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Effect of Phosphate Ore on Biochemical Compositions in Muscle Tissues for some Species of Red Sea Fishes

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Abstract: *The aim of the present research was to evaluate the biochemical alterations induced by phosphate ore shopping operations in marine ports, and its effects on the quality of fish, the bioassay was estimation the change of total DNA level and protein contents. The research were carried out in two sites in Red Sea area, El-Hamrawein port (site 1), represents the affected area with phosphate shipping operations, and El-Shalateen (site 2) as control area. DNA and protein contents in muscle tissues of three species of Red Sea fishes (Lethrinus borbonicus, Siganus rivulatus* and *Mulloidichthys flavolineatus, were estimated by using inter-simple sequence repeats (ISSRs) and SDS-PAGE markers. Results illustrated that, the DNA content in muscle tissues of the three species showed distinct changes, Siganus rivulatus and Mulloidichthys flavolineatus were revealed decrease in DNA level, whereas Lethrinus borbonicus was showed slight increase in DNA level compared to those of the control, and it showed that the muscle protein contents significantly decreased over the control in all tested species of fishes. These results revealed that, phosphate shipment operations in ports result in genotoxic effects on marine livings.*

Keywords: *Phosphate ore, Red Sea fish, muscle, DNA, protein, ISSR and SDS*

I. Introduction

The world suffers from deficiency of protein sources. Fish considered one of important sources of animal protein. Fish contain lipids also, which supply the body with energy and essential fatty acids that are necessary for life and play an important role in regulation of the cardiovascular system and for reducing cholesterol level in the blood. Moreover, fish are rich in fat-soluble vitamins, iodine and phosphorous (Mohammed, 1999). Humans today have a strong influence on almost every major aquatic ecosystem, and their activities have dramatically altered the quality of receiving waters worldwide. Thus, there is a continuous need to develop and apply effective technologies to detect, and correct human-induced degradation of aquatic systems (Rodriguez *et al.*, 2007). Pollution is the changes that occur in chemical, physical and biological characters of the environmental system. Environmental contamination of air, water, soil and food have been still the most important subject now, because it causes threat extend to many plants and animals and may ultimately threaten the survival of humanity (Salem, 2003). Phosphate ore is one of environmental contaminants that is commonly polluted the aquatic environment. It reaches the aquatic environment of Red Sea through phosphate shipment operations ports along the Egyptian Red Sea coast. Studying the effect of phosphate shipping operations in El-Hamrawein port is a very important subject due to the damage influences of phosphate shipping operations on the marine fauna in this region. Phosphate loading operations at El-Hamrawein port form immense clouds of dust directly fall in the water (McMurtry *et al.*, 2007). This dust is leading to contaminating in fish's environment. This study have been undertaken to understand the biochemical alterations by phosphate ore on exposure to sub-lethal concentrations on some fishes in muscle tissue exposed, from El-Hamrawein port area, and its compare to the control species from Al-Shalateen area, and the calculated values for total proteins and percent changes over control. The effect of phosphate ore on fish were selected for this study because, the phosphate shipping operations used in many ports in Egypt and the fish is consumed by people as staple diet (Randall and Heemstra, 2009).

II. Material and methods

Study area:

El-Hamrawein port is one of the old phosphate shipping ports on Egyptian Red Sea coast which was represented investigated area, it is located about 20km north of Quseir City. El-Shalateen City as a control area, it is located about 380km south of Quseir City.

Sample collection and DNA extraction:

To study the effect of phosphate ore on the Red Sea fish, three species of Red Sea fishes ((*Lethrinus borbonicus*, *Siganus rivulatus* and *Mulloidichthys flavolineatus*) were selected as an experimental animals. Five males of healthy fishes were chose of each species. The body weight of the samples are ranging between 100-120mg and total length of 20-25cm, the tested species were fished from two sites, El-Shalateen area as a control site and El-hamrawin area as investigated site. Muscles were stored at -20 ºC until DNA extraction, DNA was extracted used DNeasy Kit Qiagen protocol.

