Effects of exposure to CdSe/ZnS Quantum Dots on the Neurophysiological Performance in Developing Zebrafish

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Abstract

The present research investigated the effects of exposure to sublethal concentrations of CdSe/ZnS quantum dots (QDs) on the neurophysiological performance of larval zebrafish (*Danio rerio*). The fish were exposed to 0 – 100 µg/L QDs from 0 to 5 days post-fertilization, and whole body metal content, physiological conditions, and neurobehavioural responses were examined. The results suggested that exposure to QDs for 5 days increased whole body content of cadmium (Cd). QDs exposure did not affect survival rate, gross morphology, hatching rate, and body length of larvae. However, larvae exposed to QDs exhibited a significant reduction in locomotor activities. To examine the anxiety-like behaviours of larvae, a sudden light-to-dark transition test and a zone preference test (i.e., light/dark zone preference) were performed. The results suggested that the lowest QDs exposure concentration inhibited larval anxiety-related response, measured by examining effects on the wall-hugging behaviours of larvae in light followed by sudden darkness. This behaviour was unaffected when larvae were exposed to higher concentrations. On the other hand, control larvae displayed a dark avoidance behaviour (i.e., more time spent in the light zone), whereas QDs-exposed larvae exhibited an increase in the percentage of time spent in the dark zone. When compared to the control, the QDs-exposed larvae were also shown to have changed their swimming velocities, in both the zones. These findings indicated that QDs exposure attenuated the normal dark-avoidance behaviours of larvae.

Acknowledgements

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I want to thank Dr. Mohammad Naderi for helping me improve my understanding of statistics. I appreciate his patience and assistance throughout my data analyses. I would also like to thank my lab members for creating such a blissful environment, in which everyone helps one another and enjoys working as a team. Lastly, I want to thank my family and friends for supporting me through this journey and being there for me whenever I needed encouragement and moral support. This research would not have been possible without these people and I hope to have made all of them proud.

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List of Abbreviations

Ag	Silver
Al_2O_3	Aluminum oxide
Au	Gold
BBB	Blood-brain barrier
Cd	Cadmium
CdS	Cadmium Sulphide
CdSe	Cadmium Selenide
$CdSO_4$	Cadmium Sulfate
CdTe	Cadmium Tellurium
Cu	Copper
$CuInS_2$	Copper Indium Sulfide
DMT1	Divalent metal-ion transporter 1
dpf	Days post fertilization
ENMs	Engineered Nanomaterials
Fe	Iron
H_2O	Water
HCO^{-3}	Bicarbonate

hpf	Hours post fertilization
ICP-MS	Inductively coupled plasma-mass spectrometry
LC50	50% lethal concentration
L/D	Light/Dark
LED	Light emitting diode
NPs	Nanoparticles
nrf2	Nuclear factor erythroid 2- related factor 2
QD	Quantum Dot
RO	Reverse Osmosis
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
S	Sulphur
Se	Selenium
TEM	Transmission electron microscopy
TiO_2	Titanium dioxide
ZnO	Zinc oxide

ZnS	Zinc Sulfide
$ZnSO_4$	Zinc Sulfate

1. Introduction

1.1. Engineered Nanomaterials (ENMs)

The exponential growth in the development of nanotechnology has created benefits as well as new challenges on the environment. Because of the unique functional advantages of ENMs (e.g. small particle size, high surface area, good electrical conductivity, strong mechanical strength), they are being widely used in various consumer and industrial products. For example, ENMs can be found in clothing, paints, automotive, personal care products, agrochemicals, medicine, and electronics [1]. There are a variety of types and structures of ENMs, such as carbon-based nanomaterials (e.g., fullerenes) and metal-based nanomaterials (e.g, metal oxides, nanogold, nanosilver, quantum dots). To date, it is estimated that the global production of ENMs is over 300,000 tons per year. The high varieties of ENMs and progressive increase in their uses and disposal have posed great challenges in monitoring and assessing their release and ecological impacts on the environments. ENMs can enter environments via multiple pathways. It is predicted that approximately 2% of the total global production of ENMs is released into the environments during the manufacturing process. 63-91% and 8-28% of ENMs are released to landfills and soils, respectively, and about 7% of ENMs are expected to end up in the aquatic environments [2].

Using environmental modelling and probabilistic material flow analysis [3], it is estimated that the concentrations of ENMs in surface waters and industrial municipal wastewaters could range from 0.01 ng/L to 10 µg/L. However, the precise concentrations of ENMs in the aquatic environment are not well understood. This is because there is a

lack of a standardized analytical method to measure ENMs in environmental samples; ENMs are often difficult to distinguish from environmental matrices, and typical instrumental analyses are unable to measure ENMs because of their extremely small sizes and low mass concentrations [4]. In addition, measurements of ENMs could be interfered by the presence of organic matters in the waters, which could interact with ENMs and alter their physicochemical characteristics and environmental transport [5]. Detection and characterization of environmental ENMs are a growing research field, and recent advances on the enrichment and separation of ENMs from environmental samples appear to prove useful in quantifying ENMs [5]. To reliably evaluate the safety of ENMs, it is critical to advance analytical methods to perform accurate measurements of ENMs in the environment and to understand the potential ecological impact of ENMs' exposure on biota.

1.2. Ecological risks of ENMs

The effects of ENMs are highly dependent on their physical-chemical properties and transformation in the environments [6]. For example, several types of ENMs are known to undergo chemical transformation (e.g., degradation) in the environment, which may alter their surface chemistry. The structural properties and the types of coating on the ENMs are known to exhibit different effects on organisms [7]. The effects of nanoparticles (NPs) on aquatic organisms are also reported to be influenced by water chemistry such as water pH and the amount of dissolved organic matters [4]. Furthermore, several previous studies on metal-based ENMs have shown that the toxicity of ENMs can be different from their bulk counterparts (i.e., metal counterparts) [8]. Several previous studies have examined the effects of metal-based ENMs on aquatic animals. For instance, delayed and abnormal

development, and failure in reproduction, were observed in African clawed tadpoles (*Xenopus laevis*) and harlequin flies (*Chironomus riparius*) following exposure to iron (Fe)- or silver (Ag)-containing nanoparticles (NPs) [9] [10] [11] [12]. In larval zebrafish (*Danio rerio*), exposure to copper (Cu) oxide NPs was found to result in a decrease in hatching rates [13]. Although the underlying mechanism of the toxic effects of metal-based NPs is not fully understood, it is generally believed that their adverse effects are partly associated with the increased production of reactive oxygen species (ROS) following the exposure [14] [15] [16] [17]. These studies provide important information on the toxic effects of metal-based ENMs on aquatic animals; however, the potential effects of most of the ENMs remain largely unknown.

1.3. Quantum dots (QDs)

QDs are a novel type of ENMs, which are commonly 1-100 nm in size and can be excited upon exposure to light or electricity. Depending on their size and shape, QDs can emit various light frequencies after excitation [18]. For example, small QDs generate light with short wavelengths (e.g., blue light) while large QDs generate light with long wavelengths (e.g., red light) following excitation. Most QDs possess a shell/core structure, in which the shell is often composed of zinc sulfide (ZnS) with semiconducting metals inside the core. The shell acts to protect the core and to enhance the optical characteristics of QDs [19]. It also minimizes cytotoxicity and maximizes biocompatibility [20].

QDs tend to aggregate together and display hydrophobic characteristics. Therefore, post-synthetic modifications are commonly applied to the surface of the shell to increase water solubility and colloidal stability. The manipulations typically include

coatings of the shell with amphiphilic, phospholipid polymers, and/or hydrophilic ligands [21] [22] **(Figure 1.1).**

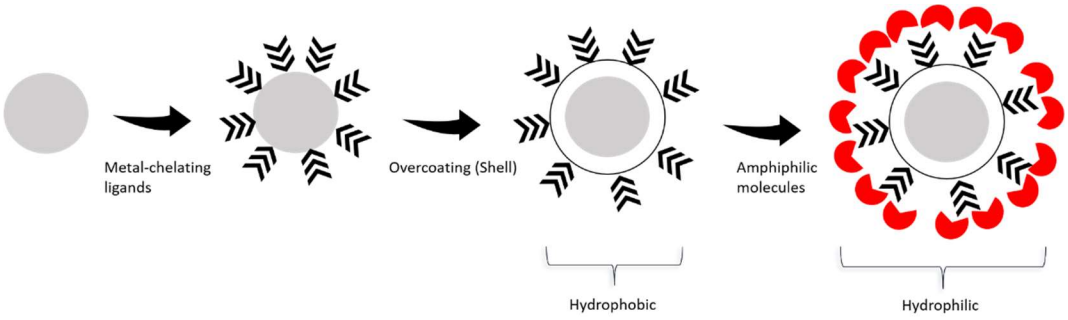


Figure 1.1 The QD core (gray sphere) undergoes manipulations to contain metal-chelating ligands conjugated to its surface. Shell coating is performed on the surface of the QD to protect it and to further enhance its optical characteristics. Different types of polymers (e.g., amphiphilic polymer) can be attached to the ligands to transform the hydrophobic QDs to hydrophilic particles.

QDs have many advantages over most fluorophore dyes, such as their high fluorescence intensity and great stability. For example, QDs are approximately 20 times brighter and 100 times more stable than traditional fluorophores [18]. Moreover, QDs are considerably more energy-efficient and have significantly wider excitation profiles. Additional photoluminescent characteristics are their long excited-state lifetime, and endurance against photobleaching and chemical degradation, which is often not seen in traditional fluorescent dyes [23].

Biological applications of QDs

Due to the unique optical characteristics and the great stability of QDs, they are being developed for various biological applications, including cellular and molecular labelling

and tracking, drug discovery, and disease detection [24]. QDs can be biofunctionalized to recognize specific molecules *in vivo* to study various intracellular activities [25]. The small size of QDs also enables them to cross various biological barriers for drug delivery (e.g., they can cross the blood-brain barrier (BBB) to deliver a drug that targets behavioural illnesses)[26]. For example, through linkage with biotin-streptavidin and specific antibodies, the QD-antibody complex can recognize a specific antigen of a cell under *in vivo* conditions (**Figure 1.2**). These characteristics allow potential disease diagnosis and targeted drug delivery via QDs (as drug carriers) [27].

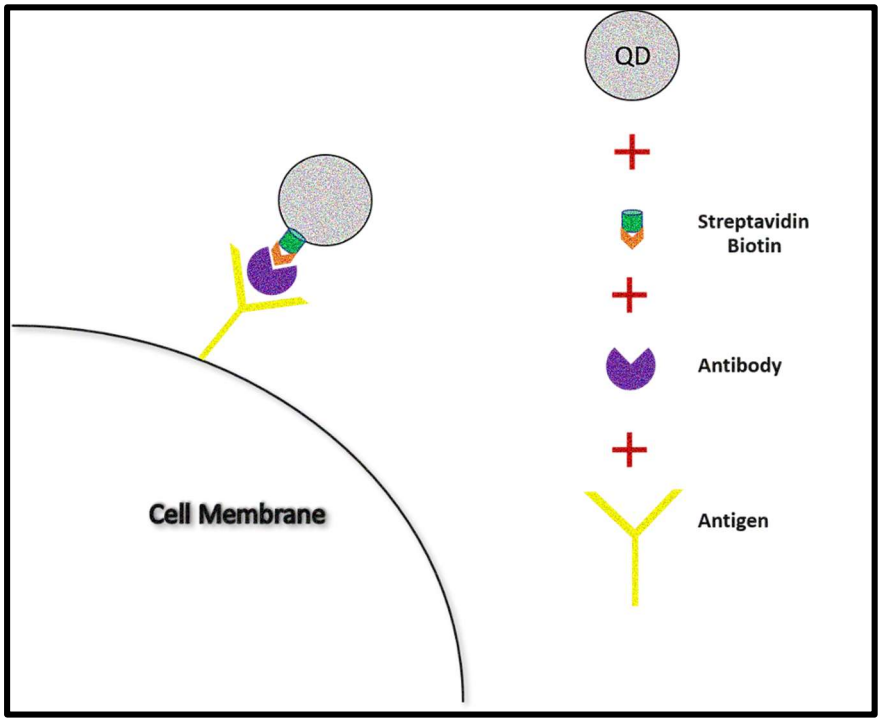


Figure 1.2. Illustration of QD-mediated cell targeting via streptavidin-biotin and antibody linkage.

Economic growth and environmental concerns of QDs

Because QDs are energy efficient with superior optical performance and stability, QDs are being increasingly used in various consumer products. For example, QDs can be found in displays (LED TV), cameras, laptops, and mobile phones and devices [28]. The QDs industry is one of the fastest-growing sectors worldwide. In 2019, the global QDs market value was USD 3.5 billion, and the value is estimated to grow, reaching over USD 10.0 billion by 2025 [29]. Although QDs offer invaluable societal and economic benefits, the growing demand for QDs likely increases the release of these materials into the environments. The introduction of QDs into the environment may occur during their synthesis or use from industries [2]. Disposal of QD materials and their leakages from the products (e.g., landfills) are also potential sources of QDs into the environments. Although little information exists regarding the environmental levels of QDs, environmental exposures likely become a significant concern because of the stability of QDs and their increased demand and use in various sectors. Additionally, certain QDs contain hazardous materials (e.g., Cd), which may pose a threat to the environments following their release. Currently, however, there is insufficient research on the potential ecological impacts of QDs on aquatic environments [30]. Understanding their effects on the environments are important for their sustainable development and uses to meet the needs of today and the future. The current study focuses specifically on the cadmium selenide-containing QDs (CdSe QDs), which are commonly used in various products such as colour displays, photovoltaic cells, solar cells, and transistors [28].

1.4. Current understanding of the effects of QDs on aquatic animals

Many studies have investigated the effects of exposure to QDs in aquatic organisms. In zooplankton (*Acartia tonsa*), exposure to a high level of QDs (2.5 μM) was found to reduce fecundity and survival rate [31]. In developing zebrafish, exposure to ≥ 0.1 mg/L CdTe QDs or CdS QDs resulted in a reduced hatching rate and an increase in the expression of oxidative stress response genes (e.g., *nrf2*) [32]. In juvenile and adult zebrafish, accumulation of QDs was observed in the head and intestinal tract, after QD exposures via aqueous or dietary routes [33] [34]. Exposure to high concentrations of QDs (0.05 to 31.25 mg/L) has also been shown to cause developmental malformation and cellular apoptosis in developing zebrafish [35]. QDs have also shown to have the potential to be maternally transferred to the offspring. For example, an elevated level of Cd was observed in the embryos of Mummichogs (*Fundulus heteroclitus*) following exposure to Cd-containing QDs [36]. It was possible that QDs was deposited in the liver, where the egg yolk protein precursor, vitellogenin, was synthesized, and that the vitellogenin acted as a carrier to transport the metal into the ova [37]. These findings suggested that QDs could have an impact on both the exposed animals and their offspring.

