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Toxicity of nickel oxide nanoparticle in *Capoeta fusca, using* bioaccumulation, depuration, and histopathological changes

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ABSTRACT

BACKGROUND AND OBJECTIVES: The nanoparticles has been discussed in terms of their adverse global impacts on health and health inequity. Metal oxide nanoparticles and their salts can have varied toxic effects on different tissues in the aquatic environments. The aim of present study was to assess the toxicity of nickel oxide nanoparticles in relation to different nickel salts.

METHODS: Acute toxicity of nickel oxide nanoparticles, nickel sulfate, nickel nitrate and nickel chloride, in black fish was investigated. A total of 125 fish were randomly assigned to a control group (n=25) and four exposure groups (n=25 per group). After 28 days of exposure, the bioaccumulation of nickel oxide nanoparticles in gill, intestine, liver, and kidney of the fish was determined by killing half of them in each group and dissecting their tissues. The remaining fish were placed in clean water for another 28 days and the depuration rate was estimated.

FINDINGS: The $LC_{50\text{-}96}$ values reported for nickel oxide nanoparticles, nickel sulfate, nickel nitrate and nickel chloride were 195, 120, 138 and 91 milligrams per liter, respectively. Therefore, nickel chloride had a higher toxicity compared to nickel oxide nanoparticles, nickel sulfate and nickel nitrate. The highest rate of nickel oxide nanoparticles bioaccumulation was observed in the gill (0.40±0.08 microgram per gram), intestine (41.82±16.95 microgram per gram), liver (2.16±1.82 microgram per gram), and kidney (2.16±1.26 microgram per gram) of the fish. The highest depuration rate of nickel oxide nanoparticles, nickel sulfate, nickel nitrate, and nickel chloride was recorded in the intestinal tissue of the fish. The lowest depuration rate of nickel oxide nanoparticles, nickel sulfate and nickel nitrate was observed in the kidney tissue of the fish. Also, the lowest depuration rate of nickel chloride was witnessed in the gill tissue of the fish. Histopathological anomalies were detected in the fish exposed to nickel oxide nanoparticles. These anomalies were fusion of lamellae, lamellar synechiae, curvature and oedema in the gill; increased number of goblet cells and cell swelling; and degradation of villi structure and expansion of villi structure in the intestine.

CONCLUSION: The study conclusively demonstrated that nickel oxide nanoparticles were eco-toxic and harmful to aquatic organisms. Strong global nickel oxide nanoparticles regulations must be enforced to prevent further environmental contamination with nanoparticles.

NUMBER OF REFERENCES	NUMBER OF FIGURES	NUMBER OF TABLES
44	5	5

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INTRODUCTION

Every year, millions of tons of nanoparticles are produced and released by powerful global industries into the environment (Giese *et al.,* 2018), while there is little to no regulations restricting the nanoparticles (NPs) environmental release. However, recently, in a welcomed turn of events, European Commission has banned a common nanoparticle, which is used as food additive, and a few nanoparticles frequently used in cosmetics. In general the multi-billion-dollar nanotechnology industry, runs wild with no regulations to restrict its risky activities. Measured in billionths of a meter, nanoparticles are comparable in size to viruses, proteins, and antibodies that can freely pass cell membrane and impact the molecules inside each cell, leading to serious allergic reactions in humans (Borgsteede *et al.,* 2021). Additionally, carcinogens, mutagens, and reproductive toxicants (collectively referred to as CMRs) have the potential to cause asthma (The UK Health, and Safety Executive). Additive toxicity can stem from NP reactions with other pollutants (Iftikhar *et al.,* 2021; Kamyab *et al.,* 2022). NPs have the potential to increase environmental toxicity because of their uncertain shape, size, and chemical composition (Dunphy Guzmán *et al.,* 2006). NPs have even been discussed in terms of their adverse impacts on global health and health inequity (Salamanca-Buentello and Daar, 2021). Riverine, freshwater, and marine ecosystems are the final depository for many NPs (Balaraman *et al.,* 2022). They are ommonly found in solid waste, wastewater effluent, direct industrial discharges, and accidental spills. Nickel oxide nanoparticles (NiO NPs) are of interest in this study because they are carcinogens with low solubility and high stability in water. They can easily be ingested by aquatic organisms (Gong *et al.,* 2016). NiO NPs also have high production rate and are widely used in multiple industries including solar and fuel cell manufacturing, automobile catalytic converters, lithium-ion batteries, and biosensor devices (Salama *et al.,* 2020). Gill, intestine, liver, and kidney, which readily absorb and accumulate contaminants, are appropriate tissues for histopathological examination. NiO NPs damage to fish internal tissue can be accurately assessed by histopathological examinations (Bais and Lokhande, 2012; Alijani Ardeshir *et al.,* 2017). Current state of knowledge suggests that metal oxide nanoparticles and their salts can have varied toxic effects on different tissues in the aquatic environments. For instance, Se-NPs are more toxic than Se salts in gill and liver tissues of iridescent shark catfish (*Pangasianodon hypophthalmus*) (Kumar *et al.,* 2018), and silver nanoparticles are more toxic than silver salts to gill and liver of European carp (*Cyprinus carpio*) (Liaqat *et al.,* 2021). However, iron nitrate [Fe $(NO_3)_3$] salt is more toxic than iron NPs in different tissues of black fish (Sayadi *et al.,* 2020). Based on the available information, no study yet have been carried out on the comprehensive ecotoxicological evaluation of the effects of NiO NPs in relation to their salts in black fish. The present study strives to compare the toxicity of NiO NPs and different nickel salts in black fish. In fact, the aim of this study was to compare the acute toxicity, chronic toxicity, and histopathological alterations of NiO NPs to different nickel salts in the fish. The uptake and depuration kinetics of different nickel compounds in the varied tissues of the fish were also investigated. This study has been carried out in University of Birjand, Birjand city, Iran in 2021.

MATERIALS AND METHODS

NiO NPs and nickel salts characterization

NiO NPs with 99.5 percent (%) purity was obtained from Twig Leaf Lane, Houston, TX, USA. Nickel salts (nickel sulfate, [$NiSO_4$], nickel nitrate [Ni $(NO_3)_2$], and nickel chloride [$NiCl_2$]) were procured from Merck, Germany. Energy Dispersive Spectroscopy (EDS) was used to determine the presence of NiO NPs; scanning electron microscopy (SEM) was applied to illustrate the morphology of NiO NPs; and X-ray Diffraction (XRD) and X-ray Photoelectron Spectra (XPS) were utilized to investigate the properties of NiO NPs. X-ray signals are arranged from lower to higher energy according to their atomic number. Elements in each sample were semi-quantitatively identified. Each peak was assigned to a specific atom. Higher peaks signify higher concentrations of an element in the sample. XRD was used to examine the properties of crystals. XPS is a surface-sensitive method which analyzes the chemicals on sample surface. This method was used to assess the purity and chemical composition of NiO NPs.

Black fish (Capoeta fusca) and test conditions

Black fish, native to eastern Iran, were collected from Eshkaftook aqueduct (59°09.6878'N 32°97.9754'W) in April and June 2021. Fiberglass aquarium containing 200-liter water from the aqueduct, with an aeration system, was used to transfer the fish to Birjand

University Limnology Laboratory. To keep the water clean and free of bacteria and other pathogens, 50% of aquarium water was replaced with clean water until the start of the experiment (10 days). During this period, the average water temperature was kept constant and the fish were fed twice a day (1% body weight).

