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Acute and Chronic Toxicity and Oxidative Stress by Metallic Nanoparticles in Early Life Stage Fathead Minnows (*Pimephales promelas*)

[1,2]*C. W. Theodoraki, [1]K. O'Shaughnessy, [1]G. A. Chavez, [1]K. Brendam, [2]Z. Mu, & [3]G. P. Cobb
Departments of [1]Biological Sciences and [2]Environmental Sciences,
[1,2] Southern Illinois University Edwardsville, Edwardsville, Illinois 62026, USA
[3]Department of Environmental Science, Baylor University, Waco, Texas 79798, USA
*Correspondence: ctheodo@siue.edu

ABSTRACT

The objective of this research was to determine the toxicity of nZnO, $nTiO_2$, nFe_2O_3, nCu and nCuO to larval *Pimephales promelas* after 96-h and 28-d exposures. Endpoints included survival, growth, spinal curvature, and oxidative stress (total glutathione, GSH [reduced glutathione], GSSG/GSH ratio [GSSG = oxidized glutathione], and TBARS [Thiobarbaturic Acid Reactive Substances; a measure of lipid peroxidation]). The lethal concentration 50s (LC50s) for nFe_2O_3 (28 mg/L) and nCuO, (0.66 mg/L) were greater than those for Fe^{+3} (0.039) and Cu^{+2} ions (0.005), but the LC50 for nCu (0.009 mg/L) was similar to Cu^{+2}. There was no evidence of acute toxicity of dissolved ions in the nanoparticle suspensions, nor for nZnO or $nTiO_2$. In chronic exposures, both mortality and growth rate were increased at the lowest concentration of nFe_2O_3 (350 μg/L), while mortality and axial spinal curvature was the most sensitive indicator of chronic nCuO toxicity (the Lowest Observed Effect Concentration [LOEC]= 32.5 μg/L for both). The levels of TBARs and glutathione-related parameters suggested that oxidative stress increased in nFe_2O_3-exposed fish,but were decreased by nCuO exposure. Because of logistic constraints, chronic tests for nCu were not carried out. The LC10 and LC50 for nCu and LOEC concentrations of nCuO fall within the range of predicted concentrations for high-level exposure scenarios, so these effects may occur at environmentally-relevant concentrations. These nanoparticles were more toxic in fathead minnows than in other species from previous studies, and differential toxicity of pure nanoparticles vs. commercial formulations is discussed as a possible reason for difference between the present and previous works.

Keywords: Survival, engineered nanomaterials, malformations, hormesis, Darwinian fitness, development.

INTRODUCTION

Metal oxide nanoparticles have been the subject of much aquatic toxicological research over the previous decade (Banerjee and Roychoudhury 2019, Kumari et al. 2019). Previous research focused mostly on nAg and $nTiO_2$ (Callaghan and MacCormack 2017; Banerjee and Roychoudhury 2019), with fewer studies on the toxicity of other nanoparticles like nCu, nCuO, and nFe_2O_3. This is significant, because the latter three types of nanoparticles have numerous industrial and commercial applications (Stark et al. 2015). Plus, most fish nanotoxicity studies have focused on zebrafish (*Danio rerio*: Haque and Ward 2018; Chakraborty, et al. 2019). However, there is less information on native North American warmwater fishes – e.g., fathead minnows (*Pimephales promelas*; see Hall et al. 2009; Laban et al. 2010; Song et al. 2015). This hinders ecological risk assessments in North America. In addition, the American Toxic Substances and Disease Registry recommends *P. promelas* as a standard test organism (ATSDR 2011). Also, while some studies have examined chronic $nTiO_2$ and nAg toxicity on fitness parameters (growth, development, and survival; Hall et al. 2009; Chen et al. 2011), chronic effects of other metallic nanoparticles have received less attention. Thus, there is a need for more studies of the effects of nanoparticles on these ecologically-relevant endpoints (Callaghan and MacCormack 2017). Finally, although the mechanism of nanoparticle toxicity is not fully understood, oxidative stress is thought to play a role (Callaghan and MacCormack 2017). However, previous studies have focused on acute exposures, while there is less information on oxidative stress during chronic exposures. This is important, because chronic oxidative stress is more environmentally-realistic, and may affect fitness components (Hõrak and Cohen 2010). Therefore, the objectives of this study are to determine acute and chronic toxicity and oxidative stress endpoints in fathead minnows exposed to nFe_2O_3, nCuO, nCu, nZnO, and $nTiO_2$. It was hypothesized that both acute and chronic toxicity and oxidative stress are affected by nanoparticle exposure.

MATERIALS & METHODS

Nanoparticles. Nanoparticles were purchased as aqueous dispersions. TiO_2 (rutile) and Fe_2O_3 (red) were purchased from Nanostructured and Amorphous Materials, Inc. (Houston, TX, USA; nominal average particle sizes (APS) 30-50 and <100 nm, respectively). Copper oxide (NanoArc®) and ZnO (NanoTek®) were synthesized by Nanophase, Inc. (Romeoville, IL, USA; nominal APS 29 and 40 nm, respectively). A "proprietary surface treatment technology" (www.nanophase.com) was used as a stabilizer. Metallic Cu nanoparticles with a nominal APS of 25 nm were purchased from Sun Innovations (Fremont, CA, USA).

Acute Tests. Acute tests used larvae within 24 hours of hatching that were purchased from Aquatic Biosystems (Fort Collins, CO) or Aquatic Research Organisms (Hampton, NH). Methods followed ASTM (2003). A standard static-renewal system was used, and half of the water was exchanged with fresh testing solution daily. Fish were kept at 16:8 light:dark cycle and fed brine shrimp (*Artemia* spp.) nauplii (< 48 hours old) daily. Fathead minnows start feeding exogenously as early as 24 hph (Jeffries et al,. 2013), so feeding began on the first day of the test. Tests were conducted in reconstituted water (deionized water mixed with 60 mg/L Instant Ocean® sea salts). There were 15 fish per replicate beaker, three replicates per treatment, and 7 test concentrations plus control. Ninety-six h Lethal Concentration 50 (LC50) was determined using the US EPA program PROBIT.

Two different strategies were used to test the hypothesis that toxicity was due to dissolved ions rather than the nanoparticles. First, toxicity of nanoparticles was compared to that of $FeCl_3$ and $CuSO_4$. Second, the nanoparticles were suspended in test water and allowed to age for 96 h. Nanoparticles were then removed from test solutions by centrifugation at 10000 X G for 1 h and vacuum-filtration through 1000 Da MW cutoff ultrafiltration disc membranes (nominal pore size ca. 5 nm; EMD Millipore Corporation, Billerica, MA, USA). The filtrate was used for toxicity testing as described above.

