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Effect of sublethal concentrations of heavy metals (cadmium, lead, and copper) on the soluble nitrogen and phosphorus excretion of marine brown mussel (*Perna perna*) (Linnaeus, 1758) (Mollusca; Bivalvia)

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Abstract
Physiological response (nitrogen and phosphorus excretion) of the brown mussel (*Perna perna*) exposed to four sublethal concentrations of cadmium (Cd) and lead (Pb) (50, 100, 150 and 200 μgL^{-1}) and three sublethal concentrations of Cu (5, 10 and 15$\mu g.L^{-1}$), were monitored for 72 hours. The results show a positive correlation between metal exposure and excretory products. Moreover, when compared to the control group, cadmium, lead and copper presented a significant ($p < 0.01$) alteration of pattern of ammonia-N, nitrate, nitrite and phosphate excretion after 24, 48 and 72 hours. This result suggests that metabolites were affected by heavy metal exposure.

Keywords: *Perna perna*, heavy metals, excretion, nitrogen and phosphorus.

1. Introduction
Introducing pollutants to the marine environment are menace to the biota and may affect marine habitats by physical, chemical and biological interactions on different temporal and spatial scales [1]. Among the pollutants that are more common to contaminate marine environment are heavy metals [2-4]. Moreover, in Algeria, metal traces, in surface and seawaters and sediments harbours, originate from industrial activities, namely tanneries and paper mills (Cd, Zn, Cu, Hg, Cr, and Ni), wastewater (Zn, Cu, Cd, Pb, and Ni), and from agriculture by improper use of mineral pesticides [5-10]. Furthermore, pollution by heavy metals is a serious problem due to their toxicity and ability to accumulate in the biota, and persist in water and sediments [11, 12]. Besides heavy metals have also significant impacts on the physiological changes in animals if their concentrations are above the critical threshold [13-15]. Cadmium (Cd) and Lead (Pb) are persistent heavy metals in aquatic environments. They have highly toxicity to aquatic animals [16-20]. Copper (Cu) is an essential metal that participates in normal physiological process of aquatic animals but becomes toxic when present above the critical level [19-23].

However, mussels release their excretion in form of soluble ammonium, nitrite, nitrate and phosphate, and insoluble faeces, which precipitate and deposit on sea bed. Feces materials are biodegradable. These nutrients can be released back into seawater [24]. In addition highly soluble molecules such as nitrate, which are generated from animal-derived nitrogenous compounds (e.g., urea), could prove to be toxic to fish and invertebrates [25]. Similarly, levels of ammonia, nitrite, and nitrate, derived from human activities [26], can impair the ability of aquatic animals to survive, grow, and reproduce [27].

Moreover marine bivalves can be used as biofilter in estuarine area to improve seawater quality due to the low cost operation and maintenance [24], and are considered to be good bioindicators resulting in their use in marine water biomonitoring programs [28-30]. Besides, excretion represents an important factor in assessing physiological status of marine animals and it is essential in quantifying an organisms energetic balance [31-35]. Though, the use of this physiological modification as endpoint for the evaluation of toxicity of these pollutants was largely discussed in various marine organisms [3, 32-37]. However, until now a little work has investigated the effect of heavy metals on nitrogen and phosphorus excretion by intertidal

bivalves [33, 38-42]. This study chose *Perna perna* as the test animal and aimed to investigate the effect of sublethal concentrations of cadmium, lead and copper on soluble nitrogen (ammonia-N, nitrate and nitrite) and phosphorus (phosphate) excretion, and their correlation during 24, 48 and 72 h of exposure.

