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Toxic effect of different metal bearing nanoparticles

(ZnO NPs, TiO_2 NPs, SiO_2 NPs, Ag NPs) toward marine phytoplankton

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Abstract

The advent of nanotechnology and the commercialization of several nanoparticle-containing-products call to a thorough assessment of the environmental risks derived from the exposure to these new materials. The most important criticisms of new nano-structured materials are represented by the emerging properties, the absence of a dedicate regulation, the increasing world-market, the implementation of the application fields. At "nano" size, materials show different physicochemical properties compared to the same material of larger size (bulk material), particularly with respect to conductivity, density, hardness, surface area and surface layer composition. At the same time, these novel properties of nanoparticles (NPs) generate special concerns about their potential hazards to humans and other organisms when released into the environment.

In this context, studies on the potential toxicity of NPs in different biological systems are urgently needed in order to define adequate guidelines for toxicity studies and to harmonize the production of new and safe materials. Since marine environment represents the ultimate sink for any materials discharged into the environment, the effect on marine organisms should be considered a critical point in the definition of NP toxicity.

In coastal ecosystems, microalgae play a key role as primary producers and, being at the base of the aquatic food web, any modification of their growth could affect higher trophic levels additionally, phytoplankton represents an excellent aquatic model for the study of the effects of pollutant exposure at population level due to a short generation time and high sensitivities. For all these reasons, they could be considered as key targets for NPs toxicity.

In this PhD thesis marine phytoplankton have been used in order to assess the potential toxicity and the mode of action of different metal bearing NPs: ZnO, SiO_2, TiO_2, and Ag. Several endpoint such as population growth inhibition, microscopy observations, cytotoxicity and evaluation of DNA damage are evaluated in the aim of understand the different interaction among algae/NPs and how this interaction could be related to the toxic mechanisms.

The comparison among the tested nanomaterial toxicity pattern highlighted that the algae population growth inhibition occurred through specific pathways related to different physicochemical NP behavior in seawater.

ZnO seems to exert its toxic action upon algae by a punctual and continuous ion release from aggregates in proximity of algae cell wall. In addition, in the case of Ag NPs, the toxicity is related to the ion release but to a greater extend respect to ZnO NP. For SiO_2 a cascade of effects (ROS production-DNA damages-growth inhibition) are evidenced suggesting a toxicity starting from oxidative stress generation. TiO_2, instead, firstly acts on DNA structure and then, being not soluble in seawater, after internalization during cell division or cell wall destruction, gives place to activation of cellular signals destabilizing DNA structure. These results underline the importance and the necessity of further long-term toxicological experiments. In addition, more attention should be paid on how the toxic effects induced by NPs has impact on the food chain.

Abstract

Lo sviluppo della nanotecnologia e la commercializzazione di diversi prodotti contenenti nanoparticelle (NP) richiede un'approfondita valutazione del rischio ambientale derivante dall'uso di questi nuovi materiali.

La maggiore criticità di questi materiali nanostrutturati è rappresentata dalle particolari caratteristiche chimico-fisiche, dall'assenza di una regolamentazione dedicata, dal crescente mercato mondiale, l'implementazione dei campi di applicazione. I nanomateriali mostrano proprietà chimico fisiche differenti rispetto allo stesso materiali di taglia più grande (bulk), soprattutto rispetto alla conducibilità, densità, durezza, area di superficie e composizione degli strati superficiali. Allo stesso tempo queste nuove proprietà delle NP generano preoccupazione circa il loro potenziale pericolo per l'uomo e l'ambiente.

In particolare, poiché l'ecosistema marino rappresenta il bacino ultimo di raccolta di qualsiasi materiale emesso in ambiente, la valutazione degli effetti delle nanoparticelle su organismi marini dovrebbe essere considerata come un punto cruciale nella definizione della tossicità dei nanomateriali.

Nell'ambiente costiero, le microalghe rappresentano un gruppo chiave in quanto alla base della rete trofica acquatica e pertanto qualsiasi effetto tossico subito potrebbe ripercuotersi su livelli trofici più elevati. Dunque le microalghe possono essere considerate come eccellenti organismi target per lo studio degli effetti derivanti dall'esposizione a diversi contaminanti a livello di popolazione grazie ai brevissimi tempi di generazione e all'elevata sensibilità. Per tutti questi motivi possono essere considerate come gli organismi ideali per la definizione del rischio derivante dall'esposizione alle nanoparticelle.

In questa tesi, il potenziale effetto tossico ed i meccanismi d' azione di diverse tipologie di NP (ZnO, SiO_2, TiO_2, Ag) sono stati valutati prendendo in considerazione come organismo target le microalghe marine.

Sono stati valutati diversi parametri: inibizione della crescita algale, morfologia, citotossicità e danno al DNA allo scopo di comprendere le differenti interazioni tra alghe/nanoparticelle e come queste interazioni siano collegate ai meccanismi di tossicità.

Il confronto tra i risultati ottenuti permette di evidenziare che la tossicità è strettamente connessa al comportamento chimico fisico delle diverse NP in una matrice complessa come l'acqua di mare.

Le NP di ZnO sembrano esercitare la loro azione tossica sulle cellule algali mediante un rilascio di ioni dagli aggregati continuo e puntuale in prossimità della parete cellulare. Anche nel caso delle NP di Ag la tossicità è strettamente collegata al rilascio di ioni ma in misura maggiore rispetto a ZnO.

Per SiO_2 sono stati evidenziati degli effetti a cascata (Produzione di ROS- Danno al DNA-inibizione della crescita) suggerendo che la tossicità potrebbe avere origine a partire dallo stress ossidativo provocato dalle NP. Il TiO_2 invece agisce prima di tutto sulla struttura del DNA ed essendo insolubile in acqua di mare, si può ipotizzare che la destabilizzazione della struttura del DNA sia conseguente a una sua internalizzazione durante la divisione cellulare o a causa della distruzione della parete cellulare.

Questi risultati sottolineano l'importanza e la necessità di ulteriori esperimenti di tossicità a lungo termine che pongano una maggiore attenzione anche sull'effetto prodotto dalle NP lungo la catena trofica.

Preface

Nanotechnology is one of the most promising and emerging technologies today. The amazing potential of this new technology, however, also comes with novel risks and uncertainties. The assessment of risks evolving from a new technology is a great challenge and should be carried out in parallel to the technological developments.

The present research is focused on the comprehension of the (eco)toxicology of a specific category of inorganic engineered nanoparticles (metal nano) upon marine phytoplankton. Different parameters and endpoint are taken into account in order to evaluate not only the NP toxic effect but also the mode of action of each material.

In Chapter 1, the main criticisms related to the production, diffusion and potential release and fate of these compounds in the marine environment are discussed.

Chapter 2 describes the aim of the project, which intends to evaluate the potential biological effects of four representative transition metal/oxides nanoparticles (TiO_2, SiO_2, ZnO and Ag NPs) on marine microalgae.

In section 3.1 (and related Supporting information) the diverse toxic effect of SiO_2 and TiO_2 nanoparticles toward the marine microalgae *Dunaliella tertiolecta* are reported and discussed.

In section 3.2, the genotoxic and cytotoxic effects of ZnO nanoparticles for *Dunaliella tertiolecta* in comparison with geno-cytotoxic SiO_2 and TiO_2 effects at population growth inhibition levels are discussed.

Section 3.3 focused on the population growth inhibition of three species of marine microalgae exposed to different sizes of Ag NPs and to coating agent PVP/PEI.

Finally, in Chapter 4 and 5, a general discussion is reported together with the main conclusions on the NP toxicity in marine environment has been delineated.

1. State of the art

1.1 Nanotechnology and nanomaterials: definition and application fields

Nanotechnology is an emerging technology that promises revolutionary improvement of products and materials for new applications. Many scientists call nanotechnology the key technology of the 21st century. By some estimates, nanotechnology even promises to far exceed the impact of the Industrial Revolution (Lyle et al., 2015). However, what is nanotechnology? By definition, nanomaterials (NM) have structures with at least one dimension in the range of 1 to 100 nm (e.g. Lespes and Gigault, 2011, Moore 2006, Stone et al., 2010, Weinberg et al., 2011, Wiesner et al., 2009). This is a very arbitrary definition since 100 nm do not represent a physicochemical threshold that justifies the distinction of NM and larger (bulk) materials. Therefore, another definition says that, in order to be a NM, it must have properties that are different from the bulk material of the same chemical composition (Zänker and Schierz 2012). These "non-bulk" properties usually only occur in dimensions under 30 nm (Auffan et al., 2009).

Hence nanoparticles (NPs) possess properties that are "qualitatively or quantitatively distinctly different from their other physical forms" (SCENIHR, 2007), such as those of larger-sized particles (bulk particles) made from the same materials and their water-soluble/ionic form. Size-related differences in particle properties may be due to the larger surface area per mass, resulting increased ratio of surface-to-core atoms and increased number of corner and edge atoms.

This results in increased reactivity (Feldheim et al., 2007) or increased ion release (Elzey and Grassian, 2010), which enables their use in novel applications.

Engineered NPs are classified as a group separate from naturally occurring nanoparticles and anthropogenic incidentally produced nanoparticles (Oberdörster et al., 2005).

Nanoscale materials have always existed and originate from both natural and anthropogenic sources (Klaine et al., 2008). Aquatic colloids, fumes originating from volcanic activity or from forest fires and atmospheric dusts, all contain naturally. Other nanomaterials are unintentionally produced and released into nature by industrial activity such as car exhaust, industrial emissions and welding fumes (Ostiguy et al., 2006; Nowack and Bucheli, 2007).

Manufactured or engineered nanomaterials (ENMs), however, are deliberately produced to take advantage of the novel properties at the nanoscale.

Based on their chemical composition, ENMs can be classified into broad categories such as carbon-based NM, which include carbon nanotubes (CNTs), fullerene C60 and graphene; metal-bearing NPs, including metal NPs such as silver (Ag), metal oxides, such as titanium dioxide (TiO_2), or semiconductor nanocrystals, also known as quantum dots, and finally, polymer-based nanomaterials such as polyethylenglycol and latex NPs (Pan and Xing, 2010; Buzea et al., 2007; Handy et al., 2008).

To document the penetration of nanotechnology in the consumer marketplace, the Woodrow Wilson International Center for Scholars and the Project on Emerging Nanotechnology created the Nanotechnology Consumer Product Inventory (CPI). (Vance et al., 2015).

In table 1, the growth of the CPI since 2005 is listed. In 2011, the CPI described 1314 products. The new total of 1814 products as of March 2015 represents a thirty-fold increase over the 54 products originally listed in 2005 – which is not a complete representation of the growth of this market. Products come from 622 companies located in 32 countries. United States is the major producer of nanotechnology-based consumer products followed by Europe East Asia and other countries as Australia, Canada, Mexico and Israel. (www.nanotechproject.org). In figure 1 the main nanotechnologies application fields together with the number of available products over time (since 2007) in each major category are reported. The Health and Fitness category includes the largest listing of products in the CPI, comprising 42% of listed products. Within the Health and Fitness category, Personal Care products (e.g., toothbrushes, lotions, and hairstyling tools and products) comprise the largest subcategory (39% of products). The number of consumer products containing NPs in their formulation is expected to reach 3400 by 2020 under current trends www. nanotechproject.org/news/archive.

Table 1: Number of products in the CPI over time. (Vance et al., 2015)

Year	Total products	Products added	Products archived	Data collection notes
2005	54	54	0	Beginning of CPI as a static pdf document.
2006	356	302	0	Launch of the online CPI.
2007	580	278	0	Nanoscale silver emerged as most cited nanomaterial.
2008	803	223	0	Health and fitness products represented 60% of the inventory.
2009	1015	212	107	Added archiving function to the CPI.
2010	1015	0	0	No data collected.
2011	1015	0	0	No data collected.
2012	1438	426	0	Beginning of CPI 2.0 project, focus on adding new products.
2013	1628	190	288	Launch of crowdsourcing component. Extensive effort put into adding and archiving products.
2014	1814[a]	238[a]	223[a]	Extensive effort put into adding and archiving products.

[a]The CPI now has crowdsourcing capabilities, so these numbers are a snapshot in time and will not represent the CPI at the time of reading.

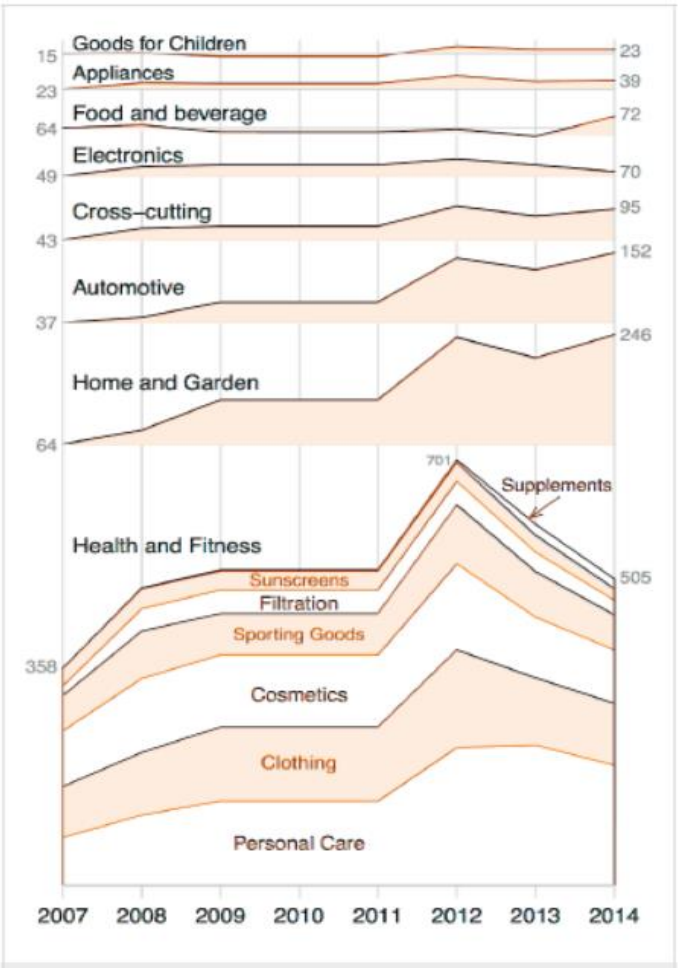

Figure 1: Number of available products over time (since 2007) in each major category and in the Health and Fitness subcategories. (Vance et al., 2015)

Comparing worldwide production of different NPs, TiO_2 (10,000 t/year), SiO_2 (10-10,000 t/year), ZnO (100-1000 t/year) are the most produced NM (Piccinno et al., 2012; Keller and Lazareva, 2013). Ag NPs are produced in moderate quantities (55 t/year) and the global annual production of silver NPs represents only 2% of that of TiO_2. However, silver NPs are the most popular advertised NM in the CPI, present in 438 products (24%). NPs such as CeO_2, FeO_x, AlO_x and quantum dots are produced between 100 and 1000 t/year (Piccinno et al., 2012).

