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Departamento de Química

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Mecanismos dos efeitos imunotóxicos dos nanomateriais nos peixes

Mechanisms of immunotoxic effects of nanomaterial in fish

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And one more step.

There were times when I didn't believe in my abilities. And I even wondered if it would be worth it or if it would be all in vain. The work was intense, frenetic, stressful…so many sleepless nights.

It is true that I may have lost some pleasures in life, such as a simple night out with my colleagues. Although invitations declined one after another, I could always count on the support and friendship of all of them.

Along this journey, in which we live, we had good times and bad times, which will be forever, remembered and recorded. To avoid forgetting someone, to everyone who shared with me these moments, a thank you.

My family was another important pillar. They showed that was with me in times of need and that despite the difficulties of life, I couldn't give up. So, I leave a special thanks to my mother, my father and sister for having put up with me this whole time, and above all, for the patience they had when I was grumpy.

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I can say that I learned that in spite of adversity and tribulations that life can bring, we should always give our best, instead of letting us down without having given fight.

After so many sacrifices, barriers overcome, from this battle I didn't come out defeated, only as a winner!

I dedicate this work to my parents, sister and my friends Bruno, Victorina, Rute, Joana, Ana Filipa, Rui, Tiago, André, César and Inês.

o júri

Presidente

Prof. Doutor Artur Manuel Soares da Silva
Professor catedrático do Departamento de Química da Universidade de Aveiro

Eng. Carlos Alberto Garcia do Vale
Investigador Coordenador do Instituto Português do Mar e da Atmosfera

Prof. Doutora Maria Eduarda da Cunha Pereira
Professora Auxiliar do Departamento de Química da Universidade de Aveiro

Keywords Immunotoxicity; reactive oxygen species; antioxidants; *Anguilla anguilla* L.; nanomaterials; mercury

Abstract

Information and knowledge related to nanotechnology raise new challenges to the scientific community mainly in terms of human health risks and environmental implications associated to nanomaterials. In this perspective, the contamination of aquatic environments cannot be overlooked since it is an ultimate repository of the contaminants where this emerging appears as part of a cocktail of different classes of contaminants. Thus, the major task of this work was to connect gaps in current knowledge with a comprehensive sequence of biological responses toward environmentally relevant concentrations of engineered nanomaterials (IONM - silica coated iron oxide nanomaterial functionalized with dithiocarbamate group) and their interaction with other conventional anthropogenic contaminant (Hg - mercury), outlining the interaction with the innate immune system of fish. The research was divided into following steps: i) phagocytes macrophages were isolated from peritoneum (P-phagocytes), gill (G-phagocytes), head kidney (HK-phagocytes) and spleen (S-phagocytes) of European eel *Anguilla anguilla* L. in order to evaluate whether, and how can IONM and its co-exposure to Hg modulate phagocytes status and function; ii) determine the changes in phagocytes activation and their association to peroxidative damage (OBA - oxidative burst activity; LPO - lipid peroxidation); iii) to assess the impact of IONM on phagocytes enzymatic (CAT - catalase; GPX - glutathione peroxidase; GR - glutathione reductase; and GST - glutathione S-transferase) and non-enzymatic (NP-SH - non-protein thiols; and TGSH - total glutathione) antioxidants protection overtime. It was hypothesized that

IONM can cause measurable changes in fish immune response and oxidative stress modulation.

A period of exposure-dependency was exhibited by IONM alone and IONM+Hg joint exposures accrued impacts on *A. anguilla* phagocytes. IONM exposure alone lead to an acute response in terms of viability increase in P-phagocytes and modulated phagocytic activity in P-, and S-.phagocytes during 2 hours of exposure; whereas, IONM lead to a chronic immunotoxicity during 72 hours exposure only in S-phagocytes. However, IONM+Hg exposure lead to both acute and chronic response in terms of modulated phagocytic activity with no change in viability in P-, HK- and S-phagocytes only. Increase in the period of exposure to Hg disrupted phagocytic activity of P-, HK- and S-phagocytes, an increase in P- and decrease in HK- and S-phagocytes was perceptible at late hours of exposure. The occurrence of synergism between IONM and Hg was evidenced at 72 hours by significantly increasing trends of phagocytosis increase.

A differential extent of OBA and LPO induction at the end of different period of exposure to IONM, Hg or IONM+Hg was also perceptible. The OBA induction and its concomitant association to LPO induction were observed only in gill after exposure to Hg (8 and 48 hours) and IONM+Hg (8 hours). At late hours of exposure, an induction was observed in G- phagocytes (OBA) after exposure to IONM+Hg suggesting that the concomitant exposure was unable to mitigate the Hg-accrued negative impacts. *A. anguilla* also displayed that the damage was accompanied with a differential modulation of enzymatic and non-enzymatic antioxidants in P, G-, HK- and S-phagocytes. Under IONM alone exposure, no LPO induction along the time was observed probably due to efficient induction of, GR and GST providing a better protection to IONM exposed phagocytes. However,

antioxidants protection responses displayed hours of exposure dependency in G- and S-phagocytes where, an insufficiency of elevated CAT, GPX, GST, NP-SH and TGSH was clearly depicted for the maintenance of pro- and antioxidant balance optimum for scavenging ROS and protecting membrane lipids against IONM impact. As increased LPO was observed under IONM alone exposure condition, the joint action of IONM+Hg led to elevated damages to membrane-lipids at 4 and 8 hours (G-phagocytes), 2 hours (HK-phagocytes) and 24 hours (S-phagocytes) of exposure. These responses together, point towards the antioxidants defense failure for the protection of membrane lipids during those periods of exposure to IONM+Hg. However, it is important to underline here that during the late hours of exposure (72 hours), the results imply the positive effect of the concomitant exposure (IONM+Hg) which significantly mitigated the said negative impacts of Hg.

Overall, the observations of this study open up new insight into the areas of evaluation of immune defense mechanisms in fish exposed to IONM and recommend that the interactions between IONM and other conventional anthropogenic contaminants should be considered while interpreting the fish immunotoxicity responses to IONM exposure in a multi-pollution state.

Palavras-chave Imunotoxicidade; espécies reativas com o oxigénio; antioxidantes; *Anguilla anguilla* L.; nanomateriais; mercúrio

Resumo

Informações e conhecimentos relacionados com a nanotecnologia colocam novos desafios à comunidade científica, principalmente em termos de riscos para a saúde humana e alterações ambientais. A contaminação dos ambientes aquáticos não pode ser ignorada, uma vez que é um repositório final dos contaminantes onde estes aparecem como parte de um conjunto complexo de diferentes tipos. O objetivo principal deste trabalho foi relacionar lacunas no conhecimento atual com uma sequência de respostas biológicas, para concentrações ambientalmente relevantes de nanomateriais (ONM - nanomateriais de óxidos de ferro revestidos com sílica e funcionalizados com grupos de ditiocarbamato) e avaliar a sua interação com outros contaminantes antropogénicos, nomeadamente mercúrio (Hg), na interação com o sistema imunitário de peixes. A investigação desenvolvida foi dividida nos seguintes passos: i) os macrófagos fagocitados foram isolados da cavidade peritoneal (P-fagócitos), das guelras (G-fagócitos), da "cabeça do rim" (HK-fagócitos) e do baço (S-fagócitos) da enguia europeia *Anguilla anguilla* L., a fim de avaliar como podem os ONM e a sua co-exposição com o mercúrio modular o estado das fagocitoses e a sua função; ii) avaliar as alterações na ativação das fagocitoses e a sua associação ao dano peroxidativo (OBA – atividade respiratória oxidativa; LPO - peroxidação lipídica); III) avaliar o impacto das ONMs ao longo do tempo nos antioxidantes, nomeadamente fagocioses enzimáticas (CAT - catalase; GPX - glutationa peroxidase; GR - glutationa redutase; e GST - Glutationa S-transferase) e não enzimáticas (NP-SH

- proteína não-tiol; e TGSH - glutationa total). É apresentada a hipótese de que as ONMs podem causar mudanças na resposta imunológica de peixe e modular o stress oxidativo.

Uma dependência do período de exposição foi observada para as ONMs isoladas e também uma sucessão de impactos sobre as fagocitoses da *Anguilla Anguilla*. A exposição isolada às ONMs parece poder induzir uma resposta aguda em termos de aumento de viabilidade em P-fagócitos e uma atividade fagocítica modulada em P e S-fagócitos após 2 horas de exposição; as ONMs podem induzir imunotoxicidade crónica durante uma exposição de 72 horas, apenas em S-fagócitos. A coexposição a ONM+Hg induziu uma resposta aguda e crónica em termos de atividade fagocítica modulada, com nenhuma mudança na viabilidade em P, HK e S-fagócitos. O aumento do período de exposição a Hg interrompeu a atividade fagocítica de P-, HK - e S-fagócitos. Porém, um aumento de P - e diminuição de HK - e S-fagócitos foi percetível nas 72 horas. A ocorrência de sinergismo entre as ONMs e o Hg foi evidenciado às 72 horas pela tendência do aumento significativo das fagocitoses.

Diferença nas induções de OBA e LPO para diferentes períodos de exposição, às ONMs, Hg e ONMs+Hg foi também percetível. Uma indução de OBA paralela à resposta do LPO foi observada unicamente nas guelras por exposição ao Hg (8 e 48 horas) e ONMs+Hg (8 horas). Para períodos mais longos de exposição, foi observada uma indução nas G-fagócitos (OBA) depois da exposição a ONMs+Hg, sugerindo que a contaminação ONMs+Hg é capaz de mitigar os impactos negativos do Hg-acumulado ao longo do tempo. *A. Anguilla* evidenciou danos ao nível da modulação diferencial de antioxidantes enzimáticos e não enzimáticos em P, G, HK e S-fagócitos. Sob exposição simples às ONMs, não foi observada nenhuma indução do LPO ao longo do tempo, talvez devido à indução eficiente de GR e GST, proporcionando uma melhor proteção para os fagócitos expostos às ONMs. No entanto, as respostas de proteção dos antioxidantes

exibiram dependência das horas de exposição em G - e S-fagócitos onde uma insuficiência elevada em CAT, GPX, GST, NP-SH e TGSH foi claramente observada para a manutenção do equilíbrio antioxidante e eliminação de ROS, protegendo os lipídios da membrana contra o impacto das ONMs. A ação conjunta das ONM+Hg conduziu a elevados danos nos lipídios da membrana às 4 e 8 horas (G-fagócitos), 2 horas (HK-fagócitos) e 24 horas (SP-fagócitos) de exposição. Estas respostas conjuntas, apontam para o fracasso da defesa dos antioxidantes na proteção dos lipídios da membrana durante os períodos de exposição com ONM+Hg. De salientar que para exposições de 72 horas, os resultados evidenciam efeitos positivos da exposição concomitante a ONM+Hg o que atenuou impactos negativos do Hg.

As observações deste estudo dão novas ideias sobre a avaliação dos mecanismos de defesa imunotoxicológica em peixes expostos a ONMs e indicam que as interações entre ONMs e outros contaminantes antropogénicos devem ser consideradas na interpretação das respostas imunotóxicas de peixe expostos às ONMs em situações contaminação múltipla.

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"A pessimist sees the difficulty in every opportunity; an optimist sees the opportunity in every difficulty."

Winston Churchill

(British Orator, Author and Prime Minister during World War II)

INTRODUCTION

1. Introduction

The nanotechnology as the *XXI Science,* has gained worldwide attention. The prefix "nano" derived from Greek "nano" meaning "dwarf". Nanotechnology consists in manipulation and application of engineered particles (such as metal oxides, carbon nanotubes, fullerenes and others) or systems that have the nano-dimensions ranging from 1-100 nanometers (nm) in length (Arora et al., 2011). The materials used in this new technology are known as nanomaterials (NMs) whose main constituents have dimensions between 1 and 100 nanometers, and they are used in various applications and products such as electronic chips, solar cells, batteries, paints, clothing, etc. About the controversy of definitions between NMs and nanoparticles (NPs), the European Scientific Committee recommended the 'term' nanomaterial (NM) to be used in European Union legislation and programs concerning products of nanotechnologies (Lidén, 2011) in order to cover the potential health, safety and environmental risks that stem from NMs. The proposed definition was set on three parameters: i) size of internal structural elements; ii) size distribution; and iii) surface area. Overall, NMs are small particle size with large surface area, low cost and easy to prepare (Petrova et al., 2011).

Over the past 7 years, it has been a major concern on the impact of NMs on human health and environmental safety. On the 7th June 2005, an action plan for Europe 2005-2009 was adopted to immediate implementation of a safe, integrated and responsible strategy for nanosciences and nanotechnologies. It was defined by the European Commission that there should be a contributions on: i) the research and development in nanotechnology and nanoecotoxicology, via quantitative results for NMs safety and risk assessment; and ii) the development of a database related to toxicological, ecotoxicological and epidemiological investigation (Kahru et al., 2010).

The ecotoxicology is a set of two fields of study: toxicology and ecology. The ecotoxicology appear in 1969 by René Truhaut that defined as "the branch of toxicology that was concerned with the study of toxic effects, caused by natural or synthetic contaminants, in particular, in the different ecosystems (microorganisms, plant, animal and human) (Altenburger, 2010). Therefore, this scientific branch is studying the effects and influences of toxic agents on the various levels of biological organization and comprises three areas of study: i) study of emissions and sorption of contaminants in the environment, such as its distribution and fate; ii) qualitative and quantitative study of the toxic effects of

contaminants in ecosystems and, in turn, how it interacts with man; and iii) study of the fate of contaminants in the biosphere, focusing on contamination of food chains (Kahru et al., 2010). In this perspective, there is a great concern of how the advancement of nanotech industries has a negative impact on human health and in the environment (Green et al., 2011).

The engineered NMs behavior in the environment such as water, sediment and soil involves multiple complex processes mainly: i) chemical aggregation and the ability of NMs to form stable dispersions in liquids such as water; ii) the effects of shape, size, surface area and surface charge on ecotoxicity and chemical aggregation; iii) NMs adsorption on the surfaces of different organisms; and iv) effect of other abiotic factors, such as the influence of the changes on pH, salinity, hardness of the water and the presence of organic matter (Handy, 2008 a).

The aquatic environment is an ultimate reservoir of various contaminants including NMs. Hence, a considerable interest has been generated in recent times to assess the extent of aquatic pollution related to NMs and their impact on aquatic life and ultimately on humans. Among the aquatic environment, estuaries and coastal areas have unique features because of their ecological importance through the primary and secondary production, nutrient cycles and with their different breeding habitats, as well as, migration and feeding (Saintilan et al., 2012). Estuaries are a transition zone of saltwater with freshwater (Selleslagh et al., 2012), where freshwater brings nutrients that stimulate the production of phytoplankton and benthic microalgae, which are important sources of autotrophic production for estuarine consumers (Yoshino et al., 2012; Saintilan et al., 2012). Furthermore, estuaries are characterized by enormous changes of physical and chemical conditions of the surrounding environment and, thus the existing fauna and flora have to adjust to varying degrees of salinity conditions. Moreover, human activities also affect on the aquatic environment leading to water deterioration and on the quality of biota (Saintilan et al., 2012).

Therefore, over the last few decades, defense strategies to protect aquatic environments through the development of policies have been implemented by the European Water Framework Directive and by the European Marine Strategy identifying the toxic contaminants concentrations in different aquatic environments (Selleslagh et al.,

2012). In an attempt to recognize the environmental impact provoked by NM, it was found that the knowledge on NMs uptake, transport and exposure in the aquatic organisms is extremely primitive and is not sufficient for making accurate predictions, hence need more attention to understand the full mechanism involved in the NMs toxicity. Another observation drawn from the literature revealed that there is a need to focus more on the evaluation of NMs toxicity in fish, because of their virtual presence everywhere in the aquatic environment and playing a major ecological role in the aquatic food-webs. Fish sorb contaminants not only by the trophic chain, but also through the direct contact of gills and its scales and thus affecting human health when ingested.

2. Potential environmental risk associated with nanomaterials

Recent literature revealed that despite the growing evidence of the effects of NMs in fish, the results still cannot clarify the basic mechanisms of NMs exposure (Jovanovic, 2012). In toxicity studies in fish, the most common NMs are titanium dioxides [(TiO_2)-NMs], zinc oxide [(ZnO)-NMs], silver [(Ag)-NMs], gold [(Au)-NMs], dendrímers, carbon nanotubes, among others. Different NMs are divided in the following classes: i) carbonaceous compounds: ii) metal-oxides, iii) semiconductor materials (including, quantum dots); iv) nanopolymers (dendrímers) and v) zero-valent metals such as iron (Fe), silver (Ag) and gold (Au) (Green et al., 2011).

2.1. Toxicological effects of carbonaceous compounds - The carbonaceous compounds are a family of new compounds that includes fullerenes (C_{60}) (figure 1), carbon nanotubes (CNTs) and their derivatives. Fullerenes are ball-shaped carbon molecules, on a type model like cage structure with different quantities of carbon atoms. C_{60} advantages are consisting on NMs with a low cost, high purity, exhibit an exceptional physicochemical stability and had the ability to encapsulate small molecules (e.g H_2 inside the carbon cage). The CNTs consist in a tubular shape with a large length/diameter ratio. CNTs are depending on the number of carbon shells, as well as potential uses in different applications such as composite materials, hydrogen storage, electronic devices, plastics, water purification systems, sensors, automotive and aerospace industry, among others. Thus, CNTs designation are single (SWCNT) (figure 1), double (DWCNT) (figure 1) or multi-walled (MWCNT) (figure 1) (Hartmann, 2011). Annually, the production of

derivatives of carbon NMs raises approximately 1500 tones/year, thus increasing the risk to the environment, aquatic organisms and humans. In aquatic systems, the carbon NMs are subject to precipitation and aggregation, because of their inherent hydrophobicity (Klaine et al., 2008). Another important issue is that the CNTs tend to aggregate and produce different ecotoxicological effects when a solvent is excluded (Oberdörster et al., 2007). The problem can be resolved using a solvent (sodium dodecyl sulphate (SDS)) with low toxicity to the fish that was fairly good at dispersing CNTs and applied sonication technique (Smith et al., 2007).

On the perspective of the toxicity, the functionalized CNTs have increased solubility in water with a possibility to pass to the cells. Macrophages damage was observed with non-functionalized nanotubes. Further, the toxicity results with non-functionalized and functionalized nanotubes are not very clear due to different functional groups; and thus, require more studies to clarify this area of study in order to predict the impacts associated with these NMs in the aquatic ecosystem (Klaper et al., 2010). The CNTs in aquatic environments are biological active, promoting oxidative stress (Nel et al., 2006; Oberdörster, 2004). SWCNT toxicity has also been reported in estuarine copepod (*Amphiascus tenuiremis*), Daphnia and rainbow trout (Roberts et al., 2007; Smith et al., 2007; Templeton et al., 2006). In a study considering 48 hours of exposure to six NMs (i.e., ZnO, TiO_2, Al_2O_3, C_{60}, SWCNTs, and MWCNTs) of Daphnia magna, a dose dependent acute toxicity was demonstrated, using immobilization and mortality as toxicological endpoints (Zhu et al., 2009). Concerning 6-18 hours toxicity assessment, Oberdörster et al. (2006) observed 100% of mortality for fathead minnows when exposed to 1 mg L^{-1} of organic solvent tetrahydrofuran, a neurotoxin, with C_{60} (THF-C_{60}). On the other hand, fathead minnows exposed to 1 mg L^{-1} water-stirred C_{60} showed no mortality up to 96 hours (Oberdörster et al., 2006).

Oberdörster et al. (2004) observed that manufactured NMs (Fullerenes, C_{60}) induce oxidative stress in the brain of juvenile largemouth bass. It was also observed that 0.5 mg L^{-1} C_{60} dissolved in THF causes the increase of lipid peroxidation (LPO) in brains of Largemouth Bass (*Mikropterus salmoides*). Moreover, the results depicted a decreased LPO in the gills and liver with no significant protein oxidation in the liver and gills. However, an increase of glutathione in the liver was perceptible. In a study based on the C_{60} sublethal toxicity without agitation, 100% mortality was observed after 18 hours' time

interval using fish, Pimephales Minnows; whereas, the time of the fish mortality was increased (96 hours) when the 1 mg L^{-1} C_{60} was agitated in water (Oberdörster et al., 2006). Several studies report LPO or changes in tissues (Oberdörster, 2004; Smith et al., 2007; Zhu et al., 2006). Smith et al. (2007) observed an increase in total glutathione (TGSH) in the gill and intestine during CNTs exposure over 10 days. Lee et al. (2007) observed that when the fish was exposed to UV radiation, the blood chemistry was modified and it a decrease in the immune system was observed. An increase of lipid damage and a decrease in GSH in gills split with C_{60} was observed by Oberdörster (2004). Therefore, the UV radiation activates toxic effects of CNTs on fish bodies.

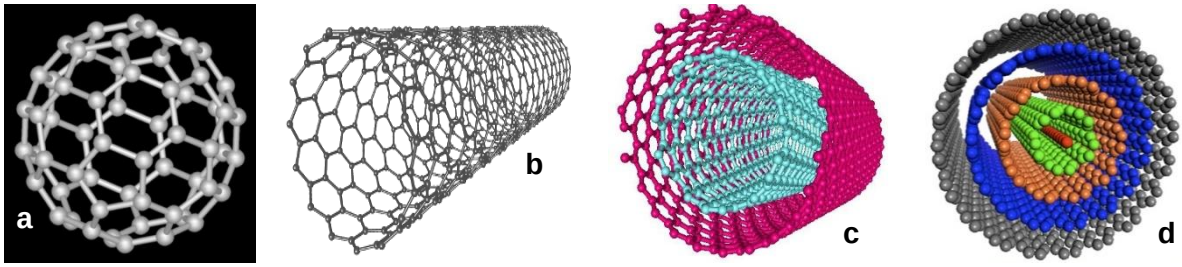

Figure 1. **a)** Fullerenes (C_{60}), **b)** Single carbon nanotubes (SWCNT), **c)** Double carbon nanotubes (DWCNT) and **d)** Multi-walled carbon nanotubes (MWCNT) (adapted from web site [1][2][3] and [4])

2.2. Toxicological effects of metals oxides

2.2.1. Titanium dioxide nanomaterials [(TiO_2)-NMs] - The metals oxides have enormous applications in industry and the most common metals oxide NMs are: titanium dioxide (TiO_2)-NMs and zinc oxide (ZnO)-NMs. The (TiO_2)-NMs and (ZnO)-NMs are exploited for their photocatalyst properties. The (TiO_2)-NMs is used in wastewater treatment due to non-toxicity and greatly enhanced surface area (Zhang et al., 2008). Other applications are in sunscreens, cosmetics, due to its particular features action in blocking ultraviolet rays and the visible transparency of nanoparticulate form. Thus, (TiO_2)-NMs have the potential to induce photo and non-reactive oxygen production of photocatalytic species leading to oxidative damage to cells. Moreover, *in vitro* (TiO_2)-NMs exposure to rainbow trout (*Oncorhyncus mykiss*) gonadal tissue have also shown its potential to induce cytotoxicity and genotoxicity in fish (Vevers et al., 2008). Lee et al. (2012) observed no chromosomal damage (measured by counting the micronucleus) after exposure to (TiO_2)-

NMs, in the cells of rainbow trout. Ma et al. (2012) reported (TiO_2)-NMs phototoxicity to Japanese medaka relating it with band gap energy of 3.2 eV (λ=388 nm). Thus, phototoxicity can lead to substantial underestimation of the risk associated with the release of (TiO_2)-NMs to natural environments. Concentration-dependent mortality in Japanese medaka exposed to (TiO_2)-NMs was also observed under laboratory lighting. Toxicity of (TiO_2)-NMs with the presence of solar UV radiation was enhanced by almost 100 times as compared to laboratory lighting, demonstrating significant phototoxicity of the (TiO_2)-NMs to Japanese medaka (Ma et al., 2012).

2.2.2. Zinc oxide nanomaterials [(ZnO)-NMs] - (ZnO)-NMs are used in solar panels, paints and coatings. Adams et al. (2006) conducted a study with different NMs, such as TiO_2, ZnO, and SiO_2 NMs and it was observed that (ZnO)-NMs were the most toxic to *Bacillus subtilis.* In a study on (ZnO)-NMs toxicity in rodent lung and zebrafish embryo's, data indicated reduced toxicity in the latter system upon doping of iron (Fe) in ZnO (Xia et al., 2011). Zhu et al. (2008) reported 96 hours LC_{50} (1.79 mg L^{-1}) and 84 hours EC_{50} (2.06 mg L^{-1}) for (ZnO)-NMs (20 nm). Also, in the same study it was perceptible less toxicity to (TiO_2)-NMs and (Al_2O_3)-NMs instead to the (ZnO)-NMs (Zhu et al., 2008). Bai et al. (2009) has shown that different concentrations such as 50 and 100 mg L^{-1} were capable to cause mortality in zebrafish embryos. Also, it was observed retard growth and malformation after 96 hours of exposure to the (ZnO)-NMs suspension. Zhu et al. (2009) observed an increase in intracellular ROS on zebrafish embryos exposed to (ZnO)-NMs. (ZnO)-NMs (30 nm) induced a 96 hours LC_{50} (3.97 mg L^{-1}). Moreover, Yu et al. (2011) observed that the aggregation and sedimentation of the (ZnO)-NMs reduced toxicity on zebrafish.

Zinc ions (Zn^{2+}) from (ZnO)-NMs have a substantially contribute to the cytotoxicity (Brunner et al., 2006; Heinlaan et al., 2008). The concentration of 50 mg L^{-1} of (ZnO)-NMs was not lethal to the carp (*Cyprinus carpio*) but caused significant oxidative stress. Some fish at the highest concentration of 50 mg L^{-1} of (ZnO)-NMs showed some unusual behaviours in the first few days. The phenomenon indicated that physiological changes of carps were affected by higher concentrations of (ZnO)-NMs. Also, Hao et al. (2012) justify, that gradually carps could be capable to adjust the self-balance *in vivo*. Furthermore, it was also observed a decrease in activity of several anti-oxidative enzymes

on organs, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) and increase in LPO (Hao et al., 2012).

2.3. Toxicological effects of quantum dots - Quantum dots (QDs) have particular properties, i.e., reactive core which controls QDs optical properties. The typical cores are constituted with metals or others compounds such as cadmium selenide (CdSe), cadmium telluride (CdTe), indium phosphide (InP), or zinc selenide (ZnSe).

Normally, the reactive semiconductor cores (figure 2) are surrounded by a shell (silicon dioxide, SiO_2) or a ZnS monolayer that protects the core from oxidation and enhances the photoluminescence yield. The cores are produced from a nucleation reaction of the metal versus semiconductor material in a high-temperature solution synthesis phase. In solution, the surfactants form an organic cap that stabilizes the particle. The cap can be modified or exchanged with other materials. The QDs can be used in telecommunications, solar cells and others (Klaine et al., 2008). Regarding the QDs toxicity, available study in recent database reflected that CdTe QD induced ROS induction in mussels as evidenced by increased LPO in gill. Also, CdTe QDs were detected in digestive gland (Gagné et al., 2008). Thus, more studies are needed to confirm these findings and to be done in other organism such as fish.

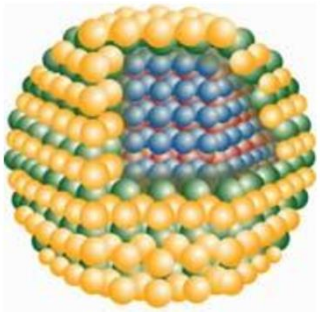

Figure 2. Core – shell: Quantum Dots (adapted from web site [5])

2.4. Toxicological effects of dendrimer - The dendrimer is a nanopolímer and consists in molecules with a molecular size on the range of 2 to 10 nm and a molecule inner core surrounded by a series of branched chains (figure 3). The molecules of the nucleus and branched chains can be synthesized from different materials. The terminal groups of each branch can be chemically modified, in order to synthesize new biologically compatible surfaces, hydrophobic, hydrophilic or charged surfaces. In this way, dendrimers

presents unique structures, and it can be controlled in size, topology, flexibility and the molecular weight. Dendrimers are applied in biology to form engineering materials, with chemical sensors, transfer of DNA agents, hydrogels and others (Klaine et al., 2008; Scown, 2009). The toxicity reports about dendrimers are very less. In this context, Heiden et al. (2007) published the first article on zebrafish embryos treated with polyamidoamine (PAMAM) dendrimers, which reflected an increased mortality at 200 µM. Also, the dechorionated embryos exposed to PAMAM dendrimers for 8 hourss did not show increased mortality. However, there is a possibility that the lack of toxicity occurs because the PAMAM dendrimers are not taken up by the embryos following aquatic exposure.

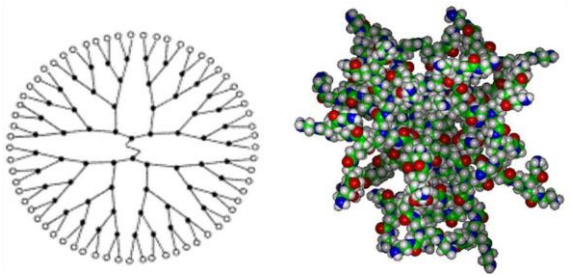

Figure 3. G4-OH, dendrimer with 64 terminal groups (adapted from web site [6])

2.5. Toxicological effects of zero-valent metals - Zero-valent metals (nZVI) include the (Au)-NMs, (Ag)-NMs and iron. The NMs do not dissolve in solution, but form a colloid dispersion. The colloid is described as a suspension of a material with sizes comprised of 1 nm to 1 µm (Handy, 2008 a). Typically, nZVI are formed by reduction of the solutions with metals salts (Klaine et al., 2008). Colloidal elements of Au have been widely used in medical diagnostic applications (e.g., detection of tumors). The detection methods for the (Au)-NMs are absorption spectroscopy, fluorescence spectroscopy, Raman spectroscopy, electrochemistry and electron microscopy (Algar et al., 2009).

2.5.1. Gold nanomaterials [(Au)-NMs] - The toxicity results for (Au)-NMs on aquatic organism are very little. Few scientific databases are available mainly in rainbow trout and *Mytilus edulis*. Farkas et al. (2010) observed that (Au)-NMs did not cause cytotoxic responses on rainbow trout fish. (Au)-NMs and Au ions (Au^{3+}) had a detectable adverse effect on membrane integrity or metabolic activity of rainbow trout hepatocytes after 48 hours. (Au)-NMs and Au^{3+} was shown to be a potent inducer of ROS after 2 hours. The ROS levels caused by Au exposure concentrations (17.4 mg L^{-1}) were not high enough

to overcome antioxidant defense mechanisms and thus, cause an immediate cytotoxicity. Tedesco et al. (2010) observed that the (Au)-NMs were accumulated in digestive gland which is known as a key site for xenobiotic and metal detoxification (Moore et al., 2006; Viarengo et al., 1989). *Mytilus edulis* exposed to (Au)-NMs exhibited decreased thiol-containing proteins. Moreover, (Au)-NMs (~5 nm) also caused significant LPO in digestive gland. Oxidative stress and cytotoxicity effects were increased in (Au)-NMs with lower diameters (~5 nm) than larger diameters (Tedesco et al., 2010).

2.5.2. Silver nanomaterials [(Ag)-NMs] - Silver is known for more than 2000 years for its medicinal properties. The Greeks and the Romans used Ag pots to hold drinking water. With the advance of the centuries, Ag began to be used as a bactericidal. Over the past decade, Ag has been modified and it was developed (Ag)-NMs. In this way, the (Ag)-NMs feature a maximized surface area, and lists high values of activity with the weight ratio. With these new properties, the (Ag)-NMs offer new market perspectives, as well as in research field. Some of the best known applications are as antibacterial agents/antifungals agents (examples: pillows, detergents, shampoos and others). Thus, (Ag)-NMs have been widely used in the disinfection of water supplies, dressings for wounds, medical devices, among others (Khaydarov et al., 2009). However, there are trade-offs for (Ag)-NMs, because there is the possibility of toxic effects to the environment and human health. It is yet unknown if the toxicity is derived from the NMs or if it comes from the release of Ag ions (Ag^+). The Ag^+ are known as toxic metals to aquatic organisms as depicted in *in vivo* studies on the zebrafish showing that the (Ag)-NMs has the ability to pass the protective membrane of the embryos. Consequently, the (Ag)-NMs are incorporated into the development of organs and lead to deaths and deformities (Buzea et al., 2007). In addition, the known results indicate that the (Ag)-NMs have the ability to penetrate into the cell membranes, and that can lead to cellular, acceleration of the generation of reactive oxygen species (ROS) and thus lead to cytotoxic and genotoxic effects. Chemical reduction is one method for the preparation of (Ag)-NMs as stable, colloidal dispersions in water or organic solvents (Sharma et al., 2009; Tao et al., 2006). The reduction of Ag^+ in aqueous solution usually yields colloidal Ag with particle diameters of several nanometers (Sharma et al., 2009).

Wise et al. (2010) observed interesting results with (Ag)-NMs about its cytotoxic nature to cells of medaka fish. The results achieved showed cytotoxicity to the different

(Ag)-NMs concentration: 0.3, 0.5, 0.05, 3 and 5 µg cm^{-2}, which induced 80, 45.7, 24.3, 1 and 0.1% relative survival. With the results obtained it was possible to achieve the LC_{50} of Ag i.e. 0.33 µg cm^{-2}. Other study revealed that 20-30 nm of (Ag)-NMs powder induced the 48 hours LC_{50} when given for 7.07-7.20 µg mL^{-1} in zebrafish. Another interesting result with (Ag)-NMs was the change in gene and embryotoxicity that can lead to the death (Wise et al., 2010). Kalbassi et al. (2011) assessed the relationship between salinity and toxicity of (Ag)-NMs in rainbow trout and it was observed that while increasing the salinity the acute toxicity is reduced. Comparatively, it was observed that high toxicity of (Ag)-NMs is due to Ag^{+} emitted from the surface of NMs (Kittler et al., 2010). The mechanisms of toxicity of (Ag)-NMs are very similar to mechanisms of toxicity of Ag^{+}. There are chemical parameters of water that can reduce the toxicity of Ag^{+} in the aquatic environment, such as dissolved organic matter, calcium carbonate, thiosulfate, and chloride.

The information obtained about the influence of salinity on reducing the toxicity of (Ag)-NMs in fish has been widely published (Ferguson et al., 1998; Nichols et al., 2006; Webb et al., 2000). It was also shown (Kalbassi et al., 2011) that the salinity is an important chemical factor to reduce the toxicity of Ag, due to tension forces of chlorine ions (Cl^{-}), which will reduce the toxicity of Ag^{+}. The study concluded that the (Ag)-NMs in freshwater can be classified as moderately toxic substances (96 hours LC_{50}) for 6±0.3 and 12±0.2 salinity of the water, and that they can classified as slightly toxic to fish rainbow trout (see table 1) (Kalbassi et al, 2011).

Table 1: U.S.EPA on Ecotoxicity categories of materials for aquatic organisms. (Adaptation of Kalbassi et al., 2011)

Concentration (mg L^{-1})	**Toxicity Category**
<0.1	very highly toxic
0.1-1	highly toxic
>1-10	moderately toxic
>10-100	slightly toxic
>100	practically nontoxic

2.5.3. Iron oxides nanomaterials [(Fe_2O_3)-NMs)] - Zero-valent irons (nZVI) are a family of NMs that include iron oxides. Iron oxides are used for the remediation of contaminated waters and sediments, as well as for the removal of nitrates from the soil. In particular, iron oxides NMs are used in pigments, catalysis, sensors, high sensitivity biomolecular magnetic resonance imaging, tumor therapy, drug and gene transfer to cells and labeling of macromolecules (Zhu et al., 2012). The increasing production and use of nZVI draw attention towards a greater exposure risk for aquatic environment and humans. Consequently, it has become essential to understand and study the potential health and environmental effects of nZVI in aquatic organisms including fish. Until the moment, very few studies have investigated the ecotoxicity of iron NMs, particularly in aquatic organisms (Garcia et al., 2011; Li et al., 2009). The iron oxides NMs, when exposed to a transition metal or light, promote the formation of pro-oxidants and this causes destabilization between biological systems and the ability to produce and detoxify ROS (Ozmen et al., 2004).

The magnetic NMs comprise the iron oxides such as the magnetite (Fe_3O_4) and the maghemite (γ-Fe_2O_3), which present the phenomena of super-paramagnetism, and thus have the benefit of reducing the risk of particle aggregation (Mahmoudi et al., 2011; Petrova et al., 2011). An important application of iron oxide NMs is that they can be used as an adsorbent in the removal of metals such as mercury or arsenic from aqueous solutions (Grover et al., 2012; Zhang et al., 2009). The most common iron oxide NMs are the Fe_3O_4 and γ-Fe_2O_3 (Girginova et al., 2010) with low toxicity, reduced cost, and exhibiting a high surface to volume ratios depending on the size of the particle and on the modification of the surface chemistry for a greater sorption of metals for water treatments. The iron nanomaterial can be coated in order to stabilize, to prevent its oxidation and, finally, introduce features that can be selective for the sorption of ions. One of the most used is (Fe_3O_4)-NMs coated with silica, that has brought fruitful results in extraction of Hg^{2+}, Cd^{2+}, Cu^{2+}, among others. On the perspective of its toxicity, Zhu et al. (2012) reported iron oxides (α-Fe_2O_3)-NMs toxicity in fish. The (α-Fe_2O_3)-NMs on Indian major carp (fish) showed that a low concentration (1 mg L^{-1} of (α-Fe_2O_3)-NMs), was able to affect the hematological, biochemical, ion-regulatory, and enzymological parameters and for *Labeo rohita*, only at hours static exposure. Thus, the results suggest a high potential

toxicity of iron oxide NMs in aquatic environments (e.g. fish species) (Saravanan et al., 2011).