2. Materials and Methods

Mussels *Perna perna* (4.5-5.5 cm shell length) were collected from natural beds at Figuier, Boumerdes, Algeria (36°46' 05.38' N, 3°28' 37.92' E). After collection, mussels were immediately transported to the laboratory and acclimatized in tanks with aerated natural filtered seawater for a period of 15 days. Stock solutions of cadmium, lead and copper were freshly prepared by dissolving the proper metal salts of cadmium sulfate, lead (II) nitrate, and copper (II) chloride dihydrate in deionized (double distilled) water. Fresh stock solutions were prepared daily. These solutions were serially diluted to get the experimental concentration for the toxicity test. The experimental method includes static renewal (24-hour renewal) test. Moreover, for each tests, the mussels were divided into rectangular polystyrene trays 42 x 28 x 19 cm (15 mussels per tray,) in a total volume of 20 L of seawater/tray. After 2 weeks of adaptation, the specimens were exposed to cadmium (50,100,150 and 200 µg.L^{-1}), lead (50,100,150 and 200 µg.L^{-1}) and copper (5, 15 and 25 µg.L^{-1}) for up to 72 h. Used seawater and a tray containing seawater with mussels had served as controls. These control readings were used to estimate the effect of heavy metals on excretion by the animals after exposure.

2.2 Some Physicochemical Properties of Aqueous Medium

Physical properties (temperature (°C), salinity (Psu), dissolved oxygen (mg.L^{-1}) and pH were measured daily in medium before and after each water renewal by using a multiparametric YSI 556.

2.3 Determination of nitrogen and phosphorus excretion

Before and after each water renewal, one liter of seawater was drawn from the animal tray and filtered (0.42 µ) to remove suspended particles. The contents of ammonia-N, nitrate, nitrite and phosphate in the medium of exposed mussels, controls and ambient seawater were estimated by following standard analytical methods [43]. The sensitivity of the methods is 0.70, 0.14, 0.70 and 0.62 µg. L^{-1} for ammonium, nitrate, nitrite and phosphate, respectively.

2.4 Statistical Analysis

All data were processed using SPSS 17.0 for Windows software. All parameters detected were statistically compared by one-way analysis of variance and LSD multiple comparison test amongst the means. Statistical analysis was expressed as means ± SD, with $p < 0.05$ being considered significant, and $p < 0.01$ being considered extremely significant.

3. Results and Discussion

3.1 Some physicochemical properties of aqueous medium

Table 1. Shows any significant differences of the average difference of physicochemical properties of seawater before and after the tests. Temperature variations were almost identical and stable throughout the period of study for all series of experiments. Salinity variation was also noted to be quasi-null, in the interval of optimal salinity for mussel growth. PH and dissolved oxygen variations were stables throughout the period of study. No difference was, therefore, observed between the reference and contaminated groups, and the bio-tests revealed that those four physicochemical parameters did not affect the metabolic activities of the indicator species used. In fact, the importance of salinity has often been highlighted in recent research, and its influence on nitrogen and phosphorus excretion responses is also well established in the literature [44, 45]. Masilamoni *et al*. [32] have reported that the lowering of salinity resulted in a reduced rate of nitrate, nitrite, phosphate excretion and fecal matter production in *Perna viridis* except for ammonia. Several recent studies have also reported on the effects of temperature, salinity, pH and dissolved oxygen on heavy metals accumulations and metabolic responses in mussels [2, 46, 47]. Besides, Charrid Resgalla *et al*. [48] have reported that ammonium excretion of *Perna perna* increased with increase in the temperature and salinity. Moreover, Wenguang and Maoxian [49] have reported that the reduction in seawater pH likely affected the metabolic process (food intake, oxygen consumption, and ammonia excretion) of three species of the bivalves (*P. viridis, P. fucata* and *C. nobilis*). Wang *et al*. [47] have reported that the clearance rate (CR), absorption efficiency (AE), respiration rate (RR) and scope for growth (SFG) decreased with decreasing salinity and dissolved oxygen concentration (DO), while excretion rate (ER) increased with decreasing salinity and increasing DO in *Perna viridis* for 4 weeks under different combinations of dissolved oxygen concentration (1.5, 3.0 and 6.0 mg O_2 L^{-1}) and salinity (15, 20, 25 and 30 psu)

