

Bioaccumulation and effects of metal-based nanomaterials using a multi-species approach

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‘God keeps his promise, and he will not allow you to be tested beyond your power to remain firm; at the time you are put to the test, he will give you the strength to endure it and so provide you with a way out’. – 1 Corinthians 10:13

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SUMMARY

Metal-based nanomaterials are being manufactured at increasing rates and are used in a large variety of consumer products and services, from biological labelling to cosmetic products. An increase in the use of these materials also means that there will be multiple opportunities for them to be released into the environment. Environmental concentrations can be modelled but there is little quantitative data available or will even exist until monitoring and analytical capabilities are improved. However, the impact of these materials on the environment is still of concern and improving toxicity knowledge to predict affects is of critical importance to ensure the use of these materials can grow sustainably. Previous results suggest that there are possibly different modes of uptake and mechanisms of toxicity between different metal-based nanomaterials (Bosch et al., 2017). Composition, particle coatings and size affect nanomaterial toxicity and should be considered and integrated into nanoparticle risk assessment. One of the key factors in determining the potential toxicity of any chemical, including a nanoparticle, is the extent to which these particles reach tissues within an organism and accumulate. The aims of this study were to evaluate the Physicochemical characteristics of three metal-based NMs nanomaterials (nanogold - nAu, nano copper oxide – nCuO and cadmium tellurium quantum dots- QDs) in the exposure media, to determine toxicity and biodistribution of the metal-based NMs and their ionic equivalents in two model organisms (*Caenorhabditis elegans* and *Danio rerio*) using standard tests, and to evaluate the efficacy of a traditional TK model after uptake assessment in adult zebrafish.

To understand the toxicity and bioaccumulation of NMs, characterization of NMs in exposure media was done. When stock concentrations of NMs were diluted in different media there was a clear difference in characteristics observed. All three materials formed large aggregates and agglomerates as soon as they were diluted in exposure media and size increased over time due to homo-aggregation in simulated soil pore water (SSPW) and embryo media (E3 media) as confirmed by dynamic light scattering (DLS), and potential hetero-aggregation/ corona formation in Zebtec system water (ZSW). Dissolution was the highest in ZSW with the lowest stability, and only QDs had metal dissolution higher than 1%, indicating these materials are not as stable in environmental media as nCuO and nAu. The dissolution of Te from QDs was the highest of all the materials and is potentially due to ligand-promoted stress dissolution or interference of K^+ with GF-AAS measurements.

To determine toxicity range finding exposure tests were done to determine the 96 h reproduction EC_{50} for the nematodes and LC_{50} for the zebrafish embryos using standard OECD and ISO protocols where ionic metals and blank controls were included. It was found that ionic materials

were overall more toxic than their nano equivalents, except in the case of QDs in *Danio rerio* embryos where the ionic metals Cd and Te were equally as toxic as the QDs. In both organisms QDs was the most toxic NM. This is attributed to the size and behaviour of QDs in the media increasing bioavailability and uptake. Dissolution was negligible and darkfield imaging confirmed nAu and QD uptake in both organisms, thus metal uptake and toxicity are attributed to whole particle uptake rather than ion uptake. Tellurium resulted in the highest dissolution, but total tellurium dissolution is not enough to account for the toxicity observed based on the EC₅₀ and LC₅₀ values of ionic Te in both organisms. To assess possible routes of exposure, metabolomics was assessed. Metabolic pathway analysis showed differences in the perturbation patterns of the metabolome in both organisms exposed to each material. These results indicate that there is not one specific metabolic biomarker for nanomaterial exposure and that the mode of toxicity of nanomaterials are different than that of their ionic equivalents.

Adult *Danio rerio* were used to further assess *in vivo* particle distribution, as well as to determine if metal uptake from nanomaterial exposure can be modelled using a standard toxicokinetic model. Results showed that nanomaterials are most likely taken up orally and ionic metals are taken up through the gills. Overall, NMs tend to accumulate in the intestine and muscle as is the case for QDs, most likely due to their small size. Ionic metals accumulated in all organs, but certain metals favoured certain organs. Overlapping uptake concentrations despite exposure concentration differences allude to the regulation of metals by the fish and the entrapment of NMs in the mucus of the gills. Slow metal elimination such as Cu suggests greater assimilation to organs by this metal. Due to bimodal uptake patterns the model could not fit the data accurately after a certain concentration. However, bioconcentration factors did predict high bioaccumulation potential of Cu in the gills from nano and ionic treatment, QD-Cd in the liver; and nCuO and QDs in the intestine. Overall NM toxicity is lower than ionic metals however, their toxicity and bioaccumulation is attributed to nano-specific pathways rather than metal toxicity.

Keywords: nCuO, Nanogold, Quantum dots, Uptake, Activity, Metabolomics, Zebrafish, *Caenorhabditis elegans*

LIST OF ABBREVIATIONS

Abbreviation	Description
% O ₂	Percentage dissolved oxygen
°C	Degrees Celsius
μL	Microgram per litre
μm	Micro meter
AAS	Atomic absorption spectroscopy
ADP	Adenosine diphosphate
Ag	Silver
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BCF	Bioconcentration factor
bpm	Beats per minute
C ₀	Concentration
C _t	Concentration at time point
Ca	Calcium
Cd	Cadmium
CdCl ₂	Cadmium chloride
CeO ₂	Cerium dioxide
Cu	Copper
CuCl ₂	Cupric chloride
Cu ₂ O	Copper oxide

CL	Confidence limit
CV	Cross validation
Da	Dalton
DLS	Dynamic light scattering
DO	Dissolved oxygen
e	Euler's number
E3	Embryo media for zebrafish
EC	Effect concentration
EDS	Energy-dispersive spectroscopy
g	Grams
GF-AAS	Graphite furnace atomic absorption spectrometry
h	Hours
HCl	Hydrochloric acid
HNO ₃	Nitric acid
hpf	Hours post fertilization
ICP-MS	Inductively coupled plasma mass spectrometry
Int	Intestine
kDa	Kilodalton
K _e	Depuration rate
K _u	Waterborne metal uptake rate
LC	Lethal concentration
min	Minutes

mg/L	Milligram per litre
mL	Millilitre
MSA	Mercaptosuccinic acid
MTs	Metallothioneins
mV	Millivolts
NaCl	Sodium chloride
nAu	Nanogold
nCuO	Nano copper oxide
NH ₃	Ammonia
nm	Nanometre
NMs	Nanomaterials
PaB	Pharynx anterior bulb
PCA	Principal component analysis
PLS-DA	Partial least squares-discriminant analysis
PpB	Pharynx posterior bulb
ppm	Parts per million
QDs	Quantum dots
r ²	Coefficient of determination
RCF	Relative centrifugal force
ROS	Reactive oxygen species
SEM	Standard mean of the error
SSPW	Simulated soil pore water

ZSW	System water
t	Time
TD	Toxicodynamic
TDS	Total dissolved solids
Te	Tellurium
TeCl ₄	Tellurium chloride
TiO ₂	Titanium dioxide
TK	Toxicokinetic
Zn	Zinc
ZP	Zeta potential

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CHAPTER 1 STUDY RATIONALE

1.1 Brief introduction to NMs

NMs have shown considerable promise in the fields of medicine, energy generation, environmental remediation and almost all aspects of life due to their unique properties (Domingos et al., 2011; Sousa de Almeida et al., 2021). NMs (NMs), produced in various forms on the nanoscale scale (1-100 nm), are currently present in over 1300 commercial goods and are widely used as medication carriers, molecular probes, catalysts, cosmetics, antibiotics, microelectronic components, image contrast agents, opacifiers, and fillers for plastics and rubber (Hou et al., 2013). Due to their wide range of uses they can end up in the environment through various phases of production and use. They can be released incidentally during synthesis, commercial product usage, or be released from industrial usage such as the direct application of NMs as pesticides and can enter the environment directly by run off or deposition or through wastewater. They may be accidentally or intentionally released into the aquatic ecosystem. Due to their occurrence in the aquatic environment they could pose a threat to aquatic organisms (Zhao et al., 2013). Natural NMs annual flux to the environment is around 97% of the total and is significantly higher than synthetic NMs. However, synthetic NMs are considered to have a detrimental effect on the environment (Malakar et al., 2021).

There are various types of metal-based NMs, including but not limited to inert materials, oxides and bimetallic NMs. Nanogold (nAu), commonly considered to be inert, has shown great potential as diagnostic agents (Umair et al., 2016). Literature has revealed that many inert NMs have toxic potential under specific conditions. Toxicity such as inflammation, oxidative stress and intracellular signalling interference occur when these relatively inert NMs are used (Bondarenko et al., 2013). In a cytotoxicity study, nAu particles as small as 1.4 nm have shown signs of oxidative stress and damage to mitochondrial integrity and are usually taken up into cells by endocytosis (Umair et al., 2016; Sousa de Almeida et al., 2021). However, larger particle clusters in the exposure media can cause greater uptake of total metal compared to smaller particles due to these agglomerates with higher metal content being mistaken for food and ingested. They could also cause blockages in the intestine of microscopic organisms such as *C. elegans*. Nano copper oxide (nCuO) has been used as a biocide and antimicrobial agent in wood preservation, paints and textiles (Grigore et al., 2016; Bondarenko et al., 2013). Studies have revealed that nCuO is more cytotoxic than other metal oxide NMs and toxicity is greatly size dependant (Karlsson et al., 2015). Quantum dots (QDs) are semiconductor bimetallic NMs, in this case cadmium (Cd) tellurium (Te) QDs. In an excited state they produce distinguishable highly sensitive fluorescence emission helpful for genotype determination, image-guided surgery, and molecular diagnostics

(Sharma et al., 2020). A study by Chen et al. (2012) showed that CdTe QD toxicity not only comes from the release of Cd²⁺ ions but also intracellular distribution of QDs in cells and the associated nanoscale effects. Organic coatings on certain QD improve their dispersion in water and aid in their targeting of biological targets (Domingos et al., 2011; Makvandi et al., 2021). For this study QDs functionalised with methylamine (NH₃) was chosen based on previous work showing that this functional group was the most toxic of the three tested (carboxylated, methylaminated and PEGylated) (Chen et al., 2018; Tatsi et al., 2020).

Gold NMs are used in resonance scattering dark-field microscopy for the detection of microbial cells and their metabolites, the bio-imaging of tumor cells, and for the detection of receptors on their surface, and for the study of endocytosis (Dykman & Khlebtsov, 2011). Nano CuO are mainly utilized as antimicrobial agents. They are used in hospitals due to their antimicrobial ability to kill more than 99.9% of Gram-positive and negative bacteria within 2 h of exposure, if a suitable dose is applied (Grigore et al., 2016). Cadmium telluride (CdTe) quantum dots (QDs) is a typical nanomaterial, which has been the subject of various studies due to its large bandwidth and excitation binding energy, which make it suitable for various purposes like anti-inflammatory, wound healing, antioxidant, antifungal and antibacterial applications (Akbari et al., 2020).

At the whole-body level, the biodistribution, accumulation and selection of NMs on target organs act differently from bulk materials. Nanomaterial exposure routes, which play a critical part in toxicity, are one of the first elements explored during NMs toxicokinetic investigation (Wu and Tang, 2017). Different exposure routes could affect different organ systems and cause different adverse effects. For example, NMs that are taken up by the gills in fish would have different effected pathways than those taken up via the intestinal lumen. Several *in vivo* studies have shown NMs may be systemically distributed and accumulated in organs and tissues, which may determine their absorption, distribution, metabolism, and excretion effects (Lee et al., 2014; Wu and Tang, 2017). Generally, the liver and kidneys are the most common accumulation organs regardless of exposure routes, animal models, and physicochemical properties of NMs used. When NMs translocate across biological barriers and enter the bloodstream after various exposure routes, these organs all become potential accumulation targets, where they could exert toxic effects.

The toxicity of metal-based NMs can be induced by the particles or by the dissolved metal ions and, is largely related to the bioaccumulation and tissue distribution in aquatic organisms (Khan et al., 2015; Zarco-Fernández et al., 2016). As a result, assessing the risk of NMs in comparison to their dissolved components is a topic of interest due to difficulties in the associated regulation. This is complicated by the fact that each type of NM has a unique composition (core and ligands), and the possible degradation products generated will be determined by the NMs final application

and use (Caixeta et al., 2021). Understanding the Physicochemical features of the NMs, particularly dispersion and dissolution, is critical for assessing their environmental impact (Domingos et al., 2011). These parameters depend on the NM itself, the physicochemical parameters of the dispersion medium and the concentration of the NM in the medium (OECD, 2017). Environmental risk assessments involve persistence, bioaccumulation and toxicity, as well as the potential for exposure (Hou et al., 2013). However, little is known about the bioavailability of metal-based NMs and their subsequent accumulation and body systems effects in e.g. fishes (Shaw et al., 2012; Khan et al., 2019). The uptake and biodistribution of NMs has been largely assessed in dietary exposures and has found metal levels to remain in the stomach, intestine and liver after depuration, while no bioaccumulation potential was observed for titanium dioxide NMs (TiO₂), cerium dioxide (CeO₂) and silver NMs (Connolly et al., 2022, Clark et al., 2021). Čaloudová et al. (2021) also notes that NMs can pass through the intestinal lumen after dietary exposure in fish.

The aquatic ecosystem is the ultimate sink for all pollutants, due to the eventual run-off in to the rivers and ocean and thus, plays an important role in the full life cycle of NMs due to their intentional and unintentional release (Zhao et al., 2017). Consequently, the interaction and behaviour of NMs in an aquatic ecosystem has become an urgent issue concerning their risk assessment. There remains limited knowledge about mechanisms of uptake, biological fate, toxicological effects, and modes of action of NMs in waterborne exposure scenarios. Technological breakthroughs in nanotechnology and its numerous uses have resulted in the introduction of metal pollutants in the form of NMs, with uncertain effects on aquatic ecosystems. Understanding the physical and ecotoxicological features of NMs in aquatic systems is crucial for building appropriate regulatory frameworks to safeguard aquatic ecosystems from their negative effects (Jackson et al., 2012; Schmutz et al., 2020). In order to address this in the present study three different NMs were selected and tested using two established model aquatic species: namely the nematode *Caenorhabditis elegans* and two life stages, embryo and adult, of the freshwater fish, *Danio rerio*.

1.2 Study hypothesis

The bioavailability, uptake and distribution of NMs are dependent on the exposure media, and their physicochemical characteristics due to how these aspects influence agglomeration, charge and dissolution (Figure 1-1). Bioaccumulation is determined by NM behaviour in environmental matrices and not by species-specific responses. Toxicity of free ionic metals is higher than the NM equivalents due to the nature of free metal ion toxicity. Metal uptake from NMs can be modelled using a toxicokinetic (TK) model.

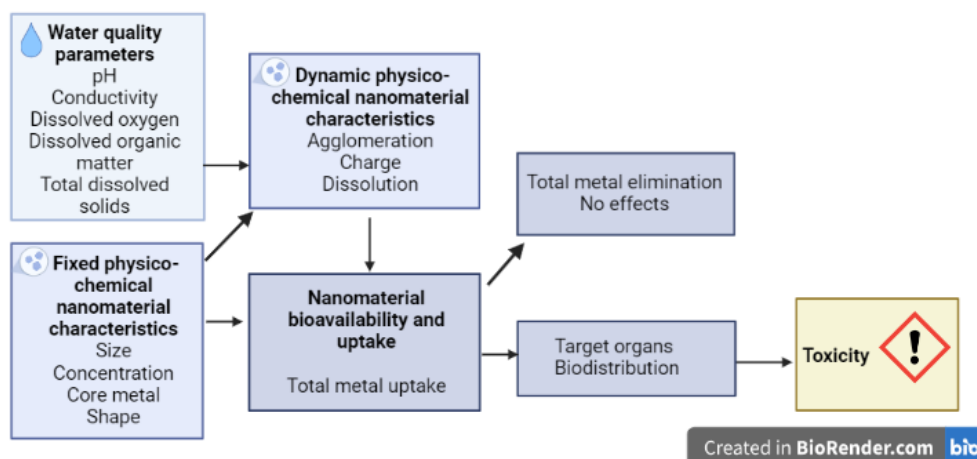


Figure 1-1 : The linkages between physicochemical parameters and nanomaterial toxicity.

1.3 Aim and objectives

The aims of this study were to evaluate the physicochemical characteristics of three metal-based NMs in the exposure media, to determine toxicity and biodistribution of the metal-based NMs and their ionic equivalents in two model organisms using standard tests, and to evaluate the efficacy of a traditional TK model after uptake assessment in adult zebrafish.

To meet the aim, the following objectives were set:

1. Determine the characterization (size, agglomeration, dissolution) of nAu, nCuO and QDs in the aquatic media (E3 media, simulated soil pore water and zebrafish system water) using transmission electron microscopy (TEM), dynamic light scattering (DLS) and graphite furnace atomic absorption spectroscopy (GF-AAS).
2. Determine the developmental toxicity of the NMs and their ionic metal equivalents (Au, Cu, Cd, Te) using the fish embryo toxicity test (FET – OECD 236) and the water quality reproductive test of *C. elegans* (ISO 10872).
3. Establish NM biodistribution in model organisms using CytoViva® dark field microscopy.
4. Track metal uptake and biodistribution where possible in model organisms using GF-AAS.
5. Determine NM and ionic metal sublethal exposure effects on *Danio rerio* embryo heart rate, activity, blood flow and respiration using Danioscope software and PreSense software.
6. Determine which metabolic pathways are affected in model organisms after exposure to sublethal concentrations of both NMs and ionic metals.
7. Test a toxicokinetic (TK) model for NM and ionic metal uptake in adult zebrafish.

CHAPTER 2 CHARACTERIZATION OF NANOMATERIALS IN ENVIRONMENTAL EXPOSURE MEDIA

2.1 Introduction

Nanomaterials (NM) that end up in the environment interact with their surroundings in ways that alter their form, fate, bioavailability, mode of uptake and possible toxicity to organisms. According to Lowry et al. (2012) the small sizes of these materials result in a higher percentage of surface atoms that in turn result in novel properties and reactivity compared to their larger particulate and ionic counterparts. It is important to understand how engineered NMs are transformed in the exposure media in order to evaluate their fate, bioavailability and toxicity. Nanomaterials can be transformed in the environment by redox reactions, dissolution, aggregation (strong chemical bonds), agglomeration (weak physical interactions), photochemical reactions, adsorption of other molecules to the surface and affinity for other surfaces (Lowry et al., 2012; Luoma et al., 2014; Lead et al., 2018). The type of reaction will depend on the physicochemical water quality parameters of the environment, the characteristics of the materials themselves and their metal composition.

Environmental water quality parameters that are used to ensure protection of aquatic organisms include dissolved oxygen, total dissolved solids, dissolved organic matter, pH and conductivity. The DLVO theory suggests that both van der Waals forces and electrostatic forces cause aggregation of particles in a suspension (Adair et al., 2001). Thus, factors such as pH and conductivity have a large influence on these aggregation reactions. Physicochemical water quality parameters included for this study comprise of pH, electrical conductivity and dissolved oxygen, as these are controlled parameters within the exposure media. Buffers such as citrate in the case of the nAu prevents aggregation of particles due to negative charges. The citrate layer causes electrostatic interparticle repulsion causing them to stay in suspension (enhances stability), but a factor such as pH can disrupt that negative charge resulting in aggregation of particles (Adem, 2014; Tatsi et al., 2020). This is because the lower the pH the more protonated citrate becomes. Full protonation occurs between a pH of zero and two. Waterborne electrolytes, such as sodium chloride (NaCl) and divalent cations, affect both aggregation and surface charge (Chinnapongse et al., 2011; Ortelli et al., 2017). Na⁺ cations would act as electrostatic bridges between particle aggregates to promote the aggregation process and divalent cations have a high ionic strength, causing counter ions to diffuse around the NM surface compressing and dampening the surface charge and electric double-layer around the particles, destabilising them (Shih et al., 2012; Wang et al., 2017). Total dissolved solids are comprised of inorganic salts (principally calcium, magnesium, potassium, sodium, bicarbonates, chlorides, and sulphates) and some small

amounts of organic matter that are dissolved in water. According to Handy et al. (2022) some of the key factors that influence particle aggregation are ionic strength, divalent ions (such as Ca^{2+}) and organic matter. Thus, by measuring the mobile charge in a solution as conductivity is a way to interpret aggregation causation and measuring zeta-potential of the NM solution is a way to measure the net surface charge of NMs in solution by measuring surface charge and flow dynamics near the surface of the NM in solution (Handy et al., 2008).

According to Lead et al. (2018) transformation rates and behaviour of NMs in solution are heavily dependent on the original properties of the particles. For hazard assessment it is important to understand the original and transformed properties of the NMs, as they influence uptake and toxicity. These properties include charge, size, aggregates, agglomerates, functionalization and dissolution. For example, aquatic snails that were exposed to nanosilver (nAg) (Croteau et al. 2014; Khan et al. 2015; Stoiber et al. 2015) and nCuO (Croteau et al. 2014) experienced higher uptake due to dissolution of metal from the particles. This means that dissolution influences total metal uptake. Aggregation and agglomeration can also affect bioavailability. It can change the main exposure route from water to sediment or even food by removing NMs from the water column due to sedimentation, this can cause elevated exposure to benthic organisms relative to pelagic organisms (Selck et al., 2016; Dai et al., 2022). Aggregated NMs can be ingested by deposit-feeders and grazers and become bioavailable in that way.

The use of capping agents to functionalize NMs can reduce metal ion bioavailability by curbing the release of metal ions into the aquatic environment (Balmuri et al., 2017). For example, Dobias and Bernier-Latmani (2013) showed that when determining the dissolution of nAg, particle size and capping agent were more important than different water quality conditions. Similar results were reported for QDs and nCuO in soil (Tatsi et al., 2018; Tatsi et al., 2020). Many studies also suggest that metal composition, dissolution and solubility play a role in toxicity for both ions and particles (Hartmann et al., 2014; Leclerc and Wilkinson, 2014), possibly where the ions have the direct biological impact, but NMs increase metal transport to the cell via endocytosis and increase localized ionic metal concentrations.

Key to that evaluation is a determination of whether intact NMs (not transformed) are able to cross biological membranes. The surface potential and the force acting between them is generally discussed based on the DLVO theory, in which van der Waals and electrostatic double-layer forces are incorporated as dominant factors (Nabika and Unoura, 2016). This is a complex interaction as it is also dependent on the chemical and physical characteristics of the NM and the membrane surface. Their ability to interact will be affected by for instance surface charge/zeta potential (ZP) of the membrane and particles. With semiconductor particles such as QDs, cellular uptake is greatly determined by particle size and charge, due to the negative charge of the lipid

bilayer. However, these aspects vary in importance depending on the type of particle and organism exposed. It is well known that small (<20 nm) nAu can enter the lipid bilayer of a cell (Rasch et al., 2010; Nabika and Unoura, 2016). Metal oxide NMs have also been shown to pierce the bilayer or be covered by it (Nabika and Unoura, 2016). Uptake of CeO₂ nanomaterials by *C. elegans* was found to be strongly charge-dependent. When exposed to positively charged CeO₂ NMs, accumulation was 20 - 25 times higher than uptake from exposure to neutral and negatively charged CeO₂ NMs (Collin et al., 2014).

Protein coronas can form on the outer surface of the NM particle, due to interactions such as hydrophobic bonds, electrostatic bonds and hydrogen bonds between NMs and proteins in the exposure media (referred to as hetero-aggregation) form (Rampado et al., 2020; Barbero et al., 2017). This is one type of aggregation where particles interact with natural colloids in the media. A small layer of soluble proteins has the potential to stabilize the suspension as the NMs are exposed to more proteins over time that bind more permanently to the NM surface reducing the potential of NMs to bind to each other (Barbero et al., 2017). However, if this protein layer builds up steric hindrance may occur causing particle settling. This depends on chain length and the thickness of the protein corona. Homo-aggregation is another type of aggregation that can occur when the same NM particles interact with and aggregate to one another. Thus, hydrodynamic particle size can change over time especially in media that contain proteins.

To verify these important NM characteristics, a suite of well used laboratory procedures is used. But it is important to note there are only a limited number of methods that can be directly applied to aqueous samples, due to high matrix salt concentrations, sample stability due to dynamic nature of aggregation dynamics, polydispersity and lack of standards for calibration (Delay and Frimmel, 2012). Due the tendency of NMs to aggregate and agglomerate in suspension, ultrasonic energy is applied to break up these large agglomerates (Delay and Frimmel, 2012). Sonication uses sound waves that incorporate small vacuum bubbles in liquid media, that collapse violently once they attain a critical volume. It has proven to be a relatively energy efficient method to rapidly break up large agglomerates into fairly monodisperse and stable suspensions of small agglomerates (Cohen et al., 2018; Taurozzi et al., 2011). Sonication is applied in order to create a homogeneous stock dispersion to ensure correct sample concentrations are extracted for dosing experiments. For particle hydrodynamic size dynamic light scattering (DLS) is used by applying Brownian motion to measure aggregate sizes in an aqueous suspension. To determine ZP of the NMs in the exposure media, laser Doppler electrophoresis is used combined with DLS by introducing gold electrodes in to the solution that attract particles of the opposite charge at a certain velocity. This gives information about the charge of the outer layer of the NM in suspension. To measured total metal chemical composition of NMs inductively coupled plasma

mass spectrometry (ICP-MS) and atomic absorption spectroscopy (AAS) is used, and for particle primary size determination, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) is used (Delay and Frimmel, 2012).

The interrelated drivers of NM bioavailability are the concentration of NMs, the physicochemical characteristics of the environment, the physicochemical attributes of the NMs and the biological attributes of the organism (Luoma et al., 2014; Falfushynska et al., 2022). It is important to conduct characterization of NMs in all experimental media due to the nature of the matrix and the NMs. Characterization in ultrapure water is not a sufficient indication of possible outcomes of NMs in solution, as there are no other ions, colloids or salts in ultrapure water that may affect aggregation and dissolution patterns. " The aim of the chapter was to examine the influence of different standard aquatic toxicity testing media on the transformation on nAu, nCuO, and QDs.

2.2 Materials and Methods

2.2.1 Stock solutions

The nAu stock solutions (1000 mg/L) were produced and supplied by the company MINTEK (TMU14G). This solution was made up of 14 ± 2 nm nAu (as stated by the supplier) and prepared by standard citrate reduction techniques and were sterilised using a filtration method. Ionic gold (Chloroauric acid) stock solutions were supplied by Sigma Aldrich (Lot# MKCM0914). Quantum dots in the form of CdTe QD-NH₃ (henceforth referred to as QDs) nanopowder (4 nm size) was produced by PlasmaChem GmbH (Lot# YF140402) and supplied through the Nanosolutions project. Cadmium chloride (Lot# MKBM1769V) and tellurium tetrachloride (Lot# MKBL8016V) solutions were purchased from Sigma Aldrich. The nCuO powder (14 ± 4 nm size) was also supplied by PlasmaChem. The copper (II) chloride powder for ionic exposures was purchased from Sigma Aldrich (Lot# BCBZ9334). Five milligrams of the NMs in powder form were added to 50 mL MilliQ water to make 100 mg/L stock solutions. The 50 mL nano stock solutions were sonicated at 60 °C in a bath ultra-sonicator (Model E-UC6-HD-D, Scientech, India) for 1 h at 20 Hz prior to diluting in the three different environmental media for exposures.

2.2.2 Media Preparation

The standard synthetic porewater (SSPW) for the nematode exposures was prepared according to Tyne et al. (2013) by adding 40 mL NaHCO₃ (0.1 M), 10 mL KNO₃ (0.1 M), 12.5 mL Ca(NO₃)₂ (0.1 M), 5 mL MgSO₄ (0.1 M), 0.01 mL Na₂HPO₄ (0.1 M), 10 mL HNO₃ (0.1 M), 0.559 mL iron standard (1 g/L) in 2 % HNO₃ and 0.27 mL aluminium standard (1 g/L) in 5 % HNO₃ (Certipur®, Merck) to 750 mL deionized water and made up to 1 L. This media was then aerated overnight and resulted in a stable pH of 8.1 ± 0.3 . For *Danio rerio* embryo exposures standard E3 media

was used (Cunliffe, 2003). This was made by dissolving 34.8 g NaCl, 1.6 g KCl, 5.8 g CaCl₂·2H₂O and 9.78 g MgCl₂·6H₂O in 2 L of deionized water, 16.5 mL of this stock and 100 µL of 0.05% methylene blue was then added to 1 L of water to make the media and aerated overnight with a stable pH of 7.4 ± 0.1. Methylene blue is added to prevent fungal contamination. Zebtec system water (ZSW) was used for adult *Danio rerio* tests, this was made automatically by using reverse osmosis water and dosing with sodium bicarbonate to a stable pH of 7.5 ± 0.5 and concentrated sea salt (Vibrant Sea, Seachem) to conductivity (EC) of 600 ± 50 µS/cm.

To make up the desired exposure concentrations the relevant volumes of sonicated NM stock solutions (100 mg/L) were added to the exposure mediums. Nominal NM concentrations used for characterization experiments are shown in Table 2-1. Concentrations were selected based on the test concentrations used in the specific organism's exposure bioassays based on their sensitivity. These concentrations were selected to ensure biological response and sufficient level of NMs to be able to detect and quantify materials. Since the all NMs were dosed by mass of the whole material, it was expected that the measured total Cd or Te concentrations of QDs would be a fraction of the total dose.

Table 2-1 : Concentrations of nanomaterials used for characterization in each of the different media.

Exposure media	Nominal nanomaterial concentrations (mg/L Nanomaterial)		
	nAu	QDs	nCuO
SSPW	20, 40, 70, 90, 160, 200	0.5, 5, 10, 20, 30, 50	5, 10, 30, 50, 60
E3	3, 10, 20, 30, 50, 60	0.5, 2, 6, 20, 40, 60	1, 3.5, 10, 20, 30, 60
ZSW	5.1, 20	2.1, 20	0.35, 20

2.2.3 Measurement of physicochemical parameters

Physicochemical water quality parameters were measured at the start of each test and monitored every 24 h thereafter. The pH, EC, oxygen saturation (% O₂) and total dissolved solids (TDS) were measured using calibrated Eutec ExStik II® handheld meters. All characterization measurements were conducted without organisms present.

2.2.4 Exposure concentrations

Actual total metal concentrations were determined for each of the nominal NM concentrations. Nominal concentrations refer to concentrations measured by mass of the whole nanomaterial. To measure exposure concentrations of total metal each concentration was made up in ultrapure water in polypropylene tubes to 11 mL in replicates of three. Nanogold samples (11 mL) were acidified with 4 mL aqua regia (3 mL 37% hydrochloric acid (HCl) and 1 mL 65% nitric acid (HNO₃)) to ensure all the gold was in ionic form. Copper oxide NMs and QDs samples (11 mL) were

acidified with 3 mL 65% nitric acid. Samples were then diluted by 50% and analyses were done using a GF-AAS (PerkinElmer, AAnalyst 600) equipped with Zeeman-effect background correction. Calibration of the instrument was performed using a series of 11 dilutions of the standard solution. With this, the concentrations of the sample analytes were calculated using regression lines with a correlation factor of 0.998. According to Grotti et al. (1996) salts from the media have the potential to interfere with Te readings on the GF-AAS, however no double peaks, that are indicative of interference, were observed in the present study. Sample blanks with only the media were run to determine the background metal concentrations for each media used.

2.2.5 Characterization of nanomaterials

To determine hydrodynamic size distribution and zeta potential (ZP) of all three NMs in solution, dynamic light scattering (Malvern Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, United Kingdom) was used. These measurements were taken at 0 h and at 48 h in each exposure media. In order to warm up the apparatus, it was switched on 30 min prior to analysis. During this time, stock suspensions were sonicated and prior to every measurement the required volume of the stock was added to 15 mL media to make up the required concentration for every material. To measure the zeta potential, the cell was rinsed with 1 ml of the dilution and emptied before injecting 1 ml once again into the cell for analysis. Care was taken to avoid bubble formation by first injecting half of the sample and once the sampled reached the halfway mark, the cell was then rotated and filled with the other half. The cell was closed with two stoppers and placed in the measurement chamber to start the zeta potential analysis. Each sample was measured in triplicate.

The primary particle diameter of initial nanomaterial particles was determined using transmission electron microscopy (TEM) (FEI Tecnai G2). One drop of 20 mg/L solution of each individual nanomaterial was added to a carbon grid separately, allowed to settle and dried, thereafter it was examined at high resolution (200 kV).

Dissolution of metal ions from the NMs were determined according to methods by Tatsi et al. (2018). Two concentrations were analysed per NM in each media (the EC₅₀/ LC₅₀ for each organism and 20 mg/L). Two concentrations were used due to laboratory constraints and practicality. The EC/LC50 for each organism in that media was used, as well as 20 mg/L to compare to the other data and between materials. Briefly, 8 mL of a sonicated stock of each material was added to a dialysis tube (product code: D9777, cellulose membrane with molecular weight cut off at 12 000 Da, Sigma-Aldrich Ltd, Dorset, UK) which was then secured on both ends using cable ties to prevent leakage. Each bag was then placed in a beaker containing 492 mL of the appropriate media. Samples (15 mL) of the external media from each beaker were taken after

the standard exposure period of the organisms (*C. elegans* 96 h and *D. rerio* embryos 96 h). In the case of ZSW samples were taken at 48 h, as this is when 100% water changes were done in the adult exposures. Samples were acidified with 300 µL of 65% nitric acid or aqua regia in the case of gold samples and analysed using GF-AAS.

The samples were injected in a pyrolytic graphite furnace tube using the autosampler AS 70 and ran under optimized operating parameters (See Supplementary Information Table S2-1 to S2-4). The calibration was performed by matrix adapted calibration where the concentrations in each sample were calculated by fitting a linear regression line with a correlation factor of ≥ 0.998 to the points defined by the spiked concentration values. Detection limits and sample blank concentrations are reported in Table 2-2.

Table 2-2 : Detection limits and blank media concentrations of each metal analysed using GF-AAS in simulated soil pore water, embryo media and zebrafish system water.

Element	Detection limits (µg/L)			Sample blank concentrations		
	SSPW	E3 media	ZSW	SSPW	E3 media	ZSW
Au	0.18	0.18	0.82	0.22 ± 0.01	$0.08 \pm 0.02^*$	1.431 ± 0.21
Cu	0.1	0.1	0.12	1.73 ± 0.029	1.49 ± 0.078	4.85 ± 1.36
Cd	0.11	0.11	0.1	0.32 ± 0.025	0.78 ± 0.012	0.76 ± 0.1
Te	1.15	1.38	0.01	1.28 ± 0.14	2.12 ± 0.49	17.25 ± 1.92

ⁱSamples with an asterisk indicate concentrations below detection limits.

2.2.6 Statistical analysis

The data are presented as mean $\pm$ standard error of the mean (SEM) (unless otherwise stated). Significant differences (at $p < 0.05$) were tested using one-way ANOVA followed by Tukey's post hoc test. All data were tested for normality (Shapiro-Wilk's test) and homogeneity of variance (Levene test) prior to performing the ANOVA.

2.3 Results

2.3.1 Metal concentrations

The total metal concentrations in each NM concentration was measured pertaining to the specific bioassay's exposure concentrations (Table 2-2). The total Cu content in the nCuO NMs was closest to the nominal concentrations. The Au content ratio in nAu treatments was 1:2 of the nominal concentrations, while the Cd content ratio in the QDs was between 1:3 and Te content was very low ($<1\%$ of QD mass) in the QDs.

Table 2-3 : Mean $\pm$ standard deviation of measured total metal concentrations (mg/L unless otherwise stated) in metal-based nanomaterials used in the different bioassays measured in ultrapure water.

NMs	nAu		nCuO		QDs		
Concentrations (mg/L)	Nominal exposure	Measured Au	Nominal exposure	Measured Cu	NM mass	Measured Cd	Measured Te ($\mu\text{g/L}$)
1		0.3 ± 0.04^a	1	0.9 ± 0.1^a	2	0.1 ± 0.04^a	5.4 ± 0.6^a
10		2.6 ± 0.5^b	3.5	3.8 ± 0.5^a	4	0.3 ± 0.03^b	6.5 ± 0.2^b
50		22.9 ± 5.6^{ab}	10	10.4 ± 0.5^{ab}	6	0.5 ± 0.02^c	9.8 ± 2.9^c
100		48.3 ± 0.3^{abc}	30	31.4 ± 1.7^{ab}	20	1.6 ± 0.05^{abc}	56.2 ± 4.8^d
600		258.9 ± 7.5^{abc}	100	100.8 ± 1.5^{ab}	100	30.7 ± 0.3^{abc}	5828.3 ± 79^{abcd}

ⁱ All values in the same column with the same letter in superscript are significantly different ($p < 0.05$).

2.3.2 Simulated soil pore water (SSPW)

Physicochemical water quality parameters

The temperature during exposures were held at a constant 21°C. The pH, DO and EC did not change significantly between exposures. The highest pH was 8.87, the DO was always above 86%. The EC ranged between 622 and 630 $\mu\text{S/cm}$.

Table 2-4 : Physicochemical water quality parameters (mean $\pm$ standard error of the mean) of simulated soil pore water used for *Caenorhabditis elegans* bioassays.

Nanomaterial	Temp. ($^{\circ}\text{C}$)	pH	EC ($\mu\text{S/cm}$)
Control	21 ± 0.4	8.8 ± 0.2	625 ± 11
nAu	21 ± 0.3	8.8 ± 0.1	622 ± 14
nCuO	21 ± 0.3	8.9 ± 0.2	641 ± 13
QDs	21 ± 0.4	8.9 ± 0.5	630 ± 18

Table 2-5 : Salts concentrations present in simulated soil pore water.

Cl (mg/L)	SO ₄ (mg/L)	Alkalinity (mg/L NO ₃ ⁻)	Na (mg/L)	Mg (mg/L)	K (mg/L)	Ca (mg/L)
-	40	217	92	12	39	50

Characterization of NMs in media

Dynamic light scattering showed that the lowest two nAu concentrations (40 and 70 mg/L) in SSPW had agglomerates greater than 200 nm, with the average size decreasing as the concentrations increased. Larger agglomerates were seen after 48 h (Table 2-6). All nCuO concentrations formed agglomerates greater than 200 nm, but there were no significant size differences between concentrations. The QD groups showed agglomerates greater than 500 nm and the size decreased as concentrations increased. After 48 h the agglomerate sizes increased to greater than 1000 nm in size (Figure 2-1). The nAu in SSPW ZP ranged between -19.63 mV and -21.43 mV, with no significant difference between concentrations and exposure periods. The nCuO ZP ranged between -17.43 mV and -23.2 mV. The NM concentration once again did not have an effect on ZP, but after 48 h, readings were significantly less negative in the high concentrations. The ZP of the QD group in SSPW ranged between -12 and -5 mV, becoming less negative with an increase in concentration and after 48 h. There was no Au dissolution from nAu in SSPW. The nCuO had less than 1% dissolution and QDs also had less than 1% dissolution of Cd and less than 7% Te.

Table 2-6 : Characterization presented as mean values $\pm$ standard deviation conducted in simulated soil pore water.

Material concentration (mg/L)	Manufacturer information	Primary particle size ^a	Hydrodynamic size (nm) ^b		Zeta potential (mV) ^b		Dissolution (% of metal concentration) ^c
		0 h	0 h	48 h	0 h	48 h	96 h
nAu 20	Lot# TMU14G 14 nm	15.1 $\pm$ 3.1	214.5 $\pm$ 77.6	274.6 $\pm$ 27.2	-20.7 $\pm$ 2.4	-20.1 $\pm$ 0.7	0
nAu 70			239.1 $\pm$ 22.9	200.1 $\pm$ 15.7	-20.1 $\pm$ 3.	-20.8 $\pm$ 1.7	-
nAu 90			80.6 $\pm$ 50.3	129.9 $\pm$ 26.3	-19.6 $\pm$ 1.7	-20.1 $\pm$ 1.9	-
nAu 200			60.6 $\pm$ 17.7	91.4 $\pm$ 1.1	-20.2 $\pm$ 0.5	-21.4 $\pm$ 1	0
nCuO 3.5	Lot# YF1510141, 10-20 nm		207.5 $\pm$ 9.1	285.5 $\pm$ 13.5	-23 $\pm$ 0.6	-19.6 $\pm$ 0.5	-
nCuO 10			207 $\pm$ 4.3	254 $\pm$ 6.7	-21.5 $\pm$ 0.6	-17.4 $\pm$ 2.2	-
nCuO 20		14.1 $\pm$ 5.9	203.4 $\pm$ 2	200.1 $\pm$ 15.5	-13.2 $\pm$ 0.6	-3.7 $\pm$ 0.2	0.02 $\pm$ 0.003
nCuO 50			212.1 $\pm$ 3.1	224.8 $\pm$ 20.6	-23.2 $\pm$ 0.7	-21.5 $\pm$ 0.3	0.02 $\pm$ 0.003

Material concentration (mg/L)	Manufacturer information	Primary particle size ^a	Hydrodynamic size (nm) ^b		Zeta potential (mV) ^b		Dissolution (% of metal concentration) ^c
		0 h	0 h	48 h	0 h	48 h	96 h
QD 0.5	Lot# YF140402, ± 4 nm		895.6 ± 138.1	1144.4 ± 132.6	-12.3 ± 0.5	-10.8 ± 1.4	-
QD 5			560.7 ± 18.1	1666.3 ± 95.1	- 5.67 ± 1.28	- 9.59 ± 2.28	-
QD 20		3.4 ± 0.9	519.3 ± 20.3	2017 ± 342.1	-13.1 ± 0.9	-8.1 ± 0.7	Cd 0.5 ± 0.3 Te 6.9 ± 1.4
QD 50			761.1 ± 40.2	3577.7 ± 117.2	-9 ± 0.5	-1.1 ± 0.3	-

a - measured 20 mg/L stock using a TEM at 0 h. Large agglomerates of the QDs group prevented primary particle size measurements.

b – measured using a zetasizer at 0 h and 48 h.

c – measured using a graphite furnace AAS. Results are shown after blank media samples were subtracted from results. Values given as 0 indicate results lower than sample blanks. Values with “-” were not measured.

[†] Values in bold indicate significant difference between 0 h and 48 h.

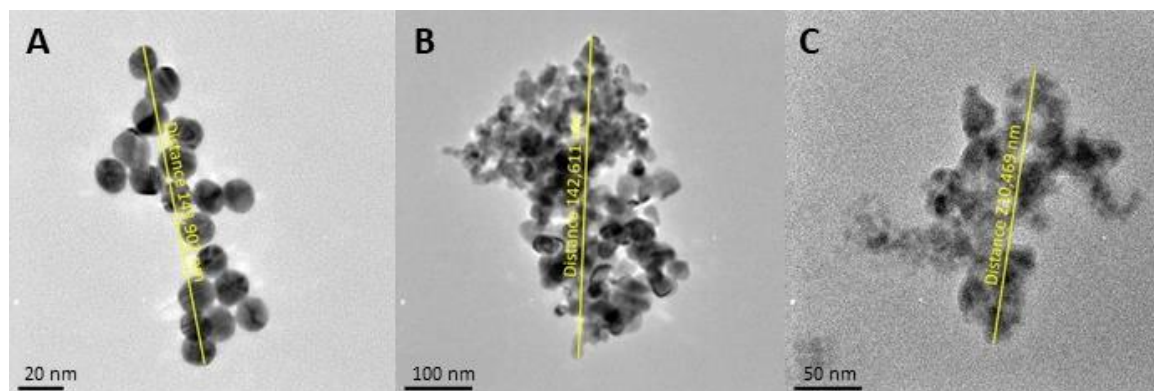


Figure 2-1 : Transmission electron microscope images visualizing primary particle size of 20 mg/L nAu (A), nCuO and QDs in simulated soil pore water exposure medium (pH 8.8).

2.3.3 E3 embryo media

Physicochemical water quality parameters

The physicochemical water quality parameters remained constant over all exposures and the addition of NM suspension did not influence EC or pH (Table 2-7). The media was aerated over

night before use and no further measurements were taken over the exposure period due to the small size of the exposure wells.

Table 2-7 : Physicochemical water quality parameters presented as mean values for Danio rerio embryo bioassays conducted in E3 media.

Nanomaterial	Temp. (°C)	pH	EC (µS/cm)
Control	28 ± 0.3	7.2 ± 0.1	923 ± 26
nAu	28 ± 0.1	7.2 ± 0.4	923 ± 22
nCuO	28 ± 0.2	7.3 ± 0.7	918 ± 31
QDs	28 ± 0.2	7.5 ± 0.2	912 ± 36

Table 2-8 : Salt concentrations present in E3 embryo media.

Cl (mg/L)	SO ₄ (mg/L)	Alkalinity (mg/L HCO ₃ ⁻)	Na (mg/L)	Mg (mg/L)	K (mg/L)	Ca (mg/L)
207	32	-	115	8	7	13

Characterization of NMs in media

In E3 media, all groups and concentrations caused aggregates greater than 200 nm (Table 2-9). All materials caused a decrease in size as concentrations increased. Overall aggregates increased in size over time, except in the lowest concentrations of the nCuO and QD groups (Figure 2-2). The QD group formed the largest aggregates and the nCuO group formed the smallest aggregates in the E3 media. For nAu in E3 media the ZP ranged between -6.63 mV and -19.07 mV and became less negative over time (Table 2-9). For nCuO in E3 media the ZP became more negative as concentrations increased and became less negative over time (Table 2-9). The ZP of the QD groups become less negative as the concentrations increased, but there was no difference in ZP after 48 h. The nAu group showed no Au dissolution from the NM in E3 media. The nCuO group had less than 1% dissolution and the QD group had less than 2% dissolution of Cd and less than 12% Te.

Table 2-9. Characterization presented as mean values $\pm$ standard deviation conducted in embryo media.

Material concentration (mg/L)	Primary particle size ^a	Hydrodynamic size (nm) ^b		Zeta potential (mV) ^b		Dissolution (% of metal concentration) ^c
	0 h	0 h	48 h	0 h	48 h	96 h
nAu 3		499.7 $\pm$ 106.3	614.2 $\pm$ 33.9	-17.7 $\pm$ 0.9	-16.7 $\pm$ 1.4	-
nAu 10		325.3 $\pm$ 42.2	320.9 $\pm$ 22.9	-18.7 $\pm$ 2.2	-14.2 $\pm$ 1.7	-
nAu 20	14.9 $\pm$ 1.3	285.1 $\pm$ 13.8	320.9 $\pm$ 22.9	-12.1 $\pm$ 1.2	-17 $\pm$ 2	0
nAu 51		171 $\pm$ 14.8	337.9 $\pm$ 13.1	-3.5 $\pm$ 0.7	-6.6 $\pm$ 20.9	0.00004 $\pm$ 0.00008
nCuO 3.5		369.1 $\pm$ 3.5	289.9 $\pm$ 19.8	-17.9 $\pm$ 2	-17.9 $\pm$ 0.4	0.004 $\pm$ 0.004
nCuO 10		217 $\pm$ 4.3	222.9 $\pm$ 2.4	-20.9 $\pm$ 0.2	-19.4 $\pm$ 0.2	-
nCuO 20	25.9 $\pm$ 8.7	212 $\pm$ 2.1	212.6 $\pm$ 0.8	-23.1 $\pm$ 0.5	-18.7 $\pm$ 0.7	0.02 $\pm$ 0.001
nCuO 60		211.5 $\pm$ 0.8	204.6 $\pm$ 2	-22.6 $\pm$ 1.2	-20.4 $\pm$ 0.6	-
QDs 0.5		880.3 $\pm$ 91.3	769.3 $\pm$ 53.8	-22 $\pm$ 3.9	-22.2 $\pm$ 1.7	-
QDs 2		643.3 $\pm$ 12.4	696.5 $\pm$ 50.1	-21.4 $\pm$ 2.6	-22.8 $\pm$ 0.5	-
QDs 6		436.5 $\pm$ 16.8	492.3 $\pm$ 11.8	-7 $\pm$ 10	-23.1 $\pm$ 0.4	-
QDs 20	22.6 $\pm$ 7.9	629.7 $\pm$ 29.1	221.6 $\pm$ 49.2	-7.8 $\pm$ 2.7	-7.8 $\pm$ 2.6	Cd 1.6 $\pm$ 0.1 Te 11.3 $\pm$ 2.8

a - measured 20 mg/L stock using a TEM at 0 h. Large aggregates of the QDs group prevented primary particle size measurements.

b – measured using a zetasizer at 0 h and 48 h.

c – measured using a graphite furnace AAS. Results are shown after blank media samples were subtracted from results. Values given as 0 indicate results lower than sample blanks. Vales with “-” were not measured.