Early life stage (ELS) was performed on zebrafish (*Danio rerio*) embryos and larvae to understand the ecotoxicity mechanisms of iron oxide NMs (Zhu et al., 2012). Currently, the ELS are known as the one of the most important tools in environmental science research, mainly for investigating the toxicity and teratogenicity of chemicals that could significantly affect environmental and human health (Fraysse et al., 2006; Zhu et al., 2008). The zebrafish are demersal fish that can settle to the bottom of the water column, which allows a mimicking of the direct contact between benthic biota and NMs in the sediment. (α-Fe_2O_3)-NMs aggregates ($\geq$ 10 mg L^{-1}) tend to be toxic to zebrafish embryos and larvae, causing a dose-dependent mortality and hatching inhibition (Zhu et al., 2012). Other characteristics of toxicity was observed like developmental abnormalities, such as pericardial edema, malformation and tissue ulceration, in group of zebrafish embryos exposed to 50 and 100 mg L^{-1} of (α-Fe_2O_3)-NMs.

The organisms in the early stages of embryonic development are usually more sensitive to NMs induced toxicological effects, and thus the evaluation of the sublethal effect of NMs distinguish the nature of the toxicological effect (e.g., neural toxicity and genotoxicity) (Hallare et al., 2004; Takeda et al., 2009). On the zebrafish embryos it was found that the direct adherence and/or sorption of aggregates, of iron oxides on the surface can cause a reduction in gas exchange and, thus results in hypoxia of embryos. Also, the adherence or aggregates of (α-Fe_2O_3)-NMs sorption induced excessive production of ROS *in vivo*, resulting in oxidative stress of embryos. The accumulation of (α-Fe_2O_3)-NMs can induce toxic mechanisms, in particular, lead to an imbalance in homeostasis and aberrant cellular responses, including cytotoxicity, DNA damage, oxidative stress, epigenetic events, and inflammatory processes. The results obtained were processed in a range of concentrations of (α-Fe_2O_3)-NMs, 0.1 to 100 mg L^{-1}, in order to be able to ensure a range of acceptable exposure levels (Zhu et al., 2012).

3. Challenges associated in working with nanomaterials

In the year 2008, a publication of Behra and Krug, in Nature Nanotechnology (Kahru et al., 2010), drew the attention on three challenges that can influence NMs associated scientific studies: i) the choice and the tests (e.g. physico-chemical analysis,

aggregation, sedimentation) to feature the NMs for their use in biological experiments; ii) there is a need to understand the mechanism of sorption of NMs by different bodies (fish and mammalians), in different environments, in particular to understand the behavior of NMs in the food chain of the various animals; and iii) the choice of agencies (e.g. Research Institute, Universities, Private Institutes) for the study. Moreover, there is also a need to comprehend the routes of exposure to NMs (Fabrega et al., 2011). Consequently, it is necessary to develop the appropriate *in vitro* and *in vivo* models for the NMs toxicity studies in living organisms including fish (Arora et al., 2011).

NMs are inadvertently released into environment and may occur at numerous points during a product's life cycle including production, transport, manufacturing, consumer use, recycling, and disposal. Release of NMs to the environment during recycling and disposal is of particular concern for NMs incorporated into limited use and/or disposable products. Once released, these NMs would readily go through transformations via biotic and abiotic processes. Understanding environmental transformations and fate of engineered NMs will be a new step for the development of new strategies in design of environmentally benign NMs. Moreover, NMs can be used as environmental tracers, in environmental sensing and in contaminant remediation. Metz et al. (2009) demonstrated a biomimetic hydroquinone based on Fenton reaction which provides a new method to characterize transformations of NMs expected to occur under oxidative environmental conditions.

Nowadays, the new technologies, such as computational techniques are being used to study interactions of NMs with biological systems (Makarucha et al., 2011). The studies could also be used to complement the experimental data on toxicity. In this perspective, a new challenge appeared to the scientific community in particular, to the nanoecotoxicologists is the toxicity evaluation of the bulk chemical as an environmental hazard (Baun et al., 2008; Handy et al., 2008 b; Klaine et al., 2008). The toxicological risks of NMs must be minimized up to the extent that it should not be compromising with environment and the human health (Singh et al., 2009). Thus, the nanotoxicology studies would require a standard set of protocols for *in vitro, in vivo* toxicity (including: genotoxicity, teratogenecity, ecotoxicity) (Arora et al., 2011). The organization of quantitative data on the toxicological effects of NMs is still very scarce. Ecotoxicological information on NMs is required for a single organisms, simplified communities and whole

ecosystems and to evaluate the risk assessment and regulatory purposes. Recently, Kahru and Dubourguier (2010) observed that the fate of NMs and their impact on animals, plants and soil communities has not been investigated in situ. However, it would be necessary to validate models proposed for environmental risk assessment of NMs. The gap on effects of long term exposure to NMs requires more studies. Consequently, the physico-chemical characteristics of NMs after they react with cultured cells *in vitro* needs to be evaluated.

In nanoecotoxicology, there are several challenges, namely in the validation of *in vitro* tests with appropriate analytical control for *in vivo* effects in different organisms and environments (Arora et al., 2011). To confirm exposure to NMs, Handy et al. (2007) developed a methodological procedure consisting of: i) "total concentration" of the nanomaterial (e.g., mg L^{-1} of NMs); ii) the information of nanomaterial and/or nanoparticle on size, shape, surface area, and purity of the product; iii) optical measurements to confirm dispersion of the test NMs in the aquarium water; and iv) example measurements of NMs size in the test solution made on the electron microscope, and others. Related problems still must be overcome, such as a lack of certified reference materials and deciding what solvent should be used. Also, simple and sensitive methods (<1 mg L^{-1}) for determining NMs concentration in natural water and fish are needed. Consequently, methods of dispersion and sonication techniques are a particular dilemma when using fish.

In this way, it is important to reveal relationship between NMs size, composition, crystallinity, and morphology and their electromagnetic response properties, reactivity, aggregation and kinetics. Also, it must be considered that properties of NMs are still being discovered, such as magnetism in NMs made of materials that are non-magnetic in bulk form. The studies on kinetics and biochemical interactions of NMs still needs research: i) on NMs translocation pathways; ii) accumulation; iii) short and long term toxicity; iv) NMs interactions with cells; v) the receptors and signaling pathways involved; vi) cytotoxicity, and vii) NMs surface functionalization for an effective phagocytosis (Buzea et al., 2007). In fact, it is known that NMs exposures is able to modulate the response of the immune system to different diseases, but the knowledge on the effects of NMs exposure on the lymphatic, immune systems and in organs of organisms are sparse. Thus, the nanoscale characterization techniques should be used in to extent identification to NMs at disease sites in affected organs or tissues, and to establish pertinent interaction mechanisms.

The pathways to reduce NMs toxicity must be studied, such as antioxidants provided from dietary sources and supplements, metals chelators, anti-inflammatory agents. A multidisciplinary approach is associated to the challenge to the work on NMs. In this way, it is necessary a dialogue between those that are involved with the NMs fabrication and their effects, but also with the scientists, chemists, toxicologists, epidemiologists, environmental scientists, industry, and policy makers. To measure environmental relevant concentrations of NMs it is necessary to develop more sensitive techniques, as well as to ensure correct interpretation of ecotoxicity test results for risk assessments keeping in view rapid and reliable measurement methods for NMs in the tissues of organisms to understand bioavailability and uptake (Handy et al., 2012). Moreover, it is also necessary to differentiate the effects of manufactured and natural NMs on fish. Thus, the current development of nanoecotoxicological field revealed several opportunities to apply existing tools and techniques for the fundamental studies of fish toxicology such as the use of perfused organs and fish cell culture systems.

4. Assays for evaluating nanomaterials toxicity

In estuarine fish species, recent literature revealed almost none in relation to the NMs association to biological responses. In recent years toxicity evaluation in fish has been dealt successfully for metals or nonmetal toxicity assessment (Ahmad et al., 2006, 2008, 2011, 2012; Maria et al., 2009). A parallel approach based on the imbalance between pro-oxidant and antioxidant status as well as on its consequences namely DNA damage, LPO and enzyme inhibition, has shown its utility in providing more accurate results and avoiding their misinterpretation generated by individual (damage or protection) aspect (Ahmad et al., 2008, 2011; Oliveira et al., 2009). Nevertheless, the role of NMs in the production of ROS in fish is lacking. When the balance between oxidants and antioxidants (enzymes or substrates) is disturbed in favor of the former, free radicals cause havoc in biological system by damaging DNA, altering biochemical compounds, corroding cell membranes and killing cells out rightly.

Oxidative damage to DNA can disrupt normal transcription and replication and induce mutations. Reports indicate the genotoxic role of NMs on fish but the measurement of product of DNA oxidation 8-hydroxyfuanosine (8-OHG) is lacking as a mechanism of damage if mediated by the oxidation processes (Oliveira et al., 2010). The determination of

genotoxicity in different cells and target tissues/organs will contribute to elucidate about the susceptibility of different organs to genotoxicity. The assays used to measure NMs cytotoxicity (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT), Neutral red release (NRR), Colony forming assay and Flow cytometric analysis) are most sensitive and accurate (Fatokis et al., 2006; Mosmann, 1983), but the evaluation of cytotoxicity using doped NMs is currently not available. To combat the continuous generation of ROS, the biological systems develop various defense mechanisms, which include antioxidants (enzymes: CAT, GPX, SOD; non-enzymes: glutathione, ascorbic acid, total thiols, non-protein thiol, metallothionein) and immune (humoral or cellular) response (Ahmad et al., 2003, 2011; Fátima et al., 2001).

Immune system biomarkers have demonstrated their utility in the pollution monitoring programs (Bekesi et al., 1979; Lee et al., 1985). The level and duration of exposure play an important role and a holistic approach has to be adopted while designating a particular biological response as biomarker of pollution. Contaminant-induced immune function alterations reflect the toxic potential of the contaminant to the immune system and the health status of the affected organism. An abnormal functioning of the immune system will result in a disease state, causing events including microbial pathogenicity. The phagocytosis cells are also potential targets of environmental toxicants (such as NMs) encountered by the fish. These cells are used as short-term and long-term responses of circulating phagocytes of fish peritoneal cavity. In fish, kidney is a major lymphoid organ, which is recognized as a source of stem cells and possibly as a specialized microenvironment for the generation of immune cells (Agbede et al., 2012; Manning, 1994). Pulsford et al. (1994) and Rijkers et al. (1980) reported a higher number of antibody-secreting cells in peripheral blood and suggested that proliferation and differentiation of immune cells take place in spleen.

Most of the reports involving study of effects of environmental chemicals such as NMs on fish immune functions are concerned with the evaluation of nonspecific immunity parameters, *e.g.*, macrophage functions as indicators of altered fish immune functions (Ahmad et al., 1998; Rice et al., 1996; Zelikoff, 1994). The major leukocyte types described in fish are lymphocytes, monocytes, neutrophils, and thrombocytes (Palić et al., 2005 b). Neutrophils were reported to perform respiratory burst, degranulation of primary granules, and neutrophil extracellular trap release (NET) (Palić et al., 2005 a; 2006; 2007).

Thus, neutrophils performs relies on the reduced nicotinamide adenine dinucleotide phosphate 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 et al., 1999). ROS facilitate pH increases in phagocytic vacuoles and entry of potassium ions (K^+), stimulating the release of the digestive enzymes responsible for microbial death (Segal, 2005). Moreover, ROS can be dismutated into 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 et al., 1999).

The final stage of the neutrophil defense mechanism is the release of neutrophil extracellular traps (NETs) through the recently described process of a cell death mechanism (NETosis) activated 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).

In this way, the immune system is the first to respond to an intrusion of an external material and the analysis of the effects of NMs, in the cells of the immune system can be accomplished by macrophages. Macrophages must show adequate information of how these materials affect the animal, both vertebrate and invertebrate animals. The immune system is different between the vertebrates, in particular between the fish and mammals. Consequently, the organisms of vertebrate animals react differently and therefore we can't generalize the results obtained for the fish (Klaper et al., 2010).

Endocytosis is a process in which cells incorporate a material showing two types of activities: phagocytosis and pinocytosis. The phagocytosis is related to large particles such as microorganisms and is held by many single-celled organisms and macrophages. The pinocytosis is related to smaller molecules dissolved in water, being carried out by almost all cells (figure 4). The phagocytosis is a process of immune system response that can absorb large particles (0.5 μm) (Maderna et al., 2003). The intermediates of phagocytosis are the phagosomes that consist of a vesicle formed near the particle absorbed by phagocytosis and the vacuole that is formed by the fusion of the membrane around the particle (Flannagan et al., 2012). The phagosome then binds to the lysosome, which

contains digestive enzymes (Aderem et al., 1999; Lee et al., 2010). The process of phagocytosis begins as target recognition, through a characteristic signal on the surface of the cell. A phagocyte recognizes the signal and transcribes it in order to be processed by the cellular mechanism. Cell recognition by phagocytes depends on the rearrangement of the lipids in the plasma membrane of target cells.

Fish phagocytes form an important integral immunological defense network to neutralize invading pathogens and particulate materials (Ellis, 1999; Robertson, 1999). Like mammals, fish phagocytes engulf invading foreign agents and normally kill them by the release of cytotoxic ROS. Activated phagocytes undergo a respiratory burst phenomenon. During respiratory burst, toxic oxygen products such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radicals ($^{\cdot}OH$) are released in the surrounding environment (Ellis, 1999; Nagelkerke et al., 1990). Exposure to environmental chemicals can lead to the release of a large amount of ROS by activated phagocytes, and this can cause local tissue damage as well as direct contaminant induced oxidative damage (Fatima et al., 2000; Zelikoff et al., 1996). Although many responses involving cellular structure and function such as LPO (Kappus, 1987; Nakano et al., 1999), methemoglobin formation (Hardig and Hoglund, 1983; Hardig et al., 1988), protein damage (Bainy et al., 1996; Stern, 1985), and DNA damage are associated with the high reactivity of ROS ($^1O_2$, $O^{\cdot}$-, $^{\cdot}OH$, $RO^{\cdot}$, $ROO^{\cdot}$), the most typical reaction to ROS induced damage involves the peroxidation of unsaturated fatty acids (Fátima et al., 2000; Kappus, 1987).

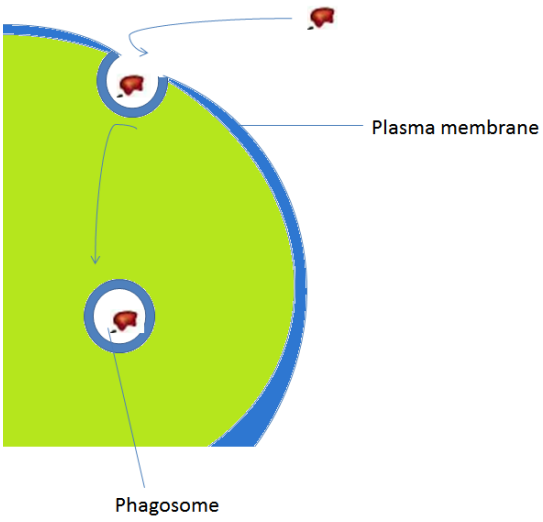

Figure 4. Phagocytosis (adapted from web site [7])

Oxidative stress is a response of the body as an effect of cellular respiration, metabolism and inflammation. Over ROS production by nanomaterial can originate from several sources:

i) ROS can be generated directly from the surface of the particles, when oxidants and free radicals are present on the surface of the particles.
ii) NMs of transition metals (iron, copper, chromium, vanadium, among others) can form ROS, acting as catalysts in Fenton-type reactions. The formation of hydroxyl radical ($^{\cdot}OH$) is extremely reactive, since it attacks the biological molecules, in diffusion range.
iii) In some published studies, it was found significant changes in mitochondria. These changes were caused by NMs that penetrate inside cells, causing injuries and thus contributing to oxidative stress (Li et al., 2012).
iv) Inflammation or inflammatory process consists of a body's response to an attack, specifically, processes that cause tissue damage or in cells. In addition, the cells involved in the inflammatory process can be distinguished, according to the site of injury. The inflammation, while it is moderately, stimulates the regeneration of healthy tissue, but when the action of inflammation is too extensive, can lead to a disease. The cells inflammatory activation may result on reactive oxygen and nitrogen species, inducing by phagocytosis of the nanomaterial.

An organism can endure oxidative stress through several different mechanisms (Usenko et al., 2008). In addition, the properties of the NMs are dependent on the size and shape and that can culminate in ROS generation. The ROS includes radicals as the superoxide anion ($O_2^{\cdot -}$), hydroxyl radicals ($^{\cdot}OH$) and the non-radical hydrogen peroxide (H_2O_2), who, typically, are constantly form inside the cells under normal conditions, as a consequence of aerobic metabolism. In abnormal conditions, such as exposure to a toxic contaminant, the production of ROS is anomalous inside cells. The cells have the ability to generate defense mechanisms against the production of ROS species, with the synthesis of antioxidants. Cell division of antioxidants is known as primary and secondary defense mechanisms. The primary defense mechanisms are superoxide dismutase, glutathione peroxidase, catalase and thioredoxin reductase and the secondary defense mechanism is

reduced glutathione. The superoxide dismutase (SOD) has the function of converting the high radicals of superoxides in less reactive species (H_2O_2), which can be destroyed by CAT or GPX. CAT is an extremely enzyme which converts reactive H_2O_2 into water and molecular oxygen. GPX catalyzes several species of ROOH and H_2O_2 through its reduction, with the glutathione (GSH). In addition, protects animal cells against oxidative damage and cellular lipid hydroperoxides reduction. Under normal conditions, a large percentage of GSH in cells is reduced and thus has an intracellular environment, extremely, reducer. However, the abrupt decrease of GSH leads to cell reduction capacity and then may induce oxidative stress without the intervention of the ROS. Free radicals are also precursors in the attack on the free fatty acids in the cells membrane forming lipid hydroperoxides. As described above, ROS can also react with DNA, proteins, carbohydrates, in order to cause cell death. The main affected molecules are macromolecules (genes or proteins) which have important roles in oxidative stress, DNA damage, inflammation or damage to the immune system (Arora et al., 2011).

To understand the negative consequences of NMs toxicity for organisms (such as malformations in embryos, overproduction of ROS, among others) it is required immunological testing. Organism's defense mechanisms have the ability to adapt over time, in order to recognize specific pathogens more efficiently. Thus, inflammatory diseases, cancer, among others are a result of affected immune system. The contaminants with a greater relevance currently are NMs.

5. Identification and justicication of the objectives of the thesis

The aquatic environment, in particular, is at risk from accumulation of a vast array of anthropogenic compounds and the continuing expansion of the nanotechnology industry will certainly release NMs into the aquatic environment. In these environments, organisms synergistic/antagonistic effects are hardly interpreted and predicted exclusively from the chemical analyses; some contaminants strongly accumulate in tissues without inducing toxic effects, while others are characterized by elevated toxicity at low levels of exposure. Moreover, the impracticality to analyze all the individual chemicals pooled in a mixture of contaminants also increases the problem of aquatic contaminant characterization. Thus, during the past two decades, the use of biological responses (biomarkers) on particular test species has become relevant in toxicological assessments since it allows the early detection of overall effects of contaminants, providing information, even at the sub-lethal level,

which reflects eventual chemical interactions (Goksøyr and Forlin, 1992; Livingstone, 1993; Passino, 1984; Peakall and Shugart, 1993).

At present, the impacts of NMs are merely speculative and unfounded. Consequently, the knowledge of the harmful effects of NMs is very limited and almost non-existent in aquatic animals. In order to clarify this aspect, a multi-disciplinary approach must be adopted to characterize the behavior of NMs and in aquatic environment. NMs and NPs tendency to aggregate and their longevity in the water column, and interaction with conventional contaminants in the aquatic environment must be better understood in order to predict the NMs toxicity in aquatic organisms. Thus, taking into account the knowledge gaps identified in the previous sections and the persistence of inadequacy of the standard toxicological models and testing methods for the assessment of the NMs potential impacts, a clear information paucity occurrence on the comparative account of pervasive contaminant behavior in aquatic system and their fate in aquatic organisms lead us to raise the question of NMs toxicity assessment in estuarine species and the development of a biologically based knowledge on macromolecules damage mechanisms. Thus, the specific objective of the present research is to evaluate NMs immunotoxicity, reactive oxygen species production, peroxidative damage as markers of NMs toxicity as well as the antioxidants evaluation as protection/adaptation markers. The present dissertation has been divided into four (1 through 4) chapters in the following manner:

Chapter 1: *A. anguilla* L. phagocytes responses following *in vitro* exposure to silica coated iron oxide nanomaterial functionalized with dithiocarbamate with or without mercury co-exposure;

Chapter 2: The role of *A. anguilla* L. phagocytes in reactive oxygen species production following *in vitro* exposure to silica coated iron oxide nanomaterial functionalized with dithiocarbamate with or without mercury co-exposure;

Chapter 3: *A. anguilla* L. phagocytes enzymatic antioxidants responses following *in vitro* exposure to silica coated iron oxide nanomaterial functionalized with dithiocarbamate with or without mercury co-exposure;

Chapter 4: *A. anguilla* L. phagocytes non-enzymatic antioxidants responses following *in vitro* exposure to silica coated iron oxide nanomaterial functionalized with dithiocarbamate with or without mercury co-exposure.

The experiments were conducted considering silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) (IONM) on the European eel (*A. Anguilla* L.*)* (figure 5). The European eel is found in rivers or estuarine areas and is one of highest commercial value in the cultured aquatic environment. This fish is characterized by tolerance on variation of the temperature, salinity and high stocking densities, which make it suitable species for culture (Barker et al., 2000). On the other hand, it is sensitive to environmental stresses (toxicity with metals and non-metals; parasitic, fungal and bacterial diseases), and fulfil most requirement as bioindicator. *In vitro* toxicity assays were considered because they have advantages over traditional *in vivo* system for sacrifice a small number of organisms, the best control of the variable environment, the possibility of simultaneous or repeated sampling over time, the use of minute amounts of chemical compounds (xenobiotics), allowing the study of a specific toxicity reaction under strict controlled conditions (Moore and Simpson, 1992).

Figure 5. European eel (*Anguilla anguilla* L.) (adapted from web site [8])

6. References

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Chapter 1

Anguilla anguilla L. phagocytes responses following *in vitro* exposure to silica coated iron oxide nanomaterial functionalized with dithiocarbamate with or without mercury co-exposure

Abstract

The study aimed to assess the immunotoxic effects of *in vitro* silica coated iron oxide nanomaterials functionalized with dithiocarbamate (IONM) exposure and its interference with mercury (Hg) co-exposure on European eel (*Anguilla Anguilla* L.) phagocytes isolated from peritoneum (P-phagocytes), gill (G-phagocytes), head kidney (HK-phagocytes) and spleen (S-phagocytes). The phagocytes maintained in culture medium were used for the assessment of phagocytes viability, phagocytic index and phagocytic capacity after exposure to IONM, Hg and IONM+Hg concomitantly for 0, 2, 4, 8, 16, 24, 48 and 72 hours. *A. anguilla* phagocytes displayed differential responses of both viability and innate immune function under all the exposure conditions. Phagocytes viability either increased (P-phagocytes) or showed no change (G-, HK- and S-phagocytes) at early hours (2 hours), then reaches to the normal levels at late hours (72 hours) indicating that cells are metabolically active and tolerant to IONM exposure. However, IONM chronic exposures scenario was linked to increased risk of immunomodulation, since phagocytes reflected modulatory responses over time on phagocytic index and phagocytic capacity except HK-phagocytes where only decrease was observed. The occurrence of synergism in viability at 8 hours (P-phagocytes), 16 hours (G-phagocytes) and antagonism at 8 hours (HK-phagocytes) was observed; moreover, a period of exposure-dependent antagonism/or synergism in phagocytic index and phagocytic capacity was also perceptible in all the studied phagocytes implying that the Hg co-exposure has the potential to modulate effects induced by IONM exposure alone. Overall, a fine-tuning among innate immune functions was revealed controlling IONM+Hg interactive impacts successfully.

1. Introduction

Despite the growing evidences of potential risk associated with releasing nanomaterials (NMs) into the aquatic environment, very few reports reflect potentially harmful toxic effects on aquatic organisms including fish that play a prominent role in the aquatic food web. In addition, no report on NMs behavior in the presence of other chemical compounds, mainly those that exhibit high level of toxicity and are persistent in nature, make this area of research completely unexplored. Nanomaterials and their interaction with other contaminants are very likely to occur in the aquatic system resulting synergistic or antagonistic toxic effects due to their interaction.

It has been documented that exposure to aquatic contaminants interferes with the development and functioning of a healthy immune response (Ahmad et al., 2004). Recent concerns about the sustainability of fish populations in the aquatic environment, have implicated chemical contaminants as a contributing factor in altering the physiology/biochemistry of ecological biota. For example, metal agents–resulting from occupational, inadvertent, therapeutic, environmental, and biological exposures–have been implicated in both reducing and augmenting immune function (Burrell, 1993; Trizio et al., 1988). Thus, undesirable immunomodulation of fish in response to NMs can provide information not only on the interference with the body's immune protection against cancerous cells and bacterial/viral pathogens, but also on undesirable immunosuppression as an important part for metal nanomaterial sustainable preparation and their applications. The great potential of NMs engineering is that particles may be manipulated to reduce/eliminate immunosuppression while retaining their environmental friendly potential.

The immune system has a specialized subset of cells, names professional phagocytes, equipped for rapidly and efficiently ingesting invading microorganisms at sites of inflammation. Phagocytosis plays an essential role on host defense mechanisms through the uptake and destruction of pathogens, and contributes to the inflammation and the immune response. Phagocytosis has shown its utility in detecting interference with the development and functioning of immune response after exposure to metals and non-metals. However, no available report reflects its utility in nanomaterial risk assessment in the aquatic environment. Thus, keeping in view the previously described lacunae, the current study has been hypothesized as: can NMs activate or suppress fish phagocytes response inappropriately? Moreover, are NMs able to regulate the magnitude and specificity of a competent immune response in the presence of other contaminants? It may be postulated that NMs induced phagocytes response may be further modulated by the concomitant co-exposure to other contaminant resulting reduced or augmented protection against pathogens and infectious diseases.

To accomplish the said hypothesis, magnetic iron oxide NMs such as (Fe_3O_4@SiO_2/SiDTC, hereafter called IONM were chosen as stressors because of their multidisciplinary applications and may enter aquatic system via their intensive use in environmental cleanup. The coating or encapsulation of Fe_3O_4-NMs with adequate

chelating agents (Li et al., 2008; Mohan and Pittman, 2007) or thiol containing polymers (Shin and Jang 2007) has been used to enhance metal ions removal efficiency. Nevertheless earlier, the coating of Fe_3O_4-NMs with silica has been found successful for efficient extraction of Cd^{2+}, Cu^{2+}, Hg^{2+} and Pb^{2+} (Girginova et al., 2010; Huang and Hu 2008). It is to be underlined here that the previous aspects have largely ignored the consideration of Fe_3O_4-NMs fate after use and/or its potential toxicity on the biota. Additionally, the literature search reflects a credible number of articles focusing mainly on the synthesis, characterization, and surface properties of Fe_3O_4-NMs; thus, reflecting the existence of a significant knowledge gap on a complete toxicological profile of Fe_3O_4-NMs which is, in fact, a pre-requisite for safe and efficient Fe_3O_4-NMs multidisciplinary applications (Mahmoudi et al., 2012). In addition, Hg was chosen as co-contaminants due to its pervasive nature in the aquatic environment and a known metal carcinogen. It is important to know how the resultant toxic effects (synergistic, antagonistic) are appeared due to contaminants interaction. Immonotoxicity endpoints viz. cells viability and phagocytosis (Phagocytic index, phagocytic capacity) were evaluated in phagocytes isolated from peritoneum (P-phagocytes), gill (G-phagocytes), head kidney (HK-phagocytes) and spleen (S-phagocytes) of *A. Anguilla* L. taking into account their ability to mirror the potential biological impacts of various metals and non-metals (Ahmad et al., 2003; 2004).

2. Materials and methods

2.1. Chemicals

Giemsa solution, hank's balanced salt solution (HBSS), L-15 medium, methanol, nitroblue tetrazolium (NBT), phosphate buffered saline (PBS), potassium phosphate mono- and di-basic (KH_2PO_4, K_2HPO_4), RPMI-1640 medium, trypan blue solution, zymosan, used for this study were of analytical grade and were purchased from Sigma.

2.2. Equipment

- Cell dissociation sieve-Tissue Grinder Kit from Sigma-Aldrich
- Centrifuge 5415 R from Eppendorf to centrifuge the samples
- Environmental Shaker from Incubator ES-20 for shaking the samples.

- Microscope from Olympus BX41 for phagocytosis count.

2.3. Silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) (IONM) group and mercury (Hg) test solution preparation

Based on the study performed by Tavares et al. (2013) where the authors evidenced a high efficiency of IONM concentration (2.5 mg L^{-1}) for the removal of Hg up to concentration of 50 µg L^{-1}, in the current bio-experiment, 2.5 mg L^{-1} and 50 µg L^{-1} were chosen as test concentrations respectively for IONM and Hg. In the current bio-experiment, a stock solution (10 mg L^{-1}) of previously prepared IONM was made and sub-diluted to the final concentration of 2.5 mg L^{-1} in RPMI medium. In order to prevent the IONM agglomeration, the L-15 dispersed IONM were mixed using vortex for 20 seconds and sonicated for 2 x 20 seconds with a 20 seconds pause in between the sonication. In parallel, the stock solution of Hg (10 mg L^{-1}) was made and diluted in order to get the required concentration of Hg 50 µg L^{-1} in different exposure groups.

2.4. Experimental protocols

European eel *Anguilla anguilla* L. (n=3) with an average weight of 50 ± 5 g were collected from the Aveiro Lagoon, Murtosa (Portugal). The eels were transported in anoxia and acclimated to laboratory conditions in aerated, filtered, and dechlorinated tap water in 50-L aquarium for 1 week, at 20°C according to the methods adopted by Santos and Pacheco, (1996). Fish were not fed during acclimatization period.

2.5. Isolation of phagocytes from peritoneum and gill cavity

Peritoneum and gill-adhered phagocytes were isolated using method previously described by Ahmad et al. (2003). Briefly, the fish blood was drained by severing the caudal vein to avoid the contamination of red blood cell with peritoneal and gill exudates. An abdominal incision was made and peritoneal cavity and gill were thoroughly rinsed into siliconized test tubes, transferred to separate unsiliconized petri-dishes with cold Hanks balanced salt solution (HBSS) and kept incubated during 1 hour for adherence. These unsiliconized petri-dishes supernatant was decanted and the cells detached with the help of a cell scraper in fresh cold HBSS (pH 7.2). Detached cells were centrifuged in siliconized tubes at 2500 rpm for 10 minutes (x3) and the final cell pellet re-suspended in 1 mL of

medium RPMI-1640. Cells were counted using a Neubauer hemocytometer. Differential counts were performed to assess the phagocytes population in the cell suspension to avoid red blood cells contamination.

2.6. Isolation of phagocytes from head kidney and spleen

Head kidney (pronephros) and spleen in fish were dissected out and cells were isolated according to the method described by Fatima et al. (2000) and Tellez- Bañuelos et al. (2009). Briefly, it was minced using a cell dissociation sieve tissue grinder kit (Sigma) and the cells were separated from tissue in RPMI-1640. The suspension was layered onto Histopaque-1077 and centrifuged at 1800 rpm for 30 minutes. The cell layer was collected, washed twice with RPMI-1640 and used for experiments.

2.7. Phagocytes processing and contaminants exposure

Phagocytes isolated from peritoneum, gill, head-kidney and spleen in RPMI medium were centrifuged and the phagocytes were maintained at a density of 1 x 10^6 cells mL^{-1}. Four aliquots of 2 mL according to the concentration of 1 x 10^6 cells mL^{-1} medium were processed for each fish. In 1 mL aliquot of 1 x 10^6 cells mL^{-1} density, 1 mL of RPMI of the equal cell density was added and referred as control group. The second group, termed as IONM group, consisted of 2 mL of IONM (2.5 mg L^{-1}) concentration and cells density according to 2 x 10^6 cells mL^{-1}. The third aliquot of 2 mL (1 x 10^6 cells mL^{-1} medium) was consisted of 2 x 10^6 cells and Hg (100 µg L^{-1}) and termed as Hg group. The remaining 4^{th} group, termed as IONM+Hg, was build up by mixing 2 x 10^6 cells in 1 mL of Hg (100 µg L^{-1}) and 1 mL of IONM (5 mg L^{-1}). After 24 hours and 48 hours, the medium of mentioned four groups were renewed by centrifugation at 3000 rpm. The pellet obtained in each group was re-suspended in fresh RPMI medium and the Hg concentrations were maintained up to 50 µg L^{-1}. Samples were collected immediately after incubation at 0, 2, 4, 8, 16, 24, 48 and 72 hours from each group and stored at -80 °C for various biochemical estimations. A little sample (10 µL) was immediately processed for the gross estimation of phagocytic viability and slides preparation for phagocytosis from each group.

2.8. Phagocytes viability

The viability of phagocytes was determined by trypan blue dye exclusion method of Raisuddin et al. (1993). Briefly, the assay consisted of 25 µL of trypan blue solution, 15 µL of PBS, 10 µL cell suspensions and 10 µL of zymossan. The mixture was allowed to stand for 15 minutes. Then, a little amount of the suspension was transferred onto cover slide and counted in duplicate under the light microscope (Olympus BX40) at a magnification of 400x. Cell viability was expressed as percentage by applying the following equation: Cell viability (%) = number of viable cells/number of viable cells + number of dead cells x 100.

2.9. Phagocytosis

Phagocytes isolated from peritoneum, gill, head-kidney and spleen in RPMI medium were centrifuged and the phagocytes were maintained at a density of 1 x 10^6 cells mL^{-1}. Four aliquots of 2 mL according to the concentration of 1 x 10^6 cells mL^{-1} medium were processed for each fish. In 1 mL aliquot of 1 x 10^6 cells mL^{-1} density, 1 mL of RPMI of the equal cell density was added and referred as control group. The second group, termed as IONM group, consisted of 2 mL of IONM (2.5 mg L^{-1}) concentration and cells density according to 2 x 10^6 cells mL^{-1}. The third aliquot of 2 mL (1 x 10^6 cells mL^{-1} medium) was consisted of 2 x 10^6 cells and Hg (100 µg L^{-1}) and termed as Hg group. The remaining fourth group, termed as IONM + Hg, was build up by mixing 2 x 10^6 cells in 1 mL of Hg (100 µg L^{-1}) and 1 mL of IONM (5 mg L^{-1}). After 24 hours and 48 hours, the medium of mentioned four groups were renewed by centrifugation at 3000 rpm. The pellet obtained in each group was re-suspended in fresh RPMI medium and the Hg concentrations were maintained up to 50 µg L^{-1}. Samples were collected immediately after incubation at 0, 2, 4, 8, 16, 24, 48 and 72 hours from each group, fixed and stored at -80°C for various biochemical estimations (see Chapter 2, 3 and 4). A little sample was immediately processed for the gross estimation of phagocytic viability and slides preparation for phagocytosis (Chapter 1) and oxidative burst activity (Chapter 2) from each group.

2.10. Cell morphology

A. anguilla L. phagocytes where placed in a six well plates and incubated with IONM, Hg, IONM+Hg for 72 hours. Phagocytes without any toxicant were taken as control. The altered cell morphology was recorded at regular intervals (24, 48 and 72 hours) using a Nikon Eclipse TS 100 microscope.

2.11. Statistical Analysis

Microsoft excel software and SPSS (PASW statistics 18) for Windows was used for statistical analysis of data. All of the data were first tested for normality and homogeneity of variance to meet statistical demands. The t-test was applied for significant differences in order to compare results within and between the groups (control, IONM, Hg and IONM+Hg). A significance level of 0.05 was ascertained in all test procedures.

3. Results

Results depiction 'given below' has been considered describing first phagocytes inter-group variations within the same hours of exposure followed by inter-hours comparisons within the same group.

3.1. Peritoneum exudates phagocytes (P-phagocytes) responses

3.1.1. Inter-group comparisons

The results based on viability and phagocytosis has been depicted in figure 6. Effects of *in vitro* exposure of IONM on *A. Anguilla* P-phagocytes resulted a significant increase in phagocytes viability at 2, 8 and 16 hours in comparison to control. A significant increase in phagocytosis (as represented by the phagocytic index) was observed at 4, 8, 16 and 48 hours, whereas a significant decrease was perceptible at 48 hours, when compared to control. Concerning, the phagocytosis capacity, a significant decrease was observed at 2 hours, whereas at 4, 8, 48 and 72 hours, a significant increase was exhibited in comparison to control.

Exposure to Hg alone resulted in a significant increase in viability at 8 and 16 hours. In terms of phagocytosis index, a significant decrease at 8 hours and increase at 16 hours was perceptible; whereas in terms of phagocytic capacity, an increase was perceptible at 2, 24, and 72 hours, respectively.

Effects of IONM and Hg concomitant exposure resulted in a significant viability decrease at 4 hours and increase at 8 hours in comparison to control. A significant viability increase at 8 hours was also perceptible when the co-exposed group was compared with the group exposed to IONM alone. No significant decrease was observed in P-phagocytic index in comparison to control. However, in comparison to IONM alone exposure, a significant decrease in phagocytic index at 16 and 48 hours and increase at 24 hours were perceptible. In terms of phagocytosis capacity, both decrease (2 hours) and increase (24, 48 and 72 hours) was observed when compared to control. Moreover, in comparison to IONM exposure alone, a significant decrease on phagocytosis capacity was perceptible at 4, 8 and 16 hours; whereas, a significant increase was observed at 72 hours. On comparison of IONM+Hg coexposed group with Hg exposure alone, a significant decrease at 2 hours and an increase at 8 and 48 hours was observed.