Furthermore, a very weak fluctuations in the variation (seemed negligible) of nutrients, contents of used seawater were observed for all experiments ((Figures 1(a, b, c), 2 (a, b, c), 3(a, b, c)). Reddy and Menon [50] have reported that the elevation of ammonia and ammonium in seawater possibly affected the metabolic process such as buyssogenesis in *P. viridis*. This led to the conclusion that the abiotic factors may have less important effects when compared to the stress level of the heavy metals exposure for our specimens. In fact, the measurements obtained of the excretion patterns (nitrogen and phosphorus**)** of mussels *P. perna* are the direct result of the effect of metals contamination.

Table 1: Means of some physicochemical properties of the aqueous medium before and after the renewal. (+): Augmentation, (-): Diminishment.

Parameters	Control Cd	Test Cd	Control Pb	Test Pb	Control Cu	Test Cu
ΔT (°C)	+0.436	-0.550	+1.350	-0.300	-0.110	-0.062
ΔpH	+0.206	+0.192	+0.006	+0.047	+0.090	+0.315
ΔSalinity (Psu)	-0.141	+0.125	-0.016	-0.135	+0.320	+0.237
ΔDissolved oxygen	+0.030	+0.060	+0.070	+0.226	+0.030	+0.025

3.2 Ammonia-N excretion

Perna perna exposed to cadmium, lead, and copper showed changes in ammonia-N excretion. Furthermore, all treatment groups show the ammonia-N excretion significantly increased with increase in concentration in the medium (Figures 1(a, b, c)). Moreover, after 24 h exposure, a very significant increase ($P < 0.01$) in ammonia-N excretion was observed at 150, 150, and 25 µg·L^{-1} cadmium, lead, and copper groups respectively (32.00 ± 2.00, 58.00 ± 20.00, and 495 ± 37.00 µg·L^{-1}, resp.), compared to the control group and ambient seawater.

Moreover, Vosloo *et al*. [22] have reported that the ammonia-N

excretion of *Exopalaemon carinicauda* may be copper dependent. In present study, the alteration pattern of ammonia-N excretion of *Perna perna* possibly metal-dependent. The coefficient of correlation, R^2 values between exposure concentration and ammonia-N excretion for Cd, Pb and Cu were 0.252, 0.547 and 0.916, respectively after 24 h of exposure. Similarly, after 48 h exposure, the coefficient of correlation, R^2 values between exposure concentration and ammonia-N excretion for Cd, Pb and Cu were 0.863, 0.061 and 0.963, respectively. Also, after 72 h the coefficient of correlation, R^2 values between exposure concentration and ammonia-N excretion for Cd, Pb and Cu were 0.610, 0.396 and 0.530, respectively.

Moreover, Widdows [51]. have reported that the excretion of ammonia-N in mussels may be regarded as the rate of protein and aminoacid catabolism. Diffusion is the principal mode of ammonia excretion in marine invertebrates since its concentration in the body fluids is considered higher than that in the outside environment [52]. However, Wu and Chen [16]. have reported that when exposed the white shrimp (*L. vannamei*) to lethal concentrations of heavy metals, dysfunction of ammonium excretion control follows gill damage, and the out flow of ammonium from the hemolymph to the ambient water results in higher concentrations of ammoniumin the water and a lower osmotic pressure in the hemolymph.

