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Full Length Research Paper

Screening of genomic DNA and analysis of heavy metals to identify mutations in the genes of *Ciona intestinalis* (Linnaeus, 1767) collected from the Mediterranean Sea - Alexandria, Egypt

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Ascidians are particularly vulnerable to hazardous accumulation of heavy metals in the marine ecosystem. Specimen of *Ciona intestinalis* were collected from three localities of the Mediterranean Sea of Alexandria in July 2009. Animals were dissected and frozen at -20°C. RAPD–PCR analysis showed natural differences or polymorphism among individuals of *Ciona intestinalis,* so investigations upon the effect of pollution on DNA level was not possible, where DNA was similar between individual samples. Genomic DNA was screened for damage using five primers. Only two primers gave fragments, suggesting very little genetic variation and extensive genetic exchange. Copper, iron, magnesium, cadmium, zinc and tributyl tin (TBT) accumulations were measured three times in both sea water and in the tissues of *Ciona intestinalis* using the atomic absorption spectrophotometer. The levels of lipid peroxidation, superoxide dismutase, catalase, glutathione transferase, glutathione reductase, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase as well as proteins were recorded as well. This study concluded that the Western harbour (Abu Kir) of Alexandria is the most polluted study area and that heavy metals accumulate in the tissues of the ascidian *Ciona intestinalis* which enters the food chain, and finally reaches man.

Key words: hazardous accumulation, marine ecosystem, RAPD, PCR analysis, heavy metals, *Ciona intestinalis*, biomonitor.

INTRODUCTION

Community stability and survival of sensitive species are threatened from anthropogenic activities that introduce significant amounts of pollutants into the Mediterranean Sea. The effluent discharge of pollutants, including heavy metals, occurs at the vicinity of the coastal cities of Egypt.

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Abbreviations: ALP, Alkaline phosphatase; **AST,** aspartate aminotransferase; **GSH,** glutathione reductase; **GST,** glutathione transferase; **CAT,** level of catalase.

The main sources of copper in the sea water are the antifouling paints that are being used on small (under 25 m length) vessels (Claisse and Alzein, 1993), sewage discharges (El-Shebly, 1993; Reichelt-Brushett and Harrison, 2000; El Gendy et al., 2003), fungicides and herbicides which are used in coastal agricultural crops (Moudon et al., 2006). Sub-lethal effects of metals may have drastic repercussions at an ecological level when they alter biological processes of the organisms. For example, a pollutant may kill half of the individuals of a species population with little or no ecological significance, whereas a pollutant that does not kill organisms but retards their development may have a considerably higher ecological impact (Berthet et al., 2005; Moriarty,

1983). Coastal marine and estuarine environments have long been used as disposal areas for industrial and mining wastes. They are also subjected to transient toxicant releases that may occur in the form of spills or runoff from agricultural and urban areas, as well as antifouling biocides. Mediterranean coastal areas are highly contaminated by heavy metals (Palanques et al., 1995; Puig et al., 1999), which produce harmful effects on marine organisms because of their toxicity and bioaccumulation through the trophic chains. Sessile benthic invertebrates as sponge, sea urchins and ascidians are especially susceptible to heavy metal pollution because of their suspension (Au et al., 2001) - or filter-feeding habitat (Moriarty, 1983) and their reduced motility, which prevents them from escaping from toxicants released to a given area (Perez et al., 2005).

Although heavy metals appear to be noxious for adults (Cebrian et al., 2003), they seem to be innocuous or even beneficial for larvae and settlers (Cebrian and Uriz, 2007). The effects of pollution in the toxic Mediterranean sponge *Crambe crambe* and in the colonial ascidians *Pseudodistoma crucigaster* and *Botryllus schlosseri* at organismal and suborganismal levels have been thoroughly studied (Agell et al., 2001; Parrinello et al., 2008). Marine invertebrates are unable to avoid stress and must adapt to variable environments. The genetic basis of copper pollution resistance across multiple environments was studied using the embryonic stages of the marine invertebrate, *Styela plicata* (Galletly et al., 2007). Increasing copper concentration caused a decrease in hatching success and survival rate. However, hatching success appeared to have a different genetic basis in high copper concentration. This study suggests that marine organisms may adapt to varying intensities of pollution through different genetic mechanisms (Schröder et al., 2000; Werner and Hinton, 2000).

Three molecular markers have been identified to address the invasion of *Didemnum vexillum* (Hess et al., 2009). Authors suggested that these markers, specifically microsatellite locus D6 and nuclear sequence locus Dnr1, are variable enough to be useful to genetically differentiate *D. vexillum* populations and potentially characterize invasion pathways. Different fields of study are present in the available literature in an attempt to understand the biology of ascidians. The Molecular characterization of *Ciona intestinalis* proteins, the deutoplasmogenesis (vitellogenesis) in the oocyte of *Styela plicata* (Lesuaer, 1823), *Styela partita* (Stimpson, 1852) and *Ciona intestinalis,* flourescence axiomicroscopy and SEM studies of metamorphosis of *Ciona intestinalis* (Linnaeus, 1767) and the role of NO/cGMP signaling with the molecular chaperone heat shock protein 90 (HSP90) activity in *Phallusia mammilata* (Cuvier, 1815), the development of the neural complex of *Ascidia mentula* E/ M investigation, the gene expression profiles in young adult *Ciona intestinalis* have been studied (El Moselhy, 1999; Nishikata et al., 2001; Dehal et al., 2002; Fujiwara et al., 2002; Kusakabe et al., 2002; Ogasawara et al., 2002; Satou et al., 2002; Michael et al., 2008; Saad et al., 2008; Saad, 2008, 2010; Saad and Hamed, 2009). The different tissues of the investigated ascidians specially the branchial chambers have different colour intensities in the different localities of the Mediterranean Sea. This observation prompted the use of *C. intestinalis* to measure and analyzes the different pollutants in their tissues on chemical procedures and at a molecular level. Our strategy included integration of method validation and qualification. In this study, RAPD marker assay is based on the PCR amplification of random locations. The DNA amplification protocol was performed according to Williams et al. (1990), with some modifications. It should be noted that Ascidians have not been investigated as bio-monitors of pollution in our locality before.

Aim of the work

The present study investigates whether heavy metals pollution with (Cu, Fe, Mn, Cd, Zn and TBT) affects the genetic variation of *C. intestinalis* along the coast of Alexandria, Egypt, in different locations; Eastern Harbour, Western Harbour (Abu Kir) and Al-Asafra beach respectively.

MATERIALS AND METHODS

Specimens of *C. intestinalis* were collected by snorkelling from the Western harbour of Alexandria, Eastern harbour of Alexandria and Al-Asafra beach in July 2009 (Figures 1 and 2). Thirty specimens were collected per locality. Immediately, these samples were stored in an insulated box containing ice cubes and transferred to deep freeze (-20°C) until the time for metal analysis. For seawater sampling surface, water samples were collected at

30 cm below the water surface to avoid contamination whereas the bottom water samples were collected at 30 cm above the bottom to avoid disturbance of the sediments (EPA Environmental Monitoring Management Concil, Washington (1993). DC, Nov. 18, 1993. "Format for Method Document"). Three animals having the same size (length and width) from the three collections were dissected. Three samples of tissues were taken in each locality and analysed for heavy metal accumulations. Each trail was made three times. Trace metals Cu, Fe, Mn, Cd, Zn and TBT were determined using Graphite Furnace Atomic Absorption Spectroscopy (Perkin-Elmer model 2300) under the recommended condition limits (DL) in the manual for each metal. All values are reported as mg/g wet wt for *Ciona* and mg/l for water. Water quality criteria; Dissolved Oxygen (DO) were measured as per procedures according to Grasshoff et al. (1983), Methods of seawater analysis. Verlag Chemie, Weinheim, Germany.

Analysis of heavy metals in the tissues

The soft tissues from the dissected samples of *Ciona intestinalis* were measured using the atomic absorption spectrophotometer. These tissues were digested at 120°C for 3 in the nitric perchloric acids mixture (3:1). Five metals; Cu, Fe, Mn, Cd, Zn and TBT were measured in the sea water and in the tissues following the method

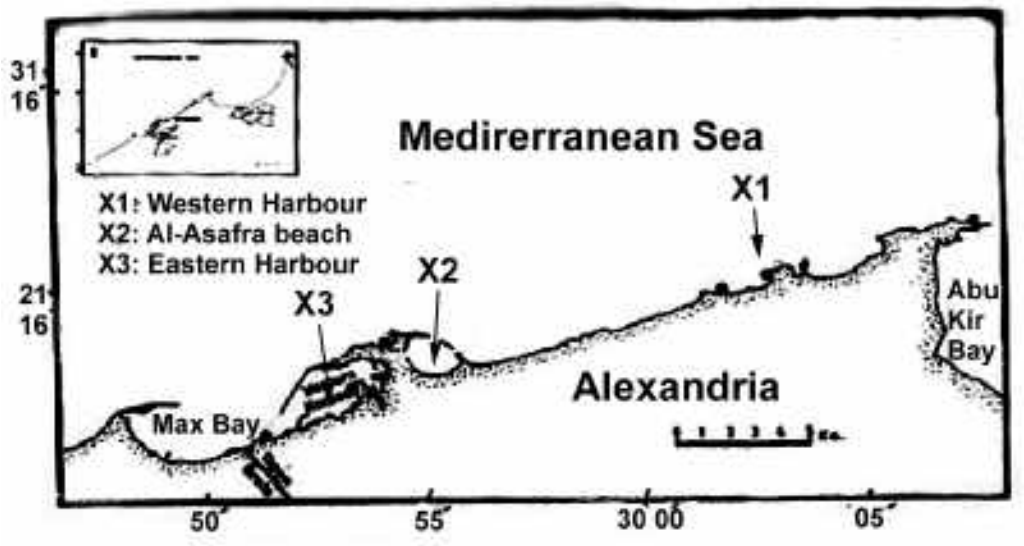

Figure 1. Area of study and sampling locations.

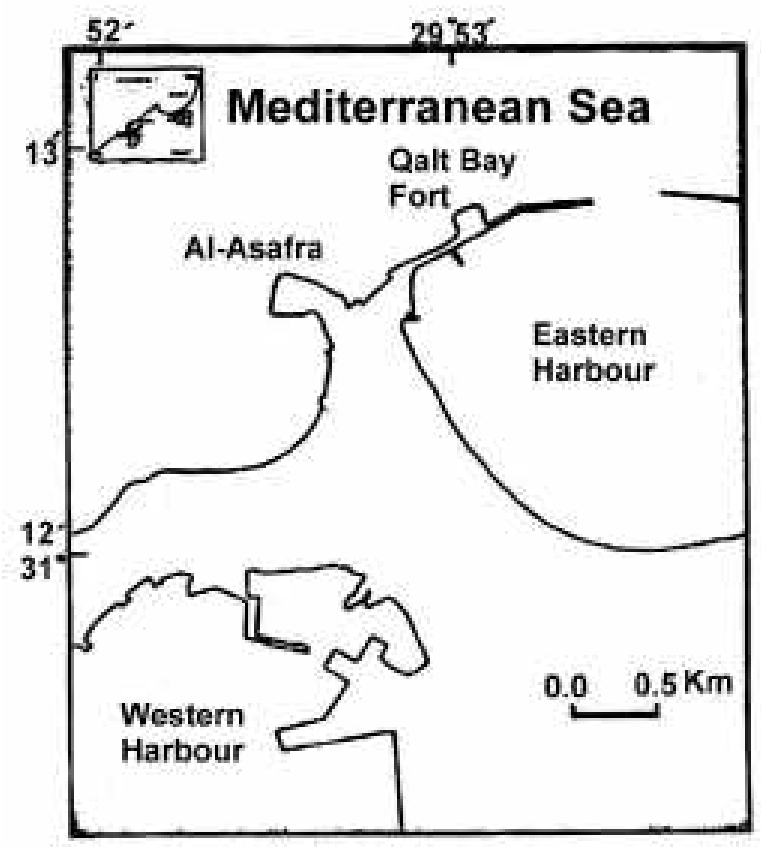

Figure 2. Enlarged map of the area of ascidians collection.