Chronic Tests. Chronic tests followed ASTM (1999) and used larvae within 24 hours of hatching (eggs were purchased from Aquatic Research Organisms, Hampton, NH, USA). The endpoints were survival, growth, and percent deformities. The percent mortality in the controls for the nCu chronic tests consistently exceeded 20%, so the tests were deemed invalid, and results were not reported. Fish were kept at a 16:8 light:dark cycle and fed brine shrimp (*Artemia* spp.) nauplii (< 48 hours old) daily, and were tested in reconstituted water as above. For each test there were five treatments (four nanoparticle concentrations plus control), with ten replicate test vessels per treatment. Test solutions used for dosing were kept in 9.5 L aquaria ("dosing chambers"). Water filter pumps were used to circulate the water in the dosing chambers. Fresh stock and test solutions were formulated daily. Each test vessel was a 1-L beaker fitted with a drainpipe – constructed from a 6.35 mm O.D. T-type polypropylene hose connector and inserted ¾ of the way up the side of the beaker. This pipe was fitted with 40-micron mesh netting over the intake to prevent escape of the fish. There was a total of 750 mL test solution in each test vessel. At the start of the exposure, twenty fish were added to each test vessel. The test solutions were dispensed from the 9.5 L dosing chambers into the test beakers using a multi-channel peristaltic pump (Waters Inc. economy L/S pump, Milford, MA, USA) with microbore tubing. The flow rate into each test beaker was adjusted to be approximately 2250 ml/day (i.e. 3 water changes per day). Concentrations of nanoparticles were designed so that the highest concentration was no more than 10% of the 96 h LC50 value (ASTM 1999). Mortality was determined by the number of fish surviving at the end of the exposure. Growth rate was determined by measuring average fish mass per beaker and assuming there were no significant differences in fish mass at the beginning of the exposures. Presence or absence of abnormalities was determined by visual inspection under a dissecting scope.

Lipid Peroxidation and Glutathione Parameter. At the end of the 28-day exposures, fish were euthanized in MS222 and weighed. The fish were centrifuged and the water was removed, they were snap frozen in liquid nitrogen, and stored at -80° C until analysis. Lipid peroxidation was measured as thiobarbaturic acid reactive substances (TBARS) using a fluorometric microplate assay following Theodorakis et al. (2017). Procedures for fluorometric determination of total, reduced (GSH) and oxidized (GSSG) glutathione followed Theodorakis et al. (2017), and used naphthalene-2-3-dicarboxaldehyde as the fluorogen. The amount of total, oxidized and reduced glutathione was normalized to the wet mass of the sample.

Water Chemistry, Nanoparticle Characterization, and Metals Analysis. Water temperature, pH, dissolved O_2, and conductivity were determined using an YSI-80 meter (YSI Instruments, Inc., Yellow Springs, OR). Total NH4 was determined calorimetrically using aquarium ammonia testing kits (API® Fishcare, Chalfont, PA, USA) and the amount of total ammonia was quantified by measuring absorbance at 670 nm and using ammonium chloride as the standard. Nanoparticle hydrodynamic diameter was determined by dynamic light scattering using a Nanotrparticle Size Analyzer (Microtrac Inc., York, PA; acute tests) or a Malvern Instruments Zetasizer (Westborough, MA; chronic tests). Because the concentration of the test water in the chronic tests was too low to able to detect the nanoparticles with this instrument, hydrodynamic diameter was determined in an aliquot of each stock solution each week. In order to determine total metal concentrations, test solutions were acidified to pH of 2 by adding 150 µL trace-metal grade concentrated HNO3 to 30 mL of sample. The samples were then filtered through a 200-micron filter and analyzed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS ; Agilent Technologies, Series 7500, Santa Clara, CA; limit of quantitation = 0.01 μg/L).

Statistical Analyses. The data did not fit a normal distribution model according to a Kolmogorov–Smirnov test. Because the data did not seem to have the same distribution for all treatments, which precluded determination of what transformation would be appropriate. For these reasons, and because of the small sample sizes, differences between the control and each treatment group were tested using the Kruskal-Wallis test with multiple comparisons contrasting treatments vs. control (Hollander and Wolfe 1973). The Krukall-Wallis test was performed

in StatisXL (https://www.statistixl.com/). A routine for conduction the multiple comparisons was written in Visual BASIC, using formulae from Hollander and Wolfe (1973).

RESULTS

Water Quality, Nanoparticle Characterization, and Metal Quantification. In order to limit the number of pages in this manuscript, these data are not reported here, but can be found in the Supplementary Information at the end. Measured metal concentrations were approximately 63-82% of nominal for acute nCu tests, 34-48% of nominal for acute nCuO, 9.3-10.8% for acute nFe_2O_3, 54-71% for chronic CuO, and 11.8-18.6% of nominal for chronic nFe_2O_3 tests (Table S5, Supplementary Information).

Acute Tests. The LC10 and LC50 values for nanoparticles, nanoparticles filtrate, and metal salts are reported in Table 1. The LC50 for nCu, nCuO, and nFe_2O_3 were greater than that their corresponding salts or ions (Table 1). There was no mortality for $nTiO_2$ or nZnO, or for the filtered stock solutions. LC50 values for the metal salts were less than their corresponding nanoparticles. The data suggest that iron oxide, copper and copper oxide nanoparticles are toxic to fathead minnow larvae (Table 1).

Chronic Exposure: Fitness Parameters. For CuO, the Lowest Observed Effect Concentration (LOEC) was 35 μg/L for mortality and 65 μg/L for growth (Figure 1). Axial spinal curvature was the only deformity noted. The percent of fish with axial spinal curvature was higher in the 32.5 and 65 μg/L treatment than in controls (i.e., the LOEC was 32.5 μg/L; Figure 1). For the Fe_2O_3 experiment, the percent mortality and average final fish mass were significantly greater than control for all treatments except 1.05 mg/L (LOEC for each = 0.35 mg/L; Figure 1). Fe_2O_3 did not cause axial spinal curvature.

Chronic Exposure: Oxidative Stress. For the nCuO exposure, the LOEC for TBARS was 32.5 μg/L (Figure 2). For nFe_2O_3, only the 7 mg/L treatment had statistically-significantly higher TBARS concentrations than the control (i.e., LOEC – 7 mg/L; Figure 2). GSH concentrations were significantly higher than control in fish treated with 8.13 and 6.26 μg/L CuO (Figure 2). The GSSG/GSH ratios were lower than controls for all nCuO treatments (LOEC = 8.13 μg/L: Figure 2). Fish exposed to Fe2O3 had lower total glutathione (LOEC = 7 mg/L), GSH (lowest observed effect concentration [LOEC] = 0.350 mg/L), and higher GSSG/GSH ra-

Table 1. LC10 and LC50 values (mg/L) for metal oxide nanoparticles, nanoparticle filtrate, and metal salts using fathead minnows.