While QDs can be hazardous to the organisms due to their nanoscale properties, it is also believed that their toxicity may be due to the release of toxic constituents from the QDs. Many have studied the effects of Cd, Se, and Zn ions, which are the metal constituents of the CdSe/ZnS QDs. The results suggested that some of the toxic effects of QDs could be attributed to Cd toxicity [38]. For example, Cd can increase the production

of reactive oxygen and nitrogen species (ROS, RNS), leading to oxidative stress and an imbalance in the redox system of the body [39]. The buildup of ROS and RNS can cause DNA damages and degradation of proteins, lipids, or other cellular components [40]. Similarly, a few studies with Cd-containing QDs have demonstrated that their exposure can increase oxidative stress responses [38]. On the other hand, a study with larval zebrafish has shown that the LC_{50} of Cd ion is 1.1 mg/L, whereas the LC_{50} of CdSe QDs is 2.0 mg/L, indicating that Cd ion and Cd-containing QDs exhibit different levels of toxicity [35].

1.5. Effects of QDs on behavioural performance

Currently, there is limited information on the effects of QDs on neuronal functions, even though QDs could cross the blood-brain barrier and enter the brain. Several studies have examined the effects of QDs exposure on locomotion, escape responses, learning, foraging, and predation behaviours. Several significant findings related to QD neurotoxicity are highlighted in this section.

In Wistar rats, intraperitoneal injections of CdSe/ZnS QDs were found to impair their spatial memory processes and decrease their learning efficiency [41]. A study on nematodes (*Caenorhabditis elegans*) has shown that exposure to CdTe QDs reduced locomotion function and affected their foraging behaviours [42]. In marine ragworms (*Hediste diversicolor*), exposure to CdS QDs reduced body undulation frequency without affecting their burrowing kinetics [43].

In larval zebrafish, exposure to 0.15 and 0.45 mg/L CdSe QDs did not affect their swimming speed, but almost 60% decrease in their swimming activity was observed,

when the larvae were exposed to 1.35 mg/L CdSe QDs [35]. Using a sudden dark challenge, the study also demonstrated alterations in their normal responses to external stimuli following the CdSe QDs exposure. Although findings from these studies provide vital information on the neurotoxic effects of QDs, the concentrations used in these studies are unlikely to be environmentally relevant [44] [35]. The sub-lethal effects of ecologically relevant concentrations of QDs await further investigations.

1.6. Zebrafish as a model organism

Zebrafish are an emerging model organism for neurotoxicological research. Compared to many other fish model species (e.g., rainbow trout, European eel), zebrafish are small in size with high fecundity and rapid development. Therefore, they are well suited for high-throughput analysis. Research with zebrafish also has greater translational relevance to humans because of their high similarity in genetic homology (>70%) and neuroanatomy [45]. Additionally, optimum conditions for their breeding and maintenance are well established [50]. Furthermore, zebrafish at early life stages are transparent, making them suitable for various *in vivo* imaging techniques [46].

Zebrafish behavioural analysis

It has been shown that zebrafish possess approximately 200 sophisticated behaviours, from which a high percentage is shared with mammals [51]. Various research has used zebrafish as a model organism to understand the effects of environmental contaminants on anxiety, depression, aggression, and social interactions [53]. At 4 days post-fertilization (dpf), zebrafish have already developed all their neuronal cell types, proceeding to develop their functional anatomical and sensory-motor systems in a period of 24 hours.

At 4-5 dpf, zebrafish have an inflated swim bladder and begin to swim freely, which allows examination of their locomotor activities and functions [52]. Larval zebrafish at 5 dpf are also able to respond to changes in illumination in their surroundings, and this response is thought to be associated with their fear responses (e.g., presence of predators). Understanding the behavioural effects of environmental contaminants such as ENMs in the zebrafish model may facilitate the development of relevant chemical hazard assessment approaches in evaluating the ecological risk of ENMs in the environments.

1.7. Hypothesis and research objectives

The present study was designed to understand the potential neurophysiological effects of QDs following exposure to sublethal concentrations of CdSe/ZnS QDs, using developing zebrafish as a model system. The CdSe/ZnS QDs contain Cd and Se in the core, and Zn and S in the shell (**Figure 1.3**). The QDs consist of a monolayer of amphiphilic polymer coating and are conjugated with carboxylic acid groups. The organic coatings added to the inorganic core of these nanocrystals are 4 nm in size. The zeta potential of the QDs is from -30mV to -50 mV, and they can be excited at 560 nm wavelength. They are very stable in aqueous media with a pH ranging from 5 to 10.

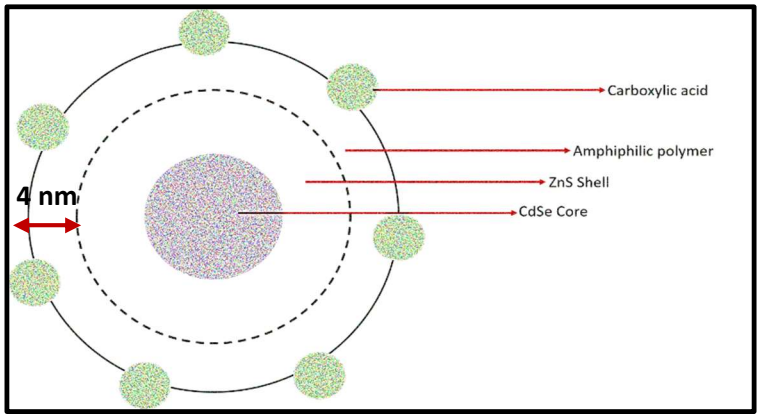


Figure 1.3. Simplified illustration of the general components of a quantum dot. The organic outer layer, consisting of the amphiphilic polymer and the carboxylic acid functional groups, is 4 nm for the specific QD used in this study.

A previous study has shown that exposure to mg/L ranges of CdSe/ZnS QDs increased Cd body burden in larval zebrafish [47]. However, whether exposure to µg/L ranges of CdSe/ZnS QDs concentrations, which are likely more environmentally relevant, increases Cd content in zebrafish was not known. Additionally, whether exposure to CdSe/ZnS also increases Se and Zn contents in fish had not been thoroughly examined previously. Furthermore, the effects of exposure to sublethal concentrations of CdSe/ZnS QDs on the physiological performance of fish had not been fully characterized. The first phase of my study was designed to test the hypothesis that exposure to sublethal concentrations of CdSe/ZnS QDs would increase whole body Cd contents and would adversely affect the physiological conditions of larval zebrafish. I performed a dose-response experiment to determine the effects of exposure to 0 - 100 µg/L CdSe/ZnS QDs on whole body contents of metals (e.g., Cd, Se, and Zn). The metal levels were analyzed using inductively coupled plasma mass spectrometry (ICP-MS). General physiological conditions, such as hatching rate, mortality, body length, and gross morphology, were also examined in this study.

Because of the very small size of QDs, QDs are known to cross the blood-brain barrier and enter the brain [26]. Several previous studies with various animal models have also shown that exposure to metal-containing QDs could affect their locomotor functions and behavioural responses [48] [49] [15]. On the other hand, increased levels of metals such as Cd and Se in the brain are known to affect the neurobehavioural functions in mammals [50] as well as fish [51]. To this end, I hypothesized that exposure to CdSe/ZnS QDs would negatively affect the swimming activities of larval zebrafish. I also hypothesized that CdSe/ZnS QDs exposure would affect the anxiety-like behaviours (i.e., thigmotaxis) in zebrafish. In thigmotaxis, larval zebrafish tend to avoid the centre of an arena and move around the periphery of the environment. Following sudden darkness, zebrafish increase their locomotor activities and the level of changes is typically attributed to their anxiety level [52]. In my study, I investigated the swimming performance of CdSe/ZnS QD-exposed larval zebrafish using a high throughput behavioural tracking system. The anxiety-like behaviours were investigated using a light-to-dark transition assay. The light/dark zone preferences and the wall-hugging behaviour were evaluated in my study. This multi-tier experimental strategy would allow me to understand the possible pathways that could be influenced by exposure to CdSe/ZnS QDs. For clarity, CdSe/ZnS QDs will be referred to as QDs except mentioned otherwise.

2. Materials and Methods

2.1. Zebrafish husbandry and embryo collection

All adult zebrafish were maintained in a recirculating water system (Aquaneering) with a pH of 7.4, and a temperature of 27° C (**Figure 2.1**). In this system, water is continuously circulating to maintain the optimal conditions for the fish. 10% of the circulating water is replaced by new reverse osmosis (RO) water daily. Water quality is measured weekly to adjust its hardness (120ppm), alkalinity (75 ppm), and conductivity (740 µS/m) to the optimal levels. The RO water circulating in the system contains sea salt (Instant Ocean) and bicarbonate (HCO^{-3}). All adult fish were habituated to a light/dark cycle of 14h/10h. System water was used to prepare all exposure waters.

Figure 2.1. The Aquaneering water system used for housing zebrafish. Every tank has a small hose dispensing water into them and an opening for water flowing out of them.

Adult zebrafish were set up to breed in their standard tanks within a breeding trap placed inside the tank. Breeding took place in a 1:2 ratio of male to females separated by a mesh divider in each tank. The divider separated the male and the females for approximately 18 hours before breeding. Dividers were removed at 8 AM to start the breeding process. Immediately after removing the divider, the trap was tilted inside the tank to simulate a shallow water effect for the fish (**Figure 2.2**). Zebrafish normally spawn in shallow waters in nature, hence this simulation results in a more productive breeding session [53]. Embryos were collected in egg water composed of the system water (~ 800 mL) containing ~0.8 mL of 1% methylene blue.

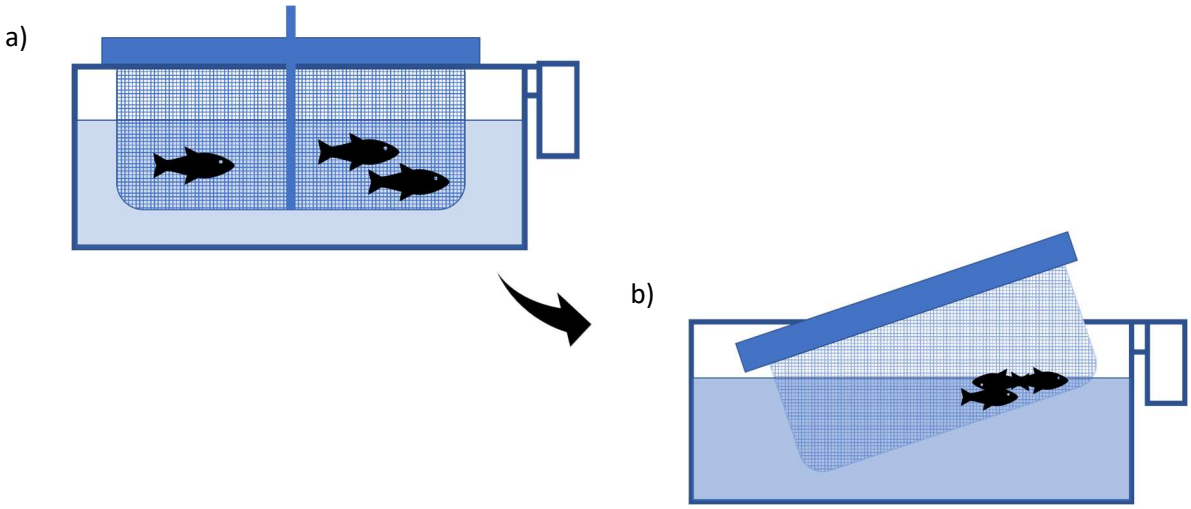


Figure 2.2 Orientation of zebrafish and the trap before and during spawning. a) Illustration of male and female fish kept separated using a mesh divider up to the spawning time b) Subsequent to removal of the divider, trap is tilted to create shallow water effects for fish to spawn in.

2.2. Exposure regimes

The stock solution of QDs (as CdSe/ZnS QDs) was purchased from Sigma-Aldrich (CAT#900225, Ocean NanoTech; 1 mg/mL in H_2O, which is equivalent to 2 µM). Six hours post fertilization (hpf), embryos were exposed to 0 (control), 1, 10, and 100 µg/L QDs in system waters. These concentrations were chosen based on a previous study that 50 µg/L of similar QDs showed less than 10% mortality [32]. Additionally, the environmental concentrations of NPs had been estimated to be in the µg/L range in NP-contaminated freshwater environments [5]. Embryos were collected in several rounds to ensure all embryos would be exposed at 6 hpf. For every round of collection, fish spawned roughly for 15 minutes and the embryos were collected for exposure. Each batch of embryos was labelled with their time of collection and kept inside the incubator until use. A 6-well plate was used for each exposure concentration group with 5-10 embryos were placed in each well of the plate. Fish analyzed from the same well were considered as one replicate (n=1). This resulted in 6 replicates per concentration group if only one plate was used per concentration. Exposure waters were renewed every 24 hours to add new QDs until 5 dpf.

2.3. Physical properties of QDs in the exposure waters

System water containing 100 µg/L QDs was selected for transmission electron microscopy (TEM) imaging. 5-10 µL of the exposure water was dispersed on a TEM grid, blotted dry, and was observed under the microscope. Hydrodynamic diameters were reported as frequency distribution. TEM imaging was performed at the Sickkids hospital, Toronto.

2.4. Assessment of physiological conditions

During the exposure period, embryos/larvae were monitored daily until 5 dpf to check for mortality, deformity, and hatching. The deformity was defined as the presence of edema (i.e., pericardial or yolk sac edema) and bent spine/tail. Hatching rate was calculated by counting the number of hatched embryos daily. Furthermore, larval body length was measured at 3 dpf and 5 dpf following euthanasia with an overdose of MS-222. Images were taken using a camera-equipped microscope and the standard body length (from the tip of snout to the end of the caudal peduncle) was recorded.

