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Immunotoxicology of titanium dioxide and hydroxylated fullerenes engineered nanoparticles in fish models

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Immunotoxicology of titanium dioxide and hydroxylated fullerenes engineered
nanoparticles in fish models

by

Boris Jovanović

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Co-majors: Toxicology; Fisheries Biology

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2011

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This thesis is dedicated to Dr. Evrim Baran as acknowledgement of hers invaluable and unconditional support, tolerance and love, and without whom this thesis would ever evolved to its present state.

Auguries of Evolution*

By Boris Jovanović – January 14th 2010

I have searched the mountain top,
Looked at every single drop
Both in drizzle and in ocean -
Searching with extreme devotion,
Twenty years and even more -
Cherub nymph to adore.

I have crept in every cave,
Looked beneath the highest wave;
East and West, close and far,
Searched at every Turk bazaar
For a very special star.
Twenty years and even more -
Cosmic star to adore.

I have crossed the desert sand,
Barren moors and open land.
I took a walk amidst the clouds,
Between the quiet and the louds.
I have searched in midnight news
Any hint to find my muse.
Twenty years and even more -
Little muse to adore.

Then I saw her, I was stunned,
She was standing beneath the sun.
Playing scrabble in the shade,
Wearing necklace made of jade.
Graceful as a songbird lark,
Profound eyes with wondrous spark.
Little cunning moving lips,
And angelic complete hips.

For those lips, I would swallow burning coal,
Sold to devil my pity soul.
Cross all mountains till Middle-East
Fight Hashishins and a deadly beast.
Bring the golden rose souvenir
On my journey to Izmir –
Ancient Smyrna if you please
Just to squat on my knees.
Kiss her hand, rub her feet
To offer my love – complete.

If no more, for all the way I have come,
I deserve to know thy name?
Evolution.
Evolution she was called, Evolution was her name;
Invigorating an ancient flame
And I knew that she will ever
In my heart be forever.

* In Turkish language the word for Evolution is Evrim

"To see a world in a grain of sand
And a heaven in a wild flower,
Hold infinity in the palm of your hand,
And eternity in an hour."

William Blake (1757 - 1827)

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ABSTRACT

Nanoparticles have the potential to cause adverse effects on the fish health, but the understanding of the underlying mechanisms is limited. Major task of this dissertation was to connect gaps in current knowledge with a comprehensive sequence of molecular, cellular and organismal responses toward environmentally relevant concentrations of engineered nanoparticles (titanium dioxide – TiO₂ and hydroxylated fullerenes), outlining the interaction with the innate immune system of fish.

The research was divided into following steps: 1) create cDNA libraries for the species of fathead minnow (*Pimephales promelas*); 2) evaluate whether, and how can nanoparticles modulate neutrophil function in *P. promelas*; 3) determine the changes in expression of standard biomarker genes as a result of nanoparticle treatment; 4) expose the *P. promelas* to nanoparticles and appraise their survival rate in a bacterial challenge study; 5) assess the impact of nanoparticles on neuro-immunological interface during the early embryogenesis of zebrafish (*Danio rerio*). It was hypothesized that engineered nanoparticles can cause measurable changes in fish transcriptome, immune response, and disease resistance.

The results of this dissertation are: 1) application of environmentally relevant concentration of nanoparticles changed function of fish neutrophils; 2) fish exposed to nano-TiO₂ had significantly increased expression of interleukin 11, macrophage stimulating factor 1, and neutrophil cytosolic factor 2, while expression of interleukin 11 and myeloperoxidase was significantly increased and expression of elastase 2 was significantly decreased in fish exposed to hydroxylated fullerenes; 3) exposure to environmental estimated concentration of nano-TiO₂ significantly increased fish mortality during *Aeromonas hydrophila* challenge. Analysis of nano-TiO₂ distribution in fish organism outlined that the nano-TiO₂ is concentrating in the fish kidney and spleen; 4) during the early embryogenesis of *D. rerio* exposure to nanoparticles caused shifts in gene regulation response patterns. Significant effects on gene regulation were observed on genes involved in circadian rhythm, kinase activity, vesicular transport and immune response.

CHAPTER 1. GENERAL INTRODUCTION

1.1. Introduction

Recent expansion of the nanotechnology use in industry and households is followed by increased introduction of various engineered nanoparticles to aquatic environments (Nowack and Bucheli, 2007). Nanoparticles have the potential to cause adverse effects on the fish immune system and health, but the understanding of the underlying mechanisms is limited. The major gaps in current knowledge can be addressed with a comprehensive sequence of studies to connect molecular, cellular and organismal responses in otherwise well described and environmentally relevant animal model – the fathead minnow (*Pimephales promelas* Rafinesque, 1820).

The major scientific problem addressed in this dissertation is: Are engineered nanoparticles (titanium dioxide and hydroxylated fullerenes) immunotoxic to fish and whether they can cause changes in the neutrophil function resulting in significantly lower chances of fighting a bacterial infection.

P. promelas is a small (<10 cm) cyprinid species, abundant in North American freshwater ecosystems and frequently used as standard model organism in environmental and aquatic toxicology assessments and studies (USEPA, 1987; Ankley and Villeneuve, 2006). Recently, *P. promelas* was also used as a model to study the innate immune responses and immunomodulators (Palić et al., 2006). The benefits of using *P. promelas* as a model include easy and fast isolation of neutrophils from anterior kidney (Palić et al., 2005a) existence of standardized assays of monitoring the neutrophil function for this species (Palić et al., 2005b) and a tolerance of wide range of basic water quality characteristics (Ankley and Villeneuve, 2006).

The overall hypothesis of the dissertation was: Engineered nanoparticles (titanium dioxide and hydroxylated fullerenes) can cause measurable changes in fish transcriptome, immune response, and disease resistance.

To achieve the goal and test the hypothesis several specific aims were developed:

Specific aim 1. Create a pool of cDNA libraries for *P. promelas*. Although the *P. promelas* is the most used fish species in aquatic toxicology, there were no attempts to sequence its genome. The current expressed sequence tag (EST) records for *P. promelas* at the National Center for Biotechnology Information (NCBI) have reached nearly a quarter of a million with nearly all of the ESTs coming from a US Department of Energy Joint Genome Institute *P. promelas* EST project (Richardson et al., unpublished), but they are still not annotated and therefore for the most part useless. Furthermore, all ESTs come from unstimulated tissues, and EST libraries may be lacking important pathogen associated molecular patterns (PAMP) regulated genes that are observed during simulated disease challenges. Therefore, as explained in chapter 3, *P. promelas* was stimulated with lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid Poly(I:C) in an effort to isolate immune-related genes from cDNA libraries using an EST approach.

Specific aim 2. Determine the sublethal dose for nanoparticles of interest in *P. promelas* (to be used in specific aims 3, 4 and 5) using acute aquatic toxicity tests. Sublethal concentrations of engineered nanomaterials were determined using Fish Embryo Toxicity Assay (FET), Fish Acute Toxicity Test and a series of intraperitoneal injections.

Specific aim 3. Determine effects of engineered nanoparticles on neutrophil function. A comprehensive immunological and clinical assessment was used to determine effects of nanoparticle exposure on responses of *P. promelas* adults. An array of cellular innate immune responses (degranulation of primary granules, neutrophil extracellular trap release, and respiratory burst) was used to assess the phagocytic cell function both *in vitro* and *in vivo* using the concentrations from the specific aim 2.

Specific aim 4. Determine the impact of nanoparticles on immunogenomics of *P. promelas* and establish a set of biomarkers to be used in evaluation of nanoparticle effects on fish health. Analysis of gene expression response was performed (using the data from specific aim 1) and genes with potential to be used as biomarkers of nanoparticle exposure were identified.

Specific aim 5. Evaluate the impact of nanoparticles on immune response during the bacterial challenge. The survivability of *P. promelas* was assessed after exposure to *Aeromonas hydrophila* bacterial pathogens with or without the presence of nanoparticles (nanoparticle concentrations were identified in specific aim 3 as the concentrations that were changing neutrophil function).

Specific aim 6. Determine the effects of titanium dioxide and hydroxylated fullerene nanoparticles on neuro-immunological interface during the early embryogenesis of fish. Zebrafish (*Danio rerio* Hamilton, 1822) embryos were exposed by microinjection technique to nanoparticle concentrations identified in specific aim 3.

1.2. Dissertation organization

The dissertation is organized in the alternative format, including six manuscripts that have been published, accepted for publication, or are in review. Each of the manuscripts is represented as a separate thesis chapter in the form prepared for publication:

1. Jovanović, B., Palić, D. (in review). Immunotoxicology of non-functionalized engineered nanoparticles in aquatic organisms – a review of current knowledge. *Ecotoxicology*.
2. Jovanović, B., Goetz, F.W., Goetz, G.W., Palić, D. (2011). Different immunological stimuli change expression of genes in fathead minnow (*Pimephales promelas* Rafinesque, 1820). *Journal of Fish Biology* 78 (4), 1054-1072.
3. Jovanović, B., Anastasova, L., Rowe, E., Palić, D. (2011). Effects of nanosized titanium dioxide on innate immune system of fathead minnow (*Pimephales promelas* Rafinesque, 1820). *Environmental Toxicology and Safety* 74, 675-683.
4. Jovanović, B., Anastasova, L., Rowe, E., Palić, D. (2011). Hydroxylated fullerenes inhibit neutrophil function in fathead minnow (*Pimephales promelas* Rafinesque, 1820). *Aquatic Toxicology* 101, 474-482.

5. Jovanović, B., Kimura, K., Palić, D. (in review). Titanium dioxide nanoparticles increased mortality of fish exposed to bacterial pathogen. *Aquatic Toxicology*.
6. Jovanović, B., Ji, T., Palić, D. (In press). Gene expression of zebrafish embryos exposed to titanium dioxide nanoparticles and hydroxylated fullerenes. *Environmental Toxicology and Safety*. doi:10.1016/j.ecoenv.2011.04.012.

In addition to six manuscript chapters, a chapter with a general introduction as well as a closing chapter with conclusions and recommendations for future research is included in the dissertation. References are listed at the end of each chapter.

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CHAPTER 2. LITERATURE REVIEW - Immunotoxicology of non-functionalized engineered nanoparticles in aquatic organisms – review of current knowledge

A paper submitted to Ecotoxicology

Boris Jovanović and Dušan Palić

Boris Jovanović is the first and corresponding author of this paper.

2.1. Abstract

The rapid increase in nanotechnology products is constantly increasing the presence of metal, metal-oxide and carbon-based nanoparticles in the aquatic environment. These non-functionalized engineered nanoparticles can easily interact with the immune system of fish and invertebrates and tip the ecological balance of population sustainability, possibly driving some species toward extinction. Most nanoparticle types that already are highly concentrated in the aquatic environment, such as titanium dioxide, do not exhibit or have very low direct toxicity but instead display silent, concealed sub-lethal effects on the immune system with serious implications. There is a gap in current available information regarding the immunotoxic potential of engineered nanoparticles toward aquatic organisms. Due to the fact that aquatic animals live their entire lives surrounded by nanoparticles suspended in water thus being chronically exposed to nanoparticles, the immunotoxicological effects of nanoparticles are even of greater concern than for terrestrial or semi-terrestrial species. Therefore there is a critical need to address this major gap and provide the first comprehensive review of the effects of engineered non-functionalized nanoparticles on the immune system of aquatic animals.

Keywords: Fish, nanoparticles, titanium dioxide, fullerenes, innate immunity, immunotoxicity

2.2. Introduction

The application of nanotechnology in various fields, such as biomedical science and the electronic, cosmetic and pharmaceutical industries, is likely going to increase the release of nanoparticles into the environment in the near future. Nanotechnology is overtaking the market at an increasing pace; it has been estimated that nanotechnology was “incorporated into US\$30 billion [worth of] manufactured goods in 2005” (Lux Research, 2006). By 2015 the worldwide market for products with nanotechnology will reach its first trillion dollars (Roco, 2005). EU and U.S. regulatory agencies are supporting the industry’s development and, with limited progress in developing regulations regarding nanoparticle release into the environment, a clear position on the safety of nanotechnology has not yet been firmly established (EPA, 2007; EPA, 2008).

In the meantime, nano-products are rapidly accumulating in the aquatic environment (Nowack and Bucheli, 2007) and their potential for exhibiting environmental toxicity is growing. Several recent review articles dealing with nanoparticle toxicity to aquatic organisms focus on nanoparticle absorption, distribution, metabolism and excretion by hydrobiota as well as ecotoxicological potential. The majority of the review articles indicate a gap in current knowledge and missing information about the immunotoxic potential of engineered nanoparticles toward aquatic organisms (Handy et al., 2008a; Handy et al., 2008b; Handy et al., 2008c; Klaine et al., 2008; Moore, 2006). Recent reviews (Dobrovolskaia et al., 2008; Dobrovolskaia and McNeil, 2007) have presented a discussion of the nanoimmunotoxicological information regarding human beings and other mammals but not aquatic animals such as fish and other lower vertebrates or invertebrates. Because aquatic animals could experience their entire life cycle in the presence of nanoparticles suspended in water, the immunotoxicological effect of nanoparticles presents a significant concern, even greater than for terrestrial or semi-terrestrial species. We will use current literature in an attempt to address the identified knowledge gap and provide the first comprehensive review of the effects of engineered nanoparticles on the immune system of aquatic animals.

The EPA has classified nanomaterials into four major types and defined them as: (1) Carbon-based materials, such as fullerenes and carbon nanotubes, that are composed almost entirely of carbon; (2) Metal-based materials, including quantum dots, nano-gold and nano-silver and metal-oxides, such as titanium-dioxide; (3) Dendrimers or nano-sized polymers built from branched units that contain interior cavities in which other molecules can be placed and are exploited for drug delivery; and (4) Composites such as nanoclay or titanium–DNA combinations between two or more nanoparticles or between a nanoparticle and a bulk-type material (EPA, 2007). Dendrimers, composites and quantum dots can be easily functionalized with various chemical groups or compounds, therefore creating an endless variety of themes of the same nanoparticle with completely different properties and different effects toward immune system. Because dendrimers, composites and quantum dots are almost nonexistent in the aquatic environment and apply mainly to biomedical research, this review will not focus on their potential effects on aquatic life. We will focus on non-functionalized metallic, metal-oxide and carbon-based nanoparticles, which have significant potential for causing ecotoxicological concerns in aquatic ecosystems and are potent modulators of immune system responses using similar mechanisms despite the difference in their chemical composition.

Among all nanoparticles, titanium dioxide nanoparticles (nano-TiO₂) are the biggest ecotoxicological concern due to the rapid increase of anthropogenic input into the environment. Estimated environmental concentrations of nano-TiO₂ are in the range from 0.0007 to 0.0245 µg mL⁻¹ (Mueller and Nowack, 2008; Pérez et al., 2009). Nano-TiO₂ has the most industrial applications compared to any other nanoparticle. It is used as a major constituent of sunscreens, soaps, shampoos, toothpastes and other cosmetics (Melquiades et al., 2008) and in paper products, plastics, ink and food as a food color (Ortlieb, 2010). Regulations in the USA allow nano-TiO₂ as the only inorganic UV filter, besides nanozinc-oxide, to be used in the cosmetic industry with a maximum product content of 25% (FDA, 1999). Four million tons of nano-TiO₂ are consumed annually worldwide just in the form of pigment (Ortlieb, 2010). Nano-TiO₂ is also used as an additive in paint and building materials (Chen and Poon, 2009), from which it easily leaches and is transported to aquatic ecosystems (Kaegi et al., 2008). As an ingredient in food products nano-TiO₂ is often listed

as E171 and commonly is used for whitening skim milk (Ortlieb, 2010). Many synthetic vitamin tablets and over-the-counter pain relief drugs also contain nano-TiO₂ (Luft et al., 2010) as do numerous capsulated antidepressants and antibiotic products. Nano-TiO₂ is already being piloted for use as an additive of drinking water in water treatment plants, and the EPA is considering a full-scale usage of nano-TiO₂ in water treatment plants for removal of arsenic from water (EPA, 2010). Therefore nano-TiO₂ can be identified as the most commonly engineered nanoparticle on Earth (also the number one nanoparticle in aquatic ecosystems) for which numerous researchers have pointed out its immunotoxic properties. The secondary aim of this review article is to discuss the potential immunotoxicity of nano-TiO₂ toward aquatic animals.

2.3. External and mucosal innate immunity and interaction with engineered nanoparticles

The very first line of fish immune defensive mechanisms against invading pathogens and parasites is the mucus membrane layer of the gills, skin and intestines. This layer provides both mechanical barriers for invading microbes as well as the first antimicrobial response by lysozyme, lectins, complement system, pentraxin, IgM and proteolytical enzymes (which will be discussed later in the text) present in the mucous layer. A mechanical barrier is secreted in the form of mucus by goblet cells (Bols et al., 2001). The mucus is composed mainly of water, mucopolysaccharides and mucoproteins, sialic acid, electrolytes and various other soluble materials (Shephard, 1994) in addition to the above-mentioned immune response components. Nanoparticle movement in water is characterized by Brownian motion, and nanoparticles can easily penetrate the mucous layer by peri-kinetic forces (Handy et al., 2008a). Once inside the mucus layer, nanoparticles bind to the mucoproteins due to their surface charge and electrostatic properties, thus becoming entrapped (Handy et al., 2008a). The common response of the mucus layer toward an encounter with toxicants is excessive mucus production (especially on the gill epithelium) followed by a change in the number of goblet cells (Bols et al., 2001). Excessive mucus production by the gills of rainbow trout after exposure to nanoparticles has been documented

(Smith et al., 2007). However, due to the fact that the nanoparticles form aggregates inside the mucus layer and that their rate of passage into the epithelium layer is slow and often insignificant (Handy et al., 2008a), once entrapped by the mucus layer the nanoparticles act as a chronic irritant stimulus affecting the goblet cells' rapid mucus release, as demonstrated by the silver nanoparticles model (Jeong et al., 2010). Eventually, this exhausts the supply of mucus and strips the fish of its protective barrier. In addition, it is possible for nanoparticles to cause direct apoptosis of goblet cells (Kumar et al., 2003), although this has never been tested with metallic/metal-oxide or carbon-based nanoparticles, only with chitosan-coated nanoparticles. The decrease in the number and density of goblet cells is an important biomarker used in aquatic toxicology (Bols et al., 2001). During the whole process, a fish's chance of becoming immunocompromised is doubled. Periods of a severe increase in mucus production can enhance the infection potential with pathogenic *Vibrio* strains, which use mucus as a carbon source (Bordas et al., 1996), and later, during the mucus exhaustion period, the fish is more susceptible to a variety of pathogens.

Considering the physical properties of the discussed nanoparticles, the authors believe that there is significant potential for nanoparticles to influence changes in alarm cell responses, possibly altering fish immune responses in the mucosal layer of the skin and also interfering with the ecological dynamics of the affected population. However, there is no available peer-reviewed information about this potential problem. The alarm cells are a specialized defensive mechanism of fish belonging to superorder Ostariophysi as well as of some isolated members of order Perciformes (which account more than 70% of all freshwater fish species, more than 30% of fish species in the world) (Smith, 1992). Alarm cells have a club-like shape, are present in the mucus layer directly underneath the goblet cells (Chivers et al., 2007) and are packed with various chemicals that, upon release, serve as alarming agents.

The ecological purpose of an alarm cells is multiple. Being one of the closest cells to the surrounding environment, alarm cells are the first cells to be damaged by any kind of predator, after which their contents leak into the environment. The olfactory-detectable contents of these cells serve as a warning to other members in the school to flee from predatory danger, but they also serve as a feeding signal to secondary predators. Once the

primary predator has captured its prey, an attempt by the secondary predator to pirate the prey may disrupt the feeding behavior of the primary predator, which often leads to the escape of the prey (Chivers et al., 1996; Smith, 1992). Alarm cells possess an immunological function for epidermal integrity and promote skin healing, protecting the fish organism from fungi, parasites and UVB radiation (Chivers et al., 2007). An alarm cell cannot release its contents voluntarily; the release is induced solely by mechanical abrasion, irritation or rupture of alarm cells by foreign particles, pathogens, parasites or predators (Chivers et al., 2007). Because nanoparticles can cause mechanical abrasion and apoptosis for other cell types present in the mucus layer (Kumar et al., 2003), they likely can cause abrasion and damage to alarm cells as well, especially given that alarm cells' physiological function makes them sensitive specifically to mechanical injury, which causes immediate content release. If this is true, the abrasive action of nanomaterials could have multiple consequences.

One consequence is that nanoparticles could become trapped in the fish's mucus layer (Handy et al., 2008a) and cause chronic damage to alarm cells, sending constant feeding signals to predators to attack and eat the nanoparticle-contaminated individual. Such preferential eating could promote biomagnification of nanoparticles in the food chain (Werlin et al., 2010) or cause avoidance of contaminated individuals by other members of the school, presenting behavioral and social problems during the mating season. Finally such an individual would be immunocompromised, with a reduced number of alarm cells in the mucus layer, allowing for greater skin damage by UV radiation and increased incidence of colonization of the skin by pathogenic microorganisms.

2.4. Nanoparticle entrance, distribution and first encounter with the internal immune system

Aggregation of nanoparticles, as well as the difficulties in passing the mucus barrier and the inefficient use of ion channels, indicates that nanoparticle absorption via the gills or skin is not the preferred uptake route. Although the nanoparticles can be absorbed by the gills and skin of aquatic animals, the absorbed amount is insignificant compared to the

potential of uptake through diet (Handy et al., 2008a). A dietary intake route was determined to be preferred over the respiratory route in fish that were exposed to metal-oxide nanoparticles including nano-TiO₂ (Johnston et al., 2010). Nanoparticles enter the gastrointestinal (GI) tract via drinking water or by preying upon the lower trophic levels biota with bioaccumulated and biomagnified levels of nanoparticles (Werlin et al., 2010).

Once inside the GI tract, the nanoparticles interact with enterocytes and the primordial intestinal immune system (Monhanraj and Chen, 2006). Most teleost fish do not have distinctive GI immune areas, such as Peyer's patches, but rather have a diffuse gut immune system composed of intestinal neutrophils, occasional macrophages, immunoglobulin-positive leukocytes scattered in the epithelium and small non-organized lymphoid aggregates (Brandtzaeg et al., 2008; Mestecky, 2005). These differences suggest that the mechanism of nanoparticle interaction with GI immune cells may be different than that of mammals. Although Peyer's patches can be the target for negatively charged and hydrophilic nanoparticles in mammalian systems (Monhanraj and Chen, 2006), fish enterocytes would serve as a primary target for both hydrophilic and hydrophobic particles, including neutral, positive or negative charges. The main difference between fish and mammalian enterocytes (and the main concern in risk assessment analyses that is very often disregarded) is that fish enterocytes are capable of the uptake of macromolecules many times larger in size than the molecules that can be endocytosed by mammalian enterocytes (Handy et al., 2008a; Ostrander, 2000). Therefore, absorption of aggregated nanoparticles as well as nanoparticle size in fish enterocytes is likely not restricting the interactions as may be case in human beings or other mammals.

Recently, it was demonstrated that nano-TiO₂ model nanoparticles do not kill enterocytes or disrupt the functional complexes of live cells but simply pass through the live cell via transcytosis without interfering with the enterocyte function (Koeneman et al., 2010). In aquatic vertebrates, after transcytosis across gut epithelium nanoparticles are transferred via the hepatic portal system throughout the body but predominantly to the liver (Moore, 2006) and kidney (Scown et al., 2009). In aquatic invertebrates, nanoparticles are stored in the hepatopancreas (Moore, 2006). Once inside the adipocytes or kidney reticular cells and renal hemopoietic tissue, nanoparticles are likely permanently stored within

lysosomes, the endoplasmatic reticulum or the Golgi apparatus and can serve as focal points in inducing oxidative damage and lipid peroxidation (Moore, 2006). There was no apparent clearance of nano-TiO₂ from the rainbow trout's liver and kidney for as long as 90 days after treatment (Scown et al., 2009). The renal function of rainbow trout kidneys in this study was not impaired, and the glomerular filtration rate was unchanged. However, it is important to note that fish kidneys (especially anterior kidneys) also have a major role in hematopoiesis (Zapata, 1979) and chronic exposure of developing immune cells can result in lysosomal overload by nanoparticles and increased potential for immune cell change of function (Jovanović et al., 2011a; Jovanović et al., 2011b). The newly developed immune cells transport the nanoparticles in their endo-lysosomal compartments through tissues and the blood stream throughout the body, without the ability to digest them, having the ability to lose them only by the process of exocytosis along the way or to release them after immune cell apoptosis, thus repeating the process again and again. A similar scenario is expected to occur in invertebrate species, which possess a hematopoietic hepatopancreas (Symonová, 2007).

2.5. Internal innate immunity

The innate immunity of fish and aquatic invertebrates is an essential component for fighting pathogens and overcoming diseases due to poikilotherms' limited capacity of acquired immunity (low number of antibodies and slow lymphocyte proliferation). Innate immunity is nonspecifically targeted by nanoparticles due to the phagocytic nature of their cells or their physical (rather than chemical, although chemical interaction is common feature of functionalized nanoparticles) interaction with cell receptors or free proteins in plasma. Innate immunity can be divided into humoral and cellular components for the purpose of a more concise presentation.

Humoral immunity

Complement system

The complement system is a biochemical cascade of small proteins found in blood plasma or mucus, predominantly synthesized by the liver hepatocytes and circulating in its inactive form. In vertebrates, the system is composed of about 30 proteins, which upon activation by antigens can form either the hollow cylinder complex that penetrates the pathogenic cell causing its death by extracellular influx and change in osmotic pressure or can coat the pathogen opsonizing it for the phagocytic cells' uptake (Janeway et al., 2008). In aquatic vertebrates the complement system can be activated through three pathways: the classical pathway (triggered by acute phase proteins or antibody binding), the alternative pathway (activated only by pathogens without the presence of antibodies), and the lectin pathway (activated when manose-binding lectin binds to the mannan of the bacterial membrane) (Whyte, 2007). Aquatic invertebrates have a smaller number of complement-related proteins as well as less complex alternative and lectin activation pathways compared to their vertebrate counterparts (Smith et al., 1999).

Once nanoparticles come into contact with a body fluid or mucus with proteins they instantly begin the formation of a protein corona. A protein corona is an association (loose or tight) of nanoparticles with proteins or other biopolymers due to the electrostatic properties as well as the surface charge of the nanoparticles (Lundqvist et al., 2008). It is a fairly fast process that can occur within seconds and, as a result, the nanoparticle is coated with organic material. If, before its ingestion, a nanoparticle spends some time in an aquatic ecosystem environment that is rich in organic matter, it can carry numerous foreign molecules and pathogens from the surrounding environment into the body of an aquatic animal. For example, it is well known that nano-TiO₂ can act as a carrier for lipopolysaccharide (LPS—a component of the outer membrane of gram-negative bacteria that acts as an endotoxin, causing severe immune reaction) (Ashwood et al., 2007) thus presenting the LPS to the complement system as well as to phagocytic cells. The size and surface properties of nanoparticles play a crucial role in forming the coronas; the type of nanomaterial is of lesser importance. Neutral (nonfunctionalized) nanoparticles, such as

most metal and metal-oxide nanoparticles, can form a corona predominantly with fibrinogen, immunoglobulin, albumin, and most of the complement system proteins, except for the complement inhibiting proteins (Lundqvist et al., 2008). The corona formation can continuously stimulate the complement system and prime the nanoparticles for phagocytosis, therefore initiating further inflammatory reactions. Binding of albumin proteins (and likely the complement system) to readily aggregative particles (such as nano-TiO₂) may be attributed primarily to their dispersion in the blood plasma (Ji et al., 2010), increasing both the transport rate and the surface area of nanoparticles presented to the immune system.

The composition of the protein corona is determined by the concentration of the biopolymer in the fluid and separately by the equilibrium binding constants of each biopolymer and each nanoparticle. The corona does not necessarily reach equilibrium immediately, and the initially fast-bound proteins of higher concentration may be replaced later with proteins of lower concentration and higher affinity (Cedervall et al., 2007). This can be especially important when nanoparticles are transported between different organ systems or locations within a body while carrying activated complement proteins. A likely possibility is that such complement system protein-coated nanoparticles can bind to the transmembrane receptors of the nearest cells, such as lipoproteins, for which the corona has high affinity (Lundqvist et al., 2008). Such cells would be primed for attack by professional phagocytes, followed by the initiation of the inflammatory processes. The best known example is the activation of the complement system by carbon-based nanotube particles (Salvador-Morales et al., 2008; Salvador-Morales et al., 2006). Carbon nanotubes can bind complement and activate both classical and alternative pathways. This activation leads to the generation of pro-inflammatory proteins such as C3a, C4a and C5a. The C5a molecule is a potent neutrophil chemotactic factor and causes the accumulation of neutrophils around the nanotubes. Because neutrophils are unable to phagocytize the nanotubes due to their extreme length, they will release granular enzymes and cause tissue damage and inflammation (Salvador-Morales et al., 2006).

Lysozyme

Lysozyme (muramidase or N-acetylmuramide glycanhydrolase) is an enzyme that can damage the bacterial cell wall by catalyzing hydrolysis of the glycosidic bond of bacterial peptidoglycans. Lysozyme is abundant in various secretions (mucus, saliva, tears, etc.), blood plasma and lymphoid tissues as well as in granules of granulocytic cells and hemocytes. Lysozyme can also act as an opsonin and activate the complement system and phagocytes. Lysozymes levels and activity in plasma or serum are standard ecotoxicological biomarkers in fish and aquatic invertebrate studies (Magnadóttir, 2006; Whyte, 2007). The influence of nanoparticles on lysozyme activity and its release in mussel models recently attracted a lot of attention. Metal-oxide nanoparticles, such as nano-TiO₂ and nano-SiO₂, as well as carbon-based nanoparticles (fullerenes) can induce a concentration-dependent increase in extracellular lysozyme activity and its release from hemocytes of the marine blue mussel *Mytilus galloprovincialis* Lamarck, 1819 (Canesi et al., 2010a). Similar results were obtained with plain carbon black nanoparticles as well (Canesi et al., 2008), indicating that the type of nanomaterial does not play a crucial role in stimulating lysozyme activity but, rather, it is a rather conserved type of immune reaction toward all nanoparticle types. Increased levels of lysozyme after acute exposure to nanoparticles (Canesi et al., 2010a) could lead to an inflammatory response (Mir, 1977), although lysozyme can express anti-inflammatory effects by suppressing the super-oxide generation of immune cells (Gordon et al., 1979). This could be a mediated response toward increased reactive oxygen and nitrogen species production by hemocytes after exposure to nanoparticles (Canesi et al., 2008; Canesi et al., 2010a).

Lysozyme has a strong affinity to form coronas around metal-oxide nanoparticles (nano-SiO₂ and nano-ZnO) by binding to them (Chakraborti et al., 2009; Vertegel et al., 2004), after which lysozyme activity is drastically inhibited (Vertegel et al., 2004). The binding of lysozyme to a nanoparticle depends on the pH (lower pH decreases binding), but once bound to a nanoparticle, lysozyme undergoes a permanent change in conformation, losing α -helices (Vertegel et al., 2004). Due to the fact that a nanoparticle corona is a dynamic structure constantly exchanging bound proteins (Cedervall et al., 2007), as described in section *Complement system*, it is possible that chronic exposure to nanoparticles

may lead to a significant loss of lysosomal activity as well as exhaustion in the release of lysosome, making animals more prone to bacterial infection (Ganz et al., 2003).

Acute phase proteins

Acute phase proteins (APP) are proteins in plasma or serum whose levels change during infection, inflammation or tissue damage and therefore are characterized as immune-related proteins. They are usually secreted by liver hepatocytes, and the most common include C-reactive protein, fibrinogen, transferrin, albumin, serum amyloid A, ceruloplasmin, haptoglobin, etc. (Whyte, 2007). APPs have not been studied intensively in the past from the perspective of nanoparticle interaction, but a recent study (Higashisaka et al., 2011) suggests that haptoglobin, serum amyloid A and C-reactive protein can be used as biomarkers of animal exposure to metal-oxide nanoparticles, as their levels increase in the plasma after exposure. Because APPs are present as free proteins in plasma, the biggest concern about interaction with nanoparticles would be their conformational change and inactivation. The small size and relatively large surface area of nanoparticles favors the covalent bonds and absorption to APP, whereas the physical–chemical properties of nanoparticles (especially metallic and metal-oxide nanoparticles), such as charge and hydrophobicity, can influence the structure of bound protein. In fact it is very likely that APPs adsorbed on the nanoparticles (see section *Complement system*) will undergo secondary and tertiary conformational changes resulting in the complete loss of α -helices (Fei and Perrett, 2009) and diminished protein activity (Vertegel et al., 2004). Although the protein adsorbed to the nanoparticle still retains most of its structure, such proteins are sensitive to degradation by urea (Shang et al., 2007). Keeping this in mind, cartilaginous fishes, as ureotelic organisms with the highest concentration of urea in blood plasma among all vertebrates, would have the highest risk for protein inactivation by nanoparticles (Ballantyne, 1997; Yancey, 2005). Either by being degraded or with inhibited activity, APP will fail in eliciting the proper response toward bacterial infection, inflammation or tissue damage and will immunocompromise its host.

Cell-mediated immunity

Phagocytic cells—uptake of nanoparticles

Phagocytosis of nanoparticles by phagocytic cells is well documented and supported by images of nanoparticle uptake and colocalization within the immune cell compartments (Krpetic et al., 2010). However, until recently it was thought that the underlying mechanism of nanoparticle uptake by immune cells is through caveolae-mediated endocytosis, clathrin-mediated endocytosis (Moore, 2006) and receptor-mediated phagocytosis including the mannose receptor, complement receptor, Fc γ receptor and scavenger receptor (Dobrovolskaia and McNeil, 2007). Although this is true, and nanoparticles can arrive to the immune cells via all of these pathways, the quantity of internalized nanoparticles is insignificant as compared to the route of macropinocytosis (Bartneck et al., 2010), which recently has emerged as the main uptake route.

This new understanding of nanoparticle arrival to immune cells is of immense value in considering the nanoimmunotoxicity of aquatic organisms. Caveolae-mediated endocytosis and clathrin-mediated endocytosis are capable of internalizing particles only up to 100 nm in size and receptor-mediated phagocytosis requires antibody coating on the nanoparticle, whereas macropinocytosis can uptake particles in the range of several μ m without the need for antibody coating (Bartneck et al., 2010; Dobrovolskaia and McNeil, 2007). This means that the aggregates of nanoparticles in an aquatic environment will be available for interaction with the hydrobiota immune system and that the size of the particle and structure of the protein corona is not that important in eliciting a toxic effect as previously thought. Keeping this in mind, from a risk assessment perspective, the classic definition of a nanoparticle being a particle smaller than 100 nm in at least one dimension (EPA, 2007) should be avoided and reformulated.

Macrophages

Macrophages are innate immunity phagocytic cells that circulate among tissues and usually are among the first immune cells, besides neutrophils, to respond to pathogen invasion. Macrophage action against the presence of foreign organisms or material is based

on detection through pathogen-associated molecular pattern recognition receptors, phagocytosis, and cytokine secretion for recruitment of other cell types as well as antigen-presenting cell service (Janeway et al., 2008).

Nanoparticles are capable of interfering with almost every aspect of macrophage function. A recent study with carbon-based nanoparticles used functionalized and non-functionalized nanotubes to assess the impact of nanotubes on rainbow trout macrophages *in vitro* (Klaper et al., 2010). Nanotubes did not stimulate the expression of interferon α (IFN) in trout macrophages, indicating that phagocytosis of nanotubes cannot stimulate the antiviral response by fish macrophages. On the contrary, all types of tested nanotubes, water soluble and non-soluble, have induced the transcription of interleukin 1 β showing the same response of macrophages as compared to the LPS control. This indicates that carbon nanotubes may elicit the same response in macrophages as do gram-negative bacteria.

