EFFECTS OF SILVER NANOPARTICLES AND HYDROXYLATED FULLERENES ON EARLY LIFE STAGE OF THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*): METABOLOMIC APPROACH

by

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(under the Direction of Marsha Black)

ABSTRACT

Silver nanoparticles and carbon-based nanoparticles are two main categories of engineered nanoparticles and are massively produced and used in industrial and consumer products. Silver nanoparticles and hydroxylated fullerenes could both cause toxicity in aquatic organisms, however, the mechanisms of their toxicity remain unclear. We examined the effects of silver nanoparticles and hydroxylated fullerenes on fathead minnow embryos (*Pimephales promelas*). Silver nanoparticles caused both mortality and sublethal effects in exposed embryos. Hydroxylated fullerenes caused neither mortality nor meaningful metabolomic responses. Proposed modes of action of silver nanoparticle exposure included disturbance in osmoregulation and energy metabolism, induction of reductive glutamine metabolism and phospholipids biosynthesis. Our study demonstrated that NMR-based metabolomics can be an ideal approach for determining the toxic mechanisms of nanomaterials.

INDEX WORKDS: Silver nanoparticles, Hydroxylated fullerenes, Fathead minnow, Embryos, Toxicity, Metabolomics, Modes of action

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Overview

Nanomaterials are one of the more well-known emerging contaminants likely being inadvertently released into the environment before much is understood of their toxicity or toxic mechanisms. Because of the increase in the applications of nanomaterials in industry and consumer products, there is the potential for nanomaterials or nanoparticles to enter the natural environment and elicit toxicity. Metabolomics is proposed in this study as a more sensitive method to measure the toxic effects and determine modes of action of nanomaterials at environmentally relevant concentration. This study focused on the toxicity of silver nanoparticles and hydroxylated fullerenes in developing fathead minnow (*Pimephales promelas*) embryos and their metabolomic responses following the exposure. Background information concerning nanomaterials will be presented first to define nanomaterials and nanoparticles, their market and application, and their environmental toxicity in general. This is followed by reviews of silver nanoparticles and fullerenes, including their fate and transport, applications and environmental toxicity. Also, a review of the value of the embryonic toxicity test with fathead minnow will be presented, followed by an overview of the metabolomic approach used in this research project. Lastly, a brief statement of the research objective and goals follows the literature review.

Nanomaterials background

Nanomaterials are defined as materials with one dimension under 100 nm and nanoparticles are defined as materials with at least two dimensions between 1 and 100 nm (ASTM 2006). Nanoparticles have been found existing in all kinds of environments. In urban atmospheres, nanoparticles are released from the combustion of diesel and gasoline, which could cause adverse effects on human health (Shi et al., 2001). In aquatic environments, the size of natural aquatic colloids ranges from 1 nm to 1 μm. Nanoscale colloids are capable of binding to organic and inorganic pollutants because of their small size and high surface area (Lead et al., 1997). In soils, natural nanoparticles include clays, organic matter, iron oxides, and other minerals. Soil colloids have been reported to facilitate the movement of pollutants (Ouyang et al., 1996).

Engineered nanomaterials have been a major concern in recent years because of their increased production. Nanotechnology is predicted to be a $1 trillion market by 2015 (Roco, 2001). Increased applications and production of nanomaterials make exposure of these contaminants to the natural water more probable (Mueller and Nowack, 2008). Engineered nanomaterials are increasingly used in sporting goods, tires, stain-resistant clothing, sunscreens, cosmetics, and electronics. Research is also looking at applying nanotechnology to disease diagnosis, medical imaging, and drug delivery (Mueller and Nowack, 2008). Nanomaterials are designed to have unique physical and chemical properties; however, these novel properties raise concerns about adverse effects on human and environmental health (Nel et al., 2006). The toxic mechanisms of most nanomaterials are not well understood. Possible toxic mechanisms include the disruption of cell membranes or cell membrane potential, protein oxidation, genotoxicity, interruption of energy transduction, formation of reactive oxygen species, and the release of

toxic constituents (Klaine et al., 2008). Recently, oxidative stress responses were predicted to be appropriate toxicological paradigms to assess hazards of nanomaterial. Nanomaterials could induce oxidative stress responses at different cellular levels, from anti-oxidant defense, to inflammation, to cytotoxicity. Differing physicochemical properties of nanomaterials could lead to varying types and levels of cellular injuries, which could result in adverse health outcomes in whole organisms (Meng et al., 2009; Nel et al., 2006).

Engineered nanomaterials could be released to the environment from either liquid or solid waste during their production. Depending on their applications, they could also be intentionally released to the environment. Some nanoparticles have applications in groundwater remediation, such as zero-valent iron, and then enter the water system. Nanoparticles also have applications in paint, sun screen, and fabric production, and thus enter the environment by different methods and in a range of concentrations. Nanoparticles could also be released through the abrasion, erosion, and decomposition in most nanotechnology involved products (Klaine et al., 2008). Once nanoparticles enter the environment, they have the potential to contaminate soils, surface and ground water systems. The nanoparticle contamination will not be restricted to the original contaminated site and will thus increase the difficulty in regulation and remediation (Klaine et al., 2008).

Nanomaterials can be classified as carbon based nanoparticles (NPs), metal and metal-containing materials, semiconductors, and dendrimers (Klaine et al., 2008). Metal NPs and carbon based NPs are the two major categories of engineered nanomaterials currently in use. In this study, silver NPs, as the most widely used metal NPs, and fullerenes, as the basic unit of carbon based NPs, are investigated.

Silver nanoparticles

Silver nanoparticles (AgNPs) are involved in the widest range of nanotechnology- based consumer products. Because of their antimicrobial properties, silver nanoparticles are widely used in detergents, fabrics, air filters, tooth paste, vacuum cleaner, and washing machines. (Project on Emerging Nanotechnologies Inventory, Woodrow Wilson International center for scholars; Washington, DC). In 2008 the worldwide production of silver nanoparticles was estimated around 500 tons per year (Mueller and Nowack, 2008). The rapid expansion of AgNPs in commercial use raises concerns for aquatic exposure and resultant toxicity to aquatic organisms.

Silver nanoparticles could be released to the environment during the production, useful life, and during disposal processes (Gottschalk and Nowack, 2011). Recent studies have shown that AgNPs could be released to the environment from consumer products such as socks and other fabrics during the household washing process (Benn and Westerhoff, 2008). When AgNPs enter a wastewater treatment plant (WWTP), the AgNPs will react with reduced sulfur and precipitate as silver sulfide in the sludge with chloride and adsorb to biomass (Kim et al., 2010). Concerns about the presence of silver nanoparticles in the effluent of WWTP are reduced, but potential contamination to soil can result following application of sewage sludge materials on agricultural land as soil amendment or used in other land application, which may contain Ag_2S (Kim et al., 2010). However, there is still concern about the fraction of AgNPs directly released into water systems without wastewater treatment. Larger silver nanoparticles (>50 nm) have the potential to persist in the aquatic environment for an extended period of time and serve as a continuous source of silver ions (Dobias and Bernier-Latmani, 2013). Currently, there is not a reliable analytical technique to quantify concentrations of silver nanoparticles in the

environment. However, a recent study reported predicted environmental concentrations of silver nanoparticles in aquatic environments as being between 0.03 and 0.08 μgL^{-1} (Mueller and Nowack, 2008).

The chemical properties of silver nanoparticles can be used in applications requiring antimicrobial properties. AgNPs can interact with bacterial cell membranes and be transported into cells, resulting in membrane damage, the release of reactive oxygen species, and induction of stress response in the bacterial cell (Choi and Hu, 2008; Morones et al., 2005). AgNPs have been reported to release silver ions and cause adverse effects on the neurodevelopment of developing zebrafish (*Danio rerio*). Lee et al. (2007) demonstrated AgNPs were transported across chorion pore canals of zebra fish embryos and accumulate inside embryos at each development stages. The dissolution of silver ions from silver nanoparticles also impairs embryonic survival, growth, and development at high concentrations, and at low concentrations, depresses larval swimming behavior (Powers et al., 2010). Exposure to silver nanoparticles caused acute mortality in both adult and juvenile zebrafish with the 48-hour LC50 values reported for the adult zebrafish being 7.07 mg/L and for juveniles being 7.20 mg/L (Griffitt et al., 2008). Laban et al. (2010) reported that the 96-hour LC50 value of two different commercial AgNPs in fathead minnow embryos were 1.25 mg/L and 1.36 mg/L. Fathead minnow embryos exposed to AgNPs exhibited high mortality and a greater chance of malformation, including edema, hemorrhage, and lordosis (Laban et al., 2010).

In juvenile and adult fish, the uptake of silver nanoparticles is chiefly mainly via the gills. The adverse effects on the gills could result from the attachment of silver ions from the AgNPs' surfaces or released from AgNPs to the solution (Griffitt et al., 2009). The contact of silver ions with the fish gills could potentially cause the inhibition of basolateral Na/K-ATPase activity and

compromise osmoregulation (Wood et al., 1999). Following exposure to silver nanoparticles zebrafish and rainbow trout (*Oncorhynchus mykiss*) have exhibited oxidative damage, impairment of the normal expression of oxyradical-scavenging enzymes, apoptosis, and impairment of metabolism and normal toxicological response to metals (Choi et al., 2010; Scown et al., 2010).

Fullerenes

Fullerenes are closed cage, hollow and nearly spherical molecules composed of 60-carbon atoms that have wide applications in a variety of industrial and consumer products. The discovery and manufacture of fullerenes provided the possibility of transforming the pure carbon units into a new structure with unique physical and chemical properties (Dresselhaus et al., 1996). Fullerenes are currently compounds of drug delivery systems, lubricants, solar cells, catalyst, and polymer modification. Fullerenes are also applied in consumer products, such as anti-wear surfaces, cosmetics, and sporting goods (Aschberger et al., 2010). Fullerenes are the basic building unit of carbon nanotubes (CNTs), of which the global production is expected to exceed 12,300 tons in 2015 with a market value of approximately $1.3 billion (Innovative Research and Products, Inc. 2011). The annual production of fullerenes in U.S. is estimated between 2 to 80 tons per year (Hendren et al., 2011).

Fullerenes display both electron donating and accepting properties and both unmodified and modified fullerenes can release oxyradicals in vitro (Kamat et al., 2000; Sayes et al., 2004). Following accumulation, fullerenes can cause oxidative damage in aquatic organisms (Oberdorster, 2004; Usenko et al., 2008) and affect gene expression of pathways involved in regulation of oxidative stress (Henry et al., 2007;). When adult fathead minnows were exposed to

0.5 mg/L of fullerenes, they showed significantly elevated lipid peroxidation in the brain and gills, and significantly increased expression of CYP2 family isozymes in the liver (Zhu et al., 2006). Hydroxylated fullerenes [$C_{60}(OH)_{24}$] possess a high water solubility and stability in solution, which increases their industrial and medical use (Da Ros and Prato, 1999). Hydroxylated fullerenes displayed less cytotoxicity then unmodified fullerenes (Sayes et al., 2004), serving mainly as antioxidants and inducing mild ROS-independent pro-apoptosis activity in vitro (Isakovic et al., 2006). As for the toxicity of $C_{60}(OH)_{24}$ in an *in vivo* model, Usenko et al. (2007) reported $C_{60}(OH)_{24}$ caused significant mortality and edema in zebrafish embryos but was less toxic than robust fullerenes. Hydroxylated fullerenes did not cause significant mortality in the fathead minnow embryo toxicity assay (FET), however, they did induce pericardial edemas and yolk coagulation starting at 0.01 mgL^{-1} (Jovanović et al., 2011a). Adult fathead minnows exposed to hydroxylated fullerenes exhibited suppressed neutrophil function and altered immune gene expression (Jovanović et al., 2011a). A significant alteration in genes involved in regulating circadian rhythm, kinase activity, vesicular transport and immune response was found in zebrafish embryos exposed to hydroxylated fullerenes (Jovanović et al., 2011b).

Fish embryo toxicity test

Fish embryo toxicity testing will be useful in detecting toxicity of nanoparticles. Fish are the major vertebrate class in freshwater and marine ecosystems and have been traditionally considered to be an indispensable test species. Fish mortality is the usual method to assess the effects of chemical spills, industrial pollution, and overall environmental health in aquatic ecosystems. Additionally, monitoring the overall health of fish populations is a common method

to evaluate water quality in surface waters, which are important water sources for humans. Thus, several standardized toxicity tests have been developed to measure the pollutant toxicity to fish.

The embryo-larval and early juvenile life- stages are the most sensitive life stages to pollutants. Toxicity tests using early life stage of fish can be used to predict long-term toxicity and create water quality criteria (McKim, 1977). Embryonic toxicity tests using zebrafish have been demonstrated to be more sensitive than cytotoxicity tests using permanent fish cell lines (Castaño et al., 1996). Early life stage toxicity tests begin with embryos prior to the eyed stage and continue until the juvenile stage (McKim, 1977). Compared to whole life stage chronic toxicity tests, early life stage testing can reduce time and culturing effort while increasing the total number of test samples.

The fathead minnow belongs to the *Cyprinidae* family, which is the largest group of freshwater fish in North America (Scott and Crossman, 1973). The popularity of this species as a baitfish and forage fish has led to widespread introductions into the wild. Fathead minnows are easily cultured in the laboratory and have one prolonged spawning period throughout the year. The fathead minnow embryo-larval survival and teratogenicity tests were developed as a standardized test to estimate the chronic toxicity of whole effluents and receiving waters (USEPA, 2002).

Metabolomics

Metabolomic response, as a major component of ecotoxicogenomics, can be used as a biomarker of exposure to nanoparticles. Ecotoxicogenomics was recently proposed as a method to integrate transcriptomics, proteomics and metabolomics into ecotoxicology to study the gene and protein expression in non-target organisms in response to environmental toxicant exposures

(Snape et al., 2004). Traditionally, toxicity endpoints have primarily focused on phenotypical responses, using mortality, growth and reproduction as endpoints. However, these traditional endpoints provide little understanding of the mechanisms of toxicity. Also, traditional endpoints lack sensitivity to elicit a noticeable response to many chemicals or metals at environmentally relevant concentrations. Ecotoxicogenomics provides a more holistic insight required for a mechanistic understanding of aquatic ecotoxicology (Snape et al., 2004).

Metabolomics measures the dynamic metabolic responses of an organism to a toxic or physiological challenge (Nicholson et al., 1999). Xenobiotics could interact with tissue and extracellular components in a living organism and have effects on gene expression, protein production, and cellular biochemical regulation. In some cases, a xenobiotic will cause fluctuations of endogenous biochemicals by affecting key enzymes and nucleic acids that regulate metabolism. Therefore, using metabolomics, toxicity effects can be investigated by analyzing subtle alterations in endogenous metabolites in blood, plasma, urine, bile, or tissue samples (Nicholson et al., 1999).

Metabolomics typically either utilizes a mass spectrometry (MS) platform or is based on a nuclear magnetic resonance spectroscopy (NMR) platform. Generally, using MS is more sensitive and more suitable for discovering novel biomarkers. On the contrary, NMR provides an approach that is open-ended, has excellent cross-platform reproducibility, and less sample bias (Keun et al., 2002). NMR-based metabolomics can be used to measure stress-induced biochemical changes in living organisms. Information about a variety of metabolic pathways can be accessed simultaneously, often with little effort in sample preparation. This information-rich method accompanied with pattern recognition algorithms and chemometric methods can maximize information recovery (Gartland et al., 1991).

High-resolution ^{1}H NMR spectroscopy has frequently been used as a rapid, noninvasive method to measure metabolites present in biofluids (Gartland et al., 1989; Nicholson and Gartland, 1989). Biochemical changes can be measured in biofluids with ^{1}H NMR and provide information for probing organ toxicity and discovery of biomarkers of toxic exposure and effect (Holmes et al., 2001). In order to interpret NMR data in metabolomic studies, principal components analysis (PCA) is a common method of multivariate analysis that reduces a large number of variables into smaller subsets of uncorrelated principal components. These principal components are linear combinations of the original (weighted) variables. For example, a 2000 variable data set can be reduced to two or three variables and be represented as a point in the PCA scores plots. The first principal component explains the greatest variability in the data, the second principal component is independent of the first component and explains the second greatest amount of variability in the data. This analysis has been successfully utilized to correlate tissue-specific toxicity with a combination of metabolite changes (Griffin et al., 2000; Holmes et al., 2001). The characteristic metabolomic changes can be indicative of the possible mechanisms behind the toxicological effects.

