Risks of Cadmium Nanoparticles on Estuarine Organisms

Ecotoxicological Effects of Engineered Cadmium Nanoparticles through Biochemical and Behavioral Responses in Two Marine Invertebrates, *Nereis diversicolor* and *Scrobicularia plana*

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Abstract:

There is an increasing concern over the safety of engineered nanoparticles (ENPs) to humans and the environment. It is likely that the environmental risks of these particles will have to be tested under research and regulatory schemes such as NanoReTox under FP7 NMP Work Programme. Due to their unique properties and the fact that their detection and characterization in complex matrices is challenging, classic analytical methods and test approaches for assessing environmental risk may not be appropriate for ENPs. In this article I present the challenges associated with ENPs exposure to the estuarine environment and the testing of a chosen ENPs to generate data on ecotoxicity in the test estuarine organisms for further consideration of risk assessment of marine environment. Careful consideration was given to the selection of the test materials (benthic organisms *Nereis diversicolor* and *Scrobicularia plana*), the test system and the test exposure conditions (CdS ENPs and aqueous CdS added to filtered natural seawater with a concentration of 10 µg L⁻¹). Evaluation of the exposure effects was carried out by behavioral tests (burrowing kinetics and feeding rate) and biochemical responses (quantification of biomarkers). Multispecies Freshwater Biomonitor® (MFB) tests and GST analysis results show significant differences in between control group and CdS NPs exposure one, indicating that CdS NPs are potential to cause sublethal effects in test organisms. Our knowledge in environmental risk assessment of ENPs is still limited. Coordinated research is required to gain a better understanding of the factors and processes affecting ENP fate and effects in the environment as well as to develop more usable, robust and sensitive methods for characterization and detection of ENPs in environmental systems.

Keywords: cadmium sulphide nanoparticles, ecotoxicity, behavior, biomarker, oxidative stress, *Nereis diversicolor, Scrobicularia plana*
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1. Introduction

Although the definition of nano size materials remains some debate, European Commission (2011) has recently adopted that a “nanomaterial” is “A natural, incidental or manufactured material containing particles, e.g. water and air, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.”. Nanoparticles (NPs) are included within the group of NM that have at least two dimensions between 1 and 100 nm (American Society for Testing and Materials, 2006). Nano size materials (NM)' properties differ significantly from those at a larger scale, due to different physico-chemical features, e.g. unique optical, electrical, chemical properties). This has innovated industrial technology breakthroughs, along with increasing hopes from the public in this field, ever since the publication of Richard Phillips Feynman’s inspiring work in 1959 (Edwards, 2006). NM have the potential to improve the quality of life and to contribute to industrial competitiveness. Inevitably, the corresponding increase in the use of NM is seen in products in every sector of society, covering various sources of diversified applications from cosmetics, catalysts, textiles, to biomedicine (Christian et al., 2008).

Specifically, nanometer sized semi-conductor nanocrystals, or quantum dots (QDs), have their size ranges between 2 and 10 nm, possessing a reactive core consisted of metal or semi-conductor, e.g. Cadmium selenide (CdSe), which controls their optical and electrical properties (Schmid, 2004; Logothetidis, 2006). QDs have unique magnetic and catalytic properties, high luminescence and stability against photobleaching, e.g. the emission wavelength of QDs depends on their crystal dimension, unlike the bulk materials (Hoshino et al., 2004). Current research thus has made out QDs in controllable size for the application in solar cells and photovoltaics (Kumar & Chandra, 2005).

However, uncertainties still exist regarding the environmental fate of these particles. Too few published research results on ecotoxicological consequences of nanoparticles on different organisms are known (Colvin, 2003; Owen & Depledge, 2005). Considering the importance of estuarine ecosystem to marine environment, a more systematic approach is needed to evaluate the processes that determine hazards, exposure and risk and for validated models predicting the release, transport, transformation, accumulation and uptake of engineered NPs, or ENPs (Maynard et al.,
This work aims to address an integrated method of behavior and biochemical tests for the purpose of environmental risks assessment of cadmium sulphide (CdS) ENPs. CdS ENPs has excellent performance in photoconductivity and electroluminescence, thus it has been applied in manufacturing a great variety of consumer goods (Pathania et al., 2011; Upadhyay et al., 2012).

The increase of production and usage of ENPs, along with their derived products, inevitably lead to increasing ENMs end up in the environment, e.g. in water, sediments and soil (Nowack & Bucheli, 2007). The unique properties of ENPs, including size, large specific surface area, reactivity, shape, etc. enable them to enter organisms and transport through tissues, cells and even into cell organelles in ways that larger particles may not do (Kovochich et al., 2007). This has raised concerns of ENMs effect on public health and environment (Royal Commission on Environmental Pollution, 2008; Norwegian Pollution Control Agency (NPCA), 2008; European Commission, 2009). Ecotoxicity measurements are conducted on different trophic levels including microorganisms, plants, invertebrates and vertebrates, and test systems have been standardized for some organisms and for some exposure conditions. Standardized protocols approved by OECD or ISO show how to test the adverse effects of pollutants (e.g. heavy metals or pesticides) towards organisms like earthworms, daphnia or zebra fish (NPCA, 2008).

In marine ecosystems, the contamination is exacerbated by coastal runoff and atmospheric deposition. Discharges into estuarine and marine systems make it very likely that pollutants, in this case including NPs, end up in coastal systems that turn out to be the ultimate sink (Klaine et al., 2008). Generation of reactive oxygen species (ROS) induced by NMs, is believed to indirectly cause membrane damage and further consequences (Hoffmann et al., 2007). Many ecotoxicological researchers employ microorganisms (mainly bacteria, but also fungi, protozoa and algae) as test organisms. It is believed that coastal systems are the ultimate sink of pollutants, including any NMs, considering deliberately or purposely discharge of effluents into the marine environment typically ending up there (Kaegi et al., 2008; Klaine et al., 2008). Only a few ecotoxicity studies were performed on limited numbers of marine organisms: Very few mammalian models have been studied (Handy & Shaw, 2007) with primary focus on respiratory toxicology and inflammation reactions to ENP exposure (Auchincloss et al., 2008; Miller et al., 2009); Test organisms have mostly been limited to crustaceans (Templeton et al., 2006), algae (Nielsen et al., 2008), and
filter-feeding species like mussels (Tedesco et al., 2008; 2010); Also some research focus on sediment dwelling marine polychaetes (Galloway et al., 2009; García-Alonso et al., 2011; Cong et al., 2011). A lot of published studies on ecotoxicity of NPs were conducted with rather high NP concentrations, e.g. probably a few hundred fold higher than current environmental levels as shown by environmental predicted concentrations, while it is concerned that high levels are unrealistic to be handled in an ecotoxicological study (Tiede et al., 2009). A wider range of test organisms are expected to fill in the information gap of NMs’ environmental consequences.

For the purpose of monitoring risk assessment of ENPs, the choice of suitable test organisms is particularly important. In our case, the ragworm Nereis diversicolor and the bivalve mollusc Scrobicularia plana are chosen from the estuarine system. Based on related studies, these two organisms are good candidates and frequently used as sentinel species in marine environmental monitoring (Byrne & Halloran, 2001; Bonnard et al., 2009; Solé et al., 2009; García-Alonso et al., 2011; Buffet et al., 2011).