However, there is a big concern about this conclusion, because sodium deoxycholate, used as a suspending agent of non-water soluble nanotubes, caused the same effect on interleukin 1 β alone without the presence of nanotubes. Therefore the effects of non-water soluble nanotubes can not be separated from the effects of detergent. Although an antiviral response was not induced in the previously mentioned study with carbon-based nanoparticles, mammalian macrophage exposure to nano-TiO₂ and nano-ZrO₂ led to upregulation of expression of viral TLR3 and TLR7 receptors. TLR10 was also upregulated with these nanoparticles, whereas nano-SiO₂ downregulated the expression of TLR9 (Lucarelli et al., 2004). This is a very important finding suggesting that metal-oxide nanoparticles may affect cell reactivity to infections by altering the expression of TLR receptors required for viral-dependent stimulation, which are not active under normal conditions. However, it is hard to transfer these finding to aquatic animals, especially fish. In addition to TLR3, fish have a novel TLR22, which acts as a dsRNA-recognizing pattern receptor recruiting TICAM-1 to induce IFN and maintain a protective role against viral infection (Matsuo et al., 2008). Thus, fish have a dual dsRNA recognition system. TLR22 is not present in mammals. TLR3 resides in the endoplasmic reticulum and recognizes short-sized dsRNA, whereas receptor TLR22 can discern long-sized dsRNA on the cell surface (Matsumoto and Seya, 2008; Matsuo et al., 2008). It is not known how the fish dual

TLR3/TLR22 system would react to metal-oxide nanoparticles, and there is only room for speculation until relevant data are obtained.

Regardless of the speculation of the effects on the receptors, nano-TiO₂ and perhaps other metallic and metal-oxide nanoparticles likely causes an inflammatory reaction of macrophages through a cathepsin B-mediated mechanism (Hamilton et al., 2009), which is a conserved protease in mammals, fish and other organisms (Bonete et al., 1984). The mechanism of cytotoxicity is governed by the loss of lysosomal integrity and the consequent release of cathepsin B, initiating apoptotic pathways of the macrophage cell. In addition, production of reactive oxygen species (ROS) by macrophages after exposure to nano-TiO₂ is significantly increased, causing peroxidation of the cell membrane lipids (Hamilton et al., 2009).

Neutrophils

Neutrophils are the primary innate immunity defensive mechanism of vertebrates against bacterial, viral and fungal infections, and neutrophil function is essential for the normal development and survival of an animal population (Segal, 2005). The neutrophil mechanism of pathogen killing relies on the NADPH oxidase system to ferry electrons over the membrane (during the process of respiratory burst) and deliver them to the oxygen present in the phagosomal compartment, creating ROS (Dahlgren and Karlsson, 1999). ROS facilitates an increase in pH in the phagocytic vacuole and the entry of potassium ions, stimulating the release of digestion enzymes responsible for microbial killing (Segal, 2005). ROS can be further dismutated to hydrogen peroxide and, with the help of halide and a myeloperoxidase (MPO) catalyzer, form hypochlorous acid, which is toxic to invading pathogens (Freitas et al., 2009). The final stage of the neutrophil defense mechanism is the release of neutrophil extracellular traps (NETs) through the recently described process of NETosis. NETosis is a cell death mechanism characterized by the disintegration of all membranes and the release of NETs (composed of DNA, histones, and granule proteins), which entrap and kill pathogens even beyond the neutrophil's lifespan (Fuchs et al., 2007).

Neutrophils are likely to be the primary target of the nanotoxicity of the majority of nanoparticle types due to the neutrophils' biology (phagocytosis, diapedesis, maturation in

kidney) as well as their sheer number in fish organisms compared to other phagocytic cell types. At least in case of nano-TiO₂, there is more available information about the immunotoxic effects on neutrophils (Fig. 1), and it is likely that the other metallic, metal-oxide and at least some carbon-based nanoparticles act in the same manner.

The NADPH oxidase system is composed of core and stabilizing small proteins p21^{rac} and p40^{phox}. The core is composed of p47^{phox}, p67^{phox} and flavocytochrom b₅₅₈ (p22^{phox} + gp91^{phox} heterodimer) components (Segal, 2005). Flavocytochrom, particularly the gp91^{phox} component, is responsible for forming a conduit of electrons that are pumped from the NADPH in the cytosol onto the molecular oxygen in the vacuole, thus creating O₂⁻. However flavocytochrom can be activated only upon binding and interaction with p47^{phox} and p67^{phox} phosphoproteins, which need to be phosphorylated by kinases prior to interaction with flavocytochrom. Upon phosphorylation *phox* subunits move from the cytoplasm to the phagosomal membrane and interact with the flavocytochrom (Segal, 2005; Wientjes and Segal, 1995).

Many stimuli (physiological and non-physiological) can lead to the activation of *phox* subunits by kinases. In general, stimulation of neutrophil Fcγ receptors during phagocytic uptake of a pathogen or complement-coated and opsonized nano-TiO₂ after a cascade of events yields a phospholipase C-β cleavage product diacylglycerol (DAG), which activates protein kinase C (PKC) (Janeway et al., 2008). PKC is responsible for phosphorylation and the translocation of p47^{phox} and p67^{phox} and activation of the NADPH oxidase (Dekker et al., 2000; el Benna et al., 1994; Quinn and Gauss, 2004). p47^{phox} and p67^{phox} can be phosphorylated through other kinase pathways as well such as extracellular signal-regulated kinases-1/2 (ERK1/2) and p38 mitogen-activated protein kinase (p38 MAPK) (Dewas et al., 2000; Guichard et al., 2005; Quinn and Gauss, 2004). Interleukin 8 (IL-8) is a cytokine produced in response to a pathogen encountered by a variety of blood cells and tissues with target specificity for neutrophil recruitment and activation in inflammatory regions (Janeway et al., 2008). IL-8 primes the respiratory burst by inducing early phosphorylation of p47^{phox} by ERK1/2 and late p67^{phox} phosphorylation by p38 MAPK (Guichard et al., 2005). Nano-TiO₂ can rapidly induce the phosphorylation of p38 MAPK and Erk-1/2 and slowly increase the production of IL-8 (Gonçalves et al., 2010). Similar

studies using blue mussel hemocytes confirmed nano-TiO₂ phosphorylation induction of p38 MAPK and Erk-1/2 but outlined that other nanoparticles, such as plain nano-carbon black, fullerenes and nano-SiO₂, have the same mechanism of action (Canesi et al., 2008; Canesi et al., 2010a). TiO₂ has a strong binding affinity for phosphorylated proteins (Larsen et al., 2005; Thingholm et al., 2006; Thingholm and Larsen, 2009), which can explain its biochemical mode of action, and many phosphorylated proteins are involved in intracellular signaling and gene regulation. Rapid tyrosine phosphorylation is evident as early as after 15 seconds of neutrophil exposure with a peak after 60 seconds of neutrophil exposure to nano-TiO₂, indicating that nano-TiO₂ probably does not even need IL-8 (the increase of which was noted after 24 hours of exposure to nano-TiO₂) to induce phosphorylation of p47^{phox} and p67^{phox} through ERK1/2 and p38MAPK and start respiratory burst (Gonçalves et al., 2010).

In fact, we recently demonstrated that nano-TiO₂ does induce respiratory burst in fish neutrophils *in vitro* (Jovanović et al., 2011b). Within 1 hour of exposure to nano-TiO₂ *in vitro* neutrophils of fathead minnow expressed a strong increase in respiratory burst compared to the non-treated control. Furthermore, nano-TiO₂ treatment in addition to neutrophils pre-stimulated with phorbol myristate acetate (PMA, a DAG analog and activator of PKC) further increased respiratory burst when compared to the PMA control, suggesting that nano-TiO₂ does not participate in the PKC phosphorylation pathway of *phox* subunits and acts only as an ERK1/2 and p38MAPK activator. The serious ecotoxicological implication of this study can be seen from the four times greater concentration of nano-TiO₂ than the environmentally estimated one induced respiratory burst (Jovanović et al., 2011b). Keeping in mind that nano-TiO₂ of up to at least 3 µm can be phagocytized by mammalian neutrophilic granulocytes and can be stimulated to produce ROS and perform respiratory burst (Kumazawa et al., 2002), the size of nano-TiO₂ particles (nano vs. aggregates) in the aquatic environment is once again unimportant for eliciting an immunotoxic response.

During respiratory burst O₂⁻ delivered to the vacuole will spend an H⁺ proton from the vacuole content, forming hydrogen peroxide with the help of super oxide dismutase (SOD) and raising the pH of the vacuole, which opens channels for potassium and chlorine ions and stimulates the release of elastase and cathepsin G (Segal, 2005). Due to the fact that

nano-TiO₂ particles cannot be digested by releasing elastase and cathepsin G from granules as pathogens can (Segal, 2005), nano-TiO₂ stay in the phagosome compartment and chronically stimulate the neutrophil to perform respiratory burst. Because respiratory burst does not cease, neutrophil elastase translocates to the nucleus and partially degrades histones, promoting chromatin decondensation (Papayannopoulos et al., 2010). The excess of hydrogen peroxide together with the chlorine ion is converted to hypochloric acid using MPO as a catalyzer, a process during which MPO is released from granules (Freitas et al., 2009; Segal, 2005). Subsequently, MPO binds to uncoiled chromatin enhancing its decondensation and promoting a trigger of nucleus disintegration and cell rupture (Papayannopoulos et al., 2010) resulting in the extracellular release of NETs (Fuchs et al., 2007).

Upon release of NETs, the neutrophil dies and nano-TiO₂ is spit out from the remains of the phagosome, back to the biological system, and is available to interact with the next neutrophil it encounters along the way. The release of NETs by fish neutrophils after exposure to nano-TiO₂ has been demonstrated both *in vitro* and *ex vivo* (Jovanović et al., 2011b). It appears that the NETs release is a standard defensive attempt by neutrophils to remove nanoparticles from the system, as nano-gold particles also induce NETs formation (Bartneck et al., 2009). Besides neutrophils, monocytes and macrophages can be stimulated by nanoparticles to release NETs (or METs) through similar signaling pathways. However, they are of less biological importance considering their small number as well as fact that only 4% of monocytes and 1.5% of macrophages in the system can release NETs as opposed to 25% of the neutrophil population (Bartneck et al., 2009). The 25% of neutrophils that can release NETs are mature neutrophils, as only mature neutrophils are capable of NETs release (Martinelli et al., 2004). The NET release stimulation by nanoparticles suggests that a significant number of the mature neutrophil population inside the circulatory system of aquatic organism will die after exposure to nano-TiO₂ or other nanoparticles. This removal of the mature neutrophil population may interfere with organismal defenses because less mature neutrophils may not be fully functional, resulting in the reduction of neutrophil *in vivo* functional responses (Jovanović et al., 2011b). Neutrophils that are not fully mature have significantly lower levels of respiratory burst and other functions, and different

developmental stages of neutrophils differ in granular content (Borregaard et al., 2001; Martinelli et al., 2004).

Acute exposures of rainbow trout and fathead minnow to nano-TiO₂ did not cause observable changes in blood neutrophil counts (Federici et al., 2007; Jovanović et al., 2011b; Ramsden et al., 2009), whereas prolonged acute exposure to TiO₂ did cause some hematological changes in flounder (Larsson et al., 1980). No change of neutrophil count in the blood suggests that more immature or nearly mature neutrophils are released from fish hematopoietic depots to potentially supplement the depletion of the mature neutrophil population that interacted with the nanoparticles.

In fish, the kidney is one of the major organs for nano-TiO₂ bioaccumulation with low nano-TiO₂ clearance even 90 days post exposure (Scown et al., 2009). But the fish kidney also has a major role in hematopoiesis and serves as a neutrophil depot (Zapata, 1979). Therefore, the neutrophil population that is continuously produced and stored in the anterior kidney can be chronically exposed to nano-TiO₂ and further disseminate the particles throughout the organism. The observed reduction in neutrophil function after exposure to nanoparticles *in vivo* (Jovanović et al., 2011b) suggests that potential interactions between nano-TiO₂ and neutrophils in the anterior kidney could affect the neutrophils' ability to control bacterial infections, causing decreased disease resistance in fish populations exposed to nano-TiO₂.

The potential for nano-TiO₂ to interfere with disease resistance in fish appears significant. We recently exposed fathead minnows to a low dose of the pathogen *Aeromonas hydrophila*, causing an approximately 15 % mortality in the challenged population. However, pre-exposure to a sub-lethal concentration of nano-TiO₂ increased the mortality of the challenged *A. hydrophila* group to 85 % of the experimental population, whereas the nano-TiO₂ treated group without the pathogen did not experience any mortality (Jovanović et al., unpublished). This finding suggests a serious environmental impact, indicating that the presence of nano-TiO₂ in the aquatic environment has the potential to obliterate fish populations by preventing proper neutrophil response toward possible pathogen outbreaks in the ecosystem.

Hemocytes

Hemocytes are the phagocytic cells of invertebrates. Although not nearly as exploited as the phagocytic cells of vertebrates in studying the nanoparticle interaction with the immune system, several nanoimmunotoxic effects on mussel hemocytes have been documented. A major effect of metal, metal-oxide and carbon-based nanoparticles is lysosomal release by hemocytes (as described in section *Lysozyme*) (Canesi et al., 2008; Canesi et al., 2010a).

After *in vivo* exposure to the same nanoparticle types, mussel hemocytes demonstrated an alteration in lysosomal membrane stability (Canesi et al., 2010b; Tedesco et al., 2010). The nature of the lysosomal membrane alteration by carbon-based nanoparticles indicates hemocyte cell injury signifying the toxic potential of nanoparticles to the endo-lysosomal systems of immune cells (Moore et al., 2009). CdTe quantum dots without coating caused the same effect in mussels, resulting in a significant decrease of hemocytes capable of performing phagocytosis (Gagné et al., 2008).

Nanoparticles can significantly induce hemocyte production of extracellular oxyradicals and nitric oxide and phosphorylate various stress kinases (as described in section *Neutrophils*), demonstrating the ability of nanoparticles to induce an inflammatory response in mussel hemocytes (Canesi et al., 2008; Canesi et al., 2010a). Based on the nano-gold model, it appears that nanoparticles accumulate predominantly in the hemocytes of the hepatopancreas where they induce lipid peroxidation and oxidative stress within 24 hours of exposure (Tedesco et al., 2010).

Hemocytes are a crucial component of the invertebrate innate immune system. In mollusks the hepatopancreas is responsible for food phagocytosis and intracellular digestion with the aid of the lysosome content (Van Weel, 1974). Increased ROS production and lysosomal destabilization in the hemocytes of aquatic snails also has been observed in response to carbon black nanoparticles (Harley, 2010). Their lysosomes contain a battery of approximately 60 digestive enzymes as a response toward ingested food or pathogens, and they also accumulate diverse toxic organic and inorganic materials which, when present as an overburden, cause oxidative damage and lysosome damage resulting in hemocyte injury and death by autophagy (Moore, 1990). Therefore nanoparticles can severely impair the

innate immune system of aquatic invertebrates both directly through hemocyte death, reduced phagocytosis and oxidative damage and indirectly by interfering with the lysosomes of the hepatopancreas, which participate in food processing.

Dendritic cells

In vertebrates, dendritic cells serve as a connection between the innate and adaptive immune response. They serve as primary antigen presenting cells (APC) and, upon pathogen encounter and engulfment, they process the pathogen material and present pathogen peptides on the cell surface. These peptides are displayed by integral membrane proteins that are encoded by the major histocompatibility complex class II (MHC class II) molecules. After the display, dendritic cells carry sample pathogen peptides bound to MHC class II proteins to the secondary lymphoid tissues, stimulating the T-lymphocyte response (Janeway et al., 2008).

Teleosts have one of the earliest identifiable adaptive immune responses, and recently it was discovered that some fish possess dendritic cells (Lovy et al., 2008; Lugo-Villarino et al., 2010). Dendritic cells of fish are formed within the anterior kidney and spleen (Lovy et al., 2008). Although there have been no attempts to identify the impact of nanomaterials on dendritic cells in aquatic model species, mammalian studies indicate various effects. However, the majority of the studies utilized a variety of functionalized nanoparticles (whose presence is dubious in an aquatic environments), exploring the possibility of vaccine and pathogen peptide delivery to dendritic cells (Klippstein and Pozo, 2010).

A few studies using bare nanoparticles, such as nano-TiO₂, indicated that nano-TiO₂ can lead to an increase in maturation and expression of co-stimulatory molecules on dendritic cells, which can drive priming, activation and proliferation of naïve CD₄⁺ cells (Schanen et al., 2009). On the contrary, dendritic cells exposed to ionic titanium showed a decrease in the expression of MHC class II proteins as well as several co-stimulatory molecules but had increased stimulatory ability toward lymphocyte activation and proliferation, which is in accordance with the above study (Chan et al., 2009). Production of Th1 cytokine IL-12 by dendritic cells increased significantly after the treatment with

titanium (Chan et al., 2009), indicating an inflammatory response. Titanium, and likely nano-TiO₂, is taken by dendritic cells and accumulate inside by binding to phosphorus contained in the molecular structures of the cytoplasm, membrane and nucleus (Chan et al., 2009) as both titanium and titanium dioxide have a strong binding affinity for phosphorylated proteins (as described in section *Neutrophils*), and many phosphorylated proteins are involved in the intracellular signaling of dendritic cells.

The peptides presented by MHC class II proteins do not come from cytoplasm, but rather, they arise by the degradation of proteins inside the endosome–lysosome complex internalized by endocytosis (Janeway et al., 2008), which is the ultimate storage for nanoparticles (Moore, 2006; Moore et al., 2009). It is possible for the foreign phosphorylated protein complexes of pathogens delivered to dendritic cells with accumulated Ti/nano-TiO₂ to be processed differently, which explains the alteration in MHC class II expression on the cell surface. Also, phosphorylated complexes bound to nano-TiO₂ might be processed differently, yielding new antigenic properties.

Thrombocytes

Fish thrombocytes are evolutionary precursors of mammalian platelets. In mammals, platelets do not have nuclei and are developed from megakaryocyte fragmentation. In fish, thrombocytes are formed by the division of thromboblasic cells. They retain nuclei and do not progress to platelet status but do perform fairly similar functions as platelets (Ellis, 1977; Jagadeeswaran et al., 1999; Jain, 1993; Meseguer et al., 2002). Just like platelets, thrombocytes of fish play the same roles in hemostasis; inflammation; chemotaxis; phagocytosis of particulate matter, viruses, bacteria and parasites; wound healing and regeneration and reparation of tissues (Jagadeeswaran et al., 1999; Meseguer et al., 2002). Platelets/thrombocytes also participate in recruitment of endothelial progenitor cells as well as monocytes and neutrophils in circulation, inducing their activation, which leads to the production of cytokines, proteolysis and respiratory burst (Ferdous et al., 2008; Freedman, 2008; May et al., 2008).

Fathead minnows stimulated with nano-TiO₂ and hydroxylated fullerenes showed a strong increase in transcription of interleukin 11 (IL-11), a cytokine responsible for

thrombocyte recruitment (Jovanović et al., 2011a; Jovanović et al., 2011b). One of the mechanisms of nanoparticle phagocytosis includes formation of nanoparticle-induced thrombocyte aggregates. Nanoparticles can then be phagocytized, either by thrombocytes and removed from the system in the form of redistribution to the blood clot or by macrophages and likely neutrophils, which phagocytize the whole thrombocyte–nanoparticle aggregate (Movat et al., 1965). Upregulation of IL-11 indicates the activation of a phagocytic defensive mechanism against nanoparticles and could be used as a potential biomarker in field studies and risk assessment.

Other studies also support the role of platelet/thrombocyte activation and participation in the response toward exposure to nanoparticles. Carbon-based nanoparticles stimulate platelet aggregation and accelerate the rate of vascular thrombosis (Radomski et al., 2005). The platelet aggregation induced by nanoparticles in this study was PKC independent, further supporting the notion that nanoparticles do not target PKC pathways (see section *Neutrophils*). In the case of hydroxylated fullerenes, these nanoparticles facilitated the adenosine-diphosphate (ADP) stimulated platelet aggregation and operated as a competitive inhibitor of the ADP receptor antagonist (Niwa and Iwai, 2007). It appears that matrix metalloproteinase 9 (MMP-9) is the crucial regulator of nanoparticle induction of platelet aggregation. MMP-9 is comprehensively expressed and is released from platelets and macrophages upon stimulation with nanoparticles, which is probably related to the nanoparticle phagocytosis process rather than inflammation (Chellat et al., 2005; Niwa and Iwai, 2007; Radomski et al., 2005). The change in balance between MMP-2 and MMP-9 is what appears to drive nanoparticle induction of platelet aggregation (Radomski et al., 2005).

Fish thrombocytes also possess MMP-9 (Lang et al., 2010), which can explain the hemostasis in zebrafish after exposure to nanoparticles (McLeish et al., 2010). It is interesting to note that the more hydrophobic and negatively charge nanoparticles are, the more platelet/thrombocyte aggregation potency they have. Hydrophilic particles without negative charge have less potency for platelet/thrombocyte aggregation (Miyamoto et al., 1989). In conclusion, engineered nanomaterials have the potential to interfere with thrombocytes of aquatic organisms, inducing their activation, adhesion, aggregation and

release of intracellular contents and affecting the recruitment of other immune cells and the phagocytosis rate as well as blood hemostasis.

2.6. Acquired immunity

Unfortunately, effects of non-functionalized nanoparticles on the acquired immunity of aquatic animals are unknown. All the studies are based exclusively on functionalized nanoparticles and their potential usage as adjuvant and carriers in vaccine designs (Rajesh Kumar et al., 2008; Sinyakov et al., 2006; Tian and Yu, 2011). One early study demonstrated a decreased number of lymphocytes in fish exposed to TiO₂ industrial effluent, probably as a result of stress-induced lymphopenia (Larsson et al., 1980), and a later study indicated that various metal-oxide nanoparticles can modulate B-lymphocyte morphology and proliferation in a catfish model (Ortega et al., 2010). Based on section *Dendritic cells*, nanoparticles have the potential to cause impairment of acquired immunity in fish, thus more research is needed on this topic. The available scientific literature of the impact of nanomaterials on the mammalian acquired immune system is not applicable for drawing conclusions in fish models due to the notable differences between the mammalian and the fish acquired immune system (Litman et al., 2010).

2.7. Miscellaneous effects that can immuno-compromise the host

Numerous effects caused by nanoparticles can have an indirect influence on the immune system of aquatic animals. For example, bioaccumulation of cadmium (Cd) is drastically enhanced when fish are exposed simultaneously to nano-TiO₂. The bioconcentration factor for the whole-body Cd of carp rises from 65, when fish are exposed to Cd alone, to 605, when fish are exposed to Cd and nano-TiO₂ simultaneously (Zhang et al., 2007). The likely reason is facilitated transport—adsorption of Cd on TiO₂ particles. Naturally occurring sediment microparticles did not caused the observed effect in this study. Cd is a well known immunotoxin of aquatic animals (Zelikoff et al., 1995), and its mode of action is predominantly as an immunosuppressant (Chatterjee et al., 2008).

Nano-TiO₂ can significantly decrease the activity of superoxide dismutase, catalase and peroxidase enzymes in juvenile fish (Hao et al., 2009), and these enzymes are required for normal processes in immune cells (see section 2.5.). Their reduced activity can further lead to lipid peroxidation and oxidative damage caused by the excess of ROS “leaking” from the cells (Hao et al., 2009). Similarly, nano-TiO₂ caused an increase in total glutathione in fish gills but depletion of hepatic glutathione (Federici et al., 2007). Nano-TiO₂ also caused an increase in glutathione as well as an increase in the activity of glutathione-S-transferase and glutathione reductase in embryos of zebrafish (Yeo and Kang, 2009). Fullerenes exhibited similar effects on the embryos of marine teleost *Fundulus heteroclitus* and on the liver tissue of adults (Blickley and McClellan-Green, 2008). Other carbon-based nanoparticles, such as nanotubes, also increased levels of total glutathione in fish tissues after exposure (Smith et al., 2007). On the contrary, chronic exposure of freshwater fish *Carassius auratus* to fullerenes led to a decrease in glutathione levels in all tissues (Zhu et al., 2008). Elevated levels of oxidized glutathione in fish gills were also measured after exposure to cadmium sulfide quantum dots (Sanders et al., 2008), and total glutathione was elevated in the liver of zebrafish after treatment with silver nanoparticles (Choi et al., 2010). Besides the main role in detoxification of xenobiotics in phase 2 of biotransformation and ROS scavenging, glutathione is essential for the immune system to exert its full potential. It modulates antigen presentation to lymphocytes, thus influencing cytokine production and enhancing proliferation of lymphocytes while enhancing the killing activity of cytotoxic T cells and NK cells and regulating apoptosis. Its deficiency can severely impair macrophage and T-lymphocyte function (Dröge and Breitkreutz, 2000; Robinson et al., 1993).

Nano-TiO₂ can inhibit Na⁺-K⁺ ATPase by 50% in the fish brain (Ramsden et al., 2009), gills and intestine (Federici et al., 2007). Na⁺-K⁺ ATPase, besides its main function as a sodium-potassium pump, serves as a signal transducer of the immune response (see section 2.5.), inducing the MAPK pathway, activating phospholipase C and inositol triphosphate and inducing mitochondrial ROS production (Yuan et al., 2005).

Recently we discovered that embryonic zebrafish exposed to nano-TiO₂ and hydroxylated fullerenes have a whole set of suppressed gene expression for circadian

rhythm (Jovanović et al., 2011c). In zebrafish circadian system is composed of retinal receptors, the pineal gland, and melatonin. The pineal gland responds to the circadian clock by secreting melatonin (Kazimi and Cahill, 1999). Besides its various effects on homeostasis, melatonin has a strong effect on the immune system. In zebrafish levels of melatonin are directly related to the phagocytic ability of leukocytes; an increase in melatonin levels during the night phase increases phagocytic ability (Kaplan et al., 2008). Therefore the transcriptional decrease of genes involved in circadian rhythm after exposure to nanoparticles might lead to an increase in melatonin release by retinal cells and pinealocytes and modulate phagocytosis.

Finally, a recent pilot study suggested that the sunscreen products based on UV filter formulation can induce coral bleaching (Danovaro et al., 2008). Corals also possess innate immunity that helps them to defend themselves against potentially pathogenic organisms. The cellular immune response consists of fixed or circulating amoeboid phagocytes that ingest microscopic organisms and kill them by exposure to proteolytic enzymes and free oxygen radicals (Reed et al., 2010). These cells have evolved further in other aquatic invertebrates and go by different names in different phyla, e.g., hemocytes in mollusks, coelomocytes in echinoderms, but they still share the mechanisms of phagocytosis and response to phagocytized material (Mullen et al., 2004). Thus coral amoebocytes should phagocytize nano-TiO₂ from sunscreens in the same manner as do phagocytic cells of later evolutionary relatives. The external and internal epithelial layers of coral polyps consist of several types of cells that also have a role in capturing and digesting food. They also support algal cells that have a symbiotic relationship with the corals, and algal cells are phagocytized into vacuoles within the gastrodermal cells but are not digested (Mullen et al., 2004). We believe that nano-TiO₂ phagocytized by these cells will retain reflective properties and be able to act as a UV shield. Reduced intensity of sun light can undermine the ability of, or prevent algae from performing photosynthesis as well as induce a lytic viral cycle in algae with latent infections (Danovaro et al., 2008). A disturbed balance of algal populations within a coral can in turn induce stress and activate the immune response of the polyp, resulting in algal expulsion observed as bleaching accompanied by rapid changes in the transcription of crucial immune-related genes (Hoegh-Guldberg, 1999; Palmer et al., 2010).

2.8. Figure captions and figures

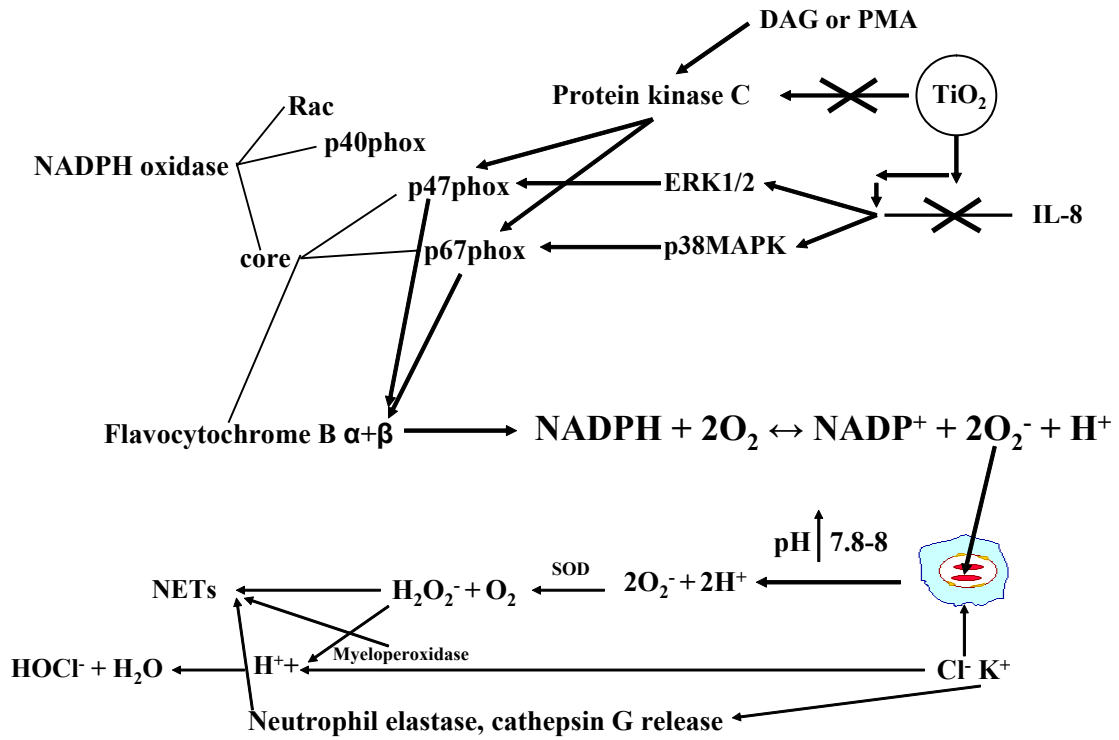
Figure 1. Effect of nano-TiO₂ on neutrophils.

Figure 1

2.9. References

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CHAPTER 3. Immunological stimuli change expression of genes and neutrophil function in fathead minnow (*Pimephales promelas* Rafinesque, 1820)

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3.1. Abstract

Fathead minnows *Pimephales promelas* were exposed to lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid [Poly(I:C)] to observe immunological responses during simulated bacterial and viral challenge at the level of gene expression and granulocyte function. Complementary DNA libraries were created from LPS and Poly(I:C) treated fish and approximately 5000 expressed sequence tags (ESTs) were sequenced. The ESTs were subjected to BLASTx analysis and 1500 genes were annotated, grouped by function, and twenty immune genes were selected for expression studies by real-time PCR. LPS treatment significantly downregulated expression of Interferon regulatory factor 2 binding protein 1 (9 fold), Chemokine (C-X-C motif) ligand 12a (3 fold), and TNF-related apoptosis inducing ligand - TRAIL (2 fold). In Poly(I:C) treated fish a significant upregulation was observed for IFN-inducible and antiviral proteins belonging to the family of Mx proteins (73 fold), and chemokine CCL-C5a (28 fold). Blood neutrophil count was significantly increased in Poly(I:C) treated fish at 24 and 48 h post-injection. Neutrophil extracellular trap release and respiratory burst of kidney granulocytes was suppressed in Poly(I:C) treated fish, while degranulation of primary granules was not affected significantly by the treatment. The changes in gene expression and neutrophil function in *P. promelas* exposed to LPS and Poly(I:C) support the use of this species as an alternative model for studies of pathogen effects on the innate immune system of fishes.

Keywords: fish immunology, lipopolysaccharide, polyinosinic-polycytidylic acid, cDNA libraries.

3.2. Introduction

The initial recognition of pathogens and pathogen associated molecular patterns (PAMPs) involves activation of pathogen recognition receptors (PRRs), resulting in the stimulation of the innate immune system, inflammatory reaction, and initiation of the appropriate adaptive immune response (Aderem & Ulevitch, 2000; Medzhitov, 2001; Janeway & Medzhitov, 2002). Toll-like receptors (TLRs) are a major class of PRRs that can respond to a variety of PAMPs including lipopolysaccharides (LPS), peptidoglycans, and viral nucleic acids. They can be activated either directly or after interaction with serum and membrane-bound factors like lipopolysaccharide binding protein and CD14, and transduce signals to various intracellular adaptor molecules that can elicit pathogen-specific responses.

LPS is a major component of the outer membrane of Gram-negative bacteria that contributes to its structural integrity and protects the membrane from chemical attack (Jirillo *et al.*, 2002; Triantafilou & Triantafilou, 2005). LPS acts as the prototypical endotoxin because it binds the CD14/TLR4/MD2 receptor complex and promotes the secretion of pro-inflammatory cytokines in various mammalian cell types. Interaction of LPS with macrophages and granulocytes leads to the production of numerous inflammatory mediators that contribute to early innate and accompanying adaptive anti-bacterial responses (Beutler, 2002; Beutler *et al.*, 2003; Freudenberg *et al.*, 2008). In fishes, however, LPS may act in other ways to mediate pro-inflammatory cytokine expression (Iliev *et al.*, 2005; MacKenzie *et al.*, 2010).

Polyinosin-polycytidylic acid [Poly(I:C)] is a synthetic analog of double stranded RNA (dsRNA) and is used to simulate viral infections under laboratory conditions (Bahl *et al.*, 2006). Stimulation of cells with Poly(I:C) in mammals activates TLR3, followed by induction of type I interferon (IFN) synthesis, inflammatory cytokine/chemokine production, and dendritic cell maturation via the Toll-IL-1R homology domain-containing adaptor protein 1 (TICAM-1, or TRIF) (Matsumoto & Seya, 2008). In addition to TLR3, fishes have a novel TLR22 that acts as a dsRNA-recognizing pattern receptor, recruiting TICAM-1 to induce IFN and maintain protection against viral infection (Matsuo *et al.*, 2008). Thus, fishes have a dual dsRNA recognition system; TLR22 is not present in mammals. TLR3

resides in the endoplasmic reticulum and recognizes short dsRNA, while receptor TLR22 can discern long-sized dsRNA on the cell surface (Matsumoto & Seya, 2008; Matsuo *et al.*, 2008).

The fathead minnow (*Pimephales promelas* Rafinesque, 1820) is a small (<10 cm) cyprinid species, abundant in North American freshwater ecosystems and frequently used as standard model organism in environmental and aquatic toxicology assessments and studies (USEPA, 1987; Ankley & Villeneuve, 2006). Recently, *P. promelas* was also used as model to study innate immune responses to stress (Palić *et al.*, 2006a) and immunomodulators (Palić *et al.*, 2005a; Palić *et al.*, 2006b) as well as in toxico/immunogenomics studies (Biales *et al.*, 2007; Mager *et al.*, 2008; Villeneuve *et al.*, 2008). The major leukocyte types have been described in *P. promelas* as lymphocytes, monocytes, neutrophils, and thrombocytes (Palić *et al.*, 2005b). Neutrophils were reported to perform respiratory burst, degranulation of primary granules, and NET - neutrophil extracellular trap release (Palić *et al.*, 2005a; Palić *et al.*, 2006a; Palić *et al.*, 2007a). The mentioned trio of neutrophil features relies on the NADPH oxidase system to ferry the electrons over the membrane (during the process of respiratory burst) and deliver them to oxygen present in the phagosomal compartment, creating reactive oxygen species (ROS) (Dahlgren & Karlsson, 1999). ROS facilitate pH increases in phagocytic vacuoles and entry of potassium ions, stimulating the release of the digestive enzymes responsible for microbial death (Segal, 2005). ROS can be further dismutated to hydrogen peroxide and, with the help of halide and myeloperoxidase catalyzer (released in phagocytic vacuole by degranulation process of primary granules), form the HOCl molecule, which is toxic to invading pathogens (Dahlgren & Karlsson, 1999). The final stage of the neutrophil defense mechanism is the release of NETs through the recently described process of NETosis. The NETosis is a cell death mechanism triggered by an excess of hydrogen peroxide, characterized by total membrane disintegration and release of NETs (composed of DNA, histones, and granule proteins) which continue to entrap and kill pathogens beyond the neutrophil's lifespan (Fuchs *et al.*, 2007).