Inter simple sequence repeat polymerase chain reaction (ISSR-PCR) amplification:

DNA of each species of the fishes was amplified using thirty primers, out of which nineteen produced ISSR implicons (Table 1).

Table1: Characteristics of selected ISSR primers

Primer	Primer sequence (5'-3')
P1	(AG)8YC
P2	(AG)8YG
P3	(AC)8YT
P4	(AC)8YG
P5	(GT)8YG
P6	CGC(GATA)4
P7	GAC(GATA)4
P8	(AGAC)4GC
P9	(GATA)4GC
P10	(GACA)4AT
P11	(TA)10G
P12	(AC) 9T
P13	(AC) 9C
P14	(GA) 9A
P15	(GA) 9T
P16	(GA) 9C
P17	(TA) 5GT
P18	(CA) 6T
P19	(CA) 8A

PCR amplifications were performed in 25µL of reaction mixture containing 30ng of genomic DNA, 1 U Taq DNA polymerase, 1µM primer, 0.2µM dNTP, and 1X PCR buffer (containing 1.5mM $MgCl_2$). Amplifications were carried out in a thermal cycle. According to manufacture instructions as follow: the initial denaturation temperature 94ºC for 5 min, followed by 40 cycles denaturation at 94ºC for 1 min, then an annealing step at 36ºC for 1 min, and extension at 72 ºC for 1.5 min. Followed by a final extension step for 7 min at 72 ºC. Amplification products were separated by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5µg/ml) in 1X TBE buffer at 95 volts, a 1kb DNA ladder was used as a molecular size standard.

Data analysis:

The banding patterns generated by ISSR-PCR marker analysis compared to determine the genetic variation of the samples under study. Distinct amplification products scored as '1' for presence and '0' for absence of bands.

Estimation of total protein SDS-PAGE

The SDS-PAGE was performed to analyze protein profile in muscle of control and phosphate ore exposed tissues in the three tested species of fishes on the base of molecular weight by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Sharaf-Eldeen *et al*., 2006). Total protein content from muscles of three species of Red Sea fishes were estimated by SDS-PAGE. Pellets were collected by centrifugation at 12000 rpm at 4 ºC, washed once with distilled water, then with 1 ml of 1mM NaCl containing 5mM EDTA. Then, it was heated in the present of low molecular weight thiol (2-meracoptoethanol) and SDS denatured total cellular protein from fish's muscle cells. One volume of the cell suspension was mixed with one volume of 2X treated buffer (0.25M Tris-HCL PH 6.8, 4% SDS, 20% glycerol and 10% 2- meracoptoethanol) and boiled in a water bath for 90 s then quickly transferred to ice water and kept until loading the gel. After electrophoresis, the gel was stained in 50ml of staining solution (0.125% Coomassie blue R-250, 50% methanol and 10% acetic acid). The presence or absence of each band was treated as binary character in a data matrix that is, coded 1 and 0, respectively.

III. Result

Inter-simple sequence repeats (ISSR) amplification

Photos (1, 2 and 3) represented the produced banding patterns by using of ISSR techniques on three species of Red Sea fishes. Tables (2, 3 and 4) represented DNA bands and percentage of the total polymorphism.

