Mechanistic insight on the impact of nanomaterials and nanocoronas on freshwater and *in vitro* systems

This thesis is presented for the degree of

Doctor of Philosophy

By

Dimitrios Kakavas M.Sc.

Under the supervision of

Ass. Prof. Konstantinos Gkrintzalis,

Ass. Prof. Keith D. Rochfort

School of Biotechnology,

Dublin City University

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List of Abbreviations

NP(s)	Nanoparticle(s)
NM(s)	Nanomaterial(s)
D. magna	Daphnia magna
AgNP(s)	Silver nanoparticle(s)
AuNPs	Gold Nanoparticles
ROS	Reactive oxygen species
OECD	Organisation for Economic Co-operation and Development
ECx	Effective concentration x
MS	Mass Spectrometry
LC-MS	Liquid Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
PFP	Pentafluorophenyl
HRMS	High resolution Mass Spectrometer
BGAL	β-galactosidase
LIP	Lipase
ALP	Alkaline Phosphatase
ACP	Acetic Phosphatase
PEP	Peptidase
GST	Glutathione-S-transferase
RT	Reduced Thiols
LDH	Lactate Dehydrogenase
U	Media unconditioned by daphnids
C7	Media conditioned by age day 7 daphnids
C14	Media conditioned by age day 14 daphnids
ANOVA	Analysis of Variables
PCA	Principal Component Analysis
Gly	Glycine

Pro	Proline
Val	Valine
Asn	Asparagine
Phe	Phenylalanine
Gln	Glutamine
Tyr	Tyrosine
Trp	Tryptophan
Orn	Ornithine
Met	Methionine
Kyn	Kynurenine
Cit	Citrulline
Ala	Alanine
Ser	Serine
Thr	Threonine
Leu	Leucine
Ile	Isoleucine
Glu	Glutamic Acid
Lys	Lysine
Arg	Arginine
His	Histidine
Glc-6P	Glucose 6-phosphate
α-KG	Alpha-Ketoglutarate
Glu	Glutamate
Asp	Aspartate
Frc-6P	Fructose 6-Phosphate
Ribu5P	Ribulose 5-Phosphate
Ribose5P	Ribose 5-phosphate
Sedu7P	Sedoheptulose 7-phosphate
Gro-3P	Glycerol 3-phosphate
3-PG	3-Phosphoglycerate

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1. Animal crowding affects the molecular responses of daphnids following exposure to silver nanoink.

Chapter 1

Introduction

Thesis abstract

Mechanistic insight on the impact of nanomaterials and nanocoronas on freshwater and in vitro systems

Dimitrios Kakavas

Nanomaterials (NMs) are a class of materials with at least one dimension in nanoscale that their application and production has increased over the past decades. Subsequently, the presence of NMs in the environments is steadily increasing, leading to questions over the potential influence they present to ecosystems. In the context of water monitoring and risk assessment, traditional 'spot' or 'grab' sampling approaches are increasingly supplemented by effect-based methods, which, however, may overlook important factors such as biota. A potential solution lies in integrating modern approaches, such as those employed in predictive ecotoxicology and adverse outcome pathway (AOP) frameworks, which utilize model species like *Daphnia magna* to assess the effects of nanomaterials and offer critical insights into their toxicity mechanisms and biological interactions. In this thesis, a holistic approach is employed by utilizing *in vivo* (daphnids) to better evaluate nanomaterial toxicity mechanisms as well as their impact on aquatic life. Silver nanoparticle impact was assessed using a range of phenotypic markers such as mortality, feeding rate, enzyme kinetics and metabolomics, which consistently showed significant impact on exposed individuals. Furthermore, significant findings were discovered in the context of the daphnia model experimental design, as well as metabolite coronas of silver nanoparticles that could improve and optimize toxicity testing for nanomaterials. Specifically, the feasibility of a miniaturized daphnia setup, as well as the importance of vessel geometry, animal crowding and nanocorona presence are discussed in their respective chapters.

1 Nanomaterials: an introduction

The first scientist to ever observe nanoparticles (NPs) was Richard Adolf Zsigmondy, the winner of the 1925 Nobel Prize in Chemistry. Zsigmondy was investigating the optical properties of colloid solutions when he first discovered NPs, and was the first scientist to use the term “nanometer” explicitly to characterize the size of these particles (Bundschuh et al., 2018a). Since Zsigmondy’s landmark work on colloidal systems, the study of nanomaterials (NMs) has developed dramatically, driven by foundational contributions from key figures such as Richard Feynman—whose visionary 1959 lecture 'There’s Plenty of Room at the Bottom' helped ignite interest in nanoscale—as well as pioneers like Sumio Iijima, who discovered carbon nanotubes (CNTs) (Iijima, 1991), and Chad Mirkin, whose work on NP assembly transformed biomedical applications (Mirkin et al., 1996).

Nanotechnology is the research field focusing on the intentional and controlled production or modification of materials in nanoscale. Owing to its vast potential across disciplines—from electronics and energy to medicine and environmental science, NMs have become the subject of a distinct field of study. Nanotechnology not only investigates the unique physicochemical properties that emerge at the nanoscale but also develops strategies for modifying these materials for specific functions in real-world applications (Khan et al., 2022). In general, NMs must have at least one dimension within size between 1 and 100 nm. Nanotechnology is defined by interdisciplinary discoveries, drawing from physics, chemistry, materials science and engineering. Advancements in the field now see the custom engineering of nanoparticles for a variety of applications. The disciplinary convergence has allowed for nanotechnology to tackle novel challenges such as targeted drug delivery, environmental monitoring and remediation and more, standing out as one of the most influential, collaborative and versatile fields today (Haleem et al., 2023, Khan et al., 2022).

The study and production of NMs revolves around their physical (incredibly small size yet large surface area), and also chemical (material-dependent) properties. At such small scales, quantum effects are prevalent in material behaviour, altering the mechanical, optical, electrical and magnetic properties of NMs. Their inherent large surface area makes NMs more efficient as catalysts, which can be a useful adjustable parameter thanks to the ability to modify and tailor NMs to serve many application needs. Their surface can be further modified to create new shapes with new properties (Acharya et al., 2024, Kaymaz et al., 2023). These tunable properties in certain instances enable NMs to facilitate interactions with certain molecules, notwithstanding biological matter and aquatic organisms, making them dangerous for aquatic ecosystems and human health (Canesi et al., 2015, Bundschuh

et al., 2018b, Sahu and Hayes, 2017). One such iteration of NMs are NPs; a particle version of NMs that are characterised by small scale (usually of 1-100 nm) but NPs can refer to larger two-dimensional shapes such as fibres of 100-500 nm.

1.2 Nanomaterial categories

There are a number of approaches to categorise NMs. One of them is categorisation based on dimensionality, or more specifically, how many of their dimensions are within the nano scale (Joudeh and Linke, 2022). Zero-dimensional NMs have all their dimensions in the nanoscale, like for example quantum dots and nanoparticles (NPs) (Wang et al., 2020). One-dimensional NMs have one dimension in nanoscale like nanofilms and nanofibers, two dimensional NMs have two dimensions like nanofilms while three-dimensional NMs (or bulk NMs) describe materials not confined to nanoscale in any dimension (Handy et al., 2008, Joudeh and Linke, 2022).

NPs can consist of two structural types, either single material or layered (Mahdavi et al., 2020, Ediriwickrema et al., 2014). The first layer (outer layer) has the ability to bind a variety of small molecules such as metallic ions, polymers, metabolites and others (Yan et al., 2011). The second layer is the shell layer that has different chemical composition compared to the core, which is the main part of the NP and the final layer is the NP core (Figure 1).

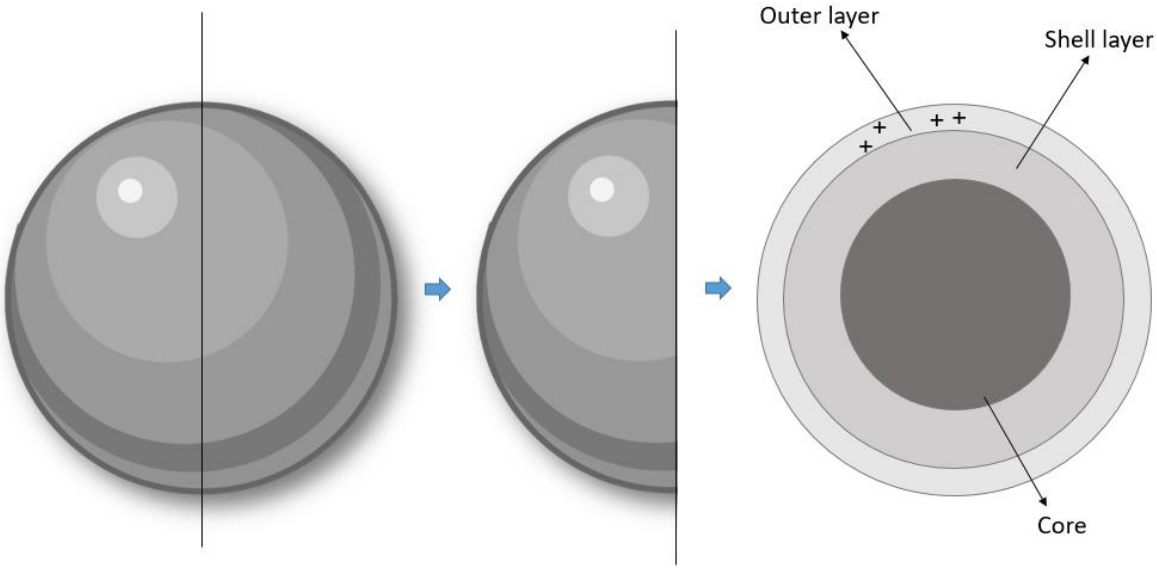

Figure 1. Nanoparticle whole (A) and cross-sectional (B + C) structural layers. The black arrows in (C) indicate the three distinct layers, outer, shell and core in NP structure. '+' denotes the presence of surface charge on the outer layer. Original figure created by the author.

Another approach to categorize NMs is based on composition which dictates their physical and chemical properties (Khan, 2020). A very commonly used category of NPs is carbon-based NPs, with their two main subcategories being ‘fullerenes’ and ‘CNTs’ (Shah et al., 2021). CNTs have elongated structures of 1-2 nm structures in diameter, and they can have single or multi-layered walls (Wang et al., 2024a, Kiang et al., 1995). Fullerenes are a class of carbon NMs in which atoms are arranged in closed hollow cages, typically composed of pentagons and hexagons, however most famous example, C_{60} ‘Buckminsterfullerene’, resembles a soccer ball and is shaped like an icosahedron (Lungerich et al., 2021). Ceramic NPs are inorganic, and non-metallic NPs that can have many different shapes such as dense, porous, polycrystalline or amorphous (Singh et al., 2016). Lipid-based NPs (LBNPs) have parts of, or full lipid molecules in their structures. Usually, their shape is spherical with diameter that ranges from 10 up to 1000 nm. LBNPs can be made to different assortments of their spatial structures as well as composition which enhance their capacity as drug delivery systems (More et al., 2024). Polymeric NPs are typically organic NPs made with suitable properties such as responsive behavior of polymers to specific chemical or physical changes (like pH-sensitive or enzyme degradable) (Bae et al., 2003) and surface charge modifications that enhance membrane interactions (He et al., 2010), to facilitate drug targeting and delivery. Shape-wise, they are comprised of nanocapsules or nanospheres which are spherical shaped polymeric NPs with either a ‘reservoir’ capacity or solid mass respectively. Finally, there are metallic NPs, composed of either pure metals or metal-based compounds (Mody et al., 2010). They possess special magnetic, electrical and optical properties which are influenced by the localized surface plasmon resonance (LSPR). LSPR describes the collective oscillation of conduction electrons on NP surfaces because of incident light (Sui et al., 2019). As an example of their optical properties, depending on the aggregation or size of metallic NPs, gold nanoparticle (AuNP) solutions can present as different colors (Figure 2) (Pluchery et al., 2023).

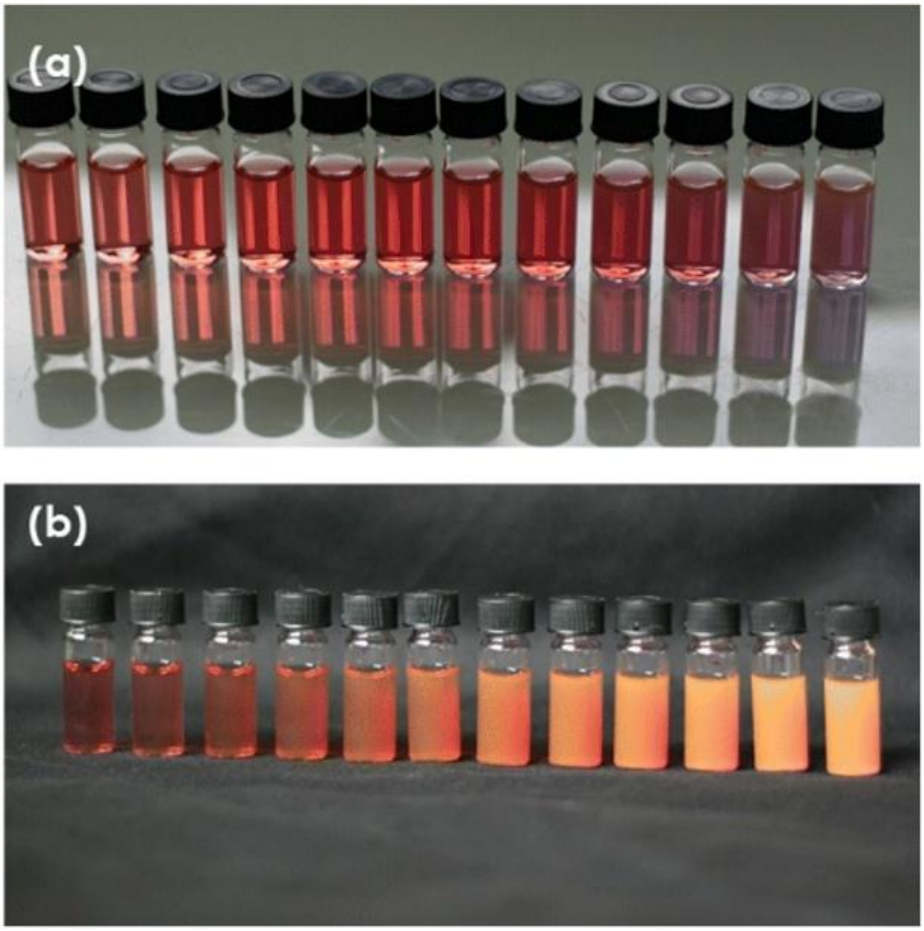

Figure 2. Transmission (A) and reflection (B) or twelve AuNP dispersions. Moving from left to right the size of AuNPs increases from Au^{16} to Au^{108}. Picture from (Pluchery et al., 2023).

1.3 Physicochemical characterisation of nanoparticles

Determining the properties of nanoparticles in liquid media is of utmost importance given how these materials are typically deployed in an application sense. As such, nanoparticle preparations are typically in solutions and commonly characterised as colloids, which are mixtures where one substance (insoluble particles) is microscopically dispersed throughout another (usually a liquid medium). To fully understand and describe the behaviour of NP solutions, a few key physicochemical measurements that will be mentioned below are required.

1.3.1 Z potential

In the context of characteristics that influence the electrical properties of NPs, z potential (or zeta potential) describes the electrostatic potential of the outer surface of NPs (Figure 3) (Clogston and Patri, 2011), and the point of zero charge describes the pH value at which the net surface charge of NPs is zero (usually referred to as pH_{pzc}) (Adamson A., 1997). The increase of the z potential describes the increase of the repellent forces between the NPs which in turn, impacts the formation of agglomerates. If the z potential has a value of 0 the NPs can create agglomerates inside the solution due to the Van der Waals forces (Table 1.) (Kumar A., 2017).

Table 1. Stability of NPs in solution as a function of z potential (Kumar A., 2017).

Z potential	Solution stability
0 to ±5	Rapid particle coagulation
±10 to ±30	Low stability
±30 to ±40	Average stability
±40 to ±60	Good stability
>60	Very good stability

The point of zero charge is an essential characteristic that provides information on the pH value at which the electrostatic repulsion is at its minimum, allowing for easier aggregation of particles, impacting the adsorption process changes based on the surface charge of the adsorbent (Ahmad and Ejaz, 2023).

1.3.2 Polydispersity index

The polydispersity index (PDI) is also routinely used for NP characterization, describing the size distribution of NPs in a solution (Joudeh and Linke, 2022). The PDI can have a numerical value ranging between 0.0 and 1.0, with PDI being equal to 0.0 when the solution is completely homogeneous, and 1.0 when the solution has intense heterogeneity, with respect to particle size in both instances (Figure 3) (Hoseini et al., 2023).

1.3.3 Surface chemistry

Additionally, surface chemistry and modifications also play an important role in the colloidal stability of NPs as well as their performance in biological systems. NP functionalization allows for a plethora of functional groups, targeting ligands, polymers or other molecules to be attached to the NP surface, subsequently altering their properties (Ahmad. F. et al., 2022). Some examples include but are not limited to: carboxyls, amines or thiols which can be used for specific moiety targeting, polyvinyl alcohol for stability purposes during synthesis and PEGylation (which describes the attachment of polyethylene glycol) serving to reduce adsorption of non-specific molecules (Patil et al., 2023).

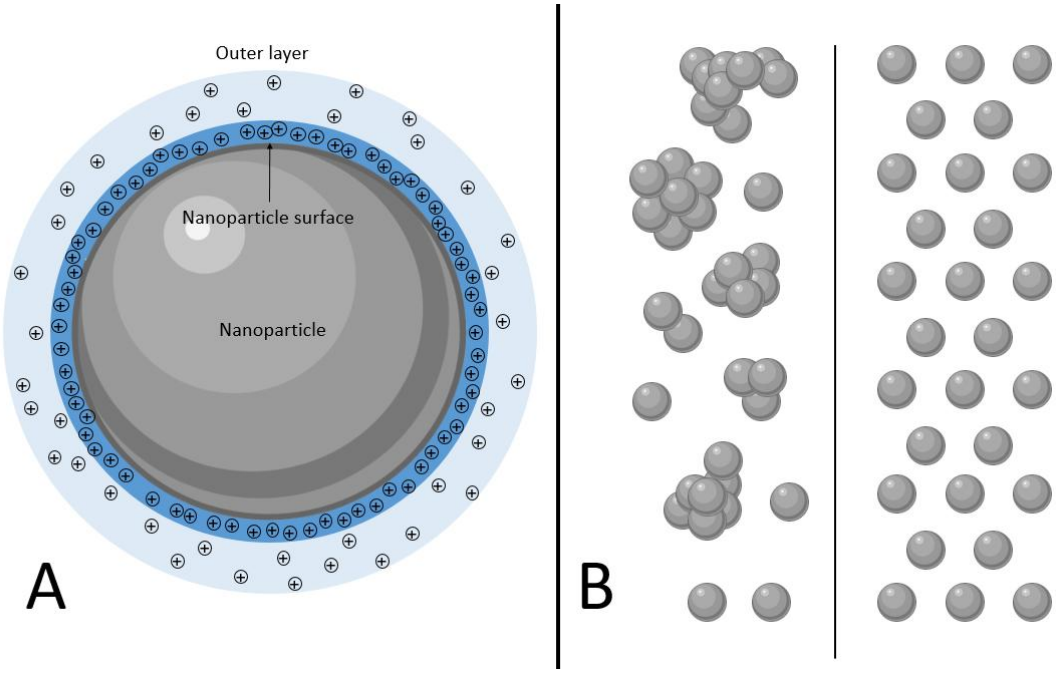

Figure 3. (A) Generation of z potential on NP surfaces. Arrow indicates the area around a NP that has a positive charge due to the z potential. Difference of particles inside a solution based on the PDI (figure 2B). (B) Left: solution with a PDI that has a value close to 1.0. (B) Right: Solution that has a PDI equal to 0.0. Original figure created by the author.

1.3 Applications of nanoparticles

NPs can exhibit tuneable physicochemical properties relative to their bulk counterparts (Table 2). As these properties -such as increased surface area, reactivity and optical or magnetic behaviour- can vary so widely, NPs have a broad range of industrial (Santos et al., 2015) and commercial applications (Altammar, 2023), which are present in several pharmaceuticals, cosmetics, and medical products (Calzolai et al., 2012).

Table 2. Applications of NMs categorized by their special physicochemical properties compared to bulk counterparts.

Category	Properties	Applications
Optic	Surface plasmon resonance and quantum confinement lead to modifiable adsorption and emission spectra.	Anti-reflective surfaces, modification of a surface's refractive index, sensors that are used in medical diagnosis (Park et al., 2021).
Magnetic	Prevention of magnetic agglomeration and can reduce remanence.	Modification of storage density of various media, improvement of Magnetic Resonance Imaging (MRI) (Materón et al., 2021).
Thermal	Enhanced heat conductivity and dissipations thanks to increased surface to volume ratio.	Heat transfer enhancement in solar panels, nanofluids for efficient cooling, increased efficiency of refrigerants and transformers (Eastman et al., 2001).
Mechanical	Increased material strength and durability thanks to Hall-Petch effect.	Improved corrosion resistance, creation of new lighter materials (Eastman et al., 2001).
Electrical	Quantum size can influence electrical conductivity.	Improved efficiency and decrease in size of many parts that we find in electronic devices such as mobile phones, creation of new materials with high conductivity (Cui et al., 2001).
Energy	Enhanced catalytic activity due to large surface to volume ratio.	Creation of new, more durable and of increased capacity energy storage batteries, many applications in hydrogen fuel cells, many applications as electro catalysts in fuel cells, improved efficiency of combustion engines (Wang and Song, 2006).
Biological	Enhanced interactions with biomolecules, increased cellular uptake and functionalisation	Coating of surfaces to give them anti-microbial properties, diagnostic sensors, new drug delivery systems in tissue – level precision (Salata, 2004).

Owing to the increased prevalence and application of NPs in commercial and industrial products, there is a correlative increase in NP presence in the environment. The earliest report of NP contamination originating from cosmetic products was released in 2009 (2009), focusing on titanium dioxide (TiO_2) NPs found in sunscreens. A year-long survey of a freshwater lake in 2014 showed the increase of TiO_2NP concentration due to routine sunscreen application, confirming the role of cosmetics in NP pollution (Gondikas et al., 2014). Between 2005 and 2010, the rapid growth in NP research and commercialization contributed to a 25-fold increase in the number of products containing and/or requiring NPs. Continued growth has led to broader use but also raised concerns about the potential environmental release and impact of such NPs (Kuehr et al., 2020).

1.3.1 Medicine

In the biomedical field, NPs are employed as tools for drug and gene delivery, as well as diagnostics and imaging. Carbon-based NPs and specifically CNTs have been used as carriers for genetic (Zare et al., 2021) and drug material (Mostafavi and Zare, 2022). Specifically, in genetic material transfer, special nanotubes that are specifically designed for this task can attach to a single stranded DNA and insert themselves inside alive cells. Ceramic NPs are applied in bone regeneration, antibacterial coatings, and ocular drug delivery, including conditions such as glaucoma and bacterial infections (Wang et al., 2021, Thomas et al., 2015). Graphene-based nanoparticles have been shown to act as targeted anticancer drug delivery candidate, showing promising results in both in vitro and in vivo systems (McCallion et al., 2016, Zhao and Zhu, 2016). Additionally, carbon nanomaterials have been extensively employed in biomedical imaging techniques such as fluorescence imaging, two-photon fluorescence, Raman imaging, magnetic resonance imaging, computed tomography, photoacoustic imaging), and nuclear medicine imaging including positron emission tomography and single-photon emission computed tomography (Lin et al., 2016). Additionally, NPs can be engineered to act as ‘therapeutic carriers’; delivering treatments directly to specific cells and/or tissues (Cordani, 2020, Mitchell et al., 2021). Polymeric NPs are also being utilized as drug carriers due to their polymers being non - toxic and biodegradable, ensuring the delivery of the drugs to the target cells. LBNPs are one of the most advanced non-viral gene delivery platforms, able to efficiently transport nucleic acid into target cells while they show promising potential in cancer research as targeted drug carriers (Garcia-Pinel et al., 2019). Similar to LBNPs, polymeric NPs composed of biodegradable and non-toxic polymers have shown promise in therapeutic delivery thanks

to their ability for selective targeting and sustained release of drugs (Rajabi and Mousa, 2016).

1.3.2 Environmental

Beyond medicine, NPs have diverse applications in environmental and energy-related technologies. As NPs can be made from many different materials, their applications vary accordingly to their unique properties imparted by their composition. Iron-based NPs are used in filtration systems for pollutant removal from the environment, while graphene-based NPs are explored for use in catalysis, fuel cell technology, and hydrogen storage. In addition to these, titanium dioxide nanoparticles (TiO_2NPs) have applications in photocatalytic degradation of organic pollutants, especially in wastewater treatment, due to their strong oxidative properties under UV light. Silver (Au) and (ZnO) zinc oxide NPs can be integrated into antimicrobial coatings and water purification membranes to inhibit bacterial growth and biofilm formation, the quality of drinking water. Furthermore, cerium oxide NPs (CeO_2NPs) can be utilized as redox catalysts for the remediation of industrial effluents as well as the degradation of persistent organic pollutants due to their oxygen storage capacity and reversible redox behavior. Carbon-based NMs, including CNTs and fullerenes, have shown promise in heavy metal and organic pollutant adsorption thanks to their high surface area and tunable surface chemistry. Magnetic nanoparticles, particularly those based on magnetite (Fe_3O_4), are frequently used in magnetic separation techniques to recover or remove contaminants from aqueous environments, allowing for efficient recycling and minimal environmental disruption. Additionally, silica (SiO2) NPs serve as carriers for enzymes and reactive agents in pollutant breakdown systems, facilitating controlled degradation processes and site-specific delivery of bioremediation agents. In the context of atmospheric applications, NPs can be integrated into air purification systems to capture and neutralize volatile organic compounds (VOCs), fine particulate matter, and other gaseous pollutants such as NO_x and SO_x. Plasmonic nanoparticles, such as gold and silver, are being researched for solar-driven environmental cleanup methods, including photothermal destruction of hazardous materials. Additionally, FeNPs and manganese oxide (MnO) NPs are involved in groundwater remediation by transforming toxic metal ions, such as arsenic and chromium, into less mobile or less toxic forms through redox reactions. Another use of NPs is incorporation into smart coatings and sensors for real-time environmental monitoring where nanosensors can detect trace amounts of environmental toxins, pathogens, or changes in pH and temperature, offering critical data for early intervention as well as generally pollution

assessment and management. Lastly, in agriculture and soil remediation, engineered NPs such as nanoclays and nanozeolites are applied to enhance nutrient delivery, immobilize contaminants, and improve soil health, showing their great potential in sustainable land management and ecosystem restoration efforts (Kianfar, 2019, Uddin et al., 2024).

1.3.3 Electronics

In electronics and industrial sectors, metallic NPs offer a broad spectrum of applications, dependent on their elemental composition and synthesis method (Khan et al., 2019). Aluminium-based NPs serve as additives in fuels and explosives (Wei et al., 2021) and as protective coatings in electronic devices (Samardzija et al., 2022). Silver nanoparticles (AgNPs) are employed for their antimicrobial properties, as well as in batteries and consumer electronics. Nickel and manganese NPs are used in catalysts, conductive materials, and battery components. Zinc oxide and titanium dioxide NPs are found in sunscreens, paints, cosmetics, antimicrobial surfaces, and photocatalytic systems (Singh et al., 2023, Dréno et al., 2019). Gold and silicon-based NPs are used in thermal and electrical insulation, molecular sieves, and drug delivery systems (Khan, 2020). Semiconductor NPs are also being actively researched for applications in light-emitting devices, laser technologies, and biosensors, though many of these uses remain at the pre-commercial or early development stage (Kumar A., 2017). The most widespread application of NPs in electronics lies in conductive inks and films, particularly those incorporating AgNPs. These are extensively used in the fabrication of flexible and printed electronics, including touch screens, photovoltaic cells, and sensors. Their exceptional electrical conductivity, compatibility with inkjet printing technologies, and low-temperature processing make them ideal for integration into various electronic components, especially in emerging flexible and wearable electronics. In addition to AgNPs, quantum dots represent another prominent nanoparticle category used widely in optoelectronics, such as displays, photodetectors, and light-emitting diodes (QLEDs). Their tuneable bandgaps and superior luminescence efficiency contribute to advanced colour purity and brightness in modern display technologies.

1.4 Methods of nanoparticle production

There are many different manufacturing processes by which NPs can be created, however, all processes are categorised as one of two main approaches: ‘top-down’ or ‘bottom-up’

(Figure 3) (Indiarto et al., 2022). A top-down approach typically utilises the mechanical reduction of larger source material into smaller sized particles in order to produce NPs. This method is often employed in the production of ceramic and metallic NPs. While the means for employing a top-down approach are more accessible overall, it is a less precise method, as it produces a comparatively broad range of particle sizes and does not allow for full control of NP shape. Furthermore, it is energy intensive, exposing both the source material and the milling equipment to thermal stress over lengthy processing times. This can result in the abrasion of the milling equipment, which risks contaminating the product (Raab, 2011). Bottom-up approaches to NP production involve the construction of NPs via chemical processing (de Oliveira et al., 2020). As NPs produced in this way are built up from the atomic or molecular level, it allows for the construction of much more complex structures, as well as better selectivity in terms of particle size and shape (Raab, 2011). This can include approaches such as aerosol processes which is used for producing ultrafine particles (Wang et al., 2005), precipitation reactions (commonly used in pharmaceutical NPs) and sol-gel processes which are used for metal oxide NPs. Microemulsion methods are used for LBNPs where NPs are formed in small droplets of immiscible fluids (Waheed et al., 2024), while for carbon tubes and quantum dots chemical vapor deposition (CVD) is employed. CVD describes the process through which, a gaseous precursor reacts or decomposes on a heated substrate to form NPs (Chen and Zhang, 2014). Additionally, NP biological (green) synthesis is also used, utilizing living organisms or extracts from them to reduce metal ions to NPs (Gour and Jain, 2019). This approach is generally described by low toxicity and biocompatibility making it ideal for biomedical applications. Post synthesis, NPs usually undergoing further processing steps like purifications, drying or surface functionalization based on the desired characteristics.

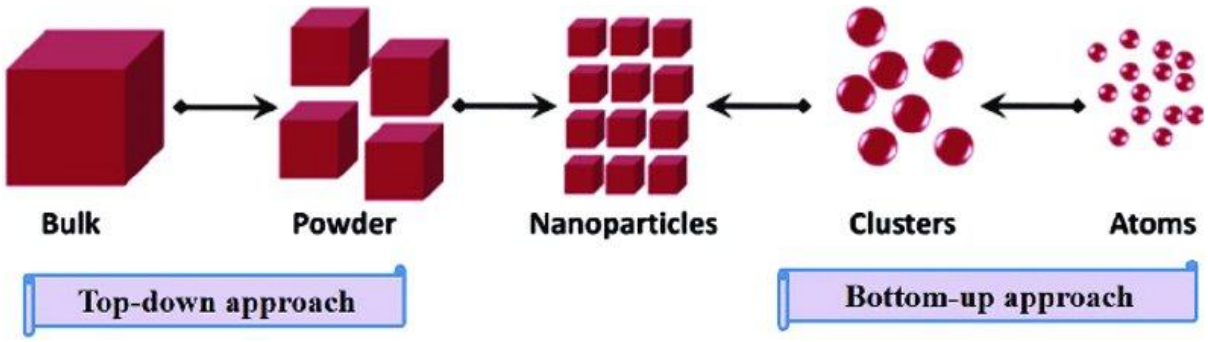

Figure 4. Overview of basic top-down and bottom-up nanoparticle production processes (Khan et al., 2019).

1.5 Nanoparticles as environmental pollutants

The toxic effects of NPs and NMs on living organisms is widely recognised as evidenced in the growing body of literature on the topic. NPs and NMs have been shown to exert direct effects on living organisms through either/both direct exposure and/or acting as carriers for other pollutants (Efthimiou et al., 2021, Handy et al., 2008, Quigg et al., 2013). Given the annual increase in NP applications, their release rate into ecosystems is correlatively increasing. The risk NPs pose to the environment is intrinsically linked with their tunable properties, resulting in biomagnification phenomena and the devaluation of their impact on an ecosystem (Hu et al., 2012, Zhang et al., 2008). Data on pollutant properties, toxicity and bioavailability, as well as how these parameters are impacted by the environment is of paramount importance.

There are several properties of NPs that tie in with their exerted toxicity and bioavailability. To begin with, surface properties that NPs have because of functionalization are critical in this context. As mentioned, these surface structures can affect their ability to interact with living organisms or other biomolecules (Navarro et al., 2008). For example, CNTs can absorb a wide range of organic compounds when dissolved in water, which typically bind on their outer cylinder surface (Pan and Xing, 2008, Chen et al., 2008). In contrast, fullerenes bind molecules on their inside, encapsulating small atoms or ions – physically locking them in (Yang et al., 2006).

There are two distinct sources of NP release to the environment, natural NPs which are generated in the environment (NNPs) and anthropogenic NPs which are engineered (ANPs) (Lespes et al., 2020). Many varieties of NNPs are present naturally in the environment, created by a wide variety of different processes like chemical weathering of minerals, mineralization, physical fragmentation over a period of time, nucleation in the atmosphere and more (Sharma et al., 2015) (Figure 5). Large quantities can be introduced in the environment due to natural geological processes, like for example the erosion of rocks and minerals, volcanic eruptions which release nanoscale particles in the ash generated during eruptions. Biological systems can also produce NNPs, also known as green nanoparticles, via the metabolic pathways of microorganisms such as bacteria, yeast or fungi (Velusamy et al., 2016).

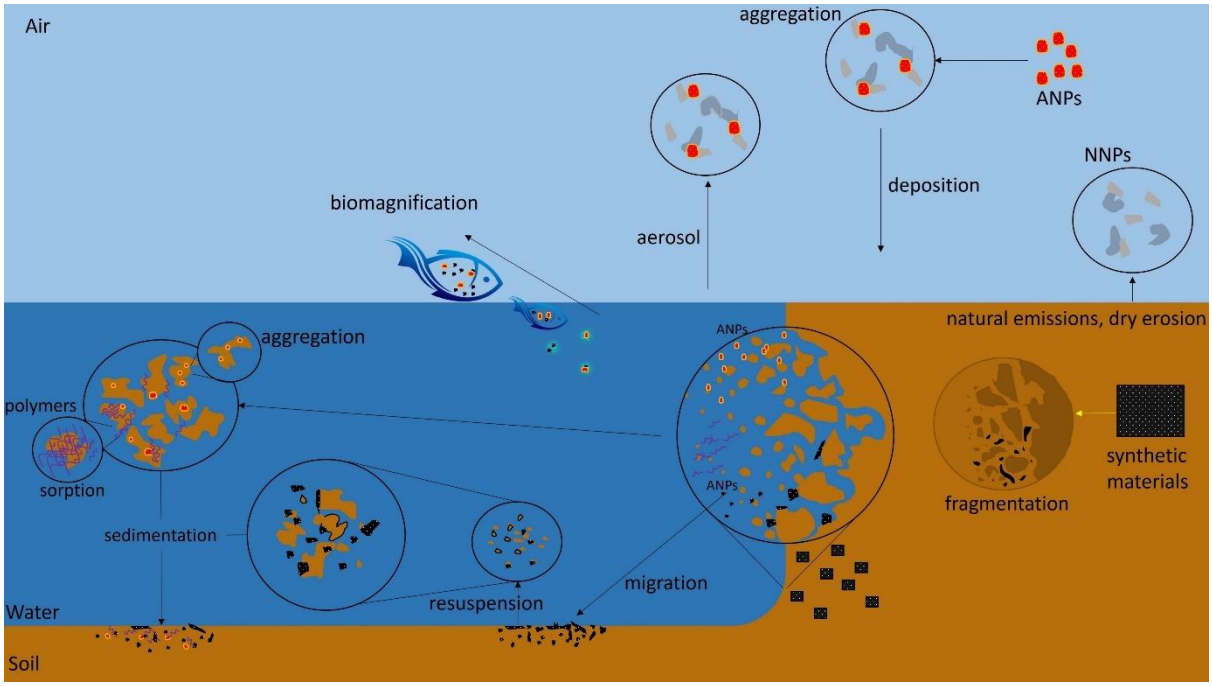

Figure 5. Environmental pathways, transformation processes and deposition destinations of NPs. Some of the processes that NPs undergo when they enter the environment can lead to a new direction as indicated by the arrows. Circles highlight specific environmental processes and interactions of NPs. In aquatic systems, NPs may also be taken up by organisms leading to biomagnification phenomena. Abbreviations: ANPs: anthropogenic nanoparticles, NNPs: natural nanoparticles. Original figure created by the author.

The main source of ANPs is the accidental release of them into the environment or as a result of an industrial process, transportation or construction for urban development. Examples of such activities are ore processing; where mechanical grinding can create metallic and oxide NPs (Nowack and Bucheli, 2007), and the biodegradation of plastics which leads to formation of nanoplastics (Lespes et al., 2020, Pilapitiya and Ratnayake, 2024). Significant contribution of ANP release comes from combustion-related processes, where incomplete fuel combustion can generate carbon-based NPs along with metal and metal oxide NPs from fuel additives (Kumar et al., 2012). Secondary NP formation can occur through chemical processes where gaseous pollutants undergo oxidation and nucleation reactions, resulting in nanoscale aerosol particles (Buzea et al., 2007, Farre et al., 2009, Kulmala and Kerminen, 2008). The fate of NPs released in the environment differs depending on what processes they have been through, such as aggregation or functionalization or chemical transformation. Chemical transformations of the NPs include but are not limited to: alteration of their surface properties by precipitation, adsorption or desorption, or alteration in chemical speciation and degradation (Bundschuh et al., 2018a). These transformations change the properties and behavior of NPs in biological systems or

the environment, highlighting the need to properly assess the impact of NP exposure to living organisms. While much of the current understanding comes from in vitro and bioindicator studies, it is equally critical to investigate how NPs behave in vivo following exposure, including their absorption, distribution, and toxicological effects.

1.6 Nanopollution risk assessment for the aquatic environment

Traditional approaches to monitor pollution focus on the quantification of pollutants in the aquatic environment and their comparison with water quality standards. These approaches are based on the detection of pollutants and their interactions with the fauna and flora of an ecosystem, however, there are many disadvantages in the traditional approaches for pollution assessment (Escher et al., 2020). Analytical quantifications have high cost and can only detect a fraction of the plethora of pollutants that can enter an aquatic ecosystem. Furthermore, there are commonly cases in which pollutants can be in concentrations bellow the detection limit or the currently used methods might not have a broad spectrum of detectable pollutants (Yu et al., 2023, Enfrin et al., 2021). These shortcomings also highlight the inability to predict future impact before the ecological damage to the environment has occurred.

1.6.1 Systems biology

When dealing with complex effects such as those caused by nanopollution in an aquatic ecosystem, a simple reductionist approach can often prove insufficient towards identifying the root cause of the problem, as it can become difficult to connect molecular interactions with biological change on a higher level. Thus, a holistic and integrative approach, viewing organisms as a series of connected and integrated systems from the molecular level to the whole organism is required to properly assess nanopollution in aquatic systems. This approach is in the framework of systems biology (Kitano, 2002). Coupled with environmental omics, a group of technologies which include (epi)genomics, transcriptomics, proteomics and metabolomics, systems biology allows for a comprehensive assessment of biological response to pollutant exposure. These approaches facilitate the understanding of mechanisms of toxicity as well as mapping the molecular pathways affected (Fröhlich, 2017). As systems biology links metabolomics with other omic data, molecular information

in toxicological key events is used to develop new adverse outcome pathways (AOPs) (Ankley and Edwards, 2018).

1.6.2 Systems toxicology

Systems toxicology uses the approach and tools of systems biology to study molecules (which can be pollutants) and their toxic effects on living organisms (Sturla et al., 2014). This holistic approach allows for new techniques, markers of oxidative stress and molecular markers of toxicity to be introduced in the pool of available tools for environmental monitoring and (Sauer et al., 2015). In essence, systems toxicology aligns the perspective of toxicology towards a mechanistic, network-based understanding of adverse effects and molecules of interest (e.g. pollutants).

1.6.3 Adverse outcome pathways

Adverse outcome pathways (AOPs) are used to characterize the environmental impact of a pollutant on living organisms and represent an arrangement of biological events leading to adverse effects, relating the severity of exposure to pollutant risk assessment. The advent of powerful omics technologies greatly speeds up the establishment of new AOPs, which allow for comprehensive analyses and detection of subtle changes in the genome, proteome, transcriptome, or metabolome of an organism. Each domain offers unique information in the context of pollutant assessment: genomics investigates inherent susceptibility by analyzing the genetic function and structure (McGuire et al., 2020), transcriptomics allows to capture the expression of genes (Monzó et al., 2025) and proteomics provide functional insight by measuring protein levels or modifications to reflect the cellular response (Cui et al., 2022).

1.7 Nanoparticle exposure and toxicological effects

Owing to the large number of sources that contribute to the presence of nanoparticles in different environments, inhabitants of the respective ecosystems experience varying degrees of contact with said nanoparticles relative to their physiology. For example, the human body has many different mechanisms by which it isolates the vital organs from any external

intrusions. These mechanisms are essentially the first line of defense, relying on physical barriers, such as skin and mucus, as well as immunological defenses which can detect and neutralize foreign particles. However, NPs are characterized by their nanoscale size which allows them to bypass these protective mechanisms and potentially infiltrate the human body. The entry pathways of NPs into the human body and their subsequent distribution to internal organs follows a structured physiological progression. This progression can be divided into three stages: exposure pathway organs, internal body pathways, and final destination within vital organs (Figure 6). Each stage can impact the biological fate, bioavailability, and potential toxicological impact of NPs due to subsequent transformations that the NPs go through, making their study essential in nanotoxicology and nanomedicine.

1.7.1 Primary organ targets upon initial nanoparticle exposure

When discussing routes of transmission, studies have shown NPs can enter the human body through the typical routes of absorption; namely the skin, nasal cavity, respiratory tract and the gastrointestinal tract (GIT), with each route involving distinct penetration mechanisms and pathways. The skin, while an effective barrier under most conditions, can permit NP penetration particularly when the barrier function is compromised (e.g., through abrasions or diseases). NPs can infiltrate through hair follicles or intercellular lipid layers and enter systemic circulation (Larese Filon et al., 2009). The nasal cavity is a crucial portal of entry for airborne nanoparticles, especially in occupational and urban environments. NPs can deposit on nasal, alveolar and tracheobronchial regions due to diffusion, and they are able to directly infiltrate the brain via the olfactory nerve, bypassing the blood-brain barrier (Oberdörster et al., 2004). This direct access raises significant neurotoxicological concerns, as many of these metals can cause neurotoxicity (Li et al., 2021a). The respiratory tract is another extensively studied exposure route due to the high surface area and vascularization of the alveoli, which thanks to the small size of NPs allow them to penetrate deep into the lungs and potentially cross into the bloodstream or lymphatic system (Geiser and Kreyling, 2010). An additional exposure route is the GIT, particularly through the ingestion of food or water contaminated with NPs. Once NPs infiltrate the GIT they can be absorbed through intestinal epithelial cells via transcellular or paracellular pathways, or even M cells, facilitating their transfer to systemic circulation (Ensign et al., 2012, Steffansen et al., 2004, Maisel et al., 2015).

