > 0.05$) (Table 1, Figure 2B). Furthermore, the finding indicates that LPO was relatively less in low salinity with an average of 1.28-µM MDA/mg w.wt contrary to 1.38-µM MDA/mg w. wt. in high salinity. Like the site, the LPO levels between salinities were not significantly different as it was revealed by ANOVA ($p > 0.05$) (Table 1, Figure 2C). Contrary to site and salinity, LPO levels of *A. occidentalis* tissues were significantly different, hepatopancreas observed to have high levels of LPO followed by gills and muscles (Table 1, Figure 2D).

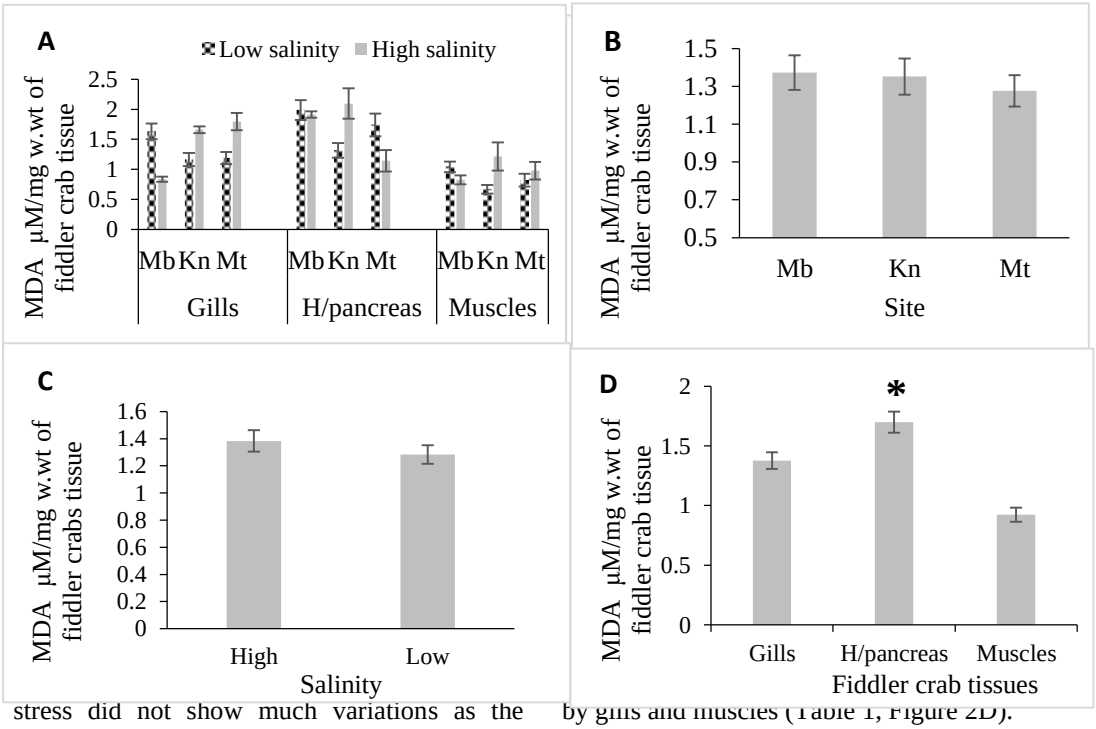

Figure 2: LPO levels in µM MDA /mg w.wt of *A. occidentalis* tissues collected from Mbweni (Mb), Kunduchi (Kn) and Mtoni Kijichi (Mt) along the Dar es Salaam coast. A) Variation of LPO levels by tissues, site and salinity n = 6, H/pancreas = hepatopancreas. B) Variation of LPO by sites n = 36; C) Variation of LPO levels by salinity n = 54 and D) LPO level by tissues of fiddler crabs n = 36. Data are mean ± SEM (SEM = standard error of the mean), * significant difference from the others ($p < 0.05$).

