Chemosphere 310 (2023) 136894

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Bioaccumulation of CuO nanomaterials in rainbow trout: Influence of exposure route and particle shape

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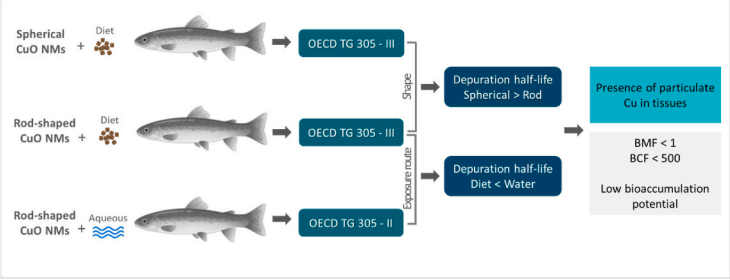
HIGHLIGHTS

- Fish were exposed to spherical and rod-shaped CuO NMs via diet and water.
- Via diet, the rod-shaped particles depurated faster than the spherical shape NMs.
- The rod-shapes particles depurated faster when exposed via the diet than via water.
- Particulate forms of Cu were detected in fish tissues.
- Biomagnification and bioconcentration factors showed low bioaccumulation potential.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling editor: Eduardo Almeida

Keywords:
Fish
Exposure route
Nanoform
Bioconcentration
Biomagnification
Single-particle ICP MS

ABSTRACT

The bioaccumulation potential of spherical and rod-shaped CuO nanomaterials (NMs) was assessed in rainbow trout (*Oncorhynchus mykiss*) exposed via water and diet following the OECD Test Guideline No. 305. Fish were exposed via diet to both NMs at concentrations of 70 and 500 mg Cu/kg for 15 days, followed by 44 days of depuration. For water-borne exposure, only the rod-shaped CuO NMs were tested at 0.08 and 0.8 mg Cu/L for 28 days, followed by 14 days of depuration. The concentration of Cu was determined in fish whole body to derive biomagnification and bioconcentration factors (BMF and BCF). Different tissues were sampled to investigate the total Cu biodistribution and target organs as well as the particle number-based bioaccumulation of CuO NMs. Estimated BMF and BCF values were below the thresholds of concern. However, shape and route influenced depuration. Following dietary exposure, there was a higher depuration of Cu from fish exposed to the rod-shaped compared to the spherical CuO NMs. A higher depuration was also observed for rod-shaped CuO NMs following the dietary exposure compared the aqueous one. Despite the much higher dietary exposure concentrations of rod-

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https://doi.org/10.1016/j.chemosphere.2022.136894
Received 29 April 2022; Received in revised form 24 September 2022; Accepted 11 October 2022
Available online 18 October 2022

shape CuO NMs, similar Cu body burdens were reached via water. Cu was found in particulate form in different tissues.

Although these NMs had a low bioaccumulation potential, differences in distribution and elimination patterns of Cu were observed depending on the exposure route and particle shape. Careful consideration of the most relevant exposure route is needed when designing a bioaccumulation experiment for testing NMs.

1. Introduction

Knowledge on the potential adverse effects of nanomaterials (NMs) has been expanding in recent years. NMs, like conventional chemicals are subject to environmental risk assessment. Testing the bioaccumulation potential is a key component of the assessment of the potential of persistence, bioaccumulation and toxicity (PBT) of a substance to fulfil regulatory requirements (EC, 2006) and to classify the substance for its potential to bioconcentrate (EC, 2008). Bioaccumulation potential is generally determined using fish following the standardized OECD test guideline (TG) 305 (OECD, 2012). This TG describes methods for bioaccumulation testing of chemicals via water and via diet to determine the bioconcentration factor (BCF) (bioaccumulation after water exposure) and the biomagnification factor (BMF) (bioaccumulation after dietary exposure). To date there have been few BCF and/or BMF values reported for NMs, likely due to uncertainties over the applicability of the TG for NM testing and any additional considerations needed (Handy et al., 2022; Connolly et al., 2022).

Within the Horizon 2020 Gov4Nano project, the applicability of the current TG for bioaccumulation testing of NMs in fish following aqueous and dietary exposure is being studied (Connolly et al., 2022). To address key issues that need to be considered when using TG 305, CuO NMs were selected. Limited information is available regarding the bioaccumulation potential of this material as a function of its particle shape and exposure route, and it represents a metal oxide NM that may undergo dissolution. The uptake and depuration kinetics of spherical CuO NMs were tested in rainbow trout (*Oncorhynchus mykiss)* only via diet due to its instability in aquarium water. We further focused on the rod-shaped CuO NMs due to their stability in aquarium water allowing comparative testing of bioaccumulation kinetics via water and diet. Moreover, the influence of NM shape on bioaccumulation could be addressed as well by this experimental design. There are indications that shape may influence bioaccumulation potential of NMs such as TiO_2 and Au NMs (Yeo and Nam, 2013; Abdolahpur Monikh et al., 2020) but this needs to be investigated further for other types of NMs.

Advanced methods such as single-particle inductively coupled plasma mass spectrometry (spICP-MS) are promising tools to detect particles in tissues and to quantify/measure particles in terms of number-based concentration, thus allowing a better understanding of the fate of NMs within organisms after particle isolation. So far, only few studies have enabled the comparison of mass and number based bioaccumulation of metallic NMs in fish tissues (Abdolahpur Monikh et al., 2019, 2020, 2021; Clark et al., 2021; Lu et al., 2022), but none of them have dealt with CuO NMs. Singe-particle ICP-MS was therefore applied in this study to shed some light on the form (particulate and ions) of any accumulated Cu in fish tissues following exposure to CuO NMs.

2. Materials and methods

2.1. Preparation of stock suspensions and NM characterization

Spherical (<50 nm manufacturer reported particle size, surface area of 29 m^2/g, CAS: 1317-38-0, product number: 544868) and rod-shaped (manufacturer reported diameter × length: 10–12 nm × 75–100 nm, surface area 60–100 m^2/g, CAS: 1317-38-0, product number: 792004) powdered CuO NMs were purchased from Merck (Madrid, Spain). Stock suspensions of both NMs were prepared by applying the NANOREG-ECOTOX dispersion protocol (Booth and Jensen, 2015). NMs were dispersed in Milli-Q water (18.2 MΩ cm at 25 °C) at concentrations of 350 mg Cu/L and 2500 mg Cu/L and sonicated for 15 min using a probe sonicator (6 mm horn, 10% amplitude continuous mode, 7.35 ± 0.05 Watt, Vibra-Cell™ VCX 130, Sonics, Newton, CT, USA). These stock suspensions were used for feed spiking immediately after preparation. In addition, the rod-shaped CuO NMs (2500 mg Cu/L) were dispersed in the aquarium water at a concentration of 8 mg Cu/L for hydrodynamic size measurement using dynamic light scattering (DLS). A stock solution (2500 mg Cu/L) was used to disperse the particles in the exposure media.