Material	LC10 (95 % Confidence Interval)	LC50 (95 % Confidence Interval)
nCuO	0.23 (0.073-0.35)	0.66 (0.49 – 0.87)
nCu	0.002 (0.001-0.004)	0.009 (0.006 – 0.013)
nFe_2O_3	23 (11-26)	28 (25-36)
$nTiO_2$	>1000	>1000
nZnO	>1000	>1000
nCuO Filtrate[a]	>1000	>1000
nCu Filtrate[a]	>1000	>1000
nFe_2O_3 Filtate[a]	>1000	>1000
$FeCl_3$	0.040 (0.029-0.056)	0.089 (0.079-0.095)
Fe^{+3} ion[b]	0.018 (0.012-0.025)	0.039 (0.033-0.042)
$CuSO_4$	0.0007 (0.0004-0.001)	0.0013 (0.0005-0.0023)
Cu^{+2} ion[b]	0.0003 (0.0002-0.0004)	0.0005 (0.0002-0.0009)

[a]"Filtrate" are toxicity tests conducted with nanoparticle solutions that were centrifuged at 8000 x G for 1 h, and filtered through 1000 MW cutoff ultrafiltration membrane. The "concentrations" correspond to the concentration of nanoparticles in the unfiltered suspensions. [b]LC10 or LC50 based on concentration of metal ion, rather than the salt. Acute toxicity tests were not done with zinc or titanium salts.

Table 2. Acute toxicity values for various fish species exposed to selected metallic nanoparticles.

Nano particle	Species and Life Stage	Endpoint	Conc. (mg/L)	Reference
Fe_2O_3	Zebrafish (*Danio rerio*) larvae	96 h LOEC	100	Zhu et al. (2012)
	Zebrafish embryo-larvae	96 h LC50	>1600	Kovrižnych et al. (2013)
	Zebrafish adults	96 h LC50	>1600	Kovrižnych et al. (2013)
	Zebrafish larvae	96 h LC50	99.2	Villacis et al. (2017)
Cu	Zebrafish larvae	48 h LC50	1.56	Griffit et al. (2007)
	Zebrafish juveniles	96 h LC50	0.71	Shaw and Handy (2011)
	Zebrafish embryo-larvae	96 h LC50	3.8	Kovrižnych et al. (2013)
	Zebrafish adults	96 h LC50	24	Kovrižnych et al. (2013)
	Fathead minnow (*Pimephales promelas*) juveniles	96 h LC50	0.28	Song et al. (2015)
	Zebrafish juveniles	96 h LC50	0.22	Song et al. (2015)
	Rainbow trout (*Oncorhynchus mykiss*) juveniles	96 h LC50	0.68	Song et al. (2015)
	Grass carp (*Ctenopharyngodon della*) juveniles	96 h LC50	14	Vafedarnejad et al. (2018)
	Common carp (*Cyprinus carpio*) juveniles	96 h LC50	4.44	Noureen et al. (2021)
CuO	Zebrafish embryo-larvae	96 h LC50	400	Kovrižnych et al. (2013)
	Zebrafish adults	96 h LC50	840	Kovrižnych et al. (2013)
	Zebrafish	96 h LC50	53	Pereira et al. (2023)
	Common roach (*Ritulus ritulus*) adults	96 h LC50	2.19	Jahanbakhshi et al. (2015)
	Nile tilapia (*Oreochromis niloticus*) juveniles	96 h LC50	150	Abdle-Khalek et al. (2018)
	Serpae tetra (*Hyphessobrycon eques*) adults	96 h LC 50	0.214	Mansano et al. (2018)
	Dwarf cichlid (*Apistogramma agassizii*) juveniles	96 h LC 50	116.6	Braz-Mota et al. (2018)
	Cardinal tetra (*Paracheirodon axelrodi*) juveniles	96 h LC 50	139.2	Braz-Mota et al. (2018)
	Rohu (*Labeo rohita*) juveniles	96 h LC 50	353.98	Aziz and Abdullah (2023)
TiO_2	Fathead minnow larvae	96 h LC50	500	Hall et al. (2009)
	Zebrafish adults	96 h LC50	124.5	Xiong et al. (2011)
	Zebrafish embryo-larvae	96 h LC50	>1600	Kovrižnych et al. (2013)
	Zebrafish adults	96 h LC50	>1600	Kovrižnych et al. (2013)
	Nile tilapia juveniles	96 h LC50	165	Vidya and Chitra (2017)
ZnO	Zebrafish embryo-larvae	96 h LC50	1.793	Zhu et al. (2008)
	Zebrafish adults	96 h LC50	4.92	Xiong et al. (2011)
	Common carp juveniles	96 h LC 50	4.9	Subashkumar and Selvanayagam (2014)
	Caspian roach juveniles	96 h LC 50	48	Khosravi-Katuli et al. (2018)
	Blackfish (*Capoeta fusca*) juvenile	96 h LC 50	4.9	Sayadi et al. (2022)

tios (LOEC = 0.350 mg/L) than control fish (Figure 2).

DISCUSSION

It should be noted that the above results are based on nominal concentrations, and the concentrations of total metals are much lower. This may be due to agglomeration and precipitation of the nanoparticles in the test vessels. In fact, a layer of sediment the same color as the nanoparticles was observed in all test vessels, suggesting that much of the nanoparticles agglomerated and precipitated out of solution. But reporting results based on measured concentrations is problematic, because it is not known how much of the metal is in the dissolved vs. particulate phase. Therefore, it has been suggested that nominal concentrations be reported in nanoparticle tests (Shaw et al. 2016).

The data suggest that iron oxide, copper and copper oxide nanoparticles are toxic to fathead minnow larvae (Table 1). The LC10 and LC50 concentrations for iron oxide may not be environmentally relevant except at sites with heavy contamination. Thus, the environmental hazard of these three nanoparticles decreases in the order of Cu > CuO > Fe_2O_3. Because Cu nanoparticles are so much more toxic than CuO, it is unlikely that the majority of the Cu nanoparticles were oxidized to CuO, although there may have been a coating of CuO at the surface of the nanoparticles. The data do not, however, indicate that TiO_2 or ZnO are toxic to fathead minnow larvae.

It could also be argued that toxicity of the nanoparticle suspensions was due to dissolved substances in the water – e.g., free metal ions or dispersing agents – and not the nanoparticles themselves (Shaw and Handy 2011). The fact that the toxicity of the metal salts was lower than the nanoparticles them-selves does not refute this hypothesis. However, the present study found that filtered nanoparticle solutions were not toxic to fathead minnow larvae, and this is not consistent with the hypothesis that toxicity was due mainly to dissolved metal ions.

