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Copper and Lead Accumulation in Tissues of a Freshwater Fish *Tilapia zillii* and Its Effects on the Branchial Na,K-ATPase Activity

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Fish are able to uptake and retain heavy metals dissolved in water via active or passive processes. Toxic effects of metals occur after excretory, metabolic, storage and detoxification mechanisms are no longer able to match to uptake rates (Heath 1987; Langston 1990; Roesijadi and Robinson 1994). The gill of teleost fish plays an important role on ion regulation, gas exchange, acid-base balance and nitrogenous waste excretion, which means it has a key role at the interface of fish with its environment. Because branchial epithelium of teleost gills is a tissue where both active and passive exchange occurs between animals and its environment it is also likely to be a site action of heavy metals. Branchial Na,K-ATPase is found in the gill epithelial cells and involved in the active electrolyte transport across the gills of both marine and freshwater teleost which makes it a key enzymes for osmoregulation.

Studies have shown that Na,K-ATPase activity in aquatic animals is sensitive both *in vitro* and *in vivo* exposure to heavy metals (Watson and Beamish 1981; Stagg and Shuttleworth 1982; Pinkney et al.1989; Thaker et al. 1996; Canli and Stagg 1996; Morgan et al. 1997). *In vitro* exposure of heavy metals generally cause a decrease in its activity whereas *in vivo* effects are not so clear, probably various compensatory mechanisms may also involve in the homeostatic mechanisms to recover enzyme loss. Because inhibition of this enzyme occurs before gross osmoregulatory dysfunction, this would point the use of Na,K-ATPase activity as an early warning of pollutant induced damage to the ionic and osmoregulatory system (Stagg et al. 1992a). Therefore, the activity of Na,K-ATPase in fish gill may be a sensitive indicator of metal exposure, as it first faces the environmental metals. There is no study to our knowledge that shows the response of this enzyme to copper and lead in freshwater fish *Tilapia zillii.* The aim of this study is to measure accumulation of copper and lead in the gill, liver and muscle of *Tilapia zilii* and investigate effects of these metals on branchial Na,K-ATPase activity.

MATERIALS AND METHODS

Tropical freshwater fish *Tilapia zillii* were obtained from fish culturing pools at the Çukurova University and transferred to the laboratory where the experiments were held. The animals were first acclimatised to the new conditions for one month and then exposed to metals separately, using three replicate for each metal exposure and control.

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The experiments were conducted in glass aquariums sized 30*40*120 cm, each containing 10 fish in 100 L of contaminated test solution or tap water only (for controls). Fish were exposed to nominal concentration of 0.5, 1,2 and 4 mg/L of lead and copper as ($Pb(NO_3)_2$) and ($CuSO_4 . 5H_2O$) for 14 days. The experimental room was air conditioned (25±1.1°C) and illuminated with two fluorescent lamps (daylight 65/80 W) for 12 hours. The tap water used for the experiment had a pH value of 7.8±0.3 and a total hardness of 211±2.2 mg $CaCO_3$/L. The aquariums were aerated with air stones attached to an air compressor to saturate with oxygen (7.1±0.6 mg O_2/L). Water in the control and metal containing aquariums was changed every two days to minimise decreases in metal concentrations.

At the end of the 14 days exposure period, 3-4 fish from each replicate tank (a total of 10 fish for each metal exposure and control) were sampled and killed by a blow to the head and their fork lengths were measured to the nearest mm. Mean fork length and associated standard deviation of fish used in this study was 16.3±1.4 cm. There was no statistical difference among the treatments and control regarding the size of the fish. This may be important because metal accumulation and ATPases activity can be higher in smaller animals compared to larger ones (Canli and Furness 1993; Canli and Stagg 1996). The animals were dissected with clean equipments to obtain muscle, gill and liver tissues. Tissue samples from two fish of the same exposure groups were pooled to make 5 subsamples and then divided for the determination of the metals, proteins and Na,K-ATPase activity. For determination of Na,K-ATPase activity, gill flaments were separated from the gill arches and weight to nearest mg and frozen at -30 until use (approx. three weeks).