There are almost a quarter of a million expressed sequence tag (EST) records for *P. promelas* at the National Center for Biotechnology Information. For the most part, the EST records have been derived from tissues or animals that had not been exposed to any

particular treatment. Therefore, the EST library may be lacking important PAMP-regulated genes that are observed during simulated disease challenges (Iliev *et al.*, 2006). The *P. promelas* in the current study were stimulated with LPS and Poly(I:C) in an effort to isolate immune-related genes from cDNA libraries using an EST approach. The changes in the expression of the selected genes from EST libraries, obtained after LPS and Poly(I:C) treatment, were evaluated with quantitative PCR (qPCR). Information about gene expression during initial phases of the immune response is of relevance for the future use of *P. promelas* as a model in studies of immune stress and disease.

3.3. Materials and methods

Animal care

Juvenile *P. promelas* with a mass of 1.2 - 1.8 g were maintained in the Department of Natural Resource Ecology and Management, Iowa State University, Ames, Iowa, USA. Fish were held in 40-120 l tank recirculation systems supplied with dechlorinated tap water at 20 °C and fed twice daily with live brine shrimp larvae and dried flake food (Aquatox[®], Zeigler Bros Inc, www.zeiglerfeed.com). *Pimephales promelas* were cared for in accordance with approved Iowa State University animal care guidelines.

Fish injection, anterior kidney cell culture and total RNA extraction

A total of 20 fish per treatment were used. Fish were anesthetized as previously described (Palić *et al.*, 2006a). Briefly, fish were quickly netted from the tanks and placed in 4 l glass beakers with 2 l of a 100 mg l⁻¹ of aerated and buffered (sodium bicarbonate, pH 8.0) solution of tricaine methane sulphonate (MS-222, Argent Laboratories, www.argent-labs.com). Upon entering the third stage of anaesthesia (Palić *et al.*, 2006a), fish were weighed and injected with LPS *E. coli* 0111:B4 (Sigma-Aldrich Corp, www.sigmaaldrich.com) and Poly(I:C) (InvivoGen, www.invivogen.com) at 10 µg or 2 µg g⁻¹ body mass, respectively. The chosen LPS dose is a standard for PAMP EST library creation with fish models (MacKenzie *et al.*, 2004). The chosen Poly(I:C) dose is routinely used in fish immunogenomics (Lockhart *et al.*, 2004; Yu *et al.*, 2009; Tian *et al.*, 2010).

Injected fish were transferred to 40 l recirculation tanks to recover from anaesthesia and were observed for 48 h for mortalities or behavioural changes (no mortalities or changes were observed). Ten fish per treatment and ten fish from non-injected controls were randomly collected at 24 h and 48 h post injection, placed individually in a 15 ml polypropylene tube, flash frozen in liquid nitrogen for 30 seconds, and stored at -80 °C.

Total RNA extraction was performed according to the acid guanidinium thiocyanate-phenol-chloroform extraction protocol (Chomczynski & Sacchi, 2006) using TRI Reagent® (Molecular Research Center, Inc., www.mrcgene.com) as an improved version of the original single-step total RNA isolation developed by Chomczynski & Sacchi (1987). Following thawing, internal organs (excluding brain) and gills from individual fish were collected in a 50 mL tube containing 7 mL of Tri-Reagent and total RNA extracted from the tissue of each fish.

Anterior kidneys were isolated from 40 additional non-injected control fish and the anterior kidney tissue from each fish was dispersed through nylon mesh (200 μ m) into individual wells of a 24 well plate containing Dulbecco's Modified Eagle Medium (cat.# 11971-025, Invitrogen, www.invitrogen.com) containing high glucose, 10% heat inactivated fetal bovine serum (Invitrogen, cat.# 16140-071) and penicillin/streptomycin (100 units and 100 μ g ml⁻¹ respectively; Invitrogen). Plates were coated with poly D-lysine. The wells were treated with 25 μ g LPS ml⁻¹ for 18 h after which the medium was removed and the cells extracted by adding TRI Reagent directly to the well.

For library construction, messenger RNA was extracted from total RNA using the PolyAtract mRNA isolation kit (Promega Corporation, www.promega.com) and the concentration assessed using a Nanodrop spectrophotometer (NanoDrop products, www.nanodrop.com)

Library construction

Five different libraries were constructed using mRNA from: a) pooled tissues of LPS-stimulated fish ($n = 6$) for 24 and 48 h; b) pooled tissues from Poly(I:C)-stimulated fish ($n = 6$) for 24 and 48 h and; c) LPS-stimulated anterior kidney cells. For each library, complementary DNA produced from mRNA was fractionated with sephacryl SF500, and the

two largest cDNA size classes were ligated in separate reactions with the Zap Express vector (Stratagene, www.stratagene.com). Ligations were packaged separately and all libraries were mass excised to polyomavirus BK-cytomegalovirus (pBK-CMV) phagemids according to the manufacturer's protocol.

Sequencing and data analysis

The XLOLR *Escherichia coli* Castellani and Chalmers 1919 strain (Stratagene) was used to propagate plasmids and to recover excised cDNA in phagemid form from a phage cDNA library. Both LPS and Poly(I:C) stimulated libraries were plated on lysogeny broth (LB) agar plates with kanamycin ($100 \mu\text{g ml}^{-1}$). Individual colonies were randomly picked and grown overnight in 96-well microtitre plates. Plasmid DNAs were extracted and sequenced from the 5' end using the dideoxy chain termination method with BigDye Terminator (Applied Biosystems Inc., www.appliedbiosystems.com) and the BK reverse vector primer. The reactions were precipitated and resuspended in Hi-Di Formamide (Applied Biosystems) and run on an ABI Prism 3730 automated sequencer (Applied Biosystems). Sequenced data were analyzed as previously described (Goetz *et al.*, 2004) and the genes of interest were identified based on their sequence homology (Table 1) established through blastx and blastn against the non-redundant GenBank protein and nucleotide databases at: www.ncbi.nlm.nih.gov/BLAST/.

Reverse transcription, PCR and real time PCR

Total RNA was obtained from stimulated (LPS/Poly(I:C)) and unstimulated fish as described above for RNA extraction. The RNA was reverse transcribed using reverse transcriptase and oligo-dT primer (Promega Corporation). The cDNA was used as a template for conventional PCR and real time PCR with primers designed for selected gene sequences (Table 1). Both forward and reverse primers (Integrated DNA Technologies, www.idtdna.com) were 20 nucleotides in length, and 50% percent guanosine/cytosine content. For conventional PCR analysis, GreenGoTaq Master Mix (Promega) was used for amplification. Samples were analyzed on ethidium bromide stained gels after 38 cycles to determine optimal annealing temperatures for each primer. All real-time PCR reactions were

created as master mixes, and individual reactions contained the following: 12.5 µl of Power SYBR[®] Green PCR Mix 2x (Applied Biosystems), 0.2 µM of forward and reverse gene specific primer, 2.5 µl of cDNA and 9 µl of nuclease-free water. Fluorescence measurements were performed in a Stratagene MX 3000 system with the following parameters: one cycle at 95 °C for 10 min and 40 cycles at 95 °C for 30 s each followed by one min at the gene specific annealing temperature (Table 1). Fluorescence readings were taken at the end of each cycle and negative controls containing water instead of cDNA template were included for each primer set. Immediately after cycling, a melting curve protocol was run consisting of one cycle at 95 °C for one min, 55 °C for 30 s and 95 °C for 30 s. Temperature was increased from 55 °C to 95 °C at a rate of 0.2 °C s⁻¹ with fluorescence readings taken every 0.5 °C increase.

Completed QPCR datasets were processed with Real-time PCR Miner (Zhao & Fernald, 2005). Quantification was performed by calculating the relative mRNA concentration (R_0) for each gene for each individual using the following equation:

$$R_0 = 1/(1+E)^{Ct}$$

where E is the average gene efficiency and Ct is the cycle number at threshold. The R_0 for each gene was normalized by dividing R_0 values of the gene of interest with R_0 values for elongation factor 1-alpha (normalization gene). The process was repeated for each individual ($n = 6$ per group) separately in both the stimulated (24 and 48 h) and unstimulated groups. For the purpose of data presentation, the mean of the normalized R_0 from all individuals in the group is expressed as the percentage of the control (norm. R_0 of stimulated fish / R_0 of unstimulated x 100%).

The treatment (LPS or Poly(I:C)) RNA used for QPCR analysis of selected genes, was initially based on a tentative gene annotation. Thus, genes that were more likely to be involved in inflammatory processes were tested initially with LPS-stimulated RNA whereas genes potentially involved in antiviral responses were tested with Poly(I:C). Genes that exhibited significant regulation with the initial treatment RNA were then tested with the other treatment RNA for comparison.

Neutrophil function assays

Twenty four fish per treatment were weighed and injected with LPS and Poly(I:C) at 10 μg or 2 $\mu\text{g g}^{-1}$ body mass, respectively, as described above. Injected fish were transferred to 40 l recirculation tanks and fed twice daily to satiation. After 48 h the fish were euthanized and a kidney cell suspension prepared according to (Palić *et al.*, 2005a) with modifications. Briefly, the cell suspension was obtained by pushing the kidney tissue through 70 μm mesh cell strainer, washed in Hank's Balanced Salt Solution without Calcium, Magnesium and Phenol Red (HBSS^{CMF}, Sigma-Aldrich Corp.) cell suspension adjusted to 2×10^7 cells ml^{-1} and used in all neutrophil function assays. A total of 12 samples per treatment, including control, were used, each sample containing kidney tissue from two individual fish exposed to the same treatment

Degranulation of primary granules and respiratory burst assays were performed according to established protocols (Hermann *et al.*, 2004; Palić *et al.*, 2005a; Palić *et al.*, 2007a). Respiratory burst assay was further modified so that the 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy- H_2DCFDA) was replaced with 5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate (carboxy- H_2DFFDA) allowing for improved photostability according to the manufacturer (Invitrogen) when compared to chlorinated fluorescein derivatives. The Neutrophil extracellular traps (NETs) release assay was performed as described in Palić *et al.* (2007a,b) with modifications as per Chuammitri *et al.* (2009) and temperature optimization for fish cells. Briefly, 10 μl of standard neutrophil suspension was seeded into 96-well plates and stimulated with 1 $\mu\text{g ml}^{-1}$ PMA (phorbol myristate acetate, Sigma-Aldrich Corp.) or Hank's Balanced Salt Solution with Calcium and Magnesium (HBSS, Sigma-Aldrich Corp., background control) and incubated at room temperature for 2 h. NETs generated by stimulated neutrophils were digested with 500 mU ml^{-1} Micrococcal Nuclease (MNase; Worthington Biochemical, www.worthington-biochem.com) for 20 min at 37 °C with 5% CO_2 . The nuclease activity was inactivated with 5 mM ethylenediaminetetraacetic acid (EDTA) and the supernatants were collected for DNA quantification using Picogreen dsDNA kit (Invitrogen) according to the manufacturer's instructions. Plates were read in a fluorescence plate reader (SpectraMAX GeminiXS,

Molecular Devices, www.moleculardevices.com; excitation 492 nm, emission 520 nm; with SOFTMax PRO software 4.0).

Blood cell count

Eight fish per treatment were weighed and injected with LPS and Poly(I:C) at 10 μg or 2 $\mu\text{g g}^{-1}$ body mass, respectively, and cared for as previously described. At 24 and 48 h, 4 fish per time point and per treatment (including control) were euthanized, the caudal peduncle was severed and blood collected from the caudal vein in heparinized microhaematocrit tubes. Blood smears were prepared in duplicate for each fish. Blood smears were stained with the Diff Quick kit (Sigma-Aldrich Corp.) and leukograms were determined as total number of lymphocytes, monocytes, neutrophils and thrombocytes per mm^3 (Blaxhall & Daisley, 1973) and presented as % of control (% of control HBSS injected fish = $[\text{TCN in experiment} / \text{TCN in control}] \times 100$) where TCN stands for total cell number of the particular leukocyte type.

Statistical analysis

All data were analyzed for significance using one-way analysis of variance (ANOVA). Significantly upregulated genes observed in Poly(I:C) treatments by one-way ANOVA were analyzed by two-way factorial ANOVA to compare gene expression of the same gene, between LPS and Poly(I:C) treated fish. Levene's test for equality of variance and Tukey HSD *post hoc* comparison of means were also performed. In neutrophil functional assays, data were compared by Student's t-test for independent samples. A *P*-value equal to or less than 0.05 was considered statistically significant.

3.4. Results

A total of 5061 sequences >100 bp were sequenced from all libraries (Table 2), of which 4442 were successfully aligned against the non-redundant GenBank protein database at a BLASTX score of $E 10^{-5}$ or higher.

The majority of sequences were similar to metabolic genes and genes associated with gametes and gonads (Fig. 1). Most of the gonad-related sequences were similar to zona pellucida (**GH711349**, **GH711997**) and vitellogenin precursor proteins (**GH712352**). Metabolic genes that were represented more than 10 times included apolipoproteins (**GH713800**, **GH713151**), ATPase (**GH714327**, **GH713927**), cytochromes (**GH715322**, **GH714896**), ferritin (**GH714171**), heat shock proteins (**GH714170**, **GH714191**), globins (**GH715188**, **GH713917**), NADH dehydrogenase (**GH715059**) and superoxide dismutase (**GH713674**). The most abundant structural gene ESTs were linked to β -actin (**GH714868**), tubulins (**GH711852**), keratins (**GH715176**) and myosin (**GH712430**). Of the proteases/antiproteases, the most dominant was the antiprotease α -2-macroglobulin (**GH712494**) followed by cathepsins and various metalloproteinases. A large number of EST copies were observed for elongation factor 1-alpha (**GH713815**) and eukaryotic translation initiation factors (**GH713700**).

The sequences that were considered to be immune-related (Fig. 1) formed a diverse category of genes that are functionally related to immune responses, but are also active in other physiological roles such as transcription/translation factors (NF-kappaB inhibitor alpha-like protein B - **GH714322**). The most common immune-related ESTs belonged to the complement system and complement control protein genes. Other immune sequences represented in increasing numbers included tax1-binding protein 1 (**GH714800**), neutrophil cytosolic factor 1 (**GH714699**), LGALS3BP lectin (**GH715009**), NF- κ B inhibitor alpha-like protein B (**GH714322**), C-type lectin (**GH713209**), fibrinogen (**GH713822**), MHC class I and II (**GH715164** and **GH712694**), and - transferrin variant A1 (**GH713680**).

Selected immune genes and gene expression under immune stimulation

Of the annotated immune genes, 20 were selected for specific expression analysis in either LPS or Poly (I:C) treated fish (Table 1). These genes included chemokines, cytokines, cytokine receptors, intracellular signal transduction modulators, and carbohydrate recognition molecules. Both treatments significantly changed gene expression compared to non-treated control and to other treatment (Table 3, Fig. 2).

In LPS-treated fish, significant downregulation was observed for chemokine (C-X-C motif) ligand 12a, TNF-related apoptosis inducing ligand (TRAIL) and interferon regulatory factor 2 binding protein 1 (Table 3). LPS treatment did not upregulate any of the genes from the selected group. Significantly upregulated genes from the Poly(I:C) treated fish included chemokine CCL-C5a, and IFN-inducible and antiviral protein (Fig. 2), but Poly(I:C) did not downregulate any of the genes from the selected group. In the LPS treatment group, the greatest downregulation (9 fold decrease) was observed with the interferon regulatory factor 2 binding protein 1 both 24 h and 48 h after stimulation. The most significant upregulation in the Poly(I:C) treatment occurred in the expression of IFN-inducible and antiviral protein with a 73 fold increase in gene transcript expression after 24 h, although no significant regulation was observed for this gene at 48 h (Fig. 2B). Significantly regulated genes from the initial analysis (Table 3) were then selected for expression analysis with RNA from the second pathogen mimic. These genes were then examined using two-way factorial ANOVA and the results are presented in Table 4. All genes from the initial pathogen mimic stimulation, except TRAIL, were still significantly regulated with the two-way ANOVA, but none was affected with the second pathogen mimic stimulation.

Neutrophil function assays

Neutrophil function was assessed with respiratory burst, neutrophil extracellular trap (NET) release, and degranulation of primary granules 48h after the exposure to pathogen mimics. The respiratory burst and NETs release were significantly reduced in Poly(I:C) treated fish compared to control and LPS treatment (t-test, d.f. = 22, $P < 0.05$; Fig. 3), but degranulation of primary granules was not affected by the treatments.

Leukogram

A significant increase in blood circulating neutrophils was observed in the Poly(I:C) treated fish at 24 h (t-test, d.f. = 6, $P < 0.01$) and 48h (t-test, d.f. = 6, $P < 0.05$), but not in the LPS treatment (Fig. 4). There were no significant changes in lymphocyte, monocyte or thrombocyte counts (Fig. 4).

3.5. Discussion

In the present study, the analysis of all ESTs from *P. promelas* tissues revealed a total of 7.4 % of immune-related genes. Other similar studies have reported on average 11% immune-related ESTs (Goetz *et al.*, 2004; Iliev *et al.*, 2006; Roberts *et al.*, 2009) when based exclusively on macrophage culture or haemocyte profiles. A total of 18 % of the immune-related ESTs were most closely aligned with sequences from non-piscine vertebrates and 19% of the closest alignments were with sequences from non-cyprinid fishes. Of the sequenced genes, 3% did not align with either the protein or nucleotide databases and therefore may be novel.

The interferon regulatory factor 2 binding protein 1 (IRF-2), which was significantly downregulated in the LPS treated fish, belongs to a class of antiviral cytokines and is expressed upon viral infection (Mamane *et al.*, 1999; Taniguchi *et al.*, 2001; Shi *et al.*, 2010). IRF-2 is considered to be a transcriptional repressor and regulatory protein that can affect TLR gene expression of murine macrophages upon LPS stimulation (Taniguchi *et al.*, 2001; Nhu *et al.*, 2006). The downregulation of IRF-2 in LPS- treated *P. promelas* may have changed the TLR expression to accommodate an appropriate immune response to bacterial stimuli (Nhu *et al.*, 2006). However, no major TLRs were observed in the ESTs for further real-time PCR analysis, and although IRF-2 has a function in an antiviral response, the upregulation was not observed in the Poly(I:C) treated *P. promelas*, contrary to the reported observations for several fish species (Sun *et al.*, 2006; Jia & Guo, 2008; Shi *et al.*, 2010).

Chemokine (C-X-C motif) ligand 12a was downregulated by the LPS in *P. promelas*. CXCL chemokines play an important role in leukocyte chemotaxis and are stimulated and upregulated by LPS in mammals (Kerr *et al.*, 2009). However, fish CXCL12 was constitutively expressed at the same level in both immune and non-immune related tissues in the channel catfish, *Ictalurus punctatus* Rafinesque 1818, and could not be induced by *Edwardsiella ictaluri* challenge (Baoprasertkul *et al.*, 2005), or by LPS and phorbol myristate acetate applied to the anterior kidney of common carp *Cyprinus carpio* L. (Huising *et al.*, 2004). Since *P. promelas* is a cyprinid, and closely related to *C. carpio*, it

appears that they share the same constitutively expressed, non-inducible CXCL12a. The significant decrease in expression of this gene in LPS-treated *P. promelas* could potentially be explained by variation among individuals (Huisling *et al.*, 2004).

The fact that IFN-inducible and antiviral protein was upregulated at 24 h post treatment with Poly(I:C), but no increase was observed at 48 h, suggests that this gene is involved in the early immune response of *P. promelas* against viral infections. Interferon antiviral inducible proteins belong to Mx type proteins with a primary role as potent antiviral agents, whose transcription is initiated after stimulation by type I (IFN $_{\alpha}$ and IFN $_{\beta}$) or type III (IFN $_{\gamma}$) interferon (Holzinger *et al.*, 2007). IFNs with structural and functional properties similar to mammals and higher vertebrates have been described in fishes (Robertsen, 2006) and it has been indicated that the two types of fish IFNs are distinct from the α, β, γ mammalian IFN system and form a separate cluster with unique properties (Robertsen, 2006). However, it appears that fish IFNs utilize the same JAK-STAT molecular pathways as in mammalian systems (Leu *et al.*, 2000; Zhang & Gui, 2004a;b) to initiate transcription of Mx IFN inducible proteins (DeWitte-Orr *et al.*, 2007). The observed peak after 24h of IFN-inducible and antiviral protein gene expression in Poly (I:C) treated *P. promelas* is in agreement with published data (Kileng *et al.*, 2008) and indicates that this species can be used as a model in viral disease studies.

Expression of chemokine CCL-C5a increased in Poly(I:C)-treated fish. However, individual variation was evident in the early (24 h) transcription response of this gene to Poly(I:C) treatment, in which expression in individual fish varied from no increase to a 65 fold increase (28 fold increase on average). This variation was reduced to a 0 to 7 fold increase by 48 h (an average increase of 2.5 fold). Three out of four subfamilies of mammalian chemokines (CXC, XC, and CC) have been described in fishes, which appear to lack CX3C chemokines (Pease & Williams, 2006; Nomiya *et al.*, 2008) The CC chemokine ligand 5 (CCL-C5), also known as RANTES (regulated on activation, T-cell expressed and secreted), plays an important role in leukocyte recruitment into inflammatory sites (Pease & Williams, 2006). CCL-C5 is a ligand for CCR1, CCR3 and CCR5 receptors (Pease & Williams, 2006) and its primary targets are eosinophils, basophils and TH1 cells (Berkman *et al.*, 1996; Kuna *et al.*, 1998). Furthermore, overexpression of CCL-C5 can be a

sign of ischemia (Kraaijeveld *et al.*, 2007). In zebrafish (*Danio rerio* Hamilton, 1822), CCL-C5a is expressed from the earliest embryonic stages until adulthood, while CCL-C5b is active only in adult fish (Nomiyama *et al.*, 2008).

The oxidative burst and NETs release were reduced in anterior kidney neutrophils harvested from fish treated with Poly(I:C). The blood neutrophil count at 24 - 48 h post injection was significantly increased, indicating possible mobilization of mature inducible neutrophils from the kidneys to the circulation. Poly(I:C) transfection of human neutrophils induced multiple cytokine transcription and complex changes in signalling pathways (Tamassia *et al.*, 2008). Therefore, it is also possible that Poly(I:C) treatment caused neutrophil mobilization and migration to the bloodstream, and primed them for extravasation, effectively rendering neutrophils refractory to PMA stimulation of oxidative burst. Immature neutrophils and neutrophil precursors have limited superoxide production, explaining the decrease in respiratory burst observed in anterior kidney derived cells (Janeway *et al.*, 2008). The ROS production appears to be a crucial signal in initiating the NETosis cell death program (Papayannopoulos & Zychlinsky, 2009) and immature neutrophils of human neonates can not generate NETs (Yost *et al.*, 2009). Therefore, in *P. promelas*, immature neutrophils with reduced ROS production, or neutrophils refractory to PMA induced oxidative burst, generated less NETs than the controls. Mx proteins are expressed and upregulated in fish neutrophils after Poly(I:C) stimulation (Kileng *et al.*, 2008) and their transcription is initiated after the upregulation of IFN γ (Holzinger *et al.*, 2007). The IFN γ primes macrophages and neutrophils for an enhanced respiratory burst (Grayfer & Belosevic, 2009) but, in the current study, the effect of Poly(I:C) was the opposite. Taken together, the potential explanation for reduced oxidative burst and NET release in anterior kidney neutrophils is that Poly(I:C) induced mobilisation of mature neutrophils from the kidney tissue into the bloodstream, leaving less active or immature cells in the kidneys.

The stimulation with LPS did not cause significant changes in neutrophil function. Major proinflammatory cytokines (e.g., TNF α , IL-1, 2, 6 and 8) have not been sequenced from *P. promelas* and no significant upregulation of genes involved in bacterial recognition and killing was observed in the LPS-treated fish, suggesting that different gene transcripts

and functional endpoints need to be considered to monitor LPS effects on the immune response of this species. Since fish may not recognize Gram-negative LPS by the TLR4 mediated pathway characterized in mammals (Iliev *et al.*, 2005), different products such as monophosphoryl lipid A (MPL) (Henricson *et al.*, 1993; De Becker *et al.*, 2000; Arigita *et al.*, 2005), various lipoarabinomannans (LAMs) (Fäldt *et al.*, 2001; Nigou *et al.*, 2003) or peptidoglycans (MacKenzie *et al.*, 2010) can potentially be tested in the *P. promelas* model as alternatives to LPS. In addition to bacterial and viral simulation, β -glucan showed a significant effect on neutrophils of *P. promelas* and may be a good choice for simulation of fungal pathogens in further studies (Palić *et al.*, 2006b).

In conclusion, this study indicates that *P. promelas* can be successfully employed as a bioindicator species of immune viral stress. Poly(I:C) treatment caused changes in gene expression, leukogram and neutrophil function, indicating potential for this model system to be used in studies of the effects of environmental stressors on disease resistance. The LPS treatment did not cause significant effects on any of the monitored endpoints, suggesting a different route of response to bacterial infection in *P. promelas*, which has yet to be determined. In order to exploit the immune model fully, future studies should sequence and characterize the pro-inflammatory and anti-inflammatory cytokines in this species, to complement the existing cellular innate immune function toolbox.

3.6. Tables

Table 1. Genes used in comparative SYBRGreen qPCR expression analysis

Accession #	Best annotated hit (blast x)	Hit Accession #	% of identity	E value	Primer sequence	°C
<u>AY643400</u>	Elongation factor 1-alpha [<i>Pimephales promelas</i>].	gb AAT91089.1	100%		5'-AGCTTCTTGGCTCAGGTCAT-3' 3'-CTCATGTCACGCACAGCAA-5'	58 58
<u>GH711063</u>	Suppressor of cytokine signaling 5-like [<i>Danio rerio</i>]	ref NP_001107269.1	97% (134/137)	5.00E-76	5'-ACGCTACAATCGTTCAGTGC-3' 3'-TGAGGAAGTCCCTGCATGGT-5'	61 61
<u>GH715114</u>	TNF-related apoptosis inducing ligand TRAIL [<i>Ctenopharyngodon idella</i>]	gb AAW22592.1	97% (86/88)	1.00E-42	5'-ATCATTCGGTCAGCGGATCT-3' 3'-CCGCATTTCCACATCCCAAT-5'	61 61
<u>GH714755</u>	Natural killer cell enhancing factor [<i>Cyprinus carpio</i>]	dbj BAA32086.1	96% (192/198)	1.00E-109	5'-TGTGGAAGACAGCCGAAGAA-3' 3'-AACAGAGGCACCAATGACCT-5'	58 58
<u>GH713605</u>	IFN-inducible and antiviral protein [<i>Carassius auratus</i>]	gb AAP68824.1	93% (245/263)	1.00E-143	5'-ATCACTGCTCTGAACCCAGT-3' 3'-ACTTGATGGCCTCTTCCACA-5'	62 62
<u>GH714009</u>	Neutrophil cytosolic factor 1 [<i>Cyprinus carpio</i>]	dbj BAF73666.1	93% (264/283)	1.00E-156	5'-TTCTTTCCAGCCAGCACTA-3' 3'-ACAAGCTGACAGCGAGAGAT-5'	61 61
<u>GH714503</u>	Chemokine (C-X-C motif) ligand 12a (stromal cell-derived factor 1) [<i>Danio rerio</i>]	ref NP_840092.1	92% (90/97)	1.00E-46	5'-AGAGCAACAAGGAAGTGTGC-3' 3'-ACATGGGTGGTGTATGATGGA-5'	61 61
<u>GH711173</u>	Il17rd protein [<i>Danio rerio</i>]	gb AAI63933.1	88% (234/264)	1.00E-139	5'-AGTGCCAGCACATGATCCTA-3' 3'-GGCGTGGTCAAACACAACAT-5'	58 58
<u>GH711586</u>	Interferon regulatory factor 2 binding protein 1 [<i>Homo sapiens</i>]	ref NP_056464.1	83% (85/88)	4.00E-30	5'-TCCCAGAATTCAACGCCTCA-3' 3'-ACCATGTGTCGCCTGGAAGAT-5'	61 61
<u>GH714489</u>	NF-kappaB inhibitor alpha-like protein A [<i>Danio rerio</i>]	gb AAO26405.1	83% (232/278)	1.00E-133	5'-TGTCAGAATGACCCGTTTCT-3' 3'-ATGTCCGCTGTAGTTTGGGA-5'	61 61
<u>GH714322</u>	NF-kappaB inhibitor alpha-like protein B [<i>Danio rerio</i>]	gb AAO26406.1	82% (233/284)	1.00E-131	5'-AGCAACGACGACCCGTTTGA-3' 3'-TGATGACGGCCAAATGCAAG-5'	61 61
<u>GH714221</u>	MHC class I antigen [<i>Ctenopharyngodon idella</i>]	dbj BAD01529.1	73% (185/251)	1.00E-108	5'-AGACTGGAGCAACAGATGGT-3' 3'-TCTGCAACAGACACCTGA-5'	58 58
<u>GH713311</u>	Chemokine CCL-C5a [<i>Danio rerio</i>]	ref NP_001076375.2	68% (65/95)	9.00E-34	5'-GCAGTTTAAAGGTGACGGCT-3' 3'-AGGCAATGCTGAGTGAGGAA-5'	61 61
<u>GH713308</u>	Interferon regulatory factor 6 [<i>Danio rerio</i>]	ref NP_956892.1	66% (187/282)	1.00E-98	5'-TTCCAGATCCCGTGGAAACA-3' 3'-CATACGTGGTCACTGAACCT-5'	58 58

Table 1. (continued)

<u>GH715816</u>	MHC class II beta chain [<i>Cyprinus carpio</i>]	emb CAA88847.1	57% (141/244)	4.00E-80	5'-AGAAGCAGCCGTCTACAACA-3' 3'-TAGACCATGGGCTTGCTGAA-5'	58 58
<u>GH713209</u>	C-type lectin [<i>Cyprinus carpio</i>]	dbj BAA95671.1	54% (90/166)	6.00E-48	5'-TGCTGAGTCTGTTGCCTTCT-3' 3'-TGGTTAAACACGGCTCGCTA-5'	59 59
<u>GH714782</u>	Interleukin 13 receptor, alpha 1 [<i>Danio rerio</i>]	ref NP_001104307.1	54% (118/215)	7.00E-62	5'-TTGACATGGAAAGAGCCGGA-3' 3'-TTGTGTTCTGTCACCAGCCT-5'	61 61
<u>GH715146</u>	Interleukin 12a [<i>Danio rerio</i>]	ref NP_001007108.1	45% (80/176)	3.00E-34	5'-TGAAGTCCTACAAGCCCACA-3' 3'-TGTGAGCGACATCACTTCCT-5'	60 60
<u>GH712297</u>	Tumor necrosis factor receptor 1 [<i>Xenopus laevis</i>]	ref NP_001108251.1	35% (30/85)	2.00E-07	5'-AGGAAACGTGCATTGTCAGC-3' 3'-AGTTCCGGTGGTCTTGTTCT-5'	61 61
<u>GH714178</u>	Toll-like receptor 5 [<i>Bos taurus</i>]	gb AAT48489.2	34% (100/292)	1.00E-38	5'-CGTGACTCTGCAAGACATGA-3' 3'-TCGCCTCAAGACCCTTGAAT-5'	59 59
<u>GH712136</u>	Interleukin 16 [<i>Gallus gallus</i>]	ref NP_989683.3	30% (65/214)	6.00E-12	5'-TGACCCCTGGGTAAAGCTGA-3' 3'-TAATCCCAAAGGTCCTGCCA-5'	58 58

Table 2. Characteristics of the ESTs from cDNA libraries of *Pimephales promelas* tissues and anterior kidney cells.

Library	LPS	Poly(I:C)	Anterior kidney
Type	Stimulated ^a	Stimulated*	Cells: LPS
Sequences > 100bp	1825	1123	2113
# of annotated sequences			
1.00E-05 cutoff	1508	983	1951
Average seq. length	750	790	755
# of Contigs	194	140	260
# of Singletons	984	640	965
% Redundancy	46	43	54

*combined data for 24 and 48 hour stimulations

Table 3. Immune genes significantly regulated by lipopolysaccharide (LPS) or polyinosinic-polycytidylic acid (Poly(I:C)).

Gene	Treatment	<i>P</i>	Post hoc comparisons of means (Tukey HSD)		% of the control
Chemokine (C-X-C motif) ligand 12a	LPS	+	24h vs. 48h	NS	
			24h vs. control	NS	50
			48h vs. control	+	30
TNF-related apoptosis inducing ligand TRAIL	LPS	+	24h vs. 48h	NS	
			24h vs. control	NS	61
			48h vs. control	+	39
Chemokine CCL-C5a	Poly(I:C)	+	24h vs. 48h	+	
			24h vs. control	+	2829
			48h vs. control	NS	238
Interferon regulatory factor 2 binding protein 1	LPS	+	24h vs. 48h	NS	
			24h vs. control	+	13
			48h vs. control	+	12
IFN-inducible and antiviral protein	Poly(I:C)	+	24h vs. 48h	+	
			24h vs. control	+	7298
			48h vs. control	NS	1480

NS, not significant; +, $P < 0.05$ by one way ANOVA.

Table 4. Immune genes regulation by lipopolysaccharide (LPS) or polyinosinic-polycytidylic acid (Poly(I:C)). Only significantly regulated genes and significant post hoc comparison are presented.

Gene	Treatment		Post hoc comparisons of means (Tukey HSD)	
	Effect	<i>P</i>	Effect	<i>P</i>
Chemokine CCL-C5a	Treatment	+	Poly(I:C) 24h X Poly(I:C) 48h	++
	Time	+	Poly(I:C) 24h X Poly(I:C) control	++
	Treatment x time	+	Poly(I:C) 24h X LPS 24h	++
			Poly(I:C) 24h X LPS 48h	++
			Poly(I:C) 24h X LPS control	++
IFN-inducible and antiviral protein	Treatment	++	Poly(I:C) 24h X Poly(I:C) 48h	+
	Time	++	Poly(I:C) 24h X Poly(I:C) control	++
	Treatment x time	+	Poly(I:C) 24h X LPS 24h	++
			Poly(I:C) 24h X LPS 48h	++
			Poly(I:C) 24h X LPS control	++
Chemokine (C-X-C motif) ligand 12a	Treatment	NS	LPS 48h vs LPS control	+
	Time	++		
	Treatment x time	NS		
Interferon regulatory factor 2 binding protein 1	Treatment	NS	LPS 24h vs LPS control	+
	Time	++	LPS 48h vs LPS control	+
	Treatment x time	NS		

NS, not significant; +, $P < 0.05$; ++, $P < 0.01$ by two-way ANOVA.

3.7. Figure captions and figures

Figure 1. Classification of cDNA sequences obtained from lipopolysaccharide - LPS and polyinosinic-polycytidylic acid - Poly(I:C) treated *Pimephales promelas*, based on function or structural similarity. “Unknown” indicates genes with significant (<95%) identities to GenBank sequences for which the function has not yet been identified. “Similar or predicted, but unknown” indicates genes with significant identities to GenBank sequences, but vaguely labelled as “hypothetical protein”, “similar to” or presented only with a code from which a function could not be inferred). “Novel” indicates genes with no significant blastx or blastn alignment; “Other” indicates genes that had significant identities to

GenBank entries of known function but could not be classified into any of the categories on the figure.