Metabolomic techniques can be a novel method to investigate the toxicity of nanomaterials. Metabolomics has been previously utilized to investigate metabolic response after chemical exposures in aquatic species and mammals, including Japanese medaka (*Oryzias latipes*) embryos exposed to trichloroethylene and dinoseb (Viant et al., 2005; Viant et al., 2006a), mice exposed to varying sizes of silica nanoparticles (Lu et al., 2011) and to TiO_2 NPs (Tang et al., 2010), and rats exposed to Ag NPs (Hadrup et al., 2012). NMR metabolomics has been applied previously in fish embryo toxicity studies to investigate the toxicity mechanisms and developmental metabolic trajectories (Viant et al., 2005; Viant et al., 2006a; Viant et al.,

2006b). The change of metabolomic profiles has been proven to be a more sensitive biomarker than traditional toxicity endpoints in some cases. Metabolomic responses to trichloroethylene exposure were more sensitive than mortality and hatching success in Japanese medaka embryos (Viant et al., 2005). Metabolomic profiles taken at different developmental stages illustrated a dynamic change occurring as an organism develops (Huang et al., 2013). Metabolic changes throughout embryogenesis can help determine the stage-specific modes of action, which can be correlated with known morphological events and thus explain potential adverse effects in organs. Transient exposure to xenobiotics can also be assessed by determining if perturbed metabolic trajectory returns to the normal course of development for that organism (Viant et al., 2005). Moreover, transcriptomic and proteomic data can be associated with metabolomic profiles to provide a deeper insight into the molecular toxic mechanisms. Applying metabolomic techniques to embryonic toxicity testing can serve as the basis for toxicological and developmental studies in the 21st century.

Research Objectives

The aim of the research in this thesis was to study the toxicity and toxic mechanisms of two kinds of nanoparticles, silver nanoparticles and hydroxylated fullerenes in early life stages of fathead minnow. By using embryonic toxicity testing and an innovative metabolomic approach, significant advances can be made in understanding morphological toxicity and toxic mechanisms of these two nanoparticles to fathead minnow embryos.

We hypothesize that, first, AgNPs and hydroxylated fullerenes can cause effects on mortality or morphological abnormalities in early life stages of fathead minnow. And secondly, that, ^{1}H NMR-based metabolomics can provide more sensitive biomarkers of toxicity than the

fathead minnow embryo toxicity assay and can be used to determine the mechanisms of action of AgNPs and hydroxylated fullerenes.

Chapter two of this thesis will focus on delineating the effects of silver nanoparticles and hydroxylated fullerenes by measuring mortality, teratogenicity, and metabolomic profiles. This research will illustrate possible toxic mechanisms in response to different stages of embryogenesis and evaluate the benefit of using metabolomic responses as novel biomarkers of nanoparticles exposure in aquatic systems. Chapter three will put into context the ecotoxicological implications of these findings and propose future directions of nanotoxicity research with a metabolomics approach.

Reference

American Society for Testing and Materials., 2006. Standard terminology relating to nanotechnology. E 2456-06. West Con-shohocken, PA.

Aschberger, K., Johnston, H.J., Stone, V., Aitken, R.J., Tran, C.L., Hankin, S.M., Peters, S.A.K., Christensen, F.M., 2010. Review of fullerene toxicity and exposure – Appraisal of a human health risk assessment, based on open literature. Regulatory Toxicology and Pharmacology 58, 455-473.

Benn, T.M., Westerhoff, P., 2008. Nanoparticle Silver Released into Water from Commercially Available Sock Fabrics. Environmental science & technology 42, 4133-4139.

Castaño, A., Cantarino, M.J., Castillo, P., Tarazona, J.V., 1996. Correlations between the RTG-2 cytotoxicity test EC50 and in vivo LC50 rainbow trout bioassay. Chemosphere 32, 2141-2157.

Choi, J.E., Kim, S., Ahn, J.H., Youn, P., Kang, J.S., Park, K., Yi, J., Ryu, D.-Y., 2010. Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. Aquatic Toxicology 100, 151-159.

Choi, O., Hu, Z., 2008. Size dependent and reactive oxygen species related nanosilver toxicity to nitrifying bacteria. Environmental science & technology 42, 4583-4588.

Da Ros, T., Prato, M., 1999. Medicinal chemistry with fullerenes and fullerene derivatives. Chemical Communications, 663-669.

Dobias, J., Bernier-Latmani, R., 2013. Silver release from silver nanoparticles in natural waters. Environmental science & technology 47, 4140-4146.

Dresselhaus, S., Dresselhaus, G., Eklund, P.C., 1996. Science of Fullerenes and Carbon Nanotubes. Academic Press.

Gartland, K.P., Beddell, C.R., Lindon, J.C., Nicholson, J.K., 1991. Application of pattern recognition methods to the analysis and classification of toxicological data derived from proton nuclear magnetic resonance spectroscopy of urine. Molecular pharmacology 39, 629-642.

Gartland, K.P., Eason, C.T., Wade, K.E., Bonner, F.W., Nicholson, J.K., 1989. Proton NMR spectroscopy of bile for monitoring the excretion of endogenous and xenobiotic metabolites: application to para-aminophenol. Journal of pharmaceutical and biomedical analysis 7, 699-707.

Gottschalk, F., Nowack, B., 2011. The release of engineered nanomaterials to the environment. Journal of Environmental Monitoring 13, 1145-1155.

Griffin, J.L., Walker, L.A., Garrod, S., Holmes, E., Shore, R.F., Nicholson, J.K., 2000. NMR spectroscopy based metabonomic studies on the comparative biochemistry of the kidney and urine of the bank vole (Clethrionomys glareolus), wood mouse (Apodemus sylvaticus), white toothed shrew (Crocidura suaveolens) and the laboratory rat. Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology 127, 357-367.

Griffitt, R.J., Hyndman, K., Denslow, N.D., Barber, D.S., 2009. Comparison of Molecular and Histological Changes in Zebrafish Gills Exposed to Metallic Nanoparticles. Toxicological Sciences 107, 404-415.

Griffitt, R.J., Luo, J., Gao, J., Bonzongo, J.C., Barber, D.S., 2008. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. Environmental toxicology and chemistry 27, 1972-1978.

Hadrup, N., Lam, H.R., Loeschner, K., Mortensen, A., Larsen, E.H., Frandsen, H., 2012. Nanoparticulate silver increases uric acid and allantoin excretion in rats, as identified by metabolomics. Journal of applied toxicology : JAT.

Hendren, C.O., Mesnard, X., Dröge, J., Wiesner, M.R., 2011. Estimating Production Data for Five Engineered Nanomaterials As a Basis for Exposure Assessment. Environmental science & technology 45, 2562-2569.

Henry, T.B., Menn, F.M., Fleming, J.T., Wilgus, J., Compton, R.N., Sayler, G.S., 2007. Attributing effects of aqueous C60 nano-aggregates to tetrahydrofuran decomposition products in larval zebrafish by assessment of gene expression. Environmental health perspectives 115, 1059-1065.

Holmes, E., Nicholson, J.K., Tranter, G., 2001. Metabonomic Characterization of Genetic Variations in Toxicological and Metabolic Responses Using Probabilistic Neural Networks. Chemical research in toxicology 14, 182-191.

Huang, S.-M., Xu, F., Lam, S.H., Gong, Z., Ong, C.N., 2013. Metabolomics of developing zebrafish embryos using gas chromatography- and liquid chromatography-mass spectrometry. Molecular bioSystems 9, 1372-1380.

Innovative research and products inc., 2011.. Production and applications of carbon nanotubes, carbon nanofibers, fullerenes, graphene and nanodiamonds: a global technology survey and market analysis., 531

Isakovic, A., Markovic, Z., Todorovic-Markovic, B., Nikolic, N., Vranjes-Djuric, S., Mirkovic, M., Dramicanin, M., Harhaji, L., Raicevic, N., Nikolic, Z., Trajkovic, V., 2006. Distinct Cytotoxic Mechanisms of Pristine versus Hydroxylated Fullerene. Toxicological Sciences 91, 173-183.

Jovanović, B., Anastasova, L., Rowe, E.W., Palić, D., 2011a. Hydroxylated fullerenes inhibit neutrophil function in fathead minnow (Pimephales promelas Rafinesque, 1820). Aquatic Toxicology 101, 474-482.

Jovanović, B., Ji, T., Palic, D., 2011b. Gene expression of zebrafish embryos exposed to titanium dioxide nanoparticles and hydroxylated fullerenes. Ecotoxicology and environmental safety 74, 1518-1525.

Kamat, J.P., Devasagayam, T.P., Priyadarsini, K.I., Mohan, H., 2000. Reactive oxygen species mediated membrane damage induced by fullerene derivatives and its possible biological implications. Toxicology 155, 55-61.

Keun, H.C., Ebbels, T.M., Antti, H., Bollard, M.E., Beckonert, O., Schlotterbeck, G., Senn, H., Niederhauser, U., Holmes, E., Lindon, J.C., Nicholson, J.K., 2002. Analytical reproducibility in (1)H NMR-based metabonomic urinalysis. Chemical research in toxicology 15, 1380-1386.

Kim, B., Park, C.-S., Murayama, M., Hochella, M.F., 2010. Discovery and Characterization of Silver Sulfide Nanoparticles in Final Sewage Sludge Products. Environmental science & technology 44, 7509-7514.

Klaine, S.J., Alvarez, P.J.J., Batley, G.E., Fernandes, T.F., Handy, R.D., Lyon, D.Y., Mahendra, S., McLaughlin, M.J., Lead, J.R., 2008. Nanomaterials in the environment: Behavior, fate, bioavailability, and effects. Environmental Toxicology and Chemistry 27, 1825-1851.

Laban, G., Nies, L.F., Turco, R.F., Bickham, J.W., Sepulveda, M.S., 2010. The effects of silver nanoparticles on fathead minnow (Pimephales promelas) embryos. Ecotoxicology 19, 185-195.

Lead, J.R., Davison, W., Hamilton-Taylor, J., Buffle, J., 1997. Characterizing Colloidal Material in Natural Waters. Aquatic Geochemistry 3, 213-232.

Lee, K.J., Nallathamby, P.D., Browning, L.M., Osgood, C.J., Xu, X.H., 2007. In vivo imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. ACS nano 1, 133-143.

Lu, X., Tian, Y., Zhao, Q., Jin, T., Xiao, S., Fan, X., 2011. Integrated metabonomics analysis of the size-response relationship of silica nanoparticles-induced toxicity in mice. Nanotechnology 22, 055101.

McKim, J.M., 1977. Evaluation of Tests with Early Life Stages of Fish for Predicting Long-Term Toxicity. Journal of the Fisheries Research Board of Canada 34, 1148-1154.

Meng, H., Xia, T., George, S., Nel, A.E., 2009. A Predictive Toxicological Paradigm for the Safety Assessment of Nanomaterials. ACS nano 3, 1620-1627.

Morones, J.R., Elechiguerra, J.L., Camacho, A., Holt, K., Kouri, J.B., Ramirez, J.T., Yacaman, M.J., 2005. The bactericidal effect of silver nanoparticles. Nanotechnology 16, 2346-2353.

Mueller, N.C., Nowack, B., 2008. Exposure Modeling of Engineered Nanoparticles in the Environment. Environmental science & technology 42, 4447-4453.

Nel, A., Xia, T., Madler, L., Li, N., 2006. Toxic potential of materials at the nanolevel. Science 311, 622-627.

Nicholson, J.K., Gartland, K.P., 1989. 1H NMR studies on protein binding of histidine, tyrosine and phenylalanine in blood plasma. NMR in biomedicine 2, 77-82.

Nicholson, J.K., Lindon, J.C., Holmes, E., 1999. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica; The Fate Of Foreign Compounds In Biological Systems 29, 1181-1189.

Oberdorster, E., 2004. Manufactured nanomaterials (fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth bass. Environmental health perspectives 112, 1058-1062.

Ouyang, Y., Shinde, D., Mansell, R.S., Harris, W., 1996. Colloid-enhanced transport of chemicals in subsurface environments: A review. Critical Reviews in Environmental Science and Technology 26, 189-204.

Powers, C.M., Yen, J., Linney, E.A., Seidler, F.J., Slotkin, T.A., 2010. Silver exposure in developing zebrafish (Danio rerio): persistent effects on larval behavior and survival. Neurotoxicology and teratology 32, 391-397.

Roco, M.C., 2001. International strategy for Nanotechnology Research. Journal of Nanoparticle Research 3, 353-360.

Sayes, C.M., Fortner, J.D., Guo, W., Lyon, D., Boyd, A.M., Ausman, K.D., Tao, Y.J., Sitharaman, B., Wilson, L.J., Hughes, J.B., West, J.L., Colvin, V.L., 2004. The Differential Cytotoxicity of Water-Soluble Fullerenes. Nano Letters 4, 1881-1887.

Scott, W.B., Crossman, E.J., 1973. Freshwater fishes of Canada. Fisheries Research Board of Canada Bulletin 184.

Scown, T.M., Santos, E.M., Johnston, B.D., Gaiser, B., Baalousha, M., Mitov, S., Lead, J.R., Stone, V., Fernandes, T.F., Jepson, M., van Aerle, R., Tyler, C.R., 2010. Effects of Aqueous Exposure to Silver Nanoparticles of Different Sizes in Rainbow Trout. Toxicological Sciences 115, 521-534.

Shi, J.P., Evans, D.E., Khan, A.A., Harrison, R.M., 2001. Sources and concentration of nanoparticles (<10 nm diameter) in the urban atmosphere. Atmospheric Environment 35, 1193-1202.

Snape, J.R., Maund, S.J., Pickford, D.B., Hutchinson, T.H., 2004. Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. Aquatic Toxicology 67, 143-154.

Tang, M., Zhang, T., Xue, Y., Wang, S., Huang, M., Yang, Y., Lu, M., Lei, H., Kong, L., Yuepu, P., 2010. Dose dependent in vivo metabolic characteristics of titanium dioxide nanoparticles. Journal of nanoscience and nanotechnology 10, 8575-8583.

Usenko, C.Y., Harper, S.L., Tanguay, R.L., 2007. In vivo evaluation of carbon fullerene toxicity using embryonic zebrafish. Carbon 45, 1891-1898.

Usenko, C.Y., Harper, S.L., Tanguay, R.L., 2008. Fullerene C60 exposure elicits an oxidative stress response in embryonic zebrafish. Toxicology and applied pharmacology 229, 44-55.

USEPA, 2002. Short Term Methods for Estimating The Acute Toxicity of Effluents and Receiving Water to Freshwater organisms, Fifth Edition. October 2002. Unted States Environmnental Protection Agency. Office of Water , Washington, D.C., EPA 821-R-02-012

Viant, M.R., Bundy, J.G., Pincetich, C.A., de Ropp, J.S., Tjeerdema, R.S., 2005. NMR-derived developmental metabolic trajectories: an approach for visualizing the toxic actions of trichloroethylene during embryogenesis. Metabolomics 1, 149-158.

Viant, M.R., Pincetich, C.A., Hinton, D.E., Tjeerdema, R.S., 2006a. Toxic actions of dinoseb in medaka (Oryzias latipes) embryos as determined by in vivo 31P NMR, HPLC-UV and 1H NMR metabolomics. Aquat Toxicol 76, 329-342.

Viant, M.R., Pincetich, C.A., Tjeerdema, R.S., 2006b. Metabolic effects of dinoseb, diazinon and esfenvalerate in eyed eggs and alevins of Chinook salmon (Oncorhynchus tshawytscha) determined by 1H NMR metabolomics. Aquat Toxicol 77, 359-371.