1.7.2 Secondary organ targets following exposure

Following their bypassing of the initial line of defence, NPs utilise the pathways of the body in order to infiltrate other organs and systems; namely the blood and lymphatic systems. Both systems can act as distribution systems for NPs to be deposited throughout the body. NPs that have gained access through the mucosal barriers, can circulate freely and/or associate with plasma proteins, forming a "protein corona" that influences their biodistribution, cellular uptake, and immune response (Cedervall et al., 2007). Once in circulation, NPs can interact with various tissues, penetrate cellular membranes via endocytosis, and accumulate in organs depending on their size, shape, surface charge, and composition (Wang et al., 2013). Alternatively, the lymphatic system serves as significant secondary route, where the lymphatic uptake of NPs occurs predominantly from interstitial spaces following transdermal or mucosal exposure and leads to accumulation in lymph nodes, which may trigger immune responses (Sestito and Thomas, 2021, Tang et al., 2024). This pathway is particularly relevant in nanomedicine and particularly in vaccine delivery and cancer therapy where targeted lymphatic transport is desired.

1.7.3 Terminal NP deposition

Finally, while many NPs are captured by physiological defenses, the fraction that evades these barriers eventually leads to the deposition of NPs across the body organs/tissues across the body organs/tissues. These penultimate deposition sites be separated to two broad destinations; the brain and all organs, each with unique physiological implications. The brain is particularly vulnerable to NP accumulation, since NPs can translocate directly from the nasal cavity via the olfactory nerve or cross the blood-brain barrier through vesicular trafficking across blood vessels (Wohlfart et al., 2012, Mistry et al., 2015). This permeability profile has significant implications for both therapeutic delivery and potential neurotoxicity, as studies have shown that NP accumulation in the brain can induce adverse effects such as oxidative stress, inflammation and more (Kreuter, 2012). As for other organs, NPs can be filtered out by phagocytes in liver and spleen, but can still evade and distribute broadly depending on their characteristics. Similarly, kidneys, lungs, and even reproductive organs can accumulate NPs, raising concerns about systemic toxicity, organ-specific effects, and long-term accumulation (Schleh et al., 2012).

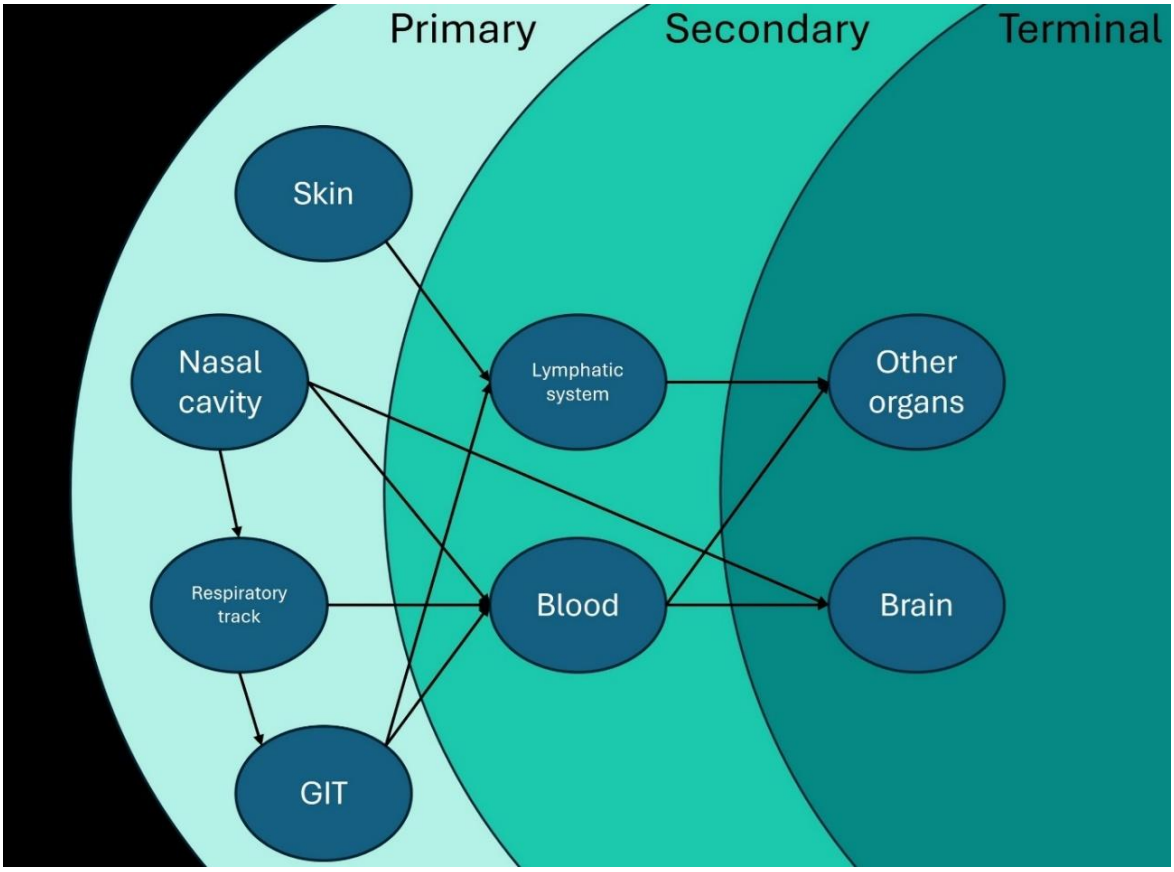

Figure 6. Schematic representation of NP distribution pathways into the human body. Anatomical regions are categorized into three stages: initial contact sites (primary), intermediate transport systems (secondary) and final accumulation sites (terminal). Arrows indicate potential routes of NPs between sites. Abbreviations: GIT: gastrointestinal tract. Original figure created by the author.

1.8 Toxicity mediated effects of nanoparticles

In the context of nanotoxicity, some important parameters that will determine the biological effect of exposure to NPs (as mentioned previously) are dimensionality, functionalization, form and composition. These are also properties that make NMs in general appealing for industrial, biomedical, and other uses and applications. Notably, many of the factors that impact the toxicity of NPs are intertwined in their effects and influence each other (Sun-young Park, 2010). Overall, the toxicological impact of NP exposure is shaped by a dynamic interaction system among certain properties, experimental parameters and interactions (Figure 7). For example, non-metallic NPs have shown toxicity effects to living organisms (Zhu et al., 2009), however, the metallic and metallic oxide NPs are generally more toxic (Sengul and Asmatulu, 2020). This can be explained from the fact that besides the mechanistic damage NPs can inflict on critical organelles or RNA/DNA strands, they have great capacity to interact with biomolecules and can under certain condition solute metal ions which induce toxic effect (Lowry et al., 2012). Toxicity of NPs in living

organisms can be attributed to a few categories of different toxicity mechanisms which include oxidative stress, genotoxicity, coordination effects, homeostasis disturbance effects, and coronas.

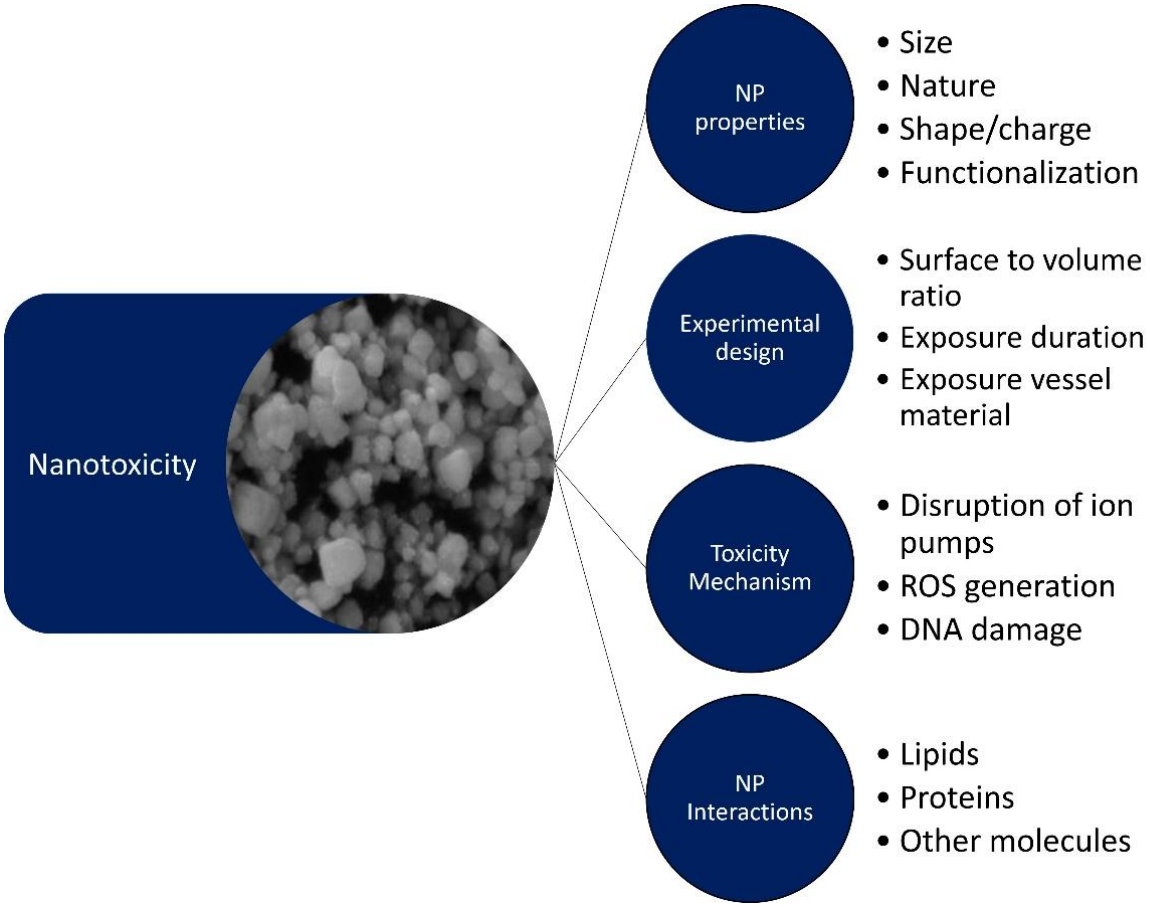

Figure 7. The multifactorial nature of NP toxicity. Interconnected elements show the complex nature of nanotoxicity and some of the factors that can modulate it. Original figure created by the author.

1.8.1 Oxidative stress

The presence of NPs can lead to ROS thanks to their high surface reactivity combined with a large surface-to-volume ratio catalyzes electron transfer reactions that generate ROS and can serve as a nanotoxicity mechanism (Manke et al., 2013, Nel et al., 2006). Oxidative stress describes the condition that the production of reactive oxygen or nitrogen species (ROS/RNS) is greater than the antioxidant defense mechanisms of living cells (Pizzino et al., 2017). ROS include high energy species with uncoupled electrons which make them very reactive. Many metallic NPs can also induce toxic effects via the generation of excess ROS within a cell. The primary ROS is superoxide radical that generates hydrogen peroxide and

the very reactive hydroxyl radical, which can cause damage to key biochemical molecules such as lipids, proteins, and nucleic acids, thereby resulting in cell injury such as mitochondrial membrane depolarization and impairment of the electron transport chain (Dudev and Lim, 2008). When NPs interact with oxidative organelles (e.g., mitochondria) or are exposed to acidic conditions (e.g., within lysosomes after endocytosis), they can induce ROS generation through reactions with biomolecules that form superoxide radicals (O_2^-), which, if accumulated, lead to oxidative stress (Chang et al., 2012, Guo et al., 2015).

1.8.2 Genotoxicity

As is the case with all NP-associated mechanisms of toxicity, NO-induced genotoxicity can vary based on the respective characteristics/properties of the NPs in question. However, there is limited evidence on the exact mechanisms invoked by NPs which cause said genotoxicity with further research required (Shukla et al., 2021). This lack of evidence is because genotoxicity can be induced via ROS which are produced in response to NP presence, by the direct interaction of genetic material with NPs or by the metallic ions that NPs release to their surroundings (Barnes et al., 2008, Kisin et al., 2007). Since some of these metallic ions play a role in regular cellular homeostasis, the presence of NPs can the increase or decrease in their concentration can disrupt regular cell homeostasis (Chang et al., 2012). NP aggregates also play an important role in NP-induced genotoxicity because, NP clumps can accumulate at the nuclear envelope and exert mechanical stress that impacts nuclear morphology. During mitosis, NP aggregates they can modify the shape or completely deform the nucleus of cells, damage or break chromosomes and adversely affect micronuclei formation (Di Virgilio et al., 2010, Alund and Xia, 2025).

1.8.3 Coordination effects

Coordination effects refer to the numerous interactions between NPs and proteins in vivo. Some of the outcomes of said interactions include but are not limited to: structural changes that can consequently lead to changes in the functions and properties of the proteins in question. This may start as interfering in the folding behavior of the protein, with subsequent knock-on effects for aspects such as protein binding and/or protein activity, relative to the NPs in the questions (Mukhopadhyay et al., 2018). Almost half of all the proteins are metalloproteins which means they contain a metal ion cofactor (Bertini I., 1998). Therefore,

the exchange of electrons between the metallic NPs and the atoms on the active site of a protein is not unlikely to occur. This interaction of biomolecules with the NPs can lead to the deactivation of inhibition of active proteins or the activation or inactive proteins (Corbo et al., 2016). The resulting disruption of normal physiological processes can extend beyond the cellular level leading to either death or chronic effects on the organism that is exposed to NPs (Mao et al., 2018). Moreover, coordination effects can also indirectly illicit other auspices of NP toxicity. For example, genotoxicity whereby the metallic ions released can in turn degrade mRNA by interaction with the mRNA stabilizing proteins (Dudev and Lim, 2008), or by interacting with signaling molecules such as purines (Miller et al., 2010, Ficerman et al., 2022).

1.9 Daphnids as bioindicators in molecular ecotoxicology

D. magna species is a bioindicator organism which has been characterized as a model organism in molecular ecotoxicology for the study of the effects of many pollutants. With many analytical techniques' chemical and physical capabilities limited in the profiling of NPs, bioindicators are defined as organisms or biological processes which can be utilized to detect or measure changes or alterations in environmental conditions. In an ecotoxicology context, the use of an appropriate bioindicator can provide vital information on the ecological health of an ecosystem, and the levels and type of pollution present within it. Within the order of Cladocera, *D. magna* are well established as an excellent bioindicator species, as their development is subject to both biotic (limited food, predator/prey interactions etc.) and abiotic factors (temperature, salinity, pollutants etc.) (Reilly et al., 2023). Thus, they are highly sensitive to almost any change in their environment, making them ideal for detecting and monitoring the impacts of pollution on a freshwater ecosystem (Samiyappan, 2019). Furthermore, pollutant-induced molecular changes in *D. magna* can be used to predict human health hazards due to the fact that over 70% of gene families linked to adverse outcomes are evolutionarily conserved between *D. magna* and humans (Colbourne et al., 2022).

The entire *D. magna* testing model falls under the three R principles (replacement, reduction and refinement). *D. magna* is one of the most commonly used species in toxicological research, due to its sensitivity to toxicants and the physiological and behavioral properties that are easily observable and quantifiable. Some other *Daphnia* species used in ecotoxicology are *Daphnia carinata* (Haney et al., 1995) and *Daphnia pulex* (Borgatta et al.,

2015). *D. magna* as a species has a high reproduction rate, is also easy to cultivate and preserve large populations in laboratory conditions and there is extensive knowledge available about the animal's physiology which is also easily evaluated (Koivisto, 1995). Additionally, *D. magna* under favorable conditions has the ability to produce clonal populations which is very helpful when studying the molecular effect of different pollutants (Siciliano et al., 2013, Reilly et al., 2023). The high reproductive rate is an excellent characteristic of the organism that allows for easy maintenance of high-numbered populations in laboratory conditions. *D. magna* has also an easy-to-use culturing protocol and low cost of maintenance. Some of the most common tests with *Daphnia* are immobilization, lethality and reproduction tests, because daphnid movements are easy to observe. Its transparent carapace also allows for easy evaluation of *Daphnia*'s physiological parameters such as feeding activity (Villegas-Navarro et al., 2003), heart rate, general movement, eye activity and more (Bownik, 2020). Additionally, some behavioral parameters that can be evaluated are the general swimming activity which can be further categorized in swimming speed, time and hoping frequency (Bownik, 2017).

1.10 Anatomy and physiology of *D. magna*

D. magna (figure 8) is a small, planktonic crustacean with the size of an adult individual ranging from 1.5 to 5.0 mm. It is found in fresh and brackish waters, with its ideal temperature for growth ranging between 18°C and 22°C. The shape of their body is nephroid and is surrounded by a transparent carapace consisting mostly of chitin. As their carapace is transparent, they usually present with the same color with the food they consume; for example, *D. magna* fed with *Spirulina sp.* will have a light green color. This characteristic is very helpful as a camouflage mechanism in order to avoid their predators. They have two pairs of branching antennae, one big which is for swimming purposes and one small which transfers food into the *Daphnia*'s mouth. Besides the second pair of antennae, six thoracic appendages with setae (hairs) are enclosed at the front side of the animal by the carapace. These generate a flow of water that besides transferring food to the mouth of the daphnia also helps with the transfer of oxygen towards daphnia's gills (Tkaczyk et al., 2021).

D. magna has one simple and one compound eye. Both of them help with the animals' orientation during swimming. It also has open type circulatory system with the heart located in the dorsal area of the body in front of a special chamber where the eggs are incubated. Hemoglobin is the protein responsible for oxygen transportation in the body of *D. magna*

(Zeis et al., 2004). *D. magna* has a tubular intestine which is made from three separate parts: the oesophagus, the mesentery and the hindgut. The food residue is removed from the hindgut thanks to peristaltic movements where the abdominal claw has a major part, and also the accumulation of new food particles inside the gut that push the older ones out. Physiology between males and females is different, with the females having a larger size and also an incubation chamber for the eggs. Males have larger antennae and modified thoracic appendages in order to have a better grab on the female during the mating (Tkaczyk et al., 2021).

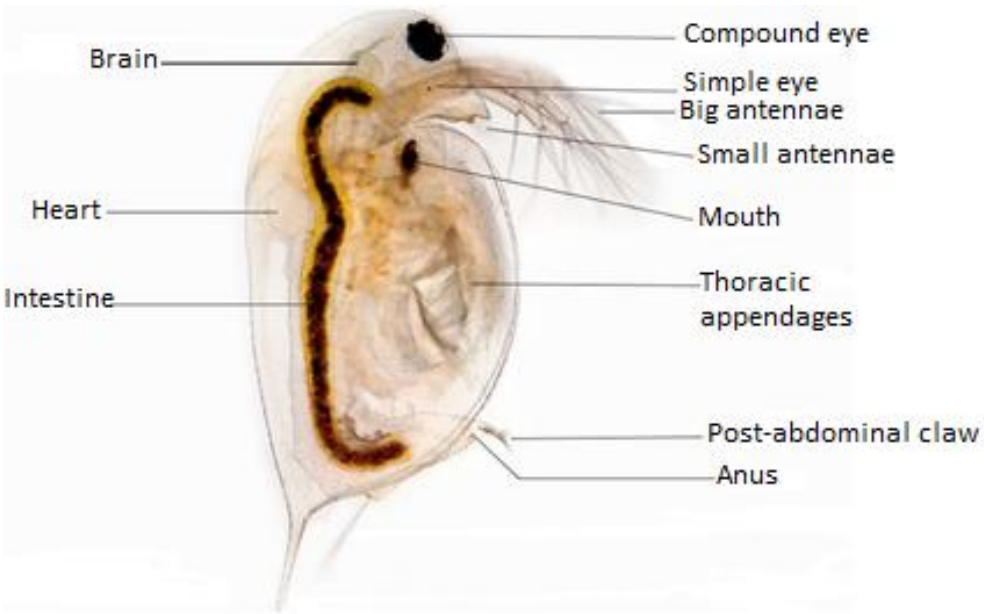

Figure 7. Basic morphological characteristics of a *D. magna*.

1.10.1 Growth and life cycle of *D. magna*

The life cycle of *D. magna* is affected greatly by the environmental conditions with most strains under favorable environmental conditions reproducing in a clonal parthenogenetic cycle with the females producing eggs which are identical clones of the initial mother. Daphnids hatch inside the incubation chamber of their mother and then are released to the environment where neonates grow to adulthood and following are considered reproductively mature.

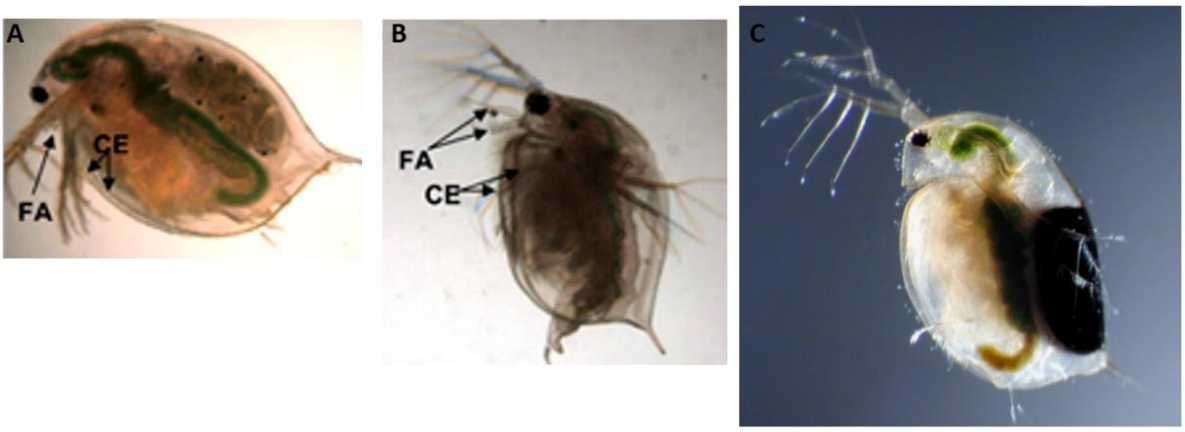

Figure 8: Morphological characteristics of daphnids. Female (A) and male (B) *D. magna*. Female with ephippium egg (C). Figure adopted from (Ebert, 2005).

As previously mentioned daphnids can grow in a female clonal population, however, the two sexes also exist in nature for many strains. The differentiating sex characteristics include the pair of minute first antennae (FA) of the females, which are elongated in the males. The female-like diminutive FA is obscured by the male-like elongated FA in the micrograph. The bivalved-like carapace of the female has two uniform, symmetrical edges (CE). Both CEs of the male are asymmetrical and are edged by setae. Under unfavorable conditions, *D. magna* has a sexual mode of reproduction (Figure 10). The females produce either male individuals or ephippia which are black, hardened protective casings that protect the eggs. In order for the eggs to hatch, it must have been preceded by mating with a male person who fertilizes the ephippium. The eggs enter dormancy before hatching and when the environmental conditions improve, the eggs hatch with the young females releasing through the ephippium (Ebert, 2005).

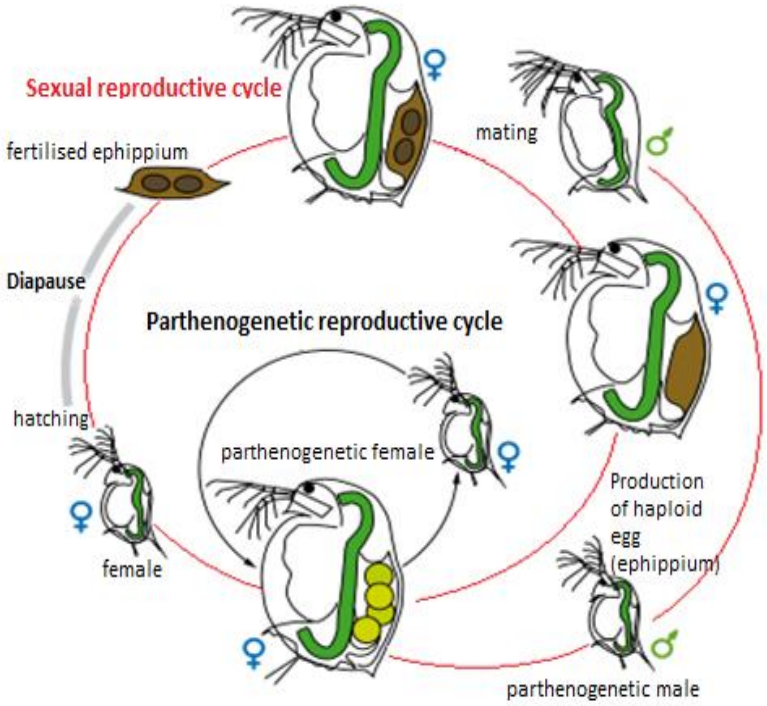

Figure 9. Sexual (red arrows) and parthenogenetic (black arrows) reproductive cycle of *D. magna*.

The average lifespan of *D. magna* is greatly affected by temperature, the oxygen levels in the water and food availability. For example, in the ideal temperature of 20°C the average lifespan for *D. magna* is 56 days (with the assumption that all the other factors are favorable and stable) while in the 25°C the average lifespan is reduced to 40 days. Due to the intense effect of environmental conditions to the *D. magna* its populations increase and decrease alternately over the seasons.

In its natural habitat, *D. magna* lives in groups and is abundant in number of individuals. Although populations do not show any type of societal hierarchy structure, the *D. magna* species is antagonistic towards other *Daphnia* species to secure resources such as food. The way daphnids swim, with the fast movement of their antennae results in a fast movement of the individual like the way fleas move (hence the common name for daphnids is water flea). All different species of *Daphnia* do vertical migrations (diel vertical migration) in the water column (Hahn and Von Elert, 2020) moving in the upper layers of the water column during the night to feed, and in the lower layers of the water column during the day to avoid their predators. Daphnids are filtering primary consumers with their food being plankton, protozoans, bacteria, algae, yeast, fungi, fungal conidia or spores and detritus. They can also perceive changes in the quality and the direction of a light source and exhibit phototactic

behaviour, usually moving towards or away the increasing intensity of light (Consi et al., 1990, Hanski and Ranta, 1983).

1.11 Environmental metabolomics

Metabolomics allow for the opportunity to determine a prime target for analysis, as changes at the metabolic level are the primary adaptation mechanism in organisms. To fulfil its role in an organism, the metabolism must respond and adapt swiftly to environmental stimuli, allowing the analysis and interpretation for the response of a model organism on the molecular pathway level. These metabolic alterations can be useful biomarkers and serve as a more rapid and efficient alternative to traditional biological endpoints (Jeong and Simpson, 2019). However, rather than replacing traditional methods, a multi-omic framework is critical to identify AOPs, linking early events with later stage phenotypic alterations. Integration of well-established bioindicator species such as *D. magna*, metabolomics stands out as a useful tool for pollution assessment, especially for novel or low-concentration pollutants. These changes may be visible in their metabolome as a disruption of their normal metabolic pathways by pollutants will result in a detectable change in endogenous metabolites. For example, metabolomic methods have already shown the ability to detect disruption in *D. magna* due to sub-lethal levels of perfluorooctane sulfonate (PFOS), and provide some measure of insight into the toxicity pathway of this pollutant (Kariuki et al., 2017).

Several separation techniques suitable for metabolic analysis are already well established. These include separation analytical techniques such as gas chromatography (GC), high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE), often coupled with other sensitive platforms such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). NMR spectroscopy and MS are the most used techniques for metabolomic analysis for the identification of metabolites (Nguyen et al., 2024). NMR spectroscopy allows the samples chemical profile analysis while retaining sample integrity as well as determining structures and concentrations (Pacholczyk-Sienicka et al., 2021). On the other hand, MS is critical for the detection and quantification of a range of metabolites and can function in several forms like the direct infusion mass spectrometry (DIMS) which directly inserts samples in the mass analyses without a chromatographic separation. Additionally, the tandem usage of techniques (e.g. GC-MS, HPLC-MS) vastly improves the analytical sensitivity and selectivity because it separates samples into

individual molecules prior to MS analysis, allowing for the detection and identification of hundreds of metabolites from a single sample (Karahalil, 2016). These platforms are enabling predictive, pathway-based ecotoxicological risk assessment, which in an ever-increasing polluted world is of critical importance.

In metabolomics analysis there are two main approaches followed; targeted and untargeted methods. Targeted methods focus on specific groups of known metabolites, while untargeted analyses capture as many metabolites as possible including unknown ones (Tareq et al., 2024, Couacault et al., 2024, Nguyen et al., 2024). Identifying unknown metabolites (or features) relies on comparisons with mass spectra derived from samples from reference libraries, which can be enhanced when coupled with other techniques such as fragmentation and derivatization. Fragmentation refers to the process of breaking down molecules into smaller known fragments in controlled conditions which can provide information on the composition and distinction of similar-sized metabolites. Derivatization is the process of chemically altering substances at specific groups and aims to improve detection, stability or volatility (Garreta-Lara et al., 2021, Jiang et al., 2016). No matter the methods, a critical aspect of metabolomics analysis is the initial sample extraction process which serves as the main step to minimize sample contamination or degradation while maintaining the concentration of metabolites. Extraction methods are mainly categorized in solvent-based and solid-phase. Solvent-based extraction methods utilize solvent mixtures (e.g. methanol-water) to separate metabolites based on polarity, allowing for the extraction of both hydrophilic and hydrophobic molecules (Dunn and Winder, 2011). Extractions with non-polar solvents end up in biphasic extractions which can further concentrate organic molecules such as lipids in the case of chloroform or dichloromethane. Solid-phase extraction focuses on capturing metabolites on the column, removing any unwanted components and enhancing sample purity and analysis sensitivity (Sitnikov et al., 2016).

The typical process after the chromatographic separation of metabolites which is followed by their identification is to generate a multidimensional dataset with many metabolites and features. Since all metabolites are interconnected via the metabolic network, multivariate and univariate statistical analyses for biological samples are used to study these multi-dimensional datasets. Data analysis includes peak normalization and scaling to ensure all data is comparable, and typical analyses include the principal component analysis (PCA) or the supervised methods of partial least squares analysis as well as clustering algorithms (Broadhurst and Kell, 2006). In tandem with other statistical analysis such as one or two-way ANOVA helps to identify statistically significant differences in metabolite abundance

across samples, and all together allow the classification of metabolomic profiles or identification of patterns which may provide critical insights. The overall aim is to generate biological information and reconstruct metabolic networks and analyze perturbations in specific pathways.

1.12 Nanocoronas

The NP interactions with living matter take place mainly between them and the constituents of the NP outer surface the NP outer surface. NPs coronas are a product of their environment, and given aquatic environments can vary significantly relative to the above influences it is imperative there are appropriate means to assess and validate water quality in ecosystems given the aforementioned risks associated with contact with such. As a result, the formation of coronas on the NP surface that are comprised of several molecules bound to such, is an overlooked but very important parameter directly related to the toxicity mediated by NPs (Xuan et al., 2023). The formation of a corona may play an important role in the mechanisms of NPs toxicity (e.g. their bioavailability to cells) while also modulating their physicochemical properties (size, agglomeration etc.) which will in turn affect their toxicity (Egbuna et al., 2021). So far, the interest in the corona formation is mainly focused on proteins adsorbed on the surface of NPs (Corbo et al., 2016) and several proteomic methods of purification have been applied to answer the question of kinetics of protein binding (Docter et al., 2015). The protein corona plays a crucial role in recognition of NPs by cells, thus impacting their cellular uptake, distribution, and toxicity (Liu et al., 2020, Li et al., 2021b). The composition of this corona is determined by NP properties such as size, shape, and surface modifications, in conjunction with those biomolecules available for binding within the local biological matrix (Chetwynd et al., 2020). For example, it is thought to be highly likely that the presence of metabolites plays a significant role in the formation of the protein corona, as many commercially produced NPs are stabilized by metabolites such as oleic acid, citric acid, or tannic acid, to name a few (Chetwynd and Lynch, 2020).

1.12.1 Metabolite Nanocoronas

Existing literature deals only scarcely with this neglected parameter, namely, the possible formation of a small molecule metabolite corona. Many varieties of metabolite can be found in the environment, originating from diverse sources. Furthermore, wastewater treatment

plants release vast quantities of waste able to bypass treatment and enter into aquatic systems such as rivers and oceans, much of it containing both proteins and metabolites or other molecules such as pharmaceuticals. Endogenous metabolites are many orders of magnitude smaller than proteins, and much greater in number while functionally, play a critical role within cell signalling cascades. For example, interaction with cellular receptors is known to play a key role in cellular uptake of NPs, meaning it is likely that different metabolites composing the metabolite corona may have a significant impact on NP bioavailability, and thus toxicity and bioaccumulation. Some metabolites with signalling roles e.g. steroids, can interact with nuclear receptors within the cell, resulting in altered gene expression (Levin and Hammes, 2016). Thus, it is clear that the adsorption of metabolites to NPs, may represent a major factor influencing of NP toxicity (Chetwynd and Lynch, 2020). The characterization of this metabolite corona could provide significant insights into the mechanisms of NP toxicity (Liu et al., 2023). This component has only recently been characterized (Grintzalis et al., 2019, Chetwynd et al., 2020), and prior studies from our laboratory have proven its ecological impact on polystyrene NPs (Grintzalis et al., 2019).

1.13 Research focus, hypothesis and scientific aims of this thesis

The main focus of this research thesis is the mechanistic assessment of nanotoxicity using freshwater and *in vitro* systems approaches. This is to address the hypothesis that due to the unique NP characteristics they interact with certain experimental parameters causing significant deviations in observed results. Additionally, we hypothesize that one of these parameters can be other molecules that can form environmental eco-coronas which cause significant changes in the properties and therefore toxic effects of NPs. In the same context, these eco-coronas can be formed by a wide variety of molecules and if these molecules are biologically active (e.g. metabolites) therefore the formation of these coronas can expose these molecules to new sites causing new and unpredictable effects.

NMs are used increasingly in a wide variety of applications, therefore their contribution to freshwater pollution is to be in high priority as water security is increasingly worsening worldwide (Wang et al., 2024b). Their presence in freshwater environments is steadily increasing, posing questions over the potential influence they present to ecosystems (Bundschuh et al., 2018a). A key initial step is to incorporate modern approaches of pollution assessment, such as those employed in predictive ecotoxicology and adverse outcome pathway (AOP) frameworks, to explore the effects of NMs and understand their toxicity

mechanisms and biological interactions. The focus of this thesis is three-fold: evaluate certain aspects of NP toxicity mechanisms such as metabolite corona, address the differences encountered in available literature and assess the feasibility of a miniaturized experimental setup. It aims to improve our understanding on how NPs exert toxicity to exposed organisms and what NP characteristics might be more significant on that exerted toxicity. Using a range of phenotypic markers such as mortality, feeding rate, enzyme kinetics and metabolomics, which have been consistently used in reported literature we focused on describing the exposure impact in depth to better assess mechanistically the exposure effects on daphnia physiology. The markers employed in this thesis focused mainly on biochemical approaches as well as metabolomics, while for the metabolite corona generation we selected daphnia that were raised in strictly monitored conditions. While this choice might not represent very accurately the metabolites that would be excreted by wild daphnia as they as not facing ideal conditions, it allowed to limit the parameters that could affect metabolite conditioning of nanocoronas and therefore potentially limit the findings on how metabolite coronas can affect exerted toxicity. A miniaturized version of daphnia toxicity assays is focused on this thesis as a means to address result inconsistency across studies and lack of a unified approach in nanotoxicity research at the same time. Finally, A549 cells were employed as a means to potentially link some of the environmental findings of this thesis to a more human-centric aspect, however, they were ultimately not included in the final version to maintain better cohesion across the major findings.

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Chapter 2

The second chapter is focused on the development of sensitive and accurate metrics for the mechanistic understanding and prediction of nanopollution levels in freshwater ecosystems that will require less amount of NMs and animals, generate less waste, and maintain a high level of reproducibility. A key research gap being addressed with this chapter is the lack of method refinement when it comes to nanopollution. We also addressed the fact that the lack of a unified testing method which can lead to statistically significant differences in comparison of results across studies. Furthermore, we proved the viability of a miniaturized toxicity testing assay that could be a suitable candidate for using in nanopollution assessment. The generated insights can clearly set a new approach to nanopollution as it should be treated with extra care as a pervasive and non-degradable form of pollution.

Miniaturizing Nanotoxicity Assays in Daphnids

Dimitrios Kakavas [1], Konstantinos Panagiotidis [1], Keith D. Rochfort [1] and Konstantinos Grintzalis [1,*]

[1] School of Biotechnology, Dublin City University, D09 Y5NO Dublin, Ireland; dimitrios.kakavas2@mail.dcu.ie (D.K.); konstantinos.panagiotidis@dcu.ie (K.P.)

* Correspondence: konstantinos.gkrintzalis@dcu.ie; Tel.: +353-17007391

Simple Summary: Applications of nanoparticles as well as their use have increased over the past decades without sufficient research on their environmental impact. Focusing on *Daphnia magna*, a widely adopted aquatic organism employed to evaluate the adverse effects of pollutants, we investigated several aspects of the experimental design used in *D. magna* toxicity testing which could impact the observed effect of exposure to nanoparticles. Furthermore, we evaluated the feasibility of a miniaturized version of *D. magna* toxicity test as a potential candidate for nanoparticle toxicity assays. Results showed that the exposure vessel and its characteristics can affect the impact of nanoparticles and, therefore, skew the observed effects. Additionally, the miniaturized exposure showed that for physiology markers such as toxicity and feeding rate it can be a good alternative to the traditional setups, as it vastly reduces the amount of media and number of nanoparticles required as well as generated waste.

Abstract: The rapid progress of the modern world has resulted in new materials and products created at an accelerating pace. As such, nanoparticles have widespread applications and often find their way into the aquatic ecosystem. In the case of freshwater ecosystems, one of the commonly used bioindicators species used for pollution assessment is *Daphnid magna*. The Organization for Economic Co-operation and Development (OECD), and other organizations such as the European Chemicals Agency (ECHA) and Environmental Protection Agency (EPA), have set guidelines for acute toxicity testing in daphnids that are severely lacking in terms of information on the characteristics of the exposure vessel when studying the adverse effects of nanoparticles (NPs). Understanding the toxicity mechanisms of nanomaterials is imperative given the scarcity of information on their adverse effects. Furthermore, miniaturization of nanotoxicity assays can reduce the number of daphnids used, as well as the cost and nanomaterial waste, and provide results even at the individual animal level with enhanced reproducibility of testing. In this study, the impact of the exposure vessel on the observed physiological changes of daphnids was investigated for a silver nano ink. Exposures in eleven commercially available vessels; nine made of plastic and two made of glass were compared for 24 h. The effect of surface to volume ratio of the exposure vessel and the animal number or “crowding” during exposure

was investigated in the context of miniaturizing biomarker assays as alternatives to traditional experimental setups in *Daphnid magna*. Toxicity curves showed differences depending on the vessel used, while a novel feeding rate assay and the activity of key enzymes were assessed as physiology endpoints.

Keywords: *Daphnia magna*; nanoparticles; silver nano ink; toxicity; miniaturization; enzyme

kinetics; mortality; feeding rate

2.1. Introduction

Nanomaterials (NMs) present an emerging threat in the ecosystem as a result of their increased use over the last number of decades. Commercial use of NMs has increased exponentially since 1990 (Sharifi et al., 2012, Bundschuh et al., 2018, Altammar, 2023), with NMs present in many consumer products; therefore, increasing the possibility of accidental release into the environment. Industry and medical domains are the most prevalent in terms of NM applications (Georgiou et al., 2008, Tang et al., 1996, Baig et al., 2021, Hussein, 2023). In relation to their entry into the environment, the primary routes are through sewage effluents (industrial or municipal), insufficient waste treatment, handling of products containing NMs and accidental spills (Holder et al., 2013). Material degradation and surface wear can also result in creating additional nanoparticles (NPs) that ultimately enter the environment. The unintentional generation of NPs is a matter of grave concern, since there is evidence of NP bioaccumulation and toxicity phenomena while there is little understanding of the factors that can affect NP bioavailability and toxicity mechanisms to aquatic life. With scarce information over the impact of engineered NMs on aquatic life, their release poses a novel environmental threat. Multiple characteristics of NPs influence their toxicity to aquatic life and these include their physicochemical properties related to their shape, size, surface charge and coating (Sanpradit and Peerakietkhajorn, 2023, Sukhanova et al., 2018, Abbasi et al., 2023).

Metallic NPs in the form of nano inks are currently used in 3D printed electronics, a relatively new industrial domain that has gathered significant traction thanks to its potential to mass produce low-cost, highly customizable electronics (Tan et al., 2019). Multiple studies have already reported the adverse effects of metallic and non-metallic NPs in bioindicator species; however, there is not sufficient information on the mechanistic aspect of toxicity for NPs in a solution (Asghari et al., 2012, Huang et al., 2017, Levard et al., 2012). Therefore, there is an urgent need to properly monitor these novel pollutants as well as expand the understanding of their toxicity mechanisms. Nanotoxicity assays conducted

using aquatic organisms often utilize different experimental setups across laboratories (Guo et al., 2023, Noss et al., 2013, Dabrunz et al., 2011, Conine and Frost, 2017, Hartmann et al., 2020, Hoheisel et al., 2012, Asghari et al., 2012). In many cases, high volumes of NP stock suspensions are used, while the exposure vessel volume (Baumann et al., 2014), surface-to-volume ratio (Kakavas et al., 2023) and animal number (Grintzalis et al., 2017) have not been thoroughly assessed or their potential impact on acute toxicity testing. Other problems that arise are the generation of pollutants from toxicity testing, as well as the inconclusive and divergent results of nano-ecotoxicological studies (Kelpsiene et al., 2022). In tandem with the great need to properly monitor and assess NPs as emergent pollutants, these two critical problems must be urgently addressed. Using bioindicator organisms such as *Daphnid magna* and *Danio rerio* for toxicity testing can provide critical insights into the toxicity of different NPs and presents several advantages over traditional approaches to pollution assessment (Avila et al., 2020, Westmoreland et al., 2022, Langan et al., 2023). Daphnids are key freshwater species in nanotoxicology and ecotoxicology as biological systems that can provide meaningful conclusions on the impact of pollutants (Reilly et al., 2023). Exposing bioindicator species to pollutants allows for a range of biomarkers such as immobilisation (Connors et al., 2022), growth (Sarrazin and Sperfeld, 2022), reproduction (Cho et al., 2022), feeding rate (Panagiotidis et al., 2023), survival (Kelpsiene et al., 2023) and more, to be utilized in order to evaluate the physiological impact of exposure, therefore enabling the detection pollutants at a very critical early stage (Michalaki et al., 2022). The utilization of daphnids as bioindicator species to serve as sensitive indicators for environmental changes can enable the detection and identification of potential hazards before they have the capacity to cause serious adverse effects on aquatic life in freshwater ecosystems (Abdullahi et al., 2022, Michalaki et al., 2022, O'Rourke et al., 2023). Their responses to pollutant exposure can provide valuable information on the toxicity levels of novel pollutants such as NPs, and provide critical insight on the underlying mechanisms of toxicity (Jeremias et al., 2021, Wang et al., 2023, Li et al., 2023, Pakrashi et al., 2017). Additionally, the use of bioindicator species allows for the assessment of various adverse effects on their physiology, providing a comprehensive understanding of the potential risks associated with NP pollution (Connors et al., 2022, Niyogi and Wood, 2004, Conine and Frost, 2017, Hou et al., 2017).

In this study, silver nano ink was used to study the impact of exposure in a miniaturised toxicity assay setup as a potential alternative to traditional experimental setups of *D. magna*. Additionally, exposure vessels which differ from one another with respect to their characteristics such as volume, surface-to-volume ratio (which reflects the shallowness or

depth of the exposure vessel) and the animal number (or crowding) were assessed in acute exposures. Using lethality as a surrogate measure of toxicity, a novel feeding assay and biochemical markers, the impact of nano inks on daphnids was assessed under different exposure conditions.

