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TITOLO DELLA TESI

Ecotoxicological assessment and comparison between metal and nano-metal oxides in different aquatic organisms: laboratory surveys.

Tesi di laurea in: Adattamenti degli animali all'ambiente marino

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To my Father.

To my Mother.

To my brother, Davide.

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Chapter 1: Introduction

1.1. Marine Ecotoxicity of ENMs

Nanotechnology involves the employment of engineered nanomaterial (ENMs) within products, or processes performed at the nanoscale (generally considered to be 1-100 nm). ENM's are increasingly being used in the production of consumer products and appliances, involving the continued discovery of novel properties exhibited by materials at the nanoscale. Accordingly, pharmaceutical, cosmetic, textile and electronic industries are harnessing the size-related properties of ENMs within a wide range of applications such as medicines, sunscreens, clothes, food and paints, to name a few. It is inevitable that, during their use, ENMs will be released into soils and waters [Valsami-Jones E, Handy RD, Owen R, 2008]. At the same time, the novel size-related behavior of these materials may also induce new forms of toxic response [Rosenkranz P, Chaudhry Q, Stone V, Fernandes TF; 2009]. There is therefore increasing concern over the potential impacts of ENMs in the environment on aquatic and terrestrial organisms and on human health.

As such, in order to support innovation within the emerging field of nanotechnology, but also minimize associated risks, it is necessary to address the potential adverse impacts of ENMs to well selected relevant organisms. While the effects of most of the metal oxides employed in engineer has been proved, only a range of ecotoxiclogical effects have been reported to date, including effects on microbes, plants, invertebrates and fish, for ENMs. Although a substantial body of work now exists, outcomes are often inconsistent and there remains a significant need for assessing toxicity within the sediment environment, where ENMs will ultimately accumulate.

In testing ecotoxicity of ENMs there are still many topics that need clarification or improvement such as the mechanism of the observed toxicity, the concept of safer ENM designs, the elaboration of an optimum test battery and an assessment strategy. When optimizes test systems become available, it will possible to test ENMs systematically. If a

potential risk by a specific ENM in identified, a risk-benefit analysis can be performed and, if required, risk reducing measures can be taken [Hund-Rinke K, Simon M, 2006].

This project aims to obtain key data on the ecotoxicolgy of ENMs in marine organisms. For this, four metal and nano-metal oxides were selected: Titanium dioxide (TiO_2), Zinc oxide (ZnO), Copper oxide (CuO) and Cerium dioxide (CeO_2).

To get this aim, two main objectives should be reached:

1. Study of the contamination by the metals and nano-metals in selected marine organisms (Fig. 1.1): *Paracentrotus lividus* (A), *Ampelisca brevicornis* (B) and *Ruditapes philippinarum* (C).

Figure 1.1. Species used for the ecotoxicological experiments: P.lividus (A; www.Wikipedia.org), A.brevicornis (B; www.flickr.com) and R. philippinarum (C; www.fegi.ru).

2. Making a comparison of the responses obtained between the metals in "nano form" and those in "no-nano form".

The various experiments are been conducted in the laboratory.

The following paragraphs explain which metals were used in the different experimental bioassays and the main characteristics will be identified, as well as its uses in nature.

Nowadays, the most investigated ENPs (Engineered Nano-Particles) are **TiO2** (31%), Ag (12%), **ZnO** (11%), **CuO** (6%), C60 (5%), Au (3%), **CeO2** (3%). The metals used in this study, its properties ad uses, will be described.

1.2. Titanium

Titanium is a chemical element with symbol “Ti” and it’s the ninth-most abundant element in Earth’s crust (0.63% by mass) and the seventh-most abundant metal in general.

The element occurs within a number of mineral deposits, principally Rutile and Ilmenite, which are widely distributed in the Earth’s Crust and Lithosphere, and is found in almost all living thing, rocks, water bodies and soils. The most common compound, Titanium dioxide (TiO_2), is a popular photo-catalyst and it’s used in the manufacture of white pigments [Buettner KM *et al*., 2012].

Titanium metal and its alloys oxidize immediately upon exposure to air. Titanium readily reacts with oxygen at 1,200 °C in air and 610 °C in pure oxygen, forming Titanium oxide. However, it is slow to react with water and air at room temperatures because it forms a passive oxide coating that protects the bulk metal from further oxidation.

Figure 1.2. Titanium dioxide; it.wikipedia.org.

The concentration of Titanium is about 4 picomolar in the Ocean. At 100 °C the concentration of Titanium in water is estimated to be less than 10^{-7} M at pH 7.

The identity of Titanium species in aqueous solution remains unknown because of it’s low solubility and the lack of sensitive spectroscopic methods, although only the 4^+ oxidation state is stable in air. No evidence exists for a biological role, although rare organisms are known to accumulate high concentrations of Titanium.

Because it is highly resistant to corrosion by seawater, Titanium is been used to make propeller shafts, rigging and heat exchanges in desalination plants, heater-chillers for saltwater aquariums, fishing line, leader and diver’s knives.

Nanoscale Titanium dioxide is been manufactured for specific applications:

1. UV-resistant material, chemical fiber, plastics, printing ink, coating, packing material;

2. Photocatalyst, self-cleaning glass, self-cleaning ceramics, antibacterial material, air purification, sewage treatment, chemical industry;

3. Cosmetics, sunscreen cream, natural white moisture protection cream, beauty and whitening cream, face cream, moistening refresher, vanishing cream, skin protecting cream, face washing milk, skin milk, powdered make-up;

4. Coating for papermaking industry: used for improving the impressionability and opacity of the paper and used for producing Titanium, ferrotitanium alloy, carbide alloy etc. in the metallurgical industry;

5. Astronautics industry,

6. Food industry, as a whitening material.

To achieve better dispersion properties and ensure photostability, these TiO2, moreover, are coated with other materials, such as polymers. Because of its many uses and its various forms, two commercial nano-TiO2 were chosen: an uncoated and a PVP- coated form.

1.3. Zinc Oxid and ZnO/CeO2 mixture

- **Zinc oxide** is an inorganic compound with the formula ZnO.

Figure 1.3. Zinc oxide; it.wikipedia.org.

Zinc oxide is a white powder that is insoluble in water, and it is widely used as an additive in numerous materials and products including rubbers, plastics, ceramics, glass, cement, lubricants, paints, ointments, sealants, pigments, foods, batteries, ferrites, fire retardants, and first-aid tapes [Hernandez-Battez A *et al.*, 2008].

Although it occurs naturally as the mineral "Zincite", most Zinc oxide is produced synthetically [De Liedekerke M, 2006].

Zinc is a wide-bandgap semiconductor of the II-VI semiconductor group. The native doping of the semiconductor due to oxygen vacancies or Zinc interstitials is n-type. This semiconductor has several favorable properties, including good transparency, high electron mobility, wide bandgap, and strong room temperature luminescence.

Crystalline Zinc oxide is thermochromic, changing from white to yellow when it is heated in air and reverting to white on cooling. This color change is been caused by a small loss of oxygen to the environment at high temperatures.

Zinc is an amphoteric oxide: it is nearly insoluble in water, but it is soluble in (degraded by) most acids.

Zinc decomposes into Zinc vapor and oxygen at around 1975 °C with a standard oxygen pressure. In a carbothermic reaction, heating with carbon converts the oxide into Zinc vapor at a much lower temperature (around 950 °C).

Nanostructures of Zinc can be synthesized into a variety of morphologies including nanowires, nanorods, tetrapods, nanobelts, nanoflowers, nanoparticles.

Nanostructures can be obtained with most above mentioned techniques, at certain conditions, and with the vapor-liquid-solid method [Baruah S *et al*., 2009; Miao L *et al.*, 2007; Xu S *et al.*, 2011]. The synthesis is typically carried out at temperatures of about 90 °C, in an equimolar aqueous solution of Zinc nitrate and hexamine, the latter providing the basic environment. Certain additives, such as polyethylene glycol or polyethylenimine, can improve the aspect ratio of the ZnO nanowires. Doping of the ZnO nanowires has been achieved by adding other metal nitrates to the growth solution.

The morphology of the resulting nanostructures can be tuned by changing the parameters relating to the precursor composition (such as the Zinc concentration and pH) or to the thermal treatment (such as the temperature and heating rate) [Elen K *et al.*, 2009].

Figure 1.4. Cerium oxide; it.wikipedia.org.

- **Cerium (IV) oxide**, also known as "ceric oxide", "ceria", "cerium oxide" or "cerium dioxide", is an oxide of the rare earth metal Cerium. It is a pale yellow-white powder with the chemical formula CeO_2.

It is an important commercial product and an intermediate in the purification of the element from the ores.

The distinctive property of this material is its reversible conversion to a nonstoichiometric oxide.

Cerium occurs naturally as a mixture with other rare earth elements in its principal ores “Bastnaesite” and “Monazite”.

After extraction of the metal ions into aqueous base, Cerium is separated from that mixture by addition of an oxidant followed by adjustment of the pH.

This step exploits the low solubility of CeO_2 and the fact that other rare earth elements resist oxidation.

To determine the possible synergic effect and changes in the solubility, aggregation and stability of two ENM's, a commercial uncoated ZnO NP and a metal hybrid uncoated CeO2/ZnO NP's were selected. ZnO and a mixture of ZnO/CeO_2 were used to compare the non-nano size effects.

Nano-ZnO exhibits antibacterial, anti-corrosive, anti-fungal and UV filtering properties, so their applications are numerous:

- medicine
- cosmetics
- anti-corrosive coating paints

Besides various industrial applications, CeO2 NPs was, recently, found to have multi enzyme mimetic properties, including superoxide-oxidase, catalase and oxidase which produce various biological effects, such as being potentially antioxidant towards almost all noxious intracellular reactive oxygen species.

CeO2 NPs have emerged as a fascinating and lucrative material in biological fields such as bioanalysis, biomedicine, drug delivery and bio-scaffolding. It is also used in the walls of self-cleaning ovens as a hydrocarbon oxidation catalyst during the high-temperature cleaning processes.

They, also, have the potential to protect astronauts from long term exposure to radiation in Space and, perhaps, even slow the effects of aging and as a prospective replacement to Zinc and Titanium dioxide NP's in sunscreens, as it has lower photo-catalytic activity but the use of these nanoparticles in cosmetic is still uncertain as they can penetrate the body and reach internal organs, so further studies are necessary to establish safety levels.

1.4. Copper

Copper oxide or **cupric oxide** is the inorganic compound with the formula CuO.

A black solid, it is one of the two stable oxides of Copper, the other being Cu_2O. As a mineral, it is been known as "Tenorite" and "Paramelaconite".

Figure 1.5. Copper oxide; it.wikipedia.org

It is a product of copper mining and the precursor to many other copper-containing products and chemical compounds. Copper oxide is an amphoteric oxide, so it dissolves in mineral acids such as hydrochloric acid, sulfuric acid or nitric acid.

As a significant product of copper mining, copper oxide is the starting point for the production of other copper salts. For example, many wood preservatives are been produced from Copper oxide.

Cupric oxide is been used as a pigment in ceramics to produce blue, red, and green, and sometimes gray, pink, or black glazes. It is been incorrectly used as a dietary supplement in animal feed. Due to low bioactivity, negligible Copper is absorbed.

Engineered nano-sized Copper oxide particles (CuO NP) are commonly used as bacteriocides and have the potential to replace noble metal catalysts for carbon monoxide oxidation [Zhou K *et al.*, 2006]. CuO NP suspensions (nanofluids) have excellent thermal conductivity and are used as a heat transfer fluid in machine tools.

The nano-CuO was suggested as one of the most potent regarding toxicity in marine environmental.

Nanoparticles of CuO were identified has being important in ecotoxicological assays due to its relatively low dissolution rate but its potentially high toxicity towards organisms [Stone V *et al.*, 2010].

The key applications of CuO NPs are the following:

- doping materials in semiconductors
- chemical sensors
- efficient anti-micro-bacterial agent

- biological applications
- a good catalyst for different cross coupling reactions

1.5. General Applications and Uses in Marine Biology

It have been considering just these metals because they have a certain application in Marine Biology, especially for some marine organisms as those with high ability to absorb these metals or directly from the surrounding water or by indirect means, by eating contaminated food. These metals were been investigated by other scientists and are strongly known and discussed in many articles.

In recent years, they are exploring new fields including their effects in the form of nanoparticles on the environment and especially on marine organisms.

For this reason, it was been texted the contamination of these metals on 3 different types of marine organisms: *A. brevicornis*, *P. lividus* and *R. philippinarum* have been investigated.

The literature about the ecotoxicity of NPs is still emerging, and there have been several reviews on the ecotoxicity of manufactured NPs and NMs [e.g., Oberdorster E *et al.* 2006; Crane M and Handy RD, 2007; Moore MN, 2006]. At this early stage, most ecotoxicological studies have been observational or ‘‘proof of principle’’, experiments that have tried to document toxic effects and the concentrations of NPs that produce these effects in different groups of organisms.

However this is a somewhat arbitrary definition, and for ecotoxicology, it should be also consider the NMs and its properties, as distribution of particle sizes, solubility or aggregation [Handy RD and Shaw BJ 2007; Handy RD *et al.* 2008].

Nanotechnology uses nanomaterials that are most commonly defined as having at least one dimension between 1 and 100 nm [OECD, 2009], and manufactured nanomaterials may take the form of a single nanoparticle (NP), wire, or thin-walled tube. Over a thousand consumer products are currently listed to contain NPs, including sunscreens, paints, semiconductors and cosmetics [PEN, 2012].

As the use and prevalence of these particles inevitably increases, so their discharge into freshwater and marine environments will increase too. The OECD (Organization for Economic Co-operation and Development) highlighted four metal and metal oxide [Me(O)] NPs as high interest due to their inherent properties, widespread use and commercial importance, namely Cerium oxide (CeO2), Silver (Ag), Zinc oxide (ZnO) and Titanium dioxide (TiO2). Widely used as a fuel additive in diesel engines (Envirox) to reduce particulate emissions [Park et al., 2008] as purifiers of Mischmetal, and in heat-resistant coatings [EPA, 2009a].

The application of these materials (i.e., nanotechnology) is also relatively new. Clearly, the scientific debate on the environmental safety of NMs needs to adopt a multi-disciplinary approach involving physicists, chemists, material scientists, biologists, toxicologists, risk assessors, regulators and policy makers. In order to have such a debate, the Society for Environmental Toxicology and Chemistry-UK branch (SETAC-UK) recently organized a meeting called the ‘‘2nd International Conference on the Environmental Effects of Nanoparticles and Nanomaterials’’ (hosted by the Natural History Museum in London) to bring this diverse group of professionals together.

With an accurate design and implementation of a series of bioassays, the evaluation of these selected ENM's will be possible, as well as the establishing of evaluation protocols for other ENM's with similar composition and characteristics.

Increasing use of metal and metal oxide nanoparticles [Me(O)NPs] in products means that many will inevitably find their way into marine systems. Their likely fate here is sedimentation following hetero aggregation with natural organic matter and/or free anions, putting benthic, sediment dwelling and filter feeding organisms most at risk. In marine systems, Me(O)NPs can absorb to micro-organisms with potential for trophic transfer following consumption.

Currently, environmentally realistic Me(O)NP concentrations are unlikely to cause significant adverse acute health problems, however sub-lethal effects e.g. oxidative stresses have been noted in many organisms, often deriving from dissolution of Copper or Zinc ions, and this could result in chronic health impacts. Ecotoxicologists often need to handle test materials in some kind of liquid or solution phase in order to perform exposures or administer a dose. However, NPs do not necessarily dissolve in solution, but may form a colloid

dispersion. The phase ‘‘colloid’’ applies to particle sizes or suspensions of material in the 1 nm–1 lm size range [Lead JR and Wilkinson KJ, 2006].

In colloid chemistry, particles may remain dispersed, or alternatively, aggregation processes may remove the material from the liquid phase. Thus, the aggregation in seawater is more likely than in freshwater, and the pH of the water, may influence the aggregation rate depending on the surface charge of the particles involved.

In the last decade, aquatic ecotoxicological studies about ENP effects grew rapidly, stressing more on freshwater rather than salt water or terrestrial species [Corsi I *et al*., 2014; Libralato G, 2014; Minetto D *et al*., 2014; Libralato G *et al*., 2016; Lofrano G *et al*., 2016; Vale G *et al.*,2016]. Salt water is a complex matrix pushing ahead ENP instability and promoting the rapid formation of agglomerated/precipitated forms [Callegaro S *et al.*, 2015]. Despite the huge number of papers, their nano-eco-safety is still fragmentary, and the comparison of multiple studies can be difficult, since experimental designs and testing conditions are rarely consistent across studies [Salieri B *et al*., 2015].

There are many types of NMs and the scientific community is making observations on NP ecotoxicity to inform the wider debate about the risks and benefits of these materials. Similarly, knowledge of the ecotoxicology of NPs to bacteria and other microbes is limited, even though some manufactured NPs have been designed as cleaning agents with antibacterial properties [e.g., Titanium and Silver NPs, Fu GF *et al*. 2005; Duran N *et al.* 2007].

Natural NPs have been generated by a wide variety of geological and biological processes and while there is evidence that some natural NPs can be toxic, organisms have been, also, evolved in an environment containing natural NPs. There are concerns that natural nano-scale process could be influenced by the presence of pollution. However, much of the ecotoxicological data is limited to species used in regulatory testing and freshwater organism. Data on bacteria, terrestrial species, marine species and higher plants is particularly lacking. From these studies, results have highlighted a range of sub-lethal effects including reduced swimming [Asghari S *et al.*, 2012], reduced growth and reproduction [Zhao CM and Wang WX, 2011], bioaccumulation [Rosenkranz P *et al*., 2009], digestive stress and reduced feeding [Croteau MN *et al*., 2011a, 2011b]. Despite extensive research on freshwater species, little study was been directed towards marine organisms.

Use of Titanium dioxide and Zinc oxide NPs in sunscreens [Wahie S *et al.*, 2007] means a major source of these materials in the marine environment is people as they enter the sea. On average 25% of sunscreen will be washed-off on immersion [Danovaro R *et al.*, 2008], and estimates indicate the potential for some 250 tonns of sunscreen-originated NPs to enter the marine environment each year [Wong SW *et al.*, 2010]. Examination of TiO2 (27 nm), CeO2 (rods 67- 8 nm) and ZnO NPs (24 nm) in Sea Water has shown a positive correlation between NOM (Natural Organic Matter), sedimentation and ionic strength [Keller AA *et al.*, 2010].

In seawater higher concentrations of NPs (>100 mg/L) show significantly faster sedimentation than seen for lower concentrations (10 mg/L). Agglomeration speed of NPs in seawater is been related to concentration and NOM and NPs used in toxicity tests are likely to quickly form large aggregates.

Instead, both the particles and any dissolved metal ions may be highly complexed by NOM and remain in suspension as they move toward the ocean.

Further incorporation into larger homo and hetero aggregates leads to faster sedimentation, and this places benthic organisms most at risk to NOM-coated NPs.

Chapter 2: *Materials and Methods*

2.1. Acute Bioassays

Bioassays allow the detection of these effects by measuring the biological responses of marine organisms, particularly in their highly sensitive early life stages.

The biological assays or toxicity tests are carried out on exposed organisms, under standardized and controlled conditions, to a substance, to a mixture of substances or in an environmental sample. The end-point of these tests are:

-Enhancement

-Mortality

-Reproduction

Moreover, these bioassays can be prepared either in the laboratory or in the field.

There are two types of biological assays: Acute and Chronic.

In the first part of this chapter, it will mainly deal with the Acute Bioassays and analyze in detail those concerning the sea urchins on toxicity tests of *Paracentrotus lividus* species.

Acute bioassays have a shorter duration of the experiment compared to the longevity of the analyzed species.

Sea urchins are widely used for this purpose due to the easy of obtaining gametes and in vitro fertilization. Sea urchins larvae are planktonic organisms living in the water column, so this bioassay must be conducted in a liquid phase.

2.1.1. ACUTE TOXICITY SEA URCHIN TESTS

Environmental managers responsible for assessing the ecological integrity of aquatic resources rely of a number of assessment tools including chemical analysis of water, sediment and tissue; biological assessment and toxicity tests. Toxicity test are an important component for assessing the impact of chemical on aquatic ecosystem because they indicate toxic effects of complex chemical mixtures. In aquatic toxicity test, group of selected organisms are exposed to test materials (water or sediment samples) under defined

conditions to determine potential adverse effects. A number of standardized toxicity test protocols have been developed for determining toxicity of chemicals to aquatic species [Anderson B *et al*., 2004]. Several marine bioassays are already standardized. For instance, for marine water assessment fertilization test or embryo-larval development are used.

The objective of this study is to examine how the concentration and size of NPs affects their toxicity on the health of organisms and the environment by using sea urchin gametes and early embryos [Volpi Ghirardini A *et al*., 2005].

It has been chosen the Mediterranean Sea urchin *Paracentrotus lividus* as a model to investigate the effects of NPs on health. A number of studies have demonstrated the reliability of this model for studying health [Falugi C *et al.*, 2012; Matranga V and Corsi I, 2012]. Thus sea urchins has more than 70% genome homology and metabolic similarity with higher vertebrates, including humans. This model is relevant for ecotoxicological studies in the marine environment because it is representative of both benthonic (adult specimens) and planktonic (embryos and larvae) marine organisms. It also lives in shallow seawater near the coast, the marine sites that are most impacted by human activities. It has been recommended as alternative model in ecotoxicological tests for the implementation of REACH regulation ("Registration, Evaluation, Authorization and Restriction of Chemical substances") [Falugi C *et al*., 2012].

Figure 2.1. El Puerto de Santa Maria, Cadiz, Spain. Sampling area for the Sea Urchin toxicity test.

Mature specimens of *P. lividus* were obtained from a no-contaminated site in the area of "El Puerto de Santa Maria", Cadiz (Fig. 2.1). The sea urchins were collected directly in the field, making sure to remove such a number as to have a sufficient number of males and females. The sea urchin were taken to the laboratory in a properly box under controlled temperature to avoid undesirable spawn of gametes. In the laboratory, the sea urchin were directly dissected and subsequently their gametes (fertilization test) and embryos (development test) were exposed

to filtered clean sea water contaminated with 4 different types of nano and no-nano metals at different concentrations.

A serial dilution of each metal and nano-metal was done in order to test their effect in the fertilization and early stages of development of the sea urchin *P. lividus*. The selected metals that were used in these two test are those described in first chapter:

1. Zinc (ZnO)
2. Titanium (TiO_2)
3. Copper (CuO)
4. Cerium (CeO_2)

The first three metals have been used as a singles metal oxides, while Cerium oxide was used to form a mixture with Zinc oxide. Although TiO_2 (normal size) is chemically inert, TiO_2 NPs can be activated because of their phototoxic effects. [Miller R *et al*., 2012].

The normal metal oxides were purchase to Sigma-Aldrich® while nano-metal oxides were provided by Promethean Nanoparticles Ltd.

The selected concentrations were these five:

1. **0,001 mg/L**
2. **0,01 mg/L**
3. **0,1 mg/L**
4. **1 mg/L**
5. **10 mg/L**

Starting from the highest concentration, it has been taken a known amount of metal oxide and, using pipettes, the liquid was poured in Eppendorf at lower and lower concentrations to obtain dilutions desired. Only at this point it is possible to proceed with the experiment.

For both bioassays, filtered (0,020 μm) clean sea water (see annex for chemical characterization) was used as control and to make the dilutions. Furthermore, before being used, the salinity of the filtered control sea water was lowered at a value of S =35‰. In addition to salinity, there are others factors that influence this type of experiment, as temperature, dissolved oxygen, pH, etc, were controlled. Each test was run twice at least to guaranty results.

For both bioassays, 5 replicates were done for control and all the five concentrations listed above of each metal and nano metal.

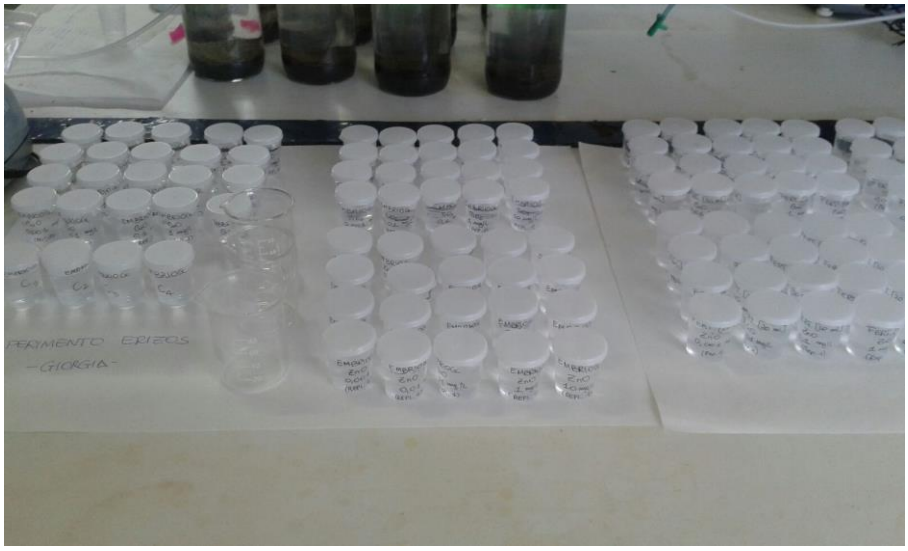


Figure 2.3. Experimental design samples of sea urchin 20 mins Fertilization. The figure shows a group of 15 samples for each metal with increasing concentrations and the control.

