USE OF THE NEMATODE, *CAENORHABDITIS ELEGANS*, TO EVALUATE BIOAVAILABILITY AND TOXICITY OF TRANSITION METALS AND MANUFACTURED ZINC OXIDE NANOPARTICLES

by

HONGBO MA

(Under the Direction of Phillip L. Williams)

ABSTRACT

The free-living nematode, *Caenorhabditis elegans*, has been suggested as an excellent model organism in ecotoxicological studies. This dissertation examines the use of *C. elegans* to evaluate bioavailability and toxicity of transition metals and manufactured zinc oxide nanoparticles. A transgenic strain of *C. elegans* using metallothionein-II promoter to drive green fluorescence protein reporter (*mtl-2::GFP*) was developed and tested for its response to several transition metals (Cd, Hg, Cu, Zn, Ni, Pb and As) in aquatic medium. Bioavailability and toxicity of manufactured zinc oxide nanoparticles in *C. elegans* were evaluated using the endpoints lethality, behavior, reproduction, transgene expression, and element spatial distribution. Phototoxicity of zinc oxide nanoparticles was also assessed under natural sunlight illumination. A preliminary study of the potential impact of ZnO nanoparticles on Cu toxicity was also conducted. Findings from these studies suggest that the *mtl-2::GFP* transgenic *C. elegans* bioassay represents an alternative approach to quantify a surrogate of metallothionein in response to Cd, Hg, Cu, Zn exposure both easily and quickly, and it may potentially be used for quantitative or semiquantitative biomonitoring for contamination of these metals in aquatic
systems. Manufactured zinc oxide nanoparticles caused similar toxicity as aqueous ZnCl₂ to *C. elegans*, and thus internal biotransformation of nanoparticles to metal ions might have occurred. Phototoxicity of ZnO nanoparticles can occur under natural sunlight illumination, and this phototoxicity is greater and the onset of action is faster than the toxicity under ambient laboratory light. Therefore, evaluation of phototoxicity of nanoparticles with photocatalytic properties should not be neglected during ecological risk assessment. Zinc oxide nanoparticles seem to be able to impact Cu toxicity and bioavailability in *C. elegans*, although further study is required. All these findings demonstrate that *C. elegans* can serve as a good model for ecotoxicological studies, both for transition metals and manufactured nanoparticles.

INDEX WORDS: *Caenorhabditis elegans*, transition metals, zinc oxide nanoparticles, bioavailability, toxicity
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by

HONGBO MA

B.E. Liaoning University of Petroleum & Chemical Technology, China, 1998
M.S. Institute of Oceanology, Chinese Academy of Sciences, China, 2001
M.S. University of South Carolina, 2004

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

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by

HONGBO MA

Major Professor: Phillip L. Williams
Committee: Travis C. Glenn
Paul M. Bertsch
Charles Jagoe
William P. Miller

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

To my parents, Shengfu Wen and Maoying Zhou, who have been the ultimate teachers in my life. To my husband, Xianben Zhu, and my daughter, Alice L. Zhu, whose love and laughter I treasure every day.
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Caenorhabditis elegans is a saprophytic nematode species that inhabits soil and leaf-litter environments in many parts of the world [1]. Although scientific reports on this species have appeared in the literature for more than one century, extensive studies on C. elegans was not started until Brenner’s seminal genetics paper was published in 1974 [2]. Since then it has emerged to be an important experimental model in a broad range of areas including neuroscience, developmental biology, molecular biology, genetics, and biomedical science. Characteristics of this animal model that have contributed to its success include its genetic manipulability, invariant and fully described developmental program, well-characterized genome, ease of culture and maintenance, short and prolific life cycle, and small and transparent body [3].

These same features have led to an increasing use of C. elegans for environmental toxicology and ecotoxicology studies since the late 1990s [3]. Although generally considered a soil organism, C. elegans lives in the interstitial water between soil particles and can be easily cultured in aquatic medium within the laboratory. It has been successfully used to study toxicity of a broad range of environmental toxicants using both lethal and sublethal endpoints including behavior (movement) [4-6], growth and reproduction [5, 7], and feeding [8]. Transgenic strains of C. elegans with specific
biomarkers have also been developed and successfully applied in assessing toxicity and bioavailability of a variety of environmental contaminants [9-14].

This dissertation examines the use of *C. elegans* to evaluate bioavailability and toxicity of transition metals and manufactured zinc oxide nanoparticles (ZnO NPs). It contains two major parts. The first part focuses on the use of a transgenic strain of *C. elegans* as a biomonitor to evaluate bioavailability and toxicity of transition metals; the second part investigates bioavailability and toxicity of manufactured ZnO NPs using *C. elegans* as a model organism, and four independent studies have been conducted, addressing the ecotoxicity of ZnO nanoparticles from different perspectives.

Chapter 2 contains a literature review pertaining to the topics studied and discussed throughout this dissertation. Chapter 3 through 7 covers five studies conducted to address the two topics described above. Chapter 8 summarizes the findings and conclusions from these studies and suggests future research directions. A brief description of experiments performed for each of the five studies is presented below.

1. **Use of a transgenic *C. elegans* to evaluate bioavailability and toxicity of transition metals**

   Heavy metal contamination may threaten both human and ecological health. Conventional chemical and physical methods can measure metal concentrations but do not address metal bioavailability or biological effects directly. Gene expression endpoints are not only sensitive in estimating the effects of toxicants on exposed organisms, but also provide insight regarding the mechanisms underlying these effects [15]. Transgenic strains of *C. elegans* have been developed and applied in environmental toxicological
studies including biomonitoring heavy metal contamination. Transgenes responsive to metals that have been developed include those that used heat shock protein (hsp) promoter and those that used metallothionein (MT) promoter [16-18]. The hsp genes are induced by a wide variety of environmental stressors, and thus those hsp-regulated transgene systems do not provide specificity for metals. In contrast, metallothionein genes are more specifically responsive to transition metals.

We aimed to apply a transgenic C. elegans using metallothionein-II promoter to drive green fluorescence protein reporter (mtl-2::GFP) to evaluate bioavailability and toxicity of certain transition metals in both aquatic and soil matrices. The mtl-2::GFP system uses mtl-2 promoter which has higher specificity for heavy metal toxicity, and GFP reporter which enables easy, quick, and accurate quantification. We investigated the response of this transgenic system to exposure to metals (e.g., Cd, Hg, Zn, Cu, Ni, Pb, and As) and compared this assay to traditional ecotoxicological endpoints, such as lethality and behavioral changes. To verify the assumption that expression of GFP is proportional to the expression of MT and, thus, that GFP can be used a surrogate for metal-induced MT for biomonitoring, we also measured the levels of mtl-2 transcription in C. elegans after exposure to each metal using real-time reverse transcription polymerase chain reaction (RT-PCR). Finally, we considered the capability and potential applications of this transgenic bioassay in environmental biomonitoring.

2. Toxicity of a commercially obtained ZnO nanoparticle (ZnO NPs) suspension

Manufactured metal oxide nanoparticles are being used in a broad-range of applications and it is anticipated that large quantities of these nanoparticles will be
released to the environment. However, information describing the possible impacts of these nanoparticles on ecological receptors is limited.

We aimed to assess the potential ecotoxicity of a commercially obtained ZnO NPs suspension (2-6 nm primary particle diameter) to the nematode *C. elegans*, and compare it to aqueous ZnCl₂. Four endpoints were used: lethality, behavior (movement), reproduction, and transgene expression. The first three are ecologically relevant, whereas transgene expression may elucidate mechanisms of toxicity. In particular, a transgenic strain of *C. elegans* with the metallothionein-2 (*mtl-2*) promoter fused to green fluorescent protein (GFP; i.e., *mtl-2::GFP*) was used. A previous study demonstrated that when exposed to metal ions, such as Cd²⁺ or Zn²⁺, transgene expression is induced and the response shows a concentration-dependent manner [19]. The *mtl-2::GFP* transgenic *C. elegans* may yield insight regarding whether the ZnO NPs are dissolving intracellularly to release Zn²⁺.

3. Spatial distribution of zinc and *mtl-2::GFP* transgene expression

As little is known about the possible mode of action or mechanism of toxicity of metal oxide nanomaterials, it is important to distinguish between toxicity arising from particle size dependent effects and that from the release of dissolution products such as free metal ions. Recently, several studies have independently reported the dissolution of ZnO NPs to metal ions to cause toxicity in a number of aquatic or terrestrial species including freshwater microalga [20, 21] and the nematode *C. elegans* [22].

In this study, we intended to determine that whether the previously observed toxicity of ZnO NPs could be explained by the release of free metal ions. We investigated zinc distribution in *C. elegans* exposed to a commercially obtained ZnO NPs suspension
and aqueous ZnCl₂ using synchrotron based X-ray fluorescence (SXRF) microscopy, and the distribution of GFP transgene expression in a mtl-2::GFP transgenic C. elegans using ultraviolet visible (UV-VIS) fluorescence microscopy. Knowledge of spatial distribution of a contaminant within an organism is important in determining the site of toxic action or mechanism of toxicity [23]. Our previous study indicated that ZnO NPs induce GFP expression in the mtl2::GFP transgenic C. elegans [19], which may suggest the dissolution of ZnO NPs to metal ions within the nematodes. A spatial resolution of GFP expression coupled with zinc spatial distribution will facilitate the understanding of the bioavailability and toxicity of ZnO NPs, and a comparison of GFP and metal distribution patterns between ZnO NPs and ZnCl₂ will bring insights to understand the possible mechanisms of toxicity. We hypothesize that toxicity of ZnO NPs in C. elegans is related to the release of zinc ions, and that the spatial distribution of Zn and GFP would be similar.

4. Phototoxicity of nanoparticulate ZnO under natural sunlight illumination

Manufactured nanoparticles may pose both direct and indirect toxic effects to ecological receptors. Direct effects are mainly determined by their chemical composition and surface reactivity, whereas indirect effects can be caused by physical restraints or the release of toxic ions (e.g., dissolution of metal and metal oxide nanoparticles) or the production of reactive oxygen species (ROS) [24]. Generation of ROS is especially relevant to photocatalytic metal oxide NPs such as ZnO or TiO₂ upon ultraviolet (UV) irradiation, and may have great implication on their toxicity. A number of studies have focused on phototoxicity of these metal oxide NPs at cellular and sub-cellular levels [25-27]; however, understanding the potential phototoxicity of these nanoparticles in whole
animal studies with ecological receptors is essential to ensure their proper use as well as understanding the implications of their release to the environment.

As an initial effort to understand the potential phototoxicity of metal oxide NPs to ecological receptors, this study evaluated phototoxicity of a manufactured nanoparticulate ZnO (nano-ZnO, 40-100 nm primary particle diameter) to *C. elegans* under natural sunlight illumination; and compared it to toxicity under ambient artificial laboratory light. Bulk ZnO (~1.5 μm primary particle diameter) and aqueous ZnCl₂ were used as reference toxicants. Our hypotheses were that phototoxicity of nano-ZnO to the nematodes under natural sunlight illumination will be greater than toxicity under artificial laboratory light illumination, and this phototoxicity will be correlated with photocatalytic activity of the nanoparticles. We also hypothesized that this phototoxicity would be greater in nano-ZnO than in bulk ZnO, as smaller particles have a greater reactive surface area and thus may be more effective in generating ROS [28]. We used natural sunlight instead of artificial UV light, thus to avoid the possible detrimental effects caused by UV light to the nematodes. It also represents a more realistic scenario under which organisms might be exposed to nanoparticles released to the environment.

5. **Impact of ZnO nanoparticles on copper toxicity**

One important aspect of understanding the environmental implication of nanoparticles is their interaction with other environmental contaminants. As a result of their remarkably high surface area to volume ratio and complexing capability, nanoparticles may adsorb pollutants, which might change the transport and bioavailability of both the nanoparticles and the pollutants in natural systems, and alter
their toxic effects [24]. Nanoparticles may affect trace-metals ion speciation and thus impact their bioavailability and potential toxicity [22, 24].

This preliminary study evaluated the possible impact of a commercially obtained ZnO NPs suspension (1.5 nm primary particle diameter) on copper (Cu) toxicity and bioavailability to *C. elegans*. We tested the impact of a range of ZnO NPs concentrations on the lethality of Cu to *C. elegans*, as well as stress protein induction by using the *mtl-2::GFP* transgenic strain of the nematode. We hypothesize that nanoparticles will alter the bioavailability and toxicity of Cu by two possible mechanisms: through competition of ZnO NPs with Cu$^{2+}$ for sensitive binding sites at cell surfaces and the second via the sorption of Cu$^{2+}$ to the highly surface active ZnO NPs.

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*Environmental Science & Technology* 41:8484-8490.


CHAPTER 2
LITERATURE REVIEW

This chapter provides an overview of the literature on two major topics: the use of transgenic strain of *C. elegans* to study metal toxicity and bioavailability; and ecotoxicology of manufactured nanomaterials (with a focus on metal oxide nanoparticles). Some of the material is presented in subsequent chapters that were submitted for publication; however, the purpose of this section is to provide a more detailed review of these topics.

I. Transgenic strains of *C. elegans* in study of metal toxicity and bioavailability

The use of *Caenorhabditis elegans* in environmental toxicology can be dated back to the early 1990s. *C. elegans* is a free-living, transparent nematode that lives in temperate soil environments. Nematodes are the most abundant animal species in soil ecosystems. Because they occupy key positions as primary and intermediate consumer in soil food webs as well as play important roles in soil nutrient cycling, nematodes may be used as bioindicators of soil conditions [1]. Evaluation and interpretation of the abundance and function of nematode faunal assemblages or community structure offers an *in situ* assessment of disruptive factors of the soil environment. These features of soil nematodes have also supported their use in ecotoxicological studies and a variety of nematodes species have been used to study environmental issues from the late 1970s [2]. Research into the molecular and developmental biology of *C. elegans* was begun in 1974.
by Sydney Brenner and it has since been used extensively as a model organism. Due to its thoroughly understood biology as well as its ecological relevance, *C. elegans* began to emerge as a representative species of nematodes for ecotoxicological studies since the early 1990s [2].

The environmental toxicants that have been studied using *C. elegans* include metals, pesticides, and other organic contaminants. The majority of these environmental studies have been performed in aquatic media due to the ease of use of the procedures; and once the toxicological endpoints are developed, they may be extended and applied to sediment and soil systems. An overview of laboratory-based environmental toxicological studies using *C. elegans* is presented in Table 2.1. In all these studies, a toxicant of environmental interest was added to a medium (water, sediment, or soil) followed by exposure to *C. elegans* and the assessment of an adverse effect. The endpoints that have been used include lethality, reproduction, behavior, growth, life span, and protein expression. To a lesser extent, *C. elegans* toxicity assays have also been used to assess real environmental samples. For example, *C. elegans* was used to test polluted sediments using growth and fertility as endpoints in Elbe River in Germany [3], and the findings from this study suggested that *C. elegans* has a great potential for use in aquatic and whole-sediment toxicity tests. *C. elegans* 72-h lethality assay had been successfully used to identify sources of water contamination and effectiveness of wastewater treatment in several industrial operations and a municipal treatment plant [4]. A summary of field studies is displayed in Table 2.2.

*Caenorhabditis elegans* has been used as a model system to elucidate toxicity and toxicological mechanisms of various transition metals, such as aluminum (Al), arsenic
(As), barium (Ba), cadmium (Cd), copper (Cu), lead (Pb), mercury (Hg), uranium (U), and zinc (Zn). Much of the early work on metal toxicity used lethality as the endpoint [5-7]. Over time, more sophisticated sublethal endpoints have been developed such as behavior [8, 9], reproduction [10, 11], growth [12], and transgene expression [13-21]. Application of transgene expression in *C. elegans* to study metal toxicity started in the late 1990s. In such transgene systems, a gene or promoter of interest is coupled to a reporter gene that is also expressed when the gene or promoter of interest is expressed. Expression is then quantified by measuring the amount of reporter protein expressed. In addition to their higher sensitivity (as compared to lethality as an endpoint) in estimating the effects of toxicants on exposed organisms, transgene expression endpoints also provide insight regarding the mechanisms underlying these effects.

To date, transgenes responsive to metals that have been constructed for *C. elegans* can be classified into two categories based on the promoters used: one uses a heat shock protein (*hsp*) promoter to control a reporter gene, and the other uses a metallothionein (MT) promoter to regulate expression of a reporter gene. Heat shock proteins are also called stress proteins, which represent one of the most primitive mechanisms of cellular protection in response to various types of environmental stresses like hyperthermia, oxygen radicals, transition metals, etc. [22]. Transgenic *C. elegans* carrying reporter genes under heat shock promoter control express reporter products under stressful conditions such as environmental contamination, microwave radiation, and immunological attack [23]. A pioneering study utilizing *hsp* controlled transgenic *C. elegans* to evaluate heavy metal toxicity was conducted by Stringham and Candido [21], in which a transgenic line of *C. elegans* carrying the fusion of the *hsp16* promoter to the
Escherichia coli lacZ reporter gene was developed. The authors found that reporter gene expression always occurred below the median lethal concentrations (LC50s) of the metals tested. Guven et al. [24] reported that several transition metals, including Cd, Zn, Hg, Mn, and Ag, caused dose-dependent transgene expression in C. elegans containing a Drosophila hsp70 promoter fused to lacZ. Transgenic strains using double reporter GFP and lacZ fused onto the hsp16 promoter have also been developed and used to monitor the effects of stressors, including heat, Cd, and microwave radiation [25].

In addition to laboratory-based studies, a few attempts have been made to apply transgenic C. elegans in metal contamination biomonitoring in field settings. For example, two different transgenic strains, PC72 (using a homologous hsp16 promoter) and CB4027 (using a heterologous Drosophila hsp70 promoter), have been used to monitor heavy metal pollution in water samples from a polluted river in England [16]. It was found that transgene (lacZ) response was inducible by mixtures of dissolved metals at concentrations actually encountered in metal-polluted watercourses. Although hsp transgenic systems are responsive to transition metals, they are induced by a wide variety of environmental stressors, and thus do not provide high specificity in heavy metal biomonitoring.

Metallothionein (MT) is a low-molecular-weight (~ 6000-8000 Da, 61 amino acid residues in mammal MTs), cysteine-rich (>30% content in mammal MTs) protein with a high affinity and binding capacity for transition metals [26]. It is ubiquitous in most animals and regulates homeostasis of essential metals, such as Zn and Cu, as well as sequesters and detoxifies toxic metals such as Cd, Hg, and Pb [27]. Due to their inducibility to transition metals, MTs are usually considered an important specific
biomarker to detect organism response to heavy metal pollutants such as Cd, Hg, Cu, Zn, etc., in aquatic environments [26]. The importance of MTs as a tool of biomonitoring is increased by the fact that they are ubiquitous proteins and therefore can be studied in most living organisms [28].

The nematode *C. elegans* has two isoforms of MT, *mtl-1* and *mtl-2* [29]. Transgenic lines of *C. elegans* incorporating genes for the two isoforms of MT, using a lacZ reporter system (*mtl-1::lacZ* and *mtl-2::lacZ*) have been constructed and tested for response to cadmium [30]. Upon cadmium treatment, the *mtl-2::lacZ* transgene was abundantly and exclusively expressed in the intestinal cells of both larvae and adult animals, and expression was not detected in the absence of metal. In contrast, the *mtl-1::lacZ* construct was constitutively expressed in the pharynx and induced by cadmium in the intestinal cells of *C. elegans* larvae; and the metal-inducible expression of *mtl-1::lacZ* was attenuated in adult nematodes. These findings suggested that *mtl-2* appeared to function in sequestration of toxic metals and protection from oxidative stress [20, 30], whereas *mtl-1* was more likely to be modulated by metals as well as developmental factors. These findings suggested the use of *mtl-2* transgene system to study metal toxicity. Cioci et al. [20] tested the toxicological response of the *mtl-2::lacZ* transgenic *C. elegans* to Cd, Hg, Zn and Ni. They demonstrated that the *mtl2::lacZ* transgene provides a more sensitive assay for metal exposure than conventional 24-h LC50 assays, as well as those transgenic systems containing hsp-based reporter transgenes. These MT transgene constructs, however, used *lacZ* as a reporter, which requires a lengthy, multi-step staining procedure to assess the reporter gene expression. The staining procedure requires death of the organisms, so time-course studies and multiple determinations are problematic.
An alternative reporter, green fluorescent protein (GFP), would avoid the problems mentioned above. GFP was originally isolated from the jellyfish *Aequorea victoria* that fluoresces green when exposed to blue light [31]. In a variety of modified forms, it serves as an excellent reporter of gene expression; and has been introduced and expressed in many types of organisms (e.g., bacteria, yeast, plant, fly, etc.) and mammalian cells. GFP expression is measured directly using fluorescence and does not require death of the organisms, staining, or other preparatory steps. Thus, a *mtl-2::GFP* transgene system takes advantage of not only the high specificity of the promoter for monitoring metal exposure but also the easy and quick quantitation of the reporter protein. Transgenic *C. elegans* using metallothionein promoters (both *mtl-1* and *mtl-2*) and GFP reporter have been developed and used to examine the functional importance of metallothioneins in cadmium trafficking [29]. A highly sensitive dose and temporal transcriptional response in the *mtl-2::GFP* transgenic *C. elegans* to Cd was observed from the Swain study. However, such transgenic strain, have not been applied to studies of heavy metal toxicity, which warrants a further exploration of this transgenic bioassay in heavy metal biomonitoring.