Acute toxicity

Acute toxicity tests were carried out under controlled laboratory conditions, and used to determine the lethal dose/concentration of a substance that causes death in 50% of the test population (lethal dose 50 or LD_{50}/LC_{50}) during a short-term exposure. In other words, LC_{50} was used as a way to measure the short-term poisoning of the substance. It is the amount of a toxicant that causes death to half of a group of test animals when given at once. NiO NPs, and other nickel salts were obtained from Merck Company (Germany) in a powder form. Using distilled water, a 1000 milligram per liter (mg/L) dilution was prepared and placed on an ultrasonic device for 30 minutes until a homogeneous and uniform solution in composition was obtained. Then, serial dilutions of NiO NPs and nickel salts were prepared to assess their acute toxicity and LC_{50}. 1, 10, 20, 30, 50, 100, 200, 300 and 400 mg/L dilutions were prepared. LC_{50} was determined for each compound. For LC_{50} determination, the fish (n=10) were exposed to nickel compounds for 24, 48, 72 and 96 hours. After dosing the fish, the dead fish were quickly removed from the aquarium and the number of the dead fish at each concentration were recorded. Mortalities at 24, 48, 72 and 96 hours were used to calculate Lethal Concentration 50 (LC_{50}). For LC_{50} calculations, average body length and average body weight were recorded (LC_{50}; OECD 1993).

Chronic toxicity studies

Chronic toxicity tests typify adverse effects following repeated administration of a test substance over a significant portion of test specie's life span. The lower sub-lethal concentration (LSC) is commonly used for chronic toxicity testing. It dosed the fish with one tenth (1/10) of LC_{50} for chronic toxicity testing as follows: NiO NPs (19.49), $NiSO_4$ (12.02), Ni $(NO_3)_2$ (13.80), and $NiCl_2$ (9.12) mg/L. Fig. 1 describes the study design for determining bioaccumulation and depuration (chronic toxicity) of NiO NPs and nickel salts. Briefly, a total of 125 fish were randomly assigned to a control group (n=25), and four exposure groups (n=25 per group). After 7, 14 and 28 days of exposure to NiO NPs and nickel salts, 3 fish were removed from each aquarium and their gill, intestine, liver, and kidney tissues were removed for analysis. The remaining fish (n=15 per group) were placed in clean water. After another 7, 14, and 28 days in clean water, 3 fish were killed, and their tissues were similarly dissected for analysis.

Acid digestion of tissues

Gill, intestine, liver, and kidney of each fish were removed and separately placed in glass tubes. 5 millilitre (mL) of nitric acid (HNO_3, 65%, Merck, Germany) and 2.5 mL of perchloric acid ($HClO_4$, 70%, Merck, Germany) were added to each tube. Glass tubes were then placed in a water bath (TW12, Julabo Co., Germany) at 100 °C for 4-6 hours. Fully digested samples were passed through a filter paper (Whatman grade 40, Nikoshimi Co, Iran). Then the paper was discarded and the remaining liquid (~ 5 mL) was washed with distilled water into another plastic tube. The volume of each tube was filled with distilled water up to 20 mL. Concentration of nickel ions in individual tissues of each fish was measured using the graphite furnace atomic absorption spectroscopy (ContrAA 700, Analytik Jena AG, Germany).

Histopathological examinations

For histopathological examination, two fish from each aquarium were removed at 28 days. Their gills and intestines were removed and fixed for 24 hours in a 10% formalin buffer. The fixed tissues were dehydrated for an hour in 90%, 95% and 100% alcohol solutions. A 30-minute treatment of the samples with xylene, rendered them transparent. The samples were then molded using liquid paraffin (60°C). The tissues were cut in 5 µm thick slices using a microtome. Afterwards, the tissue slices were placed on glass slides and kept in the oven (37 °C). Hematoxylin-eosin was used to stain slides, and a light microscope (Nikon, eclips-E200) was applied to obtain photographs from each slide. Histopathological changes of the tissue were classified into four groups: nil or no changes (-), mild (+), moderate (++) and severe (+++) (Jerome *et al.,* 2017). Tissue alterations, compared to the control, were recorded using an Axio Vision Real 4.8 software. Finally, the qualitative data were collected.

Toxicokinetic modeling

A one-compartment first-order model was used to describe Ni uptake and depuration in the fish.

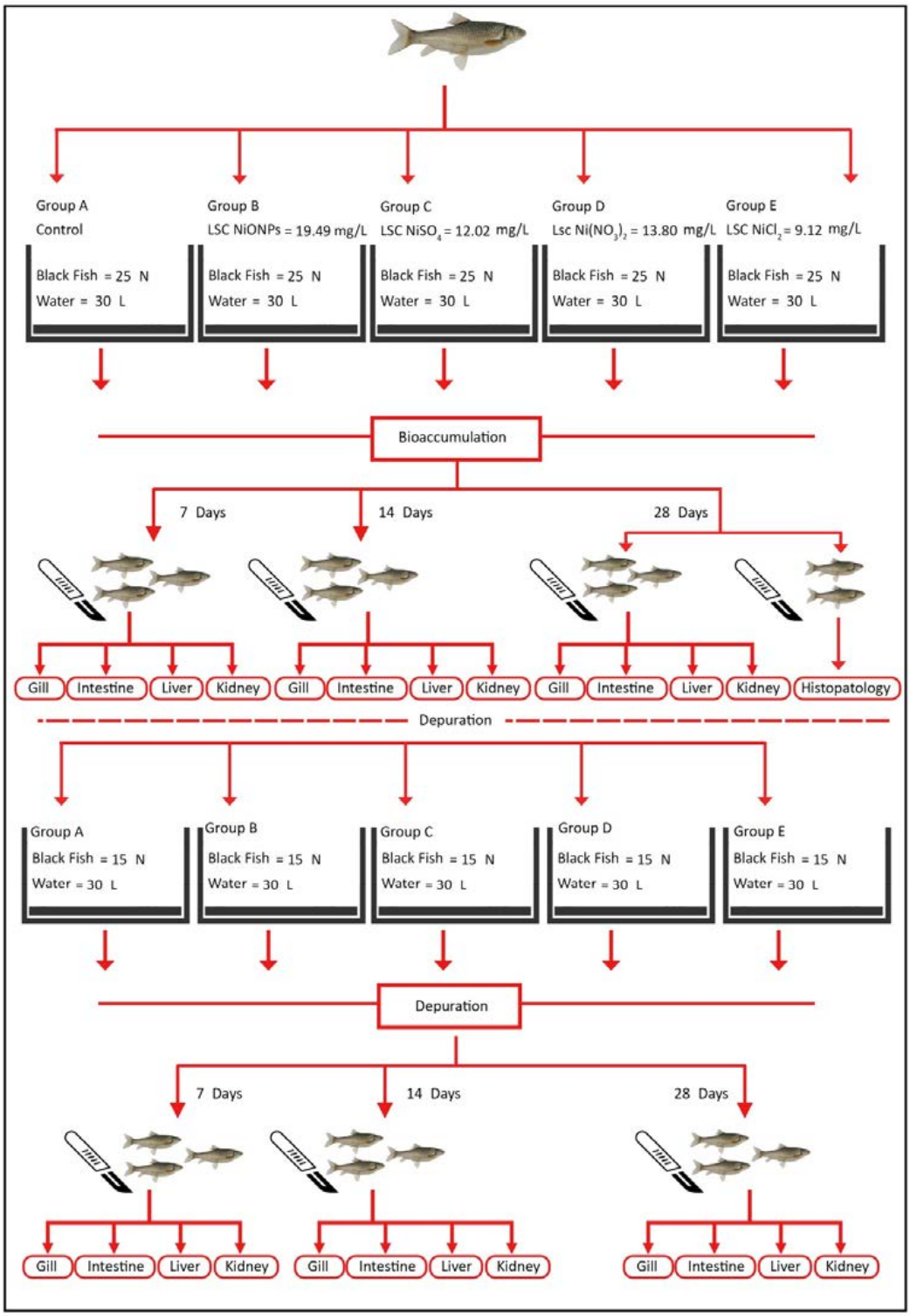

Fig. 1: Study design, including the number of fish in each group and the lower sub-lethal concentrations (or LSC) of toxicants used for determination of bioaccumulation and depuration (chronic toxicity) of NiO NPs, $NiSO_4$, Ni $(NO_3)_2$ and $NiCl_2$ in black fish (*Capoeta fusca*)

Background concentration in the fish was assumed to be constant. C_0 is the amount of toxicant in fish at time zero (Sun *et al.,* 2018). Model 1 (uptake), and model 2 (depuration) are described according to Eqs. 1 and 2, respectively.