2.5. Measurement of water and tissue metal levels

At 5 dpf, 30 larvae were collected from the 6-well plates and pooled as one sample (n=1). For each treatment, a total of 4 samples were prepared (n=4). All larvae were euthanized by exposing them to 0.2 g/L of MS-222 for 20 minutes. Euthanized larvae were washed in MiliQ water twice, and they were transferred to microcentrifuge tubes. All samples were dried on a block heater at 65° C for 2 days. After drying, 300 µl of 6N HNO_3 was added to each tube. Samples were incubated on the block heater at 65° C for another 2 days. Samples were then diluted with 5 ml of 2% HNO_3. All tissue samples were filtered by 0.45 µm filters. Water samples were prepared by acidifying the exposure waters with 2% HNO_3. Four water samples per concentration were prepared for analysis. Metal/ion measurements were performed using ICP-MS in the Water Quality Centre at Trent University.

2.6. Behavioural analysis

Following exposure to 0 – 100 µg/L of QDs, larvae at 5 dpf were analyzed for their behavioural performances. This developmental stage was chosen because, at 5 dpf, larval zebrafish were able to swim freely and respond to changes in illumination (methods described below). All behavioural tracking took place in the DanioVision chamber and the swimming activities were monitored using the EthoVision XT software (Noldus) (**Figure 2.3**). All behavioural analyses were performed between 12:30 PM to 3:30 PM to maintain consistency in time. The tracking area inside the chamber was filled with water and the temperature of the water was maintained at 28℃ using a temperature control unit. The multi-well plates were placed in the DanioVision chamber, as shown in Figure. 2.3. The chamber has an infrared backlight and an infrared-sensitive camera, therefore behavioural tracking was possible in dark conditions, as well as light.

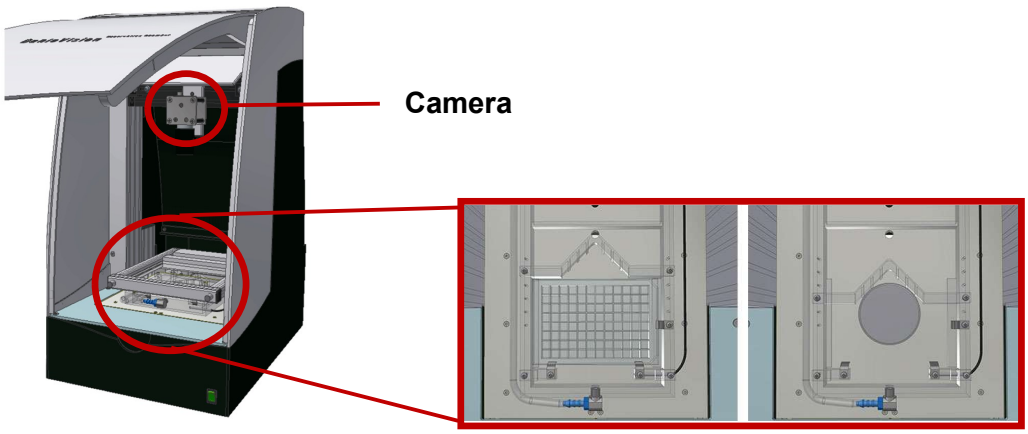


Figure 2.3 The chamber specified for plate placement is located directly under the camera. Subsequent to filling the chamber with water and correct placement of the plate, the experiment can start to run.

Spontaneous swimming activity

At 5 dpf, approximately 85-90 larvae per concentration group were transferred into 96-well plates with each well containing 1 larva. All larvae were acclimated for 5 minutes and then tracked for 30 minutes. They were monitored for distance travelled and swimming velocity every minute. The data were reported as the total distance they travelled per 5-minute time frames, total distance travelled over the entire 30 minutes of tracking duration, mean velocity per 5-minute time frames, and mean velocity over 30 minutes of tracking. The behavioural analysis took place under normal conditions of lighting with no transitions (white light).

Thigmotaxis

To assess the stress/anxiety-related responses in 5 dpf larvae, 24 larvae from each concentration group were transferred into 24-well plates with each well containing 1 larva. 24-well plate was selected in this experiment because it provides a larger space for the larva to explore and transition from zone to zone. The layout of a 24-well plate was set up on the EthoVision software to specify an outer and an inner zone for each well on the plate, as shown in **Figure 2.4 a, b**.

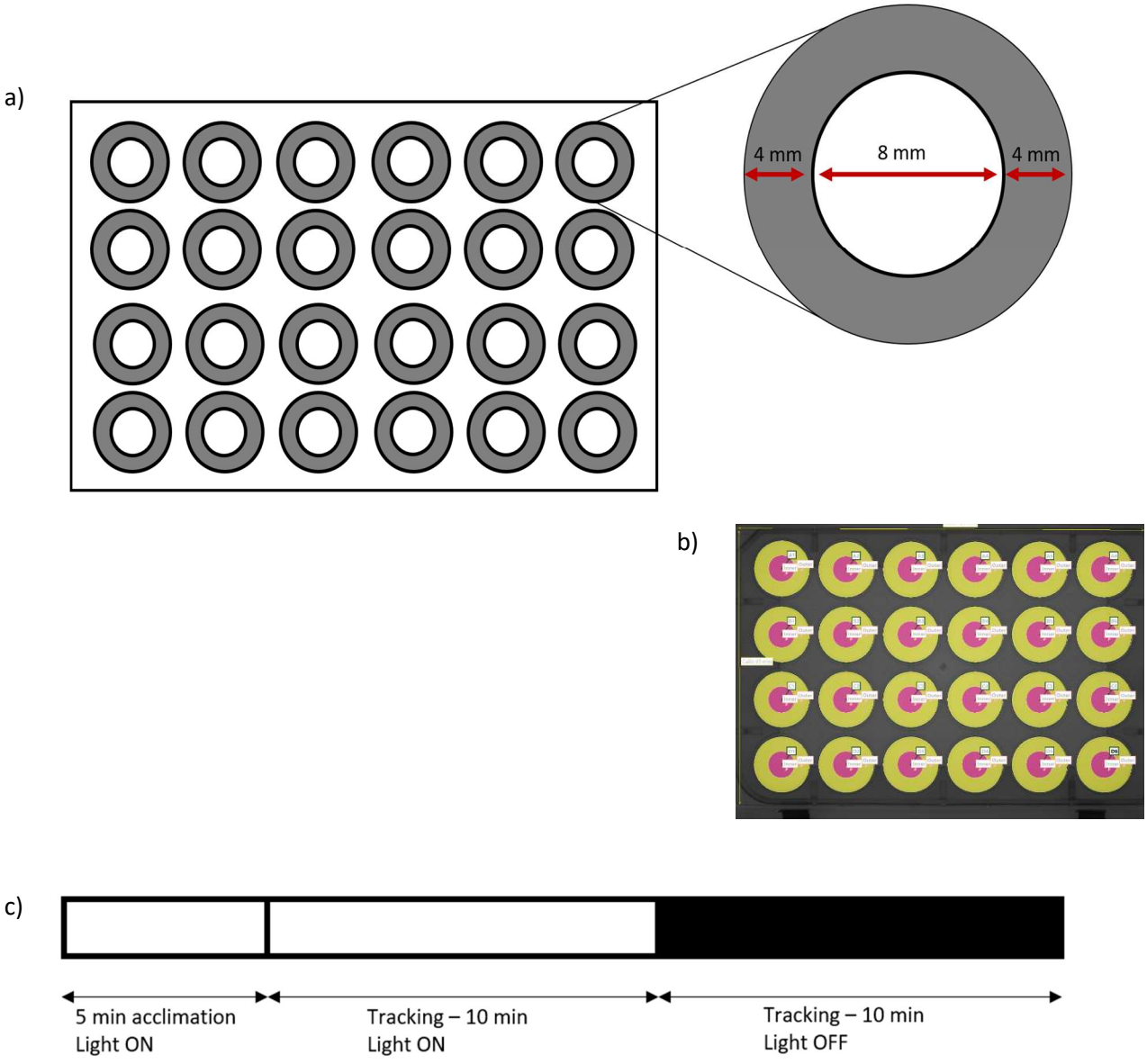


Figure 2.4 Illustration of 24-well plate layout and the tracking duration. a) Depiction of the zone layout, specifying the measurements for inner and outer zones. b) Plate layout on the EthoVision software. c) Illustration of the tracking duration with illumination transitions. After 5 min of acclimation, the zebrafish movement was tracked for 10-min with the light on and another 10-min with light off.

The experimental settings contained tracking for a total of 25 minutes, during which the white light was on for the first 15 minutes, and then was turned off for the last 10 minutes. The first 5 minutes of tracking duration was allowed for larval acclimation (**Figure 2.4 c**). Larvae at 5 dpf require a sudden light-to-dark stimulus to induce their exploratory behaviours [52], and their zone preference during the stimulation was used to evaluate their exploratory behaviour. Hence, in the results section, data were collected from the 10-min light period and the 10-min dark period of the tracking, excluding the 5-min acclimation period. This helped examine the treatment effects on thigmotaxis behaviours during light and dark periods and comparing results from the two illumination settings. Thigmotaxis behaviours in larval zebrafish were previously studied with a similar protocol as the one used in the present study [52].

Light/Dark zone preference behaviours

Larval zebrafish normally display phototaxis (dark avoidance) behaviours, which is opposite to the adult zebrafish scototaxis (light avoidance). This light/dark (L/D) preference behaviour was examined in 5 dpf zebrafish larvae, following exposure to CdSe/ZnS QDs. Their preference was tested by transferring 48 larvae from each concentration group into a 48-well plate, 1 larva per well. Each plate was set up to contain a dark and a light zone per well using a light blocker that obstructed the light in half of every well. The zones were set up on the EthoVision software to reflect the dark and lit areas of each well (**Figure 2.5 a, b**).

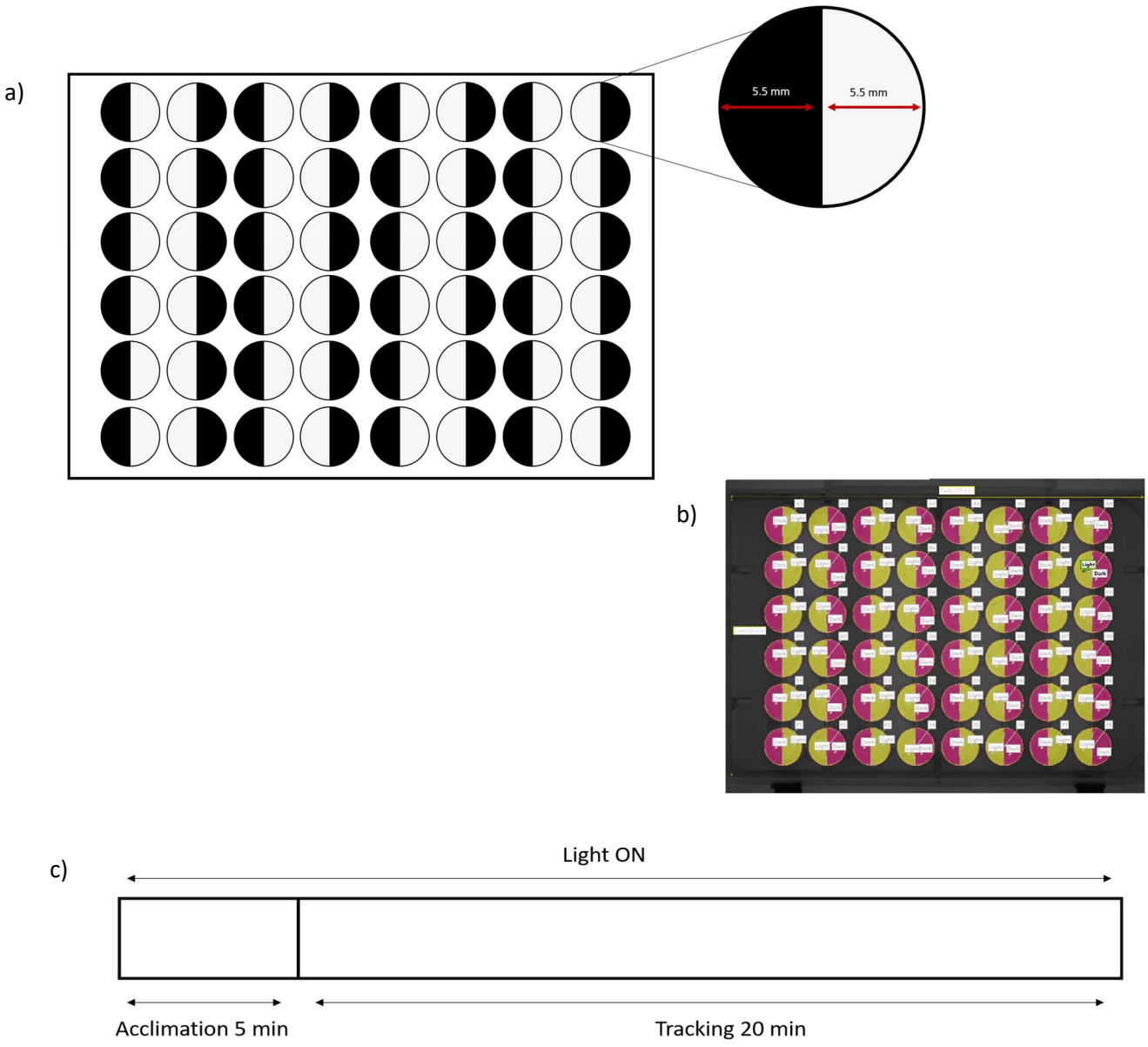


Figure 2.5 Illustration of a 48-well plate layout and the tracking duration. a) Depiction of the zones' layout, specifying the measurements in the dark and light zones. b) Plate layout on the EthoVision software. c) Experimental timeline of the Light/Dark zone preference test. Zebrafish movement was tracked and analyzed for 20 min after the 5 min of acclimation.

The experimental protocols for the L/D preference test followed a previous study [54]. L/D preference test was performed under white illumination condition (10,000 lux) throughout the entire experiment. Larvae were first acclimated for 5 minutes in the chamber, followed by a 20-minute tracking period (**Figure 2.5 c**). Swimming distance, velocity, and the time spent by larvae in each zone were measured in this study.

The preference of larvae for each zone was calculated as a Choice Index value (CI) by the following formula:

$$Choice\ Index\ (CI) = \frac{Duration\ in\ dark - Duration\ in\ light}{Duration\ in\ dark + duration\ in\ light}$$

CI = 0 → No preference for dark or light zone

CI = 1 → Only dark zone preference

CI = -1 → Only light zone preference

Theoretically, CI will lie on a spectrum from -1 to 1 showing a relative larval preference for light or dark zone.

