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Ph.D. Thesis

Toxicity evaluation of new engineered nanomaterials in model organisms

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Summary

ABSTRACT

According to the definition adopted by European Commission in 2011 a nanomaterial (NM) is *"a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm"* (European Commission, 2011/696/EU).

NM exhibit peculiar characteristics (*e.g*. small size, large surface area to mass ratio, shape, surface charge, reactive surface groups, state of agglomeration) that confer them properties substantially different from those of the bulk particles of the same composition.

Due to their widespread use in the consumer and industrial products, NMs can be released into the environment and it has been raised concern of the scientists about this question (Royal Society and the Royal Academy of Engineering, 2004).

The effects that NMs have on aquatic organisms depend on their characteristics influenced by environmental parameters. NMs enter the aquatic organisms mainly through the epithelial surfaces (such as gills, skin) or direct ingestion (Moore, 2006).

After crossing the cell membrane, NMs may be stored in vesicles, mitochondria and additional organelles within epithelial cells. They may generate reactive oxygen species, oxidative stress, cytotoxicity, apoptosis and necrosis (Oberdörster *et al*., 2005).

Ecotoxicological tests of NMs should first consider the behaviour of NMs in the aquatic environment and the conditions that may influence aggregation state. For example, some NMs are almost impossible to disperse in water by physical methods such as sonication or stirring and may require the use of a dispersing agent. The choice of dispersant is problematic since some of the best dispersants from a chemistry point of view are also toxic to organisms.

The potential for NMs to cause oxidative injury in fish and invertebrates remains controversial. Bar-Ilan *et al*., 2009 showed that silver nanoparticles (AgNPs) induced almost 100% mortality in larvae of *Danio rerio* after acute exposure and a variety of embryonic morphological malformations were observed.

A study showed that gold nanoparticles (AuNPs) are non toxic at the employed concentrations and do not cause obvious abnormalities in developing zebrafish embryos (Asharani *et al*., 2010).

Zhu *et al*. (2009) observed effects on mortality and immobility on *D. magna* in the case of titanium nanoparticles (TiO_2NPs) nanoparticles smaller than 20 nm.

Currently there is a lack of knowledge about long-term risks and potential mechanisms of toxicity of NMs; the industrial-scale application of engineered nanomaterials in many areas of daily life raises the question of the security of these systems because the nanodimensions are able to overcome natural barriers, resulting in potential biological damage.

The main aim of this *Ph.D. Thesis* was the evaluation of the potential toxic effects of several NMs on aquatic organisms used as models considering their increasing use in

the product market. Short and long-term ecotoxicological assays developed, searching for specific biomarkers of exposure by immunohistochemistry, western blotting and gene expression analysis.

In accordo alla definizione adottata dalla Commissione Europea nel 2011 per nanomateriale s'intende "un materiale naturale, casuale o prodotto contenente particelle, in uno stato slegato o come aggregato o come agglomerato e dove, per il 50% o più delle particelle nella distribuzione delle grandezze numeriche, una o più dimensioni esterne sono nell' intervallo di grandezza 1-100 nm".

I nanomateriali esibiscono peculiari caratteristiche (ad es. dimensioni piccole, area di superficie molto grande, forma, carica di superficie, gruppi reattivi presenti in superficie, stato di agglomerazione) che conferiscono loro delle proprietà molto diverse da quelle possedute dal materiale della stessa composizione da cui essi derivano. A causa dell'uso diffuso nei prodotti di consumo e nei prodotti industriali, i nanomateriali possono essere rilasciati nell'ambiente e ciò ha sollevato molte preoccupazioni da parte degli scienziati (Royal Society and the Royal Academy of Engineering, 2004).

Gli effetti che i NM esercitano sugli organismi acquatici dipendono dalle loro caratteristiche che possono essere influenzate da parametri ambientali. I NM entrano negli organismi acquatici principalmente attraverso le superfici epiteliali (come branchie, pelle, cornea) o mediante ingestione diretta (Moore, 2006). Dopo aver attraversato la membrana delle cellule, possono essere immagazzinati in vescicole, mitocondri e altri organelli all' interno delle cellule epiteliali. Possono generare specie reattive dell'ossigeno, stress ossidativo, denaturazione delle proteine, citotossicità, apoptosi e necrosi (Oberdörster *et al*., 2005).

I test ecotossicologici sui NM dovrebbero innanzitutto considerare il comportamento dei NM in ambiente acquatico e le condizioni che possono influenzare lo stato di aggregazione.

Ad esempio, alcuni NM sono quasi impossibili da disperdere in acqua con i metodi fisici quali sonicazione o agitazione e può essere richiesto l'uso di un agente disperdente. La scelta dell'agente disperdente è problematica poiché alcuni dei disperdenti migliori da un punto di vista chimico sono anche tossici per gli organismi.

La possibilità che i NM possano causare stress ossidativo nei pesci e negli invertebrati non è del tutto chiara. Bar-Ilan *et al*. (2009) hanno dimostrato che le nanoparticelle d'argento inducono quasi il 100% di mortalità in larve di *Danio rerio* dopo esposizione acuta e diverse malformazioni a carico dell'embrione sono state osservate. Uno studio ha dimostrato che nanoparticelle d'oro (AuNPs) non sono tossiche alle concentrazioni testate e non causano anomalie nello sviluppo di embrioni di zebrafish (Asharani *et al*., 2010).

Zhu *et al*. (2009) hanno osservato eventi di mortalità ed effetti di immobilizzazione su esemplari di *Daphnia magna* esposta a nanoparticelle di biossido di titanio (TiO_2NPs) aventi dimensioni inferiori ai 20 nm.

Attualmente vi è una penuria di informazioni circa i rischi a lungo termine e i potenziali meccanismi di tossicità delle nanoparticelle; l'applicazione su scala industriale dei NM in molti settori della vita quotidiana solleva la questione circa la sicurezza di tali sistemi in quanto le dimensioni nanometriche sono in grado di attraversare le barriere naturali arrecando danni ai sistemi biologici.

Scopo del presente progetto di tesi è la valutazione dei potenziali effetti tossici di nanomateriali ingegnerizzati su organismi acquatici impiegati come modello, mediante

saggi ecotossicologici a breve e lungo termine, la ricerca di specifici biomarkers di esposizione mediante analisi immunoistochimica, western-blotting e analisi dell'espressione genica.

1. INTRODUCTION

Worldwide research on nanotechnology has grown considerably over the last decades. "Nanoscience" is the ability to observe, measure and manipulate the physical matter from an atomic and molecular point of view, while "nanotechnology" is the set of processes and techniques necessary to produce nanometric materials.

Nanotechnology was born in 1959 when American Physicist Richard Feynman, during the American Physical Society's annual meeting at the Californian Institute of Technology, held a celebrated speech entitled "*There's plenty of room at the bottom*" proposing the possibility of manipulating atoms to build infinitely small objects. Later in 1986, the American Engineer Kim Eric Drexler, defined nanotechnology as "*a molecular technology that will allow us to put every atom where we want it to be. We call this ability as nanotechnology because it works on the nanometer scale of 1 billionth of a meter*". Drexler, considered the true father of nanotechnology, underlined the technological importance of nano-scale phenomena.

According to a Recommendation on the definition of nanomaterials of 18 October 2011 adopted by European Commission (2011/696/EU), Nanomaterials means a "*natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm*". Nanomaterials (NMs) have peculiar chemical and physical properties depending on their size and differ from those found in materials of conventional size (called bulk materials). NMs can have a natural origin, such as those produced by natural combustion processes (volcanoes, spontaneous fires) or have an anthropogenic origin. In this case, we distinguish those involuntarily produced (originating from vehicular traffic, in particular from diesel engines, incinerators, etc.) and those built voluntarily or engineered nanomaterials (ENM), specifically produced by nanotechnology to find application in various scientific and industrial fields (Tab. 1).

NATURALS	**MANUFACTERED**	
	Non Engineered	**Engineered**
Fires	Internal combustion engines	Carbon-based NM
Volcanoes	Power stations	Metallic NM
	Electric engines	Dendrimers
	Incinerators	Composites
	Jet airplanes	
	Fumes steel industries	

Tab. 1. Major sources of nanomaterials.

There are two fundamental ways to build ENM: bottom up (bottom to top) and top down (top to bottom).

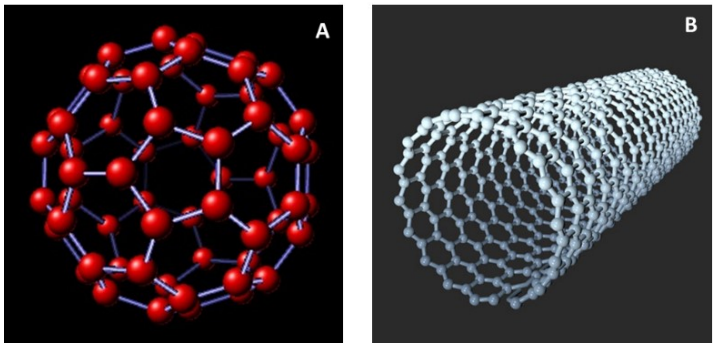
The bottom up method consists of assembling the nanostructured material from the nanoparticles that will allow the precursor to grow in dimensions and characteristics.
The top-down method, which is currently the most consolidated one, consists of making of nanostructures from massive blocking of bulk material by physical methods that reduce the size of the initial structures, bringing them to a nanometric level.

1.1. Classification of Engineered Nanomaterials (ENMs)

There are several types of engineered nanomaterials; according to a commonly used classification, it is possible to subdivide them into four groups:

- Carbon-based nanomaterials
- Metal oxide nanomaterials
- Dendrimers
- Composites.

Carbon-based nanomaterials are mainly made of carbon, usually spherical (fullerenes) or cylindrical (nanotubes). They often used for selective and controlled transport of drugs (Figs. 1);



Figs. 1. Carbon-based nanomaterials: A) Fullerene; B) Nanotube.

Metallic nanomaterials made up of metals include nanogold, nanosilver and various metal oxides such as titanium dioxide (TiO_2), zinc oxide (ZnO), silicon dioxide (SiO_2) and aluminum oxide (Al_2O_3);

Dendrimers are polymers made up of branched units with numerous terminal chains that can be engineered and/or functionalized to be used, for example, in catalysis processes (Fig. 2);

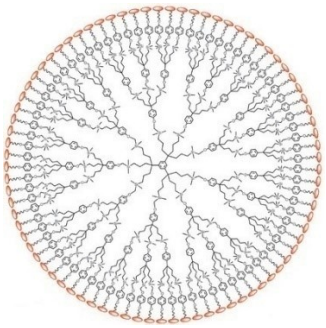


Fig. 2. Structure of a dendrimer.

Composites, obtained by combining solids of different nature bound by a matrix (metallic, polymeric or ceramic) reinforced with nanometric particles; they are used to build electronic components of cars and packaging materials.

Engineered nanomaterials are also used in sporting goods, stain-resistant clothing, sunscreens, cosmetics and electronics. The unusual physicochemical properties are attributable to their small size, chemical composition (purity, crystallinity, electronic properties, etc.), surface structure (large surface area, surface reactivity, surface groups, inorganic or organic coatings), solubility, shape and aggregation. Although impressive from a physicochemical viewpoint, the properties of NM raise concerns about adverse effects on biological systems, some studies suggest that NM are not inherently benign and that they affect biological behaviors at the cellular, subcellular, and protein levels (Oberdörster *et al*., 2005; Royal Society and Royal Academy of Engineering, 2004).
Moreover, some nanoparticles readily travel throughout the body, deposit in target organs, penetrate cell membranes and may trigger injurious responses. Manufactured nanoparticles (NPs), due to the size of the particles itself and its chemical reactivity, can lead direct toxicity, and biodegradability may be a further significant factor in governing harmful biological effects (Hoet *et al*., 2004).

1.2. Applications of nanoparticles

Because of the nanoscale nature of nanoscience and nanotechnology, they already bridge many fields including medicine, pharmaceuticals, manufacturing technologies, electronics and telecommunications (Royal Society and the Royal Academy of Engineering, 2004). In medical therapeutics, many drug candidates fail to reach their targets at appropriate concentrations, which can severely limit their effectiveness (Brigger *et al*., 2002). However, when drugs are encapsulated into nanoscale particles and treated to prevent clumping, the result is often a stable and water-soluble material, due to the very large surface to volume ratio (Panyam and Labhasetwar, 2003). Other nanoparticle-based medicines are also being investigated for use in cancer therapies and diagnosis where the nanoscale properties facilitate entry and intracellular targeting to specific sites (Brigger *et al*., 2002).

1.3. Routes of entry, uptake and bioavailability into living systems

Uptake of nanoparticles by inhalation or ingestion are likely to be the major routes in terrestrial organisms (Dowling, 2004; Warheit, 2004; Moore, 2002). However, in aquatic animals there may be other routes of entry such as direct passage across gills and external surface epithelia. At the cellular level, most internalisation of nanoparticles will occur via endocytosis (Fig. 3). Endocytotic pathways into cells can either lead to the endosomal and lysosomal compartments (Panyam and Labhasetwar, 2003). At the cellular level, prokaryotes like bacteria may well be largely protected against the uptake of many types of nanomaterials, since they do not have mechanisms for the bulk transport of supramolecular and colloidal particles across the cell wall. However, eukaryotes have highly developed processes for the cellular internalisation of nanoscale (100 nm or less) and microscale (100-100.000 nm) particles, namely endocytosis and phagocytosis respectively (Panyam and Labhasetwar, 2003; Synnes *et al*., 1999). These processes are integral to key physiological functions such as intracellular digestion and cellular immunity. In invertebrate animals, the cellular immune system, gut epithelium and hepatopancreas (digestive or midgut gland), where present, is likely to be targeted. The hepatopancreas is involved in uptake and digestion of food and storage of nutrient reserves; and is frequently specialised for intracellular lysosomal digestion of food via internalisation by endocytosis (Moore *et al*., 2004; Moore, 2002). The liver is a probable target following endocytotic transport across the intestinal epithelium into the hepatic portal blood system followed by endocytosis into hepatocytes.

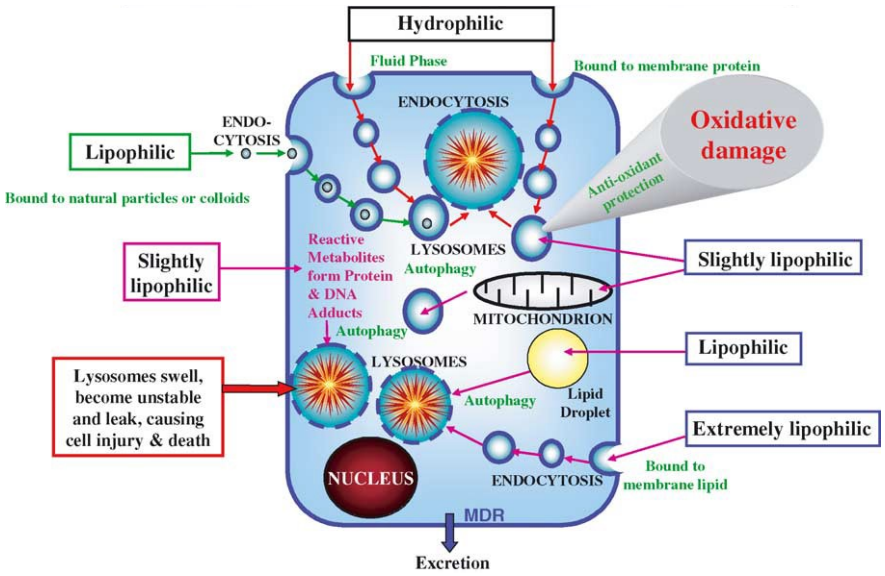


Fig. 3. Diagrammatic representation of the various routes of uptake (both diffusion and endocytotic) of xenobiotic chemicals into the cell that can result in their accumulation in the lysosomal compartment, based on their physical chemical characteristics (from Moore *et al*., 2004).

Endocytotic routes of entry to the cell may allow pollutant nanoparticles to embed themselves within the functional machinery of the cell in ways that are toxicologically quite different from conventional toxic chemicals. Nanoparticles situated in the endoplasmic reticulum, Golgi and endo-lysosomal system could conceivably act as foci of oxidative damage that could not readily be expelled from the cell; while generation of radicals could lead to organelle dysfunction.

1.4. Risks to human health via water

Accidental spillages or permitted release of industrial effluents in waterways and aquatic systems may result in direct exposure to nanoparticles of humans via skin contact, inhalation of water aerosols and direct ingestion of contaminated drinking water or particles adsorbed on vegetables or other foodstuffs (Daughton, 2004). More indirect exposure could arise from ingestion of organisms such as fish and shellfish (*i.e.* molluscs and crustaceans) as part of the human diet. Filter-feeding molluscs are prime candidates for uptake of manufactured nanoparticles from environmental releases, if it transpires that some of these nanomaterials will associate with natural particulates (Fig. 4).

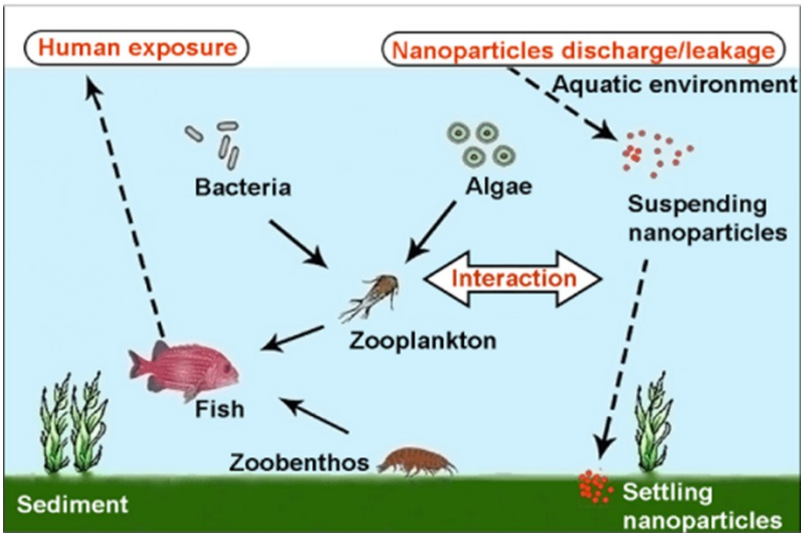


Fig. 4. In the aquatic environment, nanoparticles can react with various organisms.

1.5. Harmful effects of NPs and possible consequences on biological systems

The very large surface area of ultrafine particles can result in the direct generation of reactive oxygen species (ROS) leading to cell oxidative stress: it can cause cell injury by attacking DNA, lipids, proteins, membranes and metabolic activities. The toxic effects of ENPs essentially depend on several key factors such as their intrinsic nature and

capacity to form larger aggregations, the route of exposure, dose response, exposure time, the response of the receptor organisms to the lack of biocompatibility of ENPs, and the interactions in the mechanisms involved in the physiological process of uptake. ENPs enter the environment via different exposure routes, including solid and liquid waste from domestic sources and industrial activity, accidental spillages, and atmospheric emissions. Additional types of source that need to be assessed are those resulting from the use of products that contain ENPs (*i.e.* sunscreens and paints). Industrial products and wastes tend to end up in waterways (*e.g.* drainage ditches, rivers, lakes, estuaries and coastal waters) despite safeguards (Daughton, 2004; Moore *et al*., 2004; Moore, 2002). Consequently, it is inevitable that nanoscale products will enter the aquatic environment (Moore *et al*., 2004; Royal Society and Royal Academy of Engineering, 2004).

In natural water ecosystems, water travels over the surface of the land, through ground or underground until it reaches estuarine water (EW) or saltwater (SW), passing through conditions of increasing water salinity, ionic concentration and differences in pH. During this physical process, ENPs can be degraded, transformed, carried and accumulated. One main effect is that the ENPs could form colloidal suspensions by association with substances originating from animals or from human activity, and could undergo processes of agglomeration or self-aggregation (Lapresta *et al*., 2012). During the transport of ENPs to their final destination in the ocean, colloid mobilization is also promoted by chemical perturbations (*e.g.* ionic strength and pH) (Petosa *et al*., 2010) and by the physical conditions of the water system (*e.g.* temperature). Due to the high specific surface area and binding constants of ENPs, they tend to absorb small colloids that may aggregate and even separate out from the water column by differences in settling velocity, resulting in a transfer of chemicals (*e.g.*, metals and polymers) to the sediments where the ENPs can also be ingested by organisms. In soils, ENPs may be degraded by biotic or abiotic processes and transported to water bodies through run-off, leaching and drain flow (Boxall *et al*., 2007). Colloidal surface properties are therefore key aspects for tracking the fate and the behavior of ENPs in the environment. In the final agglomerated/aggregated entity, the particle reactivity decreases because the surface area of the individual particles and the interfacial free energy are reduced during the aggregation process (*e.g.* humic acid in water often mitigates the toxic effect of heavy metals). The aggregation of ENPs is therefore an important factor that may influence their toxicity, either mitigating or increasing the effect.

1.6. Mechanism of toxicity

One of the most important factors related to the toxicity of ENPs is oxidative stress. ROS and oxidative stress have been explained in detail by Lushchak (2011). In aquatic organisms, it is possible to measure the effect of ROS on lipids by monitoring the intermediate (primary an secondary products) species of lipid peroxidation (tightly

associated with exposure to NPs) and end products. ROS-induced DNA damage may bring physiological consequences that impair reproduction, inhibit growth, and damage both lysosomes and mitochondria. DNA damage leads to the liberation of toxic cations, inhibits algal photosynthesis and affects electron and/or ion transport. The disruption of ion transport changes membrane permeability and increases the probability of NPs gaining entry inside the cell, which may lead ultimately to cell death. The effects of ENPs on aquatic ecosystems are produced essentially by oxidative damage (internalization by cells) and interaction between ENPs and the cell membrane (without internalization). The properties of the NPs affect the uptake of NPs, which are bioaccumulated predominantly in the gills, intestine, liver, kidneys and blood (Scown *et al*., 2010). The latter three organs seem to be mainly due to internalization through the gills and the intestine. The main target of the toxicity mechanism is to disrupt the osmoregulatory function. Thus, the main adverse effects are related to an increase in the diffusive permeability of the membrane leading to a disturbed ion transport, as well as changes in cellular morphology, mitochondrial function, mitochondrial membrane potential and DNA damage-related gene expression that induce potential inflammation, necrosis and apoptosis of cells.

According to these results, ecotoxicology research is urgently required to explain and clarify the behavior of ENMs and ENPs in their different forms and their environmental impact on the ecosystems. ENPs tested in this study are: silver (AgNPs), gold (AuNPs) and titanium dioxide (TiO_2NPs), and they will be investigated hereinafter. Moreover ENMs and nanocomposites will be considered later.

1.7. Silver nanoparticles (AgNPs)

Metallic silver (Ag) is a durable transition element and because of its rarity (67th in abundance among the elements) and its attractive white metallic luster, silver has long been used as jewellery, currency coins and silverware. Silver nanoparticles (AgNPs) showed excellent performance in antibacterial application; it was reported that AgNPs show biocidal action by the slow release of Ag^+, and by multiple mechanisms (such as interaction with thiol groups in proteins and enzymes, inhibition of DNA replication, induction of oxidative stress) making it more difficult for bacteria to produce resistant strains (Luoma, 2008).

As early as 1889, Lea had reported the first synthesis of a silver colloid stabled by citrate (Lea, 1889). Though not formally registered or under the name of “nano”, literature shows that silver colloids have been used in the medical area for more than 100 years since 1897 by the name of “Collargol”. The first biocidal silver product “Algaedyn” was registered in 1954 in the U.S., which is still used in disinfectants today (Nowack *et al*., 2011). During the past two decades, the advancement of nanotechnology has opened new avenues for AgNPs. Being in the nano-scale dimension, AgNPs exhibit novel properties relative to the bulk metal, which has aroused great interest in the development of new applications.

1.7.1. Properties and applications

Pure silver has high thermal and electrical conductivity and relatively low contact resistance, which makes it a popular material in electronics. Silver nanoparticles have been used to fabricate thin-film transistor electrodes, as pastes and inks for printed circuit boards, optoelectronics, data storage devices and battery-based intercalation materials (Yu *et al*., 2013). Thanks to their extremely small size, AgNPs possess large surface area, which provide them high surface energy and more possible reactive sites. These characteristics qualify AgNPs as one of the most promising materials in catalysis. AgNPs are capable of catalyzing several reactions, such as CO and benzene oxidation, reduction of 4-nitrophenol in the presence of $NaBH_4$ (Yu *et al*., 2013).

For years, knowledge about nanosilver's ability to kill harmful bacteria has drawn extensive attention, making it popular for incorporation into various products. Nanosilver exhibits a broad spectrum of antimicrobial activity, and can inhibit the growth of both Gram-positive and Gram-negative bacteria (including *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*) (Birla *et al*., 2009). Nanosilver is also an effective fungicide: AgNPs can kill a number of fungal strains, including *Aspergillus fumigatus*, *Mucor*, *Saccharomyces cerevisiae* and *Candida tropicalis* (Wright *et al*., 1999). Nanosilver also has antiviral properties; it was reported that AgNPs synthesized in Hepes buffer could inhabit HIV-1 replication, and the anti-HIV activity (98%) was much higher than that of gold nanoparticles (6-20%) (Sun *et al*., 2005).

1.7.2. Environmental concern

The widespread application of AgNPs in our daily life will inevitably increase human and ecosystem exposure. Many literature reports have shown data on the leaching and fate of AgNPs. Benn and Westerhoff revealed that AgNPs and Ag^+ could be easily leached into water by simply immersing commercial AgNPs containing socks into water with shaking. Some brands of socks could even lose nearly 100% of the total silver contents after four consecutive washings (Benn and Westerhoff, 2008). Consumer silver nanotextiles were subjected to surfactants, oxidizing agents, different pH, washing machines and simulated perspiration fluids to test the normal laundering process and human skin sweat on the release of AgNPs (Geranio *et al*., 2009). It was shown that low pH, mechanical stress and the presence of bleaching largely enhanced the dissolution of Ag, and the nature of incorporation determined the amount and form of Ag release. The considerable release of Ag in simulated perspiration fluids suggested the potential human risk in the use of textiles containing AgNPs. As AgNPs are widely used in consumer spray products for their antibacterial ability, there is a risk that nanoparticles may be directly inhaled and deposited in the respiratory tract during product use. Quadros and Marr explored the emission behavior of three consumer spray products containing AgNPs. It was shown that emitted aerosols ranged from nanoscale up to 10 µm, and 0.24-56 ng of silver could be released per

spray action. It is also estimated that up to 70 ng of silver may deposit in the respiratory tract according to the usual use of the spray products (Quadros and Marr, 2011).

1.7.3. Natural and anthropogenic source of AgNPs

The increasing use of nanosilver has generated enthusiasm in developing methods to fabricate different AgNPs, which may eventually increase the amount of nanosilver in the environment (Yu *et al*., 2013). However, it seems that not all AgNPs are produced by humans. It is reported that silver as nanoparticles was found in an old silver mining area of Mexico (Gomez-Caballero *et al*., 2010) and before the manufacture of engineered AgNPs, colloidal and particulate silver was also discovered in river and estuarine waters of Texas (Wen *et al*., 1997).

AgNPs could be formed naturally but also by anthropogenic activities. AgNPs are used as electronic and medical devices, incorporated into textiles, dressing or directly added into disinfectants. However, during the production and manufacturing of nanosilver products, they could be directly released into the environment. The synthesis process often involves mixing centrifugation and filtration steps to remove impurities and the wastewater may be directly discharged into the environment. The uncontrollable release of silver during the use, recycling and disposal process gives rise to public concerns. Ideally the released AgNPs and Ag^+ would undergo "Wastewater Treatment Plants" (WWTP) processing. Through previous study (Benn and Westerhoff, 2008) has shown that WWTP biomass is able to partition high levels of heavy metals and with treatment largely reduce the silver concentration in the effluent stream, unfortunately, in some instances untreated sewage sludge is often used as an agricultural additive or fertilizer, which results in the recycling of AgNPs. This would be an hazardous source of AgNPs to the environment.

1.7.4. Fate, transport and distribution of AgNPs in environment

Once released into the environment, AgNPs would undergo different pathways during transport. They may remain as individual particles in suspension and be delivered long distances, or tend to aggregate at high ionic strength. The behavior of the AgNPs depends on the surface properties of the nanoparticle themselves and the surrounding environment, involving electrolyte composition, solution ionic strength, pH and natural organic matter (NOM). After contact with oxygen and other oxidants, partial oxidation and Ag^+ dissolution is also expected. Most probably, AgNPs would react with sulfide, chloride or other natural substances, altering the original properties of the nanoparticles (Luoma, 2008). Due to the large surface area and high surface energy of nanoparticles, they are prone to coalesce to form larger clusters. NOM, ubiquitously existing in aquatic systems, is also a key factor influencing the fate of AgNPs. The presence of various functional groups of NOM facilitates surface binding of AgNPs, resulting in more stable AgNPs suspensions. AgNPs coated by NOM are able to stay

dispersed for months (Akaighe *et al*., 2011). As mentioned above, AgNPs can escape from the manufacturing factory during the production process, including drying the solution, mechanical grinding, mixing and packaging, resulting in the release of AgNPs into the atmosphere. Also, the widespread use of AgNPs in disinfection sprays promotes the emission of AgNPs to the air. Surface disinfectants, which can be used on walls, tables, floors, often cause AgNPs to deposit on these surfaces, and AgNPs would probably transfer to a duster cloth after cleaning and then go down the drain during laundering. Added in anti-odor sprays and used in rooms, AgNPs could also find their way in the open air by transport. According to Fick's first law that the diffusion coefficient is inversely related to the particle diameters, AgNPs would diffuse rapidly because of their small size. If they are stable enough, long distance mobility in the air would be expected. Additionally, their large surface area provides abundant reactive sites for dusts, microbes and pollution, making the AgNPs much more toxic than the original particles. The airborne particles may also coalesce to large agglomerates during transport, and deposit on surfaces by gravitation, or be washed down to terrestrial or aquatic systems by rain. AgNPs may enter the aquatic system in several ways:

- Silver leached out from nanosilver consumer products, and eventually end up in streams and rivers (Benn and Westerhoff, 2008);
- Suspended AgNPs in air finally depositing on water;
- Runoff scouring AgNPs polluted soils or landfill sewage sludge could result in AgNPs migrating to surface water.

On the other hand, divalent cations (*e.g.* Ca^{2+} and Mg^{2+}), commonly present in natural waters, could easily induce the aggregation of AgNPs. Colloidal clusters would probably deposit in the sediment, reducing the bioavailability for aquatic organisms and plants. As AgNPs are not highly stable and can easily be oxidized, a slow dissolution of Ag^{+} would be expected in aquatic environments (Liu and Hurt, 2010). In marine waters, as sodium chloride is the dominant salt, Ag^{+} associates with Cl^{-} to a great extent, and the main species observed are $AgCl^{2-}$, $AgCl^{3-}$, and $AgCl^{4-}$, making silver more mobile in seawater (Barriada *et al*., 2007). Due to the large surface area and relatively high surface energy, once released into the environment, AgNPs could be highly dynamic and different transformations such as sulfurization, chlorination will readily occur, which would greatly affect the behavior of the AgNPs. Silver sulfide nanoparticles were also discovered in the final stage sewage sludge materials of a full-scale municipal wastewater treatment plant (Kim *et al*., 2010).

1.7.5. Mechanism of toxicity

The antibacterial activity of AgNPs is a complex process, and several possible modes of action are proposed, involving:

- Production of reactive oxygen species (ROS);
- Direct attachment to cell membrane and disruption of membrane integrity;
- Changes in membrane permeability;
- Interaction with proteins and disruption of their regular function;
- Interference with DNA replication and causing DNA damage.

However, in another report that assessed the effect of AgNPs on rainbow trout hepatocytes, no excess ROS was detected after exposure. The cytotoxicity was mainly associated with the reduced mitochondrial activity and membrane integrity (Farkas *et al*., 2010). Membrane disruption related toxicity of AgNPs was also reported in other studies. The formation of pits and pores in the cell membrane of fungus *C. albicans* was observed, and the authors suggested that AgNPs may attack cell membrane lipid bilayers and destroy the membrane permeability barrier, resulting in the leakage of ions, formation of pores and cell death (Kim *et al*., 2008).

E. coli membrane damage caused by AgNPs was also confirmed by Sondi and Salopek-Sondi (2004). TEM/SEM images all showed that AgNPs accumulated on cell membranes and some were successfully incorporated into the lipid bilayer structure, forming irregular shaped pits in the outer membrane. It was speculated that AgNPs may lead to the progressive release of lipopolysaccharide molecules and proteins, causing changes in membrane integrity and permeability, and finally inducing cell disfunction and death.

Existing results have showed that AgNPs can cause potential toxicity to test animal models. Lankveld and co-workers also demonstrate that when three different sized AgNPs (20, 80, 110 nm) were intravenously injected into rats at a concentration of 23.8 μg/ml for 5 days, the concentration of Ag was reduced rapidly in the blood, and AgNPs redistributed to liver, lung, brain and all other organs, showing a systemic distribution (Lankveld *et al*., 2010). A number of non-mammals animals have also been used to test the adverse effect of AgNPs and most of these studies are based on aquatic organisms. Zebrafish or *Danio rerio*, as a correlative and predictive model, has been used in many studies to evaluate the effects of AgNPs. Earlier *in vivo* study (Lee *et al*., 2007) demonstrated that single AgNPs (11.6 ± 3.5 nm) could transport into zebrafish embryos through the chorion pore canal, and AgNPs were detected inside embryos at each developmental stage. In another study, four different sized AgNPs were synthesized to test their toxicity to zebrafish embryos, and only a few differences were observed between them. It is reported that AgNPs induced almost 100% mortality after exposure for 120 h at 250 μM, and a variety of embryonic morphological malformations were observed at a dose of 100 μM (Bar-Ilan *et al*., 2009).