Model (1) uptake:

$$Q(t) = C_0 + K_1/K_2 * C_e * (1-e^{(-k_2 * t)}) \quad (1)$$

Model (2) depuration:

$$Q(t) = C_0 + K_1/K_2 * C_e * (e^{(-k_2 * (t-t_c))} - e^{(-k_2 * t)}) \quad (2)$$

Where, Q(t) is Ni concentration in fish in microgram per gram (µg/g) at the time of sampling t; C_0 is the concertation in an organism in terms of µg/g dry body weight at time zero; K_1 is uptake rate constant (per day); K_2 is depuration rate constant (per day); C_e is the exposure concentration in an environment in terms of µg per liter (µg/L); t_c is a period when an organism is transferred to a fresh uncontaminated water (on an hourly basis); and t is the sampling time in hours. These constants allowed for bio-concentration factor (BCF) to be evaluated, because it was assumed that Q(t) has reached a stable state. The BCF and the time when the organisms needed to undergo elimination of half-life of nickel ($T_{1/2}$) were calculated using Eqs. 3 and 4 (OECD 2016).

$$BCF = K_1/K_2 \quad (3)$$

$$T_{1/2} = Ln(2)/K_2 \quad (4)$$

Statistical analysis

SPSS software version 20 was used for all the data analyses. Significance was put at p= 0.05, and One-way ANOVA with Tukey-Kramer post hoc test was used to evaluate the effects of NiO NPs and nickel salts exposure on the fish. LC_{50} values were calculated using Probit analysis with Microsoft Excel.

RESULTS AND DISCUSSION

Ecological studies often use fish response to toxicants and assess damage caused by pollutants like Ni. Changes in molecular physiology and behavior of fish, as a result of exposure to Ni, have been reported (Sibiya *et al.,* 2022). In the present study, NiO NPs and nickel salts were put under comprehensive ecotoxicological evaluation for the first time. This study aimed to increase the current knowledge of Ni and its NPs. It described acute and chronic toxicity of NiO NPs, their histopathological effects, and their uptake and depuration kinetics. These parameters were compared to elucidate the effects of nickel slats ($NiSO_4$, Ni $(NO_3)_2$, $NiCl_2$) on black fish. Such studies are essential to push and regulate these toxic compounds.

NiO NPs properties

The properties of NiO NPs were examined using the instruments such as SEM, EDS, XRD, and XPS (Fig. 2). The SEM image showed that NiO NPs had a particle size of 15-35 mm with spherical morphology, specific surface area (SSA) of 50-100 square meter per gram (m^2/g), bulk density of 0.8 gram per cubic centimeters (g/cm^3), and true density of 6.67 g/cm^3. These structures were formed at a suitable calcination temperature (450 °C) which significantly affected the particle size and indicated that the particles had been dispersed satisfactorily in nanoscale size (Fig. 2a). EDS pattern of NiO NPs confirmed the presence of Ni (percent weight 73.2(, and O (percent weight 26). Concluding that the synthesized NiO NPs were made up of Ni and O with a molecular ratio of 1:1, no other trace elements were present (Fig. 2b). Fig. 2c shows that XRD patterns of NiO NPs have crystalline structure. The diffraction peaks at 37.23°, 43.15°, 62.12°, 75.21° and 78.55° were attributed to the diffraction plates of 111, 200, 220, 311, and 222 in NiO, respectively (ICSD card no. 01-071-2194, Jabeen *et al.,* 2020). Figs. 2d and e illustrate XPS of NiO in samples, and also Ni 2p1/2, Ni 2p3/2, and O 1s binding energies. Ni 2p3/2 peak appearing at 855.6 eV complied with NiO findings. The peak of O 1s at 530.7 eV was attributed to O_2 in NiO (Salavati-Niasari *et al.,* 2009).

Acute toxicity

$LC_{50\text{-}96h}$ values for NiO NPs, $NiSO_4$, $Ni(NO_3)_2$, and $NiCl_2$ are reported in Table 1. NiO NPs ($LC_{50\text{-}24h}$ = 575.5 mg/L) had the highest toxicity during the initial 24 hours of exposure, and $NiCl_2$ had the highest toxicity ($LC_{50\text{-}96h}$ = 91mg/L) after a 96-hour exposure. LC_{50} of different Ni compounds decreased with time, but toxicity of different Ni compounds, especially $NiCl_2$, increasd with time lapse from 24 to 96 hours. Overall, black fish was more sensitive to Ni salts rather than NiO NPs. The specific metal species can also impact toxicity. In addition, fish tolerance and/or resistance to environmental changes play a significant role in toxicity of chemicals. For example, *Rutilus rutilus* and *Carassius auratus* exposed to silver nanoparticles

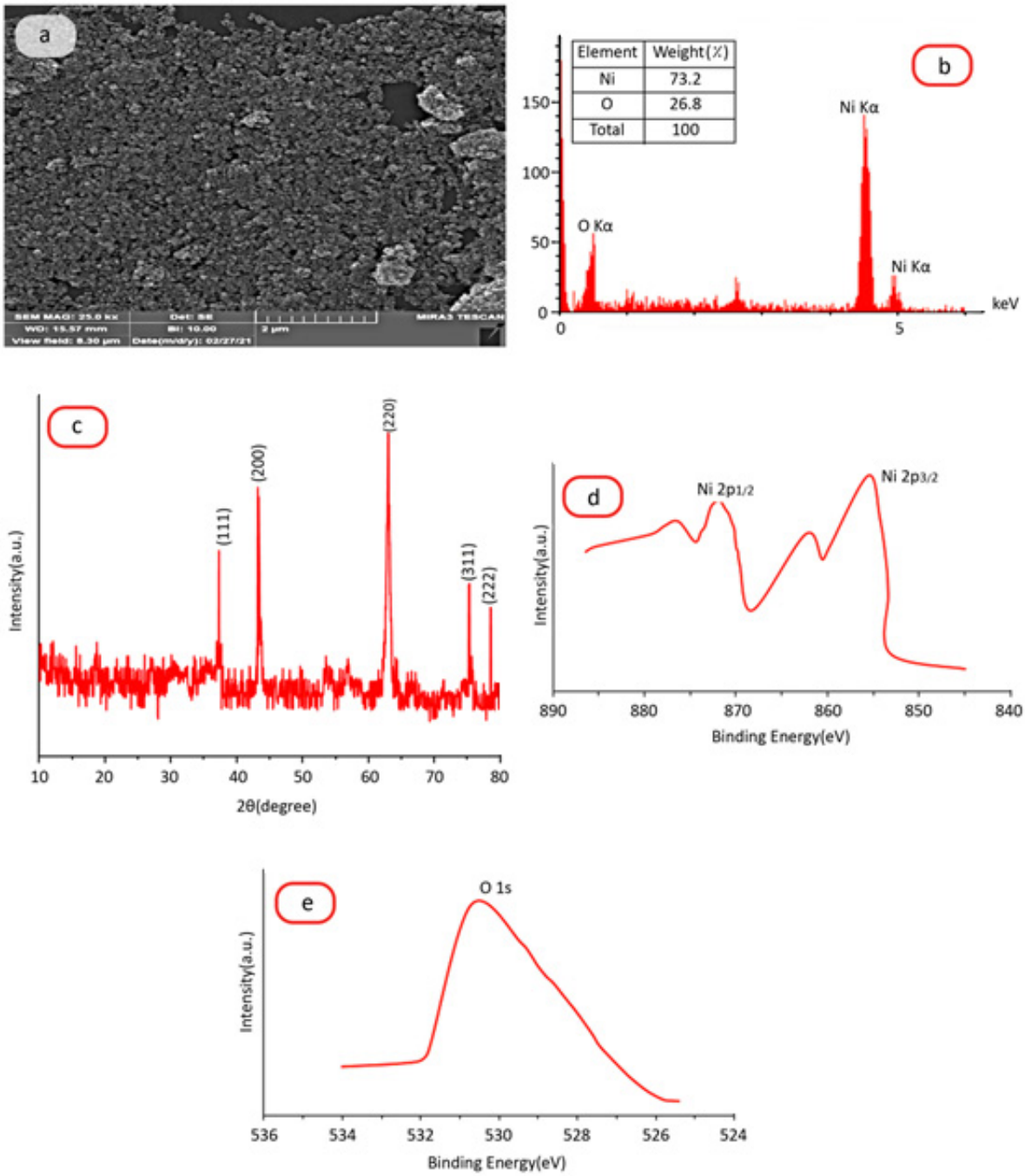

Fig. 2: Scanning Electron Microscopy (SEM) image for NiO NPs (a) , and patterns of X-ray Spectroscopic (EDS) (b), X-ray diffraction (XRD) (c), and X-Ray photoelectron Spectra (XPS) of Ni 2p (d) and O1s NiO NPs (e)

(Ag NPs) exhibited a drastically different $LC_{50\text{-}96h}$ (Yalsuyi and Vajargah, 2017). Compared to large-scale compounds, the metals in nano scale move across cell membrane and in the environment with more ease. This characteristic of NPs increases exposure and can lead to higher toxic effects (Boran and Saffak, 2018).

