# PHYSIOLOGICAL AND NEUROBEHAVIOURAL IMPACTS OF IRON OXIDE NANOPARTICLES ON DEVELOPING ZEBRAFISH

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**Abstract**

Iron oxide nanoparticles (IONPs) are widely utilized in biomedical, industrial, and environmental applications; however, their increasing release into aquatic systems raises concerns regarding their potential neurotoxic effects on aquatic organisms. This study investigated the physiological, behavioural, molecular, and electrophysiological impacts of early-life IONP exposure in zebrafish (*Danio rerio*) to elucidate concentration-dependent and developmental outcomes. Zebrafish embryos were exposed to sublethal concentrations of IONPs (0, 1, and 5 mg IONPs/L) from 4 hours post-fertilization to 6 days post-fertilization (dpf), and raised to 30 dpf under normal conditions, to assess potential persistent effects. Physiological analyses revealed no significant mortality or deformity, suggesting the absence of gross morphological toxicity. Nevertheless, trace metal analysis revealed elevated Fe accumulation in tissues, consistent with nanoparticle uptake and bioavailability. Behavioural assessments were consistent with increased locomotor activity and elevated thigmotaxis in 6 dpf larvae exposed to 1 mg/L IONPs, whereas larvae exposed to 5 mg/L exhibited locomotor activity comparable to controls while maintaining elevated thigmotaxis. Overall, behavioural effects appeared more pronounced at the lower exposure concentration. In juveniles (30 dpf), behavioural effects persisted in a non-linear manner: 1 mg/L exposure caused hypoactivity and anxiety-like responses, while 5 mg/L exposure induced hyperactivity and reduced social preference. At the molecular level, IONP exposure altered the transcript abundance of oxidative stress-related genes (*Superoxide dismutase 1, Catalase, and Glutathione S-transferase)* and dopaminergic markers (*Tyrosine hydroxylase)*). Larvae exhibited elevated *cat* and *gstp1.2* mRNA expression, consistent with increased oxidative stress, while juveniles showed *th1* upregulation and *gstp1.2* downregulation,

consistent with altered dopaminergic and antioxidant defence pathways, respectively. Electrophysiological analyses using multi-electrode arrays revealed significantly increased local field potentials, burst event frequencies and higher number of electrical spikes in 5 mg/L larvae, potentially suggesting enhanced neural excitability. Collectively, these findings demonstrate that early-life exposure to environmentally relevant concentrations of IONPs may induce lasting, life-stage- specific neurophysiological and behavioural alterations. This integrated assessment underscores the potential ecological and developmental risks of IONPs and emphasizes the need for long-term, multilevel evaluations in nanoparticle toxicology.

## Dedication

This thesis is dedicated, first and foremost, to my husband. Your presence in my life has been a steady source of strength, clarity, and perspective throughout this journey. In the long and often uncertain process of research, writing, and self-doubt, you remained constant, patient when the work felt overwhelming, encouraging when progress was slow, and unwavering in your belief that I would see this through. You have witnessed the unpolished moments behind every polished page, and you met them not with judgment, but with understanding. This work carries traces of your support in every chapter, even where your name does not appear. For your companionship, your quiet sacrifices, and the sense of balance you brought into my life when I needed it most, I dedicate this to you.

I also dedicate this thesis to my parents, whose influence reaches far beyond what can be contained in words or pages. You laid the foundation that made this work possible long before I understood what it meant to pursue knowledge at this level. Through your guidance, you taught discipline, resilience, and the value of education, not as an abstract ideal, but as something to be lived and carried forward. You gave me the freedom to question, the courage to persist, and the responsibility to take my work seriously. The path to this thesis has not been linear, and yet your support has never been conditional on ease or certainty. It has been grounded in trust, trust that I would find my way, even when it was not immediately visible.

This thesis stands, in part, as a reflection of the life my loved ones helped shape. It is a continuation of the effort, sacrifice, and belief they invested over many years, often without recognition. If this work represents any achievement, it is one that is shared with them.

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## List of Abbreviations

**Ag-NPs** – Titanium Dioxide

**BBB** – Blood–Brain Barrier

**CAT** – Catalase

**DA** – Dopamine

**DNA** – Deoxyribonucleic Acid

**ENMs** – Engineered Nanomaterials

**$Fe^{2+}$** – Ferrous Iron

**$Fe^{3+}$** – Ferrous Iron

**$Fe_3O_4$** – Magnetite (Iron(II,III) oxide)

**GST** – Glutathione S-Transferase

**GSTP1.2** – Glutathione S-Transferase Pi 1.2

**$H_2O_2$** – Hydrogen Peroxide

**ICP-MS** – Inductively Coupled Plasma Mass Spectrometry

**IONPs** – Iron Oxide Nanoparticles

**MEDs** – Microelectrode Arrays (Multi-Electrode Devices)

**MRI** – Magnetic Resonance Imaging

**mRNA** – Messenger Ribonucleic Acid

**NPs** – Nanoparticles

**PCR** – Polymerase Chain Reaction

**ROS** – Reactive Oxygen Species

**Rpl13a** – Ribosomal Protein L13a

**Rps18** – Ribosomal Protein S18

**SOD1** – Superoxide Dismutase 1

**SOD2** – Superoxide Dismutase 2

**TH1** – Tyrosine Hydroxylase 1

**TiO2** – Titanium Dioxide

**VMAT** – Vesicular Monoamine Transporter

**α-$Fe_2O_3$** – Alpha-iron(III) oxide (Hematite)

**γ-$Fe_2O_3$** – Gamma-iron(III) oxide (Maghemite)

**•$O_2^-$** – Superoxide Radical (Superoxide Anion)

**•OH** – Hydroxyl Radical

**•OH** – Hydroxide Ion

# CHAPTER 1

# Overview – General Introduction

## 1.1. Background and Rationale

### 1.1.1 Nanotechnology and Its Growing Importance

Nanotechnology, broadly defined as the manipulation of matter at the nanoscale (1–100 nm), represents one of the most transformative scientific and technological advances of the 21st century [1]. At this scale, materials exhibit unique physical, chemical, and biological properties that are often absent in their bulk counterparts, allowing them to be applied in diverse fields ranging from medicine and electronics to environmental remediation [2–5]. Over the last two decades, nanotechnology has transitioned from laboratory research to large-scale industrial applications, resulting in the widespread incorporation of engineered nanoparticles (NPs) into consumer products, biomedical tools, and environmental technologies.

Metal-based NPs have been particularly prominent in this expansion, owing to their high reactivity, catalytic potential, and multifunctionality [6]. Their ability to interact with biological and environmental systems in ways that differ from conventional materials makes them both highly promising and, simultaneously, a source of ecological concern [7,8]. As global production of metal and metal oxide nanoparticles continues to expand, with the metal nanoparticle market valued at over USD 3 billion in 2024 and projected to more than double by the early 2030s at double-digit growth rates, the probability of unintentional release into natural ecosystems, particularly aquatic environments that act as sinks for industrial and municipal waste, may likewise increase

[9,10]. The broader nanomaterials sector was estimated at USD 12.4 billion in 2023 and is projected to exceed USD 30 billion by 2030, reflecting rapidly rising production and application of nanoscale materials across industries [11] (Supplementary Figure 1).

### 1.1.2. Iron Oxide Nanoparticles (IONPs)

Among the wide spectrum of engineered nanomaterials, iron oxide nanoparticles (IONPs) have gained substantial attention due to their distinctive superparamagnetic properties, relative biocompatibility, and cost-effectiveness. Structurally, IONPs occur in several crystalline forms, including magnetite ($Fe_3O_4$), maghemite ($\gamma$-$Fe_2O_3$), and hematite ($\alpha$-$Fe_2O_3$) (Figure 1), each with distinct surface chemistry, stability, and biological interactions [6,12]. These forms also differ in oxidation state and reactivity, influencing their environmental transformations and toxicological outcomes [6]. IONPs are used across a wide array of industries. In biomedicine, they are employed as contrast agents for magnetic resonance imaging (MRI), targeted drug delivery systems, and hyperthermia-based cancer therapies. In environmental engineering, their high surface reactivity makes them valuable for wastewater treatment, catalysis, and remediation of heavy metal contamination [3]. Their capacity to adsorb and catalyze degradation of organic contaminants has placed IONPs at the forefront of nano-remediation technologies. The global market for IONPs continues to grow, reflecting their versatility and relative ease of synthesis compared to other nanomaterials [5]. Despite these benefits, concerns about unintended environmental impacts have escalated. While silver and titanium dioxide NPs are among the most extensively studied metal-based nanomaterials, IONPs have received comparatively less attention in aquatic systems. Although IONPs are often characterized as having lower acute toxicity, their redox-active properties and involvement in iron (Fe) homeostasis raise questions regarding potential sublethal

and developmental effects [8,13–15]. Their widespread use and large-scale production inevitably result in release into aquatic ecosystems through industrial effluents, wastewater treatment discharges, urban runoff, and accidental spills. Current estimates suggest that engineered NPs, including IONPs, may enter aquatic environments at levels ranging from ng/L to µg/L, with annual releases projected in the order of tens of thousands of tonnes globally [8,16,17].

a) b)

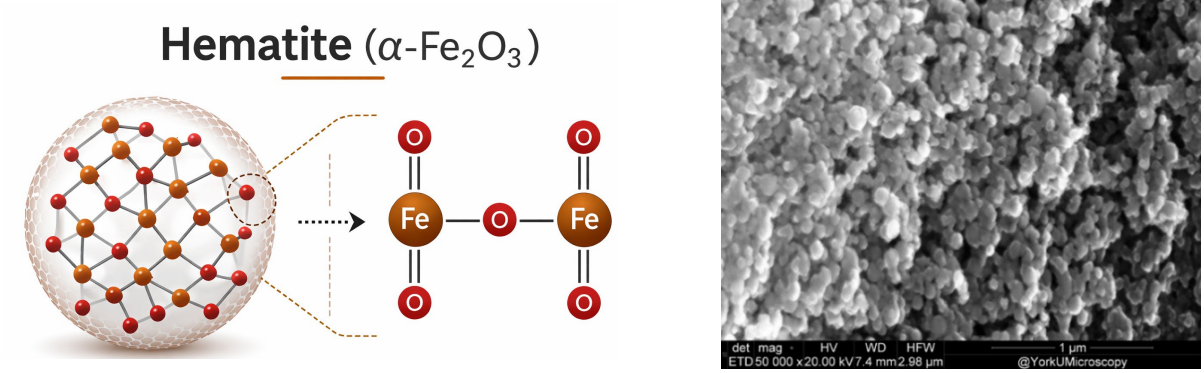



**Figure 1. Hematites** (a) Schematic representation of the crystal structure of hematite ($\alpha$-$Fe_2O_3$), illustrating the arrangement of Fe and O atoms within the lattice. (b) Scanning electron microscopy (SEM) image of hematite particles, showing their aggregated morphology and surface texture at the microscale.

### 1.1.3. Distinction Between Nanoparticulate and Ionic Iron Toxicity

It is important to distinguish between the toxicological properties of IONPs and those of dissolved ionic iron. While dissolved $Fe^{2+}$ and $Fe^{3+}$ participate in well-characterized redox reactions and are tightly regulated by biological homeostatic mechanisms, IONPs exhibit physicochemical properties that differ from their ionic counterparts [18]. As particulate materials, IONPs may undergo aggregation, surface modification, cellular internalization via endocytosis,

and localized dissolution within tissues. These properties influence biodistribution, persistence, and intracellular iron release patterns in ways that cannot be fully replicated by dissolved iron exposure alone. Consequently, NP-associated effects may reflect not only ionic iron contributions but also particle-specific characteristics such as size, surface reactivity, and cellular uptake pathways [19,20].

### 1.2. Iron Oxide Nanoparticles in Aquatic Systems

#### 1.2.1. Ecotoxicological Concerns of IONPs in Aquatic Systems:

- Environmental Transformations and Fate of IONPs

Once released into aquatic systems, IONPs undergo complex physicochemical transformations that influence their environmental persistence, bioavailability, and toxicity. These transformations include:

- Aggregation and Sedimentation: IONPs tend to aggregate due to van der Waals forces and magnetic interactions, forming larger clusters that sediment to the bottom of aquatic environments. This process influences their spatial distribution and interaction with biotic and abiotic factors [8,13].
- Dissolution: Under certain pH and redox conditions, IONPs release ferrous ($Fe^{2+}$) and ferric ($Fe^{3+}$) ions, which can participate in redox reactions such as the Fenton and Haber–Weiss cycles, generating reactive oxygen species (ROS). Particle dissolution leads to changes in chemical reactivity and biological responses hence, one of the significant factors to monitor in NP toxicity studies [13].

- Surface Modifications: Interaction with natural organic matter, dissolved ions, and other colloids alters the surface chemistry of IONPs, modifying their reactivity, aggregation behaviour, and interactions with biological membranes. While many IONPs are surface-modified, the IONPs used in this study are bare, without additional coatings, making it important to distinguish their behaviour from modified counterparts [6,12].

Identification of these transformations must be included in characterization of IONPs since they are essential when comparing results across studies. These processes are highly dependent on environmental conditions, including ionic strength, pH, temperature, and the presence of organic matter [21,22]. For example, humic substances may stabilize IONPs by forming surface coatings, whereas divalent cations (e.g., $Ca^{2+}$, $Mg^{2+}$) can promote aggregation. The environmental behaviour of IONPs is thus dynamic and context-dependent, complicating risk assessment and prediction of exposure scenarios.

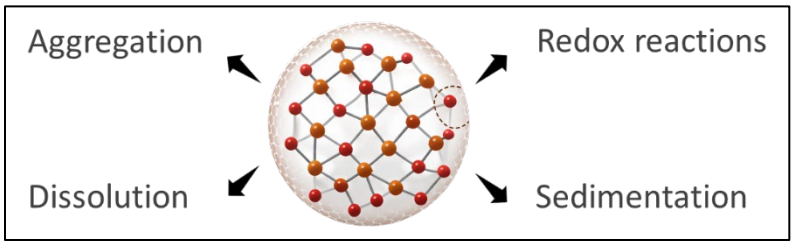



**Figure 2. Transformations IONPs may undergo in aquatic systems.**

- Detection Challenges and Environmental Monitoring

One of the major barriers to assessing IONP risk in aquatic ecosystems lies in the difficulty of detecting and quantifying nanoscale materials in complex environmental matrices. Conventional

methods for monitoring dissolved metals are not directly applicable to engineered nanomaterials (ENMs), as they fail to distinguish between ionic and particulate forms [16,23,24]. Advanced techniques such as inductively coupled plasma mass spectrometry (ICP-MS), single-particle ICP-MS, and electron microscopy have been adapted for NP detection, yet standardization remains limited [24,25]. This lack of consistent analytical protocols has hindered the accurate estimation of environmental concentrations of IONPs. Furthermore, key transformation processes, including aggregation and sedimentation, dissolution, and surface modifications, also influence detection and quantification, highlighting the importance of accounting for these dynamics in comparative studies.

Despite these challenges, existing studies report detectable concentrations of ENMs in surface waters, wastewater effluents, and sediments. For example, mass-balance models and empirical measurements suggest that metal-based NPs, including titanium dioxide ($TiO_2$) and silver nanoparticles (Ag-NPs), are present in the µg/L range in urban and industrial effluents [8]. Although direct measurements of IONPs are scarce, modeling studies predict similar or higher release rates due to their extensive production and use. The persistence of these materials raises concerns about chronic exposure scenarios for aquatic organisms, particularly in areas of high industrial activity, and highlights the importance of evaluating IONP release and biological impacts, supporting safer NP applications.

### 1.2.2. Exposure Pathways and Effects on Aquatic Systems

In aquatic systems, IONPs may enter organisms through multiple biological routes of exposure. In fish, primary routes include uptake across the gill epithelium, dermal absorption

across the skin, and gastrointestinal uptake following ingestion of contaminated water, sediment, or prey items. Gill exposure is particularly relevant due to the large surface area and direct contact with the surrounding medium, facilitating interaction with suspended NPs [26,27]. Dietary exposure through ingestion represents an additional route, especially when NPs associate with sediments or bioaccumulate in lower trophic organism [27,28]. These routes of exposure determine the extent to which IONPs interact with internal tissues and contribute to tissue distribution.

IONPs may be introduced into aquatic environments through industrial effluents, wastewater treatment plant discharges, urban runoff, and accidental releases during production or application [29]. Once in aquatic systems, IONPs can undergo physicochemical transformations that influence aggregation, sedimentation, dissolution, and bioavailability. These processes affect whether particles remain suspended in the water column, accumulate in sediments, or become available for uptake by aquatic organisms.

Although IONPs are often reported to exhibit lower acute toxicity relative to certain other ENMs, growing evidence documents sublethal molecular, physiological, and behavioural differences following exposure. Reported effects include oxidative stress-associated transcriptional responses, altered enzyme activity, and changes in locomotor or stress-related behaviours [21,30–34]. Reactive oxygen species (ROS) generation through iron-mediated redox reactions has been described in the literature as one potential mechanism contributing to these responses [35–37], although direct mechanistic pathways may vary depending on exposure conditions and particle characteristics.

Bioaccumulation studies in fish, including zebrafish, have reported the presence of Fe in tissues such as gills, liver, gastrointestinal tract, and brain following exposure [38,39]. Tissue-associated Fe has been measured alongside behavioural and physiological differences, including altered locomotor activity and stress-related responses [40–45]. Because aquatic organisms occupy interconnected trophic positions, there is ongoing investigation into trophic transfer potential and biomagnification of metal-associated NPs [46,47]. These findings highlight the importance of standardized exposure characterization and long-term ecotoxicological assessment for improving environmental risk evaluation.

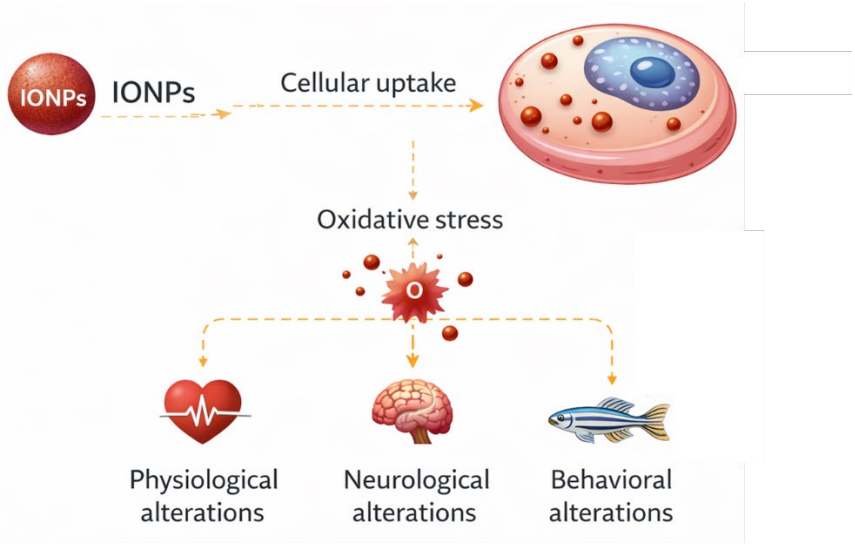



**Figure 3. Schematic illustration of the biological effects of IONPs.** Following cellular uptake, IONPs induce oxidative stress through ROS generation, which in turn leads to physiological, neurological, and behavioural alterations.

### 1.2.3. Mechanisms of IONP Toxicity

The toxicity of IONPs is reported to be multifactorial, influenced by their size, surface properties, coating, and environmental conditions [48,49]. One of the most well-documented mechanisms involves the generation of ROS through redox cycling of ferrous ($Fe^{2+}$) and ferric ($Fe^{3+}$) ions. When released into biological systems, these ions can participate in the Fenton and Haber–Weiss reactions, producing hydroxyl radicals (•OH) with high oxidative potential [37,50–52]. Excessive ROS disrupt cellular redox balance, damaging lipids, proteins, and nucleic acids, and initiating pathways leading to apoptosis or necrosis. Beyond oxidative stress, IONPs can induce toxicity through direct physical interactions with cell membranes. NPs may adhere to phospholipid bilayers, disrupt membrane integrity, or induce endocytosis, leading to altered ion permeability and signaling disruptions. Once internalized, IONPs may localize in lysosomes, where acidic conditions enhance dissolution and increase intracellular Fe levels, further amplifying oxidative stress [30–33,53].

Another critical pathway involves mitochondrial dysfunction [54,55]. Elevated ROS levels and free Fe accumulation impair mitochondrial membrane potential, reduce ATP production, and release pro-apoptotic factors. In neural tissues, these disruptions can impair neurotransmission, synaptic plasticity, and overall brain function [56–58]. The neurotoxicity of IONPs is particularly concerning given their potential ability to cross the blood–brain barrier (BBB) [59–61]. While larger aggregates are typically excluded, smaller NPs (<10 nm) and dissolved Fe ions may penetrate neural tissue [55]. Studies in rodents and fish have shown exposures to NPs that are accompanied by oxidative stress, altered neurotransmitter levels, and behavioural abnormalities [26,62]. Such findings highlight

the importance of investigating both direct NP effects and indirect ionic contributions to neurotoxicity.

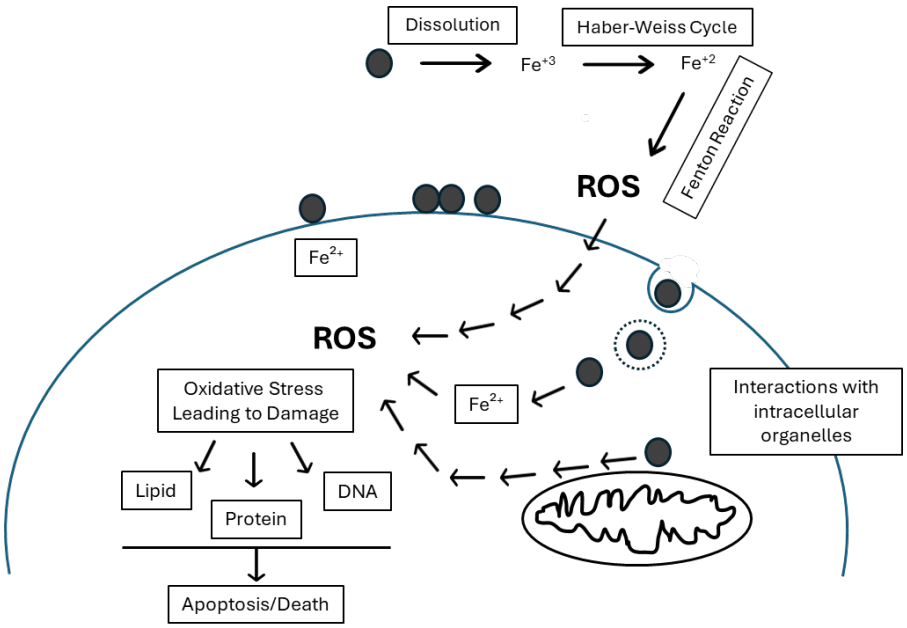



**Figure 4. Proposed pathway linking IONPs to oxidative stress.** IONPs may undergo partial dissolution, releasing ferric iron ($Fe^{3+}$) into the extracellular environment. $Fe^{3+}$ can be reduced to ferrous iron ($Fe^{2+}$) via redox cycling reactions (e.g., Haber–Weiss cycle) and may enter cells through membrane transport processes. Intracellular $Fe^{2+}$ can participate in Fenton chemistry, generating ROS. ROS production may occur both extracellularly and intracellularly. Elevated ROS levels are associated with oxidative damage to lipids, proteins, and DNA, which can contribute to cellular dysfunction and, in severe cases, apoptosis or cell death.

### 1.3. Zebrafish as a Model Organism for Neurotoxicology

Zebrafish (*Danio rerio*) have become one of the most widely used vertebrate models for studying the toxicity of ENMs, including IONPs. Several characteristics make zebrafish uniquely suited for this role:

1. Genetic homology: Approximately 70% of human genes have a zebrafish ortholog, and many genes associated with neurological and developmental disorders are conserved [63,64].
2. Rapid development: Zebrafish embryos develop externally and are optically transparent, facilitating real-time observation of morphological and physiological changes [65].
3. High reproductive output: Large clutch sizes allow for high-throughput testing and statistically robust studies [65].
4. Behavioural relevance: Zebrafish display quantifiable behaviours such as swimming activity, anxiety-like responses, social interactions, and predator avoidance, which serve as ecologically and neurologically meaningful endpoints [66].

Importantly, zebrafish have been shown to respond to neuroactive compounds in ways that parallel mammalian responses, validating their relevance for neurotoxicology research and wide application of the results. For example, anxiolytic drugs reduce thigmotaxis (wall-hugging behavior) in zebrafish in a manner similar to rodents, while psychostimulants increase locomotor activity across species [67]. These parallels strengthen the case for using zebrafish to evaluate NP-induced neurotoxicity.

Beyond their relevance for translational neurotoxicology, zebrafish also serve as an ecologically informative model species. As a freshwater teleost occupying mid-trophic levels, zebrafish share physiological and behavioural characteristics with many small-bodied aquatic vertebrates. Behavioural endpoints such as locomotion, predator avoidance, and social interaction are directly linked to survival, foraging efficiency, and reproductive success in natural environments [68–70]. Alterations in these behaviours may therefore have implications beyond individual-level responses, potentially influencing population dynamics and trophic interactions. For this reason, findings from zebrafish studies contribute not only to mechanistic toxicology but also to broader ecological risk assessment frameworks.

Together, these endpoints provide a multidimensional understanding of IONP toxicity, linking cellular events to organismal outcomes.

### 1.4. Multilevel Endpoints in IONP Toxicity

Assessing the toxicity of IONPs requires a multidimensional approach that goes beyond single endpoints. Toxicological effects can manifest at various biological levels, including molecular, physiological, behavioural, and electrophysiological responses, each providing unique insights into organismal health and ecological risk.

#### 1.4.1. Behavioural Assessments

In zebrafish behavioural analyses provide a powerful means of linking molecular and physiological disruptions to ecologically relevant outcomes. Several endpoints are commonly used to evaluate the neurotoxic effects of IONPs and other NPs:

- **Locomotor activity**: Early-life exposure to IONPs has been associated with both hypoactivity and hyperactivity in larval zebrafish, depending on concentrations and particle characteristics [41,43,71,72]. Such alterations reflect disruptions in motor circuits and neuromodulatory pathways [35,41].

- **Anxiety-Like behaviours**: Larval and juvenile zebrafish exhibit anxiety responses, such as preference for dark/light environments (scoto-/phototaxis) or wall-hugging (thigmotaxis). IONP exposure has been shown to increase anxiety-like behaviours, potentially linked to oxidative stress and altered neurotransmission [43,67].

- **Sensitivity to Stimuli**: Sensitivity to external stimuli emerges early in zebrafish development and can be assessed during larval stages. Tapping paradigms evaluate startle responsiveness and habituation, primarily mediated by hindbrain circuits including Mauthner cells (M-cells) and associated reticulospinal pathways [73]. Similarly, light–dark assays assess locomotor modulation in response to changes in illumination, reflecting early sensory processing and stress-related behavioural regulation. These stimulus-response behaviours are measurable in larvae and provide early functional endpoints for assessing neurodevelopmental variation [74–76].

- **Social Interaction:** Structured social interaction behaviours develop later in zebrafish development. While larvae exhibit functional locomotion and sensory responsiveness, organized social behaviours such as shoaling and sustained social preference typically emerge during the juvenile stage, around 2-4 weeks post-fertilization. Social interaction paradigms rely on more mature telencephalic and diencephalic circuits, including

dopaminergic pathways involved in reward processing and social recognition. As a result, assessments of social engagement are more reliably conducted in juvenile zebrafish [74,77].