However, different from previous studies that smaller particles were more toxic, some authors found that larger AgNPs (41.6 ± 9.1 nm) produced higher toxic impacts and more severely deformed zebrafish than the smaller ones (11.6 ± 3.5 nm) at the same concentration (Lee *et al*., 2012). Toxicity of AgNPs in rainbow trout gill cells showed

that nanoparticles were taken up into cells and lead to cytotoxicity related with membrane integrity disruption and oxidative stress. Furthermore, different kinds of morphological malformations such as edema, spinal abnormalities, finnfold abnormalities, heart malformations and eye defects emerged after long time exposure, indicating the developmental toxicity of AgNPs (Wu *et al*., 2010).

1.8. Gold nanoparticles (AuNPs)

Gold was one of the first metals discovered by humans, and the history of its study and application is estimated to be a minimum of several thousand years old. Looking in the past, gold has been already reported as a health adjuvant. In the Middle Ages in Europe, colloidal gold was studied and employed in alchemists' laboratories. In 1583, alchemist David de Planis-Campy, surgeon to the King of France Louis XIII, recommended his "elixir of longevity", an aqueous colloidal gold solution, as a means of life prolongation (Dykman and Khlebtsov, 2012). Chinese and Indian culture reported the use of gold compounds in the form of "Swarna Bhasma" for treatment of various health needs like increasing vital power and curing male impotence (Mahdihassan, 1985). Myocrism™ (sodium aurothiomalate) and Solganol™ (aurothioglucose) are water soluble gold complexes present which show beneficial effect in rheumatoid arthritis. Earlier in 1978 cisplatin was approved by the Food and Drug Administration (FDA) as an antitumor agent. Gold nanoparticles (AuNPs), are one of the promising inorganic nanoparticles that have attracted scientific and technological interests due to their ease of synthesis, chemical stability and excellent optical properties that make them appealing tools for cancer diagnosis and treatment (Dykman and Khlebtsov, 2012; Jain *et al*., 2012). Literature investigations have demonstrated that these inorganic nanoparticles are non toxic (Fard *et al*., 2015). Evaluation of AuNPs toxicity is essential because of broad spectrum application of these in biomedical sciences.

1.8.1. Synthesis of AuNPs

The production of nanoparticles consist of reduction of size (top-down technique). Gold nanoparticles can be produced by reduction of gold salts in the presence of stabilizing agents in order to prevent agglomeration. The gold NPs can be prepared in three different methods:

- Physical methods like ultra violet (UV) radiation, sonochemical method, laser ablation, thermolytic process (Giorgetti *et al*., 2007);
- Chemical methods in an aqueous medium using citrate or sodium borohydride as a reducing agent;
- Biological methods take into consideration microrganisms as a source used to produce NPs (Alivisatos, 2004). These ones have been used as an alternative in

order to avoid the use of organic solvents, thus making biological process an eco-friendly nanotechnological approach.

There are different types of gold NPs (Fig. 5):

Gold nanospheres: their size lie in the range of 2-100 nm and can be synthesized by controlled reduction of an aqueous solution of $HAuCl_4$ using a reducing agent like citrate (Khan *et al*., 2013);

Gold nanoshells: spherical with diameters ranging in size from 10 to 200 nm. They possess a remarkable set of optical, chemical and physical properties, which make them ideal candidates for enhancing cancer detection, cancer treatment, cellular imaging and medical biosensing (Khan *et al.,* 2013);

Gold nanocages: hollow porous gold NPs, size basically ranges in between 10 and 150 nm and consists of hollow interiors and porouswalls. They are generally produced by silver nanoparticles with chloroauric acid ($HAuCl_4$) in boiling water;

Gold nanorods: used as a drug delivery vehicle due to its low cytotoxicity, excellent stability, biocompatibility and suitable physiochemical parameters.

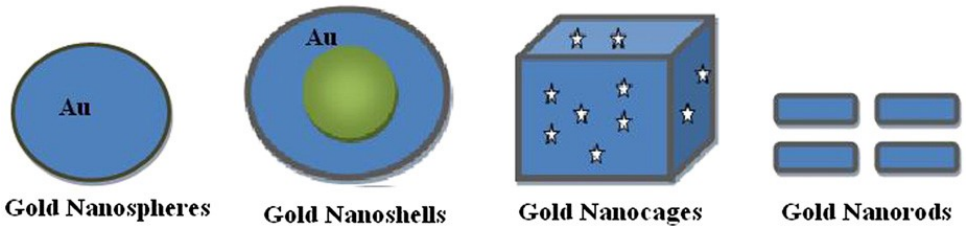


Fig. 5. Different types of gold NPs with their shapes (from Khan *et al*., 2013).

1.8.2. Role of gold NPs in biomedical applications

Several pharmacological sectors have got approval from the Food and Drug Administration (FDA) for the use and development of nanotechnology-based drugs in the last few years. AuNPs are an important tool for drug delivery, as a tracer for diagnostic imaging which will give structural information on very molecular level, and treatment of major life threatening diseases. Because of their small particle size, diversity in surface chemistry, unique physical properties, adaptable absorption, emission properties, flexibility in synthesis, AuNPs have a unique approach in biomedical technology. They are used in different fields:

- They can easily penetrate to the intracellular cell wall and, if monitored, they can give a plenty of useful information which can lead researchers for cancer diagnosis and gene therapy (Bhattacharyya *et al*., 2011);
- AuNPs have the tendency to absorb and scatter visible and near infrared (NIR) light resonantly upon excitation of surface plasmon oscillation. Detection of biological events at the single molecular level has been used for biosensing by exploiting the distance dependent scattering properties of the AuNPs. Upon getting excited plasmon resonance band can be spectrally modified over a wide range. The outcome is that the sense of light scattering signal is more profound (highly intense) and comparatively much brighter than the chemical fluorophores. This property can be useful in imaging techniques (Sönnichsen *et al*., 2002);
- Can be coated with polyethylene glycol (PEG) to impart antibiofouling properties, which extends their lifetime in the blood stream. Studies conducted by Kim *et al*. (2007) showed the advantages of AuNPs as a contrast agent compared to iodine-based compounds which show several limitations like short imaging times due to rapid renal clearance, renal toxicity and vascular permeation. The results of the X-ray absorption coefficient *in vitro* show that there is an increase in the X-ray absorption behaviour of AuNPs as compared to a iodine-based compound called “Ultravist”;
- Can be bound with biological protein and peptides for the fabrication of biosensors which can be used in new signal transduction technologies (de la Escosura-Muniz *et al*., 2009).

1.8.3. Gold nanoparticles toxicity

Gold, in its bulk form, has long been considered an inert and noble metal with some therapeutic value. However, some researches revealed the toxicity of gold NPs. As with the toxicity observed for AgNPs at the cellular level, the possible adverse effects of AuNPs can be attributed to:

- Their interaction with the cell membrane;
- Oxidative stress leading to cytotoxicity effects;
- The inhibition of metabolic activity (*e.g.*, leading to mitochondrial damage);
- Possible damage to the nuclear DNA.

To date, very few data are available on the ecotoxicity of AuNPs. A study (Lovern *et al*., 2008) made on the presence and distribution in *Daphnia magna* exposed to sub-lethal concentrations of AuNPs for 1, 6, 12 or 24 h, and the highest concentration of AuNPs was found after 12 h. Earlier reports on metal and metal oxide nanoparticles and have emphasized the deleterious effects of nanoparticles *in vivo* and *in vitro* (Zhu *et al*., 2008). It has been observed that the toxic output of nanoparticles *in vitro* differ significantly from *in vivo* models. Similarly AuNPs is considered as non-toxic *in vitro*,

whereas injection of AuNPs in mice has shown tissue damage and apoptosis in liver (Cho *et al*., 2009). A study showed that gold nanoparticles are non toxic at the employed concentrations and do not cause obvious abnormalities in developing zebrafish embryos (Asharani *et al*., 2010). Indirect human exposure is possible through the consumption of fresh water fish exposed to nanowastes. Hence it is necessary that extreme care must be taken for nanowaste disposal and *in vivo* applications of nanoparticles. The data gathered from experiments will help future researchers to unravel the mechanisms of nanoparticle toxicity, which still remains unclear.

1.9. Titanium dioxide nanoparticles (TiO_2)

Titanium dioxide (TiO_2) nanoparticles are the most extensively studied metal oxide nanoparticles (Menard *et al*., 2011; Cattaneo *et al*., 2009). One of the reasons for the large amount of ecotoxicity data on nanosized TiO_2 is the adoption of this nanomaterial by a variety of industries. It is a fine, white, crystalline, odorless, low-solubility powder which exhibit relatively low toxicity (Sager *et al*., 2008). It is a natural, thermally stable and nonflammable, nonsilicate mineral oxide found primarily in the form of the minerals rutile, anatase, brookite, and as the iron-containing mineral ilmenite ($FeTiO_3$) (Duan *et al*., 2010). Anatase phase exhibits the highest photocatalytic activity, so is used in catalysis and photocatalysis applications. Rutile is known as white pigment providing opacity to paints, papers, inks, and consumer products such as toothpaste. Because of their properties TiO_2NPs have been used in several applications, but this widespread use led to a significant environmental exposure to TiO_2 nanoparticles (Hall *et al*., 2009). With regard to its potential adverse health effects, several studies have defined TiO_2 as biologically inactive and physiologically inert in both humans and animals. Recently, pulmonary inflammation, fibrosis, epithelial hyperplasia, and tumorigenesis were reported in animals under conditions of substantial TiO_2 particle lung burden due to sufficiently high dose and/or duration of exposure. Thus, in 2006, the International Agency for Research on Cancer (IARC), classified and in 2010 reassessed TiO_2 as “possibly carcinogenic to humans” on the basis of the sufficient evidence of carcinogenicity in experimental animals and inadequate evidence in humans (Group 2B) (IARC, 2010, 2006).

1.9.1. Properties and applications

It has excellent physicochemical properties, such as good fatigue strength, resistance to corrosion, machineability, biocompatibility, whitening and photocatalysis, as well as excellent optical performance and electrical properties (Iavicoli *et al*., 2012; Chen *et al.,* 2009). Nanosized TiO_2 is used extensively in sunscreen cosmetics as an inorganic UV absorbant that can allow a film transparent to visible light to be applied to human skin (Jaroenworaluck *et al*., 2005). A surface coating, for example silica and other compounds, can also be added to nanosized TiO_2 to decrease its photoreactivity so that nanoTiO_2 can be used to protect human skin, plastic, and other objects from UV

radiation. In cosmetic products, rutile phase is used as a pigment and thickener and it is used in plastics and for its ultraviolet (UV) light absorbing properties (Mueller and Nowack, 2008). TiO_2NPs are widely used in paints, printing ink, rubber, paper, cleaning air products, decomposing organic matters in wastewater.

1.9.2. Ecotoxicological studies

Currently few data exist regarding observed environmental concentrations of TiO_2 nanoparticles (Griffitt *et al*., 2008). Evidence that TiO_2NPs can leach from exterior facade paints and discharge into surface waters has been provided. The concentrations of metallic Ti found in the surface runoff were as high as 600 mg/l (Kaegi *et al*., 2008). Two studies modelled the quantities of TiO_2NPs released into the environment (Gottschalk *et al*., 2009; Mueller and Nowack, 2008). Aggregated particles are generally less mobile and can interact with filter feeders and sediment-dwelling organisms (Farré *et al*., 2009). The extent of aggregation is governed by pH, ionic strength, and the nature of the electrolytes (Navarro *et al*., 2008). Much knowledge already exists on the effects of TiO_2 nanoparticles on biological systems. NanoTiO_2 is photoinducible, redox active and thus a generator of ROS at its surfaces. The precise mechanisms of toxicity of nanosized TiO_2 and other metal nanoparticles are largely unknown (Griffitt *et al*., 2008), but recent studies have shown that the toxicity of nanoparticles is generally governed by properties such as particle size, shape, and surface properties (Navarro *et al*., 2008). Upon irradiation by a photon possessing an energy larger than its band gap, an electron associated with the TiO_2 nanoparticle can be excited to the conduction band, thereby creating a positively charged hole. This hole can then remove an electron from a molecule (such as water) that is in contact with the surface of the TiO_2, thereby resulting in the formation of reactive oxygen species (Fujishima and Honda, 1972). Ultrafine TiO_2 nanoparticles (<20 nm) can enter the human body through different routes, including inhalation, ingestion, dermal penetration and injection (Oberdörster *et al*., 2005).

1.9.3. Toxicity of nanosized TiO_2 to freshwater and marine invertebrates

There is an emerging literature on the ecotoxicity of nanosized TiO_2 with invertebrates. According to Baun *et al*. (2008) invertebrate tests are well suited to generate reproducible and reliable nanotoxicity data. Nanoecotoxicology may take advantage of the fact that use of invertebrates as experimental animals is not subject to legal restrictions. The ecotoxicological effects of nano-TiO_2 on freshwater invertebrates have been summarised in several papers. There are a lot of data available for freshwater invertebrates especially for crustaceans like *Daphnia magna, Daphnia pulex, Ceriodaphnia dubia, Chydorus sphaericus, Thamnocephalus platyurus* (Cattaneo *et al*., 2009; Farré *et al*., 2009; Baun *et al*., 2008). Zhu *et al*. (2009) observed effects on mortality and immobility on *D. magna* in the case of particles of ≤ 20 nm. Lovern *et al*. (2007) observed significant differences in toxicity to daphnids when filtration of the

TiO_2 suspension permitted testing of smaller 30 nm particles. When the suspension of particles was no filtered, aggregates of size 100-500 nm were present and no toxicity to daphnids was observed. Sublethal effects of nanosized TiO_2 on *D. pulex* after exposure of 24 h have been observed. It was found that nanoTiO_2 at 500 mg/l elevated the activity of the antioxidant enzymes catalase and glutathione-S-transferase (Klaper *et al*., 2009). One study reported a 24-h test with mussels bivalve *Mytilus galloprovincialis* showing that nanoTiO_2 induced lysosomal membrane destabilisation in the hemocytes and digestive gland. It was also found that nanoTiO_2 induced increases in lysosomal accumulation of neutral lipids and enhancement of catalase and glutathione transferase activity in the digestive glands. No effect on catalase and glutathione transferase activities in the gills and no mortality was observed (Canesi *et al*., 2010).

1.9.4. Toxicity of nanosized TiO_2 to freshwater fish

The ecotoxicological effects of nanoTiO_2 on fish have been summarised in some papers in recent years (Farré *et al*., 2009) and toxicity data are available for *Danio rerio* (Griffitt *et al*., 2008; Zhu *et al*., 2008), *Pimephales promelas* (Hall *et al*., 2009), *Oncorhynchus mykiss* (Federici *et al*., 2007; Warheit *et al*., 2007) and *Cyprinus carpio* (Hao *et al*., 2009). Sublethal effects of nanosized TiO_2 have been reported on juvenile rainbow trout (*Oncorhynchus mykiss*) after 14 days waterborne exposure (Federici *et al*., 2007). The Na^+/K^+-ATPase activity in the gills, brain and intestine decreased after 14 days; after 8 weeks this effect was observed only in the brain. Total glutathione in the gills and liver was unaffected by nanoTiO_2. Lipid peroxidation increased in the gill, intestine and brain after 14 days, but decreased after 8 weeks. Other observed effects included gill pathologies, such as edema and thickening of the lamellae, and a few foci of lipidosis and condensed nuclear bodies in liver cells. Federici *et al*. (2007) concluded that TiO_2NPs caused respiratory distress and sub-lethal toxicity involved oxidative stress, organ pathologies and the induction of antioxidant defense system. Zhu *et al*. (2008) compared the toxicity of several metal oxide NPs with their bulk counterparts suspensions to zebrafish at early developmental stage by a 96h embryo-larval bioassay and showed that neither TiO_2NPs nor bulk TiO_2 caused any toxicity to zebrafish embryos and larvae.

1.9.5. Environmental concerns

To date, TiO_2NPs tested in ecotoxicity studies published have different physico-chemical properties, such as diameter, crystalline form, coating, surface area, zeta potential and purity. The variability of the tested material was further enhanced as a result of preparation and suspension of the nanoparticles in test media, for example by sonication, filtration, and use of different solvents and surfactants. Physicochemical characteristics of nanoTiO_2 can explain the high variability in the toxicity data for nanoTiO_2 obtained by different authors conducting similar experiments with the same

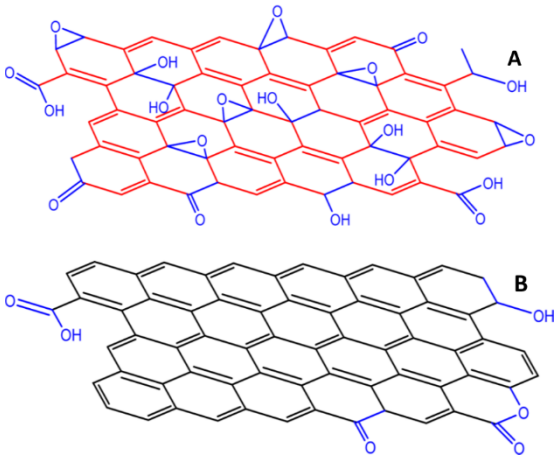
species. However, the toxicological profile of TiO_2NPs is not completely understood and several concerns have emerged on the potential undesirable effects, in regard to the harmful interactions with biological systems and the environment (Nel *et al*., 2006). The recently recognized occupational carcinogenic potential of the inhaled TiO_2NPs have ulteriourly enhanced these scientific concerns. Therefore, an appropriate assessment of the risks for the exposed population requiring TiO_2NPs hazard identification and dose-response data seems necessary (Cho *et al*., 2010). In this regard, a deeper knowledge about TiO_2NPs mechanisms, obtained from *in vitro* and *in vivo* experiments, respectively, could be useful to identify indicators of early biological events and to establish appropriate methods for their detection.

1.10. Graphene and its derivatives

Materials of the graphene family include few-layer graphene, graphene nanosheets, graphene oxide (GO) and reduced graphene oxide (rGO) (Sanchez *et al.*, 2012). Graphene-based nanomaterials vary in the layers number, lateral dimensions, surface chemistry, defect density or quality of the individual graphene sheets and purity. The structural features of these materials affect the physico-chemical properties of the resulting materials and, probably, they determine also the main biological effects (Pretti *et al.*, 2014).

The recent biomedical applications of graphene and derivatives have determined a rapid increase of the studies related to the biological interactions of these materials (Sanchez *et al.*, 2012); for example graphene dispersed in air might represent a danger to people daily handling these materials, either by contact or inhalation, and studies on this topic are therefore necessary. Nevertheless, accidental spills and effluent discharges can determine an increased risk of release of these exogenous nanoparticles (NPs) into aquatic environment and even though emissions of graphene oxide (GO) to aquatic environment should be low (if any), their expected low degradability requires adequate investigation.

Graphene is composed of sp^2-hybridized carbon atoms hexagonally arranged in a two-dimensional structure, resulting in a large surface area on both sides of the planar axis (Novoselov *et al.*, 2004). It has gained a considerable scientific interest starting from its discovery in 2004 as a result of its electrical, mechanical and optical properties proposed for use it in a wide range of applications ranging from flexible electronics to DNA sequencing (Buccheri *et al.*, 2016) (Figs. 6).



Figs. 6. Structural models of graphene: A) Graphene oxide (GO); B) Reduced graphene oxide (rGO).

Because of the formation of hydrogen bonds between polar functional groups on the GO surface and water molecules, a stable GO colloidal suspension can be reached suggesting advantages for potential biomedical applications of GO compared with other carbon-based materials (Bitounis *et al.*, 2013).

The level of toxicity that graphene might reach in a biological system and the degree of safety for its use are important to explore.

However, prior to the use of graphene-based materials, it is imperative to establish a heedful approach for these materials by evaluating their potential toxicity, which is unknown compared with that of other carbon nanostructures, such as carbon nanotubes (Seabra *et al.*, 2014).

1.10.1. Properties and application of graphene-based nanomaterials

Thanks to the surprising electrical, mechanical, thermal and reactivity, graphene-based nanomaterials are exploited in many applications:

- They are applied for bio-sensing to make preventive diagnosis of diseases and cancer;
- The use of carbon-based nanomaterials is suitable for the filtration of micro-organisms as well. In fact, graphene-based nanomaterials have been found to show antibacterial properties;
- They display the ability to adsorb heavy metals if their surfaces are adequately functionalized. In particular, GO and GO-based nanocomposites were recently proposed for the removal of pollutants (Yeh *et al.*, 2013) and for the adsorption of organic dyes from water and they are increasingly used for wastewater treatment in hybrid nanocomposite membranes (Filice *et al.*, 2015);
- Thanks to the ability to behave as a conductors or semiconductors, they are used in electronic field.

1.10.2. Origin of graphene oxide (GO)

Graphitic oxide has been known for almost a century. It was first prepared by Brodie in 1859 by repeated treatment of Ceylon graphite with an oxidation mixture consisting of potassium chlorate and fuming nitric acid. Since then, many other procedures have been devised for forming graphitic oxide nearly all dependent upon strong oxidizing mixture containing one or more concentrated acids and oxidizing materials. Fourty years after, Staudenmaier (1898) improved this procedure, but the most accomplished method is the one created by Hummers and Offeman (1958). In their method, the oxidation of graphite to graphitic oxide is governed by treating graphite with essentially a water free mixture of concentrated sulfuric acid, sodium nitrate and potassium permanganate.

GO is composed of graphene flakes mainly with hydroxyl and epoxy groups at the surfaces and carboxyl, carbonyl and hydroxyl functional groups at the edges. In particular, the presence of such oxygen functional groups makes the GO hydrophilic and highly dispersible in water (Buccheri *et al.*, 2016).

Moreover, GO shows semiconducting properties and the energy gap can be tuned by a reduction of the oxygen functional groups making the valence and conduction band of GO suitable, respectively, for O_2 and H_2 evolution from water decomposition. The reduction of the oxygen content can be achieved in several ways such as chemical or thermal treatment or UV light irradiation (Buccheri *et al.*, 2016). Moreover, literature data report that visible laser irradiation of GO flakes in water solution can cause a modification or a reduction in the oxygen content (D'Angelo *et al.*, 2015). The conductive properties of reduced graphene oxide (rGO) become similar to those of pure graphene but with lower electron mobility, because rGO flakes show defects in the deoxygenated graphene domains (Mattevi *et al.*, 2009).

1.10.3. *In vivo* studies about GO

The great majority of *in vivo* studies of graphene-related nanomaterials is based on evaluation of tissue distribution (bioaccumulation) and excretion. Zebrafish has been established as an important preclinical model for *in vivo* toxicity studies of nanomaterials due to its close homology with the human genome. Furthermore, zebrafish embryos are more sensitive to chemical agents compared with adult organisms (Seabra *et al.*, 2014). Gollavelli and Ling (2012) studied the *in vivo* toxicity of graphene to *Danio rerio* (zebrafish) embryos microinjected with multifunctionalized graphene (coated with polylactic acid and fluorescein o-methacrylate). The authors did not observe significant abnormalities or changes in the survival rate of fish embryos, although graphenes were found to be largely biodistributed in zebrafish. The results showed that the graphene complexes were not toxic toward the development from the embryo to the larvae stages. This *in vivo* biocompatibility can be explained by the presence of capping functionalities on the graphene surface. Because graphene is not a degradable material, its solubility and biocompatibility can be improved by

chemically coating it with hydrophilic polymers, such as polyethylene glycol (PEG), which will decrease its hazard (Liu *et al.*, 2013).

Recent studies indicate that graphene-based materials could be used in antimicrobial products because of their versatility. Hu *et al.* (2010) found a useful bactericidal effect of GO on *E. coli* because it caused bacterial membrane damage. Direct interaction of bacteria with graphene sheets induced the loss of bacterial membrane integrity and glutathione oxidation, suggesting that the GO antimicrobial action contributes to both membrane disruption and oxidative stress.

1.10.4. Environmental toxicity and toxicity mechanisms

Recently, the environmental and biological toxicities of graphene and graphene composites applied in environmental remediation have been discussed (Kemp *et al.*, 2013). Many biological activities were significantly impacted by the presence of GO in an activated sludge. In this sense, graphene is a promising material for environmental applications, and its phytotoxicity should be further investigated. It was suggested that the generation of ROS could be one of the responsible mechanisms for the toxic effect of GO (Ahmed *et al.*, 2013). Many reports have proposed that oxidative stress is one of the mechanisms involved in the toxic effects of carbon nanomaterials.

Due to its hydrophobic surface, graphene can significantly interact with cell membrane lipids, causing toxicity. Cell membrane damage through physical interaction with graphene possessing sharp edges is another possible mechanism of toxicity (Hu *et al.*, 2010).

Toxicity also depends on the physicochemical properties of graphene-based materials, such as the density of the functional groups, size, conductivity, and chemical nature of the reducing agent used for deoxygenation of GO, as well as on the cell types exposed to the materials.

It is clear from the recent literature that due to the increasing importance of graphene-related materials, there is a need for more detailed and accurate *in vitro* and *in vivo* studies of toxicity of the graphene family.

1.11. *Artemia salina*: a model organism in environmental field

1.11.1. Historical notes on genus *Artemia* (*Crustacea: Branchiopoda*)

The brine shrimp *Artemia* is one of the most important aquatic animals for aquaculture industry since it is highly nutritious; moreover It is easy to culture so it is widely used in biological studies (Asem *et al*., 2010). Mankind has known *Artemia* for many years. For example, Amerindian and Libyan natives have been using *Artemia* as food. Although all scientific manuscripts attribute the first report of *Artemia* to Schlösser in 1756 from Limington, England, the first scientific record belongs to an unknown Iranian author in 982 from Urmia Lake, Iran (Asem, 2008). The first Persian geography book entitled "*The Limits of The World from The East to The West*", written by an unknown geographer in 982, was dedicated to one of the Ghurid Kings that they had governed in the North East of ancient Iran (Asem, 2008). The unknown Iranian geographer had introduced *Artemia* from Urmia Lake as a "worm" that can live in the lake's saline water. Schlösser had described *Artemia* as "unknown insect" ("*un insecte peu connu*") in 1755. This first description was not accompanied by a picture (Kuenen and Baas-Becking, 1938). Schlösser's famous original description was illustrated in July 1756 (Schlösser, 1756). In 1758, Linnaeus assigned this species to the taxonomic classification of *Cancer salinus*. It was then renamed by Leach in 1819 to *Artemia salina* which is currently acknowledged as the valid classification for Mediterranean bisexual populations considered conspecific with the now extinct Lymington population (Ruebhart *et al*., 2008).

The literature on the taxonomical nomenclature of *Artemia* is still confused because systematic and phylogenetic relationships of the *Artemia* species are debatable. The taxonomic status of the genus *Artemia* is as follows (Martin and Davis, 2001):

Subphylum: *Crustacea* Brünnich, 1772
Class: *Branchiopoda* Latreille, 1817
Subclass: *Sarsostraca* Tasch, 1969
Order: *Anostraca* Sars, 1867
Family: *Artemiidae* Grochowski, 1896
Genus: *Artemia* Leach, 1819

The genus *Artemia*, once thought to be represented by a single species *Artemia salina*, is now known to be composed of several bisexual species as well as parthenogenetic populations. It is difficult identify species belong to the genus *Artemia* due to a lack of reliable morphological characters so different methods have been used for species characterization. The recognition of several *Artemia* species has been confirmed through the use of scanning electron microscopy and different genetic techniques (Triantaphyllidis *et al*., 1997; Ruebhart *et al*., 2008), comparison of biometric characteristics, cross-fertility tests and microscopic survey of the morphology such as frontal knob and gonopod (De los Ríos and Asem, 2008).

The six bisexual species are described below:

- *Artemia franciscana* Kellogg, 1906: Northern and Central America
- *Artemia persimilis* Piccinelli and Prosdocimi, 1968: Southern America
- *Artemia salina* (Leach, 1819): Mediterranean area
- *Artemia sinica* Cai, 1989: Central and Eastern Asia
- *Artemia tibetiana* Abatzopoulos, Zhang and Sorgeloos, 1998: China (Tibet)
- *Artemia urmiana* Günther, 1899: Iran (Urmia Lake; West Azerbaijan).

It is important to point out that parthenogenetic populations are present all over the world (Europe, Africa, Asia and Australia) and are grouped under the binomen *Artemia parthenogenetica* (Triantaphyllidis *et al*., 1998) for taxonomic convenience.
There are numerous published data on biochemical, toxicological and other aspects of *Artemia*, all using the name *A. salina* for any population in this genus. Assigning correct species identification, as the identity of the test organism, is an important parameter in assuring the validity of the results of the assay. Correct species identification allows comparison of test results across studies.

1.11.2. Morphological features of Class *Branchiopoda*

Artemia salina (Leach, 1819) popularly known as brine shrimp, is a small widespread saltwater crustacean and belongs to class *Branchiopoda*. The Brachiopods are the most anciently known shellfish since the Lower Devonian. The most primitive characters lie in the filopodial appendages trunk, little or no differentiated between them. They make a variety of functions: swimming, breathing, feeding; their movement, determined by variation of haemolymph turgor, causes a swirling motion of the water, which transports the organic substance in suspension. In fact *Branchiopoda* are essentially filter feeder crustaceans. As regards the cephalic appendages, antennules are usually slight and consist of a single article; the antennas are varied and much reduced; mandibles have not palpus; the jaws of the first and second pair are very small, sometimes they are missing. The number of segments of the trunk varies greatly according to the groups. The abdomen usually does not possess appendages and generally ends with a caudal furca. According to the presence or not of the carapace, the branchiopods are divided in different orders (Baccetti *et al*., 1991).

1.11.3. Morphological features of Order *Anostraca*

They have elongated body but not carapace. In the body, we can recognise after a cephalic region, a thoracic region which consists of 11-19 segments having filopodial appendages, and an abdominal region of 4-9 segments, apoda, which ends with a furca consisting of two single articled branches. The head has a dorsal nauplius eye dorsal and two lateral compound eyes. The males have antennas whose last part has a hook to grab the female during copulation. The first two abdominal segments are merged

together to form the genital area and constitute the ovisac in females, a pair of penises in males. *Anostraca* swim upside down. After fecundation, the eggs in the ovisac start the segmentation that at one point stops. The restart of the development depends on environmental factors such as humidity, temperature, salinity because *Anostraca* live in waters subdue to long periods of aridity. Chorion ornamentation on the eggs surface represent the diagnostic character to differentiate different species (Baccetti *et al*., 1991).

1.11.4. *Artemia salina* Leach, 1819

This crustacean is able of living and reproducing in water to widely fluctuating environmental conditions with regard to temperature (6-35 °C), salinity and ionic composition, oxygen levels. *Artemia salina* is a non-selective filter feeder of organic detritus, microscopic algae as well as bacteria. *Artemia salina* therefore, is only found at salinities where its predators cannot survive (>70 g/l). Its physiological adaptations to high salinity provide a very efficient ecological defense against predation (Van Stappen, 1996) as brine shrimp possess:

- A very efficient osmoregulatory system;
- The capacity to synthesize very efficient respiratory pigments to cope with the low O_2 levels at high salinities;
- The ability to produce dormant cysts when environmental conditions endanger the survival of the species.

Adult of *Artemia salina* has an elongated body (± 1.5-2 cm in length) and its trunk has no carapace. We can subdivide its body into three regions: cephalic, thoracic and abdominal ones.

- Cephalic region consists of a dorsal median eye called “nauplius eye” and two lateral stalked compound eyes. Between the lateral eyes, there are two pairs of antennae: the first set of antennae are small, cylindrical and thin in both male and female species; the second set of antennae are different in male and female forms. In male *A. salina,* they are in the form of hooks for grabbing females during mating and inject their sperm; in females, they are cylindrical and less enlarged.
- Thoracic region (trunk) consists of into 11 segments bringing paired lobular appendages called thoracopods. The thoracopods have locomotory and feeding function because they create a water flow to the mouth letting *Artemia* to eat suspended organic material. They are differentiated into three functional parts: the telopodites and endopodites (locomotory and filter feeding), and the membranous exopodites (gills).
- Abdominal region consists of 4-9 segments; in males the first two segments are merged together to form the genital region (copulatory organ); in females there is a brood pouch which contains the eggs (Baccetti *et al*., 1991).

The male has a paired penis in the posterior part of the trunk region; female can easily be recognized by the brood pouch or uterus situated just behind the eleventh pair of thoracopods. Eggs develop in two tubular ovaries in the abdomen, once ripe they become spherical and migrate via two oviducts into the unpaired uterus. In the circulatory system the heart is a pulsating dorsal vessel which collects, through the ostia, the blood from the peripheral parts of the body and conveys it to the gills; the gills are laminar expansions of the limbs. The nervous system consists of a gangliar chain. The digestive system consists of a mouth, a plurilobed stomach, a front intestinal tract and the terminal one. The excretory system consists of two maxillary glands.

Reproduction in the genus *Artemia* performs under two modalities:

1) Anfigonic sexual reproduction which produces a larval stage called nauplius;
2) Parthenogenetic reproduction that produces cysts or eggs.

Fertilized eggs normally develop into free-swimming nauplii (ovoviviparous reproduction) released by the mother.

The female organisms, produced by parthenogenetic reproduction, can generate only female species. After its fertilization, the egg undergoes a fast segmentation and when it reaches a stage of development named gastrulation enter the diapause (a quiescence period that can be very long depending on environmental factors).

The genomic (DNA set) consists of 42 chromosomes in the sexual forms and 84 chromosomes in the parthenogenetic forms.