Wood, C.M., Playle, R.C., Hogstrand, C., 1999. Physiology and modeling of mechanisms of silver uptake and toxicity in fish. Environmental Toxicology and Chemistry 18, 71-83.

Zhu, S., Oberdorster, E., Haasch, M.L., 2006. Toxicity of an engineered nanoparticle (fullerene, C60) in two aquatic species, Daphnia and fathead minnow. Marine environmental research 62 Suppl, S5-9.

CHAPTER 2

[1]EFFECTS OF SILVER NANOPARTICLES AND HYDROXYLATED FULLERENES ON EARLY LIFE STAGES OF THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*): METABOLOMIC APPROACH

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Abstract

Concerns about engineered nanoparticles as emerging contaminants have increased in recent years. Silver nanoparticles and carbon-based nanoparticles are two main categories of engineered nanoparticles and are massively produced and used in industrial and consumer products. Both silver nanoparticles and hydroxylated fullerenes could cause toxicity in exposed organisms, however, the mechanisms of their toxicity remain unclear. We examined the effects of exposure to silver nanoparticles or hydroxylated fullerenes on fathead minnow embryos (*Pimephales promelas*). In 120-h exposure, the LC50 of silver nanoparticles was 1.63 mgL^{-1} and the lowest effect concentration of fathead minnow embryos for morphological abnormalities and mobility was observed at 0.75 mgL^{-1}. Hydroxylated fullerenes caused no mortality to fathead minnow embryos until 4 mgL^{-1}. Early hatching in embryos exposed to 4 mgL^{-1} hydroxylated fullerenes was observed, but not at any of the lower exposure levels. Toxic mechanisms of silver nanoparticles and hydroxylated fullerenes were investigated by measuring the change of metabolite profiles in exposed embryos. Metabolomic responses to silver nanoparticles exposure were observed at 0.75 mgL^{-1}; changing metabolites included osmolytes, amino acids, and energy related metabolites. Proposed physiological responses from these metabolite changes include disturbances in osmoregulation and energy metabolism, induction of reductive glutamine metabolism and phospholipid biosynthesis; all are mechanisms that support the whole organismal responses of edema and reduced mobility in AgNP-exposed fish. However, we were not able to correlate changing metabolites following exposure of hydroxylated fullerenes to fathead minnow embryos with any known toxic effects.

Introduction

Engineered nanomaterials are defined as materials with one dimension under 100 nm (ASTM 2006) and have gained concern as emerging contaminants with unique physical and chemical properties. Nanotechnology is predicted to be a $1 trillion market by 2015 (Roco, 2001). Increases in application and production of nanomaterials make exposure of these contaminants in natural waters more probable (Mueller and Nowack, 2008). However, information about toxicity and mechanisms of action of nanomaterials is still limited.

Silver nanoparticles (AgNPs) are the most widely used nanomaterials in consumer products. Because of their antimicrobial properties, they are currently used in 384 kinds of consumer products, such as detergents, fabrics, air filters, and washing machines (Project on Emerging Nanotechnologies Inventory, Woodrow Wilson International Center for Scholars; Washington, DC). The worldwide production of silver nanoparticles is estimated around 500 tons per year. Silver NPs could be released to the environment during their production, useful life, and during disposal processes (Gottschalk and Nowack, 2011). Continuous release of silver from socks containing AgNPs during a simulated household washing process has been shown to last over 96 continuous hours of washing for some products (Benn and Westerhoff, 2008). Through their release from multiple products, AgNPs could provide a continuous source of silver ions to aquatic environments (Dobias and Bernier-Latmani, 2013). Currently, there is not a reliable analytical technique to distinguish concentrations of silver nanoparticles from other forms of silver, e.g., colloidal silver, in the environment. However, a recent study predicted environmental concentrations of silver nanoparticles in aquatic environments to be between 0.03 and 0.08 $\mu g L^{-1}$ (Mueller and Nowack, 2008).

Silver NPs have been reported to enter zebrafish *(Danio rerio*) eggs through chorion pore canals and accumulate inside embryos (Lee et al., 2007). Fathead minnow (*Pimephales promelas*) embryos exposed to AgNPs exhibited higher mortality and a greater chance of malformation, including edema, hemorrhage, and lordosis (Laban et al., 2010). Silver NPs could slowly release ionic Ag, which is highly toxic to fish (Gagné et al., 2012; Laban et al., 2010). Abnormal neurodevelopment in developing zebrafish and impairment of survival, growth, and development were observed in zebrafish embryos exposed to Ag ions (Powers et al., 2011a). When juvenile or adult fish gills are exposed to AgNPs, silver ions released from AgNPs can cause the inhibition of basolateral Na/K-ATPase activity and compromise osmoregulation (Wood et al., 1999). Exposure to silver nanoparticles has been associated with oxidative damage, impairment of the normal expression of oxyradical-scavenging enzymes, apoptosis, and impairment of metabolism and normal toxicological responses to metals in zebrafish and rainbow trout (*Oncorhynchus mykiss*) (Choi et al., 2010; Scown et al., 2010).

Fullerenes are an important category of carbon nanotubes, and the best-known and most stable fullerene is C_{60}. The global production of carbon nanotubes is expected to exceed 12,300 tons in 2015 with a market value of approximately $1.3 billion (Innovative Research and Products, Inc., 2011). Fullerenes are currently used in drug delivery systems, lubricants, solar cells, catalysis, polymer modification, and other consumer products (Aschberger et al., 2010).

Research has shown that cytotoxicity of fullerenes was related to its ability to cause oxidative stress (Usenko et al., 2008). Oxidative stress and lipid peroxidation were observed in the brains of adult fathead minnows exposed to fullerenes (Zhu et al., 2006). Zebrafish embryos exposed to fullerenes developed significant increases in edema, malformation, and mortality (Usenko et al., 2007). Hydroxylated fullerenes ($C_{60}(OH)_{24}$) have higher water solubility and

aqueous stability than fullerenes, giving them increased industrial applications (Da Ros and Prato, 1999; Jovanović et al., 2011a). Hydroxylated fullerenes display less cytotoxicity than unmodified fullerenes, serving mainly as antioxidants while inducing reactive oxygen species (ROS)-independent cell death *in vitro* (Isakovic et al., 2006; Sayes et al., 2004). Hydroxylated fullerenes were an order of magnitude less toxic to zebrafish embryos than fullerenes, and are proposed to have different toxic mechanisms (Usenko et al., 2007). Fathead minnow embryos exposed to hydroxylated fullerenes exhibited pericardial edema, yolk coagulation, suppressed neutrophil function, and altered immune gene expression (Jovanović et al., 2011a,b).

Metabolomics measures the dynamic metabolic responses of an organism to a toxic or physiological challenge (Nicholson et al., 1999). Instantaneous fluctuations of endogenous biochemicals caused by external stress or xenobiotics can be assessed by measuring subtle alterations in endogenous metabolites detected in biofluids or tissue samples (Nicholson et al., 1999). NMR metabolomics has been applied previously in fish embryo toxicity studies to investigate the toxicity mechanisms and developmental metabolic trajectories following exposure to trichloroethylene. In these studies, metabolomic responses were demonstrated to be more sensitive than phenotypic effects (Viant et al., 2005; Viant et al., 2006). Monitoring metabolic changes throughout embryogenesis can help determine the stage-specific modes of action, which can be correlated with known morphological events and thus explain potential adverse effects.

The objective of this study was to measure the toxicity of AgNPs and hydroxylated fullerenes in early life stages of the fathead minnow and further investigate their modes of action by analyzing their metabolomic responses.

Material and Methods

Particle Characterization

AgNPs were described as 99% purity, ≤100 nm in size (Sigma-Aldrich, St. Louis, MO). Hydroxylated fullerenes (99.8% purity) were purchased from MER Corporation, Tucson, AZ. Particle sizes of nanomaterials were confirmed by Transmission Electron Microscopy (TEM) (Tecnai F20, Hillsboro, Oregon). Solutions of AgNPs (500 mg/L) and hydroxylated fullerenes (400 mg/L) were prepared in moderately hard water (MHW) (USEPA, 2002) containing 60 mg $MgSO_4$, 96 mg $NaHCO_3$, 4 mg KCl, 60 mg $CaSO_4 \cdot H_2O$ L^{-1} and sonicated for 1.5 h (AgNPs) or 20 m (hydroxylated fullerenes). A drop of either homogenous solution was placed in a copper grid with lacy carbon film (Electron Microscopy Sciences, Ft. Washington) for TEM analysis. Dynamic light scattering (DLS) (Zetasizer Nano ZS Analyzer, Marvern, Worcestershire, UK) was conducted on AgNPs (50 mg/L) and hydroxylated fullerenes (5 mg/L) prepared in MHW to determine the particle size distribution for each nanomaterial.

Embryo collection

Adult fathead minnow breeders (age 6-8 mo) were purchased from Aquatic Biosystems (Fort Collins, CO). Breeding ratios (2 males: 5 females) were maintained in 40-L, aerated, flow-through tanks (filtered dechlorinated tap water) held at 25 ± 0.5 ^{o}C with a photoperiod of 16 h light: 8 h dark. Adult fish were fed frozen brine shrimp and tetrafin flakes twice daily. Each tank was provided with 3 pieces of 4 inch diameter PVC cut in half as breeding substrates. Embryos were collected from the PVC substrates daily for use in experiments.

Embryo Toxicity test of AgNPs and Hydroxylated fullerenes

In separate tests, the seven-day fathead minnow embryo toxicity assay was performed with either six concentrations (0.01, 0.1, 0.75, 1.5, 3, 6 mgL^{-1}) of AgNPs or six concentrations (0.0001, 0.001, 0.01, 0.1, 1 and 4 mgL^{-1}) of hydroxylated fullerenes dissolved in MHW. Stock solutions (500 mgL^{-1}AgNPs; 400 mg L^{-1}hydroxyalted fullerenes) were prepared in MilliQ water (AgNPs) or MHW (hydroxylated fullerenes) and were sonicated for 1.5 h (AgNPs) or 20 min (hydroxylated fullerenes). Test solutions were prepared from stock solutions, followed by a 5-min sonication. Fathead minnow embryos less than 16 h post fertilization (hpf) were added to 12-well microtiter plates containing 6 mL test solution per well. Five embryos were placed in each well. There were six replicate wells for each concentration (total of 30 embryos per concentration). Embryos were incubated at 25^{o}C with a 16 h light, 8 h dark cycle for the 7-d test duration. Every 24 h 80% of test solutions were renewed and embryo mortality, mobility, hatching, morphological deformities, including edema and spinal curvature, were recorded.

Statistical analysis

All data were initially analyzed for significance using One Way Analysis of Variance followed by Dunnett's procedure for post hoc comparison of means between control and experimental groups. When toxicity data failed the normality test (Shapiro-Wilk), Kruskal-Wallis One Way analysis followed by Dunnett's procedure was used as an alternative method for analyzing significances between control and experimental groups. A *p*-value equal to or less than 0.05 was considered statistically significant. Toxicity endpoints and data were analyzed using SAS 9.2 and LC50 was calculated by the Trimmed Spearman Karber (TSK) method (USEPA 2002).

Fish embryo exposures for measuring metabolomic responses

Embryos less than 16 h post fertilization were added to 250 mL beakers containing 100 mL nanomaterial test solution (50 embryos per beaker). Embryos were exposed to three different concentrations (0.001, 0.01, 0.75 mgL^{-1}) of AgNPs or three different concentrations (0.0001, 0.01, 4 mgL^{-1}) of hydroxylated fullerenes. All test solutions were prepared in MHW. There were eight replicates in each group. Because of limited daily embryo production, replicate beakers originated from different collection days, with initiation of individual replicates staggered over a period of four weeks. Exposure water in all beakers was aerated and dissolved oxygen (DO) concentrations (YSI Model Pro 2030, Yellow Springs, OH) were $\geq$ 7 mgL^{-1}. Test beakers were maintained in an incubator at 25^{o}C with a 16 h light, 8 h dark cycle. Test solutions (80%) were renewed every 24 h. After 72 h or 120 h, embryos and/or hatched larvae were harvested from replicate beakers, dead embryos were discarded and living organisms (embryos or newly hatched larvae) were snap-frozen in liquid nitrogen and stored at -80^{o}C until samples were prepared for NMR spectroscopy.

Quantification of total AgNPs

Solutions of three different concentrations (0.01, 0.1, 0.75 mgL^{-1}) of AgNPs dissolved in MHW were prepared and stabilized by adding five percent of trace metal grade nitric acid (Fisher Scientific, Waltham, MA). Silver concentrations were measured by inductively coupled plasma (ICP) spectroscopy (Perkin-Elmer Optima 4300 DV or 7300 DV), following EPA Method 6010.

Processing of metabolomics samples

The extraction of samples for metabolomic analysis was conducted according to Collette et al. (2010). Briefly each sample containing approximately 50 embryos was extracted in a 96-well plate with ice-cold methanol water solution (400 parts methanol: 85 parts Milli-Q water). All samples were tissuelysed (Qiagen, Hilden, Germany) for 10 min and transferred to Qiagen 96 well plates. Ice cold chloroform was added to each column of the Qiagen plate followed by vigorously shaking for 20 min. After spinning down the Qiagen plate for 2 min, ice cold chloroform and ice cold Milli-Q H_2O (1:1 ratio) were added to each column, mixed by shaking for 10 min and centrifuged at 2000 rpm at 3^{o}C for 20 min. The polar phase (top layer) was transferred from each column to corresponding 1-mL wells in a MicroLiter 96 square well plate. The polar residue was vacuum dried overnight (16 h) in a speedvac and stored in a -80^{o}C freezer until samples were analyzed by nuclear magnetic resonance (NMR) spectroscopy.

For NMR spectroscopy, sample extracts were reconstituted with 0.1 M sodium phosphate buffered deuterium oxide (pH 7.4) that contained 50 μM sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4 (TSP). Samples were filtered through 0.45 μm 96-well filter plates (Multiscreen HTS-HV filter plates, Millipore) and run on a 600 mHz Agilent Inova spectrometer. Spectra were collected using a 1D-NOESY pulse sequence (2-S acquisition time, 16,000 data points, 512 scans per sample).

NMR data processing and analysis

NMR data processing and analysis followed previously described procedures (Collette et al., 2010). Acquired spectra were processed using ACD/1D NMR Manager (Advanced Chemistry Development, Toronto, Canada). Samples were Fourier-transformed, baseline

corrected, and referenced to trimethylsilylpropionate (TSP). Then, the spectra were segmented into 0.005 ppm-wide bins and exported to Microsoft Excel. Water (4.70-5.00 ppm) and methanol peaks (3.35-3.37 ppm) were removed. In addition, singlets at 1.24-1.25 ppm and 7.67-7.69 ppm, due to unidentified contaminants in the controls were also removed. Remaining bins in each spectrum were then normalized to unit total integrated intensity and exported to SIMCA-13.0 (Umetrics Inc., Kinnelon, NJ). Principal component analysis (PCA) was applied to compare exposure classes (high, middle, low concentrations, and controls).

T-test filtered difference spectra were generated to help identify changing metabolites following nanoparticles exposure. Difference spectra were calculated by subtracting the average bins of the control class from those of the exposed class. T-tests were used to determine if each bin in the exposed class was significantly different from the corresponding bin in the control class ($p<0.05$). If the result of a t-test was not significant, the bin value would be replaced with zero. To control for false positives, isolated non-zero bins and adjacent bins with opposite arithmetic signs were replaced with zero, because the outcomes are not compatible with NMR spectra. T-test filtered difference spectra were then plotted based on the remaining bin value. Peaks above baseline correspond to metabolites that increased after exposure, and peaks below baseline correspond to metabolites that decreased. Peaks in the difference spectrum were identified to match a corresponding metabolite, which could be considered treatment dependent.