Cadmium and cadmium compounds are known to be human carcinogens from studies, including epidemiological and mechanistic studies (NTP, 2011). Published toxicity studies of CdS NPs, a type of widely used QDs, are still few and mostly focus on the therapeutically used NPs and their effects on human health (El-Ansary & Al-Daihan, 2009; Pujalté et al., 2011). The ecotoxicity of CdS NPs is believed to be dependent on characterizations of the NMs. One of the few studies on ecotoxicity of CdS NPs illustrates the effects on photosynthetic microorganisms (Brayner et al., 2011). No study of ecotoxicity of CdS NPs on marine organisms is found to date. Thus, the project aims at evaluating the biochemical and behavioral responses to these NPs of two marine endobenthic invertebrates, N. diversicolor and S. plana.

Biomarkers of defence metallothionein or metallothionein-like protein (MT, or MTLP) and glutathione-S-transferase (GST) are used for their wide application in environmental risk assessment of chemicals (Lagadic et al., 2000; Amiard & Amiard-Triquet, 2008). NTP’s report (2011) proves that the sensitivity of cells or tissues to cadmium is likely to be linked with MT level changes, although it is through studies of mammalian cells. The activity of the terminal enzyme, lactate dehydrogenase (LDH), has critical role in anaerobic metabolism (Gagnon & Holdway, 1999; Diamantino et al., 2001). Clams (in this case, S. plana) are proved to have functional anaerobic metabolism under anoxia and hydrogen sulphide exposure (Oeschger &
Pedersen, 1994). Chemical stress can induce LDH activity as seen in studies of different species of bivalves such as *S. plana* (Boldina-Cosqueric *et al.*, 2010), *Perna viridis* (Nicholson & Lam, 2005), *Donax trunculus* (Tlili *et al.*, 2010) and polychetes such as *H. diversicolor* (Moreira *et al.*, 2006). Besides biochemical response category of biomarkers, behavioral biomarkers are also sensitive tools in assessing the impact of the contaminants at concentrations far below the lethal effect (Amiard-Triquet, 2009; Bonnard *et al.*, 2009). Utilization of a Multispecies Freshwater Biomonitor® (MFB) measurement system (LimCo International, Ibbenbüren, DE) allows *in vivo* monitoring of animal behaviors (e.g. swimming/locomotion, ventilation, inactivity) (Gerhardt *et al.*, 2007; Gerhardt, 2011). Craig & Laming (2004) studied MFB and recorded the behaviors of *Gasterosteous aculeatus* simultaneously with movements of test organisms. Stewart *et al.* (2010) tested MFB in the marine context and were able to identify 5 different behavior patterns, “walking”, “climbing”, “leg stretch”, “cleaning”, and “inactivity”.

An aqueous medium of natural seawater was selected instead of natural sediment for the purpose of a better dispersion of NPs. It is also convenient for both intra-sedimentary species freely to move and contact with pollutants in the aqueous medium. Cadmium in water tends to sink and accumulate in bottom sediments. It has an average content in the world’s oceans of between <5 and 110 ng L⁻¹ (Natural Resources Canada, 2007; ATSDR, 2008; UNEP, 2008); with higher levels reported around coastal areas and accumulated in marine phosphates and phosphorites (Morrow 2001). Boustani (2011) investigated the world wide data on environmental cadmium concentration and found maximum admissible cadmium limits set by different countries or organizations don’t exceed 10 μg L⁻¹ in United States Environmental Protection Agency, EU, WHO, New Zealand, Australian, Iranian, and the Indian standards. It is also considered of comparing the effects of NPs with ionic Cd to understand if it is the size of ENPs or the characteristic of metal’s ion release result in toxic effects. Thus an exposure concentration of Cd at 10 μg L⁻¹ was added in filtered natural seawater for both two species under the treatment of CdS ENPs or soluble Cd. Actual exposure to the organism depends on the size distribution, stability, and solubility of NPs, as well as the bioaccumulation of Cd in organisms.

To date, there is limited understanding of the hazard of manufactured NPs and not much research into human exposure to these particles yet (Seaton *et al.*, 2010). The European Commission (EC) has initiated many research projects, technology
platforms, working groups and other committees that deal with various aspects of public acceptance and risks related to nanotechnology: the 6th (2002-2006) and 7th Framework Programmes (ec.europa.eu/research/fp6, cordis.europa.eu/fp7), investigating nanotechnology risks on health and the environment; European Committee for Standardization (CEN ) (www.cen.eu), including aspects of testing NMs for safety and risks; The European Commission’s Scientific Committee on Consumer Products (SCCP) (ec.europa.eu/health/ph_risk/committees/) continuously provides information on consumer products relate to safety issues; The Scientific Committee on Consumer Safety (SCCS) (ec.europa.eu/health/scientific_committees/consumer_safety), providing opinions on health and safety risks of non-food consumer products; ETPIS – the European Technology Platform on Industrial Safety (www.industrialsafety-tp.org), focusing on assessment risks for human exposure to ENPs in industrial working environments; The European Food Safety Agency (EFSA) (www.efsa.europa.eu) completed a report of scientific opinions on potential risks of utilizing nanotechnology on food and feed safety, upon EC’s request in 2007; The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) (ec.europa.eu/health/scientific_committees/emerging/) published a report of “opinion on the appropriateness of existing methodologies to assess the potential risks of nanotechnologies" in regard to EU’s request in 2007.

2. Materials and Methods

2.1. Selection of ENPs

A majority of the currently existing cadmium comes from anthropogenic production, as it is a rare element, not found in its pure state in nature (NTP, 2011). It occurs mainly as cadmium sulfide (CdS, or greenockite) in zinc deposits. They were first listed as reasonably anticipated to be human carcinogens in the First Annual Report on Carcinogens in 1980 (NTP, 1980). Regarded as an important type of semiconductor, CdS possesses size tunable optical transitions that appreciated by engineers and producers. The optoelectronic applications include solar cells, photodiodes, light emitting diodes, nonlinear optics, photoelectrochemical cells and heterogeneous photo catalysis (Hu et al., 1998; Weller, 1993; Prabhu et al., 2005), in a great variety of products. Biological applications, e.g. fluorescent labelling of
cellular proteins and gene technology in the field of imaging and medical areas are also increasing rapidly (Jamieson et al., 2007; Sun et al., 2007).

Cadmium concentrations found in unpolluted natural waters are usually below 1 μg L⁻¹ (Boustani, 2011). This is below the maximum admissible cadmium limits set by different countries or organizations, the United States Environmental Protection Agency 2008 (5 μg L⁻¹), the EU 1998 (5 μg L⁻¹), the WHO 2008 (3 μg L⁻¹), the New Zealand (4 μg L⁻¹), the Australian 1996 (2 μg L⁻¹), the Iranian 1996 (10 μg L⁻¹), and the Indian, 2005 (10 μg L⁻¹).

Many studies of cultured mammalian cells have shown that cadmium compounds cause genetic damage, including gene mutations, DNA strand breaks, chromosomal damage, cell transformation, and disrupted DNA repair (Zhang et al., 2007; Rzigalinski & Strobl 2009; NTP, 2011). Quantum dots NMs have yet insufficient studies to date, although Gagné et al. (2008) studied the effects of cadmium telluride (Cd-Te) on a range of sublethal endpoints, such as oxidative stress, immunotoxicity, and genotoxicity. Exposures led to oxidative stress in gills and DNA damage in gills and digestive gland, with results indicating that toxic effects are associated to some extent with the dissolved phase (Gagné et al., 2008). Nevertheless, research conducted on ecotoxicity of cadmium sulphide nanoparticles remains rare (Sanders et al., 2008).