described by UNEP/FAO/IAEA/IOC (1984) in El-Sikaily et al. (2004). To avoid contamination, all the glass wares were washed with double distilled water and soaked over night in 20% nitric acid, analytical grade. All other chemicals were of highest purity. Sample preparation was undertaken in hoods to avoid any extraneous contamination. The water used in this study was double distilled in

all glass apparatus. Briefly, the whole gonad tissues of each site were homogenized in triplicate sample each 1 g were digested using 4 ml of analar nitric acid in Teflon vessel, covered tightly and allowed predigesting at room temperature over night. The digestion block was placed on a preheated hot plat at 80ºC for 3 h. The samples were cooled to room temperature and then were transferred to a 25 ml volumetric flask. Bi-distilled water was used for all preparations. All digested solutions were analyzed in triplicate by Atomic absorption spectrophotometer (Spectr AA-10 plus Varian) working with an air/acetylene flame and D2 background correction. The levels of different metals in the sea water (in the same sites and in the same seasons) were also analyzed in triplicate by Atomic absorption spectrophotometer as described above. The reagents of analytical grade were utilized for the blanks and calibration curves; precision was checked against standard reference material provided by the National Research Council of Canada, and was within the range of certified values. Recovery of all metals studied was over 97%. The absorption wavelength and detection limits were as follows: 228.8nm and 0.006 mg g-1 for Cd; 324.7 nm and 0.008 mg g-1 for Cu; 248.3 nm and 0.007 mg g-1 for Fe; 279.5 nm and 0.006 mg g-1 for Mn; 217.0 nm and 0.01 mg g-1 for Pb, respectively. All data are presented as concentrations per unit wet weight of the samples (as mg/kg).

Water Collection

Surface water samples were collected from the three locations (Eastern harbour – Al Asfraa and Western harbour (Abu- Kir Bay) in each season. At each location water samples were collected using polyethylene bottles (2-litres capacity). The polyethylene bottles were previously cleaned with detergent rinsed several times with distilled water. Soaked in 1N HCL for several days and finally rinsed with redistilled water. For dissolved oxygen determination, at each location a 150 ml dissolved oxygen bottles was firstly filled and immediately fixed, using manganous sulphate and alkaline potassium iodide solution (Grasshoff, 1976). For salinity determination hard glass bottles with tight covers were rinsed twice with sea water before filling with samples, these samples were kept in a shady place till measurement using Inductive Salinometer (Beckman mode).

Temperature measurements

This parameter was measured in the field. The surface water temperatures were measured at the time of water sampling by using an ordinary thermometer (t).

Salinity determination (S %)

Salinity was determined by measuring the electrical conductivity using an Inductive Salinometer (Beckman; model RS. 10).

Hydrogen-ion concentration (pH)

The pH-value of water sample was measured in the laboratory immediately after collection using Bench type (JENWAY, 3410 Electrochemistry Analyzer pH-meter) with reading up to 0.01 pH unit after necessary precautions in sampling and standardization processes.

Dissolved Oxygen (DO) determination

It was determined by a modified Winkler´s method (Grasshoff, 1976). Fixation of dissolved oxygen was made *in situ* using manganous sulphate and alkaline potassium iodide solutions, taking all precautions that no bubbles are formed, after complete fixation of oxygen, the precipitated manganese hydroxide is allowed to settle and then dissolved by 9N H2SO4, the liberated iodine was titrated against standard sodium thiosulphate using starch solution as indicator. Oxygen percentage saturation was calculated using (UNESCO Tables, 1973).

Statistical analysis

The data, expressed as Mean ± SE. were analyzed statistically using column statistics and one way ANOVA with Newman-Keuls Multiple Comparison Test as a post test using the computer statistics Prism 3.0 package (GraphPad Software, Inc, San Diego, CA, USA). The minimum level of statistical significance was set at $P<0.05$.

Molecular analysis

Extraction, purification and quantization of genomic DNA

Following the protocol (Dellaporte et al., 1983), Genomic DNA was isolated from the selected specimen.

Random Amplified Polymorphic DNA (RAPD)

RAPD marker assay is based on the PCR amplification of random locations in the genome of *C. intestinalis*. The DNA amplification protocol was performed according to (Williams et al., 1990) with some modifications. Primers used in RAPD analysis: Oligonucleotide sequences of the random primers used were selected from a set of Operon kits; Operon Technologies Inc.; Alameda CA. A total of 10 random primers were used to screen *Ciona intestinalis* present in the three selected locations.

Electrophoresis of PCR products

Amplified samples were analyzed by electrophoretic separation in 1.4 % agarose gel. 8 µl of each PCR product were mixed with 2 µl loading buffer and loaded into the wells of the gels. The gels were run at 90 volts for about 2 h.

A schematic diagram for the size and molecular weight of the different bands for the DNA marker (1 Kb ladder for the Lambda/Hind III-ΦX174/Hae III) are shown in Figure 3.

Optimization of randomly ampliphied polymorphic DNA (RAPD) conditions

To determine the effect of primer concentration, twenty primers were tested at 10, 20, 30 and 40 pmoles final concentration, on genomic DNA extracted from five of the bands. In addition, four $MgCl_2$ concentrations of 0.5, 1.0, 1.5, and 2.5 mM were evaluated. The effect of template concentration (12.5, 25, 50, 75 and 100 ng) of genomic DNA and *Taq* DNA polymerase concentration (0.4, 1.0, 1.6, 2.0 and 2.5 units) in a volume of 50 µl was investigated. The best results were obtained when using 25 ng of genomic DNA with 2.5 u of *Taq* polymerase at a final concentration of 10 mM Tris-Hcl (pH 8.3), 3 mM $MgCl_2$, and 0.001% gelatin and 40-pmol primer. To verify the reproducibility of the results all experiments were repeated twice at least. These parameters were kept constant throughout the experimental analysis. The ten primers that successfully generated repeatable and easy to score polymorphic markers (Table 1) were selected for further analysis.

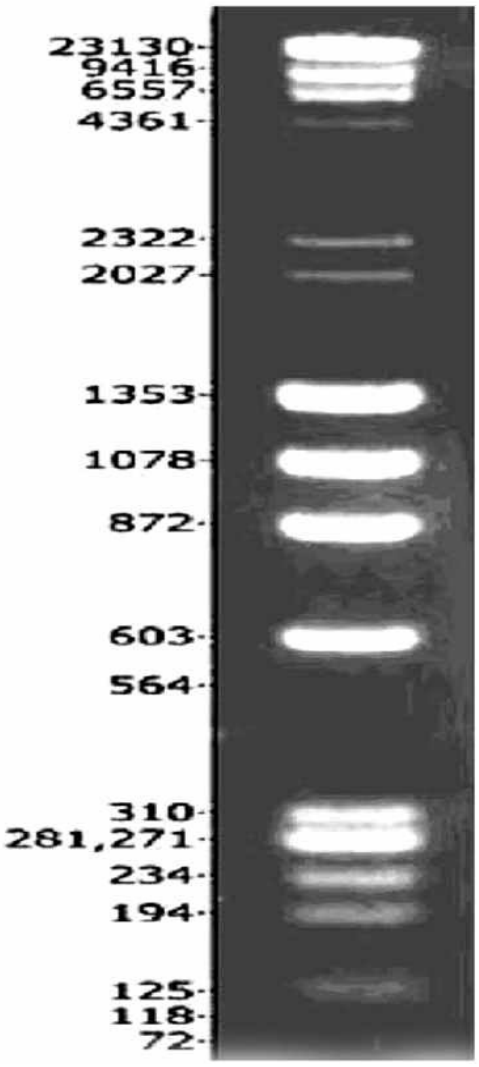

Figure 3. Showing the size and molecular weight of the different bands for the DNA marker (1 Kb ladder for the Lambda/Hind III-ΦX174/Hae III).

Table 1. Sequence of arbitrary; 10-mer, RAPD primers used for testing the effect of the pollution on the ascidian *Ciona intestinalis*.

Primers	Sequences 5` 3`	GC%
OPA-01	GGCCCTTCCC	70
OPA-20	GTTGCGATCC	60
OPB-04	GGACTGGAGT	60
OPB-17	AGGGAACGAG	60
OPB-20	GGACCCTTAC	60
OPC-16	CACACTCCAG	60
OPC-20	ACTTCGCCAC	60
OPG-15	ACTGGGACTC	60
OPG-16	AGCGTCCTCC	70
OPG-17	ACGACCGACA	60

RESULTS

Specimens of *C. intestinalis* were collected from the Western Harbor of Alexandria (Abu- Kir Bay), Eastern harbor of Alexandria and AL-Asarfa beach in July 2009 (Figures 1 and 2). Three samples of sea water were taken from each locality and analysed for heavy metal accumulations. The recovery of pollution is variable in the three studied localities. Three animals, having the same size (length and width) from the three collections were digested and the heavy metal pollutions were measured.

Molecular analysis

Polymorphisms as detected by DNA markers; the different DNA marker techniques detected the polymorphism by assaying subset of the total amount of DNA sequence variation in a genome. The level of polymorphism among the *C. intestinalis* was estimated by using PCR-based marker techniques, Random Amplified Polymorphic DNA (RAPD).

Randomly ampliphied polymorphic DNA (RAPD)

A preliminary assay was performed to assess the genetic uniformity of the different samples. In this respect, samples were collected and tested with five different primers. The results revealed that all the examined samples exhibited uniform pattern with the five examined primers. Polymorphism is detected by RAPD markers: Five primers were screened with the DNA of the *C. intestinalis*, two primers generated reproducible and easily scorable (RAPD profiles). These produced multiple band profiles with a number of amplified DNA fragments ranging from 5-15. A maximum number of 15 amplicons was amplified with primer OPG-17, while the minimal number of fragments was amplified with primer OPA-18. The size of the amplified fragments varied with different primers, ranging from (200 to 4000 bp) (Table 2).

The results revealed that DNA–PCR produced DNA of quality (sharp band) of genomic DNA as shown in (Figures 4 and 5). RAPD–PCR analysis showed natural differences or polymorphism among *C. intestinalis*.

In the present study, different concentrations of primers were tested. Genomic DNA of tunicates produced more reliable banding pattern when the primers were used at concentration of 7 pM. The best concentrations of genomic DNA were 2 ng/µl for *C. intestinalis.* Genomic DNA of *C. intestinalis* was screened for damage using five primers of arbitrary sequences. Out of these primers, two only gave fragments and three primers did not amplify the genome of the whole animal. All primers exhibited complete similarity of DNA in the tissues. Absence of a fragment presumably occurs because amplification cannot proceed on DNA strands from either

Table 2. Total number of amplicons, monomorphic amplicons and percentage of polymorphism as revealed by RAPD markers among the tested *Ciona intestinalis.*

Primer	Total number of amplicons	Monomorphic amplicons	Polymorphic amplicons	% of polymorphism
OPA-18	8	3	5	62.5
OPG-17	10	3	7	70.0
Total	18	6	12	90.0

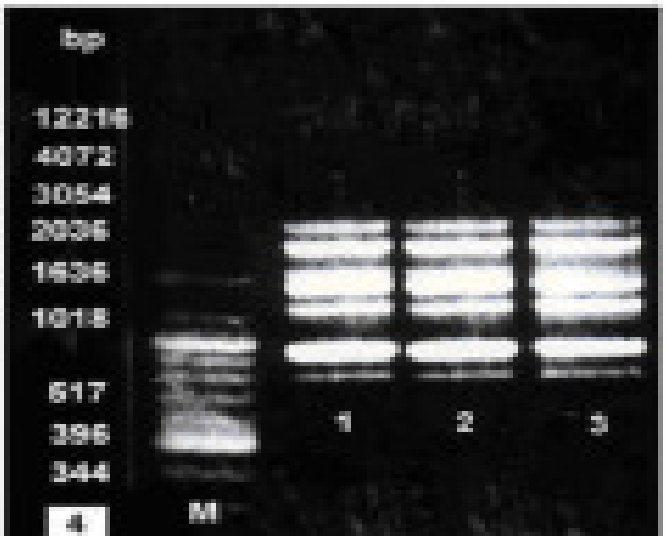

Figure 4. Showing RAPD profiles of the *Ciona intestinalis* (loc,#1, loc.#2 and loc.#3) with primer (OPG-17). MDNA molecular weight standard (1 kb pair ladder).

