

NANOTOXICOLOGY: THE IMPACTS OF 5NM SILVER NANOPARTICLES ON PROTEIN
SYNTHESIS OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

BY

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Abstract

Silver nanoparticles (nAg) are becoming increasingly prevalent in consumer products due to their unique physicochemical properties. This excessive usage is causing nanoparticulate waste to end up in the water column with the potential to affect aquatic organisms. Previous research has shown damaging effects of engineered nanomaterials (ENMs) on various fish species with the current study aimed to identify the impacts of polyvinyl pyrrolidone (PVP) capped 5 nm silver nanoparticles on biochemical stress indicators and rates of protein synthesis in rainbow trout (*Oncorhynchus mykiss*). Fish were exposed to $100 \mu\text{g L}^{-1}$ nAg or $0.22 \mu\text{g L}^{-1}$ AgNO_3 for 48 hours and tissue samples taken. Biological endpoints analyzed included hematocrit, gill sodium potassium ATPase, heart acetylcholinesterase, and cortisol and malondialdehyde content. Fractional rates of protein synthesis were determined by flooding dose method modified to use a stable phenylalanine isotope tracer. No significant differences were observed between treatment groups for any of the biologically relevant endpoints nor protein synthesis rates, although a decreasing trend was observed for sodium potassium ATPase activity in nAg-exposed fish. Results suggest that rainbow trout exposed to silver ENMs for 48 hours at the environmentally relevant point-source exposure concentration tested are not expressing signs of significant damage nor exhibiting impaired metabolism indicating that under short term conditions 5 nm PVP-capped nAg at $100 \mu\text{g L}^{-1}$ is not acutely toxic. These findings are disputable as previous research has demonstrated the toxic effects of ENMs on fish physiology, including on the endpoints analyzed in this study. These results should encourage further research into longer exposure durations and investigating other potential sublethal effects.

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Abbreviations

4EBP1 = eukaryotic translation initiation factor 4E-binding protein 1

ACh = acetylcholine

AChE = acetylcholinesterase

AgNO₃ = silver nitrate

CAIP = cholinergic anti-inflammatory pathway

eIF2- α = eukaryotic translation initiation factor 2- α

ENM = engineered nanomaterial

GC-MS = gas chromatography-mass spectrometer

GTP = guanosine triphosphate

MDA = malondialdehyde

mTOR = mechanistic target of rapamycin

nAg = silver ENM

NKA = sodium-potassium ATPase

nZnO = zinc oxide ENM

PVP = polyvinyl pyrrolidone

ROS = reactive oxygen species

TSC = tuberous sclerosis protein

Introduction

Engineered Nanomaterials

Over the past decade, engineered nanomaterials (ENMs) have become increasingly common in food, clothing, electronic technology, medicine, and cosmetics (Fabrega et al., 2011). Due to their widespread popularity, the ENM industry is predicted to grow to nearly \$80 billion by 2022 (RNCOS E-Services Private Limited, 2015). Research conducted by Vance et al. (2015) found that over 1800 consumer products from 622 companies from 32 countries contain ENMs, making this a global issue. The term nanotechnology describes the design, synthesis, characterization, and use of small materials (less than 100 nanometers in dimension) as their size causes them to have distinct physicochemical properties including unique mechanical, catalytic, optical properties, and conductivity (Fabrega et al., 2011; Klaine et al., 2008). These miniscule materials are highly reactive because of their large surface area to volume ratio, which can expose more surface functional groups and has thus enabled a technological revolution with ENMs being progressively incorporated into products (Shaw and Handy, 2011; Vance et al., 2015). Given their world-wide presence, it is inevitable that ENMs will end up in the environment and it is therefore imperative that their effects be studied to determine what kinds of ecological repercussions could be expected.

Although there is increased use of synthetically designed ENMs, nanoparticles have always existed from both natural and anthropogenic sources. Innate earthly activities including volcanic eruptions, forest fires, desert surfaces, microbial processes, and meteorite impacts causing cosmic dust all contribute to the supply of nanoparticles (Byrne and Baugh, 2008; Heiligtag and Niederberger, 2013; Strambeanu et al., 2015). Natural nanoparticles in soils include clays, organic matter, iron oxides, and varying minerals that are involved with biogeochemical processes (Klaine et al., 2008). In modern times, diesel- and gasoline-fueled engines are another main contributor as nanoparticles frequently arise from combustion reactions (Shi et al., 2001).

The broad class of nanotechnology can be subdivided into other compound classes; carbonaceous ENMs including carbon nanotubes, metal-containing materials, semiconductor nanocrystals, otherwise known as quantum dots, semi-valent metals, and nanopolymers including dendrimers (Klaine et al., 2008). Unsurprisingly, metal-containing materials are arguably the most common and have received considerable attention due to their varying applications and subsequent introduction into many everyday products from socks to sunscreen.

During the synthesis process, most ENMs undergo surface functionalization with different forms of capping agents that can be a variety of ligands incorporated onto the surface of the ENM (Mout et al., 2012; Sperling and Parak, 2010). Binding surfactant molecules to the surface can help to stabilize the nuclei and prevent larger ENMs from aggregating by a repulsive force. This process helps to control the size and shape of the ENM so that it may be useful for a specific function (Sperling and Parak, 2010). Furthermore, by reacting the surface of the ENM with the organic species, the physiochemical properties of the ENM (including improved particle dispersion or to reduce particle degradation or oxidation of the core) can be altered as well as modifying the differential affinity of the material towards proteins and cell surface molecules (Mout et al., 2012; Sperling and Parak, 2010). For example; Bajaj et al. (2010) electrostatically complexed gold ENMs with green fluorescent protein to create a material capable of differentiating between healthy, metastatic, and cancerous mammalian cells. The ability to functionalize ENMs is pivotal in determining the plethora of uses and applications they may serve.

ENMs can be capped with a variety of ligand types depending on the desired use of the product. As a result, the capping agents may be an important factor that is involved in the level of toxicity of the ENM. Some agents are labile and easily removed whereas others may be relatively refractory and not easily digested (Handy et al., 2011). Polymer capping agents such as polyvinyl pyrrolidone (PVP) are strong stabilizers of nAg as they chelate metal oxides and stabilize colloidal suspensions of ENMs through steric repulsion (Kvítek et al., 2008). The repulsion caused by the capping agent arises from the bulk size of the polymer attached to the metal interfering with electrostatic interactions between surrounding particles and thus making aggregate formation less favourable. Furthermore, PVP can prevent dissolution of ionic silver from nAg; however, the enhanced dispersion allows for the maintenance of its antimicrobial activity (Kvítek et al., 2008).

For other purposes, a variety of small molecule ligands have been incorporated onto ENM's surfaces to allow sensing of biomolecules and cells, diagnoses of diseases, and intracellular delivery (Mout et al., 2012). As an alternative to small molecule ligands, polymer coatings are also used to prevent adsorption of proteins in the blood serum, to extend circulation time, facilitate the controlled release of drugs, and increase the probability of particle permeability into tumors (Mout et al., 2012). Lastly, ENMs functionalized with biomolecules are found at the forefront of nanomedicine to control interactions with specific molecules in a targeted tissue and are an innovative mechanism for drug delivery and molecular diagnostics. Current functionalization

methods for designated drug delivery can include the addition of proteins and antibodies onto the ENM that will bind to cell surface receptors (Mout et al., 2012).

Many ENM characteristics including zeta potential (surface electrostatic potential), size distribution, state of dispersion, shape, chemical composition, surface area and surface chemistry need to be considered in toxicology studies (Jiang et al., 2009). These properties are significant when analyzing or predicting the behaviour of ENMs in the water system, how they interact with proteins or organisms, and their capabilities of being transported into cells. Surface characteristics can affect agglomeration (weak bonds between primary particles) and aggregation (hard bonds between primary particles) of ENMs, which is differentiated by the strength of the bonds resulting in different biological availability and effects (Jiang et al., 2009). The size, or hydrodynamic diameter, of the particle controls its interaction with biological systems which includes absorption, distribution, metabolism, and excretion (Jiang et al., 2009). A small change of ENM zeta potential can have an effect on the hydrodynamic size distribution as well as the uptake and translocation, such as altering the blood-brain barrier integrity and permeability (Hoshino et al., 2004; Lockman et al., 2004). The size of a particle can also be affected by other factors including aggregation, capping agents, and related water molecules. It has been noted that the higher ionic strength of seawater compared to freshwater frequently causes aggregation and evidence suggests that even small increases in salinity (2.5% above freshwater) can dramatically increase the tendency of ENMs to aggregate and precipitate (Stolpe and Hassellöv, 2007).

Environmental Implications

Due to the widespread prevalence and diversity of consumer and industrial applications of synthetic ENMs, it is inevitable that some of these particles end up in freshwater and marine environments (Keller and Lazareva, 2014). It is estimated that between 260 000 and 309 000 metric tons of ENMs are produced annually, of which 63 to 91% end up in landfills, 8 to 28% are released into soil, 0.1 to 1.5% into the atmosphere, and up to 7% (29 300 metric tons per year) end up in the water system (Keller et al., 2013). ENMs may end up in aquatic environments by air, in the water going through and bypassing wastewater treatment plants (WWTP), and landfills with over 4% of the waste likely being incorporated into waterways without treatment (Keller et al., 2013). ENMs that make up coating products account for approximately 42% of all ENM use but

contribute 89 to 97% of all ENMs aquatically released, of which up to 25% may pass through a WWTP (Keller et al., 2013).