[†] Values in bold indicate significant difference between 0 h and 48 h.

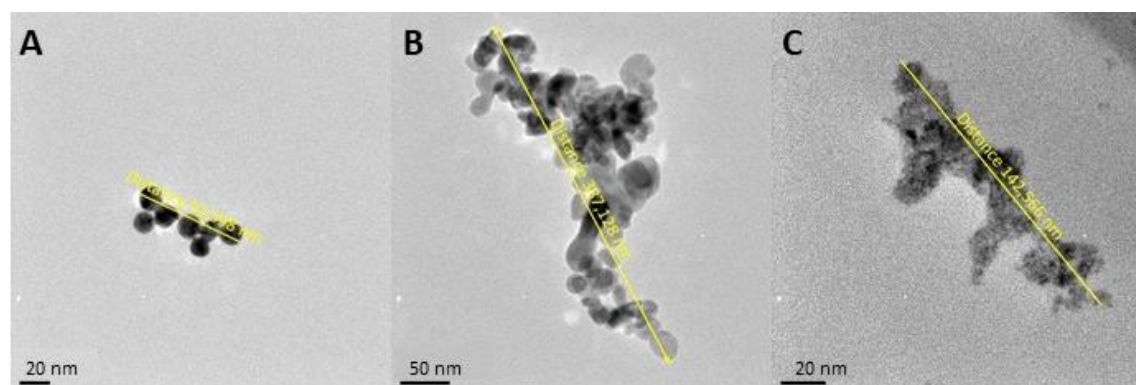


Figure 2-2 : Visualization of primary particle size of nAu (A), nCuO and QDs in E3 exposure medium (pH 7.2).

2.3.4 System water (ZSW)

Physicochemical water quality parameters

Each NM and ionic equivalent exposure in ZSW was done independently but exposure conditions were kept constant over the exposure time period, with water changes every 48 h. The addition of NM or ionic chemical did not influence the physicochemical parameters significantly. During each exposure pH was kept between 6.8 and 7.6 and EC was kept between 560 and 600 $\mu\text{S}/\text{cm}$ (Table 2-10). Ion concentrations are reported in Table 2-11.

Table 2-10 : Physicochemical water quality parameters presented as mean values for zebrafish system water.

Nanomaterial	Temp. (°C)	pH	EC ($\mu\text{S}/\text{cm}$)
Control	25 ± 0.5	7.1 ± 0.6	565 ± 12
nAu	25 ± 0.4	6.9 ± 0.8	570.3 ± 17
nCuO	25 ± 0.3	7.3 ± 0.2	568.2 ± 9.8
QDs	25 ± 0.5	7.2 ± 0.6	585.1 ± 6.3

Table 2-11 : Salts present in zebrafish system water.

Cl (mg/L)	SO ₄ (mg/L)	Alkalinity (mg/L HCO ₃ ⁻)	Na (mg/L)	Mg (mg/L)	K (mg/L)	Ca (mg/L)
111	44	55	72	9	4	5

Characterization of NMs in media

The nAu group showed a decrease in aggregates size with increasing exposure concentration and over time, forming aggregates as large as 633.6 nm at the 5 mg/L concentration after 48 h (Table 2-12). The ZP of the nAu groups became more negative as concentration increased and less negative over time. The nCuO groups showed an increase in aggregates size as concentrations increased and over time. The ZP of the nCuO groups remained the same between concentrations but became less negative over time. The QD groups showed an increase in aggregates size as concentration and time increased (Figure 2-3). The ZP became more negative with an increase in concentration and less negative after 48 h.

There was no dissolution of gold from nAu in ZSW. The nCuO showed dissolution less than 1% and the QD group showed less than 2% dissolution of Cd and 8% of Te. The lowest concentration (20 mg/L) showed the highest dissolution in the QD group.

Table 2-12. Characterization presented as mean values $\pm$ standard deviation conducted in zebrafish system water.

Material concentration (mg/L)	Primary particle size ^a	Hydrodynamic size (nm) ^b		Zeta potential (mV) ^b		Dissolution (% of metal concentration) ^c
	0 h	0 h	48 h	0 h	48 h	96 h
nAu 5		469.2 $\pm$ 49.7	633.6 $\pm$ 41.7	-6.3 $\pm$ 5.8	-5.7 $\pm$ 1.3	0
nAu 20	16.9 $\pm$ 1.786	217.2 $\pm$ 23.5	490.4 $\pm$ 9.8	-11 $\pm$ 1.1	-7.8 $\pm$ 1.3	0
nCuO 3.5		223.8 $\pm$ 14.6	417.6 $\pm$ 5.8	-16.96 $\pm$ 0.8	-5.8 $\pm$ 1.9	0
nCuO 20	20.9 $\pm$ 6.59	499.6 $\pm$ 10.2	744.1 $\pm$ 17.8	-17.4 $\pm$ 0.5	-5.9 $\pm$ 1.1	0.02 $\pm$ 0.006
QDs 20		456.3 $\pm$ 12.6	625.3 $\pm$ 8.6	-15.4 $\pm$ 2.7	-9.4 $\pm$ 1.4	Cd 1.5 $\pm$ 0.17 Te 7.8 $\pm$ 2.5
QDs 51		726.8 $\pm$ 3.6	1548.3 $\pm$ 239.5	-17.9 $\pm$ 1	-10.2 $\pm$ 1.7	Cd 0.7 $\pm$ 0.06 Te 4.8 $\pm$ 1

a - measured 20 mg/L stock using a TEM at 0 h. Large aggregates of the QDs group prevented primary particle size measurements.

b – measured using a zetasizer at 0 h and 48 h.

c – measured using a graphite furnace AAS. Results are shown after blank media samples were subtracted from results. Values given as 0 indicate results lower than sample blanks.

[†] Values in bold indicate significant difference between 0 h and 48 h.

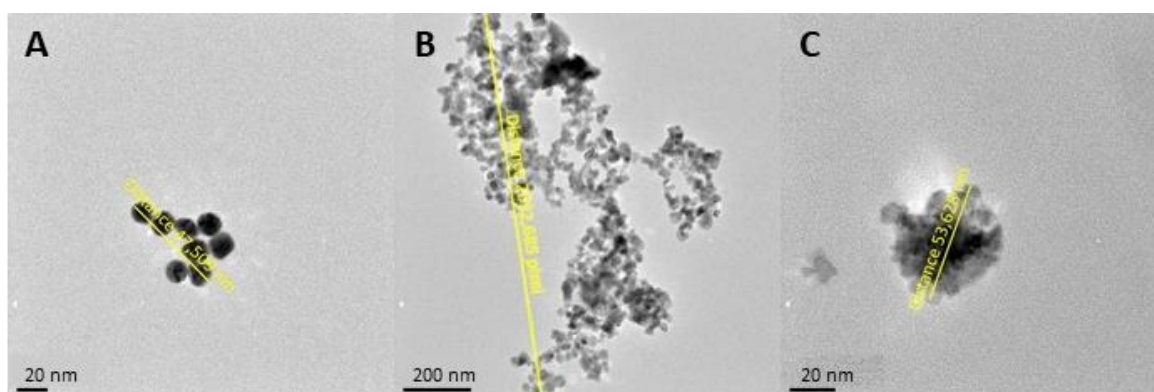


Figure 2-3 : Visualization of primary particle size of nAu (A), nCuO and QDs in system water (pH 7.5).

2.4 Discussion

Aggregation and dissolution are fundamental measurements to evaluate NM environmental risk and exposure. Whether they are in particle or dissolved ion form will influence their bioavailability and uptake patterns. According to Luoma et al. (2014) there are four interrelated drivers of bioavailability of NMs. These drivers are concentration, the environment, attributes of the nanomaterial and the organism. As the physicochemical characteristics of the exposure media remained the same, focus is placed on NM characteristics and their changes in the different media with different pH levels. In the current study nAu and QDs had the highest aggregation overall and the media causing the highest aggregation was ZSW with an average pH of 7.1. Based on the measured total metal exposure concentration dissolution of metal from the NMs were less than 2% in all three the media, but telluride ion had dissolution between 7 and 12% from QDs in the different media. Based on ZP and DLS measurements the nanomaterials tend to aggregate with a ZP closer to 0. In SSPW nAu and the low concentrations of nCuO appeared to be the most stable, in E3 media the nAu and QDs were the most stable and in the ZSW all particles were unstable, tending to aggregate. Total metal content of stock solution was not equal to the nominal NM concentrations, except for the nCuO treatment.

In many cases of NM toxicity, evidence suggests that the metal ions that dissociate from particles are the causes of toxicity. Gold is known to be relatively insoluble and this was confirmed with no dissolution measured in any of the exposure media in the present study. Dissolution from nCuO was less than 1% in all three media. Not a surprise given metal oxides are generally sparingly soluble (Odzak et al., 2014). A previous study found that media pH strongly influenced the surface potential and dissolution of nCuO via protonation or deprotonation of surface hydroxyl groups (Peng et al., 2017). Under strong acid condition, nCuO can react with high concentrations of H^+ in the solution and release a large amount of Cu^{2+} , while under weakly acidic conditions, only a small amount of H^+ forms contacts with nCuO, generating $Cu(OH)^+$ (Peng et al., 2017). In the present study cadmium dissolution from QD was less than 1% in SSPW (pH 8.8) and 1.5% in E3 media (pH 7.6) and ZSW (pH 7.2). Tellurium had the highest dissolution rate at 7% in SSPW, 12% in E3 media and 8% and less in ZSW. This is in contrast to results from Tatsi et al. (2020) that found higher dissolution of Cd than Te from QDs of the same project. This is most likely due to differences in exposure media composition. The Nanosolutions project (Vassallo et al., 2018) reports higher dissolution of Te than Cd from QDs in M7 media after 24 h, even though the total metal content of Cd is higher in QDs.

The QDs are bimetallic semiconductor particles made up of a CdTe core and coated with NH_3 functional groups. Functional group and sorption-desorption equilibria influences metal cation dissolution rates (Paydary and Larese-Casanova, 2020). It is theorised that the chemical bonds

than anchor the functional group to the core promote greater dissolution of the atoms on the exterior of the core due to mechanical stress. In the present study Te atoms could be on the exterior of the core dissociating more than Cd (Tatsi et al., 2020) or be displaced from the Cd within the core. Further experiments would be needed to explore this notion.

Copper oxide NMs have stronger affinities for various organic ligands such as endogenous glutathione (GSH), weak reactions with chloride and are more strongly influenced by major ions (such as Ca^{2+}). Agglomeration characteristically increases with increased ionic strength and concentration of particles, indicating that a matrix with a higher EC will cause greater agglomerates at higher concentrations (Ortelli et al., 2017; Barreto et al., 2020). The elevated NaCl increases the thickness of the electric double layer around the particle, and with anion exclusion (Donnan effect) causes the net surface charge to decrease (Cui et al., 2012). Lower dispersions ZP values closer to 0 will lead to aggregation, coagulation, or flocculation when the repulsive electrostatic forces are overcome by the attractive forces such as van der Waals attraction (Joseph and Singhvi, 2019; Shnoudeh et al., 2019).

The primary particle size of nAu and nCuO were approximately 14 nm, and the QD particles were around 4 nm in the present study. Low total Au (41%) compared to nominal citrate capped nAu concentrations was also observed by Barreto et al. (2020) however, no reason was given. In the case of QDs, particles are comprised of surface functional groups and possible hydrogen atoms, thus dosing total mass of QDs would be expected to have lower Cd and Te concentrations (Handy et al., 2015). Dosing on total mass of QD, then the Te and Cd concentrations would be expected to be lower than that. At certain concentrations the number of particles outcompete charge and effects of the van der Waals interaction causing varying aggregates between concentrations. Such as in the case of nAu in SSPW where higher concentrations caused smaller aggregates than the low concentrations. The same was seen for all materials in E3 media. Particles can undergo homo-aggregation between NM particles and hetero-aggregation where particle interact with organic molecules present in the water (e.g. proteins from the biofilter in ZSW), which contributes to the largest aggregates present in the ZSW for all three NMs. However, this needs to be confirmed. Smaller particles such as QD are more susceptible to aggregation under the same conditions due to larger surface areas being more reactive leading to the largest aggregates between the three materials tested (Zhang, 2014). It has also been shown that increasing ionic strength intensified the agglomeration, aggregates and sedimentation of nCuO because of the compression of electrical double layer field leading to charge density (Peng et al., 2017). This is one of the reasons for high agglomeration in ZSW as hydrodynamic size increased and pH was lower. Cations in the media will screen the NH_3^+ of QD particles. The point of zero charge will be achieved the higher the Cl^- and SO_4^- concentration, causing agglomeration and settling. As seen

in other studies an increase in concentration of NaCl and NMs leads to an increase in aggregation and agglomeration. These aggregates could reach a size that is no longer favourable for suspension and settle out resulting in low DLS readings at these higher concentrations, as observed in the QD groups (Barreto et al., 2020).

The ZSW used in the present study contains biological material such as proteins and natural organic matter due to the presence of biological filters in the Zebtec system producing the media. This could lead to the formation of a protein corona around the NMs. A study by Barbero et al. (2017) found that negatively charged particles would become more positive over time, as was also observed in the present study. They studied the formation of protein coronas around nAu and found that the largest aggregates of particles and net positively charged proteins formed at 0 h and decreased over time (48 h) as this is a dynamic process where proteins can attach and detach over time (Barbero et al., 2017; Kharazian et al., 2016). In the present study, it was seen that aggregate size increased in ZSW over time. This is most likely due to the presence of proteins and natural organic matter in the water. If there are more proteins than NMs in solution, hetero-aggregation of NMs to proteins can outcompete homo-aggregation leading to smaller clusters (Rampado et al., 2020). All three materials assessed in this study had negative ZPs, but had more positive ZPs in ZSW. This is most likely due to lower cation concentrations present and the presence of protein corona. The type of functionalization affects the types of protein that can bind to the particle and should further be investigated as this will affect how the QD is taken up by the organism (Lundqvist et al., 2008; Singh et al., 2021).

Functional groups present on the particle surface play a role in bioavailability and tissue distribution. It has been shown that in a controlled environment, citrate capped nAu can react with OH, forming strong bonds, depending on pH and chloride species (Stuart and Ando, 1996). At a pH lower than 4, AuCl_2 is most likely to form, at a pH of between 6 and 9 $\text{AuCl}(\text{OH})_3^-$ would form leading to the negative charge observed in the present study, as the highest pH observed was 8.87 in SSPW (Peng et al., 2017). At a low pH nCuO can react with the H^+ in the media that increases the release of Cu^{2+} , while nCuO can react with small amounts of H^+ that will form $\text{Cu}(\text{OH})^+$ (Peng et al., 2017). The pH levels in the present study were not considered low, thus no notable dissolution of nCuO. Amino groups attached to QD particles are basic by nature and will react in a pH <10 environment to become protonated by receiving H^+ from the environment giving the functional group a positive charge (Berg, 2002). All media had a pH lower than 10. This will result in a less stable solution as observed in the present study and could facilitate aggregation interactions with negatively charged particles in the environment, causing large aggregates observed here by DLS. Overall, SSPW resulted in the largest aggregates, most likely due to higher dissolved ions levels in this media as described by DLVO theory.

2.5 Conclusions

When stock concentrations of NMs were diluted in different media there was a clear difference in characteristics observed. These media characteristics are used to imply possible potential NM bioavailability and uptake. All three materials formed large aggregates and agglomerates when diluted in exposure media and size increased over time due to homo-aggregation in SSPW and E3 media as seen by DLS, and potential hetero-aggregation/ corona formation in ZSW. System water also had the lowest pH of the three media and this affected aggregation sizes, ZP and dissolution of metals from NMs. Dissolution was the highest in ZSW, and only QD had metal dissolution higher than 1%, indicating these materials are not as stable in environmental media as nCuO and nAu. The dissolution of Te from QDs was the highest of all the materials and is potentially due to ligand-promoted stress dissolution. Total metal content increased as NM concentrations increased, but metal to NM ratios did not remain constant over all concentrations (see Table 2-2), most likely due to NM aggregates and settling out at certain concentrations, as well as the makeup of the particles in the case of QDs. At a threshold concentration there is a potential imbalance between number of particles and attraction forces where particles can outcompete van der Waals or electrostatic interactions leading to lower aggregates and settling out. Free ions such as Cl^- and Na^+ in the media facilitate aggregation, this is confirmed by large hydrodynamic sizes in SSPW with higher concentrations of ions.

CHAPTER 3 SUB-CELLULAR AND WHOLE ORGANISM RESPONSES OF *CAENORHABDITIS ELEGANS* FOLLOWING EXPOSURE TO THREE METAL-BASED NANOMATERIALS

3.1 Introduction

Even though nanotechnology is no longer a new or emerging discipline, there is still a lack of understanding regarding the toxicity of NMs in the receiving environment (Handy et al., 2022). This is because the toxicity of NMs can be dose-dependent as in the case of traditional chemicals but may also be associated with physical properties such as size and surface engineering (Yang et al., 2012). Understanding NM transformation processes, uptake and modes of toxicity is critical for safety by design applications (Gonzalez-Moragas et al., 2015). To determine the effects of NMs on organisms it is important to make use of standardized toxicity tests so that results from different studies can be directly compared to each other (Taylor and Scroggins, 2013). *Caenorhabditis elegans* is a free-living nematode that is 1 mm in length, they can be grown on petri dishes and have a short lifespan (2-3 weeks). These factors make it an ideal test organism for chemistry, material science, environmental toxicology and medicine (Hunt, 2017). The standardized tests for *C. elegans* can be conducted in aqueous as well as sediment environments. Even though these nematodes lack specific tissues such as bones, organs such as the liver and a circulatory system, they still have a high genetic similarity to vertebrates (Gonzalez-Moragas et al., 2015). These nematodes are predominantly hermaphrodites (males will occur in 0.1% of instances) and have an alimentary system, reproductive system, nervous system, and an excretory system. They also have a 0.5 μm thick negatively charged cuticle surrounding their body, thus it is assumed that nanomaterials would be taken up orally (Minullina et al., 2011).

The similarity between *C. elegans* and vertebrates allows for the study of metabolic pathways and the possible effects of toxicants on these pathways. To date, limited studies have applied metabolomic analysis to *C. elegans* toxicology (Ratnasekhar et al., 2015; Kim et al., 2019a; Kim et al., 2019b; Yin et al., 2020; Kim and Kang, 2021). Only two of these studies focussed on metal NMs and identified affected pathways after exposure to titanium dioxide and nanopolystyrene. Exposure to titanium dioxide NMs leads to disruptions in the tricarboxylic acid (TCA) cycle, arachidonic acid metabolism and glyoxalate dicarboxylate metabolism and resulted in effects on reproduction (Ratnasekhar et al., 2015). Exposure to nanopolystyrene resulted in perturbation of metabolites related to the TCA cycle, glucose and lactic acid, and also resulted in reproduction effects (Kim et al., 2019a). Many metabolic toxicity studies on other organisms also focus mainly on titanium dioxide and silver NMs (Kim and Kang, 2021). A study by Zhang et al. (2017) revealed

time and dose dependant metabolic responses of Daphnids to silver NMs resulting in changes in amino acids, fatty acids, carbohydrates and lipids. Pathway analysis also indicated influences on reproduction and growth. Reproduction is a common output measured in toxicity studies and causal links between metabolism and reproductive toxicity can be made. Hermaphrodites can produce up to 300 embryos by self-fertilization and spermatogenesis begins during the L4 larval stage (Kim et al., 2013). A complete life cycle is 3 days, making reproduction results fast and diverse due to the range of endpoints that can be measured such as egg count, fertility and the number of offspring. Reproduction was adversely affected in *C. elegans* after exposure to mercaptosuccinic acid (MSA)-capped QDs (Hsu et al., 2012), nickel NMs (Kong et al., 2017), silica NMs (Pluskota et al., 2009), nAu (Bosch et al., 2018; Kim et al., 2013), graphene oxide (Tsai et al., 2021) and silver NMs (Jeong et al., 2018).

However, measuring effects assumes that there is uptake of particles. Uptake in *C. elegans* depends on the rate of pharyngeal pumping, which can be influenced by temperature, food and nutritional status of the nematodes (Hall and Altun, 2007; Riddle et al., 1997). Nematodes will keep pumping even in the absence of food, e.g. starved nematodes will increase pumping in the presence of low quantities of food compared to well-fed worms (Gonzalez-Moragas et al., 2015). The uptake of NMs will also depend on the properties of the materials (see Chapter 2 for a detailed description). These modes of uptake and nematode biology will affect the internal exposure and toxic effects on *C. elegans*. Thus, by determining NM localization inside the organism, relationships between distribution, metabolism and organ function can be made. Combining techniques to localize NM *in vivo* can provide a more accurate understanding of NM fate. Therefore, this chapter aimed to determine the uptake and effects of three metal-based NMs and their ionic metal equivalents on the reproduction and metabolism of *C. elegans*. The NMs selected were nAu (representing a relatively inert NM), QDs (representing a bimetallic NM) and nCuO (representing an oxide NM).

3.2 Materials and Methods

3.2.1 Standard 96 h reproduction bioassays

Stock culture maintenance

A culture of *C. elegans* N2 strain was obtained from the *Caenorhabditis* Genetics Centre at the University of Minnesota, MN, USA and is maintained at the North-West University. Nematodes are maintained in stock cultures of dauer larvae on nematode growth agar at $20 \pm 2^\circ\text{C}$ according to standard ISO procedures (ISO 10872). When age synchronized nematodes were needed for a test, a section of agar containing dauer larvae was transferred to a new agar plate containing a

fresh lawn of *Escherichia coli* OP50 as a food source. After incubation at $20 \pm 2^\circ\text{C}$ for four days the new plate contained many L1 and L2 life stage nematodes, as well as adults. According to ISO 10872, L1 stage nematodes are used for the tests. To obtain nematodes at this life stage, plates were rinsed with SSPW, this suspension was then filtered through a $5\ \mu\text{m}$ mesh size nylon net to retain larger L3-4 stage juveniles and adults. The food source *E. coli* OP50 stocks were made by flash freezing *E. coli* OP50 liquid culture with glycerine and stored at -80°C until further use.

Escherichia coli preparation

Twenty-four hours before the start of a test, 50 mL of sterile Luria Broth (ISO 10872) was inoculated with 20 μL of *E. coli* OP50, set on a shaker incubator at 37°C and 150 rpm for 17-24 h. This culture was then transferred to a sterile 50 mL polypropylene centrifuge tube and centrifuged at 2000 RCF (Eppendorf Centrifuge 5430, Hamburg, Germany) for 20 min. The bacteria formed a pellet at the bottom of the tube. The supernatant was then discarded, and the pellet was resuspended in 20 mL SSPW. This process was repeated three times. Finally, the suspension was diluted in SSPW to 50 mL and the turbidity was measured using a Spectroquant (Pharo 300, Merck Millipore, Germiston, South Africa). The suspension was then diluted to 1000 ± 50 Forzamin Attenuating Units (FAU; according to ISO). Cholesterol stock of 0.01% of the total bacterial solution volume (prepared according to ISO 10872) was added to the solution and shaken to mix properly. Five hundred microliters of this bacterial solution were then added to each well of a 12 well plate immediately at the start of the test as described below.

Reproduction test

The tests were carried out according to the standard ISO 10872 (2010) protocol. In short, 10 L1 stage nematodes were transferred to each well in a 12 well plate, in four replicates per concentration ($n = 40$). For each test replicate, 500 μL aliquot of sonicated nano and metal stock solutions at double the required test concentration was diluted with a further 500 μL of *E. coli* OP50 stock (1000 ± 50 FAU), after sonication as described in section 2.2.2, to yield the desired test concentration in a 1 mL volume, as well as the appropriate food density. The tests lasted 96 h in darkness at $20 \pm 2^\circ\text{C}$. After the exposure period, 0.5 mL of an aqueous solution of Rose Bengal (0.3 g/L) was added to each well to stain the nematodes for counting. The test was terminated by placing the plates in an oven at 80°C for 15 min. Plates were then stored at 4°C until further analysis. To count the offspring, each well was transferred to a petri dish using a pipette and the number of juveniles and adults was counted under a dissecting microscope (Nikon SMZ 1500 with a Nikon DS-Fi one camera and ImageJ software) at 7-fold magnification. The number of adult males was noted and subtracted from the number of adults, as they cannot

reproduce. The number of juveniles per hermaphrodite adult was then calculated for each replicate and expressed as a percentage of the control. An EC_{50} for reproduction was then determined by Toxrat software for each NM.

3.2.2 Biodistribution and Uptake

The EC_{50} concentrations were used as the exposure concentrations for the biodistribution and uptake; and the biological response assessment. Exposure concentrations were 230 and 0.06 mg/L for nAu and ionic Au ($HAuCl_4$); 32.5 and 0.26 mg/L for nCuO and ionic Cu ($CuCl_2$); 0.001, 0.09 and 0.005 for QDs, ionic Cd ($CdCl_2$) and ionic Te ($TeCl_4$).

Biodistribution

After exposure as described above for the reproduction test, nematodes (n=5) from each of the NM exposure treatments were dispersed onto a microscope slide without staining them. Excess liquid was removed with filter paper. Cryopreserve gel (Tissue-Tek OCT Compound) was used to mount the nematodes onto the slide, covered with a coverslip and stored at 4°C until imaging using CytoViva® 150 Unit integrated onto an Olympus BX43 microscope. Since non-fixed specimens were used, microscopy assessment was completed within 2 h of preparing the slides.

Uptake

Exposures using larger nematode biomass were necessary to determine metal uptake. For this experiment, 120 new nematode agar plates (as described in section 3.2.1) were washed with SSPW. These nematodes suspensions from the plates were collected in polypropylene centrifuge tubes to 3.5 mL. The solution was then bleached to destroy nematodes but not eggs. This bleaching solution consisted of 0.5 mL 5N NaOH and 1 mL of freshly prepared 5% NaOCl (14% NaOCl + ultrapure water). The Falcon tubes were shaken until no worms were visible anymore (~10 min) and centrifuged for 2 min at 1300 RCF. To remove the bleaching solution, the supernatant was discarded and washed with 5 mL ultrapure water twice. The remaining pellet was resuspended in 10 mL SSPW. The eggs were incubated on a 130 rpm shaker at $20 \pm 2^\circ\text{C}$ overnight for hatching. The next day all samples were pooled.

Each exposure was made up of 20 mL total media with nematodes and the required volume of each material was added to make up the final concentration (i.e. EC_{50}) for each material. Five replicates per treatment consisting of ~10 000 worms per replicate were prepared. The exposures were incubated for 96 h at 20°C on a shaker at 130 rpm. To collect the nematodes after each exposure, the samples were collected in 50 mL falcons and centrifuged at 2000 RCF for 3 min. The supernatant was discarded, and the pellet was put through a sucrose separation step as per

Johnson et al. (2017) to separate nematodes from the exposure media. In short, a sucrose gradient was created in 15 mL polypropylene tubes from the bottom 50%, 40%, 20% and then lastly 0.1 mol/L NaCl. The nematode metal pellet was then added to the top of this gradient and the tubes were centrifuged at 4°C for 5 min at 175 RCF and 5 min at 2500 RCF.

The results were that the metal particles settled out and the nematodes remained in suspension between the last two concentrations of sucrose. The nematodes were then collected with a pipette and filtered through a 5 µm mesh to remove sucrose. Nematodes were washed off the mesh with ultrapure water, collected in a polypropylene tube and centrifuged to create a nematode pellet. This pellet was then transferred to 1 mL pre-weighed micro centrifuging tubes, weighed again, frozen and freeze-dried. Freeze-dried samples were weighed again and then digested in a heat block. Shortly 1 mL of HNO₃ (sub-boiled from 65%; p.a. quality, Merck) was added to each sample and 1 mL aqua regia was added to gold samples, the heat block was then sealed and placed in a 65°C oven for 24 h. After digestion samples were diluted to 10 mL with ultrapure water and read on an AAS. Optimized AAS parameters for each metal were the same as reported in Chapter 2. Detection limits and recovery rates are reported in Table 3-1.

Table 3-1 : Recovery rates (%) and the detection limits of the metals of interest obtained for the certified reference material, as well as nematode tissue samples, respectively. Detection limits for the tissue samples were determined as three times the standard deviation of the blank measurements multiplied by the average weight of the samples.

Elements	Recovery Rates (%) Dorm-4	Limit of detection nematode tissue (µg/g dw)
Au	n.a	2.64
Cu	102	1.60
Cd	99	0.17
Te	n.a	9.86

n.a – Not available

3.2.3 Metabolomics

Exposures for metabolomics followed the same procedures as mentioned in section 3.2.2. The only difference was that the final nematode pellets were snap-frozen in liquid nitrogen and stored at -80°C until further processing. Before metabolite extraction, samples were freeze-dried and weighed. Quality control samples were also run. They were pooled samples from all treatments tested. Pooled QC samples are applied to condition the analytical platform, perform intra-study reproducibility measurements and to correct mathematically for systematic errors. Centre for Human Metabolomics

For the extraction of the whole metabolome from the Nematodes, a single-phase extraction method by Beukes et al. (2019) was applied to all experimental samples of each of the described groups. In short, starting with each dried sample in a 1.5 mL microcentrifuge tube, 50 μ L of the Internal Standard (3-Phenylbutyric acid 50 mg/L) and 1 mL extraction solution mixture composed of chloroform, methanol & water (1:3:1) was added. After the addition of a 3 mm tungsten carbide bead, the sample mixtures were shaken in a vibration mill at 30 Hz for 5 min and consequently centrifuged at 16 000 rcf for 5 min at 4°C. The supernatant was transferred into a GC vial and dried under a gentle stream of nitrogen at 40°C for 20-30 min. For derivatization purposes, 45 μ L methoxyamine HCl (150 mg in 10 mL pyridine) was added followed by an incubation step at 50°C for 90 min. Hereafter, 25 μ L BSTFA + 1% TMCS was added and the extract was re-incubated at 50°C for 60 min. The extracts were then transferred to a 250 μ L insert in a sample vial and capped before GCxGC-TOF-MS analysis. A Pegasus 4D GCxGC-TOFMS (Leco Corporation, St. Joseph, MI, USA), utilizing an Agilent 7890A GC (Agilent, Atlanta, GA) coupled to a time of flight mass spectrometer (TOFMS) (Leco Corporation, St. Joseph, MI, USA) equipped with a Gerstel Multi-Purpose Sampler (MPS) (Gerstel GmbH & co. KG, Eberhard-Gerstel- Platz 1, D-45473 Mülheim an der Ruhr) was used to undertake the untargeted metabolome analysis.

3.2.4 Statistical analysis

The dose-response function, namely, effect concentration with x% of the population affected (EC_x values), was calculated using nonlinear regression analysis in ToxRat (ToxRat Solutions GmbH, Alsdorf, Germany). To test for normal distribution, Shapiro-Wilk's test was used and the Levene test was used to test for homogeneity of variance. One-way Analysis of Variance and Tukey's multiple comparison test were performed to calculate the statistical differences ($p \leq 0.05$) of sublethal responses between the different control, NM and ionic treatments. Kruskal-Wallis test and Dunn's multiple comparison test were performed to calculate the statistical differences for all the data that were not normally distributed.

For metabolite identification, the commercial library NIST11 was used to identify the compounds. Identification could only occur once there was a spectral match of 80% similarity between the compound and library. The data was filtered, removing any compound that was not reliably measured (a CV > 50% was used), as well as any elements that were not present in all samples of at least one experimental group. Contaminant compounds were identified by comparing the compounds detected in the extraction blank and system blank to that detected in the samples and were then removed. The data were corrected for sample weight, normalized by median and auto-scaled and by default, missing values will be replaced by 1/5 of min positive values of their corresponding variables. After pre-processing the metabolomics data, uni- and multivariate statistics were performed with *MetaboAnalyst*. The most relevant variables were determined by

Analysis of Variance (ANOVA), Principal Component Analysis (PCA), followed by a *Fisher's last significant difference* posthoc test (Fisher's LSD) and Partial Least Square – Discriminant Analysis (PLS-DA). Significant differences between the control and treatment groups were tested using pairwise t-tests and pathway analysis was performed using these significant metabolites, resulting in a list of most significant and greatly impacted pathways between the two groups.

3.3 Results

3.3.1 Reproduction

Both nAu and ionic Au showed a dose-dependent decrease in reproduction (Figure 3-1). Toxicity was measured at 10 x lower concentrations in ionic Au (10 – 2000 µg/L) (B) compared to nAu (70 – 200 mg/L) (A). Ionic Cu (D) also showed a dose-dependent decrease in reproduction, but the three lowest concentrations (50 – 200 µg/L) caused a non-significant stimulation in reproduction. Nano CuO (C) showed a non-significant bimodal effect in reproduction inhibition. Quantum dots (E) and Te (G) caused a dose-dependent decrease in reproduction. Cadmium (F) also had a bimodal effect on reproduction inhibition and toxicity was observed at lower concentrations (20 and 100 µg/L) than for Te. Note the difference in the scale of the y-axes for Figure 3-1, A, F and G.

Based on the reproduction data, an EC₅₀ with upper and lower confidence limits was calculated for each material using Toxrat® software (Table 3-1). The material with the lowest EC₅₀ and therefore the most toxic was the QDs. The toxicity profile from most to least toxic is QD>Te>Ionic Au>Cd>Ionic Cu>nCuO>nAu. Many confidence limits could not be calculated due to the varying results and bimodal responses. These EC₅₀ values were used as the sublethal exposure concentrations in the following sections.

Table 3-2 : Summary of the median reproduction effective concentration (EC₅₀) for 50% effect and the subsequent 95% confidence limits (CL) for Caenorhabditis elegans after exposure to nAu, ionic Au, nCuO, ionic Cu, QDs, ionic Cd and ionic Te. N.d. is where the values could not be determined.

EC ₅₀ (mg/L)			
Material	Mean	Upper CL	Lower CL
nAu	230	n.d.	n.d.
Ionic Au (H[AuCl ₄])	0.06	0.08	0.002
nCuO	32.5	n.d.	n.d.
Ionic Cu (CuCl ₂)	0.256	1.35	0.042
QDs	0.001	0.0003	0.02
Ionic Cd (CdCl ₂)	0.085	n.d.	n.d.
IonicTe (TeCl ₄)	0.005	0	n.d.

3.3.2 Biodistribution and Uptake

CytoViva® darkfield microscopy of *C. elegans* revealed that when compared to the control medium NMs were present in the gut, pharynx and uterus of the nematode (Figure 3-2). Images also show that nCuO particles cannot be visualized using CytoViva® as they are not fluorescent but rather appear as black agglomerates. Darkfield images of stock solutions are also added to the supplementary information to show the visualization observed is NM (See Figure S3-1).

It is important to note that the exposure concentrations (based on EC₅₀ values) for the ionic equivalents of the NMs were much lower than the level of the particular metal present in the EC₅₀ exposure concentrations of the NMs. Total metal uptake of Au by the nematodes shows similar internalized Au concentrations even though the ionic Au (60 µg/L) exposure concentrations were almost 4000 fold lower than the Au present in the nAu exposure (i.e. 25 mg/L – see section 2.3.1) (Figure 3-3 A). Thus, indicating a difference in uptake and regulation of metal levels inside the nematode. The total metal concentration of ionic Cu (256 µg/L) was 100 fold lower than the Cu concentrations in the nCuO (30 mg/L) exposures, but the Cu in the nematodes was up at 10 fold higher than in the nCuO exposure group (Figure 3-3 B). There was no significant difference in uptake of QD-Cd and QD-Te compared to its ionic equivalents (Figure 3-3 C,D).

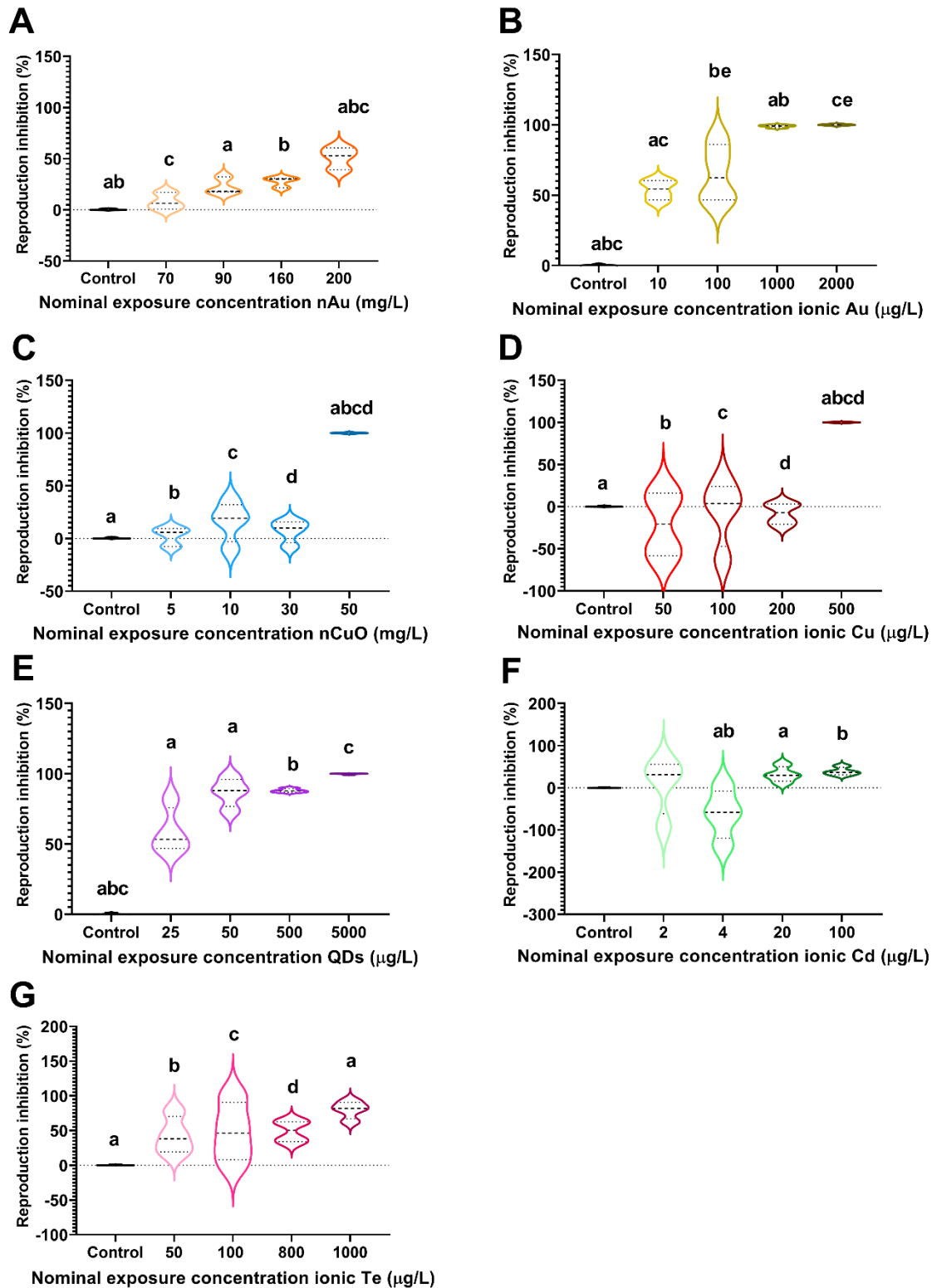


Figure 3-1 : Percentage reproduction inhibition of *Caenorhabditis elegans* ($n = 5$) after 96 h exposure to A) *nAu*, B) ionic *Au* (chloroauric acid), C) *nCuO*, D) ionic *Cu* (CuCl_2), E) *QDs*, F) ionic *Cd* (CdCl_2) and G) ionic *Te* (TeCl_4). Ionic treatments are expressed as metal concentration. Treatments that share a letter are statistically significantly different ($p \leq 0.05$, ANOVA). Dashed lines indicate the median and interquartile range. Note the difference in scale of A, F and G.

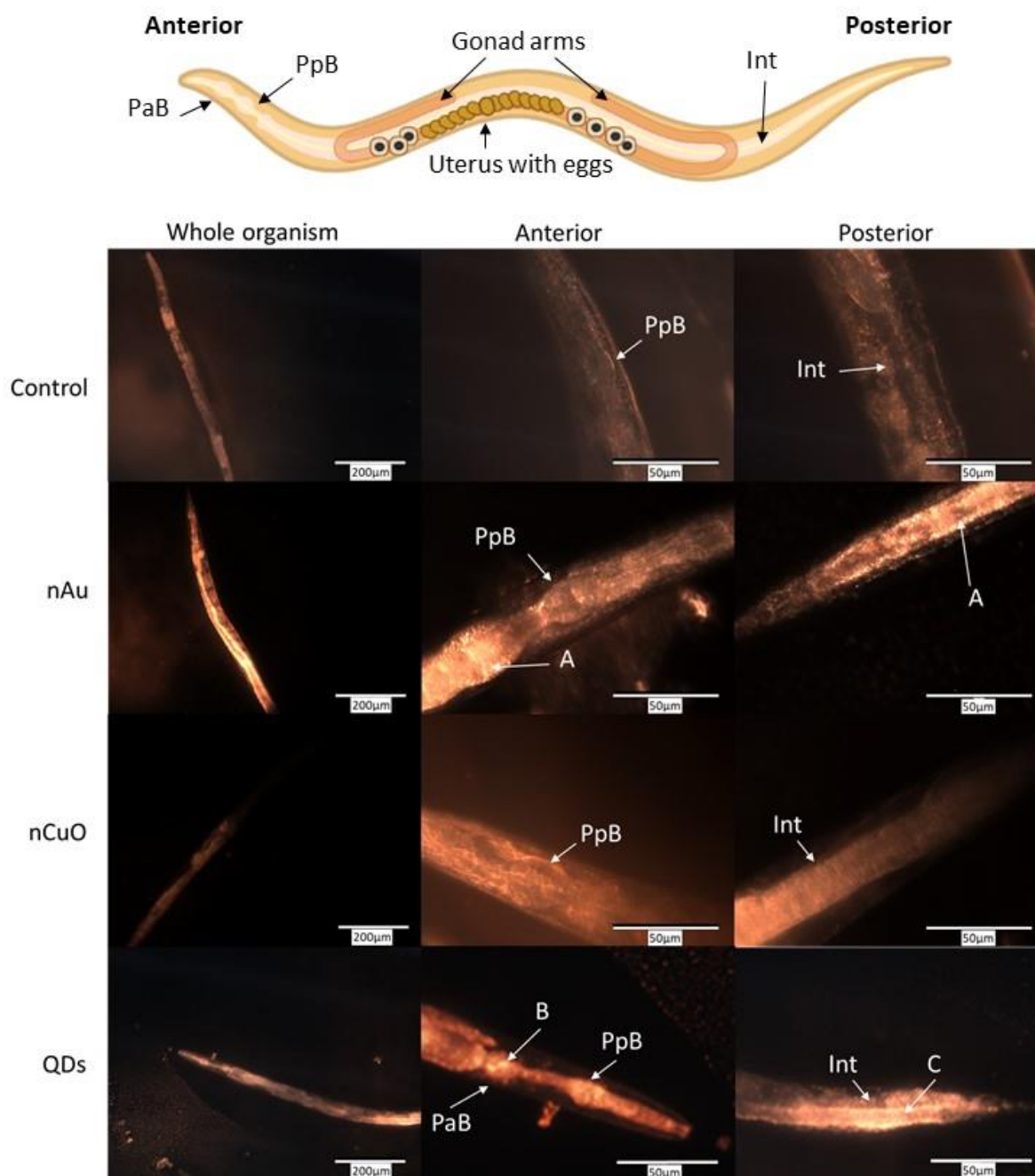


Figure 3-2 : CytoViva® dark-field imaging of *Caenorhabditis elegans* in an unexposed control, nAu, nCuO and QDs at their EC_{50} concentrations. PaB -Pharynx anterior bulb, PpB – Pharynx posterior bulb, Int – Intestine, A – agglomerates in nAu exposed nematodes, and B, C – agglomerates in QD exposed nematodes.

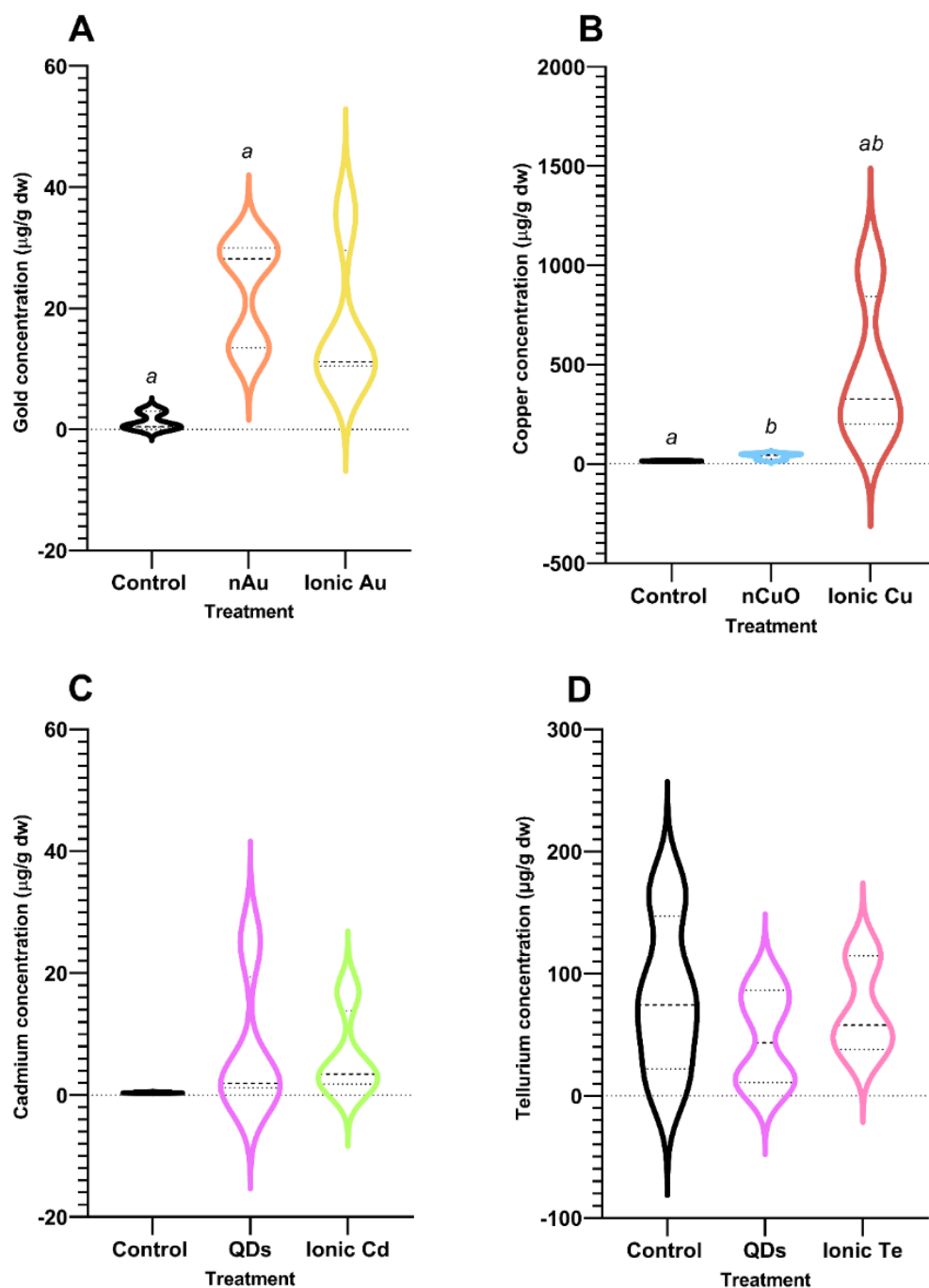


Figure 3-3 : Total metal uptake in *Caenorhabditis elegans* ($n = 3$) after 24 h exposure to EC_{50} concentrations of nanomaterials and ionic metals A) Au, B) Cu, C) Cd and D) Te. Treatments that share a letter are statistically significantly different ($p \leq 0.05$, ANOVA). Dashed lines indicate the median and interquartile range. Note the difference in scale of B and D.

3.3.3 Metabolomics

Multivariate analyses were conducted to distinguish the metabolome profiles of the control groups, different NMs and their ionic metal equivalents (Figure 3-4). For all three NMs, there were distinct differences between the NM and their ionic equivalents. For greater clarity, the controls were removed from the analyses to illustrate these differences (Figure 3-4 D-F). The validity of the metabolic profiling was demonstrated in that the three different controls were similar (plots not shown). The differences between the treatments and their controls were analysed using a pairwise *t*-test. The results of the comparison of the five most significant metabolites for each treatment to their control are presented in Table 3-5.