1.11.5. Life cycle of *Artemia salina*

At one moment of the year, *Artemia* produces cysts that float across the water surface and that are thrown ashore by wind and waves. These cysts are metabolically inactive and do not further develop as long as they are kept dry. Upon immersion in seawater, the biconcave-shaped cysts hydrate, become spherical, and the embryo resumes its interrupted metabolism. After about 24-36 h the outer membrane of the cyst bursts and the embryo appears, surrounded by the hatching membrane. While the embryo hangs underneath the empty shell (umbrella stage), the development of the nauplius is completed and within a short period of time the hatching membrane is broken and the free-swimming nauplius is born (Van Stappen, 1996).

The first larval stage or instar I (400 µm in length) has a brownish-orange colour, a red nauplius eye in the head region and three pairs of appendages: *i.e*. the first antennae (sensorial function), the second antennae (locomotory + filter-feeding function) and the mandibles (food uptake function). The ventral side is covered by a large labrum. The instar I larva does not take up food as its digestive system is not functional yet; it thrives completely on its yolk reserves. After about 8 h the animal molts into the second larval stage called instar II. Small food particles ranging in size from 1 to 50 µm are filtered out by the second antennae and ingested into the functional digestive tract (Van Stappen, 1996).

The larva grows and differentiates through about 15 molts. From the 10th instar stage on, important morphological changes take place: in males, the antennae lose their locomotory function and undergo sexual differentiation developping into hooked graspers, while the female antennae degenerate into sensorial appendages. After about 21 days from its birth, the nauplius becomes a sexually mature organism (Fig. 7).

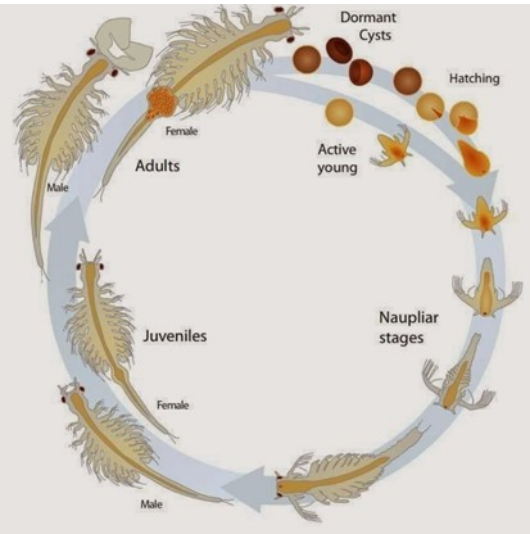


Fig. 7. Life cycle of *Artemia salina*.

1.11.6. Cysts morphology

The cysts shell, covered by chitin and lipoproteins, protects the eggs under adverse conditions. The cyst shell consists of three layers:

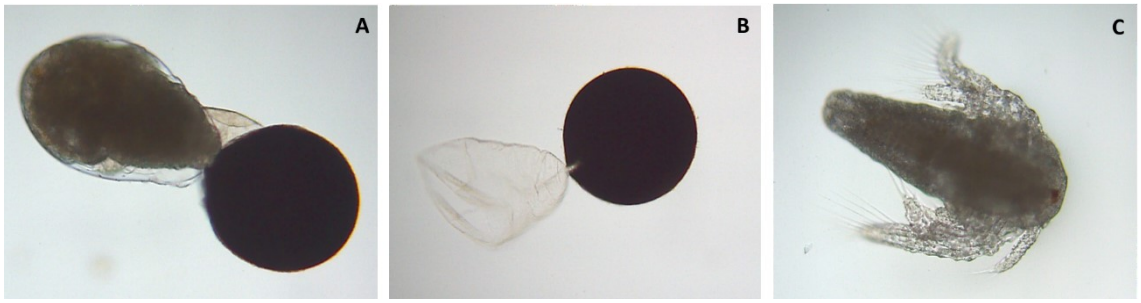
- Alveolar layer: a hard layer consisting of lipoproteins impregnated with chitin and haematin; the haematin concentration determines the colour of the shell, *i.e.* from pale to dark brown. Its main function is to provide protection for the embryo against mechanical disruption and UV radiation. This layer can be completely dissolved by oxidation treatment with hypochlorite;
- Outer cuticular membrane: a multilayer membrane acting as a permeability barrier to protect the embryo from penetration by molecules larger than CO_2 molecules;
- Embryonic cuticle: a transparent and highly elastic layer separated from the embryo by the inner cuticular membrane (develops into the hatching membrane during hatching incubation).

The cysts are available in large quantities along the shorelines of hypersaline lakes, coastal lagoons and solar saltworks scattered over the five continents. As *Artemia* is incapable of active dispersion, wind and waterfowl are the most important natural

dispersion vectors; the floating cysts adhere to feet and feathers of birds, and when ingested they remain intact for at least a couple of days in the digestive tract of birds.

1.11.7. Physiology of the hatching process

When incubated in seawater the biconcave cyst swells up and becomes spherical within 1-2 h. After that, the cyst shell (including the outer cuticular membrane) bursts (breaking stage) and the embryo surrounded by the hatching membrane becomes visible. The embryo then leaves the shell completely and hangs underneath the empty shell (the hatching membrane may still be attached to the shell). Through the transparent hatching membrane, the differentiation of the pre-nauplius into the instar I nauplius can be followed and it starts to move its appendages (Figs. 8). Shortly thereafter the hatching membrane breaks open (= hatching) and the free-swimming larva (head first) is born.



Figs. 8. Physiology of the hatching process: A) After the cyst bursts, the embryo appears, surrounded by the hatching membrane; B) the hatching membrane is broken and C) the free-swimming nauplius is born.

Dry cysts are hygroscopic and take up water at a fast rate. The aerobic metabolism in the cyst embryo ensure the conversion of the carbohydrate reserve trehalose into glycogen (as an energy source) and glycerol. Consequently, the osmotic pressure inside the outer cuticular membrane builds up continuously until a critical level is reached, which results in the breaking of the cyst envelope and all the glycerol produced is released in the hatching medium. In other words the metabolism in *Artemia* cysts previous to the breaking is a trehalose-glycerol hyperosmotic regulatory system. This means that as salinity levels in the incubation medium increase, higher concentrations of glycerol must to be built up in order to reach the critical difference in osmotic pressure which will lead to the shell burst (Fig. 9). A hatching enzyme secreted in the head region of the nauplius, weakens the hatching membrane and enables the nauplius to release itself into the hatching medium (Van Stappen, 1996).

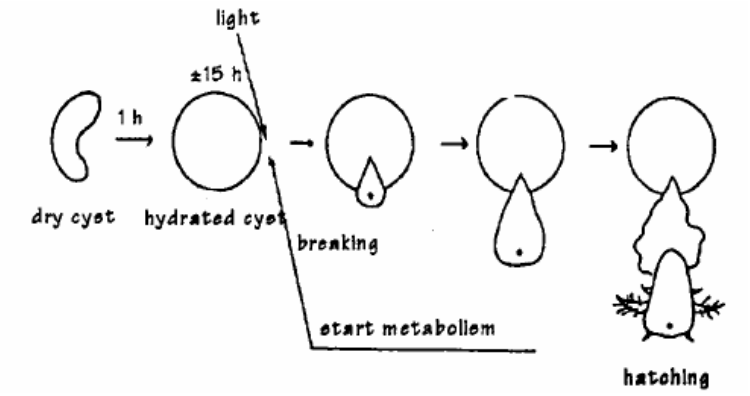


Fig. 9. Development of an *Artemia* cyst from incubation in seawater until nauplius release (from Van Stappen, 1996).

1.11.8. *Artemia salina* in aquaculture: an excellent food for newly-hatched fish larvae

Nauplii can directly be used as a nutritious live food source for the larvae of a variety of marine and freshwater organisms. During the 1940s, most commercially available brine shrimp cysts represented collections from natural saline lakes and coastal saltworks. With the growing interest for tropical hobby fish in the late 1940s, commercial value was attached to brine shrimp, thereby establishing a new industry. Early pioneers exploited in 1951 the cyst production of *Artemia* at the Great Salt Lake in Utah, USA (Van Stappen, 1996).

During the mid-1950s, commercial attention for brine shrimp was turned to controlled sources for production in the San Francisco Bay region. Here it was found that brine shrimp and their cysts could be produced as a by-product of solar saltworks. Since salt production entails management of the evaporation process, yearly cyst and biomass productions could be roughly predicted. In the 1960s, commercial provisions originated from these few sources in North America seemed to be unlimited. However, with the expansion of aquaculture production in the 1970s, the demand for *Artemia* cysts soon exceeded the offer and prices rose exponentially (Van Stappen, 1996).

During the following years, research efforts were made to prove the possibility of local production of *Artemia* in developing countries. Currently, *Artemia* is being produced and exploited on the five continents.

In the late 1970s, multidisciplinary studies showed that the nutritional value of *Artemia* was not constant but varied among strains causing unreliable outputs in marine larviculture. The causes for the nutritional variability in *Artemia* were genotypic and phenotypic variations (*i.e.* cyst size, cyst hatching characteristics, caloric content and fatty acid composition of the nauplii) therefore simple methods were developped to incorporate lipophilic compounds into the *Artemia* before being offered as a prey.

By bio-encapsulating specific amounts of emulsified products rich in highly unsaturated fatty acids in nauplii, the nutritional quality of the *Artemia* can be further tailored to suit the predators' requirements. This method of bioencapsulation, also called *Artemia* enrichment, has improved larviculture outputs, not only in terms of survival, growth of

many species of fish and crustaceans, but also with regard to their quality, *e.g.* reduced incidence of malformations, improved pigmentation and stress resistance.
Other compounds also appear to be variable from strain to strain such as total amount of free amino acids, pigments (canthaxanthin) and vitamin C:

- The ratio of free amino acids (FAA) to protein content is generally higher for instar I nauplii compared to cysts;
- Vitamin C (ascorbic acid) is considered as an essential nutrient during larviculture. It is found as ascorbic acid 2-sulfate (AAS) in cysts of brine shrimp, a very stable form but with low bio-availability. During the hatching process the AAS is hydrolyzed into free ascorbic acid, a more unstable form, but directly available in the nauplii for the predator;
- The carotenoid pattern, and more specifically the canthaxanthin contents, show qualitative differences between cysts and nauplii. In *Artemia* cysts the unusual cis-configuration is found, whereas in developing nauplii it is converted into the more stable trans-canthaxanthin.

1.12. *Danio rerio*: an emerging model organism in the environmental field

1.12.1. Morphological features of *Danio rerio*

Danio rerio (Hamilton and Buchanan, 1822), commonly known as zebrafish, is a small tropical freshwater fish which lives in rivers basins of India, Northern Pakistan, Nepal, Butan and South Asia. It belongs to the family of *Cyprinidae*, within the order of the Cypriniformes. The name *Danio* derives from the Bengali name “dhani” meaning “of the rice field”. In contrast to many other fish species, zebrafish adults are only approximately 4-5 cm long so that they can be easily managed in large numbers in the laboratory (Fig. 10).

Fig. 10. Adult specimen of *Danio rerio*.

Its body shape is fusiform and laterally compressed, shows a distinctive colour pattern based on alternating dark and light horizontal stripes (which give name of zebrafish to this species) with a terminal oblique mouth directed upwards and two pairs of barbels. The lower jaw protrudes further than the upper and the eyes are central and not visible from above. The anal fin is similarly striped, while the dorsal fin has dark blue upper edge, bordered with white.

Adults males are more slender and torpedo-shaped with blue and silver stripes alternating on their longitudinal side and coloured orange-golden on their belly and fins. The colouration of a fish is a very interesting aspect that attract people into the hobby of fishkeeping; in fact *Danio rerio* is well-known by aquarium-lovers. The colour pattern comprises three types of pigment cell: dark blue melanophores, gold xanthophores and iridescent iridophores. Females are thicker, especially before spawning.

The fin of the back is rounded and it has eight rajs, of which the first is undivided. The pectoral fins are low, and sharp above: each contains about nine rays; but they are indistinctly visible. The ventral fins are small, each containing only seven rays.

The sex of juveniles cannot be reliably distinguished without dissection and while gravid females have a more rounded body shape, the most reliable diagnostic feature is the presence of a small genital papilla in front of the anal fin origin.

Mating usually initiate at the onset of light, and spawning takes place over a short period thereafter (Nasiadka and Clark 2012). Male gonad pheromones stimulate ovulation in females; after ovulation, females release gonad pheromones to attract and stimulate males to perform courtship, which consists of abrupt turns and an elliptical swimming pattern around the female. Some pheromones released by females may also inhibit spawning of other females, playing a crucial role in competitive interactions between females (Nishimura *et al*., 2016). After three months, zebrafish reach sexual maturity.

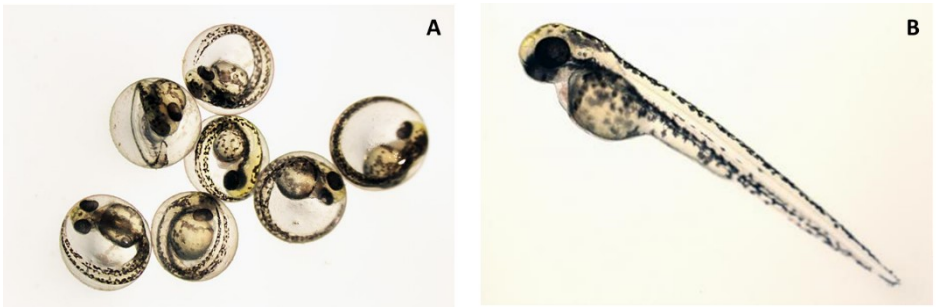
Zebrafish is one of the most important aquatic test species because of its ability to spawn the whole year, each time with huge amounts of eggs. An optimal breeding frequency for zebrafish seems to be every 10 days.

Zebrafish can tolerate a temperature range of 24.5 to 28.5 °C (Beasley *et al.,* 2012); however, the growth speed of zebrafish embryos varies according to temperature (Kimmel *et al*., 1995).

1.12.2. Embryonic development of zebrafish

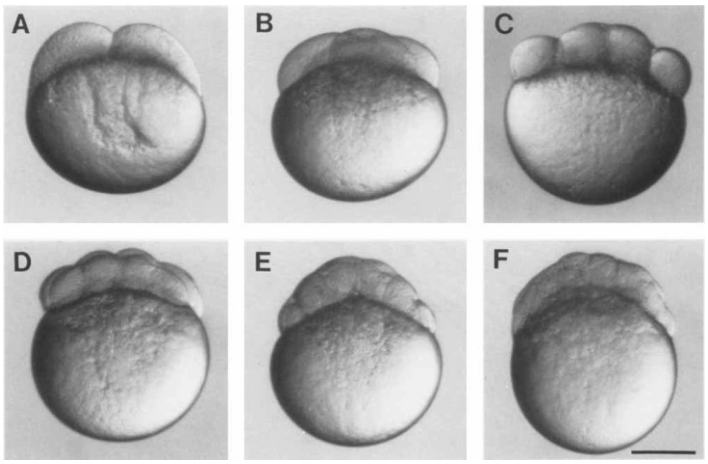
The development of the zebrafish is very similar to the embryogenesis in higher vertebrates, but, unlike mammals, it develops from a fertilised egg to an adult outside the female in a transparent egg. Zebrafish embryonic development has been well characterized (Kimmel *et al*., 1995). Moreover, the embryos themselves are transparent during the first few days of their lives because chorion is transparent (Figs. 11). Pigmentation in the embryos starts only about 30-72 hours post fertilization (Hill *et al*., 2005). Pigmentation can further be prevented *in vivo* by treatment with 0.003%

phenylthiourea or removed by bleaching after fixation, in order to extend the period of unhindered observation (Hill *et al.,* 2005).



Figs. 11. A) Zebrafish embryos with transparent chorion; B) Larva.

The first cleavage of the newly fertilized egg occurs about 40 minutes after fertilization (zygote period). Fertilization also activates cytoplasmic movements, easily evident within about 10 minutes. Nonyolky cytoplasm begins to stream toward the animal pole, segregating the blastodisc from the clearer yolk granule-rich vegetal cytoplasm. After the first cleavage the cells, or blastomeres, divide at about 15 minute intervals.
The cytoplasmic divisions are meroblastic and at the end of them a blastodisc forms. They only incompletely undercut the blastodisc, and the blastomeres, or a specific subset of them, according to the stage remain interconnected by cytoplasmic bridges. The six cleavages that comprise this period frequently occur at regular orientations, so that one can see how many blastomeres are present are by their arrangement (Figs. 12).



Figs. 12. Embryos during the cleavage period. Face views: A) Two-cell stage (0.75 h); B) Four-cell stage (1 h); C) Eight-cell stage (1.25 h); D) Sixteen-cell stage (1.5 h); E) Thirty-two cell stage (1.75 h); F) Sixty-four cell stage (2 h). Scale bar= 250 μm (from Kimmel *et al*., 1995).

The term blastula refer to the period when the blastodisc begins to look ball-like, at the 128-cell stage, and until the time of onset of gastrulation. Important processes occur during this blastula period: the embryo enters midblastula transition (MBT), the yolk syncytial layer (YSL) forms, and epiboly begins. Epiboly continues during the gastrulation period.

Blastula of zebrafish is a “stereoblastula” because blastocoele is not present. The blastula and gastrula stage of zebrafish at 28°C is equivalent to 2.25-5.25 hours post fertilization (hpf) and 5.25-10 hpf, respectively (Kimmel *et al*., 1995).

The marginal blastomeres in the early blastula lie against the yolk cell and remain cytoplasmically connected to it throughout cleavage. During cycle 10, the marginal cells undergo a collapse, releasing their cytoplasm and nuclei together into the immediately adjoining cytoplasm of the yolk cell. Thus arises the yolk syncytial layer (YSL). The YSL nuclei continue to undergo mitotic divisions in the midblastula, but the nuclear divisions are unaccompanied by cytoplasmic ones, and the yolk cell remains uncleaved and syncytial. After about three cycles, and coinciding with the beginning of epiboly, the YSL divisions abruptly cease. The YSL nuclei now begin to enlarge; possibly meaning that they are actively transcribing RNA.

The YSL, an organ unique to teleosts, may be extraembryonic, making no direct contribution to the body of the embryo. At first the YSL has the form of a narrow ring around the blastodisc edge but soon (within two division cycles) it spreads underneath the blastodisc, forming a complete “internal’’ syncytium (the I-YSL), that persists throughout embryogenesis. In this position, between the embryonic cells and their yolk stores, the I-YSL might be presumed to be playing a nutritive role.

Epiboly**,** beginning in the late blastula, is the thinning and spreading of both the YSL and the blastodisc over the yolk cell, as you might model by pulling a knitted ski cap over your head.

The morphogenetic cell movements of involution, convergence and extension occur, producing the primary germ layers and the embryonic axis.

Ballard (1981) coined the term “pharyngula” (24-48h) to refer to the embryo that has developed to the phylotypic stage, when it possesses the classic vertebrate bauplan. This is the time of development when one can most readily compare the morphologies of embryos of diverse vertebrates. The embryo is most evidently now a bilaterally organized creature, entering the pharyngula period with a well-developed notochord and a newly completed set of somites that extend to the end of a long post-anal tail. The nervous system is hollow and expanded anteriorly. With the rapid cerebellar morphogenesis of the metencephalon just preceding the pharyngula period, the brain is now sculptured into five lobes. The period name focuses attention of the primordia of the pharingheal arches develop rapidly during the second day from a primordial region. Seven arches in all develop from this primordium, a prominent boundary within it occurring between pharyngeal arches 2 and 3. This boundary is important, for later the arches anterior to it (the mandibular and hyoid arches) form the jaws and the operculum, and arches (branchial arches) posterior to it will form the gills.

Finally, in the hatching period (48-72 h) the embryo continues to grow at about the same rate as earlier. Morphogenesis of many of the organ rudiments is now rather complete and slows down considerably, with some notable exceptions including the gut and its associated organs. Much easier to see are the rapidly developing rudiments of the pectoral fins, the jaws, and the gills. During hatching period, call the creatures "embryos" until the end of the third day, and afterward "larvae", whether they have hatched or not (Kimmel *et al*., 1995).

1.12.3. Zebrafish as a model system for biomedical studies and human disease modeling

Biomedical studies employ several animal models for better understanding of pathogenesis of human diseases on cellullar and molecular level and the development of new therapeutics. Zebrafish have been used predominantly in developmental biology and molecular genetics, but their value in toxicology, drug discovery and modeling of various physiological and pathological processes has been recognized (Lieschke *et al*., 2007). Genome organization and the pathways involved into control of signal transduction appear to be highly conserved between zebrafish and humans; it should be noted that most human genes have homologues in zebrafish and functional domains of proteins such as kinase ATP-binding domains share 100% identity.

Both adult and zebrafish embryo are used for determination of acute toxicity of various compounds. The acute toxicity testing includes determination of the LC_{50} value (the concentration that is lethal to 50% of the treated fishes). Zebrafish embryo's chorion is intact until 2 or 3 days post fertilization (dpf). Pores in the chorion are about 0.5 μm in diameter, and are about 2 μm apart (Lee *et al*., 2007); it is more realistic to think of the chorion as a sieve but not a wall (Nishimura *et al*., 2016). However, chemical penetration through the chorion can vary, depending on the compound's physicochemical properties, cationic charge concentration in the aquatic test medium and electrostatic attraction between chemicals and the chorion. These factors suggest that the chorion can serve as a permeability barrier to chemicals under certain conditions. Panzica-Kelly *et al*. (2015) demonstrated that the sensitivity to detect teratogens using dechorionated embryos was higher than the sensitivity of chorion-intact embryos.

The growth speed of zebrafish embryos varies according to temperature (Kimmel *et al*., 1995). When zebrafish embryos are raised at 24.5 to 28.5 °C, they reach sphere stage at 4.1 or 3.8 hpf, and shield stage at 5.9 or 5.4 hpf (Beasley *et al*., 2012). These findings suggest that chemical exposure at a given time-point may have different effects on embryonic development, depending on the ambient temperature (Nishimura *et al*., 2016).

Zebrafish embryos have been used in studies on teratogenic effect by ethanol. The malformation of skeletal muscle caused by exposure to alcohol during the embryonic period have been observed in zebrafish (Sylvain *et al*., 2010). In zebrafish, the first somites appear around 10 hpf. Additional somites are produced at 30-min intervals in a bilaterally symmetric, anterior to posterior wave until a total of about 30 somite

pairs bracket the notochord at 24 hpf. Somites give rise to the axial skeleton and the skeletal muscle of the trunk. Muscle fibers begin to form shortly after the somite formation.

It has also been demonstrated that alcohol exposure during the embryonic period disrupts the expression of sonic hedgehog, an important regulator for the somite formation in zebrafish (Zhang *et al*., 2014). The overall process of somite development in zebrafish is similar to that in mammals; sonic hedgehog signaling was also identified as one of the key pathways in fetal alcohol syndrome in human, these findings suggest that exposure to alcohol during the embryonic period may cause the malformation of skeletal muscle through disruption of sonic hedgehog signaling in zebrafish and mammals.

Zebrafish embryos are successfully used for screening of drugs influencing angiogenesis. The process of blood vessel development is conservative in vertebrates (Zon *et al*., 2005) and it is known that increased angiogenesis is required for tumor growth and metastases.

Moreover, transgenic zebrafish, in which fluorescent proteins are expressed under the control of key genes, are a powerful tool in zebrafish-based developmental toxicity testing to detect developmental toxicities of chemicals with a high degree of sensitivity. This approach also enables us to detect tissue-specific developmental toxicity, such as the induction of green fluorescent protein (GFP) driven by the promoter of hsp70, a marker for oxidative stress response during development, in the eye of transgenic zebrafish embryos, following exposure to tert-butylhydroquinone (Hahn *et al*., 2014).

Various approaches have been used to model human diseases in zebrafish. One of them includes employment of chemical substances that induce symptoms typical for certain diseases:

- A neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, was used for modeling of Parkinsonism in zebrafish, because mechanism of its effect associated with death of dopaminergic neuron clusters is the same in zebrafish and mammals (Belyaeva *et al*., 2009).
- Zebrafish is a good models in study of glucose metabolism. Regulation of zebrafish glucose metabolism by insulin shares similarity with mammalian glucose metabolism. The main protein components of the zebrafish insulin signaling system exhibit structure functional similarity to those in mammals (Gerhard *et al*., 2003). It was shown that sensitivity of adult zebrafish to the effects of antidiabetic drugs is similar to that of mammals. Zebrafish shares high similarity with mammals in regulation of expression of phosphoenolpyruvate carboxykinase (the enzyme catalyzing one of the key stages of gluconeogenesis) by glucagon and insulin.
- *Danio rerio* is also used as a model system for studies of apoptotic mechanisms; this is very important for treatment of tumor and degenerative diseases

(Belyaeva *et al*., 2009). For example, most genes associated with apoptosis have been identified in zebrafish; these include caspases, apoptosis inhibitors, kinases, transcription factors and many other molecules involved into cell death.

- High sensitivity of zebrafish to carcinogenic agents allows using this species for investigation of carcinogenic activity of chemical compounds. There are zebrafish mutants, in which tumor development occurs in 70-90% several months after the carcinogen treatment (Spitsbergen and Kent, 2003).
- Some human tumors have been modeled in zebrafish by transgenesis. This confirms the fact that molecular mechanisms underlying development of neoplasia in mammals and man are also applicable to zebrafish. The transgenic zebrafish models suitable for studies of various forms of leukemia have been also developed (Kari *et al*., 2007).

2. ECOTOXICOLOGY

In 1969, the French toxicologist René Truhaut defined ecotoxicology as "the branch of toxicology which deals with toxic effects caused by natural or synthetic pollutants, ecosystem constituents, animals (including humans), plants and microbes, in an integral context". This new cross cutting discipline, which integrates the concepts of classical toxicology and ecology aims to study the destiny and effects of environmental contaminants in order to predict the final effects of pollution.

Its fields of investigation concern:

- Identification of pollutants;
- Monitoring and forecasting the distribution of pollutants in the environment;
- The assessment of the impact of pollutants on the biotic component;
- Identification of environmental rehabilitation and management procedures.

2.1. The ecotoxicity tests

The goal of ecotoxicological testing is to define the maximum amounts of potentially harmful compound which can be accepted in order to protect appropriately the target that is the natural ecosystems. Experiments can only be carried out on a limited number of species and then be referred to other living species known so far. There are two types of ecotoxicological tests:

- Acute toxicity tests, aimed at measuring the effect of substance exposure, which lasts between 15 minutes and 96 hours. The results obtained are then used to evaluate the toxic effects due to temporary contamination phenomena or for toxicity classifications.
- Chronic toxicity tests, aimed at calculating a toxicity threshold that is the maximum exposure level which marks the boundary between effective levels and ineffective levels for an indefinite period. Lengthy exposures are used to measure long-term responses in relation to the life of the subject being studied.

2.2. Aquatic toxicity tests

In a typical aquatic test, the organism under study assumes the contaminant, which has found in solution in the medium, through the gills or by the cell membrane of the epidermal cells. Then there is a gradual increase in concentration of the toxic substance within the body because of the balance between water and the absorbing compartments. The fate of this substance, once absorbed, is the result of the processes of distribution, metabolism and excretion. It is therefore difficult to quantify the internal concentration of the contaminant, which is why it is common to measure its toxicity in terms of substance concentration in the medium.

It is essential that the concentration of the substance remains constant throughout the test period, so it is necessary to pay attention to the persistence of the substance in the medium. There are two different approaches of exposure in aquatic toxicity tests:

Static tests: once started, the solutions of the substances to be tested and the means of controls are not renewed for the duration of the test; once started, the solutions of the substances to be tested and the means of controls are renewed with fresh solutions at specified times.

Continuous flow assays: the organism test, continuously receives an initially concentrated solution of the substance under investigation, pumped into an appropriate dilution system.

2.3. The importance of biomarkers as predictive tools in ecotoxicology

In ecotoxicology, the use of biomarkers (called also or stress indices) is increasingly used to evaluate the biological hazard of toxic chemicals and in the assessment of environmental health. The National Academy of Sciences defined biomarker as "*a xenobiotically-induced variation in cellular or biochemical components, processes, structures, or functions that are measurable in a biological system or samples"* (NAS-NRC, 1987). Such "variations" can indicate the magnitude of the organism's response to contaminants as well as provide the causal link between the presence of a chemical and an ecologic effect. This is important to quantify exposure and its potential impact by monitoring biological endpoints (biomarkers) in animals and plants as indicators of exposure to and the effects of environmental contaminants (Fossi, 1994). Pathological cellular reactions to xenobiotic can provide early-warning distress signals of injurious change in an organism. Such reactions will be of particular use if they can be shown to be precursors of pathology, since this will relate directly to the risk potential (Moore *et al*., 2004).

In general terms, the vulnerability of a species to environmental contamination depends on two main factors:

- The possibility of exposure;
- The intrinsic sensitivity of the species.

The first aspect can be checked by monitoring the habitat for the chemicals concerned. The biomarker approach may be used to assess intrinsic sensitivity. Biomarkers indicate whether or not a species is responsive, and whether or not it transforms a certain chemical into a toxic metabolite. The choice of biomarker to use should be guided by the information to be obtained; in the world of biomarker research, significant efforts have recently been made to identify potential new biomarkers (Fossi, 1994).

The ideal biomarker should have the following characteristics:

- Can be measured in readily available tissues or biological products obtainable in a non-invasive way;
- Can be related the measurement to exposure and/or degree of harm to the organism;
- Can be directly linked to the mechanism of action of the contaminants (the biomarker is specific for a particular type of exposure);

- Highly sensitive techniques that require little quantities of sample and are easy to perform and cost effective;
- Suitable for different species;
- Its use is ethically acceptable.

2.3.1. Classification of Biomarkers

Biomarkers have been classified into three general types (NAS-NRC, 1987):

- Biomarkers of exposure: suggest the presence or evidence of the parent compound, its metabolite(s) or other biological products, in physical or biochemical parameters, which can be associated with exposure to the stressor. They indicate that a certain category of substances is present and bioavailable in the environment and is potentially capable of causing damage without giving any indication of the actual toxicological effects. The exposure biomarkers analyzed in this work will be described hereafter.

- Biomarkers of effect: they represent all those responses that show the effect of a toxic compound because they point out an injury. They denote a state of functional suffering.

- Biomarkers of susceptibility: suggest the presence or change in some physical or biochemical parameter that tell us the system in question has the potential to be especially sensitive to the effects of a certain stressor should that stressor become active. They refer to the innate or acquired ability of an organism to respond to a specific xenobiotic substance.

There are also natural factors that affect the physiological status of animal model, altering the signal provided by stress indices. These factors, known as disturbance factors, are physico-chemical conditions of the environment (temperature, salinity, etc.) and biological conditions of the bioindicator (sex, developmental stage, diet, etc.). The influence of the disturbance factors can be reduced by in-depth knowledge of the test organism's life cycle. It is also a good practice to use several biomarkers at the same time to avoid a misleading analysis starting from a single biomarker.

2.4. Metallothioneins (MTs)

Metallothioneins (MTs) belong to the group of intracellular cysteine-rich, metal-binding proteins found in bacteria, plants, invertebrates and vertebrates (Vašák, 2005). They are cysteine-rich non-enzymatic proteins with low molecular weight and characterized by the absence of aromatic amino acids and histidine. They were discovered in 1957 as cadmium-binding proteins isolated from horse kidney. MTs can

be “inducible” when metal ions are present and therefore represent a specific biomarkers for this class of contaminants. These proteins are involved in several intracellular functions (Davis and Cousins, 2000), but their role in the detoxification of heavy metals and in the maintaining of essential metal ion homeostasis, due to their high affinity for these metals, is mostly investigated (Klaassen *et al*., 2009). Based on structural models, it can be assumed that the MTs molecule is composed of two binding domains, α and β, which are composed of cysteine clusters. Covalent binding of metal atoms involves sulfhydryl cysteine residues (Fig. 13). The N-terminal part of the peptide is designated as β-domain and has three binding sites for divalent ions; the C-terminal part (the α-domain) has the ability to bind four divalent metal ions.

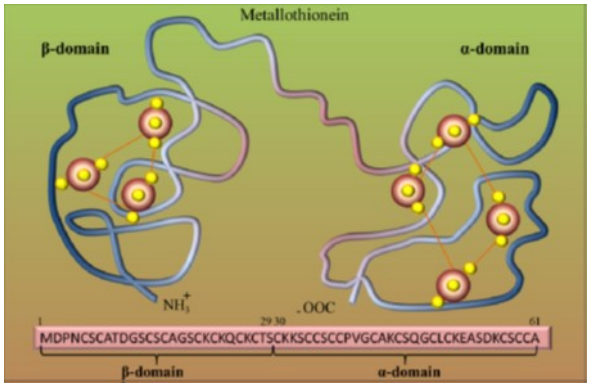


Fig. 13. Model of two binding sites of metallothionein. Red big beads are metal atoms (*e.g.*, Zn), and small yellow beads are sulfur atoms (from Ruttkay-Nedecky *et al*., 2013).

Four mammalian MT isoforms (MT-1, MT-2, MT-3, MT-4) were identified. The differences of constituent forms come mainly from post-translational modifications, small changes in primary structure, type of incorporated metal ion and speed of degradation. Despite the physical-chemical similarity of the forms, their roles in tissues vary significantly. MT-1 and MT-2 are present almost in all types of soft tissues, MT-3 is mostly expressed in brain tissue and the MT-4 gene was detected in stratified squamous epithelial cells associated with oral epithelia, esophagus, upper stomach (Ruttkay-Nedecky *et al*., 2013). Many authors described the effect of MT detoxification in aquatic organisms; It seems that proteins can be expressed differently in fish according to the tissue content of metals. The MTs induction is greater in the tissues involved in absorption and excretion of the metals such as intestine, liver and gills. Once metals accumulate and exceed the production of MTs, the detoxification system filled up and excess metals can bind to enzymes sensitive to them causing toxic effects. MTs can be also activated by a variety of stimuli. In a number of experiments, the synthesis of MTs was shown to be increased by several-fold during oxidative stress to protect the cells against cytotoxicity, radiation and DNA damage (Ruttkay-Nedecky *et al*., 2013).