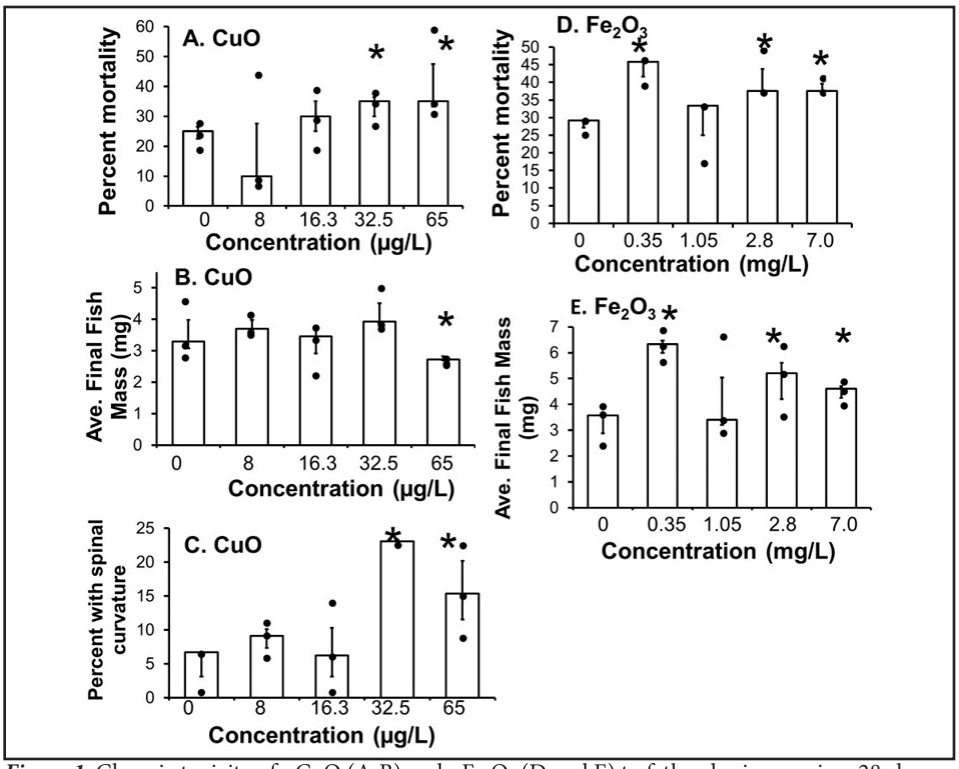

Figure 1. Chronic toxicity of nCuO (A-B) and nFe_2O_3 (D and E) to fathead minnows in a 28-day early life stage exposure. A, D) Percent mortality. B, E) Average final mass per fish. C) Percent with axial spinal curvature. Bars ± error bars represent medians ± first and third quartiles. *Significantly different from control (P < 0.05, Kruskal-Wallis test).

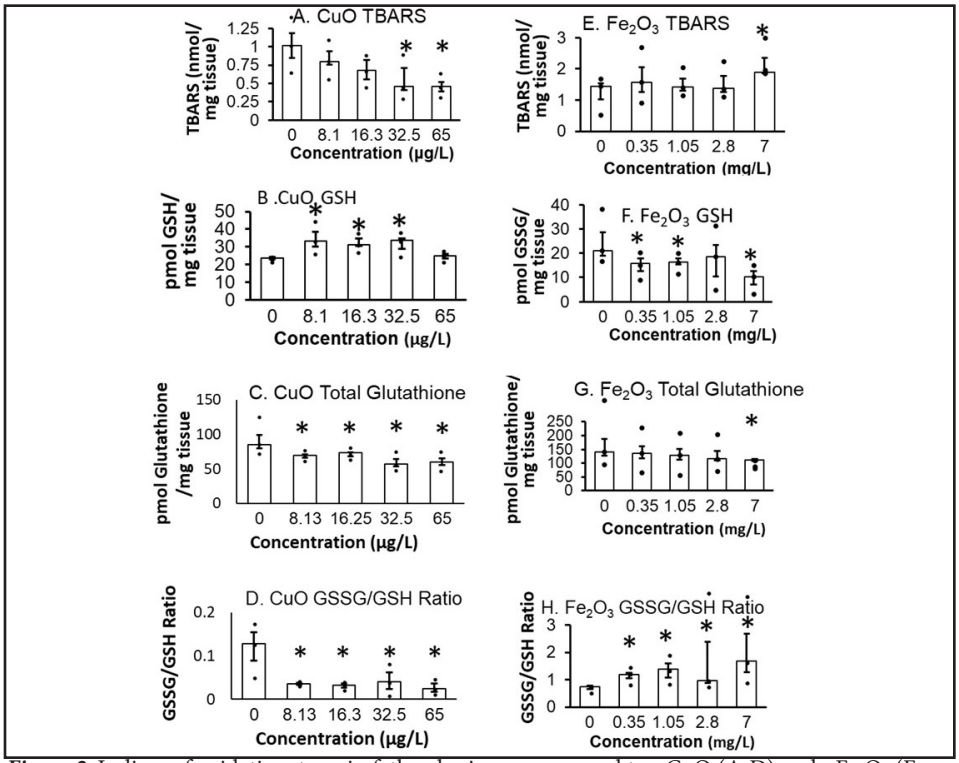

Figure 2. Indices of oxidative stress in fathead minnows exposed to nCuO (A-D) and nFe_2O_3 (E-H). A, E) Concentration of thiobarbaturic reactive substances (TBARS). B, F) concentration of reduced glutathione (GSH). C, G) concentration of total glutathione. D, H) ratio of oxidized (GSSG) to GSH. Bars ± error bars represent median ± first and third quartiles. *Significantly different from control (P < 0.05, Kruskal-Wallis test).

The LC50 values reported here are an order of magnitude lower than those reported in other studies (see Table 2). Such discrepancies may be due to species, life stage, and laboratory-specific factors. This may also be due to the fact that commercial formulations of nanoparticles were used in the present study – which include proprietary surface coatings and stabilizers to inhibit dissolution and agglomeration – while previous studies used nanoparticles synthesized or suspended (starting with dry power) in-house. But nanoparticles released to the environment may be in the form of commercial formulations, whose toxicity may differ from nanoparticles used in toxicity studies. An analogous situation occurs with pesticides, where the toxicity of the commercial formulation exceeds that of the pure compound (Nagy et al. 2020). Thus, more information is needed to assess the risk of commercial formulations vs. pure nanoparticles.

Furthermore, the results of the current study indicate that exposure to CuO and Fe_2O_3 nanoparticles can affect fitness parameters of early life stage fathead minnows in 28-day exposures. Although statistical significance is usually set at 0.05, ecological risk assessments typically assume that a chronic effect is biologically-significant when there is a 20% increase over controls (Suter et al. 2000). Thus, the statistically-significant effects on fitness parameters seen in this study would also qualify as biologically-significant by this criterion. However, larvae exposed to Fe_2O_3 actually increased apparent growth rate over controls (Figure 1). Other studies have also found that dietary exposure of fish to iron nanoparticles increases their growth rate (El-Shenawy et al. 2019). Whether this is a direct effect of the nanoparticles themselves, or due to an indirect effect (e.g., decrease pathogen load due to antimicrobial effect of nanoparticles; Shaalan et al. 2017) remains to be seen. Another finding is that CuO nanoparticles caused axial spinal curvature (Figure 1). This has also been found during acute exposure of other fish to silver nanoparticles (Wu and Zhou 2012; Kim et al. 2013). However, axial spinal curvature in fish has not been recorded before during chronic exposure to nanoparticles.