II. Ecotoxicology of manufactured nanoparticles

Manufactured nanoparticles, with sizes smaller than 100 nm in at least one dimension (SCENIHR), display unique electrical, thermal, mechanical, and optical properties because of their small size and high surface reactivity, which makes them highly desirable for applications within the commercial, medical, and environmental sectors [32]. Manufactured nanoparticles are currently produced in metric tons per year, and this quantity is expected to increase rapidly during the next decade [33]. However,
concerns about environmental, human health and safety issues related to the production, use and release of these nanomaterials have only been raised recently [34, 35].

Manufactured nanoparticles may be toxic because of their composition, shape, or unique size. Studies have suggested that nanoparticles are not inherently benign and can affect biota at the cellular, subcellular, and protein levels [36]. There has been a considerable body of knowledge on human health implications of manufactured nanoparticles. The literature on mammalian models was recently reviewed in the context of the environment and routes of human exposure to manufactured nanoparticles [37]. These reports have focused on respiratory toxicology and inflammation reactions to nanoparticles exposure (e.g., [38, 39]). In contrast, only recently have researchers begun to study the potential ecological risks and impacts of nanomaterials releases to the environment. At this early stage, most ecotoxicological studies have been observational experiments that document toxic effects and the concentrations of nanoparticles that produce these effects.

Several pioneering ecotoxicological studies on manufactured nanoparticles have focused on fullerenes (C60), a novel carbon-based nanoscale material of growing practical importance. Suspensions of nC60 have been reported to exhibit antibacterial activity, although the possible mechanisms responsible for such toxicity remain unknown [40-43]. Unlike some eukaryotic cells that can assimilate nanoparticles [44], bacteria generally cannot assimilate particles >5 nm, including nC60 [45]. Thus, antibacterial activity likely involves direct contact of nanoparticles with the cellular surface, which suggests that the surface chemistry and morphology of nanomaterials could be very influential factors in their toxicity [46].
Ecotoxicity of fullerenes has also been studied in aquatic species, and the majority of these studies have used *Daphnia*, an ecologically relevant species and key organism in regulatory testing [47]. An early study by Oberdörster [48] suggested that colloidal suspensions of fullerenes (C60) in water (nC60) may cause oxidative damage in the brains of largemouth bass. However, this is a very limited investigation as it cannot be ruled out that the effects observed were due to the toxic action of residual solvent (tetrahydrofuran, THF) used to dispense the fullerenes. Effect of THF on fullerene toxicity was later confirmed by Zhu et al [49] and Lovern et al [50], as both groups found that LC50 or EC50 in *Daphnia magna* was considerably lower for fullerenes prepared in THF than prepared by sonication or prolonged stirring. In this context, Oberdorster et al. [51] examined the acute toxicity of fullerenes in three invertebrate species *Daphnia magna*, *Hyal* *ella azteca*, and *Copepod*, by preparing the fullerene suspension by prolonged stirring in milliQ water. They found that LC50s could not be calculated because fullerene concentrations high enough to cause 50% mortality could not be reached in the exposure media. The authors concluded that acute toxicity of fullerenes to these invertebrates is not a likely scenario in most expected environmental releases. All these studies suggested that the preparation methods for solving/dispersing manufactured NPs may significantly influence the monitored toxic effects; therefore, caution should be taken during the selection for NPs sample preparation method and subsequent data interpretation.

The potential ecotoxicity of manufactured metal oxide NPs, such as TiO₂, ZnO, and Al₂O₃, have recently received great attention due to their extensive applications in a broad range of areas from electronics to personal care products. Manufactured NPs can
produce reactive oxidant species (ROS) upon interaction with organisms or with agents present in the environment (e.g., ultraviolet radiation) [52]. Generation of ROS and the subsequent oxidative damage is one of the largest concerns in nanoparticle mediated toxicity as shown for titania [53-55] and fullerene nanocrystals [56]. ROS generation is especially relevant for metal oxide NPs with photocatalytic properties such as TiO$_2$ [57] or ZnO [58] upon UV exposure. High concentrations of these radicals can cause cell injury by attacking DNA, proteins and membranes [59]. Antibacterial activity of certain photocatalytic metal oxide NPs (i.e., TiO$_2$ and ZnO) has been well recognized [60-64] and these NPs have been used as effective bactericidal materials [65, 66]. The mechanism underlying the antibacterial activity of ZnO has been outlined by Applerot et al [64]. It involves a reaction of the ZnO surface with water to generate ROS.

ROS generation may be dependent on particle size as it seems easier for smaller particles to induce oxidative damage than larger particles. For example, anatase-sized TiO$_2$ (10 and 20 nm) particles in the absence of photoactivation induced oxidative DNA damage, lipid peroxidation, and increased hydrogen peroxide and nitric oxide production in a human bronchial epithelial cell line, whereas the treatment with anatase-sized TiO$_2$ (200 and > 200 nm) particles did not induce oxidative stress in the absence of light irradiation [55]. Similarly, the study by Applerot’s [64] group found that the antibacterial activity of ZnO particles was strongly particle size dependent, with smaller particles having greater antibacterial activity. This size effect may be related to the surface area of nanoparticles, as a direct correlation between the ROS generating capability, the surface area of nanoparticles, and the inflammatory response in mammalian lungs has been established [67].
In addition to particle size, chemical composition of nanoparticles is another important decisive factor determining the formation of ROS in exposed cells or tissues. The presence of minute levels of transition metals Ti, Mn, Fe, Co (0.5 or 1.6 wt %) in relatively inert silica nanoparticles strongly changed the reaction of cultured human lung epithelial cells exposed to these materials if compared to cultures exposed to pure silica or reference cultures without particles [68]. For the silica with 1.6 wt % manganese oxide, the presence of the transition metal induced an up to 25 times increased ROS levels. The authors also conducted a systematic investigation of a series of eight iron-containing silica nanoparticles (0-10 wt % Fe/SiO₂) on their ROS generation and oxidative damage to the cells, and found that the presence of iron in the silica nanoparticles showed significant influence on ROS generation; However, there is an absence of a purely stoichiometric adverse effect on the cultures, i.e., a maximum ROS induction was observed for particles containing 1-5 wt % Fe in silica, whereas pure iron oxide even showed a statistically weaker ROS induction than doped silica. This unexpected, non-mass correlating effect can be correlated to the increased catalytic activity of transition metal sites consisting of single or few transition metals ions incorporated as clusters in an inert matrix [68]. These findings indicate that a proactive strategy for nanomaterial manufacturing and their risk assessment should consider chemical composition beyond a mere focus on physical properties such as size, shape, and degree of agglomeration.

Besides ROS generation, an important consideration concerning ecotoxicity of metal oxide NPs is the possible dissolution of the nanoparticles. Given the well-known toxicity of the ionic forms of some metals [69], the solubility of metal oxide NPs may
have great influence on their toxicity. For example, Brunner et al. [70] observed that material solubility strongly influenced cytotoxicity of several metal oxide NPs, with more soluble compounds like ZnO and FeO showing greater acute toxicity than those of lower solubility such as TiO₂ and CeO₂. A comparative toxicity study on ZnO NPs, bulk ZnO, and ZnCl₂ by Franklin et al. [71] revealed that toxicity of ZnO NPs to a freshwater alga *Pseudokirchneriella subcapitata* was attributable solely to dissolved zinc. Wang et al. [72] also reported that toxicity of ZnO NPs or bulk ZnO to the nematode *C. elegans* was partially related to the releases of dissolved Zn²⁺. It was found that cytotoxicity of CdSe quantum dots (QDs) correlated with the liberation of free Cd²⁺ ions due to deterioration of the CdSe lattice, and the CdSe QDs could be rendered nontoxic when appropriately coated [73]. Therefore, particle solubility assessment should be performed during toxicity test for these metal oxide NPs.

It has been suggested that metal oxide NPs may elicit toxicity to cells through a Trojan- horse mechanism: cell membranes provide a selective barrier against ions, preventing the dissolved metal salts from entering. However, once a metal-containing nanoparticle has penetrated a cell, metal ions can leach from the particle and generate ROS in the cell interior (http://www.nanowerk.com/news/newsid=1835.php). The study by Limbach et al. [68] demonstrated the role of nanoparticles as carriers for heavy metal uptake as the particles could efficiently enter the cells by a Trojan- horse type mechanism and provoked an up to eight times higher oxidative stress for Co₃O₄ or Mn₃O₄ as compared to reference cultures exposed to aqueous solutions of the same metals. Therefore, nanoparticle uptake into cells can be significantly increased by this Trojan- horse type mechanism, which can consequently increase the damaging action of such
materials, especially for partially soluble nanomaterials such as Co$_3$O$_4$. This Trojan-horse type mechanism should be taken into consideration when evaluating toxicity of partially soluble metal-containing nanomaterials.

Several studies have investigated ecotoxicity of metal oxide NPs to bacteria [63, 74, 75]. ZnO NPs could damage *Escherichia coli* by causing membrane disorganization, which led to accumulation of ZnO NPs in the bacterial membrane and also cellular internalization of these nanoparticles [75]. A comparative ecotoxicity study on nanosized TiO$_2$, SiO$_2$, and ZnO water suspensions using both gram-positive and gram-negative bacteria as test organisms reported that antibacterial effects increased from SiO$_2$ to TiO$_2$ to ZnO [74]. Another interesting finding from this study was that although all the NPs tested are capable of producing toxic ROS in the presence of light, the inhibitory effects observed under dark conditions suggest that additional, yet undetermined mechanisms might contribute to toxicity.

Toxicity of metal oxide NPs in aquatic species has also been studied. Most of the available acute toxicity data are on freshwater species, and mainly on species used for regulatory toxicology (e.g., *D. magna*, Lovern et al., 2006). The Lovern et al. [50] study found that TiO$_2$ caused an increase in mortality in *D. magna* with an increase in concentration. Behavioral and physiological responses of *D. magna* exposed to sublethal TiO$_2$ NPs concentrations have also been reported [76]. Most sub-lethal studies have been conducted on freshwater fish. Federici et al. [77] investigated toxicity of TiO$_2$ NPs to the main body systems of rainbow trout in terms of gill injury, oxidative stress, and other physiological effects. It was found that TiO$_2$ nanoparticles were not a major ionoregulatory toxicant, or haemolytic at the concentration and exposure times used;
however, respiratory distress and oxidative stress were observed. A more recent study by Zhu et al. [78] used the zebrafish 96-h embryo-larval bioassay to assess and compare the developmental toxicities of nano-sized ZnO, TiO$_2$, and Al$_2$O$_3$ aqueous suspensions, and their bulk counterparts. Toxicological endpoints used included embryo or larvae survival, hatching rate and malformation. The authors found that nano-sized ZnO displayed higher toxicity than TiO$_2$ or Al$_2$O$_3$.

In contrast, published reports on the ecotoxicity of manufactured NPs to soil invertebrates and other terrestrial invertebrates appear to be lacking, although these species consist of a significant portion of potential ecological recipients of NPs disposed to the environment. Toxicity of double-walled carbon nanotubes (DWNT) and C60 fullerenes to the soil-dwelling earthworm *Eisenia veneta* was assessed using both lethal and sublethal endpoints [79]. It was found that reproduction was the most sensitive endpoint, and the DWNT showed a dose-dependent effect on the reproduction of *E. veneta*; whereas there was no effect on mortality at up to 495 mg DWNT/kg and 1000 mg C60/kg dry food. Wang et al. [72] conducted a study of the toxicity of nanoparticulate and bulk ZnO, Al$_2$O$_3$ and TiO$_2$ to the nematode *Caenorhabditis elegans*, and found that both NPs and their bulk counterparts were toxic, inhibiting growth and especially the reproductive capability of the nematode. Given the ecological relevance of *C. elegans* as well as its advantageous traits as an excellent model for ecotoxicology, *C. elegans* may serve as an excellent model for toxicity studies on metal oxide NPs, as has been used in ecotoxicological studies for conventional environmental toxicants including metals.
An important consideration during risk assessment of manufactured nanomaterials is the interaction of NPs with other environmental agents, which may alter the properties and toxic effects of the NPs or the other agents. A good example of such kind of interaction is between NPs and natural organic matter (NOM). As a ubiquitous component of aquatic systems, NOM may influence the surface speciation and charge of NPs, and thus affect their aggregation/deposition properties, and consequently affect their environmental fate and toxic effects. Navarro [52] has suggested that the formation of larger aggregates by high molecular weight NOM compounds will favor the removal of engineered NPs into sediments and is likely to decrease their bioavailability. In contrast, solubilization by natural surfactants such as low-molecular-weight NOM compounds will increase their mobility and further the bioavailability of engineered NPs. Besides NOM, several artificially synthesized organic compounds have been sued to stabilize manufactured NP suspensions in aquatic systems [80, 81]. Manufactured NPs may also interact with other pollutants in the environment. Attributed to their remarkably high surface area to volume ratio and complexing capability, they may adsorb pollutants, which can change the transport and bioavailability of both the NPs and the pollutants, and alter their toxic effects. For example, the presence of manufactured TiO₂ NPs has been shown to increase the accumulation of cadmium and of arsenate in carps because of their strong sorption capacity for these elements [82, 83]. Furthermore, manufactured NPs especially oxide and oxide-coated NPs might alter trace-metal ion speciation, and thus their bioavailability and potential toxicity [52].

As ecotoxicological study of metal oxide NPs is still at its infancy, literature is being generated at an increasing rate. Toxic effects have been identified in a range of fish
and invertebrates, which raises sufficient concern that NPs in the environment, if present at high enough levels, could have adverse effect on ecological receptors [47]. There are several significant knowledge gaps in the ecotoxicology of NPs. For example, as most existing studies focused on aquatic species, information is generally lacking on ecotoxicity in terrestrial species. Furthermore, the studies reported so far are largely observational, and use high doses to ensure biological effect (“proof of principle” experiments). More sophisticated studies are required to gain mechanistic understanding of the toxic effects observed. Our knowledge of the effects of abiotic factors on the ecotoxicity of NPs is poor, and effects of factors such as pH, hardness, the presence of natural organic matter or other colloid materials also needs to be investigated.

References


Journal of Molecular Biology 341:951-959.


[77] Federici G, Shaw BJ, Handy RD. 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout (Oncorhynchus mykiss): Gill injury, oxidative stress, and other physiological effects. *Aquatic Toxicology* 84:415-430.


*Environmental Toxicology & Chemistry* 28:1311-1318.

*Environmental Toxicology and Chemistry* 16:245-250.


Table 2.1 Overview of laboratory-based environmental toxicological studies using *C. elegans*.

<table>
<thead>
<tr>
<th>Exposure Medium</th>
<th>Endpoint (test duration)</th>
<th>Chemicals tested</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>Lethality (24-96 h)</td>
<td>Ag, Hg, Be, Al, Cu, Zn, Pb, Cd, Sr, Cr, As, Tl, Ni, Sb Cd, Be, Pb</td>
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<tr>
<td></td>
<td>Movement (24-96 h)</td>
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<tr>
<td></td>
<td>Reproduction (24 h)</td>
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<td></td>
</tr>
<tr>
<td>Aqueous</td>
<td>Lethality (24 h)</td>
<td>Cu, H, Pb, Cd, pentachlorophenate various</td>
<td>[6]</td>
</tr>
<tr>
<td></td>
<td>Reproduction (96 h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lethality (72 h)</td>
<td>Cu, Cd, Ca, Ni, Hg, Mg, Mn, Pb, Zn</td>
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<tr>
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<td>Lethality (24 h)</td>
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<tr>
<td>Pore Water</td>
<td>Reproduction (72 h)</td>
<td>Cd</td>
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<td>Lethality (24 h)</td>
<td>Ethanol</td>
<td>[86]</td>
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<tr>
<td></td>
<td>Movement (24 h)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Reproduction (72 h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Movement (4 h)</td>
<td>Pb, Cu, Cd, Al, Zn</td>
<td>[87]</td>
</tr>
<tr>
<td>Aqueous</td>
<td>Lethality 24,48,72 h</td>
<td>Five gadolinium-based magnetic resonance imaging (MRI) compounds Cd, Cu, Pb</td>
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<tr>
<td></td>
<td>Movement</td>
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<tr>
<td>Aqueous</td>
<td>Movement (4 h)</td>
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<tr>
<td></td>
<td>Feeding (24 h)</td>
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<td>Growth (24 h)</td>
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<tr>
<td></td>
<td>Reproduction (72 h)</td>
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<td>Lethality (24 h)</td>
<td>DMSO, Cd</td>
<td>[88]</td>
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<td>Lethality (24 h)</td>
<td>Tannins</td>
<td>[89]</td>
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<td>Movement (4 h)</td>
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Table 2.1 (continued).

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<th>Chemicals tested</th>
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<td>Reproduction (96 h)</td>
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<td>[92]</td>
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<td>Lethality (24 h)</td>
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<td>[93]</td>
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<td>Sediment/</td>
<td>Growth (72 h)</td>
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<td>[3]</td>
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<td>Pore Water</td>
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<tr>
<td>Soil</td>
<td>Lethality (24 h)</td>
<td>Cd, Cu, Pb, Ni, Zn (Cl vs. NO₃ salts)</td>
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<td>Lethality (24 h)</td>
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<td>Growth (72 h)</td>
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<tr>
<td>Soil</td>
<td>Lethality (24 h)</td>
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<td>[90]</td>
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<td>Soil</td>
<td>Lethality (24-48 h)</td>
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<td>Hsp16-lacZ transgene, lethality</td>
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<td>Hsp70::lacZ transgene</td>
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<td>Hsp16-lacZ transgene</td>
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<td>[97]</td>
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<td>Mtl-2::lacZ transgene</td>
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<td>hsp16-GFP-lacZ transgene</td>
<td>Cd, microwave radiation</td>
<td>[25]</td>
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<td>HSP-16 reporter</td>
<td>Methanol, ethanol, isopropanol, iso-butanol</td>
<td>[98]</td>
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<td>Feeding Reproduction</td>
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<td>Lethality</td>
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<tr>
<td>Aqueous</td>
<td>Hsp16::GFP</td>
<td>Cd, Cu, Zn</td>
<td>[15]</td>
</tr>
<tr>
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<td>Cd, Pb, Cr, As</td>
<td>[19]</td>
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<tr>
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<td>Mtl-2::GFP</td>
<td>Cd, Hg, Cu, Zn, Ni, Pb, As</td>
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<th>Field site</th>
<th>Environmental samples</th>
<th>Overview</th>
<th>References</th>
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<td>Water</td>
<td>Use transgenic strains of <em>C. elegans</em> to assess metal contamination</td>
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<td>Wastewater treatment process (Georgia)</td>
<td>Industrial and municipal wastewater</td>
<td>Used <em>C. elegans</em> 72-h lethality test to identify sources of water contamination and effectiveness of water treatment</td>
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<td>Elbe River (Germany)</td>
<td>Sediments</td>
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CHAPTER 3

A TRANSGENIC STRAIN OF THE NEMATODE *CAENORHABDITIS ELEGANS* AS A BIOMONITOR FOR HEAVY METAL CONTAMINATION

Abstract

Metallothionein (MT), a protein involved in metal regulation and detoxification, has been used widely as a biomarker of metal exposure. In the present study, a transgenic strain of the free living soil nematode Caenorhabditis elegans was developed using the C. elegans MT-2 (mtl-2) promoter to control the transcription of green fluorescence protein (GFP) reporter. Response of this transgenic system to Cd, Hg, Cu, Zn, Ni, Pb, and As exposure in aquatic media was tested by quantifying GFP expression after 24 h of exposure. Response in Cd-spiked soil was tested in a similar manner. The mtl-2 transcription also was measured using real-time reverse transcription–polymerase chain reaction to gain a mechanistic understanding of the transgene expression. Green fluorescence protein is induced by Cd, Hg, Cu, and Zn in a time- and concentration dependent manner; mtl-2 transcription is consistent with the GFP response. The minimum concentrations of Cd, Hg, Cu, and Zn that induce GFP response are 2- to 1,000- fold lower than concentrations affecting traditional endpoints, such as lethality or behavioral change. The system responds to Cd in soil in a similar manner. Neither Ni nor Pb induces GFP, and neither induces mtl-2 transcription. Arsenic does not induce GFP, yet an increase in mtl-2 transcription was found, suggesting that As may interfere with GFP signaling. This mtl-2::GFP transgenic bioassay represents an alternative approach to quantify, both easily and quickly, a surrogate of MT in response to metal exposure (e.g., Cd, Hg, Cu, and Zn) in a variety of environments and potentially may be used for quantitative or semiquantitative biomonitoring of metal contamination in soils and aquatic systems.
Introduction

Heavy metal contamination may threaten both human and ecological health. Conventional chemical and physical methods can measure metal concentrations but do not address metal bioavailability or biological effects directly. Biomonitoring represents a more holistic approach to evaluate bioavailability and toxicity by directly measuring an organism’s biological responses to contaminant exposure; thus, it is a potentially powerful tool for detecting and documenting exposure to, and effects of, environmental contaminants [1]. The free-living nematode Caenorhabditis elegans has been widely adopted as a model organism in a variety of research areas because of its thoroughly understood biology and ease of culture. Toxicity tests using C. elegans have been developed, standardized, and used to evaluate metal toxicity in both soil and aqueous matrices. Assays employing lethality and sublethal endpoints, including behavior, growth, reproduction, and feeding, have been developed and applied for environmental biomonitoring [2-9].