2.5. Nitric Oxide Synthase (NOS)

Nitric oxide (NO) is a widespread intracellular molecule essential to many physiological functions and operates in several signaling pathways. NO, produced during the conversion of L-arginine to L-citrulline by the enzyme nitric oxide synthase (NOS) (Fig. 14), plays a variety of roles in mammals, ranging from regulation of blood flow, neurotransmission and immune response, and this has stimulated extensive research into the structure, characterization and evolution of NOS (Andreakis *et al*., 2011; Ghosh and Salerno 2003; Marletta 1994). In recent years, growing evidences have implicated NO signalling in various organisms spread throughout the phylogenetic scale, including invertebrates, where it has been shown to subserve a variety of functions (Palumbo, 2005).

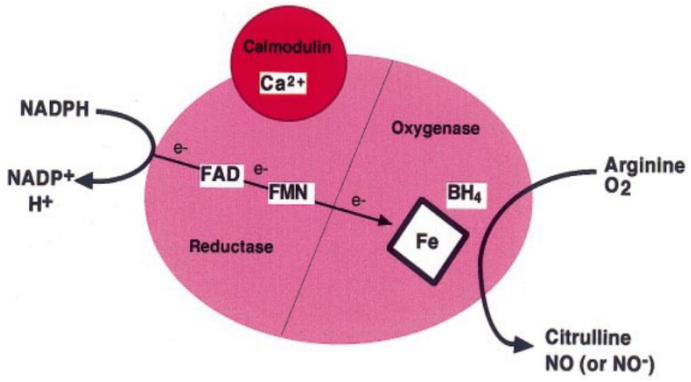


Fig. 14. NO synthesis reaction catalysed by NOS.

In mammals, three NOS isoforms have been identified as the product of three distinct genes. Two of these genes, the neuronal (abbreviated as: nNos, Nos1, NosI) and the endothelial (abbreviated as: eNos, Nos3, NosIII) Nos, are constitutively expressed, and the biochemical activity of the enzymes rely on increasing intracellular calcium concentrations to produce low levels of NO for short periods of time. The third gene, macrophage inducible Nos (abbreviated as: iNos, Nos2, NosII), operates independently of the temporal influx of calcium generating high and sustained amounts of NO (Andreakis *et al*., 2011). The neuronal Nos is also expressed in skeletal and cardiac muscle, pancreatic islets, kidney, respiratory, and gastrointestinal epithelia; the endothelial NOS has been discovered also in cardiac myocytes, brain, and kidney epithelium. The inducible NOS expression has been studied in a variety of cells, including cardiac myocytes, vascular smooth muscle cells, hepatocytes, chondrocytes (Schulz *et al*., 1992).

Vertebrate NOS isoforms share structural similarity: they are homodimeric, heme-containing flavoproteins composed of a reductase and an oxygenase domain.

The reductase domain has binding sites for flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide phosphate reduced (NADPH) and is responsible for providing electrons to the oxygenase domain.
In the oxygenase domain, heme activates oxygen through the intervention of the cofactor tetrahydrobiopterin (BH_4), with consequent production of NO from arginine.
Moreover, the reductase and oxygenase domains are linked by a calmodulin CaM-binding site that functions as a hinge to align the two domains (Ghosh and Salerno 2003).
However, inducible NOS is also up-regulated in various types of inflammatory disease, and the NO generated by the enzyme mediates various symptoms of inflammation. Although primarily identified in macrophages, expression of the enzyme can be stimulated in any cell or tissue. Once expressed, iNOS is constantly active and not regulated by intracellular Ca^{2+} concentrations. Inducible NOS, when induced in macrophages, produces large amounts of NO, which represent a major cytotoxic principle of those cells. The high levels of NO produced by activated macrophages may be toxic, when released at the wrong site, against healthy cells. *In vivo*, cell and tissue damage can be related to the NO radical itself or an interaction of NO with O_2^- leading to the formation of peroxynitrite ($ONOO^-$). The large majority of inflammatory and autoimmune lesions are characterized by an abundance of activated macrophages. Significant amounts of NO can be secreted by those cells, leading to damage of the surrounding tissue (Förstermann and Sessa, 2012).
NO and NOS protein activity has been associated to a variety of functions in invertebrates, including neurotransmission, immune responses, swimming, bioluminescence, response to environmental stress, feeding and development (Palumbo, 2005).

2.6. Heme Oxygenase (H-O)

Heme oxygenase is the rate-limiting enzyme in the catabolism of heme, a process that leads to formation of: carbon monoxide (CO), biliverdin (which is rapidly converted to bilirubin by the action of biliverdin reductase) and free iron (which leads to the induction of ferritin, an iron-binding protein) (Fig. 15).
There are 3 isoforms of Heme Oxygenase:

- HO-1 or inducible isoform;
- HO-2 or constitutive isoform;
- HO-3 recently discovered and cloned only in rats to date (McCoubrey *et al*., 1997).

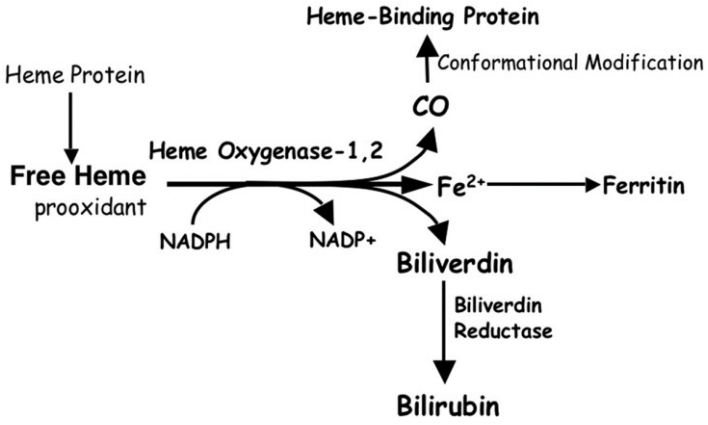


Fig. 15. HO enzymatic reaction. Heme, released from heme proteins, cleaved by HO to iron, CO and biliverdin. Biliverdin is then converted to bilirubin by biliverdin reductase.

HO-2 is constitutively expressed, whereas HO-1 is inducible in response to various stimuli, including oxidative stress, heavy metals, UV radiation and inflammation. Furthermore, HO-1 is a heat-shock protein called HSP-32 and also a stress protein induced by several agents that cause oxidative damage (Poon *et al*., 2004).

These isoenzymes are the products of two distinct genes; both HO-1 and HO-2 catalyze the same reaction (*i.e*. degradation of heme) but differ in many respects and are regulated under separate mechanisms. The most relevant similarity between HO-1 and HO-2 consists in a common 24 amino acid domain (differing in just one residue) called the "HO signature" which renders both proteins extremely active in their ability to catabolize heme (Maines, 1997). These isoforms have different localization, similar substrate, and cofactor requirements, while presenting a different molecular weight.

HO-1 serves as a protective gene by virtue of its anti-inflammatory, anti-apoptotic and antiproliferative actions, variously manifested in endothelial, epithelial, smooth muscle and other cell types (Abraham and Kappas, 2008).

HO-1 induction protect cells from the oxidative stress through different mechanisms, such as: intracellular control of "free" heme (which serves as a pro-oxidant); producing biliverdin (which shows antioxidant properties) and inducing the ferritin synthesis.

Infact, ferritin limits the generation of free radicals by binding free Fe^{2+} that would otherwise participate to promote generation of reactive oxygen species. HO-1 releases Fe^{2+} from the core of the heme molecule, leading to the rapid expression of the iron-sequestering protein ferritin as well as an ATPase pump that actively removes intracellular iron from the cell; in this way contributing to decrease the overall intracellular pool of Fe^{2+} and protecting the cells from apoptosis (Poon *et al*., 2004).

2.7. The use of *Artemia salina* in ecotoxicological testing

Artemia salina is one of the most widespread saltwater organism used in ecotoxicological testing. The use of *Artemia*'s cysts is very common bioassays, but also

larvae or adults can be cultured in laboratory. *Artemia* has many characteristics that explain the large use of this little crustacean for ecotoxicity testing, including:

- Short life cycle;
- High fecundity;
- Bisexual/parthenogenetic reproduction strategy;
- Small body size;
- Adaptability to varied nutrient resources as it is a non-selective filter-feeder;
- Adaptability to a wide range of salinities and temperatures.

According to Nunes *et al*. (2006), cysts based assays are cheap, simple and reliable in routine screening (Libralato, 2014). The *Artemia* bioassay is attractive for a variety of reasons, including:

- The commercial availability of the cysts;
- *Artemia* can be maintained indefinitely in the laboratory in their cyst form and are easily induced to hatch;
- The assay is quick, simple, and performed at low cost;
- It requires small sample volume and can be performed with microplates;
- It complies with animal ethics guidelines in many countries.

This strong adaptability to hypersaline environments, such as permanent salt lakes, coastal lagoons and man-made salt pans (where evaporation of seawater results in high sodium chloride concentrations), make the habitats in which the genus *Artemia* is found deprived of the predatory animal species. Therefore, in such environments, the evolution of *Artemia* populations is favoured by the abundance of protozoa and algae that are the basis of the *Artemia* diet. High flexibility to adverse environmental conditions explain well the wide geographical distribution of the genus.
As such, these characteristics should make *Artemia salina* as a really suitable organism for ecotoxicity testing, but several criticisms about its sensitivity have presented according to a leaning-by-doing approach. At the international level, the use of *Artemia* salina in toxicity testing is subjected to a broad discussion with supporters and detractors (Nunes *et al*., 2006). The sensitivity towards a wide range of substances is really lower than that of other species, so that the possibility to underestimate potential effects may occur. *Artemia salina* showed to be less sensitive than *Vibrio fischeri, Daphnia magna* and *Brachionus plicatili*s (Guerra, 2001), *Daphnia pulex* and *Thamnocephalus platyurus* (Nunes *et al*., 2006).
Other criticisms could be related to the use of cysts (Migliore *et al*., 1997) and cyst-hatched nauplii (Persoone and Wells, 1987) of *Artemia* for testing purposes that could introduce some variability in test results. Indeed, in the genus *Artemia* there is no cyclic bisexual versus parthenogenetic mechanism like for rotifers or cladocerans, that is generally on a seasonal basis, but cysts production may reflect the occurrence of genetic variation to increase adaptability; it is evident that genetic variability is not a desirable condition for ecotoxicity assays (Nunes *et al*., 2006).

The cytotoxicity assays are often tedious and expensive, and there is a lack of a simple and rapid screening procedure. Nowadays, brine shrimp lethality assays are extensively used in research and applied toxicology. Several endpoints can be considered with *Artemia* salina, including:

- Short-term (24-48 h) (Vanhaecke and Persoone, 1981) and long-term (several days) mortality;
- Cysts and nauplii hatchability;
- Biomass productivity including behavioural (such as swimming speed alteration) (Gambardella *et al*., 2014) and biomarker related ones (*e.g.* enzyme expression or inhibition, oxygen consumption rate);
- Bioaccumulation on larvae as well as organisms' reproductive ability (Libralato, 2014).

Recently, *A. salina* started to be used as a reference biological model in nanoecotoxicology with both inorganic and organic engineered nanomaterials. Because of the rapidity, convenience, and low cost of Artemia-based assays, the aim of this paper is to investigate the potential toxic effects of different classes of engineered nanomaterials (ENMs) using *A. salina*. Resilience of these animals makes them ideal for testing samples in the experiments.

2.8. The use of *Danio rerio* as a model vertebrate in ecotoxicological testing

There are several advantages for using zebrafish as a ecotoxicological model species.
The main benefit regards its size. Zebrafish adult is approximately 5 cm long so it can be handled without any difficulty and reduces housing space and husbandry costs.
The tiny size of the larval and adult zebrafish minimizes costs through low quantities of dosing solutions (experimental chemicals, drugs, pollutants) and thereby creates limited volumes of waste for disposal, and minimizes quantities of labware and chemicals.
Small embryos allow reasonable sample sizes to be tested together using a multiwells plate or series of Petri dishes to provide several experimental replicates at one time. From the egg stage, zebrafish embryos can survive for several days through the absorption of yolk and can be visually assessed for malformation (Hill *et al*., 2005).
The rapid maturation of zebrafish (sexual maturation occurs around 100 days) allows easy experimentation for transgenerational endpoints required for mutagenesis screening and assessing chemicals for teratogenicity.
This species shows high fecundity (single female can lay up to 200 eggs per week) and transparent embryos. The eggs hatch rapidly and organogenesis occurs quickly. As a result, the major organs are developed within few days post-fertilization (dpf) in larvae.
Zebrafish eggs remain transparent from fertilization to when the tissues become dense and pigmentation is initiated (at approximately 30-72 h post fertilization (hpf)), this

allows unobstructed observations of the main morphological changes up to and beyond pharyngulation. Therefore, using little magnification, adverse effects of chemical exposure on development of the brain, notochord, heart, jaw, trunk segmentation, and measurements of size can be assessed quantitatively.

Their optical clarity allows for identification of phenotypic traits during mutagenesis screening, and assessment of endpoints of toxicity during toxicity testing. This proves even more valuable when immunochemistry (IHC) techniques are used. There are a vast amount of histochemical markers available, allowing assessments of aberrant morphology or activation of certain signaling pathways by toxicants through the staining of specific tissues and cells types. Whole-mount larval staining can be performed rather than first having to dissect the tissue or stain sections.

Zebrafish may be considered a relatively simple organism when compared to mammals. However its genome is more complex than the human genome, because zebrafish have two more pairs of chromosomes than human. This difference occurred because, at some time in teleost evolution, there was a whole-genome duplication event that did not occur in mammals. It resulted in numerous duplicate genes (paralogs) of those found in mammals. The significance of this finding is that, where a mutation in a mammalian ortholog may cause embryonic lethality, a mutation in one of the zebrafish paralogs may show a less severe phenotype with the embryo remaining viable. This may permit analyses of gene function in mutant zebrafish that would be difficult to achieve in mutant mammals due to the associated embryo mortality (Spitsbergen and Kent, 2003). The zebrafish research community has developed a range of resources very useful to the toxicologists, including mutant strains, cDNA clone collections, and whole genome that has been sequenced a few years ago (Sanger Institute, http://www.sanger.ac.uk/Projects/D_rerio). Highly conserved signaling pathways are found both in zebrafish and mammals with a high level of genomic homology (Belyaeva *et al*., 2009).

In recent years, the use of zebrafish as an established animal model system for nanoparticle toxicity assay is growing exponentially. Different types of parameters are used to evaluate nanoparticle toxicity such as hatching achievement rate, developmental malformation of organs, damage in gills and skin, abnormal behavior (movement impairment), reproduction toxicity and finally mortality. In fact, there are an increasing number of literatures that documents the concern over toxicity for broad range of engineered nanoparticles/nanomaterials. In this regard, zebrafish as an *in vivo* model organism has attracted scientific interest because of its unique features above mentioned.

Interestingly, zebrafish behavioral response is also a sensitive indicator for abnormal change in toxicity. An experiment performed by Chen *et al*. (2011) has also shown that TiO_2 nanoparticles affect larval swimming parameters, including velocity and activity level.

The disruption of gills, skin and endocrine system by nanoparticles is another parameter to understand nanoparticle induced toxicity. It has been reported that silver ions (Ag^+) generated by AgNPs exert acute toxicity, mainly due to their interaction with

the gills. In the gills, ionic Ag^+ inhibits Na^+/K^+ - ATPase action and the enzymes related to Na^+ and Cl^- uptake, finally affecting osmoregulation (Wood *et al*., 1999).

Nanoparticle affects male and female reproductivity and fetal development. Wang *et al*. (2011) assessed the disturbance in zebrafish reproduction after the chronic exposure of TiO_2 nanoparticles.

Presently, zebrafish have become a smart vertebrate model for toxicological testing and for nanotoxicological testing of ENMs. With the use of modern technology, the zebrafish might be able to become a significant alternative of other mammalian models in next future.

The aim of this *PhD thesis* is to investigate the potential toxic effects of different classes of engineered nanomaterials using *Danio rerio*. Peculiar features of these animals makes them ideal for testing samples in the experiments.

3. MATERIALS AND METHODS

3.1. Synthesis of oxide nanoparticles by Pulsed Laser Ablation in Liquids (PLAL)

Pulsed Laser Ablation in Liquids (PLAL) is one of the most promising techniques for the synthesis of oxide nanoparticles in liquids. It is a versatile, inexpensive, compatible with industrial processes and "green" methodology: it avoids the use of chemical reagent and reaction products, solving the problem of purification of nano-materials (Pyatenko *et al*., 2009). It is a physical process that involves the use of short laser energy pulses on a substrate immersed in a solvent. The substrate absorbs the energy and release material that then condenses into nanoparticles.

The synthesis of laser ablated nanoparticles (LA-NPs) was performed by the PLAL method. A Nd:YAG (Giant G790-30) 1064 nm laser was employed as source for irradiating a titanium metal plate (Goodfellow, purity 99.9%, as rolled) with 10 ns pulses and repetition rate of 10 Hz. The beam was focused with a lens on the bottom of a teflon vessel filled with 5 ml of deionised Milli-Q water. Samples were irradiated with 350 mJ/pulse for 30 minutes with a fluence of 5 J/cm^2. The mass of the ablated material was measured by weighting the target before and after the ablation process with a micro analytical balance (Sartorius M5). The titanium concentration in solution was calculated by using the mass loss of the target during irradiation.

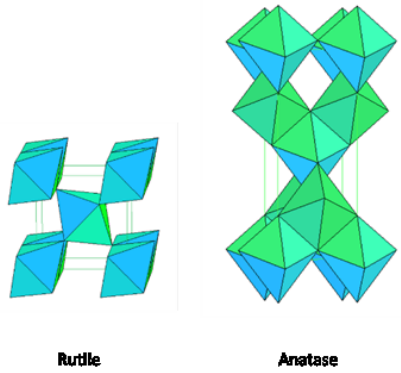
AgNPs and AuNPs described and used in this study were produced by PLAL method; on the contrary TiO_2NPs were purchased and then characterized.

3.2.Characterization of nano-TiO_2 powder

TiO_2 nanopowders (Degussa, P25) were purchased from Sigma-Aldrich. The structural characterization was performed by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) (Figs. 16).

Figs. 16. A) Scanning Electron Microscope (SEM) Zeiss Supra 25; B) Transmission Electron Microscope (TEM) Jeol JEM-2010F.

The ENPs have an average size of about 50 nm. SEM analyses were performed in plan-view by using a field emission Zeiss Supra 25 microscope. TEM analyses were performed in cross-section by using a Jeol JEM-2010F microscope operated at 200 keV and equipped with a post-column Gatan GIF 2001 energy image filter.
The nano-powder crystalline phase is mixed: 86% anatase, 14% rutile (Figs. 17). The purity of the material is 99.5%, with metal traces (according to manufacturer's information).



Figs. 17. Basic scheme of the TiO_2 crystalline phases.

3.3. Synthesis of graphene oxide (GO) and reduced graphene oxide (rGO)

Graphene oxide was synthesized by the modified Hummers method (Hummers and Offeman, 1958), which was proposed by Laura J. Cote and her research group (2009). This modified approach can be described as follows:

- 0.5 g of graphite, 0.5 g of sodium nitrate ($NaNO_3$) and 23 ml of sulfuric acid (H_2SO_4) are stirred together in an ice bath.
- 3 g of potassium permanganate ($KMnO_4$) was slowly added.
- After mixing, the solution is carried to a 35 ± 5 °C water bath and stirred for about 1 h, forming a dense paste.
- Subsequently, 40 ml of water is added and the solution is transferred to 90 ± 5 °C for 30 min (stirring).
- Then, 100 ml of water is added, followed by the addition of 3 ml of hydrogen peroxide(H_2O_2) (30%): the color of the solution will turn from dark brown to yellow.
- The solution is filtered and washed with 100 ml of water. The filter cake is then dispersed in water by mechanical agitation.
- Low-speed centrifugation is done at 1000 rpm until all visible particles are removed from the precipitates.

- The supernatant is then spin-dried by a high-speed centrifugation step at 8000 rpm for 15 min to remove small GO pieces and water-soluble byproducts.
- The final sediment is re-dispersed in water.

A field emission scanning electron microscope (Zeiss Supra 35 FE-SEM) was used to investigate the morphology of the GO flakes. The SEM image of GO sheets produced in aqueous suspensions, via oxidation processes of graphite powder, is shown in Fig. 18. The solutions were analyzed by recording the absorbance spectra using a UV-Vis AGILENT Cary 50 spectrophotometer in a wavelength range between 200 and 800 nm. The concentration of GO flakes in water was evaluated by the intensity of the absorbance peak at 231 nm.

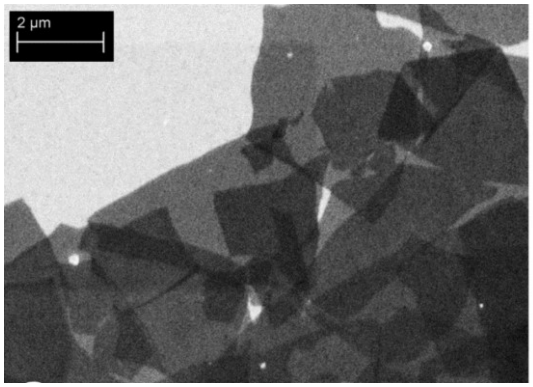


Fig. 18. SEM images of the GO flakes. Note the typical regular shape for GO sheets with an average area of several μm^2 (from Buccheri *et al*., 2016).

The obtained GO solution was subjected to a pulsed laser irradiation process, under continuous stirring, in order to modify the graphene oxide in size and in the total amount of oxygen obtaining reduced graphene oxide (rGO) (Fig. 19). For such a process, the second harmonic (532 nm) radiation of a Nd:YAG pulsed laser system (Continuum, Surelite II model) was used, operating with a pulse duration of 5 ns and a repetition rate of 10 Hz. The laser beam size was around 28 mm^2, and it was directed toward the GO solution without any focusing lens. The GO suspension was irradiated homogeneously at a constant fluence of 0.32 J/cm^2 for different times ranging between 15 and 300.

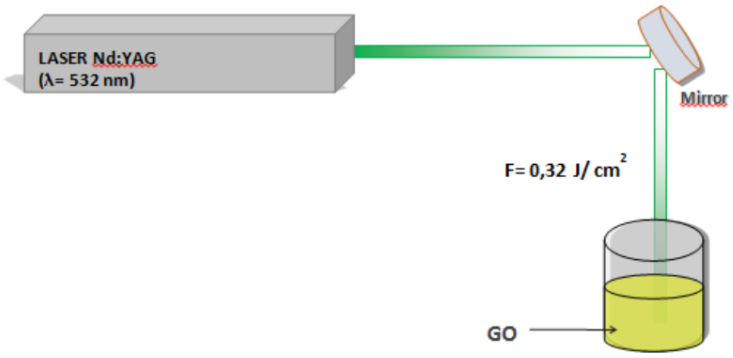


Fig. 19. Experimental setup for laser irradiation process of GO.

3.4 Atomic Layer Deposition (ALD): synthesis of engineered nanomaterials (ENMs)

Atomic layer deposition (ALD) is a technique for depositing thin films for a variety of applications. Since the ALD process is intrinsically atomic, ALD produces layers with nanometer scale thickness control (George, 2010).
A gold film, with a thickness of ~ 5 nm, was sputtered on a quartz substrate (Fig. 20).

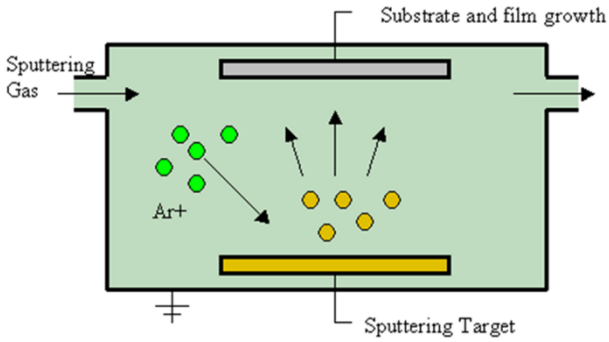
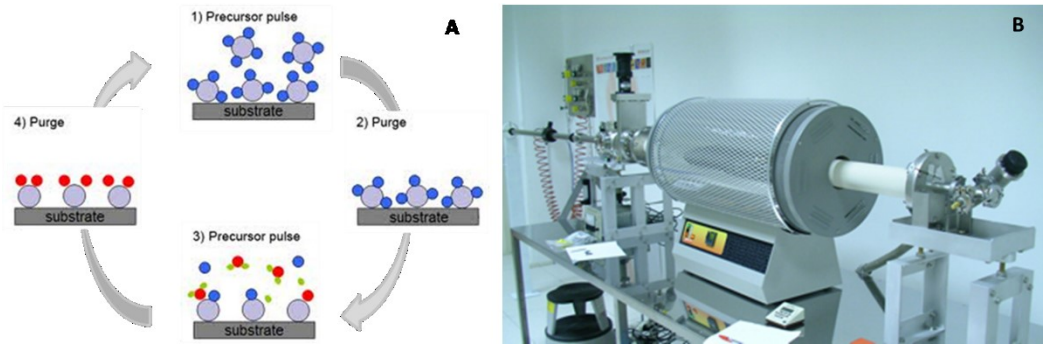


Fig. 20. Basic scheme of sputtering deposition process: target material (in this case gold) is eroded by an inert gas such as argon (Ar^+).

In order to induce the self-organization of the metal nanoparticles, the samples were annealed at 600 °C for 1 h in a conventional furnace under a controlled N_2 flux (Figs. 21).



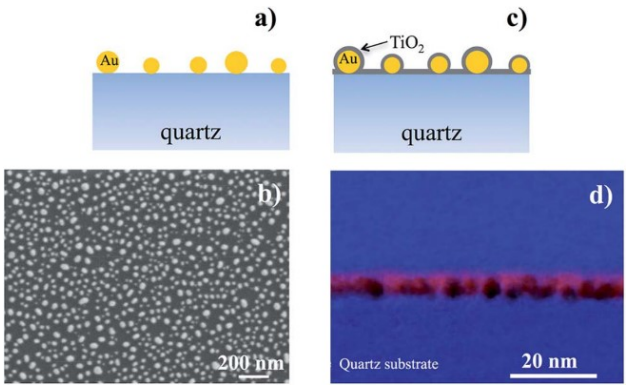
Figs. 21. A) A basic schematic of the Atomic Layer Deposition (ALD) process; B) Coventional and orizzontal furnace adopted for termical treatments (Carbolite, CNR-IMM Catania).

The synthesized gold NPs (~ 8 nm mean diameter) were covered with a thin layer of TiO_2 (~ 4 nm thick) deposited by ALD. The ALD was performed with a Beneq TFS-200 system, with $TiCl_4$ (Sigma Aldrich, 99.9%) and deionized water as precursors, at a deposition temperature of 200 °C. Nitrogen (>99.999%) from a Sirocco N_2 generator was used as a carrier gas, with a combined flow rate of 550 sccm, maintaining a reactor pressure of approximately 1.3 mbar. The deposition of the TiO_2 film on top of the gold nanoparticles avoided the photocatalytic efficiency loss due to coverage of the TiO_2 surface by the metal particles (Armelao *et al*., 2007). This sample typology will be hereafter called "TiO_2/Au" (*i.e*. TiO_2 on Au nanoparticles).

Two reference samples were also synthesized and used for comparison:

(1) a TiO_2 at film (~ 4 nm thick) deposited by ALD on a quartz substrate, hereafter simply called "TiO_2";

(2) Au nanoparticles on top of a TiO_2 film (~ 4 nm thick), realized by ALD and sputtering, on a quartz substrate, which is the configuration commonly reported in the literature (Armelao *et al*., 2007), hereafter called "Au/TiO_2" (*i.e*. Au nanoparticles on top of TiO_2) (Figs. 22).



Figs. 22. A) Scheme of the AuNPs on the quartz substrate just after the thermal process and B) the relative plan-view SEM image; C) Scheme of the sample after the TiO_2 deposition by ALD and D) the relative EF-TEM image in false colors (TiO_2 is denoted in magenta and Au nanoparticles in black) (from Scuderi *et al*., 2014).

3.5 Graphene oxide and titania loaded Nafion based nano-composites

Nanocomposite membranes were prepared using Nafion polymer combined with various fillers, such as anatase-type TiO_2 nanoparticles and graphene oxide. Nafion, as a weight ratio (wr) of 20% dispersion in water and lower aliphatic alcohols, was supplied by Aldrich. Graphene oxide in aqueous suspensions synthesized by a modified Hummers' method (Hummers and Offeman, 1958), via oxidation processes of graphite powder using sulfuric acid, potassium permanganate and hydrogen peroxide. Anatase TiO_2 nanoparticles with a nominal average diameter of 21 nm, and methyl orange (MO, 0.1 M in H_2O) were acquired from Sigma-Aldrich.

3.5.1 Membranes preparation

The preparation of hybrid nanocomposite Nafion membranes consists of the following steps: dispersing the fillers (anatase-type TiO_2 nanoparticles, GO) directly in Nafion solution, with a filler/polymer weight ratio of 3%, ultrasonicating for 1 day and stirring for another day at room temperature until a clear solution is obtained.
After that, the dispersion is cast on a Petri dish at 30 °C overnight to remove the solvents. The hybrid membranes are removed from the Petri dish by immersing the glass plate in deionized water for several minutes. To reinforce the membrane, it is sandwiched and pressed between two Teflon plates and placed in oven at 150 °C for about 25 min.

All composite membranes produced by casting are subsequently treated by rinsing in:

1) Boiling HNO_3 solution (1 M) for 1 h to oxidize the organic impurities;
2) Boiling H_2O_2 (3 vol%) for 1 h to remove all the organic impurities;
3) Boiling deionized H_2O for 40 min three times;
4) Boiling H_2SO_4 (0.5 M) for 1 h to remove any metallic impurities;
5) Boiling deionized H_2O for 40 min twice to remove excess acid.

The membranes, as well as the fillers, were analyzed by scanning electron microscopy (SEM), using a ZEISS Supra 35 field emission SEM, in order to observe their morphology, homogeneity and size.

3.6. *Artemia salina* acute toxicity test (24 h)

Commercially avalaible *Artemia salina* dehydrated cysts were purchased from local aquarium store (Artemio Pur® JBL GmBh&Co., Germany; ArtemiaCyst Blue Line, Italy). Cysts were first hydrated in artificial seawater Istant Ocean® (prepared by dissolving 35 g in 1 liter of deionised water) and then washed to separate the floating cysts from

those that sink. The sinking cysts were collected and approximately 1 g of the precleaned cysts were incubated in 1 liter of ASPM solution seawater in a conical plastic contained with graduations. A 1.500 lux daylight was provided continuously by a fluorescent lamp. Aeration was maintained by a small line extending to the bottom of the hatching device from an aquarium air pump. Under conditions of incubation at room temperature (26 ± 1 °C), gentle aeration and continuous illuminations, the nauplii hatched within 48 h. Bioassays was conducted using ASPM solution containing following salts (per liter of deionised water):

- NaCl = 26.4 g
- KCl = 0.84 g
- $CaCl_2 \cdot H_2O$ = 1.67 g
- $MgCl \cdot H_2O$ = 4.6 g
- $MgSO_4 \cdot 7H_2O$ = 5.58 g
- $NaHCO_3$ = 0.17 g
- H_3BO_3 = 0.03 g

Materials:

- Stereomicroscope (Leica M0205C, Multifocus);
- Standard 96-well plates;
- Parafilm to cover the 96-well plates;
- Incubator (26 ± 1 °C);
- pH-meter;
- Laboratory thermometer;
- Glass vials to prepare different test concentrations and dilution water;
- Sonicator for dissolving NPs solutions;
- 1500 lux illuminating system for animal housing (luximeter for measuring luminous intensity);
- Adjustable volume pipettes with tips;
- Aquarium air pump.

A stock solution of AuNPs (500 μg/ml), AgNPs (200 μg/ml) and TiO_2NPs (100 μg/ml) diluted in ASPM solution was prepared. A number of 200 nauplii were exposed for each concentration. Then, fresh suspensions with different concentrations of NPs (serial dilutions ranging from 10^{-1} to 10^{-3} μg/ml) were made starting from the stock suspensions of NPs diluted in ASPM water. This solution was vortexed for 30 seconds, and then sonicated in an ultrasonic bath for about 20 min for maximum dispersion immediately before use.

500 μl of each different concentrations of NPs solutions were added to the 96-well microplates. After that, N. 1 nauplius per well was added and incubated at 26 °C for 24 h. The numbers of surviving nauplii in each well were counted under a

stereomicroscope after 24 h. A control group was also setup with ASPM only. Larvae were not fed during bioassays.
The endpoint (immobility/death) was assessed at the end of the test by a stereomicroscope (Leica EZ4): a nauplium was considered to be immobile or dead, if it could not move its antennae after slight agitation of the water for 10 seconds.
Larvae that were completely motionless were counted as dead, and the percentages of mortality compared to the control were calculated.
The percentages of deaths organisms were calculated by comparing the number of survivors in the test and control wells. The immobilization % of the crustacean for each concentration considered was calculated as follow:

(n. dead nauplii/n. total animal treated) * 100

The collected data were analyzed for significance by one-way ANOVA test at $p < 0.05$.

Furthermore, another set of experiments designed to verify if hatching could be conditioned by nanoparticles exposure, consisted of adding 1 mg of cysts per well to the 96-well microplates (the same used for nauplii). Then, 500 µl of each different concentrations of NPs solutions were added to each well. Given that, 1 mg contains about 100 cysts, the percentages of not-hatched nauplii for each concentration considered were calculated by counting the number of whole cysts.