Result and Discussion

Particle characterization

Particle size distribution of AgNPs and hydroxylated fullerenes measured by dynamic light scattering yielded an average diameter of 67.87 nm for AgNPs and an average diameter of

35.87 nm for hydroxylated fullerenes (Fig. 1). The TEM images revealed that AgNPs can aggregate and form large particles (> 100 nm); however, some smaller-sized AgNPs still existed in the solution (Fig. 2A). The TEM images showed the presence of large, aggregated hydroxylated fullerene particles (> 200nm) in unfiltered test solution (Fig. 2B).

Embryos toxicity test

Exposure of fathead minnow embryos to AgNPs induced mortality and a variety of developmental abnormalities, including pericardial edema, yolk edema, and spinal curvature (Fig. 3). Exposure to AgNPs resulted in significant mortality compared to controls at concentrations higher than 3.0 mgL^{-1}(Kruskal-Wallis One Way analysis, $p<0.05$). The 96- and 120-hr LC50 were 3.57 (95% confidence interval, 2.54 to 5.03) mgL^{-1} and 1.63 mgL^{-1}(95% confidence interval; 1.41 to 1.88) mgL^{-1}, respectively. Laban et al. (2010) reported a 96-hr LC50 of 1.35 $mgL^{-1}$for fathead minnow embryos exposed to AgNPs, which is more toxic than our observations. Early hatching compared to control was observed in embryos exposed to 0.75, 1.5, 3 and 6 mgL^{-1} AgNPs after 96-h exposure (Fig. 4). The lowest observed effects concentration (LOEC) for early hatching was 0.75 mgL^{-1}. Concentration-dependent effects of AgNPs included decreased mobility, edema, and spinal curvature. The LOEC for edema and decreased mobility after 120-h exposure was 0.75 mgL^{-1} (Kruskal-Wallis One Way analysis, $p<0.05$) (Fig. 5).

Differences in toxicity of AgNPs to fish embryos observed among different studies could be caused by many factors. Properties of nanomaterials, including size, shape, aggregate state, solubility, and binding capacities to macromolecules and other organic materials, would affect bioavailability and toxicity of nanoparticles (Fabrega et al. 2011, Gagné et al. 2008; Gagné et al. 2012). In addition, the size of nanoparticles has been shown to be a factor in the uptake of

nanoparticles by cells (Chithrani and Chan, 2007; Chithrani et al., 2006). *In vitro* tests with mammalian cells after a 6-h exposure showed nanoparticles with an approximate diameter of 50 nm are taken up into the cell through endocytosis more readily than larger or smaller sizes (Chithrani et al., 2006; Johnston et al., 2010). AgNPs with an approximate diameter of 50 nm were also more toxic than AgNPs with smaller (20 nm) or larger (110 nm) diameter to zebrafish embryos (Bowman et al., 2012). The average diameter of AgNPs in our study was approximately 70 nm (Fig. 1). The reduced toxicity of AgNPs to fathead minnow embryos in the present study could be related to their smaller surface area which may decrease the protein adsorption to the particle and reduce the efficiency of protein-mediated endocytosis.

Increased incidence of developmental abnormalities and mortality were observed, which was supported by previous studies with zebrafish and fathead minnow embryos exposed to AgNPs (Asharani et al., 2008; Laban et al., 2010; Lee et al., 2007). The toxicity of AgNPs to fathead minnow embryos could result from silver ions released by AgNPs, although silver ions were not measured in the present study. In the zebrafish model, silver ions have been reported to have corresponding effects on neurodevelopment, embryonic survival, growth, and morphology, in addition to depressed larval swimming behavior (Powers et al., 2010). The concentration of free Ag ions could decrease in the presence of increased pH, hardness and various ligands found in solution (Hogstrand and Wood, 1998). The rate of Ag ions released from AgNPs decreased rapidly at pH values ≥ 8 (Liu and Hurt, 2010). In our test system, the pH was 7.9 and hardness was 82 mgL^{-1}; the rate of free silver ions released from AgNPs could be much slower than from $AgNO_3$. Previous research has also shown that larger AgNPs, such as the ones used in our study, released much fewer Ag ions by desorption in natural water than did smaller AgNPs (Dobias and Bernier-Latmani, 2013). Therefore, in our study, the toxicity of AgNPs could be reduced by a

decreased release of Ag ions. Previous studies reported that zebrafish embryos could accumulate AgNPs by passive diffusion through chorion pore canals (Lee et al., 2007). Therefore, the toxicity of AgNPs in our study could also result from accumulated AgNPs inside embryos, which may interact with biomolecules and further alter the homeostasis of metabolism or signaling cascades of the developing fish.

Embryos exposed to hydroxylated fullerenes did not exhibit significant developmental deformities or toxicity at concentrations ranging from 0.0001 to 4 mgL^{-1}. Although early hatching was observed at 96-h post exposure in embryos exposed to 4 mgL^{-1} hydroxylated fullerenes (Fig. 6), early-hatched embryos did not differ morphologically from normally hatched embryos. Previous research has shown that hydroxylated fullerenes could cause pericardial edema and yolk coagulation in fathead minnow embryos at concentrations higher than 0.01 mgL^{-1} (Jovanović et al., 2011a). Zebrafish embryos exposed to hydroxylated fullerenes exhibited significant mortality and pericardial edema at 5 mgL^{-1} (Usenko et al., 2007). Hydroxylated fullerenes caused less toxicity in our system compared to previous research, which may be related to the hardness and pH of water in our exposure system. Use of moderately hard water (pH 7.9 to 8.1; hardness, 80 to 100 mgL^{-1}) rather than reconstituted instant ocean in MilliQ water (hardness is estimated less than 20 mgL^{-1}) could facilitate particle aggregation, which may affect uptake of nanoparticles by embryos (Handy et al., 2008). This hypothesis is consistent with our TEM results that identified a high degree of aggregation (aggregates > 200 nm) among hydroxylated fullerenes in our exposures.

Total silver in metabolism exposures

Embryos were exposed to three different concentrations of AgNPs for measuring metabolite profiles. Nominal concentrations (low, middle, and high concentrations) were 0.001, 0.01, 0.75 mgL^{-1}, and the actual concentrations were confirmed using ICP-MS. Actual concentrations were 122.81± 9.9% of nominal concentrations at the middle concentration, 85.61± 2.32% of nominal concentration at the high concentration. The lowest concentration (0.001 mgL^{-1}) was below the instrument detection limit (0.005 mgL^{-1}).

Metabolite profiles in response to AgNPs exposure

By measuring the metabolite profile of fathead minnow embryos exposed to AgNPs, we were able to assess the putative modes of action of AgNPs. We hypothesized first, that metabolomic responses could be sensitive biomarkers of exposure; and second, that the changes in metabolite profile could provide insights into modes of action for toxicity of AgNPs exposure.

Scores plots from a PCA model were built for all four exposure classes of AgNPs: control, low concentration (0.001 mgL^{-1}), middle concentration (0.01 mgL^{-1}), and high concentration (0.75 mgL^{-1}). These three concentrations of AgNPs correspond to two no observed effect concentrations (low and middle concentrations) and one LOEC (high concentrations) in our embryo toxicity tests. Responses from the high concentration class showed a better separation along PC1 than did responses from the medium or low concentrations (Fig. 7), and the separation was more evident in the 120-h exposure group than the 72-h exposure group. However, the variation within each class was larger than we expected. In our experimental design, replicates of each class of exposed embryos were collected and combined at different times, from two independent groups with identical exposure systems and conditions. For most

classes, we found a systematic difference in metabolite profiles between these two groups, which tended to increase within-class variation.

Next, we constructed several *t*-test filtered difference spectra ($p<0.05$), wherein the average spectrum for the control class was subtracted from that of an average exposed class. These difference spectra helped determine the metabolites that were changing in response to exposure to AgNPs. We could further compare the treatment effect by comparing multiple difference spectra. If AgNPs caused a large impact on metabolite profile, numerous and intense peaks could be observed in the corresponding difference spectrum. Also, the pattern of peak changes could be regarded as specific fingerprints in response to specific modes of action. When we examined the difference spectra of both 72-h treated and 120-h treated embryos (Fig. 9A, B), there were more changing metabolites at the highest exposure concentration (0.75 mgL^{-1}), which was also the LOEC in the embryo toxicity testing, than at the middle (0.01 mgL^{-1}) or low concentrations (0.0001 mgL^{-1}). This result indicated that the metabolomics responses to AgNPs might not be more sensitive biomarkers of exposure than the embryo toxicity endpoints in our exposures. However, the increased within-class variation due to the different replicate exposure batches could have reduced the ability to observe significantly changing metabolites at lower concentrations.

Metabolites that were identified from the difference spectra of AgNP-treated embryos, either treated for 72 h or 120 h, are listed in Table 1a. For embryos exposed to AgNPs for 72 h, some changing metabolites related to energy metabolism, including lactate, fructose, and citrate, were observed. An increase in lactate is accompanied by a decrease in citrate in embryos exposed to the highest concentration of AgNPs (0.75 mgL^{-1}). This might have indicated an increase in anaerobic respiration, wherein pyruvate is converted into lactate instead of entering

the tricarboxylic acid (TCA) cycle and forming citrate. Increased lactate concomitant with decreased citrate was previously reported when cells were under hypoxia or exposed to respiratory chain inhibitors (Fendt et al., 2013). Recently published research has shown that inhibition of oxidative phosphorylation and reduced consumption of oxygen were observed in zebrafish embryos treated with AgNPs (van Aerle et al., 2013) and other mammalian cell models (Costa et al., 2010; Teodoro et al., 2011). The decrease in glutamate observed in the 72-h spectrum could indicate the conversion of glutamate to *a*-ketoglutarate, an important alternative carbon source for fatty acid synthesis that sustains rapid cell proliferation (Fendt et al., 2013). Similarly, previous research with AgNPs-treated juvenile rainbow trout has also shown an up-regulation in glutamate dehydrogenase, which was likely responsible for glutamate reduction through reversible conversion of glutamate into *a*-ketoglutarate and ammonia (Gagné et al., 2012).

Embryos exposed to AgNPs for 120 h exhibited more changing metabolites than those exposed for 72 h (Fig. 9A, B; Table 1a). An increase in glucose was observed, which could be a general stress response in fish mainly mediated by catecholamine-induced liver glycogenolysis (Wendelaar Bonga, 1997). Previous research also showed a significant increase in glucose concentration in the serum of the Nile tilapia (*Oreochromis niloticus*) following a 10-d exposure to Ag (Öner et al., 2008). The observed increase in glucose could be correlated with the observed increase in three amino acids (phenylalanine, tyrosine, and tryptophan) (Table 1a), which are related to the biosynthesis of catecholamine. Catecholamine biosynthesis is likely controlled by tyrosine level because tyrosine hydroxylase acts as a rate-limiting enzyme in the catecholamine synthesis pathway (Kanehisa et al., 2012)

Thus, elevated tyrosine could cause increases in both catecholamine and catecholamine-stimulated glucose biosynthesis (Gamperl et al., 1994; Reid et al., 1998). Increased glucose might also result from decreased glycolysis. Previous research has shown that AgNPs cause a decreased activity of pyruvate kinase, which catalyzes the rate limiting step of glycolysis that produces ATP (van Aerle et al., 2013).

Additionally, AgNPs could induce reductive glutamine metabolism in exposed embryos. Decreases in citrate and glutamine were identified after 120 h exposure to AgNPs and could have resulted from a reductive glutamine metabolism (Table 1a). Reductive glutamine metabolism involves the conversion of α-ketoglutarate to citrate and acts as a carbon source for fatty acid synthesis to support cell growth and viability (Fendt et al., 2013; Wise et al., 2011). Respiratory chain inhibitors were reported to induce reductive glutamine metabolism in mammalian cells as well as decrease the efficiency of the TCA cycle (Fendt et al., 2013). We suggest that AgNPs, while acting as a respiratory inhibitor, could induce reductive glutamine metabolism to support cell growth, viability, and proliferation in embryonic development.

We observed an increase in choline or phosphocholine in difference spectra of AgNPs (Table 1a). Increased phosphocholine following 72-h exposure to AgNPs coupled with increases in both choline and phosphocholine following 120-h exposure at 0.75 mgL^{-1} could indicate an induction of phospholipid biosynthesis. Phosphocholine is a precursor of phosphatidylcholine, which is synthesized along the cytidine diphosphate (CDP)-choline pathway. This pathway is often up-regulated in tissues experiencing cell proliferations leading to cellular increases in choline and phosphocholine levels (Southam et al., 2008). CDP-choline could attenuate lipid peroxidation and protect neurons from ROS (Fresta et al., 1994; Rao et al., 2000). Exposure of AgNPs has been reported to result in lipid peroxidation in juvenile rainbow trout (Gagné et al.,

2012); the observed increase in choline and phosphocholine could serve as a protective mechanism to counter AgNP-induced formation of ROS.

Osmolyte disturbance, including metabolic changes in trimethylamine-N-oxide (TMAO), betaine, and taurine, were observed in changing metabolites following exposure to AgNPs for 120 h (Table 1a), whereas metabolic changes in TMAO was observed following 72-h exposure. Changes in osmolytes could indicate a disturbance in osmoregulation. The primary function of these osmolytes is to adjust the salinity and maintain the osmotic balance of tissues in aquatic organisms (Wu and Wang, 2010), and they may also have cytoprotective properties (Arakawa and Timasheff, 1985). Taurine and betaine are cytoprotective antioxidants (Hagar, 2004; Jaleel et al., 2007) and TMAO can stabilize serum proteins from the effects of accumulated waste products, especially elevated concentration of urea (Arakawa and Timasheff, 1985; Cho et al., 2011). Disturbed osmoregulation could also result from the impairment of the Na/K transporter by exposure to ionic Ag released by AgNPs, which can perturb two key enzymes of ion transport in gills, Na/K-ATPase and carbonic anhydrase (Webb et al., 2001; Wood et al., 1999), limit the excretion of ammonia, and cause disturbance in ionoregulation and osmolytes (Corotto and Holliday, 1996; Morgan et al., 2005).

We proposed that metabolomic responses can be correlated with sublethal effects that we observed in the embryo toxicity assay. Edema, usually caused by an increase in fluid uptake and/or retention by tissues, was an important sublethal endpoint for AgNP exposure in present study. Edema was observed in fish embryos exposed to 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Hill et al., 2004) and polycyclic aromatic hydrocarbons (PAH) (Li et al., 2011). Mechanisms of inducing edema include disruption of the water barrier (i.e., embryonic epidermis), alteration in the osmotic gradient between interior body fluids and external water

environment (Hill et al., 2004), and inhibition of Na^+/K^+ ATPase (Li et al., 2011). In fish, silver ions appear to bind to and inhibit the gill Na^+/K^+ ATPase transporter, which reduces active Na^+ and Cl^- transport across the gills, a critical mechanism regulating osmolality in fish (Wood et al., 1999). Altered osmotic gradient correlates with our observation of altered osmolytes. Thus, in the present study, we propose that AgNPs-induced edema may result from inhibited gill Na^+/K^+ ATPase and increased osmotic gradient.

Reduced mobility was observed in AgNP exposed embryos and may also be explained by the metabolomics results. Reduced mobility can be observed when fish are exposed to a hypoxic environment and reduce their energy expenditure (Crocker and Cech, 1997). We observed that the AgNP exposure induced reductive glutamine metabolism while inhibiting oxidative phosphorylation in exposed embryos, which reduces the efficiency of aerobic respiration and cellular oxygen consumption (Fendt et al., 2013; van Aerle et al., 2013). We suggest that embryos exposed to AgNPs might experience physiological effects similar to those in organisms subjected to long-term hypoxia. Thus, reduced mobility in exposed embryos might result from inhibition of oxidative phosphorylation in order to reduce energy expenditure.