Three-way ANOVA also revealed that there were significant interactions of all factors (site vs. salinity, site vs. tissue, and site vs. salinity vs. tissue) in LPO levels with the exception for salinity vs. tissue factors (Table 1). The Tukey's post hoc test was carried out for the tissue factor only since site and salinity had no influence on the LPO, which showed that the differences in LPO levels were significant for fiddler crab tissues; hepatopancreas vs. muscles, hepatopancreas vs. gills and gills vs. muscles ($p < 0.05$).

Table 1: The effect of site, salinity, tissue, and their interaction with LPO of *A. occidentalis*

Source	Sum-of-squares	df	Mean-square	F-ratio	P	Status
Site	0.185	2	0.093	0.802	> 0.05	ns
Salinity	0.271	1	0.271	2.347	> 0.05	ns
Tissue	10.938	2	5.469	47.374	< 0.05	s
Site vs. Salinity	4.257	2	2.128	18.437	< 0.05	s
Site vs. Tissue	1.792	4	0.448	3.881	<0.05	s
Salinity vs. Tissue	0.071	2	0.035	0.307	> 0.05	ns
Site vs. Salinity vs. Tissue	3.178	4	0.795	6.882	< 0.05	s
Error	10.39	90	0.115			

Analysed by three-way ANOVA at $p < 0.05$, ns = not significant, s = significant, df = degree of freedom and P is probability at which significance was accepted.

Acetylcholinesterase activity of *A. occidentalis* tissues from different sites with different levels of salinity and environmental contamination

AChE activities in the *A. occidentalis* tissues collected from low and high salinity at Mbweni, Kunduchi and Mtoni Kijichi were determined. In Mbweni, the mean low and high salinities were 20.8‰ and 40.6‰, respectively. Kunduchi had 23.3‰ (low) and 37.5‰ (high) while in Mtoni Kijichi there was no significant difference in salinity between the two points (23.3‰).

Using three-way ANOVA ($p < 0.05$) the findings indicated that AChE activity was reduced in low salinity with an average of 4.93 nmol/min/mg protein contrary to 5.12 nmol/min/mg protein in high salinity (Figure 3A, Table 2). Furthermore, results showed that AChE activity varied significantly between the sites with *A. occidentalis* from Mbweni having the least activity followed by Kunduchi and Mtoni Kijichi ($p < 0.05$). Although the lowest AChE activity of (2.5 nmol/min/mg protein) was recorded in Mbweni, the highest was also observed from the same site with enzyme activity of 7.5 nmol/min/mg protein. Kunduchi crabs had the minimum and maximum AChE activity of 3.188 and 6.99 nmol/min/mg proteins, respectively, which is similar to the range obtained in the fiddler crabs from Mtoni Kijichi site (4.08–6.93 nmol/min/mg protein (Figure 3B). Moreover, the AChE activity in *A. occidentalis* showed significant differences between tissues, with gills and hepatopancreas having less levels of the enzyme activities than muscles ($p < 0.05$), (Figure 3C, Table 2). This tissue variation was also observed among the sites and salinity levels. There was a reduction of AChE activity in hepatopancreas and gills for the crabs collected from Mbweni and Kunduchi compared to those from Mtoni Kijichi. However, the situation was different in muscle tissues where the AChE activity was less inhibited (Figure 3D). With respect to salinity, there was a decreasing trend of AChE activity among the tissues and per sites. The low salinity points exhibited a decreased level of enzyme activity against high salinities. Interestingly, the two way-ANOVA revealed an interaction of salinity and site on AChE activity of fiddler crabs ($p < 0.05$), among the three sites. Kunduchi and Mtoni Kijichi crabs had higher AChE activity in high salinity than

low salinity points (Table 2). The Tukey's post hoc test between sites revealed that, the difference in AChE activity was significant for *A. occidentalis* from Mtoni Kijichi vs. Mbweni ($p < 0.05$) as well as for Kunduchi vs. Mtoni Kijichi ($p < 0.05$). However, there was no significant difference in AChE activity of fiddler crabs between Mbweni and Kunduchi ($p > 0.05$). The Tukey's post hoc test indicated that there were significant differences in AChE activity in all tissue pairs compared ($p < 0.05$).