Also, Wu and Chen [16]. have reported that an increasein ammonium excretion of 53.7 percent or 44.1 percent higher than the average amount of the control animals in *L. vannamei* was observed when exposure to either 3 $mg.L^{-1}$ of Cd or 3 $mg.L^{-1}$ of Zn, respectively, for 24 h. Likewise, Cd and Zn exposure in *F. paulensis* caused increases in ammonium excretion of 51.85 percent and 42.85 percent, respectively [53]. By contrast, a significant decrease in ammonium excretion was observed in *P. indicus* post larvae when exposed to Pb [40]. The authors concluded that the decrease in ammonia-N excretion can be attributed to the reduction in metabolic rate or the interaction of Pb with pathways for the production of ammonia-N. In the present study, ammonia-N excretion of mussels *Perna perna* increased significantly ($p < 0.01$) with the exposure concentrations of Cd, Pb and Cu can be attributed to the increasing in catabolism of amino acids rate or interaction of cadmium, copper and lead with pathways for the production of ammonia-N. Moreover, these results are comparable to those found in other studies, where ammonia-N excretion increases in exposed aquatic animals to cadmium, lead and copper. Furthermore, Vosloo *et al.* [22] have reported that a significant increase of ammonia excretion in *Perna perna* exposed to sublethal copper levels (at 25 and 50 $\mu g\ L^{-1}$ copper) for up to 24 h. Besides, Cheung and Richard [38] have reported that the interaction between time and concentration of Cd was responsible for the changes in the rate of ammonia excretion of *Perna viridis*. In contrast, Chinni *et al*. [40] have reported that, ammonia-N excretion decreased in *P. indicus* PL on exposure to different concentrations of lead.

Therefore, metal ions are well known inducers of oxidative stress in aquatic animals [19, 54, 55]. They can stimulate Reactive Oxygen Species (ROS) production via two different mechanisms. The first one is related with the interference of metal-related processes (such as Cd and Pb) and the

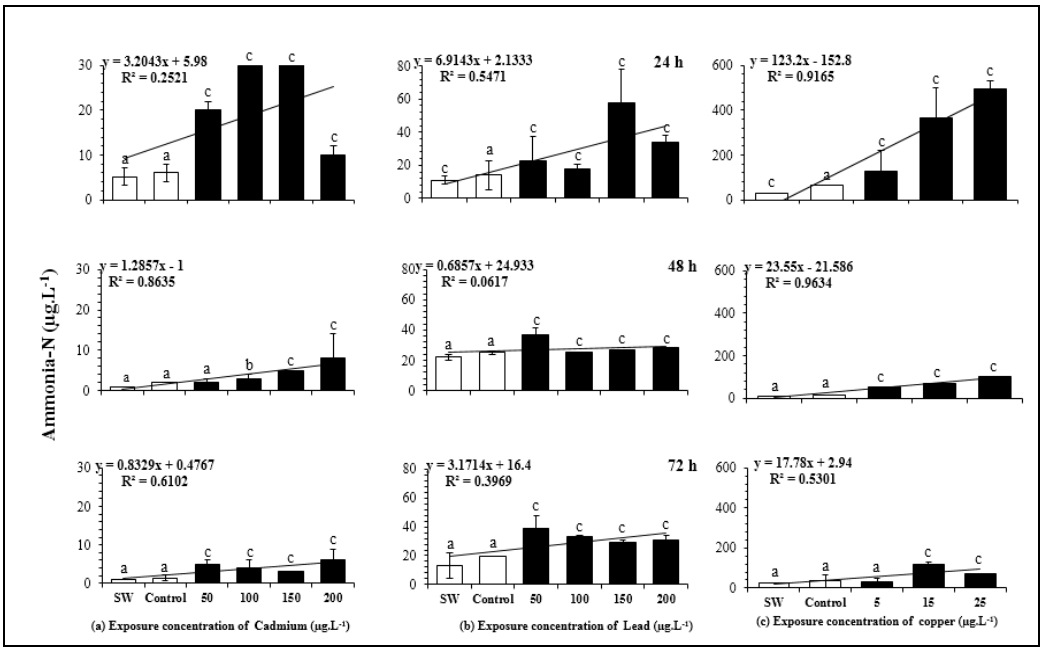

Fig 1: Mean (± SD) variation of ammonia-N levels in seawater (SW), controls and Cd-exposed (a), Pb-exposed (b), and Cu-exposed mussels in aqueous medium (c) after 24, 48 and 72 h exposure. Values followed by the same letter are not statistically different ($P > 0.05$) but values followed by the different letter are statistically significant ($P < 0.05$).