ENMs have the potential to contaminate soil, migrate into surfaces and groundwater, and are commonly transported to aquatic systems by wind or rainwater runoff (Klaine et al., 2008). As can be seen in Figure 1, ENMs may have toxic effects for many types of organisms regardless of their location in the water column. ENMs arriving via terrestrial runoff or as aerosols can pose a threat to embryos and plankton as well as sea birds and mammals at the water surface. As the suspended ENMs are diluted and transported to the open ocean they can be toxic to pelagic species. Finally, after aggregating, the ENMs may precipitate to the ocean floor and be dangerous for benthic biota (Klaine et al., 2008).

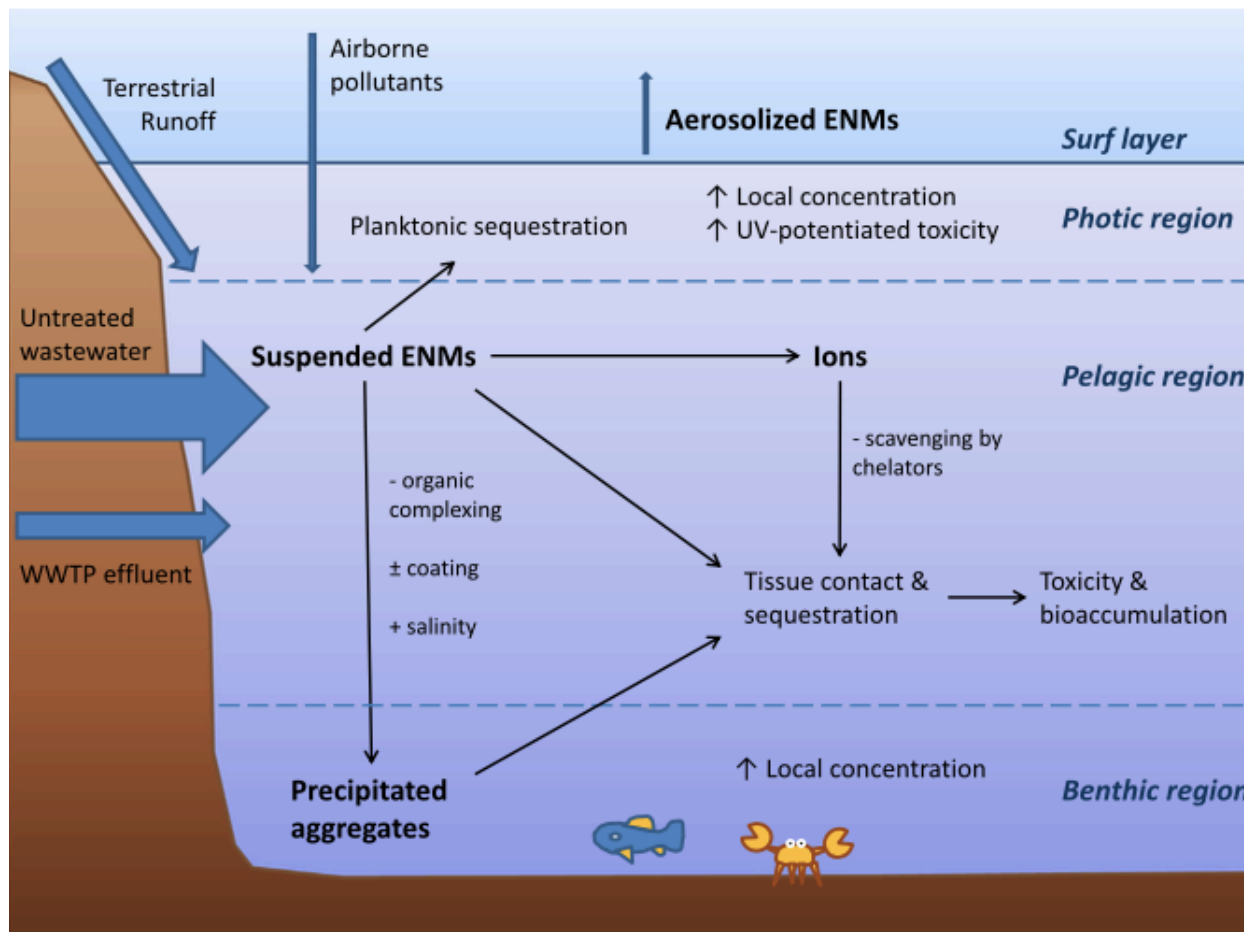


Figure 1: Schematic diagram outlining the various ENM routes into the water column and marine environment as well as indicating regions of organisms at risk of ENM toxicity. Image taken from Callaghan and MacCormack (2017).

Silver ENMs

While there are many different types of metal ENMs used throughout industry for various purposes, silver ENMs (nAg) are most commonly used with almost 450 consumer products containing nAg including clothing, plastics, wound dressings, plush animals, and air purifiers (Fabrega et al., 2011). nAg is frequently incorporated into materials due to its relatively low cost of manufacturing as well as its unique physicochemical properties and bactericidal function. These distinct physicochemical properties include high electrical and thermal conductivity, chemical stability, catalytic activity, and non-linear optical behaviour (Fabrega et al., 2011).

It is estimated that a single household can release 470 μg of ionic silver daily into the sewer from common products that contain nAg such as clothing and fabrics, washing machines, toothpaste, and shampoo (Benn et al., 2010). Benn et al. (2010) did further research and, using scanning electron microscopy, confirmed that nAg is also already present in wastewater. Before the increased use of nAg, most of the silver found in surface waters was attributed to natural leaching, mining, and the photographic industry yielding concentrations in natural and contaminated waters in the ng L^{-1} range (Purcell and Peters, 1997; Sanudo-Wilhelmy and Flegal, 1992). Current environmental nAg concentrations are estimated at 30.1 mg/kg in sediments and 2.2 $\mu\text{g L}^{-1}$ in surface waters and are expected to continue rising by approximately 1.7 times between 2014 and 2020 (Massarsky et al., 2014; Sun et al., 2016).

Ionic silver is well-known to have antimicrobial properties and the efficacy is only improved as nAg is incorporated into products (Massarsky et al., 2014). This particle's antibacterial and antifungal activity arises through membrane damage, permeabilization, and possibly through oxidative damage by creating reactive oxygen species (ROS) (reviewed by Massarsky et al., 2014). nAg has become a promising candidate for antibacterial applications due to the nature of its activity, which makes it effective against both Gram-positive and -negative bacteria (Morones et al., 2005). The bactericidal activity of nAg is due to its nanospecific effects along with the controlled release of ionic silver. nAg has already had several medical applications including in healing creams and dressings, bone and dental cements, in coatings for cardiovascular prosthetics and catheters, and is also being investigated for potential use for targeted drug delivery applications (reviewed by Massarsky et al., 2014). Part of the antimicrobial activity of nAg includes the ability to inhibit the formation of biofilms which is a polysaccharide matrix excreted

by microorganisms that provides a barrier against many antimicrobial agents (Percival et al., 2007).

While nAg has many practical and advantageous applications, if it interacts in excess with various organisms it can be potentially dangerous. After uptake into the body, the ENMs can bioaccumulate and become toxic to internal organs (Ates et al., 2013; Ramskov et al., 2014; Walters et al., 2014). The toxicity of nAg has been proposed to be caused by the penetration of silver ions into a cell and interacting with the DNA that consequently reacts with thiol group proteins, resulting in cell death (Rai et al., 2009). Other experiments show that nAg reduces ATP content in the cell, causing damage to the mitochondrial respiratory chain, producing reactive oxygen species and interrupting ATP synthesis, ultimately damaging DNA (AshaRani et al., 2009). Furthermore, nAg may physically interact with cell membranes and proteins which can result in interference with ion transport pathways (Schultz et al., 2012).

Although this experiment primarily focuses on the effects of nanoparticulate silver, ionic silver can also be detrimental to living organisms and is considered to be one of the most toxic forms of a heavy metal, surpassed only by mercury (Ratte, 1999). Ionic silver is known to be highly toxic to the sodium potassium ATPase (NKA) of gill epithelial cells. Its primary method of toxicity is through the inhibition of this highly important enzyme responsible for controlling osmoregulation. It has been elucidated that the silver ions have a tendency to bind to the Mg^{+} cofactor binding sites in the NKA enzyme which prevents the proper hydrolysis of ATP and consequently non-competitively inhibits the enzyme and disrupts the osmoregulation of the fish (McGeer and Wood, 1998).

The toxic free silver ion Ag^{+} may be released from common silver compounds such as silver nitrate ($AgNO_3$) as well as from nAg dissolution. It is possible that Ag^{+} from nAg dissolution may only be present at low concentrations in ecologically-relevant conditions because of its capacity for thiol binding (Blaser et al., 2008). While relatively harmless to humans, high exposures to silver compounds have also been known to cause argyria, which is an irreversible condition where the deposition of silver in the body tissue results in bluish coloured skin (Rosenman et al., 1979). However, dissolved silver ions in the environment are known to be highly toxic to many prokaryotes, and freshwater and marine invertebrates and fish (Fabrega et al., 2011). Silver ions tend to bioconcentrate in organisms as their chemical properties cause them to be compatible for uptake by the ion transporters in the cellular membrane (Fabrega et al., 2011).

While the mechanism of ionic silver toxicity has been well characterized, it has not been determined whether the mechanism of nAg toxicity is the same and, if so, if the ENM and/or the dissociation of ionic silver causes the effect. Morgan et al. (2004) conducted a detailed time-course analysis in rainbow trout exposed to $4.3 \mu\text{g L}^{-1} \text{AgNO}_3$ and demonstrated that there is a two-step process for the interruption in ion regulation. Firstly, within 1-2 hours of exposure, gill carbonic anhydrase activity is inhibited by 30% which is then followed by a progressive inhibition of NKA activity which is about 40% blocked by 24 hours of exposure. Schultz et al. (2012) found that in rainbow trout exposed to citrate-capped nanoparticulate silver, NKA activity was significantly inhibited by nAg but that the ENM had no effect on the carbonic anhydrase activity. While the complete mechanism has not been fully elucidated, this study was the first to show that sodium regulation is disrupted by citrate-capped nAg and results suggest nanospecific effects.