Table 2. Production/utilization quantities of ten nanomaterials in the world and in Europe in t/year). (Piccinno et al., 2012).

ENM	Worldwide (t/year) Median and 25/75 percentile	Europe (t/year) Median and 25/75 percentile	US (t/year) (Hendren et al. 2011) Range	Switzerland (t/year) (Schmid and Riediker 2008) In brackets values extrapolated to Europe
TiO_2	3,000 (550–5,500)	550 (55–3,000)	7,800–38,000	435 (38,000)[a]
ZnO	550 (55–550)	55 (5.5–28,000)		70 (6,100)
SiO_2	5,500 (55–55,000)	5,500 (55–55,000)		75 (6,500)
FeO_x	55 (5.5–5,500)	550 (30–5,500)		365 (32,000)
AlO_x	55 (55–5,500)	550 (0.55–500)		0.005 (0.4)
CeO_x	55 (5.5–550)	55 (0.55–2,800)	35–700	
CNT	300 (55–550)	550 (180–550)	55–1,101	1 (87)
Fullerenes	0.6 (0.6–5.5)	0.6 (0.6–5.5)	2–80	
Ag	55 (5.5–550)	5.5 (0.6–55)	2.8–20	3.1 (270)
Quantum dots (QDs)	0.6 (0.6–5.5)	0.6 (0.6–5.5)		

Each of these categories presents specific features. Thanks to their crystalline structure and electrical properties carbon-based, NPs are mainly used in the electronic field. Thanks to the great surface area, some of these NPs are also used for molecular absorption (i.e. gas storage). CBs are diffusely applied as pigments or strengthening agents in tires.
Organic NPs are investigated for their application in medical, biomedical and cosmetic field. Finally, inorganic NPs show a broad spectrum of properties so they are used in many applications such as catalysis, cosmetics, optic, diagnostic, and drug delivery.
Among these inorganic NPs, metal oxides are of great interest in nanotechnology (Rice et al., 2009) and represent at the same time an attractive and critical group. The reasons of this consideration (emerging properties, growing market, hazard, and (eco) toxicity) are reported in the sections below.

1.2 Metal bearing nanoparticles: characteristics and critical aspect

Metal oxides play a very important role in many areas of chemistry, physics, and materials science (Rodriguez and Garcia, 2007). Metallic elements can form a large diversity of oxide compounds. These can adopt a vast number of structural geometries with electronic structures that can exhibit metallic, semiconductor, or insulator character. In the emerging field of nanotechnology, the goal is to make nanostructures or nanoarrays with special properties with respect to those of bulk or single-particle species (Meenakshi et al., 2012).
Criticisms arise for manufactured metal nanoxides (MONs) since their dimensions at the nanoscale confer emerging properties, which differ from single atoms, individual molecules or bulk materials. For this reason, MONs should be considered as new chemical compounds, which do not obey to classical physic laws (SCENIHR, 2007; Vippola et al., 2009).
Firstly, they have a surface area to volume ratio greater than microparticles with the same chemical composition; this means that the atoms on the surface are more than in the core and the binding energy is lower if compared to the bulk material. With the reduction in size, also, the electrons can be confined in a very little space and the result can be both a quantized spectrum of energy and a quantized ability to accept and donate electrical charge (Kamat, 2002). Some MONs (e.g. TiO_2, ZnO) show the ability to generate electron-hole pairs when photo-activated: when particles with a specific size (5÷20 nm) are excited by energy greater than their band gap. A positive holes in the valence band occur because electrons are promoted to the conduction band (Hurst et al., 2011). This electronic unbalance can lead to redox processes on particle surface and further recombination reactions can occur with the subsequent loss of the absorbed energy. Another consequence of the quantum effect is the appearance of magnetic moment in materials, which do not present this property at the bulk state (Buzea et al., 2007). Emerging properties are of actual interest in the nanotechnological world. These new characteristics are usefully employed to develop new technological applications and benefits, but, at the same time, such changes in chemical-physical behaviour may determine different environmental fate and/or toxic

properties, making necessary a risk assessment on case-by-case basis. For these reasons, these chemicals should be treated as new substances and therefore regulated by a specific discipline. The main characteristics of the NPs selected for this study were reported below.

ZnO: Zinc oxide nanoparticles (ZnO NPs) (Fig. 2) is of great importance, with their annual global production to be estimated in 550 ton, classifying them third in production order after SiO_2 (5550 ton) and TiO_2 (3000 ton) (Piccinno et al., 2012). ZnO NPs are used as ultraviolent light absorbents additives in sunscreens, toothpastes and beauty products (Serpone et al., 2007), as well as in rubber manufacture, production of solar cells and LCD, pigments, chemical fibers, electronics, and textiles (Bondarenko et al., 2013; Klaine et al., 2008) due to their specific properties, e.g. transparency, high isoelectric point, biocompatibility, and photocatalytic efficiency. Finally, ZnO NPs have been also employed as antimicrobial agents (Padmavathy and Vijayaraghavan, 2008).

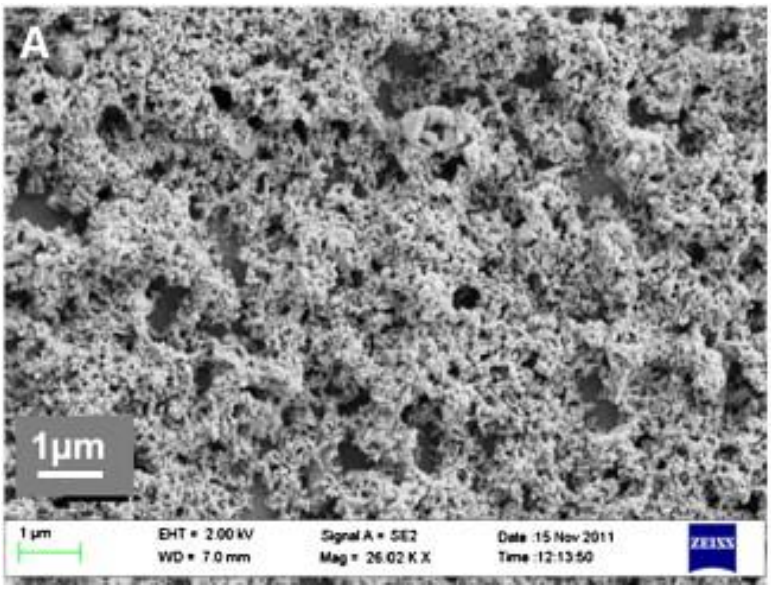

Figure 2: Zinc Oxide nanoparticles. (Manzo et al., 2013)

TiO_2: Titanium dioxide (TiO_2) NPs (Fig. 3) is the naturally occurring oxide of titanium. It has several different crystalline structures. Rutile is the most common natural form of TiO_2, whereas anatase and brookite are two more rare polymorphs. TiO_2 has been used widely in pigments, accounting for 70% of the total production volume of pigments worldwide. It provides whiteness and opacity to products such as paints, plastics, papers, inks, foods, and toothpastes. It can also be found in pharmaceuticals and cosmetic products such as sunblock due to its photocatalytic, biocidal, and/or antiproliferative properties (Chen, 2014). TiO_2 NP are also used in the decontamination of air, soil, and water.

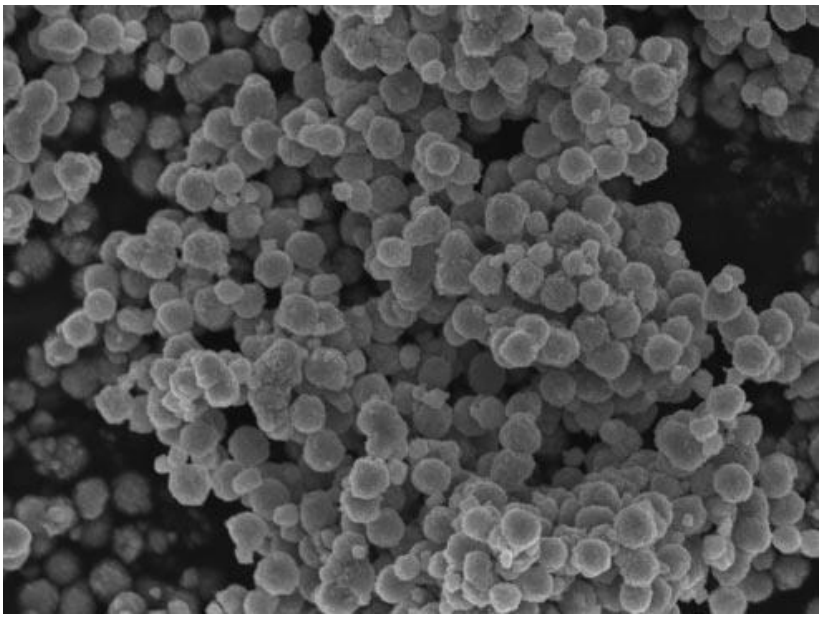

Figure 3: TiO_2 nanoparticles from (www.nanolabs.co.in)

SiO_2: Silica-based nanomaterials (fig. 4) have attracted much attention in biomedical applications as cell markers, gene transfection agents, imaging moieties, and drug carriers. They possess a variety of unique properties, such as ease of synthesis, availability of surface modification, robust mechanical properties, and relatively inert chemical composition silica (SiO_2) NPs have found extensive applications in chemical mechanical polishing and as additives to drugs, cosmetics, printer toners, varnishes, and food. In recent years, the use of SiO_2 NPs has been extended to biomedical and biotechnological fields, such as biosensors for simultaneous assay of glucose, lactate, l-glutamate (Zhang et al., 2004), biomarkers for leukemia cell identification (Santra et al., 2001), cancer therapy (Hirsch et al., 2003), DNA delivery (Bharali et al., 2005) and drug delivery (Venkatesan et al., 2005).

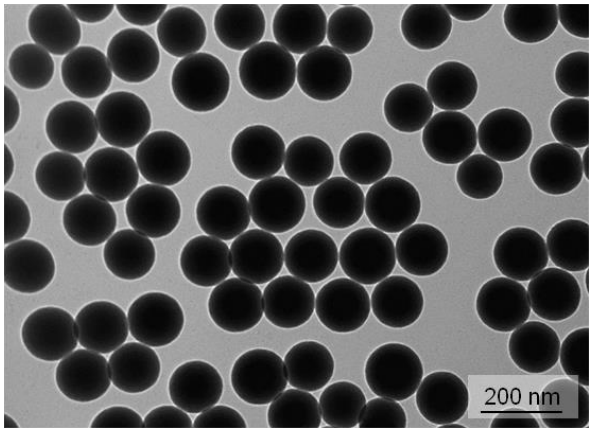

Figure 4: SiO_2 nanoparticles (www.hiqnano.com)

Ag: Engineered Silver (Ag) NPs (fig. 5) are believed to be the most commercialized nanomaterials. As a result of their wide applications, a considerable fraction of the Ag NPs will eventually find their way into aquatic ecosystems and possibly exert some negative effects, given their anti-bacterial characteristics (Miao et al., 2009) Silver nanoparticles Ag NPs are emerging as one of the fastest growing product categories in the nanotechnology industry. Due to their physico chemical properties, including a high thermos electrical conductivity, catalytic activity and non-linear optical behavior (Capek, 2004) Ag NPs have potential value in the formulation of inks, microelectronic products and medical imaging devices. Due to bactericides or fungicides, properties have found versatile applications in diverse products like household appliances, cleaners, clothing, cutlery, children's toys, and coated electronics (Luoma et al., 2008).

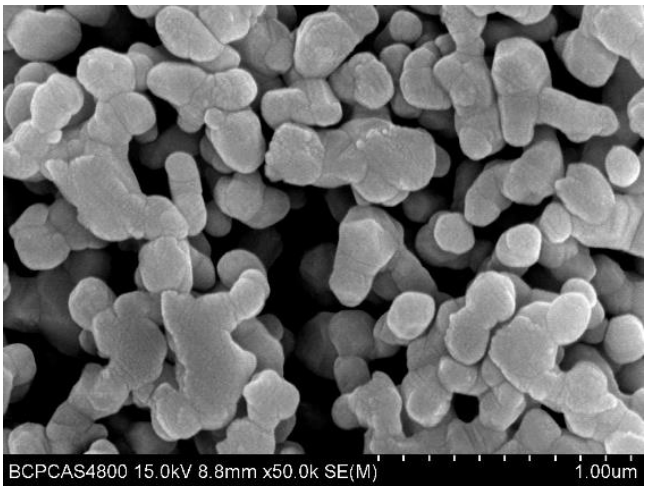

Figure 5: Silver nanoparticles (www.nanobond.com)

1.3 Fate and release of nanoparticles in environment

Nanomaterials are produced and applied for products that improve our daily life (e.g. medical products, cleaning products, cosmetics, computer technique) and for industrial applications (e.g. paintings, coatings, powders and fibers for the production of materials with new properties). However, increased production levels inevitably lead to increasing incidence of the materials in the environment.

Until a few years ago, little was known about the fate of nanomaterials in the environment, but recent studies suggest important emerging patterns (Gottschalk et al., 2009; Keller et al., 2014; Garner and Keller 2014). There are still major strategic knowledge gaps for even the most widely used nanoparticles (NPs) involving their postproduction life cycles, including entry into the environment, environmental pathways, eventual environmental fate, and potential ecotoxicological effects.

Engineered Nanomaterials ENMs are released into the environment either during their use, by spillages, by intentional release for environmental remediation applications, or as end-of-life waste (Keller et al., 2013). As already reported more than 1,800 products that are on the market today contain NPs (Bondarenko et al., 2013) and production estimates of major ENMs range from 270,000 to 320,000 metric tons per year, of which high end estimates suggest that 17 % may be release to soils, 21 % to water, and 2.5 % to air, with the balance entering landfills (Keller and Lazareva 2013). Many fate and transport processes need to be considered to understand ENM mobility, bioavailability, and ultimate fate (Fig. 6). These include ENM emissions to air, water, and soil; advection in and out of the system; diffusive transport; volatilization to air; transformation into other ENMs or compounds; aggregation; sedimentation; dissolution; filtration; and sorption to suspended particles and the subsequent deposition to sediment (Quik et al., 2011). Many processes are important to ENMs that may not be relevant to the environmental behavior of traditional contaminants (Quik et al., 2011), such as aggregation, dissolution, deposition, and attachment. These are all determined by their size, surface properties, and ambient environmental characteristics.

