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MIXTURE EFFECTS OF METALLIC NANOPARTICLES, AND ORGANIC CONTAMINANT ON *DAPHNIA MAGNA* IN AQUEOUS MEDIA

By

Mpho Rosemary Makofane

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Prof. Ndeke Musee

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Declaration

I, **Mpho Rosemary Makofane**, hereby declare that this dissertation is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature of candidate

Date

Abstract

Engineered nanoparticles (ENPs) including silver (n-Ag) and zinc oxide (n-ZnO) are among rapidly increasing contaminants of emerging concern (CECs) globally. Moreover, pesticides (e.g. atrazine (ATZ)) owing to their extensive application are present in variant ecological systems like ground and surface waters, with unintended threats to aquatic life forms. Further, increasing presence of different classes of CECs and persistent organic pollutants (POPs) has led to their co-existence as mixtures in the environmental systems; but with unknown toxicological outcomes to organisms at different biological levels of organization. Herein, freshwater crustacean *Daphnia magna* was used as a model organism to assess the biological effects of individual ENPs, their binary mixtures with or without ATZ, and ternary mixtures with ATZ. The organisms were exposed to variant concentrations of ENPs and ATZ in ISO medium (standard freshwater), and immobilization was investigated as the endpoint.

Evaluation of the physicochemical characterization of ENPs was carried out to establish plausible link between their properties, and the observed biological outcomes. Aggregation of n-Ag increased with exposure time; whereas that of n-ZnO significantly decreased over time. For the binary mixtures, results revealed irrespective of ENPs type, aggregation size increased in the presence of ATZ due to hetero-aggregation between the compounds. Results of the ternary mixtures indicated aggregation size was four-fold relative to those of ENPs binary mixtures. Dissolution of individual n-Ag and n-ZnO increased and decreased over exposure period of 48 h, respectively. The presence of ATZ enhanced the dissolution of ENPs in the binary mixtures. However, in the ternary system a reduction of the dissolution of both ENPs in the presence of ATZ was observed.

Studies on the toxicity of individual contaminants based on median effective concentration (EC_{50}) values in decreasing order were n-Ag > n-ZnO > ATZ, largely attributed to high dissolution of n-Ag in the exposure medium. For binary mixtures with n-Ag, higher toxicity was apparent, relative to other mixture systems (binary or ternary). For ternary mixtures, no toxicological outcomes were observed irrespective of the exposure concentrations. These findings demonstrated that the toxic silver ions were the primary driver of toxicity towards *D. magna*. Moreover, co-existence of both nanoscale and macroscale may lead to the chemical transformation of CECs in the exposure medium with marked reduction of individual contaminants toxic effects.

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Dedication

I dedicate this thesis to my son Lerutla. *You are my inspiration*.

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List of abbreviations and acronyms

µg/L	Micrograms per liter
ANOVA	Analysis of variance
ATZ	Atrazine
BBC	British broadcasting corporation
BPEI	Branched polyethyleneimine
C. cornuta	*Ceriodaphnia cornuta*
CA	Concentration addition
CdS	Cadmium Sulphide nanoparticles
CdSe	Cadmium Selenide nanoparticles
CEC	Commission of European Communities
CECs	Contaminants of Emerging Concern
CI	Confidence intervals
cm^3/g	Gram per cubic centimeter
CPI	Consumer products inventory
D. carinata	*Daphnia carinata*
D. galeata	*Daphnia galeata*
D. magna	*Daphnia magna*
D. pulex	*Daphnia pulex*
D. similis	*Daphnia similis*
DEFF	Department of environmental, fisheries and forestry
DIW	Deionised water
DLS	Dynamic light scattering
DLVO	Derjaguin-Landau-Verwey-Overbeek

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DTU	Technical University of Denmark
DWAF	Department of water affairs and forestry
DWS	Department of water and sanitation
EC	Electrical conductivity
EC_{50}	Median effect concentration
ECs	Emerging contaminants
EC_x	Concentration causing x% toxic effect
EDL	Electrical double layer
ENMs	Engineered nanomaterials
ENPs	Engineered nanoparticles
g/cm^3	Gram per cubic centimeter
g/mol	Grams per mole
GSH	Glutathione
HA	Humic acid
HA	Hydroxyatrazine
HDD	Hydrodynamic diameter
HM	Hoaglands media
HNO_3	Nitric acid
IA	Independent action
IACR	International Agency for Research on Cancer

ICP-MS	Inductively Coupled Plasma Mass spectrometry
IS	Ionic strength
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
K_2CrO_7	Potassium dichromate
KDa	Kilo daltons
K_{oc}	Soil adsorption coefficient
Log K_{ow}	Octanol/water partition coefficient
MDA	Malondialdehyde
mg/L	Milligrams per litre
MHRW	Moderately hard reconstituted water
MHW	Moderately hard water
mM	Millimolar
mmol/l	Millimoles per litre
mPa	Megapascal
mV	Millivolts
MW	Molecular weight
MWCO	Molecular weight cut-off
$NaCO_3$	Sodium carbonate
n-Ag	Silver nanoparticles
NaOH	Sodium hydroxide
n-Au	Gold nanoparticles
n-CeO_2	Cerium Oxide nanoparticles
NEPs	Nano-enabled products

n-Fe	Iron nanoparticles
n-Fe_2O_3	Iron oxide nanoparticles
ng/L	Nanograms per liter
n-GO	Graphene oxide
nm	Nanometre
NOM	Natural organic matter
NPs	Nanoparticles
n-SiO_2	Silica nanoparticles
n-TiO_2	Titanium Dioxide nanoparticles
n-ZnO	Zinc oxide nanoparticles
OECD	Organization for Economic Co-operation and Development
PCPs	Personal care products
PECs	Predicted environmental concentrations
PEN	Project of emerging nanotechnology
pH	Potential of hydrogen
Pi	Fractions of components
PLFA	Pony Lake fulvic acids
PPCPs	Pharmaceuticals and personal care products
PVP	Polyvinylpyrrolidone
PZC	point of zero charge
ROS	Reactive oxygen species
RPM	Revolutions per minute
SA	South Africa

SD	Standard deviation
TEM	Transmission electron microscope
Tons/yr	Tons per year
TU	Toxicity unit
TU	Toxic unit
TWQG	Target water quality guidelines
UNEP	United nations environment programme
USA	United States of America
USEPA	United states environmental protection agency
UV	Ultra violet
WHO	World health organization
WRC	Water research commission
ZP (ζ)	Zeta potential

Chapter 1: Introduction

1.1 Background

Over the past few decades, advances in technology have resulted to high volume production of chemicals to improve the quality of life, and meet needs (e.g. food, water, transport, hygiene, health, etc.) of exponentially growing global population. As a result, this has resulted to dramatic increase on the release of large chemical quantities into the environment of known and unknown deleterious effects to humans and the environment (Nel et al., 2006; Klaine et al., 2008; Baalousha et al., 2016; Dutta et al., 2016; Hartmann et al., 2018; Ouyang et al., 2018). This in turn, led to deleterious implications including dramatic reduction in biodiversity (Kumar, 2013; Sharma et al., 2015; Wilke et al., 2016; Singh et al., 2018), and human diseases (Oberdörster et al., 2005; Marambio-Jones and Hoek 2010; Bakshi et al., 2015; Dutta et al., 2016). Further, the increasing presence of different classes of contaminants of emerging concern (CECs) has led to their co-existence as mixtures in the environmental systems; but with unknown toxicological outcomes to organisms at different biological levels of organization.

The CECs are synthetic or naturally occurring contaminants, and broadly are not regulated, mostly recently detected, not routinely monitored in environmental systems, and defined by limited knowledge about their potential deleterious effects to human health and the environment (USEPA, 2008b; Noguera-Oviedo and Aga, 2016; Rimayi et al., 2018a). The CECs encompasses a wide range of chemical compounds including engineered particles (ENPs), pharmaceuticals and personal care products (PPCPs), pesticides, illicit drugs, and antibiotic resistance genes (Gavrilescu et al., 2015; Naidu et al., 2016; Salimi et al., 2017; da Silva and Abessa, 2019; Galindo-Miranda et al., 2019). At present, it is challenging to address the impacts of individual chemicals in the selected classes particularly in the environment. This challenge is further exacerbated by their co-existence as mixtures, and concomitant interactions with each other besides the influence of abiotic factors. As a result, this renders evaluation of their fate and toxicity highly complex, and multifactorial in nature. Yet, in order to effectively manage chemicals in the environment, raises the urgent necessity for systematic evaluation of their exposure and hazard as mixtures in order too ascertain actual likely implications into the aquatic organisms.

Over the past two decades, the nanotechnology industry has experienced rapid growth globally due to increasing production and incorporation of different ENPs into variant consumer products and industrial applications. Metal-based ENPs including silver (n-Ag) and zinc oxide (n-ZnO) find widespread applications in consumer products (Piccinno et al., 2012; Vance et al., 2015) e.g., personal care products (PCPs) (Maynard, 2006; Serpone et al., 2007; Becheri et al., 2008; Wiench et al., 2009; Shi et al., 2012) as well as biomedical and industrial applications (Nowack and Bucheli, 2007; Luoma, 2008; Nowack et al., 2012; Hansen et al., 2016), just to mention a few. The unique physicochemical properties of ENPs render them highly distinctive from their bulk counterparts (Oberdörster et al., 2005; Handy and Shaw, 2007; Handy et al., 2008). Consequently, for over a decade, the toxic potential of ENPs has raised concerns they may pose to human and environmental health (Nel et al., 2006; Marambio-Jones and Hoek, 2010; Garner and Keller, 2014; Bundschuh et al., 2018; Zhao et al., 2020). Therefore, to date, to close these gaps numerous studies have been carried out to support systematic risk assessment of ENPs using modelling (Mueller and Nowack, 2008; Gottschalk et al., 2009; Musee, 2017), and experimental (Oberdörster, 2004; Guzmán et al., 2006; Thwala et al., 2013) approaches.

Sources and pathways of ENPs into the aquatic environment includes accidental spills, manufacturing plants, discharges from sewage water treatment plants, run-off from land surfaces, landfill leachate, land application of biosolids, and release from consumer products (Gottschalk and Nowack, 2011; Keller and Lazareva, 2013; Garner and Keller, 2014; Musee, 2017). As a result, ENPs have been detected and/or predicted in different environmental compartments in previous studies (Gottschalk et al., 2013; Sun et al., 2016; Giese et al., 2018). To date, numerous studies, for example, have reported environmental deleterious effects of ENPs e.g., n-Ag and n-ZnO to aquatic such as daphnids (Fabrega et al., 2011; Ma et al., 2013; Cupi et al., 2015; Azevedo et al., 2017; Gonçalves et al., 2018; Khoshnamvand et al., 2020). Further, both ENPs have been ranked in the top ten for immediate toxicity testing (OECD, 2010), and also the top five most studied for their ecotoxicological properties (Kahru and Ivask, 2013) linked to their high production and toxicity.Once in the environmental systems, ENPs are transformed from their pristine state, but can also interact with other pollutants (both macro- and nano-pollutants). This is occasioned by processes such as hetero-aggregation and adsorption of other chemicals onto the ENPs surfaces. The fate and behaviour of ENPs is the primary driving force of their toxicity

(Lowry et al., 2012a, b) as regulated by inherent physicochemical properties (e.g., size, surface charge, morphology, surface coating, etc.) (Bhatt and Tripathi, 2011; Lynch et al., 2014), and environmental factors (e.g., light, temperature, natural organic matter, pH, etc.,) (Barreto et al., 2015; Ren et al., 2016; Odzak et al., 2017).

In addition to nanopollutants, micropollutants e.g., pesticides are major sources of pollution to drinking, and surface wasters (Rani et al., 2017; Hartmann et al., 2018). Pesticides are known to pose adverse effects to aquatic organisms, and consequently, harm the biodiversity (Grützmacher et al., 2008; Aguilar-Alberola and Mesquita-Joanes, 2012; Singh et al., 2018). Pesticides such as herbicides are used extensively in agriculture to boost food productivity in pursuit to feed dramatically growing world population (Miller and Spoolman, 2009; Snyder et al., 2015; Lushchak et al., 2018). Atrazine (ATZ), for example, is highly effective in weeds control in agricultural fields (Graymore et al., 2001; Religia et al., 2019); hence, it is the second most used herbicide worldwide (Kumar et al., 2013; Cheng et al., 2016). However, the extensive application of ATZ has led to large releases into ground and surface waters (Graymore et al., 2001; Baker et al., 2016; Zheng et al., 2017) with unintended concomitant threat to aquatic life.

Hence, with increasing use of n-Ag, n-ZnO, and ATZ raises the possibility for the exposure of aquatic organisms to their resultant binary and/or ternary mixtures. This is because such mixtures are highly probable for co-existence in the surface waters, for example, based current patterns of application. However, to our knowledge such mixtures are yet to be investigated to establish their likely joint effects or lack thereof. Evaluation of chemicals mixtures is essential since aquatic organisms are more likely to be exposed to a cocktail of chemicals in the environment as opposed to individual chemicals. Therefore, in this thesis, the toxicological outcomes of individual as well as binary and ternary effects of n-Ag, n-ZnO and ATZ are investigated with *Daphnia magna* as the exposure organism.

1.2 Test organism

The aquatic crustacean *D. magna* is a key freshwater organism in ecological food webs as they consume phytoplankton, and serve as food for fish (Persson et al., 2007). Also, they are considered among the most sensitive test organisms in ecotoxicology, and hence are recommended for toxicity testing by regulatory authorities including United States Environmental Protection Agency (USEPA), Organization for Economic Co-operation and Development (OECD), and International Organization for Standardization (ISO) standardized protocols (USEPA, 2002; OECD, 2004; Baun et al., 2008a; ISO, 2012;). Daphnids are highly prone to environment pollutants exposure due to their filter feeding mechanism (Lovern and Klaper, 2006; Baun et al., 2008a; Griffitt et al., 2008). Moreover, their transparent body structure (Chapman et al., 1976) render them easy to study effects on the entire physiology simultaneously (Paul et al., 1997). Further, other factors including low cost and easy to maintain culture, short life cycle (eight weeks), rapid growth, and easy to handle render daphnia an ideal organism commonly used for both nanopollutants and micropollutants ecotoxicity investigations (USEPA, 2002; OECD, 2004; ISO, 2012).

1.3 Study motivation

Modern societies have been associated with anthropogenic activities including the production and wide application of CECs (e.g., ENPs) and organic pollutants (e.g., pesticides) in order to sustain the existence of humanity (Keller et al., 2013; Hansen et al., 2016; Wilkinson et al., 2017; Sharma et al., 2019a). In the aquatic environments, chemical pollutants co-exist as mixtures with high variation in composition(s) both from spatial and temporal viewpoints. To date, the commercialization in the global markets and advances in the production of CECs exceeds the pace of data and information generation required for the purposes of environmental protection, hazards assessment and risk management. Thus, to elucidate the effects, behaviour and environmental fate of ENPs and pesticides, ecotoxicology studies are crucial to generate data necessary to support long-term responsible management.

At present, substantial data on individual toxic effects of ENPs and pesticides to *D. magna* in variant environmental and laboratory media have yielded a wide spectrum of outcome(s) as expressed based on highly endpoints e.g., for n-Ag (Zhao and Wang, 2011; Ribeiro et al., 2014; Hu et al., 2018; Khoshnamvand et al., 2020), n-ZnO (Lopes et al., 2014; Wu et al., 2019; Danabas et al., 2020), and ATZ (Palma et al., 2008; Moreira et al., 2014; Religia et al., 2019). However, data and knowledge on their mixtures key to systematic hazard assessments are lacking. This is reflected by current practices where environmental hazard guidelines on chemical pollutants rely on data from exposure assessments of individual chemicals. Yet, interactions of multiple chemicals as a complex mixture in environmental systems remain largely unaccounted though this truly reflects exposures that occur to the aquatic organisms.

Increasing investigations have reported ENPs are detected at very low concentrations in the environment (ng/L range), however, their co-occurrence and interactions with other pollutants completely modifies their toxicity potential (Hartmann and Baun, 2010; Sanchís et al., 2016; Martín-de-Lucía et al., 2019). ENPs, for example, can be carriers of organic pollutants in environmental systems (Wilke et al., 2016; Abbas et al., 2020b) with concomitant potentiation (Han et al., 2012; Soler de la Vega et al., 2019), or attenuation of toxic potential of organic pollutants (Simon et al., 2015; Molins-Delgado et al., 2016). At present, n-Ag and n-ZnO have been substantially studied in nano-ecotoxicology. However, there is paucity of data on their joint effects to aquatic organisms, with fewer than 10 accessible studies that have evaluated ENPs/ENPs joint toxicity to *D. magna*, and none for n-Ag and n-ZnO system in particular.

For example, there is evidence of growing body of scientific literature database on joint toxicity effects of ENPs, and other contaminants to *D. magna* (Baun et al., 2008b; Han et al., 2012; Kim et al., 2016; Naasz et al., 2018; Ye et al., 2018; Park et al., 2019a, b; Baek et al., 2020; Li et al., 2020). However, paucity of joint effects data in this domain remain key limitation for effective risk assessment of ENPs and other organic pollutants in the aquatic environment. This motivates a need to elucidate the fate and effects of CECs in the aquatic environment hinged on generation of robust information that can support their risk management with specific reference to mixtures. Therefore, this work seeks to make a contribution in addressing these knowledge gaps where n-Ag, n-ZnO, and ATZ are used as case study contaminants of focus.

1.4 Research aims and objectives

The aim of this study was to investigate the fate, and toxicological effects of individual ENPs and organic pollutant as well as their binary and ternary mixtures on *D. magna* in an aqueous medium. And, the specific objectives of the study were to:

- Evaluate the fate of individual ENPs (n-Ag and n-ZnO), and their binary and ternary mixtures with organic pollutant (ATZ)
- Investigate the toxicity effects of n-Ag, n-ZnO, and ATZ on *D. magna*, and
- Assess joint effects of n-Ag, n-ZnO, and ATZ for their binary and ternary mixtures on *D. magna*

1.5 Thesis outline

The thesis comprises of five chapters, and details of each chapter are outlined as follows:

Chapter 1 Provides overview of the study, background information, motivation, aim and objectives of the study, and thesis layout.

Chapter 2 provides an outline of the relevant literature on studies related to toxicity and fate of ENPs and pesticides including their variant mixtures. Mixtures to be considered will include ENPs/ENPs, ENPs/organic pollutant(s), and ENPs/ENPs/organic pollutant(s). All toxicity studies reviewed were specifically for daphnia under variant exposure media.

Chapter 3 Details and summary of materials, experimental setup, and analytical methods employed in this study. All methods were standard unless where slight modifications were made in the context of this work.

Chapter 4 Presents results and discussions on the fate and toxicity of n-Ag, n-ZnO, and ATZ as individual contaminants as well as their binary and ternary mixtures in ISO media.

Chapter 5 Summarizes the key study findings, concluding remarks and several recommendations for further work in pursuit to fill several knowledge gaps that were outside the scope of this work.

Chapter 2: Literature review

2.1 Nanotechnology and engineered nanomaterials

Nanotechnology is defined as the creation and utilization of materials, devices and systems through the manipulation of matter at scales of less than 100 nanometers (BBC Research, 2012). It encompasses the building blocks generically referred to as engineered nanomaterials (ENMs) or devices with the smallest functional organization at the nanometer scale (1–100 nm) (Auffan et al., 2009). Another definition suggests nanomaterials as any natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate/ agglomerate and where, 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1-100 nm (European Commission, 2011). This includes but not limited to engineered nanoparticles (ENPs) with at least one dimension between 1 and 100 nm (BBC Research, 2012), nanofibres and nanotubes, nanocomposites, and nanostructured materials.

Basically, ENPs are distinguished from their bulk or metal salts counterparts by their unique physico-chemical properties. These includes size, shape, specific surface area, surface charge and chemical reactivity (Handy et al., 2008; Klaine et al., 2008, 2012). ENPs are further categorized into variant broad classes, namely: carbon-based materials that includes fullerenes and carbon nanotubes; inorganic metal oxides (e.g., zinc oxide (n-ZnO), iron oxide (n- Fe_2O_3), titanium dioxide (n-TiO_2), silica (n-SiO_2), and cerium oxide (n-CeO_2), etc.); metals (e.g., gold (n-Au), silver (n-Ag) and iron (n-Fe), etc); and quantum dots (e.g., cadmium sulphide (CdS) and cadmium selenide (CdSe)).

2.2 Production of ENPs

Technological advances in the 21st century has been linked to increasing production of ENPs at industrial scale, and as a result are widely incorporated into numerous consumer products and industrial applications including cosmetics, food, clothing, agriculture, medical devices, automotive, and electronics (Piccinno et al., 2012; Vance et al., 2015; Hansen et al., 2016; PEN, 2019; The Nanodatabase, 2020). Estimates suggest the production and application of ENPs into numerous fields to grow by about half a million tons by 2020 (Piccinno et al., 2012). ENPs produced in higher quantities includes metal-based ENPs (e.g., n-SiO_2, n-TiO_2, n-ZnO, and n-Ag) (Piccinno et al., 2012; Vance et al., 2015; Hansen et al., 2016). Table 2.1 summarizes global, regional and country productions of n-Ag in the range of 2.8 and 450 tons/yr (Hendren et al., 2011; Piccinno et al., 2012; Future markets, 2012) and 15750 to 34000 tons/yr for the n-ZnO (Future markets, 2012). Although production volumes data are not easily accessible to the public (Keller et al., 2013), reported production figures (Table 2.1) were mostly estimated from surveys of industries and experts using questionnaires and interviews (Piccinno et al., 2012; Future Markets, 2012).

Table 2.1: Examples of annual productions of ENPs (tons/yr) globally from variant studies.

ENPs	**Global**	**Regional**	**Country**
n-Ag	55[a], 360-450[b]	180-225[a], 2,8-20[c]	0.085[d], 3.1[e], 3.6-4.5[b]
n-ZnO	550[a], 31500-34000[b]	15750-17000[b]	70[e], 165-178[b]

(a) Piccinno et al., 2012 (b) Future markets, 2012 (c) Hendren et al., 2011 (d) Musee, 2011 (e) Schmid and Riediker, 2008.