Metal determination was carried out with an atomic absorption spectrophotometer (Perkin Elmer AS 3100) using the similar method explained earlier (Canli and Furness 1993). Protein determination was carried out with the method of Lowry et al. (1951) using the bovine serum albumin as standard. The activity of Na,K-ATPase was measured by determination of the inorganic phosphate (Pi) liberated from the hydrolysis of the substrate adenosine triphosphate (ATP) at 37 °C. For the measurement of Na,K-ATPase activity, frozen gill flaments (approx. 200-250 mg) were first homogenised for 90 seconds in 0.3 M sucrose buffer (pH=7.4). Homogenates were centrifuged at 1000 g to remove the debris for 15 minutes. ATPase activity was carried out with supernatant immediately on the resulting supernatant. Final assay concentrations of chemicals used here (Analar grade or Sigma) were (in mmol/L) tris-HCl (pH=7.4) 135, NaCl 100, KCl 10, $MgCl_2$ 6, ATP (vanadium free) 3, EDTA 0.1 and ouabain 3.

After pre-incubation of the medium for 5 minutes, reaction was started by adding the samples and ATP appropriately. The reaction was continued for 30 minutes and during this time the incubation medium was shook well with a shaker at 100 rev/min (Gallenkamp, England). The reaction was stopped after putting the samples on ice and addition of a lubrol-molybdate mixture (1:1). Samples were then vortexed and kept at room temperature for 10 minutes to form the soluble yellow complex. Inorganic phosphate was determined at 340 nm using 1 ml aliquots of the incubated mixtures. All assays were carried out in triplicate and run with enzyme and reaction blanks. ATPase activity was normalised by protein concentration in crude homogenate and expressed as µmol Pi/mg protein/hr. Similar method is given elsewhere in detail (Canli and Stagg 1996).

Data analyses were carried out using the SPSS statistical package. Mann-Whitney U test was used for data with two groups while Kruskall-Wallis one-way Analysis of Variance was used to compare data with more than two groups. Regression analyses were carried out to examine the relationships between metal concentrations and branchial Na,K-ATPase activity or protein concentrations in the tissues.

RESULTS AND DISCUSSION

Mean accumulation values of copper and lead in the tissues of *Tilapia zillii* are shown in table 1, together with the statistical comparisons. This table shows that when compared to control values both copper and lead accumulated significantly in the tissues, except copper in the muscle where there was no significant accumulation (P>0.05). Highest copper accumulation occurred in the liver and was followed by the gill and muscle, whereas highest lead accumulation occurred in the gill and was followed by the liver and muscle.

Table 1. Mean metal concentrations (µg metal/g dry weight) and associated standard deviations in the tissues of *Tilapia zillii* exposed to copper and lead for 14 days. Results of statistical comparisons (Kruskall-Wallis one-way Anova) are also given in the table. NS = not significant (P>0.05). ND = not detected (< 0.05 µg/g d.w.).

	MUSCLE		GILL		LIVER	
Exp. (mg/L)	Copper	Lead	Copper	Lead	Copper	Lead
Control	5.38±0.78	ND	7.58±1.75	ND	29.7±19.6	ND
0.5	4.33±1.28	4.33±0.57	39.4±7.17	22.7±7.13	158.8±33.9	9.87±3.47
1	5.79±0.82	4.28±0.65	71.4±33.2	63.7±41.3	228.1±64.4	19.8±13.2
2	6.56±2.64	11.5±5.13	74.8±36.5	70.2±30.4	198.1±117.9	20.0±15.0
4	6.93±1.53	9.67±5.80	78.0±3.89	101.6±31.5	278.3±92.6	16.5±8.03
P value	NS	< 0.001	<0.002	< 0.001	<0.003	<0.05

Figure 1a.