2.2 Materials and Methods

2.2.1. Culturing of Daphnids and Toxicity Exposures

Cultures of daphnids were maintained in glass beakers in aqueous media (final concentrations 0.29 g $CaCl_2.2H_2O$/L, 0.123 g $MgSO_4.7H_2O$/L, 0.065 g $NaHCO_3$/L, 0.0058 g KCl/L, 2 µg Na_2SeO_3/L, pH 7.7) under a 16 h:8 h of light:dark photoperiod at 20 °C, at a density of 80 adults per 4 L of media. Media were renewed every 5 days and cultures were fed daily with an algal (*Chlamydomonas rheinhartii*) suspension and 15 mL of an organic seaweed extract (*Ascophylum nodosum*) only upon media renewal. For nano exposures, neonates (<24 h) were collected and cultured until they were 4 days old and fed daily with algae (60 million cells). On the fourth day, animals were relocated to different vessels for exposure and mortality was assessed following 24 h. The vessels used were a 6-well plate (6wp), a 12-well plate (12wp), a 24-well plate (24wp), a 48-well plate (48wp), a 96-well plate (96wp), a cuvette (C), a Petri dish 50 mL (P50), a Petri dish (100 mL), a centrifuge tube (f), a glass vessel 50 mL (G50), and a glass vessel 100 mL (G100). Toxicity curves were plotted using the four-parameter logistic (4PL) model, following the equation Span = Top − Bottom and Y = Bottom + (Top − Bottom)/(1 + 10^((LogIC$_{50}$ − X) × HillSlope)), using the GraphPad software 10.2. The EC values were calculated and a concentration of 0.5 µL nano ink/L (derived from the data of the toxicity curves) was used as sub-lethal exposure concentration for the enzyme activity and feeding rate exposures of 24 h. Silver nanoparticle ink was purchased from Sigma-Aldrich to conduct the exposure experiments (Product No. 719048). The silver nanoparticle ink with this product code has been altered to wt 20%; however, when originally purchased the product was different with wt 50%. In this study, we selected to work with four-day-old daphnids based on other studies (Kakavas et al., 2023, Michalaki et al., 2022, O'Rourke et al., 2023) focusing on older animals compared to neonates which would be more sensitive to pollutants. In addition, four-day-old daphnids provide more tissue for biochemical assays and are easier due to their size to work with, while they can also give critical information on their response to pollutants compared to an early adolescent life stage of the organism.

2.2.2. Sample Homogenization and Biochemical Assays

Four-day-old daphnids were exposed to nano ink (0.5 μL/L) for 24 h. Following exposure, 30 daphnids were combined and homogenized in 0.5 mL of phosphate buffer (pH 7.2) using an Eppendorf pestle. The homogenate was cleared by centrifugation (12,000 × g for 10 min at 5 °C) and the clear supernatant was collected and immediately assayed for protein and enzyme activity (Grintzalis et al., 2022, Michalaki et al., 2022). The highly concentrated homogenate was further diluted as it is necessary to obtain unit values within a reliable linear range for each kinetic assay. Protein quantification was performed using a sensitive Bradford method (Georgiou et al., 2008, Grintzalis et al., 2015) to normalise enzyme activity in units/μg protein.

Phosphatase activity was determined for the released *p*-nitrophenol utilizing *p*-nitrophenyl phosphate (*p*NPP) as substrate, in 100 mM citric acid pH 4.5 (for ACP) or 100 mM boric acid pH 9.8 (for ALP) (Grintzalis et al., 2022). The reaction volumes were set to 200 μL sample appropriately diluted in buffer and mixed with 50 μL 8 mM *p*NPP. The reaction was alkalized after 30 min with the addition of 50 μL 4 M NaOH, and the absorbance was measured at 405 nm and converted to *p*-nitrophenol from the corresponding standard curve. Similar to the aforementioned endpoint kinetics, the activities of β-galactosidase (BGAL) and lipase (LIP) were determined, by measuring the generation of nitrophenol resulting from the catalysis of *o*-nitrophenyl-β-galactoside and *p*-nitrophenyl butyrate, respectively, in 50 mM phosphate buffer pH 7.2.

The activity of γ-glutamyl transferase (peptidase) was quantified using L-leucine-4-nitroanilide as a substrate (van Acker et al., 1998). For the quantification, 200 μL of appropriately diluted sample in 50 mM phosphate buffer pH 7.2 was mixed with 50 μL of 8 mM substrate in 100% DMSO, and the release of the product *p*-nitroanilide was monitored continuously at 418 nm. The activity of GST was determined by monitoring the formation of a complex between reduced glutathione and 1-chloro-2,4-dinitrobenzene and measured photometrically at 340 nm (Fernandez et al., 2002). The activity of LDH was quantified by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH. The substrate employed was a 1:1 mixture of 40 mM pyruvate and 0.5 mM NADH. For the reaction, 200 μL of the sample appropriately diluted in phosphate buffer was mixed with 50 μL of 20 mM pyruvate and 0.25 mM NADH, and the oxidation of NADH to NAD^+ was continuously monitored at 340 nm. Reduced thiols (RT) were quantified through their reaction with aldrithiol following the protocol of Grintzalis et al. (Grintzalis et al., 2014).

2.2.3. Feeding and Imaging of Daphnids

The feeding rate was assessed as a phenotypic physiology endpoint. Typically, methods employ algae and long incubation periods; however, in our approach we used a novel method based on the tracking of ingested fluorescent microparticles (carboxylate-modified fluorescent latex beads, CAT number: L3030), which were were purchased from Sigma Aldrich (St. Louis, USA) (Giannouli et al., 2023). Daphnids were collected following a 24-h exposure to silver nano ink (0.5 µL/L) and incubated with 18 mL of fluorescent microparticles for 30 min to evaluate the feeding rate of daphnids. For the feeding assay, 4 replicates of 15 animals each were utilized to evaluate the impact of exposure on the feeding rate. Fluorescence from microparticles was measured at Ex/Em 560/590 nm using a TECAN plate reader (Männedorf, Switzerland). Fluorescence measurements from the media were converted to the amount of ingested microplastics using a standard curve and expressed as feeding rate per animal.

2.2.4. Statistical Analysis

Results were presented as mean ± standard deviation (SD) and were analysed and plotted with GraphPad Prism software 10.2. Statistically significant differences were identified using one-way ANOVA and a Student's *t*-test with a Welch's correction over the unexposed control condition with a p value of 0.05.

2.3. Results

2.3.1. Exposure to Silver Nano Ink in Different Vessels and Volumes Impacts Mortality

Acute exposure of daphnids to the silver nano ink for 24 h was assessed in different volumes and vessels with full toxicity curves (Figure 1) and the EC_{50}, EC_1 and Hill slope were calculated (Table 1). Full toxicity curves were generated with a minimum of three independent experiments per exposure vessel conducted, to reduce any possible difference between batches of daphnids in the observed mortality. The lowest EC_{50} value (0.221 µL/L of the nano ink stock) was observed in the P100 (Petri 100 mL) exposure condition while the highest (5.515 µL/L of nano ink stock) was observed in the cuvette condition (Table 1).

Hill Slope values also deviated across the tested conditions, ranging from 2.248 (centrifuge tube) to 7.321 (Petri 50 mL), showing great differences in steepness and the range of observed toxicity to the exposed daphnid. There were no visible signs of precipitation of AgNPs observed during the exposures. The characteristics of the vessel significantly

impacted the observed effect on mortality during exposure; however, mortality does not follow any specific pattern among the tested vessels (Figure 2). For the following exposures and assessment of other biomarkers, 0.5 µL/L was selected as the exposure concentration due to being close to the equivalent EC_1 values for the f (centrifuge tube), 96wp (96-well plate) and the g50 (glass 50 mL) exposure conditions.

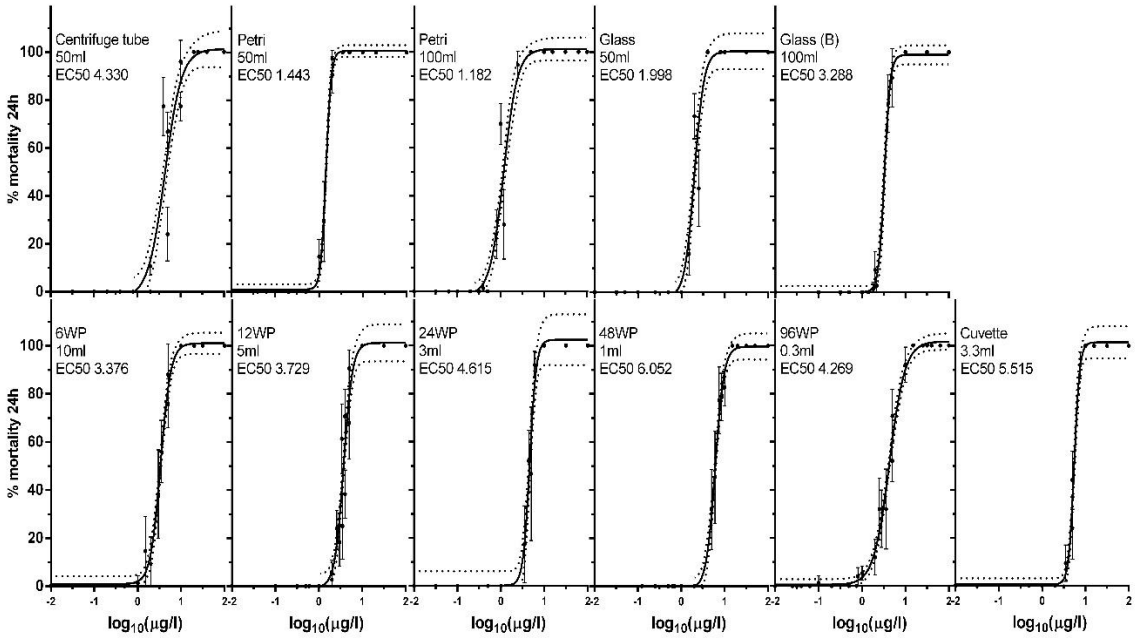

Figure 1. Acute toxicity curve for AgNP nano ink in 11 exposure vessels. EC_{50} values are in µL/L. Fifteen 4−day−old daphnids per replicate of each concentration tested were exposed to AgNPs nano ink for 24 h. Data represent average ± SD (N = 5). Using the four-parameter logistic (4PL) model, the EC_{50} values were calculated.

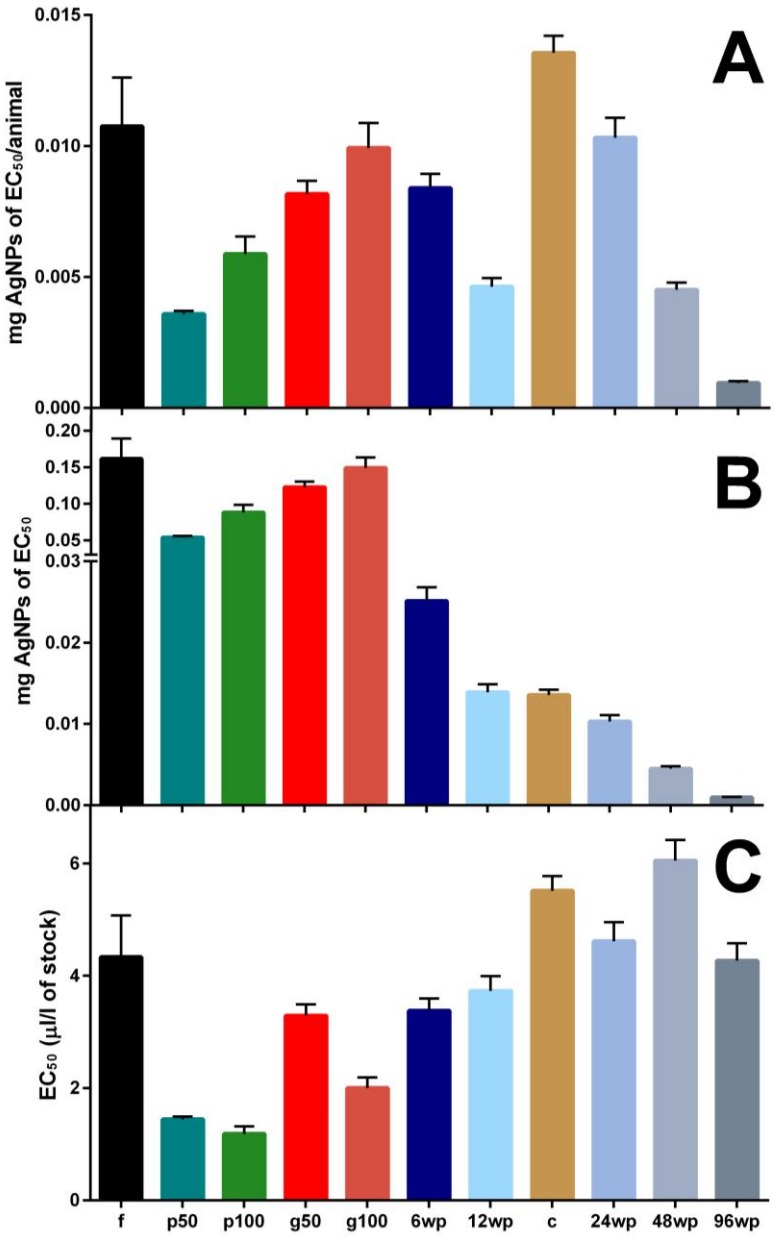

Figure 2. EC_{50} values of all 11 vessels tested were calculated with the four-parameter logistic (4PL) model. (**A**,**B**) show the mg of AgNPs calculated that are equivalent to the EC_{50} for each vessel tested, per replicate and per animal, respectively. (**C**) depicts the amount of silver nano ink in µL/L equivalent to the EC_{50} for each vessel tested. The error bars in the figure depict the range of data, with each bar indicating the minimum and maximum values (Table 1).

The top figure 2 (mg AgNPs/daphnid) shows that tall vessels require more NPs per daphnid during exposure in order to achieve the same % of lethality in a tested group. The NP amount per daphnid that is calculated as equivalent to the EC50 for each condition tested shows great variation across all conditions. The miniaturisation approach for the experimental setup results in a significant decrease in the NP amount that is equivalent to the EC50, potentially presenting a more accurate and realistic depiction of the AgNPs exposure effect when focusing on single individuals of D. magna compared to larger test

vessels. The 96wp required the smallest amount of AgNPs that is equivalent to the EC_{50} concentration during exposures. The middle figure 2 (mg AgNPs equivalent to EC_{50}) shows that the AgNP amount of EC_{50} in each condition follows a decrease that mostly correlates to the decrease in the test vessel's volume. These observations also confirm that smaller vessels require fewer AgNPs to produce the same effect of exposure. The miniaturisation approach of using small volume vessels shows a trend of decrease for the mg of AgNPs equivalent to the EC_{50} of each condition that correlates and matches to the decrease in the exposure volume in all exposure vessels. In the bottom figure 2, we can observe the differences in amount of silver nano ink stock required in each test condition. Furthermore, in the large exposure vessels with a high S:V (petri 50 mL, petri 100 mL) the stock amount required is also significantly less when compared to other large vessels. The only exception is a miniaturised exposure vessel, the 96wp which, in spite of having a high S:V, requires more silver nano ink stock for the EC_{50} concentration when compared to vessels with a smaller S:V.

The initial assessment of the different exposure vessel mortality during AgNP nano ink exposure revealed that the higher-volume vessel does not always require greater amounts of AgNPs compared to a miniaturised approach, as seen by the C (cuvette) condition (Figure 3). However, the deviation in EC_{50} values in tested vessels clearly shows an influence of the different vessel characteristics on the toxicity of AgNPs. During the exposure period, no mortality was observed in control exposures that had no AgNPs added. These conditions were added to ensure the good quality of the brood during exposure. The lowest EC_1 value calculated was in the petri 50 mL exposure vessel (0.221 µL/L of silver nano ink stock), and the highest was calculated in the cuvette condition (2.807 µL/L of silver nano ink stock). The differences in vessel characteristics for S:V, animals, animals/mL and volume for the 11 conditions tested can be observed in Table 1.

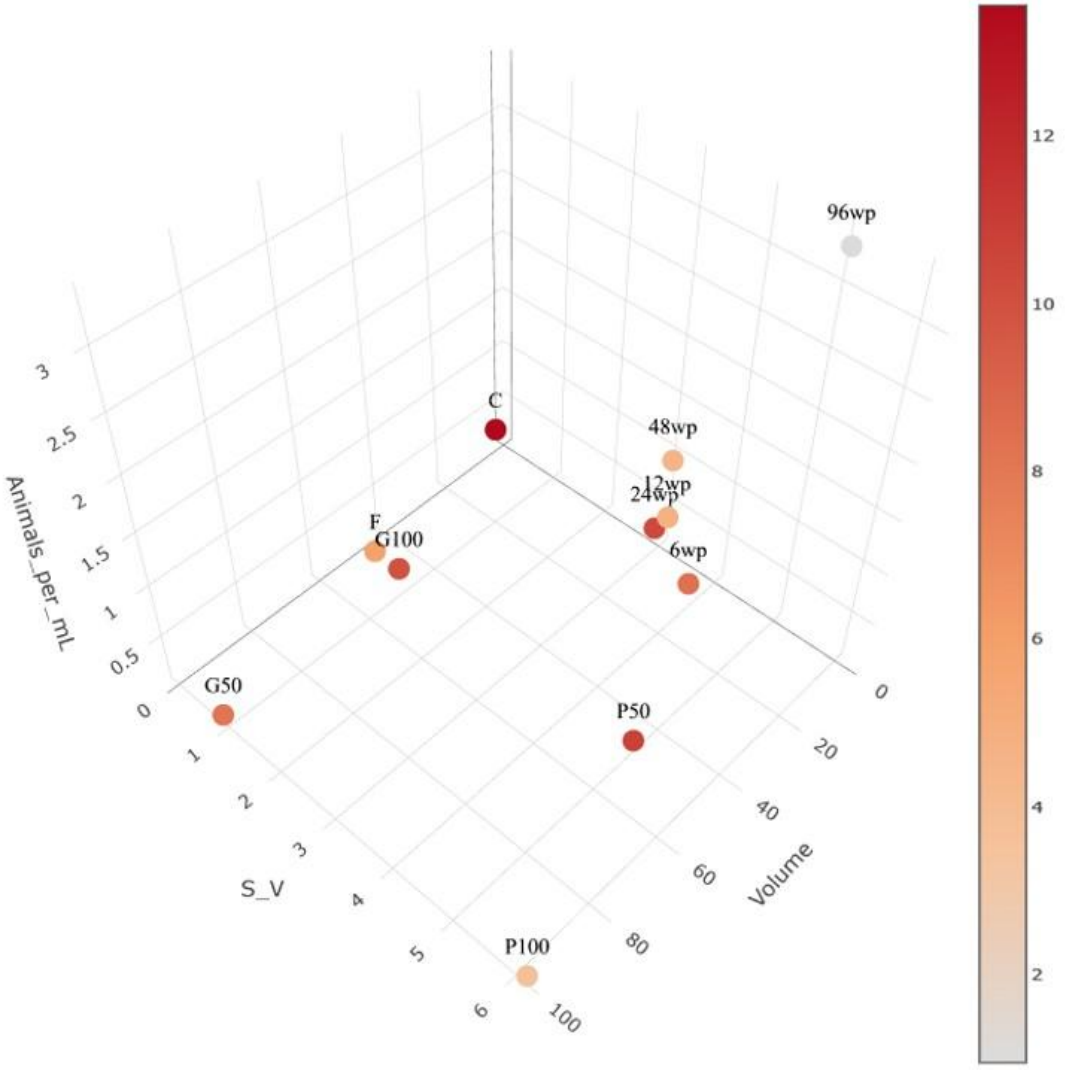

Figure 3. The impact of vessel on mortality. Points in the figure correlate to each exposure vessel with axes used to plot the characteristics of the vessel. Colour intensity represents the amount of AgNP nano ink (mg/daphnid in the exposure vessel) for the EC50. A supplementary version of this image with free rotation is provided.

Table 1. EC values (in µL/L) for silver nano ink acute exposures.

Vessel	6-Well Plate	12-Well Plate	24-Well Plate	48-Well Plate	96-Well Plate	Cuvette	Petri Dish (50 mL)	Petri Dish (100 mL)	Centrifuge Tube	Glass Vessel (100 mL)	Glass Vessel (50 mL)
Abbreviation	6wp	12wp	24wp	48wp	96wp	C	P50	P100	f	G100	G50
Volume (mL)	10	5	3	1	0.3	3.3	50	100	50	100	50
Animals	3	3	1	1	1	1	15	15	15	15	15
Animals/mL	0.3	0.6	0.33	1	3.33	0.3	0.3	0.15	0.3	0.15	0.3
S:V	3.98	3.32	3.03	3.14	5.13	0.03	5.09	6.16	0.49	0.92	0.96
EC_1	0.974	1.264	2.060	2.042	0.629	2.807	0.77	0.221	0.561	0.615	1.605
EC_{50}	3.376	3.729	4.615	6.052	4.269	5.515	1.443	1.182	4.330	1.998	3.288
(min–max)	(3.17–3.60)	(3.43–4.00)	(4.3–4.96)	(5.71–6.42)	(3.98–4.58)	(5.27–5.78)	(1.36–1.49)	(1.06–1.32)	(3.69–5.08)	(1.82–2.19)	(3.1–3.49)
Hill slope	3.697	4.246	5.696	4.230	2.400	6.804	7.321	2.744	2.248	3.898	6.407

2.3.2. Physiology Responses following Exposure to the Silver Nano Ink

Daphnids were exposed to 0.5 μL/L of silver nano ink and responses in key enzyme activity were recorded (see Supplementary Tables). Comparisons between control and nano exposed but also across the exposed conditions reveal statistically significant differences. Biochemical responses (Figure 4) showed differences between the centrifuge tube, 96-well plate and glass vessel on the comparison of observed enzyme activity levels but also when exposure vessels were compared to their respective controls. The statistically significant differences in enzyme activity across the eight tested enzymes do not follow a specific trend and change both in the enzymes and observed intensity across the tested conditions. The greatest difference in the enzyme activity was observed in the activity of LDH in the glass exposure condition (+34%) and the lowest for the reduced thiols (−22%). The miniaturised approach in the 96wp shows a significant decrease in ACP activity (−22%) and an increase in reduced thiols (+9%). Furthermore, the centrifuge tube exposure condition shows a significant increase in GST activity (+13.5%) as well as in the activity of BGAL (+10%).

Statistically significant differences in the eight enzymes and compounds tested show a different profile of exposure response to the AgNPs in exposed individuals. A different toxicity mechanism of AgNPs in the 96wp condition could explain the difference in GST and reduced thiols when comparing the 96wp with the centrifuge tube or the glass exposure conditions (Figure 4). Additionally, in the glass exposure condition GST and LDH activity were significantly impacted by the exposure whereas in the centrifuge tube exposure condition, GST activity is impacted less and LDH significantly less when compared to the equivalent enzymes in the glass exposure condition. According to our results, GST activity and reduced thiols in both the centrifuge tube and the glass greatly deviated from their corresponding values in the 96wp exposed conditions.

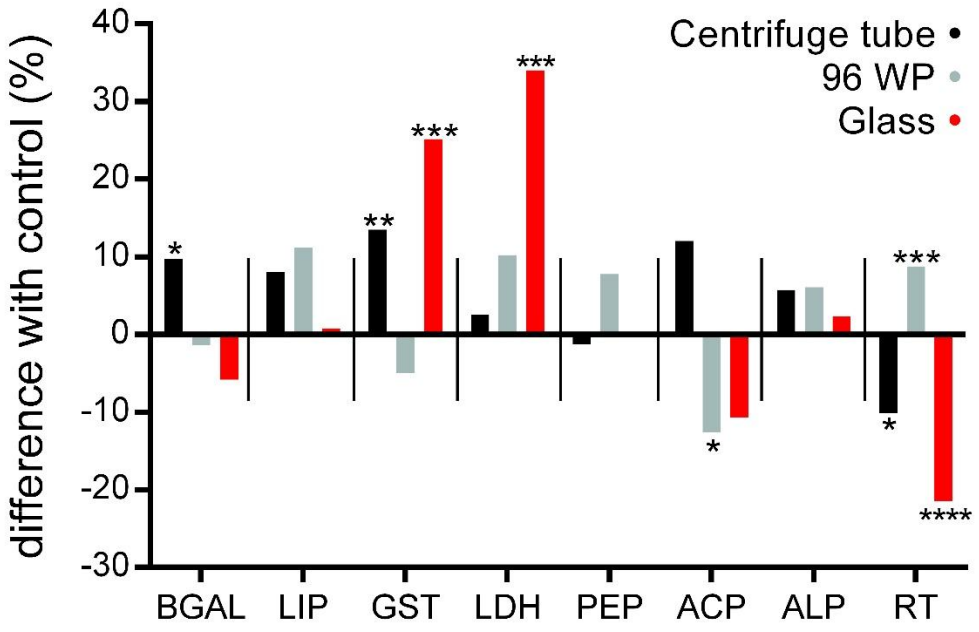

Figure 4. Percentage of difference in enzyme activity due to exposure to silver nano ink (0.5 µL/L, 24h) in different exposure vessels (each vessel was compared to their corresponding control). Data represent average ±SD (N = 4) replicates for each condition. Statistically significant changes identified by one-way ANOVA between control and exposed conditions with *p*-values of $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). All data values are provided in Supplementary Table. Abbreviations: BGAL: β−galactosidase, LIP: lipase, GST: glutathione S−transferase, LDH: lactate dehydrogenase, PEP: peptidase, ACP: acidic phosphatase, ALP: alkaline phosphatase, RT: reduced thiols.

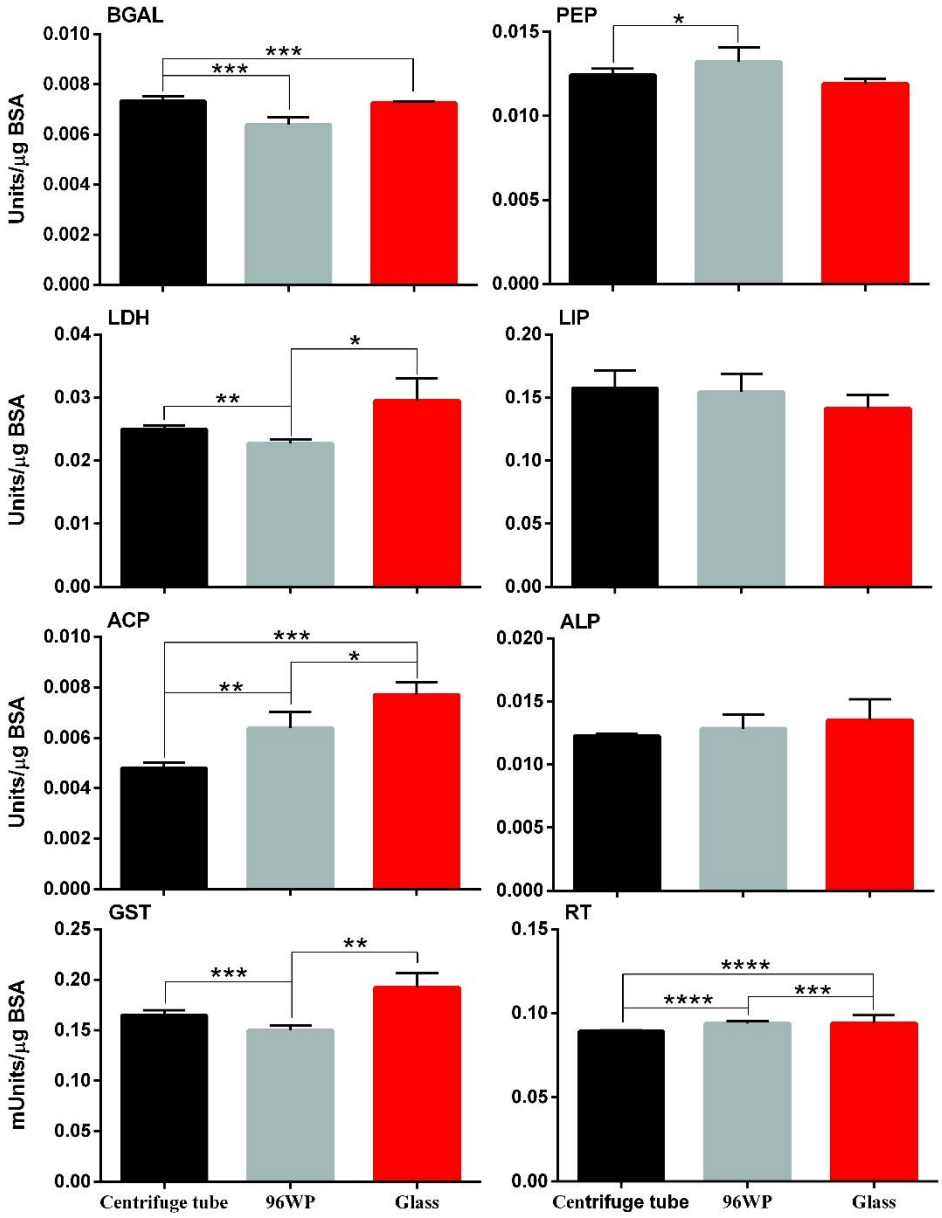

Figure 5. The impact of miniaturization and vessel material on controls. Comparisons of enzyme activities between the unexposed controls of a centrifuge tube, glass and 96-well plate exposure vessels. Data represent average ± SD (N = 4) replicates for each condition. Statistically significant changes identified by one-way ANOVA between control and exposed conditions with *p*-values of $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). All data values are provided in Supplementary Tables. Abbreviations: BGAL: β-galactosidase, LIP: lipase, GST: glutathione S-transferase, LDH: lactate dehydrogenase, PEP: peptidase, ACP: acidic phosphatase, ALP: alkaline phosphatase, RT: reduced thiols.

To provide a more holistic image on the effect of the exposure vessel on the physiology of daphnids, the enzyme activity of eight different enzymes was tested in the absence of silver nano ink (Figure 5). We observed statistically significant different enzyme amounts in six out of eight tested enzymes and compounds, with ALP and LIP being the two that displayed none. These observations confirm that the impact of the exposure vessel on the

physiology of daphnids could in part explain the differences observed in other tested biomarkers.

However, the feeding rate showed no statistically significant differences between the tested conditions. These findings suggest the absence of impact on the daphnid feeding rate in the sub-lethal concentrations that were tested and could indicate that a miniaturized approach in the feeding rate assessment is feasible.

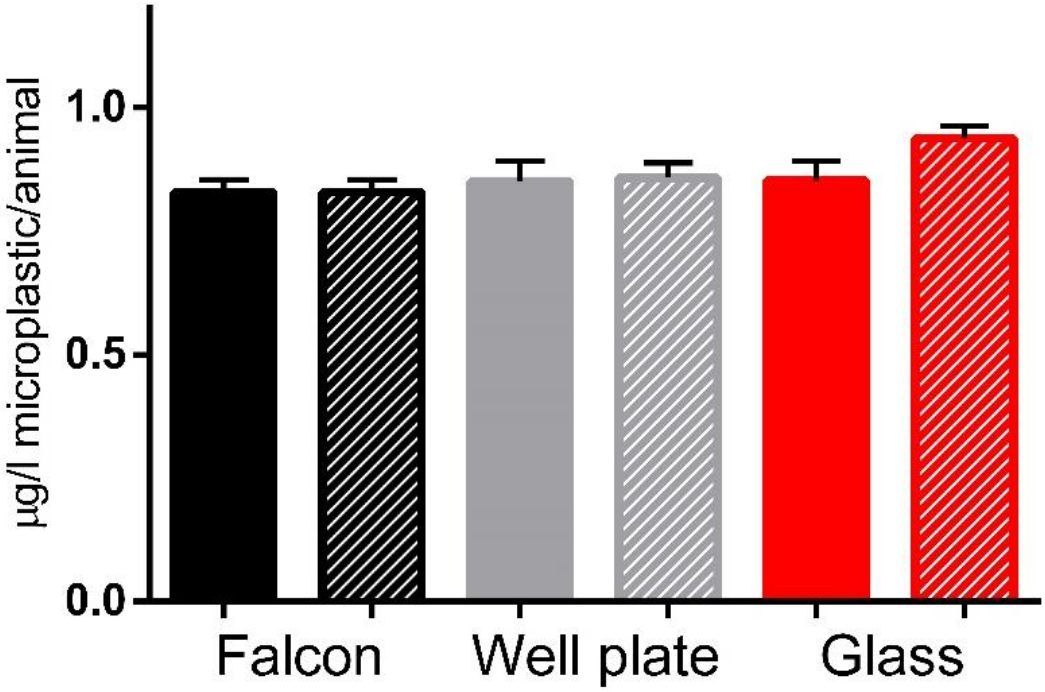

Figure 6. The impact of silver nano ink (indicated by striped bars, exposed at 0.5 µL/L for 24 h) compared to each vessel group's control (non-striped bars) on the feeding rate of daphnids under different exposure vessels. Data represent the mean ± standard deviation (N = 4). No statistically significant differences using a Student's *t*-test were found across the comparisons of exposure to control condition in the tested vessel groups. No statistically significant differences using one-way ANOVA were found in the comparisons among the control (solid) or among the exposed (striped) groups.

2.4. Discussion

In this study, the effects of different exposure vessels on the toxicity of AgNPs to *D. magna* were investigated on several biomarkers. Additionally, we investigated a miniaturized approach as a better alternative to traditional *D. magna* toxicity testing experimental setups, as well as studied how miniaturization can affect the observed impact in certain biomarkers of daphnid physiology (Ulm et al., 2015, Zhang and Tan, 2023). The dynamic interactions between the exposure vessel and pollutants may affect the physicochemical properties of NPs and, therefore, their impact on exposure to daphnids. This also highlights the importance of the standardization of exposure vessels and protocols

used across laboratories for *D. magna* toxicity testing. Our study hypothesizes that as NP solutions are colloidal in nature, when exposure vessels with different characteristics are used for pollutant assessment NPs can potentially interact with the vessel itself and the exposed daphnid in distinct ways. Consequently, certain vessel characteristics could influence the observed impact of NP exposure to bioindicator species used to study pollution such as *D. magna* (Grintzalis et al., 2017). There is a clear gap in knowledge concerning the influence of the exposure vessels on the observed impact of exposure on biomarkers of daphnid physiology, which are used to assess responses to pollutants such as mortality, feeding rate, etc. To the best of our knowledge, this is the only study that focuses on the impact of exposure vessels to observed toxicity and other biomarkers of daphnid physiology except for Gkrintzalis et al. (Grintzalis et al., 2017), in which the researchers used a wide range of exposure vessels to evaluate the impacts of systematically varying the total media volume, the surface-to-volume ratio, and the animal density for the acute toxicity testing of cadmium. However, in said study, only mortality was used as a marker to evaluate the effect of different vessel characteristics. In this study, 11 vessels were assessed, selected with the criteria of being (1) commercially available, (2) used in ecotoxicological studies with daphnids, and (3) good candidates to test the viability of miniaturising acute exposures (Castro et al., 2018, Lekamge et al., 2019). The vessel characteristics studied were the volume, number of animals in each vessel (crowding), S:V and vessel material (glass/plastic) on their impact to the observed effect of AgNP nano ink exposure to mortality, enzyme activity and feeding rate. According to our results (Figure 3), the higher to S:V, the observed mortality is lower but not in all vessels. This could be attributed to the fact that a NP solution is colloidal and therefore in vessels with capacity for large volumes (e.g., 50 mL) and that are tall, NPs will sink over time to the bottom of the vessel. However, the *D. magna* exposure to AgNPs results in an observable effect of exposure, since part of the toxicity effect of AgNP exposure is attributed to the leeching of Ag+ ions in the surrounding media (Ghobashy et al., 2021). The vessel volume alone is not a defining factor for the observed mortality in vessels but vessels with more volume generally resulted in higher observed toxicity to exposed *D. magna*, which can be seen when comparing the following groups in the context of their different volumes in Figure 3: p50/p100/96wp, g50/g100/96wp or instead of 96wp p50/p100/c, etc. Furthermore, the range of AgNPs concentration for the toxicity curve of each condition which can be correlated to the Hill Slope (Table 1) shows that the toxicity levels of AgNPs are impacted by the difference in the tested vessel characteristics, but do not follow a specific trend. The impact of animals per mL, or crowding, was also studied

and according to mortality data (Figure 3), we cannot reach any clear conclusions regarding the impact of animal crowding.

According to our previous study (Kakavas et al., 2023), S:V impacts the toxicity of AgNPs. For example, the Petri dish as a shallow vessel, when compared to the centrifuge tube allows for easier access to AgNPs, possibly due to the large S:V, where the daphnid can only swim close to the bottom of the vessel while AgNPs will sink over the time of the acute exposure. This hypothesis is supported by the findings in Figure 2A,B where the amount of AgNPs in the centrifuge tube is equivalent to the EC_{50} for a single replicate and per animal is less when compared to the Petri dish. Additionally, the same observation can be made when comparing the AgNP amount of the AgNP silver nano ink concentration equivalent to EC_{50}/animal for the 24wp and the cuvette, or the miniaturised version of 96wp when compared to any other exposure vessel (Figure 2A). The hypothesis we posit for this observation is the same as in our previous study which focused only on the S:V (Kakavas et al., 2023), and is that the restricted vertical movement of daphnids inside the exposure vessel, in tandem with the fact that the exposure media is a colloid solution is the reason for the increased impact of AgNPs on daphnids exposed in vessels with higher values of S:V, or more specifically, with a geometry that will restrict the daphnid to swim close to the bottom of the vessel during the exposure and possibly increase the availability of AgNPs. The impact of the vessel material can be observed when comparing the mortality in the f and g50 exposure conditions, where exposure in g50 seems to result in increased daphnid mortality. A possible explanation for this observation is that the AgNPs do not interact in the same way with glass and plastic, and this might result in different amounts of NPs being available in the media for the exposed daphnid, resulting in a different observed impact. However, there is very limited information on the adsorption capacity of glass surfaces and plastic surfaces in regard to AgNPs (Polowczyk et al., 2015), and more research is required in order to fully understand the interactions between AgNPs and glass or plastic exposure vessels. Regarding the vessel impact on observed mortality, Figure 3 underscores a significant problem with the use of various vessels across studies: even with remarkably similar EC_{50} values, the true quantity of AgNPs per exposed individual can exhibit considerable variation. Therefore, a range of outcomes in terms of the observed effect of exposure can be expected for other, more sensitive biomarkers of daphnid physiology such as enzyme activity, survival assays or omics analysis. Another crucial aspect that needs to be considered is the geometry of exposure vessels which can inflict additional stress on the exposed daphnid as our results in Figure 5 highlight. The observed effect on the studied biomarkers shows that the choice of exposure vessels used in laboratory studies can result in significantly varying distributions

of AgNPs to the exposed daphnid and therefore observed effect of exposure among different studies, even when the concentrations of the initial NP stock are similar.

After assessing the impact of various vessels on the observed mortality, three vessels were identified for additional investigation: f, g50 and 96wp. The selection was made based on the differences in vessel material and with the aim to further investigate the feasibility of miniaturization. The feeding rate was not impacted by the different exposure setups as it showed no differences between them. This absence of observed differences is a good indicator that a miniaturised experimental setup for *D. magna* exposures will not affect this commonly used biomarker in environmental studies. However, this is not the case for more sensitive biomarkers such as enzyme activity, which clearly show a distinctively different response in many key enzymes of *D. magna* physiology and therefore invalidate any comparison of such results across studies unless the same exposure vessels were used among them. According to (Kelpsiene et al., 2022), biochemical assays are a viable tool to examine on a more precise level the effect of pollutant exposures. Regarding the results of the enzyme activity assays (Figure 5), it is noteworthy that all control conditions exhibited statistically significant differences in the activity levels of six out of eight enzymes tested. The most notable variations were observed in the comparisons between the centrifuge tube and 96wp, with statistically significant differences detected in the activity levels of four enzymes (BGAL, PEP, LDH, ACP, GST) and reduced thiols. Comparison of the glass and the centrifuge tube control conditions displayed the fewest differences, with three out of eight enzymes having statistically significant differences, which we hypothesize is because the centrifuge tube and glass have the same exposure vessel volume (Figure 3). The fact that the controls of each tested vessel displayed a different pattern of statistically significant differences when compared to their respective exposed condition shows that in the context of studying the effect of AgNP exposure to very sensitive markers of daphnid physiology the vessel used for the exposures cannot be changed in favour of a miniaturized approach as this can result in inflicting additional stress or differences on a biochemical level for the exposed daphnid (Figure 3), skewing the observed. In the context of less sensitive markers such as mortality and the feeding rate, which in our study showed no statistically significant differences, the miniaturisation setup is a good potential alternative to traditional *D. magna* exposure setups. Furthermore, it may be actually a more accurate alternative for pollution assessment specifically for NPs, which due to their unique features require an alternative approach that is not affected by the S:V of the vessel or other vessel characteristics. Regarding the differences between the centrifuge tube and glass exposure vessels, the different factors between these two exposure vessels are the S:V and the vessel material.

According to our previous study (Kakavas et al., 2023), we know that the difference in S:V can result in differences in observed toxicity as well as the activity of the eight enzymes tested. However, the S:V of the centrifuge tube and glass exposure vessels does not differentiate much in order to be the only responsible factor for any differences observed. Therefore, the vessel material may also be responsible for the deviation in enzyme activity. To the best of our knowledge, there is no available information that covers how different vessel material can influence NP toxicity, although there has been some research (Sakka et al., 2017) which suggests that the test vessel's parameters could impact the observed effect but this is focused mainly on the impact of surface stabilizers or dispersants.

In both centrifuge tube and glass exposure vessels, we observed either an increase in the case of GST activity or a decrease in the case of reduced thiols whereas the opposite is true for the 96wp. Since AgNPs are known to cause ROS and DNA damage to living organisms, the deviation in the observed enzyme activity values for GST and RT between centrifuge tube/glass and 96wp we hypothesize is observed because in the 96wp daphnid are more encumbered by the exposure to NPs in terms of oxidative stress and possibly hinting at a different toxicity mechanism (Jozefczak et al., 2012, Harris and Hansen, 2012). Although the differences in the control values of enzyme activity indicate additional stress may be caused to daphnids during exposure due to the vessel characteristics, the miniaturized approach may be a more precise alternative to standard testing in daphnids regarding NP pollutants. Due to the geometry of the vessel, daphnids are limited in terms of their movements very close to NPs in the exposure media. Therefore, more realistic results can be obtained in terms of the true impact of AgNP exposure and how dangerous an NP pollutant is, due to the fact that the amount of NPs required to achieve these results is considerably less when compared to larger volume vessels (Figure 2). Additional research is required to fully evaluate the feasibility of a miniaturized approach of daphnid toxicity testing for more sensitive markers of daphnid physiology.

In conclusion, our study highlights the critical influence of exposure vessel characteristics on the observed toxicity of AgNPs to *D. magna*. These findings underscore the importance of standardizing exposure vessel parameters in ecotoxicological studies to ensure the reliability and comparability of toxicity assessment data for NP pollutants across studies. Our study also shows that certain biomarkers can be reliably used for NP pollution assessment in a miniaturised approach which potentially produces more accurate results compared to traditional experimental setups. This new approach vastly reduces the required amount of NPs for toxicity testing and shows that the required NP amount to observe adverse

effects after exposure is significantly less compared to when using larger exposure vessels. Moving forward, further research is necessary to further explore the underlying mechanisms driving the observed correlations of toxicity, vessels characteristics and observed effect, and to develop comprehensive guidelines for the selection and use of exposure vessels in the toxicity assessment of NP pollutants. By addressing these considerations, we can improve the accuracy and relevance of NP toxicity assessments, ultimately contributing to more effective environmental risk assessment and management strategies.

2.5. Conclusions

The presented data highlight the absence of information relating to the impact of the characteristics of the exposure vessel on the observed effect of NP, and, therefore, the urgency for a standardized testing for the assessment of NP pollutants. Additional research is required to determine how much impact each individual test vessel parameter actually has, as well as for the possibility of using a miniaturized exposure setup in order to reduce generated pollutants during testing. This new miniaturized approach to the NP pollution assessment may also result in a more holistic understanding of the toxicity mechanisms of NPs without compromising the reproducibility of tests.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Supplementary Table S1: The acute effect of miniaturization on multiple enzymatic markers of daphnids upon exposure to silver nano ink; Supplementary Table S2: The acute effect of miniaturization on multiple enzymatic markers of daphnids. Supplementary Figures S3 with rotation for all expressions of nano ink.