Gene expression endpoints are sensitive and useful in estimating the effects of toxicants on exposed populations, and they also provide insight regarding the mechanisms underlying these effects. In many cases, results from transgenic biomonitors can be obtained within hours of exposure. Transgenic strains of C. elegans have been developed for a variety of purposes, including applications in environmental toxicology. In many such strains, a gene of interest is coupled to a reporter gene that also is expressed when the gene of interest is expressed. Expression is then quantified by measuring the amount of reporter protein. To date, transgenes responsive to metals that have been constructed for C. elegans can be classified into two categories based on the promoters
used: One uses a heat shock protein (hsp) promoter to control a reporter gene, and the other uses a metallothionein (MT) promoter to regulate expression of a reporter gene. As an example of the former, a transgenic line of *C. elegans* carrying the fusion of the *hsp16* promoter to the *Escherichia coli lacZ* reporter gene has been developed and used to examine the effects of a wide variety of chemical stressors, including several transition metals [10]. In that study, reporter gene expression always occurred below the median lethal concentrations (LC50s) of the test substances. Similar transgenic lines have been used to monitor heavy metal pollution in water samples from a polluted river [11]. Guven et al. [12] reported that several transition metals, including Cd, Zn, Hg, Mn, and Ag, caused dose-dependent transgene expression in *C. elegans* containing a *Drosophila hsp70* promoter fused to *lacZ*. Another strain carrying a transgene of green fluorescent protein (GFP) reporter coupled to a *hsp16* promoter demonstrated that heavy metal stress occurs within 5 h of exposure [13]. Transgenic strains using double-reporter GFP and *lacZ* fused onto the *hsp16* promoter also have been developed and used to monitor the effects of stressors, including heat, Cd, and microwave radiation [14]. Because the hsp genes are induced by a wide variety of environmental stressors, those hsp-regulated transgene systems do not provide specificity in environmental biomonitoring for metals.

Metallothionein-regulated transgene systems represent a more specific biomonitor for metal exposure. Metallothionein is a low-molecular-weight, cysteine-rich protein with a high affinity for metals such as Hg, Zn, Cu, and Cd. It is ubiquitous in most animals and regulates homeostasis of essential metals, such as Zn and Cu, as well as sequestering and detoxifying transition metals [15]. The nematode *C. elegans* has two isoforms of MT, *mtl-1* and *mtl-2* [16]. Transgenic lines of *C. elegans* incorporating genes for the two
isoforms of MT, using a lacZ reporter system (mtl-1::lacZ and mtl-2::lacZ) have been constructed and tested. The mtl-2 isoform is induced in the gut in response to Cd during all life stages, and it appears to function in sequestration of toxic metals and protection from oxidative stress [7, 17]. In addition, Cioci [7] has suggested that C. elegans containing mtl-2::lacZ transgenes can function as sensitive toxicological indicators of metals. These MT transgene constructs, however, used lacZ as a reporter, which requires a lengthy, multistep staining procedure to assess the reporter gene expression. The staining procedure requires death of the organisms, so time-course studies and multiple determinations are problematic. An alternative reporter using GFP avoids these problems. The GFP expression is measured directly using fluorescence and does not require death of the organisms, staining, or other preparatory steps. Thus, a mtl-2::GFP transgene system takes advantage of not only the high specificity of the promoter for monitoring metal exposure but also the easy and quick quantitation of the reporter protein.

The present study reports on the development and testing of a mtl-2::GFP transgenic C. elegans in aqueous and soil matrices. The mtl-2::GFP construct is similar to, but independent of, that reported by Swain et al. [16]. In particular, we did not use the rol-6 mutation/rescue system, because it affects mobility of the worms and, thus, would limit the use of behavior as an assay for toxicity testing. Instead, we used the lin-15 strain of the N2 Bristol strain. Our exposure scenarios also are different, because our goal was to investigate the response of this transgenic system to exposure to metals (e.g., Cd, Hg, Zn, Cu, Ni, Pb, and As) and then compare this assay to traditional ecotoxicological endpoints, such as lethality and behavioral changes. To verify the assumption that expression of GFP is proportional to the expression of MT and, thus, that GFP can be
used a surrogate for metal-induced MT for biomonitoring, we also measured the levels of mtl-2 transcription in worms after exposure to each metal using realtime reverse transcription polymerase chain reaction (RTPCR). Finally, we considered the capability and potential applications of this transgenic bioassay in environmental biomonitoring.

Materials and Methods

Development of the mtl-2::GFP transgenic C. elegans

The transgenic strain was developed in 2004, as reported by Humphries (2004, Master’s thesis, University of South Carolina, Columbia, SC, USA). Briefly, the mtl-2::GFP transgene (324-bp promoter region of the mtl-2 gene) was inserted in a plasmid vector (L3781, pPD 118.15; a gift from Fire [18]). The plasmid was then coinjected with a pL15EK rescue gene plasmid into the adult gonad of the phenotypic mutant C. elegans lin-15, which expresses multiple genital pores. Offspring of injected worms were screened for normal phenotype, which indicates successful incorporation of the rescue gene. Normal-appearing individuals were collected and screened for GFP, which indicates the possible presence of the MT vector. The transgene was integrated into the genome by gamma-irradiating the selected young adult C. elegans to cause chromosomal breakage and repair. During repair, some copies of plasmid DNA were incorporated into the DNA of developing eggs. Integrants showing strong GFP fluorescence were selected and backcrossed to wild-type N2 males for a total of seven times to achieve a stable line with desired properties. This final, established line also was assayed by PCR to verify the presence of the mtl-2::GFP transgene.

Toxicity testing in aqueous media

The 24-h LC50s were measured for six metals (Cd, Cu, Hg, Ni, Pb, and Zn) and one metalloid (As) using both wildtype N2 and the mtl-2::GFP transgenic C. elegans. The N2 worms were obtained from the Caenorhabditis Genetics Center. Four-day-old worms,
from age-synchronized cultures prepared using the methods described by Donkin and Williams [2], were exposed to test conditions in 24-well plastic plates. Five or six concentrations plus a control were tested for each metal, with three wells per concentration. Ten nematodes were added to each well and then exposed for 24 h without food. The test was independently repeated three times for each metal. All metals used were reagent-grade chloride salts except for Ni and As, which were in the forms of sulfate and sodium arsenite, respectively (Sigma-Aldrich). All metal solutions were prepared in K-medium (0.051 M NaCl and 0.032 M KCl [19]), because nematodes suffer osmotic stress in deionized water. At the end of the exposure period, nematodes were counted and scored as live or dead; they were judged to be dead if they did not respond to touch using a small, metal wire.

**mtl-2::GFP transgene expression and quantitation**

Five to eight sublethal concentrations were selected for each metal based on the LC50 results. In general, a concentration of 5 to 25% of the LC50 for each metal was selected as the upper limit of the concentration range used for the GFP induction experiment. Costar plates with 96 wells, a clear bottom, and black sides (Corning Incorporated Life Sciences) were used for exposure and measurement. Nematodes (n = 35 ±1) and test solution (250 μl) were added to each well, with three wells per concentration, and incubated at 20°C. At a series of time points, GFP intensity was measured using a Synergy HT microplate reader (BioTek Instruments) with a 485-nm excitation filter and a 528-nm emission filter. The GFP intensity is reported as relative fluorescence units based on calibration using a standard recombinant EGFP (BioVision). The assay for each metal was independently repeated three times.
Aquatic exposure for quantitation of \textit{mtl-2} transcription

The procedure for \textit{mtl-2} quantitation was similar to that described above except that a large quantity of worms (~0.1–0.25 ml of worm pellet) and 24-well tissue culture plates were used. Because of the labor intensity and cost of the assay, only one concentration of each metal was analyzed. After 24 h of exposure, the metal solution was removed, and worms were washed at least three times with M9 buffer, then immediately frozen at -80°C until RNA extraction and quantitative PCR analysis.

\textit{Reverse transcription–polymerase chain reaction}

Total RNA was extracted using RNeasy Mini Kit (Qiagen). The RNA concentrations were determined spectrophotometrically using NanoDrop ND-1000 (NanoDrop Technologies), and RNA integrity was verified by an absorption ratio of optical density at 260 nm to optical density at 280 nm of greater than 1.95. Total worm RNA (~1 µg) was reverse-transcribed using iScript\textsuperscript{TM} Select cDNA Synthesis Kit (Bio-Rad Laboratories), and real-time PCR was performed using the primers of J.D. Coolon et al. (unpublished data) for the target gene \textit{mtl-2} (forward primer, 5-
CTGCCAGTGAGAAGAAATGC-3; reverse primer, 5-
CGAACAATATCAATTAGTAGGAATTTG-3) and reference gene \textit{ubq-1} (forward primer, 5-
CACTTGGTTCTTCGTCTTAG-3; reverse primer, 5-
CCTCCTTGTCTTGAATCTTG-3). Real-time PCR was run at the optimized annealing temperature of 58°C on a Mastercyclerep realplex thermocycler (Eppendorf) using ABsolute QPCR SYBR Green Mix (Thermo Fisher Scientific). The relative quantification of the \textit{mtl-2} gene in comparison to the reference \textit{ubq-1} gene was determined using the method described by Pfaffl [20], and the final results were
expressed as the relative expression ratio (between target gene and reference gene) in the treatments as compared to the ratio in the control.

**mtl-2::GFP transgene expression with metal exposure in soil**

Cecil soil, a well-characterized Ultisol collected from the Piedmont region of Georgia (USA), was spiked with a range of Cd concentrations and allowed to equilibrate for 7 d. Soil moisture was adjusted to 35%, and soils were placed into 100×15-mm, polystyrene Petri dishes for exposure. Approximately 100 nematodes were added to each soil dish and exposed for 24 h. Nematodes were then recovered using centrifugation/flotation in a colloidal silica suspension as described previously [3]. Recovered organisms were rinsed three times with K-medium, then transferred to 96-well plates for GFP quantification as described above.

**Data analysis**

Data in the figures are expressed as the mean ± standard deviation of three independent experiments unless otherwise noted. Median lethal concentrations were calculated by probit analysis using Toxstat 3.2 software (Statistics Unlimited). Comparison of LC50s between wild type N2 and the mtl-2::GFP transgenic strain for each metal were performed by oneway analysis of variance using SAS® (Ver 9.1; SAS Institute). The minimum detectable increase in GFP intensity relative to controls was calculated using Tukey’s multiple comparisons in SAS. The lowest metal concentration where the GFP fluorescence was significantly higher (α=0.05) than in controls was considered to be the minimum detectable response concentration for mtl-2::GFP induction.
Results

Comparison of lethality: Transgenic and wild-type N2 C. elegans

The LC50s for the six metals and As in wild-type N2 and mtl-2::GFP transgenic C. elegans were determined to test whether transgenic and wild-type nematodes had significantly different sensitivities to acute metal toxicity. All LC50s were comparable to those reported previously for wild-type C. elegans [8]. No statistical difference in LC50s was found between the two strains except for Pb and As (Fig. 3.1), for which the LC50s in the transgenic strain were lower than those in wildtype N2 (p<0.05). Despite their statistical significance, these differences (5,350 vs 2,944 μM As and 172 vs 113 μM Pb for wild type vs transgenic, respectively) may not represent a meaningful difference from a biological standpoint.

mtl-2::GFP transgene response to Cd, Hg, Cu, and Zn

Visual examination of the exposed organisms under a fluorescent microscope indicated a strong GFP signal in the intestinal region for four of the seven elements tested (Cd, Hg, Cu, and Zn). Expression of mtl-2::lacZ transgene to these metals exclusively in intestinal cells was reported previously by Cioci [7]. The intensity of mtl-2::GFP expression varied in a time- and concentration-dependent manner (Fig. 3.2a to d). Significant GFP expression was detected after 4 h of exposure and continued to increase with exposure time. Based on these observations, the standard exposure time in the present study was set at 24 h, both because GFP expression is abundant at this time and because 24 h approaches the maximum time that C. elegans can survive without stress when no food source is available. A statistically significant increase (p<0.01) in GFP was measured with increasing metal concentration for all four metals within the
concentration range tested. At an identical molar concentration, Cd and Hg induced higher transgene expression than Cu and Zn. Statistical analysis revealed that the minimum concentrations of metals that produced a GFP significantly greater than that of controls were 5 μM Cd, 1 μM Hg, 50 μM Cu, and 250 μM Zn.

**mtl-2::GFP transgene response to Ni, Pb, and As**

Neither Ni, Pb, nor As exposure induced significant GFP expression, as shown in Figure 2e to g. Nickel as high as 1,200 μM did not induce a detectable GFP increase compared to control organisms after 24 h of exposure; in fact, GFP expression compared to controls decreased somewhat over the exposure period (Fig. 3.2e). Although GFP increased slightly as Pb concentration increased, this increase was not statistically significant. In all tests, control worms had background GFP expression with a magnitude of 100 to 200 relative fluorescence units; also, controls had a slightly increased background GFP expression after 24 h of exposure.

**mtl-2 transcription response to metal exposure**

Changes in *mtl-2* transcription, presented as fold-change after 24 h of exposure to each metal, are shown in Figure 3.3. Nematodes exposed to Cd, Hg, Cu, Zn, and As exhibited upregulation of *mtl-2* transcription, with fold-changes in treatment being significantly greater than those in controls (*p*<0.05), whereas Pb and Ni were down-regulated, with foldchanges in treatment being significantly less than those in controls (*p*<0.05).

**mtl-2::GFP transgene response to Cd in soil**

In Cd-spiked soils, a significant increase in GFP induction occurred with increased Cd concentration from 10 to 100 μM (Fig. 3.4). The organisms exposed to 10 μM Cd in
soil did not have significantly higher GFP fluorescence than the control organisms, but GFP was significantly higher in exposed organisms than in controls at the other soil concentrations tested.

**Discussion**

Metallothionein plays an important role in detoxification of many transition metals. In general, metal toxicity results from two major categories of insults: Replacement of essential metals at protein active sites by toxic metals, and the generation of reactive oxygen species that damage macromolecules, including DNA and proteins, and thus impair cellular function [13]. As a protective mechanism in response to such insults, cells modulate expression of genes encoding stress-response proteins. For example, Cd has been shown to increase the expression of most stress-related genes, including *hsp16* and *mtl-2* in *C. elegans* [21]. The induction of MT gene expression has been suggested as a potential indicator of metal contamination in the environment [22], and transgenic systems using readily measurable reporter gene expression (e.g., GFP and *lacZ*) have provided an excellent alternative for MT protein or mRNA measurement. In the present study, we test a *mtl-2::GFP* transgenic strain of *C. elegans* with six metals and As. Four of the six metals—namely, Cd, Hg, Cu, and Zn—induce GFP expression in a time- and concentration-dependent manner. The minimum concentration of Cd that produces a detectable response from control over a 24-h time period is 5μM, which is comparable to the 2.5 μM Cd that has been reported in another *mtl-2::GFP* transgenic strain using a semiquantitative PCR technique [16]. As suggested by many studies, Cd is an excellent MT inducer, and the induction of MT plays an essential role in protecting the organisms/cells from Cd toxicity. Our findings confirm this, given that Cd has an aquatic LC50 as high as 15,000 μM in *C. elegans*. Mercury induces GFP in the transgenic nematodes with fluorescence intensity comparable to that of Cd, yet at considerably lower concentrations. The minimum concentration that produces a detectable increase in fluorescence is 1 μM Hg. This is consistent with the observation that the LC50
of Hg is thousands of times lower than that of Cd. Metallothionein has been found to play a significant role in protecting against the cytotoxic effects of various forms of Hg, including elemental Hg vapor [23] and Hg\(^{2+}\) [24].

The GFP induced by Cu or Zn is considerably lower than that with either Cd or Hg, and higher concentrations of Cu and Zn are required to induce the levels of GFP produced by relatively low concentrations of Cd. Other studies also have found that Zn and Cu were poor MT inducers compared to Cd in *C. elegans* [16]. It may be that MT is more critical for Cd detoxification than for Zn or Cu detoxification. As essential elements, Zn and Cu are actively regulated in many species of aquatic invertebrates, although the regulation strategies differ among species [25]. Cadmium is not an essential element, and MT likely serves a solely protective function with Cd exposure. A study of Cd toxicity in rats has shown that MT is the major protein synthesized to protect against Cd toxicity, with 85% of Cd in renal cytosol bound to MT after Cd exposure; in contrast, only 40 to 45% of Zn or Cu was bound to MT, suggesting the importance of other stress-inducible regulatory proteins [26]. Although those findings were based on mammalian responses, similar systems may exist in *C. elegans*. Higher minimum detectable concentrations of Zn and Cu suggest that the sensitivity of this transgenic bioassay for Zn or Cu is not as high as that for Cd or Hg.

The MT transcriptional response, as indicated by *mtl-2* transcription to the four metals (Cd, Hg, Cu, and Zn), is consistent with the GFP response described above. After 24 h of exposure, all four metals induced elevated *mtl-2* transcription compared to controls, with Cd and Hg inducing greater transcription than Cu and Zn. This confirms that Cd and Hg are better MT inducers than Cu and Zn, as suggested by other studies with *C. elegans* [16]. These data verify our proposition that a positive correlation exists
between the expression of GFP reporter and the transcription of the gene of interest, \textit{mtl-2}. Thus, GFP can be used as a surrogate for metal-inducible MT transcription in this \textit{mtl-2::GFP} transgenic strain of \textit{C. elegans}, although the exact correlation coefficient between the GFP reporter and MTL-2 protein remains unknown.

Neither Ni nor Pb induces GFP expression in these \textit{mtl-2::GFP} transgenic nematodes, and neither induces \textit{mtl-2} transcription. Previous work has suggested that Ni induces expression of -\textit{galactosidase reporter (LacZ) in a mtl-2::lacZ transgenic strain [7]. Failure to detect the mRNA of mtl-2 after 24 h of exposure to Ni in the present study may not necessarily indicate that Ni does not induce \textit{mtl-2} transcription; it may be that transcription has occurred but that the mRNA has been degraded after 24 h. A time-course study will be needed to clarify this. Furthermore, because the Ni concentrations used in the present study (50–1,000 \text{ \textmu M}) are quite different from those of Cioci’s study (10–200 \text{ \textmu M}), it is difficult to make direct comparisons between the two transgene systems regarding their response to Ni. Nevertheless, these findings suggest that this \textit{mtl-2::GFP} transgenic \textit{C. elegans} cannot be used to biomonitor Ni or Pb.

Although As does not induce GFP in the \textit{mtl-2::GFP} strain, \textit{mtl-2} transcription is induced (Fig. 3). A possible explanation could be that the cloned length of \textit{mtl-2} promoter lacks distant upstream transcription binding sites, enhancers, or repressors; therefore, the GFP expression would not be transcriptionally controlled. The native transcript, however, can be detected by PCR. Alternately, it may be that As interferes with auto-oxidation and/or protein-folding processes that are necessary for GFP to become fluorescent. Similar findings have been reported in previous studies: Candido [27] demonstrates strong \textit{hsp16-1::lacZ} response to As, whereas the response of gene expression levels in a
transgenic strain using the same promoter *hsp16-1* but a GFP-based reporter is not sensitive [21]. Thompson and de Pomerai [28] found a similar problem in relation to alcohol toxicity: Using the *hsp16-1::GFP::lacZ* double-reporter strain, *lacZ* induction was strong whereas GFP signals were inconsistent, and expression levels were sometimes reduced at higher test concentrations. All these findings suggest that GFP reporters may not be as sensitive as *lacZ* for certain toxicants.

When using *C. elegans* for metal biomonitoring, a major advantage of the *mtl-2::GFP* transgenic bioassay is the great improvement in sensitivity compared to conventional toxicological endpoints. For metals that induce GFP expression, a significant increase in fluorescence occurs at concentrations much lower than the LC50s and median effective concentrations (EC50s) (Table 3.1). For example, the lowest-observed-effect concentration (the minimum concentration that causes a significant response compared to the control) for Cd in this *mtl-2::GFP* transgenic system was 5 μM, which is 18-fold lower than that for behavior change [8] and 1,000-fold lower than that for lethality. This confirms that the *mtl-2::GFP* transgenic strain provides a very sensitive approach for Cd biomonitoring, as suggested by other studies [7, 16]. Likewise, the response of GFP induction for Hg is 10-fold more sensitive than behavioral change and 15-fold more sensitive than lethality. It should be noted that these ratios of the lowest-observed-effect concentrations only represent an estimate for the difference in sensitivity among different endpoints, and these ratios can vary, depending on the concentrations selected in a particular experiment. Detection for Zn and Cu is not as sensitive as that for Cd or Hg, with minimum detectable concentrations of 250 μM Zn and 10 μM Cu. These concentrations, however, are still considerably lower compared to
the EC50s or LC50s for these elements. In each case, MT induction is related, both
directly and causally, to metal exposure and, thus, represents a mechanistically relevant
response to environmental metals.