1.4.2. Molecular Assessments

At the molecular level, IONP exposure has been associated with changes in the expression of mRNAs involved in oxidative stress, apoptosis, and neurotransmitter regulation [62,78,79]. These genes were selected to capture complementary and sequential components of the antioxidant defense system. Superoxide dismutase 1 and 2 (SOD1 and SOD2) represent the first line of defense against superoxide radicals, catalyzing their conversion into hydrogen peroxide, while catalase (CAT) facilitates the breakdown of hydrogen peroxide into water and oxygen. Glutathione S-transferase (GST) functions downstream by detoxifying secondary oxidative byproducts, including lipid peroxides. Together, these markers provide a pathway-level assessment of oxidative stress responses rather than isolated gene-level changes [79,80]. Altered expression of mRNAs of these genes is consistent with oxidative imbalance and the activation of molecular mechanisms in response to NP-induced redox cycling.

The selection of dopaminergic-associated markers in this study was guided by both mechanistic relevance to NP-induced neurotoxicity and their established roles in regulating the specific behavioural endpoints assessed. Dopamine is a key neuromodulator of locomotor activity, anxiety-like behaviour, and social interactions in zebrafish, making it particularly relevant to the behavioural assays employed in this work [81,82]. Moreover, dopaminergic neurons are known to be highly sensitive to oxidative stress due to their high metabolic demand and reliance on tightly regulated redox balance, rendering them especially vulnerable to disruptions caused by

Fe-based NPs capable of generating ROS. Within this system, dopaminergic function was assessed using mRNA expression levels of tyrosine hydroxylase-1 (TH1), a rate-limiting enzyme in dopamine synthesis that provides a direct indication of dopaminergic activity, and slc18a2 (vesicular monoamine transporter, VMAT), which mediates vesicular transport of monoamines and is essential for neurotransmitter storage and release. While VMAT is not specific to dopamine and is involved in the packaging of multiple monoamine neurotransmitters (e.g., serotonin and norepinephrine), it provides a broader indicator of monoaminergic vesicular transport capacity [83].

The combined assessment of *th1* and *slc18a2* mRNA expressions therefore enables evaluation of both dopamine-specific synthesis and general monoaminergic transport processes, providing complementary insight into neurochemical pathways that may be disrupted by IONP exposure. Disruption of these genes is highly relevant to neurobehavioural outcomes, as monoaminergic systems, including dopaminergic pathways, play a central role in regulating locomotor activity, reward processing, stress responses, and social behaviours [81,82]. Furthermore, the focus on oxidative stress- and monoaminergic-related genes reflects a targeted approach based on their known sensitivity to metal-induced redox imbalance and their established involvement in regulating locomotor and anxiety-related behaviours in zebrafish, thereby enabling a more integrated understanding of how IONP exposure may link cellular stress responses to alterations in neural function and behaviour.

### 1.4.3. Electrophysiological Assessments Using Multi-Electrode Array Devices (MEDs)

In addition to behavioural and molecular endpoints, previous studies in zebrafish and other model organisms are consistent with sensitivity of MEDs for detecting neurotoxic effects of environmental contaminants, pharmaceuticals, and NPs. MEDs allow non-invasive, real-time recording of extracellular action potentials across multiple neurons, providing insights into excitability, synaptic transmission, and network dynamics that complement traditional endpoints [84]. In vivo studies on mice or rat forebrains and neuron-like PC12 cells collectively show disruptions in firing patterns, reduce synchrony, impair connectivity, or alter electrophysiological activity in ways consistent with oxidative stress and neurotransmitter imbalance, when exposed to metals or NPs [85–87]. While behavioural and molecular endpoints provide valuable information about the consequences of IONP exposure, they do not directly measure neural activity. Neurons communicate through electrical impulses, and alterations in this activity occurs in parallel with many of the observed behavioural and molecular disruptions. Thus, electrophysiological techniques are essential for directly linking exposure to functional changes in the nervous system.

- MED's Role in Assessing NP Neurotoxicity

The development of MEDs has revolutionized the ability to study neural network activity in vitro and in vivo. MEDs consist of grids of microelectrodes that record extracellular action potentials from multiple neurons simultaneously, enabling real-time monitoring of firing rates and intensities, bursting patterns, and network synchrony. These electrophysiological assessments capture dynamic neural activity, providing insights into how environmental stressors affect brain function [84].

Previous toxicological studies have applied MEDs to assess neurotoxicity of metals and NPs. For example, exposure to mercury has been shown to reduce spike frequency and impair network connectivity in cultured neurons [88]. Silver NPs were reported to disrupt burst firing and reduce electrical excitability synchrony, effects attributed to oxidative stress and mitochondrial dysfunction [86]. These findings highlight the sensitivity of MEDs in detecting subtle neurotoxic effects that may not be immediately apparent in molecular assays alone. In zebrafish research, MEDs are Increasingly being used to investigate developmental neurotoxicity. Zebrafish embryos and larvae can be placed on microelectrode arrays to record spontaneous and evoked neural activity, providing functional endpoints that complement morphological and behavioural observations.

### 1.5. Knowledge Gaps and Research Rationale

Although a growing body of research has examined the toxicity of IONPs in zebrafish, the majority of studies are restricted to acute exposure scenarios during early larval development, typically terminating at or before 5 dpf. These studies predominantly focus on overt endpoints such as mortality, deformities, or short-term behavioural changes, which provide limited insight into whether early-life exposure leads to persistent or evolving neurobiological effects [43,44]. As a result, current understanding of IONP toxicity remains largely confined to immediate responses, with insufficient consideration of longer-term developmental trajectories.

This gap is not merely incidental but reflects broader methodological and conceptual limitations within NP toxicology. Longitudinal studies require extended animal maintenance, increased experimental complexity, and integration of multiple endpoints across developmental

stages, which are less amenable to high-throughput screening approaches commonly used in early toxicological assessments. Consequently, the field has largely prioritized short-term evaluations, leaving the persistence and progression of sublethal neurotoxic effects poorly understood.

Addressing this limitation is critical, as early-life exposure to neuroactive contaminants may not manifest as immediate toxicity but instead produce delayed or stage-specific effects that emerge as neural systems mature. In zebrafish, the transition from larval to juvenile stages involves significant development of dopaminergic circuits, sensory integration, and social behaviour. Disruptions occurring during early development may therefore lead to persistent or altered phenotypes that are not detectable during initial exposure windows [55,89]. From an ecological perspective, such latent neurobehavioural impairments could have direct consequences for predator avoidance, foraging efficiency, and social interactions, ultimately affecting survival and population dynamics.

This thesis directly addresses these gaps by employing a longitudinal, multi-level approach that integrates behavioural, molecular, and electrophysiological endpoints across developmental stages. By tracking zebrafish from early embryonic exposure through to the juvenile stage, this work provides insight into both the persistence and evolution of IONP-induced neurotoxicity. This design enables direct evaluation of whether early-life exposure leads to transient, adaptive, or progressively detrimental outcomes, thereby advancing current understanding beyond short-term toxicity frameworks.

### 1.5.1. Significance of an Integrated Approach

Understanding the neurotoxic potential of IONPs requires more than isolated molecular or behavioural observations; it demands an integrated framework that connects cellular mechanisms to whole-organism outcomes. This thesis adopts such an approach by combining complementary endpoints to generate a comprehensive assessment of IONP-induced neurotoxicity in zebrafish.

1. Behavioural endpoints: Measuring locomotor activity, anxiety-like responses, sensitivity/adaptation to stimuli, and social interactions in larval/juvenile zebrafish.
2. Molecular endpoints: Longitudinal assessment of oxidative stress and dopaminergic pathway mRNA expressions across life stages.
3. Electrophysiological endpoints (MEDs): Recording neural activity of larval zebrafish brain to detect functional disruptions in neural circuits.

By combining these complementary approaches, this thesis addresses a central challenge in nanotoxicology: connecting molecular mechanisms to ecologically relevant outcomes, which is particularly important for environmental risk assessments.

### 1.5.2. Relevance to Environmental and Human Health

The ecological significance of IONP toxicity stems from their potential to impair the health and fitness of aquatic organisms. Neurobehavioural impairments in fish, such as reduced swimming ability, heightened anxiety, or impaired predator avoidance, can directly affect survival and ecosystem balance [90,91]. If larval zebrafish exposed to IONPs exhibit persistent behavioural

deficits, this suggests that even acute environmental exposures may disrupt fish populations and biodiversity over extended life stages and potentially future generations.

From an ecological and human health perspective, the effects of IONPs on aquatic organisms extend beyond individual toxicity to potential disruptions of entire ecosystems. Fish and other aquatic species play critical roles in maintaining trophic balance, nutrient cycling, and water quality [30,92]. Aquatic ecological imbalances such as, compromised feeding efficiency, reproduction, and survival, can have cascading impacts on human populations that depend on aquatic systems for food security, livelihood, and ecosystem services. Moreover, bioaccumulation and trophic transfer of nanoparticles through aquatic food webs may result in human exposure via seafood consumption, posing additional health risks [93]. Understanding how IONPs affect the neurodevelopment and behaviour of aquatic organisms is therefore crucial not only for protecting biodiversity but also for safeguarding ecosystem integrity and human well-being.

### 1.6. Aims and Objectives of This Thesis

The overarching aim of this thesis is to investigate the long-term neurobehavioural, molecular, and electrophysiological consequences of early-life exposure to IONPs in zebrafish. By integrating behavioural assessments with molecular and electrophysiological analyses across developmental stages, this work seeks to elucidate mechanistic pathways underlying IONP-induced neurotoxicity and to evaluate the persistence of these effects beyond early development.

#### 1.6.1. Hypotheses

This thesis is guided by the following hypotheses:

- Early-life exposure to IONPs before full development of the BBB increases vulnerability in developing zebrafish, resulting in alterations in neurobehavioural performance, including locomotor activity, anxiety-like behaviour, and sensitivity to external stimuli.
- Behavioural impairments induced by IONP exposure are mediated by disruptions from increased oxidative stress and altered dopaminergic signalling, which interfere with normal neurodevelopmental processes.
- Neurobehavioural and molecular disturbances arising from early-life IONP exposure persist into juvenile stages, reflecting long-lasting effects of nanoparticle-induced stress.
- At the neural network level, IONP exposure alters patterns of brain activity, including changes in neural firing dynamics and burst signalling, providing a functional link between molecular disruptions and behavioural outcomes.

### 1.6.2. Experimental Strategy and Objectives

To test these hypotheses, this thesis employs an integrative experimental framework encompassing behavioural, molecular, and electrophysiological approaches in both larval and juvenile zebrafish. Behavioural analyses were conducted to characterize neurobehavioural outcomes following early-life IONP exposure. These included assessments of locomotor activity and anxiety-like behaviour in larval and juvenile zebrafish, sensitivity to stimuli in larvae (6 days post-fertilization; dpf), and social interaction behaviours in juveniles (30 dpf). To investigate the molecular mechanisms underlying observed behavioural changes, mRNA expression analyses were performed focusing on oxidative stress–related and dopaminergic markers in both

developmental stages. This approach aimed to determine whether behavioural alterations were accompanied by disruptions in key neurodevelopmental and neurotransmitter-related pathways. Electrophysiological recordings were conducted using MED to evaluate neural network activity in 6 dpf exposed larvae. These measurements provided functional insight into how molecular perturbations translate into altered neural signalling and network-level activity.

Hence, this thesis integrates behavioural, molecular, and electrophysiological endpoints to examine developmental outcomes associated with early-life IONP exposure. The work is organized into three interconnected projects that address distinct but complementary aspects of nanoparticle exposure across developmental stages:

**Project 1**: Toxicological assessment of IONPs in developing zebrafish: Physiological performance and neurobehavioural responses

**Project 2**: Prolonged effects of IONPs in juvenile zebrafish: Physiological performance and neurobehavioural responses

**Project 3:** Non-invasive electrophysiological assessment of larval zebrafish brain following IONP exposure

# CHAPTER 2

## Effects of Iron Oxide Nanoparticles on Larval Zebrafish Neurobehaviour and Molecular Endpoints

### 2.1. Chapter Overview

This chapter investigates the effects of IONP exposure during early zebrafish development, focusing on larval stages up to 6 days post-fertilization (dpf). Building upon the general context established in Chapter 1, this study examines whether sublethal IONP exposure alters physiological development, metal accumulation, neurobehavioural performance, and molecular pathways related to oxidative stress and dopaminergic signaling. This chapter represents the first experimental component of the thesis and provides a mechanistic foundation for subsequent longitudinal and electrophysiological analyses presented in later chapters.

### 2.2. Introduction

#### 2.2.1. Early-Life Exposure to Nanoparticles in Aquatic Organisms

ENMs are increasingly detected in aquatic environments due to industrial activity, consumer product use, and waste streams, including electronic waste [8,94,95]. Among these materials, IONPs are of particular concern because of their redox-active properties, environmental persistence, and widespread use [3]. Early developmental stages of aquatic organisms are especially vulnerable to NP exposure due to rapid organogenesis, immature detoxification systems, and heightened sensitivity of the nervous system [19,96].

#### 2.2.2. Larval Zebrafish as a Model for Developmental Neurotoxicity

Zebrafish larvae are a well-established vertebrate model for assessing developmental neurotoxicity due to their rapid development, genetic tractability, and well-characterized nervous system [97–99]. By 4–5 days post-fertilization (dpf), larvae possess differentiated neuronal cell types, functional sensory-motor circuits, and coordinated swimming behaviour, allowing robust responses to environmental stimuli such as changes in illumination and mechanical perturbation [100,101]. These characteristics enable assessment of neurobehavioural alterations arising from chemical or NP exposure during early development [102–104]. Importantly, the BBB at this stage is not fully mature and remains more permeable than in later life stages, potentially increasing larval susceptibility to neuroactive contaminants [17]. This developmental immaturity may permit greater access of NPs to neural tissues, thereby enhancing vulnerability to neurotoxicity. Consequently, behavioural endpoints such as spontaneous locomotion, thigmotaxis, habituation, and escape responses serve as integrative indicators of neural circuit function and sensory processing, making larval zebrafish a biologically relevant and sensitive system for evaluating neurotoxic effects during critical windows of development [67,105].

### 2.2.3. Oxidative Stress and Dopaminergic Regulation as Targets of IONP Toxicity

IONPs have the capacity to influence biological systems through both their particulate properties and their redox-active Fe core. Iron-containing NPs can catalyze the generation of reactive oxygen species (ROS) via the Haber–Weiss and Fenton reactions, linking IONP exposure to oxidative stress. Oxidative imbalance during early development is of particular concern, as excessive ROS can disrupt neural signaling, synaptic plasticity, and downstream behavioural outputs [34,40]. In aquatic organisms, exposure to iron-based NPs has therefore been associated with alterations in antioxidant defense pathways and neurophysiological function. In addition to

redox-mediated mechanisms, dopaminergic pathways play a central role in modulating behaviours such as anxiety, fear responses, learning, and social interactions, making the investigation of its homeostasis essential for understanding the neurobiological mechanisms underlying behavioural changes [73,77,106]. DA synthesis, transport, and receptor-mediated signaling are tightly integrated with sensorimotor circuits and escape responses, including those mediated by reticulospinal neurons such as M-cells [73,83,107]. Disruptions to oxidative balance or dopaminergic homeostasis during critical developmental windows may impair sensory processing, habituation, and adaptive behavioural responses. Consequently, examining both oxidative stress-responsive pathways and DA-related molecular markers provides an essential framework for understanding the mechanistic basis of IONP-induced neurobehavioural alterations in developing zebrafish.

Overall, this chapter will examine the following hypothesis:

Early-life exposure to IONPs in developing zebrafish will result in alterations in neurobehavioural performance, and behavioural impairments induced by IONP exposure are mediated by disruptions from increased oxidative stress and altered dopaminergic signalling, which interfere with normal neurodevelopmental processes.

Objectives of Chapter 2:

- Characterize the physicochemical properties and stability of IONPs in exposure waters.
- Evaluate developmental and physiological outcomes following early-life IONP exposure.
- Quantify tissue metal accumulation and ionic balance.
- Assess neurobehavioural performance at the larval stage.

- Examine molecular markers associated with oxidative stress and dopaminergic regulation.

## 2.3. Materials and Methods

### 2.3.1. IONP Characterization

Iron(III) oxide nanoparticles (IONPs; non-coated $Fe_2O_3$, <50 nm, O=[Fe]O[Fe]=O, molecular weight 159.69 g/mol; Sigma-Aldrich), commonly referred to as hematite or ferric oxide, are finely milled particles with sizes typically under 50 nm. These NPs occur naturally as minerals but can also be synthesized under controlled laboratory conditions. In this study, a 1000 mg IONPs/L stock suspension was prepared by dispersing IONP powder in Milli-Q water, and exposure solutions were obtained by diluting the stock with water from the Aquaneering zebrafish housing system and sonicating them to avoid formation of aggregates. Given the dynamic physicochemical properties of NPs in aqueous environments, detailed characterization was conducted to evaluate their morphology, dispersion, and dissolution, all of which are critical for understanding bioavailability and interactions with biological systems.

The morphology and dispersion of IONPs in exposure water were assessed using scanning electron microscopy (SEM) at the York University scanning transmission electron microscopy (STEM) Imaging Facility. For SEM, 10 µL of a 100 µg/L IONP exposure solution was deposited onto carbon-coated TEM grids and imaged to evaluate particle shape, surface structure, and aggregation state. In parallel, the hydrodynamic diameter of IONPs in suspension was measured using Dynamic Light Scattering (DLS) with a DynoPro DLS plate reader (Wyatt Technology) at the Hospital for Sick Children (Toronto). Data were processed with Dynamics software to determine

particle size distribution and to assess whether particles remained evenly dispersed in the medium or exhibited agglomeration, adhesion to surfaces, or sedimentation.

To further examine solubility and potential dissolution, a 10 mg/L IONP suspension was subjected to a membrane diffusion assay using dialysis tubing (cut-off 12,000 Da; Sigma-Aldrich). A total of 12 mL of the suspension was loaded into the tubing, sealed with medical clamps, rinsed to remove residual particles from the outer surface, and placed in 18 mL of Milli-Q water for 20 minutes. The surrounding Milli-Q water was then collected for subsequent inductively coupled plasma mass spectrometry (ICP-MS, performed at Trent University) analysis to quantify dissolved iron. This procedure allowed assessment of NP stability in aqueous environments and provided insights into the extent of ionic release.

2.3.2. Zebrafish Husbandry

Adult zebrafish (TL strain) were housed in a recirculating water system (Aquaneering, CA, USA) maintained at 28 °C with a pH of 7.4. Water quality parameters, including hardness (150 mg/L as $CaCO_3$), alkalinity (65 mg/L), and conductivity (70 µS/m), were monitored weekly to ensure optimal conditions. The system water was prepared by mixing reverse osmosis (RO) water with commercial sea salt (Instant Ocean) and bicarbonate ($HCO_3^-$) ions to achieve the desired ionic and metal composition. The adults were kept under a 14-hour light and 10-hour dark cycle and were fed twice daily with brine shrimp in the morning and a commercial zebrafish diet (Zeigler, PA, USA) in the afternoon. Breeding was conducted in tanks equipped with breeding traps, maintaining a male-to-female ratio of 1:2. Males and females were separated by a mesh divider for approximately 18 hours prior to breeding, and dividers were removed at 8:00 AM to initiate spawning. Traps were positioned at a diagonal angle to create a

shallow end for fish to breed in, as their preference. Embryos were collected in system water containing ~0.0002% methylene blue to prevent fungal growth.

For larval rearing, 15 embryos were placed in Petri dishes containing exposure waters (described below) and maintained at 28 °C until 6 dpf. Larvae were monitored daily for health and developmental milestones, with any mortalities or abnormalities recorded to ensure cohort integrity. All procedures were performed in compliance with the Canadian Council of Animal Care guidelines and were approved by the York University Animal Care Committee (2017-2 R1).

### 2.3.3. Exposure Regime

A semi-static exposure regime with daily water renewal was employed to evaluate the effects of IONPs on developing zebrafish from early embryogenesis through the juvenile stage. Exposures began at 4 hours post-fertilization (hpf), following the elimination of unfertilized or defective embryos. Previous research has shown that IONP concentrations above 10 mg/L induce severe developmental toxicity in zebrafish [108]. Therefore, this study employed lower, sublethal concentrations of 0, 0.1, 1.0, and 5.0 mg/L, which also reflect environmentally relevant Fe levels reported in aquatic ecosystems [109].

For behavioural assays, 15 embryos were allocated to each Petri dish, with duplicate dishes prepared for each exposure concentration. Each dish contained 40 mL of exposure water and was incubated under controlled conditions (28 °C; 14-hour light/10-hour dark cycle). Exposure waters were refreshed daily with freshly prepared IONP solutions. For physiological assessments, embryos were randomly placed in 6-well plates, with 5–10 embryos per well and 8–9 mL of exposure water added to each well. Samples from the same well were considered a single sample (n = 1). Plates were maintained under the same incubating conditions as the Petri

dishes, and ~90% of the exposure water was renewed every 24 hours. Exposures continued until 6 dpf, at which point the larvae were collected for analysis or transferred to rearing tanks.

2.3.4. Assessment of Physiological Conditions

Throughout the exposure period, zebrafish embryos and larvae were monitored daily until 6 dpf to assess lethal and sublethal physiological outcomes, including mortality, deformities, and hatching rates. The sublethal IONP concentrations used in this study were selected to minimize severe developmental toxicity while allowing assessment of more subtle disruptions. Deformities were defined as pericardial or yolk sac edema, as well as malformations of the spine or tail. Hatching rates were evaluated by recording the daily number of hatched embryos relative to the total fish per Petri dish, providing a measure of potential developmental delays or deviations. In this experiment, six samples per exposure group were used. All samples were maintained at 28 °C under a 14-hour light/10-hour dark cycle.

2.3.5. Measurement of Water and Tissue Metal Levels

The preparation and analysis of major ions and trace metals in zebrafish tissues and exposure waters were performed following established protocols [36]. At the larval stage (6 dpf), whole-body samples were collected and analyzed.

30 larval zebrafish per sample were pooled, with each pooled group treated as a single sample (n=4 per exposure concentration). After euthanization [110], larvae were washed twice with Milli-Q water and then dried in microcentrifuge tubes at 65 °C for two days. After drying, all tissue samples were digested in 300 µL of 6N nitric acid ($HNO_3$) and incubated at 65 °C for an additional two days to ensure complete breakdown. Digested samples were diluted with 5 mL of 2% $HNO_3$ and filtered through 0.45 µm syringe filters prior to analysis. Water samples from each

IONP exposure concentration were processed in parallel using identical acidification and dilution procedures to maintain consistency across sample types.

Trace metal and major ion concentrations were quantified using inductively coupled plasma triple quadrupole mass spectrometry (ICP-QQQ-MS, Agilent 8800) at the Water Quality Centre, Trent University. The analytes included Fe, Mn, Cu, Zn, Na, Mg, K, and Ca, depending on sample type. Quality assurance and control were maintained using NIST SRM 1640a (trace elements in natural water), with all results falling within 5% of the certified values, confirming the accuracy of the analytical process.

#### 2.3.6. Neurobehavioural Analyses

- Spontaneous and Stress-Related Activities

Behavioural assays were conducted at the larval stage to evaluate immediate neurobehavioural effects of IONP exposure. Larval behaviour was assessed at 6 dpf, a stage at which zebrafish possess well-developed neural circuits for motor and sensory functions and are highly responsive to changes in illumination due to stress or fear responses [103,104]. All behavioural trials were conducted using the DanioVision Chamber (Noldus Information Technology) under controlled conditions (28 °C with a thermal regulation unit; ~300-350 lux light intensity). Fish were acclimated prior to tracking, and activity was recorded and analyzed using EthoVision XT software (Noldus). To minimize variability due to circadian influences, all assays were performed between 12:30 PM and 3:30 PM, ensuring temporal consistency across groups.

24 larval zebrafish per concentration group were individually placed in the wells of 24-well plates and acclimated for 20 minutes before tracking (n=24). Their spontaneous locomotor

activity was then monitored for 10 minutes, with distance travelled and swimming velocity recorded for each larva. Data were reported as mean distance travelled per 10-minute interval, alongside corresponding velocity measurements, to provide an assessment of baseline swimming activity.

For thigmotaxis analysis, larvae were exposed to a light-dark cycle designed to elicit stress-induced exploratory behaviour. Following a 10-minute acclimation period, each fish underwent 10 minutes of light and 10 minutes of darkness, during which swimming preference for the outer zone ("wall-hugging") versus the inner zone of the well was quantified. Outputs included the percentage of time spent and percentage of distance travelled in the outer zone relative to the total tracking duration and overall distance. The experimental timeline and the calculations for both assays are shown in Figure 5.

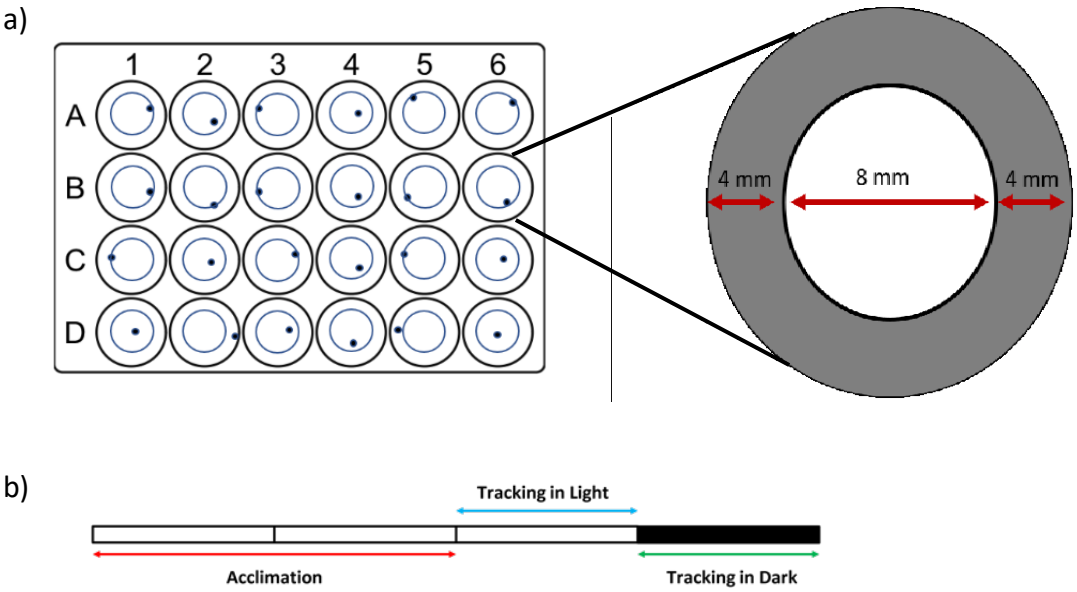



**Figure 5. Experimental outline for spontaneous swimming and thigmotactic behaviours.** a) Zone layout for larval assays, showing the outer and inner zones of each well with precise measurements of each zone. b) Timeline of the tracking duration. After a 20-min acclimation period, fish movement was tracked for 10 min under light and 10 min under dark conditions.