3.1.2. Inter- hours comparisons

Inter-hours comparisons revealed no statistically significant difference between the controls during the 72 hours exposure period. IONM exposure alone lead to significantly decreased viability at 8 hours (vs. 2 hours) and 16 hours (vs. 2 hours), respectively. In terms of phagocytic index, an increase was observed at 8 hours (vs. 2 hours), 16 hours (vs. 2 and 4 hours) and 48 hours (vs. 2, 4, 16 and 24 hours), whereas a decrease was perceptible at 24 hours (vs. 8 hours) and 72 hours (vs. all exposure period). Concerning phagocytic capacity, an increase was observed at 4 hours (vs. 2 hours), 8 hours (vs. 2 and 4 hours) 16 hours (vs. 2 hours) and 48 hours (2, 4, 8, 16 and 24 hours).

Hg exposure alone decreased viability at 8 and 16 hours (vs. 2 hours) and phagocytic capacity at 24 hours (vs. 2 hours) and 48 hours (vs. 4, 8, 16 and 24 hours); whereas increased phagocytic index and phagocytic capacity were observed at 16 hours (vs. 8 hours) and 72 hours (vs. 2, 16, 24 and 48 hours), respectively.

Concomitant IONM and Hg exposure revealed increased viability at 8 (vs. 4 hours) and phagocytic capacity at 24 hours (vs. 2, 4 and 16 hours), 48 hours (vs. 2, 4, 16 and 24 hours) and 72 hours (vs. 2, 4, 16, 24 and 48 hours); whereas, a decrease was perceptible in viability at 8 hours (vs. 2 hours) and phagocytic capacity at 8, 24, 48 and 72 hours in comparison to 8 hours.

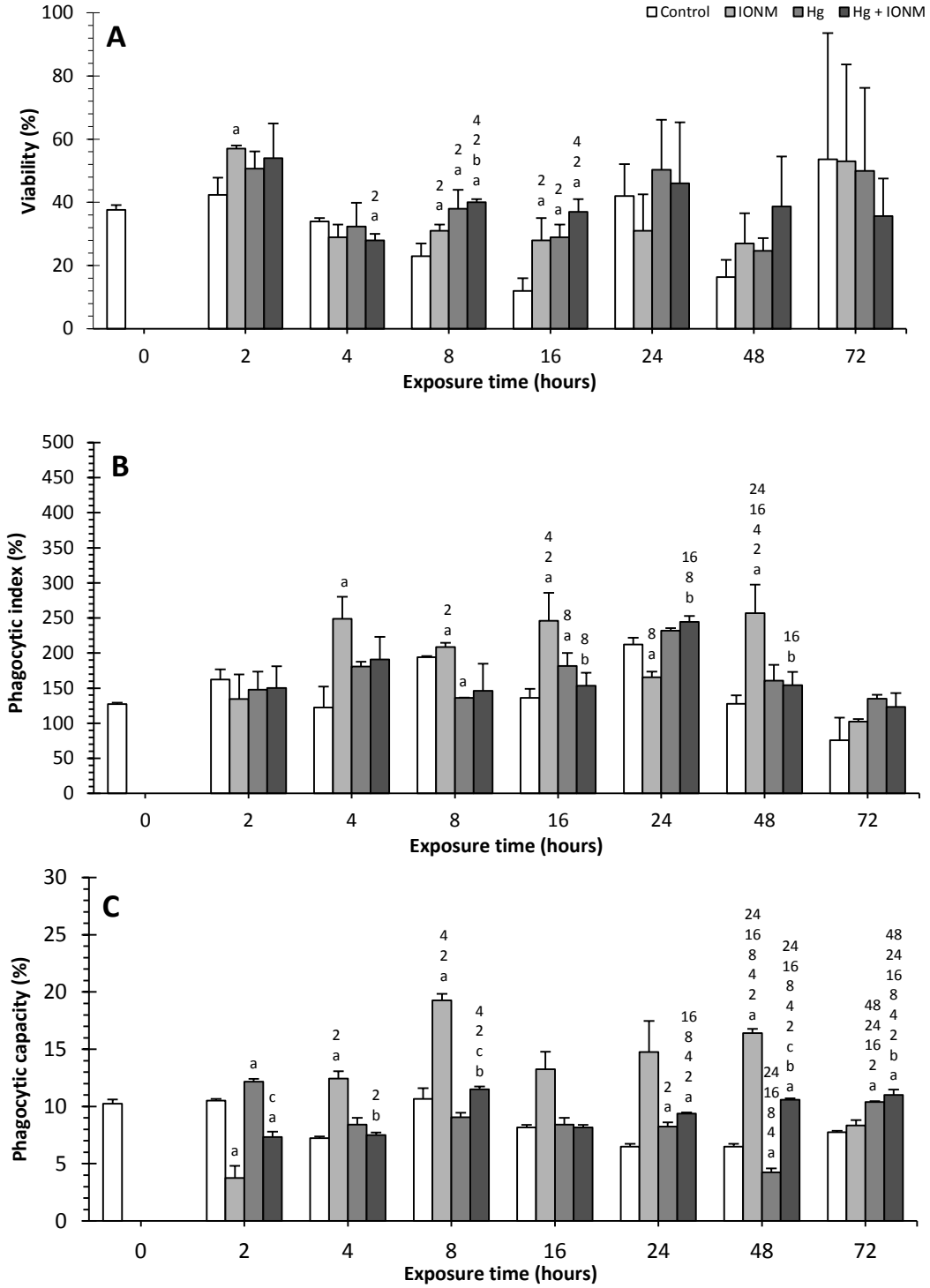

Figure 6. *Anguilla anguilla* L. *in vitro* peritoneum exudates phagocytes: viability (%) (A), phagocytic index (%) (B), phagocytic capacity (%) (C) under exposure to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone or concomitantly for a period of 72 hours. Inter-group significant differences have been denoted by letters: a (vs. control), b (vs. IONM), c (vs. Hg) and inter-hours significant differences have been marked by numbers: 2 (vs. 2 hours), 4 (vs. 4 hours), 8 (vs. 8 hours), 16 (vs. 16 hours), 24 (vs. 24 hours) and 48 (vs. 48 hours).

3.2. Gill-adhered phagocytes (G-phagocytes) responses

3.2.1. Inter-group comparisons

The results based on viability and phagocytosis has been depicted in figure 7. *A. Anguilla* L. G-phagocytes revealed a significant increase in viability after exposure to IONM alone at 16 hours when compared to control. A significant increase in phagocytic index at 8 hours was also observed, whereas at 16 hours, the G-phagocytes showed a significant decrease. To the other, phagocytic capacity significantly increased at 16 hours and decreased at 48 hours.

G-phagocytes exposed to Hg alone exhibited a decrease in cell viability at 8 hours in comparison to control. In terms of phagocytic index, a significant increase at 8 and 48 hours was perceptible; whereas a significant increase in phagocytic capacity was observed at 16, 24 and 48 hours.

Effects of IONM and Hg concomitant exposure displayed a significant increase in cell viability at 8 and 16 hours, phagocytic index at 4 and 8 hours as well as phagocytic capacity at 2 and 24 hours in comparison to control. Effects comparison of concomitant exposure with IONM exposure alone revealed a significant increase in viability at 16 hours and phagocytic index at 4 and 8 hours. Moreover, a decrease in viability at 4 hours and phagocytic capacity at 2 hours was also perceptible when IONM+Hg group was compared with IONM alone. Concomitant effects comparison with Hg alone exhibited increase in viability at 4, 8 and 16 hours and phagocytic index at 4 hours; whereas a decrease in phagocytic index at 8 and 48 hours and phagocytic index at 2 and 24 hours.

3.2.2. Inter- hours comparisons

Inter-hours comparisons revealed no statistically significant difference between the controls during the 72 hours exposure period. Effects of IONM *in vitro* exposure alone on G-phagocytes revealed an increase in phagocytic index at 8 hours (vs. 2 hours) and phagocytic capacity at 16 hours (vs. 8 hours), whereas a decrease in viability at 16 hours (vs. 2, 4 and 8 hours), phagocytic index at 16 hours (vs. 8 hours) and phagocytic capacity at 48 hours (vs. 24 hours).

Concerning Hg exposed G-phagocytes, a significant increase in phagocytic index at 8 hours (vs. 2 and 4 hours) and 48 hours (vs. 2, 4, 8, 16 and 24 hours), phagocytic capacity

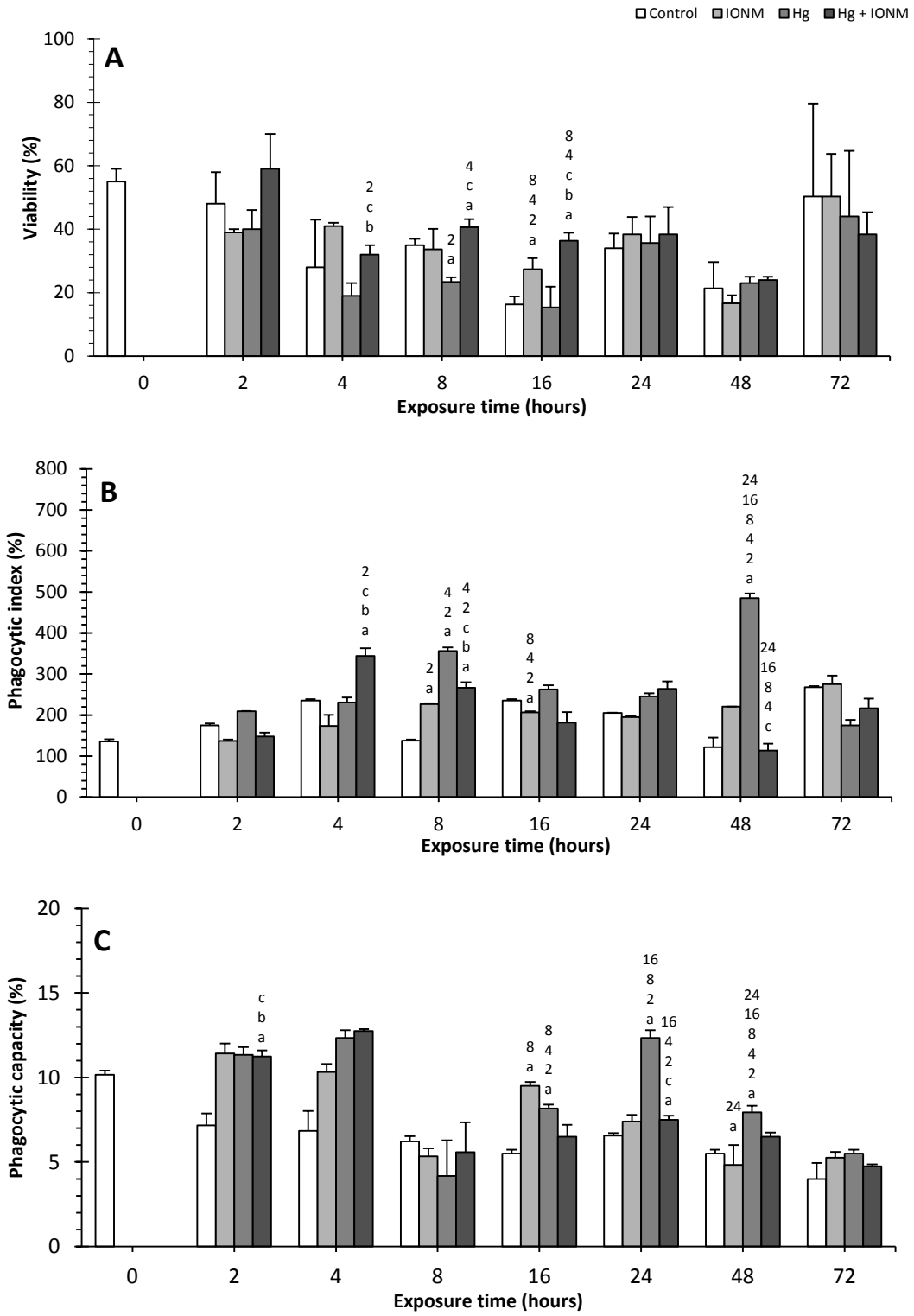

Figure 7. *Anguilla anguilla* L. *in vitro* gill-adhered phagocytes: viability (%) (A), phagocytic index (%) (B), phagocytic capacity (%) (C) under exposure to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone or concomitantly for a period of 72 hours. Inter-group significant differences have been denoted by letters: a (vs. control), b (vs. IONM), c (vs. Hg) and inter-hours significant differences have been marked by numbers: 2 (vs. 2 hours), 4 (vs. 4 hours), 8 (vs. 8 hours), 16 (vs. 16 hours), 24 (vs. 24 hours) and 48 (vs. 48 hours).

at 16 hours (vs. 8 hours) and 48 hours (vs. 8 hours) was observed; whereas, a decrease was perceptible in phagocytic capacity at 48 hours (vs. 2, 4, 16 and 24 hours).

G-phagocytes concomitantly exposed to IONM and Hg revealed a significant increase in viability at 8 and 16 hours (vs. 4 hours), phagocytic index at 4 hours (vs. 2 hours) and 8 hours (vs. 2 hours), and phagocytic capacity at 24 hours (vs. 16 hours); whereas a decrease was observed in viability at 16 hours (vs. 8 hours), phagocytic index at 8 hours (vs. 4 hours) and phagocytic capacity at 24 hours (vs. 2 and 4 hours).

3.3. Head kidney resident phagocytes (HK-phagocytes) responses

3.3.1. Inter-group comparisons

The results based on viability and phagocytosis has been depicted in figure 8. Effects of IONM exposure alone on *A. Anguilla* HK-phagocytes exhibited a significant increase only in viability at 8 hours in comparison to control; whereas decrease in viability at 16 hours and phagocytic index at 16 and 24 hours was perceptible.

Effects of Hg exposure alone versus control revealed no induction in any of the studied parameters; however, a significant decrease was observed in viability at 8 and 16 hours, phagocytic index at 2, 4 and 8 hours, and phagocytic capacity at 2, 8, 48 and 72 hours.

Effects of IONM and Hg concomitant exposure on HK-phagocytes displayed a significant increase in phagocytic index at 2 and 4 hours, and phagocytic capacity at 2 and 72 hours when compared to control; whereas, a decrease was perceptible in viability at 16 hours, phagocytic index and phagocytic capacity at 4 and 16 hours. Effects of IONM exposure alone versus concomitant exposure revealed a significant increase in phagocytic index at 48 hours and phagocytic capacity at 2 and 72 hours; whereas, decrease was recorded in viability at 8 hours, phagocytic index at 4 and 16 hours, and phagocytic capacity only at 16 hours. Concomitant effects comparison with Hg alone exhibited increase in viability at 8 hours, phagocytic index at 4 hours, and phagocytic capacity at 2 and 72 hours.

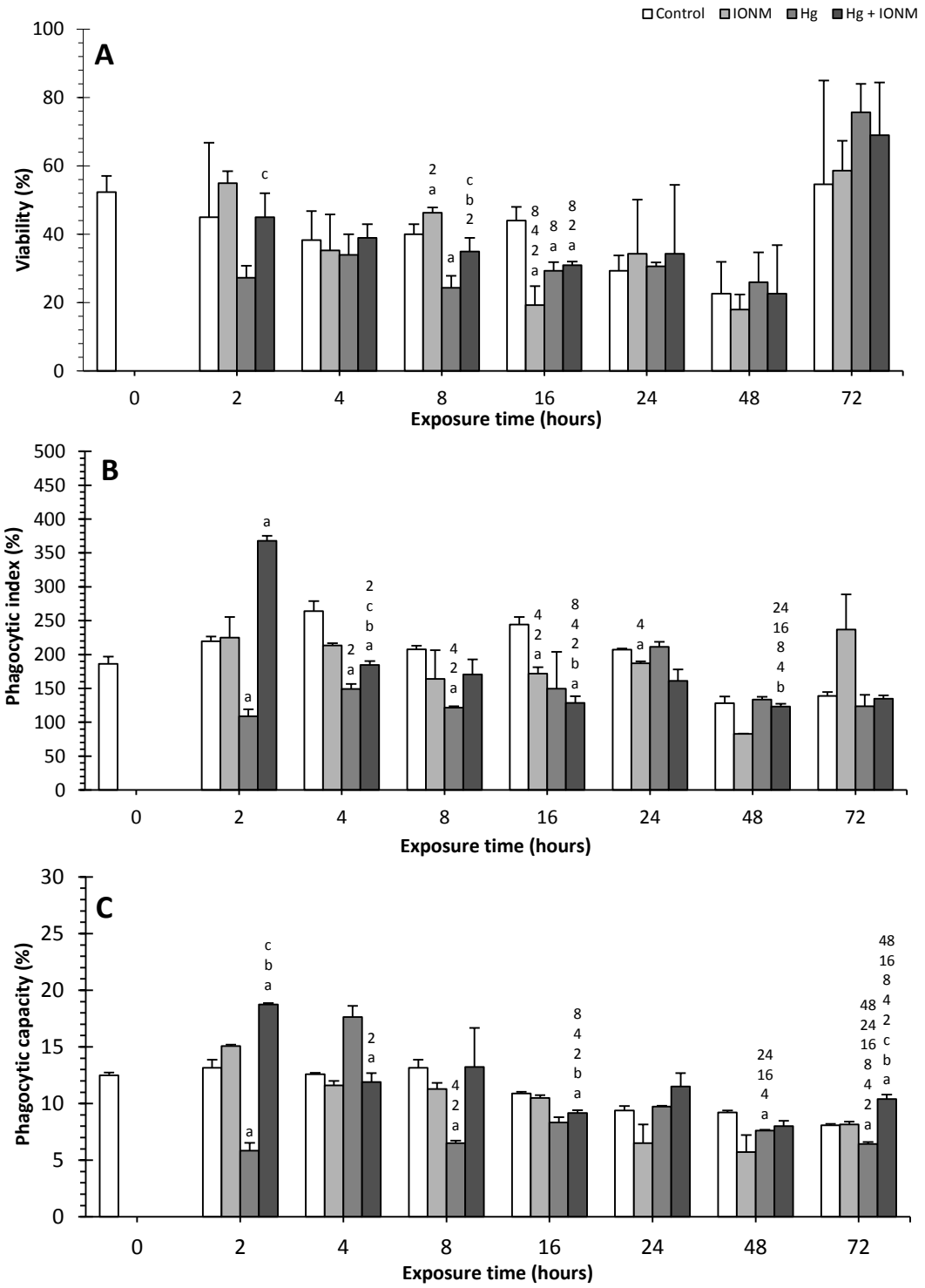

Figure 8. *Anguilla anguilla* L. *in vitro* head kidney resident phagocytes: viability (%) (A), phagocytic index (%) (B), phagocytic capacity (%) (C) under exposure to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone or concomitantly for a period of 72 hours. Inter-group significant differences have been denoted by letters: a (vs. control), b (vs. IONM), c (vs. Hg) and inter-hours significant differences have been marked by numbers: 2 (vs. 2 hours), 4 (vs. 4 hours), 8 (vs. 8 hours), 16 (vs. 16 hours), 24 (vs. 24 hours) and 48 (vs. 48 hours).

3.3.2. Inter- hours comparisons

Inter-hours comparisons revealed no statistically significant difference between the controls during the 72 hours exposure period. Effects of IONM *in vitro* exposure alone, on G-phagocytes revealed a decrease in viability at 8 hours (vs. 2 hours), 16 hours (vs. 2, 4 and 8 hours) and phagocytic index at 16 hours (vs. 2 and 4 hours) and 24 hours (vs. 4 hours).

Concerning Hg exposed G-phagocytes, a significant increase in viability at 16 hours (vs. 8 hours), phagocytic index at 4 hours (vs. 2 hours) and 8 hours (vs. 2 hours), phagocytic capacity at 8 hours (vs. 2 hours) was observed; whereas, a decrease was perceptible in phagocytic capacity at 8 hours (vs. 4 hours), 48 hours (vs. 4, 16 and 24 hours) and 72 hours (vs. all exposure period).

G-phagocytes concomitantly exposed to IONM and Hg revealed a significant increase only in phagocytic capacity at 72 hours (vs. 48 hours); whereas a decrease was observed in phagocytic index at 4 hours (vs. 2 hours), 16 hours (vs. 2, 4 and 8 hours) and phagocytic capacity at 4 hours (vs. 2 hours), 16 hours (vs. 2, 4 and 8 hours) and 72 hours (vs. 2, 4, 8 and 16 hours).

3.4. Spleen resident phagocytes (S-phagocytes) responses

3.4.1. Inter-group comparisons

The results based on viability and phagocytosis has been depicted in figure 9. Effects of IONM exposure alone on *A. Anguilla* HK-phagocytes exhibited a significant increase in phagocytic index at 4 and 72 hours and phagocytic capacity at 24 and 48 hours in comparison to control; whereas decrease in phagocytic index at 2 and 8 hours, and phagocytic capacity at 2, 8 and 72 hours was perceptible.

Effects of Hg exposure alone versus control revealed increase in viability at 16 hours, phagocytic index at 24 and 72 hours, phagocytic capacity at 48 hours; a significant decrease was observed only in phagocytic capacity at 2 and 72 hours.

Effects of IONM and Hg concomitant exposure on HK-phagocytes displayed a significant increase in viability at 8 hours, phagocytic index at 4 and 24 hours, and phagocytic capacity only at 4 hours when compared to control; whereas, a decrease was perceptible only in phagocytic capacity at 2 and 48 hours. Effects of IONM exposure alone versus concomitant exposure revealed a significant increase in phagocytic index at 24

hours and phagocytic capacity at 8 hours; whereas, decrease was perceptible in phagocytic index at 4 and 16 hours, and phagocytic capacity at 24 and 48 hours. Concomitant effects comparison with Hg alone exhibited increase only in phagocytic capacity at 72 hours; whereas a decrease was observed in phagocytic index at 16 and 24 hours and phagocytic capacity at 2 and 48 hours.

3.4.2. Inter- hours comparisons

Inter-hours comparisons revealed no statistically significant difference between the controls during the 72 hours exposure period. Effects of IONM *in vitro* exposure alone on G-phagocytes revealed an increase in phagocytic index at 4 hours (vs. 2 hours) and 72 hours (vs. 8 hours) and phagocytic capacity at 8 hours (vs. 2 hours), 24 hours (vs. 2 and 8 hours), 48 hours (vs. 2 hours) and 72 hours (vs. 2 hours); whereas, a decrease was recorded in phagocytic index at 8 hours (vs. 2 and 4 hours) and 72 hours (vs. 2 and 16 hours), and phagocytic capacity at 48 hours (vs. 24 hours) and 72 hours (vs. 24 and 48 hours).

Concerning Hg exposed G-phagocytes, a significant increase in viability at 16 hours (vs. 8 hours), phagocytic index only at 24 hours (vs. 2, 4 and 16 hours), phagocytic capacity at 48 hours (vs. 2, 4, 8, 16 and 24 hours) was observed; whereas, a decrease was perceptible in phagocytic index at 4 hours (vs. 2 hours) and 72 hours (vs. all exposure period) and phagocytic capacity at 72 hours (vs. all exposure period).

G-phagocytes concomitantly exposed to IONM and Hg revealed a significant increase in phagocytic index at 4 hours (vs. 2 hours), 24 hours (vs. 2 and 16 hours) and phagocytic capacity at 4 hours (vs. 2 hours) and 48 hours (vs. 2 hours); whereas a decrease was observed in viability at 8 hours (vs. 2 hours), phagocytic capacity only at 48 hours (vs. 8, 16 and 24 hours).

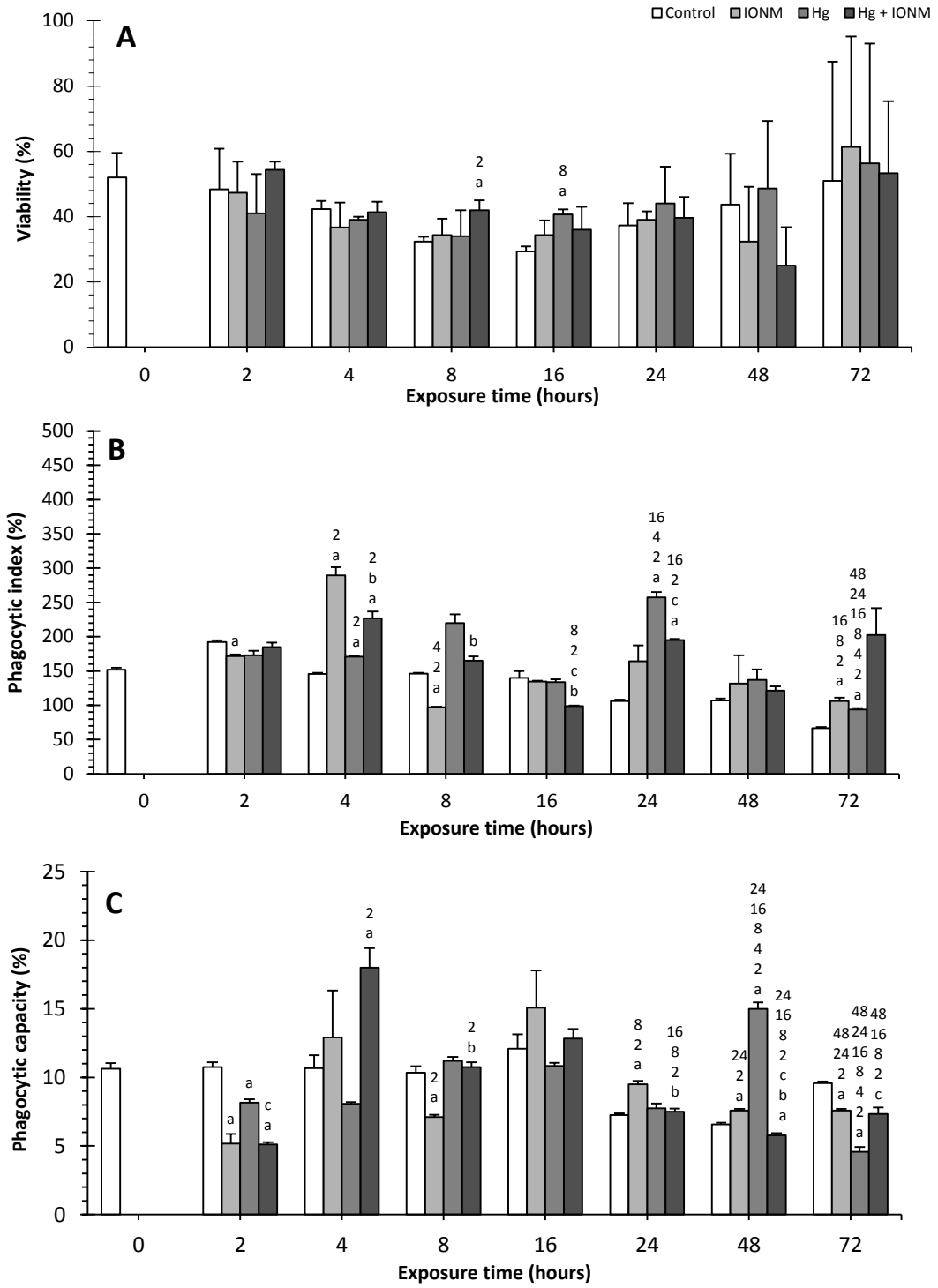

Figure 9. *Anguilla anguilla* L. *in vitro* spleen resident phagocytes: viability (%) (A), phagocytic index (%) (B), phagocytic capacity (%) (C) under exposure to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone or concomitantly for a period of 72 hours. Inter-group significant differences have been denoted by letters: a (vs. control), b (vs. IONM), c (vs. Hg) and inter-hours significant differences have been marked by numbers: 2 (vs. 2 hours), 4 (vs. 4 hours), 8 (vs. 8 hours), 16 (vs. 16 hours), 24 (vs. 24 hours) and 48 (vs. 48 hours).

3.5. Cell morphology

3.5.1. Effect on cell morphology in P-, G- and HK-phagocytes

The results based on cell morphology have been depicted in figure 10, 11 and 12. Light microscopic images of phagocytes isolated from peritoneum, gill and head kidney after exposure to IONM, Hg and IONM+Hg showed no morphological changes suggesting that the phagocytes were of same lineage.

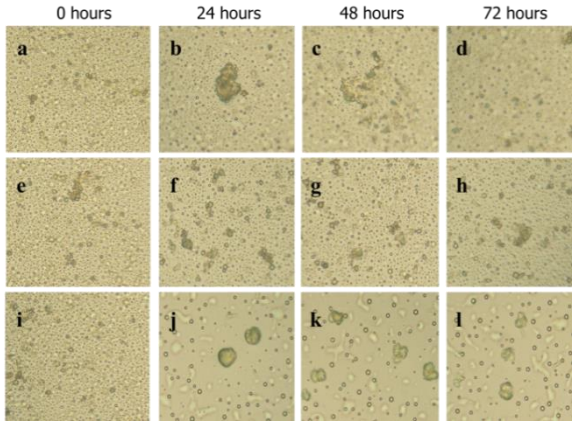

Figure 10. *Anguilla Anguilla* L. peritoneum exudates phagocytes morphology showing lamellopedia in response to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone (a-d for IONM; e-h for Hg) and concomitant exposure (i-l for IONM+Hg) for 24, 48 and 72 hours in comparison to control (0 hours).

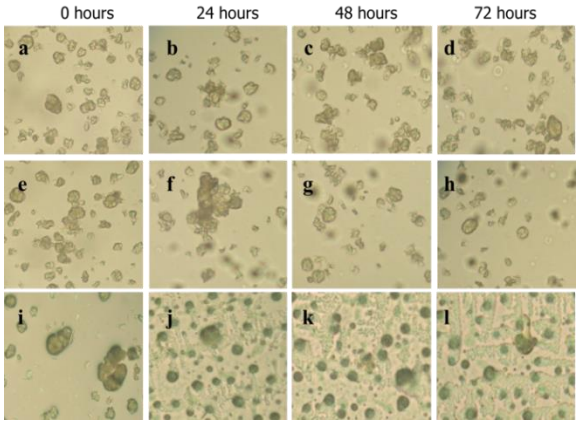

Figure 11. *Anguilla Anguilla* L. gill-adhered phagocytes morphology showing lamellopedia in response to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone (a-d for IONM; e-h for Hg) and concomitant exposure (i-l for IONM+Hg) for 24, 48 and 72 hours in comparison to control (0 hours).

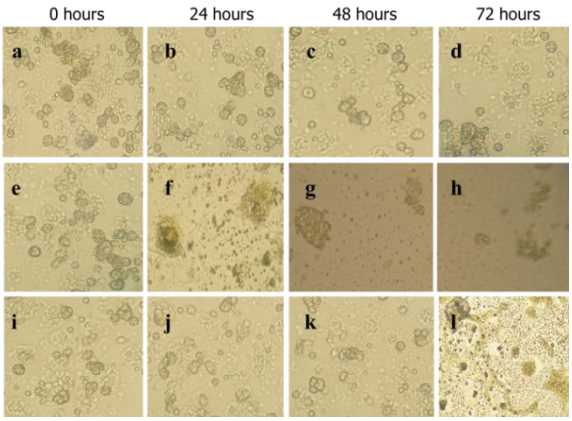

Figure 12. *Anguilla Anguilla* L. head kidney resident phagocytes morphology showing lamellopedia in response to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone (a-d for IONM; e-h for Hg) and concomitant exposure (i-l for IONM+Hg) for 24, 48 and 72 hours in comparison to control (0 hours).

4. Discussion

4.1. Peritoneum exudates phagocytes (P-phagocytes) responses

In the current study, increased P-phagocytes viability (2 and 8 hours) clearly indicates that no apparent cytotoxicity is perceptible for IONM suggesting that cells are metabolically active and resistant to IONM exposure. This result is consistent with other reports that nanomaterials do not show obvious acute lethal toxicity to cells within 24 hours of incubation (Lima et al., 2011; Semete et al., 2010). However, no change after 24, 48 and 72 hours may suggest that cell viability was uncompromised (100%) after 24 hours exposures to IONM. IONM can either bind to the external cell membrane or can be internalized into the cytoplasm. IONM that are bound externally do not affect cell viability, although, they may interfere with cell-surface interactions or may simply detach from the cell membrane (Bulte et al., 2004 a) However, since the current study includes phagocytes having property of engulfing and attacking particles that have been signaled for removal by various mechanisms in the host, the current viability increase may strengthens the explanation that IONM have been internalized within the cells with no deleterious effects on the P-phagocytes (Bulte et al., 2004 b). Phagocytosis process with nanomaterials has been very well document and is capable of internalizing particles up to 100 nm in size (Bartneck et al., 2010; Dobrovolskaia and McNeil, 2007). This means that the aggregates of NMs in an aquatic environment may be available for interaction with the hydro-biota immune system after their uptake by the organism.

Increase P-phagocytes activation (4, 8, 16 and 48 hours) was observed in the current study indicating that the IONM has the potential to induce innate immune response similar to that of the lower concentration of conventional metals, non-metals and industrial effluents in fish (Ahmad et al., 1998; 2003; 2004; Santos et al., 2006). Since IONM is characterized by both metals and organic compounds having known toxic potential, the stimulation of phagocytes may be attributed to the combination of different components of IONM because of their molecular size acting as elicitor of phagocytes. At the initial (2 hours) and end (72 hours) of the exposure, no response was observed; however, the decrease was observed at 24 hours. Thus, it is clear that since the decrease did not arise from a drop in the cells viability induction, phagocytic activity may be emerged or induced on the following exposure as is the case of the current study, supposedly as a consequence of the limited ingestion capacity arising from the interaction along the time. Moeller et al. (2012) described this phenomenon in humans with incubation of macrophages (100 nM SRL (rapamycin) nanomaterial (≈91 μg L^{-1})), which caused a reduction of phagocytosis by 10–20%.

Similarly, increase or decrease in viability, phagocytosis was observed for Hg exposure; however at different point of exposure duration. Thus a decrease in phagocytosis was observed after 2 hours of exposure to Hg; whereas, an increase in viability and phagocytosis index was perceptible after 16 hours. According to Sauvé et al. (2002), *in vitro* toxicity of Hg may exhibit: i) lack of cell cytotoxicity and stimulation of the phagocytosis; ii) lack of cell cytotoxicity and normal phagocytosis; iii) relatively low metal-related cytotoxicity and dramatic inhibition of phagocytosis; and iv) relatively high cytotoxicity accompanied by a drastic inhibition of the phagocytosis in bivalves. Thus, the previous explanation seems to be applicable in the current *in vitro* study on fish reflecting a specific interaction along the time producing synergistic and antagonistic effects in fish phagocytes. A decrease in phagocytic index and capacity may strengthen the explanation given above for the no induction in cells viability and phagocytosis modulation along the time. Moreover, a decrease in phagocytosis is well documented in the literature following either *in vivo* or *in vitro* Hg exposure (Fournier et al., 2001).

Under IONM and Hg concomitant exposure, a decrease in cell viability was observed only at 4 hours of exposure; thereafter an increase reaching normal at the end of exposures was perceptible. As observed in IONM and Hg alone, the decrease in viability

clearly indicates that the interaction of IONM with Hg can influence bioavailability/bio-accessibility as well as toxicity potential of either IONM and/or Hg. The adoption of the strategy of phagocytosis decrease with no viability increase was not applicable here since no index and capacity modulation was perceptible in the current study. However, compared to 2 hours of IONM+Hg exposure, significantly increased phagocytic capacity point towards a continuous existence of IONM+Hg immune response inducing potential along the time.

It is important to underline here that during the late hours of exposure (72 hours), though the viability and phagocytosis with IONM and Hg were at par with that of control but significantly induced trend was displayed by phagocytosis capacity with IONM+Hg when compared to Hg-exposed group. However during early hours of exposure (2 hours), the concomitant exposure was unable to mitigate the Hg-accrued negative impacts, which was evidenced by significantly decreased phagocytic capacity. These results imply the positive effect of the concomitant exposure (IONM+Hg), which significantly mitigated the said negative impacts of Hg at early hours. Hence, the occurrence antagonism between IONM and Hg cannot be ignored which is further evidenced as a principle of IONM-mediated Hg-IONM complex formation (Girginova et al., 2010; Tavares et al., 2013).

4.2. Gill-adhered phagocytes (G-phagocytes) responses

Phagocytes isolated from gill revealed similar patterns of response in terms of viability and phagocytosis which may be related to the similarities in the lineage (figure 10, 11 and 12) of these cells (Ahmad et al., 1998). It seems that the current observation on G-phagocytes viability increase after exposure to IONM is unique of its kind and specific to phagocytes only since the available reports are mostly based on the gill cells of non-phagocytic nature and show a reduction in cell viability in this perspective (He et al., 2012). In phagocytosis, no further increase after a period of exposure dependent decrease in phagocytic index (16 hours) and phagocytic capacity (24 hours) was observed strengthening the explanation as suggested above for the P-phagocytes that the decrease in phagocytosis did not arise from a drop in the cells viability induction, supposedly as a consequence of the limited ingestion capacity along the time.

On Hg exposure alone, a decrease in viability (8 hours) and increase phagocytosis (8 and 48 hours) was observed. Thus, an opposite trend is perceptible in G-phagocytes in

comparison to P-phagocytes suggesting that phagocytosis response is at the drop of viability decrease after Hg exposure. Since, Hg is known immunotoxic substance and its effect on phagocytic functions is well recognized (Brousseau et al., 2000), the immune response modulation observed in the current study seems obvious.

