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The Effects of Chitosan on Aluminium Accumulation in the Gill, Liver and Muscle of Freshwater Fish (*Oreochromisniloticus*)

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ABSTRACT
In this study, accumulation of aluminium (Al) in the gill, liver and muscle of *Oreochromis niloticus* were determined following exposure to Al (0, 1, 2 and 4 ppm) alone and in combination with chitosan (10 ppm) for 7, 14 and 21 days. Aluminium concentrations in the tissues were measured by ICP-MS. There were no fish mortality and apparent morphological or behavioural changes after 21 days of exposure duration. Al concentrations of the tissues increased significantly ($P<0.05$) in both Al alone exposures and Al+chitosan combination exposures and following order was found in Al accumulation among the tissues; Gill>Liver>Muscle. Data also showed that chitosan significantly ($P<0.05$) reduced the accumulation of Al in the tissues. This study suggests that chitosan may be used as an effective chelate in Al contaminated waters and emphasizes Al burdens in commercial fish species from contaminated waters for human health point of view.

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Alüminyum'un *Oreochromis niloticus*'un Solungaç, Karaciğer ve Kas dokularındaki Birikimi ile Kitosan'ın Doku Alüminyum Birikimi Üzerine Etkileri

ÖZET
Bu çalışmada, alüminyum (Al)'un tek başına (0, 1, 2 ve 4 ppm) ve kitosan (10 ppm) ile birlikte etkilerine 7, 14 ve 21 gün süre ile maruz kalan *Oreochromis niloticus*'un solungaç, karaciğer ve kas dokularında Al birikimi belirlenmiştir. Dokulardaki alüminyum düzeyleri ICP-MS ile ölçülmüştür. Yirmi bir gün sonunda balıklarda ölüm ve belirgin bir morfolojik değişim veya davranışsal bozukluk görülmemiştir. Hem tek başına Al etkilerinde hem de Al+kitosan kombinasyonlarında dokuların Al düzeyleri anlamlı ($P<0.05$) olarak artarken, dokular arasında Al birikimini bakımından şu sıralamada görülmüştür; Solungaç>Karaciğer>Kas. Veriler ayrıca kitosan'ın dokularda Al birikimini önemli ölçüde ($P<0.05$) azalttığını da göstermiştir. Bu çalışma, kitosan'ın Al ile kirlenmiş sular için etkili bir şelat olarak kullanılabileceğini öne sürmekte ve Al kirlenmesinin olduğu alanlarda insan tüketimi açısından balık dokularındaki Al yükünün toksisitesini vurgulamaktadır.

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INTRODUCTION
Developments in science and technology have increased the use of heavy raw metal materials in industry and consequently resulting an increasing their discharges into aquatic systems. Because the aquatic systems are the main receivers for contaminants released to the environment, the environmentalists worry about the increase in the contaminant loads of waters. The uncontrolled discharges of wastes to the aquatic environments can

cause significant changes in the physical and chemical qualities of waters and finally affect the health of aquatic biosystem (Sidra et al., 2018). Some metals such as Cd, Hg, Pb and As do not have any known function in biological systems and they can be very toxic to fish even at low concentrations. On the other hand, fish need the essential metals (Cu, Zn, Fe, Mn, Mo, Co, Se) at trace levels for their metabolisms. However, even the essential metals can be toxic to fish above certain threshold levels (Abbasi and Khayatzadeh, 2012).

Al is one of the most abundant metal in the earth's crust and used widely in different areas of industry including automotive, aerospace, packaging, construction materials, catalysts, ammonium nitrate explosives and waste water treatment plants. Al can directly enter to the aquatic ecosystems via various discharges and also indirectly enter the aquatic systems by washing up the soil and rocks due to the acid rains (Wood et al., 2012). Studies conducted with aquatic organisms showed that Al inhibited the active ion uptake by inhibiting ATPases and altered the ion regulation, causing development disorder and reduced swimming performance (Authman, 2011; Azmat et al., 2012). It was also shown that Al can cause the lipid peroxidation and alter the biochemical and haematological parameters in the blood (Camargo et al., 2009).