Table 1: Lethal Concentration (LC) of NiO NPs and nickel salts in black fish (*Capoeta fusca*)

NPs/salts	LC	Concentration (mg/L)			
		24 h	48 h	72 h	96 h
NiO NPs	LC_{50}	575.5	302	282	195
$NiSO_4$	LC_{50}	1862	371.5	155	120
Ni $(NO_3)_2$	LC_{50}	2884	219	182	138
$NiCl_2$	LC_{50}	1071.5	199.5	182	91

Higher toxic effects of Ni NPs compared to Ni salts was expected. However, Ni salts were much more toxic than NiO NPs to the fish. LC_{50} of all nickel compounds decreased with the increase of time from 24 to 96 hours, but its toxicity increased with time, since a lower concentration of nickel compounds caused death to the fish as it has been documented in other studies. Zinc oxide nanoparticles and graphene (a carbon-based nano material) toxicity to black fish increased with the increase of time from 24 to 96 hours (Sayadi *et al.,* 2021). In similar a study, Sayadi *et al.* (2020) stated that $LC_{50\text{-}96h}$ values of iron oxide nanoparticles (Fe_3O_4 NPs), iron nitrate ($Fe(NO_3)_3$), iron chloride ($FeCl_3$) and iron sulfate ($FeSO_4$) salts in blackfish were 32.3, 1.6, 2.3 and 2.5 mg/L, respectively. Based on these findings, toxicity of iron nitrate ($Fe(NO_3)_3$) was higher than that of iron nanoparticles, iron chloride and iron sulfate. With the time lapse from 24 hours to 96 hours, when the fish were exposed to different iron compounds, the toxicity of the compounds, especially $Fe(NO_3)_3$, increased. Boran and Saffak (2018) stated that $LC_{50\text{-}96h}$ value of NiO NPs and $NiCl_2$ in fish larvae (Zebrafish) were 122.2 and 32.6 mg/L, respectively, and concluded that the toxicity of $NiCl_2$ was higher than that of NiO NPs. Ghosh *et al.* (2018) reported the $LC_{50\text{-}96h}$ values of nickel in crustacean (*Diaptomus forbesi*) and fish (*Cyprinus carpio*) as 5.43 and 14.70 mg/L, respectively, and found that *Cyprinus carpio* was more sensitive than *Diaptomus forbesi* to nickel.

Bioaccumulation and depuration trend of NiO NPs and nickel salts in black fish tissues

Gill, intestine, liver, and kidney tissues of the fish were examined to determine bioaccumulation and depuration trend of NiO NPs, $NiSO_4$, Ni $(NO_3)_2$, and $NiCl_2$ (Fig. 3). It was found that the bioaccumulation of NiO NPs (intestine > kidney > liver > gill); $NiSO_4$ (kidney > liver > intestine > gill); $Ni(NO_3)_2$ and $NiCl_2$ (kidney >intestine > liver > gill) during their 7-to-28-day exposures had a downward trend. High accumulation of NiO NPs occurred in the intestine, and kidney showed the highest accumulation of $NiSO_4$, Ni $(NO_3)_2$, and $NiCl_2$. Gills accumulated the least amount of NiO NPs, $NiSO_4$, Ni $(NO_3)_2$ (Table 2). However, studies on toxicity and bioaccumulation of NPs and their metal salts show conflicting results. Some reports imply that, compared to nano forms, metal salts have higher absorption and toxicity (Sayadi *et al.,* 2020). While others suggest the opposite and assert that toxicity and accumulation in nano forms are higher (Wang *et al.,* 2015). Bioaccumulation of all the compounds in the fish tissue indicated that some were highly concentrated in the intestine (NiO NPs), while others accumulated more in the kidney ($NiSO_4$, Ni $(NO_3)_2$ and $NiCl_2$). Interestingly, although NiO NPs bioaccumulated more than $NiCl_2$, it was $NiCl_2$ that caused more tissue damage in the fish (Table 2). This could be due to sequestration of NiO NPs in the intestinal tissues (contributing to reduction of its toxicity), while the metal salts, which were not sequestered early on in the digestive tract, had time to circulate in the body and inflict more damage. High metabolic activity in intestine and kidney could be behind high accumulation rates of NiO NPs in these tissues. Studies on *Cyprinus carpio* confirm the findings of this study (Khoei, 2021). However, still some reports are conflicting in terms of what tissue is more prone to metal NP bioaccumulation. Results of this study matches those of other studies (Yin *et al.,* 2020). The depuration of NiO NPs, $NiSO_4$ and Ni $(NO_3)_2$ had a trend of intestine > gill > liver > kidney, and the depuration of $NiCl_2$ had a trend of intestine > kidney > liver > gill. In general, the highest depuration of NiO NPs, NiSO4, Ni $(NO_3)_2$ and $NiCl_2$ was in the intestinal tissue, the lowest depuration of NiO NPs, $NiSO_4$, Ni $(NO_3)_2$ was in the kidney tissue, and the lowest depuration of $NiCl_2$ was in the gill tissue of the fish (Table 2). The detection limit and relative standard deviation (RSD%) of graphite furnace atomic absorption instrument for nickel were 0.55 g/L and 2% respectively. In ecotoxicological studies, intestine is a suitable bioindicator tissue, reflecting dietary metal uptake (Mijosek *et al.,* 2021). The intestine was the main tissue where rapid depuration of all tested