Looking further at potential variations in effects of ionic versus nanoparticulate silver, studies of gene expression in rainbow trout liver indicated that ionic silver can trigger the activation of genes involved with the oxidative stress pathway and protein stability whereas exposure to nAg was found to influence genes involved with general inflammation (Gagné et al., 2012). Research conducted by Gagné et al. (2012) elucidated that ionic silver is more bioavailable than nAg as significantly higher levels of hepatic Ag^+ were seen after the exposure, and that nAg and aggregates may be capable of stimulating the immune (complement C1) system. Gene expression studies indicating that nanoparticulate silver may elicit a stress response based on the regulation of specific pathways highlights the potential toxicity of this nanomaterial.

Biological impacts of nAg in aquatic species

Given increased ENM production, the potential for release into the environment and resulting effects on aquatic ecosystems is increasingly being studied and analyzed as species' physiologies are constantly being put at risk of detrimental effects (Klaine et al., 2008). ENMs are known to be toxic and therefore the interaction between ENMs and critical proteins is an area of concern. This interaction could be detrimental as bioactivity may occur through the denaturation of proteins resulting in loss of function (Dieni et al., 2016; Maccormack et al., 2012). Similar to how mammals can inhale air-borne ENMs, aquatically suspended ENMs are equally likely to interact with the gills of fish, and potentially cause severe damage to organs and whole animal physiology. Much research has been done showing ENM toxicity in gills and it has therefore become an important area of concern (reviewed by Handy et al., 2008).

The organism of interest for this project is rainbow trout (*Oncorhynchus mykiss*), a freshwater salmonid fish species commonly found in lakes and streams (Northcote, 1962). This organism was chosen as it is a model teleost fish that is abundant throughout many freshwater systems around the globe (MacCrimmon, 1971). As ENMs are continually being leached into the water column, it is imperative to determine their effects on common aquatic animals such as salmonids. Previous studies show that acute Ag^+ and nAg exposure decreases sodium uptake, thus disrupting ionoregulation (Schultz et al., 2012; Wood et al., 1999), which allows a platform for a more in-depth assessment as to the mechanisms of action and other potential effects of nanoparticulate silver.

Many studies have been done exposing organisms to ENMs to evaluate the uptake of the ENMs and their subsequent effects to assess ENM's impact in an environmental situation. The exact mechanism of ENM uptake is still a focus of research, but studies show that ENMs can enter cells by diffusing through the membrane, endocytosis, and adhesion (Geiser et al., 2005; Kim et al., 2006; Klaine et al., 2008; Lin et al., 2006). In aquatic organisms, the primary points of nAg entry include the gill epithelium, gastrointestinal epithelium, and the dermal layer (Bennat and Müller-Goymann, 2000; reviewed by Handy et al., 2008). Although the method by which ENMs inhibit important enzymes in key organs is not fully known, it has been established that ENMs accumulate a shell of associated proteins in biological tissues known as a "protein corona" and, if entering a cell in a manner other than endocytosis, could stay intact and interact with other proteins in the cell (Lundqvist et al., 2008).

Gills are highly sophisticated and delicate organs that make up more than half of a fish's total surface area and contains a suite of proteins that are involved with gas exchange as well as ionoregulation, acid-base homeostasis, and nitrogenous waste excretion (Evans et al., 2005; Hogstrand and Wood, 1998). Due to the substantial surface area, small diffusion distances, and numerous membrane proteins exposed to the environment, the gill tissue is highly sensitive to damage by toxins (Evans, 1987). Studies show that in freshwater fish, silver toxicity is caused by a disturbance of osmoregulation at the gill, where the organism has an inability to maintain proper Na^+ and Cl^- concentrations in the blood plasma which, in turn, affects the Na^+ and Cl^- transport across the gills (Hogstrand and Wood, 1998; Wood et al., 2012). Teleost gills also contain neuroepithelial cells which sense changes in environmental oxygen and regulate cardiorespiratory function to control uptake of oxygen and its subsequent delivery (Burlison, 2009).

The effects of various ENMs on the ionoregulatory mechanisms in fish can be difficult to predict as their effects are varied. Studies conducted by Federici et al. (2007) on TiO₂ ENMs and by Schultz et al. (2012) on nAg showed that gill NKA is inhibited whereas Smith et al. (2007) found that exposure to single-walled carbon nanotubes enhanced its activity. NKA is a highly important ATP-driven enzyme found in higher eukaryotes that is responsible for translocating sodium and potassium ions across the plasma membrane (Lingrel and Kuntzweiler, 1994). This translocation yields both a chemical and electrical gradient across the membrane which is essential for maintaining the resting potential as well as excitable activity of muscles and nerves (Lingrel and Kuntzweiler, 1994). As it is so critical for energy-dependent ion transport, the enzyme uses a significant amount of energy and it is estimated that 23% of ATP in humans at rest is used by this enzyme (Lingrel and Kuntzweiler, 1994).

Toxicants in the water can cause structural damage to the gill epithelium and, due to the highly important function of gills, it unsurprisingly leads to increased diffusion distances, impaired gas exchange, gill damage, loss of blood oxygen tension, and finally suffocation (Hogstrand and Wood, 1998; Mallatt, 1985). Exposure to ENMs most frequently has serious impacts on the gills, resulting in increased mucus production, influences sodium-potassium ATPase activity, and can cause epithelial damage or epithelial remodeling that will undoubtedly reduce the aerobic scope (Callaghan et al., 2016). Research done by Bilberg et al. (2010) has already shown such effects by demonstrating the increased sensitivity of fish to changes in available oxygen after being exposed to nAg.

When exposed to hypoxic conditions, gill chemoreceptors are stimulated resulting in a depolarization of neuroepithelial cells and the enhancement of parasympathetic input to the cardiac pacemaker via the vagus nerve. This process eventually releases acetylcholine at the sinoatrial node of the heart, thus slowing heart rate (Callaghan et al., 2016). This bradycardia is frequently accompanied by a decrease in total cardiac output, although may increase in some species to compensate for the decreased heart rate (Gamperl and Driedzic, 2009; MacCormack and Driedzic, 2007). If the gill tissue were damaged upon ENM exposure, it is possible that the neuroepithelial cell function may be altered and consequently affect the parasympathetic nervous control over the cardiac pacemaker (Bessemmer et al., 2015; Callaghan et al., 2016). If silver ENMs cause a decrease in cardiac function while simultaneously reducing the efficiency of the gill, it is likely that there will be a significant, and potentially detrimental, effect on aerobic metabolism of the whole animal.

The aerobic scope is defined as the difference between the maximum and minimum rates of oxygen consumption ($\dot{M}_{O_2\max} - \dot{M}_{O_2\min}$) and describes the amount of oxygen available to an organism for aerobic activity (Clark et al., 2011). A reduction of the aerobic scope will cause the fish to have less energy available to devote to routine practices such as foraging, reproduction, hunting, or predator avoidance (Clark et al., 2011). Research conducted by Callaghan et al. (2016) found a 9% decrease in the aerobic scope of white suckers (*Catostomus commersonii*) upon exposure to zinc oxide ENMs, however, the difference was small, the duration of the exposure was short (25 hours), and the authors state that it is possible that the effects would be magnified if fish were treated in a more environmentally relevant chronic exposure situation.

Due to the many parts of the gill that can be affected by ENMs, it is assumed that under sufficiently high concentrations the fish will fall into a hypoxemic state as it fails to get enough oxygen. To counteract these conditions, animals reduce their metabolic rates by reducing the activity of energetically expensive metabolic processes. The maintenance of ionic gradients via NKA and protein turnover (including synthesis and degradation) are considered to be the two most energetically demanding metabolic processes (Wieser and Krumschnabel, 2001). Protein synthesis alone accounts for between 20 and 40% of all oxygen consumption in fish (Carter et al., 1993; Houlihan et al., 1988) making it likely that this process could be influenced by hypoxemia or metabolic depression.

Signaling pathways involved with protein synthesis

Cassidy et al. (2018) determined for the first time in fish that the decrease in protein synthesis during hypoxia is likely controlled by signaling molecules 4EBP1 and eIF2- α and not solely due to a lack of energy (ATP). In ideal growth conditions, mechanistic target of rapamycin (mTOR) is activated and subsequently phosphorylates the downstream signaling protein target 4EBP1, thus increasing protein synthesis (Johnston et al., 2011). Phosphorylation of protein kinase B in the mTOR pathway can trigger the phosphorylation of 4EBP1 leading to increased protein synthesis, and the dephosphorylation should act in opposite fashion (Lamarre et al., 2016).

Under hypoxic conditions, mTOR is inhibited in multiple ways which slows protein synthesis. In fish, AMP-activated protein kinase, which is an adenylate energy charge sensor, is stimulated during hypoxia which concurrently triggers the tuberous sclerosis protein (TSC1-TSC2) complex (Liu et al., 2006). The activation of any of these various pathways can potentially lead to a decrease in phosphorylated 4EBP1 and result in a decreased rate of protein synthesis. The

signaling protein eIF2- α is a eukaryotic initiation factor that is critical for the commencement of most types of eukaryotic translation and a means of controlling protein synthesis through phosphorylation (Kimball, 1999). This important protein mediates the binding of the transfer RNA (tRNA) containing the initial amino acid, tRNA^{Met}, to the small ribosomal subunit using guanosine triphosphate (GTP) energy. The phosphorylation of this protein prevents the formation of the critical eIF2-GTP-tRNA-Methionine complex and stops protein synthesis (Kimball, 1999).