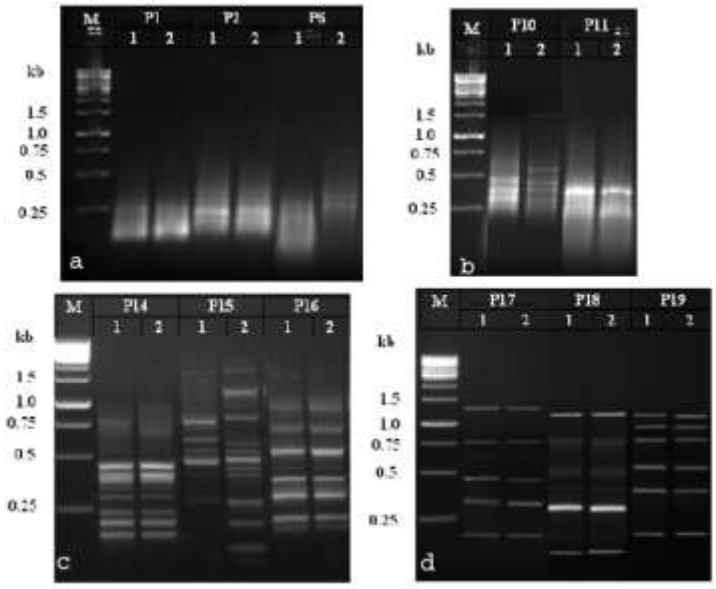

Photo 1. The produced DNA profiles of muscles of *Lethrinus borbonicus* fish using ISSR amplification technique, generated by P1, P2, P6, P10, P11, P14, P15, P16, P17, P18 and P19 primers, codes are in lanes 1is the control and 2 is the exposure sample, M =DNA Marker

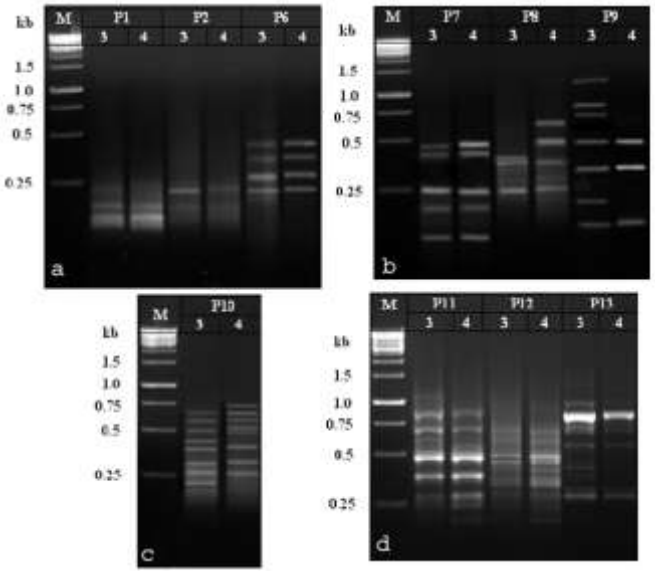

Photo 2. The produced DNA profiles of muscles *of Siganus rivulatus* fish using ISSR amplification technique, generated by P1, P2, P6, P7, P8, P9, P10, P11, P12, and P13 codes are in lanes 1 is the control and 2 is the exposure sample, M =DNA Marker

PCR-based techniques, such as ISSRs, have previously allowed the discrimination and estimation of change in genetic material to genotoxic elements.The exposure animals to the pollutant areas by genotoxic. Agents will give rise to alterations in DNA structure that can lead tohappening abnormal changes of DNA fingerprints. Therefore, the authors have applied Inter-simple sequence repeats (ISSRs) method to evaluate the genotoxic effects in muscle tissues of fish that exposed to phosphate ore dust in their environments.

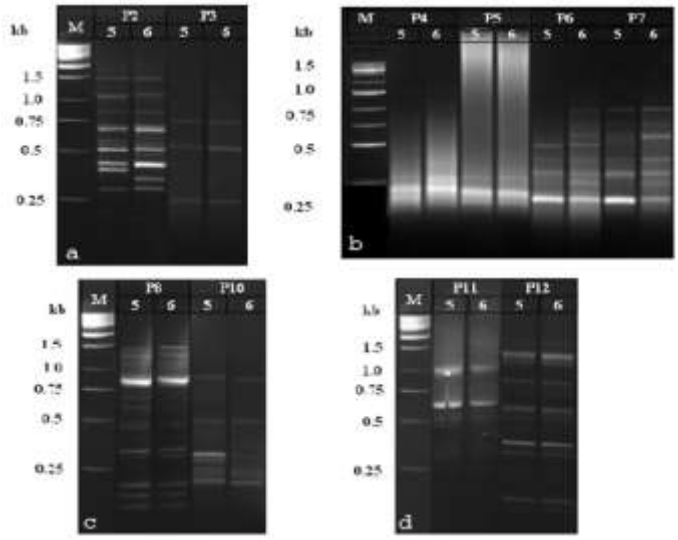

Photo 3. The produced DNA profiles of muscles *of Mulloidichthys flavolineatus* fish using ISSR amplification technique, generated by P2, P3, P4, P5, P6, P7, P8, P10, P11and P12 codes are in lanes 1 is the control and 2 is the exposure sample, M =DNA Marker.