Once released, ENMs will interact with the environment in several ways. These interactions are controlled by the inherent properties of the ENMs (solubility in water, colloidal stability, reactivity, etc.) and the properties of the environment into which they are released (temperature, flows of air, water, and solids, and the physicochemical characteristics of each phase) (Garner and Keller, 2014). Properties such as ionic strength (IS), pH, the presence of organic matter, and compartment composition are all important parameters that will modify ENM behavior (Keller et al., 2010; Lowry et al., 2012a, b; Zhou et al., 2012b). It is important to understand both how ENMs interact with their environment and how their environment alters the expected interactions.

Current predictions indicate that globally as much as 66,000 metric tons of ENMs are released directly to surface waters every year (Keller and Lazareva 2013).

ENMs release in the aquatic environment largely depends on the chemical properties of the water. Differences in aquatic characteristic can significantly affect the rate of many fate and transport processes. Studies of ENM fate in realistic aquatic media indicates that in general, ENMs are more stable in freshwater and storm water than in seawater or groundwater, suggesting that transport may be higher in freshwater than in seawater.

For example, the IS and concentration of natural organic matter NOM present in seawater versus freshwater will impact rates of aggregation, sedimentation, and dissolution for some ENMs. Variations in surface charge, surface coating, and shape can also alter the fate of ENMs in the environment. Transformations processes such as oxidation, sulfidation, and interactions with phosphate, all frequently present in aquatic systems, will also have a significant effect on aggregation, dissolution, and as a result toxicity.

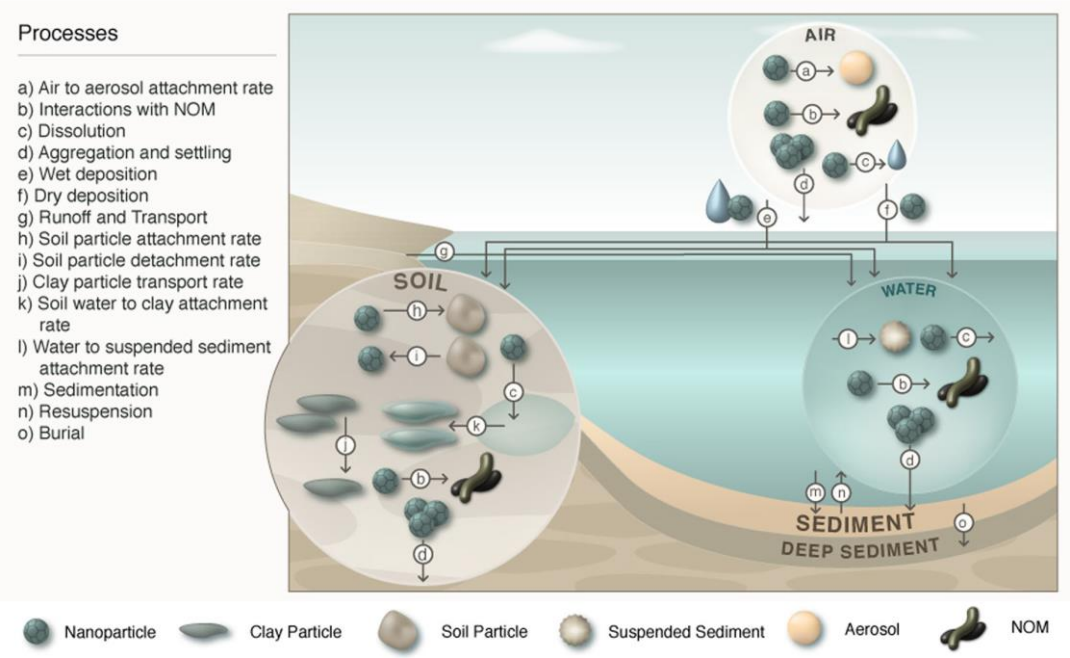

Figure 6: Fig. 1 Conceptual model of key ENM fate processes. Diagram by Anastasiya Lazareva. This shows how nanoparticles are transported between environmental compartments and how they may interact with other constituents in the environment as well as with themselves. (Garner and Keller, 2014)

1.4 Toxicity of nanoparticles

The unique physic-chemical properties of engineered NPs derived from their small size, surface area and surface reactivity (inorganic or organic coatings etc.), chemical composition, solubility, shape and aggregation state are crucial factors that determine their toxicity. Together with the development of nanotechnology a new area of toxicology rise up: nanotoxicology. Nanotoxicology focuses on the understanding of the relationship between the toxicity of NPs depending on their dose levels and physicochemical properties such as size, shape, reactivity and material composition (Paur et al., 2011). In general, the evaluation of NP toxicity was focused on in vitro cells. NPs may be taken up by, and induce effects in, organisms in many different ways, however the exact method for this is entirely particle specific (Bhatt and Tripathi, 2011). In the first instance, NPs may adhere to a cell and block essential pores and membrane functions. Alternatively, they could also enter the cell by endocytosis, via diffusion through pores (with the potential for pore stretching or damage), or via ion transport systems. Having entered the cell, the NPs can potentially interfere with electron transport processes, or facilitate reactive oxygen species (ROS) production by hampering organelle functions. ROS production may lead to nucleic acid damage, protein oxidation or disruption of cell membranes. (Xia et al., 2015)

1.5 Toxic effect on the aquatic organisms

Most of the currently available ecotoxicological data regarding NPs are limited to species used in regulatory testing or freshwater species (Lovern and Klaper, 2006; Federici et al., 2007; Warheit et al., 2007; Handy et al., 2008a,b; Blaise et al., 2008) including phytoplankton (Navarro), *Daphnia magna* (waterflea), *Lymnaea stagnalis* (pond snail) and *Caenorhabditis elegans* (nematode). From these studies, results have highlighted a range of sub-lethal effects including reduced swimming (Boyle et al., 2013)), reduced growth and reproduction (Zhao and Wang, 2011), bioaccumulation (Rosenkranz et al., 2009), digestive stress and reduced feeding (Croteau et al., 2011a, 2011b).

Despite extensive research on freshwater species, little study has been directed towards marine organisms. Published data at this time are available for just eight phyla and, of these, many reports are limited to a single class, order or species. It is not yet clear how best to extrapolate freshwater data for marine organisms given that the properties of NPs will change according to exposure media, as will the biological, behavioral and respiration characteristics of marine organisms.

In general, three primary biological targets can be identified in the marine environment:

1) filter feeders, which can be exposed to high ENP concentrations present in surface waters released by terrestrial and atmospheric sources or existing aggregates;

2) pelagic species ranging from phytoplankton to fish and mammals, including deep sea species exposed during vertical migration of the particles;
3) benthic species that are exposed to ENPs deposited in sediment biofilms (Matranga and Corsi, 2012).

1.6 Microalgae as target organisms

In coastal ecosystems, microalgae play a key role as primary producers and, being at the base of the aquatic food web, any modification of their growth could affect higher trophic levels (Rioboo et al., 2007). Additionally, phytoplankton represents an excellent aquatic model for the study of the effects of pollutant exposure at population level (Chen C. et al., 2012), due to a short generation time and high sensitivities. The evaluation of NP effects upon marine phytoplankton is a necessary step to predict their potential impact on coastal marine food webs and overall ecosystems they support.

Zn2þ for example has been shown to interfere with silica uptake in diatoms. ZnO NPs (10 mg/L) have been shown to inhibit growth of the diatoms *Chaetoceros gracilis* and *Thalassiosira pseudonana* with accumulation in *T. pseudonana* resulting in mortality (Peng et al., 2011), although not at relevant concentrations. *Phaeodactylum tricornutum* displayed fewer effects with growth only slightly inhibited, suggesting a lower nutrient demand for silica than the other species. Larger ZnO NPs (20-30 nm spheroids) have been shown to inhibit growth of *T. pseudonana* at only 0.5 mg/L, but the diatom *Skeletonema marinoi*, the chlorophyte *Dunaliella tertiolecta* and the prymnesiophyte *Isochrysis galbana* at concentrations of 1 mg/L over 96 h (Miller et al., 2010). ZnO NPs showed significant toxicity to *T. pseudonana* and *Skeletonema costatum* over 96 h (Wong et al., 2010), generating an LC50 of 2.36 e 6.65 mg/L. Similar EC50 values for exposure of *D. tertiolecta* to $ZnCl_2$, ZnO NPs (100 nm) and micron ZnO and have been recorded as 0.65 mg/L, 1.94 mg/L and 3.57 mg/L respectively (Manzo et al., 2013). That significant reduction in growth was seen at 0.23 mg/L for $ZnCl_2$, 1 mg/L for ZnO NPs and 3 mg/L for micron ZnO highlights dissolution as the primary driver of toxicity. However, these values are far above environmental relevance meaning that only highly acute, point source discharges are likely to affect marine algae. Dissolution of Ag ion is also believed to be the driver of toxicity for Ag NPs. 50% inhibition (IC50) in the growth of *P. tricornutum* has been recorded at 2380±1880 and 3690±2380 µg/L for ionic Ag, citrate-capped (14 nm) and PVP-capped (15 nm) Ag NPs respectively (Angel et al., 2013). Referencing the particles to their dissolution rate shows equivalent Ag concentrations for each IC50 value. Similar conclusions were drawn comparing Ag NPs (PVP capped, 10 nm) and Ag ions on the photosystem quantum yield of the coastal diatom *Thalassiosira weissflogii* (Miao et al., 2009). Dissolved Ag ion was seen to form AgCl complexes that adsorbed to the diatoms' surface, thereby making algae vectors for AgCl transport to higher trophic organisms. To date, only one study exists on the effects of NPs on macroalgae, on the sea lettuce *Ulva lactuca* (Turner et al., 2012). A 48 h exposure to Ag NPs (58 27 nm, PVP-capped) only reduced the yield of photosystem II at concentrations above 55 mg/L, however $AgNO_3$ exposures showed negative effects at only 2.5 mg/L, suggesting dissolution of Ag ion as the main driver of toxicity. Evidence of bioaccumulation was strongly associated with surface adsorption rather than internalization, however this could still provide a toxic substrate for surface grazers.

TiO_2 and SiO_2 NPs were observed to be able to inhibit the growth of varieties of algae (Fujiwara et al., 2008; Van Hoecke et al. 2008; Hall et al. 2009). Van Hoecke et al. (2008) showed that different sizes of SiO_2 were toxic to *Pseudokirchneriella subcapitata*, with an EC 20 for the growth rate in the range of 20.0–28.8 mg/L. Ji et al. (2011), in a study about the green algae *Chlorella*, reported that SiO_2 had no significant toxicity while TiO_2 NPs (HR3, anatase) greatly inhibited the algal growth with an EC30 of 30 mg/L.

Data about TiO_2 are various and effects were generally found at concentrations >10 mg/L (Hund-Rinke and Simon 2006; Menard et al., 2011). A very recent study of Xia et al., (2015) reported for *Nitzschia closterium* population (96 h) EC50 values of 88 and 118 mg/L for 21 and 60 nm TiO_2 NPs, respectively. Actually lower EC50 values were observed for *P. subcapitata* (Aruoja et al., 2008, Lee et al., 2013) and for different marine algae; Li et al., 2015 reported TiO_2 EC50 values of 10 mg/L for *Karenia brevis* and 7 mg/L for the diatom *Skeletonema costatum* while 1–3 mg/L TiO_2 was reported to exert a significant adverse effect upon some marine phytoplankton population (*Thalassiosira pseudonana, Skeletonema costatum, Dunaliella tertiolecta, and Isochrysis galbana*).

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2. Aims of the study

Nanotechnology is one of the most promising and emerging technologies today. The amazing potential of this new technology however is associated with many uncertainties regarding risks posed by nanomaterials, especially in marine environment that represents the ultimate sink for any substance deliberately or purposely discharged into the environment.

In this view, the present thesis wants to elucidate the ecotoxicological impacts of a set of metal bearing nanoparticles (NPs) to a class/group key organism in marine environment such as marine microalgae.

The evaluation of NP effects upon marine phytoplankton is a necessary step to predict their potential impact on coastal marine food webs and overall ecosystems they support.

The work presented in this thesis aims to collect new knowledge about NP ecotoxicity. Additionally, this thesis explores the diverse mode of action of the several NPs by different kind of endpoints and tests.

The main hypotheses of the present work are:

- NP toxic action for algae is not solely ascribable to ion releasing.
- The NP physic-chemical characteristics in environmental media influence the effects upon algae
- The NP ecotoxic action is the results of different effects valuable at cellular and DNA levels by COMET assay.
- The presence of a capping agents could largely influence the toxicity

These tests might provide a complementary tool in environmental risk assessment of metal bearing NPs in marine ecosystems and might reveal if the toxic action of NPs occur through cellular mechanisms involving oxidative stress, genotoxicity and damage to different cellular compartments.

In order to achieve these objectives and to proof this hypotheses true, the present work attempts to address the following general objective:

- to evaluate the population growth inhibition, cell viability, oxidative stress, DNA damage, morphological modifications of microalgae exposed to ZnO, TiO_2, SiO_2, and Ag NPs.

This general objective has been subdivided into the three specific objectives shown below, which are addressed in each chapter of the Results section and in the General Discussion section:

- AIM Section 3.1

To evaluate the population growth rate alterations of *Dunaliella tertiolecta* exposed to SiO_2 and TiO_2. The cytotoxicity is also assessed by the analysis of cell viability and ROS production.

- AIM Section 3.2

To assess the genotoxic (COMET assay) and cytotoxic effects (ROS production and cell viability) of ZnO NPs towards *D. tertiolecta*. Genotoxic effects were also compared to those exerted by other metal oxide nanomaterials such as SiO_2 and TiO_2 NPs at levels of population growth inhibition, in order to disclose the diverse mode of action.

- AIM Section 3.3

To assess a preliminarily screening in order to understand the sensitivity of microalgae belonging to different classes exposed to different size of Ag NP. Interactions of algae cells surface with Ag NPs were also studied by microscopy analysis.