In general, metabolic changes following exposure of AgNPs to fathead minnow embryos showed a possible mechanism of action of AgNPs, including a perturbed energy metabolism (i.e., switch to reductive glutamine metabolism and induced catecholamine biosynthesis), increased phospholipid biosynthesis, and several disturbed osmolytes (Fig. 10). In addition, the number of changing metabolites increased with extended exposure time (120 h). Although mechanisms of proposed 72 h and 120 h exposures to AgNPs follow the same trend, there were no changing metabolites following either 72-h or 120-h exposure to AgNPs at 0.001 or 0.01 mgL^{-1}. If we consider all changing metabolites as a biomarker of exposure, the metabolomic

LOEC will be 0.75 mgL^{-1}, which is the same as the LOEC for sublethal effects from embryo toxicity testing.

Metabolites profiles in response to hydroxylated fullerenes exposure

The separation among each hydroxylated fullerene-treated classes and control class along PC1 was not significant (Fig. 8). Few meaningful interpretations could be made from metabolomics analysis of embryos exposed to hydroxylated fullerenes (Fig. 9 c, d; Table 1b). Decreases in glycogen and glucose following 72-h exposure of 4 mgL^{-1} hydroxylated fullerenes to fathead minnow embryos could indicate an induction in glycolysis. Previous research has shown that hydroxylated fullerenes could inhibit the genes responsible for transporting lactate out of the cell (Jovanovic et al., 2011). Perhaps the increase in lactate was only observed at the lowest concentration (0.0001 mgL^{-1}) because lactate couldn't be transported outside the cells at the increased exposure concentration (0.01 and 4 mgL^{-1}) of hydroxylated fullerenes. Increases in the osmolyte TMAO was observed at 0.01 and 4 mgL^{-1} hydroxylated fullerenes. However, with extended exposure time from 72 to 120 h, none of these changing metabolites persisted, except for lactate. The lack of metabolic responses to hydroxylated fullerene exposures correlated with the absence of observable morphological abnormalities or mortality in the embryo toxicity assay. Without further metabolomic information, the mechanism of action of the only toxicity endpoint, early hatching, remains unclear.

Conclusion

In the present study, the toxicity of AgNPs and hydroxylated fullerenes was assessed by a fathead minnow embryo toxicity assay. We found that AgNPs exposures were consistent with

mortality and incidence of several morphological deformities in exposed embryos. Proposed mechanisms of action were identified from metabolomic responses to AgNP exposure. We detected physiological stress to fathead minnow embryos that correlated with increased glucose levels, possibly regulated by increased catecholamine biosynthesis. We also proposed that AgNPs can act as a respiratory chain inhibitor and induce reduced glutamine metabolism to sustain the need for cell growth and proliferation. In addition, data suggest that AgNPs cause induced phospholipids biosynthesis and altered osmoregulation. All these above responses were more significant with a longer exposure time. In addition, these metabolomic responses following exposure to AgNPs were correlated with the sublethal effects we observed in embryo toxicity testing. For hydroxylated fullerenes, we found no mortality or morphological abnormalities following exposure to hydroxylated fullerenes, which was consistent with the lack of meaningful metabolomic responses.

This is the first study applying metabolomic profiling to assess fish embryo toxicity following exposure to nanoparticles. The metabolomic approach described here adds valuable mechanistic information to known genetic responses to help determine modes of action for nanomaterial exposure.

Acknowledgement

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Reference

American Society for Testing and Materials., 2006. Standard terminology relating to nanotechnology. E 2456-06. West Con-shohocken, PA

Arakawa, T., Timasheff, S.N., 1985. The stabilization of proteins by osmolytes. Biophysical Journal 47, 411-414.

Aschberger, K., Johnston, H.J., Stone, V., Aitken, R.J., Tran, C.L., Hankin, S.M., Peters, S.A.K., Christensen, F.M., 2010. Review of fullerene toxicity and exposure – Appraisal of a human health risk assessment, based on open literature. Regulatory Toxicology and Pharmacology 58, 455-473.

Asharani, P.V., Wu, Y.L., Gong, Z., Valiyaveettil, S., 2008. Toxicity of silver nanoparticles in zebrafish models. Nanotechnology 19, 255102.

Benn, T.M., Westerhoff, P., 2008. Nanoparticle Silver Released into Water from Commercially Available Sock Fabrics. Environmental science & technology 42, 4133-4139.

Bowman, C.R., Bailey, F.C., Elrod-Erickson, M., Neigh, A.M., Otter, R.R., 2012. Effects of silver nanoparticles on zebrafish (Danio rerio) and Escherichia coli (ATCC 25922): A comparison of toxicity based on total surface area versus mass concentration of particles in a model eukaryotic and prokaryotic system. Environmental Toxicology and Chemistry 31, 1793-1800.

Chithrani, B.D., Chan, W.C.W., 2007. Elucidating the Mechanism of Cellular Uptake and Removal of Protein-Coated Gold Nanoparticles of Different Sizes and Shapes. Nano Letters 7, 1542-1550.

Chithrani, B.D., Ghazani, A.A., Chan, W.C.W., 2006. Determining the Size and Shape Dependence of Gold Nanoparticle Uptake into Mammalian Cells. Nano Letters 6, 662-668.

Cho, S.S., Reddy, G., Straub, J.E., Thirumalai, D., 2011. Entropic Stabilization of Proteins by TMAO. The Journal of Physical Chemistry B 115, 13401-13407.

Choi, J.E., Kim, S., Ahn, J.H., Youn, P., Kang, J.S., Park, K., Yi, J., Ryu, D.-Y., 2010. Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. Aquatic Toxicology 100, 151-159.

Collette, T.W., Teng, Q., Jensen, K.M., Kahl, M.D., Makynen, E.A., Durhan, E.J., Villeneuve, D.L., Martinovic-Weigelt, D., Ankley, G.T., Ekman, D.R., 2010. Impacts of an anti-androgen and an androgen/anti-androgen mixture on the metabolite profile of male fathead minnow urine. Environmental science & technology 44, 6881-6886.

Corotto, F.S., Holliday, C.W., 1996. Branchial Na, K-ATPase and osmoregulation in the purple shore crab, Hemigrapsus nudus (Dana). Comparative Biochemistry and Physiology Part A: Physiology 113, 361-368.

Costa, C.S., Ronconi, J.V., Daufenbach, J.F., Goncalves, C.L., Rezin, G.T., Streck, E.L., Paula, M.M., 2010. In vitro effects of silver nanoparticles on the mitochondrial respiratory chain. Molecular and cellular biochemistry 342, 51-56.

Crocker, C., Cech, J.J., Jr., 1997. Effects of environmental hypoxia on oxygen consumption rate and swimming activity in juvenile white sturgeon, Acipenser transmontanus, in relation to temperature and life intervals. Environmental Biology of Fishes 50, 383-389.

Da Ros, T., Prato, M., 1999. Medicinal chemistry with fullerenes and fullerene derivatives. Chemical Communications, 663-669.

Dobias, J., Bernier-Latmani, R., 2013. Silver Release from Silver Nanoparticles in Natural Waters. Environmental science & technology 47, 4140-4146.

Fabrega, J., Luoma, S.N., Tyler, C.R., Galloway, T.S., Lead, J.R., 2011. Silver nanoparticles: Behaviour and effects in the aquatic environment. Environment International 37, 517-531.

Fendt, S.M., Bell, E.L., Keibler, M.A., Olenchock, B.A., Mayers, J.R., Wasylenko, T.M., Vokes, N.I., Guarente, L., Vander Heiden, M.G., Stephanopoulos, G., 2013. Reductive glutamine

metabolism is a function of the alpha-ketoglutarate to citrate ratio in cells. Nature communications 4, 2236.

Fresta, M., Puglisi, G., Di Giacomo, C., Russo, A., 1994. Liposomes as In-vivo Carriers for Citicoline: Effects on Rat Cerebral Post-ischaemic Reperfusion#. Journal of Pharmacy and Pharmacology 46, 974-981.

Gagné, F., Gagnon, C., Blaise, C., 2008. Aquatic nanotoxicology: a review. Res.Trends, Curr. Top. Toxicol. 4, 1–14.

Gagné, F., André, C., Skirrow, R., Gélinas, M., Auclair, J., van Aggelen, G., Turcotte, P., Gagnon, C., 2012. Toxicity of silver nanoparticles to rainbow trout: A toxicogenomic approach. Chemosphere 89, 615-622.

Gamperl, A.K., Vijayan, M.M., Boutilier, R.G., 1994. Experimental control of stress hormone levels in fishes: techniques and applications. Rev Fish Biol Fisheries 4, 215-255.

Gottschalk, F., Nowack, B., 2011. The release of engineered nanomaterials to the environment. Journal of Environmental Monitoring 13, 1145-1155.

Hagar, H.H., 2004. The protective effect of taurine against cyclosporine A-induced oxidative stress and hepatotoxicity in rats. Toxicology letters 151, 335-343.

Handy, R.D., Henry, T.B., Scown, T.M., Johnston, B.D., Tyler, C.R., 2008. Manufactured nanoparticles: their uptake and effects on fish--a mechanistic analysis. Ecotoxicology 17, 396-409.

Hill, A.J., Bello, S.M., Prasch, A.L., Peterson, R.E., Heideman, W., 2004. Water Permeability and TCDD-Induced Edema in Zebrafish Early-Life Stages. Toxicological Sciences 78, 78-87.

Hogstrand, C., Wood, C.M., 1998. Toward a better understanding of the bioavailability, physiology, and toxicity of silver in fish: Implications for water quality criteria. Environmental Toxicology and Chemistry 17, 547-561.

Isakovic, A., Markovic, Z., Todorovic-Markovic, B., Nikolic, N., Vranjes-Djuric, S., Mirkovic, M., Dramicanin, M., Harhaji, L., Raicevic, N., Nikolic, Z., Trajkovic, V., 2006. Distinct Cytotoxic Mechanisms of Pristine versus Hydroxylated Fullerene. Toxicological Sciences 91, 173-183.

Jaleel, C.A., Manivannan, P., Kishorekumar, A., Sankar, B., Gopi, R., Somasundaram, R., Panneerselvam, R., 2007. Alterations in osmoregulation, antioxidant enzymes and indole alkaloid levels in Catharanthus roseus exposed to water deficit. Colloids and Surfaces B: Biointerfaces 59, 150-157.

Johnston , H., Hutchison, G., Christensen, F., Peters, S., Hankin, S., Stone, V., 2010. A review of the in vivo and in vitro toxicity of silver and gold particulates: Particle attributes and biological mechanisms responsible for the observed toxicity. Critical Reviews in Toxicology 40, 328-346.

Jovanović, B., Anastasova, L., Rowe, E.W., Palić, D., 2011a. Hydroxylated fullerenes inhibit neutrophil function in fathead minnow (Pimephales promelas Rafinesque, 1820). Aquatic Toxicology 101, 474-482.

Jovanovic, B., Ji, T., Palic, D., 2011b. Gene expression of zebrafish embryos exposed to titanium dioxide nanoparticles and hydroxylated fullerenes. Ecotoxicology and environmental safety 74, 1518-1525.

Kanehisa, M., Goto, S., Sato, Y., Furumichi, M., Tanabe, M., 2012. KEGG for integration and interpretation of large-scale molecular data sets. Nucleic Acids Research 40, 109-114.

Laban, G., Nies, L.F., Turco, R.F., Bickham, J.W., Sepulveda, M.S., 2010. The effects of silver nanoparticles on fathead minnow (Pimephales promelas) embryos. Ecotoxicology 19, 185-195.

Lee, K.J., Nallathamby, P.D., Browning, L.M., Osgood, C.J., Xu, X.H., 2007. In vivo imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. ACS nano 1, 133-143.

Li, R., Zuo, Z., Chen, D., He, C., Chen, R., Chen, Y., Wang, C., 2011. Inhibition by polycyclic aromatic hydrocarbons of ATPase activities in Sebastiscus marmoratus larvae：Relationship with the development of early life stages. Marine environmental research 71, 86-90.

Liu, J., Hurt, R.H., 2010. Ion Release Kinetics and Particle Persistence in Aqueous Nano-Silver Colloids. Environmental science & technology 44, 2169-2175.

Morgan, T.P., Guadagnolo, C.M., Grosell, M., Wood, C.M., 2005. Effects of water hardness on the physiological responses to chronic waterborne silver exposure in early life stages of rainbow trout (Oncorhynchus mykiss). Aquat Toxicol 74, 333-350.

Mueller, N.C., Nowack, B., 2008. Exposure Modeling of Engineered Nanoparticles in the Environment. Environmental science & technology 42, 4447-4453.

Nicholson, J.K., Lindon, J.C., Holmes, E., 1999. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica; The Fate Of Foreign Compounds In Biological Systems 29, 1181-1189.

Öner, M., Atli, G., Canli, M., 2008. Changes in serum biochemical parameters of freshwater fish Oreochromis niloticus following prolonged metal (Ag, Cd, Cr, Cu, Zn) exposures. Environmental Toxicology and Chemistry 27, 360-366.

Powers, C.M., Slotkin, T.A., Seidler, F.J., Badireddy, A.R., Padilla, S., 2011a. Silver nanoparticles alter zebrafish development and larval behavior: distinct roles for particle size, coating and composition. Neurotoxicology and teratology 33, 708-714.

Powers, C.M., Yen, J., Linney, E.A., Seidler, F.J., Slotkin, T.A., 2010. Silver exposure in developing zebrafish (Danio rerio): persistent effects on larval behavior and survival. Neurotoxicology and teratology 32, 391-397.

Rao, A.M., Hatcher, J.F., Dempsey, R.J., 2000. Lipid Alterations in Transient Forebrain Ischemia. Journal of neurochemistry 75, 2528-2535.

Reid, S.G., Bernier, N.J., Perry, S.F., 1998. The adrenergic stress response in fish: control of catecholamine storage and release. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 120, 1-27.

Roco, M.C., 2001. International strategy for Nanotechnology Research. Journal of Nanoparticle Research 3, 353-360.

Sayes, C.M., Fortner, J.D., Guo, W., Lyon, D., Boyd, A.M., Ausman, K.D., Tao, Y.J., Sitharaman, B., Wilson, L.J., Hughes, J.B., West, J.L., Colvin, V.L., 2004. The Differential Cytotoxicity of Water-Soluble Fullerenes. Nano Letters 4, 1881-1887.

Scown, T.M., Santos, E.M., Johnston, B.D., Gaiser, B., Baalousha, M., Mitov, S., Lead, J.R., Stone, V., Fernandes, T.F., Jepson, M., van Aerle, R., Tyler, C.R., 2010. Effects of Aqueous Exposure to Silver Nanoparticles of Different Sizes in Rainbow Trout. Toxicological Sciences 115, 521-534.

Southam, A.D., Easton, J.M., Stentiford, G.D., Ludwig, C., Arvanitis, T.N., Viant, M.R., 2008. Metabolic Changes in Flatfish Hepatic Tumours Revealed by NMR-Based Metabolomics and Metabolic Correlation Networks. Journal of proteome research 7, 5277-5285.

Teodoro, J.S., Simoes, A.M., Duarte, F.V., Rolo, A.P., Murdoch, R.C., Hussain, S.M., Palmeira, C.M., 2011. Assessment of the toxicity of silver nanoparticles in vitro: a mitochondrial perspective. Toxicology in vitro : an international journal published in association with BIBRA 25, 664-670.

Usenko, C.Y., Harper, S.L., Tanguay, R.L., 2007. In vivo evaluation of carbon fullerene toxicity using embryonic zebrafish. Carbon 45, 1891-1898.

Usenko, C.Y., Harper, S.L., Tanguay, R.L., 2008. Fullerene C60 exposure elicits an oxidative stress response in embryonic zebrafish. Toxicology and applied pharmacology 229, 44-55.

USEPA, 2002. Short Term Methods for Estimating The Acute Toxicity of Effluents and Receiving Water to Freshwater organisms, Fifth Edition. October 2002. Unted States Environmnental Protection Agency. Office of Water , Washington, D.c., EPA 821-R-02-012

van Aerle, R., Lange, A., Moorhouse, A., Paszkiewicz, K., Ball, K., Johnston, B.D., de-Bastos, E., Booth, T., Tyler, C.R., Santos, E.M., 2013. Molecular Mechanisms of Toxicity of Silver Nanoparticles in Zebrafish Embryos. Environmental science & technology 47, 8005-8014.