Three of the five significant metabolites in the ionic Au group were down-regulated. In the ionic Cu group, all significant metabolites were upregulated and in the Cd group, all significant metabolites were down-regulated. In the Te group, four of the five significant metabolites were down-regulated. Heptadecanoic acid was present and down-regulated in the ionic Au, Te and Cd groups. Eicosapentaenoic acid was present and down-regulated in the Te and Cd groups. When comparing the NMs to the control, nAu caused an upregulation in three of the five significant metabolites. Exposure to both QDs and nCuO resulted in down-regulation of the most important metabolites as shown. For the QDs exposure group, only three of the significant metabolites could be identified. Eicosapentaenoic acid and phenylacetic acid were the only metabolites that were common between different NMs and ionic metals, and this was for nCuO, Cd and Te. Only oleic acid was the common metabolite influenced by nCuO and QDs. When comparing the metabolites of the ionic metals to their specific nano equivalents, ionic Au caused a down-regulation of all five metabolites compared to nAu, ionic Cu caused an upregulation of all five metabolites compared to nCuO; and Cd and Te caused a down-regulation of four and three metabolites, respectively. Comparing Te to Cd, only one identifiable significant metabolite was up-regulated in the Te group.

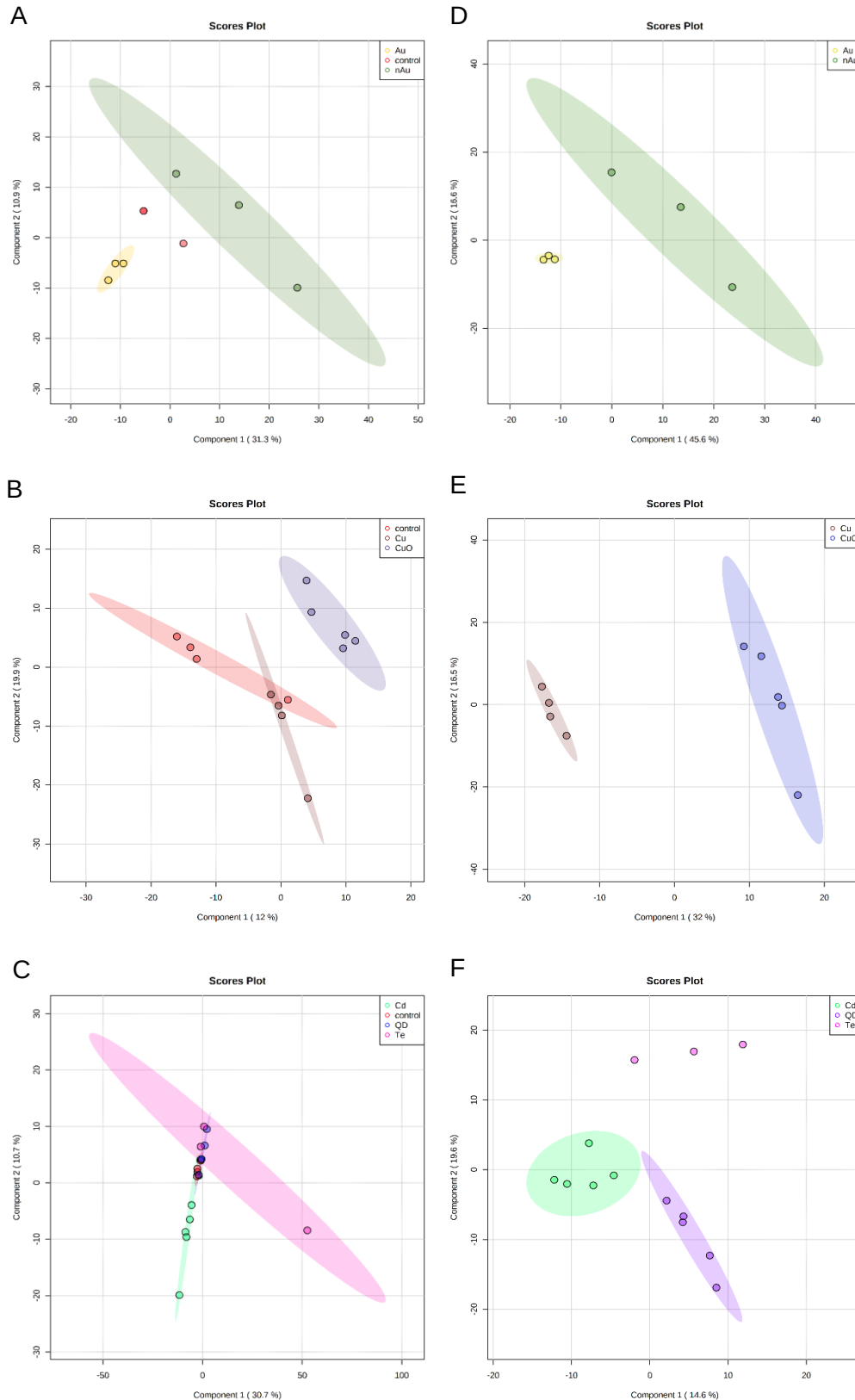


Figure 3-4 : Partial Least Square – Discriminant Analysis scores-plots of metabolic profiles ($n=5$) of *Caenorhabditis elegans* exposed to the three different nanomaterials and their ionic metal equivalents. with the highest intensities with (A-C) and without (D-F) the control.

Table 3-3 : Pairwise comparison of the five most significant ($p \leq 0.05$) metabolite concentrations in *Caenorhabditis elegans* following exposure to three different NMs and their ionic metal equivalents.

Group	Control	nAu	nCuO	QD	Cd
Control		Arabinitol ↑ 6,10,14-Trimethyl-2-pentadecanol ↑ Methyl α-D-ribofuranoside ↑ 7-Acetyl-6-ethyl-1,1,4,4-tetramethyltetralin ↓ 2-Propenoic acid ↓	Eicosapentaenoic acid ↓ Phenylacetic acid ↓ Linolenic-Acid ↓ Oleic Acid ↓ Pentadecanoic acid ↓	3-(hexadecyloxy)propane-1,2-diol ↓ Diacetone alcohol ↓ Oleic Acid ↓	Eicosapentaenoic acid ↓ Heptadecanoic acid ↓ Linolenic acid ↓ Phenylacetic acid ↓ Ethyl decyl ether ↓
Au	Heptadecanoic acid ↓ Tetracosane ↓ 6,10,14-Trimethyl-2-pentadecanol ↑ 2-Ketovaleic-Acid ↓ 2,3-bis((Trimethylsilyl)oxy)propyl (Z)-heptadec-10-enoate ↑	2,3-Butanediol ↓ Glyceric acid ↓ Butanoic acid ↓ Cyclobutane ↓ Hexanedioic acid ↓			
Cu	Methyl galactoside ↑ Acetin ↑ L-(+)-Threose ↑ 2-Hydroxyethyl palmitate ↑ D-(+)-Talofuranose ↑		2-Hydroxyethyl palmitate ↑ D-(+)-Talofuranose ↑ D-(-)- Erythrofuranose ↑ D-Xylopyranose ↑ Methyl galactoside ↑		
Cd				9-Tetradecenoic-Acid ↓ N-Acetyl-D-glucosamine ↓ Gluconolactone ↓ Glucoheptonolactone ↑ 9-Octadecenoic acid ↓	
Te	Eicosapentaenoic acid ↓ Heptadecanoic acid ↓ Levoglucosan ↑ Tetracosanol ↓ Glucoheptonolactone ↓			Glucoheptonolactone ↑ N-Acetyl-D-glucosamine ↓ 9-Tetradecenoic-Acid ↓ à-L-Galactopyranoside ↑ acetic acid ↓	à-L-Galactopyranoside ↑

i Arrows indicate upregulation or downregulation of metabolites when comparing the groups in rows to the groups in columns. Unidentified metabolites were excluded from the table. Grey sections indicate comparisons were not run.

Pathway analysis

Pathway results originate from pairwise comparisons, showing the differences between a treatment group and the control. Formal pathway analysis with Metaboanalyst was only performed between the control group and Cu, control and Te, control and nAu, and control and Au (Figure 3-5) since these were the four significant responses. Depending on the number and role of the detected metabolites in the respective pathways, as well as pathway importance, “Impact factors” were calculated (values ranging between 0 and 1, 1 being the highest). The rest of the groups did not have enough significant metabolites for pathway analysis, however their significant metabolites and their corresponding affected pathways are reported in Table 3-3.

Ionic Au had the most significant impact on the galactose metabolism and the fatty acid biosynthesis pathway and had the most significant difference in metabolite concentrations compared to the control (Figure 3-5 A). Nanogold caused a significant impact on the glycine, serine and threonine metabolism, with the aminoacyl-tRNA biosynthesis pathway the most significant influence by metabolite concentrations (Figure 3-5 B). The predominant pathway affected by ionic Cu was the nicotinate and nicotinamide metabolism (Figure 3-5 C), and after Te exposure, the post predominantly affected pathways were the glycerolipid metabolism and the nicotinate and nicotinamide metabolism in terms of pathway impact as well as metabolite concentrations (Figure 3-5 D).

Commonly affected pathways of nCuO, QD and Cd treatments included the biosynthesis of unsaturated fatty acids, the fatty acid metabolism and lipid metabolism (Table 3-3). The longevity regulating pathway was also affected in both nCuO and QD treatments. The phenylalanine metabolism and xenobiotic biodegradation pathways were only affected in the nCuO group and Cd group through the down-regulation of phenylacetic acid.

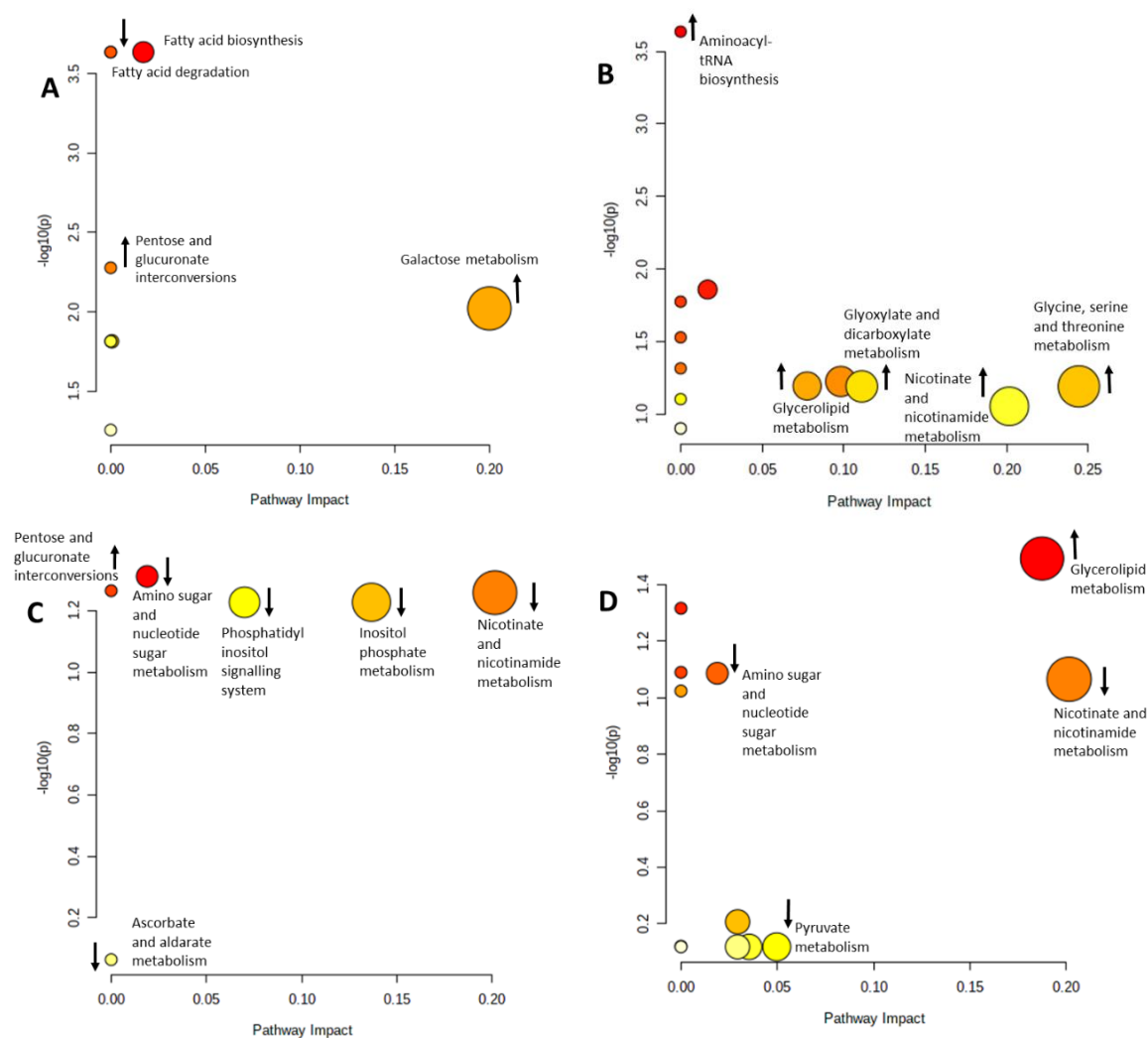


Figure 3-5 : The most significant and greatest impacted metabolic pathways calculated by Metaboanalyst between the control group and ionic Au (A), nAu (B), ionic Cu (C) and ionic Te (D). The identified pathways are ranked after their importance and the role of the detected metabolite within the pathway to calculate the impact factor (x-axis). Differences in metabolite concentration between the treatment and the control are represented on the y-axis. Relation of significance and impact factor determine size of pathway circle.

Table 3-4 : The most significant metabolites and corresponding pathways calculated between the control group nCuO, QDs and Cd.

Group	Significant Metabolites	P-values	Pathway
nCuO	Eicosapentaenoic acid ↓	3.27E-05	Biosynthesis of unsaturated fatty acids
	Phenylacetic acid ↓	0.00022	Phenylalanine metabolism, xenobiotics biodegradation
	Linolenic acid ↓	0.00125	Lipid metabolism
	Oleic acid ↓	0.00645	Fatty acid metabolism, longevity regulating pathway
	Pentadecanoic acid ↓	0.00583	Fatty acid metabolism
QD	3(hexadecyloxy)propane-1,2-diol ↓	0.01204	-
	Diacetone alcohol ↓	0.03419	Secondary metabolite
	Oleic acid ↓	0.04219	Fatty acid metabolism, longevity regulating pathway
Cd	Eicosapentaenoic acid ↓	4.99E-05	Biosynthesis of unsaturated fatty acids
	Heptadecanoic acid ↓	8.50E-05	Fatty acid metabolism
	Linolenic acid ↓	0.00017	Lipid metabolism
	Phenylacetic acid ↓	0.00022	Phenylalanine metabolism, xenobiotics biodegradation
	Ethyl decyl ether ↓	0.00037	-

- No metabolic information found.

3.4 Discussion

This chapter shows the difference in uptake and effects (metabolic responses and reproduction) of *C. elegans* following exposure to nAu, nCuO, QDs and their ionic metal equivalents. The 96 h reproduction test and subsequent EC₅₀ results indicate that ionic metals are more toxic than their nano equivalents, and uptake and exposure concentrations together with dissolution results from Chapter 2 indicate that NM toxicity is not necessarily linked to free metal ions. Of the three NMs tested QDs were the most toxic, most likely due to their size and ZP (see Chapter 2 section 2.3.2 for the detailed physicochemical characterization of the three NMs).

Uptake and bioaccumulation

The reproduction EC₅₀ was used as exposure concentration to determine the biodistribution of NMs and uptake for each material. CytoViva® confirmed particle agglomerate uptake in nAu and QD treatments and revealed the localization of assumed particles in the pharynx, intestine and uterus. The limitation here is that EDx could not be applied thus, the presence of metal or elemental composition could not be confirmed. The presence of particles in the pharynx and intestine is to be expected as the main uptake mechanism is through the mouth of the nematode (Gonzalez-Moragas et al., 2015). Studies by Meyer et al. (2010), Kim et al. (2013) and Qu et al.

(2011) found that NMs such as nAu, silver NMs and CdTe QDs can translocate and influence the reproductive system of *C. elegans*. (Scharf et al., 2013). Particles present in the reproductive organs such as the uterus may directly impact reproduction. According to Fakhruddin et al. (2021) noble metal NMs such as nAu result in stronger images due to enhanced light absorption and scattering by surfaces conducting electrons. Nano CuO could not be observed via CytoViva® and should thus be fluorescently labeled for future studies. This is shown in Figure S3-1. Fluorescently labelled silica-NMs were also observed to translocate to the vulva and spermatheca in *C. elegans*. The QDs have unique electronic and fluorescent characteristics, such as narrow emission spectra, high photochemical stability, and continuous absorption spectra (Zia-ur-Rehman et al., 2018; Jackson et al., 2012), which also make them easier to visualize using CytoViva®. Qu et al. (2011) demonstrated that after a 12 h exposure period, CdTe QDs were mostly excreted and only some were present in the reproductive system. Longer-term exposures (3 d) resulted in QDs being transferred from the intestine to the reproductive system. The findings of the present study support these observations. Uptake of NMs into the body was likely a result of the grinding of food in the pharynx which resulted in the large particle agglomerates breaking up and translocating to the uterus as seen by the Cytoviva images.

In the present study, uptake exposures were carried out without a food source to eliminate the effect it would have on sample sizes, as the *E. coli* would add non-targeted mass to each sample. Food would also cause nematodes to grow and reproduce changing the life stage composition of the samples, which would not be desired for comparability. This means that worms were starved for the 24 h period of exposure, with the control being treated in the same manner. Starved worms will continue pumping water into the pharynx looking for food and this could increase the uptake of materials compared to non-starved nematodes like those found in the normal reproduction exposure that contained a food source (Gonzalez-Moragas et al., 2015).

The toxicity of nanoparticles is highly dependent on the uptake and retention of the particles by the organism (Rossbach et al., 2020). Uptake was similar in the nAu treatment and the ionic Au treatment groups (23 µg/g and 17 µg/g), even though the ionic Au exposure concentration (0.06 mg/L) was 1000 times lower than the nAu exposure concentration (50 mg/L Au) as seen in Chapter 2. Kleiven et al. (2018) showed high intake but low retention of silver NMs in *C. elegans* after depuration, indicating low cellular uptake of NMs. Due to the low dissolution of Au from the NMs in the media and CytoViva® images we assume the uptake seen in the present study is in the form of particles and not free metal ions. This means that the nematodes internalised much less Au from nAu compared to ions in the ionic exposure groups.

In the case of nCuO and ionic Cu, it can be seen that the exposure concentration of ionic Cu is 100 times lower (0.23 mg/L) than the nCuO exposure concentration (32 mg/L), but uptake was

10 times higher in the ionic Cu treatment (457 µg/g) than nCuO treatment (38 µg/g). This could be due to dissolved ion uptake in the ionic Cu group. The main site for dietary copper absorption is the intestinal epithelium (Yuan et al., 2018). Copper is exported from the cells in the intestine and other organs by a Cu exporter CUA-1 (Chun et al., 2016). Copper is exported from the cells in the intestine and other organs by a Cu exporter CUA-1 (Chun et al., 2016).

The non-significant uptake of Cu in the nCuO exposure group is most likely due to a more negative ZP and hydrodynamic size reducing the uptake potential of nCuO (see Chapter 2 of this thesis for an explanation of the influence of these physicochemical characteristics on the bioavailability of NMs). This means that nCuO is not taken up by the nematode or something is preventing its uptake. The nematodes have been reported to cease the pharyngeal pumping to reduce the uptake of toxicants (Jones and Candido, 1999). Moreover, a synchrotron X-ray study indicated that water-borne copper was not taken up by nematodes, and the external surface (cuticle) was the primary pathway of water-borne copper (Jackson et al., 2005). Even though uptake after 24 h and biodistribution of nCuO could not be confirmed, some reproduction effects were seen at the selected exposure concentration. Reproductive effects were seen at concentrations 10 times lower than in the ionic Cu treatment. A study by Heinlaan et al. (2011) showed no translocation of nCuO from the intestine of *Daphnia magna* but sub-lethal effects indicated that there was severe impairment of digestive enzymes due to extensive bacterial colonization in the gut. It is also known that nCuO can cause the formation of reactive oxygen species (ROS) and damage cellular membranes of bacteria which could cause gut microbial diversity to change and affect the nematode (Ivask et al., 2014). The main site for dietary copper absorption is the intestinal epithelium (Yuan et al., 2018). A study by Eom et al. (2019) confirmed silica NM uptake in *C. elegans* via clathrin-mediated endocytosis in the gut resulting in reproductive toxicity. This suggests this is a possible route of exposure for other NMs such as nCuO.

The selected exposure concentrations for the QDs, Cd and Te were very varied, but no significant uptake was seen. This may be related to the shorter exposure period since the EC₅₀ was derived following 96 h exposures, while the uptake exposures were only conducted for 24 h due to the absence of food in the assay. Experiments conducted by Ellegaard-Jensen et al. (2012) with silver NMs found that their LC₅₀ values were more than three times higher in exposures without food. This is due to NMs adhering to the bacteria surface leading to enhanced oral uptake when the food source is present. Thus, in the present study uptake of NMs will be lower in the uptake exposure groups than in the reproduction assay groups due to the absence of food. Studies found that QDs can be internalized into the lysosomes of cells in *C. elegans* (Qu et al., 2011).

Gold (nAu and ionic Au) toxicity

The reproduction inhibition results show a dose-dependent relationship when exposed to ionic Au and nAu. As discussed in Chapter 2, nAu particle sizes decrease as concentration increases and particles smaller than 200 nm at the higher exposure concentrations would be taken up easier by the nematodes when pumping for food. It was evident that the ionic metal was more toxic than the NMs and nAu was the least toxic of the three all materials tested in this study ($EC_{50} = 230$ mg/L). Other studies confirmed greater reproductive toxicity of ionic materials compared to NMs (Kleiven et al., 2018; Scharf et al., 2013; Rossbach et al., 2020). In the present study, there was no dissolution of Au from nAu and the ZP in the nAu group did not change between concentrations, thus it is assumed that all uptake and toxicity in the nAu exposure is particle related. This is confirmed by Gonzalez-Moragas et al. (2017), Pedrosa et al. (2015) and Hu et al. (2018). These studies found that nAu particles had negligible dissolution and that smaller particles caused higher toxicity regardless of the metal concentration found in the organisms.

At the cellular level, no similarity in significant pathways for nAu and ionic Au groups was seen. This suggests different modes of toxicity of ionic Au and nAu. In the nAu treatment the glycine, serine and threonine metabolism was affected with higher metabolite concentrations than the control, with the aminoacyl-tRNA biosynthesis pathway being downregulated. These two pathways are linked to one another. The function of aminoacyl-tRNA synthesis is to precisely match amino acids with tRNAs containing the corresponding anticodon, which means if this pathway is affected it can cause genetic mutations (Ibba and Soll, 2000). An increase in glycine in the glycine metabolism was observed in the present study and this can probably be linked to the mechanisms to counteract an increase in intracellular oxidative stress (Farré and Jha, 2020). This could therefore result in a decrease in reproduction as seen in the present study due to energy being used to counteract oxidative stress, as well as cellular and genetic toxicity.

The ionic Au treatment resulted in significant downregulation effects in the galactose metabolism, pentose and glucuronate interconversion pathway and upregulation in the fatty acid metabolism. Galactose metabolism is responsible for converting galactose into glucose derivatives to make it accessible for glycolytic catabolism and energy production (Watts and Ristow, 2017). Studies by Li et al. (2015a) and Li et al. (2015b) found that silver NMs induced nano-specific metabolic responses in *Daphnia magna* and earthworms compared to ionic silver treatments. Overlapping metabolites included downregulated heptadecanoic acid between ionic Au, Cd, Te and the control. While previous knowledge about the physiological roles of heptadecenoic (a saturated fatty acid) is very limited, it has been recognized as a minor constituent of ruminant fats and used as a potential marker of microbial biomass (Li et al., 2018). The FATH-1 gene in *C. elegans* is essential for maintaining normal development and survival and works through heptadecenoic acid to carry out its functions (Li et al., 2018). Thus, the ionic metals (Au, Cd and Te) all seem to have

this mode of toxicity. There are only a limited number of studies exploring the metabolic effects of nAu and most of them are *in vitro* studies (Farré and Jha, 2020). Studies show that nAu influences the amino acid metabolism and carbohydrate metabolism *in vitro*, not seen in the present study (Lindeque et al., 2018; Gioria et al., 2016). This indicates that whole organism exposure can have different effects compared to cells due to varying modes of uptake and exposure scenarios. This highlights the novelty of the present study to look at environmental impacts and using metabolomics as early indicators of NM toxicity.

Copper (nCuO and ionic Cu) toxicity

No significant size distribution patterns were observed in the nCuO treatment group alluding to the fact that the bimodal reproduction responses are likely due to variations in the ZP and size observed over all the concentrations. The influence of possible dissolved metal ion uptake is negligible due to the low dissolution (discussed in Chapter 2). This corresponds to results by Wei et al. (2020) who found less than 1% Cu ion release from nCuO and no significant effect at that concentration of ionic Cu. However, low metal concentrations released from NMs at the cell surface could cause a high local concentration in the glycocalyx. The ionic Cu showed reproduction stimulation at the lowest concentration (50 µg/L), where after there is a concentration-dependent decrease in reproduction. Reproduction stimulation is ascribed to a hermetic response after adapting to low levels of stress. This response has been reported for multiple materials and organisms (Iavicoli et al., 2018). Copper is an essential metal needed for biological functions, however, an overload of essential metals can be detrimental to organisms. Thus, no significant effect on reproduction at low ionic Cu concentrations are to be expected as seen in the present study, due to this essential metal being used for biological processes up to an exposure concentration where biological overload occurs (between 200 and 500 mg/L).

At the biochemical level, there was some overlap in metabolites between the ionic Cu and nCuO exposures. Exposure to nCuO resulted in downregulation of eicosapentaenoic acid, phenylacetic acid and oleic acid. Eicosapentaenoic acid is a polyunsaturated fatty acid forming part of the biosynthesis of unsaturated fatty acids and is an important component of endocannabinoids, which are ethanolamide derivatives of phospholipids that bind to endocannabinoid receptors to regulate neural function and appetite (Kulas et al., 2008; Watts, 2016). Phenylacetic acid is an important metabolite for several pathways such as the phenylalanine metabolism and styrene degradation, and undergoes degradation during a subsequent catabolic route, which leads to the formation of the central metabolites acetyl-CoA and succinyl-CoA. It also leads to the citrate cycle, which is the metabolic hub for energy production (Oelschlägel et al., 2018; Ratnasekhar et al., 2015). This suggests that nCuO acts through pathways that influence feeding and neural signalling, as well as indirect effects to energy metabolism through CoA deficiency. Oleic acid

is a polyunsaturated fatty acid that is linked to longevity in *C. elegans* (Ackerman and Gems, 2012).

Significant metabolites associated with ionic Cu treatment are by-products or formation products of the nicotinate and nicotinamide metabolism and inositol phosphate metabolism. The nicotinate and nicotinamide metabolism generates the coenzyme nicotinamide-adenine dinucleotide (NAD⁺) which plays an essential role in many fundamental biological processes, such as metabolism, cell signaling, gene expression, and DNA repair (Lee et al., 2020). The inositol phosphate metabolism influences longevity in nematodes. The impairment of this pathway by ionic Cu influences reproductive effects measured by reducing the longevity of the nematodes. These do not correspond with the nCuO treatment pathways such as the fatty acid metabolism, lipid metabolism and phenylalanine metabolism, suggesting different modes of toxicity between ionic Cu and nCuO. Results by Ratnasekhar et al. (2015) also found significant alterations of fatty acids in *C. elegans* after exposure to TiO₂ NMs. Low concentrations of these NMs can cause neural pathologies and growth defects, which would link to reproduction inhibition through sexually undeveloped larval stages not reproducing in the time frame of this study. Fatty acids in general are precursor molecules for all lipid classes, including storage lipids, membrane lipids, and signalling lipids (Choi et al., 2021). Fatty acid metabolism as affected by nCuO plays a crucial role in regulating the lifespan of worms with reduced reproduction. Under nutrient-poor and oxidative stress conditions, fatty acids intermediate the balance of lipid stores between the soma and germline, suggesting a key role of fatty acids in harmonizing reproduction and somatic aging (Choi et al., 2021). Fatty acids were most significantly altered and downregulated in the nCuO treatment group. Farré and Jha (2020) suggested the use of phenylalanine or valine as biomarkers for cellular stress by NMs. A study testing the metabolic responses of *Enchytraeus crypticus* after exposure to nCuO and ionic Cu also found early responses in the amino acid metabolism after nCuO exposure and lipid metabolism responses in the ionic Cu treatment (Farré and Jha, 2020).

Cadmium and Tellurium (QDs and ionic Cd and Te) toxicity

The reproduction inhibition results show a dose-dependent relationship when exposed to QDs and ionic Cd. In the case of QDs, the size distribution ranged between 519 nm and 895 nm (see Chapter 2) and particles around 500 nm are taken up easier by nematodes than particles at 200 nm in size due to a combination of the size of their mouthparts and the pharyngeal pumping motions (Suzuki et al., 2019; De et al., 2018). As the cuticle of the nematode is negative, a more positive ZP such as that of QDs compared to the other NMs may result in particles being taken up more readily resulting in toxicity (Minullina et al., 2011). Low dissolution of Cd and Te from the QDs was observed. The concentrations (5 and 70 ng/L for Cd and Te respectively) are much

lower than the concentrations used in the ionic exposures, where toxicity was observed. Thus, toxicity due to free ions in the QD exposures is unlikely. Ionic Cd also showed a pattern of hormesis at the lower concentrations, as seen in the ionic Cu treatment group. This represents an overcompensation for mild environmental stress (Kouda and Iki, 2010).

When looking at EC₅₀ results for each material it is evident that the QDs were more toxic than both ionic Cd and ionic Te. Of the three NMs tested QDs were the most toxic (EC₅₀ = 0.001 mg/L). This is in agreement with results from Wei et al. (2020). It is well described that NMs with smaller primary particle sizes have a greater potential to induce higher oxidative stress due to their extensive surface areas, such as the QDs in this study (Hu et al., 2018; Wu et al., 2012). Even though it is widely stated that QD toxicity is due to the core metals, results from the present study would indicate that toxicity is rather related to the particle rather than the metals or due to combined exposure to both metals in the composite. In addition, uptake of QDs was also confirmed by CytoViva® even though there was no significant metal uptake. As the detection limits were 0.17 and 9.86 µg/g dw for Cd and Te respectively, total metal uptake must have been less than this. Studies by Zhao et al. (2015) and Hsu et al., (2012) also found nano-specific toxicity of QDs in *C. elegans*. It was also found that QDs can cause neurotoxicity in *C. elegans* by means of oxidative stress and DNA damage, which could result in reproductive toxicity observed in the present study (Sinis et al., 2019). This involves deposition at the site of neurons, perturbation of neural development and abnormal neurotransmission. In the present study QDs were localized in the pharynx and intestine of the nematode, but further translocation needs to be investigated. The toxicity mechanisms of QDs are extremely complicated due to the nano-size effects being greater than larger particles and composite heavy metal components (Hu et al., 2017). Therefore, the toxicity differences observed in the present study may be explained by a combination of the size difference, surface difference, combined metal toxicity and altered uptake rate due to agglomeration difference.

At the cellular level, QDs induced some similar toxicity to nCuO as mentioned above, which involves oleic acid which is linked to longevity and fatty acids affecting reproduction through oxidative stress-induced lipid store depletion. Diacetone alcohol was downregulated in the QD treatment only. This is a secondary metabolite that is linked to signalling pathways. Both ionic Cd and ionic Te downregulated eicosapentaenoic acid and heptadecanoic acid, thus acting via similar modes of toxicity not related to the other ionic metals. These metabolites are linked to the biosynthesis of unsaturated fatty acids and the fatty acid metabolism. Fatty acid profiles differ according to life cycle or metabolic activity and are altered by stress conditions such as toxicity, solvent exposure, or hunger (Ruess et al., 2002). According to pathway analysis, the nicotinate and nicotinamide metabolism and the glycerolipid metabolism were the most significantly

impacted pathways by ionic Te. Nicotinate and nicotinamide are essential for organisms as the precursors for the generation of coenzymes, NAD⁺ and NADP⁺, which are essential for redox reactions and carry electrons from one reaction to another (National Center for Biotechnology Information, 2022). Ionic Te also impacted more pathways than both QDs and ionic Cd. This means that ionic Te causes unique and more severe toxicity than ionic Cd, as confirmed by the EC₅₀ values. This has not been shown in other studies. It suggests that should QDs enter an organism and *in vivo* dissolution of metals occur, the possible toxicity of ionic Te should not be neglected but should further be investigated. Other significant metabolites were present in the QD group that were not present in the Cd and Te groups such as diacetone-alcohol which is a secondary metabolite responsible for signalling.

Finally, when considering both QDs and nCuO, fatty acid oleic acid seems to be a good early biomarker for metabolic responses in *C. elegans* following NM exposure. It should be noted that this metabolite was not affected in the nAu treatment thus, there is most likely not a one size fits all biomarker for NM exposure. Heptadecanoic acid, eicosapentaenoic acid and metabolites associated with the nicotinate metabolism seem to be good choices for biomarkers of exposure to the ionic metals in this study. Overall, there seem to be different metabolic pathway perturbation for all three NMs and no significant overlap between NMs and their ionic metal equivalents indicating nano-specific effects. In the present study, the phenylalanine pathway was affected but only in the nCuO and Cd treatments indicating it might not be a one size fits all biomarker. It is important to note that metabolomic exposures were only 24 h thus, no knock-on pathway effects were observed but, the results here highlight the capability of metabolomics to reveal an early metabolic response to stress induced by NMs.

3.5 Conclusions

Particle uptake and exposure were confirmed by CytoViva® in both nAu and QDs treatments. The exposure concentrations for ionic Cd and Te in the uptake study were higher than the concentrations of these metals in the exposure concentration of QDs, but uptake was not significant from one another. This suggests that reproductive effects in these materials were not metal-related. From the results in this chapter ionic Cu, nCuO and nAu showed the lowest reproductive toxicity (high EC₅₀ values) and the only significant metal uptake, indicating that metal uptake and related effects can only be seen at high exposure concentrations (higher than 0.1 mg/L). Exposure concentrations based on reproduction EC₅₀ values indicated that QDs and ionic Te showed the same range in toxicity which is a novel finding. Reproductive toxicity probably occurred through indirect pathway effects such as genetic mutations, oxidative stress and lower energy availability.

To further assess possible modes of toxicity GC-MS based untargeted metabolomics was utilized to determine potential biomarkers for *C. elegans* response to NMs. Multivariate component analysis (PLS-DA) revealed perturbations in the metabolome of *C. elegans* after exposure to the EC₅₀-based exposure concentrations. Minimal cluster overlap suggests that metabolome perturbations are nano-specific and not linked to ion toxicity. Pathways affected by nAu treatment indicate modes of toxicity linked to genetic mutations (aminoacyl-tRNA biosynthesis) and oxidative stress (glycine metabolism). From the results QDs, ionic Te and nAu were found to be the most toxic materials. Interestingly these three materials showed no significant metal uptake compared to the control, thus indicating possible regulation of these metals *in vivo*. Some overlapping metabolites such as oleic acid between QDs and nCuO suggest the possibility of use as biomarkers for NM exposure, but further studies are needed to confirm this. The high number of non-overlapping metabolites suggests that there might not be a one size fits all metabolic biomarker for NM vs ionic metal exposure, but results do indicate that NMs and metals act through different effect pathways.

CHAPTER 4 USING *DANIO RERIO* EARLY LIFE STAGES AS A VERTEBRATE MODEL FOR NANOMATERIAL EXPOSURE

4.1 Introduction

Fish are the most phylogenetically diverse group of vertebrates and have been valuable models for the understanding of fundamental processes in vertebrate evolution, development and disease (Vicario-Parés et al., 2014). The zebrafish (*D. rerio*) is one of the most widely used models among fish species. Studies using zebrafish have increased exponentially due to the multiple advantages it offers as a toxicological model such as easy housing and care, fast development, development outside of the body, genetic similarity to humans, lots of offspring are produced and they share many major organs with humans (Vicario-Parés et al., 2014; Su et al., 2021; Johann et al., 2021; Arteaga et al., 2021). The zebrafish embryo is a useful research model because it is small in size and transparent, easy to maintain in laboratory conditions, has a rapid embryogenesis timeline and continuous reproduction is possible in adult fish (Zhao et al., 2013). Zebrafish eggs (1.0–1.2 mm in diameter) have two membranes, the chorionic and vitelline membrane with a perivitelline space in between. The chorionic membrane has a thickness of 3.5 μm with numerous pore canals of 0.5–0.7 μm in diameter (Ganesan et al., 2016). Consequently, the size of the materials influences their ability to cross the chorion membrane before embryo hatching at 72 h post-fertilization (hpf). Embryos with a chorion are less prone to become affected by waterborne chemicals when compared with larvae and adults (Osaki et al., 2006). The chorion of the egg is also reported to be negatively charged attracting more positively charged particles to the surface (Böhme et al., 2017).

The fish embryo toxicity test (FET) is used to study malformations, developmental defects and mortality to calculate LC_{50} values for each material. To address the limitations of the FET test, investigations have led to sublethal endpoints that would increase its sensitivity and allow for the estimation of sublethal adverse effects and chronic toxicity (Rothe et al., 2021, Krzykwa et al., 2019). Respiration, embryo activity and heart rate are considered important sublethal endpoints and can be linked to metabolic responses (Haque and Ward, 2018). It has also been established that zebrafish embryos can survive in the absence of cardiac function by diffusing oxygen from the water (Mably and Childs, 2010), however cardiac function is essential to vascular flow and development and is worth investigating. This also means that at the larval stage metabolic activity, respiration and cardiac activity are not yet coupled (Pelster and Bagatto, 2010). Souders et al. (2018) reported that total respiration of zebrafish increased with age and mass of the embryo and that early embryogenesis corresponded to a higher rate of non-mitochondrial respiration and possible metabolic deficiencies.

The interaction between organisms and NMs is mediated by their respective surfaces. Surface charge and particle size are recognized as important parameters determining NMs interaction with the biological membranes, influencing cellular internalization, immune response and overall toxicity. These attributes are dynamic, especially in aquatic environments (Massarsky et al., 2013; Martinez et al., 2019). It is therefore not surprising that zebrafish are often utilised as model aquatic organism to elucidate sublethal effects of NMs in aquatic media. These sublethal endpoints have been used in some studies such as a study by Patibandla et al. (2018) that proved that Au nanorods decreased heart rate in zebrafish embryos and a study by Ahmad et al. (2015) saw an increase in heart rate after exposure to cobalt ferrite NMs. In contrast to these findings another study found no significant effect on heart rate and blood flow in zebrafish embryos after exposure to nAg (Kannan, 2011). Huang et al. (2013) has also used oxygen consumption in developing zebrafish embryos as a measure of metabolic change.

Chemical profiling of metabolic function in fish embryos is rapidly becoming an important method for assessing hazard (Souders et al., 2018). Metabolomics provides valuable information regarding the overall changes of metabolites and biochemical pathways that might be altered in response to NMs and particular metal-based NMs. However, only a limited number of studies have been carried out until now (Farré and Jha, 2020; Revel et al., 2017) and even less using zebrafish larvae metabolomics to determine NM toxicity. Studies by Ganesan et al. (2018) and Ganesan et al. (2021) studied the metabolic effects of TiO₂ NMs and carbon nanotubes respectively on zebrafish. Marana et al. (2022) also studied metabolic disruption in zebrafish exposed to plastic nanoparticles.

The present study has utilized the zebrafish embryo as a model for NM exposure to evaluate the developmental toxicity of nAu, QDs and nCuO, as well as their ionic metal equivalents. Uptake and biodistribution were also determined to confirm the uptake of materials. Heart rate, blood flow, activity and respiration were measured in developing embryos to assess sublethal toxicity. Furthermore, to better understand the stress process induced by these materials at the molecular level, metabolite profiles were determined. Therefore, this chapter aimed to confirm metal uptake and NM biodistribution *in vivo* and to determine the effects of the three metal-based NMs and ionic exposure on *D. rerio* larvae development, heart rate, blood flow, activity, respiration and metabolomics.

4.2 Materials and Methods

4.2.1 Fish embryo acute toxicity test

Fish husbandry and breeding protocol

Long-fin wild-type adult *D. rerio* breeding stocks were housed and bred in toxicant-free zebrafish system water (see Chapter 2) in the National Aquatic Bioassay facility at the North-West University (Potchefstroom, South Africa). Twenty-four hours prior to initiating a bioassay, adults were transferred to Tecniplast iSpawn spawning chambers (Tecniplast, Italy) in a ratio of two males to three females, males and females were divided by a mesh insert. On the morning of an exposure, the mesh separating the fish was removed, lights were ZSWitched on and the water level was dropped to 2 cm. This allowed for the fish to spawn and the eggs to drop to the chamber floor. After approximately 2 h, eggs were collected and placed in an incubator at 27°C for another 2 h. The *D. rerio* embryo acute toxicity test (OECD 236, 2013) was carried out using 4 hpf embryos according to methods by Cassar et al. (2019), Kumar et al. (2020) and Martinez et al. (2019). Viable fertilized eggs at similar cell stages were then sorted out using a stereomicroscope e (Zeiss, Germany), separated from unhealthy eggs and placed directly into E3 media (see Chapter 2 for E3 media).

Exposures

Test concentrations of each NM (ass mass of NM) and metal salts (as concentration metal) were prepared by adding the required amount of the stock solution to each well in a 6-well cell culture plate (Corning® Costar®, Sigma Alrich Merck, Darmstadt, Germany) and filling it up to 5 mL with E3 media. Negative controls were prepared consisting of only E3 media with no NMs added. Five viable 4 h old eggs were then transferred to each well so that each treatment consisted of 4 replicates of 5 eggs ($n = 20$). The six-well plates were sealed with self-adhesive, oxygen-permeable sealing film (BRAND®, Sigma Aldrich, Merck, Darmstadt, Germany) to prevent evaporation from taking place. Unless otherwise stated, data for the NMs are shown as a nominal mass concentration of NM per unit volume and not normalized to the concentration of metal in the particle. This is because the dissolution rates (see Chapter 2) of NMs were relatively low and therefore NMs were the dominant form of metal in water throughout NM exposures, which better reflects the study goal of understanding particle toxicity. The plates were then incubated at 28°C for 96 h in a 12 h light/12 h dark cycle in an incubator (IncoCool, Labotec., South Africa). Observations performed on each tested embryo included: coagulation of embryos, lack of somite formation, non-detachment of the tail, and lack of heartbeat (See Supplementary Information, Table S4-1). These observations are used as endpoints for the determination of lethality (LC_{50}):

Coagulation of the embryo: Coagulated embryos are milky white and appear dark under the microscope. The number of coagulated embryos was determined after 24, 48, 72 and 96 h.

Lack of somite formation: A normally developed embryo shows spontaneous movements. Spontaneous movements indicate the formation of somites. The absence of somites was

recorded after 24, 48, 72 and 96 h. Non-formation of somites after 24 h might be due to general retardation of development. Somites should be formed by 48 h and if not, the embryos are considered dead (see Supplementary Information, Figure S4-1).

Non-detachment of the tail: In a normally developing zebrafish embryo, detachment of the tail from the yolk is observed following posterior elongation of the embryonic body. The absence of tail detachment is recorded after 24, 48, 72 and 96 h.

Lack of heartbeat: In a normally developing zebrafish embryo at 26 ± 1 °C, the heartbeat is visible after 48 h.

Additionally, hatching was recorded in treatment and control groups daily starting from 48 hpf. All observations were recorded every 24 h, until the end of the test at 96 h, when larvae are humanely killed in iced water and frozen per each following method. The resultant LC₅₀ concentrations were used as the exposure concentrations for the biodistribution and uptake, behaviour, metabolic and physiological response assessment. Figure 4-1 illustrates the developmental stages of *D. rerio* at different time points and when different toxicity endpoints were measured. Activity and respiration were measured at 24 hpf, respiration was measured again at 48 hpf, heart rate and blood flow were measured at 72 hpf and uptake were measured at 96 hpf at the end of the whole exposure period.

Animal ethics

All breeding, maintenance and experimental treatments were conducted as approved by the North-West University animal care ethics committee - AnimCare (NWU-00173-18-A5). These are all covered by AnimCare approved standard operating protocols; SOP_NABF_006.

Zebrafish Developmental Timeline

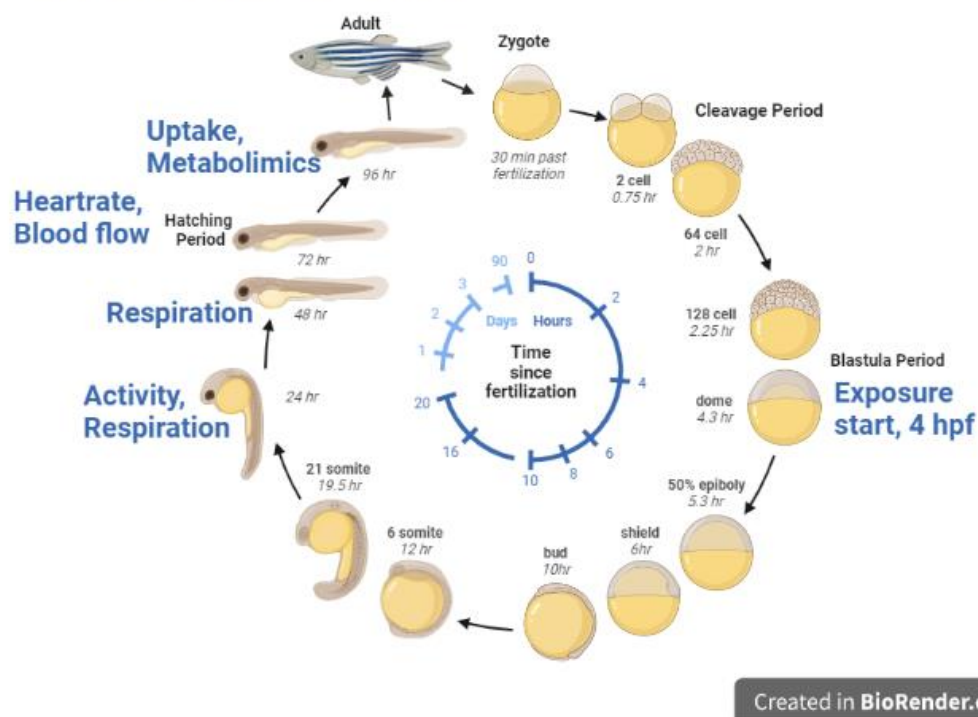


Figure 4-1 : Zebrafish developmental stages and toxicity endpoints measured, illustrated in blue.

4.2.2 Biodistribution and uptake

To assess biodistribution and uptake, additional exposures were conducted as described in section 4.2.1, where 96 h old larvae were exposed the LC_{50} of each NMs and humanely killed in ice water after 96 h exposure. The LC_{50} exposure concentrations were: 51.22 mg/L nAu, 0.85 mg/L ionic Au; 3.54 mg/L nCuO; 0.45 mg/L ionic Cu; 20.61 mg/L QDs; 20.9 mg/L ionic Cd and 22.4 mg/L ionic Te.

Biodistribution

To prepare samples for biodistribution determination, five larvae per treatment ($n=5$) were washed with ultrapure water and dispersed onto a microscope slides without staining them. Excess liquid was removed with filter paper. Cryopreserve gel (Tissue-Tek OCT Compound) was used to mount the larvae onto the slide, the slide was covered with a cover slip and stored at 4°C until same-day imaging using CytoViva® 150 Unit integrated onto an Olympus BX43 microscope at a magnification of 4X could be undertaken.

Uptake

Exposures with larger numbers of embryos were necessary to determine uptake after 96 h. For this step, exposures were conducted as described in section 4.2.1 but fertilized embryos were exposed in groups of 20 individuals (n=40) exposed in 30 mL of exposure media in beakers to the relevant LC₅₀ of each NM and ionic metal, individually. After 96 h larvae that had not hatched were manually dechorionated using a pair of forceps to eliminate possible NMs adhering to the chorion surface. In instances of mortality, the dead larvae were discarded and not used for uptake determination. All larvae were humanely killed in ice water and washed twice in ultrapure water. Ten larvae per replicate, with a minimum of 3 replicates per treatment were frozen in 1 mL Eppendorf microcentrifuge tubes.