second one with the generation of free radicals by ions with changeable valence (such as Cu) via Haber-Weiss reaction [19]. In our study, ammonia-N excretion response to Cu exposure was higher than observed for Cd and Pb. It can be attributed to the nature of metals tested. Moreover, cadmium and lead are not essentials metals, whose toxic effects are induced mainly by acting on the sulfhydryl groups of the proteins; on the contrary, copper (metals essential) indirectly induces ROS production and lipid peroxidation by affecting the antioxidant systems and affects a physiology of mussels [19, 54]. In this study, the degree of alteration pattern of ammonia-N excretion in *Perna perna* can be classified as follows: Ammonia-N (Cu^{2+}) > Ammonia-N (Pb^{2+}) > Ammonia-N (Cd^{2+}).

3.3 Nitrate and Nitrite excretion

When exposed to sublethal concentrations, there was a change in the pattern of nitrate and nitrite excretion (Figures 2 (a, b, c) and 3 (a, b, c)). Moreover, all treatment groups show the nitrite and nitrate significantly increased with increase in concentration in the medium when compared with controls animals and ambient seawater. Moreover, a maximum amount of nitrate excretion was observed at the sublethal concentration of 50, 150 and 15 µg.L^{-1} after an exposure of 24, 24, and 48 h for Cd, Pb and Cu respectively. In our study, the alteration pattern of nitrate excretion may be metal-dependet. The coefficient of correlation, R^2 values between exposure concentration and nitrate excretion for Cd, Pb and Cu were 0.538, 0.814 and 0.206, respectively after 24 h of exposure. Similarly, after 48 h the coefficient of correlation, R^2 values between exposure concentration and nitrate excretion for Cd, Pb and Cu were 0.755, 0.421 and 0.925, respectively. Also, after 72 h the coefficient of correlation, R^2 values between exposure concentration and nitrate excretion for Cd, Pb and Cu were 0.836, 0.899 and 0.810, respectively.

Furthermore, higher levels of nitrite excretion were observed with an exposure of 24, 48 and 72 h at sublethal concentrations of cadmium, lead and copper. Increased nitrite excretion was statistically significant ($P < 0.01$) for 24, 48 and 72 h. The coefficient of correlation, R^2 values between exposure concentration and nitrite excretion for Cd, Pb and Cu were 0.717, 0.878 and 0.939, respectively after 24 h of exposure. Similarly, after 48 h the coefficient of correlation, R^2 values between exposure concentration and nitrite excretion for Cd, Pb and Cu were 0.907, 0.072 and 0.936, respectively. Also, after 72 h the coefficient of correlation, R^2 values between exposure concentration and nitrite excretion for Cd, Pb and Cu were 0.509, 0.363 and 0.838, respectively. In contrast, Valarmathi & Azariah [33]. have reported that the rates of nitrite excreted by Crab *Sesarma quadratum* (*Fabricius*) were negatively correlated to the sublethal concentrations of the copper chloride and chlorine. Reduced rates of excretion of nitrite were observed in Crab *Sesarma*