In the aquatic systems, some organic and inorganic materials such as EDTA (Ethylene diamine tetra acetic acid), NTA (Nitrilo tri acetic acid), DTPA (Diethylene triaminepenta acetic acid), DFO (Deferroxamine), DFP (Deferiprone), zeolite, clinopylolite and chitosan are widely used as a chelate. Chitosan is a linear amino polysaccharide obtained from deacetylation of chitin in the exoskeleton of crustaceans and arthropods in alkaline environment. It has been determined that chitosan has the capacity to strongly adsorb heavy metals such as Hg, Cu, Ni and Zn (McKay et al., 1989). Chitosan has low toxicity, biological applicability, easy to obtain and low cost. These characteristics of chitosan could make it widely used remediation material in contaminated aquatic systems (Samarakoon et al., 2003).

Freshwater fish *O. niloticus*is widely distributed in fresh waters in the tropical and subtropical climate zone. It has high resistant to diseases, salinity and pollutants that make them desirable culture fish. Therefore, tilapias are widely consumed fish species in the tropical and subtropical regions. However, resistance to pollutants may cause great amounts of metal accumulations in their tissues that end up in the feed of humans. Because the chronic exposures of subletal concentrations of metals cause their accumulations in fish tissues and chitosan is an effective chelate in removing metals from the aquatic systems, this work was undertaken to investigate the chronic accumulation of Al in tissues of *O. niloticus* and effects of chitosan in tissue accumulation of Al.

MATERIALS and METHODS

In this study, freshwater fish *O. niloticus* (13.0 ± 3.0 cm in length and 35.0 ± 1.2 g in weight) were used for the experiments. Fish were obtained from the Aquaculture Unit of the Faculty of Fisheries (Mersin University) and transported to the laboratory where the experiments were carried out (the Basic Sciences Research Laboratory). Fish were kept in glass aquariums (40x100x40 cm) for one month until they were adapted (12 h light/ 12 h dark) to the experimental conditions. Some physical and chemical characteristics of experimental waters were measured daily and presented in Table 1. In the experiments, 5 fish were randomly allocated to 7 glass aquariums (40x100x40 cm) for each experimental period (7, 14 and 21 days) and a total of 21 aquariums (105 fish in total) were used for all experiments. About 120 L of 1, 2 and 4 ppm Al solutions were added in the first three out of 7 aquaria and the other three aquaria was filled with the 1, 2 and 4 ppm alüminium solutions together with 10 ppm chitosan solutions. The last aquarium was filled with the same amount (120L) of tap water and used as control. Sublethal concentrations of aluminium (0, 1, 2, 4 ppm Al) were determined using our preliminary studies and also literature data guided in determination of the test concentrations (Noureen, 2017; Canli et al., 2018; Canli and Canli, 2020). Fish were exposed to Al concentrations alone and also together with 10 ppm chitosan. An acetic acid solution (1%) was used in preparing the stock solution of chitosan (Aldrich, GR, Deacetylation ≥ 75). The aquaria of control fish did not contain Al or chitosan. Experiments were carried out using the semi-static test protocol, renewing exposure media every day. Fish were fed once a day with a commercial fish food (Pellets No: 2, Izmir, Turkey) during the experiments, serving them approximately 2% of their total biomass at the same day of water renewal.