3. Results and discussion

3.1 The diverse toxic effect of SiO_2 and TiO_2 nanoparticles toward the marine microalgae *Dunaliella tertiolecta*

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Abstract

Nanoparticles (NPs) are widely used in many industrial applications. NP fate and behavior in seawater are a very important issue for the assessment of their environmental impact and potential toxicity. In this study, the toxic effects of two nanomaterials, silicon dioxide (SiO_2) and titanium dioxide (TiO_2) NPs with similar primary size (~20 nm), on marine microalgae *Dunaliella tertiolecta* were investigated and compared. The dispersion behavior of SiO_2 and TiO_2 NPs in seawater matrix was investigated together with the relative trend of the exposed algal population growth. SiO_2 aggregates rapidly reached a constant size (600 nm) irrespective of the concentration while TiO_2 NP aggregates grew up to 4±5 μm. The dose–response curve and population growth rate alteration of marine alga *D. tertiolecta* were evaluated showing that the algal population was clearly affected by the presence of TiO_2 NPs. These particles showed effects on 50 % of the population at 24.10 [19.38–25.43] mg L^{-1} (EC50) and a no observed effect concentration (NOEC) at 7.5 mg L^{-1}. The 1% effect concentration (EC1) value was nearly above the actual estimated environmental concentration in the aquatic environment. SiO_2 NPs were less toxic than TiO_2 for *D. tertiolecta*, with EC50 and NOEC values one order of magnitude higher.

The overall toxic action seemed due to the contact between aggregates and cell surfaces, but while for SiO_2 a direct action upon membrane integrity could be observed after the third day of exposure, TiO_2 seemed to exert its toxic action in the first hours of exposure, mostly via cell entrapment and agglomeration.

Introduction

Engineered nanomaterials (ENMs) are an important emerging class of contaminants, with potential wide-ranging ecological impacts due to their small size and high reactivity. Silicon dioxide and titanium dioxide are the most commonly employed among the 10 major ENMs in various industrial sectors (production of >100 t/year) (Future markets, 2012).

Nanostructured TiO_2 is mainly used for protection against UV ray exposure in many sunscreens and cosmetics, while SiO_2 nanoparticles (NPs) are mainly used in paints and coatings for an improved rheology, attachment, and scratch resistance (Rittner 2003; Mizutani et al. 2006; Zappa et al. 2009).

As so, these nanomaterial-based products are expected to end up in waterbodies mainly via urban and industrial sewage. In particular, SiO_2 and TiO_2 could represent, respectively, the 7 and 53 % of the predicted engineered NM emissions in waterbodies (Keller et al. 2013). As a result, NPs could reach the marine environment and therefore the coastal systems, which are likely to be the ultimate sink for any NM deliberately or purposely discharged into the environment (Klaine et al. 2008). In this view, marine algae, which are highly diffused in coastal ecosystems (Behrenfeld et al. 2006) and are particularly susceptible to contaminants associated with anthropogenic pollution, can be regarded as a suitable indicator for marine water pollution by ENMs. The evaluation of NP effects upon marine phytoplankton is indeed a necessary step to predict their potential impact on coastal marine food webs and on the whole ecosystems they support.

Recently, some studies regarding the effects of nanomaterials such as ZnO, TiO_2, Ag, and SiO_2 upon marine algae and diatoms were published (Bielmyer-Fraser et al. 2014, Li et al. 2005, Xia et al. 2015), showing that this is still an emerging field. TiO_2 and SiO_2 NPs were observed to be able to inhibit the growth of varieties of algae (Fujiwara et al. 2008; Hall et al. 2009; Van Hoecke et al. 2008). Van Hoecke et al. (2008) showed that different sizes of SiO_2 were toxic to *Pseudokirchneriella subcapitata*, with an EC20 for the growth rate in the range of 20.0–28.8 mg L^{-1}; Ji et al. (2011), in a study about the green algae *Chlorella*, reported that SiO_2 had no significant toxicity while TiO_2 NPs (HR3, anatase) greatly inhibited the algal growth with an EC30 of 30

mg L^{-1}. The same authors emphasize the contribution of the crystalline structure to the toxicity due to surface properties and reactivity, and a greater toxic effect was generally reported for anatase in comparison with rutile (Clément et al. 2013, Ji et al. 2011).
Data about TiO_2 are various and effects were generally found at concentrations >10 mg L^{-1} (Hund-Rinke and Simon 2006; Menard et al. 2011). A very recent study of Xia et al. (2015) reported for *Nitzschia closterium* population (96 h) EC50 values of 88 and 118 mg L^{-1} for 21 and 60 nm TiO_2 NPs, respectively. Actually lower EC50 values were observed for *P. subcapitata* (Aruoja et al. 2008, Lee et al. 2013) and for different marine algae; Li et al 2015 reported TiO_2 EC50 values of 10 mg L^{-1} for *Karenia brevis* and 7 mg L^{-1} for the diatom *Skeletonema costatum* while 1–3 mg L^{-1} TiO_2 was reported to exert a significant adverse effect upon some marine phytoplankton population (*Thalassiosira pseudonana, S. costatum, Dunaliella tertiolecta, and Isochrysis galbana*) only under natural levels of ultraviolet radiation (Miller et al. 2012). In the main, studies about SiO_2 and TiO_2 NP toxicity toward microalgae are hardly comparable because of several differences in testing matrices, test organisms, and standardized experimental conditions (Minetto et al. 2014).
Another important issue is the NP tendency to aggregate in aquatic environments. The formation of micrometer-sized particles modifies the surface properties and the influence of particle size and shape on their ecotoxicity (Handy et al. 2008; Limbach et al. 2005). In particular, TiO_2 aggregates so rapidly in seawater that the predicted residence times are in the range of hours (Garner and Keller, 2014). A fast sedimentation and a short residence time in the water column (i.e., within hours to days) result in low exposure doses to species living in the water column but also in a corresponding accumulation in sediment (Klaine et al. 2008). In this context, SiO_2 presents a noteworthy behavior: it is rather stable even in high-ionic strength media and its aggregates in seawater show a long residence time with a consequent slower sedimentation (i.e., multiple weeks or longer) and potentially greater transport distances (Zhang et al. 2009). In this work, we focused on the different behavior of well investigated materials, such as TiO_2 (anatase) and SiO_2 in pristine nanometric powders (~20 nm), in a marine environment over a 4-day testing time. A test organism particularly sensitive to NP exposure as *D. tertiolecta* was used (Miglietta et al. 2011). The population growth rate alterations were evaluated and determined the no observed effect concentration (NOEC) and EC50 for SiO_2 and TiO_2. The cell damages were also evaluated by the analysis of cell viability and ROS production.
The different aggregation trends in standard seawater were highlighted and related to the ecotoxicological effects.

Materials and method

Chemicals

Commercial silicon dioxide nanoparticles (nominal purity 99.5 %, primary particle size 10–20 nm) and titanium dioxide (anatase, nominal purity 99.7 %, primary particle size 25 nm) were purchased from Sigma-Aldrich. Artificial seawater (ASW) was prepared according to the ASTM method (NaCl 0.4 M, $MgCl_2*6H_2O$ 0.053M, Na_2SO_4 0.02 M, $CaCl_2*H_2O$ 0.01 M, KCl 9 mM, $NaHCO_3$ 2 mM, KBr 0.8 mM, H_3BO_3 0.4 mM, $SrCl_2*6H_2O$ 0.09 mM, $NaSiO_3*9H_2O$ 0.07 mM) and filtered through 0.22 μm (pH 8.0) (ASTM 1998).

Organisms

D. tertiolecta (Chlorophyceae: Chlamydomonadales) (CRIAcq Laboratory, (Na) Italy) algae were maintained in a sterilized standard medium (Guillard 1975) made with ASW.
Microalgae were incubated under cool continuous white fluorescent lights until log phase growth prevailed (about 58 μmol photons m−2 s−1 at 24±1 °C with aeration for 5–7 days) to provide inocula for experiments. Cell density was measured by a hemocytometer.

Particle dispersion

Stock suspensions of SiO_2 and TiO_2 NPs were prepared by dispersing dry powders into artificial seawater to the final concentration of 2000 mg L−1 for SiO_2 and 1000 mg L−1 for TiO_2, respectively. The NP suspensions were bath sonicated in a low-power ultrasonic bath (Elma Transsonic Digital S) for 30 min. Stock dispersions were properly diluted at concentrations ranging between 5 and 200 mg L−1 for SiO_2 and 1 and 100 mg L−1 for

TiO_2. After dilution, all the suspensions were bath sonicated again for 10 min. The dilutions were vortexed briefly before the addition of micronutrients and test organism.

Particle characterization

The average particle size of only few of the diluted suspensions (i.e., 200, 125 mg L−1 for SiO_2 and 100, 20, 7.5 mg L−1 for TiO_2) was analyzed by dynamic light scattering (DLS) in order to monitor the particle aggregation at an early stage (first 180 min) and for the next 4 days using the Zetasizer Nano ZS (Malvern Instruments). This instrument employs a 4-mWHe–Ne laser, operating at wavelength 632.8 nm with the measurement angle set at 173° using a Non-Invasive Back Scatter (NIBS) patented technology. Samples were measured at 25 °C. The electrophoretic mobility was measured with the Zetasizer (Nano ZS, Malvern Instruments Ltd., UK) and converted to ζ potentials by the instrument software (Dispersion Technology Software, version 5.1, Malvern Instruments Ltd., UK) using Henry's equation: $Ue=2\varepsilon\zeta f(ka)/3\eta$, where Ue is the electrophoretic mobility, ε is the dielectric constant, ζ is the zeta potential, η is the viscosity of the dispersant, and f (ka) is the Henry function. For high-ionic-strength media was used the Smoluchowski approximation f (ka) =1.5.

Toxicity test

Algal growth inhibition test

Algal bioassays were performed according to IRSA-CNR (IRSA-CNR 1978). All glassware was acid washed, rinsed with purified water Milli-Q, and autoclaved before use. Algal cells (with a final density of 103 cells L^{-1}) were first filtered (0.22 μm) and rinsed three times with filtered autoclaved seawater. The algal cells were then added to each treatment and control (standard culture media, Guillard medium) together with nutrients. Test plates (10 mL) were kept in a growth chamber constantly illuminated with a white fluorescent lamp (enhanced irradiation between 400 and 500 nm), at a temperature of 24 ± 1 °C for 4 days. The growth inhibition was expressed in percent effect with respect to the control. The concentrations of the testing solutions were defined on the basis of a preliminary screening (Miglietta et al. 2011) and were 100, 50, 40, 30, 20 10, 7.5, 5, and 1 mg L^{-1} for TiO_2 and 200, 175, 150, 125, 100, 75, 50, and 5 mg L−1 for SiO_2.

Growth rate determination

During the experiments, 0.5 mL algal cells were taken daily and cell quantity was counted with a Burker chamber counting the cells under a microscope to determine the growth rate. The growth rate was calculated according to the equation described by Xiong et al. (2005):

$$U= (\ln Nt-\ln N0)/ (t-t0)$$

where U (cell number/h) is the growth rate; Nt and N0 are the cell quantity at times t and 0, respectively; t (h) is the sample time for counting cell quantity; and t0 (h) is the origin time of the treatment.

Microscope observations

The algal cells were subjected to microscope analysis (fluorescence microscope ZEISS Axioskop 50) for preliminary observation of the nature and extent of the damage (optical) followed by a more specific observation through fluorescence microscopy.
Optical observations were carried out on 50–100 algal cells treated with the highest TiO_2 (100 mg L−1) and SiO_2 (200 mg L−1) concentrations, and the 10 most representative images were recorded (Axiovision REL 4.8 by Axiocam/cm1 ZEISS).

- Viability assay by AO staining:
  The Acridine orange (AO) staining was carried out in triplicate on *D. tertiolecta* exposed to TiO_2 (7.5, 20 and 100 mg L^{-1}) and SiO_2 (200 and 125 mg L^{-1}) suspensions by adding 25 μL of 7.5 mg mL^{-1} of the

dye solution to 0.5 mL of samples (particle exposed and untreated control). The treatment was applied for 5, 24 and 96 h. The observation was performed using a 460-490 nm excitation filter.

- Qualitative evaluation of intracellular ROS
  Qualitative ROS production was carried out in triplicate on *D. tertiolecta* exposed to TiO_2 (7.5, 20 and 100 mg L^{-1}) and SiO_2 (200 and 125 mg L^{-1}) NP suspensions for 24 and 96 h. 0.5 mL of SiO_2 samples was centrifuged for 5 min at 3000 rpm washed with sterile saline solution (8 g L^{-1} NaCl, 0.2 g L^{-1} KCl) and re-supended in 1mL of the saline. Then, 5 µL of 2 mM DCFH-DA (Sigma-Aldrich) and 50 µL of 10 mM Na_2EDTA (permeabilization agent) were added to cell suspensions for 1 h at room temperature under dark condition. The stained cells were analysed by microscopy using a 460-490 nm excitation filter.

- MTT assay
  Cell viability was measured in triplicate by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay (Pakrashi et al. 2013, modified). Briefly, after 96 h of interaction of the microalgae with the SiO2 (200 and 125 mg L−1) and TiO2 (7.5, 20, and 100 mg L−1) NP suspension, 10 mL of samples and untreated control were added with 400 µL MTT solution (5 mg MTT in 1 mL in phosphate buffer solution filtered at 0.22 µm) and incubated in the dark for 4 h. The suspensions were centrifuged at 8000 rpm for 8 min. The pellets were washed with 5 mL of ASW and then 4 mL of DMSO was added. The absorbance was measured at 570 nm using a Spectrophotometer (Varian Cary 1E).

Data analysis

Analysis of variance (ANOVA) was applied, using raw data, in order to test for significant differences in effects among treatments (the significance level was always set at p=0.05).
The 50 % effect concentration (EC50) and the 1 % effect concentration (EC1) were calculated using the linear interpolation method (inhibition concentration procedure or ICp) (Cesar et al. 2004; US EPA 1993). The bootstrap method was used to obtain the 95 % confidence interval because standard statistical methods for confidence interval calculations were not applicable. No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) were determined by Dunnett's test. Concentration–response functions were statistically determined by applying a best-fit procedure (Scholze et al. 2001). With this approach, different regression models (Boltzmann, logistic, exponential), provided by Origin 8 SR2 (Northampton, MA) statistical software, were applied to each data set in order to determine, on the basis of statistical criteria, the regression model that better described the trend observed in the toxicity data. Differences in growth inhibition (comparisons between the control group and each of the experimental groups) were tested for significance using the multiple comparisons Dunnett's procedure US EPA (1989).