Fish are frequently exposed to hypoxic conditions; however, the mechanisms responsible for the response, specifically concerning protein metabolism, has not been extensively studied. When an animal is exposed to hypoxia, the three major signaling pathways involved are the mTOR, hypoxia-inducible factor, and unfolded protein response pathways (Wouters and Koritzinsky, 2008). By analyzing the quantities and characteristics of these two signaling molecules (4EBP1 and eIF2- α) after being exposed to nanoparticles inducing hypoxic conditions, results may indicate the correlation between fluctuating quantities of molecules and their relationship to rates of protein synthesis, and elucidating the potential mechanism of nanotoxicity.

It is known that common ENMs can trigger metabolic depression, cardiorespiratory alterations, and gill remodeling in fish in a manner similar to a hypoxia response; however, there are many hypotheses as to the underlying mechanisms. Other pathways that may affect metabolism and thus, protein synthesis, include the cholinergic anti-inflammatory pathway (CAIP) as well as thyroid hormones. As seen in Figure 2 outlining some of the potential mechanisms of toxicity of ENMs on aquatic species, these nanomaterials frequently result in oxidative stress and inflammation. The normal response involves the activation of signal transduction pathways for the regulation of the inflammatory responses and limiting tissue damage (Callaghan and MacCormack, 2017; Rosas-Ballina and Tracey, 2009). The CAIP is critical in neurally controlling the inflammatory response to curtail tissue damage and was discovered to be a novel function of the efferent vagus nerve (Pavlov et al., 2003). It is associated with the release of acetylcholine (ACh) which can affect the hearts of teleost as they are strongly influenced by cholinergic signaling as a stress response. The muscarinic ACh receptor-linked potassium channels drastically affect the heart rate and stroke volume under varying conditions (Abramochkin and Vornanen, 2017).

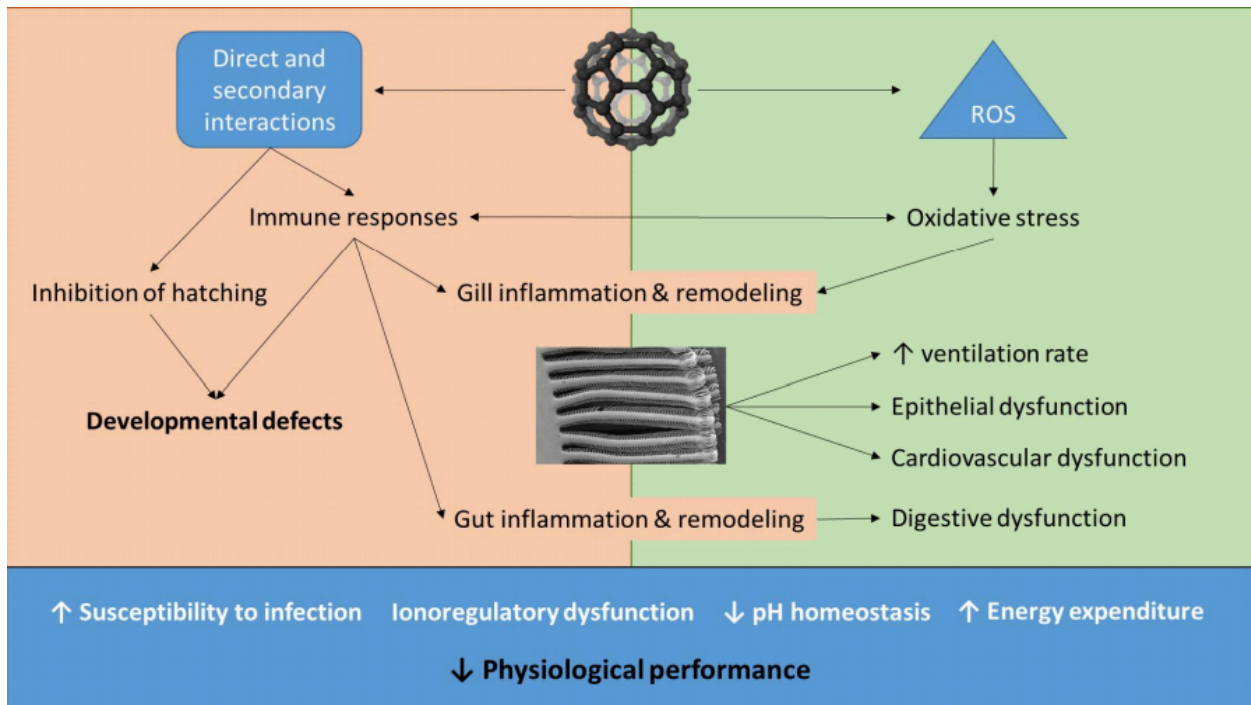


Figure 2: Potential mechanisms of toxicity of ENMs in aquatic species. Reactive oxygen species can be generated directly or through an immune response and the downstream effects of the oxidative stress may include tissue damage and/or epithelial remodeling, commonly in the gill or gut. Interactions between ENMs and proteins may stimulate immune responses that may trigger localized or systemic inflammation. Furthermore, the hatching processes may be inhibited leading to developmental defects. All of these responses may eventually impact the metabolism, protein synthesis rate, and the physiology of the whole animal thus effecting their potential for survival. Image taken from Callaghan and MacCormack (2017).

Acetylcholinesterase (AChE) is a highly important enzyme that is involved in termination of neuronal signaling to the musculature and controls muscle contraction by breaking down ACh. The level of AChE in an organism is so critical it is often used as an important biomarker for environmental toxicity among vertebrates (Lionetto et al., 2013). AChE hydrolyzes the ACh neurotransmitter allowing for uptake of free choline into the presynaptic neuron and is found primarily at neuromuscular junctions as well as cholinergic synapses. Increased amounts of ACh results in ceaseless stimulation of the post-synaptic muscle neuron, causing an increase in cardiovascular and respiratory responses that can lead to long-term damage (Čolović et al., 2013). Research done by Katuli et al. (2014) found that nAg inhibited erythrocyte AChE activity and, in concert with increased ACh release, these metabolite levels could prove detrimental. As ENM-induced oxidative stress causes an increase in ACh release and decreased AChE activity, the resulting bradycardia by the CAIP may be related to decreased metabolism and protein synthesis.

Research Objectives

Although there is continued proliferation of ENM-containing products, research on their safety is ongoing and therefore few regulations have been implemented pertaining to the use, pre-release treatment, and release of ENMs and their byproducts (Callaghan and MacCormack, 2017). For this reason, it is imperative that people continue to study the effects ENMs may have on aquatic organisms as they are increasingly being exposed. Research conducted in the MacCormack Lab by Campbell et al. (2017) found that killifish (*Fundulus heteroclitus*) exposed to 80 $\mu\text{g L}^{-1}$ nAg showed a 30% decrease in oxygen consumption. These results inspired several follow-up projects in this lab developed to determine the cause of the suppressed metabolism and potentially establish a mechanism of toxicity. A larger fish species was needed to be able to assess the potential effects of exercise performance and to be conducive for protein synthesis studies. This study focuses on the potential toxicity of PVP-capped nAg in rainbow trout to determine whether a 48-hour exposure to 100 $\mu\text{g L}^{-1}$ nAg will elucidate discernable biochemical changes. Potential silver ENM toxicity was analyzed through measuring key biological endpoints including hematocrit count, heart acetylcholinesterase activity, plasma cortisol levels, gill MDA content, and gill NKA activity as well as rates of protein synthesis as an indicator of metabolism. It is hypothesized that, because of nAg's known disruption of ionoregulation, oxygen consumption may be impaired thus impacting the metabolic rate and protein synthesis as it is an important and highly regulated energy-consuming process. Understanding the toxicity and repercussions of ENMs in the wild will hopefully help encourage managers to implement critical regulations surrounding the use and waste management of ENMs to help protect our environment.

Materials and Methods

Experimental Animals

A variety of both male and female rainbow trout (*Oncorhynchus mykiss*) were acquired from Fraser's Mills Fish Hatchery (Fraser's Mills, NS). The fish were housed in a 750 L tank held at 17°C with recirculating freshwater and were acclimated for at least 2 weeks prior to experimentation. The trout were fed approximately 3% of their body weight every 1-2 days with 3-Optimum sinking food pellets (Corey Aquafeeds; Fredericton NB). Fish were not fed 24 hours prior to experimentation to limit metabolic rate deviations caused by specific dynamic action.

Procedures were performed in accordance with the Mount Allison University Animal Care Committee (Protocol #102197).

ENM Characterization

Nanoparticulate silver was purchased from nanoComposix (CA, USA) as a stock solution and particles were advertised by the manufacturer as having a 5 nm core and capped with PVP. Previous work on these particles was done to characterize the hydrodynamic diameter (19.3 nm) and zeta potential (-12.5 mV) of the nAg using dynamic light scattering with a Zetasizer Nano ZS (Malvern Instruments, Ltd, Malvern, UK). The rate of dissolution of ionic silver from nAg was also measured by doing a dialysis of nAg in freshwater and Ag^+ in the dialysate was quantified. From 1.0 mg nAg loaded into the cassette, a concentration of $8.64 \mu\text{g L}^{-1}$ was present in the dialysate. These results indicate that 0.22% of total silver in the nAg will be released as Ag^+ into aqueous solution over a 48-hour period. In this experiment, it was therefore assumed that at an exposure dose of $100 \mu\text{g L}^{-1}$ nAg, free Ag^+ would be present in solution at a concentration of $0.22 \mu\text{g L}^{-1}$.