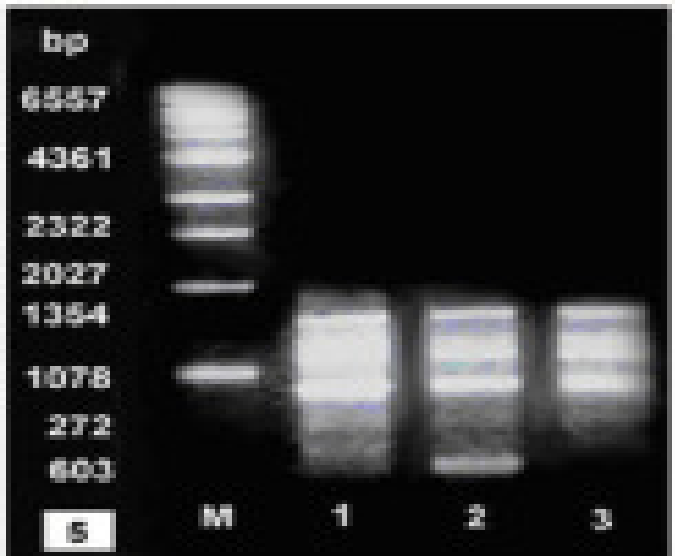

Figure 5. Showing RAPD profiles of the *Ciona intestinalis* (loc,#1, loc.#2 and loc.#3) with primer (OPG-17). MDNA molecular weight standard (1 kb pair ladder).

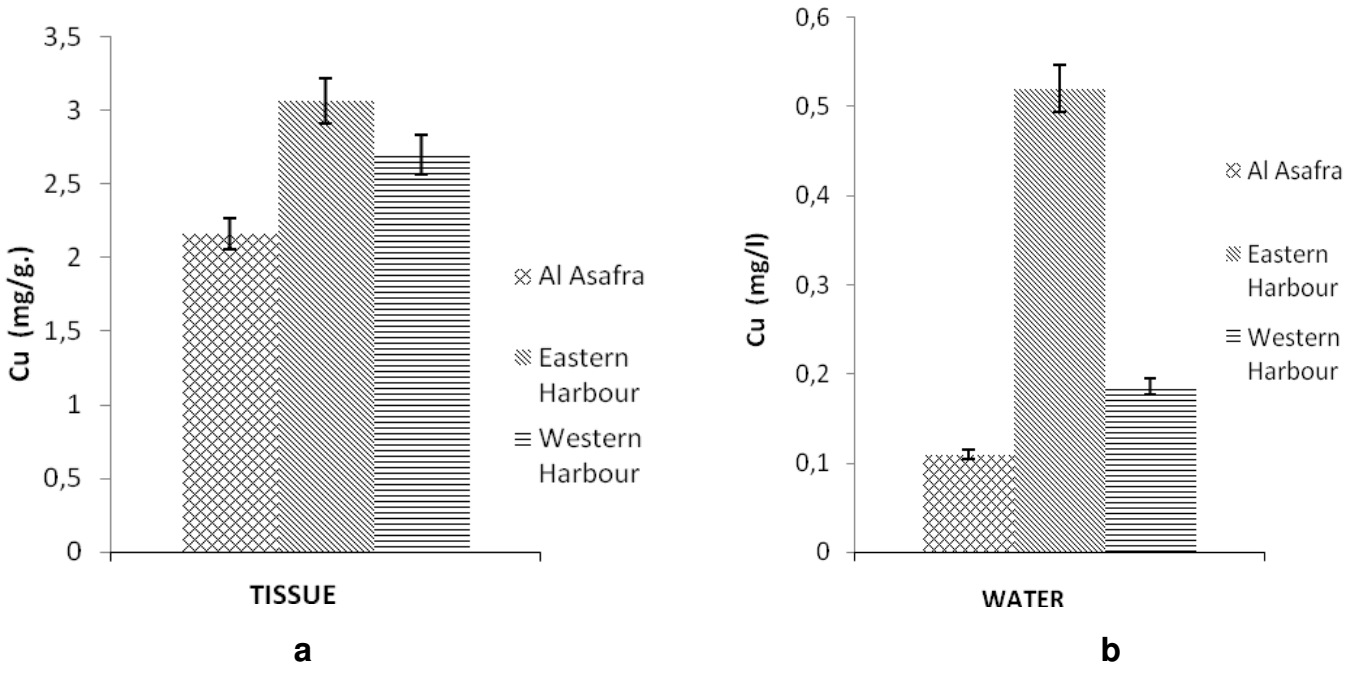

Figure 6. Histogram showing measurement of copper concentration in the sea water and in tissues of *Ciona intestinalis*

of the homologous chromosomes in an individual. This can occur through point of mutation at one or both primer annealing sites on a DNA strand, inversion surrounding a site of insertion that separate the annealing sites at a greater distance than can be amplified. Results of the present study revealed that synthesis of stress proteins belong to different protein subfamilies according to molecular weight has been occurred. Some peptides were common between *C. intestinalis* in the three locations, while other new peptide spots appeared on the gel due to pollution stress. However, the pollution affected the disappearance of some peptides. Such compounds (Five metals; Cu, Fe, Mn, Cd, Zn and TBT) can cause a potential increase of mutagenicity and carcinogenicity. Mutagenicity can increase the frequency of delirious mutants in next generation. However, genotoxic effects can be tested by recent techniques. Several genotoxic assay are now available, but it is often difficult to decide which are the most suitable for screening the genotoxic effect of a given chemical because there are considerable differences between the sensitivity of the test methods and between the effects of different classes of chemicals (Hess et al., 2009). Current awareness of the potential hazards of pollutants has stimulated much interest in the use of *C. intestinalis* as indicators for monitoring environmental mutagens. The present work planned to employ *Ciona intestinalis* genome as sensitive monitor for possible genotoxic effect induced by the aquatic contaminants. The results obtained revealed that two primers were capable in closing the genotoxic effect induced by marine pollution. This work indicates the importance of the study of the amount and distribution of genetic diversity for a better exploration of *C. intestinalis*.

Heavy metals analysis in water and tissues of Ciona intestinalis and enzymes in tissues of ciona

Copper accumulation is maximal in the tissues of *C. intestinalis* collected from the Eastern Harbour measuring 3.1 mg/g whereas it is 2.7 mg/g in samples collected from the Western Harbour (Abu- Kir Bay) and 2.1 mg/g in samples collected from Al-Asafra beach (Figure 6a). Copper dissolved in the sea water of the three localities showed negligible differences (Figure 6b).

Iron accumulation is maximal in the tissues of *C. intestinalis* collected from the Western harbour(Abu- Kir Bay) measuring 4.5 mg/g. whereas it is 3.90 mg/g. in samples collected from the Eastern Harbour and 2.35 mg/g. in samples collected from Al-Asafra beach beach (Figure 7a). Iron dissolved in the sea water in the Western Harbour was 350 mg/l and in the Eastern Harbour was 220 mg/l while 190 mg/l in Al-Asafra beach (Figure 7b).

Manganese accumulation is maximal in the tissues of *C. intestinalis* collected from the Eastern harbour measuring 6.9 mg/g, whereas it is 6.2 mg/g in samples collected from the Western Harbour (Abu- Kir Bay) and 3.1 mg/g in samples collected from Al-Asafra beach (Figure 8a). Manganese dissolved in the sea water in the Western Harbour was 3.9 mg/l and in the Eastern Harbour

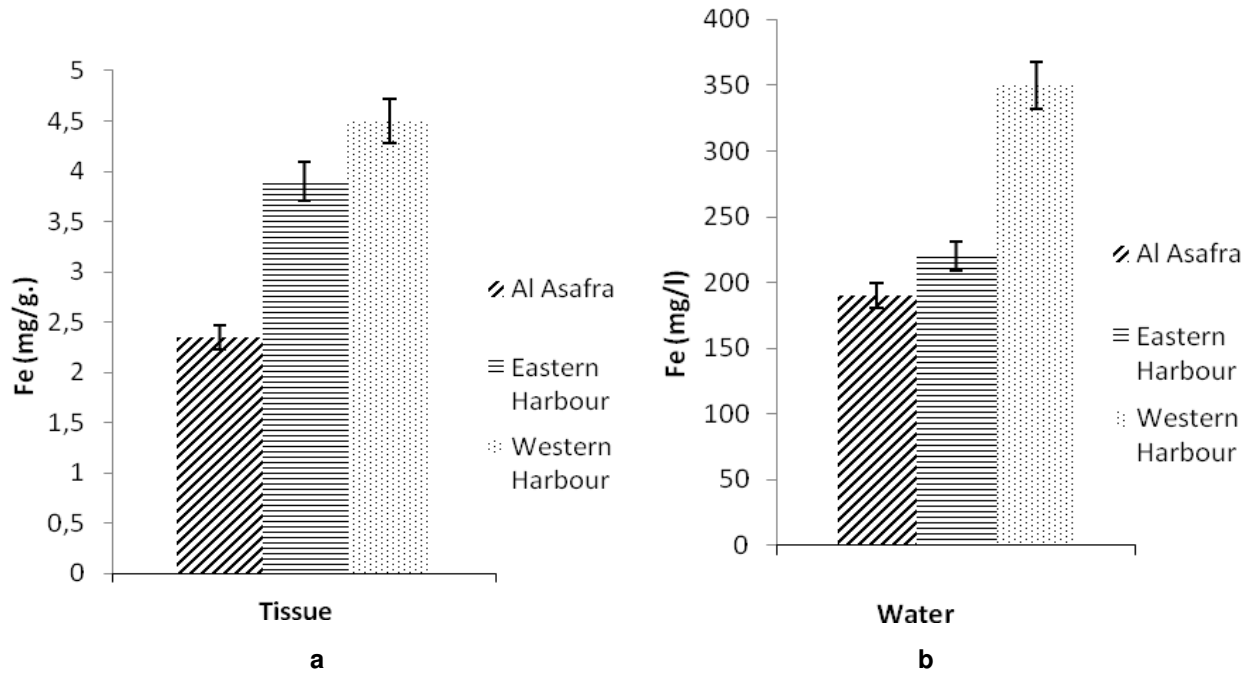

Figure 7. Histogram showing measurement of iron concentration in the sea water and tissues of *Ciona intestinalis.*