For analysis of the total metal concentrations in exposed larvae, thawed samples were subjected to 24 h acid digestion in a heat block at 65°C to generate homogeneous solutions (Böhme et al. 2017). The acid digestion protocol was adjusted to the physicochemical properties of the individual NMs and ionic metals. Samples exposed to Cd/Te and Cu (NMs or ionic metals) were digested with 1 mL nitric acid (65%, suprapure, Merck), and those exposed to Au (NMs or ionic metals) were digested in 1 mL aqua regia (v/v HCl/HNO₃, 3/1) and diluted to 10 mL with 1% nitric acid. For all experiments, untreated organisms were used as negative controls (blank) and the blank concentrations (natural background) were subtracted from the concentrations measured in the exposed organisms. Concentrations were expressed as the total metal concentration/dry weight of the sample. Recovery rates and detection limits are reported in Table 4-1.

Table 4-1 : Recovery rates (%) and the detection limits of the metals of interest obtained for the certified reference material, as well as 96 h larvae tissue samples, respectively. Detection limits for the tissue samples were determined as three times the standard deviation of the blank measurements multiplied by the average weight of the samples.

Elements	Recovery Rates (%) Dorm-4	Detection limits larvae tissue (µg/g dw)
Au	n.a	1.73
Cu	102	4.8
Cd	99	0.03
Te	n.a	0.21

4.2.3 Embryo activity, cardiac output and respiration

The activity of unhatched embryos was measured after 24 h of exposure and heart rate and blood flow was measured in hatched larvae after 72 h of exposure. Both endpoints were evaluated by

recording 1 min video recordings (x15 frames per second) using a Zeiss Primo Star stereo microscope (Zeiss, Germany) for activity and heart rate, and a light microscope for blood flow. These microscopes were mounted with Zeiss AxioCam ERc 5s cameras and recordings were taken with Zeiss Zen 3.3 software using a tablet to ensure no disruptions from movement by the researcher. The recordings were then analysed using Noldus (Danioscope™, Netherlands) software. Recordings for bursts of movement were one minute long per individual embryo and each treatment had three embryos ($n = 3$). Heart rate and blood flow recordings were done for at least 10 seconds, but as long as possible before the larvae moved. Recordings were done in triplicate for each embryo in each treatment replicate. The activity was expressed as the frequency of bursts of movement, duration of bursts, and heart rate was expressed as beats per minute (bpm). Blood flow recordings were also analysed by using Noldus (Danioscope™, Netherlands) software. Recordings were taken at the same point on the tail of each larvae so that flow results are comparable between individuals. The camera was mounted perpendicular to the microscope sample and measurements are made by the software reading pixel changes in the selected area. Data are expressed as a percentage of the control as each treatment group had a corresponding control group.

Respiration of unhatched embryos was measured at 24 h and 48 h by removing embryos from exposure media, washing them twice with clean E3 media and randomly placing three embryos per replicate in each well of a 96-well Loligo® microplate system (Denmark) containing 80 μ L of clean aerated E3 media and sealed. Each concentration had at least 3 replicates ($n = 3$). Measurements were taken using PreSens software (Version 3.2, Denmark) every 5 seconds for at least 50 min, were after embryos were carefully removed from the wells and returned to the exposure media and incubated to continue with the test. Respiration was calculated by multiplying the slope of the data by 60 seconds and dividing it by the nr of embryos per well. Data is expressed as mg oxygen per embryo per minute.

4.2.4 Metabolomics

To assess metabolic changes in the zebrafish larvae after exposure, the same protocol was followed as described in section 4.2.2. However, the larvae were not placed in ice water but rather flash-frozen in microcentrifuge tubes using liquid nitrogen after weighing them and stored at -80°C until further analyses. Samples were prepared for analysis by freeze-drying and reweighing the samples. Extraction was done using a method by Beukes et al. (2019) as described in Chapter 3 Section 3.2.3. Metabolomic sample preparation and analysis was done by the Centre for Human Metabolomics at the NWU.

The commercial library NIST11 was used to identify the compounds. Identification could only occur once there was a spectral match of 80% similarity between the compound and library. The data were filtered, removing any compound that was not reliably measured (a coefficient of variation > 50% was used), as well as any elements that were not present in all samples of at least one experimental group. The data were corrected for sample weight, normalized by median and auto-scaled.

4.2.5 Statistical analysis

The dose-response function, namely, effect concentration with 50% of the population died (LC_{50} values) was calculated using nonlinear regression analysis (Cumulative Guassian distribution) in the Toxicity Relationship Analysis Program (TRAP). Both lethal and sublethal endpoints were used for this calculation. To test for differences between the control and different endpoints, data were first tested for normal distribution using Shapiro-Wilk's test and the Levene's test was used to test for variance in homogeneity. One-way Analysis of Variance followed by Tukey's post hoc test was performed to calculate the statistical differences ($p \leq 0.05$) between the different concentrations of the NM, as well as any significant differences between the control and the NM. Kruskal-Wallis test followed by Dunn's post hoc test was performed to calculate the statistical differences for all the data that were not normally distributed.

After metabolomic data pre-processing, uni- and multivariate statistics were performed with MetaboAnalyst. The most relevant variables were determined by Analysis of Variance (ANOVA), Principal Component Analysis (PCA), followed by a Fisher's last significant difference posthoc test (Fisher's LSD) and Partial Least Square – Discriminant Analysis (PLS-DA). Significant metabolites between two groups (control vs exposed) at a time were identified using t-tests and pathway analysis was performed using these significant metabolites, resulting in a list of most significant and greatly impacted pathways between two groups.

4.3 Results

4.3.1 Fish embryo acute toxicity test

Mortality

From Figure 4-2 it can be seen that mortality commonly occurs in the first 48 hpf. In the nAu treatment group 1 mg/L, 30 mg/L and 50 mg/L, resulted in the highest mortality (20% and 27%) and the 100 mg/L treatment resulted in the lowest mortality. In the ionic Au treatment group, only the 1 mg/L concentration resulted in significant mortality at 80%. In the nCuO treatment group, 10 mg/L caused the highest mortality of 55% followed by the 1 mg/L concentration group at 35%

mortality. The ionic Cu treatment resulted in the highest mortality at 500 µg/L and 100 µg/L (55% and 40%). The QDs treatment showed that the lowest concentration (0.5 mg/L) causing the highest mortality of 52%, in the ionic Te treatment 50 mg/L resulted in the highest mortality of 100% and in the ionic Cd group the highest mortality was seen in the 20 mg/L concentration at 48%. Thus, both nAu and QDs showed a bimodal response. See Table S4-2 to S4-8 for statistics.

Hatching success

In general, all groups except ionic Cd and ionic Te treatment embryos only started hatching after 48 hpf (Figure 4-3). In the nAu treatment (Figure 4-3 A) the 30 mg/L concentration had the lowest percentage of hatching success (50% after 96 hpf) and in the ionic Au treatment (Figure 4-3 B) the 1 mg/L concentration resulted in no hatching success. In the nCuO treatment (Figure 4-3 C) the 10 mg/L groups resulted in no hatching success, while the other concentrations resulted in delayed hatching success. In the ionic Cu treatment (Figure 4-3 D) concentrations of 50 µg/L and higher resulted in delayed hatching, with 200 and 500 µg/L resulting in 50% and 45% hatching success after 96 hpf. In the QDs group (Figure 4-3 E) there was no hatching success in the 2 mg/L group, which is the second lowest concentration, while the highest concentration (20 mg/L) had the highest hatching success. No hatching impairment was seen in the ionic Te treatment (Figure 4-3 F). Both ionic Te and ionic Cd showed an accelerated hatching success compared to other groups, but important to note is that hatching was also accelerated in this control group. No hatching impairment was seen in the ionic Cd treatment (Figure 4-3 G) but a delay was seen in the 10 and 20 mg/L groups. See Table S4-9 to S4-15 for statistics.

Figure 4-4 shows the percentage embryos that were considered to be dead based on the lethal criteria of mortality and lack of hatching success at each concentration after 96 h. The line of best fit was determined by the Cumulative Gaussian distribution and used to calculate the LC₅₀ values seen.

Malformations

In relation to the control group, only pericardial oedemas and spinal deformations were observed in all exposures except in the ionic Au group which saw several developmentally retarded larvae but no spinal deformations (Supplementary Information, Figure S4-1). Due to large variations in numbers, no significant developmental effects were seen in the nAu treatment (Figure 4-5 A), however, it can be observed that nAu caused a greater incidence of spinal deformations than oedemas. In the ionic Au treatment, 100 mg/L resulted in a significant (ANOVA, $p < 0.05$) percentage of oedemas compared to the control; and the only developmental retardations observed were in the mid-concentration ranges (10 and 50 mg/L) where no oedemas were

observed. In the nCuO treatment, a significant (ANOVA, $p < 0.05$) percentage of oedemas were observed in the 5 and 10 mg/L concentrations compared to the control and the lower concentrations and no significant spinal deformations. In the ionic Cu group, oedemas and spinal deformations were observed but not at significant percentages. The QDs treatment resulted in significant (ANOVA, $p < 0.05$) percentages of both oedemas and spinal deformations compared to the control; and oedema percentages seem to decline as the concentration increases. No significant malformations could be seen in both the ionic Cd and ionic Te groups and most groups excluding nCuO and QDs concentration did not induce malformations.

Both mortality and hatching success data were combined to calculate LC_{50} values for each material (Table 4-2). Unhatched embryos at 96 h were considered dead and added to the mortality count. The material that has the lowest LC_{50} and is, therefore, the most toxic is ionic Cu while the most toxic NM was nCuO. The toxicity profile from most to least toxic is ionic Cu > ionic Au > nCuO > QDs > ionic Cd > ionic Te > nAu. Many CL (confidence limits) could not be calculated due to the varying results and bimodal responses.

Table 4-2 : Summary of the median lethal concentrations (LC_{50}) based on 50% lethality (mortality and lack of hatching success) and the subsequent 95% confidence levels (CL) for Danio rerio embryos after exposure to nAu, ionic Au, nCuO, ionic Cu, QDs, ionic Cd and ionic Te.

Material	LC_{50} (mg/L)		
	Mean	Upper CL	Lower CL
nAu	51.22	n.d.	n.d.
Ionic Au ($H[AuCl_4]$)	0.85	1852.25	389.49
nCuO	3.54	n.d.	n.d.
Ionic Cu ($CuCl_2$)	0.45	n.d.	n.d.
QDs	20.61	n.d.	n.d.
Ionic Cd ($CdCl_2$)	20.9	n.d.	n.d.
IonicTe ($TeCl_4$)	22.4	n.d.	n.d.

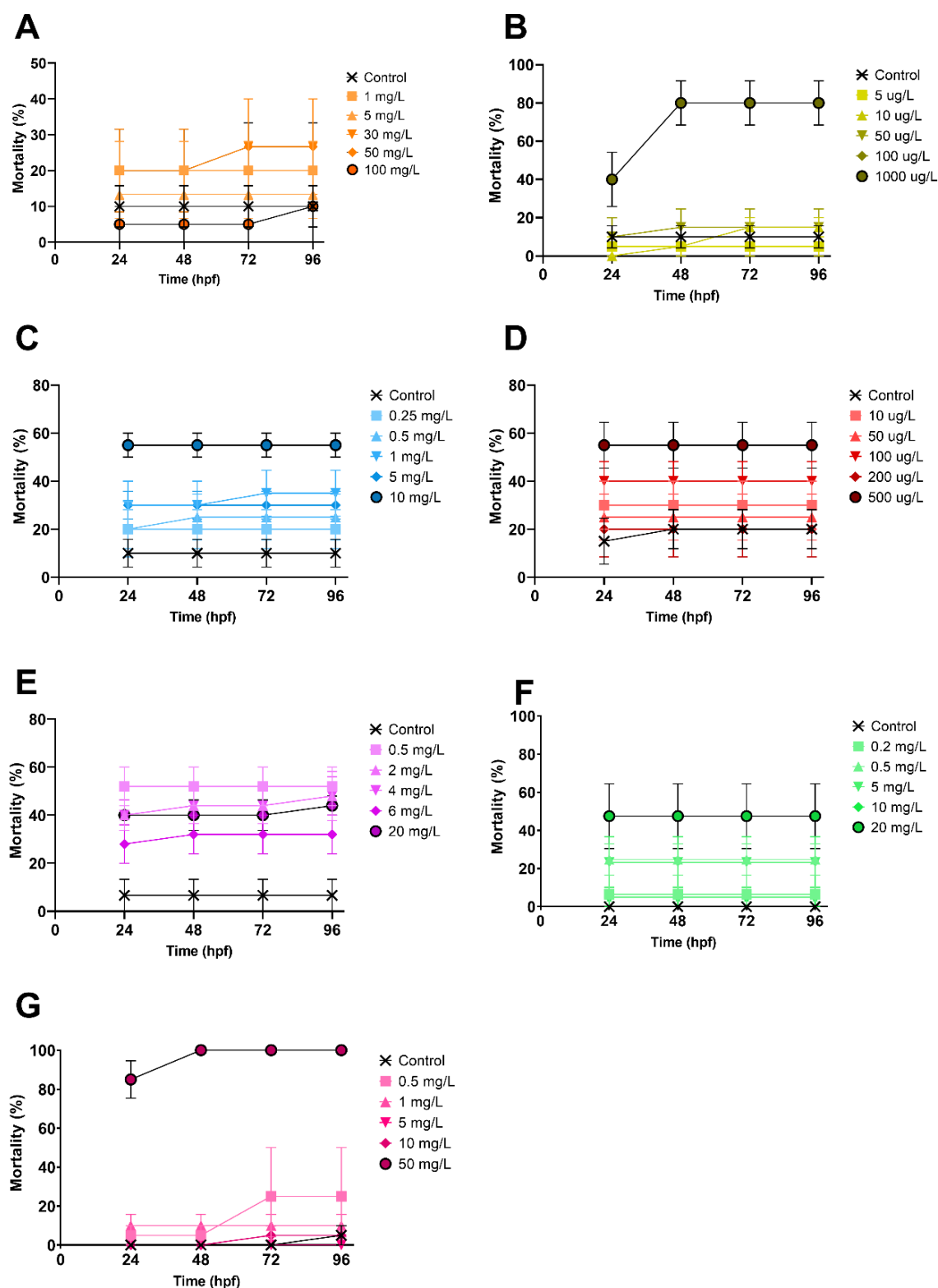


Figure 4-2 : Percentage mortality of *Danio rerio* embryos over time during 96 h exposures in nAu (A), ionic Au (B) as chloroauric acid, nCuO (C), ionic Cu (D) as CuCl_2 , QDs (E), ionic Cd (F) as CdCl_2 and ionic Te as TeCl_4 (G). Nanomaterial concentrations are expressed as mass NM and ionic treatments are expressed as metal concentration. Mortality (%) was calculated based on the number of coagulated individuals per replicate ($n = 4$).

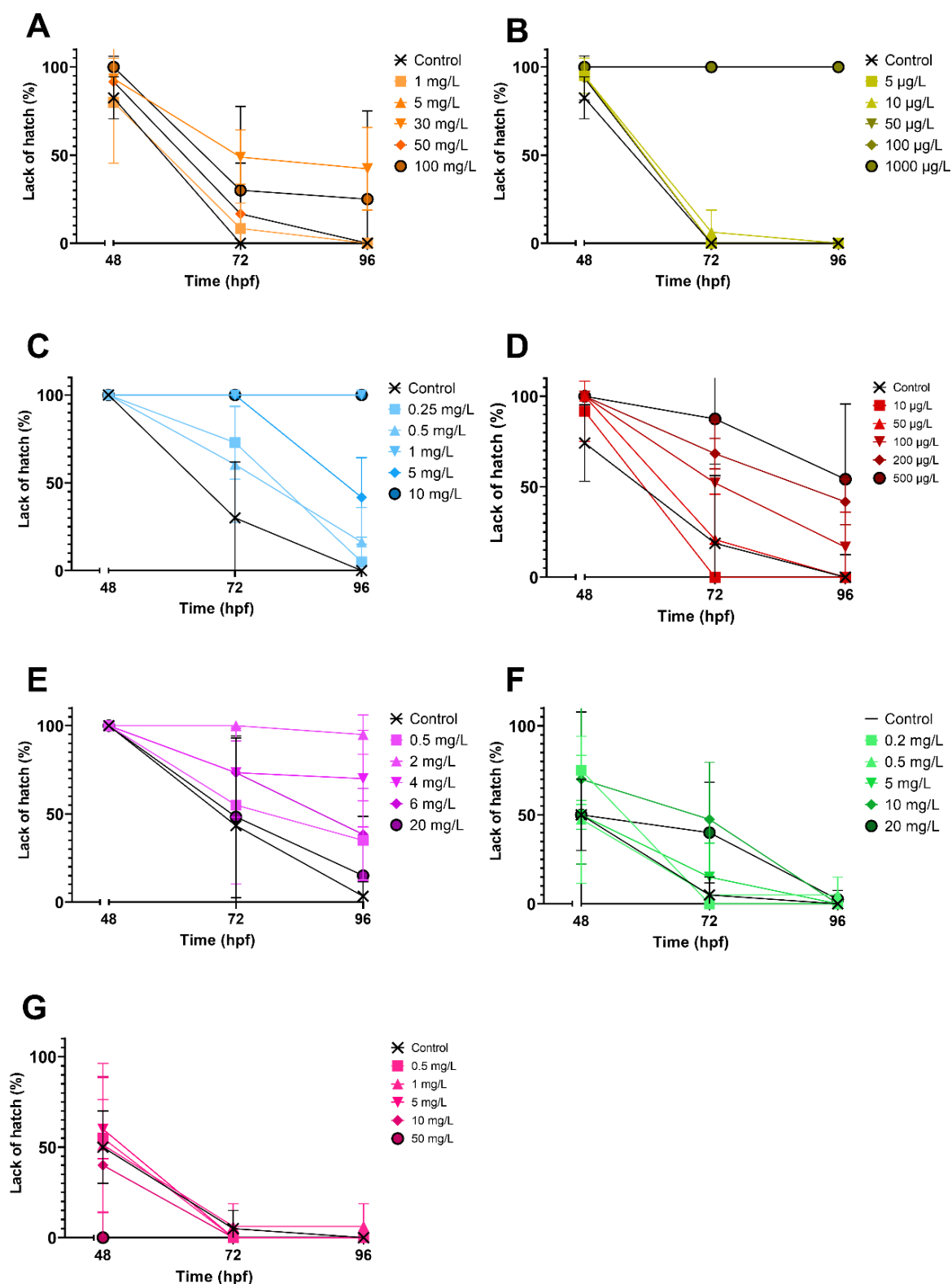


Figure 4-3 : Lack of hatching success of *Danio rerio* embryos over time during 96 h exposures in nAu (A), ionic Au (B) as chlorauric acid, nCuO (C), ionic Cu (D) as CuCl_2 , QDs (E), ionic Cd (F) as CdCl_2 and ionic Te as TeCl_4 (G). Nanomaterial concentrations are expressed as mass NM and ionic treatments are expressed as metal concentration. Lack of hatch (%) was calculated as the number of unhatched individuals as a percentage of all exposed individuals per replicate ($n = 4$).

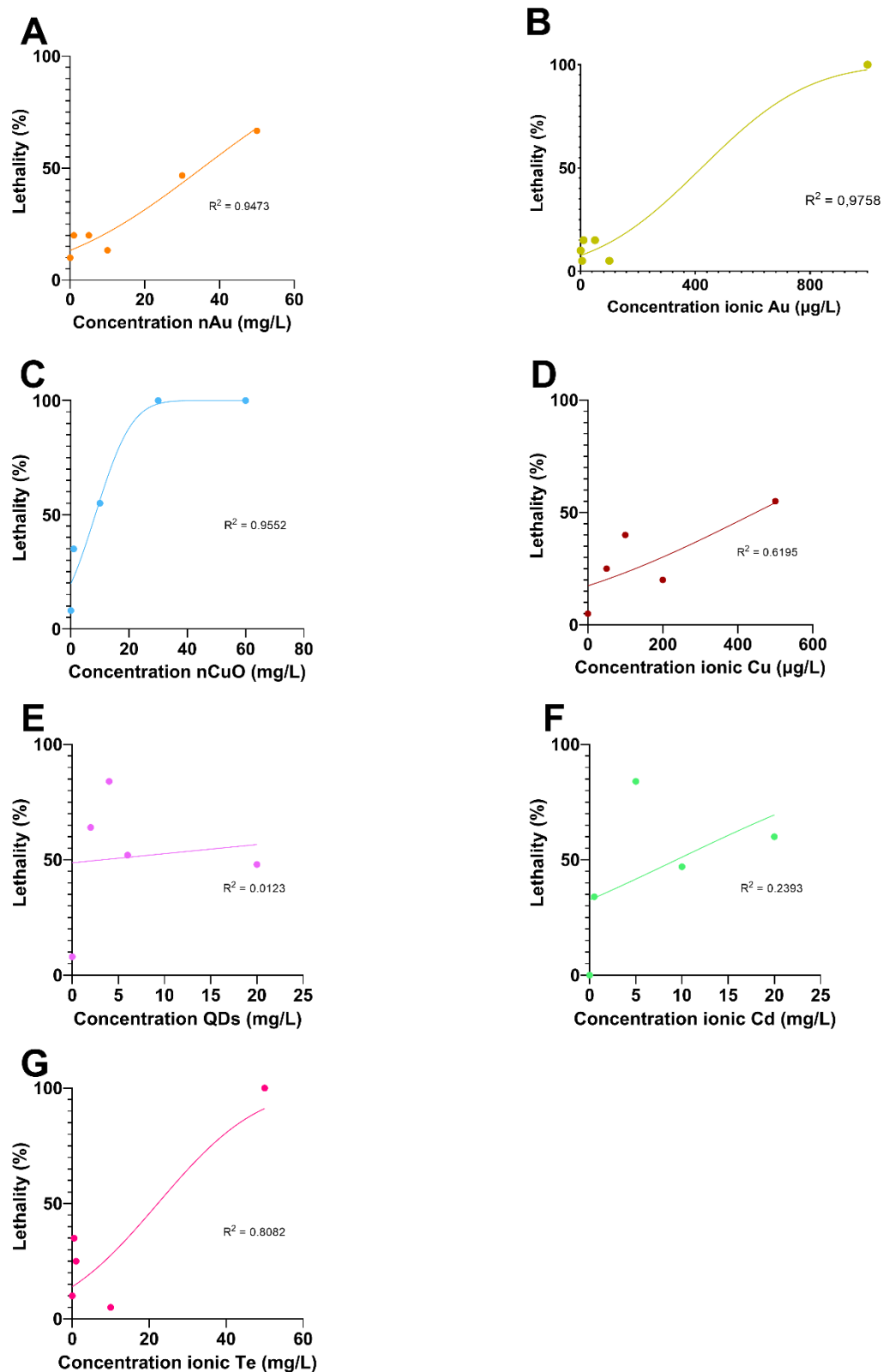


Figure 4-4 : The mean lethality (mortality and lack of hatching success) for each material after 96 h exposure to nAu (A), ionic Au (B), nCuO (C), ionic Cu (D), QDs (E), ionic Cd (F) and ionic Te (G). The line of best fit represents the Cumulative Gaussian distribution used to calculate the LC_{50} for each material (see Table 4-1).

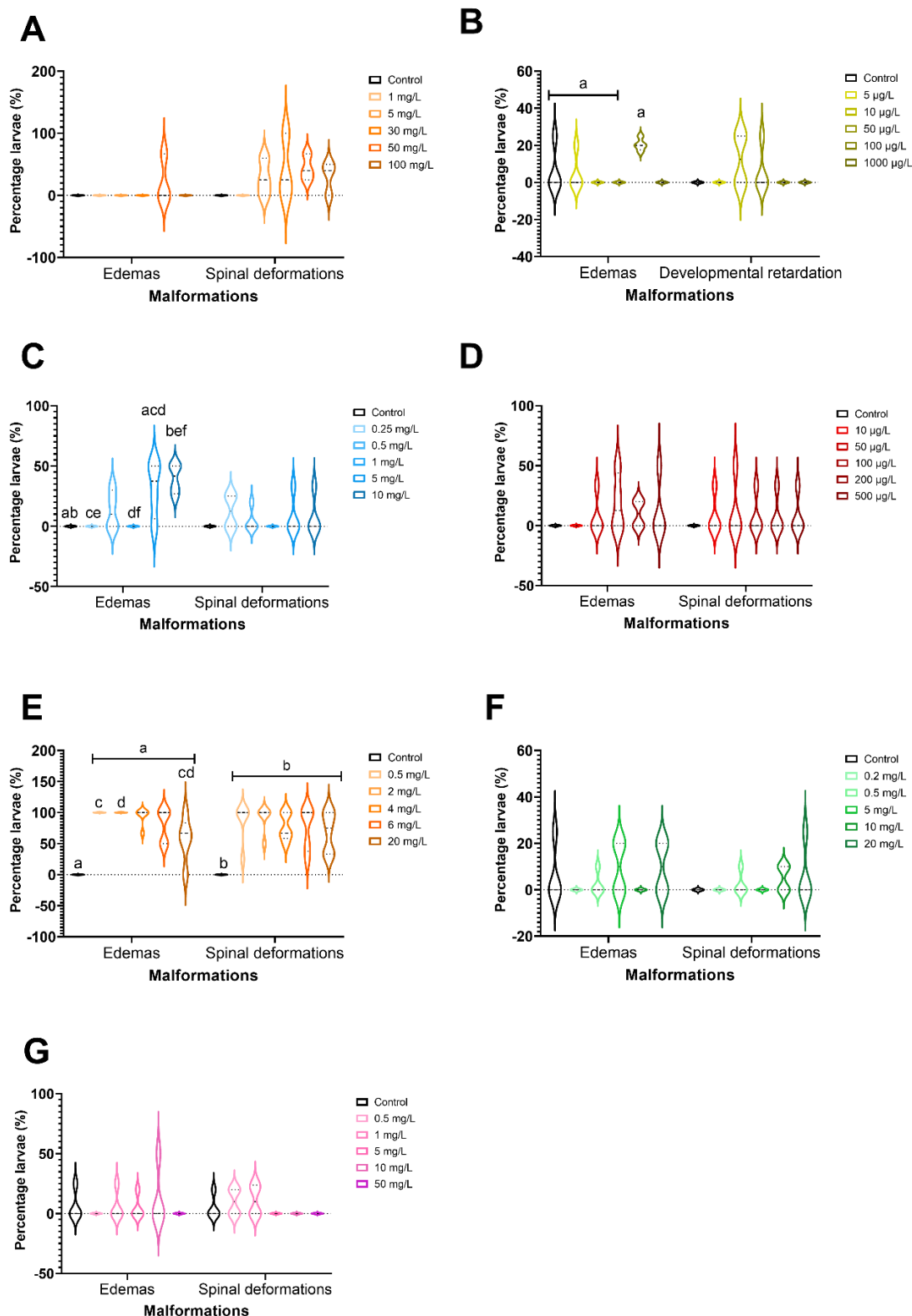


Figure 4-5 : Percentage malformations of living *Danio rerio* embryos following 96 h exposure to nAu (A), ionic Au (B) as chlorauric acid, nCuO (C), ionic Cu (D) as CuCl_2 , QDs (E), ionic Cd (F) as CdCl_2 and ionic Te as TeCl_4 (G). Nanomaterial concentrations expressed as material mass and ionic treatment concentrations are expressed as metal concentration. The violin plot represents the mean $\pm$ SEM of at least 4 independent replicates. Superscript letters denote significant differences between treatments (ANOVA, $p < 0.05$).

4.3.2 Biodistribution and uptake

General observations made during exposures noted that only the QDs agglomerates tended to adhere to the external surface of the embryo chorion (Figure 4-6) and was not observed for the nAu and CuO. CytoViva® darkfield microscopy of *D. rerio* larvae (96 hpf) revealed that when compared to the control, NMs were present in and around the eyes, spinal cord and caudal muscles (Figure 4-7). Images also show that nCuO particles cannot accurately be visualized using CytoViva® as they are not fluorescent. Some agglomerates could be visualized on the exterior of the head and tail of the organism, but are only partially distinguishable from the pigmentation spots.

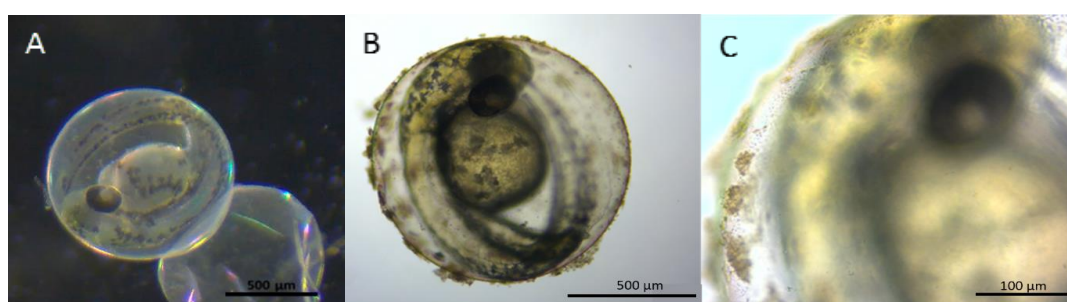


Figure 4-6: Stereo microscope images of 48 hpf *Danio rerio* embryos indicating no particle agglomerates on the chorion of the control (A) and QD agglomerates covering the outside of the chorion (B,C).

Uptake

Total metal uptake shows significantly higher ($p < 0.05$, ANOVA) concentrations of Au in larvae exposed to ionic Au (0.85 mg/L nominal concentration) but not in nAu (51.22 mg/L nominal concentration) compared to the control (Figure 4-8). No significant difference in Au concentrations was seen between nAu and ionic Au even though exposure concentrations differed by > 100 magnitude. The same trend was seen in the Cu groups where ionic Cu larvae had significantly higher ($p < 0.05$, ANOVA) concentrations of Cu present than both the control and nCuO treatments. Here nCuO also had significantly higher ($p < 0.05$, ANOVA) Cu concentrations when compared to control. In the Cd group, both QDs and ionic Cd caused a significant uptake of Cd but the concentrations did not differ significantly between QDs and ionic Cd even though exposure concentrations varied by $\times 10$ magnitude when comparing the ionic concentration to the Cd concentration present in the QD treatment concentration (see Chapter 2). Tellurium concentrations were the highest and both ionic Te and QDs, and resulted in a higher Te uptake, but not significantly higher than the control group.

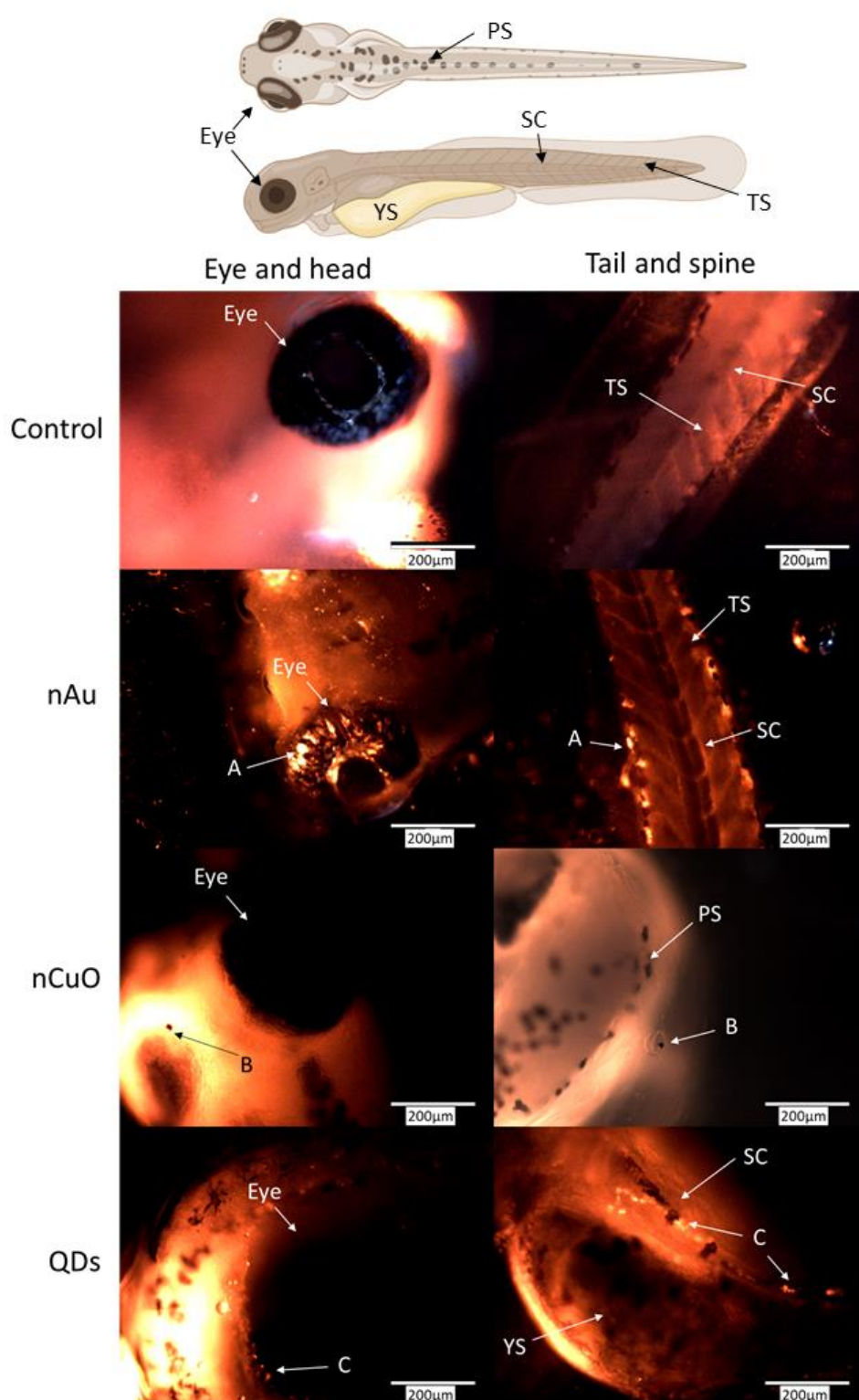


Figure 4-7 : CytoViva® dark-field imaging of *Danio rerio* larvae 96 hpf in an unexposed control, nAu, nCuO and QDs at their LC_{50} concentrations. PS – Pigmentation spots, SC – Spinal cord, TS – Tail somites, YS – Yolk sack, A – nAu, B – nCuO and C – QD.

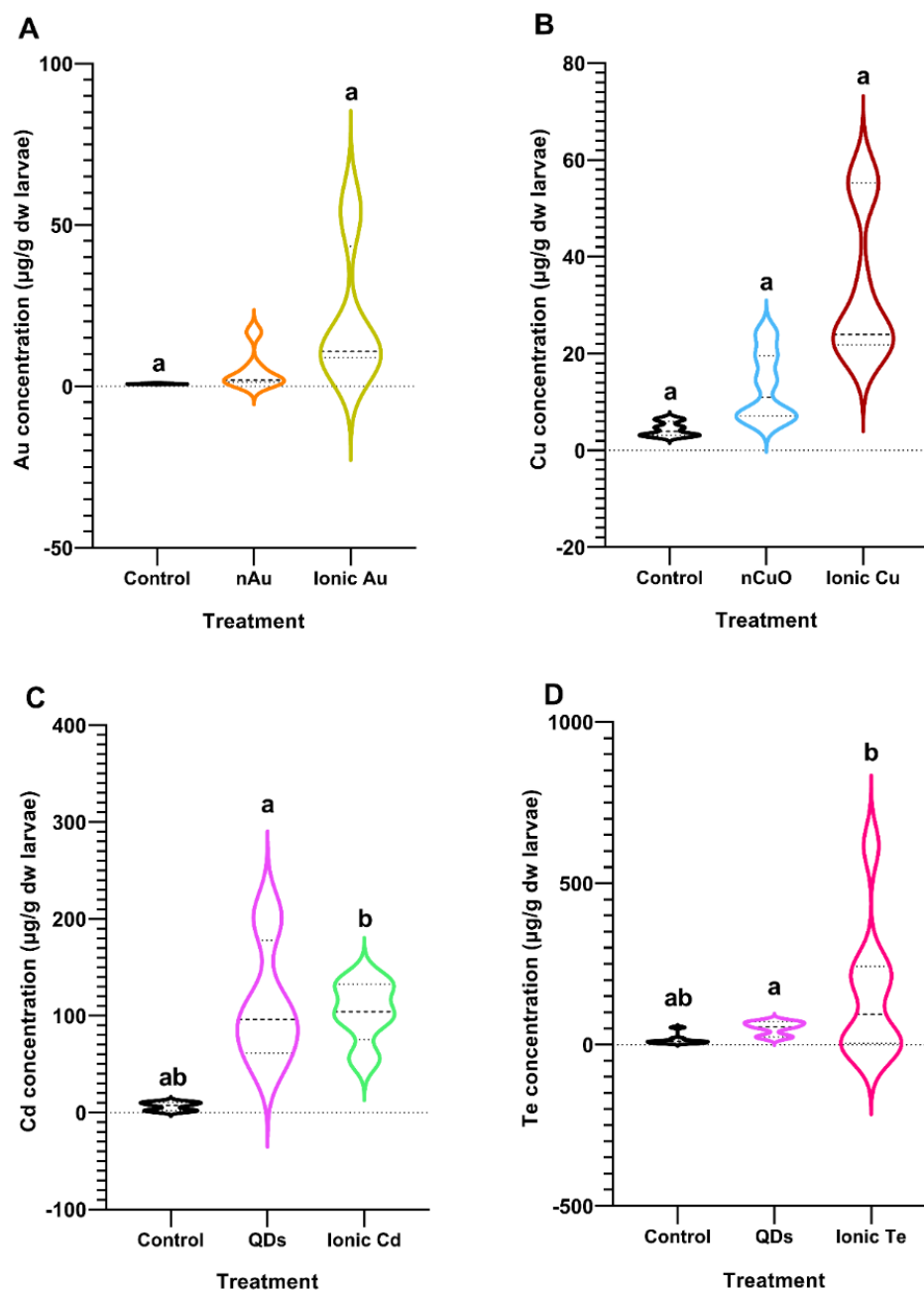


Figure 4-8 : Total metal concentrations of Au (A), Cu (B), Cd (C) and Te (D) in 96 hpf *Danio rerio* larvae after exposure to nAu, CuO, QDs, ionic Au, ionic Cu, Cd and Te at their LC₅₀ concentrations. The graph represents the mean $\pm$ SEM of at least 4 independent replicates. Superscript letters denote significant differences between treatments ($p < 0.05$, ANOVA). Note the difference in scale between treatments A and D.

4.3.3 Embryo activity, cardiac output and respiration

Embryo activity was measured at 24 hpf as embryos were still in the chorion. Tellurium was the only ionic treatment resulting in a significant decrease ($p < 0.05$, T-test) in activity compared to the control (Figure 4-9). The distribution of data suggests that burst duration and burst activity could be affected by NM exposure, as both nAu and nCuO show lower duration and activity compared to the control. Ionic Cu showed a large variation in data due to 40% of embryos being greatly active while the rest were not active at all.

The heart rate of 72 hpf larvae shows significant effects in the NM groups (Figure 4-10). Both nAu and QDs showed a significant increase ($p < 0.05$, T-test) in heart rate and nCuO showed a significant ($p < 0.05$, T-test) decrease in heart rate compared to the control. No significant effects on the blood flow were seen. Only Te caused a significant decrease in blood flow.

The respiration results are presented on graphs comparing the treatment to the treatment matched control. There were no significant effects on respiration after 24 hpf and respiration increased significantly after 48 hpf (Figure 4-11). Quantum dots, Cd and Cu resulted in a decrease in respiration after 48 hpf (not significant). Ionic Au, ionic Te, nAu and nCuO resulted in an increase in respiration compared to the control, but only the nCuO treatment was significant.

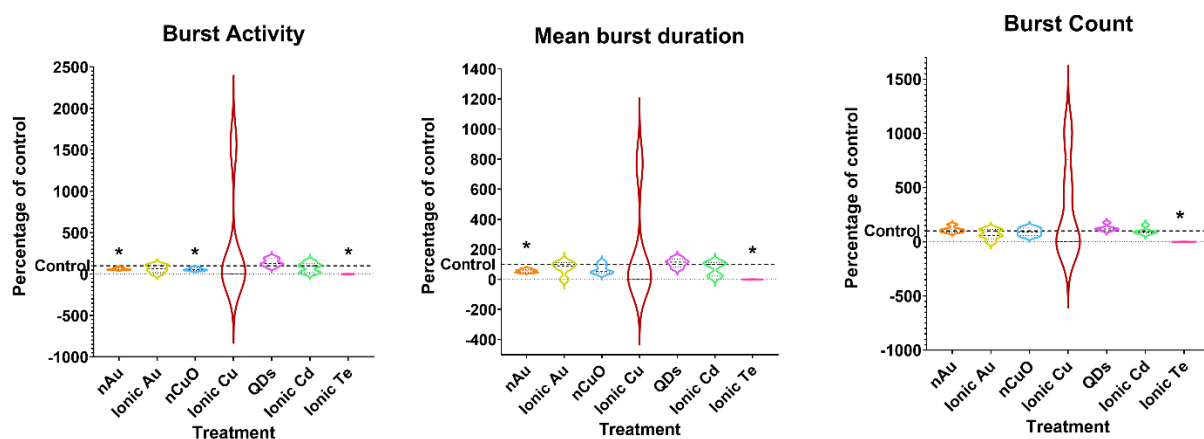


Figure 4-9 : Burst activity, mean burst duration and burst count/minute of 24 hpf *Danio rerio* embryos exposed to LC_{50} concentrations of nAu, ionic Au, nCuO, ionic Cu, QDs, ionic Cd and ionic Te. The graph represents the mean $\pm$ SEM of at least 5 independent replicates. Asterisk denotes a significant difference between treatments and the control indicated with a dashed line ($p < 0.05$, T-tests).

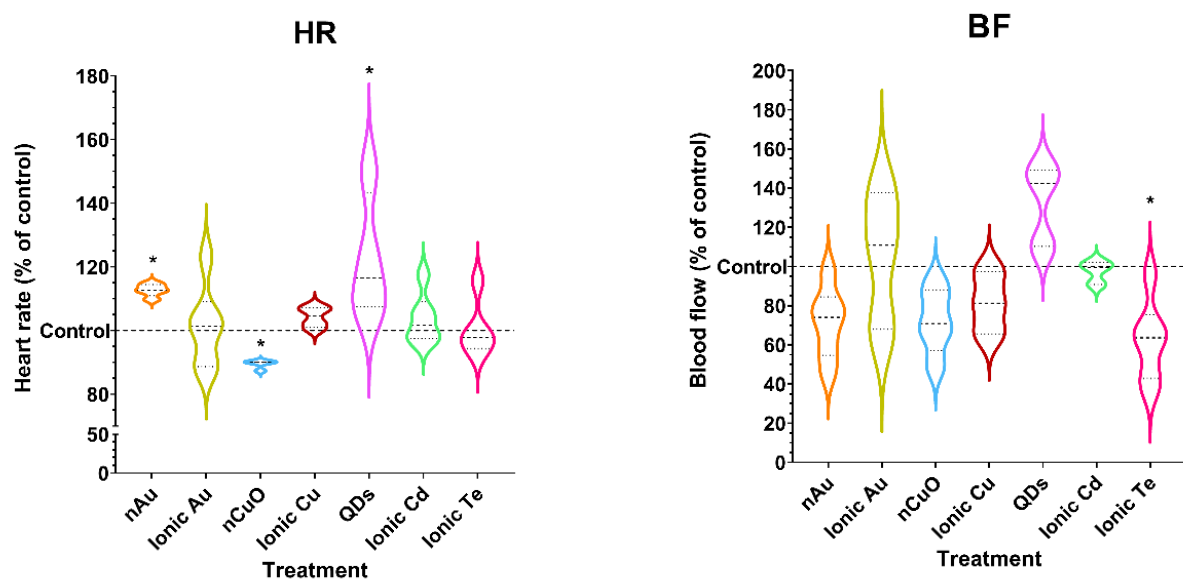


Figure 4-10 : Heart rate (HR) and blood flow (BF) of 72 hpf *Danio rerio* larvae exposed to LC_{50} concentrations of nAu, ionic Au, nCuO, ionic Cu, QDs, ionic Cd and ionic Te. The graph represents the mean $\pm$ SEM of at least 5 independent replicates. Asterisk denotes a significant difference between treatments and the control ($p < 0.05$, T-test).

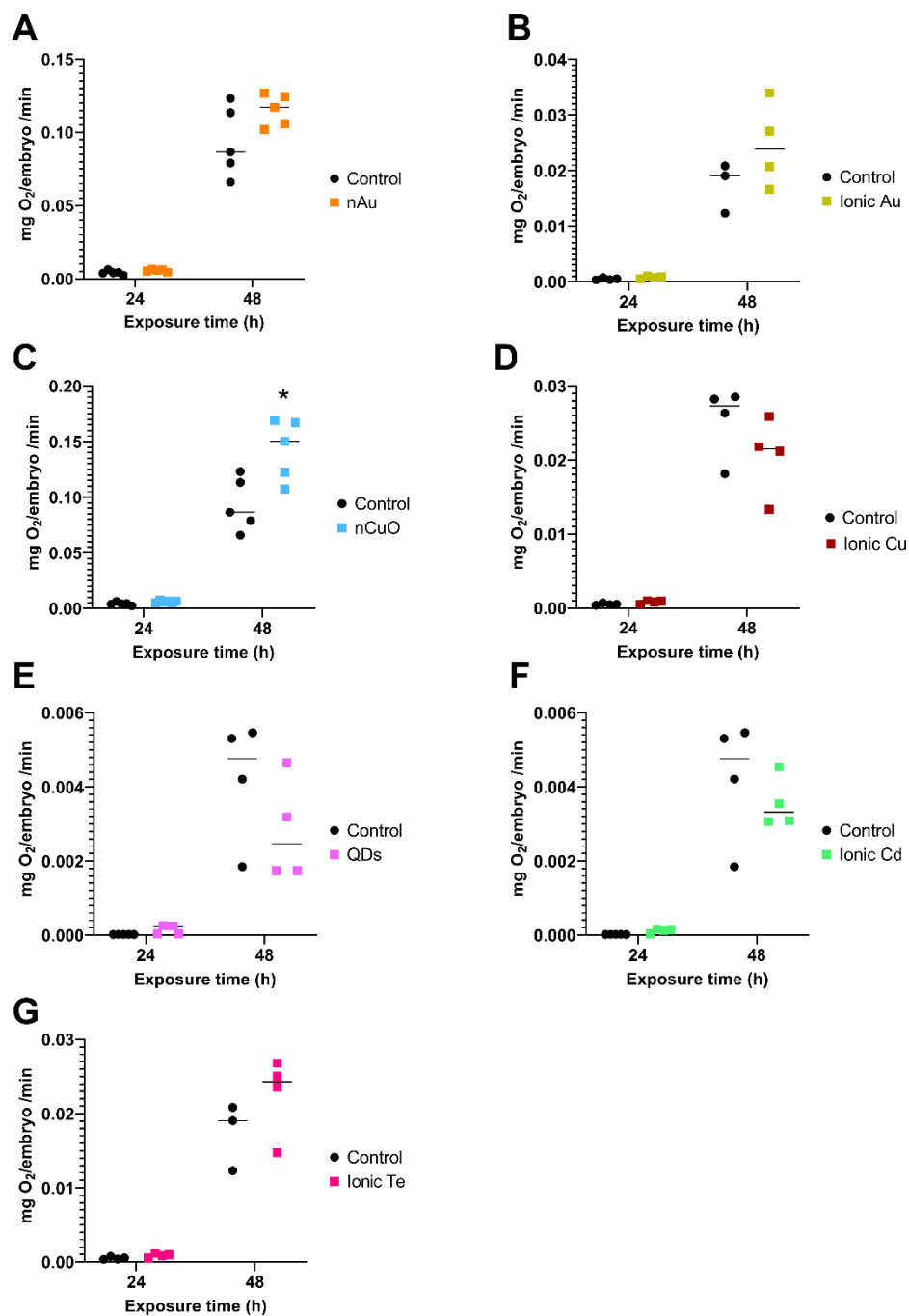


Figure 4-11 : Respiration of *Danio rerio* embryos 24 hpf and 48 hpf. Graphs A-G denotes each treatment with their control group. The graph represents the replicates and mean of each treatment. Asterisk denotes a significant difference between treatments and the control ($p < 0.05$, T-tests).