Table 2: ISSR amplicons of *Lethrinus borbonicus* sample

Primer	Total bands	Polymorphic bands	Polymorphism %	Amblicon size range (kb)
P1	6	0	0	0.2- 0.24
P2	6	0	0	0.23- 0.26
P6	8	2	25	0.2- 0.3
P10	22	4	18.2	0.24- 1.5
P11	7	1	14.3	0.24- 0.37
P14	22	2	9.1	0.2- 1.0
P15	19	7	36.8	0.2- 1.7
P16	19	1	5.3	0.2- 1.0
P17	10	0	0	0.2- 1.7
P18	16	0	0	0.2- 1.2
P19	16	0	0	0.23- 1.2
Sum.	151	17	11.3	

Table 3: ISSR amplicons of *siganus rivulatus* sample

Primer	Total bands	Polymorphic bands	Polymorphism %	Amblicon size range (kb)
P1	8	2	25	0.2 - 0.27
P2	8	0	0	0.21- 0.27
P6	9	1	11.1	0.23- 0.5
P7	10	0	0	0.21- 0.4
P8	8	4	50	0.22-1.5
P9	13	5	38.5	0.22- 0.4
P10	21	5	23.8	0.22- 0.75
P11	26	2	7.7	0.24- 1.25
P12	21	1	4.8	0.2- 1.0
P13	18	0	0	0.27- 1.2
Sum.	142	20	14.1	

The molecular biological results of this study revealed that, phosphate ore was able to induce DNA fragmentation in tested species muscles (Table 2, 3 and 4). The PCR products generated from ISSR analysis were used to estimate the genetic changes between investigated fishes and its control. Nineteen primers were selected from thirty primers were used (Table 1). A total of 463 clear and distinguishable ISSR bands (151 bands for *Lethrinus borbonicus* species, 142 for *Siganus rivulatus* and 170 for *Mulloidichthys flavolineatus*) were generated by the nineteen ISSR primers that used (Table 1), 11.3 %, 14.1 % and 5.9% are Polymorphic bands scored for the nineteen primers in the three species of fishes respectively. Polymorphic bands ranged from 1 to 7 per optimized Primer, and the PCR products ranged from 0.2 kb to1.7 kb. The number of bands per primer, showed variant range among the three species.

Table 4: ISSR amplicons of *Mulloidichthys flavolineatus* sample.

Primer	Total bands	Polymorphic bands	Polymorphism %	Amblicon size range (kb)
P2	22	2	9.1	0.28-1.5
P3	8	0	0	0.25- 0.7
P4	11	1	9.1	0.16 - 0.4
P5	10	0	0	0.15 – 0.4
P6	14	2	14.3	0.15 - 0.7
P7	18	2	11.1	0.16 -1.0
P8	28	0	0	0.15 - 1.5
P10	19	3	15.8	0.22- 0.8
P11	16	0	0	0.3- 1.0
P12	24	0	0	0.15- 1.5
Sum	170	10	5.9	

In *Lethrinus borbonicus* species, ranged from 6 to 22, in *Siganus rivulatus* species, ranged from 8 to 26 bands and in *Mulloidichthys flavolineatus* observed bands ranged from 8 to 28. Primers 6, 10, 11, 14, 15 and 16 amplified polymorphic markers for *Lethrinus borbonicus* species, primers 1, 6, 8, 9, 10, 11 and 12 revealed polymorphic markers for *Siganus rivulatus* species and primers 2, 4, 6, 7 and 10 amplified polymorphic markers for *Mulloidichthys flavolineatus* in (Tables 2, 3 and 4 respectively).