Particle size distributions of stock suspensions were determined by DLS using a Zetasizer Nano Series (Malvern Instruments, UK). The morphology of the particles was determined by means of transmission electron microscopy (TEM). For TEM analysis, samples were prepared by placing a drop of a stock suspension on nickel TEM grids and they were allowed to evaporate at room temperature before analysis. NMs were observed in a JEOL 1400 Plus TEM (JEOL Ltd., Japan). The dissolution of the CuO NMs in the Milli-Q and exposure medium were assessed using an ultra-centrifugal filter unit (Amicon® Ultra-15 device, 3 kDa, 8000×*g* for 30 min centrifugation). In addition, the stability of rod-shaped NMs was tested in Milli-Q water and in the exposure medium following the OECD TG 318 (OECD, 2017a). The stability of the particles against aggregation in stock suspensions is shown in Supplementary data S.1. The spherical NMs underwent aggregation and sedimentation after 10 min of preparation.

2.2. Fish bioaccumulation tests

Fish bioaccumulation studies were performed with both spherical and rod-shaped CuO NMs and, when possible, by aqueous and dietary routes. The following tests were conducted: 1) spherical CuO NMs were tested following a full dietary bioaccumulation test (TG 305 – III). Bioaccumulation potential of spherical CuO NMs was tested only via diet due to its instability as indicated in the previous paragraph. 2) the uptake and depuration kinetics of rod-shaped CuO NMs were investigated following a full dietary and minimised aqueous (TG 305 – II) bioaccumulation test. Before performing these assays, a preliminary dietary uptake study was conducted with spherical CuO NMs (Supplementary data S.2). This preliminary study was performed to set the methodology for feed spiking, the appropriate concentrations to test and to identify the time to steady state. It also served as a means for treated tissue samples to be collected to determine the presence and amount of nanoparticles in the fish tissues by spICP-MS.

2.2.1. Full dietary bioaccumulation assay

Full dietary bioaccumulation tests with the spherical and rod-shaped CuO NMs were performed on two separate occasions following the OECD TG 305-III (2012). Two independent experiments were carried out under identical experimental conditions. It was not possible to perform the experiments with the same stocks of fish. However, as detailed below there were no significant differences in fish size and background Cu concentrations.

Fish used in the treatments with the spherical and rod-shaped CuO NMs were supplied from the fish farms Río Mundo S.L.U. (Albacete, Spain) and Felechosa (Asturias, Spain), respectively and acclimated in our stock aquaria. For each assay, 240 rainbow trout (*O. mykiss*) were randomly distributed in 3 tanks (control tank, 70 mg Cu/kg feed

exposure tank, and 500 mg Cu/kg feed exposure tank) (120 L capacity) filled with 110 L recirculating filtered tap water supplemented with a mixture of salts (Aquadur, JBL), with 80 fish per tank. Fish initial weight and length (mean ± SD) were 1.4 ± 0.3 g and 4.7 ± 0.2 cm for the spherical, while 1.2 ± 0.3 g and 4.6 ± 0.4 cm for the rod-shaped CuO NMs assays, respectively. Fish were acclimatized in the tanks for 11 and 13 days (spherical and rod-shaped CuO NMs assays, respectively) prior to the start of the experiment. According to the results of the preliminary study an uptake phase of 15 days was set (Supplementary data S.2). During the uptake phase, fish were fed daily with 70 and 500 mg Cu/kg CuO NM spiked diet at a feeding rate of 2% body weight/day which equates to nominal exposure doses of 1.4 and 10 mg Cu/kg body weight fish, respectively. These concentrations were used taking into consideration palatability, background levels, sensitivity of the analytical technique, a practical range of between 1 and 1000 mg/kg (OECD TG 305), and (for comparative purposes) concentrations used previously in studies with CuO NMs (Boyle et al., 2021) and Cu ions (Clearwater et al., 2002). A pellet size of 1.9 mm and a lipid content of 18% (Inicio Plus 887, BIOMAR Iberia, S.A., Dueñas, Spain) were used. A direct addition and mixing technique was applied for feed spiking (see Supplementary data S.2), with the difference being that the ultra-centrifugal filtration step of the stock suspensions was omitted as very low dissolution of Cu was observed. In the case of the spherical CuO NM experiment, the entire feed used to expose fish was spiked using a stock suspension prepared at the start of the experiment, whereas rod-shaped CuO spiked feed was prepared every day from freshly prepared stock suspensions.

With no previous knowledge on the elimination kinetics of CuO NMs, an extended depuration phase of 44 days was used to ensure the estimation of depuration rate constants. Over this period fish were fed with clean (unspiked with any NMs) diets at the same rate. Faeces were removed daily 1 h after feeding and water was reconstituted (20%) every day. Water parameters were checked once a week during the acclimatization period and on days of sampling thereafter (Supplementary data S.3). The photoperiod was set to 12 h light:12 h dark. The behaviour of fish was observed daily, and mortalities recorded as well as growth performance at each sampling point (Supplementary data S.3).

At the start of the experiment, 3 fish were taken from the stock to determine background Cu concentrations. Five fish were sampled for total body Cu level analysis from control and treatment groups on days 2, 11, 13, 15 during uptake and days 16, 18, 22, 29, 44, 59 during depuration. Additionally, 5 fish per treatment were taken for tissue analysis (liver, intestine, stomach, gill, muscle, carcass, and brain) on day 8 during uptake and days 16, 29 and 59 during depuration. For lipid analysis, 1 fish from each group was sampled at the start of the experiment (day 0) and at the end of the uptake (day 15) and depuration period (day 59). Fish were not fed for 24 h before sampling. Fish were euthanized with Tricaine methanesulfonate (MS222) (250 mg/L), weights and lengths recorded and thereafter stored at −30 °C until analysis.

Under the same experimental conditions, in parallel to the full dietary study, 5 fish were fed 500 mg Cu/kg feed spiked with rod-shaped CuO NMs for 15 days and tissues (liver, intestine, stomach, gill, muscle and brain) were taken for spICP-MS analysis and stored at −80 °C until analysis.

2.2.2. Minimised aqueous exposure bioaccumulation test

No existing information regarding the toxicity and bioaccumulation following aqueous exposure of specifically CuO NMs in rainbow trout could be found. Therefore, as a first approach, using a minimal number of animals, and to determine concentration dependence, a minimised test design was used. Although some studies reported no mortality in other fish species (*Cyprinus carpio* and *Danio rerio*) up to 100 mg/L CuO NMs (Zhao et al., 2011; Boyle et al., 2020; Song et al. (2015) estimated a 96-h LC_{50} of 0.68 ± 0.15 mg/L and a 96-h LOEC of 0.17 mg/L for Cu NMs (spherical, 50 nm) in rainbow trout. Considering this information, exposure concentrations of 0.08 and 0.8 mg Cu/L of rod-shaped CuO NMs were selected for the minimised aqueous exposure bioaccumulation test following the OECD TG 305-II. The rod-shaped CuO NMs were chosen for aqueous exposure testing according to maintenance of hydrodynamic size distribution and low dissolution over 24 h (see results section 3.1).