4.3.4 Metabolomics

When comparing NMs to their ionic equivalents, as well as the control the PLS-DA bi-plots indicate clear groupings and discrimination between the treatments (Figure 4-12). The only slight overlap can be seen between the QDs and ionic Te when the control is present (Figure 4-12 C). There is no clear indication of which of the two component axes explain the variation in the data best. It is evident that both metabolic profiles between treatments as well as variation within treatments are apparent.

Table 4-3 presents the five most significant metabolites that account for the differences ($p < 0.05$, ANOVA) between treatments. Four out of five metabolites were up-regulated in the ionic Au group compared to the control. All metabolites were up-regulated in the ionic Cu group compared to the control and three out of five metabolites were up-regulated in the ionic Cd treatment. In the ionic Te treatment group, four of the five metabolites were down-regulated compared to the control. Most metabolites were up-regulated in the NM treatments compared to the control except two in the nAu and two in the QDs treatments. When comparing ionic treatment to NM treatment most metabolites were up-regulated in the ionic groups. Three metabolites were down-regulated in the ionic Au group compared to nAu, two metabolites in ionic Cd compared to QDs and one in ionic Te compared to QDs. There were no significant differences between the metabolites of the ionic Cu and nCuO exposures. Overlapping metabolites for ionic Te exposure include 2-Aminomalonic acid, undecanoic acid, methoxyamin and pentasiloxane. Overlapping metabolites for ionic Au exposure include palmitic acid, D-xylose, 2-aminomalonic acid and D-arabinose. Palmitic acid and D-arabinose are also present in the QDs treatment group. All nAu metabolites are unique and only D-xylose, urea and methyl 9,10-dihydroxystearate overlap between nCuO treatment and other treatment groups. Phosphoric acid and 2,3,4,5-tetrahydroxypentanoic acid-1,4-lactone are overlapping metabolites for Cd exposure.

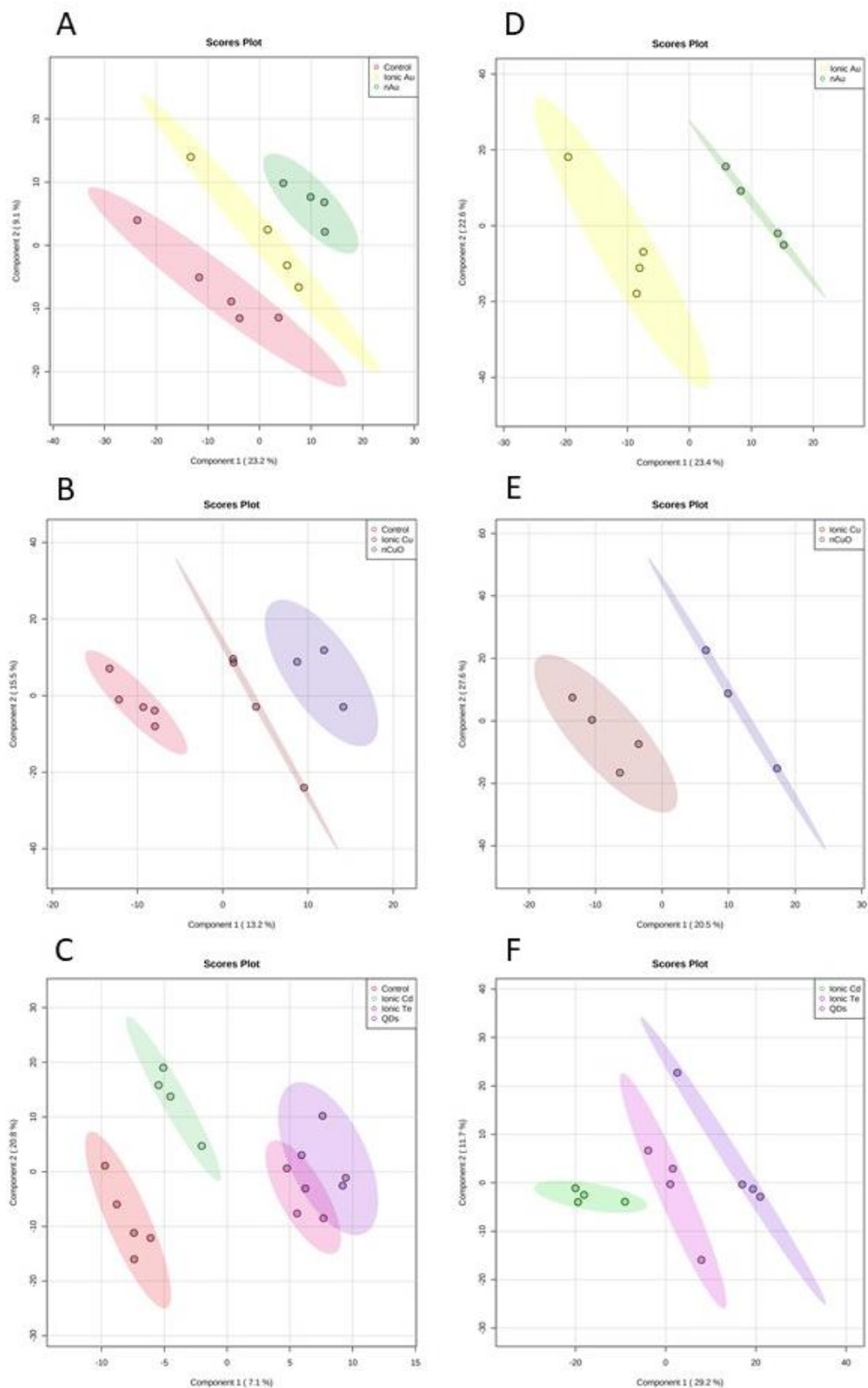


Figure 4-12 : PLS-DA scores-plots based on highest metabolite intensities for Au (A), Cu (B) and QDs (C) with their respective controls and without (D-F) the control.

Table 4-3 : Pairwise comparison of the five most significant ($p < 0.05$) metabolite concentrations in *Danio rerio* following exposure to three different NMs and their ionic metal equivalents.

Group	Control	nAu	nCuO	QD	Ionic Cd
Control		L-Aspartate ↑ Alloxanoic acid ↓ 4-Pentanoic acid ↓ 2,2,6-Trimethyl-2H,5H-pyrano[3,2-c]quinolin-5-one ↓ Silanamine ↑	Urea ↓ Aucubin ↓ Methyl 9,10-dihydroxystearate ↓ D-Xylose ↓ 3-Hydroxyproline ↓	Decane ↓ D-Arabinose ↓ Silane ↑ 2-Butenedioic acid ↑ Palmitic acid ↓	Phosphoric acid ↓ 2,3,4,5-Tetrahydroxypentanoic acid-1,4-lactone ↓ Urea ↓ Propane ↑ L-Hydroxyproline ↑
Ionic Au	Palmitic acid ↑ D-Xylose ↓ 2-Aminomalonic acid ↑ D-Arabinose ↑ Ritalinic acid ↑	1-Dodecanol ↓ Palmitic acid ↑ Aucubin ↓ 2-Aminomalonic acid ↑ 4-Pyridinol ↓			
Ionic Cu	Palmitic Acid ↑ Nonadecane ↑ D-Xylose ↑ 2,2,6-Trimethyl-2H,5H-pyrano[3,2-c]quinolin-5-one ↑ Aucubin ↑		6 unidentified metabolites		
Ionic Cd				Phosphoric acid ↓ 2,3,4,5-Tetrahydroxypentanoic acid-1,4-lactone ↑ Urea ↑ L-Glutamic acid ↓ Methyl 9,10-dihydroxystearate ↑	
Ionic Te	Undecanoic acid ↓ Methoxyamine ↓ 2,2'-Bipyridine ↓ Heptadecane ↓ Pentasiloxane ↑			Undecanoic acid ↓ 2-Propanol ↑ Methoxyamine ↑ 2-Aminomalonic acid ↑ Pentasiloxane ↑	Phosphoric acid ↑ L-Proline ↑ 2,3,4,5-Tetrahydroxypentanoic acid-1,4-lactone ↓ 2-Aminomalonic acid ↑ Urea ↓

i Arrows indicate up regulation or down regulation of metabolites when comparing the groups in rows to the groups in columns. Unidentified metabolites were excluded from the table. Grey sections indicate comparisons were not run.

Pathway analysis

Pathway analyses were based on the metabolites identified from pairwise comparisons using Metaboanalyst (Figure 4-13 and Figure 4-14). Depending on the number and role of the detected metabolites in the respective pathways, as well as pathway importance, “Impact factors” were calculated (values ranging between 0 and 1, with 1 being the highest). Nanogold had the most significant impact on the alanine, aspartate and glutamate metabolism and the aminoacyl-tRNA biosynthesis pathway had the most significant difference in metabolite concentrations compared to the control (Figure 4-13 A). All significant metabolites in these pathways were lower than the control. Ionic Au caused the greatest impact on the pentose and glucuronate interconversions pathway, with the fatty acid and unsaturated fatty acid biosynthesis pathways having the largest difference between metabolite concentrations (Figure 4-13 B). Pathway statistical results are reported in Tables S4-20 to S4-25.

The predominant pathway affected by nCuO is the phenylalanine, tyrosine and tryptophan biosynthesis pathway, with the most significant metabolite concentrations in the tyrosine and phenylalanine metabolism pathways (Figure 4-13 C). Significant metabolites in these pathways were lower than in the control. The greatest affected pathways in the ionic Cu treatment group include the phenylalanine, tyrosine and tryptophan biosynthesis and tyrosine metabolism also present in the nCuO treatments (Figure 4-13 D). Other pathways affected include the D-Glutamine and D-glutamate metabolism, alanine, aspartate and glutamate metabolism and the fatty acid metabolism.

For QD treatments the pathway that was affected the most was the fatty acid biosynthesis pathway, and others that had significant metabolites were the aminoacyl-tRNA biosynthesis pathway, the biosynthesis of unsaturated fatty acids and the selenocompound metabolism pathway (Figure 4-14 A). The latter pathway is unique to the QDs exposure group. The ionic Cd treatment resulted in the largest number of affected pathways (Figure 4-14 B). Notable pathways include D-glutamine and D-glutamate metabolism, glycine and serine and threonine metabolism. The arginine and proline metabolism, alanine, aspartate and glutamate metabolism and aminoacyl-tRNA biosynthesis were also significantly affected and some of the contributing metabolites were up-regulated and some were down-regulated. Ionic Te treatment resulted in the downregulation of metabolites in the phenylalanine, tyrosine and tryptophan biosynthesis pathway, tyrosine metabolism, aminoacyl-tRNA biosynthesis pathway and the phenylalanine metabolism (Figure 4-14 C).

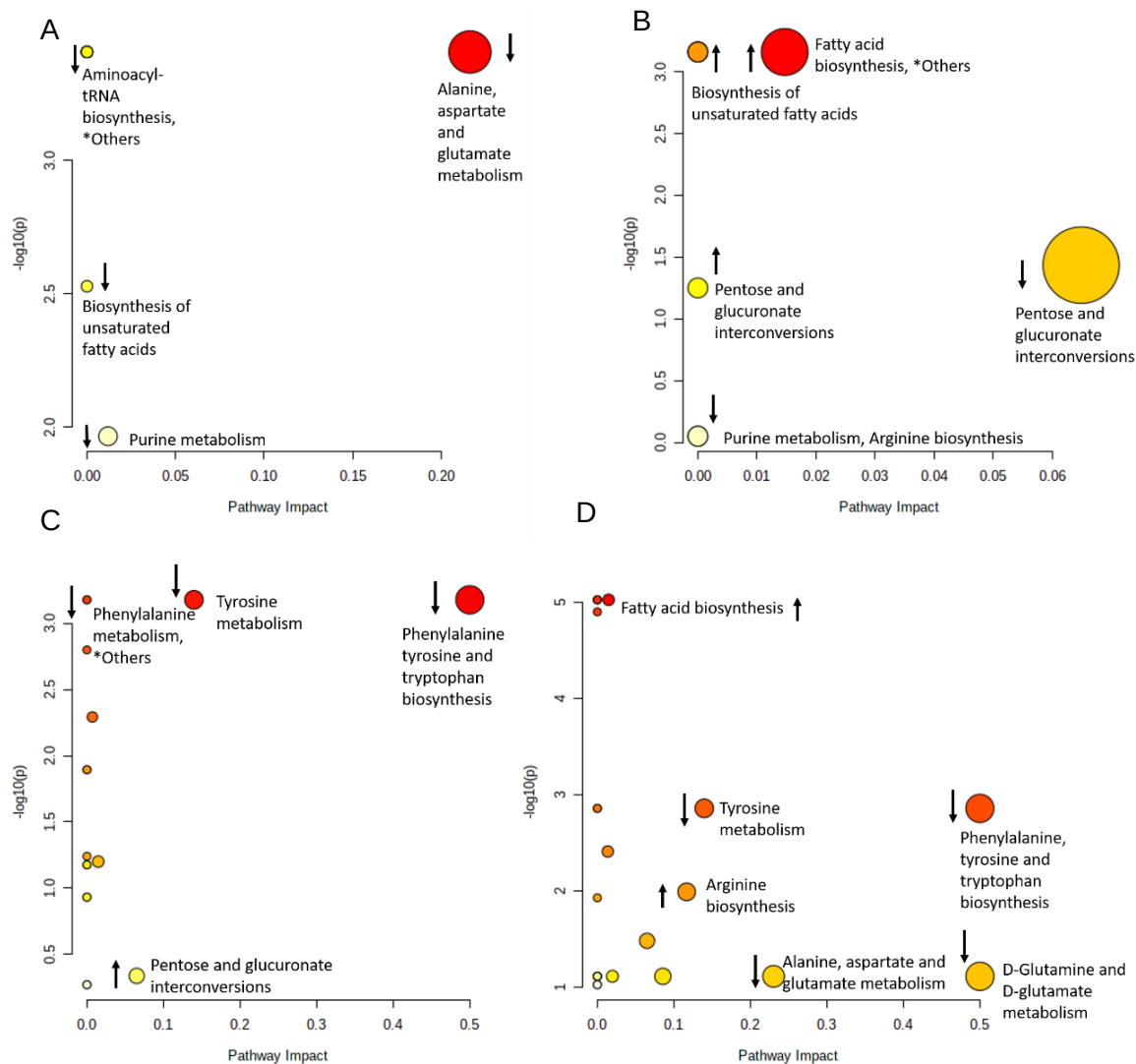


Figure 4-13 : The most significant and greatest impacted metabolic pathways calculated by Metaboanalyst between the control group and nAu (A), ionic Au (B), nCuO (C) and ionic Cu (D). The identified pathways are ranked after their importance and the role of the detected metabolite within the pathway to calculate the impact factor (x-axis). Differences in metabolite concentration between the treatment and the control are represented on the y-axis. Relation of significance and impact factor determined the size of pathway circles. Pathway circles noted with *others contain multiple pathways with the same impact factor and metabolite concentration and can be viewed in Supplementary Information Tables S4-2 to S4-8.

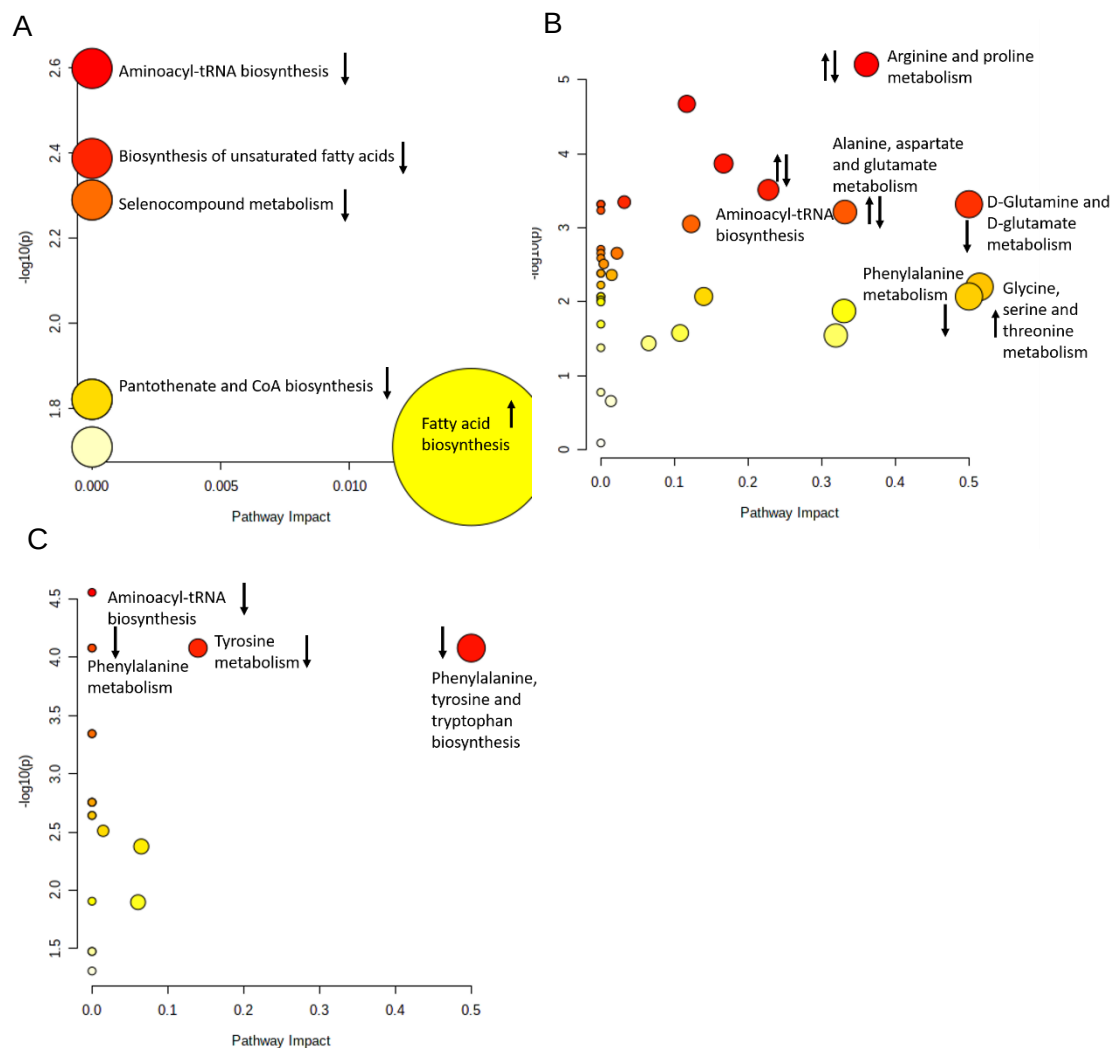


Figure 4-14 : The most significant and greatest impacted metabolic pathways calculated by Metaboanalyst between the control group and QDs (A), ionic Cd (B) and ionic Te (C). The identified pathways are ranked after their importance and the role of the detected metabolite within the pathway to calculate the impact factor (x-axis). Differences in metabolite concentration between the treatment and the control are represented on the y-axis.

4.4 Discussion

This chapter investigated the differences in toxicity of nAu, CuO and CdTe QDs, and their ionic metal equivalents (ionic Au, Cu, Cd, Te). Uptake was confirmed by CytoViva® darkfield microscopy and metal analysis that indicated the same concentrations of total metals in the organisms from the different types of test substances (nano and ionic) despite exposure concentration differences. Endpoints such as mortality and hatching success showed bimodal responses after exposure to nAu and QDs and dose dependant responses of organisms exposed to nCuO and all the ionic metals. The sublethal endpoints such as pericardial oedemas and spinal deformations did not show a dose-dependent effect, except in ionic Au and nCuO groups. This study also has presented some evidence for the first time that there are differences in metabolic profiles in zebrafish larvae exposed to nAu, nCuO and QDs, as well as their between NMs and their ionic metal equivalents.

Biodistribution and uptake

In this study an attempt at measuring NM biodistribution was made in larvae exposed to the LC₅₀ of each material using CytoViva, but not all organs can be detected. Only total metal uptake could be quantified GF-AAS and not particle uptake. The *in vivo* distribution of metal-based NMs is poorly understood and hindered by a limited number of methods for quantifying NMs in tissues (Coty and Vauthier, 2018; Laux et al., 2017). Gold has been found in zebrafish brain and liver after dietary exposure however, only brain, liver and muscle samples were analysed (Geffroy et al., 2011). In the present study, it was established that nAu were found around the eyes, head and muscle/somites in the caudal region of the larvae. A study by Heath, 1987 reported most of the copper was found in the gills, kidney, brain, liver, and skeletal muscle of the fish. In the present study biodistribution of nCuO could not confidently be established, but some agglomerates could be seen around the outer surface of the head and tail. A study by Heinlaan et al. (2016) confirmed the accumulation of nCuO on the body of the embryo, especially around the eyes and the yolk sack after hatching. Cadmium has been reported to bioaccumulate most significantly in the kidney followed by liver and gills of catfish. In the present study biodistribution of QDs were found around the eyes, spinal cord and yolk sac.

A study by Böhme et al. (2017) tested nAu, nano-silver, nCuO and nano zinc oxide in zebrafish embryos and found the highest accumulation of metal in the nAu treatment group, with the highest metal concentration found around the chorion. However, Bar-Ilan et al. (2009) reported several studies confirming the transport of nAu through chorion pore canals into the embryo before hatching after 72 hpf. Studies have shown this is size and coating-dependent. However, in the current study hatched larvae were analysed for uptake and biodistribution after 96 hpf. Thus, for

two days larvae did not have the protection of the chorion after hatching, assisting in the uptake of NMs after hatching. Malhotra et al. (2020) has reported many studies that indicate the gills as a primary organ for Cu uptake from salt and NM exposures. After hatch the gills are most likely the uptake route for metal in the present study. Adsorption of NMs onto the gill surface will involve similar processes in the gill microenvironment and mucus layer as other substances, but NM uptake by epithelial cells is more likely to occur via vesicular processes (e.g., endocytosis) than uptake on membrane transporters or diffusion through cell membranes (Handy et al., 2008).

Absolute exposure concentrations were significantly lower in the ionic Au (0.85 mg/L measured Au) group than in the nAu group (25 mg/L measured Au), but total metal uptake was much higher in the ionic Au group (21 µg/g) compared to the nAu group (5 µg/g) indicating that nAu was not taken up as readily by the embryo. This is most likely due to NM aggregates settling out reducing bioavailability and uptake through the gills. This was also found in another study that tested ionic Au and different sizes of nAu. They found that ionic Au and 5 nm nAu were taken up by cells in larvae of golden perch (Shuster et al., 2021). The same results were seen for nCuO and ionic Cu. However, accumulation of total Cd and Te, QDs and the ionic metal treatments was the same, despite exposure concentration differences between the ionic materials and the metal concentrations in the QDs. This suggests that for dissolved Cd^{2+} which binds very well and irreversibly to mucus, it was simply trapped in the perivitelline fluid and not bioaccessible to the fish embryo inside. Quantum dots can also bind to Cl^- in the media to form complexes that cause aggregation rather than diffusion of NMs through the paracellular route (Shaw et al., 2011). Another explanation may be that the Ca in the media slows the uptake of Cd by outcompeting Cd for interaction with the plasma membrane Ca^{2+} -transporting ATPase of fish gills (Verbost et al., 1988). Zarco-Fernández et al. (2016) reported that QDs and thus, the Cd concentration seemed to be more homogeneous distributed along the larvae surface. Their results suggest that QDs are not integrated into the organism (Jackson et al., 2012) and that the analysed Cd could correspond to the fraction of ionic Cd present in the solution. However, in the present study larvae were washed with ultrapure water to reduce the likelihood that there were no particles adsorbed to the external surface and that the measured metal concentrations were internal concentrations. It is also important to note that we assume total metal uptake in the NM treatments was due to particle uptake as dissolution in the media was very low and particles can be confirmed in the CytoViva® images.

By attempting to determine the uptake and biodistribution of NMs it is evident that developmental toxicity can be attributed to metal and NM uptake and not solely toxicity related to the ion as suggested by other studies (Aksakal and Ciltas, 2019; Duan et al., 2013). As the dissolution of metals from the particles is so low (Chapter 2) it is assumed that metal uptake in the NM

treatments is due to particle uptake and that toxicity is not solely linked to free ion uptake. This was also found in a study by Zhu et al. (2009), where the highest toxicity was found in concentrations with the lowest dissolution. King-Heiden et al. (2009) also found that Cd released from QDs was not sufficient to contribute to the total Cd body burden, indicating that Cd uptake reflected uptake of intact QDs.

Gold (nAu and ionic Au) toxicity

Ionic Au toxicity is expected to be higher than that on the nAu. Griffitt et al. (2008) reported that the highest dose (10 mg/L) was not elevated enough to establish an LC₅₀ for adult zebrafish or larvae. Ionic Au mortality and decreased hatching success were concentration dependant, but in the nAu group this was not the case. The lower concentrations resulted in the highest mortality. This corresponds to results by Lovern and Klaper (2006) who found lower concentrations of fullerene NMs showed higher levels of toxicity in *Daphnia magna*. They also found that sonication of suspensions resulted in varying toxicity between concentrations. From the characterization (see Chapter 2) it can be seen that lower concentrations of nAu resulted in larger suspended agglomerates in the exposure media, thus it is likely that once hatched the older larvae ingest these large agglomerates due to accidental ingestion through gulping water (Roch et al., 2020; Marana et al., 2022). This results in higher NM uptake in the lower concentrations, but the present study did not test uptake over all concentrations and needs further tests to confirm. Although samples were sonicated it is also possible that because of the uneven distribution of NMs and agglomerates, the ratio between large and small agglomerates could be different in some replicates (see Chapter 2). Nanogold resulted in more spinal deformations than pericardial oedemas and no significant concentration effects were seen, suggesting these endpoints are not closely linked to total metal in the organism. In the ionic Au group, there was a significant number of pericardial oedemas in the 100 µg/L treatment. This is in contrast to A study by Shuster et al. (2021) found that 5 nm nAu caused yolk sac oedemas that indirectly caused spinal deformations in golden perch larvae, more so than ionic Au. Some larvae were developmentally retarded showing delayed development in the 10 and 50 µg/L treatments and are reported instead. Thus, in the case of the inert nAu spinal deformations may be a nano-specific reaction.

According to the resulting LC₅₀ values, ionic Au and Cu are more toxic than their nano equivalents. However, due to bimodal results LC₅₀ values for nAu, ionic Cu, QDs, ionic Cd and ionic Te are not reliable and should be used as a guideline (based on the R² values of the fit. This has been seen in studies by Ganesan et al. (2016), Zhao et al. (2013), Massarsky et al. (2013) and Vicario-Parés et al. (2014). In the present study, nAu was shown to be only mildly toxic compared to the other NMs as previously also reported by Pereira et al. (2019), most likely due to the nature of

the particle being inert. The greater toxicity of the ionic metals compared to the NMs is most likely due to the formation of ROS as was found by Botha et al. (2019).

The mortality and higher incidence of physical deformities could be at least partially attributed to the disrupted heart rate observed in this study. The heart is the first organ formed in zebrafish embryonic development and the measurement of heart rate during the embryonic period is an important toxicological parameter (Aksakal and Ciltas, 2019). The significant increase in heart rate of the nAu group at 72 hpf can be linked to the increase in respiration observed at 48 hpf. Elevated heart rate and in turn respiration are common signs of stress (Svendsen et al., 2021). However, this was not seen in the ionic Au treatment group in the present study. A non-significant increase in respiration was observed but no effects on cardiac output (heart rate and blood flow) were seen. Rombough (2007) reports that gills are only completely formed after 5dpf thus, metabolism, heart rate and respiration are not yet linked at the time points in the study. In the present study, there were slight decreases in burst activity of zebrafish embryos after 24 hpf exposure. This could be a sign of neurotoxicity, as lower levels of some nanoparticles have been shown to induce an increase in caspase activity and cytokines secretion from astrocytes, leading to apoptosis and neuroinflammation, respectively (Teleanu et al., 2019). It could also be a direct result of nAu in the muscle of the tail as observed by CytoViva® as a result of thiol in the muscle directly reacting with the oxidative gold surface to form Au–S bonds via the oxidation–reduction reaction, or simply a stress response due to increased oxygen use (Kataba et al., 2022).

When linking these endpoints to effects on the biochemical level, it can be seen that nAu and ionic Au affect different metabolites significantly, leading to different modes of toxicity. Distinctive significant metabolites for nAu in the present study were the down-regulation of L-aspartate and the upregulation of alloxanoic acid. The only distinctive metabolite for ionic Au was ritalinic acid and it was up-regulated. Commonly affected pathways between all materials resulting from revealed pathways such as the fatty acid biosynthesis and degradation pathways (not affected by nAu treatment), alanine, aspartate and glutamate metabolism (not affected by ionic Au treatment) and the aminoacyl-tRNA biosynthesis pathway (also not affected by ionic Au exposure). A study by Zhang et al. (2017) also showed responses of *Daphnia* to silver NMs included down regulation of aminoacyl-tRNA biosynthesis. This is part of the first step in protein synthesis and amino acid production and is a common response to toxicant exposure. Disruption of this pathway may cause growth defects such as the spinal deformations (Zhang et al., 2017), as observed in the nAu group but not in the ionic Au group in the present study. The metabolism of amino acids, energy and nucleotides are influenced by SWCNT which might indicate a disturbance in metabolic reaction networks (Ganesan et al., 2021). All exposures were carried out at the LC₅₀. It is interesting to

note that this pathway was not influenced in the ionic Au treatment group indicating different modes of toxicity that would need further studying.

Nicotinate and nicotinamide metabolism was influenced in the nAu treatment and not in the ionic Au treatment. This pathway is responsible for forming coenzymes to catalyse cellular reactions and also links to the alanine, aspartate and glutamate metabolism which is responsible for the metabolism of amino acids (Genome.jp, 2022). The metabolite L-aspartate forms part of this metabolism and was seen to be down-regulated in the nAu treatment. The alanine, aspartate and glutamate metabolism was the most significantly affected pathway due to a decrease in L-aspartate from nAu exposure. Aspartate is an amino acid that is a precursor for proteins and an essential energy source for fish (Francesca et al., 2020). A decrease in glutamate metabolism will reduce L-glutamate levels that can increase heart rate as observed in the nAu group in the present study. Amino acids are essential because fish utilize these macromolecules continuously to replenish existing proteins for tissue repair and to produce new proteins during growth and reproduction. This alludes to a possible disruption in both these pathways due to nAu exposure and the possible knock-on effect on the histidine metabolism is also affected in the nAu treatment and not the other treatments. This affects normal growth and energy as seen in the sublethal effects of the present study such as increased heart rate and developmental toxicity.

The ionic Au treatment also influenced fatty acid metabolism responsible for the formation of cellular membranes and part of the lipid metabolism, an increase in degradation metabolites here could mean this pathway is not functioning correctly and could lead to cellular defects corresponding to cellular damage from ROS as well, which is likely the reason for developmental retardation observed in the present study. The ROS can also cause neurological abnormalities by DNA damage that can cause the increase in burst activity seen in the present study. The initiation of antioxidant activity in response to ROS formation after exposure of zebrafish to nAu was confirmed by Botha et al. (2019). The purine metabolism was affected by both nAu and ionic Au and can thus be a biomarker for Au exposure. The purine metabolism, including the anabolism/catabolism of ATP, ADP, AMP, inosine, and xanthine, was significantly influenced by TiO₂ NMs exposure in zebrafish, which may indicate some genotoxicity. Additionally, increasing the dose and time of exposure led to metabolic disturbances in glycerophospholipid metabolism and the Krebs cycle (Farré and Jha, 2020, Raja et al., 2018).

Copper (nCuO and ionic Cu) toxicity

In Chapter 2, it was shown that NM size decreased as concentration increased resulting in smaller agglomerates available for uptake. The LC₅₀ results indicate that ionic Cu is more toxic than nCuO. As described above this is most likely due to ROS formation and has been observed in

other studies. Both nCuO and ionic Cu resulted in dose-dependent mortality and hatching inhibition, suggesting that nCuO acts more like its ionic metal than the other NMs. Other studies have also reported dose-dependent responses to NMs (Ganesan et al., 2016; Zhao et al., 2013; Chen et al., 2020; Hansjosten et al., 2022). Our results are in good agreement with those obtained by Lin et al. (2012), who observed a reduced hatching rate at 72 hpf in embryos exposed to concentrations over 0.5 mg/L of nCuO. Griffitt et al. (2007) found nCuO to be acutely toxic with a 48-LC₅₀ of 1.5 mg/L. They also reported dose dependent gill pathologies (proliferation of epithelial cells and oedema of primary and secondary filaments), inhibition of branchial Na⁺/ K⁺-ATPase activity. Toxic effects suggested to be unexplained by the effects of dissolved Cu alone. In our study, effects on hatching were registered as delayed hatching, similar to studies conducted by (Vicario-Parés et al., 2014) on CuO and zebrafish. In previous studies, significant concentration-dependent hatching retardation was observed in nCuO treated embryos exposed to 60 mg/L (Ganesan et al., 2016). Similar results were found by Aksakal and Ciltas (2019). It was hypothesized that inhibition of hatching was a result of the interference of Cu ions with the hatching enzyme, chorionase in fish (Zhao et al., 2013; Massarsky et al., 2013; Aksakal and Ciltas, 2019). This suppression was demonstrated in another study where Cu ions also delayed hatching and explains why both nCuO and ionic Cu resulted in delayed hatching and not by other ionic metals (Vicario-Parés et al., 2014). Various other NMs have delayed hatching in zebrafish embryos (Bar-Ilan et al., 2009; Zhu et al., 2011; Lee et al., 2012). In these studies, scanning electron microscopy with EDS revealed the accumulation of NMs in the embryos and this accumulation inhibited the hatching process, where after hatching retardation can also directly disrupt normal embryo growth as seen in the present study.

Common observed effects of nCuO exposure are heart malformations, pericardial oedema, spinal curvature, end tail malformation, scoliosis and head malformations; and that small agglomerates at low concentrations and large agglomerates at high concentrations affected the embryo development (Ganesan et al., 2016). The present study saw significant dose-dependent pericardial oedema formation and the presence of spinal deformations in the nCuO treatment. Stressors and mutations cause severe pericardial oedemas and circulatory collapse in developing zebrafish (Chen, 2013). Cardiac output of nCuO treatments was found to be lower than that of the control and can be attributed to the presence of pericardial oedemas at that concentration (See Figure 4-5) (Chen, 2013; Sun et al., 2021). A significant increase in respiration was seen in the nCuO treatment group but was not linked to a decrease in heart rate due to oedemas, as respiration as these systems are not yet linked. Whereas the ionic Cu group did not have a significant impact on cardiac output (heart rate and blood flow). Ionic Cu had no effect on respiration, also not linked to an increase in heart rate. According to Pelster and Bagatto (2010) at the early stages of development in zebrafish embryos metabolic activity, oxygen consumption

and circulation/blood flow are not yet coupled in the early stages of zebrafish embryo development, and the direct relationship is established later in development after gills are formed 5dpf. This could explain the absence of corresponding data patterns at 48 h seen between heart rate, respiration and blood flow.

At the cellular level, it was found that for nCuO, the up-regulation of aucubin and D-xylose were distinctive metabolites of these exposures. D-xylose is part of the glucose metabolism that forms part of the carbohydrate metabolism responsible for energy production (Steele, 1981). Palmitic acid, nanodecane and aucubin up-regulation were distinctive of ionic Cu treatment. From pathway analysis, it was confirmed that the fatty acid metabolism was greatly impacted by all treatments except nAu. Plastic NMs were also found to disrupt fatty acid pathways in zebrafish (Marana et al., 2022). The fatty acid metabolism includes the digestion, transport, synthesis and oxidation of fatty acids (Oliveras-Rubio and Vega-López, 2016). Lipids and their constituent fatty acids are the principal organic constituents of fish, along with proteins, and they serve important roles as sources of metabolic energy for growth, reproduction and movement (Tocher, 2003). The effect on pentose and glucuronate interconversion pathway was unique to nCuO exposure and is responsible for detoxification processes, and the D-glutamine and D-glutamate metabolism are responsible for cell membrane stability (Sun et al., 2018).

The effect of nCuO and ionic Cu on metabolomics of *Enchytraeus crypticus* also influenced amino acids. It was found that the underlying mechanisms of nCuO toxicity (e.g., neurotransmission, nucleic acid generation, cellular energy, and immune defence) differ from ionic Cu toxicity pathways (lipid metabolism and fewer to amino acids) (Farré and Jha, 2020). In the present study, some corresponding pathways were influenced by both nCuO and ionic Cu, i.e., the phenylalanine, tyrosine and tryptophan biosynthesis pathway, as well as the phenylalanine metabolism. This corresponds to results by Ganesan et al. (2021) who found similar toxicity outcomes as observed in the present study with regards to hatching success and mortality linked to these pathways.

Cadmium and tellurium (quantum dots and ionic) toxicity

Similarly, to nAu, the QDs group showed the highest mortality at the lowest concentration (0.5 mg/L) and the lowest hatching rate at 2 and 4 mg/L. The light microscopy images that show QD agglomerates on the outer surface of the chorion at 20 mg/L were also reported in studies by Böhme et al. (2017), Zhao et al. (2013), Massarsky et al. (2013) and Zolotarev et al. (2012). This could cause decreased uptake since the NMs cover the chorion and prevent further particle uptake, which in turn impacts upon the transport of molecules between the internal and the external environments resulting in a delayed hatching or decreased survival (Massarsky et al.,

2013). Bimodal responses at the lower QD concentrations were noted for mortality, hatching success and oedemas. This is most likely attributed to smaller NMs crossing the chorion membrane and eliciting direct toxicological responses, whereas larger agglomerates at higher exposure concentrations blocking the membrane pores and eliciting indirect toxicity. Passage of NMs through chorion pores is random and consequently toxic effects are stochastic (Olasagasti et al., 2014). This bimodal variation in NM size as concentrations increase was demonstrated for the characterization of QDs in Chapter 2.

There appears to be a threshold concentration where the NM concentration is high enough to have an effect but low enough to not agglomerate and settle out. Quantum dots can show a possible threshold as oedema decreased at the high QD concentration, and mortality and lack of hatching success were also highest at the lower concentration. The present study saw QDs, Cd and Cu causing an increase in pericardial oedemas but at different concentrations indicating that metal exposure concentration does not significantly influence oedema formation. Similar to the present study Zhang et al. (2012b) found spinal deformations in embryos exposed to CdTe QDs and this did not correspond to Cd malformations, thus it is hypothesized that spinal deformation is unique to the QD particle exposure and not the Cd ion. Duan et al. (2013) found that QDs were responsible for the observed developmental toxicity and attributed the pericardia and yolk sac malformations, as well as bradycardia to accumulation of QDs in the intestine. According to LC₅₀ values of the present study, QDs were more toxic than ionic Cd and Te and supports the findings by Duan et al. (2013), King-Heiden et al. (2009), Xiong et al. (2011) and Zhang et al. (2012b) who also found that NMs were more toxic and produced toxicological endpoints distinct from that of ionic metal release. This is attributed in part to the smaller size of the QD, thus its bioavailability and cytotoxicity, as well as higher Te dissolution in the media attributed to toxicity as indicated by ionic Te exposures.

Eventhough dissolved Cd²⁺ does depolarise nerves and cause a tetanus contraction that you would see as a spasm in the embryo, quantum dots and ionic Cd had no significant effect on burst activity in the embryos. However, ionic Te significantly reduced embryo activity. Cadmium exposure in zebrafish embryos has been shown to inhibit the commitment of neural progenitor cells during brain development (Chow et al., 2008). Not much is known on NM induced behavioural changes but in other cases, silver NMs have been known to interfere with the normal activity of cardiac muscle, causing reduced blood flow to the brain and the spinal cord starving the cells of essential nutrients and gases leading to decomposition (Massarsky et al., 2013). It should be noted that respiration is a very sensitive parameter and technical differences may have contributed to these different values. If measured in a closed respirometer such as in the present

study, the critical oxygen concentration may be affected by the speed with which the oxygen declines in the respirometer chamber.

Considering that the heart rate patterns between the nanomaterials and their ionic metals were opposite from each other it can be assumed that it was not dependent on exposure to the ionic metal. Especially in the case of QDs, Cd and Te, the ionic concentrations of both metals were much higher (21 and 22 mg/L respectively) than the total metals measured in the QDs (1.7 and 0.06 mg/L from Chapter 2) but they had comparable uptake. This was also seen in studies by Griffitt et al. (2007), Xiong et al. (2011) and Thit et al. (2017). Possible metal release from particles in the gut of the organism at a low pH should not be ignored. The metal concentration in the target organ of the organism is also important to the observed effects. Unfortunately, the present study could not measure metal uptake in organs of such small organisms.

At the cellular level, QDs resulted in the down regulation of decane and palmitic acid and the up-regulation of silane. All significant pathways following QD exposure corresponded to ionic Cd and ionic Te treatments, indicating that modes of toxicity in the QD treatment are a combination of both metals, however, both ionic groups did alter more metabolites than QDs alone. The reason for this is unclear. Valine, leucine and isoleucine biosynthesis was significantly influenced in the QD treatment and not by ionic Cd and Te exposure. This pathway is responsible for the production of branched-chain amino acids that are building blocks for proteins, and leucine acts as a signalling molecule that activates the muscle protein synthetic machinery, which can cause malformations (Gorissen and Phillips, 2019) as observed in the present study. A study of QD and Cd exposure to mice postulated that due to metabolic effects, the toxicity of QDs was associated with other factors and not just dissolution toxicity from Cd (Khoshkam et al., 2018). Thus, particle toxicity should not be ruled out, as described in the previous paragraph.

4.5 Conclusions

Increased exposure concentration and ions from the media increases NM agglomeration, leading to varying uptake, which results in a bimodal toxicity response. Nanomaterials were found inside embryo tissues and the eyes however, CytoViva® was not effective in showing nCuO uptake and distribution. Effects of QDs on respiration are attributed to agglomerates on the chorion surface. The total Au uptake from nAu treatment was not significant from the control, but heart rate and respiration suggests toxicity via different modes other than direct metal exposure. Nano Au seems to act via pathways such as the glutamate metabolism and fatty acid metabolism to influence heart rate and activity. Copper uptake from both nCuO and ionic Cu was significantly different from the control but not from each other suggesting particles agglomerate in the mucus of the organism and only the dissolved fraction is translocated in the larvae. Nano CuO seems to act

through the glucose metabolism to affect energy production, while ionic Cu acts through fatty acid metabolism to influence energy production. Total metal uptake of through QDs and ionic Cd suggests that their uptake is influenced by a combination of bioavailability of the QD particles and mucus sequestration mechanisms. Quantum dots were the most toxic, followed by nCuO and then nAu. The highest total metal uptake was seen in the QD treatment and resulted in significant oedemas and spinal deformations. However, the different metabolic responses induced by the NMs and ionic Cd suggest that there are different modes of toxicity for these two Cd treatments. QDs influenced the valine, leucine and isoleucine biosynthesis resulting in protein and DNA damage linked to morphological effects seen in the study.

Metabolomic analysis indicate that there is not a one size fits all metabolite or pathway that can be ascribed to NM toxicity. Further studies need to be done to be able to establish definite metabolic biomarkers of exposure in zebrafish. Suggested pathways to focus on would be the fatty acid pathways, aminoacyl-tRNA biosynthesis, phenylalanine metabolism, purine metabolism, pentose and glucuronate interconversion pathway and the valine, leucine and isoleucine biosynthesis. The CoA biosynthesis, was only affected by the bimetallic NM treatment and should thus also be considered for QDs metabolic toxicity. Based on the endpoints studied in this chapter it is clear that sublethal exposures to NMs and ionic metals elicit some shared modes of toxicity, while other mechanisms may be unique to NMs or metal ions.

CHAPTER 5 BIOACCUMULATION KINETICS AND TISSUE DISTRIBUTION OF METAL-BASED NANOMATERIALS IN ADULT ZEBRAFISH

5.1 Introduction

Nanomaterials do not form a homogeneous solution when added to water; instead, they form a colloidal suspension that can aggregate, agglomerate, sediment out of the water column and adsorb to nutrients, all of which can impact their bioavailability when compared to ionic metals (Handy et al., 2008). Despite multiple investigations of NM toxicity in fish, there remains a crucial gap in knowledge about NM toxicokinetics, such as bioavailability, tissue distribution, accumulation, and depuration of total metal in fish after exposure to NMs. Metabolism and excretion studies are critical for enhancing our understanding of the effects of NM exposure and directing future toxicological investigations toward target tissues for further research (Jang et al., 2014). Direct passage through the gills or the epithelial cells of the intestine is the uptake route considered most likely in fish. Unlike metal ions, NMs are too large for ion, protein, or transepithelial transporters (Shaw and Handy, 2011). Rather, the accumulation and toxicity of NMs were found to correlate with several physicochemical properties of the NMs such as the shape, specific surface area, the primary particles size, surface potential (zeta-potential), and surface chemistry of the nanoparticles.

In fish, metal ions are removed from the circulation through the liver, which serves as the primary compartment for metal metabolism, with urine losses being relatively minor (Shaw and Handy, 2011). Metal ions may also be excreted from the systemic circulatory system by active efflux via ion transport channels in the gills, however, this would not be the case for NMs due to their size. Glomerular filtration by the vertebrate kidney has a molecular weight cut-off of around 60 kDa, indicating that NMs would not pass through. As a result, metal-based NMs are more likely to be excreted through the liver (Shaw and Handy, 2011; Handy et al., 2008; Barreto et al., 2020). Limited research on mammalian models has shown bioaccumulation of nAu highest in liver, kidney, and heart of rats (Rambanapasi et al., 2016; Abdelhalim, 2013). Furthermore, the capacity of NMs to reach interstitial spaces in organs is typically limited by large particle size and poor permeability across the capillary endothelium, particularly in organs with continuous or tight capillary junctions (e.g., brain, muscle, skin) (Aborig et al., 2019). They tend to accumulate in organs with sinusoidal or open capillary connections (e.g., the liver, spleen, and tumors) (Aborig et al., 2019). In zebrafish, the highest bioaccumulation of silver NMs was found in the liver (Xiao et al., 2020) and the clearance of metal-based NMs (nano-Fe₂O₃ and nano-Fe₃O₄) was

demonstrated to have a high elimination rate, 24 d post-exposure (Zhang et al., 2015b). Unlike mammals, fish are in constant contact with the exposure media and once excreted, NMs could be taken up by the fish again.

One of the most commonly studied metal-based NM in aquatic exposure media is nTiO₂ (Zhang et al., 2007; Zhu et al., 2010a). Due to the nature and variability of the NM distribution in exposure media, exposure time is critical when determining the harmful effects of metal-based NMs on aquatic organisms. The bioaccumulation of nTiO₂ and nZnO, and the ensuing mortality of brine shrimp (*Artemia salina*) increased dramatically following 96 h exposure compared to 24 h (Ates et al., 2013a; Ates et al., 2013b). Furthermore, following a 3 d exposure period, both cubic and octahedral Cu₂O micro/nanocrystals demonstrated Cu accumulation in the midgut of *Daphnia magna* (Fan et al., 2012). Given that aquatic organisms are likely to be exposed to NMs for an extended time, the uptake and accumulation by aquatic organisms will influence the overall toxicity of NMs. As a result, time-dependent exposure of aquatic organisms to metal-based NMs, as well as their clearance processes, should be investigated (Zhang et al., 2015a).

Modelling can be used to predict certain outcomes or effects and is useful because it can simulate processes to predict outcomes and minimize experimental costs. Thus, models can assist in regulatory decision-making. Toxicokinetic (TK) models together with the toxicodynamic (TD) aspects are traditionally used for pharmacological studies, but have successfully been used for quantitatively assessing the uptake, depuration and bioconcentration processes of NMs in aquatic organisms via water or dietary exposure (Gao et al., 2019; Grech et al., 2017; Larisch and Goss, 2018). Toxicokinetics links the external exposure concentration to the concentration at the target site, which is the biologically effective dose and helps to quantify toxicity and better understand the causes of variable toxicity between species or exposure compounds (Ashauer and Escher, 2010). The exceptional physicochemical characteristics and the TK process of NMs in biological media should be taken into account in toxicity studies (Gao et al., 2019). In contrast to traditional small molecule drugs, most NMs exhibit complex TKs *in vivo* due to the variety of these physicochemical and biological factors. Thus, the uncertainties in these factors limit model assessments and therefore biokinetic parameters depicting the interactions between NMs and aquatic organisms are limited (Xiao et al., 2020).