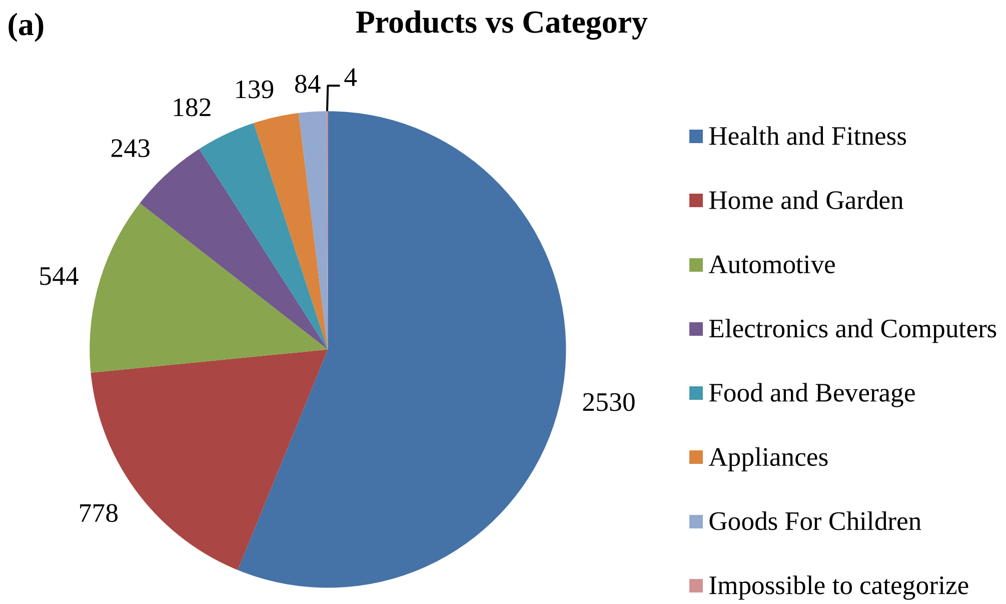
2.3 Application of ENPs in consumer products

Incorporation of ENPs into a wide spectrum of nano-enabled products (NEPs) has increased exponentially over the years. This is to meet the growing demands on improving performance and functionality of common consumer products such as medical devices, electronics, PCPs, etc (Figure 2.1a). Hence, this has led to the establishment of consumer products inventories (CPI) to track and archive available products at country level (e.g., Denmark, USA), regional (Europe), or globally. Examples of such CPIs incudes Project of Emerging Nanotechnology (PEN) database from the Wilson Woodrow Center for Scholars, The Nanodatabase by the Technical University of Denmark (DTU), the Danish Ecological Council and Danish Consumer Council (PEN, 2020; The Nanodatabase, 2020). Both n-Ag and n-ZnO are among most applied ENPs in the market based on these databases. n-Ag, for example, are incorporated in applications including textiles, food preservation (Luoma, 2008) and biomedical applications (Nowack et al., 2012; Keller et al., 2013; Pourzahedi et al., 2017) chiefly linked to their excellent antifungal and antibacterial properties. Further, n-ZnO find wide applications in sunscreens for radiation protection (Wiench et al., 2009; Shi et al., 2012), coatings (Blinova et al., 2010), environmental remediation processes (Wong et al., 2010), ceramics and biosensors (Brayner et al., 2010), medical and sanitary products as antifungal agent (Keller et al., 2013), among others. Notably, n-Ag is the most applied in NEPs, however, least produced in tonnage. Conversely, n-ZnO is the third most produced and one of the most applied ENPs (Table 2.1; Figure 2.1b).

According to The Nanodatabase (2020), there are over 4 365 NEPs largely in eight product categories, including: Appliances, Automotive, Electronics and Computers, Food and Beverage, Goods for Children, Health and Fitness, Home and Garden as well as miscellaneous (as is "impossible to categorize") (Figure 2.1a). Further, data show that n-Ag accounts for about 10 % of the total NEPs in the database. Health and fitness accounts for 60% NEPs, with PCPs as a predominant product category. Moreover, in South Africa, a study by Moeta and co-workers (2019) established six categories of NEPs (health and fitness, electronics and computers, home and garden, appliances, automotive and food beverages) with 264 products with health and fitness; as the dominant category (66.7%). Hence, their results showed similar trends as those reported in the existing databases (CPI; The Nanodatabase) irrespective of countries or regions of focus (PEN, 2020; The Nanodatabase, 2020). The remainder of the NEPs in other categories in descending order were: electronics and computers > home and garden > appliances > automotive > food and beverage (Moeta et al., 2019). Their results showed n-TiO_2 and n-Ag were the most incorporated ENPs in NEPs found in the South African commerce.

2.4 Environmental concentrations of ENPs

As stated earlier, following the use of NEPs results to eventual release of ENPs into variant environmental compartments (e.g. surface water, soils, etc.) at different stages of their lifecycle (e.g. production, usage, disposal, etc.,) (Musee, 2011; Keller et al., 2013; Giese et al., 2018; Musee, 2017). Most ENPs are released from NEPs during the use and disposal lifecycle phases (Mueller and Nowack, 2008; Gottschalk and Nowack, 2011; Keller et al., 2013; Vance et al., 2015; Musee, 2017). At present, ENPs are increasingly being detected in different environmental systems such as aquatic ecosystems (Selck et al., 2016; Bäuerlein et al., 2017; Giese et al., 2018).



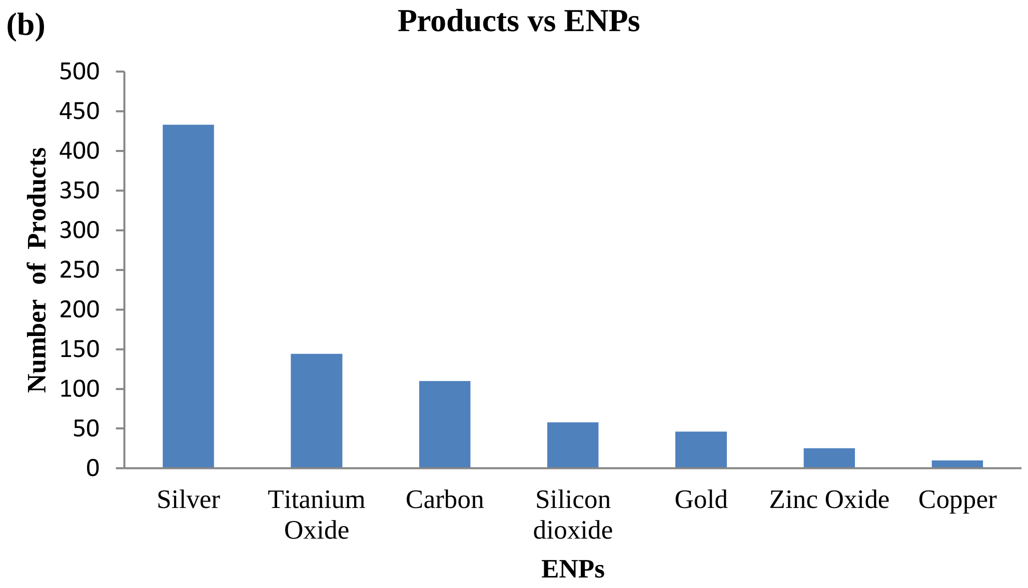


Figure 2.1: Summary of (a) categories of NEPs widely found in the market and (b) the distribution of NEPs containing different ENPs (Source: The Nanodatabase 2020, with permission).

In addition, several studies have reported variant predicted environmental concentrations of ENPs in different regions across the globe (Musee, 2011; Keller et al., 2013; Sun et al., 2014; 2016), and illustrative examples are listed in Table 2.2. Concentrations of n-Ag and n-ZnO were in the ranges of 0.1–619 ng/L, and 0.001–0.38.5 ug/L, respectively (Table 2.2). Conversely, concentrations of 0.3–3200 ng/l n-Ag (Peters et al., 2018; Loula et al., 2019), and 0.09–212 µg/L n-ZnO (Sun et al., 2014; Choi et al., 2018) have been detected in the actual environments.

The lack of accurate data on the production volumes (Hendren et al., 2011; Tiede et al., 2016) and reporting on chemical composition of ENPs in NEPs by manufacturers severely compromises the predictions of environmental concentrations and hazard assessments (Hansen et al., 2016). Therefore, data reported herein only represents a fraction of what is likely to be in the actual environment because manufactures do not declare the content of ENPs in consumer products. Challenges on the quantification of ENPs in the environment are exacerbated by lack of well-developed analytical tools especially in complex environmental matrixes. Such data are crucial in generating information for risk assessment of ENPs.

Table 2.2: Predicted environmental concentrations of ENPs in variant aquatic environments.

ENPs	**Global**	**Regional**	**Country**
n-Ag (ng/l)	0.03-2.79[a]	0.002-0.3[b], 0.088-0.428[c], 0.51-0.94[d], 0.588-2.16[c], 0-3[e], 0.87-7.84[f]	0.0001[g], ≈0.0006[h], 0-0.044[i], 0.37-0.73[d], 0.555-2.63[c], 3.3-58.9[i], 30-80[j], 2.80-619[k]
n-ZnO (µg/L)		1[c], 10[c], 90[d], 380[f]	120[d]

a. Giese et al., 2018 b. Dumont et al., 2015, c. Gottschalk et al., 2009, d. Sun et al., 2014, e. Johnson et al., 2011, f. Sun et al., 2016, g. Liu and Cohen, 2014, h. Gottschalk et al., 2015 i. O'Brien and Cummins, 2010, j. Mueller and Nowack, 2008, k. Musee, 2011.

2.5 Fate of ENPs in aquatic environments

Following their release into the aquatic environments, ENPs undergo numerous physical, chemical and biological transformation processes (Lowry et al., 2012b; Amde et al., 2017). These processes include aggregation/agglomeration, dissolution, adsorption, sulfidation, and sedimentation (Lowry et al., 2012b; Xiao et al., 2018). This, in turn, controls the ENPs' fate, mobility, bioavailability and eventually toxicity to the aquatic organisms. For example, transformation processes may alter the physicochemical properties of ENPs (Lowry et al., 2012b; Peng et al., 2017; Abbas et al., 2020a), and ultimately influence their observed toxicity to the aquatic organisms (Zhang et al., 2018a, b; Abbas et al., 2020b). The transformation processes are chiefly influenced by ENPs inherent physicochemical properties (size, morphology, surface charge, etc.,), and exposure media chemistry (pH, ionic strength (IS), light, temperature and NOM) (Lowry et al., 2012a, b; Amde et al., 2017; Odzak et al., 2014; 2017).

Elucidating ENPs transformation processes in the aquatic systems is essential for toxicity studies, and hence, meaningful hazard assessment of nano-pollutants in variant environmental matrixes as pictorially depicted in Figure 2.2. Transformation of ENPs occur following their contact with aqueous media, whereby they either dissociate into ions, or form agglomerates/aggregates (Lin et al., 2010; Cheng et al., 2011; Odzak et al., 2014). Aggregation and dissolution are key processes as they regulate the toxicity of ENPs in the aquatic systems. This is because their formed aggregates, or alternatively their soluble state are key underpinning drivers for the observed potential toxicity effects as previously demonstrated (Peijnenburg et al., 2015; Abbas et al., 2020b). Here, key transformation processes including reference to their contribution to metallic ENPs toxicity are presented in the following sections.

2.5.1 *Aggregation/Agglomeration kinetics*

Aggregation is an irreversible phenomenon whereby a primary material with nano-scale dimensions fuses to form larger particles of the same material (Stone et al., 2010; Nowack et al., 2012; Hartmann et al., 2014). The particles in aqueous media agglomerates or forms aggregates due to particle collision as a result of combined influence of the attractive van der Waals force and repulsive electrostatic force) in accordance to the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (Tourinho et al., 2012; Amde et al., 2017).

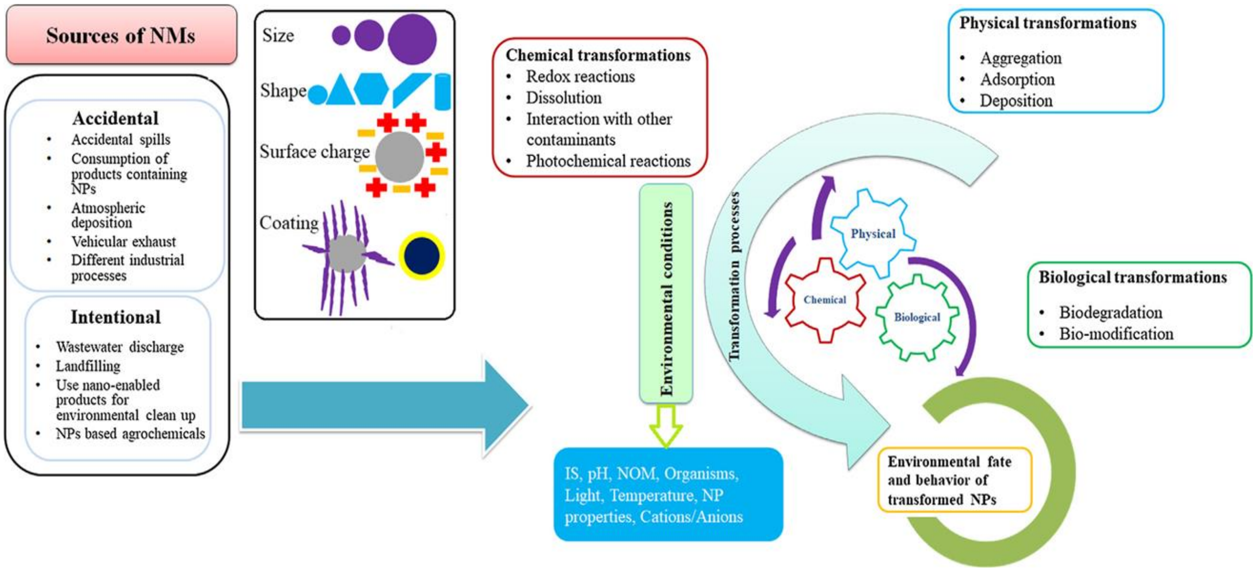


Figure 2.2: Sources of ENPs and their variant transformation processes in the aquatic environments. (Source: Abbas et al., 2020b with permission).

Aggregation in aqueous media systems occur in two ways. First, ENPs may adhere to one another, and generically regarded as homoaggregation. The second way entails the interaction between ENPs and a cocktail of other environmental components (referred as heteroaggregation) (Hotze et al., 2010; Lowry et al., 2012b; Quik et al., 2014). Notably, heteroaggregation is the most dominant process owing to large presence of a cocktail of other chemical components in the aquatic environment relative to ENPs (Quik et al., 2014; Schultz et al., 2015). Aggregation kinetics of ENPs like other transformation processes is dependent on exposure media chemistry, and inherent physicochemical properties of ENPs (Lowry et al., 2012b; Cross et al., 2015; Amde et al., 2017) mainly pH, IS and size as mentioned earlier, which determines their potential biological effects (Peng et al., 2017). In addition, ENPs concentration in the aqueous solution also greatly influences the aggregation. High concentrations of ENPs enhances particles collision frequency leading to rapid aggregation driven by thermodynamics of the particles; thus, forming larger aggregates due to their reduced surface energies (Amde et al., 2017). For instance, Yung et al. (2015) reported faster aggregation rate of n-ZnO (20 nm) at high concentration of 100 mg/L in sea water.

In aqueous media, pH is among main regulators of ENPs surface charge (ζ-potential), and stability (Lin et al., 2010). Aggregation of a colloidal suspension is at maximum around the point of zero charge (PZC) pH as the stability of the colloidal suspension is at the minimum due to the absence of mobility and surface charge of the particles (Zhou and Keller, 2010; Zhang et al., 2016; Peng et al., 2017). Stabilized ENPs in aqueous media have a ζ-potential value of ± 30 mV; whereas ζ-potential values between 0 and ± 5 mV indicate highly unstable ENPs characterized by rapid agglomerates formation (Tan et al., 2018). Lin and co-workers observed high formation of agglomerates of n-TiO_2 in aqueous media at PZC of pH 6, and the particles ζ-potential were pH dependent; where at pHs of < 4 and > 8 the particles were stabilized with ζ-potential of +31 mV and -30 mV, respectively (Lin et al., 2017). For metal-based ENPs, aggregation decreases their concentration in solution and surface area leading to increased particle size (Lowry et al., 2012b; Levard et al., 2012) with eventual sedimentation (Quik et al., 2012); which in turn partially accounts for their reduced toxicity (Hu et al., 2018).

2.5.2 *Dissolution kinetics*

Dissolution is a process whereby a solid ENP dissolves (release of individual ions or molecules) in an aqueous media (Stone et al., 2010; Nowack et al., 2012). Dissolution in aquatic environment is among fundamental drivers of fate and toxicity of ENPs (Lowry et al., 2012b; Cross et al., 2015). Among the highly soluble ENPs includes n-Ag, n-CuO, and n-ZnO. The extent to which ENPs undergo dissolution is dependent on their inherent physicochemical properties (e.g., size, zeta potential, shape, etc.), and environmental factors mostly the NOM and IS (Odzak et al., 2014; 2017; Wang et al., 2016c).

Particle size can significantly influence the dissolution of ENPs in aqueous media. For instance, Ivask et al. (2014) investigated the influence particle size of n-Ag (10 and 80 nm) on dissolution at 1 mg/L. Dissolution rate decreased from 1.2 % to 0.3 % with increasing size, with smaller size particles yielding enhanced dissolution due to their higher surface area to mass ratio than larger particles (Soenen et al., 2015). Further, the presence of NOM in the aqueous media may promote or inhibit dissolution. For example, adsorption of NOM on ENP surfaces modifies their dissolution resulting in a more stable aqueous suspension, due to steric stabilization or electrostatic repulsion between particles (Delay et al., 2011). The presence of NOM in the environment mediates the dissolution, by complexation reaction with metal ions, and by destabilizing the agglomerated particles (El Badawy et al., 2012; Domingos et al., 2013). For instance, Yang and co-workers (2014) observed the dissolution of citrate-coated n-Ag in aqueous media was enhanced by the presence of NOM Pony Lake fulvic acids (PLFA) (10 mg/L) using concentrations of 1-10 mg-C/L. The dissolved Ag ions were observed to have increased from 250 to 450 nM.

Another influencing factor of dissolution in aqueous media is IS. High IS lowers dissolution of ENPs in aqueous media due to precipitation and sedimentations of the particles (Bian et al., 2011). Yang and co-authors (2019) assessed the influence of IS on the dissolution of PVP n-Ag (25 and 75 nm) in EPA media. It was observed that dissolution was lower (61% and 49%) compared to deionized (DI) water, for 25 nm and 75 nm respectively. Overall, dissolution of metal based ENPs largely influences their resultant observed toxicity to aquatic organisms.

2.6 Toxicity of ENPs to Daphnia

The potential toxicity effects of ENPs to daphnia species have widely been investigated, and the data is excellently reviewed based covering a wide breadth of exposure media and toxicological outcomes (Fabrega et al., 2011; Ma et al., 2013; Skjolding et al., 2016; Hou et al., 2018; Zhang et al., 2019b). For example, n-Ag and n-ZnO toxicity effects towards daphnia were investigated for end-points including immobility (Azevedo et al., 2016; Hu et al., 2018), mortality (Xiao et al., 2015; Chae and An, 2016), reproduction (Ribeiro et al., 2014; Sakka et al., 2016), and bioaccumulation (Khan et al., 2015) as endpoints both in natural and artificial media. As an example, Cupi et al. (2016) investigated the effects of n-Ag (17.5 nm) and n-ZnO (151 nm) to *D. magna* (for immobility end-point) in M7 media at concentrations range 10–1000 µg/L and 0.01–20 mg/L, respectively. The results showed that n-Ag was the most toxic with observed EC_{50} of 66 µg/L and 4.9 mg/L n-ZnO, respectively. Further, both ENPs were characterized by high aggregation and low dissolution due to particle collision. Furthermore, Chae and An (2016) investigated mortality of 10 μm and 20 μm PVP n-Ag (± 41 nm) nanowires (NWs) and observed mortality was higher for the longer NWs than the shorter ones.

To date, substantial data useful for risk assessment on human and environmental health have been reported. Several species of daphnia are used as test organism due to their sensitivity to pollution in natural water (Baun et al., 2008a; Griffitt et al., 2008), and includes *D. magna, Ceriodaphnia cornuta, Ceriodaphnia dubia, Daphnia carinata, Daphnia galeata, Daphnia pulex,* and *Daphnia similis.* In the case of ENPs as environmental pollutants, *D. magna* is the most commonly investigated species based on results listed in Table 2.3 accounting for 84.2% (32 of total 38 studies).

Analysis of the studies show that the 48 h median effect concentration (EC_{50}) of *D. magna* are widely variable irrespective of ENPs type (e.g., in Tables 2.3 and 2.4 for n-Ag and n-ZnO, respectively). For example, the reported EC_{50} values for n-Ag differ markedly by about 270-folds (in the range of 1.1–296.85 μg/L) for *D. magna*. The wide variations of EC_{50} were attributed to variant ENPs physicochemical properties, and media chemistry. For example, Blinova et al. (2013) investigated the toxicity of n-Ag with different coatings in artificial and natural freshwater exposure media. Results revealed the PVP coated-Ag were more toxic than protein coated ones, and the exposure media composition had significant influence on the observed toxicity irrespective of n-Ag type. Overall, lower toxicity was observed in natural freshwater relative to the artificial exposure media. Conversely, Heinlaan and co- authors (2016) observed a different trend following an assessment of the media chemistry influence on n-Ag. Their findings demonstrated higher toxicity in natural water relative to the artificial media. These findings among others suggest the toxicity exerted by ENPs to *D. magna* is dependent on numerous factors, and in turn, the interplay on the interactions of ENPs inherent properties and exposure media chemistry are defined by infinite permutations; and hence it is infeasible to generalize the likely observable effects from set of conditions to another.

Although n-ZnO is detected at higher concentrations in the environmental compartments than n-Ag, however, aquatic organisms are more sensitive to latter. Table 2.4 summarizes the acute effects of n-ZnO to *D. magna*, and the observed EC_{50s} ranged from 0.047 to 9 mg/L. Seo et al. (2014)

illustrated the influence of media composition e.g., hardness in mitigating the toxicity of n-ZnO to *D. magna*. Findings summarized in Tables 2.3 and 2.4 indicate that based on apical endpoint immobilization, n-Ag is more toxic relative to n-ZnO irrespective of daphnia species, with *D. Similis* as most sensitive species.

2.7 Mechanisms of ENPs toxicity

Numerous investigations have demonstrated that the toxicity of ENPs to aquatic organisms is largely dependent on environmental conditions (e.g., pH, IS, NOM, etc.) (Zhang et al., 2016; Hou et al., 2017b; Li et al., 2018) as earlier mentioned. Aquatic organisms may be exposed to ENPs through various pathways including ingestion and their external epithelia (Oberdörster et al., 2005). As a result, ENPs may induce toxicity to organisms by means of variant modes. For example, toxicity associated with soluble ENPs results in inducing oxidative stress due to the release of toxic ions, and through cell membrane permeability as the primary nanotoxicity mechanisms (Zhao et al., 2017a; Das et al., 2018; Buchman et al., 2019). Zhao et al. (2020) illustrated that nanotoxicity mechanism occurs via three stages, namely: external toxicity, interface interaction, and intracellular response as shown in Figure 2.3.

For example, dissolution has been directly associated to the toxicity of n-Ag (Shen et al., 2015; Liu et al., 2018) and n-ZnO (Tong et al., 2015; Bacchetta et al., 2016). For example, Shen et al. (2015) investigated the contribution of various Ag species (total Ag, free Ag^+, total Ag^+, dissolved Ag) in n-Ag citrate (9.2-56.3nm) and polyvinylpyrrolidone (PVP) (28.1-95.5nm) exposure mixtures to the toxicity towards *D. magna* following 8h exposure in different media. The findings demonstrated that Ag^+ was exclusively responsible for the observed acute toxicity of n-Ag to *D. magna*. In another study, Bacchetta et al. (2016) investigated the toxicity of ionic and particulate Zn. It was concluded that Zn^{2+} was the key driver of the observed cytotoxicity to *D. magna*. These studies and many others demonstrate that release of ions play a significant role on the toxicity of ENPs with appreciable dissolution in aqueous media.