ENM Exposures

Rainbow trout were exposed to an environmentally relevant point-source exposure of silver ENM at concentration $100 \mu\text{g L}^{-1}$. Prior to adding the nAg stock solution to the water, the solution was sonicated with a Fisher Scientific Series 60 Sonic Dismembrator Model F60 (Fisher Scientific Company, NH, USA) to disrupt aggregates and improve dispersion. The nAg was then transferred to 30 L of fresh water in the containment vessels. The holding tanks were well oxygenated and temperature was kept constant at 16°C using a recirculating refrigerated water bath. Individually, fish were exposed for 48 hours to either fresh water as a control, $100 \mu\text{g L}^{-1}$ nAg, or $0.22 \mu\text{g L}^{-1}$ silver nitrate (AgNO_3) which is equivalent to the amount of Ag^+ dissolution from nAg. To ensure fish would not suffer from ammonia build-up, 50% of the water in the holding tanks was exchanged after 24 hours. Following the exposures, the fish were immediately sampled.

Tissue Extraction

Fish were sacrificed by exposure to 0.38 g L^{-1} tricaine methanesulfonate (MS-222; Aqualife, Syndel Laboratories, Nanaimo, BC) buffered with 0.76 g L^{-1} NaHCO_3 until ventilation ceased. Blood samples were collected from the caudal vein using a needle and syringe treated with

heparinized modified Cortland saline containing NaCl (143 mmol L⁻¹), CaCl₂ (0.88 mmol L⁻¹), MgSO₄ (0.90 mmol L⁻¹), KCl (3.35 mmol L⁻¹), NaH₂PO₄ (2.25 mmol L⁻¹), NaHCO₃ (5.50 mmol L⁻¹), HEPES (0.010 mmol L⁻¹), and heparin ammonium salt from porcine intestinal mucosa (100 U mL⁻¹, pH 7.5). Hematocrit was measured in single with a capillary tube and centrifuge and remaining blood was spun at 13 200 RPM for 5 minutes to separate the plasma and red blood cells. Heart, liver, and gill samples (first arch) were extracted and all samples were flash frozen in liquid nitrogen and stored at -80°C.

Acetylcholinesterase Assay

The activity of heart acetylcholinesterase (AChE) was assayed following the methods of Ellman et al. (1961). For this spectrophotometric kinetic assay, the acetylcholine analog acetylthiocholine iodide is used as the substrate for AChE which will cleave it into acetate and thiocholate. The thiocholate subsequently reacts with dithiobisnitro-benzoate (DTNB) to produce 5-thio-2-nitrobenzoate which absorbs at 405 nm. Heart samples were sonicated with a Fisher Scientific Series 60 Sonic Dismembrator Model F60 (Fisher Scientific Company, NH, USA) in homogenization buffer (100 mM KPO₄ buffer (pH 7.4) with 1% Triton X). Homogenate dilutions were initially prepared as 1/4 (w/v) and further diluted with buffer to 1/16 (w/v) to obtain the best kinetic readings. The assay was done in a 96-well plate each containing 170 µL of Ellman's reagent (100 mM KPO₄ and 0.5 mM DTNB), 10 µL of the diluted sample, and 20 µL of 20 mM AChI (in milli-Q H₂O) giving final concentrations of 85 mM KPO₄, 4.25 mM DTNB, and 0.2 mM AChI. The plate was read at 405 nm every 15 seconds using a Spectromax 190 microplate reader (Molecular Devices LLC, CA, USA). A DC protein assay using the BioRad kit (BioRad Laboratories, CA, USA) was performed on the samples to standardize the enzyme activity per unit protein.

Sodium Potassium ATPase Assay

The protocol for this assay was modified from that previously described by McCormick (1993). During the tissue extraction, one set of gill samples were flash frozen and stored in SEI buffer containing 250 mM sucrose, 10 mM Na₂ ethylenediaminetetraacetic acid (EDTA), and 50 mM imidazole. For the spectrophotometric kinetic assay, the tissue was diluted 1/4 (w/v) and homogenized with a Polytron PT 10-35 GT tissue homogenizer (Kinematica, NY, USA) in SEI

buffer with 0.5% deoxycholic acid (SEID). Samples were spun at 5000 x g for 30 seconds in an Eppendorf 5430R centrifuge (Eppendorf Canada, ON, Canada). To run the assay, two assay mixtures were created: one with ouabain and one without, which was replaced by imidazole buffer. Each well contained 50 mM imidazole, 2.0 mM phosphoenolpyruvate (PEP), 0.5 mM adenosine triphosphate (ATP), 0.16 mM nicotinamide adenine dinucleotide (NADH), 3.3 U mL⁻¹ lactate dehydrogenase, 3.6 U mL⁻¹ pyruvate kinase, and 0.5 mM of ouabain solution (or further imidazole). A salt solution was created according to protocol and added to the assay mixture. Sample (10 µL) was loaded onto the plate along with assay mixtures both with and without ouabain. The assay plate was performed at 25°C, shaken to mix, and read every 10 seconds for 15 minutes at 340 nm using a Spectromax 190 microplate reader (Molecular Devices LLC, CA, USA). Figure 3 shows the coupling of the reagents to be able to detect the loss of NADH as the reaction progresses to be able to track the activity of the NKA. Given that ouabain is a specific inhibitor of the NKA, the activity of this enzyme can be determined by analyzing the difference between the assays run with and without ouabain and standardized by protein content with a BioRad DC assay kit (BioRad Laboratories, CA, USA).

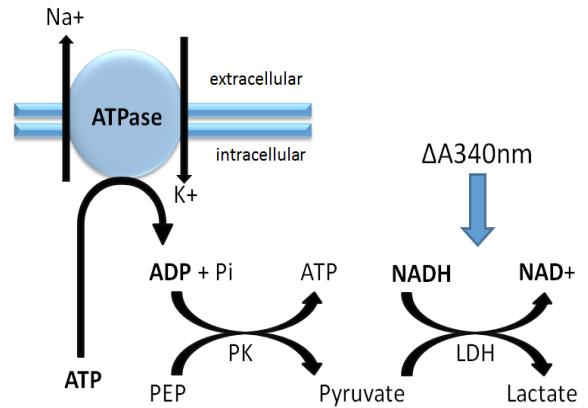


Figure 3: The coupling of reagents to detect the transition of NADH to NAD⁺ which is equivalent to the activity of NKA.

Cortisol Assay

Levels of cortisol in rainbow trout plasma were quantified following Neogen Cortisol ELISA Kit (Neogen, MI, USA). Plasma was combined with ethyl acetate and vortexed in a glass tube. The organic phase (top) was removed and solvent was evaporated using a stream of nitrogen (N₂). Residue was resuspended with the provided diluted extraction buffer and the extract was further diluted 100-fold and vortexed. Samples, along with standards, were all assayed in duplicate. Cortisol standards used included 0, 0.04, 0.1, 0.2, 0.4, 1.0, 2.0, and 10 ng mL⁻¹. The diluted enzyme conjugate was added to the wells, the plate was shaken, covered with Parafilm, and incubated at room temperature for an hour. The plate was washed thrice with diluted wash buffer. Substrate was then added to each well, the plate was shaken, incubated at room temperature for a further 30 minutes, shaken again and read at 650 nm using a Spectromax 190 microplate reader (Molecular

Devices LLC, CA, USA). Analysis of the results was done using online software provided by the company in paid partnership with MyAssays.com.

Malondialdehyde assay

Levels of malondialdehyde (MDA) in gill samples were quantified following OxisResearch Bioxytech MDA-586 assay protocol (OXIS International Inc., CA, USA) that was modified to be used with a microplate. Gill tissue was homogenized 1/4 with homogenization buffer (50 mM Na₃PO₄, 5mM EDTA, 5 mM EGTA, 50 mM β-glycerophosphate, pH 7.2) using a Polytron PT 10-35 GT tissue homogenizer (Kinematica, NY, USA). Homogenate was transferred to a new tube and centrifuged in a Thermo Scientific Heraeus Multifuge X3 FR centrifuge (Thermo Fisher Scientific, MA, USA) at 4°C for 10 minutes at 10 x g. Provided standards consisted of 1,1,3,3-tetramethoxypropane (TMOP) in TRIS-HCl and diluted to the following concentrations: 0.5, 1.0, 2.0, 4.0, 6.7, 8.9, 11.9, 15.8, 21.1, 28.1, 37.5, 50.0 μM. Standards and samples were added to new tubes and probucol, R1 diluted with methanol, and R2 were added in turn and vortexed upon addition. All tubes were heated in block at 45°C for one hour, vortexed, centrifuged under the same conditions. Standards were assayed in duplicate while samples were in triplicate and all were read at 586 nm.

Samples were standardized per unit protein using a Thermo Scientific bicinchoninic acid assay (BCA assay) kit (Thermo Fisher Scientific, MA, USA). Standards ranging from 0.02 to 2 mg mL⁻¹ were prepared and samples were diluted to 1/64 to fit within the range of the standards. Working reagent from the kit was added to the samples and standards were incubated, covered with Parafilm, at 37°C for 30 minutes before being read at 562 nm with a Spectromax 190 microplate reader (Molecular Devices LLC, CA, USA).

Protein Synthesis

Exposure and Tissue Extraction

The fractional rate of protein synthesis was determined using the flooding dose method that was modified to use with a stable isotope tracer (Lamarre et al., 2016). Fish weighed 37.06 ± 10.98 g and were exposed in the same three treatment groups as before; control, 0.22 μg L⁻¹ silver-exposed (as AgNO₃), and 100 μg L⁻¹ nAg-exposed. After two days of being held in coolers in the given conditions, each fish received an intrperitoneal injection of a 150 mM solution of phenylalanine (PHE) composed of 50% ring-D₅ L-phenylalanine (D₅-PHE; Cambridge Isotope

Laboratories, Tewksbury, MA) at a dosage of 1 mL/100 g body mass. The animals were immediately returned to their respective containers following the injection and subsequently sampled approximately 2 hours after the tracer injection. Fish were killed by anesthetic in 0.38 g L⁻¹ buffered MS-222 and severing the spinal cord. Blood samples were collected from the caudal vein using a needle and syringe treated with heparinized modified Cortland saline as previously mentioned. Tissue samples were collected including the brain, heart, liver, white muscle, red muscle, and gill and the blood was centrifuged as above to separate red blood cells from plasma. All samples were frozen in liquid nitrogen and stored at -80°C until further use.