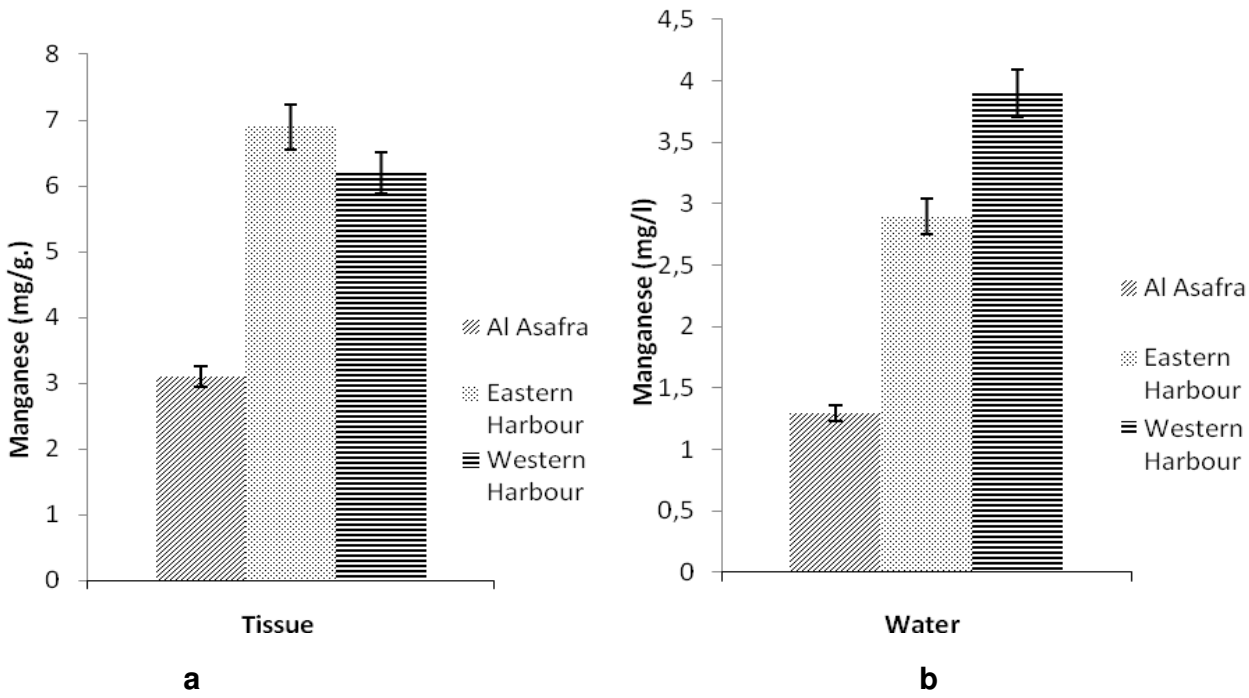

Figure 8. Histogram showing measurement of Manganese in the sea water and tissues of *Ciona intestinalis.*

was 2.9 mg/l while 1.3 mg/l in Al-Asafra beach (Figure 8b).

Cadmium accumulation is maximal in the tissues of *C. intestinalis* collected from Al-Asafra beach measuring 10.6 mg/g. whereas it is 10.2 mg/g. in samples collected from the Western Harbour(Abu- Kir Bay) and 5.2 mg/g. in samples collected from the Eastern Harbour (Figure 9a). Cadmium dissolved in the sea water in the Western

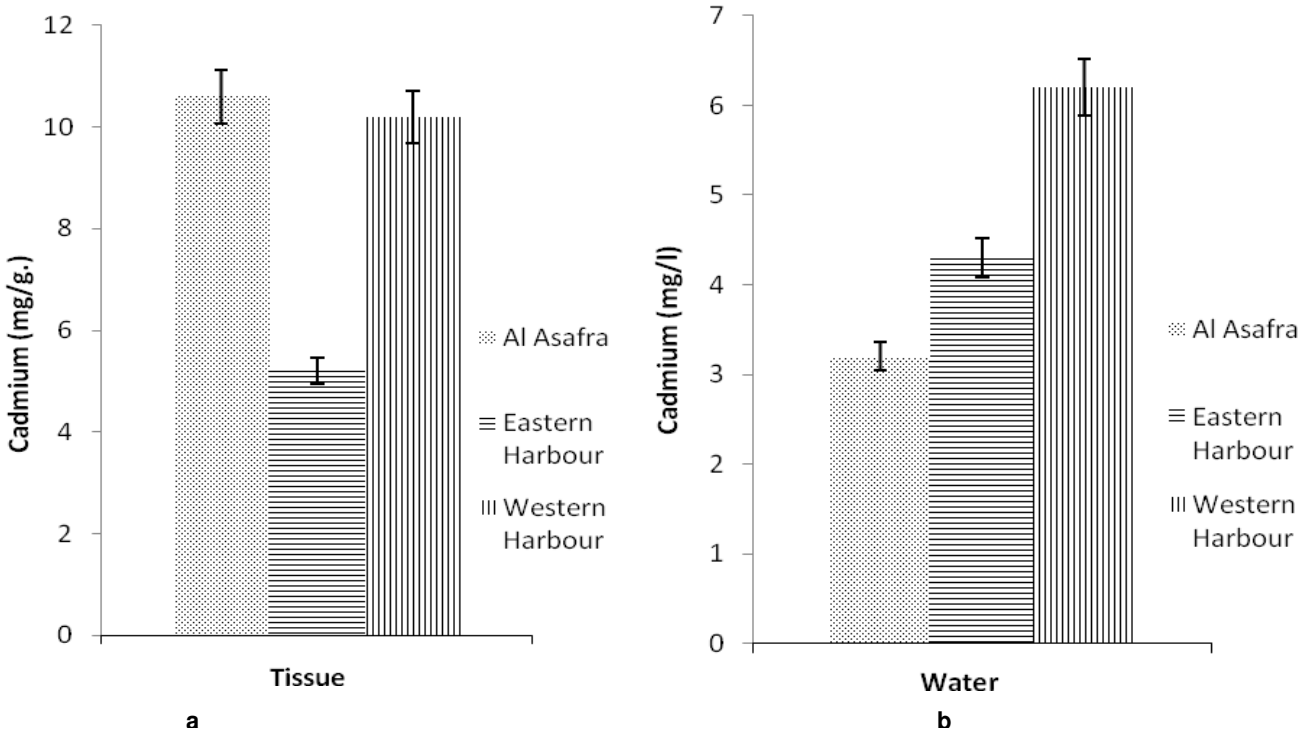

Figure 9. Histogram showing measurement of Cadmium in sea water and animal tissue in *Ciona intestinalis.*

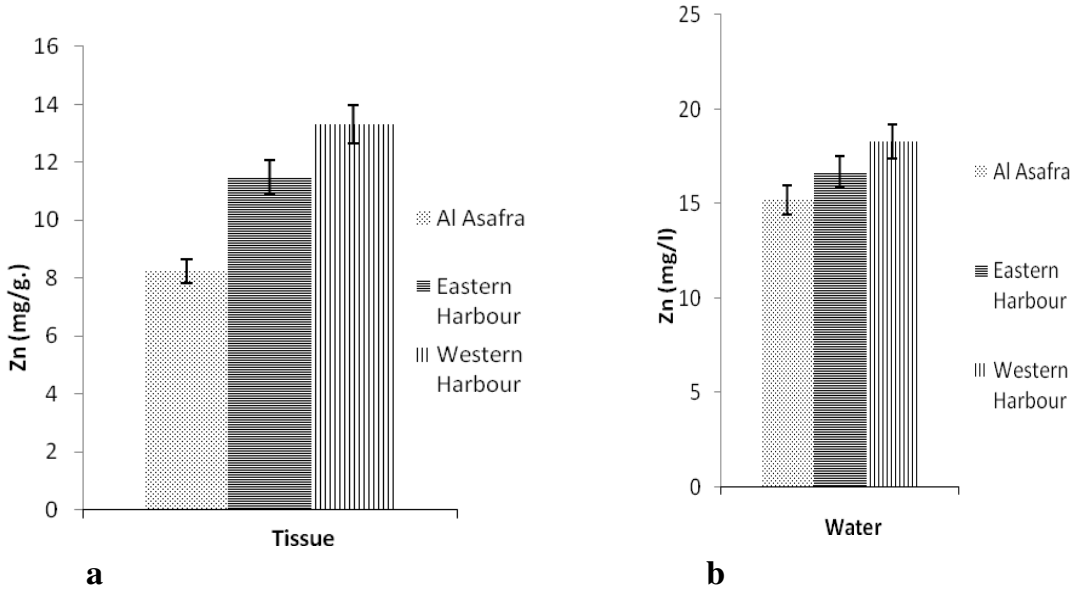

Figure 10. Histogram showing measurement of Zinc in sea water and animal tissue of *Ciona intestinalis.*

Harbour was 6.2 mg/l and in the Eastern Harbour was 4.3 mg/l while 3.2 mg/l in Al-Asafra beach (Figure 9b).

Zinc accumulation is maximal in the tissues of *C. intestinalis* collected from the Western harbour (Abu- Kir Bay) measuring 13.3 mg/g whereas it is 11.5 mg/g in samples collected from the Eastern Harbour and 8.2 mg/g in samples collected from Al-Asafra beach (Figure 10a). Zinc dissolved in the sea water in the Western Harbour was 18.3 mg/l and in the Eastern Harbour was 16.7 mg/l while 15.2 mg/l in Al-Asafra beach (Figure 10b).

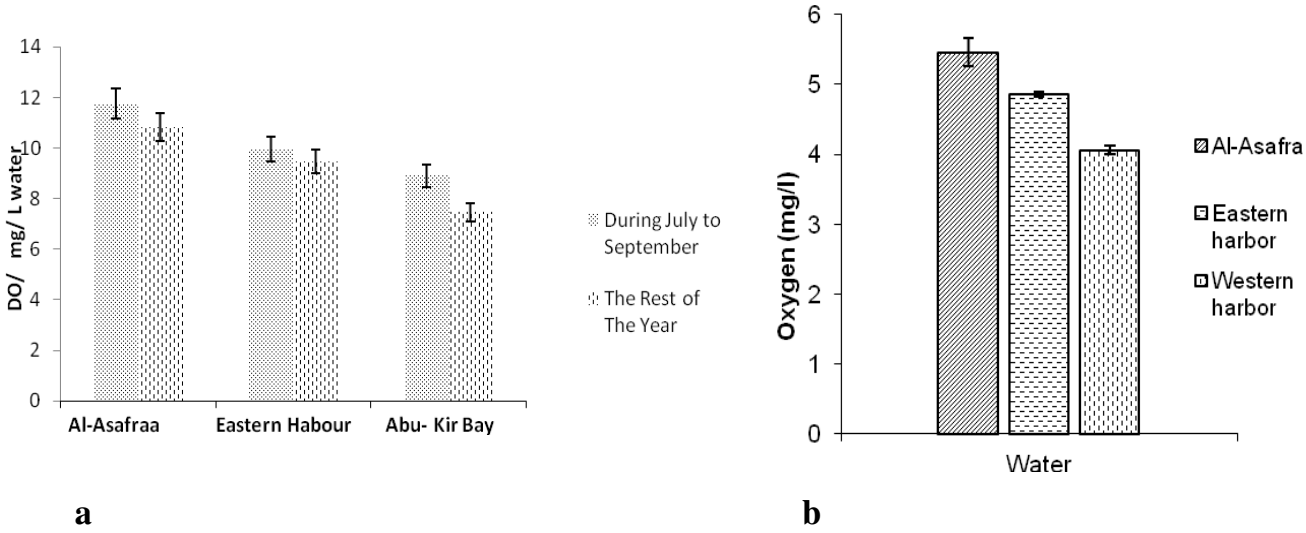

Figure 11. Histogram showing measurement of dissolved oxygen in sea water. The level of dissolved oxygen (DO) in the sea water obtained from the three studied locations, Al-Asafraa ,Eastern harbour and Abu- Kir Bay in July to September and in the rst of the year. Note, The level of dissolved oxygen (DO) in the sea water obtained from Abu- Kir Bay was significantly decreased (*P<0.01*) than those obtained from Al-Asafraa.The level of dissolved oxygen (DO) in the sea water obtained from Abu- Kir Bay was significantly decreased (*P<0.001*) than those obtained from Eastern harbour.The level of dissolved oxygen (DO) in the sea water obtained from Eastern harbour was significantly increased (*P< 0.001*) than those obtained from Al-Asafraa.

