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Effect of Probiotic on Copper Nanoparticle Accumulation in *Dreissena polymorpha*

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A B S T R A C T

Materials with dimensions between 0.1 and 100 nm are called nanoparticle (NP) materials. In recent years, the usage areas and quantities of NP materials have increased in parallel with the development of the industry. The need and usage areas of heavy metals such as Cu have also expanded in NP sizes. All these developments have led to problems on the ecosystem that are becoming more difficult to compensate. In this study, Zebra Mussel (*Dreissena polymorpha*) was chosen as a model to investigate the effect of probiotics on CuNP heavy metal accumulation. The model organism was exposed to three different concentrations of CuNP (5, 10, 50 mg/L) with probiotics and directly for 24 and 96 hours. CuNP accumulation amounts in *D. polymorpha* tissues treated directly and with probiotics were compared. The amount of accumulation in the test organism directly exposed to CuNP was higher compared to the groups administered with probiotics, but a statistically significant difference ($p<0.05$) was found only in the treatment group with the highest 24-hour concentration (50 mg/L). As a result, according to the findings obtained from the study, it has been determined that probiotics have positive developmental effects on aquatic organisms, as well as beneficial in the elimination of their accumulation in the organism.

Keywords: *Dreissena polymorpha*, Copper Nanoparticle, probiotic, bioaccumulation

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Probiyotiğin *Dreissena polymorpha*'da Bakır Nanopartikül Birikimi Üzerine Etkisi

Öz: Boyutları 0,1-100 nm arasında olan materyaller nanopartikül (NP) malzemeler olarak adlandırılmaktadır. Son yıllarda endüstrinin gelişmesine paralel olarak NP materyallerin kullanım alanları ve miktarları da artmıştır. Cu gibi ağır metallerin de NP boyutlarında gereksinimi ve kullanım alanları genişlemiştir. Tüm bu gelişmeler beraberinde ekosistem üzerinde telafisi zorlaşan sorunları doğurmuştur. Yapılan bu çalışmada probiyotiklerin CuNP ağır metal birikimi üzerine etkisini araştırmak için model canlı olarak Zebra Midye (*Dreissena polymorpha*) seçilmiştir. Model organizma probiyotikli ve doğrudan olmak üzere CuNP'ün üç farklı konsantrasyonuna (5, 10, 50 mg/L) 24 ve 96 saat süreyle maruz bırakılmıştır. Doğrudan ve probiyotik ile muamele edilen *D. polymorpha* dokularındaki CuNP birikim miktarları kıyaslanmıştır. CuNP'ye doğrudan maruz bırakılan test organizmasındaki birikim miktarı probiyotik uygulanan gruplara kıyasla daha fazla olduğu, ancak yalnızca 24 saatlik en yüksek konsantrasyon (50 mg/L) olan uygulama grubunda istatistiksel açıdan anlamlı ($p < 0,05$) fark bulunmuştur. Sonuç olarak çalışmadan elde edilen bulgulara göre, probiyotiklerin sucul canlılar üzerinde olumlu gelişimsel etkilerinin yanısıra organizmada birikim miktarlarının eliminasyonunda faydalı olduğu tespit edilmiştir.

Anahtar kelimeler: *Dreissena polymorpha*, bakır nanopartikül, probiyotik, biyobirikim