Results and discussion

Aggregation trends

As expected in a high-ionic-strength medium such as seawater, the analysis of the particle size of the SiO_2 and TiO_2 clearly shows hydrodynamic sizes larger than their primary size (Fig. 1a) (Keller et al. 2010; Metin et al. 2011; Zhang et al. 2008). Already, in the first 120 min, SiO_2 (at 125 mg L^{-1}) showed an average aggregate size of around 1300 nm which increased up to nearly 2µm at a higher particle concentration. These dispersions show ζ potential values of −12.2±0.6 and −10.3±0.7 mV, respectively. These magnitudes indicate that the repulsive energy among the particles is smaller compared to the van der Waals attraction energy, and so the particles show a marked tendency to flocculate. A similar behavior was already described in a work by Zhang and co-workers for metal oxide nanoparticles and addressed to a destabilization effect provoked by the presence of a high concentration of electrolytes in solution, especially double charged cations like Mg^{2+} (Zhang et al. 2008). In fact, the presence of a high content of electrolytes (as in seawater) can result in the compression of the electrical double layer surrounding the particles with the consequent decrease in its repulsive energy; in this way, the net repulsive energy barriers between nanoparticles become negligible and aggregation occurs.

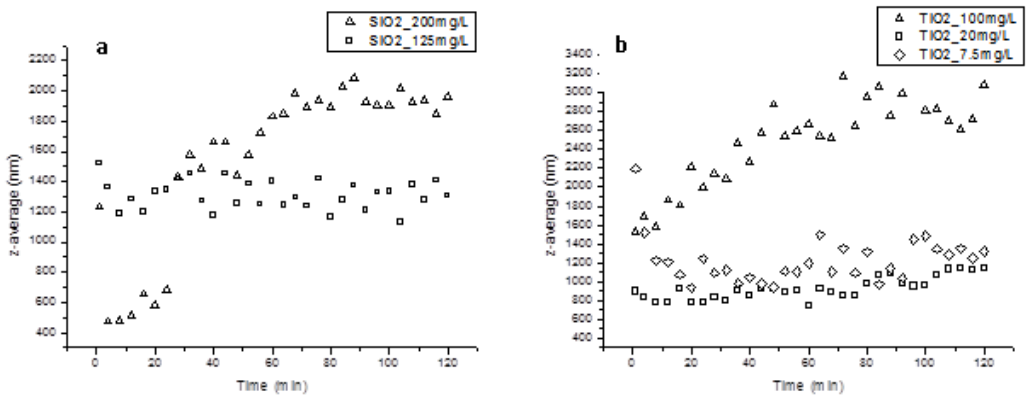

Figure 1. Changes in nanoparticle aggregate sizes in ASW as measured by DLS over time for a SiO_2 at 200 and 125 mg L^{-1} and b TiO_2 at 100, 20, and 7.5 mg L^{-1}. The average indexes of polydispersity were 0.49±0.08 for 125 mg L^{-1} and 0.34±0.14 for 200 mg L^{-1} of SiO_2, and 0.45±0.1 for 100 mg L^{-1}, 0.46±0.07 for 20 mg L^{-1}, and 0.63±0.07 for 7.5 mg L^{-1} of TiO_2.

It was also reported that, above a critical electrolyte concentration, an increase in nanoparticle concentration shortens the average distance travelled by a particle between collisions, resulting in an increase in aggregation rate (Metin et al. 2011). Eventually, this means that in our experimental conditions, the agglomeration behavior is affected primarily by the initial concentration.

As a consequence of particle aggregation, around 50 % of the total original mass of SiO_2 nanoparticle aggregates settled out of the water within 2 h of sedimentation (data not shown).

However, after 24 h, the nanoparticles did not settle out of the water efficiently: 20–30 % still remained in the settled water and were observed as aggregates with an average size of around 600 nm (Fig. 2).

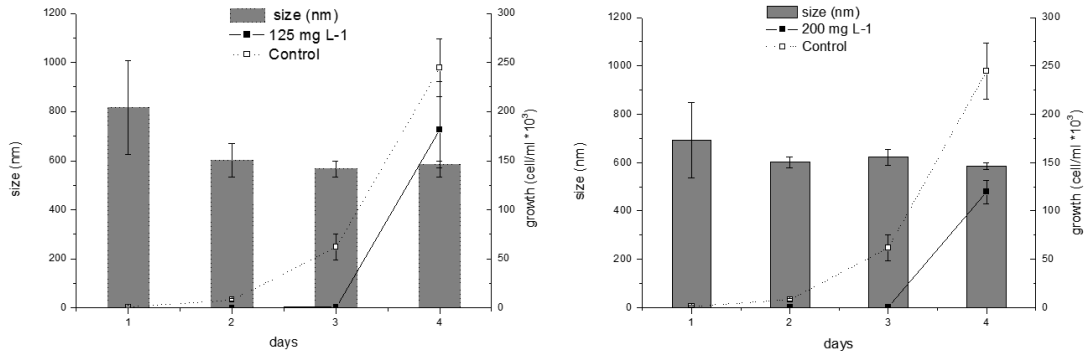

Figure 2. Mean size (±SD) of the particle aggregates, at two SiO_2 concentrations (125 and 200 mg L^{-1}), during the 4 days of the algal bioassay together with the relative trend of the exposed (straight line) and control (dotted line) algal population growth (10^3 cells mL^{-1}).

This experimental finding could be related to the reduced residual concentrations of particles in the aqueous solution with a consequent decrease of the average size of suspended agglomerates.

Analogously, TiO_2 NPs aggregated rapidly to around micrometer-sized particles even at relatively low (20– 7.5 mg L^{-1}) concentrations (Fig. 1b). At a higher particle concentration (100 mg L^{-1}), the increased probability of collisions between particles affected the aggregation rate. These TiO_2 dispersions showed a negative ζ potential from−4.7±0.9 to −10.7±0.3 mV (at 100 and 7.5 mg L^{-1}, respectively). The absolute values of this parameter indicate that the dispersions were rather unstable and support the observation of an increasing tendency for flocculation and settling with increasing initial concentration.

During the 96 h of the ecotoxicological assay, TiO_2 aggregates confirmed the aggregation trend observed in the first hours (Fig. 3). After 48 h, the aggregates were around 5 μm and afterwards settled out and drastically reduced their concentration in the water column even at the lower initial particle concentration.

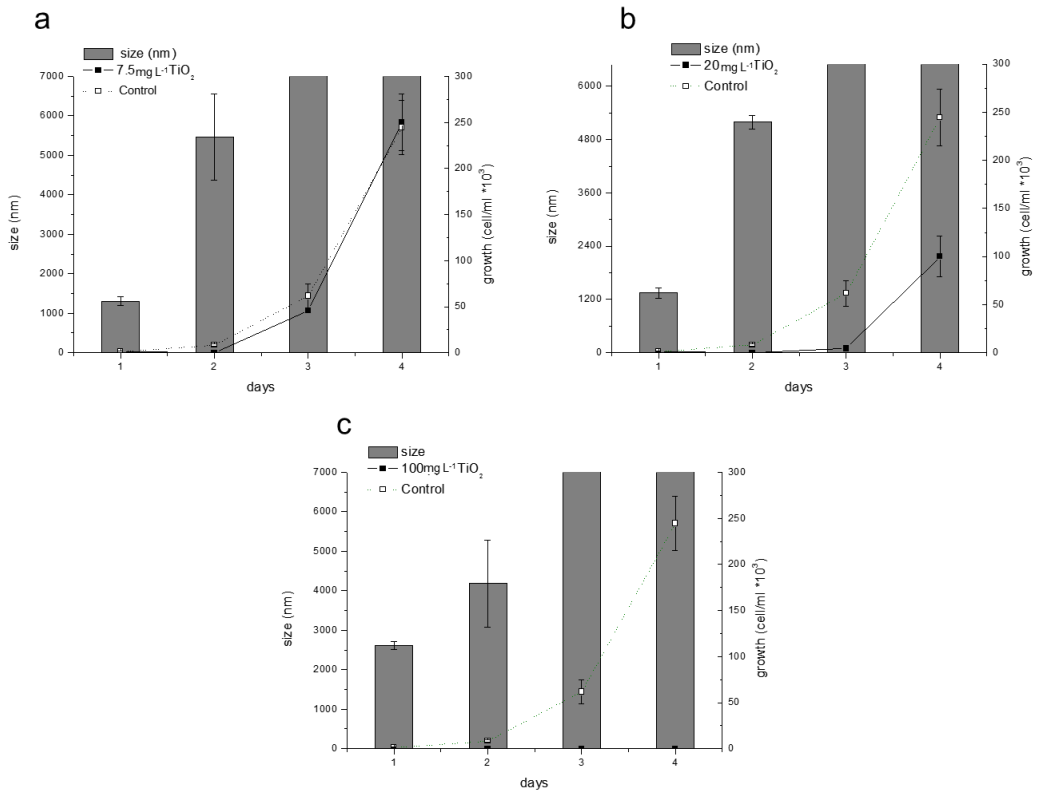

Figure 3. Mean size (±SD) of the particle aggregates, at three TiO_2 concentrations (7.5, 20, and 100 mg L−1), during the 4 days of the algal bioassay together with the relative trend of the exposed (straight line) and control (dotted line) algal population growth (10^3 cells mL−1).

Toxic effects of SiO_2 and TiO_2 aggregates

SiO_2 toward *D. tertiolecta* (Fig. 4). Figure 4 reports the effects on the growth of the algal population caused by the presence of TiO_2 and SiO_2 particles. A complete dose–response curve was recorded in the tested concentration range of TiO_2 while SiO_2 appeared less toxic than TiO_2. In fact, though having observed a wider concentration range, only EC50 and NOEC values were measured for SiO_2. In Fig. 2 was reported the mean size of the particle aggregates, at SiO_2 NOEC and EC50 values (125 and 200 mg L^{-1}, respectively), during the 4 days of the algal bioassay together with the relative trend of the exposed algal population growth. As previously reported, within 24 h, mainly aggregates with sizes around 600 nm were present in suspension. The average size of particles available in the water column was the same at both concentrations. This indicates that SiO_2 formed a stable population of homoaggregates and that, by increasing the SiO_2 concentration, only the overall number of these aggregates increased.

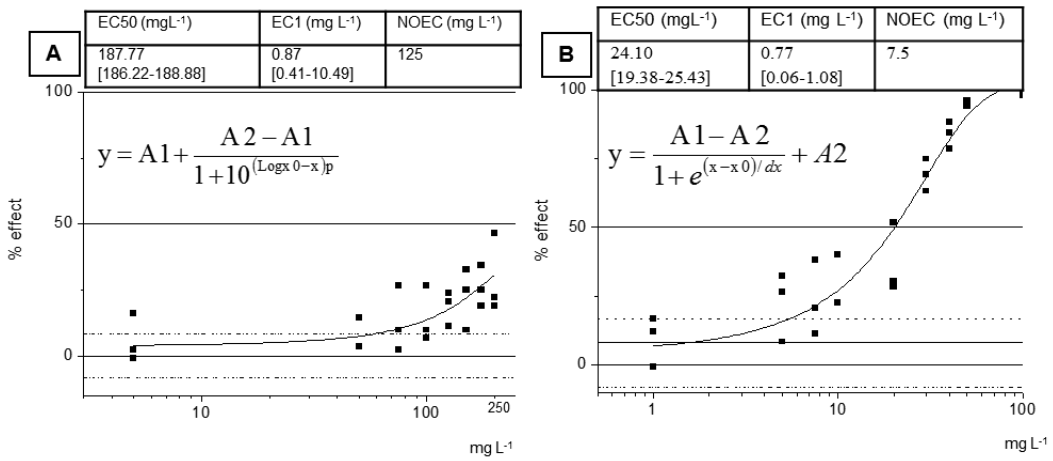

Figure. 4 Toxic effects (EC 50%, EC 1%and NOEC) of *D. tertiolecta* cells (96 h exposure time), together with the corresponding regression fit curve functions: SiO_2 (a) and TiO_2 (b). The best-fit function of toxicity data (n= 6) was sigmoid growth functions. Horizontal lines indicate the 95 % confidence limits of the control mean (n=6). EC 50 and 100 % are also represented with lines. *See "Data analysis".

In the first 24–48 h, independently by the tested concentration, the algae were covered by aggregates (Fig. 5); however, no clear toxic effects upon algal cell number, viability, and ROS production were evident (Fig. 6). On the other hand, it is likely that the cell surfaces covering by the aggregates can induce a certain inhibition of the photosynthetic activity due to the reduction of the light availability (Navarro et al., 2008; Wei et al., 2010). This sharp tendency for strong heteroagglomerations between SiO_2 aggregates and algal cells was also reported, in experimental conditions (i.e., pH and IS) close to ours in a recent work by Ma et al. (2015). Thereafter, SiO_2 interacted with cell surfaces producing the measured effect. In particular, after 72 h of exposure, cell numbers were reduced; after 96 h, the growth inhibition became evident with respect to the control (SI Fig. S1) also with appreciable differences related to the tested concentration. At 125 mg L^{-1} and after 96 h of exposure, optical microscopic observations (Fig. 5c) showed that algal cells were completely covered by aggregates but still no alterations of cell shape and/ or integrity were evident. Signs of cell morphology alteration and cytoplasmatic cell membrane damages were observed instead at 200 mg L^{-1} (Fig. 5f). Several authors have already reported this same behavior

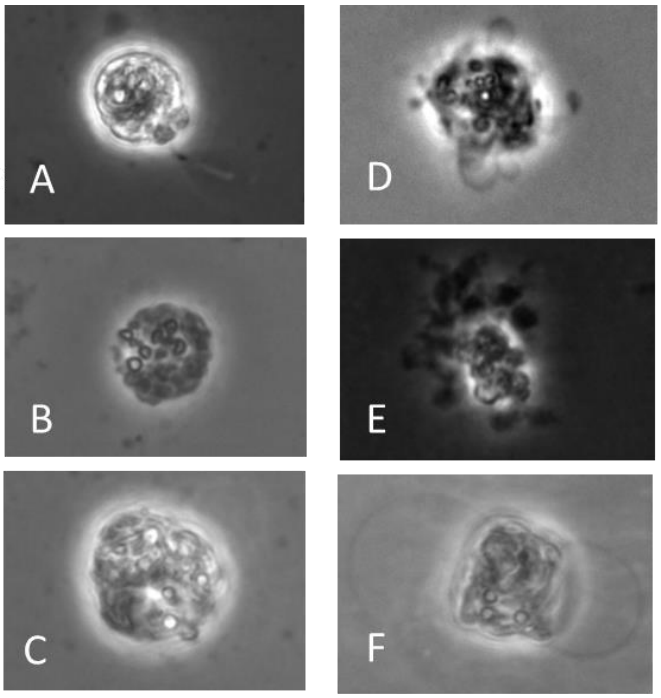

Figure 5 Phase contrast microscopy images (×40) of *D. tertiolecta* cells exposed to selected concentrations of SiO_2 NP at different times of exposure. SiO_2 125 mg L−1 5 h (a), 24 h (b), and 96 h (c); SiO2 125 mg L−1 5 h (d), 24 h (e), and 96 h (f).