Table 1. Some physical and chemical properties of water in experimental aquariums
Çizelge 1. Deney akvaryumlarındaki suyun bazı fiziksel ve kimyasal özellikleri

Temperature	22 ± 1 °C
Total alkalinity	331 ± 0.5 mg $CaCO_3$/L
Total harness	259.3 ±5.82 ppm $CaCO_3$
Dissolved Oxygen	6.6 ± 0.5 mg/L
pH	7.4 ± 0.7

Heavy Metal Analysis

At the end of the exposure periods, fish were removed from exposure aquariums and anesthetized with an anesthetic substance MS 222 (Tricaine methane - sulphonate 75 mL/L) (Cicik et al., 2004). They were

rinsed with tap water and dissected using clean equipment. Liver, gill and muscle tissues were taken out carefully from each fish and put into petri dishes and placed in an oven set at 150 °C. The tissues were kept in the oven for 48 hours until they reached to constant weights. Then, the tissues were weighed to the nearest mg and put into the digestion tubes. All the tissues were digested in 2 ml of nitric acid (HNO_3, 65%, s.g. 1.40, Merck) and 1 ml perchloric acid ($HClO_4$, 60%, s.g. 1.53, Merck) mixtures on a hot plate set to 120 °C for 3 h (Muramoto, 1983). Digested samples were transferred to polyethylene tubes and ultrapure water was added onto them to obtain a final volume of 10 ml. Al levels in the tissues were measured using an ICP-MS and IAEA-407 (International Atomic Energy Agency) samples prepared from fish tissue homogenate were used as reference material to check the validity of the measurements (IAEA, 2003).

Statistical Analyses

A statistical package program SPSS v.16.0 (IBM Corp., Armonk, NY, USA) was used to analyse data. Before statistical tests, the homogeneity of variance was checked. One-way ANOVA test was first applied to data and significant ($P<0.05$) results were re-analysed by post-hoc tests (SNK) to estimate groups differing from controls (see Figure 1, 2, 3).

RESULTS and DISCUSSION

The present data demonstrated that significant ($P<0.05$) accumulation of Al occurred in the gill, liver and muscle of *O. niloticus* comparing to controls (Figs. 1, 2, 3). Additionally, tissue accumulation of Al increased in relation to increases in exposure concentrations and exposure periods. However, the presence of chitosan in the exposure mediums reduced significantly ($P<0.05$) the accumulation of Al in the tissues (Figs. 1, 2, 3). The present data demonstrated that chitosan affected the uptake of Al by fish, possibly reducing bioavailability of Al and suggested that it could be an effective chelate for Al contaminated waters. As it is well known, heavy metal accumulation in fish tissues is determined as a results of the uptake, excretion, storage and transformation processes which are regulated by homeostatic mechanisms. However, continuous exposures to metals deactivate homeostatic mechanisms and accumulation increases in tissues, eventually causing toxic effects (Javed and Usmani, 2017). The gill plays vital roles in the uptake of metals as it is responsible for the respiration and osmoregulation in fish and is also the main target organ for toxic substances (Heath, 1995). The effects of different chelates in Al accumulation by *Cyprinus carpio* (Muramoto, 1981). The authors demonstrated that exposure of fish to the subletal concentrations of different Al compounds ($AlCl_3.6H_2O$ and $Al_2(SO_4)_3.18H_2O$) increased Al accumulation in tissues, though Al levels in tissues reduced sharply when fish were exposed Al and chelates (EDTA or NTA) combinations. Their findings were similar to the present data, as the highest accumulation occurred in the gill following exposure to Al alone and together with chitosan (Figure 1A). This is apparently due to the fact that the gills are in direct interaction with exposure waters, so that the gill accumulate more Al compared to the other tissues. The other reason for this might be the retention of Al by binding to sialic acid residues in the mucus covering the gill surface (Muramoto, 1981).

Effects of toxic xenobiotic on mortality in the aquatic organisms vary depending on various factors such as toxic potential of xenobiotic, duration and concentration of exposure, chelates, temperature and biology of species in concern. Although there was no fish mortality in the present study, Rao and Kumar (2014) demonstrated that there was fish mortality (*Channa punctatus*) following exposure to 0.001 M Al on the 30th day and 80% of the fish died at the end of 60th of exposure period. In a study conducted with *O. niloticus*, the 96 hour LC50 value for Al was found to be 68.03±0.86 mg/L and the lethal concentration was 111.00±10.02 mg/L (Noureen, 2017). Chelates used to remove pollutants from the medium can also cause fish mortality if they are used in high levels. The lethal effects of EDTA (0.5 g/L) in *O. niloticus* (Janes et al., 1998) and chitosan (0.075, 0.75 ppm) in *Oncorhynchus mykiss* (Bullock et al., 2000) were evident, as the fishes died in a few days. However, no fish mortality occurred following exposures to Al alone or together with 10 ppm chitosan up to 21 days in the present study, indicating the concentrations of both Al and chitosan were not in the range of lethal concentrations for tilapias.