2.2. NPs used in this experiment

CdS nanoparticles were provided by one of our NanoRetox EU 7th Framework Programme partners, JRC in Ispra, Italy. The original sample is a yellow aqueous solution containing CdS at a mass concentration of 0.9 mg/ml corresponding to a Cd concentration of 0.7 mg/ml. The particles (5~6 nm in diameters by DLS) have been thoroughly washed using centrifugal ultrafiltration. A stock suspension of CdS nanoparticles from the original package was prepared in deionized water (DIW) at a concentration of 25 mg L⁻¹, stored at 20 ºC and used for testing within 2 weeks. Then 5 μL of this stock solution were added into natural seawater (UV treated and filtered through 0.45 μm filter. It is the same for all natural seawater mentioned in materials and methods) to achieve a final concentration of 10 μg L⁻¹ CdS.
2.3. Selection of test organisms

Two estuarine organisms, the intra sedimentary *Nereis diversicolor* and the deposit-feeder bivalve *Scrobicularia plana*, are selected to investigate environmental effects of engineered NPs (Figure 1). Playing important roles in connecting benthic food webs through sediment reworking, they are often used as sentinel species in marine environmental assessment and monitoring (Sarkar *et al.*, 2006; Amiard-Triquet *et al.*, 2007; Galloway *et al.*, 2009).

![Figure 1. Ragworm *Nereis diversicolor* (left), deposit-feeder polychaete, able to make long tunnels through the sand; *Scrobicularia plana* (right), filter feeder-bivalves, possess long siphons and are able to bury up to 20 cm deep in sand or mud.](image)

2.4. Animal collection and acclimatization

Ragworms (*N. diversicolor*) and bivalves (*S. plana*) were collected by hand from an intertidal mudflat (upper 20 cm depth) in the Bay of Bourgneuf (46° 56' 23.08"N, 2° 4' 40.60"W) (46.939744, -2.077944), located on the West Atlantic coast (France) (Figure 2), in November 2011. This site is monitored by the French “Mussel Watch” Programme (Réseau National d’Observation, 2006) and is documented as relatively clean (Kalman *et al.*, 2009). Bivalves with shell length ranging from 15 to 20 mm were selected for MFB tests, as well as for avoiding any potential influence of sexual maturity. Bigger sizes of bivalves ranging from 20 mm to 35 mm were selected for other behavioral tests, including burrowing kinetics and feeding rates, and biomarker analysis. Worms were selected from individual length from 20 mm to 35 mm. Special attention was paid to individual’s color to avoid sexual maturity (Mouneyrac *et al.*, 2010). Then, *N. diversicolor* and *S. plana* were transported to the laboratory (Cold chamber 15°C) in cool boxes with sediment from the collection site.
In the laboratory, animals were allowed to eliminate their gut contents and acclimatize for 48 h in aerated natural seawater (UV treated and filtered through 0.45 μm), with a stable salinity 29.1-32.5‰ (Tankoua et al., 2012). Acclimatization conditions were chosen based on Poirier et al. (2006) and Burlinson & Lawrence (2007).

Figure 2. Location of the sampling site, Bay of Bourgneuf, France. From left to right: two photos clipped from Google Earth with the place situation, the third photo was taken at the sampling site.

2.5. Exposure protocol

Bivalves (S. plana) were placed into 2.2 L polypropylene aquaria (15 individuals per tank) filled with 2 L seawater and ragworms (N. diversicolor) were introduced individually in plastic beakers of 100 mL (one individual per beaker, 15 individuals per tank) filled with 50 mL natural seawater. Concentration relevant to environmental data (Tiede et al., 2009; Boustani, 2011) was considered in the design. Three treatments: (1) natural seawater only as control; (2) soluble cadmium 10 μg Cd²⁺ L⁻¹ (prepared from Fluka™ Analytical’s Cadmium Standard for AAS, 1000 mg L⁻¹ Cd in nitric acid), and (3) CdS NPs: 10 μg CdS NPs L⁻¹, were carried out in a replicate design (four tanks per treatment for each species). A semi-static exposure regime, in the dark and at the same temperature as N. diversicolor and S. plana can have in their sediment of origin at this period of the year (15 ºC), was applied. The experimental media (water and contaminant) were renewed every two days. In order to avoid interferences between food and the fate of NPs, invertebrates remained unfed during the first two-week period of the experiment. The last two-week period, bivalves were fed with algae (Nitzschia sp. that were allowed to grow in a culture media with 10 μg L⁻¹ soluble cadmium or 10 μg L⁻¹ CdS NPs) and were put in the containers of 2 L glass flask instead of the plastic tank. N. diversicolor were fed with
shredded pieces of ragworms that were exposed to contamination regime in the first two-week period.

2.6. Behavioral experiments

Behavioral experiments included the estimation of feeding rate and burrowing kinetics for both organisms (Figure 3). In addition, Multispecies Freshwater Biomonitor (MFB), which is an impedance converter (Gerhardt et al., 2002), was used to record behavioral patterns in a sensitive and quantitative way.

Test organisms were put back to their respective test conditions after finishing the behavioral experiments. This procedure is acceptable since Burlinson & Lawrence (2007) have shown that burrowing organisms were not affected by consecutive behavioral assays.

Figure 3. Overview of the behavioral tests protocols used in this experiment

2.6.1. MFB tests

MFB Multispecies Freshwater Biomonitor® (LimCo International, Ibbenbüren, DE) is a flexibly designed, animal behavioral recording and monitoring machine. It allows locally-adapted and optimal-alarm evaluation for almost all kinds of aquatic (in)vertebrate organisms. Based on the quadruple impedance conversion technology (Gerhardt et al., 1994), MFB can record living organisms’ behavioral responses by producing electrical signals simultaneously that transmitted to the recording device. Thus MFB has been explicitly developed to provide a rapid warning of the occurrence of contaminants at concentrations which could be of immediate threat to living organisms. As a modular system, MFB may contain several test chambers, ranging from 8 to 96, which are flow-through cells with the two electrode pairs placed on the chamber walls. Each cell contains usually one organism, and there may be different
species in one experiment, as well as one cell acts as control, while it is required to calibrate the system when changing to new species. An alternating current is applied between electrodes at opposite walls of the test chamber. Movements of the animals will change the conductivity and the electrical field between a second pair of electrodes and thus generate specific electrical signals, at different frequency ranges for different kinds of behavior (swimming/locomotion, ventilation, inactivity) (Gerhardt et al., 2007).

In my experiment settings, I applied MFB with 8 test chambers (2 cm diameter and 5 cm length). I selected test organisms by their lengths and also avoided sexual matured N. diversicolor by picking red-colored ones mainly. The N. diversicolor or S. plana were put individually into the test chamber, containing sediments sampled from the original site moistured with natural seawater. After an acclimatization period of 30 min the measurement was started. Behavior of S. plana was recorded constantly for 12 hours (overnight) in intervals of 10 min and for duration of 4 min, while N. diversicolor was recorded constantly for 8 hours (daytime) in same intervals and duration. The test chambers were sealed with a PP-foil for N. diversicolor. Each test chamber was placed in a 150 ml plastic container with about 3-cm deep natural sediments and topped with 2-cm deep natural seawater to simulate the benthic condition of estuarine environment. Natural sediments taken from the original sampling site were homogenized and checked to remove any small animals when used in MFB tests. In order to get maximized recorded animal behavior from the MFB test, I placed the chambers differently for different organisms: chambers vertically for N. diversicolor and horizontally for S. plana (Figure 6). Containers with chambers were later transferred to a 5 L-big plastic tank on steady surface to maintain undisturbed. Each round of MFB test was composed by 7 individuals of one species from different exposure conditions in 7 chambers. One chamber was left with sediments and natural seawater only to be used as a control. Sediments and natural seawater were replaced with fresh ones after each round. First two-week (water T7 and T14)’s MFB tests were for test organisms exposed to control or contaminated natural seawater (soluble Cd or CdS NPs). The second two-week period (food T7 and T14) was for test organisms exposed via contaminated food. Behaviors of 7 individuals of each species from different experimental conditions were recorded every week during the exposure period (water T7, T14; food T7, T14).