(Wei et al. 2010; Van Hoecke et al. 2008), and in some cases, the strong SiO_2 interaction with the cell led to the production of holes that allowed the cytoplasmatic materials to come out (Lin and Xing 2008). In the same timetable, cell viability assays corroborate these results. AO staining as well as MTT assay and ROS production highlighted the effect of particle concentration on the viability and on the oxidative stress extent (Fig. 6). SiO_2 cytotoxicity was then dependent on exposure time. Accordingly, other studies with different species showed that longer exposure time to silica caused higher toxicity due to irreparable damages accumulated kinetically (Napierska et al. 2010; Vo et al. 2014). These damages could be linked to NP interaction with cell surfaces or by NP internalization (Von Moos et al. 2013). Although the relatively rigid cell wall is known to be an efficient barrier that prevents ENM internalization (Ma and Lin 2013), the permeability of cell walls also changes during the delicate phase of cell division in the course of which the cell wall is newly synthesized (Wessels 1993; Ovecka et al. 2005; Navarro et al. 2008; Wang et al. 2011). On the other hand, it is unlikely that algal cells could internalize silicon ions because of the extremely low solubility of SiO_2 (Brunner et al. 2006).

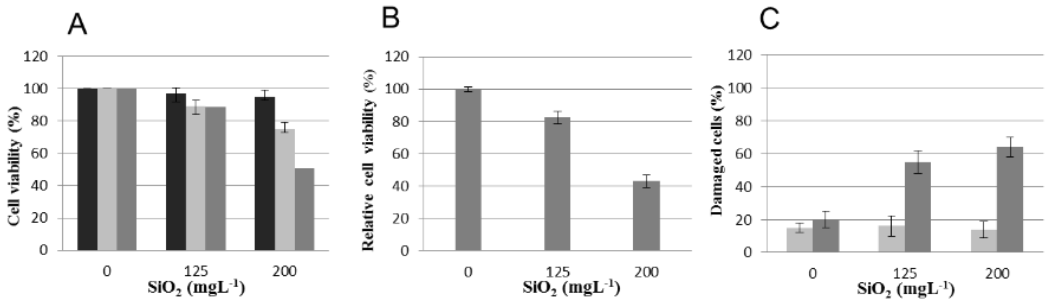

Figure 6 Graphs showing percent viability and percent of damaged algal cells, treated with increasing SiO_2 concentrations, at different times of exposure 5 h (black bars), 24 h (light gray bars) and 96 h (dark gray bars) measured by acridine orange (a), MTT (b), and DCFH-DA (c)

In Fig. 3, the relative trend of the exposed algal population growth is reported together with the mean size of the particle aggregates, at three concentrations of TiO_2 that represent NOEC, EC50, and EC100 values for the investigated algae. Figure 3a shows the effects of exposure at 7.5 mg L−1 of TiO_2 (NOEC). During this test, a slight (not statistically significant) reduction in microalgae growth was observed in the second and third day, but it was completely recovered at the end of the test (96 h). Accordingly, growth rate values were quite similar to those of the control up to concentrations as high as 20 mg L^{-1} (SI Fig. S2). Similarly, Wang et al. (2008) observed that the growth of algae *Chlamydomonas reinhardtii* exposed to 10 mg L^{-1} of TiO_2 was inhibited until the third day but was recovered at the end of the test. Also, Xia et al. (2015) reported a similar trend for the *N. closterium* exposed at 5 mg L−1 TiO_2 (anatase). Starting from the second day of exposure to 20 mg L^{-1} of TiO_2, a growth reduction was observed which evolved in a marked effect at the fourth day (Fig. 3b). At TiO_2 100 mg L^{-1}, *D. tertiolecta* growth resulted completely inhibited (EC 100 %) since the first day of exposure (Fig. 3c). As can be seen, at the three selected concentrations, the size of the TiO_2 aggregates rapidly increased from a few microns and, since the second day, it was always higher than 4000 nm.

However, increasing the particle concentrations increases the probability of collisions not only between particles (homoaggregation) affecting the aggregation rate but also between aggregates and algal cells, thus increasing the number of potential cells injured by the nanomaterial. This heteroaggregation phenomenon was previously described (Wang et al. 2008, Li et al. 2015, Ma et al. 2015, Xia et al. 2015), and the rate of agglomeration seemed to be faster in the presence of algae (Sadiq et al. 2011). The highest cell/TiO_2 aggregation occurred in cultures spiked with 100 mg L^{-1}.

Figure 7 shows indeed how large aggregates entrapped algal cells. The same was already observed by Huang et al. (2005) which showed how *P. subcapitata* cells adsorbed TiO_2 nanoparticles carrying up to 2.3 times their own weight. Actually, AO staining after 5, 24, and 96 h of exposure at 100 mg L^{-1} of TiO_2 evidenced entrapped cells still viable up to 5 h of exposure while, after 24 h, microalgae nuclei were coming out, provoking cell death (Figs. 8 and 9). These results highlighted that the toxic effects always appeared after at least 5 h of incubation/ exposure at 20 and 100 mg L^{-1} TiO_2 concentrations (Fig. 7).

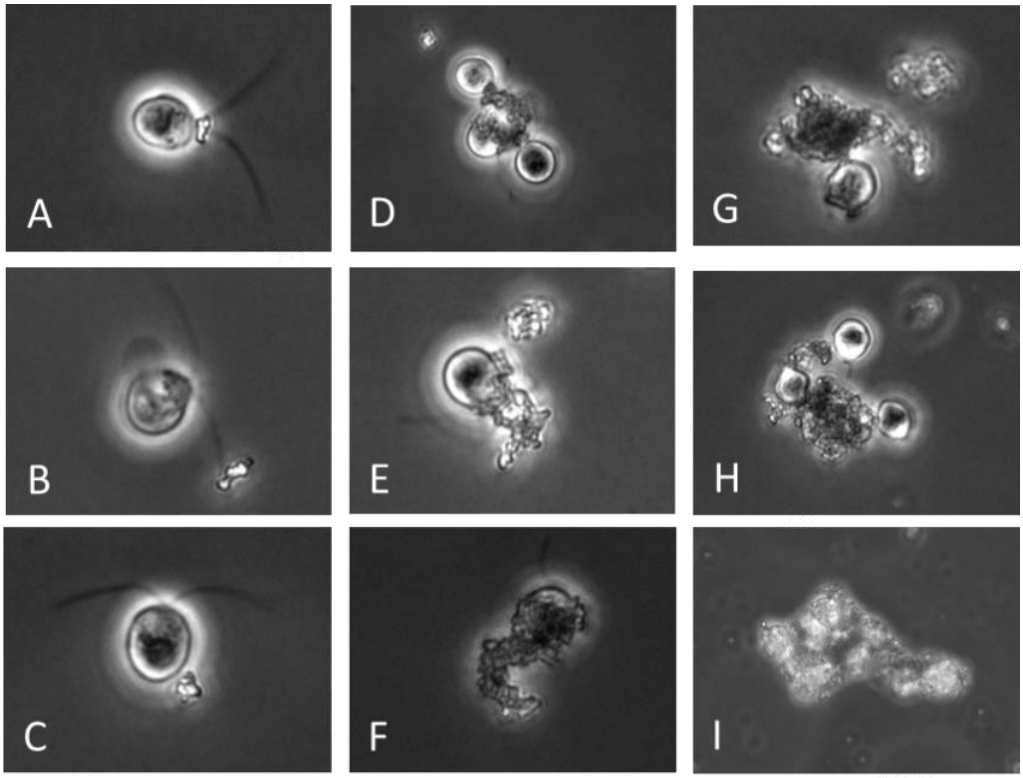

Figure 7 Phase contrast microscopy images (×40) of *D. tertiolecta* cells exposed to increasing concentrations of TiO_2 NP at different times of exposure. TiO_2 7.5 mg L^{-1} 5 h (a), 24 h (b), and 96 h (c); TiO_2 20 mg L^{-1} 5 h (d), 24 h (e), and 96 h (f); TiO_2 100 mg L^{-1} 5 h (g), 24 h (h), and 96 h (i).

Since it was observed that the TiO_2 aggregates were already few microns sized in these first hours, it could be envisaged that the first step of the toxic action is the entrapment of the algal cells by means of very large particle aggregates. Then, the close interaction between aggregates and cell membranes induced an oxidative stress as ROS production (Thill et al. 2006; Hartmann et al. 2010) that actually provoked damages in around 80 % of the cell population exposed to 100 mg L^{-1} of TiO_2 (Fig. 9). Although in the aggregation process pristine NP characteristics may be lost, the peculiar TiO_2 anatase reactivity resulting in an evident cytotoxicological effect (Braydich-Stolle et al. 2009), should be taken into account.

On the other hand, the alteration of the cell membrane due to the cell entrapment may affect cellular uptake of nutrients and energy transduction mechanisms (related to ATP synthesis) (Yeung et al. 2009; Hartmann et al. 2010).

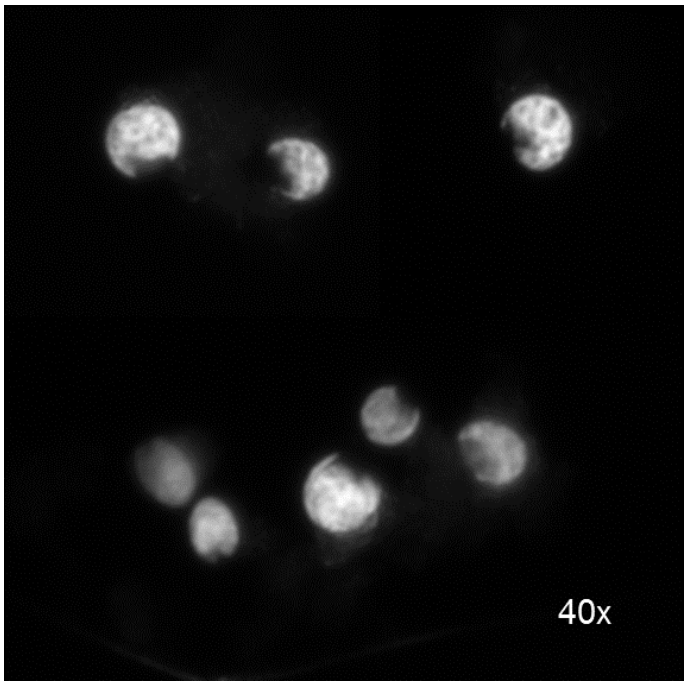

Figure 8 Fluorescence microscopy images showing *D. tertiolecta* cellular damage observed after TiO_2 exposure (100 mg L^{-1}) (AO staining, ×40).

Although the mechanisms of TiO_2 toxicity were not completely known, considering that the dissolution of TiO_2 NP was negligible in water, the effect seems mainly ascribable to the ROS production, in response to the particle interaction with algae cells and in some cases to their internalization and accumulation in the chloroplast (Xia et al. 2015, Li et al. 2015, Iswarya et al. 2015). Recently, Iswarya et al. (2015) showed that the TiO_2 crystalline structure highly influenced ROS generation in exposed freshwater algal cells. Besides, a different toxicity cellular target for anatase (nucleus and cell membrane) and rutile (internal organelles) was also reported.

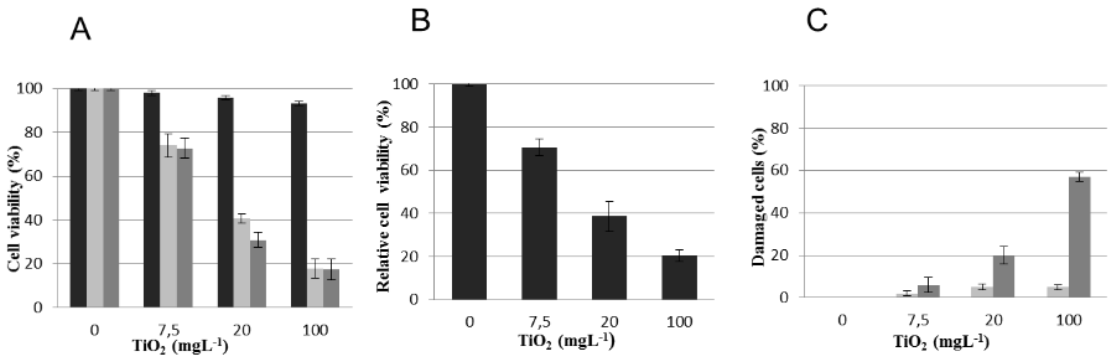

Figure 9 Graphs showing percent viability and percent of damaged algal cells, treated with increasing TiO_2 concentrations, at different times of exposure 5 h (black bars), 24 h (light gray bars) and 96 h (dark gray bars) measured by acridine orange (a), MTT (b), and DCFH-DA (c)

Conclusion

The aim of this investigation was to evaluate and compare the potential toxicity of two nanoparticles, SiO_2 and TiO_2, that were largely used in various industrial sectors, toward the marine microalgae *D. tertiolecta*. Our results showed that the NP aggregation was a key phenomenon and it could be relevant for toxicity in the marine environment. SiO_2 aggregates rapidly reached a value (600 nm) which was independent of the concentration, while TiO_2 NPs underwent a completely different aggregation behavior in the seawater matrix: TiO_2 aggregates grew up to 4±5 µm, and reaching such a large size, they deposited as sediment. *D. tertiolecta* showed different sensitivities to tested NPs and TiO_2 was the most toxic one. The overall toxic action seemed due to the contact between aggregates and cell surfaces, but while for SiO_2 a direct action upon membrane integrity could be also observed after 72–96 h of exposure, TiO_2 seemed to exert its toxic action mostly via cell entrapment and agglomeration in the first hours of exposure. The results of this study and in particular the EC1 values, compared with the current maximum predicted release concentrations, highlighted the actual potential risk of these ENMs in the marine environment. However, SiO_2 could be more hazardous than TiO_2 toward phytoplankton species due its longer residence time as a suspension in the water column.