- <u>Sensitivity and Escape Responses</u>

Larval zebrafish exhibit sensitivity to external stimuli through rapid escape responses and behavioural adjustments to repeated stimulation. Alterations in these responses are commonly used as functional indicators of sensorimotor integration and neurodevelopmental integrity [111,112]. The ability to appropriately respond to threatening cues while adapting to repeated non-threatening stimuli is essential for maintaining efficient energy use and effective predator avoidance in dynamic aquatic environments [105]. In the present study, stimulus sensitivity was assessed using a tapping device that delivered mechanical stimuli of progressively increasing intensity and frequency (Figure 6).

During each trial, larvae were placed individually into wells, and their behavioural responses to the tapping stimuli were recorded using video tracking. The primary parameters measured

included larval swimming velocity and the frequency of freezing bouts, both of which provide indicators of activity levels and adaptive capacity throughout the different phases of the assay. For freezing bouts measurements, EthoVision XT software (Noldus) automatically detects immobility based on pixel change thresholds between consecutive video frames. A freezing bout was defined as a period during which the zebrafish's movement fell below the predefined activity threshold (typically <0.2–0.5 cm/s) for a continuous duration exceeding the minimum bout length (e.g., ≥1 s). Both velocity and freezing bout measurements allowed for comparison of stimulus sensitivity between exposure groups, with the objective of detecting alterations potentially attributable to environmental factors, including IONP exposure.

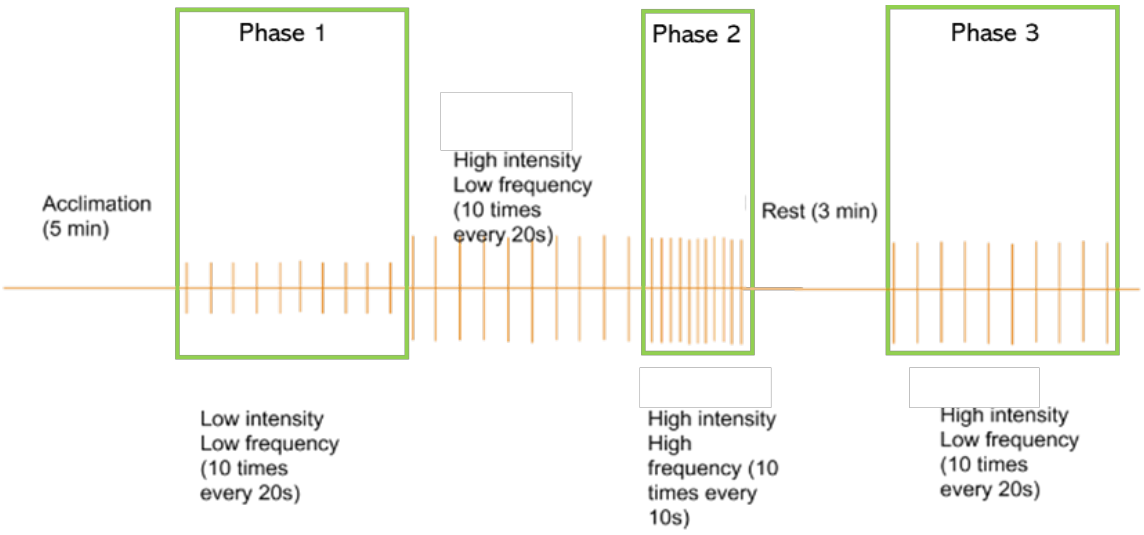



**Figure 6. Sensitivity responses of larvae towards tapping stimuli**. Lines represent the tapping events. Shorter lines represent lower intensity, and longer lines represent higher intensity of stimulation. High frequency is shown by lines that are closer together and low frequency is shown by lines that are further apart. Precise timings of stimulation are demonstrated on this figure. Low frequency and intensity stimuli were applied during "phase 1" for 10 total repeats with 20 second intervals in between. Low frequency and high intensity stimuli were applied next for total of 10 repeats with 20 second intervals in between. High frequency and intensity stimuli were applied during "phase 2" for total of 10 repeats with 10 second intervals in between. During the 3 min rest, no stimulation was applied. Low frequency and high intensity stimuli were applied last during "phase 3", for a total of 10 repeats with 20 second intervals in between.

### 2.3.7. Evaluation of mRNA Expression Levels Using Droplet Digital PCR (ddPCR)

DA homeostasis and antioxidant defense systems, as possible mechanisms underlying the toxic effects of IONPs, were examined in this study by inspecting the mRNA expression levels of genes related to dopaminergic, and broader monoaminergic signaling, including *th1*, a key enzyme in dopamine synthesis, and *slc18a2*, which mediates vesicular transport of multiple monoamine neurotransmitters; and oxidative stress responses, *sod1*, *sod2*, *cat*, and *gstp* (Table

1). Assessing the expression of these targets provides a framework towards linking behavioural changes to underlying molecular disruptions.

For larval zebrafish, mRNA expression level was assessed at 6 dpf, immediately following the exposure period. Each sample consisted of 20–25 pooled larvae, with three biological samples prepared per exposure concentration (n = 3). Total RNA was extracted using the Monarch Total RNA Miniprep Kit (New England Biolabs) and subsequently reverse-transcribed into cDNA with the iScript cDNA Synthesis Kit (Bio-Rad). mRNA expression level was quantified using droplet digital PCR (ddPCR) on a QX200 system (Bio-Rad). The thermal cycling conditions were as follows: initial denaturation at 95 °C for 5 minutes, followed by 40 cycles of denaturation at 95 °C for 3 minutes and annealing/extension at 58 °C for 1 minute. Post-cycling, signal stabilization was performed at 4 °C for 5 minutes and 90 °C for 5 minutes. Droplets were subsequently read using the QX200 Droplet Reader and analyzed with QuantaSoft software (Bio-Rad). For mRNA analyses, normalization of expression was performed against a panel of validated housekeeping genes, including elongation factor 1 alpha (*ef1a*), ribosomal protein s18 (*rps18*), and ribosomal protein L13a (*rpl13a*) [36,113].

**Table 1. Primer sequences used in the ddPCR analysis**

| Gene | Accession number | Fwd primers (5' – 3') | Rev primers (5' – 3') |
|---|---|---|---|
| *th1* | NM_131149.1 | CCTATGGAGAGCTGGTGCATT | CACAGAAAACGGTCGCTTGA |
| *slc18a2* | NM_001256225.2 | GCTAGGGATTGCATTTGTGCC | TAAGTGAGCACAGCCATCTCC |
| *Sod1* | NM_131294.1 | GGTGACAACACAAACGGCTG | AGGTCTCCGACGTGTCTCA |
| *Sod2* | NM_199976.1 | GAACCACAGGGTGAGCTGTT | GCTGCAATCCTCAATCTTCCG |
| *Cat* | NM_130912.2 | TCTCCTGATGTGGCCCGATA | TTTGCACCATGCGTTTCTGG |
| *gstp* | NM_131734.3 | CTTCGCAGTCAAAGGCAGATG | CGCCCTTCATCCACTCTTCA |
| *Rps18* | NM_173234.1 | CCCTCGTCATCCCAGAGAAGT | CGCCTTCCAACACCCTTAATAG |
| *Rpl13a* | NM_212784.1 | GTATTTGGCTTTCCTCCGCA | ACCATGCGCTTTCTCTTGTC |

### 2.3.8. Statistical Analysis

All data analyses were performed using IBM SPSS software (version 23.0, IBM SPSS Inc., USA), and results were expressed as mean ± standard error of the mean (SEM). Graphs were generated in GraphPad Prism. Prior to statistical testing, datasets were evaluated for normality using the Kolmogorov–Smirnov one-sample test and for homogeneity of variance using Levene's test to ensure compliance with the assumptions of parametric analyses.

For data meeting assumptions of normality and homogeneity, one-way analysis of variance (ANOVA) was employed, followed by Tukey's post hoc test for multiple comparisons. In cases where heteroscedasticity was detected, Welch's ANOVA was applied, with Games–Howell or Dunnett's t (two-sided) post hoc tests used for pairwise comparisons. Repeated measures one-way ANOVA was used to assess treatment effects on locomotor activity (distance traveled and mean velocity) across time intervals.

Independent Student's t-tests were conducted to evaluate specific comparisons, including treatment-related effects on locomotion and differences in thigmotactic behaviour under light versus dark conditions. A p-value of $< 0.05$ was taken as the threshold for statistical significance for all analyses.

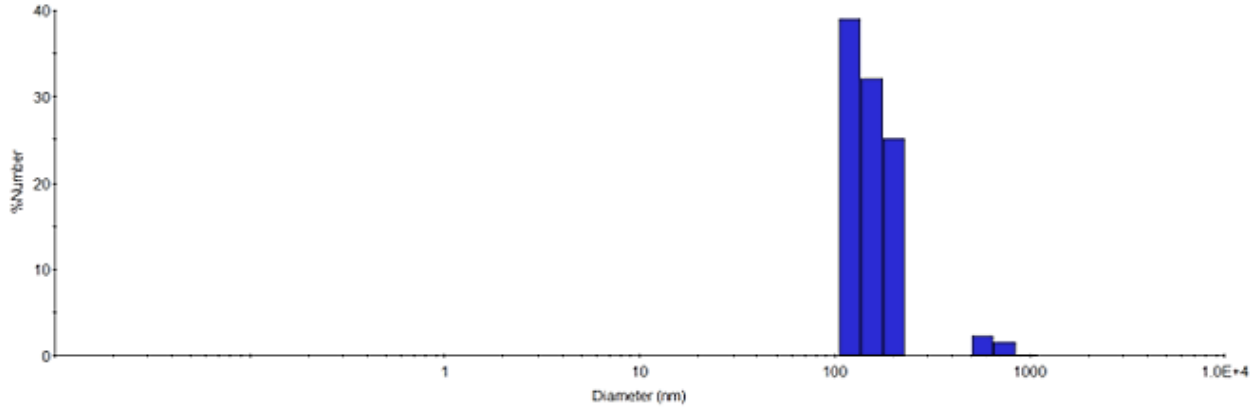
## 2.4. Results

### 2.4.1. Characteristics of IONPs

Characterization of the IONPs used in this study was performed to determine their physicochemical properties and ensure consistency across experiments with both larval and juvenile zebrafish (experiments described in Chapter 3). DLS analyses revealed two distinct particle populations, with the primary peak at 154 nm representing the dominant fraction (96.1% by number), and a secondary peak at 651.7 nm accounting for a smaller proportion (3.9% by number), reflecting minor aggregation and a hydrodynamic diameter of approximately 154 nm (Figure 7b and Table 3). DT results showed minimal dissolution and negligible release of free Fe ions (Table 2). These findings suggested that the particles remained largely intact throughout the duration of the experiments.

Further assessments using DLS and scanning transmission electron microscopy (STEM) were employed to evaluate particle stability and morphology. At low to moderate exposure concentrations, IONPs remained uniformly dispersed within the exposure water, with minimal aggregation. STEM imaging confirmed a predominantly spherical morphology of the NPs (Figure 7a, consistent with expectations for hematite-based $Fe_2O_3$ particles.

a)




b)

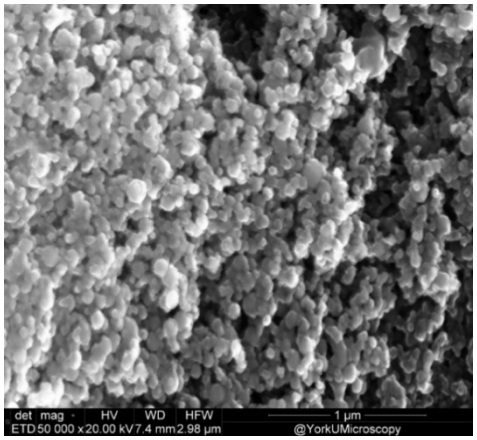



**Figure 7. Physicochemical characterization of IONPs**. a) DLS analysis plotted as the hydrodynamic size distribution, showing the percentage of various particle sizes. b) STEM image showing the surface morphology of the IONPs.

**Table 2. Results of DLS analysis, showing the two distinct peaks of hydrodynamic diameters with corresponding percent polydispersity, intensity, and mass of particles.**

| Peak | Diameter (nm) | %Polydispersity | %Intensity | %Mass | %Number |
|---|---|---|---|---|---|
| Peak 1 (True) | 154 | 21.39 | 68.5 | 2.60E+01 | 96.1 |
| Peak 2 (True) | 651.7 | 14.66 | 31.5 | 7.40E+01 | 3.9 |

**Table 3. Results of DT-based dissolution of 10 mg/L IONP solution**

| Tested solution | [Fe] mean (µg/L) |
|---|---|
| **Outside the dialysis tube** | 4.513 ±0.392 |
| **Inside the dialysis tube** | 2898.255 ±293.66 |

### 2.4.2. Water and Tissue Metal/Ion Content

Concentrations of trace metals in exposure waters were analyzed using ICP-MS (Table 4 and Figure 8). A broad range of IONP concentrations (0–10 mg/L) was initially tested to establish a concentration-response relationship and facilitate comparative analyses across exposure levels. This approach enabled the selection of optimal concentrations for subsequent experiments. Analyses revealed that the lowest concentration tested (0.1 mg/L) resulted in no significant Fe accumulation in zebrafish tissues (Results not shown), suggesting that this concentration was insufficiently absorbed by zebrafish. Conversely, the highest concentration (10 mg/L) led to the formation of visible IONP precipitation in the exposure water, even after sonication. This precipitation likely reduced the bioavailability of the NPs to larvae and compromised the precision of exposure, thereby limiting the interpretability of results. Based on these outcomes, 0, 1, and 5 mg/L were selected as the working concentrations for further studies. Whole-body Fe content in larval zebrafish increased with increasing IONP exposure concentration. Larvae exposed to 1 and 5 mg/L IONPs exhibited a marked elevation in Fe accumulation compared with the control group.

**Table 4. Fe content in exposure waters, zebrafish whole body larval tissue**

| [IONPs](mg/L) | Water (mg/L) | Larva (µg/larva) |
|---|---|---|
| **0** | u.d. * | 0.009±0.004[a] |
| **1** | 0.110±0.005[b] | 0.033±0.002[b] |
| **5** | 1.009±0.020[c] | 0.089±0.013[b] |

*u.d. Under the Detection limit
Value labelled with different letters represents a statistical difference across treatments (Welch, post hoc Games-Howell, $p<0.001$, $n=4$).

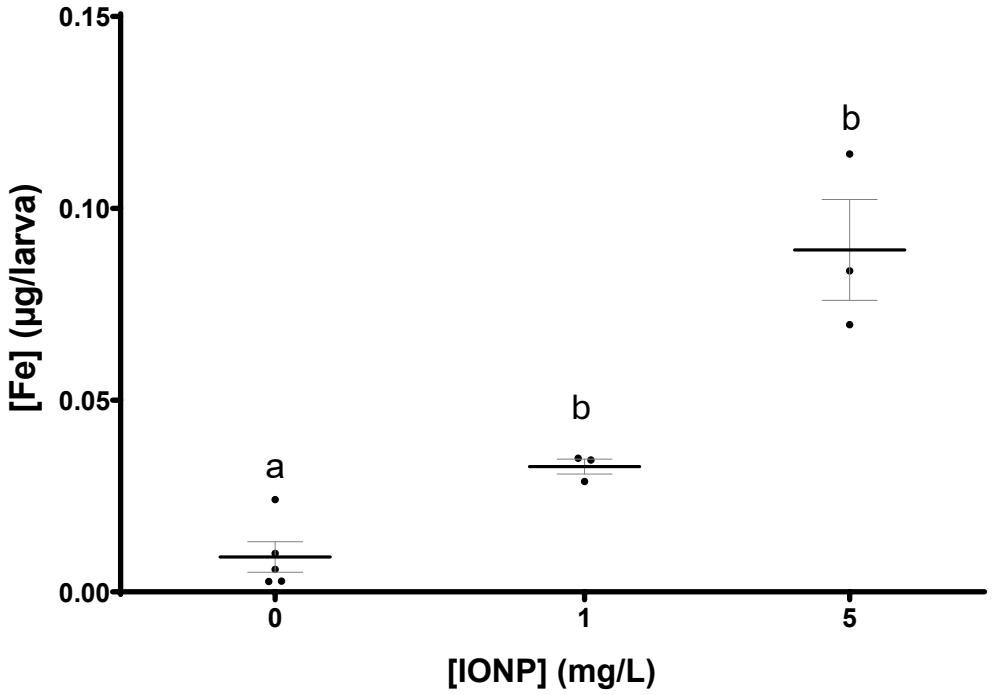



**Figure 8. Iron accumulation in larval zebrafish following exposure to IONPs**. Whole-body iron content ([Fe], µg per larva) was quantified in zebrafish larvae exposed to 0, 1, and 5 mg/L IONPs. Individual data points represent single larvae. Statistical differences are shown with groups not sharing the same letter (One-Way ANOVA, post-hoc Tukey HSD $p \leq 0.002$ for both 0 and 1 mg/L vs 5 mg/L, N=3-5, Mean±SEM).

### 2.4.3. Assessment of Physiological Conditions

Throughout the IONP exposure period (0–6 days post-fertilization, dpf), zebrafish embryos and larvae were monitored daily for mortality, deformities, and hatching success to evaluate potential developmental toxicity. No statistically significant differences were observed among exposure groups in any of these parameters, indicating that the selected IONP concentrations (0–5 mg/L) were within a sublethal range that avoided overt or lethal toxicity ($p>0.05$, Figure 9). Deformities, when present, were limited to mild edema or minor axial malformations and did not differ in frequency across treatments. Only larvae that appeared morphologically normal at the end of the 6-dpf exposure period were selected for subsequent behavioural and molecular analyses to ensure that results reflected functional, rather than developmental, disturbances.

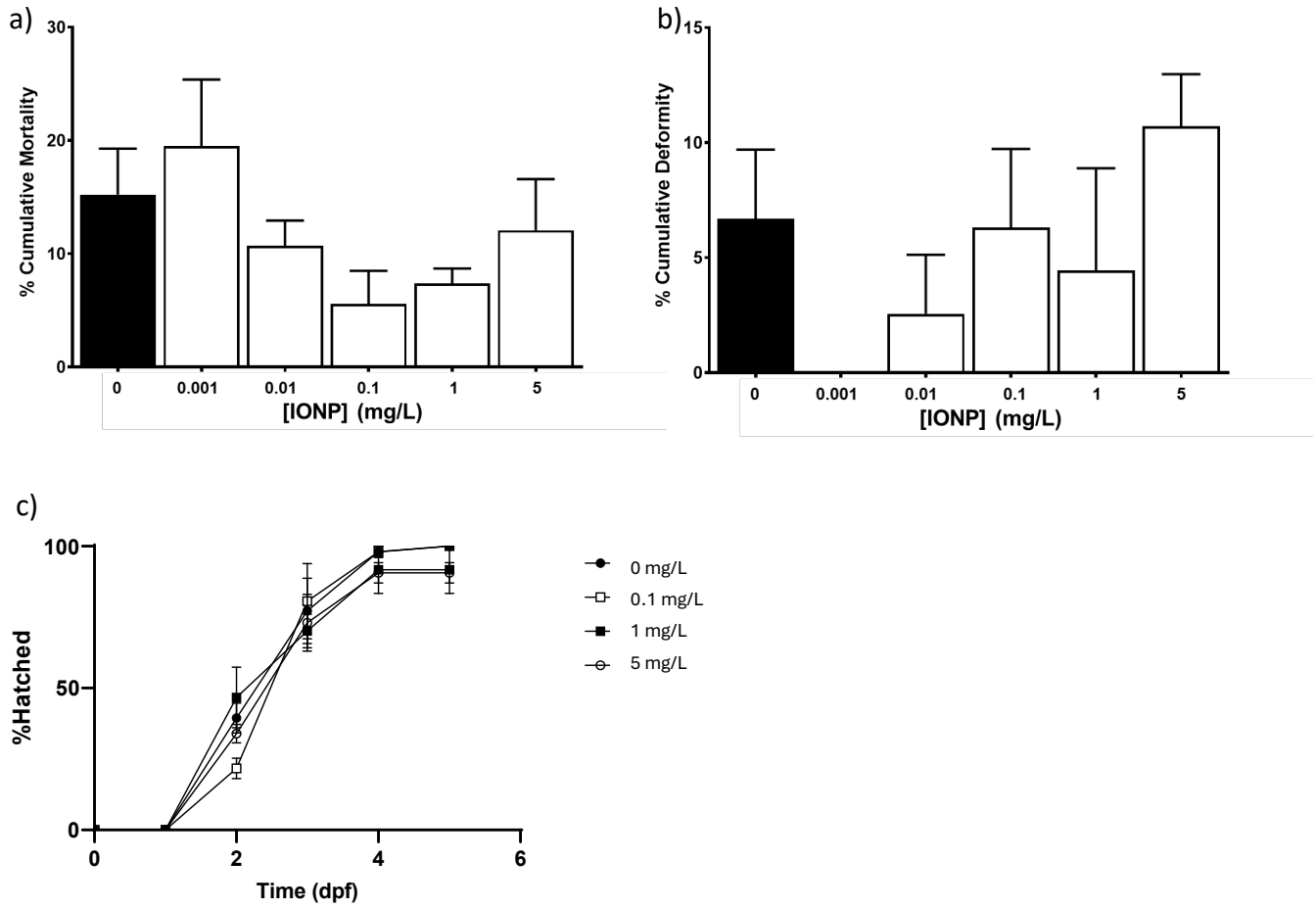



**Figure 9. Effects of IONP exposure on the physiological conditions of zebrafish larvae.** a) Cumulative mortality; percentage of dead larvae throughout the monitoring period (One-way ANOVA $p>0.05$). b) Cumulative deformity; percentage of deformed larvae throughout the monitoring period (One-way ANOVA $p>0.05$). c) Hatching rate of larvae from 0 to 6 dpf (two-way ANOVA, $p>0.05$). Data are Mean ± SEM, n=6 (each sample consisted of 5-10 larvae)

### 2.4.4. Spontaneous Swimming Activities and Stress-Related Behaviours

To investigate the effects of IONPs on behavioural performance, both spontaneous swimming activity and thigmotactic behaviour were assessed in zebrafish larvae. At the larval stage, zebrafish exposed to 1 mg/L IONPs exhibited a significant increase in cumulative distance traveled and mean swimming velocity compared to the control group ($p \leq 0.001$ for both; Figure 10). Larvae in both the 1 and 5 mg/L exposure groups also demonstrated a higher ratio of time spent in the outer zone of their arenas compared to controls, indicating elevated thigmotactic behaviour (Figure 11). Additionally, larvae exposed to 5 mg/L IONPs display decreased time spent in the outer zone of their arenas under dark conditions compared to light, while the control and 1 mg/L groups did not show this. Distances travelled by larvae in the control and 5 mg/L groups in the outer zone were greater under light conditions, compared to dark, while no significant difference was observed in the 1 mg/L group.

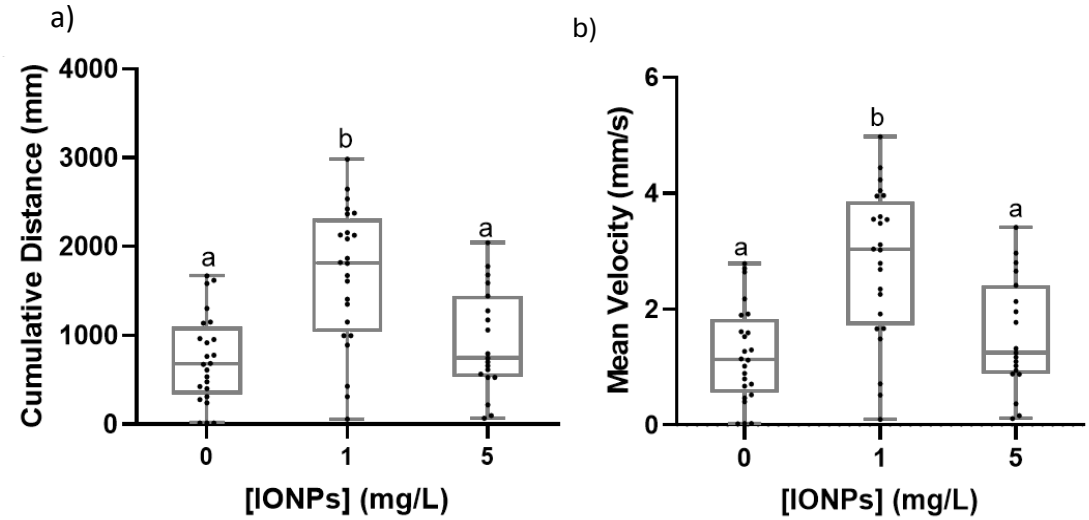



**Figure 10. Spontaneous swimming activities in larval zebrafish**. a) Cumulative distance travelled by larvae. b) Mean velocity of larvae throughout the tracking duration. Min-Max showing all data points, statistical differences are shown with groups not sharing the same letter (One-way ANOVA, tukey's post-hoc, $p \leq 0.001$ for both, N=24).

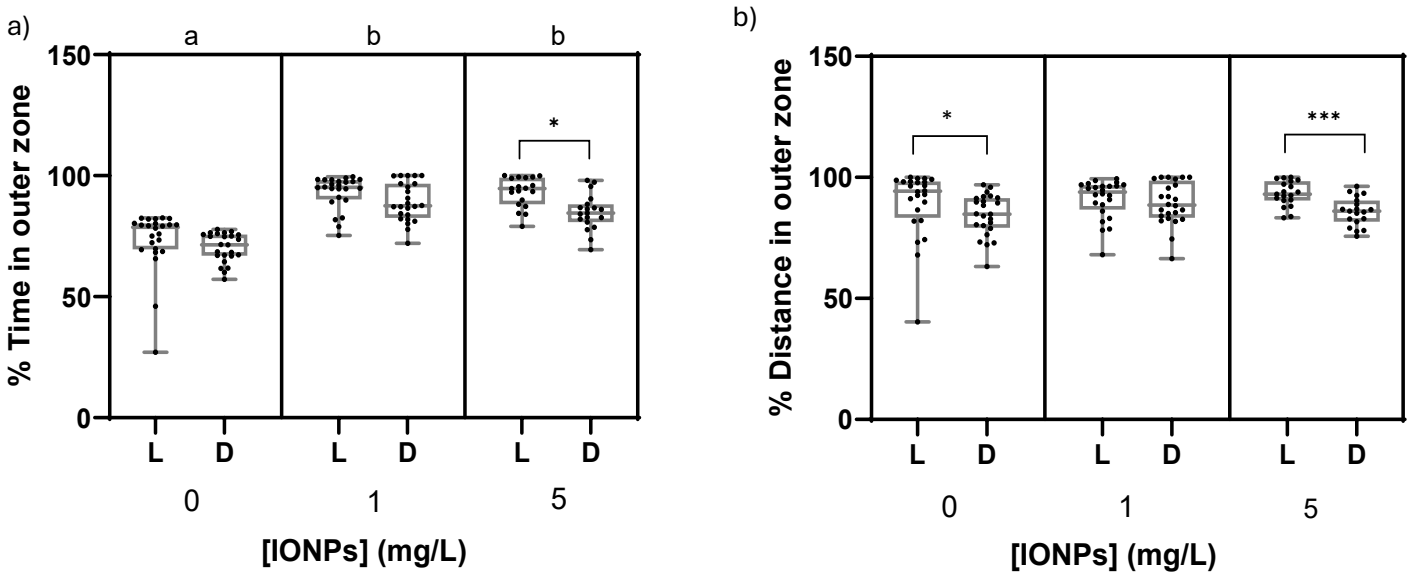



**Figure 11. Thigmotactic behaviour in larval zebrafish**. a) Ratio of time spent by larvae in the outer zone of the swimming arena over the total tracking duration, shown in percentage (Two-way ANOVA, 1 mg/L and 5 mg/L vs. Control, $p<0.001$ for both; Independent-samples T test, in 5 mg/L light (L) vs. dark (D), $p\leq0.001$, n=24). b) Ratio of distance travelled by larvae in the outer zone of swimming arena. Change in illumination conditions are shown as L (light) and D (dark) periods on the x-axis (Independent-samples T test, Control and 5 mg/L, L vs. D, $p=0.029$, and $p <0.001$, respectively, n=24). All ratios are calculated as percentages. Min-Max showing all data points. Statistical differences are shown with groups not sharing the same letter. Number of asterisks denote levels of statistical significance based on p-values.