Bioaccumulation is critical in defining NM toxicity; however, the dynamics of NM bioaccumulation in aquatic species are poorly known (Xiao et al., 2020). The TK model is an accumulation model traditionally used to study trace metal uptake in organisms and is beneficial in quantifying the absorption, depuration, and bioconcentration processes of NMs in aquatic species via water or food exposure (Fan et al., 2018; Khan et al., 2015). However, little is known about how the physicochemical properties (e.g., partitioning coefficients and water solubility) affect protein

binding and biotransformation. Studies that focus on biotransformation in relation to bioconcentration in fish are scarce (Gao et al., 2019). Thus, the objectives of this chapter were to evaluate the bioaccumulation kinetics and tissue distribution of three metal-based NMs in fish by applying a basic TK model for three NMs (nAu, nCuO and QDs and their ionic equivalents Au, Cu, Cd and Te) in the gills, liver, intestine and muscle of zebrafish.

5.2 Materials and Methods

5.2.1 Experimental design

Fish Care

Wild-type adult zebrafish were maintained in the National Aquatic Bioassay Facility at the North-West University. Fish were fed dry feed (400-ZM, ZM systems, UK) twice daily. Fish were housed in 3.5 L capacity Techniplast fish tanks in bioactive system water and compressed air aeration. Water quality parameters were as follows for flow-through tanks: DO was 8.5–8.9 mg/L; pH was between 7.5 and 7.8 and EC was maintained between 620 and 660 μ S/cm. The water temperature was 27°C. Fish were maintained on a natural 12:12 light-dark cycle.

Experimental procedure

Nanomaterial stock solutions were sonicated for one h prior to use. Static renewal exposures were conducted consisting of a 6 d uptake period and a 6 d depuration period to determine accumulation and elimination kinetics of selected NMs and ionic metals in zebrafish. All exposures were conducted in Zebtec tanks with a total capacity of 3.5 L filled with 2.8 L exposure media (reconstituted system water) and aerated with air stones. The experimental set-up consisted of three tanks for each of the NM and metal treatments and ten fish (5 males and 5 females of 0.4 ± 0.2 g) were randomly added into each tank. Water quality parameters were kept constant throughout the exposure as mentioned in the section above. The fish were acclimated to test condition 24 h before the test. Ten fish per treatment, in triplicate ($n = 30$ fish) were exposed to NM suspensions for 6 d and then were placed in clean water for a further 6 d. Exposure concentrations were selected as a 10th of the larval zebrafish LC₅₀ as determined in this study, except for ionic Cu where the exposure concentration was selected as 20th of the LC₅₀ since the 10th would have been in an acutely toxic range of Cu for adult zebrafish as reported in the literature (Chen et al., 2011, Leung et al., 2014). Nanomaterial stock suspensions were sonicated as described in Chapter 2 and the appropriate amount of stock was added to each tank to make up the desired concentration.

Table 5-1 : Adult zebrafish exposure concentrations for each material based in embryo LC₅₀ values.

Material	Nominal exposure concentration ($\mu\text{g/L}$)
nAu	5100
Ionic Au (HAuCl_4)	85
nCuO	350
Ionic Cu (CuCl_2)	25
QDs	2100
Ionic Cd (CdCl_2)	2100
Ionic Te (TeCl_4)	2200

The exposure water was replaced (100% water change) every 48 h to ensure that NM suspensions remained relatively constant during the accumulation phase. Similarly, the water was exchanged with clean system water every 48 h during the depuration phase to prevent the accumulation of fish waste product in the water. All wastewater was collected and disposed of through standard wastewater disposal procedures. Fish were fed on alternate days, between medium renewal, with dry food (~2 mg dry food per fish). Test medium renewal took place after sampling on the day. The experiments were performed at room temperature ($27 \pm 1^\circ\text{C}$) with a natural 12:12 light-dark cycle.

Samples ($n=3$) were taken after the following time intervals: for uptake (0, 6, 24, 48, 96 and 144 h) and elimination (150, 168, 240 and 288 h). At each time interval, three zebrafish were randomly sampled (one from each tank), humanely killed by applying a light blow to the head and immediately severing the spinal cord. Fish were rinsed and dissected with clean instruments to remove the gill lamellae and arch, liver, intestine (posterior portions only) and a fillet of anterior muscle. Samples were frozen at -20°C until analyses. Water samples (11 mL) were collected from the mid-region of each tank for metal analyses at the same time interval that the fish were removed. In addition to the NM and metal treatment groups, a control group (NM-free system water) was set up, water and three fish were sampled on day 6 to determine the background values of total metal content in the fish tissues and exposure media.

5.2.2 Metal analyses in fish tissue and exposure media

For metal analysis in each organ, samples were weighed, freeze-dried and subjected to 24 h acid digestion in a heat block at 65°C (Böhme et al., 2017). The acid digestion protocol was adjusted for the different NMs and bulk metals following optimization of metal recoveries during the characterization of the materials. The Cd/Te and Cu (NMs and ionic metals) treatments were digested with 1 mL nitric acid (65%, suprapure, Merck), and Au (NMs and ionic metals) treatments were digested with 1 mL aqua regia (v/v HCl/HNO_3 , 3/1) and diluted to 10 mL with 1% nitric acid.

Concentrations are expressed as the average of the total metal concentration/dry weight of the tissue sample.

Water samples (11 mL) acidified with 250 μ L aqua regia for Au samples and 250 μ L 65% HNO₃ for other samples as described in Chapter 2. Analyses were done using a GF-AAS (PerkinElmer, AAnalyst 600) equipped with Zeeman-effect background correction. Tellurium samples were red on a single particle ICP-MS. Calibration of the instrument was performed using a series of 11 dilutions of the standard solution. With this, the concentrations of the sample metals were calculated using regression with a resultant correlation factor of 0.998. Both Cu and Cd showed recovery rates within the 20% deviation range based on the certified reference material (CRM DORM-4 fish protein homogenate). Detection limits for the samples were determined as three times the standard deviation of the blank measurement divided by the slope of the calibration curve multiplied by the average weight of each type of tissue.

Table 5-2 : Recovery rates of metals in the Certified Reference Material.

Metals	Recovery rate (%)	Mean (n = 3) detection limits and standard deviation for each organ and exposure media (μ g/g DW)				
	DORM-4	Gills	Liver	Intestine	Muscle	Media (μ g/L)
Au	n.a	0.74 $\pm$ 0.29	0.72 $\pm$ 0.27	1.89 $\pm$ 0.71	2.76 $\pm$ 1.04	0.82
Cu	102	2.75 $\pm$ 1	1.61 $\pm$ 0.76	5.41 $\pm$ 2.25	12.16 $\pm$ 4.08	0.12
Cd	99	0.03 $\pm$ 0.005	0.02 $\pm$ 0.005	0.05 $\pm$ 0.01	0.08 $\pm$ 0.005	0.1
Te	n.a	0.012	0.012	0.012	0.012	0.012

n.a - Not available.

5.2.3 Kinetic rate constants and bioconcentration factors

A prediction model implies that metals via different pathways contribute additively to total internal concentration, allowing for a process-based description of chemical concentrations in organisms' tissues or organs due to dosing concentration and exposure time (Tsai et al., 2013). Metal concentrations in different tissues were measured, followed by the model determination and testing. The present study focused only on the uptake of total metal via waterborne exposure routes and the actual measured metal concentration in the water was used as the exposure concentration and not the nominal exposure concentration. The following equations represent the TK model implemented in this study.

The elimination rate constant (k_e) was determined by fitting a nonlinear regression to the equation

$$C_t = C_0 e^{-k_e t}$$

where C_0 is the concentration of total metal in the tissue at the beginning of the depuration phase, C_t is the concentration at time t , and t is time (hours). The depuration phase started at time 150 h. The uptake rate constant (k_u) was calculated by fitting a nonlinear regression to the equation

$$C_t = (k_u/k_e)C_0(1 - e^{-k_e t})$$

using the values of k_e determined above and the bioconcentration factor (BCF) was calculated from k_u/k_e for each time point for the single concentration used (Tsai et al., 2013, Jang et al., 2014, Fan et al., 2018). This was calculated for gill, liver, intestine and muscle tissue, and for the linear phase as well as over the whole exposure period. The linear phase TK characteristics describe the greatest capacity of the organism to absorb, depurate and accumulate the chemical without being susceptible to saturation kinetics at very high concentrations or extended exposure time. Thus, the linear phase is modelled to the highest point of uptake. Parameters computed from the whole steady-state exposure describe accumulation curves measuring the asymptotic process of metal kinetics at larger concentrations or longer periods (Tsai et al., 2013). Thus, whole exposure period were modelled as well.

5.2.4 Statistical analysis

Quantitative data are reported as a mean $\pm$ standard deviation. Analytical replicates that returned values below the detection limit were replaced with half of the detection limit. Following the testing for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test), analysis of variance (one- or two-way ANOVA) with Tukey's test was conducted to determine the significance of differences in the metal accumulation profile datasets between the treatment and control groups. Results were considered statistically significant when p values were less than 0.05. Nonlinear regression was used to perform all curve fittings to raw data and to calculate the coefficient of determination (r^2), k_u and k_e using Python software. The fit was forced through 0 at the intercept.

5.3 Results

Exposure media

The Au water concentrations were stable over time (Figure 5-1 E). Ionic Au remained constant over time and stabilised following each water exchange and material replacement. The nAu concentrations were stable over time, however a significant decrease in nAu concentration can be seen after 144 h in the uptake phase. This is accompanied by a significant increase in Au concentration in the intestine (Figure 5-1 C). The Cu concentrations in the ionic exposures

remained constant (22 µg/L) for the duration of the uptake phase, while during the depuration phase it reduced to the same as the control. The Cu levels were significantly lower during all uptake nCuO exposure periods compared to the initial Cu in the nCuO that was added to the exposure media (Figure 5-2 E). These Cu levels also stabilised after 24 h exposure. The Cd concentration in the water of the QD treatment was stable at around 600 µg/L, for the first 48 h of exposure and then decreased significantly ($p < 0.05$) to 300 µg/L after 96 h, just before the water change. During the following 48 h the levels of Cd increased to 800 µg/L at 144 h (Figure 5-3 E). Following the depuration phase the Cd levels decreased to 20 µg/L Cd. The ionic Cd treatment resulted in a water concentration of 2 mg/L during treatment and depuration resulted in an average residual Cd concentration of 50 µg/L. The Te concentrations in the water increased with exposure time to 200 µg/L Te in the QD treatment and 2 mg/L in the ionic Te treatment groups (Figure 5-4 E). During depuration, all measured Te concentrations at the same levels as the control.

5.3.1 Metal uptake, elimination and biodistribution

The control group showed very low levels of Au in all organs (between below detection in the intestine and muscle and 0.4 µg/g in the liver). Copper in the control group ranged between 5 µg/g in the intestine and 75 µg/g in the gills. Cadmium in the control ranged from 0.9 µg/g in the muscle to 11 µg/g in the gills. Tellurium ranged between 5 µg/g in the muscle and 46 µg/g in the liver.

Uptake and elimination

Gold

The organs measured showed the same level of uptake and depuration concentrations between nAu and ionic Au despite a large variation in exposure concentrations between them. In the gills, nAu showed the highest uptake of metal and most Au depurated over time (Figure 5-1 A). An increase in metal concentration was seen in the gills as the water was changed and spiked (every 48 h), then there is a decrease in concentration over time. The liver showed high initial uptake but gradually decreased until the water was replenished and spiked again (Figure 5-1 B). At the end of the uptake phase, nAu showed a higher concentration in the liver compared to ionic Au (See Table S5-1). After depuration gold was removed from the liver to levels lower than the control. The concentrations of Au increased over time in the intestine and the highest concentration was seen in the nAu treatment (Figure 5-1 C). However, complete Au depuration was immediate (within 30 min) after transferring fish to clean water and they were only fed at 24 h depuration after samples were taken. High initial Au uptake was seen in muscle tissue for both ionic and nAu

treatments, however concentrations decreased over time in the ionic Au group but spiked in the end at 144 h in the nAu group (Figure 5-1 D). Higher concentrations of Au remained in muscle tissue of the ionic group (0.12 µg/L) at the end of the depuration phase than in the nAu group (0.01 µg/L).

Copper

The organs measured in this treatment also showed the same level of uptake concentrations between nCuO and ionic Cu despite exposure concentrations being very different. The gills showed an increase in total Cu concentration 24 h after water was replenished and spiked (48 h and 96 h) (Figure 5-2 A), while depuration is more gradual compared to the previous Au treatments. The liver showed an increase in concentration over uptake time in both groups and a gradual depuration of Cu over depuration time (Figure 5-2 B). Both groups showed a stable level of metal in the intestine over the uptake time and a steady decrease over depuration (Figure 5-2 C). The highest Cu concentration in the intestine was seen in the nCuO treatment group. An increase in Cu concentration was seen in the muscle over time with a steady decrease during depuration (Figure 5-2 D). Nano CuO resulted in the highest concentration Cu left in the muscle after depuration (2 µg/g), but all less than the control sample. See Table S5-2

Cadmium and tellurium

Cadmium concentrations in the gills increased over time in both QD and ionic Cd treatments (Figure 5-3 A). Depuration resulted in a decrease in concentration however, all Cd was not removed from the gills and both treatments resulted in a residual Cd concentration of 28 µg/g. Ionic Cd treatment resulted in the highest Cd concentration in the liver and the highest concentration left after depuration (25 µg/g) (Figure 5-3 B). Depuration in both treatments gradually decreased Cd over time. Both groups showed an initial spike of Cd concentration in the intestine but then decreased over time (Figure 5-3 C). Both resulted in an average concentration of 73 µg/g Cd in the intestine after depuration. The Cd concentration in the muscle tissue increased slightly over time but did not decrease in the ionic Cd treatment during the depuration phase as expected (Figure 5-3 D). See Table S5-3 and 4 for statistics.

Uptake and depuration of Te in the gills were higher in the QD treatment group than in the ionic Te group (Figure 5-4 A). Overall, the Te concentration decreased over time in both treatment groups. The liver showed significantly higher Te concentrations during QD treatment, but during depuration, concentrations were immediately decreased to control levels at 6 h depuration (Figure 5-4 B). Ionic Te treatment did not show significant uptake compared to the control. The intestine had much higher Te concentrations in the ionic Te treatment than in the QD treatment groups

(Figure 5-4 C). It was observed that the TeCl_4 suspension would make white residue flakes in the water, but would eventually dissolve after approximately 10 min. It was observed that the fish would feed on these flakes as the water was spiked and this would cause the high concentrations observed in the intestine. After depuration, the Te levels measured were the same as the control. In the muscle tissue, QDs resulted in the highest Te concentration but were efficiently depurated compared to the ionic Te treatment which resulted in more Te remaining in the muscle tissue (Figure 5-4 D). After 144 h depuration, Te concentrations in all organs were the same or lower than the control.

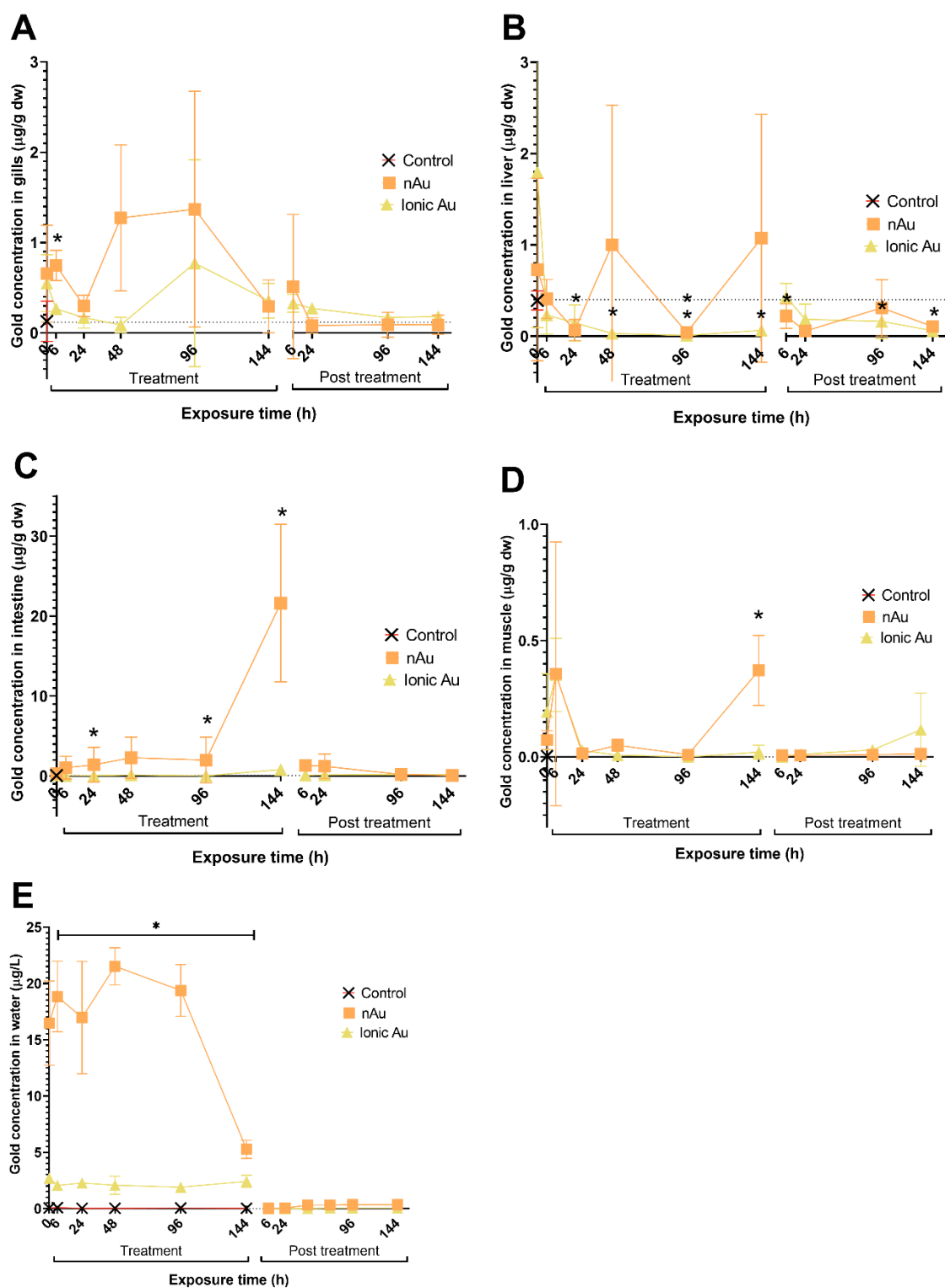


Figure 5-1 : Total Au from exposures to nAu and ionic Au treatment in the gills (A), liver (B), intestine (C), muscle (D) and water (E) of adult zebrafish. The error bars represent the standard error of replicate samples ($n = 3$). In the figure, for a given treatment, points with an asterisk indicate significant differences from the control at different time points determined by the two-way ANOVA followed by Tukey's test ($p < 0.05$).

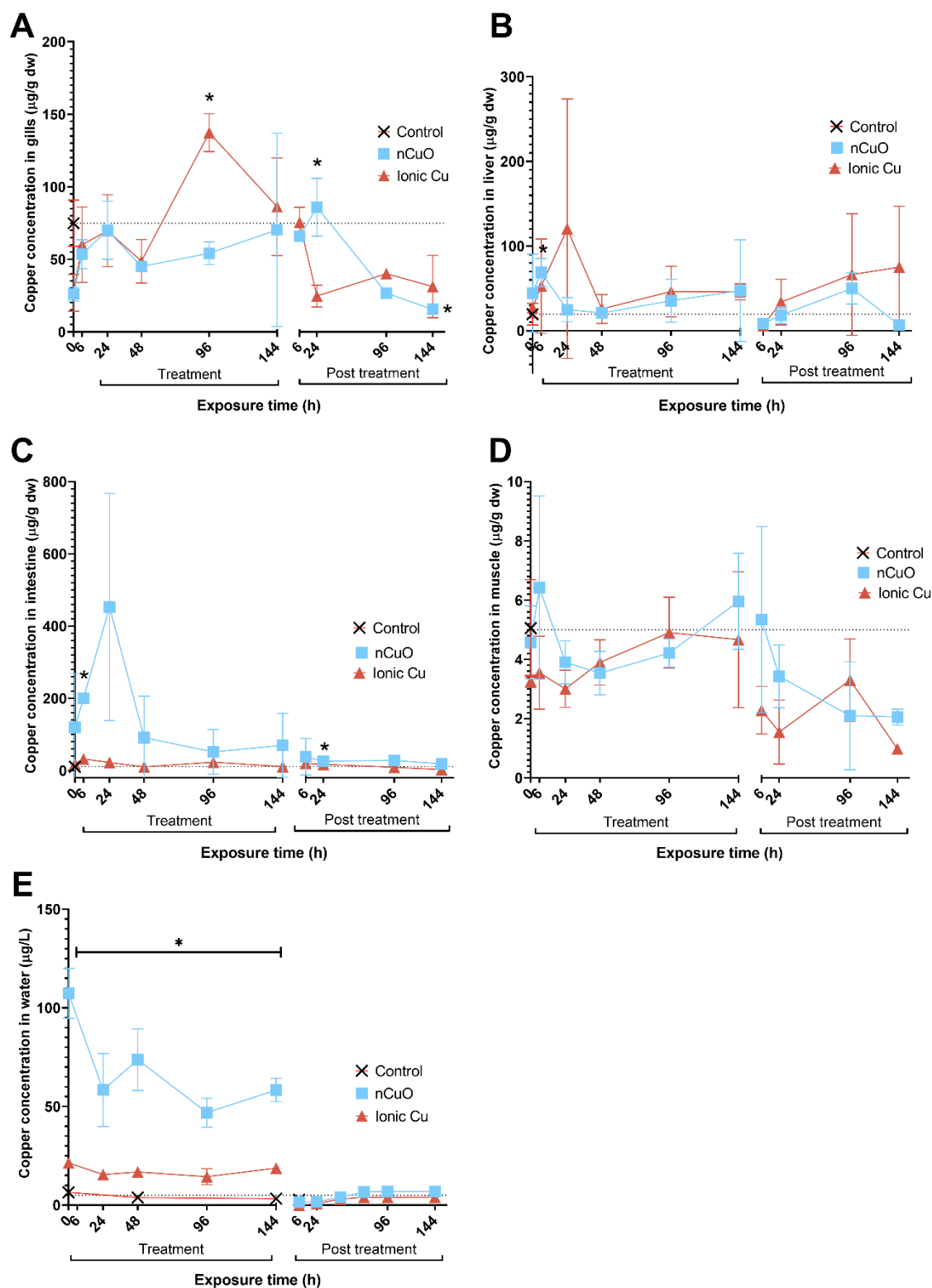


Figure 5-2 : Total Cu from exposures to nCuO and ionic Cu treatment in the gills (A), liver (B), intestine (C), muscle (D) and water (E) of adult zebrafish. The error bars represent the standard error of replicate samples ($n = 3$). In the figure, for a given treatment, points with an asterisk indicate significant differences from the control at different time points determined by a two-way ANOVA followed by Tukey's test ($p < 0.05$).

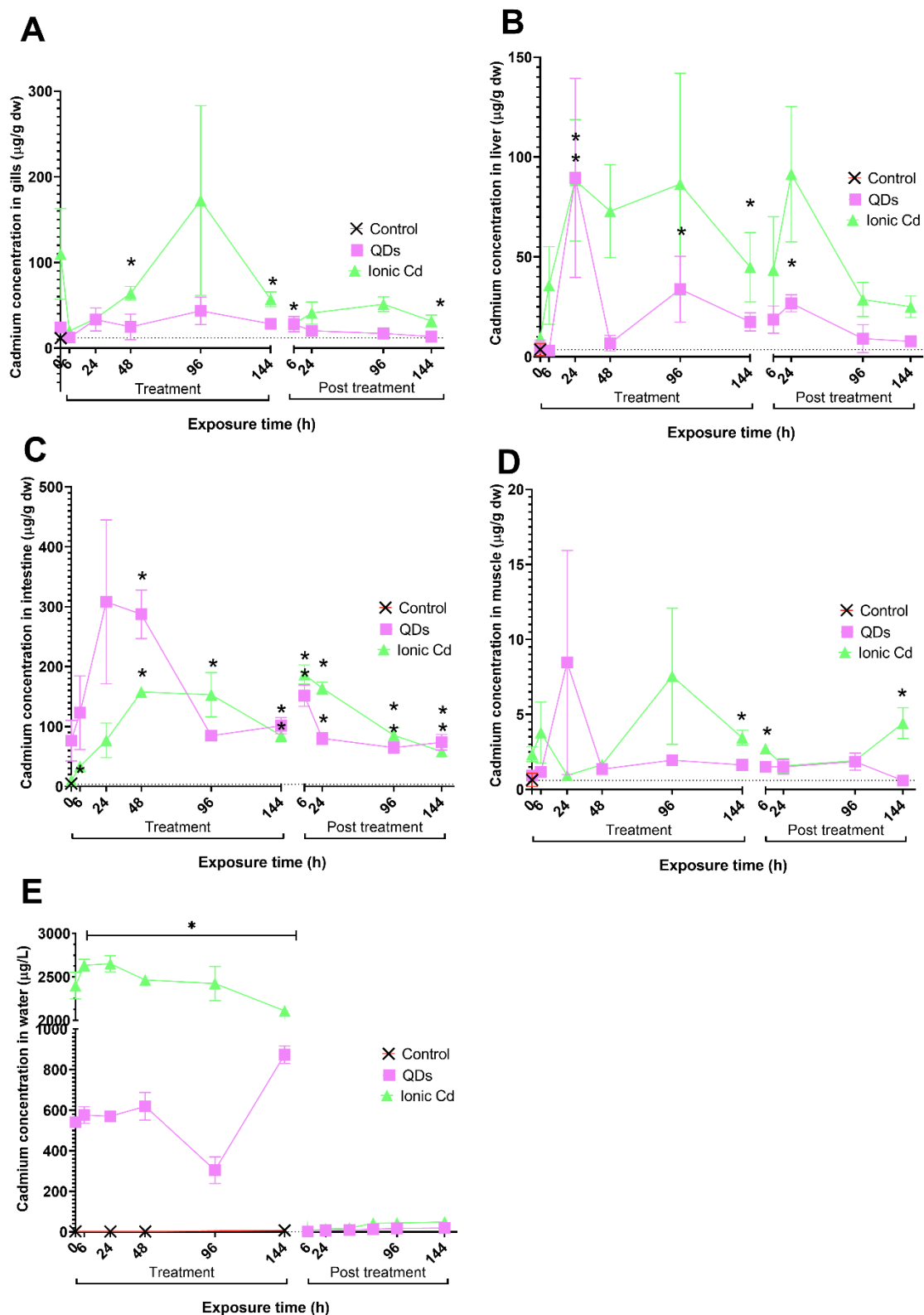


Figure 5-3 : Total Cd from exposures to QDs-Cd and ionic Cd treatment in the gills (A), liver (B), intestine (C), muscle (D) and water (E) of adult zebrafish. The error bars represent the standard error of replicate samples ($n = 3$). In the figure, for a given treatment, points with an asterisk indicate significant differences from the control at different time points determined by a two-way ANOVA followed by Tukey's test ($p < 0.05$).

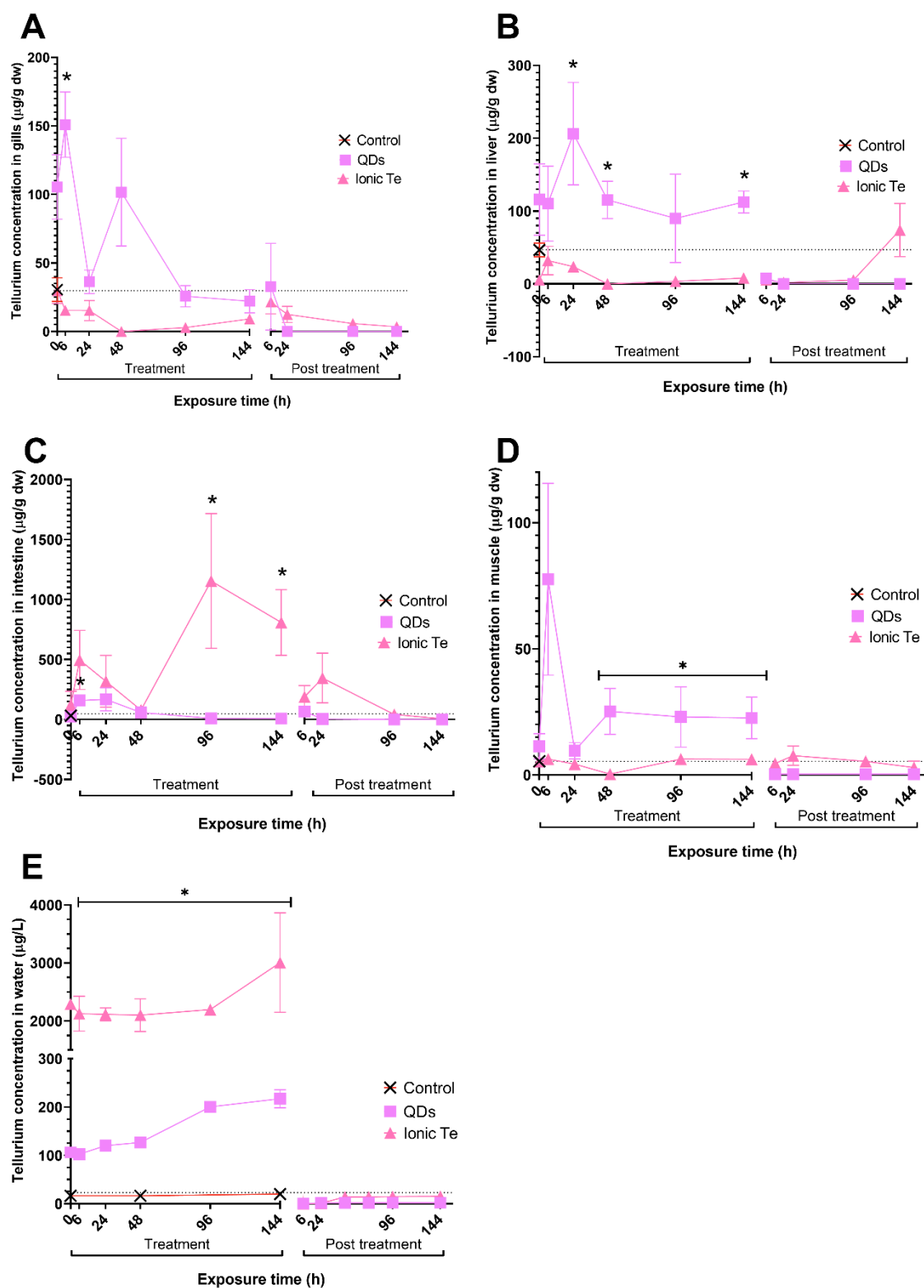


Figure 5-4 : Total Te from exposure to QDs-Te and ionic Te treatment in the gills (A), liver (B), intestine (C), muscle (D) and water (E) of adult zebrafish. The error bars represent the standard error of replicate samples ($n = 3$). In the figure, for a given treatment, points with an asterisk indicate significant differences from the control at different time points determined by a two-way ANOVA followed by Tukey's test ($p < 0.05$).

Tissue distribution of metals

Figure 5-5 indicates the total metal uptake after the uptake phase in all four organs for each treatment, as well as the ratio of metal between these organs. Total uptake was higher in the nAu group (Figure 5-5 A) than in the ionic Au (Figure 5-5 B) group most likely due to the higher exposure concentration in the nAu treatment. Overall metal was highest in the intestine and liver for nAu treatment and intestine and gills for ionic Au treatment. Total Cu uptake between nCuO (Figure 5-5 C) and ionic Cu (Figure 5-5 D) only differed by 44 µg/g, but distribution showed the highest uptake in the gills and intestines after nCuO treatment and in the gills and liver for ionic Cu treatment. Ionic Cd (Figure 5-5 F) and QDs (Figure 5-5 E) showed similar total Cd uptake. Both QDs and ionic Cd showed high concentrations in the intestine, but ionic Cd showed similar distributions between the liver and gills. Ionic Te (Figure 5-5 H) showed much higher total Te uptake but 97% of this was due to the high concentration in the intestine, if the intestine is excluded QDs (Figure 5-5 G) resulted in higher uptake of Te, where 67% was found in the liver. This is the highest fraction of metal found in the liver and muscle tissue of all treatments. The same fraction of Te was found in the gills, liver and muscle of the ionic Te group (See Tables S5-3,4).

5.3.2 Toxicokinetic Model

Figure 5-6 and 5-7 reveals the comparison between the fitting curves of the bioaccumulation model to the linear phases of the metal profiles and their predictions for the entire exposure duration in the selected tissues/organs. For each organ, the model only fit the nAu intestine, ionic Au intestine, ionic Cu liver, ionic Cd liver, ionic Cd intestine and ionic Te intestine profiles well ($r^2 = 0.63$ to 0.90). Even though the model did fit the data well, the model predictions deviated from the experimental data when metal concentrations exceeded a certain level in the gills, and muscle. From the linear uptake phases (referring to the section of the curve where maximum uptake occurs), values of organ-specific k_u ranged between 0.00005 and 0.213 L/g/h. The highest k_u occurred in the muscle for QD-Te, followed by the liver for ionic Cu and Te-QDs, and the gills for ionic Cu and Te-QDs (Table 5-3). For nAu, the highest k_u was in the intestine and for ionic Au the highest k_u was in the muscle. Nano CuO showed the highest in the intestine and for ionic Cu the highest was in the liver. Cd-QDs the highest was in the intestine, for ionic Cd all organs had the same k_u , for Te-QDs the highest was in the muscle and for ionic Te the highest was in the intestine. Overall, ionic Cu and Te-QDs had the highest k_u .

Only 9 BCF values of the target organs were above one in the linear phase, while the values ranged from 0.002 to 11.5 (Table 5-3). showing that their potential to accumulate these different materials is low and can vary. The $BCF > 1$ is indicated in grey. The intestine is characterized by

the highest average BCF values (ranging from -0.25 to 9.14). The highest BCF value was found for ionic Cu in the gills (11.33) and nCuO in the intestine (60.51). In general, the muscle had the lowest ability to accumulate but had the highest BCF for both Cd and Te from QD treatments. Results showed that the k_u , k_e , and BCF of fish tissues/organs were dependent upon the treatment and exposure duration. Negative k_e values (increase in concentration during depuration) were seen in both Cu groups in the liver, ionic Te in the liver, ionic Au in the intestine and ionic Cd in the muscle. No general pattern was observed in the change in k_u of organs, only that uptake potential was higher for the ionic metals than the NMs, except QDs that had higher k_u values than ionic Cd and Te. See Tables S5-1 to S5-4 at 96 h for statistics.

The relatively higher r^2 values of the model fits for the intestine and liver suggested that the ionic metal concentrations in these organs were well described by the model. This indicated that the uptake rate and depurate rate constants for the intestine and liver were relatively insensitive to changes in ionic metal concentration. The results revealed that the assumption of constant TK processes could lead to incorrect predictions by overestimating (linear phase) or underestimating (entire exposure) the metal concentrations in most tissues/organs of zebrafish under short exposure scenarios, especially in NM treatments due to varying uptake patterns that are not linear with time.

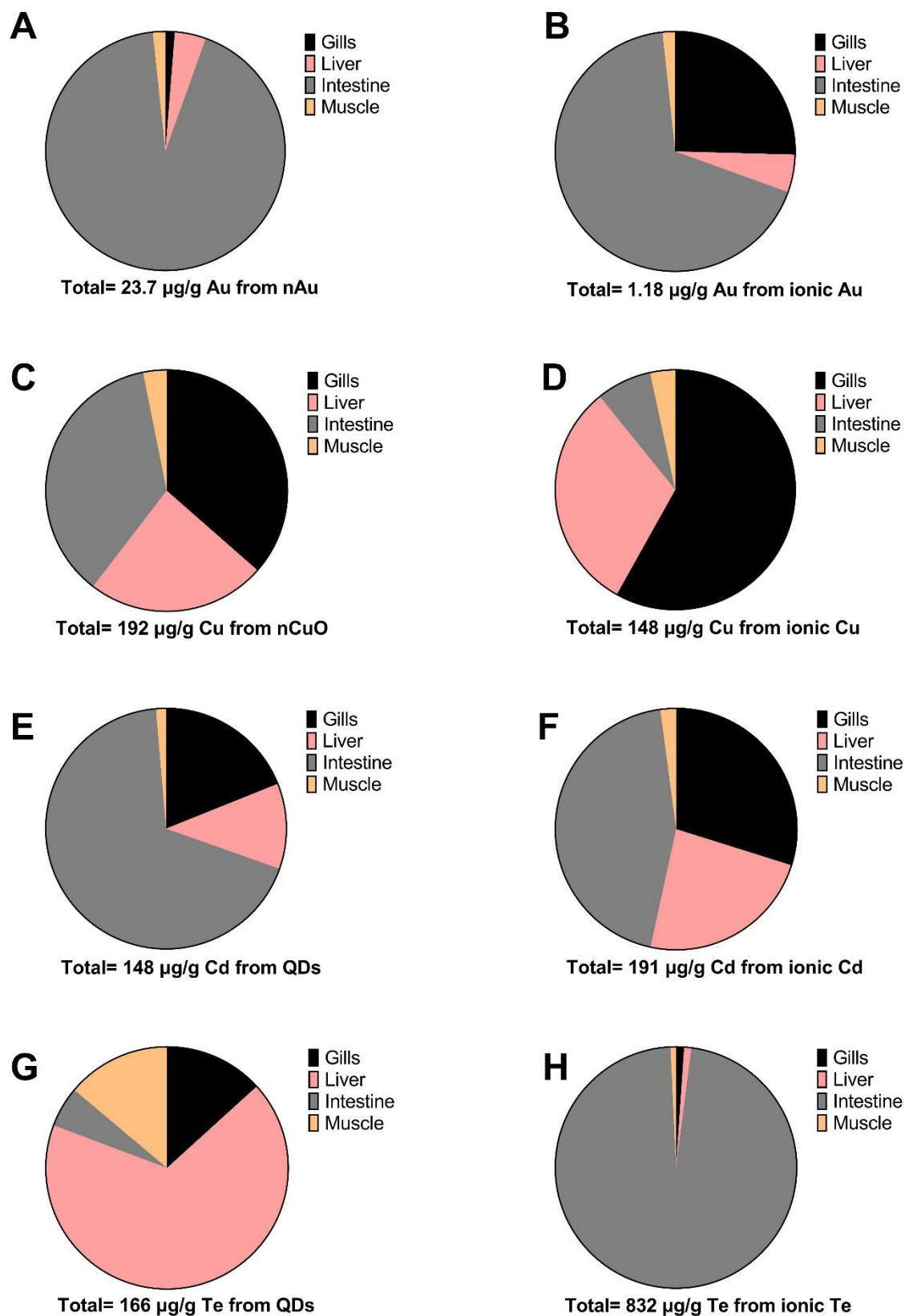


Figure 5-5 : The total metal concentration ($\mu\text{g/g dw}$) in the gills, liver, intestine and muscle samples of zebrafish treated with nAu (A), ionic Au (B), nCuO (C), ionic Cu (D), Cd-QDs (E), ionic Cd (F), Te-QDs (G) and ionic Te (H) at the end of the 6-day uptake period.

Table 5-3 : Comparison between estimated values (mean $\pm$ SE) of waterborne metal uptake rate (k_u), depuration rate (k_e), and bioconcentration factor (BCF) in selected organs of adult zebrafish in various waterborne treatment levels for the entire 12-day exposure duration and all linear accumulation phases.

Organ	Linear phase ^a				Entire exposure		
	Fitted hours ^b	k_u (L/g/h)	k_e (/h)	BCF ^c (L/g)	k_u (L/g/h)	k_e (/h)	BCF ^c (L/g)
Gills							
nAu	0-96	0.003 $\pm$ 0.002	0.019 $\pm$ 0.031	0.158	0.002 $\pm$ 0.001	0.019 $\pm$ 0.027	0.084
Ionic Au	0-24	0.005 $\pm$ 0.0004	0.005 $\pm$ 0.003	1	0.002 $\pm$ 0.001	0.005 $\pm$ 0.003	0.492
nCuO	0-96	0.067 $\pm$ 0.002	0.007 $\pm$ 0.001	9.03	0.04 $\pm$ 0.001	0.007 $\pm$ 0.001	5.71
Ionic Cu	0-144	0.102 $\pm$ 0.032	0.009 $\pm$ 0.002	11.33	0.086 $\pm$ 0.008	0.009 $\pm$ 0.002	9.81
QDs Cd	0-144	0.001 $\pm$ 0.001	0.006 $\pm$ 0.005	0.189	0.001 $\pm$ 0.0005	0.006 $\pm$ 0.005	0.151
Ionic Cd	0-24	0.001 $\pm$ 0.0001	0.004 $\pm$ 0.002	0.167	0.0003 $\pm$ 0.0001	0.004 $\pm$ 0.002	0.078
QDs Te	0-24	0.185 $\pm$ 0.187	0.374 $\pm$ 0.443	0.495	0.151 $\pm$ 0.153	0.374 $\pm$ 0.383	0.404
Ionic Te	0-24	0.0005 $\pm$ 0.0003	0.025 $\pm$ 0.024	0.02	0.0001 $\pm$ 7E-05	0.025 $\pm$ 0.024	0.004
Liver							
nAu	0-144	0.001 $\pm$ 0.001	0.003 $\pm$ 0.002	0.333	0.0004 $\pm$ 0.0002	0.003 $\pm$ 0.002	0.144
Ionic Au	0-24	0.004 $\pm$ 0.004	0.029 $\pm$ 0.022	0.138	0.001 $\pm$ 0.0003	0.029 $\pm$ 0.023	0.026
nCuO	0-24	0.036 $\pm$ 0.001	-0.008 $\pm$ 0.001	-4.84	0.011 $\pm$ 0.0003	-0.008 $\pm$ 0.001	-1.38
Ionic Cu	0-96	0.192 $\pm$ 0.255	-0.018 $\pm$ 0.001	- 10.67	0.005 $\pm$ 0.002	-0.018 $\pm$ 0.001	-0.309
QDs Cd	0-24	0.005 $\pm$ 0.005	0.005 $\pm$ 0.005	1	0.001 $\pm$ 0.0001	0.005 $\pm$ 0.004	0.11
Ionic Cd	0-144	0.001 $\pm$ 0.0003	0.008 $\pm$ 0.001	0.131	0.001 $\pm$ 9.9E-05	0.008 $\pm$ 0.001	0.073
QDs Te	0-24	0.185 $\pm$ 0.187	0.374 $\pm$ 0.443	0.495	0.151 $\pm$ 0.153	0.374 $\pm$ 0.383	0.404
Ionic Te	0-24	0.0003 $\pm$ 0.0001	-0.004 $\pm$ 0.007	- 0.075	2E-05 $\pm$ 5E-06	-0.004 $\pm$ 0.006	-0.005
Intestine							
nAu	0-144	0.014 $\pm$ 0.007	0.026 $\pm$ 0.025	0.538	0.014 $\pm$ 0.006	0.026 $\pm$ 0.021	0.529
Ionic Au	0-144	0.001 $\pm$ 0.001	-0.004 $\pm$ 0.002	-0.25	0.001 $\pm$ 0.001	-0.004 $\pm$ 0.002	-0.277
nCuO	0-24	0.83 $\pm$ 0.019	0.014 $\pm$ 0.016	60.51	0.113 $\pm$ 0.004	0.014 $\pm$ 0.014	8.24
Ionic Cu	0-24	0.064 $\pm$ 0.033	0.007 $\pm$ 0.002	9.14	0.014 $\pm$ 0.005	0.007 $\pm$ 0.003	1.95
QDs Cd	0-24	0.027 $\pm$ 0.01	0.009 $\pm$ 0.005	3	0.004 $\pm$ 0.001	0.009 $\pm$ 0.005	0.488
Ionic Cd	0-144	0.001 $\pm$ 0.0003	0.008 $\pm$ 0.001	0.127	0.001 $\pm$ 9E-05	0.008 $\pm$ 0.001	0.071
QDs Te	0-24	0.103 $\pm$ 0.057	0.055 $\pm$ 0.001	1.87	0.026 $\pm$ 0.012	0.055 $\pm$ 0.001	0.473
Ionic Te	0-144	0.005 $\pm$ 0.001	0.009 $\pm$ 0.01	0.556	0.004 $\pm$ 0.001	0.009 $\pm$ 0.009	0.501
Muscle							
nAu	0-144	0.011 $\pm$ 0.014	0.374 $\pm$ 0.443	0.029	0.005 $\pm$ 0.006	0.374 $\pm$ 0.383	0.0143
Ionic Au	0-24	0.085 $\pm$ 0.028	1 $\pm$ 0	0.085	0.036 $\pm$ 0.012	1 $\pm$ 0	0.036
nCuO	0-144	0.007 $\pm$ 0.0002	0.01 $\pm$ 0.005	0.71	0.003 $\pm$ 0.001	0.01 $\pm$ 0.004	0.367
Ionic Cu	0-144	0.005 $\pm$ 0.003	0.001 $\pm$ 0.0006	5	0.003 $\pm$ 0.001	0.001 $\pm$ 0.0004	1.85
QDs Cd	0-144	0.001 $\pm$ 0.001	0.334 $\pm$ 0.471	0.003	8.2E-04 $\pm$ 0.001	0.334 $\pm$ 0.471	0.002
Ionic Cd	0-144	2E-05 $\pm$ 2E-05	-0.001 $\pm$ 0.004	-0.03	1E-05 $\pm$ 8E-06	-0.001 $\pm$ 0.003	-0.011
QDs Te	0-24	0.213 $\pm$ 0.215	0.667 $\pm$ 0.471	0.319	0.002 $\pm$ 0.001	0.019 $\pm$ 0.027	0.084
Ionic Te	0-96	6E-05 $\pm$ 5E-05	0.005 $\pm$ 0.003	0.012	3E-05 $\pm$ 4E-06	0.005 $\pm$ 0.003	0.005

^a The linear phase is defined as the metal concentration profile from the beginning of exposure (day 0) to the highest data point when the accumulative metal concentration is statistically judged as steady-state status

^b Maximum time from three replicates

^c Organ-specific bioconcentration factor (BCF) can be calculated as $BCF = k_u/k_e$

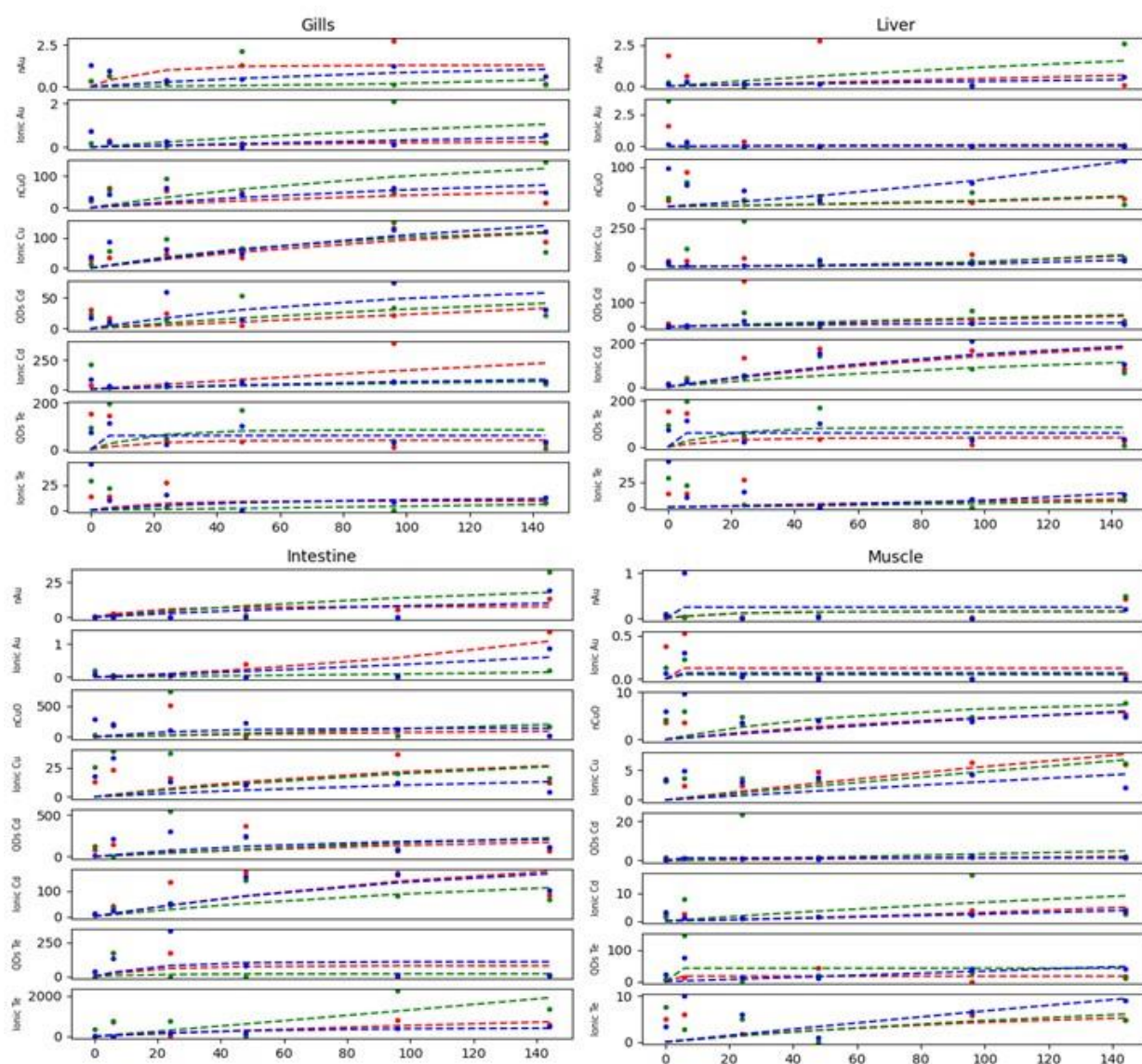


Figure 5-6: Time series of metal accumulation ($n=3$ individually plotted) in the gills, liver, intestine and muscle of zebrafish adults exposed to selected waterborne nAu, ionic Au, nCuO, ionic Cu, QDs, ionic Cd and ionic Te for 6 days. Fitted lines show the comparison between the TK model predictions for the linear phase (dashed lines)

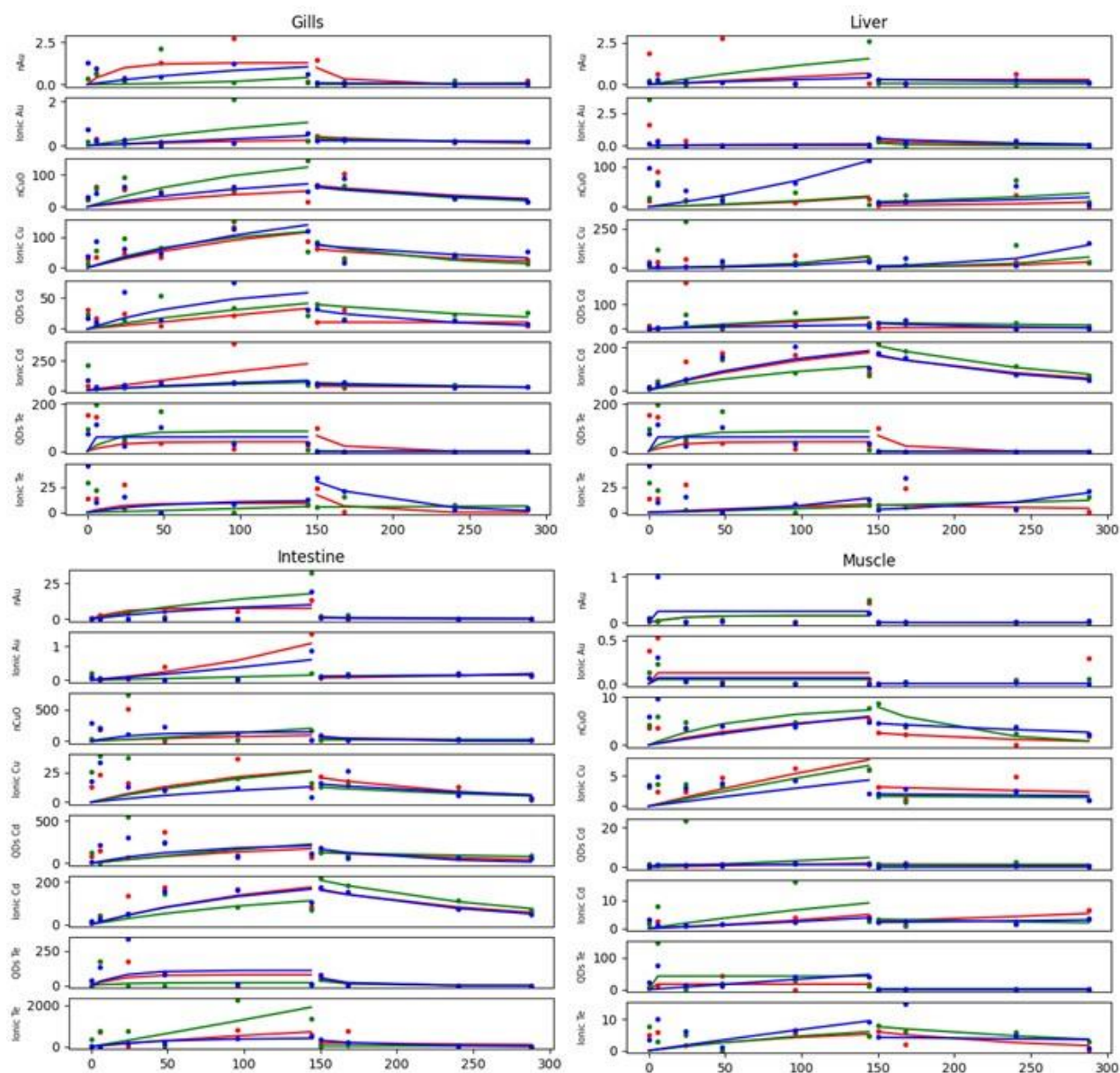


Figure 5-7: : Time series of metal accumulation and depuration ($n=3$ individually plotted) in the gills, liver, intestine and muscle of zebrafish adults exposed to selected waterborne nAu, ionic Au, nCuO, ionic Cu, QDs, ionic Cd and ionic Te for 6 days and 6 days depuration. Fitted lines show the comparison between the TK model predictions for the entire exposure duration (solid lines).