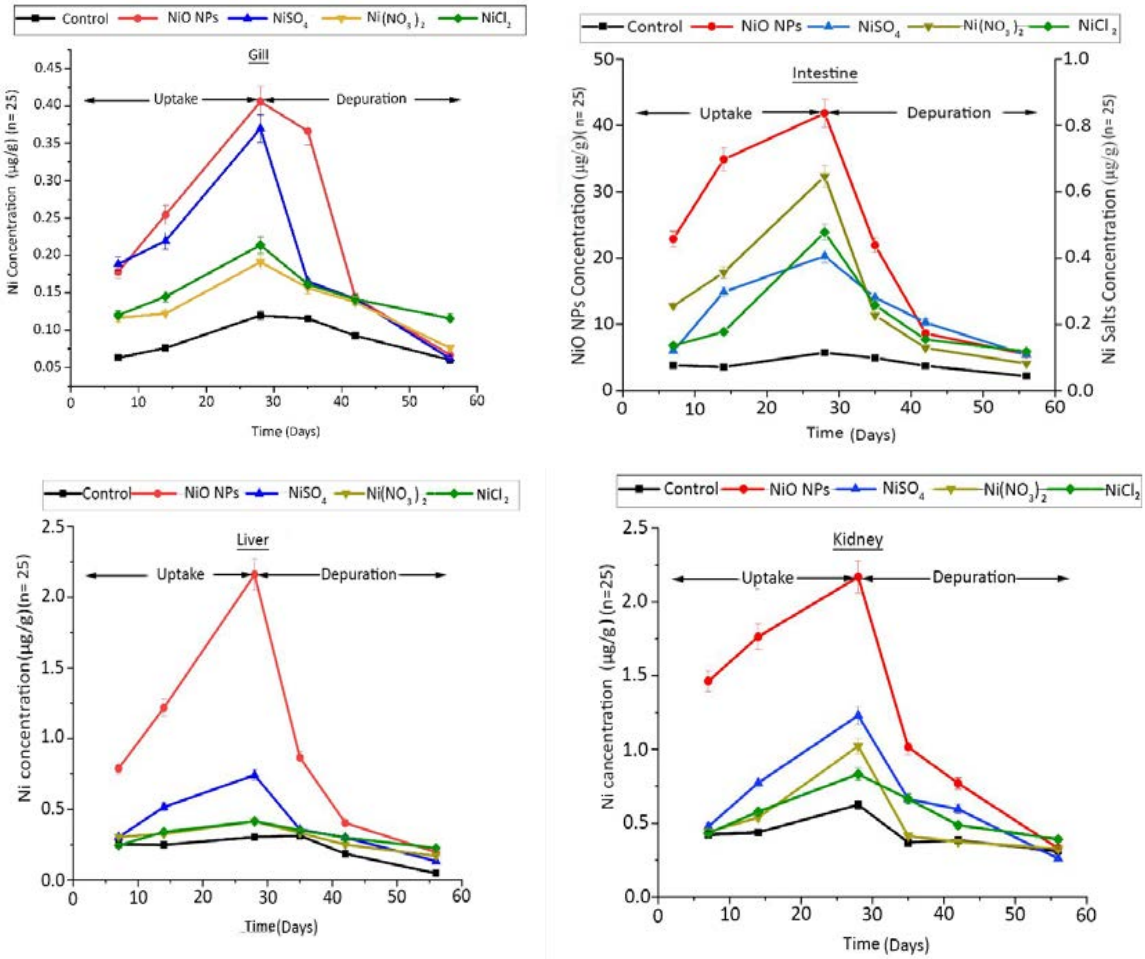

Fig. 3: Accumulation and depuration of Ni (µg/g) in gill, intestine, kidney, and liver of black fish (*Capoeta fusca*) exposed to NiO NPs, $NiSO_4$, Ni $(NO_3)_2$ and $NiCl_2$

compounds occurred. Ni compounds, in this study, had a short biological half-life in the intestine of 1-2 days (Table 2), and intestinal sequestration and excretion of NiO NPs from this tissue were high. This phenomenon may be a protective mechanism in fish to reduce the exposure to toxicants. In addition, clean water can contribute to fish protection against Ni exposure since it facilitates depuration of this metal (Kalay and Canli, 2000). However, the available data suggest that intestinal depuration of NiO NPs depends, mainly, on its concentration and the duration of depuration (Sayadi *et al.*, 2020). Khoei (2021) reported that the highest bioaccumulation rate of occurred in liver and intestine tissues of fish (*Cyprinus carpio*), and it increased with the increase of Fe_3O_4 NPs and $FeCl_3$ concentrations. Sayadi *et al.* (2021) stated that the bioaccumulation pattern of ZnO NPs in different tissues of blac kfish was intestine> gill> kidney > liver. They also claimed that the bioaccumulation of ZnO NPs in different tissues of black fish increased with the increase of time. Hwang *et al.* (2016) stated that lead depuration trend in different fish (*Platichthys stellatus*) tissues with the increase of time from 4 weeks to 6 weeks was brain (85%)> muscle (79.1%)> intestine (69.1%)> spleen (66.5%)> liver (64.4%)> kidney (58.8%)> gill (49.1%).

Uptake and depuration kinetics of Ni

Kinetic parameters of Ni ion activity were examined and recorded using a one-compartment first-order model (Table 3). The uptake rate constant (K_1) and

Table 2: Bioaccumulation and depuration of Ni (µg/g) in gill, intestine, liver, and kidney tissues of black fish (*Capoeta fusca*)

Tissues	Time in Days	Bioaccumulation					Depuration				
		Control	NiO NPs	$NiSO_4$	$Ni(NO_3)_2$	$NiCl_2$	Control	NiO NPs	$NiSO_4$	$Ni(NO_3)_2$	$NiCl_2$
Gill (n=3)	7	0.06±0.04	0.17±0.08	0.18±0.08	0.11±0.07	0.11±0	0.11±0.04	0.36±0.10	0.16±0.01	0.15±0.03	0.16±0.03
	14	0.07±0.04	0.25±0.07	0.21±0.10	0.12±0.00	0.14±0	0.09±0.03	0.14±0.06	0.14±0.04	0.13±0.01	0.14±0.02
	28	0.11±0.02	0.40±0.08	0.36±0.04	0.19±0.01	0.21±0.12	0.05±0.02	0.06±0	0.06±0.01	0.07±0	0.11±0
p-value		0.07	0.0003*	-	-	0.04*	0.04*	-	0.07	0.04*	0.07
Intestine (n=3)	7	0.07±0.03	22.89±7.33	0.12±0.02	0.25±0.06	0.13±0	0.09±0.04	21.94±7.60	0.28±0	0.22±0.13	0.25±0
	14	0.07±0.01	34.88±7.11	0.29±0.15	0.35±0.07	0.17±0.01	0.07±0.04	8.67±1.11	0.20±0.06	0.12±0	0.15±0.02
	28	0.11±0	41.82±16.95	0.40±0.27	0.64±0.57	0.47±0.15	0.04±0	5.49±1.01	0.10±0	0.08±0	0.11±0
p-value		-	-	-	0.04*	-	0.07	-	0.03*	-	-
Kidney (n=3)	7	0.42±0.02	1.46±0.43	0.47±0.01	0.44±0.05	0.43±0.13	0.37±0	1.01±0.06	0.66±0.02	0.41±0.14	0.66±0.19
	14	0.43±0.04	1.76±0.47	0.77±0.05	0.53±0.12	0.57±0.11	0.38±0.15	0.76±0.67	0.59±0.01	0.37±0.11	0.48±0.10
	28	0.62±0.07	2.16±1.26	1.23±0.48	1.02±0.27	0.83±0.09	0.31±0.16	0.32±0.13	0.26±0	0.32±0.03	0.39±0.18
p-value		-	0.06	0.04*	-	0.02*	-	0.01*	-	0.07	-
Liver (n=3)	7	0.25±0.08	0.78±0.36	0.30±0.07	0.30±0	0.24±0.04	0.31±0.03	0.86±0.04	0.35±0.02	0.33±0	0.35±0.04
	14	0.24±0.18	1.21±0.43	0.51±0.07	0.32±0.07	0.33±0.05	0.18±0.03	0.40±0.25	0.30±0.02	0.24±0.04	0.29±0.01
	28	0.30±0.21	2.16±1.82	0.74±0.32	0.41±0.02	0.41±0.08	0.04±0	0.19±0.05	0.13±0.03	0.16±0	0.22±0
p-value		-	0.01*	-	0.09	-	-	-	0.04*	-	0.04*