A summary of the experiments carried out in this study, including a timeline, is shown in **Figure 2.6**.

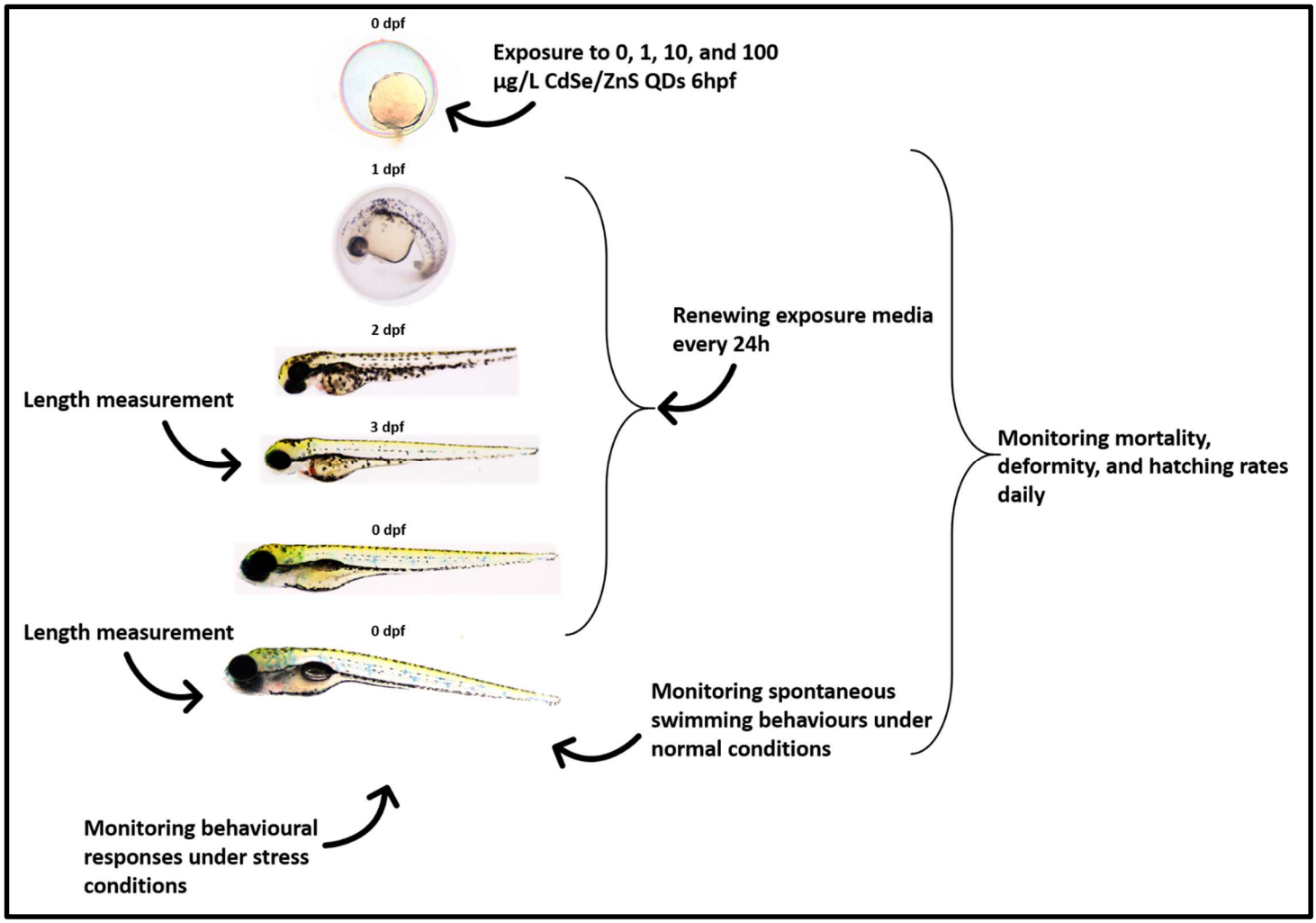


Figure 2.6 Experimental outline illustrating embryonic and larval stages of zebrafish and the corresponding experiments.

Statistical Analysis:

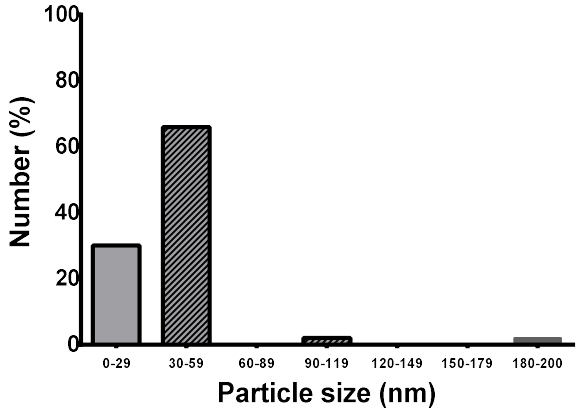
Data were analyzed using SPSS software (version 23.0, IBM SPSS Inc., USA) and presented as mean ± the standard error of the mean (SEM). GraphPad Prism was used to plot graphs. The data were checked for normality and homogeneity of variance using the Kolmogorov−Smirnov one-sample test and Levene's test, respectively. Statistical analysis was performed on parametric data using a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons. In the case of heteroscedasticity, the Welch's test followed by the Games−Howell post hoc test was conducted. When data were percentages (thigmotaxis, light/dark preference, mortality, and total deformities), they were normalized using an arcsine square root transformation. One-way repeated measure was used to determine the effects of treatment on the distance moved over time and mean velocity over time. Independent sample T-test was used to determine significant changes among larval lengths from different days, and to compare the treatment effects on thigmotactic behaviours in light to dark conditions.

3. Results

3.1. Physical properties of QDs in exposure waters

According to the size distribution analysis, the most frequently appearing particle size was 30-59 nm in hydrodynamic diameter. No significant aggregations of QDs were observed in the exposure water (**Figure 3.1**).

a)



b)

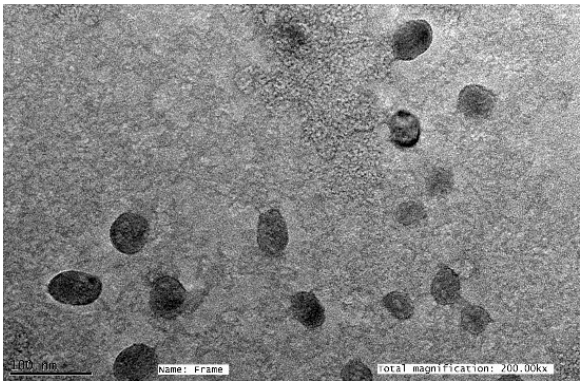


Figure 3.1 Characterization of QD particles in exposure waters. a) Particle size distribution showing most QDs are in the range of 30-59 nm. b) Representative TEM image of QDs at x200,000 magnification (100 µg/L QDs exposure water).

3.2. The concentration of trace metals in exposure waters

Concentrations of trace metals in exposure waters (0, 1 µg/L, 10 µg/L, and 100 µg/L of QDs) were tested using ICP-MS, and the results are summarized in **Table 3.1.** Differences observed in Cd concentration in the exposure waters were found statistically significant ($F_{3,12}$ = 596.28, $p<0.001$). Exposure waters containing 10 µg/L and 100 µg/L

of QDs had significantly higher Cd levels (both $p<0.001$) compared to the other treatment groups. Cd concentration in the 1 µg/L QD exposure water was only significantly different from 10 and 100 µg/L QD treatment groups, and not from the control water (1 µg/L compared to control, $p=0.98$).

Se concentration in the exposure waters was statistically significant among treatment groups ($F_{3,12} = 93.84$, $p<0.001$). Se levels in the 1 µg/L and 10 µg/L QD exposure waters were under the detection limit and were not significantly different from the control water. A significant increase in Se levels was observed in the 100 µg/L QD exposure water when compared to the control water ($p<0.001$).

No significant change in the concentrations of Zn, Fe, and Ca was observed between the different exposure waters.

Table 3.1 Concentrations of Cd, Se, Fe, and Ca in exposure waters.

[QDs]	[Cd] µg/L	[Se] µg/L	[Zn] µg/L	[Fe] µg/L	[Ca] mg/L
0 µg/L	u.d.*[a]	u.d.[a]	23.56±9.63	24.37±8.85	6.73±0.17
1 µg/L	0.21±0.02[a]	u.d.[a]	23.88±6.93	3.93±1.23	6.60±0.07
10 µg/L	3.10±0.56[b]	0.08±0.01[a]	50.86±34.44	6.16±2.93	6.47±0.06
100 µg/L	15.08±0.17[c]	0.74±0.07[b]	68.74±23.80	13.52±4.97	6.33±0.15

*u.d. = under the detection limit. Values are mean ± SEM, n=4. Values labelled with different letters represent a statistical difference ($p < 0.05$).

3.3. Metal/ion body burden in larval zebrafish

Whole body burden of Cd, Se, Zn, Fe, and Ca in 5 dpf larval zebrafish following QDs exposure was measured using ICP-MS. Whole body Cd content in 5 dpf larvae was statistically different among treatment groups ($F_{3,12} = 406.52$, $p<0.001$). Cd contents in larvae exposed to 10 and 100 µg/L QDs (were higher than that in control- and 1 µg/L QDs-treated larvae (For both $p<0.001$). No statistical differences in the whole body level of Se ($F_{3,12} = 2.57$, $p=0.10$), Zn ($F_{3,5.17} = 4.78$, $p=0.06$), Fe ($F_{3,12} = 3.00$, $p=0.07$), and Ca ($F_{3,12} = 3.00$, $p=0.07$) were observed following QDs exposure (**Figure 3.2a – e**).

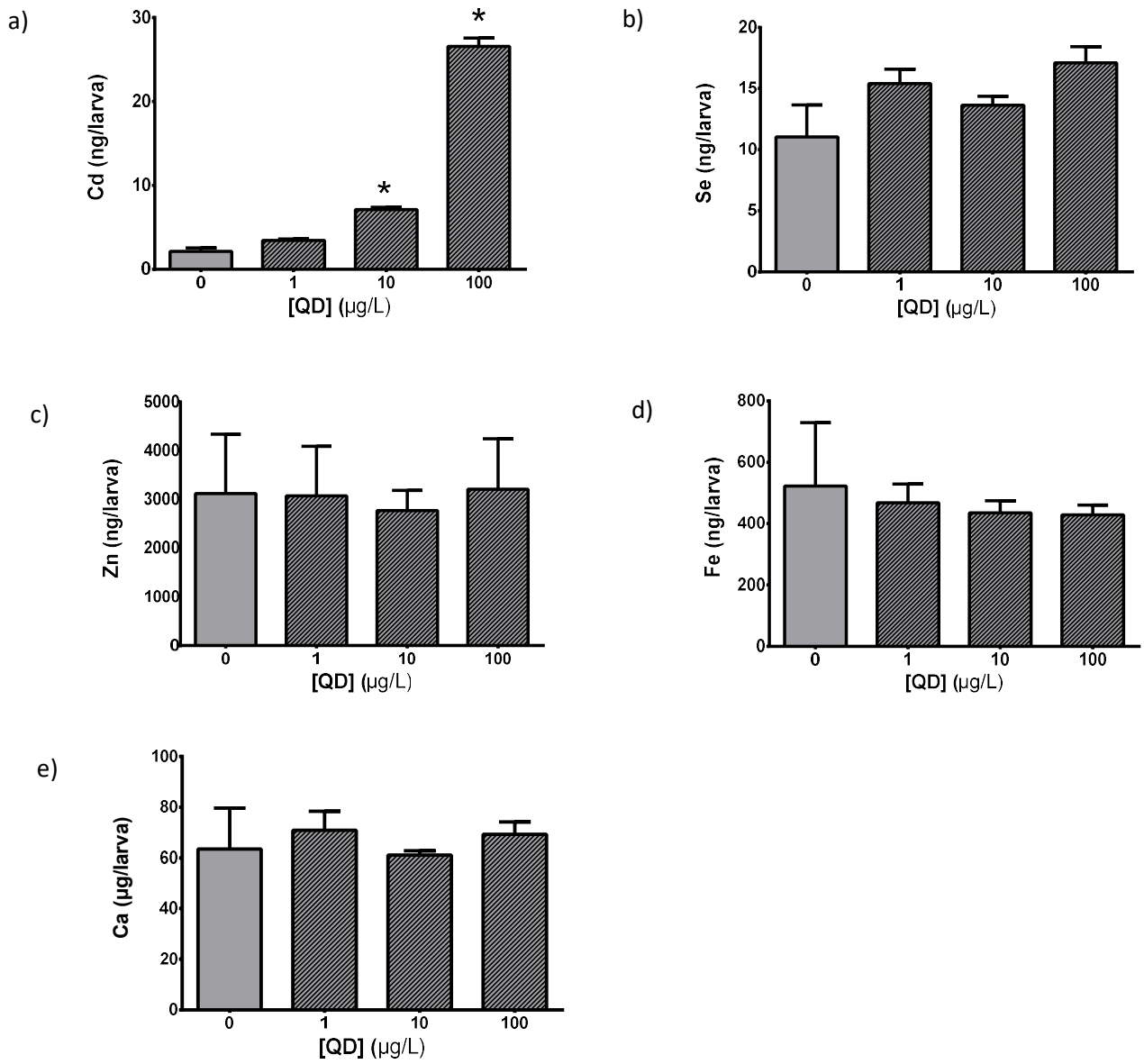


Figure 3.2 Whole body Cd, Se, Zn, Fe, and Ca contents in 5 dpf larval zebrafish. a) Cd content in larvae increased as QD concentration in exposure waters increased (Welch test followed by a post-hoc of Games-Howell, $p<0.001$). b) Se contents showed no significant differences among treatment groups (One-way ANOVA followed by a Tukey's post-hoc, $p=0.10$). c) Zn contents showed no significant variations among treatment groups (One-way ANOVA followed by a Tukey's post-hoc, $p=0.06$). d) Fe metal content showed no significant variations among treatment groups (Welch test followed by a Games-Howell post-hoc, $p=0.07$). e) Ca ion content showed no significant differences among treatment groups (One-way ANOVA was run followed by a Tukey's post-hoc, $p=0.07$). All data are mean ± SEM, N=4 (each replicate consisted of 30 pooled larvae).