Figure 2. Effects of lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid (Poly(I:C)) treatment, at 24 and 48 h, on changes in *Pimephales promelas* immune gene expression. The changes in expression are presented as normalized R_0 values (y-axis), with S.E.M. (boxes) and S.D. (whiskers) for $n = 6$. Asterisk indicates statistically significant effect ($P < 0.05$).

Figure 3. Effect of lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid (Poly(I:C)) treatment on neutrophil function in *Pimephales promelas*, measured as oxidative burst, neutrophil extracellular trap release (NET release), and degranulation of primary granules (degranulation). Bars represent mean value for $n = 12$ samples presented as % of non-stimulated control (exposed to Hank's balanced salt solution with calcium and magnesium, HBSS). Color of the vertical bars represents different treatments: LPS (dark grey), Poly(I:C) (light grey), HBSS (white). Error bars indicate standard error of means (S.E.M.); different letters indicate significant difference ($P < 0.05$).

Figure 4. Effect of lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid (Poly(I:C)) treatment, 24 and 48 h, on blood neutrophil count in *Pimephales promelas*. The neutrophil count (y-axis) is presented as the percentage of average neutrophil number observed in control fish, with S.E.M. (boxes) and S.D. (whiskers) for $n = 8$. Asterisk indicates a statistically significant effect ($P < 0.05$).

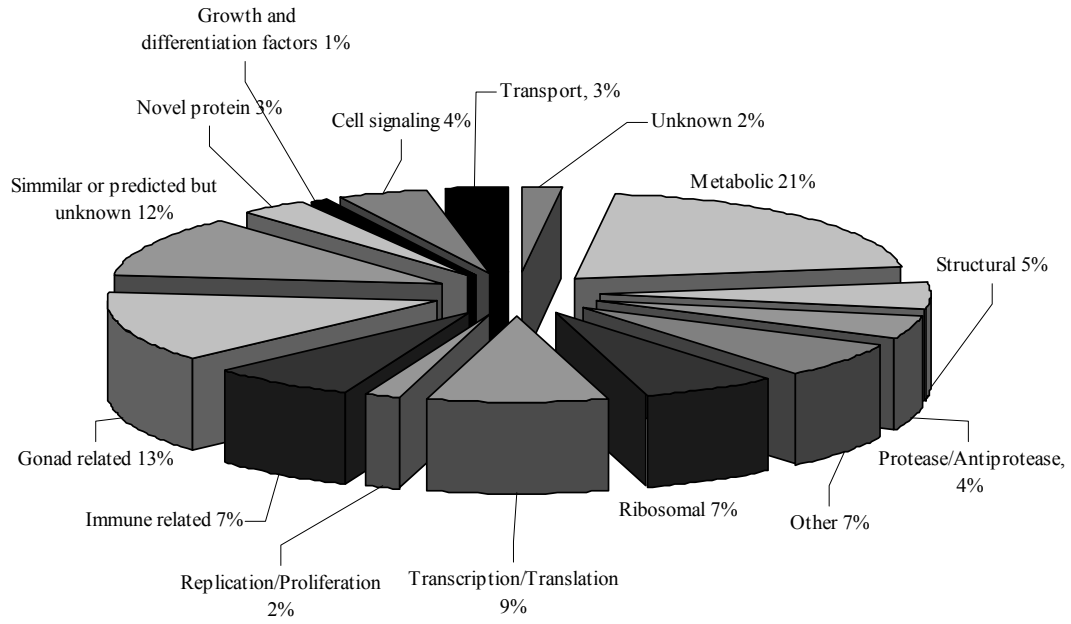


Figure 1.

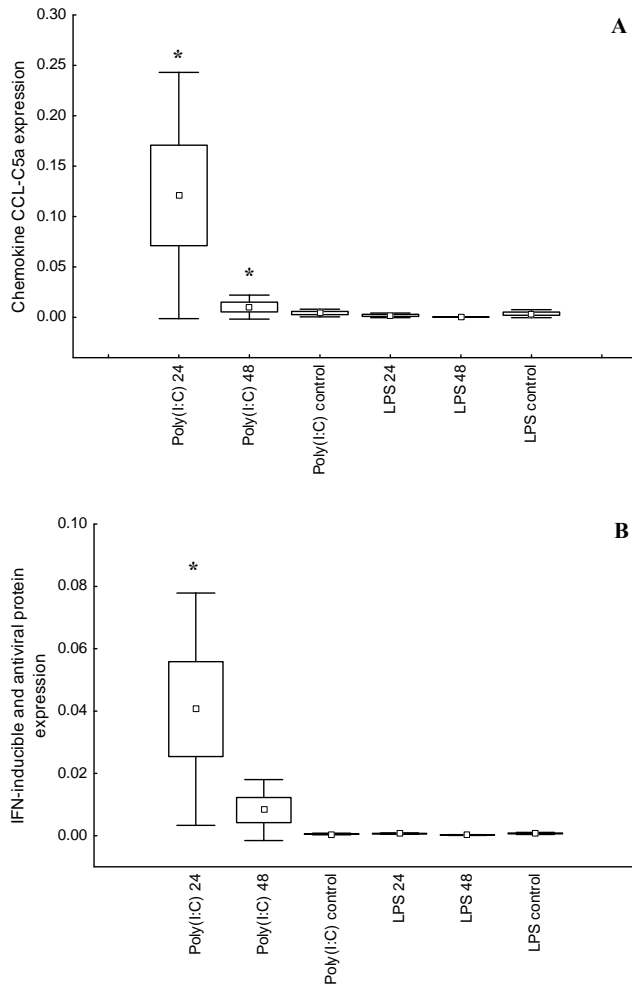


Figure 2.

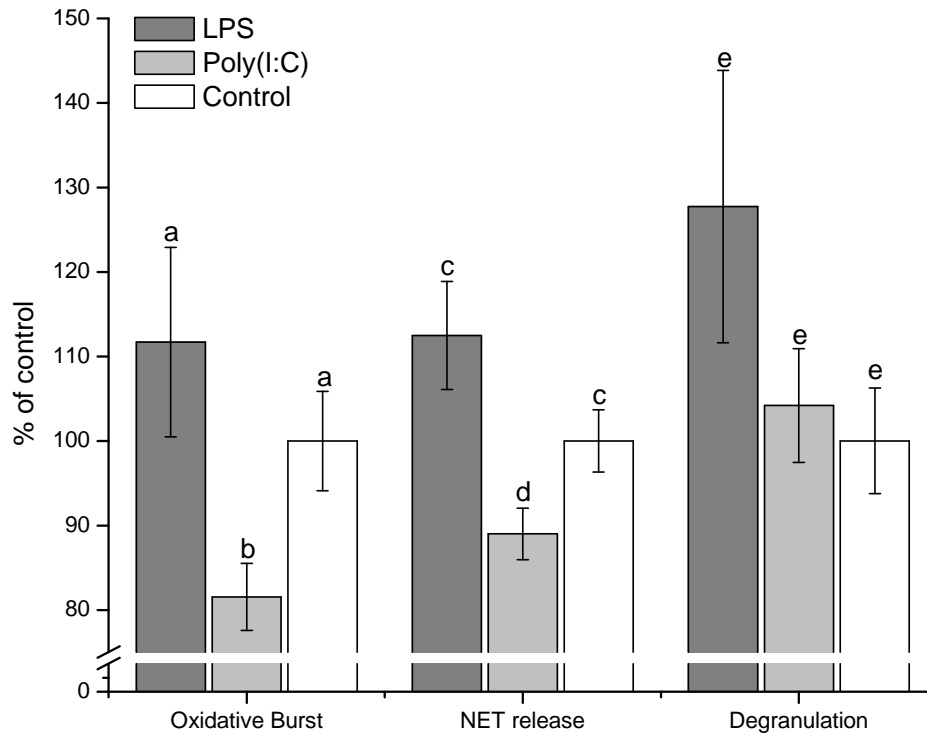


Figure 3.

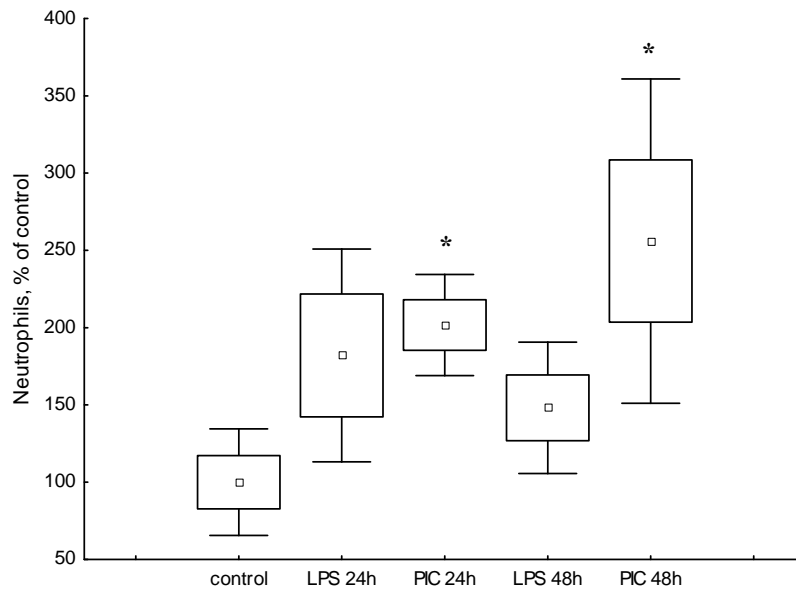


Figure 4.

3.8. References

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CHAPTER 4. Effects of nanosized titanium dioxide on innate immune system of fathead minnow (*Pimephales promelas* Rafinesque, 1820)

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4.1. Abstract

Effects of nanosized (<100 nm) titanium dioxide (TiO₂) particles on fish neutrophils and immune gene expression was investigated using the fathead minnow (*Pimephales promelas*). Expanded use of TiO₂ in the cosmetic industry has increased the potential exposure risk to aquatic ecosystems and human health. Effects of nano-TiO₂ on neutrophil function of the fathead minnow was investigated using oxidative burst, neutrophil extracellular traps (NETs) release and degranulation of primary granules. The innate immune gene expression was determined with quantitative PCR (qPCR). Application of 0.1 µg mL⁻¹ of nano-TiO₂ *in vitro* stimulated oxidative burst and NET release. Intraperitoneal injection of 10 µg g⁻¹ of nano-TiO₂ caused a significant decrease in oxidative burst, NETs release and degranulation (21%; 11%; and 30%, decrease respectively). Fish exposed to nano-TiO₂ for 48 h *in vivo* had significantly increased expression of interleukin 11, macrophage stimulating factor 1, and neutrophil cytosolic factor 2 (4; 2.5; and 2 fold increase, respectively). Nano-TiO₂ has potential to interfere with the evolutionary conserved innate immune system responses, as evidenced with observed changes in gene expression and neutrophil function. This finding encourages the use of fish models in the studies of nanoparticle immunotoxicity. The lowest significant response concentration studied *in vitro* is four times greater than the estimated environmental concentration for TiO₂ (0.025 µg mL⁻¹) causing concern about potential impact of nano-TiO₂ on aquatic animals and ecosystems.

Keywords: titanium dioxide nanoparticle, immunotoxicity, fish, neutrophil, immune function, gene expression.

4.2. Introduction

The increased application of nanotechnology in biomedical science, electronics, cosmetics and pharmaceutical industry has potential to increase the release of nanoparticles into environment. By 2015 the worldwide market for products with nanotechnology will reach its first trillion dollars (Roco, 2005) and it is expected that production of engineered nanoparticles is going to reach approximately 60 000 tons in 2011, increasing 30 fold from an estimated 2004 production of 2000 tons (Nowack and Bucheli, 2007).

Although some acute effects of several nanoparticle types on aquatic organisms have been reported (Gagné et al., 2008; Lovern and Klaper, 2006; Reeves et al., 2008) it is likely that chronic exposure may be of equal ecological importance. Exposure to sublethal pollutant concentrations has been determined to interfere with normal life processes of aquatic organisms such as feeding, reproduction, and defense (Sprague, 1971). Current sublethal exposure information of nanoparticles on aquatic vertebrates is limited, and potential for long-term ecological impacts of nanoparticle exposure remains unknown.

The opacity and whitening properties of metal-oxide nanoparticles have made these pigments desirable for a variety of industrial applications, such as sunscreens, soaps, shampoos and other cosmetics (Melquiades et al., 2008), and they are also used as additives to paint and building materials. Among all nanoparticles, titanium dioxide (TiO₂) nanoparticles are of biggest ecotoxicological concern due to the rapid increase of anthropogenic input of nano-TiO₂ into the environment. Estimated environmental concentrations of titanium dioxide in nanoparticle are in the range from 0.0007 to 0.0245 µg mL⁻¹ (Mueller and Nowack, 2008; Pérez et al., 2009). These estimates were made for the Nordic countries which only account for 0.68% of global nanoparticle production. The U.S., Japan, EU, South Korea and China currently lead in global nanoparticle production (Mueller and Nowack, 2008). There is no available data about estimated environmental concentration

for TiO₂ in the U.S. which accounts for 20% of global nanoparticle production (Diamond, Personal communication).

In aquatic organisms, the lethal threshold of TiO₂ is correlated to particle size (Lovern and Klaper, 2006). When TiO₂ was filtered through a 0.22 µm filter a concentration of 10 µg mL⁻¹ caused 100% mortality of *D. magna*, while unfiltered nanoparticles at 500 µg mL⁻¹ caused only 9% mortality. Since metal-oxide particles behave like ice crystals in solution, removing aggregates by filtering prevents them from rejoining each other, thus individual particles have a greater total surface area to interact with cells or a living organism compared to the aggregate form. Regulations in the USA allow TiO₂ and zinc-oxide (ZnO) as the only two inorganic UV filters that can be used in the cosmetic industry at 25% maximum product content (FDA, 1999). This regulation coupled with the widespread use of nano-TiO₂ in industry allows for continuous exposure of aquatic organisms to nano-TiO₂.

In fish, proper immune system function is closely intertwined with the environment, and changes of the cellular innate immune responses can lead to serious failure in organismal defenses (Magnadóttir, 2006; Whyte, 2007). Some nanomaterials appear to induce toxic responses at both the molecular and organismal levels (Farré et al., 2009; Khan et al., 2007), but the great diversity of nanoparticles and lack of research data does not allow for complete understanding of observed interactions. Therefore, mechanistic studies are needed to evaluate the toxic potential of different nanoparticles and their impact on immune system function and fish populations. There is limited information about nano-TiO₂ immunotoxicity, and there is even less information on the toxic effects of nano-TiO₂ on innate immunity, especially neutrophils in aquatic vertebrates. Recently described effects of nano-TiO₂ on mammalian neutrophils and inflammation include the ability of neutrophils to phagocytize and absorb nano-TiO₂ (Kumazawa et al., 2002), and induction of neutrophil apoptosis (Wang et al., 2002). Nano-TiO₂ also induced activation of neutrophils by phosphorylation of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinases-1/2 (Erk-1/2) (Gonçalves et al., 2010). Previously, neutrophil extracellular trap release (NET) was induced by nano-gold particles (Bartneck et al., 2009) but the nano-TiO₂ stimulation of NET release have for the first time been reported here.

The fathead minnow (*Pimephales promelas* Rafinesque, 1820) is a small (<10 cm) cyprinid species, abundant in North American freshwater ecosystems and frequently used as a standard model organism in environmental and aquatic toxicology assessments and studies (Ankley and Villeneuve, 2006; USEPA, 1987; Villeneuve et al., 2008). Recently, *P. promelas* was also used as a model to study innate immune responses to stress (Palić et al., 2006b) and immunomodulators (Palić et al., 2006a) as well as in toxico/immunogenomics studies (Biales et al., 2007; Mager et al., 2008; Villeneuve et al., 2008). In fathead minnows the major leukocyte types have been described as lymphocytes, monocytes, neutrophils, and thrombocytes (Palić et al., 2005a); and neutrophils were reported to perform respiratory burst, exocytosis of primary granules, and neutrophil extracellular trap release (Palić et al., 2005b; Palić et al., 2006b; Palić et al., 2007b).

Neutrophils are an important component of host defense against many bacterial, viral and fungal infections, and the evaluation of neutrophil function is valuable for assessment of the health status of individuals and animal populations (Smith and Lumsden, 1983). Their ability to phagocytize and kill microorganisms and cellular debris is essential for normal development and survival of animal populations (Segal, 2005). In response to inflammatory stimuli, neutrophils migrate from the circulating blood into infected tissues, where they efficiently bind, engulf, and kill bacteria by proteolytic enzymes, antimicrobial proteins, and produce reactive oxygen species (Dalmo et al., 1997). Fish neutrophils are generally considered to perform similar function as their mammalian counterparts and phagocytic, chemotactic, and bactericidal functions have been described (Whyte, 2007).

To date, no apparent effort has been made to evaluate the impact of nanoparticles on fish neutrophils or other aspects of the fish innate immune system function. This manuscript aimed for the first time to investigate changes in neutrophil function of fish exposed to nanosized titanium dioxide and investigates the expression of genes involved in innate immune response and detoxification processes.

4.3. Materials and methods

Animal care

Adult fathead minnows (average weight 4.5 g) were maintained in the Iowa State University College of Veterinary Medicine Laboratory Animal Resources Facility, Ames, Iowa, USA. Fish were housed in a water recirculation system supplied with dechlorinated tap water at 20 °C in 120 L tanks, and fed twice daily with live brine shrimp larvae and dried flake food (2:1 w/w mixture of Aquatox[®] and Plankton/Krill/Spirulina flake food, Zeigler Bros Inc, PA, USA). Fathead minnows were cared for in accordance with approved Iowa State University animal care guidelines.

Nanoparticle characterization

Nanoparticles were characterized using the light scattering technique with Malvern Zetasizer Nano ZS-90 instrument (Malvern Instruments Ltd, Malvern, Worcestershire, UK). Size distribution, zeta potential and conductivity were determined for nano-TiO₂ particles suspended in all types of media used in the experiments. (HBSS without Ca, Mg and Phenol Red; HBSS with Ca, Mg no Phenol Red; egg water).

Toxicity tests

In order to determine the range of sublethal concentrations for further immunotoxicogenomic tests an alternative methodology considering Fish Embryo Toxicity Assay (FET) was used (Braunbeck et al., 2005; Braunbeck and Lammer, 2006; OECD, 2006). Recently, use of fish embryos in nanomaterial toxicity testing has been accepted as standard methodology for studying the absorption, accumulation, distribution and toxicity of nanomaterials in animals (Kashiwada, 2006). The absorption and toxicity of nano-TiO₂ (Yeo and Kang, 2009; Zhu et al., 2008) and other metallic nanoparticles (Asharani et al., 2008; Lee et al., 2007; Xiaoshan and et al., 2009) have been studied using zebrafish embryo toxicity assays. Briefly, the seven day static renewal acute toxicity test was performed with six different concentrations (0.01, 0.1, 1, 10, 100, and 1000 µg mL⁻¹) of nano-TiO₂ (anatase, nanopowder, <25nm, 99.7% metals basis; Sigma-Aldrich Corp, St. Louis, MO,

USA) using fathead minnow embryos less than 24 hours post fertilization (hpf) at test start. Nano-TiO₂ particles were introduced to 12 well microtiter plates (6 ml, 5 embryos per well). Nano-TiO₂ suspension in fish egg water (60 µg of sea salt [Instant Ocean, Kingman, AZ, USA] per mL of deionized water) (Westerfield, 2000) was sonicated for 60 min and filtered through 0.22 µm general purpose filter (Costar, Cambridge, MA, USA, # 8110) to remove aggregated nanoparticles larger than 220 nm prior to loading wells following previously suggested standard procedure (Lovern and Klaper, 2006). Each group test was performed with six replicas (total of 30 embryos per group), embryo mortalities were recorded and 80% of the test solution was renewed daily. Embryos were incubated in wells with the nanoparticle suspension for seven days at 25 °C and a 14 h light, 10 h dark cycle. Positive and negative controls (8 mg L⁻¹ 3,4-dichloroaniline (Sigma-Aldrich Corp) (Call et al., 1987); no active substance, respectively) were performed.

In vitro neutrophil function assays

Adult fathead minnows were euthanized, kidneys from eight fish were pooled as a single sample, and cell suspensions were prepared (Palić et al., 2005b). A total of 16-24 samples have been used per assay and readings were performed either in duplicates (respiratory burst, degranulation) or triplicates (NETs release). The average myeloperoxidase (MPO) positive to MPO negative cell ratio (Palić et al., 2005a) in the final suspension was 82%, indicating that majority of the cells were from myelopoietic lineage. Cell suspensions were adjusted to 2×10^7 cells mL⁻¹ and used in all neutrophil function assays. Cells were exposed to standard stimulants (phorbol myristate acetate, PMA, 1 µg mL⁻¹; and calcium ionophore A23187, CaI, 5 µg mL⁻¹; Sigma-Aldrich Corp.), nano-TiO₂ at 0.1, 1, 10, 100, and 1000 µg mL⁻¹, a combination of nano-TiO₂ and standard stimulants, or negative control (Hanks Balanced Salt Solution with Ca, Mg, no Phenol Red, HBSS; Mediatech – CellGro, AK, USA). Nano-TiO₂ was suspended in HBSS with Ca, Mg and no Phenol Red and prepared as described in section *Toxicity tests*. The process of absorption and uptake of nano-TiO₂ particles in saline solution by neutrophils has been described earlier (Kumazawa et al., 2002).

Degranulation of primary granules and respiratory burst assays were performed according to established protocols (Hermann et al., 2004; Palić et al., 2005b). The respiratory burst assay was further modified so that the 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) was replaced with 5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate (carboxy-H₂DFFDA) allowing for improved photostability according to the manufacturer (Invitrogen) when compared to chlorinated fluorescein derivatives. The neutrophil extracellular trap release assay was performed as described (Palić et al., 2007a; Palić et al., 2007b) with modifications as per (Chuammitri et al., 2009) and temperature optimization for fish cells. Briefly, 10 µL of standard neutrophil suspension was seeded into 96-well plates and stimulated with 1 µg mL⁻¹ PMA (standard stimulant – positive control), HBSS with Ca, Mg, no Phenol Red (background control), or nano-TiO₂ (0.1, 1, 10, 100, and 1000 µg mL⁻¹), and incubated at room temperature for two hours. NETs generated by stimulated neutrophils were digested with 500 mU mL⁻¹ Micrococcal Nuclease (MNase; Worthington Biochemical, USA) for 20min at 37 °C with 5% CO₂. The nuclease activity was inactivated with 5mM EDTA and the supernatants were collected for DNA quantification using Picogreen dsDNA kit (Molecular Probes, USA) according to manufacturer's instructions. Plates were read in a fluorescence plate reader (SpectraMAX GeminiXS, Molecular Devices, USA; excitation 492 nm, emission 520 nm; with SOFTMax PRO software 4.0). The NET-DNA release was analyzed using GraphPad Prism version 5.0 (GraphPad Software, USA). The NET-DNA release index was calculated using the following formula:

$$\text{NET-DNA release index} = \text{DNA}_{\text{stim}} / \text{DNA}_{\text{non-stim}}$$

where DNA is the average amount of DNA content released from neutrophils.

In vivo neutrophil function assays

To investigate the effects of *in vivo* application of nano-TiO₂, adult fathead minnows (two years old; mixed sex; 4.5 g average body weight) were quickly netted from the tanks and anesthetized with 100 mg L⁻¹ of aerated and buffered (sodium bicarbonate, pH 8.0) solution of the tricaine methane sulphonate (MS-222, Argent Laboratories, Redmond WA, USA). Upon entering the third stage of anesthesia (Palić et al., 2006b), fish were weighed

and injected intraperitoneally with $10 \mu\text{g g}^{-1}$ of fish body weight nano-TiO₂ suspended in HBSS without Ca, Mg and Phenol Red and prepared as previously described in section *toxicity tests*. The $10 \mu\text{g g}^{-1}$ was the lowest dose that caused observable effects on NETs release (Fig. 1-C) during *in vitro* study. Intraperitoneal injection (I.P.) of nanoparticles in fish models allows for direct interaction of immune cells with the nanoparticles (Scown et al., 2009), and has been an accepted approach for toxicological, disease challenge, and immunological studies in various species (Janeway et al., 2008). Injected fish were transferred to 40 L recirculation tanks and fed twice daily to satiation. After 48 h the fish were euthanized, kidneys from four fish pooled as a single mixed sex sample, and kidney cell suspensions prepared according to (Palić et al., 2005b) with modification. Briefly, the cell suspensions were obtained by pushing the kidney tissue through a 70 μm mesh cell strainer, washed and adjusted to final concentration of 2×10^7 cells mL^{-1} . Sixteen samples per each treatment and control (fish injected with HBSS) were used in neutrophil function studies. The time point of 48 h post injection to test for neutrophil function changes was based on studies of stress and immunomodulatory responses in this fish species (Palić et al., 2006a; Palić et al., 2006b) as the earliest time point where observable effects were expected. The data is presented as % of neutrophil activity in control (HBSS injected) fish using the following formula:

$$\% \text{ of control} = \text{Mean of [Average activity of nano-TiO}_2 \text{ injected fish / Activity of HBSS injected fish]} \times 100$$

Activity refers to stimulation index or % degranulation as described in section *In vitro neutrophil function assays*.

Total RNA extraction

Six adult minnows per treatment and control each were anesthetized and intraperitoneally injected with nano-TiO₂ as described in section *In vivo neutrophil function assays*. Injected fish were transferred to 40 L recirculation tanks to recover from anesthesia, fed twice daily up to satiation, and were observed for 48 h for mortalities or behavioral changes (no mortalities or changes were observed). Fish were collected and euthanized at 48

h post injection, and the anterior kidney, liver, spleen, and gills were collected from each individual. Total RNA extraction was performed according to the acid guanidinium thiocyanate-phenol-chloroform extraction protocol (Chomczynski and Sacchi, 2006) using TRI Reagent® as an improved version of the original single-step total RNA isolation developed by Chomczynski (Chomczynski and Sacchi, 1987). All collected organs for each individual fish were pooled in a 1.5 mL centrifuge tube containing 1 mL of Tri-Reagent (Molecular Research Center, Inc. Cincinnati, OH, USA), total RNA extracted and the concentration was assessed using a Nanodrop spectrophotometer (NanoDrop products, Wilmington, DE, USA). The pooling of tissues to study immune gene expression at the organismal level in fish and qPCR analysis of targeted genes in mixed tissues/whole fish homogenates has been described in similar studies (Balcazar et al., 2007; Hyyti et al., 1998; Sæle et al., 2009).

Reverse transcription PCR and real time PCR

Total RNA was obtained from stimulated (TiO₂) and unstimulated (HBSS without Ca, Mg, and Phenol Red) fish as described above for RNA extraction. The RNA was reverse transcribed using reverse transcriptase and oligo-dT primer (Promega Corporation, Madison, WI, USA). The cDNA was used as a template for conventional PCR and real time PCR with primers designed for selected gene sequences (Tab 1). Both forward and reverse primers (Integrated DNA Technologies, Coralville, IA, USA) were 20 nucleotides in length, and 50% percent guanine/cytosine content. For conventional PCR analysis, GreenGoTaq Master Mix (Promega) was used for amplification. Samples were analyzed on ethidium bromide stained gels after 38 cycles to determine optimal annealing temperatures and verify product for each primer. All real-time PCR reactions were created as master mixes, and individual reactions contained the following: 12.5 µL of Power SYBR® Green PCR Mix 2x (Applied Biosystems), 0.2 µM of forward and reverse gene specific primer, 2.5 µL of cDNA and 9 µL of nuclease-free water. Fluorescence measurements were performed in a Stratagene MX 3005 system with the following parameters: one cycle at 95 °C for 10 minutes and 40 cycles at 95 °C for 30 seconds each followed by one minute at the gene specific annealing temperature (Tab. 1). Fluorescence readings were taken at the end of each cycle and

negative controls containing water instead of cDNA template were included for each primer set. Immediately after cycling, a melting curve protocol was run consisting of one cycle at 95°C for one minute, 55°C for 30 seconds and 95°C for 30 seconds. Temperature was increased from 55°C to 95°C at a rate of 0.2°C/second with fluorescence readings taken every 0.5°C increase.

Completed QPCR datasets were processed with Real-time PCR Miner (Zhao and Fernald, 2005). Quantification was performed by calculating the relative mRNA concentration (R_0) for each gene for each individual using the following equation: $R_0 = 1/(1+E)^{Ct}$, where E is the average gene efficiency and Ct is the cycle number at threshold. The R_0 for each gene was normalized by dividing R_0 values of gene of interest with R_0 values for elongation factor 1-alpha and β actin (Zhao and Fernald, 2005). Gene regulation was considered significant only when both the normalization to elongation factor 1-alpha and β actin showed that there was a significant difference between control and the treatment. For graph presentation purposes only graphs using the normalization to elongation factor 1-alpha were used, as there is no difference between the normalization to β actin. The process was repeated for each group (n=6 per group) separately in both the stimulated and unstimulated groups.

Leukogram

Six adult fathead minnows per treatment and control group were individually weighed and intraperitoneally injected with TiO₂ nanoparticles at 10 $\mu\text{g g}^{-1}$ of fish body weight and cared for as described in section - *In vivo neutrophil function assays*. At 48 h post injection minnows were euthanized, blood was collected from caudal vein in heparinized microhematocrit tubes, and blood smears were prepared in duplicate for each fish. Blood smears were stained with a Diff Quick kit (Sigma-Aldrich Corp) and leukograms were determined as total number of lymphocytes, monocytes, neutrophils and trombocytes per mm^3 (Blaxhall and Daisley, 1973) and presented as % of control using the following formula:

$$\% \text{ of control HBSS injected fish } N = (\text{TCN in experiment} / \text{TCN in control}) \times 100$$

TCN: total cell number of particular leukocyte type.

Statistical analysis

All data were analyzed for significance using One-Way ANOVA followed by Dunnett's procedure post hoc comparison of means between single control and multiple experimental groups, and a *P*-value equal to or less than 0.05 was considered statistically significant. In addition to Dunnett's procedure student t-test was performed according to accepted statistical methodology for testing multiple pairs of control/treatment effects on neutrophil function in human and animal models, which also provide confidence in results to be further used as relevant clinical information (Hayashi et al., 2003; Kumazawa et al., 2002).

4.4. Results

Nanoparticle characterization

The zeta potential of nano-TiO₂ particles suspended in HBSS without Ca, Mg, and Phenol Red; HBSS with Ca, Mg, no Phenol Red; and egg water was -8.87, -5.61 and -9.75 mV respectively, while the conductivity was 15.4, 15.35 and 3.33 mS cm⁻¹ in the same order of appearance. Size characterization revealed that the particle size is unimodal following a normal distribution with a bell shaped curve as seen in Fig. 1A and B, but multimodal in Fig. 1C. Average diameter of nano-TiO₂ particles dissolved in HBSS without Ca, Mg, and Phenol Red was 86 nm with a single peak at 88 nm and area intensity of 100%. Particles suspended in HBSS with Ca, Mg, no Phenol Red had average diameter of 66 nm with a single peak at 66 nm and area intensity of 100%. Particles suspended in egg water had an average diameter of 43 nm with 3 peaks at 1 nm, 7 nm and 75 nm with respective area intensity of 36, 29.4 and 34.5%.

Toxicity tests

Nano-TiO₂ did not cause significant mortality in the fish embryo toxicity assay, and the mortality rate was < 10% seven days post exposure for all tested concentrations. 3,4-dichloroaniline was used as a positive control and caused 40% mortality after 96 h and 70% mortality seven days post exposure, as expected (Call et al., 1987). There were no

observable defects during development of embryos exposed to nano-TiO₂.

Neutrophil function assays

Nano-TiO₂ caused a significant increase in respiratory burst compared to non-stimulated controls at all tested concentrations *in vitro* ($P < 0.05$). Increases in respiratory burst were observed both when a combination of nano-TiO₂ and PMA (Fig. 2A) or nano-TiO₂ alone was used (Fig. 2B). A significant increase in neutrophil extracellular trap release was observed when PMA was used together with 10 $\mu\text{g mL}^{-1}$ of nano-TiO₂ ($P < 0.05$; Fig 2C), and the significance level at higher nano-TiO₂ concentrations was at $P < 0.001$. At the two lowest concentrations a near significant increase in NET release ($P = 0.07$) was also observed. Nano-TiO₂ alone did not induce NETs release *in vitro* in unstimulated neutrophils (Fig. 2D) and nano-TiO₂ did not affect the degranulation of primary neutrophil granules in stimulated/unstimulated neutrophils.

Nano-TiO₂ significantly suppressed neutrophil function after *in vivo* exposure ($P < 0.05$). The respiratory burst was decreased by 21%, NETs release by 11% and degranulation of primary granules by 29% (Fig. 3).

Gene expression

Exposure to TiO₂ nanoparticles caused a significant upregulation of genes for interleukin 11 (4 fold increase), macrophage stimulating factor 1 (2.5 fold increase) and neutrophil cytosolic factor 2 (2 fold increase), (Fig. 4 A-C). Other investigated genes (Tab 1) were not significantly regulated by the treatment.

Blood cell count

There was no difference between the control and treatment groups in the number of circulating lymphocytes, monocytes, neutrophils and trombocytes. In control group average number (\pm SEM) of lymphocytes, monocytes, neutrophils and trombocytes was $132,580 \pm 29,361$; $3,372 \pm 2,437$; $63,648 \pm 29,435$; and $575,200 \pm 92,105$ per μL of blood, respectively. The average number of blood leukocytes in the group exposed to nano-TiO₂

was $123,540 \pm 21,970$; $8,202 \pm 4,160$; $85,457 \pm 22,917$; and $456,800 \pm 72,016$ per μL , presented in the same order as above.

4.5. Discussion

Our *in vitro* data have shown that concentration of $0.1 \mu\text{g mL}^{-1}$ of TiO_2 nanoparticles can stimulate fish neutrophil respiratory burst. This concentration is only four times greater than the estimated environmental concentration of $0.025 \mu\text{g mL}^{-1}$ (Mueller and Nowack, 2008; Pérez et al., 2009). Titanium particles of up to $3 \mu\text{m}$ can be phagocytized by mammalian neutrophilic granulocytes and stimulate them to produce reactive oxygen species (ROS) and perform oxidative burst (Kumazawa et al., 2002). Although the route for phagocytic uptake of nanoparticles is not fully explained, one of the possibilities involve phagocytosis mediated through $\text{Fc}\gamma$ receptor engagement and activation (Dobrovolskaia and McNeil, 2007). Engagement of $\text{Fc}\gamma$ receptors during phagocytosis initiates activation of protein kinase C and subsequent formation of NADPH oxidase complex responsible for delivering ROS into the phagosome (Dekker et al., 2000).

Exposure of stimulated neutrophils to nano- TiO_2 *in vitro* significantly increased the release of neutrophil extracellular traps, indicating a potential shift to NETosis cell death pathway. NETs release is closely related to the process of respiratory burst and ROS production appears to be a crucial signal involved in initiating the NETosis cell death program (Papayannopoulos and Zychlinsky, 2009). Superoxide ions formed via respiratory burst can dismutate to hydrogen peroxide and dioxygen, spontaneously or through catalyzation. The presence of hydrogen peroxide in the phagosome is responsible for inducing downstream signaling leading to NETs release and NETosis (Fuchs et al., 2007). The observed increase in oxidative burst in neutrophils exposed to nano- TiO_2 is consistent with increase of NETs release in neutrophils exposed to similar assay conditions.

The release of NETs indicates that neutrophils have undergone a novel cell death pathway that was described recently and dubbed “NETosis” (Fuchs et al., 2007). In short, during the sequence of events leading to the release of NETs; cellular membrane integrity is disturbed; DNA long strands migrate from the nucleus to the cytoplasm, mix with granular

content and are released through plasma membrane into the extracellular space (Brinkmann et al., 2004; Fuchs et al., 2007). Superoxide and other by-products of the respiratory burst serve as critical signaling components in activation of NETosis, but once this cell death pathway is initiated, the respiratory burst activity is decreased or stopped. Thus, a nano-TiO₂ dose dependent increase in NET release can correlate with observed initial increase, followed by decrease in the respiratory burst, causing absence of linear dose-response of respiratory burst to nano-TiO₂ exposure (Fig. 2-A).