Fish react to changing environmental conditions by changing their behaviour. There were behavioural changes such as impaired swimming movements, lack of food intake, orientation to the aquarium surface and increase in operculum movements in studies conducted with *O. mykiss* (Rod et al., 1992) and *Brachydanio rerio* (Anandhan and Hemalatha, 2008). In the present study, similar behavioural changes were observed in fish after exposure to Al and Al+chitosan combinations at the beginning of the experiments, but these changes returned to normal at the end of the exposures. This can be attributed to the response of fish to changing environmental conditions and adaptation.

Heavy metals absorbed through the gills are primarily transported to the liver via the circulatory system. The liver is a metabolically active organ in which toxic substances are detoxified (Heath, 1995). Azmat et al. (2012) found that there were significant accumulations in the liver of fishes (*Catla catla, Labeo rohita, Cirrihinus mrigala*) after exposure to Al. Similarly, the present data also demonstrated that there were

significant accumulations of Al in the tissues of *O. niloticus* following exposure to Al alone and in combination with chitosan (Fig 2). Accumulation of great amount of Al in the liver may result from the retention of Al in the liver by binding to metal-binding proteins such as metallothionein and glutathione.

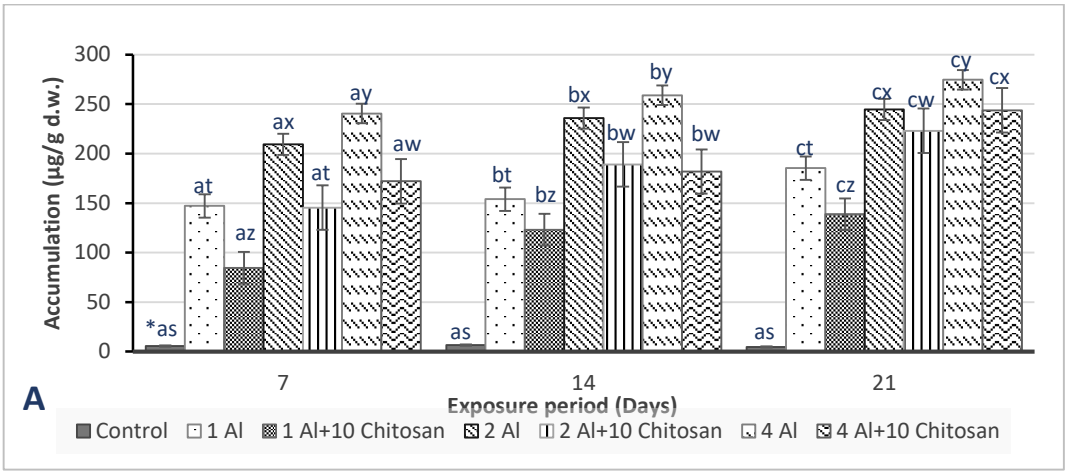

Figure 1. Accumulation of aluminium in the gill tissues of *O. niloticus* following exposure to Al alone and Al+chitosan mixture for 7, 14 and 21 days.

*=SNK; Letters s, t, x, y, w, z and a, b, c show differences among concentrations and exposure periods at a given tissue. Data shown with different letters are significant at the P<0.05 level.

Şekil 1. Al'un tek başına ve kitosan ile birlikte 7, 14 ve 21 gün sürelerle etkisinde O. niloticus'un solungaç dokularındaki birikim düzeyleri.