Sixty rainbow trout (mean ± SD) 1.5 ± 0.2 g in weight and 5.5 ± 0.2 cm in length (supplied from the Felechosa fish farm and acclimatized in our stock aquaria) were distributed in 3 tanks (control tank, 0.08 mg Cu/L exposure tank, and 0.8 mg Cu/L exposure tank) (50 L capacity) filled with 36.5 L filtered tap water supplemented with the same salts used in the dietary studies, with 20 fish per tank. Fish were acclimatized for 7 days in the experimental tanks prior to the start of the experiment and thereafter exposed to the CuO NMs for 28 days, followed by 14 days of depuration. Fish were fed daily at a feeding rate of 2% body weight/day and the behaviour and mortality of the fish was recorded. The growth, weight and related parameters were recorded at each sampling point (Supplementary data S.4). The renewal of exposure concentrations and water conditions are also described in Supplementary data S.4.

At the start of the experiment, 3 fish were taken from the stock to determine background Cu concentrations. Four fish were sampled from control and treatment groups on days 14 and 28 during uptake and on days 35 and 42 during depuration to determine accumulated Cu concentrations. For lipid analysis 1 fish from each group were sampled at the start of the experiment (day 0), end of the uptake (day 28), and at the end of depuration (day 42). Fish were not fed 24 h before sampling and were euthanized and stored as described above.

To determine Cu concentrations in the test medium, water samples were taken before addition of fish (background Cu in aquarium water) and on days 0, 7, 14, 21, 28 during the uptake phase (measured exposure concentrations) and weekly during the depuration. A further characterization of the particles with regards to sedimentation/stability over time was performed by taking additional water samples directly following renewal and redosing at 0, 1, 2, 4, 6 and 24 h on days 7, 14 and 15 and on day 28 (at 0 and 24 h). This also served to check any variations in Cu concentrations during the uptake phase. Samples were acidified with nitric acid (4% final acid concentration) and stored at 4 °C until analysis. Under the same experimental conditions, in a separate tank, 5 fish were exposed to 0.8 mg Cu/L of rod-shaped CuO NMs for 28 days and tissues (liver, intestine, stomach, gill, muscle and brain) were taken for spICP analysis.

2.3. Cu analysis in water, feed and tissues

For Cu analysis, whole fish bodies were dried to constant weight at 105 °C for 48 h, ground in a mortar with a pestle and 50 mg was weighed in triplicate in 15 mL polyethylene DigiTUBEs (SCP SCIENCE, Canada). Tissue samples were directly dried in polyethylene DigiTUBEs at 105 °C for 24 h. Feed samples (100 mg) were weighed in triplicate in DigiTUBEs and dried at 105 °C for 24 h. Samples were digested in DigiPrep blocks with temperature ramping using 1.33 mL nitric acid and 0.1 mL hydrofluoric acid (75 °C, 15 min) and 0.33 mL hydrogen peroxide (115 °C, 60 min). Following digestion, samples were diluted to 10 or 15 mL with Milli-Q water before being analysed by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) or Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Water samples were acidified with nitric acid (4% final acid concentration) prior to analysis. All acids used for sample preparation were analytical grade and purchased from Merck (Spain).

Determination of Cu at ppm-ppb levels was performed by ICP-OES using an Agilent 5900 synchronous vertical dual view (SVDV) ICP-OES, and at levels below limit of quantification of this technique by ICP-MS using an i-CapRQ mass spectrometer (Thermo Fisher Scientific) with quadrupole analyzer and dual mode secondary electron multiplier (SEM) as detection system. It incorporates a collision cell (CCT) with kinetic energies discrimination (KED) mode to eliminate impurities. Instrumental and acquisition parameters are summarized in

Supplementary data S.5.

To measure the number of Cu nanoparticles in the tissue, spICP-MS was applied following a method described previously (Abdolahpur Monikh et al., 2021). Briefly, the tissue samples were dissected and digested using 30% hydrogen peroxide for 1 h at 80 °C in a water bath. The tissues were dissolved, and the particles were isolated from the biological matrices. The dispersions were immediately diluted with Milli-Q water and sonicated for 3–5 min using a bath sonicator (Elma Schmidbauer, Singen, Germany). The samples were measured using ICP-MS (PerkinElmer NexION 350D ICP-MS, Germany) operating in a single particle mode. The setup for the instrument is summarized in Supplementary data S.6, Table S4.

The sample preparation method for extraction of the nanoparticles from the tissues was validated in-house using the procedures described in the published protocol (Abdolahpur Monikh et al., 2021). Briefly, a dispersion of the particles in Milli-Q water was prepared and measured using spICP-MS. To understand the influence of background matrices (fish tissues) on the particle extraction methods, clean tissue samples were spiked with 1 mL of 0.1 mg/L of CuO NMs and homogenized using a bath sonicator. The homogenized samples were diluted to a final volume of 10 mL, and the particles were extracted from the spiked tissues using the same method as applied for the main samples. The extracted particles were measured using spICP-MS. The influence of the digestive fluid on the particles was also investigated (Supplementary data S.6, Table S5).

2.4. Statistical analysis and calculations of BCF and BMF

All data are expressed as mean ± standard error of the mean (SEM), unless otherwise stated. All statistical analyses were performed using Sigma Plot (version 12.0, Systat Software, Inc., Chicago, IL, USA). The program checks automatically for homogeneity of variance. The normality of the distribution was confirmed with the Shapiro-Wilk test. Statistical comparisons ($p < 0.05$) between multiple treatments were assessed using one-way analysis of variance (ANOVA) followed by Holm-Sidak post hoc test. For non-parametric data, where data transformation was not possible, Kruskal-Wallis one-way ANOVA on ranks was used on untransformed data followed by the Dunn's post hoc test. BMF and BCF factors were estimated using equations described in TG 305. BCF and BMF were corrected for growth dilution and for lipid content (BCF_{mkgL}, BMF_{kgL}). The lipid content was measured following the method described by Bligh and Dyer (1959). The feed lipid content was stated by the supplier as 18%.

3. Results and discussion

3.1. Characterization of stock suspensions and water exposure suspensions

The hydrodynamic diameters of CuO NMs dispersed in Milli-Q water show the presence of two sized populations with a main particle size ranging from 300 to 323 nm and a smaller proportion of aggregated/agglomerated particles (Supplementary S.7, Table S6). However, the aggregates were predominant for the spherical CuO at the lower concentration. DLS measurements indicated one size population (263 nm) for the rod-shaped CuO in aquarium water with minimal change in size over 24 h (334 nm).

The spherical and rod-shaped CuO NMs prepared in Milli-Q water showed very low dissolution (<0.1%) after 1 h. About 4% of the rod-shaped CuO dissolved after 24 h in aquarium water.

The spherical CuO NMs in the stock suspension in Milli-Q water precipitated in less than 10 min (Fig. S1) whereas the rod-shaped CuO NMs showed low-intermediate stability in Milli-Q water (23 and 19 %of the initial Cu concentration remaining in the upper layer for the low and high dose, respectively) and high-intermediate in aquarium water (65 and 52% for the low and high dose, respectively) after 24 h under static conditions (OECD TG 318, 2017) (Supplementary data S.8).

TEM images revealed that both NMs form aggregates/agglomerates (Supplementary data S.9, Fig. S4). No remarkable differences in size or shape for CuO nanorods were found between Milli-Q or aquarium water dispersion, or at different time points.