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Institutional Review Board Statement: Ethical review and approval were waived for this study, because daphnids are regarded as “animals” in terms of being members of the kingdom Animalia; however, they are not “animals” as defined in regulation SI543 of 2012 on the protection of animals used for scientific purposes. Therefore, the study does not require authorization from the Health Products Regulatory Authority (HPRA), while it is also in line with the aim of working under the 3Rs (reduce, refine, replacement) strategy, since daphnids are commonly used in ecology and ecotoxicology as replacements for more evolutionary advanced species (i.e., fishes), posing no ethical implications.

Informed Consent Statement: Not applicable.

Data Availability Statement: All raw data from this study will be provided upon request. Additionally, data from Figures 2 and 3 are provided in Supplementary Materials in table format.

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Chapter 3

The third chapter investigates the impact of exposure vessel geometry on the observed effect of silver nanoink exposures, using a range of exposure vessels with varying surface to volume ratios. Notably, a number of physiology metrics was significantly affected by changes in surface to volume ratios of the exposure vessels which had been noticed before to a certain extent in a different setup, which underlines the significance of setting a standardized approach in nanopollution assessment. We confirm similar findings to prior research focused on miniaturizing daphnid toxicity tests where we observed significant differences exerted on exposed individuals due to changes in surface to volume ratio.

Surface-to-Volume Ratio Affects the Toxicity of Nanoinks in Daphnids

Dimitrios Kakavas [1], Konstantinos Panagiotidis [1], Keith D. Rochfort [2,†] and Konstantinos Grintzalis [1,*,†]

[1] School of Biotechnology, Dublin City University, D09 Y5NO Dublin, Ireland; dimitrios.kakavas2@mail.dcu.ie (D.K.); konstantinos.panagiotidis@dcu.ie (K.P.)
[2] School of Nursing, Psychotherapy and Community Health, Dublin City University, D09 NA55 Dublin, Ireland; keith.rochfort@dcu.ie
* Correspondence: konstantinos.gkrintzalis@dcu.ie; Tel.: +353-17007391
† These authors contributed equally to this work.

Abstract: The Organization for Economic Co-operation and Development (OECD) has set widely used guidelines that are used as a standardized approach for assessing toxicity in a number of species. Given that various studies use different experimental setups, it is difficult to compare findings across them as a result of the lack of a universally used setup in nano-ecotoxicology. For freshwater species, Daphnia magna, a commonly used filter feeding crustacean, can generate significant molecular information in response to pollutant exposure. One factor that has an effect in toxicity induced from nanomaterials in daphnids is the surface-to-volume ratio of the exposure vessels; however, there is limited information available about its impact on the observed effect of exposure. In this study, daphnids were exposed to silver nanoparticle ink in falcon tubes and Petri dishes for 24 h. Toxicity curves revealed differences in the observed mortality of daphnids, with animals exposed in Petri dishes displaying significantly higher mortality. Differences in the activities of a number of key enzymes involved in the catabolism of macromolecules and phosphate were also observed across the exposure setups, indicating possible differences in the toxicity mechanism of silver nano-ink. Understanding the impact of factors relevant to experimental setups in ecotoxicology can increase the reproducibility of testing, and also reduce experimental costs, time, generated waste, and daphnids used in research.

Keywords: *Daphnia magna*; nanoparticles; silver nano-ink; toxicity; enzyme kinetics; mortality; feeding rate; surface-to-volume ratio

3.1 Introduction

Nanoparticles (NPs) are currently present in an increasing number of products used in our daily lives, and their commercial applications are increasing every year. The market of NPs reached more than USD 2 billion in the U.S. alone by 2021 (2021), with most NPs being

used in the industrial, medical, and food sectors (Stark et al., 2015). Titanium dioxide, silica, and carbon nanotubes are among the most commonly used products, of which are there many (Mohajerani et al., 2019). With more NPs being applied annually, more and more products find their way into the terrestrial and aquatic environment as emerging pollutants (Islam et al., 2021), and knowledge of their true impact on the environment is severely lacking. Crucial information on the toxicity of NPs and their environmental impact on the aquatic environment can be obtained with toxicity testing using bioindicator species, e.g., crustaceans such as *Daphnia magna* (*D. magna*), mussels such as *Mytilus galloprovincialis* (Efthimiou et al., 2021), and other species that are less advanced in their evolutionary development, which are used as alternative approaches to replace the use of higher animals in research (Avila et al., 2020, Westmoreland et al., 2022). The planktonic crustacean *D. magna* is one of the most commonly used bioindicator species in toxicity testing and environmental monitoring (Reyes et al., 2022). Other daphnia species are also used in both acute and chronic toxicity testing (Connors et al., 2022, Duchet et al., 2023). A variety of biomarkers can be used to assess the impact of pollutant exposure to the physiology of exposed individuals (Sanpradit and Peerakietkhajorn, 2023, Kim et al., 2023, He et al., 2023, Duchet et al., 2023), providing the means to detect any kind of adverse effect (e.g., genotoxicity and cytotoxicity) on aquatic life.

The negative impact of metallic NPs, such as silver NPs, on in vivo and in vitro systems has been well documented in multiple studies (Marambio-Jones and Hoek, 2010, Navarro et al., 2008a, Navarro et al., 2008b, Stevenson et al., 2013, Yin et al., 2011, Zhao et al., 2021, Lekamge et al., 2018, Tortella et al., 2020, Michalaki and Grintzalis, 2023). In the available literature, there is a plethora of evidence that illustrates the adverse effects of silver nanoparticles (AgNPs) on aquatic life. AgNPs have been documented to generate oxidative damage and tissue damage to fish (Sibiya et al., 2022, Pecoraro et al., 2017), which can impair the survival and growth of invertebrates such as *D. magna*, as well as other aquatic life, including algae (Din et al., 2023, Andrade et al., 2022, Manikandan et al., 2023). However, it is worth noting that in the available literature, studies on the ecotoxicological impact of AgNPs have shown inconsistent results (Cui et al., 2017, Hou et al., 2017, Ribeiro et al., 2014, Seitz et al., 2013, Reilly et al., 2023), where the EC_{50} values of AgNPs vary significantly across studies due to the inconsistency of experimental designs across laboratories. Therefore, this makes the evaluation of the impact of AgNPs in the ecotoxicological context very difficult. One possible explanation for such discrepancies is the multitude of NP characteristics that impact its toxicity (e.g., size (Seitz et al., 2015), coating (Kim et al., 2016), and shape (Auclair and Gagne, 2022)); however, there is little-

to-no available information on the fate of NPs in the test system. By increasing the focus on the test system itself, we may be able to better understand the mechanistic aspects of nano-toxicity and eliminate the way in which factors which are unrelated to pollutants (NPs) themselves impact the study.

Phenotypic and enzyme markers of physiology were used to evaluate the impact of S:V on the physiology of daphnids following exposure to nano-inks. In relation to NP toxicity testing, this study demonstrates that the traditionally varying experimental setup of toxicity exposures can generate wildly divergent results due to differences in the S:V of exposure vessels alone. Furthermore, we observed that S:V can alter the certain endpoints of animal physiology, even in the absence of nano-ink pollutants.

3.2 Results

Shallow vessels such as Petri dishes have a high S:V ratio, while tall or deeper vessels such as Falcon tubes have a lower S:V ratio. The impact of S:V was investigated here in the context of how it affected mortality and biochemical and phenotypic markers of physiology of daphnids in response to exposure to silver nano-inks.

3.2.1 Acute Exposure to Silver Nanoparticle Ink in Different Vessels

The toxicity of silver nano-ink was assessed in three exposure conditions: falcon tubes with 50 mL of media, and Petri dishes with 50 mL or 100 mL of OECD media. Toxicity curves were plotted and the EC values were calculated in a similar order of magnitude; however, the falcon tube had the highest EC_{50} value of 4.33 µL of nano-ink/L, whereas the EC_{50} values of the Petri dish were 1.443 µL/L and 1.182 µL nano-ink/L for 50 and 100 mL of media, respectively (Figure 1). EC_1 values of the toxicity curves were calculated and used for the selection of exposure concentrations for daphnids (EC_1^{Falcon}: 0.561 µL/L, EC_1^{Petri}: 0.77 µL/L). The non-lethal exposure concentration selected was used for the assessment of silver nano-ink exposure to the activity of key enzymes in daphnid metabolism. To reduce the number of experimental variables throughout the exposures, the 50 mL Petri dish was selected as a comparison vessel to the 50 mL Falcon tube. The decision to employ this approach was based on the intention of avoiding any alterations to other vessel parameters, such as volume and animals/mL (the crowding of daphnids) in the vessel comparison, in order to eliminate their potential impact on silver nano-ink toxicity.

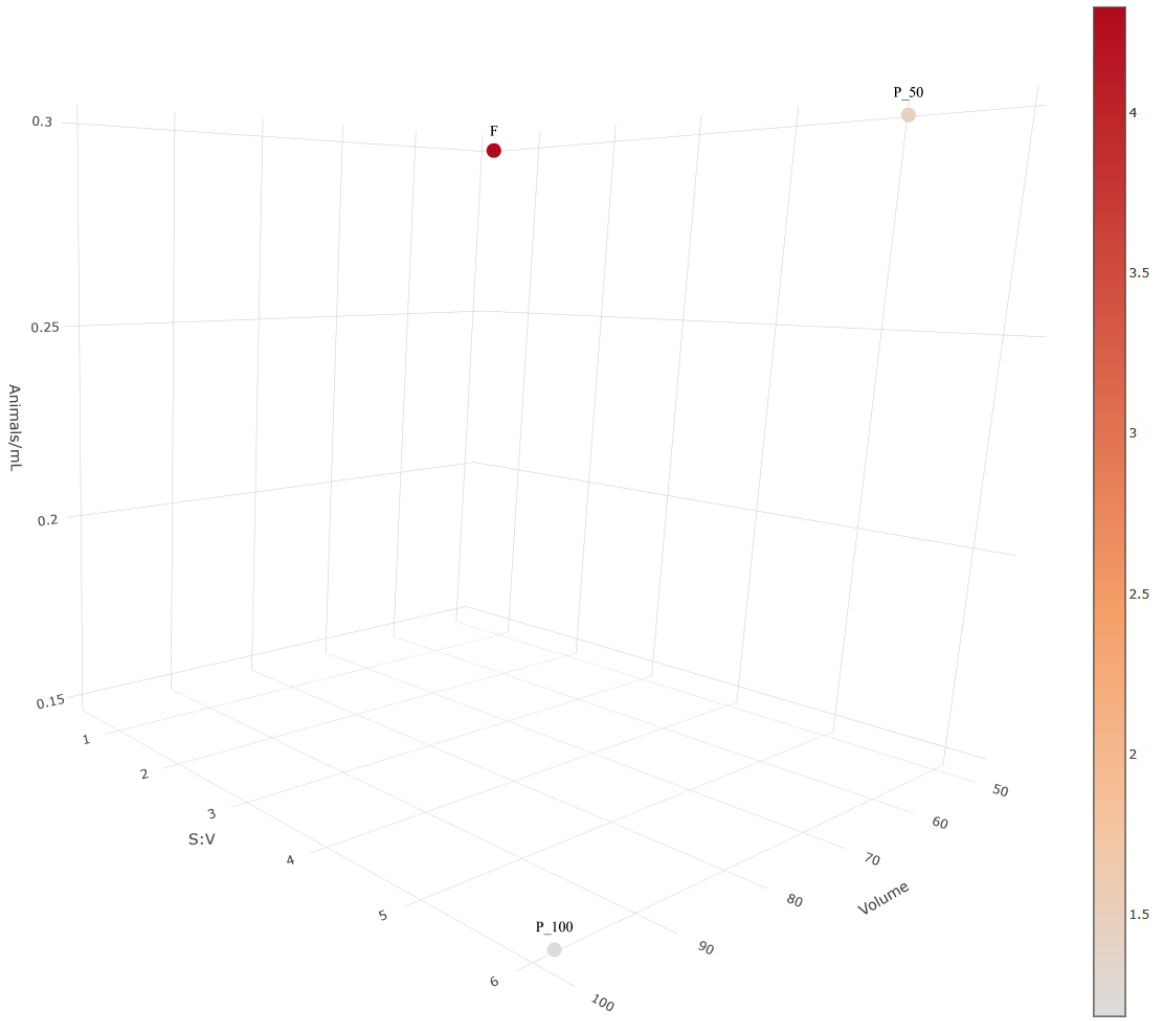

Figure 1. The impact of S:V, volume, and animals/mL on the toxicity of silver nanoparticle ink. EC_{50} values are expressed in µL/L in the color scale. An interactive version is provided in Supplementary Materials.

3.2.2 Acute Biochemical Responses to Nanomaterials

Exposure to the silver nano-ink in the falcon tube and the Petri dish at a constant volume of 50 mL triggered different responses in the activity of enzymes (Figure 2). Exposure in the falcon tube resulted in a different profile of enzyme activities in the exposed individuals, when compared to the enzyme activities of daphnids in the Petri dish exposure condition. For the Falcon tube exposure condition, the exposure of daphnids in 0.5 µL of nano-ink/L generated statistically significant changes in the activity of β-galactosidase (BGAL) and lactate dehydrogenase (LDH) with an observed increase of 8.84% and a decrease of 8.09%. In the exposure concentration of 1 µL of silver nano-ink/L, the activity of BGAL, peptidase (PEP), acidic phosphatase (ACP), and alkaline phosphatase (ALP) increased by 12.12%, 4.95%, 29.27%, and 41.69%, respectively. A dose-depended effect can be observed for β-galactosidase, ACP, ALP, and reduced thiols in the Falcon tube exposure condition. In the Petri dish exposure condition of 0.5 µL silver nano-ink/L, statistically significant differences

were observed for the activity of LDH (62.66%). Interestingly, this was the largest change in enzyme activity recorded in this study. For the 1 µL/L silver nano-ink exposure condition, a significant increase in LDH activity (56.17%) was observed, followed by a significant decrease in the enzyme activity of peptidase (−11.25%), ACP (−33.45%), and ALP (−20.43%). Dose-dependent enzyme activity changes were also observed for LDH, ACP, and ALP in a concentration-dependent profile.

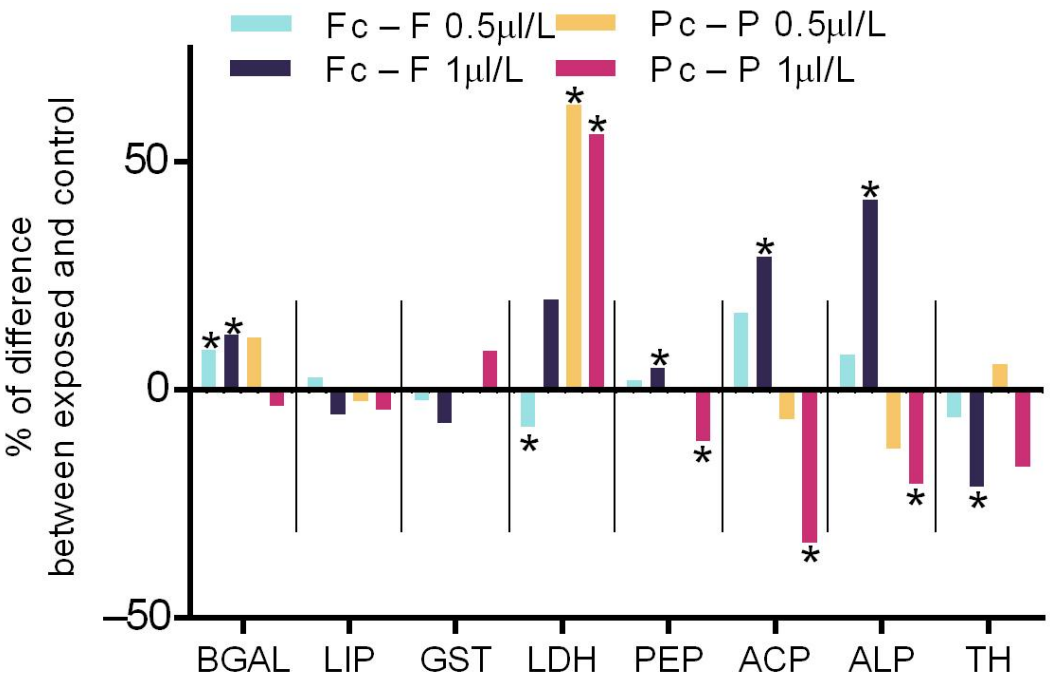

Figure 2. The impact of silver nano-ink in enzyme activity in relation to S:V difference. The data represent the percentage of difference in the enzyme activity of daphnids exposed to silver nano-ink compared to their unexposed control ($N = 4$). The Mann Whitney U test (*) denotes significant differences between the two conditions. Abbreviations: Fc: falcon control, F: falcon exposed, Pc: Petri control, P: Petri exposed, BGAL: β-galactosidase, LIP: lipase, GST: glutathione-S-transferase, LDH: lactate dehydrogenase, PEP: peptidase, ACP: acidic phosphatase, ALP: alkaline phosphatase, TH: reduced thiols.

The impact of S:V on daphnids in the absence of silver nano-ink was assessed using an array of biochemical assays to compare the two control conditions (Figure 3). The unexposed controls for the Petri resulted in statistically significant changes in the activity of BGAL (+5.55%), LIP (−11.1%), and ALP (+27.6%), and a non-statistically significant decreasing trend for LDH, ACP, and reduced thiols compared to the control in the falcon tubes.

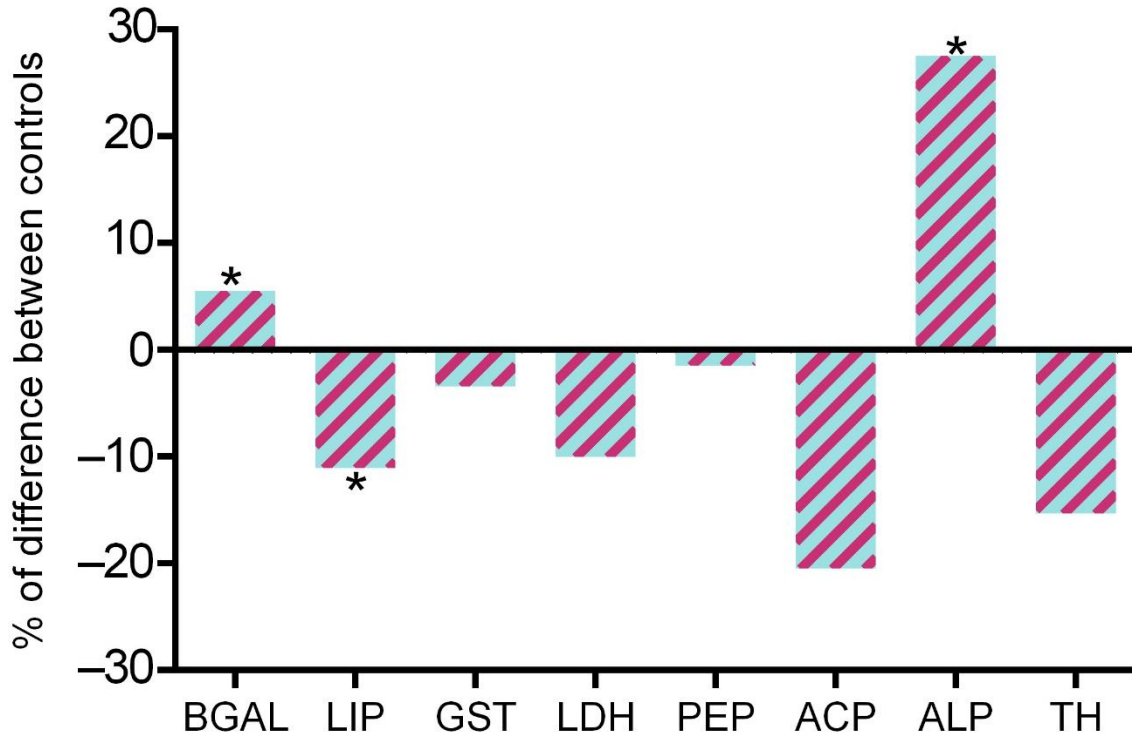

Figure 3. The impact of SV on unexposed controls. Comparisons of enzyme activity between the unexposed controls of the Falcon tube and the Petri dish. The data represent the average (N = 4) replicates for each condition. The Mann Whitney U test (*) denotes significant differences between the two conditions. Abbreviations: BGAL: β-galactosidase, LIP: lipase, GST: glutathione-S-transferase, LDH: lactate dehydrogenase, PEP: peptidase, ACP: acidic phosphatase, ALP: alkaline phosphatase, TH: reduced thiols.

3.2.3 Feeding and Microscope Images

The impact of nano-inks was evaluated with non-invasive tests on the feeding rate of exposed daphnids. The feeding rate is a phenotypic endpoint used in multiple studies to evaluate the physiology of daphnids (Gutierrez et al., 2021, Kelpsiene et al., 2020, Liu et al., 2019). The fluorescent microparticles used were selected because they have a mean particle size of 2.0 μm and D. magna feeds non-selectively on a wide range of particles with sizes ranging from 1 to 50 μm, which would allow microparticles to be tracked with fluorescence microscopy (Ebert, 2005). In addition, toxicity testing revealed that the microparticle concentration used for the assay was not toxic to daphnids. The feeding rate of daphnids exposed in Petri dishes was significantly more impacted compared to daphnids exposed in Falcon tubes. Furthermore, differences in the feeding rate when compared to their respective controls were higher for daphnids exposed in Petri dishes (Figure 4).

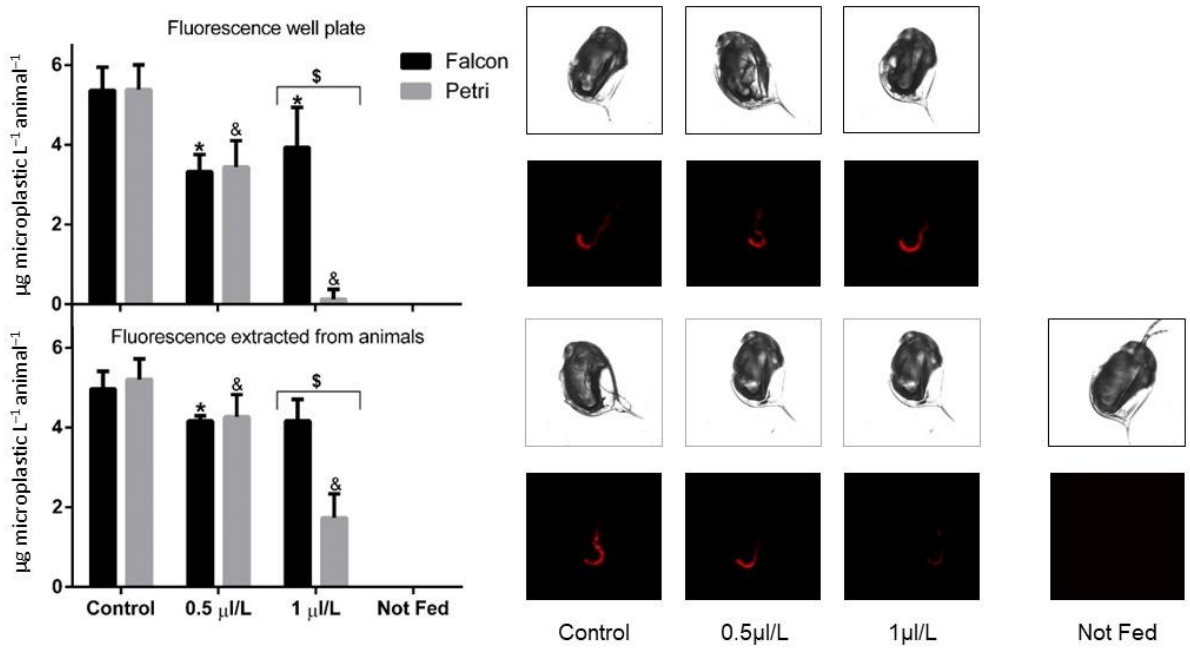

Figure 4. The impact of silver nano-ink exposure on the feeding rate of daphnids. The ingestion of fluorescent microparticles was visualized using bright field and fluorescence microscopy (panel A). The feeding rate was quantified by the fluorescence of ingested microparticles in the incubation media or extracted from daphnids after 30 min (panel B). The data represent the mean ± standard deviations (N = 4). The *, & symbol indicates statistically significant differences found by the Mann Whitney U test, from the unexposed control to silver nano-ink, and from 0.5 µL/L to 1 µL/L, respectively. The '$' on the bracket symbol indicates statistically significant differences between the same concentration of silver nano-ink in the two different exposure vessels. "Not Fed" displays the fluorescence measured in daphnids that were not incubated with microplastic before the start of the start of the feeding assay to acquire any background fluorescence.

3.3 Discussion

In this study, we investigated the impact of vessel S:V in the context of nanotoxicology by exposing daphnids to AgNPs in two vessels with different S:V ratios. Most laboratory studies that use NPs do not use the same, or sometimes even similar, vessels for the toxicity assays, which can create a discrepancy in the results. In any experimental setup for toxicity testing for NPs, it is mandatory that the physicochemical properties are considered. NPs are never fully dissolved in solution; hence, an NP solution is characterized as a colloidal suspension. Consequently, the S:V of the exposure vessel can change the toxicity of pollutants that create colloidal suspensions (Levard et al., 2012) because it could influence the availability of NPs to exposed individuals (Lekamge et al., 2019, Seitz et al., 2013). According to the available literature (Grintzalis et al., 2017), S:V can impact several parameters of daphnid physiology, such as mortality and feeding rate; however, information on the true impact of S:V on NP toxicity remains extremely scarce. To the best of our knowledge, there are only two studies that have investigated the impact of S:V in a

toxicological context (Baumann et al., 2014, Grintzalis et al., 2017). In a study by Grintzalis et al. (Grintzalis et al., 2017), the impact of S:V was investigated under the prism of a non-NP pollutant. The S:V range that was investigated in different vessels was much smaller (0.12–0.90 cm^2 mL^{-1}) than the range used in this study (Falcon tube S:V: 0.493 cm^2 mL^{-1} and Petri dish S:V: 5.089 cm^2 mL^{-1}). According to Grintzalis et al. (Grintzalis et al., 2017), S:V, as a factor in the experimental design, did not impact the toxicity of $CdCl_2$ to daphnids, while our findings indicate that S:V has an insoluble contaminant impact on the daphnids. Baumman et al. (Baumann et al., 2014) reported that S:V impacted the toxicity of AgNPs in a miniaturized toxicity approach compared to its control. However, the experimental design in the study by Baumman et al. resulted in changes in the daphnid crowding inside the exposure vessels, which could result in additional factors besides the vessel S:V impacting the observed effect. The changes to daphnid crowding could have also influenced the potential impact of S:V as there is no available information on the synergistic effects of experimental design factors, such as S:V, volume, and daphnid crowding, on NP exposure. Furthermore, the S:V vessel differences that were investigated in the study by Baumman et al. were smaller (2.08–3.50 cm^2 mL^{-1}) compared to the difference of S:V of vessels investigated in this study. Furthermore, the uniformity of volume, the number of daphnid individuals per replicate, and the vessel material were maintained across all tested conditions. Additionally, the sole experimental parameter that changed across the exposure vessels was the S:V.

One plausible explanation for the observed impact of S:V is that daphnids undergo changes in the activity of many key enzymes merely by being placed in a flattened or shallow exposure vessel with a small water column, such as a Petri dish, even without presence of a pollutant (Figure 3). The reason for these key changes could be attributed to the restriction of the diel vertical migration of daphnids (Ebert, 2005); however, no available information exists in the literature on the impact of restricting diel migration in daphnids. What is known so far is that diel vertical migration is related to the presence of predators, and that chemical cues such as kairomones are responsible for "alerting" changes in the daphnid's environment (Hahn et al., 2019).

The profile of enzyme activity for individuals in the two exposed conditions was divergent when they were compared with their respective controls, although there were a few similarities as well. According to Choi et al. (Choi et al., 2010), pollutants with similar modes of toxicity have similar profiles of gene expression, and, for AgNPs, it is known to be a case for aquatic organisms such as *Danio rerio* (Choi et al., 2010), as well as other

Cladocera species such as *Chydorus sphaericus* (Wang et al., 2012) and *Daphnia magna* (Poynton et al., 2012). According to the available literature (Poynton et al., 2012), AgNPs and Ag^+ generate different gene expression patterns in exposed daphnids. In a solution that contains AgNPs, daphnids are exposed to NPs as well as Ag^+ ions because of the dissolution (Lee et al., 2018); therefore, one hypothesis states that daphnids exposed in Falcon tubes may ‘’interact’’ more with silver in its ionic form rather than silver NPs due to the possible NP sedimentation (Devasena et al., 2022). Our findings support this hypothesis, as the observed enzyme activity in daphnids exposed in Falcon tubes or Petri dishes had distinct differences (Figure 2). According to the available literature, there is evidence that suggests the differentiating mechanism of toxicity on a molecular level for AgNPs and Ag^+ ions in daphnia (Sun-young Park, 2010). Research on the mechanism of toxicity for AgNPs reports that silver ions cause inhibitory changes to the activity of the Na^+,K^+-ATPase, which in turn disrupts the normal function of the gills (Bianchini and Wood, 2003, Hou et al., 2017). Another suggested mechanism of toxicity for the AgNPs is the disruption of the RNA polymerase via ion binding on specific sites, according to the biotic ligand model for daphnia (Niyogi and Wood, 2004, Janes and Playle, 1995), as well as the protein digestion and absorption pathways of the RNA polymerase (Sgrignani and Magistrato, 2012). Furthermore, Poynton et al. (Poynton et al., 2012) discovered that AgNPs can disrupt proteolysis, which corresponds with our findings showing reduced enzyme activity in both acetic and alkaline phosphatases, as well as peptidases for Petri exposure conditions (Figure 2).

This study posits that the inhibition of the proper phosphorylation of crucial proteins in energy production pathways is balanced out, to some extent, by an elevation in LDH activity (Figure 2), which is likely a compensatory response by daphnids to counteract the energy loss via anaerobic metabolism. The present investigation lends support to this hypothesis as a notable distinction between the two exposed conditions was observed with regard to enzyme activity for ACP, ALP, and LDH. Our results align with prior research, which has reported an increase in LDH activity in acute exposure of neonates to AgNPs (Ulm et al., 2015). In contrast to the daphnids exposed in Falcon tubes that exhibited increased phosphatase enzyme activity, daphnids exposed in Petri dishes manifested reduced phosphatase activity and increased LDH activity, also hinting at a distinctive mode of toxicity for the two vessels (Kim et al., 2011, Sun-young Park, 2010).

The exposure of daphnids to silver nano-ink was further evaluated with measurements of their feeding rate (Conine and Frost, 2017). The feeding rate of daphnids is an important

endpoint used in daphnid toxicity testing, and, according to the available literature, other crustacean species such as *Gammarus* sp. silver nanoparticles (AgNPs) have even been reported to cause disturbances in their feeding rate (Andrei et al., 2016, Dedourge-Geffard et al., 2009). Silver nano-ink exposures impacted daphnids exposed in the two tested vessels differently (Figure 4). Dose-dependent effects were not observed for the feeding rate in the Falcon tube as both 0.5 µL/L and 1 µL/L exposure conditions displayed the same impact. The Petri dish exposure condition displayed a dose-depended effect, as the 0.5 µL/L and 1 µL/L exposure conditions had statistically significant differences with their control in terms of observed feeding rate, which further emphasizes the different impact that silver nano-ink had in the two exposure conditions. In contrast to the key enzyme activity endpoint, the control conditions for the feeding endpoint did not display any statistical significant differences. This shows that the two exposure vessels tested do not have any impact on the daphnid feeding rate endpoint before the exposure to pollutants. The findings of Ribeiro et al. (Ribeiro et al., 2014), which report that AgNPs exposure results in a greater impact on the feeding rate of daphnids compared to the impact of ionic silver exposure, also support this interpretation of results. Our hypothesis for this effect is based on the different dimensions of the two exposure vessels. In OECD media, AgNPs have been reported to rapidly aggregate (Romer et al., 2013); therefore, the two exposed groups did not interact with the same form of silver during their exposure periods due to the S:V differences in their respective vessels. There is already evidence in the available literature which suggests that there are differences in silver uptake in daphnia when comparing ionic silver and AgNPs (Khan et al., 2015, Zhao and Wang, 2010).

In conclusion, our study highlights the importance of critical thinking in the experimental design of nano-pollutants. However, the knowledge gap in nano-pollutants remains a matter of grave concern. Exploring the importance of different factors in nanotoxicity testing could lead to the development of an experimental design framework for nano-pollutants if the importance of different factors in nanotoxicity testing is explored further. A uniform experimental design for nano-pollutants could greatly benefit research and improve the relevance of the generated results.

3.4 Materials and Methods

3.4.1. Culturing Daphnids and Exposures

Cultures of daphnids were maintained in glass beakers in OECD media (final concentrations of 0.29 g of $CaCl_2.2H_2O$/L, 0.123 g of $MgSO_4.7H_2O$/L, 0.065 g of $NaHCO_3$/L, 0.0058 g of KCl/L, and 2 µg of Na_2SeO_3/L, pH 7.7) under a 16 h:8 h light–dark photoperiod at 20 °C. All chemicals used in this study were of the highest purity >99.9% and quality. KCl, Na_2SeO_3, latex beads, carboxylate-modified polystyrene, fluorescent red, bovine serum albumin, brilliant blue G, *p*-nitrophenyl butyrate, 2-nitrophenyl-B-D-galactopyranoside, 1-chloro-2,4-dinitrobenzene, L-glutathione reduced, sodium phosphate dibasic were purchased from Sigma-Aldrich. 4,4'-dithiodipyridine, $CaCl_2 \cdot 2H_2O$, $MgSO_4.7H_2O$, $NaHCO_3$, HCl, *p*-nitrophenyl phosphate, boric acid, ammonium acetate and NaOH were purchased from Fisher Scientific. The media were renewed every five days and the cultures were fed daily with *Chlamydomonas rheinharti* suspension and an organic seaweed extract (*Ascophylum nodosum*) only upon media renewal. For silver nanoparticle ink exposures, the neonates (<24 h) were collected from the third brood of their mothers and cultured until the age of four days old. Animals were exposed for 24 h in Falcon tubes and Petri dishes (Figure 5). Fifteen daphnids were used per replicate of each vessel in 50 or 100 mL of volume and full toxicity curves were obtained with a minimum of three experiments conducted to reduce differences between the different batches of animals in mortality (Figure 1). All plots were calculated using the four parameter logistic (4PL) model, following the equation Span = Top − Bottom and Y = Bottom + (Top-Bottom)/(1 + 10^((LogIC50-X) × HillSlope)) using GraphPad software. The top and bottom parameters were commonly fixed to 100 and 0, accordingly.

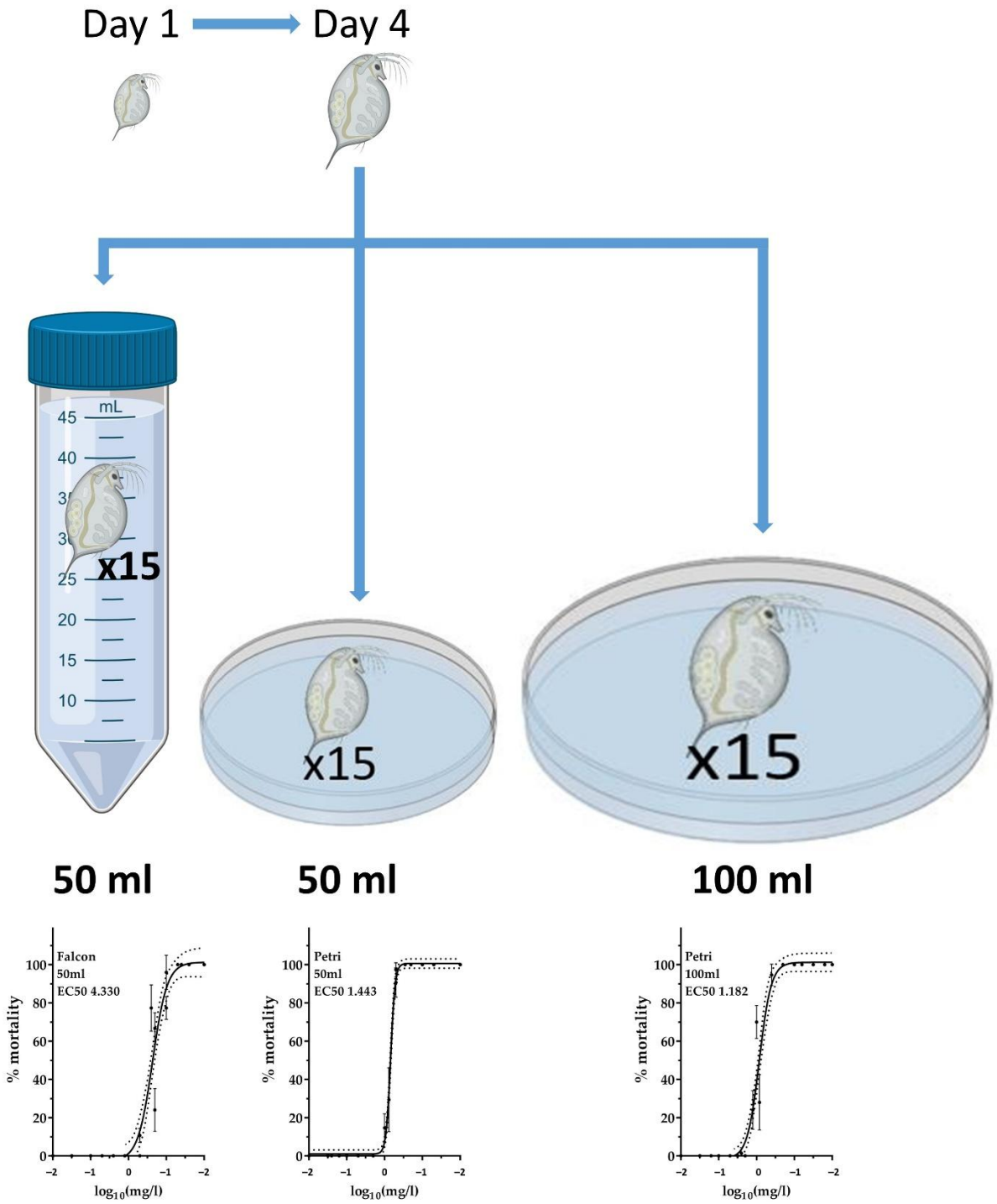

Figure 5. Experimental design for exposures of daphnids to silver nano-ink in Falcon tubes and Petri dishes.

3.4.2. Exposure of Daphnids to Silver Nanoink and Markers of Physiology

Daphnids (four days old) were exposed to 0.5 µL/L and 1 µL/L of silver nanoparticle ink for 24 h. Thirty daphnids were pooled together and homogenized in a 0.5 mL buffer using a pestle homogenizer. The homogenate was centrifuged and the clear supernatant was

collected and assessed for protein and enzyme activity. The protein was quantified using a sensitive Bradford method (Kontoghiorghes, 2020) to normalize the enzyme activity. The activity of phosphatases was assayed in 100 mM citric acid (pH 4.5) (for ACP) or 100 mM boric acid (pH 9.8) (for ALP) using the substrate *p*-nitrophenyl phosphate. The reaction was alkalined and the absorbance of produced *p*-nitrophenol was measured at 405 nm and converted to units per protein. Similarly, the activities of BGAL and LIP were quantified via the generation of nitrophenol from the catalysis of *o*-nitrophenyl-β-galactoside or *p*-nitrophenyl butyrate, respectively, in a phosphate buffer (pH 7.2). The activity of the peptidase (specifically γ-glutamyl transferase) was quantified using L-leucine-4-nitroanilide as a substrate. Then, 200 µL of the sample, appropriately diluted in a 50 mM phosphate buffer (pH 7.2), was mixed with 50 µL of the 8 mM substrate in 100% DMSO, and the release of the product (*p*-nitroanilide) was measured by continuous kinetics at 418 nm (van Acker et al., 1998). GST activity was measured via the formation of a complex between reduced glutathione with 1-chloro-2,4-dinitrobenzene at 340 nm (Fernandez et al., 2002). The activity of LDH was quantified by monitoring the decrease in absorbance at 340 nm caused by the oxidation of NADH. The substrate used was a 1:1 mixture of 40 mM pyruvate and 0.5 mM NADH. In addition, 200 µL of the sample, appropriately diluted in the 50 mM phosphate buffer (pH 7.2), was mixed with 50 µL of the substrate. The resulting reaction was measured via continuous kinetics at 340 nm.

3.4.3. Feeding Assay

Carboxy microplastics (L3030, Sigma Aldrich, St. Louis, MO, USA) were used in this study to assess the ingestion (feeding) rate of daphnids. These microplastics were tested initially using 1-day-old neonates as they are more sensitive, and no toxicity was recorded for up to 10 h of exposure to microplastics. The concentrations used for this assessment ranged up to 52 mg/L; however, for the feeding assay, a concentration of 13 mg/L was used. The fluorescence from microparticles was measured as Ex/Em 560/590 nm using a TECAN plate reader (Männedorf, Switzerland). The ingested microparticles were measured twice, first as fluorescence in the media during the feeding assay, and the fluorescence difference in the media from the initial time of incubation was converted to the amount of ingested microplastic using a standard curve and expressed per animal. Then, the feeding rate of daphnids was measured after 30 min from the ingested carboxy microplastics following the homogenization of animals and the extraction of microparticles in 0.5 mL of ddH_2O. The ingestion of microparticles was also confirmed with fluorescence microscopy using the

TRITC filter (Nikon Eclipse TS100). A separate pool of animals was incubated in the absence of microplastic as a negative control for any animal background fluorescence.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1.

Author Contributions: Conceptualization, K.G. and K.D.R.; methodology, K.G., D.K., and K.P.; formal analysis, investigation, D.K. and K.P.; writing—original draft preparation, D.K. and K.G.; resources, writing—review and editing, supervision, project administration, and funding acquisition, K.G. and K.D.R. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All raw data from this study will be provided upon request.

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Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; and in the writing of the manuscript (however, in compliance with the open access policy and availability of results in science, we are committed to open access publications).

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Chapter 4

The work included in the fourth chapter focuses on another experimental parameter of exposures which is animal crowding using a range of daphnid physiology markers to quantify its impact. Our findings are in accordance with existing literature and confirm significant impact on a number of used markers of physiology following changes in animal crowding. In the context of nanopollution increased crowding results in decreased mortality which is a novel finding that is not adherent to the trend of increased stress due to increased crowding. Some potential reasons as to why this is the case with nanopollutants are also discussed in this chapter. Nanopollution assessment is an ever-evolving fiend with the refinement of the experimental approaches used also being addressed to an extend by this work.

Animal crowding affects the molecular responses of daphnids following exposure to silver nanoink

Dimitrios Kakavas[1,2], Beatrice Engelmann[3], Martin Krauss[4], Ulrike E. Rolle-Kampczyk[3], Martin von Bergen[3,5], Keith D. Rochfort[1,2], Konstantinos Grintzalis[1,2]*

[1]School of Biotechnology, Dublin City University, Republic of Ireland
[2]Life Sciences Institute, Dublin City University, Republic of Ireland
[3]Department of Molecular Toxicology, Helmholtz-Centre for Environmental Research - UFZ, Leipzig, Germany
[4]Department of Effect-Directed Analysis, UFZ - Helmholtz Centre for Environmental Research, Leipzig, Germany
[5]Institute of Biochemistry, Leipzig University, Leipzig, Germany

*Corresponding author: Konstantinos Grintzalis, Email: konstantinos.gkrintzalis@dcu.ie

Abstract: Nanomaterials have been increasingly used in many commercial products since the turn of the millennium. However, the presence of nanomaterials in freshwater ecosystems poses a critical threat to aquatic life and requires assessment and monitoring to prevent further degradation. Due to its unique characteristics, nanomaterials circumvent traditional water testing methods, making their detection and risk assessment challenging. Bioindicator species such as waterfleas can be used as sensitive biomarkers to assess the impact of nanomaterial pollution, providing evidence to understand the underlying toxicity mechanisms. This study focused on the influence of animal crowding as the experimental setup parameter, as this might result in additional changes in biomarkers used to assess environmental pollution. It was shown that the animal number can promote deviations in several physiological endpoints, even in the absence of any pollutant, thus underlining the urgency for revising existing pollution assessment methods.