The experimental set-up is shown below in Figure 4: a, b, c, and d.
2.6.2. Burrowing kinetics

Test of burrowing kinetics followed the same protocol as described in Bonnard et al. (2009) and Buffet et al. (2011). Bivalves (S. plana) and worms (N. diversicolor) were picked out after being previously exposed in the laboratory for 4 days to seawater only (controls), CdS NPs or dissolved Cd\(^{2+}\).

20 bivalves were collected from each experimental condition and randomly chosen from the tank replicates with similar sizes. Burrowing experiments were carried out in 5 L plastic containers filled with about 3 cm of natural sediments and topped up with 2 L of seawater. The sediments were taken from the original sampling site and homogenized a few hours before experimentation. Burrowing behavior was studied by placing individuals on the surface of the sediment and observing the number that had burrowed at frequent intervals; I checked once every 5 min in the 1\(^{st}\) hour, then every 10 min in the 2\(^{nd}\) hour, later every 20 min in the 3\(^{rd}\) and 4\(^{th}\) hour, and finally once every hour until 6\(^{th}\) or 7\(^{th}\) hour of test.
For *N. diversicolor*, twenty individuals from each experimental condition were chosen for burrowing kinetic tests as well. Burrowing environment was set up by using 100 ml plastic clean containers that filled with 5 cm of wet sediment from the site of origin and homogenized. Twenty ragworms were placed separately in each beaker on top of the sediment and their visibility (remained on top, or burrowed in deep of the sediments) were recorded every 120 sec during a 30 min period.

2.6.3. Feeding rate

Feeding rate was estimated for ragworms (*N. diversicolor*) and bivalves (*S. plana*) after the exposure period of 7 or 14 days under seawater only (Controls), CdS NPs, or soluble cadmium. Methodology to examine the feeding rate of *S. plana* followed Worrall & Widdows (1983) and Buffet *et al.* (2011).

Bivalves in all experimental conditions were involved (n = 45 per condition distributed in three replica plastic tanks of 2 L (60*30*10 cm)) for feeding rate tests. *Algae Tetraselmis suecica* supplied by Ifremer, French Research Institute for Exploration of the Sea, were used as food at a concentration of 10 000 cells mL\(^{-1}\) in each tank (three replicates). The number of algae left in each tank that were not taken by bivalves was determined after 1 hour.

Ragworms in all experimental conditions that remained alive and healthy were chosen for the feeding rate tests. Quantification of the feeding rate referred to the methodology described by Moreira *et al.* (2006) and Buffet *et al.* (2011). Twenty ragworms were fed 100 *Artemia salina* larvae into their individual plastic beakers containing 50 mL of natural seawater. They were left undisturbed in dark in cold chamber for 1 h, then the remaining larvae were collected and counted and results were expressed as the number of larvae ingested by each ragworm per hour.

2.7. Biochemical analysis

Enzymes and proteins play important roles in defence, detoxification, and elimination in trace metal contamination, especially as their biochemical changes are usually the first detectable responses to changes in the environment, e.g. existence of contaminants (Stegeman *et al.*, 1992). Thus it is possible to use them as biochemical biomarkers.
In this experiment, several defence biomarkers were chosen, metallothionein-like protein (MTLP), glutathione-S-transferase (GST) and lactate dehydrogenase (LDH) activity.

For trace metals, the most important group of scavenging proteins is metallathioneins (MT), of which a variety of forms exist, namely metallathionein-like protein (MTLP). MT is involved in a variety of processes concerned with metal metabolism including the regulation of the uptake of essential metals (especially Zn and Cu) and metal detoxification (Stegeman et al., 1992). MT appears to be a promising indicator of exposure to Cd, Cu, Zn, and mercury (Hg) (Stegeman et al., 1992). However, practical application of MT for monitoring can be influenced by numerous confounding factors such as sexual maturation, temperature, and nutritional status (Benson et al., 1990; Stegeman et al., 1992). MT induction has been well studied in fish (Olsson et al., 1998; Roeva et al., 1999), molluscs (Langston et al., 1998; Isani et al., 2000), and other organisms (Poirier et al., 2006; Buffet et al., 2011).

Glutathione-S-transferases (GST) bind to contaminant metabolites, mostly oxygenated organic contaminants, and appear to be elevated in fish, crabs, and mussels from sites contaminated with PAHs (Stegeman et al., 1992).

Lactate dehydrogenase (LDH) is an important glycolytic enzyme being present in virtually all tissues (Kaplan & Pesce, 1996). It is associated with metabolic state – enables energy production in hypoxic (low oxygen) conditions. Under low oxygen, or hypoxic conditions, energy produced by cells is primarily generated by glycolysis in the cytoplasm, and levels of LDH may increase. It is found that under exposure to various concentrations of cadmium telluride (Morgan et al., 1995), cadmium (Hassoun and Stohs, 1996) or oxygen stress (Wu and Lam, 1997), normal LDH activities was interfered.

Before starting biochemical analysis of test organisms, I selected 10 ragworms and bivalves respectively for each biomarker from the three different tanks (n = 3 or 4 taken randomly from each replica) corresponding to each experimental condition. After exposure period (water T14 and food T14), the length and the total weight of bivalves were recorded. The shells of the bivalves were removed, their soft tissues were wiped carefully with absorbent paper, and then stored these soft tissues at -80 ºC until biochemical analysis. Ragworms after exposure were as well handled with absorbent paper to remove extra liquid, weighed individually and stored at -80 ºC until biochemical analysis. The measurements were carried out individually (n = 10
for each biomarker of MTLP, n=7~10 for LDH and GST biomarkers). MTLP analyses were performed only in the bivalves (S. plana), since previous studies revealed that metallothionein-like protein (MTLP) determination was not a relevant biomarker of metal exposure in N. diversicolor (Poirier et al., 2006; Amiard-Triquet et al., 2007).

For MTLP analysis, the whole soft tissues of S. plana were homogenised at 4 °C in 20 mM TRIS, 105 mM b-mercaptoethanol, 0.1 mM Phenylmethanesulfonyl Fluoride (PMSF), 150 mM NaCl adjusted to pH = 8.6 (4 mL g⁻¹ soft tissue). The soluble (S1) and insoluble (P1) fractions were separated by centrifugation at 30 000 g for 30 min at 4 °C. An aliquot of the soluble fraction (S1) was heated at 75 °C for 15 min. Then MTLPs (i.e. heat-stable thiolic compounds) were isolated by centrifugation (15 000 g for 10 min at 4 °C) and determined by Differential Pulse Polarography (DPP) analysis (Mouneyrac et al., 2002). The standard addition method was used for calibration with rabbit liver MT (Sigma Chemical Co., St. Louis, MO) in the absence of a marine bivalve MT standard.