3.2. Full dietary bioaccumulation assay

3.2.1. Cu concentrations in feed

As no specific guidance for NM feed spiking is available, one of our aims within the Gov4Nano project was to establish a spiking methodology aligning closely with the technique outlined in the TG 305. We have given particular attention to the CuO NM stock suspensions prepared prior to spiking, to ensure consistent NM size distributions and Cu concentrations at the time of spiking. We used the same dispersion protocol for both CuO NMs to prepare the stock suspensions. To spike the feed, we applied the same suspension volume to feed spiking ratio for both NMs. Therefore, all efforts have been made to generate comparable results from the two dietary assays. However, the effects of possible transformations of NMs in spiked feed during storage are not yet understood and must be taken into consideration. According to the TG 305 (OECD, 2012) appropriate storage conditions should be selected to maintain stability of test substances. The feed spiked with spherical CuO NMs was prepared at the start of the exposure period and stored in refrigeration in a tube capped and sealed with parafilm. This was done to prevent any gain of humidity of the feed which could favour microbial growth, ion release or interactions with feed constituents. To avoid any effect from refrigerated storage, daily fresh suspensions of rod-shaped CuO NMs and feed spiking were prepared to explore the reproducibility of feed spiking within days and the feasibility of such an approach.

Background Cu concentrations in control feed used for the spherical and rod-shaped CuO assays were 10.8 ± 3.9 and 7.0 ± 0.1 mg Cu/kg, respectively. The measured concentrations of Cu in feed spiked with spherical and rod-shaped CuO NMs showed a homogenous distribution (<15% of relative standard deviation). At the start of the uptake period, spiking recoveries of spherical CuO NMs (based on background corrected concentrations) were 94 and 80% of the nominal concentrations for the low and high doses, respectively. Dietary Cu concentrations for the spherical CuO NMs were calculated taking the average of the concentrations measured in spiked feed at the start and end of the uptake period and resulted in (mean ± SD, n = 3) 67 ± 18 mg Cu/kg and 375 ± 55 mg Cu/kg for the low and high treatments, respectively. For the rod-shaped CuO NMs, Cu concentrations measured in spiked feed demonstrated lower average recoveries (44 and 61% of the nominal Cu concentrations for the low and high dose, respectively). Dietary Cu levels (mean ± SD) from 7 feed preparations (day 1–7 of the uptake period) were 38 ± 6 and 312 ± 24 mg Cu/kg for the low and high treatments, respectively. Variations among the preparations were less than 20%, showing a good reproducibility between days. Although the TG 305 and its guidance document (OECD No. 264, OECD, 2017b) provide some information on feed preparation, no specific guidance for an efficient NM feed spiking method (recovery >85%) with a minimal loss (<10%) of NMs from feed prior to ingestion is currently available. Such a high recovery was not achieved in the present study (due to cumulative losses from stock preparation and spiking method), yet it was possible to obtain a good homogeneity and reproducibility of spiking (<15%). Our results indicated that both methods of spiking (single batch at the start or daily) provide homogeneous and stable concentrations, however recoveries were generally lower than 85% in both cases. As BMF values are dependent on the concentration of NMs in the feed, it is essential to determine the exact concentration to ensure an accurate estimation. It is also essential to measure the leaching of the NM from the spiked feed. Release of Cu from rod-shaped spiked feed immersed in aquarium water for 5 min was 13 and 11% for the low and high dose, respectively showing a similar leaching profile as for the spherical CuO NM spiked

feed (see Supplementary data S.2).

3.2.2. Cu concentrations in fish whole body

In the case of spherical CuO NMs, during the uptake phase there was only a significant increase in Cu concentration in the whole bodies of fish fed high dose diets, although higher levels with respect to controls were also reached with the low dose (Fig. 1). Steady state was reached already at the second day of uptake and significant amounts of Cu remained in the body up to 14 days after depuration in fish fed high dose diets. This could be related with the ability of fish to regulate essential elements such as Cu in their bodies (Boyle et al., 2021).

In the case of rod-shaped CuO NMs, there were significant increases in Cu concentration in the whole bodies of fish fed low and high dose diets (Fig. 1). Similarly to the spherical CuO NMs, in the high treatment group steady state was reached by day 2. However, steady state was achieved after 11 days of exposure in fish from the low treatment. Cu eliminated from the fish fed low dose diet very quickly, i.e. within one day after depuration, whereas Cu concentrations in fish from the high treatment only returned to control levels 7 days after depuration.

Where it was possible, both steady state and kinetic BMFs were estimated for Cu uptake via the diet (Table 1). BMF values were below the threshold (<1) for classification as bioaccumulative for both CuO NMs. A higher depuration half-life for the spherical CuO NMs (28.8 day) indicated slower depuration when compared to the rod-shaped CuO NMs (2.8 day). Despite the differences observed between concentrations of both NMs, concentration dependency was not evidenced according to the BMF values (Table 1).

Although BMF values used for classification are based on whole body concentrations, none of the studies with CuO NMs report measurements made directly on the whole organism basis, instead they report only organ specific BMFs. There is, however, one study that estimated a whole body BMF value of 0.004 (based on dry weight and assuming steady-state) calculated from the sum of the total Cu concentrations of individual organs and remaining carcass of rainbow trout exposed to another type of CuO NMs via the diet (Handy et al., 2022). This value is lower than steady-state BMFs from the present study when converted to dry weight (0.03) for comparative purposes. Both values are however below BMF values of concern and the difference is likely related to different experimental testing conditions.

Despite being an important property of NMs, little research has been conducted on the effects of NM shape on bioaccumulation and toxicity in