Viant, M.R., Bundy, J.G., Pincetich, C.A., de Ropp, J.S., Tjeerdema, R.S., 2005. NMR-derived developmental metabolic trajectories: an approach for visualizing the toxic actions of trichloroethylene during embryogenesis. Metabolomics 1, 149-158.

Viant, M.R., Pincetich, C.A., Hinton, D.E., Tjeerdema, R.S., 2006. Toxic actions of dinoseb in medaka (Oryzias latipes) embryos as determined by in vivo 31P NMR, HPLC-UV and 1H NMR metabolomics. Aquat Toxicol 76, 329-342.

Webb, N.A., Shaw, J.R., Morgan, J., Hogstrand, C., Wood, C.M., 2001. Acute and chronic physiological effects of silver exposure in three marine teleosts. Aquat Toxicol 54, 161-178.

Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiological Reviews 77, 591-625.

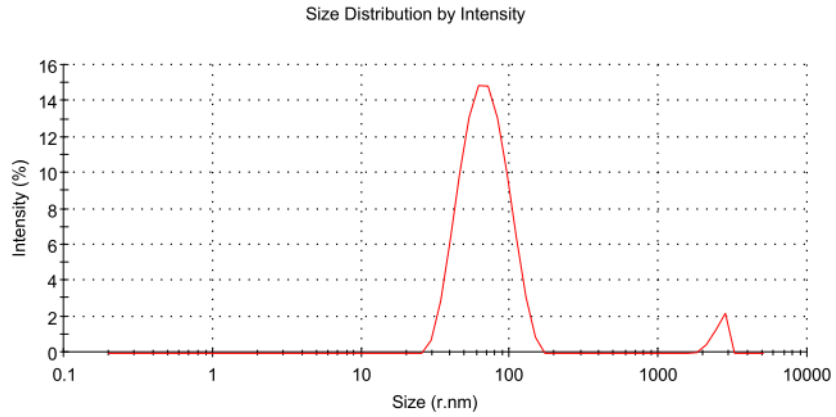
Wise, D.R., Ward, P.S., Shay, J.E., Cross, J.R., Gruber, J.J., Sachdeva, U.M., Platt, J.M., DeMatteo, R.G., Simon, M.C., Thompson, C.B., 2011. Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of alpha-ketoglutarate to citrate to support cell growth and viability. Proceedings of the National Academy of Sciences of the United States of America 108, 19611-19616.

Wood, C.M., Playle, R.C., Hogstrand, C., 1999. Physiology and modeling of mechanisms of silver uptake and toxicity in fish. Environmental Toxicology and Chemistry 18, 71-83.

Wu, H., Wang, W.-X., 2010. NMR-based metabolomic studies on the toxicological effects of cadmium and copper on green mussels Perna viridis. Aquatic Toxicology 100, 339-345.

Zhu, S., Oberdorster, E., Haasch, M.L., 2006. Toxicity of an engineered nanoparticle (fullerene, C60) in two aquatic species, Daphnia and fathead minnow. Marine environmental research 62 Suppl, S5-9.

A



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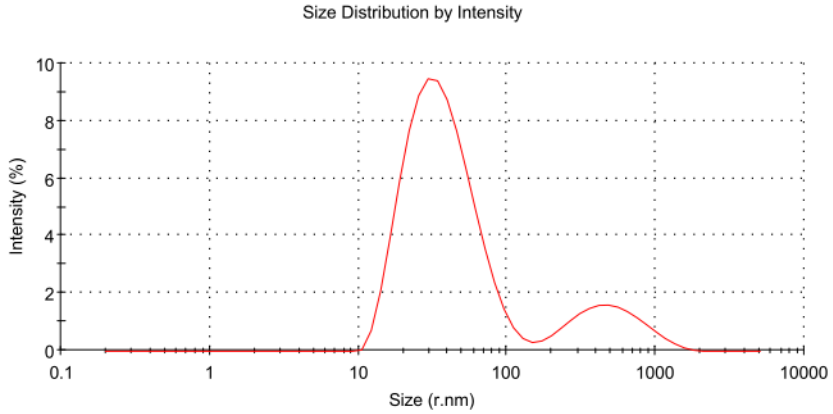


Fig. 1. Particle size distribution of (**A**) AgNPs and (**B**) hydroxylated fullerenes. The average diameter for AgNPs and hydroxylated fullerenes were 67.87 and 35.87 nm, respectively.

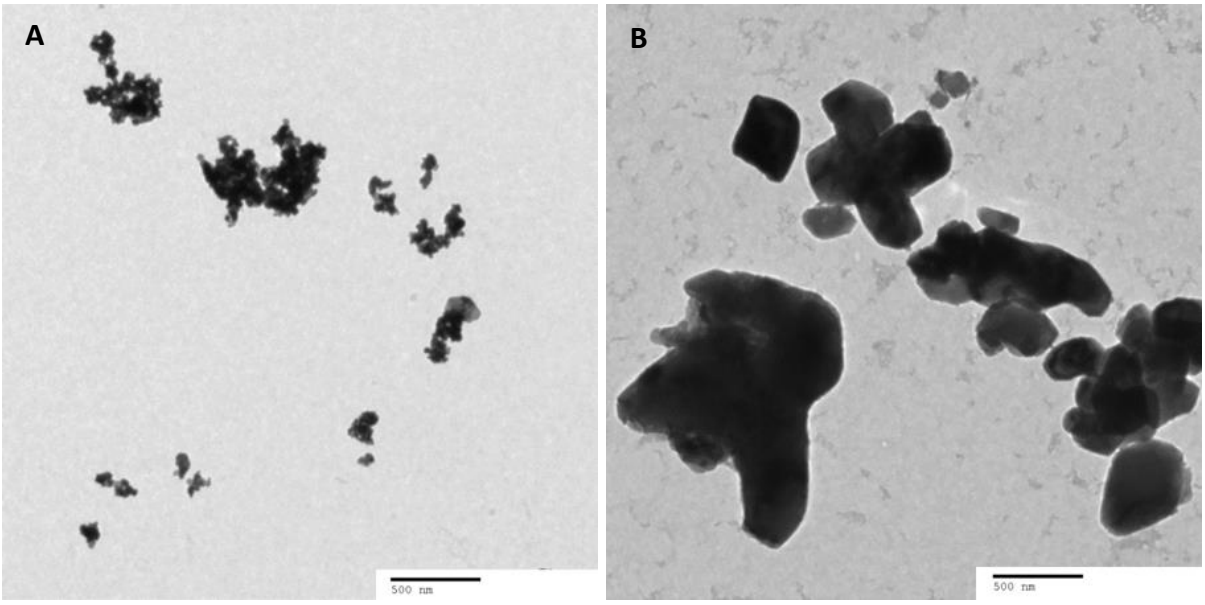


Fig. 2. Transmission electron microscopy of the nanoparticles used in this study. (**A**) AgNPs, (**B**) hydroxylated fullerenes

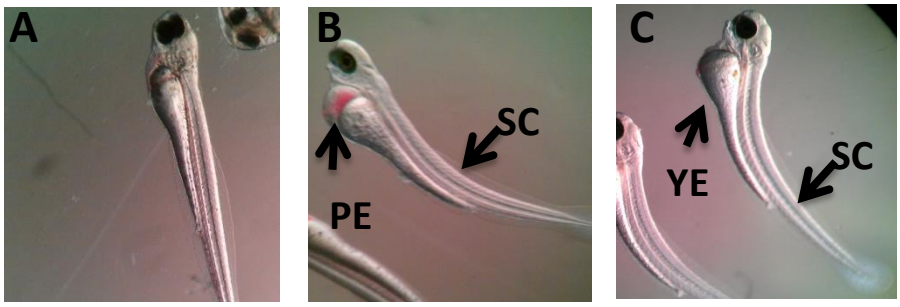


Fig. 3. Representative larval fathead minnow (*Pimephales promelas*) after 96 h exposure: (**A**) control larvae, (**B** and **C**) embryos exposed to 1.5 mg/L AgNPs. AgNP-exposed embryos exhibited sublethal effects, including pericardial edema (PE), yolk edema (YE) and spinal curvature (SC).

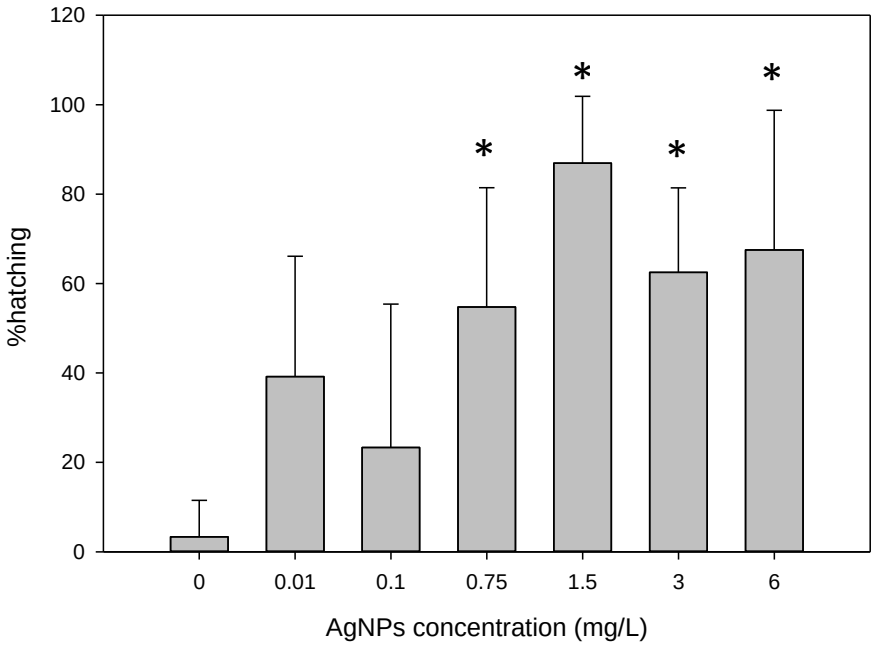


Fig. 4. Effects of silver nanoparticles on hatching of fathead minnow embryos following 96-h exposure (mean ± SD). The LOEC for early hatching was 0.75 mg/L. Asterisks denote treatment that differs significantly from the MHW control group (One-Way ANOVA Dunnett's test $p<0.05$).

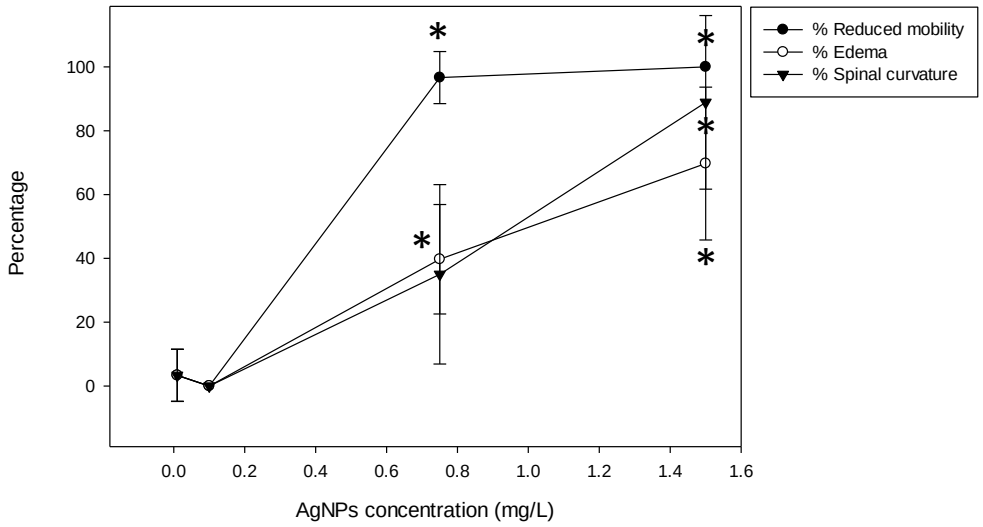


Fig. 5. Effects of silver nanoparticles on fathead minnow embryos included reduced mobility, edema, and spinal curvature following 120-h exposure (mean ± SD). Asterisks denote treatment that differs significantly from the MHW control group. The LOEC for reduced mobility and edema were 0.75 mg/L (Kruskal-Wallis One Way Dunnett's test, $p<0.05$).

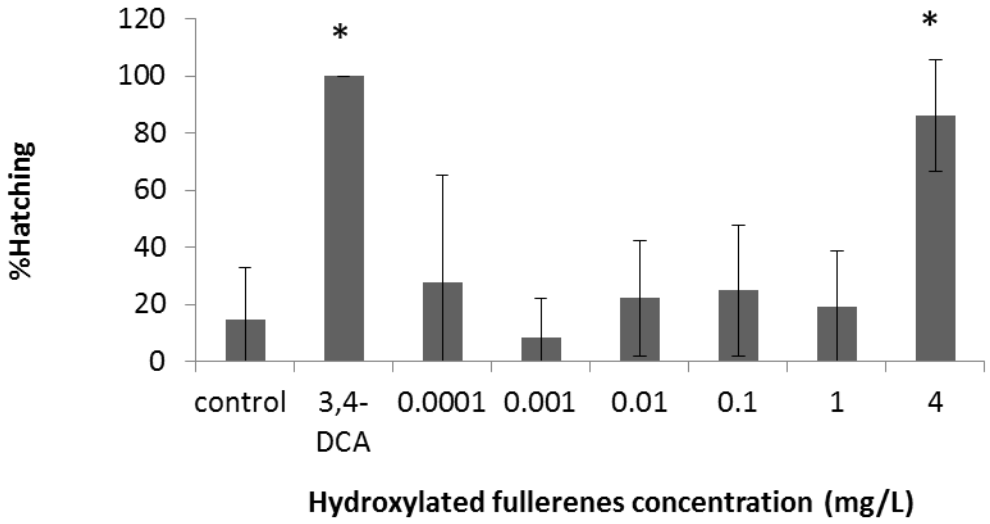


Fig. 6. Percentage of hatching in fathead minnow embryos following 96-h exposure to hydroxylated fullerenes ($C_{60}(OH)_{24}$). (mean ± SD). Early hatching was observed at 4 mg/L $C_{60}(OH)_{24}$ exposure. 3,4-dichloroaniline (3,4-DCA) exposures serve as positive control. Asterisks denote treatment that differs significantly from the MHW control group (One-Way ANOVA Dunnett's test, $p<0.05$).