3.6.1. SEM protocol on *Artemia salina* nauplii

Nauplii were rinsed in PBS, then fixed in 2.5% PBS-buffered glutaraldehyde (1:10, Merck) for 1 h at room temperature (r.t.). Samples were washed in PBS (3 x 10 min), dehydrated in ascending alcohols series (35°, 50°, 70°, 95°) for 10 min each one and absolute ethanol (20 min), and placed in hexamethyldisalazane (HMDS) ($C_6H_{19}NSi_2$, Merck) for 5 minutes. Then, they were air dried at room temperature, mounted on SEM stubs, metal coated and observed by SEM-EDX analysis (Cambridge Stereoscan 360C Instruments - Energy Dispersive X-ray Analysis, INCA Oxford).

3.7. Fish embryo toxicity (FET) test on zebrafish with GO and nanocomposites

The FET test is a modern toxicity test representing an effective alternative to an acute test with adult fish. This model has become increasingly common for the investigation of developmental toxicity mechanisms, especially in environmental studies. Zebrafish of the AB strain (wild-type, wt) were obtained from the Center of Experimental Ichthyiopathology of Sicily (CISS), University of Messina (Italy), where they are kept in a “Fish facility” (Stand Alone Unit, Tecniplast), a closed-loop system that allows the continuous monitoring of vital parameters. They were raised in a circulating aquarium system in an environmentally controlled room (28 °C, 80% humidity), with the

photoperiod adjusted to a 14 h light/10 h dark cycle. The larval and adult zebrafish were fed with brine shrimp (hatched from 2 g of eggs in 1 liter of artificial sea water) daily. All embryos used in the experiments derived from the same spawn of eggs.

Materials

- Fish tanks made of chemically inert material (*e.g.*, glass) and with 10 liter capacity;
- Glass marbles or other inert material to protect the eggs laid;
- Stereomicroscope (Leica M0205C, Multifocus);
- Standard 24-well plates;
- Parafilm to cover the 24-well plates;
- Incubator (26 ± 1 °C);
- Pipettes with widened openings to collect eggs;
- Glass vessels to prepare different test concentrations and dilution water or to collect eggs;
- Sonicator.

According to OECD guidelines for the testing of chemicals (2013, Test Guideline N° 236), Fish Embryo Toxicity (FET) test was performed to determine acute toxicity of some different Engineered Nanomaterials (ENMs), nanoparticles and nanocomposites, adopting some preventing measures. Before starting treatment, NPs solutions were sonicated by an ultrasound probe (sonicator), for effectively dispersing NPs in aqueous solution (it is very important breaking down the aggregates that could be formed because of sedimentation). Test solutions, prepared by dilution of a stock solution, were dispensed into multiwells plates. If more than one dead embryo is observed in the internal plate control, the plate is rejected. Moreover, a positive control is performed with a 4 mg/l 3,4-dichloroaniline (DCA) in water (as provided by OECD guidelines). Zebrafish fertilized eggs are collected immediately after fertilization (within 4 h post fertilization (hpf)) and transferred gently one by one in each well through glass Pasteur pipettes. Specifically, embryos were exposed to GO, irradiated GO for 1 and 4 hour, respectively named iGO-2h and iGO-4h at different concentrations (10, 20, 40, 80, 160 mg/l) for 96 hours. The GO and iGO solutions were renewed; the embryonic/larval mortality and hatching rate were evaluated every 24 h. Healthy embryos were placed in 24-well culture plates (10 embryos in 2 ml solution/well). Each group had five replicate wells. Each experiment was replicated four times. During the exposure period, photographs of the embryos were captured under a stereomicroscope (Leica M0205C, Multifocus) and the percentage of abnormal embryos was counted every 24 h. Apical observations performed on each tested embryo include: coagulation of embryos, lack of somite formation, non-detachment of the tail, and lack of heartbeat. These observations are used for the determination of

lethality: Any positive outcome in one of these observations means that the zebrafish embryo is dead. Observations are recorded every 24 hrs, until the end of the test.

Eggs can be distributed to well plates in the following way:

- 20 eggs on one plate for each test concentration;
- 20 eggs as positive control on one plate;
- 4 eggs in dilution water as internal plate control on each of the above plates;
- 24 eggs in dilution water as negative control on one plate.

Distribution of eggs over the 24-well plates is illustrated in Fig. 23.

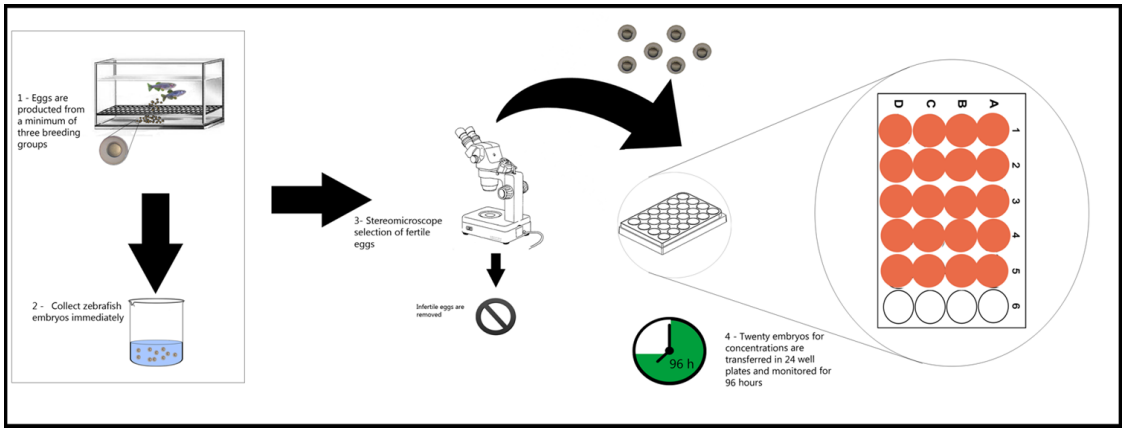


Fig. 23. Freshly spawned eggs are collected. Under the dissecting microscope, fertilized eggs (differentiated from non-fertilized eggs) are transferred into the 24 well plates pre-incubated with the test solutions.

3.7.1 Fish Embryo Toxicity (FET) Test with Nanomaterials "TiO_2/Au" and "Au/TiO_2" compared to Au and TiO_2 free NPs

Fish Embryo Toxicity (FET) test was effectuated according to OECD (2013). Zebrafish embryos exposed to "TiO_2/Au" and "Au/TiO_2" nanomaterials, to Au and TiO_2 free NPs at different concentrations (10^9, 10^{10}, and 10^{11} in 5 ml of freshwater) for 4-96 hpf were used to measure potential toxic effects of new engineered nanomaterials.
The experiment was carried out on the nanomaterials just produced (day 0) and then placed for 12 days (day 12) in water on an orbital shaker (Stuart Scientific SO3, Bibby). The TiO_2 and Au solutions were renewed and embryonic/larval mortality and hatching rate were evaluated every 24 h. Healthy embryos were placed in 24-well culture plates (10 embryos in 2 ml solution/well).
Each group had five replicate wells. Each experiment was replicated four times. During the exposure period (4-96 hpf), photographs of the embryos were captured under a

stereomicroscope (Leica M0205C, Multifocus) and the percentage of abnormal embryos was counted every 24 h.

The toxicity of free gold NPs has been also tested in salt water solution in order to discriminate the stability of the nano-composite and of the free nanoparticles. The Au nanoparticles were synthesized by PLAL method; Dynamic light scattering technique evidenced a hydrodynamic diameter of the gold nanoparticles of 30 ± 2 nm.

Statistical Analysis was performed with Prism Software (Graphpad Software Inc., La Jolla, CA, USA). Data were expressed as mean ± SEM. Statistical analysis was carried out by ANOVA test. A p value < 0.05 was considered statistically significant between experimental and control groups.

3.7.1.1 SEM Analysis (Day 0/Day 12) of the Nanomaterials "TiO_2/Au" and "Au/TiO_2"

The structural characterization of the nanostructured materials was performed by SEM. The analyses, in plan view, were acquired by a field emission Zeiss Supra 25 microscope. The size distribution of the gold nanoparticles, deposited on the surface of the TiO_2 film, was calculated by using the Digital Micrograph program by Gatan. In particular, several micrographs were taken into account, each of about 1700 × 1200 nm in size, containing ~ 2000 nanoparticles. The structural characterization and the relative gold nanoparticles distribution were performed before and after 12 days dipping in aquatic environment, in order to evaluate the stability of the gold nanoparticles.

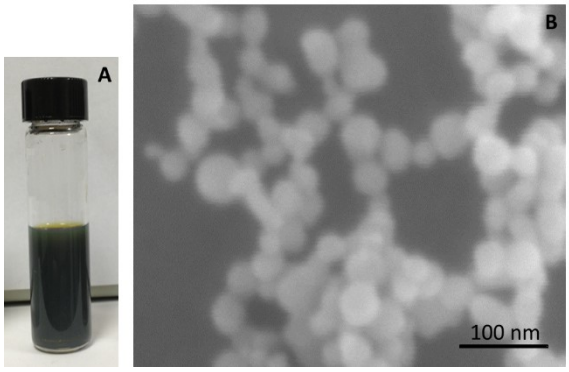
3.8. Ecotoxicological long-term testing on adult zebrafish with AgNPs

Long term toxic effects by silver nanoparticles were studied on adult zebrafish provided by the Center of Experimental Ichthyiopathology of Sicily (CISS) of the Department of Veterinary Science (University of Messina, Italy) which has provided zebrafish specimens. The Italian Health Ministry (authorization n°1244/2015-PR) approved the use of N. 40 adult animals set up for this trial. Before starting experimentation, animals housed for a long time in the Fish Facility (Zeb Tech, Stand-Alone), fed and natural physical/chemical conditions were provided daily (photoperiod 14:10 light:dark; temperature 27 °C; pH7.5) ensuring the highest animal welfare as it established by the World Organisation for Animal Health. Three concentration of AgNPs (8, 45, 70 µg/l) were evaluated for an exposure time of 30 days. Three different solutions were prepared daily starting from stock particle solution, added to the standalone facility water and sonicated for 4 cycles. Each cycle was 15 minutes with 5 min of break using a probe sonicator (FALC Labsonic LBS2) with a frequency of 40 kHz under extractor fan. Zebrafish, randomly selected and mixed between male and female, have been subdivided in four groups (CTRL, NPa, NPb, NPc), each one of them

composed of 10 fishes and introduced in tanks, equipped with aeration, 2 litres of volume containing AgNPs solutions. The fish behaviour was evaluated visually after 0, 3, 6, 24 hours after exposure to silver nanoparticles in order to verify changes in swimming speed, loss of balance, respiration or other abnormal behaviour. Every day, fish mortality recorded.

3.8.1 AgNPs Characterization

Silver nanoparticles was synthesized by the PLAL method (as described above). The examination by SEM reveals the characteristic spherical shape of about 25 nm in diameter, negatively charged at pH 7 with a Z-potential of approximately -40 mV (Figs. 24).



Figs. 24. A) Vials of AgNPs just produced; B) SEM images of silver nanoparticles.

SEM images acquired by using a Field Emission SEM (Gemini Zeiss SUPRA™25) at working distance of 5-6 mm, using an electron beam of 5 KeV and an in-lens detector. The crystalline structure of the samples was determined by glazing angle (0.5°) X-Ray Diffraction by using a Bruker D-9000 instrument (Cu Kα) and Bruker diffraction suite software for the diffraction analysis. The instrumental resolution was less than 0.01°. UV spectrum were carried out by a Perkin-Elmer Lambda 35 spectrometer in the wavelength range 300-700 nm. It exhibited a characteristic absorption maximum at 410 nm (Fig. 25).
The AgNPs solution kept in the darkness at the temperature of 4 °C before use, due to the photosensibility of these nanoparticles.

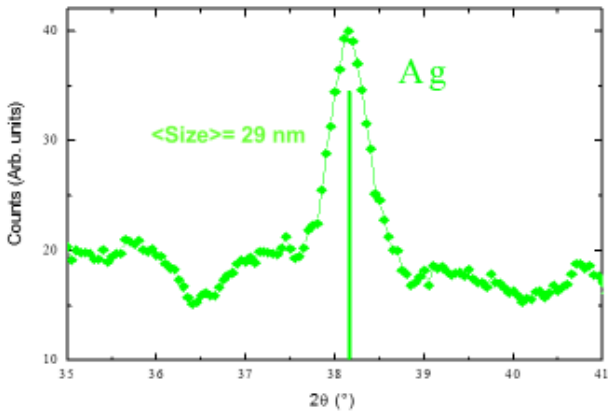


Fig. 25. XRD spectrum of AgNPs.

3.8.2 Silver quantification through ICP-MS analysis

Metal silver extraction performed by microwave digestion (Milestone Ethos 1 FKV, Bergamo, Italy).

- AgNPs solutions sonicated for 2 hours;
- 0.5 g of each analytical sample weighed with an analytical scale (Mettler A30) into a TFM vessel and mineralized in a microwave system with a digestion solution prepared using 7 ml of 65% nitric acid (HNO_3) and 2 ml of 35% hydrogen peroxide (H_2O_2, J.T. Baker);
- After mineralization (Microwave power 1500 W, temperature 200 °C), the vessels were opened, the solutions transferred into flasks and ultrapure water (Millipore Merck) was added to the samples up to final volume. A standard Silver ICP (Sigma-Aldrich, Milan, Italy) and the calibration curve used with the method of the standard solution diluted to various concentrations with a range 1-100 ppb (μg/l).

Ag content carried out by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) under following conditions:

- Octapolo ICP-MS Agilent 7500 (Santa Clara, CA, USA) equipped with a Autosampler ASX 500;
- Radio frequency of 1.5 kW;
- Make-up gas at a flow rate of 0.24 l/min;
- Carrier gas at a rate of 0.88 l/min;
- Argon as the plasma gas with a flow rate of 15 l/min.

^{45}Sc, ^{89}Y, ^{182}W were used as internal standard, at a concentration of 100 mg/kg.
The quantitative analysis performed using a calibration curve (R^2 > 0.997). The detection limit for silver was < 1μg/kg. The SEM analysis conducted on polished graphite plates through the drying of a few drops of AgNPs mother solution after

appropriate sonication.

3.8.3. Istological protocol on adult zebrafish

At the end of the experimental period, adult of zebrafish (N. 40) have been carefully euthanized by anesthesia with a dose of 0.7 g/l tricaine methane sulfonate (MS-222) buffered. Gills, liver, gut were excised and immediately fixed in 4% formaldehyde (Bio-Optica) in PBS buffered (Phosphate Buffered Saline, Sigma Life Science) at room temperature for 24 h.

Gills were decalcified, prior to processing, with a decalcifier agent (Biodec R, Bio-Optica) for 2 h at room temperature. Histological examinations were performed according to our standard laboratory procedures. All tissues were washed three times with PBS (0.1 M, pH 7.4), gradually dehydrated in ascending alcohols series (35°, 50°, 70°, 95°, absolute ethanol) for 20 min each one and clarified in xylene for 1 h at room temperature, subsequently embedded in paraffin wax (Medite tissue wax 56-58 °C). Five µm thick histological sections cut by microtome (Reichert Jung 1150 Autocut) and collected on glass slides (Menzel Gläser, Thermo Scientific). At least ten slides of each tissue were collected. The sections were stained with Haematoxylin-Eosin (HE) (Bio-Optica) and observed under optical microscope (Leica DM750) to identify potential morphological alterations. Photographs were produced using an optical microscope (Leica DMLB) equipped with a digital camera (Leica DFC500).

3.8.4. Immunohistochemical protocol on zebrafish adult

Immunohistochemistry (IHC) is a technique to identify discrete tissue components by the interaction of target antigens with specific antibodies tagged with a visible label. IHC makes it possible to visualize the distribution and localization of specific cellular components within cells and in the proper tissue context. In this work, conjugation of a secondary antibody fluorochrome-conjugated that binds a primary antibody resulting in a fluorescence emission was used.

This protocol performed on gills, gut and liver tissue sections of zebrafish adults, in order to detect Metallothioneins. Negative controls for immunodetection performed using 1% bovine serum albumin (BSA, EMS) in PBS buffered in place of primary antibody. Immunoglobulins non-specific binding sites were saturated using normal goat serum (Vector Laboratories, 1:10) in PBS for 1 hour in a humid chamber. Primary antibody is anti-mouse MT (Santa Cruz Biotechnology, CA, USA, 1:1000), secondary antibody is TRITC-conjugated goat anti-mouse IgG secondary antibody (1:2000, Sigma-Aldrich).

Sections (5 µm thick) of zebrafish liver, gills and gut dewaxed in xylene overnight, were rehydrated in descending alcohols series (absolute ethanol, 95°, 70°, 50°, 35°) until tap water 2 min each one. Then, they were incubated with hydrogen peroxide (H_2O_2) and methanol (CH_3OH, J. T. Baker) (1:10) in humid chamber (to deactivate the endogenous

peroxidase eventually present) at 4°C for 20 minutes. After three washings in PBS (0.1 M, pH 7.4) and incubation with normal serum, sections incubated with primary antibodies overnight to 4 °C. Then, sections washed twice in PBS and incubated with secondary antibodies to 4 °C for 1 hour in darkness. Finally, sections rinsed twice in PBS and rapidly dehydrated in ascending alcohols series (70°, 80°, 95°), mounted with Antifade Mounting Medium containing DAPI (Vectashield, Vector Laboratories). Photographs were carried out using a microscope ZEISS AXIO Observer Z1 with Apotome2 system, equipped with the ZEN PRO software.

3.9. Immunofluorescence protocol on zebrafish larvae

This protocol performed only on zebrafish larvae in order to detect biomarkers of exposure by immunofluorescence, including:

- Metallothioneins (MTs);
- inducible Nitric Oxide Synthase (iNOS);
- Heme Oxygenase (HO-1).

Materials

- Phosphate buffered saline (PBS tablet) (Sigma Life Science);
- Paraformaldehyde (J.T. Baker);
- Triton X-100 (Chemsolute);
- PBS-Tween20 (J. T. Baker);
- Primary Antibodies:
  Anti-mouse Metallothionein (MT) (1:1000, Santa Cruz Biotechnology, CA, USA);
  Anti-rabbit Heme Oxygenase (HO-1) (1:1000, Enzo Life Sciences);
  Anti-rabbit Nitric Oxide Synthase (iNOS) (1:1000, Santa Cruz Biotechnology, CA, USA);
- Secondary Antibodies (respectively indicated to their Ab I correlated):
  TRITC-conjugated goat anti-mouse IgG secondary antibody (1:2000, Sigma-Aldrich);
  FITC-conjugated goat anti-rabbit IgG secondary antibody (1:2000, Sigma-Aldrich);
- DAPI Vectashield (Vector Laboratories);
- Rubber Cement (Victor Product, Inc).

Samples washed in PBS (1 x for 3 minutes) at room temperature and fixed in Paraformaldehyde for 20 minutes at r.t. They rinsed in PBS (2 x 5 minutes) at r.t. and permeabilized in TRITON (0.5% in PBS 1X) for 15 minutes at r.t. Larvae were placed on slides and incubated in primary antibody over night at 4 °C. Wash in PBS-Tween20 (2 x 5 minutes) at r.t. and incubate in secondary antibody fluorescein isothiocyanate (FITC) conjugated IgG for 1 hour at 4 °C in dark condition. Samples washed in PBS-Tween20 (2

x 5 minutes) at r.t., dehydrated in 70°, 80°, 95° alcohol for 1 minutes each one, air dried at room temperature and stained with DAPI. Mount with rubber cement.
Negative controls for immunofluorescence labeling were incubated only in PBS-Tween20 over night at 4 °C (without primary antibody). Observations were carried out using a microscope ZEISS AXIO Observer Z1 with Apotome2 system, equipped with the ZEN PRO software.

3.10. Protein expression protocol performed by Western blot

Several proteins determination in larvae and in tissue of zebrafish adult can be conducted by Western blotting analysis.

Materials

- iBlot® Transfer Stack, nitrocellulose and system;
- Novex® Mini Gels, Bolt® Bis-Tris Plus Gels, NuPAGE® Mini Gels;
- Sample Bolt™ LDS sample buffer (4X);
- Bolt™ Reducing Agent (10X);
- Deionized water total volume;
- Odyssey protein marker
- Licor blocking buffer
- Metallothionein Antibody (FL-61): sc-11377
- IRDye® 800CW Goat anti-Rabbit IgG, LICOR

Samples (~ 5 mg piece of tissue) placed in round bottom microfuge tubes of Eppendorf tubes and added ~ 300 µl lysis buffer (M-PER cell lysis buffer, THERMO Scientific, USA) to extrac total proteins. Centrifuge for 20 minutes at 12.000 rpm at 4 °C. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice, discarding the pellet.

3.10.1. Performing electrophoresis using Bolt™ Mini Gels (Life Technologies)

- Prepare 1X Buffer: each chamber of the tank requires 400 ml of 1X SDS Running Buffer (mix 20 ml of 20X Bolt™ MES or MOPS SDS Running buffer with 380 ml of deionized water and Heat samples at 70 °C for 10 minutes;
- Loading up 20-40 µl of each sample in a predetermined order into the bottom of wells;
- Attach the electrophoresis apparatus to an electric power supply (the positive electrode should be connected to the bottom buffer reservoir);
- Running at 200 V for 45-60 minutes;

- Remove the glass plates from the electrophoresis apparatus and place them on a paper towel. Using a spatula, pry the plates apart;
- Mark the orientation of the gel by cutting a corner from the bottom of the gel
- Remove the gel from the electrophoresis apparatus;
- Proceed to the Western transfer protocol with iBlot® Gel Transfer Stacks, Regular Kit (Life Technologies);
- After transfer to a PVDF membrane, use a 1:1 mixture of Licor blocking buffer and 1X PBS for the blocking solution (Licor Odyssey). Incubate the PVDF membrane in the blocking solution for 1 h at room temperature on the rocker (This step can also be done overnight on the rocker in the cold room);
- Make the solution for primary antibody. The solution is made of blocking solution plus 0.01% of Tween 20;
- Incubate the membrane in primary antibody for 1h at room temperature, or overnight at 4 °C in seal-a container, preferably a sealed mealbag;
- Wash the membrane 4 x 5 min on the rocker with a washing solution made of 1X PBS + 0.01% Tween20;
- Incubate in secondary antibody (1:3000, IRDye® 800CW Goat anti-Rabbit IgG, LICOR) for 1 h at room temperature on the rocker;
- Wash 4 x 5 min in washing solution;
- Wash 2 x 5 min with 1X PBS only to get rid of the tween (avoiding background);
- Open the Odyssey scanner (Odyssey® Fc, Imaging System, Licor) and add the membrane facing down on the glass and on the bottom left side of the grid;
- Read the membrane.

The nitrocellulose membranes were incubated with mouse monoclonal primary antibody anti-MTs (1:500, Abcam, ab57287) over night at 4 °C. The complex protein-primary antibody was detected using a HRP-conjugated Ig-G anti-mouse secondary antibody (1:1000, Abcam) by chemiluminescent method. Blots were scanned and quantified by aspecific software (ImageJ).

3.11. Gene expression protocol performed by Real-Time PCR method

Real-Time Polymerase Chain Reaction (RT-PCR) was used to assess m-RNA espression of following genes:

- Metallothioneins (MTs);
- inducible Nitric Oxide Synthase (iNOS);
- Heme Oxygenase (HO-1).

Materials

- Oligonucleotide Primers-Gene specific (shown in Tab. 2);
- SYBR Green PCR master mix, 200 reactions (Life Technologies);
- Optical plates and cover for (Life Technologies);
- High-Capacity cDNA Reverse Transcription Kit (Life Technologies);
- 7900HT Fast Real-Time PCR System (Life Technologies).

All the primers are desalted and both UV absorbance and capillary electrophoresis are used to assess the quality of primer synthesis.

Gene	Primer Forward	Primer Reverse
MT	GCTGGAGCCACAGGAAT	CTGCGAATGTGCCAAGACT
HO-1	ACGCTTACACCCGCTACCTC	ATCCCCTTGTTTCCAGTCAG
iNOS	CCTCCTCATGTACCTGAATCTCG	GCTCCTGCTTTAGTATGTCGC
β-actin2	AAGCAGGAGTACGATGAGTC	TGGAGTCCTCAGATGCATTG

Tab. 2. Primer sequences used in gene expression.

3.11.1 Reverse Transcription

Reverse Transcription is carried out with the High-Capacity cDNA Reverse Transcription Kit (Life Technologies). The following procedure is based on Life Technologies' protocol.

Prepare the following RNA/primer mixture in each tube (see also Tab. 3):

- Pipette 10 µl of 2X RT master mix into each well of a 96-well reaction plate;
- Pipette 10 µl of RNA sample into each well, pipetting up and down two times to mix;
- Seal the plate o tubes;
- Briefly, centrifuge the plate or tubes to spin down the contents to eliminate any air bubbles;
- Place the plate or tubes on ice until you are ready to load the thermal cycler and setting these conditions are optimized for use with the High-Capacity cDNA Reverse Transcription Kit;
- Store the 1st strand cDNA at -20 °C until use for real-time PCR.

REAGENTS	WITH RNase INHIBITORS
10X RT Buffer	2.0 μl
25X dNTPs Mix (100 mM)	0.8 μl
10X RT Random Primers	2.0 μl
MultiScribe™ Reverse Transcriptase	1.0 μl
RNase inhibitor	1.0 μl
Nuclease-free H_2O	3.2 μl
Total per reaction	10.0 μl

Tab. 3. Reverse transcription reagent.

3.11.2 Real Time PCR preparation

You must amplify each of your samples in triplicates.

- Calculate how many reactions you have for each primer set (including the controls);
- Make a cocktail of SYBR Green Master Mix and Forward/Reverse Primers that is enough for all the reactions. Prepare the following master mix:
  0.5 μl Forward Primer (50 uM solution)
  0.5 μl Reverse Primer (50 uM solution)
  10.5 μl H_2O
  1 μl cDNA
  12.5 μl SYBR Green PCR Master Mix
- Add 24 μl of the cocktail to each well;
- Add 1 μl cDNA or water to each well, so that the total volume is 25 μl (make sure you do not cross contaminate your samples);
- Cover reaction plate with adhesive cover;
- Centrifuge reaction plate briefly to collect all liquid at the bottom of the plate;
- Load the plate in the instrument. Perform PCR using the Real-Time instrument for the manufacturers' protocol and calculate the fold change of the Mt-1 expression by the Comparative Ct Method using the $2^{-\Delta\Delta Ct}$.

4. RESULTS and DISCUSSIONS

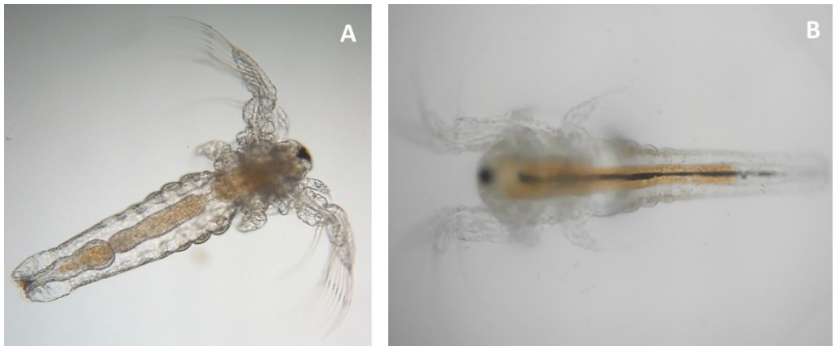
Nanotechnology is the manipulation of matter on a atomic scale to produce new materials and devises. The essence of nanotechnology is the synthesis of engineered nanoparticles (NPs), *i.e.* particles between 1 and 100 nanometers (2011/696/EU) in size, that exhibit characteristics, such as small size, large surface area to mass ratio, shape, crystallinity, surface charge, reactive surface groups, dissolution rate, state of agglomeration, or dispersal that confer them properties substantially different from those of the bulk particles of the same composition. For these reasons, ENPs have gained great scientific interest and benefits of nanotechnology are widely publicised; however the discussion of the potential effects of their widespread use in the consumer and industrial products has been raised concern of the scientists (Royal Society and the Royal Academy of Engineering, 2004).
The results obtained at the end of the experiments on the different engineered nanomaterials tested with related discussions are presented hereafter.

4.1. Discussion on short-term toxicity test with *Artemia salina*

Several areas of scientific knowledge dispense informations related to the genus *Artemia*. In the last three decades, *Artemia* nauplii have been employed to assay a large number of different substances. It continues to be used extensively and it represents one species of invertebrates suitable for standard ecotoxicological tests. It is also the most commonly live food in aquaculture.
Research on *Artemia* ecotoxicology began in 1975 at the State University of Ghent in Belgium, to evaluate the usefulness and reliability of different published toxicity testing methods with brine shrimp until then (Vanahaecke *et al*., 1980).
Generally, several criticisms against the use of *Artemia* have been presented in spite of its current practical use. Persoone and Wells (1987) summarized in a remarkable way the standpoint of criticism against the inclusion of *Artemia* as test organism in standardized bioassays. It is not present in the sea, thus it is not a natural or endemic marine organism. However it is an euryhaline crustacean and it can be cultured at wide range of salinities. Since it is not competitive with other zooplankton, it is mainly found in high salinity biotopes, not those of typical estuaries and coastal waters.
A second reason is that *Artemia* because of its specialized tolerance to high salinities, is presumed not to be very sensitive to contaminants. This is usually correct for the mortality criterion, especially compared to other crustacean as *Daphnia magna* (Guerra, 2001), *Daphnia pulex* and *Thamnocephalus platyurus* (Nunes *et al*., 2006).
The third reason (probably it is the most invalid for rejecting a valuable reference test organism) is that some experiments have not had success probably due to incorrect techniques for hatching the cysts and manipulating the nauplii.

However, many characteristics that turn this organism into a suitable species for use in Ecotoxicology may also be pointed out including a wide geographic distribution, small body size, relatively simple laboratory culture and maintenance, high adaptability to adverse environmental conditions, short life-cycle, large offspring production and the existence of a considerable amount of information about this species (Libralato, 2014).
Recently, *Artemia* has started to be used as a biological model in nanoecotoxicology. Currently, several papers highlight the main output in the investigation of engineered nanomaterials (ENMs) effects.
Ates *et al*., (2013) investigated the potential toxic effects of $nTiO_2$ (10, 50 and 100 mg/l) on *Artemia salin*a after acute exposure. Nauplii and adults accumulated the nanoparticles in their gut within 24 h of exposure. No significant mortality or toxicity occurred within 24 h at any dose. These results suggested that suspensions of the TiO_2 NPs were non toxic to *Artemia*, most likely due to the formation of benign TiO_2 aggregates in water.
The effect of gold nanoparticles were studied by Karthik *et al*. (2013). Altough no information is available about testing organisms and exposure conditions, authors carried on the toxicity tests (lethality) showing no mortality up to 8 mg/l of gold nanoparticles.
Vijayan *et al*. (2014) assessed the effect of silver and gold nanoparticles against *A. salina* mortality (24 h). The toxicity test included several exposure concentrations (40, 60, 80, 100, 120, 140 and 160 mg/l) e for both nanoparticles. Exposures occurred in 24-well plates containing 1 ml of sterilised seawater with ten nauplii per well were exposed for 24 h at 25 °C. Silver nanoparticles mortality was found to be proportional to increasing exposure concentrations, while no ecotoxicological data were reported about gold nanoparticles.
In this study, potential toxic effects about different ENMs on microcrustacean *Artemia salina* were evaluated. Sonication has been used to achieve better dispersion and to prevent aggregation (Zhu *et al*. 2010). Similar strategy was used in this work and NPs were exposed to ultrasounds in a sonicator bath for 20 min to improve dispersion in water.
Aggregation in aqueous suspensions has been reported previously in other studies (Adams *et al*. 2006; Zhu *et al*. 2008, 2010). Although aeration provided effective means of mixing to maintain homogeneity of the suspensions, aggregation occurred at all concentrations tested. *Artemia* is a non-selective filter-feeder organism that can readily ingest fine particles smaller than 50 µm (Zhu *et al*. 2010). Accumulation of NPs was performed qualitatively on each group at the conclusion of the exposure under a stereomicroscope (Leica M0205C, Multifocus) equipped with a digital camera. Compared with the controls, the guts of the exposed *Artemia* were filled with particles, especially in *Artemia* exposed to gold NPs (Figs. 26 B). The ingested particles appeared as a long strip of particles suggesting that even larger aggregates formed inside the guts. This effect was thought to be due to the reduced surface area as the NPs aggregated or agglomerated to microscale particles in solution and inside the guts.

However, these effects could be due to the lack of food uptake since the guts were completely filled with the aggregates of NPs.



Figs. 26. Nauplii of *Artemia salina*: A) control; B) larva exposed to the highest concentration of AuNPs visible inside the gut as a long strip of dark particles.

In this study, *Artemia salina* dehydrated cysts purchased from local aquarium store (Artemio Pur® JBL GmBh&Co., Germany) were used. Another type of cysts (ArtemiaCyst Blue Line, Italy) were used as a replicate. Larvae that were completely motionless were counted as dead, and the percentages of mortality compared to the control were calculated.

The results obtained with *Artemia* nauplii "JBL" are not significant at $p < 0.05$ neither for TiO_2NPs nor AgNPs (respectively p value $p= 0.3155$ and $p= 0.0502$) altough the latter is at the limit of significance. The results are not significant for AuNPs also ($p= 0.5000$).