➢ Fertilization

Mature specimens of *P. lividus* were collected from the Puerto of Santa Maria (Cadiz), transported to the wet laboratory of the Sea and Environmental Sciences Faculty of the University of Cadiz, where they were maintained in filtered seawater until their use in the bioassays. Adult samples were brought to the laboratory in a refrigerated box, wrapped in tissues wet with seawater from the collected area.

Figure 2.4. Female specimen of *P. lividus* that is emitting the eggs (red circle)

To carry on the fertilization bioassay, several sea urchins were dissected in order to collect eggs and sperm, respectively from females and males. The eggs were been placed in a glass cylinder with clean seawater, suitably filtered.

Sperm, instead, was extracted and added to an Eppendorf tube and kept dry on ice because otherwise could degrade. The gametes were used in less than one hour once they were extracted.

A mix of at least the gametes of three males and three females was used to guaranty the variability. After checking the quality of the eggs and the sperm, the best were chosen and mix in a pool of sperm and a pool of eggs.

When the sperm was collected in sufficient quantities, 10 μL were taken and added to each test tubes containing filtered seawater and metal at a specific concentration (NP and no-nano).

The sperm was contaminated for 1 hour while, in the meantime, the eggs were analyzed under a microscope and their density was calculated to put the optimal concentration of 30 eggs/mL approximately. After the eggs density is adjusted, the eggs were taken from the glass cylinder and added to the samples vials containing only filtered clean sea water (10 mL per vial).

While the eggs were being added to the samples, the glass cylinder had to be appropriately agitated because the eggs tend to decant with the risk to withdraw with the pipette only water or an inappropriate density of eggs.

After an hour had passed from sperm's exposure to metals, the contaminated sperm was added to no-contaminated eggs in the samples vials of the experimental set containing eggs (in the density of 30 eggs/mL) and 10 mL of clean seawater.

Later, the eggs on contact with contaminated sperm are been fixed after 20 min after fertilization, time enough to let the fertilization in case the sperm is viable.

The fixation process is been occurred by adding 0.5 mL of Formaldehyde at 40% in each replication of each metal concentration.

Fertilization test was performed to evaluate the following aspects: the presence/absence of fertilization membrane measured in 200 individuals counted randomly under inverse microscope, expressed as percentage, in order to calculate how much each metal had effects on fertilization, also depending on the concentration.

➢ Development

The eggs and sperm were extracted by the same process, direct dissection, which involved the fertilization with the only difference that, in this case, the extract sperm from males curly has not been exposed to metals for a certain time before being added to the eggs not contaminated, but an "*in vitro*" fertilization was done.

For this aim, a drop of sperm, kept on ice, was added directly to the eggs immersed in filtered clean seawater inside the glass cylinder.

After a gentle shaking, 3 drops of 10 μL were extracted and placed on the microscope slide inverted so as to further control the density of the eggs and even if fertilization had occurred: in that case it would be noticed a transparent double membrane around the egg cell.

Samples vials, containing 20 mL of each concentration metal per 5 replicates were already prepare and fertilized eggs were add in a 30 eggs/mL optimal density.

Even in this case, every time the eggs were added to the samples, the glass cylinder had to be appropriately agitated in order to avoid the decantation of the eggs and the risk to withdraw the pipette with an incorrect eggs density.

The samples should be incubated under controlled laboratory conditions, as shown in the figure below (Fig. 2.2).

Parameter	Conditions
Temperature	20ºC
Salinity	33-35 ppt
Photoperiod	darkness
Water renew	None
Organisms conditions	Check quality of biological material (eggs and sperm)
Incubation density	20-30 fertilized eggs / mL
Replicates	5
Water quality	Check temperature, Salinity, pH and dissolved oxygen (sulphurs and ammonium if necessary)
Incubation period	48 hours
Biological response: endpoints	Embryogenesis success, registered as percentage of normal pluteus (see figure 1)
Observations per replicate	100

Figure 2.2. Optimal conditions to conduct the bioassay with embryos of *Paracentrotus lividus* sea-urchin.

These samples were always fixed with 0.5 mL of formaldehyde to 40% after 48 h after fertilization.

As regards the larval growth had made reference to a table in which are recorded the main stages of growth normally found in nature, as in the figure 2.5.:

Figure 2.5. Embryological development of *P. lividus* sea-urchin in relation to the concentration of the toxic element; PhD Thesis of Nuria Fernandez Rodriguez (2002).

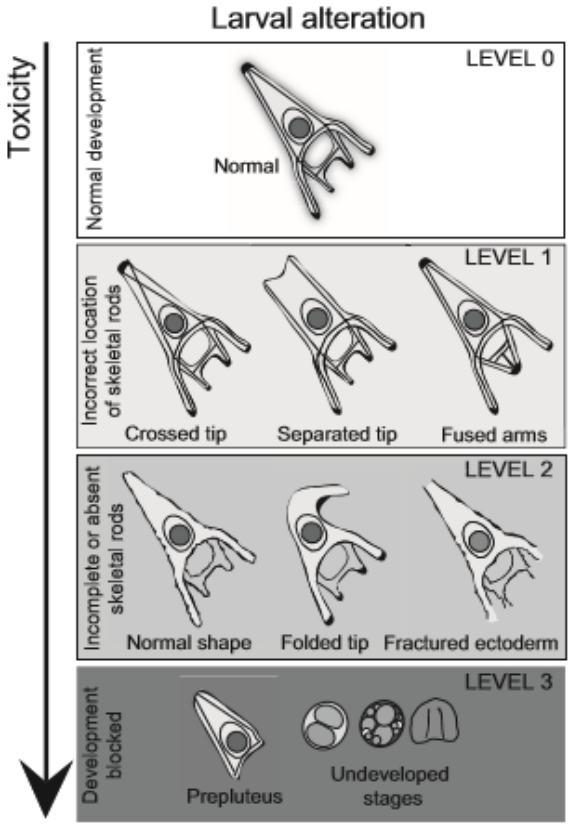


Figure 2.6. [Carballeira C *et al*, 2011]. Classification of larval malformations according to degree of alteration, in order to establish the severity of toxicity.

Instead, to recognize malformations had referred to a further table, below, where you can recognize the most common malformations of exposure to metals.

The figure shows four Levels:

Level 0 it represents a larva properly formed;

Level 1 it depicts larvae that don't have a high level of defect but have only peculiarity in the spicules;

Level 2 represents larvae with a higher level of malformation in which the spicules not have been formed;

Finally (**Level 3**) it can see the most contaminated forms in which it does not have a ghost, but you may find the lower-level growth stages ranging from Morula to the Pre-Pluteus [Carballeira C *et al*., 2011].

2.1.2. CHARACTERIZATION OF METAL OXIDES FOR Paracentrotus lividus BIOASSAYS

In order to characterize the nano-metals, Transmission Electron Microscopy (TEM) samples were prepared for all the nano-metal oxides.

TEM samples were prepared by partially drying a drop of the NPs solution or the NPs in seawater on a copper mesh 400 holey carbon film (Agar scientific) at room temperature [Romer I *et al.*, 2013].

The grid was then carefully washed several times with UHP water and re-dried. Images were obtained using a JEOL 1200EX (accelerating voltage 80 kV), and recorded using Gatan Digital Micrograph software. Energy dispersive X-ray spectrometer (EDX) was measured with a JEOL 2100 200 kV LaB6 TEM with Oxford INCA EDX.

Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES) analysis was carried out with Optima 8000 (Perkin Elmer). The concentration of the different NPs in seawater was measured using a simulated exposure (no organisms present), for dissolution at different times as well as total concentration. Methods for the analysis of zinc, copper, zinc/ceria and titanium at different concentrations were set up and utilized accordingly. Analysis took place using radial detection for the particles with a concentration higher than 1ppm and axial detection for samples with concentrations on the ppb range, and a 70 second delay time. The final result was the average of three replicate readings that were obtained for each sample analyzed.

For dissolution measurements at each sampling point 5ml of the NPs in seawater were taken and put directly into Amicon 15 centrifugal filtration unit (sourced from Millipore, with regenerated cellulose acetate membrane with 3kDa cut off value and PP filter housing, maximum filtration volume 10ml). The cap was closed and the units were placed in a centrifuge (Eppendorf 5804-R). The units were spun for 30min at maximum speed which for this centrifuge was 5000 rpm. The membrane hosting unit was removed after spinning the samples and the filtrate was poured into a clean ICP-OES tube. Concentrated HNO_3 acid was added to obtain a 2% final acid concentration. For the total concentration of the NPs in seawater, a sample was taken at the end of the exposure and 20% HNO3 was added and left for 24 hours. For ICP-OES measurements the concentrated acid solution was diluted to obtain a 2% acid concentration. To dissolve the TiO2 NPs in seawater a 3:1 solution of 70% HNO_3 and H_2O_2 was used and later diluted [Osborne OJ *et al*., 2016].

2.2. Chronic Bioassays

The ecotoxicologists are constantly looking for new "improvements" assays, which allow that to obtain more realistic information and ecologically relevant, possibly by a significant and concomitant savings in time and money. An example of improvements in scientific field of application is been given by the bioassays.

Bioassay (commonly used shorthand for biological assay or assessment), or “biological standardization” is a type of scientific experiment. A bioassay involves the use of live animal or plant (*in vivo*) or tissue or cell (*in vitro*) to determine the biological activity of a substance, such as a hormone or drug. Bioassays are, typically, conducted to measure the effects of a substance on a living organism and are essential in the development of new drugs and in monitoring environmental pollutants. Both are procedures by which the potency or the nature of a substance is been estimated by studying its effects on living matter. Furthermore, a bioassay can be used to determine the concentration of a particular constitution of a mixture that may cause harmful effects on organisms or the environment.

Chronic bioassay is been defined as such if it covers the entire life cycle of the organism or at least a part of it. As in acute bioassays, they can be developed for different substances or mixtures and various substrates such as water and sediment, among the most relevant.

Water and sediment are excellent substrates for the bioaccumulation analysis for in the chronic bioassays.
The major chemical components of produced water are all natural products that occur at low concentrations in seawater, marine sediments and the tissues of marine organisms. The form of a chemical in the environment has a marked effect on the extent to which it can be taken up into the tissues of organisms and on the extent to which it can interact with the tissues to cause various harmful biological effects in the organisms themselves and their consumers [Jerry M.Neff, 2002].
The presence of some heavy metals in ecosystems (including seas and oceans) can have deleterious effects because:

1. They do not degrade and have long half-lives.
2. They may bioaccumulate in living tissues, giving rise to symptoms of toxicity.

Heavy metals are poorly soluble in water, tending to absorb onto suspended particulate matter in the sea and affect marine organisms.

Toxic effects do not normally manifest themselves immediately after the toxin enters the environment and organisms [Danis B *et al*., 2006].

The duration of an organism's exposure period, the amount of a metal deposited in an organism may be considerable [Radenac G *et al*., 2000].

The vast majority of H-M is stored in tissues and organs. The bioaccumulation factor (BAF) is characterized by total amount of contaminant that has entered the organism by all possible pathways, for example via food intake, respiratory pathways, penetration through the skin [Deforest DK *et al.*, 2007].

Anthropopressure on the sea's resources is increasing and can influence bioaccumulation in the tissues of marine organisms.

In order to determine biological and chemical effects in these bioassays, it' necessary have a "good test". A good test, be it Chronic or Acute, is defined as such only if it meets certain characteristics. Among the most important in the scientific world are found:

- Rear organisms (or readily available)
- Simple
- Not so expensive
- Short term
- Standardized
- Sensible and Discriminating
- Precise
- Reproducible
- Significant from an ecological point of view

Chronic bioassays are so defined in that they provide a greater duration of the experiment than acute. Among the chronic bioassays that exist in the scientific literature, the experiments that will be reported in the following paragraphs relate to two particular species of marine organisms: *Ampelisca brevicornis*, amphipod, and juvenile (12 mm average long length) of *Ruditapes philippinarum* clam. Both bioassays were done twice time to guaranty the obtained results.

2.2.1. TOXICITY TEST WITH AMPHIPODS

Amphipods are widely used for whole-sediment toxicity assessment because they are sensitive indicators for sediment pollution. In addition, amphipods are abundant, widely distributed and ecologically important in estuarine and marine benthic communities.

On the other hand, the main exposition route for some chemical substances is the liquid phase (interstitial water or pore water), and its concentration will be determined by the substance affinity to the solid phase (mainly to the organic carbon).

The toxicity test is been conducted with the species of *A. brevicornis* for texting its response to metals, nano and no-nano size.

At first, amphipods and sediment, substrate conducive to their survival, were been taken a clean area from the Cadiz Bay (see annex for sediment analytical characterization).

Amphipods collected at filed were brought to the laboratory where they were acclimated at least for one week under laboratory controlled conditions.

An aquarium was prepared with the sediment collected from the same area than amphipods and filtered-clean seawater. Initial salinity was adjusted from 40‰ to 35‰ to recreate the same living conditions of organisms, as if they were still in their natural environment.

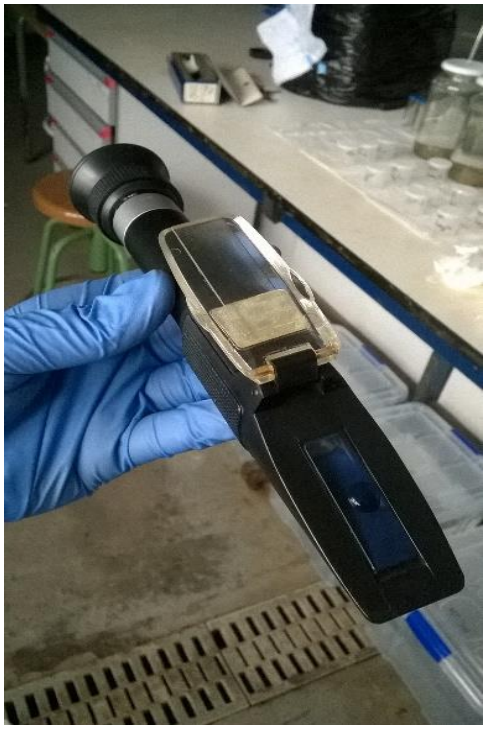


Figure 2.7.The refractometer, an instrument for testing Salinity of Sea Water

The salinity was been adjusted using a tool called "refractometer" (Fig.2.7) in which was placed a drop of water to be tested and, looking in the direction of the light, it was observed a graduated indicator showing the salinity of the water sample.

If the salinity was too high compared to the value that you wanted to get then had to be added to distilled water, low in salts. In case where the salinity is too low it had to add seawater so that the value came to that desired.

Once the aquariums for acclimatization were ready with sediment from the collection area, filtered clean seawater and aeration to ensure the saturation of oxygen, the amphipods were inserted into them, and

were left in the acclimatization to the laboratory conditions for 7 days. During the acclimatization period, the amphipods were fasted.

This toxicity test was characterized by specific parameters, listed in the following table (Tab. 2.1):

Table 2.1. Parameters and conditions to develop the test using crustacean amphipods in the laboratory.

Parameters	Conditions
1. Test type	Static; on whole sediment
2. Temperature	15–20°C (depending on the species)
3. Salinity	32–40
6. Photoperiod	Natural of the season; also continuous light
7. Test chambers	Glass, 2 L (recommended cylindrical and covered)
8. Volume of sediment	250 mL (or 1:4 sediment/water)
9. Volume of overlying water	1 L (or 1:4 sediment/water)
10. Water renewal	No
11. Size and state of organisms	*Ampelisca brevicornis*, 3–5 mm; *Corophium volutator*, 5 mm or larger; *Mycrodeutopus gryllotalpa*, 5 mm or larger; *Corophium multisetosum*, 10 mm
12. Number of organisms per chamber	20
13. Number of replicates	3–5
14. Feeding regime	No
15. Aeration	12 h before introducing the organisms, to ensure dissolved oxygen concentrations equal or higher than 90% saturation
16. Overlying water	Clean seawater
17. Water quality	pH, ammonia, salinity and dissolved oxygen at the beginning and at the end of the test
18. Test duration	10 days
19. Endpoints	Survival
20. Test acceptability	90% survival in the negative toxicity control

The preparation of the toxicity tests took place several days: initially the experimental design was set up which consisted in the preparation of a Control (C) and 2 metals, each with 4 different concentrations and all with 3 replications each.

The toxicity test with no nano metals, was been conducted using two metals at the concentrations inserted in the Table 2.2.

Table 2.2. Table of metals used in the toxicity test on amphipods. In the table, there are the four concentrations too.

METALS	CONCENTRATION (mg/L)			
ZnO	0,1	0,5	1	1,5
CuO	0,5	1	2,5	5

The samples were thus prepared: in each jar were poured 150 g of dry sediment, weighed directly with the scale and always with the same instrument were added 800 mL of filtered clean seawater and with a salinity of 35‰.

Figure 2.8. Two different images: at left, we can see the balance with 150 gr of sediment; at right we can see the dry sediment.

Subsequently, the metals were added in different quantities according to the indicated concentration, as shown by the following model (Table 2.3 and Table 2.4).

Table 2.3. Zinc table: in the left column lists the four concentrations in which it was carried out the experiment; in the middle column are indicated the amount of metal poured; the last column lists the amount of distilled water. The initial concentration of ZnO was 1%.

ZnO		
CONCENTRATION (mg/L)	**Quantity of Metal**	**Distilled Water**
0,1	80 uL	4,920 mL
0,5	0,4 mL	4,600 mL
1	0,8 mL	4,200 mL
1,5	1,2 mL	3,800 mL

Table 2.4. Copper table: in the left column lists the four concentrations in which it was carried out the experiment; in the middle column are indicated the amount of metal poured; the last column lists the amount of distilled water. The initial concentration of CuO was 1%.

CuO		
CONCENTRATION (mg/L)	**Quantity of Metal**	**Distilled Water**
0,5	80 uL	4,920 mL
1	0,8 mL	4,200 mL
2,5	2 mL	3,000 mL
5	4 mL	1,000 mL

The metal used in no-nano form were first dissolved. They were weighed, with as much precision as possible, 1 gr for each of the two metals by means of the precision balance, and this amount was subsequently dissolved in 1 L of distilled water.

These metals, however, were quite insoluble in water so they were heat and stirring high to facilitate the dissolution.

The same experiment was conduct with the same metals NPs but were purchased in dissolution and ready to use them. The concentrations of metal oxides NPs are shown in the Table 2.5:

Table 2.5. Concentrations of ZnO and CuO NPs used in the amphipod bioassay.

METAL OXIDE NPs	**CONCENTRATIONS mg/L**				
ZnO NPs	0,1	0,5	1	1,5	2,5
CuO NPs	0,5	1	2,5	5	10

Oxygen was continuously supply from 24 h of the start of the bioassay and until the end of the exposure period (Fig.2.9).

Figure 2.9. Image of the aeration system with the complete experiment (bottles, sediment and water).

The amphipods were removed from the aquarium of acclimatization withdrawing the sediment, which has been properly sieved.

For each replication were included 20 amphipods. The selected organism should seem in good health state (they present movement when receive a stimulus and they are able to bury themselves in the sediment). Any amphipods that does not appear to be in good health should be replaced in the first few hours of exposure. By the time they were entered into exposure chambers, officially started the experiment lasting 10 days.

During this time, regularly controls on certain parameters such as salinity, pH and ammonium should be done at days 2nd and 7th of the experiment.

Salinity was tested with the help of the refractometer; however, the pH was tested with the use of the pHmeter, scientific instrument that must first be calibrated with two specific tester and then immersed in water until the screen freezes for a few seconds to a value that will be one indicating the pH effective solution. The pH must be close to seawater value that is 8.3-8.5.

The ammonium control, instead, was done through a kit for aquariums. It can provide a quickly, and not very much expensive, value of the amount of ammonium and other nitrates species, providing the trend of the ammonium as alternative of other analytical alternatives with higher costs in time or money.

The test involves several steps: withdraw 5 mL of water to each sample, place them in the test tube and add 5 drops of three reagents, one at a time, and stir for about 5 seconds.

The solution will assume a specific color which depending on the card included in the kit, will indicate a certain value of ammonium (Fig.2.10).

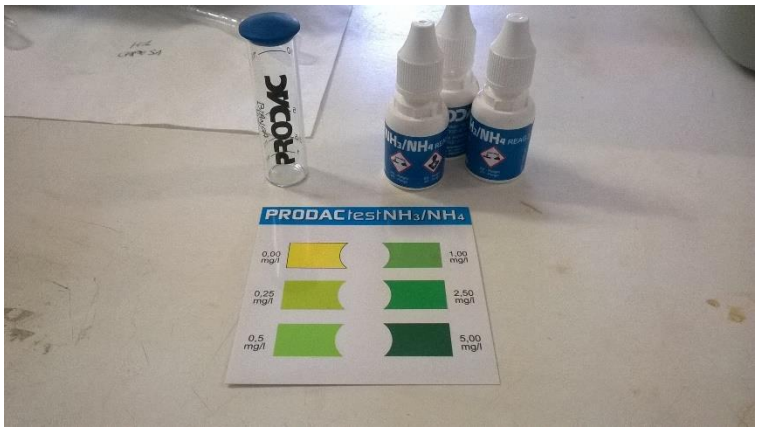


Figure 2.10. Complete kit of Ammonium with three reagents, plastic chamber and graduate colorimetric tab.

The last day (Day 10) amphipods were taken again from each chamber and its survival was evaluated.

Each replicate is been emptied on a sieve and were counted as many amphipods of the 20 we initially put were alive and how many had died.

To measure the bioaccumulation of each metal and nano metal oxide, the amphipods survivors were placed in an Eppendorf for each replication of each concentration, appropriately labeled, and were put in the freezer at -80 ° to be stored until they have been lyophilized and used to conduct the analysis.

Amphipods are coastal animals that live sunken in the sediment and they are excellent bioindicators of the metal and no-nano metal oxides presence in the sediment. In order to predict the effects of metals bioaccumulation, amphipods were analyzed. The effects on the survival at different concentrations were investigated.

2.2.2. CHARACTERIZATION OF METAL OXIDES FOR Ampelisca brevicornis BIOASSAY

Transmission electron microscopy (TEM) samples were prepared by partially drying a drop of the NPs solution or the NPs in seawater on a copper mesh 400 holey carbon film (Agar scientific) at room temperature [Romer I *et al.,* 2013].The grid was then carefully washed several times with UHP water and re-dried. Images were obtained using a JEOL 1200EX (accelerating voltage 80 kV), and recorded using Gatan Digital Micrograph software.

Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES) analysis was carried out with Optima 8000 (Perkin Elmer).

The concentration of the different NPs in seawater was measured under simulated conditions; no organisms were present, two exposure concentrations were chosen and sediment was also included. The same exposure was performed using ZnO and CuO salts. Dissolution at 30mins, 1h, 6h, 24h, 4 days and 10 days was measured as well as total concentration at time 0h and 10 days and sediment element content after 10 days.

Sediment was digested in a 2ml mixture of 1:4 of H_2O_2:HNO_3 in a microwave system (CEM Mars-5 Microwave accelerated reaction system) [Dogra Y *et al*., 2016], and diluted to 10ml with UHP water. The solutions were filtered using a syringe filter with 0.2µm Supor® membrane and finally measured using an ICP-OES.

Methods for the analysis of zinc and copper at different concentrations were set up and utilized accordingly. Analysis took place using radial detection for the particles with a concentration higher than 1ppm and axial detection for samples with concentrations on the ppb range, and a 60 second delay time. The final result was the average of three replicate readings that were obtained for each sample analyzed.