There was also an indication that the Fe_2O_3 nanoparticles in the present study induced oxidative stress. This was seen for by and increase in TBARS – an indicator of lipid peroxidation a decrease in reduced glutathione (GSH) and an increase in the GSSG/GSH ratio (Hayes and McLellan 1999). These nanoparticles have also been found to cause oxidative stress in other studies (Sarkar et al. 2014; Gürkan 2018), and oxidative stress may be a mechanism of nFe_2O_3 toxicity (Naqvi et al. 2010).

However, there are some limitations and areas for future research for this study. First, it is not known if the effects are due to uptake of nanoparticles and toxic effects caused by solid nanoparticles themselves, uptake of metals after dissolution of the nanoparticles in the test water, or due to metal ion toxicity from dissolution of nanoparticles after uptake. Approaches such as filtering the test water through filters with 1 nm (1000 kDa) filters before analysis, measuring body burdens in the animals, and transmission electron microscopy of the fish can provide data to help resolve this. Second, the sample sizes used here are very small, so a follow-up study with larger sample sizes would have more statistical power. Third, the approaches such as examining gene expression (e.g., for glutathione-metabolizing enzymes) and actual ROS production in fish tissues (using ROS-reactive fluorescent dyes) could provide more mechanistic information.

CONCLUSIONS & SUMMARY

There is a paucity of information on the levels of nanoparticles that may be present in the environment and expected environmental exposure levels of Fe_2O_3 and CuO nanoparticles have not yet been estimated with environmental fate models. However, the nCu LC50 and the LOEC concentrations for mortality fall within the range of simulated environmental concentrations predicted for metallic nanoparticles (Boxall et al. 2007). Also, although some have suggested that surface waters concentrations may be in the ng/L range, it has also been suggested that concentrations of nanoparticles in areas heavily impacted by multiple effluents – as well as areas where nanoparticles are directly applied for applications such as remediation – may exceed 10 mg/L (Callaghan and MacCormack 2017). Also, it has been found that nFe_2O_3 has insecticidal properties, but the LC50 to mosquito larvae is 4.1-20.9 mg/L (Murugan et al. 2018). Thus, any application of these nanoparticles for mosquito control would be at or near the concentrations shown to elicit adverse effects in the present study. Hence the chronic effects of Fe_2O_3 and CuO nanoparticles seen here may occur at environmentally-relevant concentrations, especially if one considers that the actual concentrations are much lower than the nominal. Therefore, any intentional application of nanoparticles for remediation or mosquito control should be viewed with caution. Finally, attention needs to be paid to the effects of commercial formulations vs. pure nanoparticles for more realistic risk assessments.

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LITERATURE CITED

Abdel-Khalek, A.A., Badran, S.R. and Marie, M.A.S. (2016) Toxicity evaluation of copper oxide bulk and nanoparticles in Nile tilapia, *Oreochromis niloticus*, using hematological, bioaccumulation and histological biomarkers. *Fish Physiology and Biochemistry*, 42: 1225-1236.

Agency for Toxic Substances and Disease Registry (ATSDR). (2011). *EPA Reportable Quantity Methodology Used to Establish Toxicity/Environmental Scores for the 2011 Substance Priority List*. https://www.atsdr.cdc.gov/spl/resources/atsdr_tes_methodology.pdf (Accessed 30 January 2022).

ASTM (1999). *Standard Practice For Conducting Chronic Toxicity Tests with Early Life Stages of Fish*. E-1241-98. Annual Book of ASTM Standards. 11.04. American Society for Testing and Materials.

ASTM. (2003). *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians*. E 729-96. Annual Book of ASTM Standards. 11.04. American Society for Testing and Materials.

Aziz, S. and Abdullah, S. (2023) Evaluation of Toxicity Induced by Engineered CuO Nanoparticles in Freshwater Fish, *Labeo rohita*. *Turkish Journal of Fisheries and Aquatic Sciences*, 23(8) DOI : 10.4194/TRJFAS18762

Banerjee, A., and Roychoudhury, A. (2019). Nanoparticle-induced ecotoxicological risks in aquatic environments: concepts and controversies. *Nanomaterials in Plants, Algae and Microorganisms*: 129-141

Boxall, A, Chaudhry, Q, Sinclair, C, Jones, A, Aitken, R, Jefferson, B, and Watts, C (2007). Current and future predicted environmental exposure to engineered nanoparticles. Central Science Laboratory, Department of the Environment and Rural Affairs, London, UK :9 http://randddefragovuk/Documentaspx?Document=CB010)98_6270_FRPpdf (Accessed 30 January 2022).

Braz-Mota, S., Campos, D.F., MacCormack, T.J., Duarte, R.M., Val, A.L. and Almeida-Val, V.M. (2018) Mechanisms of toxic action of copper and copper nanoparticles in two Amazon fish species: Dwarf cichlid (*Apistogramma agassizii*) and cardinal tetra (*Paracheirodon axelrodi*). *Science of the Total Environment*, 630: 1168-1180.

Callaghan, N. I., and MacCormack, T. J. (2017). Ecophysiological perspectives on engineered nanomaterial toxicity in fish and crustaceans. *Comparative Biochemistry and Physiology* Part C: Toxicology and Pharmacology, 193: 30-41

Chakraborty, C., Sharma, A. R., Sharma, G., and Lee, S. S. (2016). Zebrafish: A complete animal model to enumerate the nanoparticle toxicity. *Journal of Nanobiotechnology*, 14:1-13.

Chen, J, Dong, X, Xin, Y, Zhao, M (2011). Effects of titanium dioxide nano-particles on growth and some histological parameters of zebrafish (Danio rerio) after a long-term exposure. *Aquatic Toxicology*, 101: 493-499.

El-Shenawy, A. M., Gad, D. M., and Yassin, S. A. (2019). Effect of Iron Nanoparticles on the Development of Fish Farm Feeds. *Alexandria Journal for Veterinary Sciences*, 60, pp 102-115.

Griffitt, R.J., Luo, J., Gao, J., Bonzongo, J., and Barber, D.S. (2008) Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. *Environmental Toxicology and Chemistry* 27: 1972-1978.

Gürkan, M. (2018). Effects of three different nanoparticles on bioaccumulation, oxidative stress, osmoregulatory, and immune responses of *Carcinus aestuarii*. *Toxicological and Environmental Chemistry*, 100: 693-716.

Hall S., Bradley T., Moore J. T., Kuykindall T., and Minella L. (2009). Acute and chronic toxicity of nano-scale TiO_2 particles to freshwater fish, cladocerans, and green algae, and effects of organic and inorganic substrate on TiO_2 toxicity. *Nanotoxicology*, 3: 91-97.

Haque, E., and Ward, A. C. (2018). Zebrafish as a model to evaluate nanoparticle toxicity. *Nanomaterials*, 8: 561-578.

Hayes, J.D., and McLellan, L.I. (1999). Glutathione and glutathione-dependent enzymes repre-sent a coordinately regulated defense against oxidative stress. *Free Radical Research*, 31, 273-300.