Under IONM and Hg concomitant exposure, an increase in cell viability was observed at 8 and 16 hours; thereafter, reaching to normal at the end of exposures. As observed in IONM and Hg alone, the increase in viability clearly indicates that the interaction of IONM with Hg can influence the toxicity potential of either IONM and/or Hg. The adoption of the strategy of phagocytosis decrease with no viability increase was not applicable here since both increase in viability and phagocytosis was in the current study. It is important to underline here that during the late hours of exposure (72 hours), though the viability and phagocytosis with IONM and Hg were at par with that of control but significantly decreased trend up to normal was displayed by phagocytic capacity with IONM+Hg when compared to Hg-exposed group. These results imply the positive effect of the concomitant exposure (IONM+Hg), which significantly mitigated the said negative impacts of Hg at late hours. Hence, the occurrence antagonism between IONM and Hg cannot be ignored which is further evidenced as a principle of IONM-mediated Hg-IONM complex formation (Girginova et al., 2010; Tavares et al., 2013).

4.3. Head kidney resident phagocytes (HK-phagocytes) responses

A. anguilla HK-phagocytes displayed a differential response of viability at the end of different periods of exposure to IONM. Though the extent of viability was significantly high at 8 hours of IONM exposure (compared to control), its decrease (16 hours) with increase in the period of exposure to IONM is also observed. This result is not in the agreement of the previous reports that nanomaterials do not show obvious acute lethal toxicity to cells within 24 hours of incubation (Lima et al., 2011; Semete et al., 2010) and may suggest a different origin of tissue resident phagocytes in comparison to those collected from peritoneum and gill. However, further increase in viability shows the ability of retaining metabolic activity of HK-phagocytes and their resistance to IONM exposure. In addition the explanation that viability decreases may be the cause of phagocytic activity decrease also not applicable in HK-phagocytes since no decrease in viability was observed at 24 hours despite of being a decrease in phagocytosis at 16 hours.

On Hg exposure alone, a decrease in viability (8 and 16 hours) and decrease in phagocytosis (2, 4, 8, 48 and 72 hours) was observed. The results are in agreement of the reports reflecting that mercuric compounds represents huge problem to immune and physiologic functions in fish (Low et al., 1998). Sarmento et al. (2004) showed a decrease at lower Hg chloride concentration in the head kidney macrophages integrity (apoptosis and necrosis) and functions (respiratory burst capacity and phagocytic activity).

Under IONM and Hg concomitant exposure, a decrease in cell viability was observed at 16 hours; thereafter, reaching to normal at the end of exposures. As observed in IONM and Hg alone, the viability decrease clearly indicates that the interaction of IONM with Hg induce the toxicity potential of either IONM and/or Hg. The adoption of the strategy of phagocytosis decrease with no viability increase is clearly applicable here since both parameters were decreased in the current study. It is important to underline here that during the late hours of exposure (72 hours), though the viability and phagocytosis with IONM and Hg were at par with that of control but significantly increasing trend up to normal was displayed by phagocytic index and capacity with IONM+Hg when compared to Hg-exposed group. These results imply the positive effect of the concomitant exposure (IONM+Hg), which significantly mitigated the said negative impacts of Hg at both early and late hours; thus, the occurrence of synergism between IONM and Hg cannot be ignored.

In fish, the trend of induction to the contaminant co-exposure IONM and Hg exhibited an increase and decrease pattern in viability and phagocytosis. Concerning, the present study an induction in viability (2, 8 and 16 hours), but when compared to IONM and Hg exposure alone; whereas a decrease after 8 hours, when compared to IONM exposure. On phagocytosis index an induction was revealed after 2 hours when compared to control. However, at different time interval the co-exposure group revealed a decrease (4 and 16 hours) when compared to IONM, whereas an increase after 48 hours. On co-exposure group an induction was observed after 4 hours, when compared to Hg exposure. On the other hand, on phagocytosis capacity after 2 and 72 hours an induction was observed for all parameters studied. This pattern was not similar with P and G-phagocytes. Moreover, a decrease was revealed after 4 and 16 hours, when compared to control. After 16 hours, in comparison to IONM a decrease was observed. The hypothesis to explain these results can be observed in the previous sections.

4.4. Spleen resident phagocytes (S-phagocytes) responses

A clear absence of S-phagocytes viability and decrease or increase in phagocytosis (phagocytic index and capacity) after exposure to IONM alone suggest that phagocytic response is not at the drop of viability increase and thus the modulation in phagocytosis responses according to the increase in exposure time may be obvious. Various reports reflect only increase in splenic cells phagocytic activity upon exposure to organic contaminants such as endosulfan (Christin et al., 2004; Tellez-Bañuelo et al., 2009). However, the current response on increase or decrease in phagocytosis of spleen phagocytosis should be directed towards the combination of both metal and dithiocarbamate of IONM material as a biomarker of exposure.

On Hg exposure alone, an increase in viability (16 and 24 hours) and increase in phagocytosis (4, 24 and 72 hours) was observed; however, not to the same exposure time indicating Hg is innate immune modulation capability in S-phagocytes. Moreover, Sauvé et al. (2002) revealed that Hg have the potential to induce stimulation in phagocytosis in fish.

Under IONM and Hg concomitant exposure, an increase in cell viability was observed at 8 hours; thereafter, reaching to normal in subsequent exposures periods. As observed in IONM and Hg alone, the increase in viability clearly indicates that the interaction of IONM with Hg can influence on the reduction of toxicity potential of either IONM and/or Hg as depicted by phagocytic activity. The adoption of the strategy of phagocytosis decrease with the drop in viability was not applicable here since phagocytosis modulation was there without change in viability. It is important to underline here that during the late hours of exposure (72 hours), though the phagocytosis with IONM and Hg were at par with that of control but significant modulation up to normal was displayed by phagocytic index and capacity with IONM+Hg when compared to Hg-exposed group. These results imply the positive effect of the concomitant exposure (IONM+Hg), which significantly mitigated the said negative impacts of Hg during early and late hours. Hence, the occurrence of antagonism between IONM and Hg at early hours and synergism at late hours cannot be ignored.

5. Conclusion

Major findings of the present investigation may be summarized in the following

points:

- *A. anguilla* phagocytes displayed a differential extent of immunotoxicity at the end of different periods of exposure to IONM, Hg or IONM+Hg, where a differential modulation in viability and phagocytosis was perceptible. In general, viability was induced only in P- and G-phagocytes, whereas an overall decrease in phagocytosis was observed in HK-phagocytes suggesting that the phagocytosis modulation was independent to the drop in viability in phagocytes;
- A period of exposure-dependency was exhibited by IONM alone and IONM+Hg joint exposures accrued impacts on *A. anguilla* phagocytes;
- IONM exposure alone lead to an acute response in terms of viability increase in P-phagocytes and modulated phagocytic activity in P- and S-phagocytes during 2 hours of exposure; whereas, IONM lead to a chronic immunotoxicity during 72 hours exposure only in S-phagocytes. However, IONM+Hg exposure lead to both acute and chronic response in terms of modulated phagocytic activity with no change in viability in P-, HK- and S-phagocytes only;
- Increase in the period of exposure to Hg disrupted phagocytic activity of P-, HK- and S-phagocytes, an increase in P- and decrease in HK- and S- phagocytes was perceptible at late hours of exposure;
- The occurrence of synergism between IONM and Hg was evidenced at 72 hours by significantly increasing trends of phagocytosis increase, which imply the positive effect of the concomitant exposure (IONM+Hg) against Hg-accrued negative impacts when compared to early hours of exposure (2 hours).

Overall, a fine-tuning among innate immune functions was revealed controlling IONM+Hg interactive impacts successfully.

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Chapter 2

The role of *Anguilla anguilla* L. phagocytes in reactive oxygen species production following *in vitro* exposure to silica coated iron oxide nanomaterial functionalized with dithiocarbamate with or without mercury co-exposure

Abstract

The study aimed to assess the immunotoxic effects of *in vitro* silica coated iron oxide nanomaterials (NMs) functionalized with dithiocarbamate (IONM) exposure and its interference with mercury (Hg) co-exposure on European eel (*Anguilla Anguilla* L.) phagocytes isolated from peritoneum (P-phagocytes), gill (G-phagocytes), head kidney (HK-phagocytes) and spleen (S-phagocytes). The phagocytes maintained in culture medium were used for the assessment of phagocytes oxidative burst activity (OBA) and lipid peroxidation (LPO), after exposure to IONM, Hg and IONM+Hg concomitantly for 0, 2, 4, 8, 16, 24, 48 and 72 hours. *A. anguilla* phagocytes displayed a differential extent of OBA and LPO induction at the end of different periods of exposure to IONM, Hg or IONM+Hg. Moreover, the OBA induction was concomitantly associated with LPO induction only in gill after exposure to Hg (8 and 48 hours) and IONM+Hg (8 hours). Observation of no reactive oxygen species (ROS) production (measured as OBA induction) and LPO increase or vice e versa, suggest that the innate immune modulation is well tuned with the phagocytes function responses in maintaining the overall cells tolerance. A period of exposure-dependency was exhibited by IONM alone and Hg joint exposures accrued impacts on *A. anguilla* phagocytes. Along the time it was observed that there is either no OBA induction (P- and S-phagocytes) or LPO, which may suggest that in the presence of lower ROS levels, adaptive mechanisms might have been activated, providing a better protection to IONM and Hg co-exposed phagocytes. At late hours of exposure, an induction was observed in G- phagocytes OBA after exposure to (IONM+Hg) suggesting that the concomitant exposure was unable to mitigate the Hg-accrued negative impacts. Moreover, the results imply that the negative effect of the concomitant exposure (IONM+Hg), significantly increased the said negative impacts of Hg at early hours. In addition, phagocytes isolated from gill and spleen was more vulnerable to the negative effect (LPO increase at 8 hours for G- and at 4, 24 hours for S-phagocytes) of the concomitant exposure (IONM+Hg) in comparison to the Hg exposure alone.

1. Introduction

Studies have shown that nanomaterials (NMs) may induce toxicity in different organs of different fish species (Garcia et al., 2011; Li et al., 2009). For example, Zhu et al. (2012) in Zebrafish (*Danio rerio*) reported that the accumulation of (α-Fe_2O_3)-NMs can

induce toxic mechanisms leading to an imbalance in homeostasis and aberrant cellular responses, including cytotoxicity, DNA damage, oxidative stress, epigenetic events, and inflammatory processes. Moreover, another study revealed that iron oxide nanomaterials located on the surface of *Escherichia coli* damaged the cell wall and outer membrane (He et al., 2011). However, to the authors, no report is available in terms of immune functions alteration taking considering IONM in particular.

Phagocytes or macrophages and tissue damage are important to recognize the self-defense mechanisms (biochemical and immunological) in fish to elucidate the effects of environmental contaminants on these vital processes (Luo et al., 2010; Von Bernhardi et al., 2010). Fish phagocytes form an important integral immunological defense network to neutralize invading pathogens and particulate material. Moreover, the induction of circulating fish phagocytes has been associated with peroxidative damage related to various industrial effluent exposures and their utility has been clearly demonstrated in water pollution monitoring programs (Ahmad et al., 1998, 2000, Fatima et al., 2001; Oikari and Niittyla, 1985).

The cell damage of the aquatic organisms (i.e, estuarine fish), including minor lesions, ulceration and necrosis, is ascribed mainly to the non-specific disturbance of cell membrane caused by the lipophilic xenobiotics (Ercal et al., 2001; Hoet et al., 2004). The cells aberrations may facilitate the invasion and growth of infectious agents (Moore et al., 2010). Moreover, the mechanism of toxic action of environmental chemicals is not usually thought to be restricted to one specific process, but rather multiple sites and mechanisms are involved.

Thus, taking into account the previous knowledge gaps on NMs toxicity mechanisms, the current study is aimed to assess i) how fish phagocytes respond to IONM exposure? Moreover, ii) is there any association between phagocytes responses and peroxidative damage in the exposed fish? In addition, since fish in the aquatic environment in general comes into contact with more contaminants including persistent contaminant such as Hg, the IONM co-exposure with Hg was also considered. Oxidative burst activity (OBA) was performed as a marker of reactive oxygen species production; whereas lipid peroxidation (LPO) was performed as a damage marker in fish phagocytes isolated from peritoneum (P-phagocytes), gill (G-phagocytes), head kidney (HK-phagocytes) and spleen (S-phagocytes) of *A.Anguilla* L.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), bradford reagent, butylated hydroxytoluene (BHT), diethylenetriaminepentaacetic acid (DTPA), dimethyl sulfoxide (DMSO), methanol, nitroblue tetrazolium (NBT), phosphate buffered saline (PBS), potassium hydroxide (KOH), RPMI-1640 medium tricloroacetic acid (TCA), trizma hydrochloride reagent grade (Tris-HCl), 2-thiobarbituric acid (TBA), zymosan used for this study were of analytical grade and were purchased from Sigma. The IONM, considered for this experiment, were kindly provided by Tavares et al. (2013) University of Aveiro, Portugal. The details pertaining to characterization of IONM can be obtained elsewhere (Tavares et al., 2013).

2.2. Equipment

- Centrifuge 5415 R from Eppendorf to centrifuge the samples;
- Environmental Shaker from Incubator ES-20 for shaking the samples;
- Spectra max – 384 Plus from Molecular devices.

2.3. Experimental design

The previously collected samples were processed for different antioxidant enzymes in peritoneal, gill, head kidney and spleen phagocytes. The experimental protocols, test animal, synthesis of IONM and statistical analysis for the chapter 2 were the same as described in chapter 1.

2.4. Oxidative burst activity (OBA) assay

The previously collected samples were processed for oxidative burst activity in peritoneal, gill, head kidney and spleen phagocytes according to the method of Fujiki and Yano. (1997) with some modifications as adopted by Ahmad et al. (2004). Briefly, an aliquot (20 µL) of phagocyte suspension was mixed with 100 µL PBS containing 0.1% NBT and zymosan suspension (1 mg mL^{-1}). The mixture was incubated at 25ºC for 2 hours with continuously shaking. After the incubation, the suspension was centrifuged at 3000 rpm for 10 minutes. The cell pallet was washed two times, first with 200 µL of PBS and then centrifuge at 3000 rpm during 10 minutes and the second with 70% methanol, air

dried at 60ºC for 15 minutes and suspended with 100 µL of 2M KOH and 10 µL of DMSO. The mixture was well suspended using the vortex for 30 s. After centrifugation at 3000 rpm for 3 minutes, the absorbance of supernatant was measured at 630 nm, which denotes the NBT reduction potential of phagocytes. The OBA was expressed as A_{630} nm 10^{-6} cells.

2.5. Estimation of lipid peroxidation (LPO)

LPO was determined in the previously prepared cells suspension. Briefly, to a 50 µL cell suspension, 500 µL of 12% TCA in aqueous solution, 450 µL of Tris-HCl buffer (60 mM, pH 7.4 and 0.1 mM DTPA) and 500 µL 0.73% TBA was added with a final volume 1.5 mL. The mixture was heated for 15 minutes in water bath set at boiling temperature. The eppendorfs were then removed and cooled to room temperature. The contents from each eppendorf (1.5 mL) were decanted into 2 mL microtubes and centrifuged at 12000 rpm for 3 minutes. To the absorbance of the plate was measured at 535 nm. The rate of LPO was expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed per milligram of protein using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

2.6. Protein concentration measurement

Total protein was determined according to the Bradford method (1976) using bovine serum albumin (BSA) as standard. The assay mixture contained 990 µL of Bradford reagent, 10 µL of diluted BSA (2.5 mg mL^{-1}) and 10 µL miliQ-water. The absorbance of plate was measured at 595 nm after incubate during 10 minutes in dark.

3. Results

Results depiction ‘given below’ has been considered describing first phagocytes inter-group variations within the same hours of exposure followed by inter-hours comparisons within the same group.

3.1. Peritoneum exudates phagocytes (P-phagocytes) responses

3.1.1. Inter-group comparisons

The results based on reactive oxygen species production has been depicted in figure 13. Effects of IONM exposure alone on *A. Anguilla* HK-phagocytes exhibited a significant increase only in oxidative burst activity (OBA) at 8 hours in comparison to control; whereas a decrease was perceptible only in LPO at 2 and 72 hours.

Effects of Hg exposure alone versus control revealed an increase in OBA at 8 hours; however, a significant decrease was observed in OBA at 24 hours and LPO at 16 hours.

Effects of IONM and Hg concomitant exposure on HK-phagocytes displayed only significant decrease in OBA at 48 hours and LPO at 2 and 72 hours when compared to control. Effects of IONM exposure alone versus concomitant exposure revealed a significant decrease in OBA at 8 hours and LPO at 2 and 72 hours. Concomitant effects comparison with Hg alone exhibited a decrease in OBA at 8 hours and LPO at 2 hours.

3.1.2. Inter-hours comparisons

Inter-hours comparisons revealed no statistically significant difference between the controls during the 72 hours exposure period. Effects of IONM *in vitro* exposure alone on G-phagocytes revealed an increase in OBA at 4 hours (vs. 2 hours) and 8 hours (vs. 2 and 4 hours) and LPO at 8 hours (vs. 2 and 4 hours); whereas, a decrease was displayed in OBA at 16 and 24 hours (vs. 4 and 8 hours) and 48 and 72 hours (vs. 8 hours) and LPO at 72 hours (vs. 2, 8 and 16 hours).

Concerning Hg exposed G-phagocytes, a significant increase was observed in OBA at 4 and 16 hours (vs. 2 hours) and 8 hours (vs. 2 and 4 hours) and LPO at 8 and 16 hours (vs. 2 hours); whereas, a decrease was perceptible in OBA at 24 hours (vs. 4, 8 and 16 hours) and LPO at 16 hours (vs. 2 and 8 hours) and 24 hours (vs. 8 hours).

G-phagocytes concomitantly exposed to IONM and Hg revealed a significant increase in LPO at 4, 8 and 72 hours (vs. 2 hours); whereas, a decrease was displayed in OBA at 48 hours (vs. 4 hours) and LPO at 16 and 72 hours (vs. 8 hours) and 48 hours (vs. 2, 8 and 16 hours).

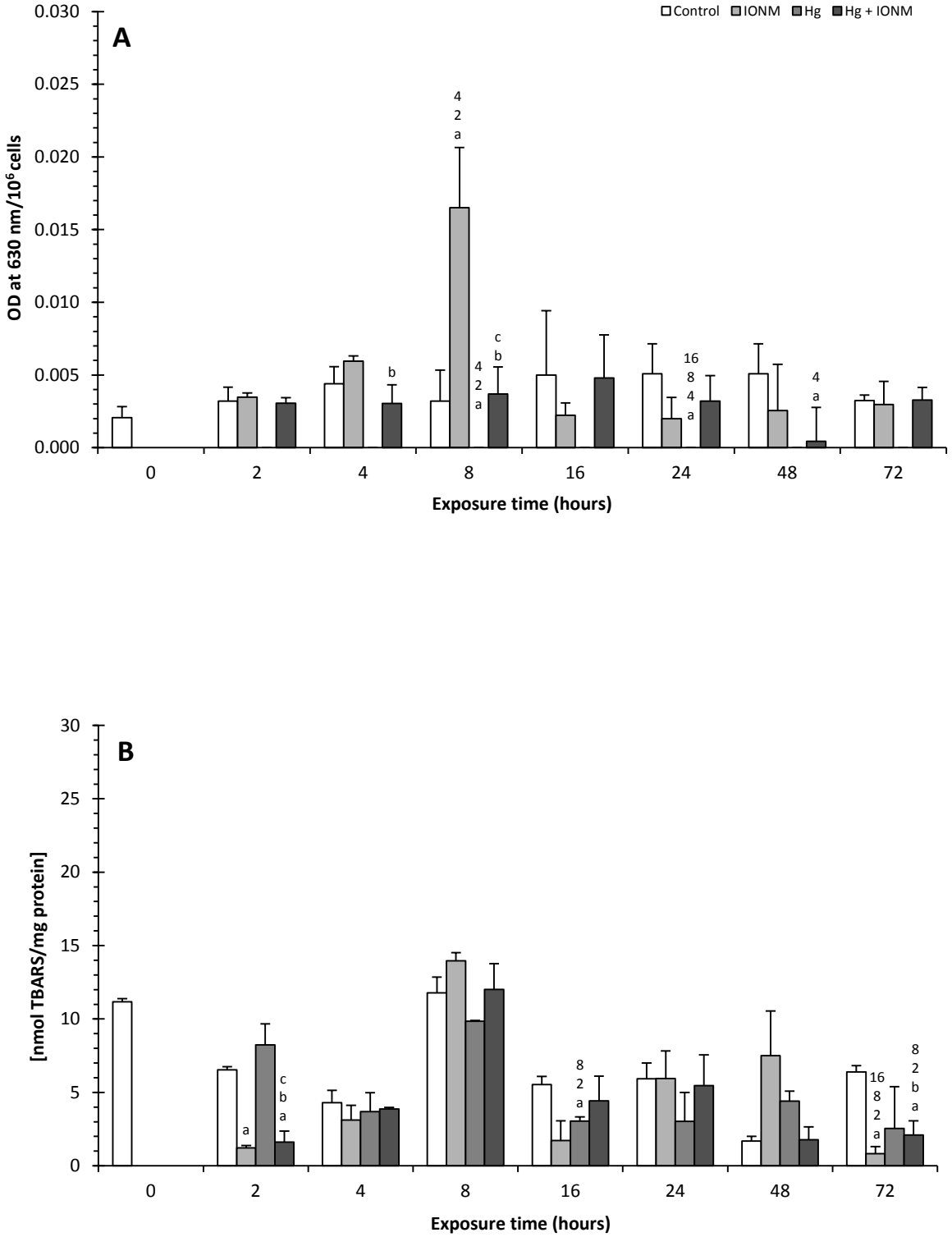

Figure 13. *Anguilla anguilla* L. *in vitro* peritoneum exudates phagocytes: oxidative burst activity (A) and lipid peroxidation (B) under exposure to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone or concomitantly for a period of 72 hours. Inter-group significant differences have been denoted by letters: a (vs. control), b (vs. IONM), c (vs. Hg) and inter-hours significant differences have been marked by numbers: 2 (vs. 2 hours), 4 (vs. 4 hours), 8 (vs. 8 hours), 16 (vs. 16 hours), 24 (vs. 24 hours) and 48 (vs. 48 hours).

3.2. Gill-adhered phagocytes (G-phagocytes) responses

3.2.1. Inter-group comparisons

The results based on reactive oxygen species production has been depicted in figure 14. Effects of IONM exposure alone on *A. Anguilla* G-phagocytes exhibited a significant increase in both oxidative burst activity (OBA) and LPO at 8 hours in comparison to control; whereas a decrease was perceptible only in LPO at 16 hours.

Effects of Hg exposure alone versus control revealed increase in OBA at 2, 8 and 48 hours, and LPO at 8 and 48 hours; however, a significant decrease was observed only in OBA at 4 hours.

Effects of IONM and Hg concomitant exposure on G-phagocytes exhibited a significant increase in OBA at 8 and 72 hours and LPO at 4 and 8 hours when compared to control. Effects of IONM exposure alone versus concomitant exposure revealed a significant increase in OBA at 72 hours and LPO at 4 and 8 hours. Concomitant effects comparison with Hg alone depicted an increase in OBA at 72 hours and LPO at 4 and 8 hours; whereas, a decrease was noted in LPO at 8 and 48 hours.

3.2.2. Inter- hours comparisons

Inter-hours comparisons revealed no statistically significant difference between the controls during the 72 hours exposure period. Effects of IONM *in vitro* exposure alone on G-phagocytes revealed an increase in OBA at 8 hours (vs. 2 and 4 hours), 24 hours (vs. 2 hours) and 72 hours (vs. 2 and 24 hours) and LPO at 8 hours (vs. 2 hours), 16 and 24 hours (vs. 2 and 4 hours); whereas, a decrease was displayed in OBA at 16 hours (vs. 8 hours), 24 hours (vs. 8 and 16 hours), 48 and 72 hours (vs. 8 hours) and LPO at 48 hours (vs. 8 hours).

Concerning Hg exposed G-phagocytes, a significant increase was observed in OBA at 8 hours (vs. 2 and 4 hours) and 24 hours (vs. 2 and 4 hours), and LPO at 8 hours (vs. 2 and 4 hours) and 16 hours (vs. 2 hours); whereas, a decrease was perceptible in OBA at 16 hours (vs. 8 hours), 24 hours (vs. 8 and 16 hours) and 72 hours (vs. 8 and 48 hours) and LPO at 16 hours (vs. 8 hours), 24 hours (vs. 8 hours) and 72 hours (vs. all exposure period). G-phagocytes concomitantly exposed to IONM and Hg revealed a significant increase in OBA at 8 hours (vs. 2 and 4 hours), 48 hours (vs. 2 ad 4 hours) and 72 hours

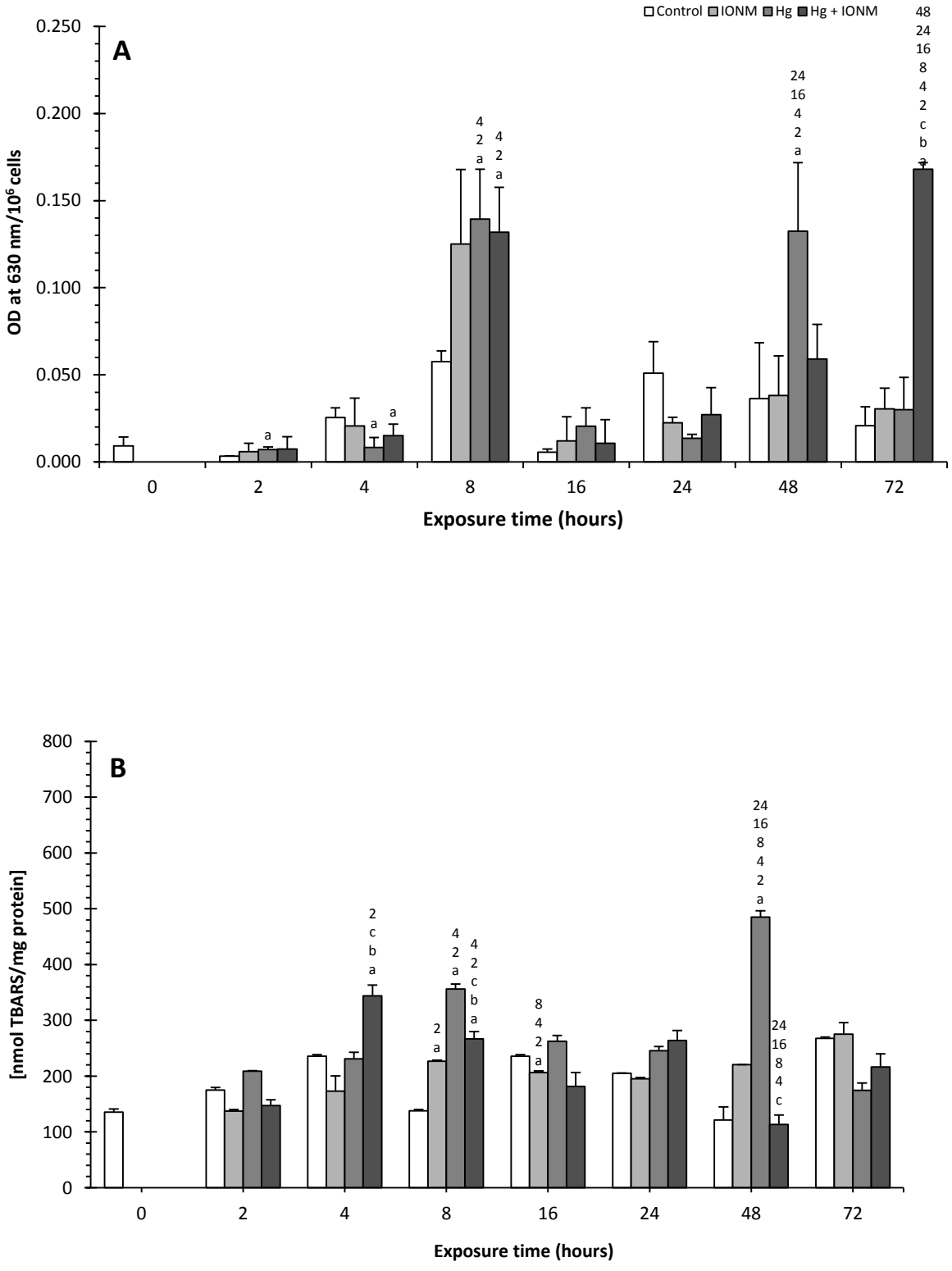

Figure 14. *Anguilla anguilla* L. *in vitro* gill-adhered phagocytes: oxidative burst activity (A) and lipid peroxidation (B) under exposure to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone or concomitantly for a period of 72 hours. Inter-group significant differences have been denoted by letters: a (vs. control), b (vs. IONM), c (vs. Hg) and inter-hours significant differences have been marked by numbers: 2 (vs. 2 hours), 4 (vs. 4 hours), 8 (vs. 8 hours), 16 (vs. 16 hours), 24 (vs. 24 hours) and 48 (vs. 48 hours).

(vs. all exposure period) and LPO at 4, 8 and 24 hours (vs. 2 hours); whereas, a decrease was observed in OBA at 16, 24 and 48 hours (vs. 8 hours) and LPO at 8 hours (vs. 4 hours), 24 hours (vs. 4 hours), 48 hours (vs. 8, 16 and 24 hours) and 72 hours (vs. 4, 8 and 48 hours).

3.3. Head kidney resident phagocytes (HK-phagocytes) responses

3.3.1. Inter-group comparisons

The results based on reactive oxygen species production has been depicted in figure 15. Effects of IONM exposure alone on *A. Anguilla*, HK-phagocytes exhibited only significant decrease in oxidative burst activity (OBA) at 4 hours and LPO at 16 and 24 hours in comparison to control.

Effects of Hg exposure alone versus control revealed a significant decrease was displayed only in LPO at 2, 4 and 8 hours.

Effects of IONM and Hg concomitant exposure on HK-phagocytes displayed significant increase in OBA at 4 and 8 hours and LPO at 2 hours when compared to control; whereas, a decrease was observed only in LPO at 4 and 16 hours. Effects of IONM exposure alone versus concomitant exposure revealed a significant increase only in OBA at 4 and 8 hours; whereas, a decreased was perceptible in LPO at 4, 16 and 48 hours. Concomitant effects comparison with Hg alone exhibited increase in OBA at 4 and 8 hours and LPO at 4 hours; whereas, a decrease was noted only in LPO at 4 hours.

3.3.2. Inter- hours comparisons

Inter-hours comparisons revealed no statistically significant difference between the controls during the 72 hours exposure period. Effects of IONM *in vitro* exposure alone on G-phagocytes revealed an increase in OBA at 72 hours (vs. 4 hours); whereas, a decrease was displayed in OBA at 2, 16, 24, 48 and 72 hours (vs. 2 hours) and LPO at 16 hours (vs. 2 and 4 hours), 24 hours (vs. 4 hours) and 48 hours (vs. 2, 4, 8, 16 and 24 hours).

Concerning Hg exposed G-phagocytes, a significant increase was observed in OBA at 72 hours (vs. 48 hours) and LPO at 4 hours (vs. 2 hours), 8 hours (vs. 2 hours) and 24 hours (vs. 2, 4 and 8 hours), 48 hours (vs. 2 and 8 hours); whereas, a decrease was perceptible in OBA at 4 and 8 hours (vs. 2 hours) and LPO at 4 hours (vs. 2 hours), 8 hours (vs. 4 hours), 48 hours (vs. 4, 16 and 24 hours).

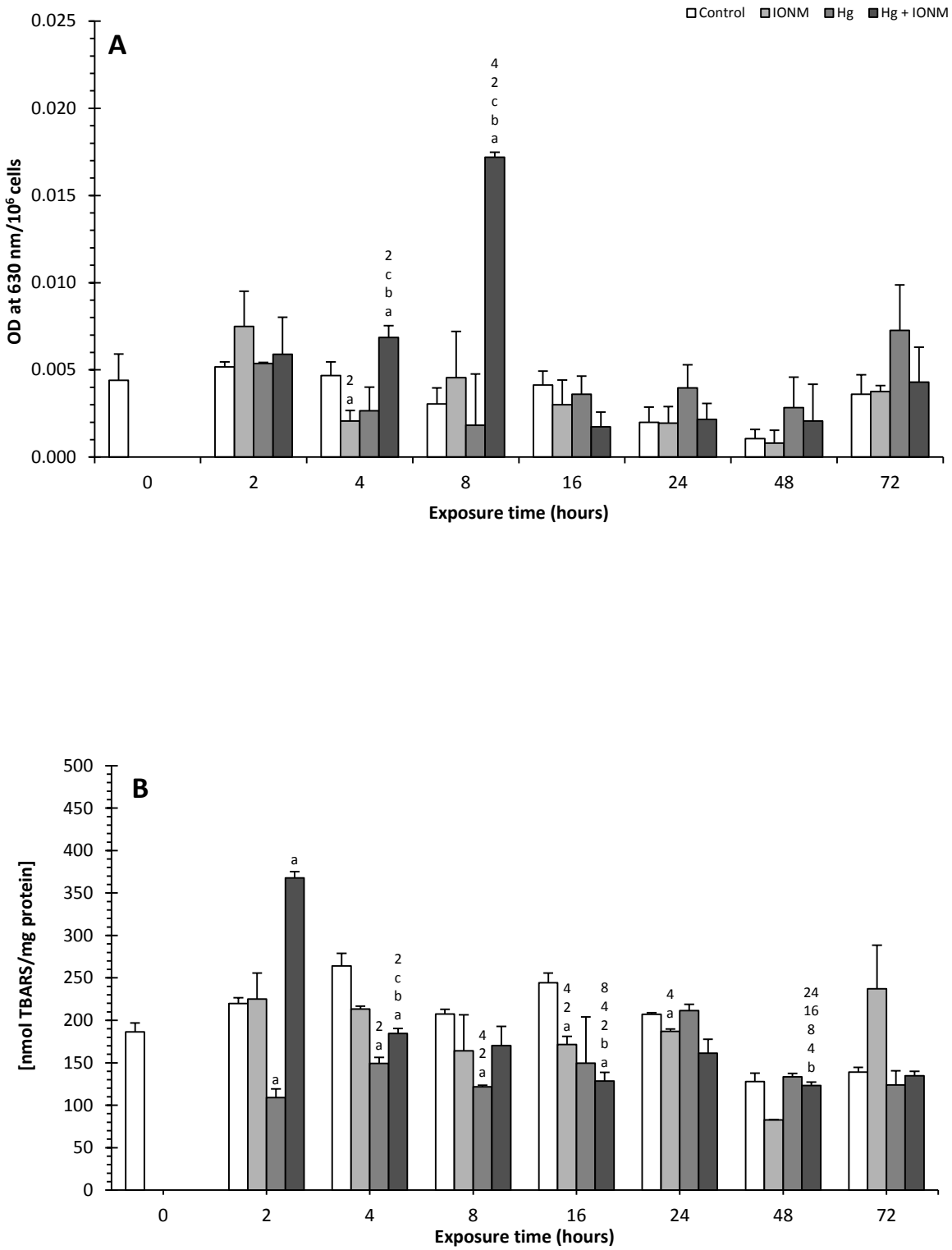

Figure 15. *Anguilla anguilla* L. *in vitro* head kidney resident: oxidative burst activity (A) and lipid peroxidation (B) under exposure to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone or concomitantly for a period of 72 hours. Inter-group significant differences have been denoted by letters: a (vs. control), b (vs. IONM), c (vs. Hg) and inter-hours significant differences have been marked by numbers: 2 (vs. 2 hours), 4 (vs. 4 hours), 8 (vs. 8 hours), 16 (vs. 16 hours), 24 (vs. 24 hours) and 48 (vs. 48 hours).

G-phagocytes concomitantly exposed to IONM and Hg revealed a significant increase in OBA at 4 hours (vs. 2 hours), 8 hours (vs. 2 and 4 hours) and 72 hours (vs. 48 hours) and LPO at 24 hours (vs. 16 hours); whereas, a decrease was observed in OBA at 16 and 24 hours (vs. 2, 4 and 8 hours), 48 hours (vs. 2 and 8 hours) and 72 hours (vs. 8 hours) and LPO at 4 hours (vs. 2 hours), 8 hours (vs. 2 hours), 16 hours (vs. 2and 4 hours) 24 hours (vs. 2 and 4 hours), 48 hours (vs. 4, 8, 16 and 24 hours) and 72 hours (vs. 2, 4, 8 and 24 hours).

3.4. Spleen resident phagocytes (S-phagocytes) responses

3.4.1. Inter-group comparisons

The results based on reactive oxygen species production has been depicted in figure 16. Effects of IONM exposure alone on *A. Anguilla* HK-phagocytes exhibited significant increase only in LPO at 4 and 72 hours; whereas, a significant decrease was displayed in OBA at 2, 4 and 72 hours and LPO at 2 and 8 hours in comparison to control.

Effects of Hg exposure alone versus control revealed a significant increase only in LPO at 24 and 72 hours; whereas, a significant decrease was displayed only in OBA at 4 and 16 hours.

Effects of IONM and Hg concomitant exposure on HK-phagocytes displayed significant increase only in LPO at 4 and 24 hours; whereas, a significant decrease was observed only in OBA at 2, 4 16 and 24 hours when compared to control. Effects of IONM exposure alone versus concomitant exposure revealed a significant increase in OBA only at 4 hours and LPO at 4 and 8 hours; whereas, a decreased was perceptible in LPO at 16 hours. Concomitant effects comparison with Hg alone exhibited only decrease in LPO 16 and 24 hours.