For dissolution measurements at each sampling point 5ml of the NPs in seawater were taken and put directly into Amicon 15 centrifugal filtration unit (sourced from Millipore, with regenerated cellulose acetate membrane with 3kDa cut off value and PP filter housing, maximum filtration volume 10ml). The cap was closed and the units were placed in a centrifuge (Eppendorf 5804-R). The units were spun for 30min at maximum speed which for this centrifuge was 5000 rpm. The membrane hosting unit was removed after spinning the samples and the filtrate was poured into a clean ICP-OES tube. Concentrated HNO_3 acid was added to obtain a 2% final acid concentration. For the total concentration of the NPs in seawater, a sample was taken at the end of the exposure and 20% HNO_3 was added and left for 24 hours. For ICP-OES measurements the concentrated acid solution was diluted to obtain a 2% acid concentration [Osborne OJ *et al*., 2016].

For the preparation for TEM analysis, samples were first fixed by using 2.5% glutaraldehyde in 0.1 M sodic cacodilate at 4° C for 4 hours. After this, 0.1 M cacodilate was used to wash the samples 3 times for 15 mins each. 1% osmium tetroxide in 0.1 M cacodilate was added and left for 1 hour. After this, the cacodilate was repeated.

The samples were then dehydrated by using: 70% ethanol for 30 minutes, 90% ethanol for 30 minutes, 100% ethanol for 30 minutes for 3 times and 100% ethanol + propylene oxide (1:1) for 5 minutes, 100% propylene oxide for 10 minutes for 2 times. Next, infiltration was

performed by using propylene oxide + epon (1:1) for 45 minutes, the epon had been left for one night in the fridge.

The final step was embedding, which was achieved by adding fresh epon and keeping the samples in an oven at 700°C for 24 hours. The blocks were trimmed and sectioned with an ultramicrotome. Copper grids were used and no stains were used.

2.2.3. CLAMS TOXICITY TEST

The experiment is based on the long -term exposure of juvenile of the species *R. philippinarum*, to four types of metal oxides: ZnO, CuO, TiO_2 and CeO, in both sizes, nano and the standard size. The metal oxides were provides by two different ways, direct in the media and throw the feeding. To supply the metal oxides through the feed, two species of microalgae were cultured in a medium containing these metal oxides in known concentrations.

2.2.3.1. MICROALGAE CULTURES

The metals can be absorbed by the clams of the selected species *R. philippinarum* mainly by filtration of nutrients. These marine organisms eat microalgae. For this bioassay, two microalgae *Phaeodactylum tricornutum* and "*Tetraselmis* sp", which are respectively a diatom and a green algae. These microalgae were grown in two different ways in the laboratory: a clean, not contaminated by the metals, culture of each microalgae, while, for the other way involves the contamination of the algae with metals.

From an uncontaminated culture of each microalgae, different new cultures were made for each metal and nano metal oxides at 1 mg/L and 10 mg/L of metal concentration in the media. At least 5 generations of each culture were made precious to their use in the bioassay. Cleans cultures were made as control and for its use for feeding those samples than received the metals directly in the media (versus through the food of contaminated microalgae). When the cultures reached a good density, 20-30 mL of each culture from both species were taken and were provided to the clams from each sample. After each use of the microalgae for feeding the clams, the culture (and the metals in each case) were renewed to let the microalgae grow again. Each culture was made per duplicate to guaranty the quality. If one culture was suspected of contamination, it was discarded and new one was made.

2.2.3.2. EXPERIMENTAL DESIGN AND TEST

The experimental design of this test involved the preparation of: a control, feeding with clean microalgae; samples feeding with contaminated microalgae and samples feeding with clean microalgae but where metal and nano metal were thrown at same time than food. Each sample and the control were run per triplicate and 30 juvenile clams were put in each replicate.

In each sample chamber, characterized by a glass jar of 1 L, were added 850 mL of clean seawater, suitably filtered, and with salinity adjusted to 35‰, and the 30 clams in the juvenile stage.

Before starting the experiment, it was set up an aeration system to allow proper oxygenation to the organisms. Exposure period was 10 days.

Briefly, samples for metal characterization and clams were collected the days 1, 4, 7 and 10 to exposure. These days, the clams were food (with their correspondence microalgae clean or contaminated) and a period of at least 3 h was left to ensure the clams filtered of the volume of water. After this period, the samples were taken and the water of the all the chamber was removed and change by filtered clean seawater. Metal and nano metal oxides were added at the same time that the clean microalgae in the samples of direct contamination. Control samples were only feeding with clean microalgae from both cultures.

Figure 2.11. Clams toxicity test and aeration system with four microcosms.

So, in the direct contamination design, 10 mL of green algae and 10 mL of diatom no-contaminated were added to each replicate sample. Separately were added 15 mL of each metal dissolved in MQ water at its correspondence concentration to each replicate in order to filter the microalgae at the same time with the metal.

After feeding all of samples, about 3 hours were left to ensure that the clams ate all the introduced algae or at least have enough time for doing it.

The second part of the experiment included the change of the contaminated water and simultaneously the removal of five clams from each replicate cultures (for biomarkers characterization, not showing in this work due its extension). These clams were kept, properly labeled, at the -80° freezer until the homogenization and analyses.

Contaminated algal cultures had also to be freshly prepared and they had to be grown in three days that existed between the successive phases of the experiment.

This procedure was repeated at days 1, 4, 7 and 10, but with the exception that the last day of the experiment all the remaining 15 clams were removed and samples were divided for bioaccumulation and biomarker characterization.

Samples collected in four days described, were frozen and subsequently taken to proceed with the dissection.

Only after dissection were placed in criovials, lyophilized and analyzed for bioaccumulation.

The bivalve *R. philippinarum* may be appropriately deployed as a bioindicator in monitory transitional environments, in term of bioaccumulation potential. The bivalves are filter-feeders and they are able to accumulate in their organs and tissues without any apparent effects. In order to predict the effects of bioaccumulation, different metal concentrations were determined.

2.3. Statistical analysis

For the embryo-larval development, significance differences between the percentage of abnormal larvae and differences between fecundated and no-fecundated eggs in metal oxide and nano netal oxides were determined by one-way ANOVA, followed by a Dunnet test for multiple comparison. Two level of significance were established: $p<0.01$ and $p<0.05$. The same statistical analysis (one-way ANOVA) was performed for bioaccumulation (amphipods and clams) and survival for amphipods. All the statistics test were performed with SPSS Software (version 17.0).

Graphics and tables for sea urchin, amphipods and clams were created with Excel program.

Chapter 3: *Results*

3.1. Acute bioassay results: *Paracentrotus lividus*

In this work it was studied the effects of CuO, ZnO, TiO_2 and ZnO/CeO_2 NPs and salt on *P. lividus* for fertilization and larvae development. Seawater samples were taken for characterization of the nanoparticles (size, distribution, etc.) by Transmission Electron Microscopy (TEM) and for bioaccumulation.

3.1.1. *CHARACTERIZATION OF METAL DISSOLUTION*

Table 3.1. Dissolution results of ZnO, TiO2, CuO and ZnO/CeO2. TiO2 and CeO2 have a value b.d.l. (Below Detection Limit).

Particle		Total concentration	Ionic concentration				
			30mins	1h	6h	24h	48h
ZnO		20. 2 ± 0.3	15.1 ± 0.2	15.22± 0.01	15.59 ± 0.07	15.5 ± 0.2	15.1 ± 0.8
		1.05 ± 0.07	0.480 ± 0.001	0.508 ± 0.002	0.50 ± 0.01	0.55 ± 0.01	0.559 ± 0.007
CuO		10.5 ± 0.4	**Below detection limit (2ppb)**				
		1.08 ± 0.3	5.2 + 0.9	9.1 ± 0.4	9.6 ± 0.4	10.538 ± 0.004	10.3 ± 0.4
TiO_2 uncoated		9.6± 0.5	Below detection limit (1ppb)				
		8.9± 0.2					
TiO_2 PVP		10.2± 0.3	Below detection limit (1ppb)				
		9.7± 0.4					
ZnO/ CeO_2	**ZnO**	5. 2 ± 0.3	2.7 ± 0.3	3.3± 0.4	2.4 ± 0.1	2.2 ± 0.1	2.25 ± 0.07
		5.05 ± 0.06	35.1 ± 0.1	41.0 ± 0.4	63.4 ± 0.3	69.1 ± 0.1	94.5 ± 0.2
	CeO_2	5.1 ± 0.4	**Below detection limit (1ppb)**				
		5.03 ± 0.04	**Below detection limit (1ppb)**				

Table 3.1 shows the following results:

-It notes that the value of ZnO ppb decreases in the first 30 mins and varies significantly for the entire time-period of the test. ZnO in ppm, instead, shows a decrease of 50% that it remains stable from beginning (30 mins) to the end of the test (48 h).

- CuO ppm shows a slight decrease (1%) to the end of 48h. CuO ppb, as for the CeO_2, presents values below the b.d.l. (2 ppb).

-We observed that the dissolution of the ZnO NPs decreased significantly in the Zn/Ce particles, at a ppm concentration from 50% when only Zn was present to 2% when CeO_2 NPs were present, and from 75% at a ppb concentration to 60% when CeO_2 NPs were present. As it regards the dissolution of CeO_2 in the mixture with ZnO, it denotes that after few minutes its concentration decreases so much as cross the b.d.l., even if the initial concentration was approximately equal to concentration of ZnO in the same mixture. To confirm this, Dogra Y *et al*. (2016) say that the dissolution of Ce has been shown to be extremely low in seawater.

-Furthermore, it has been observed that a decrease in the toxicity of ZnO NPs to zebrafish embryos was occurred with the addition of TiO_2 NPs, which have shown very low solubility, [Hua J *et al*., 2016] and a similar effect could be observed in the case of the mixed ZnO/CeO_2 NPs used. Hua J *et al.* (2016) found that the ZnO NPs formed larger aggregates with addition of TiO_2 NPs and the toxicity of these metal-based NPs was thus decreased. Titanium not presents, nor for the form NPs and nor for that PVP coated, values that exceed the minimum level of b.d.l. (1 ppb).

- Nanomaterials

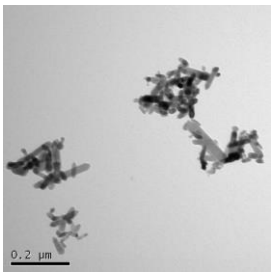
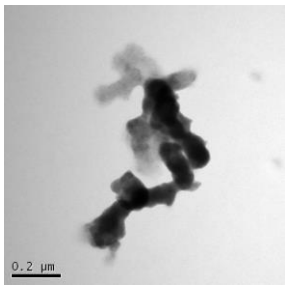
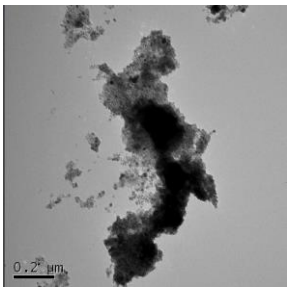
Commercial stock suspensions (TiO_2 PVP, TiO2 uncoated, CuO, ZnO and ZnO/CeO_2) were used as received (from Promethean Particles Ltd.).

Properties are the following:

Table 3.2. Properties of commercial suspension: TiO_2 PVP, TiO2 uncoated, CuO, ZnO and ZnO/CeO_2.

Particle	**Concentration**	**Size TEM (nm)**
TiO_2 uncoated	1 %	8 ± 4
TiO_2 PVP	1%	10 ± 3
CuO	1%	N/A
ZnO	1%	100 – 200nm
ZnO/CeO_2	1%	N/A

- TEM data

Particle	pristine		ppb	ppm
ZnO		1h		

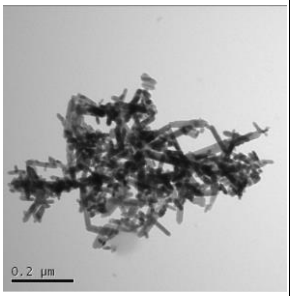
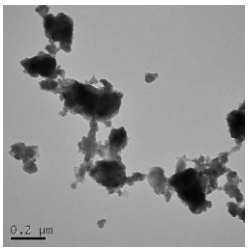
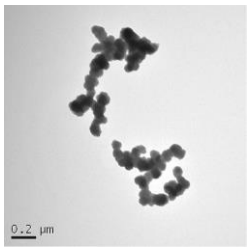
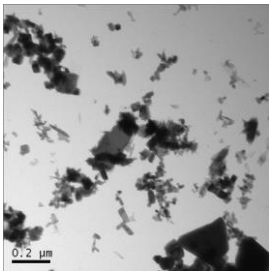
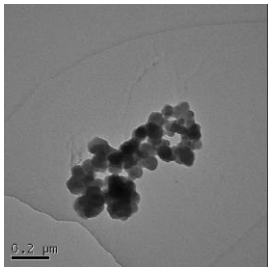
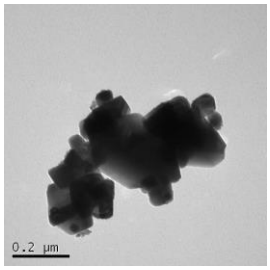
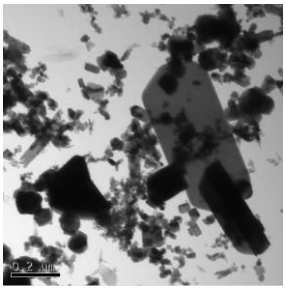
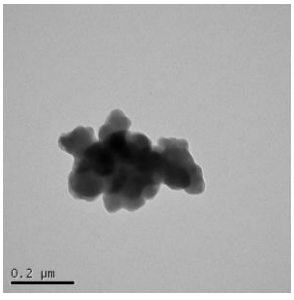
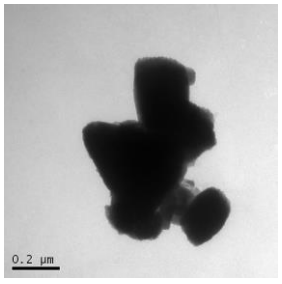
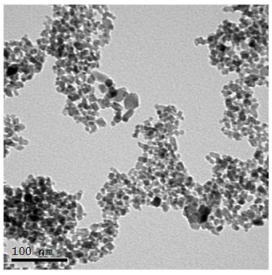
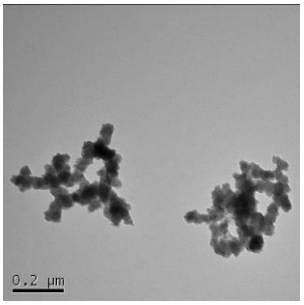
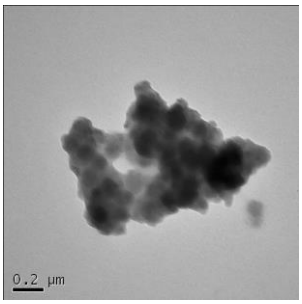
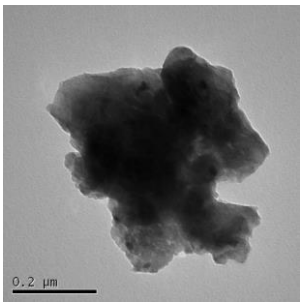
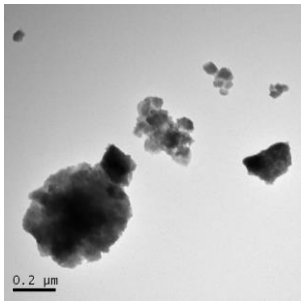
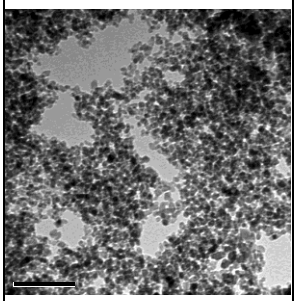
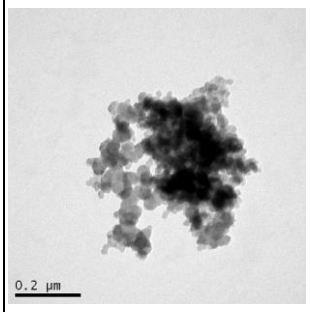
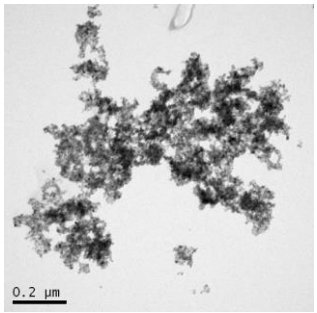
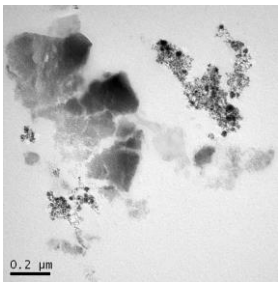
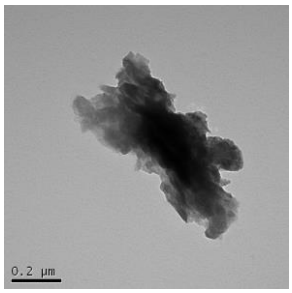
		48h		
CuO		1h		
		48h		

Figure 3.1. Imagines of pristine metals (ZnO and CuO) and ppb/ppm imagines after 1 h and 48 h.

Particle	pristine		ppb	ppm
TiO_2 uncoated		1h		
		48h		
TiO_2 PVP		1h		

TiO_2 PVP		48h		

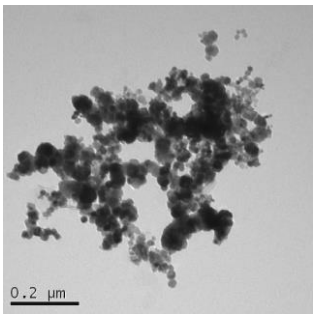
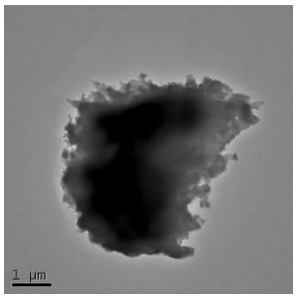
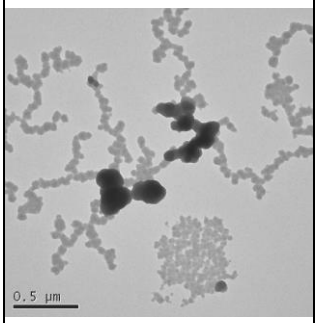
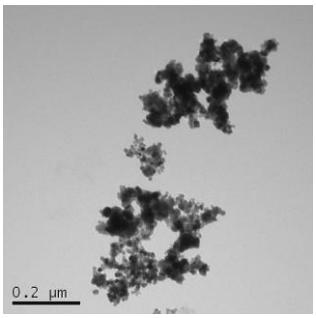
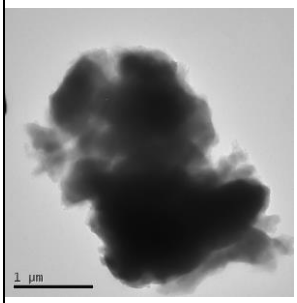
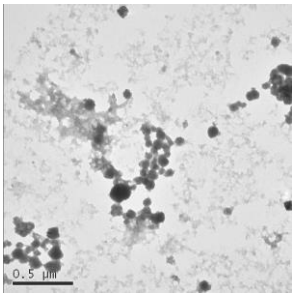
Particle	pristine		ppb	ppm
ZnO/CeO_2		1h		
		48h		

Figure 3.1: Continued (ZnO/CeO_2 and TiO_2).

ZnO in pristine shape appears partially aggregated after 1 h but even more united after 48 h in this time span, there are differences between ppb e ppm: in the first case, it is switching from an aggregated form a more detached and filamentous form; in ppm, it is switching from very compact mass to a more detached form.

CuO pristine appears aggregated and homogeneous. In ppm and ppb, it observes the same passage of compacting of the metal over time.

Uncoated TiO2 and TiO2 PVP not appear very different under the microscope in pristine shape. The first from 1 ppb to ppb at 48 h become more homogeneous; on the contrary, the ppm tends to break down slightly, even though it retains a compact form. TiO2 PVP appears as the inverse.

As regards the mixture of ZnO/CeO_2 it denotes a non-linear trend. Not contaminated metals, present a filamentous aspect and partially aggregated, in seawater. In ppb and ppm it denotes a clear aggregation of particles.

Being a mixture let's see in details how it behaves each metals: ZnO is represented by darker spots and has a tendency towards aggregation; the CeO_2 instead is represented by lighter areas and is more conductive to the break with more stringy appearence.

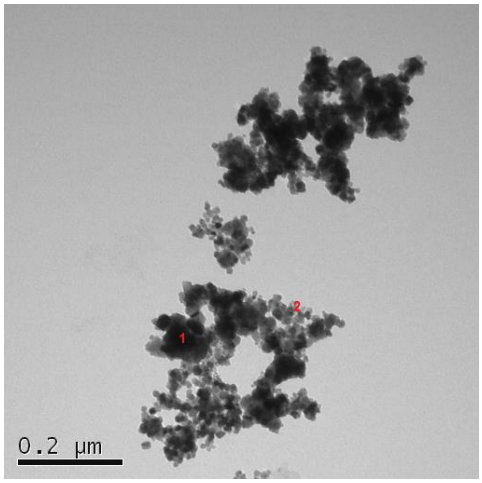


Figure 3.2. EDX Results of pristine particles: the darker parts (1) seem to be a mix of Ce/Zn and the lighter areas (2) only have Ce

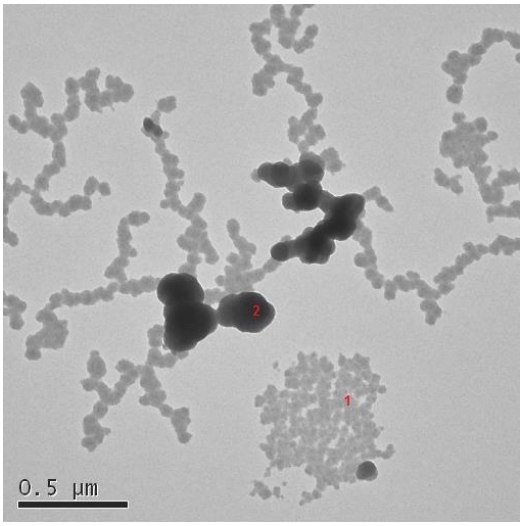


Figure 3.3. EDX Results of pristine particles: the darker parts (2) only have Zn present and the lighter part (1) only have Ce.

3.1.2. FERTILIZATION AND LARVAL DEVELOPMENT RESULTS

- Titanium

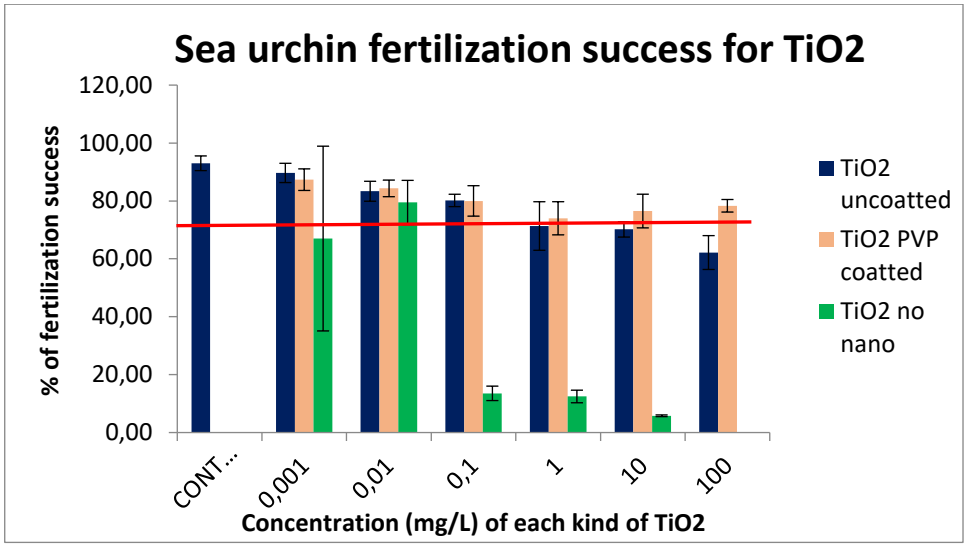


Figure 3.4. Results of success of fertilization of the *P. lividus* sperm exposed (1h, 20ºC) to different concentration of TiO2 (nano uncoated, nano PVP coated and no nano size). Red line indicates the limits under which the concentration is considered toxic according to 70% success of fertilization respect to the results of Control group.