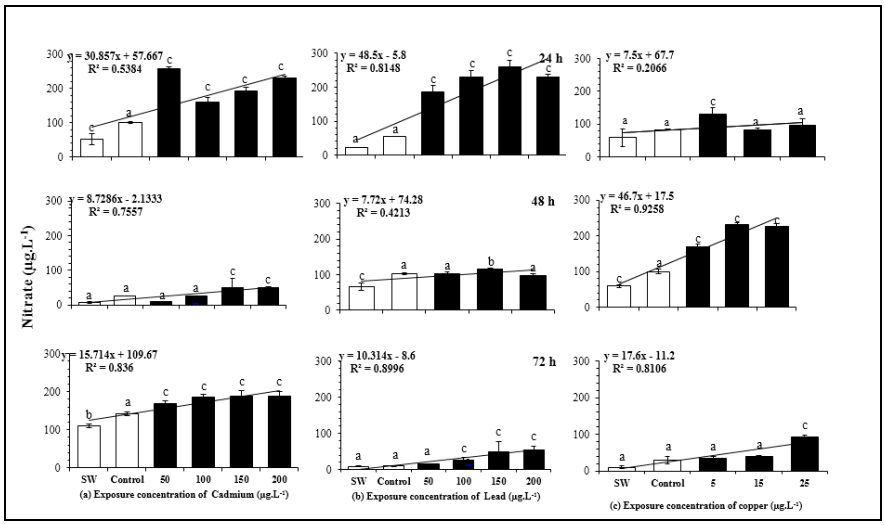

Fig 2: Mean (± SD) variation of nitrate levels in seawater (SW), controls and Cd-exposed (a), Pb-exposed (b), and Cu-exposed mussels in **aqueous** medium (c) after 24, 48 and 72 h exposure. Values followed by the same letter are not statistically different ($P > 0.05$) but values followed by the different letter are statistically significant ($P < 0.05$).

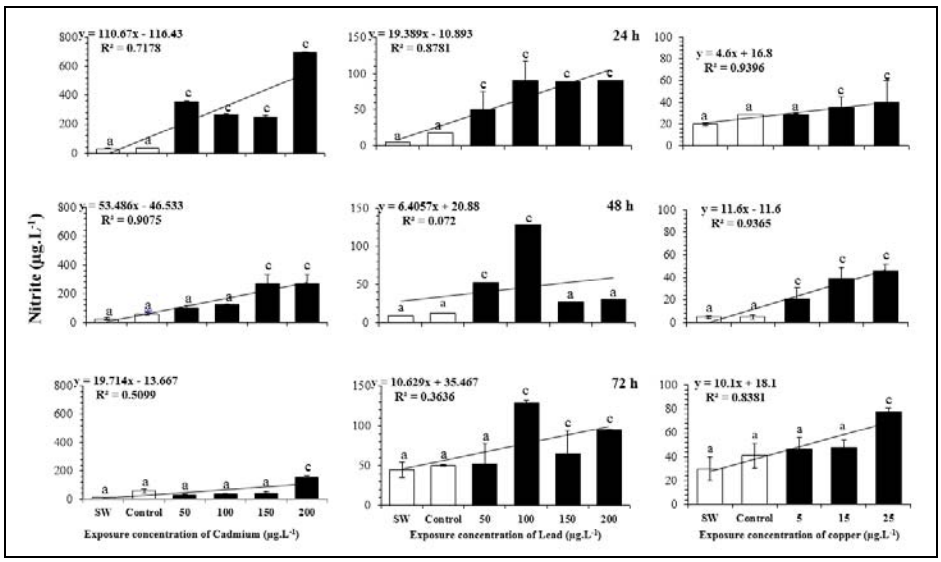

Fig 3: Mean (± SD) variation of nitrite levels in seawater (SW), controls and Cd-exposed (a), Pb-exposed (b), and Cu-exposed mussels in aqueous medium (c) after 24, 48 and 72 h exposure. Values followed by the same letter are not statistically different ($P > 0.05$) but values followed by the different letter are statistically significant ($P < 0.05$).

quadratum (Fabricius) when exposed to these sublethal concentrations of copper chloride and chlorine [33].