5.4 Discussion

Uptake and biodistribution

In the present study the uptake, depuration and biodistribution of total metal from nAu, nCuO and QDs in some organs, and their ionic metal equivalents was determined. Results indicate similar metal concentrations are accumulated from NMs and ionic exposures, regardless of the initial exposure concentration in the external media. Griffitt et al., 2009 also reported copper

accumulation in the gill was similar following exposure to both soluble and nanoparticulate Cu; and the authors interpreted this as uptake of dissolved Cu from the Cu-NP. Organs directly linked to routes of exposure such as the gills and intestine show high initial uptake but immediate depuration of all metals when the fish was transferred to clean water. This means they were surface bound, easily washed out and not indicative of true uptake. When considering biodistribution, exposure to both NM and ionic metals resulted in metal accumulation primarily in the gills, liver and intestine. However, differences in biodistribution between NMs and ionic metals can be seen. Since agglomerates of various sizes were present in the media after exposure, the heterogeneity in NM size distribution could have had an influence on metal accumulation by individual fish within the same treatment. Although sonication before exposure resulted in a homogeneous dispersion of NMs, there was rapid agglomeration and subsequent sedimentation in the water (see in Chapter 2). Aeration of the exposure media during the exposure can also contribute to agglomeration.

The concentrations of Au were lower in tissues of zebrafish from the ionic Au treatment group compared to the nAu treatment. However, despite the large differences in exposure concentrations (5.1 mg/L nAu and 85 µg/L ionic Au), there weren't any significant differences in total Au uptake. This was also seen in the other metal-based NMs and ionic equivalents in this study. It is hypothesised that this can be due to particles trapped in the mucus and only the dissolved ion fraction is taken up by the fish. Botha et al. (2019) demonstrated that following initial exposure to nAu, there was a 6-fold increase in the expression of MT genes with subsequent sequestration in the musculature of zebrafish. This active regulation and detoxification of Au in the present study can be seen in the gills, where there is an initial spike in concentration followed by a decrease over time. An alternative is that particles in the gill mucus are slowly deposited as mucus is replenished due to gill irritation. Barreto et al. (2020) suggests that it is also likely that agglomeration of NMs at these concentrations make them less bioavailable for uptake than ionic Au. The liver showed a bimodal accumulation pattern while Au in both the intestine and muscle showed a significant increase in Au concentration after 144 h of exposure to nAu. It is possible that the gut mucosa acts as a barrier trapping the NMs and only ionic metal is taken up to a certain point leading to a bimodal response as exposure media is replenished. This corresponds to results by Zhu et al. (2010b) that found the highest Au accumulation in the intestine and gills of Japanese medaka treated with 4 differently functionalized nAu materials in exposure water. Gold has been found in zebrafish brain and liver after dietary exposure however, only brain, liver and muscle samples were analysed (Geffroy et al., 2011). In zebrafish exposed for 20 d to sediment spiked with 14 nm nAu, Au was detected in the gills and intestine but not in the brain and muscle (Dedeh et al., 2015).

The increased Au in the muscle tissue of zebrafish is ascribed to the reaction of the gold surface to form Au–S (thiol) bonds via the oxidation–reduction reaction, (Kataba et al., 2022)., while the increased levels in the intestine is explained by increased uptake through feeding. It has been established that metal concentrations in the digestive tract rise due to dietary intake and will remain high until the fish are placed in clean water and depuration takes place (Jezierska and Witeska, 2006). Besides the intestine, the largest percentage of total Au uptake from nAu after 144 h was seen in the liver. This corresponds to a study by Geffroy et al. (2011) that found the highest concentration of nAu in the liver of zebrafish after dietary exposure to nAu. Barreto et al. (2019) have also reported unexpectedly high levels of Au in the liver after nAu treatment. This is most likely due to the transportation of metal via via blood through the hepatic portal vein from the gut to the liver (Eliason et al., 2007). The low Au accumulation in the intestine from the ionic Au exposure group suggested uptake through the gills. Total Au uptake from the ionic Au treatment after 144 h also showed distribution in the intestine and liver. Similar findings were also reported in the seabream after ionic Au treatment (Barreto et al., 2020).

Copper is an essential micronutrient that serves as a catalytic cofactor in enzymes involved in various vital processes, such as cytochrome c oxidase, which plays a key role in the electron transport chain for cellular respiration, and Cu/Zn superoxide dismutase, which is crucial for the removal of free radicals (Leung et al., 2014). Despite no significant uptake compared to the control the results do show clear uptake and depuration patterns of Cu in both nCuO and ionic Cu treatments. The Cu depuration rate is more gradual than the Au depuration rate, this slow elimination rate suggests that the absorbed Cu is highly bound to tissues (Zarco-Fernández et al., 2016). Distribution patterns show that Cu accumulates in the gills and intestine for nCuO treatment and the gills and liver for ionic Cu treatment. Copper is actively transported from the gut epithelial cells into the blood via the vesicular Cu-ATPase and chloride-dependent efflux pathways (Handy et al. 2000). It is also transported from the gills via the blood to the liver in the case of NMs (Handy et al., 2008). In other studies, gill Cu levels were also similar in nCuO treatments (likely from dissolved Cu from NMs) and ionic Cu treatments indicating low cellular uptake of nCuO (Griffitt et al., 2007; Shaw and Handy, 2011). Ates et al. (2015) showed accumulation of Cu following nCuO exposure in the intestine, liver and gills of goldfish, however no significant increase in concentrations in muscle, heart, or brain after dietary (or waterborne) exposure. Ultimately, the accumulation of NMs in the gills may impair the efficiency of the gills as respiratory organs due to gill damage such as hyperplasia and telangiectasia (Utembe et al., 2018). The results in the present study also corresponds to results by Shaw et al. (2012) that also found no significant uptake in organs other than the gills of rainbow trout. Differences in observed uptake between nCuO and ionic Cu are likely due to NMs being too large for solute transporters,

such as those used for Cu ions (Ctr1 and Cu-ATPases), and any intact NMs would be taken up by slower endocytosis pathways (Lutsenko, 2016).

That was particularly evident for Cu and Cd. Metal levels in the liver rapidly increase during exposure and remain high even during the depuration phase (Jezierska and Witeska, 2006). This can be seen in both Cu groups and Cd groups in the present study. Significantly high concentrations of Cd were seen in both QD and ionic Cd treatments in the liver and intestine. In both the nAu and QD treatments, the total Au and Cd content in the zebrafish intestine was higher than that in the gills, suggesting that the uptake via oral ingestion outcompeted gill uptake and is the dominant pathway for the bioaccumulation of these materials in the zebrafish. Similar findings were reported for nAg in zebrafish (Xiao et al., 2020).

In the bimetallic CdTe QDs dissolution results (see Chapter 2) showed that there was a higher Te dissolution. Thus, one would expect the Te accumulation to be due to Te ions, while Cd uptake would be predominantly due to NM-based exposure. The slow elimination rate suggests that the Cd is tightly bound to possibly MTs and MT-like proteins (Peyrot et al., 2009) and sequestered in tissues, especially the muscle tissue. A study by Zarco-Fernández et al. (2016) showed similar results with Cd be highest in muscle tissue of zebrafish following exposure to CdSe QDs. Notable was that Cd concentrations in the different tissues were very similar even though the actual Cd measured in the exposure media differed by an order of magnitude. This would suggest that irrespective of the external Cd exposure concentration, uptake was most likely mediated by mucus sequestration.

Background Te concentrations were found to be quite high for a non-essential element (40-50 µg/g) in the gills and liver of untreated control fish and support the findings of Zare et al. (2017). A study by Owkhonda (2016) reported Te levels in *Tilapia guineensis* and *Sarotherodon melanotheron* from the Bodo River at 12 µg/g and 7 µg/g respectively. However, not much is known about the biological relevance of Te but notable concentrations of Te in humans were traced in the bone tissue, muscle and liver (Vávrová et al., 2021; Zare et al., 2017; Ogra, 2017). After two weeks of tellurium dioxide administration in rats, the largest concentrations of Te were found in the heart, followed by the kidney, spleen, bones, and lungs, with amounts barely detectable in the brain, liver, and muscles (Taylor, 1996). In the present study, the most significant Te uptake was seen in the liver for QDs and the intestine for ionic Te treatments. Both materials were immediately depurated from all organs once fish were placed in clean water, indicating that Te was not highly bound to tissues as was the case for Cu and Cd. The high Te concentrations found in the intestine of the zebrafish in the ionic Te treatment is attributed to direct oral uptake (consumption) of the white TeCl₄ precipitate that formed with initial dosing. Interestingly the gills seemed to be the main uptake route for Te during the QD treatment assay. This is a phenomenon

that is usually associated with exposure to metal ions and uptake is across the respiratory surface and then distributed to the other tissues via the circulatory system (Chen et al., 2017). Oral ingestion formed an important uptake route for QDs since fish may have mistaken the precipitated NM agglomerates as food as was also reported for zebrafish following exposure to AgO (Xiao et al., 2020). The high concentrations of Te in the liver suggests that following uptake by the gill (for the QD-bound Te) and intestine (for the precipitated Te salts), it is transported through the circulatory system to the liver.

In this study the total metal concentrations were measured in the organs. Nanomaterial absorption, toxicokinetics, and biotransformation by organisms all influence their environmental fate. There is potential dissolution of QDs and release of Cd and Te ions in the extra- and intercellular media in the aquatic environment. This can occur under the acidic conditions of the digestive system or inside the lysosomes as demonstrated for bivalves and fish, thereby promoting different exposure scenarios after QD interaction with organisms (Rocha et al., 2017). Boyle et al. (2020) demonstrated this further when it was found that dissolution of nCuO was higher at a low pH in the gut resulting in increased uptake of free ions from the gut lumen rather than uptake of intact NMs. Thus, exposure to both QDs and nCuO could lead to free ion uptake due to the dissolution of NMs in the gut.

Toxicokinetics

The bioaccumulation kinetics of nAu, nCuO, QDs and their ionic metals were evaluated by using data from the uptake and depuration study to construct a TK model for the selected zebrafish organs (gills, liver, intestine and muscle). The model did not fit the data in some cases indicating a different model should be used other than first order kinetics. R^2 is low in the gills and intestine. Indicating that more than a third of the variation is caused by a factor not measured in the expt. This is a common problem with curve fitting.

Often divalent metals that change valency with uptake cannot be described as by first order kinetics because there is dissociation of Cu^{2+} to Cu^{+} , then another step of Cu^{+} uptake at the membrane, then different efflux pathways. So it can be second order or even third order kinetic fit instead. This is a crucial limitation that needs to be addressed.

Results indicated that uptake potential was highest for ionic metals, except in the case of QDs. According to the model the intestine had the highest bioaccumulation ability and muscle had the lowest accumulation ability, except for QDs. It could be that the intestine was not rinsed thoroughly enough to get rid of loosely bound material. The model only fits well for ionic metals in the intestine and liver. Probably because the data points are spread out enough and detectable enough to get

a fit. Thus, assumptions made by the model regarding uptake kinetics that remain the same over time, bimodal uptake of NMs lead to underestimation of metal in organs/tissues. The model also assumes a constant level of exposure and does not consider physicochemical changes of NMs over time. It is also important to note that any BCF for NMs is calculated on the basis of total metal concentration in the tissue (Handy et al., 2022) due to the lack of quantitative imaging techniques for NMs in tissues as previously mentioned. Bioaccumulation is also influenced by NM shape, size, functionalization and concentration, as well as organism physiological characteristics (Utembe et al., 2018). This means that these results are unique to these NMs in adult zebrafish at the specific exposure concentration. Further BCF studies testing the different concentrations of the same NM should be done to acquire a clearer image of the kinetics of these NMs in zebrafish.

These findings revealed that the TK model underestimated metal concentrations, particularly over prolonged exposure periods. Because the TK model does not yet consider potential TK regulations for fish (i.e., changes in the values of k_u , k_e , and BCF). The US EPA guidance on bioaccumulation of metals indicates not to use the kinetic BCF for regulatory purposes because it is not a constant (U.S. EPA., 2007). So it would give a false impression in the risk assessment unless the exposure concentration and time are reported for that specific BCF. Other studies found poor modelling for metal accumulation in freshwater fish, such as evident variations in Cd accumulation mechanisms in wild yellow perch (Kraemer et al., 2008). Once the metal concentrations in these organs reached a certain level, yellow perch lowered the Cd content in the gills and intestines. In this study, the successful match between the predicted and actual data was evident solely in the liver and intestine of ionic metal treatment groups. This was also seen in a study by Tsai et al. (2013) that only found accurate predictions of Cu uptake in tilapia in the liver. Metals and NMs present complex internal dynamics of uptake (specific channels in the cell membrane, active transport, or endocytosis) and storage (as detoxified forms, such as liver granules or bound to MT proteins, active elimination) (Cuello et al., 2012). Shaw et al. (2012) also report no significant elevations of Cu concentrations in the spleen, brain, or muscle of fish from both NM and ionic Cu waterborne treatments. Fish actively regulate tissue Cu concentrations, and the lack of Cu accumulation in the muscle is consistent with previous reports (Shaw et al., 2012). The increase of Cu in the liver that led to negative elimination factors, can be due to the flux of natural Cu over time adding to internal Cu concentrations, as control fish were not sampled over time this cannot be confirmed. Copper is an important compound for the maintenance of red blood cells, nerve cells, and the immune system and thus, naturally fluctuates over time (Malhotra et al., 2020, Festa and Thiele, 2011, Grosell, 2011).

In the present study, the highest BCF was seen for ionic Cu in the gills. Following aqueous exposure, dissolved copper from metal salts accumulates first in the gills, reflecting the route of exposure, and later in the internal organs (Shaw et al., 2012). It therefore stands to reason that the tissue displaying the highest toxicokinetic activity will also be subjected to toxicodynamic interactions due to the direct effects of Cu on the structural integrity of the gill epithelium and loss of branchial ion-regulatory functions (Wood, 2001; Shaw et al., 2012). Overall, the intestine had the highest BCF for NMs indicating slow excretion most likely due to intact NMs in the gut lumen after ingestion or washing impairment (Khan et al., 2015; Khan et al., 2014; Petersen et al., 2009; van der Zande et al., 2020). Elimination rates have been linked to NM size and hydrophobicity (Fan et al., 2018). In the present study, QDs have the smallest initial particle size (Chapter 2), influencing uptake and distribution and can be the reason for high QD metal association with muscle and general higher uptake potential observed compared to the other NMs.

This suggests that it is questionable whether the classic TK model explicitly illustrates metal concentrations, especially under prolonged exposure. The extrapolation of metal concentrations between different treatments with a constant TK rate estimated from a single treatment is also questionable because Tsai et al. (2013) found that the uptake and elimination rate constants showed dependence on exposure duration, but also on the metal concentration of the water. Although metal concentrations in the present study were kept relatively constant, there were drops in concentrations before water changes. The present study has assessed metal as a measure of nanodistribution due to the lack of equipment and methods available to measure particle uptake. This method has its limitations, as these values are derived for solutes that form equilibria between the compartments. Nanomaterials are colloids, and that equilibrium theory does not apply to particles. But, for other reasons the particles might give apparent uptake and excretion curves, so we should be cautious and call them apparent kinetics.

As an alternative option quantitative structure-activity relationships (QSAR) models can also be used to predict the octanol/water distribution coefficient (K_{ow}), which can be used to predict bioaccumulation of some NMs such as fullerenes and polyamidoamine dendrimers based on their nano specific characteristics. However, this promises challenges for NMs such as carbon nanotubes (Utembe et al., 2018). In our study, we found that uptake was varying resulting in challenges regarding finding the best fit for the model. As NM uptake is greatly linked to particle characteristics in the media this should be linked to the TK model to find a better model fit and incorporate nano-specific variables. It is also difficult to build a model with minimum information and multiple routes of uptake. This model only takes into account one route of uptake (i.e. from the water to the gills) and incorporating uptake through ingestion proves challenging due to the unknown concentration.

5.5 Conclusions

Results indicate different uptake patterns and biodistributions between NMs and ionic metals. Higher uptake of NMs in the intestine suggests oral consumption of intact NMs and higher uptake of ionic materials in the gills suggest uptake of ions through the gills. It is also possible that the intestine was not washed properly, leading to artefact concentrations. Overlapping uptake concentrations despite exposure concentration differences allude to the regulation of metals by the fish, as well as mucus deposition of NMs in organs such as the gills and intestine. Slow metal elimination such as Cu suggests greater assimilation to organs by this metal. Overall, NMs tend to accumulate in the intestine and muscle as is the case for QDs, most likely due to size. Ionic metals accumulated in all organs, but certain metals favoured certain organs. Due to the proposed dissolution of oxide and bimetallic particles in the intestine, further translocation and uptake could be due to free ions, but further studies are needed to confirm this hypothesis. Thus, TKs are dependent on the organ type, time, concentration, and material and should be assessed with regard to all these parameters. However, the model was only successfully applied to ionic materials in the liver and intestine, indicating that assumptions from this model regarding metal-based NMs are not as accurate due to uptake fluctuations and exposure fluctuations, and underestimate metal concentrations in many cases. Thus, future studies should consider including NM age and transformation and exposure concentrations. It would also be beneficial to have more replicates over longer periods of time to be able to predict concentrations more accurately, this was however not included due to the 4Rs employed in the ethical approach of this study. A more accurate model should also consider multiple routes of uptake via waterborne exposures. To the best of our knowledge, this is the first study to investigate the time-dependent uptake, biodistribution, and elimination of total Au, Cu, Cd and Te in fish after exposure to aqueous NMs, and the results will provide quantitative information to help in the formulation of ecological and human health risk assessments.

CHAPTER 6 FINAL CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Nanomaterial characterization

Aquatic environments are extremely complex and dynamic, influencing both NM uptake pathways and adverse biological effects. Thus, the first objective was met by the use of standardised tests makes it possible to eliminate some of these complex parameters that would be present in the natural environment in order to narrow down the modes and variables of NM toxicity. Nanomaterial specific variables such as size, shape, charge and metal content also influence uptake and toxicity. For the present study these parameters were measured, and primary particle size was chosen at 14 nm for both nAu and nCuO to minimize primary size influence. The nAu were obtained from MINTEK (South Africa). The nCuO purchased from a commercial supplier, PlasmaChem. The quantum dots primary particle size was 4 nm as was produced and provided through the EU-FP7 Nanosolutions Project. The total Au in nAu was 50 mg/L, which is 50% of the nominal ionic gold exposure concentration. The ionic Au dissolution from nAu was 0%. For 100 mg/L nCuO the total Cu was 100 mg/L and dissolution was 0.02%. For 100 mg/L QDs the Cd concentrations was 31 mg/L and dissolution was 2%; and the Te concentration was 6 mg/L with 11% dissolution. In this study three different environmental media were used, i.e., SSPW for *C. elegans*, E3 media for zebrafish embryos and ZSW for adult zebrafish. There was agglomeration of all NMs studied but the exposure concentration to agglomeration relationship is different for different NMs in different environmental media. Overall QDs showed the greatest agglomerate sizes. Smaller particles such as QD are more susceptible to agglomeration under the same conditions due to larger surface areas being more reactive leading to the largest hydrodynamic particle sizes among the three materials tested. The largest agglomerates for all three NMs were also seen in the SSPW which was a more complex media containing more free ions leading to agglomeration and settling of NMs. All ZP values were negative, becoming more positive as the exposure concentration increased, except in ZSW where they became more negative with an increase in concentration. System water had the same pH as the E3 media indicating that the differences are likely due to the salt (electrical conductivity) and other organic ligand differences between the two media. The large agglomerate sizes and increase in negative ZP are most likely due to more complexing salts and the presence of a protein corona in ZSW. However, changes in size and charge were not completely linear and fluctuations are most likely due to particles outcompeting van der Waals or electrostatic interactions at certain concentrations, leading to less agglomeration and settling out. Dissolution was the highest in ZSW and only QD had metal

dissolution higher than 1%, indicating these materials are not as stable in solution as nCuO and nAu.

Biological responses to nanomaterial exposures

To understand the hazard potential of the NMs and their ionic metals EC₅₀ reproduction and LC₅₀ embryo toxicity tests were done for *C. elegans* and *D. rerio*, respectively. Thus, objective two was to determine the developmental toxicity of the NMs and their ionic metal equivalents (Au, Cu, Cd, Te) using the fish embryo toxicity test (FET – OECD 236) and the water quality reproductive test of *C. elegans* (ISO 10872). Ionic Cu and Cd stimulated reproduction in *C. elegans* at lower exposure concentrations, while nCuO induced a bimodal reproduction inhibition response. An increase in reproduction compared to the control is most likely due to hormesis. This is a dose-response relationship where low doses cause stimulation and high doses cause inhibition and indicates an overcompensation to mild stress (Kouda and Iki, 2010). The bimodal response is attributed to the lower exposure concentrations having sufficient NMs in suspension. This leads to less agglomeration and precipitation so that they still elicit an effect and is a common response to NM exposure. This type of response could also be seen in the mortality and hatching success results for the embryo test of both nAu and QDs treatments. The NMs and their ionic metals did not show the same patterns in developmental toxicity. Malformations in the embryos were more prevalent in the QDs treatment group regardless of concentration, with a significant increase in both spinal deformations and oedemas relative to the control. Exposure to nCuO resulted in more oedemas and nAu resulted in more spinal deformations.

The resulting EC₅₀ and LC₅₀ values indicate that the ionic metals were more toxic than their nano counterparts, except in the case of QDs. In both organisms tested, QDs were more toxic than ionic Cd and Te. It appears that the toxicity of QDs is linked to smaller particles of 4 nm being taken up more readily causing NM specific toxicity rather than metal-based toxicity. There are many kinds of QDs with unique properties and it has been found that some QDs elicit cytotoxicity after their core ligands are degraded (Hardman, 2006). Very little is however known about the degradation of QDs in vertebrate organisms and possible effects of photolytic or oxidative conditions on the degradation *in vivo*. Research does suggest that QDs can be degraded *in vivo* depending on the surface coating (Gao et al., 2004). The metals present in the present QD exposures are significantly lower than the metal concentration needed to evoke responses in the organisms during the ionic metal exposures, even if the metals were degraded from the QDs *in vivo*. *In vivo* studies indicate that, regardless of the type of QD, vertebrate systems tend to detect QDs as foreign and subsequently eliminating them through the major excretory organs/systems, i.e. the liver, spleen, and lymphatic systems (Hardman, 2006). However, this is a broad generalization, and there are variations in the literature. In the nematode, ionic Te was more toxic

than ionic Cd. This is a noteworthy finding, as not many studies to date have assessed Te toxicity. In the embryo tests, nCuO was found to be the most toxic NM, which was expected, due to Cu being a known hazard to fish species and has been reported to delay hatching due to interference with the hatching enzyme chorionase leading to mortality. Overall, nAu was the least toxic NM to both organisms, due to this being an inert NM there was no dissolution in the exposure media and low uptake due to larger agglomerates with higher exposure concentrations.

Exposure to metals and NMs resulted in a number of sublethal effects at whole organism level in zebrafish embryos. This was objective 5. Nano Au caused an increase in heart rate and respiration, which was not present following ionic Au exposure. This was attributed to the influence that the NM particles had on the external surface of the embryo chorion. Other particle-related responses were the effects of the NMs in the musculature of the embryos (as visualised through the darkfield spectroscopy), which was associated with the duration of burst activities observed. In the oxide NM treatment, there was a decrease in heart rate and an increase in respiration. The decreased heart rate was attributed to the development of pericardial oedemas. Only ionic Te exposure had reduced burst activity in the embryos and can be linked to the significantly reduced blood flow seen in the ionic Te treatment, resulting in reduced blood flow to muscle impairing movement. The QDs and ionic Cd caused an increased cardiac output and a decrease in respiration. It was observed that QDs covered the surface of the chorion, impairing respiration indirectly.

To establish links between total metal body burden and adverse effects, metal uptake and NM distribution were determined. This was objective 3. One exposure concentration (based on the EC₅₀ for nematodes and LC₅₀ for embryos) of each treatment was selected. CytoViva® dark field images indicated the presence of nAu and QDs in the pharynx, gut and reproductive organs of *C. elegans*. In the zebrafish embryos, Au and QD NMs agglomerated around the eyes, spinal cord and tail somites, while nCuO could not be effectively visualised using this method. However, limitations of this method was that not all organs could be assessed. This means that particle presence in certain organs can be confirmed, but the exclusion of particles in others organs not observed cannot be confirmed.

Objective 4 was to quantify uptake with GF-AAS. When comparing total Au uptake in the nematode following exposure to nAu and ionic Au, it was evident that levels were very similar even though the nominal exposure concentrations were 1000 times higher in the nAu group. This is attributed to larger particles in the intestine being excreted by the nematode and only ions being taken up further into the body. Based on the CytoViva® darkfield images intact nAu was found inside the organism, however the possibility that Au dissolution from the NMs occurs during the digestion process and even degradation of the NMs as part of digestion should be considered

and studied further. This is also the case for the QDs and nCuO groups which showed no significant metal uptake at the EC₅₀ concentrations. This means that effects seen at this concentration are indirect effects of exposure. It should be noted that the uptake exposure only lasted 24 h in order to avoid the effects of food and reproduction on sample biomass. Thus, uptake time and reproduction exposure time are not the same. Significant uptake is also influenced by the large variation in data points (n=approx. 10 000 nematodes per group).

In the fish embryos, uptake of ionic Au and Cu was significantly higher compared to their nano equivalents, even though exposure concentrations were lower. The low metal uptake from NMs is attributed to the influences the NMs had on hatching success. This means that embryos had the protection of the chorion for a longer duration during exposure due to many not hatching at 72 h. The levels of Cd and Te were the same irrespective of whether the exposure was through the ion or QDs. This is surprising since the Te in the QDs are much lower than the ionic Te exposure concentrations. It is therefore likely that irrespective of external exposure concentrations, the fish embryos are able to regulate the internal Cd and Te concentrations. This is in contrast with what was observed for Cu uptake, as both nCuO and ionic Cu had significant uptake with varying mortality between them. This is most likely due to homeostasis being maintained by metallothionein detoxification since Cu is an essential element, but this was not directly assessed in this study. As the measured dissolution of the NMs was so low we presume metal uptake of NM treatments are due to particle uptake and that toxicity is not solely linked to free ion uptake. The evidenced metal uptake of metals from the NMs treatments cannot be attributed to ionic metal uptake following dissolution from the particle alone. Since dissolution was less than 1% for Au, Cu and Cd from nAu, nCuO and QDs, respectively (see Chapter 2), it is highly likely that uptake of NMs contributed to the total metal load. Indicating that metal uptake reflected uptake of intact NMs.

Metabolomics was used to assess changes in metabolite profiles and corresponding pathways in both the nematodes and the zebrafish embryos following exposure to the NMs and their ionic equivalents. This was objective 6. Results of the nematode exposures indicate that the aminoacyl-tRNA biosynthesis pathway and glycine, serine and threonine metabolism was influenced by nAu. Significant metabolites included the upregulation of arabinitol and down regulation of 2-propenoic acid, among others. Results of embryo exposures indicate that the purine metabolism was influenced by nAu and ionic Au treatment and literature suggests this could indicate genotoxicity. Nanogold had the most significant impact on the alanine, aspartate and glutamate metabolism and the aminoacyl-tRNA biosynthesis pathway as seen in the nematode as well. Distinctive significant metabolites for nAu exposures in the fish embryos were the down regulation of L-aspartate and the upregulation of alloxanoic acid. Aspartate is an amino acid that are precursors

for proteins, an essential energy source for fish (Francesca et al., 2020). In the nematodes and embryos ionic Au influenced the fatty acid metabolism that can cause cellular damage, resulting in the developmental retardation that was observed. Ionic Au also influenced the galactose metabolism in the nematodes. Significant metabolites for ionic Au exposure in the nematodes were heptadecanoic acid and tetracosane, among others and in the embryos they were palmitic acid and D-xylose. The purine metabolism was affected by both nAu and ionic Au and can thus be a biomarker for Au exposure in zebrafish embryos.

The nCuO exposure influenced fatty acid metabolism, longevity regulating pathway and xenobiotic biodegradation pathways in the nematode exposures. In the fish embryos, aucubin and D-xylose were unique metabolites influenced by nCuO exposure. D-xylose is a component of the glucose metabolism, which is responsible for energy synthesis in the carbohydrate metabolism (Steele, 1981). The ionic Cu treatment in the nematodes resulted in the disruption of the nicotinate and nicotinamide metabolism and the inositol phosphate metabolism due to disruption of metabolites such as acetin and methyl-galactose. In the embryos, ionic Cu treatment caused an increase in palmitic acid, nanodecane, and aucubin. The phenylalanine, tyrosine and tryptophan biosynthesis pathway was effected in both NM and ionic Cu treatments and can be an indicator of Cu toxicity in the embryos. No other pathways that correspond with both nCuO and ionic Cu were identified, indicating that distinct metal and NM related toxicity pathways were present.

Quantum dot treatment in the nematodes resulted in the significant down regulation of only three metabolites (3-(hexadecyloxy)propane-1,2-diol, diacetone alcohol and oleic acid) that are either secondary signalling metabolites or part of the fatty acid metabolism and longevity regulating pathway such as oleic acid. In the embryos the bimetallic NM resulted in the down regulation of decane and palmitic acid and the up regulation of silane at the cellular level. The majority of changes in metabolic pathways were associated with ionic Cd and ionic Te exposures. Most of the same pathways were also influenced by QD exposure, indicating the effect of the metals in the QDs. The disruption of biosynthesis of valine, leucine, and isoleucine was the only pathway associated with the QD treatments in the zebrafish, indicating unique particle toxicity. Also, no observed overlap was found between affected metabolites and pathways of QD exposure to nematodes and zebrafish. Due to the large variation (FDR) in *C. elegans* metabolomics data attributed to variation in sample size (nr of individuals), the use of this nematode for metabolomics analysis is not ideal and methods need to be optimised to retain more sample size while excluding NMs from pellet. Omic and bioinformatic tools offer substantial promise for discovery of gene, protein, and/or metabolite alterations indicative of the mode of action (MOA) of NMs and improved understanding of mechanisms in prospective studies. Knowing the MOA can reduce uncertainties

in NM risk assessments, providing, for example, a basis for extrapolating effects across species (Aggelen et al., 2010). This proves that there is a place for metabolomics in early risk assessment of NMs, especially when evaluating ionic vs nano toxicity, but the organism and sample size is of crucial importance to generate reliable and reproducible results.

To meet objective 7 the biodistribution of total metal concentrations was determined in adult zebrafish following a six-day uptake and six-day depuration period. There was evidence of different routes of uptake and patterns of biodistribution between the metals and NMs. Overall, higher uptake of NMs was seen in the intestine indicating oral uptake, while higher levels of metals were found in the gills following exposure to ionic metals. Copper and Cd was eliminated more slowly than other metals, indicating greater binding affinity in organs. However, natural metabolism of total Cu in the fish were not taken into account in this study. As seen from *ex vivo* gut sac studies, transepithelial transport of nCuO is limited and the gut pH and amino acids cause severe dissolution in the gastrointestinal tract leading to dissolved Cu accumulation in fish (Boyle et al., 2020). It is therefore also likely that dissolution of metals from oxide and bimetallic particles in the intestine, resulted in further translocation and uptake of the free ions. It was not possible to make this distinction in this study and therefore it is not clear whether total metal concentration in the gut is due to ion accumulation or the presence of NMs. The toxicokinetic model results indicated that uptake potential was highest for ionic metals, except in the case of QDs. The intestine had the highest bioaccumulation ability and muscle had the lowest accumulation ability, except for once again QDs. Nanomaterials tend to accumulate in the intestine and muscle and ionic metals tend to accumulate in all organs. However, the model was only able to fit the data ionic metals in the intestine and liver. Our findings revealed that the TK model underestimated metal concentrations, particularly over prolonged exposure periods and that it does not account for NM physicochemical characteristics, changes in aquatic media and the body, different concentrations and TK changes over time due to natural homeostatic metal regulation in the fish. This suggests that it is questionable whether the classic TK model explicitly illustrates metal concentrations, especially under prolonged exposure. However, artefacts from washing of intestinal samples could have impaired the uptake results as well as K^+ interference with metal readings and should be considered in future publications. A more inclusive and trusted model could be established by including the rest of the fish carcass in to uptake determination. As certain models did not fit well in the specific organ this implies that the “wrong” equation was used. Some organs do not have first order kinetics.

Overall, the inert nAu was the least toxic NM, as it showed no dissolution, but uptake and internal distribution was confirmed. Nano CuO, the oxide material, was the most toxic in the fish due to known high Cu ion toxicity. This toxicity, however, was not observed in the nematodes. Quantum

dot exposure was the most toxic NM to the nematodes, while ionic Cd and ionic Te toxicity was comparable to QD toxicity in the fish embryos. Based on the metal dissolution, total metal uptake results and the ensuing variation in metabolic profiles, it was evident that there were both metal ion-induced modes of toxicity as well as NM specific modes of action at work. Adult zebrafish exposures also confirmed different modes of uptake and biodistribution patterns among NMs and ionic metals. Aminoacyl-tRNA biosynthesis was a common pathway effected by both NMs and their ionic equivalents in the nematodes and embryos. The fatty acid metabolism was commonly affected in the ionic treatment groups of both organisms and also in the nCuO and QDs treatments of the nematodes, indicating some ionic metal toxicity. However, results show many non-corresponding pathways and metabolites indicating a combination of ionic and NM toxicity and strengthening the hypothesis that there is not a one size fits all metabolic biomarker of toxicity for different types of NMs.

Based on the results obtained in this study the **data do not support Hypothesis 1** that NM bioavailability and distribution are dependent on ionic dissolution of metals from the particles. It was further evident that the two model organisms reacted differently to NM and ionic metal exposure indicating that there are species-specific bioaccumulation considerations and therefore the **data do not support Hypothesis 2** that bioaccumulation and biodistribution is determined by NM behaviour in environmental matrices and not by species-specific responses. The EC₅₀ and LC₅₀ values indicate that for all materials except QDs the ionic metals were more toxic than the NMs, thus **supporting Hypothesis 3** that the toxicity of ionic metals is higher than the NMs. Results from the model indicate an over and underestimation of bioaccumulation in fish organs when concentrations reach a certain level and can thus, not accurately model NM uptake in zebrafish and **does not support Hypothesis 4**, that metal uptake from NMs can be modelled using a toxicokinetic (TK) model.

6.2 Recommendations

Due the fact that metal ions can form complexes in environmental media it is advised that characterization of ionic metals in media should also be done. This will help in evaluating exposure routes of ionic metals. Matrix matching electrolyte composition for metal analysis would also be beneficial to eliminate uncertainty in metal readings. It is also recommended that characterization be done with and without organisms in the media to better understand the behaviour of NMs in a complex environment.

In the present study it was found that QDs was the most toxic material in the nematodes and that for zebrafish the ionic Cd and ionic Te were just as toxic as QDs. Many studies relate the toxicity

of NMs to small particle sizes (i.e., 4 nm), as they are taken up more readily and distributed throughout the body and cells causing NM specific toxicity. This should further be tested by comparing uptake and toxicity of different sizes QDs that have metal cores with low dissolution.

The dissolution of particularly Au from / or movement of nAu through the gastrointestinal tract should be investigated using the *ex vivo* gut-sac methodology in combination with Cytoviva darkfield spectroscopy. In addition, the indirect effects of particle exposure such as mechanical abrasion of gut microbiota should be studied. Possible links can then be made to the effects on metabolism as clearly demonstrated by the changes in metabolomic profiles.

One of the possible confounding factors in the nematode assays was that the uptake and metabolomics exposures were only 24 h to avoid the effects of food and reproduction on sample biomass. There was therefore a difference in the exposure periods between the uptake and reproduction assays. It is therefore recommended to also conduct longer nematode uptake exposures and include *E. coli* control groups in order to be able to conclude whether reproductive effects are related to NM or metal concentration. Large variation in data from *C. elegans* suggests until methods are optimized this is not an ideal model for metabolomics or uptake studies.

In this study a single sublethal exposure concentration was selected to demonstrate sublethal effects. Since many of the endpoints analysed in this study are not traditional toxicity endpoints it is recommended that more exposure concentrations should be selected and ascertained whether a dose/concentration – response relationship is present. For future studies more exposure concentrations should be used to determine if observed results are due to metal uptake or NM specific toxicity. It was suggested that the zebrafish is regulating the uptake of these metals and there is a threshold limit concentration, because the same trend was not seen with ionic uptake.

There was much speculation on the role of metallothionein or similar cysteine-rich proteins in detoxifying or sequestering metallic NMs and this should be verified in future studies. In order to better understand the pathways of toxicity other omics techniques should be used to link with the metabolomics results in the present study so that a clear adverse outcome pathway can be established and links/ divergences can be made between different types of NMs.

It has been suggested that a first order bioaccumulation model developed for organic contaminants can be used to predict NM bioaccumulation, as well QSAR models. Whole organism toxicity endpoints, such as ZSWimming behaviour or biomarkers, should also be measured at each uptake time point and in that way a toxicodynamic aspect can be added to the model creating a complete uptake and effect TD-TK model which can be incorporated in to a physiologically-based pharmacokinetic model. It is therefore more applicable to use a first-order bioaccumulation

model, which was developed for organic contaminants as it takes decreasing chemical levels (bioavailability and potential transformation) during exposure into account.

$$C_i = C_i(0)e^{-k_e t} + \frac{k_u \times C_w}{k_e - \lambda} \times (e^{-\lambda t} - e^{-k_e t})$$

where λ refers to the rate constant (in days) that allows for decreasing chemical levels throughout the exposure (Tsai et al., 2013; Higgins et al., 2009). However, this has not been validated for metals but is an option. However, to implement it one would have to include exposure time, NM transformation *in vivo* and NM characteristics.

Overall, it is recommended to evaluate NM characteristics in exposure media rather than ultrapure water due to the effects of DLVO theory. In the case of quantum dots, the nematode was more sensitive to exposure than the zebrafish embryo. For both nAu and nCuO the embryo was more sensitive. This strengthens the case that for the best results and broader picture for risk assessment it is beneficial to use multiple model organisms. Using metabolomics and other omics tools are of critical importance to establish modes of toxicity and distinguish between NM and ionic metal toxicity for risk assessment. It is recommended that a combination of first and second order kinetics be used to better the toxicokinetic model in future studies.

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SUPPLEMENTARY INFORMATION

Table S2-1 : Optimized operating parameters for gold analysis by GF-AAS

Temp (°C)	Ramp Time (s)	Hold Time (s)	Argon flow (psi)
110	1	30	250
160	15	30	250
1100	10	20	250
2200	0	3	0
2450	1	3	250

Table S2-2 : Optimized operating parameters for copper analysis by GF-AAS

Temp (°C)	Ramp Time (s)	Hold Time (s)	Argon flow (psi)
110	1	30	250
130	15	30	250
1200	10	20	250
2000	0	5	0
2450	1	3	250

Table S2-3 : Optimized operating parameters for cadmium analysis by GF-AAS

Temp (°C)	Ramp Time (s)	Hold Time (s)	Argon flow (psi)
110	1	30	250
130	15	30	250
500	10	20	250
1500	0	4	0
2450	1	3	250

Table 2-4 : Optimized operating parameters for tellurium analysis by GF-AAS

Temp (°C)	Ramp Time (s)	Hold Time (s)	Argon flow (psi)
110	1	30	250
130	15	30	250
1200	10	20	250
1800	0	5	0
2450	1	3	250

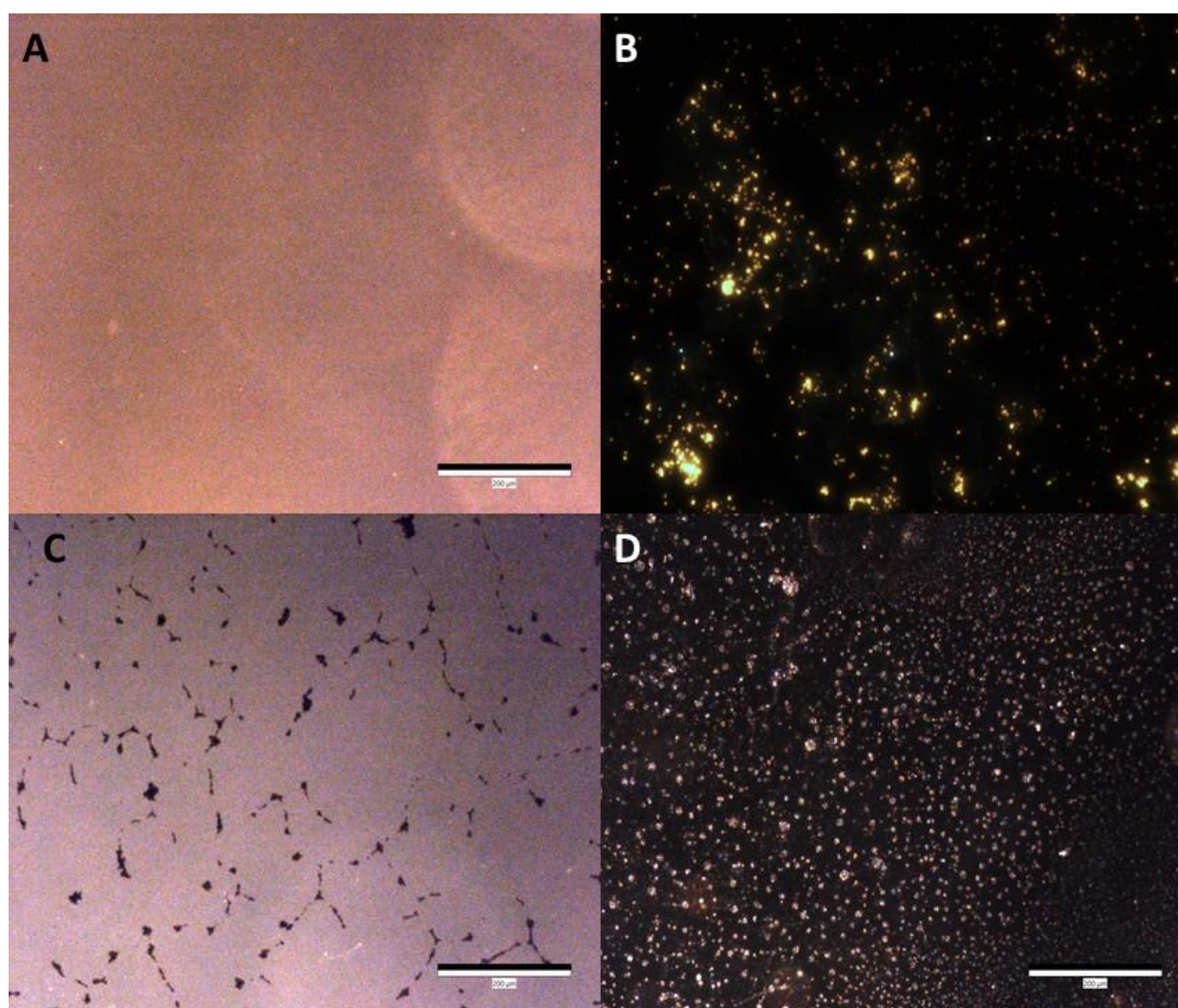


Figure S3-1 : CytoViva images of ultrapure water (A), nanogold (B), nano CuO (C) and quantum dots (D) suspensions.