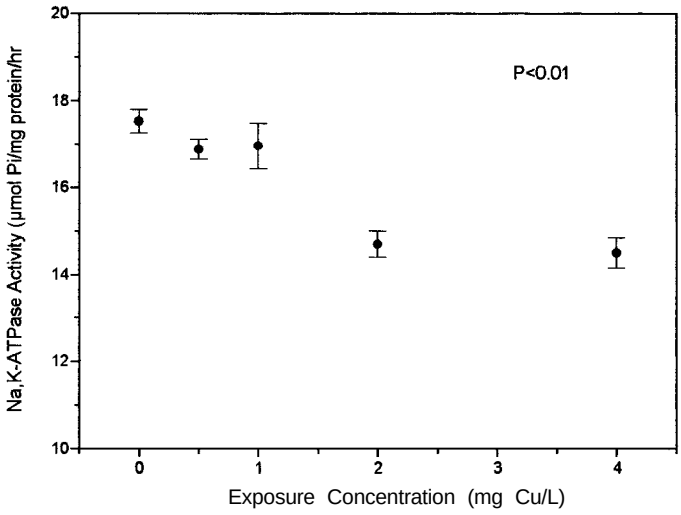

Figure 1b

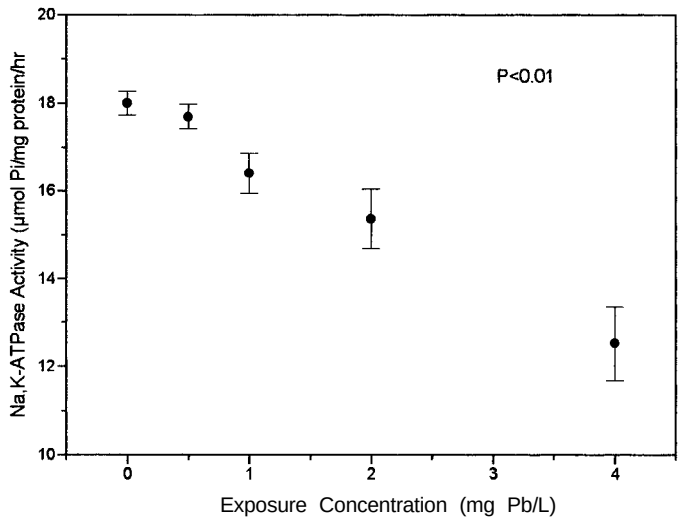

Figure 1. Effects of copper **(a)** and lead **(b)** exposures on the activity of Na,K-ATPase in the gill of *Tilapia zillii.* Mean enzyme activity and associated standard errors are given in these figures together with the results of statistical analyses (Kruskall-Wallis one-way Anova).

Figure 2a.

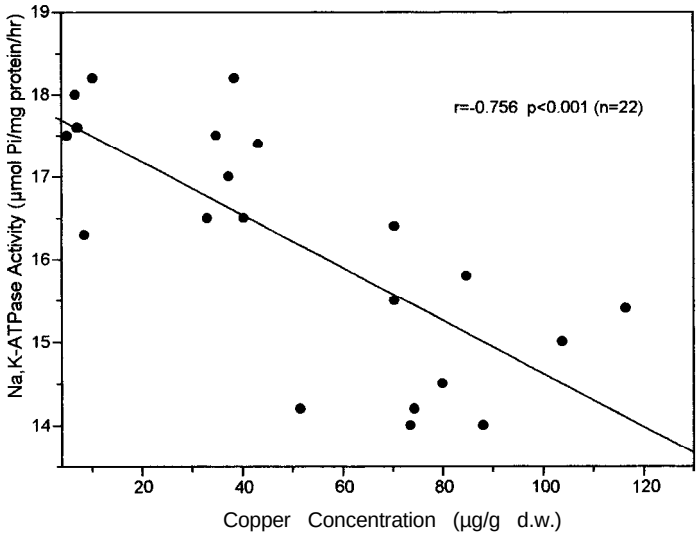

Figure 2b.

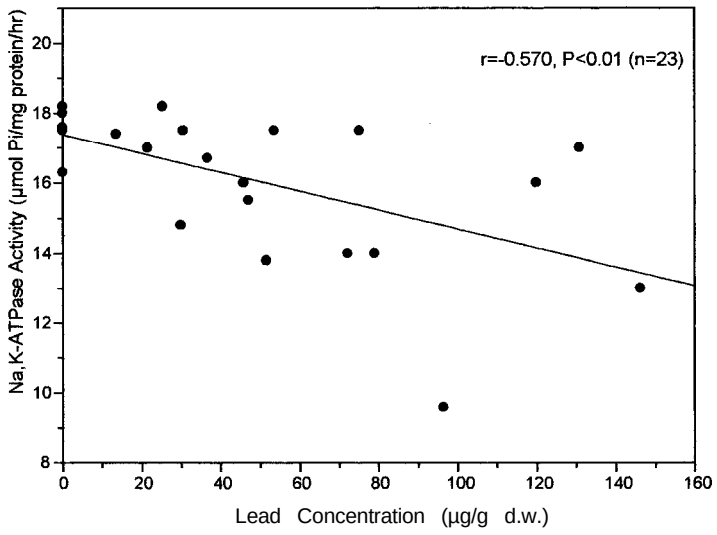

Figure 2. The relationships between Na,K-ATPase activity and copper **(a)** or lead **(b)** concentrations in the gill of *Tilapia zillii.* Results of regression analyses are also given in these figures.