Results from experiments with Cd-spiked soil in the laboratory demonstrate that this
assay also works well in soil matrices, although the minimum concentration of Cd that
produces a detectable increase in GFP fluorescence is higher than that in aquatic media.
Soils are far more complex than dilute aqueous media, which may influence Cd speciation
and bioavailability as well as organismal responses. For example, Cd can sorb to soil
particles or complex with soil organic ligands, altering bioavailability. Other trace elements
present in the soils may possibly modulate GFP expression. Further work is needed to
evaluate responses of the \textit{mtl-2::GFP} system to other metals in soils, including Hg, Cu, and
Zn. The concentrations of metals in soil that produce detectable increases in GFP
fluorescence likely will be higher than those in aquatic media. This expectation is based on
the observation that lethal and behavioral responses of \textit{C. elegans} to various metals occur at
higher concentrations in soils than in waters [9]. Indeed, this transgenic nematode will
facilitate investigation of bioavailable metals in a variety of simulated and real-world soils to
determine their characteristics and how their constituents interact to produce toxicity.

It should be noted that background GFP expression always occurs in this \textit{mtl-2::GFP}
transgenic \textit{C. elegans}, even when the organisms are not exposed to any metals or
stressors. Because the \textit{mtl} 2 gene is native to \textit{C. elegans} and is closely associated with
homeostasis of essential metals, some level of MTL-2 and, thus, of GFP may be
constitutively expressed. Another possibility is that this basal level of expression could
simply reflect leaky expression, because upstream transcription binding sites, enhancers,
or repressors are missing. Nonetheless, as long as this baseline expression remains stable
and quantifiable, it does not impair or mask the response to metals of interest. We have
found that this GFP background is constant over time both within and among experiments. We also notice that GFP increases in control nematodes over a 24-h exposure period, with a magnitude of approximately 100 to 200 relative fluorescence units (Fig. 3.2). Transcription of MT is controlled not only by cell-specific factors and metal-responsive pathways but also by developmentally modulated pathways [29]. Three-day-old worms were used in the present study. At this age, the nematodes have just completed their final molt and entered the mature, adult life stage; moreover, a 24-h period represents a significant fraction of the life span of *C. elegans*, which typically lives for approximately 14 d. Changes in developmental and reproductive stages during a 24-h period may influence *mtl-2* transcription and, thus, GFP expression. Furthermore, stress associated with starvation during the 24-h exposure also may contribute to *mtl-2* transcription. As with the background GFP expression discussed above, if the change in GFP expression in controls over time is constant and relatively small compared to that induced by metals, then this response in controls should not compromise the sensitivity of the assay.

We independently repeated each metal exposure three times, and we observed considerable variation among the replicates. This could be attributed to biological variation, but *C. elegans* strains are inbred, with little genetic variation. Instead, the variation likely relates to the methods used for measuring fluorescence. When measuring total fluorescence in a group of individuals, the relative positions and movement of individuals during the measurement process affects the signal. We have tried different ways, including using larger numbers of worms per well, preparing multiple wells for exposure with each concentration, and taking multiple readings over time for each well, to minimize such effects. Counting large numbers of worms, however, can be cumbersome and time-consuming. We also measured GFP based on total protein, using tens of thousands of worms per exposure. This
method yielded a concentration–response relationship almost identical to that obtained using smaller number of worms, and it did not significantly enhance the sensitivity of the assay. Future work, however, would benefit from use of sorters capable of handling eukaryotic organisms, such as the COPAS Biosorter, which can accurately deliver large and reproducible numbers of worms to individual wells of plates as well as measure the fluorescence of individual worms [30].

In summary, our results have demonstrated the $\textit{mtl-2::GFP}$ transgenic strain of $\textit{C. elegans}$ provides a rapid, noninvasive assay to measure MT induction in response to environmental metal exposure. This system can be employed to evaluate aquatic toxicity and bioavailability of transition metals, including Cd, Zn, Cu, and Hg, with higher sensitivity and specificity compared to other toxicity endpoints, such as lethality or behavioral changes, in $\textit{C. elegans}$. Potential applications include quantitative or semiquantitative biomonitoring of metal concentrations and bioavailability in soils and aquatic systems contaminated with metals. The system also will facilitate experimentation regarding environmental factors that modulate MT induction, because the assay provides a much faster, cheaper, and easier method of quantifying MT expression compared to existing assay methods based on techniques such as immunoassay or metal saturation. Future applications of this assay might include assessment of the interactive effects of metals in mixtures, the role of speciation and complexation in metal bioavailability and MT induction, the impact of age or developmental state or other biological variables on MT expression, and the role of MT in metal sequestration and toxicity under different exposure scenarios.

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*Fundamentals of Ecotoxicology.* CRC. Boca Raton, FL, pp 118-120.


Fig 3.1 Median lethal concentrations (LC50s) for wild-type N2 and mtl-2::GFP transgenic Caenorhabditis elegans (a 1:100 scale is applied to Cd, Zn, Ni, and As). Error bars represent the standard deviation (n = 3). Bars with the same lowercase letters are not significantly different (p < 0.05).
Fig 3.2 Induction of green fluorescence protein (measured as relative fluorescence units) in transgenic Caenorhabditis elegans in response to six metals and arsenic as a function of exposure time and metal concentration: (a) Cd, (b) Hg, (c) Cu, (d) Zn, (e) Ni, (f) Pb, and (g) As. Error bars represent the standard deviation (n = 3).
Fig 3.2 (continued).
Fig 3.3 Metallothionein transcription in the mtl-2::GFP transgenic Caenorhabditis elegans in response to six metals and arsenic. Relative expression ratio (between mtl-2 target gene and ubq-1 reference gene) in treatments are normalized to the control. Error bars represent the standard deviation (n = 3).
Fig 3.4 Induction of green fluorescence protein (measured as relative fluorescence units) in the mtl-2::GFP transgenic Caenorhabditis elegans in response to Cd in soil. Error bars represent the standard deviation (n = 3).
Table 3.1 Comparison of different endpoints used to evaluate metal toxicity and bioavailability in the nematode Caenorhabditis elegans.

<table>
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<th>Behavior</th>
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<td>LOEC (μM)</td>
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<sup>a</sup>All behavior data from Dhawan et al.[9] except for Hg. EC50 = concentration at which movement is reduced by 50% compared with controls after 24 h of exposure with food; GFP = green fluorescence protein; LC50 = median lethal concentration; lowest-observed-effect concentration.
CHAPTER 4

TOXICITY OF MANUFACTURED ZINC OXIDE NANOPARTICLES IN THE NEMATODE *CAENORHABDITIS ELEGANS* 2

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Abstract

Information describing the possible impacts of manufactured nanoparticles on human health and ecological receptors is limited. The objective of the present study was to evaluate the potential toxicological effects of manufactured zinc oxide nanoparticles (ZnO-NPs; 1.5 nm) compared to aqueous zinc chloride (ZnCl₂) in the free-living nematode Caenorhabditis elegans. Toxicity of both types of Zn was investigated using the ecologically relevant endpoints of lethality, behavior, reproduction, and transgene expression in a mtl-2::GFP (gene encoding green fluorescence protein fused onto the metallothionein-2 gene promoter) transgenic strain of C. elegans. Zinc oxide nanoparticles showed no significant difference from ZnCl₂ regarding either lethality or reproduction in C. elegans, as indicated by their median lethal concentrations (LC₅₀; \( p=0.29, n=3 \)) and median effective concentrations (EC₅₀; \( Z=0.835, p=0.797 \)). Also, no significant difference was found in EC₅₀s for behavioral change between ZnO-NPs (635 mg Zn/L; 95% confidence interval [CI], 477–844 mg Zn/L) and ZnCl₂ (546 mg Zn/L; 95% CI, 447–666 mg Zn/L) (\( Z=0.907, p=0.834 \)). Zinc oxide nanoparticles induced transgene expression in the mtl-2::GFP transgenic C. elegans in a manner similar to that of ZnCl₂, suggesting that intracellular biotransformation of the nanoparticles might have occurred or the nanoparticles have dissolved to Zn²⁺ to enact toxicity. These findings demonstrate that manufactured ZnO-NPs have toxicity to the nematode C. elegans similar to that of aqueous ZnCl₂.
Introduction

The nanotechnological revolution promises to bring major advances in numerous areas, including medicine, manufacturing, electronics, sensor development, energy production, and environmental remediation. Manufactured nanoparticles, defined as particles with one or more dimensions of less than 100 nm [1], display unique electrical, thermal, mechanical, and optical properties because of their small size and high surface reactivity, which makes them highly desirable for applications within the commercial, medical, and environmental sectors [2]. Manufactured nanomaterials currently are produced in metric tons per year, and this quantity is expected to increase rapidly during the next decade [3]. Potential occupational and public exposure to manufactured nanoparticles also is expected to increase in the near future because of anticipated increase in utilization. It is anticipated that large quantities of nanoparticles will be released to the environment during the coming decades, either via waste disposal or by accidental discharge. Thus, understanding the human health implications and ecological consequences of exposure to nanoparticles is essential before the commercial benefits of these materials can be fully realized [4]. Information describing the possible impacts of manufactured nanoparticles on human health and the environment, however, is lacking. This is particularly true for the fate and transport of nanoparticles in the environment as well as for their potential ecological effects and risks [3]. Therefore, research on bioavailability, uptake, tissue distribution, food-chain transport, and effects of nanoparticles in ecological receptors from both aquatic and terrestrial systems has been identified as a near-term priority by the U.S. Environmental Protection Agency [5].
Manufactured nanoparticles may be toxic because of their composition, shape, or unique size. Studies have suggested that nanoparticles are not inherently benign and can affect biota at the cellular, subcellular, and protein levels [6]. A considerable body of data is available regarding the toxicological effects of nanoparticles in mammalian systems, particularly with respect to the respiratory and cardiovascular systems. Toxicological effects identified include oxidative stress, inflammation, and cell injury [7]. For example, a mouse spermatogonial stem cell line has been used as a model to assess the toxicity of a variety of nanoparticles, including Ag, Al, and MoO₃ in the germline in vitro, and all the nanoparticles tested have shown a concentration-dependent cytotoxicity [8]. Fewer studies have been conducted regarding the possible adverse effects of nanoparticles to ecological receptors, such as microorganism, invertebrates, and vertebrates from terrestrial and aquatic environments. An investigation concerning the potential ecotoxicity of nanosized TiO₂, SiO₂, and ZnO suspensions on bacteria suggested they all displayed harmful effects, and the antibacterial activity increased with particle concentration [9]. Acute toxicity tests on TiO₂ and fullerenes using *Daphnia magna* found that mortality increases with exposure concentrations [10]. Oberdorster [11, 12] and Zhu et al. [13] conducted pioneering work in testing toxicological effects of fullerenes (C₆₀) on some aquatic species, including a freshwater crustacean (*D. magna*), a marine harpacticoid copepod (*Hyalella azteca*), fathead minnow (*Pimephales promelas*), and largemouth bass (*Micropterus salmoides*), using both acute toxicity assays and sublethal endpoints. Their findings provided the first evidence that uncoated fullerenes may cause oxidative damage and glutathione depletion in vivo in those aquatic species.
The purpose of the present study was to assess the toxicological effects of manufactured ZnO nanoparticles (ZnO-NPs), as compared with those of aqueous ZnCl₂, to the free living nematode *Caenorhabditis elegans*. Manufactured ZnO-NPs has been categorized as one of the richest families of nanostructures, with a broad range of applications from chemical sensors to personal care products [14]. It is reasonable to assume that significant quantities of ZnO-NPs will be introduced into the environment in the near future. Nematode species are of ecological significance and have important roles in nutrient cycling in soil. The nematode *C. elegans* feeds on soil microorganisms and has been used to represent the nematode phylum [15]. Furthermore, *C. elegans* is one of the most thoroughly studied animals, with its complete genome sequenced and its cell lineage described [16]. Transgenic lines of *C. elegans* also have been developed and applied successfully in a variety of environmental studies to address toxicological effects from molecular to individual levels [17-19]. *Caenorhabditis elegans* has a short life cycle, is easy to culture in the laboratory, and can be used in both aqueous and soil matrices. All these advantageous traits make *C. elegans* an excellent candidate for ecotoxicological studies for a wide range of environmental toxicants. Both lethal and sublethal endpoints, including behavior, growth, feeding, and reproduction, have been used in environmental toxicological studies [15, 17, 20].

In the present study, toxicity of ZnO-NPs, as compared to that of ZnCl₂, to *C. elegans* was evaluated using lethality, behavior (movement), reproduction, and transgene expression as endpoints. The first three endpoints are ecologically relevant, whereas transgene expression may elucidate mechanisms of toxicity. In particular, a transgenic strain of *C. elegans* with the metallothionein-2 (mtl-2) promoter fused to green
fluorescent protein (GFP; i.e., \( mtl-2::GFP \)) has been used. A previous study demonstrated that when exposed to metal ions, such as \( \text{Cd}^{2+} \) or \( \text{Zn}^{2+} \), transgene expression is induced and the response shows a concentration-dependent manner [21]. The \( mtl-2::GFP \) transgenic \( C. \ elegans \) may yield insight regarding whether the ZnO-NPs are dissolving intracellularly to release \( \text{Zn}^{2+} \) and, thereby, trigger transgene expression.

**Materials and Methods**

**ZnO-NPs and \( \text{ZnCl}_2 \)**

PinnacleAF ZnO-NPs suspension, with a reported primary particle size of 2 to 6 nm, was purchased from Applied Nanoworks (http://www.appliednanoworks.com). Chemical analysis confirmed the presence of Zn (56,000 mg/L) as well as a significant amount of acetate (2.33 M). Received samples had pH 6.25. Particle images were taken using a Philips/FEI Tecnai 20 transmission-electron microscope (TEM) operating at 200 kV (FEI/Philips Electron Optics). Size determination by TEM found the particles to be between 1 to 2.5 nm, with an average size of 1.5 nm (Fig. 4.1). It would have been ideal to use bulk ZnO (~1 \( \mu \text{m} \)) as a positive control for all toxicological testing in the present study; however, the lack of stability of bulk ZnO in the test medium (because of flocculation/settling) made it impossible to provide consistent exposure to the bulk ZnO. Thus, only \( \text{ZnCl}_2 \) was used for comparison of toxicity with ZnO-NPs. Because aggregation occurs when the stock ZnO-NPs is diluted by unbuffered K-medium (0.032 M KCl and 0.051 M NaCl) [22], acetic acid/acetate-buffered K-medium (0.032 M KCl, 0.051 M NaCl, and 0.14 M acetate; pH 6.0) was used as the diluent for experiments using ZnO-NPs. Reagent- grade \( \text{ZnCl}_2 \) (Sigma-Aldrich) was used, and various concentrations were made in both buffered and unbuffered K-medium for toxicity test.
Caenorhabditis elegans culture maintenance and synchronization

The wild-type N2 strain of *C. elegans* originally was obtained from the Caenorhabditis Genetics Center and was maintained as a dauer larva stock in M9 buffer, replenished monthly [23]. All cultures were maintained at 20°C. Four-day-old worms from age-synchronized cultures were prepared using the methods described by Donkin and Williams [24]. Briefly, dauers from the stock were transferred to Petri dishes containing nematode growth medium covered with a lawn of OP50 (a uracil-deficient strain of *Escherichia coli*) and incubated; within 3 d, eggs were harvested and isolated from adults by treatment for 10 min in a 1% (v/v) Clorox® (Clorox) and 0.013 M NaOH solution, then transferred to nematode growth medium plates with an established lawn of OP50 and incubated at 20°C for 4 d. The worms were transferred onto a fresh OP50 plate 1 d before the testing.

*Lethality test*

For the 24-h lethality test, exposure was conducted in 24-well tissue culture plates (Corning Costar). Each test consisted of five to six ZnO-NPs or ZnCl$_2$ concentrations (range, 325–1,625 mg Zn/L) and a control, with three replicate wells for each concentration and the control. A 1.0-ml aliquot of test solution was added to each of the wells, which was subsequently loaded with 10 (±1) worms (~30 worms for each concentration). The worms were exposed for 24 h at 20°C. Following exposure, the wells were observed under a dissecting microscope, and the worms were counted and scored as live or dead. The nematodes were judged to be dead if they did not respond to stimulus using a small, metal wire. The test was independently repeated three times. The same exposure procedure was followed to conduct the 4-h lethality test except that the
concentrations of ZnO-NPs or ZnCl₂ used were approximately 10-fold higher. Copper chloride was used as a reference toxicant for the duration of the study [25]. All median lethal concentrations (LC50s) for CuCl₂ were within two standard deviations of the control LC50 of 63 (41–97) mg Cu/L, ensuring the health of the organisms and their statistically constant response to toxicants.

**Movement tracking**

Dilutions of ZnO-NPs were made using buffered K-medium, and dilutions of ZnCl₂ were made using both buffered and unbuffered K-medium. Five to six concentrations of ZnO-NPs or ZnCl₂ (range, 50–1,000 mg Zn/L) were tested. Twelvewell, sterile tissue culture plates were used for exposure before movement tracking. A 5-μl worm pellet (~100 worms) was loaded into a single well containing 1 ml of metal solution for each concentration and a control well in the absence of a food source. The exposure plates were placed in an incubator at 20°C for 4 h. The movement tracking procedure was adopted from that described by Boyd et al. [26]. Immediately following the 4-h exposure, the worms were transferred into 2-ml glass centrifuge tubes with a Pasteur pipette. The worms were allowed to gravity settle into a pellet, rinsed with approximately 1.5 ml of fresh K-medium, and gently mixed by creating bubbles with a Pasteur pipette. This washing process was repeated three times. Approximately 50 to 80 worms were then transferred in 5-μl aliquots to a cooled, 2-ml, 1% agar pad on a clear, 100×200-mm glass slide. The worms were allowed to disperse on the agar pad inverted over a Petri dish filled with water to avoid desiccation. Movement tracking was started at exactly 1 h after the end of exposure. The individual glass slides were placed in a tracking chamber with a gentle, water-saturated airstream. Using a video camera interfaced with a Macintosh®
(Apple) computer that contained a modification of the National Institutes of Health tracking software [27], the movements of individual worms were tracked and recorded to an Excel® (Microsoft) spreadsheet. A macro was used to calculate the average movement per worm per second.

**Reproduction**

Reproduction was tested using the 72-h assays described by Dhawan et al. [28]. The test solutions consisted of five concentrations of ZnO-NPs or ZnCl₂ (range, 10–200 mg Zn/L) and a control. One adult worm from an age-synchronized culture was placed in each 1 ml of test solution. Three wells were used for each concentration and exposed under the same conditions as described for the lethality test. Three days later, at 72 (±1) h, the number of offspring at all stages beyond the eggs was determined [29]. For each test concentration and control, the average number of progeny from three wells was obtained for each test replicate, and the testing was repeated three times.

**GFP expression in transgenic C. elegans**

The *mtl-2::GFP* transgenic *C. elegans* was developed in 2004, as reported by Humphries (2004, Master’s thesis, University of South Carolina, Columbia, SC, USA). Based on the LC50s determined from the lethality test, five to six sublethal concentrations of ZnO-NPs or ZnCl₂ (range, 5–130 mg Zn/L) were selected for exposure to induce GFP expression. Costar 96-well plates with a clear bottom and black sides (Corning Incorporated Life Sciences) were used for exposure and measurement. Nematodes (35±3) and test solution (250μl) were added to each well, with three wells for each concentration. At a series of time points after exposure, GFP was measured using a Synergy HT microplate reader (Bio-Tek Instruments) with a 485-nm excitation filter and
a 528-nm emission filter. The GFP intensity is reported as relative fluorescence units.

The assay was repeated three times for both ZnO-NPs and ZnCl₂. The relative fluorescence unit was calibrated using a standard recombinant EGFP protein (BioVision).