### 2.4.5. Sensitivity and Fear-Related Responses in Larval Zebrafish

The effects of IONP exposure on the sensitivity of larval zebrafish to external stimuli were evaluated by examining their behavioural responses to tapping-induced mechanical stimulation (Figure 12). The analysis revealed a significant decrease in velocity in larvae from the control group during Phase 2 of the test ($p=0.046$). Larvae exposed to IONPs exhibited lower velocities than the control group during Phase 1. To further investigate the effects of IONPs on fear-related behaviours, the frequency of freezing bouts was quantified across the different experimental phases. Larvae exposed to 1 mg/L IONPs were the only group to exhibit a significant change in freezing behaviour between phases, showing a markedly higher frequency of freezing bouts during Phase 2 ($p=0.003$).

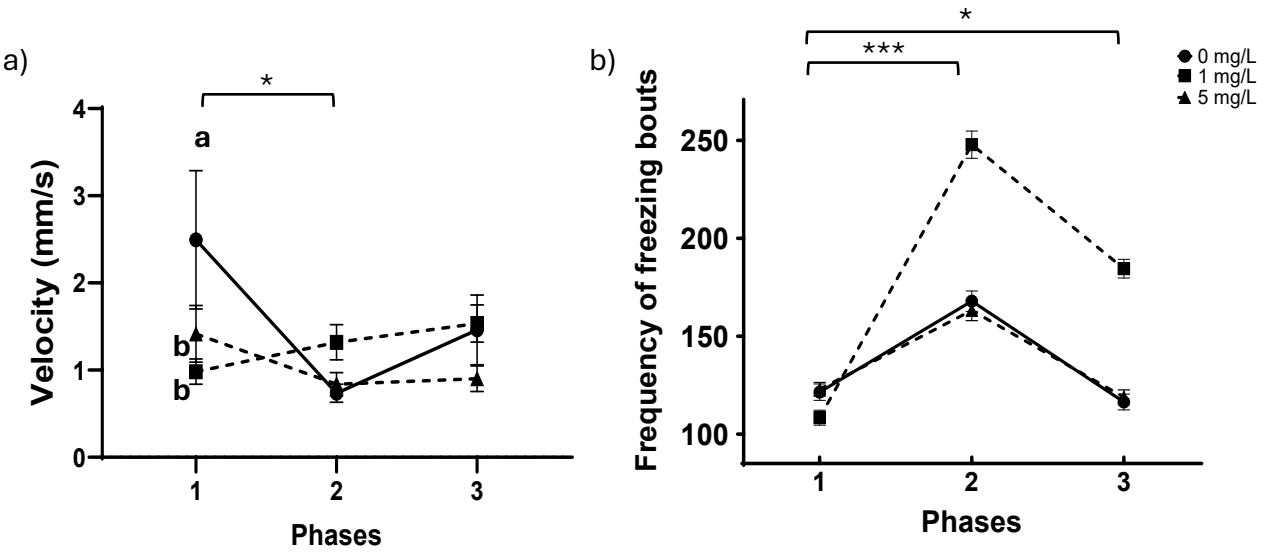



**Figure 12. Sensitivity of larvae to tapping stimuli**. According to the stimuli, three different phases of low intensity and low frequency (1), high intensity and high frequency (2), and high intensity and low frequency (3) were chosen to represent the variations in sensitivity towards stimuli. a) Demonstration of mean velocity of larvae during different phases of the assay (Two-way ANOVA treatment x phase p=0.04, One-way ANOVA for treatments and phases separately, Control vs Exposed in phase 1, p=0.01, Phase1 vs phase2 in Control p=0.046). b) Demonstration of mean of total number of freezing bouts per phase (Welch and Games-Howell post-hoc for treatments and phases separately, Phase1 vs 2 p=0.003, Phase 1 vs 3 p=0.039). Statistical differences are shown with groups not sharing the same letter. Number of asterisks denote levels of statistical significance based on p-values. Mean±SEM, n=24.

2.4.6. ddPCR analysis of oxidative stress responsive genes and DA-related genes

To further investigate the molecular mechanisms underlying the observed behavioural alterations in zebrafish, the effects of IONPs on oxidative stress responses and dopaminergic signalling pathways were examined in 6 dpf larvae using ddPCR Figure 13). IONP exposure produced distinct concentration-dependent changes in antioxidant mRNAs expression. A significant reduction in *sod1* mRNA expression level was observed in the 1 mg/L exposure group ($p=0.048$). In contrast, larvae exposed to 5 mg/L IONPs exhibited significant increases in the relative mRNA expression levels of both *cat* and *gst* genes compared to the control group ($p=0.011$ and $p=0.016$, respectively). No significant differences were observed among exposure groups in the expression levels of dopaminergic pathway-related mRNAs (*th1* and *slc18a*).

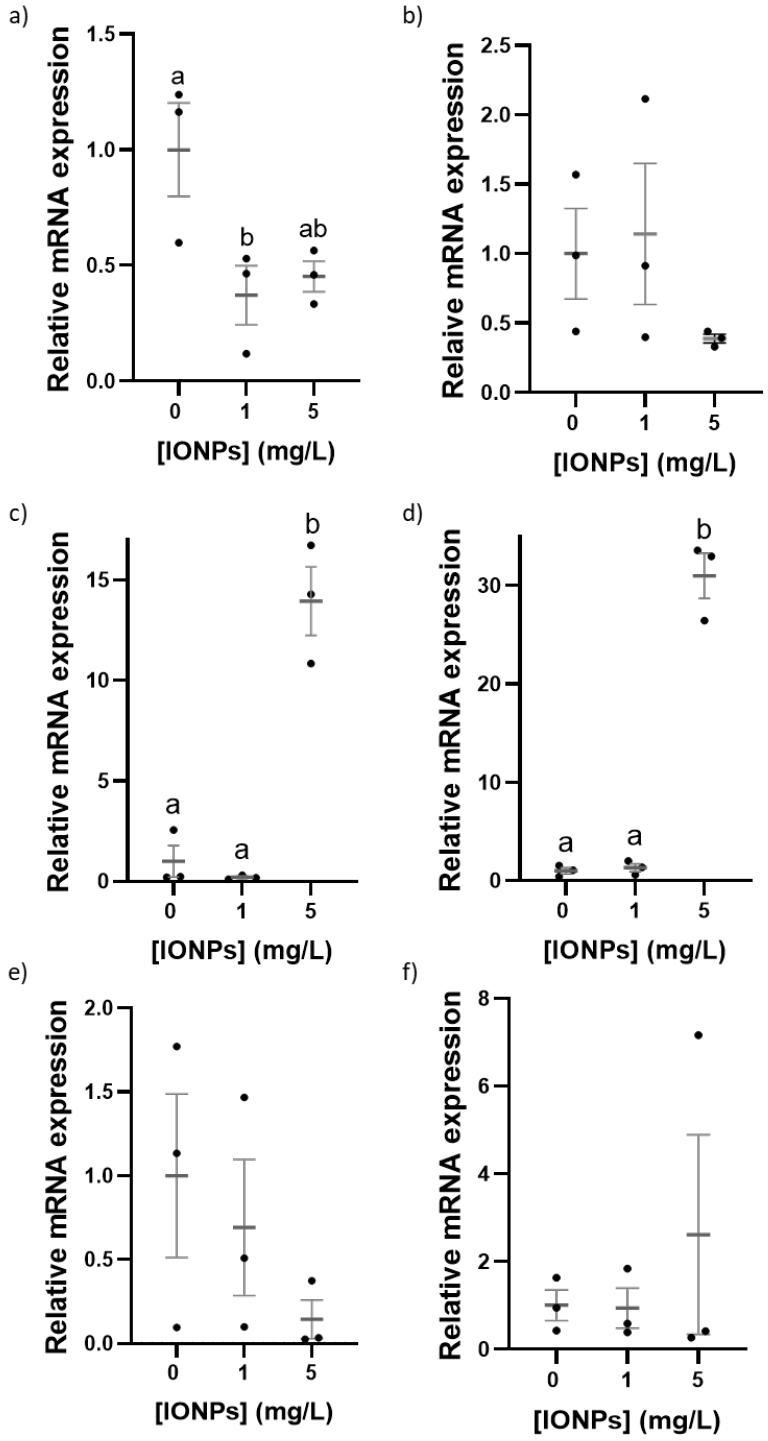



**Figure 13. Quantification of mRNA expression of genes involved in oxidative stress-related response and DA homeostasis using ddPCR.** Relative mRNA expression levels of a) *sod1*(1 mg/L vs. Control, p=0.048), b) *sod2*, c) *cat (*p=0.011), d) *gst (*p=0.011 and p=0.016), e) *slc18a*, and f) *th1*. All values are expressed relative to the control group and are normalized by *ef1a*, *rps18*, and *rpl13a*. All data are mean SEM, n=3 (one sample consisted of 20 pooled larvae). Treatment groups that do not share a letter are statistically different from each other (ANOVA with Tukey HSD, or Welch with Games-Howell post hoc test).

## 2.5. Discussion

This study investigated the effects of IONP exposure on larval zebrafish to evaluate physiological, molecular, and neurobehavioural responses. Exposure to 5 mg/L IONPs resulted in increased whole-body Fe accumulation without affecting tality, deformity, or hatching rates, indicating sublethal exposure conditions. Despite the absence of gross physiological toxicity, exposed larvae exhibited pronounced alterations in swimming behaviour, anxiety-like responses, and sensitivity to external stimuli, accompanied by changes in the mRNA expression of genes associated with oxidative stress. Together, these findings suggested sublethal neurotoxicity potentially induced by IONPs during early development, disrupting behavioural and molecular pathways in larval zebrafish even in the absence of gross developmental abnormalities.

### 2.5.1. Stability, Bioavailability, and Uptake of IONPs During Early Development

In the present study, IONPs remained dispersed in the exposure waters with minimal observable aggregation under the experimental conditions. Particle dispersion was evaluated to confirm stability of the exposure suspensions throughout the experimental period. The average hydrodynamic diameter of IONPs in the exposure water was approximately 154 nm, which falls well below the zebrafish chorion pore diameter (0.5–0.7 µm), suggesting that particle penetration through the chorion was feasible during early embryogenesis [40,114]. Dialysis tubing analyses detected minimal Fe in the surrounding solution during the assay period, indicating limited measurable dissolution of IONPs under the exposure conditions employed in this study. Previous studies have reported that Fe release from uncoated IONPs can vary depending on environmental factors such as pH, synthesis method, and surface characteristics; however, the present study did

not manipulate these parameters and therefore only describes dissolution behaviour within the specific experimental medium used here [21,22]. The observed stability and minimal aggregation are consistent with persistence of IONPs primarily as suspended particulate forms rather than as free ions, which is a critical consideration in evaluating their bioavailability and toxicity potential.

Exposure of larval zebrafish to IONPs led to a concentration-dependent increase in Fe levels in exposure waters as well as in larval tissues at 1 and 5 mg/L IONPs, as determined by ICP-MS analysis. This finding suggested the uptake and bioaccumulation of Fe in the form of IONPs, supporting the notion that IONPs can be internalized by developing organisms. Overall, these results demonstrate IONP bioavailability and provide a foundation for examining their potential longer-term effects in zebrafish following developmental exposures.

### 2.5.2. Effects of IONPs on Physiological Conditions

Given the potential for IONPs to penetrate zebrafish embryo chorions due to their nanoscale dimensions, it remains uncertain whether the particles remain intact within embryonic and larval tissues or undergo partial dissolution, releasing Fe ions. ICP-MS analysis quantifies total Fe content, encompassing both the Fe cores of intact IONPs and any Fe ions that may arise from NP degradation within biological systems. Previous studies have shown that both particulate and ionic Fe forms can influence early-life physiological parameters, including mortality, deformity, and hatching rates in zebrafish embryos and larvae[115,116]. They have also reported that hatching success can vary depending on the type, concentration, and route of exposure to Fe or Fe-containing NPs, making this an important endpoint for evaluating early developmental effects [115,116]. Excessive Fe ion exposure, particularly in the 0.5–5 mg/L range, comparable to the Fe

concentration present in the IONPs used here, has been linked to developmental abnormalities, delayed hatching, and increased mortality [115,117,118]. However, the physiological effects of IONPs often depend strongly on factors such as concentration, exposure duration, NP type, and surface functionalization [59,79,119]. In the present study, IONP exposures ranging from 0 to 5 mg/L did not induce significant mortality, deformity, or changes in hatching rate among larval zebrafish. This lack of physiological disruption suggested that the tested IONP concentrations were within a sublethal range and that the particles may have remained relatively stable in the biological environment, since comparable Fe concentrations have previously shown to cause adverse physiological impacts. The minimal dissolution of Fe detected in the exposure waters supports the notion that IONPs likely persisted as NP forms rather than being degraded into free Fe ions. These findings indicate that IONPs, under the current experimental conditions, did not produce gross developmental toxicity. Nonetheless, future studies employing advanced imaging and chemical speciation techniques are needed to confirm the integrity and localization of IONPs within zebrafish tissues and to elucidate the extent of potential particle transformation over time.

### 2.5.3. Behavioural Disruptions and Stress Phenotypes in Larval Zebrafish

The study of IONPs and their effects on neurobehavioural performance is particularly critical given their nanoscale dimensions and their potential to interact with neural tissue [39,120,121]. This is especially concerning in developing organisms such as larval zebrafish, whose BBB remains underdeveloped and more permeable than that of adults, increasing their vulnerability to NP-induced neurotoxicity [17,59,60]. By 4–5 dpf, zebrafish larvae possess a fully functional nervous system and sensory-motor networks that regulate locomotion, stress, and fear-related responses [67,103,104]. Thus, alterations in spontaneous swimming and thigmotaxis could offer sensitive

measures of neurobehavioural disturbances. In the current study, exposure to IONPs was associated with behavioural differences in larvae, particularly in the 1 mg/L group, which exhibited increased locomotor activity and elevated thigmotaxis, consistent with heightened stress [122,123]. Whole-body Fe accumulation at 1 mg/L was also statistically different from the control group, indicating presence of biological effects. Behavioural endpoints can be sensitive to subtle physiological or molecular shifts that may or may not be proportionally reflected in bulk Fe measurements. In contrast, larvae exposed to 5 mg/L displayed high thigmotaxis with locomotor activity values comparable to controls. This pattern reflects a concentration-specific difference in behavioural profile rather than a strictly linear dose response. This behavioural pattern has previously been consistent with differential Fe uptake and accumulation pathways, stress thresholds and energy conservation in response to stress [124,125]. Non-linear behavioural responses to environmental stressors have been reported in previous studies and may occur under sublethal exposure conditions where different endpoints vary in magnitude across concentrations [120,126].

Further in the thigmotaxis assay, larvae exposed to 1 mg/L IONPs did not exhibit sensitivity to light changes, as no significant differences in time spent or locomotor activity were observed between the light and dark phases. This suggests that these larvae did not engage in increased exploration of the arena under dark conditions, which can be consistent with either a reduced drive to investigate their surroundings when the illumination changed, or stress-driven hypoactivity [71]. In contrast, larvae from the control and 5 mg/L IONP exposure groups displayed less locomotion and time in the outer zone during the dark phase, indicating enhanced exploration of the entire arena under reduced illumination. Previous research has shown that larval zebrafish typically display phototactic behaviour, whereas juvenile and adult zebrafish tend

to exhibit scototaxis [127]. In the present study, however, this expected pattern of light preference was not clearly observed. Several factors may account for this discrepancy. First, the development of phototactic and scototactic responses can vary across zebrafish strains and developmental stages. It is possible that TLxTL larvae at 6 dpf have not yet established a consistent illumination preference. In addition, the behavioural assay employed here included only a 10-minute dark phase, which represents an acute light-dark transition rather than sustained nocturnal conditions. The protocol was designed to assess stress-related and exploratory responses rather than circadian phototactic behaviour across an extended dark period. Therefore, the absence of a clear phototactic pattern may reflect the temporal structure of the assay rather than a deviation from previously reported developmental trends.

Together, these findings are consistent with stage- and concentration-dependent neurobehavioural alterations in zebrafish, potentially induced by IONP exposures. These specific behavioural changes may reflect differences in neurodevelopmental sensitivity and possibly adaptive responses to NP-induced stress.

### 2.5.4 Sensory Processing and Escape Responses in Larval Zebrafish

Disruptions in escape behaviour and stimulus responsiveness can influence an organism's ability to respond to environmental threats [73]. In zebrafish, M-cells are not the only but the central components of the fast escape circuitry and are involved in rapid startle responses and behavioural adaptation to repeated stimuli [128]. DA has been reported to modulate arousal and excitability within these networks, contributing to the regulation of response probability and behavioural adjustment [129,130]. In the present study, control larvae exhibited a reduction in

velocity across repeated tapping stimuli, consistent with habituation and intact sensory filtering. In contrast, larvae exposed to IONPs did not show a comparable reduction in locomotor velocity with increasing stimulus intensity. The 1 mg/L group displayed a significantly higher frequency of freezing bouts during phase 2, despite no significant difference in overall velocity relative to controls. This pattern reflects an alteration in response profile rather than a reduction in motor capacity [131]. Larvae retained the ability to swim but showed increased immobility events under repeated stimulation. The observed behavioural pattern is compatible with variation in sensorimotor integration or escape circuit modulation [111,112]. Previous research has demonstrated that modulation of M-cell networks and associated neuromodulatory pathways can influence habituation, freezing behaviour, and escape probability [128] however, the present study did not directly measure M-cell activity, synaptic transmission, or neural excitability. Therefore, any interpretation regarding specific circuit-level mechanisms remains inferential. Furthermore, although dopaminergic systems are known to participate in regulating arousal, startle responsiveness, and behavioural adaptation, the current larval analyses did not detect significant transcriptional changes in dopaminergic markers. As such, behavioural differences cannot be attributed to specific neurotransmitter disruptions based on the present data alone. The behavioural changes reported here occur within a broad neurobiological framework but do not establish direct alterations in M-cell function. To begin to localize the dysfunction and explore whether altered excitability of M-cells is consistent with the observed behavioural shift, electrophysiological assessment of brain areas corresponding to M-cell activity is essential.

Collectively, the findings indicate that early-life IONP exposure was associated with altered responsiveness to repeated sensory stimulation, characterized by reduced habituation and

increased freezing events in certain exposure groups. These differences occurred in the absence of overt motor impairment, suggesting that basic locomotor capacity was preserved. Further studies incorporating direct electrophysiological or neurochemical measurements would be required to determine whether changes in escape circuitry excitability or neuromodulatory balance underlie the behavioural profiles observed here.

2.5.5. Oxidative Stress and Dopaminergic Pathways as Potential Underlying Mechanisms

Given that exposure to Fe-based NPs has previously been associated with the induction of oxidative stress responses, the mRNA expression levels of oxidative stress-related genes were examined [119,132–136]. Superoxide dismutase 1 (SOD1), catalase (CAT), and glutathione S-transferase P1.2 (GSTP1.2) are three critical antioxidant enzymes that operate at different points along the oxidative defense cascade, forming a coordinated system that mitigates the damaging effects of ROS. In the current research, the IONPs consisted of Fe in its ferric state ($Fe^{3+}$), which readily participates in the Haber–Weiss and Fenton reactions, chemical processes that amplify ROS generation. In the Haber–Weiss cycle, $Fe^{3+}$ is reduced by superoxide anions ($\bullet O_2^-$) to ferrous iron ($Fe^{2+}$), which subsequently reacts with hydrogen peroxide ($H_2O_2$) in the Fenton reaction to regenerate $Fe^{3+}$ along with hydroxyl radicals ($\bullet OH$) and hydroxyl ions ($OH^-$) (Supplementary Formula 1). These hydroxyl radicals are among the most reactive oxygen species and can inflict oxidative damage on lipids, proteins, and DNA [137].

In larval zebrafish, IONP exposure was associated with a significant downregulation of *sod1*, despite presence of stress-related behavioural alterations. Since an increased accumulation of Fe was observed in larval tissues, the observed reduction in *sod1* mRNA expression levels may

be associated with altered redox dynamics following IONP exposure. $Fe^{3+}$ can participate in the Haber–Weiss reaction, consuming $\bullet O_2^-$ and thereby modifying the redox environment in which SOD1 normally functions. Changes in superoxide availability and sustained oxidative challenge may reduce reliance on SOD1 activity, contributing to transcriptional downregulation through broader redox-sensitive regulatory mechanisms. In addition, under conditions of prolonged or Fe-mediated oxidative stress, antioxidant systems may become overwhelmed or maladaptive, leading cells to shift toward downstream antioxidant defenses such as CAT and GSTP1.2-dependent pathways. Consistent with this observations, NP exposures have previously been reported to suppress specific antioxidant enzymes, including SOD1, while concurrently impacting other components of the antioxidant response in various ways [138,139]. Downregulation of *sod1* coincided with altered locomotor behaviour in larvae exposed to 1 mg/L IONPs, characterized by increased activity alongside elevated freezing bouts, a response commonly associated with stress or fear. Comparable behavioral phenotypes, including locomotor dysregulation and heightened stress sensitivity, have been reported in SOD1-deficient invertebrate models (*C. elegans*), supporting a link between impaired antioxidant defense and altered neural function [140]. In contrast, *cat* mRNA expression level was significantly elevated in larvae exposed to 5 mg/L IONPs, suggesting activation of antioxidant pathways commonly associated with increased oxidative challenge. CAT catalyzes the conversion of $H_2O_2$ to water and oxygen, and increased *cat* mRNA expression level has frequently been reported under conditions of elevated $H_2O_2$ availability, which may influence the extent of downstream redox reactions, including Fenton chemistry. Similarly, *gstp1.2* mRNA expression was upregulated in the 5 mg/L group, aligning with the established role of glutathione S-transferases in detoxifying secondary oxidative byproducts such

as lipid peroxides and reactive aldehydes generated during radical-mediated cellular damage [137,141]. Together, these transcriptional changes indicate molecular responsiveness to higher IONP exposure levels, even in the absence of pronounced behavioural alterations at this developmental stage. This pattern suggested that molecular changes precede or differ temporally from behavioural outcomes, particularly under higher nanoparticle concentrations. At higher exposure concentrations, IONPs have been reported to exhibit an increased tendency to agglomerate within biological media, a process that can alter their effective surface area and apparent bioavailability, as well as their interaction with cellular membranes and physiological barriers [142]. Such physicochemical changes may be associated with reduced immediate interaction with neural tissues, which has been observed in some studies as a relative attenuation of acute phenotypic responses [143]. Nonetheless, longer-term processes, including changes in particle dispersion, cellular internalization, or partial release of Fe species, have been documented in biological systems and may coincide with alterations in redox balance and neurotransmission. These delayed processes have been associated with the emergence of neurobehavioural effects at later time points in other nanoparticle exposure studies.

Parallel to oxidative stress, DA regulation was also assessed, given DA's central role in modulating anxiety, sensory responsiveness, and escape behaviour. *th1* and *slc18a2* were examined as key markers of DA synthesis and vesicular transport [63,83,144]. In larvae, no significant differences were observed in *th1* or *slc18a2* mRNA expression levels. This indicated that, under the conditions tested, transcriptional regulation of these dopaminergic markers was not altered following IONP exposure. Previous studies have shown that behavioural alterations can occur in the absence of transcriptional changes in DA-related genes, potentially reflecting modulation at

post-transcriptional, receptor, or circuit levels [100,145–147]. Moreover, anxiety- and stress-related behaviours in zebrafish are regulated by multiple interacting neural pathways, and variability in mRNA expression of dopaminergic genes may reflect the engagement of broader neuromodulatory systems rather than a simple dose-dependent transcriptional effect [148,149].

### 2.6. Conclusion

This study provides an integrated understanding of how IONPs affect zebrafish neurodevelopment across molecular, behavioural, and electrophysiological levels. Sublethal, early-life exposure to IONPs (1–5 mg/L) did not induce overt physiological toxicity but caused significant, concentration- and stage-specific neurofunctional alterations. Larval fish showed elevated oxidative stress responses, which were accompanied by hyperactivity, altered thigmotaxis, and impaired sensitivity, and altered oxidative mRNA expression, all changes that have been linked to heightened stress. Analysis of oxidative stress- and DA-related genes via their mRNA expression levels, revealed that IONP exposure was associated with concentration-specific transcriptional changes in select antioxidant pathways, while dopaminergic markers remained unchanged at the transcriptional level in larval zebrafish. The observed downregulation of *sod1* at lower exposure levels and upregulation of cat and *gstp1.2* at higher concentrations are consistent with differential engagement of antioxidant responses depending on IONP dose. These molecular responses occurred alongside behavioural alterations, suggesting that molecular and behavioural sensitivity both exist, even though the quantity of effects may not be concentration-dependent during early development. Together, these results characterize IONP exposure as being associated with concentration- and stage-specific molecular and behavioural at environmentally relevant concentrations. From an ecological standpoint, such disruptions could

impair survival-related behaviours, with potential consequences at the population level. Future research should quantify wider range of DA and oxidative damage markers, and employ electrophysiology and imaging to localize affected circuits, thereby establishing links between NP-induced oxidative stress, dopaminergic modulation, and altered neural function. Additionally, integration of time-resolved molecular, behavioural, and physiological assessments would be required to clarify how these responses interact across developmental stages.

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# CHAPTER 3

# Prolonged Effects of IONPs in Juvenile Zebrafish: Physiological Performance and Neurobehavioural Responses

## 3.1. Chapter Overview

This chapter examined the longer-term physiological, molecular, and neurobehavioural consequences of early-life IONP exposure in juvenile zebrafish. ICP-MS analysis revealed a non-linear pattern of brain Fe accumulation, with alterations in ionic levels observed at the higher exposure concentration, highlighting stage- and dose-dependent differences in NP handling and ionic regulation. Despite the absence of overt physiological toxicity, juvenile zebrafish displayed persistent and concentration-specific alterations in locomotor activity, anxiety-like behaviour, and social preference. Molecular analyses further demonstrated developmental shifts in oxidative stress and dopaminergic mRNA expression profiles relative to larvae, potentially suggesting that early IONP exposure can lead to distinct neurochemical signatures later in development. Collectively, these findings underscore the complexity of NP-organism interactions and support the presence of delayed and stage-specific neurofunctional effects following developmental IONP exposure.

## 3.2. Introduction

### 3.2.1. Developmental Progression Following Early-Life Nanoparticle Exposure

Engineered NPs are increasingly detected in aquatic environments as a consequence of industrial activity, consumer product use, and waste streams. Early-life exposure to these materials has received considerable attention due to the heightened sensitivity of developing

organisms during periods of rapid growth and differentiation. However, exposure during early development does not necessarily result in immediate or overt toxicity, and growing evidence suggests that some effects may emerge only later in life, following developmental maturation or physiological reorganization. Assessing organisms beyond the larval stage is therefore critical for understanding whether early NP exposure is associated with persistent or delayed biological effects that may not be evident during initial exposure windows.