It is generally accepted that metal accumulation in tissues of aquatic animals is dependent upon exposure concentration and period as well as some other factors such as salinity, temperature, interacting agents and metabolic activity of tissue in concern. Similarly, it is also known that metal accumulation in tissues of fish is dependent upon the rate of uptake, storage and elimination (Heath 1987; Langston 1990; Roesijadi and Robinson 1994). In most cases, the tissues of *Tilapia zillii* accumulated copper and lead from the medium following the accumulation protocol described above and tissue concentrations rose sharply, although the levels of metal accumulation differed considerably among the tissues. The liver and gill showed highest accumulation of copper and lead, while the muscle lowest. Similar pattern of lead accumulation was also shown in other studies carried out with different fish species (Reichert et al. 1979; Amiard et al. 1987; Tulasi et al. 1992; Allen 1994). Copper also accumulated in the tissues of *Tilapia zillii* primarily in the liver. Copper is an essential element and tends to accumulate to a greater extent than other essential elements, such as zinc and iron (Heath 1987; Roesijadi and Robinson 1994). The induction of low molecular weight metal-binding proteins, such as metallothionein is closely related to heavy metal exposure and metals taken up from the environment can be detoxificated by binding on these proteins (Heath 1987; Roesijadi and Robinson 1994; Canli et al. 1997). Therefore, tissues like the liver which is a major producer of metal-binding proteins shows high concentrations of most heavy metals (Thomas et al. 1983; Amiard et al. 1987; Roesijadi and Robinson 1994; Allen 1994). As the present study also showed, accumulation of lead in the gill is also high but in the liver lead accumulation is relatively low, especially when one compares accumulation levels of other non-essential metals (Tulasi et al. 1992; Allen, 1994; Roesijadi and Robinson 1994). There is lack of evidence on the induction of lead-binding proteins related to metallothioneins in fish tissues (Reichert et al. 1979; Roesijadi and Robinson 1994) and this may be the reason for the relatively low accumulation of lead in the liver.

The activity of Na,K-ATPase activity in the gill of *Tilapia zillii* was inhibited significantly ($P<0.001$) by both copper and lead (Figure la and b). These figures shows that there was an exposure dependent decrease in the activity of this enzyme after 14 days exposure to copper and lead. Regression analyses between Na,K-ATPase activity and metal concentrations in the gill (Figure 2a and b) showed that there were negative relationships between Na,K-ATPase activity and copper ($r = -756$, $P<0.001$) and lead ($r = -570$, $P<0.01$).

This study showed that the branchial Na,K-ATPase of *Tilapia zillii* is sensitive to both copper and lead exposure as its activity was significantly inhibited following *in vivo* exposure to these metals. Additionally, these inhibitions were exposure dependent and enzyme activity showed negative relationships with both metal. However, there is no clear tendency on the behaviour of branchial Na,K-ATPase in the literature as its activity showed considerable variations among different fish species, metals and exposure conditions as well as differences *in vivo* and *in vitro* applications. Thaker et al. (1996) found significant inhibitions of Na,K-ATPase in the gills, intestine and kidney of coastal teleost *Periophthalmus dipes*, with a general dose-and duration dependent inhibitory trend following exposure to chromium (VI). They indicated that exposure duration is more important then dose in the inhibitory activity of the enzymes and, Morgan et al. (1997) showed that activity of brachial Na,K-ATPase in the rainbow trout *Oncorhychus mykiss* was inhibited (85%) following exposure to 10 ppb Ag in 48 hours. Similarly, exposure of bluegill sunfish *Lepomis macrochirus*, fathead minnow *Pimephales promelas*

and golden shiner *Notemigonus crysoleucas* to cadmium (10 and 100 ppb) caused an inhibition in the the activity of branchial Na,K-ATPase activity, but in the golden shiners the activity was not influenced by cadmium exposure Watson and Benson 1987). They related this to high background levels of cadmium in golden shiners. Exposure of yearling coho salmon (*Oncorhynus kisutch*) to sublethal concentrations of zinc (between 0.1-2.5 ppm) in freshwater did not make significant alteration, although acute and chronic exposure to sublethal concentrations of copper (between 5-30 ppb) in freshwater had a deleterious effects on gill Na,K-ATPase activity (Lorz and McPherson 1976). Inhibition of Na,K-ATPase activity in fish in relation to metal concentration was also shown from the field. Stagg et al. (1992a) carried out an investigation to determine the relationship between mercury contamination and Na,K-ATPase activity in the gill of the flounder *Platichtys flesus*. Results showed that branchial Na,K-ATPase activity was lower in contaminated areas than found in relatively less contaminated areas, also indicating that the enzyme activity showed a strong negative correlation between mercury concentration and Na,K-ATPase activity. Considering the exposure concentrations of metals in this study and from the other studies, results suggest that the gill Na,K-ATPase of *Tilapia zillii* is less sensitive to heavy metals than the gill Na,K-ATPases of other fish species. This is probably because differences between metabolic activities of *Tilapia zillii* and other fish species as a result of adaptation to different ecological conditions in the nature.