**Data analysis**

The concentration–response relationships for lethality, behavior, and reproduction were generated from three independent replicate tests. The median lethal concentrations and 95% confidence interval (CI) were determined using Toxstat® 3.4 (Western Ecosystems Technology) with a probit transformation. The data were tested for normality with the chi-square test and for homogeneity of variance with the Bartlett test. Any data that did not pass these tests were not used to estimate LC₅₀. Analysis of variance was performed to test for differences among the mean LC₅₀s for the three chemicals, with a significance level of \( \alpha = 0.05 \) using SAS software (SAS Institute). Median effective concentrations (EC₅₀s; concentration producing a 50% reduction in movement or offspring compared to control) and corresponding 95% CIs for movement and reproduction were estimated using nonlinear-regression models supplied by Environment Canada [30] in SYSTAT 11.0 (Systat Software): A hormesis model was used for movement data, because the data showed hormetic effects. A logistic model was used to derive EC₅₀s for reproduction. Normality was assessed using the Shapiro–Wilk test, and homogeneity was evaluated using the Levene test. Comparison of EC₅₀s between chemicals was performed using the Zajdlik ad hoc method (B.A. Zajdlik, unpublished data), which is based on the two sample Z test. Assessment for significant difference (\( \alpha = 0.05 \)) between all treatment levels and control for GFP expression was conducted with analysis of variance followed by the William test.
Results

LC50s for ZnO-NPs and ZnCl2

Exposure of *C. elegans* to ZnO-NPs or ZnCl2 in buffered K-medium caused an increase in mortality (10–100%) with increasing concentration of Zn (325–1,625 mg/L). Mortality in control worms was less than 10% in all cases. The LC50 (mean±standard error, n=3) was 789±103 mg Zn/L for ZnO-NPs and 884±106 mg Zn/L or ZnCl2, with no significant difference between them (p=0.29) (Fig. 4.2). In unbuffered K-medium, ZnCl2 displayed a similar concentration response relationship, but with a significantly lower LC50 (348±67 mg Zn/L) than that of ZnCl2 in buffered medium (p<0.001) (Fig. 4.2). At 4 h, LC50s were considerably higher than those obtained at 24 h in buffered K-medium for both ZnO-NPs and ZnCl2, with values of 5,173±775 and 5,434±889 mg Zn/L, respectively. In unbuffered K-medium, precipitation started to occur at 500 mg Zn/L and greater, and the 4-h LC50 for ZnCl2 was determined to be 5,768±1,286 mg Zn/L.

EC50 for ZnO-NPs and ZnCl2

*Behavior.* The 4-h movement–concentration and survival–concentration plots were constructed as shown in Figure 4.3. The average movement per worm per second in the control worms was 3.5 μm, consistent with previous studies [20]. For both ZnO-NPs and ZnCl2 in buffered K-medium, movement responses (from 100 to 20% of control movement) occurred across one order of magnitude in concentration, and survival response occurred across less than one order of magnitude in concentration, with those concentrations being approximately one order of magnitude higher than those for movement. For ZnO-NPs, the EC50 was 635 mg Zn/L (95% CI, 477–844 mg Zn/L), and for ZnCl2, the EC50 was 546 mg Zn/L (95% CI, 447–666 mg Zn/L). The two forms of Zn
showed similar concentration–response patterns with hormetic effects (low-dose stimulation). In unbuffered K-medium, movement responses to ZnCl\(_2\) also span an order of magnitude of ZnCl\(_2\) concentration, with an EC\(_{50}\) of 906 mg Zn/L (95% CI, 832–988 mg Zn/L).

**Reproduction.** The change in number of offspring in the nematodes as a function of ZnO-NPs or ZnCl\(_2\) concentration is plotted in Figure 4.4. The average number of offspring for the controls was 122 (±12), comparable to previous studies [29]. The worms showed a significant decrease in reproduction with an increase of ZnO-NPs or ZnCl\(_2\) concentration (10–200 mg Zn/L). Reproduction was affected at much lower concentrations as compared to survival or behavior. The EC\(_{50}\) was 46 mg Zn/L (95% CI, 36–59 mg Zn/L) for ZnO-NPs and 59 mg Zn/L (95% CI, 42–83 mg Zn/L) for ZnCl\(_2\).

**GFP transgene expression**

Green fluorescent protein expression was induced by ZnO-NPs and ZnCl\(_2\) in both buffered and unbuffered K-medium (Fig. 4.5). All treatments showed similar concentration–response patterns, with GFP increasing as Zn concentration increased. The minimum Zn concentration that induced a significantly greater GFP expression compared to control was 33 mg Zn/L in all three cases. The maximum GFP expression after 24 h of exposure was approximately 2,000 relative fluorescence units, which is approximately threefold higher than that for the controls. The organisms in the control also showed an increase in GFP expression after a 24-h exposure, but this increase was much lower in intensity. At a given concentration, no significant difference in GFP intensity was found between ZnO-NPs and ZnCl\(_2\) (either in buffered or unbuffered K medium) (\(p=0.35–0.69\)).
Discussion

Acute toxicity testing with an environmentally relevant species is an essential first step in understanding the possible adverse ecological effects of manufactured nanoparticles released to the environment. It has been suggested that ecotoxicity of nanoparticles can be assessed using methods comparable to the procedures applied for assessing soluble chemicals [31]. In the present study, toxicity testing of manufactured ZnO-NPs was conducted using the nematode *C. elegans*, which has been used widely in ecotoxicological studies. The effect of manufactured ZnO-NPs on *C. elegans* mortality is almost identical to that of ZnCl₂, with no significant difference in their respective LC50s ($n=3, p=0.29$). Toxicity of metal ions, including ZnCl₂, usually involves inhibition of the activity of important enzymes or disruption of the integrity of the cell membrane, or competing with and displacing some essential cations, such as Cu, Ca, and Fe [32], whereas a small size, a large surface, and an ability to generate reactive oxygen species appears to play an essential role for the nanoparticles to induce toxicity, as suggested by toxicity studies on nanoparticles, including TiO₂ and carbon fullerenes (C60) [11, 12, 17, 33]. In the present study, however, the mechanism of toxicity or mode of action of ZnO-NPs on the nematodes remains unknown. A recent study by Franklin et al. [34] has revealed that ZnO-NPs can actually dissolve to Zn²+ to enact toxicity in a freshwater alga. The ZnO-NPs dissolution also might have occurred in the test medium during the present study, and a future study using equilibrium dialysis will help to elucidate this.

To determine if the acetic acid/acetate-buffered K-medium impacts ZnCl₂ toxicity, testing also was conducted in unbuffered K-medium. Zinc chloride exhibited considerably higher toxicity in unbuffered compared with buffered K-medium, as
indicated by its higher LC50s in buffered K-medium. Bioavailability and toxicity of transition metals are controlled by a number of extrinsic factors, including pH and complexation by inorganic and organic ligands. The free ionic form of metals generally is considered to be the most toxic to biota, whereas precipitated, sorbed, and complexed forms generally appear to be less toxic [35]. To examine how the buffered K-medium may affect the toxicity of ZnCl₂, speciation of Zn in the two medium systems was modeled using Visual MINTEQ (Ver 2.40; KTH, Land and Water Resources Engineering), and the concentration–response relationship for different forms of Zn was plotted (Supporting Information). When expressed as total Zn concentration, the concentration–response relationships showed different patterns between the two solution systems; however, the relationships were almost identical when the concentration was expressed as free Zn²⁺ ion. This suggests that the free Zn²⁺ ion is the major contributor to the ZnCl₂ toxicity. The apparently decreased toxicity of ZnCl₂ in buffered K-medium, as suggested by its higher LC50, is actually attributed to the decreased free Zn²⁺ ion concentration, which is caused by complexation of Zn with other species in the solution.

Behavioral change represents a sublethal endpoint that is more sensitive than lethality in evaluating the toxicity of a chemical to an organism. Movement has been used successfully to assess behavioral toxicity in *C. elegans* for a number of chemical classes, including transition metals [15, 27, 36]. In the present study, the acute behavioral toxicity of ZnO-NPs to *C. elegans* was investigated by tracking the movement of the nematodes after exposure. A 4-h exposure time was selected, both because previous studies have demonstrated that movement is sensitive to a 4-h exposure for a variety of toxicants [36] and because short exposure times obviate the requirement for food by the nematodes,
simplifying measurement of the tracking process. A 4-h lethality test also was conducted to make a direct comparison of sensitivity between the two endpoints. Movement is reduced after a 4-h exposure to ZnO-NPs or ZnCl₂ in buffered K-medium, with EC50s of 635 mg Zn/L (95% CI, 477–844 mg Zn/L) and 546 mg Zn/L (95% CI, 447–666 mg Zn/L), respectively, which do not show significant differences (Z = 0.907, p = 0.834). These findings suggest the two forms of Zn have similar toxicity on the behavior of C. elegans. Four-hour LC50s were approximately 10-fold higher than these 4-h EC50s in all cases, confirming that movement is a much more sensitive endpoint than mortality.

A higher EC50 for movement was found for ZnCl₂ in unbuffered K-medium (906 mg Zn/L) than in buffered K-medium (558 mg Zn/L), which seems to contradict the fact that ZnCl₂ is more toxic in unbuffered than in buffered K-medium, as indicated by their 24-h LC50s. In fact, the ZnCl₂ concentrations used for the 4-h lethality tests were so high that precipitation occurred in unbuffered K-medium (both observed during the experiment and predicted by MINTEQ speciation modeling), which decreased the effective free ionic Zn concentration dramatically. This precipitation also explains the slightly higher LC50 for ZnCl₂ in unbuffered K-medium during a 4-h exposure. The differences in these apparent EC50s or LC50s are caused by speciation of Zn in different medium systems; the effective free ionic Zn concentrations required to cause a certain toxicity effect are actually the same, as can be verified by speciation modeling using MINTEQ (Supporting Information). These findings suggest that caution should be taken when reporting or interpreting toxicity data for tests conducted in different medium systems.
Toxicity of ZnO-NPs on the reproduction of *C. elegans* did not show a significant difference from that of ZnCl₂, as suggested by their similar EC50s and overlapping 95% CIs ($Z = 0.835$, $p = 0.797$). Reproduction is more sensitive than behavior in evaluating toxicity of ZnO-NPs or ZnCl₂ to *C. elegans*, given that EC50s for behavior are approximately 10-fold greater than those for reproduction.

Compared to conventional toxicological endpoints, such as lethality or behavior, transgene expression can provide more specific information regarding the bioavailability of a toxicant to an organism from a mechanistic perspective. Transgenic strains of *C. elegans* have been developed and applied successfully in toxicological studies, including those using a promoter from a stress-response gene (e.g., heat shock protein gene [*hsp-16*]) or metallothionein gene with GFP as the reporter [17, 37, 38]. When the GFP-tagged gene is up-regulated following exposure to a chemical of interest, the resulting marker can be measured, and a concentration–response relationship can be established that is both sensitive and specific to certain cellular processes. A *mtl-2::GFP* transgenic strain of *C. elegans* using metallothionein-2 as promoter and GFP as reporter was used to evaluate ZnO-NPs toxicity by transgene expression in the present study. Previous studies in our laboratory have indicated that ZnCl₂ induces GFP expression in this transgenic strain and that the GFP response is concentration dependent within a Zn concentration range of up to 130 mg/L; the lowest concentration of Zn that has induced significant GFP expression is 16 mg/L [21]. In the present study, ZnO-NPs induce GFP expression in a pattern similar to that of ZnCl₂, with GFP increasing as the Zn concentration increases, and the minimum Zn concentration that induces a significant GFP response is 33 mg/L. These findings suggest similar bioavailability between ZnO-NPs and ZnCl₂ to *C. elegans*. 
It may be that biotransformation of the nanoparticles (i.e., intracellular dissolution) occurred after ingestion. This biotransformation might partially explain why ZnO-NPs are not more toxic than their counterpart aqueous species. To confirm this, however, requires a more comprehensive investigation regarding the absorption, transport, and distribution of the nanoparticles within the organism, and these studies are ongoing in our laboratory. We are in the process of examining Zn elemental distributions in ZnO-NPs- and ZnCl₂-exposed worms by employing advanced, synchrotron-based, x-ray microanalyses to image elemental distribution and speciation in the organism and then comparing this to spatial patterns in GFP expression as elucidated by epifluorescence microscopy. Initial studies suggest that differences exist in the spatial distribution of Zn between treatments. An alternative hypothesis is that ZnO-NPs dissolve before absorption by the organism, which has been found with ZnO-NPs having a larger particle size (i.e., 30 nm) [34].

Many factors, including size, shape, surface area, surface chemistry, and aggregation, should be considered when evaluating the potential toxicity of nanoparticles [39], because the interaction of nanoparticles with the surface of biological tissues is determined by surface chemistry and reactivity [40]. Aggregation of primary particles may occur during synthesis or once nanoparticles are introduced into various solutions, where electrostatic interactions or chemical bonding can lead to the formation of aggregates and this may affect the toxicity of nanoparticles by restricting their uptake into living cells. No significant aggregation was visually observed for the ZnO-NPs solutions tested in the present study, which also was confirmed by TEM imaging. In addition, it
was determined that the acetic acid/acetate buffer system used to stabilize the ZnO-NPs does not cause mortality of the nematodes.

Findings from the present study demonstrate that manufactured, nanosized ZnO particles can cause toxicological effects in the nematode *C. elegans*. Using a variety of endpoints, the nanoparticles were determined to have toxicity comparable to that of ZnCl₂ to *C. elegans*. Future work aims to examine the toxicity and bioavailability of larger ZnO-NPs (i.e., 50 nm) and to determine if particle size influences toxicity. The present study also demonstrates that *C. elegans* is a useful model for toxicity testing of nanomaterials, both because it has been applied widely for toxicological studies for conventional compounds and because several ecologically relevant endpoints can be easily measured in the same exposure system.

**Supporting information**

**Fig. S1.** Impact of Zn speciation on toxicity of ZnCl₂ to *Caenorhabditis elegans*:

Concentration–response relationships for mortality for total Zn (▲), free Zn²⁺ (□), and Zn²⁺ activity (■) in (a) buffered K-medium and (b) unbuffered K-medium.

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Fig 4.1 Transmission-electron micrographs of zinc oxide nanoparticles (ZnO-NPs): (A) Cryogenic preparation of original stock ZnO-NPs; (B) 1:1,000 dilution of stock ZnO-NPs in acetic acid/acetate-buffered K-medium. Original magnification: × 35,000
Fig 4.2 Concentration–response relationships for mortality of Caenorhabditis elegans after exposure to zinc oxide nanoparticles (ZnO-NPs; ●) or ZnCl₂ (buffered K-medium, Δ; unbuffered K-medium, □). Error bar denotes the standard error (n = 3): Median lethal concentration (LC50; mean ± standard error) was 789 ± 103 mg Zn/L for ZnO-NPs, 884 ± 106 mg Zn/L for ZnCl₂ in buffered K-medium, and 348 ± 67 mg Zn/L for ZnCl₂ in unbuffered K-medium.
Fig 4.3 Concentration–response relationships for movement and survival in Caenorhabditis elegans for a 4-h exposure to zinc oxide nanoparticles (ZnO-NPs; ●), ZnCl₂ in buffered K-medium (Δ), or ZnCl₂ in unbuffered K-medium (□). Error bar denotes the standard error (n = 3).
Fig 4.4 Concentration–response relationships for reproduction of Caenorhabditis elegans on exposure to zinc oxide nanoparticles (ZnO-NPs; ●) or ZnCl$_2$ in buffered K-medium (△). Error bar denotes the standard error ($n = 3$). Median effective concentration (EC50) was 46 mg Zn/L (95% confidence interval [CI], 36–59 mg Zn/L) for ZnO-NPs and 59 mg Zn/L (95% CI, 42–83 mg Zn/L) for ZnCl$_2$. 
Fig 4.5 Concentration–response relationships for mtl-2::GFP transgene (gene encoding green fluorescence protein fused onto the metallothionein-2 gene promoter) expression after a 24-h exposure to zinc oxide nanoparticles (ZnO-NPs; ●), ZnCl₂ in buffered K-medium (Δ), or ZnCl₂ in unbuffered K-medium (□). Error bar denotes the standard error (n = 3).
Fig. S1. Impact of Zn speciation on toxicity of ZnCl₂ to Caenorhabditis elegans: concentration-response relationships for mortality for total Zn (▲); free Zn²⁺ (○); and Zn²⁺ activity (■); (a) buffered K-medium and (b) unbuffered K-medium.
CHAPTER 5

BIOAVAILABILITY OF ZINC OXIDE NANOPARTICLES AS REFERENCED TO AQUEOUS ZINC CHLORIDE: ZINC SPATIAL DISTRIBUTION IN THE NEMATODE CAENORHABDITIS ELEGANS

Abstract

In this study, we evaluated the bioavailability and toxicity of manufactured ZnO nanoparticles (ZnO-NPs, 1.2 nm, in suspension) as compared to aqueous ZnCl$_2$ ($\text{Zn}^{2+}_{\text{aq}}$) in the nematode Caenorhabditis elegans, by employing synchrotron based X-ray fluorescence microscopy to image Zn spatial distribution. We also examined distribution of GFP transgene expression induced by these two forms of Zn in a transgenic strain of C. elegans using the metallothionein II promoter to drive the expression of green fluorescent protein, using ultraviolet-visible fluorescence microscopy. Wild-type nematodes were exposed to both materials for 24 h, washed out and preserved in formalin. Two-dimensional Zn distribution was mapped at the microprobe beamline X26A at the National Synchrotron Light Source. Transgenic nematodes were exposed to both materials and GFP was visualized using an Olympus BX61 microscope at different time points. Consistent with previous studies, we found that ZnO-NPs showed overall similar bioavailability and toxicity to Zn$^{2+}_{\text{aq}}$, however, there were subtle differences in the internal distribution of Zn in the nematodes. Expression of GFP was limited to the intestinal tissue, and few differences were observed in distribution of GFP fluorescence.
among treatments. We suggest that ZnO-NPs may be taken up by *C. elegans* and biotransformed releasing Zn$^{2+}_{aq}$.

**Introduction**

The revolution in nanotechnology has brought advantages in a variety of areas in human life from engineering to medical diagnostics, which necessitates large scale production of nanoparticles with new formulations and surface properties to meet novel demands [1]. The potential human health and environmental impacts of these manufactured nanomaterials has become a concern to both general public and governmental regulation agencies. As numerous studies have investigated the human health implications of nanoparticles, only recently have researchers begun to study the potential ecological risks and impacts of nanomaterial released to the environment [2].

Zinc oxide nanomaterials have been widely used in cosmetics and sunscreens as well as UV protectants in coatings, fabrics and polymers. Zinc oxide can also be grown into a diverse array of nanoscale shapes and structures, making it one of the more diverse and versatile nanomaterials [3]. Given these characteristics, and the potential for Zn toxicity, ZnO nanomaterials may eventually become a contaminant of potential concern.

In an earlier study, we have found that a commercially obtained ZnO nanoparticulate suspension (ZnO-NPs) exhibited similar bioavailability and toxicity to aqueous ZnCl$_2$ in the nematode *C. elegans* using a series of ecological endpoints including lethality, movement, reproduction, and transgene expression [4]. However, little is know about the possible mode of action or mechanism of toxicity of these metal oxide nanomaterials. There is great interest in distinguishing between toxicity that arises from particle size dependent effects and toxicity arising from the release of dissolution products such as
free metal ions, because differentiating between these mechanisms is an important step towards identifying key issues associated with environmental toxicity and risks of metal-based nanomaterials. Recently, several studies have independently reported the dissolution of ZnO nanoparticles to metal ions to cause toxicity in a number of aquatic [5, 6] and terrestrial species [7].

In this study, we aimed to determine whether the previously observed toxicity of ZnO-NPs could be explained by the release of free metal ions. We investigated the Zn distribution in the nematode Caenorhabditis elegans exposed to a commercially obtained ZnO-NPs suspension and ZnCl₂ by using synchrotron based X-ray fluorescence (SXRF) microscopy, and the distribution of GFP transgene expression in a mtl-2::GFP transgenic C. elegans using ultraviolet visible (UV-VIS) fluorescence microscopy. Knowledge of spatial distribution of a contaminant within an organism is important in determining the site of toxic action or mechanism of toxicity [8]. Using SXRF, Jackson [8] investigated the Cu and Pb spatial distribution in nematode C. elegans and found that Cu was homogeneously distributed throughout the body of the nematode, whereas Pb was exclusively located in the anterior pharynx region with a high density of neurons. This variation in distribution pattern between the two metals coincided with their difference in toxicity: Pb is a neuron toxicant whereas Cu can impact many cell types. The spatial distribution of hsp16 expression in response to a number of trace metal cations in a hsp16-lacZ transgenic C. elegans has been reported [9]. Several distinct patterns of transgene expression were observed in response to various transition metals (Pb, Hg, Zn, Cu and Cd), suggesting that certain tissues may be especially vulnerable to specific toxic agents. Caenorhabditis elegans are soil dwelling nematodes which feed primarily on
bacteria and other microorganisms. Attributed to their genetic manipulability, fully described developmental program, well-characterized genome, ease of maintenance, short and prolific life cycle, *C. elegans* has emerged as an important animal model in a variety of fields including neurobiology, development biology, and genetics [10]. They have also gained acceptance and use in toxicological testing serving as a model for both mechanistic studies and high-throughput screening approaches in environmental and human exposures [10].

Our previous study indicated that ZnO-NPs induce GFP expression in the *mtl2::GFP* transgenic *C. elegans* [4], which may suggest the dissolution of ZnO-NPs to metal ions within the nematodes. A spatial resolution of GFP expression coped with Zn spatial distribution will facilitate the understanding of the bioavailability and toxicity of ZnO-NPs, and a comparison of GFP and metal distribution patterns between ZnO-NPs and ZnCl₂ will bring insights to understand the possible mechanisms of toxicity. We hypothesized that toxicity of ZnO-NPs in *C. elegans* is related to the release of zinc ions, and that the spatial distribution of Zn and GFP would be similar. The hypothesis was tested by comparing the relative distribution of GFP and Zn fluorescence between ZnO-NPs and ZnCl₂ exposed nematodes.