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Introduction

Copper (Cu), which is one of the basic nutrients for humans and other living things, also forms an important part of the oxidation-reducing enzyme system (Muralisankar et al. 2016). Cu has an important role in connective tissue, iron metabolism and the central nervous system (Turnlund 1994; Watanabe et al. 1997; Lall 2002). Some studies have revealed Cu requirements in various aquatic organisms (Lorentzen et al. 1998; Lee and Shiau 2002; Wang et al. 2013; Shao et al. 2012). However, it has been reported that high Cu levels can be toxic to aquatic organisms as they increase reactive oxygen species (ROS) and oxidative stress (Clearwater et al. 2002). The effects of nano-based products may differ due to the nature of nanoparticles (NP), the characteristics of the environment, the way NPs are administered and the immune system of the living organism, and the production of aquatic organisms in different environments (for example, freshwater and saltwater or tropical and temperate regions) (Katuli et al., 2017). Considering the widespread use of NPs, attention should be paid to the environment and human health (Çimen et al. 2020). A product that is harmless on a larger scale can create a reactive and toxic state at the nanoscale. Problems with NPs have emerged with the use of NPs in human life (Dagani 2003; Dreher 2004; Hoet et al. 2004). The mixing of NPs in the aquatic environment leads to the accumulation of metals in organisms and produces long-lasting effects (Yu et al. 2020). It occurs when NPs enter the body through permeable membranes (such as gills) and interfere with different reactions by differentiating the natural metal ions of metabolic enzymes, causing disruption of protein structures and forming DNA cross-links that can disrupt the cell cycle (Garai et al. 2021). As a result of human consumption of NPs in aquatic organisms, similar effects such as neuronal, hepatic, renal and reproductive damage and cardiovascular and peripheral vascular diseases occur in humans (Azeh et al. 2019).

Feed additives such as prebiotics and probiotics are effective against NPs and pathogens by maintaining intestinal barriers and healthy intestinal bacterial count (Yukgehnaish et al. 2020; Arun et al. 2021; Yaqoob et al. 2021). Prebiotics and probiotics are used in fish, shrimp, etc. to improve growth performance, increase utilization of feed, strengthen the immune system, produce inhibitory compounds against pathogens, suppress virulence genes and reduce the expression of proinflammatory cytokines such as interleukin (IL). It is widely applied in the cultivation of aquatic organisms (Kakade et al. 2023).

The zebra mussel (*Dreissena polymorpha*) (Figure 1) is a reference species for ecotoxicological studies in aquatic ecosystems. These mussels are mainly distributed in lakes and reservoirs in Turkey. As a species that is not endangered and can be encountered continuously in nature, has stable behavior and sufficient body size, it is easier to sample than other species (Binelli 2015; Serdar 2021; Erguven et al. 2022) and has been preferred because it is not selective in food intake (Serdar 2021).

Figure 1. *Dreissena polymorpha*

In this study, it was aimed to investigate the effect of CuNP heavy metal on the accumulation amount with probiotic supplementation in the aquatic organism *D. polymorpha*.

Materials and Methods

Model Organism Supply and Adaptation

D. polymorpha individuals were collected from the Euphrates River (38º48'25” N, 38º43'51” E) (Figure 2) and quickly brought to the laboratory in plastic bottles. The temperature was set at 18 °C for 30days before using it in the experiments. Then, it was adjusted to laboratory conditions, which were similar to the natural living conditions, using 200 L oxygen-supplemented tanks. The aquatic environment was also refreshed by the addition of rested tap water. During the adaptation, a photoperiod of 12:12 light:dark cycle was applied and the organisms were fed with microalgae. Organisms at similar developmental stages were selected for the study and were not fed during the experimental study.

CuO Nanoparticles

The metal-based Cu-NP (60-80nm) chemical (Sigma-Aldrich) was purchased, and CuO-NP

(40nm) was purchased from SkySpring (Houston, TX, USA). No purification or analytical reagent classification has been done for Cu-CuO NP chemicals. In the bioassay studies, the shape and size data declared by the manufacturer were taken as a reference.