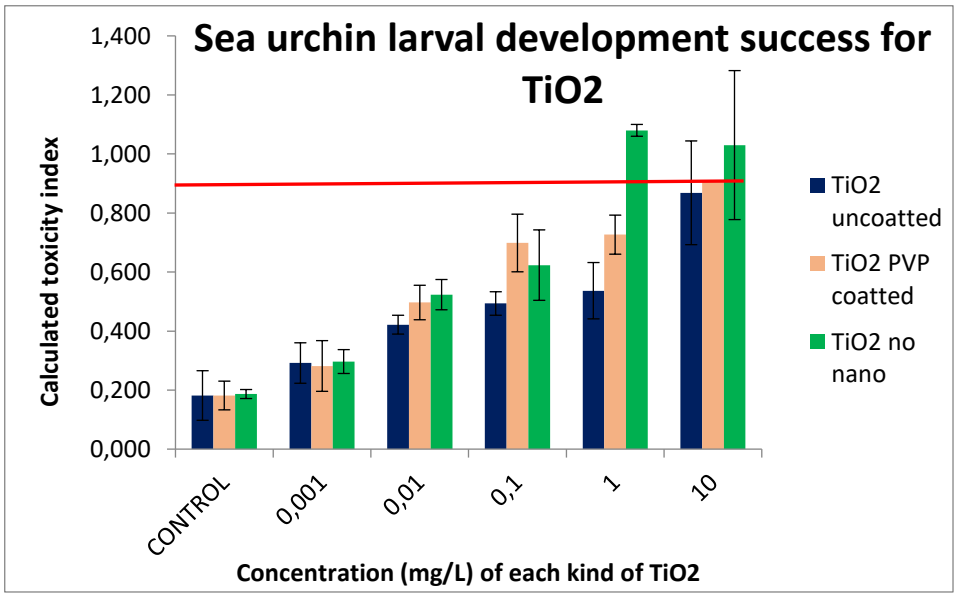


Figure 3.5. Summarized results of success of embryogenesis of *the P. lividus* embryos exposed (48h, 20ºC) to different concentration of TiO2 (nano uncoated, nano PVP coated and no nano size). Red line indicate the limits below which the concentration is considered toxic according to 70% of success (after calculated toxicity index) respect to the results of Control group.

The first graph shows the percentage of the fertilization success in relation to the Titanium concentration; the second shows the index above which the metal is toxic in the larvae formation.

The red line, in the fertilization, determines the limit beyond which the eggs have been successfully fertilized and so, the influence of metal in the fertilization. As it can see, for uncoated TiO_2 as for TiO_2 PVP, an elevated percentage of eggs has been successfully fertilized in all concentrations, instead for TiO_2 no-nano when the concentration increases, the fertilized % decreases drastically.

In the following graph, the limit between normally formed larvae and severely malformed is between 0 and 3 and it represented of the red line at value 0.9: the IT (index of toxicity) weights the degree of deformations by the frequency (%) observed as follows: IT = [0 x % Level 0 + 1 x % Level 1 + 2 x % Level 2 + 3 x % Level 3]/100. The IT for each discharge ranged from 0 (no toxicity) to 3 (high toxicity) [Carbelleira C *et al.*, 2011]. In the all three types of Titanium, the malformed larvae increases with the elevated concentration but the values remain under the toxicity line. Only the more elevated concentrations (from 1 mg/L) of TiO_2 no-nano exceed this limit, resulting toxic.

- Copper

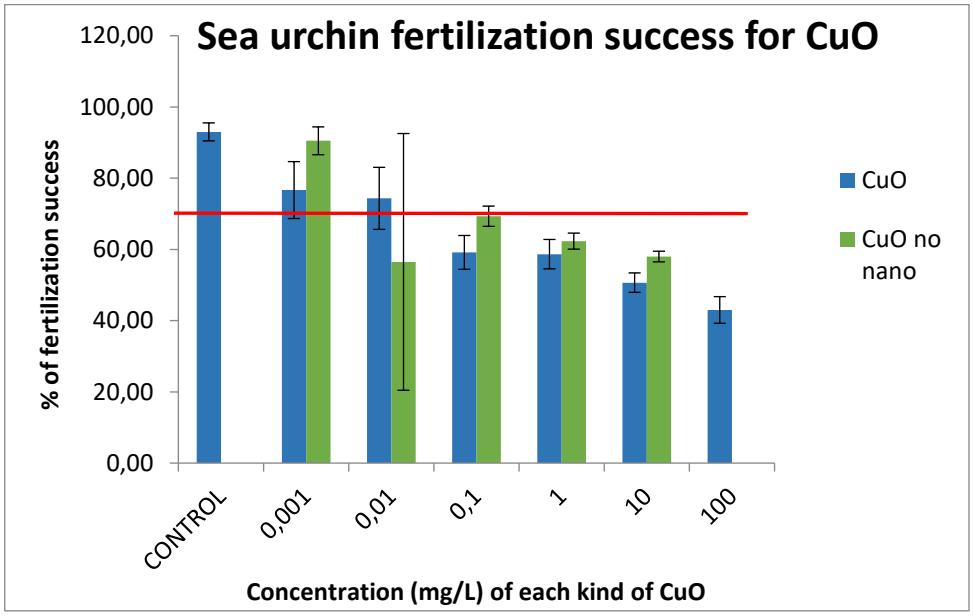


Figure 3.6. Results of success of fertilization of the *P. lividus* sperm exposed (1h, 20ºC) to different concentration of CuO (NPs and no nano size). Red line indicates the limits under which the concentration is considered toxic according to the success of fertilization respect to the results of the Control group.

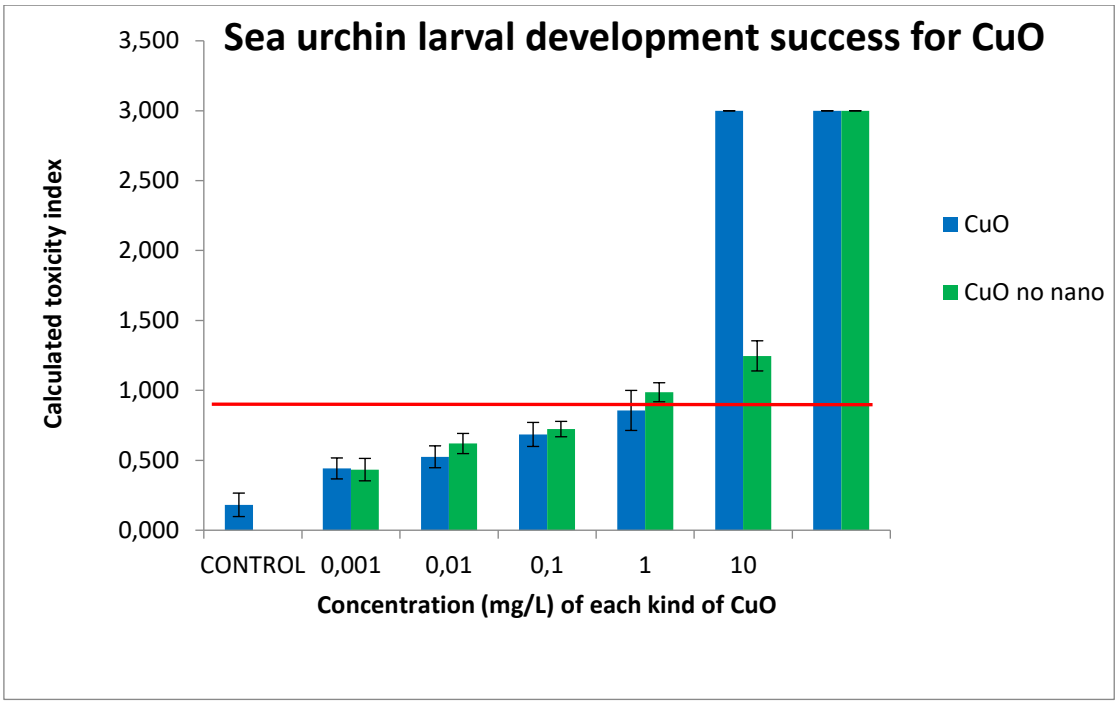


Figure 3.7. Summarized results of success of embryogenesis of the *P. lividus* embryos exposed (48h, 20ºC) to different concentration of CuO (NPs and no nano size). Red line indicate the limits below which the concentration is considered toxic according to 70% of success (after calculated toxicity index) respect to the results of Control group.

As regards CuO, the reproductive success is been standardized to 70% respect to the Control. It denotes that there is an elevated percentage of fertilization for the lower concentrations but the percentage decreases under the control line when the concentrations increase.

The larval development detects a slight increase of the malformations when the concentrations increase without exceeding the toxicity line. Only for two highest concentrations, a spike in values ca be reported, at larval level, on the malformations, mostly for CuO no-nano. If the results are correlated with the percentage of fertilized eggs, it denotes that a lower number of fertilized eggs matches a highest level of malformations, confirming an elevated toxicity to highest values of CuO.

- Zinc

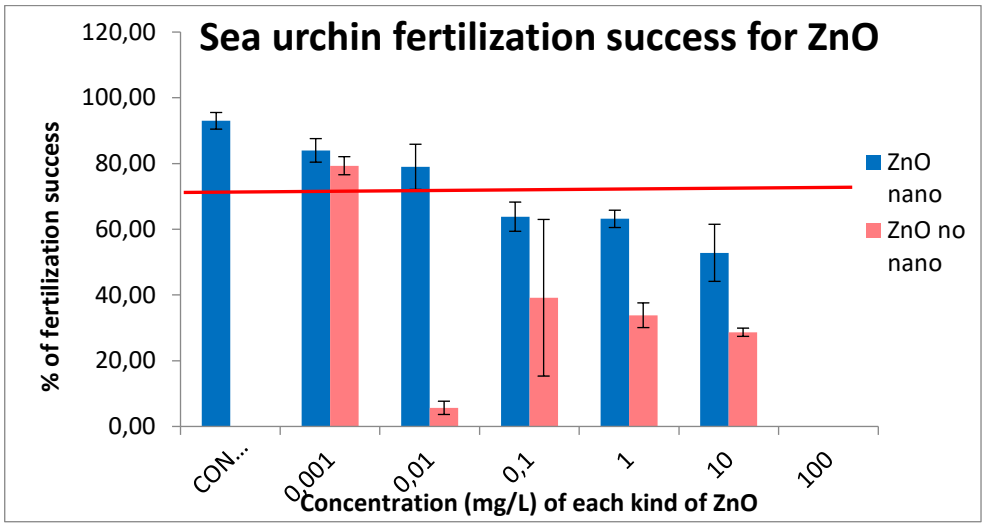


Figure 3.8. Results of success of fertilization of the *P. lividus* sperm exposed (1h, 20ºC) to different concentration of ZnO (NPs and no nano size). Red line indicates the limits under which the concentration is considered toxic according to the success of fertilization respect to the results of the Control group.

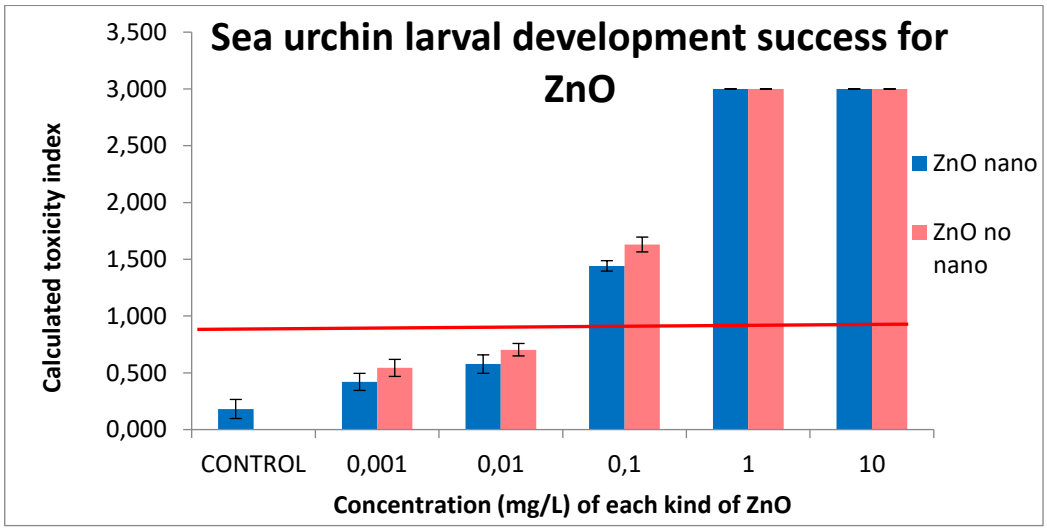


Figure 3.9. Summarized results of success of embryogenesis of the *P. lividus* embryos exposed (48h, 20ºC) to different concentration of ZnO (NPs and no nano size). Red line indicate the limits below which the concentration is considered toxic according to 70% of success (after calculated toxicity index) respect to the results of Control group.

The line of reproductive success is to 70% respect to the control. It denotes that the eggs has been fertilized at the lower concentrations for both metal forms, instead at elevated values, the percentage decreases under the red line but it remains around high values. Only anomaly is for ZnO no-nano at 0,001 mg/L, in which a little percentage of eggs was been fertilized.

As regards the larval development: the level of malformation increases in both forms of ZnO when the concentrations increase, confirming an eventually toxicity of the metal at this concentration.

- Zinc/Cerium

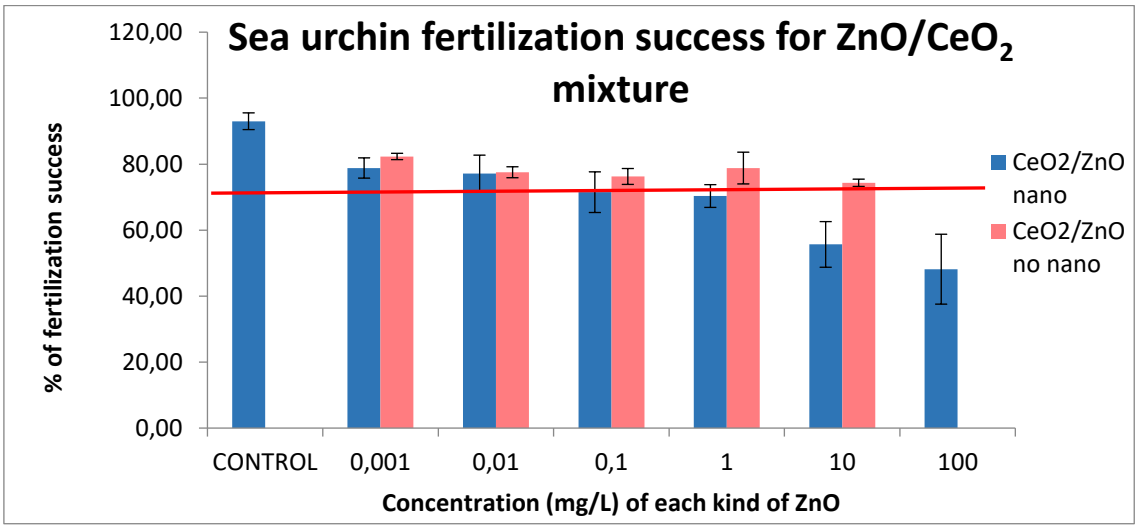


Figure 3.10. Results of success of fertilization of the *P. lividus* sperm exposed (1h, 20ºC) to different concentration of ZnO/CeO2 (NPs and no nano size). Red line indicates the limits under which the concentration is considered toxic according to the success of fertilization respect to the results of the Control group

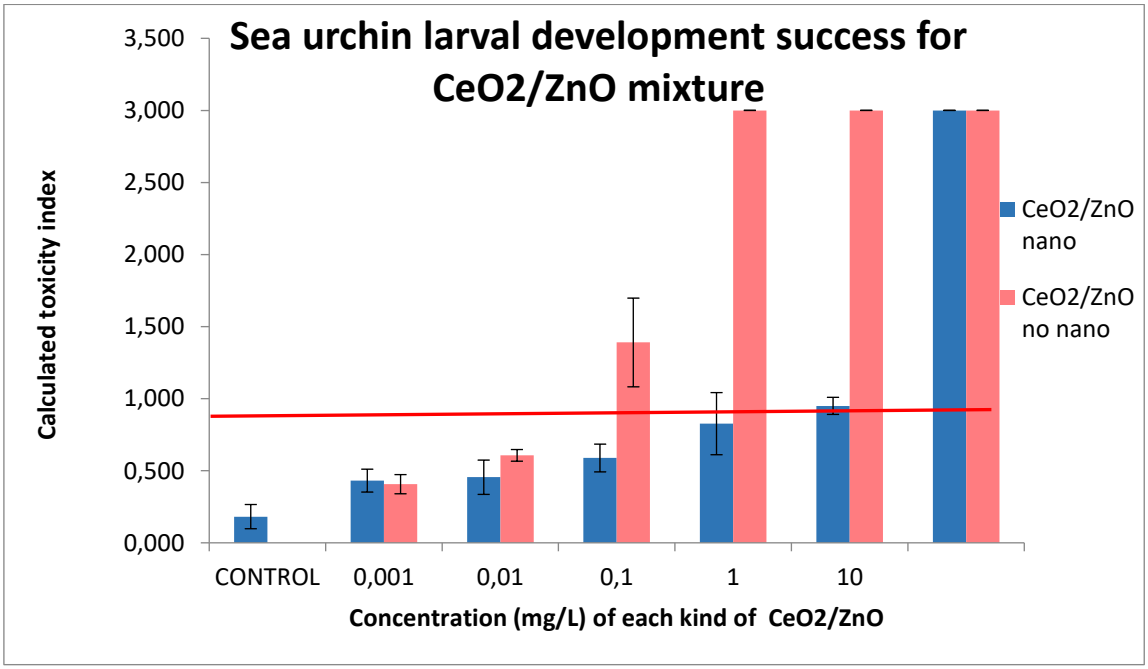


Figure 3.11. Summarized results of success of embryogenesis of the *P. lividus* embryos exposed (48h, 20ºC) to different concentration of ZnO/CeO2 (NPs and no nano size). Red line indicate the limits below which the concentration is considered toxic according to 70% of success (after calculated toxicity index) respectto the results of Control group.

In this case, the percentages of eggs fertilized are close to the red line values or they are above the red line, indicating a low toxicity of the ZnO/CeO_2 mixture.

The larval development shows that the high concentrations haven't a much malformations (low toxicity) but from 0.1 mg/L of mixture, mostly in the no-nano form, the values are raised to the level 3.

3.2. Chronic bioassay results: *Ampelisca brevicornis*

The effects of nanoparticles CuO and ZnO, as well as salts of CuO and ZnO, were studied in this projection *A. brevicornis* over an exposure time of 10 days. Samples of the living organisms were taken for study, to be analyzed by Transmission Electron Microscopy (TEM), and for bioaccumulation.

3.2.1. CHARACTERIZATION OF METAL DISSOLUTION

- Nanoparticles in Seawater

Table 3.3. Concentration of Zn NP and Cu NP in seawater at different time points under simulated conditions.

Particle	Total concentration (ppm)	Ionic concentration (ppm)						Total conc. 10days (ppm)
		30mins	1h	6h	24h	4d	10d	
ZnO	1.02 ± 0.03	0.58 ± 0.1	0.530± 0.006	0.49 ± 0.02	0.480 ± 0.007	0.42 ± 0.2	0.20 ± 0.03	0.24 ± 0.01
	2.54 ± 0.08	1.43 ± 0.06	1.39 ± 0.03	1.33 ± 0.06	1.29 ± 0.03	1.22 ± 0.03	0.54 ± 0.02	0.58 ± 0.06
	(ppm)	(ppb)						(ppb)
CuO	1.04 ± 0.07	5.4 ± 0.6	6.2 ± 0.2	23 ± 1	29.5 ± 0.6	33 ± 3	35 ± 1	160 ± 20
	10.5 ± 0.6	53.1 ± 0.8	57.3 ± 0.3	94.3 ± 0.5	108 ± 1	364 ± 6	452 ± 7	404 ± 36

In seawater, two concentrations were chosen to have a low and a high concentration used in the bioassays. It can be observed (Table 3.3.) that after 30 mins > 50% of the ZnO NPs has dissolved (58% for 1 ppm and 56% for 2.5 ppm), but these ionic concentrations decreased with time. It believes that this effect, as well as the low final concentration, was due to the absorption to the container and possibly sedimentation of aggregates. After 10 days, the measured total concentration was 0.24 ppm and the ionic concentration was 0.2 ppm. In the case of CuO NPs, very little dissolution was observed at both concentrations after 10 days (3.5% for 1 ppm and 4.5% for 10 ppm). The concentration of dissolved CuO increased with time, we did not see the same effect as for the ZnO NPs, but we also found that the final total CuO concentration after 10 days was low, due to aggregate sedimentation.

- No-nanos in Seawater

Table 3.4. Metals in no-nano form: ZnO and CuO in seawater. Total concentrations are expressed in ppm for ZnO and mg/L for CuO and ionic concentration were calculated in 2 times.

Compound	Total concentration (calculated) (ppm)	Ionic concentration	
		1h	10d
ZnO	0.1	(41.2 ± 0.7) ppb	(36.7 ± 0.2) ppb
	1.5	(0.491 ± 0.006) ppm	(0.49 ± 0.02) ppm
	(ppm)	(ppb)DI	
CuO	0.5	24.5 ± 0.2	12.61 ± 0.08
	5	251 ± 3	44.0 ± 0.2

For the CuO and ZnO salts in seawater (Table 3.4.), it was observed a reduction in the total concentration after 30 mins, which did not change significantly for ZnO, but was decreased by 50% for the 1 ppm concentration of CuO and by 82% for 10 ppm.

- Concentration in soil

Table 3.5. Concentration of Zn and Cu NPs and salts in soil after 10 days of simulated exposure.

Compound	Concentration in the exposure (ppm)	Concentration after 10 days	Concentration in soil (μgKg^{-1})
ZnO NP	1.02 ± 0.03	(0.24 ± 0.01) ppm	4.3 ± 0.3
	2.54 ± 0.08	(0.58 ± 0.06) ppm	7.2 ± 0.7
CuO NP	1.04 ± 0.07	(160 + 20) ppb	4.3 ± 0.4
	10.5 ± 0.6	(404 ± 36) ppb	40.3 ± 0.5
ZnO salt	0.1	(36.7 ± 0.2) ppb	7.3 ± 0.5
	1.5	(0.49 ± 0.02) ppm	4.5 ± 0.4
CuO salt	0.5	(12.61 ± 0.08) ppb	1.5 ± 0.4
	5	(44.0 ± 0.2) ppb	9.3 ± 0.1

Table 3.5 shows the sediment with CuO and ZnO content after 10 days (after normalization with the blank). It can be observed that in all cases the concentration was very low (between 1 and 40 μgKg^{-1}), which leads us to believe that the aggregates were deposited on the surface of the sediments and were removed when the seawater was removed to dry the sediment.

- TEM data

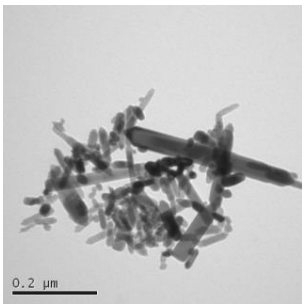
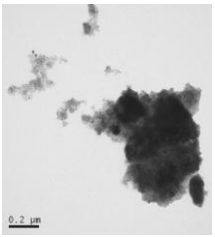
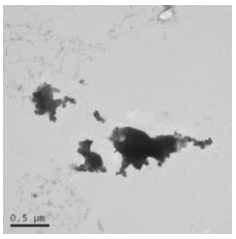
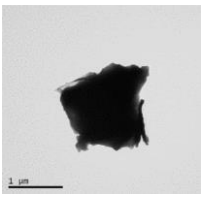
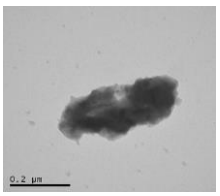
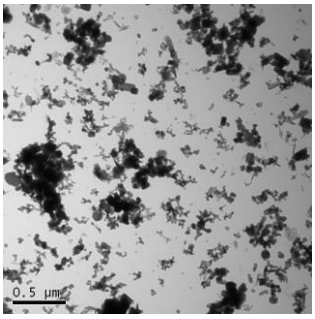
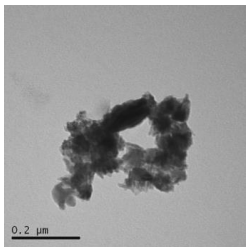
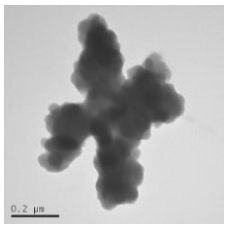
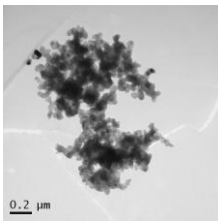
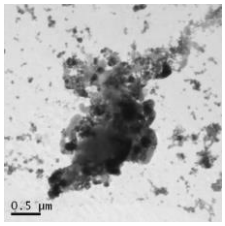
<table>
<tr><th>Particle</th><th>pristine</th><th></th><th>1ppm</th><th>2.5ppm</th></tr>
<tr><td rowspan="2">ZnO</td><td rowspan="2">  </td><td>24h</td><td>  </td><td>  </td></tr>
<tr><td>10days</td><td>  </td><td>  </td></tr>
<tr><td rowspan="3">CuO</td><td rowspan="3">  </td><td rowspan="2">24h</td><td>1ppm</td><td>10ppm</td></tr>
<tr><td>  </td><td>  </td></tr>
<tr><td>10days</td><td>  </td><td>  </td></tr>
</table>

Figure 3.12. TEM Imagines of pristine ZnO and CuO after 24 h and 10 days.