Besides Chen *et al*. [56] have described the excretion of nitrite and nitrate by animals as the intrinsic mechanism for detoxification of ammonia in the blood and maintaining ionic stability inside the animal. Furthermore Arumugman *et al*. [57]. have reported that the production of superoxide and nitric oxide (as nitrite and nitrate) *in vitro* by *Mytilus galloprovincialis* haemocytes upon incubation with PMA or laminarin or during yeast phagocytosis. Moreover, stimulation of either NADPH oxidase or NO synthase activity in mussels by various stimuli has been mentioned in a lot of studies in the last decades. In mussels, haemocytes basal NO production is a consequence of the enzymatic activity of a constitutive NO synthase [58, 59]. Dailianis *et al*. [6]. have reported that heavy metals, such as Cd, have the ability to enhance superoxides ($\bullet O_2^-$) and nitric oxide (NO) production (as nitrites) in haemocytes of mussel *Mytilus galloprovincialis* as well as the possible involvement of Na^+/H^+ exchanger (NHE) in the induction of NADPH oxidase and NO synthase activity. According to the results of the present study, increased levels of NO_3^- and NO_2^- measured in mussels medium exposed to Cd, Pb and Cu, thus suggesting a possible activation of NO synthase in haemocytes of mussels exposed to sublethal concentrations of Cd, Pb and Cu.

In addition, nitrifying bacteria then readily convert the ammonium to nitrite, which can be oxidized to nitrate depending on redox conditions. The nitrate is then reduced to nitrite, which can be converted to nitrogen gas by denitrifying bacteria with the aid of mussel-derived organic carbon (feces and pseudofeces) [61]. In the present study the increasing rate of ammonia-N, nitrate and nitrite excretion might be influenced not only by the effect of heavy metals studied on metabolism of the animal but also by the availability of these nitrogen nutrients in the medium. Besides, the degree of alteration pattern of nitrate and nitrite excretion in *Perna perna* can be classified as follows: Nitrate (Cu^{2+}) > Nitrate (Pb^{2+}). > Nitrate (Cd^{2+}); Nitrite (Cu^{2+}) > Nitrite (Pb^{2+}) > Nitrite (Cd^{2+}).

3.4 Phosphate excretion

In all the metals tested, a positive relationship was observed between exposure concentration and phosphate excretion. The level of phosphate excretion was found to be increased as the exposure concentration was higher when compared with control animals and ambient seawater (Figure 4. (a, b, c)).

Phosphate ions play an important role in euryhaline organisms, and the animals exhibit a tendency for retention of phosphate ions in response to decreased salinity [62]. The excretion of phosphate ions by marine animals contributes to the phosphate levels in the water bodies. Richard & Dankers (1988) [63] have reported that the increase in the phosphate level in the Wadden Sea is due to