### 3.2.2. Juvenile Zebrafish as a Model for Post-Exposure Neurodevelopmental Assessment

Juvenile zebrafish at 30 dpf represent an important developmental stage for evaluating outcomes following early-life exposure. By this stage, zebrafish have undergone substantial neurodevelopment, including refinement of neural circuits governing locomotion, stress responsiveness, and social behaviour. Compared with larvae, juveniles display more complex and stable behavioural repertoires, allowing assessment of higher-order functions such as anxiety-related responses and social interactions. In addition, the BBB is more developed by 30 dpf, providing a physiologically relevant context for examining brain-associated impacts and molecular markers following earlier exposure. Studying juveniles therefore enables evaluation of developmental progression and supports investigation of whether early-life NP exposure is associated with measurable outcomes after end of exposure.

### 3.2.3. Metal Homeostasis and Behavioural–Molecular Endpoints in Juvenile Neurotoxicity Assessment

Following early developmental exposure, juvenile zebrafish represent a critical stage for evaluating the persistence, modification, or resolution of NP-induced effects. By 30 dpf, zebrafish

have undergone substantial neurodevelopment, including maturation of central nervous system architecture, refinement of neural circuits, and increased physiological regulation [103,104]. Importantly, key protective and regulatory systems, including mechanisms governing metal transport and ion homeostasis, are more established at this stage, allowing assessment of neurobiological outcomes under conditions that more closely resemble later-life vertebrate physiology[96,150]. Regulation of Fe within the brain is tightly controlled during juvenile development, as deviations in metal balance may influence neural metabolism, redox status, and cellular signaling. In parallel, maintenance of essential ions is fundamental for neural excitability, action potential propagation, and synaptic transmission [151]. Alterations in metal or ionic homeostasis at the juvenile stage therefore provide insight into potential disruptions of neurophysiological stability that may arise following earlier nanoparticle exposure.

Juvenile zebrafish also display broader collection of complex behaviours compared to larvae, including refined locomotor patterns, context-dependent anxiety responses, and social interactions [152]. Behavioural endpoints such as spontaneous swimming activity, thigmotaxis, and social preference integrate sensory processing, motor control, and neuromodulatory regulation across distributed neural networks. When interpreted alongside tissue-specific metal and ion measurements and targeted molecular markers, these behaviours provide a systems-level framework for evaluating whether early-life IONP exposure is associated with altered neurodevelopment that persist into later stages.

Overall, this chapter will examine the following hypothesis:

The neurobehavioural and molecular disturbances from early-life IONP exposures will persist into the juvenile stage, reflecting long-lasting effects of NP-induced stress.

Objectives of Chapter 3:

- Quantify brain metal and ion concentrations in juvenile zebrafish.
- Assess spontaneous locomotion and anxiety-related behaviour at the juvenile stage.
- Evaluate social preference and 1fear-related responses using a plus-maze paradigm.
- Examine persistence or modification of oxidative stress and dopaminergic gene expression in juvenile brain tissue.

## 3.3. Materials and Methods

### 3.3.1. Post-Exposure Rearing and Juvenile Husbandry

Following completion of the initial exposure period, zebrafish larvae were either collected at 6 dpf for experimental analyses or transferred to larger plastic rearing tanks (14 × 21 × 10 cm) to allow continued growth to the juvenile stage. The tank dimensions provided sufficient space to support normal development and minimize density-related stress. Housing density was maintained at approximately 10–12 fish per tank, consistent with standard zebrafish husbandry practices. Juvenile rearing tanks were maintained at 28 °C under a 14 h light/10 h dark photoperiod, with water quality and husbandry conditions consistent with established zebrafish rearing protocols. Fish were monitored daily throughout the post-exposure period for general health, survival, and visible developmental abnormalities. Any instances of delayed mortality or malformation were noted to ensure the integrity of experimental cohorts. Fish were maintained under these conditions until 30 dpf, at which point juveniles were used for subsequent behavioural and molecular analyses. Continued monitoring during this rearing phase enabled

assessment of potential delayed or cumulative effects following early-life IONP exposure. The juvenile physiological monitoring component was conducted solely to verify experimental consistency and data reliability; therefore, these observations were not subjected to statistical analysis or graphical presentation.

### 3.3.2. Brain Metal and Ion Quantification

At 30 dpf, zebrafish were euthanized and brain tissues were dissected for metal and ion analysis. For each exposure group, brain tissues from 5–7 juveniles were pooled per sample, with three biological samples prepared per concentration (n = 3). Pooling was employed to obtain sufficient tissue mass for reliable quantification while minimizing inter-individual variability. Following dissection, brain samples were dried at 65 °C for two days. Dried tissues were then digested in 300 µL of 6N nitric acid ($HNO_3$) and incubated at 65 °C for an additional two days to ensure complete tissue digestion. After digestion, samples were diluted with 5 mL of 2% $HNO_3$ and filtered through 0.45 µm syringe filters prior to analysis.

Metal and ion concentrations were quantified using inductively coupled plasma triple quadrupole mass spectrometry (ICP-QQQ-MS; Agilent 8800) at the Water Quality Centre, Trent University. The analytes measured included iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), sodium (Na), magnesium (Mg), potassium (K), and calcium (Ca), depending on sample type. Quality assurance and quality control procedures were implemented throughout the a$^{na}$lytical process. Certified reference material (NIST SRM 1640a; trace elements in natural water) was analyzed alongside samples, and all measured values fell within 5% of certified concentrations, confirming analytical accuracy and instrument performance.

To ensure consistency across developmental stages, the same batch of IONPs, exposure concentrations, digestion procedures, and analytical methods used for larval whole-body metal analysis were applied to juvenile brain samples. This methodological continuity allows direct comparison of metal and ion profiles across developmental stages.

### 3.3.3. Neurobehavioural Assessments in Juvenile Zebrafish

- Spontaneous and Stress-Related Activities

Juvenile behavioural assessments were conducted at 30 dpf to evaluate the persistence of neurobehavioural effects following early-life IONP exposure. At this stage, zebrafish have undergone substantial neurodevelopment, enabling assessment of more complex behavioural domains, including spontaneous locomotion, stress-related responses, and exploratory behaviour. Behavioural testing protocols were adapted from those described for larval assessments (Chapter 2), with modifications appropriate for juvenile size and behavioural capacity. All behavioural trials were performed using the DanioVision Observation Chamber (Noldus Information Technology) under controlled conditions. The testing environment was maintained at 28 °C using a dedicated thermal regulation unit, with light intensity set to approximately 300–350 lux. Fish were acclimated prior to recording, and behavioural activity was captured and analyzed using EthoVision XT software (Noldus Information Technology). To minimize variability associated with circadian rhythms, all assays were conducted between 12:30 PM and 3:30 PM, ensuring temporal consistency across experimental groups.

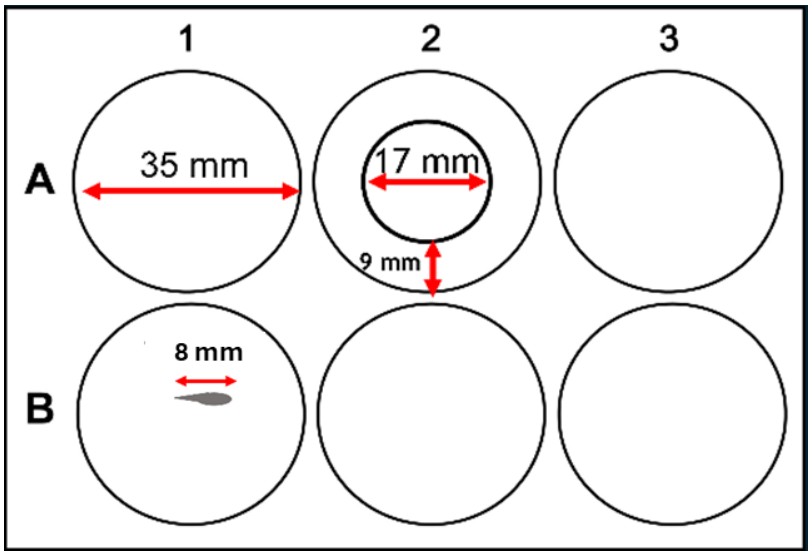
Spontaneous swimming behaviour was assessed as an indicator of baseline locomotor function and neurodevelopmental integrity. Fish previously exposed to IONPs during the larval

stage, were individually placed into the wells of a 6-well plate and allowed to acclimate for 20 minutes within the DanioVision chamber. Locomotor activity was then recorded for 20 minutes (10 min light and 10 min dark, recording under the light condition was used for spontaneous activity analysis). Behavioural parameters quantified included cumulative distance traveled and mean swimming velocity for each individual (n = 9–12 per exposure group). These metrics provide an integrated measure of general motor output and activity levels, allowing comparison of baseline locomotion across exposure concentrations.

In addition to spontaneous locomotion, anxiety-like behaviour was evaluated through analysis of thigmotaxis, defined as a preference for swimming along the periphery of the arena ("wall-hugging"). Thigmotactic behaviour was quantified using predefined inner and outer zones within each well, configured in EthoVision XT software (Figure 14). Following an initial 20-minute acclimation period, juveniles were subjected to sequential 10-minute light and 10-minute dark phases designed to elicit stress- and exploration-related responses, consistent with the protocol described for larval assessments in Chapter 2. Behavioural outputs were calculated as the percentage of time spent and the percentage of distance traveled in the outer zone relative to total tracking duration and total distance traveled, respectively (Supplementary Formula 1).

Together, these behavioural assays enabled assessment of long-term effects of early IONP exposure on locomotor performance, exploratory behaviour, and stress responsiveness at the juvenile stage. By applying a methodologically consistent framework across developmental stages, this approach supports direct comparison between larval and juvenile behavioural outcomes while capturing stage-specific neurobehavioural features.

a)




b)

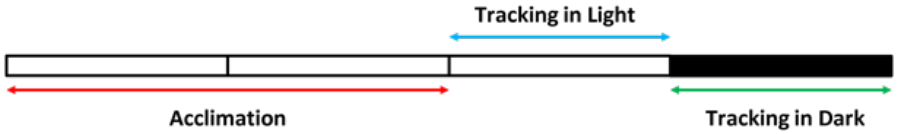



**Figure 14. Experimental setup of neurobehavioural assessments in juvenile zebrafish** a) Depiction of the zone layout for the zone preference test (inner and outer zones). Precise dimensions of the wells and zones are shown along with the average size of juvenile zebrafish at the time of the assessment. b) Illustration of the tracking timeline (Each block represents one 10-min period). After zebrafish acclimate to the wells for 20 min, their movement was tracked for 10 min with the light on and then another 10 min with the light off.

- Social Preference and Fear-Related Behaviour

To further investigate neurobehavioural performance, including fear- and social preference-related responses, a series of assays were conducted using a custom-built plus maze specifically designed to evaluate zebrafish interactions with conspecific cues. The plus maze was selected over the more commonly used rectangular tank format due to its capacity to enhance exploratory behaviour and improve both the sensitivity and specificity of social preference measurements. To provide a controlled social stimulus, a mirror was incorporated within one arm of the maze, allowing for direct comparison between attraction to a social cue and the absence of such a stimulus. This configuration facilitated a more refined assessment of zebrafish decision-making processes while ensuring that mirror-induced interactions were concentrated within a defined and reproducible environment.

The plus maze used in this study was custom-designed, with dimensions adapted from a previous investigation of the relationship between stress levels and depth preference in juvenile zebrafish [153]. To our knowledge, this represents the first application of this specific protocol and maze configuration for assessing juvenile zebrafish behaviour. The experimental setup is depicted in figure 15. A mirror was positioned at the distal end of one of the maze arms, designated as the "M arm," to serve as the social stimulus. This approach has been used in similar behavioural assessments and has been reported to reliably elicit social preference behaviours [154]. The three remaining arms of the maze were randomly assigned as Arms 1, 2, or 3, and the orientation of the maze was randomized for each trial to vary the position of the M arm and the numbered arms. This randomization was implemented to eliminate potential directional bias and to minimize confounding factors in behavioural analysis.

Two versions of the plus-maze experiment were conducted: one with the mirror present in the M arm and another without the mirror. The no-mirror version served as a control condition to determine whether mirror placement specifically elicited social preference behaviours. The spatial configuration of the maze, including the numbering of the arms, remained identical across both conditions to ensure consistency. During each trial, juvenile zebrafish (30 dpf) were individually introduced into the plus maze. Fish were allowed a 10-minute acclimation period, after which behavioural activity was recorded for 15 minutes. Trials were conducted with six fish tracked simultaneously, with one fish placed per maze. For social preference analysis, data were quantified as the ratio of time spent in the M arm relative to the total time spent across all arms. Fear-related behaviours were assessed by measuring the latency to first entry into the M arm as well as the frequency of entries into the M arm across the trial duration.

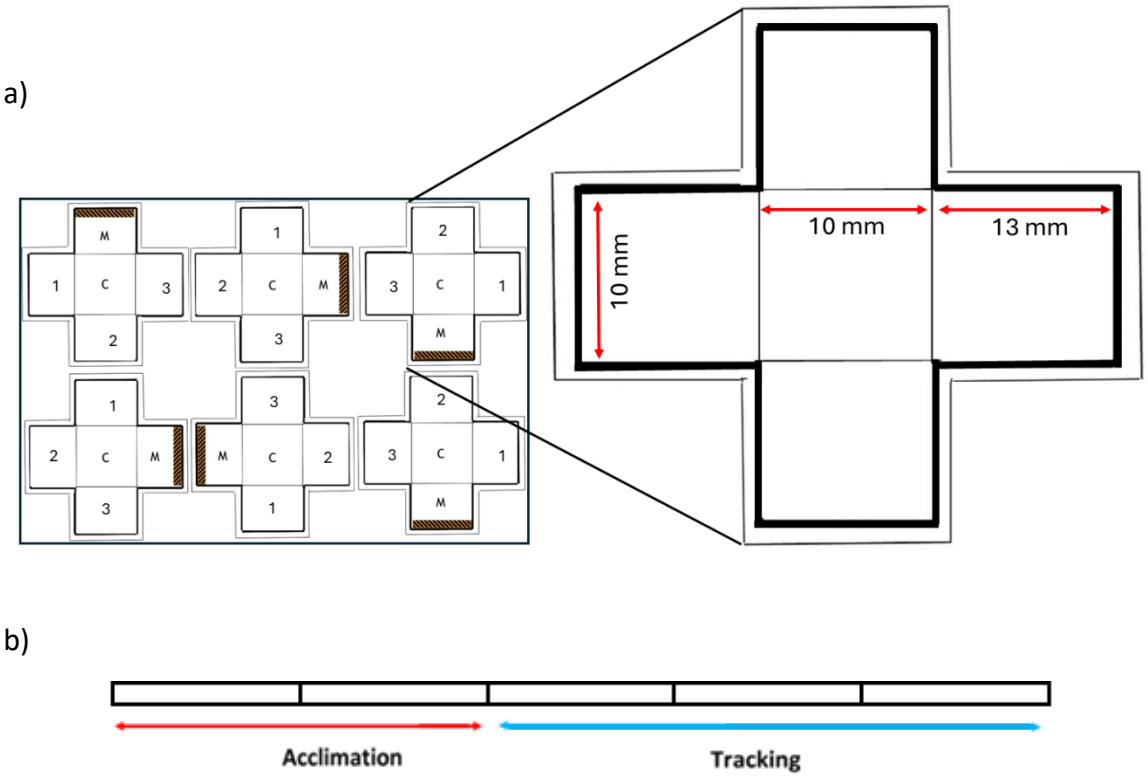



**Figure 15. Schematic representation of the experimental setup for behavioural testing using a plus-maze apparatus**. The left panel illustrates the arrangement of six plus-mazes within the tracking chamber of DanioVision system, each containing labeled compartments: central zone (C), arms (1, 2, 3), and an arm containing a mirror (M) indicated by shaded regions. The magnified view of a single cross-maze shows precise dimensions (10 mm × 10 mm for the central zone and 13 mm × 10 mm for each arm). This setup is used to assess spatial exploration, preference in interaction with conspecific, and decision-making in the test subjects. b) Illustration of the tracking timeline (Each block represents one 5-min period). After fish acclimate to the wells for 10 min, their movement was tracked for 15 min.

### 3.3.4. mRNA Expression Analysis in Juvenile Brain Tissue

To evaluate whether molecular alterations induced by early-life IONP exposure persist into later developmental stages, mRNA expression analyses were conducted in juvenile zebrafish at 30 dpf. This assessment builds directly upon the larval mRNA expression analyses described in Chapter 2 and was designed to examine the long-term status of molecular pathways implicated in neurobehavioural regulation.

As in the larval stage, the focus was placed on genes associated with dopaminergic homeostasis and oxidative stress responses, given their established roles in modulating neural function, stress responsiveness, and behaviour. Target genes included *th1* and *slc18a2* as markers of DA synthesis and vesicular transport, respectively, as well as *sod1, sod2, cat*, and *gstp* as key components of the antioxidant defense system. Assessment of these targets provides a molecular framework for interpreting behavioural outcomes observed at the juvenile stage in relation to earlier developmental exposure. For juvenile analyses, brain tissues were dissected from 6–7 zebrafish per sample, with three biological samples prepared for each exposure concentration (n = 3). Following euthanization, brain tissue was processed for RNA extraction, cDNA synthesis, and ddPCR quantification using the same protocols described for larval samples in Chapter 2, with minor methodological adaptations to optimize RNA recovery from brain tissue. Normalization of mRNA expression levels was performed using a panel of validated housekeeping genes, including *ef1a*, *rps18*, and *rpl13a*. This normalization strategy was applied consistently across both larval and juvenile datasets to ensure accuracy and comparability of mRNA expression measurements across developmental stages. Primer sequences and assay details for all target and reference genes are provided in Table 1.

Together, these methods enabled precise, absolute quantification of molecular markers related to oxidative stress and dopaminergic regulation in juvenile brain tissue. By directly comparing mRNA expression profiles between larvae and juveniles, this approach allows evaluation of the persistence, modulation, or resolution of IONP-induced molecular effects over development, thereby providing insight into the long-term neurobiological consequences of early-life nanoparticle exposure.

### 3.3.5. Statistical Analysis

All statistical analyses for juvenile-stage experiments were conducted using IBM SPSS software (version 23.0, IBM SPSS Inc., USA). Data are presented as median (Max and Min) or mean ± standard error of the mean (SEM), and graphical representations were generated using GraphPad Prism.

Prior to hypothesis testing, all datasets were evaluated for normality using the Kolmogorov–Smirnov one-sample test and for homogeneity of variance using Levene's test to confirm suitability for parametric analyses, as described for larval assessments in Chapter 2. When data met assumptions of normality and equal variance, one-way analysis of variance (ANOVA) was applied, followed by Tukey's post hoc test for multiple comparisons. In cases where variance heterogeneity was detected, Welch's ANOVA was used, with Games–Howell or Dunnett's t (two-sided) post hoc tests applied as appropriate.

Repeated-measures one-way ANOVA was employed to assess treatment-related effects on locomotor activity parameters across time or experimental phases where applicable.

Independent Student's t-tests were used for targeted pairwise comparisons, including assessment of treatment effects under different lighting conditions and behavioural contexts.

For all statistical tests, a p-value of $< 0.05$ was considered indicative of statistical significance.

## 3.4. Results

### 3.4.1. Physiological Outcomes During Juvenile Development

The physicochemical characterization of the IONPs used in this study has been described in detail in Chapter 2 (Section 2.4.1). As the same batch of NPs and identical exposure regime were applied across both larval and juvenile experiments, the particle properties relevant to exposure, including hydrodynamic diameter, stability, and minimal dissolution, are considered consistent between developmental stages. Dynamic light scattering (DLS) and dissolution testing (DT) analyses demonstrated that the iron oxide nanoparticles (IONPs) had a mean hydrodynamic diameter of approximately 150 nm and showed negligible release of free Fe ions under the specified exposure conditions. Additional DLS measurements and electron microscopy results were consistent with well-dispersion of particles at low to moderate concentrations, with minimal aggregation or sedimentation (see Chapter 2, Figure 7, Table 1 & 2).

During this juvenile rearing phase, fish were monitored daily for delayed mortality or the emergence of visible morphological abnormalities. No additional mortality, growth impairment, or external deformities were observed beyond the larval stage, indicating that early-life exposure to IONPs at concentrations up to 5 mg/L did not result in overt physiological toxicity during juvenile development. These observations are consistent with the larval findings reported in

Chapter 2 (Section 2.4.3), which demonstrated the absence of significant effects on mortality, deformity incidence, or hatching success during early development.

### 3.4.2. Brain Metal and Ionic Homeostasis

Quantification of metal content in juvenile zebrafish brain tissue revealed exposure-dependent differences in Fe accumulation. Brain Fe concentrations were significantly higher in the 1 mg/L exposure group compared to the 5 mg/L group, while control values were intermediate and not significantly different from either treatment. This pattern differed from the concentration-dependent increase in Fe previously observed in whole-body larval tissues. Comparison between larval whole body and juvenile brain tissue Fe content is shown in table 5.

In addition to Fe, several other metals and major ions were measured in juvenile brain samples, including Mn, Cu, Zn, Na, Mg, K, and Ca (Table 6). Among these, $Na^+$ concentrations were significantly reduced in the 5 mg/L exposure group compared to controls. $Na^+$ levels in the 1 mg/L group were intermediate and not significantly different from either the control or high-dose group. No statistically significant differences were detected across exposure groups for Mn, Cu, Zn, Mg, K, or Ca.

**Table 5. Fe content in exposure waters, zebrafish whole body larval tissue, and juvenile brain tissue**

| [IONPs](mg/L) | Water (mg/L) | Larva (µg/larva) | Brain (µg/brain) |
|---|---|---|---|
| **0** | <0.001±0.001[a] | 0.009±0.004[a] | 0.496±0.029[ab] |
| **1** | 0.110±0.005[b] | 0.033±0.002[b] | 0.611±0.081[a] |
| **5** | 1.009±0.020[c] | 0.089±0.013[b] | 0.311±0.036[b] |

Value labelled with different letters represents a statistical difference across treatments (Welch, post hoc Games-Howell, $p<0.01$, n=3-4).

**Table 6. Additional metal/ion content in zebrafish brain tissue**

| [IONP] | 0 mg/L | 1 mg/L | 5 mg/L |
|---|---|---|---|
| [Metals] (µg/Brain) | | | |
| Mn | 0.016±0.001 | 0.019±0.002 | 0.014±0.001 |
| Cu | 0.137±0.010 | 0.142±0.002 | 0.121±0.001 |
| Zn | 0.978±0.094 | 1.020±0.087 | 0.883±0.064 |
| [Ions] (µg/Brain) | | | |
| Na | 29.062±2.502[a] | 26.571±1.080[ab] | 21.041±0.945[b] |
| Mg | 4.010±0.385 | 3.914±0.153 | 3.204±0.107 |
| K | 37.502±4.297 | 37.296±1.714 | 32.345±1.156 |
| Ca | 90.221±8.294 | 93.604±12.290 | 79.464±4.224 |

Value labelled with different letters represents a statistical difference across treatments (Welch, post hoc Games-Howell, $p<0.01$, n=4).

### 3.4.3. Spontaneous Locomotion and Anxiety-Related Behaviour

In juvenile zebrafish, assessment of locomotor activity revealed a more complex dose-dependent pattern. Fish exposed to 1 mg/L IONPs showed a significant decrease in cumulative distance traveled compared to controls ($p = 0.014$), whereas the 5 mg/L group exhibited a markedly greater cumulative distance traveled ($p < 0.001$). Similarly, mean swimming velocity was significantly elevated in the 5 mg/L exposure group compared to controls ($p < 0.001$), while no significant difference in velocity was observed in the 1 mg/L group (Figure 16). Thigmotactic behaviour in juveniles also demonstrated differential responses across exposure concentrations. The percentage of time spent in the outer zone of the swimming arena was significantly greater in the 1 mg/L exposure group compared to the control ($p = 0.044$), while the percentage of distance traveled in the outer zone was significantly reduced in the 5 mg/L group ($p = 0.006$). Across all groups and conditions, thigmotactic activity was consistently higher during the dark phase than during the light phase (Figure 17, all groups, $p \leq 0.035$).

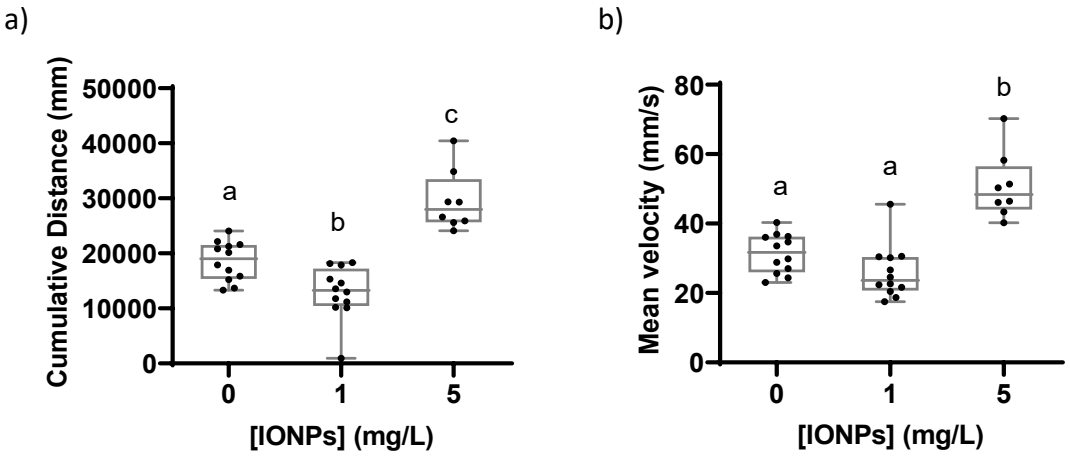



**Figure 16. Spontaneous swimming activities in juvenile zebrafish**. a) Cumulative distance travelled by juvenile fish (One-Way ANOVA, tukey's post-hoc, 1 and 5 mg/L vs. Control, p=0.014 and p≤0.001, respectively). b) Mean velocity of juvenile fish over the tracking duration (One-Way ANOVA, tukey's post-hoc, 5 mg/L vs. Control, p≤0.001). Min-Max showing all data points, statistical differences are shown with groups not sharing the same letter, N=9-12.