3.4.2. Inter- hours comparisons

Inter-hours comparisons revealed no statistically significant difference between the controls during the 72 hours exposure period. Effects of IONM *in vitro* exposure alone on G-phagocytes revealed an increase in OBA at 16, 24 and 72 hours (vs. 4 hours) and LPO only at 4 hours (vs. 2 hours); whereas, a decrease was displayed in OBA at 8 hours (vs. 2 and 4 hours) and 48 hours (vs. 4 and 16 hours) and LPO at 8 hours (vs. 2 and 4 hours), 16

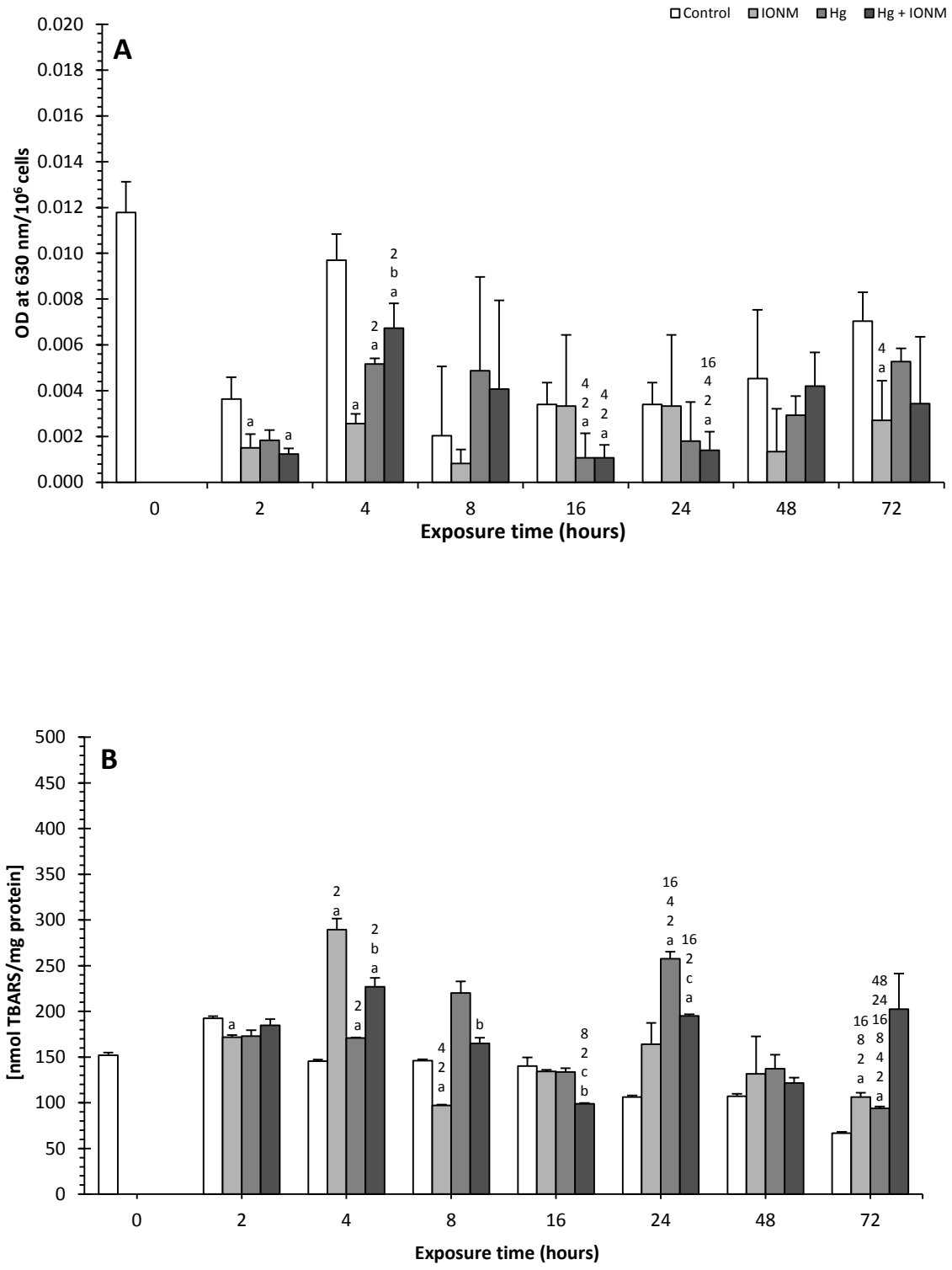

Figure 16. *Anguilla anguilla* L. *in vitro* spleen resident phagocytes: oxidative burst activity (A) and lipid peroxidation (B) under exposure to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone or concomitantly for a period of 72 hours. Inter-group significant differences have been denoted by letters: a (vs. control), b (vs. IONM), c (vs. Hg) and inter-hours significant differences have been marked by numbers: 2 (vs. 2 hours), 4 (vs. 4 hours), 8 (vs. 8 hours), 16 (vs. 16 hours), 24 (vs. 24 hours) and 48 (vs. 48 hours).

hours (vs. 2 and 4 hours), 24 and 48 hours (vs. 4 hours) and 72 hours (vs. 2, 8 and 16 hours).

Concerning Hg exposed G-phagocytes, a significant increase was observed in OBA at 4 hours (vs. 2 hours), 16 hours (vs. 4 hours) and 72 hours (vs. 2, 16, 24 and 48 hours) and LPO at 24 hours (vs. 2 and 16 hours); whereas, a decrease was perceptible in OBA at 16 hours (vs. 4 hours), 24 and 48 hours (vs. 4 hours) and 72 hours (vs. 4 hours) and LPO at 4 hours (vs. 2 hours), 16 hours (vs. 2, 4 and 8 hours), 48 hours (vs. 8 and 24 hours) and 72 hours (vs. all exposure period).

G-phagocytes concomitantly exposed to IONM and Hg revealed a significant increase in OBA at 4 hours (vs. 2 hours) and 48 hours (vs. 16 hours) and LPO at 2 hours (vs. 2 hours), 24 hours (vs. 2 and 16 hours) and 72 hours (vs. 8 hours); whereas, a decrease was observed in OBA at 16 and 24 hours (vs. 2 and 4 hours), 48 and 72 hours (vs. 4 hours), and LPO only at 16 hours (vs. 2 and 8 hours).

4. Discussion

4.1. Peritoneum exudates phagocytes (P-phagocytes) responses

As a cornerstone of the innate immune response, phagocytes actively seek out, ingest, and destroy pathogenic microorganisms (Bowser et al., 1994; Filho et al., 1992; Novoa et al., 1996; Zelikoff et al., 1991, 1993). To achieve this essential role in host defense, phagocytes deploy a potent arsenal that includes oxidants having a dual function (Niazi et al., 2009). On one hand, they function as potent antimicrobial agents by virtue of their ability to kill microbial pathogens directly. On the other hand, they participate as signalling molecules that regulate diverse physiological signalling pathways in phagocytes. Increased P-phagocytes activation as reported in the previous chapter is clearly associated to the excess production of ROS to the same exposure time (8 hours).The results are in accordance of Zhu et al. (2012) who observed that the adherence and/or adsorption of iron oxides nanomaterials aggregates may cause excessive production of reactive oxygen species resulting in oxidative stress in fish. No association of phagocytic induction with ROS production may be directed to the change in oxidation state of iron oxides nanomaterials and protein-iron oxides nanomaterials interaction as suggested by Berry et al. (2003, 2004) and Mahmoudi et al. (2009, 2010). With the increase in exposure time, the modulation of phagocytes induction is perceptible reaching the value of control at the end

of exposure time. Concerning, the previous findings they are corroborating with the observation of no ROS production (except 8 hours) and no LPO increase, suggesting that the innate immune modulation is well tuned with the phagocytes function responses in maintaining the overall cells tolerance. Iron oxides nanomaterials (Magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3)) can reveal different cellular responses because of their ability to experience oxidation/reduction reactions. For example, Fe_3O_4 has been shown to cause higher levels of oxidative damage in A549 human lung epithelial cell line in the absence of decreased cell viability as compared to γ-Fe_2O_3 remaining to its potential to undergo oxidation (Karlsson et al., 2008, 2009). Moreover, Singh et al. (2010) hypothesised that the toxicity of iron oxides nanomaterials can may be decreased by coating magnetite particles resulting in fewer oxidative sites that are less reactive and thereby produce less macromolecule damage (Mahmoudi et al., 2009).

Similar to the response of IONM, Hg exposed phagocytes reflected increased OBA (at 8 hours) and no LPO suggesting the same kind of phagocytes response towards Hg maintaining the cells viability. Phagocytic induction induced by IONM alone at 4, 8, 16 and 48 hours reached to the normal level with Hg co-exposure. OBA increase at 8 hours, also went to normal with Hg co-exposure to the same exposure length. The occurrence of no OBA induction and either LPO decrease or no LPO along the time can suggest that in the presence of lower ROS levels, adaptive mechanisms might have been activated, providing a better protection to IONM and Hg co-exposed phagocytes.

4.2. Gill-adhered phagocytes (G-phagocytes) responses

In the current study, statistically insignificant OBA induction during the entire exposures length (more pronounced at 8 hours) was observed. However, a significant LPO increase was perceptible at 8 hours as clear indication of the phagocytic induction and its association to peroxidative damage. Thus, the current observation strengthens the explanation that both gill and peritoneum phagocytes have the same lineage (figure 10 and 11).

On the perspective of Hg exposure, we found that Hg exposure has a deleterious membrane-damaging effect on G-phagocytes since after 2, 8 and 48 hours, the LPO response was significantly increased in gill. Moreover, a significant increase was also perceptible in LPO to the same exposure length (insignificant at 2 hours) indicating the

peroxidative damage as a cause of ROS production. Early reports have shown that the release of large amounts of ROS by activated phagocytes and its association with peroxidative damage may become a potential cause of these differences in antioxidants besides the direct oxidative damage inflicted by the Hg (Ahmad et al., 2003, 2004).

Under IONM and Hg concomitant exposure, a decrease OBA was observed only at 4 hours of exposure; thereafter an increase at 8 and 72 hours was perceptible. As observed in IONM and Hg alone, the increase in OBA over time (72 hours) clearly indicates the interaction of IONM with Hg and its influence on bioavailability/bioaccessibility as well as toxicity potential of either IONM and/or Hg. The adoption of the strategy of OBA increase with increase in LPO was applicable only at 8 hours. The occurrence of OBA induction at 72 hours with no LPO along the time can suggest that in the presence of higher ROS levels, adaptive mechanisms might have been activated, providing a better protection to IONM and Hg co-exposed phagocytes. It is important to underline here that during the late hours (72 hours) the concomitant exposure was unable to mitigate the Hg-accrued negative impacts, which was evidenced by significantly increased OBA. These results imply the negative effect of the concomitant exposure (IONM+Hg), which significantly increased the said negative impacts of Hg at early hours. Hence, the occurrence synergism between IONM and Hg cannot be ignored.

4.3. Head kidney resident phagocytes (HK-phagocytes) responses

In the current study, HK-phagocytes exposed to IONM and Hg reflected a refusal to go along with increased OBA and LPO induction. Moreover, a significant decrease was also observed at 4 hours (OBA), 16 hours (LPO) and 24 hours (LPO). Though, Ahmad et al. (2003) and He et al. (2011) observed HK-phagocytes OBA increase to naphthalene and iron oxide nanomaterial exposure, our results are in contradiction with the previous findings on IONM. Taking into account the observation of phagocytic activity decrease in response to IONM and Hg exposure to the same time (Chapter 1), the current seems obvious. However, under IONM and Hg concomitant exposure, an increase in OBA (4 and 8 hours) and LPO (2 hours) clearly indicates that the interaction of IONM with Hg induce the toxicity potential of either IONM and/or Hg. The adoption of the strategy of phagocytic activity increase and its association to LPO was depicted only at 2 hours probably due to interaction of IONM and Hg producing antagonistic and synergistic effects. It is important

to underline here that during the late hours of exposure (72 hours), though the OBA and LPO with IONM and Hg were at par with that of control no significant trend was displayed compared to early hours strengthening the explanation that cells did not compromise at late hours of exposure.

4.4. Spleen resident phagocytes (S-phagocytes) responses

In the current study, no significant induction, in IONM exposure, in S-phagocytes OBA to IONM exposure was observed, but a significant LPO induction was perceptible at 4 and 72 hours. The adoption of the strategy of phagocytic activity increase and its association to LPO was clearly depicted for 4 and 72 hours of exposure. Similarly, Hg alone exposure revealed an increase in phagocytes induction and its association to LPO at 4, 24 and 72 hours. Phagocytosis phenomenon produces ROS, having high chemical reactivity of radicals and their intermediates where various components in the organism constantly undergo chemical changes in a more or less random manner. Thus, the intensity of these processes in the organism is controlled by a variety of radicals and their intermediates that can neutralize the produced ROS or prevent their formation (Margarita et al., 2009).

Under IONM and Hg concomitant exposure, decrease in OBA at all the exposure time intervals with no association to LPO (except at 4, 8 and 24 hours) indicate S-phagocytes incapability to induce ROS due to a high concentration of IONM component specific to ROS inhibition. However, increase in LPO may be suggested to the increased stimulation of phagocytes to the same exposure time. As observed in IONM and Hg alone, significant increase in OBA (4 hours) and less increased LPO compared to IONM (4 hours) and Hg (16 hours) alone clearly indicates that the interaction of IONM with Hg induce the toxicity potential of either IONM and/or Hg. It is important to underline here that during the late hours of exposure (72 hours), though the OBA and LPO with IONM and Hg were at par with that of control but significantly decreasing trend up to normal was displayed by LPO with IONM+Hg when compared to Hg-exposed group. These results imply the negative effect of the concomitant exposure (IONM+Hg), which significantly enhanced the said negative impacts of Hg at early hours (4 hours) and mitigated the negative response of the Hg at late hours (72 hours); thus, the occurrence of both synergism (4 hours) and antagonism (72 hours) between IONM and Hg cannot be ignored.

5. Conclusion

Major findings of the present investigation may be summarized in the following points:

- *A. anguilla* phagocytes displayed a differential extent of ROS production and LPO induction at the end of different periods of exposure to IONM, Hg or IONM+Hg. Moreover, the OBA induction was concomitantly associated with LPO induction only in gill after exposure to Hg (8 and 48 hours) and IONM+Hg (8 hours). Observation of no ROS production and LPO increase or viz., suggest that the innate immune modulation is well tuned with the phagocytes function responses in maintaining the overall cells tolerance.
- A period of exposure-dependency was exhibited by IONM alone and Hg joint exposures accrued impacts on *A. anguilla* phagocytes.
- Along the time it was observed that there is either no OBA induction (P- and S-phagocytes) or LPO which may suggest that in the presence of lower ROS levels, adaptive mechanisms might have been activated, providing a better protection to IONM and Hg co-exposed phagocytes.
- At late hours of exposure, an induction was observed in G- phagocytes OBA after exposure to (IONM+Hg) suggesting that the concomitant exposure was unable to mitigate the Hg-accrued negative impacts. Moreover, the results imply that the negative effect of the concomitant exposure (IONM+Hg), significantly increased the said negative impacts of Hg at early hours. Moreover, phagocytes isolated from gill and spleen was more vulnerable to the negative effect (LPO increase at 8 hours for G- and at 4, 24 hours for S-phagocytes) of the concomitant exposure (IONM+Hg) in comparison to the Hg exposure alone.

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Chapter 3

Anguilla anguilla L. phagocytes enzymatic antioxidants responses following *in vitro* exposure to silica coated iron oxide nanomaterial functionalized with dithiocarbamate with or without mercury co-exposure

Abstract

The study aimed to assess the effects of *in vitro* silica coated iron oxide nanomaterials functionalized with dithiocarbamate (IONM) exposure on enzymatic antioxidant responses and its interference with mercury (Hg) co-exposure on European eel (*Anguilla Anguilla* L.) phagocytes isolated from peritoneum (P-phagocytes), gill (G-phagocytes), head kidney (HK-phagocytes) and spleen (S-phagocytes). The phagocytes maintained in culture medium were used for the assessment of catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione S-transferase (GST) after exposure to IONM, Hg and IONM+Hg concomitantly for 0, 2, 4, 8, 16, 24, 48 and 72 hours. *A. anguilla* phagocytes displayed a differential extent of antioxidants enzymes induction at the end of different periods of exposure to IONM, Hg or IONM+Hg. Under IONM alone exposure, enzymatic antioxidants protection responses displayed hours of exposure dependency in G- and S-phagocytes where, compared to control, an insufficiency of elevated CAT, GPX, GST, non-protein thiols (NP-SH) and total glutathione (TGSH) was clearly depicted for the maintenance of pro- and antioxidant balance optimum for scavenging reactive oxygen species (ROS) and protecting membrane lipids against IONM induced lipid peroxidation (LPO). The adoption of the strategy of enzymatic antioxidant modulation in *A. anguilla* G-phagocytes at 4 hours was reflected by significantly decrease in CAT and increase in GR, GPX and GST; whereas at 8 hours, the significantly decrease in CAT and GPX and increase in GR and GST was perceptible. The strategy adopted for HK-phagocytes at 2 hours was reflected by decrease in all the studied enzymes. In addition, strategies adopted by S-phagocytes at 24 hours were reflected by an increase in CAT, GR, GST and NP-SH. These responses together, point towards the enzymatic antioxidants defense failure for the protection of membrane lipids during those periods of exposure to IONM+Hg. However, it is important to underline here that during the late hours of exposure (72 hours) the results imply the positive effect of the IONM+Hg exposure, which significantly mitigated the said negative impacts of Hg. Hence, the occurrence antagonism between IONM and Hg cannot be overlooked.

1. Introduction

The iron's ability to catalyse the formation of radicals via the well-known Fenton reaction is well known in fish. Catalytic amounts of iron are sufficient to yield hydroxyl radicals ($OH^{\cdot}$) from superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) and reactive oxygen

intermediates (e.g ROS). Free radicals are highly reactive species, which can affect peroxidation of membrane lipids, modification of nucleic acids, and eventually cause cell death and tissue injury (Müller et al., 2011; Papanikolaou et al., 2005). Various *in vitro* and *in vivo* toxicity of zero-valent iron showed oxidative damage by redox cycling of iron and ROS generation, thus leading to tissue injury and cell death (Chen et al., 2010; Kadar et al., 2010; Li et al., 2009; Phenrat et al., 2009).

Fish also possess defense mechanisms to neutralize the impact of ROS (Alexander et al., 1992; Ellis, 2001). The overall enhancement or reduction of contaminant-induced toxicity greatly depends on the imbalance between pro-oxidant and antioxidant status as a result of xenobiotic interferences. Hence, enhanced contaminant oxygenation rates may increase toxicity via oxidative stress, rendering fish antioxidants less effective. In this perspective, a number of studies confirmed the successful employment of antioxidant enzymes modulation in identifying environmental stress (Regoli et al., 1998; Ahmad et al., 2000, 2004, 2005; Livingstone, 2001; Santos et al., 2004). These defense mechanisms have been viewed by some as an adaptive propensity. However, on the perspective of IONM, the information is meagre. IONM is silica coated iron oxide nanomaterial combined with dithiocarbamate with high oxidant potential. Our previous studies on immune cells have established that IONM can activate fish phagocytes (Ahmad et al., 2004; Santos et al., 2006). It has been shown that over-activation of phagocytes could be associated with the release of reactive oxygen species contributing to the overall oxidative stress in fish phagocytes. Additionally, we have observed a differential response of LPO in various phagocytes isolated from peritoneum, gill, head kidney and spleen. Thus, the current study was focussed on the assessment of various enzymatic antioxidants in fish (*A. anguilla* L.) in order to find out possible causes of such phagocytes response differences.

Antioxidant enzymes viz. CAT, GPX, GR and GST were studied in various phagocytes of fish exposed to IONM for short and long durations. Moreover, since fish in the aquatic environment in general comes into contact with more contaminants including persistent contaminant such as Hg, the IONM co-exposure with Hg was also considered. It may be postulated that nanomaterial induced phagocytes antioxidant responses may be further modulated by the concomitant co-exposure to other contaminant resulting reduced/ or augmented protection against pathogens and infectious diseases.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), Bradford reagent, 1-chloro-2,4-dinitrobenzene (CDNB), diethylene triamine pentaacetic acid (DTPA), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA/Na_2), glutathione reductase from Baker's Yeast, hydrogen peroxide 30% from Merck, L-glutathione oxidized (GSSG), L-Glutathione reduced (GSH), nicotinamide adenine dinucleotide phosphate (NADPH), potassium phosphate dibasic (K_2KPO_4), potassium phosphate monobasic (KH_2PO_4) and sodium azide (NaN_3) (analytical grade) were purchased from Sigma. Other routine chemicals and reagents (analytical grade) were purchased from local sources. The IONM, considered for this experiment, were kindly provided by Tavares et al. (2013) University of Aveiro, Portugal.

2.2. Equipment

- Centrifuge 5415 R from Eppendorf to centrifuge the samples;
- Environmental Shaker from Incubator ES-20 for shaking the samples;
- Spectra max – 384 Plus from Molecular devices.
- Analytic balance E425 from Gibertini
- pH meter 720 from WTW

2.3. Experimental design

The previously collected samples were processed for different antioxidant enzymes in peritoneal, gill, head kidney and spleen phagocytes. The experimental protocols, test animal, synthesis of IONM and statistical analysis for the chapter 3 were the same as described in chapter 1.

2.4. Enzymatic antioxidants

2.4.1. Catalase (CAT) activity measurement

CAT activity was assayed according to the methods adopted by Ahmad et al. (2011) with little modifications. Reaction mixture consisted of 100 µL phosphate buffer (0.05 M, pH 7.0), 50 µL H_2O_2 (50 mM) and 10 µL of sample in a final volume of 160 µL.

The absorbance of each plate was measured at 240 nm, during 3 minutes. CAT activity was calculated in terms of μmol H_2O_2 consumed $min^{-1}mg^{-1}$ protein using a molar extinction coefficient of 43.5 $M^{-1}cm^{-1}$.

2.4.2. Glutathione peroxidase (GPX) activity measurement

GPX activity was assayed according to the methods adopted by Ahmad et al. (2011) with little modifications. Briefly, reaction mixture consisted of 60 μL of phosphate buffer (0.1 M, pH 7.0), 20 μL of sample, 20 μL of GR (2.40 U mL^{-1}), 20 μL of GSH (10 mM), 20 μL sodium azide (1 mM), 20 μL EDTA/Na_2 (10 mM), 20 μL of NADPH, 20 μL of H_2O_2 (1.5 mM) in total volume of 200 μL. Oxidation of NADPH was recorded spectrophotometrically at 340 nm during 30 minutes with 30 seconds time interval, at room temperature. The enzyme activity was calculated as nmol NADPH oxidized min^{-1} mg^{-1} protein using a molar extinction coefficient of 6.22 x 10^3 $M^{-1}cm^{-1}$.

2.4.3. Glutathione reductase (GR) activity measurement

GR activity was assayed in sample according to the method described by Mohandas et al. (1984) with some modifications. Briefly, the assay mixture contained 0.0086 g of NADPH, 0.0327 g of GSSG (oxidized glutathione), 0.0098 g DTPA and then dissolved in 25 mL of distilled water. The final volume was made 50 mL by adding 25 mL of phosphate buffer (0.05 M). Then 150 μL of reaction mixture and 10 μL of sample were mixed during some minutes. The absorbance of each plate was measured at 340 nm during 3 minutes with 30 seconds time interval. The enzyme activity was calculated as nmol NADPH oxidized/min/mg protein using a molar extinction coefficient of 6.22 x 10^3 M^{-1} cm^{-1}.

2.4.4. Glutathione S-transferase (GST) activity measurement

GST activity was assayed in sample using the method of Habig et al. (1974) with some modifications. The reaction mixture consisting 125 μL buffer (before adding the buffer was kept in the hot water bath at 30°C), 10 μL sample, 8 μL reduced glutathione (20 mM) and 8 μL CDNB (20 mM) in total volume of 151 μL was assayed. The change in absorbance was recorded at 340 nm and the enzyme activity calculated as mmol CDNB conjugate formed min^{-1} mg^{-1} protein using a molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

3. Results

Results depiction 'given below' has been considered describing first phagocytes inter-group variations within the same hours of exposure followed by inter-hours comparisons within the same group.

3.1. Peritoneum exudates phagocytes (P-phagocytes) responses

3.1.1. Inter-group comparisons

The results based on enzymatic antioxidants responses have been depicted in figure 17. *A. anguilla* P-phagocytes *in vitro* exposure to IONM alone in comparison to control revealed a significant increase in CAT at 4, 16 and 24 hours, GPX at 4, 16 and 24 hours, GR at 2, 4, 8 and 72 hours, GST at 2, 4 and 24 hours; whereas a decrease in CAT at 2, 8 and 48 hours, GPX at 2, 48 and 72 hours, GR at 24 and 48 hours, and GST at 48 hours.

Effects of Hg exposure alone versus control on P-phagocytes revealed an increase in CAT at 4, 16, 24 and 48 hours, GPX at 4 and 16 hours, GST at 2, 4, 8, 24 and 72 hours; whereas a decrease only in GPX at 2 and 8 hours.

Effects of IONM and Hg concomitant exposure on P-phagocytes displayed a significant increase in CAT at 4, 8, 16, 24, 48 and 72 hours; GPX at 4 and 16 hours, GR at 2, 8 and 72 hours and GST at 2, 4, 16 and 24 hours; whereas a decrease in CAT at 2 hours, GPX at 2, 8 and 48 hours, GR at 4, 16, 24 and 48 hours. Effects of IONM exposure alone versus concomitant exposure revealed a significant increase in CAT at 2, 4, 8, 16 and 48 hours, GPX at 4, 48 and 72 hours, GR at 2, 8 and 72 hours and GST at 16, 24, 48 and 72 hours; whereas a decrease in CAT at 24 hours, GPX at 2, 8, 16 and 24 hours, GR at 4 and 48 hours. Concomitant effects comparison with Hg alone exhibited an increase in CAT at 4, 8, 16, 48 hours, GPX at 4 hours, GR at 2, 4, 8 and 72 hours and GST at 24 hours; whereas a decrease in CAT at 2 hours, GPX at 2 and 48 hours, GR at 16, 24 and 48 hours, GST at 8 and 72 hours.

3.1.2. Inter-hours comparisons

Inter-hours comparisons revealed no statistically significant difference between the controls during the 72 hours exposure period. Effects of IONM exposure on P-phagocytes revealed a significant increase in CAT at 8 hours (vs. 2 and 4 hours), 16 hours (vs. 2, 4 and 8 hours), 24 hours (vs. 2, 4, 8 and 16 hours), GPX at 4 and 16 hours (vs. 2 hours), GR at 48

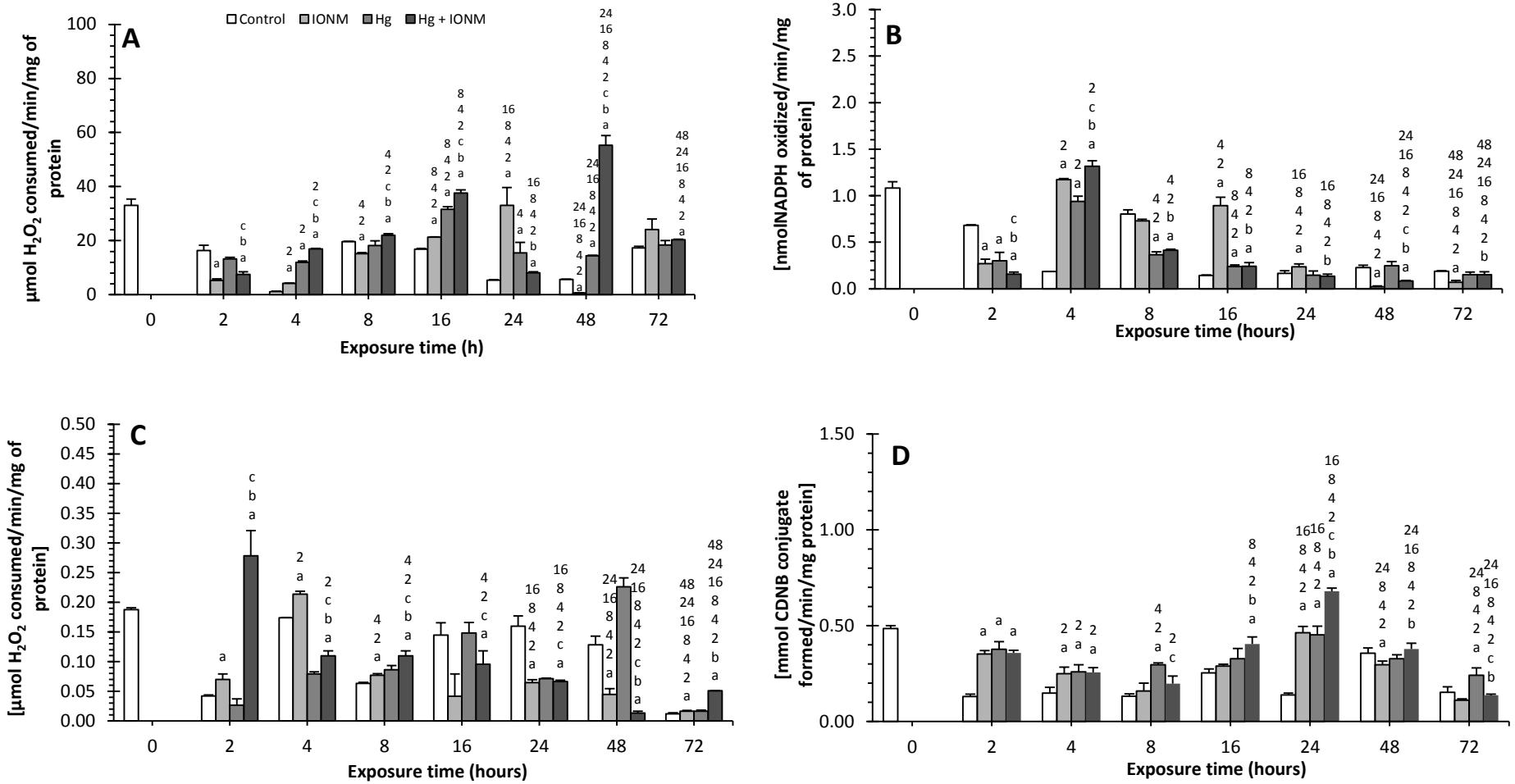

Figure 17. *Anguilla anguilla* L. *in vitro* peritoneum exudates phagocytes: Catalase (A), Glutathione Peroxidase (B), Glutathione reductase (C) and Glutathione S-transferase (D) under exposure to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone or concomitantly for a period of 72 hours. Inter-group significant differences have been denoted by letters: a (vs. control), b (vs. IONM), c (vs. Hg) and inter-hours significant differences have been marked by numbers: 2 (vs. 2 hours), 4 (vs. 4 hours), 8 (vs. 8 hours), 16 vs. (16 hours), 24 (vs. 24 hours) and 48 (vs. 48 hours).

hours (vs. 16 hours) and GST at 24 hours (vs. 2, 4, 8 and 16 hours), 72 hours (vs. 4 and 8 hours); whereas a decrease was observed in CAT at 4 hours (vs. 2 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours), GPX at 16 hours (vs. 2 hours), 24 hours (vs. 2, 4, 8 and 16 hours), 48 and 72 hours (vs. 2, 4, 8, 16 and 24 hours), GR at 24 hours (vs. 2, 4, 8 and 16 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours) and 72 hours (vs. 2, 4, 8, 16, 24 and 48 hours), and GST at 4 hours (vs. 2 hours), 48 hours (vs. 2 and 24 hours) were observed.

P-phagocytes exposed to Hg alone revealed a significant increase in CAT at 16 hours (vs. 2, 4 and 8 hours), 24 hours (vs. 4 hours), 48 hours (vs. 2 and 4 hours), GPX at 4 hours (vs. 2 hours) and GST at 8 hours (vs. 4 hours), 24 hours (vs. 2, 4, 8 and 16 hours); whereas a decrease was perceptible in CAT at 4 hours (vs. 2 hours), 48 hours (vs. 8, 16 and 24 hours), GPX at 8 hours (vs. 4 hours), 16 hours (vs. 2, 4 and 8 hours) and GST at 4 and 8 hours (vs. 2 hours). Concerning, GR no significant changes was observed.

Effects of IONM and Hg concomitant exposure on P-phagocytes displayed an increase in CAT at 4 and 24 hours (vs. 2 hours), 8 hours (vs. 2 and 4 hours), 16 hours (vs. 2, 4 and 8 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours), 72 hours (vs. 2, 4 and 24 hours), GPX at 4, 8 and 16 hours (vs. 2 hours), 72 hours (vs. 48 hours), GR at 72 hours (vs. 48 hours) and GST at 16 hours (vs. 2, 4 and 8 hours), 24 hours (vs. 2, 4, 8 and 16 hours). Moreover, in CAT at 24 hours (vs. 2, 8 and 16 hours), 72 hours (vs. 8, 16 and 48 hours), GPX at 8 hours (vs.4 hours), 16 hours (vs. 4 and 8 hours), 72 hours (vs. 2, 4, 8, 16 and 24 hours), GR at 4 hours (vs. 2 hours), 8 hours (vs. 4 hours), 16 hours (vs. 2 and 4 hours), 24 hours (vs. 2, 4, 8 and 16 hours), 48 and 72 hours (vs. 2, 4, 8, 16 and 4 hours) and GST at 4 hours (vs. 2 hours), 72 hours (vs. 2, 4, 8 and 24 hours) were observed a significant decrease.

3.2. Gill-adhered phagocytes (G-phagocytes) responses

3.2.1. Inter-group comparisons

The results based on enzymatic antioxidants responses have been depicted in figure 18. *A. anguilla* G-phagocytes *in vitro* exposure to IONM alone in comparison to control revealed a significant increase in CAT at 2 until 72 hours, GPX at 2, 4, and 24 hours, GR at 16 hours, and GST at 2, 4, 8, 16, 24 and 72 hours; whereas a decrease was observed in GPX at 16 and 48 hours and GR at 4 and 24 hours.

Exposure to Hg alone, G-phagocytes revealed a significant increase in CAT at 4, 8, 16, 24 and 72 hours, GPX at 2, 4, 8 and 48 hours, GR at 2 until 72 hours and GST at 2, 4, 24, 48 and 72 hours; whereas a significant decrease was observed in CAT at 48 hours and GPX at 16, 24 and 72 hours, in comparison to control.

Effects of IONM and Hg concomitant exposure resulted a significant G-phagocytes increase in CAT at 2, 8, 16 and 24 hours, GPX at 2, 4 and 72 hours, GR at 2 until 72 hours and GST 2 until 72 hours; whereas a decrease was observed in GPX at 8, 16 and 24 hours, when compared to control. Effects of IONM exposure alone versus concomitant exposure revealed a significant increase in CAT at 24 hours, GPX at 2, 48 and 72 hours, GR at 4, 8, 24 48 and 72 hours, and GST at 8, 48 and 72 hours; whereas a decrease was observed in CAT 4, 16, 48 and 72 hours, GPX at 24 hours, GR at 2 and 16 hours, and GST at 16 and 24 hours. Concomitant effects comparison with Hg alone depicted an increase in CAT at 24 and 48 hours, GPX at 2, 4 and 72 hours, and GST at 8, 16 and 48 hours; whereas a decrease was observed in CAT at 4, 8 and 72 hours, GPX at 8, 24 and 48 hours, GR at 2, 8, 16, 48 and 72 hours and GST at 24 and 72 hours.

3.2.2. Inter-hours comparisons

Inter-hours comparisons revealed no statistically significant difference between the controls during the 72 hours exposure period. Effects of exposure IONM alone revealed a significant increase in CAT at 4 and 8 hours (vs. 2 hours), 16 hours (vs. 2 and 4 hours), 24 hours (vs. 2, 8 and 16 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours) and 72 hours (vs. 2, 8 hours), GPX at 4 and 16 hours (vs. 2 hours), 24 hours (vs. 2, 8 and 16 hours), GR at 16 and 24 hours (vs. 4 and 8 hours), 72 hours (vs. 2, 8, 16, 24 and 48 hours), and GST at 4 and 8 hours (vs. 2 hours), 16 hours (vs. 2, 4 nad 8 hours), 24 hours (vs. 2, 4, 8 and 16 hours) and 72 hours (vs. 2, 4, 8, 16 and 48 hours); whereas a decrease was perceptible in CAT at 8 and 24 hours (vs. 4 hours), 72 hours (4, 16, 24 and 48 hours), GPX at 16 and 72 hours (vs. 4 and 8 hours), 24 hours (vs. 4 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours), GR at 4 and 16 hours (vs. 2 hours), 24 hours (vs. 2 and 16 hours), 72 hours (vs. 4 hours) and GST at 8 hours (vs. 4 hours), 48 hours (vs. 4 and 24 hours) and 72 hours (vs. 24 hours).

