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The Effect of Phosphate Ore on the Immunomodulatory Activities of Haemocytes of the Marine Snail, *Planaxis sulcatus* (Born, 1778) in the Gulf of Aqaba, Jordan

Saleem Aladaileh, Sulaiman Alnaimat, Amir Alhroob, Abdel-Rahman Al-Tawaha and Nidal Odat

Department of Biology, Al-Hussein Bin Talal University P.O. Box: (20), Ma'an - Jordan

Abstract: Phosphate is one of the main sources of pollution in the Gulf of Aqaba. Our study investigates the effects of phosphate ore on the marine snail, *P. sulcatus* (Born, 1778) immune system. The study site was at the Phosphate loading port (PLP). A total of Three locations were chosen around the phosphate port (L1 an L2) with a control/reference site located about 2 km away, near the marine station (L3). Phenoloxidase and phagocytic activities, total and differential haemocytes frequencies, as well as superoxide production were measured after two weeks of exposure to phosphate pollution. All of the immunology parameters were enhanced significantly ($P<0.05$) except the phagocytic ability which decreased significantly as a result to phosphate exposure. The impact of the phosphate pollution on these immune parameters was correlated to the concentration of pollutant. The most severe impact was within the PLP location followed by L1 and L2. The results obtained suggest that phosphate ore modulates a range of immunological functions in marine snail, *P. sulcatus*. Therefor it is suggested that further monitoring should be conducted using such biomarker.

Key words: *Planaxis sulcatus* · Phosphate ore · Gulf of aqaba · Immune parameters

INTRODUCTION

The global expansion in industrial activities has resulted in an increase in the discharge of chemical waste into the environment [1, 2]. Most of the waste we create eventually ends up in oceans, either through deliberate dumping activities or through wind, water drains and rivers. It has been shown that anthropogenic activities affect a wide range of ecosystems.

The Gulf of Aqaba is one of the world's most biologically diverse environments. Its marine waters and shoreline habitats host some 2,000 species of plants and animals [3, 4]. The gulf measures a length of 180 km and expands to a width of 25 km with 27 km of the shoreline belonging to Jordan. Phosphate is one the main sources of pollution in the Gulf of Aqaba. In 2012, about $5x10^6$ tons of phosphate ore have been exported though the port of Aqaba [5]. It has been estimated that ~ 500 tons/year of phosphate ore are lost as fine particulate matter during the phosphate loading process [3]. It is reported that some of the phosphate does dissolve in the seawater and cause an increase in the concentration of phosphate nutrients in the seawater. It has been found that the Phosphate Port area exhibited the highest total phosphorus concentration [6, 7]. The nutrients increase may cause a phase-shift, from coral to algal dominated communities [8-10]. In addition to that, corals have been significantly affected by siltation from settling phosphate dust and the rate of necrosis in coral colonies have increased significantly in the phosphate polluted areas [10, 11].

Pollution could be detected directly by quantifying the amount of contaminants in the environment [7, 12] or by measuring the direct effect of pollution on micro and macro fauna assemblages. There is evidence that a number of man-made chemicals can cause serious problems for marine species, such as suppression to the immune system, behavioral problems and reduced fertility [13-17].

Among various biochemicals, cellular and physiological systems, innate immune responses are considered perfect biomarkers for monitoring biological

Corresponding Author: Saleem Aladaileh, Department of Biology, Al-Hussein Bin Talal University, P.O. Box: (20), Ma'an–Jordan. Tel: +962-775620563.

effects of pollution [18, 19]. It has been widely accepted that only the innate immune system is present among invertebrates. However, recent studies have shown that some defense systems of invertebrates associated with acquired immunity [20-24].

Despite these recent discoveries, most information on invertebrates pertains to non-specific, innate immune responses. These responses include cellular and humoral responses. Invertebrate cellular defenses are mediated by haemocytes or coelomocytes. Although many studies have characterized invertebrate haemocytes, their classification often remains ambiguous. Haemocytes are usually divided into two types, hyalinocytes and granulocytes. The two types of cells have been identified in various mollusca species, such as *Saccostrea glomerata*, *Scrobicularia plana*, *Anodonta cygnea*, *Meretrix lusoria*, *Argopecten irradians* and *Babylonia areolata* [25-29]. Haemocytes are involved in various defense responses, such as phagocytosis, nodule formation, encapsulation and the production of reactive oxygen intermediates (superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) [30-35]. Humoral responses are also initiated during infections, including the activation of phenoloxidase (PO) and excretion of antimicrobial peptides [36, 37].