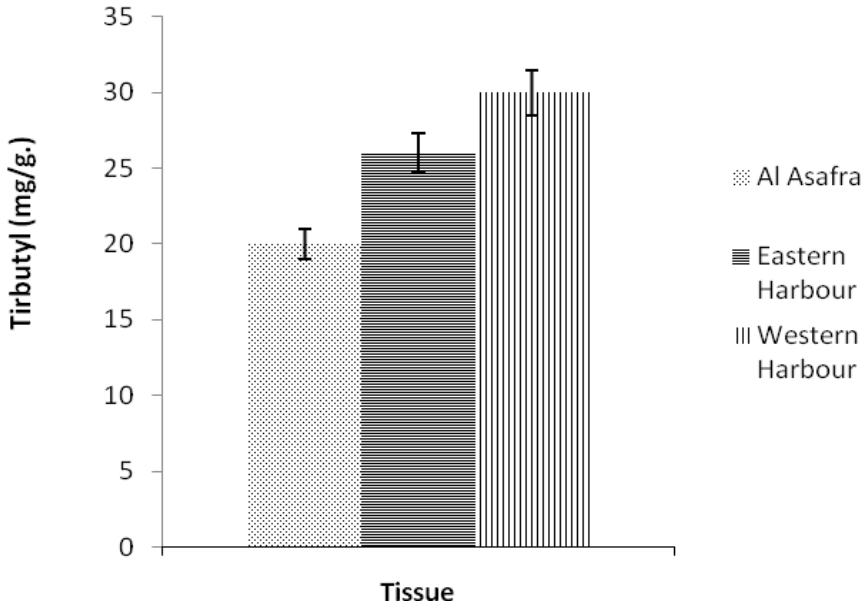

Figure 12. Histogram showing measurement of Tributyl tin (TBT) in tissues of *Ciona intestinalis.*

The dissolved Oxygen in the sea water of Al-Asafra beach is 5.8 mg/l while it is 4.9 mg/l in the Eastern Harbour and 4.0 mg/l in the Western Harbour (Figure 11).

Tributyl tin (TBT) accumulation is maximal in the tissues of *C. intestinalis* collected from the Western harbour (Abu- Kir Bay) measuring 30 mg/g whereas it is 26 mg/g in samples collected from the Eastern Harbour and 20 mg/g in samples collected from Al-Asafra beach (Figure 12).

The level of lipid peroxidation (MDA) in tissues of *C.*

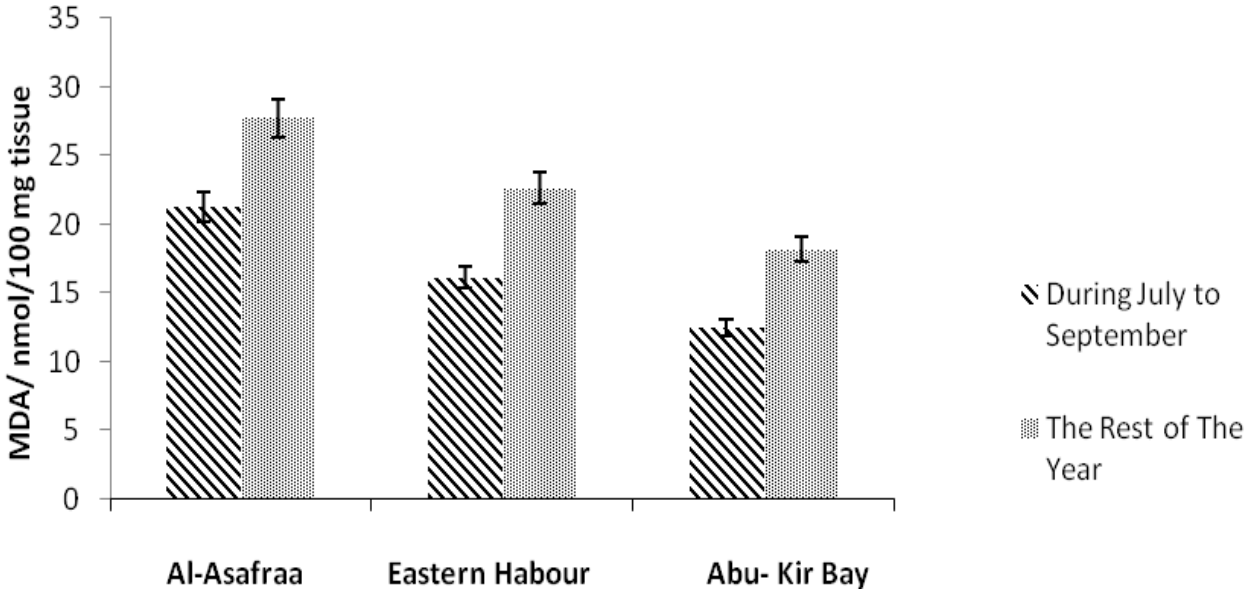

Figure 13. Histogram showing the level of lipid peroxidation (MDA) in the tissues of *Ciona intestinalis* obtained from the three studied locations, Eastern harbour, Al- Asafraa coatal water and Abu- Kir Bay. The level of lipid peroxidation (MDA) obtained from the tissues in Abu- Kir Bay was significantly increased (*P<0.05*) than those obtained from Eastern harbour. The level of lipid peroxidation (MDA) obtained from the tissues of *Ciona in* Abu- Kir Bay (b) was significantly increased (*P<0.001*) than those obtained from Eastern harbour.The level of lipid peroxidation (MDA) obtained from the tissues of *Ciona in* Eastern harbour was significantly decreased (*P<0.001*) than those obtained from Al- Asafraa coastal water.

intestinalis recorded its highest level in Western Harbour (Abu- Kir Bay) (Mean ± SE in the rest of the year was 55.50 ± 4.058, while in July to September it was 41.75 ± 1.52). On the other hand, *Al-Asafraa* recorded the least MDA levels (Mean ± SE in the rest of the year was 27.75 ± 1.6, while in July to September it was 28.38 ±1.5). Eastern harbor recorded medium MDA levels (Mean ± SE in July to September was 44.38 ± 2.4, while in July to September it was 35.88 ±1.45). It is worth highlighting that MDA levels across all areas were higher in the rest of the year than in the July to September (Figure 13).

The level of superoxide dismutase (SOD) in tissues of *C. intestinalis* recorded its highest level in *Al-Asafraa* (Mean ± SE in the rest of the year was 21.25 ± 1.278, while in July to September it was 27.75 ± 1.176). On the other hand, Western Harbour (Abu- Kir Bay) recorded the least SOD levels (Mean ± SE in the rest of the year was 12.49 ± 0.465, while in the July to September it was 18.13 ± 0.833). Again, Eastern harbor recorded medium levels between the other two areas (Mean ± SE in the rest of the year was 16.13 ± 0.953, while in July to September it was 22.63 ± 1.179). It should be noted that SOD levels across all areas were higher in the July to September than in the rest of the year (Figure 14).

The level of catalase (CAT) in tissues of *C. intestinalis* recorded its highest level in *Al-Asafraa* (Mean ± SE in the rest of the year was 123.1 ± 3.3, while in July to September it was 113.6 ±3). On the other hand, Western Harbour (Abu- Kir Bay) recorded the least CAT levels (Mean ± SE in the rest of the year was 88.3 ± 5, while in July to September it was 87.5 ± 3). Again, Eastern harbor recorded medium levels between the other two areas (Mean ± SE in the rest of the year was 101.8 ± 4, while in July to September it was 98.6 ± 3). It should be noted that CAT levels across all areas were higher in July to September than in the rest of the year (Figure 15).

The level of glutathione transferase (GST) in tissues of *Ciona intestinalis* recorded its highest level in *Al-Asafraa* (Mean ± SE in the rest of the year was 22 ± 1.9, while in July to September it was 22.5 ± 0.8). On the other hand, Western Harbour (Abu- Kir Bay) recorded the least GST levels (Mean ± SE in the rest of the year was 13 ±1.1, while in July to September it was 16.5 ±0.5). Again, Eastern harbor recorded medium levels between the other two areas (Mean ± SE in the rest of the year was 18 ± 1.2, while in the July to September it was 19.8 ±0.44). It should be noted that GST levels across all areas were higher in July to September than in the rest of the year (Figure 16).

The level of glutathione reductase (GSH) in tissues of *C. intestinalis* recorded its highest level in *Al-Asafraa* (Mean ± SE in the rest of the year was 49.25 ± 1.8, while in July to September it was 44.13 ±1.4). On the other hand, Western Harbour (Abu- Kir Bay) recorded the least GSH levels (Mean ± SE in the rest of the year was 37.75 ± 1.2, while in July to September it was 33.88 ± 1.15). Again, Eastern harbor recorded medium levels between the other two areas (Mean ±SE in the rest of the year

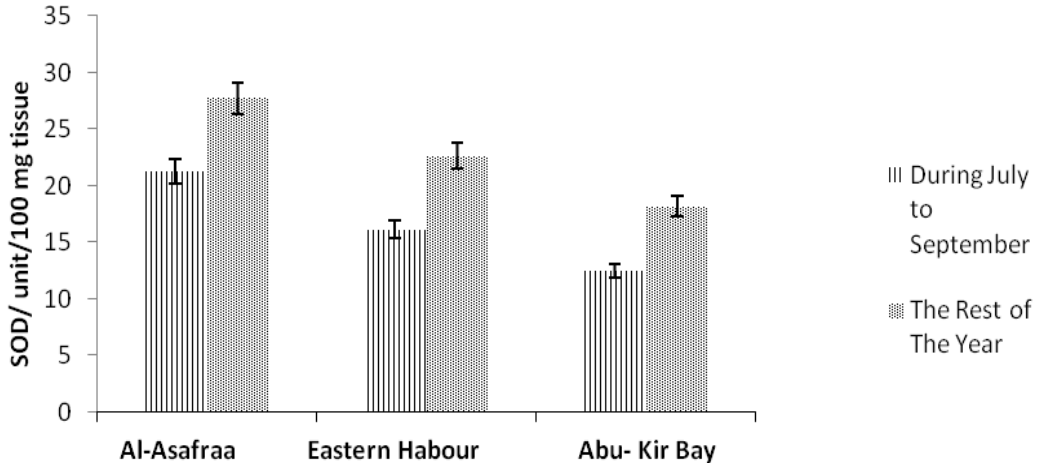

Figure 14. Histogram showing the level of superoxide dismutase (SOD) in the tissues of *Ciona intestinalis* obtained from the three studied locations, Eastern harbour, Al- Asafraa coatal water and Abu- Kir Bay.The level of superoxide dismutase (SOD) obtained from the tissues of Ciona in Abu- Kir Bay was significantly decreased (P<0.05) than those obtained from the Eastern harbour. The level of superoxide dismutase (SOD) obtained from the tissues of Ciona in Abu- Kir Bay was significantly decreased (P<0.001) than those obtained from Al-Asafraa coastal water. The level of superoxide dismutase (SOD) obtained from the tissues of Ciona in Eastern harbour was significantly increased (P<0.01) than those obtained from Al-Asafraa coastal water.