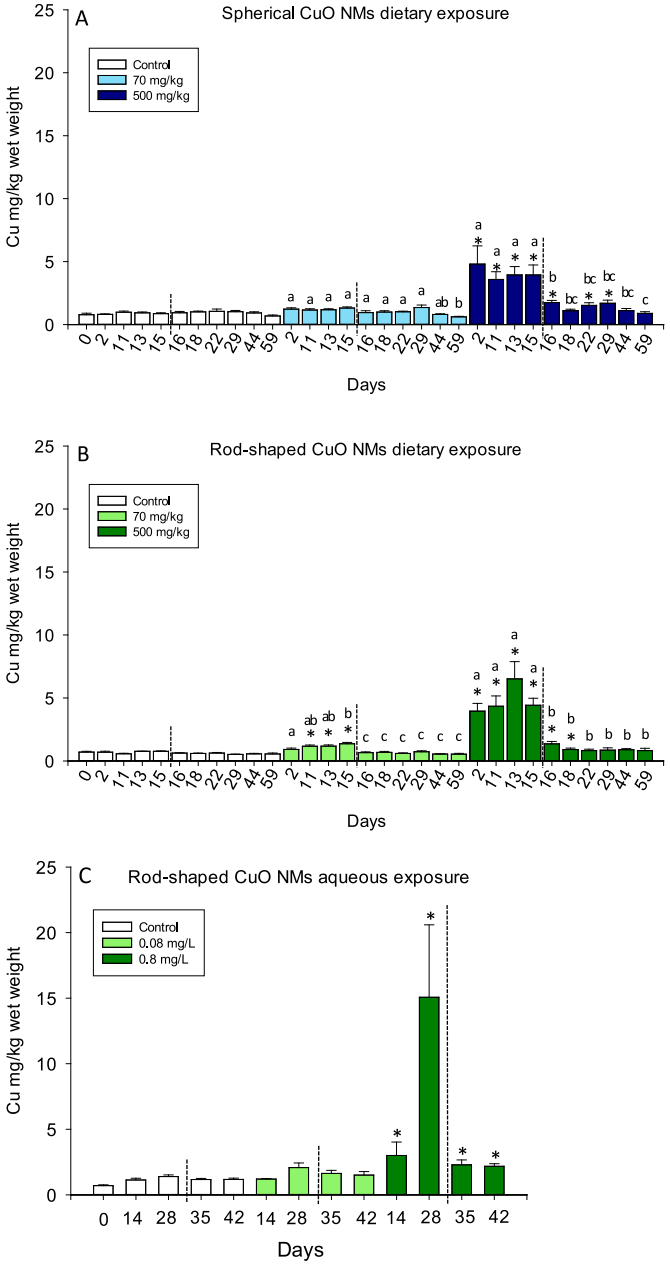

Fig. 1. Cu concentrations in the whole bodies of fish fed control diets or spiked diets with spherical (A) and rod-shaped (B) CuO NMs for 15 days, followed by 44 days of depuration (from day 16). (C) Cu concentrations in fish whole bodies following 28 days of aqueous exposure to control or rod-shaped CuO NMs and 14 days of depuration. Asterisks indicate significant differences between the control and each treatment groups at the same respective sampling day ($p < 0.05$). Different letters indicate significant differences between the sampling days within treatment ($p < 0.05$). Data are mean ± SEM. (Dietary: n = 5, Aqueous: n = 4).

Table 1
Parameters characterizing the bioaccumulation potential of spherical and rod-shaped CuO NMs in fish whole body following dietary and aqueous exposure. BMF: Biomagnification factor; BMF_{ss}: BMF at steady state; BMF_{ssL}: BMF at steady state lipid corrected; BMF_k: Kinetic BMF; BMF_{kL}: Kinetic BMF lipid corrected; BMF_{kgL}: Kinetic BMF growth and lipid corrected; BCF: Bioconcentration factor; BCF_{mss}: minimised BCF at steady state (assumed); BCF_{mssL}: assumed minimised BCF at steady state lipid corrected; BCF_{mk}: kinetic BCF; BCF_{mkL}: kinetic BCF lipid corrected; BCF_{mkg}: kinetic BCF growth corrected; BCF_{mkgL}: kinetic BCF growth and lipid corrected; k_2: Depuration rate constant; k_{2g}: Depuration rate constant growth corrected; $t_{½}$: Depuration half-life.

	Dietary			Aqueous
	Spherical	Rod-shaped		Rod-shaped
Low dose	70 mg Cu/kg	70 mg Cu/kg		0.08 mg Cu/L
BMF_{ss}	0.008	0.013	BCF_{mss} (L/kg)	13.4
BMF_{ssL}	0.037	0.048	BCF_{mssL} (L/kg)	40.2
k_2 (1/day)	n.a	n.a*	k_2 (1/day)	0.187
$t_{1/2}$ (day)	<1	<1	$t_{1/2}$ (day)	3.7
k_{2g} (1/day)	n.a	n.a*	k_{2g} (1/day)	0.149
BMF_k	n.a	n.a*	BCF_{mk} (L/kg)	16.9
BMF_{kL}	n.a	n.a*	BCF_{mkL} (L/kg)	14.1
BMF_{kg}	n.a	n.a*	BCF_{mkg} (L/kg)	21.2
BMF_{kgL}	n.a	n.a*	BCF_{mkgL} (L/kg)	17.7
High dose	500 mg Cu/kg	500 mg Cu/kg		0.8 mg Cu/L
BMF_{ss}	0.008	0.012	BCF_{mss} (L/kg)	33.0
BMF_{ssL}	0.045	0.039	BCF_{mssL} (L/kg)	97.3
k_2 (1/day)	0.024	0.247	k_2 (1/day)	n.a**
$t_{1/2}$ (day)	28.8	2.8	$t_{1/2}$ (day)	>14
k_{2g} (1/day)	0.007	0.231	k_{2g} (1/day)	n.a**
BMF_k	0.008	0.003	BCF_{mk} (L/kg)	n.a**
BMF_{kL}	0.041	0.009	BCF_{mkL} (L/kg)	n.a**
BMF_{kg}	0.027	0.003	BCF_{mkg} (L/kg)	n.a**
BMF_{kgL}	0.147	0.010	BCF_{mkgL} (L/kg)	n.a**

n.a: not applicable due to non-significant uptake respect to control.
n.a*: not applicable due to fast depuration.
n.a**: not applicable due to incomplete depuration.

different organisms. Contrary to our findings, in the polychaete (*Capitella teleta*) rod-shaped CuO NMs (different from the one used in this study) was preferentially accumulated from spiked sediment over spherical CuO NMs during the uptake phase, but the elimination followed the same pattern (Dai et al., 2015). The same research group found that accumulation of Cu was species specific, as snails (*Potamopyrgus antipodarum*) exposed to sediments spiked with rod-shaped CuO NMs were the ones not accumulating Cu to any significant level higher than control snails (Ramskov et al., 2014). Thus, while we have shown that shape has an influence on bioaccumulation kinetics in fish, further investigation is needed to draw general conclusions on an increased/decreased risk to environmental organisms according to NM shape.

3.2.3. Cu concentrations in tissues

Tissue Cu levels of fish fed low dose diets did not differ significantly from those measured in control fish (Figs. 2 and 3), except for in the intestine of fish in the assay with the rod-shaped CuO NMs on the first day of depuration (day 16). A significant amount of Cu was taken up from both CuO NMs in the liver of fish fed the high dose diet. However, in the case of spherical CuO NMs a significant increase in Cu concentration occurred during depuration and Cu remained in the liver for 14 days, showing a slow elimination thereafter. On the contrary, spherical CuO NMs depurated quicker from the intestine, stomach and gill than did rod-shaped CuO NMs. Significant amounts of Cu were taken up from both CuO NMs in the carcass also and there was no difference in the depuration pattern for the two NMs tested. There was also no significant increase in Cu content in the muscle or brain compared to the control in fish from both assays.