Given the clear relationship between contamination and altered immune function in invertebrates, in the present work, effects of phosphate ore on immune activities of haemocytes of the marine snail, *Planaxis sulcatus* were investigated. The changes in the phenoloxidase activity, phagocytosis, superoxide production, total hemolymph protein content and circulating haemocyte frequencies will be measured.

MATERIALS AND METHODS

Study Location and Experiment Design: The study area is located between 29°35′ and 29°5′ N latitudes and between 34°95′ and 35°00′ E longitudes around the phosphate port in the Gulf of Aqaba, Jordan. The study site was at the Phosphate loading port (PLP) (Fig. 1). This port is handling the export of phosphate ore, Diammonium phosphate fertilizer and potash. A total of Three locations were chosen around the phosphate port (L1 and L2) with a control/reference site located about 2 km away, near the marine station (L3) (Fig. 1). The distance between each location was approximately 300 m. From earlier studies on marine pollution levels in the Gulf of Aqaba , the phosphate port has been found to have the highest sedimentation rate, highest organic carbon content and highest total phosphorus concentration [6]. Prior to the beginning of the study, mature snails, were collected about 4 km away from the phosphate port. The snails were brought to the laboratory, placed in large plastic tanks with running filtered seawater (FSW, 25°C) and fed a mixture of algae cells. The mixture contained *Nannochloropsis* sp. and *Tetraselmis* sp. (Reed Mariculture Inc., USA). After 1 week, 40 animals of about similar sizes (~1-1.2cm) were chosen and placed in each of the plastic cages prepared for the exposure study. All locations were chose to reflect the natural habitat of *P. sulcatus.* A nested sampling design was used in this study. To ensure sufficient replication, at each location, two sites were randomly selected within 20 meters of each other and two cages were placed at each site. After 2 weeks, snails were randomly sampled from the cages and brought to the laboratory where the cellular and humoral responses of their innate immune system were determined.

Haemolymph Collection: Each snail shell was cleaned with 70% alcohol and air dried for two minutes. The haemolymph was harvested by a cardiac puncture using a 22-gauge needle fitted to a 5 ml syringe. To avoid cell agglutination, the whole haemolymph was collected and diluted with an equal volume of marine anti-coagulant (MAC; 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 10 mM EDTA, 0.45 M NaCl, pH 7.0). Haemolymph samples were immediately transferred to polypropylene tubes and held in ice.

Phenoloxidase Assays: An assay for phenoloxidase (PO) activity was run according to Peters and Raftos [38]. Phenoloxidase activity in serum or whole haemolymph was measured spectrophotometrically by using the L-3,4-dihydroxyphenylalanine (L-DOPA, ICN, Irvine, CA, USA), as a diphenol substrate and the hydroquinine monomethyl ether (4HA, Fluka, Switzerland) as a monophenol substrate. The chromogenic nucleophile, 3-methyl-2-benzothiazolinone hydrazone (MBTH, Sigma Aldrich) was added to both types of substrate solutions. PO activity was quantified by monitoring the formation of dopachrome. One hundred µl of serum or whole haemolymph (adjusted to 1×10^6 cells ml^{-1}) was added per well to 96 well flat bottom microtitre plates followed by the addition of 100 µl of L-DOPA (4 mg/ml) or 4-HA (5 mM) and 1 mM MBTH. Instantly after the addition of the substrates, the absorbance was measured at 490.0 nm using a microplate spectrophotometer (BioRad, Regents Park, NSW). After that, the Plates were covered and incubated at room temperature for 1 hour before a second