For GST, soft tissues were homogenised at 4 °C to prevent enzyme or tissue degradation in TRIS buffer (TRIS 50 mM, NaCl 150 mM, DTT 1 mM, antiprotease mixture (Sigma P8340, diluted in 1/1000) adjusted to pH 7.4 in a 1:3 ratio (weight: volume) using a motor-driven glass-Teflon homogenizer at 500 rpm. The homogenates were then centrifuged for 25 min at 9 000g. Supernatants kept as 50 µL aliquots on ice were prepared for biomarker analysis. An aliquot of the homogenate was centrifuged (9 000 g for 30 min at 4 °C) and the resulting supernatant was used directly in the enzyme assay. GST activity was determined spectrophotometrically at 340 nm (ε = 9.6 mM⁻¹ cm⁻¹) by monitoring the formation of 1-glutathion-2,4-dinitrobenzene, resulting from the conjugation of the substrate, 1-chloro-2,4-dinitrobenzene (CDNB), with glutathione reduced form (GSH), as described by Habig et al. (1974). 96-well microplates were applied for the analysis. Results were expressed as nmoles of glutathione conjugate produced per min and per mg protein (nmoles min⁻¹ mg⁻¹ protein).

For LDH determination, the same aliquots as GST of the homogenate were prepared but centrifuged at 3 300 g for 5 min at 4 °C. By applying microplate (96-well), LDH activity was determined spectrophotometrically at 340 nm (ε = 9.6 mM⁻¹ cm⁻¹) as well, following methodology described in Diamantino et al. (2001). Proteins were quantified in the supernatants according to Bradford (1976). Results were
expressed as nmoles of lactate dehydrogenase decomposed per min and per mg protein (nmoles min\(^{-1}\) mg\(^{-1}\) protein).

2.8. **Statistical analysis**

Refer to Buffet *et al.* (2011), the burrowing kinetic curves were ln-transformed to obtain a linearized curve for better fit to linear regression model, which were later compared by using analysis of covariance (ANCOVA) between regression coefficients of the least-square best-fit regression lines. Otherwise, results are presented as mean ± SD. Significant differences were established by using one-way analysis of variance (ANOVA) or non-parametric Mann-Whitney U test comparison tests when variances of groups were different. Level of significance was established at \( p = 0.05 \) (95% confidence level). Statistical analyses were performed by using XLSTAT Pro 7.5 (Addinsoft), R (GNU project), and JMP® 9.0.2 (SAS Institute).
3. Results and Data Analysis

3.1. Behaviors analysis

3.1.1. MFB tests

Behaviors of *N. diversicolor* and *S. plana* in MFB test chambers were recorded in the form of oscilloscope signals with different strength. This was used to distinguish the behavior patterns of the test organisms.

For *N. diversicolor*, movements that induce signals within the range of 0.5 Hz were identified as undulation, and signals in between 1.0 Hz and 2.0 Hz indicated its head movement and feeding activity (Figure 5).

For *S. plana*, movements that induce signals within the range of 0.5 Hz were identified as burrowing activities with foot movements, and signals in between 1.0 Hz and 2.5 Hz indicated feeding and ventilation siphon movements (Figure 6). Burrowing activities were seen as the major ones, while ventilation occurred only occasionally.

Examples of 4-minute movements recorded by MFB are shown in Figure 7 and Figure 8 for *N. diversicolor* and *S. plana*, respectively.
Figure 5. Example of 40s signals of *N. diversicolor* for behaviors such as undulation (Upper, frequency ~0.5 Hz) and head movement and feeding activity (Bottom, frequency ~1.0 Hz).

Figure 6. Example of 40s signals of *S. plana* for behaviors of foot movement (Upper, frequency ~0.5 Hz) and feeding with siphon, (Bottom, frequency ~1.0 Hz). Image was taken by web camera simultaneously with MFB records, showing the white-to-transparent foot and siphon part, respectively.
Figure 7. Example of 4-minute recording of Fast Fourier Transformation (FFT) (%; left) histograms of measurements and motility signals (Voltage, right) of *N. diversicolor* in the MFB test chamber after two-hour of measurement (Above: *N. diversicolor* in control condition with filtered natural seawater; Middle: *N. diversicolor* in seawater contaminated with soluble cadmium for 7 days; Bottom: *N. diversicolor* in seawater contaminated with CdS NPs for 7 days). Declined signal frequency from *N. diversicolor* in contaminated environment for 7 days can be seen after two-hour measurement, comparing to the control group in seawater.

Figure 8. Example of 4-minute recording Fast Fourier Transformation (FFT) (%; left) histograms of measurements and motility signals (Voltage, right) of *S. plana* in the MFB test chamber after two-hour of measurement (Above: *S. plana* in control condition with natural seawater; Middle: *S. plana* in seawater contaminated with soluble cadmium for 14 days; Bottom: *S. plana* in seawater contaminated with CdS NPs for 14 days). Declined signal frequency from *S. plana* in contaminated environment for 14 days can be seen after two-hour measurement, comparing to the control group in seawater.
Recorded signals from MFB test were processed by calculating the mean value of signal numbers in different ranges (for *N. diversicolor*, 0.5 Hz and 1.0~2.0 Hz; for *S. plana*, 0.5 Hz and 1.0~2.5 Hz) for each test organisms (Table 1). The difference of individuals in different treatment was shown by a non-parametric statistical analysis of Mann-Whitney that compares treatment groups in pairs (Table 2). Impaired activities frequencies can be observed in *N. diversicolor* after 7 days of contamination regime in CdS NPs, both water and food, and only via water for soluble Cd exposure. Bivalve *S. plana* showed a relative latent response to contamination that decreased activities were seen after 14 days of contamination regime via water exposure in CdS NPs (Figure 9).

### Table 1. Mean frequency (Mean), range (Min~Max), and Standard Deviation (SD) of signals recorded from test organisms (n = 7) in MFB test chambers. Ctrl: Control, NP: CdS NPs, Sol: Soluble Cd.

<table>
<thead>
<tr>
<th></th>
<th><em>Nereis diversicolor</em></th>
<th><em>Scrobicularia plana</em></th>
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<tbody>
<tr>
<td></td>
<td>0.5 Hz</td>
<td>1.0~2.0 Hz</td>
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<tr>
<td></td>
<td>Min~Max</td>
<td>Mean±SD</td>
</tr>
<tr>
<td><strong>WATER</strong></td>
<td></td>
<td></td>
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<tr>
<td>T7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl</td>
<td>40.63~70.35</td>
<td>50.85±10.65</td>
</tr>
<tr>
<td>NP</td>
<td>8.33~58.82</td>
<td>24.29±18.48</td>
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<tr>
<td>Sol</td>
<td>2.94~63.63</td>
<td>29.03±21.86</td>
</tr>
<tr>
<td>T14</td>
<td>15.63~82.96</td>
<td>49.13±27.36</td>
</tr>
<tr>
<td><strong>FOOD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl</td>
<td>21.56~86.00</td>
<td>57.53±23.64</td>
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<tr>
<td>NP</td>
<td>15.76~72.42</td>
<td>36.29±21.82</td>
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<tr>
<td>Sol</td>
<td>15.86~91.92</td>
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</tr>
<tr>
<td>T14</td>
<td>10.76~75.40</td>
<td>52.28±21.25</td>
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</table>
| **Mann-Whitney U test results for non-parametric statistics of test organisms’ MFB records in different treatment pairs, showing some pairs with differences at 95% confidence level with p values, respectively.**