Table 2.3: Summary of acute toxicity effects of n-Ag to Daphnia species under different exposure conditions.

Daphnia species/age	**Exposure media**	**Exposure concentrations (µg/L)**	**End point**	**Obtained EC/LC$_x$ (µg/L)**	**Protocol**	**Size/coating type of n-Ag**	**Refs**
D. magna < 24h	COMBO Media	0-20	Mortality 48h	48h LC_{50}: 5 (without food) 48h LC_{50}: 17 (with food)	USEPA (2002)	10 nm carboxy-functionalized polyacrylate capped n-Ag	[1]
D. Similis < 24h	Artesian well water	0.15-1500	Immobilization 48h	24h EC_{50}: 0.34 48h EC_{50}: 0.26	OECD Part 1 (1984b)	2-18 nm PVP n-Ag	[2]
D. magna < 24h	ASTM hard water	0.2-20	Immobilization 48h	24h, 48h EC_{50}: 11.41, 11.02 (without food) 24h, 48h EC_{50}: 81.83, 72 (with food)	OECD (2004)	3-8 nm alkaline coated n-Ag	[3]
D. magna < 24h	ISO Medium	Not reported	Immobilization 48h	48h EC_{50}: 54	OECD (2004)	8.4 nm PVP n-Ag	[4]
	Natural Water (RW1;RW2;LW1;LW2;LW3)			48h EC_{50}: 191, 98.7, 176, 236.3, 162			
D. magna < 24h	ISO Medium		Immobilization 48h	48h EC_{50}: 49.4		12.5 nm Collargol n-Ag	
	Natural Water (RW1;RW2;LW1;LW2;LW3)			48h EC_{50}: 59.4, 40.2, 74.9, 50.8, 65.7			
D. magna < 24h	MHW	100-500	Immobilization 48h	24h $EC_{10,50}$: 168.087, 336.07	OECD (2004)	42 nm PVP coated (10 µm NWs)	[5]
				48h $EC_{10,50}$: 141.682, 256.16			
			Mortality 48h	24h $LC_{10,50}$: 201.464, 383.61			
				48h $LC_{10,50}$: 182.127, 296.85			
		150-300	Immobilization 48h	24h $EC_{10,50}$: 175.997, 262.52	OECD (2004)	42 nm PVP coated (20 µm NWs)	
				48h $EC_{10,50}$: 167.812, 247.08			
			Mortality 48h	24h $LC_{10;50}$: 218.598, 283.9			
				48h $LC_{10,50}$: 196.569, 263.17			
D. pulex adults	MHW	Not reported	Immobilization 48h	48h EC_{50}: 40	ASTM (2002)	20-30 nm n-Ag	[6]
C. dubia <24h				48h EC_{50}: 67			
D. magna 10d	Reconstituted water (RW)	1-10	Immobilization 48h	No reported mortality @ 10µg/L	Not reported	10 and 40 nm PVP n-Ag	[7]
D. magna < 24h	ASTM hard water	50 – 250	Immobilization 48h	48h EC_{50}: 79	OECD (2004)	< 10 nm powder nanospheres	[8]

D. magna < 24h	Elendt M7 medium	16-80	Immobilization 48h	24h EC_{50}: 130 48h EC_{50}: N.D	OECD (2004)	29.9 nm citrate n-Ag	[9]
C. cornuta < 24h	Standard synthetic freshwater	5-50	Immobilization 24h	24h EC_{50}: 7.94	USEPA (2002)	23 nm Sodium alginate n-Ag	[10]
D. magna < 24h	AFW	0.6- 1000	Immobilization 48h	24h EC_{50}: 7.94; 48h EC_{50}: 3.0	OECD (2004)	21 nm PVP n-Ag	[11]
	Lake Greifen water Lake Lucerne water		Immobilization 48h	48h EC_{50}: 2.2 48h EC_{50}: 1			
	ADaM water Lake Greifen water (2014/2015) Lake Lucerne water (2014/2015)	1-5	Immobilization 48h	48h EC_{50}: 3.4 48h EC_{50}: 5.5, 1.1 48h EC_{50}: 2.1, 1.9			
D. Similis < 24h	ASTM hard water	1-6	Immobilization 48h	48h EC_{50}: 1.59	OECD (2004)	20 nm n-Ag	[12]
D. magna < 24h	Elendt M7 medium	10-100	Immobilization 48h	48h $EC_{10,50}$: 42, 66	OECD (2004)	17.5 nm n-Ag in TWEEN 20, at 4%	[13]
	VS EPA medium			48h $EC_{10,50}$: 32, 51			
D. magna < 24h *D. galeata* < 24h	COMBO Medium	5.92-28.56	Immobilization 48h	48h EC_{50}: 2.43 48h EC_{50}: 2.16	OECD (2004)	79.9 nm uncoated nanocolloids	[14]
D. magna < 24h	ASTM reconstituted hard water	0.1-62.5; 10-160; 479-750; 750-1200	Immobilization 48h	48h EC_{50}: 3.9-33.4 48h EC_{50}: 28.9-80.6 48h EC_{50}: 77.6-91.6 48h EC_{50}: 185.9-216.1	OECD (2004)	140 nm uncoated n-Ag 20 nm Citrate n-Ag 60 nm Citrate n-Ag 100 nm Citrate n-Ag	[15]
D. magna < 24h	Elendt M7 medium	10-200	Immobilization 48h	24h EC_{50}: 61.2, 200, 304 (no food; low food; high food) 48h EC_{50}: 47.6, 184, 302 (no food; low food; high food)	OECD (2004)	30 nm Citric acid n-Ag	[16]
D. magna < 24h	EPA Synthetic hard water	0-10	Immobilization 24h	24h LC_{50}: 48.60	USEPA (2002)	15.5 nm citrate n-Ag	[17]
D. magna 6-24h	Surface water	1-100	Immobilization 48h	24h,48h EC_{50}: 270, 270	OECD (2004)	8.6 nm citrate n-Ag	[18]
	M4 medium	1-100	Immobilization 48h	24h,48h EC_{50}: 105, 110			
D. magna < 24h	MHW	30-60	Immobilization 48h	48h EC_{50}: 44.83	USEPA (2004)	56.6 nm PVP n-Ag	[19]
		100-500		48h EC_{50}: 256.16		41.32 nm AgNWs-10	
		150-300		48h EC_{50}: 247.08		42.1 nm AgNWs-20	
		15-75		48h EC_{50}: 27.92		30 nm AgNPLs	

D. galeata < 24h	Saponin-MHW	30-60	Immobilization 48h	48h EC_{50}: 35.51		56.6 nm PVP n-Ag	
		100-300		48h EC_{50}: 193.4		41.32 nm AgNWs-10	
		100-300		48h EC_{50}: 139.42		42.1 nm AgNPs-20	
		10-45		48h EC_{50}: 31.42		30 nm AgNPLs	
D. magna 7d	SM7	10-50	Survival 48h	48h LC_{50}: 28.7	USEPA (2002)	< 100 nm lactate n-Ag	[20]
		1-8		48h LC_{50}: 2.0		10-20 nm PVP n-Ag	
		1-5		48h LC_{50}: 1.1		10-20 nm Sodium dodecylbenzene sulfonate n-Ag	
D. magna < 24h		5-100	Immobilization 48h		OECD (2004)	17.5 nm Tween 20 n-Ag	[21]
	M7			0h $EC_{10,50}$: 23.5, 32.6 24h $EC_{10,50}$: 27.9, 39.3 48h $EC_{10,50}$: 24.7, 41.3			
	M7+ Suwannee River NOM			0h $EC_{10,50}$: >100, >100 24h $EC_{10,50}$: >100, >100 48h $EC_{10,50}$: >100, >100		Stock I	
	M7+ Suwannee River NOM			0h $EC_{10,50}$: >100, >100 24h $EC_{10,50}$: >100, >100 48h $EC_{10,50}$: >100, >100		Stock II	
D. magna < 24h	EPA Water	10-10 000	Mortality 96h	96h LC_{50}: 100	USEPA (2002)	35 nm n-Ag	[22]
D. magna < 24h	Elendt M7 medium	1-3	Immobilization 48h	48h EC_{50}: 1.86	OECD (2004)	Alcea rosea biosynthesized n-Ag	[23]
D. magna < 24h	MHRW	1000-32 000	Immobilization 24h	24h EC_{50}: 90-3844.1	USEPA (2002)	< 100 nm n-Ag	[24]
				24h EC_{50}: 4.2-1404.6		< 150 nm n-Ag	
D. magna < 24h	M4	1-10	Immobilization 48h	48h $EC_{10,50,90}$: 3, 4, 5	OECD (2004)	5-25 nm spherical colloidal n-Ag	[25]
		1-32		48h $EC_{10,50,90}$: 1.5, 2, 3		16.6 nm spherical colloidal n-Ag	
		100-320		48h $EC_{10,50,90}$: 140, 187, 251		20 nm nanopowder	
D. magna 6-7d	Adapted M7 medium	0-8	Bioaccumulation 48h	48h LC_{50}: 5.9	OECD (2004)	10.8 nm PVP n-Ag	[26]
		0-22.5		48h LC_{50}: 13.4		10.3 nm PEG n-Ag	
		0-14.5		48h LC_{50}: 8.5		11.6 nm Citrate n-Ag	
D. magna < 24h	Not reported	10.1-29	Immobilization 48h	48h EC_{50}: 13.8	OECD (2004)	11.7 nm citrate (Fractionated) n-Ag	[27]
		4.9-25		48h EC_{50}: 6.1		11.7 nm Citrate (Colloidal) n-Ag	

D. magna < 24h	M4		Immobilization 48h	48h EC$_{50}$: 10.3 48h EC$_{50}$: 0.95	OECD (2004)	13.3 nm citrate-stabilized: Sol-type SNC 60- 100 nm Citrate-stabilized: Powder-type (SNC)	[28]
D. magna 7d	M7	20-500	Survival 48h	No mortality at 500 µg/L (48-h LC$_{50}$: > 500 (with 1 µM cysteine)	OECD (2004)	< 220 nm carbonate-coated n-Ag	[29]
D. magna < 24h	ASTM reconstituted water	25-125	Immobilization 96h	96h EC$_{50}$: 113.8	OECD (2004)	15.5nm n-Ag dispersions	[30]
D. magna < 24h	MHRW	Not reported	Mortality 48h	48h LC$_{50}$: 1.8, 5.8, 5.3, 5.4, 11.1, 14.9, 17.7, 97	OECD (2004)	31 nm SNC , (10 nm; 20 nm ;30 nm; 80 nm) AgNPs, 29 nm citrate stabilized, 36 nm EDTA stabilized, 41 nm PVP stabilized	[31]
D. magna < 24h	COMBO Media	0.5-5.5	Immobilization 48h	48h LC$_{50}$: 2.75	OECD (2004)	1-10nm carboxy-functionalized polyacrylate capped n-Ag	[32]
D. carinata < 24h	MHW	0.97-15.56	Mortality 48h	24h,48h LC$_{50}$: 3.56, 1.75	USEPA (2002)	PVP n-Ag	[33]
	CUE		Mortality 48h	24h,48h LC$_{50}$: 10.81, 4.61			
D. magna < 24h	M4	1- 1000	Mortality 48h	48h LC$_{10,50,90}$: 7, 12, 17	OECD (2004)	5-25 nm citrate n-Ag	[34]
				48h LC$_{50}$: 117;139;160		60 nm AgNWs	
D. magna < 24h	7 Lakes water	2- 475	Survival 24h	24h LC$_{50}$: 34-292	OECD (2004)	30-50 nm PVP n-Ag	[35]
	MHW	0.01-40.3	Mortality 48h	48h LC$_{50}$: 0.41	USEPA (1987)	10 nm BPEI n-Ag	[36]
				48h LC$_{50}$: 2.88		56 nm Citrate n-Ag	
				48h LC$_{50}$: 4.79		72 nm PVP n-Ag	
D. magna < 24h	M4	2.5-10	Mortality 48h	48h LC$_{10,50}$: 60.3, 121	OECD (2004)	< 20 nm PVP coated	[37]
D. pulex		1.25-5		48h LC$_{10,50}$: 4.37, 8.95			
D. galeata		1.25-10		48h LC$_{10,50}$: 11.0, 13.9			
D. magna < 24h	MHW; 1.46mg C/L SRDOC; 10.43 mg C/L SRDOC	0.5-20	Mortality 48h	48h LC$_{50}$: 3.41, 2.27, 13.08	USEPA (2002)	4.7 nm PEG n-Ag	[38]
	MHW; 1.46mg C/L SRDOC; 11.30 mg C/L SRDOC	1-10		48h LC$_{50}$: 3.16, 2.14, 3.48		6 nm GA n-Ag	
	MHW;	5-75		48h LC$_{50}$: 14.81, 14.04, 14.09		25 nm PVP n-Ag	
	1.46mg C/L SRDOC; 9.61 mg C/L SRDOC	5-75		48h LC$_{50}$: 14.81, 14.04, 14.09		25 nm PVP n-Ag	

References: [1] Conine and Frost (2017) [2] Becaro et al., 2015 [3] Ribeiro et al., 2014 [4] Blinova et al., 2013 [5] Chae and An (2016) [6] Griffitt et al., 2008 [7] Li et al., 2015 [8] Azevedo et al., 2017 [9] Sørensen et al., 2016 [10] Vijayakumar et al., 2016 [11] Heinlaan et al., 2016 [12] Zhang et al., 2017a [13] Cupi et al., 2016 [14] Sakamoto et al., 2015 [15] Seitz et al., 2015 [16] Mackevica et al., 2015 [17] Kim et al., 2016 [18] Hu et al., 2018 [19] Cui et al. (2017) [20] Zhao and Wang (2012) [21] Cupi et al., 2015 [22] Gaiser et al., 2012 [23] Khoshnamvand et al., 2020, [24] Jo et al., 2012 [25] Asghari et al., 2012 [26] Khan et al., 2015 [27] Park et al., 2014 [28] Lee et al., 2012 [29] Zhao and Wang 2011 [30] Galhano et al., 2020 [31] Kennedy et al., 2010 [32] Das et al., 2013 [33] Qin et al., 2015 [34] Sohn et al., 2015 [35] Conine et al., 2017 [36] Silva et al., 2014 [37] Völker et al., 2013 [38] Newton et al., 2013.

Abbreviations: ADaM – Aachener Daphnienmedium, AFW – Artificial fresh water, AgNPs – Silver nanoparticles, ASTM - American Society for Testing and Materials, BPEI- branched polyethyleneimine-coated, CUE – Predator conditioned water, DIW - deionized water, EPA – Environmental Protection Agency, GA- gum Arabic, ISO - International Organization for Standardization, LW – Lake Water, MHRW -moderately hard reconstituted water, MHW – Moderately hard water, $NaCO_3$ – Sodium Carbonate, NOM – Natural organic matter, PEG -polyethylene glycol, POE – Polyoxyethylene, PVP –polyvinylpyrrolidone, RW – River Water, SM7 – Soft M7 medium, SNC – Silver nanocoloids, SRDOC -Suwannee River dissolved organic carbon, VS EPA – Very soft Environmental Protection Agency medium

Table 2.4: Summary of acute toxicity effects of n-ZnO to Daphnia species under different exposure conditions.

Daphnia species/age	**Exposure media**	**Exposure concentrations (mg/L)**	**Endpoint**	**Obtained (EC/LC$_x$) (mg/L)**	**Protocol/ Guideline**	**Size/coating type of n-ZnO**	**Refs**
D. magna < 24h	ASTM hard water	0.5-1.3	Immobilization 48h	48h LC_{50}: 1.29	OECD (2004)	Powder tetrapods	[1]
D. magna < 24h	M4	0.01-100	Immobilization 48h	48h $EC_{10,50}$: 5.2, 7.5	OECD (2004)	< 200 nm uncoated n-ZnO	[2]
C. dubia < 24h	Lake water	0.05-0.5	Immobilization 48h	48h LC_{50}: 0.431 48h LC_{50}: 0.605	OECD (2004)	50 nm n-ZnO 100 nm n-ZnO	[3]
D. magna < 24h	ASTM hard water	0.5-5	Immobilization 48h	48h LC_{50}: 0.7	OECD (2004)	30 nm nanopowder	[4]
D. magna < 24h	VS EPA water M7	0.01-20	Immobilization 48h	48h $EC_{10,50}$: 0.026, 0.047 48h $EC_{10,50}$: 0.60, 4.9	OECD (2004)	151 nm nanopowder	[5]
D. magna < 24h	AFW	Not reported	Immobilization 48h	48h EC_{50}: 2.6	Daphtoxkit^{TM} magna OECD (2004)	70 nm n-ZnO	[6]
	Natural River water (R1;R2;R3;R4;R5;R5;R6)	0.01-10		48h EC_{50}: (3.3; 9.0; 1.7; 3.5; 2.8; 3.4)			
C. cornuta	Standard synthetic water	5-50	Immobilization 24 h	42%	USEPA (2002)	n-ZnO powder	[7]

D. magna < 24h	Synthetic freshwater	Not reported	Immobilization 48h	48h $EC_{20,50}$: 2.45, 3.2	Daphtoxkit^{TM} magna (1996)-OECD (2004)	50-70 nm n-ZnO	[8]
D. magna < 24h	Hard water	0.01-5	Immobilization/mortality 48h	48h EC_{50}: 0.622 48h LC_{50}: 1.51	OECD (2004)	20 nm n-ZnO	[9]
D. magna < 24h	Commercial mineral water	0.1-10	Immobilization 48h	48h $EC_{10,50,90}$: 1, 1.9, 3.6 48h $EC_{10,50,90}$: 0.85, 3.1, 11.5	OECD (2004)	< 50 nm nanopowder < 100 nm nanopowder	[10]
D. magna < 24h	ASTM hard water	0.25-10; 0.25-10; 1.2-2.4	Mortality 48h	48h LC_{50}: 1.02, 1.10, 0.89	OECD (2004)	30nm; 80-100 nm; > 200 nm nanopowders	[11]
D. magna < 24h	ISO Medium	1-50	Immobilization 48h	48h $EC_{10, 50}$: 0.88, 1.9 48h $EC_{10, 50}$: 6.9, 15.5 48h $EC_{10,50}$: 4.1,13.7	ISO (2012)	30 nm nanopowder 30 nm ZnO-OH nanopowder 30 nm ZnO-octyl nanopowder	[12]
D. magna 10d	MHRW	2.2-9.0	Immobilization 48h Mortality 24h	24h $LC_{10,25,50}$: 3.7, 9, 22	USEPA (2002)	20 nm uncoated n-ZnO powder	[13]
D. magna < 24h	M7	2.5-20	Immobilization 48h	0h $EC_{10,50}$: 1.8, 6.7 24h $EC_{10,50}$: 0.05, 1.7 48h $EC_{10,50}$: 1.5, 4.7	OECD (2004)	151 nm nanopowder	[14]
	M7+ Suwannee River NOM			0h $EC_{10,50}$: N.D; N.D 24h $EC_{10,50}$: 0.2, 1.1 48h $EC_{10,50}$: 0.3, 1.5	OECD (2004)	Stock I	
	M7+ Suwannee River NOM			0h $EC_{10,50}$: N.D, N.D 24h $EC_{10,50}$: N.D, N.D 48h $EC_{10,50}$: 0.4, 2.2	OECD (2004)	Stock II	
D. magna < 24h	Daphnia medium	0.2-50	Mortality 72h	72h LC_{50}: 89.409	OECD (2004)	10–30 nm n-ZnO	[15]
D. magna < 24h	MHW	1-10	Mortality 96h	24h $LC_{10,50,90}$: 1.12, 6.58, 38.66 48h $LC_{10,50,90}$: 0.4, 3.23, 25.99 72h $LC_{10,50,90}$: 0.28, 1.19, 7.71 96h $LC_{10,50,90}$: 0.2, 0.64, 2.05	OECD (2004)	10-30 nm n-ZnO	[16]
D. magna 5d	MHRW	0.31-10	Mortality 72h	24h,48h,72h LC_{50}: 1.9, 1.4, 1.06	USEPA (2002)	20-30 nm Uncoated dry powder	[17]

D. magna 4-5d	MHW	0.25-5	Mortality 48h	48h LC$_{50}$: 0.59 (SSR); 1.53 (Lab)	USEPA (2002)	< 100 nanopowder	[18]
				48h LC$_{50}$: 0.51 (SSR); 0.54 (Lab)		30-50 nm n-ZnO suspensions	
D. magna < 24h	MHW	0.78-25	Immobilization 48h	48h EC$_{50}$: 1.57	USEPA (2002)	20-30 nm n-ZnO dry powder	[19]
	HW			48h EC$_{50}$: 8.22	OECD (2004)		
	ISO medium			48h EC$_{50}$: 3.12			
D. magna < 24h	M4	0.4-2.6	Immobilization 48h	48h LC$_{50}$: 0.99	OECD (2004)	43 nm n-ZnO	[20]
D. magna < 24h	Not reported	0.01–10	Immobilization 48h	48h EC$_{50}$: 0.76	ISO (2012)	48 nm n-ZnO	[21]
D. magna < 24h	ISO medium	1.3-5.2	Immobilization 48h	48h EC$_{50}$: 2.67	ISO (2012)	32 nm n-ZnO	[22]
				48h EC$_{50}$: 3.12		26 nm n-ZnO@AF	
				48h EC$_{50}$: 3.02		20 nm ZnO NRs	
				48h EC$_{50}$: 2.04		22 nm ZnO NR@ AF NPs	
D. magna < 24h	M7	0.5-8	Immobilization 48h	48h LC$_{50}$: 1.78	OECD (2004)	30 nm n-ZnO suspensions	[23]
		1-10		48h LC$_{50}$: 6.73		50 nm n-ZnO suspensions	
D. magna < 24h	ISO medium	1.84-7.37	Immobilization 48h	48h EC$_{50}$: 3.97	ISO (2012)	25 nm ZnO NRs	[24]
		1-10		48h EC$_{50}$:3.12		32nm ZnO@ AF NRs	

References: [1] Azevedo et al., 2017, [2] Wiench et al., 2009, [3] Bhuvaneshwari et al., 2016, [4] Azevedo et al., 2016, [5] Cupi et al., 2016, [6] Blinova et al., 2010, [7] Vijayakumar et al., 2017, [8] Heinlaan et al., 2008, [9] Zhu et al. (2009), [10] Santo et al., 2014, [11] Lopes et al., 2014, [12] Skjolding et al., 2014, [13] Poynton et al., 2011, [14] Cupi et al., 2015, [15] Danabas et al., 2020, [16] Khoshnood et al., 2016, [17] Kim et al., 2017, [18] Ma et al., 2014, [19] Seo et al., 2014, [20] Xiao et al., 2015, [21] Hai-zhou et al., 2012, [22] Melegari et al., 2019, [23] Liu et al., 2014, [24] Gonçalves et al., 2018.