Although it seems that metals inhibit brachial Na,K-ATPase *in vitro* (Watson and Beamish 1981; Stagg and Shuttleworth 1982; Pinkney et al. 1989), inhibition of this enzyme by heavy metals *in vivo* can be compensated by homeostatic regulation and its activity can return to normal. This recovery may possibly occur by increasing the number of enzyme and/or increasing the turnover rates of the enzyme present in order to compensate the activity of lost enzymes. Stagg and Shuttleworth (1982) found that there was marked reduction in the activity of gill Na,K-ATPase in the gill of flounder *Platichthys* flesus following exposure to copper (200 ppb) *in vivo*. But, they indicated that this response of the enzyme was modulated in the sensivity returned to the control level, indicating the involvement of a compensatory mechanism for the activity of this enzyme. Lauren and McDonald (1987) exposed rainbow trout *Salmo gairdneri* to 55 ppb copper for 28 days. Gill Na,K-ATPase activity was inhibited by 33 % within 24 hr of copper exposure and returned to the normal by day 14. Stagg et al. (1992b) studied Na,K-ATPase activity in dab *Limanda limanda* caught at different stations from German Bight and they found that enzyme activity was similar in all stations, but measurement of ouabain binding showed that although levels of the enzyme were elevated the turnover rates of the enzyme were lower at the contaminated sites. However, recovery of enzyme lost may only be possible if exposure concentrations are not high enough to make irreversible change in the enzyme structure. For example, Verma et al. (1983) studied sublethal (between 0.0176-0.088 ppm) concentrations of mercury chloride (30 days) on the Na,K-ATPase in tissues of *Notopterus notopterus* and found that Na,K-ATPase activity was inhibited significantly by mercury exposure in most tissues of the fish and the enzyme activity did not return to the normal except the lowest exposure concentration. Haya et al. (1983) exposed the lobster *Homarus americanus* to relatively high concentration of zinc and found that brachial Na,K-ATPase activity inhibited significantly and this inhibition was irreversible after 168 h of depuration period.

Metals may alter enzyme activity or function by binding to a number of sites on proteins, especially on sulphydryl groups that cause conformational changes and this may prevent substrate binding. Inhibition of branchial Na,K-ATPase may be severe for the animal healt point of view. Na,K-ATPase is located on the basolateral membranes of the ion-

transporting cells of the branchial epithelium and actively transport Na^+ from these cells into the blood. Morgan et al. (1997) showed that acute exposure of rainbow trout *Oncorhychus mykiss* to silver resulted 100% inhibition of Na^+ and Cl^- influxes within 8 hours, although effluxes of these ions were much less affected. They indicated that a disturbance of branchial osmoregulation, as a result of inhibition of branchial enzymes involved in ion transport, is the principal mechanism of the physiological toxicity of silver to freshwater fish. Thaker et al. (1996) explained the mechanism of the inhibition in the gill of coastal teleost *Periophthalmus dipes* exposed to chromium. In their model, chromium blocked the active transport system of the gill epithelial as well as chloride cells, glomerular and epithelial cells of the tubules and thus altered the osmoregulatory mechanism of the fish. Because ion-dependent ATPases are known to regulate the influx and efflux of ions across the membrane to maintain the physiological requirements of the cells, the inhibition of Na,K-ATPase in gills probably disturb Na^+ and K^+ pump, resulting in an uncontrollable entry of Na^+ into the cell along the concentration gradient and the water molecule follows along the osmotic gradient (Thaker et al. 1996). However, Bouquegneau (1977) found that Na,K-ATPase activity in the gill of mercury exposed eels *Anguilla anguilla* inhibited largely in an exposure dependent manner and Na^+ and Cl^- concentrations in the plasma increased. Lauren and McDonald (1986) indicated that the inhibition and induction of Na,K-ATPase is the most likely explanation for the inhibition and recovery of sodium uptake, pointing out the importance of conducting *in vivo* studies whenever possible as *in vitro* assays do not necessarily reflect *in vivo* conditions and/or metals may affect the other components of the sodium uptake mechanism. Nevertheless, the results of present study and the other studies mentioned above, there seem to be needs to carry out more studies to understand better the mechanism of the inhibition on branchial Na,K-ATPase by heavy metal ions which would also help to set up its implication in natural monitoring studies.

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