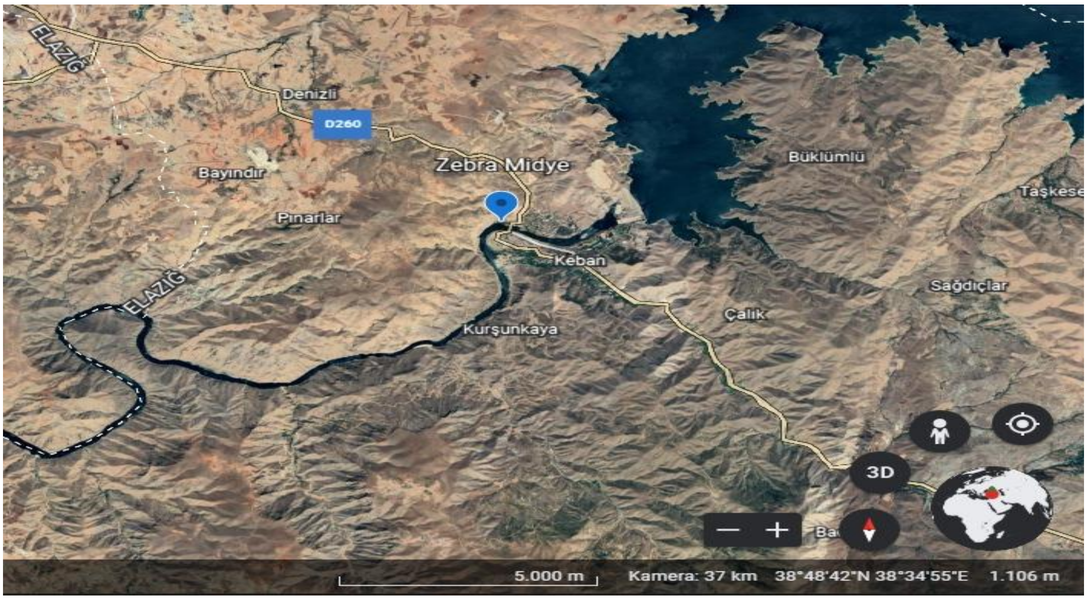

Figure 2. The area where living material is collected, Euphrates River (38º48'25" N, 38º43'51")

Probiotic Supply

The probiotic utilized in the study was acquired from Uğur Aqua Aquaculture Food Industry Trade. Bacteria species in probiotic content: *Lactobacillus plantarum, Lactobacillus casei, Enterococcus faecium, Bacillus subtilis, Pediococcus spp*. Bacterial colony count (CFU/ml): 2.2 x 10^{11}.

Acute Toxicity Tests (LC50)

Acute toxicity tests were not performed since the damage caused by pollutants to the environment was taken into account. Subacute values were determined by a literature review (Cimen and Serdar, 2022).

Experiment Design

After a 30-day adaptation period, 100 individuals with similar development and body size were selected from *D. polymorpha* individuals obtained from the Euphrates River and placed in 80-liter aquariums. Metric meristic data of the mussels used in the trial design (weight; 1.005+-0.29 g, length; 20.28+-2.09 mm, width; 10.04+-0.96 mm, height 9.74+-1 It was recorded as .07 mm). Dried microalgae was fed twice a day during the adaptation period. The stock aquarium/tanks were supplied with oxygen by aquarium air motors. Physico-chemical parameters of the ambient water during the experimental application; ambient water temperature 18 ± 1 °C; dissolved oxygen: 11.04 ± 0.15 mg/L; pH:8.12 ±0.45; electrical conductivity: 461 ± 43 µS cm-1; salinity: 0.11 ± 0.01 g/L were measured and recorded regularly daily.

Two different experimental design groups were created to investigate the effect of CuNp accumulation on probiotic use. In the first experimental group created, CuNp was applied at the determined concentrations without using probiotics (Figure 3).

CuNp was applied to *D. polymorpha* organisms in the 2nd experimental group at the determined concentrations of 3% of the water volume for 21 days. After the probiotic application, CuNp application and Probiotic + CuNp applications were applied simultaneously and in triplicate in both experimental groups.

As in all toxicological studies, application concentrations were determined by considering the application concentrations determined in CuNp application study, the rates of release to the environment and the values in this range. CuNP concentrations of 5, 10, 50 mg/L were established as sublethal concentrations.