Abbreviations: AF- Amine-functionalized, ASTM - American Society for Testing and Materials, HW - Hard water, ISO - International Organization for Standardization, MHW – Moderately hard water, Lab- laboratory light, ND - Not determined, NR – Nanorods, R – River, SSR- simulated solar radiation, VS EPA – Very soft Environmental protection agency medium.

Toxicity of metal-based ENPs has been largely attributed to oxidative stress (Auffan et al., 2009; Chang et al., 2012; Skjolding et al., 2016; Ates et al., 2020) linked to the release of dissolved ions (Yang et al., 2012; Xie et al., 2019; Leareng et al., 2020). Further, other studies have reported that the toxicity of metal based ENPs as due to both the ionic species of the dissoluting ENPs, and their particulates (Thwala et al., 2013; Adam et al., 2014a, b; Huang et al., 2017). In the following sections, key mechanisms that account for the toxicity of ENPs in aqueous media are briefly summarized.

2.7.1 *Release of dissolved ions*

The dissolution of ENPs to toxic ions is a crucial process in the aquatic environments as it can alter their size, shape and surface charge in aqueous media (Zhang et al., 2018b). This may in turn significantly affect aquatic organisms' health. The released metal ions from highly soluble ENPs e.g., n-Ag and n-ZnO are the central mechanism of metallic ENPs toxicity (Adam et al., 2014a; Chen et al., 2017). Dissolved metal ions can bind onto organisms and exert toxicity at various interfaces as illustrated in Figure 2.3 based on their ion composition. These effects include, but not limited to induction of oxidative stress (Sharifi et al., 2012; Skjolding et al., 2016; Buchman et al., 2019), inhibition of cellular functions (Barras and Fontecave, 2011; Tong et al., 2015), and genetic material damage (Dupont et al., 2011; Leareng et al., 2020).

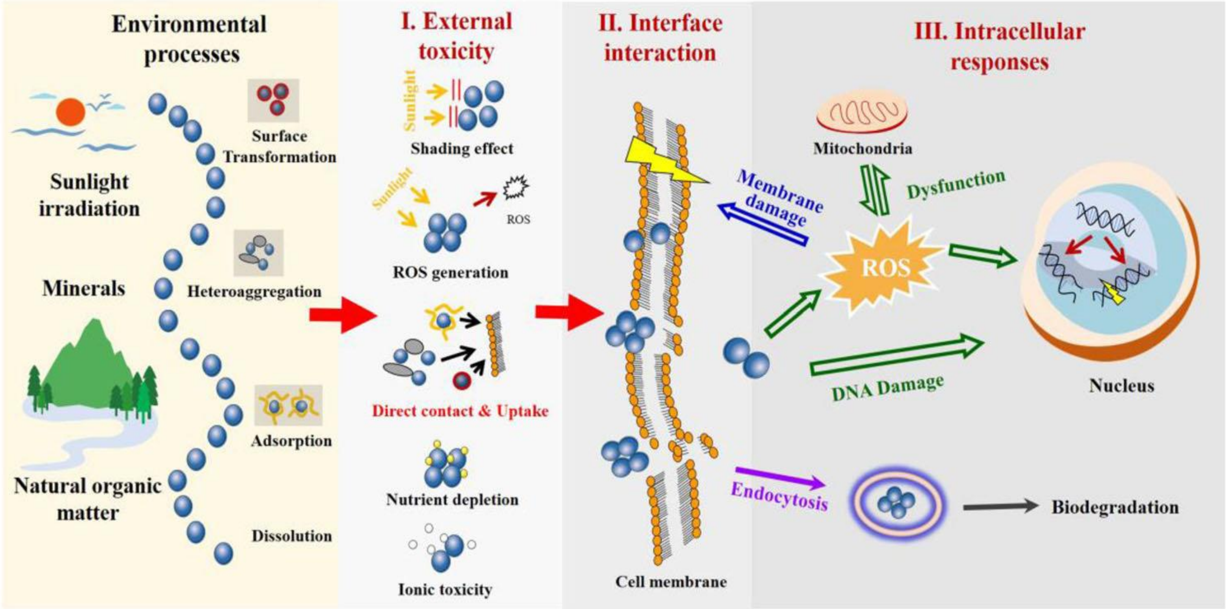


Figure 2.3: ENPs toxicity mechanism and related transformation processes as regulated by environmental factors. (Source: Zhao et al., 2020, reproduced with permission).

2.7.2 *Oxidative stress from ROS*

Production of ROS and free radicals causes oxidative stress, and consequent DNA and membrane damage (Nel et al., 2006) (Figure 2.3). Excessive ROS generation, for example, can induce lipid peroxidation (Sharifi et al., 2012; Leung et al., 2016), and in turn, genotoxic effects ultimately leading to cell death (Mukherjee et al., 2014; Buchman et al., 2019). Oxidative stress has been demonstrated to be a possible important mechanism for the observed for the toxicity of n-ZnO (Ma et al., 2014; Skjolding et al., 2016; Ates et al., 2020), and n-Ag (El Badawy et al., 2010; Hou et al., 2017a; Jiang et al., 2017; Ellis et al., 2020). For example, in a study undertaken by Ates et al. (2020), n-ZnO (10–30 nm) were administered to *D. magna* over 72h at concentrations of 0.2–50 mg/L. Glutathione (GSH) and malondialdehyde (MDA) were analysed as biomarkers of oxidative stress. Results indicated that both GSH activity and MDA levels increased with increasing n-ZnO concentrations; hence, an indication that n-ZnO induced oxidative stress to *D. magna*.

2.8 Factors influencing toxicity of ENPs in Aquatic systems

Numerous factors exert influence on the fate and toxicity of ENPs to aquatic organisms discussed in sections 2.5. Interactions of ENPs with the exposure media chemistry play a crucial role in their overall toxicity (Ben-Moshe et al., 2013), for example. In addition to concentration and exposure media chemistry, other factors including inherent properties and environmental conditions also play a key role (Barreto et al., 2015). Physicochemical properties of ENPs including size, coating, zeta potential, and morphology are regarded among key drivers of ENPs toxicity to aquatic organisms in the environmental systems (Lynch et al., 2014; Hou et al., 2017a; Zhang et al., 2019b). Conversely, environmental factors e.g., pH, dissolved oxygen (DO), IS, and NOM have been reported to influence the toxicity of ENPs to aquatic organisms (Ren et al., 2016; Peng et al., 2017). In this work, few illustrative examples are discussed in the following sections.

2.8.1 *Physicochemical properties of exposure media*

2.8.1.1 Size and morphology

The primary particle size is the main driver of ENPs induced toxicity (Sotiriou and Pratsinis, 2010; Park et al., 2011; Hou et al., 2017a). The smaller-sized ENPs are highly toxic to aquatic organisms due to their high dissolution rate (Ma et al., 2013; Gliga et al., 2014), and enhanced interactions with the organisms in focus. Different shapes may also display variant toxicity effects; however, toxicity of ENPs can be partially influenced by shape (Zhang et al., 2019b). Various shapes of ENPs includes wires, rings, tubes, rods, spheres, cylindrical, and cubes. A study by Sohn and colleagues (2015) compared the potential toxicity effects of two types of n-Ag with different morphologies (n-Ag spheres (5-25 nm); n-Ag nanowires (60 nm diameter × 10 µm length) towards *D. magna*. The findings revealed the smaller sized n-Ag to be more toxic to Daphnia relative to larger counterparts. The observed toxicity ranked based on shape parameter in descending order were: > n-Ag spheres > n-Ag nanowires.

2.8.1.2 Surface coating

Surface coatings such as citrate, PVP, or Tween-80 are used as stabilizing agents for ENMs. Surface coating can alter transformation processes e.g., aggregation and dissolution (El Badaway et al., 2010; Zhang et al., 2019b), and have been observed to mitigate ENMs adverse effects by blocking the release of toxic ions (Kirchner et al., 2005; Pereira et al., 2018). For instance, Angel et al. (2013) reported the effect of surface coatings on PVP- (size 15 nm) and citrate-coated (10 nm) n-Ag to *C. dubia*. Results showed that the coating type exerted influence on observed toxicity with citrate coated n-Ag being relatively more toxic than PVP-coated n-Ag to *C. dubia*.

2.8.1.3 Zeta potential

Surface charge of ENPs can influence their toxicity as it determines plausible cellular uptake, and interactions with biological systems (El Badawy et al., 2011). The fate of ENPs in aquatic systems is largely affected by ζ-potential as described in section 2.5.1 by regulating processes including adsorption of ENPs on organisms or organic macromolecules, and colloidal behaviour (Georgieva et al., 2011). As surface charge is a major determinant of colloidal behaviour, it specifically influences the observed organism response following exposure to ENPs driven by changing their shape and size through aggregate or agglomerate formation (Hoshino et al., 2004; Jo et al., 2015). Positively charged ENPs can be taken up easily into organisms' cells, however, adsorption of proteins by ENPs modifies their ζ-potential to negative ζ-potential irrespective of their original charge (Yue et al., 2011; Tenzer et al., 2013).

For instance, Silva et al. (2014) investigated the effect of surface charges (positive or negative) for PVP (10 nm)-, Citrate (56 nm)-, branched polyethyleneimine-coated (BPEI) (72 nm) coated n-Ag on *D. magna* mortality. The results demonstrated that smaller-sized particles exhibited higher toxic effects compared to larger ones. The reported ζ-potentials were +28.8, -20.08, and -7.49 mV for the PVP, citrate, and BPEI coated n-Ag, respectively. In addition, positively charged n-Ag showed greater mortality relatively to negatively charged ones. These findings suggest that the cytotoxicity of n-Ag is strongly influenced by their particle size and surface charge. Therefore, for exposure studies of ENPs in aqueous media, role of their inherent physicochemical properties should be considered in pursuit to elucidate and predict their potential adverse effects on aquatic biota.

2.8.2 *Environmental factors*

2.8.2.1 Ionic strength

Ionic strength is a key determinant of dissolution and aggregation kinetics in environmental systems (French et al., 2009; Keller et al., 2010; Abbas et al., 2020b). High IS in aqueous media generally can result to increased aggregation of ENPs, and this in turn, further can induce reduced ζ-potential. In addition, divalent cations have higher IS relative to monovalent cations. For instance, owing to higher IS in aqueous with dominant divalent cations resulted in enhanced compression of the electrical double layer (EDL) may lead to a reduction in electrostatic repulsive force promoting inter-particle interaction, thus resulting to high aggregation and subsequent sedimentation (Domingos et al., 2010; Peng et al., 2017). The effect of high IS on aggregation ultimately influences the observed toxic effects of ENPs in question. For instance, Liu et al. (2015) investigated the influence of IS on the potential biotoxicity effects of n-TiO_2 towards *D. magna*. Results demonstrated that high IS mitigated against bioaccumulation of n-TiO_2 by 10–26 % linked to presence of Cu^{2+} as responsible for enhanced agglomeration and sedimentation.

2.8.2.2 pH

Several transformation processes governing the potential effects of ENPs are mainly influenced by the exposure media pH (Liu et al., 2011). pH is regarded as a key environmental factor that influences toxicity of n-Ag in aqueous media (Oukarroum et al., 2015). This is because pH can influence oxidative dissolution of n-Ag in aqueous media (Zhang et al., 2019a) with resultant higher toxicity. Other works have demonstrated that exposure media pH can affect aggregation kinetics (Lin et al., 2010), and dissolution rate of ENPs (Baalousha et al., 2008; Bian et al., 2011). For example, at pH 6.5 and pH 8, citrate coated n-Ag (size 20-100nm) was studied for acute toxicity on *D. magna* under acidic and basic conditions (Seitz et al., 2015). Results indicated a reduction in toxicity at higher pH, and larger size particles; hence, the observed toxicity was found to be pH dependent (Seitz et al., 2015).

2.8.2.3 NOM

The NOM consists of polydispersed mixtures of natural high molecular weight polymer macromolecules derived from the debris of organisms ubiquitous in the aquatic environments (Ren et al., 2016). Interactions of NOM with ENPs have been observed to alter the particles transformation processes including their fate, transportation, bioavailability and toxicity (Ansari et al., 2014; Wang et al., 2016c; Amde et al., 2017; Sharma et al., 2019b). Presence of NOM in aqueous media can stabilize ENPs, for instance, by coating their surfaces with excess charges and blocking their oxidative sites (Kittler et al., 2010), with concomitant dissolution by destabilizing agglomerates as described in section 2.5.1. It should be noted, however, the observed toxicity of ENPs can be either enhanced or reduced by NOM by increasing their accumulation and dispersion on organisms, or enhancing agglomeration, respectively (Pokhrel et al., 2013; Zhao et al., 2020). Further, NOM can scavenge free radicals, and also induce a reduction in bioaccumulation of ENPs on organisms (Gao et al., 2012; Zhang et al., 2019b). In summary, ENPs-NOM interactions have been reported to alter nanotoxicity in variant ways, including: (i) suspension stability of ENPs, (ii) electrostatic repulsion between NPs and organisms/cells, (iii) steric hindrance, (iv) generation of ROS, and (v) bioavailability of dissolved metal ions (Wang et al., 2016c).

Cupi et al. (2015) investigated the influence of Suwannee River NOM (20 mg/L) on the toxicity of n-Ag and n-ZnO towards *D. magna*. Results demonstrated that the presence of NOM can alleviate the toxicity of n-Ag to *D. magna*, but conversely, NOM stabilized n-ZnO without mitigating the resultant toxicity. Toxicity was reduced by the adsorption of the NOM onto the released Zn ions eliminating their bioavailability to the organism. In another work, however, Zhang and co-workers (2019a) observed enhanced cytotoxicity of n-Ag (30 nm) in the presence of HA (10 mg/L) to bacteria. The authors attributed the findings to increasing accumulation of Ag particles, or their ionic species on to bacteria. Generally, based on large body of published literature the presence of NOM in aqueous media reduces the toxicity of ENPs as opposed to enhancing their toxicity to aquatic taxa.

2.9 Pesticides

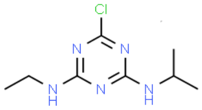
Pesticides covers a wide breadth of any substance or mixture of substances whose function is to prevent, destroy, repel or mitigate pests, or intended for use as plant regulators, etc. (USEPA, 2006; 2008a). Among the most common types and major categories of pesticides includes herbicides, insecticides, fungicides, and bactericides (Zhang et al., 2011; Meffe and de Bustamante, 2014). In South Africa, pesticides are among key pollutants in surface waters (Ansara-Ross et al., 2012) owing to their extensive use and applications including in agriculture, forestry, transportation (through control of flora and fauna), and various industries. As a result, pesticides have been classified by United Nations Environment Programme (UNEP) as one of major sources of Persistent Toxic Substances in the Sub-Saharan Africa (Burger and Nel, 2008). Owing to the numerous number of pesticides in each of the four main categories, in this work, the focus will be on herbicide atrazine (6-chloro-N2-ethyl-N4-isopropyl-1,3,5-triazine-2,4-diamine, ATZ) widely used in agriculture.

A herbicide is defined as any substance, individually or in a mixture whose function is to control, destroy, repel or mitigate the growth of weeds in a crop (Vasilescu and Medvedovici, 2005), and is regarded as one of the main agents of intoxication amongst the pesticides (Marin-Morales et al., 2013). Herbicides are reportedly among the most widely used chemicals globally. This is because they are highly effective for weeds control in agriculture, and in turn, with concomitant improved crop yields per unit area (He et al., 2012; Moreira et al., 2014; Kim et al., 2017; Lushchak et al., 2018).

ATZ is a pre- and post-emergence triazine herbicide widely used in agriculture to control broadleaf and grass weeds in crops production including maize, sorghum, sugarcane, pineapple, and orchards

(Solomon et al., 1996; Graymore, 2001; Kumar and Singh 2016; Zhao et al., 2017b). Further, ATZ is an odourless non-volatile, non-reactive, and non-flammable white crystalline powder. Among the key characteristics of ATZ includes moderate solubility in water, moderate octanol-water partition coefficient (log Kow), among other properties (Balci et al., 2009; PubChem, 2019) as listed in Table 2.5.

Table 2.5: Summary of key physicochemical properties of ATZ.

Chemical structure	Chemical formula	Molecular weight (g/mol)	Log K_{ow}	K_{oc} (cm^3/g)	Vapour Pressure (mPa) (20 °C)	Water solubility (mg/L) (20 °C)	Density (g/cm^3)	Water half-life
	$C_8H_{14}ClN_5$	215.68	2.7	100	0.039	35	1.23	75d

2.9.1 *Production and application of pesticides*

Advances in agricultural practices have also triggered excessive production and use of pesticides worldwide to feed burgeoning global population. This is chiefly to protect crops against pests, enhance both the crops' quality and quantity, optimize profits (Abhilash and Singh, 2009; Zhang et al., 2015; Devi et al., 2017), and contribute towards the global economy (Abate et al., 2000; Sharma et al., 2019a). For example, Hou et al. (2017c) reported that the annual production of ATZ at about 70000–90000 tons. Further, to produce adequate food to meet steepening demand occasioned by rapidly growing global population, use of pesticides associated with agricultural and industrial sectors are expected to be on increase into the foreseeable future. Miller and Spoolman (2009) projected the global population to reach 9.3 billion by 2050. As a consequence, this heightens the production, and concomitant application of pesticides to sustain food security to feed exponentially growing global population (Devi, 2009; Snyder et al., 2015).

About 2 billion tons per annum of pesticides are used globally, with herbicides accounting for over 40% (De et al., 2014; World Atlas, 2017), and estimated to increase to some 3.5 billion tons by

2020 (Zhang 2018) with South Africa and China amongst the main producers of ATZ (Burger and Nel, 2008; Zheng et al., 2017). Africa has the lowest global consumption of pesticides (Table 2.6) with China, USA, and Brazil among the top pesticides consuming countries (World Atlas, 2017; Zhang 2018; Sharma et al., 2019a). For example, ATZ were applied annually to crops in USA (30000–38000 tons), Brazil (24000–29000 tons), and China (2000–1807000 tons) (Table 2.6). In South Africa, on average the estimated usage is over 1 000 tons of ATZ for crop treatment by 2009 (Dabrowski, 2015a, b).

In addition, South Africa is amongst the top pesticides users and exporter in Africa (Dalvie et al., 2009; Agnews, 2016). It should be noted, however, that in South Africa actual production data of ATZ are lacking due to two-fold reasons. First, poor or lack thereof of declaration by users and registration by manufactures (Quinn et al., 2011; Dabrowski et al., 2014; Dabrowski, 2015a), and secondly, absence of publicly accessible data sources (Dabrowski et al., 2014; Dabrowski, 2015a). Further, ATZ is the second most widely used pesticide globally (Kumar et al., 2013; Cheng et al., 2016, and ranked among the top 25 pesticides in South Africa (Dabrowski et al., 2014; Dabrowski, 2015a, b). Other large users of ATZ includes USA, India, as well as many countries in South America, and Africa (Pascal-Lorber and Laurent, 2011; Ansara-Ross et al., 2012;FAO, 2017, Sighn et al 2018,). However, in the European Union has ATZ among its member states have been banned (Ackerman 2007; Hakim, 2015).

Table 2.6: Examples of annual consumption of ATZ globally.

Country	ATZ (tons/yr)	Reference
India	0.26–340	Kadian et al., 2008; Solomon et al., 2013a, b; Singh et al., 2018.
South Africa	1118.2	Dabrowski 2015a, b
Ethiopia	40	Teklu et al., 2015
Argentina	17637	De Gerónimo et al., 2014
China	2000–1807000	Zheng et al., 2017; FAO (2017)

Brazil	24.731–29 762.4	IBAMA, 2014; Brasil, 2019
USA	30000–38250	Land Stewardship Project, 2010; Pascal-Lorber and Laurent, 2011

2.9.2 *Occurrence of pesticides in aquatic systems*

Both intensive use of pesticides in agricultural and industrial practices are the key sources with resultant release into variant aquatic environments (Ansara-Ross et al., 2012; Xue et al., 2014; Glinski et al., 2018). Variant pathways and sources of pesticides includes runoff, agricultural or industrial effluents, drainage, municipal waste, manufacturing, and chemical spills (Solomon et al., 1996; Xue et al., 2014; Sun et al., 2017). This, in turn, has resulted to their high concentrations in the aquatic environment. This is because only a fraction of the pesticide applied to crops reaches the target organisms with the rest ending up into the environment.

To date, numerous studies have demonstrated that pesticides can induce deleterious effects to non-target aquatic taxa, and pollute surrounding environments e.g., water resources (He et al., 2012; Handford et al., 2015). Further, long-term harmful impacts of pesticides are not confined to aquatic life, but also may adversely cause severe human deleterious effects, and exhibits high persistence in variant environmental compartments (Dutta et al., 2016; Hartmann et al., 2018; Singh et al., 2018). As a result, pesticides have been found to severely impact on biodiversity (Grützmacher et al., 2008; Zhang et al., 2011; Kumar et al., 2013). For example, pesticides may interfere with species composition and diversity, nutrient cycling patterns, modify food chains, among other adverse impact. (Pérez et al., 2011; Lin et al., 2016). Consequently, this can give rise to far reaching implications including food insecurity, contamination of water resources, and extinctions of certain species at variant levels of biological organization.

ATZ is ubiquitous in the environment due to its high persistence and mobility (Solomon et al., 1996; Jablonowski et al., 2011; Dabrowski et al., 2014; Sun et al., 2017), and hence, is widely detected in surface and ground waters (Graymore et al., 2001; Baker et al., 2016; Zheng et al., 2017) from very low (ng/L) to very high (mg/L) concentrations (Table 2.7). Results of ATZ usage

(Table 2.6) showed close agreement with findings of detected concentrations with respect to countries of focus. As such, South Africa, USA, China, and Brazil as major users of ATZ (Table 2.6) were also found to collaborate the highest detected concentrations in freshwater systems (Table 2.7). For example, figures show that the highest environmental concentrations were detected in Brazil (250–15000 ng/L), and China (120–6285 ng/L) (Table 2.7). According to the World Health Organization (WHO), the maximum allowable concentration of ATZ is 100 µg/L and its metabolites in drinking water (WHO, 2017). In addition, 0.1 and 3 µg/L of ATZ is the permissible drinking water limit in USA and Europe, respectively (USEPA, 2009; Omotayo et al., 2011). In the case of South Africa, the allowed target water quality range (TWQR) is 0–2 µg/L for domestic use, and 10 µg/L in the aquatic systems (DWAF, 1996a, b).

Table 2.7: Measured environmental concentrations of ATZ in freshwater systems (surface water).