Keywords: *Daphnia magna*; NPs; crowding; silver nanoink; enzyme kinetics; mortality; metabolomics

4.1 Introduction

Use of nanoparticles (NPs) began to gain traction in the medical and industrial domains between the end of 1990s and early 2000s (Bayda et al., 2019, Ramsden, 2011). NPs are a category of nanomaterials (NMs) characterised as particles of sizes ranging from 1 to 100 nm. There is a plethora of NP applications including but not limited to their uses in electronics industry; as bulk material in the manufacturing of computer chips and solar cells,

as well as in medicine; where NPs are used to improve drug delivery and imaging (Han et al., 2019, Mitchell et al., 2021, Taha et al., 2022, Wang et al., 2022). The unique properties of NPs, and specifically the high surface area to volume ratio (S:V) and size-dependent optical and electronic properties, make them appealing for numerous applications. As a result of their fast and widespread adoption in multiple domains, there are still many gaps of knowledge on their potential environmental impact (Williams and Bannon, 2022). Due to their unique properties, NPs can accumulate in the environment and are responsible for the adverse effects on living organisms exposed to them (Rajput et al., 2020).

Conventional methods to monitor water pollution may not be ideal to measure NP presence in the environment. These methods are based on analysing specific water characteristics like pH, phosphate and nitrate presence (Isiuku and Enyoh, 2020), however, there are several limitations regarding NP pollution specifically. For example, total dissolved solids (TDS) or chemical analysis for the presence of heavy metals (Rahmanian et al., 2015, Adjovu et al., 2023) , which might not be sensitive enough to detect the presence of NPs accurately and lead to underestimation or complete omission of true NP pollution levels (Hairom et al., 2021, Janani et al., 2022). Conventional approaches also lack any means to provide information on the adverse effects of NPs following exposure, which is of imperative importance for pollution assessment by providing critical knowledge on bioaccumulation or other related phenomena. Additionally, chemical analysis methods are often time-consuming and expensive, and the results may not always map on a biological framework from which we can assess the environmental impact of the pollutant. Alternatively, new approach methodologies (NAMs) in environmental toxicology provide the means for understanding complex chemical interactions in aquatic ecosystems, which, in an ever-increasingly polluted planet, is of imperative importance to preserve the environment. NAMs harness technologies such as omics, microphysiological systems, as well as computational modelling to evaluate exposure risks, optimize existing testing protocols, and reduce reliance on conventional animal testing (Zolkefli et al., 2020). The advancement of these methods enhances our ability to assess pollution by improving predictions of toxic outcomes and assessment of long-term environmental impacts (Schmeisser et al., 2023).

NAMs like multiple omics techniques have been used extensively to characterise the effects of nanomaterial. Omics technologies, such as transcriptomics, epigenomics, proteomics, metabolomics, and lipidomics, are powerful tools for assessing the toxicity of inorganic nanoparticles (Li, 2024). These methods allow for a comprehensive analysis of the

molecular changes that occur in biological systems upon exposure to nanomaterials (Shin et al., 2018b). By identifying alterations in genes, proteins, and metabolites, researchers can uncover the pathways and functions that are dysregulated by nanoparticles (Li et al., 2024). Omics studies provide mechanistic insight by identifying key genes and gene products that mediate toxicological effects, as well as endogenous functions and pathways dysregulated by nanoparticles (Li et al., 2024). This provides a deeper understanding of the mechanisms underlying the biological responses to nanomaterials (Nguyen and Falagan-Lotsch, 2023). Omics can be used to identify biomarkers of toxicity (Fortino et al., 2022, Gioria et al., 2016). These biomarkers can aid in the development of integrated approaches to testing and assessment (IATA) (Bannuscher et al., 2020).

Bioindicators species like the freshwater crustacean *D. magna*, is a promising approach for assessing the environmental impact of nanoparticle pollution in freshwater (Guilhermino et al., 2021, Lovern et al., 2007, Samadi et al., 2022, Valsesia et al., 2021, Li et al., 2019). Bioindicators can provide critical information on the toxicity and bioavailability of NPs in aquatic ecosystems, as well as on bioaccumulation and biomagnification phenomena observed in food chains (Parmar et al., 2016, Azzazy, 2020). Furthermore, bioindicator species are often more sensitive to environmental changes and pollutants than traditional chemical analyses, offering a means to detect the presence of NPs and other pollutants outside of their respective ranges (Carignan and Villard, 2002). In the context of pollution assessment *D. magna* is a commonly utilized organism, using an array of biomarkers which can provide vital information of the adverse effects of exposure and improve the understanding of the toxicity mechanisms. In recent years, multiple advances in biology and analytical science led to a paradigm shift when it comes to pollution monitoring and assessment (Krewski et al., 2020). This utilization shows that bioindicator use also supports many different approaches while using the same reference which can significantly enhance pollution assessment and monitoring strategies. *D. magna* is also widely used in metabolomics, with about 6500 publications relevant to metabolomics alone since 2015. Metabolomics is the study of metabolites with low molecular weight that originate in biological samples and provide information on the changes in the metabolome of the affected organism as a result of stressor exposure. Thanks to the sensitivity of metabolomics for detecting the actual phenotype, it has been utilized successfully to provide critical information on the toxicity mechanisms behind certain pollutants and has been relevant to understanding better how NMs interact differently with organisms based on their characteristics (Li et al., 2015, Kelpsiene et al., 2023). However, according to available literature, the lack of a standardized experimental setup – especially when it comes to novel

pollutants such as NPs, makes the comparison of results across studies challenging and inconsistent (Martinez et al., 2022, Kakavas et al., 2024, Shin et al., 2018a). The absence of a framework for experimental setups specifically for NMs, allows discordance of information gathered from apical endpoints, which is amplified to an extend when involving more sensitive biomarkers like omics. Therefore, the refinement of the experimental design aimed to assess NP impact on freshwater ecosystems is of imperative importance.

This study focused on the influence of crowding in the experimental set-up as a factor that has not been investigated in relation to NMs when using daphnids as test species. The findings are also relevant for other pollutants, as the biological changes inflicted on daphnids might be reflected in tested biomarkers usually employed for pollution assessment. This was achieved by changing the number of exposed individuals and comparing the observed impact of NP exposure on different endpoints of daphnia physiology. Phenotypic and enzyme markers of physiology of daphnids were used in tandem with toxicity testing and metabolomics. In relation to the impact of crowding, we demonstrated that changes in the number of animals exposed to silver nanoinks will lead to deviations on the observed effect on a number of daphnia physiology endpoints, even in the absence of the pollutant.

4.2 Materials and methods

4.2.1 Culturing daphnids and crowding exposure conditions

Daphnids were cultured in aqueous media under a under a 16h:8h of light:dark photoperiod at 20 °C as previously described (cite your Animals article). Media was renewed every five days in the parent cultures and animals were fed daily with *Chlamydomonas reinhartii* suspension and an *Ascophylum nodossum* extract only upon media renewal. For experiments with NPs, neonates (<24 hours) were collected from the third brood of their mothers and cultured until the age of four days old. Following the culturing phase, 15 four days old daphnids were used per replicate of each vessel in varying volumes of media to simulate the changes in animal crowding, ranging from 5 daphnids up to 60 daphnids per 50 ml (Figure 1).

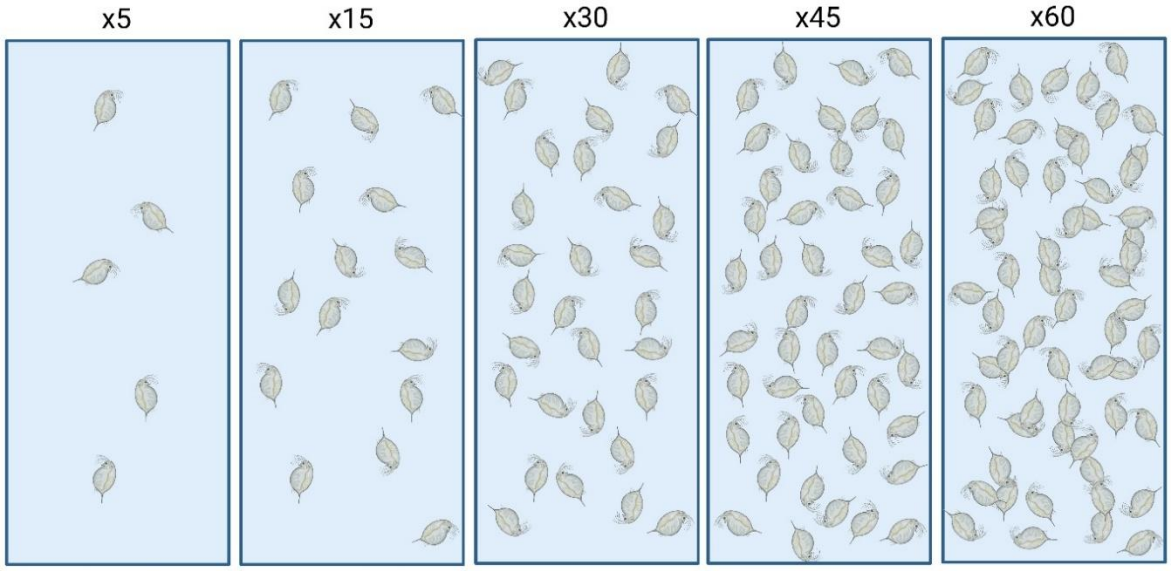

Figure 1. Different crowding of daphnids for exposure groups. Five different numbers of daphnids per 50 ml vessel (seen in white box, from 5 to 60) were used to generate the toxicity curves.

Complete toxicity curves were calculated using four replicates per concentration for each crowding condition. All plots were calculated using the four-parameter logistic (4PL) model, following the equation Span = Top − Bottom and Y = Bottom + (Top-Bottom)/(1 + 10^((LogIC$_{50}$-X)*HillSlope)) using the GraphPad software. The parameters top and bottom were commonly fixed to 100 and 0, accordingly.

4.2.2 Exposures and markers of physiology

Day four daphnids were exposed to 0.15 μl/L and 0.45 μl/L (for metabolomic analysis) of silver nanoparticle ink for 24 hours. The silver nanoink used in these experiments was obtained from Sigma-Aldrich (Product No. 719048). Thirty individuals were pooled together per biological replicate and homogenized in 0.5 ml buffer (assay dependent, see below) using a pestle homogenizer. Following centrifugation of the homogenate, the clear supernatant was collected and assessed immediately for protein and enzyme activity (Michalaki et al., 2022, Grintzalis et al., 2022). Protein was quantified following a sensitive Bradford assay to normalize enzyme activity. For the quantification of protein, 0.2 ml of the sample diluted with water was mixed with 0.05 ml of the CBB:2M HCl reagent and absorbance was measured at 610 nm and converted to equivalents of protein using a BSA standard curve (2-20 μg BSA/ml) (Georgiou et al., 2008, Grintzalis et al., 2015). Phosphatase activity was

measured in 100 mM citric acid pH 4.5 for acidic phosphatase - ACP or 100 mM boric acid pH 9.8 for alkaline phosphatase – ALP, using the substrate *p*-nitrophenyl phosphate (*p*NPP) and measuring the produced *p*-nitrophenol at 405 nm. Reactions of 0.2 ml sample appropriately diluted in each buffer were initiated with 0.05 ml 8 mM *p*NPP and incubated for 30 minutes at room temperature and then alkalined with 0.05 ml 4M NaOH to measure absorbance at 405 nm. Similarly, the activities of lipase (LIP) and β-galactosidase (BGAL) were quantified by the generation of nitrophenol from the catalysis of *p*-nitrophenyl butyrate or *o*-nitrophenyl-β-galactoside, respectively, in 100mM phosphate buffer pH 7.2. Activity of peptidase (specifically γ-glutamyl transferase) was quantified using L-leucine-4-nitroanilide as a substrate. Sample (0.2 ml) diluted appropriately in 50 mM phosphate buffer pH 7.2 was mixed with 0.05 ml 8 mM substrate (prepared in 100% DMSO) and the release of *p*-nitroanilide was measured by continuous kinetics at 418 nm and converted to units of enzyme activity (van Acker et al., 1998). The activity of lactate dehydrogenase (LDH) was quantified by monitoring the oxidation of NADH as the decrease in absorbance at 340 nm. Samples (0.2 ml in phosphate buffer) were mixed with 0.05 ml of 40 mM pyruvate and 0.5 mM NADH (in phosphate buffer) and reaction was measured by continuous kinetics at 340 nm. Glutathione-S-transferase (GST) activity was measured by the formation of a complex between reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Fernandez et al., 2002). Briefly, 0.2 ml sample (in phosphate buffer) was mixed with 0.05 ml 6 mM GSH and 2 mM CDNB. Formation of the GSH-CDNB complex was converted to units using the extinction coefficient of the complex. Reduced thiols were quantified by the reaction of aldrithiol or 4,4-dithiopyridine and the formation of a complex at 325 nm according to the protocol by Grintzalis et. al (Grintzalis et al., 2014).

4.2.3 Targeted LC-MS/MS analysis

To analyse amino acids and central carbon metabolism metabolites, groups of 15 daphnids were frozen immediately in liquid nitrogen following acute exposure. Sample homogenization occurred in 0.6 ml ice-cold methanol: water (HPLC-grade in 8:2 ratio) using an Eppendorf pestle homogenizer to quench metabolic reactions. To clear the homogenates, centrifugation was used, set at 10,000× g, 4 °C for 5 min and the 100 µL of the clear supernatant was vacuum dried with a speedvac (SpeedVac, Eppendorf) and stored at −80 °C for further analysis.

Samples were resuspended in 120 µl water. For amino acids derivatization, 10 µl were evaporated to dryness, resuspended in 50 µl 5% phenyl isothiocyanate (PITC) in ethanol: H_2O: pyridine (1:1:1, v/v/v) and incubated for 25 min at RT. Subsequently, samples were dried to remove excess PITC and resuspended in 10 µl 5 mm ammonium acetate in methanol. Following incubation (10 min, 14000 rpm), 90 µl H_2O: ACN + 0.2% formic acid were added. Central carbon metabolites were analysed using a targeted LC-MS/MS data of each extract was acquired using a QTRAP® 6500+ system (Sciex, Framingham, MA, USA) coupled on-line with an HPLC-system. Initially, 10 µL were injected onto an Agilent 1290 II infinity UPLC system (Agilent Technologies Inc., Santa Clara, CA, USA). Chromatographic separation was achieved using a XSelect HSS T3 XP column (2.1 × 150 mm, 2.5 µm, 100 Å; Waters, Milford, MA, USA). Metabolites were eluted at a flow rate ranging from 0.4 ml/min to 0.15 ml/min with a non-linear 33 min gradient. Mobile phases A and B were 10 mM tributylamine, 10 mM acetic acid, 5% methanol, 2% 2-propanol (pH 7.1) and 100% 2-propanol, respectively. Autosampler was maintained at 5°C with the column oven set at 40 °C. Mass spectrometric measurement was performed in negative ionization mode.

In regards to amino acid abundance, each derivative was diluted 1:25 and 10 µl were injected onto a Waters Acquity UPLC system coupled on-line with a QTRAP® 5000 mass spectrometer (Sciex, Framingham, USA). Chromatographic separation was achieved with an Agilent Zorbax Eclipse XDB-C18 column (3.5µm, 3.0 x 100 mm) using a constant flowrate of 0.5 ml/min and water + 0.2% formic acid and ACN + 0.2% formic acid as mobile phases A and B, respectively. The linear LC gradient was as follows: 0-0.5 min at 0% B, 0.5-4 min 0-70% B, 4-5.3min 70% B, 5.3-5.4 min 70-0% B, 5.4-7.3 min 0% B. The autosampler was maintained at 10 °C, the column oven was set at 50 °C and the QTRAP was set up to positive ionization mode. Identification and quantification were based on specific MRM transitions used for both amino acids and central carbon metabolites. External calibration curves were measured for linear regression. Peak integration was determined in SciexOS® software (Version 3.0.0). Data was normalized as a fold change over the average of the respective control, principal component analysis was performed using the Multiple Experiment Viewer (Saeed et al., 2003) and heatmaps were made using the GraphPad Prism version 10.

4.2.4 Statistical analysis

Results were presented as mean ± standard deviation (SD) and were analysed and plotted with the GraphPad Prism software. Statistically significant differences were identified using one-way and two-way ANOVA when suited for specific comparisons as well as Student's *t*-test with Welch's correction with a *p* value of 0.05.

4.3 Results

4.3.1 The effect of crowding on the toxicity of silver nanoparticle ink

The toxicity of silver nanoink was impacted by the number of exposed daphnids in each crowding condition as can be seen in Figure 2. The calculated EC_{50} values varied between 0.24 µl/L for 5 daphnids/50ml and 0.48 µl/L for the 60 daphnids/50ml group. The observed mortality values remained within the same order of magnitude for all conditions and a trend of decreased mortality can be observed as daphnid crowding increases, as for both 24h and 48h the order of observed EC_{50} values for the 5 exposure conditions remained the same: $EC_{50}^{60d/50ml} > EC_{50}^{45d/50ml} > EC_{50}^{30d/50ml} > EC_{50}^{15d/50ml} > EC_{50}^{5d/50ml}$. Additionally, this observed divergence of toxicity among tested groups was reduced in 48h of exposure, with an exception for the group of 5 daphnids/50ml which was the most sensitive to exposure group.

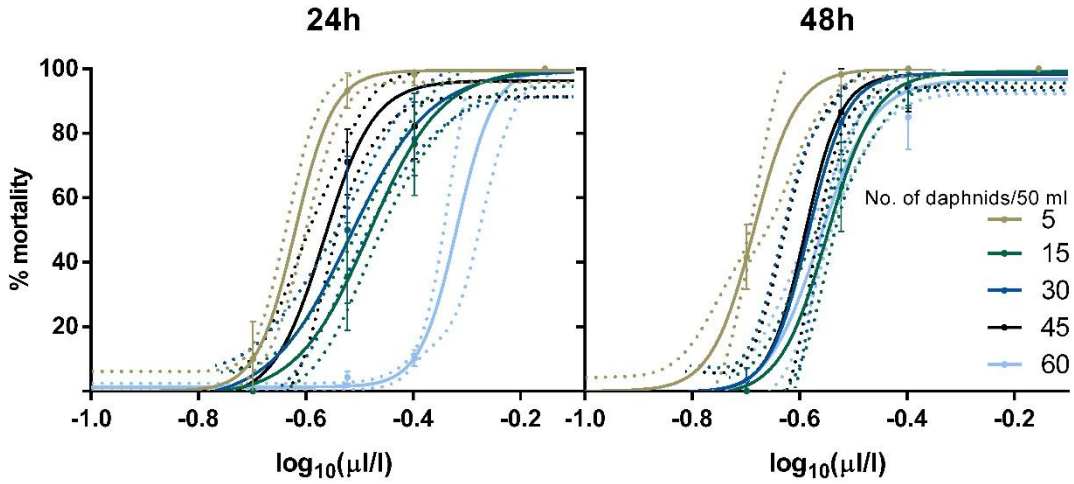

Figure 2. Acute toxicity curve for silver nanoparticle (AgNP) nanoink in three exposure conditions for 24 hours of exposure. Data represents average ±SD (N=4) replicates for each concentration. EC50 values depict the AgNPs concentration in µl/L of silver nanoink.

4.3.2 The impact of crowding on key enzyme activities among control and exposed groups

To evaluate the impact of animal crowding to the toxicity of silver nanoink, key enzymes of daphnid physiology were also investigated. The groups selected were 5, 15, 30 and 45 daphnids, while the 60 daphnid group was omitted for this biomarker as it had already greatly differentiated in observed toxicity during exposures. In absence of NPs, control groups showed statistically significant differences in enzyme activity (Figure 3), with PEP and LDH showing the most significant differences among tested groups, followed by LIP and GST.

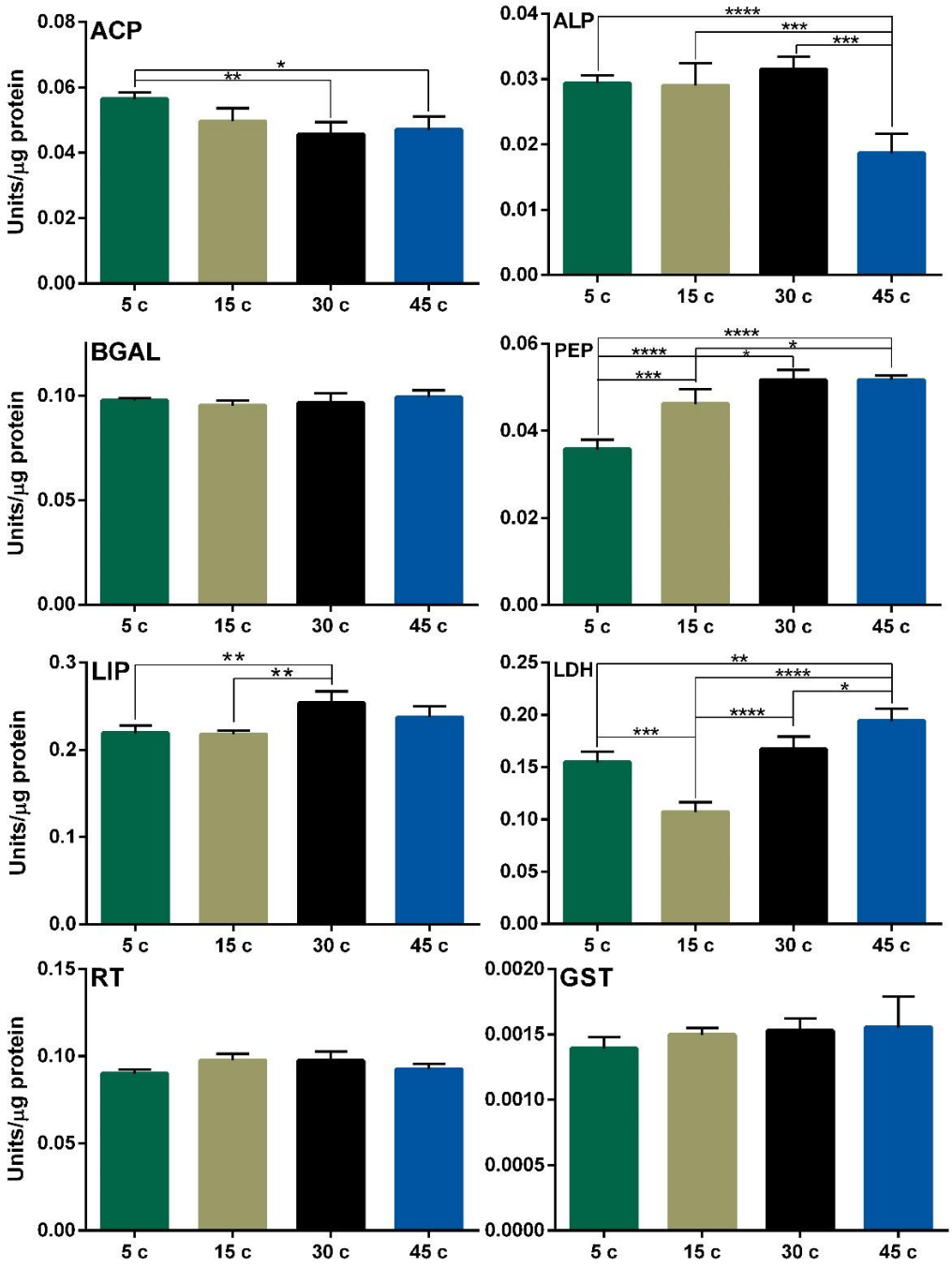

Figure 3. Impact of daphnid crowding on key enzyme activity among controls. Data represents average ±SD (N=4) replicates for each crowding condition. Statistically significant by one-way ANOVA (*) denotes significant difference among control groups with *p*-values of $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). Abbreviations: 'C' denotes control group of daphnid crowding for 5, 15, 30 and 45 daphnia respectively. BGAL: β-galactosidase, LIP: lipase, GST: glutathione S-transferase, LDH: lactate dehydrogenase, PEP: peptidase, ACP: acidic phosphatase, ALP: alkaline phosphatase, RT: reduced thiols.

4.3.3 Impact of crowding on key enzyme activity among exposed groups

Statistically significant differences among exposed groups show clear differentiation of NP impact due to crowding. Different crowding groups were exposed to silver nanoink using a sub-lethal exposure concentration of 0.15µl/l derived from their toxicity curves. PEP and RT had the most variation, followed by LIP, ACP, ALP and LDH as enzyme activity showed statistically significant differences among exposed groups in all but two enzymes (BGAL, GST). Differences observed generally tended to be more pronounced in comparisons among groups with higher differences in daphnid crowding (e.g. 45 daphnids vs 5 daphnids).

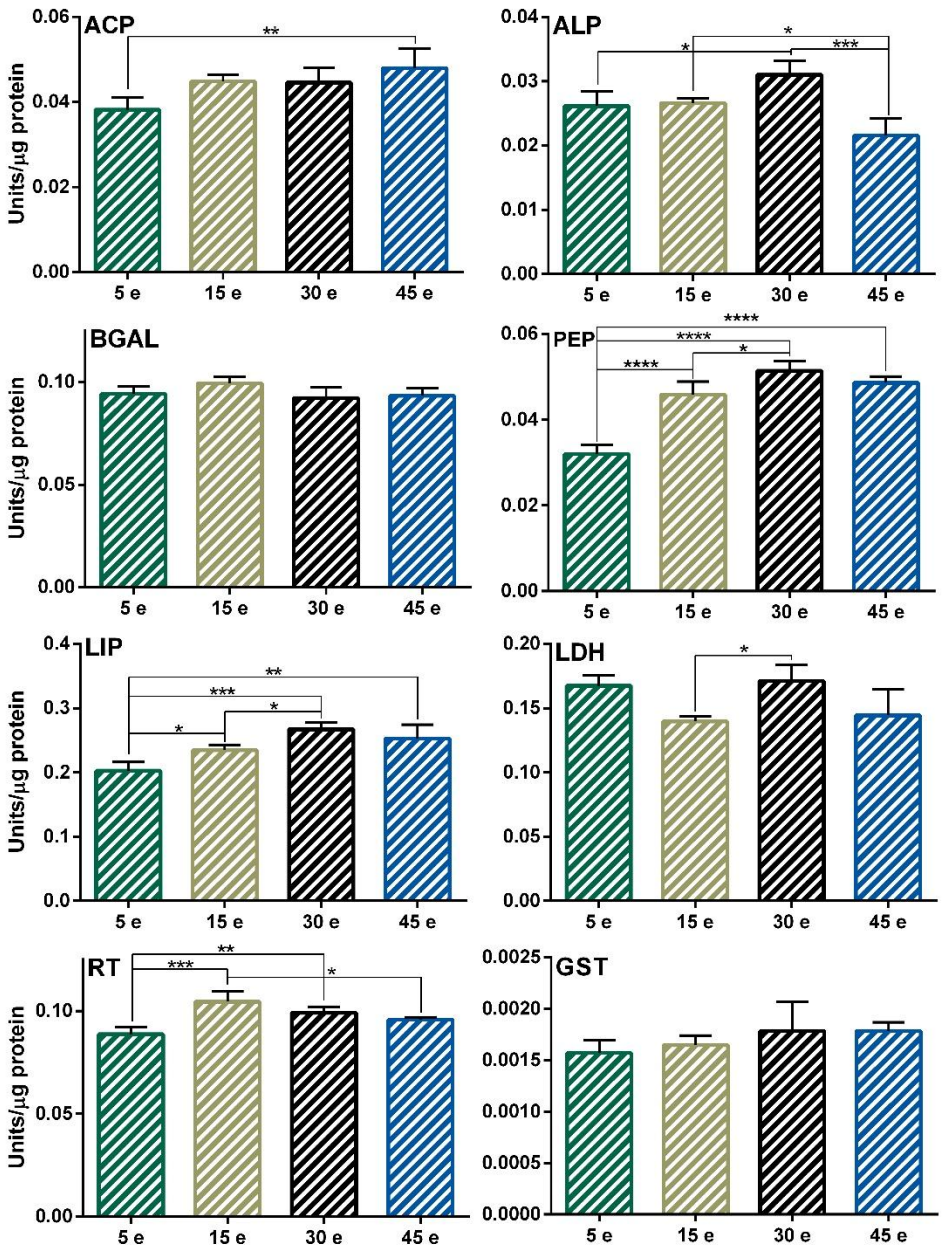

Figure 4. Impact of daphnid crowding on key enzyme activity among exposed groups. Data represents average ±SD (N=4) replicates for each condition. Statistically significant by one-way ANOVA (*) denotes significant difference among control groups with p-values of $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). Abbreviations: 'E' denotes exposed group of daphnid crowding for 5, 15, 30 and 45 daphnia respectively. BGAL: β-galactosidase, LIP: lipase, GST: glutathione S-transferase, LDH: lactate dehydrogenase, PEP: peptidase, ACP: acidic phosphatase, ALP: alkaline phosphatase, RT: reduced thiols.

4.3.4 Impact of silver nanoink exposure in different crowding setups

When compared to their respective controls, crowding conditions showed differences in their response profile following acute exposure. Statistically significant changes were

observed in comparisons of exposed conditions to their respective controls (Figure 5) for the activity of LIP for 15d (15 daphnids) (+7.85%), GST for 5d (5 daphnids) (+12.62%) and 15d (+10%), LDH for 15d (+30.35%) and 45d (-25.62%), PEP for 5d (-5.96%), as well as for the activity of ACP for 5d (-32.34%) and 15d (-9.66%) exposure condition (Figure 4). Similarly with previous analyses, the differences among crowding setups appear intensified following a reduction in daphnid crowding.

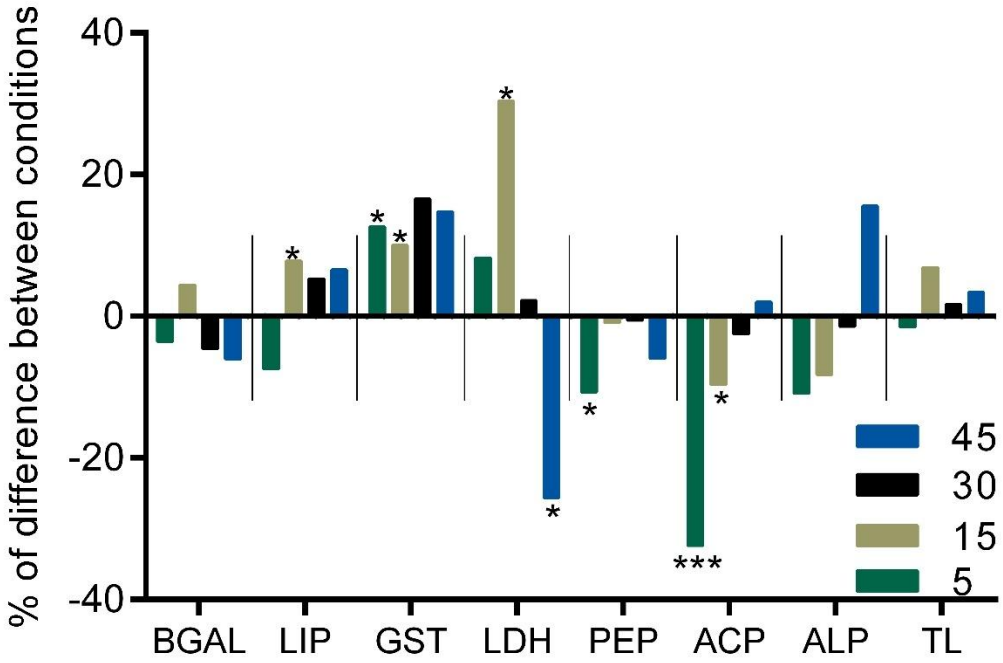

Figure 5. Percentage of difference in enzyme activity in different crowding conditions following acute exposure to silver nanoink (0.15 µl/l, 24h). Each exposure condition was compared to their corresponding control. Data represents average (N=4) replicates for each condition. Statistically significant changes identified by Student's *t*-test with Welch's correction between control and exposed conditions with *p*-values of $p \leq 0.05$ (*) and $p \leq 0.001$ (***). Abbreviations: BGAL: β-galactosidase, LIP: lipase, GST: glutathione-S-transferase, LDH: lactate dehydrogenase, PEP: peptidase, ACP: acidic phosphatase, ALP: alkaline phosphatase, RT: reduced thiols.

4.3.5 The impact of silver nanoink on metabolism of daphnids with different crowding

With the aim to evaluate daphnid crowding in the context of environmental studies as well as the impact observed during acute NP exposure, two exposure concentrations of 0.15 µl/l and 0.45 µl/l were used in tandem with two crowding groups of 15 and 45 daphnia. Exposed daphnid response displayed a divergent profile in control-exposed comparisons between groups with the same crowding (Figure 6 left heatmaps). For metabolites of the central carbon metabolism (CC) we observed statistically significant differences with the

highest fold change in the 45 daphnid group (C_{45}-0.45) for pyruvate (Pyr), while the less crowded (15 daphnids) group had more metabolites with statistically significant differences albeit less pronounced in terms of fold change. Regarding the abundances of amino acids (AA), C_{45}-0.45 had the most statistically significant differences following exposure, which is the opposite case for the rest of the exposed groups. The impact of crowding as seen on the right heatmaps in Figure 6, shows statistically significant differences for many metabolites in CC, with ribulose-5-phosphate (Ribu5P) showing the highest observed difference in terms of fold change in the $0.45_{45/15}$ comparison, followed by ribose5-phosphate (Ribo5P). We observed that the change in daphnid crowding results in more statistically significant differences in higher NP exposure concentrations, except for methionine (Met) which appeared to follow the opposite trend. Principal component analysis also depicts the differences that result from changes in animal crowding for AA and CC.

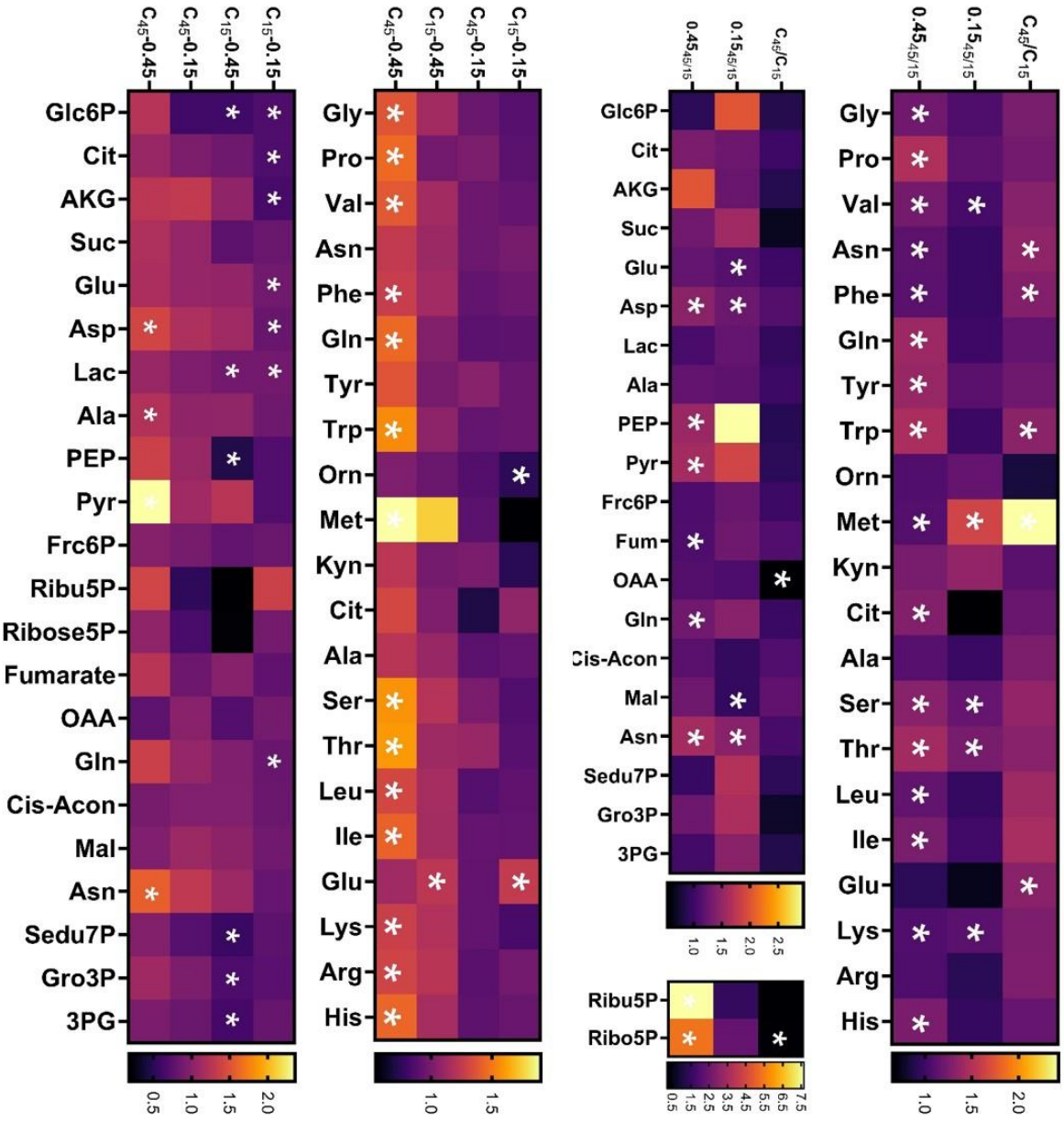

Figure 6. Effect of exposure to silver nanoink (0.15 µl/l and 0.45 µl/l) for 15 and 45 daphnids. Left heatmap corresponds to the ratio of exposed over the corresponding control group for 15d and 45d respectively. Right heatmap corresponds to the ratio of 45d over 15d for each exposure group (control, 0.15 µl/l and 0.45 µl/l) to visualise the impact of daphnid crowding. Data represents average fold values (N=5) of each group. Statistically significant changes were identified by Student's *t*-test with Welch's correction (*) between control and their respective exposure conditions (left heatmap), or two-way ANOVA (*) among crowding groups with their respective exposure conditions (right heatmap).

Abbreviations: glycine (Gly), proline (Pro), valine (Val), asparagine (Asn), phenylalanine (Phe), glutamine (Gln), tyrosine (Tyr), tryptophan (Trp), ornithine (Orn), methionine (Met), kynurenine (Kyn), citrulline (Cit), alanine (Ala), serine (Ser), threonine (Thr), leucine (Leu), isoleucine (Ile), glutamic acid (Glu), lysine (Lys), arginine (Arg), and histidine (His), Glc-6P: Glucose 6-phosphate, α-KG: Alpha-ketoglutarate, Glu: Glutamate, Asp: Aspartate, Frc-6P: Fructose 6-phosphate, Ribu5P: Ribulose 5-phosphate, Ribose5P: Ribose 5-phosphate, Sedu7P: Sedoheptulose 7-phosphate, Gro-3P: Glycerol 3-phosphate, 3-PG: 3-Phosphoglycerate.

4.5 Discussion

The results of this study suggest that crowding has a substantial effect on both apical and more sensitive endpoints. Due to the lack of information on the effect of daphnid crowding, as most research is focused on the bioindicator or the pollutant itself, the effects of the experimental setup appear to be significant in the context of pollution assessment. This highlights the importance of a standardized experimental setup in daphnid toxicity testing to improve the available tools to combat pollution.

Increased densities of daphnids can influence the physicochemical behaviour of AgNPs in the exposure medium as for example, higher concentrations of excreted organic matter, such as metabolites and biological macromolecules. The presence of these molecules can potentially functionalize NPs, altering their aggregation, bioavailability and toxicity (Liu et al., 2013). A study demonstrated that the presence of natural organic matter leads to less aggregation of ZnO NPs, increasing their dispersion in the medium and potentially affecting their bioavailability (Lee et al., 2020). At higher population densities, daphnids may excrete increased amounts of protective compounds, such as proteins and other organic molecules, into the surrounding medium, which can interact with AgNPs or free silver ions, forming new complexes. We posit that to an extent this might be one of the explanations for the correlation of increased crowding with reduced toxicity, as research has shown that dissolved organic carbon can mitigate the toxicity of silver nanoparticles by complexing with silver ions (Newton et al., 2013). According to the available literature, the study of Lowes showed that the different number of daphnids as individuals vs. groups may influence the observed mortality of *D. magna* neonates exposed to copper in the first day of life (Lowes et al., 2021). Furthermore, other daphnid biomarkers such as swimming behaviour that are used to measure the impact of pollutants to daphnids can also be impacted by crowding and impairing their response to pollutants (Bownik, 2017). One possible explanation to the observed deviation of EC_{50} values for the different crowding conditions is that the behaviour and interspecies interactions of social groups might alter with changes in crowding of daphnids (Viaene et al., 2015) and in turn influence the observed toxicity. This can also be the case for other bioindicator species (Foit et al., 2012, Knillmann et al., 2012, Sloman et al., 2002).

The observed differences among the control groups (Figure 3) could potentially provide additional insights on the influence of crowding on the baseline response for certain markers employed in pollution assessment. Crowding-induced variation in baseline enzyme activity without silver nanoink exposure that enzyme activity levels varied significantly between

different crowding groups of daphnids. PEP and LDH showed the most pronounced differences followed by LIP and GST, suggesting that crowding itself induces physiological changes or adaptation mechanisms that affect certain metabolic processes (Kos, 2023, Oh et al., 2014, Olivecrona, 2016). This baseline variation may be relevant to how these groups respond differently to nanoparticle exposure, therefore highlighting the importance of revising existing standards of *D. magna* testing. Crowding influences the impact of silver nanoink exposure on enzyme activities (Figure 4), with statistically significant differences across most enzymes observed between different crowding conditions, except for BGAL and GST. Higher crowding (e.g., 45 daphnids) tended to result in more pronounced deviations in enzyme activity compared to lower crowding (e.g., 5 daphnids). This deviation in responses of exposed groups strongly suggests that the previously observed baseline variation (Figure 3) can lead to variation in responses of exposed groups. Variability of enzyme response profiles between different crowding groups (Figure 5) suggests that as the most pronounced changes are observed in the 5-daphnid and 15-daphnid groups, potentially as a result of higher sensitivity to silver nanoink exposure in these groups compared to the more crowded groups. This variability among different exposure groups underlines the need for further investigation on the interspecies interactions aspect of experimental setups using *D. magna*, especially for nanomaterials due to their unique properties and ability to form nanocoronas (Nasser and Lynch, 2016, Miclăuş et al., 2016). Higher crowding may induce a baseline stress response that primes daphnids to be more resilient to additional stressors such as AgNP exposure and potentially result in greater excretion of metabolites, enhancing nanocorona formation which mitigates toxicity (Newton et al., 2013, Ekvall et al., 2021). Certain enzymes, such as LDH and PEP, show different regulation because of changes in crowding, influencing the ability of daphnids to manage the stress of nanoink exposure (Legrand et al., 1992, Kos, 2023). Decreased mortality observed in more crowded groups, aligns with the observed differences in enzyme activities as groups with higher crowding (e.g., 45 daphnids) seem to be partly protected from the exposure effect, as shown by both reduced toxicity and less pronounced changes in key enzyme activity.