Neutrophil function (oxidative burst, NETs release, degranulation of primary granules) was significantly decreased after 48h of exposure to nano-TiO₂ *in vivo*. The findings of increased cell NETosis upon *in vitro* exposure to nano-TiO₂ suggest that after 48 h of *in vivo* exposure, the mature population of circulating neutrophils might have been depleted since only mature neutrophils are capable of NETs release (Martinelli et al., 2004). Total circulating leukocyte counts indicated that no observable change in neutrophil numbers occurred, further suggesting that the observation of decreased function could be attributed to either inhibition of the function after longer term (48 h) exposure to nano-TiO₂, or the decreased number of mature neutrophils in anterior kidney. Exposure of rainbow trout to nano-TiO₂ did not cause any effect on neutrophil migration or blood neutrophil counts (Federici et al., 2007). In fish, the major organ for titanium dioxide nanoparticle bioaccumulation is the kidney, and no apparent nano-TiO₂ clearance was observed for up to 90 days post exposure (Scown et al., 2009). The fish kidney also serves as a major lymphopoietic site (Kobayashi et al., 2006). Therefore, neutrophil population that is continuously produced and stored in the anterior kidney can be chronically exposed to titanium nanoparticles. Observed reduction in neutrophil function suggests that potential interactions between nano-TiO₂ and neutrophils in the anterior kidney could affect the neutrophils ability to control bacterial infections and cause decreased disease resistance in fish populations exposed to nano-TiO₂.

Expression of interleukin 11 was upregulated four fold upon exposure to nano-TiO₂. Interleukin 11 is the key regulator of multiple events in hematopoiesis especially in megakaryocyte maturation (Paul et al., 1990) and platelet production (Aribi et al., 2008; Gordon, 1996). Another common mechanism of nanoparticle phagocytosis, besides

neutrophil uptake, includes formation of nanoparticle-induced trombocyte/platelet aggregates which are phagocytized either by trombocytes/platelets and removed from the system in the form of redistribution to the blood clot, or the whole trombocyte/platelet-nanoparticle aggregate is phagocytized by macrophages (Movat et al., 1965). Upregulation of interleukin 11 indicates activation of a phagocytic defensive mechanism against nanoparticles, and could be used as a potential biomarker in field studies and risk assessment.

Macrophage stimulating factor 1 (MSF-1) was significantly upregulated in nano-TiO₂ treated fish. This factor is responsible for macrophage differentiation and is increased during inflammatory processes that require mobilization and activation of monocytic cell lineage in organismal defenses (Valledor et al., 1998). The increase in expression of MSF-1 could be related to the increased need for phagocytosis and removal of platelet-nanoparticle aggregates or to scavenging the remains of neutrophils that have undergone the process of NETosis induced by nanoparticles.

Neutrophil cytosolic factor 2 (NCF2) was also significantly upregulated in nano-TiO₂ treated fish. This protein is an integral part of the NADPH oxidase system responsible for oxidative burst delivered to the lumen of the phagosome (Wientjes and Segal, 1995). Observed increases in neutrophil respiratory burst during *in vitro* nano-TiO₂ exposure suggests that in the early stages of nanoparticle exposure, neutrophil activity such as increased phagocytosis of nanoparticles, or platelet-nanoparticle aggregates may be increased. This demand could result in an increased expression of NCF2, similar to bacterial infections (Sancho-Shimizu and Malo, 2006).

In conclusion, exposure of fish to TiO₂ nanoparticles can result in significant changes in innate immune function at the levels of gene expression and cellular function. Furthermore, the immunotoxic effects have been observed with concentrations near the estimated environmental concentration values. The fathead minnow has potential to become a model for testing the immunotoxicity of nanoparticles and development of molecular biomarkers for future assessment of aquatic ecosystem health.

4.6. Acknowledgements

We are thankful to Nada Pavlović and Tom Skadow for the technical help provided. This work was partially funded by Iowa Center for Advanced Neurotoxicology.

4.7. Tables

Table 1. List of genes used in comparative SYBRGreen qPCR expression analysis

Biomarker type	Accession #	Best annotated hit (blast x)	Organism source	Primer sequence (forward and reverse primer)	annealing T (°C)
Normalization genes	<u>AY643400</u>	Elongation factor 1-alpha	<i>Pimephales promelas</i>	5'-AGCTTCTTGGCTCAGGTCAT-3'	58
				5'-CTCATGTCCACGCACAGCAAA-3'	58
Oxidative or metabolic stress	<u>GH714199</u>	Beta actin	<i>Pimephales promelas</i>	5'-AGGACCTGTATGCCAACACT-3'	58
				5'-GAAGCATTGCGGTGGACAA-3'	58
	<u>GH712103</u>	Cytochrome P450, family 2, subfamily J, polypeptide 2, B	<i>Pimephales promelas</i>	5'-TGTTGTCACAGCTGATGGGT-3'	60
				5'-AGTGAGCAGTATGCAAGGCT-3'	60
	<u>GH713989</u>	Glutathione S-transferase alpha	<i>Pimephales promelas</i>	5'-TCCCTGTGTTTGAGAAGGGT-3'	60
				5'-AACACCTCCTTCACCACGTT-3'	60
<u>GH714428</u>	Glutathione reductase	<i>Pimephales promelas</i>	5'-CCTGCGTGAATGTTGGTTGT-3'	60	
			5'-TTCAACGGTAGGTTTCAGGGT-3'	60	
<u>GH712625</u>	Catalase	<i>Pimephales promelas</i>	5'-AGGCGACGGATCAGATGAAA-3'	60	
			5'-ACATGCTCGAACACTTTGGC-3'	60	
Heat shock proteins	<u>GH711955</u>	HSP60	<i>Pimephales promelas</i>	5'-AGATGGTGTACGGTTGCTA-3'	58
				5'-TGTTGTGACTGGCTTGAGA-3'	58

Table 1. (continued)

	<u>GH711278</u>	Heat shock 70kDa protein 5	<i>Pimephales promelas</i>	5'-ACATTCACTGCGTTCAAGGC-3'	60
				5'-ATGAGGGAGTGATGCGGTTT-3'	60
Inflammation	<u>DT285264</u>	Interleukin 17 D	<i>Pimephales promelas</i>	5'-TTCAGCTTGGTTGCGTGTTTC-3'	59
				5'-TCTCCATGTAGGAATGACGACC-3'	59
	<u>GH711173</u>	Il17rd protein	<i>Pimephales promelas</i>	5'-AGTGCCAGCACATGATCCTA-3'	58
				5'-GGCGTGGTCAAACACAACAT-3'	58
	<u>NM 001003</u> <u>414</u>	NFKB activating protein-like (nkpl)	<i>Danio rerio</i>	5'-AAGCAGTCAGAAGACAGCGA-3'	60
				5'-GGCAGTAAAGCATGGCCAAA-3'	60
	<u>GH714703</u>	NF-kappaB inhibitor alpha-like protein B	<i>Pimephales promelas</i>	5'-AACGCTCAGGAGCAGTGTA-3'	58
				5'-TCTTCATAGTCCTCGTCGCT-3'	58
	<u>AJ632159</u>	Interleukin 11	<i>Cyprinus carpio</i>	5'-TGTTAGCATCTGCCTTCCCT-3'	62
				5'-TCGTTCTGTTTCAGCCACTCA-3'	62
	<u>DT361081</u>	Interleukin 6	<i>Pimephales promelas</i>	5'-AGTGCTGAGTTGACATGGGA-3'	60
				5'-TGAGGCAAACCACGACTACA-3'	60
	<u>GH715146</u>	Interleukin 12a	<i>Pimephales promelas</i>	5'-TGAAGTCCTACAAGCCCACA-3'	60
				5'-TGTGAGCGACATCACTTCCT-3'	60
Antioxidants	<u>NM 001007</u> <u>281</u>	glutathione peroxidase 1a	<i>Danio rerio</i>	5'-GCTTCGAGCCCAAATTCCAA-3'	58
				5'-TGTCGATGGTGAGGAACCTT-3'	58
	<u>GH713840</u>	Peroxioredoxin 2	<i>Pimephales promelas</i>	5'-AGTTTGTCTGCTGGATGGA-3'	60
				5'-TTCAGTGCATGAGAGACGCT-3'	60

Table 1. (continued)

	<u>DT350430</u>	Myeloid-specific peroxidase (mpx, mpo)	<i>Pimephales promelas</i>	5'-TGTCTGCAACAACAGGAGGA-3'	60
				5'-TGCTTGGTGAGTTGGGTGTA-3'	60
Ribosomal proteins	<u>GH715111</u>	Ribosomal protein L23a	<i>Pimephales promelas</i>	5'-GCCAAGAAAGCTGTGCTGAA-3'	58
				5'-CAGTTTCTTGACGGCGTGTT-3'	58
ROS metabolism	<u>DT188783</u>	NADPH oxidase 2 (NOX2) aka cytochrome b-245, beta polypeptide	<i>Pimephales promelas</i>	5'-CGGCATCAATGCGTTTCTCT-3'	60
				5'-TAGGCCACCAGTTTGTGGAA-3'	60
	<u>NM_015760</u>	NADPH oxidase 4 (Nox4)	<i>Mus musculus</i>	5'-TACCGATGCATCAGGAGCAA-3'	58
				5'-ACTTGATGGAGGCAGTAGCA-3'	58
	<u>AJ242906</u>	Inducible nitric oxide synthase (iNOS)	<i>Cyprinus carpio</i>	5'-TGGCAGAATTCAGTGGGCTA-3'	60
				5'-TGAAGTCAACACTGGCAGGA-3'	60
	<u>GH712134</u>	Rho GDP dissociation inhibitor (GDI) alpha	<i>Pimephales promelas</i>	5'-ATAAACTCGCAGTACCCGCA-3'	58
				5'-AGTTAGCCGTGTCACCTGAA-3'	58
	<u>GH714009</u>	Neutrophil cytosolic factor 1	<i>Pimephales promelas</i>	5'-TTCTTTCCCAGCCAGCACTA-3'	58
				5'-ACAAGCTGACAGCGAGAGAT-3'	58
	<u>DT145606</u>	Neutrophil cytosolic factor 2	<i>Pimephales promelas</i>	5'-CTTTCCGTTGCATTGACCCT-3'	60
				5'-GCCAAGGAAGGCTTGGATAA-3'	60
	<u>DT152670</u>	Superoxide dismutase 2, mitochondrial precursor	<i>Pimephales promelas</i>	5'-TGCACTTGAGCCTCACATCT-3'	58
				5'-TAATGGCCTCCAACAGCTCA-3'	58
	<u>GH713798</u>	Superoxide dismutase 3	<i>Pimephales promelas</i>	5'-AGTCAAGGCAGCATCACTCT-3'	60
				5'-TTCTGCCACAGACTTGGAGT-3'	60

Table 1. (continued)

Other immune markers	<u>GH711273</u>	Na ⁺ /K ⁺ ATPase alpha subunit isoform 8	<i>Pimephales promelas</i>	5'-AAGCAGAAGGACATGGACGA-3'	60
				5'-TGGATTCCGTATGCCAGGAA-3'	60
	<u>GH713354</u>	Macrophage stimulating 1 (hepatocyte growth factor like)	<i>Pimephales promelas</i>	5'-TGATTGCCTTCCGGTCTCT-3'	58
				5'-ACAACTACTGCAGGAACCCA-3'	58
	<u>GH713807</u>	Pannexin 1	<i>Pimephales promelas</i>	5'-TGCTGGTGGCGATATCAGTT-3'	62
				5'-AGAGAAGCGTTTGGTCCTCA-3'	62
	<u>DT092840</u>	Elastase 2 - neutrophil elastase	<i>Pimephales promelas</i>	5'-ATCGTGCATGAGAACTGGGA-3'	60
				5'-ATGAGGTTGGTCACGAGGTT-3'	60

4.8. Figure captions and figures

Figure 1. Size distribution of nano-TiO₂ in different media: **A** - Hank's Balanced Salt Solution without Ca, Mg, no Phenol red; **B** - Hank's Balanced Salt Solution with Ca, Mg, no Phenol red; **C** – Egg water.

Figure 2. *In vitro* effect of nano-TiO₂ on neutrophil respiratory burst (A-B) and neutrophil extracellular trap release (C-D) in fathead minnows. Neutrophils were stimulated with PMA (phorbol-myristate acetate), and background control was exposed to HBSS (Hank's Balanced Salt Solution with Ca, Mg, no phenol red). **a**, **b**: indicate that the function is significantly different from control ($P < 0.05$, student t-test or Dunnett's procedure post hoc comparison of means respectively). Asterisk (*): indicates near significant effect ($P < 0.07$). Boxes refer to standard errors and whiskers refer to standard deviations.

Figure 3. *In vivo* 48 h exposure effect of nano-TiO₂ on neutrophil respiratory burst, extracellular trap release and degranulation of primary granules presented as % of neutrophil function activity of the control injected with HBSS (Hank's Balanced Salt Solution with Ca, Mg, no Phenol red). **a**, **c**: indicate that the function is significantly different from control with student t-test ($P < 0.05$, $P < 0.001$ respectively).

Figure 4. Nano-TiO₂ effect on fathead minnow immune gene expression after 48 h of exposure. The changes in expression are presented as normalized R₀ values to elongation factor 1-alpha (y-axis) and are unitless. **a**, **c**: indicate statistical significance of the treatment compared to HBSS injected control ($P < 0.05$, $P < 0.001$ respectively). Boxes refer to standard errors and whiskers refer to standard deviations.

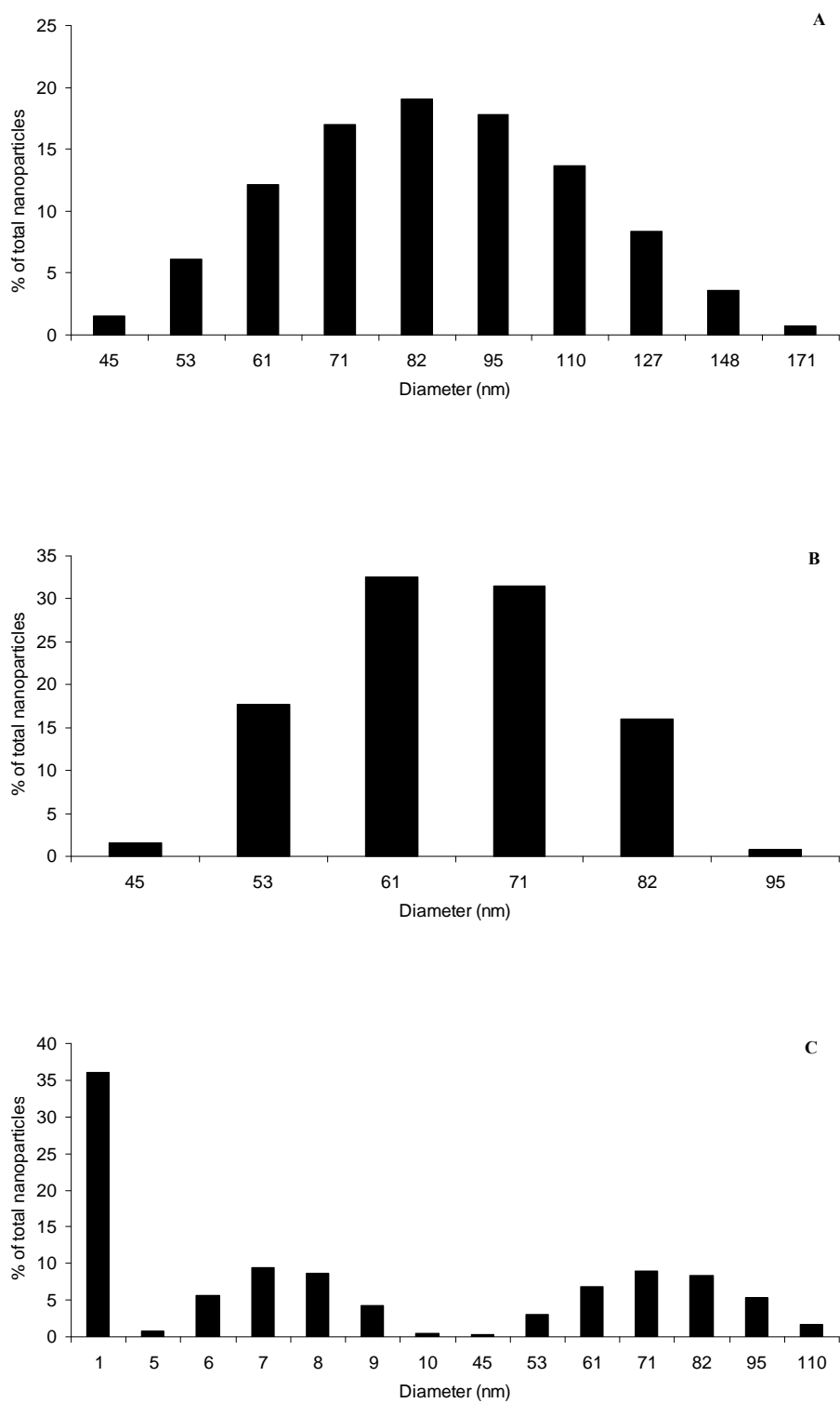


Figure 1

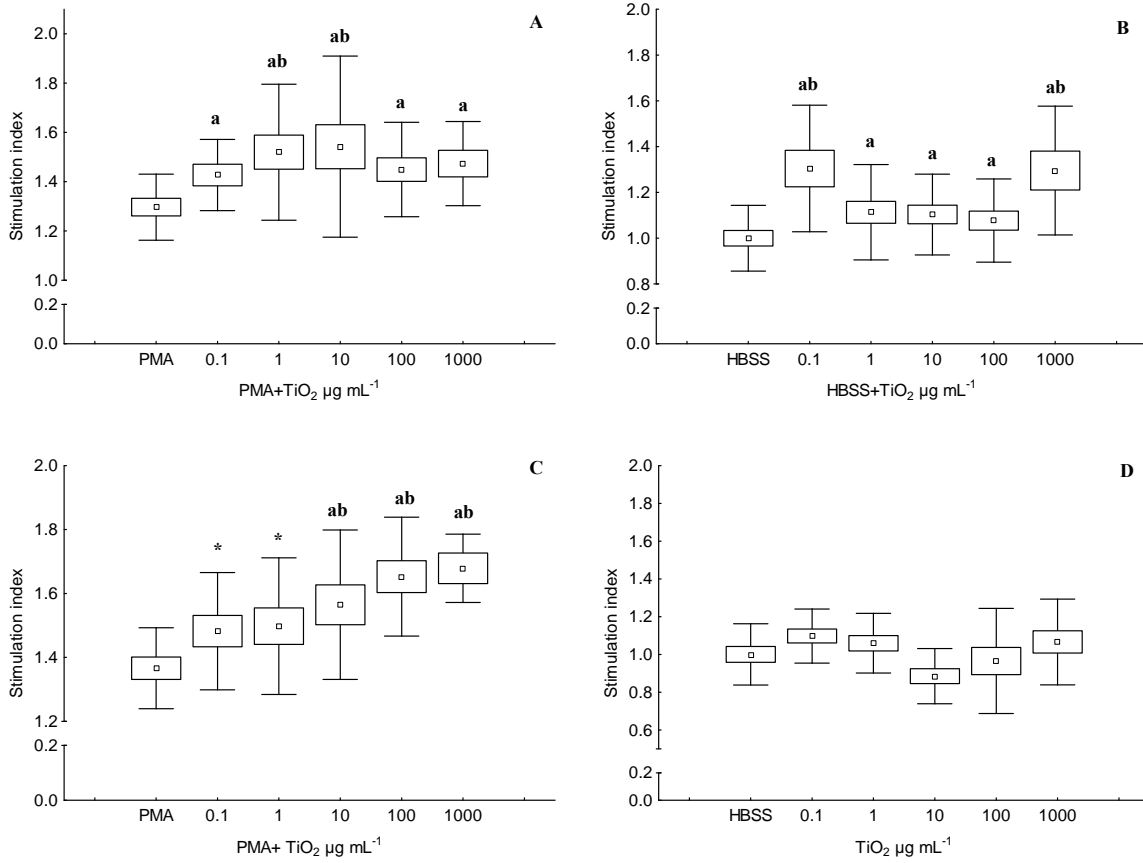


Figure 2

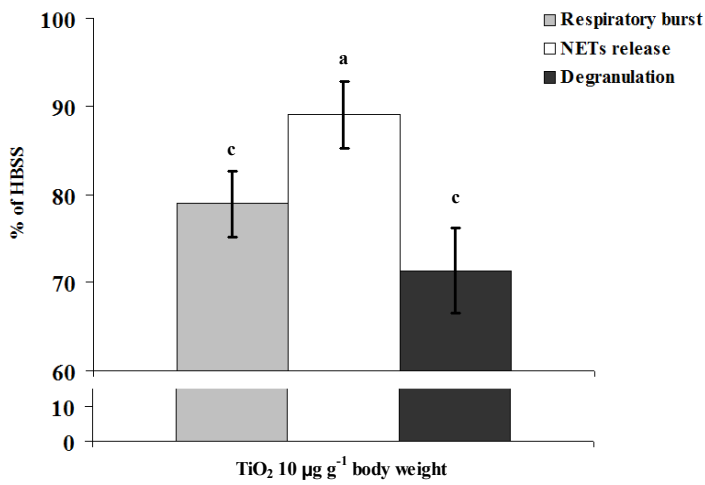


Figure 3

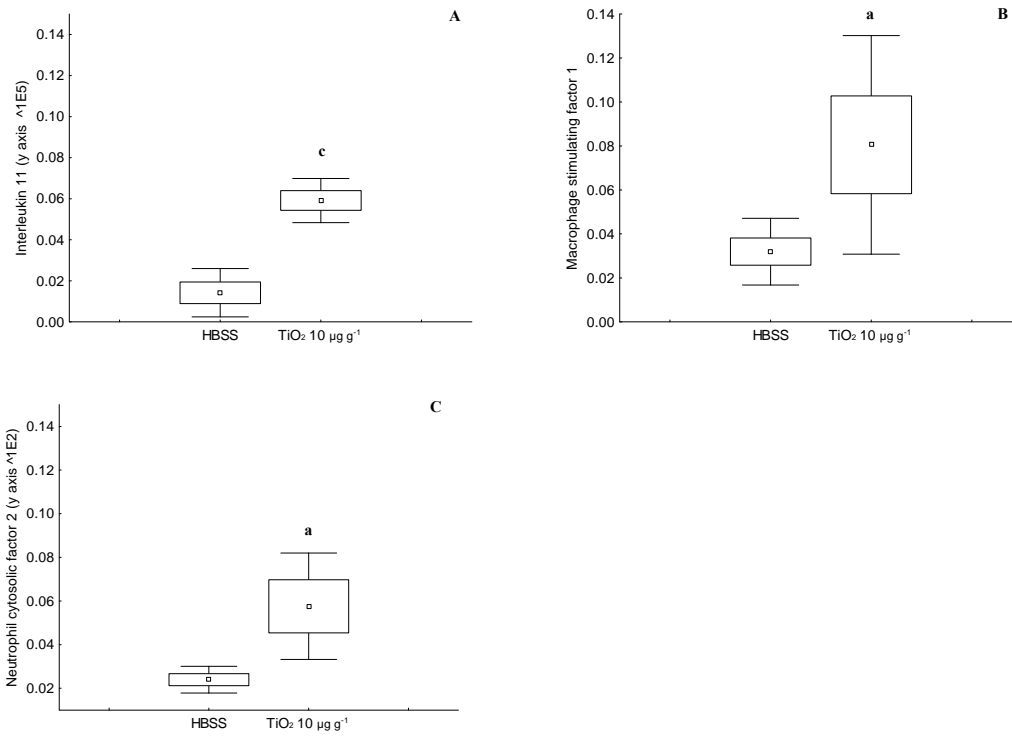


Figure 4

4.9. References

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**CHAPTER 5. Hydroxylated fullerenes inhibit neutrophil function in fathead minnow
(*Pimephales promelas* Rafinesque, 1820)**

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5.1. Abstract

Hydroxylated fullerenes act as a potent inhibitor of cytochrom P450-dependent monooxygenases, and are reported to be very strong antioxidants quenching reactive oxygen species (ROS) production. Effects of nanosized hydroxylated fullerenes on fish neutrophil function and immune gene expression was investigated using fathead minnow (*Pimephales promelas*). Neutrophil function assays were used to determine the effects of fullerene exposure *in vitro* and *in vivo* on oxidative burst, degranulation and extracellular trap (NETs) release, and the innate immune gene expression was determined with quantitative PCR (qPCR). Application of fullerenes (0.2 - 200 $\mu\text{g mL}^{-1}$ *in vitro*) caused dose dependent inhibition of oxidative burst and suppressed the release of NETs and degranulation of primary granules (up to 70, 40, and 50 % reduction in activity compared to non-treated control, respectively). Expression of interleukin 11 and myeloperoxidase was significantly increased and expression of elastase 2 was significantly decreased in fish exposed to hydroxylated fullerenes for 48 h *in vivo* (12 and 3 fold increase, and 5 fold decrease, respectively). Observed changes in gene expression and neutrophil function indicate potential for hydroxylated fullerenes to interfere with the evolutionary conserved innate immune system responses and encourages the use of fish models in studies of nanoparticle immunotoxicity.

Key words: neutrophils, innate immunity, nanoparticles, hydroxylated fullerenes, fathead minnow.

5.2. Introduction

Fullerenes are a heterogeneous group of carbon allotropes shaped as hollow spheres (buckyball) or tubes composed most dominantly of C₆₀ or C₇₀ molecules, and are increasingly used in industry, research, and medicine (Farré et al., 2009). The uptake of fullerenes can cause significant intracellular toxic effects in aquatic organisms, including oxidative damage (Oberdorster, 2004) followed with up-regulation of genes involved in oxidative damage control (Henry et al., 2007). Fullerene exposure is known to cause increases in lipid peroxidation activity in the fish brain and induced expression of CYP2 family proteins in the liver (Haasch et al., 2005). The association between the C₆₀ molecule and DNA is more favorable than the association between two C₆₀ molecules in water (Zhao et al., 2005), indicating potential for genotoxic effects (Dhawan et al., 2006). Electron donating and accepting properties of modified fullerenes can be used to enhance the release of oxyradicals in *in vitro* systems (Kamat et al., 2000) to inactivate viral envelopes, mediate electron transport across lipid bi-layers, and modulate immune function by forming lipid peroxides.

Recently developed hydroxylated fullerenes (fullerenols; C₆₀(OH)₂₄) have an increased potential for industrial use (Guirado-Lopez and Rincon, 2006) with water solubility of up to 100 g L⁻¹ (Hirsch, 2003) and stability in solution for at least nine months (Deguchi et al., 2001). Hydroxylated fullerenes exhibit easy uptake at organismal and cellular level (Handy et al., 2008; Moore, 2006). They act as a potent inhibitor of cytochrom P450-dependent monooxygenases (Ueng et al., 1997), and are reported to be very strong antioxidants (Cai et al., 2008; Dobrovolskaia and McNeil, 2007; Markovic and Trajkovic, 2008) suppressing the reactive oxygen species (ROS) production.

Neutrophils are an important component of the host defense against many bacterial, viral and fungal infections, and the evaluation of neutrophil function is valuable for assessment of the health status of individuals and animal populations (Smith and Lumsden, 1983). Their ability to phagocytize microorganisms and cellular debris is essential for normal development and survival of animal population (Segal, 2005). Neutrophil respiratory burst relies on NADPH oxidase system ability to shuttle the electrons over the plasma

membrane and transfer them to oxygen molecules in phagosomal compartment producing reactive oxygen species (ROS) (Dahlgren and Karlsson, 1999). ROS facilitates a pH increase in phagocytic vacuole and entry of potassium ions stimulating the release of digestion enzymes responsible for microbial killing (Segal, 2005). ROS can be further dismutated to hydrogen peroxide, and with the help of halide and myeloperoxidase catalyzer (released in phagocytic vacuole by degranulation process of primary granules) form HOCl molecule toxic to invading pathogens (Dahlgren and Karlsson, 1999). The final stage of neutrophil defense mechanisms is the release of neutrophil extracellular traps (NETs) through the recently described process of NETosis (Fuchs et al., 2007). NETosis is a cell death mechanism triggered by excess of hydrogen peroxide and is characterized by cellular membrane disintegration resulting in the release of NETs (composed of DNA, histones, and granule proteins), which entrap and kill pathogens (Brinkmann et al., 2004; Fuchs et al., 2007).

The fathead minnow is a small (<10 cm) freshwater cyprinid fish, used extensively as a model organism in environmental, aquatic toxicology (Ankley and Villeneuve, 2006; USEPA, 1987), and toxico/immunogenomics studies (Biales et al., 2007; Mager et al., 2008; Villeneuve et al., 2008). The innate immune responses to stress and immunomodulators were recently described in this species, and neutrophils were reported to perform respiratory burst, degranulation of primary granules, and neutrophil extracellular trap (NET) release (Palić et al., 2005b; Palić et al., 2006; Palić et al., 2007b).

Endocytic and phagocytic routes of fullerene entrance to the immune cells and their storage in endosomes, lysosomes, and phagosomes have been described (Dobrovolskaia and McNeil, 2007; Moore, 2006). However, the question remained if hydroxylated fullerenes can abolish respiratory burst and suppress the neutrophil function after entering the phagocytic vacuole of neutrophils due to their antioxidant abilities (Cai et al., 2008; Dobrovolskaia and McNeil, 2007; Markovic and Trajkovic, 2008). The purpose of this study is to investigate effects of hydroxylated fullerene exposure on fish neutrophil function and immune gene expression.

5.3. Materials and methods

Animal care

Adult fathead minnows (average weight 4.5 g) were maintained in the Iowa State University College of Veterinary Medicine Laboratory Animal Resources Facility, Ames, Iowa, USA. Fish were housed in a water recirculation system supplied with dechlorinated tap water at 23.5 °C in 120 L tanks and fed twice daily to satiation with live brine shrimp larvae and dried flake food (2:1 w/w mixture of Aquatox[®] and Plankton/Krill/Spirulina flake food, Zeigler Bros Inc, PA, USA). Fathead minnows were cared for in accordance with approved Iowa State University animal care guidelines.

Hydroxylated fullerene characterization

Hydroxylated fullerene C₆₀(OH)₂₄ (MER Corporation, Tuscon, AZ, USA, cat# MR16, 98.8% purity) characterization was performed using dynamic light scattering on a Malvern Zetasizer instrument (Malvern Instruments Ltd, Malvern, Worcestershire, UK). Size distribution, zeta potential and conductivity was determined for hydroxylated fullerenes in all the media used in the experiments (HBSS without Ca, Mg and Phenol Red; HBSS with Ca, Mg no Phenol Red; egg water).

Toxicity tests

Sublethal concentrations of hydroxylated fullerenes for immunotoxicogenomics were determined using Fish Embryo Toxicity Assay (FET) (Braunbeck et al., 2005; Braunbeck and Lammer, 2006; OECD, 2006), Fish Acute Toxicity Test (EPA, 1996) and a series of intraperitoneal injections.

Seven day FET assay was performed with six different concentrations (0.0001, 0.001, 0.01, 0.1, 1 and 4 mg L⁻¹) of hydroxylated fullerenes dissolved in fish egg water (60 µg mL⁻¹ of Instant Ocean salt in deionized water, Instant Ocean, Kingman, AZ, USA) (Westerfield, 2000). The use of fish embryos has been established as a standard methodology for testing the toxicity of fullerene species (Usenko et al., 2007). Fathead minnow embryos less than 24 hours post fertilization (hpf) were added to 12 well microtiter

plates (6 ml volume, 5 embryos per well). Each group test was performed with six replicas (total of 30 embryos per group), embryo mortalities were recorded and 80% of the test solution was renewed daily. Embryos were incubated at 25 °C and a 14 h light, 10 h dark cycle for the whole duration of the test. Positive and negative controls (8 mg L⁻¹ 3,4-dichloroaniline (Sigma-Aldrich Corp) (Call et al., 1987); no active substance, respectively) were performed.

Fourteen day static renewal acute toxicity test was performed with six 10-fold dilutions starting from 2 x 10⁻¹ to 2 x 10⁻⁶ mg L⁻¹ of hydroxylated fullerenes in dechlorinated tap water. Twenty fathead minnows at 5 weeks of age were randomly collected from a stock tank, placed in an aerated 1 L glass chamber, and 80% of the test solution was renewed daily. Each test group and control was repeated in triplicate (3 x 20 fish in each treatment and control group). Fish were kept at 23.5 °C and 14 h light, 10 h dark cycle for the duration of the test. Positive and negative controls (8 mg L⁻¹ 3,4-dichloroaniline (Call et al., 1987); no active substance, respectively) were included in the test.

Eighteen day flow-through acute toxicity test was performed with three different concentrations (0.2, 2 and 20 µg g⁻¹ body weight) of hydroxylated fullerenes in Hank's Balanced Salt Solution without Ca, Mg and Phenol Red (HBSS; Mediatech – CellGro, AK, USA). Adult fathead minnows (3-5 g body weight) were quickly netted from the tanks and anesthetized with 100 mg L⁻¹ of aerated and buffered (sodium bicarbonate, pH 8.0) solution of the tricaine methane sulphonate (MS-222, Argent Laboratories, Redmond WA, USA). Upon entering the third stage of anesthesia (Palić et al., 2006), fish were weighed and injected intraperitoneally. Injected fish were transferred to 40 L flow-through tanks (pH=8 and water temperature 23.5 °C) and fed twice daily to satiation for 18 days. Control group was injected with HBSS without Ca, Mg and Phenol Red. Each test group had 21 fish and was performed in duplicates.

In vitro neutrophil function assays

Adult fathead minnows (3 years old; 4-7 g body weight; mixed sex) were euthanized, kidneys from eight fish were pooled as a single sample, and cell suspensions were prepared (Palić et al., 2005b). The average myeloperoxidase (MPO) positive to MPO negative cell

ratio (Palić et al., 2005a) in the final suspension was 82%, indicating that majority of the cells were from myelopoietic lineage. Cell suspensions were adjusted to 2×10^7 cells mL⁻¹ and used in all neutrophil function assays. A total of 24 samples have been used per assay on three separate days. Duplicate (respiratory burst, degranulation) or triplicate wells (NETs release) were used in each assay treatment. Cells were exposed to standard stimulants (phorbol myristate acetate, PMA, 1 $\mu\text{g mL}^{-1}$; and calcium ionophore A23187, CaI, 5 $\mu\text{g mL}^{-1}$; Sigma-Aldrich Corp.), hydroxylated fullerenes at 0.2, 2, 20 and 200 $\mu\text{g mL}^{-1}$ in HBSS with Ca, Mg, no Phenol Red, a combination of hydroxylated fullerenes and standard stimulants at listed concentrations, or to HBSS (negative control).

Degranulation of primary granules and respiratory burst assays were performed according to established protocols (Hermann et al., 2004; Palić et al., 2005b). The respiratory burst assay was further modified so that the 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) was replaced with 5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate (carboxy-H₂DFDA) allowing for improved photostability according to the manufacturer (Invitrogen) when compared to chlorinated fluorescein derivatives. The neutrophil extracellular trap release assay was performed as described (Palić et al., 2007a; Palić et al., 2007b) with modifications as per (Chuammitri et al., 2009) and optimal temperature for fish cells (20 °C).

The NET and respiratory burst-stimulation index was calculated using the following formula:

Stimulation index = PMA + hydroxylated fullerene exposed neutrophils fluorescence / PMA stimulated neutrophils fluorescence; as well as hydroxylated fullerene exposed neutrophils fluorescence / HBSS exposed neutrophils fluorescence. Similar calculations were performed for degranulation assay using CaI as a stimulant.