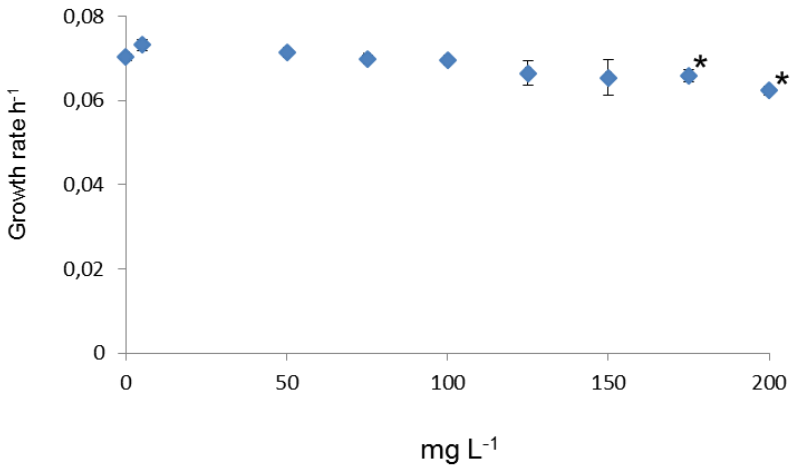

Figure S1- Growth rate (mean ±SD) of *D. tertiolecta* population at different SiO_2 concentrations. *Values significantly different from control ($p<0.05$).

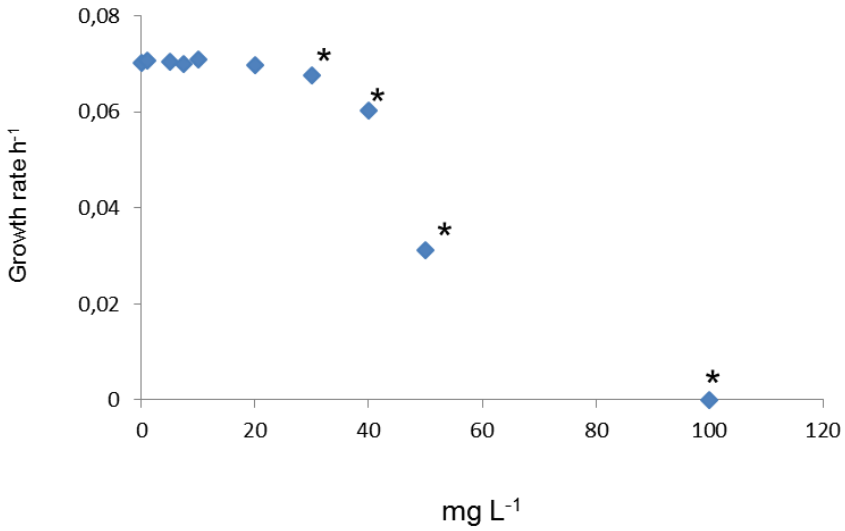

Figure S2- Growth rate (mean ±SD) of *D. tertiolecta* population at different TiO_2 concentrations. *Values significantly different from control ($p<0.05$).

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3.2 Genotoxic and cytotoxic effects of ZnO nanoparticles for *Dunaliella tertiolecta* and comparison with SiO_2 and TiO_2 effects at population growth inhibition levels.

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Abstract

The increasing use of oxide nanoparticles (NPs) in commercial products has intensified the potential release into the aquatic environment where algae represent the basis of the thropic chain.
NP effects upon algae population growth were indeed already reported in literature, but the concurrent effects at cellular and genomic levels is still largely unexplored.
Our work investigates the genotoxic (by COMET assay) and cytotoxic effects (by qualitative ROS production and cell viability) of ZnO NPs toward marine microalgae *Dunaliella tertiolecta*. A comparison at defined population growth inhibition levels (50% Effect Concentration, EC50, and No Observed Effect Concentration, NOEC) with SiO_2 and TiO_2 genotoxic effects and previously investigated cytotoxic effects (Manzo et al. 2015) was performed in order to elucidate the diverse mechanisms leading to algae growth inhibition.
After 72 h exposure, ZnO particles act firstly at level of cell division inhibition (EC50:2 mg Zn/L) while the genotoxic action is evident starting from 5 mg Zn/L. This outcome could be ascribable mainly to the release toxic ions from aggregate of ZnO particle in the proximity of cell membrane.
In the main, at EC50 and NOEC values for ZnO NPs showed the lowest cytotoxic and genotoxic effect with respect to TiO_2 and SiO_2. Based on Mutagenic Index (MI) the rank of toxicity is actually: TiO_2>SiO_2>ZnO with TiO_2 and SiO_2 showing similar MI values at NOEC and EC50 concentrations.
The results presented herein suggest that up to TiO_2 NOEC (7.5 mg/L), the algae DNA repair mechanism is efficient and the DNA damage does not result in an evident algae population growth inhibition. A similar trend for SiO_2, although at lower effect level respect to TiO_2, is observable.
The comparison among the tested nanomaterial toxicity pattern highlighted that the algae population growth inhibition occurred through specific pathways related to NPs different physicochemical behavior in seawater.

1. Introduction

In the last years, the production volume of engineered nanomaterials (ENMs) as well as their use in several applications continue to grow rapidly (Keller et al. 2013). Personal care products represent one of the most significant applications of ENMs that could have environmental implication. ZnO, TiO_2 and, to a less extent, SiO_2 are commonly used in skin care and sunscreens for UV protection, facial moisturizers and foundation (Keller et al. 2014). Therefore, an increasing release, currently estimated less than 1 μg/L (Keller et al. 2014), of these particles can be expected in the coastal marine waters, as ultimate sink for any ENM discharged into the environment. Once released, ENMs will interact with the environment in several ways. These interactions are controlled by the inherent properties of the ENMs (solubility in water, colloidal stability, reactivity, etc.) and by the properties of the environment into which they are released such as ionic strength, pH, the presence of organic matter (Keller et al. 2010; Lowry et al. 2012).
In coastal ecosystems, microalgae play a key role as primary producers and, being at the base of the aquatic food web, any modification of their growth could affect higher trophic levels (Rioboo et al. 2007). Additionally, phytoplankton represents an excellent aquatic model for the study of the effects of pollutant exposure at population level (Chen C. et al. 2012), due to a short generation time and high sensitivities.
The action of nanoparticles (NPs) upon microalgae is usually evaluated by parameters that integrate and reflect sublethal effects at population level such as growth rate, biomass, chlorophyll fluorescence and primary production (Aruoja at al. 2009; Ji et al. 2011; Chen X. et al. 2012; Ma et al. 2013). However to shed light on the NP mode of toxic action it could be useful to combine the investigation of the cellular response, as cell viability, ROS production, with genotoxicity (Dalai et al. 2013; Gunawan et al. 2013; Bhuvaneshwari et al. 2015; Demir et al. 2014; Golbamaki et al. 2015; Suman et al. 2015). Due to their small size and high surface area, coupled to other physicochemical features, such as ion dissolution and charged surfaces, NPs may indeed interfere with replication, transcription and cell division mechanisms (Singh et al. 2009; Xia et al. 2015).

Actually, if small enough, NPs may pass through cellular membranes/walls and gain access to the nucleus and DNA (Singh et al. 2009) or be internalized during cell division (Magdolenova et al. 2013) or through holes in cell wall (Navarro et al. 2008; Chen X. et al. 2012; Li et al. 2015).
Alternatively they may indirectly cause DNA damage by promoting oxidative stress and cellular inflammatory responses (Golbamaki et al. 2015).
The most part of genotoxic studies were conducted in vitro (Falck et al. 2009; Apoorva et al. 2013) although more relevant aspects such as uptake, metabolism and repair mechanisms could be taken into account only by in vivo studies (Gonzalez et al. 2008; Rocco et al. 2015). Nevertheless, the NP genotoxicity toward some environmentally relevant organisms, up to now is still little explored.
Some studies were conducted upon cells of aquatic organisms such as fishes and mussels (Lee et al. 2009; Clemente et al. 2013; Gomes et al. 2013; Isani et al. 2013; D'Agata et al. 2014), very few ones upon plant cells (Kumari et al. 2011; Demir et al. 2014; Pakrashi et al. 2014) and, to the best of our knowledge, none about microalgae (Akcha et al. 2008). In this frame, a useful and sensitive technique for the assessment of genotoxicity is the alkaline single-cell gel electrophoresis, also known as comet assay. Despite other genotoxicity tests (e. g. micronucleus test), the comet assay is applicable to any kind of eukaryotic cell and it is independent of cell proliferation or cell cycle status. This method, generally used to test NPs, as well as other genotoxic agents (Kumar et al. 2011; Shukla et al. 2011) is a suitable tool for measuring primary DNA damage also in microalgae (Akcha et al. 2008; Prado et al. 2009).
Herein, the genotoxic (COMET assay) and cytotoxic effects (ROS production and cell viability) of ZnO NPs towards *D. tertiolecta* were investigated. Genotoxic effects were also compared to those exerted by other metal oxide nanomaterials such as SiO_2 and TiO_2 NPs at levels of population growth inhibition, in order to disclose the diverse mode of action. To this purpose we relied on the growth inhibition dose-response curves for *D. tertiolecta* exposed to ZnO, SiO_2 and TiO_2 NPs and the relative no observed effect concentration (NOEC) and EC50 (50% Effect concentration) values defined in some previous works (Manzo et al. 2013a; Manzo et al. 2015). Cytotoxic effects of ZnO were compared with those previously reported for SiO_2, TiO_2 (Manzo et al. 2015).

2. Materials and methods

Organisms

Dunaliella tertiolecta (Chlorophyceae: Chlamydomonadales) is a marine green flagellate with a cell size of 10−12 μm. Algal cells were maintained in sterilized standard medium (Guillard, 1975) made with filtered Artificial SeaWater (fASW, ASTM, 1998, pH 8.0, 35‰ of salinity, 0.22 μm filtered).
To provide inoculant for experiments, microalgae were incubated under cool continuous white fluorescent lights (about 58-μmol photons m−2 s−1) at 24±1 °C with aeration for 5-7 days until log phase growth prevailed. Cell density was measured by hemacytometer.

Material characterization and particle dispersions in exposure medium

Commercial silicon dioxide nanoparticles (nominal purity 99.5%, primary particle size 10–20 nm) and titanium dioxide (anatase, nominal purity 99.7%, primary particle size 25 nm) were purchased from Sigma-Aldrich. Bare zinc oxide (ZnO cod. 544906, particle size 100 nm, surface area 15–25 m^2/g) were purchased from Sigma-Aldrich. Artificial seawater (ASW) was prepared according to the ASTM method (NaCl 0.4 M, $MgCl_2$*6H2O 0.053 M, Na_2SO_4 0.02 M, $CaCl_2$*H_2O 0.01 M, KCl 9 mM, $NaHCO_3$ 2 mM, KBr 0.8 mM, H_3BO_3 0.4 mM, $SrCl_2$*$6H_2O$ 0.09 mM, $NaSiO_3$*$9H_2O$ 0.07 mM) and filtered through 0.22 μm (pH 8.0) (ASTM, 1998).
Stock suspensions of SiO_2, TiO_2 and ZnO NPs were prepared by dispersing dry powders into fASW to the final concentration of 2000 mg/L for SiO_2, 1000 mg/L for TiO_2 and 1000 mg Zn/L for ZnO. The NP suspensions were bath sonicated in a low-power ultrasonic bath (Elma Transsonic Digital S) for 30 min. Stock dispersions were properly diluted at NOEC (No Observed Effect Concentration) and EC50 (concentration that causes 50% effect relative to the control) obtained for SiO_2 TiO_2 and ZnO in our previous studies for *D. tertiolecta* growth inhibition test (Manzo et al. 2013a; Manzo et al. 2015): 125 mg/L (NOEC) and 200 mg/L (EC50) for SiO_2; 7,5 mg/L (NOEC) and 20 mg/L (EC50) TiO_2; 0.1 mg Zn/L (NOEC) and 2 mg Zn/L (EC50) for ZnO NP. In addition, for ZnO NP the complete dose-response curve (5, 10, 25, 50, 100 mg Zn/L) was also evaluated.
After dilution, all the suspensions were bath sonicated again for 10 min. The dilutions were vortexed briefly before the addition of micronutrients and test organisms.

Particle characterization

The physico-chemical characterizations of the materials in the exposure medium were previously evaluated (Manzo et al. 2013a; Manzo et al. 2015) and are now briefly summarized in Table 1. In the main, these physicochemical features highlight the instability of all the nanomaterials tested in seawater, which result in the formation of large, micrometric sized aggregates with a faster or slower tendency to sediment also in relation to the initial particle concentrations. The Z-potential magnitudes indicate indeed that the repulsive energy among the particles is smaller than van der Waals attraction energy, and so the particles have a marked tendency to flocculate.

Table 1- Physic-chemical characterizations of SiO_2 TiO_2 and ZnO Nanoparticles, in filtered (0.22 µm) ASW (T 18°C , pH: 8; Salinity 35‰).* reported in Manzo et al. 2015, ^ reported in Manzo et al. 2013

NPs	[NPs] (mg/L)	Size (nm)	pH	Z-pot (mV)
SiO_2*	125	1300±100	7.8	-12.15±0.63
	200	1800±180	7.9	-10.31±0.81
TiO_2*	7.5	1300±110	8.0	-10.7±0.28
	20	1350±115	8.0	-9.40±0.35
ZnO	1	470±45	8.0	-10.35±0.83
	5	1040±70	8.0	-10.51±1.43

Algae cells treatment

Algal cells (with a final density 10^6 cells L^{-1}) were filtered (0.22 µm) and rinsed three times with autoclaved fASW. The algal cells were then added to each treatment and control (standard culture media, F/2 medium) together with nutrients. All the experiments were performed in triplicate. For positive control the algal cells were exposed to H_2O_2 100 µM. Two different concentrations of a non-nano control (ZnSO4) were also used. Test plates (10 mL) were kept in a growth chamber with continuous light (about 58 µmol photons m^{-2} s^{-1}), at a temperature of 24 ± 1°C for 3 days.