Hg exposure alone increased in CAT at 4 and 8 hours (vs. 2 hours), 24 hours (vs. 16 hours) and 72 hours (vs. 2, 16, 24 and 48 hours), GPX at 4, 16 and 24 hours (vs. 2 hours), 8 hours (vs. 2 and 4 hours), 48 hours (vs. 2 and 24 hours), 72 hours (vs. 2, 16, 24 and 48 hours), GR at 16 and 24 hours (vs. 4 and 8 hours), 48 hours (vs. 8 hours), GST at 4

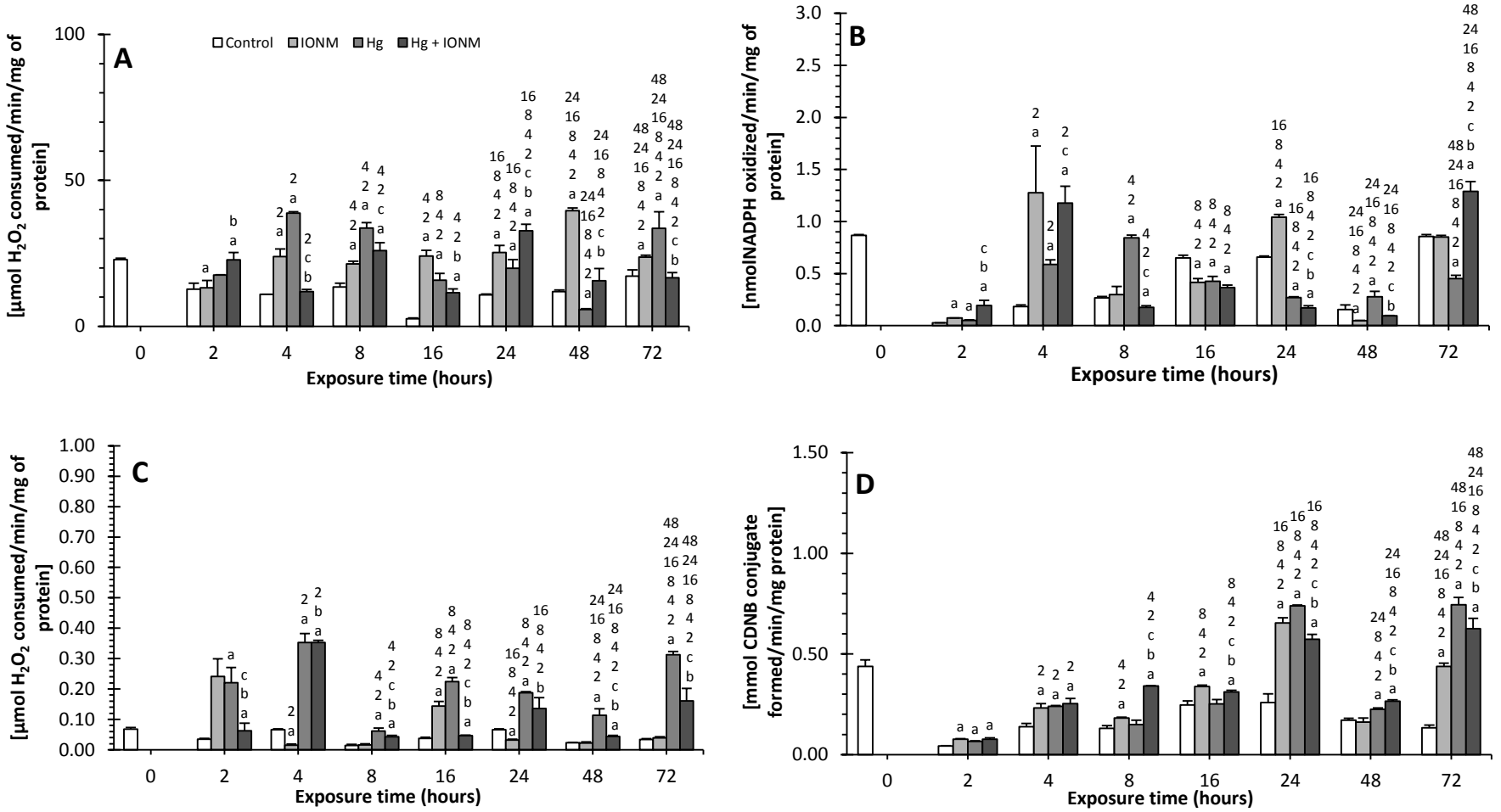

Figure 18. *Anguilla anguilla* L. *in vitro* gill-adhered phagocytes: Catalase (A), Glutathione Peroxidase (B), Glutathione reductase (C) and Glutathione S-transferase (D) under exposure to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone or concomitantly for a period of 72 hours. Inter-group significant differences have been denoted by letters: a (vs. control), b (vs. IONM), c (vs. Hg) and inter-hours significant differences have been marked by numbers: 2 (vs. 2 hours), 4 (vs. 4 hours), 8 (vs. 8 hours), 16 (vs. 16 hours), 24 (vs. 24 hours) and 48 (vs. 48 hours).

hours (vs. 2 hours), 24 hours (vs. 2, 4, 8 and 16 hours), 48 hours (vs. 2 and 8 hours) and 72 hours (vs. 2, 4, 8, 16 and 48 hours). Moreover, G-phagocytes revealed a significant decrease in CAT at 8 hours (vs. 4 hours), 16 hours (vs. 2, 4 and 8 hours), 24 hours (16 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours), 72 hours (vs. 4 and 8 hours), GPX at 16 and 72 hours (vs. 4 and 8 hours), 24 hours (vs. 4 hours), 48 hours (vs. 4, 8 and 16 hours), 72 hours (vs. 4 and 8 hours), GR at 8 and 24 hours (vs. 2 and 4 hours), 16 hours (vs. 4 and 8 hours), 48 hours (vs. 2, 4, 16 and 24 hours) and GST at 48 hours (vs. 2 and 24 hours).

Concomitant IONM and Hg exposure revealed increased CAT at 8 (vs. 2 and 4 hours), 24 hours (vs. 2, 4, 8 and 16 hours), GPX at 4 hours (vs. 2 hours), 72 hours (vs. 2, 4, 8, 16, 24 and 48 hours), GR at 4 hours (vs. 2 hours), 48 hours (vs. 8 hours) and GST at 4 hours (vs. 2 hours), 8 and 48 hours (vs. 2 and 4 hours), 16 hours (vs. 8 hours), 24 hours (vs. 2, 4, 8 and 16 hours) and 72 hours (vs. 2, 4, 8, 16, 24 and 48 hours); whereas a significant decrease at CAT at 16 hours (vs. 2 and 4 hours), GPX at 8 hours (vs. 2 and 4 hours), 16 hours (vs. 4 hours), 24 hours (vs. 2, 4, 8 and 16 hours), GR at 8 hours (vs. 2 and 4 hours), 16 hours (vs. 2,4 and 8), 48 hours (vs. 2, 4, 16 and 24 hours) and GST at 16 hours (vs. 8 hours) and 48 hours (vs. 8, 16 and 24 hours).

3.3. Head kidney resident phagocytes (HK-phagocytes) responses

3.3.1. Inter-group comparisons

The results based on enzymatic antioxidants responses have been depicted in figure 19. Effects of IONM exposure alone on *A. anguilla* HK-phagocytes exhibited a significant increase in CAT at 4, 8, 24, 48 and 72 hours, GPX at 2, 4, 16 and 48 hours, GR at 2, 4, 8, 16 and 72 hours and GST at 2, 8, 16 and 24 hours. Moreover, a decrease was also observed in CAT at 16 hours, GPX at 24 and 72 hours, GR at 48 hours and GST at 4 hours, in comparison to control.

Effects of Hg exposure alone versus control revealed in HK-phagocytes an increase in CAT at 48 hours, GPX at 2, 4, 8, 16 and 72 hours, GR at 2, 4, 24, 48 and 72 hours, and GST at 2, 4, 8, 16, 24 and 72 hours; whereas a significant decrease in CAT at 2, 4 and 8 hours, and GPX at 24 hours.

Effects of IONM and Hg concomitant exposure on HK-phagocytes displayed a significant increase in CAT at 4, 16, 24 and 48 hours, GPX at 4, 8 and 16 hours, GR at 2, 8, 16, 24 and 48 hours, and GST at 8, 16, 24 and 48 hours; whereas a decrease in CAT at 2

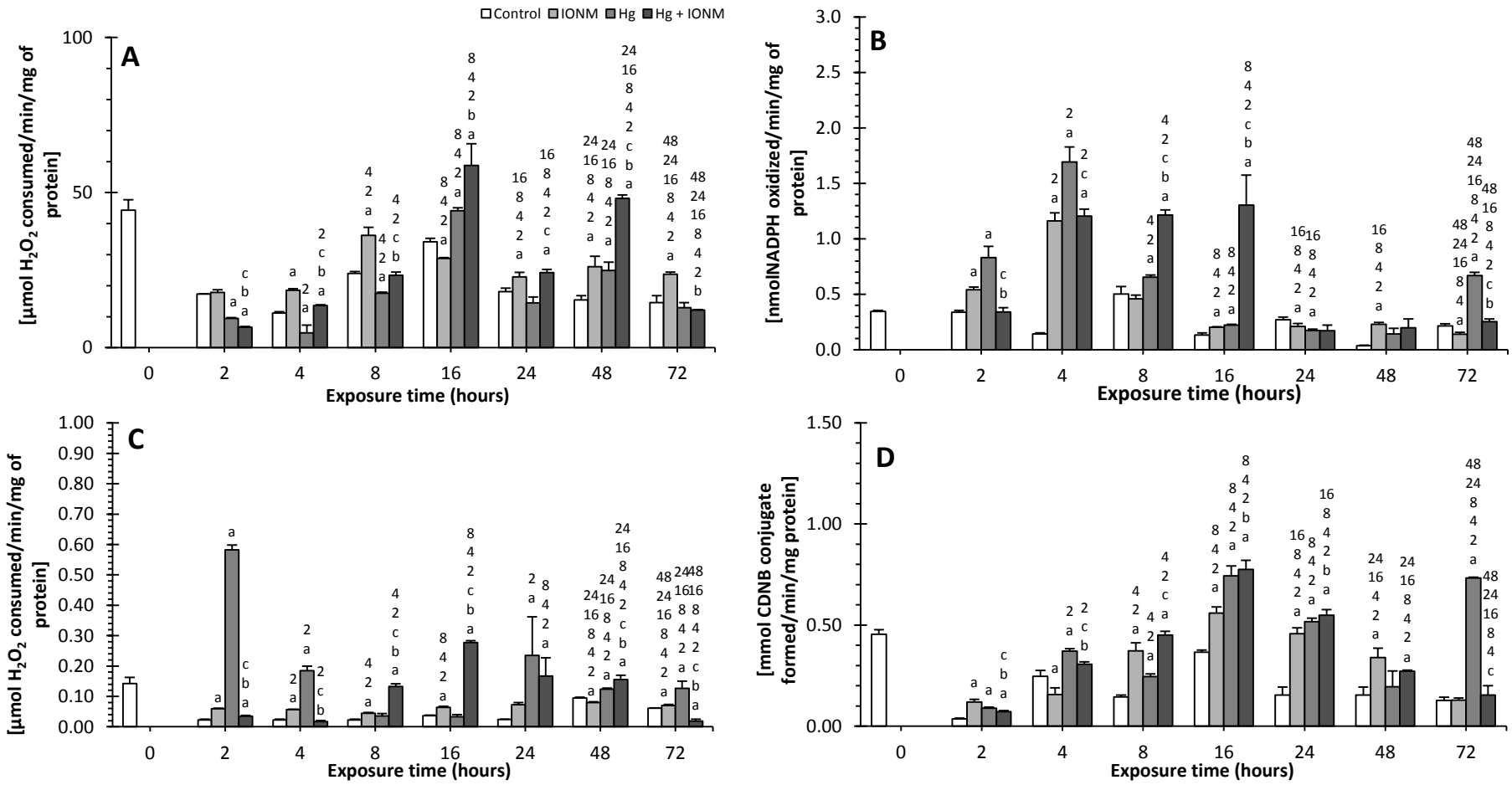

Figure 19. *Anguilla anguilla* L. *in vitro* head kidney resident phagocytes: Catalase (A), Glutathione Peroxidase (B), Glutathione reductase (C) and Glutathione S-transferase (D) under exposure to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone or concomitantly for a period of 72 hours. Inter-group significant differences have been denoted by letters: a (vs. control), b (vs. IONM), c (vs. Hg) and inter-hours significant differences have been marked by numbers: 2 (vs. 2 hours), 4 (vs. 4 hours), 8 (vs. 8 hours), 16 (vs. 16 hours), 24 (vs. 24 hours) and 48 (vs. 48 hours).

hours and GR at 72 hours. Effects of IONM exposure alone versus concomitant exposure revealed a significant increase in CAT at 16 and 48 hours, GPX at 8, 16 and 72 hours, GR at 8 and 48 hours and GST at 4, 16 and 24 hours; whereas a decrease in CAT at 2, 4, 8 and 72 hours, GPX at 2 hours, GR at 4 and 72 hours, and GST at 2 hours. Concomitant effects comparison with Hg alone exhibited an increase in CAT at 4, 8, 16, 24 and 48 hours, GPX at 8 and 16 hours, GR at 8 and 48 hours, and GST at 8 hours; whereas a decrease in CAT at 2 hours, GPX at 2, 4 and 72 hours, GR at 2, 4 and 72 hours, and GST at 2, 4 and 72 hours.

3.3.2. Inter-hours comparisons

Inter-hours comparisons revealed no statistically significant difference between the controls during the 72 hours exposure period. Effects of exposure IONM alone revealed a significant increase in CAT at 8, 16 and 24 hours (vs. 2 and 4 hours), 48 and 72 hours (vs. 2, 4 and 24 hours), GPX at 4 hours (vs. 2 hours), 24 and 48 hours (vs. 16 hours), GR at 16 hours (vs. 2, 4 and 8 hours), 48 and 72 hours (vs. 2, 4, 8, 16 and 24 hours) and GST at 8 and 48 hours (vs. 2 and 4 hours), 16 and 24 hours (vs. 2, 4 and 8 hours); whereas a decrease in CAT at 16 hours (vs. 8 hours), 24 and 48 hours (vs. 8 and 16 hours), 72 hours (vs. 8, 16 and 48 hours), GPX at 16., 24 and 48 hours (vs. 2, 4 and 8 hours), 72 hours (vs.4, 8, 16, 24 and 48 hours), GR at 4 hours (vs. 2 hours), 8 hours (vs. 2 and 4 hours), 72 hours (vs. 48 hours) and GST at 24 hours (vs. 16 hours) and 48 hours (vs. 16 and 24 hours).

Hg exposure alone increased in CAT at 8 hours (vs. 2 and 4 hours), 16 hours (vs. 2, 4 and 8 hours), 48 hours (vs. 2, 4 and 24 hours), GPX at 4 hours (vs. 2 hours), 72 hours (vs. 16, 24 and 48 hours), GR at 48 and 72 hours (vs. 8 and 16 hours), and GST at 4 and 8 hours (vs. 2 hours), 16 and 24 hours (vs. 2, 4 and 8 hours), 72 hours (vs. 2, 4, 8, 24 and 48 hours); whereas, a decrease in CAT at 4 hours (vs. 2 hours), 48 hours (vs. 8 and 16 hours), GPX at 8 hours (vs. 2 and 4 hours), 16 and 72 hours (vs. 2, 4 and 8 hours), 24 hours (2, 4, 8 and 16 hours), GR at 4 and 24 hours (vs. 2 hours), 48 and 72 hours (vs. 2, 4 and 24 hours), and GST at 8 hours (vs. 4 hours) and 24 hours (vs. 16 hours).

Concomitant IONM and Hg exposure revealed increased CAT at 4 hours (vs. 2 hours), 16 and 24 hours (vs. 2, 4 and 8 hours), 48 hours (vs. 2, 4, 8 and 24 hours), GPX at 4 hours (vs. 2 hours), 8 hours (vs. 2 and 4 hours), 16 hours (vs. 2, 4 and 8 hours), GR at 8 hours (vs. 2 and 4 hours), 16, 24 and 48 hours (vs. 2, 4 and 8 hours), 72 hours (vs.4 hours) and GST at 8 hours (vs. 2 and 4 hours), 16 and 24 hours (vs. 2, 4 and 8 hours) and 48 hours

(vs. 2 hours); whereas a decrease in CAT at 24 and 48 hours (vs. 16 hours), GR at 48 hours (vs. 16 and 24 hours) and 72 hours (vs. 2, 8, 16, 24 and 48 hours), and GST at 24 hours (vs. 16 hours) and 48 hours (vs. 4, 8, 16 and 24 hours).

3.4. Spleen resident phagocytes (S-phagocytes) responses

3.4.1. Inter-group comparisons

The results based on enzymatic antioxidants responses have been depicted in figure 20. Effects of IONM exposure alone on *A. anguilla* S-phagocytes displayed a significant increase in CAT at 4, 8, 16, 24, 48 and 72 hours, GPX at 4, 8, 16, 24, 48 and 72 hours, GR at 8 and 48 hours, and GST at 16, 24 and 48 hours; whereas a decrease in GR at 2, 4, 16 and 24 hours, and GST at 4 hours, when compared to control.

Effects of Hg exposure alone versus control revealed in S-phagocytes an increase in CAT at 2, 4, 8, 16 and 72 hours, GPX at 2, 4, 16, 24 and 72 hours, GR at 2, 4 and 24 hours, and GST at 8 and 24 hours; whereas a significant decrease in CAT at 24 and 48 hours and GST at 4, 16 and 48 hours.

Effects of IONM and Hg concomitant exposure on S-phagocytes displayed a significant increase in CAT at 2, 4, 16 and 24 hours, GPX at 2, 4, 8, 16, 24 and 48 hours, and GST at 8 and 16 hours; whereas a decrease in CAT at 48 hours, GR at 2 until 72 hours, and GST at 2, 4 and 48 hours. Effects of IONM exposure alone versus concomitant exposure revealed a significant increase in GPX at 2 and 48 hours, GR at 4 hours and GST at 4 and 8 hours; whereas a decrease in CAT at 8 and 48 hours, GPX at 24 and 72 hours, GR at 8, 16, 24 and 48 hours, and GST at 2 hours. Concomitant effects comparison with Hg alone exhibited an increase in CAT at 24 hours, GPX at 2, 8 and 48 hours, and GST at 4, 16 and 48 hours; whereas a decrease in CAT at 4 and 16 hours, GPX at 72 hours, GR at 2, 4, 16 and 24 hours, and GST at 2, 24 and 72 hours.

3.4.2. Inter-hours comparisons

Inter-hours comparisons revealed no statistically significant difference between the controls during the 72 hours exposure period. Effects of exposure IONM alone revealed a significant increase in CAT at 16 and 24 hours (vs. 2, 4 and 8 hours), 48 hours (vs. 4 and 8 hours), 72 hours (vs. 2, 4, 8, 24 and 48 hours), GPX at 4 and 8 hours (vs. 2 hours), 72 hours (vs. 48 hours), GR at 8 hours (vs. 2 and 4 hours), 16 hours (vs. 2 and 4 hours), 24

hours (vs. 2, 4, 16 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours) and GST at 4 and 72 hours (vs. 2 hours), 16 hours (vs. 2, 4 and 8 hours) and 24 hours (vs. 2, 4, 8 and 16 hours); whereas a decrease in CAT at 4 hours (vs. 2 hours), 24 and 72 hours (vs. 16 hours), 48 hours (vs. 2, 16 and 24 hours), GPX at 8 and 16 hours (vs. 4 hours), 24 hours (vs. 4, 8 and 16 hours), 48 and 72 hours (vs. 2, 4, 8, 16 and 24 hours), GR at 4 hours (vs. 2 hours), 16 and 24 hours (vs. 8 hours), and GST at 72 hours (vs. 4, 8, 16, 24 and 48 hours).

Hg exposure alone displayed an increase in CAT at 8 hours (vs. 2 and 4 hours), 16 and 72 hours (vs. 24 and 8 hours), 48 hours (vs. 24 hours), GPX at 4 hours (vs. 2 hours), 72 hours (vs. 16, 24 and 48 hours), GR at 24 hours (vs. 8 and 16 hours) and GST at 4 hours (vs. 2 hours), 8 hours (vs. 2 and 4 hours) and 24 hours (vs. 2, 8 and 16 hours); whereas a decrease in CAT at 4 hours (vs. 2 hours), 24 hours (vs. 16 hours), 48 hours (vs. 2, 4, 8 and 16 hours), 72 hours (vs. 2, 4, 8 and 16 hours), GPX at 16 and 24 hours (vs. 2, 4 and 8 hours), GR at 4 hours (vs. 2 hours), 24 hours (vs. 2 and 4 hours) and 72 hours (vs. 2, 4, 24 and 48 hours), and GST at 16 hours (vs. 2, 4 and 8 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours).

Concomitant IONM and Hg exposure revealed increased CAT at 16 hours (vs. 2, 4 and 8 hours), 24 hours (vs. 2, 4, 8 and 16 hours), GPX at 4 and 8 hours (vs. 2 hours), GR at 8 hours (vs. 4 hours), 16 hours (vs. 2, 4 and 8 hours), 48 and 72 hours (vs. 2, 4, 8, 16 and 24 hours) and GST at 4 and 48 hours (vs. 2 hours), 8 hours (vs. 2 and 4 hours), 16 hours (vs. 2, 4 and 8 hours), 24 hours (vs. 2, 4, 8 and 16 hours); whereas a decrease in CAT at 4 hours (vs. 2 hours), 24 hours (vs. 2, 4, 8 and 16 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours), GPX at 8 hours (vs.4 hours), 16 and 72 hours (vs. 2, 4 and 8 hours), 24 hours (vs. 2, 4, 8 and 16 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours), GR at 4 and 8 hours (vs. 2 hours), 24 hours (vs. 2, 4, 8 and 16 hours) and 72 hours (vs. 48 hours) and GST at 48 hours (vs. 4, 8, 16 and 24 hours).

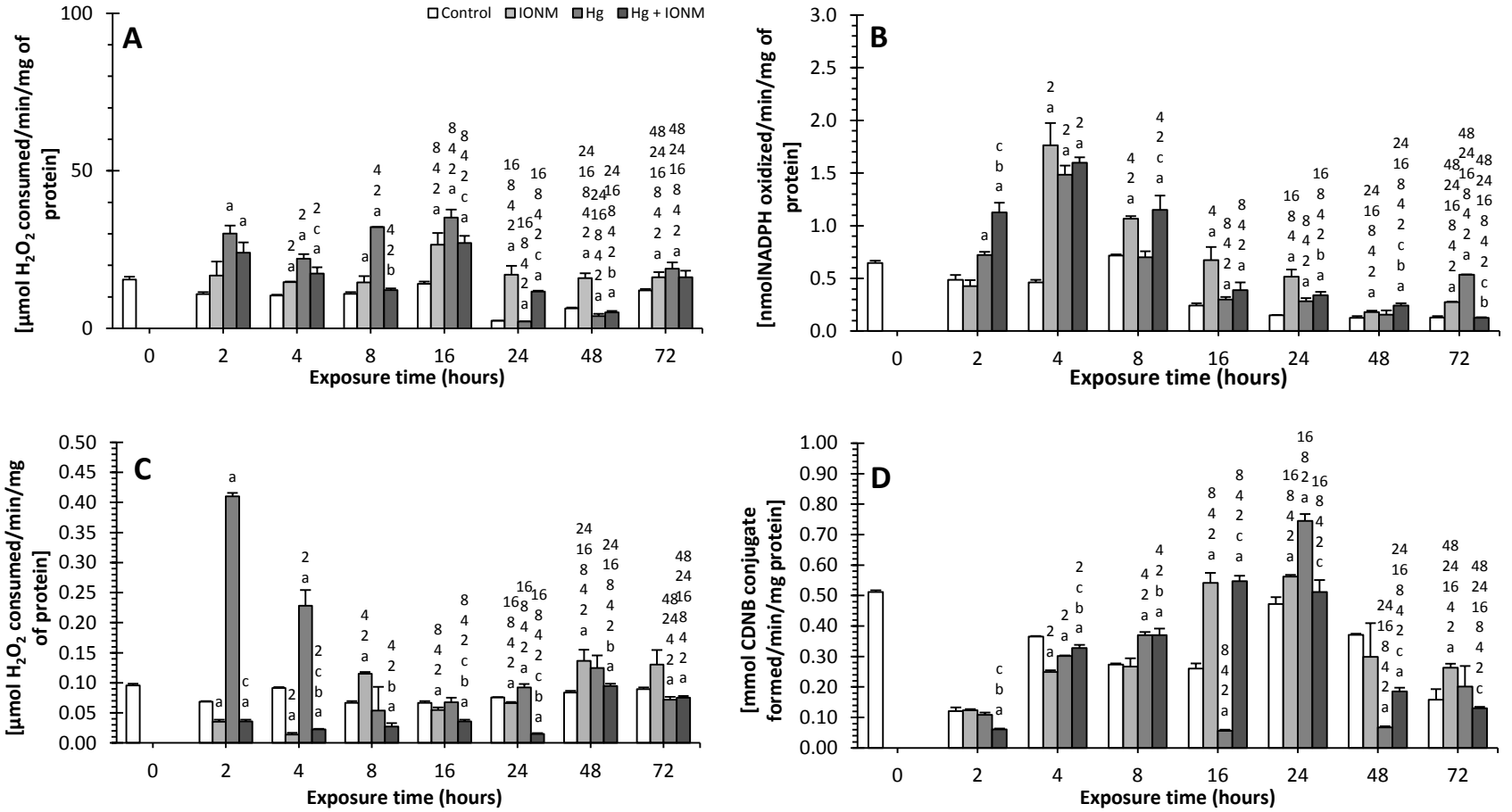

Figure 20. *Anguilla anguilla* L. *in vitro* spleen resident phagocytes: Catalase (A), Glutathione Peroxidase (B), Glutathione reductase (C) and Glutathione S-transferase (D) under exposure to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone or concomitantly for a period of 72 hours. Inter-group significant differences have been denoted by letters: a (vs. control), b (vs. IONM), c (vs. Hg) and inter-hours significant differences have been marked by numbers: 2 (vs. 2 hours), 4 (vs. 4 hours), 8 (vs. 8 hours), 16 (vs. 16 hours), 24 (vs. 24 hours) and 48 (vs. 48 hours).

4. Discussion

4.1. Peritoneum exudate phagocytes (P-phagocytes) responses

A. anguilla P-phagocytes displayed a differential response of defence endpoints at the end of different periods of exposure to IONM. Concerning damage endpoints response, no LPO increase was observed with increase in the period of exposure to IONM (as depicted in Chapter 2). However, the damage endpoint was accompanied with modulation of the enzymatic antioxidants contributing to the control of cellular redox by scavenging harmful intracellular ROS under normal and adverse conditions. Thus, at the end of the 2 hours of exposure, a decrease in CAT and GPX was observed; whereas an increase was perceptible in GR and GST. Our report coincides with early studies revealing that fullerenes (C_{60}), carbon nanotubes (CNT), and metal oxides are capable to induce oxidative stress (Bonner et al., 2007; Thannickal et al., 2000). Thus, CAT and GPX decrease provides an indication of the higher H_2O_2 amount production at the end of the 2 hours exposure; hence, fish exposed to IONM seem to cope efficiently with H_2O_2 production only through GR and GST as no peroxidative damage was observed. At late hours of exposure, P-phagocytes revealed increased GR and decreased GPX suggesting that GR is still restoring GSH to scavenge ROS preventing peroxidative damage.

On the perspective of Hg exposure alone, at the end of the 2 hours of exposure, a decrease in GPX and increase in GST was observed suggesting that the protection against ROS induction is occurring only through the GST activity. Increased GST activity has been associated with metal exposure in several fish species (Mager et al., 2008). The increase in GST activity may be due to the fact that GST is used as a cofactor for GPX, thus the decrease of GPX resulting from oxidative stress induced by Hg requires more co-factor and hence, the levels of GST may also enhanced. With the increase in exposure time the GST activity in the Hg alone exposed phagocytes is remained constant for a period of 72 hours. Similar kind of increase in the GST activity was observed in the kidney of fish (*Liza aurata*) (Mieiro et al., 2011). Thus, GST is considered a good indicator of the presence of contaminants, such as Hg, in the aquatic environment (Elia et al., 2003).

In concomitant exposure, similar trend of antioxidants enzymes was perceptible as observed for IONM alone; thus suggesting the same explanation as described above for IONM alone exposure at the end of 2 hours. However, the activity of CAT and GPX is less when compared to control and Hg alone exposed phagocytes; whereas, GR activity was

significantly increased. The toxicity of Hg has caused the elevation of GR in concomitant exposure at the same time the interference of IONMs has tried to normalize the GR activity by keeping its activity up to the control value. The induction of CAT and GR at late hours of exposure might be due to a continuous ROS generation and their scavenging as reported by Huang et al. (2010). Concomitant induction of GST reaching to normal at late hours of exposure, along with decline in TGSH content (chapter 4) may indicate that these enzymes are glutathione dependent and decline in their activities is obvious.

4.2. Gill-adhered phagocytes (G-phagocytes) responses

A. anguilla G-phagocytes displayed induction in all the studied enzymatic antioxidants at the end of the early hours exposure. With the increase in exposure time, CAT and GST remained constant; whereas, GPX and GR showed a modulatory response reaching to normal at late hours of exposure. Concerning damage endpoints response, an LPO increase was observed only at 8 hours of exposure to IONM (as depicted in Chapter 2). The LPO increase at 8 hours with enzymatic antioxidants induction may strengthen the explanation of Cossu et al. (2000) that LPO cannot be predicted only on the basis of antioxidants modulation. Thus, the depletion of other glutathione independent antioxidants can also be suggested for the current observation. Our report coincides with early studies revealing that fullerenes, CNT, and metal oxides are capable to induce oxidative stress (Bonner et al., 2007; Thannickal et al., 2000).

On the perspective of Hg exposure alone, similar antioxidants induction was observed at initial hours of exposure and remained constant with the increase of exposure time (except decrease in GPX at 16 and 24 hours). Interestingly, the increase in antioxidant enzymes was corroborated with OBA and LPO increase suggesting that the antioxidants are not efficient to protect the cells against ROS. The induction of enzymes is coincided with the findings of Zhu et al. (2008 a, b) who revealed that fullerene exposed for 32 days in goldfish gill cells (*Carassius auratus*) induced antioxidant enzymes superoxide dismutase and catalase. Regarding, LPO induction viz. glutathione dependent enzymes induction, Scown (2009) hypothesised that the mechanism of nanomaterial-induced lipid peroxidation is via the dissociation of nanomaterial ions into the culture media, maybe as a cause of glutathione dependent enzymes induction since enzymes catalyses the conjugation of zinc ions to glutathione sulfhydryl groups. Regarding GST, early report revealed that Hg

significantly increased gill GST activity (Vieira et al., 2009). Some authors (Liu et al., 2006; Sanchez et al., 2005; Vieira et al., 2009) shared some hypotheses for the induction of GST when exposed Hg to fish gill cells. Thus, (i) since GST is a cofactor for glutathione peroxidase, the increase of GPX to face oxidative stress requires more co-factor and, thus, the levels of GST are also enhanced, and (ii) since GST determinations were performed in gills that constitute a first barrier against the entrance to toxicants in fish body and GST have the capability of bind and/or store or transport substances, the increase of GST levels observed may correspond to a first attempt to overcome metal stress by producing a high amount of enzyme that will be then available to bind Hg, decreasing their local concentration and, therefore, their uptake by the organism.

In concomitant exposure, initially induced enzymes reached either to normal or increase in comparison to early hours of exposure revealing the same pattern of response as observed for Hg alone. However, in comparison to Hg, a synergism is perceptible at early hours in GPX and remained increased at the end of the exposure length (72 hours) suggesting an increased toxicity of IONM when come in contact with Hg co-exposure. On the other, an antagonism was observed at early and late hours of exposure in GR activity; whereas, in CAT and GST only at the end of exposure. Thus, Hg induced negative effects seem to be inhibited with the increase in exposure time, however not to the normal level at the end of the exposure length. These results imply the positive effect of the concomitant exposure (IONM+Hg), which significantly mitigated the said negative impacts of Hg at both early (GR) and late hours (CAT, GR, GST); thus, the occurrence of antagonism between IONM and Hg can be emphasized.

4.3. Head kidney resident phagocytes (HK-phagocytes) responses

A. anguilla HK-phagocytes displayed either no (CAT) or induced (GPX, GR, GST) antioxidants responses at the end of the early and late hours of IONM exposure; thus, suggesting the same explanation for HK-phagocytes as revealed for G- phagocytes. Since the head kidney is the main hemopoietic organs of phagocytes origin, the same response to IONM exposure seems to be applicable in HK resident phagocytes. However, on Hg exposure alone, a CAT decrease at early hours (2, 4 and 8 hours) reaching to normal at the end of 72 hours exposure was perceptible; whereas an increase in rest of the enzymes at the end of both early and late hours of exposure may indicate that the over-production of ROS

is overwhelming the antioxidants protection. In concomitant exposure, initially induced enzymes reached either to normal or decrease in comparison to early hours of exposure. Moreover, in comparison to Hg, an antagonism is perceptible at both early and late hours suggesting a decrease in toxicity of Hg when come in contact with Hg co-exposure. These results imply that the Hg induced negative effects were inhibited with the increase in exposure time, however in terms of H_2O_2 production an opposite trend is perceptible since a decrease in CAT was observed at the end of the exposure.

4.4. Spleen resident phagocytes (S-phagocytes) responses

In the current study, *A. anguilla* S-phagocytes displayed a differential response of defence endpoints at the end of different periods of exposure to IONM. After 2 hours of exposure, a decrease was perceptible only in GR among all the studied enzymes; however, with the increase of exposure time, no or increased levels of studied enzymes was observed suggesting IONM's enzyme-inducing effect of H_2O_2 derived from O^2. LPO increase with no corroboration with enzymatic antioxidants modulation further validate the explanation given by Cossu et al. (2000) as described above. On the perspective of Hg exposure alone, the antioxidant enzymes inducing pattern was same as observed for IONM. However, under co-exposure of IONM and Hg, modulatory response of enzymes CAT, GPX, GR and GST at both early and late hours of exposure clearly indicates that the interaction of IONM with Hg alter the toxicity potential of either IONM and/or Hg. In concomitant exposure, initially induced enzymes reached either to normal or decrease in comparison to early hours of exposure. Moreover, in comparison to Hg, an antagonism is perceptible in GR and GST at early hours and GPX and GST at late hours suggesting a decrease in toxicity of Hg when come in contact with IONM co-exposure. These results imply that the Hg induced negative effects were inhibited with the increase in exposure time, however in terms of H_2O_2 production; an opposite trend was perceptible since a decrease in GR was observed at the end of the exposure.

5. Conclusion

Major findings of the present investigation may be summarized in the following points:

- *A. anguilla* phagocytes displayed a differential extent of antioxidants enzymes (CAT,

GPX, GR and GST) induction at the end of different periods of exposure to IONM, Hg or IONM+Hg. Under IONM alone exposure, enzymatic antioxidants protection responses displayed hours of exposure dependency in G- and S-phagocytes where, compared to control, an insufficiency of elevated CAT, GPX and GST was clearly depicted for the maintenance of pro- and antioxidant balance optimum for scavenging ROS and protecting membrane lipids against IONM impact.

- As no or increased LPO was observed under IONM alone exposure condition, the joint action of IONM+Hg led to elevated damages to membrane-lipids at 4 and 8 hours (G-phagocytes), 2 hours (HK-phagocytes) and 24 hours (S-phagocytes) of exposure (Chapter 2). The adoption of the strategy of enzymatic antioxidant modulation in *A. anguilla* G-phagocytes at 4 hours was reflected by significantly decrease in CAT and increase in GR, GPX and GST; whereas at 8 hours, the significantly decrease in CAT and GPX and increase in GR and GST was perceptible. The strategy adopted for HK-phagocytes at 2 hours was reflected by decrease in all the studied enzymes. In addition, strategies adopted by S-phagocytes at 24 hours were reflected by an increase in CAT, GR, GST and NP-SH.

-These responses together, point towards the enzymatic antioxidants defense failure for the protection of membrane lipids during those periods of exposure to IONM+Hg.

- However, it is important to underline here that during the late hours of exposure (for example 72 hours) the results imply the positive effect of the concomitant exposure (IONM+Hg) which significantly mitigated the said negative impacts of Hg. Hence, the occurrence antagonism between IONM and Hg cannot be overlooked.

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Chapter 4

Anguilla anguilla L. phagocytes non-enzymatic antioxidants responses following *in vitro* exposure to silica coated iron oxide nanomaterial functionalized with dithiocarbamate with or without mercury co-exposure

Abstract

The study aimed to assess the effects of *in vitro* silica coated iron oxide nanomaterials functionalized with dithiocarbamate (IONM) exposure on non-enzymatic antioxidant responses and its interference with mercury (Hg) co-exposure in European eel (*Anguilla Anguilla* L.) phagocytes isolated from peritoneum (P-phagocytes), gill (G-phagocytes), head kidney (HK-phagocytes) and spleen (S-phagocytes). The phagocytes maintained in culture medium were used for the assessment of non-protein thiol (NP-SH) and total glutathione (TGSH) after exposure to IONM, Hg and IONM+Hg concomitantly for 0, 2, 4, 8, 16, 24, 48 and 72 hours. *A. anguilla* phagocytes displayed a differential extent of toxicity at the end of different periods of exposure to IONM, Hg or IONM+Hg where a differential thiol redox system components modulation was perceptible. In P-phagocytes after exposure to IONM, an initial increase followed by a loss of cellular TGSH was a sign of oxidative stress which could be a consequence of several processes including: (a) impaired synthesis of TGSH, (b) accelerated export of TGSH and/or (c) conjugation of TGSH to applied compounds. A synergism is perceptible at early hours in G-phagocytes (NP-SH and TGSH) and remained induced at the end of the exposure length (72 hours) in concomitant exposure when compared to Hg suggesting a modulatory response of IONM when come in contact with Hg co-exposure. A negative increase in toxicity was also observed at early and late hours of exposure in TGSH content in P-, HK- and S-phagocytes. Increase in the period of exposure to Hg, a modulation of GSH redox system in P-, G-, HK- and S-phagocytes phagocytes is perceptible suggesting an adaptation to increased detoxification activity. Moreover, in P-phagocytes, increase in the period of exposure to Hg continuously reflected no signal of lipid peroxidation (LPO) induction. Thus, P-phagocytes cells might have stimulated glutathione peroxidase (GPX) and glutathione S-transferase (GST) activities in order to scavenge Hg-lead increased reactive oxygen species (ROS) using reduced TGSH pool as a substrate, resulting ultimately into a difference in the GSH pool.