Country/Region	ATZ (ng/L)	References
China	183	Kong et al., 2015
USA	1650	Glinski et al., 2018
Canada	120	Byer et al., 2011
Hungary	500-15000	Székács et al., 2015
Spain	19	Pitarch et al., 2016
China	130	Yu et al., 2018
Brazil	7000-15000	Sousa et al., 2016
Brazil	250-9300	Nogueira et al., 2012
Brazil	2000-9950	Arraes et al., 2008
South Africa	390-9300	Du Preez et al., 2005
China	175-6285	Zheng et al., 2017
South Africa	780-3760	Burger and Nel, 2008
South Africa	< 25-230	Bollmohr et al., 2008
South Africa	< 250-1190	Meintjies et al., 2000
South Africa	< 5- 1570	Rimayi et al., 2018b
USA	150	Tagert et al., 2014

2.10 Toxicity of ATZ to aquatic organisms

To date, numerous studies have reported the effects of ATZ to aquatic biota at different levels of organization including cellular, molecular, and whole organisms. For example, ATZ was demonstrated to block the electron transport in photosystem II, and inhibits photosynthesis in plants cells (Allen et al., 1983). ATZ has also been observed to induce severe adverse effects to non-target organisms with arthropods as most vulnerable species (He et al., 2012). This implies, ATZ may effect aquatic taxa at lower trophic levels with adverse outcomes to whole body end-points including growth, survival, and reproduction; thus, inducing disruption to the sustainability of the ecosystems (Solomon et al., 1996; Graymore et al., 2001; Singh et al., 2018). Further, other works have reported ATZ as an endocrine disruptor (Kucka et al., 2012; Morales-Pérez et al., 2016) and carcinogenic (IARC, 1999; Rohr and McCoy, 2010; Mahler et al., 2017) pollutant.

Further, at molecular level, ATZ was shown to induce DNA damage, genotoxicity, mutagenicity, immunotoxicity (Hayes et al., 2002; Freitas and Rocha, 2012; Papoulias et al., 2014). For example, studies have demonstrated that ATZ has the potential to reduce cellular metabolism, and exerts influence on the formation of ROS with resultant alteration of antioxidant activity in *D. magna* (Schmidt et al., 2017; Stara et al., 2018). The potential toxicity effects of ATZ to aquatic taxa including crustaceans like daphnia have been widely reported (Palma et al., 2008; He et al., 2012; Moreira et al., 2014). Among daphnia species studied are *D. magna*, *D. carinata*, and *Ceriodaphnia silvestrii* have been investigated for acute toxicity as results in listed Table 2.8 show. Table 2.8 lists immobilization studies of ATZ, and derived EC_{50} values. Most studies used technical grade ATZ, with a few using analytical grade under variant exposure media conditions e.g., artificial (Moreira et al., 2014; Sengupta et al., 2015) and river water (Phyu et al., 2004; Wan et al., 2006). ATZ $L(E)C_{50s}$ are in the range of 9 – > 1 000 mg/L. Thus, according to the EU-Directive 93/67/EEC (CEC 1996) based on the reported $L(E)_{50}$ values, ATZ can be classified as harmful (10-100 mg/L), or not harmful (> 100 mg/L). For instance, Moreira et al. (2014) assessed the immobilization over 48h of technical grade ATZ to *D. magna* in artificial media at exposure concentrations of 2.25 –144 mg/L. An EC_{50} of 50.4 mg/L was obtained. In contrast, Klementová et al. (2019) studied the effects of HPLC grade ATZ towards *D. magna* exposed to 0.01–6.1 mg/L in artificial media. No mortality was observed because at environmentally relevant concentrations ATZ is known to induce no effects on aquatic organisms. Although ATZ is less toxic relative to

ENPs, it may be associated with long-term effects and high values detected in the environment, which in turn render it to be a pseudo-persistent pollutant. Thus, it is likely to pose health threats to the environment and humans. Herein, in this study, the focus is on the effects of ATZ at organism level with immobilization as the endpoint of focus.

2.11 Mixture toxicity of ENPs and other pollutants to aquatic organisms

As stated earlier, myriad contaminants including PCPs, pesticides, heavy metals, ENPs among others, are emitted into the environments concurrently and sequentially. This, in turn, leads to combined toxicity of chemical cocktails to the aquatic organisms arising from their co-occurrence. Hence, at present there is large body of scientific literature on risk assessment of both individual and chemical mixtures in pursuit to understand, and mitigate their possible environmental impacts (Han et al., 2012; Cupi et al., 2016; Heys et al., 2016; Park et al., 2017; Hou et al., 2018; Naasz et al., 2018; Li et al., 2020).

To date, studies on the risk assessment of compounds mostly focuses on individual chemicals, with only a few on chemical mixtures (Heys et al., 2016). The risks posed by chemical mixtures may not be predicted from single chemical studies. Thus, mixture toxicity information is necessary to avoid inaccurate estimation (under- or over-estimation) of actual hazards of pollutants to the aquatic organisms at different levels of biological organization. Effects of chemical pollutants in the environment have been extensively reviewed including ENPs (Segner et al., 2014; Heys et al., 2016; Naasz et al., 2018; Li et al., 2020), and therefore, will not be comprehensively covered herein, save the most salient points for the purposes of this research.

Table 2.8: Summary of acute toxicity effects of ATZ to Daphnia species under different exposure conditions.

Daphnia species/age	**Exposure media**	**Exposure concentrations (mg/L)**	**End point**	**Obtained EC/LC_x(mg/L)**	**Protocol**	**ATZ Type**	**Refs**
D. magna < 24h	Dilution water (EPA 2001)	0-10	Immobilization/Mortality 48h	$EC_{5,10,15,50}$: 126.3, 285.5, 389.2, 618.6	USEPA (2001)		[1]
D. magna < 24h	Aged tap water	0.1-6.1	Immobilization/Mortality 48h	No effects/mortality observed	OECD (2004)	ATZ HPLC quality	[2]
C. silvestrii	Reconstituted Water	2.25-72	Immobilization 48h	48h EC_{50}: 14.3	ABNT (2004)	ATZ Atanor 500SC (500g/l)-commercial	[3]
D. magna < 24h	Reconstituted Water	2.25-144	Immobilization 48h	48h EC_{50}: 50.4	ABNT (2004)	ATZ Atanor 500SC (500g/l)-commercial	
D. magna < 24h	ASTM Hard water	30-100	Immobilization 48h	48h EC_{50}: 35.5	ISO (1996)	ATZ 97.4%	[4]
			Immobilization/Mortality 48h	48h LC_{50}: 9.07	USEPA (2002)	ATZ 98.6%	
D. magna < 24h	MHW	0.5-9	Immobilization/Mortality 48h	48h LC_{50}: 9	USEPA (2002)	ATZ 98.9% purity in DMSO 99.7%	[5]
D. magna < 24h	Fresh well water		Immobilization 24h	24h LC_{50}: >250		ATZ 98% technical grade in acetone	[6]
D. magna < 24h	Fresh well water		Immobilization 48h	48h LC_{50}: 75		ATZ 98% technical grade in acetone	
D. magna < 24h	Fresh well water		Immobilization 24h	24h LC_{50}: >1000		ATZ500® (48.5%) +other triazines	
D. magna < 24h	Fresh well water		Immobilization 48h	48h LC_{50}: >1000		ATZ500® (48.5%) +other triazines	
D. carinata < 24h	Cladoceran water	(-25)	Immobilization 24h	48h EC_{50}: 24.6	ASTM (1992)	ATZ ≥ 97% purity-technical grade in acetone99% purity	[7]
D. carinata < 24h	River water	(3-25)	Immobilization 48h	48h EC_{50}: 22.4	ASTM (1992)	ATZ ≥ 97% purity-technical grade in acetone99% purity	
D. carinata < 24h	MHW	0-120	Mortality 48h	48h EC/LC_{50}: 60.6	OECD (2004)	ATZ 99.1%	[8]

References: [1] Han et al., 2012, [2] Klementová et al., 2019, [3] Moreira et al., 2014, [4] Palma et al., 2008, [5] Sengupta et al., 2015, [6] Wan et al., 2006, [7] Phyu et al., 2004, [8] He et al., 2012.

At present, studies have investigated the toxicity of ENPs/ENPs and ENPs/pesticides mixtures using different daphnia strains e.g., *D. magna*, *D. carinata* and *C. dubia* as test organisms (He et al., 2012; Simon et al., 2015; Molins-Delgado et al., 2016; Ye et al., 2018; Martín-de-Lucía et al., 2019). To date, the available published literature on ENPs-pesticides mixture toxicities using *D. magna* species are too few to aid our ability to draw definitive conclusions on their plausible implications to the ecological health. For illustrative purposes, the few accessible examples on the joint toxicity of ENPs and pesticides for binary mixtures on daphnia are presented in Table 2.9. The most mixtures studies are for carbon based ENPs and organics e.g., antibiotics. However, there are data deficits on ENPs/ENPs mixtures especially those of n-Ag and n-ZnO, yet both ENPs are among widely used in NEPS as described in section 2.3..

To our knowledge, based on the accessible literature, there is no study, which has investigated the potential joint toxicity effects of n-Ag and n-ZnO to *D. magna*. Previously, only few ENPs/ENPs mixture studies (Iswarya et al., 2016; Azevedo et al., 2017; Ye et al., 2018) have investigated immobilization and/or mortality of combined toxicity of n-Ag coated ZnO tetrapod nanostructure on *D. magna*. Results indicated synergism of the interaction between the two ENPs, and their induced effects were higher relative to the predicted mixture toxicity. Ye et al. (2018) investigated binary mixtures of n-ZnO (14 nm) and graphene oxide nanoplatelets (n-GO) (0.5-3 μm) on *D. magna* with immobilization as the endpoint over 48 h. The binary mixtures resulted in additive effects to *D. magna*. Antagonistic effects were reported from a study by Iswayra et al. (2016) investigating immobilization over 48 h for *C. dubia* under visible irradiation using n-TiO_2 with two types of crystalline phases: anatase (size < 25 nm) and rutile (size < 100 nm).

A significant number of binary mixture studies for ENPs/organics including pesticides (Baun et al., 2008b; Molins-Delgado et al., 2016; Martín-de-Lucía et al., 2019) have been reported. However, none has assessed the joint toxicity effects of ATZ with either n-Ag or n-ZnO. Such studies are essential for holistic risk assessment of ENPs in the aquatic environments. This is because, studies have demonstrated that presence of ENPs in a mixture may enhance (Han et al., 2012; Soler de la Vega et al., 2019), or reduce (Baun et al., 2008b; Simon et al., 2015; Molins-Delgado et al., 2016) the overall combined mixture toxicity. Using nTiO_2 and organic pollutants

(UV filters and parabens), Soler de la Vega et al. (2019) found ENPs to enhance the joint toxicity of the resultant mixtures. Binary mixtures of n-Ag and UV filters showed reduced joint effects to *D. magna* in artificial freshwater (Molins-Delgado et al., 2016). This means, at present it is not plausible to generalize the overall mixture toxicity especially where ENPs is one of the constituent component for a given mixture in question. To the author's knowledge, for example, there is no data either for binary (ATZ/ENPs), or ternary (ENPs/ENPs/ATZ) effects to daphnia. Hence, in pursuit to fill some of these data and knowledge deficits forms part of this work to investigate the potential effects thereof.

2.12 Mechanism of mixture toxicity

The presence of various environmental pollutants in aquatic systems leads to interactions with ENPs, and as consequence, alter the ENPs physicochemical properties (Wang et al., 2016c). Adsorption on ENPs is a critical physicochemical process at the ENPs-water interface. This is because ENPs often acts as carriers for other pollutants including organics and heavy metals owing to their small particle size, and larger active surface area (Wilke et al., 2016; Amde et al., 2017; Deng et al., 2017; Abbas et al., 2020b). Either ENPs can adsorb onto other surfaces, or other substances may adsorb onto ENPs surfaces (Hartmann et al., 2014). The interaction between ENPs and organic pollutants is a key controlling factor for their fate and transport in the aquatic environment. As a result, such interactions significantly influences their physicochemical transformation characteristics e.g., aggregation and dissolution (Hartmann et al., 2014; Zhao et al., 2014), and in turn, their observed toxicity on aquatic organisms (Zhao et al., 2014; Besha et al., 2019).

Adsorption occurs via van der Waals interaction, chelation, ligand exchange, bridging, and hydrogen bonding, and electrostatic mechanisms (Von Moos et al., 2014; Lata et al., 2016; Abbas et al., 2020b). ENPs such as n-ZnO are widely used to degrade environmental contaminants e.g., pesticides (Dehghani and Fadaei, 2012; Khan et al., 2020), and heavy metal ions (Shyni et al., 2016). For instance, adsorption modifies the properties and toxicity of the ENPs as well as the adsorbed pollutant (Zhao et al., 2014; Amde et al., 2017). This can affect organisms via a complex particle by means of the "Trojan horse" effect, which as a result, may increase their bioavailability and toxicity (Hartmann and Baun, 2010; Abbas et al., 2020b). Sorption behaviour primarily depends on various aspects e.g., ENPs properties, chemical properties, exposure media, and test organism (Naasz et al., 2018). Han et al. (2012), for an example, observed ATZ was adsorbed onto n-CeO_2. As a consequence, ATZ accumulation and toxicity in *D. magna* was enhanced by adsorption onto n-CeO_2, whereby the reproduction of daphnia was significantly reduced.

2.13 Concluding remarks

Herein, the production, fate, occurrence, effects of n-Ag, n-ZnO and ATZ as well as their toxicity mechanisms for individual and mixtures of ENPs and organic pollutants in the aquatic environment are outlined. Findings indicate numerous studies have investigated the individual effects of n-Ag, n-ZnO and ATZ to crustaceans especially daphnia in the aquatic media both in synthetic and natural exposure systems. Broadly, acute toxicity in the descending order for the three contaminants is, thus: n-Ag (very toxic) > nZnO (toxic) > ATZ (harmful/not harmful). Further, key research and knowledge gaps were identified regarding joint effects of ENPs and organic contaminants to important test organisms e.g., daphnia – an organism known to be sensitive to variant classes of contaminants.

From the review, the following propositions are made. First, elucidate the joint effects of ENPs, and their interactions with organic pollutants e.g., ATZ in aquatic environments need to be prioritized for risk assessment given the wide use of the macro-pollutant globally. This is because CECs may exhibit plausible unique modes of action, and in turn, render them exhibit different pathways affecting the aquatic organisms. Secondly, water chemistry and physicochemical parameters of ENPs should form an integral part of understanding their fate, behavior and toxicity mechanisms in aquatic systems as well as their toxicological outcomes. Thirdly, despite ENPs and ATZ being detected at very low concentrations in the aquatic systems e.g., less than their median affects concentration, however, following their interactions may induce joint effects following modification of individual constituent components toxicity mechanism(s). Hence, these aspects merit further investigations. Finally, because transformation processes play a key role in the ENPs toxicity outcomes, raises the need to pay attention in investigating environmentally relevant concentrations using actual environmental matrixes (e.g., river water, sea water, sediments, etc.). The data derived from such studies would aid to understand toxicological outcomes of these CECs reflective of actual aquatic environments. It is in this context, this work focuses on addressing partly some of the data and knowledge deficits identified with specific reference to joint effects of selected ENPs (n-Ag and n-ZnO), and organic pollutant (ATZ) to daphnia.

Table 2.9: Summary of mixture toxicity to Daphnia species under different exposure conditions.

Compounds	**Daphnia Species**	**Endpoints**	**Observations**	**Refs**
Ag/ZnO nanostructure	*D. magna*	Immobilization 48h	The observed induced toxicity was higher than predicted toxicity.	[1]
Functionalized Fullerenes (*f*C60), and bifenthrin and tribufos	*D. magna*	Survival 48h; Reproduction 70d bifenthrin and 21d tribufos; Growth rate 10d	*f*C60 Increased acute toxicity of the pesticides and no significant effect on chronic endpoints	[2]
n-TiO_2 and Copper (Cu)	*D. magna*	Bioaccumulation 3d	n-TiO_2 enhanced accumulation of Cu in *D. magna*.	[3]
Cu and n-TiO_2	*D. magna*	Biotoxicity	Cu induced higher levels of oxidative stress and physiological damage to *D. magna* in the presence of n-TiO_2.	[4]
n-TiO_2 and Cu	*D. magna*	Bioaccumulation 48h	n-TiO_2 decreased accumulation of Cu in *D. magna*	[5]
Permethrin, ʎ-cyhalothrin, and chlorpyrifos	*D. magna*	Environmental mesocosm long term effects	*D. magna* was the second most sensitive species to acute toxicity effects induced by the pesticides.	[6]
n-CeO_2, n-TiO_2, and Lead (Pb)	*C. dubia*	Uptake/Mortality 24h	ENPs enhanced overall toxicity of Pb	[7]
Butachlor and atrazine	*D. carinata*	Immobilization/mortality	No significant synergism	[8]
Multi-walled carbon nanotubes (MWCNT) and Cu	*D. magna*	Mortality/bioavailability	The bioavailability and toxicity of Cu to *D. magna* was influenced by the presence of NOM and MWNTs.	[9]
Citrate n-Ag and Arsenate (As(V)), Cadmium (Cd), Cu.	*D. magna*	Toxicity/bioaccumulation	Citrate n-Ag increased both toxicity and bioaccumulation of Cd to *D. magna*. No significant effect on the toxicity of As (V) and Cu and reduced their bioaccumulation.	[10]

Cu and Lysophosphatidylcholine single-walled carbon nanotubes (LPC-SWNT)	*D. magna*	Mortality 96h/uptake 48h	LPC-SWNT enhanced the uptake and toxicity of Cu with additive effects	[11]
Anatase and rutile n-TiO_2	*C. dubia*	Immobilization/mortality	Antagonism on *C. dubia* joint effects under visible irradiation, and additive effect under UV-A irradiation.	[12]
n-TiO_2 and As(V)	*D. magna*	Bioaccumulation/toxicity	n- TiO_2 increased the uptake of As (V), increased accumulation with increasing n-TiO_2 concentrations, and resulted in dual toxicity effects.	[13]
Carbon nanotubes (CNT) and Cd	*D. magna*	Mortality/bioaccumulation	CNT reduced the accumulation and the toxicity of Cd to *D. magna*.	[14]
TiO_2 and Cu^{2+}	*D. magna*	Bioaccumulation	Cu^{2+} enhanced agglomeration and reduced bioaccumulation of TiO_2, TiO_2 increased Cu Bioaccumulation	[15]
UV-filters and n-Ag	*D. magna*	Immobilization 48h	Reduced toxicity effects on UVFs/UVfs, AgNPs/4MBC, AgNPs/EHMC, and AgNPs/BP3 binary mixtures ; Increased toxicity on AgNps/BP1 and AgNps/EtPABA binary mixtures.	[16]
$AgNO_3$ and n-ZnO	*D. magna*	Immobilization 48h	Mixture toxicity resulted in synergism at higher Ag ions concentrations and antagonism at higher Zn concentrations	[17]
Hydroxyl functionalized MWCNT (OH-MWCNTs) and Pb	*D. magna*	Mortality 24h	OH-MWCNTs induced acute toxicity of Pb(II) to *D.magna*, joint effects resulted in synergism	[18]
n-TiO_2 and Heavy metals (Ag, As, Cu)	*D. magna*	Immobilization/uptake 72h	n-TiO_2 increased toxicity of Ag and decreased the toxicity of As and Cu.	[19
MWCNT and triclocarban (TCC)	*D. magna*	Population test/Survival 93d	MWCNT reduced the toxicity effects of TCC	[20]

Polyacrylate-coated n-TiO$_2$ (PAA-n-TiO$_2$) and Cd	*D. magna*	Bioaccumulation/uptake 48h, Toxicity 24h	PAA-n-TiO$_2$ adsorbed Cd and influenced its bioaccumulation by *D. magna*, ameliorated Cd toxicity and induced Cd uptake at high Ca concentrations.	[21]
n-C60 and Copper	*D. magna*	Bioaccumulation/mortality 48h	n-C60 lowered the LC$_{50}$ enhanced the 48 h-toxicity and accumulation of copper ions on *D. magna*.	[22]
Penta-BDE and n-TiO$_2$	*D. magna*	Immobilization	n-TiO$_2$ enhanced acute toxicity of Penta-BDE.	[23]
n-Al2O$_3$ and As (V)	*C. dubia*	Mortality 48h	n-Al2O$_3$ enhanced the toxic effect of As (V)	[24]
		Uptake 24h	The adsorption of As(V) on the nano-Al2O$_3$ surface induced the uptake and exposure to by *C. dubia*	
n-TiO$_2$ and As (V)	*C. dubia*	Mortality 48h	The toxicity of As (V) increased in the presence of n-TiO$_2$ even at low concentrations.	[25]
(OH-MWCNTs) and Nickel (Ni)	*D. magna*	Immobilization 48h	OH-MWCNTs increased the Ni toxicity depending on concentration and pH Ni adsorption onto the OH-MWCNTs and the uptake of OH-MWCNTs increased the exposure of *D. magna* to Ni.	[26]
CNTs and Cadmium	*D. magna*	Immobilization 24h	CNTs enhances the toxicity of Cd to *D. magna*	[27]
		Accumulation 24h	CNTs increased the Cd accumulation in *D. magna*.	
n-C60 and atrazine	*D. magna*	Reproduction 14d	n-C60 and atrazine joint effects reduces reproduction of *D. magna*	[28]
n-C60 and flouranthene	*D. magna*	Immobilization (photo-induced toxicity) 1d Ultra structural examination 1,10-21d	Phototoxicity and the effects of Flouranthene were reduced in the presence of n-C60	[29]

			Long-term exposure (21 d) of low-level n-C60 caused significant cellular damage in the *Daphnia magna* alimentary canal	
CNT (Functionalized and Non-functionalized) and metals (Cd/Zn)	*D. magna*	Bioaccumulation/ uptake	CNTs facilitated metal accumulation on *D. magna*; F-CNTs increase metal accumulation to daphnids; non-functionalized metal uptake by daphnia	[30]
Functionalized fullerenes (*f*C60) and metals (Cadmium and Zinc)	*D. magna*	Uptake/depuration	*f*C60 enhances metal accumulation by daphnia, and slower depuration of metals.	[31]
TiO_2 and Cu	*D. magna*	Immobilization 48h	Cu adsorbed onto n-TiO_2, toxicity of Cu reduced.	[32]
Graphite-diamond and thiabendazole	*D. magna*	Immobilization 48h	ENP/Fungicide mixtures resulted in synergism at low concentrations and antagonism at high nanoparticle concentration.	[33]
n-TiO_2 and UV filter and parabens (oxybenzone (BP3), methylparaben, propylparaben, benzylparaben (BzP))	*D. magna*	Immobilization 48h	n-TiO_2 increased the overall joint toxicity effects when combined the organic pollutants, antagonistic effects were observed for BP3+BzP binary mixture.	[34]
MWCNT, kaolinite , and Cd	*D. magna*	Mortality 48h/ Adsorption kinetics	Cd toxicity reduced by CNTs in binary mixtures and higher percentage mortality in ternary mixtures; Kaolinite negatively affected adsorption process of Cd to MWCNT influencing overall toxicity	[35]
$ZnSO_4$ and n-PVP-$Fe3O_4$	*D. magna*	Immobilization 48h	Synergistic effects were dependent on $ZnSO_4$ concentrations and high combination concentrations and PVP-Fe_3O_4 induced antagonistic effects	[36]
n-ZnO, n-TiO2, and Triton X-100 (Surfactant)	*D. magna*	Immobilization 48h	The presence of the surfactant increased the toxicity of the ENPs	[37]

n-ZnO, n - TiO_2 and Ag^+	*D. magna*	Immobilization 48h	Ag+ induced synergistic effects in the mixtures with the metal oxides	[38]
Atrazine n-CeO_2	*D. magna*	Accumulation/Reproduction 15d	Atrazine accumulation in *D. magna* increased in the presence of CeO_2/ significant reduction in *D. magna* reproduction due to atrazine/ CeO_2 co-exposure.	[39]
n-TiO_2 and Cd	*D. magna*	Immobilization/uptake	n-TiO_2 facilitated the increased uptake of Cd by *D. magna*; no significant toxicity effects	[40]
Imidacloprid, thiacloprid, nickel chloride, and chlorpyrifos	*D. magna*			[41]
Imidacloprid/thiacloprid		Immobilization 48h	Synergistic joint effects	
		Feeding inhibition 24h	Antagonistic joint effects	
Imidacloprid/chlorpyrifos		Immobilization 48h	Antagonistic joint effects	
		Feeding inhibition 24h	Antagonistic joint effects	
Nickel chloride/chlorpyrifos		Immobilization 48h	Synergistic joint effects	
		Feeding inhibition 24h	Antagonistic joint effects	
Imidacloprid, thiacloprid, nickel chloride	*D. magna*	Reproduction/Survival 21	Synergistic joint effects	[42]
Imidacloprid/thiacloprid		Body length Reproduction/Survival 21	Synergistic behaviour observed in reproduction test, body length best estimated with CA model	
Imidacloprid/nickel chloride		Body length	Synergism observed in low doses, antagonism observed in high doses	