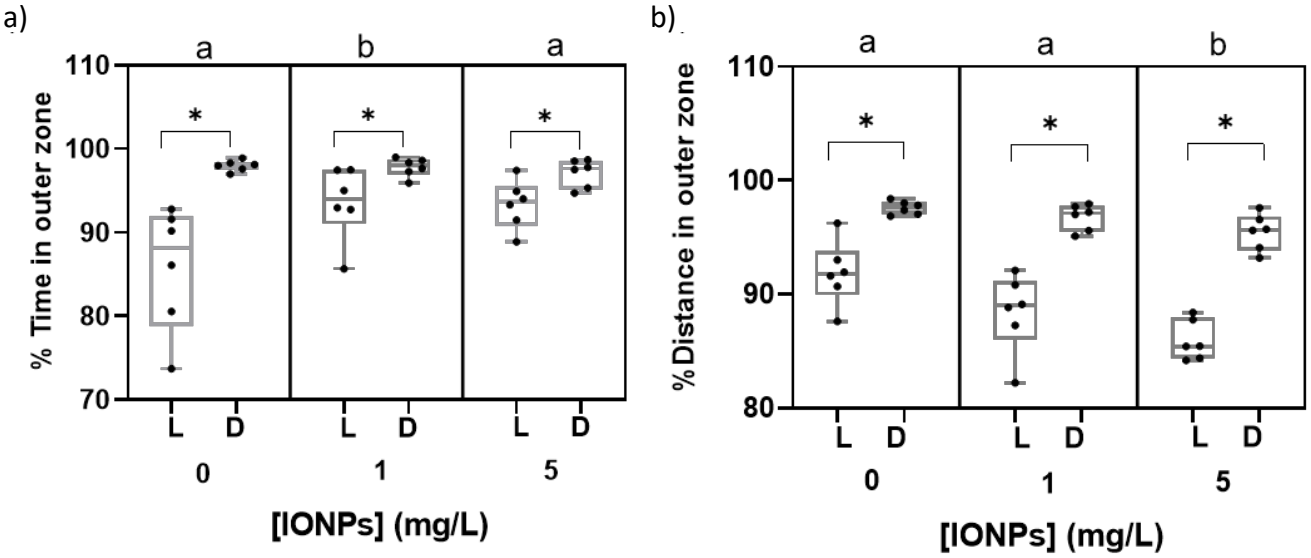



**Figure 17. Thigmotactic behaviour in juvenile zebrafish**. Change in illumination conditions are shown as L (light) and D (dark) periods on the x-axis. a) Ratio of time spent by fish in the outer zone of the swimming arena over the total tracking duration, shown in percentage (Two-way ANOVA, 1 mg/L vs Control, $p = 0.044$; Independent-samples T test, L vs. D in all groups, $p \leq 0.035$). b) Ratio of distance travelled by fish in the outer zone of swimming arena (Two-way ANOVA, 5 mg/L vs Control, $p = 0.006$). All ratios are calculated as percentages. Statistical differences are shown with groups not sharing the same letter. Asterisks denote statistical significance between illumination conditions (Min-Max showing all data points, n=9-12).

### 3.4.4. Fear and Social Preference-Related Behaviour in Juvenile Zebrafish

The behavioural responses of juvenile zebrafish to mirror-based social and fear-related stimuli were evaluated across different IONP exposure groups. Under baseline conditions, where no mirror was present, control fish exhibited no statistically significant differences in the time spent across the four arms of the maze, including the arm later designated as the “M” arm. Upon introduction of a mirror into the “M” arm, control fish spent significantly more time in the “M” arm compared to arms 1, 2, and 3, indicating a clear preference for the social stimulus. A similar behavioural pattern was observed in the 1 mg/L IONP exposure group, where fish also spent significantly more time in the “M” arm relative to the other arms. In the 5 mg/L IONP exposure group, fish likewise spent significantly more time in the “M” arm compared to the remaining arms. When comparing across exposure groups, the time spent in the “M” arm was significantly higher in both the control and 1 mg/L exposure groups assessed under mirror-present conditions than in the control group tested in the absence of a mirror. In contrast, fish exposed to 5 mg/L IONPs did not display a significant difference in the time spent near the mirror compared to the no-mirror condition. Furthermore, when the same parameter was compared across all three exposure groups under both mirror-present and mirror-absent conditions, significant increases in time spent in the “M” arm were observed for the 0 and 1 mg/L groups when the mirror was present. However, no such difference was detected for the 5 mg/L exposure group (Figure 18).

Representative heatmaps of the plus-maze tracking chamber (Figure 20) illustrate spatial movement patterns of fish across experimental conditions, derived from six individual trials per group. These images demonstrate increased presence in the “M” arm under mirror-present conditions, with this effect being most pronounced in the control and 1 mg/L exposure groups.

Fear-related behaviours were further examined by quantifying the latency to first enter the “M” arm and the frequency of entries into the “M” arm (Figure 19). Across all exposure groups (0, 1, and 5 mg/L), the latency to first enter the “M” arm was significantly longer under mirror-present conditions compared to the no-mirror condition, suggesting an initial hesitation or exploratory delay induced by the mirror stimulus. This pattern remained consistent across all exposure concentrations, with no significant main effects of IONP treatment. In contrast, the frequency of entries into the “M” arm was significantly higher in the mirror condition for both the control and 1 mg/L exposure groups, whereas no significant difference was observed for the 5 mg/L group.

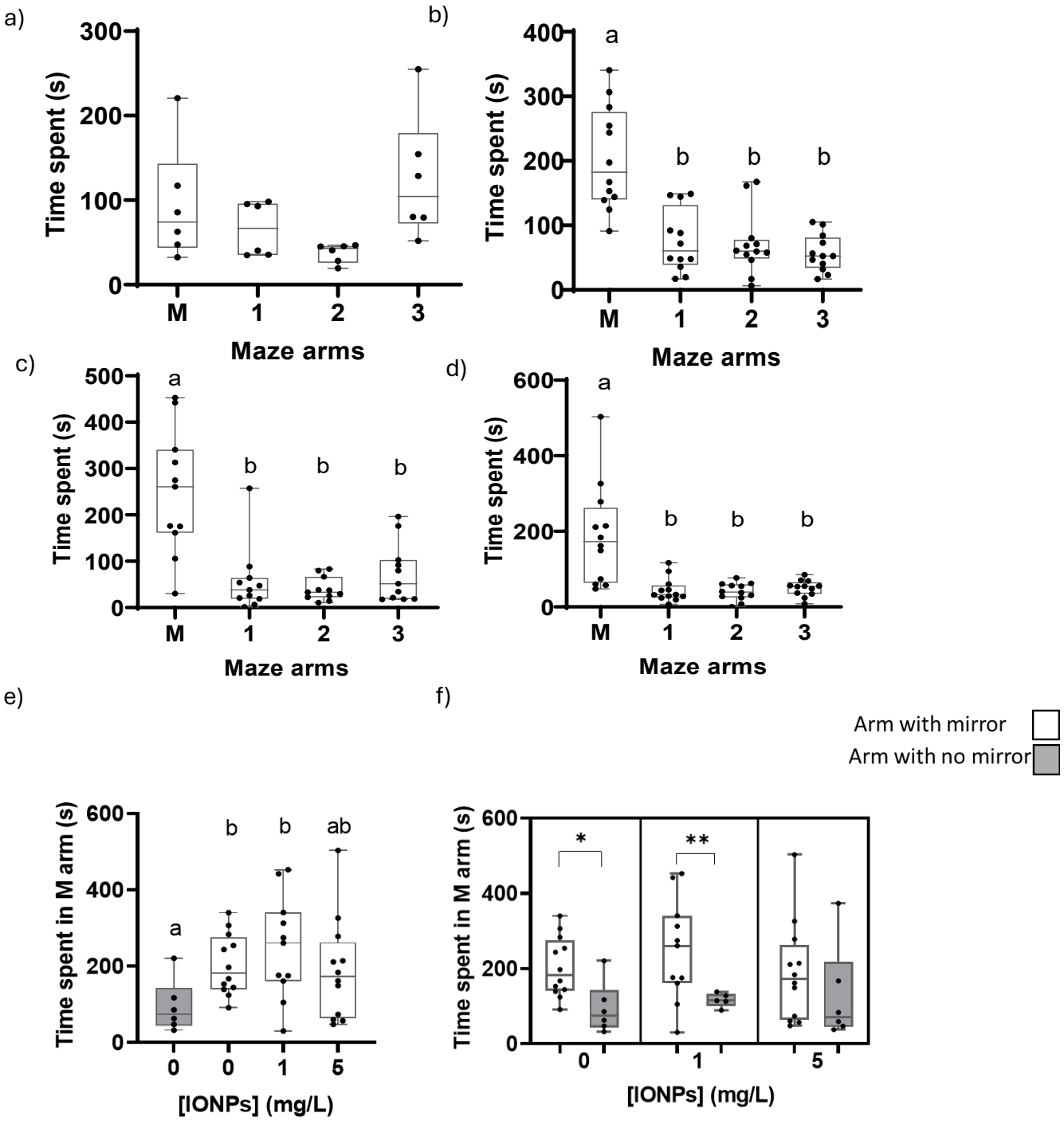

a)
b)
c)
d)
e)
f)
Time spent (s)
Maze arms
M
1
2
3
Time spent in M arm (s)
[IONPs] (mg/L)
0
1
5
Arm with mirror
Arm with no mirror

**Figure 18. Social preference-related behaviour in juvenile zebrafish at 30 dpf**. a) Depicts the duration spent by control group fish in each arm of the plus maze, under baseline conditions, where no mirror was present in any arm. The arm labeled “M” corresponds to the location where a mirror was subsequently introduced. Panels b), c), and d) depict the duration spent in each arm of the plus maze by the fish in control (One-Way ANOVA, M vs other arms, $p \leq 0.001$), 1 mg/L (One-Way ANOVA , M vs other arms, $p \leq 0.001$), and 5 mg/L (One-Way ANOVA , M vs other arms, $p \leq 0.001$) exposure groups, respectively. The arm labeled “M” corresponds to the arm where a mirror was present. e) Compares the duration spent in the “M” arm among exposure groups. The shaded box represents the control fish in mazes with no mirror (Welch and Games-Howell post hoc, Control and 1 mg/L with mirror vs Control without mirror, $p=0.047$ and $p=0.031$, respectively). f) Compares between the duration fish spent in the “M" arm in presence or absence of the mirror, for all exposure groups (Two-Way ANOVA, Independent T-test, in Control and 1 mg/L, mirror vs. no mirror, $p=0.011$ and $p=0.009$, respectively). Statistical differences are shown with groups not sharing the same letter. Number of asterisks denote levels of statistical significance based on p-values, Min-Max showing all data points, N=6-12).

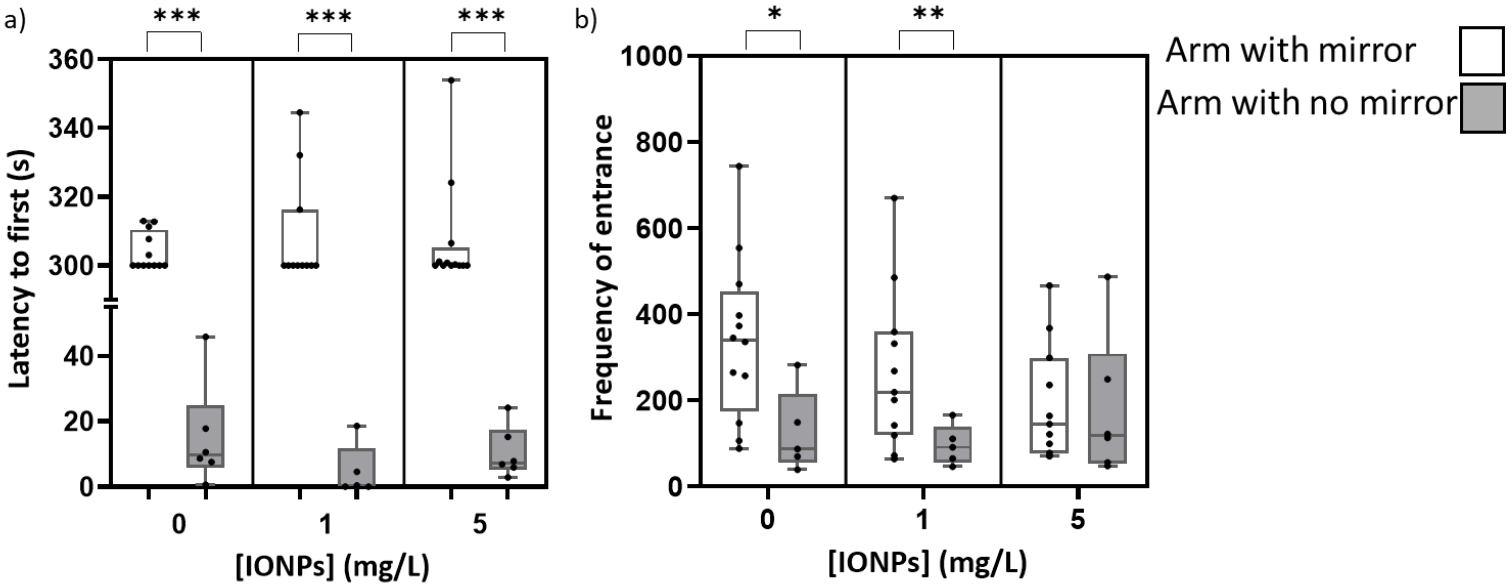



**Figure 19. Fear-related behaviour in juvenile zebrafish at 30 dpf**. a) Depicts the duration taken for the fish to first enter the "M" arm. Comparison is made across different exposure groups, under two conditions of presence and absence of mirror in "M" arm (Two-way ANOVA, Independent-samples T test, for all groups $p \leq 0.001$). b) Depicts the frequency of entrance into the "M" arm. Comparison is made across different exposure groups, under two conditions of presence and absence of mirror in "M" arm (Two-way ANOVA, Independent-samples T test, Control and 1 mg/L, M vs no M, $p=0.008$ and $p=0.014$, respectively). Statistical differences are shown with groups not sharing the same letter. Number of asterisks denote levels of statistical significance based on p-values (Min-Max showing all data points, two-way ANOVA, Independent-samples T test, n=6-12).

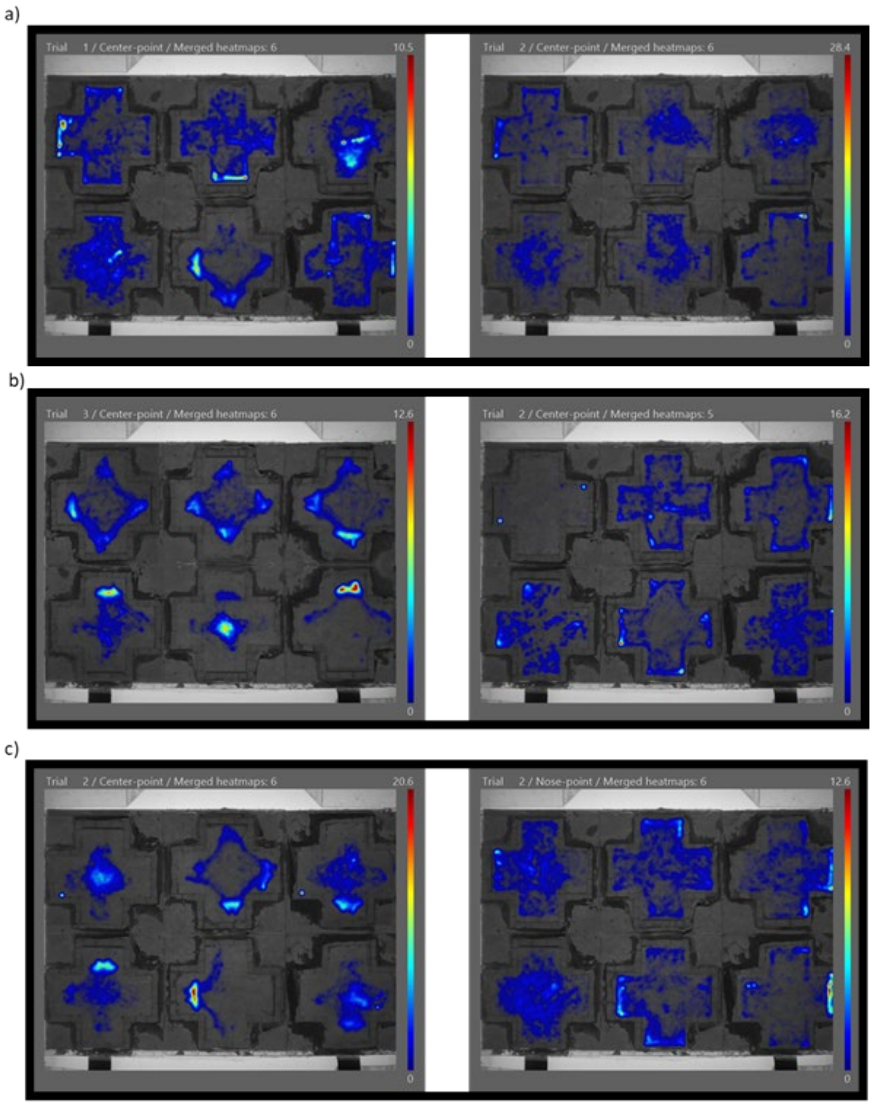



**Figure 20. Heatmaps showing spatial distribution of juvenile zebrafish** movement in a plus-maze across different IONP exposure concentrations and mirror conditions. Panels a–c display merged heatmaps generated using EthoVision XT software, representing the cumulative spatial location data of fish from six individual trials per condition. Each heatmap illustrates the amount of time spent in each region of the plus maze, with warmer colors (blue to red) indicating increasing time. The color bar to the right of each heatmap ranges from 0 (minimum time in seconds), in blue, to the maximum time at a specific location, in red, reflecting the highest localized duration across all subjects in a merged condition. a) Control group in mazes containing a mirror in one of the arms (left) vs. no mirror condition (right). b) 1 mg/L IONP-exposed fish in mirrored maze (left) vs. no-mirror maze (right). c) 5 mg/L IONP-exposed fish in mirrored maze (left) vs. no-mirror maze (right). The mirrored arm "M" in mirrored conditions is the same maze arm across all groups, permitting consistent comparison. The position of the “M” arm can be found in figure 12.

### 3.4.5. ddPCR Analysis of Oxidative Stress Responsive and DA-Related Genes in Juvenile Zebrafish Brain

To assess molecular responses associated with oxidative stress regulation and dopaminergic signaling at the juvenile stage, mRNA expression levels of selected antioxidant and DA-related genes were quantified using ddPCR. Target genes included *sod1, sod2, cat*, and *gstp1.2*, as well as *th1* and *slc18a2*.

Juvenile zebrafish displayed a mRNA expression profile that differed from that observed at the larval stage (Figure 21). A significant reduction in *gstp1.2* mRNA expression was detected in the 5 mg/L IONP exposure group compared to the 1 mg/L and the control group ($p=0.02$ and $p=0.05$, respectively). In contrast, *th1* mRNA expression was significantly elevated in the 5 mg/L exposure group relative to the control ($p=0.049$). No statistically significant differences were observed among exposure groups for *sod1, sod2, cat*, or *slc18a2* mRNA expressions (all $p > 0.05$).

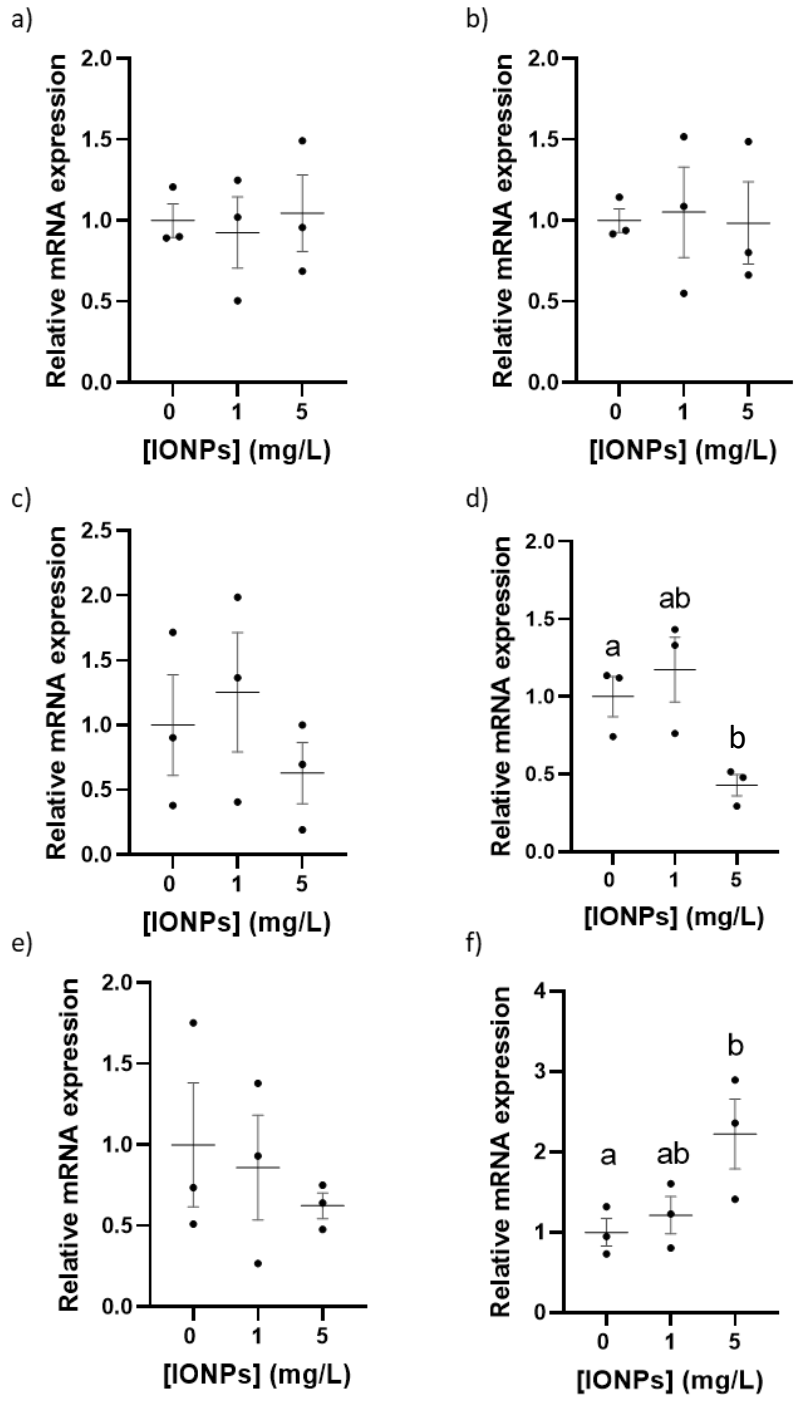



**Figure 21. Relative mRNA expression levels** of (a) *sod1*, (b) *sod2*, (c) *cat*, (d) *gst (*5 mg/L vs. 1 mg/L and Control, p=0.02 and p=0.05, respectively), (e) *slc18a*, and (f) *th1* (5 mg/L vs. Control, p=0.049) genes in the juvenile zebrafish brain tissue under different concentrations of IONP exposure. Statistical differences are shown with groups not sharing the same letter (ANOVA and post hoc of Dunnette t (2-sided), all data are Mean±SEM, n=3).

## 3.5. Discussion

This chapter shows that early-life exposure to IONPs at sublethal concentrations may not produce overt physiological toxicity through juvenile development but is potentially associated with stage- and concentration-specific differences in metal distribution, ionic balance, behaviour, and molecular regulation. While larval zebrafish showed concentration-dependent whole-body Fe accumulation, juvenile brain tissue exhibited a non-linear Fe profile alongside selective Na depletion. Behavioural assessments revealed persistent and emergent alterations in locomotion, anxiety-related responses, and social engagement at 30 dpf, with phenotypic expression differing by exposure concentration and developmental stage. Molecular analyses further indicated developmental shifts in antioxidant and dopaminergic mRNA expression, highlighting that transcriptional responses to early-life IONP exposure evolve over time. Collectively, these findings underscore the importance of longitudinal assessment for identifying subtle, stage-specific neurobiological variation following developmental NP exposure.

### 3.5.1. Developmental Shifts in IONP Accumulation, Physiological Resilience, and Ionic Homeostasis

Consistent with the larval exposure findings described in Chapter 2, no overt physiological abnormalities were detected in zebrafish following IONP exposure during early development. Monitoring of the same cohort through 30 dpf revealed no significant differences in survival, gross morphology, or general somatic development among exposure groups, indicating that the selected IONP concentrations (0–5 mg/L) remained within a sublethal range throughout post-exposure growth. As in the larval study, physiological assessments at the juvenile stage were

conducted primarily to verify experimental consistency and cohort integrity rather than to quantify subtle functional impairments. The continued absence of detectable physiological toxicity through 30 dpf supports the conclusion that early-life IONP exposure did not compromise overall developmental viability under the conditions tested.

Despite the lack of overt physiological effects, tissue-specific metal analyses revealed distinct developmental differences in Fe handling between larval and juvenile stages. Whereas larval exposures were associated with a concentration-dependent increase in whole-body Fe accumulation (Chapter 2), ICP-MS analysis of juvenile brain tissue demonstrated a non-linear dose-response pattern. Juveniles exposed to 5 mg/L IONPs exhibited significantly lower brain Fe concentrations than those exposed to 1 mg/L. This pattern contrasts with larval accumulation profiles and reflects stage-specific differences in Fe distribution between early and later developmental periods [55,155].

The BBB, which is functionally established in zebrafish by approximately 30 dpf, likely represents an important factor influencing brain Fe levels at the juvenile stage. Compared to larvae, juveniles possess more developed regulatory mechanisms governing metal transport and retention within neural tissues. The observed reduction in brain Fe at the higher exposure concentration, despite elevated Fe burdens at earlier stages, is consistent with the presence of developmental processes that may limit cerebral accumulation, including restricted BBB permeability, redistribution of Fe to peripheral tissues, or enhanced clearance mechanisms [156]. Similar time-dependent changes in NP distribution and clearance have been reported in mammalian systems, supporting the concept that NP fate in vivo is dynamic and developmentally regulated [157].

In addition to Fe, analysis of other metals and ions revealed selective ionic alterations in juvenile brain tissue. Among the measured ions, $Na^+$ levels were significantly reduced in the 5 mg/L exposure group relative to controls, while concentrations of Mn, Cu, Zn, Mg, K, and Ca did not differ significantly among treatments. $Na^+$ is essential for neural excitability, action potential propagation, synaptic transmission, and osmotic balance, and its depletion indicates altered ionic homeostasis within the juvenile brain. No causal relationships between changes in $Na^+$ levels and other behavioural and molecular alterations were established here however, comparable ionoregulatory changes have been reported along with other physiological and behavioural disruptions, following exposure to Fe-based NPs [62,158].

Collectively, these findings demonstrate that although early-life IONP exposure was not associated with overt physiological toxicity through juvenile development but was accompanied by tissue-specific and developmentally distinct differences in metal and ion distribution. The non-linear pattern of brain Fe accumulation and the selective reduction in $Na^+$ levels underscore the variability of nanoparticle distribution patterns across developmental stages and support the inclusion of longitudinal analyses when assessing potential neurobiological effects of nanoparticle exposure.

### 3.5.2. Persistent and Emergent Behavioural Phenotypes

At the juvenile stage, spontaneous locomotion assays revealed a non-linear pattern of behavioural responses across IONP exposure concentrations. Juveniles previously exposed to 1 mg/L IONPs exhibited reduced overall locomotor activity accompanied by increased thigmotaxis, whereas individuals from the 5 mg/L group displayed elevated locomotor activity

and reduced thigmotaxis. The co-occurrence of reduced movement and increased thigmotaxis in the 1 mg/L group is consistent with behavioural profiles commonly interpreted as anxiety-associated hypoactivity or freezing-like behaviour in zebrafish, as reported in previous studies [72]. Such behavioural patterns have been described under conditions of heightened stress responsiveness and may reflect altered behavioural state rather than impaired motor capacity. Similar non-linear and concentration-specific behavioural profiles were also observed during the larval stage (Chapter 2), indicating continuity in behavioural sensitivity across development.