**Materials and Methods**

**ZnO Nanoparticles**

PinnacleAF ZnO nanoparticles suspension (ZnO-NPs) was purchased from Applied Nanoworks (Rensselaer, NY USA). The product data sheet reports the primary particle size to be 2-6 nm, and the surface area to be (250 m²/g). Chemical analysis confirmed the presence of zinc (56,000 mg/L Zn) as well as significant amount of acetate (2.33 M).
Received samples had a pH of 6.25. Particle size was determined using two independent methods. Transmission electron microscopy (TEM) was performed to determine particle morphology and $d_{h}$ using a Philips/FEI Technai 20 (Amsterdam, Netherlands) electron microscope operating at 200 keV. Dynamic light scattering (DLS) was conducted using a photon correlation spectrophotometer (DynaPro, Wyatt Technology, Santa Barbara, CA, USA) to determine the $d_{h}$ of the particles in the stock suspension. Observation of the $d_{h}$ at the actual test concentrations in the exposure medium was not possible because the small particle size, small refractive index and low concentration yielding poor light scattering.

Nematode Exposure

Wild type N2 *C. elegans* were originally obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN). Three-day-old, age-synchronized cultures were derived from egg plates following the methods of Williams and Dusenbury [11]. Two concentrations (expressed in terms of [Zn]) of ZnO-NPs or ZnCl$_2$ were selected for nematode exposure: the highest concentration that caused no mortality (100 mg Zn/L) and a concentration that caused 25% mortality (500 mg Zn/L) as determined previously [4]. The ZnO-NPs and ZnCl$_2$ were diluted in K-medium (32 mM KCl, 51 mM NaCl) as described by Williams and Dusenbury [11], but modified by buffering with 140 mM sodium acetate/acetic acid, pH 6.0 to avoid nanoparticle agglomeration/aggregation. This buffer was not expected to significantly affect responses because *C. elegans* tolerates a wide range of pH and ionic strength conditions [12]. Nematodes were added to 1 mL of test solutions or control medium in 12-well tissue culture plates and exposed for 24 h at 20°C in the dark. Following each exposure nematodes were rinsed with K-medium 2-3 times, preserved in 4% formaldehyde, and stored at 4°C prior to X-ray analysis.
The *mtl2*:GFP transgenic *C. elegans* was developed in 2004, as reported by Humphries (2004, Master’s thesis, University of South Carolina, Columbia, SC, USA). Three-day-old, age-synchronized cultures were derived from egg plates and used for exposure following the same procedure as described above. For UV-Vis fluorescence analysis for GFP expression, after 24 h exposure to either 6.5 or 130 mg Zn/L as ZnO-NPs or ZnCl₂, nematodes were rinsed with K-medium, and mounted on 25mm×75mm microscope glass slides in 70% ethanol for imaging. Lower Zn concentrations were chosen for this experiment because *mtl-2*:GFP induction occurs at much lower concentrations than lethal responses [13].

**SXRF Microscopy**

Synchrotron based X-ray fluorescence microscopy was conducted at beam line X-26A, National Synchrotron Light Source (NSLS), Brookhaven National Laboratory (Upton, NY, USA). The incident X-ray beam was monochromatized to 12.5 keV. Preserved *C. elegans* were mounted on polyimide film (Kapton, DuPont, Willmington, DE, USA) which was mounted in the X-ray beam. Energy-dispersive XRF data were collected using a combination of a 9-element Ge array detector (Canberra, Merridian, CT, USA) and a single element Si drift detector (SDD;Vortex EX, SII NanoTechnology, Tokyo, Japan). It was extremely difficult to convert the fluorescence intensity directly into equivalent Zn concentrations in the nematodes because of variations in and uncertainty about the geometry and density of individual nematodes and a lack of suitable matrix matched standards. Therefore, raw fluorescence intensities (counts per second) were normalized to current through an ion chamber which was used to measure the beam flux. Image analysis was performed using a custom Interactive Data Language (IDL) program (X26A Plot).
**GFP Fluorescence Microscopy**

GFP fluorescence distribution images of transgenic *C. elegans* after exposure to ZnO-NPs and ZnCl2 were obtained using an BX-61 epifluorescence microscope (Olympus, Center Valley, PA, USA) equipped with a Peltier cooled, charge-coupled device camera (Orca-ERII, Hamamatsu, Yokohama, Japan). Both differential interference contrast (DIC) and epifluorescence images were taken. Filter set with maxima of $\lambda_{ex} = 420 \text{ nm} / \lambda_{em} = 500 \text{ nm}$ was used for visualization of GFP, and filter set with $\lambda_{ex} = 350 \text{ nm} / \lambda_{em} = 460 \text{ nm}$ (Chroma Technology, Rockingham, VT, USA) was used for visualization of *C. elegans* autofluorescence.

**Results**

Analysis by TEM demonstrated an average size of 1.5 nm (n=10 particles), which was slightly different from what was reported. The hydrodynamic diameter ($d_h$) determined by DLS was found to be 1.2 nm. The correspondence between the non-invasive DLS results and the TEM results indicate that the primary particle diameter averaged around 1.2-1.5 nm.

Both ZnO-NPs and ZnCl2 treated nematodes showed greater mean pixel intensities than control animals, although significant differences were not observed between equal concentrations of ZnO-NPs and ZnCl2 (Figure 5.1). In control animals, high pixel intensity of Zn was localized in the posterior region. The areas of high pixel intensity spread out towards the anterior end of the nematode as ZnO-NPs or ZnCl2 concentration increased from 100 to 500 mg Zn/L. The maximum pixel intensity in the ZnCl2 treated nematodes was nearly twice as great as in the ZnO-NPs treated ones. A visual assessment of the images indicated a more even distribution of Zn fluorescence with fewer foci of
elevated fluorescence in the ZnO-NPs treated nematodes relative to the ZnCl₂ treated nematodes, particularly at 500 mg Zn/L.

GFP transgene expression was observed both in control and ZnO-NPs or ZnCl₂ treated nematodes, and few differences were observed in distribution of GFP fluorescence among treatments. In all cases, GFP expression was limited to the intestinal tissue. The Zn treated nematodes showed greater GFP intensity than control animals as indicated by the “brightness” of the GFP distribution pattern (Figure 5.2). A remarkable increase in autofluorescence was observed in all treatments compared to controls. In control animals, autofluorescence was only observed in a small region immediately posterior of the pharynx, whereas in both ZnO-NPs and ZnCl₂ treated nematodes, autofluorescence extended nearly the entire length of the body (Figure 5.2).

**Discussion**

Metal accumulation in organism depends on both physicochemical properties of metals and the physiology of organisms. Essential and nonessential metals may have distinguished metal accumulation strategies: essential metals are subject to strict regulation either by limiting uptake or inducing active excretion or storage in an inert form, whereas for nonessential metals, excretion and internal storage are the major strategies [14]. Zinc is an essential metal and plays an important role in diverse biological processes [15]. The functional diversity of zinc suggests highly sophisticated mechanisms in regulation of its uptake and distribution with an organism. The widespread existence of Zn within the control nematodes confirmed its multiple biological functions in a wide range of cells and tissues in the organism, whereas an elevated Zn intensity localized in the posterior region may suggest the strategy that the nematodes accumulate Zn in an
environment with relatively high Zn concentration. This localization of Zn may indicate the storage of this essential metal in the nematode, which may function as a “reservoir”: it stores extra Zn and release it to support the organism’s need once a Zn-deficiency situation occurs.

The distribution of Zn in all treatments was consistent with absorption of both materials within the gut rather than through the cuticle, which has also been observed for Pb [8]. The change in spatial distribution of Zn as exposure concentration increases provides insights into the strategy that the nematodes deal with elevated Zn exposure. At lower concentrations (3 and 100 mg Zn/L for both ZnO-NPs and ZnCl2), the maximum intensity of Zn is localized in the posterior region. None of these concentrations caused mortality in nematodes during 24 h exposure. Previous studies have reported that waste nodules in which metals were detoxified and stored was identified in the posterior portion of earthworms using atomic absorption and X-ray fluorescence spectrometry [16]. As exposure concentration increased to 500mg Zn/L, which caused approximately 25% mortality, Zn distribution spread out toward the anterior end of the nematode with hotspots detected in intestinal region. This elevated Zn exposure induced toxicological effects in a number of cells and tissues, which led to mortality. Although no significant differences in Zn distribution pattern was observed between nematodes treated by ZnO-NPs and ZnCl2, there was a trend toward lower average maximum pixel intensities for ZnO-NPs treated nematodes than their ZnCl2 treated counterparts. Taken as a whole, the SXRF images suggest that the overall bioavailability of Zn is similar between ZnO-NPs and ZnCl2, but there were subtle, yet consistent, differences in the distribution of Zn between the two treatments. To statistically resolve these differences requires observation.
of a very large number of individuals, which is not possible the time constraints for using the SXRF microprobe beamline.

We observed few differences in distribution of GFP fluorescence among treatments. GFP was exclusively distributed in the gut of the nematodes, which was consistent with the Zn “hotspots” on SXRF images. Distribution pattern of certain stress protein (i.e., heat shock protein, metallothionein) after exposure to environmental toxicants can assist to the underlying mechanism of toxicity of those toxicants. In a hsp16::lacZ transgenic strain of C. elegans, transgene expression exhibited distinct patterns in response to a number of transition metals including Hg, Zn, Pb, Cd, Cu and As, suggesting that certain tissues may be especially vulnerable to specific toxic agents [9]. The GFP distribution patterns we observed may indicates that the intestine is a major target tissue for Zn toxicity to the nematodes under this circumstance.

The spatial extent of GFP fluorescence appeared to be decreased in the nematodes treated with higher concentrations of both ZnO-NPs and ZnCl2. This is likely explained by reabsorption of the fluorescence by eggs in individuals that were matricidal hatching. The presence of eggs in the ZnO-NPs or ZnCl2 treated nematodes is demonstrated by the outline of oval objects throughout the body which do not autofluoresce. Matricidal hatching refers to intra-uterine egg hatch with resulting death of parent, and it represents a stress response in C. elegans. It occurs when the animals are subject to starvation or other stress (e.g., high salt concentrations), and is thought to be a strategy for the nematodes to enhance progeny survival and dispersal under stress [17]. It appears that this behavior increases with increasing exposure concentration, and is more prevalent in the ZnO-NPs treated than in the ZnCl2 treated animals.
Autofluorescence was observed in nematodes from all treatments. This autofluorescence is likely to be linked with lipofuscin. Lipofuscin is a naturally generated age- or stress-pigment granules which is produced in cells by oxidative attack on lipoproteins [18]. It is commonly observed in *C. elegans* using DAPI-type filters and is usually considered a confounding factor when imaging GFP expression. In the present study, as in other studies [19], we are exploiting it as a stress biomarker. Correlations among lipofuscin, decreased lifespan and exposure to Ca, Al and Fe in *C. elegans* have been reported in a previous study [20]. We only observed autofluorescence in a small region immediately posterior of the pharynx in control nematodes, but more widespread autofluorescence was observed in either ZnO-NPs or ZnCl₂ treated animals. This suggests accelerated aging and/or oxidative stress in the exposed animals.

In summary, this study showed subtle, but consistent differences between the spatial distributions of Zn in *C. elegans* exposed to ZnO-NPs versus ZnCl₂. Similarities in the toxicity of ZnO nanomaterials and ZnCl₂ was observed independently in one of our previous studies [13] and the study of Wang et al [7], although the ZnO-NPs materials tested in these two studies were very different. Both studies suggested a role for Zn²⁺aq in toxicity. The evidence that similar distribution of GFP transgene expression was induced by these two materials tends to reinforce this conclusion. Furthermore, we suggest that biotransformation of the nanomaterials may occur internally within *C. elegans*, and the observed effects could be explained by biotransformation of ZnO-NPs releasing Zn²⁺.
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Fig 5.1 Representative Zn distribution (Zn K\textsubscript{α1} fluorescence) images of Caenorhabditis elegans exposed to two concentrations of ZnCl\textsubscript{2}, a commercial ZnO-NPs suspension, or control medium. The units for the scale bars are normalized counts per second. The pixel sizes are approximately 10 x 10 μm.
Fig 5.1 (continued).
Fig 5.2 Differential interference contrast and corresponding fluorescence micrographs of mtl-2::GFP transgenic Caenorhabditis elegans exposed to two concentrations of ZnCl₂, ZnO-NPs suspension, or control medium. Green fluorescence corresponds to green fluorescent protein. Blue fluorescence likely corresponds to lipofuscin or “age pigment”.
CHAPTER 6

PHOTOTOXICITY OF NANOPARTICULATE ZINC OXIDE UNDER NATURAL SUNLIGHT IRRADIATION IN THE NEMATODE CAENORHABDITIS ELEGANS

Ma, H, Kabengi, NJ, Bertsch, PM, Glenn, TC, Unrine J and Williams, PL. To be submitted to Environmental Science & Technology.
Abstract

The present study evaluated phototoxicity of a manufactured nanoparticulate ZnO (nano-ZnO, 40-100 nm primary particle diameter) to an environmentally relevant species the free living nematode, Caenorhabditis elegans, under natural sunlight illumination and compared it to toxicity under ambient artificial laboratory light. Bulk ZnO (~1.5 μm primary particle diameter) and aqueous ZnCl₂ were used as reference toxicants. Particle agglomeration for both nano-ZnO and bulk ZnO was significant in the test medium as revealed by transmission electron microscopy, dynamic light scattering, and differential interference contrast microscopy. Both nano-ZnO and bulk ZnO exhibited significant phototoxicity with 2-h LC₅₀s of 39±8 (mean±SEM, n=3) and 67±8 mg Zn/L, respectively. Photocatalytic activity of nano-ZnO and bulk ZnO measured by methylene blue degradation showed strong positive correlation with mortality in the nematodes, suggesting the mode of action of the phototoxicity is closely associated with photocatalytic activity of the metal oxide. Toxicity in the presence of ambient artificial laboratory light was much less than in the presence of sunlight for both nano-ZnO and bulk ZnO. The 24-h exposure under ambient laboratory light caused less lethality than 2-h exposure under natural sunlight illumination. Toxicity in the presence of ambient light was probably not related to photocatalytic ROS generation as neither nano-ZnO nor bulk ZnO degraded methylene blue under these conditions. Aqueous ZnCl₂ showed no lethal toxicity over the concentration range tested. Our findings suggest that phototoxicity of ZnO nanoparticles may occur under natural sunlight illumination, and this phototoxicity is greater and the onset of action is faster than toxicity under ambient laboratory light. We
have also demonstrated that primary particle size is more important than agglomerate size in determining such phototoxicity.

**Introduction**

Nanoparticulate metal oxides are an important class of materials with useful optical, magnetic, and electronic properties, which renders them useful for a great variety of applications such as catalysts, sensors, novel optical and electrical materials, and magnetic storage devices [1]. However, the implications of these novel properties of nanoparticles for the environment and human health are largely unknown. With more widespread use of these manufactured nanoparticles, concerns about their potential impact on the environment and human health have been raised [2]. In particular, zinc oxide nanoparticles (nano-ZnO) have recently received much attention due to their increasing use as a pigment, a UV absorber in personal care products such as sunscreens, as well as commodity materials including composites, coatings and paints [3].

Studies have suggested that manufactured nanoparticles are not inherently benign and can pose both direct and indirect toxic effects to ecological receptors [4]. Direct toxic effects are mainly determined by the chemical composition and surface reactivity of the engineered nanomaterials, as the greater surface area per mass of nanoparticles compared with their bulk counterparts usually renders them more biologically reactive [5]. Indirect effects can be caused by the release of toxic ions (e.g., dissolution of metal and metal oxide nanoparticles) or the production of reactive oxygen species (ROS) by the nanoparticles [6]. These two categories are not exclusively differentiated and can sometimes overlap. Several studies on toxicity of nanoparticles have attributed toxicity to
the surface reactivity of nanoparticles, which is primarily dependent on the particle size and surface chemistry [5]. More recent studies have identified that dissolution of nanoparticulate metal oxide to metal ions plays an important role in eliciting toxicity [7, 8]. There have been fewer studies examining the toxic effects of ROS generation by photocatalytic metal oxide nanoparticles such as TiO$_2$ and ZnO [9, 10].

Manufactured nanoparticles may produce ROS upon their interaction with organisms or with agents present in the environment (e.g., ultraviolet (UV) radiation). Reactive oxygen species production by nanoparticles interacting with environmental agents such as UV radiation is especially relevant for photocatalytic nanoparticles such as TiO$_2$ [11] or ZnO upon UV exposure [12]. When a photocatalyst is illuminated by the light stronger than its band gap energy, electron-hole pairs form on the surface of the metal oxide and transform the surrounding oxygen or water molecules into reactive oxygen species such as hydroxyl (OH$^-$) or superoxide (O$_2^-$) radicals [13]. High concentrations of these radicals can damage DNA, cell membranes, and cellular proteins, and may lead to cell death [10]. Damaging effects of TiO$_2$ nanoparticles to bacteria have been shown to be enhanced substantially by sunlight or UV illumination, presumably due to generation of ROS, especially hydroxyl radicals [9, 14]. Sunlight illuminated TiO$_2$ catalyses DNA damage both in vitro and in human cells [15], and the damaging effect increases as irradiation time increases [16]. Jones et al. [14] reported that ZnO nanoparticles display a wide range of antibiotic effects to various microorganisms under normal visible light conditions, and the authors proposed the possibility that ZnO is activated by the small (<4%) UV component associated with normal laboratory fluorescent lighting. All of these studies have focused primarily on the toxic effects
resulting from nanoparticle phototoxicity at the cellular and sub-cellular levels; however, understanding the potential phototoxicity of metal oxide nanoparticles in whole animal studies with ecologically relevant receptors is essential to ensure their proper use as well as the implications of their release to the environment.

As an initial effort to understand the potential phototoxicity of metal oxide nanoparticles to ecological receptors, the current study aims to evaluate acute phototoxicity of manufactured nano-ZnO to a free living nematode, *Caenorhabditis elegans* under natural sunlight illumination and compare it to toxicity under ambient artificial laboratory light. Under each type of illumination, the toxicity of nano-ZnO was compared to toxicity of bulk ZnO and aqueous ZnCl₂. *Caenorhabditis elegans* serves as a good model for both terrestrial and aquatic receptors [17]. Our hypotheses are that phototoxicity of nano-ZnO to the nematodes under natural sunlight illumination will be greater than toxicity under artificial laboratory light illumination, and this phototoxicity will be correlated with photocatalytic activity of the nanoparticles. We also hypothesize that this phototoxicity is greater in nano-ZnO than in bulk ZnO, as smaller particles have a greater reactive surface area and thus may be more effective in generating ROS [10]. We used natural sunlight instead of artificial UV light as illumination source for two reasons. First, previous studies in our laboratory found that one-hour irradiation by a germicidal UV lamp (254 nm) caused mortality as high as 30% in control animals, making the interpretation of nano-ZnO toxicity under this UV irradiation regime problematic, whereas *C. elegans* exposed to natural sunlight for a few hours did not cause any observed detrimental effects. Second, exposure under natural sunlight is a more
realistic scenario under which organisms might be exposed to nanoparticles released to
the environment.

Materials and Methods

Nano-ZnO and bulk ZnO. Powdered nanoparticulate ZnO (NanoGard® zinc oxide) was purchased from Alfa Aesar (Ward Hill, MA, USA) with a stated size of 40-100 nm. Stock suspensions of nano-ZnO and bulk ZnO (~1.5 μm, ACS Reagent grade, Mallinckrodt, Phillipsburg, NJ, USA) at a concentration of 100 mg particles/L (80% Zn) were prepared by sonication for 2 h using a Bransonic B-32H sonicator (Danbury, CT, USA). Specific surface areas (SSA) of nano-ZnO and bulk ZnO were determined from a multipoint Brunauer-Emmet-Teller (BET) N₂ adsorption isotherms obtained with a Micromeritics ASAP 2000 surface area analyzer. Dried samples were out-gassed overnight prior to measurement. The specific surface areas of nano-ZnO and bulk ZnO were found to be 17.0 and 4.2 m²/g, respectively. Reagent grade ZnCl₂ (Mallinckrodt, Phillipsburg, NJ, USA) was used to make ZnCl₂ stock solution at an equivalent concentration of 80 mg Zn/L. Test solutions for all experiments were freshly diluted from the stock, which was sonicated for 2 h prior to making dilutions to ensure proper dispersion of the materials. All solutions were made in K-medium (0.032 M KCl, 0.051 M NaCl) [18], an aqueous medium used for C. elegans based bioassays. The pH of the test solutions was measured using an ORION® Benchtop 720A pH/ION Meter (Boston, MA, USA).

Transmission Electron Microscopy (TEM). Particle images were taken using a Philips/FEI Tecnai 20 Transmission Electron Microscope (TEM) operating at 200 kV (FEI/Philips Electron Optics, Eindhoven, The Netherlands). Subsamples at various
concentrations were shaken vigorously to break up visible agglomerates prior to TEM analysis. Samples were prepared by depositing a drop (6 μL) of the suspensions on a carbon-coated copper specimen grid and allowing the water to evaporate in a laminar flow hood. All sample analyses included at least four different magnifications and at least three fields of view. Samples from aqueous medium blanks were also analyzed by TEM to check for particles that may have been present in the reagents.