Primers P10 and P14 was generated the highest number of bands (22 bands) for the species *Lethrinus borbonicus*, P11was generated (26 bands) for the species *Siganus* rivulatus and P8 (28 bands) for the *Mulloidichthys flavolineatus* (Photos 1, 2 and 3). The highest percentage of polymorphism (36.8 %, 50 % and 15.8 %) for the species *Lethrinus borbonicus*, *Siganus rivulatus* and *Mulloidichthys flavolineatus* respectively, which were generated from the primers P15, P8 and P10 respectively. The number of polymorphic bands produced per primer for *Lethrinus borbonicus* ranged between one (P11 and P16) to seven (P15). In the species *Siganus rivulatus* ranged from one (P6 and P12) to five (P9 and P10), and for the species *Mulloidichthys flavolineatus* ranged from one (P4) to three (P10) out of 17, 20 and 10 polymorphic bands for *Lethrinus borbonicus*, *Siganus rivulatus* and *Mulloidichthys flavolineatus* respectively. The profiles generated by ISSR primers showed polymorphism among the control and the investigated species from polluted environment with phosphate ore of the three species of fishes. The extent of the polymorphism varied from species to the other, the percentage polymorphic bands calculated to be 11.3%, 14.1% and 5.9% for *Lethrinus borbonicus*, *Siganus rivulatus* and *Mulloidichthys flavolineatus* respectively.

The present biochemical study showed that phosphate-loading operations have a strong influence on the chemical structure of DNA of the fish, which may be influence on the quality of the fish.

SDS-PAGE analysis:

The product SDS-protein profiles of the three species shown in (Photo. 4). A maximum number of 117 bands detected at approximately molecular weights ranging between 11 KDa and 400 KDa. The protein in muscles profile showed distinct polymorphism among the three species compared to its controls. The electrophoretogram (Photo. 4) represents decrease in the intensity of protein subunits of the three species exposure tissue samples compared to their controls. The proportion for the decrease in protein subunits of muscles are variant between the tested fishes, *Lethrinus borbonicus* showed more decrease proportion in muscle protein subunits (14.3%) than *Mulloidichthys flavolineatu*s and *Siganus rivulatus* samples (9.8% and 6.5%) respectively. After electrophoresis of cellular muscle proteins, 35 bands were observed in *Lethrinus borbonicus* species, by comparison of molecular weight to control sample five bands were disappeared in exposure phosphate ore tissue, at molecular weights (265, 240, 135, 125 and 18 KDa). Whereas, in *Mulloidichthys flavolineatus* 51 protein subunit bands were produced through the SDS-PAGE analysis, with seven polymorphic bands compared to the control, five bands were disappear in exposure sample at molecular weight (400,125, 120, 18 and 14 KDa) and two new bands were appeared at (135 and 16 KDa) molecular weight, as indicated by the decrease of band intensity. In *Siganus rivulatus* species, 31 bands observed with two polymorphic bands, the two bands were disappeared at molecular weight (400 and 17 KDa) in phosphate exposure samples. These results indicate that the phosphate ore may be toxic, and Pollutant marine environment with phosphate ore lead to change in protein content of the fish muscles, this study showed that there are disappearance of some protein subunits in fishes and sometimes appears new subunits compared to the control.