After 21 days of probiotic application;

Groups of 8 organisms were formed in 1000 ml aquariums at 5, 10, 50 mg/L direct and CuNP and probiotic applied concentrations, and each concentration was repeated 3 times. 8 animals were used for each experimental group and hour (24 and 96). No changes were made in the water environment and the number of living things during the trial period (96 hours). A certain number of samples were taken from the application groups at 24 and 96 hours, labeled and stored at -86^{0}C until the accumulation amounts were measured.

C1: CuNP 5 mg/L
C2: CuNP 10 mg/L
C3: CuNP 50 mg/L
C4: Probiotic (30 ml) + CuNP 5 mg/L
C5: Probiotic (30 ml) + CuNP 10 mg/L
C6: Probiotic (30 ml) + CuNP 50 mg/L

Figure 3. Experiment design

Dissolving Process

For the dissolution process, a microwave dissolution treatment system (Berghof, Germany) was used to thaw *D. polymorpha* samples. Approximately 0.15 g of *D. polymorpha* sample was taken into the digestion vessel and 4 mL of concentrated nitric acid (HNO_3) and 1 mL of hydrogen peroxide (H_2O_2) were added (Table 1). The mixture was shaken carefully and after waiting for at least 20 minutes, the container was closed and the disintegration program was applied (Serdar et al. 2019). After centrifugation, the clear solutions were diluted to 15 mL (Pala et al. 2019). It was mixed with ultrapure water and CuNP ion concentrations in solution were measured by electrothermal atomic absorption spectrophotometer (ETAAS).

The amount of *D. polymorpha* CuNP measurement values, which is a living material, was calculated with the calibration curve obtained from known standard solutions (Figure 4).

Table 1. *D. polymorpha* Sample Digestion Method

Biological Sample	Weight (gr)	Solvent	Volume(ml)	Temp.(°C)	Pressure (Atm)	Time(Min)
D.Polymorpha	0,15	HNO_3	4	150	15	4
		H_2O_2	1	180	25	5

Results

In treatment groups directly exposed to CuNP heavy metal (5, 10 and 50 mg/L), *D. polymorpha*. The amount of accumulation in *D. polymorha* tissues was measured at 24 and 96 hours. It was found that there was a linear increase between increasing concentration and application time, but the calculated increase was statistically insignificant ($P < 0.05$) (Figure 5).

CuNP accumulation amounts were measured in *D. polymorha* tissues treated with probiotics for 24 and 96 times. It was found that there was a linear increase between increasing concentration and application time, but the calculated increase was statistically insignificant ($P < 0.05$) (Figure 5).

When the amount of CuNP accumulation in *D. polymorha* tissues treated directly and with probioticswas compared 24 and 96 hours, it was found that there was a statistically significant ($P < 0.05$) difference only in the 24-hour highest concentration (50 mg/L) application group ($P < 0.05$) (Figure 5).