(*) Statistical significance is indicated by an asterisk

the depuration rate constant (K_2) for $NiSO_4$ in gill were higher compared to other treatments, and it followed the pattern of NiO NPs > (Ni $(NO_3)_2$ and > $NiCl_2$. In the intestine and kidney, the highest uptake and depuration rate constants were observed in NiO NPs treatment group, and it followed the pattern of Ni $(NO_3)_2$ > $NiCl_2$ and > $NiSO_4$. In the liver, uptake and depuration rate constants pattern was NiO NPs > $NiCl_2$ > Ni $(NO_3)_2$ > $NiSO_4$. Since zero state stable equilibrium was obtained in all the treatments, the bioconcentration factor (BCF) was calculated using K_1 and K_2 ratio values. For all the tested compounds, the maximum BCF in gill, intestine and liver was in the following order: $NiCl_2$ > Ni $(NO_3)_2$ > $NiSO_4$ > NiO NPs. The maximum BCF in kidney followed the order of Ni $(NO_3)_2$ > $NiCl_2$ > $NiSO_4$ > NiO NPs, and the estimated time required for elimination half-life ($t_{1/2}$) of Ni was between 1 day for NiO NPs to 7 days for $NiCl_2$. In addition, the maximum BCF in gill, intestine, and liver followed the pattern of $NiCl_2$ > Ni $(NO_3)_2$ > $NiSO_4$ > NiO NPs. Kidney followed the different order of Ni $(NO_3)_2$ > $NiCl_2$>$NiSO_4$ > NiO NPs. It was also found that half-life ($T_{1/2}$) of nickel in black fish was between 1 day for NiO NPs to 7 days for $NiCl_2$ (Table 3). In a similar study, Sayadi *et al.* (2020) stated that the uptake rate constant K_1 and the depuration rate constant K_2 were higher in the intestinal tissue compared to the other tissues (*viz.* gill, liver and kidney) of black fish. They suggested that the maximum BCF for $FeCl_3$, Fe_3O_4 NPs and $FeSO_4$ groups was observed in the liver tissue and for Fe $(NO_3)_3$ group was observed in the gill tissue, whereas the lowest BCF occurred for $Fe(NO_3)_3$, $FeCl_3$ and $FeSO_4$ in the kidney tissue and for Fe_3O_4 NPs occurred in the intestinal tissue. They also stated that the approximate time required for elimination half-life ($T_{1/2}$) or iron in black fish was estimated to be between 15 days for Fe_3O_4 to 93 days for Fe $(NO_3)_3$. Comparison of these reports showed that Ni cleared from the black fish body much faster than Fe NPs. Mansouri *et al.* (2016) reported that the BCF value in the mixed treatment of copper oxide nanoparticles (CuO NPs) and titanium dioxide nanoparticles (TiO_2 NPs) was higher compared to the control group. Also, the highest BCF level for copper oxide nanoparticles was detected in liver > gill > intestine > muscle tissues. They also showed that the copper uptake rate increased in different tissues of common carp in the presence of titanium dioxide nanoparticles. López-Serrano Oliver *et al.* (2015) found that the BCF value of titanium ion was higher in 0.1 mg/L concentration than in 0.9 mg/L concentration, the BCF value of titanium dioxide nanoparticles was higher in 2 mg/L concentration than in 10 mg/L concentration, and the BCF value of titanium ion (Ti) was higher than that of TiO_2 NPs in Zebrafish embryonic fetus.

Physical and behavioral changes in exposed fish

Behavioral changes in black fish after 28 days of exposure to NiO NPs, $NiSO_4$, Ni $(NO_3)_2$ and $NiCl_2$ are tabulated in Table 4. These findings confirm the earlier reports (Sayadi *et al.,* 2020). A guide to observed changes is provided as: -: none, +: 25%, ++: 50%, +++: 75% and ++++: 100% of black fish showed behavioral changes. In short, the fish exposed to $NiCl_2$, and Ni $(NO_3)_2$ exhibited "breathing at the surface" and "spiral swimming". The $NiCl_2$-exposed fish lost their coloration. NiO NPs exposure caused rapid gill movement and high mobility. The fish exposed to $NiSO_4$ exhibited weakness and lethargy. It swam in spirals and around air pockets. The fish behavior was negatively impacted by exposure to water contaminants. It has been argued that damage to fish organs and tissues result in such behavioral anomalies (Kienle *et al.,* 2008). This knowledge has even allowed us to use fish behavioral anomalies as a marker of water contamination level (Li *et al.,* 2014). The fish exposed to Ni compounds in this study exhibited a range of behavioral anomalies including rapid gill movement, surface breathing, mucus secretion on the gill surface, lethargy, and imbalance. Increased time of exposure resulted in intensified levels of such abnormalities (Table 4). Gill is the first tissue to be exposed to water contaminants. Rapid gill movement is due to fish hypoxia which occurs as a result of pollutant exposure (Sloman *et al.,* 2003). Continuous exposure to contaminants leads to higher mucus production and secretion in gill tissue, a protective response which would cause hypoxia if prolonged. These findings have been approved by others who reported rapid swimming, loss of balance, seizure, and increased fish activity in TiO_2 NPs-contaminated waters (Pirsaheb *et al.,* 2019).

Damage to gill and intestinal tissue

Severe histopathological lesions were caused by exposure to Ni compounds in gill and intestinal tissues (Figs. 4 and 5). The extent of tissue alteration was measured by Axio Vision Real 4.8 software compared to the control and the data were presented qualitatively. The fish exhibited fusion of lamellae, lamellar synechiae, curvature and oedema. $NiCl_2$ exposure of gill caused the highest rate of lamellar synechiae and curvature.

Table 3: Bioaccumulation and depuration kinetics of Ni in gill, intestine, kidney, and liver of black fish (*Capoeta fusca*)

	Gill				Intestine			
Parameters	NiO NPs	$NiSO_4$	$Ni(NO_3)_2$	$NiCl_2$	NiO NPs	$NiSO_4$	$Ni(NO_3)_2$	$NiCl_2$
K_1	0.21±0.03	0.24±0.04	0.11±0.02	0.09±0.01	0.62±0.43	0.29±0.06	0.37±0.07	0.36±0.07
K_2	0.01±0	0.01±0	0.005±0	0.004±0	0.030±0.019	0.012±0.002	0.015±0.002	0.013±0.002
$T_{1/2}$	3.27	2.87	6.42	7.88	1.11	2.38	1.85	1.89
BCF	16.3	17.21	21.60	22	23.00	24.25	24.93	28.15
	Kidney				Liver			
K_1	0.51±0.12	0.23±0.04	0.44±0.07	0.35±0.07	0.67±0.12	0.25±0.04	0.31±0.05	0.37±0.07
K_2	0.02±0	0.01±0	0.01±0	0.01±0	0.03±0	0.01±0	0.01±0	0.01±0
$T_{1/2}$	1.34	2.99	1.55	1.94	1.03	2.78	2.25	1.84
BCF	19.89	23.20	31.85	29.83	21.50	24.90	25.66	26.86

K_1: uptake kinetic constant (l/kg/day); K_2: elimination kinetic constant (1/ day); $T_{1/2}$: half-life (days); BCF: bioconcentration factor.

The maximum histopathological anomalies, fusion of lamellae, occurred when the fish were exposed to NiO NPs (Table 5). Fusion of lamellae and lamellar synechiae were observed in gill of the fish exposed to $NiSO_4$ and $Ni(NO_3)_2$ (Fig. 4). Gas exchange and osmotic regulation occur in the gill where pollutants first come into contact with the organism and can therefore be absorbed (Capaldo *et al.,* 2019). Fish gills are highly sensitive to exposure to environmental contaminants, since the respiratory epithelium has a large surface area and a high perfusion rate that facilitates the entry of pollutants into the gill (Santos *et al.,* 2014; Aghamirkarimi *et al.,* 2017). For this reason, gill's morphological changes, caused by toxic compounds, are widely used in biomonitoring of metal toxicity. Compared to NiO NPs, $NiCl_2$ caused much more severe damage to the

Table 4: Behavioral changes in black fish (*Capoeta fusca*) after exposure to NiO NPs, $NiSO_4$, $Ni(NO_3)_2$ and $NiCl_2$. None (-), Mild (+), Moderate (++) and Severe (+++)

Parameter	Control	NiO NPs	$NiSO_4$	$Ni(NO_3)_2$	$NiCl_2$
Hitting the wall of the aquarium	-	++	++	++	+++
Lightening of body color	-	+	++	+	+++
Rapid opening and closing of the gills	-	++++	+++	++	+++
Sleeping on back	-	++	+	+	+++
Loss of the ability to navigate in water	-	+	+	+	++
Swimming and high activity	-	+++	+	++	+++
The lack of balance	-	+	++	+	++
Mucus secretion	-	++	+	++	+++
Swim around the air rocks	-	++	+++	++	+++
Breathing from the water surface	-	+	++	++	+++
Sudden spins	-	+	+	+	++
Spiral swimming	-	++	++++	++	+++
Weakness and lethargy	-	+	++++	++	++
Protruding eyes	-	-	-	-	++