<table>
<thead>
<tr>
<th></th>
<th><em>Nereis diversicolor</em></th>
<th><em>Scrobicularia plana</em></th>
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<tbody>
<tr>
<td></td>
<td>0.5 Hz</td>
<td>1.0~2.0 Hz</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>Food</td>
</tr>
<tr>
<td>T7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl vs. NP</td>
<td>0.021*</td>
<td>0.055*</td>
</tr>
<tr>
<td>Ctrl vs. Sol</td>
<td>0.030*</td>
<td>0.125</td>
</tr>
<tr>
<td>T14</td>
<td>0.443</td>
<td>0.371</td>
</tr>
<tr>
<td>Ctrl vs. Sol</td>
<td>0.443</td>
<td>0.609</td>
</tr>
</tbody>
</table>
Figure 9. One-way ANOVA image of groups that have significant (or close to significant) difference between Control groups and contaminated treatments in MFB test chambers: a) Recorded average 0.5 Hz signals from *N. diversicolor* individuals in WATER contaminated environment for 7 days. Difference of Ctrl vs. NP (p=0.021 at 95% confidence level) and Ctrl vs. Sol (p=0.030 at 95% confidence level); b) Recorded average 1.0~2.0 Hz signals from *N. diversicolor* individuals in WATER contaminated environment for 7 days. Difference of Ctrl vs. NP is close to significant (p=0.055 at 95% confidence level), and Ctrl vs. Sol is p=0.125 at 95% confidence level; c) Recorded average 1.0~2.0 Hz signals from *N. diversicolor* individuals in FOOD contaminated environment for 7 days. Difference of Ctrl vs. NP is significant (p=0.041 at 95% confidence level), and Ctrl vs. Sol is p=0.371 at 95% confidence level; d) Recorded average 0.5 Hz signals from *S. plana* individuals in WATER contaminated environment for 14 days. Difference of Ctrl vs. NP is significant (p=0.041 at 95% confidence level), and Ctrl vs. Sol is p=0.443 at 95% confidence level. Control: individuals in filtered seawater without any contamination; NP: individuals in water contaminated with CdS NPs or food contamination with food previously exposed to CdS NPs; Sol: individuals in water contaminated with soluble cadmium or food contamination with food previously exposed to soluble cadmium.

### 3.1.2. Burrowing kinetics

The burrowing behavior test results of *N. diversicolor* and *S. plana* (individuals exposed to experimental contaminated conditions and control condition for 7 days) is shown in figures below (Figure 10). Burrowing kinetic curves were compared using ANCOVA to see the difference of their slopes (Table 3).

There is no significant difference in terms of burrowing kinetics of *N. diversicolor* among treatments, showing their burrowing competence were not impaired to the extent that it could be observed. Burrowing kinetics of *S. plana* was significantly different, indicating a severely impaired burrowing behavior through contamination.
Figure 10. Burrowing kinetic curves show percentage of unburrowed individuals change over time: a) Burrowing curve of 20 individuals of *N. diversicolor* followed WATER contamination regime for 7 days ahead of test in a total time period of 14 minutes; b) Burrowing kinetic curve of 20 individuals of *S. plana* followed WATER contamination regime for 7 days ahead of test in a total time period of 6 hours; c) Burrowing curve of 20 individuals of *N. diversicolor* followed FOOD contamination regime for 7 days ahead of test in a total time period of 14 minutes; d) Burrowing kinetic curve of 20 individuals of *S. plana* followed FOOD contamination regime for 7 days ahead of test in a total time period of 6 hours. No significant difference found for *N. diversicolor*. Ctrl: Control; Sol: soluble cadmium; NP: CdS NPs.

Table 3. Slopes and determination coefficients ($R^2$) of the best fit linear regression models obtained after ln-transformed of the raw data shown in Figure 12. No significant difference of slope pairs found in *N. diversicolor*. For *S. plana*, slope pairs with significant differences: WATER exposure with Ctrl vs. NP has a p value of 0.007 at 95% confidence level, Ctrl vs Sol has p value of 0.601 at 95% confidence level; FOOD exposure with Ctrl vs. NP and Ctrl vs. Sol both have the difference in significances p <0.0001 at 95% confidence level. Ctrl: Control; Sol: soluble cadmium; NP: CdS NPs.

<table>
<thead>
<tr>
<th></th>
<th><em>Nereis diversicolor</em></th>
<th><em>Scrobicularia plana</em></th>
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<tbody>
<tr>
<td></td>
<td>Slopes</td>
<td>$R^2$</td>
</tr>
<tr>
<td><strong>WATER</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl</td>
<td>-0.2661</td>
<td>0.9183</td>
</tr>
<tr>
<td>NP</td>
<td>-0.3297</td>
<td>0.9373</td>
</tr>
<tr>
<td>Sol</td>
<td>-0.3165</td>
<td>0.9123</td>
</tr>
<tr>
<td><strong>FOOD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl</td>
<td>-0.4349</td>
<td>0.8601</td>
</tr>
<tr>
<td>NP</td>
<td>-0.3903</td>
<td>0.9673</td>
</tr>
<tr>
<td>Sol</td>
<td>-0.4979</td>
<td>0.9159</td>
</tr>
</tbody>
</table>
3.1.3. Feeding rates

Feeding rates of *N. diversicolor* and *S. plana* were tested on individuals following the experimental contamination regime (controls, soluble cadmium, CdS NPs) for 7 days or 14 days. For *N. diversicolor*, no significant change of feeding rates was found, indicating that ragworms were not affected by experimental contamination conditions (no significant difference among the groups in any pair of treatments). During the first hour of feeding test, highest feeding rate was observed, 5600 ± 386 cells h⁻¹, on *S. plana* in the control medium. For the water contamination regime, there was significant decrease of feeding rate in CdS NPs groups compared to control groups, as well as for soluble cadmium groups (Figure 11), showing that bivalves’ feeding rates were severely impaired under contamination conditions.

![Boxplot of feeding rates test results of *S. plana* individuals](image)

Figure 11. Boxplot of feeding rates test results of *S. plana* individuals: a) under WATER contamination regime for 14 days ahead of test. Significant difference found in the pair of Ctrl vs. NP (p=0.040 at 95% confidence level), and Ctrl vs. Sol (p=0.005 at 95% confidence level) treatment groups; b) under FOOD contamination regime for 14 days ahead of test. Significant difference found in the pair of Control vs. NP treatment groups (p= 0.0044 at 95% confidence level). At 95% confidence level, Ctrl vs. Sol p=1.000. Ctrl: Control, NP: CdS NPs, Sol: Soluble Cd. Y axis: scaled Feeding rates per hour.

3.2. Biochemical analysis

Measurements of biomarkers of defences in ragworms (*N. diversicolor*) and bivalves (*S. plana*) are illustrated in Table 4 and descriptive boxplots of significative biomarker responses in Figure 12. GST levels of Cd NPs in both contamination regime and organisms were seen an increase compared with control group, but only few are significant. For water contamination rout, significant elevated GST levels were seen in *N. diversicolor* individuals exposed to soluble cadmium (p=0.0158 at...
95% confidence level). For bivalves exposed to CdS NPs, both GST and LDH levels were significantly high (respectively: \( p=0.0143 \) and \( p=0.0003 \) at 95% confidence level), comparing to control condition. MTLP concentrations increased in bivalves exposed to both forms of cadmium (soluble Cd, CdS NPs), although without significant results. Food exposure with contaminants did not show significant elevation of neither GST nor LDH level among treatment groups in both organisms, indicating no significant damages to GST or LDH activities under this contamination regime.