It was also observed that for both types of NPs large aggregates were mainly observed (Figure 3.12.), as indicated by the TEM measurements. It can be observed that the pristine NPs showed high polydispersity indexes and hydrodynamic diameters when measured by DLS.

It was no possible to determine a size distribution by TEM due to the sample's polydispersity in both cases, particles ranged from 10 to 200 nm in the case of the ZnO NP's, and from 10 to 500 nm in the case of CuO. The ZnO NP's were needle shaped, while the CuO NPs ranged in shape, from squares, rectangles, spheres and needles. Particle aggregation and dissolution of the pristine was measured under simulated conditions by TEM and ICP-OES, respectively.

Buffet PE *et al.* (2011) found that CuO NPs (with an initial size ranging from 10 to 100nm) in seawater highly aggregated/agglomerated and that the hydrodynamic size (or Z-average) measured by dynamic light scattering increased rapidly to values of around 1000 nm [Buffet PE *et al.*, 2011]. In addition, these has been observed in previous studies on CuO NPs, indicating aggregation in seawater [Gomes T *et al.*, 2011].

3.2.2. BIOACCUMULATION AND SURVIVAL OF AMPHIPODS

- Zinc

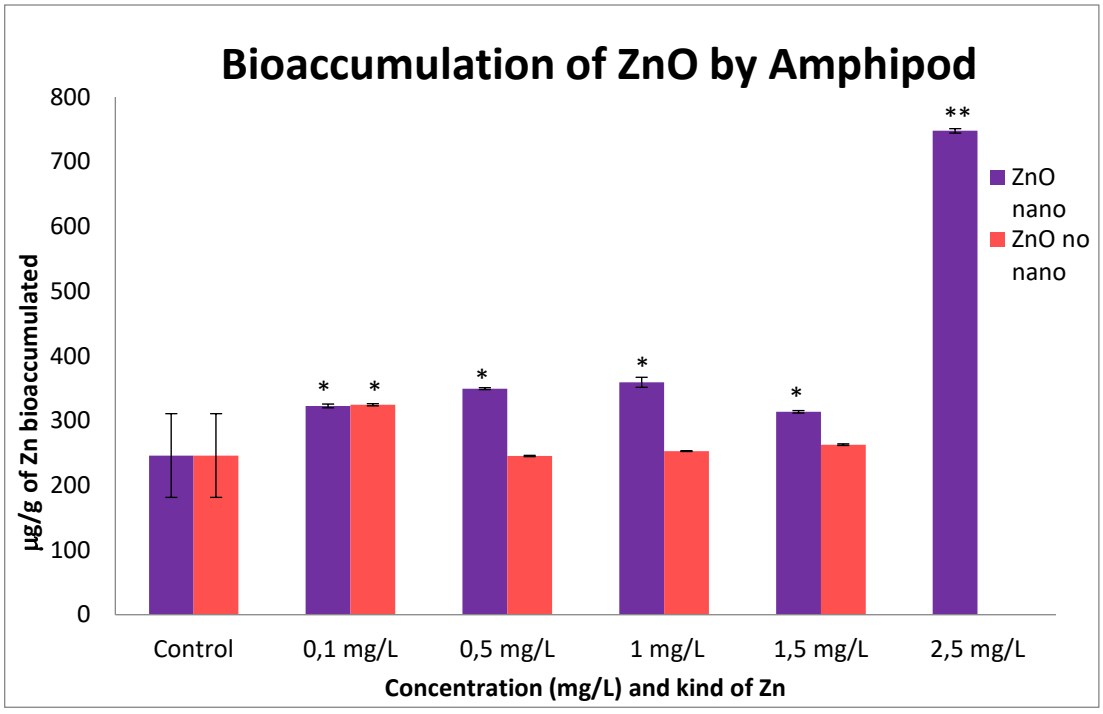


Figure 3.13. ANOVA statistical significance (*p<0.05, **p<0.01) for ZnO concentration (micrograms per grams of dry weigh measured in the whole organisms) respect to the Day 0, Control and groups of individuals.

The following graph shows the results of analyzes concerning the bioaccumulation of ZnO. It denotes a fairly constant trend between the accumulation of ZnO nano size and ZnO

standard for all concentrations, except for the highest concentration in which it has a peak with respect to the ZnO NPs.
From this concentration, also the amount of bioaccumulated ZnO increases significantly.

Instead, as regards the resistance of amphipods exposure to Zn (Fig. 3.3.), there is an important decrease of survival of 1.5 mg/L and 2.5 mg/L of ZnO NPs and size normal ZnO.

In general, ZnO NPs presents a greater potential to bioaccumulate; instead, ZnO no-nano results to be more lethal if we compare the same concentrations.

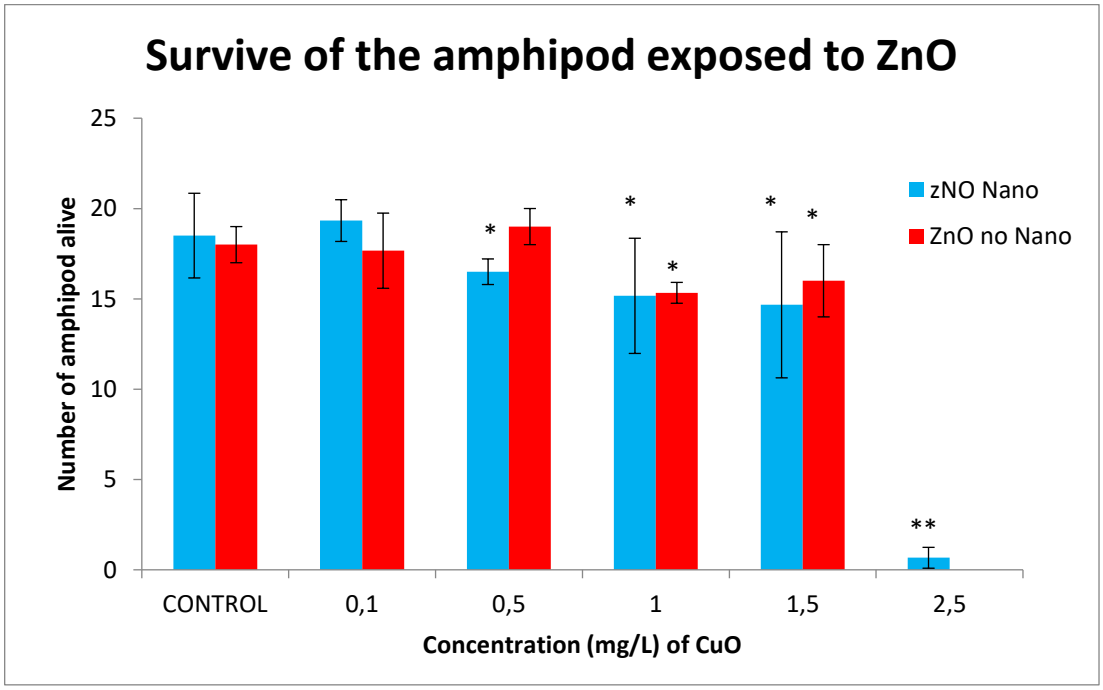


Figure 3.14. Concentration of ZnO NPs and ZnO no nano in relation to the number of alive amphipods, after 10 days of exposure to the metal. The asterisks (*; **) indicate values statistically significant by ANOVA.

- Copper

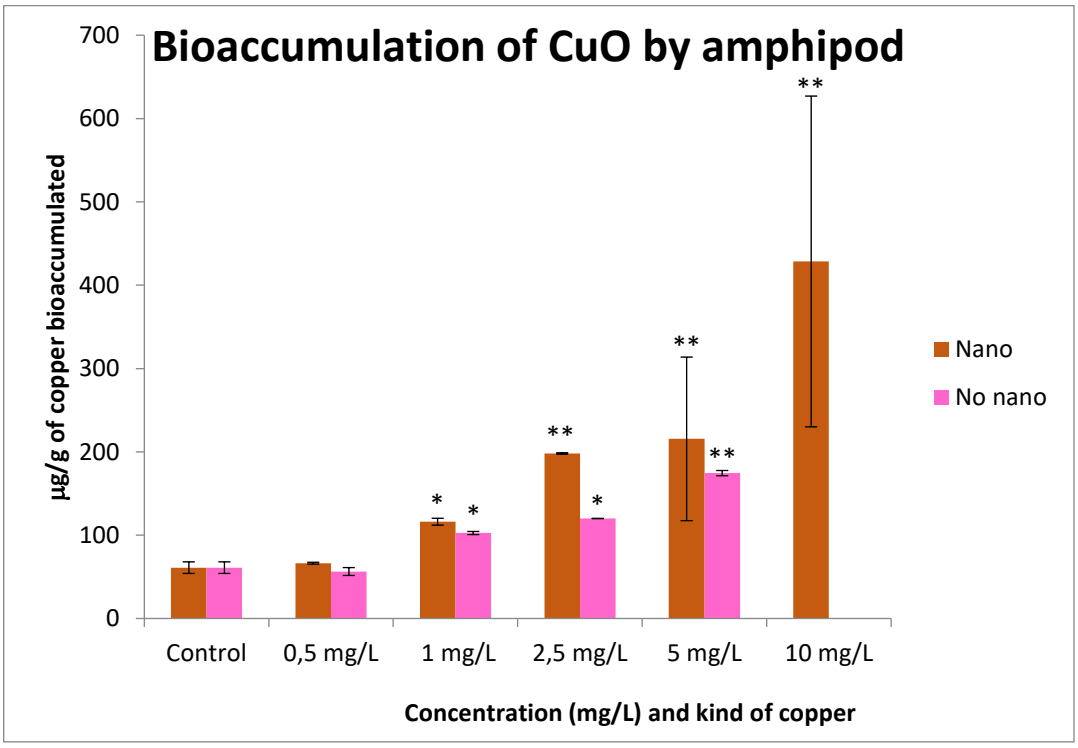


Figure 3.15. ANOVA statistical significance (*p<0.05, **p<0.01) for each CuO concentration (micrograms per grams of dry weigh) measured in the organism respect to the Control and groups of individuals.

This bar graph (Fig. 3.15.) shows that there are significant differences compared to the values of the Control from the concentration of 1 mg/L for the CuO no nano as well as for the CuO NPs. In general, the quantity of CuO accumulated in amphipods tissues increases in accordance to the concentration that was added in the medium, both for size standard CuO as for CuO NPs.

However, there is a not clear tendency for the tested concentration of CuO in the case of survival (Fig. 3.16.), in fact, it was not possible to realize the experiment with CuO of normal size, due to poor dissolution of the same metal to 10 mg/L. This fact, it gives an idea of that classical bioassays, such as survival, should be supplemented with other studies to assess the effects of the metals, as they may have some resistance to some of them (do not die, so they do not seem to have no effect), however, there are effects to other levels (as in the case of bioaccumulation).

The highest concentrations of CuO NPs have a lethal effect on the amphipods with the increase of the concentration (5 mg/L and 10 g/L).

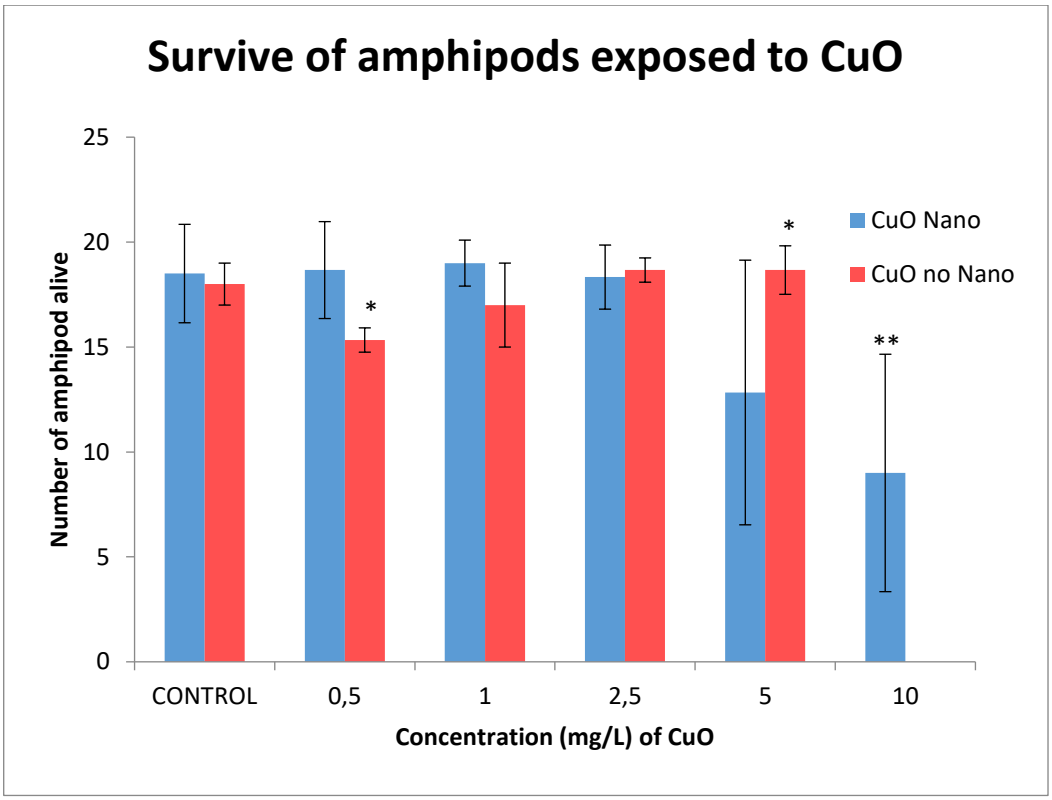


Figure 3 .16. Concentration of CuO NPs and CuO no nano in relation to the number of alive amphipods, after 10 days of exposure to the metal. The asterisks (*; **) indicate values statistically significant by ANOVA.

Hanna SK *et al.* (2013) shows that, through his study, between ZnO and CuO, in the survival experiments of amphipods, about 63% of ZnO NPs it dissolves in seawater in about 3 days, while only 21% of CuO NPs it dissolves in about 90 days. In general, ZnO NPs dissolves much faster than CuO NPs. In addition, the results of this study show that the mortality of amphipods increases in a dose dependent manner with ZnO and CuO NP exposure and in generally linear manner.

3.3. Chronic bioassay results: *Ruditapes philippinarum*

In this work, the effects of metal oxides NPs and no-nano were studied on *R. philippinarum*. Samples of the survival organisms were taken for study, to be analyzed by Transmission Electron Microscopy (TEM), and for bioaccumulation. The analysis of metal dissolution and the characterization of the metal oxides were been conducted also in the microalgae cultures.

3.3.1. CHARACTERIZATION OF METAL DISSOLUTION OF CLAMS AND ALGAE

- Clams ZnO

Table 3.6. Total concentrations and dissolution, in 3 times, of ZnO and ZnO salt.

Particle	Total concentration 0h (ppm)	Total concentration 5mins (ppm)	Dissolution 5mins (ppm)	Total concentration 3h (ppm)
ZnO	1.0 ± 0.5	0.46 ± 0.05	0.30 ± 0.06	0.37 ± 0.03
	10.1 ± 0.2	4.9 ± 0.3	0.9 ± 0.1	3.9 ± 0.1
ZnO salt	1.01 ± 0.07	0.523 ± 0.03	N.M	0.166 ± 0.009

As regards ZnO NPs and ZnO standard, it denotes that the total concentrations at time 0 in all three cases decreases of 50% only after 5 mins and further decreases after 3 h. Instead, the dissolution is determined after 5 mins and, respect to initial concentration, shows lower levels that change, depending on the cases: ZnO NPs, with lowest concentration, shows a decrease of 70%; ZnO NPs with highest concentration, shows a decrease of 90%; ZnO salt shows such a high reduction as not to be detected by the instrument.

- Algae ZnO

Table3.7. Values of Concentration and dissolution of the algae contaminated with ZnO NP and salt.

Particle	Total concen. 0h (ppm)	Total concen. 5min (ppm	Dissol. 5mins (ppm)	Total Conc. 3d (ppm)	Dissol. 3d (ppm)
ZnO NP	1.02 ± 0.03	0.097 + 0.001	0.090 ± 0.001	N.M	0.032 ± 0.003
	10.05 ± 0.07	0.49 ± 0.02	0.48 ± 0.03	N.M	0.08 ± 0.02
ZnO salt	1.01 ± 0.05	0.470 ± 0.002	N.M	0.103 ± 0.03	N.M
	10.1 ± 0.9	2.61 ± 0.06	N.M	0.53 ± 0.02	N.M

On algae, values of ZnO NP at lower concentrations remain approximately equal after 5 mins, both for concentration and for dissolution; lower concentration shows a decrease of 50%. After 3 days (the period of culture of the microalgae), the total values have not been determined, instead for the dissolution there is a significance increase.

ZnO salt, after 5 mins, has a total concentration lower than 50%, for the lowest value, and lower than 70% for the highest value. Values decrease after 3 days.

- Clams ZnO/CeO_2

Table 3.8. Total concentrations and dissolution, in 3 times, of ZnO/CeO2 NP's and salt.

Particle	Total concentration 0h (ppm)	Total concentration 5mins (ppm)	Dissolution 5mins (ppm)	Total concentration 3h (ppm)
ZnO	1.0 ± 0.7	0.310 ± 0.001	0.18 ± 0.04	0.13 ± 0.01
	10.05 ± 0.05	0.161 ± 0.006	0.028 ± 0.006	0.042 ± 0.002
ZnO salt	1.02 ± 0.03	0.33 ± 0.03	N.M	0.10 ± 0.02
Particle	**Total concentration 0h (ppm)**	**Total concentration 5mins (ppm)**	**Dissolution 5mins (ppm)**	**Total concentration 3h (ppm)**
CeO_2	1.1 ± 0.8	0.0652 ± 0.0008	bdl	0.0188 ± 0.0008
	10.2 ± 0.4	0.49 ± 0.04	0.022 ± 0.003	0.016 ± 0.006
CeO_2 salt	1.04 ± 0.05	0.028 ± 0.005	N.M	bdl

Bdl: below detection limit, 0.01ppm

In the ZnO/CeO_2 mixture, it denotes that, for both NPs metals, values of total concentration steadily decrease over time; the ZnO and CeO_2 salt dissolution is not determinable; instead, CeO_2 NPs at lowest initial concentration has a value lower to b.d.l. (0.01 ppm).

- Algae ZnO/CeO_2

Table 3.9. Values of Concentration and dissolution of the algae contaminated with ZnO/CeO2 NP's and salts.

Particle	Total concen. 0h (ppm)	Total concen. 15min (ppm	Dissol. 15mins (ppm)	Total Conc. 3d (ppm)	Dissol. 3d (ppm)
ZnO NP	1.03 ± 0.05	0.039 + 0.002	0.016 ± 0.002	N.M	0.015 ± 0.002
	10.1 ± 0.1	0.034 ± 0.001	0.0180 ± 0.0005	N.M	bdl
ZnO salt	10.06 ± 0.08	0.80 ± 0.02	N.M	0.420 ± 0.0009	N.M
CeO_2	1.07 ± 0.03	bdl	bdl	N.M	bdl
	10.1 ± 0.1	0.14 ± 0.0004	bdl	N.M	bdl
CeO_2 salt	10.05 ± 0.03	0.23 ± 0.03	N.M	bdl	N.M

Algae: ZnO NPs represents decreasing values for the total concentrations over time but, after 3 days, ithe values are not determinable. Dissolution not changes (highest concentration shows values under b.d.l.). CeO_2 NPs always shows values not determinable or under b.d.l.

A similar trend occurs for salt metals.

- TiO2 clam

Table 3.10. Total concentrations, in 3 times, of TiO2 NP's, PVP and salt.

Particle	Total concentration 0h (ppm)	Total concentration 5mins (ppb)	Total concentration 3h (ppb)
TiO_2	1.02 ± 0.06	16 ± 1	2.1 ± 0.2
	10.03 ± 0.07	236.8 ± 0.8	430 ± 10
TiO_2 PVP	1.05 ± 0.03	10 ± 1	3.806 ± 0.004
	10.08 ± 0.03	117 ± 1	29 ± 3
TiO_2 salt	1.01 ± 0.07	74 ± 4	10.7 ± 0.6

TiO_2 in clams: the lowest concentration of TiO_2 NPs increases after 5 mins but decreases after 3 h. The highest concentration presents a gradual increase. TiO_2 PVP has an opposite trend. TiO_2 salt presents an increase and, than, a decrease.

- TiO_2 Algae

Table 3.11. Values of Concentration and dissolution of the contaminated algae with TiO2 NP, PVP and salt.

Particle	Total concentration 0h (ppm)	Total concentration 5 mins (ppb)	Total concentration 3days (ppb)
TiO_2	1.01 ± 0.04	1.9 ± 0.5	1.6 ± 0.5
	10.09 ± 0.06	3.22 ± 0.09	2.7 ± 0.3
TiO_2 PVP	1.02 ± 0.04	3.8 ± 0.1	1.92 ± 0.07
	10.05 ± 0.06	3.1 ± 0.4	2.82 ± 0.07
TiO_2 salt	10.04 ± 0.06	50.5 ± 0.9	3.9± 0.3

Algae, to the lowest concentration of TiO_2 NPs PVP and salt, show an increase that remain stable over time except for TiO_2 salt, in which the decrease is significance. The initial values of highest concentrations show a certain stability.

- CuO clams

Table 3.12. Total concentrations, in 3 times, of CuO NP's and salt.

Particle	Total concentration 0h (ppm)	Total concentration 5mins (ppm)	Dissolution 5mins (ppb)	Total concentration 3h (ppm)
CuO	1.16 ± 0.09	1.1 ± 0.1	43 ± 3	1.0 ± 0.1
	10.05 ± 0.08	7.86 ± 0.02	76 ± 4	5.9 ± 0.2
CuO salt	1.06 ± 0.02	1.00 ± 0.02	N.M	1.0 ± 0.1

As regards the dissolution of CuO in clams, the values after 5 mins are elevated except CuO salt, in which the values are not determinable. The values most low of CuO concentration are normal but CuO NPs at initial concentration (10 ppm) represents a decrease over time (40%).

- CuO algae

Table 3.13. Values of Concentration and dissolution of the algae contaminated with CuO NP's and salt.

Particle	Total concentration 0h (ppm)	Total concentration 15mins (ppm)	Dissolution 5mins (ppb)	Total concentration 3days (ppm)	Dissolution 3 days (ppb)
CuO	1.19 ± 0.05	1.17 ± 0.07	24.1 ± 0.9	N.M	11.8 ± 0.3
	10.1 ± 0.6	1.5 ± 0.3	41 ± 2	N.M	36.1 ± 0.2
CuO salt	10.2 ± 0.9	1.8 ± 0.1	N.M	1.14 ± 0.05	N.M

On algae, the dissolution trend is similar to the clam trend but, in this case, there is a decrease after 3 days; instead, in the salt form there is a not determinable value. Total contamination values decrease over time until they become unmeasurable in CuO NPs.