UV-filters (ethylhexyl methoxycinnamate, octocrylene, and avobenzone)	*D. magna*	Immobilization 48h	Predicted mixture toxicity was higher than the observed toxicity.	[43]
n-ZnO and graphene oxide nanoplatelets (n-GO)	*D. magna*	Immobilization 48h	Binary mixtures resulted in additive effects to *D.magna*.	[44]
Atrazine, triclosan and DHA	*D. magna*	Immobilization/Mortality - 48 h	Atrazine reduced the acute toxicity of triclosan and DHA to *D. magna*	[45]
Atrazine, triclosan, parathion, endosulfan, and p-nonylphenol.	*D. magna*	Immobilization/Mortality - 48 h	Atrazine decreased toxicity of triclosan in the mixture, increased the toxicity of parathion, endosulfan, and p-nonylphenol.	[46]
n-C60 and xenobiotic organic compounds. • C60/phenanthrene • C60/pentachloropenol (PCP) • C60/methyl parathion • C60/atrazine	*D. magna*	Immobilization/Mortality - 48 h Bioaccumulation	Decreased toxicity effects of phenanthrene and PCP in the presence of n-C60; no significant toxicity effect difference of methyl parathion and atrazine in the presence of n-C60 n-C60 increased phenanthrene uptake by *D. magna*	[47]

References: [1] Azevedo et al., 2017, [2] Brausch et al., 2010, [3] Fan et al., 2011, [4] Fan et al., 2012, [5] Fan et al., 2016, [6] Hasenbein et al., 2016, [7] Hu et al., 2012, [8] He et al., 2012, [9] Kim et al., 2009, [10] Kim et al., 2016, [11] Kim et al., 2010, [12] Iswarya et al., 2016, [13] Li et al., 2016, [14] Liu and Wang (2015), [15] Liu et al., 2015, [16] Molins-Delgado et al., 2016, [17] Baek et al., 2020, [18] Qin et al., 2014, [19] Rosenfeldt et al., 2014, [20] Simon et al., 2015, [21] Tan et al., 2017, [22] Tao et al., 2013, [23] Tian et al., 2014, [24] Wang et al., 2011a, [25] Wang et al., 2011b, [26] Wang et al., 2014, [27] Wang et al,. 2016b, [28] Yan et al., 2010, [29] Yang et al., 2010, [30] Yu and Wang (2013), [31] Yu and Wang (2014), [32] Rosenfeldt et al., 2015, [33] Martín-de-Lucía et al., 2019, [34] Soler de la Vega et al., 2019, [35] Lee et al., 2019, [36] Park et al., 2019b, [37] Renzi and Blašković, 2019, [38] Park et al., 2019a, [39] Han et al., 2012, [40] Hartman et al., 2012, [41] Loureiro et al., 2010, [42] Pavlaki et al., 2011, [43] Park et al., 2017, [44] Ye et al., 2018, [45] Sengupta et al., 2015, [46] Schmidt et al., 2017, [47] Baun et al., 2008b.

Chapter 3: Materials and methods

3.1 Test chemicals

Commercial n-Ag (40 nm, sodium citrate stabilized, 0.02 mg/mL dispersion in aqueous buffer, CAS No. 7440-22-4), and n-ZnO (< 100 nm, 20 % dispersion in H_2O, CAS No. 1314-13-2) suspensions were purchased from Sigma-Aldrich (Johannesburg, South Africa). ATZ purity > 99% (CAS No. 1912-24-9) was obtained from Industrial Analytical (Johannesburg, South Africa). Dimethyl sulfoxide (DMSO) with purity > 99% was sourced from Merck (Johannesburg, South Africa), and used as a carrier solvent for studies entailing ATZ (Barbosa et al., 2003). n-Ag and n-ZnO stock suspensions were prepared in 100 ml volumetric flasks using Milli-Q water, and thereafter sonicated for 15 min at 20 ºC. Exposure solutions were prepared by diluting ENPs stock solutions in ISO medium (described in section 3.2) prior to exposure experiments on *D. magna*. A stock solution of ATZ was prepared by, first, by dissolving ATZ powder in DMSO, and then diluted to 100 mg/L in Milli-Q water. Finally, the solution was sonicated for 30–45 min at 20 ºC, and stored in the dark at 4 °C prior to use.

3.2 Exposure media preparation

The composition and physico-chemical characteristics of the ISO media are listed in Table 3.1. The media was prepared following ISO 6341 guideline (ISO, 2012). The ISO media was used both as a control, and dilution media to prepare the working ENPs and ATZ dispersions. Among water chemistry parameters determined included pH, temperature, dissolved oxygen (DO), and electrical conductivity (EC) in the test media prior to test exposures as well as after 24 and 48 h. All values for the measured parameters were within the prescribed ranges as shown in Table 3.1.

3.3 Test organism

Daphtoxkit F^{TM} (MicroBioTest Inc., Gent, Belgium; DM427; DM15061) was purchased from ToxSolutions (Johannesburg, South Africa). Ephippia of *D. magna* were hatched at room temperature (20–22 °C) under 6000 lux continuous light illumination for 72 h prior to testing. All preparations were done following the manufacturer’s instructions. After eggs development, neonates (< 24 h) were fed with spirulina 2 h prior to the toxicity testing in accordance to the Daphtoxkit F magna standard operating procedure.

3.4 Characterization of ENPs

The ENPs size and morphology were characterised using High-Resolution Transmission Electron Microscope (HRTEM; JEOL JEM 2100, Japan operating at 200 kV). ENPs suspensions were prepared using Milli-Q (15 MΩ/cm), thereafter were then vortexed for 60s, dipped in carbon coated copper grid, and then finally dried overnight at room temperature (*ca* 25°C) before analysis. ImageJ software (National Institutes of Health, USA) was used to determine particle size diameter from several TEM micrographs using at least 100 ENPs. ENPs hydrodynamic diameters (HDD) and ζ-potential in ISO media were characterized using dynamic light scattering (DLS) on a Malvern Zetasizer Nano series instrument (Model ZEN 3600; Malvern Instruments, UK). The highest and lowest concentrations of the exposure suspensions were used to characterize HDD and ζ-potential. These parameters were measured both for single ENPs, and the resultant mixtures (binary and ternary) as described in Section 3.5.1. All analysis was done in triplicates, with measurements done three times per replicate post sonication at 0, 24, and 48 h for each exposure suspension.

Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) was utilized to determine the dissolved ionic species of ENPs in ISO media. Samples were prepared by filtrating the suspensions using Amicon ultra-15 centrifugal filters (MWCO 3 kDa; Merck Millipore, Darmstadt, Germany), and the suspensions were thereafter centrifuged for 1 h at 4000 rpm (Eppendorf 5810 R, Eppendorf, Germany). Further, the supernatant was collected and preserved with 5% nitric acid (HNO_3). Only the highest concentrations used for toxicity exposure studies were analysed for dissolution (exposure concentrations are discussed in Section 3.5). Suspension concentrations used for the dissolution studies are listed in Table 3.2. Samples were prepared at 24h and 48h, and kept at -80 °C until analysis. Samples were then analysed in triplicates to determine the total dissolved Ag and Zn using the ICP-MS (ICPE-9820, Shimadzu, Japan).

Table 3.1: Composition and physicochemical characteristics of ISO medium (OECD, 2004).

Component	**MW**	**mg/L**	**Mg^{2+}**	**Ca^{2+}**	**Cl^{-}**	**HCO^{3-}**	**$SO4^{2-}$**	**Na^{+}**	**K^{+}**	**mmol/L**
CaCl2.2H2O	147.01	294	-	80.15	141.8	-	-	-	-	2
MgSO4.7H2O	246.47	123.25	12.15	-	-	-	48.04		-	0.5
NaHCO3	84.01	64.75--	-	-	-	47.03	-	17.72	-	0.77
KCL	74.55	5.75			2.73				3.02	0.077
Totals, mg/L			12.15	80.15	144.53	47.03	48.04	17.72	3.02	
mmol/L			0.5	2	4.08	0.771	0.5	0.771	0.077	
Physico-chemical properties										
Media temperature		20-22 °C								
Dissolved oxygen		6.8-8.0 mg/L								
pH		7.2-7.8								
IS		12.5 mM								

Reference: OECD, 2004.-Adapted from Skeaff et al., 2011.

Table 3.2: Nominal concentrations for Aggregation and dissolution kinetics of individual and ENPs mixtures.

	Nominal concentrations for aggregation (mg/L)	**Nominal concentrations for dissolution (mg/L)**
Single ENPs		
n-Ag	0.031; 0.5	0.5
n-ZnO	0.4; 40	0.4
Mixtures		
n-Ag + n-ZnO	0.24; 3.91	0.07 Ag ; 3.84 Zn
n-Ag + ATZ	3.56; 57.04	0.07 Ag
n-ZnO + ATZ	3.80; 60.81	3.84 Zn
n-Ag + n-ZnO + ATZ	3.81; 60.88	0.07 Ag ; 3.84 Zn

3.5 Acute toxicity studies

3.5.1 *Outline of exposure studies*

Single and mixture studies were conducted following the ISO Guidelines 6341 (ISO, 2012). ISO media was used as the exposure media as described in section 3.2. All toxicity experiments were performed in four replicates each with five neonates (< 24 h old) in the exposure solutions, and control media (without ENPs or ATZ) using a six well test plates (Cellstar®, Greiner Bio-one, Germany) containing 10 mL of each exposure solution. Each test plate was covered and incubated at 20 ± 2 °C in the dark. After 24 h and 48 h, immobile and dead neonates were recorded. Neonates unable to swim or move after 15 sec following gentle shaking of the beaker were considered immobile, even if they could still move their antenna. In addition, daphnids unable to move appendages and antennas were considered dead.

Tests were considered valid if mortality or immobility in the controls was ≤ 10 %. Each test was repeated three times for the purposes of results reproducibility. DMSO solution was used as a solvent control for studies performed entailing ATZ test exposures. The maximum concentrations of DMSO used in the test solutions did not exceed 0.01%. Exposures with serial dilutions in the range from 0.06 to 1 mg/L of DMSO were tested. Notably, at this range of DMSO concentrations were observed to induce no observable toxic effects to daphnia. A reference toxicant toxicity test was also run in parallel using potassium dichromate ($K_2Cr_2O_7$) per batch of daphnids to monitor their health and sensitivity. Daphnia was exposed to concentrations in the range of 0.32–3.2 mg/L $K_2Cr_2O_7$ over 48h, and the calculated EC_{50} values were all within the quality control thresholds of 0.6–2.1 and 0.94 mg/L at 24 h and 48 h EC_{50}, respectively.

3.5.2 *Acute toxicity for single chemicals*

Acute immobilization test for single chemicals was conducted according to ISO guideline 6341 (ISO, 2012) as explained in Section 3.5.1. Exposure concentrations for n-ZnO and ATZ were determined following the Daphtoxkit F^{TM} definitive test protocol for chemicals (MicroBioTest Inc, Gent, Belgium). The values in parenthesis represents the nominal concentrations used (in mg/L) were 0.031, 0.063, 0.125, 0.25, 0.5 for n-Ag; 0.4, 1.2, 4, 12.8, 40 for n-ZnO; and 5, 9, 16, 28, 50, 100 for ATZ. After 24 h and 48 h exposures, immobilized neonates were recorded.

3.5.3 *Acute toxicity for mixture chemicals*

Binary and ternary mixtures were conducted in accordance to ISO 6342 (ISO, 2012) similar to experiments carried out for the single chemicals (Section 3.5.2). Concentrations for the mixture exposures were determined following the CA Model expression defined in Equation 3.1. The model was used to estimate fractions of each component (*pi*) in the mixture based on the EC_{50} derived from single chemicals exposures (Table 3.3). After 24 h and 48 h exposure, immobilized neonates were recoded. The median effective concentration (EC_{50}) values and the 95% confidence intervals (CI) of individual ENPs and ATZ including their combined exposures. All calculations were carried using probit and Spearman-Karber method for the ENPs and ATZ, respectively. To evaluate the combined effect of the binary and ternary mixtures of the ENPs and ATZ, the evaluations were done at different concentrations by predicting EC_x for the mixtures using the expression:

$$ECx_{mix} = \sum_{i=1}^{n} \left(\frac{pi}{ECx_i}\right)^{-1} \tag{3.1}$$

where ECx_{mix} is the predicted toxic effect of the mixture, *pi* is the fraction of component *i* in the mixture, and ECx_i is the individual effect concentrations when applied individually.

Further, to determine the interactions of constituent component chemicals in binary and ternary mixture systems, toxic units (TU) were calculated following the method by Altenburger et al. (2003). Briefly, the relative contributions of mixture components to the joint toxicity can be added when expressed as TU. The toxic units of the mixture (TUmix) were calculated using the expression:

$$\mathrm{TU_{mix}} = \sum_{i=1}^{n} \frac{C_i}{EC_{x0i}} \tag{3.2}$$

where C_i is the concentration of the chemical compound *i* in the mixture with *n* chemicals, and EC_{50i} is the effect concentration for the component compound *i* that result in the same effect (x%) as the mixture. The mixture interactions were classified as formalized by Broderius et al. (1995), thus: TU < 0.8 indicates synergism; TU > 1.2 signifies antagonism; and 1.2 > TU > 0.8 represents simple addition.

Table 3.3: Nominal test concentrations for mixture exposure tests on *D. magna*.

Binary and ternary mixtures	**Fraction of component (*pi*) for each chemical in the mixtures**			**Nominal concentrations for mixtures (mg/L)**				
	n-Ag	n-ZnO	ATZ					
n-Ag + n-ZnO	0.017	0.983	-	0.24	0.49	0.98	1.96	3.91
n-Ag + ATZ	0.001	-	0.999	3.56	7.13	14.26	28.52	57.04
n-ZnO + ATZ	-	0.063	0.937	3.80	7.60	15.20	30.41	60.81
n-Ag + n-ZnO + ATZ	0.001	0.063	0.936	3.81	7.61	15.22	30.44	60.88

(-) did not contribute

3.6 Statistical analysis

Data herein are presented as means (n = 3) ± standard deviations (SD). Statistical differences between exposures treatments were evaluated by two-way analysis of variance (ANOVA) followed by *post hoc* Tukey's multiple comparison tests using GraphPad Prism V9 software (GraphPad Prism software, La Jolla, CA, USA). Differences were considered statistically significant when $p < 0.05$.

Chapter 4: Results and discussion

4.1 Characterization of ENPs

Results for the ENPs primary size and morphologies are shown in Figure 4.1. n-Ag had spherical shapes with an average size of 40 ± 12 nm (40 nm). n-ZnO exhibited a mixture of hexagonal-, pentagonal-, rod- and spherical-like shapes owing to the asymmetry of the morphology with larger diameters in the range of 60–136 nm (< 100 nm). The results herein are in agreement with the manufacturer's specifications for both ENPs. Values in the parentheses are the manufacturers' specified sizes.

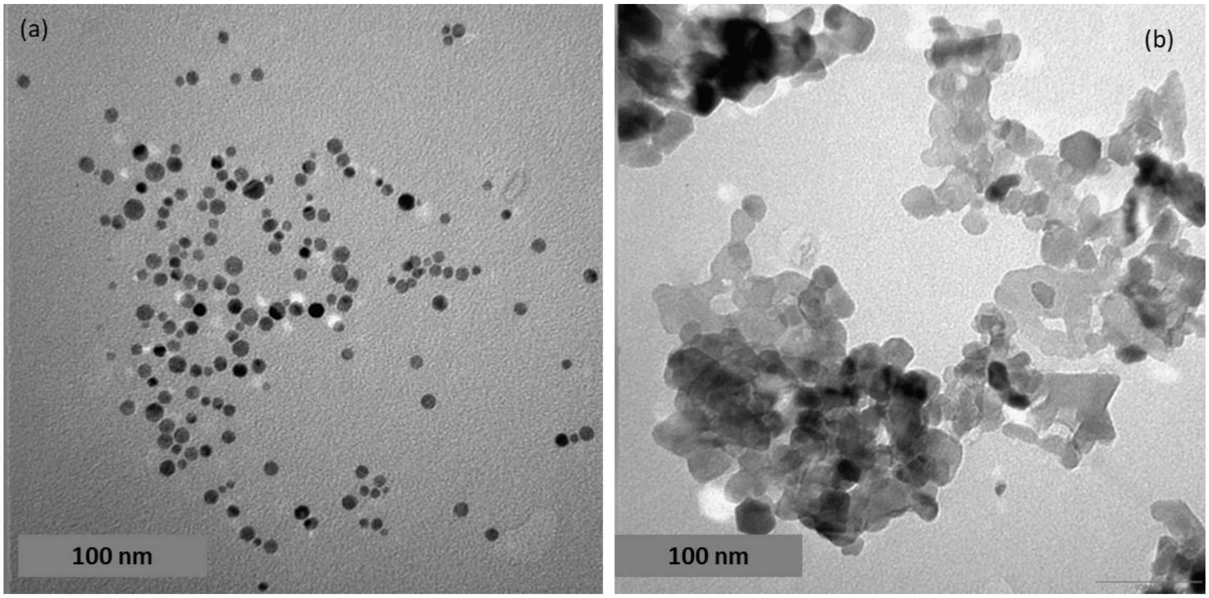


Figure 4.1: TEM images of (a) n-Ag, and (b) n-ZnO as measured in pristine state and Milli-Q water prior to exposures, respectively.

Zetasizer results for HDD and ζ-potential for the ENPs in ISO exposure media are listed in Table 4.1. HDD of n-Ag at high concentration increased with time in ISO exposure media from 133–151 nm, and consistent with similar trends previously observed in earlier works (Zhao and Wang, 2012; Park et al 2014; Sakka et al., 2016; Hu et al., 2018). Results showed that HDD at low n-Ag concentration increased over the first 24 h to 674 nm; and thereafter, a slight reduction was observed to 486 nm after 48 h. Sakka and colleagues (Sakka et al., 2016) observed a decrease in HDD of sterically stabilized n-Ag after 48 h. This was due to the sedimentation of larger agglomerates, which in turn, led to the exclusion of larger particles in the obtained measurements.

Further, the HDD of n-Ag in the higher concentration (0.5 mg/L) was relatively lower than at the low concentration of 0.03 mg/L. This is likely owing to the settling of larger particles through the sedimentation process caused by the complexation of carboxyl groups of the n-Ag coating with Ca^{2+} present in the ISO media (El Badaway et al., 2010; Peng et al., 2017). And as a result, plausibly sedimentation accounts for the detection of lower sized-particles at the higher exposure concentration. Mackevica et al. (2015) and Cupi et al. (2016) also observed similar trends in their aggregation studies for n-Ag. The increased agglomeration of n-Ag was largely due to the high IS (12.5 mM) of the ISO media (Delay et al., 2011), and the presence of divalent ions e.g., Ca^{2+} and Mg^{2+} in the ISO media (Blinova et al., 2013) that may reduce the EDL. Similar observations have also been highlighted in earlier works linked, for example, to high concentration of electrolytes like Ca^{2+} (Domingos et al., 2009; El Badaway et al., 2010; Mackevica et al., 2015; Cupi et al., 2016) as is the case in this study.

Negatively charged low ζ-potential was observed that spanned over narrow range during the exposure period irrespective of exposure concentration. For instance, results of ζ-potential at n-Ag concentrations of 0.03 and 0.5 mg/L were -11.8 to -9.5 mV, and -5.5 to -7.4 mV, respectively. Hence, ζ-potential was not concentration dependent. Electrostatic stability of ENPs is attained at higher ζ-potential of ± 30 mV (Hitchman et al., 2013; Cupi et al., 2015). Therefore, the high observed aggregation of the n-Ag was associated with low ζ-potential (< ± 30 mV), and hence, being indicative of unstable suspensions.

Aggregation kinetics of n-ZnO was characterized by an increase in size during the first 24 h, and a narrow drop after 48h irrespective of exposure concentration (low or high). Moreover, ζ-potential of nZnO at low concentration of 0.4 mg/L (– 10 to – 8.5 mV) decreased after every 24 h. Conversely, at the highest exposure concentration of 40 mg/L, the ζ-potential increased from negatively to positively charged after 48h (– 6.27 to + 3.4 mV). Similar to n-Ag, the high agglomeration of nZnO was attributed to low ζ-potential; thus, signifying the instability of the particles in the ISO media. Kim et al. (2017), for instance, reported highly fluctuating HDD of n-ZnO. However, the authors did not account for the underpinning drivers, and the aggregation trends were reported to be independent of concentration, or exposure time.

The average HDD of n-ZnO was larger relative to that of n-Ag plausibly due to two-fold reasons. First, owing to likely hydroxylation precipitation of n-ZnO in aqueous media (Bian et al., 2011), and secondly, as a result of fairly high exposure concentrations used in this study. The high exposure concentrations may have in turn, enhanced particle interaction and collisions in the exposure media (Keller et al., 2010; Kennedy et al., 2010; Cupi et al., 2016). Overall, aggregation kinetics of both ENPs were mainly influenced by the media chemistry primarily the high IS of the ISO media (Cupi et al., 2016; Peng et al., 2017), and the presence of divalent cations e.g., Ca^{2+} and Mg^{2+} in the exposure media. Numerous findings have demonstrated that divalent cations play a key role in the formation of large-sized ENPs aggregates in aqueous media (Zhang et al., 2009; El badaway 2010; Baalousha et al., 2013; Wang et al., 2016a).

Table 4.1: Summary of HDD and ζ-potential results of n-Ag and n-ZnO in ISO media.