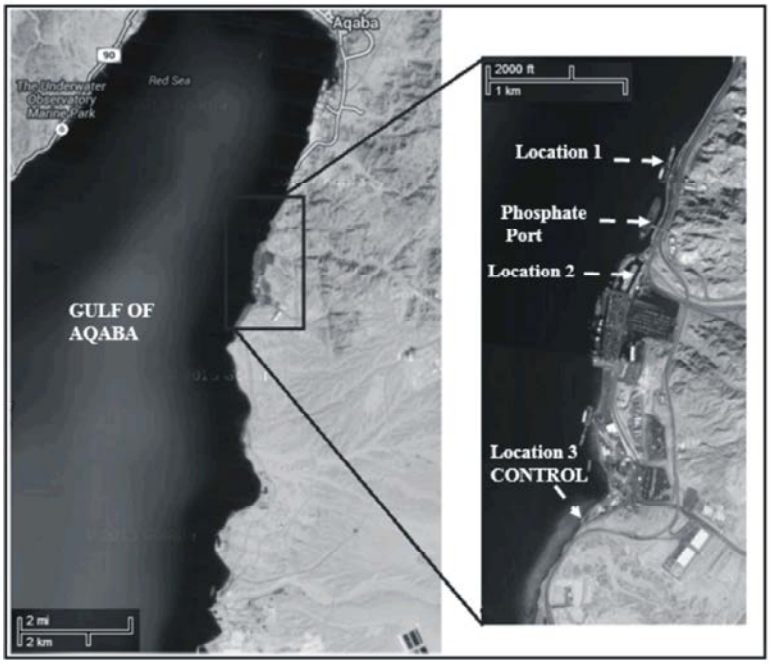

Fig. 1: Map of Gulf of Aqaba showing the three sampling sites around the phosphate port (L1 and L2) and the control site (L3) located about 2 km away, near to the marine station

reading was made. Phenoloxidase activity was determined as the results of the subtraction of the initial readings from 60 min readings. Enzyme activities are expressed as the change in optical density (OD) at 492nm (OD_{492}).

Measurement of Superoxide Production: Nitroblue tetrazolium (NBT) assay was used to determine the production of superoxide anions [39]. One hundred µl of treated and control haemolymph were added to wells of 96 well plates then was followed by the addition of 100 µl NBT (2 mg ml^{-1} in FSW). The plates were overed with aluminum foil and incubated for 1 hour at room temperature. The formation of the blue formazan deposits were stopped by adding 100 µl methanol. After that, the plates were centrifuged at 300×*g* for 10 min. The formazans deposits in each well were washed twice with 70% methanol before being air dried and dissolved in a mixture of 120 µl 2 M KOH and 140 µl DMSO. The absorbance of dissolved formazans deposit was measured at 620 nm using a microplate spectrophotometer.

***In vitro* Phagocytosis Assay:** The phagocytic activity of haemocytes was quantified by microscopic methods of Cooper, *et al*. [40]. The baker's yeast, *Saccharomyces cerevisiae* (Sigma Aldrich) was used as target cells in this assay. Yeast cells (5×10^7 cells ml^{-1} in FSW) were autoclaved in the presence of filtered Congo red (Sigma Aldrich; 0.8% in FSW) for 10 min (120°C). The stained yeast were then washed extensively with FSW and stored frozen (-20°C, $5x10^6$ yeast ml^{-1}). One hundred microliter haemocytes suspensions (1×10^6 cells ml^{-1} in FSW) were incubated on cleaned glass cover slips (22x22 mm) for 30 min in a humidified chamber at room temperature (25°C). The adhered haemocytes were then washed in FSW before being overlaid with 100 µl of Congo red stained yeast ($0.3x10^6$ ml^{-1}) and incubated in a dark moist chamber for 30 min at room temperature(25° C). Non-phagocytosed yeast were removed by extensive washing in FSW. The percentage of haemocytes that had ingested at least one yeast cell was then determined microscopically by inspecting a minimum of 200 haemocytes on each coverslip.

Total and Differential Haemocyte Counts: Total haemocyte counts (THCs) were determined by applying diluted haemolymph (1:1 in MAC) to a Neubauer hemocytometer. Differential haemocyte counts that identified granulocytes and hyalinocytes were calculated using haemocyte monolayers stained with Giemsa. The light microscopy was used to differentiate between haemocyte types depending on the presence or absence of cytoplasmic granules. Two hundred haemocytes were counted and the ratio of two different cell types was calculated.

Total Protein Content of Haemocyte Lysates: Protein concentrations were quantified by the Bradford method using a BioRad protein assay kit (BioRad). Haemocytes were collected by centrifugation at 2000×*g* for 5 min at room temperature. The pellets were then resuspended in 300 µl PBS before being freeze-thawed twice (-80°C) and centrifuged at 5,000×g for 30 min at 4°C to remove cellular debris. Twenty microliter of haemocyte lysates, FSW or BSA standards of known concentration (bovine serum albumin, 2µg/ml to 500µg/ml) were added to 96 well plates followed by the addition of 80 µl of Bradford reagent (1:5 dilution in distil water) to each well. The plates were then read with a spectrophotometer at 595 nm. The FSW and BSA standards were used to create standard curves so that the amount of protein could be interpolated.