Table 4. Means and Standard Deviation (SD) (in brackets below mean value) of biomarkers quantified after 14 days of exposure experiments (3 treatments: controls with natural seawater (Ctrl), soluble Cd (Sol), and CdS NPs (NP). Units: MTLP (\( \mu \text{mol min}^{-1} \text{mg}^{-1} \text{protein} \)); GST and LDH (nmol min\(^{-1} \text{mg}^{-1} \text{protein} \)).

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Nereis diversicolor</th>
<th>Scrobicularia plana</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Ctrl</td>
<td>NP</td>
</tr>
<tr>
<td><strong>WATER</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTLP</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>GST</td>
<td>(3.18)</td>
<td>12.03</td>
</tr>
<tr>
<td>LDH</td>
<td>(88.16)</td>
<td>257.63</td>
</tr>
<tr>
<td><strong>FOOD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTLP</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>GST</td>
<td>(8.74)</td>
<td>16.05</td>
</tr>
<tr>
<td>LDH</td>
<td>(37.78)</td>
<td>243.23</td>
</tr>
</tbody>
</table>

23
Figure 12. Boxplots of measurement of biochemical markers GST, and LDH, with p values less than 0.05 at 95% confidence level: a) GST level of *N. diversicolor* after 14-day WATER contamination regime. One-way ANOVA test at 95% confidence level: Ctrl vs. NP, p=0.092; Ctrl vs. Sol, p=0.016; b) GST level of *S. plana* after 14-day WATER contamination regime. One-way ANOVA test at 95% confidence level: Ctrl vs. NP, p=0.014; Ctrl vs. Sol, p=0.364; c) LDH level of *S. plana* after 14-day WATER contamination regime. One-way ANOVA test at 95% confidence level: Ctrl vs. NP, p=0.0003; Ctrl vs. Sol, p=0.918. Ctrl: Control, NP: CdS NPs, Sol: Soluble Cd.
4. Discussion
4.1. Environmental fate of ENPs

The ultimate recipients for any non-volatile compound or particle spreading in the environment will be sediments and soils, in this case including ENPs (NPCA, 2008). However, little is known about how ENPs interact with soils and sediments (Oberdörster et al., 2006; Wiesner et al., 2006). Environmental factors, such as pH and ionic strength (Brandt et al., 2005; Luthy et al., 1997), as well as physico-chemical properties, structure and concentration of ENPs (Huuskonen, 2002; Schwarzenbach et al., 2003), may determine whether ENPs are bound within or transported out of soils and sediments. Interactions with dissolved constituents may also affect their mobility. Hyung et al. (2007) showed that the interaction of dissolved organic matter (DOM) with carbon nanotubes (CNTs, a major group of nanoparticles) in a way may enhance the latter’s dispersion and transport. Most ENPs have low solubility and are rather persistent in the environment, thus they are more likely to accumulate in the environment than to disappear, although this feature varies among different types. Carbon-based ENPs like CNTs are non-polar and do not easily disperse or dissolve in water, but mineral ENPs like ZnO are prone to weathering and dissolution, e.g. ZnO ENPs dissolve over time in common environmental conditions (NPCA, 2008). ENPs have considerably high biodegradability as nanopolymers; various capping or coating technologies (e.g. surfactants and organic coatings), are used to gain controllable degradability (Roberts et al., 2007).

In natural waters, behavior and effects of ENPs will be highly dependent on their characteristics (e.g. surface area concentration, size distribution, state of aggregation and fraction of ENP in the dissolved form), the environmental conditions and the ENP concentrations (Hardman, 2006). In marine ecosystems, coastal runoff and atmospheric deposition have resulted in a great variety of organic matter and colloids in sea water. Varying with the types and amount of diffuse inputs, it is expected that organic matter in the estuarine coastal zone has higher concentration than pristine oceanic water (Wurl & Obard, 2004; Middelburg & Hennan, 2007; Yamashita et al., 2007). Changes of environmental factors, e.g. temperature, may influence aggregation and colloid chemistry in the ocean (Wurl & Obard, 2004). Stolpe & Hassellöv (2007) noticed dramatically decreased colloid concentrations in the condition of slightly increased salinity of freshwater (~2.5‰) due to aggregation and precipitation processes and concluded that high ionic strength that characterize
seawater makes it more likely to cause aggregation than freshwater. When the salinity changes for freshwater organisms that enter the estuarine zone, the significant change of colloid loss happens no matter how much the seawater is diluted (Klaine et al., 2008). This changing state of aggregation also alters the status of precipitation of NPs to the sea bottom, indicating higher probability that NPs can be taken by benthic dwelling organisms (Fabrega et al., 2011). Thus the risk of NPs on benthic estuarine organisms, in our cases N. diversicolor and S. plana, is of certain potential to result in biological effects.

4.2. Toxicity and Ecotoxicological effects

NPs can enter into the gut cells by diffusion through cell membranes (Lin et al., 2007), through endocytosis (Kim et al., 2006) and adhesion (Geiser et al., 2005) as shown in Figure 13. Possible mechanisms of NMs include disruption of membranes or membrane potential, oxidation of proteins, genotoxicity, interruption of energy transduction, formation for reactive oxygen species, and release of toxic constitutes (Klaine et al., 2008).

One of the important toxicity mechanisms of NMs, generation of reactive oxygen species (ROS), is believed to indirectly cause membrane damage (Hoffmann et al., 2007). ROS can oxidize double bonds on fatty acid tails of membrane
phospholipids in a process known as lipid peroxidation. This increases membrane permeability and fluidity, resulting in cells getting more vulnerable to osmotic stress or hindering nutrient uptake (Cabisco et al., 2000). Peroxidized fatty acids can trigger reactions that generate other free radicals, leading to consequences of more damage to cell membranes, cellular organelles, and nucleic acids contained in DNA and RNA.

ROS generation also happens where cadmium is toxic to organisms. Free cadmium ions concentration plays key role in the toxicity of cadmium to estuarine organisms (Engel & Fowler, 1979). Cadmium ions work in a way that induce an oxidative stress, causing oxidative deterioration of biological macromolecules, depletion of glutathione and protein-bound sulfhydryl groups, resulting in enhanced production of ROS (Stohs et al., 2001). On the other hand, cadmium sulfide, which is only soluble in acid, has the aqueous-phase equilibrium between Cd\(^{2+}\) and S\(^{2-}\) (i.e., \(\text{H}_2\text{S}\) or HS\(^{-}\)), which determines its actual toxicity (Daskalakis & Helz, 1992). Factors like changing pH in the surrounding water column may also alter the equilibrium. In higher salinities of waters, decreased toxicity of dissolved cadmium was found for a variety of marine animals (Engel & Fowler, 1979). Test organisms under the CdS NPs’ exposure showed more adverse effects than cadmium ions in some occasions (behavioral tests: burrowing kinetics of \(S.\) plana under both WATER and FOOD exposure, and feeding rates under FOOD exposure; biochemical analysis of \(S.\) plana under WATER exposure: GST and LDH results), but few of these difference are significant. Thus it is less clear whether it is due to the reason of more toxic cadmium in nanoparticles, or simply due to insufficient replica of test organisms under experimental conditions.