1. Introduction

Non-enzymatic compounds such as non-protein thiol and glutathione also serve as an important biological defense against environmental pro-oxidant conditions. However, literature revealed an insufficient exploration of the role of non-enzymatic antioxidants in

fish, not accomplishing a more holistic approach towards the understanding of antioxidant system function and regulation in relation to both endogenous and exogenous sources of ROS. No report reflects the non-enzymatic antioxidants variations in fish in relation to nanomaterials to mobilizing adaptive processes in this context.

The principal toxic effects of nanomaterials may involve interactions with a number of cellular processes including the formation of complexes with thiol groups, which not only regulate the enzymes but regulate also the cellular redox status. The most commonly observed manifestation of thiol depletion is its accompaniment with the alteration of reduced glutathione (GSH) towards an oxidized (GSSG) state (Hoffman et al., 2002). GSH is the most abundant NP-SH involved in the detoxification of metals and oxy-radicals in aquatic organisms (Canesi et al., 1999). The available literature also revealed enzymes reacting with potentially toxic endogenous compounds and xenobiotics using GSH (Forman et al., 2008; Wendel et al., 1990). It is known that metal forms covalent bonds with GSH and a single metal ion can bind to and cause irreversible excretion of two glutathione molecules (Franco et al., 2009). Moreover, it was also reported that, in the presence of reducing compounds such as metal-GSH complexes are not stable, instead it release metal ions, which disturb GSH metabolism and exerts cell-damaging effects.

Taking into account the knowledge gaps as identified above, the current study focused on: 1) to investigate the role of non-enzymatic antioxidants, such as NP-SH, TGSH against to IONM exposure, non-enzymatic protection viz. NP-SH and GSH were studied in various phagocytes of fish exposed to IONM in different time intervals. Moreover, since fish in the aquatic environment in general comes into contact with more contaminants including persistent contaminant such as Hg, the IONM co-exposure with Hg was also considered. It may be postulated that nanomaterial induced phagocytes antioxidant responses may be further modulated by the concomitant co-exposure to other contaminant resulting reduced/or augmented protection against pathogens and infectious diseases.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), Bradford reagent, 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), glutathione reductase from Baker’s Yeast, nicotinamide adenine dinucleotide

phosphate (NADPH), potassium phosphate dibasic (K_2KPO_4), potassium phosphate monobasic (KH_2PO_4) and sulphosalicydic acid. Chemicals and reagents (analytical grade) used for this study were purchased from Sigma. The IONM, considered for this experiment, were kindly provided by Tavares et al. (2013) University of Aveiro, Portugal.

2.2. Equipment

- Centrifuge 5415 R from Eppendorf to centrifuge the samples;
- Environmental Shaker from Incubator ES-20 for shaking the samples;
- Spectra max – 384 Plus from Molecular devices.
- Analytic balance E425 from Gibertini
- pH meter 720 from WTW

2.3. Experimental design

The previously collected samples were processed for different non-enzymatic antioxidants in peritoneal, gill, head kidney and spleen phagocytes. The experimental protocols, test animal, synthesis of IONM and statistical analysis for the chapter 4 were the same as described in chapter 1.

2.4. Non-enzymes tests

2.4.1. Total non-protein thiols (NP-SH)

Non-protein thiols were measured as described by methods adopted by Ahmad et al. (2008) with a little modification. Briefly, the sample 25 µL was precipitated with a solution of 25 µL (1:1) of sulphosalicydic acid (5%) in eppendorf, vortexed for few seconds and were left in the ice for 45 minutes to precipitate. Afterward, all the eppendorfs were centrifuged at 10000 rpm during 5 minutes. The assay mixture contained 30 µL of filtered aliquot, 150 µL of phosphate buffer (0.1 M, pH 7.4) and 15 µL DTNB (10 mM) in a total volume of 195 µL. The optical density of reaction product was read immediately at 412 nm on a spectrophotometer and the results were expressed as µmol TNB formed/mg of protein using a molar extinction coefficient 136 x 10^3 mM^{-1} cm^{-1}.

2.4.2. Total glutathione (TGSH)

TGSH contents were measured as described by the methods adopted by Ahmad et al. (2008) with a little modification. Briefly, the sample of 25 µL was precipitated with a solution of 25 µL (1:1) of sulphosalicydic acid (5%) in eppendorf, vortexed for few seconds and were left in the ice for 45 minutes to precipitate. Afterward, eppendorfs were centrifuged at 10000 rpm during 5 minutes. TGHT content was determined in the resulting supernatant adopting the enzymatic recycling method using GR excess, whereby the sulfhydryl group of GSH reacts with DTNB producing a yellow coloured TNB. The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the concentration of GSH in the sample. The formation of TNB was measured at 412 nm and the results were expressed as nmol TNB formed/min/mg protein using a molar extinction coefficient 14.1 x $10^3 M^{-1}$ cm^{-1}.

3. Results

Results depiction 'given below' has been considered describing first phagocytes inter-group variations within the same hours of exposure followed by inter-hours comparisons within the same group.

3.1. Peritoneum exudates phagocytes (P-phagocytes) responses

3.1.1. Inter-group comparisons

The results based on non-enzymatic antioxidants responses have been depicted in figure 21. *A. anguilla* P-phagocytes, *in vitro* exposure to IONM alone in comparison to control revealed a significant increase in NP-SH at 4 and 8 hours, TGSH at 2 and 48 hours; whereas an decrease at NP-SH at 2, 24, 48 and 72 hours, and TGSH at 8, 24 and 72 hours.

Effects of Hg exposure alone versus control on P-phagocytes revealed an increase in NP-SH at 48 hours and TGSH at 16, 24 hours; whereas a decrease only in NP-SH at 2, 4, 8, 48 and 72 hours and TGSH at 4 and 24 hours.

Effects of IONM and Hg concomitant exposure on P-phagocytes displayed a significant increase in NP-SH at 4 and 24 hours; whereas a decrease in NP-SP at 2, 16, 48, 72 hours and TGSH at 4, 24, 48 and 72 hours. Effects of IONM exposure alone versus concomitant exposure revealed a significant increase in NP-SH at 24 hours, TGSH at 8, 16 hours; whereas a significant decrease in NP-SP at 2, 8 and 48 hours and TGSH at 2, 4, 48

and 72 hours. Concomitant effects comparison with Hg alone exhibited an increase in NP-SP at 4 hours; whereas a decrease in NP-SP at 2, 4 and 72 hours and TGSH at 48 and 72 hours.

3.1.2. Inter-hours comparisons

Inter-hours comparisons revealed no statistically significant difference between the controls during the 72 hours exposure period. Effects of IONM exposure on P-phagocytes revealed a significant increase in NP-SP at 8 hours (vs. 2 and 4 hours), 48 hours (vs. 8 and 24 hours), 72 hours (vs. 24 hours), TGSH at 24 hours (vs. 8 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours) and 72 hours (vs. 8 and 24 hours); whereas a significant decrease in NP-SH at 24 hours (vs. 2, 4 and 8 hours), 48 hours (vs. 2 hours) and 72 hours (vs. 2, 4, 8 and 48 hours) and TGSH at 8 and 24 hours (vs. 2 and 4 hours) and 72 hours (vs. 2, 4 and 48 hours).

P-phagocytes exposed to Hg alone revealed a significant increase in NP-SH at 16 hours (vs. 2, 4 and 8 hours), 24 hours (vs. 4 and 8 hours), 48 hours (vs. 8 hours), 72 hours (vs. 4, 8 and 48 hours), TGSH at 4 hours (vs. 2 hours) and 48 hours (vs. 2, 4, 8, 16 and 24 hours); whereas in NP-SH at 4 and 8 hours (vs. 2 hours), 24 hours (vs. 2 and 16 hours), 48 and 72 hours (vs. 2, 16 and 24 hours) and TGSH at 24 hours (vs. 4 hours).

Effects of IONM and Hg concomitant exposure on P-phagocytes displayed an increase in NP-SH at 4 hours (vs. 2 hours), 24 hours (vs. 2, 4 8 and 16 hours) and 72 hours (vs. 16 hours) and TGSH at 48 hours (vs. 4, 8 and 24 hours). Moreover, in NP-SH at 8 hours (vs. 2 hours), 16 hours (vs. 2, 4 and 8 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours), 72 hours (vs. 2, 4, 8, 24 and 48 hours) and TGSH at 24 hours (vs. 4 and 8 hours) and 72 hours (vs. 2, 4, 8, 16, 24 and 48 hours) were observed a significant decrease.

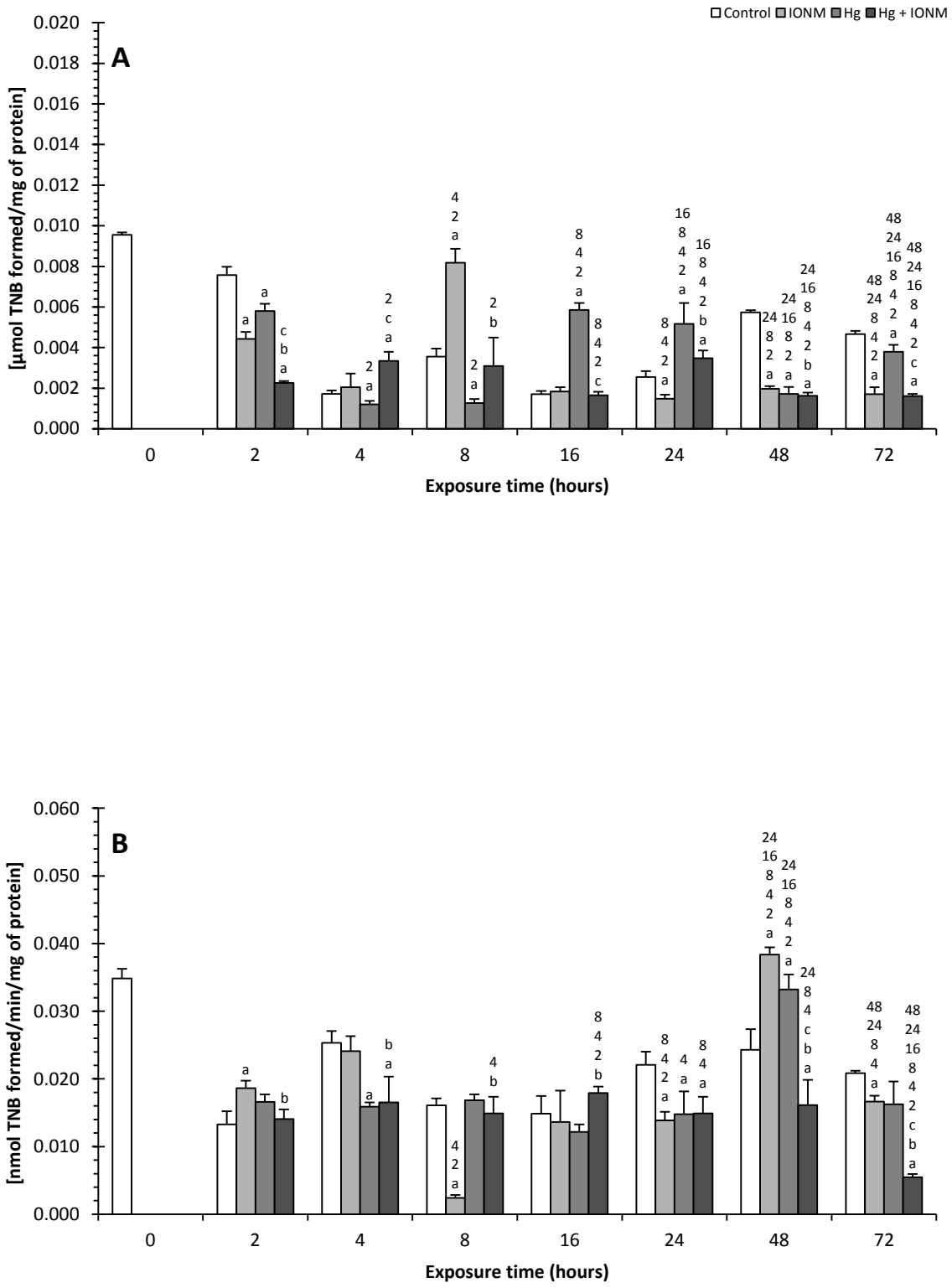

Figure 21. *Anguilla anguilla* L. *in vitro* peritoneum exudates phagocytes: Total non-protein thiols (A) and Total glutathione (B) and under exposure to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone or concomitantly for a period of 72 hours. Inter-group significant differences have been denoted by letters: a (vs. control), b (vs. IONM), c (vs. Hg) and inter-hours significant differences have been marked by numbers: 2 (vs. 2 hours), 4 (vs. 4 hours), 8 (vs. 8 hours), 16 (vs. 16 hours), 24 (vs. 24 hours) and 48 (vs. 48 hours).

3.2. Gill-adhered phagocytes (G-phagocytes) responses

3.2.1. Inter-group comparisons

The results based on non-enzymatic antioxidants responses have been depicted in figure 22. *A. anguilla* G-phagocytes *in vitro* exposure to IONM alone in comparison to control revealed a significant increase in NP-SH at 2, 8, 48, 72 hours and TGSH at 4, 8, 16, 24 and 48 hours. Concerning, TGSH and NP-SH no significant decrease was observed.

Effects of Hg exposure alone versus control on G-phagocytes revealed an increase in NP-SH at 2, 4, 8, 48 and 72 hours, TGSH at 8, 48 hours; whereas a significant decrease in NP-SH at 16 hours and TGSH at 2 hours.

Effects of IONM and Hg concomitant exposure on G-phagocytes displayed a significant increase in TGSH at 8, 48, 72 hours, NP-SH at 2, 4, 8, 24, 48 and 72 hours; whereas NP-SH at 24 hours. Concerning, Concerning, TGSH no significant decrease was observed.

Effects of IONM exposure alone versus concomitant exposure revealed a significant increase in NP-SH at 2, 4, 24 hours, TGSH at 8 and 72 hours; whereas a decrease in NP-SH at 48 hours and TGSH at 4, 24 hours. Concomitant effects comparison with Hg alone exhibited an increase in NP-SP at 2, 16, 72 hours, TGSH at 8, 16, 72 hours; whereas a decrease in NP-SP at 4, 8 and 48 hours, and TGSH at 48 hours.

3.2.2. Inter-hours comparisons

Inter-hours comparisons revealed no statistically significant difference between the controls during the 72 hours exposure period. Effects of IONM exposure on G-phagocytes revealed a significant increase in NP-SH at 8 and 48 hours (vs. 4 hours), 72 hours (vs. 2, 4, 8 and 48 hours), TGSH at 4, 16 and 24 hours (vs. 2 hours), 8 hours (vs. 2 and 4 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours), 72 hours (vs. 2, 4 and 24 hours); whereas a decrease in TGSH at 16 hours (vs. 4 hours), 24 hours (vs. 4, 8 and 16 hours), 72 hours (vs. 8, 16 and 48 hours) and NP-SP at 8 hours (vs. 2 hours), 48 hours (vs. 2, 8, 16 and 24 hours).

G-phagocytes exposed to Hg alone revealed a significant increase in TGSH at 8 hours (vs. 2 and 4 hours), 24 hours (vs. 2 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours), NP-SH at 8 and 16 hours (vs. 4 hours), 72 hours (vs. 4 and 48 hours); whereas a decrease in TGSH at 24 hours (vs. 4, 8 and 16 hours), 72 hours (vs. 8, 16 and 48 hours) and, NP-SH

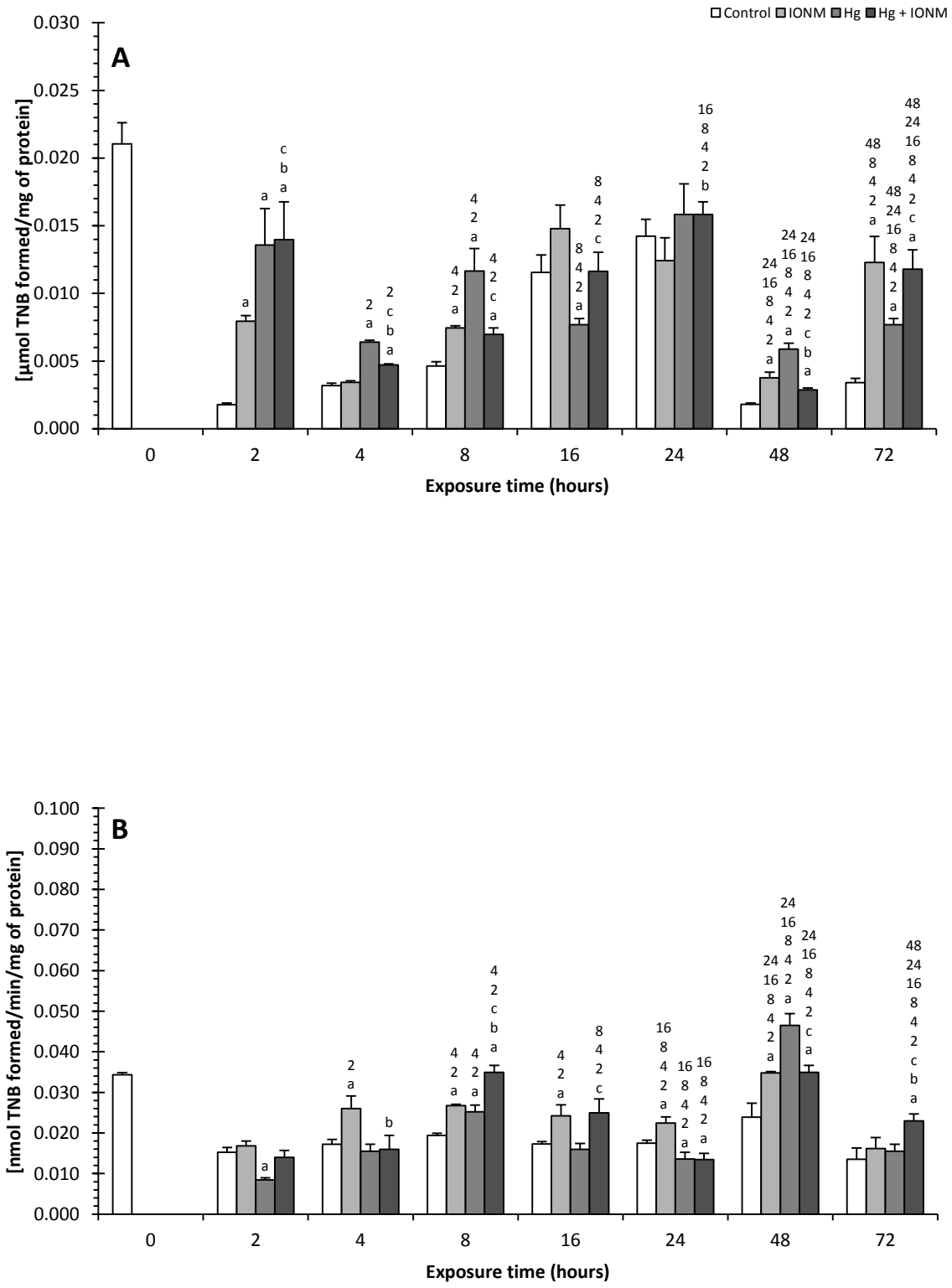

Figure 22. *Anguilla anguilla* L. *in vitro* gill-adhered phagocytes: Total non-protein thiols (A) and Total glutathione (B) under exposure to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone or concomitantly for a period of 72 hours. Inter-group significant differences have been denoted by letters: a (vs. control), b (vs. IONM), c (vs. Hg) and inter-hours significant differences have been marked by numbers: 2 (vs. 2 hours), 4 (vs. 4 hours), 8 (vs. 8 hours), 16 (vs. 16 hours), 24 (vs. 24 hours) and 48 (vs. 48 hours).

at 4 and 8 hours (vs. 2 hours), 16 hours (vs. 2 and 8 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours) and 72 hours (vs. 2, 8, 16 and 24 hours).

Effects of IONM and Hg concomitant exposure on G-phagocytes displayed an increase in NP-SH at 8 hours (vs. 4 hours), 72 hours (vs. 4, 8, 16 and 48 hours), TGSH at 8 hours (vs. 2 and 4 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours), and 72 hours (vs. 2, 4 and 24 hours). Moreover, in NP-SH at 4 and 8 hours (vs. 2 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours) and 72 hours (vs. 2 and 24 hours) and TGSH at 24 hours (vs. 2, 4, 8 and 16 hours) and 72 hours (vs. 8, 16 and 48 hours) were observed a significant decrease.

3.3. Head kidney resident phagocytes (HK-phagocytes) responses

3.3.1. Inter-group comparisons

The results based on non-enzymatic antioxidants responses have been depicted in figure 23. *A. anguilla* L. HK-phagocytes *in vitro* exposure to IONM alone in comparison to control revealed a significant increase in NP-SH at 4 hours, TGSH at 8, 48 hours; whereas a significant decrease in NP-SH at 2 and 16 hours and TGSH at 24 and 72 hours.

Effects of Hg exposure alone versus control on HK-phagocytes revealed an increase in NP-SH at 8, 16, 48 hours, TGSH at 2, 4, 8, 16, 48 hours; whereas a significant decrease in NP-SH at 2 hours, and TGSH at 24 and 72 hours.

Effects of IONM and Hg concomitant exposure on G-phagocytes displayed a significant increase in NP-SH at 24, 48, 72 hours, TGSH at 8, 16, 72 hours; in NP-SH at 2, 8 and 16 hours and TGSH at 48 hours. Effects of IONM exposure alone versus concomitant exposure revealed a significant increase in NP-SH at 2, 4, 16, 24, 48 and 72 hours, TGSH at 2, 4, 72 hours; whereas a decrease in NP-SH at 8 hours and TGSH at 24 and 48 hours. Concomitant effects comparison with Hg alone exhibited an increase in NP-SP at 4, 24 and 72 hours, TGSH at 72 hours; whereas a decrease in NP-SP at 8, 16 and 48 hours, and TGSH at 16, 24 and 48 hours.

3.3.2. Inter-hours comparisons

Inter-hours comparisons revealed no statistically significant difference between the controls during the 72 hours exposure period. Effects of IONM exposure on HK-phagocytes revealed a significant increase in NP-SH at 4 hours (vs. 2 hours), TGSH at 48

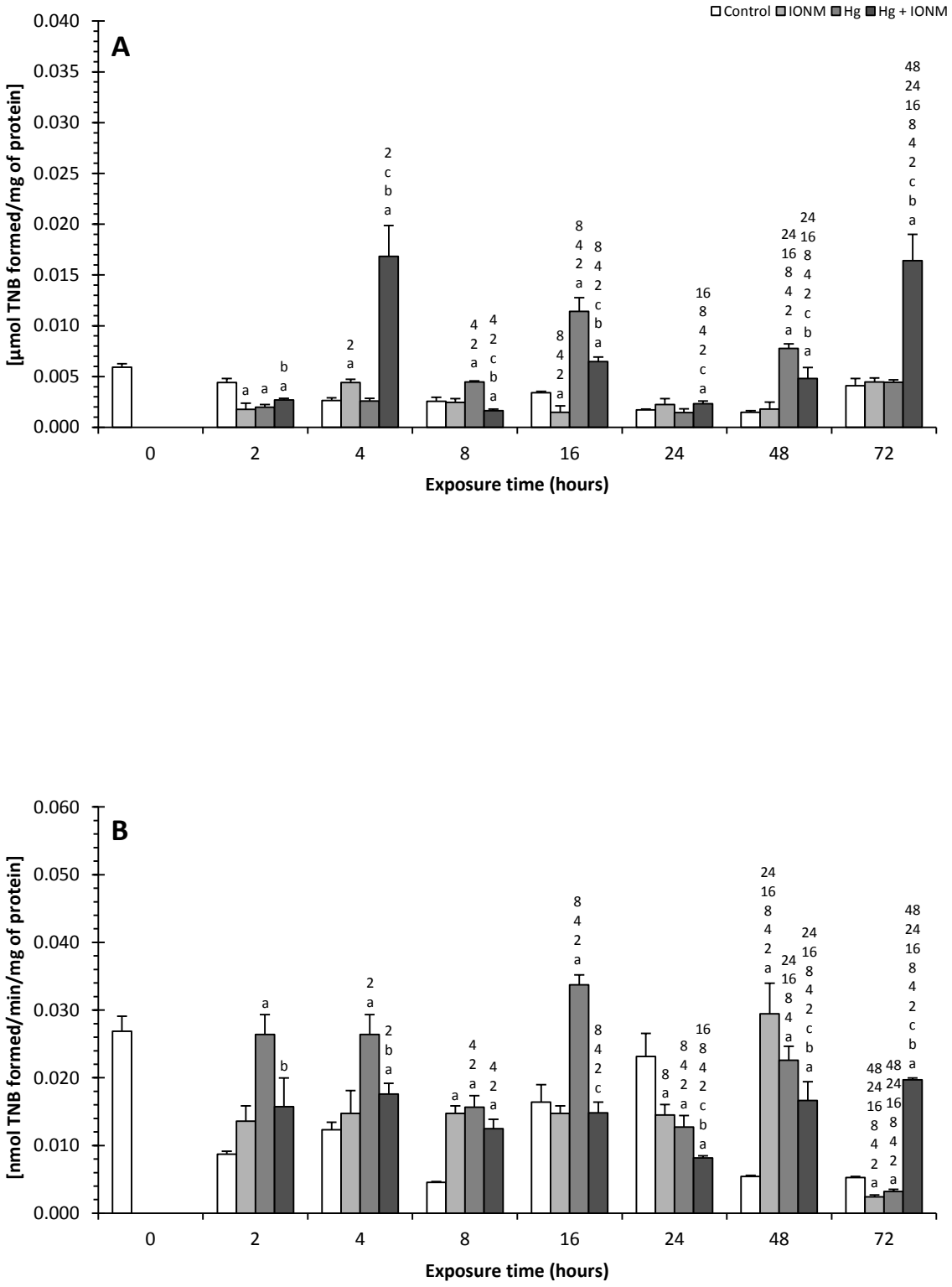

Figure 23. *Anguilla anguilla* L. *in vitro* head kidney resident phagocytes: Total non-protein thiols (A) and Total glutathione (B) under exposure to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone or concomitantly for a period of 72 hours. Inter-group significant differences have been denoted by letters: a (vs. control), b (vs. IONM), c (vs. Hg) and inter-hours significant differences have been marked by numbers: 2 (vs. 2 hours), 4 (vs. 4 hours), 8 (vs. 8 hours), 16 (vs. 16 hours), 24 (vs. 24 hours) and 48 (vs. 48 hours).

hours (vs. 2, 4, 8, 16 and 24 hours); whereas a decrease in NP-SP at 16 hours (vs. 2, 4 and 8 hours) and TGSH at 24 hours (vs. 8 hours) and 72 hours (vs. 2, 4, 8, 16, 24 and 48 hours).

HK-phagocytes exposed to Hg alone revealed a significant increase in NP-SH at 8 hours (vs. 2 and 4 hours), 16 hours (vs. 2, 4 and 8 hours), 48 hours (vs. 2, 4, 8 and 24 hours), TGSH at 4 hours (vs. 2 hours), 16 hours (vs. 2, 4 and 8 hours), 48 hours (vs. 24 hours); whereas a decrease in NP-SH at 48 hours (vs. 16 hours), TGSH at 8 hours (vs. 2 and 4 hours), 24 hours (vs. 2, 4 and 8 hours), 48 hours (vs. 4, 8 and 16 hours) and 72 hours (vs. 2, 4, 8, 16, 24 and 48 hours).

Effects of IONM and Hg concomitant exposure on HK-phagocytes displayed an increase in NP-SH at 4 hours (vs. 2 hours), 16 hours (vs. 2 and 4 hours), 48 hours (vs. 2, 8 and 24 hours), 72 hours (vs. 2, 4, 8, 16, 24 and 48 hours), TGSH at 4 hours (vs. 2 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours), 72 hours (vs. 2, 4, 8, 16, 24 and 48 hours). Moreover, in NP-SH at 8 hours (vs. 2 and 4 hours), 16 hours (vs. 8 hours), 24 hours (vs. 2, 4 and 16 hours), 48 hours (vs. 4 and 16 hours) and TGSH at 8 hours (vs. 2 and 4 hours), 24 hours (vs. 2, 4, 8 and 16 hours) were observed a significant decrease.

3.4. Spleen resident phagocytes (S-phagocytes) responses

3.4.1. Inter-group comparisons

The results based on non-enzymatic antioxidants responses have been depicted in figure 24. *A. anguilla* P-phagocytes *in vitro* exposure to IONM alone in comparison to control revealed a significant increase in NP-SH at 2 hours, TGSH at 2, 4 hours; whereas a significant decrease in NP-SH at 4, 8, 16 and 24 hours, and TGSH at 24 and 48 hours.

Effects of Hg exposure alone versus control on S-phagocytes revealed an increase in NP-SH at 2, 8, 72 hours, TGSH at 2, 4, 8, 16 and 48 hours; whereas a significant decrease in NP-SH at 4, 16 and 48 hours, and TGSH at 24 and 72 hours.

Effects of IONM and Hg concomitant exposure on S-phagocytes displayed a significant increase in NP-SH at 16, 48 and 72 hours, TGSH at 4, 8, 24 and 72 hours; whereas a decrease in NP-SH at 2, 4 and 8 hours, and TGSH at 16 hours. Effects of IONM exposure alone versus concomitant exposure revealed a significant increase in NP-SH at 8, 16, 24, 48 and 72 hours, TGSH at 24, 48 and 72 hours; whereas a decrease in NP-SH at 2

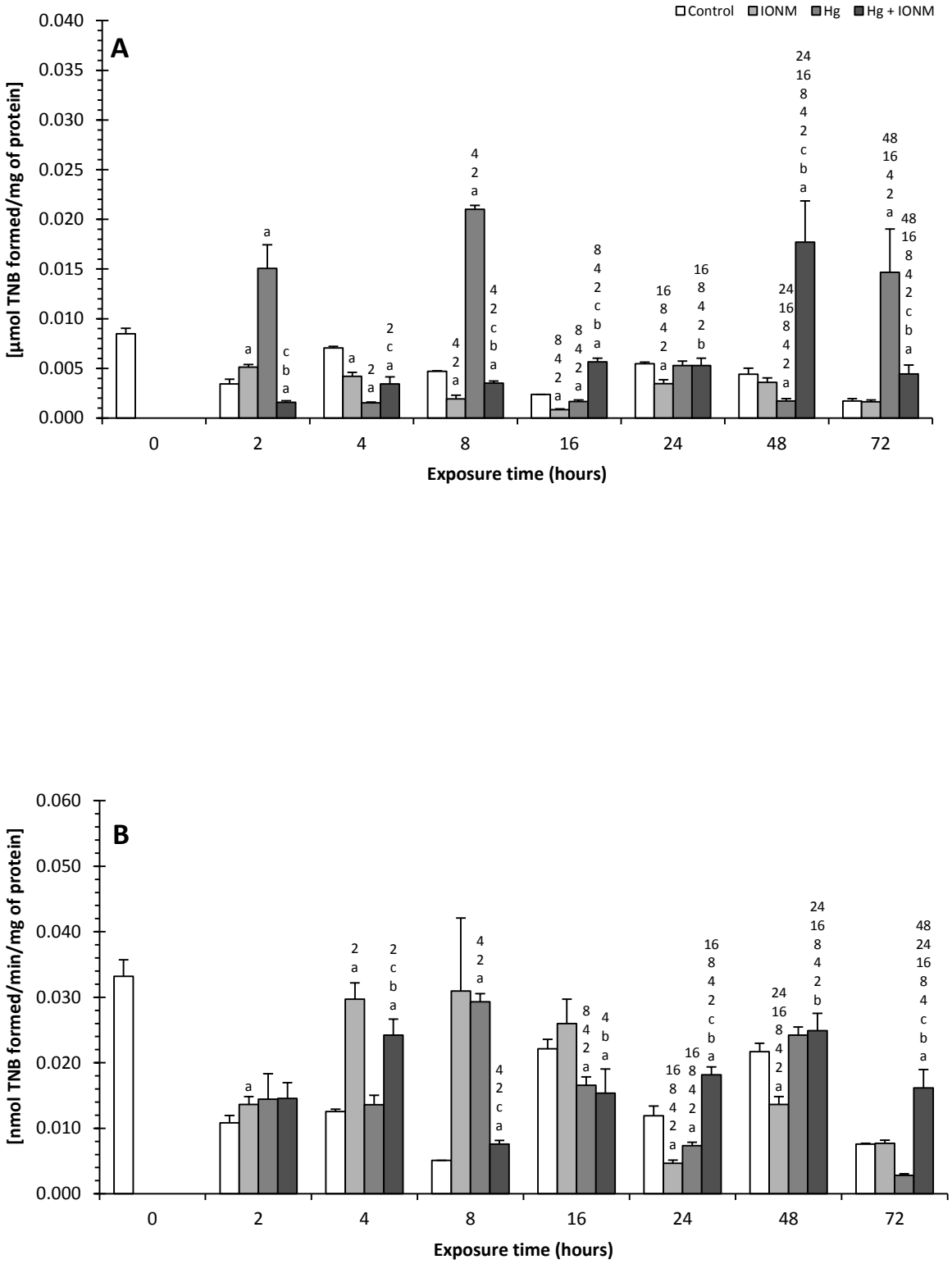

Figure 24. *Anguilla anguilla* L. *in vitro* spleen resident phagocytes: Total non-protein thiols (A) and Total glutathione (B) under exposure to silica coated Fe_3O_4 nanomaterial functionalized with dithiocarbamate (Dtc) groups (IONM) and mercury (Hg) alone or concomitantly for a period of 72 hours. Inter-group significant differences have been denoted by letters: a (vs. control), b (vs. IONM), c (vs. Hg) and inter-hours significant differences have been marked by numbers: 2 (vs. 2 hours), 4 (vs. 4 hours), 8 (vs. 8 hours), 16 (vs. 16 hours), 24 (vs. 24 hours) and 48 (vs. 48 hours).

hours, and TGSH at 4 and 16 hours. Concomitant effects comparison with Hg alone exhibited an increase in NP-SP at 16, 48 and 72 hours, TGSH at 4, 24 and 72 hours; whereas a decrease in NP-SP at 2, 4 and 8 hours, and TGSH at 8 hours.

3.4.2. Inter-hours comparisons

Inter-hours comparisons revealed no statistically significant difference between the controls during the 72 hours exposure period. Effects of IONM exposure on S-phagocytes revealed a significant increase in NP-SP at 24 hours (vs. 4, 8 and 16 hours), TGSH at 4 hours (vs. 2 hours); whereas a decrease in NP-SP at 8 hours (vs. 2 and 4 hours), 16 hours (vs. 2, 4 and 8 hours) and 24 hours (vs. 2 hours) and TGSH at 24 and 48 hours (vs. 2, 4, 8 and 16 hours).

S-phagocytes exposed to Hg alone revealed a significant increase in NP-SH at 8 hours (vs. 2 and 4 hours), 16 hours (vs. 4 hours), 48 hours (vs. 16 hours), 72 hours (vs. 2, 4, 16 and 48 hours), TGSH at 8 and 16 hours (vs. 2 and 4 hours), 8 hours (vs. 2 and 4 hours), 16 hours (vs. 4 hours), 48 hours (vs. 16 hours), 72 hours (vs. 2, 4, 16 and 48 hours); whereas a decrease in NP-SH at 4 hours (vs. 2 hours), 16 hours (vs. 2 and 8 hours), 48 hours (vs. 2, 4, 8 and 24 hours) and TGSH at 16 hours (vs. 8 hours), 24 hours (vs. 2, 4, 8 and 16 hours), 48 hours (vs. 4, 8 and 16 hours) and 72 hours (vs. 2, 4, 8, 16, 24 and 48 hours).

Effects of IONM and Hg concomitant exposure on S-phagocytes displayed an increase in NP-SH at 4 hours (vs. 2 hours), 8 hours (vs. 2 and 4 hours), 16 and 72 hours (vs. 2, 4 and 8 hours), 24 hours (vs. 4, 8 and 16 hours), 48 hours (vs. 2, 4, 8, 16 and 24 hours), 72 hours (vs. 2, 4, 8, 16, 24 and 48 hours) and TGSH at 4 hours (vs. 2 hours), 24 hours (vs. 2, 8 and 16 hours), 72 hours (vs. 8 and 16 hours). Moreover, in NP-SH at 24 hours (vs. 2 hours) and 72 hours (vs. 16 and 48 hours) and TGSH at 8 hours (vs. 2 and 4 hours), 16 hours (vs. 4 hours), 24 hours (vs. 4 hours) and 72 hours (vs. 4, 24 and 48 hours) were observed a significant decrease.