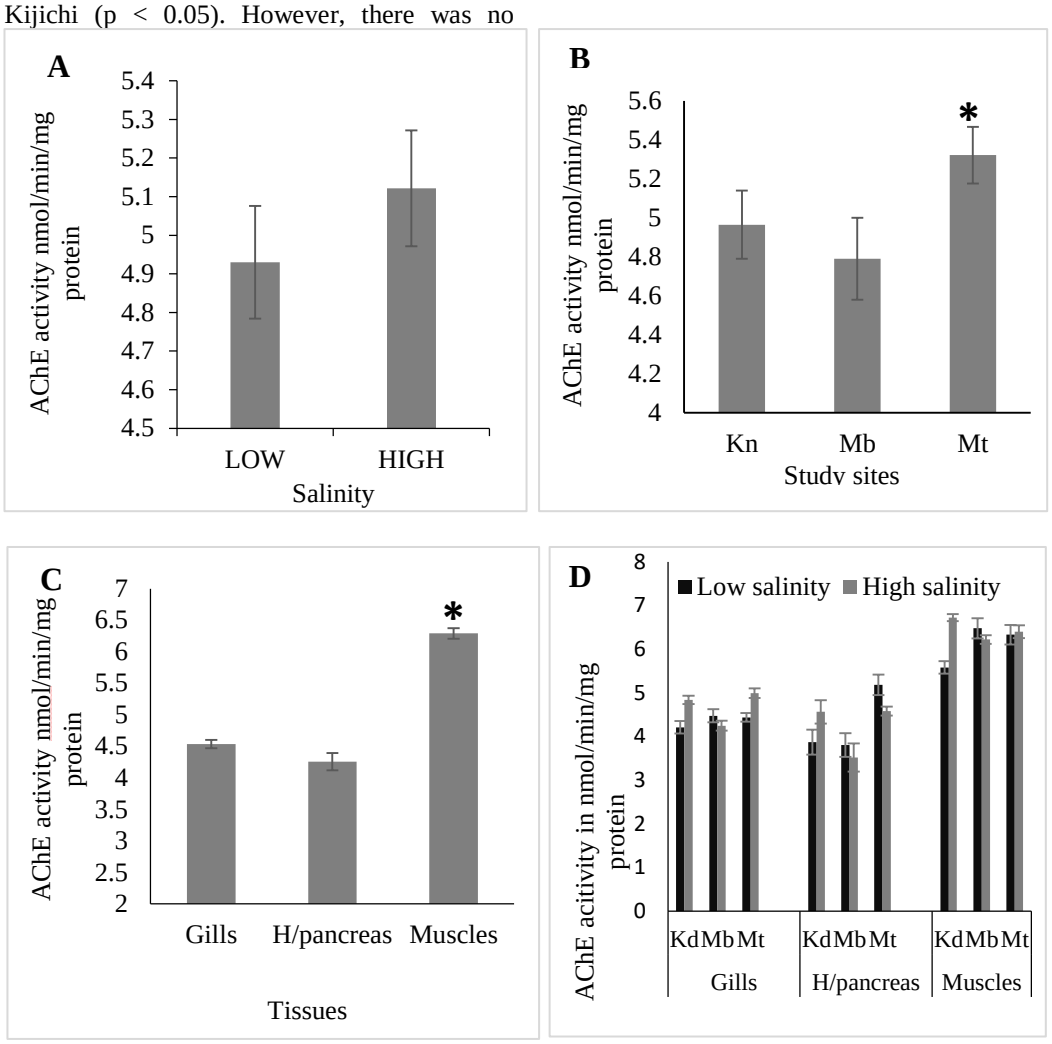

Figure 3: AChE activity in *A. occidentalis* tissues collected from Mbweni (Mb), Kunduchi (Kn) and Mtoni Kijichi (Mt) along the Dar es Salaam coast at low and high salinity. A) AChE activity between low salinity and high salinity, n = 54 determination. B) AChE activity variations among the sampling site, n = 36. C) AChE activity variation in the *A. occidentalis* tissues, H/pancreas = hepatopancreas, n = 36. D) AChE activity variation by tissues, site and salinity n = 6. Data are expressed as mean ± SEM (SEM standard error of the means), * mean significantly different from the others, $p < 0.05$.