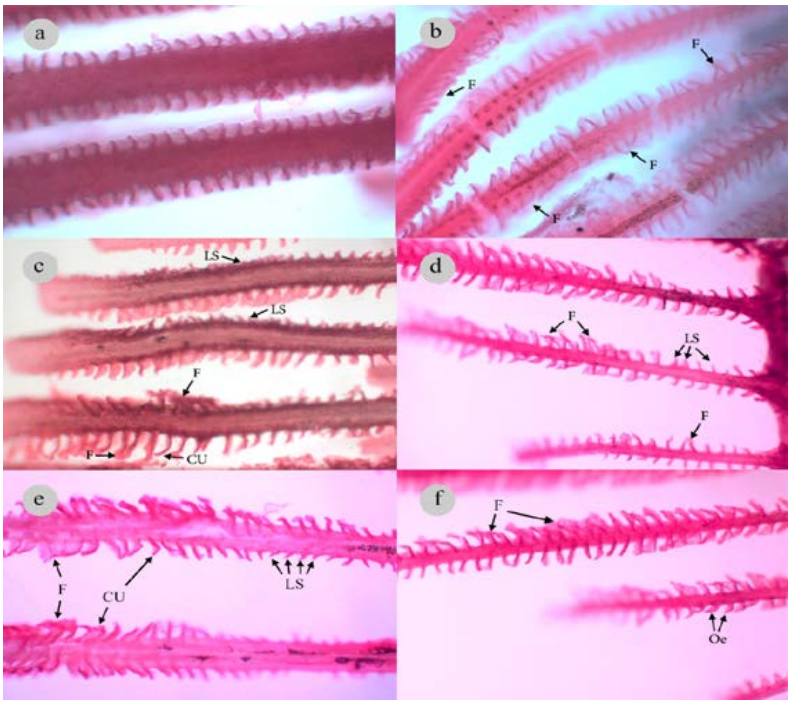

Fig. 4: Gill morphology in control (a) and black fish (*Capoeta fusca*) chronically exposed to nickel compounds: NiO NPs (b), $NiSO_4$ (c), $Ni(NO_3)_2$ (d), and $NiCl_2$ (e) (f). The injuries documented are: fusion of lamellae (F), lamellar synechiae (LS), curvature (CU), and oedema (Oe).

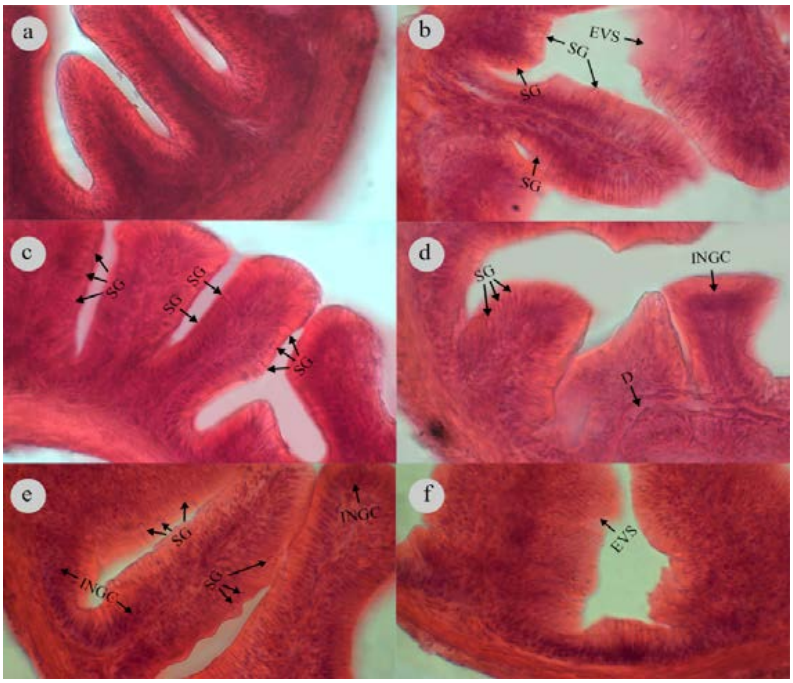

Fig. 5: Intestinal tissue of black fish (*Capoeta fusca*) exposed to NiO NPs and nickel salts (X400); Control (a), NiO NPs (b), $NiSO_4$ (c), Ni $(NO_3)_2$ (d), and $NiCl_2$ (e) (f). The injuries documented are: swelling of goblet cells (SG), higher number of goblet cells (INGC), degeneration of villi structure (D), and expansion at villi structure (EVS).

fish gill (fusion of lamellae, lamellar synechiae and gill curvature) and intestine. The curvature and adhesion of epithelium in gill's secondary lamellae is often the first sign of toxic effects. Fusion of lamellae is caused by cellular proliferation that gradually fills the space between gill layers (Mansouri *et al.,* 2016). Similar to mucosal secretion, fusion of gill lamellae is a protective mechanism to help limit exposure and tissue damage (Beegam *et al.,* 2020). The findings comply with results reported by others showing that hyperplasia, fusion of lamellae and aneurism in fish are due to the exposure to TiO_2 NPs, ZnO NPs, ZnO NPs and Cu NPs (Pirsaheb *et al.,* 2019; Sayadi *et al.,* 2021). Additive toxic effects of Fe on TiO_2 NPs and reduced toxicity of ZnO NPs when combined with NGs have been reported by the same authors. In the control group, intestinal structure was normal, and epithelia and Goblet cells were properly arranged. After 28 days of exposure to NiO NPs and nickel salts, swelling of Goblet cells (SG), increased number of Goblet cells (INGC), degradation (D), and expansion at villi structure (EVS) were observed. The highest rate of these abnormalities occurred when the fish were exposed to $NiCl_2$. However, the gill and intestine abnormalities were much higher in the fish exposed to nickel salts than in the fish exposed to NiO NPs (Fig. 5). It was not possible to study histopathology of the fish intestine exposed to NiO NPs because the tissue had been destroyed. Intestine plays an important role in braking down of the environmental chemicals, and there is clear evidence that metabolism of pollutants modulates the toxicity for the host, emphasizing the protective role that intestine plays against toxic exposures. Moreover, pathophysiological changes in the gastrointestinal tissue caused by ingestion of toxins, have led to systemic adverse effects (Claus *et al.,* 2017). The intestinal epithelium consists of a mucosal layer with villi that synthesizes and secretes mucin and a goblet cell layer (Cheng, 1974). Mucin plays a critical role in trapping toxicants, particularly metals. The results showed the severe damage to the intestinal tissue of the fish. Compared to NiO NPs, $NiCl_2$ was more damaging to the intestinal tissue and the tissue lesions became progressively worst in time. $NiCl_2$ exposure led to goblet cell hypertrophy and swelling. Goblet

Table 5: Semi-quantitative evaluation of lesions recorded in the gill and the intestine of black fish (*Capoeta fusca*) exposed to NiO NPs and nickel salts

Tissues	Histopathological changes			
Gill	**Oe**	**LS**	**F**	**CU**
Control	-	+	-	-
NiO NPs	+	+++	+	-
$NiSO_4$	+	++	++	-
$Ni(NO_3)_2$	-	++	++	-
$NiCl_2$	++	++	+++	+
Intestine	INGC	EVS	D	SG
Control	-	-	-	-
NiO NPs	-	+	-	+++
$NiSO_4$	-	-	-	+++
$Ni(NO_3)_2$	++	-	+	++
$NiCl_2$	++	++	-	+++

Odema (Oe), Lamellar synechiae (Ls), Lamellar fusion (F) and Curvature (CU) in gill; and Swelling of Gobletcells (SG), Degeneration (D), Expansion at Villi Structure (EVS), and Increase in the Number of Goblet Cells (INGC) in intestine. None (-), Mild (+), Moderate (++) and Severe (+++)

cell hypertrophy is described as the first protective response to toxic exposure, and it can temporarily reduce toxic effects. The findings confirm the results of other studies showing the intestinal damage as a result of exposure to metal NPs (Kakakhel *et al.,* 2021). Liu *et al.* (2019) reported that cadmium caused histopathological lesions erosion of villi, necrosis in the mucosal layer, hyperplasia and swelling of goblet cells in the fish (*Carassius auratus*). They demonstrated that the severity of anomalies increased with the increase in the cadmium concentration. Rahmani *et al.* (2019) reported that mercury and Ag NPs increased the number of goblet cells and swelling of goblet cells in the intestine of fish (*Cyprinus carpio*). Pirsaheb *et al.* (2019) reported the histopathological anomalies caused by titanium oxide nanoparticles in the intestinal tissue of fishes (*Carassius auratus*) and (*Cyprinus carpio*) as necrosis and erosion, degeneration, increase in the number of goblet cells, swelling of goblet cells, and increase in the number of lymphocytes.