- TEM Results

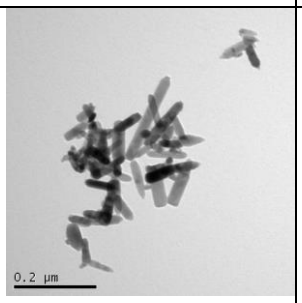
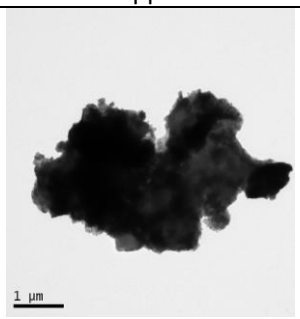
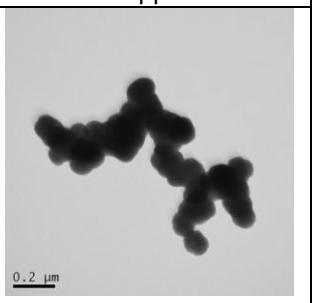
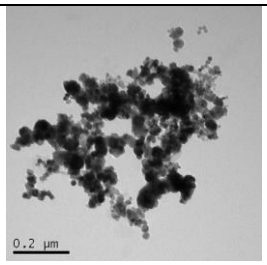
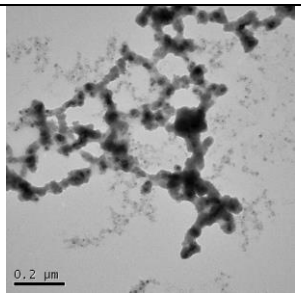
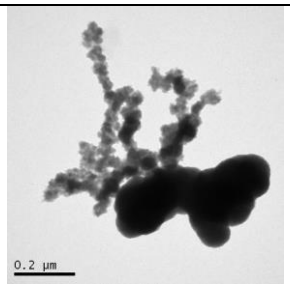
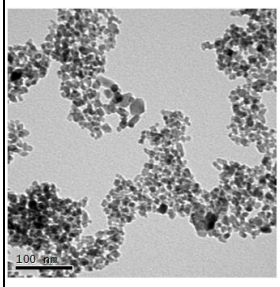
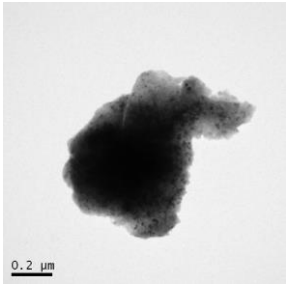
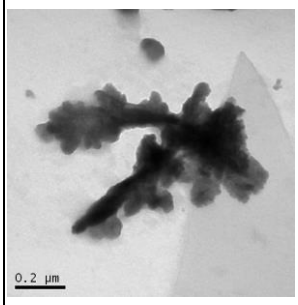
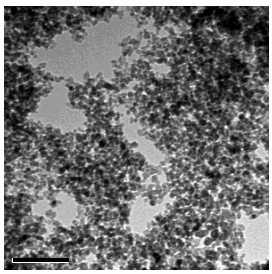
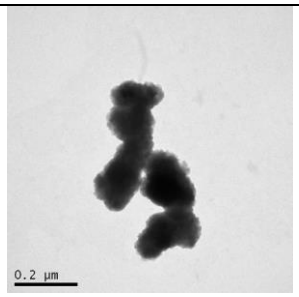
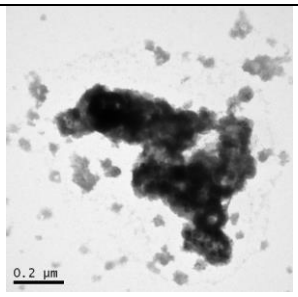
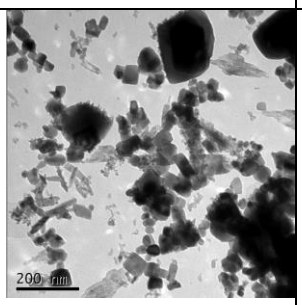
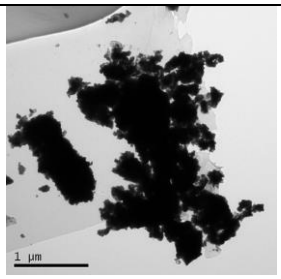
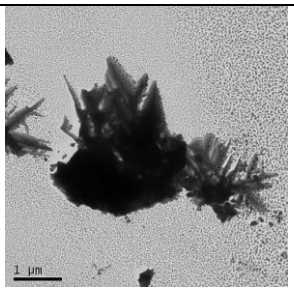
Particle	Pristine	1ppm	10ppm
ZnO			
ZnO/CeO_2			
TiO2			
TiO2 PVP			
CuO			

Figure 3.17. TEM imagines of pristine particles at 1 and 10 ppm for clams

ZnO and TiO_2 at 1 ppm appear most aggregated on respect to pristine form, but at 10 ppm appear most disaggregated to 1 ppm. For the other metals (ZnO/CeO_2, TiO_2 PVP and CuO), at 1 ppm the images show metals less aggregated or similar to the pristine form; at 10 ppm they appear most aggregated, in fact, CuO presents a crystallized form.

-TEM data

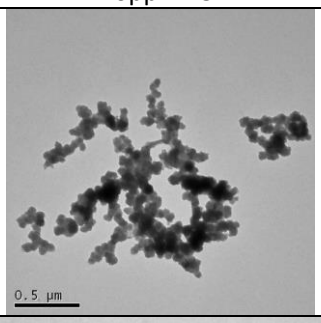
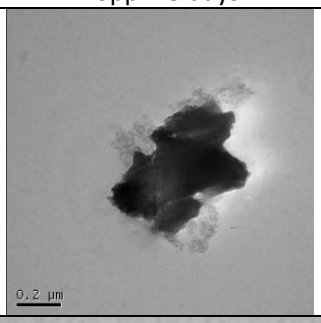
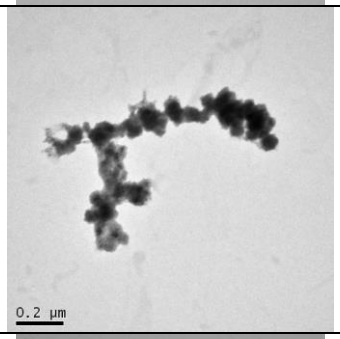
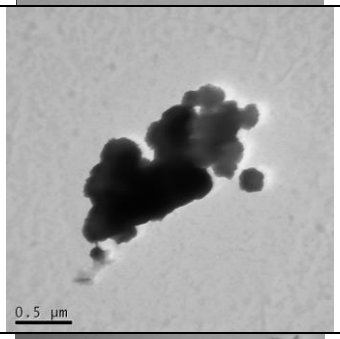
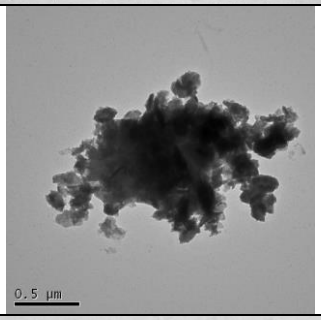
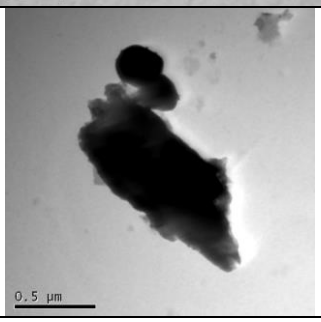
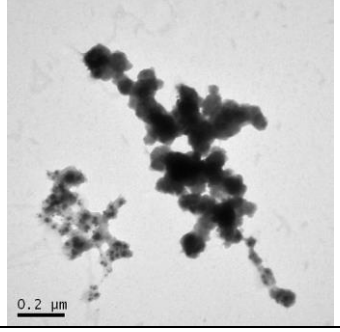
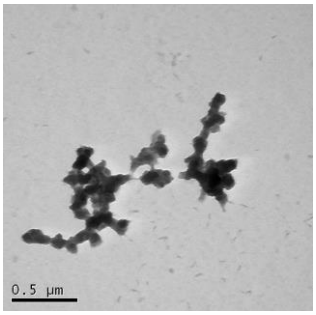
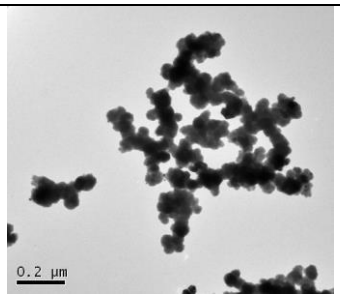
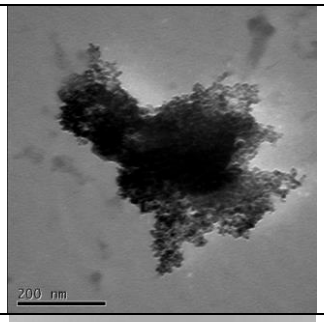
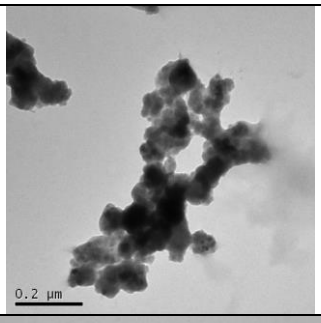
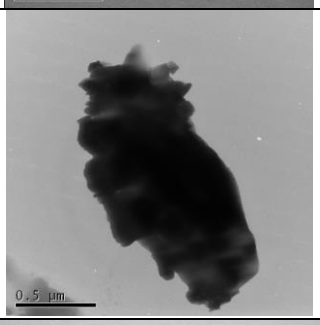
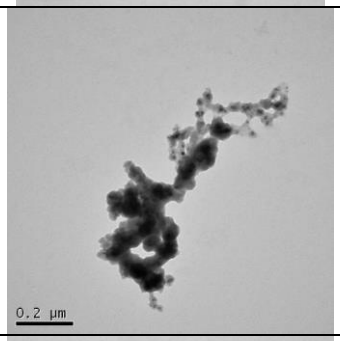
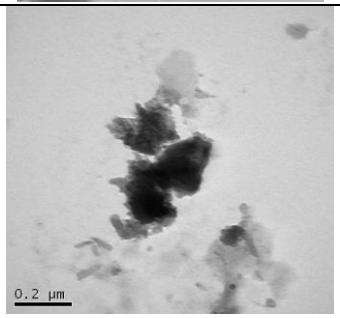
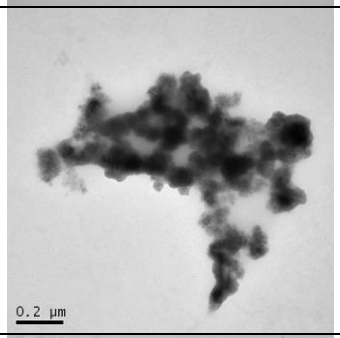
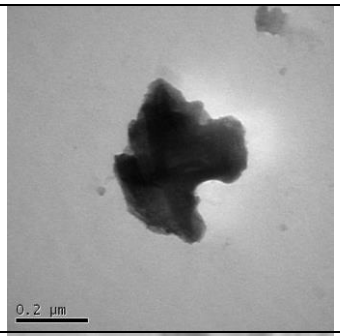
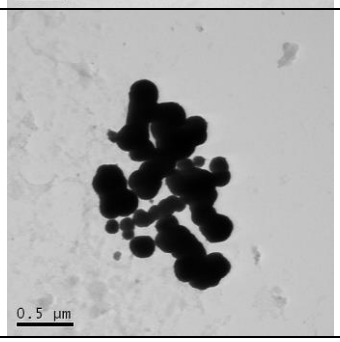
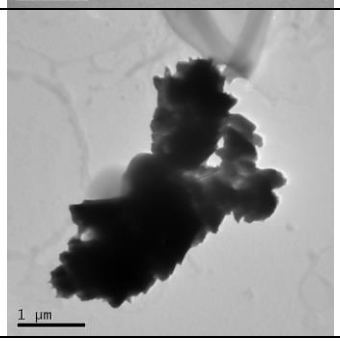
Particle	type	10ppm 15’	10ppm 3 days
ZnO	phaedo		
	tetra		
ZnO/CeO_2	phaedo		
	tetra		

Figure 3.18. TEM imagines of pristine particles at 10 ppm after 15 mins and 3 days for *Phaedodactylum tricornutum* and *Tetraselmis sp.* microalgae.

TiO_2	phaedo		
	tetra		
TiO_2 PVP	phaedo		
	tetra		
CuO	phaedo		

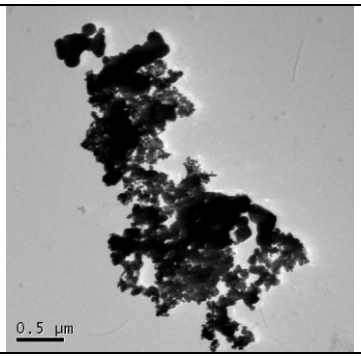
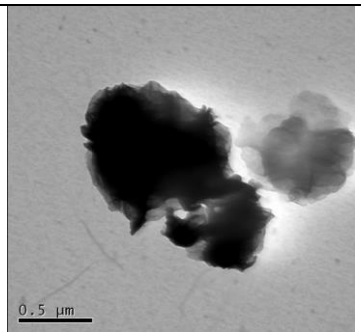
	tetra		

Figure 3.18.: Continued.

Two types of algae were been used in the experiment with clams: *Phaedodactylum tricornutum* and *Tetraselmis sp*. For ZnO of both algae at 10 ppm, after 15 mins and 3 days, the aspect of metal changes from branched to a compact mass.

A similar trend is also found in *Phaedodactylum* of ZnO/CeO_2, but not in *Tetraselmis*, in which metals results still distinguishable and they have an approximatively spherical form.

TiO_2 and TiO_2 PVP have a branched/spherical form and after present a compact/aggregated mass. The same is for CuO in both algae.

3.3.2. BIOACCUMULATION RESULTS

- Zinc

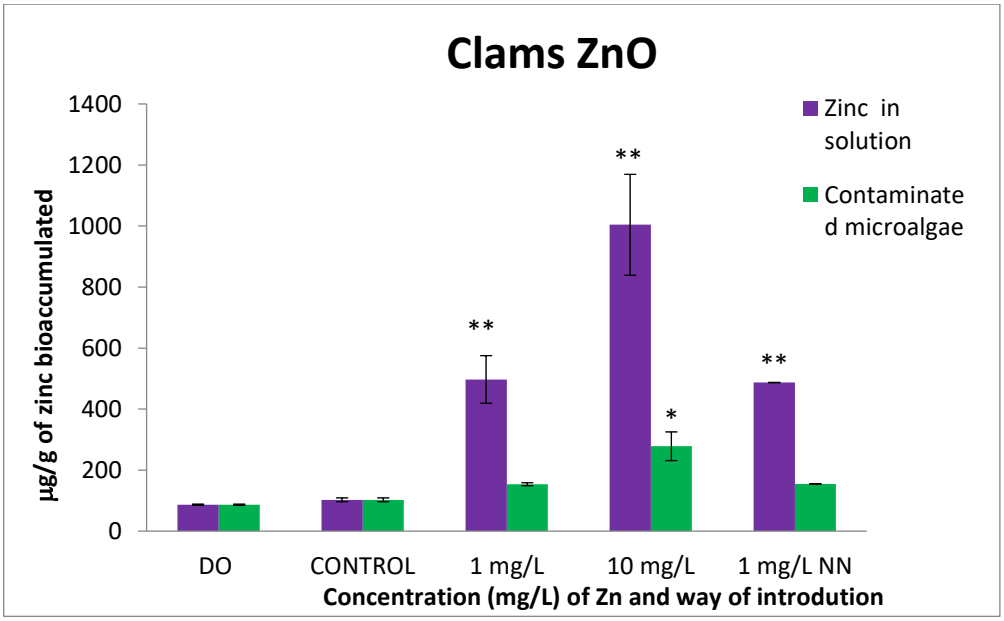


Figure 3.19. ANOVA statistical significance (*p<0.05, **p<0.01) for ZnO concentration (micrograms per grams of dry weigh measured in the whole organisms) respect to the Day 0, Control and groups of individuals.

The following bars graph shows in parallel the results of absorption of metal in the liquid phase (purple bars) and dietary intake of contaminated algae (green bars). It denotes that in the control there are not big differences of absorption between metal and contaminated algae, instead to the other concentrations it denotes an increased absorption of Zn in solution compared to absorption accumulated from algae. High concentrations of Zn inhibit the filtration rates of the algae and make them less attractive for clams.

It is also noted a similarity in 1 mg/L of Zn standard and 1 mg/L of Zn NPs, confirming the initial hypothesis in which was thought that they would not be distinct differences in the results.

From a statistical point of view, after 10 days of exposure to Zn NPs and no-nano, bioaccumulation with significance differences is been observed, compared to Control for both ZnO (nano and no-nano). Instead, there are no significance statistical differences between Zn NPs and ZnO of normal size at the same concentrations the major bioaccumulation is been observed at the highest concentration (10 mg/L).

- Zinc/Cerium

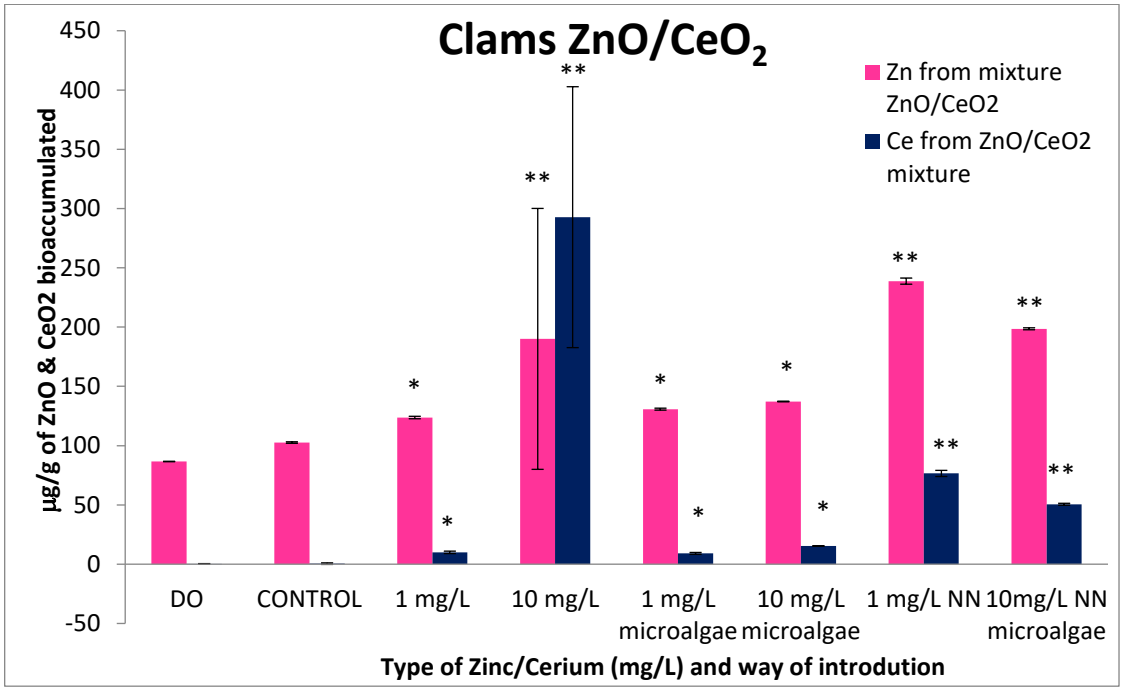


Figure 3.20. ANOVA statistical significance (*p<0.05, **p<0.01) for ZnO/CeO_2 mixture (micrograms per grams of dry weigh measured in the whole organisms) respect to the Day 0, Control and groups of individuals.

In this graph is reported the result of contaminated clams bioaccumulation with a mixture of ZnO/CeO2. In general, ZnO remains always at highest levels of absorption compared to CeO_2, for standard and NPs forms, and the same is for microalgae.

Only exception results in the mixture with the highest concentration on standard size (10 mg/L), in which the interaction between two metals makes that CeO_2 is bioaccumulated much more compared to ZnO; this fact it can be explained because CeO_2 increases his solubility at high concentrations. This phenomenon does not happen in microalgae, in which the absorption of CeO_2 is also smaller at high concentrations.

From a statistical perspective, everything is relevant result in relation to the fact that the proportion of metals in the mixture was 1: 1 for which we had expected levels of bioaccumulation almost 50% and this not so obvious difference.

- Copper

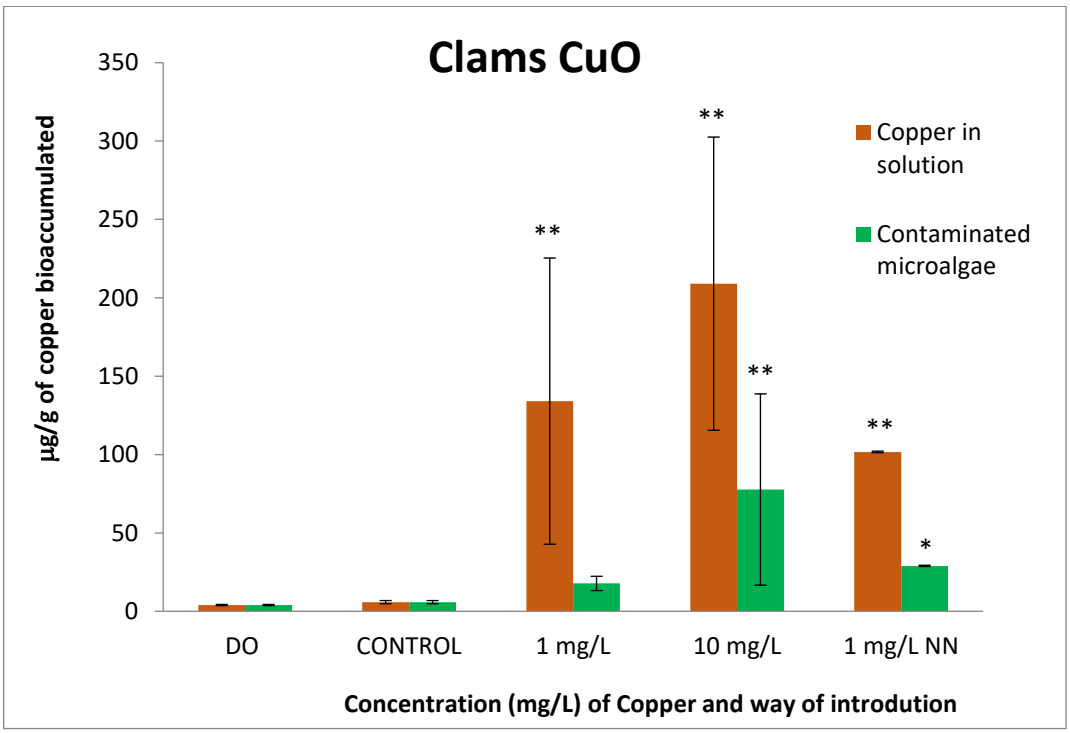


Figure3.21. ANOVA statistical significance (*p<0.05, **p<0.01) for CuO concentration (micrograms per grams of dry weigh measured in the whole organisms) respect to the Day 0, Control and groups of individuals.

In this following graph, the Control presents optimal values of contamination. As it increases the metal concentrations, introduced in the microcosm, in solution or by algae, its absorption increases in a clear way, especially in the dissolved phase. A slightly lower trend is been observed for values of 1 mg / L NPs dissolved, compared to the same in standard form, but always within normal limits. Contamination by microalgae results at minimum levels but there is an exception for the concentration 10 mg/L of CuO standard.

After 10 days of exposure to CuO NPs and no-nano, is been observed a bioaccumulation statistically significance differences compared to the Control, for both CuO (NPs and no-nano), even if is lower for CuO NPs (at the same concentration). Highest bioaccumulation is represented by highest concentration in which the amphipods were exposed (10mg/L).

As regards to bioaccumulation of CuO in the trophic chain, the highest value of bioaccumulation is been produced by highest concentration of CuO NP.

- Titanium

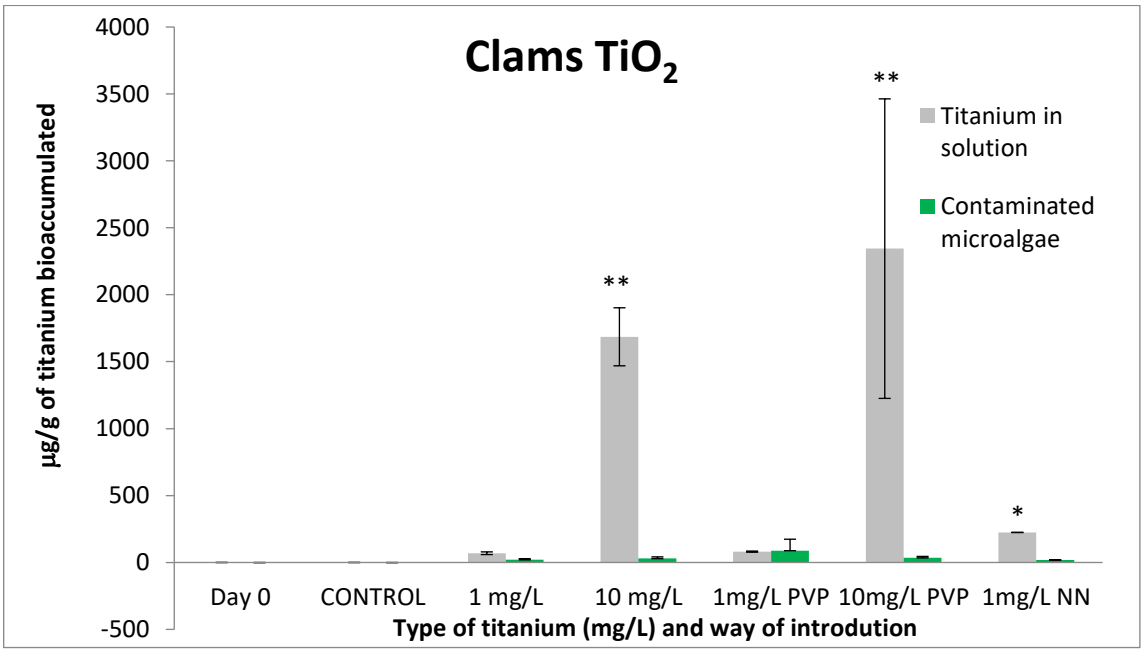


Figure 3.22. ANOVA statistical significance (*p<0.05, **p<0.01) for each TiO2 concentration of the micrograms per grams of dry weigh measured in the organism respect to the Day 0, Control and groups of individuals.