Table 2: The effects of salinity, site, tissue and their interactions on AChE activity of *A. occidentalis*

Parameter	Sum of squares	df	Mean square	F	p	Status
Site	5.267	2	2.633	12.256	< 0.05	S
Salinity	0.991	1	0.991	4.61	<0.034	S
Tissue	87.7	2	43.85	204.093	< 0.05	S
Site vs. Salinity	5.682	2	2.841	13.224	< 0.05	S
Site vs. Tissue	4.822	4	1.206	5.611	< 0.05	S
Salinity vs. Tissue	0.876	2	0.438	2.038	> 0.05	Ns
Site vs. Salinity vs. Tissue	1.604	4	0.401	1.866	> 0.05	Ns
Error	19.34	90	0.215			

Three-way ANOVA at $p < 0.05$, ns = not significant, s = significant, df = degree of freedom and P is probability at which significance was accepted.

Discussion

The present study investigated the levels of LPO and AChE in *A. occidentalis* tissues in mangroves with different status of environmental contamination in Dar es Salaam coast. The findings of this study showed that LPO levels were generally low in Mtoni Kijichi as compared to Mbweni and Kunduchi. This is contrary to the findings of previous studies (Machiwa 1992, Kruitwagen et al. 2008) that showed sediments from Mtoni Kijichi estuary were highly polluted including the presence of heavy metals which are believed to induce oxidative stress. Thus, it was expected to have high levels of LPO in *A. occidentalis* from Mtoni Kijichi. On the contrary, the tested animals from Mtoni Kijichi showed low levels of LPO. This indicates that *A. occidentalis* from Mtoni Kijichi were either less stressed or the effects were cancelled by heavy rainfall during the sampling, which in turn washed out the contaminants and reduced the variations of salinity. In addition, the activities that were contributing to the causes of high levels of contamination in Mtoni Kijichi in the past have been reduced substantially. For instance, the industry (Karibu Textiles Mills Ltd) that was emptying the industrial wastes to Mtoni Kijichi has been closed and the area is now isolated having no human activities. However, the high levels of LPO were recorded in Mbweni followed by Kunduchi. These findings could be associated to human activities conducted in the study areas. Mbweni and Kunduchi are currently dominated by active fishing activities and human settlement encroachments to the coast. However, statistically there was no influence of site as well as salinity, though; slightly high LPO was observed in high salinity in relation to the low salinity.

Oxidative stress of *A. occidentalis* was more manifested in hepatopancreas followed by gills and lastly in the muscles. This implies that, concerning tissues, hepatopancreas is more suitable for oxidative stress studies in *A. occidentalis* than other tissues as revealed in this study. These findings are in line with the study by Franco et al. (2018), who reported elevated levels of oxidative stress in the hepatopancreas of fiddler crabs in the higher concentration of oil. The high levels of lipid peroxidation may be due to the activation of detoxification mechanisms. This involves the redox reactions that provide electrons to molecular oxygen and hence results in formation of reactive oxygen species, resulting in high levels of lipid peroxidation because of oxidative damage to membrane lipids (Dorts et al. 2009). Oxidative damage can also be due to the stress induced by salinity variations, as it has been associated with enhanced ROS generation (Liu et al. 2007). It can be assumed that oxidative stress may be responsible for adaptation of organisms to a broad range of environmental stressors (Lushchak 2011). The Environmental stressors such as heavy metals

and other contaminants are well known inducers of oxidative stress by stimulating ROS production (Ghedira et al. 2011).