The percentage of immobilization for TiO_2NPs were 4.0%, 2.5% and 1.5% respectively for the highest, medium and minimum concentration tested. The percentage of immobilization for AgNPs were 34.5%, 20.5% and 10.5% respectively for the highest, medium and minum concentration tested. The percentage of immobilization for AuNPs were 6.0%, 6.0% and 4.0% respectively for the highest, medium and minum concentration tested.

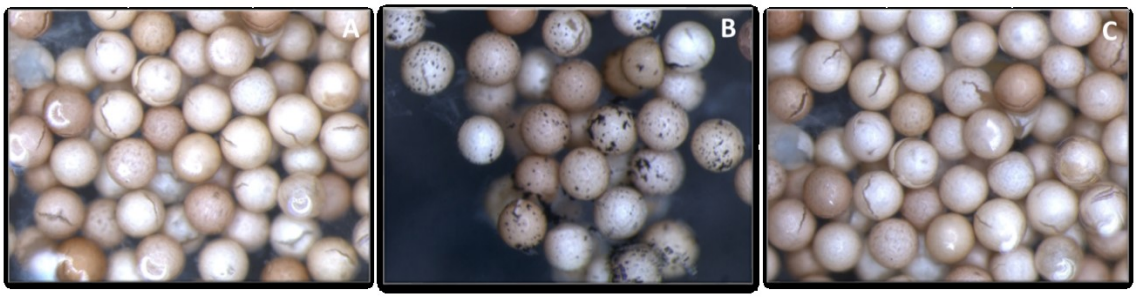
The results obtained with *Artemia* nauplii "Blue Line" are not significant at $p < 0.05$ neither for TiO_2NPs nor AgNPs (respectively $p= 0.9803$ and $p= 0.5818$). The results are not significant for AuNPs also ($p= 0.9548$).

The percentage of immobilization for TiO_2NPs were 4.0%, 3.5% and 4.0% respectively for the highest, medium and minimum concentration tested. The percentage of immobilization for AgNPs were 21.0%, 21.5% and 15.5% respectively for the highest, medium and minum concentration tested. The percentage of immobilization for AuNPs were 4.0%, 4.5% and 4.5% respectively for the highest, medium and minum concentration tested.

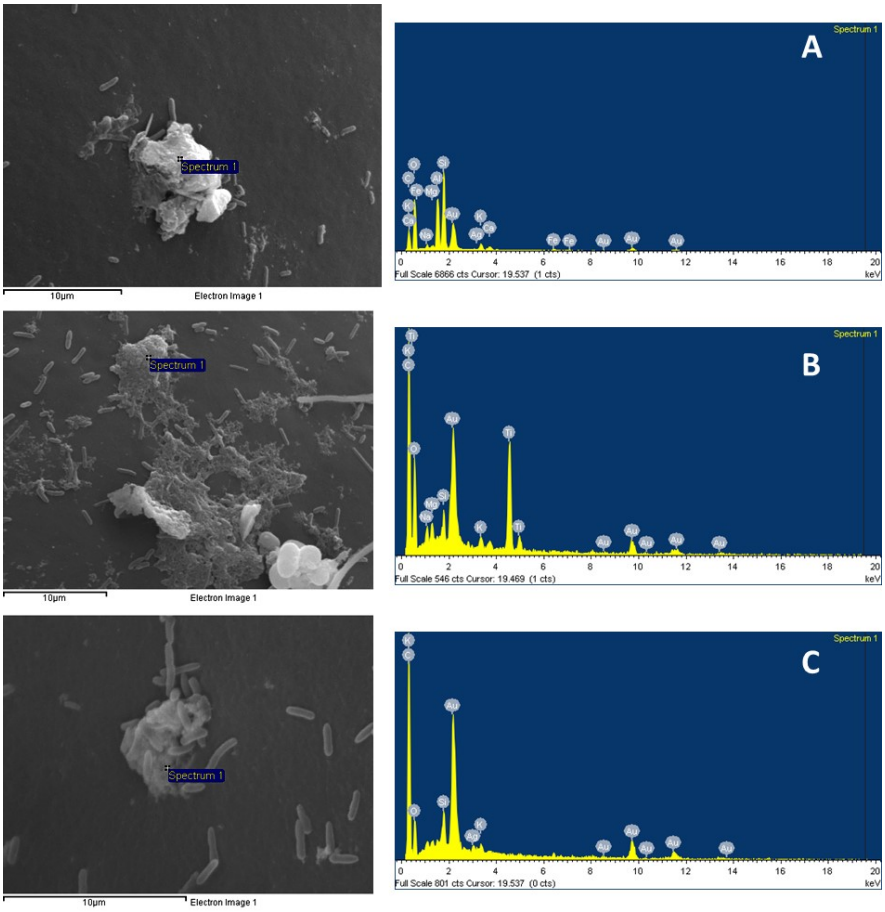
Although the percentages of immobilisation are relatively modest, the nanoparticles are absorbed and internalized by the crustacean (as shown in Figs. 26) and it is

comparable to results found in other papers present in the literature (Ates *et al*., 2013; Zhu *et al*. 2010).

The experiments conducted with cysts showed that all three types of nanoparticles used (Au, Ag and TiO_2 nanoparticles) adhered to the ovular surface of cysts as well shown in Figs. 27 A, B, C and 28 A, B, C.



Figs. 27. Several cysts of *Artemia salina* exposed to A) TiO_2NPs; B) AuNPs; C) AgNPs.



Figs. 28. SEM-EDX analysis shows different NPs on cysts' ovular surface: A) AgNPs; B) TiO_2NPs; C) AuNPs.

The results obtained with *Artemia* cysts "JBL" showed that the percentage of not hatched cysts for TiO_2NPs were 4.0%, 3.5%, 2.0% at respectively for the highest, medium and minimum concentration tested. The percentage of not hatched cysts for AuNPs were 44.0%, 39.0%, 27.0%, and the percentage of not hatched cysts treated with AgNPs were 44.5%, 40.5%, 40.0% at respectively for the highest, medium and minimum concentration tested.
As regard *Artemia* cysts "Blue Line" the results obtained showed that the percentage of not hatched cysts for TiO_2NPs were 3.0%, 1.5%, 1.5% at respectively for the highest, medium and minimum concentration tested. The percentage of not hatched cysts for AuNPs were 37.0%, 28.0%, 24.0%, and the percentage of not hatched cysts treated with AgNPs were 41.0%, 37.0%, 23.0% at respectively for the highest, medium and minimum concentration tested.

According to the literature data, I can affirm the non-toxicity of titanium dioxide nanoparticles on *Artemia salina* larvae as shown by low percentage of immobilization and on cysts because they didn't affect hatching.
Gold nanoparticles exert toxic effects on hatching of the cysts, but doesn't affect larvae development as well as TiO_2NPs.
Otherwise, silver nanoparticles are toxic either on larvae and on cysts as shown by results obtained.

Finally, it is important highlight that most of the papers present in the literature referred to a different protocol. This suggests the existence of several approaches that could not present common actions. It is evident that the absence of a standardised method to refer to this microcrustacean in nanoecotoxicology is a main gap that should be filled as soon as possible to make *Artemia salina* an official standard biological model. On the contrary, standardized protocols (*i.e*. ZFET) give us the possibility to compare results obtained in several researches.
A full revision of these protocols will be essential to elucidate the main gaps of each procedure in order to formulate a proposal for a standard protocol. The available data cannot help to support or detract such a biological model. Anyway, they contribute to effectively understand which informations must be provided in a scientific paper in order to make them employable such as in toxicity or hazard assessment of ENMs.

4.2. Discussion on FET test with Nanomaterials ("TiO_2/Au" - "Au/TiO_2") versus free Au and TiO_2 nanoparticles

In order to verify the toxicity of the nano-systems, we tested the effect of the material directly on zebrafish larvae. It is well known that *Danio rerio* is considered an excellent model for biomonitoring of aquatic environment, and the Zebrafish Embryo Toxicity Test (ZFET) represents one of the most important method of acute toxicity testing. The

FET test is included in the FDA and ICH guidelines to perform toxicity test for pharmaceutical products, in EPA and OECD for chemical substances (2013). For this reason, zebrafish larvae were exposed to different concentrations of nanoparticles of TiO_2 and Au and new nanomaterials to understand how engineered nanomaterials can interfere on biological activities of living systems. As biomarkers of exposure, we evaluated the expression of metallothioneins by immunohistochemistry analysis and western blotting analysis also.

Several nanoparticles are traditionally considered as chemically inert and biocompatible, in particular, titanium dioxide and gold nanoparticles were identified as valuable tools in several research areas (Iavicoli *et al*., 2012; Dykman and Khlebtsov, 2012; Jain *et al*., 2012).

The proposed method involves the development an innovative TiO_2/Au nano-composite synthesized by sputtering and atomic layer deposition (ALD) techniques. The peculiarity of this system is the covering of gold nanoparticles ($\sim$ 8 nm mean diameter) with a thin layer of TiO_2 ($\sim$ 4 nm thick) (Scuderi *et al*., 2014). The ALD technique is one of the few techniques able to realize films with a controlled thickness of few nanometers. This effectively contributes to the photoreactions, avoiding any wastage of the material. This optimized design takes advantage on the presence of metal nanoparticles (in terms of electron trapping), without losing any TiO_2 exposed surface. In addition, the form of thin film eliminates the problem of the particle filtration step after the water treatment.

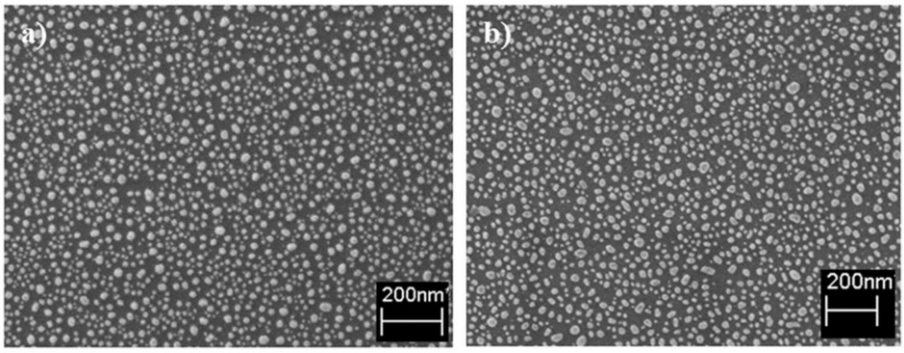
A large number of studies carried out on AuNPs have produced conflicting results. In fact, despite the constant attempts to establish a correlation between the structure of the particles and their interactions with biological systems, the toxicological profile of gold nanoparticles has not yet been fully clarified. Among these investigations, a large numbers of authors has confirmed the non-toxicity of AuNPs (Patra *et al*., 2007; Bar-Ilan *et al*., 2009; Asharani *et al*., 2010) conversely, others have observed the toxicity of AuNPs (Tedesco *et al*., 2010; Pan *et al*., 2007; Cho *et al*., 2009).

Despite, some authors showed low or neither acute toxicity of TiO_2NPs to fish survival (Chen *et al*., 2011; Zhu *et al*., 2008), several studies demonstrated that exposure to high concentrations of TiO_2 particles was able to induce lung tumors formation after 2 years in rats (Iavicoli *et al*., 2012). In 2006, the International Agency for Research on Cancer (IARC), classified and in 2010 reassessed TiO_2 as “possibly carcinogenic to humans” on the basis of the sufficient evidence of carcinogenicity in experimental animals and inadequate evidence in humans (Group 2B).

The innovative nano-material proposed here could help overcome the problems of the toxic effects of nanoparticles without affecting their performance.

SEM images of the Au nanoparticles on the TiO_2 film show that before the immersion (Fig. 29 A) the samples consisted of a homogeneous distribution of gold nanoparticles with a TiO_2 coverage about $\sim$ 36%. After 12 days in aquatic environment, the analyses indicated a reduction of the TiO_2 coverage about $\sim$ 36-28% (Fig. 29 B). Thus, the liquid environment induced detachment of the Au nanoparticles from the TiO_2 underneath film. The same investigation was done with the samples with the TiO_2 film on top of

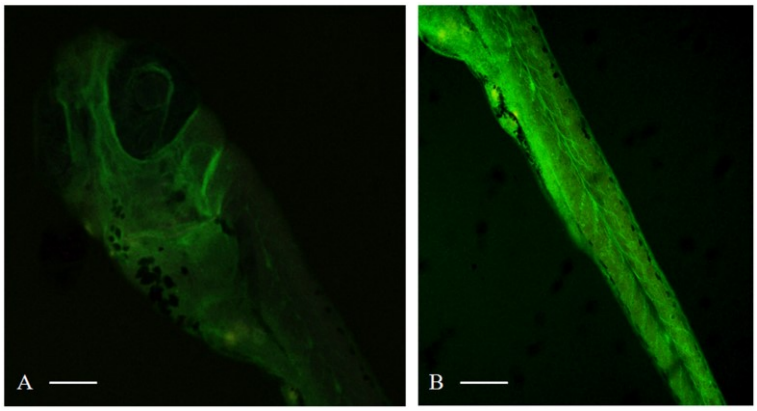
the gold nanoparticles. In this case, the SEM analyses showed the same gold distribution, before and after the 12 days dipping, indicating a strong stability of the embedded AuNPs.



Figs. 29. Nanocomposed of Au nanoparticles on TiO_2 flat film A) before and B) after a 12 days dipping in aquatic environment (from Brundo *et al*., 2016).

The NMs ("TiO_2/Au" and "Au/TiO_2") were immersed in water until 12 days in agitator. The Zebrafish larvae were added to aquatic sample the first day (Time 0) and after 12 (Time 12) days for 96 hpf.

Immunohistochemical analysis performed in larvae exposed to "TiO_2/Au," "Au/TiO_2" in aquatic samples at Time 0 and Time 12, and free TiO_2, showed the presence of MTs only in head region (Fig. 30 A), as well as to control samples (untreated). Moreover, the zebrafish exposed to free AuNPs showed a positive response to anti-MT in whole body (Fig. 30 B).



Figs. 30. Larvae zebrafish 96 hpf treated with anti-MT antibody: A) Larvae exposed to free TiO_2 showed the presence of MTs only in head region; B) Zebrafish exposed to AuNPs showed a positive response to anti-MT in whole body. Scale bar: 150 µm (from Brundo *et al*., 2016).

In addition, we evaluated the mRNA and protein expression of MTs. The data are expressed as mean ± SEM. Statistical analysis was carried out by one way ANOVA test. A *p*-value of less than 0.05 ($p < 0.05$) was accepted as statistically significant between experimental and control groups. It was observed that the free TiO_2, "TiO_2/Au" and "Au/TiO_2" nanosystems did not able to induce the mt mRNA expression, while free gold NPs increased mt levels of about 20-fold ($p < 0.0001$) respect to untreated sample. This result was confirmed by western blotting analysis (Fig. 31; Figs. 32 A, B).

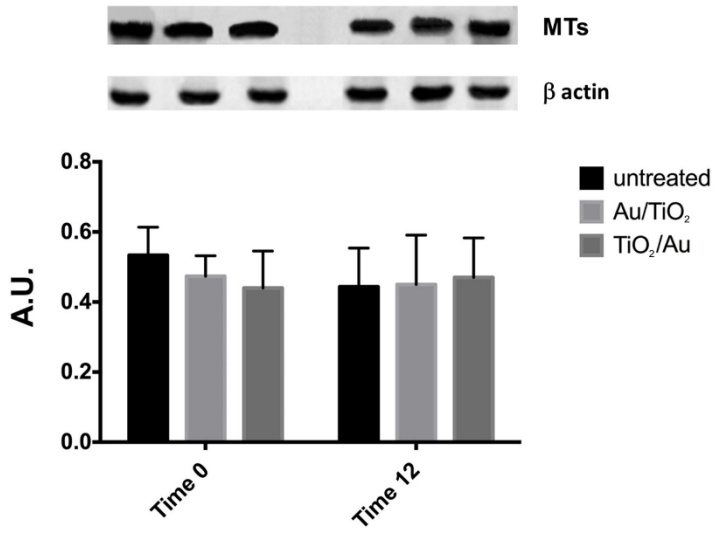
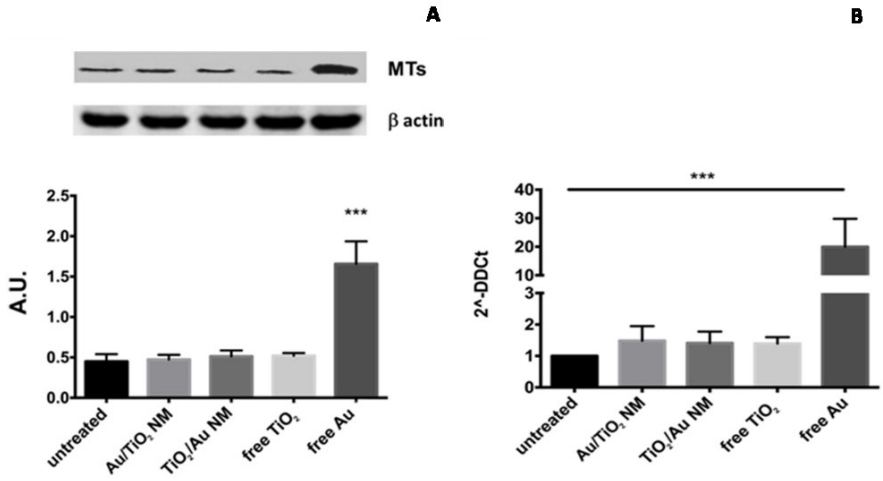


Fig. 31. Western blotting analysis. The MTs level, in untreated samples and in larvae exposed to nanomaterials before (Time 0) and after a 12 days (Time 12) dipping in aquatic environment was evaluated (from Brundo *et al.*, 2016).



Figs. 32. A) Western blotting analysis; B) mRNA gene expression of mt in zebrafish after exposure to free nanoparticles and nanomaterials. The mt mRNA expression was increased only in free Au treatment. Bars represent the mean ± SEM of three independent experiments. ***$P < 0.0001$ vs untreated sample. (Calculated value of $2^{-\Delta\Delta Ct}$ in untreated sample was 1) (from Brundo *et al.*, 2016).

The use of MTs as biomarkers of exposure is supported by several studies. Some authors showed that MT proteins could be a potential biomarkers for metal contamination in aquatic environment (De Domenico *et al*., 2011; Copat *et al*., 2012). Moreover, some studies demonstrated that during oogenesis the MTs level in zebrafish slowly decreases during the early stages of development (Riggio *et al*., 2003; Chen *et al*., 2004). In particular, they demonstrated a gradual decrease in the level of MT transcripts during the early embryonic stages (Chen *et al*., 2004). Chen *et al*. (2002) also observed a strong ubiquitous expression of metal-responsive transcription factor (MTF-1), the responsible for Mt gene expression (Günes *et al*., 1998).

Furthermore, the expression patterns of MT (Chen *et al*., 2004) and MTF-1 (Chen *et al*., 2002) at prim-5 stage are in opposite manner: the former expressed well in the tail region while the latter in head region. For this reason, the authors suggest that MT may be involved in urinary and digestive system whereas, MTF-1 not and the expression of MT in the tail region may be due to other regulatory factors. The MT expression pattern is more comparable with MTF-1, during early hatching period (Chen *et al*., 2004).

In particular, after hatching, no significant MT signal could be observed in the tail region. On 1 dph, clear as well as distinct MT expression signals are seen in common cardinal vein and chloride cells. The signals observed on 1 dph at lateral and dorsal views become stronger on 2 dph. The intensity of signals at common cardinal vein, pronephric region, and retina almost vanished on 3 dph. The intensity of staining due to MT expression at cerebellum and pronephric region on 4 dph becomes increased and maintains until 12 dph, show that MT expression plays a role on zebrafish embryogenesis and early larval development (Chen *et al*., 2004).

In this experimental study, the MTs expression shows a high susceptibility to gold free metallic nanoparticles, highlighting the importance of their trapping inside the new engineered nanomaterials. This could be useful to make maximum use of potential of NPs removing the possible risk of damage the environment. However, the synthesized material with Au nanoparticles on top, showed a dispersion of Au nanoparticles in the liquid environment, due to their instability in the aqueous solution that clearly represents an environmental contamination issue. For this reason, in the near future, the possibility of wrapping Au nanoparticles in a TiO_2 film could have great potential as eco-friendly materials.

4.3. FET test with graphene oxide (GO) and Nafion polymer combined with various fillers

In the last years, carbon-based nanomaterials have attracted considerable attention in different areas of nanotechnology research and several Authors have conducted studies on graphene. Graphene oxide (GO) is the most important derivative of

graphene with the presence of oxygen functional groups such as carboxylates, epoxides, and hydroxyls at the basal planes and edges of graphene sheets (Dreyer *et al*., 2010).

GO and GO-based nanocomposites were recently proposed for the removal of pollutants (Yeh *et al*., 2013) and for adsorption of organics dyes from water (Sharma *et al*., 2013) and they are increasingly used for wastewater treatment in hybrid nanocomposite membranes (Filice *et al*., 2015). GO, infact, is a hydrophilic compound and highly dispersible in water.

For this reason, several studies have been proposed to verify graphene toxicity. The developing zebrafish larvae is increasingly used as alternative animal model for evaluating different toxicities, but few studies exhist about graphene toxicity in zebrafish. Liu *et al*. (2014) investigated the toxic effects of 3 nanomaterials (multi-walled carbon nanotubes, graphene oxide and reduced graphene oxide) on zebrafish embryos. They observed a decreased length of the hatched larvae at 96 hpf, but no obvious morphological malformation and mortality were observed in exposed zebrafish embryos. Also Alday *et al.* (2016) have seen that graphene oxide does not affect external morphology but could be neurotoxic for larvae zebrafish because locomotor activity decreased after GO treatment.

Buccheri *et al*. (2016) investigated structural, morphological, chemical properties of GO and GO irradiated (iGO) by laser treatment. Pulsed laser irradiation of GO is an environmentally friendly process that avoids the use of chemicals. The Authors have demostrated a marked antibacterial activity (at concentration of 20 mg/l) was correlated to the morphology and the size reduction of the iGO flakes that became similar to the bacteria size. They performed a FET test showed that neither mortality nor sublethal effects were caused by the different kinds of GO tested at concentration eight times larger than 20 mg/l.

In this study, nanocomposite membranes prepared using Nafion polymer combined with various fillers, such as anatase-type TiO_2 nanoparticles and graphene oxide were tested. The toxicity of these nanocomposites was established by Zebrafish Embryo Toxicity Test. As biomarkers of exposure, it was evaluated the expression of Heme-Oxygenase 1 (HO-1) and Nitric Oxide Synthases (iNOS) by immunohistochemistry and gene expression.

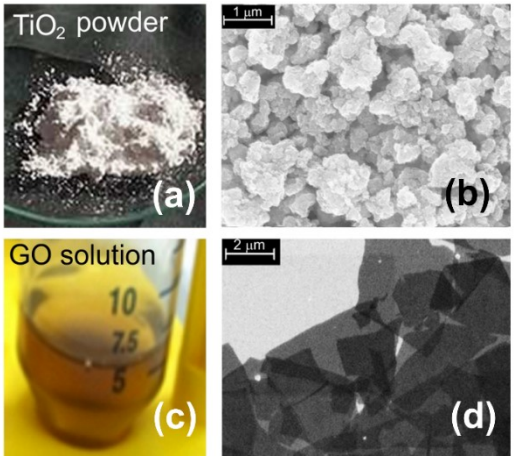
In Figs. 33 A and 33 C are reported, respectively, the photos of the materials used for the present study both as fillers or as free particles: TiO_2 nanoparticles and GO in water solution. SEM images of some aggregates of TiO_2 nanoparticles and GO flakes deposited on silicon substrates are shown in Figs. 33 B and 33 D.

Figs. 34 A and 34 C show the nanocomposite membranes (Nafion-TiO_2 and Nafion-GO, respectively) prepared by drop casting and Figs. 34 B and 34 D report the same membranes observed by SEM in cross section. The fillers are homogeneously dispersed in the polymer, as also verified by energy dispersive X-ray analysis in a previous work (Filice *et al*., 2015).

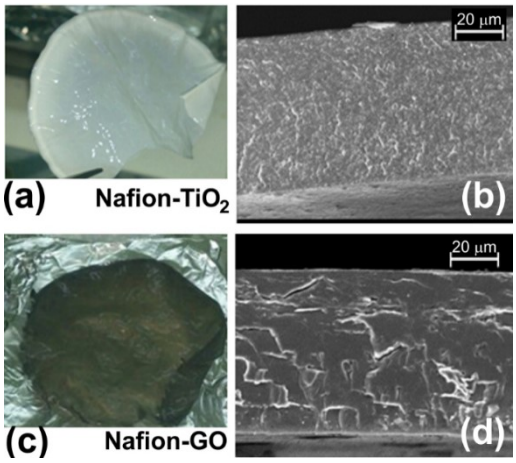
The FET test showed that neither mortality nor sublethal effects were caused by the different nanomaterials and free nanoparticles tested (Figs. 35). In particular, the

following different endpoints were evaluated: viability, growth (larval length), brain morphology, pharyngeal arches and jaw, other craniofacial structures, heart, fins, notochord, somites, tail, body shape, cardiovascular function, yolk sac and locomotor function and touch response. Significant differences were detected, however, in the expression of biomarkers in larvae exposed to the nanocomposites versus to the free nanoparticles. Infact, immunohistochemical analysis performed in larvae exposed to nanocomposite membranes, did not show the presence of biomarkers, as well as control samples (untreated). On the contrary, the zebrafish exposed to GO flakes showed a positive response to anti HO-1 and iNOS in the whole body (Figs. 36 A, B). These results were confirmed by gene expression analysis (Figs. 37 and 38).

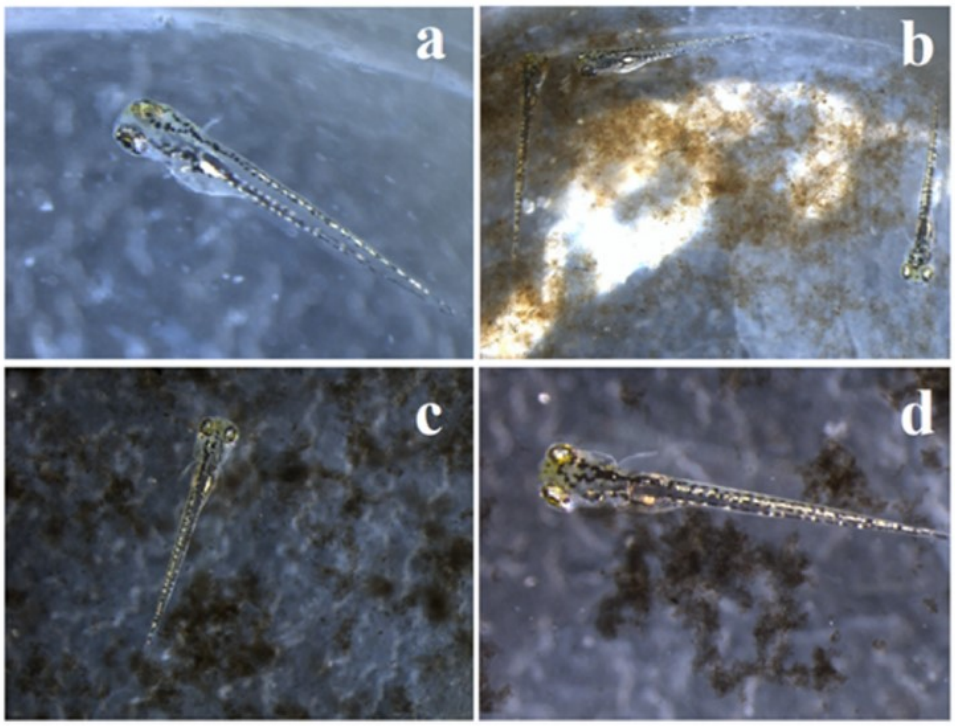
The efficient photocatalytic rate, the antibacterial properties and the absence of toxicity make GO a promising green option for possible applications dedicated to water purification.



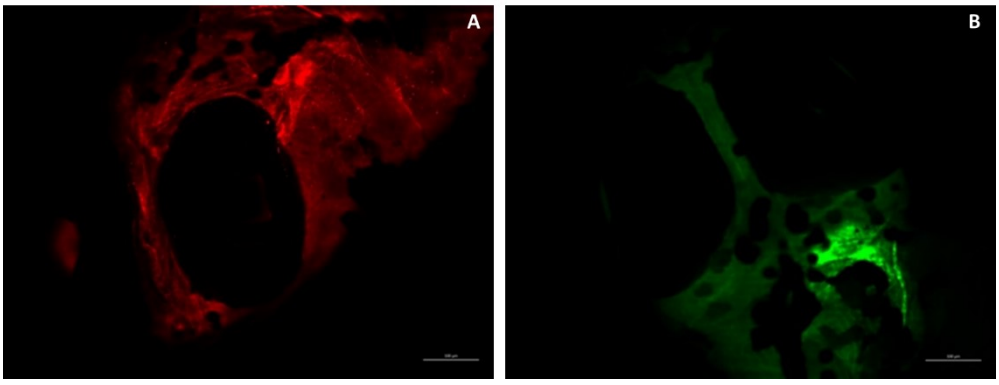
Figs. 33. A) Picture of TiO_2 powder; B) SEM image of aggregates of TiO_2 nanoparticles; C) Picture of GO dispersed in water solution; D) SEM image of GO flakes (from Pecoraro *et al*., 2017).



Figs. 34. A) Picture of a Nafion-TiO_2 membrane; B) SEM image of a cross section of the membrane; C) Picture of a Nafion-GO membrane; D) The same membrane observed by SEM in cross section (from Pecoraro *et al*., 2017).



Figs. 35. Zebrafish larval 72 hpf, x32. A) Untreated; B) GO, 160 mg/l; C) iGO2, 160 mg/l; D) iGO4, 160 mg/l (from Buccheri *et al*., 2016).



Figs. 36. Larvae zebrafish 96 hpf free GO treated: A) Anti-HO-1 and B) Anti-iNOS. Scale bar:150 µm (from Pecoraro *et al*., 2017).

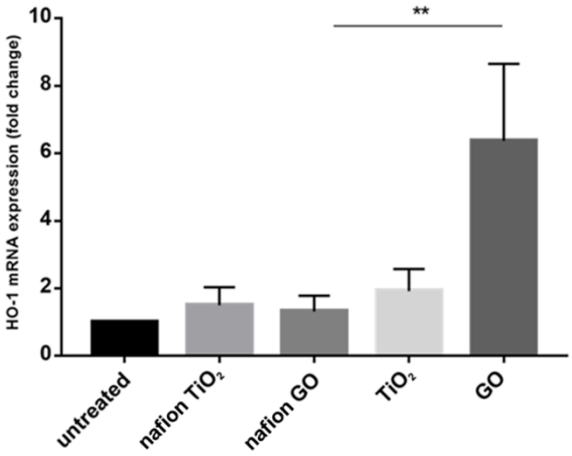


Fig. 37. mRNA gene expression of HO-1 in zebrafish after exposure to free nanoparticles and nanocomposites. The HO-1 mRNA expression was increased only in free GO treatment. Bars represent the mean ± SEM of three independent experiments. ***P* < 0.05 vs. untreated sample. (Calculated value of $2^{-\Delta\Delta Ct}$ in untreated sample was 1) (from Pecoraro *et al.*, 2017).

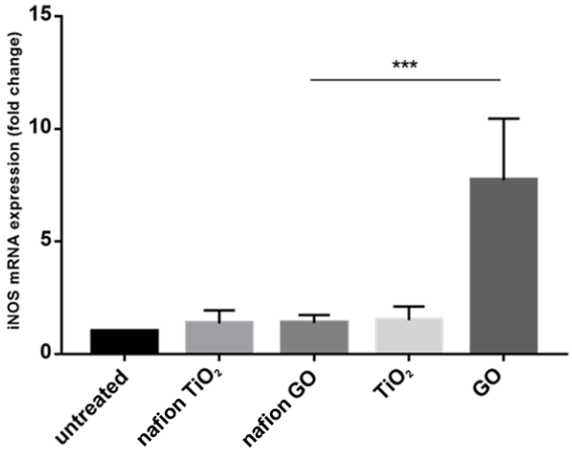


Fig. 38. mRNA gene expression of iNOS in zebrafish after exposure to free nanoparticles and nanocomposites. The iNOS mRNA expression was increased only in free GO treatment. Bars represent the mean ± SEM of three independent experiments. ****P* < 0.05 vs. untreated sample. (Calculated value of $2^{-\Delta\Delta Ct}$ in untreated sample was 1) (from Pecoraro *et al.*, 2017).

4.4. Discussion on long term exposure with AgNPs and adults of zebrafish

Studies demonstrate that NPs can be released in the environment and cause harmful effects on all living organisms (Owen, 2005). Several *in vivo* and *in vitro* studies have demonstrated that the engineered nanomaterials can experimentally cause a wide variety of toxic effects. The toxicity depends on their chemical-physical properties and,

in particular, by their chemical composition, the diameter and shape, and the possible state of aggregation/agglomeration (Borm *et al*., 2006; Nel *et al*., 2006).

It is known that most of the industrial and urban waste end up in the rivers and coastal waters, due to the extremely small size of the nanoparticles. Release of nanoparticles into the sea raise new doubts that need to be studied in the next future. The potential routes of entry of nanoparticles into aquatic organisms include the direct ingestion, diffusion through the gills and skin (Handy *et al*., 2008).

Recently, *in vivo* studies performed on *Mytilus galloprovincialis*, bioindicator species used for testing exposure to xenobiotic substances, showed that titanium dioxide nanoparticles (TiO_2NPs) accumulate in various target organs, especially in gills and digestive glands. The genotoxicity test carried out through Comet Assay revealed a significant dose and time-dependent lesion to the DNA. In addition, TEM analysis showed cellular damages supporting the initial inflammatory response by the cells of the target organs leading to apoptosis (Gornati *et al*., 2016).