Hollander, M., and Wolfe, A. (1973). *Nonparametric statistical methods*. John Wiley & Sons.

Hõrak, P., & Cohen, A. (2010). How to measure oxidative stress in an ecological context: methodological and statistical issues. *Functional Ecology*, 24: 960-970.

Jahanbakhshi, A., Hedayati, A. and Pirbeigi, A. (2015) Determination of acute toxicity and the effects of sub-acute concentrations of CuO nanoparticles on blood parameters in *Rutilus rutilus*. *Nanomedicine Journal*, 2: 195-202.

Jeffries, M. K. S., Stultz, A. E., Smith, A. W., Stephens, D. A., Rawlings, J. M., Belanger, S. E., and Oris, J. T. (2015). The fish embryo toxicity test as a replacement for the larval growth and survival test: a comparison of test sensitivity and identification of alternative endpoints in zebrafish and fathead minnows. *Environmental Toxicology and Chemistry*, 34: 1369.1381.Khosravi-Katuli, K., Lofrano, G., Nezhad, H.P., Giorgio, A., Guida, M., Aliberti, F., Siciliano, A., Carotenuto, M., Galdiero, E., Rahimi, E. and Libralato, G. (2018) Effects of ZnO nanoparticles in the Caspian roach (*Rutilus rutilus caspicus*). *Science of the Total Environment*, 626: 30-41.

Kim, J. Y., Kim, K. T., Lee, B. G., Lim, B. J., and Kim, S. D. (2013). Developmental toxicity of Japanese medaka embryos by silver nanoparticles and released ions in the presence of humic acid. *Ecotoxicology and Environmental Safety*, 92: 57-63.

Kovrižnych J.A., Sotníková R., Zeljenková D., Rollerová E., Szabová E., and Wimmerová .S (2013) Acute toxicity of 31 different nanoparticles to zebrafish (*Danio rerio*) tested in adulthood and in early life stages–comparative study. *Interdisciplinary Toxicology*, 6: 67-73.

Kumari, K., Singh, P., Bauddh, K., Mallick, S., and Chandra, R. (2019). Implications of metal nanoparticles on aquatic fauna: A review. *Nanoscience and Nanotechnology-Asia*, 9: 30-43..

Laban G., Nies L.F., Turco R.F., Bickham J.W., and Sepúlveda M.S. (2010). The effects of silver nanoparticles on fathead minnow (*Pimephales promelas*) embryos. *Ecotoxicology*, 19: 185-195.

Mansano, A.S., Souza, J.P., Cancino-Bernardi, J., Venturini, F.P., Marangoni, V.S. and Zucolotto, V. (2018) Toxicity of copper oxide nanoparticles to Neotropical species *Ceriodaphnia silvestrii* and *Hyphessobrycon eques*. *Environmental Pollution*, 243: 723-733.

Murugan, K., Dinesh, D., Nataraj, D., Subramaniam, J., Amuthavalli, P., Madhavan, J., Rajasekar, A., Rajan, M., Thiruppathi, K. P., Kumar, S. and Higuchi, A. (2018). Iron and iron oxide nanoparticles are highly toxic to *Culex quinquefasciatus* with little non-target effects on larvivorous fishes. *Environmental Science and Pollution Research*, 25: 10504-10514.

Nagy, K., Duca, R. C., Lovas, S., Creta, M., Scheepers, P. T., Godderis, L., and Ádám, B. (2020). Systematic review of comparative studies assessing the toxicity of pesticide active ingredients and their product formulations. *Environmental Research*, 181, p 108926.

Naqvi, S., Samim, M., Abdin, M., Ahmed, F. J., Maitra, A., Prashant, C., and Dinda, A. K. (2010). Concentration-dependent toxicity of iron oxide nanoparticles mediated by increased oxidative stress *International Journal of Nanomedicine*, 5: 983-989.

Nations, S., Long, M., Wages, M., Cañas, J., Maul, J. D., Theodorakis, C., and Cobb, G. P. (2011a). Effects of ZnO nanomaterials on *Xenopus laevis* growth and develop-

ment. *Ecotoxicology and Environal Safety*, 74: 203-210.

Nations, S., Long, M., Wages, M., Maul, J. D., Theodorakis, C. W., and Cobb, G. P. (2015). Subchronic and chronic developmental effects of copper oxide (CuO) nanoparticles on *Xenopus laevis*. *Chemosphere*, 135: 166-174.

Nations, S., Wages, M., Cañas, J.E., Theodorakis, C., and Cobb, G. P. (2011b). Acute effects of Fe_2O_3, TiO_2, ZnO and CuO nanomaterials on *Xenopus laevis*. *Chemosphere*, 83: 1053-1061.

Noureen, A., Jabeen, F., Tabish, T.A., Ali, M., Iqbal, R., Yaqub, S. and Shakoor Chaudhry, A., (2021) Histopathological changes and antioxidant responses in common carp (*Cyprinus carpio*) exposed to copper nanoparticles. *Drug and Chemical Toxicology*, 44: 372-379.

Pereira, S.P., Boyle, D., Nogueira, A. and Handy, R.D (2023) Differences in toxicity and accumulation of metal from copper oxide nanomaterials compared to copper sulphate in zebrafish embryos: Delayed hatching, the chorion barrier and physiological effects. *Ecotoxicology and Environmental Safety*, 253: 114613, https://doi.org/10.1016/j.ecoenv.2023.114613.

Sarkar, A., Ghosh, M., and Sil, P. C. (2014). Nanotoxicity: Oxidative stress mediated toxicity of metal oxide nanoparticles. *Journal of Nanoscience and Nanotechnology*, 14: 730-743.

Sayadi, M.H., Pavlaki, M.D., Loureiro, S., Martins, R., Tyler, C.R., Mansouri, B., Kharkan, J. and Shekari, H., (2022) Co-exposure of zinc oxide nanoparticles and multi-layer graphenes in blackfish (*Capoeta fusca*): Evaluation of lethal, behavioural, and histopathological effects. *Ecotoxicology*, 31:425-439.

Shaalan, M. I., El-Mahdy, M. M., Theiner, S., El-Matbouli, M., and Saleh, M. (2017). In vitro assessment of the antimicrobial activity of silver and zinc oxide nanoparticles against fish pathogens. *Acta Veterinaria Scandinavica*, 59, 1-11.

Shaw, B. J., and Handy, R. D. (2011). Physiological effects of nanoparticles on fish: a comparison of nanometals versus metal ions. *Environment International*, 37: 1083-1097.

Shaw, B. J., Liddle, C. C., Windeatt, K. M., and Handy, R. D. (2016). A critical evaluation of the fish early-life stage toxicity test for engineered nanomaterials: Experimental modifications and recommendations. *Archives of Toxicology*, 90: 2077-2107.

Song, L., Vijver, M. G., Peijnenburg, W. J., Galloway, T. S., and Tyler, C. R. (2015). A comparative analysis on the in vivo toxicity of copper nanoparticles in three species of freshwater fish. *Chemosphere*, 139: 181-189.