Ex vivo neutrophil function assays

To investigate the effects of hydroxylated fullerenes on neutrophils *ex vivo*, adult fathead minnows (3 years old; 4-7 g body weight; mixed sex) were intraperitoneally injected with 2 $\mu\text{g g}^{-1}$ of fish body weight, as described in section *Toxicity tests*. Injected fish were transferred to 40 L recirculation tanks and fed twice daily to satiation. After 48 h the fish

were euthanized with MS-222, kidneys from four fish pooled as a single sample, and kidney cell suspensions prepared according to section *In vitro neutrophil function assays*. Sixteen samples per each treatment and control (fish injected with HBSS), were used in neutrophil function studies. Results were expressed as % of control.

Total RNA extraction

Six adult minnows per treatment and control each were anesthetized and intraperitoneally injected with hydroxylated fullerenes as described in section *Ex vivo neutrophil function assays*. Fish were collected and euthanized at 48 h post injection, and the anterior kidney, liver, spleen, and gills were collected from each individual. Total RNA extraction was performed according to the acid guanidinium thiocyanate-phenol-chloroform extraction protocol (Chomczynski and Sacchi, 2006) using TRI Reagent®. All collected organs for each individual fish were pooled in a 1.5 mL centrifuge tube containing 1 mL of Tri-Reagent (Molecular Research Center, Inc. Cincinnati, OH, USA), total RNA extracted and the concentration was assessed using a Nanodrop spectrophotometer (NanoDrop products, Wilmington, DE, USA).

Reverse transcription PCR and real time PCR

RNA extracted from fish exposed to hydroxylated fullerenes or HBSS without Ca, Mg, and Phenol Red was processed as described before (Jovanović et al., *In press-b*). Briefly, RNA was reverse transcribed using reverse transcriptase and oligo-dT primer (Promega Corporation, Madison, WI, USA), the cDNA was used as a template for conventional PCR and real time PCR with primers designed for selected gene sequences (Tab 1) that have been established as designated *P. promelas* biomarkers for nanoparticle immunotoxicogenomic studies (Jovanović et al., *In press-a*). Both forward and reverse primers (Integrated DNA Technologies, Coralville, IA, USA) were 20 nucleotides in length, and 50% percent guanine/cytosine content. Optimal annealing temperature was determined, product was verified with conventional PCR/ethidium bromide electrophoresis, and real-time PCR reactions were performed. Fluorescence measurements, gene normalization, and

processing of completed QPCR datasets with Real-time PCR Miner were performed as described (Jovanović et al., *In press-a*).

Leukogram

Six adult fathead minnows per treatment and control group were intraperitoneally injected with hydroxylated fullerenes as described in section *Ex vivo neutrophil function assays*. At 48 h post injection minnows were euthanized, blood was collected from the caudal vein in heparinized microhematocrit tubes, and blood smears were prepared in duplicate for each fish. Blood smears were stained with a Diff Quick kit (Sigma-Aldrich Corp) and leukograms were determined as total number of lymphocytes, monocytes, neutrophils and thrombocytes per mm^3 (Blaxhall and Daisley, 1973) and presented as % of control using the following formula: % of control HBSS injected fish $N = (\text{TCN in experiment} / \text{TCN in control}) \times 100$ where TCN stands for total cell number of particular leukocyte type.

Statistical analysis

All data were analyzed for significance using One-Way ANOVA followed by Dunnett's procedure for post hoc comparison of means between single control and multiple experimental groups and a *P*-value equal to or less than 0.05 was considered statistically significant except when otherwise noted.

5.4. Results

Hydroxylated fullerene characterization

The zeta potential of hydroxylated fullerenes dissolved in HBSS without Ca, Mg and Phenol Red; HBSS with Ca, Mg no Phenol Red; and egg water was -19.1, -16.7 and -41.3 respectively, while the conductivity was 15.2, 14.9 and 0.17 mS cm^{-1} in the same order of appearance. Size characterization revealed that the particle size is uniform following normal distribution with bell shaped curve as seen in Fig. 1. Size range distribution of particles dissolved in HBSS without Ca, Mg and Phenol Red had two peaks at 235 and 1370

nanometers while particles dissolved in HBSS with Ca, Mg no Phenol Red and egg water had only one peak at 814 and 1250 nanometers respectively. Such size distribution suggested that small aggregates of 2-10 individual particles might form. This was confirmed with microscopic imaging (Fig. 2).

Toxicity tests

Hydroxylated fullerenes did not cause significant mortality in the Fish Embryo Toxicity Assay (< 10% at seven days post exposure for all tested concentrations including negative control). Positive control, 3,4-dichloroaniline caused 40% mortality after 96 h and 70% mortality seven days post exposure, as expected (Call et al., 1987). Starting at the 48 h of exposure, the concentrations of 0.01 mg L⁻¹ and higher induced significantly increased development of severe pericardial edemas and yolk coagulation in up to 25% of experimental embryos (Fig. 3) comparing to the control (Chi-Square test, $P=0.05$). Similar edemas attributed to hydroxylated fullerenes were described previously in zebrafish model (Usenko et al., 2007), however the affected fathead minnows did not die before the completion of the assay and were not counted as mortalities in the FET assay data analysis.

In the Fish Acute Toxicity test mortality, malformations, or behavior changes were not observed in any of the hydroxylated fullerenes treated groups or negative control. Positive control, 3,4-dichloroaniline caused 55% mortality after 96 h.

Intraperitoneal injections of fullerenes at 0.2 and 2 µg g⁻¹ body weight and control treatment (HBSS) did not cause any mortality during 18 days observation period. Treatment with 20 µg g⁻¹ body weight caused a significant (12%) increase in mortality (Chi-Square test, $P<0.01$). All mortalities occurred within the first 36 h of the 18 days observation period.

Neutrophil function assays

Hydroxylated fullerenes caused a significant decrease in respiratory burst *in vitro* compared to non-stimulated controls starting at concentration of 2 µg ml⁻¹ and higher ($P<0.05$ and $P<0.001$ for higher concentrations). Respiratory burst decrease was observed both when a combination of hydroxylated fullerenes and PMA (Fig. 4A) or hydroxylated fullerenes alone were used (Fig. 4B). A significant response of neutrophil extracellular trap

release forming bell-shaped dose-response curve was observed with concentration of $2 \mu\text{g ml}^{-1}$ increasing the NETs release ($P<0.05$), while concentrations of $20 \mu\text{g ml}^{-1}$ and higher decreased the NETs release ($P<0.001$). This phenomenon was observed both when PMA was used together with hydroxylated fullerenes (Fig. 4C) and when hydroxylated fullerenes were used alone (Fig. 4D). Hydroxylated fullerenes caused a significant decrease in degranulation of primary neutrophil granules starting at dose of $20 \mu\text{g ml}^{-1}$ ($P<0.001$) compared to controls. The decrease was observed both when CaI was used together with hydroxylated fullerenes (Fig. 4E) and when hydroxylated fullerenes were used alone (Fig. 4F).

Hydroxylated fullerenes significantly suppressed neutrophil function after *in vivo* exposure (Fig. 5). Degranulation of primary granules was reduced by 25% ($P<0.001$), and 10% reduction in respiratory burst and NETs release was significant at the level of $P<0.1$.

Gene expression

Exposure to hydroxylated fullerenes caused a significant upregulation of genes for interleukin 11 (12 fold increase), myeloperoxidase (3 fold increase) and a significant downregulation for neutrophil elastase 2 (5 fold decrease) compared to control (Fig. 6 A-C) ($P<0.05$). Expression of other monitored genes (Tab. 1) did not differ between treatment and control.

Blood cell count

There was no difference between the control and treatment groups in the number of circulating lymphocytes, monocytes, neutrophils and thrombocytes.

5.5. Discussion

The results confirmed our hypotheses that hydroxylated fullerenes will inhibit neutrophil function. Respiratory burst was inhibited after application of hydroxylated fullerenes. In the process of respiratory burst ROS are accumulated inside phagocytic vacuoles (Segal, 2005). It was previously argued that hydroxylated fullerenes can be

phagocytized by neutrophils through Fc γ receptor engagement and activation (Dobrovolskaia and McNeil, 2007) thus being stored in phagocytic vacuoles where they can potentially be metabolized (Kagan et al., 2010). Since hydroxylated fullerenes can scavenge the ROS in various types of cells and cellular compartments, (Cai et al., 2008; Dobrovolskaia and McNeil, 2007; Markovic and Trajkovic, 2008) we believe that the ROS produced in the process of respiratory burst were scavenged in the phagocytic vacuole of neutrophils. Size distribution of hydroxylated fullerenes did not pose any barrier for neutrophil phagocytosis. The largest aggregates that were formed in our solution had a diameter of 2 μm , while the neutrophils can easily phagocytized nanoparticle aggregates up to 3 μm (Kumazawa et al., 2002).

Our data indicates that hydroxylated fullerene antioxidant properties are hampering the dismutation process, reducing the amount of starting available ROS thus reducing the gain in hydrogen peroxide and preventing signaling in the process of degranulation (Dahlgren and Karlsson, 1999).

Neutrophil extracellular traps release was induced with the fullerene concentration of 2 $\mu\text{g ml}^{-1}$.in accordance with observations of NETs induction by nanogold particles (Bartneck et al., 2009). Induction of NETs release depends on the excess of hydrogen peroxide and will not be triggered if the concentration of H_2O_2 is below a certain threshold (Fuchs et al., 2007), and it is known that the gain in hydrogen peroxide depends on the ROS production during respiratory burst (Dahlgren and Karlsson, 1999). The increase in concentration of hydroxylated fullerenes significantly reduced respiratory burst (Fig. 2-B). It appears that hydroxylated fullerene concentration of 20 $\mu\text{g ml}^{-1}$ is the first dose to be associated with reduction of hydrogen peroxide gain, thereby preventing the release of NETs. The observed bell-shaped dose response of NETs release showing NETs increase at $\leq 2 \mu\text{g ml}^{-1}$ and decrease at $\geq 20 \mu\text{g ml}^{-1}$ of hydroxylated fullerenes (Fig. 4-D) is in accordance with the suggested mechanism of NETosis (Fuchs et al., 2007).

Our data indicate that a nonlethal hydroxylated fullerene dose of 2 $\mu\text{g g}^{-1}$ body weight *in vivo* is significantly reducing neutrophil function in fish as soon as 48 h after the exposure. Fish treated with fullerenes did not display any changes in blood neutrophil count, suggesting that the observed decrease in neutrophil function could be attributed to direct

action of fullerenes via their potent antioxidant capabilities. Further studies are required to fully explain the mechanism of hydroxylated fullerene inhibition of neutrophil function and potential effects on disease resistance. However, awareness about potential unwanted side effects on innate immune system should be raised regarding not only fish and aquatic ecosystem health, but also biomedical applications since hydroxylated fullerenes are considered as experimental drugs in the treatment of Parkinson disease (Cai et al., 2008; Dugan et al., 2001; Markovic and Trajkovic, 2008).

Expression of interleukin 11 was upregulated twelve fold upon exposure to hydroxylated fullerenes. Interleukin 11 is the key regulator of multiple events in hematopoiesis especially in megakaryocyte maturation (Paul et al., 1990) and trombocyte/platelet production (Aribi et al., 2008; Gordon, 1996). Besides uptake by neutrophils, nanoparticle can be phagocytosed by trombocytes/platelets and removed from the system in the form of redistribution to the blood clot, or nanoparticle-induced trombocyte/platelet aggregates can be formed which are phagocytized by macrophages (Movat et al., 1965). Upregulation of interleukin 11 indicates activation of a phagocytic defensive mechanism against hydroxylated fullerenes, and could be used as a potential biomarker in field studies and risk assessment.

Expression of myeloperoxidase was upregulated three fold after the application of hydroxylated fullerenes. Myeloperoxidase is expressed in early stages of neutrophil development (Handin et al., 2002) and it may be an indicator of increased myelopoiesis in the anterior kidney of fathead minnow. Recently, a team of scientists showed that neutrophil myeloperoxidase catalyzes degradation of carbon nanotubes (Kagan et al., 2010). Both the buckyballs and the nanotubes are essentially very similar in their physical-chemical properties (Guldi et al., 2006) and it is very likely that hydroxylated fullerenes can be metabolized in a similar way, potentially explaining an increase in transcription of myeloperoxidase product.

Neutrophil elastase 2 is a serine protease stored in azurophil granules of neutrophils with a major role in bacterial killing both during oxidative and non-oxidative pathways (Belaouaj, 2002; Segal, 2005). In our study, gene expression for neutrophil elastase 2 was downregulated five times. It has been reported earlier that strong antioxidants including

water soluble fullerenes act as elastase enzyme inhibitors (Lai et al., 1999), but this is the first report to demonstrate hydroxylated fullerene effects on elastase gene expression and downregulation of elastase 2 gene. The mechanism behind elastase 2 downregulation is not been fully understood, but it is known that transcription of elastase occurs at a different developmental stage than myeloperoxidase during neutrophil hemopoiesis (Borregaard et al., 2001). The observed increases in MPO and decrease in elastase 2 transcription therefore may be reflecting a specific time point in neutrophil development induced by action of hydroxylated fullerenes at 48 hours post application.

In conclusion, application of hydroxylated fullerenes significantly suppressed neutrophil function in fish models by hampering the process of respiratory burst, preventing neutrophil extracellular trap release and degranulation of primary granules, and possibly decreasing neutrophil elastase transcription. The hydroxylated fullerenes may pose an environmental threat to aquatic ecosystem by immunocompromising its inhabitants due to their water solubility of up to 100 g L^{-1} (Hirsch, 2003) and stability in solution for at least nine months (Deguchi et al., 2001). In 2008 environmental estimated concentration of engineered carbon based nanotubes in aquatic environment was $0.0000008 \text{ } \mu\text{g mL}^{-1}$, and $0.00031 \text{ } \mu\text{g mL}^{-1}$ for fullerenes (Mueller and Nowack, 2008; Pérez et al., 2009). These concentrations are nearly 1000-fold less than the concentration of $0.2 \text{ } \mu\text{g mL}^{-1}$ that was the lowest observable concentration in this study to elicit immunotoxicity. However, it is not known what percent of environmental estimated concentration can be attributed to non-hydroxylated buckyball fullerenes, and what percentage can be attributed to the hydroxylated fullerenes. Previous studies have shown that non-hydroxylated fullerenes are more toxic than hydroxylated and that non-hydroxylated fullerenes can elicit same toxic effects with significantly smaller concentration (Sayes et al., 2004; Usenko et al., 2007). This, coupled with the constant increase in nanoparticle production and discharge, further raise the issue and need for risk assessment studies in aquatic ecosystems.

5.6. Acknowledgements

We are thankful to Ning Fang and Wei Sun for the help with nanoparticle characterization and to Nada Pavlović and Tom Skadow for the technical help provided. This work was partially funded by Iowa Center for Advanced Neurotoxicology.

5.7. Tables

Table 1. List of genes used in comparative SYBRGreen qPCR expression analysis

Biomarker type	Accession #	Best annotated hit (blast x)	Organism source	Primer sequence (forward and reverse primer)	annealing T (°C)
Normalization genes	<u>AY643400</u>	Elongation factor 1-alpha	<i>Pimephales promelas</i>	5'-AGCTTCTTGGCTCAGGTCAT-3'	58
				5'-CTCATGTCACGCACAGCAAA-3'	58
Oxidative or metabolic stress	<u>GH714199</u>	Beta actin	<i>Pimephales promelas</i>	5'-AGGACCTGTATGCCAACACT-3'	58
				5'-GAAGCATTGCGGTGGACAA-3'	58
	<u>GH712103</u>	Cytochrome P450, family 2, subfamily J, polypeptide 2, B	<i>Pimephales promelas</i>	5'-TGTTGTCACAGCTGATGGGT-3'	60
				5'-AGTGAGCAGTATGCAAGGCT-3'	60
	<u>GH713989</u>	Glutathione S-transferase alpha	<i>Pimephales promelas</i>	5'-TCCCTGTGTTTGAGAAGGGT-3'	60
				5'-AACACCTCCTTCACCACGTT-3'	60
<u>GH714428</u>	Glutathione reductase	<i>Pimephales promelas</i>	5'-CCTGCGTGAATGTTGGTTGT-3'	60	
			5'-TTCAACGGTAGGTTTCAGGGT-3'	60	
Heat shock proteins	<u>GH712625</u>	Catalase	<i>Pimephales promelas</i>	5'-AGGCGACGGATCAGATGAAA-3'	60
				5'-ACATGCTCGAACACTTTGGC-3'	60
	<u>GH711955</u>	HSP60	<i>Pimephales promelas</i>	5'-AGATGGTGTACGGTTGCTA-3'	58
				5'-TGTTGTGACTGGCTTGGAGA-3'	58
<u>GH711278</u>	Heat shock 70kDa protein 5	<i>Pimephales promelas</i>	5'-ACATTCCTGCGTTCAAGGC-3'	60	
			5'-ATGAGGGAGTGATGCGGTTT-3'	60	

Table 1. (continued)

Inflammation	<u>DT285264</u>	Interleukin 17 D	<i>Pimephales promelas</i>	5'-TTCAGCTTGGTTGCGTGTTTC-3'	59
				5'-TCTCCATGTAGGAATGACGACC-3'	59
	<u>GH711173</u>	Il17rd protein	<i>Pimephales promelas</i>	5'-AGTGCCAGCACATGATCCTA-3'	58
				5'-GGCGTGGTCAAACACAACAT-3'	58
	<u>NM 001003 414</u>	NFKB activating protein-like (nkapl)	<i>Danio rerio</i>	5'-AAGCAGTCAGAAGACAGCGA-3'	60
				5'-GGCAGTAAAGCATGGCCAAA-3'	60
	<u>GH714703</u>	NF-kappaB inhibitor alpha-like protein B	<i>Pimephales promelas</i>	5'-AACGCTCAGGAGCAGTGTA-3'	58
				5'-TCTTCATAGTCCTCGTCGCT-3'	58
	<u>AJ632159</u>	Interleukin 11	<i>Cyprinus carpio</i>	5'-TGTTAGCATCTGCCTTCCCT-3'	62
				5'-TCGTTCTGTTTCAGCCACTCA-3'	62
Antioxidants	<u>DT361081</u>	Interleukin 6	<i>Pimephales promelas</i>	5'-AGTGCTGAGTTGACATGGGA-3'	60
				5'-TGAGGCAAACCACGACTACA-3'	60
	<u>GH715146</u>	Interleukin 12a	<i>Pimephales promelas</i>	5'-TGAAGTCCTACAAGCCCACA-3'	60
				5'-TGTGAGCGACATCACTTCCT-3'	60
	<u>NM 001007 281</u>	glutathione peroxidase 1a	<i>Danio rerio</i>	5'-GCTTCGAGCCCAAATTCCAA-3'	58
				5'-TGTCGATGGTGAGGAACCTT-3'	58
	<u>GH713840</u>	Peroxiredoxin 2	<i>Pimephales promelas</i>	5'-AGTTTGCCTGCTGGATGGA-3'	60
				5'-TTCAGTGCATGAGAGACGCT-3'	60
	<u>DT350430</u>	Myeloid-specific peroxidase (mpx, mpo)	<i>Pimephales promelas</i>	5'-TGTCTGCAACAACAGGAGGA-3'	60

Table 1. (continued)

				5'-TGCTTGGTGAGTTGGGTGTA-3'	60
Ribosomal proteins	<u>GH715111</u>	Ribosomal protein L23a	<i>Pimephales promelas</i>	5'-GCCAAGAAAGCTGTGCTGAA-3'	58
				5'-CAGTTTCTTGACGGCGTGTT-3'	58
ROS metabolism	<u>DT188783</u>	NADPH oxidase 2 (NOX2) aka cytochrome b-245, beta polypeptide	<i>Pimephales promelas</i>	5'-CGGCATCAATGCGTTTCTCT-3'	60
				5'-TAGGCCACCAGTTTGTGGAA-3'	60
	<u>NM_015760</u>	NADPH oxidase 4 (Nox4)	<i>Mus musculus</i>	5'-TACCGATGCATCAGGAGCAA-3'	58
				5'-ACTTGATGGAGGCAGTAGCA-3'	58
	<u>AJ242906</u>	Inducible nitric oxide synthase (iNOS)	<i>Cyprinus carpio</i>	5'-TGGCAGAATTCAGTGGGCTA-3'	60
				5'-TGAAGTCAACACTGGCAGGA-3'	60
	<u>GH712134</u>	Rho GDP dissociation inhibitor (GDI) alpha	<i>Pimephales promelas</i>	5'-ATAAACTCGCAGTACCCGCA-3'	58
				5'-AGTTAGCCGTGTCACCTGAA-3'	58
	<u>GH714009</u>	Neutrophil cytosolic factor 1	<i>Pimephales promelas</i>	5'-TTCTTTCCCAGCCAGCACTA-3'	58
				5'-ACAAGCTGACAGCGAGAGAT-3'	58
	<u>DT145606</u>	Neutrophil cytosolic factor 2	<i>Pimephales promelas</i>	5'-CTTTCCGTTGCATTGACCCT-3'	60
				5'-GCCAAGGAAGGCTTGGATAA-3'	60
	<u>DT152670</u>	Superoxide dismutase 2, mitochondrial precursor	<i>Pimephales promelas</i>	5'-TGCACTTGAGCCTCACATCT-3'	58
				5'-TAATGGCCTCCAACAGCTCA-3'	58
	<u>GH713798</u>	Superoxide dismutase 3	<i>Pimephales promelas</i>	5'-AGTCAAGGCAGCATCACTCT-3'	60
				5'-TTCTGCCACAGACTTGGAGT-3'	60

Table 1. (continued)

Other immune markers	<u>GH711273</u>	Na ⁺ /K ⁺ ATPase alpha subunit isoform 8	<i>Pimephales promelas</i>	5'-AAGCAGAAGGACATGGACGA-3'	60
				5'-TGGATTCCGTATGCCAGGAA-3'	60
	<u>GH713354</u>	Macrophage stimulating 1 (hepatocyte growth factor like)	<i>Pimephales promelas</i>	5'-TGATTGCCTTTCCGGTCTCT-3'	58
				5'-ACAACACTGCAGGAACCCA-3'	58
	<u>GH713807</u>	Pannexin 1	<i>Pimephales promelas</i>	5'-TGCTGGTGGCGATATCAGTT-3'	62
				5'-AGAGAAGCGTTTGGTCCTCA-3'	62
	<u>DT092840</u>	Elastase 2 - neutrophil elastase	<i>Pimephales promelas</i>	5'-ATCGTGCATGAGAACTGGGA-3'	60
				5'-ATGAGGTTGGTCACGAGGTT-3'	60

5.8. Figure captions and figures

Figure 1. Size distribution of hydroxylated fullerenes in different media: **A** - Hank's Balanced Salt Solution without Ca, Mg, no Phenol red; **B** - Hank's Balanced Salt Solution with Ca, Mg, no Phenol red; **C** - Egg water.

Figure 2. Hydroxylated fullerenes in Hank's Balanced Salt Solution without Ca, Mg, no Phenol red imaged under the microscope. Bar in the bottom left corner equals 300 nanometers. Black arrow points to aggregate; white arrow to an individual particle.

Figure 3. Pericardial edemas and yolk coagulation in fathead minnow (*Pimephales promels*) embryos 120 h post fertilization exposed to hydroxylated fullerenes. Top: control fish (not exposed); Bottom: five day semistatic renewal exposure to 2 mg L⁻¹ of C₆₀(OH)₂₄. Right arrow points to pericardial edema, left arrow point to yolk coagulation areas. Scale bar on the bottom right = 0.5 mm.

Figure 4. *In vitro* effect of hydroxylated fullerenes on neutrophil respiratory burst (A-B), neutrophil extracellular traps release (C-D) and degranulation of primary granules (E-F) in fathead minnows. Neutrophils were stimulated with PMA (Phorbol-Myristate Acetate) in respiratory burst and neutrophil extracellular trap release assays, or CaI (Calcium Ionophore A23187) in degranulation assay. Background control was exposed to HBSS (Hank's Balanced Salt Solution with Ca, Mg, no phenol red). **a**, **b**, **c**: indicate that the function is significantly different from control ($P < 0.05$, $P < 0.01$, $P < 0.001$ respectively). Boxes refer to standard errors and whiskers refer to standard deviations.

Figure 5. *In vivo* 48 h exposure effect of hydroxylated fullerenes on neutrophil respiratory burst, extracellular trap release and degranulation of primary granules presented as % of neutrophil function activity of the control injected with HBSS (Hank's Balanced Salt Solution without Ca, Mg, no Phenol red). **c**: indicate that the function is significantly

different from control ($P < 0.001$); * indicate that the function is different from the control at the level $P < 0.1$.

Figure 6. Hydroxylated fullerenes effect on fathead minnow immune gene expression after 48 h of exposure. The changes in expression are presented as normalized R_0 values to elongation factor 1 alpha (y-axis) and are unitless. **a:** indicate statistical significance of the treatment compared to HBSS injected control ($P < 0.05$). Boxes refer to standard errors and whiskers refer to standard deviations.

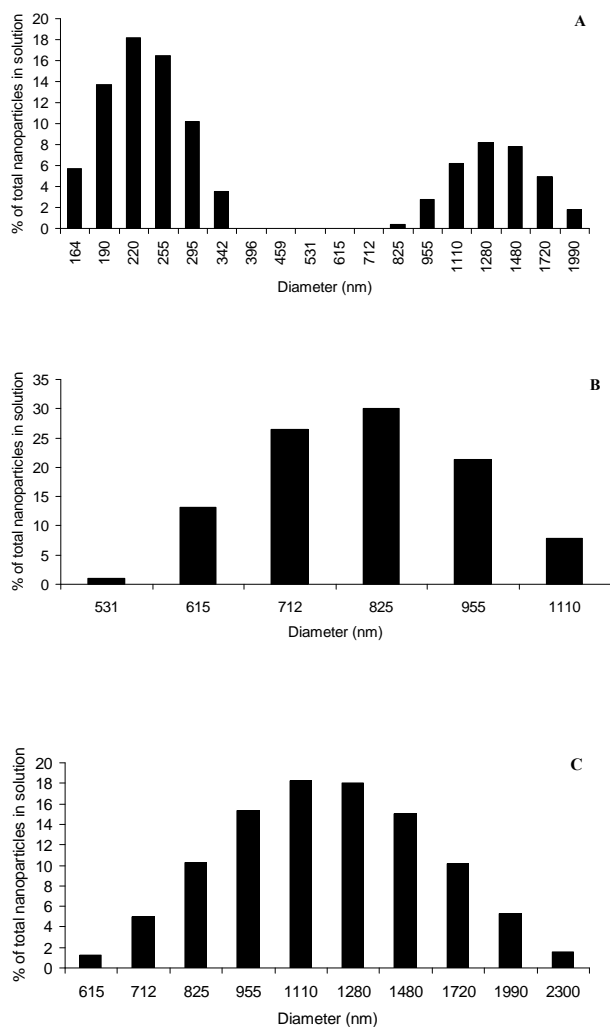


Figure 1

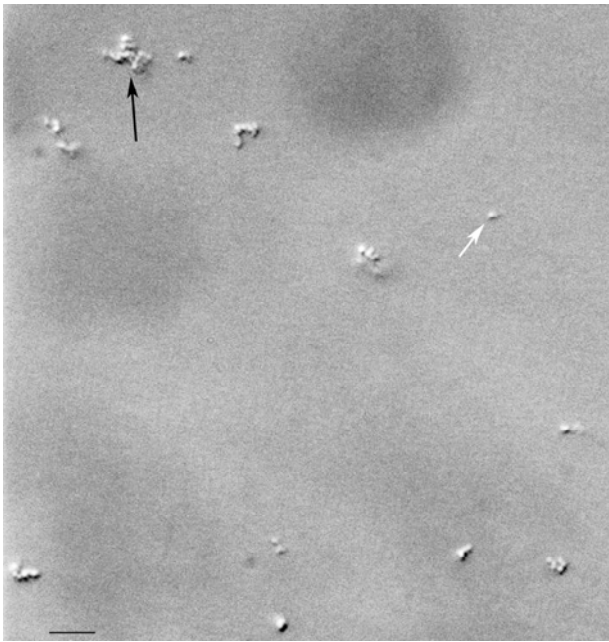


Figure 2

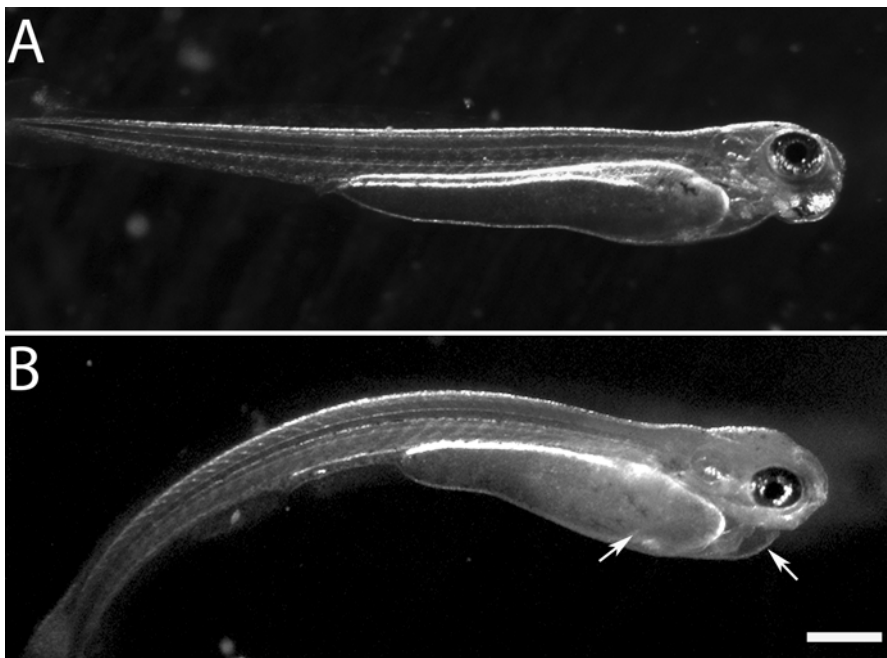


Figure 3

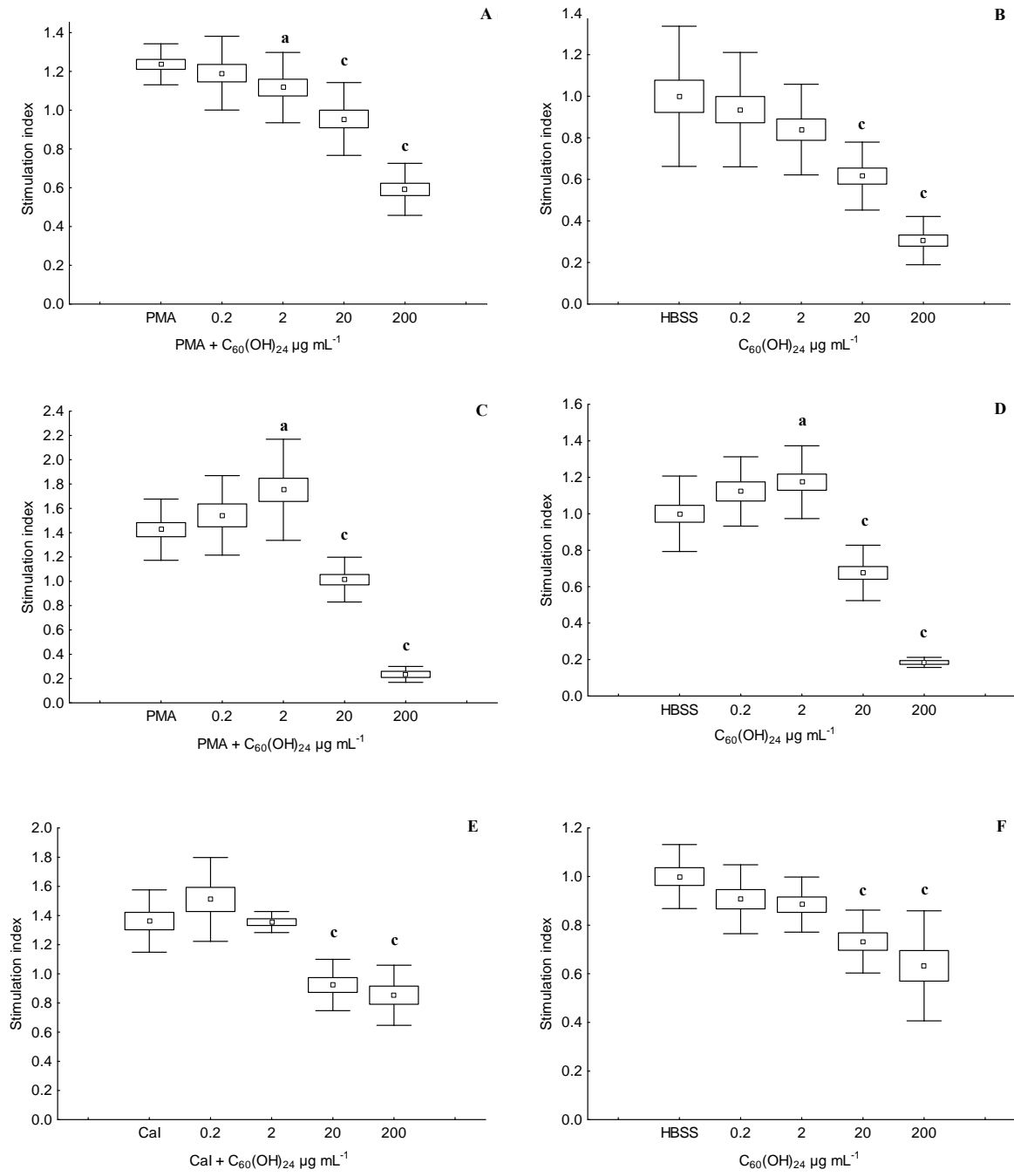


Figure 4

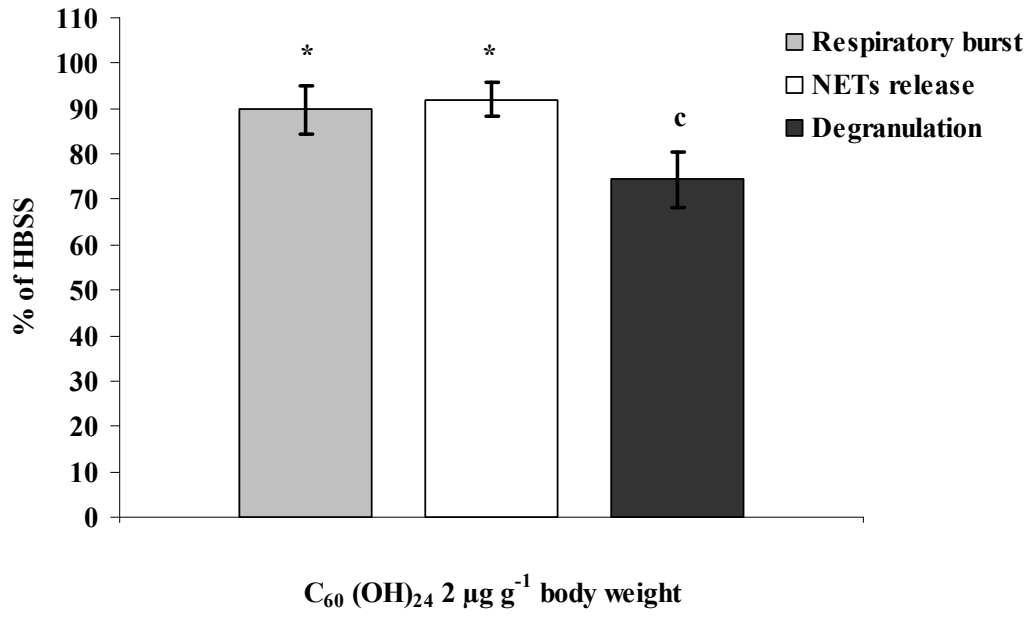


Figure 5

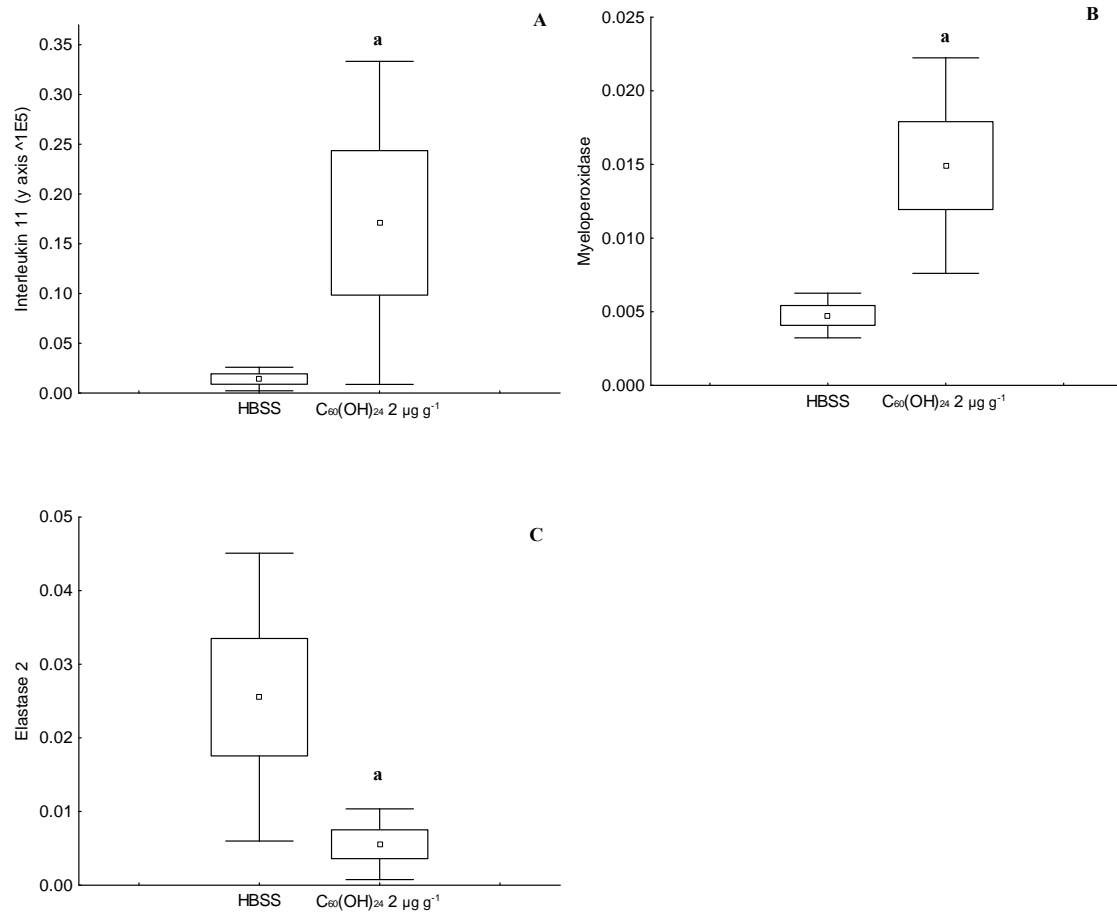


Figure 6

5.9. References

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CHAPTER 6. Titanium dioxide nanoparticles increased mortality of fish exposed to bacterial pathogen

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6.1. Abstract

Titanium dioxide nanoparticles (nano-TiO₂) can interact with fish neutrophils and modulate their function both *in vitro* and *in vivo*. Fathead minnows (*Pimephales promelas*) were exposed to low, environmentally relevant concentration of nano-TiO₂ (2 ng g⁻¹ body weight) and challenged with a common fish bacterial pathogen, *Aeromonas hydrophila*. Exposure to nano-TiO₂ significantly increased fish mortality during *A. hydrophila* challenge: 60% of nano-TiO₂ treated and challenged fish died, compared to 13.5% in group challenged with *A. hydrophila*, but not exposed to nano-TiO₂. The fish exposed to nano-TiO₂ alone presented no mortality or morbidity. The ICP-MS analysis of nano-TiO₂ distribution in fish organism with particular outline on hematopoietic organs was performed. The nano-TiO₂ is concentrating in the fish kidney and spleen. Efforts to better understand the possible interplay between nanoparticle exposure, immune system and infectious disease pathogenesis in any animal model have not been described before. The environmentally relevant concentration of manufactured nano-TiO₂ has potential to interfere with disease resistance in fish populations by modulating immune responses against bacterial pathogens in a disease outbreak.