3.4. Physiological conditions

Mortality and deformity were monitored daily and their cumulative rates at 5 dpf were calculated. The results suggested that treatment with different concentrations of QDs did not affect mortality rate ($F_{3,20} = 0.28$, $p=0.84$) nor deformity rate ($F_{3,20} = 0.432$, $p= 0.733$). Treatment with different QDs levels also did not affect the hatching rate of larvae ($F_{1.9, 9.56} = 1.82$, $p=0.21$) (**Figure 3.4a-c**).

a) b)

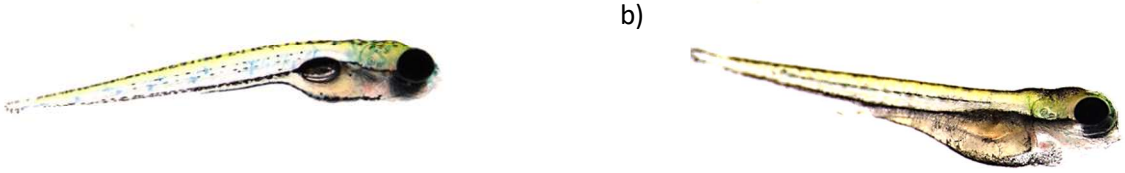
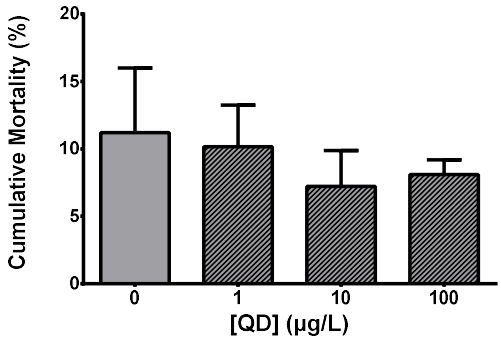


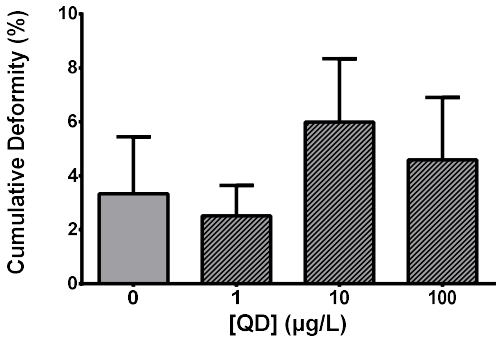
Figure 3.3. Comparing normal larva to deformed larva at 5 dpf. a) Represents a normally developed larva. b) Represents a deformed larva with pericardial edema, shortened length, and slightly bent tail.

Exposure to different concentrations of QDs did not affect standard body length of larvae at 3 dpf ($F_{3,22} = 2.69$, $p=0.07$) nor at 5 dpf ($F_{3,20} = 1.99$, $p=0.15$). The body length at 5 dpf was significantly higher than that at 3 dpf in all the treatment groups ($t_{11,\ 9.10} = -2.26$, $p=0.04$, $t_{10,\ 9.14} = -2.16$, $p= 0.03$, $t_{11,10.98} = -4.97$, $p<0.001$, and $t_{11,\ 8.12} = -5.14$, $p<0.001$, for 0, 1, 10, and 100 µg/L treatment groups, respectively).

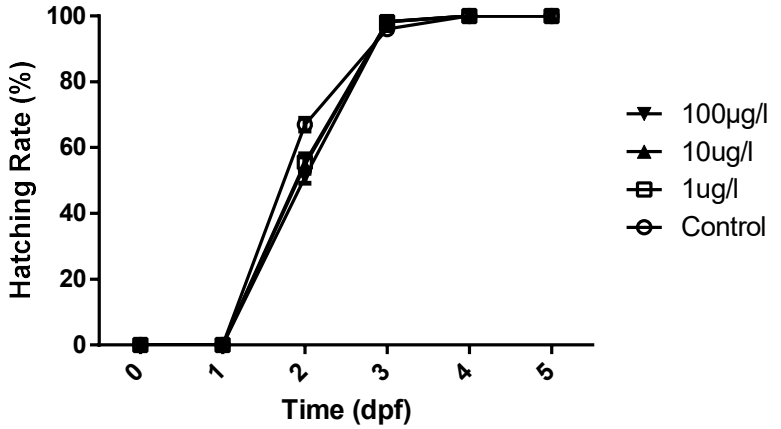
a)


Cumulative Mortality (%)
20
15
10
5
0
0
1
10
100
[QD] (µg/L)

b)


Cumulative Deformity (%)
10
8
6
4
2
0
0
1
10
100
[QD] (µg/L)

c)


Hatching Rate (%)
100
80
60
40
20
0
0
1
2
3
4
5
Time (dpf)
100µg/l
10ug/l
1ug/l
Control

d)

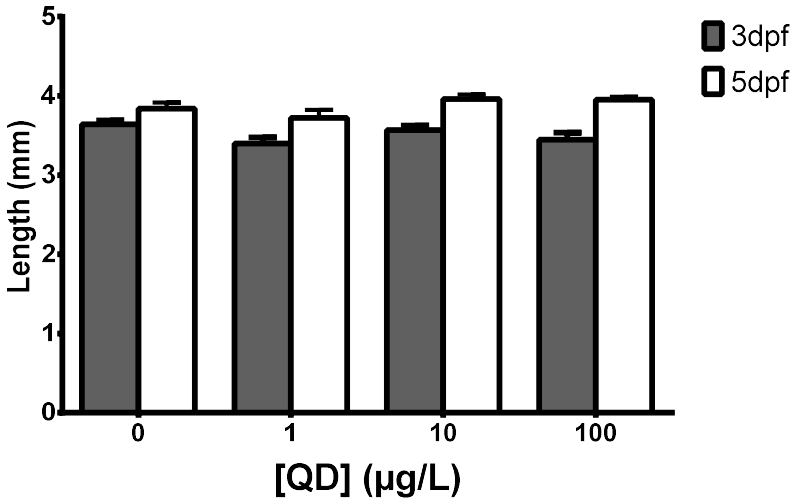

Length (mm)
5
4
3
2
1
0
0
1
10
100
[QD] (µg/L)
3dpf
5dpf

Figure 3.4. Effects of QDs exposure on the physiological conditions of zebrafish larvae. a) Cumulative mortality (%) of larvae at 5 dpf showed no significant differences among treatment groups (One-way ANOVA followed by a Tukey's post-hoc, p=0.84). Data are mean ± SEM, N=6 (each replicate consisted of 20 larvae). b) Cumulative deformity (%) showed no significant differences (One-way ANOVA test followed by a Tukey's post-hoc, p= 0.733). Data are mean ± SEM, N=6 (each replicate consisted of 20 larvae). c) The body length of larvae at 3 and 5 dpf. There were no significant differences when comparing 3 dpf data to each other and 5 dpf data to each other (One-way ANOVA was performed on both sets of data, followed by a Tukey's post-hoc, p=0.07 and p=0.15, respectively). There were significant differences between 3 dpf and 5 dpf within each treatment group. An independent samples T-test was performed (p=0.04, p=0.03, p<0.001, and p<0.001 for 0, 1, 10, and 100 µg/L groups, respectively). Data are mean ± SEM, N=7.

3.5. Spontaneous swimming activities

Locomotion activities including swimming distance and swimming velocity were measured for 30 min in 5 dpf larvae following exposure to 0 – 100 µg/L QDs. Results were reported as average distance moved, and average swimming velocity in 5-min intervals. Average cumulative distance moved and average swimming velocity over the 30-min tracking period were also reported.

Effects of QD treatment on locomotion over time

A two-way repeated measures ANOVA was performed to determine the effects of QD treatments on distance travelled over time (**Figure 3.5a**). There was no significant interaction between time and treatments ($F_{1.57, 130.13} = 1.25$, p=0.28). Therefore, the main effects of time and treatment were investigated separately. There was no significant effect on distance travelled as a result of the QD treatments ($F_{1.02, 84.65}=3.03$, p= 0.085). In

contrast, distance travelled was significantly differed in all treatment groups among different time frames ($F_{1.60, 132.01}$=19.53, p<0.001).

Distance travelled decreased from 0-5 min to 5-10 min, increased during 10-15 min time frames. All three changes in locomotion were statistically significant (p<0.001, p<0.001, and p=0.005, respectively). Changes in distance travelled during the 5-10 min time bin were significant compared to all other time bins (p<0.005). Hence, the decrease in the distance travelled by larvae during this period was significant. On the other hand, when comparing the 0-5 min time frame to the last two timeframes, there were no significant differences among them (p=0.37 and p=1.00, respectively). The last 10 min of distance travelled was significantly different from 5-10 and 10-15 min time frames (for both p<0.001). Therefore, distance moved appeared to increase towards the end of the tracking duration in all treatment groups.

Effects of QD treatments on average cumulative distance travelled

Results from a one-way ANOVA revealed that QD treatments had a significant effect on average cumulative distance moved over the 30-min tracking period ($F_{3, 340}$ = 10.09, p<0.001) (**Figure 3.5 b**). Tukey's post hoc test was then performed to compare results from each treatment group to the control group. Cumulative distance moved in larvae exposed to 1 µg/L or 100 µg/L QDs was significantly reduced when compared to the control group (p=0.005 and p=0.003, respectively). In contrast, the 10 µg/L QD treatment group did not significantly differ from the control group (p=0.78). Representative locomotion heatmaps and track visualizations of the wells are shown in **Figure 3.6**.

Effects of QD treatment on swimming velocity over time

A two-way repeated measures ANOVA was performed to determine the effect of different QD treatments over time on velocity (**Figure 3.5 c**). There was a statistically significant interaction between treatment and time on velocity ($F_{3.99,327.30}$ =3.69, p=0.006). The velocity was significantly different among treatment groups during the 0-5 minute time frame ($F_{2.05, 168.41}$=7.17, p≤0.001). Pairwise comparisons further revealed that velocity in the groups treated with 1 and 100 µg/L QDs was higher compared to the control group during this time frame (both p≤0.002). At 5-10 minutes, a significant difference in velocity was observed among the treatment groups ($F_{1.53, 125.18}$= 3.42, p=0.048). There was a significant difference in 1 µg/L and 100 µg/L treated groups compared to control (both p<0.005). Larvae exposed to 100 ug/L of QDs showed the highest velocity at this time frame. In contrast, at 10-15, 15-20, 20-25, and 25-30 minutes, there was no significant differences among different QD treatments ($F_{1.53, 125.15}$ = 2.04, p=0.15, $F_{1.68, 137.60}$ = 2.64, p=0.08, $F_{1.65, 135.42}$ = 3.26, p=0.05, $F_{1.57, 14116.39}$ = 2.92, p=0.07). Overall, there were significant differences in average swimming velocity among different time frames ($F_{2.01, 164.54}$ = 22.50, p<0.001).

Effects of QD treatments on average swimming velocity

Results from a one-way ANOVA analysis suggested that there were no significant differences in average swimming velocity among the QD treatment groups ($F_{3,343}$ = 0.5, p=0.68) (**Figure 3.5d**).

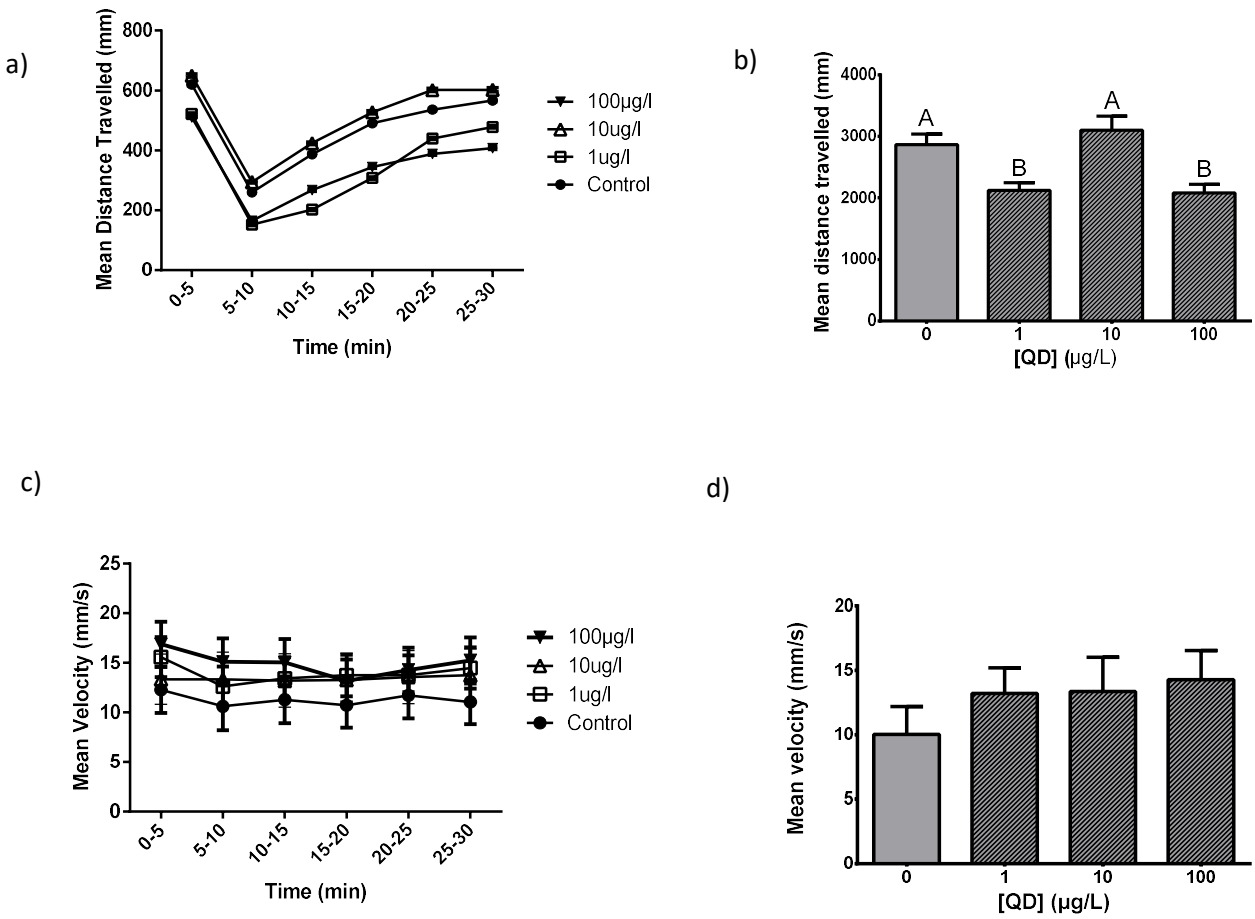


Figure 3.5 Effects of QDs exposure on spontaneous swimming activities of zebrafish larvae at 5 dpf. a) Distance travelled over a 30-min tracking period. Data were collected over 5-min time frames. There were no differences among treatments, but significant differences were observed among time frames. Two-way ANOVA repeated measures was performed (treatment effects on locomotion over time $p=0.28$, treatment effects $p= 0.085$, and time effects $p<0.001$). b) Cumulative distance travelled by larvae over the 30-min period. Significant decreases in distance travelled in 1 and 100 µg/L QD treatment groups were observed, compared to the control group (Welch test followed by a post-hoc of Games-Howell, $p<0.001$. c) Mean velocity over a 30-min tracking period. Data were collected over 5-min time frames. There were significant treatment effects on the velocity at different time frames. Two-way repeated measures ANOVA was performed (Effects of treatments $p=0.006$: effects of time: $p<0.001$). d) Mean velocity There were no significant differences among treatment groups. One-way ANOVA was performed with a Tukey's post-hoc ($p=0.68$). All data are mean ± SEM, N=85.