Changes in metabolic activity of two crowding groups (15 and 45 daphnids), show clear differences among exposed (0.15µl/l, 0.45µl/l) and control groups in central carbon metabolism and amino acids abundances which has been also shown in available literature to be the result of AgNP exposure (Poynton et al., 2012, Zhang et al., 2017, Shen et al., 2023, Galhano et al., 2022). To focus on the effect of silver nanoink exposure and the effect of crowding, the changes in metabolite abundance were shown using heatmaps with the fold change of exposed over corresponding control group or the ratio of 45d over 15d respectively

(Figure 6). Impact on the abundance of central carbon metabolites in C_{15}-0.15µl/l comparison like for example the significant increase of more than two-fold for pyruvate in the C_{45}-0.45µl/l comparison, strongly suggest that changes in crowding results in very different responses even when the same ratios of daphnids and pollutant were used. In amino acids, The C_{45}-0.45µl/l resulted in the most observed changes, notably with methionine where a nearly two-fold significant increase could be observed. Many others such as arginine, lysine, glutamate, glutamine, histidine and glycine also showed significant changes which is in accordance with results seen in existing literature for *D. magna* exposures to AgNPs (Li et al., 2015, Xiang et al., 2024, Zhang et al., 2017). Using the 45 over 15 daphnids ratio (right heatmap Figure 6), the effect of crowding on the central carbon metabolism and amino acids showed significant differences among all three tested conditions – 0.45µl/l, 0.15µl/l and control. Most notable was the impact on ribulose5-phospate and ribose5-phosphate with a significant increase of seven-fold and five-fold respectively while for amino acids methionine showed the most profound increase of more than two-fold for controls. The most differences were observed in the 0.45µl/$l_{45/15}$ comparison for amino acids, with only ornithine, alanine and glutamic acid not showing significant changes, and the same number of significant changes in amino acids were observed for both 0.15µl/l and control group comparisons for 45/15 daphnids. The difference between controls showed that changes in crowding can alter the baseline of certain biomarkers used to evaluate pollutants, highlighting the importance of revising existing pollution assessment protocols using *D. magna*. The higher crowding group (45 daphnids) showed a more uniform response to increased nanoparticle concentration (0.45 µl/l) across multiple metabolites, especially in central carbon metabolism. The PCA results support this by showing clearer separations for higher concentration exposures, both in AA and CC profiles. Interestingly, both AA and CC PCA (supplementary figure 1) showed the separation of the two groups that had the same ratios of daphnids and silver nano ink (0.15µl/l-15 daphnids and 0.45µl/l-45 daphnids), indicating the importance of evaluating the markers used for pollutions assessment in the context of comparing results across studies that use different experimental setups. In general, our results show that higher crowding resulted in exposed daphnids becoming more resilient to silver nanoink but certain biomarkers were more strongly affected by more crowding, reinforcing the hypothesis that higher crowding provides some protective mechanism, potentially through changes in interspecies interactions. Available literature indirectly supports this claim, as it has been shown that changes in crowding lead to substantial alterations in daphnid growth, reproduction, morphology and feeding which would explain the changes in daphnid metabolism (Burns, 1995, Burns, 2000, Lürling et al., 2003).

Consequently, these results clearly show the unique way each crowding condition responded to silver ink nanoparticle exposure. Because of this, and the observed differences in the generated toxicity curves, our study shows that daphnid crowding dramatically influences the observed impact of acute NP exposure on a number of key biomarkers. This is highlighting the need for updating the currently used guidelines for environmental studies that use *D. magna* as a model organism. Further research is imperative to gather additional information on the impact of daphniid experimental design, since very little information exists on how the setup will exert itself on the exposures to pollutants. A novel, rigid design on pollution assessment setups with bioindicator species may be required for novel pollutants like NPs, and more information is required on which markers can be affected by certain experimental design aspects.

Data availability

Data will be made available upon request. The matrix of the metabolomic data and processed data are provided in the Supplementary Materials. The metabolomic datasets of central carbon pathways (CC) and amino acids (AA), raw data are provided in Excel files with their processing (sheet Raw data Processed). In this sheet we scaled with the fold over the unexposed control per group of crowding and relative standard deviations (coefficient of variances) were calculated to assess variance due to mass spectrometry analysis. The values that we used for the heatmaps with the fold over each comparison are provided in the same sheet. In the second sheet (Processed PCA) we provide the final matrix used for the principal component analysis which are also illustrated as supplementary figure 1.

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CRediT authorship contribution statement

Dimitrios Kakavas: Investigation, Methodology, Formal Analysis, Writing-Original Draft Preparation, Writing-Review & Editing. **Beatrice Engelmann:** Investigation, Methodology, Formal Analysis, Visualisation, Data curation, Writing-Review & Editing. **Ulrike E. Rolle-Kampczyk:** Formal Analysis, Writing-Review & Editing, Resources. **Martin von Bergen:** Writing-Review & Editing, Resources, Funding acquisition, Supervision. **Keith D. Rochfort:** Conceptualization, Methodology, Writing-Original Draft Preparation, Writing-Review & Editing, Funding acquisition, Supervision, Project administration. **Konstantinos Grintzalis:** Conceptualization, Methodology, Investigation, Visualization, Writing-Original Draft Preparation, Writing-Review & Editing, Funding acquisition, Supervision, Project administration.

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Chapter 5

The fifth chapter is focused on the impact of metabolite coronas on nanotoxicity as well as the differences in observed effect due to differences in the metabolites it is composed of. The presence of nanocoronas excreted by living organisms has been shown to modulate the biological responses of exposed individuals. Furthermore, we demonstrate that the environmental context in which nanopollution is encountered greatly impacts the observed adverse effects, and that there is a potential for underestimation of risk when it comes to nanopollutants. So far, protein coronas had been the main focus for how nanocoronas may affect the impact of nanopollutants, however we demonstrated that metabolite coronas can also have a modulative effect on exposure.

Excreted metabolite nanocoronas impact metabolic and toxicity responses of daphnids to silver nanoink

Dimitrios Kakavas[12], Beatrice Engelmann[3], Martin Krauss[4], Ulrike E. Rolle-Kampczyk[3], Keith D. Rochfort[1,2], Konstantinos Grintzalis[1,2]*

[1]School of Biotechnology, Dublin City University, Republic of Ireland

[2]Life Sciences Institute, Dublin City University, Republic of Ireland

[3]Department of Molecular Toxicology, Helmholtz-Centre for Environmental Research - UFZ, Leipzig, Germany

[4]Department Exposure Science, - Helmholtz Centre for Environmental Research - UFZ, Leipzig, Germany

*Corresponding author: Konstantinos Grintzalis, Email: konstantinos.gkrintzalis@dcu.ie

Abbreviations

NP(s), Nanoparticle(s); D. magna, Daphnia magna; NOM, Natural organic matter; AgNP(s), Silver nanoparticle(s); ROS, Reactive oxygen species; OECD, Organisation for Economic Co-operation and Development; U, Media unconditioned by D. magna; C7, Media conditioned by age day 7 daphnids; C14 media conditioned by age day 14 daphnids; ECx, Effective concentration; TCA, Tricarboxylic Acid Cycle; PPP, Pentose Phosphate Pathway; MS, Mass Spectrometry; LC-MS, Liquid Chromatography-Mass Spectrometry; HPLC, High Performance Liquid Chromatography; PFP, Pentafluorophenyl; HRMS, High resolution Mass Spectrometer; BGAL, β-galactosidase; LIP, Lipase; ALP, Alkaline Phosphatase; ACP, Acetic Phosphatase; PEP, Peptidase; GST, Glutathione-S-transferase; RT, Reduced Thiols; LDH, Lactate Dehydrogenase; ANOVA, Analysis of Variables; PCA, Principal Component Analysis; Gly, Glycine; Pro, Proline; Val, Valine; Asn, Asparagine; Phe, Phenylalanine; Gln, Glutamine; Tyr, Tyrosine; Trp, Tryptophan; Orn, Ornithine; Met, Methionine; Kyn, Kynurenine; Cit, Citrulline; Ala, Alanine; Ser, Serine; Thr, Threonine; Leu, Leucine; Ile, Isoleucine; Glu, Glutamic Acid; Lys, Lysine; Arg, Arginine; His, Histidine; Glc-6P, Glucose 6-phosphate; α-KG, Alpha-Ketoglutarate; Glu, Glutamate; Asp, Aspartate; Frc-6P, Fructose 6-Phosphate; Ribu5P. Ribulose 5-Phosphate; Ribose5P, Ribose 5-phosphate; Sedu7P, Sedoheptulose 7-phosphate; Gro-3P, Glycerol 3-phosphate; 3-PG, 3-Phosphoglycerate

Abstract: The rapid advancements of the modern world led to the creation of new materials and products at an unprecedented rate. Among these, nanoparticles have found extensive applications and frequently enter freshwater ecosystems. Daphnia magna is a

widely used bioindicator species for the assessment of aquatic pollution. This study explores the impact of excreted metabolites on the toxicity mechanisms of silver nanoparticle ink using D. magna metabolite conditioned media. Two different life stages of daphnids were used to condition aqueous media and form metabolite-based nanocoronas with different compositions. The primary objective was to examine how these metabolite nanocoronas affect the observed impact of exposure on several physiological markers in daphnids including mortality, key enzyme activities and core metabolism. Conditioned media was also characterised to compare similarities in composition to assess the impact of silver nanoink. The response profiles to acute exposure varied among the tested media groups, with unfunctionalized nanoparticles exerting noticeably different responses on the investigated markers of physiology. Lethality was significantly reduced by the presence of excreted metabolites compared to unconditioned exposure to the silver nanoink, while the presence of metabolites had a more significant effect on key enzyme activities and metabolism of daphnids.

Keywords: *Daphnia magna*; silver nanoink; toxicity; excreted metabolites; eco-corona; enzyme activity; metabolomics

5.1 Introduction

Nanoparticles (NPs) are increasingly being studied in ecotoxicology as their unique properties alter the way they interact with, and how they exert toxicity on aquatic life (Khan et al., 2019). Diverse applications of NPs include but are not limited to electronics (Payal and Pandey, 2022, Ghiyasiyan-Arani, 2024b), coatings (Zhu et al., 2022) and modification of materials for enhanced properties (Ghiyasiyan-Arani and Sobhani, 2025, Ghiyasiyan-Arani, 2024a), drug delivery (Mitchell et al., 2021), cosmetics (Gupta and Mohapatra, 2022) and more. The NPs that enter the environment can be difficult to remove and can degrade very slowly, ultimately leading to degradation of habitats as well as the disruption of the local fauna and flora over time, negatively impacting the stability of aquatic ecosystems. A steady increase in their applications presents a threat to freshwater ecosystems as the main entry pathways of NPs to the aquatic ecosystems are via industrial effluents, wastewater and product disposal (Wang and Liu, 2022). Fate of NPs can also be difficult to predict as they have demonstrated phenomena of bioaccumulation (Devasena et al., 2022), and there have been studies on their transfer through the food web among organisms (Werlin et al., 2011, Gardea-Torresdey et al., 2014, Tran et al., 2024). Relative to the number of types of nanomaterials that are currently produced, the adverse effects of NP exposure to aquatic life are insufficiently studied and understood which is reflected in contrasting results across studies. While numerous studies have investigated the effects of NP exposure – such as inflammation, oxidative stress, apoptosis, developmental and reproductive disruptions (Yu

et al., 2020, Yin et al., 2013, Fan et al., 2023), relatively little is known about the interactions of NPs and specific metabolic pathways, especially when coated with biologically derived coronas. The nature of the nanocorona can modulate NP cellular uptake and bioavailability (Monopoli et al., 2012), however, the nature of interactions with core metabolic processes (such as the Krebs cycle) remains underexplored. A widely used category of metallic NPs are silver nanoinks which are utilized in printed electronics and antimicrobial coatings, raising concerns about their release into freshwater ecosystems via wastewater and product degradation (Badawy et al., 2010, Wang et al., 2024). They can undergo transformation once in the aquatic environment, including dissolution to Ag^{+} ions (Sotiriou et al., 2012) and surface modification through interactions with other molecules or natural organic matter (NOM) (Wei et al., 2023). Filter-feeding species like *D. magna* can be particularly vulnerable to such contaminants due to their constant exposure to suspended particles, or particle ingestion due to passive feeding. Investigating the ecotoxicological impact of environmentally relevant nanoinks thus provides critical insights into real-world exposure scenarios, which can also provide critical information in the context of pollution assessment for other NPs (Reilly et al., 2023b).

Over the past number of decades, the use of bioindicator species to assess the potential environmental impact of certain pollutants has resulted in new biological markers through which these pollutants are evaluated. There are a few commonly used species such as *Mytilus galloprovincialis* (Kristan et al., 2014, Politakis et al., 2018), *Danio rerio* (Çelebi et al., 2023) and *Daphnia magna* (Tkaczyk et al., 2021), which have already spearheaded research on pollution assessment. This new approach has many benefits over the traditional testing methods, such as the detection of the effects of pollutants even at concentrations lower than the detection level, therefore offering more effective assessment methods (Schmeisser et al., 2023). *D. magna* as a bioindicator species offers critical information on the mechanisms through which a pollutant exerts toxicity to aquatic life as there are many endpoints available to use for pollution assessment such as immobilisation, feeding rate, survival, enzyme activity, and more (Samanta et al., 2020, O'Rourke et al., 2023, Panagiotidis et al., 2023, Kakavas et al., 2023, Michalaki et al., 2023). Additionally, activities of key enzymes can guide more expensive and holistic analyses such as omic experiments which can show adverse effects at sub-lethal concentrations. *D. magna* is among the most widely used bioindicator species for pollution assessment thanks to its ecological significance and sensitivity to pollutants. In tandem with many other beneficial characteristics such as small life span, reproductive rate and more, *D. magna* has proved as a very useful tool to monitor freshwater ecosystems (Reilly et al., 2023a).

Nanocoronas are the formations of protein and metabolite layers on the outside surface of NPs. The environmentally relevant nanocoronas could have an antagonistic effect on the toxicity exerted by NPs by reducing the available surface (Bashiri et al., 2023, Liu et al., 2022). In the aquatic environment, excreted metabolites can coat the surface of NPs and play an important role in the surface-mediated effects of NP exposure (Liu et al., 2022, Zhang et al., 2022, Chetwynd et al., 2020, Wheeler et al., 2021, Grintzalis et al., 2019). Furthermore, the interactions between proteins and metabolites can lead to scenarios where the presence of proteins can induce the addition of metabolites to the nanocorona, or vice versa. NOM can interact with NPs in an aquatic environment, forming complexes which in turn can alter their physicochemical properties (Grassi et al., 2020, Liu et al., 2023, Khort et al., 2024). Ultimately, this process can result in a multi-layered complex nanocorona which is dynamic in nature, with unpredictable changes over time to the effect of exposure. Nanocoronas have also shown that they can induce 'Trojan-horse' effects, bypassing the defensive mechanisms of the organism and promoting NP uptake (Hsiao et al., 2015, Park et al., 2010, Sabella et al., 2014). These nanocorona characteristics make accurate environmental risk assessment very challenging, considering how many different metabolites can have an adverse effect to aquatic life (Pawlik-Skowrońska and Toporowska, 2019, Świacka et al., 2022, You et al., 2022, Chetwynd et al., 2020). Due to the dynamic nature of the formation of nanocoronas in the aquatic environment, more research is required to shed light on how these interactions might impact the aquatic environment in the context of NP toxicity.

The reason why excreted metabolites that coat NPs may affect the impact of NP exposure is related to the mechanisms through which they exert their interactions or toxicity with exposed organisms. Nanocoronas are composed of molecules which could create physical barriers around silver nanoparticles (AgNPs), limiting direct contact with the medium which can in turn limit the dissolution rate into Ag_+ ions that exert toxicity to exposed organisms (Adamczyk et al., 2016, Dong et al., 2021, El Badawy et al., 2011, Liu and Hurt, 2010, Khort et al., 2024). Additionally, nanocoronas can change some of the surface properties of AgNPs, like charge, reactivity or hydrophobicity (Dols-Perez et al., 2023, Sanità et al., 2020). By reducing the surface reactivity for example, nanocoronas could influence ROS (reactive oxygen species) generation and oxidative stress (Lynch and Dawson, 2008). The presence of nanocoronas could also reduce the adhering capacity of AgNPs to daphnia cell membranes or their ability to penetrate cellular barriers leading to, coated AgNPs entering cells in smaller amounts or at a reduced rate, delaying the onset of observed toxicity (Menichetti et al., 2023, Ekvall et al., 2021b). These findings underscore the importance for further research into the dynamics of nanocorona formation and its impact on aquatic ecosystems. Given the

complexity of metabolite coronas, their potential role in interacting with cellular metabolism – especially processes such as the Krebs cycle require further assessment to elucidate sublethal and pathway-specific adverse effects.

In this study, we investigated the effects of silver nanoink coated with environmentally relevant molecules excreted from a well-characterized freshwater model system. We assessed how excreted metabolites originating from two different *D. magna* life stages affect the exposure outcomes. Using markers of physiology such as lethality, key enzyme activities and more sensitive metabolomic analyses and characterization of the conditioned media, we evaluated the responses from functionalized and unfunctionalized silver nanoink. This approach enabled us to assess how environmentally relevant coronas impact NP toxicity in a sentinel aquatic species.

5.2 Materials and methods

5.2.1 Culturing daphnids

Daphnids were maintained in parent cultures as 20 animals per L of media using OECD standard medium (final concentrations of 0.29 g $CaCl_2 \cdot 2H_2O$/L, 0.123 g $MgSO_4 \cdot 7H_2O$/L, 0.065 g $NaHCO_3$/L, 0.0058 g KCl/L, and 2 µg Na_2SeO_3/L, pH 7.7) at a photoperiod of 16h:8h light:dark and 20ºC. Daphnids were fed daily with 2 mL/L of a *Chlamydomonas reinhardtii* algae suspension (6 million cells per mL). Culture media was refreshed every five days, with the addition of an organic seaweed extract derived from *Ascophyllum nodosum* added following media change. These cultures provided animals to start experiments (conditioning and exposures) from their third brood.

5.2.2 Conditioning media with excreted metabolites and exposure

To condition media, two ages were selected; 7 and 14 days as they would represent different life stages and excretion of metabolites. Starting with neonates (<24 hours) from parent cultures, 200 individuals in 4L of media were cultured until 7 or 14 days old, fed daily with algae (60 million cells). 100 animals (7 or 14 days old) were collected and transferred in 1L OECD media and fed with algae (1 mL/L – concentration 60 million cells) to prevent any starvation-related stress and allow them to "condition media" with excreted molecules over 24h. After conditioning, media was filtered (using a Thermo Scientific, Nalgene rapid

flow 0.2 µm 1L bottle top filter) to remove debris and any remaining algae. The media was then divided into clean bottles; one incubating with and one without silver nanoink, to correspond to media for the "exposure" and "control" groups, respectively. The bottles were left on a table shaker for 24h to keep the media stirring during the coating phase. In this study, these media will be considered conditioned from 7 or 14 days old daphnids (C7 and C14, respectively). At the same time, OECD media in the absence of animals followed the same preparation steps and incubation time of 24 hours, and this media will be considered as unconditioned (U) (Figure 1).

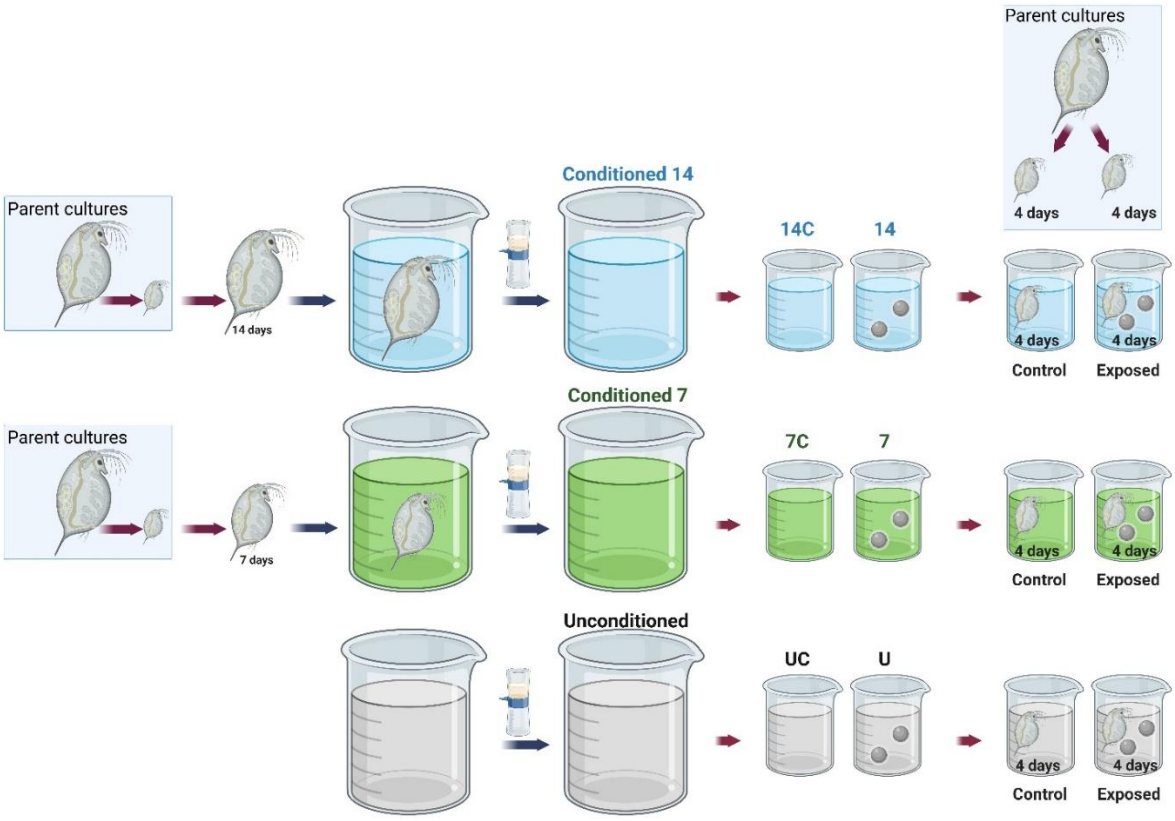

Figure 1. Experimental design for culturing daphnids and conditioning media. From parent cultures daphnids were cultured to 7 and 14 days of age. Daphnids were transferred in clean media to condition it for 24h. Clean media without animals were considered as "unconditioned". All media were filtered and subsequently incubated with the silver nanoink (or without for the controls) for 24h prior to exposure. Following this, media was kept for characterization by LC-MS or was used for exposures using four-days-old daphnids which were separately cultured.

5.2.3 Characterization of conditioned media with LC-MS

For media characterization, unconditioned media (U) and conditioned media (from 7 and 14 days old animals) were characterized by LC-MS/MS. Each media group was prepared in

the same way, with eight 50 mL centrifuge tubes, each with 50 mL media dried in the oven (60 ºC).

For the untargeted analysis, metabolites were resuspended in 0.5 mL of 95%LC-MS grade water: 5% LC-MS grade methanol, vortexed, centrifuged and the clear supernatant was collected. 10 µl were analysed by HPLC (Thermo Ultimate 3000 System) coupled to a QExactive Plus high resolution mass spectrometer (HRMS; Thermo). For the chromatography, a PFP column (Thermo Accucore, PFP, 150 x 2.0 mm, 2.6 µm) was used with mobile phase A of 0.1% formic acid and mobile phase B of methanol containing 0.1% formic acid. After injection of 10 µl eluent A was held for 5 minutes at 100% followed by a linear increase to 95% B for 5 minutes and isocratic elution at 95% B for 2 minutes and a re-equilibration to the initial conditions for 5 minutes. Separate runs were conducted for positive and negative ion mode using electrospray ionization. The HRMS data acquisition combined a MS1 full scan with a m/z range of 100-1500 at a nominal resolving power of 70,000 (referenced to m/z 200) and four data-dependent MS/MS scans for the most intense MS1 ions at a resolving power of 35,000 and at two normalised collision energy steps of 35 and 60 a.u. The Thermo raw data was converted to mzML format using ProteoWizard version 3.0.18265 (Chambers et al., 2012) and peak detection was conducted in MZmine 3.1 (Schmid and Heuckeroth, 2023) using the steps mass detection, ADAP chromatogram deconvolution (Myers et al., 2017) smoothing, local minimum feature resolver and join aligner. The aligned feature (peak) list were exported as .csv files for further processing. For filtering peaks, the unconditioned media was used as a background to remove any peaks resulting from non-biological (conditioning) origin. A strict filtering criterion was used to identify only peaks that were present in >75% (6 out of 8 replicates) of the samples analyzed for each media group and simultaneously were absent from the unconditioned media and, thus, were considered as true excreted features of biological (daphnid) origin. These features were plotted with Venn diagrams to visualize the number of unique and common peaks among media groups. Furthermore, only for the common peaks between the two conditioned media (7 and 14), the fold change of 14/7 was used to compare the abundance of features.

5.2.4 Exposure to conditioned and unconditioned media

For exposure to silver nanoink, unconditioned and conditioned media was incubated for 24h to allow for sufficient coating of NPs. Following the incubation, four-day-old animals (started from neonates and cultured to four-day-old) were collected and exposed to AgNPs

for 24h, after which, mortality rates were recorded. Toxicity curves were performed with 15 animals in 50 mL volume (four replicates per concentration) and plotted using the four-parameter logistic (4PL) model (Hill model). An EC_5 concentration of 0.5 µL silver nanoink/L was selected as a sub-lethal exposure level for all experiments. The silver nanoink used in these experiments was obtained from Sigma-Aldrich (Product No. 719048). In this study, four-day-old daphnids were chosen as the preferred daphnid age to work with, as they represent a development stage considered less sensitive in the daphnid life cycle that aligns with the objective of the study and the investigation of toxicity mechanisms. Additionally, daphnids in this life stage are easier to work with and offer more tissue mass for biochemical assays than younger neonates do.

5.2.5 Sample homogenization and biochemical assays

For biochemical assays, four-day-old daphnids were exposed to silver nanoink (0.5 µl/L) for 24h. Following exposure, 30 daphnids were pooled and homogenized in 0.5 mL of appropriate buffer (pH 7.2) using an Eppendorf pestle homogeniser. The clear supernatant was collected from homogenate following centrifugation (12,000 *g* for 10 minutes at 5 °C) to remove any carapace parts and assayed for protein and enzyme activity immediately (Grintzalis et al., 2022, Michalaki et al., 2022). Protein quantification was performed using a sensitive Bradford method (Georgiou et al., 2008, Grintzalis et al., 2015) to normalise enzyme activity in units/µg protein.

The activities of β-galactosidase (BGAL) and lipase (LIP) were determined, by measuring the generation of nitrophenol resulting from the catalysis of *o*-nitrophenyl-β-galactoside and *p*-nitrophenyl butyrate, respectively, in 50 mM phosphate buffer pH 7.2. The reaction was stopped by alkalization after 30 minutes with the addition of 50 µl 4 M NaOH, and the absorbance was measured at 405 nm and converted to nitrophenol using a standard curve. In a similar approach, activity of phosphatases was determined for the released *p*-nitrophenol utilizing *p*-nitrophenyl phosphate (*p*NPP) as substrate, in 100 mM boric acid pH 9.8 or 100-mM citric acid pH 4.5 for ALP and ACP respectively. The activity of peptidase (γ-glutamyl transferase, PEP) was assessed with the formation of *p*-nitroanilide (with L-leucine-4-nitroanilide as a substrate) with kinetics at 418 nm (van Acker et al., 1998). Absorbance was converted to p-nitroanilide and expressed as rate. Glutathione-S-transferase (GST) activity was determined by monitoring the formation of a complex between reduced glutathione and 1-chloro-2,4-dinitrobenzene and measured

photometrically at 340 nm (Fernandez et al., 2002). Reduced thiols (RT) were quantified through their reaction with aldrithiol following the protocol of Grintzalis et. al (Grintzalis et al., 2014). The activity of LDH was quantified from the decrease in absorbance at 340 nm resulting from the oxidation of NADH. A 1:1 mixture of 40 mM pyruvate and 0.5 mM NADH was used as a substrate. For the reaction, 200 µl of the sample appropriately diluted in phosphate buffer were mixed with 50 µl of 20 mM pyruvate and 0.25 mM NADH. The oxidation of NADH to NAD^+ which was continuously monitored at 340 nm.

5.2.6 Targeted LC-MS/MS analysis

For metabolomic analysis, groups of 15 animals were frozen immediately in liquid nitrogen following exposure. Samples were homogenized in 0.6 mL ice-cold methanol: water (HPLC-grade in 8:2 ratio) using an Eppendorf pestle homogenizer to quench metabolic reactions. The homogenates were cleared by centrifugation at 10,000× g at 4 °C for 5 min and the 100 µL of the clear supernatant was vacuum dried using a speedvac and stored at −80 °C until further analysis.

Each sample was resuspended in 120 µl water. For derivatization of amino acids, 10 µl were evaporated to dryness (SpeedVac, Eppendorf), resuspended in 50 µl 5% phenyl isothiocyanate (PITC) in ethanol: H_2O: pyridine (1:1:1, v/v/v) and incubated for 25 min at RT. Subsequently, samples were dried to remove excess PITC and resuspended in 10 µl 5 mm ammonium acetate in methanol. After incubation (10 min, 14000 rpm) 90 µl H_2O: acetonitrile + 0.2% formic acid were added. For the analysis of central carbon metabolites, targeted LC-MS/MS data of each extract was acquired using a QTRAP® 6500+ system (Sciex, Framingham, MA, USA) coupled on-line with an HPLC-system. Initially, 10 µL were injected onto an Agilent 1290 II infinity UPLC system (Agilent Technologies Inc., Santa Clara, CA, USA). Chromatographic separation was achieved using a XSelect HSS T3 XP column (2.1 × 150 mm, 2.5 µm, 100 Å; Waters, Milford, MA, USA). Metabolites were eluted at a flow rate ranging from 0.4 mL/min to 0.15 mL/min with a non-linear 33 min gradient. Mobile phase A and B were 10 mM tributylamine, 10 mM acetic acid, 5% methanol, 2% 2-propanol (pH 7.1) and 100% 2-propanol, respectively. The autosampler was kept at 5°C with the column oven set at 40 °C. Mass spectrometric measurement was performed in negative ionization mode.

Prior to measurement of the amino acids, each derivative was diluted 1:25 and 10 µl were injected onto a Waters Acquity UPLC system coupled on-line with a QTRAP® 5000 mass

spectrometer (Sciex, Framingham, USA). Chromatographic separation was achieved with an Agilent Zorbax Eclipse XDB-C18 column (3.5µm, 3.0 x 100 mm) using a constant flowrate of 0.5 mL/min and water + 0.2% formic acid and ACN + 0.2% formic acid as mobile phases A and B, respectively. The linear LC gradient was as follows: 0-0.5 min at 0% B, 0.5-4 min 0-70% B, 4-5.3min 70% B, 5.3-5.4min 70-0% B, 5.4-7.3 min 0% B. The autosampler was kept at 10 °C, the column oven was set at 50 °C and the QTRAP was set up to positive ionization mode.

Identification and quantification were based on specific MRM transitions used for both amino acids and central carbon metabolites. External calibration curves were measured for linear regression. Peak integration was determined in SciexOS® software (Version 3.0.0).

To assess the impact of acute exposure in amino acid levels among the tested conditions, we used the control unconditioned group (Uc) as the "baseline". Data normalization was performed by dividing individual replicates with the mean of Uc for each metabolite. Principal component analysis was performed using the Multiple Experiment Viewer (Saeed et al., 2003), and heatmaps were made using the GraphPad Prism version 10. Significant identified metabolites were used to reconstruct and illustrate these changes in the metabolic network.

5.2.7 Statistical analysis

Results were presented as mean ± standard deviation (SD) and were analysed and plotted with the GraphPad Prism software. Statistically significant differences were identified using one-way ANOVA and Student's *t*-test with Welch's correction with a *p* value of 0.05.

5.3 Results

5.3.1 The composition of conditioned media with liquid chromatography

With the aim to investigate if the age difference between the two groups used for conditioning demonstrates different metabolite composition in the conditioned media, an untargeted analysis using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was performed to characterize their composition.

The metabolite composition of conditioned media (C7 and C14) in both negative and positive ion modes showed distinct patterns of the excreted metabolites (Figure 2). The

number of peaks detected in the chromatograms of the C7 and C14 conditioned media were 58 and 100 features, respectively, for the negative ion mode, whereas in the positive ion mode 77 and 151 features were measured. Additionally, these peaks represent only the peaks that did not appear in any replicate (N=8) in the unconditioned media (U), ensuring the accuracy of the readings by excluding any potential background. A fold change analysis revealed that 17 out of 33 common features in the negative ion mode and 31 out of 48 in the positive ion mode were more abundant in C7 media.

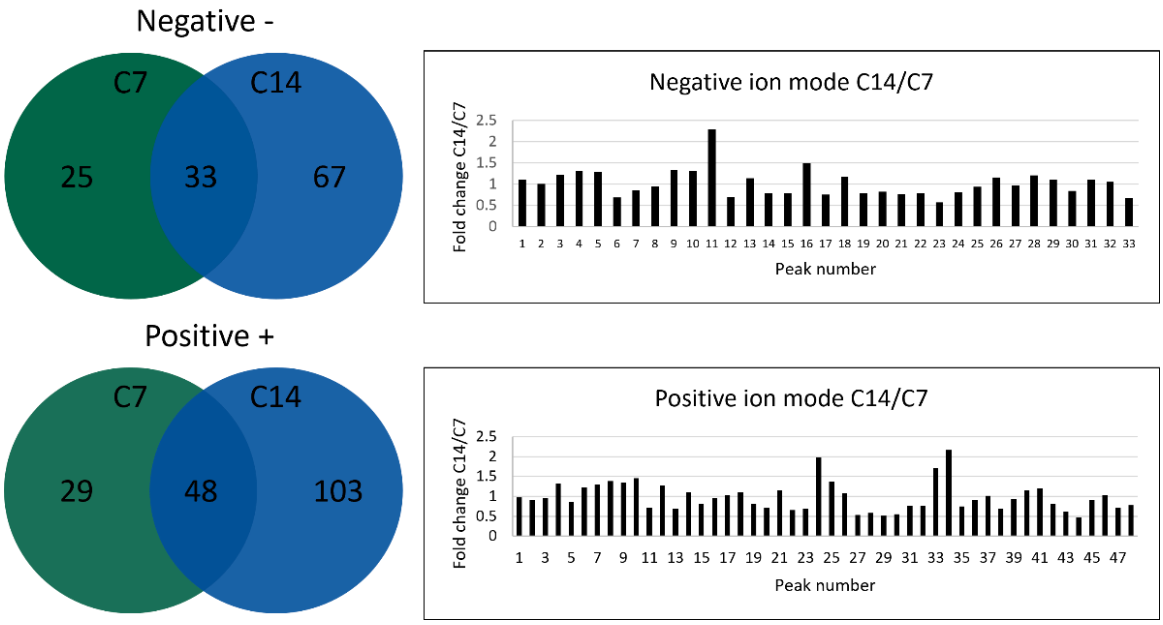

Figure 2. Venn diagram displaying the number of unique and common peaks in negative and positive ion modes, following an untargeted LC-MS analysis. The fold change C14/C7 was calculated using the average (N=8) samples per media condition, for their common peaks.

5.3.2 The impact of excreted metabolites on mortality and physiology of daphnids following exposure to silver nanoink

Acute exposures of *D. magna* to silver nanoink in three different exposure conditions showed significant divergence of the generated toxicity curves due to the presence of coated silver nanoink (Figure 3). Exposure to unconditioned silver nanoink had a lower EC_{50} (0.3 µl/l) compared to their coated counterparts of C7 (1.422 µl/L) and C14 (1.332 µl/L). Toxicity curves of silver nanoink conditioned C7 or C14 showed a similar pattern for 24 and also 48h (data not shown) of exposure which did not differentiate from each other. Furthermore, conditioning of the silver nanoink with C7, C14 media, resulted in a much steeper increase in mortality observed in exposed groups when compared to unconditioned silver nanoink

(Supplementary Table 1), while simultaneously maintaining the same silver nanoink concentration at which the exposed population mortality reaches 100%. To investigate further the impact of metabolite coronas on the exposure of silver nanoink, daphnids were exposed to a concentration of 0.5 µl silver nanoink/l. Responses in key enzyme activities of control and exposed individuals were recorded (Supplementary Table 2).

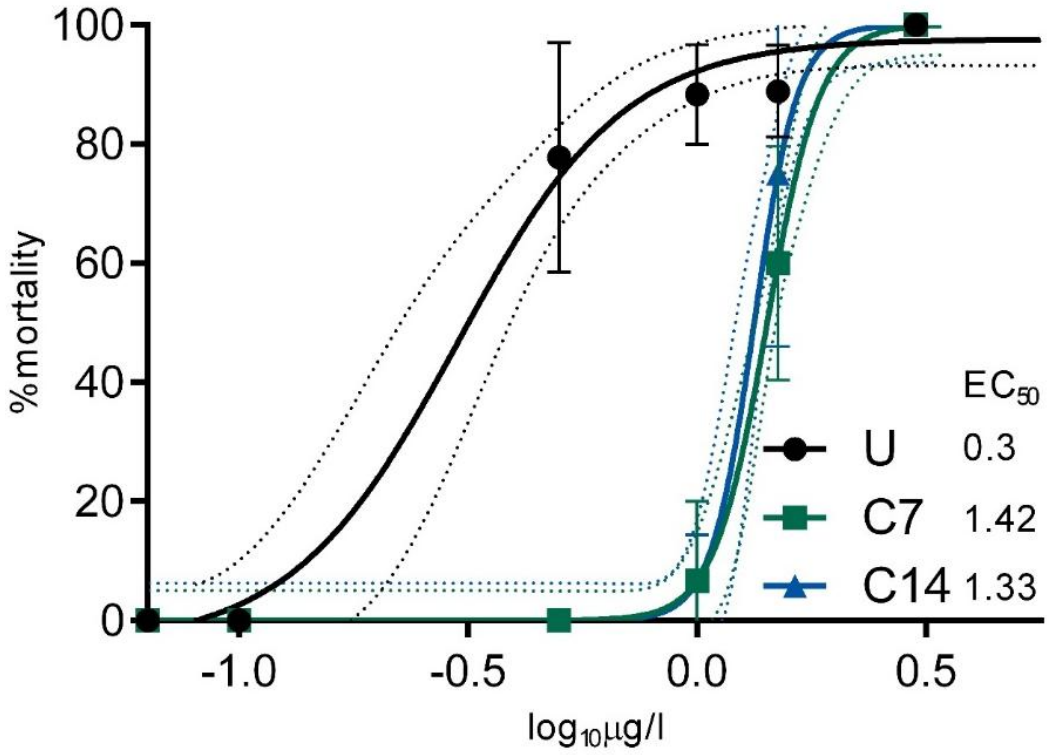

Figure 3. Acute toxicity curve for AgNP nanoink in three exposure conditions for 24 hours of exposure. Data represents average ±SD (N=4) replicates for each concentration. EC_{50} values depict the AgNPs concentration in µl/L of silver nano-ink. Abbreviations: Unconditioned exposure media with AgNP nanoink (U), AgNPs conditioned by day 7 media (C7) and AgNPs conditioned by day 14 media (C14).

5.3.3 The impact of media on enzyme activity: Responses to "conditioned" and "unconditioned" media

Initial assessment on the impact of media conditioning to D. magna following a 24h exposure without the presence of silver nanoink shows that the presence of excreted metabolites in the exposure media results in statistically significant differences upon comparison of enzyme activity between the conditioned media and the unconditioned media for the control conditions (Figure 4). LDH and GST showed the highest differences between conditioned and unconditioned media, followed by LIP and BGAL while PEP showed a significant increase in activity only in C14 media.

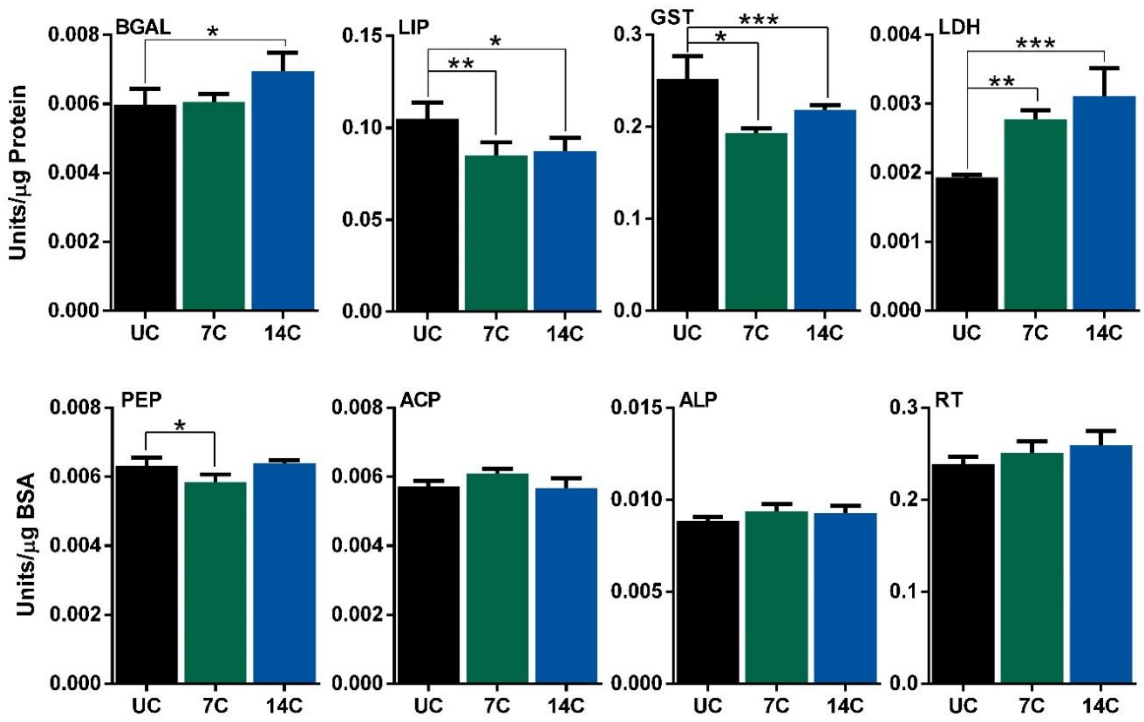

Figure 4. The impact of media conditioning on acute exposure, in the absence of silver nanoink. Comparisons of enzyme activities among the unexposed controls when compared to unconditioned media. Data represents average ± SD (N=4) replicates for each condition. Statistically significant changes identified by one-way ANOVA between control conditions 7C, 14C when compared to UC, with *p*-values of $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***). Abbreviations: 'C' denotes a control condition with no silver nanoink present, UC: unconditioned exposure media, 7C: media conditioned by day 7 daphnids, 14C: media conditioned by day 14 daphnids. BGAL: β-galactosidase, LIP: lipase, GST: glutathione S-transferase, LDH: lactate dehydrogenase, PEP: peptidase, ACP: acidic phosphatase, ALP: alkaline phosphatase, RT: reduced thiols.

5.3.4 The impact of conditioning media on enzyme activities in the presence of nanoink

The presence of metabolites during acute exposure reveals distinct responses between silver nanoink with different metabolite coronas and silver nanoink without coronas (Figure 5). Silver nanoink exposure had an impact on all enzyme groups, leading to notable differences between the coated (conditioned) and uncoated conditions. Enzyme activities varied significantly across all tested conditions, with LDH, BGAL, and LIP showing the most pronounced differences, emphasizing that NP functionalization has a significant impact at the enzymatic level.