In contrast, juveniles from the 5 mg/L exposure group demonstrated increased locomotor output alongside reduced thigmotaxis, suggesting a distinct behavioural phenotype compared to both the control and 1 mg/L groups. Notably, these behavioural outcomes occurred in parallel with measured differences in brain Fe and $Na^+$ levels (Section 3.4), although no direct causal relationship is inferred. $Na^+$ is essential for neural excitability, neurotransmitter cycling, and osmotic regulation, and alterations in $Na^+$ balance have been associated with zebrafish locomotor behaviours previously, even though no evidence of that was introduced in this thesis [159]. Hence, ion homeostasis is potentially a relevant contextual factor in interpreting juvenile behavioural outcomes.

Thigmotaxis analyses further demonstrated that juveniles across all exposure groups spent significantly more time and distance in the outer zone during dark conditions relative to light conditions. This pattern indicates increased stress-related or reduced exploratory behaviour under darkness across treatments. While larval zebrafish are generally reported to exhibit phototactic behaviour and juveniles to display scototaxis, this expected transition was not clearly reflected in the present study [127]. Similar deviations from expected light-dark

preferences were also observed in larvae (Chapter 2). One possible explanation is that the ontogeny of phototactic and scototactic responses varies across zebrafish strains and developmental timelines. In the TLxTL strain used here, phototactic behaviour may emerge later than 6 dpf, while scototactic behaviour may not yet be fully established by 30 dpf.

Taken together, the juvenile behavioural outcomes reflect stage- and concentration-specific alterations that align with, but are not identical to, those observed at the larval stage. The persistence and divergence of behavioural profiles across development suggest that early-life IONP exposure is associated with lasting changes in behavioural regulation, with phenotypic expression differing according to developmental stage and exposure concentration. These findings underscore the importance of assessing neurobehavioural outcomes across multiple life stages to capture both persistent and emergent behavioural patterns following developmental NP exposure.

### 3.5.3. Social Behaviour in Juvenile Zebrafish Following Early-Life IONP Exposure

At 30 dpf, zebrafish display more complex social and emotional behaviours, allowing evaluation of social responsiveness following early-life IONP exposure [74,160]. Social preference was assessed using a mirror-based plus-maze paradigm, which elicited clear differences among exposure groups. Control juveniles previously exposed to 1 mg/L IONPs spent significantly more time in the mirror-containing (M) arm relative to other arms, indicating preserved mirror-directed social engagement. In contrast, juveniles exposed to 5 mg/L IONPs showed a reduced time spent in the M arm, reflecting a diminished mirror-oriented behavioural response.

Fear-related or defensive behaviour was evaluated by measuring latency to first entry into the M arm and the frequency of entries. Across all exposure groups, latency to first entry was significantly longer in mirror-present trials compared to mirror-absent conditions, with no statistically significant differences among treatments. This pattern is consistent with baseline social vigilance and exploratory hesitation commonly observed in juvenile zebrafish [161] and suggests that early-life IONP exposure did not markedly alter initial approach behaviour or defensive responses toward a conspecific-like stimulus.

Differences among exposure groups became more apparent when examining entry frequency into the M arm. Only the control and 1 mg/L exposure groups exhibited an increased frequency of entries under mirror-present conditions, whereas juveniles exposed to 5 mg/L IONPs did not show a comparable increase. This divergence indicates variation in sustained engagement with the social stimulus across treatments. Comparable alterations in social responsiveness following developmental exposure to engineered nanomaterials have been reported in juvenile zebrafish in previous studies [121], supporting the sensitivity of social behaviours as indicators of neurofunctional variation at later life stages. The persistence of altered social interaction patterns weeks after the cessation of exposure parallels behavioural findings observed earlier in development. While social behaviours were not assessed at the larval stage, larval assays revealed altered sensory responsiveness, habituation, and freezing behaviour (Section 2.5.4), indicating that early-life IONP exposure was associated with changes in behavioural regulation across multiple functional domains. Together, these observations suggest continuity in behavioural sensitivity across development, with phenotypic expression differing according to behavioural context and developmental stage.

Social behaviours in zebrafish are regulated by distributed neural circuits involving reward processing, motivation, and social recognition, with dopaminergic signalling playing an important modulatory role [154]. Although the present study did not directly assess social circuitry or neurotransmitter activity, the observed group-level differences in mirror-directed behaviour occur alongside documented molecular and ionic alterations at the juvenile stage, providing relevant biological context for these behavioural patterns without implying direct causality.

Collectively, the juvenile social preference data indicate exposure- and concentration-specific differences in social engagement that persist beyond early development. In the absence of overt physiological toxicity, these findings support the interpretation that early-life IONP exposure is associated with subtle but measurable alterations in higher-order behavioural phenotypes. When considered alongside larval behavioural outcomes, the results emphasize the value of longitudinal behavioural assessment for identifying persistent and emergent neurobehavioural variation following developmental NP exposure.

### 3.5.4. Oxidative Stress and Dopaminergic Modulation in Juveniles

Exposure to Fe-based NPs has been widely associated with alterations in oxidative stress-responsive pathways across aquatic and vertebrate systems [119,132–136]. Accordingly, transcriptional markers of antioxidant defense were examined in juvenile zebrafish to evaluate whether molecular responses observed during early development persisted or diverged at later life stages. SOD1, SOD2, CAT, and GSTP1.2 represent key components of the cellular antioxidant network, acting sequentially to limit the accumulation and downstream effects of ROS. In parallel, the $Fe^{3+}$ core of IONPs has the capacity to participate in redox cycling via the Haber-

Weiss and Fenton reactions, processes that can generate highly reactive •OH capable of damaging lipids, proteins, and nucleic acids [137].

In contrast to the transcriptional profile observed in larvae (Section 2.5.4), juvenile zebrafish did not exhibit significant differences in *sod1, sod2*, or *cat* mRNA expressions across exposure groups. However, *gstp1.2* mRNA expression was significantly reduced in juveniles exposed to 5 mg/L IONPs relative to the control group. GST enzymes play a critical role in conjugating and detoxifying secondary oxidative byproducts, including lipid peroxides and reactive aldehydes generated during ROS-mediated damage [162,163]. Reduced *gstp1.2* mRNA expression in the high-exposure group therefore indicates a distinct molecular response pattern at the juvenile stage compared to larvae, where *gstp1.2* was upregulated at higher concentrations (Section 2.5.4). This divergence underscores a developmental shift in antioxidant gene regulation following early-life IONP exposure.

These stage-specific differences in mRNA expression of antioxidant genes align with the broader concept that oxidative stress responses are dynamically regulated across development and may vary temporally following NP exposure. While larval zebrafish exhibited evidence of early antioxidant pathway engagement, the juvenile transcriptional profile suggests a different regulatory state at 30 dpf, potentially reflecting altered redox demands, changes in tissue Fe handling, or longer-term adjustments in antioxidant system regulation [35,127,142,144].

In addition to oxidative stress markers, dopaminergic gene mRNA expression was evaluated given the established role of DA in regulating locomotion, anxiety-related behaviour, and sensory responsiveness in zebrafish [144]. At the juvenile stage, *th1* mRNA expression was

significantly elevated in the 5 mg/L exposure group compared to controls, whereas *slc18a2* mRNA expression remained unchanged across treatments. TH, the rate-limiting enzyme in DA synthesis, requires Fe as a catalytic cofactor, establishing a biochemical link between dopaminergic function and metal homeostasis [89,164]. The observed increase in *th1* mRNA expression at higher exposure concentrations therefore coincides with the altered brain metal and ionic profiles reported earlier in this chapter and differs from the larval stage, where no transcriptional changes in dopaminergic markers were detected.

Taken together, these molecular findings indicate that juvenile zebrafish exhibit a distinct oxidative stress and dopaminergic mRNA expression profile relative to larvae following identical early-life IONP exposure. The combination of *gstp1.2* downregulation and *th1* upregulation at 30 dpf highlights a developmental shift in transcriptional responses affecting both redox regulation and neuromodulatory pathways. These patterns are consistent with stage- and concentration-specific molecular modulation and support the interpretation that transient developmental exposure to IONPs is associated with persistent, but evolving, neurochemical signatures across life stages. Further longitudinal studies incorporating time-resolved molecular and neurochemical analyses will be necessary to clarify the temporal progression and functional significance of these regulatory changes.

### 3.6. Conclusion

Taken together, the results of this chapter show that early-life exposure to IONPs was associated with exposure- and stage-specific differences in metal distribution, ionic balance, behavioural outcomes, and molecular markers in zebrafish. Although no overt physiological

toxicity was detected through juvenile development, tissue-specific analyses identified non-linear patterns of brain Fe accumulation and selective reductions in Na levels at 30 dpf. These findings are consistent with stage-dependent variation in NP handling and ion regulation and highlight the importance of considering developmental context when evaluating NP fate in vivo.

Behavioural assessments at the juvenile stage identified exposure-associated differences in locomotor activity, thigmotaxis, and social engagement that differed in expression from those observed during larval development. While behavioural sensitivity was evident at both life stages, the specific phenotypic patterns varied according to developmental stage and exposure concentration, indicating that behavioural outcomes may evolve over time following early-life exposure.

At the molecular level, juvenile zebrafish exhibited a transcriptional profile distinct from that observed in larvae, characterized by reduced *gstp1.2* mRNA expression and elevated *th1* mRNA expression at higher exposure concentrations, with no significant changes in other antioxidant or dopaminergic markers. These stage-dependent differences in mRNA expression indicate that oxidative stress- and dopaminergic-related pathways may be differentially regulated across development following identical early-life exposure conditions.

Overall, this chapter documents exposure-associated variation across physiological, behavioural, and molecular endpoints in the absence of overt developmental toxicity. When integrated with larval findings, the results support the value of longitudinal, multi-level assessment for capturing both persistent and emergent biological variation following developmental NP exposure. Further studies incorporating time-resolved molecular,

neurochemical, and imaging approaches will be necessary to better define the temporal dynamics and biological significance of these observations.

Acknowledgments

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# CHAPTER 4

# Non-invasive electrophysiological assessment of larval zebrafish brain post IONP exposure

## 4.1. Chapter Overview

This chapter describes the electrophysiological assessment of 6 dpf larval zebrafish neural activity following early-life exposure to increasing concentrations of IONPs. Using MED-based recordings, spontaneous brain activity was quantified across multiple parameters to evaluate potential concentration-dependent effects on neural function. When integrated with the behavioural and molecular findings presented in chapters 2 and 3 of this thesis, these electrophysiological data contribute to a more complete understanding of how sublethal IONP exposure influences neurodevelopment at the level of neural circuits.

## 4.2. Introduction

### 4.2.1. Rationale for Electrophysiological Analysis

A comprehensive evaluation of neurotoxicity requires approaches that extend beyond behavioural and molecular endpoints to directly assess neural function. While changes in mRNA expression and behaviour provide valuable insight into downstream consequences of neurotoxic exposure, they do not directly capture how neural circuits operate in real time. Because neural activity is closely linked to both molecular signaling and behaviour, electrophysiological measurements are essential for establishing functional context alongside molecular and behavioural findings.

NP exposure presents a particular challenge in this regard. Effects through mechanisms such as oxidative stress, altered ion homeostasis, and neurotransmitter dysregulation, which may not immediately manifest as overt toxicity, can nevertheless alter neural excitability, firing dynamics, and network synchrony, especially during early developmental windows when neural circuits are still forming [165]. Electrophysiological approaches therefore provide a sensitive means of detecting early functional disturbances that may precede, or later contribute to, behavioural and molecular phenotypes.

4.2.2. Larval Zebrafish as a Model for Functional Neurodevelopment

Larval zebrafish are well suited for electrophysiological investigations of developmental neurotoxicity. By the larval stage, the zebrafish nervous system exhibits organized spontaneous neural activity and functional connectivity across major brain regions, while remaining highly plastic and responsive to environmental perturbations [166]. The small size and optical transparency of larvae allow intact, whole-organism recordings that preserve physiological circuit architecture and regional interactions [167]. Importantly, electrophysiological recordings in larval zebrafish can be conducted without invasive procedures that disrupt neural networks. This enables the assessment of brain-wide activity patterns under near-physiological conditions, providing a robust platform for evaluating how early-life exposures influence neural function during critical periods of neurodevelopment [168].

4.2.3. Multi-Electrode Arrays (MEDs) as Functional Endpoints in Neurotoxicity

MEDs enable non-invasive, extracellular recording of neural activity from multiple sites simultaneously, offering high temporal resolution and broad spatial coverage. This technology allows quantification of local field potentials (LFPs), action potentials, and burst dynamics,

capturing both individual neuronal firing and coordinated network-level activity. As such, MEDs provide a powerful functional complement to molecular and behavioural assessments. Previous neurotoxicological studies have used electrophysiological parameters measured by MEDs as sensitive endpoints to chemical and particulate exposures [168], often revealing functional alterations that are not immediately apparent through other endpoints. Changes in spike frequency, bursting behaviour, and network synchrony have been reported following exposures, underscoring the utility of MED-based recordings for detecting subtle disruptions in neural excitability and connectivity. In zebrafish research, MEDs are increasingly applied to investigate developmental neurotoxicity, allowing functional assessment of neural circuits in intact embryos and larvae.

#### 4.2.4. Relevance to IONP Exposure

In earlier chapters of this thesis, exposure to IONPs was shown to elicit molecular responses related to oxidative stress and neurotransmitter regulation, alongside measurable behavioural changes across developmental stages. While these findings suggest that IONPs influence neural systems, they do not directly address whether neural activity itself is altered, nor how such effects are distributed across the developing brain.

Electrophysiological assessment therefore represents a critical next step in evaluating the neurodevelopmental impact of IONPs. By directly measuring spontaneous neural activity, MED-based recordings allow investigation of whether IONP exposure modifies baseline excitability, firing patterns, or network coordination during early development. Such functional changes may help explain the emergence of behavioural phenotypes observed later in life and provide

mechanistic insight into how early molecular alterations translate into impacts on the neural circuit function.

Overall, this chapter will examine the following hypothesis:

At the neural network level, IONP exposure alters patterns of brain activity, including changes in neural firing dynamics and burst events, providing functional link between molecular disruptions and behavioural outcomes.

Objectives of Chapter 4:

- Implement a non-invasive MED-based recording of larval zebrafish.
- Quantify spontaneous LFP, spike, and burst activity following early-life IONP exposure.
- Compare neural activity across exposure concentrations and brain regions.
- Evaluate electrophysiological endpoints alongside behavioural and molecular findings.

## 4.3. Materials and Methods

### 4.3.1. Exposure Protocol and Sampling

Larvae used for electrophysiological recordings were derived from the same exposure cohort described in Chapter 2. Briefly, zebrafish embryos were exposed to IONPs beginning at 4 hpf under a semi-static exposure regime with daily renewal of exposure water. Exposure concentrations included 0, 1, and 5 mg/L IONPs, selected based on sublethal conditions established in earlier experiments. Embryos and larvae were maintained at 28 °C under a 14 h light/10 h dark photoperiod throughout the exposure period. Exposure continued until 6

dpf. At 6 dpf, larvae were randomly selected from each exposure group and immediately prepared for MED electrophysiological recordings as described below.

4.3.2. Preparation and Mounting

Electrophysiological recordings were performed using a MED64 system (Alpha MED Sciences, Osaka, Japan) equipped with MED probes containing a 61-electrode recording array (20 µm electrode diameter, 70 µm inter-electrode spacing, ~10 kΩ impedance). Prior to first use, probes were rendered hydrophilic following the manufacturer's instructions by overnight coating with 0.1% polyethyleneimine (PEI) in 25 mM borate buffer (pH 8.4). Probes were subsequently rinsed multiple times with distilled water and stored submerged in distilled water at 4 °C until use. A 1.5% (w/v) low-melting-point agarose solution was prepared in E3 embryo medium (50 mL total volume) and maintained at 95–100 °C on a hotplate until required. For mounting, approximately 1 mL of molten agarose was transferred to a 15 mm Petri dish and allowed to cool for 1–2 min to approximately 35–40 °C. The cooled agarose was then transferred onto the MED probe surface, followed by the placement of individual larval zebrafish transferred in E3 medium.

Under a stereomicroscope, larvae were positioned dorsal side down using a fine eyelash tool, ensuring that the head and upper spinal cord were aligned flat across the electrode grid (Figure 19). Larvae were held in position until agarose solidified (approximately 10 s). Additional agarose cooled to 35–40 °C was applied to fill the recording well to approximately half its volume, providing mechanical stability while minimizing dilution by E3 medium and preventing thermal damage to the electrodes. An agarose-only well was included in each experimental session as a recording control.

4.3.3. Electrophysiological Recording and Data Acquisition

Three experimental groups were assessed: 0 mg/L, 1 mg/L, and 5 mg/L IONPs, with six larvae recorded per group (n = 6). Following mounting, each larva was allowed to acclimate on the probe for 5 min prior to recording. Spontaneous neural activity was recorded for 5 min per larva at room temperature using MED64 hardware and Mobius software. The integrated probe heater was set to maintain preparations at 28 °C. Wide-band extracellular signals were acquired at a sampling rate of 20 kHz per channel. For spike detection, signals were high-pass filtered at ≥100 Hz. Active channels were defined as electrodes exhibiting spontaneous activity in contact with the head or upper spinal cord regions, confirmed both visually under a microscope and within the recording software before and after each session. For each larva, local field potential (LFP) intensity (<0.01 mV) and spike intensity (>0.01 mV) were quantified across all active channels. Burst activity was characterized by calculating total burst events and burst percentage, with bursts defined as ≥4 spikes per second within 1-s bins exceeding twice the standard deviation of the mean spike rate [168]. Data were averaged across all active channels per larva for group comparisons.

Quality control procedures were applied prior to analysis, and wells exhibiting high baseline noise (>8 µV) were excluded. Following recordings, larvae were removed using a gentle stream of E3 medium to avoid electrode damage and were euthanized in iced tricaine methane sulfonate (MS-222), in accordance with institutional animal care guidelines. Probes were inspected between experiments and reused only if electrode integrity and acceptable noise levels were maintained (Figure 22).

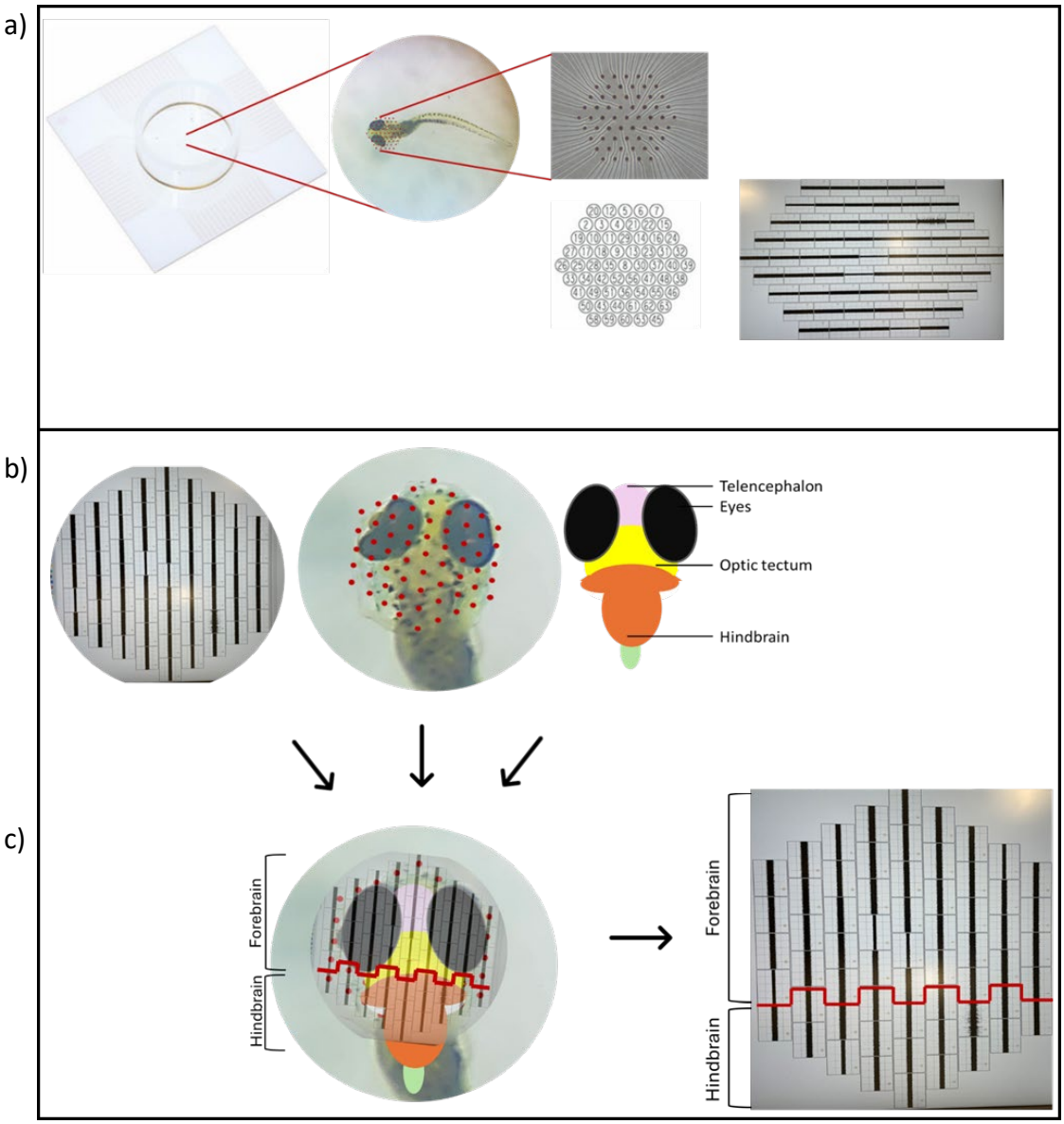


**Figure 22. Schematic overview of MED-based electrophysiological recording setup for intact larval zebrafish**. The figure illustrates the configuration and workflow used for microelectrode array (MED) recordings of neural activity in live. a) The MED probe layout showing the circular electrode array region where the larva is positioned. Magnified view of the electrode grid and the electrode numbering map used for data acquisition are also shown here. b) Example of electrode arrangement and contact points across the larval brain surface is illustrated in addition to a map of brain regions that match with the mounted larva. c) Overlay of recorded regions on the actual larval head demonstrating spatial correspondence between electrode placement and major brain structures and illustration of signal acquisition across multiple electrodes.

## 4.4. Results

All electrophysiological measurements were obtained from larval zebrafish recorded on MED probes. Baseline electrical activity was assessed using agarose-only wells (“empty probe”) to quantify background system noise in the absence of larvae.

### 4.4.1. Baseline and Recording Validation

Recordings obtained from empty probes exhibited low-amplitude baseline electrical signals, confirming minimal background noise under experimental conditions (Figure 23). In contrast, probes containing larvae displayed substantially higher signal amplitudes across all electrophysiological measures, indicating successful detection of larval neural activity. An axis break is included in the LFP figures to accommodate the large separation between empty-probe baseline values and larva-associated recordings.

### 4.4.2. Local Field Potential Intensity

Average LFP intensity increased with increasing IONP exposure concentration (Figure 23). In the total brain analysis, LFP intensity was highest at the 5 mg/L exposure group. Regional analyses revealed similar patterns. In the fore/midbrain, LFP intensity was significantly higher in larvae exposed to 5 mg/L IONPs compared to the 0 mg/L and 1 mg/L conditions. In the hindbrain, LFP intensity also increased across exposure concentrations, with the highest mean values recorded in the 5 mg/L group.

4.4.3. Burst and Spike Activity

Analysis of burst activity revealed an exposure-dependent increase in the average number of burst events recorded from the total brain (Figure 24a). Mean burst events were highest at 5 mg/L, with statistically significant difference compared to both control and 1 mg/L groups.

Spike count analysis showed that the total number of spikes recorded was greatest in larvae exposed to 5 mg/L IONPs (Figure. 24b). This pattern was observed in both fore/midbrain and hindbrain regions, where higher spike counts were measured at the highest exposure concentration. The magnitude of the increase in spike counts in 5 mg/L group was higher in the fore/midbrain region compared to the hindbrain regions.

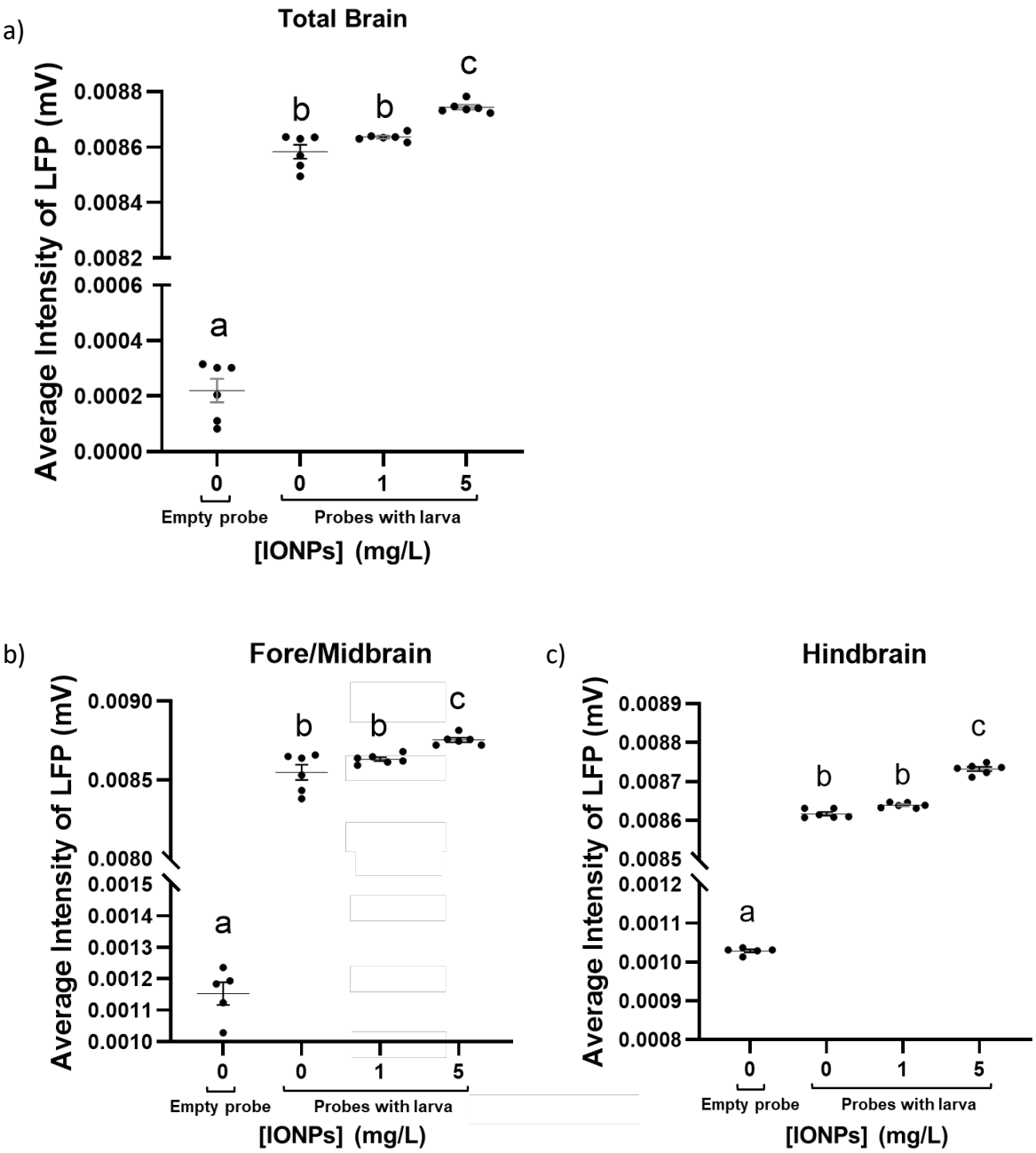


**Figure 23. Average LFP intensities recorded from larval zebrafish brains following exposure to varying concentrations of IONPs**. a) Average intensity of LFPs recorded from the total brain (All groups vs empty probe, p≤0.001, 5 mg/L vs Control and 1 mg/L, p≤0.003). (b) Average LFP intensity recorded from the fore-/midbrain region (All groups vs empty probe, p≤0.001; 5 mg/L vs Control and 1 mg/L, p≤0.028). (c) Average LFP intensity recorded from the hindbrain region (All groups vs empty probe, p≤0.001; 5 mg/L vs Control and 1 mg/L, p≤0.001). The "empty probe" condition indicates baseline electrical activity in the absence of larvae. Statistical differences are shown with groups not sharing the same letter (One-way ANOVA and Tukey's post hoc test, or Welch and Games-Howell post hoc, data represent mean ± SEM, n=6).