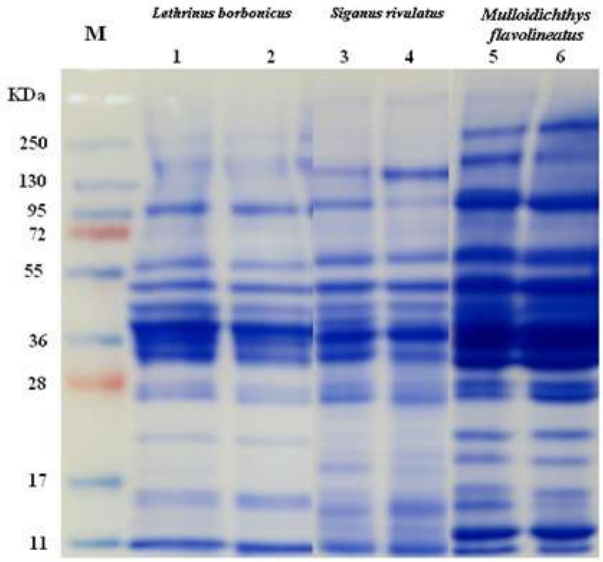

Photo 4: Changes in protein subunits in muscle tissues of the three species of fishes, exposed to phosphate ore, *Lethrinus borbonicus*, *Siganus rivulatus* and *Mulloidichthys flavolineatus*, using SDS-PAGE technique. Lanes 1, 3 and 5 Muscle controls and 2, 4 and 6 phosphate ore exposed respectively for the three species.

Because of Phosphorus is an essential element of the structure of DNA. Phosphorus at different energy states is essential to the operation of metabolism, and catabolism. The present study showed that, both SDS-protein and ISSR primers revealed that, the tested fish represented various degree of sensitivity to the pollutant environment with phosphate ore that indicate genetic damage, this appeared by variations in averages of DNA and protein fragments among species compared to the controls.

IV. Discussion

Nowadays, pollution of aquatic environments is widespread problems. Scanty information is available on molecular and biochemical alteration produced in DNA structure and protein content of exposed fish to phosphate ore in there aquatic environment. The aim of this study was to determine the effect of pollution with phosphate shipping operations on Red Sea fish that live near Phosphate loading operations at ports. The authors were selected three species of Red Sea fishes belonging to three families to achieve this research and they were choose two sites, El-Hamrawin area as observation site and El-Shalateen area as a control site. The present study represents the first study achieved to determine influence of phosphate shipping operations in port environments on fish in Egypt.In general, results of the present study revealed that, phosphate ore induced variant effect in DNA and decrease in protein content in muscle tissues of the three tested species. The decrease of DNA level was statistically significant in muscle tissues of two Red Sea fish, *Siganus rivulatus* and *Mulloidichthys flavolineatus* under the effect of phosphate components, but *lethrinus borbonicus* fish showed slight increasing in DNA level. This explanation may be supported by the finding of (Thenmozhi *et al*., 2011) stated that, organophosphate pesticide was affected on nucleic acid contents in different tissues of muscles, liver, and gill, which showed significant decrease in the DNA and RNA content. Our results agree with the finding of (Nagaraju and Rathnamma, 2013) they also added freshwater fish *Labeo rohita* under the exposure to profenofos (an organophosphorus) showed decrease in the DNA content in muscles. And this is in accordance with (Altman *et al*., 1972) who demonstrated that, the decrease in DNA content may be due to reduction the essential factors controlling DNA synthesis which are the substrates (4-Deoxyribonucleosude triphosphat), enzymes (polymerase) template activity of deoxyribonucleic-protein and activators like Mg2+ and other divalent ions. Recently this experimentally shown by (Nagaraju and Rathnamma, 2013) who reported, this is possible that the enzyme necessary for DNA synthesis might have been inhibited by phosphate component.

Moreover, (Rathnamma and Nagaraju, 2013) reported that, the DNA content in fish muscle and brain were decreased under exposure to sublethal concentration of quinalphos (an organophosphorus). Our results revealed that, phosphate ore compositions caused variability effects in the DNA content in different muscle tissues of the three tested species of fishes, where it was concluded the decrease in DNA level of two species *Siganus rivulatus* and *Mulloidichthys flavolineatus* but increase in *lethrinus borbonicus* species. In (1981, Sastry and Sharma), reported that, organophosphate inhabit acid phosphatase and alkaline phosphatase activity in different tissue of fishes which may adversely affect nucleic acid synthesis.Our study was revealed that the exposed *lethrinus borbonicus* species to phosphate ore compositions showed an increase in DNA content of