Very little is known about ecotoxicity of cadmium NPs to date, however some metal NPs are able to pass from the water column to the marine food web (Ferry et al., 2009) and induce oxidative stress on marine organisms (Tedesco et al., 2008; 2010). Specific toxicity effects are expected through various biomarkers. MTLP, known to be provoked to defence oxidative stress (Viarengo et al., 1999), were observed an increased level induced by both CuO NPs and soluble CuO on the estuarine bivalve, \(S.\) plana (Buffet et al., 2011). Significant contribution of MTLP to the detoxification of estuarine organisms was not seen in this experiment, though slight increase was seen in water contamination regime to \(S.\) plana. The GST level had a general increase in all Cd NPs treatment groups of both organisms, indicating the activation of antioxidant systems of defence (Almeida et al., 2007). Depletion of
glutathione due to cadmium ions may have the strongest impact on this. LDH activity is usually seen as the organism’s energy requirement under anaerobic conditions (Moreira et al., 2006), it was proved in *S. plana*’s water contamination regime for Cd NPs by significant elevated LDH level. Nevertheless, the defence mechanism of GST, LDH, and MTLP did not seem to compensate the impairment of behaviors efficiently. This is in accordance with Buffet et al. (2011) who also found occasions of insufficient defense mechanisms due to saturation of biochemical markers like MTLP.

Behaviors recorded by MFB showed decreased activity for *N. diversicolor* in undulation and feeding starting from the first week, while *S. plana* gave a relatively latent response in the second week. For burrowing kinetics, they were significantly affected in both CdS NPs and soluble Cd groups but only for *S. plana*. This shows that MFB tests allowed to register movements in a continuously and efficient way (Craig & Laming, 2004; Stewart et al., 2010). Feeding rate of *S. plana* was seen more significantly impaired in soluble cadmium treatment in water contamination regime than under food contamination regime. Brayner et al. (2011) investigated how Cd$^{2+}$ ions affect the photosynthetic activity of microalgae, *Euglena gracilis*, yet results showed no effect in a period of more than 1 month. Cadmium exists in aqueous phase could have been more toxic to bivalves in terms of releasing more Cd$^{2+}$ directly than CdS NPs. In the research of toxicity of cadmium QDs to filter feeding bivalves, oysters (*Crassostrea virginica*), Ringwood et al. (2006) showed an increased degradation of QDs with salinity increase. Gagné et al. (2008) and Peyrot et al. (2009) later proved that more than 80% of NPs in aqueous phase in freshwater are aggregated colloidal forms, indicating that under the salty circumstances like estuaries, QDs are easily degraded and form bigger size colloides that can be ingested by organisms. Bivalves in CdS NPs exposure showed impaired feeding behaviors as well for both water and food contamination regime. It is assumed that there is a consistent toxicity of CdS NPs in food chain from the autotrophic algae through the trophic cascade up to filter feeding organism *S. plana*.

Both sediment dwelling organisms, *N. diversicolor* and *S. plana* have different strategies (longevity of lifespan, intake patterns of external food, digestion mechanisms, etc.) in combating oxidative stresses, resulting in different responses at different levels. While *S. plana* in general gave a better idea in the ecotoxicological effects, which was being impaired through the observed reduced behavioral results and elevated biochemical levels. At the same time, it was more obvious to see
imperative through behavioral tests than biochemical tests. Ringwood et al. (2006) proved the toxicity of cadmium QDs to hepatopancreatic cells of oyster, under the concentration of QDs from 0.01 to 1.0 ppm (µg/L). The behavioral impairment of stickleback, Gasterosteus aculeatus, under cadmium QDs exposure was found at the concentration of 500 µg/L (Sanders et al., 2008). The feeding environment with algae for bivalves may have altered the pH of the water column (Dubinsky & Rotem, 1974) that help facilitate the degradation and colloidal formation of CdS NPs (Ringwood et al., 2006), resulting in more severe deleterious effect of cadmium both in soluble Cd and Cd NPs. While dissolved Cd\(^{2+}\) is more toxic to organisms than in particle forms, there is less than 20% of cadmium QDs found in dissolved phase (Gagné et al., 2008; Peyrot et al., 2009). Estimation of the dissolved fraction of metals by using DGT (Diffuse Gradients in Thin film) can be studied following the method raised by Davidson & Zhang (1994). In Buffet et al. (2011), DGT results showed that no measurable release of labile Cu from CuO NPs occurred during the time of experiment. Thus DGT was involved in this project as well, but the analysis is still ongoing.

**4.3. Environmental risk assessment**

Risk assessment is the task of characterizing a level of risk, usually in terms of a relative score or ranking. The goal of performing a risk assessment is to provide information that will help evaluate alternatives and for decision making (Calow, 1998). Risk assessment of ENPs has started relatively early with studies on hazards and exposure routes for humans with occupational exposure, for being the most concerned among public (Kreyling et al., 2006; Lam et al., 2006).

Classic approaches for common chemicals in aquatic environmental risk assessment is less applicable to NMs. ENPs might not be as toxic as other pollutants in the traditional sense, taking the beneficial effects of ENPs into consideration (NPCA, 2008). Current approaches for environmental risk assessment of chemicals basically compare their existing or predicted environmental concentrations to no adverse effect levels. Similar strategy may be applied when evaluating ENPs. However, speciation and concentrations may not be matched with the classic basis for estimating hazards and environmental effects.

New methodologies integrating various classic techniques (Klaine et al., 1996), as well as new ones, emerged in recent years for better evaluation of environmental
pollutants, to fulfil the needs of integrated risk assessment of pollutants (Maynard et al., 2006; Klaine et al., 2008). Biomarkers can be used as early indicators in organisms exposed to polluted environments, possible to detect even under low concentration of pollutants. Living organisms integrate exposure to contaminants in their environment and respond in some measurable and predictable way. It can be identified at different organizational levels, such as binding to a receptor in between biomolecules, biochemical responses among cells, physiological alterations in organs, or effects on individuals (e.g. behavioral changes) (Walker et al., 2001). Biological responses at higher organizational levels, such as populations, communities and ecosystems, are considered as bioindicators (Walker et al., 2001). Behavioral responses e.g. in locomotion is exclusively valuable due to its close linkage with fitness-related parameters like food seeking and predator avoidance (Little et al., 1990), indicating behavior’s great ecological importance in population and community scales of study (Kruzynski & Birtwell, 1995; Roast et al., 2000; Weis et al., 2001). Ideally, biomarkers can provide not only evidence of exposure to a broad spectrum of anthropogenic chemicals, but also a temporally integrated measure of bioavailable contaminant levels. It is applicable to have a suite of biomarkers that are evaluated over time to determine the magnitude of the problem and the possible consequences (NPCA, 2008).
5. Conclusions

Even at a low concentration of Cd that is environmentally correlated, CdS NPs, as well as Cd in ionic form, are still able to induce significant sublethal effect in the locomotion and biochemical activities of test organisms. Not only GST biomarker was evoked, but also MTLP. However, the induced effect in biochemical biomarker was relatively limited to be detected in a significant level. Behavioral impairment was proved to be most significant, especially through MFB tests, although burrowing kinetics showed the expected effect only in *S. plana*. No obvious effect was shown in feeding rate test, either. All together this suggests a specific adverse effect of nanoparticles brought by CdS NPs, which is more important considering that it induces the same or even worse consequences. The usage of MFB and experimental settings were suitable for this kind of ecotoxicity studies, and yet it can be improved for further research. The chosen marine invertebrates, *N. diversicolor* and *S. plana*, were proved to be useful and informative in providing ecotoxicity information of the studied estuarine site, especially bivalves *S. plana*, thus it is highly recommend their use for nanoparticle ecotoxicology. Integrated methodology combining biological responses with chemical analysis is an important asset for current marine environmental risk assessment. Further improvements of the work include dissolved fraction of metals via DGT method and subsequent chemical and DNA analysis of the tissues from the test organisms.
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