TG 305 specifically advises reporting BCF/BMF values in wet weight. Conversion of wet weight Cu concentrations to dry weight was needed for proper comparison with other published data. Tissue Cu levels measured in this study were comparable with those measured by Boyle et al. (2021) in liver, intestine (mid and hinder), gill, brain, and carcass of *O. mykiss* after 14 days of dietary exposure to CuO NMs (750 mg/kg dry weight, PlasmaChem GmbH, Germany, 18 nm), as well as with concentrations measured after the 14 days of depuration. Moreover, in agreement with our findings for the spherical CuO NMs, Cu levels remained elevated in the liver during depuration indicating the involvement of this organ in Cu metabolism and elimination. As well as levels remaining in the liver, Cu was not completely eliminated from the brain and kidney in this case. Boyle et al. (2021) also investigated the link between the tissue Cu levels and biochemical effects and observed induction of metallothionein in the hind intestine and liver as well as some depletion in total glutathione in the liver at the end of the exposure. In general, lower tissue Cu burdens were determined by Johari et al. (2020) in the gill, intestine and liver of common carp (*C. carpio*) fed diets containing 100 and 1000 mg/kg CuO NMs (US Research Nanomaterials, Inc., 88 nm) for 21 days. Dose dependent Cu uptake was observed in the tissues of carp, with the highest Cu contents in the liver (low dose diet) and intestine (high dose diet). Cu eliminated from the tissues after the 21 days recovery period, except for the liver, in which again Cu concentrations did not return to control levels. Only one study was found considering the influence of NM shape on tissue distribution in fish (Abdolahpur Monikh et al., 2021). Trophic transfer of differently sized (10, 60 and 100 nm spherical) and shaped Au NMs (10 × 45 nm rod) was investigated in this study in an aquatic food chain in which Au NM-exposed algae were fed to daphnids and zebrafish were then fed these exposed daphnids. There was a size and shape dependent distribution of Au NMs in fish tissues, with the brain and liver identified as target organs for spherical and rod shaped NMs, respectively.

3.2.4. Number of particles in fish tissues

The number of particles in each tissue was measured using spICP-MS (Fig. 4). When fish were exposed to spherical CuO NMs via diet, the highest number of particles were measured in the stomach followed by the liver (Fig. 4A). Whereas, fish exposed to the rod-shaped CuO NMs accumulated a significantly higher number of particles in the brain (Fig. 4B). This was also observed in previous findings that showed that the brain accumulates the highest number of Au NMs compared to other tissues of zebrafish when the particles enter the organisms through the diet (Abdolahpur Monikh et al., 2021). We did not test whether the rod-shaped CuO NMs could pass the blood-brain barrier, penetrate the brain and accumulate in the tissue. It is likely that the particles enter the brain by two pairs of large blood vessels and, then, enter the small vessels, where, they are retained and cannot be excreted from the brain.

3.3. Aqueous exposure bioaccumulation tests

3.3.1. Cu concentrations in water

According to the measurements over the renewal periods (24 h) on days 7, 14 and 15, Cu concentrations followed the same pattern of exponential decay in both low and high treatment tanks during the uptake phase (Supplementary data S.10). The average initial and final Cu concentrations show approximately a 30 and 40% loss of Cu in the low and high treatments, respectively. The same pattern was expected for the Cu exposure concentrations in the treatment tanks for the rest of the days during the uptake phase. Therefore, following the recommendations of the OECD guidance document No. 317 (2020) for NMs, time-weighted average concentrations (TWA) were calculated from Cu concentrations measured at the start and end of renewal periods on days 7, 14, 15 and 28 to get the most relevant exposure concentration. Values of TWA were 0.08 and 0.44 mg Cu/L, corresponding to 95 and 55% of the nominal Cu concentrations, for the low and high treatments, respectively.

To maintain a stable exposure concentration (i.e., <20% change in mass concentration) can be extremely difficult for many NMs. Some approaches have been proposed to improve the dispersion stability in test media, such as the application of turbines and air stones or the addition of natural organic matter (NOM). Their appropriateness is however questioned when testing NMs, and the modifications may even have a direct influence on the bioaccumulation (Lu et al., 2017). Given that the derivation of BCF is based on the actual exposure concentrations, monitoring of the exposure concentrations over the entire experiment is highly recommended to be able to decide, for example, on the frequency of media renewal or the averaging methods to determine the most relevant exposure concentrations (Schwirn et al., 2020).

3.3.2. Cu concentrations in fish whole body

Elevated levels of Cu were found in fish from both treatment groups during the uptake phase. However, significant increases in Cu concentrations compared to controls occurred only in fish exposed to a high dose of Cu (Fig. 1). Concentration dependence was evidenced when comparing assumed steady state BCFs values (40.2 versus 97.3 L/kg, Table 1). Although Cu remained in the body over the depuration period of 14 days, estimated BCF values indicated low bioaccumulation potential (BCF <500 L/kg) for the rod-shaped CuO NMs (Table 1). However, this should be verified by exposing fish until steady state is reached.

Among the available aquatic bioaccumulation studies with CuO NMs in fish only one reported Cu concentrations in the whole body following 30 days exposure to 100 mg/L CuO NMs in juvenile carp (*C. carpio*) (Zhao et al., 2011). Despite the high Cu levels (2480 mg/kg dry weight) measured, the estimated steady-state BCF (~4.96, wet weight basis) was lower than in the present study. Again, these differences are likely related to the different experimental conditions and species used for bioaccumulation assessment. Other studies found in the literature in different fish species report Cu distribution, mainly in the liver (Abdel-Khalek et al., 2016; Mansouri et al., 2016; Tunçsoy and Erdem, 2018) and gill (Shahzad et al., 2018). On the contrary, Auclair et al. (2020) found no increase in Cu concentrations in the gills of juvenile rainbow trout following aqueous exposure to CuO NMs (25–55 nm) up to 0.5 mg Cu/L, however authors reported increased glutathione