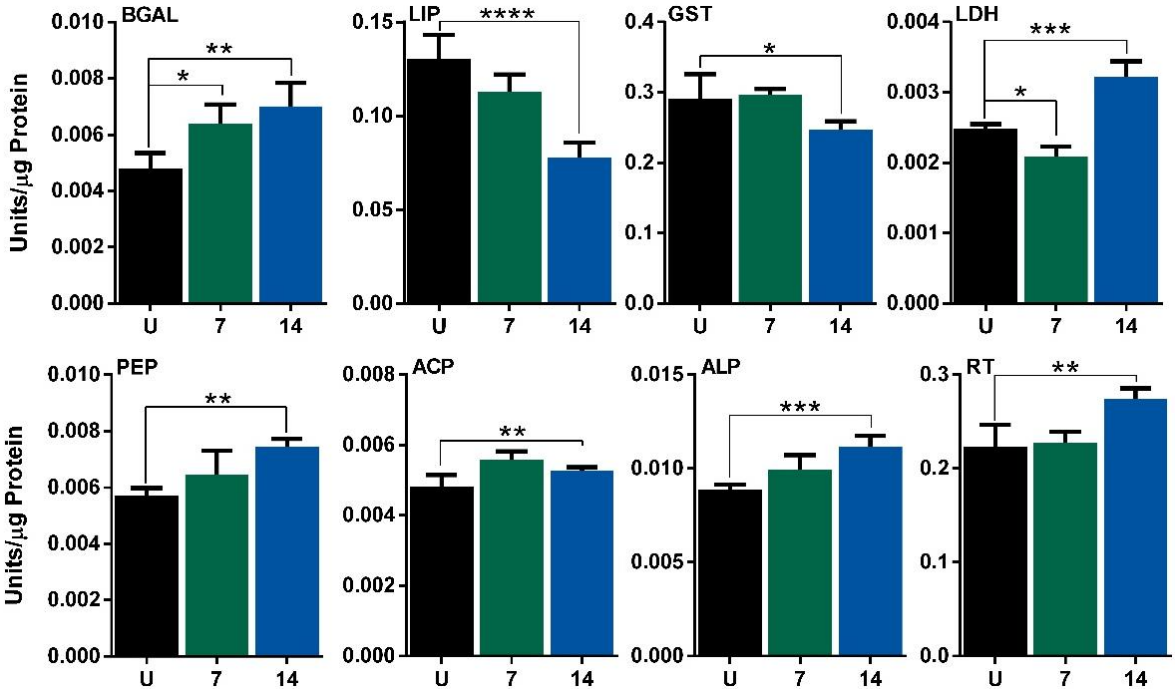

Figure 5. The impact of media conditioning on acute exposure to silver nanoink. Comparisons of enzyme activities among the exposed coated groups when compared to unconditioned media. Data represents average ± SD (N=4) replicates for each condition. Statistically significant changes identified by one-way ANOVA among tested conditions with p-values of $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). Abbreviations: U: unconditioned silver nanoink exposure media, 7: exposure media with AgNPs conditioned by day 7 daphnids, 14: exposure media with silver nanoink conditioned by day 14 daphnids. BGAL: β-galactosidase, LIP: lipase, GST: glutathione S-transferase, LDH: lactate dehydrogenase, PEP: peptidase, ACP: acidic phosphatase, ALP: alkaline phosphatase, RT: reduced thiols.

5.3.5 The impact of exposure to silver nanoink on enzyme activity for each media

Upon comparison of exposed conditions to their respective controls, the statistically significant differences showed a different response profile for each condition. The presence of metabolite coating on the surface of silver nanoink is responsible for the differences between the response of the coated tested condition and uncoated condition. The % of difference values (Figure 6) shows that U condition impacted LDH activity the most (+28%), followed by LIP (+24.3%), BGAL (-19.8%), ACP (-15.4%), PEP (-9.8%). C7 media group had the most impact in GST activity (+53.4%) followed by LIP (+33%), LDH (-24.7%) and ACP (-8.4%). The C14 group exposures resulted in significant increases in the activity of ALP (+20.5%), PEP (+16.4%) and GST (+13.5%).

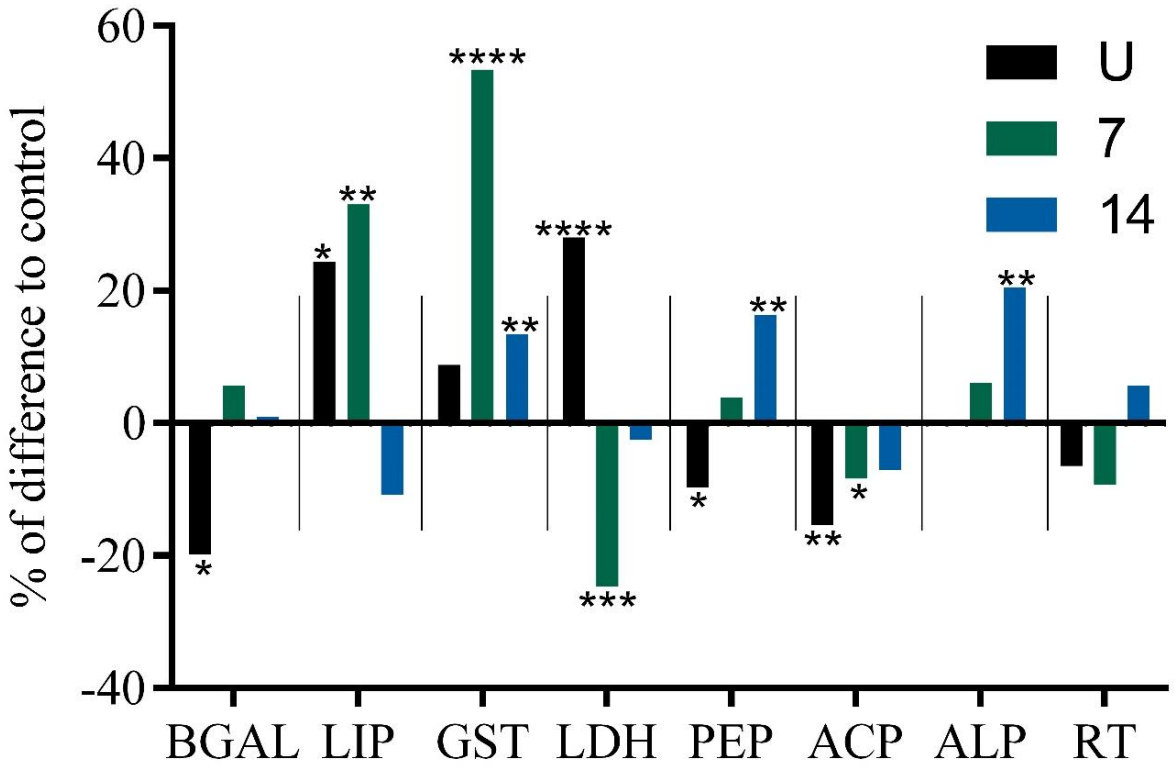

Figure 6. Percentage of difference in enzyme activities due to acute exposure to conditioned and unconditioned silver nanoink (0.5 µl/l, 24h). Each exposure condition was compared to their corresponding control. Data represents average (N=4) replicates for each condition. Statistically significant changes identified by Student's *t*-test with Welch's correction between control and exposed conditions with *p*-values of $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). Abbreviations: BGAL: β-galactosidase, LIP: lipase, GST: glutathione S-transferase, LDH: lactate dehydrogenase, PEP: peptidase, ACP: acidic phosphatase, ALP: alkaline phosphatase, RT: reduced thiols. Unconditioned AgNP nanoink (U), media conditioned by day 7 media (C7) and media conditioned by day 14 media (C14).

5.3.6 Metabolic perturbations induced by conditioning of media and exposure to silver nanoink

The impact of metabolite coronas on amino acid levels in exposed daphnids was also investigated after acute exposure. In the U exposure group, ornithine and histidine showed a significant decrease following acute exposure. In the C7 media exposure group, isoleucine and proline displayed significant increases, while arginine, histidine, lysine, methionine, ornithine, and phenylalanine exhibited significant decreases. For the C14 media group, no amino acids showed a decrease; however, a significant increase was observed in arginine, asparagine, glutamic acid, alanine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, and valine.

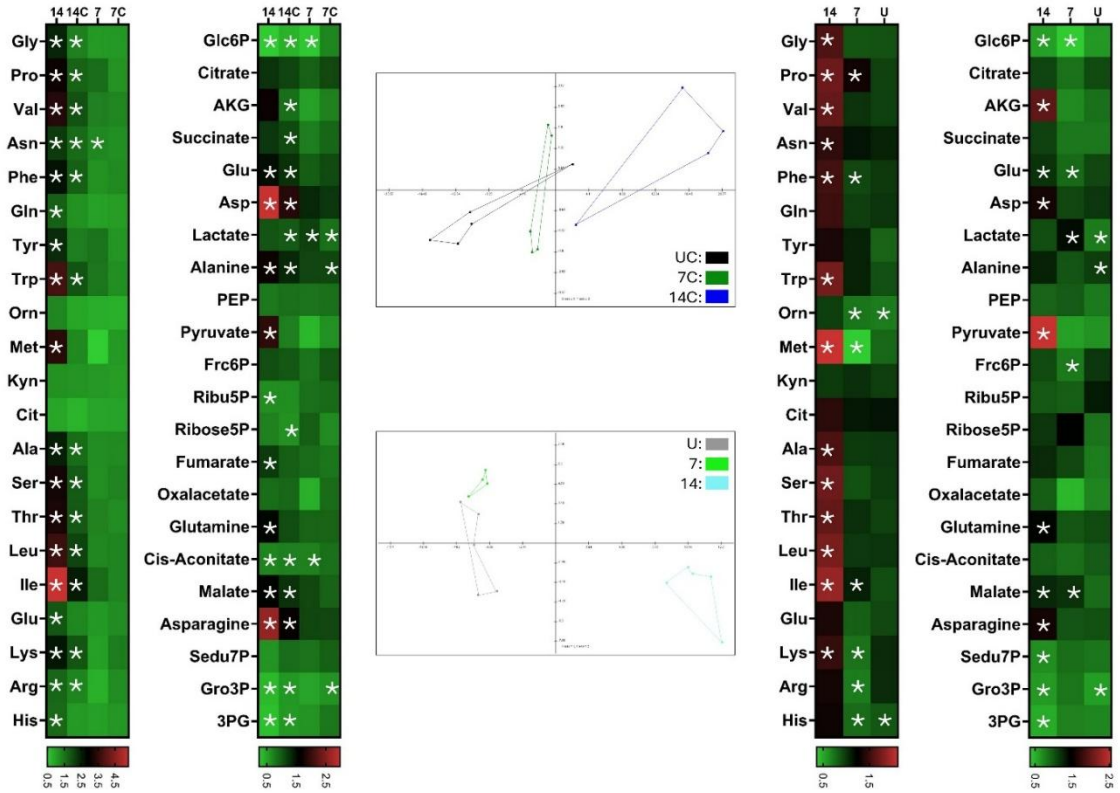

Figure 7. Effect of exposure to U, C7, C14 for 24h on metabolism of daphnids. Left heatmap corresponds to the ratio of conditioned over the corresponding unconditioned media (14/U, 14c/Uc, 7/U, 7c/Uc). Right heatmap corresponds to the ratio of exposed over control for each media (14/14c, 7/7c, U/Uc) to visualise the impact of the silver nanoink in each media. Data represents average fold values (N=5) of each group. Statistically significant changes were identified by Student's *t*-test with Welch's correction between control and their respective exposure conditions, or one-way ANOVA among exposure groups (*). Abbreviations: glycine (Gly), proline (Pro), valine (Val), asparagine (Asn), phenylalanine (Phe), glutamine (Gln), tyrosine (Tyr), tryptophan (Trp), ornithine (Orn), methionine (Met), kynurenine (Kyn), citrulline (Cit), alanine (Ala), serine (Ser), threonine (Thr), leucine (Leu), isoleucine (Ile), glutamic acid (Glu), lysine (Lys), arginine (Arg), and histidine (His), Glc-6P: Glucose 6-phosphate, α-KG: Alpha-ketoglutarate, Glu: Glutamate, Asp: Aspartate, Frc-6P: Fructose 6-phosphate, Ribu5P: Ribulose 5-phosphate, Ribose5P: Ribose 5-phosphate, Sedu7P: Sedoheptulose 7-phosphate, Gro-3P: Glycerol 3-phosphate, 3-PG: 3-Phosphoglycerate. Middle: Principal component analysis (PCA). Top PCA shows the grouping of controls (UC, 7C, 14C) using the amino acid metabolites. Bottom PCA shows the exposed to silver nanoink groups (U, 7, 14) using the central carbon metabolites. All metabolites measured are provided in supplementary as txt files and as a bar chart (Figure S1).

Glycolysis and the pentose phosphate pathway in C7 and C14 are also differently affected, with significant changes for Glc-6P in C7 and C14 media group, significant increase for pyruvate and decrease in C14 media group. Regarding metabolites involved in the central carbon metabolism, there were many differences observed among the conditioned media groups following acute exposure (Figure 8). In the U group, alanine was the only metabolite to show a significant increase, while lactate and glycerol-3-phosphate showed a

decrease. In the C7 group, significant increases were observed in lactate and malate, while glucose-6-phosphate, glucose, and fructose-6-phosphate exhibited changes. For the C14 group, several molecules α-ketoglutarate, glutamic acid, aspartate, alanine, pyruvate, glutamine, malate, and asparagine displayed significant increases compared to the control, whereas, glucose, glycerol 3-phosphate, and 3-phosphoglyceric acid were significantly decreased following exposure (Figure 5 supplementary). Principal component analysis (PCA) showed a distinct profile of the samples for the C14 group in both control (top) and silver nanoink exposed (bottom) groups. It was clear that the C7 group was separated from the U group for the exposed conditions but not in the controls. These observations together suggest additional metabolic perturbations induced by the silver nanoink on top of the impact of conditioning, which is stronger for the C14 groups.

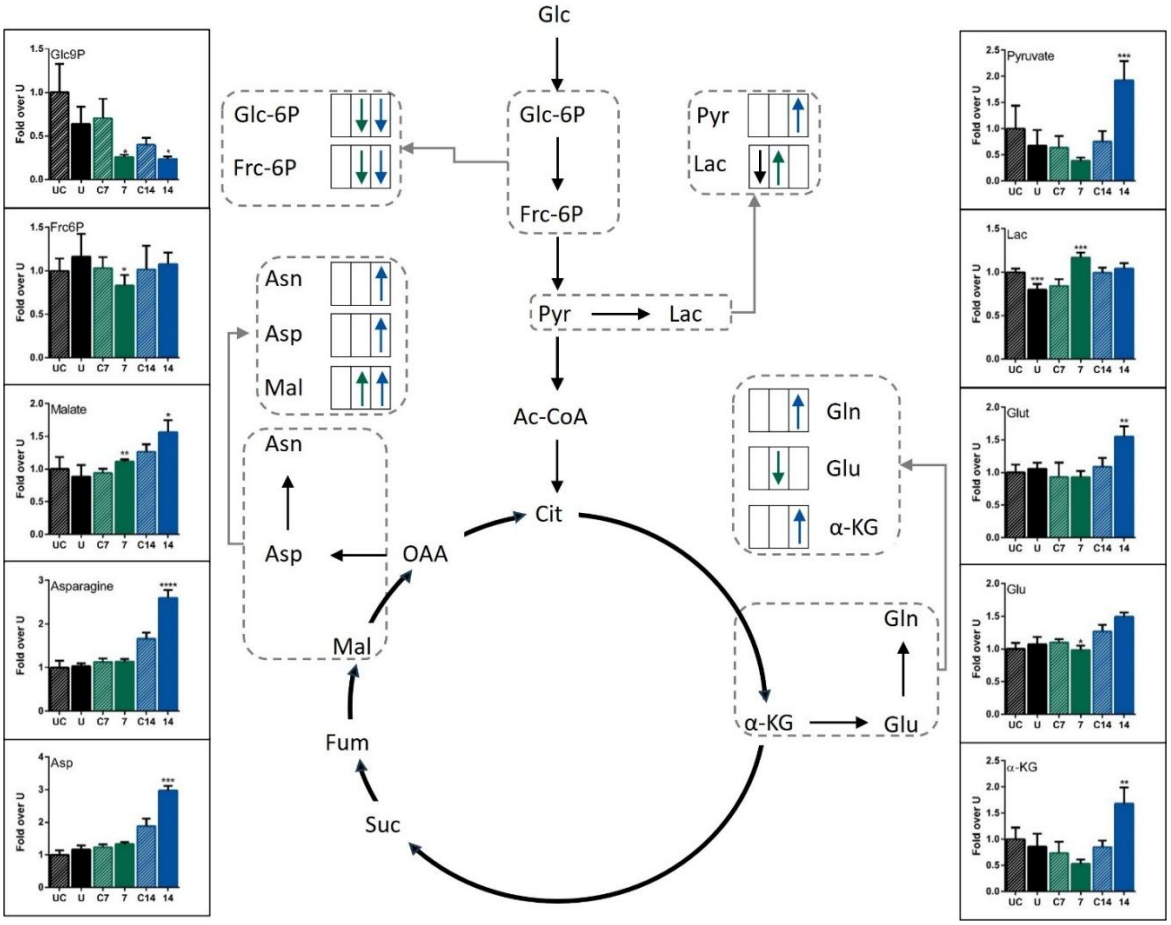

Figure 8. Illustration of the exposure impact of U, C7, C14 for 24h on the metabolism of *D. magna* levels in control (striped) and exposed (non-striped) groups. Each condition was compared to their corresponding control group. Data represents average fold values (N=6) of each replicate over unexposed control group, for each condition. Statistically significant changes identified by Student's *t*-test with Welch's correction between control and exposed conditions with p-values of $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). Abbreviations: Glc-6P: Glucose 6-phosphate, Cit: Citrate, α-KG: Alpha-

ketoglutarate, Suc: Succinate, Glu: Glutamate, Asp: Aspartate, Lac: Lactate, Ala: Alanine, Pyr: Pyruvate, Frc-6p: Fructose 6-phosphate, Ribu5P: Ribulose 5-phosphate,

Ribose5P: Ribose 5-phosphate, Fum: Fumarate, OAA: Oxaloacetate, Gln: Glutamine, Cis-A: Cis-Aconitate, Mal: malate, Asn: asparagine, Sedu7p: Sedoheptulose 7-phosphate, Gro-3P: Glycerol 3-phosphate, 3-PG: 3-Phosphoglycerate. All metabolites measured are provided in supplementary as txt files and as a bar chart (Figure S1).

5.4 Discussion

This study investigates the effect of excreted metabolite and coronas on the observed impact of silver nanoink on daphnids. The AgNP functionalization by environmentally relevant nanocoronas could potentially shed light to the fate of NPs and their true impact as pollutants once they enter the environment. Although literature is scarce, there are a number of recent studies that highlight the importance of eco-coronas in nanopollution assessment (Table 1).

Table 1. Key questions of excreted and selected metabolites and their implication to small molecule coronas.

Research question	Reference	Relevance to our work
Proof for the formation of metabolite nanocoronas	(Grintzalis et al., 2019)	A small molecule (metabolite) nanocorona can form and modulate physiological responses such as feeding, following acute exposure to sub-lethal concentrations of microparticles in daphnids.
Eco-corona presence alters NM interactions and toxicity.	(Nasser et al., 2020)	Macromolecules of interest can attach on NMs and create an eco-corona, altering the inherent characteristics of NMs in the context of acute exposure.
Highlighting the importance of the metabolite corona following exposure.	(Chetwynd and Lynch, 2020)	This critical review is in alignment with our findings, underlining the scarcity of information on metabolite nanocoronas as well as their potential as a key factor that significantly modulates nano-exposures.
Metabolites adsorb on NM surface based on surface chemistry.	(Chetwynd et al., 2020)	Chetwynd et al. showed that metabolite nanocoronas are dynamic and difficult to predict in the context of composition and formation, highlighting the need for further research.
Nanoinformatics approach on some factors that impact	(Varsou et al., 2021)	Using predictive models, this work predicted that the presence of an ecological corona may reduce the toxicity of NP exposure in accordance with the results of our work.

nanotoxicity for Ag and Ti NPs.		
Investigation on the impact of eco-coronas on tungsten carbide cobalt and cobalt NP toxicity	(Ekvall et al., 2021a)	This work showed the reduction of toxicity during acute exposure to NPs following the formation of eco-coronas on NPs of interest.
Investigation of eco-corona relevance on nanoplastic characteristics	(Zhu et al., 2023)	This study shows that eco-coronas will modulate the properties of nanoplastics as well, which is another important pollutant in the nano scale.

Conditioned media characterization showed several common peaks between C7 and C14 in both negative and positive ion modes, indicating that the same metabolic pathways in the two age groups were affected by media conditioning (Campos et al., 2018, Ebert, 2022, Bojadzija Savic et al., 2019) (Figure 2). According to the C14/C7 fold change in the common peaks, there is a clear decrease in the relative abundance of common metabolites excreted by the two age groups, suggesting a reduction of activity in certain metabolic pathways as the animal ages. However, it is unclear whether the reduction in the abundance of these metabolites reflects changes associated with growth or other early developmental processes. These findings highlight the variance of the metabolic profile of *D. magna* through different stages of life, which can have important implications regarding their study, and interactions with their environment.

Regarding the impact on acute toxicity, a significant difference is observed when comparing the effects of uncoated vs coated AgNPs, with coated AgNPs displaying reduced toxicity (Figure 3). These effects were also observed even after 48h of exposure, with the tested conditions displaying the same trend as the 24h exposure, showing that the presence of nanocoronas is severely reducing the observed mortality on acute exposures (Li et al., 2022, Khort et al., 2024) (Suppl.1). These findings also show that the initial toxicity threshold of AgNP nanoink was increased when we compare coated AgNPs vs uncoated AgNPs exposure conditions, highlighting the fact that NP presence in the environment can be masked to a certain extent by the presence of nanocoronas. The nanocorona slows down the onset of toxicity providing a certain level of protection to the exposed individual from the adverse effects of exposure which, however, remains effective only up to a certain

concentration of AgNPs of about 1µl/l. The sharp increase in observed toxicity once the threshold has been exceeded could mean the nano corona delays but doesn't mitigate the inherent AgNP toxicity. Furthermore, the amount of AgNP nanoink required to observe 100% mortality in the exposed population remained the same in both coated and uncoated groups, suggesting that the presence of the nano corona is affecting the kinetics of interaction but not the underlying mechanism through which toxicity is exerted.

Following the initial assessment of the presence of nano coronas, the impact on key enzyme activities was investigated to provide more information on a biochemical level. Enzyme activity can be used to assess the acute impact of exposure to daphnids as it 'captures' the actual response in the metabolome of an exposed individual (Michalaki et al., 2022, O'Rourke et al., 2023, Panagiotidis et al., 2023, Michalaki et al., 2023). Initial analysis on the impact of media conditioning upon acute exposure to *D. magna* among control groups (Figure 4) shows that key enzyme activity is impacted by the presence of metabolites in the media. Comparisons of C7 and C14 media to U, which was considered in this study as the 'baseline' as it includes only individuals which were placed for 24h in clean OECD media, showed a different response, with C14 media exposure resulting in a more pronounced effect on enzyme activity than C7. The differences of C7 and C14 prove that the media contents were not just NOM but bioactive molecules with the capacity to impact a key physiology endpoint in *D. magna*. Furthermore, this impact on enzyme activities also underlines the ability of NPs to adsorb molecules that have the capacity to further influence the impact of exposure, hinting at the existence of a dynamic system of NPs and bioactive molecules that can form a nanocorona in freshwater environment. This system would ultimately be very difficult to correctly assess in terms of interactions with freshwater organisms and the severity it impacts freshwater ecosystems in general.

The comparison of individuals exposed to unconditioned AgNPs with individuals exposed to AgNPs with nanocorona (Figure 5) aims to show if the changes in nanocorona composition can impact the enzyme activities in exposed *D. magna*. To assess this, AgNPs conditioned by C7 or C14 media was compared to a 'baseline' which is the U exposure group, showing the difference in which enzymes were affected by different nanocoronas versus the impact on enzyme activity by AgNPs without nanocoronas. The C14 corona creates a broader and more intense response in exposed *D. magna*, suggesting that the C14 corona is more biologically active compared to C7 corona. This could be due to C14 being more enriched in certain biomolecules that may intensify the AgNPs interaction with

exposed individuals, which is supported by our findings on media characterisation (Figure 2).

To assess if the mechanism through which conditioned AgNPs exert toxicity to exposed *D. magna*, the response profile of each media group was assessed. By comparing the exposed to the respective control conditions in each group there is a clear distinction in the response profile following acute exposure (Figure 6). BGAL activity was decreased only in U, suggesting AgNP interference in carbohydrate metabolism or glycoside breakdown pathways (Lu et al., 2020, Juers et al., 2012), and according to available literature, a decrease in BGAL activity has been related as a response to stress in plants (Hoang et al., 2023). LIP activity was significantly increased in U and C7 conditions, possibly reflecting an effort to manage or repair cell membrane damage (Tam et al., 2010) induced by the acute exposure (Xiang et al., 2024). These findings indicate that uncoated AgNPs have greater impact on digestive enzyme activity than uncoated AgNPs, likely due to their ingestion by *D. magna*, as AgNPs have been detected in their digestive tract following acute exposure (Lekamge et al., 2019, Zhao and Wang, 2012, Mackevica et al., 2015). GST is a major enzyme in management of oxidative stress (Mazari et al., 2023, Röth et al., 2011), showing a significant increase in the conditioned groups. These results suggest that the presence of the C7 and C14 nanocorona causes an upregulation of detoxifying pathways (Lakkim et al., 2023, Khan et al., 2024, Dickinson and Forman, 2002, Masip et al., 2006, Xu et al., 2019, Carmel-Harel and Storz, 2000), with C7 media group's stronger response indicating that metabolites originating in younger *D. magna* result in increased oxidative stress response when compared to metabolites originating from older *D. magna*. The significant increase in LDH activity in U following exposure shows elevated anaerobic glycolysis (Dhillon and Kaur, 2016) or cellular damage (Kumar and Ameeramja, 2021) which is a result of cell stress (Yao et al., 2019, Song et al., 2012). In contrast, the significant decrease in C7 may indicate either a reduction of cellular damage or that the presence of the C7 nanocorona prevents the need for anaerobic pathways. In either case, these results suggest that C7 media results to *D. magna* coping with AgNP induced stress differently than when exposed to C14 metabolites. PEP activity was significantly reduced in U while significantly increased in C14, implying a degradation of damaged proteins in response to AgNP exposure (Kos, 2023) in U while in C14 cellular repairs appears to be supported, further underlining the notion that these metabolites have to a certain extend mitigate the impact of exposure. ACP activity was impaired in U and C7 groups, suggesting an impairment of lysosomal (Ashtari et al., 2016, Bull et al., 2002, Hánsch and Emmerling, 2010) or other functions, while impairment of ACP activity is in accordance with the exposure effects of AgNPs according to available

literature (Assar et al., 2023, Kamaszewski et al., 2023). The lack of significant change in C14 suggests that older *D. magna* metabolites may help preserve lysosomal function. ALP activity was increased in C14 indicating improved cellular repair mechanisms or generally compensatory membrane responses to acute AgNP exposure (Krötzsch et al., 2005, Harris et al., 2004). Another important consideration in NP research related to environmental pollution is that these results highlight the variability introduced by coronas, which may go unnoticed in studies using only unconditioned particles.

Metabolomic analysis of *D. magna* following acute exposure focusing on the impact of exposure on amino acid levels in exposed individuals clearly showed a differentiation among the three media groups. The U media exposures likely result in general oxidative stress and a more non-specific impact on the metabolome of exposed individuals. Increased LDH activity, a marker of cellular damage (Legrand et al., 1992) and anaerobic metabolism (Farhana and Lappin, 2024), and decreased PEP activity, which is related to energy metabolism, align with observed metabolic disturbances of amino acids, like decreased ornithine and histidine, indicating a more generalized metabolic disruption (Pereira et al., 2024). The significant increase in LIP also suggests altered lipid metabolism (Farhana and Lappin, 2024), possibly as a broader stress response, which is less focused compared to the effects seen in nanocorona exposures. The presence of a nanocorona conditioned by C7 media appears to induce a more specific stress response. Elevated GST levels indicate heightened detoxification activity, likely due to reactive oxygen species (ROS) induced by AgNPs. This aligns with observed amino acid shifts, such as the decrease in arginine (Cao et al., 2001) and increase in proline (Liang et al., 2013, Hayat et al., 2012), which are often involved in stress responses. The reduced LDH activity suggests that, unlike in the U group, cellular energy pathways in C7 might adapt better to AgNP exposure, potentially due to the protective nature of a simpler nanocorona. This is consistent with the results of acute toxicity tests which demonstrated a significant reduction in observed mortality aligning with available literature (Ekvall et al., 2021a) (Figure 3). The nanocorona of C14 media appears to intensify interactions with *D. magna* physiology, driving a more complex stress response. Elevated ALP activity, associated with membrane stability and repair (Krötzsch et al., 2005, Harris et al., 2004), alongside increased PEP activity which is linked to energy metabolism (Kos, 2023), suggests cellular stress that subsequently leads to adaptive responses. The increase in amino acids such as Gly, Glu, and Ala may reflect an upregulation of pathways related to energy production (Chaudhry and Varacallo, 2024) and nitrogen metabolism (Yadav et al., 2022) to compensate for the acute exposure to AgNPs. Enhanced GST activity, paired with the diverse metabolic shifts observed (e.g., increased Ile, decreased Asn),

suggests that AgNPs in C14 media invoke strong oxidative and metabolic stress, pointing to a more targeted stress response in exposed individuals.

Metabolomic analysis of the investigated media groups revealed significant differences among the comparisons of exposed to control conditions for each respective group, showing a diverse response. For the U group, significant decreases are observed in lactate, which could imply a disruption in lactate metabolism suggesting a less direct metabolic inhibition by AgNPs when no nanocorona is present (Figure 8). In contrast, C7 and C14 exhibit unique patterns not seen in U, with significant increases in key TCA intermediates (e.g., α-KG in C14 and Asp in C14) suggesting an adaptive or compensatory upregulation of the TCA cycle, possibly due to altered cellular stress or energy demands caused by the coronas. Glycolysis & PPP in C7 and C14 are also differently affected as seen in Figure 8. These differences indicate that the nanocoronas are modulating interactions that lead to shifts in energy production and/or antioxidant responses. The increase in LDH aligns with the significant decrease in lactate in the U group, suggesting a potential feedback where elevated LDH might reflect increased lactate consumption or generally impacted lactate metabolism due to AgNP stress (Figure 8) (Oh et al., 2014). This points to a disruption in energy production pathways (glycolysis and lactate metabolism), as indicated by both the metabolite and enzyme shifts. The increase in GST suggests enhanced antioxidant defence in C7, possibly in response to oxidative stress mediated by the presence of a nanocorona. This increase may also relate with the observed increase in lactate in C7, hinting at elevated energy demand to support antioxidant defences. The contrasting LDH response in C7 versus U could indicate that C7-conditioned AgNPs shift toxicity away from glycolysis and lactate pathways toward lipid and antioxidant metabolism that is supported by the significant increases in LIP (Olivecrona, 2016) and GST activity (Figure 7). Furthermore, in the C14 group, the upregulation of ALP and PEP aligns with observed increases in TCA metabolites (e.g., α-KG and Asp) in C14, suggesting improved energy metabolism (Wu et al., 2016) and stress adaptation (Azevedo et al., 2006) during the exposure. Significant increase of GST activity (Figure 7) further supports improved oxidative stress response, while elevated TCA metabolites and ALP activity showing additional metabolic shifts due to the presence of the nanocorona.

5.6 Conclusions

The presence of excreted metabolites can significantly impact the mechanisms through which nanotoxicity is exerted on *D. magna* exposed to AgNPs. This is indicated by the numerous differences observed across multiple markers of daphnid physiology, from reducing acute toxicity following exposure to distinct response profiles emerging for enzyme activity and metabolism based on the presence and composition of a metabolite nanocorona. While the exact reason as to why there is a reduction of observed adverse effect of exposure is unclear, we assume that the ion generation of Ag^{+} is mitigated by the presence of the nano corona coating the AgNPs. Additionally, these changes in the composition of nanocoronas had a significant impact on the enzyme activity and metabolism, highlighting the importance of nanocoronas in the context of studying NPs for freshwater ecosystem pollution assessment and monitoring. However, organism-derived metabolite coronas remain largely unexamined compared to protein coronas, despite their relevance in ecotoxicological contexts. Our findings underline the overlooked influence of organism-excreted metabolites on silver nano ink behaviour and toxicity, suggesting the existence of a dynamic system of NPs and nanocoronas composed of bioactive molecules not currently captured in existing models. The lack of chronic exposure studies and omics-based data further limits our understanding of adverse outcome pathways related to AgNP exposure, potentially underestimating the ecological impact of these materials.

Data availability

Data will be made available upon request. The matrix of the metabolomic data and processed data are provided in the Supplementary Materials.

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CRediT authorship contribution statement

Dimitrios Kakavas: Investigation, Methodology, Formal Analysis, Writing-Original Draft Preparation, Writing-Review & Editing. **Beatrice Engelmann:** Investigation, Methodology, Formal Analysis, Visualisation, Data curation, Writing-Review & Editing. **Martin Krauss:** Investigation, Methodology, Formal Analysis, Data curation, Writing-Review & Editing, Resources. **Ulrike E. Rolle-Kampczyk:** Formal Analysis, Writing-Review & Editing, Resources. **Keith D. Rochfort:** Conceptualization, Methodology, Writing-Original Draft Preparation, Writing-Review & Editing, Funding acquisition, Supervision, Project administration. **Konstantinos Grintzalis:** Conceptualization, Methodology, Investigation, Visualization, Writing-Original Draft Preparation, Writing-Review & Editing, Funding acquisition, Supervision, Project administration.

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Chapter 6

Conclusions

The aim of this research was to investigate unexplored aspects of nanotoxicology and nanocoronas by using *in vivo* (daphnids) and *in vitro* (A549 cells) models as 'representatives' for environmental and human health respectively. The main focus of this work was the effects of silver nanoink toxicity, and in an environmental perspective this is reflected in the daphnia chapters. Silver nanoink was investigated as a 'model' NP, as it is known from available literature that AgNPs are toxic to exposed aquatic organisms and are also known to be an environmental pollutant (Ramirez et al., 2022, Ipek et al., 2023). Conventional toxicological assessment frameworks do not take into account the NP properties that make them different from their bulk counterparts (Hanazato and Dodson, 1995). The NP small size and large surface area are the main culprits for NP contaminants bypassing monitoring and removal interventions, while the distinct NP shape, presence of nanocorona and NP aggregation/agglomeration profile cumulatively determines the overall impact (Turan et al., 2019, Ramirez et al., 2022). Therefore, the inability of a conventional approach to accurately measure the effects of nanopollution only highlights the need for a more in depth understanding of nanotoxicity mechanisms as well as which parameters might impact more the observed effect of exposure on markers of physiology. This work uses a holistic approach by combining different models and key physiological endpoints used in toxicology with metabolomic data to describe changes induced by NP exposure. Despite using one NP to study nanotoxicity mechanisms, the shared characteristics of silver nanoink with other NPs ensure the applicability of this work to other NP pollutants in different contexts, as the presence of these emerging contaminants in environment is sure to persist.

6.1 Major Findings

In freshwater ecotoxicology, the *D. magna* remains the most widely adopted and ecologically relevant bioindicator model thanks to its high sensitivity to environmental pollutants, ease of culturing and well characterized physiology and genome (Reilly et al., 2023). Its responsiveness to pollutant exposure in a range of biomarkers such as feeding behavior, enzyme activity, growth and more, can provide critical insights into toxicity mechanisms (Tkaczyk et al., 2021). Despite this wide adoption of the daphnia model, the use of daphnia in nanotoxicity testing can be limited due to the absence of standardized testing protocols. Variability in experimental setups have led to inconsistent and sometimes contradictory results, complicating study comparisons and interpretation.

Chapter 2

The findings of this work underscore the critical importance of standardizing exposure vessel parameters in ecotoxicological studies to ensure that toxicity assessment data for NPs pollutants are both reliable and comparable across studies. By reducing the volume of test media required, the miniaturized assays not only reduce NP laboratory waste, but also minimize variability. Moreover, our results highlight the urgent need for a universally adopted miniaturized daphnid toxicity test for NPs pollutants.

- The work on miniaturization demonstrates that biomarkers such as key enzyme activity and feeding rate can be employed reliably in a miniaturized testing approach, which can yield greater data precision than traditional setups (Kakavas et al., 2024). This is accomplished by specifically accounting for the unique aggregation, sedimentation, and dissolution behaviors of NPs (Krug and Nau, 2022).
- Protocol standardization would streamline endpoints across laboratories, facilitate meta-analyses, and provide researchers with robust benchmark data for pollution risk assessment (Krug and Nau, 2022).

We identified two reliable physiological markers; feeding rate and key enzyme activity that can detect stress responses using sub-lethal concentrations of NPs, which is also the case for other pollutants as well according to available literature (Michalaki and Grintzalis, 2023, Michalaki et al., 2022, O'Rourke et al., 2023). To strengthen environmental risk management strategies and ecological relevance, it is essential to address remaining gaps in

our understanding of NPs toxicity mechanisms. In particular, systematic investigation of the influence of vessel volume, material composition, hydrodynamics, and nanocoronas on toxicological outcomes of different NMs can shed light on how a variety of parameters may modulate observed effects of exposure.

Chapter 3

Several markers of physiology were used to assess the impact of silver nanoink using exposure vessels with different S:V ratios in an effort to induce diverging responses from exposure groups – proving that the comparison of results across studies using different S:V setups is confounded.

- Results showed that an increase in S:V of exposure vessels enhanced acute toxicity of silver nanoink as well as the observed effect of exposure on key enzyme activity but failed to inflict any changes in feeding rates of exposed daphnia.
- As evidenced by altered baseline enzyme activities in control groups that were not exposed to any pollutants and showed statistically significant changes in markers of physiology after being placed in vessels with S:V differences, changes in vessel geometry can potentially have a significant impact on observed results.
- Comparative analyses across studies that employ different vessel geometries risk misattributing experimental variability to pollutant properties rather than to test design.

More research is needed to evaluate the impact of S:V in daphnia toxicity testing, especially given the fact that NPs are colloid solutions that behave differently compared to other pollutants, therefore it is of utmost importance to accurately distinguish between which parameters are affected due to the uniqueness of NPs as a pollutant and which are affected due to the characteristics of the animal model used. For example, high S:V vessels may restrict diel vertical migration (Ebert, 2005), a natural behavior that organisms use to regulate light exposure and predation risk; therefore, inducing physiological stress even in the absence of NPs due to confinement in such environments. Our data therefore calls for the incorporation of S:V guidelines into existing standard methods, and for future research to elucidate the mechanistic links between vessel geometry, organismal behavior, and NP bioavailability.

Chapter 4

The study on daphnid crowding demonstrates that differences in the density of daphnids during exposure to silver nanoink significantly change observed toxicity, enzyme responses, and core metabolism, even in the absence of pollutant.

- Increased crowding resulted in reduced acute toxicity of AgNPs while it induced significant changes in toxicity, activities of enzymes such as PEP and GST, indicative of energy and oxidative stress disruption respectively.

- Triggered significant shifts in amino-acid profiles and metabolic pathways associated with core metabolism.

- Our findings underline that stress management mechanisms can be greatly influenced by the exposed animal population.

The variety of markers that showed significant changes highlights the importance of this experimental parameter in the context of nanotoxicity assessment as well as cross-study result comparison. Future protocols should integrate rigorous criteria for crowding instead of typically broad ranges for organism density, such as fixed per-capita water volume, to ensure reproducible results for nanopollutants.

Chapter 5

Our findings on nanocoronas showed that organism-derived metabolites significantly influence how AgNPs exert toxicity in *D. magna* through the formation of dynamic nanocoronas (Kakavas et al., 2025). Although the precise nanotoxicity mechanisms are not clear (Mahmoudi et al., 2023), we propose that metabolite nanocoronas adsorb to NP surfaces, creating steric barriers to dissolution and altering surface charge, thereby modulating cellular uptake and bioreactivity.

- Exposure to NP suspensions conditioned with daphnid released metabolites resulted in reduced acute toxicity compared to freshly prepared suspensions, indicating that metabolite nanocoronas can mitigate ion release and particle reactivity.
- Detailed analyses of enzyme activities and metabolic changes revealed distinct response profiles that depend on the composition of the nanocoronas while certain metabolic byproducts attenuated stress markers of physiology endpoints.

- These findings highlight the critical role of metabolite nanocoronas, a largely unexamined field compared to protein coronas in freshwater pollution assessment, as they reflect environmentally relevant interactions between NPs and biota.

The lack of chronic-exposure studies and integrated omics-based data on AgNP interactions with these coronas limits our understanding of nanotoxicity mechanisms and may lead to underestimation of long-term ecological impacts (Chetwynd and Lynch, 2020). Bridging these knowledge gaps through a holistic approach, coupled with transcriptomic, proteomic, and metabolomic profiling, will be essential for developing AOPs and accurate predictive models of NP behaviour in complex ecosystems. By integrating vessel design, ecology, organism characteristics, and coronas into nanopollution assessment, we can accurately capture the impact of nanopollution.

The investigation of nanotoxicity mechanisms with an *in vitro* model resulted in promising findings, however, there is room for further optimisations for A549 cell model use in nanotoxicology research. Possible NP interference due to high reactivity is the main suspect for variability of results – indicating that for nano cytotoxicity studies a different cell model may be more suitable. Enzyme activity assays demonstrated that key physiological enzymes can reliably detect statistically significant changes following sub-lethal NP exposures, even in more 'simple' *in vitro* systems, mirroring their effectiveness in the environmental part of this thesis. Therefore, optimisation of its employment in cytotoxicity research can be a very useful tool as an intermediate step between conventional toxicity assays and more in-depth approaches such as genomics or metabolomics. The generated data can also be critical in understanding mechanisms of nanotoxicity on a molecular level as enzyme activity captures the metabolic state caused by pollutant exposure and can be used in investigating early stress responses to nanopollutants.

6.2 Mechanistic insights of nanotoxicity

The ecotoxicological impact of NMs, arises not merely from their physicochemical toxicity but from their unique physicochemical interactions at the nano-bio interface (Martinez et al., 2022). These interactions can modulate cellular processes through mechanisms including oxidative stress, membrane disruption, and protein-NP interactions. There are a number of established endpoints utilized in toxicology, however, in an environmental context the use of sensitive bioindicator species is critical to gather data on nanotoxicity mechanisms and molecular disruptions induced by NP exposure.

- Enzymatic biomarkers serve as mechanistic proxies for these underlying processes, converting molecular disruptions into quantifiable physiological responses (Michalaki and Grintzalis, 2023, Michalaki et al., 2023, Michalaki et al., 2022, O'Rourke et al., 2023, O'Rourke et al., 2023).
- This work utilizes ALP, ACP, LDH, LIP, GST, PEP, BGAL and RT as biomarkers of daphnids and A549 cells to investigate nanotoxicity mechanisms. These biomarkers successfully showed significant changes in enzyme activity following exposure to AgNPs that were accurate, quantifiable and can be further employed as a precursor marker to metabolomics.
- Metabolite nanocorona formation significantly affects the adverse effects of exposure to AgNPs on enzymatic and metabolomic markers.

For the works included in this thesis, enzyme activity patterns were systematically used to uncover aspects of the mechanistic basis of nanotoxicity, including the role of nanocorona formation. The enzyme activity was then used with the *D. magna* model and A549 cells as means to quantify changes in a range of phenotypic endpoints like mortality, feeding, enzyme activities and metabolic activity. Plenty of core biological processes like energy metabolism, digestion and detoxification are modulated by the activity of these enzymes while at the same time, these processes have also been shown in available literature to be adversely affected by NP exposure. Additionally, numerous metallic NPs have been shown to induce oxidative stress by the generation of ROS (Kessler et al., 2022) and there are many instances of mechanically induced DNA damage if they bypass the nuclear membrane (Rim et al., 2013).