Keywords: titanium dioxide nanoparticles, immune response, disease resistance, fish,

6.2. Introduction

Among all nanoparticles, titanium dioxide nanoparticles (nano-TiO₂) present the biggest ecotoxicological concern due to the rapid increase of anthropogenic input into the environment. Estimated environmental concentrations of nano-TiO₂ range from 0.7 to 24.5 ng mL⁻¹ (Mueller and Nowack, 2008; Pérez et al., 2009). Nano-TiO₂ is used as a major constituent in sunscreens, soaps, shampoos, toothpastes and other cosmetics (Melquiades et al., 2008), as well as in paper products, plastics, ink, and food as a food color (Ortlieb, 2010). Most known use of nano-TiO₂ is to serve as the only approved inorganic UV-filter (besides nano-zinc-oxide) in sunscreens with product content of up to 25% (FDA, 1999). However, nano-TiO₂ is also used as an additive in paint and building materials (Chen and Poon, 2009), from which it easily leaches and is transported to aquatic ecosystems (Kaegi et al., 2008). As an ingredient in food products nano-TiO₂ is often listed as E171 and commonly is used for whitening skim milk (Ortlieb, 2010). Many synthetic vitamin tablets and over-the-counter pain relief drugs also contain nano-TiO₂ (Luft et al., 2010) as do numerous capsulated antidepressants and antibiotic products. Nano-TiO₂ is being examined for use as an additive of drinking water in water treatments plants, and the EPA is considering approval of large-scale use of nano-TiO₂ in water treatment plants for removal of arsenic from water (EPA, 2010).

Nano-TiO₂ has a strong bactericidal effect and has been used to efficiently kill fish pathogens such as *Edwardsiella tarda* and *Streptococcus iniae* *in vitro* (Cheng et al., 2008; Cheng et al., 2009). Therefore, addition of nano-TiO₂ particles to the water of fish farms has been recommended in order to prevent bacterial outbreaks (Cheng et al., 2008). However, methods that are successfully used for bacterial killing *in vitro* are frequently not useful or as efficient when applied to *in vivo* bacterial killing, likely due to the differences in the intracellular environment, as well as the specific function of phagocytic cells (Segal, 2005). It was recently demonstrated that nano-TiO₂ acts as a strong immunomodulator of fish neutrophil function *in vitro* and *in vivo* (Jovanović et al., 2011). A review of immunomodulating properties of nano-TiO₂ in aquatic organisms has recently become available (Jovanović and Palić unpublished).

Aeromonas hydrophila, a gram negative motile rod and one of the most important bacterial pathogens of aquatic animals in temperate water (Angka, 1990; Esteve et al., 1993) has been employed here as a model for infection of fathead minnow (*Pimephales promelas* Rafinesque, 1820). Pathological condition of *A. hydrophila* pathogenesis may include dermal ulceration, tail or fin rot ocular ulceration, erythrodermatitis, hemorrhagic septicemia, red sore disease, red rot disease and scale protrusion disease (Cipriano, 2001). In the acute form of disease rapid septicemia is the most common outcome of mortality and systemic infection includes eyes, liver and kidney, which are the target organs of an acute septicemia (Cipriano, 2001). The primary mechanism of *A. hydrophila* lethal effects in systemic infections is caused by the kidney cells death, due to the cytotoxic and haemolytic activities of the bacterial extracellular polysaccharides (Rodríguez et al., 2008). It is important to note that *A. hydrophila* is a member of normal intestinal flora of healthy fish (Trust et al., 1974). The presence of the bacteria itself in fish does not indicate the disease *per se* and the stress is often considered to be a contributing factor in the outbreaks of disease caused by *A. hydrophila* (Cipriano, 2001). Stress in fish is closely connected to the modulation of immune response and change in neutrophil function (Palić et al., 2006a; Palić et al., 2006b).

The potential of nano-TiO₂ to interfere with infectious disease resistance as a consequence of the ability to modulate immune responses has not been studied, and there are no available reports addressing possible outcomes of nanoparticle exposure and bacterial challenge in any organism. The aim of this manuscript was to determine if the outcome of a bacterial challenge will be different between fish that were exposed to environmentally relevant concentrations of nanoparticles known to modulate cellular innate immune response, and the fish that were not exposed to the nanoparticles. Our hypothesis was that fish exposed to nano-TiO₂ will have higher mortality than non-exposed fish after challenge with pathogenic bacteria.

6.3. Materials and methods

Bacterial culture

Aeromonas hydrophila (fish pathogen group, outbreak strain, USDA) was plated on the trypticase soy agar (TSA) with 5% of sheep blood plates and incubated at 37 °C overnight. A single colony was picked in the morning and the swab was rinsed into the liquid soy broth sterile tube. The tube was incubated at the 37 °C and the optical density was checked every 30 minutes using spectrophotometer with 450 nm setting until the bacteria proliferated to the desired concentration and were diluted to 5.5×10^7 colony forming units (CFU) mL⁻¹. Standard curve was previously determined with known bacterial concentrations. Sample of adjusted concentration was plated on TSA sheep blood plates for manual colony counts and confirmation of the CFU number after 24 h of incubation, and the rest was immediately used for intraperitoneal injections of *P. promelas*.

Animal care

Juvenile fathead minnows (average weight 2.5 g) were maintained in the Iowa State University College of Veterinary Medicine Laboratory Animal Resources Facility, Ames, Iowa, USA. Fish were housed in a water recirculation system supplied with dechlorinated tap water at 20 °C in 120 L tanks, and fed twice daily with live brine shrimp larvae and dried flake food (2:1 w/w mixture of Aquatox[®] and Plankton/Krill/Spirulina flake food, Zeigler Bros Inc, PA, USA). Fathead minnows were cared for in accordance with approved Iowa State University animal care guidelines.

Nanoparticle characterization and distribution in fish tissues

The nano-TiO₂ (anatase, nanopowder, <25nm, 99.7% metals basis; Sigma-Aldrich Corp, St. Louis, MO, USA) was used in all the experiments. For injection, the nano-TiO₂ was suspended in the Hank's Balanced Salt Solution (HBSS) without Ca, Mg and Phenol Red. The suspension of nanoparticles was used as non-filtered and as filtered through 220 nm general purpose filter. The non filtered nano-TiO₂ suspension was highly polydispersed to provide any accurate distribution of particle size using dynamic light scattering

methodology due to the various sizes of the aggregates. After the filtration, the particle size distribution followed unimodal normal distribution with a bell shaped curve and average particle diameter of 86 nm, zeta potential of -8.87 mV and conductivity of 15.4 mS cm⁻¹ was recorded (Jovanović et al., 2011).

To determine the distribution of nano-TiO₂ particles in fish organs, fathead minnows were injected with 10 µg g⁻¹ body weight with nonfiltered nano-TiO₂ as described below. Nano-TiO₂ negative control was injected with HBSS without Ca, Mg and Phenol Red and both groups fed *ad libitum* for 48 h. After 48 h fish were euthanized with an overdose of tricaine methane sulphonate (MS-222, Argent Laboratories, Redmond WA, USA) (Palić et al 2006b) and kidney, spleen and liver were dissected out from three individuals each per treatment and control. Organs were weighed and digested in nitric acid. In addition five whole individuals were also digested with nitric acid. After digestion concentration of Ti for the isotopes 47 and 49 was measured with inductively coupled plasma mass spectrometry (ICP-MS) using a scandium (Sc) internal standard (m/z=45). The preparation standard used for this analysis was created by spiking on the blank samples with Ti and Sc. Results were corrected for the Ti isotopes natural abundance, averaged between the two Ti isotopes measured and converted to TiO₂ concentration as µg g⁻¹ of sample weight.

Nanoparticle treatment and challenge with Aeromonas hydrophila

To investigate the effects of *in vivo* application of nano-TiO₂ on the fish ability to fight bacterial infection fathead minnows were quickly netted from the tanks and anesthetized with 100 mg L⁻¹ of aerated and buffered (sodium bicarbonate, pH 8.0) solution of the MS-222. Upon entering the third stage of anesthesia (Palić et al., 2006b), fish were weighed and injected intraperitoneally with nanoparticles or with Hank's Balanced Salt Solution (HBSS) without Ca, Mg and Phenol Red. The fish were randomly divided in five groups (two nanoparticle treatment groups, and three control groups).

The first treatment group was injected with 10 µg g⁻¹ body weight of nonfiltered nano-TiO₂ containing lots of aggregates suspended in HBSS without Ca, Mg and Phenol Red. The second treatment group was injected with nano-TiO₂ suspended in HBSS without Ca, Mg and Phenol Red and filtered through 220 nm general purpose filter to remove the

aggregates. The concentration of nano-TiO₂ in suspension after the filtration was determined with ICP-MS and the fish were administered with final concentration of 2 ng g⁻¹ body weight of nano-TiO₂ to simulate the lower end of the estimated environmental concentrations of nano-TiO₂ in aquatic ecosystems (0.7 to 24.5 ng mL⁻¹) (Mueller and Nowack, 2008; Pérez et al., 2009).

Two control groups were injected with 10 µL g⁻¹ body weight of HBSS without Ca, Mg and Phenol Red and no-injection control was also included. All treatment and control groups had 30 individual fish per group. After the injections, fish were transferred to 10 gallon tanks (one tank per treatment or control group), and fed to satiation for 48 h. We have previously determined that 48 h after the administration of nano-TiO₂ to fathead minnows, their neutrophil function (respiratory burst, degranulation and neutrophil extracellular trap release) is diminished (Jovanović et al., 2011). After the 48 h incubation period has ended, fish from two treatment groups and one control group (positive control) were anesthetized again as described above and injected intraperitoneally with 10 µL per gram body weight of 5.5 X 10⁷ CFU mL⁻¹ of live *A. hydrophila* culture suspension (determined to cause 10-15% mortality). Negative control group was injected with 10 µL of sterile soy broth g⁻¹ body weight.

In a subsequent experiment, *A. hydrophila* was inactivated by exposure to 58 °C for 30 minutes. Inactivation was confirmed as no colonies grew on TSA sheep blood plate. Injections of fish were performed as described above.

After the injections fish were returned to 10 gallon tanks and fed to satiation for 21 days. Mortality events were recorded twice per day (every 12 h) to the end of experiment. Dead fish were sampled for bacterial culture (sores, intraperitoneal cavity, and kidney) and in all instances a monoculture of live *A. hydrophila* colonies was isolated on TSA sheep blood plates after 24 h.

Statistics

The statistical difference in mortality rate between experimental groups and control was calculated using the Chi-Square Test. The differences of nano-TiO₂ bioconcentrations

in the organs as opposed to the whole fish sample were evaluated with Analysis of Variance (ANOVA). Only the values of $P \leq 0.05$ were considered statistically significant.

6.4. Results

Nanoparticle distribution in fish tissues

ICP-MS analysis confirmed the injected concentration, and upon 48 h of injection with $10 \mu\text{g g}^{-1}$ body weight of nonfiltered nano-TiO₂ an average of 9.04 ± 0.76 (standard error of the mean – SEM) $\mu\text{g g}^{-1}$ body weight of TiO₂ was detected in the whole fish sample. Analysis of targeted organs revealed that the TiO₂ has the biggest potential of bioconcentration in kidney: $78.7 \pm 28.3 \mu\text{g g}^{-1}$ kidney weight followed by the concentration in the spleen: $46 \pm 0.9 \mu\text{g g}^{-1}$ spleen weight and the concentration in the liver: $8.6 \pm 3.2 \mu\text{g g}^{-1}$ liver weight (Fig. 1). The bioconcentration of nano-TiO₂ in kidney and spleen was significantly higher comparing to the concentration measured in the whole body ($P < 0.01$ and $P < 0.05$ respectively). On the contrary concentration measured in the liver was not statistically different from the average concentration of the whole body. In the control samples that did not receive any injections of nano-TiO₂, TiO₂ concentration was below the detection limit of the instrument in all instances.

*Nanoparticle treatment and challenge with *Aeromonas hydrophila**

The positive control (fish treated with HBSS without Ca, Mg and Phenol Red and then challenged with *A. hydrophila*) had cumulative mortality of 13.5 %, as expected (Fig. 2). The fish in the group that was pre-treated with 2 ng g^{-1} body weight of filtered nano-TiO₂ and challenged with *A. hydrophila* had 60 % cumulative mortality. The fish in the group pre-treated with $10 \mu\text{g g}^{-1}$ body weight of nonfiltered nano-TiO₂ and challenged with *A. hydrophila* had 82.5 % of cumulative mortality. Both non-filtered and filtered nano-TiO₂ treatments, followed by *A. hydrophila* challenge, caused statistically significant increase in mortality comparing to the positive control (not exposed to nano-TiO₂; challenged with *A. hydrophila*) (Chi-Square, $P < 0.05$). There were no mortalities, and no clinical signs were observed in fish from negative (nano-TiO₂ treated; no challenge) and no injection control

groups. Visual examination of fish that were pre-treated with nano-TiO₂ and challenged with *A. hydrophila* revealed development of bigger and more severe hemorrhages than in the fish that were subjected to challenge only (Fig. 3). In fish injected with heat inactivated *A. hydrophila*, there were no mortalities observed in any of the treatment or control groups.

6.5. Discussion

The results of this study are in accordance with the current understanding of nano-TiO₂ potential to cause immunotoxicity in fish neutrophils (Jovanović et al., 2011; Jovanović and Palić, unpublished). The *in vitro* upregulation of neutrophil extracellular trap (NET) release by nanoparticles suggests that significant number of the mature neutrophil population inside the circulatory system of aquatic organism could be depleted via NETosis (Fuchs et al., 2007) after exposure to nano-TiO₂ or potentially other nanoparticles (Bartneck et al., 2009). The removal of the mature neutrophil population may interfere with organismal defenses because immature neutrophils may not display the same functional activity (Borregaard et al., 2001; Martinelli et al., 2004), potentially causing the reduction of neutrophil *in vivo* functional responses (Jovanović et al., 2011). As nano-TiO₂ particles are indigestible by neutrophils, after the nanoparticle is released from the remains of the phagosome, it becomes available to interact with the next neutrophil it encounters along the way (Jovanović and Palić, unpublished).

Neutrophil ability to phagocytize and kill microorganisms and cellular debris is essential for normal development and survival of animal populations (Segal, 2005). In response to inflammatory stimuli, neutrophils migrate from the circulating blood into infected tissues, where they efficiently bind, engulf, and kill bacteria by proteolytic enzymes (Dalmo et al., 1997; Segal, 2005). This study outlined that the kidney is one of the major organs for nano-TiO₂ bioaccumulation as soon as 48 h after parenteral administration. Similar study with rainbow trout exposure to nano-TiO₂ also demonstrated high bioaccumulation within the fish kidney with almost no clearance even 90 days post exposure (Scown et al., 2009). Fish kidney has a major role in hematopoiesis and serves as a neutrophil depot (Zapata, 1979). Therefore, the neutrophil population that is continuously

produced and stored in the fish anterior kidney can be chronically exposed to nano-TiO₂ immunotoxicity and undergo changes in development that may interfere with normal function (Jovanović et al., 2011). Such potential changes in neutrophil function could explain the increase in mortality and severity of clinical symptoms during bacterial challenge in fish pre-exposed to nano-TiO₂.

Heat inactivated *A. hydrophila* did not cause mortality in any of the experimental groups. This is consistent with the previous findings (Rodríguez et al., 2008). The mechanism of *A. hydrophila* infection in fish is based on the fact that viable bacteria (but not heat inactivated) are causing cell death in kidney due to the cytotoxic and haemolytic activities of the bacterial extracellular polysaccharides (Rodríguez et al., 2008). Nano-TiO₂ can act as carrier of bacterial lipopolysaccharides (LPS) in the process of protein corona formation (Ashwood et al., 2007). The absence of mortality in the experimental groups exposed both to nano-TiO₂ and heat inactivated *A. hydrophila* suggests that potential introduction of LPS by nanoparticle to the kidney cells could not cause mortality, or that LPS structure was damaged during heat-inactivation and lost the ability to interact with immune system. The observed increase in mortality with live *A. hydrophila* pretreated with nano-TiO₂ suggests direct immunotoxic effects of nano-TiO₂ to predominantly granulocytic population of cells in fish kidney, and possibly to other kidney cells as well (Jovanović et al., 2011). The results suggest that nano-TiO₂ presence modulates the pathogenesis of *A. hydrophila* infection as the fish with immunocompromized kidney white cell depot can not elicit a proper immune response and fight the infection.

The interaction of nano-TiO₂, immune response, and bacterial infection increased mortality and morbidity of the fish. The significant increase in mortality during bacterial challenge was observed after the administration of 2 ng g⁻¹ body weight of nano-TiO₂. This dose is within the estimated environmental concentration of nano-TiO₂ in aquatic ecosystems of 0.7 to 24.5 ng mL⁻¹ (Mueller and Nowack, 2008; Pérez et al., 2009), indicating the potential of engineered nano-TiO₂ to affect fish survival by interfering with immune responses during possible disease outbreaks in the aquatic ecosystems.

6.6. Acknowledgments

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6.7. Figure captions and figures

Figure 1. Distribution of nano-TiO₂ in *P. promelas* organs 48 h after intraperitoneal injection of nonfiltered nano-TiO₂ 10 µg g⁻¹ body weight. Concentration was determined by ICP-MS.

Figure 2. Cumulative mortality rate of *P. promelas* exposed to nano-TiO₂ and challenged with *Aeromonas hydrophila*. Filled squares represent negative control (HBSS without Ca, Mg and Phenol Red + soy broth); Filled triangles represent positive control (HBSS without Ca, Mg and Phenol Red + 5.5 X 10⁷ CFU mL⁻¹ of live *A. hydrophila*); Empty squares represent group treated with filtered nano-TiO₂ (2 ng g⁻¹ body weight + 10 µL per gram body weight of 5.5 X 10⁷ CFU mL⁻¹ of live *A. hydrophila*); Empty triangles represent group treated with nonfiltered nano-TiO₂ (10 µg g⁻¹ body weight + 10 µL per gram body weight of 5.5 X 10⁷ CFU mL⁻¹ of live *A. hydrophila*). At 0 h time point fish were anaesthetized and intraperitoneally injected with either HBSS or nano-TiO₂ (treatment or control, respectively). The dashed line at 48 h time point indicates administration of *A. hydrophila* and reset of x axis to 0 h of exposure to *A. hydrophila*. The experiment was run for 21 days and since no changes in survival rate occurred at a later time point, the last time point presented on x-axis is at 168 h. * indicates that the effect of nano-TiO₂ on survivability rate is statistically significant when compared to positive control (Chi – Square, *P* < 0.05).

Figure 3. Gross lesions observed in fish after challenge with *Aeromonas hydrophila*. A: Positive control treated with HBSS without Ca, Mg and Phenol Red and challenged with 10 µL per gram body weight of 5.5 X 10⁷ CFU mL⁻¹ of live *A. hydrophila*; B: fish treated with filtered nano-TiO₂ (2 ng g⁻¹ body weight) and challenged with 10 µL per gram body weight

of 5.5×10^7 CFU mL⁻¹ of live *A. hydrophila*. Photographs were taken 36 h after the challenge.

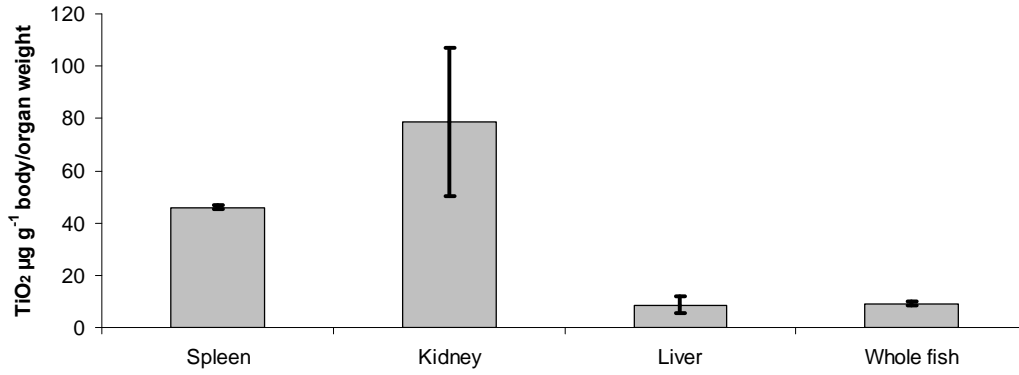


Figure 1.

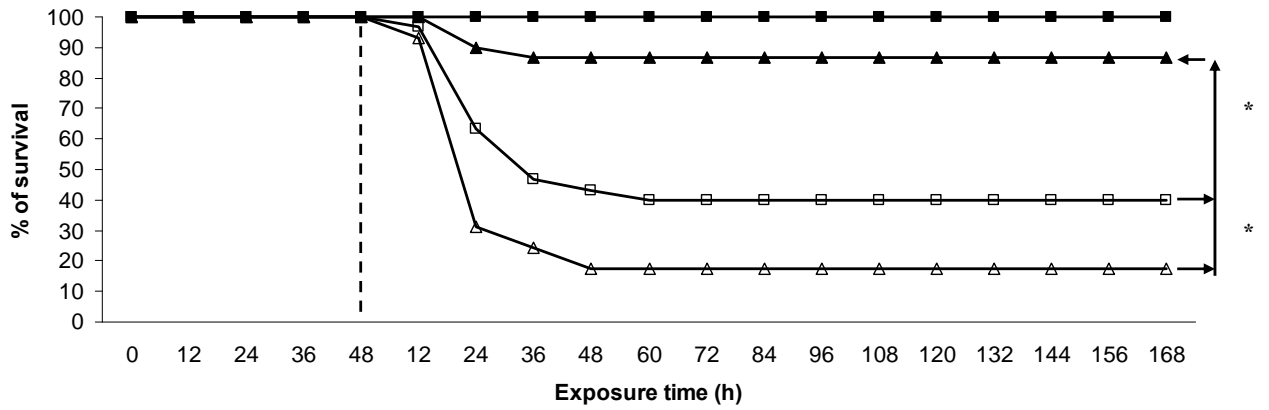


Figure 2.



Figure 3.

6.8. References

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CHAPTER 7. Gene expression of zebrafish embryos exposed to titanium dioxide nanoparticles and hydroxylated fullerenes

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7.1. Abstract

Increased release of engineered nanoparticles to the environment suggests a rising need for the monitoring and evaluation of potential toxicity. Zebrafish frequently have been used as a model species in human and aquatic toxicology studies. In this study, zebrafish embryos were microinjected in the otic vesicle with a sublethal dose of engineered nanoparticles (titanium dioxide/TiO₂ and hydroxylated fullerenes/C₆₀(OH)₂₄). A gene microarray analysis was performed on injected and control embryos to determine the potential for nanoparticles to change the expression of genes involved in cross talk of the nervous and immune systems. The exposure to TiO₂ and hydroxylated fullerenes caused shifts in gene regulation response patterns that were similar for downregulated genes but different for upregulated genes. Significant effects on gene regulation were observed on genes involved in circadian rhythm, kinase activity, vesicular transport and immune response. This is the first report of circadian rhythm gene deregulation by nanoparticles in aquatic animals, indicating the potential for broad physiological and behavioral effects controlled by the circadian system.

Keywords: engineered nanoparticles, zebrafish embryos, circadian rhythm, vesicular trafficking, microarray

7.2. Introduction

Titanium dioxide nanoparticles (nano-TiO₂) have the most industrial applications compared to any other nanoparticles. They are a major component of sunscreens, soaps,

shampoos, toothpastes and other cosmetics (Melquiades et al., 2008), and regulations in the USA allow TiO₂ as the only inorganic UV filter, besides zinc-oxide, to be used in the cosmetic industry at maximum product content of 25% (FDA, 1999). Worldwide, four million tons of TiO₂ are consumed annually in uses as a pigment (Ortlieb, 2010). Nano-TiO₂ is also used as an additive in paint and building materials (Chen and Poon, 2009), from which it can leach and run off into aquatic ecosystems (Kaegi et al., 2008). It is found frequently in paper products, plastics and ink and is used commonly as a food additive (E171 ingredient) for the whitening of skim milk (Ortlieb, 2010). Synthetic vitamin tablets, over-the-counter pills, and prescription drugs, including antidepressant and antibiotic capsulated products, contain TiO₂ in various quantities (Luft et al., 2010). Estimated environmental concentrations of nano-TiO₂ in aquatic ecosystems range from 0.0007 to 0.0245 µg mL⁻¹ (Mueller and Nowack, 2008; Pérez et al., 2009). Recently the International Agency of Research on Cancer (IARC) classified TiO₂ as a possible group 2B human carcinogen (IARC, 2006) due to the possibility of it causing lung cancer by exposure through inhalation. Applications of TiO₂ in cosmetics, pharmaceuticals and the food industry were debated by IARC, but the final report was inconclusive, as outlined by Jacobs and colleagues (2010) in the journal *NanoEthics*.

Hydroxylated fullerenes (fullerenols; C₆₀(OH)₂₄), as well as other water soluble fullerene species, are increasingly used in biomedical research and appear to have therapeutic and diagnostic potential (Cataldo and da Ros, 2008). They are readily absorbed at the organismal and cellular level (Handy et al., 2008; Moore, 2006), can act as a potent inhibitor of cytochrom P450-dependent monooxygenases (Ueng et al., 1997) and are strong antioxidants (Markovic and Trajkovic, 2008). Due to their suppression of reactive oxygen species (ROS) production, hydroxylated fullerenes are being investigated as experimental drugs in the treatment of Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (Cai et al., 2008; Cataldo and da Ros, 2008; Dugan et al., 2001). Research performed on zebrafish models indicate that water soluble fullerenes can be used as effective cytoprotective and antiapoptotic drugs (Beuerle et al., 2007). Other fullerene species are considered as photosensitizer agents in photodynamic therapy of bacterial, viral and neoplastic diseases (Cataldo and da Ros, 2008).

Medicinal and therapeutic applications of hydroxylated fullerenes have been investigated so far using a set of studies that focus on desirable effects and efficacy, with limited or no information about potential off-target effects and safety concerns. Furthermore, the increase of industrial manufacturing of hydroxylated fullerenes may pose an environmental threat to aquatic ecosystems due to their water solubility of up to 100 g L^{-1} (Hirsch, 2003) and stability in solution for at least nine months (Deguchi et al., 2001). In 2008 the estimated concentration of fullerenes in aquatic environments was $0.00031 \mu\text{g mL}^{-1}$ (Pérez et al., 2009). Recently, it was shown that the administration of hydroxylated fullerenes leads to rapid uptake by immune granulocytic cells and significant reduction of the innate immune response in a fish model (Jovanovic et al., 2011), suggesting further studies are needed to better estimate the potential risk of hydroxylated fullerenes for biomedical use as well as environmental effects.

This study examined the potential of zebrafish (*Danio rerio* Hamilton, 1822) embryo microarray analysis in nanotoxicology. Zebrafish is widely used as a model in biomedical research because of its rapid reproduction rate and the abundant genetic information from recent sequencing of the zebrafish genome. These characteristics make the zebrafish an excellent model for studies in developmental biology, genetics and toxicology (Berman et al., 2003; Chen et al., 2010). Zebrafish are also increasingly used in immunological research (Trede et al., 2004) and offer a unique opportunity to discover and study novel genes required for the control of normal vertebrate hematopoiesis and functioning of adult blood cells in health and disease. The immune system of zebrafish has many features in common with the mammalian innate immune responses (Palić et al., 2007; Renshaw et al., 2006; Traver et al., 2003). Similarities of inflammatory processes are used in the zebrafish model to study inflammation resolution related to lung pathology (Renshaw et al., 2007). The optical clarity, ease of maintenance, chemical administration and sensitivity make zebrafish embryos an irreplaceable tool in drug screening and toxicity testing (Parng, 2005; Parng et al., 2002; Rubinstein, 2006).

Gene microarrays are used frequently in large scale quantitative studies of mechanisms of disease, disease progression prediction, predicting activities of new compounds and grouping genes into functional pathways (Stoughton, 2005). Gene

microarrays of zebrafish embryos have been used increasingly in place of adult zebrafish genomic studies (de Jong et al., 2010; Mathavan et al., 2005). However, most gene microarray nanoparticle studies are based solely on adult zebrafish, and limited attempts have been made to adapt zebrafish embryo microarrays to study the mechanisms of nanotoxicology. The few exceptions (Usenko et al., 2008; Yeo and Kim, 2010) have exposed the embryos through immersion in nanoparticle solutions without the ability to measure the concentration of nanoparticles that was actually entering the embryo and was responsible for observed transcriptomic changes. Although fish embryos can uptake nanoparticles from a water column (Kashiwada, 2006), the primary mechanism of nanoparticle uptake by adult fish is not through their skin or gills, but through the gut (Handy et al., 2008) and after the uptake nanoparticles are transported to target organs. Nanoparticles also have a limited ability to penetrate through mammalian skin as well (Baroli et al., 2007; Kokura et al., 2010; Xia et al., 2010). Due to the tender nature of the embryo epidermal layer, nanoparticle immersions can cause mechanical abrasions on embryo integument and hinder the detection of important molecular pathways. Such abrasions may explain the unusual expression of genes for keratin and β -actin in zebrafish microarray embryo studies (Usenko et al., 2008). Therefore these changes in gene expression might not be the result of intrinsic toxicity feature of nanoparticles, but rather reflect mechanical damage and tissue regeneration. The zebrafish embryo microinjection technique is used to precisely deliver a known concentration of chemical to the target organ or developmental area (Xu, 1999), therefore bypassing the fragile integument exposure through immersion. Injections are also a standard model for evaluating toxicology and hematology parameters not only in fish models but also in mammals, including humans (Janeway et al., 2008).

Due to the rising concerns about nanoparticle effects on the immune and nervous systems (Dobrovolskaia and McNeil, 2007; Yang et al., 2010) the aim of this study was to investigate transcriptomic pathways at the level of the neuro-immunological interface of zebrafish embryos microinjected with nano-TiO₂ and hydroxylated fullerenes in the otic vesicle which is in the close proximity of the brain.

7.3. Materials and methods

Animal care

Adult zebrafish were maintained in the Iowa State University College of Veterinary Medicine Laboratory Animal Resources Facility, Ames, Iowa, USA. Fish were housed in an AHAB Benchtop system from Aquatic Habitats (Apopka, FL, USA) supplied with dechlorinated tap water at 28 °C. Fish were cared for in accordance with standard zebrafish protocol (Westerfield, 2000), approved by the Animal Care and Use Committee of Iowa State University.

Nanoparticle characterization

Nano-TiO₂ (anatase, nanopowder, <25nm, 99.7% metals basis; Sigma-Aldrich Corp, St. Louis, MO, USA) and hydroxylated fullerenes C₆₀(OH)₂₄ (MER Corporation, Tucson, AZ, USA, cat# MR16, 98% purity) were suspended in Hanks Balanced Salt Solution without Ca, Mg, no Phenol Red, (HBSS; Mediatech – CellGro, Manassas, VA, USA). Full characterization of both particles' suspension in HBSS and suspension preparation is described in our previous research (Jovanovic et al., 2011; Jovanovic et al., In Press). Briefly, nano-TiO₂ and hydroxylated fullerenes with mean particle diameters of 86 nm and 409 nm and zeta potential of -8.87 mV and -19.1 mV, respectively, were used in the experiments.