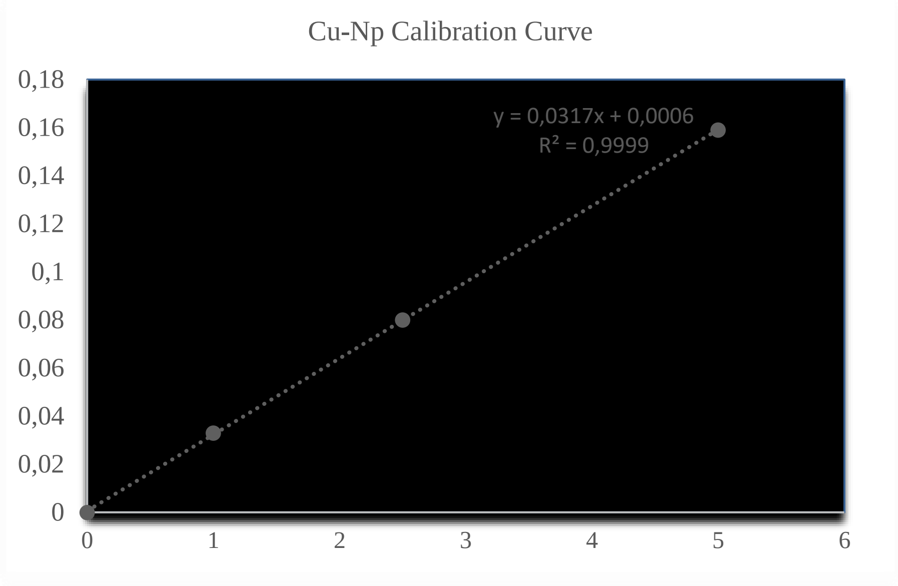

Figure 4. Calibration curve of Cu-Np

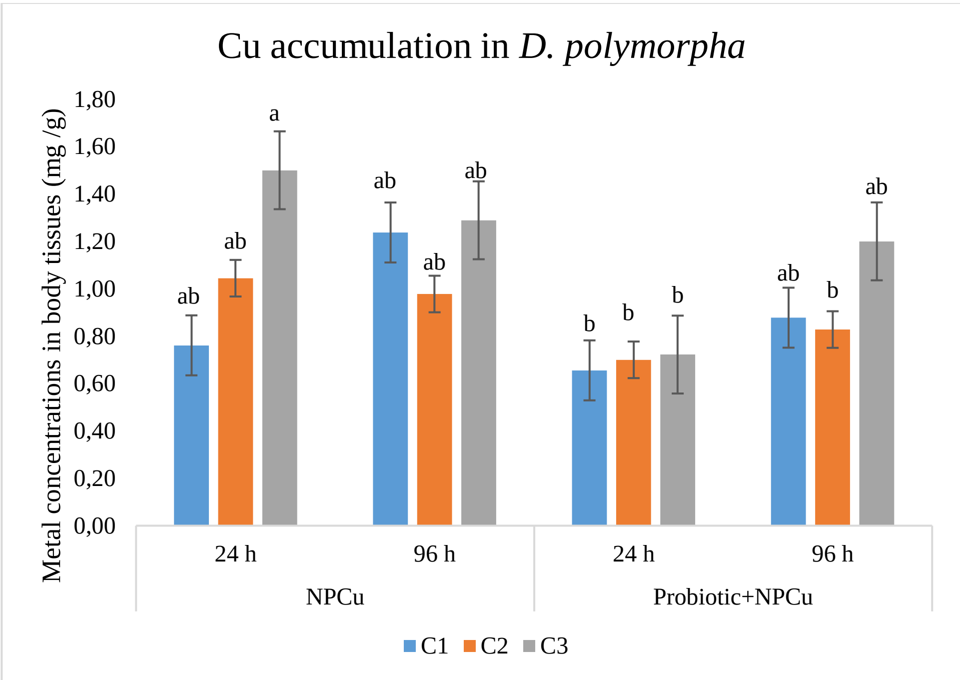

Figure 5. Cu accumulation in *D. Polymorpha*

Discussion

Çimen et al. (2020) investigated the accumulation rates of Cu (60–80 nm) and CuO (40 nm) nanoparticles in *Artemia salina*, and as a result, there was a difference in the accumulation and elimination rates of both NPs in parallel with the increase in concentration and at each application time they indicated. Dağlıoğlu and Öztürk (2016) evaluated single-celled freshwater algae (*Desmodesmus multivariabilis*) test organism for the bioaccumulation of boron nanoparticles. As a result, they observed that nano and micro boron particles accumulated in different amounts in algae. Le et al. 2021 investigated Cu accumulations under chronic exposure at various pH and sodium (Na) concentrations in *D. polymorpha* and stated that pH and Na have a significant effect on Cu uptake and accumulation rates in the body. According to Clayton et al. (2000), D. polymorpha showed a high tendency to accumulate copper and tributyltin (TBT) contaminants. When pooled, the replicated samples exhibited significant differences from the controls, despite the presence of relatively high inter-individual variability. Mersch et al. (1993) evaluated the potential of the freshwater organisms *D. polymorpha* and *Rhynchostegium riparioides* as indicators of heavy metal contamination, consequently, exposure of both metals to Cu and Cd over a 27-day period resulted in the accumulation of both metals in *R. riparioides*. They stated that it was fast and it was not observed that it was slower for *D. polymorpha*. It has also been observed that *D. polymorha* individuals exposed to CuO NP accumulate in the model organism depending on time and concentration.