Titanium shows an anomalous trend compared to the other metals. Control at Day 0 has values near 0. The lower concentrations have minimum values of bioaccumulation for the metal (standard size, PVP coated and NPs) in dissolved phase and for contaminated microalgae.

Bioaccumulation analysis of Titanium shows a statistical significance for 10 mg/L of TiO_2 (standard size and PVP coated), for 1 mg/L of TiO_2 PVP and 1mg/L for TiO_2 NP, in solution.

After 10 days of exposure to TiO_2 (NPs, NPs PVP coated and TiO_2 standard size), were not found significance differences compared to Control for TiO_2 NPs.

TiO_2 standard accumulates much more respect to TiO_2 NPs, in the clam tissues.

In general, TiO_2 solubility is slow in all its form but this difference can be explained by the capability of TiO_2 forming aggregates.

Comparing TiO_2 PVP coated and TiO_2 not coated, TiO_2 PVP is been accumulated much more in the clam tissues for the same concentrations tested, being particularly evident the difference in the maximum concentration, both in the dissolved phase and in the microalgae.

On regards to TiO_2 by the diet, by the culture of two types of microalgae, in which were added 1 mg/L and 10 mg/L of TiO2 (with PVP, without PVP and standard size), the highest accumulations were in TiO_2 PVP NPs at 1 mg/L concentration. The growth of the algae is affected by high concentrations; in fact, the maximum bioaccumulation is with TiO_2 PVP in the sample of 1 mg/L.

Chapter 4: Discussions

4.1. Fertilization and development of sea urchin

The results obtained from toxicity on eggs and larvae of *P. lividus* tests, show that the fertilized eggs with contaminated semen are less sensitive to metal toxicity because a good percentage has been, successfully, fertilized; on the contrary, the larvae seem to suffer more because of the toxicity show gradual increases in skeletal deformations in relation to the increase of the concentrations of metals. Even in this case, no observed differences in responses between the metals in nano form that in those in no-nano form.

Below are mentioned other studies that confirm this thesis: such standardization would enable detection of effluents of relatively low toxicity, thus avoiding the high variability in toxicity classification associated with the sea urchin embryo development test, resulting from the different toxicity criteria used when counting deformities. Toxicity was characterized by use of two parameters: ECs and alterations in larval development. Identification of sea urchin malformations was carried out by applying the classical and the skeletal criteria. Both toxicity criteria identified the discharges from fish farm I and IV as the most and least toxic respectively. However, the results showed that the skeletal criterion was much more sensitive than the classical criterion for the assessing the toxicity of fish farm effluents. The skeletal criterion indicated subtle alterations in the larval structure that enabled abnormalities to be correlated with toxicant dilutions, and calculation of the effective concentrations [Carballeira C *et al*., 2011]. The effects of pollutants and the other stressors may be reflected in the sea urchin skeleton apparatus, either by the absence or the incorrect location of the skeletal rods, or by inactivation of the gene regulatory system underlying the development of the embryonic skeleton [Sharma T and Ettensohn CA, 2010]. In many studies the toxic effects of metal oxides NPs were largely ascribed to metal ions released into the suspension, this effect was also claimed for sea urchin exposed to ZnO NP [Fairbairn EA *et al*., 2011]. On the other hand, some authors observed that the adverse impacts of ZnO NP could not be completely explained by the release of metal ions [Miao L *et al*., 2010], and that metal NPs may be more toxic than both their ionic forms and their parent compounds [Navarro E *et al.,* 2008; Farré M *et al.,* 2009].

Another study suggested that the similarity of effects between ZnO NPs and ZnO (arrested development, skeletal abnormality) suggested ion dissolution was the main driver of toxicity in 96 h studies on embryo development of the white sea urchin *Lytechinus pictus* [Fairbairn *et al*., 2011]. Exposure to insoluble CeO_2 and iron oxide NPs at 10 mg/L resulted in total mortality after only 2 days [Falugi C *et al.*, 2012]. All types of NPs tended to agglomerate and were present in the digestive, immune and reproductive systems.

Despite not, specifically, using nanomaterials, the potential for CeO_2 toxicity to urchins has been demonstrated [Oral R *et al.*, 2010]. Similarly, exposures of *P.lividus* embryos exposed to various concentrations resulted in 100% mortality [Radenac G *et al.*, 2001].

Certainly, CeO_2 NPs are considered insoluble, however the dissolution potential of Cu^{2+} and Zn^{2+} form causes concern regarding exposure of sea urchin embryos. Some of the recorded effects on echinoderm are at the concentrations within the regulatory limit. That this effects are on embryos has implications for population growth and stability, especially in coastal waters where inputs will be less diffuse.

4.2. Metal and nano metal oxides effects on amphipods survival

The experiment results on amphipods of the species *A. brevicornis*, show that ZnO and CuO, in NPs component and the components in the standard sizes, does not adversely affect survival compared to mortality.
On the contrary, an experiment conducted by Dai L *et al.* (2012), shows that clam *Macoma balthica*, exposed to CuO NPs through spiked sediment at 200 microg/g for 35 days, has a mortality ranged between 2.4% and 15.5%, and clams burrowed more slowly and irregularity. The difference can be explained with a greater resistance of amphipods to the contamination by these specific metals, or they need a higher degree of intoxication to suffer the effects.

Many ENPs and especially metal oxides ENPs aggregates rapidly to the micro scale in seawater and settle from the water column [Keller AA *et al.*, 2010], where they accumulate in sediments [Buffet PE *et al*., 2013]. Commonly used metal oxides ENPs composed of ZnO, TiO_2 and CeO_2 have rates of aggregation and sedimentation that can vary with ENP concentrations [Keller AA *et al.*, 2010]: for example, ZnO ENPs that enter marine environments will settle and dissolve rapidly [Fairbairn EA *et al.*, 2011]. A study of the

species *Leptocheirus plumulosus* indicates that the mortality of amphipods can increase if you leave time to accumulate metals in the sediment. Amphipods play a key role in estuarine and coastal ecosystems as prey and bioturbators, and our study suggests that ENPs that build up in sediments over time will be accumulated by amphipods and reduce their survival, which would directly impact higher trophic levels by reducing their food supply and exposing them to ENPs [Hanna SK *et al.*, 2013].
Even in this case, however, it has been confirmed the hypothesis that there are no differences in the response between NPs metals and those of the normal size.

4.3. Bioaccumulation discussion of *Ruditapes philippinarum*

Regarding bioaccumulation, each individual metal analyzed shows a specific trend. ZnO for example, to each level and for all tester-organisms, not presents absorption peaks but has a linear trend that it seem not to be influenced by the concentration. The same thing is for CuO, although other studies have demonstrated that CuO tends to accumulate much more in NPs form: molluscs represent the most investigated taxonomic group with a special focus on mussels and clams. Gomes T *et al.* (2011), Gomes T *et al.* (2012) and Gomes T *et al.* (2013a) exposed adult *Mytilus galloprovincialis* at 0.01 mg/L of CuO NP for 15 days. They observed significant accumulation of Cu in soft tissues. According to Gomes T *et al.* (2012), since only a small fraction seemed to be related to the nano-form with aggregation playing a key role. In the experiment conducted by Dai L *et al.* (2013), *M.balthica* was been exposed to CuO NPs and in soft tissues Cu increased approximately 10 fold compared to the Control, while the condition index generally decreased over time, due to metal exposure, causing consumption and lack of nutrition.

Hence, independent of direct toxic effects, the presence of TiO_2 NPs may cause indirect effects, for example by influencing toxicity and bioaccumulation of other pollutants present in the aquatic environment.

The limited trace metal bioaccumulation is likely to be due to an enforced adaptation capability involving regulation mechanisms in the uptake, metabolism and elimination of metals within certain concentrations of contaminants [Moschino V *et al.*, 2012].

The interaction between the algal cells and the particles may be different due to the presence of a semi-permeable cell wall surrounding the algal cells. If the cell wall is penetrated,

passage through the plasma membrane by endocytosis may be possible [Navarro E *et al*., 2008]. This may directly lead to cell damage due to the effect of TiO_2, but could also facilitate the transport of the other environmental pollutants through cell membranes.

Also, according to Hartmann NB *et al*. (2010) a combination of light and TiO_2 can influence on the algal growth.

The differences in the effects of three particle types might be attributed mainly to differences in particle size, but also other parameters such as the crystalline composition, are expected to influence the toxicity of TiO_2.

As the results, inhibition found in the standard test cannot be claimed, with confidence, to be only dependent on TiO_2 concentration, and further experimental studies are needed to separate different influencing parameters (size, aggregation, aggregate structure, shape, crystallinity). Decreased growth rate in algal tests may also be caused by adhesion of TiO_2 to the cell surface. In another study by Aruoja V *et al*. (2009) nanosized particles were observed to cover the algal surfaces to a larger extent than the bulk particles, for which more particle-free algal cells were seen.

Different types of ENMs, such as TiO_2 standard and NPs, accumulate preferentially in the digestive system of bivalves [Ward JE and Kach DJ, 2009; Hull MS *et al.*, 2011; Zuykov M *et al.*, 2011a; Wegner A *et al.*, 2012; Al-Sid-Cheikh M *et al*., 2013]. The longer gut retention time usually indicates that the ENMs undergo extensive extracellular digestion or are transported to the digestive gland for complete intracellular digestion [Ward JE and Kach DJ, 2009; Hull MS *et al.*, 2011; Al-Sid-Cheikh M *et al.*, 2013].

As regards the ZnO/CeO_2 mixture, there are not many studies in regard and, for this aspect, the general idea, by analyzing results of the present study is that the absorption of ZnO is not influenced by the presence of CeO_2 but that it is precisely CeO_2 to be absorbed in a different way when it is associated with ZnO in two dimensions tested. The NP may adhere to a cell and block essential pores and membrane functions. Alternatively, it could also enter the cell by endocytosis, via diffusion through pores, or via ion transport-systems and all these effects can result with a range of sub-lethal effects including reduced growth and bioaccumulation.

4.4. General characterization of metal and nano metal oxides

The tests were designed to examine the responses of three model-organisms exposure to metals in the NPs form and standard size (salt metal oxides dissolved in distiller water), and compare the results with each other to confirm or refute the hypothesis, according to which there are no differences between the metals in the two forms tested.

As we have seen from the results of the test conducted in the laboratory, the four metal used (ZnO, ZnO/CeO_2 mixture, TiO_2 and CuO) have a pattern almost identical in both NPs form and in the form of salt but, also, in all three model-organisms. In particular, ZnO tends to decr and it tends to increase its dissolution over time. TiO_2 increases the level of dissolution so that a few times appears under the limit-values of determinability. In the case of ZnO/CeO_2 mixture in *A. brevicornis*, the values of ZnO increase; instead, the values of CeO_2 decrease but, in clams and microalgae both decrease; on the contrary, CuO tends to increase and, so, decrease the dissolution on amphipods and in sea urchins but, in clams and contaminated microalgae, values presents inverse trend. This results are identical both for aqueous matrix and for the sediment. Several studies have shown that CuO NPs do not readily dissolve in different aqueous media, including freshwater and seawater [Gomes T *et al.*, 2011], as it is often less than 3% of the original mass added that is reported to be in dissolved form [Griffitt RJ *et al.,* 2008; Misra SK *et al.,* 2012; Buffet PE *et al.*, 2014; Thit A *et al*., 2015]. Misra SK *et al.* (2014) reported a dissolution of 2.5 wt% from spherical CuO NPs, of 1.1 wt% for rod-shapped NPs and 0.8 wt% for spindle shaped CuO NPs [Misra SK *et al*., 2014].

Recent studies have shown that CuO NPs have a distinct biological effect not caused entirely by the release of soluble metal ions [Griffitt RJ *et al.,* 2009; Heinlaan M *et al.,* 2011].

The presence of organisms can affect particle dissolution in exposure medium [Griffitt RJ *et al.,* 2008].

Buffet PE *et al.* (2011) studied the fate and effects of CuO NPs from the same batch in seawater, and found that CuO NPs with initial size ranging from 10 to 100 nm highly aggregated/agglomerated [Buffet PE *et al*., 2011], and this has been observed in other publications [Gomes T *et al*., 2011].

In aquatic media particles tend to aggregate, and the extent of this aggregation is dependent on surface charge, particle shape and size, and pH of the medium. In saltwater, the increasing

salinity reduces the negativity of electrophoretic mobility of the particles to encourage aggregation [Batley GE *et al*, 2013]. Aggregation is correlates positively with concentration, presumably due to more particles being available for interaction [Fairbairn EA *et al.*, 2011; Miller RJ *et al*., 2010]. Aggregation reduces surface area and therefore dissolution potential, especially at high concentrations. Size also affects dissolution rates [Baker TJ *et al.*, 2013].

4.5. Conclusions

Nanoparticle toxicology in aquatic systems is complex. In the first instance particle size, shape, chemistry and capping agents will all play a role regarding the stability, and thus bioavailability, of the NP within any media. The major ways in which nanoparticles and no-nano particles may interact with an organism are: adsorption to the surface (cell, organ or body), cellular internalization, dissolution of ions from the NP and mechanistic nano-effect.

Current anthropogenic discharge of NPs and no-nano particles is not a volume that chronic exposure would be expected to be harmful to marine organisms. Acute exposure may have an extremely localized impact, but the diffuse nature of the oceans means effects will not be widespread. It is realistically only on beaches where continuous use of sunscreen may expose a large number of organisms to raw NPs following immersion, yet such scenarios are understudied. A fuller understanding of the long term consequences of these particles in the marine environment is warranted and, importantly, also a need to relate the effects and release of these NPs to biomarkers in ecologically relevant species that can be used to inform on potential impacts on marine systems. Moreover in the scientific field, the studies on no-nano metal oxides and their effects on marine organisms was very frequent but the innovation of this study is that they show similar effects of the nanoparticles. Providing a comparison between these two toxic sources in marine environment may be useful for improving ecotoxicological knowledge. The important thing that emerges from this study is that there is no significant differences between the size of the metals used, but it seems that the effects on organisms are related with increasing concentrations. The presence of the metals in transition environments is due to several factors, such as the presence of man that pours large amounts of harmful substances in water and these substances have a long residence time and therefore can be toxic to the organisms.

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Never stop dreaming!

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Annexes:

For 35 1981 - 2016 Years, Innovation has been at the heart of everything we do

INFORME Nº16/SEV/25717 Pag. 1/1

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11510 - Puerto Real - Cádiz

Sevilla 28-febrero-2016

Descripción muestra: Tres botes falcon con un total aproximado de 100 mL de agua marina.
Recepción muestra: EMPRESA MENSAJERIA

N/Referencia: FE/SEV/00629/16 S/Referencia: Agua Mar Casem.
Fecha recepción: 17-febrero-2016 12:29 Fecha inicio: 18-febrero-2016 Fecha finalización: 25-febrero-2016

RESULTADOS ANALÍTICOS:

- METALES Inicio: 18-febrero-2016 Final: 25-febrero-2016

Arsénico	<0.05 mg/L LC: 0.05 mg/L	Cadmio	<0.05 mg/L LC: 0.05 mg/L	Cerio	<0.05 mg/L LC: 0.05 mg/L
Cinc	<0.05 mg/L LC: 0.05 mg/L	Cobalto	<0.05 mg/L LC: 0.05 mg/L	Cobre	<0.05 mg/L LC: 0.05 mg/L
Cromo	<0.05 mg/L LC: 0.05 mg/L	Estaño	<0.05 mg/L LC: 0.05 mg/L	Hierro	<0.05 mg/L LC: 0.05 mg/L
Manganeso	0.50 mg/L LC: 0.05 mg/L	Mercurio	<0.05 mg/L LC: 0.05 mg/L	Níquel	<0.05 mg/L LC: 0.05 mg/L
Plata	<0.05 mg/L LC: 0.05 mg/L	Plomo	<0.05 mg/L LC: 0.05 mg/L	Titanio	<0.05 mg/L LC: 0.05 mg/L

Técnica analítica: ICP/MS
Método analítico: PNTe/LQM/FYQ/140

Dtor. Laboratorio: Luis Jesús Moreno López

Laboratorio Químico Microbiológico, S.A. SEVILLA

Laboratorio Autorizado por la Consejería de Agricultura y Pesca de Andalucía Nº A-206-AU. Toma de muestra no realizada por inspección oficial.
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LC: Límite de Cuantificación expresado en las mismas unidades y cifras significativas que el parámetro informado.

Análisis y Control Alimentario

Fecha impresión: 28/02/2016

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11510 - Puerto Real - Cádiz

Sevilla 5-febrero-2016

Descripción muestra: Sedimento marino en envase de plástico con 2Kg de peso aprox.
Recepción muestra: EMPRESA MENSAJERIA

N/Referencia: EN/SEV/00053/16 S/Referencia: REF: Ca-1
Fecha recepción: 11-enero-2016 11:08 Fecha inicio: 11-enero-2016 Fecha finalización: 5-febrero-2016

RESULTADOS ANALÍTICOS:

- ANALISIS DE SUELOS
Inicio: 18-enero-2016 Final: 22-enero-2016

Materia Orgánica Total 0.91 %
Técnica analítica: Varias
Método analítico: Métodos Oficiales del Ministerio de Agricultura, Pesca y Alimentación.

- BIFENILOS POLICLORADOS
Inicio: 11-enero-2016 Final: 12-enero-2016

PCB 28	<5,0 µg/Kg LC: 5,0 µg/Kg	PCB 52	<5,0 µg/Kg LC: 5,0 µg/Kg	PCB 101	<5,0 µg/Kg LC: 5,0 µg/Kg
PCB 118	<5,0 µg/Kg LC: 5,0 µg/Kg	PCB 138	<5,0 µg/Kg LC: 5,0 µg/Kg	PCB 153	<5,0 µg/Kg LC: 5,0 µg/Kg
PCB 180	<5,0 µg/Kg LC: 5,0 µg/Kg				

Técnica analítica: GC-MSD-QqQ
Método analítico: PNTe/LQM/FYQ/044

- GRANULOMETRIA
Inicio: 18-enero-2016 Final: 18-enero-2016

Particulas con tamaño mayor de 0.500 mm	3.50 % LC: 0.10 %	Del 18/01/16al 18/01/16
		Método analítico: PNT/LQM/FYQ/0189
		Técnica analítica: Tamización
Particulas con tamaño entre 0.250 y 0.500 mm	51.30 % LC: 0.10 %	Del 18/01/16al 18/01/16
		Método analítico: PNT/LQM/FYQ/0189
		Técnica analítica: Tamización
Particulas con tamaño entre 0.125 y 0.250 mm	43.50 % LC: 0.10 %	Del 18/01/16al 18/01/16
		Método analítico: PNT/LQM/FYQ/0189
		Técnica analítica: Tamización
Particulas con tamaño inferior a 0.125 mm	1.70 % LC: 0.10 %	Del 18/01/16al 18/01/16
		Método analítico: PNT/LQM/FYQ/0189
		Técnica analítica: Tamización

- HIDROCARBUROS AROMÁTICOS POLICÍCLICOS
Inicio: 11-enero-2016 Final: 12-enero-2016

Acenafteno	<5,0 µg/Kg LC: 5,0 µg/Kg	Acenaftileno	<5,0 µg/Kg LC: 5,0 µg/Kg	Antraceno	<5,0 µg/Kg LC: 5,0 µg/Kg
Benzo (a) antraceno	<5,0 µg/Kg LC: 5,0 µg/Kg	Benzo (a) pireno	<5,0 µg/Kg LC: 5,0 µg/Kg	Benzo (b) fluorantreno	<5,0 µg/Kg LC: 5,0 µg/Kg
Benzo (g,h,i) perileno	<5,0 µg/Kg LC: 5,0 µg/Kg	Benzo (k) fluorantreno	<5,0 µg/Kg LC: 5,0 µg/Kg	Criseno	<5,0 µg/Kg LC: 5,0 µg/Kg
Dibenzo (a,h) antraceno	<5,0 µg/Kg LC: 5,0 µg/Kg	Fenantreno	<5,0 µg/Kg LC: 5,0 µg/Kg	Fluorantreno	<5,0 µg/Kg LC: 5,0 µg/Kg
Fluoreno	<5,0 µg/Kg LC: 5,0 µg/Kg	Indeno (1,2,3) pireno	<5,0 µg/Kg LC: 5,0 µg/Kg	Naftaleno	<5,0 µg/Kg LC: 5,0 µg/Kg
Pireno	<5,0 µg/Kg LC: 5,0 µg/Kg				

Técnica analítica: CG-MSD-QqQ
Método analítico: PNTe/LQM/FYQ/040

Análisis y Control Alimentario

Fecha impresión: 05/02/2016

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Laboratorio Químico Microbiológico, S.A.