**=SNK; s, t, x, y, w, z derişimleri; a, b, c harfleri etkide kalma süreleri arasındaki farklılıkları gösterir. Farklı harflerle gösterilen veriler arasında P<0.05 düzeyinde istatistik ayrım vardır.*

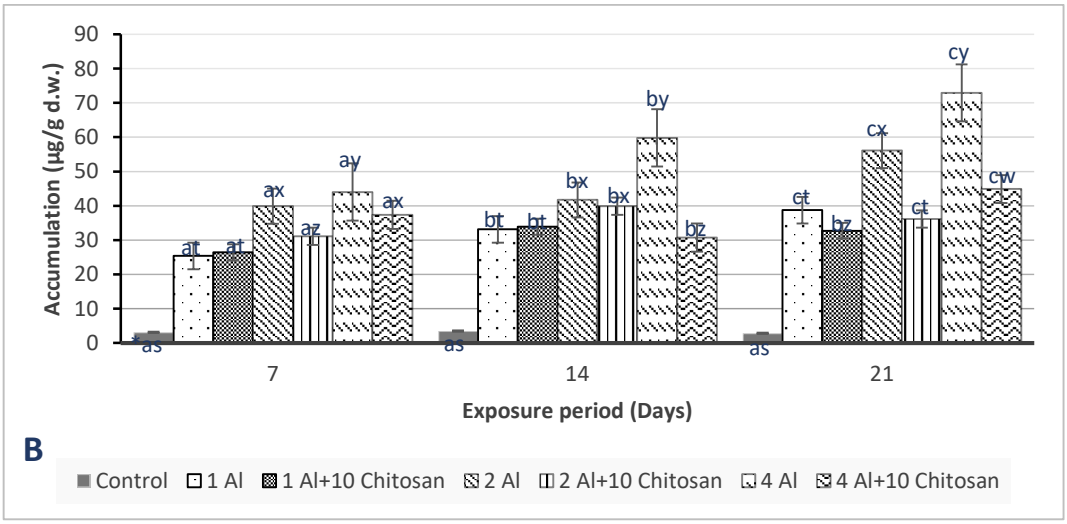

Figure 2. Accumulation of aluminium in the liver tissues of *O. niloticus* following exposure to Al alone and Al+chitosan mixture for 7, 14 and 21 days.

*=SNK; Letters s, t, x, y, w, z and a, b, c show differences among concentrations and exposure periods at a given tissue. Data shown with different letters are significant at the P<0.05 level.

Şekil *2. Al'un tek başına ve kitosan ile birlikte 7, 14 ve 21 gün sürelerle etkisinde O. niloticus'un karaciğer dokularındaki birikim düzeyleri.*

**=SNK; s, t, x, y, w, z derişimleri; a, b, c harfleri etkide kalma süreleri arasındaki farklılıkları gösterir. Farklı harflerle gösterilen veriler arasında P<0.05 düzeyinde istatistik ayrım vardır.*

As it is well known, the muscle is the main consumable part of fish by humans. Although muscle tissue is not an active in terms of metal accumulation, it is very important for public health due to the transport of metals to the diet of humans through the food chain. It was determined that muscle tissue of *B. rerio* showed the lowest Al accumulation following exposure to Al (5.69 and 17.08 ppm) up to 28 days (Anandhan and Hemalatha, 2009). Similarly, Al nanoparticles accumulated in *O. niloticus*, lowest accumulation occurring in muscle tissues (Abdel-Khalek et al., 2020). Similarly, the present data also demonstrated that the lowest Al accumulation occurred in muscle tissue (Figure 3) compared to the gill and liver, suggesting relatively lower metabolic activity of the muscle.