Sandeva et al. (2016) observed a slight improvement in some parameters (nitrates, nitrites, permanganate) by applying probiotics for 4 weeks in *Cyprinus carpio*. Sharifuzzaman et al. (2011) evaluated the efficacy of the cellular components of probiotics Kocuria SM1 and Rhodococcus SM2 to protect rainbow trout (*Oncorhynchus mykiss*) against vibriosis and stated that probiotics provide significant protection. Sami et al. (2020) evaluated the growth performance and survival rates of the examined species by adding probiotics to fresh algae, yeast and bacteria in *Ruditapes decussatus*. They reported that the highest specific growth rate and growth gain in total weight of test organisms were in mixed algae-probiotic feeding, and the highest in wet weight and dry weight was in the group fed with bacteria-probiotics. Giri et al. (2018) investigated the protective effects of the probiotic *Lactobacillus reuteri P16* against the toxicity caused by Pb exposure in *Cyprinus carpio* and stated that *L. reuteri P16* reduced the accumulation of Pb in tissues. Daisley et al. (2019) stated that *Lactobacillus rhamnosus GR-1* probiotic reduces the separation of Pb and Cd and their absorption through the intestinal epithelium. Tawwab et al. (2010) aimed to evaluate the growth response and resistance to water-borne copper toxicity of *Sarotherodon galilaeus* probiotic in *Galilee tilapia*, and as a result, it reduced Cu absorption; indicated that it had a positive effect on growth and feed utilization. Yu et al. (2020) examined the copper-induced accumulation and histopathological-biochemical parameters of dietary microbial flox in *Rhynchocypris lagowski dybowski*, and stated that their diet reduced Cu accumulation. Madreseh et al. (2018) investigated the accumulation and some parameters of some heavy metals (cadmium, lead, nickel, zinc) in *Oncorhynchus mykiss* tissues of feeding with encapsulated *Lactobacillus fermentum* and Lactulose; As a result of the examination, they stated that *L. fermentum* prevented the absorption and accumulation of heavy metals in the liver and gills of rainbow trout. Kargar and Shirazi (2020) examined the removal rates of Cu and Zn ions from the aquatic environment of *L. fermentum* and *Lactobacillus plantarum* probiotics for Cu and Zn in their study; indicating that both metals and both species showed rapid biosorption. Bhakta et al. (2012) examined water samples of Cd and Pb resistant lactic acid bacteria from some regions where aquaculture is used as probiotics, and as a result, these probiotics have the potential to remove heavy metals from the fish intestinal environment to control the progressive bioaccumulation of heavy metals in fish. They stated that they can be used as probiotic strains.

The data obtained from this study show parallelism with the information in the literature. It was determined that there was less Cu-Np accumulation in the groups treated with probiotics compared to the groups not given probiotics. In this respect, the study is similar to the information in the literature.

With this study, the effect of probiotics on heavy metal accumulation in the body was investigated and it was observed that they play an important role in positive excretion and elimination from the body, preventing or reducing the amount of accumulation. According to these results, the study is consistent with previous research in this field and is expected to lead the studies to be done in the future. It is believed that probiotics have an impact on the excretory system of the body and they are resistant to heavy metal accumulation in the body in this respect. *D. polymorha* is a suitable biomonitor to quantify the bioavailable of Cu-Np in freshwaters besides a continuous biomonitoring system as part of ecological risk assessment. Furthermore, this model can be used as a predictive tool to evaluate the quality of the environment.

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