Stark, W. J., Stoessel, P. R., Wohlleben, W., & Hafner, A. J. C. S. R. (2015). Industrial applications of nanoparticles. *Chemical Society Reviews*, 44: 5793-5805.

Subashkumar, S. and Selvanayagam, M. (2014) First report on: Acute toxicity and gill histopathology of freshwater fish *Cyprinus carpio* exposed to Zinc oxide (ZnO) nanoparticles. *International Journal of Scientific and Research Publications*, 4: 1-4.

Suter, G.W., Jr, Efroymson, R.A., Sample, B.E., and Jones, D.S. (2000). *Ecological risk assessment of contaminated sites*. CRC Press.

Theodorakis, C. W., Adams, S. M., Smith, C., Rotter, J., Hay, A., and Eslick, J. (2017). Effects of military activity and habitat quality on DNA damage and oxidative stress in the largest population of the federally threatened gopher tortoise. *Ecotoxicology*, 26: 1344-1357.

Theodorakis, C., D'surney, S., Bickham, J., Lyne, T., Bradley, B., Hawkins, W., Farkas, W., McCarthy, J., and Shugart, L. (1992). Sequential expression of biomarkers in bluegill sunfish exposed to contaminated sediment. *Ecotoxicology*, 1: 65-73.

Vafadarnejad, M., Gharaei, A., Mirdar, H., and Miri, M. (2018) Determining of copper oxide nanoparticles (CuO NPs) LC50 in grass carp (*Ctenopharyngodon della*) and its effects on hematological and liver enzymes activity indices. *Journal of Fisheries (Iranian Journal of Natural Resources*), 71: 11-21.

Vidya, P.V. and Chitra, K.C. (2017) Assessment of acute toxicity (LC50-96 h) of aluminium oxide, silicon dioxide and titanium dioxide nanoparticles on the freshwater fish, *Oreochromis mossambicus* (Peters, 1852). *International Journal of Fisheries and Aquatic Studies*, 5: 327-332.

Villacis, R.A., José Filho, S., Pina, B., Azevedo, R.B., Pic-Taylor, A., Mazzeu, J.F. and Grisolia, C.K. (2017) Integrated assessment of toxic effects of magnetite (γ-Fe_2O_3) nanoparticles in zebrafish. *Aquatic Toxicology*, 19: 219-225.

Wickliffe, J., and Bickham, J. (1998). Flow cytometric analysis of hematocytes from brown pelicans (*Pelecanus occidentalis*) exposed to planar halogenated hydrocarbons and heavy metals. *Bulletin of Environmental Contamination and Toxicology*, 61: 239-246.

Wu, Y., and Zhou, Q. (2012). Dose-and time-related changes in aerobic metabolism, chorionic disruption, and oxidative stress in embryonic medaka (*Oryzias latipes*): Underlying mechanisms for silver nanoparticle developmental toxicity. *Aquatic Toxicology*, 124: 238-246.

Xia, J., Zhao, H. Z., and Lu, G. H. (2013). Effects of metal oxide nanoparticles on multiple biomarkers in *Carassius auratus*. *Biomedical and Environmental Sciences*, 26: 742-749.

Zhu, X., Tian, S., and Cai, Z. (2012) Toxicity assessment of iron oxide nanoparticles in zebrafish (*Danio rerio*) early life stages. *PloS One 7*, e46286 doi1013)71/journalpone004)6286.

Zhu, X., Zhu, L., Duan, Z., Qi, R., Li, Y., and Lang, Y. (2008) Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio*) early developmental stage. *Journal of Environmental Science and Health*, A 43:278-284.

Supplementary Information

Table S1. Mean (± SD) water quality parameters for acute toxicity tests of metal oxide nanoparticles using fathead minnows.

Nanoparticle	Nominal Concentration (mg/L)	pH	Temp (°C)	$\%O_2$	Salinity (mg/L)	Conductivity (µS)	Total ammonia (mg/L)
Fe_2O_3	0	8.07 (±0.06)	21.3 (±0.28)	51.8 (±10.61)	4.06 (±0.42)	6.3 (±0.57)	1.28 (±0.18)
	17.5	8.1 (±0.11)	20.7 (±0.28)	48.9 (±21.21)	5.48 (±0.77)	6 (±0.99)	1.27 (±0.2)
	21	8.21 (±0.01)	19.7 (±0.57)	62.5 (±0.85)	4.75 (±1.39)	4.9 (±1.13)	1.12 (±0.08)
	22.75	8.15 (±0.05)	20.25 (±1.2)	49.5 (±6.93)	4.6 (±0.51)	3.95 (±1.06)	1.23 (±0.26)
	24.5	8.15 (±0.08)	19.95 (±1.06)	51.6 (±10.75)	5.09 (±0.59)	2.7 (±0.14)	1.25 (±0.25)
	26.25	8.07 (±0.07)	19.2 (±0.42)	35.65 (±9.55)	4.49 (±1.2)	2.95 (±0.07)	1.31 (±0.2)
	28	8 (±0.03)	19.55 (±0.07)	28 (±0.85)	4.09 (±0.75)	3.05 (±1.06)	1.2 (±0.03)
	29.75	8.01 (±0.07)	20.25 (±1.06)	36.65 (±14.21)	3.61 (±0.28)	4.25 (±1.91)	1.16 (±0.11)
	31.5	7.98 (±0.02)	19.35 (±0.21)	27.6 (±1.41)	3.65 (±0.34)	2.7 (±0.28)	1.09 (±0.11)
CuO	0	8.12 (± 0.04)	20.75 (± 0.4)	30.7 (± 5.89)	2.77 (± 0.51)	607.68 (± 236.31)	1.31 (±0.23)
	0.267	8.15 (± 0.05)	20.83 (± 0.5)	31.5 (± 5.76)	2.8 (± 0.49)	850 (± 3.83)	1.31 (±0.22)
	0.35	8.17 (± 0.11)	20.8 (± 0.64)	33.88 (± 4.8)	3.03 (± 0.4)	848.5 (± 6.76)	1.26 (±0.27)
	0.474	8.16 (± 0.09)	20.88 (± 0.61)	31.98 (± 7.75)	2.84 (± 0.66)	717.88 (± 217.18)	1.25 (±0.24)
	0.633	8.11 (± 0.04)	20.85 (± 0.53)	31.25 (± 6.01)	2.78 (± 0.5)	524.85 (± 13.08)	1.26 (±0.24)
	0.844	8.11 (± 0.05)	20.65 (± 0.55)	28.9 (± 8.44)	2.58 (± 0.73)	466.78 (± 145.51)	1.27 (±0.23)
	1.125	8.1 (± 0.08)	20.78 (± 0.38)	29.33 (± 6.2)	2.62 (± 0.53)	419.25 (± 65.36)	1.11 (±0.07)
	1.5	8.03 (± 0.14)	20.78 (± 0.33)	30.2 (± 4.76)	2.79 (± 0.58)	518.3 (± 227.24)	1.09 (±0.05)
	2	7.97 (± 0.14)	20.33 (± 0.34)	22.93 (± 7.07)	2.06 (± 0.6)	542.4 (± 172.5)	1.22 (±0.23)

Table S2. Mean (± SD) water quality parameters for chronic toxicity tests of metal oxide nanoparticles using fathead minnows.