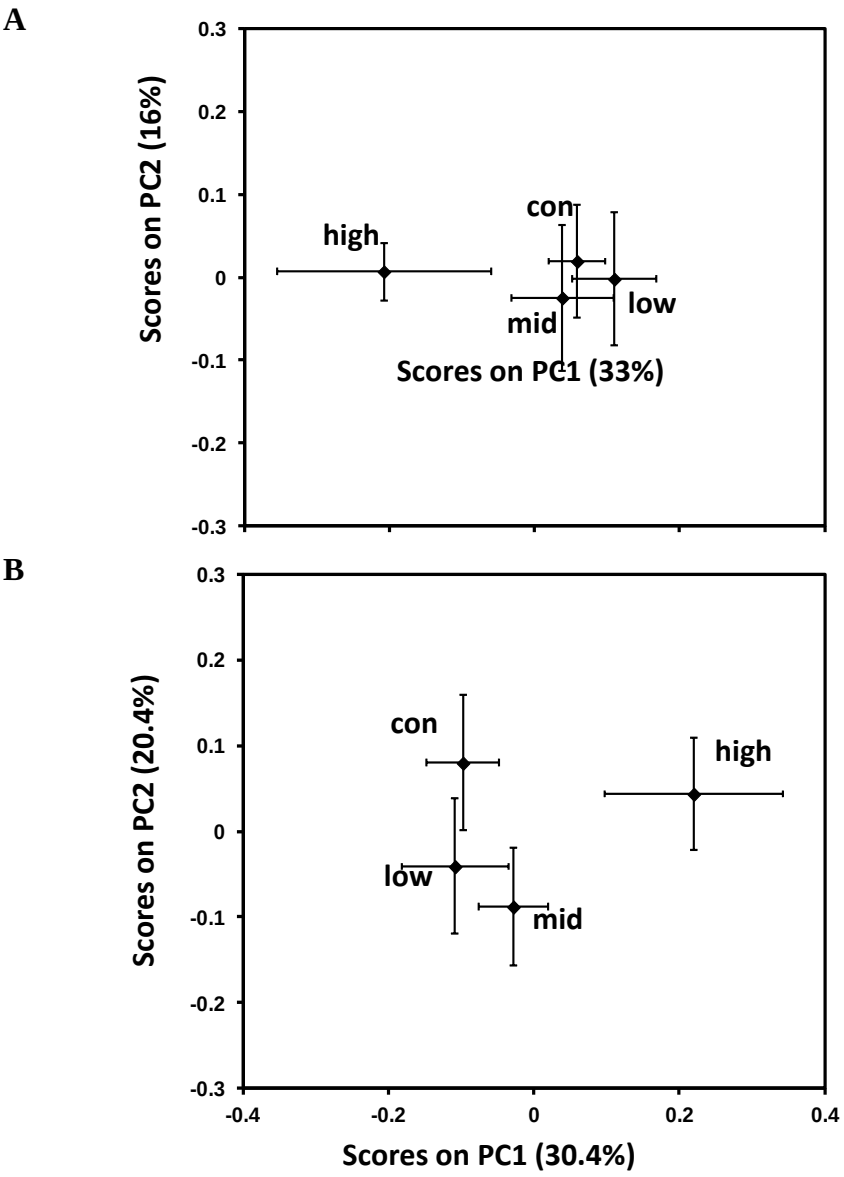
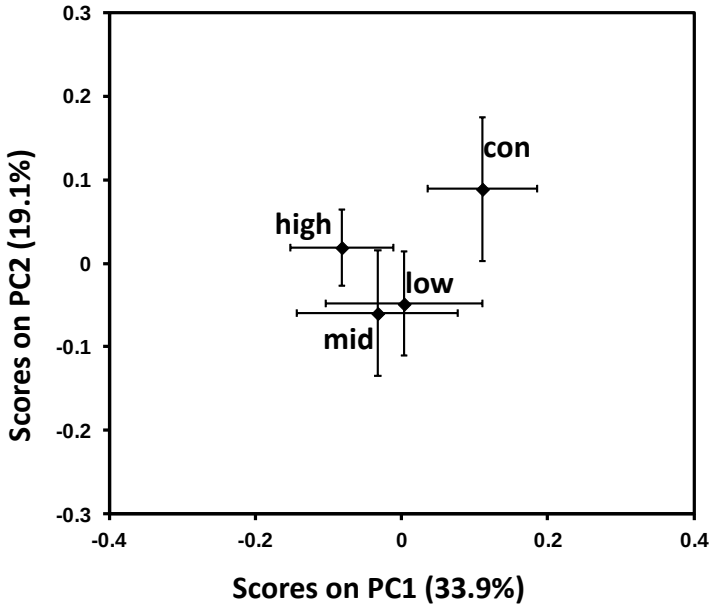


Fig. 7. Two-component scores plot from principal component analysis (PCA) of NMR spectra from unexposed fathead minnow embryos (con, control) and those exposed to AgNPs at low concentration (low, 0.001 mg/L), middle concentration (mid, 0.01 mg/L), and high concentration (high, 0.75 mg/L) for (A) 72 h or (B) 120 h. Each marker is the mean score value for a given treatment with standard error. The amount of variations included in each component is shown in the axis title.

A



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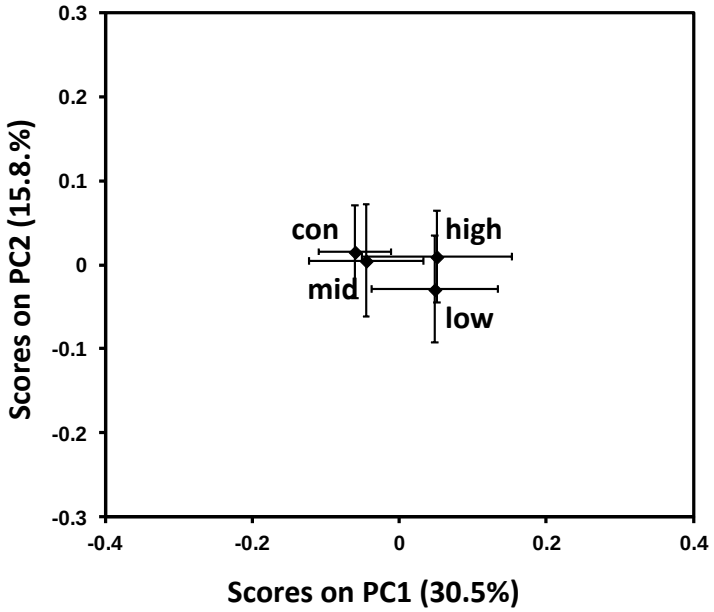
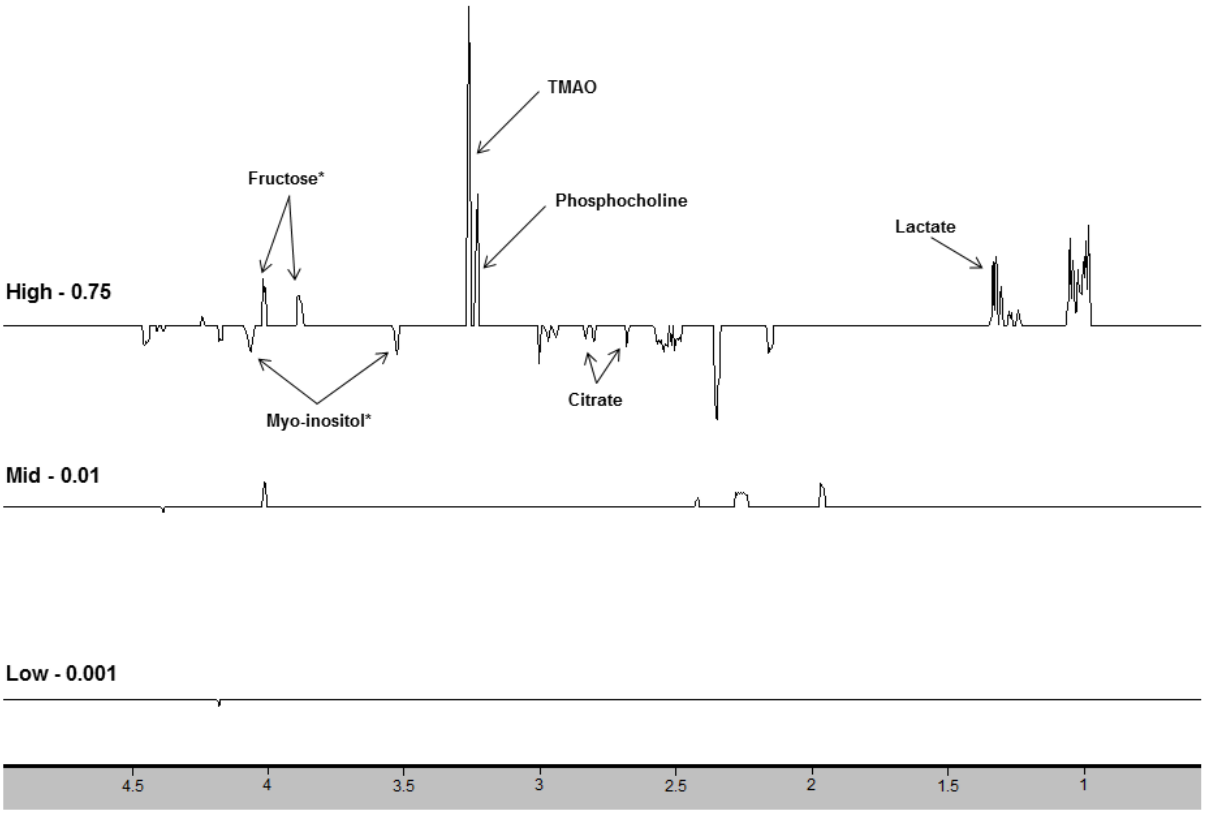


Fig. 8. Two-component scores plot from principal component analysis (PCA) of NMR spectra from unexposed fathead minnow embryos (con, control) and those exposed to hydroxylated fullerenes at low concentration (low, 0.0001 mg/L), middle concentration (mid, 0.01 mg/L), and high concentration (high, 4 mg/L) for (**A**) 72 h or (**B**) 120 h. Each marker is the mean score value for a given treatment with standard error. The amount of variations included in each component is shown in the axis title.

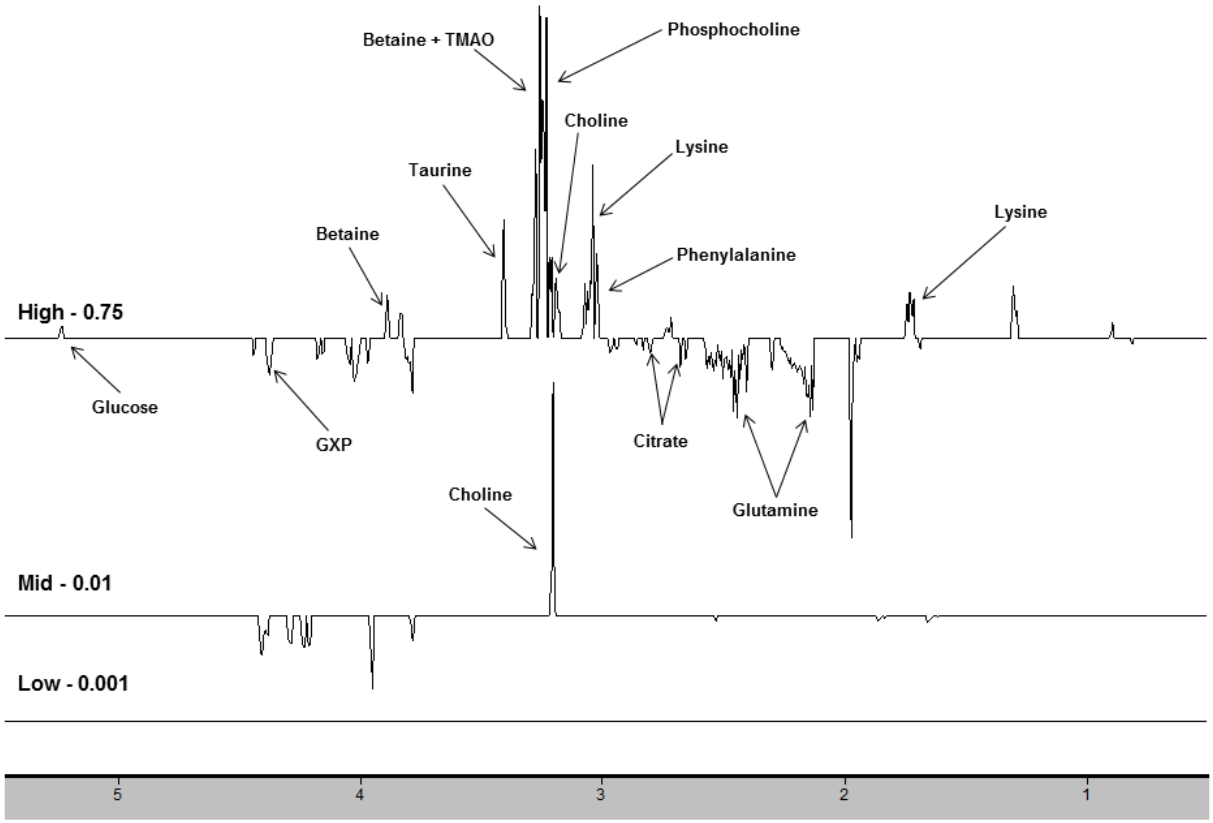
A



Chemical shift (ppm)

Fig. 9A.

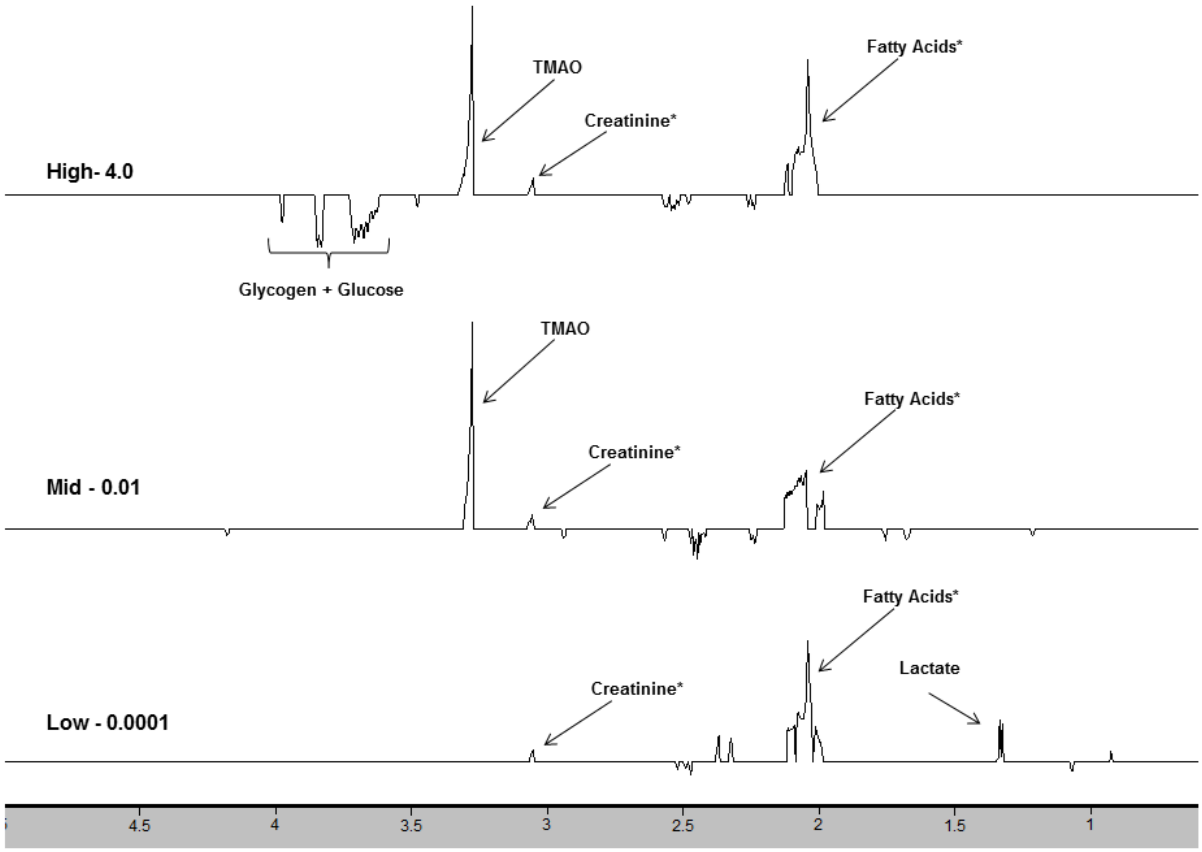
B



Chemical shift (ppm)

Fig. 9B.

C



Chemical shift (ppm)

Fig. 9C.