4. Discussion

4.1. Peritoneum exudates phagocytes (P-phagocytes) responses

Glutathione represents the bulk of NP-SH content of the cells, but it was demonstrated that other thiols, namely N-acetyl-L-cysteine, can also play an important role as antioxidant (Jones et al., 1995). Thus, in order to clarify the involvement of other NP-SH, the assessment of both NP-SH and TGSH contents is recommended. Herein, at the end of 2 hours of exposure, IONM caused a significant decrease in NP-SH and increase in TGSH compared to control. However, compared to 2 hours of exposure, significantly decreased pool of TGSH was perceptible during 8 and 24 hours, whereas a decrease in NP-SH was observed at all the exposure time (except 8 hours). A decreased level of cellular TGSH has been reported earlier in MRC-5 cell line cultures (Radu et al., 2010) and cancer cells (human hepatocellular carcinoma, HepG2; human lung adenocarcinoma, A549) (Ahamed et al., 2013) under IONM exposure. Nevertheless, a loss of cellular TGSH has earlier been considered as a sign of oxidative stress which could be a consequence of several processes including: (a) impaired synthesis of TGSH, (b) accelerated export of TGSH and/or (c) conjugation of TGSH to applied compounds (Hirrlinger and Dringen, 2010). Similar to the observations of Radu et al. (2010) reported in MRC-5 cell line cultures, in the current investigation also, compared to control, elevated activities of GSH-utilizing enzyme namely GPX may explain the IONM - mediated decreased level of TGSH pool under 2, 4, 16 and 48 hours of exposure. Also during these hours of exposure, elevated GR activity (vs. control) reflected its insufficiency to maintain TGSH pool-optimum for scavenging reactive oxygen species and protecting membrane lipids and cellular proteins against IONM impact.

In the present investigation, increase in the period of exposure to Hg continuously reflected no signal of LPO induction. Our observations disagree with the findings of Filipak Neto et al. (2008) where the authors reported significant increases in LPO in methyl-Hg exposed *Hoplias malabaricus* hepatocytes. On the perspective of thiol redox system component's modulation, in the present investigation, increase in the period of exposure to Hg disrupted TGSH redox system. Significantly increased TGSH pool was perceptible initially. Moreover, compared to early hours (2 hours) of exposure, TGSH content significantly decreased at 8 and 24 hours but again it increased at 48 reaching to normal at 72 hours. Immune cells are well-known for its oxidative stress sensitivity where

it exhibits insufficiency in handling ROS (Ahmad et al., 2004). A decreased TGSH pool under Hg exposure is in close agreement with the observations reported in the tissue of methyl-Hg exposed mouse (Roos et al., 2009; Stringari et al., 2008) and Hg-exposed fish (Mieiro et al., 2010) where the authors suggested it as an expression of Hg-accrued toxicity (Merad-Boudia et al., 1998). It is possible that enhanced activities of both GST and GPX continuously utilized the reduced TGSH pool during their scavenging reaction with Hg-accrued elevated ROS. However, it is in disagreement with the findings of Mieiro et al. (2010) where the authors observed inhibited activities of GPX and GST in Hg exposed fish. It can further be explained that TGSH may covalently bind with Hg and latter TGSH-Hg conjugates irreversibly get excreted (Franco et al., 2009). The release of TGSH-Hg conjugates may disturb TGSH metabolism (Franco et al., 2009). Since no elevated levels of LPO was noted with the increase in Hg exposure period in the current study, phagocytes cells might have stimulated GPX and GST activities in order to scavenge Hg-lead increased ROS using reduced TGSH pool as a substrate, resulting ultimately into its depleted pool.

As observed under IONM alone exposure, the joint action of IONM+Hg led no elevated damages to membrane-lipids at all hours of exposure. The adoption of the strategy of non-stimulation of thiol-redox system by IONM+Hg-exposed *A. anguilla* P-phagocytes was reflected by significantly decreased trend of NP-SH content and no definite trend of variation in TGSH with increase in the period of exposure. However, compared to 2 hours of IONM+Hg exposure, significantly decreased NP-SH coincided with decreased TGSH content (at 48 and 72 hours). These responses together, point towards the increased use of thiol-redox system for the protection of membrane lipids during increased period of exposure to IONM+Hg when compared to 2 hours. It is important to underline here that during the late hours of exposure (for example 72 hours) significantly decreased trends were displayed by NP-SH traits with IONM+Hg when compared to Hg-exposed group. However during early hours of exposure (for example 2 hours), the concomitant exposure was unable to mitigate the Hg-accrued negative impacts. Hence, the occurrence synergistic decrease between IONM and Hg cannot be ignored.

4.2. Gill-adhered resident phagocytes (G-phagocytes) responses

The trend for thiols increase (NP-SH and TGSH) after IONM exposure observed in G-phagocytes unlike what others have showed after *in vitro* exposure of nanomaterials to both macrophages and transformed human bronchial epithelial cells (Li et al., 2003; Ramkumar et al., 2012). During, the initial hours of exposure, IONM caused a significant increase in NP-SH and no significant changes in TGSH, when compared to control. However, compared to 2 hours of exposure, significantly increased pool of TGSH was perceptible during 8, 24 and 48 hours, whereas no significant decrease in NP-SH was observed at all the exposure time. These findings are identically with early reports, for example, Federici et al. (2007) investigated oxidative stress in rainbow trout (*Oncorhynchus mykiss*) exposed to (TiO_2)-NMs revealing that the concentration of TGSH in the gills of rainbow trout significantly increased after (TiO_2)-NMs exposure.

On the perspective of Hg exposure alone, at the end of the 2 hours of exposure, a decrease was observed in TGSH, whereas NP-SH displayed an increase. With the increase in exposure time, both NP-SH and TGSH content were modulated reaching to either normal (TGSH) or increased (NP-SH) at the late hours of exposure. This biphasic response can be considered as an adaptation to increased detoxification activity. A decreased TGSH pool under Hg exposure is in close agreement with the observations reported in the tissue of methyl-Hg exposed mouse (Roos et al., 2009; Stringari et al., 2008) and Hg-exposed fish (Mieiro et al., 2010) where the authors suggested it as an expression of Hg-accrued toxicity (Merad-Boudia et al., 1998). In contrast, we found a time-dependent increase in thiols level suggesting that increased thiols levels in G-phagocytes serve as an indicator of degree and duration of exposure to Hg. Protective and adaptive roles of GSH against oxidative stress-induced toxicity are well established in aquatic animals (Ahmad et al., 2000).

In concomitant exposure, initially induced NP-SH reached to increased level at the end of the exposure length in comparison to early hours of exposure revealing the same pattern of response as observed for Hg alone. However, in comparison to Hg, a synergism is perceptible at early hours in NP-SH and remained induced at the end of the exposure length (72 hours) suggesting an increased toxicity of IONM when come in contact with Hg co-exposure. Antagonism was also observed at early and late hours of exposure in TGSH content. Thus, Hg induced negative effects seem to be inhibited with the increase in

exposure time, however not to the normal level at the end of the exposure length. These results imply the positive effect of the concomitant exposure (IONM+Hg), which significantly mitigated the said negative impacts of Hg at both early and late hours; thus, the occurrence of antagonism between IONM and Hg should be emphasized.

4.3. Head kidney resident phagocytes (HK-phagocytes) responses

A. anguilla HK-phagocytes displayed decreased NP-SH at 2 hours exposure to IONM; thereby, reaching normal at the end of exposure length (72 hours); whereas, TGSH reflected an opposite trend than NP-SH with the increase of exposure length. Similar observations were perceptible on thiols in Hg exposed HK-phagocytes. Thus, the explanation based on the adaptation to increased detoxification activity as suggested for G-phagocytes can be implied for these biphasic responses of HK-phagocytes NP-SH and TGSH content. These findings are also corroborated with Guo et al. (2011) showing that MWCNTs were capable to affect cellular redox status producing cytotoxic effects in endothelial cells (ECs).

In concomitant exposure, initially no induced TGSH and decreased NP-SH reached to normal at the end of exposure (72 hours). Moreover, the thiols increased were significant in comparison to early hours of IONM+Hg exposure. Thus, in comparison to Hg, an antagonism (TGSH) is perceptible at 72 hours in TGSH and synergism in NP-SH suggesting an increase in toxicity of IONM when come in contact with Hg co-exposure.

4.4. Spleen resident phagocytes (S-phagocytes) responses

After 2 hours of exposure, a significant increase was perceptible in TGSH and NP-SH reaching to normal at the end of the exposure. On the perspective of Hg exposure alone, the TGSH showed an initial increase with no decrease at the end of exposure. Thus, the explanation based on the adaptation to increased detoxification activity as suggested for G-phagocytes can be implied for these modulatory responses of HK-phagocytes NP-SH and TGSH content. However, under co-exposure of IONM and Hg, modulatory response of TGSH early and late hours of exposure clearly indicates that the interaction of IONM with Hg alter the toxicity potential of either IONM and/or Hg. Thus, compared to 2 hours of IONM+Hg exposure, significantly increased TGSH was observed. Moreover, in comparison to Hg, an antagonism is perceptible at both early and late hours of exposure

suggesting a decrease in toxicity of Hg when come in contact with IONM co-exposure. However, on the perspective of NP-SH content, a synergistic response was perceptible at late hours of exposure suggesting that the Hg induced negative effects were induced with the increase in exposure time.

5. Conclusion

Major findings of the present investigation may be summarized in the following points:

- *A. anguilla* phagocytes displayed a differential extent of toxicity at the end of different periods of exposure to IONM, Hg or IONM+Hg where a differential thiol redox system components modulation was perceptible.
- A period of exposure-dependency was exhibited by IONM alone and IONM+Hg joint exposures-accrued impacts on *A. anguilla* phagocytes.
- A synergism is perceptible at early hours in G-phagocytes (NP-SH and TGSH) and remained induced at the end of the exposure length (72 hours) in concomitant exposure when compared to Hg suggesting a modulatory response of IONM when come in contact with Hg co-exposure. A negative increase in toxicity was also observed at early and late hours of exposure in TGSH content in P-, HK- and S-phagocytes.
- In P-phagocytes after exposure to IONM, an initial increase followed by a loss of cellular TGSH was a sign of oxidative stress which could be a consequence of several processes including: (a) impaired synthesis of TGSH, (b) accelerated export of TGSH and/or (c) conjugation of TGSH to applied compounds.
- Increase in the period of exposure to Hg, a modulation of GSH redox system in P-, G-, HK- and S-phagocytes phagocytes is perceptible suggesting an adaptation to increased detoxification activity. Moreover, in P-phagocytes, increase in the period of exposure to Hg continuously reflected no signal of LPO induction. Thus, P-phagocytes cells might have stimulated GPX and GST activities in order to scavenge Hg-lead increased ROS using reduced TGSH pool as a substrate, resulting ultimately into a difference in the GSH pool.

6. References

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GENERAL DISCUSSION

The general discussion has been made taking into account the critical analysis of the overall thesis as well as specific discussion of each chapter.

1. *In vitro* laboratory study

The works analysis in this point includes chapters 1, 2, 3 and 4 and corresponds to the *in vitro* laboratory studies in *A. anguilla* L. phagocytes (P-, G-, HK- and S-phagocytes) with contaminants IONM, Hg and IONM+Hg: viability, phagocytosis index and capacity (Chapter 1); lipid peroxidation (LPO) and respiratory oxygen burst (OBA) (Chapter 2); catalase (CAT), glutathione peroxidation (GPX), glutathione reductase (GR), glutathione S-transferase (GST) (Chapter 3) and non-protein thiols (NP-SH) and total glutathione (TGSH) (Chapter 4).

1.1. Immonotoxicity endpoints viz. cells viability and phagocytosis (Phagocytic index, phagocytic capacity) in fish

This point correspond the *Anguilla anguilla* L. phagocytes responses following *in vitro* exposure to silica coated iron oxide nanomaterial functionalized with dithiocarbamate with or without mercury (Hg) co-exposure (chapter 1), rewarding one of the objectives whose advantage had previously been presented in point 5 of the general introduction. Immonotoxicity endpoints viz. cells viability and phagocytosis (phagocytic index, phagocytic capacity) were evaluated in phagocytes isolated from peritoneum (P-phagocytes), gill (G-phagocytes), head kidney (HK-phagocytes) and spleen (S-phagocytes) of *A. Anguilla* L. taking into account their ability to mirror the potential biological impacts of various metals and non-metals (Ahmad et al., 2003; 2004).

A. anguilla P-, G-, HK-and S-phagocytes displayed a differential extent of immunotoxicity at the end of different periods of exposure to IONM, Hg or IONM+Hg, where a differential modulation in viability and phagocytosis was perceptible. In general, viability was induced only in G- and P-phagocytes. In P-phagocytes viability was induced (2 and 8 hours) and, clearly, indicates that no apparent cytotoxicity was perceptible for IONM suggesting that cells were metabolically active and resistant to IONM exposure. This result was consistent with other reports that nanomaterials do not show obvious acute lethal toxicity to cells within 24 hours of incubation (Lima et al., 2011; Semete et al., 2010). G-phagocytes revealed similar patterns of response in terms of viability and

phagocytosis which may be related to the similarities in the lineage (figure 10, 11 and 12) of these cells (Ahmad et al., 1998). An overall decrease in phagocytosis was observed in HK-phagocytes suggesting that the phagocytosis modulation was independent to the drop in viability in phagocytes.

Similarly, increase or decrease in viability in phagocytosis was observed for Hg exposure; however at different point of exposure duration (P-, G-, HK- and S-phagocytes). An opposite trend is perceptible in G-phagocytes in comparison to P-phagocytes suggesting that phagocytosis response is at the drop of viability decrease after Hg exposure. Concerning, P-phagocytes a decrease in phagocytosis was observed after 2 hours of exposure to Hg; whereas, an increase in viability and phagocytosis index was perceptible after 16 hours. According to Sauvé et al. (2002), *in vitro* toxicity of Hg may exhibit: i) lack of cell cytotoxicity and stimulation of the phagocytosis; ii) lack of cell cytotoxicity and normal phagocytosis; iii) relatively low metal-related cytotoxicity and dramatic inhibition of phagocytosis; and iv) relatively high cytotoxicity accompanied by a drastic inhibition of the phagocytosis in bivalves. Thus, the previous explanation seems to be applicable in the current *in vitro* study on fish reflecting a specific interaction along the time producing synergistic and antagonistic effects in fish phagocytes. A decrease in phagocytic index and capacity may strengthen the explanation given above for the no induction in cells viability and phagocytosis modulation along the time. Moreover, a decrease in phagocytosis is well documented in the literature following either *in vivo* or *in vitro* Hg exposure (Fournier et al., 2001). HK- and S-phagocytes revealed opposite scenarios in viability and phagocytosis. Thus, in viability an increase in S-phagocytes (16 and 24 hours) and decrease in HK-phagocytes (8 and 16 hours) as well as in phagocytosis an increase in S-phagocytes (4, 24 and 72 hours) and decrease (2, 4, 8, 48 and 72 hours) was observed.

Under IONM and Hg concomitant exposure it was observed periods of exposure-dependency exhibited by IONM alone and IONM+Hg joint exposures accrued impacts on *A. anguilla* phagocytes. During, IONM exposure alone was leading to an acute response in terms of viability increase in P-phagocytes and modulated phagocytic activity in P- and S-phagocytes during 2 hours of exposure; whereas, IONM lead to a chronic immunotoxicity during 72 hours exposure only in S-phagocytes. However, IONM+Hg exposure lead to both acute and chronic response in terms of modulated phagocytic activity with no change

in viability in P-, HK- and S-phagocytes only. Concerning, exposure to Hg, an increase in the period of disrupted phagocytic activity of P-, HK- and S-phagocytes; whereas an increase in P- and decrease in HK- and S- phagocytes was perceptible at 72 hours of exposure. During early hours of exposure (2 hours), the concomitant exposure was unable to mitigate the Hg-accrued negative impacts, which was evidenced by significantly decreased phagocytic capacity. These results imply the positive effect of the concomitant exposure (IONM+Hg), which significantly mitigated the said negative impacts of Hg at early hours. Hence, the occurrence antagonism between IONM and Hg cannot be ignored which is further evidenced as a principle of IONM-mediated Hg-IONM complex formation (Girginova et al., 2010; Tavares et al., 2013).

1.2. The role of *A. anguilla* L. phagocytes in reactive oxygen species production following *in vitro* IONM with or without mercury co-exposure

A. anguilla phagocytes displayed a differential extent of reactive oxygen species (ROS) production and lipid peroxidation (LPO) induction at the end of different periods of exposure to IONM, Hg or IONM+Hg. In P-phagocytes, no ROS production (except 8 hours) and no LPO increase, when exposed to IONM was observed suggesting that the innate immune modulation is well tuned with the phagocytes function responses in maintaining the overall cells tolerance. Karlsson et al. (2008, 2009) revealed that magnetite (Fe_3O_4) has been shown to cause higher levels of oxidative damage in A549 human lung epithelial cell line in the absence of decreased cell viability as compared to maghemite (γ-Fe_2O_3) remaining to its potential to undergo oxidation In G-phagocytes, statistically insignificant oxidative burst activity (OBA) induction during the entire exposures length (more pronounced at 8 hours) was observed. However, a significant LPO increase was perceptible at 8 hours as clear indication of the phagocytic induction and its association to peroxidative damage. Thus, the current observation strengthens the explanation that both gill and peritoneum phagocytes have the same lineage (figure 10 and 11). Regarding, along the time it was observed that there is either no OBA induction (P- and S-phagocytes) or LPO which may suggest that in the presence of lower ROS levels, adaptive mechanisms might have been activated, providing a better protection to IONM and Hg co-exposed phagocytes.

Similar to the response of IONM, Hg exposed phagocytes reflected increased or decrease OBA and LPO. In P-phagocytes was observed a decrease in OBA (at 8 hours) and no LPO suggesting the same kind of phagocytes response towards Hg maintaining the cells viability. However, in G-phagocytes was observed a deleterious membrane-damaging effect on G-phagocytes since after 2, 8 and 48 hours, the LPO response were significantly increased in gill. Moreover, a significant increase was also perceptible in LPO to the same exposure length (insignificant at 2 hours) indicating the peroxidative damage as a cause of ROS production. These findings were reported by Ahmad et al. (2003, 2004) and it was observed that the release of large amounts of ROS by activated phagocytes and its association with peroxidative damage may become a potential cause of these differences in antioxidants besides the direct oxidative damage inflicted by the Hg.

Under IONM and Hg concomitant exposure, the OBA induction was concomitantly associated with LPO induction in gill IONM+Hg (8 hours). Observation of no ROS production and LPO increase or vice e versa, suggest that the innate immune modulation is well tuned with the phagocytes function responses in maintaining the overall cells tolerance. Concerning, P-phagocytes, OBA increase at 8 hours went to normal with Hg co-exposure to the same exposure length. The occurrence of no OBA induction and either LPO decrease or no LPO along the time can suggest that in the presence of lower ROS levels, adaptive mechanisms might have been activated, providing a better protection to IONM and Hg co-exposed phagocytes. During, the late hours of exposure, an induction was observed in G-phagocytes OBA after exposure to (IONM+Hg) suggesting that the concomitant exposure was unable to mitigate the Hg-accrued negative impacts. Moreover, the results imply that the negative effect of the concomitant exposure (IONM+Hg), significantly increased the said negative impacts of Hg at early hours. Moreover, phagocytes isolated from gill and spleen was more vulnerable to the negative effect (LPO increase at 8 hours for G- and at 4, 24 hours for S-phagocytes) of the concomitant exposure (IONM+Hg) in comparison to the Hg exposure alone. In S-phagocytes was observed negative effect of the concomitant exposure (IONM+Hg), which significantly enhanced the said negative impacts of Hg at early (4 hours) and mitigated the negative response of the Hg at late hours (72 hours); thus, the occurrence of both synergism (4 hours) and antagonism (72 hours) between IONM and Hg cannot be ignored. Overall, it was observed

in P-, G-, HK- and S- phagocytes a period of exposure-dependency was exhibited by IONM alone and Hg joint exposures accrued impacts on *A. anguilla* phagocytes.

1.3. *A. anguilla* L. phagocytes enzymatic antioxidants responses following *in vitro* IONM with or without mercury co-exposure

A. anguilla phagocytes displayed a differential extent of antioxidants enzymes (CAT, GPX, GR and GST) induction at the end of different periods of exposure to IONM, Hg or IONM+Hg. In P-phagocytes displayed a differential response of defence endpoints at the end of different periods of exposure to IONM. However, the damage endpoint was accompanied with modulation of the enzymatic antioxidants contributing to the control of cellular redox by scavenging harmful intracellular ROS under normal and adverse conditions. Thus, at the end of the 2 hours of exposure, a decrease in CAT and GPX was observed; whereas an increase was perceptible in GR and GST. Our report coincides with early studies revealing that fullerenes, CNT, and metal oxides are capable to induce oxidative stress (Bonner et al., 2007; Thannickal et al., 2000). Comparing, the results of G- and HK-phagocytes with previous description these cells with the increase in exposure time, CAT and GST remained constant; whereas, GPX and GR showed a modulatory response reaching to normal at late hours of exposure. Concerning damage endpoints response, no LPO increase (P-phagocytes) was observed with increase in the period of exposure to IONM; whereas LPO increase (G-phagocytes) was observed only at 8 hours of exposure to IONM (as depicted in Chapter 2). The LPO increase with enzymatic antioxidants induction may strengthen the explanation of Cossu et al. (2000) that LPO cannot be predicted only on the basis of antioxidants modulation. Thus, the depletion of other glutathione independent antioxidants can also be suggested for the current observation. Our report coincides with early studies revealing that fullerenes, CNT, and metal oxides are capable to induce oxidative stress (Bonner et al., 2007; Thannickal et al., 2000).

On the perspective of Hg exposure alone, in P-phagocytes at the end of the 2 hours of exposure, a decrease in GPX and increase in GST was observed suggesting that the protection against ROS induction is occurring only through the GST activity. Similar, in G-phagocytes antioxidants induction was observed at initial hours of exposure and remained constant with the increase of exposure time (except decrease in GPX at 16 and

24 hours). Interestingly, the increase in antioxidant enzymes was corroborated with OBA and LPO increase suggesting that the antioxidants are not efficient to protect the cells against ROS. The induction of enzymes is coincided with the findings of Zhu et al. (2008 a, b) who revealed that fullerene exposed for 32 days in goldfish gill cells (*Carassius auratus*) induced antioxidant enzymes superoxide dismutase and catalase.

The increase in GST activity may be due to the fact that GST is used as a cofactor for GPX, thus the decrease of GPX resulting from oxidative stress induced by Hg requires more co-factor and hence, the levels of GST may also enhanced. With the increase in exposure time the GST activity in the Hg alone exposed phagocytes is remained constant for a period of 72 hours. Similar kind of increase in the GST activity was observed in the kidney of fish (*Liza aurata*) (Mieiro et al., 2011). Thus, GST is considered a good indicator of the presence of contaminants, such as Hg, in the aquatic environment (Elia et al., 2003). Increased GST activity has been associated with metal exposure in several fish species (Mager et al., 2008). In particular in G-phagocytes was reported the Hg significantly increased by Vieira et al. (2009). Moreover, other authors hypothesised some points for the understanding of induction of GST when exposed Hg to fish gill cell such as (i) since GST is a cofactor for glutathione peroxidase, the increase of GPX to face oxidative stress requires more co-factor and, thus, the levels of GST are also enhanced, and (ii) since GST determinations were performed in gills that constitute a first barrier against the entrance to toxicants in fish body and GST have the capability of bind and/or store or transport substances, the increase of GST levels observed may correspond to a first attempt to overcome metal stress by producing a high amount of enzyme that will be then available to bind Hg, decreasing their local concentration and, therefore, their uptake by the organisms (Liu et al., 2006; Sanchez et al., 2005; Vieira et al., 2009).

Under IONM alone exposure, enzymatic antioxidants protection responses displayed hours of exposure dependency in G- and S-phagocytes where, compared to control, an insufficiency of elevated CAT, GPX, GST, NP-SH and TGSH was clearly depicted for the maintenance of pro- and antioxidant balance optimum for scavenging ROS and protecting membrane lipids against IONM impact. The adoption of the strategy of enzymatic antioxidant modulation in G-phagocytes at 4 hours was reflected by significantly decrease in CAT and increase in GR, GPX and GST; whereas at 8 hours, the significantly decrease in CAT and GPX and increase in GR and GST was perceptible. The

strategy adopted for HK-phagocytes at 2 hours was reflected by decrease in all the studied enzymes. In addition, strategies adopted by S-phagocytes at 24 hours were reflected by an increase in CAT, GR, GST and NP-SH. Thus, these responses together, point towards the enzymatic antioxidants defense failure for the protection of membrane lipids during those periods of exposure to IONM+Hg. Though, it is important to underline here that during the late hours of exposure in P-, G-, HK- and S-phagocytes (for example 72 hours) the results imply the positive effect of the concomitant exposure (IONM+Hg) which significantly mitigated the said negative impacts of Hg. Hence, the occurrence antagonism between IONM and Hg cannot be overlooked.

1.4. *A. anguilla* L. phagocytes non-enzymatic antioxidants responses following *in vitro* IONM with or without mercury co-exposure

A. anguilla phagocytes displayed a differential extent of toxicity at the end of different periods of exposure to IONM, Hg or IONM+Hg where a differential thiol redox system components modulation was perceptible. In P-phagocytes after exposure to IONM, an initial increase followed by a loss of cellular TGSH was a sign of oxidative stress which could be a consequence of several processes including: (a) impaired synthesis of TGSH, (b) accelerated export of TGSH and/or (c) conjugation of TGSH to applied compounds. Glutathione represents the bulk of NP-SH content of the cells, but it was demonstrated that other thiols, namely N-acetyl-L-cysteine, can also play an important role as antioxidant (Jones et al., 1995). In P-phagocytes, at the end of 2 hours of exposure, IONM caused a significant decrease in NP-SH and increase in TGSH compared to control. However, compared to 2 hours of exposure, significantly decreased pool of TGSH was perceptible during 8 and 24 hours, whereas a decrease in NP-SH was observed at all the exposure time (except 8 hours). A decreased level of cellular TGSH has been reported earlier in MRC-5 cell line cultures (Radu et al., 2010) and cancer cells (human hepatocellular carcinoma, HepG2; human lung adenocarcinoma, A549) (Ahamed et al., 2013) under IONM exposure. Moreover, a period of exposure-dependency was exhibited by IONM alone and IONM+Hg joint exposures-accrued impacts on *A. anguilla* phagocytes. The trend for thiols increase (NP-SH and TGSH) after IONM exposure observed in G-phagocytes unlike what others have showed after *in vitro* exposure of nanomaterials to both macrophages and transformed human bronchial epithelial cells (Li et al., 2003; Ramkumar et al., 2012). Federici et al.

(2007) investigated oxidative stress in rainbow trout (*Oncorhynchus mykiss*) exposed to (TiO_2)-NMs revealing that the concentration of TGSH in the gills of rainbow trout significantly increased after (TiO_2)-NMs exposure. In case of HK-phagocytes displayed decreased NP-SH at 2 hours exposure to IONM; thereby, reaching normal at the end of exposure length (72 hours); whereas, TGSH reflected an opposite trend than NP-SH with the increase of exposure length.

On the perspective of Hg exposure alone, phagocytes revealed an increase or decreased trend in NP-SH and TGSH. P-phagocytes at the end of the 2 hours of exposure, a decrease was observed in TGSH, whereas NP-SH displayed an increase. With the increase in exposure time, both NP-SH and TGSH content were modulated reaching to either normal (TGSH) or increased (NP-SH) at the late hours of exposure. A decreased TGSH pool under Hg exposure is in close agreement with the observations reported in the tissue of methyl-Hg exposed mouse (Roos et al., 2009; Stringari et al., 2008) and Hg-exposed fish (Mieiro et al., 2010) where the authors suggested it as an expression of Hg-accrued toxicity. In contrast, we found a time-dependent increase in thiols level suggesting that increased thiols levels in G-phagocytes serve as an indicator of degree and duration of exposure to Hg. Protective and adaptive roles of GSH against oxidative stress-induced toxicity are well established in aquatic animals (Ahmad et al., 2000). G-phagocytes compared to early hours (2 hours) of exposure, TGSH content significantly decreased at 8 and 24 hours but again it increased at 48 reaching to normal at 72 hours. Immune cells are well-known for its oxidative stress sensitivity where it exhibits insufficiency in handling ROS (Ahmad et al., 2004). Moreover, no elevated levels of LPO was noted with the increase in Hg exposure period in the current study, phagocytes cells might have stimulated GPX and GST activities in order to scavenge Hg-lead increased ROS using reduced TGSH pool as a substrate, resulting ultimately into its depleted pool. Our observations disagree with the findings of Filipak Neto et al. (2008) where the authors reported significant increases in LPO in methyl-Hg exposed *Hoplias malabaricus* hepatocytes. Consequently, increase in the period of exposure to Hg, a modulation of GSH redox system in P-, G-, HK- and S-phagocytes phagocytes is perceptible suggesting an adaptation to increased detoxification activity. Moreover, in P-phagocytes, increase in the period of exposure to Hg continuously reflected no signal of LPO induction. Thus, P-phagocytes cells might have stimulated GPX and GST activities in order to scavenge Hg-

lead increased ROS using reduced TGSH pool as a substrate, resulting ultimately into a difference in the GSH pool.

As observed under IONM alone exposure, the joint action of IONM+Hg led no elevated damages to membrane-lipids at all hours of exposure. The adoption of the strategy of non-stimulation of thiol-redox system by IONM+Hg-exposed *A. anguilla* P-phagocytes was reflected by significantly decreased trend of NP-SH content and no definite trend of variation in TGSH with increase in the period of exposure. A synergism is perceptible at early hours in G-phagocytes (NP-SH and TGSH) and remained induced at the end of the exposure length (72 hours) in concomitant exposure when compared to Hg suggesting a modulatory response of IONM when come in contact with Hg co-exposure. A negative increase in toxicity was also observed at early and late hours of exposure in TGSH content in P-, HK- and S-phagocytes.

2. Final considerations and future perspectives

Based on the results of this dissertation *Anguilla anguilla* L. appears to be a useful model for investigating immunotoxicological effects of IONM and their interference with the co-exposure of a pervasive environmental contaminant such as Hg. This dissertation provided essential information about fish innate immune responses to IONM; determined the potential biomarkers of IONM presence in the fish organism and mode of actions; and evaluated realistic impact scenarios of environmentally relevant concentration of iron oxide nanomaterials.

Complementary immune cells from different organs (peritoneum, gill, head kidney and spleen) were isolated from *A. anguilla* and relevant cells (phagocytes) showing adherence property. Phagocytes were *in vitro* deployed for 2 until 72 hours for the assessment of effects of IONM on phagocytes function of the *A. anguilla* through viability, phagocytosis (phagocytic index and phagocytic capacity) and oxidative burst activity.

A. anguilla phagocytes displayed a differential extent of immunotoxicity at the end of different periods of exposure to IONM, Hg or IONM+Hg, where a differential modulation in viability and phagocytosis was perceptible. In general, viability was induced only in P- and G-phagocytes, whereas an overall decrease in phagocytosis was observed in HK-phagocytes suggesting that the phagocytosis modulation was independent to the drop in viability of phagocytes

A period of exposure-dependency was exhibited by IONM alone and IONM+Hg joint exposures accrued impacts on *A. anguilla* phagocytes. IONM exposure alone lead to an acute response in terms of viability increase in P-phagocytes and modulated phagocytic activity in P-, and S-.phagocytes during 2 hours of exposure; whereas, IONM lead to a chronic immunotoxicity during 72 hours exposure only in S-phagocytes. However, IONM+Hg exposure lead to both acute and chronic response in terms of modulated phagocytic activity with no change in viability in P-, HK- and S-phagocytes only. Increase in the period of exposure to Hg disrupted phagocytic activity of P-, HK- and S-phagocytes, an increase in P- and decrease in HK- and S- phagocytes was perceptible at late hours of exposure. The occurrence of synergism between IONM and Hg was evidenced at 72 hours by significantly increasing trends of phagocytosis increase, which imply the positive effect of the concomitant exposure (IONM+Hg) against Hg-accrued negative impacts when compared to early hour of exposure (2 hours).

As a cornerstone of the innate immune response, phagocytes actively seek out, ingest, and destroy pathogenic microorganisms. To achieve this essential role in host defense, phagocytes deploy a potent arsenal that includes oxidants having a dual function. On one hand, they function as potent antimicrobial agents by virtue of their ability to kill microbial pathogens directly. On the other hand, they participate as signalling molecules that regulate diverse physiological signalling pathways in phagocytes. Increased P-phagocytes activation may be related more to the ability of phagocytes to interact with IONM directly since excess production of ROS was observed in the current study only at 8 hours of exposure. Moreover, the OBA induction was concomitantly associated with LPO induction only in gill after exposure to Hg (8 and 48 hours) and IONM+Hg (8 hours) displaying a differential extent of ROS production and LPO induction at the end of different periods of exposure to IONM, Hg or IONM+Hg. Observation of no ROS production and LPO increase or viz. carbon nanotubes, suggest that the innate immune modulation is well tuned with the phagocytes function responses in maintaining the overall cells tolerance. A period of exposure-dependency was exhibited by IONM alone and Hg joint exposures accrued impacts on *A. anguilla* phagocytes. Along the time it was observed that there is either no OBA induction (P- and S-phagocytes) or LPO which may suggest that in the presence of lower ROS levels, adaptive mechanisms might have been activated, providing a better protection to IONM and Hg co-exposed phagocytes. At late hours of

exposure, an induction was observed in G-phagocytes OBA after exposure to (IONM+Hg) suggesting that the concomitant exposure was unable to mitigate the Hg-accrued negative impacts. Moreover, the results imply that the negative effect of the concomitant exposure (IONM+Hg), significantly increased the said negative impacts of Hg at early hours. Moreover, phagocytes isolated from gill and spleen was more vulnerable to the negative effect (LPO increase at 8 hours for G- and at 4, 24 hours for S-phagocytes) of the concomitant exposure (IONM+Hg) in comparison to the Hg exposure alone.

A. anguilla displayed damage viz. OBA and/or LPO induction at the end of different periods of exposure to IONM, Hg or IONM+Hg which was accompanied with a differential modulation of enzymatic and non-enzymatic antioxidants in P-, G-, HK- and S-phagocytes. Period of exposure-dependent responses of both damage and defense endpoints were revealed under IONM alone and IONM+Hg joint exposures.

Under IONM alone exposure, the occurrence of no OBA induction and either LPO decrease or no LPO along the time can suggest that in the presence of lower ROS levels probably due to induction of GR and GST, adaptive mechanisms might have been activated, providing a better protection to IONM exposed phagocytes. However, antioxidants protection responses displayed hour of exposure dependency in G- and S-phagocytes where, compared to control, an insufficiency of elevated CAT, GPX, GST, NP-SH and TGSH was clearly depicted for the maintenance of pro- and antioxidant balance optimum for scavenging ROS and protecting membrane lipids against IONM impact. As no or increased LPO was observed under IONM alone exposure condition, the joint action of IONM+Hg led to elevated damages to membrane-lipids at 4 and 8 hours (G-phagocytes), 2 hours (HK-phagocytes) and 24 hours (S-phagocytes) of exposure. The adoption of the strategy of thiol-redox system modulation in *A. anguilla* G-phagocytes at 4 hours was reflected by significantly decrease in CAT and increase in GR, GPX and GST; whereas at 8 hours, the significantly decrease in CAT and GPX and increase in GR and GST was perceptible. The strategy adopted for HK-phagocytes at 2 hours was reflected by decrease in all the studied enzymes and NP-SH. In addition, strategies adopted by S-phagocytes at 24 hours were reflected by an increase in CAT, GR, GST and NP-SH. These responses together, point towards the antioxidants defense failure for the protection of membrane lipids during those periods of exposure to IONM+Hg. However, it is important to underline here that during the late hours of exposure (for example 72 hours) the results

imply the positive effect of the concomitant exposure (IONM+Hg) which significantly mitigated the said negative impacts of Hg. Hence, the occurrence antagonism between IONM and Hg cannot be ignored which is further evidenced as a principle of IONM-mediated Hg-IONM complex formation. Overall, the observations of this study open up new insight into the areas of evaluation of immune defence mechanisms in fish exposed to IONM and recommend that the interactions between IONM and other conventional anthropogenic contaminants should be considered while interpreting the fish immunotoxicity responses to IONM exposure in a multi-pollution state.

2.1. 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 nano-immunotoxicology; ecological risk assessment of nanomaterials in aquatic ecosystems; and eco-friendly engineering of nanomaterials. For example results from the Chapter 1 and 2 can lead to future investigations of general innate immune responses in *A. anguilla*. Future studies can be directed in investigating phagocytes response toward different preparations of lipopolysaccharides and their most common contaminants – peptidoglycans with the goal of understanding how do fish recognize antigen. In order to exploit the immune model of *A. anguilla* 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 3 and 4 have revealed the enzymatic and non-enzymatic antioxidants responses for investigating the effect of environmentally relevant concentrations of IONM. 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 *A. anguilla* would receive the dose of IONM through food and water, while the outbreak of antigen is closely monitored in control and experimental mesocosms.

Finally, Chapter 1-4 provided a complete mode of action of IONM immunotoxicity towards fish phagocytes. Future research should utilize this information in engineering eco-friendly IONM which would replace the nanomaterials that are currently used in various industrial products and applications.

3. Remarks

The findings of the above research work will be submitted within a month in the peer-reviewed journals "Acta biomaterial" and "Chemosphere" with the title given below.

3.1. Papers under preparation

- *Anguilla Anguilla* L. phagocyte innate immune responses modulation in different organs following silica-coated iron oxide nanoparticles exposure with or without mercury co-exposure **(Acta biomaterial)**

- *Anguilla anguilla* L. phagocytes glutathione redox system significance for the control of silica-coated magnetite nanoparticles with or without mercury co-exposures mediated oxidative stress **(Chemosphere)**

3.2. Paper submitted

- Mohmood I., Ahmad, I., Saleem, M., Costa L., Lopes, C. B., Trindade, T., Duarte, A. C., Pereira, E. Silica-coated iron oxide nanoparticles *in vitro* genotoxicity assessment and its interference with mercury co-exposure in European eel *Anguilla anguilla* L. (**Science of the Total Environment (MS# STOTEN-D-13-02938))**

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