Component	**Concentration (mg/L)**	**HDD (nm)**			**ζ-potential (mV)**		
		0	**24**	**48**	**0**	**24**	**48**
Control	0.0	640 ± 15	866 ± 122	708 ± 188	-11.3 ± 1.7	-11.4 ± 2.0	-27.8 ± 2.1
n-Ag	0.031	410 ± 105	674 ± 131	486 ± 170	-11.8 ± 2.9	-10.6 ± 1.2	-9.5 ± 1.7
	0.5	133 ± 23	135 ± 16	151 ± 20	-5.5 ± 0.6	-7.0 ± 0.7	-7.4 ± 0.4
n-ZnO	0.4	937±181	1074 ± 440	1047 ± 308	-10.0 ± 0.5	-8.6 ± 0.5	-8.5 ± 0.6
	40	2158 ±132	2931±161	2095 ± 66	-6.3 ± 2.0	1.5 ± 1.0	3.4 ± 0.6

Figures 4.2 (a and b) summarizes the aggregation kinetics of ENPs binary mixtures (i.e. n-Ag and n-ZnO). Results indicate that HDD increased throughout at the lower concentration (528–616 nm), but decreased at higher concentration (807 nm to 610 nm) over 48 h. Further, both concentrations exhibited low negatively charged ζ-potential values, which decreased slightly with increase over exposure time (Figure 4.2b). The trend exhibited by the n-Ag/n-ZnO binary mixture resembled that of individual ENPs i.e. formation of aggregates was influenced by the media chemistry, and in turn, led to the observed their instability as evidenced by low ζ-potentials.

Additionally, HDD and ζ-potential of ENPs was also determined in the presence of ATZ as shown in Figure 4.2 (c–h). First, the binary mixture results of n-Ag and ATZ showed a reduction in HDD over exposure time at low concentration from 383 to 310 nm. However, at higher exposure concentration, HDD increased over exposure time from 698 to 874 nm (Figure 4.2c). Moreover, similarly to the ENPs/ENPs mixture, negatively charged ζ-potential values were observed for both concentrations (Figure 4.2d).

Secondly, aggregation kinetics of n-ZnO and ATZ binary mixtures were also assessed, and the results are summarized in Figures 4.2 (e and f). The ζ-potential was low and negatively charged at both exposure concentrations (3.8 and 60.81 mg/L). The ζ-potential decreased more at the low concentration of 3.80 mg/L (-10.3 – -4.6 mV) over exposure time, relative to the higher concentration of 60.81 mg/L (-16.7 – -15.5 mV) (Figure 4.2 f). Although the variability of ζ-potential irrespective of the exposure concentration were not significant, but slightly higher increase in HDD (923-1383 nm) at low exposure concentration may be linked to higher reduction in ζ-potential relatively to the change at higher concentration (1497–1584 nm) (Figure 4.2 e).

For the ternary mixture of n-Ag, n-ZnO, and ATZ, results of HDD and ζ-potential are shown in Figure 4.3 (g and h), respectively. Results indicate the formation of larger aggregates in all concentrations largely influenced by the presence of the organic compound. The interaction of the ENPs and ATZ led to heteroaggregation process in the mixture. The observed HDDs were relatively high in particular considering the concentrations used for all the contaminants. Figure 4.2 g illustrates that the HDD values fluctuated with time at the high concentration and decreased with exposure time in the low concentration (1512–1353 nm). All observed ζ-potentials were negative throughout the exposure period (Figure 4.2h).

Overall, aggregation kinetics of ENPs in binary and ternary mixtures was largely influenced by the composition of the ISO media similar to the trends observed for individual ENPs. Formation of aggregates was observed to immediate following the introduction of the ENPs in the ISO media with or without the presence of ATZ. The ENPs were characterized by instability throughout as indicated by the low ζ-potential values and the large agglomerates; hence resulted to larger HDDs.

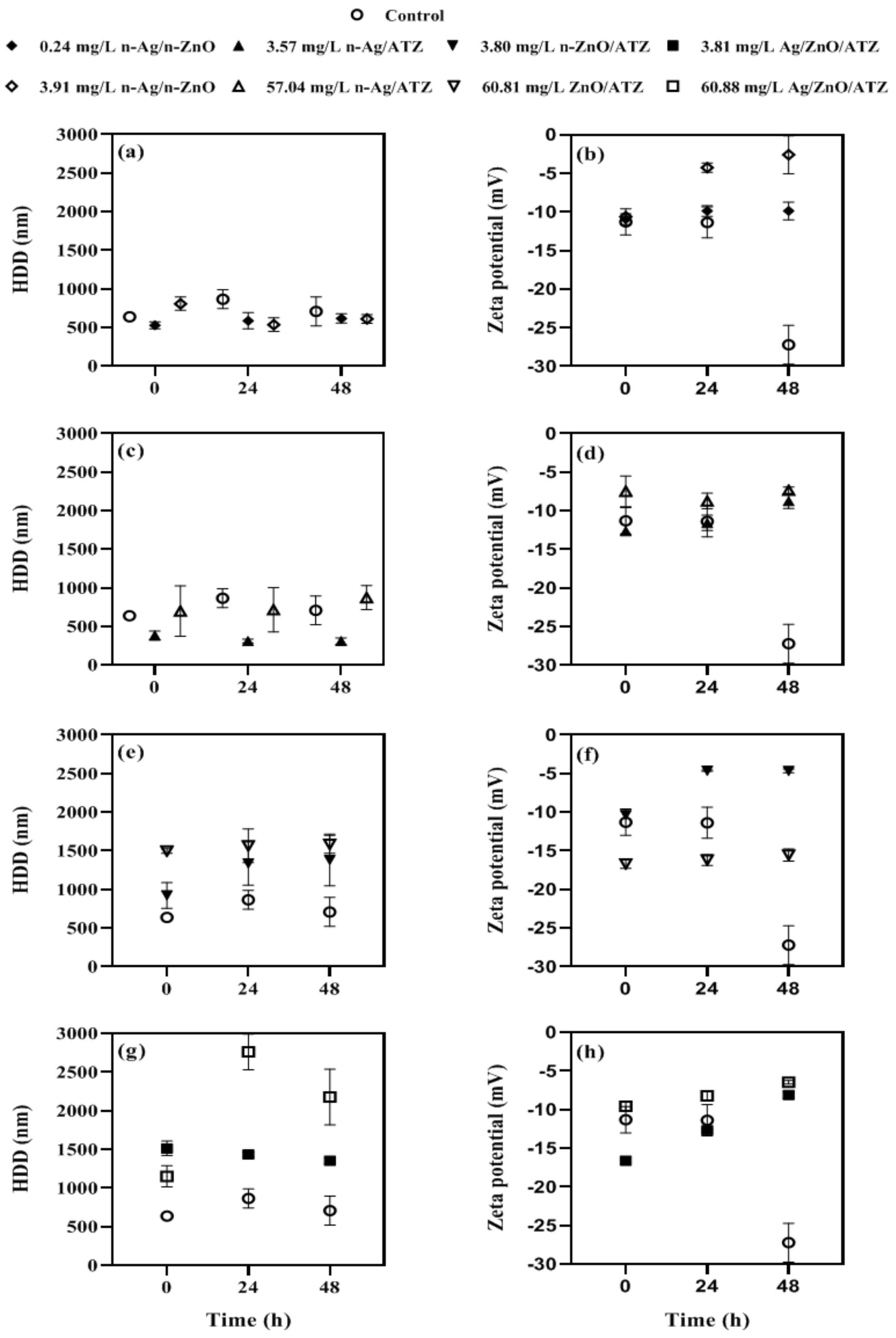


Figure 4.2: HDD and ζ-potential of binary and ternary mixtures of (a, b) n-Ag/n-ZnO; (c, d) n-Ag/ATZ; (e, f) n-ZnO/ATZ; and (g, h) n-Ag/n-ZnO/ATZ in ISO Media. ISO media without ENPs or ATZ was used as a control.

4.2 Dissolution of ENPs

4.2.1 *Dissolution of individual ENPs*

The ICP-MS analytical results for ENPs in ISO media are summarized in Figure 4.3. As mentioned earlier, results herein only reports the dissolution of ENPs only at the highest exposure concentrations linked to their observed induction of toxicity to *D. magna*. At a nominal concentration of 0.5 mg/L of n-Ag, measured dissolved silver ions were 54 and 63 μg/L at 24 h and 48 h, respectively (Figure 4.3a). Thus, the observed data herein amounts to *ca* 11 and 13 % Ag^+ of the initial nominal n-Ag concentration. Further, dissolution of n-Ag in ISO media appeared to increase with exposure time. This trend was inconsistent with earlier reported works (Sakka et al., 2016; Mertens et al., 2019).

It should be noted that the high exposure concentration used in this study may have had a direct influence on the final dissolution results. However, studies have also reported the dissolution of n-Ag to be concentration dependant (Hu et al., 2018) with the observed dissolution increasing with exposure concentrations in the synthetic media. For example, in M4 EPA media dissolution increased as exposure concentrations increased (Cupi et al., 2016; Park et al., 2014) as it has similar composition to ISO media used in this study. Park et al. (2014) analysed the dissolution of fractionated n-Ag at concentrations of 13.1 and 29 μg/L in MHW. The authors observed higher dissolution at higher concentration of 29 μg/L (2%) relative to 13.1 μg/L (1.5%). Cupi and colleagues (Cupi et al., 2016) observed higher dissolution of 25% at 100 μg/L, and 6% at 20 μg/L. Therefore, our results herein are in good agreement with earlier works as pertains to the dissolution of n-Ag, and the high aggregation rate observed in Section 4.1 may be among the driver of the observed dissolution rate. And, percentage dissolution values in this study are within ranges reported elsewhere of 1–25 % (Lee et al., 2012; Cupi et al., 2016; Kim et al., 2016; Hu et al., 2018).

The dissolution of n-Ag in this study was chiefly influenced by the ENP transformation processes e.g., the aggregation kinetics. This observed behaviour was chiefly attributed to ENP surface charge (low ζ-potential), and the media composition i.e. high IS content which regulated the aggregation rate by causing electrostatic destabilization (El Badaway 2010; Peng et al., 2017). Previously, high IS and electrolytes concentration as well as nutrient (Na, K) content of the ISO media were reported to cause n-Ag to form complex with anions (Cl^-); and in turn, leading to the sedimentation of larger agglomerates (Unrine et al., 2012; Yu et al., 2013).

Results in Figure 4.3b demonstrates the dissolution of n-ZnO at the highest exposure concentration of 40 mg/L. Seven and six per cent of the dissolved n-ZnO was observed, respectively, after 24 h and 48 h. The slight drop on dissolution between these exposure times were attributed to the observed increase in agglomeration (based on HDD results in Section 4.1) over the same period. Similar observations were reported by Gonçalves et al. (2018) with their results linked to the influence of ENPs characteristics, and the exposure media water chemistry.

Additionally, this trend could also have been facilitated by the positive ζ-potential increase over time observed in this study, and similarly as observed in earlier work of Gonçalves and co-authors (2018). Myriad of previous studies have observed n-ZnO to exhibit higher dissolution at low concentrations as opposed to high concentrations (Adam et al., 2015; Thwala et al., 2013; Cupi et al., 2016; Kim et al., 2017). For example, using Hoaglands media (HM) and MHW (Thwala et al., 2013; Kim et al., 2017) observed < 10% dissolution at high concentrations of 10 and 1000 mg/L. n-ZnO has been reported to dissolve rapidly once in contact with aqueous medium (Adam et al., 2014a). However, media composition in this case, water hardness and IS as well as the electrolytes constituting the ISO media played a key role on the final observed dissolution. The larger particle sizes of the agglomerates, and the low positive ζ-potential were considered as drivers of the low dissolution observed at the high concentration of n-ZnO. This is because of the significant adsorption of the ISO media electrolytes onto the n-ZnO surfaces, and less surface reactivity of larger particles (Thwala et al., 2013). Zn ions plausibly may have adsorbed onto larger sedimented particles; hence, could further account for the observed low dissolution of n-ZnO at high exposure concentrations.

Similarly, release of zinc ions is also media chemistry and inherent characteristics of n-ZnO dependent (Leung et al., 2012; Wang et al., 2016a; Odzak et al., 2017); similar to the discussions for the dissolution of n-Ag. For n-ZnO, increased aggregation as evidenced by formation of larger aggregates was observed relative to n-Ag. This was attributed to the high IS of the exposure media, and presence of monovalent and divalent electrolytes; which in turn, enhanced the inter-particle interaction of the n-ZnO. This resulted to the reduction of ζ-potential and enhanced aggregation of n-ZnO due to compression of the EDL, and reduction of the inter-particle repulsion as results in Table 4.1 indicates (Musee et al., 2014; Odzak et al., 2014; Wang et al., 2016c; Peng et al., 2017). Further, presence of anions in the ISO media may have adsorbed on the ENPs surfaces leading to reduction of ζ-potential, and consequent formation of larger aggregates leading to low dissolution because of the reduced available surface area (Li et al., 2013). Similar findings as outlined in this study have been reported elsewhere on the formation of larger agglomerates with sizes exceeding 1000 nm (Cupi et al., 2015; Kim et al., 2017).

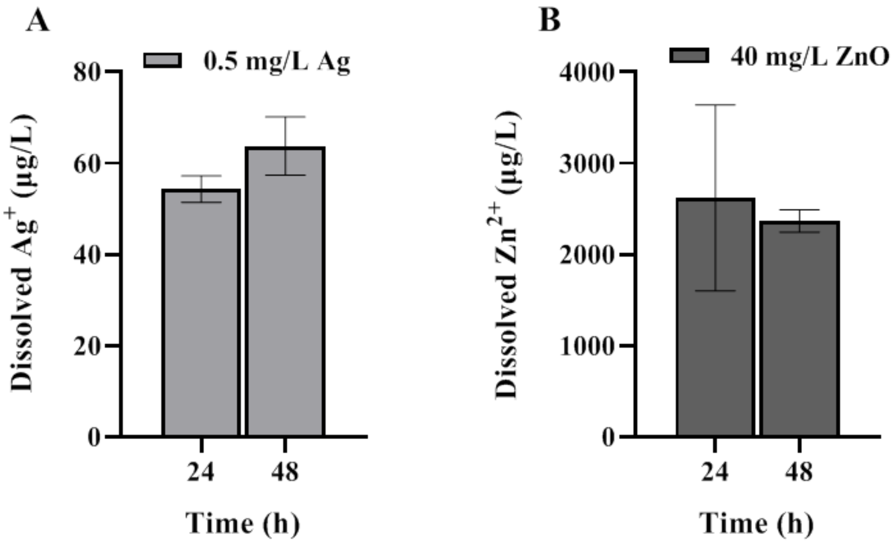


Figure 4.3: Dissolution of n-Ag (a) and n-ZnO (b) in the ISO media after 24 and 48 h. Error bars denote standard deviation (n = 3). Nominal exposure concentrations used were 0.5 and 40 mg/L for n-Ag and n-ZnO, respectively.

4.2.2 *Dissolution of binary mixtures*

The dissolution results of ENPs outlined herein are only for the highest exposure concentrations. This is because these were the concentrations at which toxicity of the ENPs to *D. magna* was observed based on 48h-EC_{50s} results described in 3.5.3. The concentrations used were 0.07 mg/L and 3.84 mg/L for n-Ag andn-ZnO, respectively. Notably, both concentrations were used in all binary and ternary mixture investigations including in systems with ATZ containing mixtures. Results showed ATZ concentration that induced observable toxicity was 56.97 mg/L, and hence, was used in both binary and ternary mixture studies.

Figure 4.4 summarizes dissolution results of n-Ag and n-ZnO in the mixture, and exhibited similar trends as observed for the individual ENPs (Section 4.3.1), and consitent with earlier works (Sakka et al., 2016; Gonçalves et al., 2018). Dissolution of n-ZnO was relatively higher to that of n-Ag in the mixture based on percentage recoveries plausibly because of the former's considerably higher initial concentration (3.84 mg/L). Further, the presence of n-Ag and subsequent formation of Ag^+ may have promoted the dissolution of n-ZnO. This is likely due to low aggregation as observed for the mixture in question Figure 4.2a. The findings herein are in aggregement with earlier investigation of Azevedo et al. (2017) where the dissolution of n-ZnO were higher relative to that of n-Ag in a mixture. Most Ag^+ may have adsorbed unto Zn^{2+}, although substancial Ag^+ were still detected in the mixture (Park et al., 2019a; Baek et al., 2020). There was no significant increase of dissolution over time, however, rather the dissolution of the metal ENPs was observed to be concentration dependent.

Due to low concentration of n-Ag used, dissolution was in the range 42–44% over 48 h (Figure 4.4). Dissolution exhibited time dependancy, and showed similar traits that of individual n-Ag. In the mixture, dissolution of n-ZnO was 38–42%, and showed slight increase after 48h (Figure 4.4). Overall, both the dissolution rate and dissolution behaviour of the ENPs in the mixture were similar to patterns observed for individual component of ENPs. Ions of Ag can be adsorbed onto the surface of ZnO NPs, which in turn, may have rendered them less available, thus: exerting affect on the equilibrium of dissolved and adsorbed Zn ions as well as the competition of ionic Zn and Ag in the media. In general, the release of Zn ions from n-ZnO into the test media may have resulted into ionic species interaction or competition with not only ionic Ag, but also other cations like Ca^{2+} and Mg^{2+} present in the media for the binding sites, consequently reducing its bioavailability to *D. magna*.

Figure 4.5 (a and b) illustrates dissolution of binary mixtures of ENPs and ATZ. The dynamics observed differed markedly from the results of the mixtures between ENPs only (Figure 4.4). For example, for the binary mixture of n-Ag and ATZ the dissolution of n-Ag ranged from 68 to 83 % at 0.07 mg/L (Figure 4.5a). Hence, the presence of ATZ in the mixture could have enhanced the dissolution of n-Ag as the resultant concentration of released Ag ions was higher relative to that of the binary mixture of n-Ag and nZnO (Figure 4.4).

Similarly to n-Ag/ATZ mixture, the presence of ATZ significantly influenced the dissolution of n-ZnO in the binary n-ZnO/ATZ mixture. Figure 4.5 (b) show dissolution increased from 1365 to 2217 µg/L, and ranged from 36 to 58%. ATZ did not completely adsorb all Zn ions and supress their availability as evidenced by higher dissolution results. Results demonstrated that the dissolution of both ENPs/ENPs and ENPs/ATZ binary mixture systems were time dependant, and higher with n-Ag. These results are consistent with the fact that organic compound(s) are known to enhance the dissolution for ENPs (Domingos et al., 2013; Miao et al., 2015) as are known to act as chelating agents to ENPs and/or ions (Wang et al., 2016c). This, partly in turn, may account for slightly higher dissolution compared to that of ENPs/ENPs binary mixture system.

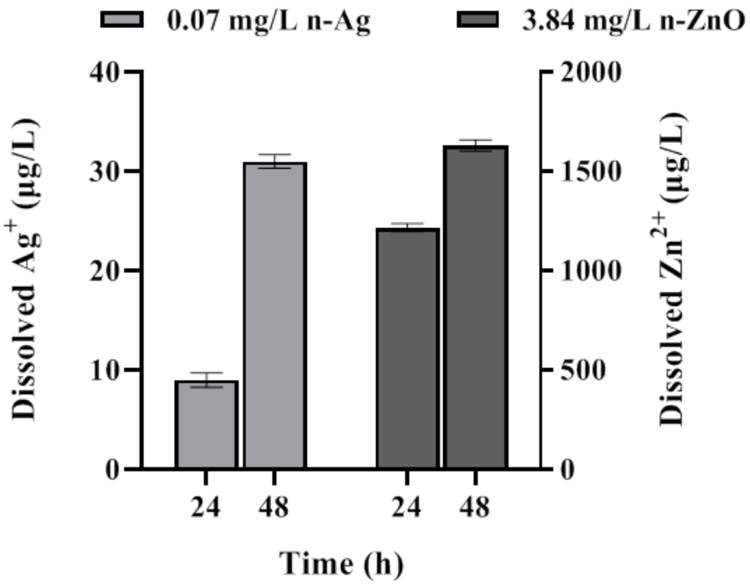


Figure 4.4: Dissolved concentrations of n-Ag/n-ZnO binary mixtures in ISO media. Error bars denote standard deviation (n = 3). Nominal exposure concentrations used were 0.07 mg/L n-Ag and 3.84 mg/L n-ZnO.

4.2.3 *Dissolution of ternary mixture*

Analysis of dissolved ions in the ternary system of n-Ag, n-ZnO, and ATZ was carried out and results are summarized Figure 4.6. Results demonstrate the trend for the dissolution of both ENPs increased over time, similarly as observed in the binary mixtures with or without ATZ (Figure 4.4 and 4.5). Further, the dissolution of both ENPs was lower in the ternary mixture, similar to that of individual ENPs. For example, dissolution of n-Ag was 30 µg/L, and for n-ZnO results were comparable to that of n-ZnO/ATZ mixture (42%) at 48h. The dissolution of n-ZnO may have been influenced by the interaction of the released Zn ions with cations such as Mg^{2+}, Ca^{2+}, and Ag^{+} (Wang et al., 2016a, c; Park et al., 2019a; Baek et al., 2020). The lower dissolution of n-Ag in the ternary mixture was likely due to the adsorption of released Ag^{+} onto n-ZnO, and this in turn, may have effected the total Zn^{2+} concentration in the mixture (Park et al., 2019a; Baek et al., 2020).

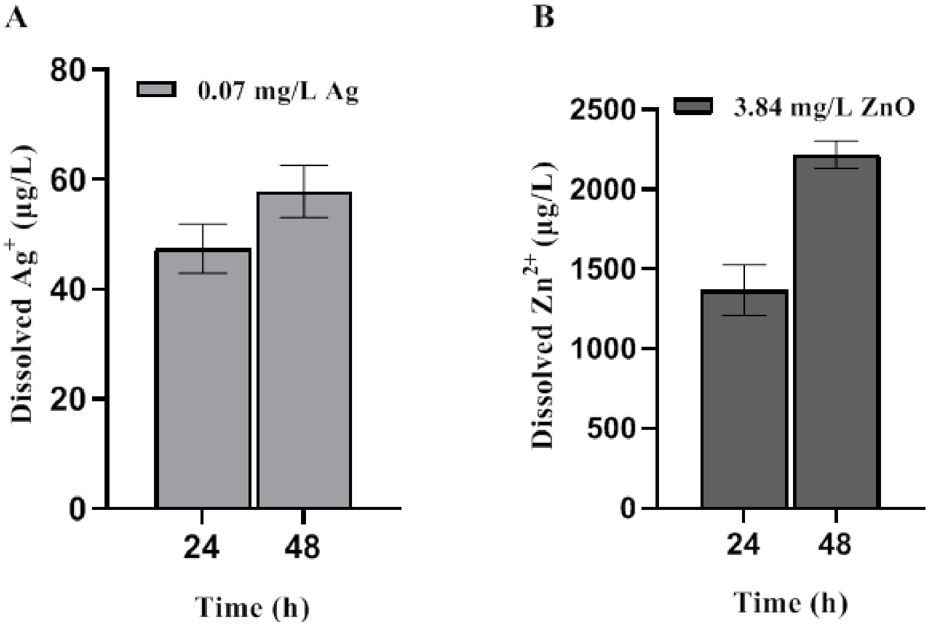


Figure 4.5: Dissolved concentrations of (a) n-Ag/ATZ and (b) n-ZnO/ATZ binary mixtures in ISO media at ATZ concentration of 56.97 mg/L. Error bars denote standard deviation (n = 3). Nominal exposure concentrations used for n-Ag and n-ZnO were 0.07 and 3.84 mg/L, respectively.