Homogenization

Approximately 0.075 g of tissue was homogenized in 2.0 M perchloric acid (PCA) with a Polytron PT 10-35 GT tissue homogenizer (Kinematica, NY, USA). Samples were centrifuged for 5 minutes at 13 000 rpm and the supernatant, containing the free-pool sample, was collected and stored at -20°C. The remaining pellet was resuspended in PCA and sonicated using the Fisher Scientific Series 60 Sonic Dismembrator Model F60 (Fisher Scientific Company, NH, USA) and centrifuged under the same conditions as previously mentioned and was repeated. Once washed thrice with PCA, pellet was resuspended in acetone using sonicator, spun under the same conditions, and supernatant was discarded. 6 M hydrochloric acid (HCl) was added to the pellet to dislodge it, and the pellet-HCl mixture was transferred to a capped glass tube containing 5 mL of 6 M HCl. The glass tubes were incubated at 110°C overnight in a heating block (Fisher Scientific Company, NH, USA) to hydrolyze peptide bonds.

Solid Phase Extraction

Solid phase extraction is done to purify the sample by separating the free and protein-incorporate phenylalanine pools from the rest of the homogenate using centrifugation and silica gel. For the free-pool samples, 5 M KOH was added to the tubes and left to sit for 3 minutes to allow the perchlorate to precipitate. 6 M HCl was added to the samples and caps were opened repetitively over 3 minutes to allow for gas to escape. Tubes were spun at 13 000 rpm using Eppendorf 5415D centrifuge (Eppendorf Canada, ON, Canada) for 30 seconds.

Both free-pool and protein-pool samples were then passed through silica-containing Bond Elut C18 solid phase extraction (SPE) columns (Agilent Technologies, CA, USA). Columns were activated with 100% methanol (MeOH) followed by 1 M HCl, 250 µL of liver sample or 500 µL

of any other sample, HCl a second time and finally eluted with 30% MeOH into a tube. Samples were left open in heating block at 110°C until all liquid was evaporated and were stored at room temperature.

Derivatization

Distilled water was added to the evaporated samples and tubes were vortexed (Avantor Inc., PA, USA). In a 2 mL gas chromatography-mass spectrometer (GC-MS) identified vial (Agilent Technologies, CA, USA), phosphate buffer (pH 8.0), sample, and pentafluorobenzyl bromide (PFBBBr; 261 mg per 10 mL GC-MS-grade acetone) was added and vortexed. Vials were incubated in a heating block (Fisher Scientific Company, NH, USA) at 60°C for 45 minutes. Once cool, approximately 330 µL of hexane was added to each vial, which were then vortexed again. 200 µL of the organic phase was collected and added in the 250 µL glass inserts (Agilent Technologies, CA, USA) which were placed inside the vials. Samples were read with GC-MS (model 5977B, Agilent Technologies, CA, USA).

Calculating Rates of Protein Synthesis

The equations below were used to calculate the fractional rates of protein synthesis of each tissue. The phenylalanine enrichment was determined by analyzing the ratios between the deuterated and protiated amino acid. The fractional rate was calculated by comparing the protein and free pool of each sample and standardized by accounting for the duration of the isotope incorporation period after the injection.

Phenylalanine enrichment	=	$\frac{[D5-Phe]}{[D5-Phe]+[Phe]}$	<i>free & protein pool</i>
Fractional rate of protein synthesis	=	$\left[\left(\frac{\text{protein pool}}{\text{free pool}} \right) \times \frac{1440}{\text{time (min)}} \right] \times 100\%$	

Statistical Analyses

Statistical analyses were performed using PRISM5 software (GraphPad Software, San Diego, California, USA). One-way ANOVAs were conducted on all data sets to determine significant differences between treatment groups. P values below 0.05 are considered significant and below 0.1 are indicative of a trend. Results are represented as mean \pm standard error of the mean.

Results

Biochemical Stress Indicators

Hematocrit

The hematocrit was tested on the blood samples of each fish used and no difference of statistical significance ($p = 0.154$). These results, as seen in Figure 4, indicate that the ENMs have a limited effect on the amount of red blood cells and thus the oxygen carrying capacity of the samples following a 48-hour exposure. Hematocrit results seen here are highly comparable to results found by Imani et al. (2015) who found that the average rainbow trout hematocrit fell around 40%. In that study, fish were exposed to different concentrations of silver ENMs; 0.1 mg L^{-1} , 0.2 mg L^{-1} , and 0.4 mg L^{-1} and the only statistically significant difference seen was after 8 days at the highest concentration. Further research conducted by Webb and Wood (1997) looked at the effects of $9.2 \text{ } \mu\text{g L}^{-1}$ ionic silver on rainbow trout and also found that there was no significant difference in hematocrit values between Ag-exposed and control fish.

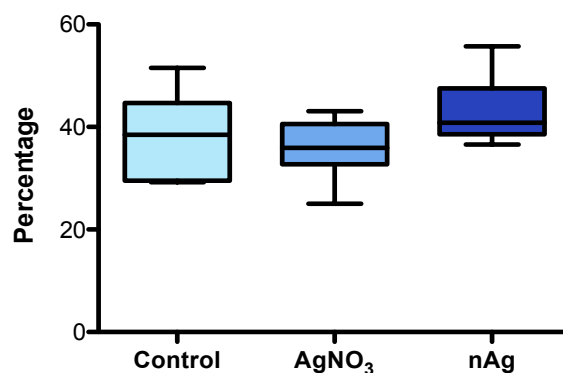


Figure 4: Hematocrit content (percentage) determined from centrifuged blood samples of *O. mykiss* for control and after 48-hour exposure to $0.22 \text{ } \mu\text{g L}^{-1}$ AgNO₃ and $100 \text{ } \mu\text{g L}^{-1}$ nAg. Mean values \pm SEM are shown with sample sizes, left to right, of $n=6$, $n=8$, and $n=8$, respectively. No difference of statistical significance was observed.

Acetylcholinesterase Activity

Activity of heart AChE activity was greatly varied between samples. Although triplicates remained tight, samples from fish in each group had large variability yielding variations in data that is not significantly different ($p = 0.320$). These results, as seen in Figure 5, indicate that nAg did not have an inhibitory effect on this enzyme's activity in the heart. Limited literature exists on heart AChE activity in rainbow trout; however, research done by Tierney et al. (2007) found that the activity of brain acetylcholinesterase of a different salmonid (coho salmon) ranged from 50-150 $\text{nmol mg}^{-1} \text{min}^{-1}$. Although higher, results are similar, and differences may be attributed to differences in tissues and species. Katuli et al. (2014) found that gill acetylcholinesterase in zebrafish was significantly impacted by 0.2 mg L^{-1} silver ENMs after just 7 days, but concentration and duration were significantly longer than exposure conditions used in this study.

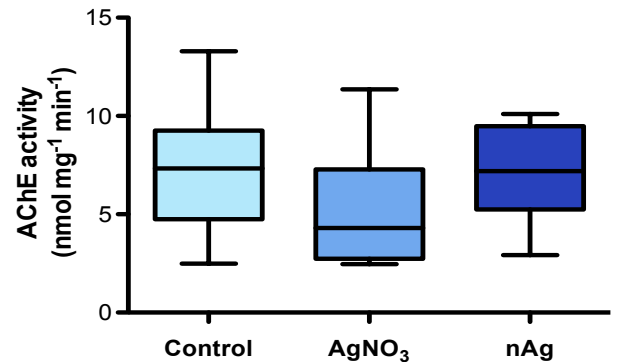


Figure 5: Acetylcholinesterase activity ($\mu\text{mol mg}^{-1} \text{min}^{-1}$) in heart tissue of *O. mykiss* for control and after 48-hour exposure to 0.22 $\mu\text{g L}^{-1}$ AgNO₃ and 100 $\mu\text{g L}^{-1}$ nAg. Mean values \pm SEM are shown, with each sample size of $n=8$. No difference of statistical significance was observed.

Sodium Potassium ATPase Activity

Data obtained from this project shows that there is a trend that nAg influences the activity of gill sodium potassium ATPase (NKA; $p = 0.081$). Results, as seen in Figure 6, show the trend towards less activity in samples exposed to nAg as well as ionic silver. Adams et al. (1973) found that the NKA activity of steelhead trout at 15°C ranged from approximately 10 to 20 $\mu\text{mol mg}^{-1} \text{hr}^{-1}$; highly comparable to our results. Research conducted by Schultz et al. (2012) done on juvenile rainbow trout found that 1 mg L^{-1} citrate-capped silver ENMs had a significant effect in decreasing NKA activity. Further research done by

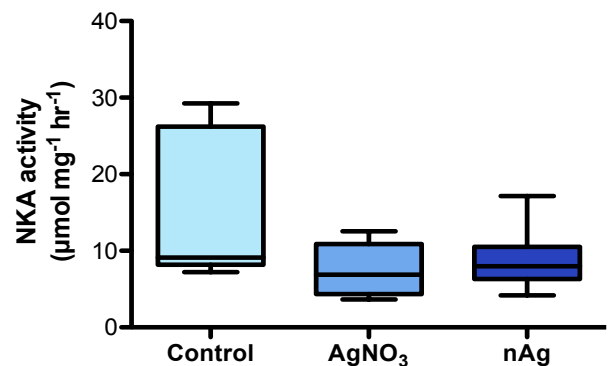


Figure 6: Sodium potassium ATPase (NKA) activity ($\mu\text{mol mg}^{-1} \text{hr}^{-1}$) in gill tissue of *O. mykiss* for control and after 48-hour exposure to 0.22 $\mu\text{g L}^{-1}$ AgNO₃ and 100 $\mu\text{g L}^{-1}$ nAg. Mean values \pm SEM are shown, with each sample size of $n=8$. A trend in decreased NKA activity caused by nAg can be seen however no difference of statistical significance was observed.