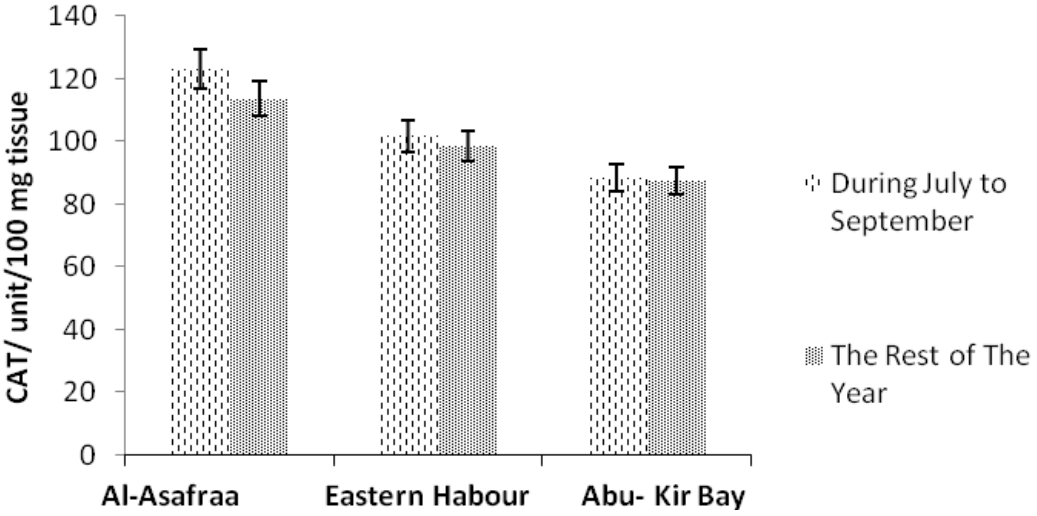

Figure 15. Histogram showing the level of catalase (CAT) in the gonads of tissues of *Ciona intestinalis* obtained from the three studied locations, Eastern harbour, Al- Asafraa coatal water and Abu- Kir Bay. The level of catalase (CAT) obtained from the gonads of tissues of *Ciona intestinalis* in Abu- Kir Bay was significantly decreased (*P<0.05*) than those obtained from Al-Asafraa coastal water. The level of catalase (CAT) obtained from the gonads of tissues of *Ciona intestinalis* in Abu- Kir Bay was significantly decreased (*P<0.001*) than those obtained from Eastern harbour. The level of catalase (CAT) obtained from the gonads of tissues of *Ciona intestinalis* in Eastern harbour was significantly increased (*P<0.01*) than those obtained from Al-Asafraa coastal water.

was 43 ± 1.2, while in July to September it was 38.25 ± 0.9). It should be noted that GSH levels across all areas were higher in the July to September than in the rest of the year (Figure 17).

The level of aspartate aminotransferase (AST) in tissues of *C. intestinalis* recorded its highest level in *Al-Asafraa* (Mean ± SE in the rest of the year was 308.3 ±12.6, while in July to September it was 128.1 ± 4). On

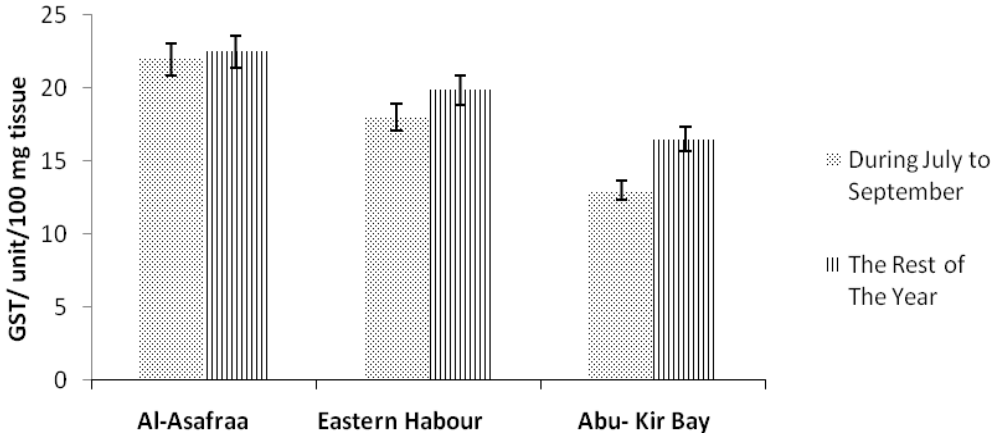

Figure 16. Histogram showing the level of glutathione transferase (GST) in the tissues of *Ciona intestinalis* obtained from the three studied locations, Eastern harbour, Al- Asafraa coatal water and Abu- Kir Bay.The level of GST)obtained from the tissues of *Ciona intestinalis* in Abu- Kir Bay was significantly decreased (*P<0.05*) than those obtained from Al-Asafraa. The level of GST obtained from the tissues of *Ciona intestinalis* in Abu- Kir Bay was significantly decreased (*P<0.001*) than those obtained from Eastern harbour. The level of glutathione transferase (GST) obtained from the tissues of *Ciona intestinalis* in Eastern harbour was significantly increased (*P< 0.05*) than those obtained from Al-Asafraa.

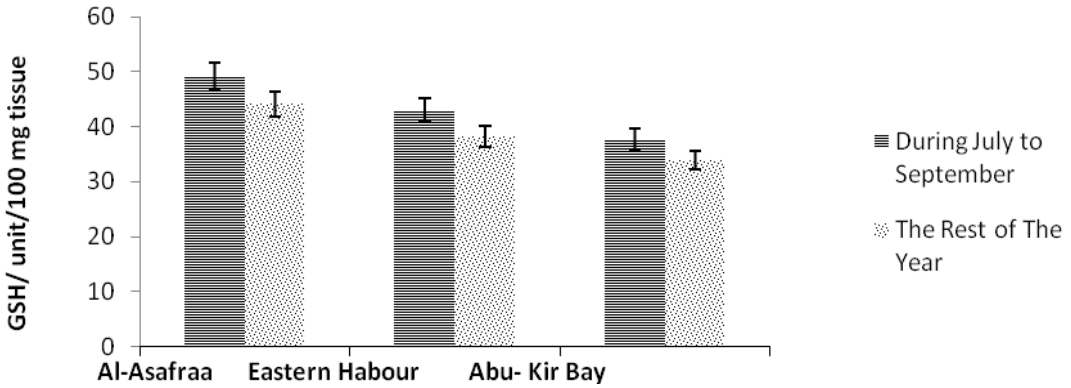

Figure 17. Histogram showing the level of glutathione reductase (GSH) in the in the tissues of *Ciona intestinalis* obtained from the three studied locations, Eastern harbour, Al- Asafraa coatal water and Abu- Kir Bay.The level of GSH obtained from the tissues of *Ciona intestinalis* in Abu- Kir Bay was significantly decreased (*P< 0.05*) than those obtained from Al-Asafraa.The level of GSH obtained from the the tissues of *Ciona intestinalis* in Abu- Kir Bay was significantly decreased (*P<0.001*) than those obtained from Eastern harbour.The level of GSH obtained from the tissues of *Ciona intestinalis* in Eastern harbour was significantly increased (*P< 0.01*) than those obtained from Al-Asafraa**.**

the other hand, Western Harbour (Abu- Kir Bay) recorded the least AST levels (Mean ± SE in the rest of the year was 243.6 ± 0.7, while in July to September it was 107 ± 5.4). Again, Eastern harbor recorded medium levels between the other two areas (Mean ± SE in the rest of the year was 271.3 ± 11.04, while in July to September it was 95.7 ± 3.5). It should be noted that AST levels across all areas were higher in the rest of the year than in July to September (Figure 18).

The level of alanine aminotransferase (ALT) in tissues of *C. intestinalis* recorded its highest level in *Al-Asafraa* (Mean ± SE in the rest of the year was 219.9 ± 14, while in July to September it was 67.88 ± 3.8). On the other hand, Western Harbour (Abu- Kir Bay) recorded the least ALT levels (Mean ± SE in the rest of the year was 164.6 ± 13.8, while in July to September it was 51.38 ± 3.4). Again, Eastern harbor recorded medium levels between the other two areas (Mean ± SE in the rest of the year

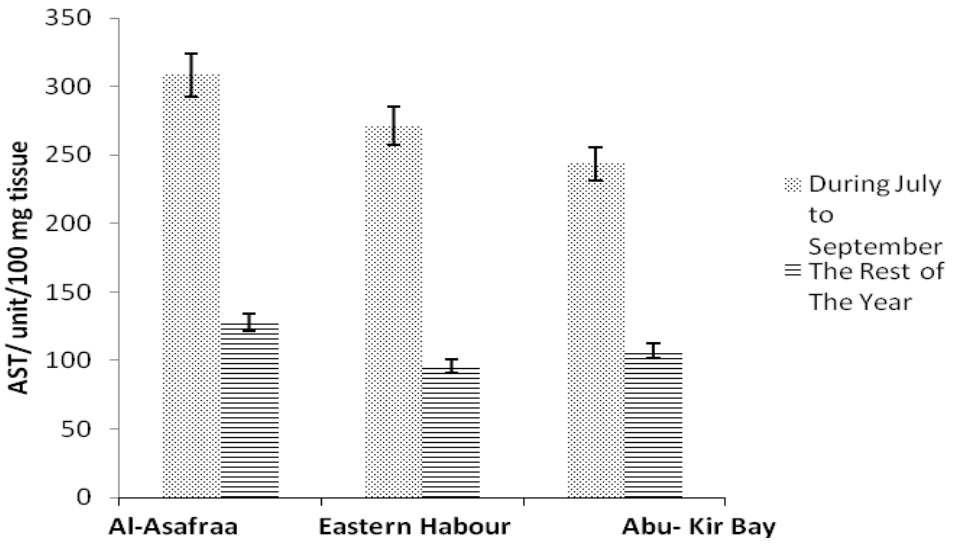

Figure 18. Histogram showing the level of aspartate aminotransferase (AST) in the tissues of *Ciona intestinalis* obtained from the three studied locations, Eastern harbour, Al- Asafraa coatal water and Abu- Kir Bay. The level of aspartate aminotransferase (AST) obtained from the tissues of *Ciona intestinalis* in Abu- Kir Bay was non-significantly decreased than those obtained from Al-Asafraa. The level of AST obtained from the tissues of *Ciona intestinalis* in Abu- Kir Bay was significantly decreased (*P<0.001*) than those obtained from Eastern harbour. The level of AST obtained from the tissues of *Ciona intestinalis* in Eastern harbour was significantly increased (*P<0.05*) than those obtained from Al-Asafraa.

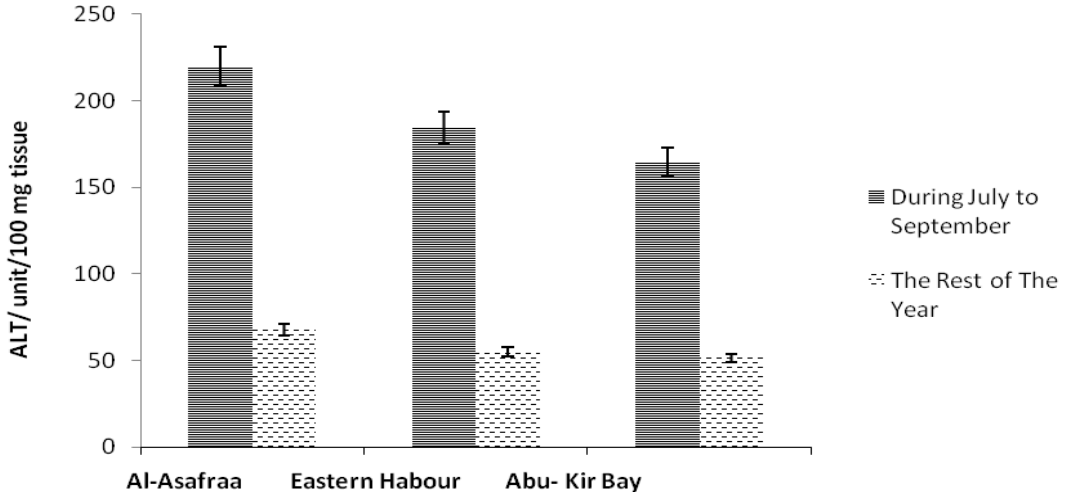

Figure 19. Histogram showing the level of alanine aminotransferase (ALT) in the tissues of *Ciona intestinalis* obtained from the three studied locations, Eastern harbour, Al- Asafraa coatal water and Abu- Kir Bay.The level of alanine aminotransferase (ALT) obtained from the tissues of *Ciona intestinalis* in Abu- Kir Bay was non-significantly decreased than those obtained from Al-Asafraa.The level of alanine aminotransferase (ALT) obtained from the tissues of *Ciona intestinalis* in Abu- Kir Bay was significantly decreased (*P<0.01*) than those obtained from Eastern harbour. The level of alanine aminotransferase (*ALT*) obtained from the tissues of *Ciona intestinalis* in Eastern harbour was significantly increased (*P<0.05*) than those obtained from Al-Asafraa.

was 184.4 ± 5.4, while in July to September it was 54.88 ± 2.7). It should be noted that ALT levels across all areas were higher in the rest of the year than in July to September (Figure 19).