Fish mating and embryo microinjections

Zebrafish mating and embryo collection was performed daily according to (Levraud et al., 2007; Westerfield, 2000). Several mating tanks were set up each day with 3 females and 2 males. After successful mating, embryos from three random mating tanks were pooled together, rinsed with fish egg water (60 µg of sea salt [Instant Ocean, Kingman, AZ, USA] per mL of deionized water) (Westerfield, 2000), transferred to a petri dish with egg water and incubated for 48 h at 28.5 °C. At 48 hours post-fertilization (hpf) 25 dechorionated embryos were randomly collected, weighed (average weight of an embryo was 0.2 mg), anesthetized (0.25 mg mL⁻¹ buffered [sodium bicarbonate, pH 7.2] solution of the tricaine

methane sulphonate [MS-222, Argent Laboratories, Redmond WA, USA] in egg water and microinjected in the otic vesicle with 10 nL of nanoparticle suspension (Levraud et al., 2007). Control group embryos were injected with 10 nL of HBSS. In order to deliver 10 nL PLI-90 Pico-Injector (Harvard Apparatus, Holliston, MA, USA) was used with the following setup: pressure at 30 psi, time at 2 s and diameter of syringe at 1 μm , according to manufacturer's protocol.

Nanoparticle injection suspensions were made fresh daily, with a concentration of 40 $\mu\text{g mL}^{-1}$ of hydroxylated fullerenes and 170 ng mL^{-1} of nano-TiO₂. Nano-TiO₂ was previously filtered through 220 nm filter and the concentration of 170 ng mL^{-1} was determined after filtration by inductively coupled plasma mass spectroscopy (ICP-MS). After the administration of 10 nL to 0.2 mg embryos, the final concentration inside the embryo was 2 $\mu\text{g g}^{-1}$ bodyweight for hydroxylated fullerenes and 8.5 ng g^{-1} bodyweight for nano-TiO₂. These concentrations were chosen based on previous findings for sublethal intraperitoneal injections in fathead minnow that were affecting the innate immune response (Jovanovic et al., 2011; Jovanovic et al., In Press). After the microinjections, embryos were incubated for 48 h at 28.5 °C. At 96 hpf embryos were collected and sacrificed with quick asphyxia, and total RNA was immediately isolated. The whole process was repeated to obtain a total of 18 samples, each consisting of 25 pooled embryos (n=6 samples per each treatment, and n=6 samples per control).

RNA isolation and microarray hybridization

Total RNA extraction was performed according to the acid guanidinium thiocyanate-phenol-chloroform extraction protocol (Chomczynski and Sacchi, 2006) using TRI Reagent® (Molecular Research Center, Inc. Cincinnati, OH, USA). Embryos were ground in a 1.5 mL centrifuge tube containing 1 mL of Tri-Reagent. Total RNA extracted and the concentration was assessed using a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE, USA) and adjusted to 100 $\text{ng }\mu\text{L}^{-1}$. Zebrafish microarrays were purchased from Affymetrix (Santa Clara, CA, USA). Each sample was hybridized on a separate array, and the arrays were not reused. Samples were labeled with the GeneChip® 3' IVT Express Kit (Affymetrix, part # 901229). This kit includes all reagents needed for the generation of

biotin-labeled target amplified RNA (aRNA). The major advantage of the 3' IVT Express Kit over the one-cycle kit is that it requires low starting total RNA input, and 250 ng of the total RNA was used for the labeling. Reagents for the hybridization, wash and sample staining were also purchased from Affymetrix (part # 900720). Reverse transcription was used to synthesize first-strand cDNA. PolyA mRNA from the 250 ng total RNA was used as a template to synthesize cDNA with a T7 oligo (dT) primer that contains a T7 promoter sequence, so that the first-strand cDNA would have a T7 sequence. The single-stranded cDNA–RNA hybrid was converted into a double stranded cDNA (dsDNA). The reaction employed DNA polymerase, RNase H and DNA ligase to simultaneously degrade the RNA and synthesize second strand cDNA. The IVT labeling master mix generated multiple copies of biotin-modified aRNA from the double stranded cDNA templates. This amplification step was conducted with 16 h of incubation at 40 °C in order to consistently obtain high aRNA yield. Finally aRNA purification was performed and aRNA concentration was measured with a NanoDrop. The labeled aRNA of 15 µg was fragmented in 1x fragmentation buffer at 94 °C for 35 minutes. The fragmented RNA was tested on an Agilent Bioanalyzer RNA nano chip to make sure the fragments were roughly 100–120 nucleotides and all the samples had similar concentration. Fragmented aRNA (12.5 µg) was mixed with hybridization controls to make a total of 250 µl of hybridization cocktail, 200 µl of which was used for hybridization with the arrays. The hybridization was conducted at 45 °C for 16 h in a GeneChip® hybridization oven 640 with constant rotation at 60 rpm. The arrays were washed and stained in the GeneChip® fluidics station 450 and scanned with GeneChip® scanner 3000 7G. Affymetrix GeneChip Command Console™ software was used to control the fluidics stations and scanner and to generate all data files (all equipment and software is produced by Affymetrix). The probe-level summarization (.CHP) files were generated by Affymetrix® Expression Console™ Software.

Data analysis and statistics

Nano-TiO₂ and hydroxylated fullerene treatments were compared separately with the control treatment for difference in gene expression. Storey and Tibshirani *q* value procedure for microarray data analysis was employed (Storey and Tibshirani, 2003) while controlling

for False Discovery Rate (FDR) at the 0.05 level (Aubert et al., 2004). Only probes with $q < 0.05$ were considered statistically significant. In addition, fold change values were taken into consideration. Only probes with $q < 0.05$ values that were upregulated or downregulated two-fold or more compared to the non-treated (HBSS-injected) control were considered biologically significant and are presented in the results section.

7.4. Results

Nano-TiO₂ treatment had 2380 significantly expressed probes, whereas hydroxylated fullerenes had 313 significantly expressed probes at the $q < 0.05$ level compared to the non-treated controls (Fig. 1). The total number of probes on the microarray chip was 15617. At the level of biological significance (defined as change in expression of ± 2 -fold or more compared to the non-treated control), the nano-TiO₂ treatment had 36 downregulated probes associated with 33 different genes with 2-fold or more downregulation (Table 1). There were 12 probes with 2-fold or more upregulation in nano-TiO₂ treatment associated with 12 different genes (Table 1). The hydroxylated fullerene treatment had 28 downregulated probes associated with 25 different genes with 2-fold or more downregulation and 11 probes belonging to 11 genes with 2-fold or more upregulation (Table 2). Out of 25 downregulated genes, 22 were also downregulated by the nano-TiO₂ treatment, whereas only 4 out of 11 upregulated genes were upregulated in both the nano-TiO₂ and fullerene treatments. By examining the function of downregulated genes it was observed that the nanoparticle treatment interfered with four areas of organismal function: circadian rhythm; cell signaling through kinase-related activities; exocytosis and trafficking of Golgi vesicles; and immune function. Due to the unknown function of most upregulated genes (Tables 1 and 2) it was not possible to group genes into clusters, but the involvement of two upregulated genes in the immune system regulation indicates a possible effect of nanoparticle treatment on immune function.

7.5. Discussion

Microarray analysis identified a similar response in gene downregulation of embryonic zebrafish exposed to different nanoparticle treatments. Two different types of nanoparticles (nano-TiO₂ and hydroxylated fullerene) appear to have induced downregulation of the specific group of genes as their common response. Results indicate the potential of nanoparticles to downregulate specific circadian rhythm pathways composed of: period homolog 2; cryptochrome 1a; cryptochrome 1b; cryptochrome DASH coupled with a downregulation of retinal light receptors and transducers phosphodiesterase 6H, cGMP-specific, cone, gamma; guanylate cyclase activator 1C and a specific gene, thyrotroph embryonic factor, involved in the development of the pituitary gland. The same pathway, circadian clock—thyrotroph, is disrupted by nano-gold particles in the mammalian model (Balasubramanian et al., 2010), but these are the first findings of the disruption of the circadian system pathway by nanoparticles in fish.

Cryptochromes are blue and ultraviolet light receptors and are integral parts of the central circadian oscillator in the brain of animals, thus controlling daily physiological and behavioral rhythm. The oscillations of the central oscillator are governed by a period homolog gene through a transcriptional feedback loop with cryptochromes (Lin and Todo, 2005). Therefore cryptochromes perform both light-dependent (in photoreceptors) and light-independent (as transcriptional repressors) functions. Retinal photoreceptors and cryptochromes act together in regulation of circadian rhythm (Lin and Todo, 2005), and downregulation of several retinal genes after exposure to nanoparticles could be related to the downregulation of the cryptochrome complex. The thyrotroph embryonic factor directly regulates the majority of circadian genes as well as DNA damage repairs and stress response processes (Gavriouchkina et al., 2010). It also has an important role in mediating xenobiotic detoxification and controls expression of genes involved in xenobiotic biotransformation, such as cytochrome P450 enzymes (Gachon et al., 2006), the expression of which was also downregulated by the treatment (Tables 1 and 2).

The circadian system of zebrafish involves retinal receptors, the pineal gland and melatonin, and the circadian rhythm is primarily affected by light intensity (Kazimi and

Cahill, 1999). Pineal gland receptors can be photostimulated directly or through retinal receptors, and both the pineal gland and the retina respond to stimulation by secreting melatonin (Kazimi and Cahill, 1999). Melatonin has different effects on homeostasis, including a role in the regulation of diurnal rhythms of immune system elements. The increase in melatonin level during the night phase increases the phagocytic ability of zebrafish macrophages (Kaplan et al., 2008). It is possible that the transcriptional decrease of genes involved in circadian rhythm after exposure to nanoparticles can be explained by the activation of an internal regulatory defensive mechanism that could lead to an increase in melatonin release by retinal cells and pinealocytes. This increase in melatonin could have a stimulative effect on zebrafish neutrophils, as well as on macrophages, enhancing their ability for phagocytosis and nanoparticle clearance. In addition, recent studies using the cyprinid fish model indicate that nano-TiO₂ can induce oxidative burst activity of fish neutrophils, suggesting the potential for increased tissue damage in areas exposed to activated neutrophils (Jovanovic et al., In Press). Neutrophils were observed in zebrafish embryos as early as 48 h, with increased migration and phagocytic activity after 72 hpf (Lieschke et al., 2001; Renshaw et al., 2006). Therefore, synergistic stimulative action of melatonin increase and nano-TiO₂ could cause widespread tissue injury and activation of downregulating mechanisms in an effort to reduce the damage.

Another possibility for circadian signaling suppression may be due to the destructive nature of nanoparticles to retinal cells and the pineal gland. The pineal gland forms at about 19–20 hpf in zebrafish, and the photoreceptors can be detected in pinealocytes as early as 24 hpf, whereas the retinal photoreceptors are fully functional at 48–50 hpf (Kazimi and Cahill, 1999). Microinjections were delivered at 48 hpf to the otic vesicle in close proximity to the pineal gland. Unlike for other parts of the brain, the pineal gland is not isolated by the blood–brain barrier (Pritchard and Alloway, 2007) therefore allowing for rapid and aggressive interaction with nanoparticles. It is possible that the nanoparticles caused mechanical damage and apoptosis to pinealocytes, therefore resulting in transcriptional decrease of its receptors and subsequent downregulation of the entire circadian clock–retina–thyrotroph development. However, there are no published data that support direct

destructive action of nanoparticles or tissue damage caused by the action of hyperactive phagocytic cells.

The second functional cluster of downregulated genes that was identified by microarray analysis as a response toward exposures to nanoparticles is made up of genes involved in cell signaling, particularly kinase-related activities (Tables 1 and 2). Examples of downregulated genes that regulate the activity of kinases include type I cytokeratin, coatmer protein, COP9, Si:ch211-195b13.1 and serum/glucocorticoid regulated kinase 1. Different types of kinases are common targets for modulation by carbon-based and metal-oxide nanoparticles. For example nano-TiO₂ can induce activation of neutrophils by phosphorylation of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinases-1/2 (Erk-1/2) (Gonçalves et al., 2010). Carbon black nanoparticles activate MAPK and JNKs stress-activated kinases in blue mussel hemocytes (Canesi et al., 2008). The same p38 MAPK stress kinase activation in the blue mussel model has been confirmed for metal-oxide nanoparticles including nano-TiO₂ (Canesi et al., 2010). Other kinase signaling pathways, such as GDFN/Fyn kinase activity, can be disrupted by metallic nanoparticles (Braydich-Stolle et al., 2010). The Src kinase pathway, an important kinase involved in the regulation of immune signaling and cancer, is suppressed by gold nanoparticles (Karthikeyan et al., 2010). The specificity of nanoparticles for kinases has advanced the field of science by creating specific microarray-based kinase inhibition assays by nanoparticle probes (Sun et al., 2006) and magnetic resonance imaging specific assays (Shapiro et al., 2009). The observed changes in the regulation of different kinase pathways in zebrafish exposed to nanoparticles is in accordance with published findings and add to the general understanding of the interaction between kinase-related gene activity and nanoparticles.

The third functional cluster of downregulated genes identified by the microarray analysis as a response toward exposure to nanoparticles includes genes involved in exocytosis, transport and vesicular trafficking such as coatmer protein, solute carrier family 16-monocarboxylic acid transporters, secretory carrier membrane protein 5, and solute carrier family 44-choline transporter. Secretory carrier membrane proteins are important proteins in the regulation of pH in vesicular organelles and the recycling of the endosomal

pool of vesicles serving as a reservoir of functional transporters (Lin et al., 2005). Coatamer proteins further mediate biosynthetic protein transport from the endoplasmic reticulum (ER) via the Golgi up to the trans-Golgi network. The preferable storage compartments of nanoparticles are vesicular organelles such as lysosomes, Golgi and ER (Moore, 2006). Therefore, it is reasonable to suspect that nanoparticle storage would interfere in the recycling process of these vesicles and diminish the transport potential, followed by effects observable on the level of gene expression.

The solute carrier family 16 gene is responsible for synthesis of proteins that carries monocarboxylates, predominantly lactate and pyruvate, across cell membranes. Lactic acid is one of the products of glycolysis that yields ATP. In order to maintain a high rate of glycolysis, lactic acid needs to be transported out of the cell by solute carrier family 16 proteins (Halestrap and Meredith, 2004). The downregulation of the gene responsible for lactate and pyruvate transport by nanoparticles could lead to glycolysis and tricarboxylic acid cycle arrest. Also, the nanoparticles can interfere with glucose catabolism by interacting with various kinases (as outlined above) that are involved in the process of glycolysis and reduce the need for byproduct transporters leading to downregulation of the solute carrier 16 gene. The other downregulated solute carrier was a family 44 member choline transporter that carries choline across the blood–brain barrier to neurons for the synthesis of the neurotransmitter acetylcholine (Allen and Lockman, 2003; Dahlin et al., 2009). Downregulation of a choline transporter will interfere with and reduce the amount of choline transported over the membrane. This deficiency of choline transportation can strongly influence circadian rhythm and the activity of the central oscillator (Lakin-Thomas, 1996; Lakin-Thomas, 1998; Morley and Charles Murrin, 1989), providing a potential connection between observed degranulation of vesicular trafficking gene cluster and circadian gene cluster through defects in choline transportation.

The fourth functional cluster of downregulated genes identified by microarray analysis as a response toward exposures to nanoparticles consists of several genes involved in immune response such as: tissue inhibitor of metalloproteinase 2b, FK506 binding protein 5, matrix metalloproteinase 9 (neutrophil elastase) and prostaglandin D2 synthase. Downregulation of these genes suggests changes at the organismal level that suppress the

proliferation of endothelial cells, inhibit thrombocyte aggregation and suppress interleukin 8 mobilization as outlined in Table 1.

The profiles of upregulated genes observed in zebrafish embryos exposed to the two treatments (nano-TiO₂ and hydroxylated fullerenes) were significantly different. The few genes with known function have a role in innate immune responses and include complement system protein and cathepsin. However, the function of most of the upregulated genes in both treatments is currently unknown. The introduction of nanoparticles in the blood plasma is followed rapidly by formation of a protein corona, an association (loose or tight) of a nanoparticle with proteins or other biopolymers due to the electrostatic properties of nanoparticles as well as their surface charge (Lundqvist et al., 2008). Neutral (non-functionalized) nanoparticles such as nano-TiO₂ form corona predominantly with proteins of a complement system (Lundqvist et al., 2008), stimulating prolonged transcription of the complement system proteins and priming the nanoparticles for phagocytosis or other interactions with the innate immune system. Such prolonged stimulation could explain the observed increase in complement gene expression.

Cathepsin belongs to a family of proteins that is part of the neutrophil granular enzymes involved in the destruction of phagocytized pathogens (Segal, 2005). Hydroxylated fullerenes can interfere with neutrophil function *in vivo* and alter the expression of granular enzymes such as myeloperoxidase (MPO) and elastase (matrix metaloproteinase 9; MMP9) at different stages of neutrophil maturation (Jovanovic et al., 2011). It is possible that an observed increase in transcription of cathepsins can be explained by the action of hydroxylated fullerenes on zebrafish neutrophil development, especially given that the period from 48 to 96 hpf has been deemed critical in granulocyte and phagocyte differentiation in zebrafish (Lieschke et al., 2001).

In conclusion, the microinjection of nanoparticles in zebrafish embryos caused significant changes in gene expression patterns that were detected with the Affymetrix[®] gene microarray chip. The nanoparticle exposure caused changes in genes related to circadian rhythm, cell kinase activity, intracellular trafficking and immune response. The zebrafish embryo model suggests that different nanomaterials may have similar suppression but different upregulation patterns of transcriptome changes. The precise delivery of

nanoparticles to the target tissue exposure model has great potential to be used in future studies of nanoparticle toxicity.

7.6. Acknowledgments

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7.7. Tables

Table 1. List of genes that were downregulated (-) and upregulated (+) by the nano-TiO₂ treatment.

Probe ID	Gene name	Regulation	Function*
Dr.24216.1.S1_at	Type I cytokeratin	-9.58	Regulate the activity of kinases such as PKC and SRC via binding to integrin beta-1 (ITB1) and the receptor of activated protein kinase C (RACK1/GNB2L1)
Dr.6754.1.A1_at	Period homolog 2	-6.61	Circadian rhythm, transcription regulation
Dr.25639.1.A1_at	Similar to LON peptidase N-terminal domain and ring finger 1 (LONRF1)	-5.73	ATP-dependent peptidase activity, metal ion binding, protein binding, proteolysis. Circadian rhythm?
Dr.18853.1.A1_at	Wu:fc22g01 - Cofilin 2 (muscle)	-5.01	Encodes an intracellular protein involved in the regulation of actin-filament dynamics. This protein is a major component of intranuclear and cytoplasmic actin rods
Dr.17061.1.A1_at	Hypothetical LOC569866	-3.92	Unknown
Dr.9849.1.A1_at	zgc:77060	-3.73	Regulation of transcription, DNA-dependent
Dr.14687.1.A1_at	Coatomer protein complex, subunit beta 2	-3.22	Cytosolic protein complex that binds to dilysine motifs and reversibly associates with Golgi non-clathrin-coated vesicles, which further mediate biosynthetic protein transport from the ER, via the Golgi up to the trans Golgi network. Is a selective binding protein (RACK) for protein kinase C, epsilon type
Dr.15281.1.A1_at	Tissue inhibitor of metalloproteinase 2b	-3.19	The encoded protein is natural inhibitor of the matrix metalloproteinases. In addition, the encoded protein has a unique role among TIMP family members in its ability to directly suppress the proliferation of endothelial cells
Dr.10293.2.S1_at	Phosphodiesterase 6H, cGMP-specific, cone, gamma	-3.02	Encodes cone-specific cGMP phosphodiesterase. Exclusively expressed in the retina; involved in the transmission and amplification of the visual signal.
Dr.10293.2.S1_a_at	Phosphodiesterase 6H, cGMP-specific, cone, gamma	-3.01	See above
Dr.14300.1.S1_at	Guanylate cyclase activator 1C	-2.71	Stimulates guanylyl cyclase 1 (GC1) and GC2 when free calcium ions concentration is low and inhibits guanylyl cyclases when free calcium ions concentration is elevated. This regulation of guanylyl cyclase is a key event in recovery of the dark state of rod photoreceptors following light exposure.

Table 1. (continued)

Dr.1603.1.A1_at	Cytochrome P450, family 51	-2.68	This endoplasmic reticulum protein participates in the synthesis of cholesterol by catalyzing the removal of the 14alpha-methyl group from lanosterol
DrAffx.2.2.S1_at	Period homolog 2	-2.58	See above
Dr.25214.1.A1_at	Cytochrome P450, family 24, subfamily A, polypeptide 1	-2.56	This mitochondrial protein initiates the degradation of 1,25-dihydroxyvitamin D3, the physiologically active form of vitamin D3, by hydroxylation of the side chain.
Dr.25135.1.A1_x_at	COP9 constitutive photomorphogenic homolog subunit 4	-2.46	This gene encodes one of eight subunits composing COP9 signalosome, a highly conserved protein complex that functions as an important regulator in multiple signaling pathways. Associated with de-ubiquitination activity and protein kinase activities
Dr.578.1.A1_at	Thyrotroph embryonic factor	-2.43	Transcription factor. During embryonic development, TEF expression appears to be restricted to the developing anterior pituitary gland, coincident with the appearance of thyroid-stimulating hormone, beta (TSHB). Circadian rhythm
Dr.21755.1.A1_at	Wu:fc51h05	-2.17	Unknown
Dr.7340.1.A1_at	Solute carrier family 16 (monocarboxylic acid transporters), member 9a	-2.14	Proton-linked monocarboxylate transporter.
Dr.2675.1.A1_at	FK506 binding protein 5	-2.13	Member of the immunophilin protein family, which play a role in immunoregulation and basic cellular processes involving protein folding and trafficking.
Dr.10329.1.S1_at	Cryptochrome 1a	-2.11	Blue light-dependent regulator of the circadian feedback loop.
Dr.7371.1.S2_at	Cryptochrome 1b	-2.11	Fairly similar as cryptochrome 1a
Dr.6618.1.A1_at	Unknown	-2.1	Unknown
Dr.25166.2.S1_at	Zona pellucida glycoprotein 3a.2	-2.08	The protein encoded by this gene is a structural component of the zona pellucida and functions in primary binding and induction of the sperm acrosome reaction
Dr.18310.2.A1_at	Cryptochrome DASH	-2.08	Circadian rhythm light-responsive regulatory processes
Dr.24146.1.S1_at	Solute carrier family 16 (monocarboxylic acid transporters), member 9b	-2.07	Fairly similar as 9a above
Dr.9528.1.S1_at	Unknown	-2.06	Unknown
Dr.22985.1.A1_at	Tissue inhibitor of metalloproteinase 2b	-2.06	Same as above

Table 1. (continued)

Dr.23741.1.S1_at	Unknown	-2.06	Unknown
Dr.9896.1.A1_at	Si:ch211-195b13.1	-2.05	ATP binding, protein phosphorylation, kinase and transferase function
Dr.19471.1.A1_at	Secretory carrier membrane protein 5	-2.05	Required for the calcium-dependent exocytosis of signal sequence-containing cytokines such as CCL5
Dr.967.1.S1_at	Matrix metalloproteinase 9	-2.03	The enzyme encoded by this gene degrades type IV and V collagens. The enzyme is involved in IL-8-induced mobilization of hematopoietic progenitor cells
Dr.10320.1.S1_at	Serum/glucocorticoid regulated kinase 1	-2.00	This gene encodes a serine/threonine protein kinase that plays an important role in cellular stress response
Dr.1192.1.S1_at	Prostaglandin D2 synthase	-2.00	Neuromodulator and trophic factor in the CNS. Involved in smooth muscle contraction/relaxation. Potent inhibitor of platelet aggregation. Preferentially expressed in brain. Development and maintenance of the blood-brain and blood-retina barrier
Dr.10441.1.S1_at	Cryptochrome 3	-2.00	Play a pivotal role in the generation and maintenance of circadian rhythms
Dr.1603.1.A1_x_at	Cytochrome P450, family 51	-2.00	See above
Dr.17570.1.S3_at	Solute carrier family 44, member 5b	-1.95	Choline-like transporter. Catalysis of the transfer of choline from one side of the membrane to the other
Dr.17591.1.S1_at	Complement component 1, q subcomponent-like 4 like	+1.99	C1q complex is potentially multivalent for attachment to the complement fixation sites of immunoglobulin
Dr.12706.2.S1_at	Si:dkey-21n12.1	+2.05	Unknown
Dr.9682.1.A1_at	Zgc:172053	+2.07	Unknown
Dr.25393.1.A1_at	Si:dkey-21n12.1	+2.18	Unknown
Dr.10476.1.A1_at	Wu:fc75d03	+2.27	Unknown
Dr.25767.1.S1_at	Unknown	+2.28	Unknown
Dr.25767.1.S1_x_at	Unknown	+2.33	Unknown
Dr.535.2.A1_at	Carboxyl ester lipase	+2.36	Catalyzes fat and vitamin absorption.
Dr.21047.1.S1_at	Activin receptor Iib	+2.39	Constitutively active kinase. Encodes activin A type IIB receptor
Dr.12770.1.S1_at	Unknown	+2.56	Unknown
Dr.9890.1.A1_at	Wu:fk54f12	+2.78	Unknown
Dr.15945.1.A1_at	Si:ch211-121a2.2 (hypothetical protein LOC564515)	+4.14	Protein tyrosine/serine/threonine phosphatase activity, hydrolase activity, proteine dephosphorylation

* Gene function was derived based on the entries in GeneCards, WikiGenes, GeneBank or IHOP database.

Table 2. List of genes that were downregulated (-) and upregulated (+) by the hydroxylated fullerenes treatment.

Probe ID	Gene name	Regulation	Function
Dr.6754.1.A1_at	Period homolog 2	-6.54	See Table 1
Dr.25639.1.A1_at	Similar to LON peptidase N-terminal domain and ring finger 1 (LONRF1)	-5.34	See Table 1
Dr.18853.1.A1_at	Wu:fc22g01 - Cofilin 2 (muscle)	-4.90	See Table 1
Dr.17061.1.A1_at	Hypothetical LOC569866	-3.8	See Table 1
Dr.9849.1.A1_at	Zgc:77060	-3.56	See Table 1
Dr.10293.2.S1_at	Phosphodiesterase 6H, cGMP-specific, cone, gamma	-3.05	See Table 1
DrAffx.2.2.S1_at	Period homolog 2	-2.85	See Table 1
Dr.10293.2.S1_a_at	Phosphodiesterase 6H, cGMP-specific, cone, gamma	-2.83	See Table 1
Dr.578.1.A1_at	Thyrotroph embryonic factor	-2.74	See Table 1
Dr.578.2.S1_a_at	Thyrotroph embryonic factor	-2.70	See Table 1
Dr.14300.1.S1_at	Guanylate cyclase activator 1C	-2.58	See Table 1
Dr.2675.1.A1_at	FK506 binding protein 5	-2.57	See Table 1
Dr.25166.2.S1_at	Zona pellucida glycoprotein 3a.2	-2.56	See Table 1
Dr.25135.1.A1_x_at	COP9 constitutive photomorphogenic homolog subunit 4	-2.52	See Table 1
Dr.19471.1.A1_at	Secretory carrier membrane protein 5	-2.51	See Table 1
Dr.25214.1.A1_at	Cytochrome P450, family 24, subfamily A, polypeptide 1	-2.45	See Table 1
Dr.10441.1.S1_at	Cryptochrome 3	-2.34	See Table 1
Dr.10329.1.S1_at	Cryptochrome 1a	-2.26	See Table 1
Dr.1192.1.S1_at	Prostaglandin D2 synthase	-2.23	See Table 1
Dr.7371.1.S2_at	Cryptochrome 1b	-2.16	See Table 1
Dr.9896.1.A1_at	Si:ch211-195b13.1	-2.16	See Table 1

Table 2. (continued)

Dr.23741.1.S1_at	Unknown	-2.09	See Table 1
Dr.9528.1.S1_at	Unknown	-2.07	Unknown
Dr.18310.2.A1_at	Cryptochrome DASH	-2.04	See Table 1
Dr.17570.1.S3_at	Solute carrier family 44, member 5b	-2.03	See Table 1
Dr.6618.1.A1_at	Unknown	-2.00	See Table 1
Dr.16174.1.A1_at	Novel protein similar to vertebrate odz, odd Oz/ten-m (Drosophila) family ODZ1	-2.00	The protein encoded by this gene belongs to the tenascin family and teneurin subfamily. It is expressed in the neurons and may function as a cellular signal transducer.
Dr.10320.1.S1_at	Serum/glucocorticoid regulated kinase 1	-1.87	See Table 1
Dr.21047.1.S1_at	Activin receptor IIB	+1.90	See Table 1
Dr.25714.2.S1_at	Cathepsin S, b.2	+1.98	Member of the peptidase C1 family is a lysosomal cysteine proteinase that may participate in the degradation of antigenic proteins to peptides for presentation on MHC class II molecules.
Dr.106.1.S1_at	Heat shock protein 47	+2.06	Heat shock protein
Dr.12770.1.S1_at	Unknown	+2.16	See Table 1
Dr.16350.1.A1_at	Unknown	+2.23	Unknown
Dr.18339.1.S1_at	Hypothetical LOC559783	+2.24	Unknown
Dr.9890.1.A1_at	Wu:fk54f12	+2.39	See Table 1
Dr.20125.1.A1_s_at	Zgc:111983	+2.44	Unknown
Dr.4744.1.S1_a_at	Wu:fk35f04	+2.61	Unknown
Dr.20125.1.A1_at	Zgc:111983	+2.63	Unknown
Dr.9682.1.A1_at	Zgc:172053	+3.46	See Table 1

7.8. Figure captions and figures

Figure 1. Venn diagram of probes expression under two treatments (nano-TiO₂ and hydroxylated fullerenes) compared to control represented as the total number of probes. Diagram intersection indicates the number of probes that were regulated in both treatments. Probes (13,177) outside of the circles were not affected by the treatments.

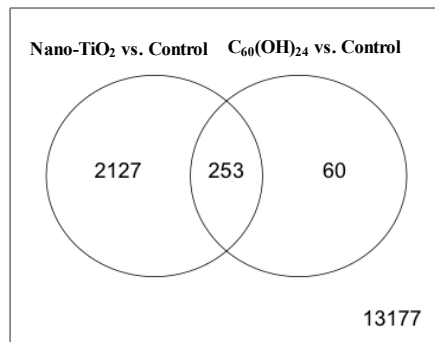


Figure 1

7.9. References

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CHAPTER 8. GENERAL CONCLUSIONS

8.1. Conclusions

Based on the results of this dissertation *Pimephales promelas* appear to be a useful model for investigating immunotoxicological effects of nanoparticles. This dissertation provided essential information about fish immune responses to nanoparticles; determined the potential biomarkers of nanoparticle presence in the fish organism and mode of actions; and evaluated realistic impact scenarios of environmentally relevant concentration of titanium dioxide nanoparticles.

Complementary DNA libraries were created for *P. promelas* species and relevant genes from libraries were identified and deployed as biomarkers of engineered nanoparticle exposure.

Effects of nano-TiO₂ on neutrophil function of the fathead minnow was investigated using oxidative burst, neutrophil extracellular traps (NETs) release and degranulation of primary granules. Application of 0.1 µg mL⁻¹ of nano-TiO₂ *in vitro* stimulated oxidative burst and NET release. *In vivo* application of 10 µg g⁻¹ of nano-TiO₂ caused a significant decrease in oxidative burst, NETs release and degranulation. Fish exposed to nano-TiO₂ for 48 h *in vivo* had significantly increased expression of interleukin 11, macrophage stimulating factor 1, and neutrophil cytosolic factor 2.

Exposure to nano-TiO₂ significantly increased fish mortality during *Aeromonas hydrophila* challenge. Analysis of nano-TiO₂ distribution in fish organism outlined that the nano-TiO₂ is concentrating in the fish kidney and spleen. The significant increase in mortality during bacterial challenge was observed after the administration of 2 ng g⁻¹ body weight of nano-TiO₂. This dose is within the estimated environmental concentration of nano-TiO₂ in aquatic ecosystems of 0.7 to 24.5 ng mL⁻¹, indicating the potential of engineered nano-TiO₂ to affect fish survival by interfering with immune responses during possible disease outbreaks in the aquatic ecosystems.

Application of fullerenes (0.2 - 200 µg mL⁻¹ *in vitro*) caused dose dependent inhibition of oxidative burst and suppressed the release of NETs and degranulation of

primary granules. Expression of interleukin 11 and myeloperoxidase was significantly increased and expression of elastase 2 was significantly decreased in fish exposed to hydroxylated fullerenes for 48 h *in vivo*.

A gene microarray analysis was performed on injected and control embryos of *Danio rerio* to determine the potential for nanoparticles to change the expression of genes involved in cross talk of the nervous and immune systems. The exposure to TiO₂ and hydroxylated fullerenes caused shifts in gene regulation response patterns that were similar for downregulated genes but different for upregulated genes. Significant effects on gene regulation were observed on genes involved in circadian rhythm, kinase activity, vesicular transport and immune response. This is the first report of circadian rhythm gene deregulation by nanoparticles in aquatic animals, indicating the potential for broad physiological and behavioral effects controlled by the circadian system.

8.2. Recommendations for future research

The results and conclusions of this dissertation can be used as a starting point for studying different research problems: from basic innate fish immunology to molecular nanoimmunotoxicology; ecological risk assessment of engineered nanoparticles in aquatic ecosystems; and eco-friendly engineering of nanoparticles.

For example results from the Chapter 3 can lead to future investigations of general innate immune responses in *P. promelas*. Future studies can be directed in investigating neutrophil response toward different preparations of lipopolysaccharides and their most common contaminants – peptidoglycans with the goal of understanding how do fish recognize bacteria. In order to exploit the immune model of *P. promelas* fully, future studies should sequence and characterize the pro-inflammatory and anti-inflammatory cytokines in this species, to complement the existing cellular innate immune function toolbox.

Chapters 4, 5 and 6 have established the models and basis for investigating the effect of environmentally relevant concentrations of titanium dioxide nanoparticles. Future research should target to answer the problems from the ecological risk assessment perspective. A mesocosm approach with a basic food chain hierarchy of species should be

utilized where the *P. promelas* would receive the dose of titanium dioxide nanoparticles through food and water, while the outbreak of *A. hydrophilla* is closely monitored in control and experimental mesocosms.

Finally Chapter 2 provided a complete mode of action of titanium dioxide nanoparticles immunotoxicity towards fish neutrophils. Future research should utilize this information in engineering eco-friendly titanium dioxide nanoparticles which would replace the nanoparticles that are currently used in cosmetic, food and pharmaceutical products.

APPENDIX

In the chapter 4 all concentrations of the nano-TiO₂ that were used in experiment refers to the concentration before filtration through the 220 nm general purpose filter. Large aggregates removed in the process of filtration contain significant mass, thus after filtration the concentration of filtered suspension can change from its original value. This appendix provides data about actual concentration of filtered suspension of nanoparticles from the chapter 4. However it is important to note that classical statement of toxicology – “it is the dose that matter” dose not apply to nanomaterials readily, as in nanotoxicology – “it is the dose in the function of surface area that matter”. Removal of hundreds of aggregates from the pool of billions of nanoparticles can change the concentration of the suspension but have very little effect on the overall surface area of the nanoparticles in the suspension.

After filtration, concentration of Ti for the isotopes 47 and 49 was measured with inductively coupled plasma mass spectrometry (ICP-MS) using a scandium (Sc) internal standard ($m/z=45$). The preparation standard used for this analysis was created by spiking on the blank samples with Ti and Sc. Results were corrected for the Ti isotopes natural abundance, averaged between the two Ti isotopes measured and converted to TiO₂ concentration as $\mu\text{g mL}^{-1}$. Table 1 provides determined concentrations of TiO₂ after the filtration.

Table 1. Concentrations of TiO₂ before and after filtration through 220 nm general purpose filter.

Concentration before filtration $\mu\text{g mL}^{-1}$	0.1	1	10	100	1000
Concentration after filtration $\mu\text{g mL}^{-1}$	0.003	0.007	0.047	0.138	0.168

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