**Dynamic Light Scattering (DLS).** DLS was performed using a photon correlation spectrophotometer (Zetasizer nano ZS, Malvern Instruments, Worcestershire, UK) to determine the $d_h$ (hydrodynamic diameter) of the particles in the stock suspension and subsamples. Subsamples from stock suspensions were vigorously shaken prior to analysis to break up visible agglomerates and resuspend any sedimented ZnO. Samples were placed in clean disposable cuvettes, and three consecutive measurements consisting of 15-40 sub-measurements were performed at 25 °C for each sample.

**Differential Interference Contrast Microscopy (DIC).** To analyze agglomerates > 1 μm in diameter, suspensions of nanomaterials were examined by DIC microscopy using a motorized microscope (Eclipse 90i, Nikon Instruments, Melville, NY, USA) equipped with a 40X objective lens and a cooled CCD monochrome camera. Liquid samples (50 μL) were mounted on pre-cleaned, particle free microscope slides. Using image analysis software (NIS Elements Basic Research, Nikon Instruments), diameters of rotation were defined around each particle in three fields of view for each sample and recorded.

**Solubility Testing.** The dissolution of nano-ZnO and bulk ZnO in suspensions was assessed by filtration through low binding Ultracel® membranes with a 3,000 nominal molecular weight limit (approximately 0.9 nm) using a centrifugal filtration device.
Three mL of nano-ZnO or bulk ZnO solutions were added to the filter units, and were centrifuged (Eppendorf 5810R; Westbury, NY, USA) for 30 min at 3220g relative centrifugation force. Filtrate were collected and analyzed for Zn concentration using an inductively coupled plasma mass spectrometer (ICP-MS; 7500 series, Agilent, USA).

**Caenorhabditis elegans Culture Maintenance.** The wild type N2 strain of *C. elegans* was originally obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN, USA), and maintained as a dauer larva stock in M9 buffer, replenished monthly [19]. All cultures were maintained at 20°C. Four-day-old worms from age-synchronized cultures were prepared using the methods of Donkin and Williams [20]. Briefly, dauers from the stock were transferred to Petri dishes containing nematode growth medium covered with a lawn of OP50 (a uracil deficient strain of *Escherichia coli*) and incubated; within three days, eggs were harvested and isolated from adults by treating for 10 min in a 1% (v/v) Clorox and 0.013 M NaOH solution, and transferred to nematode growth medium plates with an established lawn of OP50 and incubated at 20°C for four days. The worms were transferred onto a fresh OP50 plate one day prior to the testing.

**Caenorhabditis elegans Bioassay.** Acute toxicity test was conducted for three materials: nano-ZnO, bulk ZnO, and ZnCl₂. For each material, two sets of exposure were performed: one under natural sunlight illumination, and the other under ambient artificial laboratory light. All tests were conducted in K-medium (0.032 M KCl, 0.051 M NaCl, [18]) using 24-well tissue culture plates (Corning Costar, Kennebunk, ME, USA). Each test consisted of six concentrations of test substance (ranging from 4 to 80 mg Zn/L) and a control, with three replicate wells for each concentration and the control. A 1.0 ml
 aliquot of test solution was added to each of the wells, which was subsequently loaded
with 10 (±1) nematodes (~30 nematodes for each concentration). Light intensity was
measured using a Mastech Digital Luxmeter (Precision Mastech, Kowloon, Hong Kong,
China). For exposure under natural sunlight illumination, the experiment was conducted
on the window of a southwest facing laboratory on bright days (26±1.5°C average
temperature, UV index 4-5) in October in Athens, GA (33N, 83W). The plates were left
in direct sunlight through the open window for 2 h (3pm to 5pm EST) with lids off. The
average incident luminescence on the window during the test period was 1500±250 lux.
Mortality was monitored following the 2-h sunlight exposure. To examine long-term
effects induced from 2-h sunlight illumination, the exposure was continued for an
extended 22 h under ambient laboratory light after sunlight illumination by bringing back
the plates to the laboratory and continuing the exposure for 22 h. Mortality was
monitored again after the extended 22-h exposure. For exposure under ambient laboratory
light illumination, the plates were placed on a bench with lids off at ambient room
temperature (24~25 °C). The ambient laboratory lighting uses fluorescent lamps, and had
an intensity of 575±25 lux during the test period. For the purpose of comparison,
mortality was monitored at 2 h and 24 h, respectively. The plates were observed under a
dissecting microscope and the nematodes were counted and scored as live or dead; the
nematodes were judged dead if they did not respond to stimulus using a small platinum
wire.

Measure of Photocatalytic Activity. In parallel to the C. elegans toxicity assay,
evaluation of photocatalytic activity was conducted for nano-ZnO, bulk ZnO, and ZnCl₂
under both sunlight illumination and artificial laboratory light by measuring
photocatalytic degradation of methylene blue in aqueous solution [13, 21]. The mechanism leading to the degradation of methylene blue by photocatalysts such as ZnO or TiO₂ includes generation of radicals such as O₂⁻ and OH⁻ [22, 23]; therefore, degradation of the dye can be used as an indicator for the generation of ROS. For degradation experiments, a series of concentrations of each material and dissolved Zn were prepared in an identical manner as that for nematode bioassay, except that no nematodes were added; one mL of 25 mg/L methylene blue was added to each well of the plate. The plates were incubated for 30 min to allow for equilibrium for the dye sorption to particles. Prior to exposure under sunlight, the plates were gently shaken by hand for a few minutes to homogenize the solution. Methylene blue concentrations were measured at the beginning, after 2 h, and after 24 h of exposure for the tests conducted both under sunlight illumination and artificial laboratory light, using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 665 nm. As a negative control, one plate with a series of methylene blue concentrations in K-medium aqueous solution was kept under artificial laboratory light during the entire exposure and methylene blue concentration was measured at the beginning and the end of the experiment.

**Chemical Analysis.** Zinc concentrations in stock suspensions and selected subsamples were verified using inductively coupled plasma optical emission spectrometry (ICP-OES; 4300DV; PerkinElmer-Sciex, Waltham, MA USA). For ICP-OES analysis, samples were dissolved and diluted in 0.16 M ultra high purity HNO₃.

**Data Analysis.** All data reported were based on three independent experiments. Median lethal concentrations (LC50s) were calculated using TOXSTAT software
Correlation between *C. elegans* mortality and methylene blue degradation was assessed using Pearson’s correlation coefficients.

**Results**

**Particle Size of Nano-ZnO and Bulk ZnO.** TEM images of nano-ZnO and bulk ZnO at concentrations of 10 mg/L and 100 mg/L are shown in Figure 6.1. Significant particle agglomeration was observed for both nano-ZnO and bulk ZnO samples, with agglomerate size varying from a few hundred nanometers to several microns in diameter. Higher concentrations of nano-ZnO or bulk ZnO appeared denser under TEM. Primary particles were never isolated under the experimental conditions used. The primary particles for both nano-ZnO and bulk ZnO observed by TEM were predominately near-spherical to ellipsoidal in shape.

A low signal to noise ratio from large sedimenting particles during DLS analysis made it problematic to fit a negative non-linear least squares model to the data; therefore a cumulant analysis was used. The influence of these large sedimenting particles can be observed in the baselines of the correlograms (Figure 6.2). The $z$-average diameters of the 100 mg/L nano-ZnO and bulk ZnO samples were 1,683 and 1,703 nm respectively. Because DLS analysis was incapable of accurately measuring the larger, sedimenting agglomerates, we examined the suspensions using DIC microscopy. The average diameters of rotation for these agglomerates were 2,790 and 2,430 nm for 100 mg/L nano-ZnO and bulk ZnO, respectively. The size distribution was similar between the nano-ZnO and bulk ZnO (Figure 6.3).

**Toxicity of Nano-ZnO, Bulk ZnO, and ZnCl₂ to *C. elegans.*** Significant concentration-dependent lethal effects were observed in nematodes after 2-h exposure to
both nano-ZnO and bulk ZnO under sunlight illumination (Figure 6.4). At an identical concentration, nano-ZnO caused greater lethal effects than bulk ZnO. The 2-h LC50s for the two materials were $39 \pm 8$ (mean$\pm$SEM, n=3) and $67 \pm 8$ mg Zn/L, respectively. To examine long-term effects induced from 2-h sunlight illumination, the exposure was continued for an extended 22 h under ambient laboratory light after sunlight illumination. This extended 22-h exposure caused a significant increase in mortality compared to the initial 2-h sunlight exposure for both nano-ZnO and bulk ZnO (Figure 6.5). For example, nano-ZnO at 20 mg Zn/L increased mortality 40% by extending the exposure 22-h under ambient light conditions. Nano-ZnO concentrations above 40 mg Zn/L caused 100% mortality after the extended exposure. Similarly, bulk ZnO at 40 mg Zn/L caused an increase of 30% in mortality after the extended exposure. With the 2-h sunlight illumination and 22-h laboratory light exposure combined, the 24-h LC50s for nano-ZnO and bulk ZnO were determined to be $17 \pm 4$ (mean$\pm$SEM, n=3) and $38 \pm 6$ mg Zn/L, respectively.

For nematodes exposed under ambient laboratory light conditions, neither nano-ZnO nor bulk ZnO caused lethal effects after 2-h exposure (Figure 6.4). However, lethal effects were observed after 24-h exposure for both nano-ZnO and bulk ZnO (Figure 6.5), although the effects were considerably less compared to that induced by 2-h sunlight illumination followed by 22-h laboratory light exposure. Although accurate LC50s could not be determined for the 24-h laboratory light exposure because insufficient mortality was observed, an extrapolation from the concentration-response curves suggest that the LC50 for nano-ZnO is between 60-80 mg Zn/L and is even greater for bulk ZnO. No lethal effects were induced by ZnCl2 in the concentrations tested, regardless of the
exposure conditions and duration. Under all situations, mortality in control animals was below 10%.

**Methylene Blue Degradation by Nano-ZnO, Bulk ZnO, and ZnCl₂**. Methylene blue was degraded by both nano-ZnO and bulk ZnO under sunlight illumination, and the degradation was concentration dependent (Figure 6.6). After 2 h sunlight illumination, nano-ZnO at the highest (80 mg Zn/L) and lowest (4 mg Zn/L) concentrations degraded methylene blue by 63% and 7%, respectively. Similarly, bulk ZnO at the highest and lowest concentrations degraded methylene blue by 45% and 8%, respectively (Figure 6.6). There was a strong positive correlation between mortality in *C. elegans* and methylene blue degradation for both nano-ZnO (r=0.94, p=0.002, n=7) and bulk ZnO (r=0.90, p=0.005, n=7). Methylene blue concentration did not change during the extended 22 h exposure under ambient laboratory light. When illuminated under ambient laboratory light, neither nano-ZnO nor bulk ZnO degraded methylene blue. Methylene blue degradation was not observed with ZnCl₂, regardless of illumination method.

**Discussion**

Ecotoxicity of nanoparticles is largely determined by their physical and chemical properties, such as particle size, shape, surface area, solubility, and intrinsic surface chemistry or surface chemistry controlled by the types of coatings or reactive groups added to the core nanoparticle during synthesis [24], as well as the physicochemical conditions in the environment. Agglomeration is one of the most prominent behaviors of nanoparticles in aqueous environments that may have significant consequences for toxicity. It is affected by numerous abiotic factors such as pH, ionic strength, and the presence of organic matter [25]. For example, as pH approaches the point of zero charge,
repulsive forces between particles are decreased and agglomeration is expected to occur. An increase in electrolyte concentration, or counter ion valence, however, can contribute to agglomeration at any pH [26]. Therefore, appropriate characterization of the nanoparticles within a particular aqueous system is essential in evaluating their potential toxicity. We observed significant agglomeration of nano-ZnO in the current study, which is consistent with other studies conducted under varying solution conditions from distilled water [9] to complex biological media [7]. In our aqueous system, a relatively high ionic strength (0.083I) as well as the solution pH seemed to have played a key role in the agglomeration behavior observed. The point of zero charge for ZnO particles in aqueous solution, ranging from pH 8.7 to 10.3 have been reported in literature [27], suggesting that pH 7.8 measured in our aqueous system was unlikely to facilitate the formation of stable suspensions.

As TEM has the advantage of providing a direct image of particle size and morphology at high resolution, it has the inherent drawback that sample preparation with desiccation and high vacuum conditions may induce artifacts that are not related to the actual conditions in aqueous suspensions. Therefore, TEM by itself may not be able to provide a reliable measurement for the ambient particle agglomeration in suspension, and TEM data cannot be extrapolated to the actual conditions in the suspension. DLS serves as a complementary technique to TEM, which measures particle size in solution in real time with no need for sample preparation. However, determination of particle size distribution by DLS in the current study was challenging due to the extensive agglomeration of ZnO particles in the aqueous medium. However, cumulants analysis
combined with direct examination of the agglomerates using DIC microscopy seemed to indicate that both nano-ZnO and bulk ZnO formed agglomerates of similar size, and the size distribution of agglomerates was also similar.

As many studies have investigated nano-ZnO toxicity to a wide variety of environmentally relevant receptors [7, 8, 28, 29], few researchers have considered its phototoxicity associated with photocatalytic ROS generation [9]. Here we attempted to evaluate the potential phototoxicity of nano-ZnO under natural sunlight illumination to an environmentally relevant species, the nematode *C. elegans.* Phototoxicity was significant for both nano-ZnO and bulk ZnO as considerable nematode mortality was observed after 2-h exposure to these materials under sunlight illumination, whereas no mortality was observed for the two materials after 2-h exposure under ambient laboratory lighting. Photodegradation of methylene blue by nano-ZnO and bulk ZnO occurred under sunlight illumination and was a predictor of nematode mortality. A strong positive correlation between nematode mortality and methylene blue degradation suggests that photocatalytic ROS generation by nano-ZnO or bulk ZnO is involved in eliciting the lethal toxicity. There have been reports on positive correlation between ROS formation and antibacterial activity for TiO₂ nanoparticles, under both artificial UV irradiation [9] and natural UV component of sunlight [30]. Increased antibacterial activity of ZnO nanoparticles under ambient laboratory light [10] or natural sunlight [9] as compared to dark conditions has also been documented, presumably due to photoactivation of the nanoparticles. Our findings, however, demonstrated that photoactivation (ROS generation) of ZnO nanoparticles under natural sunlight illumination can cause lethal toxicity to a terrestrial and aquatic ecological receptor – the nematode *C. elegans.*
From a mechanistic perspective, ROS generation and oxidative stress represent the best developed paradigm to explain the toxic effects of inhaled nanoparticles in mammalian lungs, and adverse effects of ROS generation by nanoparticles at cellular and subcellular levels have been extensively studied in human and mammal models [6]. In the current study, although the specific mechanism for the lethal toxicity in *C. elegans* induced by nano-ZnO photoactivation remains unidentified, two possible mode of action may be proposed. First, the ROS generation and the consequent toxicity may occur at the surface of the nematodes. The concept of surface acting toxicant is not new in ecotoxicology as nanoparticles could be adsorbed to the exterior surface of the organism and elicits toxicity. This process has been implicated in TiO$_2$ nanoparticle toxicity to trout [31]. The *C. elegans* cuticle has an evenly distributed net negative charge at neutral pH [32], and this negative charge may enhance particle agglomeration on the surface of the animals via electrostatic interactions [8]. We have observed particle agglomerates attaching to the nematode cuticle during the exposure. The nematode cuticle is an extracellular matrix with a major component of collagen; it is highly impervious and acts as a barrier to protect the animal from external environment [33]. Monboisse et al. [34] has reported that ROS such as superoxide anion or hydroxyl can cause damage to calf skin collagen by degrading the protein. Therefore, it is possible that the intensive ROS generation on the surface of the nematode cuticle induced damage to the cuticle and subsequently caused toxicity to the internal organelles. Another possibility is the absorption of the nanoparticles into internal tissues.

In addition to phototoxicity, both nano-ZnO and bulk ZnO exhibited toxicity that appears to be independent of their photoactivation and ROS generation. We observed
lethality in *C. elegans* after 24-h exposure to both materials under ambient laboratory light. Jones et al. [14] have suggested the possibility that ZnO nanoparticles are activated by the small amount of UV present in emissions from conventional fluorescent lamps, as they found that antibacterial activity of ZnO nanoparticles is greater in ambient laboratory conditions than in the dark. However, this is probably not true in the present case as we did not observe any methylene blue degradation, which is an indicator for ZnO photoactivation and ROS generation. This indicates that toxicity not associated with photoactivation and ROS generation was also involved. One possibility is nano-ZnO dissolution to metal ions caused toxicity, which has been reported in freshwater alga [7] and fish embryos [35]. We evaluated the possible dissolution of nano-ZnO and bulk ZnO in suspensions and found a maximum of 5.3% and 3.6% of dissolution for nano-ZnO and bulk ZnO, respectively. This small percentage of dissolution does not seem to have significantly contributed to the observed lethal toxicity in *C. elegans*, because aqueous ZnCl₂ at much higher concentrations did not cause lethality in the nematodes. Therefore, this toxicity is more likely to be related to nanoparticle-dependent effects, i.e., stress or stimuli caused by the surface, size, and shape of the particles [8], although the exact mechanism or mode of action remains unknown. Many studies have suggested that toxic effects of nanoparticles may not be mediated solely by one mechanism, but rather multiple mechanisms or modes of action could be involved [8, 9, 36]. Our findings demonstrated this, as both photoactivation and particle-dependent effects (without photoactivation) have contributed to toxicity of both nano-ZnO and bulk ZnO to the nematodes, indicated by the nematodes mortality after 2-h exposure under sunlight illumination and 24-h exposure under ambient laboratory light. These two types of
toxicity could occur simultaneously within one single exposure, leading to synergistic toxic effects, as found in the nematodes exposed under ambient laboratory light following a 2-h sunlight exposure. As nanoparticles spilled into the environment will inevitably be exposed to sunlight, toxicity results from this exposure scenario are likely to represent the outcome when an ecological receptor encounters nanoparticles in a real environment exposure scenario.

The functional activities (chemical, catalytic or biological) of nanoparticles are heavily influenced by the size of the particles [14]. Antibacterial effects of nano-ZnO has been found to be strongly dependent on the particle size[10, 14], and particle size has been a crucial factor to determine the efficiency of ROS formation and consequently contaminant decomposition by photocatalysts such as TiO$_2$ or ZnO in water treatment [23]. We found that nano-ZnO showed greater toxicity (both photoactivation-mediated and particle-dependent) to the nematodes than bulk ZnO, although the two materials formed agglomerates of similar size in the test medium. Production of ROS is largely dependent on the surface reactivity of the particles, which is proportional to surface area and particle size. We have determined that nano-ZnO has a 3-fold greater surface area than bulk ZnO, and the former showed a 1.5-fold greater capacity in methylene blue photo degradation and consequently lethal toxicity to the nematodes than the later. Similar findings have been reported with toxicity of engineered TiO$_2$ nanoparticles to a green algae as the smallest particles showed a clear concentration-effect relationship, whereas the larger ones caused less toxicity [37]. However, the authors did not characterize particle agglomeration in their study, so it is not clear if it is particle size, or agglomerates contribute to the difference. We found that nano-ZnO and bulk ZnO formed
similarly sized agglomerates in our test system; therefore, it appears that primary particle size was more important, in terms of phototoxicity and methylene blue degradation, than agglomerate size. Although agglomeration may influence phototoxicity of the particles through altering the actual agglomerate or aggregate size and shape in solution, this effect is not as crucial as primary particle size in determining toxicity.

Photocatalytic metal oxide nanoparticles, such as ZnO, are being used in an increasingly wider range of application and it is expected that increasing quantities of these materials will be released to both terrestrial and aquatic environments. As research on toxicity of nonmaterial are expanding on daily basis, very few studies have considered phototoxicity of these metal oxide nanoparticles. The current study demonstrates that photocatalytic ZnO nanoparticles cause lethal toxicity to the nematode *C. elegans* under natural sunlight illumination, and the lethal effects occur within only a few hours. This phototoxicity is much greater than particle-dependent toxicity, with greater lethal effects occurring within shorter period of time. The phototoxicity and particle-dependent toxicity may occur simultaneously and lead to synergistic toxic effects to *C. elegans*. Our findings highlight the importance of phototoxicity evaluation for photocatalytic nanomaterials during risk assessment and management, as well as the need for caution during the use and disposal of such manufactured nanomaterials to prevent unintended environmental impacts. We have also demonstrated that primary particle size is more important than agglomerate size in determining such phototoxicity.