CONCLUSION

In recent years, nanoparticles are widely used in many fields and various sectors such as electronics, textiles, cosmetics, medical equipment, and food packaging. They are considered as a serious threat to the environment due to their toxicity. Based on chemical composition, nanoparticles can be divided into different groups such as metal nanoparticles, polymer metal oxide, semiconductor, and metal oxide. Among the metal oxide group, NiO NPs, due to their properties, are widely used in many industrial and commercial products such as solar cells, conductive coating, fuel cells, catalytic converters in cars, and biosensors. NiO NPs have low solubility and high stability in water, so that it can remain in solid form in water and attach to other particles or aquatic organisms. After entering the water, NiO NPs can be eaten by organisms or attached to their external organs or absorbed by their internal membranes, causing direct toxicity. Despite the increased use of nanomaterials in various sectors, few studies are available on the health effects and toxicity of these materials on organisms. Therefore, this study attempted to use biological indicators in monitoring and controlling the environmental pollution, since these substances eventually enter the aquatic environment. When there is a specific concentration of pollutant in the environment and this amount does not cause death, it can cause changes in the tissues and cells of organisms. The use of tissue pathology method with the help of light microscope is one of the useful ways to detect the amount of damage to the tissue caused by the environmental factors such as metals and nanomaterials. Tissue pathology can provide a correct assessment of the health of the target tissue in living organisms and reflects the effects of exposure to environmental pollutants. Intestinal and gill tissues are among the pollutant-absorbing tissues that contain a high concentration of toxic substances. The purpose of this study was to investigate the toxicity of NiO NPs compared to different nickel salts. The acute toxicity of NiO NPs, $NiSO_4$, Ni $(NO_3)_2$, and $NiCl_2$ on black fish (*Capoeta fusca*) was investigated. To explore the chronic toxicity of NiO NPs and Ni salts, first the fish were exposed to NiO NPs and its salts for 28 days to determine the amount of bioaccumulation of

various Ni compounds in gill, intestine, liver and kidney tissues. Then, the fish were placed in clean water for 28 days to calculate the rate of depuration of various Ni compounds. Various changes in hematological, biochemical and liver enzymes parameters were investigated in the fish. $LC_{50\text{-}96h}$ values for NiO NPs and salts of $NiSO_4$, Ni $(NO_3)_2$, and $NiCl_2$ were reported as 194.98, 120.22, 138.03, and 91.20 mg/L, respectively. The results showed that $NiCl_2$ was more toxic than NiO NPs, $NiSO_4$, and Ni $(NO_3)_2$. Moreover, NiO NPs had the highest bioaccumulation rate in gill tissues (0.40 ± 0.08 µg/g), intestine (41.82 ± 16.95 µg/g), liver (2.16 ± 1.82 µg/g g) and kidney (2.16 ± 1.26 µg/g). Exposure of the fish to NiO NPs and its salts caused histopathological damage such as swelling of goblet cells, increase in the number of goblet cells, destruction, and expansion of the villi structure in the intestine. The amounts of white blood cells, hemoglobin and hematocrit of the fish exposed to Ni $(NO_3)_2$ were higher compared to the control group, showing that they had polycythemia. Changes in the erythrocytes structure (deformed erythrocytes, nuclear disintegration, and micronucleus) were observed in the fish exposed to various Ni compounds. Biochemical factors in the fish blood, such as cholesterol, glucose, creatinine and uric acid, were higher compared to the control group. Liver enzymes ALP, AST and ALT blood of the fish exposed to various Ni compounds were significantly ($p < 0.05$) higher as compared to the control group, showing that the liver tissue was damaged. All the tested compounds bioaccumulated in the fish tissue, some with higher concentrations in the intestine (NiO NPs), and others with higher accumulation in the kidney ($NiSO_4$, Ni $(NO_3)_2$, and $NiCl_2$). The depuration of NiO NPs, $NiSO_4$, and Ni $(NO_3)_2$ followed the trend of intestine > gill > liver > kidney, and the depuration of $NiCl_2$ followed the trend of intestine > kidney > liver > gill. The highest depuration of NiO NPs, $NiSO_4$, Ni $(NO_3)_2$, and $NiCl_2$ was in the intestinal tissue, the lowest depuration of NiO NPs, $NiSO_4$, and Ni $(NO_3)_2$ was in the kidney tissue, and the lowest depuration of $NiCl_2$ was is in the gill tissue of the fish. The gill and intestine were found to be the most vulnerable tissues in the fish exposed to Ni contaminants. $NiCl_2$ exposure of gill caused the highest rate of lamellar synechiae and curvature. The maximum histopathological anomalies, fusion of lamellae, occurred when the fish were exposed to NiO NPs. The results showed that compared to NiO NPs, $NiCl_2$ was more damaging to the intestinal tissue of the fish, and the tissue lesions became progressively worst in time. The obtained results imply the necessity for the relevant organizations to specify limits for the amount of different nanoparticles, especially nickel, entering into the water environments. It is suggested to study the toxicity effects of multi metals in aquatic organisms in a real environment (according to mutual effects on each other) on other tissues such as skin and muscle in different environmental conditions such as pH, hardness, salinity and temperature.

AUTHOR CONTRIBUTIONS

J. Kharkan conducted the conceptualization, study design, field work, data analysis, and writing of the first draft. M.H. Sayadi performed the project administration, funding, conceptualization, review, and some rewriting of the first draft. M. Hajiani did the field work, data analysis, and feedback. M.R. Rezaei participated in the interpretation of the results and manuscript preparation. M. Savabieasfahani made a significant contribution to data illustration, enhancing the discussion section, and rewriting of the manuscript in proper English.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest regarding the publication of this work. In addition, the ethical issues including plagiarism, informed consent, misconduct, data fabrication and, or falsification, double publication and, or submission, and redundancy have been completely witnessed by the authors.

PUBLISHER'S NOTE

ABBREVIATIONS

%	Percent
BCF	Bio-concentration factor
CU	Curvature
Cu NPs	Copper nanoparticles
D	Degeneration of villi structure
EDS	Energy dispersive spectroscopy
EVS	Expansion at villi structure
F	Fusion of lamellae
Fig	Figure
Fe_3O_4 NPs	Iron oxide nanoparticles
$FeCl_3$	Iron chloride
$FeSO_4$	Iron sulfate
$Fe\ (NO_3)_3$	Iron nitrate
g/L	Gram per liter
g/cm^3	Gram per cubic centimeter
INGC	Higher number of goblet cells
K_1	Uptake rate constant
K_2	Depuration rate constant
LC_{50}	Lethal concentration 50
LD_{50}	Lethal dose 50
LSC	Lower sub-lethal concentration
LS	Lamellar synechiae
mg/L	Milligram per liter
mL	Millilitre
m^2/g	Square meter per gram
NiO NPs	Nickel oxide nanoparticles
$NiSO_4$	Nickel sulfate
$Ni\ (NO_3)_2$	Nickel nitrate
$NiCl_2$	Nickel chloride
NPs	Nanoparticles
Ni	Nickel
N	Number
NGs	Graphene nanoparticles
Oe	Oedema
RSD	Relative standard deviation
SEM	Scanning electron microscopy
SG	Swelling of goblet cells
SSA	Specific surface area
$T_{1/2}$	Half-life
TiO_2 NPs	Titanium dioxide nanoparticles
XRD	X-ray diffractive
XPS	X-ray photoelectron spectra
ZnO NPs	Zinc oxide nanoparticles
µg/g	Micrograms per gram
µm	Micrometer
µS/cm	Microsiemens per centimeter

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