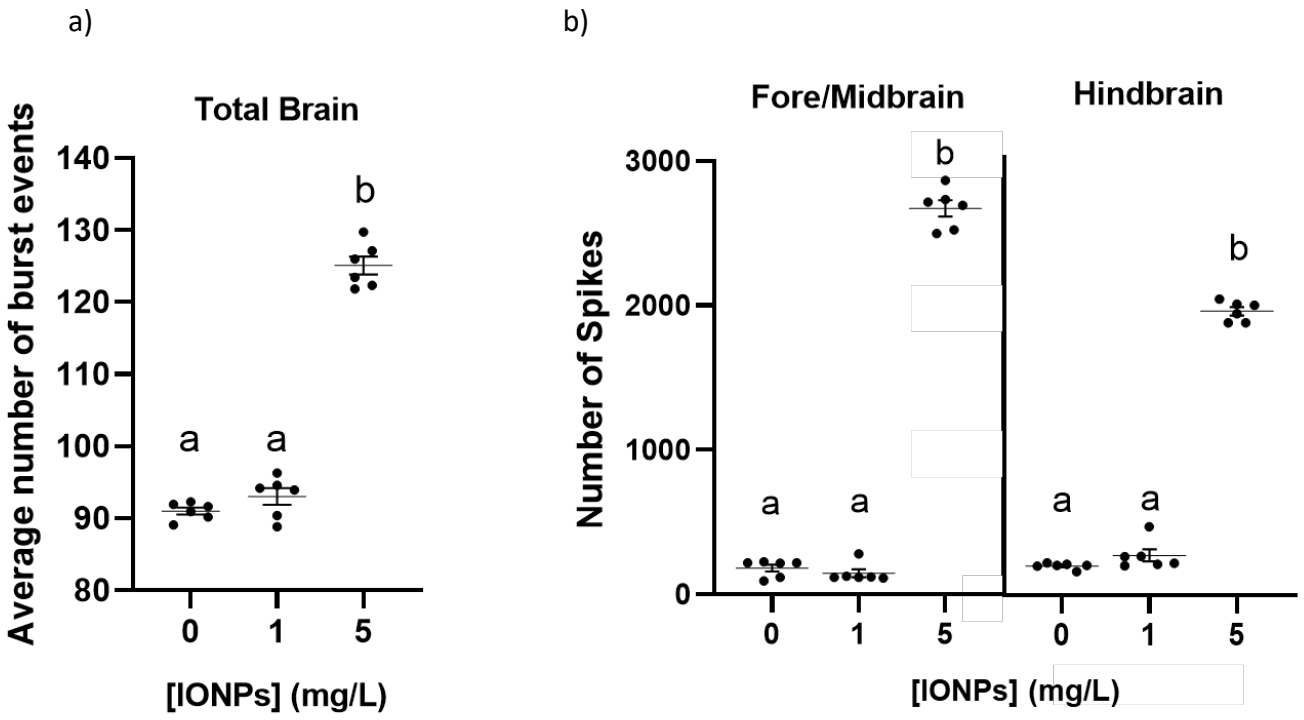


**Figure 24. Spike and burst activity recorded from larval zebrafish brains exposed to IONPs**. a) Average number of burst events recorded from the whole brain (One-way ANOVA, Tukey's post hoc test, higher number of burst events in 5 mg/L vs 0 and 1 mg/L, $p<0.001$). (b) Total number of spikes recorded in the fore-/midbrain and hindbrain regions. Statistical differences are shown with groups not sharing the same letter (One-way ANOVA, Tukey's post hoc test, higher number of spikes in 5 mg/L vs. 0 and 1 mg/L, $p<0.001$ for both; Independent Samples-T Test, Fore-/midbrain vs. hindbrain in 5 mg/L, $p<0.001$, data are presented as mean ± SEM, n=6).

## 4.5. Discussion

### 4.5.1. Overview

This section evaluates the electrophysiological outcomes of early-life IONP exposure in larval zebrafish and integrates these findings with behavioural and molecular results presented in earlier chapters. MED recordings provide a functional assessment of spontaneous neural activity at 6 dpf, enabling examination of concentration- and region-specific differences in network-level activity. These results are discussed in relation to oxidative stress-related mRNA expression and later developmental phenotypes, alongside consideration of methodological limitations. Together, the findings contribute to a multi-level characterization of exposure-associated neurobiological variation across development.

### 4.5.2. Electrophysiological Signatures Associated with IONP Exposure

Electrophysiological recordings obtained using multi-electrode arrays revealed concentration-dependent differences in neural activity across total and region-specific brain areas of larval zebrafish exposed to IONPs. Across all measured parameters, including LFP amplitude, burst event frequency, and spike count, higher values were consistently observed in larvae exposed to the highest IONP concentration. These findings provide functional evidence that early-life IONP exposure is associated with altered neural activity patterns during early neurodevelopment [169].

At 5 mg/L IONP exposure, larvae exhibited higher burst event frequency at the whole-brain level, as well as increased spike counts in both fore-/midbrain and hindbrain regions, while spike intensity remained unchanged. In parallel, LFP amplitudes were elevated across

total and regional brain analyses. The observed increases in spike frequency and burst activity are consistent with differences in measured neural firing patterns and network-level electrophysiological activity across exposure groups [170]. Elevated LFP amplitudes are commonly interpreted as reflecting increased synchronous activity across neural populations, indicating coordinated changes in network dynamics rather than isolated neural events [171].

4.5.3. Integration with Molecular Indicators of Oxidative Stress

The electrophysiological patterns observed at the larval stage align with previously reported molecular responses measured in the same developmental window. Specifically, increased expression of antioxidant-related mRNAs, including *cat* and *gstp1.2*, was detected following IONP exposure, consistent with an adaptive cellular response to elevated oxidative stress. The concurrent presence of heightened neural activity and upregulated antioxidant pathways suggests a potential association between redox homeostasis and neural excitability [172,173].

ROS have been shown to modulate neuronal membrane properties, ion channel function, and synaptic transmission. Within this framework, elevated ROS levels could increase neural sensitivity or responsiveness, manifesting as increased spike frequency and LFP amplitude, while antioxidant activation may limit cellular damage and preserve neural integrity [173]. Direct measures of ROS or ion channel function were not performed and remain to be investigated; hence these observations remain correlative and do not imply direct causation. However, they support the interpretation that electrophysiological alterations occur within a broader physiological context of oxidative stress regulation discussed in Chapter 2.

### 4.5.4. Regional Differences in Neural Activity and Behavioural Relevance

Regional analyses demonstrated that spike frequency differed across exposure conditions in both fore-/midbrain and hindbrain regions, indicating that neural activity metrics varied across anatomically distinct brain areas. The hindbrain region includes well-characterized sensorimotor structures, such as Mauthner cells and associated reticulospinal circuits, which are localized within the hindbrain and participate in rapid escape responses and habituation to repeated stimuli. These neural populations are functionally linked to stimulus detection and motor output coordination [174]. The fore/midbrain, encompassing telencephalic and diencephalic structures, contains neuronal populations involved in behavioural state regulation, sensory integration, and dopaminergic modulation [175,176]. Dopaminergic cell groups within the diencephalon contribute to locomotor regulation, arousal, and behavioural responsiveness. Fore/midbrain circuits are also implicated in anxiety-related behaviours, exploratory activity, and social interaction [177]. The telencephalic and diencephalic regions, play a central role in early forms of decision-making, social recognition, and anxiety-related behaviours in zebrafish [161,178,179]. Elevated fore/midbrain activity at the larval stage may therefore be relevant for understanding behavioural alterations observed later in development.

At the juvenile stage, molecular profiles of exposed fish shifted toward increased *th1* mRNA expression and reduced *gst* mRNA expression, consistent with altered dopaminergic tone and partial attenuation of antioxidant capacity, respectively. When considered alongside the larval electrophysiological data, these findings suggest that early changes in neural activity may precede or coincide with later molecular and behavioural alterations. Hyperactivity,

reduced thigmotaxis, and decreased social interaction observed in juveniles exposed to high IONP concentrations may therefore represent delayed manifestations of earlier neural circuit modulation rather than acute effects.

The observation that spike frequency metrics varied between these regions under the same exposure conditions is consistent with the known functional heterogeneity of the zebrafish brain. Distinct neuronal populations, circuit architectures, and neuromodulatory influences characterize fore-/midbrain and hindbrain regions, and such structural and functional differences provide context for regional variation in electrophysiological measures. The electrophysiological findings therefore describe exposure-associated differences in neural activity that are region-specific rather than uniform across the brain.

At the molecular level, larval zebrafish exposed to the highest IONP concentration exhibited increased mRNA expression levels of antioxidant-related genes (*cat* and *gstp1.2*) and decreased expression of *sod1* mRNA during the same developmental period. These transcriptional differences in exposed fish coincided with the alterations in electrophysiological recordings. The co-occurrence of regional differences in spike frequency and altered redox-related gene expression provides a multi-level description of neural variation across anatomically distinct brain regions without implying direct mechanistic linkage. Within this context, increased LFP amplitudes as well as the increased spike frequencies may reflect elevated basal excitability, with some immediate behavioural disruption, and some shift in neural circuit state that may influence later developmental trajectories [180].

### 4.5.5. Interpretation and Broader Implications

Collectively, the electrophysiological data indicate that IONP exposure is associated with concentration- and region-specific differences in neural activity in developing zebrafish. Elevated spiking and bursting activity may reflect neural responses to oxidative or ionic stress, or alternatively, early functional markers of neurotoxicity. Importantly, these interpretations are not mutually exclusive, and both mechanisms may operate concurrently during sensitive developmental windows. When integrated with molecular and behavioural endpoints assessed throughout this thesis, the electrophysiological findings provide convergent evidence that sublethal IONP exposure is associated with measurable changes in neural network function. These functional alterations may persist across developmental stages and contribute to the behavioural phenotypes observed in juveniles, underscoring the importance of incorporating electrophysiological endpoints into NP neurotoxicity assessments.

### 4.5.6. Methodological Considerations and Sources of Experimental Variability

While MED-based electrophysiology provides a powerful, non-invasive approach for assessing neural activity in larval zebrafish, several methodological factors may influence data interpretation. Signal amplitude and channel activation are sensitive to larval positioning, tissue-electrode contact quality, and agarose thickness, which may introduce variability across preparations. Although active channels were carefully identified both visually and via software, slight differences in electrode contact across larvae could contribute to inter-individual variability in recorded signals.

Additionally, MED recordings primarily capture extracellular population-level activity and do not provide direct information on specific neural subtypes or synaptic mechanisms. As a result, changes in LFPs, spikes, or bursts cannot be attributed to specific neurotransmitter systems or cell populations without complementary approaches. Environmental noise, baseline drift, and electrode impedance changes over repeated probe use may also affect signal quality, despite quality control thresholds being applied.

Future studies could mitigate these limitations by incorporating higher-density electrode arrays, automated positioning systems to improve reproducibility, and complementary techniques such as calcium imaging or pharmacological manipulation to refine mechanistic interpretation [181]. Longitudinal recordings across developmental stages may also help distinguish transient compensatory changes from persistent alterations in neural circuit function.

### 4.6. Conclusion

This chapter applied non-invasive MED recordings to quantify spontaneous neural activity in larval zebrafish following early-life exposure to IONPs. Across total brain and region-specific analyses, electrophysiological endpoints differed by exposure concentration, with the highest IONP group showing higher LFP intensity, increased burst event frequency, and elevated spike counts relative to controls. These patterns correspond to differences in measured network-level electrophysiological activity across exposure conditions.

When considered alongside Chapter 2, these electrophysiological findings provide functional context for larval-stage outcomes observed under the same exposure paradigm. Chapter 2

reported concentration-dependent whole-body Fe accumulation under sublethal conditions, alongside behavioural differences in locomotion, thigmotaxis, and sensory responsiveness as well as transcriptional variation in oxidative stress-related genes. The MED results are consistent with the presence of exposure-associated neurofunctional differences at the larval stage that co-occur with behavioural alterations and oxidative stress-related molecular responses, without implying direct causation among these endpoints.

In relation to Chapter 3, the electrophysiological data add an early functional layer to the developmental trajectory documented at 30 dpf. Chapter 3 showed that early-life IONP exposure was associated with persistent and emergent juvenile phenotypes, including non-linear behavioural profiles and altered social engagement, occurring alongside stage-specific shifts in brain metal distribution, selective $Na^+$ depletion at higher exposure concentration, and transcriptional differences in *gstp1.2* and *th1*. Together, Chapters 2-4 support a framework in which early exposure is accompanied by measurable larval neural activity differences that occur prior to, and provide biological context for, later-life behavioural and molecular variation identified in juveniles.

Overall, within this chapter, MED-based electrophysiology detected exposure-associated differences in larval neural activity under conditions where overt developmental toxicity was not observed. By integrating these functional measures with behavioural and molecular endpoints from Chapters 2 and 3, the thesis provides a more complete description of how sublethal early-life IONP exposure coincides with multi-level neurobiological variation across development.

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# CHAPTER 5

# Conclusions and Future Directions

## 5.1. Overall Conclusions

The primary objective of this thesis was to evaluate the neurodevelopmental impacts of early-life exposure to IONPs in zebrafish using an integrated, multilevel approach. By combining behavioural, molecular, physiological, and electrophysiological endpoints across larval and juvenile life stages, this work sought to move beyond assessments of overt toxicity and instead characterize subtle yet persistent alterations in neural function that may arise following sublethal NP exposure.

Collectively, the findings describe associations between early developmental IONP exposure, at environmentally relevant concentrations, and stage-dependent variation in behavioural, molecular, and electrophysiological measures, in the absence of overt morphological abnormalities or increased mortality. Importantly, although the three projects presented in this thesis employed distinct experimental approaches, they unite on a common conclusion: early IONP exposure is associated with altered neural regulation that evolves across development and manifests differently depending on life stage, neural region, and functional endpoint. Together, these projects form a unified framework for understanding how early-life NP exposure may shape neurodevelopmental trajectories.

### 5.2. Integration of Behavioural, Molecular, and Electrophysiological Findings

Project 1 established that larval zebrafish exposed to IONPs exhibit concentration-dependent alterations in locomotor behaviour, anxiety-like responses, and sensitivity to stimuli, alongside changes in the expression of oxidative stress-related and dopaminergic mRNAs. These effects occurred in the absence of overt developmental toxicity, highlighting the sensitivity of behavioural and molecular endpoints for detecting early neurophysiological perturbations. Project 2 extended these observations by examining behavioural and molecular endpoints at the juvenile stage, where exposure-dependent and non-linear patterns were observed. Juvenile zebrafish previously exposed to IONPs as embryos displayed altered locomotion, anxiety-related behaviour, and social preference, accompanied by shifts in dopaminergic and antioxidant mRNA expression. Notably, the direction and magnitude of these effects differed between exposure concentrations, suggesting that early-life IONP exposure may engage compensatory or adaptive mechanisms that evolve over time rather than producing static outcomes. Project 3 provided functional evidence that early IONP exposure is associated with changes in neural network activity at the larval stage. The electrophysiological findings add a functional layer to the molecular and behavioural observations, allowing these endpoints to be considered together in the context of neural activity.

When considered together, these projects are consistent with association of IONP exposure with coordinated changes across molecular, functional, and behavioural levels of biological organization. The convergence of these independent endpoints strengthens the overall interpretation of the findings and highlights the value of integrative approaches in developmental nanotoxicology.

### 5.3. Developmental Timing and Non-Linear Outcomes

A key insight emerging from this thesis is the importance of developmental timing in shaping the neurobiological consequences of IONP exposure. Larval zebrafish displayed molecular and electrophysiological signatures consistent with heightened neural activity and oxidative stress responses, while behavioural effects at this stage were relatively modest or transient. In contrast, juvenile fish exhibited more pronounced and functionally relevant behavioural alterations, despite the absence of ongoing exposure. These findings may suggest that early-life IONP exposure initiate subtle neural changes that are initially buffered by compensatory mechanisms but later manifest as altered behaviour as neural circuits mature and reorganize [182]. The observed non-linear dose-response relationships further indicate that higher exposure concentrations do not necessarily produce proportionally greater effects, emphasizing the complexity of NP-biological interactions during development [78]. This developmental perspective reinforces the limitation of relying solely on acute toxicity assays and highlights the need for longitudinal assessments that capture delayed and stage-specific outcomes.

### 5.4. Implications for Environmental and Neurotoxicological Risk Assessment

From an environmental toxicology standpoint, the results of this thesis have important implications for the assessment of engineered nanoparticles in aquatic ecosystems. IONPs are increasingly used in biomedical, industrial, and environmental applications, and their release into aquatic environments is likely to continue. The findings presented here consistently show that even brief, early-life exposure to sublethal concentrations can be associated with persistent functional changes that are not captured by traditional toxicity endpoints.

The findings reported in this thesis have broad relevance for how ecosystem health is monitored. Traditional biodiversity and ecosystem health assessments often focus on population abundance, species richness, or the presence of indicator taxa, with the assumption that stable or recovering population numbers reflect a relatively healthy system. However, ecological monitoring frameworks increasingly recognize that functional and behavioural indicators can provide earlier and more sensitive signals of environmental stress than population measures alone, because changes in animal behaviour or physiology can occur at lower levels of stress and before demographic effects are apparent [183]. For example, behavioural responses and other sublethal endpoints have been proposed as effective complementary indicators in ecological monitoring programs, as they may align with declines in biodiversity or habitat quality even when abundance data remain unchanged [184]. This conceptual shift is reflected in multi-tier monitoring approaches that integrate organismal performance, molecular biomarkers, and population indices to provide a more complete picture of ecological condition.

The neurobehavioural and electrophysiological differences associated with sublethal IONP exposure in this thesis illustrate how functional changes in individual organisms can be detectable in the absence of overt population decline. Over longer time scales, such functional alterations may influence fitness-related behaviours (e.g., locomotion, sensory responsiveness, social interaction), which in turn can affect reproductive success, predator–prey interactions, and ultimately demographic trends [185]. By demonstrating that behavioural and neural measures can vary under exposures that do not produce immediate mortality or reduced population growth, this work reinforces the view that ecosystem health assessments benefit from inclusion of sublethal biological endpoints alongside traditional abundance-based metrics [186].

By showing that behavioural, molecular, and electrophysiological alterations can occur independently of overt toxicity, this work supports calls for expanded testing frameworks that incorporate neurodevelopmental and functional endpoints. Such approaches are particularly relevant for contaminants like NPs, whose biological effects may arise through subtle modulation of redox balance, ion regulation, and neural excitability rather than direct cytotoxicity.

### 5.5. Future Directions

While this thesis provides a comprehensive assessment of early-life IONP exposure in zebrafish, it also highlights several areas where further investigation is warranted.

Primarily, a more comprehensive characterization of IONPs is necessary via additional analyses such as zeta potential measurements (to evaluate surface charge and colloidal stability), X-ray diffraction (XRD; to confirm crystallinity), and Fourier-transform infrared spectroscopy (FTIR; to assess surface functional groups) could be incorporated. These parameters would provide further insights into the stability, reactivity, and potential interactions of IONPs in biological and environmental contexts.

Secondly, future studies should aim to directly link electrophysiological alterations to specific neurotransmitter systems or neuronal populations. Combining multi-electrode array recordings with pharmacological manipulation, calcium imaging, or genetically encoded neural reporters would help clarify the cellular mechanisms underlying the observed changes in network activity [181]. Third, extending electrophysiological assessments to later developmental stages would provide valuable insight into whether early neural hyperexcitability persists, resolves, or transforms as circuits mature. Longitudinal recordings from the same individuals, where feasible,

would be particularly informative in addressing this question. Fourth, additional molecular analyses, including transcriptomic or proteomic approaches, could further elucidate the pathways involved in NP-induced neurodevelopmental modulation. Such approaches may help identify early biomarkers predictive of later behavioural outcomes [187]. Fifth, exploring the influence of NP physicochemical properties, such as size, coating, and aggregation state, would improve understanding of how specific material characteristics influence biological responses. This information would be valuable for both environmental risk assessment and the safer design of nanomaterials. Finally, future work should consider ecological relevance by examining how observed behavioural alterations influence survival-related outcomes, such as predator avoidance, foraging efficiency, and social integration, in more complex or semi-natural environments.

In conclusion, this thesis shows that early-life exposure to IONPs was associated with stage-dependent molecular, behavioural, and electrophysiological differences across development. By integrating multiple levels of analysis, this work provides a framework for examining how sublethal NP exposures correspond with variation in neurodevelopmental endpoints. As engineered nanomaterials continue to proliferate, integrative assessments of this kind contribute to more comprehensive evaluations of potential risks to aquatic organisms and ecosystem health.

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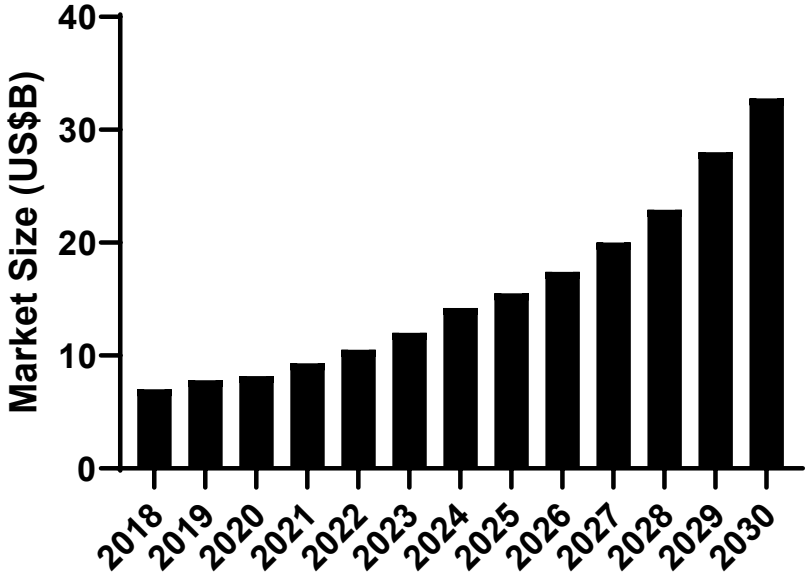
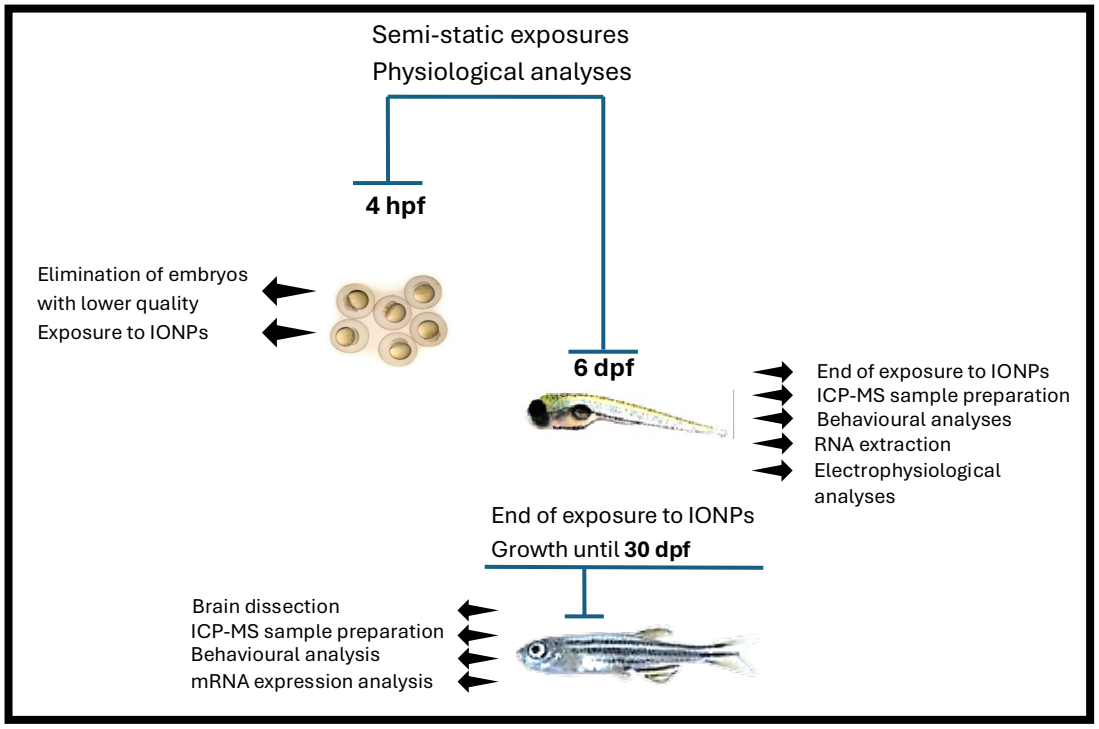
**Supplementary Figures**




**Supplementary Figure 1. Estimated global market size for nanotechnology and nanomaterials from 2018 to 2030** (USD billions), based on data reported by Grand View Research (2023). Data reproduced in graphical form from publicly available industry market analysis (Grand View Research, 2023 [11]).




**Supplementary Figure 2. Summary of experimental design and developmental stages** examined in this study. Schematic overview of the experimental workflow investigating the effects of IONP exposure across zebrafish developmental stages

**Supplementary Figure 3. Outline of the setup of each well on the EthoVision Software** for the thigmotaxis analysis. The red areas show the inner zone and the green areas show the out zone on a 24-well plate.

**Supplementary Formula 1. Calculations of Spontaneous swimming activities and Thigmotaxis**

$$\text{Mean Cumulative Distance (mm)} = \frac{\sum \text{Cumulative Distance Traveled by Each Fish}}{n}$$

$$\text{Mean Velocity (mm/s)} = \frac{\sum \text{Average Velocity per Second of Each Fish}}{n}$$

$$\text{Average \% Time Spent in Outer Zone During Light/Dark Phase} = \sum\left(\left(\frac{\text{Time Spent in Outer Zone (s)}}{\text{Time Spent in Entire Arena (s)}}\right) \text{x } 100\right)/n$$

$$\text{Average \% Distance Travelled in Outer Zone During Light/Dark Phase} = \sum\left(\left(\frac{\text{Distnce Traveled in Outer Zone (mm)}}{\text{Distance Traveled in Entire Arena (mm)}}\right) \text{x } 100\right)/n$$

**Supplementary Formula 2**. The Fenton reaction and Haber-Weiss cycle showing the fate of Fe at different states.

Fenton Reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$$

Haber-Weiss Cycle:

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

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