AChE is an enzyme responsible for termination of nerve impulse transmission and its compromised activity levels has been established as a biomarker of neurotoxicity raised from contaminants such as organophosphates, carbamates, heavy metals and other substances (Tu et al. 2012, Jebal et al. 2013). This study investigated the AChE activity of *A. occidentalis* tissues from sites facing different anthropogenic activities and revealed that AChE activity was the function of salinity, tissue and contamination levels. Its activity level was high in *A. occidentalis* tissues from high salinity, particularly muscle tissues from Mtoni Kijichi site. This was statistically shown that salinity, tissues and site had an influence separately and interactively on AChE. Previous studies, both laboratory and field have pointed out that some metallic ions such as cadmium, mercury, copper and lead depress the activity of AChE in studied animals (Lionetto et al. 2013, Jebali et al. 2013). These heavy metals have been associated with the production of strong inhibition to several enzymes that have a sulfhydryl functional group, AChE being one of such enzymes (Devi and Fingerman 1995). Among the three studied sites, AChE activity was more inhibited in crab tissues collected from Mbweni and Kunduchi than those from Mtoni Kijichi. As it was seen in LPO levels, Mtoni Kijichi site is now isolated and no direct human activities. However, the situation is different in Mbweni and Kunduchi. Pesticides such as organophosphates and carbamates are another group of contaminants that are known to inhibit the activity of AChE (Jebali et al. 2011, Tu et al 2012, Jebali et al. 2013). Some studies also have pointed the inhibition of AChE activity by the polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) that are commonly found in the environment (Kang and Fang 1997, Lionetto et al. 2013). PAHs are present in surface water, sediments, soils and urban air, which are the results of incomplete combustion of fossil fuels, wood and municipal waste incineration and from internal combustion engines (Van der Oost et al. 2003, Kruitwagen et al. 2008). Additionally, the difference in AChE activity levels in the crab tissues may be related to the difference in tissue distribution and abundance of the enzymes in the organism. This correlates with the study by Solé et al. (2012), who reported the difference in the abundance of AChE in Senegalese sole fish tissues that were collected from the same site, with brains and livers having higher AChE levels than kidneys and gills.

With respect to salinity, this study found that AChE activity was inhibited in areas with low salinity compared to high salinity levels. Also, the gills and hepatopancreas had low activity of the enzyme in low salinity. Although the effects of salinity and xenobiotic are still unclear, some studies have pointed out that, tissues such as gills increase the uptake of toxic materials such as cadmium in low salinities (O'Hara 1973) cited in Tu et al. 2012). In addition, salinity has been shown to influence the toxicity of organophosphorous and carbamate pesticides both in crustaceans and fish (El-Alfy et al. 2001, Pfeifer et al. 2005).

This study represents the first report on the effects of environmental pollutants on AChE activity in *A. occidentalis* crabs along the Dar es Salaam coast. Therefore, AChE activity can be used as a biomarker of environmental pollution by indicating neurotoxic compound contamination in the natural ecosystem (Jebali et al. 2013, Ben-Khedher et al. 2013). This view is supported by the study of Devi and Fingerman (1995), who clearly concluded that AChE activity has the potential to serve as a biomarker of pollution. Thus, future studies focusing on assessing the types and levels of environmental pollutants both in animal tissues and sediments at different seasons are recommended.

Conclusions

This study was carried out to investigate the oxidative stress responses and acetylcholinesterase activity as biomarkers in *A. occidentalis* in contaminated areas along the coastal mangrove areas of Dar es Salaam. The findings from this study indicate the potential of *A. occidentalis* as a bioindicator, particularly in mangrove ecosystems. Also, the study shows that hepatopancreas is a suitable tissue model for biomarkers analysis in invertebrates. Furthermore, among the studied sites, the study concludes that Mtoni Kijichi is currently less contaminated compared to Kunduchi and Mbweni; however, evaluation of the current possible toxicants in the given sites is recommended.

Declaration of interest

The authors report no conflicts of interest.

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