6.2.1 Miniaturized nanotoxicity testing and enzyme activity as a consistent physiology marker

The 2024 study published in the journal Animals expanded on this mechanistic framework by testing whether enzymatic responses to silver nanoink exposure remained unaffected in their ability to show the adverse effects of exposure under assay miniaturization (Kakavas et al., 2024). Results confirmed that despite drastically reduced test volumes (up to 99.4%) and animal numbers, key enzymes like ACP and RT maintained sensitivity. This was a very positive finding given the fact that for these tests, sub-lethal silver nanoink concentrations were used, equivalent to the EC_5 or lower. Additionally, there are many more enzymes such as acetylcholinesterase (Lionetto et al., 2013), catalase (Sirivarasai et al., 2015), superoxide dismutase (Vlahogianni et al., 2007) and more that can

be used as a physiological marker to quantify pollutant exposure effects, allowing for a highly customizable model that can be 'tuned' to many different pollutant categories or concentrations – even in those that would evade conventional toxicity testing assays. The mechanistic implication is that AgNPs perturb core metabolic and membrane-related processes, which can be effectively monitored through enzyme activity even when assay architecture is scaled down—critical for future high-accuracy and low-footprint nanotoxicity testing. Notably, the miniaturized 96 well plate format required the smallest quantity of AgNPs to reach EC_{50} values, suggesting that proximity between the test organism and sedimenting NPs may be a critical factor contributing to variability in nanotoxicity testing outcomes. Therefore, special variations in NP concentration that might arise over the time of exposure should be considered in nanotoxicity testing. Further investigation is warranted into the colloidal instability of NP suspensions during exposure, particularly how surface adsorption of other molecules may exacerbate sedimentation and influence test reliability.

6.2.2 Exposure vessel geometry can modulate observed nanotoxicity effects

This work investigated how surface-to-volume ratio (S:V) in exposure systems modulates NP bioactivity (Kakavas et al., 2023). From a mechanistic standpoint, the confinement of NPs in high S:V vessels increase their likelihood of interaction with biological membranes and reduces the dilution of both NPs and excreted metabolites due to sedimentation. The results indicated that restricting daphnids close to the vessel bottom enhances local NP concentration amplifying adverse effects of exposure. Enzyme biomarkers such as LDH, ACP, ALP, and PEP showed diverging activity under comparison of exposure vessels with large differences of S:V, mechanistically reflecting increased membrane disruption as indicated by significant increase in LDH activity that reaffirms findings in available literature on AgNP exposure effects, impaired phosphate signalling or ion channel interference due to ACP and ALP disruption (McCarthy et al., 2010, Michalaki et al., 2022), and reduction of defensive proteolytic responses as shown by the decrease in PEP activity. Feeding rate was also investigated to quantify changes inflicted by changes of S:V ratios, and in concurrence with previous results, increased S:V ratio of the exposure vessels enhances the nanotoxicity effects of exposure. We propose that due to the accumulation of AgNPs on the vessel bottom resulted in increased AgNP consumption which in turn led to additional reduction of feeding rate in exposed individuals. In the context of updating nanotoxicity testing guidelines these are critical findings as there is not a universally accepted experimental setup that takes into account these parameters, therefore invalidating

result comparison across nanotoxicity studies which use different S:V ratio in their exposure vessel of choice. These findings support a model where bioavailability and nanoscale reactivity are co-modulated by the test system's geometry among other characteristics, and where enzyme responses track these effects with high sensitivity and accuracy. They also provide a mechanistic rationale for the observed adverse effect amplification in vessels with large S:V ratio and affirm that nanotoxicity is inherently context-dependent, influenced by both biological and environmental factors that must be taken into account due to the unique properties of NPs. It might also highlight a potential reason as to why there is inconsistency of findings among studies relating to NPs in the context of toxicology given that we also detected significant changes in the baseline enzyme activity of exposed daphnids in absence of any nano pollutant.

6.2.3 Daphnid crowding as a modulator of observed adverse effects and toxicity pathways

In the context of daphnid nanotoxicity testing the number of exposed individuals per vessel is another unexplored aspect. In this study, daphnid crowding was introduced as a biological variable influencing nanotoxicity outcomes. Mechanistically, changes in crowding led to altered baseline enzyme activity possibly through changes in interspecies interactions, diverging response profiles of exposure groups in enzyme activity and significant differences in central carbon metabolism and amino acid levels among exposed groups as well as their baseline—all of which impact how organisms respond to AgNPs. Control groups showed significant variation in enzyme activity, especially in LDH, GST and ACP across different crowding levels. These changes suggest that crowding modulates metabolic set points and physiological readiness, which in turn influence the downstream toxicity pathways activated during AgNP exposure. Upon exposure to silver nanoinks, enzyme activity profiles diverged substantially between crowding groups: LDH increased in low-crowding groups, indicating high membrane and oxidative stress, but decreased in high-crowding groups, suggesting preconditioning or some kind of protective adaptation. Significantly reduced ACP activity in low crowding groups, indicate lysosomal membrane compromisation or cellular degradation (Kumar and Gill, 2018). PEP activity remained sensitive across conditions, consistent with proteolytic involvement in NP response (Kos, 2023). These findings imply that crowding may serve as a biological modifier of nanotoxicity by altering metabolite excretion profiles through changed interspecies interactions, which could in turn influence nanocorona formation and NP-cell interactions.

The reduced toxicity observed in high-crowding groups could be explained by nanocorona formation due to the increased number of exposed individuals (and excreted molecules), thereby acting as a natural protective mechanism. From a mechanistic perspective, this highlights the interdependence of physiology and physicochemical transformation of NPs *in situ* and calls for key updates in daphnid nanotoxicity testing.

6.2.4 Nanocoronas as modulators of NP-cell interactions and enzyme responses

The final environmental chapter of this thesis explores the role of metabolite nanocoronas on nanotoxicity using silver nanoink (Kakavas et al., 2025). Although there is scarce information in available literature, metabolite nanocoronas are an emerging and niche aspect of nanotoxicity (Chetwynd and Lynch, 2020). This study evaluated how silver nanoink toxicity and physiological responses in *D. magna* are modulated by metabolite nanocoronas formed in media conditioned by daphnids of different ages. Specifically, exposure media were unconditioned (U) to examine the responses to 'clean' silver nanoink or were preconditioned using daphnids aged day 7 (C7) and day 14 (C14) to simulate a more environmentally relevant alternative. By comparing toxicity and biomarker outcomes in these two biologically enriched systems, the work provides mechanistic insight into how NP identity and bioactivity are transformed through life-stage-specific metabolite conditioning of the aquatic environment. Exposure media were pre-conditioned by daphnids at either the age day 7 or 14, then used in silver nanoink exposures at a sub-lethal concentration; maintaining chemical parameters constant and varying only the biological age of the conditioning organisms; therefore, only the life stage–specific metabolites which resulted in changes of AgNP behaviour and toxicity. Acute exposure outcomes revealed significant differences in toxicity severity depending on if the media was conditioned or not. This nanocorona dependent divergence of observed nanotoxicity strongly supports the role of biologically derived excretions in NP toxicity outcomes and is based on NP characteristics that are known to modulate their adverse effects such as surface modifications (Rybak-Smith, 2012). Since daphnid excretions include lipids, proteins, and enzymatically active compounds (Schwarzenberger and Fink, 2018, Richardot et al., 2001), the biochemical composition of C7 and C14 media necessarily differed. This supports the interpretation that conditioned media enabled the spontaneous formation of biologically functional nanocoronas, which modulated the surface of AgNP, leading to lower reactivity, lower cytotoxicity, and selective shielding of membrane-interacting domains. In contrast, the

‘simpler’ in unconditioned media showed a different response of daphnids as if the NPs were more biologically ‘aggressive’.

The analysis of key enzymes provided additional mechanistic observations regarding nanotoxicity across exposures, clear divergence in enzyme responses was observed between C7 and C14 media and unconditioned AgNPs. In general, C14 media was shown to be more potent in influencing enzyme responses. Baseline enzyme activity in daphnids was also affected by exposure to conditioned media in the absence of silver nanoink in diverging response profiles for exposed groups. From a mechanistic perspective, these findings confirm the potential of environmentally relevant metabolites to influence enzyme activity in *D. magna* when used to condition NPs. It also reaffirms the potential of enzyme activity as a biomarker to accurately reflect functional changes in NPs, even when using sub-lethal concentrations that would otherwise evade detection in conventional toxicity testing (Schuijt et al., 2021). Furthermore, the findings regarding enzyme activity exposures among groups exposed to both types of silver nanoink exposure media (unconditioned and conditioned) also showed diverging profiles due to the presence of excreted metabolites, reaffirming the findings from control comparisons alone. By investigating both the background effects of conditioned media and the differential responses of AgNP exposure, these results show a mechanistic assessment of how environmentally realistic coronas alter nano-bio interactions and toxicological outcomes. Research on NP exposure induced effects must consider the mechanisms of nanocorona formation and their potential to add ‘downstream’ modifications of unpredictable nature, skewing the observed effect of exposure on physiology markers (Wheeler et al., 2021).

In a mechanistic context, the metabolomic analysis reflected the increased content of metabolites in C14 media which was shown by LC-MS analysis and enzyme activity data as the C14 media resulted in the most changes observed on amino acids levels and central carbon metabolism. C7 and unconditioned media resulted in far less changes in the metabolome of exposed daphnids, showing diverging response profiles. These results also reflect the significance of these nanocoronas as a potential ‘Trojan horse’ which on one hand allows exposed individuals to survive acute toxic effects but at the same time significantly alters core metabolic processes such as TCA activity (Hsiao et al., 2015). Additionally, they might induce effects completely different from unconditioned NPs which is the case for pyruvate in C14 group, or lactate in C7 group - increasing the potential of these NPs to evade detection of effect-based methods of environmental assessment. In particular to the metabolic shifts observed by the exposure groups, this study also proves that integration of

omic data in the arsenal of nanotoxicity assessment is a critical feature to differentiate the corona effect from NP exposure. Furthermore, these results show that the significant increase in pyruvate, malate, aspartate and asparagine indicate enhanced preservation of mitochondrial function (Krall et al., 2021, Koch et al., 2024, Jin et al., 2025) possibly by reducing mitochondrial membrane stress and ROS production since the presence of the nanocorona shields the AgNPs from their surroundings to a certain extend. Comparing U, C7 and C14 metabolomic data shows that the nanocorona composition directly influences NP toxicity at the metabolic level, not necessarily preventing nano-bio interactions completely but by modulating metabolic resource distribution at cell level.

6.2.5 Toward a mechanistic framework for nanotoxicology

This integrated enzymatic profile across four studies provides compelling evidence that AgNP toxicity in *D. magna* is mediated by multiple, interlinked mechanisms, including: nanocorona-induced modulation of NP toxicity, uptake, cellular signalling, and experimental factors (e.g., exposure geometry) that influence NP concentration dynamics, and which alter enzyme activity levels and core metabolism either as a baseline or post exposure. The enzymes activity assays consistently emerged as mechanistically informative biomarkers across all chapters in this thesis. Their activity patterns reveal NP-induced stress through disruptions in key processes for exposed individuals. These findings collectively support a paradigm shift where a functional reporter of mechanistic processes underlying NP toxicity is essential to investigate mechanisms of nanotoxicity and adverse effects. Going forward, integration of enzyme assays with molecular and omics-level data may be integral to provide predictive power for environmental risk assessment of emerging NMs. To conclude, this work provides direct evidence that nanotoxicity is heavily modulated by the functional identity of the nanocorona itself, which can be a product of the NM microenvironment. However, this also means that unpredictable functionalisation of NMs could also potentially result in enhanced toxic effects. As such, future NM risk assessments should not only consider chemical composition and dosage but also account for biological context as a driver of NP functionalisation and adverse effects of exposure.

6.3 Complexity in environmental nanoexposure

The assessment of NM toxicity in toxicological contexts presents unique methodological and conceptual challenges unlike any other type of pollutant. These challenges arise not only from the nanoscale properties themselves, but also from the resulting dynamic interaction systems that arise within biological complexes and chemically rich environments. A central aspect to this complexity is the phenomenon of nanocorona formation, wherein biomolecules from the surrounding environment adsorb onto the NP surface based on its properties, forming a soft or hard biocorona that redefines the NPs' biological effects (Casals et al., 2010, Monopoli et al., 2011). This transformation alters NP behaviour complicates both prediction and measurement of toxicological outcomes. A central barrier in advancing environmental risk assessment of NPs lies in the lack of reproducible exposure models that replicate environmentally relevant conditions while maintaining the ability to identify and understand specific nano-bio interactions. In natural ecosystems, NPs do not exist in isolation, as they interact with DOM, excreted metabolites, secretions and other suspended particles, all of which can contribute to the nanocorona composition (Chetwynd et al., 2020). Consequently, NPs deployed in ecotoxicological assays may bear coronas that differ dramatically in biochemical composition and toxicological function depending on subtle changes in environmental parameters (Keller et al., 2010). This introduces fundamental uncertainty into laboratory-to-field extrapolation of findings. In particular, the corona's role as an environmental modulator remains difficult to isolate, due to its dependence on biomolecular availability and the lack of standardized characterization techniques (Baimanov et al., 2022, Grintzalis et al., 2019). Real-world exposure scenarios may involve aquatic media conditioned by diverse biological sources, including senescent phytoplankton blooms, high-density invertebrate populations, or microbial biofilms. Each of these biological matrices contains distinct classes of excretions capable of nanocorona formation, therefore, these coronas may in turn mitigate or exacerbate NP toxicity (Louie et al., 2014), depending on their composition and the pathways they shield or activate during cellular interaction (Soliman et al., 2024). Furthermore, in aquatic systems, sedimentation dynamics, water column classification, and the hydrodynamic environment all affect NP distribution and corona evolution. NPs may be found in higher concentrations in benthic zones, increasing exposure for bottom-dwelling or grazing species, while also undergoing biotransformation through contact with organic-rich sediments (Louie et al., 2014). In such scenarios, NPs acquire coronas from DOM or excreted biomolecules in sediment porewaters, further diverging from their laboratory analogues. The transient and situation-

specific nature of coronas under these conditions complicates both the detection of consistent adverse outcomes and the establishment of regulatory guidelines and thresholds. However, employing consistent biological models and sub-lethal exposure paradigms has demonstrated that even sub-lethal NP exposures can induce quantifiable changes in physiological markers such as enzyme activity and core metabolism. Importantly, these changes are not solely dependent on NP dose or composition, but on environmentally driven corona assembly, which modulates NP-cell interactions at the interface of exposure. Such insights provide an empirical foundation for advancing risk assessment models beyond concentration-based approaches toward functionally integrated markers and omic analyses.

The use of metabolite-conditioned exposure media has proven especially effective in replicating more ecologically realistic exposure scenarios (Grintzalis et al., 2019, Chetwynd and Lynch, 2020). When exposure media are preconditioned by aquatic organisms at different life stages, the resulting corona composition reflects biologically plausible molecular environments. Comparative analyses of nanotoxicity effects under measurable and controlled changes in these conditions revealed that conditioning significantly alters both toxicity profiles and the underlying mechanisms of action, particularly in oxidative stress responses, membrane disruption markers, and energy metabolism (Kakavas et al., 2025). These works suggest that even common variations in the biological status of test systems—such as age, density, or prior environmental exposure—may yield coronas with a range of functional consequences (Ellis and Lynch, 2020, Briffa et al., 2018). This variability poses a critical challenge to toxicity guideline standardization efforts and highlights the need for context-sensitive nanotoxicity frameworks, especially given the possibility of these functionalisation would lead to enhanced adverse effects (Wang et al., 2011, Trevisan et al., 2020). An additional layer of complexity arises from the experimental setup itself, as the physical parameters, while often overlooked, significantly modulate the likelihood of NP uptake and biological interaction (Kakavas et al., 2024, Grintzalis et al., 2017, Kakavas et al., 2023). This complexity further highlights that nanotoxicity is not solely a property of the material but of the material-in-context. Taken together, these findings point toward a mechanistic reconceptualization of nanotoxicity assessment—one that integrates biological context, physicochemical transformation, and functional biomarker response. Enzymatic biomarkers, in particular, have emerged as practical and mechanistically rich indicators of NP induced stress, capturing accurately molecular perturbations in sub-lethal concentrations, serving as a useful intermedia between simple toxicity exposures and more focused omic approaches (Li et al., 2024). Their integration into standardized assays, particularly when paired with omics-based readouts, offers a pathway for constructing exposure models across

specific environmental scenarios (Brockmeier et al., 2017). In conclusion, environmental nanoexposure cannot be adequately understood or regulated through simplified, chemically defined systems. The role of nanocoronas as modifiers of nanotoxicity necessitates that NM risk assessments account for the complexity of the biological environment in which they may be found. This includes not only the chemical makeup of coronas but also their ecological effect and dynamic bio-nano interactions. Moving forward, mechanistic insight rather than mere compliance with conventional protocols must guide the development of robust, adaptive, accurate and environmentally relevant nanotoxicity testing strategies.

Another important parameter for research of nanotoxicity mechanisms is bridging the findings to human-health related consequences. These shortcomings can be overcome with the use of co-culture models that might be able to mimic more accurately the microenvironment of lung tissue. The monoculture nature of the cell model, where the multicellular interactions in the human alveola are not observed or considered does have a number of benefits over more complex 3D models but it might fail to account for some factors which could modulate effects of exposure. Alveolar epithelial cells interact with macrophages, endothelial cells and other parts of the immune system like dendritic cells (Fytianos et al., 2017) and neutrophils (von Schéele et al., 2014). These interactions could prove critical in transforming NPs that have infiltrated the human body, affecting their toxicity or bioavailability. Certain factors such as nanocorona formation might become more complicated, therefore careful consideration of the type of NM, the focus of the research question and different culture models is necessary to accurately capture adverse effects that result from nanotoxicity. Additionally, certain assays that might be otherwise optimal to investigate mechanisms of nanotoxicity, such as enzyme activity assays might require further optimisation or altogether replacement in *in vitro* studies. The findings of this work further reinforce that while A549 cells offer a practical and mechanistically relevant model for NP exposure studies, their response to silver nanoink under acute exposure conditions was variable, while according to available literature, certain widely adopted assays have sometimes shown induced variability due to interaction with NMs (Kroll et al., 2012). This observed variation of results may reflect the complexity of NP behaviour in biological media and the inability of monoculture systems to accurately replicate the full spectrum of *in vivo* interactions. Despite these constraints, observed changes in key enzyme activities point to early metabolic and structural adjustments by A549 cells, suggesting that enzyme-based assays, if properly optimised, hold promise for toxicity screening and early stress responses assessments. Future work should aim to build on these early findings by integrating systems that include immune cells, extracellular matrices, and air-exposure interfaces for evaluations

that better reflect real-world exposure scenarios. These advancements would facilitate a more comprehensive understanding of the mechanisms underlying NP-cell interactions and contribute to the development of refined risk assessment strategies for engineered NMs as well as nanopollution.

6.4 Future directions

Based on the findings described in this thesis, there is new knowledge in several areas of nanotoxicity research that will direct both the mechanistic understanding and practical relevance of nano risk assessment in aquatic environments.

- To complement the physiological and biochemical biomarker framework already established, integrating additional omic approaches would provide a more holistic understanding of NM-induced disruptions at multiple levels of biological organization.
- Gene expression profiling, particularly through RNA-seq methodologies (Deshpande et al., 2023), could provide critical information on activated stress response pathways that may be modulated by specific nanocoronas or NM types.

Changes in the gene expression profile in those pathways associated with mitochondrial health, oxidative stress, and immune defences for example, could be mapped alongside observed changes in enzyme activities and metabolomic analysis. Such integration would allow connecting phenotypic observations to upstream transcriptional changes, uncovering toxicity mechanisms on multiple levels. For example, by pairing metabolomic shifts like altered pyruvate, α-KG and aspartate levels which have shown the potential to reflect the changes induced by nano exposures with corresponding gene expression changes, could validate whether metabolic pathway modulation is transcriptionally driven or post-translationally regulated. Additionally, this integrative approach would support the development of adverse outcome pathways (AOPs) specific to NMs and nanocoronas.

- Another direction that could be added in a holistic approach to nanotoxicology is the expansion of bioindicator species to a multi-species framework with the goal to improve the accuracy of nanopollution assessment.

While *D. magna* remains a gold-standard freshwater model due to its sensitivity, widespread use and ecological relevance, other species could be better suited to specific environmental scenarios. For example, addition of filter feeding organisms such as *Mytilus galloprovincialis* could offer critical information on NM accumulation in tissues, especially

for organisms without the ability to evade nanopollution (Curpan et al., 2022). However, the importance of aquatic plants shouldn't be overlooked in this context, as there are plant species such as *Lemna minor* that are utilized as a tool to capture phytotoxic effects as well as bioavailability (Glavaš Ljubimir et al., 2023).

- In a different perspective on nanotoxicity research, future studies could focus on constructing nanocoronas consisting of defined molecular components or synthetic analogs to environmentally derived biomolecules.

These engineered nanocoronas could be the key to allow for systemic assessment of NMs and classification of interactions between NPs and molecules in their environment (Pinals et al., 2020). An interesting direction could also be the comparison of coronas derived from *D. magna* under different physiological states like healthy or stressed rather than different life stages, as the role of interspecies interactions is critical in the context of nanocorona formation since it modulates excreted molecules (Viaene et al., 2015). Additionally, such work could determine whether the stress-induced metabolic fingerprint alters corona composition in ways that modulate toxicity, amplifying or hiding adverse effects in a manner not captured by simplified models.

- Experimental systems could incorporate isotopically labelled or fluorescently modified metabolites to track the dynamic formation, exchange, and biological association of the nanocorona as well as its accumulation sites within tissues once nano pollutants infiltrate the body.

These tools would enhance mechanistic insights into corona–NP interactions and how they translate into cellular or organism-level responses. In turn, this approach could improve design principles in NM engineering, where coatings are intentionally selected or avoided based on their interaction potential with environmentally relevant metabolites or biological interfaces. Such research would advance the predictive capacity of nanotoxicological assessments by linking corona chemistry to functional outcomes through AOPs. Finally, while biologically enriched media represent a step toward environmental realism, current laboratory studies still fall short of capturing the full complexity of natural ecosystems where nanopollution exposures occur.

- Another important direction is the simulation of real-world exposure scenarios, which would require chronic, low-dose exposure regimens that more accurately reflect ambient NMs concentrations typically found in freshwater systems.

These conditions would permit the investigation of accumulation, bioavailability and chronic physiological effects that acute nanotoxicity testing will overlook. Environmental factors are critical in the NP fate and biological uptake. Thus, future testing environments should incorporate ecologically relevant variables through the use of mesocosms to better approximate environmental dynamics under controlled conditions (Nassar et al., 2021). Moreover, complex mixtures of pollutants, including NPs co-existing with pharmaceuticals, pesticides, and/or microplastics should be assessed to evaluate synergistic and/or antagonistic interactions that could distort toxicity responses in bioindicator species (Wang et al., 2011, Martin, 2023). By embedding nanocorona formation within these multifactorial exposure systems, biological and environmental complexity drivers of nanotoxicity can be further explored, providing key information on NM transformation and other influences of downstream toxicity (Thu et al., 2023). These efforts would substantially improve the ecological validity of current testing frameworks and could improve regulatory strategies by accounting for context-specific factors in NM behavior and risk. Ultimately, the incorporation of environmental complexity into nanotoxicology will be essential for producing mechanistically grounded and ecologically relevant assessments of nanopollution.

This thesis included an additional chapter in the pre-examination version that was submitted in which some preliminary work into A549 cells was included. The aim of including A549 cells in this thesis was for them to serve as a gateway for exploring adverse effects of NP exposure *in vitro*. Therefore, some future work could using *in vitro* models could be employed to investigate nanotoxicity.

- A549 cells could be employed to investigate nanocorona formation with specific metabolites. Some advantages that this would provide is improved focus on tissue level adverse effects as well as potentially unveiling NP fate on a cellular level in the context of accumulation.

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Appendices

Appendix A

A.1 *Daphnia magna* culturing protocols

The culturing scheme on the table below is used to produce clonal populations of mothers who will breed animals for experiments. Set new breeder cultures every Wednesday, Thursday and Friday. Every Friday double-feed the cultures that are younger than 7 days old and the cultures that have new OECD media.

<table>
<tr><th>Daphnid age (days)</th><th>Procedure</th><th>OECD</th><th>Yeast</th><th>Fresh Algae</th><th>Seaweed Extract</th></tr>
<tr><td>1</td><td>Setup new culture</td><td rowspan="21">4 L</td><td rowspan="21">2 ml</td><td rowspan="3">4 ml</td><td rowspan="12">12 ml</td></tr>
<tr><td>2</td><td rowspan="4"></td></tr>
<tr><td>3</td></tr>
<tr><td>4</td><td rowspan="4">6 ml</td></tr>
<tr><td>5</td></tr>
<tr><td>6</td><td>Change media</td></tr>
<tr><td>7</td><td rowspan="6">Remove neonates daily</td></tr>
<tr><td>8</td><td rowspan="13">8 ml</td></tr>
<tr><td>9</td></tr>
<tr><td>10</td></tr>
<tr><td>11</td></tr>
<tr><td>12</td></tr>
<tr><td>13</td><td>Change media</td><td rowspan="9">16ml</td></tr>
<tr><td>14</td><td rowspan="8">Remove neonates daily</td></tr>
<tr><td>15</td></tr>
<tr><td>16</td></tr>
<tr><td>17</td></tr>
<tr><td>18</td></tr>
<tr><td>19</td></tr>
<tr><td>20</td></tr>
<tr><td>21</td></tr>
</table>

A.1.1 OECD standard media

OECD media stocks solutions were prepared fresh and added, according to the table, to a tank filled with 50 L ddH_2O. The media were covered with cellophane membrane to prevent contamination and were aerated for at least 48 hours prior to use. The pH of the media was adjusted after the aeration to 7.77.

Chemicals	Quantity
Calcium Chloride	15.29 g $CaCl_2.2H_2O$ in 0.5 l ddH_2O
Magnesium sulphate	6.41 g $MgSO_4.7H_2O$ in 0.5 l ddH_2O
Sodium bicarbonate	3.37 g $NaHCO_3$ in 0.5 l ddH_2O
Potassium chloride	0.3 g KCl in 0.5 l ddH_2O
40 µg/mL Sodium selenite	2.6 ml
1 M HCl	23 ml

A.1.2 Seaweed extract supplement

As supplement the seaweed extract Ascophylum nodosum was added to the 4l of culture OECD media only during the media change (days 6 and 13). The extract (500 g/l) was diluted in ddH_2O to a final absorbance of 8A at 400 nm.

A. 2 Chlamydomonas reinhardtii culturing

Algae (*Chlamydomonas reinhardtii*) are the primary food for daphnids. They were cultured in *C. reinhardtii* growth medium (CGM), collected by centrifugation at 3,000 rpm for 10 min at RT and re-suspended in ddH_2O to a 7A dilution at 440 nm (equals to 6.1M cells/ml).

A.2.1 Chlamydomonas reinhardtii growth medium (CGM)

Using a volumetric cylinder, add to a Duran bottle 5.25 l ddH_2O and a magnet. Under continuous stirring add the following volumes.

Stock	Concentration g/l	ml to add
NH_4Cl	20	140
$MgSO_4.7H_2O$	8	70
$CaCl_2.2H2O$	4	70
K_2HPO_4	8.64	70
KH_2PO_4	8.4	62
EDTA/KOH	50/31	2.85
Acidified iron	4.98	5.7
Boric acid	11.42	5.7
$ZnSO_4.7H_2O$	14.12	3.56
$MnCl_2.4H_2O$	2.33	3.56
$CuSO_4.5H_2O$	2.54	3.56
$Co(NO_3)_2.6H_2O$	0.82	3.56
$Na2MoO_4.4H_2O$	1.92	3.56
Acetic acid	100%	5.7

Appendix B

B.1 First authorship publications

Kakavas, D., Panagiotidis, K., Rochfort, K. D., & Grintzalis, K. (2024). Miniaturizing Nanotoxicity Assays in Daphnids. *Animals, 14*(14). doi: Artn 204610.3390/Ani14142046

Kakavas, D., Panagiotidis, K., Rochfort, K. D., & Grintzalis, K. (2023). Surface-to-Volume Ratio Affects the Toxicity of Nanoinks in Daphnids. *Stresses, 3*(2), 488-499. doi:10.3390/stresses3020035

Kakavas, D., Engelmann, B., Krauss, M., Rolle-Kampczyk, U. E., Rochfort, K. D., & Grintzalis, K. (2025). Excreted metabolite nanocoronas impact metabolic and toxicity responses of daphnids to silver nanoink. *Environmental Research, 283*, 122131. doi: https://doi.org/10.1016/j.envres.2025.122131

B.2 Conferences

2024	‘British Toxicology Society Annual Congress, Liverpool, UK’’
2023	‘’Revisiting Nanotoxicology Tests—Miniaturized Approaches of Nanotoxicity Tests in Daphnids’’ Proceedings 92, no. 1: 52. Leung,

	Anne, Emma Rowan, Dimitrios Kakavas, Konstantinos Panagiotidis, Keith D. Rochfort, and Konstantinos Grintzalis. 2023.
2022	‘‘Miniaturizing nanotoxicity in daphniids: The impact of animal crowding and exposure vessels’’ Dimitrios Kakavas, Konstantinos Panagiotidis, Konstantinos Grintzalis Federation of European Biochemical Societies (FEBS), Lisbon 2022.
2022	‘‘Miniaturisation of nanotoxicity in daphniids’’ Dimitrios Kakavas, Konstantinos Panagiotidis, Konstantinos Grintzalis Annual Congress of British Toxicology Society, Newcastle 2022.
2021	‘‘Daphnia magna acute toxicity test for nanoparticles of Ag, ZnO, ZnO-Ag mixture’’ Dimitrios Kakavas, Konstantinos Grintzalis, Stefanos Dailianis, (online workshop) “Non-animal Approaches in Science: The Three R...evolution, JRC summer school workshop”.

Appendix C

Declarations of authorship.

Declaration of Authorship for PhD by Publication

Section 1: Candidate's Details	
Candidate's Name	Dimitrios Kakavas
DCU Student Number	A21269594
School	Biotechnology
Principal Supervisor	Konstantinos Gkrintzalis
Title of PhD by Publication Thesis	Mechanistic insight on the impact of nanomaterials and nanocoronas on freshwater and in vitro system
Section 2: Paper Details	
Title of co-authored paper included in the thesis under examination	Miniaturizing Nanotoxicity Assays in Daphnids
List of authors as in order given on accepted/published version	Dimitrios Kakavas, Konstantinos Panagiotidis, Keith D Rochfort, Konstantinos Grintzalis
Publication Details (e.g. Year of Publication, Journal Name, Issue Number, Page Numbers, Place of Publication, DOI, ISSN, URL etc.)	Animals (Basel). 2024 Jul 12;14(14):2046. doi: 10.3390/ani14142046
Publication Status (e.g. Published, Accepted)	Published

Provide a brief statement here on the disciplinary ranking of this publication and the peer-review process involved (e.g. how many reviewers, single blinded or other, initial decision, revision timeline etc and any other details that speak to the rigour of the peer review process)	The manuscript was first submitted on the 8th of June 2024. The journal has a single blinded peer review process and this involved two reviewers. The first comments were received on the 20th of June and following responses (on the first of July) that were satisfactory, the manuscript was accepted on the 8th of July and published on the 12th of July.

This paper (Chapter 2) is one of 4 co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination.

Section 3: Candidate's Contribution to the Paper

Briefly outline your contribution to the **conception** of the work described in this paper:

This paper was conceptualised in the first year of my PhD as a general idea from my primary supervisor based on previous research he had done on similar context. I discussed with my primary supervisor the main idea and I proposed bold experiments to achieve this as target worthy of further investigation based on preliminary findings I performed (80%).

Briefly outline your contribution to the **literature review** in this paper:

As the primary author, I carried out all literature review to collect the relevant literature. Although references were scarce, hence the originality of this work, with the guidance of my supervisor, I collected the relevant sources (95%).

Briefly outline your contribution to the **methodological design**:

The methodological design of this work was in line with the assays developed by our group. Following discussions with my supervisor who a better context of the feasibility and available budget, I proposed very bold experiments which required high number of animals, which were met to implement this paper (85%).

Briefly outline your contribution to **data collection**:

I contributed to the collection of data from daphnids (90%). The main challenge of this paper was to generate and perform the initial toxicity curves which required a huge number

of animals. The required numbers were met with help from my supervisor and postdoc, and this stage was completed. Following, I performed all assays and generated all samples for the study for the toxicity of silver nano ink (80%).

Briefly outline your contribution to **data analysis**:

I am the sole contributor to the analysis of data and the statistical analysis following discussion with my supervisor (95%).

Briefly outline your contribution to the **synthesis and summary of findings**:

As the main author, I wrote the first draft and summarized the findings in this work. The postdoc and my supervisors assisted in the writing and editing with improvements in the clarity of findings (95%).

Finally, outline your contribution to the **writing** and **revision** of the paper:

As the main author, I wrote the first draft of the manuscript in its entirety, and my supervisors assisted until the submission. Following, I did the revisions according to the comments of the reviewers and my supervisors assisted in the revision by providing guidance for the accuracy of my responses (95%).

Section 4: Signature and Validation

I confirm that the following statements are true:

(a) the information I have provided in this form is correct
(b) this paper is based on research undertaken during my candidature at DCU

Signature of PhD Candidate: Dimitrios Kakavas **Date:** 18/6/2025

I confirm that the information provided by the candidate is correct:

Signature of Principal Supervisor: Konstantinos Gkrintzalis **Date:** 18/6/2025

Section 1: Candidate's Details

Candidate's Name	Dimitrios Kakavas
DCU Student Number	A21269594
School	Biotechnology
Principal Supervisor	Konstantinos Gkrintzalis
Title of PhD by Publication Thesis	Mechanistic insight on the impact of nanomaterials and nanocoronas on freshwater and in vitro system

Section 2: Paper Details

Title of co-authored paper included in the thesis under examination	Surface-to-Volume Ratio Affects the Toxicity of Nanoinks in Daphnids
List of authors as in order given on accepted/published version	Dimitrios Kakavas, Konstantinos Panagiotidis, Keith D Rochfort, Konstantinos Grintzalis
Publication Details (e.g. Year of Publication, Journal Name, Issue Number, Page Numbers, Place of Publication, DOI, ISSN, URL etc.)	Stresses 2023, 3(2), 488-499; https://doi.org/10.3390/stresses3020035
Publication Status (e.g. Published, Accepted)	Published
Provide a brief statement here on the disciplinary ranking of this publication and the peer-review process involved (e.g. how many reviewers, single blinded or other, initial decision, revision timeline etc and any other details that speak	The manuscript was first submitted on the 17th of April 2024. The journal has a single blinded peer review process and this involved two reviewers. The first comments were received on the 17th of May and following extensive revisions on statistical analysis, (29th of May) the responses provided were found satisfactory, and the manuscript was accepted on the first of June and published on the 7th of June (2024).

to the rigour of the peer review process)	

This paper (Chapter 3) is one of 4 co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination.

Section 3: Candidate's Contribution to the Paper

Briefly outline your contribution to the **conception** of the work described in this paper: This paper was conceptualised based on the results from another work package that I was involved during my first year of PhD. Following a conversation with my primary supervisor we agreed to investigate this idea based on initial findings which looked promising to follow up and evidently resulted in the published manuscript (85%).

Briefly outline your contribution to the **literature review** in this paper:

I am the main author in this work and all the literature review was done mostly by me, with my supervisors providing a few suggestions for alternate references in certain points (95%).

Briefly outline your contribution to the **methodological design**:

The methodological design for the implementation of this work (i.e. the choice of physiology markers) was done together with my primary supervisor who has a better understanding of the capabilities and available budget (90%).

Briefly outline your contribution to **data collection**:

I am the main contributor to data collection for this study. The main challenge for this paper was to generate and perform the toxicity curves which required a huge number of animals. The required numbers were met with help from my supervisor and postdoc, and after this stage was completed, I performed all assays and generated all samples myself (80%).

Briefly outline your contribution to **data analysis**:

I am the sole contributor to data analysis as I was the person performing the statistical analysis for the data (95%) with advice from my supervisor.

Briefly outline your contribution to the **synthesis and summary of findings**:

As the main author, I wrote the first draft and summarized the findings in this work. The postdoc and my supervisors assisted in the writing and editing with improvements in the clarity of findings (95%).

Finally, outline your contribution to the **writing** and **revision** of the paper:

As the main author, I wrote the initial draft, and with the guidance of my supervisors we submitted this. Following the receipt of comments from reviewers, I did the revisions and my supervisors assisted by providing guidance and feedback before sending the manuscript to the journal in order to improve the quality of the work (95%).

Section 4: Signature and Validation

I confirm that the following statements are true:

(c) the information I have provided in this form is correct
(d) this paper is based on research undertaken during my candidature at DCU

Signature of PhD Candidate: Dimitrios Kakavas **Date:** 18/6/2025

I confirm that the information provided by the candidate is correct:

Signature of Principal Supervisor: Konstantinos Gkrintzalis **Date:** 18/6/2025

Section 1: Candidate's Details

Candidate's Name	Dimitrios Kakavas
DCU Student Number	A21269594
School	Biotechnology

Principal Supervisor	Konstantinos Gkrintzalis
Title of PhD by Publication Thesis	Mechanistic insight on the impact of nanomaterials and nanocoronas on freshwater and in vitro system

Section 2: Paper Details

Title of co-authored paper included in the thesis under examination	Animal crowding affects the molecular responses of daphnids following exposure to silver nanoink
List of authors as in order given on accepted/published version	Dimitrios Kakavas, Beatrice Engelmann, Martin Krauss, Ulrike E. Rolle-Kampczyk, Keith D. Rochfort, Konstantinos Grintzalis
Publication Details (e.g. Year of Publication, Journal Name, Issue Number, Page Numbers, Place of Publication, DOI, ISSN, URL etc.)	
Publication Status (e.g. Published, Accepted)	Submitted and under review
Provide a brief statement here on the disciplinary ranking of this publication and the peer-review process involved (e.g. how many reviewers, single blinded or other, initial decision, revision timeline etc and any other details that speak to the rigour of the peer review process)	The manuscript was submitted on the 5th of June to the journal Aquatic Toxicology. This journal has a single blinded peer review, and we are waiting their comments.

This paper (Chapter 4) is one of 4 co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination.

Section 3: Candidate's Contribution to the Paper

Briefly outline your contribution to the **conception** of the work described in this paper:

This paper was conceptualised based on the initial results from the first work package during the first year of my PhD. Following a conversation with my primary supervisor, I proposed this idea of crowding for further investigation based on previous findings. My contribution (80%) to the context evidently lead to an independent manuscript.

Briefly outline your contribution to the **literature review** in this paper:

As the primary author, I performed all literature review working with advice from my supervisors who provided suggestions and guidance for alternate references in certain points (95%).

Briefly outline your contribution to the **methodological design**:

The methodological design of this work was discussed with my primary supervisor who is the expert in daphnids. Following my suggestions for inclusion of experiments and taking into account the capabilities and budget available the design was similar to other studies (80%).

Briefly outline your contribution to **data collection**:

I contributed to the collection of data from daphnids (90%). I performed all toxicity curves, biochemical assays, and I generated and extracted the samples for the metabolomic analysis which was done by our collaborators in UFZ.

Briefly outline your contribution to **data analysis**:

I am the sole contributor to the analysis of data and the statistical analysis following discussion with my supervisor especially for data provided by the collaborators for this manuscript (95%).

Briefly outline your contribution to the **synthesis and summary of findings**:

I am the main contributor to the synthesis and summary of findings as I wrote the first draft which was the skeleton for this article (95%).

Finally, outline your contribution to the **writing** and **revision** of the paper:

I am the main contributor to the writing and revision of the paper (95%). I wrote the original draft and following the comments of my supervisors and collaborators I provided

the final version for submission. When we get the comments from the reviewer, I will aim to reply similar to other papers I published before to deliver the published.

Section 4: Signature and Validation

I confirm that the following statements are true:

(e) the information I have provided in this form is correct
(f) this paper is based on research undertaken during my candidature at DCU

Signature of PhD Candidate: Dimitrios Kakavas **Date:** 18/6/2025

I confirm that the information provided by the candidate is correct:

Signature of Principal Supervisor: Konstantinos Gkrintzalis **Date:** 18/6/2025

Section 1: Candidate's Details

Candidate's Name	Dimitrios Kakavas
DCU Student Number	A21269594
School	Biotechnology
Principal Supervisor	Konstantinos Gkrintzalis
Title of PhD by Publication Thesis	Mechanistic insight on the impact of nanomaterials and nanocoronas on freshwater and in vitro system

Section 2: Paper Details

Title of co-authored paper included in the thesis under examination	Excreted metabolite nanocoronas impact metabolic and toxicity responses of daphnids to silver nanoink
List of authors as in order given on accepted/published version	Dimitrios Kakavas, Beatrice Engelmann, Martin Krauss, Ulrike E. Rolle-Kampczyk, Keith D. Rochfort, Konstantinos Grintzalis
Publication Details (e.g. Year of Publication, Journal Name, Issue Number, Page Numbers, Place of Publication, DOI, ISSN, URL etc.)	2025, Environmental Research, https://doi.org/10.1016/j.envres.2025.122131
Publication Status (e.g. Published, Accepted)	Available online (Accepted, in press)
Provide a brief statement here on the disciplinary ranking of this publication and the peer-review process involved (e.g. how many reviewers, single blinded or other, initial decision, revision timeline etc and any other details that speak to the rigour of the peer review process)	The manuscript was first submitted to the journal Environmental Research (Elsevier) on the 18th of April. The peer review process was single-blinded, and we had two reviewers who recommended a minor revision without any additional experiments on the 22nd of May. After addressing the comments, on the 29th of May, our responses were satisfactory, and the manuscript was accepted for publication on 10th of June.

This paper (Chapter 5) is one of 4 co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination.

Section 3: Candidate's Contribution to the Paper

Briefly outline your contribution to the **conception** of the work described in this paper:

This paper was conceptualised together with my primary supervisor based on previous research he had done on similar context. I had also contribution in the idea and its implementation (80%).

Briefly outline your contribution to the **literature review** in this paper:

As the primary author, I carried out all literature review to collect references in the context of this work. My primary supervisor as well as the reviewers provided additional references in certain points (95%).

Briefly outline your contribution to the **methodological design**:

The methodological design of this work (i.e. the choice of physiology markers selected) was done by myself and my primary supervisor who has a better context of the capabilities and the budget available to my PhD. I made suggestions for experiments, and I had bold ideas to implement this paper (85%).

Briefly outline your contribution to **data collection**:

contributed to the collection of data from daphnids (90%). I performed all toxicity curves, biochemical assays, and I generated and extracted the samples for the metabolomic analysis which was done by our collaborators in UFZ.

Briefly outline your contribution to **data analysis**:

I am the sole contributor to the analysis of data and the statistical analysis following discussion with my supervisor especially for data provided by the collaborators for this manuscript (95%).

Briefly outline your contribution to the **synthesis and summary of findings**:

I am the main contributor to the synthesis and summary of findings as I wrote the first draft which directed the course of this manuscript (95%).

Finally, outline your contribution to the **writing** and **revision** of the paper:

I am the main contributor to the writing and revision of the paper (95%). I wrote the original draft and following the comments of my supervisors and collaborators I provided the final version for submission. Additionally, I replied to the comments of the reviewers.

Section 4: Signature and Validation

I confirm that the following statements are true:

(g) the information I have provided in this form is correct
(h) this paper is based on research undertaken during my candidature at DCU

Signature of PhD Candidate: Dimitrios Kakavas **Date:** 18/6/2025

I confirm that the information provided by the candidate is correct:

Signature of Principal Supervisor: Konstantinos Gkrintzalis **Date:** 18/6/2025