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Descripción muestra: Sedimento marino en envase de plástico con 2Kg de peso aprox.
Recepción muestra: EMPRESA MENSAJERIA
N/Referencia: EN/SEV/00053/16 S/Referencia: REF: Ca-1
Fecha recepción: 11-enero-2016 11:08 Fecha inicio: 11-enero-2016 Fecha finalización: 5-febrero-2016

- METALES PESADOS

Inicio: 11-enero-2016 Final: 5-febrero-2016

Arsénico	2.9 mg/Kg LC: 0.5 mg/Kg	Cadmio	<0.5 mg/Kg LC: 0.5 mg/Kg	Cerio	72.60 mg/Kg LC: 0.01 mg/Kg
Cinc	<20.0 mg/Kg LC: 20.0 mg/Kg	Cobalto	1.7 mg/Kg LC: 1.0 mg/Kg	Cobre	4.1 mg/Kg LC: 1.0 mg/Kg
Cromo	55.0 mg/Kg LC: 1.0 mg/Kg	Estaño	<1.0 mg/Kg LC: 1.0 mg/Kg	Hierro	1.100 % LC: 0.001 %
Manganeso	528.0 mg/Kg LC: 1.0 mg/Kg	Mercurio	<0.5 mg/Kg LC: 0.5 mg/Kg	Níquel	3.2 mg/Kg LC: 1.0 mg/Kg
Plata	<0.5 mg/Kg LC: 0.5 mg/Kg	Plomo	27.8 mg/Kg LC: 0.5 mg/Kg	Titanio	389.0 mg/Kg LC: 1.0 mg/Kg

Técnica analítica: ICP-OES
Método analítico: PNTe/LQM/FYQ/140

- MULTIRRESIDUOS PLAGUICIDAS GC-QQQ

Inicio: 11-enero-2016 Final: 12-enero-2016

35-dicloroanilina	<0.01 mg/Kg LC: 0.01 mg/Kg	4,4-Didorobenzofenona (Dicofol)	<0,01 mg/Kg LC: 0,01 mg/Kg	Acetocloro	<0,01 mg/Kg LC: 0,01 mg/Kg
Acrinatrina	<0.01 mg/Kg LC: 0.01 mg/Kg	Alaclor	<0.01 mg/Kg LC: 0.01 mg/Kg	Aldrin	<0.01 mg/Kg LC: 0.01 mg/Kg
Antraquinona	<0,01 mg/Kg LC: 0,01 mg/Kg	Atrazina	<0.01 mg/Kg LC: 0.01 mg/Kg	Azaconazol	<0,01 mg/Kg LC: 0,01 mg/Kg
Benfluralina	<0.01 mg/Kg LC: 0.01 mg/Kg	Benfuresato	<0.01 mg/Kg LC: 0.01 mg/Kg	Bifentrin	<0.01 mg/Kg LC: 0.01 mg/Kg
Bromofos Etil	<0.01 mg/Kg LC: 0.01 mg/Kg	Bromopropilato	<0.01 mg/Kg LC: 0.01 mg/Kg	Bupirimato	<0.01 mg/Kg LC: 0.01 mg/Kg
Buprofezin	<0.01 mg/Kg LC: 0.01 mg/Kg	Butafenacil	<0.01 mg/Kg LC: 0.01 mg/Kg	Butoxido de Piperonilo	<0.01 mg/Kg LC: 0.01 mg/Kg
Butralin	<0.01 mg/Kg LC: 0.01 mg/Kg	Cadusafos	<0.01 mg/Kg LC: 0.01 mg/Kg	Chinometionato	<0.01 mg/Kg LC: 0.01 mg/Kg
Cianazina	<0.01 mg/Kg LC: 0.01 mg/Kg	Cianofos	<0.01 mg/Kg LC: 0.01 mg/Kg	Cihalotrin lambda	<0.01 mg/Kg LC: 0.01 mg/Kg
Ciproconazol	<0.01 mg/Kg LC: 0.01 mg/Kg	Ciprodinil	<0.01 mg/Kg LC: 0.01 mg/Kg	Clomazone	<0.01 mg/Kg LC: 0.01 mg/Kg
Clorfenson	<0.01 mg/Kg LC: 0.01 mg/Kg	Clorfenvinfos	<0.01 mg/Kg LC: 0.01 mg/Kg	Clormefos	<0.01 mg/Kg LC: 0.01 mg/Kg
Cloropropilato	<0.01 mg/Kg LC: 0.01 mg/Kg	Clorpirifos Etil	<0.01 mg/Kg LC: 0.01 mg/Kg	Clorpirifos Metil	<0.01 mg/Kg LC: 0.01 mg/Kg
Clorprofam	<0.01 mg/Kg LC: 0.01 mg/Kg	Clortal Dimetil	<0.01 mg/Kg LC: 0.01 mg/Kg	Clortion	<0.01 mg/Kg LC: 0.01 mg/Kg
Clortiophos	<0.01 mg/Kg LC: 0.01 mg/Kg	Crimide	<0.01 mg/Kg LC: 0.01 mg/Kg	Cumafos	<0.01 mg/Kg LC: 0.01 mg/Kg
Demetrin	<0.01 mg/Kg LC: 0.01 mg/Kg	Diazinon	<0.01 mg/Kg LC: 0.01 mg/Kg	Dicaptom	<0.01 mg/Kg LC: 0.01 mg/Kg
Diclobenil	<0.01 mg/Kg LC: 0.01 mg/Kg	Diclobutrazol	<0.01 mg/Kg LC: 0.01 mg/Kg	Diclofention	<0.01 mg/Kg LC: 0.01 mg/Kg
Diclofop metil	<0.01 mg/Kg LC: 0.01 mg/Kg	Dieldrin	<0.01 mg/Kg LC: 0.01 mg/Kg	Difenilamina	<0.01 mg/Kg LC: 0.01 mg/Kg
Dimetenamida	<0.01 mg/Kg LC: 0.01 mg/Kg	Dimetoato	<0.01 mg/Kg LC: 0.01 mg/Kg	Dimoxistrobin	<0.01 mg/Kg LC: 0.01 mg/Kg
Diniconazol	<0.01 mg/Kg LC: 0.01 mg/Kg	Dioxation	<0.01 mg/Kg LC: 0.01 mg/Kg	Dipropetrin	<0.01 mg/Kg LC: 0.01 mg/Kg
Disulfoton	<0.01 mg/Kg LC: 0.01 mg/Kg	Endrin	<0.01 mg/Kg LC: 0.01 mg/Kg	EPN	<0.01 mg/Kg LC: 0.01 mg/Kg
Etaconazol	<0.01 mg/Kg LC: 0.01 mg/Kg	Etion	<0.01 mg/Kg LC: 0.01 mg/Kg	Etofumesato	<0.01 mg/Kg LC: 0.01 mg/Kg
Etrimfos	<0.01 mg/Kg LC: 0.01 mg/Kg	Famfur	<0.01 mg/Kg LC: 0.01 mg/Kg	Fenarimol	<0.01 mg/Kg LC: 0.01 mg/Kg
Fenazaquin	<0.01 mg/Kg LC: 0.01 mg/Kg	Fenbuconazaol	<0.01 mg/Kg LC: 0.01 mg/Kg	Fenclorfos	<0.01 mg/Kg LC: 0.01 mg/Kg
Fenitrotion	<0.01 mg/Kg LC: 0.01 mg/Kg	Fenpropatrin	<0.01 mg/Kg LC: 0.01 mg/Kg	Fenpropimorf	<0.01 mg/Kg LC: 0.01 mg/Kg
Fention	<0.01 mg/Kg LC: 0.01 mg/Kg	Fentoato	<0.01 mg/Kg LC: 0.01 mg/Kg	Fipronil	<0.004 mg/Kg LC: 0.004 mg/Kg
Fluatriafol	<0.01 mg/Kg LC: 0.01 mg/Kg	Fluazifop butil	<0.01 mg/Kg LC: 0.01 mg/Kg	Flucloralina	<0.01 mg/Kg LC: 0.01 mg/Kg
Fludioxinil	<0.01 mg/Kg LC: 0.01 mg/Kg	Flumetralina	<0.01 mg/Kg LC: 0.01 mg/Kg	Fluotrimazol	<0.01 mg/Kg LC: 0.01 mg/Kg
Fluquinconazol	<0.01 mg/Kg LC: 0.01 mg/Kg	Flutolanil	<0.01 mg/Kg LC: 0.01 mg/Kg	Fonofos	<0.01 mg/Kg LC: 0.01 mg/Kg
Formotion	<0.01 mg/Kg LC: 0.01 mg/Kg	Fosfamidon	<0.01 mg/Kg LC: 0.01 mg/Kg	Fosmet	<0.01 mg/Kg LC: 0.01 mg/Kg
Furalaxil	<0.01 mg/Kg LC: 0.01 mg/Kg	HCH-alfa	<0.01 mg/Kg LC: 0.01 mg/Kg	HCH-beta	<0.01 mg/Kg LC: 0.01 mg/Kg

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LC: Límite de Cuantificación expresado en las mismas unidades y cifras significativas que el parámetro informado.

Análisis y Control Alimentario

Fecha impresión: 05/02/2016

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Fecha recepción: 11-enero-2016 11:08 Fecha inicio: 11-enero-2016 Fecha finalización: 5-febrero-2016

- MULTIRRESIDUOS PLAGUICIDAS GC-QQQ [Continuación]

Inicio: 11-enero-2016 Final: 12-enero-2016

HCH-delta	<0.01 mg/kg LC: 0.01 mg/kg	Heptacloro	<0.01 mg/kg LC: 0.01 mg/kg	Heptenofos	<0.01 mg/kg LC: 0.01 mg/kg
Hexacinona	<0.01 mg/kg LC: 0.01 mg/kg	Hexaclorobenceno	<0.01 mg/kg LC: 0.01 mg/kg	Hexaconazol	<0.01 mg/kg LC: 0.01 mg/kg
Iodofenfos	<0.01 mg/kg LC: 0.01 mg/kg	Iprobenfos	<0.01 mg/kg LC: 0.01 mg/kg	Isazofos	<0.01 mg/kg LC: 0.01 mg/kg
Isofenfos	<0.01 mg/kg LC: 0.01 mg/kg	Isofenfos metil	<0.01 mg/kg LC: 0.01 mg/kg	Kresoxim Metil	<0.01 mg/kg LC: 0.01 mg/kg
Leptofos	<0.01 mg/kg LC: 0.01 mg/kg	Lindano	<0.01 mg/kg LC: 0.01 mg/kg	Malation	<0.01 mg/kg LC: 0.01 mg/kg
Mecarbam	<0.01 mg/kg LC: 0.01 mg/kg	Mefenpir dietil	<0.01 mg/kg LC: 0.01 mg/kg	Mepanipirin	<0.01 mg/kg LC: 0.01 mg/kg
Metacrifos	<0.01 mg/Kg LC: 0.01 mg/Kg	Metalaxil	<0.01 mg/kg LC: 0.01 mg/kg	Metazacloro	<0.01 mg/Kg LC: 0.01 mg/Kg
Metidation	<0.01 mg/kg LC: 0.01 mg/kg	Metolaclor	<0.01 mg/kg LC: 0.01 mg/kg	Metoprotrina	<0.01 mg/kg LC: 0.01 mg/kg
Metribuzin	<0.01 mg/kg LC: 0.01 mg/kg	Mevinfos	<0.01 mg/kg LC: 0.01 mg/kg	Miclobutanil	<0.01 mg/kg LC: 0.01 mg/kg
Napropamida	<0.01 mg/kg LC: 0.01 mg/kg	Nitrofen	<0.01 mg/kg LC: 0.01 mg/kg	Nitrotal isopropil	<0.01 mg/kg LC: 0.01 mg/kg
Nuarimol	<0.01 mg/kg LC: 0.01 mg/kg	Ometoato	<0.01 mg/kg LC: 0.01 mg/kg	Ortofenilfenol	<0.01 mg/kg LC: 0.01 mg/kg
Oxadixil	<0.01 mg/kg LC: 0.01 mg/kg	Oxadizon	<0.01 mg/kg LC: 0.01 mg/kg	Oxifluorfen	<0.01 mg/kg LC: 0.01 mg/kg
Paclobutrazol	<0.01 mg/kg LC: 0.01 mg/kg	Paration Etil	<0.01 mg/kg LC: 0.01 mg/kg	Paration Metil	<0.01 mg/kg LC: 0.01 mg/kg
Penconazol	<0.01 mg/kg LC: 0.01 mg/kg	Pendimetalina	<0.01 mg/kg LC: 0.01 mg/kg	Pentacloroanisol	<0.01 mg/kg LC: 0.01 mg/kg
Picloxistrobin	<0.01 mg/kg LC: 0.01 mg/kg	Pirazofos	<0.01 mg/kg LC: 0.01 mg/kg	Piridafention	<0.01 mg/kg LC: 0.01 mg/kg
Pirifenox	<0.01 mg/kg LC: 0.01 mg/kg	Pirimetanil	<0.01 mg/kg LC: 0.01 mg/kg	Pirimicarb	<0.01 mg/kg LC: 0.01 mg/kg
Pirimifos Etil	<0.01 mg/kg LC: 0.01 mg/kg	Pirimifos Metil	<0.01 mg/kg LC: 0.01 mg/kg	Piriproxifeno	<0.01 mg/kg LC: 0.01 mg/kg
Procimidona	<0.01 mg/kg LC: 0.01 mg/kg	Profenofos	<0.01 mg/kg LC: 0.01 mg/kg	Profluralina	<0.01 mg/kg LC: 0.01 mg/kg
Prometrina	<0.01 mg/kg LC: 0.01 mg/kg	Propacloro	<0.01 mg/kg LC: 0.01 mg/kg	Propanil	<0.01 mg/kg LC: 0.01 mg/kg
Propetamfos	<0.01 mg/kg LC: 0.01 mg/kg	Propiconazol	<0.01 mg/kg LC: 0.01 mg/kg	Propizamida	<0.01 mg/kg LC: 0.01 mg/kg
Propoxur	<0.01 mg/kg LC: 0.01 mg/kg	Protiofos	<0.01 mg/kg LC: 0.01 mg/kg	Quinalfos	<0.01 mg/kg LC: 0.01 mg/kg
Quinoxifen	<0.01 mg/kg LC: 0.01 mg/kg	Quintozeno	<0.01 mg/kg LC: 0.01 mg/kg	Simazina	<0.01 mg/kg LC: 0.01 mg/kg
Simetrina	<0.01 mg/kg LC: 0.01 mg/kg	Sulprofos	<0.01 mg/kg LC: 0.01 mg/kg	Tebuconazol	<0.01 mg/kg LC: 0.01 mg/kg
Tebufenpirad	<0.01 mg/kg LC: 0.01 mg/kg	Tebupirimfos	<0.01 mg/kg LC: 0.01 mg/kg	Tecnazeno	<0.01 mg/kg LC: 0.01 mg/kg
Teflutrina	<0.01 mg/kg LC: 0.01 mg/kg	Terbacilo	<0.01 mg/kg LC: 0.01 mg/kg	Terbufos	<0.01 mg/kg LC: 0.01 mg/kg
Terbutilazina	<0.01 mg/kg LC: 0.01 mg/kg	Terbutrina	<0.01 mg/kg LC: 0.01 mg/kg	Tetrametrina	<0.01 mg/kg LC: 0.01 mg/kg
Tetrasul	<0.01 mg/kg LC: 0.01 mg/kg	Tolclofos Metil	<0.01 mg/kg LC: 0.01 mg/kg	Triazofos	<0.01 mg/kg LC: 0.01 mg/kg
Tricloronato	<0.01 mg/kg LC: 0.01 mg/kg	Trifluralina	<0.01 mg/kg LC: 0.01 mg/kg	Vinclozolina	<0.01 mg/kg LC: 0.01 mg/kg

Técnica analítica: GC-MSD-QqQ
Método analítico: Quechers Gases PNTe/LQM/FYQ/216

- MULTIRRESIDUOS PLAGUICIDAS LC-QQQ

Inicio: 12-enero-2016 Final: 13-enero-2016

Abamectina	<0,01 mg/Kg LC: 0,01 mg/Kg	Acefato	<0.01 mg/Kg LC: 0.01 mg/Kg	Acetamiprid	<0,01 mg/Kg LC: 0,01 mg/Kg
Aldicarb	<0.01 mg/Kg LC: 0.01 mg/Kg	Aldicarb sulfona	<0.01 mg/Kg LC: 0.01 mg/Kg	Aldicarb sulfoxido	<0.01 mg/Kg LC: 0.01 mg/Kg
Atrazina	<0,01 mg/Kg LC: 0,01 mg/Kg	Azoxistrobina	<0,01 mg/Kg LC: 0,01 mg/Kg	Bendiocarb	<0.01 mg/Kg LC: 0.01 mg/Kg
Boscalida (Nicobifen)	<0.01 mg/Kg LC: 0.01 mg/Kg	Brodifacoum	<0,01 mg/Kg LC: 0,01 mg/Kg	Bromuconazol	<0.01 mg/Kg LC: 0.01 mg/Kg
Butoxicarboxim	<0.01 mg/Kg LC: 0.01 mg/Kg	Buturon	<0,01 mg/Kg LC: 0,01 mg/Kg	Carbaril	<0.01 mg/Kg LC: 0.01 mg/Kg
Carbendacima	<0,01 mg/Kg LC: 0,01 mg/Kg	Carbofuran 3-OH	<0.01 mg/Kg LC: 0.01 mg/Kg	Carbofurano	<0,01 mg/Kg LC: 0,01 mg/Kg
Cianofenfos	<0.01 mg/Kg LC: 0.01 mg/Kg	Ciazofamida	<0.01 mg/Kg LC: 0.01 mg/Kg	Cicloxidim	<0.01 mg/Kg LC: 0.01 mg/Kg

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- MULTIRRESIDUOS PLAGUICIDAS LC-QQQ [Continuación]

Inicio: 12-enero-2016 Final: 13-enero-2016

Cimoxanilo	<0,01 mg/kg LC: 0,01 mg/kg	Cletodim	<0.01 mg/Kg LC: 0.01 mg/Kg	Clofentezina	<0,01 mg/kg LC: 0,01 mg/kg
Clorbromuron	<0,01 mg/Kg LC: 0,01 mg/Kg	Cloroxuron	<0,01 mg/Kg LC: 0,01 mg/Kg	Clorsulfuron	<0,01 mg/Kg LC: 0,01 mg/Kg
Clortoluron	<0,01 mg/Kg LC: 0,01 mg/Kg	Clotianidina	<0.01 mg/Kg LC: 0.01 mg/Kg	Demeton-S-metil	<0,01 mg/Kg LC: 0,01 mg/Kg
Dietofencarb	<0.01 mg/Kg LC: 0.01 mg/Kg	Difenoxuron	<0,01 mg/Kg LC: 0,01 mg/Kg	Diflubenzuron	<0,01 mg/Kg LC: 0,01 mg/Kg
Diflufenican	<0,01 mg/kg LC: 0,01 mg/kg	Dimetomorfo	<0.01 mg/Kg LC: 0.01 mg/Kg	Diniconazol	<0,01 mg/kg LC: 0,01 mg/kg
Dioxacarb	<0.01 mg/Kg LC: 0.01 mg/Kg	Ditalimfos	<0.01 mg/Kg LC: 0.01 mg/Kg	Diuron	<0,01 mg/Kg LC: 0,01 mg/Kg
Dodina	<0.01 mg/Kg LC: 0.01 mg/Kg	E-Fenpiroximato	<0.01 mg/Kg LC: 0.01 mg/Kg	Emamectina	<0.01 mg/Kg LC: 0.01 mg/Kg
Epoxiconazol	<0,01 mg/kg LC: 0,01 mg/kg	Espiromesifeno	<0,01 mg/kg LC: 0,01 mg/kg	Espiroxamina	<0.01 mg/Kg LC: 0.01 mg/Kg
Etiofencarb	<0.01 mg/Kg LC: 0.01 mg/Kg	Etiofencarb Sulfona	<0.01 mg/Kg LC: 0.01 mg/Kg	Etiofencarb Sulfoxido	<0.01 mg/Kg LC: 0.01 mg/Kg
Etoxazol	<0.01 mg/Kg LC: 0.01 mg/Kg	Famoxadona	<0,01 mg/kg LC: 0,01 mg/kg	Fenazaquina	<0,01 mg/kg LC: 0,01 mg/kg
Fenbuconazol	<0,01 mg/kg LC: 0,01 mg/kg	Fenbutatim Oxido	<0.01 mg/Kg LC: 0.01 mg/Kg	Fenhexamida	<0,01 mg/Kg LC: 0,01 mg/Kg
Fenmedifan	<0.01 mg/Kg LC: 0.01 mg/Kg	Fenoxicarb	<0.01 mg/Kg LC: 0.01 mg/Kg	Fenpropimorfo	<0.01 mg/Kg LC: 0.01 mg/Kg
Flonicamid	<0.01 mg/Kg LC: 0.01 mg/Kg	Flufenoxuron	<0,01 mg/Kg LC: 0,01 mg/Kg	Fluometuron	<0,01 mg/Kg LC: 0,01 mg/Kg
Formetanato	<0.01 mg/Kg LC: 0.01 mg/Kg	Furatiocarb	<0,01 mg/Kg LC: 0,01 mg/Kg	Hexaflumuron	<0,01 mg/Kg LC: 0,01 mg/Kg
Hexitiazox	<0,01 mg/Kg LC: 0,01 mg/Kg	Imazalil	<0,01 mg/Kg LC: 0,01 mg/Kg	Imidaclorprid	<0,01 mg/Kg LC: 0,01 mg/Kg
Indoxacarbo	<0,01 mg/Kg LC: 0,01 mg/Kg	Iprovalicarbo	<0,01 mg/Kg LC: 0,01 mg/Kg	Isocarbofos	<0,01 mg/kg LC: 0,01 mg/kg
Linuron	<0,01 mg/Kg LC: 0,01 mg/Kg	Lufenuron	<0,01 mg/Kg LC: 0,01 mg/Kg	Mepanipirima	<0.01 mg/Kg LC: 0.01 mg/Kg
Mepronilo	<0.01 mg/Kg LC: 0.01 mg/Kg	Metalaxilo-M	<0.01 mg/Kg LC: 0.01 mg/Kg	Metamidofos	<0.01 mg/Kg LC: 0.01 mg/Kg
Metamitrona	<0.01 mg/Kg LC: 0.01 mg/Kg	Metiocarb	<0,01 mg/kg LC: 0,01 mg/kg	Metiocarb sulfona	<0,01 mg/kg LC: 0,01 mg/kg
Metiocarb sulfóxido	<0,01 mg/kg LC: 0,01 mg/kg	Metobromuron	<0,01 mg/Kg LC: 0,01 mg/Kg	Metomilo	<0,01 mg/Kg LC: 0,01 mg/Kg
Metoxifenozida	<0,01 mg/kg LC: 0,01 mg/kg	Metoxuron	<0,01 mg/Kg LC: 0,01 mg/Kg	Monolinuron	<0,01 mg/Kg LC: 0,01 mg/Kg
Monuron	<0,01 mg/Kg LC: 0,01 mg/Kg	Neburon	<0,01 mg/Kg LC: 0,01 mg/Kg	Nitempiran	<0,01 mg/kg LC: 0,01 mg/kg
Oxadiazon	<0,01 mg/kg LC: 0,01 mg/kg	Oxadixilo	<0,01 mg/Kg LC: 0,01 mg/Kg	Oxamilo	<0,01 mg/Kg LC: 0,01 mg/Kg
Oxamilo oxima	<0,01 mg/kg LC: 0,01 mg/kg	Oxydemeton metilo	<0.01 mg/Kg LC: 0.01 mg/Kg	Pimetrozina	<0,01 mg/kg LC: 0,01 mg/kg
Piraclostrobina	<0.01 mg/Kg LC: 0.01 mg/Kg	Pirimicarb	<0.01 mg/Kg LC: 0.01 mg/Kg	Promecarb	<0.01 mg/Kg LC: 0.01 mg/Kg
Prometrina	<0.01 mg/Kg LC: 0.01 mg/Kg	Propamocarb	<0.01 mg/Kg LC: 0.01 mg/Kg	Propargita	<0.01 mg/Kg LC: 0.01 mg/Kg
Propoxur	<0.01 mg/Kg LC: 0.01 mg/Kg	Prosulfocarb	<0.01 mg/Kg LC: 0.01 mg/Kg	Rotenona	<0,01 mg/Kg LC: 0,01 mg/Kg
Simazina	<0,01 mg/Kg LC: 0,01 mg/Kg	Spinosad (A+D)	<0,01 mg/Kg LC: 0,01 mg/Kg	Tebufenocida	<0.01 mg/Kg LC: 0.01 mg/Kg
Tebufenpirad	<0.01 mg/Kg LC: 0.01 mg/Kg	Teflubenzuron	<0,01 mg/Kg LC: 0,01 mg/Kg	Tiabendazol	<0,01 mg/Kg LC: 0,01 mg/Kg
Tiaclorprid	<0,01 mg/Kg LC: 0,01 mg/Kg	Tiametoxam	<0,01 mg/Kg LC: 0,01 mg/Kg	Tiobencarb	<0.01 mg/Kg LC: 0.01 mg/Kg
Tiodicarb	<0.01 mg/Kg LC: 0.01 mg/Kg	Tridemorfo	<0.01 mg/Kg LC: 0.01 mg/Kg	Triflumuron	<0,01 mg/Kg LC: 0,01 mg/Kg
Triforina	<0.01 mg/Kg LC: 0.01 mg/Kg	Zoxamida	<0.01 mg/Kg LC: 0.01 mg/Kg		

Técnica analítica: HPLC-QqQ
Método analítico: PNTe/LQM/FYQ/199

- SULFONATOS DE ALQUILBENCENO LINEALES (LAS)

Inicio: 19-enero-2016 Final: 4-febrero-2016

C-11 LAS	<5.0 µg/Kg (ms)	LC: 5.0 µg/Kg (ms)	Del 19/01/16al 04/02/16
			Método analítico: Interno LQM, S.A. - Sevilla
			Técnica analítica: HPLC-MS-QqQ
C-12 LAS	<5.0 µg/Kg (ms)	LC: 5.0 µg/Kg (ms)	Del 19/01/16al 04/02/16
			Método analítico: Interno LQM, S.A. - Sevilla
			Técnica analítica: HPLC-MS-QqQ

Laboratorio Autorizado por la Consejería de Agricultura y Pesca de Andalucía Nº A-206-AU. Toma de muestra no realizada por inspección oficial.
Las incertidumbres de cada uno de los ensayos están calculadas por el laboratorio y a disposición del cliente que las solicite.
Este informe sólo afecta a la muestra sometida a ensayo. Este informe no deberá reproducirse parcialmente sin la aprobación por escrito de este Laboratorio.
LC: Límite de Cuantificación expresado en las mismas unidades y cifras significativas que el parámetro informado.

Análisis y Control Alimentario

Fecha impresión: 05/02/2016

GLOBALG.A.P.
Miembro Asociado.
Miembro del Comité Técnico Nacional español.

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Descripción muestra: Sedimento marino en envase de plástico con 2Kg de peso aprox.
Recepción muestra: EMPRESA MENSAJERIA
N/Referencia: EN/SEV/00053/16 S/Referencia: REF: Ca-1
Fecha recepción: 11-enero-2016 11:08 Fecha inicio: 11-enero-2016 Fecha finalización: 5-febrero-2016

- SULFONATOS DE ALQUILBENCENO LINEALES (LAS) [Continuación] Inicio: 19-enero-2016 Final: 4-febrero-2016

C-13 LAS	<5.0 µg/Kg (ms)	LC: 5.0 µg/Kg (ms)	Del 19/01/16al 04/02/16
			Método analítico: Interno LQM, S.A. - Sevilla
			Técnica analítica: HPLC-MS-QqQ
C10-LAS	<5.0 µg/Kg (ms)	LC: 5.0 µg/Kg (ms)	Del 19/01/16al 04/02/16
			Método analítico: Interno LQM, S.A. - Sevilla
			Técnica analítica: HPLC-MS-QqQ

Dtor. Laboratorio: Luis Jesús Moreno López

Análisis y Control Alimentario

Fecha impresión: 05/02/2016