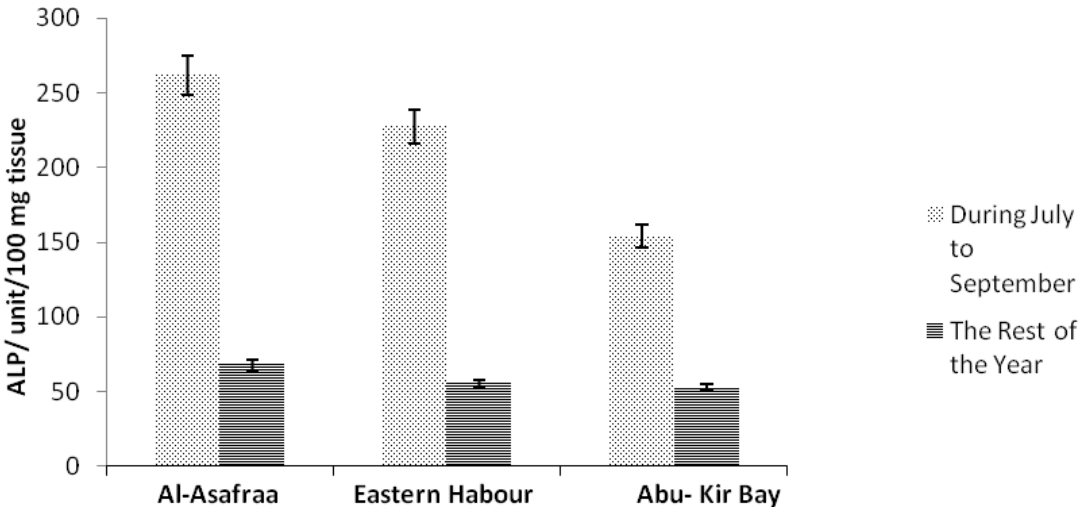

Figure 20. The level of alkaline phosphatase (ALP) in the tissues of *Ciona intestinalis* obtained from the three studied locations, Eastern harbour, Al- Asafraa coastal water and Abu- Kir Bay. The level of alkaline phosphatase (ALP) obtained from the tissues of *Ciona intestinalis* in Abu- Kir Bay was significantly decreased (*P<0.05*) than those obtained from Al-Asafraa. The level of alkaline phosphatase (ALP) obtained from the tissues of *Ciona intestinalis* in Abu- Kir Bay was significantly decreased (*P<0.01*) than those obtained from Eastern harbour. The level of alkaline phosphatase (ALP) obtained from the tissues of *Ciona intestinalis* in Eastern harbour was non-significantly increased than those obtained from Al-Asafraa.

The level of alkaline phosphatase (ALP) in tissues of *C. intestinalis* recorded its highest level in *Al-Asafraa* (Mean ± SE in the rest of the year was 261.6 ± 18, while in July to September it was 67.8 ± 3.8). On the other hand, Western Harbour (Abu- Kir Bay) recorded the least ALP levels (Mean ± SE in the rest of the year was 153.8 ± 25.6, while in July to September it was 52.7 ± 3.41). Again, *Eastern harbor* recorded medium levels between the other two areas (Mean ± SE in the rest of the year was 227.5 ±23.6, while in July to September it was 55.28 ± 2.7). It should be noted that ALP levels across all areas were higher in the rest of the year than in July to September (Figure 20).

The level of lactate dehydrogenase (LDH) in tissues of *Ciona intestinalis* recorded its highest level in *Al-Asafraa* (Mean ± SE in the rest of the year was 60.13 ± 3.5, while in July to September it was 54.38 ± 1.8). On the other hand, Western Harbour (Abu- Kir Bay) recorded the least LDH levels (Mean ± SE in the rest of the year was 26 ±1.6, while in July to September it was 40 ± 1.03). Again, *Eastern harbor* recorded medium levels between the other two areas (Mean ± SE in the rest of the year was 45.75 ± 3.4, while in July to September it was 45.5 ± 2.31) (Figure 21).

The total protein content in tissues of *Ciona intestinalis* recorded its highest level in Al-Asafraa (MeanMean ± SE in the rest of the year was 60.13 ± 3.5, while in July to September it was 54.38 ± 1.8). On the other hand, Western Harbour (Abu- Kir Bay) recorded the least LDH levels (Mean ± SE in the rest of the year was 26 ± 1.6, while in July to September it was 40 ± 1.03). Again, *Eastern harbor* recorded medium levels between the other two areas (Mean ± SE in the rest of the year was 45.75 ± 3.4, while in July to September it was 45.5 ± 2.31) (Figure 22).

Salinity level (S %) in the sea water obtained from Al Asafraa is the lowest concentration (325.2 ± 1.138 during July to September and the rest of the year). Eastern harbour showed concentration (350.8 ± 1.231 during the rest of the year and 349.2 ± 1.400 during July to September). The largest concentration is recorded in the Western harbour (Abu- Kir Bay) as (400.8 ± 1.509 during the rest of the year and 366.3 ± 1.174 during July to September) (Figure 23).

Hydrogen ion concentration (pH) in the sea water obtained from Al Asafraa is the lowest concentration (7.62 ± 1.106 during the rest of the year and 7.02 ± 0.106 during July to September and the rest of the year). Eastern harbour showed concentration (8.4 ± 0.231 during the rest of the year and 7.9 ± 1.100 during July to September). The largest concentration is recorded in the Western harbour (Abu- Kir Bay) as (8.8 ± 1.209 during the rest of the year and 8.1 ± 1.674 during July to September (Figure 24).

It was found that there is no significant difference in temperature of the sea water obtained from these three locations in the two seasons (Figure 25).

DISCUSSION

Gradual increases of heavy metals in water ecosystems

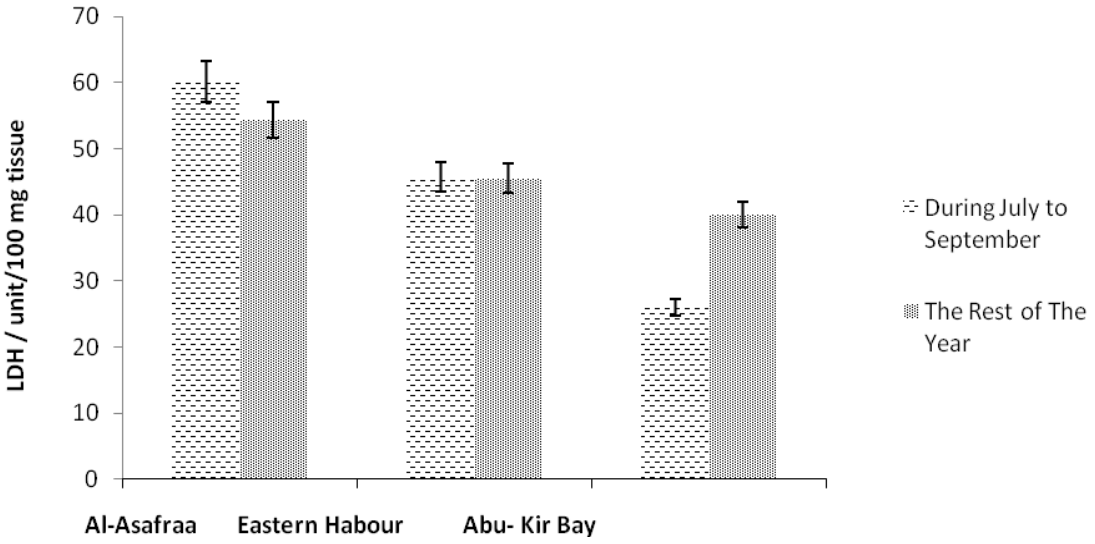

Figure 21. The level of LDH activity in the tissues of *Ciona intestinalis* obtained from the three studied locations, Eastern harbour, Al- Asafraa coastal water and Abu- Kir Bay. The level of LDH activity obtained from the tissues of *Ciona intestinalis* in Abu- Kir Bay (b) was significantly decreased (*P<0.001*) than those obtained from Al-Asafraa. The level of LDH activity obtained from the tissues of *Ciona intestinalis* in Abu- Kir Bay was significantly decreased (*P<0.001*) than those obtained from Eastern harbour. The level of LDH activity obtained from the tissues of *Ciona intestinalis* in Eastern harbour was significantly increased (*P< 0.01*) than those obtained from Al-Asafraa.

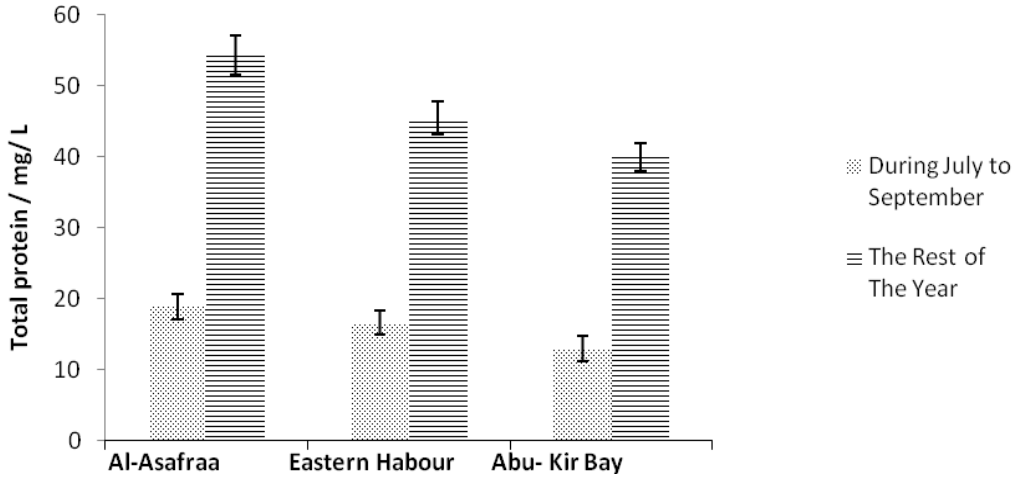

Figure 22. Histogram showing the total protein content in the tissues of *Ciona intestinalis* obtained from the three studied locations, Eastern harbour, Al- Asafraa coastal water and Abu- Kir Bay. The level of the total protein content obtained from tissues of *Ciona intestinalis* in Abu- Kir Bay was significantly decreased (*P<0.01*) than those obtained from Al-Asafraa. The level of the total protein content obtained from the tissues of *Ciona intestinalis* in Abu- Kir Bay was significantly decreased (*P<0.001*) than those obtained from Eastern harbour. The level of the total protein content obtained from the tissues of *Ciona intestinalis* in Eastern harbour was significantly increased (*P<0.05*) than those obtained from Al-Asafraa.

allow some organisms to adapt to metal concentration. The danger of heavy metals in marine environment is driven by their persistence, toxicity, and the remarkable degree of concentration. They pass through the trophic chain, thus becoming a serious danger to man. The presence of heavy metals in high concentrations causes a great danger to all marine filter feeder organisms**.** The Mediterranean Sea is surrounded by 18 countries from