Katuli et al. (2014) found that after 21 days of exposing zebrafish to 2 mg L⁻¹ of silver ENMs, significant decreases in NKA activity also resulted.

Cortisol Assay

Although the duplicates were tight, the concentration of plasma cortisol varied considerably between fish. Samples ranged from 5.1 to 48.5 ng mL⁻¹ yielding no significant differences between groups ($p = 0.438$). Results, as seen in Figure 7, indicate that the level of stress of each fish was not dependent on the exposure condition. Cortisol is a measure of general stress and studies conducted by Katuli et al. (2014) on zebrafish exposed to 2 mg L⁻¹ of silver ENMs found plasma cortisol levels significantly increased after 7 days. Research done by Webb and Wood (1997) looked at the effects of 9.2 µg L⁻¹ ionic silver on cortisol levels in rainbow trout and found that Ag-exposed fish to showed a continual increase in cortisol levels until significantly different from the control group by day 4. Finally, an experiment done by Johari et al. (2013) analyzing the plasma cortisol content in juvenile rainbow trout concluded that there is a significant difference between the control and fish exposed to 1 mg L⁻¹ after just 3 hours.

Malondialdehyde

Measuring cellular injury as an indicator of oxidative stress in gill samples revealed (Figure 8) no significant differences between treatment groups ($p = 0.787$). Studies done by Scown et al. (2010) found that the concentration of gill MDA was significantly decreased

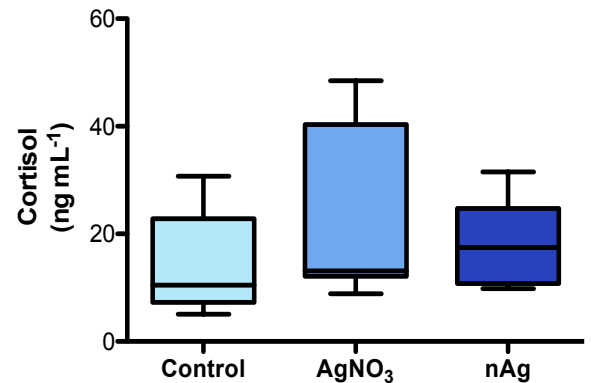


Figure 7: Concentration of cortisol (ng mL⁻¹), a physiological stress indicator, in plasma of *O. mykiss* for control and after 48-hour exposure to 0.22 µg L⁻¹ AgNO₃ and 100 µg L⁻¹ nAg. Mean values ± SEM are shown with sample sizes, left to right, of n=6, n=8, and n=8, respectively. No difference of statistical significance was observed.

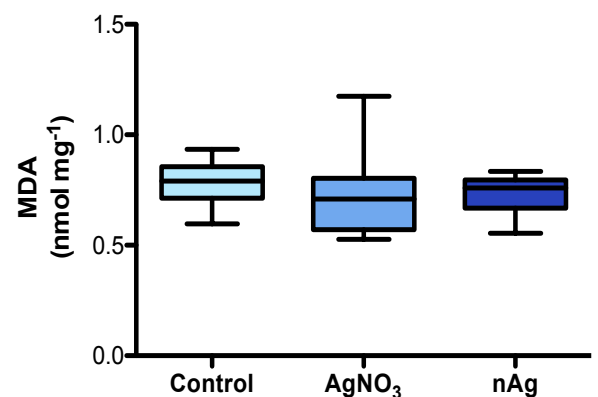


Figure 8: Malondialdehyde content (nmol mg⁻¹ of protein), an indicator of cellular stress, in gill tissue of *O. mykiss* for control and after 48-hour exposure to 0.22 µg L⁻¹ AgNO₃ and 100 µg L⁻¹ nAg. Mean values ± SEM are shown, with each sample size of n=8. No difference of statistical significance was observed.

after rainbow trout were exposed to $100 \mu\text{g L}^{-1}$ of 10 nm of nAg for 10 days. Fish exposed for the same duration to 35 nm at $100 \mu\text{g L}^{-1}$, $10 \mu\text{g L}^{-1}$ nAg at both 10 nm and 35 nm, $100 \mu\text{g L}^{-1}$ bulk ENMs (ranging from 600 to 1600 nm), and $0.1 \mu\text{g L}^{-1}$ AgNO_3 all resulted in not-significant differences of MDA levels. Research done by Bessemer et al. (2015) found that MDA levels were significantly increased in gill tissue for white suckers (*Catostomus commersonii*) after being exposed to 1.0mg L^{-1} zinc oxide ENMs (nZnO) for 25 hours.

Protein Synthesis

Eight different tissues were measured to determine differences in fractional rates of protein synthesis (K_s in % per day) and results (Figure 9) showed no significant differences in rates between control, ionic silver, and nAg-exposed groups. P-values for tissues are as follows; heart $p = 0.316$, liver $p = 0.266$, gill $p = 0.245$, white muscle $p = 0.524$, red muscle $p = 0.882$, brain $p = 0.912$, red blood cell $p = 0.740$, and plasma $p = 0.980$. Research conducted by Houlihan et al. (1986) on rainbow trout found similar K_s values for common tissues tested including gill (9.07 ± 0.57 % per day), red muscle (1.25 ± 0.09 % per day), and white muscle (0.49 ± 0.04 % per day).

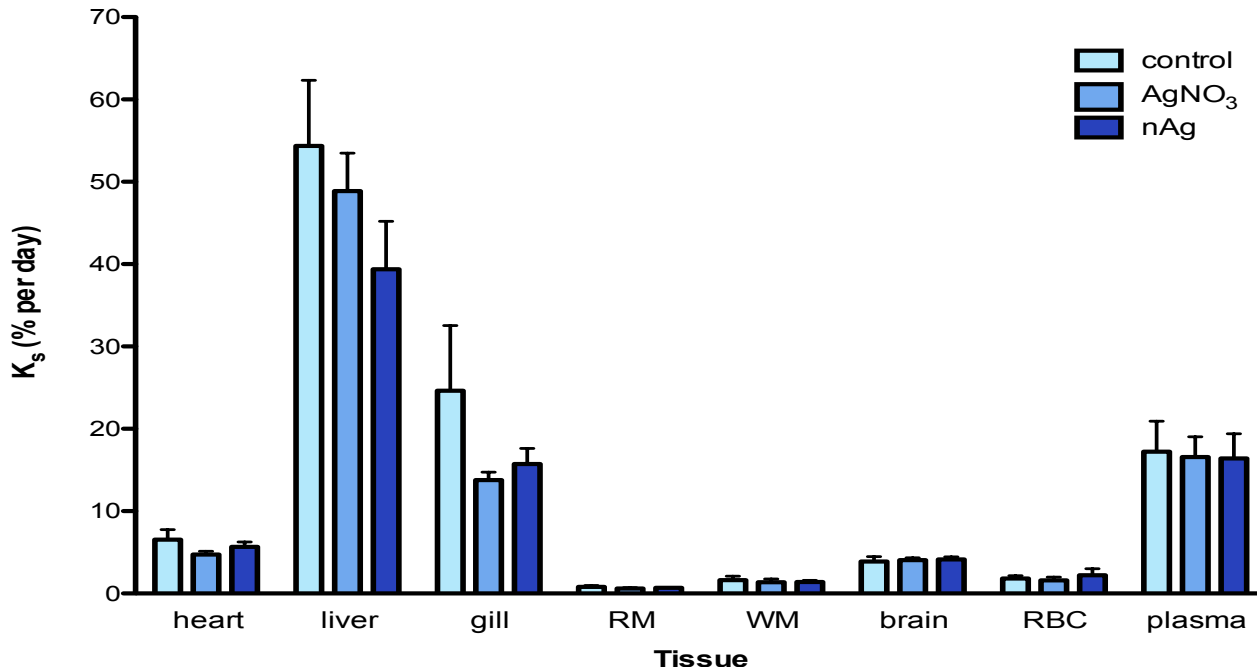


Figure 9: Fractional rate of protein synthesis (K_s in % per day) in *O. mykiss* for control and after 48-hour exposure to $0.22 \mu\text{g L}^{-1}$ AgNO_3 and $100 \mu\text{g L}^{-1}$ nAg. Various tissues were sampled including heart, liver, gill, red muscle (RM), white muscle (WM), brain, red blood cells (RBC), and plasma. Mean values \pm SEM are shown, with each sample size of $n=6$ (WM control $n=5$). No difference of statistical significance was observed.

Discussion

The purpose of this study was to determine the potential toxicity of PVP-capped nAg in rainbow trout to determine whether any discernable biochemical changes were observable after a 48-hour exposure to the environmentally relevant point-source exposure concentration of $100 \mu\text{g L}^{-1}$. The data illustrated that there were no statistically significant differences between treatment groups, with rainbow trout exhibiting no signs of significant damage nor of a decrease in protein synthesis.

Biochemical Stress Indicators

No significant changes in hematocrit were observed in fish exposed to ionic or nanoparticulate silver (Figure 4). These results indicate that neither AgNO_3 nor nAg exposure had an effect on oxygen carrying capacity under the conditions tested. Research by Imani et al. (2015) on trout found that the hematocrit averaged around 40%, which is comparable to these results. That experiment exposed fish to 0.1 mg L^{-1} which were sampled after 4 and 8 days, both with no changes in hematocrit. Furthermore, Webb and Wood (1997) determined that exposure to $9.2 \mu\text{g L}^{-1}$ ionic silver did not affect hematocrit in rainbow trout. During acute silver toxicity situations, silver ions are lost from the extracellular compartment faster than from the intracellular compartment, resulting in a compensatory fluid shift into the tissues and decreases in blood volume (reviewed by Wood et al., 2012). This causes hematocrit concentrations to rise and because this was not observed in this experiment, it is likely that the fish were not experiencing this acute silver toxicity.