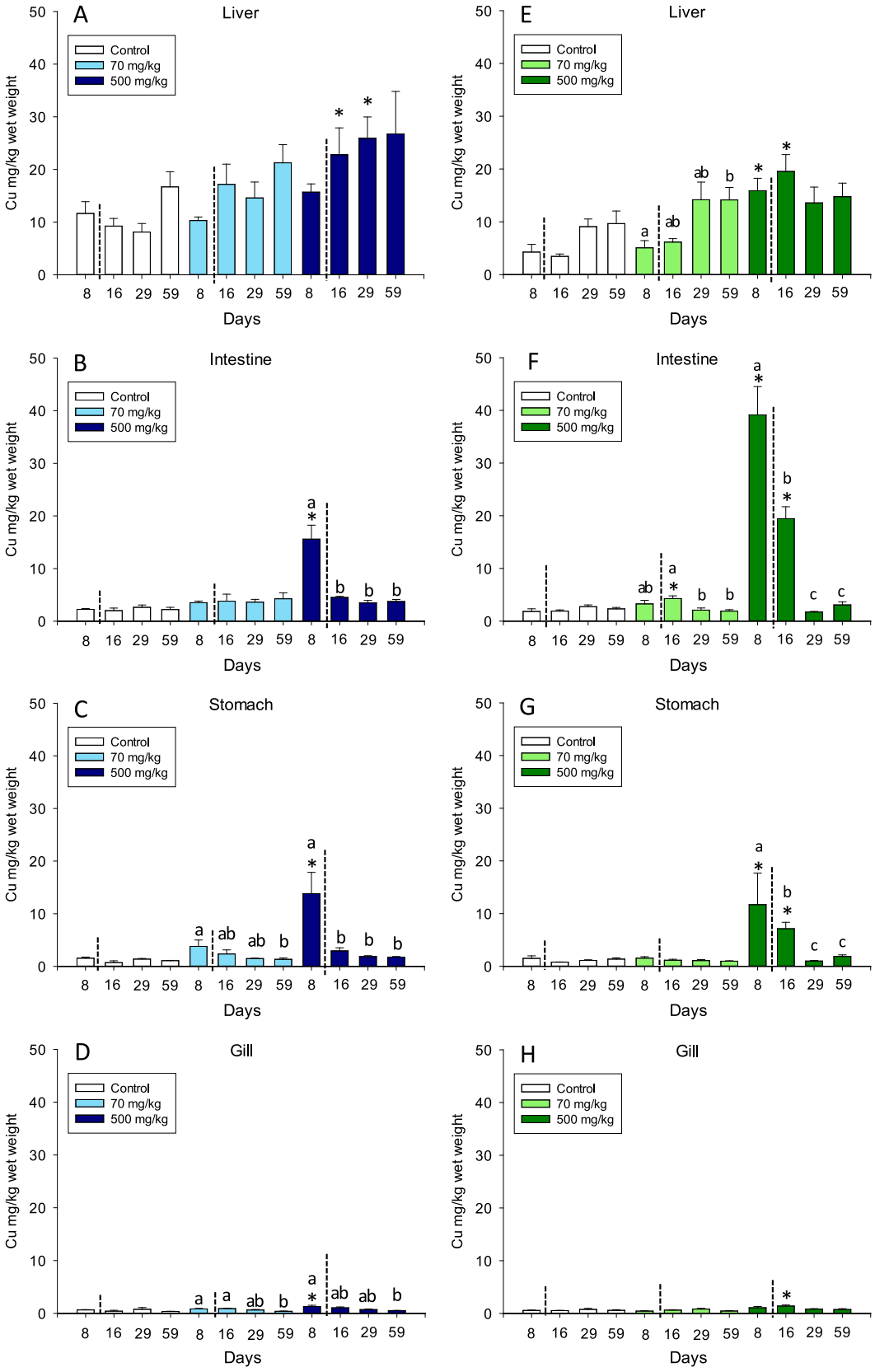

Fig. 2. Cu concentrations in liver, intestine, stomach and gill of fish sampled after 8 days of dietary exposure to control and spiked feed with spherical (A–D) and rod-shaped (E–H) CuO NMs followed by feeding of untreated diet (experimental days 16, 29 and 59 corresponding to 1, 14 and 44 days of depuration, respectively). Data are mean ± SEM (n = 5). Asterisks indicate significant differences between the control and each treatment groups at the same respective sampling day ($p < 0.05$). Different letters indicate significant differnces between the sampling days within treatment ($p < 0.05$).

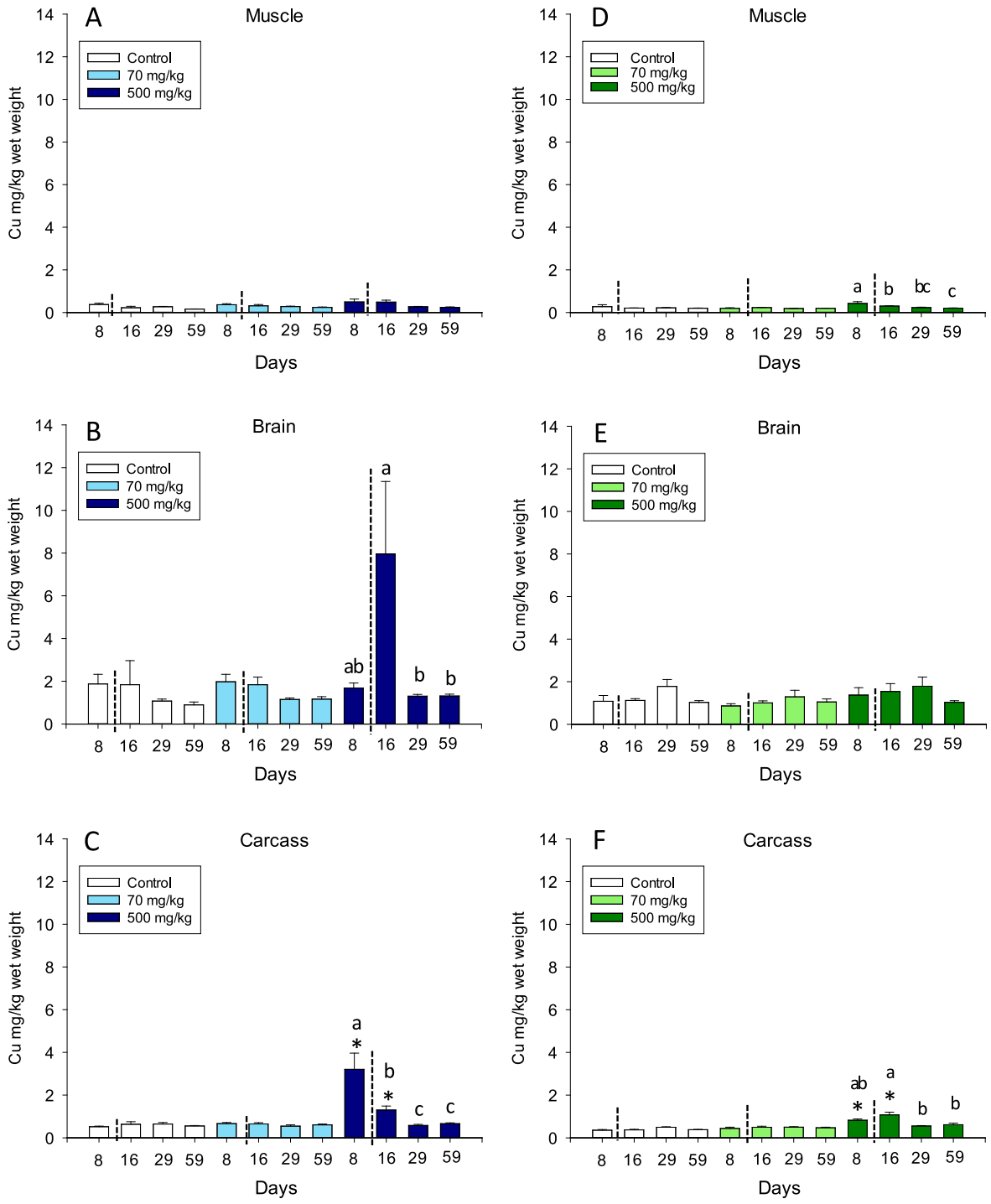

Fig. 3. Cu concentrations in muscle, brain and carcass of fish sampled after 8 days of dietary exposure to control and spiked feed with spherical (A–C) and rod-shaped (D–F) CuO NMs followed by feeding of untreated diet (experimental days 16, 29 and 59 corresponding to 1, 14 and 44 days of depuration, respectively). Data are mean ± SEM (n = 5). Asterisks indicate significant differences between the control and each treatment groups at the same respective sampling day ($p < 0.05$). Different letters indicate significant differnces between the sampling days within treatment ($p < 0.05$).

S-transferase and decreased lipid peroxidation and DNA strand breaks in the same tissues. Assessment of biochemical responses associated with the exposure can reveal specific effects of NMs, however, it was beyond the scope of the present study.

3.3.3. *Number of particles in fish tissues*

When fish are exposed to rod-shaped CuO NMs through water-borne exposure, the highest number of particles are accumulated in the liver tissue (Fig. 4C). No particle could be measured in the brain after water-borne exposure. The results also showed that a higher number of particles accumulate in the stomach when the organisms are exposed to rod-shaped CuO NMs through the water compared to exposure through the diet. This finding highlights the importance of considering the exposure pathway of NMs for their risk assessment as the pathway is shown to influence the particles distribution in fish.