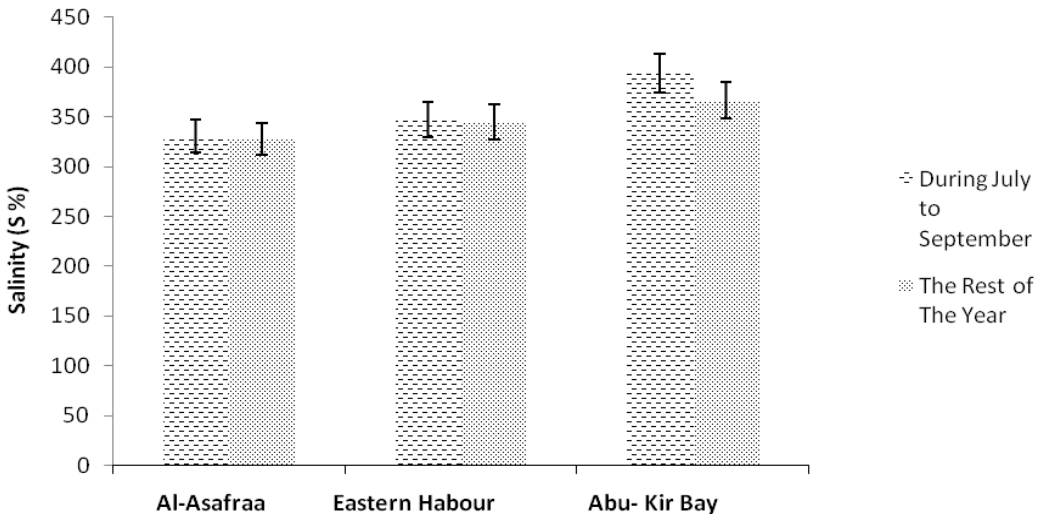

Figure 23. Histogram showing the level of salinity (S %) in the sea water obtained from the three studied locations, Al-Asafraa, Eastern harbour and Abu- Kir Bay in July to September and the rest of the year. The level of salinity (S %) in the sea water obtained from Abu- Kir Bay was significantly increased (*P<0.001*) than those obtained from Al-Asafraa. The level of salinity (S %) in the sea water obtained from Abu- Kir Bay was significantly increased (*P<0.001*) than those obtained from Eastern harbour. The level of salinity (S %) in the sea water obtained from Eastern harbour was non-significantly decreased than those obtained from Al-Asafraa.

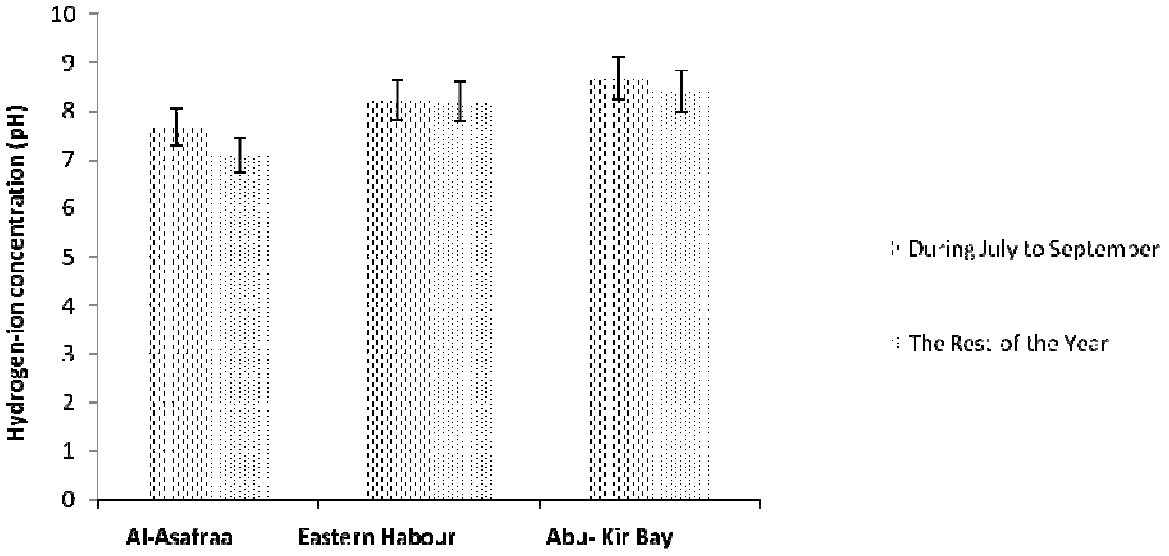

Figure 24. Histogram showing the level of hydrogen-ion concentration (pH) in the sea water obtained from the three studied locations, Al-Asafraa , Eastern harbour and Abu- Kir Bay in July to September and the rst of the year.The level of hydrogen-ion concentration (pH) in the sea water obtained from Abu- Kir Bay was significantly increased (*P< 0.01*) than those obtained from Al-Asafraa.The level of hydrogen-ion concentration (pH) in the sea water obtained from Abu- Kir Bay was significantly increased (*P<0.001*) than those obtained from Eastern harbour. The level of hydrogen-ion concentration (pH) in the sea water obtained from Eastern harbour was significantly decreased (*P<0.001*) than those obtained from Al-Asafraa.

three continents, that is, Europe, Africa and Asia (UNEP, 2002). Intense human activities from these countries produce a strong environmental impact in form of marine degradation (EEA, 2005), and cause heavy metal stress in the Mediterranean waters mainly through discharging different sources of pollutants in the coastal waters

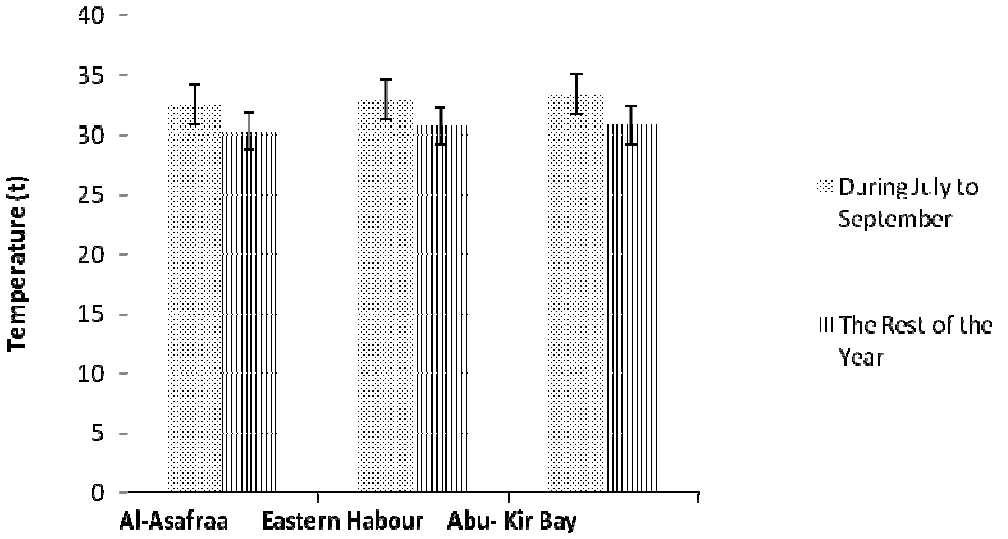

Figure 25. Histogram showing the level of temperature (t) in the sea water obtained from the three studied locations, Al-Asafraa , Eastern harbour and Abu- Kir Bay in July to September and the rest of the year. Notice, the relation and the significant difference between the three studied locations in July to September and the rest of the year. Note, there is no significant difference in the temperature of the sea water obtained from these three locations in the two seasons.

(Dellaporte et al., 1983). The Egyptian Mediterranean coast has been influenced by untreated urban and industrial effluents that caused coastline degradation (EEA, 2005; NDA, 2003), particularly in Alexandria coast due to the high population growth and rapid development (EEA, 2005). It is worthy to mention that the irregular discharge of drainage water leads to variation in heavy metals concentrations (El-Shebly, 1994; Barlas, 1999; Otsvik et al., 2000; El Gendy et al., 2003). RAPD polymorphism results from using, generally, 10 bp synthetic primers of random sequence. These oligonucleotides serve to amplify fragments from (3-10) genomic sites simultaneously (Williams et al., 1990). One of the most important features of the RAPD techniques is detecting high levels of polymorphism and this feature has been met in the present study, where five primers were screened with the DNA of *Ciona intestinalis*, out of them, two primers generated reproducible RAPD profiles. In the present study, the number of the polymorphic fragments ranged from 5-15. Thirty-six 10 mer arbitrary primers were used to detect RAPD markers in mutants of lemon. It is reported that twenty-two of the tested primers generated polymorphic profiles (Deng et al., 1995). The accuracy of genetic similarity estimates on molecular data depends on several variable factors; such as the number of markers analyzed, their distribution over the genome and the accuracy in scoring the markers (Schut and Stam, 1997). The number and size of amplification products on the complementing of sequences of the particular primer and template DNA is more or less similar (Williams et al., 1990). The banding pattern of DNA depends on the frequency of annealing sites for the primers used on the effective concentration of such primers in the reaction tube (Virk et al., 1995). Amplified fragment length polymorphism analysis is a relatively noval technique based on the selective PCR amplification of restriction fragments from a total digested genomic DNA (Coulibaly et al., 2003). Differences between genotypes are either direct or indirect representations of differences at the DNA level and are, therefore, expected to provide information about genetic relationships (Radwan et al., 2005). The range of metal concentrations is generally below acute thresholds in coastal areas where recognition of chronic sub-lethal effects is more relevant. Experimental studies were carried out on *Mytilus galloprovincialis* exposed in aquarium (5 days) to different concentrations of Cd Cl_2. Cd induced increase of DNA damage (Bolognesi et al., 1999). Mussels are sensitive towards genotoxins and are widely used in biomonitoring programmes (Viarengo et al., 198; Viarengo and Canesi, 1991; Gagné et al., 2002; O`Connor, 2002; Shi et al., 2004). Exposure of mussels in the field to water polluted by different mixtures of genotoxic contaminants induces DNA alterations (Izquierdo et al., 2003). The response of genotoxicity biomarkers showed a good discriminatory power allowing the identification of stations along a pollution gradient, and confirmed the sensitivity of these methods for coastal biomonitoring (Bolognesi et al., 2004). They suggested that mussel's bioaccumulate higher concentrations of persistent pollutants, such as heavy metals, and express higher genetic damage frequency as a result of time integrated response to cumulative exposure. Caged mussels showed higher strand breaks probably as the

result of very recent exposure to genotoxic agents. *C. intestinalis* have a very similar genetic make-up from different locations along the coast, (as estimated from RAPD profiling), suggesting very little genetic variation and extensive genetic exchange. This work indicates the importance of the study of the amount and distribution of genetic diversity for a better exploration of *C. intestinalis* genetic resources.

Heavy metals accumulation in seawater of the coast of Alexandria is generally high especially in the western harbour. There are high metal concentrations in the tissues of investigated ascidians in all locations. Cu and Zn accumulation in the tissues is more or less constant and do not differ much between locations. It is well known that many cytotoxic compounds of therapeutic interest have been extracted from marine invertebrates such as pyridoacridine alkaloids from the ascidian *Cystodytes dellechiajei* and aplidin from the ascidian *Aplidium*: These substances are anti-tumorigen and against Alzheimer disease. So it is very important to protect these animals all over the world.

Conclusion

Hazardous accumulation of heavy metals in the different tissues of the marine fouling ascidian *C. intestinalis* alters the molecular characterization proteins and exerts harmful effects. The ascidian *C. intestinalis* enters the food chain and thus the different heavy metals accumulated in the tissues will be reached to man (secondary consumer). Efforts must be exerted to solve the biological problem of marine pollution.

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