AChE is critical in termination of neuronal signaling and control of muscle contraction, so the survival of an organism is dependent on the regulated functioning of this enzyme. The activity of this enzyme is frequently used as a biological marker of toxicity for various reasons including that it shows a dose-dependent behaviour to pollutant exposure, it is sensitive, and it exhibits links to adverse health effects (Lionetto et al., 2013). Wang et al. (2009) analyzed the effects of various ENMs (SiO_2 , TiO_2 , Al_2O_3 , Al, Cu, carbon-coated copper, multi- and single-walled carbon nanotubes) on AChE activity and found that they exhibited a high affinity for the enzyme, causing inhibition through adsorption or interaction with AChE. Since nAg did not impact heart AChE levels, it is unlikely that cardiac function was impacted during exposure.

Depending on the species, exposure concentration, and tissue, studies have found different effects of ENMs on AChE activity. Tierney et al. (2007) found a correlation between swimming

performance and AChE activity in the brain of coho salmon exposed to the toxicant *chlorpyrifos*; an organophosphate insecticide. It was concluded that AChE activity decreased in a concentration-dependent manner whereas swim performance was impaired once a threshold of AChE impairment was reached. It can thus be inferred that either nAg does not affect this enzyme or that the levels of nAg in this study were not sufficient to alter the levels of AChE activity. If AChE activity decreases and crosses threshold levels for rainbow trout, it may result in potentially life-threatening acetylcholine buildup leading to swimming impairment, heart rate abnormalities, or increases in cardiovascular and respiratory responses causing long-term damage (Čolović et al., 2013).

Heart AChE activity was studied in this experiment as white suckers (*Catostomus commersonii*) exposed to 1 mg L⁻¹ nZnO showed a significant decrease in heart AChE activity (Callaghan et al., 2016) and a 35% decrease in heart rate (Bessemmer et al., 2015). It was hypothesized that a decreased heart rate would cause fish to depress their metabolism, possibly by lower rates of protein synthesis. The mechanism by which heart rate slows has not been fully elucidated, however, there are two proposed possibilities. Bessemmer et al. (2015) suggests that nZnO exposure may impact gill chemoreceptor function which, in teleosts, consist of independent, differentially innervated clusters of neuroepithelial cells (NECs) that detect environmental or arterial O₂ partial pressures. The receptors are involved with regulating heart and ventilation rates as part of the hypoxia response. When gills are exposed to the ENMs causing significant interactions or damage, this may induce a pseudo-hypoxic response causing the depolarization of NECs and the release of acetylcholine at the sinoatrial node of the heart, inducing bradycardia (Callaghan et al., 2016). The second method involves the accumulation of acetylcholine due to the depression of heart AChE activity. By reducing the rate of enzymatic degradation, acetylcholine will begin amassing in the heart and will stimulate inhibitory muscarinic acetylcholine receptors, slowing the heart rate (Callaghan et al., 2016). While heart rate was not assessed in this study, heart AChE activity was unaffected in fish exposed to nAg so it is unlikely that ACh buildup caused heart rate depression.

Based on the results seen in Figure 6, nAg and AgNO₃ tended to decrease the activity of gill NKA. Because previous studies conducted on the PVP-capped silver ENMs showed that little ionic silver is released, this trend seen in NKA activity is likely nano-specific. It is well known that ionic silver is highly toxic as the ions have a tendency to bind to the Mg⁺ cofactor binding sites in the enzyme which prevents the proper hydrolysis of ATP and consequently non-

competitively inhibits the enzyme and disrupts the osmoregulation of the fish (McGeer and Wood, 1998). With the inhibition of this critical ionoregulatory enzyme, ion levels can be altered and cause significant damage to the fish. As mentioned, when ions are lost from the extracellular compartment faster than the intracellular compartment, the fluid shift causes severe changes in the body. The blood viscosity and pressure rise, causing the release of stress hormones and accelerated heart rate leading to the release of erythrocytes from the spleen and the promotion of systemic vasoconstriction. The fish will eventually die of hypovolemic, hypertensive cardiovascular failure due to the disturbance of Na^+ and Cl^- (reviewed by Wood et al., 2012). Because changes in NKA activity or hematocrit were not observed, the dose used in this experiment was likely not sufficient to cause this interference.

It should be noted that differences in the characteristics of the exposure water may have impacted the levels of ionic silver and nAg toxicity. Several studies have been conducted on the matter as water chemistry, including hardness, pH, and ion-richness, can impact results (reviewed by Wood et al., 2012). The factors contributing to the water chemistry may offer some protection against toxicity, more strongly against acute toxicity than chronic tests. Depending on how the water chemistry of the water used for this experiment differed from similar experiments in other locations, it is possible that some protection from toxicity could have been offered. Data from this experiment suggests that at the tested concentration, exposure duration, and with this water chemistry, 5 nm nAg are not substantially impacting this critical enzyme.

Cortisol content is a measure of general stress in an animal and results show that this dose of silver and silver ENMs does not induce a significant change in cortisol levels. Katuli et al. (2014) working with zebrafish found that 2 mg L^{-1} nAg only increased cortisol significantly after 7 days. Webb at Wood (1997) found that plasma cortisol levels only increased after 4 days in rainbow trout exposed to $9.2 \text{ } \mu\text{g L}^{-1}$ ionic silver. In both circumstances, the concentration of the dose and the length of exposure were greater than in this experiment and it is therefore unsurprising that these conditions elicited no effects.

Previous research has shown variances in lipid peroxidation levels and increased oxidative stress as a result of nAg exposure in fish (Christen et al., 2013; Gagné et al., 2012). Oxidative stress is a common mechanism of ENM toxicity and was measured in this study with a gill MDA assay. The differences, as seen in Figure 8, were not statistically significant. Bessemer et al. (2015) found that gill MDA levels were significantly increased in fish exposed to 1 mg L^{-1} nZnO for 25

hours. Scown et al. (2010) found that rainbow trout exposed to various doses and sizes of nAg for 10 days showed no significant differences of MDA content, with the exception of significantly lower levels of gill MDA when exposed to 10 nm nAg at 100 $\mu\text{g L}^{-1}$. In the current experiment, the fish were exposed to the same concentration as Scown et al. (2010) for considerably less time and this could have had an influence on the level of oxidative stress in the gills. These results show that for short periods of time, the 5 nm nAg may not cause significant oxidative stress on the gills of the fish but this result does not necessarily apply for other types of ENMs.

Protein Synthesis

Campbell et al. (2017) looked at the effects of 80 $\mu\text{g L}^{-1}$ nAg on killifish (*Fundulus heteroclitus*) and found a decrease in the minimum and maximum oxygen consumption rates. This decrease in oxygen consumption demonstrated that the exposure was causing a whole organism response induced by exposure to the ENMs. Because killifish are small, weighing only a few grams, this research was conducted on rainbow trout (37 \pm 11 g) to be able to sample other tissues and more accurately measure rates of protein synthesis. As mentioned previously, nZnO has been shown to affect the respiration and heart rates of white suckers, potentially influencing the metabolism (Bessemmer et al., 2015; Callaghan et al., 2016). It was hypothesized that similar results would also be observed in rainbow trout. This was the premise of the study as a decrease in oxygen consumption or heart rate may be due to a regulated decrease in fractional rates of protein synthesis. Alternatively, a decrease in metabolic rate initiated in some other way could limit energy availability and force protein synthesis to slow.

No statistically significant differences were found in fractional rate of protein synthesis (K_s % per day) in the eight rainbow trout tissues assessed (Figure 9). Values obtained were comparable to those found in another rainbow trout protein synthesis experiment conducted by Houlihan et al. (1986), indicating validity of the data. Results from this ENM study show that rainbow trout exposed for 48 h to 5 nm nAg at 100 $\mu\text{g L}^{-1}$ do not decrease rates of protein synthesis, consistent with concurrent studies in the lab showing the maintenance of aerobic metabolic rate in this species under these conditions.

Farkas et al. (2010) found significant decreases in metabolic activity in rainbow trout after being exposed to ionic silver and silver ENMs for 48 hours at concentrations ranging from 1.9 mg L^{-1} to 19 mg L^{-1} ; far more concentrated than ENM levels assessed in this experiment. The

concentrations or exposure durations used in the current experiment were likely not high or long enough to significantly impact metabolism or protein synthesis in these animals. Further studies testing longer durations of exposure and elucidating signaling pathways involved in fluctuating protein synthesis rates should be conducted for a more complete overview of the potential toxicity of 5 nm PVP-capped silver ENMs on rainbow trout.

Conclusion

In this study, the effects of exposing rainbow trout to nAg were studied while simultaneously being compared to control and ionic silver groups. While there was a trend of decreased sodium-potassium ATPase activity for fish exposed to ionic and nanoparticulate silver, all other biological endpoints were unaffected. This research indicates that at the concentration and duration tested, nAg likely has few effects on rainbow trout and that these animals may survive in nAg-contaminated waters. Although this is positive news, wastewater must continue to be treated and managed to ensure concentrations do not rise to lethal amounts. Furthermore, other ENM formulations with different core materials or nAg with alternate capping agents may exhibit different effects. Many other experiments have indicated that higher concentrations of ENMs or exposures for longer durations can lead to detrimental effects on fish and it is therefore the responsibility of government, water treatment facilities, and consumers to mitigate these effects to help with the survival of native flora and fauna in our water systems.

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