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**References**


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Fig 6.1 TEM images of nanoparticulate ZnO and bulk ZnO in K-medium (pH=7.8): (a) 10 mg/L nano-ZnO; (b) 100 mg/L nano-ZnO; (c) 10 mg/L bulk ZnO; (d) 100 mg/L bulk ZnO.
Fig 6.2 Correlograms of cumulant analysis from DLS analysis for agglomeration of nano-ZnO and bulk ZnO suspensions.
Fig 6.3 Agglomerate size distribution of nano-ZnO and bulk ZnO suspensions using DIC microscopy. The average diameters of rotation for these agglomerates were 2790 and 2430 nm for 100 mg/L nano-ZnO and bulk ZnO, respectively.
Fig 6.4 Two-hour lethal toxicity of nano-ZnO (A) (LC50 = 39(±8) mg Zn/L) and ZnO bulk (B) (LC50 = 67(±8) mg Zn/L) to the nematode C. elegans under natural sunlight illumination (■) and artificial light in laboratory condition (□). Error bars represent standard errors (n=3).
Fig 6.5 Twenty-four-hour lethal toxicity of ZnO NPs (A) and ZnO bulk (B) to the nematode C. elegans: 2-h sunlight exposure plus 22-h laboratory exposure (■) vs. 24-h laboratory exposure (□). Error bars represent standard errors (n=3). LC50 (ZnO NPs, sunlight plus laboratory) = 17(±4) mg Zn/L, LC50 (ZnO bulk, sunlight plus laboratory) = 38(±6) mg Zn/L.
Fig 6.6 Two-hour methylene blue degradation by ZnO-NPs (a) and ZnO bulk (b) under natural sunlight illumination (■) and artificial light in laboratory condition (□). Error bars represent standard errors (n=3).
CHAPTER 7

IMPACT OF ZINC OXIDE NANOPARTICLES ON COPPER TOXICITY IN THE NEMATODE CAENORHABDITIS ELEGANS

Abstract

Nanoparticles may alter the bioavailability and toxicity of dissolved metals because of their high surface activity. This chapter describes a preliminary attempt to examine the potential impact of ZnO nanoparticles (NPs) on the toxicity of aqueous CuCl₂ to the nematode *C. elegans*. We tested the acute lethal toxicity of CuCl₂ to *C. elegans* in the presence and absence of the ZnO NPs. Three concentrations of ZnO NPs (375, 750, 1500 μM), which cause 0%, 15%, and 30% mortality in the nematodes, were used. We also measured transgene response to CuCl₂ in a *mtl-2::GFP* transgenic *C. elegans* in the presence and absence of ZnO NPs. Only one ZnO NPs concentration (500 μM) was tested. It was found that at lower concentration (375 μM Zn), ZnO NPs appeared to increase the toxicity of Cu as the LC50 (Cu) was slightly lower with the presence of ZnO NPs, although the difference was not statistically significant; whereas at higher concentrations (750 and 1500 μM Zn), the nanoparticles seemed to decrease Cu toxicity as indicated by the greater LC50 (Cu) with the presence of ZnO NPs. For GFP transgene expression, the presence of ZnO NPs enhanced GFP response to Cu at all concentrations tested, and the increase in GFP exceeded the GFP expression that would be induced by ZnO NPs alone. Aqueous ZnCl₂ showed similar effects on Cu toxicity in lethality and GFP expression. These preliminary findings suggest that ZnO NPs can impact
bioavailability and toxicity of Cu in *C. elegans*. Future work will examine the impact using more sensitive endpoints such as behavior, and reproduction; and determine the adsorption of aqueous Cu$^{2+}$ to the nanoparticles.

**Introduction**

Environmental implications of manufactured nanoparticles have received increasing attention in both scientific community and general public. One important aspect of the potential impact of nanoparticles to the environment and ecosystem is their interaction with other environmental contaminants. Nanoparticles may adsorb pollutants due to their remarkably high surface area to volume ratio and complexing capability, which might change the transport and bioavailability of both the nanoparticles and the pollutants in natural systems, and consequently alter their toxic effects [1].

Interaction between hydrophobic engineered nanoparticles such as carbon nanotubes and fullerenes (C60) with organic pollutants has been reported [2-4], which could decrease or increase the toxicity of the pollutants. For example, the toxicity of diuron to green algae was reduced in the presence of carbon-black nanoparticles [5], and fullerenes were found to decrease the toxicity of various chemicals to daphnids and algae as a result of their decreased bioavailability [6]. On the contrary, the toxicity of phenanthrene to the green alga *P. subcapitata* and the zooplankton *D. magna* increased with the presence of fullerenes, despite that 85% of the phenanthrene was adsorbed to C60 aggregates [6]. The bioavailability of phenanthrene to plant roots also increased upon its adsorption to alumina nanoparticles [7]. Furthermore, the presence of TiO$_2$ nanoparticles has been shown to increase the accumulation of cadmium and arsenate in carps because of their strong sorption capacity for these elements [8, 9].
Manufactured nanoparticles may impact the potential bioavailability and toxicity of trace metals by affecting metal ion speciation and/or metal absorption [1]. A number of studies have considered the potential interaction between metals and hydrophobic nanomaterials such as fullerenes and carbon nanotubes [10]. For example, an investigation on the potential interaction between ZnCl₂ and carbon black nanoparticles suggested that ZnCl₂ and carbon black can synergistically interact to stimulate generation of tumor necrosis factor alpha in a macrophage cell line [11]. However, interaction between metal or metal oxide nanoparticles with trace metals has not been reported.

As an initial step to understand the potential impact of manufactured metal oxide nanoparticles on bioavailability and toxicity of trace metals, we evaluated the potential impact of manufactured ZnO NPs on the toxicity of CuCl₂ in the nematode *C. elegans*. Manufactured ZnO NPs are currently used for a broad range of applications, and it is reasonable to assume that they will be introduced into the environment in sufficient quantities in the near future to present a potential risk to ecological receptors. Copper is a common environmental contaminant, and Cu²⁺ levels in the environment have increased in recent years due to anthropogenic activities, such as application of copper containing fungicides and algaecides, industrial discharges, and use of the metal in the manufacture of kitchen tools, paint pigments, and water pipes [12]. Therefore, understanding the potential interaction between ZnO NPs and copper will contribute to a thorough ecotoxicological assessment for ZnO NPs.

We tested and compared acute lethal toxicity of Cu with the presence and absence of a series of ZnO NPs concentrations. We also looked at a transgene response to Cu in the nematodes with the presence and absence of ZnO NPs. For the purpose of
comparison, the same tests were performed for ZnCl₂. We hypothesize that nanoparticles will alter the bioavailability and toxicity of Cu through two possible mechanisms: competition of ZnO NPs with Cu²⁺ for sensitive binding sites at cell surfaces (which should decrease the toxicity of Cu), and sorption of Cu²⁺ to the highly surface active ZnO NPs (which may decrease or increase the toxicity of Cu, depending on the characteristics and bioavailability of the ZnO NPs to the nematodes).

**Materials and Methods**

**ZnO Nanoparticles**

Pinnacle AF ZnO nanoparticles suspension (ZnO-NPs) was purchased from Applied Nanoworks (Rensselaer, NY USA). The product data sheet reports the primary particle size to be 2-6 nm, and the surface area to be (250 m²/g). Chemical analysis confirmed the presence of zinc (56,000 mg/L Zn) as well as significant amount of acetate (2.33 M). Received samples had a pH of 6.25. Particle size was determined using two independent methods. Transmission electron microscopy (TEM) was performed to determine particle morphology and dₘ using a Philips/FEI Technai 20 (Amsterdam, Netherlands) electron microscope operating at 200 keV. Dynamic light scattering (DLS) was conducted using a photon correlation spectrophotometer (DynaPro, Wyatt Technology, Santa Barbara, CA, USA) to determine the dₘ of the particles in the stock suspension. Observation of the dₘ at the actual test concentrations in the exposure medium was not possible because the small particle size, small refractive index and low concentration yielding poor light scattering.

**Culture of nematodes**

The wild type nematode *C. elegans* N2 strain was originally obtained from the *Caenorhabditis Genetics Center* (Minecraft, MN, USA). Four-day-old worms from age-
synchronized cultures prepared using the methods of Donkin and Williams [13], were used for toxicity test. The mtl2::GFP transgenic C. elegans was developed in 2004, as reported by Humphries (2004, Master’s thesis, University of South Carolina, Columbia, SC, USA). Three-day-old, age-synchronized cultures were derived from egg plates and used for exposure following the same procedure as described above.

Toxicity test for Cu$^{2+}$ with and without presence of ZnO NPs

For all Cu$^{2+}$ toxicity test, five concentrations of Cu$^{2+}$ plus a control were used. All tests were conducted in 24-well tissue culture plates (Corning Costar, Lowell, MA, USA). For test without ZnO NPs, one mL of Cu$^{2+}$ solution was added to each well with three wells per concentration, and 10(±1) age-synchronized nematodes were added to each well. For tests with the presence of ZnO NPs, three concentrations of ZnO NPs were prepared. Cu$^{2+}$ solutions were prepared similarly as described above except that the concentrations were doubled. One half mL of each Cu$^{2+}$ concentration was added to the plates, followed by 0.5 mL of ZnO NPs, and this was done for all three concentrations of ZnO NPs. The plate was then gently shaken for a few seconds to homogenize the exposure solution. Finally the nematodes were added. All plates were exposed in a 20˚C incubator for 24 h. Following exposure, the plates were observed under a dissecting microscope, and the worms were counted and scored as live or dead. The nematodes were judged to be dead if they did not respond to stimulus using a small, metal wire. The test was independently repeated three times for each experiment. The same exposure procedure was also performed for ZnCl$_2$. For both ZnO NPs and ZnCl$_2$ experiments, ZnO NPs or ZnCl$_2$ and Cu$^{2+}$ solutions were originally made and diluted using acetic acid/acetate buffered K-medium (0.032 M KCl, 0.051 M NaCl, 0.14 M acetate, pH=6.0) instead of regular K-medium to avoid strong aggregation and gel formation. To examine
the effect of buffered K-medium on Cu toxicity, the Cu toxicity test was also conducted in regular unbuffered K-medium. Reagent-grade ZnCl$_2$ and CuCl$_2$ were used.

*Transgene response to Cu$^{2+}$ with and without ZnO NPs*

Test procedure for transgene response to Cu$^{2+}$ with and without ZnO NPs was similar to that for lethal toxicity, except that both Cu$^{2+}$ and ZnO NPs concentrations used were lower, the volume of test solution was much less, and the test were conducted in 96-well plates with clear bottom and black sides (Corning Costar, Lowell, MA, USA). Nematodes ($n = 35 \pm 1$) and test solution (250µL) were added to each well, with three wells per concentration, and exposed at 20°C for 24 h. Upon exposure, GFP intensity was measured using a Synergy HT microplate reader (BioTek Instruments) with a 485-nm excitation filter and a 528-nm emission filter. The GFP intensity is reported as relative fluorescence units based on calibration using a standard recombinant EGFP (BioVision). Each test was independently repeated three times.

*Data analysis*

The concentration–response relationships for lethality were generated from three independent replicate tests. The median lethal concentrations and 95% confidence interval (CI) were determined using Toxstat 3.2 software (Statistics Unlimited) with a probit transformation. The data were tested for normality with the chi-square test and for homogeneity of variance with the Bartlett’s test. Any data that did not pass these tests were disregarded. Analysis of variance was performed to test for differences among the mean LC50s of different treatment, with a significance level of $\alpha = 0.05$ using SAS software (SAS Institute).
Results and Discussion

Copper in unbuffered K-medium showed greater toxicity to *C. elegans* than in buffered K-medium, as indicated by their LC50s (849±124 vs. 4344±275 μM Cu/L, n=3, p<0.0001) (Figure 7.1). The LC50 for Cu with the presence of 375 μM ZnO NPs was 3853±500 μM Cu/L, which was slightly lower than that without the presence of ZnO NPs, although the difference was not statistically significant (p=0.12). With the presence of ZnO NPs at 750 and 1500 μM Zn/L, LC50s for Cu were 5897±296 and 5904±680 μM Cu/L, respectively, which were significantly higher than that without the presence of ZnO NPs (n=3, p<0.001). Aqueous ZnCl₂ showed similar impact on Cu toxicity as compared to ZnO NPs: at 375 μM ZnCl₂, LC50 for Cu was 4041±506 μM Cu/L; at 750 μM ZnCl₂, LC50 for Cu was 6135±188 μM Cu/L; and at 1500 μM ZnCl₂, LC50 for Cu was 6102±578 μM Cu/L.

For GFP transgene expression, the presence of 500 μM ZnCl₂ enhanced the GFP response at all Cu concentrations tested (Figure 7.2), and the enhancement in GFP exceeded the GFP that was induced by 500 μM ZnCl₂.

We have observed the impact of ZnO NPs on Cu toxicity, in terms of both lethality and transgene expression. For lethality, the impact appeared to be concentration dependent. At lower concentration (375 μM ZnO NPs), the ZnO nanoparticles seemed to increase the Cu toxicity; at higher concentrations (750 and 1500 μM ZnO NPs), the nanoparticles likely decreased the Cu toxicity. For transgene expression, the presence of nanoparticles seemed to have a synergistic effect on the copper to induce transgene expression.
As no study addressing the interaction between metal oxide nanoparticles and dissolved metals has been reported so far, the initial efforts in this area may involve the adoption of some concepts or principles from our understanding on the interaction between metal ions. Although metal species in the environment almost always exist in mixtures, few studies have focused on their combined effects on living organisms [12, 14]. Toxicological significance of interactions between trace metals within an organism is sometimes debatable, partly because they were usually demonstrated under extreme experimental conditions where massive doses of the metals were administered to animals whose trace metal status was already severely compromised [15]. There have been scattered reports on synergistic or neutralizing effect of specific transition metals in biological systems, e.g., copepod Amphiascus tenuiremis [16], and nematode C. elegans [17, 18]. One explanation of trace metal interaction on a rational basis is competitive biological interaction between metals with similar chemical and physical properties, and the mutual antagonism between Zn and Cu has been regarded as a prime example of such kind of interaction [15]. Based on this concept, and given the high surface reactivity of the ZnO nanoparticles, we speculate that ZnO NPs may affect the bioavailability and toxicity of Cu to C. elegans through two possible mechanisms: competition of ZnO NPs with Cu$^{2+}$ for sensitive binding sites at cell surfaces; sorption of Cu$^{2+}$ to the highly reactive surface of the nanoparticles. However, the preliminary data from this study did not provide sufficient evidence to examine either of the two hypotheses. Nevertheless, findings from these preliminary experiments suggest that ZnO nanoparticles can have impact on Cu toxicity, and highlight the necessity for considering such secondary effects of nanoparticles in the environment during their ecological risk assessment.
**Future Work**

The next step of this study attempts to determine the sorption of Cu to ZnO NPs by Cu sorption experiments (sorption isotherms), and measure the concomitant free Cu$^{2+}$ ion concentrations in the solution. As we have observed that impact of ZnO NPs on Cu toxicity was dependent on the nanoparticles concentration (lower ZnO NPs increased Cu toxicity and higher ZnO NPs decreased Cu toxicity), it would be necessary to examine if Cu sorption to the nanoparticles have occurred under these test conditions; and if yes, how does the Cu sorption change as the nanoparticles concentration changes. Results from the sorption experiments will help to elucidate the possible underlying mechanism of the interaction between ZnO NPs and ionic Cu in terms of toxicity to *C. elegans*.

A solubility test for the ZnO NPs will also be performed. We have observed synergistic effects between the nanoparticles and Cu$^{2+}$ in transgene expression induction. Our previous study demonstrated that ZnO NPs induces transgene expression in a similar manner as ZnCl$_2$, and that Zn spatial distribution displayed similar patterns between ZnO NPs treated and ZnCl$_2$ nematodes; both suggested the possible dissolution of ZnO NPs to Zn$^{2+}$ to elicit the effects. A solubility test will help to reveal the possible mechanism underlying the synergistic interaction.

**References**


Fig 7.1 Median lethal toxicity (LC50) of Cu in unbuffered K-medium, buffered K-medium, and buffered K-medium with the presence of three different concentrations of ZnO nanoparticles. Error bars denote standard error (n=3).
Fig 7.2 GFP transgene expression in response to Cu in buffered K-medium, and buffered K-medium with the presence of 500 μM ZnO nanoparticles. Error bars denote standard error (n=3).
CHAPTER 8
CONCLUSIONS

This dissertation explored using the nematode *C. elegans* to evaluate bioavailability and toxicity of transition metals and manufactured zinc oxide nanoparticles. We hypothesize that *C. elegans* can serve as a good model organism for ecotoxicological studies for both transition metals and manufactured metal oxide nanoparticles. Five studies were conducted to test this hypothesis. First, a transgenic strain of *C. elegans* using the metallothionein-2 promoter to control the expression of green fluorescent protein (GFP) reporter (*mtl-2::GFP*) was developed and tested in both aqueous and soil matrices for its GFP transgene response to several transition metals; the sensitivity of this transgenic bioassay was then compared to some conventional ecotoxicological endpoints such as lethality and behavior. The second study examined the toxicity of a commercially obtained ZnO nanoparticles suspension (2-6 nm in diameter) in *C. elegans* using ecologically relevant endpoints including lethality, behavior, reproduction, and transgene expression in the *mtl-2::GFP* transgenic strain, and compared the toxicity of ZnO nanoparticles to aqueous ZnCl2. In the third study, we evaluated the bioavailability and toxicity of the ZnO nanoparticles as compared to ZnCl2 by examining Zn spatial distribution using synchrotron based X-ray fluorescence microscopy, and GFP distribution (in the transgenic *C. elegans*) using ultraviolet-visible fluorescence microscopy. The fourth study assessed phototoxicity of manufactured ZnO
nanoparticles (powder, 40-100 nm primary particle diameter) in *C. elegans* under natural sunlight illumination; and compared it to the toxicity under ambient artificial laboratory light illumination. Bulk ZnO (~1.5 μm primary particle diameter) and aqueous ZnCl₂ were used as reference toxicants. Finally, a preliminary study to evaluate the potential impact of ZnO nanoparticles (suspension, 2-6 nm in diameter) on ecotoxicity of copper was conducted, by testing the acute lethal toxicity of CuCl₂ to the nematodes in the presence and absence of ZnO nanoparticles. GFP transgene response to CuCl₂ in the presence and absence of the nanoparticles was also tested and compared.

The major findings and conclusions from the five studies were summarized below:

**Conclusion 1:** The *mtl-2::GFP* transgenic *C. elegans* bioassay represents an alternative approach to quantify, both easily and quickly, a surrogate of metallothionein in response to certain metals (including Cd, Hg, Cu and Zn) exposure in a variety of environments. It may be potentially used for quantitative or semiquantitative biomonitoring for contamination of these metals in soils and aquatic systems.

**Conclusion 2:** Manufactured ZnO nanoparticles can cause toxicity in *C. elegans*. Overall bioavailability and toxicity between the ZnO nanoparticles and ZnCl₂ were similar, but subtle differences exist in the internal distribution of Zn in the nematodes treated by the nanoparticles and aqueous ZnCl₂. This suggests a role of Zn²⁺ in toxicity and internal biotransformation of the nanoparticles releasing Zn²⁺ might have occurred.

**Conclusion 3:** Phototoxicity (associated with ROS generation) of ZnO nanoparticles can occur under natural sunlight illumination, and this phototoxicity in the nematode *C. elegans* is greater and the onset of action is faster than toxicity under
ambient laboratory light. Evaluation on phototoxicity of nanoparticles with photocatalytic property should not be neglected during ecological risk assessment.

**Conclusion 4:** For phototoxicity associated with ROS generation, primary particle size is more important than agglomerate size in determining toxicity.

**Conclusion 5:** Preliminary data suggest that ZnO nanoparticles may influence the bioavailability and toxicity of copper in terms of lethality and transgene expression. However, solid conclusions regarding the impact of the nanoparticles on copper bioavailability and toxicity as well as the possible underlying mechanisms could not be obtained yet.

**Conclusion 6:** The nematode *C. elegans* is a useful model for toxicity testing of nanomaterials (nanoparticles), as it has been applied widely for toxicological studies for conventional compounds, and several ecologically relevant endpoints can be easily measured in the same exposure system.

In view of these conclusions, some suggestions for future research on relevant topics are outlined below:

1. Study the response of the *mtl-2::GFP* transgenic bioassay to other metals (i.e., Hg, Cu, Zn) in soil system, as well as application of the bioassay to field settings, i.e., water samples contaminated with transition metals.
2. Evaluate the impact of particle size on toxicity of nanoparticles.
3. Examine the influence of sample preparation method (i.e., sonication vs. stirring) on toxicity of nanoparticles.
4. Investigate the underlying mechanisms of phototoxicity of ZnO nanoparticles.
5. Study of the interaction between ZnO nanoparticles and trace metals and the potential consequence of such interaction on toxicity of the nanoparticles and the trace metals.

6. Evaluate possible trophic transfer of ZnO nanoparticles in a simplified food web, i.e., *E. coli* and *C. elegans*

The unique features of *C. elegans* make it an excellent model for ecotoxicological research. As the scientific community has started data generation and collection on the ecotoxicity of manufactured nanomaterials and nanoparticles, a great challenge is to broaden the data set so that ecotoxicity is assessed on organisms from different phyla. As most of ecotoxicological studies reported have focused on aquatic species, more studies are needed on terrestrial species. *Caenorhabditis elegans* may serve as a representative organism for terrestrial species in ecotoxicity studies on nanoparticles. Furthermore, the well-characterized biology and genetic manipulability of this organism will help to understand the underlying mechanisms of nanotoxicological effects. Therefore, future ecotoxicological studies on nanomaterials should include *C. elegans* as a test species.