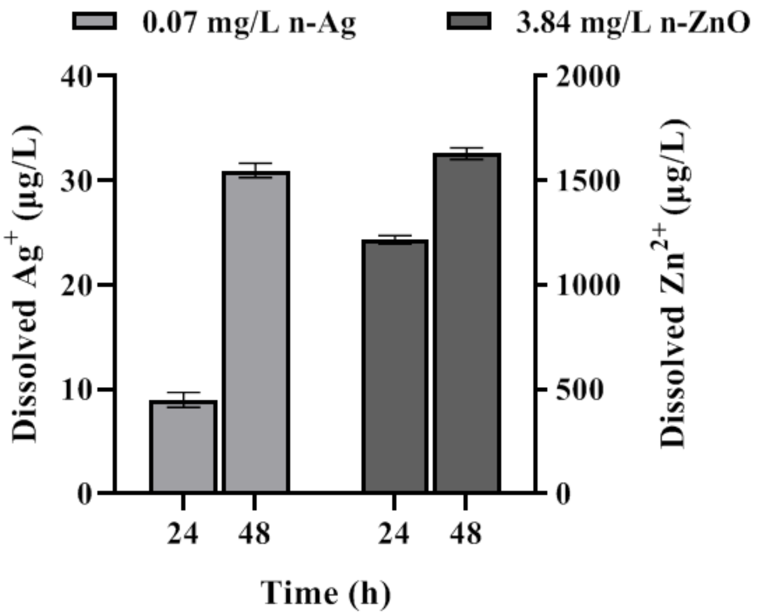


Figure 4.6: Dissolved concentrations of n-Ag/n-ZnO/ATZ ternary mixtures in ISO media at ATZ concentration of 56.97 mg/L. Error bars denote standard deviation (n = 3). Nominal exposure concentrations used for n-Ag and n-ZnO were 0.07 and 3.84 mg/L, respectively.

4.3 Acute toxicity

4.3.1 *Single toxicity tests*

Herein the results indicate highly variant acute toxicities of n-Ag, n-ZnO, and ATZ to the crustacean *D. magna* based on the calculated EC_{50} values. In descending order, the observed toxicities were n-Ag > n-ZnO > ATZ. Calculated 24 and 48 h EC_{50s} for n-Ag were 0.08 and 0.07 mg/L, respectively (Figure 4.7a), thus is extremely toxic to *D. magna*. Results herein are in agreement with previous works carried out in artificial media including ISO media and hard water. For instance, EC_{50} values at 24 and 48 h for n-Ag were in the range of 0.011–0.383 mg/L (Ribeiro et al., 2014; Becaro et al., 2015; Cupi et al., 2015; Hu et al., 2018), and 0.001–0.296 mg/L (Zhao and Wang, 2012; Seitz et al., 2015; Chae and An, 2016; Heinlaan et al., 2016; Melegari et al., 2019), respectively, and similar to those obtained from this study. Further, the observed toxicity of n-Ag was due to both ionic species and the particulates as has been demonstrated in earlier works (Liu and Hurt, 2010; Thwala et al., 2013).

Immobilization results of n-ZnO to *D. magna* are shown in Figure 4.7 (b). Fourty-eight hour EC_{50} was 3.84 mg/L, but 24h EC_{50} could not be established as no significance response was observed. Similar to n-Ag, previously the toxicity of n-ZnO towards *D. magna* in artificial water was determined for 24 and 48 h EC_{50s} found to be, respectively, in the range 1.7–22 mg/L (Poynton et al., 2011; Cupi et al., 2015), and 0.047–8.2 mg/L (Seo et al., 2014; Cupi et al., 2016). Other studies in natural water reported EC_{50s} almost in the same range with a maximum value of 9 mg/L (Blinova et al., 2010; Cupi et al., 2015). Differences in the observed toxicities of the ENPs can be attributed to the inherent ENPs characteristics (e.g., size, morphology, etc.) and exposure media properties as discussed in Section 2.8 as well as particles type.

For example, as illustrated in section 4.1, n-Ag were smaller-sized (40 ± 12 nm) relative to n-ZnO (60–136 nm), as a result were more reactive, and hence partially may account for the differences on the observed toxicity. Of the three pollutants, ATZ induced the least toxic to *D. magna* (Figure 4.8) with EC_{50} of 56.97 mg/L relative to n-Ag and n-ZnO (Figure 4.7). The majority of previous studies have reported acute toxicities of ATZ towards *D. magna* with EC_{50} values in the range of 9 – > 1000mg/L (Wan et al 2006; Han et al., 2012; Sengupta et al., 2015; Schmidt et al., 2017). Hence, our results are within earlier observed toxicities. Moreover, Klementová et al. (2019) observed no effects of ATZ to *D. magna*. Thus, based on the EU Directive 93/67/EEC (1996) classification formalism the observed toxicities of n-Ag, n-ZnO, and ATZ towards *D. magna* in this study were categorised as extremely toxic, toxic, and harmful, respectively.

4.3.2 *Mixture toxicity tests*

Results of individual contaminants as discussed in section 4.3.1 are highly unlikely to be found in the environment. Rather, because of the concurrent and sequential release of variant contaminants, they thus co-exist as a cocktail, and therefore, effects of individual contaminants can be completely altered in the environment as they interact with each even at the lowest non-toxic concentrations. In this section, the binary and mixture toxicities of three contaminants are discussed. Results on the acute toxicity of binary and ternary mixture exposures of n-Ag, nZnO and ATZ at 24 and 48 h, and predicted values following the CA model formalism are listed in Table 4.2.

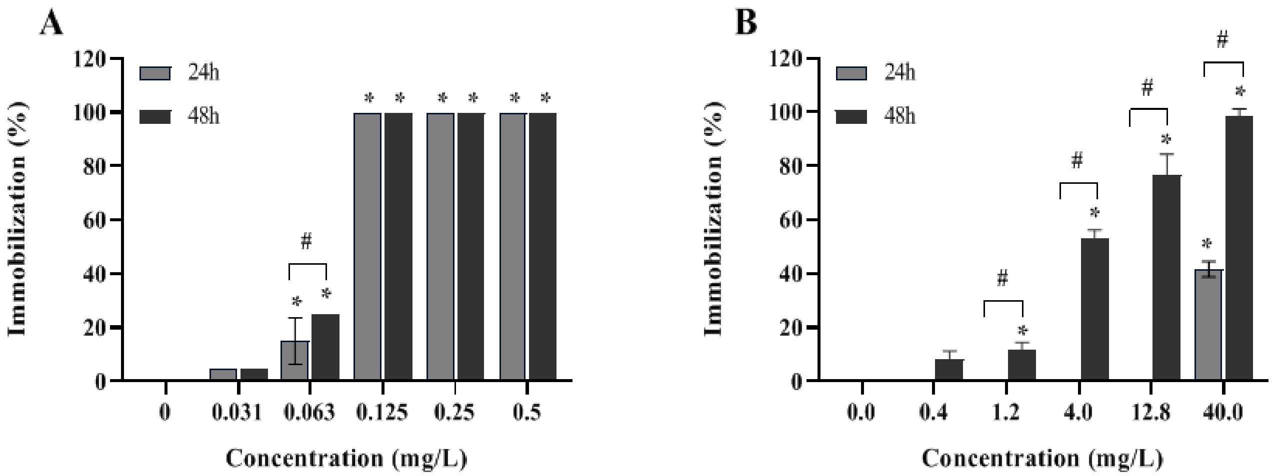


Figure 4.7: Immobilization of *D. magna* exposed to n-Ag (a) and n-ZnO (b) in ISO media. Data represents the mean ± SD (n=3), asterisk (*) denotes statistical differences relative to control (ISO media without ENPs), and # signifies statistical differences of treatments over time following two-way ANOVA, Tukey's test ($p < 0.05$).

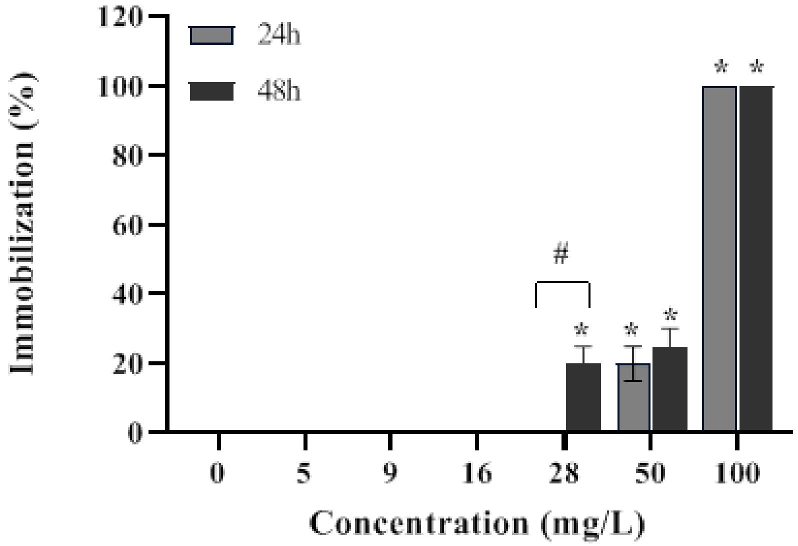


Figure 4.8: Immobilization of *D. magna* exposed to ATZ in ISO media. Data represents the mean ± SD (n=3), (*) represent significant difference of treatments to control (ISO media without ATZ); and # indicates statistical differences of treatments over time determined by Tukey's test ($p < 0.05$).

Table 4.2 Predicted and observed toxicity values, and interactions for the binary and ternary mixtures of n-Ag, n-ZnO, and ATZ.

Binary and ternary mixtures	Predicted toxicity (CA) EC_{50} (mg/L)	Observed toxicity EC_{50} (mg/L)	TU	Predicted toxicity interaction based on TU
n-Ag + n-ZnO	1.96	1.67 (1.22–2.28)	0.51	Synergism
n-Ag + ATZ	28.52	4.48 (3.26–6.09)	0.04	Synergism
n-ZnO + ATZ	30.41	ND	0.03	Synergism
n-Ag + n-ZnO + ATZ	20.29	ND	0.05	Synergism

TU= Toxicity Unit; '(brackets)' = values of 95% confidence intervals; ND: not determined.

4.3.2.1 Binary mixtures toxicity

Toxicity results for the binary mixtures of n-Ag and n-ZnO are depicted in Figure 4.9a. Variant combination of ENPs binary mixtures was observed to induce toxic effects towards *D. magna.* Predicted EC_{50} using CA model indicated synergistic effects of ENPs towards *D. magna* as further confirmed by calculated TU values (Table 4.2). Further, the observed results from the immobilization tests were in agreement with predicted values derived using the CA model. Therefore, the EC_{50} results herein and the interactions in the binary mixture point to synergistic effects. Similar results have also been observed in earlier works towards *D. magna* (Azevedo et al., 2017; Park et al., 2019a). Other mixture toxicity studies on *D. magna* have reported Ag in ionic form were observed to induce synergistic effects in mixture combinations with metallic pollutants (Kim et al., 2016), and metal oxide NPs (e.g., n-ZnO, n-TiO_2, etc.) (Park et al., 2019a; Baek et al., 2020). For example, Park et al. (2019a) observed a mixture of n-ZnO and ionic Ag yielded synergistic effects to *D. magna* at concentrations in the range of 0.5–1.5 μg/L Ag^+.

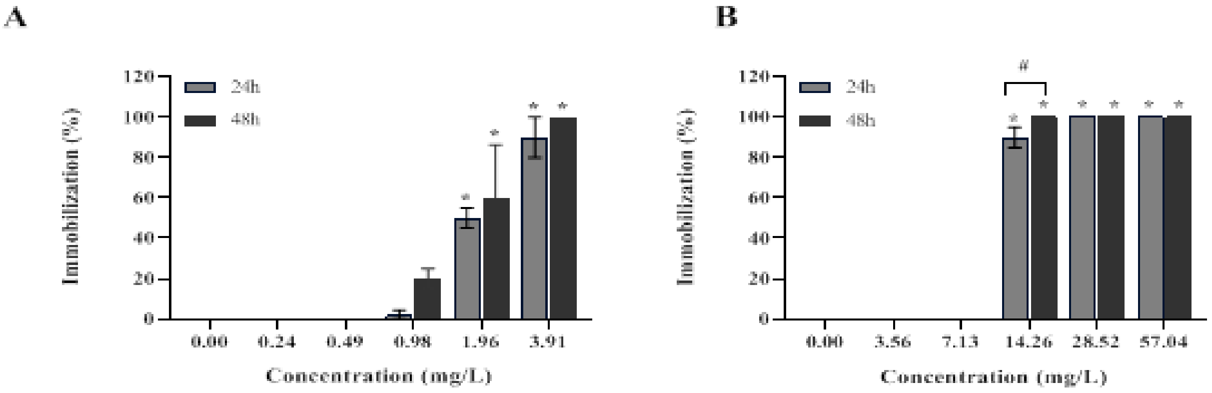


Figure 4.9**:** Immobilization of *D. magna* exposed to (a) n-Ag/n-ZnO and (b) n-Ag/ATZ binary mixtures in ISO media. (*) denotes statistical differences relative to control (ISO media without ENPs/ATZ); data represents the mean ± SD (n=3), whilst (#) represents statistical differences of treatments over time (Tukey's test, $p < 0.05$).

Figure 4.9b summarizes the toxicity effects for the binary mixtures of n-Ag and ATZ, and similarly revealed the interactions were synergistic in character. In addition, the predicted EC_{50} value was indicative of harmful toxicity level effects, however, observed EC_{50} value pointed to high toxic effects of the mixture. Notably, mixture toxicity effects of chemical contaminants are known to yield toxicity effects higher relative to those of individual constituent as recently observed by Soler de la Vega and colleagues (Soler de la Vega et al., 2019) from investigations on the joint toxicity effects of n-TiO_2 with organic UV filters and parabens. In this study, the enhanced toxicity was attributed to the adsorption of ATZ by ENPs, which in turn, increased the bioavailability and effectively the toxicity of the complexed pollutants through the Trojan horse phenomenon (Hartmann and Baun, 2010; Abbas et al., 2020b).

For example, Molins-Delgado and co-workers (2016) investigated the joint mixture toxicity effects of n-Ag and an organic pollutant used in PCPs such as UV-filters (Benzophenone 1). Results revealed enhanced toxicity of UV-filters in the presence of n-Ag. Previously, similar toxicological outcomes have also been reported, with results demonstrating the joint effects of ENPs e.g., n-TiO_2, n-C60, and organic pollutants e.g., pesticides and PCPs were higher (Brausch et al., 2010; Tian et al., 2014; Zhang et al., 2017b; Soler de la Vega et al., 2019).

Results of binary mixtures for n-ZnO/ATZ yielded no observable joint effects. Individually, nZnO and ATZ induced moderate and low toxicity, respectively, on *D. magna*. Table 4.2 show the predicted interaction of the binary mixture using the CA model, and TU results point to synergic effects. However, the observed results did not indicate synergistic effects thus highlighting the current available models are not always accurate in predicting the joint toxicity of compounds. The absence of observed response by *D. magna* to these mixture exposures suggest the organic ATZ may have modified the n-ZnO toxicity effects by adsorbing unto the n-Zn, and in turn reducing the nano-bio interaction and subsequent the toxicity on *D. magna*. Thus, the joint effects of n-ZnO and ATZ resulted in the detoxification of the compounds. This can further be explained by lower concentration of ionic species of Zn relative to the case of n-ZnO alone, or particulate forms to the *D. magna* owing to the formation of large agglomerates, which effectively reduced the dose available to the organism (Chen et al., 2020).

The low concentration may be due to ATZ adsorption of the released Zn ions, and competition for attachment with Ca^{2+} and Mg^{2+} in the exposure media. Further, owing to the high ionic strength of ISO media may have fostered the formation of larger complex aggregates of n-ZnO/ATZ mixture system. As a result, this may have led to the sedimentation of larger aggregates of n-ZnO/ATZ mixture. Subsequently, this may have led to lower degree of interactions (bioavailability) of the mixture and the *D. magna* relative to that of individual contaminants. As such, this resulted to a reduction on the toxic effects of the mixture. Similar observations as in the current study have been reported by Hackenberger et al. (2019) where no significant joint toxicity effects of n-ZnO and organic contaminant propiconazol in a mixture was apparent.

4.3.2.2 Ternary mixture toxicity

Acute toxicity response of *D. magna* to a ternary mixture assessed in this study comprised of two ENPs and a pesticide. The predictions by CA model yielded higher toxicity relative to binary mixture results, and the interactions between the compounds was predicted to be synergistic (Table 4.2). Further, the ternary mixtures response was similar to that of binary mixture system of n-ZnO/ATZ. Experimental results for the ternary mixture exposures, however, indicated no detectable response to *D. magna*. Although individual constituent component induced toxicity to *D. magna* at variant degrees, however, their ternary combination and interactions pointed to plausible mitigative effect on each other's toxic potential.

The presence of ATZ may have had an influence on the transformation of the ENPs; with resultant outcome being the detoxification of the toxic n-Ag and n-ZnO. Notably, the combination of the concentrations tested in the ternary mixture for each pollutant was observed to induce 50% lethality to the daphnia population (based on results in section 4.3.1). Hence, experimental results are indicative of likely toxicity reduction in the mixture. This can be partly accounted by the fact that n-ZnO and ATZ were the dominant constituent components in the ternary mixture as outlined in section 3.5.3. (Table 3.2) In turn, both chemicals attached to one another, hence eliminating their potential effects and therefore constituents competed for binding sites together with the media constituents e.g., divalent ions consequently eradicating toxicity of one another in the mixture. And secondly, formation of large aggregates of the ternary mixture based on the aggregation kinetics (section 4.1), may have subsequently contributed to the unavailability of toxic Zn and Ag ions or particulates to the daphnia, hence leading to no observed toxicological outcomes as experimental results depicts.

In conclusion, the results of this study herein indicate that individual toxicity tests are inadequate to offer holistic assessment of the true effects of contaminants in the environment. Evaluation of the impact of chemical mixtures in ecosystems is crucial because co-existence of contaminants may render them potentially toxic or non-toxic to aquatic organisms. The observed effects and/or lack therefore for the mixture systems are dependent on numerous influencing factors including ENPs properties, chemical properties, exposure media, test organism as observed in this study, and in previous works (Dwivedi and Ma, 2014; Naasz et al., 2018). Further, the wide variety of new and existing contaminants poses a challenge for chemical management and hazard assessment. For environmental protection and sustainability, ecotoxicology studies play an essential role. As demonstrated by the results herein, the organic pollutant ATZ altered the toxicity of metallic ENPs, a factor that prediction model techniques did not take into account. This further emphasizes the relevance and importance of using bioassays for chemicals risk assessment; together with the prediction models as means to improve our ability to assess the likely implications of variant pollutants in the ecosystems.

Chapter 5: Conclusions and recommendations

5.1 Concluding remarks

In this study, the fate, and toxicological effects of individual ENPs and organic pollutant as well as their binary and ternary mixtures on *D. magna* in an aqueous media were investigated. This entailed determination of acute toxicological effects of individual ENPs, their ENPs/ENPs mixtures as well as their mixtures with an organic contaminant on *Daphnia magna*. Results indicated that the observed effects were influenced by ENPs transformation processes, and regulated by the exposure media chemistry. To accomplish the main aim of this project, three sub-objectives were defined: (i) The first objective evaluated the fate of individual ENPs (n-Ag and n-ZnO) as well as their binary and ternary mixtures with organic pollutant (ATZ), (ii) the toxicity effects of individual pollutants, viz.: n-Ag, n-ZnO, and ATZ on *D. magna* investigated, and (iii) assessment of the joint effects of n-Ag, n-ZnO, and ATZ for their binary and ternary mixtures on *D. magna*.

Objective 1: Fate of individual ENPs (n-Ag and n-ZnO), and their binary and ternary mixtures with organic pollutant ATZ

The fate and behaviour of ENPs in artificial media ISO was observed to be chiefly controlled by the media chemistry, and inherent ENPs physicochemical properties. Findings herein indicated the constituents of the ISO media as the primary drivers of ENP aggregation and dissolution. Hence, the overall individual and mixture fate trend were demonstrated to be ENPs physicochemical properties dependent.

Objective 2: Assessment of toxicological effects of n-Ag, n-ZnO, and ATZ on *D. magna*

Effects of *D. magna* to n-Ag, n-ZnO, and ATZ was assessed and observations revealed variant toxicities dependent on the individual chemical properties. The toxicological outcomes were very toxic, toxic, and harmful for the n-Ag, n-ZnO, and ATZ, respectively, as influenced by the chemical type and its inherent properties as well as the interaction with daphnia.

Objective 3: Assessment of joint effects of n-Ag, n-ZnO, and ATZ for their binary and ternary mixtures on *D. magna*

Binary and ternary mixture toxicity was determined using the median effect concentrations of the individual contaminants. It was observed that media chemistry and the interactions among variant contaminants influenced the observed outcome of the mixture in question. It was also observed that prediction models (e.g., CA in this case) are not always accurate in predicting the outcome of binary and ternary mixtures of contaminants. For instance, findings showed synergistic effects for the binary mixtures of ENPs/ENPs, and the ability of organic contaminant to detoxify ENPs in a binary mixture system as well as in the ternary mixture system. However, such effects could not be established based on the experimental results.

5.2 Recommendations

This study aimed to establish the fate and toxicity of ENPs, and organic contaminant both as individuals, and their resultant joint binary and ternary mixtures. Understanding the fate and behaviour of ENPs and their interaction with co-contaminants is important for realistic risk assessment in the aquatic environments. Results demonstrated that the characteristics of each contaminant determine their fate and toxicity as influenced by the exposure media chemistry; hence the outcome varied case by case.

Prediction of mixture toxicity effects by reference models such as CA are not sufficient to determine the realistic effects of a mixture, and therefore, the use of bioassays is a crucial element to support studies and generation of mixture toxicity data of ENPs including their likely co-existing contaminants in environmentally realistic concentrations. Generated data can be valuable to aid policy makers e.g., in the Department of Water and Sanitation (DWS), and Department of Environment, Forestry and Fisheries (DEFF) in developing guidelines and framework necessary for resource protection, and water quality management towards the goal of maintaining biodiversity and sustainable natural resources. And, more similar mixture studies of ENPs with other classes of organic pollutants are warranted as there is dearth of data in such domain, and different levels of biological organization using real environmental matrixes e.g., river water, groundwater, or lake water. Utilization of such systems is necessary as are more reflective of actual environmental matrixes as opposed to widely published findings on the effects of individual ENPs with exposures done in synthetic media.

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