The adsorption by the intestinal structures was also demonstrated in rainbow trout (*Oncorhynchus mykiss*) after exposure to TiO_2NPs both through food (Ramsden *et al*., 2009) and through the titanium dioxide NPs water dispersion (Federici *et al*., 2007).

Brundo *et al*. (2016) demostrated the non-toxicity of new synthesized engineered nanomaterials compared with free Au and TiO_2 nanoparticles by testing the effect of the material on zebrafish larvae using Zebrafish Embryo Toxicity Test (ZFET). The results obtained showed that neither the mortality nor sublethal effects were induced by the different nanomaterials and nanoparticles tested. Only zebrafish larvae exposed to free Au nanoparticles showed a different response to anti-metallothioneins (MTs), which were correlated to the dispersion of metal ions in aqueous solution.

In this work, the long-term effects of AgNPs in adult specimens of zebrafish were evaluated.

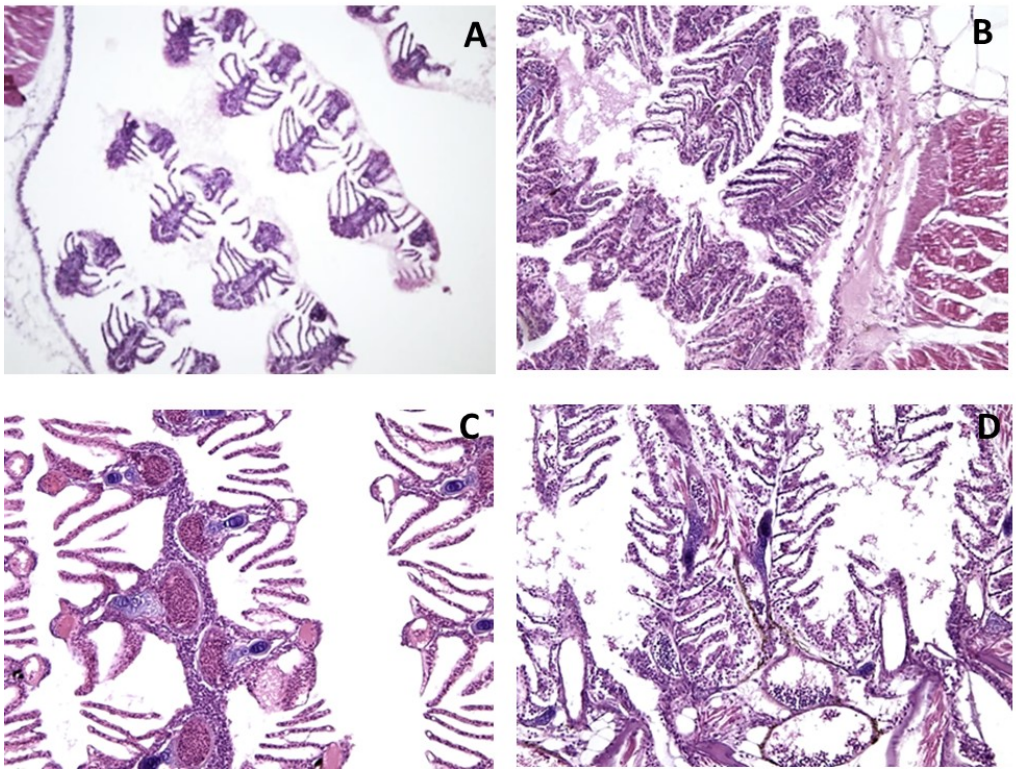
The histological examination on AgNPs treated subjects showed lesions in the digestive tract and gills.

Histological observation of tissues at the end of the experiment showed normal anatomy in the freshwater controls. Gills structure, both primary and secondary lamellae, appeared intact and well recognizable as well as chloride cells, pillar cells and mucosal ones too (Fig. 39 A). A whole intestinal lumen and well lying microvilli appeared in the gut histological sections (Figs. 40). On the contrary, silver nanoparticles caused damage to the gills structure such as sub-epithelial edema, hyperplasia (Figs. 39 B and C), lamellar fusion (Fig. 39 B), as well as teleangectasia (Fig. 39 D). The necrosis of intestinal villi with reduction of their length was also demonstrated in the gut (Figs. 40 B, C, D).

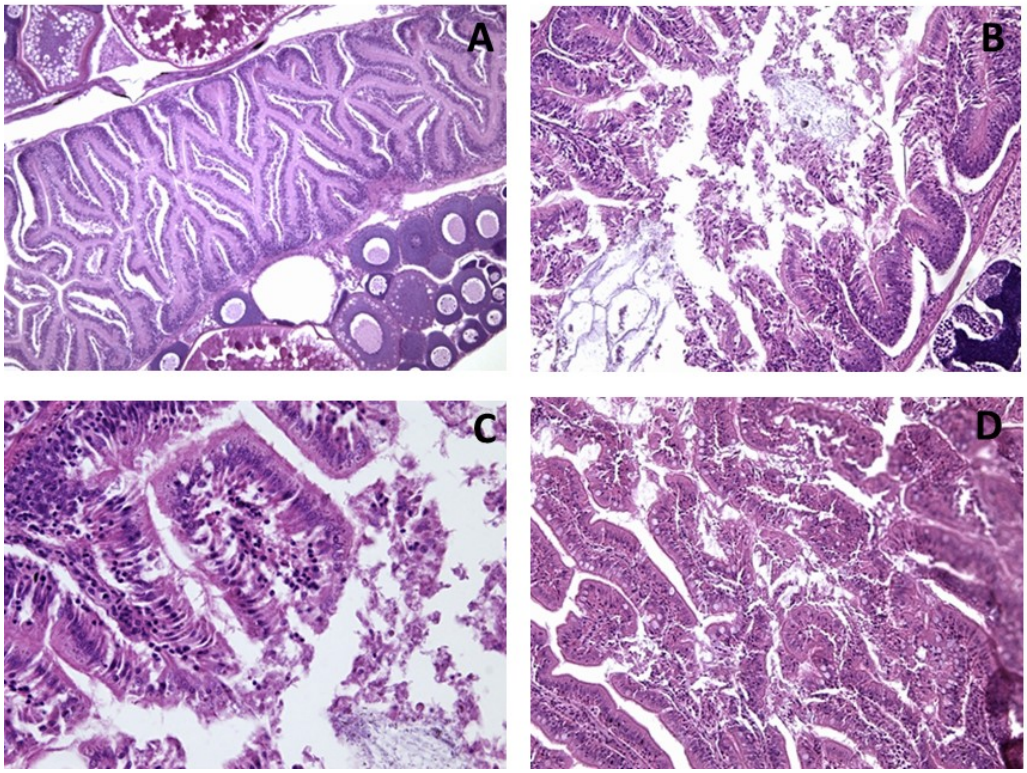
No injuries have observed in liver tissue as shown in Figs. 41. In this image, a clear distinction can be made between the male and female liver in the adult zebrafish. The female hepatocytes are very basophilic as a result of the production of vitellogenin (Menke *et al*., 2011).

However, dose dependent effects of nanosilver suspensions with significantly greater damage were observed at lower concentrations.

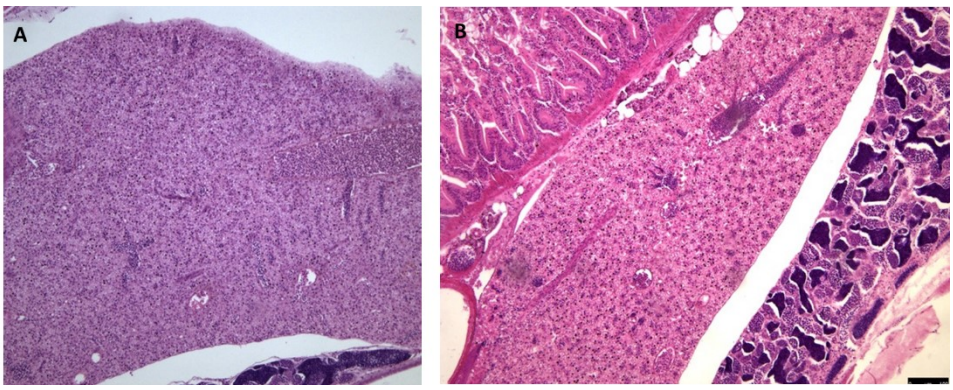
The immunohistochemical examination highlighted the presence of metallothioneins.
Using specific antibodies anti-MT, it has been showed a clear expression of these proteins in the intestinal tissues AgNPs treated (Figs. 42 B and C), compared with the control group (Fig. 42 A). The same results were obtained for the liver and the gills (Fig. 43). These results were confirmed by western blot analysis (Figs. 44).
It was also observed a higher response at lower concentration of mt mRNA expression, in all three tissue treated (Figs. 45).
Results obtained through the different applied methods showed that silver nanoparticles have caused tissue damage because they were absorbed in the body.
Assuming a purely preliminary conceptual analysis, we would expect a corresponding increase of the lesions correlated to the growing nanoparticles concentration both in gill and gut tissues.
Instead, the group of zebrafish exposed to the highest concentration had a lower degree of toxicity compared to the group treated with NPs lower concentrations. These results suggested that as high is the concentration as high is the probability that the particles aggregate, and little by little their size increase, the absorption capacity of the exposed tissues may reduce. Instead, in the solution with the lower concentration of NPs, silver nanoparticles are mono-dispersed and more easily adsorbed by gills and intestine.
The ICP-MS analysis infact shows that the amount of particles absorbed in all treated samples is almost the same. Total silver concentrations found vary from a minimum of 6 mg/l in NPa group samples to 8.2 mg/l in those belong to NPc group. The rate of absorption was respectively of 76.1% (NPa), 14.5 % (NPb) and 11.7% (NPc), demonstrating a greater capacity of assimilation of the particles in mono-dispersed phase (Tab. 4).
For this reason, it is possible affirm that the toxicity of silver nanoparticles is related to their size and degree of dispersion rather than their concentration.
Therefore, it is necessary that the NPs solutions undergo sonication during the whole period of the trial in order to prevent the possibility of aggregation and make them more bioavailable for animals used for ecotoxicological tests.
These results suggest that AgNPs can generate different degrees of toxicity. The bioavailability of silver nanoparticles, in fact, is an important parameter of toxicity to aquatic organisms and depends on many factors such as sedimentation, aggregation and particle size.



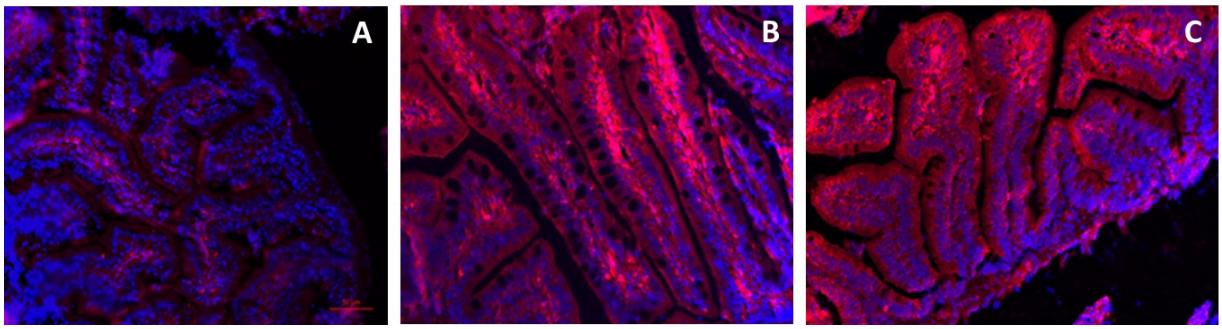
Figs. 39. Gills morphology after 30 days of exposure: A) CTRL; B) NPa; C) NPb; D) NPc.



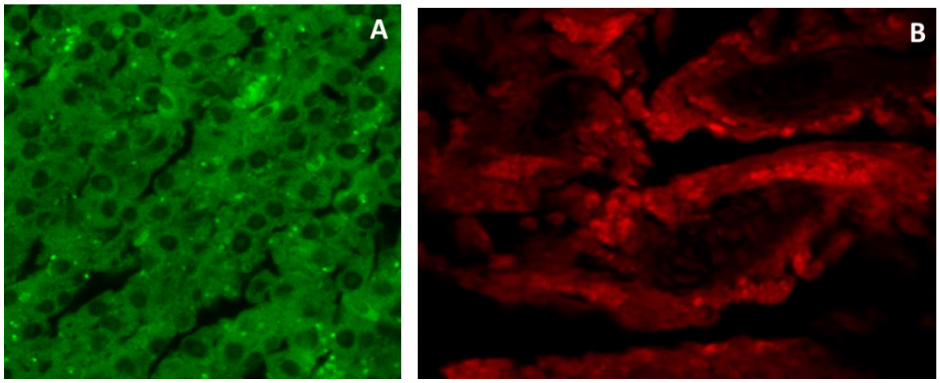
Figs. 40. Gut morphology after 30 days of exposure: A) CTRL; B) NPa; C) NPb; D) NPc.



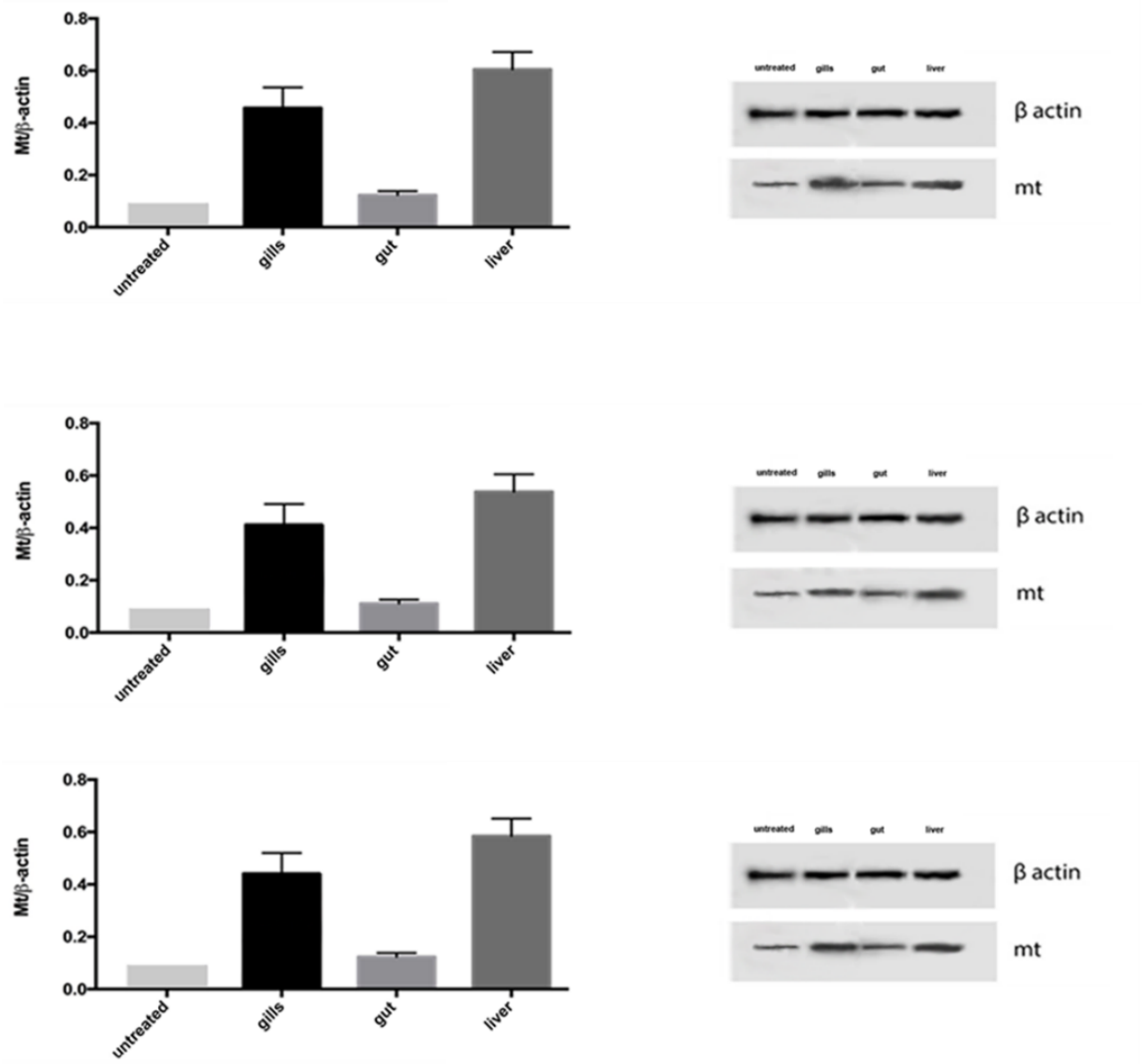
Figs. 41. Liver morphology after 30 days of exposure to 70 µg/l AgNPs: A) female; B) male.



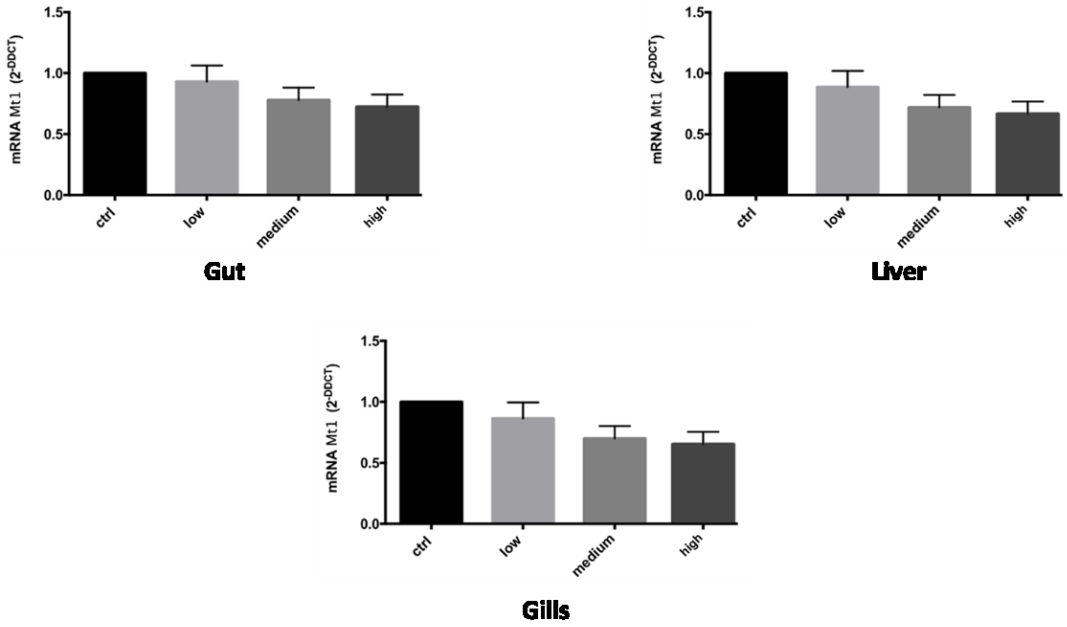
Figs. 42. MTs expression in the gut tissue: A) CTRL; B) NPa; C) NPc.



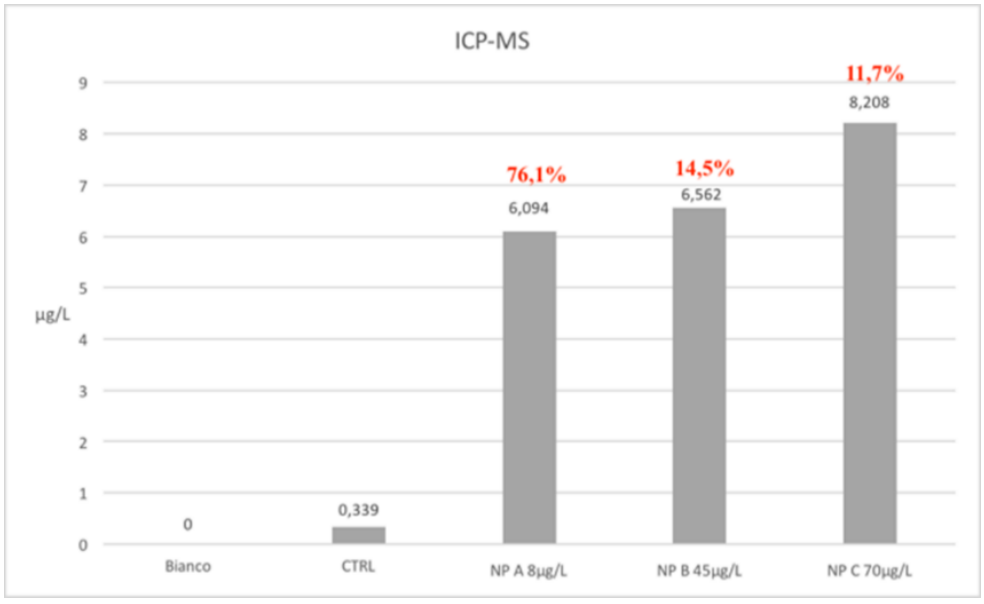
Figs. 43. MTs expression in the liver tissue A) and gills B) NPc.



Figs. 44. Western blotting analysis. The MT1 level in zebrafish after exposure to Ag free nanoparticles. The data are expressed as mean ± SEM. Statistical analysis was carried out by ANOVA test. A *p*-value of less than 0.05 ($p < 0.05$) was accepted as statistically significant between experimental and control groups (respectively, from top to bottom: NPa group= 8 μg/l, NPb group= 45 μg/l, NPc group= 70 μg/l).



Figs. 45. mRNA gene expression of MT1 in zebrafish after exposure to Ag free nanoparticles. Bars represent the mean ± SEM of three independent experiments. Statistical analysis was carried out by ANOVA test. A *p*-value of less than 0.05 ($p < 0.05$) was accepted as statistically significant between experimental and control groups. (Calculated value of $2^{-\Delta\Delta Ct}$ in untreated sample was 1).



Tab. 4. Graphic results of ICP-MS analysis.

5. CONCLUSIONS

Thanks to results obtained from several experiments discussed above, I can assert that:

- The possibility of trapping nanoparticles within nanosystems and/or nanocomposites is an advantage in nanotechnological applications. It avoids the possible dispersion of these into the aquatic medium and hence any damage to the ecosystem; The efficiency and performance for which they are designed are not compromised (Brundo *et al*., 2016);

- The use of MTs, iNOS and HO-1 as biomarkers of exposure, supported by many studies, have showed that these proteins represent a valid tool for assessing a preliminary xenobiotic contamination in aquatic environment (De Domenico *et al*., 2011; Copat *et al*., 2012) and its effects such as oxidative damage, depletion of antioxidant defences, cell dysfuntion and tissue pathology (Livingstone, 1993, 2001);

- Short-term toxicity tests are useful to estimate different degrees of toxicity, such as ZFET, which is a sensitive, specific, simple, economical and rapid assay. It also fits perfectly in the direction of the European Community, which aims to reduce the impact of *in vivo* experimentation;

- Long-term studies provide more complete information in order to evaluate substantial alterations that irreversibly compromise the normal developping of cellular functions. Conversely, long-term studies require authorizations that are often granted after a long time and this may affect the planning of experiments. To date, short-term studies outnumber long-term ones in literature, so other long-term studies are needed to demonstrate the safe use of these engineered nanomaterials because prolonged use of them can lead to serious human health and environmental problems.

6. REFERENCES

- Abraham NG, Kappas A. Pharmacological and clinical aspects of heme oxygenase. Review. *Pharmacol. Rev.*, 2008, 60:79-127.
- Adams LK, Lyon DY, Alvarez PJJ. Comparative ecotoxicity of nanoscale TiO_2, SiO_2, and ZnO water suspensions. *Water Res.*, 2006, 40:3527-3532.
- Ahmed F, Rodrigues DF. Investigation of acute effects of graphene oxide on wastewater microbial community: A case study. *J. Hazard. Mater*., 2013, 256-257:33-39.
- Akaighe N, MacCuspie RI, Navarro DA, Aga DS, Banerjee S, Sohn M, Sharma VK. Humic acid-induced silver nanoparticle formation under environmentally relevant conditions. *Environ. Sci. Technol*., 2011, 45:3895-3901.
- Alday A, Alzualde A, Alonso B, Zurutuza A, Muriana A, Quevedo C. Testing toxicity of graphene oxide in zebrafish embryos. *7th SETAC World Congress/SETAC*, North America 37th Annual Meeting, 2016.
- Alivisatos P. The use of nanocrystals in biological detection. *Nat. Biotechnol*., 2004, 22:47-52.
- Andreakis N, D'Aniello S, Albalat R, Patti FP, Garcia-Fernàndez J, Procaccini G, Sordino P, Palumbo A. Evolution of the nitric oxide synthase family in Metazoans. *Mol. Biol. Evol*., 2011, 28(1):163-179.
- Armelao L, Barreca D, Bottaro G, Gasparotto A, Maccato C, Maragno C, Tondello E, Stangar UL, Bergant M, Mahne D. Photocatalytic and antibacterial activity of TiO_2 and Au/TiO_2nanosystems. *Nanotechnology*, 2007, 18:375709.
- Asem A, Rastegar-Pouyani N, De Los Ríos-Escalante P. The genus *Artemia* Leach, 1819 (*Crustacea*: *Branchiopoda*). True and false taxonomical descriptions. *Lat. Am. J. Aquat. Res*., 2010, 38(3):501-506.
- Asem A. Historical record on brine shrimp *Artemia* more than one thousand years ago from Urmia Lake, Iran. *J. Biol. Res*., 2008, 9:113-114.
- Asharani PV, Lianwu Y, Gong Z, Valiyaveettil S. Comparison of the toxicity of silver, gold and platinum nanoparticles in developing zebrafish embryos. *Nanotoxicology*, 2010, 5(1):43-54.
- Ates M, Daniels J, Arslan Z, Farah IO. Effects of aqueous suspensions of titanium dioxide nanoparticles on *Artemia salina*: assessment of nanoparticle aggregation, accumulation, and toxicity. *Environ. Monit. Assess*., 2013, 185:3339-3348.
- Baccetti B, Baldaccini NE, Bedini C, Brandmayr P, Capanna E, Chieffi G, Cobolli M, Ferraguti M, Ghirardelli E, Ghiretti F, Giusti F, Grigolo A, Mainardi D, Minelli A, Papi F, Parrinello N, Ricci N, Ruffo S, Sarà M, Scali V, Zullin A. Trattato Italiano-Zoologia 2, Editoriale Grasso, 1991, pp. 569-572.
- Ballard WW. Morphogenetic movements and fate maps of Vertebrates. *Am. Zool*., 1981, 21:391-399.
- Bar-Ilan O, Albrecht RM, Fako VE, Furgeson DY. Toxicity assessments of multisized gold and silver nanoparticles in zebrafish embryos. *Small*, 2009, 5:1897-1910.
- Barriada JL, Tappin AD, Evans EH, Achterberg EP. Dissolved silver measurements in seawater. *Trends Anal. Chem*., 2007, 26:809-817.
- Baun A, Hartmann NB, Grieger K, Kusk KO. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. *Ecotoxicology*, 2008, 17:387-395.
- Beasley A, Elrod-Erickson M, Otter RR. Consistency of morphological endpoints used to assess developmental timing in zebrafish (*Danio rerio*) across a temperature gradient. *Reprod. Toxicol.*, 2012, 34:561-567.
- Belyaeva NF, Kashirtseva VN, Medvedeva NV, Khudoklinova YY, Ipatova OM, Archakov AI. Zebrafish as a model system for biomedical studies. Review. *Biochemistry (Moscow) Suppl. Ser. B: Biomed. Chem*., 2009, 3(4):343-350.

- Benn TM, Westerhoff P. Nanoparticle silver released into water from commercially available sock fabrics. *Environ. Sci. Technol*., 2008, 42:4133-4139.
- Bhattacharyya S, Kudgus RA, Bhattacharya R, Mukherjee P. Inorganic nanoparticles in cancer therapy. *Pharm. Res*., 2011, 28(2):237-59.
- Birla SS, Tiwari VV, Gade AK, Ingle AP, Yadav AP, Rai MK. Fabrication of silver nanoparticles by *Phoma glomerata* and its combined effect against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Lett. Appl. Microbiol*., 2009, 48:173-179.
- Bitounis D, Ali-Boucetta H, Hong BH, Min DH, Kostarelos K. Prospects and challenges of graphene in biomedical applications. *Adv. Mater*., 2013, 25:2258-2268.
- Borm PJ, Robbins D, Haubold S, Kuhlbusch T, Fissan H, Donaldson K, Schins R, Stone V, Kreyling W, Lademann J, Krutmann J, Warheit D, Oberdörster E. The potential risks of nanomaterials: a review carried out for ECETOC. *Part. Fibre Toxicol*., 2006, 3:11.
- Boxall ABA, Chaudhry Q, Sinclair C, Jones A, Aitken R, Jefferson B, Watts C. Current and future predictive environmental exposure to engineered nanoparticles. Central Science Laboratory, Department of Environment Food and Rural Affairs, York London, U.K., 2007.
- Brigger I, Dubernet C, Couvreur P. Nanoparticles in cancer therapy and diagnosis. *Adv. Drug Deliv. Rev*., 2002, 54:631-51.
- Brodie BC. On the atomic weight of graphite. *Philos. Trans. R. Soc. Lond. B. Biol. Sci*., 1859, 149:249-259.
- Brundo MV, Pecoraro R, Marino F, Salvaggio A, Tibullo D, Saccone S, Bramanti V, Buccheri MA, Impellizzeri G, Scuderi V, Zimbone M, Privitera V. Toxicity evaluation of new engineered nanomaterials in zebrafish. *Front. Physiol*., 2016, 7:130.
- Buccheri MA, D'Angelo D, Scalese S, Spanò SF, Filice S, Fazio E, Compagnini G, Zimbone M, Brundo MV, Pecoraro R, Alba A, Sinatra F, Rappazzo G, Privitera V. Modification of graphene oxide by laser irradiation: a new route to enhance antibacterial activity. *Nanotechnology*, 2016, 27:245704.
- Canesi L, Fabbri R, Gallo G, Vallotto D, Marcomini A, Pojana G. Biomarkers in *Mytilus galloprovincialis* exposed to suspensions of selected nanoparticles (nano carbonblack, C_{60} fullerene, nano-TiO_2, nano-SiO_2). *Aquatic. Toxicol*., 2010, 100:168-177.
- Cattaneo AG, Gornati R, Chiriva-Internati M, Bernardini G. Ecotoxicology of nanomaterials: the role of invertebrate testing. *Invertebrate Surviv. J*., 2009, 6:78-97.
- Chen J, Dong X, Zhao J, Tang G. *In vivo* acute toxicity of titanium dioxide nanoparticles to mice after intraperitioneal injection. *J. Appl. Toxicol*., 2009, 29(4):330-337.
- Chen TH, Lin CY, Tseng MC. Behavioral effects of titanium dioxide nanoparticles on larval zebrafish (*Danio rerio*). *Mar. Pollut. Bull*., 2011, 63:303-8.
- Chen WY, John JA, Lin CH, Lin HF, Wu SC, Lin CH, Chang CY. Expression of metallothionein gene during embryonic and early larval development in zebrafish. *Aquat. Toxicol.,* 2004*,* 69:215-227.
- Chen WY, John JAC, Lin CH, Chang CY. Molecular cloning and developmental expression of zinc finger transcription factor MTF-1 gene in zebrafish, *Danio rerio*. *Biochem. Biophys. Res. Commun.,* 2002*,* 291:798:805.
- Cho WS, Cho M, Jeong J, Choi M, Cho HY, Han BS, Kim SH, Kim HO, Lim YT, Chung BH. Acute toxicity and pharmacokinetics of 13 nm-sized PEG-coated gold nanoparticles. *Toxicol. Appl. Pharmacol*., 2009, 236:16-24.
- Cho WS, Duffin R, Poland CA, Howie SEM, MacNee W, Bradley M, Megson IL, Donaldson K. Metal oxide nanoparticles induce unique infammatory footprints in the lung: important implications for nanoparticle testing. *Environ. Health Perspect*., 2010, 118(12):1699-1706.
- Commission Recommendation of 18 october 2011 on the definition of nanomaterial. Official Journal of the European Union (2011/696/EU).