Table S3-1 : Significantly impacted metabolic pathways of *Caenorhabditis elegans* exposed to nAu compared to the control. Match status refers to the number of metabolites impacted in that pathway. P is

the significance. $-\log(p)$ is the log of the p value, FDR is the false discovery rate and impact refers to the pathway impact.

Pathway Name	Match Status	p	$-\log(p)$	Holm p	FDR	Impact
Aminoacyl-tRNA biosynthesis	2/45	2.316E-4	3.6353	0.0030108	0.0030108	0.0
Glycerophospholipid metabolism	1/32	0.013866	1.858	0.1664	0.072844	0.01653
Pentose and glucuronate interconversions	1/15	0.01681	1.7744	0.18491	0.072844	0.0
Ascorbate and aldarate metabolism	1/6	0.029636	1.5282	0.29636	0.096316	0.0
Galactose metabolism	1/15	0.048487	1.3144	0.43638	0.10268	0.0

Table S3-1 : Significantly impacted metabolic pathways of *Caenorhabditis elegans* exposed to ionic Au compared to the control. Match status refers to the number of metabolites impacted in that pathway. P is the significance. $-\log(p)$ is the log of the p value, FDR is the false discovery rate and impact refers to the pathway impact.

Pathway Name	Match Status	p	$-\log(p)$	Holm p	FDR	Impact
Fatty acid biosynthesis	1/42	2.3165E-4	3.6352	0.0020849	6.9496E-4	0.01723
Fatty acid elongation	1/37	2.3165E-4	3.6352	0.0020849	6.9496E-4	0.0
Fatty acid degradation	1/38	2.3165E-4	3.6352	0.0020849	6.9496E-4	0.0
Pentose and glucuronate interconversions	2/15	0.0052866	2.2768	0.031719	0.011895	0.0
Galactose metabolism	2/15	0.0095294	2.0209	0.047647	0.017153	0.2
Glycolysis / Gluconeogenesis	1/25	0.015348	1.8139	0.061392	0.017267	6.7E-4
Fructose and mannose metabolism	1/16	0.015348	1.8139	0.061392	0.017267	0.0
Amino sugar and nucleotide sugar metabolism	1/29	0.015348	1.8139	0.061392	0.017267	0.0
Glutathione metabolism	1/25	0.055576	1.2551	0.061392	0.055576	0.0

Table S3-2 : Significantly impacted metabolic pathways of *Caenorhabditis elegans* exposed to ionic Cu compared to the control. Match status refers to the number of metabolites impacted in that pathway. P is the significance. $-\log(p)$ is the log of the p value, FDR is the false discovery rate and impact refers to the pathway impact.

Pathway Name	Match Status	p	$-\log(p)$	Holm p	FDR	Impact
Amino sugar and nucleotide sugar metabolism	1/29	0.048777	1.3118	0.29266	0.071066	0.01887
Ascorbate and aldarate metabolism	2/6	0.054318	1.2651	0.29266	0.071066	0.0
Nicotinate and nicotinamide metabolism	1/14	0.055107	1.2588	0.29266	0.071066	0.20155
Inositol phosphate metabolism	1/26	0.059222	1.2275	0.29266	0.071066	0.13671
Phosphatidylinositol signalling system	1/26	0.059222	1.2275	0.29266	0.071066	0.07002

Table S3-3 : Significantly impacted metabolic pathways of *Caenorhabditis elegans* exposed to ionic Te compared to the control. Match status refers to the number of metabolites impacted in that pathway. P is the significance. $-\log(p)$ is the log of the p value, FDR is the false discovery rate and impact refers to the pathway impact.

Pathway Name	Match Status	p	$-\log(p)$	Holm p	FDR	Impact
Glycerolipid metabolism	1/13	0.032224	1.4918	0.38669	0.18943	0.1875
Pentose and glucuronate interconversions	1/15	0.048328	1.3158	0.53161	0.18943	0.0
Ascorbate and aldarate metabolism	1/6	0.081376	1.0895	0.81376	0.18943	0.0
Amino sugar and nucleotide sugar metabolism	1/29	0.082023	1.0861	0.81376	0.18943	0.01887
Nicotinate and nicotinamide metabolism	1/14	0.086079	1.0651	0.81376	0.18943	0.20155
Galactose metabolism	1/15	0.094714	1.0236	0.81376	0.18943	0.0

Table S4 1 Technical observation template for Danio rerio fish embryo toxicity test. Endpoints are evaluated at each time point and marked as the nr to surviving ratio.

	Replicate 1				Replicate 2				Replicate 3				Replicate 4				Replicate 5			
Hours post fertilisation (hpf)	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
Endpoints																				
Lack of somite formation																				
Coagulation of embryos																				
Non-detachment of tail																				
Spontaneous movements																				
Non-development of eyes																				
Developmental retardation																				
Lack of heart function																				
Lack of blood circulation																				
Lack of pigmentation																				
Edema formation																				
Lack of hatch																				
Spinal deformations																				
Evaluated but not used as lethal criterion																				
Not evaluated at this time point																				

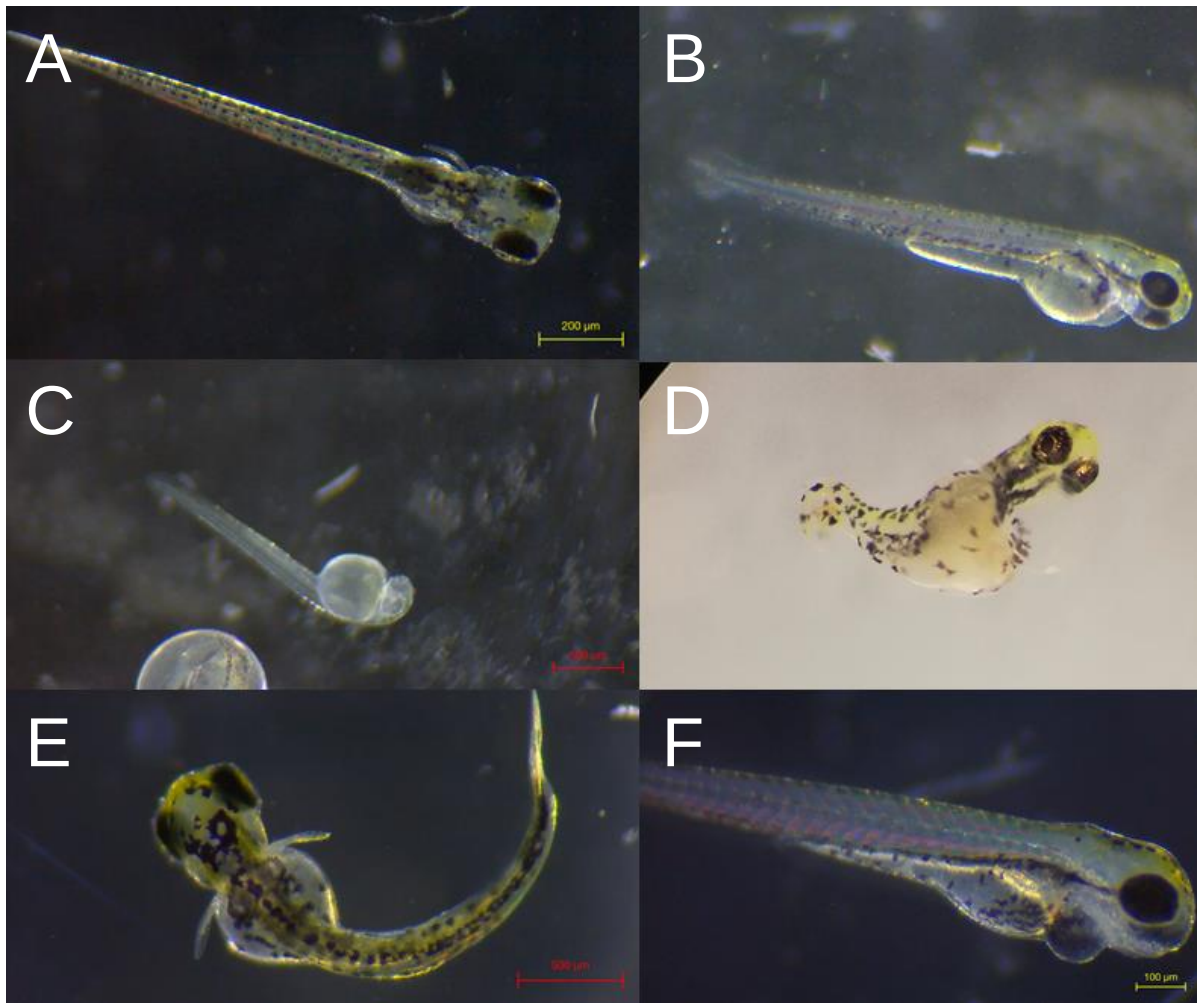


Figure S4 1 : Danio rerio larvae developmental defects A, B – Normally developed, C – lack of pigmentation, D – spinal deformation, lack of somite formation, E – spinal deformation, F – pericardial edema

Table S4 2 : Zebrafish larvae mortality significance results of nAu based on a two-way ANOVA for each time point. NS - Not significant.

nAu	24 h	48 h	72 h	96 h
Control vs. 1 mg/L	ns	ns	ns	ns
Control vs. 5 mg/L	ns	ns	ns	ns
Control vs. 10 mg/L	ns	ns	ns	ns
Control vs. 30 mg/L	ns	ns	ns	ns
Control vs. 50 mg/L	ns	ns	ns	ns
Control vs. 100 mg/L	ns	ns	ns	ns
1 mg/L vs. 5 mg/L	ns	ns	ns	ns
1 mg/L vs. 10 mg/L	ns	ns	ns	ns
1 mg/L vs. 30 mg/L	ns	ns	ns	ns
1 mg/L vs. 50 mg/L	ns	ns	ns	ns
1 mg/L vs. 100 mg/L	ns	ns	ns	ns
5 mg/L vs. 10 mg/L	ns	ns	ns	ns
5 mg/L vs. 30 mg/L	ns	ns	ns	ns
5 mg/L vs. 50 mg/L	ns	ns	ns	ns
5 mg/L vs. 100 mg/L	ns	ns	ns	ns
10 mg/L vs. 30 mg/L	ns	ns	ns	ns
10 mg/L vs. 50 mg/L	ns	ns	ns	ns
10 mg/L vs. 100 mg/L	ns	ns	ns	ns
30 mg/L vs. 50 mg/L	ns	ns	ns	ns
30 mg/L vs. 100 mg/L	ns	ns	ns	ns
50 mg/L vs. 100 mg/L	ns	ns	ns	ns

Table S4 3 : Zebrafish larvae mortality significance results of Ionic Au based on a two-way ANOVA for each time point. NS - Not significant.

Ionic Au	24 h	48 h	72 h	96 h
5 ug/L vs. 10 ug/L	ns	ns	ns	ns
5 ug/L vs. 20 ug/L	ns	ns	ns	ns
5 ug/L vs. 50 ug/L	ns	ns	ns	ns
5 ug/L vs. 100 ug/L	ns	ns	ns	ns
5 ug/L vs. 1000 ug/L	*	****	****	****
10 ug/L vs. 20 ug/L	ns	ns	ns	ns
10 ug/L vs. 50 ug/L	ns	ns	ns	ns
10 ug/L vs. 100 ug/L	ns	ns	ns	ns
10 ug/L vs. 1000 ug/L	**	****	****	****
20 ug/L vs. 50 ug/L	ns	ns	ns	ns
20 ug/L vs. 100 ug/L	ns	ns	ns	ns
20 ug/L vs. 1000 ug/L	*	****	****	****
50 ug/L vs. 100 ug/L	ns	ns	ns	ns
50 ug/L vs. 1000 ug/L	ns	****	****	****
100 ug/L vs. 1000 ug/L	*	****	****	****

Table S4 4 : Zebrafish larvae mortality significance results of nCuO based on a two-way ANOVA for each time point. NS - Not significant.

nCuO	24 h	48 h	72 h	96 h
Control vs. 0.25 mg/L	ns	ns	ns	ns
Control vs. 0.5 mg/L	ns	ns	ns	ns
Control vs. 1 mg/L	ns	ns	ns	ns
Control vs. 5 mg/L	ns	ns	ns	ns
Control vs. 10 mg/L	**	**	**	**
0.25 mg/L vs. 0.5 mg/L	ns	ns	ns	ns
0.25 mg/L vs. 1 mg/L	ns	ns	ns	ns
0.25 mg/L vs. 5 mg/L	ns	ns	ns	ns
0.25 mg/L vs. 10 mg/L	*	*	*	*
0.5 mg/L vs. 1 mg/L	ns	ns	ns	ns
0.5 mg/L vs. 5 mg/L	ns	ns	ns	ns
0.5 mg/L vs. 10 mg/L	*	ns	ns	ns
1 mg/L vs. 5 mg/L	ns	ns	ns	ns
1 mg/L vs. 10 mg/L	ns	ns	ns	ns
5 mg/L vs. 10 mg/L	ns	ns	ns	ns

Table S4 5 : Zebrafish larvae mortality significance results of ionic Cu based on a two-way ANOVA for each time point. NS - Not significant.

Ionic Cu	24 h	48 h	72 h	96 h
Control vs. 10 ug/L	ns	ns	ns	ns
Control vs. 50 ug/L	ns	ns	ns	ns
Control vs. 100 ug/L	ns	ns	ns	ns
Control vs. 200 ug/L	ns	ns	ns	ns
Control vs. 500 ug/L	*	ns	ns	ns
10 ug/L vs. 50 ug/L	ns	ns	ns	ns
10 ug/L vs. 100 ug/L	ns	ns	ns	ns
10 ug/L vs. 200 ug/L	ns	ns	ns	ns
10 ug/L vs. 500 ug/L	ns	ns	ns	ns
50 ug/L vs. 100 ug/L	ns	ns	ns	ns
50 ug/L vs. 200 ug/L	ns	ns	ns	ns
50 ug/L vs. 500 ug/L	ns	ns	ns	ns
100 ug/L vs. 200 ug/L	ns	ns	ns	ns
100 ug/L vs. 500 ug/L	ns	ns	ns	ns
200 ug/L vs. 500 ug/L	ns	ns	ns	ns

Table S4 6: Zebrafish larvae mortality significance results of QDs based on a two-way ANOVA for each time point. NS - Not significant.

QDs	24 h	48 h	72 h	96 h
Control vs. 0.5 mg/L	***	***	***	***
Control vs. 2 mg/L	*	**	**	**
Control vs. 4 mg/L	*	**	**	**

Control vs. 6 mg/L	ns	ns	ns	ns
Control vs. 20 mg/L	*	*	*	**
0.5 mg/L vs. 2 mg/L	ns	ns	ns	ns
0.5 mg/L vs. 4 mg/L	ns	ns	ns	ns
0.5 mg/L vs. 6 mg/L	ns	ns	ns	ns
0.5 mg/L vs. 20 mg/L	ns	ns	ns	ns
2 mg/L vs. 4 mg/L	ns	ns	ns	ns
2 mg/L vs. 6 mg/L	ns	ns	ns	ns
2 mg/L vs. 20 mg/L	ns	ns	ns	ns
4 mg/L vs. 6 mg/L	ns	ns	ns	ns
4 mg/L vs. 20 mg/L	ns	ns	ns	ns
6 mg/L vs. 20 mg/L	ns	ns	ns	ns

Table S4 7 : Zebrafish larvae mortality significance results of ionic Cd based on a two-way ANOVA for each time point. NS - Not significant.

Ionic Cd	24 h	48 h	72 h	96 h
Control vs. 0.2 mg/L	ns	ns	ns	ns
Control vs. 0.5 mg/L	ns	ns	ns	ns
Control vs. 5 mg/L	ns	ns	ns	ns
Control vs. 10 mg/L	ns	ns	ns	ns
Control vs. 20 mg/L	*	*	*	*
0.2 mg/L vs. 0.5 mg/L	ns	ns	ns	ns
0.2 mg/L vs. 5 mg/L	ns	ns	ns	ns
0.2 mg/L vs. 10 mg/L	ns	ns	ns	ns
0.2 mg/L vs. 20 mg/L	*	*	*	*
0.5 mg/L vs. 5 mg/L	ns	ns	ns	ns
0.5 mg/L vs. 10 mg/L	ns	ns	ns	ns
0.5 mg/L vs. 20 mg/L	ns	ns	ns	ns
5 mg/L vs. 10 mg/L	ns	ns	ns	ns
5 mg/L vs. 20 mg/L	ns	ns	ns	ns
10 mg/L vs. 20 mg/L	*	*	*	*

Table S4 8 : Zebrafish larvae mortality significance results of ionic Te based on a two-way ANOVA for each time point. NS - Not significant.

Ionic Te	24 h	48 h	72 h	96 h
Control vs. 0.5 mg/L	ns	ns	ns	ns
Control vs. 1 mg/L	ns	ns	ns	ns
Control vs. 5 mg/L	ns	ns	ns	ns

Control vs. 10 mg/L	ns	ns	ns	ns
Control vs. 50 mg/L	****	****	****	****
0.5 mg/L vs. 1 mg/L	ns	ns	ns	ns
0.5 mg/L vs. 5 mg/L	ns	ns	ns	ns
0.5 mg/L vs. 10 mg/L	ns	ns	ns	ns
0.5 mg/L vs. 50 mg/L	****	****	****	****
1 mg/L vs. 5 mg/L	ns	ns	ns	ns
1 mg/L vs. 10 mg/L	ns	ns	ns	ns
1 mg/L vs. 50 mg/L	****	****	****	****
5 mg/L vs. 10 mg/L	ns	ns	ns	ns
5 mg/L vs. 50 mg/L	****	****	****	****
10 mg/L vs. 50 mg/L	****	****	****	****

Table S4 9 : Zebrafish larvae hatching success significance results of nAu based on a two-way ANOVA for each time point. NS - Not significant.

nAu	48 h	72 h	96 h
Control vs. 1 mg/L	ns	ns	ns
Control vs. 10 mg/L	ns	ns	ns
Control vs. 30 mg/L	ns	ns	ns
Control vs. 50 mg/L	ns	ns	ns
Control vs. 100 mg/L	ns	ns	ns
1 mg/L vs. 10 mg/L	ns	ns	ns
1 mg/L vs. 30 mg/L	ns	ns	ns
1 mg/L vs. 50 mg/L	ns	ns	ns
1 mg/L vs. 100 mg/L	ns	ns	ns
10 mg/L vs. 30 mg/L	ns	ns	ns
10 mg/L vs. 50 mg/L	ns	ns	ns
10 mg/L vs. 100 mg/L	*	ns	ns
30 mg/L vs. 50 mg/L	ns	ns	ns
30 mg/L vs. 100 mg/L	ns	ns	ns
50 mg/L vs. 100 mg/L	ns	ns	ns

Table S4 10 : Zebrafish larvae hatching success significance results of ionic Au based on a two-way ANOVA for each time point. NS - Not significant.

Ionic Au	48 h	72 h	96 h
Control vs. 5 µg/L	ns	ns	ns
Control vs. 10 µg/L	ns	ns	ns
Control vs. 20 µg/L	ns	ns	ns
Control vs. 50 µg/L	ns	ns	ns
Control vs. 100 µg/L	ns	ns	ns
Control vs. 1000 µg/L	*	****	****
5 µg/L vs. 10 µg/L	ns	ns	ns
5 µg/L vs. 20 µg/L	ns	ns	ns
5 µg/L vs. 50 µg/L	ns	ns	ns
5 µg/L vs. 100 µg/L	ns	ns	ns
5 µg/L vs. 1000 µg/L	ns	****	****
10 µg/L vs. 20 µg/L	ns	ns	ns

10 µg/L vs. 50 µg/L	ns	ns	ns
10 µg/L vs. 100 µg/L	ns	ns	ns
10 µg/L vs. 1000 µg/L	ns	****	****
20 µg/L vs. 50 µg/L	ns	ns	ns
20 µg/L vs. 100 µg/L	ns	ns	ns
20 µg/L vs. 1000 µg/L	ns	****	****
50 µg/L vs. 100 µg/L	ns	ns	ns
50 µg/L vs. 1000 µg/L	ns	****	****
100 µg/L vs. 1000 µg/L	ns	****	****

Table S4 11 Zebrafish larvae hatching success significance results of nCuO based on a two-way ANOVA for each time point. NS - Not significant.

nCuO	48 h	72 h	96 h
Control vs. 0.25 mg/L	ns	***	ns
Control vs. 0.5 mg/L	ns	*	ns
Control vs. 1 mg/L	ns	****	****
Control vs. 5 mg/L	ns	****	**
Control vs. 10 mg/L	ns	****	****
0.25 mg/L vs. 0.5 mg/L	ns	ns	ns
0.25 mg/L vs. 1 mg/L	ns	ns	****
0.25 mg/L vs. 5 mg/L	ns	ns	**
0.25 mg/L vs. 10 mg/L	ns	ns	****
0.5 mg/L vs. 1 mg/L	ns	**	****
0.5 mg/L vs. 5 mg/L	ns	**	ns
0.5 mg/L vs. 10 mg/L	ns	**	****
1 mg/L vs. 5 mg/L	ns	ns	****
1 mg/L vs. 10 mg/L	ns	ns	ns
5 mg/L vs. 10 mg/L	ns	ns	****

Table S4 12 : Zebrafish larvae hatching success significance results of ionic Cu based on a two-way ANOVA for each time point. NS - Not significant.

Ionic Cu	48 h	72 h	96 h
Control vs. 10 µg/L	ns	ns	ns
Control vs. 50 µg/L	ns	ns	ns
Control vs. 100 µg/L	ns	ns	ns
Control vs. 200 µg/L	ns	**	*
Control vs. 500 µg/L	ns	****	**
10 µg/L vs. 50 µg/L	ns	ns	ns
10 µg/L vs. 100 µg/L	ns	**	ns
10 µg/L vs. 200 µg/L	ns	***	*
10 µg/L vs. 500 µg/L	ns	****	**

50 µg/L vs. 100 µg/L	ns	ns	ns
50 µg/L vs. 200 µg/L	ns	*	*
50 µg/L vs. 500 µg/L	ns	***	**
100 µg/L vs. 200 µg/L	ns	ns	ns
100 µg/L vs. 500 µg/L	ns	ns	ns
200 µg/L vs. 500 µg/L	ns	ns	ns

Table S4 13 : Zebrafish larvae hatching success significance results of QDs based on a two-way ANOVA for each time point. NS - Not significant.

QDs	48 h	72 h	96 h
Control vs. 0.5 mg/L	ns	ns	ns
Control vs. 2 mg/L	ns	**	****
Control vs. 4 mg/L	ns	ns	***
Control vs. 6 mg/L	ns	ns	ns
Control vs. 20 mg/L	ns	ns	ns
0.5 mg/L vs. 2 mg/L	ns	ns	**
0.5 mg/L vs. 4 mg/L	ns	ns	ns
0.5 mg/L vs. 6 mg/L	ns	ns	ns
0.5 mg/L vs. 20 mg/L	ns	ns	ns
2 mg/L vs. 4 mg/L	ns	ns	ns
2 mg/L vs. 6 mg/L	ns	ns	**
2 mg/L vs. 20 mg/L	ns	*	****
4 mg/L vs. 6 mg/L	ns	ns	ns
4 mg/L vs. 20 mg/L	ns	ns	**
6 mg/L vs. 20 mg/L	ns	ns	ns

Table S4 14 : Zebrafish larvae hatching success significance results of ionic Cd based on a two-way ANOVA for each time point. NS - Not significant.

Ionic Cd	48 h	72 h	96 h
Control vs. 0.2 mg/L	ns	ns	ns
Control vs. 0.5 mg/L	ns	ns	ns
Control vs. 5 mg/L	ns	ns	ns
Control vs. 10 mg/L	ns	ns	ns
Control vs. 20 mg/L	ns	ns	ns
0.2 mg/L vs. 0.5 mg/L	ns	ns	ns
0.2 mg/L vs. 5 mg/L	ns	ns	ns
0.2 mg/L vs. 10 mg/L	ns	ns	ns
0.2 mg/L vs. 20 mg/L	ns	ns	ns
0.5 mg/L vs. 5 mg/L	ns	ns	ns

0.5 mg/L vs. 10 mg/L	ns	ns	ns
0.5 mg/L vs. 20 mg/L	ns	ns	ns
5 mg/L vs. 10 mg/L	ns	ns	ns
5 mg/L vs. 20 mg/L	ns	ns	ns
10 mg/L vs. 20 mg/L	ns	ns	ns

Table S4 15 : Zebrafish larvae hatching success significance results of ionic Te based on a two-way ANOVA for each time point. NS - Not significant.

Ionic Te	48 h	72 h	96 h
Control vs. 0.5 mg/L	ns	ns	ns
Control vs. 1 mg/L	ns	ns	ns
Control vs. 5 mg/L	ns	ns	ns
Control vs. 10 mg/L	ns	ns	ns
0.5 mg/L vs. 1 mg/L	ns	ns	ns
0.5 mg/L vs. 5 mg/L	ns	ns	ns
0.5 mg/L vs. 10 mg/L	ns	ns	ns
1 mg/L vs. 5 mg/L	ns	ns	ns
1 mg/L vs. 10 mg/L	ns	ns	ns
5 mg/L vs. 10 mg/L	ns	ns	ns

Table S4 16 : Significantly impacted metabolic pathways of Danio rerio exposed to nAu compared to the control. Match status refers to the number of metabolites impacted in that pathway. P is the significance. -log(p) is the log of the p value, FDR is the false discovery rate and impact refers to the pathway impact.

Pathway Name	Match Status	p	-log(p)	Holm p	FDR	Impact
Alanine, aspartate and glutamate metabolism	1/27	3.9149E-4	3.4073	0.0031319	5.2199E-4	0.21629
Arginine biosynthesis	1/14	3.9149E-4	3.4073	0.0031319	5.2199E-4	0.0
Histidine metabolism	1/15	3.9149E-4	3.4073	0.0031319	5.2199E-4	0.0
beta-Alanine metabolism	1/18	3.9149E-4	3.4073	0.0031319	5.2199E-4	0.0
Nicotinate and nicotinamide metabolism	1/14	3.9149E-4	3.4073	0.0031319	5.2199E-4	0.0
Aminoacyl-tRNA biosynthesis	1/48	3.9149E-4	3.4073	0.0031319	5.2199E-4	0.0
Biosynthesis of unsaturated fatty acids	1/35	0.0029612	2.5285	0.0059225	0.0033843	0.0
Purine metabolism	1/66	0.010804	1.9664	0.010804	0.010804	0.01187

Table S4 17 : Significantly impacted metabolic pathways of Danio rerio exposed to ionic Au compared to the control. Match status refers to the number of metabolites impacted in that pathway. P is the

significance. $-\log(p)$ is the log of the p value, FDR is the false discovery rate and impact refers to the pathway impact.

Pathway Name	Match Status	p	$-\log(p)$	Holm p	FDR	Impact
Fatty acid biosynthesis	1/47	6.9241E-4	3.1596	0.0055393	0.0013848	0.01472
Fatty acid elongation	1/39	6.9241E-4	3.1596	0.0055393	0.0013848	0.0
Fatty acid degradation	1/38	6.9241E-4	3.1596	0.0055393	0.0013848	0.0
Biosynthesis of unsaturated fatty acids	1/35	6.9241E-4	3.1596	0.0055393	0.0013848	0.0
Pentose and glucuronate interconversions	1/19	0.036761	1.4346	0.14705	0.058818	0.06494
Pentose phosphate pathway	1/22	0.056186	1.2504	0.16856	0.074914	0.0

Table S4 18 : Significantly impacted metabolic pathways of *Danio rerio* exposed to nCuO compared to the control. Match status refers to the number of metabolites impacted in that pathway. P is the significance. $-\log(p)$ is the log of the p value, FDR is the false discovery rate and impact refers to the pathway impact.

Pathway Name	Match Status	p	$-\log(p)$	Holm p	FDR	Impact
Phenylalanine, tyrosine and tryptophan biosynthesis	1/4	6.6293E-4	3.1785	0.01127	0.0028174	0.5
Tyrosine metabolism	1/42	6.6293E-4	3.1785	0.01127	0.0028174	0.13972
Ubiquinone and other terpenoid-quinone biosynthesis	1/9	6.6293E-4	3.1785	0.01127	0.0028174	0.0
Phenylalanine metabolism	1/8	6.6293E-4	3.1785	0.01127	0.0028174	0.0
Aminoacyl-tRNA biosynthesis	2/48	0.0015853	2.7999	0.020608	0.0053899	0.0
Glutathione metabolism	1/28	0.0050933	2.293	0.06112	0.014431	0.00709
Fatty acid elongation	1/39	0.012758	1.8942	0.14034	0.027111	0.0
Fatty acid degradation	1/38	0.012758	1.8942	0.14034	0.027111	0.0

Table S4 19 : Significantly impacted metabolic pathways of *Danio rerio* exposed to ionic Cu compared to the control. Match status refers to the number of metabolites impacted in that pathway. P is the

Pathway Name	Match Status	p	-log(p)	Holm p	FDR	Impact
Fatty acid biosynthesis	1/47	9.3232E-6	5.0304	2.2376E-4	5.5939E-5	0.01472
Fatty acid elongation	1/39	9.3232E-6	5.0304	2.2376E-4	5.5939E-5	0.0
Fatty acid degradation	1/38	9.3232E-6	5.0304	2.2376E-4	5.5939E-5	0.0
Biosynthesis of unsaturated fatty acids	1/35	9.3232E-6	5.0304	2.2376E-4	5.5939E-5	0.0
Aminoacyl-tRNA biosynthesis	3/48	1.2453E-5	4.9047	2.4905E-4	5.9773E-5	0.0
Phenylalanine, tyrosine and tryptophan biosynthesis	1/4	0.0013823	2.8594	0.026263	0.0036861	0.5
Tyrosine metabolism	1/42	0.0013823	2.8594	0.026263	0.0036861	0.13972
Ubiquinone and other terpenoid-quinone biosynthesis	1/9	0.0013823	2.8594	0.026263	0.0036861	0.0
Phenylalanine metabolism	1/8	0.0013823	2.8594	0.026263	0.0036861	0.0
Glycerophospholipid metabolism	1/38	0.0038885	2.4102	0.058327	0.0093324	0.01364
Arginine biosynthesis	2/14	0.010224	1.9904	0.14314	0.022307	0.11675
Purine metabolism	1/66	0.011782	1.9288	0.15317	0.023564	0.0
Pentose and glucuronate interconversions	1/19	0.033067	1.4806	0.3968	0.061047	0.06494
D-Glutamine and D-glutamate metabolism	1/6	0.077495	1.1107	0.85244	0.08454	0.5
Alanine, aspartate and glutamate metabolism	1/27	0.077495	1.1107	0.85244	0.08454	0.23034
Arginine and proline metabolism	1/38	0.077495	1.1107	0.85244	0.08454	0.0856
Glutathione metabolism	1/28	0.077495	1.1107	0.85244	0.08454	0.01966
Histidine metabolism	1/15	0.077495	1.1107	0.85244	0.08454	0.0
Glyoxylate and dicarboxylate metabolism	1/32	0.077495	1.1107	0.85244	0.08454	0.0
Butanoate metabolism	1/15	0.077495	1.1107	0.85244	0.08454	0.0

significance. -log(p) is the log of the p value, FDR is the false discovery rate and impact refers to the pathway impact.

Porphyrin and chlorophyll metabolism	1/30	0.077495	1.1107	0.85244	0.08454	0.0
Nitrogen metabolism	1/6	0.077495	1.1107	0.85244	0.08454	0.0
Lysine degradation	1/25	0.094767	1.0233	0.85244	0.094767	0.0
Biotin metabolism	1/10	0.094767	1.0233	0.85244	0.094767	0.0

Table S3-6 : Significantly impacted metabolic pathways of *Danio rerio* exposed to QDs compared to the control. Match status refers to the number of metabolites impacted in that pathway. P is the significance. -log(p) is the log of the p value, FDR is the false discovery rate and impact refers to the pathway impact.

Pathway Name	Match Status	p	-log(p)	Holm p	FDR	Impact
Aminoacyl-tRNA biosynthesis	2/48	0.0025207	2.5985	0.025207	0.012825	0.0
Biosynthesis of unsaturated fatty acids	2/35	0.0041009	2.3871	0.036908	0.012825	0.0
Alanine, aspartate and glutamate metabolism	1/27	0.0051302	2.2899	0.041041	0.012825	0.0
Selenocompound metabolism	1/18	0.0051302	2.2899	0.041041	0.012825	0.0
Valine, leucine and isoleucine degradation	1/40	0.015071	1.8218	0.090428	0.019499	0.0
Valine, leucine and isoleucine biosynthesis	1/8	0.015071	1.8218	0.090428	0.019499	0.0
Pantothenate and CoA biosynthesis	1/18	0.015071	1.8218	0.090428	0.019499	0.0
Fatty acid biosynthesis	1/47	0.019499	1.71	0.090428	0.019499	0.01472
Fatty acid elongation	1/39	0.019499	1.71	0.090428	0.019499	0.0
Fatty acid degradation	1/38	0.019499	1.71	0.090428	0.019499	0.0

Table S4 20 : Significantly impacted metabolic pathways of *Danio rerio* exposed to ionic Cd compared to the control. Match status refers to the number of metabolites impacted in that pathway. P is the significance. $-\log(p)$ is the log of the p value, FDR is the false discovery rate and impact refers to the pathway impact.

Pathway Name	Match Status	p	$-\log(p)$	Holm p	FDR	Impact
Arginine and proline metabolism	5/38	6.2653E-6	5.2031	2.2555E-4	2.2555E-4	0.3607
Arginine biosynthesis	2/14	2.136E-5	4.6704	7.4761E-4	3.8449E-4	0.11675
Aminoacyl-tRNA biosynthesis	11/48	1.3641E-4	3.8652	0.004638	0.0016369	0.16667
Glyoxylate and dicarboxylate metabolism	4/32	3.086E-4	3.5106	0.010184	0.0021699	0.22752
Butanoate metabolism	2/15	4.5106E-4	3.3458	0.014434	0.0021699	0.03175
D-Glutamine and D-glutamate metabolism	1/6	4.822E-4	3.3168	0.014948	0.0021699	0.5
Histidine metabolism	1/15	4.822E-4	3.3168	0.014948	0.0021699	0.0
Nitrogen metabolism	1/6	4.822E-4	3.3168	0.014948	0.0021699	0.0
Porphyrin and chlorophyll metabolism	2/30	5.8432E-4	3.2333	0.016361	0.0022096	0.0
Alanine, aspartate and glutamate metabolism	4/27	6.1377E-4	3.212	0.016572	0.0022096	0.33146
Glutathione metabolism	4/28	8.9027E-4	3.0505	0.023147	0.0029136	0.12267
Valine, leucine and isoleucine degradation	3/40	0.0019523	2.7095	0.048806	0.005679	0.0
Cysteine and methionine metabolism	1/33	0.0022085	2.6559	0.053004	0.005679	0.02184
Sphingolipid metabolism	1/21	0.0022085	2.6559	0.053004	0.005679	0.0
Selenocompound metabolism	1/18	0.0025705	2.59	0.05655	0.0061691	0.0
Purine metabolism	2/66	0.0030845	2.5108	0.064776	0.0069402	0.00387
Fatty acid elongation	1/39	0.0041215	2.3849	0.08243	0.0082201	0.0
Fatty acid degradation	1/38	0.0041215	2.3849	0.08243	0.0082201	0.0
Fatty acid biosynthesis	3/47	0.0043384	2.3627	0.08243	0.0082201	0.01472
Biosynthesis of unsaturated fatty acids	6/35	0.0059307	2.2269	0.10082	0.010675	0.0
Glycine, serine and threonine metabolism	4/33	0.0062636	2.2032	0.10082	0.010738	0.51433
Phenylalanine, tyrosine and tryptophan biosynthesis	1/4	0.0084629	2.0725	0.12694	0.012187	0.5
Tyrosine metabolism	1/42	0.0084629	2.0725	0.12694	0.012187	0.13972
Ubiquinone and other terpenoid-quinone biosynthesis	1/9	0.0084629	2.0725	0.12694	0.012187	0.0
Phenylalanine metabolism	1/8	0.0084629	2.0725	0.12694	0.012187	0.0
Pantothenate and CoA biosynthesis	2/18	0.0093821	2.0277	0.12694	0.012991	0.0
Valine, leucine and isoleucine biosynthesis	4/8	0.010041	1.9982	0.12694	0.013389	0.0
Glycerolipid metabolism	2/16	0.013284	1.8767	0.12694	0.017079	0.33022
Galactose metabolism	1/27	0.020022	1.6985	0.16018	0.024855	0.0
Pyrimidine metabolism	2/41	0.026335	1.5795	0.18435	0.031602	0.10763
Sulfur metabolism	1/8	0.028402	1.5466	0.18435	0.032983	0.31915
Pentose and glucuronate interconversions	1/19	0.036253	1.4407	0.18435	0.040784	0.06494
Pentose phosphate pathway	2/22	0.041613	1.3808	0.18435	0.045396	0.0

Table S4 21 : Significantly impacted metabolic pathways of Danio rerio exposed to ionic Te compared to the control. Match status refers to the number of metabolites impacted in that pathway. P is the significance. -log(p) is the log of the p value, FDR is the false discovery rate and impact refers to the pathway impact.

Pathway Name	Match Status	p	-log(p)	Holm p	FDR	Impact
Aminoacyl-tRNA biosynthesis	3/48	2.7797E-5	4.556	5.2815E-4	3.1702E-4	0.0
Phenylalanine, tyrosine and tryptophan biosynthesis	1/4	8.3426E-5	4.0787	0.0015017	3.1702E-4	0.5
Tyrosine metabolism	1/42	8.3426E-5	4.0787	0.0015017	3.1702E-4	0.13972
Ubiquinone and other terpenoid-quinone biosynthesis	1/9	8.3426E-5	4.0787	0.0015017	3.1702E-4	0.0
Phenylalanine metabolism	1/8	8.3426E-5	4.0787	0.0015017	3.1702E-4	0.0
Arginine biosynthesis	1/14	4.5324E-4	3.3437	0.0063454	0.0012302	0.0
Purine metabolism	1/66	4.5324E-4	3.3437	0.0063454	0.0012302	0.0
Valine, leucine and isoleucine degradation	1/40	0.0017586	2.7548	0.021103	0.0033413	0.0
Valine, leucine and isoleucine biosynthesis	1/8	0.0017586	2.7548	0.021103	0.0033413	0.0
Pantothenate and CoA biosynthesis	1/18	0.0017586	2.7548	0.021103	0.0033413	0.0
Alanine, aspartate and glutamate metabolism	1/27	0.0022802	2.642	0.021103	0.0036104	0.0
Selenocompound metabolism	1/18	0.0022802	2.642	0.021103	0.0036104	0.0
Fatty acid biosynthesis	2/47	0.0030901	2.51	0.021631	0.0045163	0.01472
Pentose and glucuronate interconversions	1/19	0.0042103	2.3757	0.025262	0.005714	0.06494
Biosynthesis of unsaturated fatty acids	2/35	0.012418	1.9059	0.062092	0.015035	0.0
Arginine and proline metabolism	1/38	0.012661	1.8975	0.062092	0.015035	0.0607
Fatty acid elongation	1/39	0.033509	1.4748	0.10053	0.03537	0.0
Fatty acid degradation	1/38	0.033509	1.4748	0.10053	0.03537	0.0
Propanoate metabolism	1/23	0.049306	1.3071	0.10053	0.049306	0.0

Table S5 1 : Significant difference (2-way ANOVA) in uptake of Au in zebrafish organs after exposure to nAu and ionic Au and depuration. NS- Not significant.

Treatment	Gills	Liver	Intestine	Muscle
0 h				
Control vs. nAu	ns	ns	ns	ns
Control vs. Ionic Au	ns	ns	ns	ns
nAu vs. Ionic Au	ns	ns	ns	ns
6 h				
Control vs. nAu	*	ns	ns	ns
Control vs. Ionic Au	ns	ns	ns	ns
nAu vs. Ionic Au	ns	ns	ns	ns
24 h				
Control vs. nAu	ns	*	ns	ns
Control vs. Ionic Au	ns	ns	ns	*
nAu vs. Ionic Au	ns	ns	ns	ns
48 h				
Control vs. nAu	ns	ns	ns	ns
Control vs. Ionic Au	ns	*	ns	ns
nAu vs. Ionic Au	ns	ns	ns	ns
96 h				
Control vs. nAu	ns	*	ns	ns
Control vs. Ionic Au	ns	*	*	ns
nAu vs. Ionic Au	ns	ns	ns	ns
144 h				
Control vs. nAu	ns	ns	ns	ns
Control vs. Ionic Au	ns	*	ns	ns
nAu vs. Ionic Au	ns	ns	ns	ns
Post treatment				
150 h				
Control vs. nAu	ns	ns	ns	ns
Control vs. Ionic Au	ns	*	ns	ns
nAu vs. Ionic Au	ns	ns	ns	ns
168 h				
Control vs. nAu	ns	*	ns	ns
Control vs. Ionic Au	ns	ns	ns	ns
nAu vs. Ionic Au	ns	ns	ns	ns
240 h				
Control vs. nAu	ns	ns	ns	ns
Control vs. Ionic Au	ns	ns	ns	ns
nAu vs. Ionic Au	ns	ns	ns	ns
288 h				
Control vs. nAu	ns	ns	ns	ns

Control vs. Ionic Au	ns	ns	ns	ns
nAu vs. Ionic Au	ns	ns	ns	ns

Table S5 2: Significant difference (2-way ANOVA) in uptake of Cu in zebrafish organs after exposure to nCu and ionic Cu and depuration. NS- Not significant.

Treatment	Gills	Liver	Intestine	Muscle
0 h				
control vs. nCuO	*	ns	ns	ns
control vs. Ionic Cu	*	ns	ns	ns
nCuO vs. Ionic Cu	ns	ns	ns	ns
6 h				
control vs. nCuO	ns	*	***	ns
control vs. Ionic Cu	ns	ns	ns	ns
nCuO vs. Ionic Cu	ns	ns	****	ns
24 h				
control vs. nCuO	ns	ns	ns	ns
control vs. Ionic Cu	ns	ns	ns	ns
nCuO vs. Ionic Cu	ns	ns	ns	ns
48 h				
control vs. nCuO	ns	ns	ns	ns
control vs. Ionic Cu	ns	ns	ns	ns
nCuO vs. Ionic Cu	ns	ns	ns	ns
96 h				
control vs. nCuO	ns	ns	ns	ns
control vs. Ionic Cu	*	ns	ns	ns
nCuO vs. Ionic Cu	**	ns	ns	ns
144 h				
control vs. nCuO	ns	ns	ns	ns
control vs. Ionic Cu	ns	ns	ns	ns
nCuO vs. Ionic Cu	ns	ns	ns	ns
Post treatment				
150 h				
control vs. nCuO	ns	ns	ns	ns
control vs. Ionic Cu	ns	ns	ns	ns
nCuO vs. Ionic Cu	ns	ns	ns	ns
168 h				
control vs. nCuO	ns	ns	**	ns
control vs. Ionic Cu	*	ns	*	ns
nCuO vs. Ionic Cu	*	ns	***	ns
240 h				
control vs. nCuO	ns	ns	ns	ns
control vs. Ionic Cu	ns	ns	ns	ns
nCuO vs. Ionic Cu	*	ns	ns	ns
288 h				
control vs. nCuO	*	ns	ns	ns
control vs. Ionic Cu	ns	ns	ns	ns
nCuO vs. Ionic Cu	ns	ns	ns	*

Table S5 3 : Significant difference (2-way ANOVA) in uptake of Cd in zebrafish organs after exposure to QDs and ionic Cd and depuration. NS- Not significant.

Treatment	Gills	Liver	Intestine	Muscle
0 h				
control vs. QDs	ns	ns	ns	ns
control vs. Ionic Cd	ns	ns	ns	ns
QDs vs. Ionic Cd	ns	ns	ns	ns
6 h				
control vs. QDs	ns	ns	ns	ns
control vs. Ionic Cd	ns	ns	*	ns
QDs vs. Ionic Cd	ns	ns	ns	ns
24 h				
control vs. QDs	ns	ns	ns	ns
control vs. Ionic Cd	ns	ns	ns	ns
QDs vs. Ionic Cd	ns	ns	ns	ns
48 h				
control vs. QDs	ns	ns	*	ns
control vs. Ionic Cd	*	ns	**	ns
QDs vs. Ionic Cd	ns	ns	ns	ns
96 h				
control vs. QDs	ns	ns	*	ns
control vs. Ionic Cd	ns	ns	ns	ns
QDs vs. Ionic Cd	ns	ns	ns	ns
144 h				
control vs. QDs	*	ns	*	ns
control vs. Ionic Cd	ns	ns	*	*
QDs vs. Ionic Cd	ns	ns	ns	ns
Post Treatment				
150 h				
control vs. QDs	ns	ns	*	ns
control vs. Ionic Cd	ns	ns	*	*
QDs vs. Ionic Cd	ns	ns	ns	*
168 h				
control vs. QDs	ns	*	*	ns
control vs. Ionic Cd	ns	ns	**	ns

QDs vs. Ionic Cd	ns	ns	**	ns
240 h				
control vs. QDs	ns	ns	*	ns
control vs. Ionic Cd	ns	ns	*	ns
QDs vs. Ionic Cd	ns	ns	ns	ns
288 h				
control vs. QDs	ns	ns	ns	ns
control vs. Ionic Cd	**	ns	*	ns
QDs vs. Ionic Cd	ns	ns	ns	ns

Table S5 4 : Significant difference (2-way ANOVA) in uptake of Te in zebrafish organs after exposure to QDs and ionic Te and depuration. NS- Not significant.

Treatment	Gills	Liver	Intestine	Muscle
0 h		ns	ns	ns
control vs. QDs	ns	ns	ns	ns
control vs. Ionic Te	ns	ns	ns	ns
QDs vs. Ionic Te	ns			
6 h		ns	**	ns
control vs. QDs	ns	ns	ns	ns
control vs. Ionic Te	ns	ns	ns	ns
QDs vs. Ionic Te	ns			
24 h		ns	ns	ns
control vs. QDs	ns	ns	ns	ns
control vs. Ionic Te	ns	ns	ns	ns
QDs vs. Ionic Te	ns			
48 h		ns	ns	ns
control vs. QDs	ns	ns	ns	ns
control vs. Ionic Te	ns	ns	ns	ns
QDs vs. Ionic Te	ns			
96 h		ns	ns	ns
control vs. QDs	ns	ns	ns	ns

control vs. Ionic Te	ns	ns	ns	ns
QDs vs. Ionic Te	ns			
144 h		ns	ns	ns
control vs. QDs	ns	ns	ns	ns
control vs. Ionic Te	ns	*	ns	ns
QDs vs. Ionic Te	ns			
Post treatment				
150 h		ns	ns	ns
control vs. QDs	ns	ns	ns	ns
control vs. Ionic Te	ns	ns	ns	ns
QDs vs. Ionic Te	ns			
168 h		ns	ns	ns
control vs. QDs	ns	ns	ns	ns
control vs. Ionic Te	ns	**	ns	ns
QDs vs. Ionic Te	ns			
240 h		ns	ns	ns
control vs. QDs	ns	ns	ns	ns
control vs. Ionic Te	ns	ns	ns	***
QDs vs. Ionic Te	*			
288 h		ns	ns	ns
control vs. QDs	ns	ns	ns	ns
control vs. Ionic Te	ns	ns	ns	ns
QDs vs. Ionic Te	ns			