Statistical Analysis: The results were analyzed using Graph Pad prism (Version 5.01). One-way analysis of variance (ANOVA) was used to determine the significance of differences between the mean values. Differences were considered significant if $P<0.05$.

RESULTS

Phenoloxidase Activity: Fig. 2 shows that whole hemolymph phenoloxidase activities (monophenolase and diphenolase) were enhanced significantly ($P<0.05$) after 2 weeks of exposure to phosphate pollution. The increase in the values of hemolymph phenoloxidase activities (monophenolase and diphenolase) is most evident for animals located at PLP followed by location 1. Fig. 2B showed that animals from all locations had diphenolase activity values higher than the controls. In comparison to the value for the control of approximately 0.3631±0.06525 OD_{492}, the values of diphenolase activity of the animals located at PLP, locations 1 and 2 were about 1.106±0.16 OD_{492}, 0.8605±0.1088 OD_{492}, 0.4905±0.06702 OD_{492} respectively.

Fig. 2A showed that whole hemolymph monophenolase activity followed a similar pattern after 2 weeks of exposure. It increased significantly ($P<0.05$) compared to the control. Monophenolase activity values of the animals located at PLP, locations 1 and 2 were about 0.8185±0.07728 OD_{492}, 0.7421±0.04147 OD_{492}, 0.4904±0.05453 OD_{492} respectively compared to 0.4171±0.08931 OD_{492} for control.

Total and Differential Haemocyte Counts: The effect of two weeks of exposure to phosphate pollution on the total haemocytes count (THC) and differential haemocyte counts (DHC) in whole haemolymph of *P. sulcatus* is shown in Fig. 3 (A) and (B) respectively. Fig. 3A shows that the exposure to phosphate pollution caused a significant increase ($P<0.05$) in haemocytes number to ~ $628x10^3$ ml^{-1} compared to $333x10^3$ ml^{-1} in control animals, $582x10^3$ ml^{-1} in L1 animals and $488x10^3$ ml^{-1} in L2 animals.

In this experiment, the differential counts identified two cell types, granulocytes and hyalinocytes. There was also a significant increase in the frequencies of both cell types (Fig. 3B) in hemolymph collected from animals located at the PLP. Hyalinocyte and granulocyte frequencies were (42±5.2%) and (65±6.3%) respectively compared to (25±5.4%) and (42±3.6%) respectively for animals located at the control location.

Phagocytic Activity: Fig. 4 demonstrates that 2 weeks of exposure to phosphate pollution decreased the phagocytic ability of haemocytes. 49±5% of haemocytes from animals located at PLP had ingested yeast, compared to 78±7% of haemocytes from animals located at the control location ($P<0.05$). The frequency of phagocytic haemocytes also decreased for animals located at L1 and L2 (52±8% and 70±5% respectively).

Total Protein Concentration: In this study, there was significant increase in the total protein content of the haemocyte lysates after 2 weeks exposure to phosphate pollution. Fig. 5 shows that the total protein content of hemolymph increased significantly for animals located at PLP, L1 and L2 (0.6911±0.06255 OD_{595}, 0.4807±0.03209 OD_{492} and 0.4855±0.08367 OD_{492} respectively) compared to those located at the control location (0.2636±0.03913 OD_{492}) ($P<0.05$).

Superoxide Anion Production: Fig. 6 shows that 2 weeks of exposure to phosphate pollution increased the H_2O_2 production by haemocytes significantly ($P<0.05$) compared to control. Haemocytes from animals located at PLP showed an almost two fold increase in H_2O_2 production (0.2753±0.0299, OD_{620}), when compared to haemocytes from animals located at the control location (0.1447±0.0345, OD_{620}). Haemocytes from animals located at L1 and L2 did not show a significant increase in H_2O_2 production compared to haemocytes from the control location.

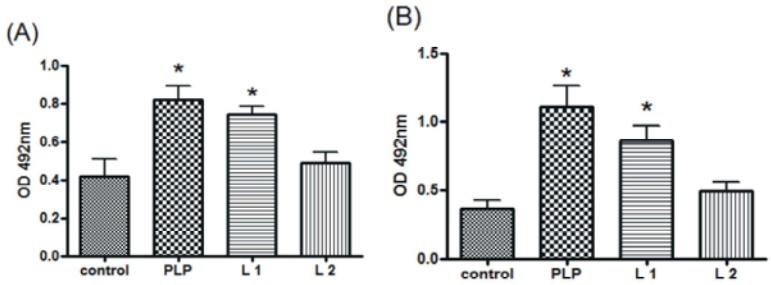

Fig. 2: Phenoloxidase activities of haemocytes of the marine snail, *Planaxis sulcatus* exposed to reference and phosphate polluted sites. (A) monophenolase acivity and (B) Diphenolase activity.
(*) indicates a significant difference at P < 0.05. n = 10, bars = SEM.