- Copat C, Brundo MV, Arena G, Grasso A, Oliveri Conti G, Ledda C, Fallico R, Sciacca S, Ferrante M. Seasonal variation of bioaccumulation in *Engraulis encrasicolus* (*Linneaus*, 1758) and related biomarkers of exposure. *Ecotoxicol. Environ. Saf.,* 2012*,* 86:31-37.
- Cote LJ, Kim K, Huang J. Langmuir-Blodgett assembly of graphite oxide single layers. *J. Am. Chem. Soc*., 2009, 131(3):1043-1049.
- D'Angelo D, Bongiorno C, Amato M, Deretzis I, La Magna A, Compagnini G, Spanò SF, Scalese S. Electron energy-loss spectra of graphene oxide for the determination of oxygen functionalities. *Carbon,* 2015, 93:1034-1041.
- Daughton CG. Non-regulated water contaminants: emerging research. *Environ. Impact Asses. Rev*., 2004, 24:711-732.
- De la Escosura-Muniz A, Sanchez-Espinel C, Freitas BD, Gonzales AG, Maltez-de Costa M, Merkoci A. Rapid identification and quantification of tumor cells using an electrocatalytic method based on gold nanoparticles. *Anal. Chem*., 2009, 81:10268-10274.
- De los Ríos P, Asem A. Comparison of diameter of the frontal knobs in *Artemia urmiana* Günther, 1899 (*Anostraca*). *Crustaceana*, 2008, 81(11):1281-1288.
- Dowling AP. Development of nanotechnologies. *Mater. Today*, 2004, 7(1):30-35.
- Dreyer DR, Park S, Bielawski CW, Ruoff RS. The chemistry of graphene oxide. *Chem. Soc. Rev.*, 2010, 39:228-40.
- Duan Y, Liu J, Ma L, Li N, Liu H, Wang J, Zheng L, Liu C, Wang H, Zhao X, Yan J, Wang S, Wang H, Zhang X, Hong F. Toxicological characteristics of nanoparticulate anatase titanium dioxide in mice. *Biomaterials*, 2010, 31(5):894-899.
- Dykman L, Khlebtsov N. Gold nanoparticles in biomedical applications: recent advances and perspectives. *Chem. Soc. Rev*., 2012, 41(6):2256-2282.
- Enotiadis A, Angjeli K, Baldino N, Nicotera I, Gournis D. Graphene-based Nafion nanocomposite membranes: enhanced proton transport and water retention by novel organo-functionalized graphene oxide nanosheets. *Small*, 2012, 8(21):3338-49.
- Fard JK, Jafari S, Eghbal MA. A review of molecular mechanisms involved in toxicity of nanoparticles. *Adv. Pharm. Bull*., 2015, 5(4):447-454.
- Farkas J, Christian P, Urrea JAG, Roos N, Hassellöv M, Tollefsen KE, Thomas KV. Effects of silver and gold nanoparticles on rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquat. Toxicol*., 2010, 96:44-52.
- Farré M, Gajda-Schrantz K, Kantiani L, Barceló D. Ecotoxicity and analysis of nanomaterials in the aquatic environment. *Anal. Bioanal. Chem*., 2009, 393:81-95.
- Federici G, Shaw BJ, Handy RD. 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): Gill injury, oxidative stress, and other physiological effects. *Aquat. Toxicol.,* 84:415-430.
- Filice S, D'Angelo D, Libertino S, Nicotera I, Kosma V, Privitera V, Scalese S. Graphene oxide and titania hybrid Nafion membranes for efficient removal of methyl orange dye from water. *Carbon*, 2015, 82:489-99.
- Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. *Eur. Heart J.*, 2012, 33:829-837.
- Fujishima A, Honda K. Electrochemical photolysis of water at a semiconductor electrode. *Nature*, 1972, 238:37-38.
- Gambardella C, Mesarič T, Milivojević T, Sepčić K, Gallus L, Carbone S, Ferrando S, Faimali M. Effects of selected metal oxide nanoparticles on *Artemia salina* larvae: evaluation of mortality and behavioural and biochemical responses. *Environ. Monit. Assess*., 2014, 186:4249-4259.
- George SM. Atomic layer deposition: An overview. *Chem. Rev*., 2010, 110:111-131.
- Geranio L, Heuberger M, Nowack B. The behavior of silver nanotextiles during washing. *Environ. Sci. Technol*., 2009, 43:8113-8118.

- Gerhard GS. Comparative aspects of zebrafish (*Danio rerio*) as a model for aging research. *Experimen. Gerontol.*, 2003, 38:1333-1341.
- Ghosh DK, Salerno JC. Nitric oxide synthases: domain structure and alignment in enzyme function and control. *Front Biosci*., 2003, 8:193-209.
- Giorgetti E, Giusti A, Laza SC, Marsili P, Giammanco F. Production of colloidal gold nanoparticles by picosecond laser ablation in liquids. *Phys. Status Solidi A*, 2007, 204(6):1693-1698.
- Gollavelli G, Ling YC. Multi-functional graphene as an *in vitro* and *in vivo* imaging probe. *Biomaterials*, 2012, 33:2532-2545.
- Gomez-Caballero JA, Villasenor-Cabral MG, Santiago-Jacinto P, Ponce-Abad F. Hypogene Ba-rich todorokite and associated nanometric native silver in the San Miguel Tenango mining area, Zacatlán, Puebla, Mexico. *Can. Mineral*., 2010, 48 (5):1237-1253.
- Gornati R, Longo A, Rossi F, Maisano M, Sabatino G, Mauceri A, Bernardini G, Fasulo S. Effects of titanium dioxide nanoparticle exposure in *Mytilus galloprovincialis* gills and digestive gland. *Nanotoxicology,* 2016, 10(6):807-17.
- Gottschalk F, Sondere T, Scholz RW, Nowack B. Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, fullerenes) for different regions. *Environ. Sci. Technol*., 2009, 43:9216-9222.
- Griffitt RJ, Luo J, Gao J, Bonzongo JC, Barber DS. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. *Environ. Toxicol. Chem*., 2008, 27:1972-1978.
- Guerra R. Ecotoxicological and chemical evaluation of phenolic compounds in industrial effluents. *Chemosphere*, 2001, 44:1737-1747.
- Günes C, Heuchel R, Georgiev O, Müller KH, Lichtlen P, Blüthmann H, Marino S, Aguzzi A, Schaffner W. Embryonic lethality and liver degeneration in mice lacking the metal-responsive transcriptional activator MTF-1. *EMBO J*., 1998, 17(10):2846-2854.
- Hahn ME, McArthur AG, Karchner SI, Franks DG, Jenny MJ, Timme-Laragy AR, Stegeman JJ, Woodin BR, Cipriano MJ, Linney E. The transcriptional response to oxidative stress during Vertebrate development: effects of *tert* butylhydroquinone and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *PLoS One*, 2004, 9(11):1-17.
- Hall S, Bradley T, Moore JT, Kuykindall T, Minella L. Acute and chronic toxicity of nano-scale TiO_2 particles to freshwater fish, cladocerans, and green algae, and effects of organic and inorganic substrate on TiO_2 toxicity. *Nanotoxicology*, 2009, 3:91-97.
- Hamilton F, Buchanan. An account of the fishes found in the rivers Ganges and its branches. Edinburgh: printed for Archibald Constable and Company, Edinburgh; and hurst, Robinson, and Co. 90, Cheapside, London, 1822.
- Handy RD, Henry TB, Scown TM, Johnston BD, Tyler CR. Manufactured nanoparticles: their uptake and effects on fish, a mechanistic analysis. *Ecotoxicolog*y, 2008, 17(5):396-409.
- Hao L, Wang Z, Xing B. Effect of sub-acute exposure to TiO_2 nanoparticles on oxidative stress and histopathological changes in juvenile Carp (*Cyprinus carpio*). *J. Environ. Sci*., 2009, 21:1459-1466.
- Hill AJ, Teraoka H, Heideman W, Peterson RE. Zebrafish as a model vertebrate for investigating chemical toxicity. Review. *Toxicol. Sci*., 2005, 86(1):6-19.
- Hoet PHM, Brüske-Hohlfeld I, Salata OV. Nanoparticles - known and unknown health risks. *Review. J. Nanobiotechnol*., 2004, 2:1-15.
- Hu W, Peng C, Luo W, Lv M, Li X, Li D, Huang Q, Fan C. Graphene-based antibacterial paper. *ACS Nano*, 2010, 4(7):4317-4323.
- Hummers WS, Offeman RE. Preparation of graphitic oxide. *J. Am. Chem. Soc*., 1958, 80(6):1339.
- IARC (International Agency for Research on Cancer) "Titanium dioxide group 2B" in IARC Monographs on the evaluation of carcinogenic risks to humans, International Agency for Research on Cancer, World Health Organization, Lyon, France, 2006, vol. 9.

- IARC (International Agency for Research on Cancer), "Carbon black, titanium dioxide, and talc," in IARC Monographs on the evaluation of carcinogenic risks to humans, International Agency for Research on Cancer, World Health Organization, Lyon, France, 2010, vol. 93.
- Iavicoli I, Leso V, Bergamaschi A. Toxicological effects of titanium dioxide nanoparticles: A review of *in vivo* studies. *J. Nanomater*., 2012, 1-36.
- Jain S, Hirst DG, O'sullivan JM. Gold nanoparticles as novel agents for cancer therapy. *Br. J. Radiol*., 2012, 85(1010):101-113.
- Jaroenworaluck A, Sunsaneeyametha W, Kosachan N, Stevens R. Characteristics of silica-coated TiO_2 and its UV absorption for sunscreen cosmetic applications. *Surf. Interface Anal*., 2005, 38:473-477.
- Kaegi R, Ulrich A, Sinnet B, Vonbank R, Wichser A, Zuleeg S, Simmler H, Brunner S, Vonmont H, Burkhardt M, Boller M. Synthetic TiO_2 nanoparticle emission from exterior facades into the aquatic environment. *Environ. Pollut*., 2008, 156:233-239.
- Kari G, Rodeck U, Dicker AP. Zebrafish: an emerging model system for human disease and drug discovery. *Clin. Pharmacol. Therapet.*, 2007, 82:70-80.
- Karthik L, Kumar G, Keswani T, Bhattacharyya A, Reddy BP, Rao KVB. Marine actinobacterial mediated gold nanoparticles synthesis and their antimalarial activity. *Nanomedicine Nanotechnol. Biol. Med*., 2013, 9:951-960.
- Kemp KC, Saema H, Saleh M, Le NH, Mahesh K, Chandra V, Kim KS. Environmental applications using graphene composites: water remediation and gas adsorption. *Nanoscale*, 2013, 5:3149-3171.
- Khan MS, Vishakante GD, S H. Gold nanoparticles: a paradigm shift in biomedical applications. *Adv. Colloid Interface Sci*., 2013, 199-200:44-58.
- Kim B, Park CS, Murayama M, Hochella MF. Discovery and characterization of silver sulfide nanoparticles in final sewage sludge products. *Environ. Sci. Technol*., 2010, 44:7509-7514.
- Kim D, Park S, Lee JH, Jeong YY, Jon A. Antibiofouling polymer-coated gold nanoparticles as a contrast agent for *in vivo* X-ray computed tomography imaging. *J. Am. Chem. Soc*., 2007, 129:7661-5.
- Kim KJ, Sung WS, Suh BK, Moon SK, Choi JS, Kim J, Lee DG. Antifungal activity and mode of action of silver nano-particles on Candida albicans. *BioMetals*, 2008, 22:235-242.
- Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev. Dyn.*, 1995, 203:253-310.
- Klaassen CD, Liu J, Diwan BA. Metallothionein protection of cadmium toxicity. *Toxicol. Appl. Pharmacol.,* 2009, 238:215-220.
- Klaper R, Crago J, Barr J, Arndt D, Setyowati K, Chen J. Toxicity biomarker expression in daphnids exposed to manufactured nanoparticles: changes in toxicity with functionalization. *Environ. Pollut*., 2009, 157:1152-1156.
- Kuenen DJ, Baas-Becking LGM. Historical notes on *Artemia salina* (L.). *Zoologische Mededelingen*, 1938, 20:222-230.
- Lankveld DPK, Oomen AG, Krystek P, Neigh A, Troost-de Jong A, Noorlander CW, Van Eijkeren JCH, Geertsma RE, De Jong WH. The kinetics of the tissue distribution of silver nanoparticles of different sizes. *Biomaterials*, 2010, 31:8350-8361.
- Lapresta-Fernández A, Fernández A, Blasco J. Nanoecotoxicity effects of engineered silver and gold nanoparticles in aquatic organisms. *Trends Anal. Chem.*, 2012, 32:40-59.
- Lea MC. Allotropic forms of silver. *Am. J. Sci*., 1889, 3(37):476-491.
- Leach WE. *Entomostraca*, *Dictionaire des Science Naturelles*, 1819, 14:524.
- Lee KJ, Browning LM, Nallathamby PD, Desai T, Cherukuri PK, Xu XHN. *In vivo* quantitative study of sized-dependent transport and toxicity of single silver nanoparticles using zebrafish embryos. *Chem. Res. Toxicol*., 2012, 25:1029-1046.

- Lee KJ, Nallathamby PD, Browning LM, Osgood CJ, Xu XHN. *In vivo* imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. *ACS nano*, 2007, 1:133-143.
- Libralato G. The case of *Artemia* spp. in nanoecotoxicology. *Mar. Environ. Res.*, 2014, 101:38-43.
- Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. *Nat. Rev. Genetics*, 2007, 8:353-367.
- Linnaeus C. *Systema naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Tomus I. Editio decima, reformata. Holmiae Impensis Direct. Laurentii Salvii*, 1758, pp. 824.
- Liu XT, Mu XY, Wu XL, Meng LX, Guan WB, Ma YQ, Sun H, Wang CJ, Li XF. Toxicity of multi-walled carbon nanotubes, graphene oxide, and reduced graphene oxide to zebrafish embryos. *Biomed. Environ. Sci*., 2014, 27(9):676-683.
- Liu CW, Xiong F, Jia HZ, Wang XL, Cheng H, Sun YH, Zhang XZ, Zhuo RX, Feng J. Graphene-based anticancer nanosystem and its biosafety evaluation using a zebrafish model. *Biomacromolecules*, 2013, 14:358-366.
- Liu JY, Hurt RH. Ion release kinetics and particle persistence in aqueous nano-silver colloids. *Environ. Sci. Technol*., 2010, 44:2169-2175.
- Livingstone DR. Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. *J. Chem. Tech. Biotechnol.*, 1993, 57:195-211.
- Livingstone DR. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Mar. Pollut. Bull*., 2001, 42(8):656-666.
- Lovern SB, Owen HA, Klaper R. Electron microscopy of gold nanoparticle intake in the gut of *Daphnia magna. Nanotoxicology*, 2008, 2:43-48.
- Lovern SB, Strickler JR, Klaper R. Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nano-C_{60}, and $C_{60}H_xC_{70}H_x$). *Environ. Sci. Technol*., 2007, 41:4465-4470.
- Luoma SN. Silver nanotechnologies and the environment: old problems or new challenges? Woodrow Wilson International Center for Scholars, Washington, DC, PEN-15 Silver 2008.
- Lushchak VI. Environmentally induced oxidative stress in aquatic animals. *Aquat. Toxicol*., 2011, 101:13-30.
- Mahdihassan S. Cinnabar-gold as the best alchemical drug of longevity, called Makaradhwaja in India. *Am. J. Chin. Med*., 1985, 13:93-108.
- Maines MD. The heme oxygenase system: a regulator of second messenger gases. *Ann. Rev. Pharmacol. Toxicol*., 1997, 37:517-554.
- Marletta MA. Nitric oxide synthase: aspects concerning structure and catalysis. *Cell*, 1994, 78:927-930.
- Martin JW, Davis GE. An updated classification of the recent *Crustacea*. Natural History Museum of Los Angeles County of Los Angeles County. *Science Series*, 2001, 39:1-124.
- Mattevi C, Eda G, Agnoli S, Miller S, Mkhoyan KA, Celik O, Mastrogiovanni D, Granozzi G, Garfunkel E, Chhowalla M. Evolution of electrical, chemical, and structural properties of transparent and conducting chemically derived graphene thin films. *Adv. Funct. Mater*., 2009, 19:2577-2583.
- McCoubrey WK Jr, Huang TJ, Maines MD. Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *Eur. J. Biochem*., 1997, 247:725-732.
- Menard A, Drobne D, Jemec A. Ecotoxicity of nanosized TiO_2. Review of *in vivo* data. *Environ. Pollut*., 2011, 159:677-684.
- Menke AL, Spitsbergen JM, Wolterbeek APM, Woutersen RA. Normal anatomy and histology of the adult zebrafish. *Toxicol. Pathol*., 2011, 39:759-775.
- Moore MN, Depledge MH, Readman JW, Leonard P. An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. *Mutat. Res*., 2004, 552:247-268.

- Moore MN. Biocomplexity: the post-genome challenge in ecotoxicology. *Aquat. Toxicol*., 2002, 59:1-15.
- Mueller NC, Nowack B. Exposure modeling of engineered nanoparticles in the environment. *Environ. Sci. Technol*., 2008, 42:4447-4453.
- Nasiadka A, Clark MD. Zebrafish breeding in the laboratory environment. *ILAR J*., 2012, 53(2):161-168.
- NAS-NRC - Committee on Biological Markers of the National Research Council. Biological markers in environmental health research. *Environ. Health Perspect*., 1987, 74:3-9.
- Navarro E, Baun A, Behra R, Hartmann NB, Filser J, Miao AJ, Quigg EA, Santschi PH, Sigg L. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. *Ecotoxicology*, 2008, 17:372-386.
- Nel AE, Xia T, Mädler L, Li N. Toxic potential of materials at the nanolevel. *Science*, 2006, 311(5761):622-627.
- Nishimura Y, Inoue A, Sasagawa S, Koiwa J, Kawaguchi K, Kawase R, Maruyama T, Kim S, Tanaka T. Using zebrafish in systems toxicology for developmental toxicity testing. Review. *Congenit. Anom.,* 2016,56:18-27.
- Novoselov KS, Geim AK, Morozov SV, Jjiang D, Zhang Y, Dubonos SV, Grogorieva IV, Firsov AA. Electric field effectin atomically thin carbon films. *Science*, 2004, 306:666-669.
- Nowack B, Krug H, Height M. 120 years of nanosilver history: implications for policy makers. *Environ. Sci. Technol*., 2011, 45:1177-1183.
- Nunes BS, Carvalho FD, Guilhermino LM, Van Stappen G. Use of the genus *Artemi*a in ecotoxicity testing. *Environ Pollut*., 2006, 144:453-462.
- Oberdörster G, Oberdörster E, Oberdörster J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ. Health Perspect*., 2005, 113:823-83.
- OECD (Organisation for EconomicCo-operation and Development). Fish Embryo Acute Toxicity (FET) Test. Test Guideline N° 236, Guidelines for the testing of chemicals, 2013, 1-22.
- Owen R, Depledge MH. 2005 Nanotechnology and the environment: risks and rewards. *Mar. Pollut. Bull*., 50:609-612.
- Palumbo A. Nitric oxide in marine invertebrates: a comparative perspective. Review. *Comp. Biochem. Physiol. Part A*, 2005, 142:241-248.
- Pan Y, Neuss S, Leifert A, Fischler M, Wen F, Simon U, Schmid G, Brandau W, Jahnen-Dechent W. Size-Dependent cytotoxicity of gold nanoparticles. *Small*, 2007, 3(11)1941-1949.
- Panyam J, Labhasetwar V. Biodegradable nanoparticles for drug and gene delivery to cells and tissues. *Adv. Drug Deliv. Rev*., 2003, 55:329-347.
- Panzica-Kelly JM, Zhang CX, Augustine-Rauch KA. Optimization and performance assessment of the chorion-off [dechorinated] zebrafish developmental toxicity assay. *Toxicol. Sci.,* 2015*,* 146:127-134.
- Patra HK, Banerjee S, Chaudhuri U, Lahiri P, Dasgupta AK. Cell selective response to gold nanoparticles. *Nanomedicine*, 2007, 111-119.
- Pecoraro R, D'Angelo D, Filice S, Scalese S, Capparucci F, Di Caro G, Scalisi ME, Salvaggio A, Tibullo D, Nicotera I, Brundo MV. Toxicity evaluation of graphene oxide and titania loaded nafion membranes in zebrafish. *Under Review*.
- Persoone G, Wells PG. *Artemia* in aquatic toxicology: a review. In: Sorgeloos P, Bengstone DA, Decleir W, Jasper E (Eds.). *Artemia* research and its applications. Morphology, genetics, strain characterisation, toxicology. Universa Press, Wetteren, Belgium, 1987, vol. 1.
- Petosa AR, Jaisi DP, Quevedo IR, Elimelech M, Tufenkji N. Aggregation and deposition of engineered nanomaterials in aquatic environments: role of physicochemical interactions. *Environ. Sci. Technol*., 2010, 44:6532-6549.
- Poon HF, Calabrese V, Scapagnini G, Butterfield A. Free radicals: key to brain aging and heme oxygenase as a cellular response to oxidative stress. *J. Gerontol. A Med. Sci*., 2004, 59(5):478-493.

- Pretti C, Oliva M, Di Pietro R, Monni G, Cevasco G, Chiellini F, Pomelli C, Chiappe C. Ecotoxicity of pristine graphene to marine organisms. *Ecotoxicol. Environmen. Saf*., 2014, 101:138-145.
- Pyatenko A, Yamaguchi M, Suzuki M. Mechanisms of size reduction of colloidal silver and gold nanoparticles irradiated by Nd:YAG laser. *J. Phys. Chem. C,* 2009, 113:9078-9085.
- Quadros ME, Marr LC. Silver nanoparticles and total aerosols emitted by nanotechnology-related consumer spray products. *Environ. Sci. Technol*., 2011, 45:10713-10719.
- Ramsden CS, Smith TJ, Shaw BJ, Handy RD. Dietary exposure to titanium dioxide nanoparticles in rainbow trout, (*Oncorhynchus mykiss*): no effect on growth, but subtle biochemical disturbances in the brain. *Ecotoxicology*, 2009, 18(7): 939-951.
- Riggio M, Filosa S, Parisi E, Scudiero R. Changes in zinc, copper and metallothionein contents during oocyte growth and early development of the teleost *Danio rerio* (zebrafish). *Comp. Biochem. Physiol. C,* 2003*,* 135:191-196.
- Royal Society and Royal Academy of Engineering. Nanoscience and nanotechnologies: opportunities and uncertainties. RS policy document 19/04. London: The Royal Society, 2004.
- Ruebhart DR, Cock IE, Shaw GR. Brine Shrimp Bioassay: importance of correct taxonomic identification of *Artemia* (*Anostraca*) species. *Environ. Toxicol*., 2008, 1-6.
- Ruttkay-Nedecky B, Nejdl L, Gumulec J, Zitka O, Masarik M, Eckschlager T, Stiborova M, Adam V, Kizek R. The role of metallothionein in oxidative stress. Review*. Int. J. Mol. Sci.,* 2013, 14:6044-6066.
- Sager TM, Kommineni C, Castranova V. Pulmonary response to intratracheal instillation of ultrafine versus fine titanium dioxide: role of particle surface area. *Part. Fibre Toxicol*., 2008, 5:17.
- Sanchez VC, Jachak A, Hurt RH, Kane AB. Biological interactions of graphene-family nanomaterials: an interdisciplinary review. *Chem. Res. Toxicol*., 2012, 25:15-34.
- Schlösser D. Extrait d'une lettre de Monsieur le Docteur Schlosser concernant un insecte peu connu. Observation periodique sur la physique, l'histoire naturille et les beaux arts, de Gautier, 1756, pp. 58-60.
- Schulz R, Nava E, Moncada S. Induction and potential biological relevance of a Ca^{2+}-independent nitric oxide synthase in the myocardium. *Br. J. Pharmacol*., 1992, 105:575-580.
- Scown TM, Santos EM, Johnston BD, Gaiser B, Baalousha M, Mitov S, Lead JR, Stone V, Fernandes TF, Jepson M, Van Aerle R, Tyler CR. Effects of aqueous exposure to silver nanoparticles of different sizes in rainbow trout. *Toxicol. Sci*., 2010, 115(2):521-534.
- Scuderi V, Impellizzeri G, Romano L, Scuderi M, Brundo MV, Bergum K, Zimbone M, Sanz R, Buccheri MA, Simone F, Nicotra G, Svensson BG, Grimaldi MG, Privitera V. An enhanced photocatalytic response of nanometric TiO_2 wrapping of Au nanoparticles for eco-friendly water applications. *Nanoscale,* 2014*,* 6:11189-11195.
- Seabra AB, Paula AJ, De Lima R, Alves OL, Durán N. Nanotoxicity of graphene and graphene oxide. *Chem. Res. Toxicol*., 2014, 27(2):159-168.
- Sharma P, Hussain N, Borah DJ, Das MR. Kinetics and adsorption behavior of the methyl blue at the graphene oxide/reduced graphene oxide nanosheet-water interface: a comparative study. *Journal of Chemical & Engineering Data*, 2013, 58(12):3477-3488.
- Sondi I, Salopek-Sondi B. Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. *J. Colloid Interface Sci*., 2004, 275:177-182.
- Sönnichsen C, Franzl T, Wilk T, Von Plessen G, Feldmann J, Wilson O, Mulvaney P. Drastic reduction of plasmon damping in gold nanorod. *Phys. Rev. Lett*., 2002, 88(7):402-406.
- Spitsbergen JM, Kent ML. The state of the art of the zebrafish model for toxicology and toxicologic pathology research - Advantages and current limitations. *Toxicol. Pathol*., 2003, 31(Suppl):62-87.
- Staudenmaier L. Verfahren zur darstellung der graphitsäure. *Ber. Dtsch. Chem. Ges*., 1898, 31:1481-1487.

- Stegeman JJ, Lech JJ. Cytochrome P-450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. *Environ. Health Perspect*., 1991, 90:101-109.
- Sun RW, Chen R, Chung NP, Ho CM, Lin CL, Che CM. Silver nanoparticles fabricated in Hepes buffer exhibit cytoprotective activities toward HIV-1 infected cells. *Chem. Commun*., 2005, 40:5059-5061.
- Sylvain NJ, Brewster DL, Ali DW. Zebrafish embryos exposed to alcohol undergo abnormal development of motor neurons and muscle fibers. *Neurotoxicol. Teratol*., 2010, 32:472-480.
- Synnes M, Prydz K, Lovdal T, Brech A, Berg T. Fluid phase endocytosis and galactosyl receptor-mediated endocytosis employ different early endosomes. *Biochim. Biophys. Acta*., 1999, 1421:317-328.
- Tedesco S, Doyle H, Blasco J, Redmond G, Sheehan D. Oxidative stress and toxicity of gold nanoparticles in *Mytilus edulis*. *Aquat. Toxicol.,* 2010*,* 100:178-186.
- Triantaphyllidis GV, Abatzopoulos TJ, Sorgeloos P. Review of the biogeography of the genus *Artemia* (*Crustacea, Anostraca*). *J. Biogeogr*., 1998, 25:213-226.
- Triantaphyllidis GV, Criel GRJ, Abatzopoulos TJ, Thomas KM, Peleman J, Beardmore JA, Sorgeloos P. International study on *Artemia*. LVII. Morphological and molecular characteristics suggest conspecificity of all bisexual European and North African *Artemia* populations. *Mar. Biol*., 1997, 129:477-48.
- Van Stappen G. Introduction, biology and ecology of *Artemia*. In: Lavens P, Sorgeloos P, editors. Manual on the production and use of live food for aquaculture. FAO Fisheries Technical Paper N° 361, 1996, Rome FAO, pp 79-123.
- Vanhaecke P, Persoone G. Report on an intercalibration exercise on a short term standard toxicity test with *Artemia* nauplii (ARC-test). *Institut National de la Sante et de la Recherche Medicale (INSERM)*, 1981, 106:359-376.
- VašáK M. Advances in metallothionein structure and functions. *J. Trace Elements Med. Biol.,* 2005, 19:13-17.
- Vijayan SR, Santhiyagu P, Singamuthu M, Kumari Ahila N, Jayaraman R, Ethiraj K. Synthesis and characterization of silver and gold nanoparticles using aqueous extract of seaweed, *Turbinaria conoides*, and their antimicrofouling activity. *Sci. World J*., 2014, 1-10.
- Wang J, Zhu X, Zhang X, Zhao Z, Liu H, George R, Wilson-Rawls J, Chang Y, Chen Y. Disruption of zebrafish (*Danio rerio*) reproduction upon chronic exposure to TiO_2 nanoparticles. *Chemosphere*, 2011, 83:461-7.
- Warheit DB, Hoke RA, Finlay C, Donner EM, Reed KL, Sayes CM. Development of a base set of toxicity tests using ultrafine TiO_2 particles as a component of nanoparticle risk management. *Toxicol. Lett*., 2007, 171:99-110.
- Warheit DB. Nanoparticles: health impacts? *Mater. Today*, 2004, 7:32-35.
- Wen LS, Santschi PH, Gill GA, Paternostro CL, Lehman RD. Colloidal and particulate silver in river and estuarine waters of Texas. *Environ. Sci. Technol*., 1997, 31:723-731.
- Wood CM, Playle RC, Hogstrand C. Physiology and modeling of mechanisms of silver uptake and toxicity in fish. *Environ. Toxicol. Chem*., 1999, 18:71-83.
- Wright JB, Lam K, Hansen D, Burrell RE. Efficacy of topical silver against fungal burn wound pathogens. *Am. J. Infect. Control*, 1999, 27:344-350.
- Wu YA, Zhou QF, Li HC, Liu W, Wang T, Jiang GB. Effects of silver nanoparticles on the development and histopathology biomarkers of Japanese medaka (*Oryzias latipes*) using the partial-life test. *Aquat. Toxicol*., 2010, 100:160-167.
- Yeh TF, Cihlář J, Chang CY, Cheng C, Teng H. Roles of graphene oxide in photocatalytic water splitting. *Mater. Today*, 2013, 16(3):78-84.
- Yu SJ, Yin YG, Liu JF. Silver nanoparticles in the environment. *Environ. Sci.: Processes Impacts*, 2013, 15:78-92.

- Zhang C, Frazier JM, Chen H, Liu Y, Lee JA, Cole GJ. Molecular and morphological changes in zebrafish following transient ethanol exposure during defined developmental stages. *Neurotoxicol. Teratol*., 2014, 44:70-80.
- Zhu X, Chang Y, Chen Y. Toxicity and bioaccumulation of TiO_2 nanoparticle aggregates in *Daphnia magna*. *Chemosphere*, 2010, 78:209-215.
- Zhu X, Zhu L, Chen Y, Tia S. Acute toxicities of six manufactured nanomaterial suspensions to *Daphnia magna*. *J. Nanopart. Res*., 2009, 11:67-75.
- Zhu X, Zhu L, Duan Z, Qi R, Li Y, Lang Y. Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to zebrafish (*Danio rerio*) early developmental stage. *J. Environ. Sci. Health A Tox. Haz. Subst. Environ. Eng*., 2008, 43:278-284.
- Zon L, Peterson RT. *In vivo* drug discovery in the zebrafish. *Nature. Rev. Drug Discov.,* 2005, 4:35-44.

RESEARCH ACTIVITY

Papers

- Toxicity evaluation of graphene oxide and titania loaded nafion membranes in zebrafish. **Pecoraro R**, D'Angelo D, Filice S, Scalese S, Capparucci F, Di Caro G, Scalisi ME, Salvaggio A, Tibullo D, Nicotera I, Brundo MV. (*Under Review*).
- Metallic nano-composite toxicity evaluation by zebrafish embryo toxicity test with identification of specific exposure biomarkers. **Pecoraro R**, Salvaggio A, Marino F, Di Caro G, Capparucci F, Lombardo BM, Messina G, Scalisi EM, Tummino M, Loreto F, D'Amante G, Avola R, Tibullo D, Brundo MV. *Current Protocol and Toxicology*. (Accepted on 2 June 2017).
- Toxicity evaluation of new engineered nanomaterials in zebrafish. Brundo MV, **Pecoraro R**, Marino F, Salvaggio A, Tibullo D, Saccone S, Bramanti V, Buccheri MA, Impellizzeri G, Scuderi V, Zimbone M, Privitera V. *Frontiers in Physiology,* 2016, 7:130.
- Modification of graphene oxide by laser irradiation: a new route to enhance antibacterial activity. Buccheri MA, D'Angelo D, Scalese S, Spanò SF, Filice S, Fazio E, Compagnini G, Zimbone M, Brundo MV, **Pecoraro R**, Alba A, Sinatra F, Rappazzo G, Privitera V. *Nanotechnology,* 2016, 27(24):245704.
- Toxic effects of zinc chloride on the bone development in *Danio rerio* (Hamilton, 1922)**.** Salvaggio A, Marino F, Albano M, **Pecoraro R**, Camiolo G, Tibullo D, Bramanti V, Lombardo BM, Salvatore Saccone, Mazzei V, Brundo MV. *Frontiers in Physiology-Aquatic Physiology*, 2016, 7:153.

Abstracts/Posters

- Toxic effects caused by a long-term exposure of *Danio rerio* to TiO_2 nanoparticles. **Pecoraro R**, Camiolo G, Droutsa A, Tibullo D, Buccheri MA, Impellizzeri G, Brundo MV, Marino F, Privitera V. *Frontiers Marine Science*, Conference abstracts ECI XV, Porto (Portugal) published on 27 November 2015.
- Valutazione della tossicità di GO E rGO sugli stadi larvali di zebrafish. **Pecoraro R**, Camiolo G, Marino F, Di Caro G, D'angelo D, Scalese S, Buccheri MA, Brundo MV, Privitera V. In Atti XXI Congresso Nazionale SIPI 07-08 ottobre 2015, Chioggia.
- Toxicity of engineered nanoparticles in *Mytilus galloprovincialis.* Brundo MV, **Pecoraro R**, Mazzei V, Ferrante M, Zimbone M, Grimaldi MG, Buccheri MA, Impellizzeri G, Privitera V. IEEE-NMDC 12-15 ottobre 2014, Acicastello (CT) Italy.
- Toxicity evaluation of gold, silver and titanium dioxide nanoparticles in *Artemia salina* larvae. Brundo MV, **Pecoraro R**, Mazzei V, Zimbone M, Grimaldi MG, Buccheri MA, Romano L, Privitera V. IEEE-NMDC 12-15 ottobre 2014, Acicastello (CT) Italy.
- Lo Z-TEST per la valutazione della tossicità di nanoparticelle di biossido di titanio. Marino F, **Pecoraro R**, Brundo MV, Puglisi F, Pappalardo H, Zimbone M, Buccheri MA, Impellizzeri G, Reina V, Di Caro G, Privitera V. In Atti XX Congresso Nazionale SIPI 18-19 Settembre 2014, Pisa.
- Valutazione della tossicità di nanoparticelle di oro, argento e biossido di titanio in *Artemia salina*. Marino F, **Pecoraro R**, Brundo MV, Puglisi F, Pappalardo H, Zimbone M, Buccheri MA, Impellizzeri G, Macrì B, Privitera V. In Atti XX Congresso Nazionale SIPI 18-19 Settembre 2014, Pisa.

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