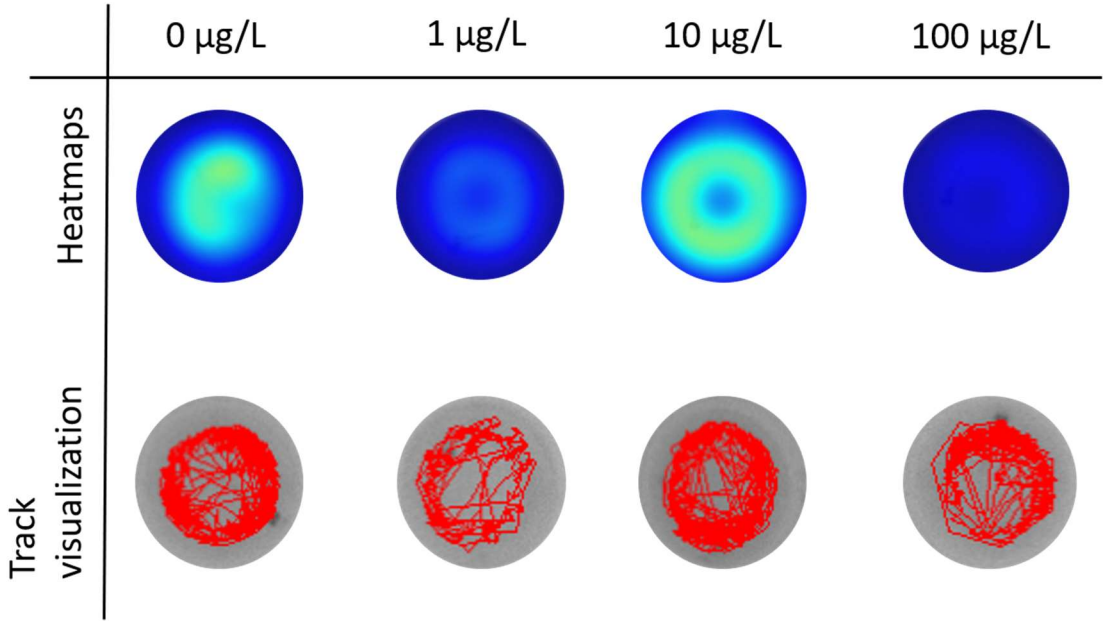


Figure 3.6 Representative heatmap and track visualization of larvae in a well from each treatment group. The tracking was performed for 30 min on a 96-well plate under a white light condition.

3.6. Thigmotaxis (wall-hugging behaviour)

Thigmotactic behaviour of 5 dpf larvae following QD treatments was examined using an inner/outer (wall-hugging) zone preference test following sudden darkness.

Percentage of total time spent in the outer zone during light and dark period

The percent of time spent (both active and inactive larvae) in the outer zone of a well during light and dark periods was measured. During the light period, there were no significant treatment effects found in the percentage of total time spent in the outer zone

($F_{3,48.22}$ = 0.83, p=0.48). All larvae spent approximately 90% of their time within the outer zone of the wells during the light period. Similarly, during the dark period, there were no significant effects of QD treatments on the percentage of time larvae spent in the outer zone of the wells ($F_{3,92}$ = 0.98, p=.0.40). All larvae spent approximately 80% of the total duration in the outer area (**Figure 3.7 a**).

When compared the time spent (%) between the light periods and the dark periods within the outer zone, control larvae spent significantly less time in the outer zone during the dark period (i.e., less wall-hugging behaviour following sudden darkness) ($t_{46,41.50}$ = 2.63, p=0.01). Similar observations were recorded for larvae treated with 10 ($t_{46,45.99}$ = 3.62, p<0.001) or 100 µg/L QDs ($t_{46,41.50}$ = 2.63, p=0.01). However, larvae treated with 1 µg/L QDs did not reduce their time spent in the outer zone following sudden darkness ($t_{44,25.48}$ = -0.21, p=0.84).

Percentage of total distance travelled in the outer zone during the light and dark period

Percentage of total distance travelled (i.e., % of time spent moving; active larva) between inner and outer zones in both light and dark periods were analyzed. During the light period, all larvae travelled approximately 80% of their total distance within the outer zone (i.e., 80% of their movement occurred in the outer zone) and QD treatment did not affect their percent distance travelled in the outer zone ($F_{3,79}$ = 0.20, p=0.89). During the dark period, all larvae also travelled approximately 80% of their total distance within the outer zone (**Figure 3.7 b**). There were no significant differences between the total distance travelled in the outer area of the wells among treatment groups ($F_{3,92}$ = 1.37, p=0.26).

When compared the percent distance travelled (%) between light periods and dark periods within the outer zone, there were no significant differences observed for all the treatment groups (control: $t_{44,31.34} = 0.13$, $p=0.89$; 1 µg/L QDs: $t_{45,31.82} = -0.74$, $p=0.46$; 10 µg/L QDs: $t_{44,29.89} = 0.63$, $p=0.63$; 100 µg/L QDs: $t_{41,22.96} = -0.009$, $p=0.99$). Representative heatmaps and track visualizations of larvae from each treatment group are shown in Figure 3.8.

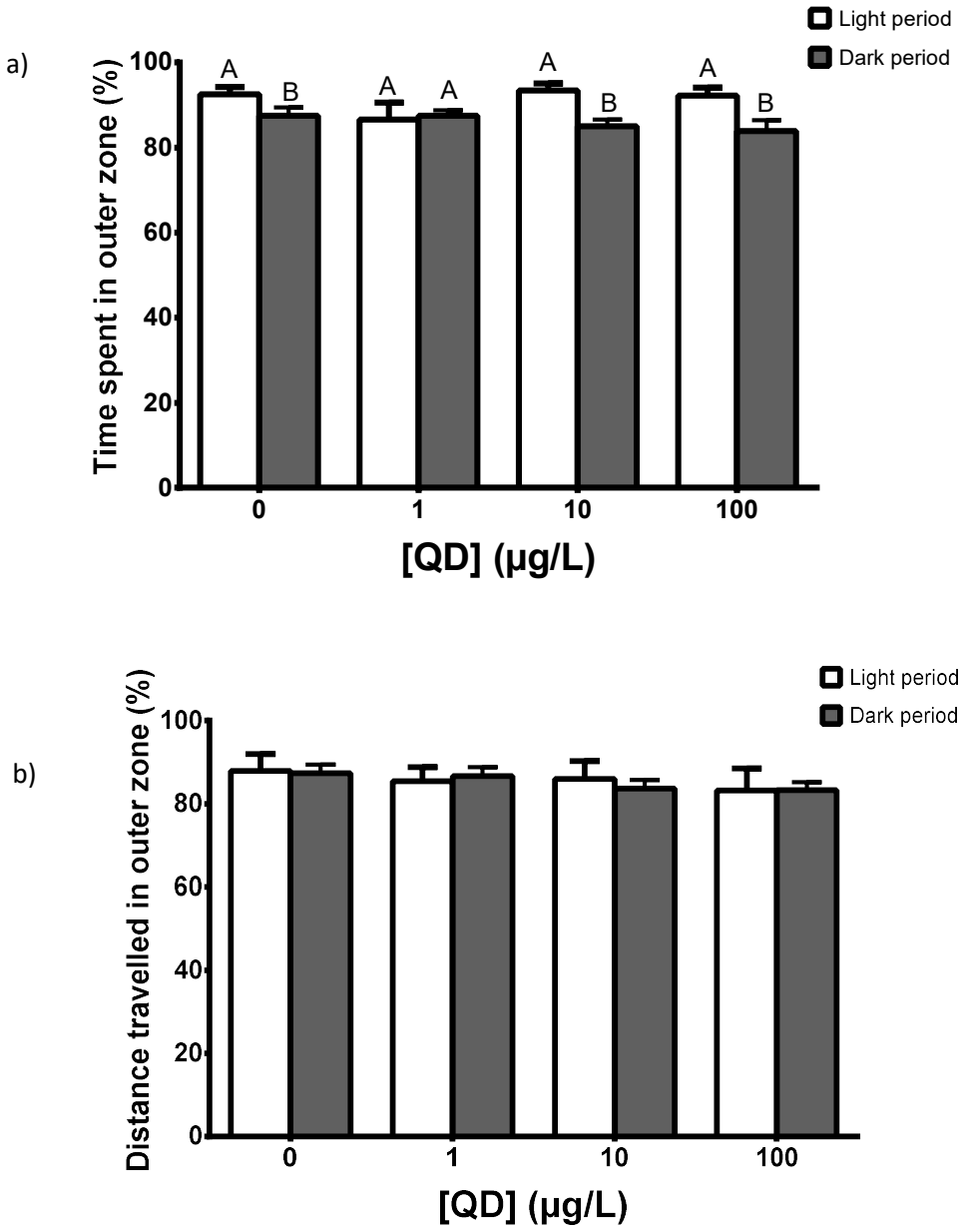


Figure 3.7 Thigmotaxis behaviour of 5 dpf larvae measured as the percentage of time spent and the percentage of total locomotion (i.e., active larvae) in the outer zone of the wells. a) Time spent in the outer zone by larvae during the light (white bars) and dark (gray bars) period. There were no significant differences among treatment groups when analyzed behaviours in light and dark separately (Welch test followed by a Games-Howell post-hoc test, p=0.48), (One-way ANOVA followed by a Tukey's post-hoc test, p=.0.40). Differences in time spent in outer zone during light and dark periods within each treatment group were significant (p=0.01, p=0.84, p<0.001, and p=0.01 for 0, 1, 10, 100 µg/L, respectively).b) Percentage distance travelled in outer zones (% time spent moving) during the light (white bars) and dark (gray bars) period. There were no significant

differences among treatment groups when analyzed behaviours in light and dark separately (One-way ANOVA followed by a Tukey's post-hoc test, p=0.89), (One-way ANOVA followed by a Tukey's post-hoc test, p=0.26). There were no significant differences in time spent in the outer zone during light and dark periods within each treatment group (Independent sample T-test, p=0.89, p=0.46, p=0.63, p=0.99 for 0, 1, 10, 100 µg/L, respectively). All data are mean ± SEM, N=24.

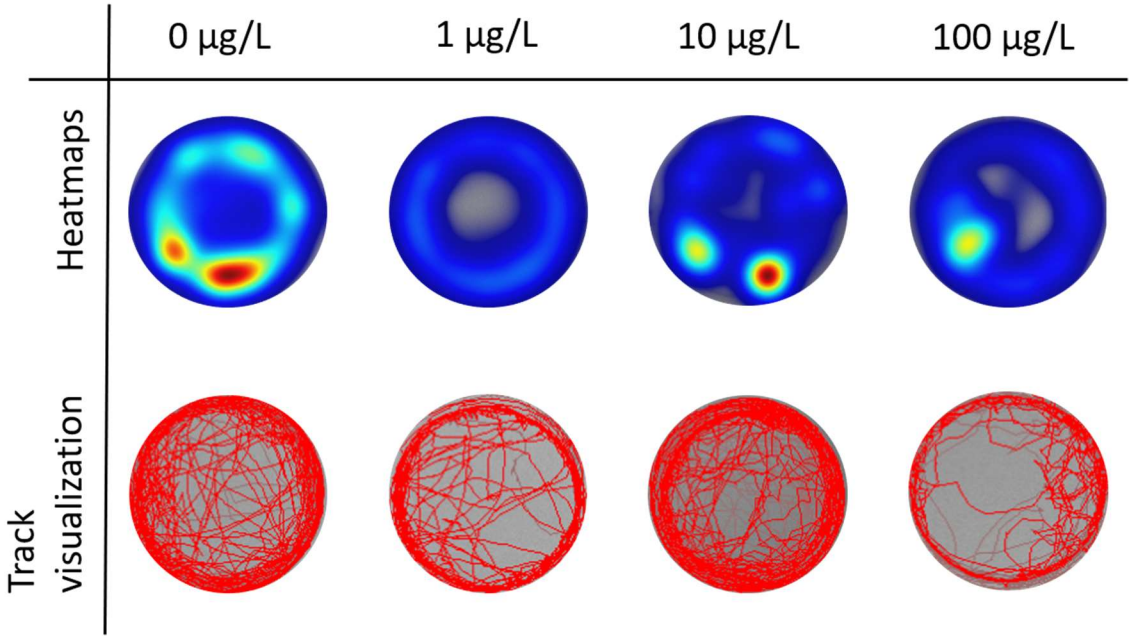


Figure 3.8 Representative heatmap and track visualization of larvae from each treatment group (combination of both light and dark periods). There was higher activity around the corners of the wells.

3.7. Light/Dark (L/D) zone preference behaviours

At 5 dpf, larvae from all treatment groups were examined for their zone preferences between light or dark arenas using a light-dark grid underneath the multi-well plates to create a light and dark half in each well. Preference behaviours were measured as the

percentage of time spent in the lit area, choice index (CI), percentage of total locomotion in the lit area, and velocity in both lit and dark areas.

Percentage of total time spent in the light zone

Effects of QD treatments on L/D preference were statistically significant ($F_{3,92.65} = 5.09$, p=0.003). When compared to control larvae, larvae treated with 10 µg/L QDs exhibited a significant reduction in their time spent in the light zone (p=0.01). Time spent in the light zone by 10 µg/L QD treated larvae also was significantly lower than that by 100 µg/L QD treated larvae (p=0.004). Treatment with 1 µg/L or 100 µg/L QDs did not affect their time spent in the light zone when compared to the control (p=0.29, p=0.93, respectively) (**Figure 3.9 a**).