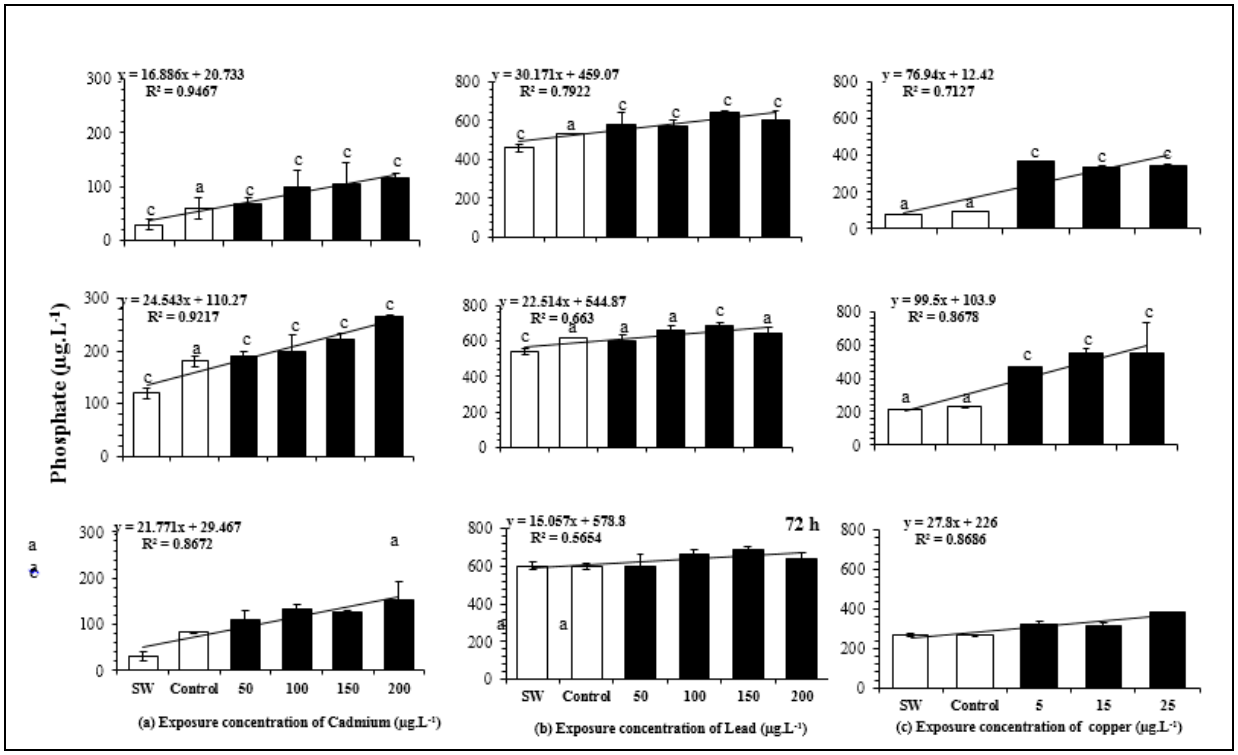

Fig 4: Mean (± SD) variation of phosphate levels in seawater (SW), controls and Cd-exposed (a), Pb-exposed (b), and Cu-exposed mussels in aqueous medium (c) after 24, 48 and 72 h exposure. Values followed by the same letter are not statistically different ($P > 0.05$) but values followed by the different letter are statistically significant ($P < 0.05$).

Excretion by mussels from the mussel bed. In *Modiolus demissus* the phosphate excretion was reported to be directly proportional to the salinity [62]. In present study, we observed that phosphate excretion by brown mussels is correlated well with exposure concentration of cadmium, lead and copper. The coefficient of correlation, R^2 values between exposure concentration and phosphate excretion for Cd, Pb and Cu were 0.946, 0.792 and 0.712, respectively after 24 h of exposure. Similarly, after 48 h the coefficient of correlation, R^2 values between exposure concentration and phosphate excretion for Cd, Pb and Cu were 0.921, 0.663 and 0.867, respectively. Also, after 72 h the coefficient of correlation, R^2 values

between exposure concentration and phosphate phosphorus excretion for Cd, Pb and Cu were 0.867, 0.565 and 0.868, respectively.

Mussels have been reported to absorb phosphorus from the environment [62-64]. Kuzenzler (1961) [62]. have reported that out of the total phosphorus absorbed by mussels, 83% was excreted as phosphate. Hence, the rate of phosphate excretion might be influenced not only by the effect of heavy metals studied on metabolism of the animal but also by the availability of phosphorus in the medium. Besides, the degree of alteration pattern of phosphate excretion in *Perna perna* can be classified as follows: Phosphate (Cu^{2+}) > Phosphate (Pb^{2+}). > Phosphate (Cd^{2+}).

In the light of the ecotoxicological information reported in the present study, the alteration of pattern of the excretion of nitrate and nitrite by bivalve under sublethal concentrations of heavy metals (cadmium, lead, and copper) were much lower than that of ammonium-N and phosphate. These results are comparable with results of Magni *et al*. [39] when reported that the excretion of nitrate and nitrite by bivalve was in most cases not significant or much lower than that of ammonium and phosphate.

4. Conclusion

In conclusion, the metabolites were affected by heavy metal exposures and strongly have the potential as indicators of heavy metal contamination and cadmium, lead and copper in specific. Besides, the alteration of pattern of ammonia-N, nitrate, nitrite and phosphate excretion in *P. perna* is possibly metal concentration-dependent. Hence, the determination of the soluble nitrogen and phosphorus excretion of marine brown mussel (*Perna perna*) may serve as a convenient approach during pollution biomonitoring programme.

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