Although TEM was not performed in the present study, it can be used along with quantification as a direct visualisation tool for complete determination of CuO NMs in tissues and CuO NPs have been identified

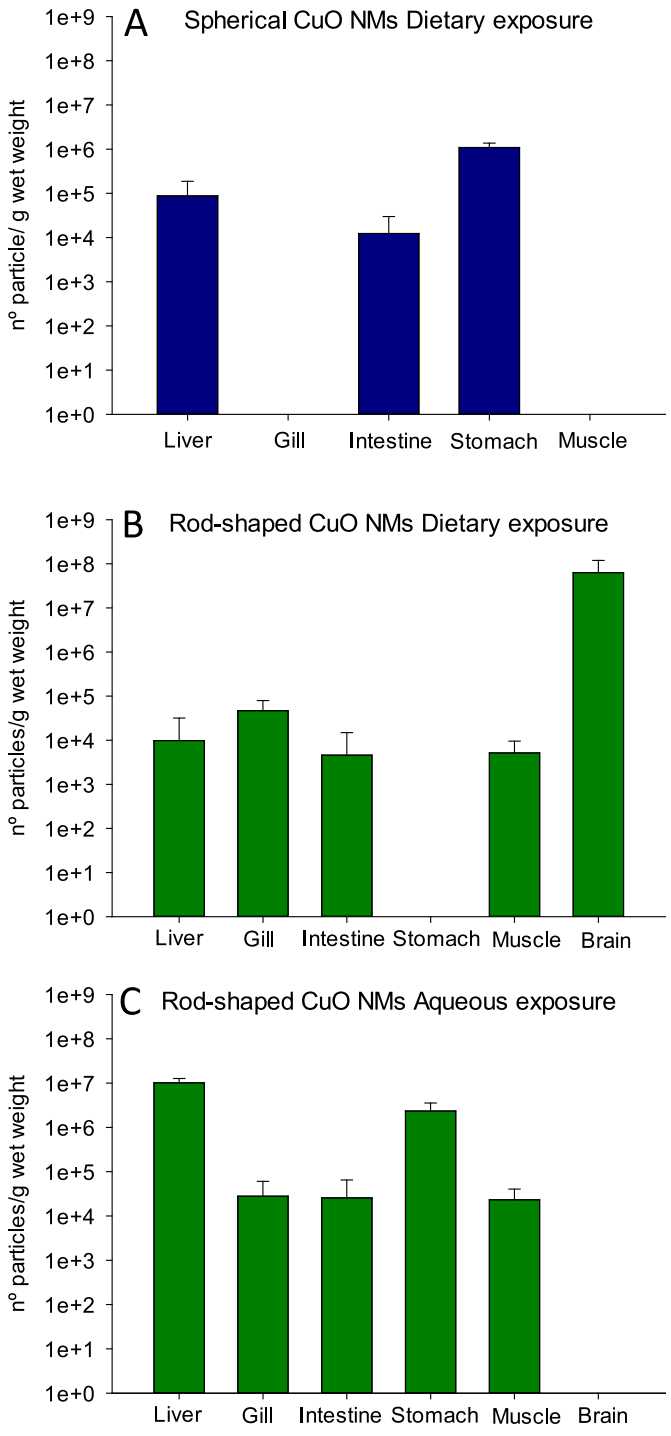

Fig. 4. Particle number (per g wet weight tissue) of CuO NMs measured in different tissues of fish fed diets with (A) spherical NMs for 37 days, (B) rod-shaped NMs for 15 days. (C) Particle number (per g wet weight tissue) of CuO NMs measured in different tissues of fish following 28 days of aqueous exposure to rod-shaped NMs. Mean ± SD. (A) n = 2; (B) n = 5; (C) n = 4.

in the muscle and liver tissues of 14 day exposed (*O. niloticus*) (Canli and Canli, 2020a,b), and even remaining in the muscle of fish after 14 days of depuration (Canli and Canli, 2020b).

4. Conclusions

In this study the bioaccumulation potential of spherical and rod-shaped CuO NMs in rainbow trout was assessed following the OECD TG 305 and including specific steps. Preliminary investigations on particle stabilities, and methodologies for feed spiking and particle identification in tissues were developed. This allowed testing NMs using aqueous and dietary exposure routes. Both the shape and exposure route had an influence on bioaccumulation kinetics. There was a different depuration pattern for the spherical and rod-shaped CuO NMs after dietary exposure, showing a slower depuration following spherical CuO NMs exposure and long residence time in liver tissues after depuration. An influence of the exposure route was also observed. The rod-shaped CuO NMs showed a slower depuration via diet. Careful consideration of the most appropriate and relevant exposure route is thus needed when designing a bioaccumulation experiment for testing NMs. A minimised aqueous study design proved adequate to determine the bioaccumulation potential of NMs. A concentration dependence was evidenced and points to the need for two concentration testing in future studies with NMs. However, the observed low BMF/BCF values indicated a low bioaccumulation potential for both NMs irrespective of the exposure concentrations used. Particles were identified at the end of the uptake phase in the different fish tissues demonstrating the assimilation and distribution of both CuO NMs via water and diet. The importance of considering not only the exposure pathway of NMs for their risk assessment but also their physicochemical properties such as shape was also noticed from the results of the spICP-MS analysis.

Author contribution statement

Judit Kalman: Conceptualization, Methodology, Formal analysis, Investigation (Full dietary tests and aqueous test), Writing – original draft, Writing – review & editing. **Mona Connolly**: Conceptualization, Methodology, Formal analysis, Investigation (preliminary test, spherical CuO NMs), Writing – original draft, Writing – review & editing. **Fazel Abdolahpur-Monikh**: Methodology, Formal analysis, Investigation (single-particle ICP-MS), Writing – original draft, Writing – review & editing. **Rocío Fernández-Saavedra**: Formal analysis, Investigation (ICP-MS, rod-shaped CuO NMs). **Ana I. Cardona-García**: Formal analysis, Investigation (ICP-OES). **Estefanía Conde-Vilda**: Formal analysis, Investigation (ICP-MS, spherical CuO NMs). **Salome Martínez-Morcillo**: Formal analysis, Investigation (spherical CuO NMs, full bioaccumulation test), Writing – original draft, Writing – review & editing. **Willie J. G. M. Peijnenburg**: Writing – original draft, Writing – review & editing. **Isabel Rucandio**: Supervision, Writing – original draft, Writing – review & editing (ICP-OES/MS). **María Luisa Fernández-Cruz**: Conceptualization, Methodology, Formal analysis, Supervision, Funding acquisition, Project administration, Writing - original draft; Writing - review & editing.

Ethics

Ethics approval was granted to perform the experiments with fish, Ref PROEX 94.2/20.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

This work was funded through the H2020 project Gov4Nano (Grant Agreement nº 814401). Authors thank all the effort done at the National Centre for Electron Microscopy (Spain) in relation to the electron

microscopy work. Mona Connolly has received financing granted by the Community of Madrid (2018T2/AMB-11392, Mode 2, Young Doctor Recruitment).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.136894.

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