Choice Index (CI)

CI lies on a spectrum ranging from 1 to -1; negative values suggest light preference and positive values suggest dark preference. CI calculated for the L/D preference was statistically significant among treatment groups ($F_{3,92.64} = 5.29$, $p<0.002$). All larvae exhibited negative CI values; however, larvae treated with 10 µg/L QDs demonstrated the least negative value (-0.06), which was significantly higher than the control group (p=0.009). The result suggested that treatment with 10 µg/L QDs decreased larval preference to the light zone. Treatment with 1 or 100 µg/L QDs did not affect the zone preference of larvae (**Figure 3.9 b**).

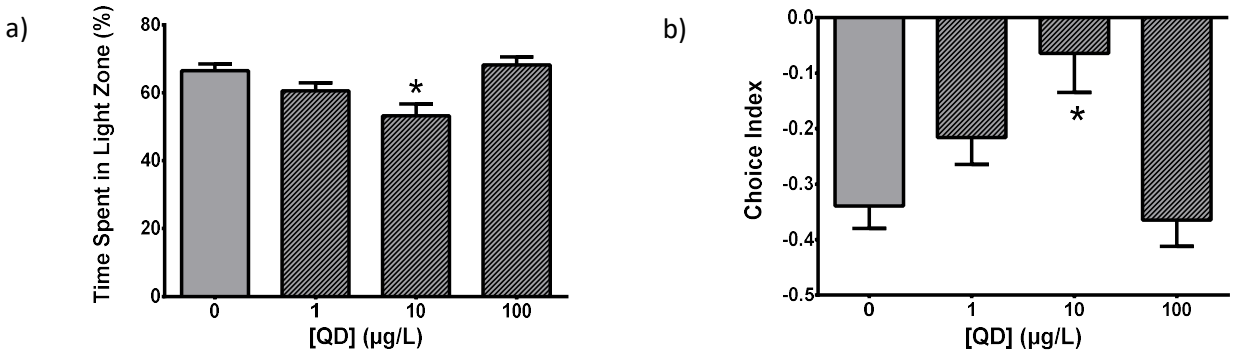


Figure 3.9 Light/Dark zone preference behaviour of 5 dpf larvae measured as the percentage of time spent in the light zone and the Choice Index (CI). a) Larvae from the 10 µg/L QDs treatment group showed a significant decrease in the time spent in the light zone (Welch test followed by a Games-Howell post-hoc, p=0.01). b) CI is a measure of the preference of the larvae for dark (CI>0) or light areas (CI<0). Significant effects were observed in larvae exposed to 10 µg/L QDs (Welch test followed by a Games-Howell post-hoc test, p=0.009). All data are mean ± SEM, N=48.

Percentage of total locomotion in light zone

The percentage of total distance travelled by larvae in the light area was not significantly different among treatment groups ($F_{3,187} = 1.11$, $p=0.35$). Approximately 60% of the locomotor activities occurred in the light zone for all the treatment groups (**Figure 3.10**).

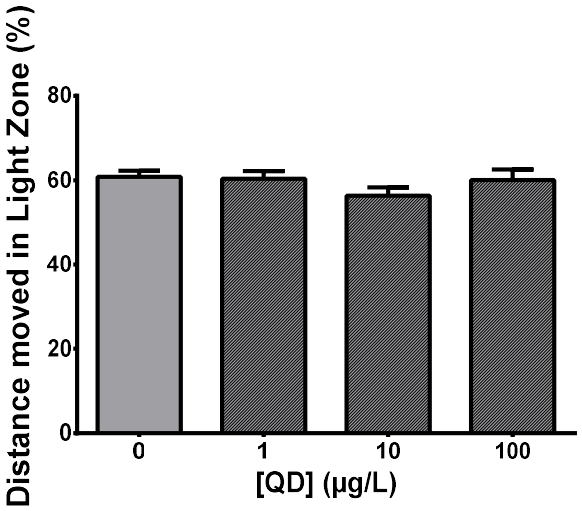


Figure 3.10 Percentage distance moved (% time spent moving) in the light area of the wells did not significantly differ among treatment groups (One-way ANOVA followed by a Tukey's post-hoc test, p=0.35). Data are mean ± SEM, N=48.

Average swimming velocity in light and dark zones

Differences in the swimming velocities between the light and dark zones were statistically significant ($F_{1,374}$ = 10.76, p=0.006); larvae appeared to have a lower swimming velocity in the light zone. Treatment with QDs did not affect swimming velocity between light and dark zones ($F_{3,374}$ = 1.95, p= 0.12) (Figure 3.11). However, there were significant differences among treatment groups when comparing velocities in light ($F_{3,187}$ = 28.36, p<0.001) or in the dark zones ($F_{3,102.43}$ = 17.70, p<0.001). In both light and dark zones, the average swimming velocity of 1 µg/L QD treated larvae was significantly higher when compared to the other treatment groups (For all p<0.05). In contrast, larvae treated with 10 µg/L QDs exhibited a reduced swimming velocity in both light and dark zones when compared to the control (p=0.02).

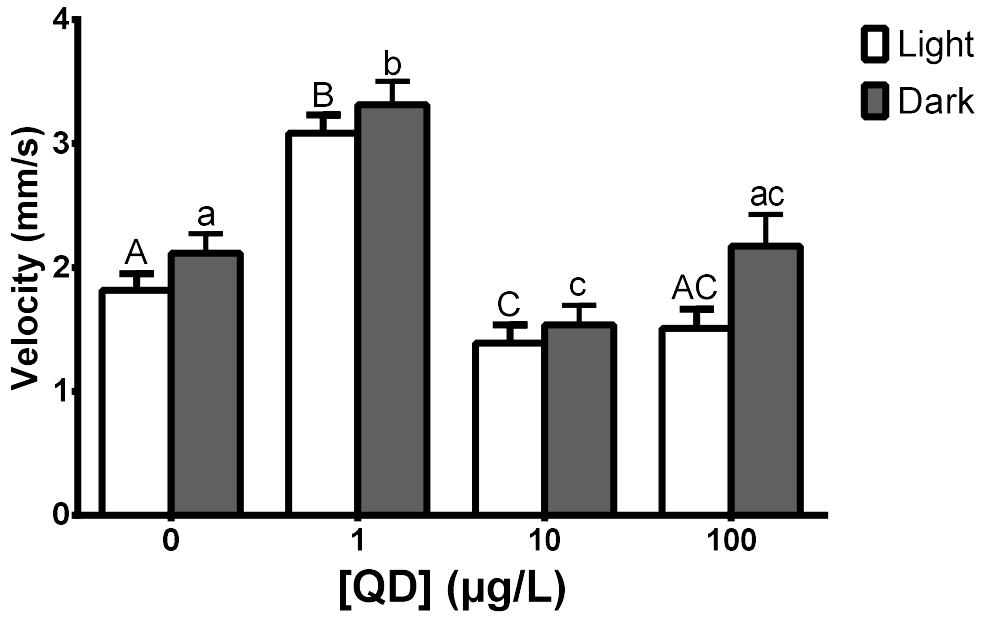


Figure 3.11 Effects of QDs exposure on mean velocity of 5 dpf larvae in the light and dark zones. There were significant differences between velocities among treatment groups when comparing light zones and dark zones separately to one another ($p<0.001$ and $p<0.001$, respectively). Mean velocity of larvae in both the light and dark zones was higher in 1 µg/L QD treatment group when compared to the control ($p<0.05$). Velocities measured from both zones were significantly lower in the 10 µg/L QD treatment group ($p=0.02$). Data are mean ± SEM, N=48.

4. Discussion

Overview

The evolution of nanotechnology poses new challenges for the environmental risk assessment of ENMs. Despite the increasing concern regarding their safety and environmental impact, metal-containing ENMs are extensively used in many consumer, medical, and industrial products. Importantly, the toxicological effects of metal-containing ENMs can be very different from that of their ionic counterparts. Among the different ENMs, QDs are at the forefront of nanotechnology owing to their unique photophysical properties. Cd is commonly used in the construction of QDs; however, their biological impact on biota has remained largely unclear. In this study, I used developing zebrafish as a model organism to examine the effects of exposure to sublethal concentrations of CdSe/ZnS QDs on neurophysiological performances. The results indicated that exposure to QDs did not appear to affect the general physiological conditions of larvae. However, alterations in swimming activities and anxiety-like behaviours were observed following the exposure.

4.1. Physicochemical properties of QDs

Using TEM technology, we observed that the hydrodynamic diameters of CdSe/ZnS QDs in the exposure water (i.e., 100 μg/L) were mostly in the range of 30-59 nm. QDs appeared to be well-dispersed in the exposure water without significant particle

aggregation. Similarly, a previous study with CdSe QDs showed that QD agglomerates started to form only at higher concentrations (~100 mg/L) [55].

Before hatching (0 to 2-3 dpf), zebrafish embryos/larvae are surrounded by the chorion. The chorion contains pore canals and the size of these pores is reported to be approximately 0.5-0.7 μm [56]. The canals are used for gas, ion, and waste exchange between the embryos and their surrounding water [49]. Considering the size of the QDs, QDs were likely able to pass through the chorion canals during the embryonic stage.

ICP-MS was used to measure metal concentrations in the exposure waters. The results revealed that the levels of Cd and Se in the exposure waters increased with increasing QD concentrations. The increased Cd and Se concentrations in the waters were owing to the presence of these metals in the QDs, and Cd and Se were likely the major metal constituents found in QDs. In control water (0 μg/L QDs), both Cd and Se were undetectable. Notably, Cd concentration was approximately 20- to 30-fold higher than Se concentration measured in the 10-100 μg/L QDs exposure waters, suggesting the core of the QDs probably contained a higher amount of Cd compared to Se.

4.2. Metal and ion homeostasis

We observed that whole body content of Cd was significantly increased in 5 dpf larvae exposed to 10 or 100 μg/L of QDs, suggesting the absorption of QDs from the waters. It is important to note here that ICP-MS analysis determines the total metal level in the sample; it could not distinguish between free Cd released from the degraded QDs and the Cd integrated within the intact QDs. Although a previous study has shown that ZnS

coating on the QDs could protect against the release of Cd from the core [20], the possible degradation of QDs in biota warrants further study.

Exposure to high concentrations of Cd is known to increase mortality in zebrafish larvae. For example, zebrafish larvae exposed to 3 to 15 µg Cd/L resulted in approximately 20% mortality [57]. In the current study, exposure to 100 µg/L QDs (equivalent to 15 µg Cd/L) for 5 days resulted in the accumulation of ~25 ng Cd/larva without affecting their mortality rate, suggesting that Cd-containing QDs were likely less toxic than Cd ions. On the other hand, the whole body Se burden was not increased by QDs exposure. This could be due to the relatively lower amount of Se present in the QDs.

Similarly, whole body Zn content was not significantly altered by QD exposure. A previous study on the effects of CdSe/ZnS QDs on larval zebrafish suggested that if QDs are completely degraded in water, QDs containing 0.2 to 200 µM-Cd equivalents (~22 - 22,400 µg/L Cd) could release approximately 0 to 8 µM of Zn [47]. They reported that larvae exposed to this range of Zn concentrations did not result in increased malformation nor mortality [47]. Our results also suggested that the amount of Zn contained in, or released from, the QDs was not sufficient to affect the Zn already existing in the larvae.

In freshwater fish, free Cd ion (Cd^{2+}) is known to induce hypocalcemia by inhibiting Ca uptake [47] [58]. A previous study with larval zebrafish has also demonstrated that waterborne Cd exposure reduced whole body Ca content [20] and reduced expression levels of Ca transporters [59]. On the other hand, it has previously been reported that mRNA expression levels of the apical Fe transporter, divalent metal transporter 1 (DMT1), and Fe exporter, ferroportin1, could be modulated by Cd exposure [14]. Hence, changes in Fe homeostasis could be a possible indicator of QD degradation and the release of Cd in the

body [60]. Findings from the current study showed that exposure to QDs did not affect whole body Ca content in larvae, suggesting no inhibition on Ca uptake by free Cd in the water. Additionally, no significant changes in Fe body burden were observed after QDs exposure. These results suggested that QDs were unlikely degraded in the exposure water nor in the body of larvae; the Cd detected by the ICP-MS was potentially still integrated within the QD particles. However, further studies are necessary to distinguish between the free Cd ions and the integrated Cd within the QD particles and to determine whether the observed effects in the larvae are due to the toxicity of Cd ions or the nanoscale properties of QDs. Because exposure and accumulation of Cd ion are known to induce the expression levels of certain oxidative stress-responsive genes (e.g., metallothionein) [61], assessing their changes may prove useful in understanding the potential release of Cd ion from QDs. In addition, individual particles can be detected via single particle ICP-MS (spICP-MS). In contrast to the ICP-MS technique used in this study, in which single elements were detected subsequent to particles' acid digestion, spICP-MS will detect all the elements inside a single particle resulting in determining the entire multi-element composition of the particle. This will help quantitate the amount of QD nanoparticles without having to digest them into their bulk components [62].

4.3. Physiological performance

Several previous studies have demonstrated that exposure to a high concentration of NPs reduces the survival rates of zebrafish larvae. For example, a study with silica-containing (Si-) NPs has shown that the mortality rate increased by 20% following exposure to 200

mg Si-NP/L [49]. In addition, studies with copper sulfate ($CuSO_4$) NPs demonstrated that the LC_{50} of $CuSO_4$-NP was 1.5 mg/L of Cu-equivalents [63]. In contrast, exposure to ZnO NPs (0 to 10 mg/L) did not affect the survival rates of larval zebrafish [15]. A few studies have also examined the effects of various types of QDs on the survival of zebrafish. Exposure to 50 and 150 µg/L CdSe/ZnS QDs was found to have no effect on the mortality of zebrafish larvae [35]. However, exposure to 0.5 µM of mercaptoacetic acid (MAA)-CdSe/ZnS QDs were reported to cause 45% mortality at 2 dpf. Notably, the larvae were exposed to these QDs via microinjection, therefore these larvae were likely to have a higher amount of QDs in their body [64]. On the other hand, exposure of zebrafish hepatocyte cells to unshelled CdTe QDs was found to significantly decrease their viability at 50 nM [65]. Exposure to as low as 5 nM of shelled, but uncoated CdTe QDs was also found to decrease cell viability [66]. In the present study, we observed that exposure to 1-100 µg/L QDs did not affect the mortality of zebrafish larvae. These results suggested that in addition exposure concentrations, the types and the coating of NPs may influence the toxicity of NPs on larvae.