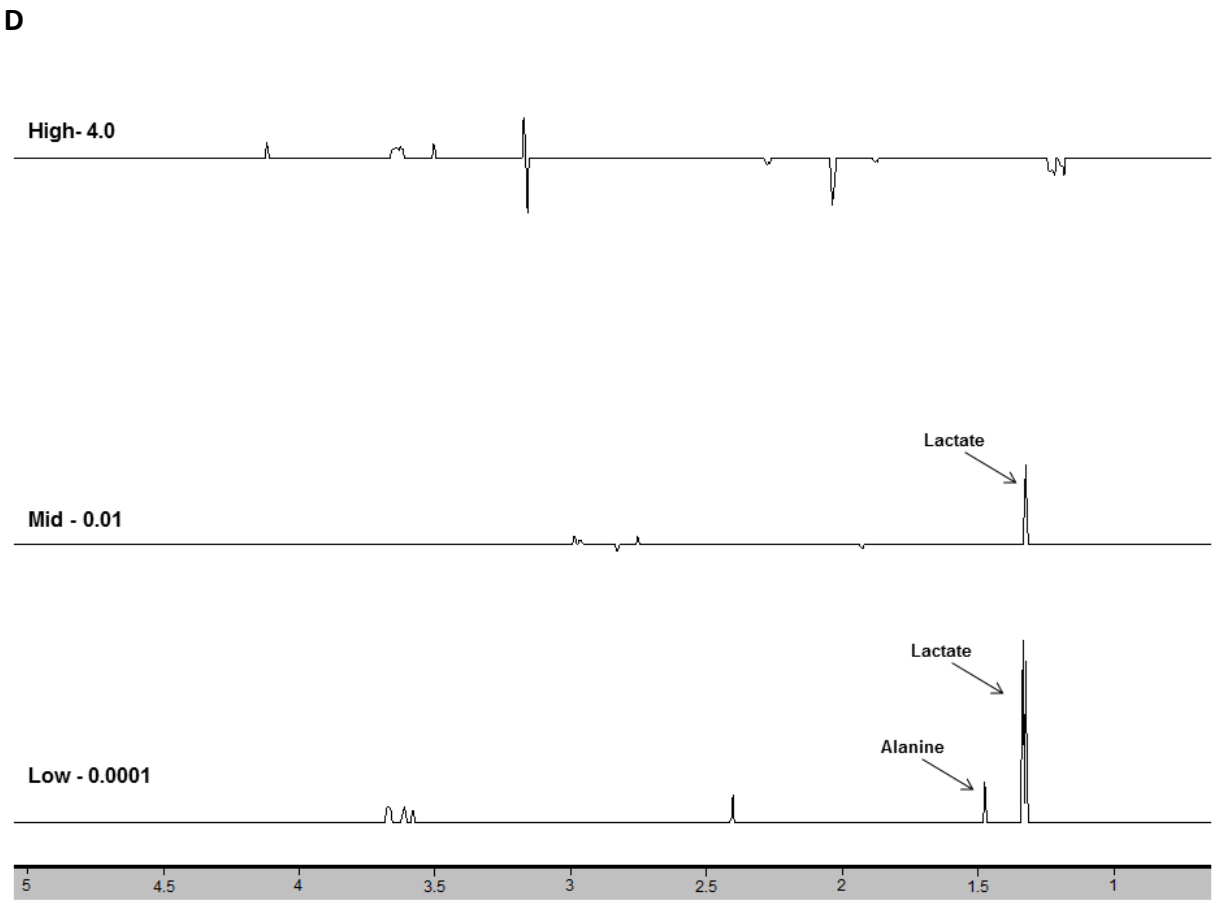


Fig. 9. Average NMR difference spectra (exposed minus control) generated from fathead minnow embryos exposed to: (**A**) AgNPs (0.001, 0.01, 0.75 mg/L) for 72 h (**B**) AgNPs (0.001, 0.01, 0.75 mg/L) for 120 h (**C**) hydroxylated fullerenes (0.0001, 0.01, 4 mg/L) for 72 h (**D**) hydroxylated fullerenes (0.0001, 0.01, 4 mg/L) for 120 h. Positive-going peaks indicate metabolites that increased upon exposure, while negative-going peaks indicate those that decrease. Each peak was determined to be significant by t-test (p<0.05). All difference spectra in each fig. are displayed using the same Y-axis scale. Changing metabolites following exposure were identified and labeled. Tentative assignments are indicated by Asterisks.

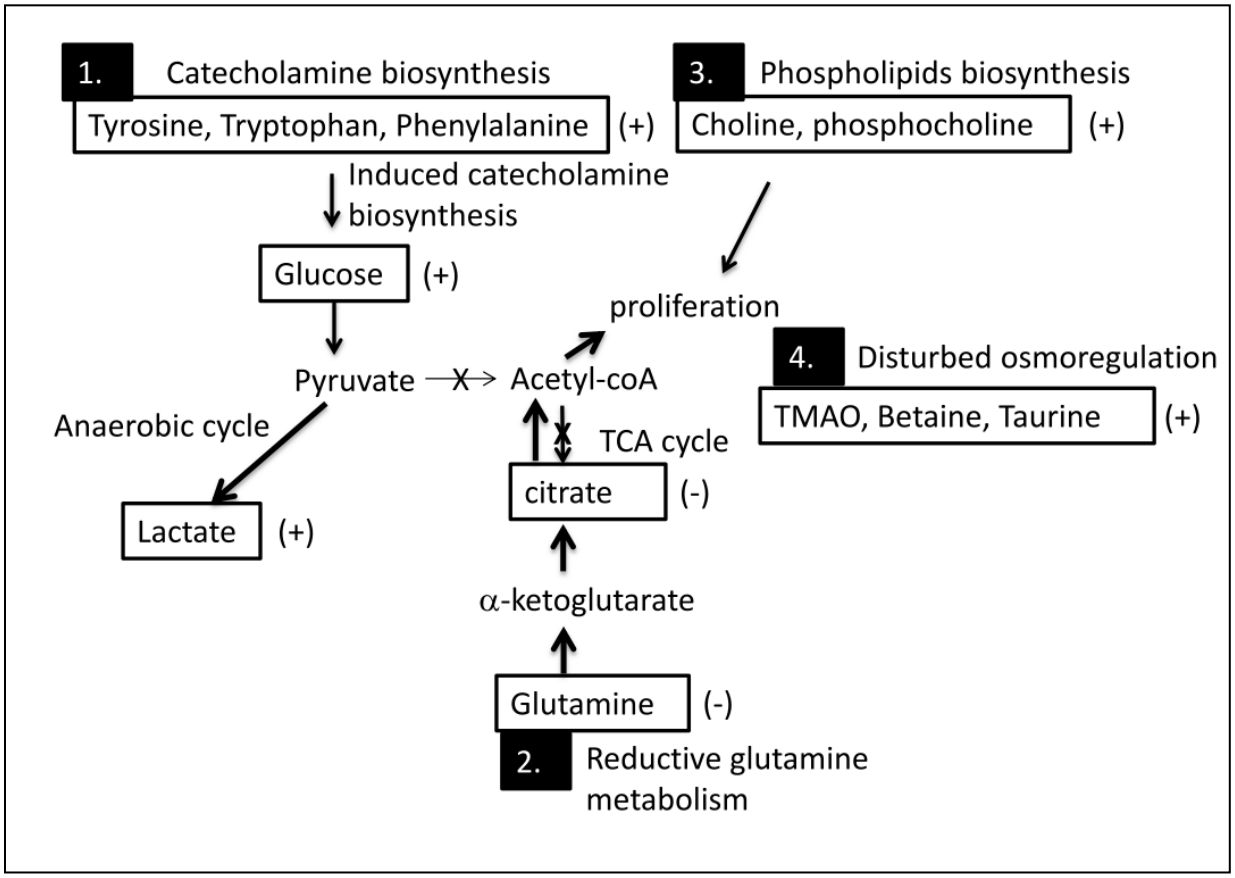


Fig. 10. Proposed modes of action based on changing metabolites following AgNP exposure. AgNPs may induce biosynthesis of catecholamines and phospholipids, reductive glutamine metabolism, and disturbed osmoregulation, while blocking the TCA cycle.

Table 1a. Metabolite profiles following exposure of fathead minnow embryos to AgNPs

Metabolites	AgNPs 72-h exposure[a]			AgNPs 120-h exposure[b]		
	Low - 0.001	Mid - 0.01	High - 0.75	Low - 0.001	Mid - 0.01	High - 0.75
Energy Metabolism related						
Glucose	-	-	-	-	-	UP
Glycogen	-	-	-	-	-	-
Lactate	-	-	UP	-	-	-
GXP	-	-	-	-	-	DOWN
Fructose	-	-	UP	-	-	-
Citrate	-	-	DOWN	-	-	DOWN
Amino Acids						
Valine	-	-	UP	-	-	-
Glutamate	-	-	DOWN	-	-	-
Lysine	-	-	-	-	-	UP
Phenylalanine						UP
Glutamine	-	-	-	-	-	DOWN
Tyrosine	-	-	-	-	-	UP
Tryptophan	-	-	-	-	-	UP
Osmolytes						
TMAO	-	-	UP	-	-	UP
Myoinositol[c]	-	-	DOWN	-	-	-
Betaine	-	-	-	-	-	UP
Taurine	-	-	-	-	-	UP
phospholipids synthsis related						
Phosphocholine	-	-	UP	-	-	UP
Choline	-	-	-	-	-	UP

[a] Following exposure to AgNPs for 72 h at low (0.001 mg/L), medium (0.01 mg/L), and high (0.75 mg/L) concentrations

[b]Following exposure to AgNPs for 120 h at low, medium and high concentrations

[c]Tentative assigned metabolites

Table 1b. Metabolite profiles following exposure of fathead minnow embryos to hydroxylated fullerenes for 72 h and 120 h

Metabolites	Hydroxylated fullernes 72-h exposure			Hydroxylated fullernes 120-h exposure		
	Low -0.0001	Mid - 0.01	High - 4	Low - 0.0001	Mid - 0.01	High - 4
Energy Metabolism related						
Glucose	-	-	DOWN	-	-	-
Glycogen	-	-	DOWN	-	-	-
Lactate	UP	-	-	UP	-	-
Amino Acids						
Alanine*	-	-	-	UP	-	-
Osmolytes						
TMAO	-	UP	UP	-	-	-
others						
Creatinine[c]	UP	UP	UP	-	-	-
Fatty Acids[c]	UP	UP	UP	-	-	-

[a]Following exposure to hydroxylated fullernes for 72 h at low (0.0001 mg/L), medium (0.01 mg/L), and high (4mg/L) concentrations
[b]Following exposure to hydroxylated fullernes for 120 h at low (0.0001 mg/L), medium (0.01 mg/L), and high (4mg/L) concentrations
[c]Tentative assigned metabolites

CHAPTER 3

CONCLUDING REMARKS

With increased applications of nanomaterials in industry and consumer market, their global production and market value will keep growing. The global market value of nanomaterials is expected to exceed 1 trillion by 2015 (Hobson, 2009). Nanomaterials were often synthesized to fit the need of specific applications. Surface modification, size, and shape of nanomaterials could greatly determine their physical and chemical properties and thus decide their application. However, these properties could also affect the bioavailability and toxicity of nanomaterials. Nanomaterials of the same type but with different coatings, surface modifications, size, or shapes could react differently with nontarget organisms, causing differences in toxicity (George et al., 2012). In 2007, a US EPA Nanotechnology White Paper advocated the importance of developing suitable toxicity test methods for nanomaterials. However, uncertainty exists when we try to use the toxicological assessment of one nanomaterial to predict the toxicity of others that are produced in different ways. Measuring the toxicity of every kind of nanomaterials is not feasible, and a toxicogenomic approach was proposed to assist in determining and classifying the modes of action of various nanomaterials (Matranga and Corsi, 2012; Robertson, 2005; Snape et al., 2004).

In the present study, we focused on studying the toxicity and toxic mechanisms of AgNPs and hydroxylated fullerenes in the fathead minnow (*Pimephales promelas*). Previous research has reported that the genetic responses corresponding to AgNP exposure to fish embryos and hydroxylated fullerenes could be used in determining possible toxic mechanisms (Jovanović et al., 2011; van Aerle et al., 2013). Compared to genomic responses, metabolomic responses are

much closer to the actual physiological effects. Based on our metabolomics results, we propose that AgNPs serve as a respiratory chain inhibitor to inhibit aerobic respiration, induce catecholamine-regulated glucose synthesis and phospholipids' biosynthesis, and also cause disturbed osmoregulation. Disturbed osmoregulation could result in yolk edema, a prominent sublethal toxicity endpoint. Thus, in our study, NMR-based metabolomics appeared to be a good method for determining the modes of action of AgNPs. However, we are not able to assess the relative sensitivity of metabolomic responses versus whole body responses following AgNP exposure because we only detected responses at the highest concentration (0.75 mgL^{-1}), the LOEC for both assessment techniques. To reduce variability and improve the sensitivity of metabolomic responses, all embryo samples should be exposed at the same time to avoid systematic differences in metabolite profiles, and adding exposure concentrations at closer intervals should also be considered. Few changing metabolites in response to hydroxylated fullerene exposure were consistent with results from embryo toxicity testing; however, variations within exposure group could result in reducing statistical significance between exposed and control groups. By combining whole embryo toxicity results with metabolite profiles, we can predict the long-term toxicity of specific nanoparticles and make prediction about their modes of action.

Future research should compare the toxicity and modes of action between AgNPs and Ag ions. Silver ions released by AgNPs could cause toxicity to aquatic organisms, however, recently published research reported that Ag ions induced different modes of action in Japanese medaka (*Oryzias latipes*(Chae et al., 2009). Silver NP exposures resulted in DNA damage, oxidative stress and induction of a metal detoxification pathway, while exposure to silver ions only induced some inflammatory responses. NMR-based metabolomics would provide a method to

confirm and refine these findings. In addition, conducting AgNP exposures in the presence of natural matter (NOM) would create a more realistic and natural exposure scenario, but also help isolate effects of AgNPs from those of Ag ions. Gao et al (2009) reported decreased release of silver ions by AgNPs in the presence of NOM, which could result in differences in uptake, toxicity and modes of action for AgNP-NOM exposures.

Metabolomics can assess all the metabolic alterations in response to nanomaterial exposure in specific tissues or whole organisms (e.g., fish embryos) at the same time. In combination with traditional toxicity testing, this information-rich method has the potential to be a timely and cost effective system to provide health risk assessment information for an increasing number of nanomaterial types.

Reference

Chae, Y.J., Pham, C.H., Lee, J., Bae, E., Yi, J., Gu, M.B., 2009. Evaluation of the toxic impact of silver nanoparticles on Japanese medaka (Oryzias latipes). Aquatic Toxicology 94, 320-327.

Gao, J., Youn, S., Hovsepyan, A., Llaneza, V.L., Wang, Y., Bitton, G., Bonzongo, J.C., 2009. Dispersion and toxicity of selected manufactured nanomaterials in natural river water samples: effects of water chemical composition. Environmental science & technology 43, 3322-3328.

George, S., Lin, S., Ji, Z., Thomas, C.R., Li, L., Mecklenburg, M., Meng, H., Wang, X., Zhang, H., Xia, T., Hohman, J.N., Lin, S., Zink, J.I., Weiss, P.S., Nel, A.E., 2012. Surface defects on plate-shaped silver nanoparticles contribute to its hazard potential in a fish gill cell line and zebrafish embryos. ACS nano 6, 3745-3759.

Hobson, D.W., 2009. Commercialization of nanotechnology. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology 1, 189-202.

Jovanović, B., Anastasova, L., Rowe, E.W., Palić, D., 2011. Hydroxylated fullerenes inhibit neutrophil function in fathead minnow (Pimephales promelas Rafinesque, 1820). Aquatic Toxicology 101, 474-482.

Matranga, V., Corsi, I., 2012. Toxic effects of engineered nanoparticles in the marine environment: Model organisms and molecular approaches. Marine environmental research 76, 32-40.

Robertson, D.G., 2005. Metabonomics in toxicology: a review. Toxicological sciences : an official journal of the Society of Toxicology 85, 809-822.

Snape, J.R., Maund, S.J., Pickford, D.B., Hutchinson, T.H., 2004. Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. Aquatic Toxicology 67, 143-154.

van Aerle, R., Lange, A., Moorhouse, A., Paszkiewicz, K., Ball, K., Johnston, B.D., de-Bastos, E., Booth, T., Tyler, C.R., Santos, E.M., 2013. Molecular mechanisms of toxicity of silver nanoparticles in zebrafish embryos. Environmental science & technology 47, 8005-8014.