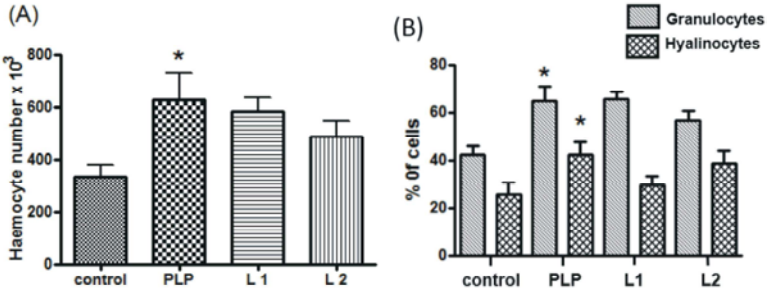

Fig. 3: Total haemocytes count (A) and differential haemocyte counts (B) in whole haemolymph of the marine snail, *Planaxis sulcatus* exposed to reference and phosphate polluted sites.
(*) indicates a significant difference at P<0.05. n = 10, bars = SEM.

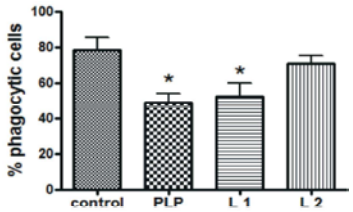

Fig. 4: Percentage of haemocytes that had ingested one or more yeast (% phagocytic cells) in the marine snail, *Planaxis sulcatus* exposed to reference and phosphate polluted sites.
(*) indicates a significant difference at P<0.05. n = 10, bars = SEM.

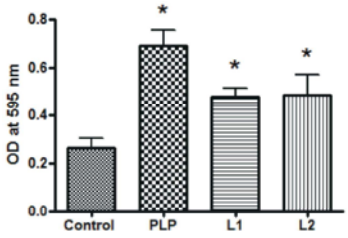

Fig. 5: Total protein concentration of whole haemolymph in the marine snail, *Planaxis sulcatus* exposed to reference and phosphate polluted sites.
(*) indicates a significant difference at P<0.05. n = 10, bars = SEM.

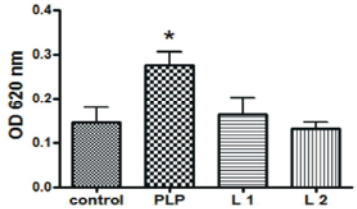

Fig. 6: Superoxide anion production in the marine snail, *Planaxis sulcatus* exposed to reference and phosphate polluted sites.
(*) indicates a significant difference at P<0.05. n = 10, bars = SEM

DISCUSSION

A number of studies documented that environmental stressors, like salinity, temperature and water pollution reduce immunological activity among invertebrates and increase susceptibility to pathogens [41-46]. For example, in Sydney rock oysters, exposure to low salinity has been shown to decrease PO activity [38, 47]. It has also been found that starvation and chemical pollution decrease many immunological responses in Sydney rock oysters, such as PO activity and superoxide and peroxide production [48, 49]. Böttger and McClintock [50] found

that exposure to phosphates decreased the antimicrobial defenses of sea urchin, *Lytechinus variegatus*.

Although the Gulf of Aqaba waters were considered to be relatively, clean, eutrophication problems started emerging in the last few years. Rapid coastal development in the Gulf of Aqaba has caused substantial ecological stress and some coastal areas have shown signs of ecological imbalance associated with multiple stressors caused by pollution. Major sources of pollution in the Gulf of Aqaba include Phosphate ore, Diammonium phosphate fertilizer and potash.

The results showed that phosphate exposure decreased phagocytosis ability significantly, while phenoloxidase activity, THC and DHC, total protein concentration and superoxide anions production increased significantly with respect to controls. Our results indicate that whole hemolymph phenoloxidase activities (monophenolase and diphenolase) were enhanced significantly ($P<0.05$) after 2 weeks of exposure to phosphate pollution. The results showed that the effect of phosphate pollution is mainly localized in the PLP and L1. This can be explained by the localization of the phosphate pollution in the loading berth [7]. Many studies showed that the animals exposed to pollution had significantly enhanced phenoloxidase activities relative to controls [45, 51-53]. On the other hand, other studies showed that contaminated sediment had either no significant effect or significant inhibition on phenoloxidase activity [54, 55].