Like mortality rate, hatching rate and cumulative deformity were not affected by QD treatments in this study. Studies on metal oxide NPs, such as zinc oxide (ZnO), showed no significant deformity (pericardial edema, tail deformity, and spine curvature) among treatment groups at low exposure concentrations. However, exposure concentrations ranging from 10 to 100 mg/L of ZnO NPs caused significant malformations in the larvae [67] [68]. Meanwhile, a significant delay on larval hatching rate was observed at an exposure concentration of 1 mg/L of ZnO NPs [68]. Higher ZnO concentrations (10 mg/L) resulted in no hatching at all, and eventual larval death [55]. Exposure to other metal-oxide

NPs, such as TiO_2 and AL_2O_3, did not pose significant toxicity effects on hatching rates and deformity, at 1-1000 mg/L concentration range [68]. Furthermore, Cd-containing NPs, such as CdS, have shown to significantly delay hatching rate in zebrafish larvae, when exposing them to 100 µg equivalent Cd/L of CdS [69]. Uncoated CdTe QDs caused a significant decrease in hatching rate at concentrations ≥5 nM when examined 48 hpf-144 hpf [66]. On the other hand, coated CdTe QDs did not cause any delay in larval hatching rates up to a 100 nM concentration; however, there was a concentration-dependent decrease in hatching rate when exposed to concentrations greater than 100 nM CdTe [70]. Finally, larval zebrafish exposed to 10 mg/L coated CdSe QDs were examined 72 hpf and showed approximately 37% significant decrease in hatching rate compared to the control group. At higher concentrations (100 mg/L), larvae died unhatched [55]. Exposures to coated CdSe/ZnS QDs caused delayed hatching in zebrafish exposed to 0.45 mg/L QDs; hatching rate further decreased in a concentration-dependent manner [35]. These QDs caused no significant deformity in the larvae exposed to concentrations up to 4.05 mg/L [35]. Overall, various NPs showed varied effects at different concentrations on zebrafish development and hatching rates. Delayed hatching rates and morphological deformities seem to occur mainly at higher concentrations, compared to the concentration range tested in the present study.

Measuring larval lengths can quantify the potential effects of QDs on larval growth. Study on Gold-containing NPs (AuNPs) showed an increase in the length of adult zebrafish after exposure to a concentration range of 0.01-0.05 µg/L, during early developmental stages [71]. In contrast, CdTe QDs caused a significant decrease in the length of 5dpf larvae exposed to concentrations greater than 25 nM [70]. Interestingly,

there were both decreasing and increasing effects on larval lengths as a result of exposure to ZnS-shelled Copper Indium Sulfide ($CuInS_2$/ZnS) QDs. An increase in the length was observed at 50 nM, then it started to decrease at exposure concentrations ≥100nM, in a concentration-dependent manner [72]. Meanwhile, larval zebrafish exposed to 1 and 10 mg/L CdSe NPs did not show any significant differences in their head to tail measurements compared to the control group [55]. In the current study, there were no significant alterations in the larval lengths measured at 3 and 5 dpf, among treatment groups. According to the data provided by previous research, it may be concluded that any length alterations depend on the NPs type and concentration. Various concentrations may induce, hinder, or generate no effects at all on larval growth, depending on the type of NPs. Moreover, exposure concentrations used in the present research seem to be well below the concentrations tested previously for Cd-based NPs therefore, it is suggested that the amount of QDs in the present study was not enough to alter larval length growth.

4.4. Behavioural analysis

Zebrafish have been used in many behavioural studies due to their wide range of complex performances such as locomotor responses, anxiety-related behaviours, including spatial and photo-recognition. Behavioural changes are more sensitive parameters to measure toxicity effects, compared to physiological parameters [71]. Hence, larval zebrafish were examined for their spontaneous swimming activities under normal conditions, and their anxiety-related responses, as a result of exposure to QDs.

Spontaneous swimming activities

NPs such as TiO_2 have shown effects on larval locomotion and mean velocity of rainbow trout. Larvae displayed hypoactivity after exposures to 0.1-1 mg/L TiO_2; however, higher concentrations (5-10 mg/L) did not have an impact on their locomotor activities [48]. In addition, silica-containing NPs (Si-NPs) were shown to significantly reduce larval locomotor activities at 100 - 200 mg/L exposure concentration for up to 96 hpf [49]. Moreover, 5-10 mg/L of ZnO NPs declined mean velocity, as well as locomotor activity, in larval zebrafish [15]. It is important to study the effects of Cd ion, as the main metal component of QDs, on larval zebrafish locomotion as well. In larvae exposed to 9-72 μM (~1.6-13.2 mg/L) concentrations of CdCl2, there were significant decreases in the total distance moved and mean velocity, in a concentration-dependent manner [73]. Further studies revealed a reduction in free-swimming distance and velocity observed after exposure to Cd ion concentrations as low as 0.1 μM (11.24 ug/L) [51]. To put the Cd exposure concentrations previously studied in perspective with the amount of Cd detected in the exposure waters in the present study, there were 27.6 and 134.3 nM Cd detected in 10 and 100 ug/L exposure waters, respectively. This Cd concentration range has previously shown to affect larval locomotion and velocity. In comparison, Cd-containing NPs have also been examined for their possible behavioural effects. Larval zebrafish exposed to coated CdTe QDs were examined at 144 hpf; they displayed lower velocities at 4 and 16 nM exposure concentrations, compared to the control group [70]. There has been limited research on CdSe QD behavioural effects on the developing zebrafish. A study on coated CdSe/ZnS QDs, however, reported no changes in larval locomotor activities as a result of exposures up to 1.35 mg/L QDs. Though,

concentrations equal to or greater than 1.35 mg/L elicited a significant decrease in the swimming speed [35]. In comparison to the QD concentrations in the present study, 1.35 mg/L is a much higher exposure concentration however, the exposure duration took place at 6 hpf through 72 hpf, as opposed to 6-120 hpf in the present research, hence larvae were exposed to QDs for a longer period of time. In addition, differences in QD characteristics such as surface coatings and hydrodynamic sizes exist among studies, which create varied results.

In the present research, larvae showed a significant decline in the total distance they travelled as a result of exposure to 1 and 100 µg/L of QDs. Furthermore, there were no significant alterations in larval mean velocities among treatment groups. The variations observed in locomotion and velocity within the first 10 minutes of the tracking duration were most likely due to the lack of sufficient acclimation time. Results obtained from the total locomotor activities were comparable with previous findings, as there was a decline observed in larval locomotion. However, the behavioural response generated appeared to have an inverted U-shape dose-response pattern. This type of pattern is often observed under the effects of neuroactive chemicals, in which the lowest and the highest concentrations display similar effects [74 75]. A similar dose-response trend was previously observed in larval zebrafish exposed to Si-NPs as well. Even though behavioural responses, such as decreases in locomotor activities, maybe due to impacts on the central nervous system (CNS) of larvae, they may also be due to effects on the eye development or damages to larval muscles. Impacts on the eyes and muscles may lead to hindered ability to see the illumination of the surrounding environment and the inability to move properly, respectively [49]. On the other hand,

swimming velocities in this study were not affected in similar ways as previous studies, in which NPs caused a decline in larval swimming speeds. This would potentially be an indication that muscles' abilities are still intact during declined larval locomotion.

Thigmotaxis and L/D preference behaviours

To further investigate and specify the effects of QDs on the CNS of the larvae, or other possible target organs, anxiety-related responses were examined after QD exposures. Larval zebrafish at 4-5 dpf have already acquired a broad range of behaviours that can be measured. Light/dark preference/avoidance, thigmotaxis, startle responses, etc. are among those behaviours. In the present study, thigmotaxis and light/dark preference behaviours have been selected as anxiety parameters to examine [76]. These behaviours have shown to be affected by neuroactive drugs, previously. The thigmotactic behaviours were heightened in larvae when they were exposed to an anxiogenic drug such as caffeine. This means anxious larvae spent more time in the periphery of their habitat [52]. In the present study, larvae showed no significant differences in the percentage of time spent and the percentage of distance travelled in the outer zone of the wells, among various treatment groups. However, larval thigmotactic behaviour was different when transitioning from light to sudden dark. During the light period, larvae spent approximately 90% of their time in the outer zone, while during the dark periods, they spent 80% of their time in the outer zone. The decrease in outer zone preference in the dark conditions occurred in all treatment groups, except for 1 µg/L, showing no response to the transition in illumination. Based on previous findings, however sudden darkness promotes exploratory behaviours in larval zebrafish, during which larvae

explore their entire space (i.e., hyperactively). In addition, due to their phototactic characteristic, the anxiety resulted from the dark conditions contributes to hyperactivity during this period. This leads to an increase in thigmotactic behaviours in larvae [52]. This is in contrast with the results obtained from the current study, in which larvae in the control group and the 10 and 100 µg/L treatment groups all showed decreased thigmotaxis in dark. Several factors contribute to this discrepancy. Differences in the strain of zebrafish used could be a factor contributing to the altered thigmotactic response here [77]. Other factors such as media content, handling of the larvae, light intensities applied, and parents' husbandry conditions, all could play important roles in anxiety-related responses in the developing zebrafish. It is important to further investigate the underlying reason for the unresponsiveness of 1 µg/L treatment group to the change in illumination settings. Whether QDs have hindered anxiety-related responses in the exposed larvae, is a question that requires more research.

Light/dark preferences were examined in larvae from all treatment groups. Larvae exposed to 10 µg/L of QDs spent less time in and displayed a lower IC value for the light zone. Moreover, larvae exposed to 1 µg/L and 10 µg/L showed higher and lower mean velocities, respectively, in both light and dark zones. It has been previously reported that larval zebrafish prefer light zones over dark zones, avoiding dark under normal conditions [78]. In the present study, all larvae have displayed preference in light over dark zones. There is limited research on larval zebrafish L/D preference behaviours after NP exposures; however, their preference behaviours have been examined under other conditions. Larval zebrafish were previously exposed to cold,

heat, and UV radiations, as stressors, to examine potential effects on their L/D preference behaviours. There was an increase in the percentage of time spent in the light area as a result of exposure to each of the stressors, which was believed to reflect anxiety in larvae. In contrast, larvae treated with anxiolytic drugs, such as chlordiazepoxide and buspirone, showed decreased dark avoidance [54]. This decrease in light preference suggested that the larvae were not responsive to the illumination settings, possibly due to impacts on the development of larval CNS. On the other hand, sensory deficits were also suggested to be a potential cause, inhibiting proper recognition of incoming light from the surrounding environment. The decrease in light preference of 10 µg/L larval group and the decline in their mean velocity in the light and the dark zones might be due to damages to the eye (unable to sense the light properly) or to the neuromuscular system (unable to move properly) developments. However, the response pattern created for larval velocities in light and dark zones, once again, is an inverted U-shape dose-response pattern. A possible pathway underlying the effects on mean velocities may be muscle fiber activation in larval zebrafish. There are two types of skeletal muscle fibers in zebrafish, fast and slow fibers. Fast muscle fibers elicit a faster movement, as opposed to slow muscle fibers that result in slower movements in larvae, upon activation [79]. Under anxiety-related conditions, 1 µg/L of CdSe QDs may induce activation of fast muscle fibers and result in an increase in larval velocity, while 10 µg/L QDs may activate slow muscle fibers causing slow movements. Overall, the inverted U-shape dose-response pattern [49] was a recurring pattern of larval responses in the present study, which potentially suggests the neuroactivity of QDs in larval zebrafish.

Although monitoring anxiety-related phenotypes are useful in understanding the effects of QDs on neuroactivity and anxiogenic responses, there are several limitations to these approaches. Behavioural endpoints are highly sensitive to environmental and procedural factors, which may cause discrepancies between results [80]. In addition, differences in fish strains create data variabilities among studies. Furthermore, anxiety-related behavioural analyses, such as ones performed in the present study, are unable to quantify the amount of stress/anxiety caused by specific NPs under study. Larvae with a varied amount of anxiety may elicit different types of behavioural responses. Cortisol is the main mediator of the anxiety-related responses and it is strongly connected to the physiological reaction to stress in fish. Measuring whole-body cortisol levels in larvae may help link the amount of stress to the anxiety-related response elicited [81].

Conclusions and Future perspectives

Overall, having looked at various research findings on QD toxicity, many aspects have been considered while studying their effects. Firstly, it is important to characterize the physical properties of QDs, including their components, coating, size, particle aggregation, and state. QD characteristics play a key role in their uptake, accumulation, and toxic effects. Secondly, QD concentration, exposure duration, and exposure route would also influence their effects on organisms. In the present study, 6 hpf larval zebrafish were exposed to carboxylated CdSe/ZnS QDs for a duration of 0-5 dpf. Considering that QDs have previously shown the ability to cross the BBB and penetrate

the organisms' brain raises the speculation of their potential impacts on CNS. Further observing the inverted U-shape dose-response pattern repeatedly in the current research, suggests possible neuroactivity of these particles in the developing zebrafish, affecting their neurobehavioural performances. In addition to CNS, the observed effects may be due to sensory or neuromuscular dysfunctions. The latter may be ruled out after further examination of locomotor activities under stress/anxiety-related conditions. Increased locomotion as a stress-related response would potentially show the proper development and functioning of the neuromuscular system in larvae [70].

The present study demonstrated the potential neurotoxic effects of QDs in larval fish; however, future study should also examine the anxiety-related behaviours with various strains of zebrafish and under different environmental conditions. Moreover, further research is required to investigate the possible tissue-specific accumulation of QDs and to confirm their penetration into the CNS. Although previous studies on functionalized QDs have revealed their distribution in the brain via the systemic blood circulation [26 82], there is still limited information on the biological fate of CdSe QDs with various characteristics. This study demonstrated the neurophysiological impacts that QDs may impose on fish, even at extremely low exposure concentrations. Spatial recognition, preference in light/dark, and startle responses, are important behavioural responses for the survival of fish (e.g. escape from predators, hunting for preys, etc). Alteration in these behaviours may lead to complications in their survival and eventually cause devastating consequences to the lives of fish. Furthermore, it is necessary to advance our analytical methods to measure QDs in aquatic environments and to

improve our understanding of their biological effects in order to better assess their potential risks to aquatic animals.

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