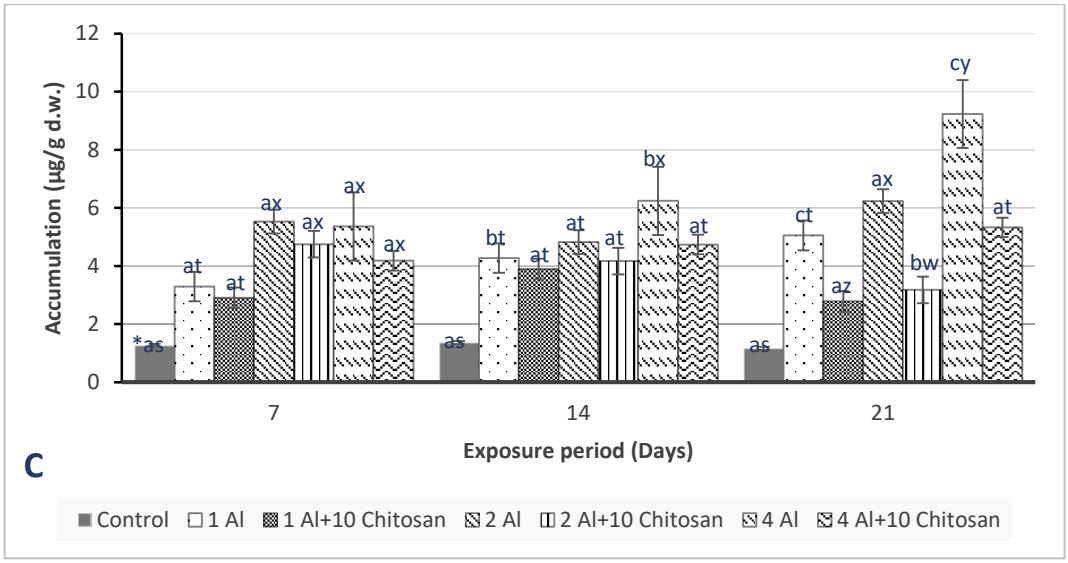

Figure 3. Accumulation of aluminium in the muscle tissues of *O. niloticus* following exposure to Al alone and Al+chitosan mixture for 7, 14 and 21 days.

*=SNK; Letters s, t, x, y, w, z and a, b, c show differences among concentrations and exposure periods at a given tissue. Data shown with different letters are significant at the P<0.05 level.

Şekil 3. Al'un tek başına ve kitosan ile birlikte 7, 14 ve 21 gün sürelerle etkisinde O. niloticus'un kas dokularındaki birikim düzeyleri.

**=SNK; s, t, x, y, w, z derişimleri; a, b, c harfleri etkide kalma süreleri arasındaki farklılıkları gösterir. Farklı harflerle gösterilen veriler arasında P<0.05 düzeyinde istatistik ayrım vardır.*

Chitosan is a very strong adsorbent for heavy metals due to the nitrogen content of amino groups (Qin et al., 2003). The effects of adsorbents on metal toxicity differ. It has been determined that while chitosan increases copper accumulation in the gill tissue in *Clarias gariepinus*, it reduces the accumulation in the liver (Tunçsoy et al. 2016). Similarly, it has been determined that DFO and DFP in *C. mrigala* reduced Al accumulation in liver, kidney, gill and muscle tissues (Sivakumar and Khatiwada, 2012). The present data are in accord with the literature data, as chitosan reduced the accumulation of Al in the gill, liver and muscle tissues. This shows that chitosan forms a complex with Al and prevents its binding to active surfaces and finally reduces the uptake by fish.

CONCLUSION

The effects of Al (1, 2 and 4 ppm) alone and together with chitosan (10 ppm) in different exposure periods (7, 14 and 21 days) resulted significant accumulation of Al in the gill, liver and muscle tissues of *O. niloticus*. Highest accumulation occurred in the gill, while the lowest accumulation was in the muscle. The effect of Al together with chitosan reduced the accumulation of Al in the tissues compared to Al alone exposures. Finally, this study suggests that chitosan may be used as an effective chelate in contaminated waters.

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Authors' Contributions

Authors declares the contribution of the author's is equal.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

The ethics committee approval was obtained from Mersin University Animal Experiments Local Ethical Committee by decision number 14/41 dated 04/11/2016.

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