In Mollusca, many studies showed that the exposure to pollution has often caused an increase in haemocytes number [56-58]. In this study, total and differential haemocytes (THC, DHC) were counted in *P. sulcatus* after being exposed to phosphate pollution. We have found that phosphate exposure significantly increased both the THC and DHC. Several studies have investigated the effects of environmental contaminants on invertebrates' defense mechanisms. Manganese (Mn), a naturally abundant metal in marine sediments, increased the number of circulating coelomocytes in sea star, *Asterias rubens* [59]. Also, Bianchi *et al.* [57] showed that sewage water pollution increased haemocyte number and phagocytic activity in freshwater mussel, Diplodon chilensis. However, Gagnaire *et al.* [55] Studied the effects of cadmium and mercury on defence mechanisms of Crassostrea gigas and found that mercury severely decreased the number of circulating haemocytes by inducing haemocyte mortality. Mayrand *et al.* [58] suggested that in *Mytilus edulis* transplanted in contaminated site, the circulating haemocytes have the ability to rapidly increase their number through proliferation. Therefore, we suggest that the increase in haemocytes number in this experiment may be explained by the rapid proliferation of haemocytes rather than cell migration. However, Pipe *et al.* [60] explained the increase in haemocytes number in response to a chemical stressor by cell migration from tissues rather than haemocyte proliferation. They believed that the increase is too fast to be a result of cell proliferation.

Our results showed that the exposure to high levels phosphate contamination decreased the phagocytic ability. The level of reduction of phagocytosis appears to depend on the concentration of pollutant, because the reduction in phagocytic ability happened only in animals transplanted in the PLP location. Many studies have investigated the effects of environmental contaminants on phagocytic activity. In marine gastropod *Haliotis tuberculata*, Mottin *et al.* [53] reported that exposure to zinc induced a significant reduction in phagocytic activity and reactive oxygen species production. In addition to that, polycyclic aromatic hydrocarbons (PAHs) were found to inhibit phagocytosis of haemocytes from M. edulis [61]. Despite the increase in the haemocytes number, the phagocytic ability was weak compared to the control. This can be explained through the apparent reduction in the haemocytes efficiency caused by the rapid proliferation which in turn produces less efficient haemocytes [58].

Results from the current study showed that the levels of superoxide anion production by the phosphate-exposed animals were higher than the levels in the control animals. In earlier studies, it has been observed that superoxide anion production was significantly higher in animals from contaminated sites [62-64]. In oysters *Crassostrea virginica*, Fisher *et al.* [62] reported xenobiotic chemicals enhanced superoxide anion production in haemocytes collected from oysters with higher tissue concentrations of xenobiotic chemicals. Contrary to what we have found, the mussel, *D. chilensis,* which were exposed to sewage water pollution, showed lower reactive oxygen species productivity in haemocytes compared to mussels from an unpolluted area [57]. Haberkorn *et al.* [65] also showed that oysters exposed to metals have lower reactive oxygen species production than those in the clean sites.

In the current study, we have shown that the phosphate-exposure caused a significant increase in the total protein content of the haemocyte lysates. Again, it is obvious that the increase in the total protein content is correlated to the concentration of pollutant, because the increase in the total protein content is observed in snails transplanted in the PLP, L1 and L2. Similar response has been associated with environmental pollution. For instance, in mussels *Mytilus galloprovincialis* collected from several polluted locations, stress-70 proteins, metallothioneins and total cytochrome P450 were significantly induced in the polluted locations [66, 67]. Gorinstein *et al.* [68] showed qualitative changes in secondary and tertiary structures in *M. galloprovincialis* proteins in the samples collected from polluted sites.

Based on the results of this study, the impact of the phosphate pollution on immune parameters of haemocytes of the marine snail, *Planaxis sulcatus* was correlated to the concentration of pollutant. The most severe impact was within the PLP location followed by L1 and L2. Snails placed within these areas were severely affected compared to the control after 2 weeks of exposure. The data showed that phosphate pollution modulates a range of immunological functions. Phosphate inhibited the phagocytic ability but increased PO activities, haemocyte frequencies, superoxide anion production and total protein content.

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