Nanoparticle	Nominal Concentration (mg/L)	pH	Temp (°C)	$\%O_2$	Salinity (mg/L)	Conductivity (µS)	Total ammonia (mg/L)
Fe_2O_3	0	8.47 (± 0.01)	19.73 (± 0.06)	64.77 (± 6.95)	5.29 (± 0.47)	887.67 (± 317.61)	1.21 (±0.11)
	0.35	8.49 (± 0.01)	19.67 (± 0.06)	60.57 (± 4.86)	4.83 (± 0.36)	1191 (± 1.73)	1.34 (±0.42)
	1.05	8.44 (± 0.05)	19.6 (± 0)	50.33 (± 9)	3.96 (± 0.59)	1154.67 (± 77.78)	1.19 (±0.07)
	2.8	8.56 (± 0.03)	19.7 (± 0.17)	59.93 (± 2.54)	4.79 (± 0.42)	1201.33 (± 7.37)	1.13 (±0.09)
	7	8.59 (± 0.01)	19.9 (± 0.1)	55.3 (± 4.16)	4.67 (± 0.19)	1122.67 (± 71.81)	1.2 (±0.08)
CuO	0	8.21 (± 0.04)	21.27 (± 0.06)	61.67 (± 3.87)	5.27 (± 1.18)	907.33 (± 321.94)	1.28 (±0.18)
	0.008	8.39 (± 0.04)	21.4 (± 0.1)	57 (± 5.57)	5.02 (± 0.34)	882.33 (± 308.89)	1.23 (±0.26)
	0.016	8.21 (± 0.13)	21.37 (± 0.06)	51.33 (± 9.71)	4.47 (± 0.74)	968.67 (± 177.25)	1.2 (±0.03)
	0.033	8.45 (± 0.06)	21.17 (± 0.21)	56 (± 8.66)	4.92 (± 0.9)	1063 (± 25.16)	1.05 (±0.04)
	0.065	8.46 (± 0.06)	21.23 (± 0.06)	60.67 (± 6.81)	5.64 (± 0.08)	1078 (± 2)	1.32 (±0.18)

Table S4. Average hydrodynamic diameter, size range, and percentage of particles ≤ 100 nm for metal oxide nanoparticles in stock solutions used in chronic toxicity tests for fathead minnows.

Nano particle	Week	Average Size (nm)	Size Range	Percentage of Particles ≤ 100 nm Diameter
CuO[a]	1	76	36-243	90 %
	2	86	43-243	83 %
	3	82	36-204	86 %
	4	105	51-344	65 %
Fe_2O_3	1	113	15-687	65 %
	2	86	21-486	84 %
	3	103	30-486	68 %
	4	92	30-289	73 %

[a]Stock concentration 1 g/L. [b]Stock concentration 10 g/L.

Table S3. Average hydrodynamic diameter[a] of metal oxide nanoparticles used in acute toxicity tests for fathead minnows.

Nano particle	Concentration (mg/L)	First day[b]		Last day[b]	
		Size	Percent	Size	Percent
Fe_2O_3	17.5	76.8	100	74.9	100
	21	45	44.5	68.9	100
		14.93[d]	55.5		
	22.75	89.6	100	-	-
	24.5	13.97[d]	100	64.9	100
	26.25	12.16[d]	100	-	-
	28	15.94	100	69.3	100
	29.75	122.3	53.8	76.6	60.6
		38.6	46.2	33.1	39.4
	31.5	50.2	100	-	-
CuO	0.267	66	100	70.1	100
	0.35	73.6	100	71.8	100
	0.4.74	72.1	100	76.9	100
	0.633	80.9	100	77.7	100
	0.844	86.6	100	84.2	100
	1.125	79.7	100	86	100
	1.5	87.3	100	120.6	100
Cu	0.000925	93.6	100	1117	1.1
				32.7	98.9
	0.00185	140.9	78	210.6	78.3
		38	22	72	21.7
	0.002775	75.2	100	1065	1.3
				46.4	98.7
	0.0037	51.6	100	980	3.5
				63.9	96.5
	0.00555	48.1	100	20.15	100
	0.00925	18.59	100	1054	4.6
				46.7	95.4
	0.0185	73.1	100	1092	1.6
				29.88	98.4
	0.02775	1007	1.8	1032	5.2
		68.4	98.2	65.6	94.8
	0.037	936	2.5	1071	3.2
		78.4	97.5	50.6	96.8

[a]Measured using Dynamic Light Scattering. [b]Water samples were taken from test beakers on the first and last day of exposure. Data represent average size and percent of the nanoparticles in that particular size class.

Table S5. Metal concentrations in test vessels for chronic toxicity tests of metal oxide nanoparticles using fathead minnows.

Nanoparticle	Nanoparticle Nominal	Metal Nominal	Metal measured ($\bar{X}$ ± SD)
A. Acute			
Cu	0.0008	0.0008	0.0005 (±0.00007)
	0.0016	0.0016	0.0011 (±0.0001)
	0.0024	0.0024	0.0014 (±0.0003)
	0.0032	0.0032	0.0025 (±0.0004)
	0.0047	0.0047	0.0037 (±0.0005)
	0.0079	0.0079	0.0065 (±0.0008)
	0.0158	0.0158	0.0101 (±0.0016)
	0.0237	0.0237	0.0152 (±0.0026)
	0.0316	0.0316	0.0254 (±0.0038)
CuO	0.267	0.213	0.084 (±0.008)
	0.35	0.28	0.135 (±0.015)
	0.474	0.379	0.181 (±0.019)
	0.633	0.506	0.242 (±0.030)
	0.844	0.674	0.324 (±0.041)
	1.125	0.899	0.330 (±0.028)
	1.5	1.199	0.413 (±0.037)
	2	1.598	0.591 (±0.066)
Fe_2O_3	17.5	12.23	1.268 (±0.067)
	21	14.69	1.378 (±0.090)
	22.75	15.90	1.481 (±0.115)
	24.5	17.13	1.733 (±0.113)
	26.25	18.35	1.862 (±0.123)
	28	19.57	2.078 (±0.151)
	29.75	20.80	2.048 (±0.117)
	31.5	22.02	2.262 (±0.169)
	35	24.47	2.609 (±0.179)
B. Chronic			
CuO	0.008	0.006	0.003 (±0.0006)
	0.0163	0.011	0.007 (±0.0016)
	0.0325	0.023	0.016 (±0.0023)
	0.065	0.045	0.025 (±0.0044)
Fe_2O_3	0.35	0.24	0.037 (±0.004)
	1.1	0.77	0.092 (±0.011)
	2.8	1.96	0.231 (±0.029)
	7	4.89	0.909 (±0.112)