muscle tissue compared with control species, that in agreement with (Mustafa, 1977) who reported that, there was an increase in DNA content of muscle tissue in *Clarias batrachus* under the effect of organophosphate.
In (2000, Das and Mukherjee), reported that, DNA levels were elevated in Indian *Labeo rohita* tissues when exposed to quinolphos (an organophosphate) for 15, 30 and 45 days, this result was in agree with our result of *lethrinus borbonicus* species. They added the alteration in DNA levels may be due to the disturbances in the normal synthesis and turnover rate of DNA besides degenerative changes. Overall, variation of change in DNA structure as decreased or increased values noted in DNA in muscle tissue of exposed fish to phosphate ore. Searches' regarding the effect of phosphate ore on food value of fish is scanty. Therefore, this research was undertaken to investigate the effect of phosphate ore on certain biochemical by estimation the DNA and protein contents in muscles of tested species of fishes, where fish forms an important food item of most people in Egypt.

The three species of tested fishes showed distinct decrease in muscle protein under exposure to dust of phosphate shipment operations in El- Hamrawein port compared to its control species, this results agreement with (Borah and Yadav, 1996b and Thenmozhi *et al.,* 2011) who stated that, the pollutant might decrease the synthesis of protein. The decrease in the protein content of exposure fish to phosphate suggests the disruption of protein and protein synthesis machinery and inhibition of ATP synthesis. The changes in biochemical parameters such as DNA and proteins are important to indicate the susceptibility of organ systems to pollutant by altering their function as indicated by (Verma *et al.*, 1983 and Fahmy, 2012). Proteins can be stressed organisms. Our results of the present study showed that, the fish exposure to phosphate ore, the protein content in the muscles found to have decreased. The reduction of protein may be due to proteolysis and increased metabolism under toxicant stress, that agreement with (Venkatramana, *et al*., 2006). Inhibition of DNA synthesis, might affect both protein and amino acid level by decreasing the content of RNA in protein synthesis machinery (Nagaraju and Rathnamma, 2013). Muley *et al.* (2000) and Tripathi and Singh (2003), reported that, the enzyme necessary for DNA synthesis might have been inhibited by the insecticide. On compilation of the results, it appears that the disruption of DNA synthesis might have affected RNA synthesis and consequently protein synthesis; this explanation supported our results especially in the two species, *Siganus rivulatus* and *Mulloidichthys flavolineatu*. The decrease in protein content of exposure fish to pollutant area by phosphate ore indicate the physiological adaptability to the fish to compensate for phosphate stress. To overcome the stress the animal requires high energy, this energy element may be lead to the stimulation of protein catabolism. Several other investigations also revealed a decrease in protein profiles with organophosphorus compounds. All these investigations support the present study of decreasing trend to protein in the muscle tissue of the tested fish exposed to pollutant environment with phosphate shipping operations in El-Hamrawein port.Palanichamy *et al.* (2004) reported that, the low level of protein content estimated in the phosphate compound exposed fishes. This may be due to the pollution stress posted to the fish mobilization protein from muscle to blood, to compensate to certain acidosis caused by the lactate accumulation. Rathnamma and Nagaraju (2013) reported that, the significant decrease in both protein and nucleic acids would suggest that pollutant impair the process of protein synthesis in the tissues of fishes exposed to pesticides organophosphorus.Kavitha and Binukumari (2014) reported that, the biochemical alteration observed in fish *Cirrhins mrigala* under physiological stress can correlated with the structure and functional changes of cellular protein. Decrease in intensity of protein in herbicide glyphoste treated fish *Cirrhins mrigala* that indicates, these proteins were highly study finding that the change in the protein levels are decreased protein degradation and synthesis are sensitive over a wide range of conditions. And (Priya *et al*. 2014) study the sublethal effect of organophosphate detergents on the *Catla catla* and reported that, fishes treated with organophosphate detergents were showed more decreased value of protein in muscle.

In the present investigation, it can concluded that under exposure to phosphate ore, the DNA level showed distinct change in the muscles of fishes, leading to change in protein synthesis and cellular degradation. The present data happens to constitute the first report in Egypt to study the effect of phosphate shipping operations in ports which causing alteration in the DNA and protein content in the marine fish. Therefore, the authors advise peoples to avoid eating from fish of pollutant areas by phosphate ore dust resulted from phosphate shipping operations that may be influence on health human.

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