Genotoxicity of ZnO, TiO_2 and SiO_2 NPs

The comet assay was carried out on the algal cells after 24 and 72 h of NP exposure, based on our previous results, as described in Akcha et al. 2008. For each replicate, two slides were prepared. 30 µL of the cell suspension (50 x 10^6 cells/L) were added to 225 µL of 0.5% low melting point (LMP) agarose in PBS. Then, 85 µL of this mixture were deposited on a slide pre-coated with 0.5% normal melting point (NMP) agarose in PBS. The slides were immediately placed on ice, in the dark, for 1 min to allow the agarose to solidify. Then, 90 µL of the LMP agarose solution were deposited on the slide. Once the last layer was solidified, the slides were immersed in a glacial lysis buffer (NaCl 2.5 M, Na_2EDTA 0.1 M, Tris base 0.01 M, N-sarcosinate 1%, DMSO 10%, Triton X-100 1%, pH 10) for 1 h at room temperature, in dark. DNA unwinding was performed

by pre-incubating the slides (15 min at room temperature in the dark) in freshly prepared electrophoresis buffer (NaOH 0.3 M, EDTA 0.001 M, pH 13). DNA migration was performed in the same buffer for 20 min at 23 V (390 mA, E= 0.66 V/cm). At the end of electrophoresis, slides were washed for three times for 5 min in Tris base 0.4 M, pH 7.5 and fixed with methanol. The slides were then stored at 4 °C in the dark.
For DNA staining, 75 µL of ethidium bromide solution (20 µL/mL) was placed on each slide. The slides were observed using an optical fluorescence microscope coupled to a camera and 100 cells per replicate were analyzed by Comet Score TM program to measure the length of the tail and the percentage of DNA in the head. The percentage of damaged nucleus (number of damaged cells/100 cells) was calculated. The DNA damage was quantified by classification of cells into five scores corresponding to tail length (Collins, 2004; Kalantari et al. 2012) that is Score 0: no tail, Score 1: tail shorter than the diameter of the head (nucleus), Score 2: tail length 1 to 2x the diameter of the head, Score 3: tail longer than 2x the diameter of the head and Score 4: no head.
The Mutagenic Index was then calculated according the following formula:

$$MI = \sum_{i=0}^{4} \frac{i*NCSi}{N}$$

Where:

i= 0-4 score

N= number of total cells

Cytotoxicity of ZnO NP

Cell viability

Cell viability was measured by MTT (3-(4.5-Dimethylthiazol-2-yl)-2.5-Diphenyltetrazolium Bromide) assay (Pakrashi et al. 2013 modified). After 24 and 72 h of interaction, 10 mL of the algae cell suspensions were added to 400 µL of MTT solution (5 mg MTT in 1 mL phosphate buffer solution) and incubated in dark for 4 h. The suspension was then centrifuged at 8000 rpm for 8 min.
The pellet obtained was washed with 5 mL of fASW and then 4 mL of DMSO (dimethyl sulfoxide) was added. The absorbance was measured at 570 nm using spectrophotometer (Varian Cary 1E).
Cell viability was also evaluated by Acridine Orange (AO) staining. The assay was carried out on the untreated and treated algal cells after 24 and 72 h of exposure: 25 µL of the dye solution (7.5 mg/mL) was added to 0.5 mL of sample. The stained cells were analyzed by microscopy using a 460–490 nm excitation filter (fluorescence microscope ZEISS Axioskop 50).

Qualitative evaluation of intracellular ROS

The production of intracellular reactive oxygen species (ROS) was determined using 2.7-dichlorofluorescin diacetate (DCFH-DA), as described in Pakrashi et al. 2013. 0.5 mL of samples were collected after 24 h and 72 h of exposure, and incubated in the dark at room temperature for 1 h with DCFH-DA solution (5 µL of 2 mM DCFHDA and 50 µL of 10 mM Na_2EDTA). The stained cells were analyzed by microscopy using a 460–490 nm excitation filter (fluorescence microscope ZEISS Axioskop 50).

Data analysis

All data presented in this study are reported as mean ± SD. One-way ANOVA was applied in order to est for significant differences between treatments and control (significance level was always set at p = 0.05). Results were also recorded as effect percentage with respect to the control by using the Abbott's formula (Abbott, 1925). The error relative to the mutagenic index was calculated with this formula:

$$\left[\left(\frac{SDc}{Xc}\right) + \sum_{i=0}^{4}\left(\frac{SDi}{Xi}\right)\right] * MI$$

SDc= standard deviation control

X= means control

SDi= standard deviation samples

Xi= means samples

MI= Mutagenic Index

3. Results and discussion

The genotoxicity results obtained after 24 and 72 hours exposure to the investigated oxide nanoparticles are reported in Fig. 1 for ZnO and in Fig. 2 for SiO_2, TiO_2. The cytotoxic effects (ROS production and cell viability) already investigated for SiO2 and TiO2 (Manzo et al. 2015) were reported for ZnO in figure 3.
The cytotoxic (as % viable cells) and genotoxic effects (as % MI) obtained at (ZnO, SiO_2, TiO_2) EC50 and NOEC levels of population growth inhibition were summarized in Table 2. Due to the relevant differences among investigated particles and related effects, the section was arranged in four subsections: one for each nanoparticle and a final comparison among them.

Table 2. ZnO, TiO_2, SiO_2 genotoxicity and cytoxicity (as % effect) at EC50 and NOEC values measured for *D. tertiolecta* population growth inhibition (Manzo et al. 2013a; Manzo et al. 2015)

		ZnO		TiO_2		SiO_2	
		NOEC (0.1 mg Zn/L)	EC50 (2 mg Zn/L)	NOEC (7.5 mg/L)	EC50 (20 mg/L)	NOEC (125 mg/L)	EC50 (200 mg/L)
Genotoxicity	**Comet** (% Effect)	5	7.5	95	95	40	56.25
Cytotoxicity	**MTT** (% Effect)	3	2	29.3	61	17.6	57
	Acridine Orange (% Effect)	-1.03	0	25	72	12	49
	DCFH-DA (% Effect)	-1.14	2.27	6	20	43.75	55

ZnO

Figure 2 showed the level of DNA damage after 24 h of algae exposure. No effects were evaluable up to 5 mg Zn/L, where a 40% of damaged nuclei was registered (Score 4), similarly to that obtained at 10 mg Zn/L (40% of cells classified as score 3). At 25 mg Zn/L, the highest effect was observed with the 55% of nuclei seriously damaged (Score 4), while at 50-100 mg Zn/L, the lowest percentage of injured cells was obtained (33%-20% respectively). The Mutagenic Index (MI) also corroborated these results: the highest value (2.4) was detected at 25 mg Zn/L, while intermediate values (2 and 1.6) for 5-10 mg Zn/L respectively and low values (0.99 and 1.2) for 50 and 100 mg Zn/L were obtained (fig. 3a).
Analogously, after 72 of exposure, no effects were evaluable up to 5 mg Zn/L, where instead a decreasing percentage of injured nuclei respect to the 24 h was observed (fig. 4a). In the range 10- 100 mg Zn/L an increasing number of damaged nuclei were present and in particular at 25 mg Zn/L the highest percentage of damaged nuclei was observed (fig. 4a) together with the maximum MI (3.64) (fig. 3a). Under the applied experimental conditions, untreated *D. tertiolecta* cells showed a compact nucleus with a well-defined head region without any tail. Damaged algae cells, instead, showed comets with undefined head regions and tails of DNA fragments as reported by the microscope images in figure 1.

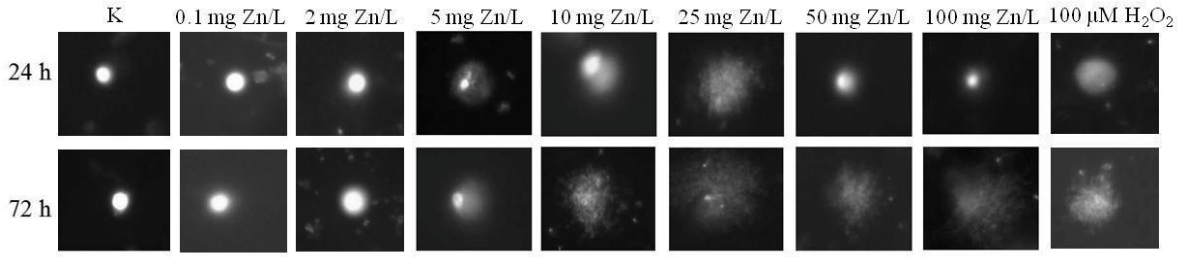

Figure 1. Fluorescence microscopy images showing DNA damage of *D. tertiolecta* exposed to increasing concentration of ZnO NP and to negative and positive control after 24 and 72 h of exposure

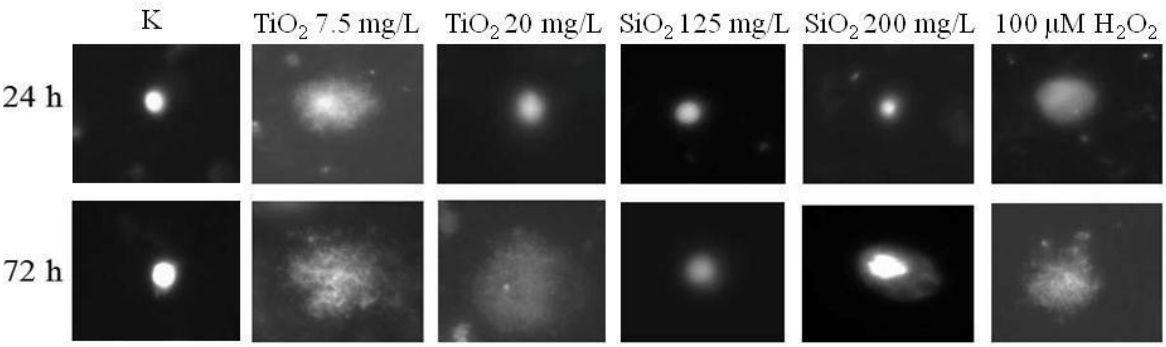

Figure 2. Fluorescence microscopy images showing DNA damage of *D. tertiolecta* exposed to increased concentration of TiO_2, SiO_2 NP and to negative and positive control after 24 and 72 h of exposure.

Several studies reported that ZnO particles are capable of inducing genotoxic effects on human cells (Gopalan et al. 2009; Mu et al. 2014) earthworms (Hu et al. 2010), freshwater snail (Ali et al. 2012) and plants root cells (Demir et al. 2014) in the range of 10-1000 mg/L.

It is worth to note that after 24 h of *D. tertiolecta* exposure to ZnO suspensions, a non monotonic dose-response relationship was obtained with a decreasing trend starting from 25 mg Zn/L. Kitchin et al. 2011, explained a similar non-monotonic trend, observed in the TiO_2 NP genotoxicity dose response curve, with a substantial concentration-dependent agglomeration occurring during the first hour after sonication (i.e. higher concentrations-larger agglomeration-less reactive surface area-less DNA damaged cells-higher cell hetero-agglomeration and consequent loss of cell vitality).

However, an efficient DNA repair process (Rinna et al. 2015) could be assumed up to a certain threshold dose as we observe at 5 mg Zn/L (higher genotoxic effect at 24 h respect to 72 h). The viability of algal cells exposed to ZnO particles for 24, 72 h and measured by AO staining, was shown in Fig. 3b. After 24 h the action of ZnO particles upon cell viability became evident in the range of 10-25 mg Zn/L with a clear worsening between 50 and 100 mg Zn/L. At 72 h, the toxic effects became more evident, with very low percentage of viable cells (32 % and 26% at 50 and 100 mg Zn/L, respectively).

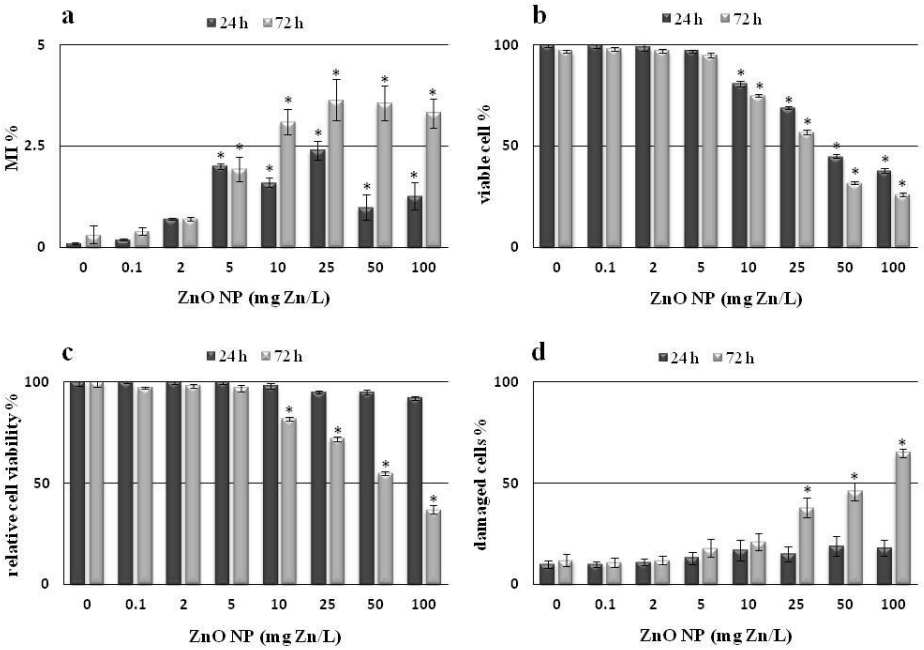

Figure 3. Genotoxicity and cytotoxicity of algal cells exposed to different concentrations of ZnO NP for different exposure times: Mutagenic Index (A); Cell viability (AO staining) (B); Relative Cell viability (MTT) (C); Intracellular ROS (DCFH-DA) (D). *Statistically different with respect to the control. (p=0.05).

Actually, at 72 h of exposure, the amount of relative viable cells (fig. 3c) measured by MTT assay, was even higher (46% at 100 mg Zn/L) showing a noticeable action upon the level of cell metabolism. Likewise, the intracellular ROS production, evaluated by DCFH-DA, similar at all concentrations after 24 h, was significantly increased after 72 h of exposure to concentrations above 10 mg Zn/L (fig. 3d). To explain these findings it is necessary to recall some of the peculiar physicochemical properties of the NPs in seawater. Once in testing medium, NPs undergo to complex chemical transformations that lead to presence of ions/complexes, suspended/agglomerated NPs (Misra et al., 2012) whose bioavailability, uptake rates and toxicity can be largely different. In particular, in seawater, ZnO NPs tended both to rapidly form aggregates of considerable size, depending on time and ZnO NP concentration, and to dissolve with a maximum solubility around 25 mg Zn/L after 70 h, as evidenced in our previous work for a saturated ZnO NPs suspension (Manzo et al., 2013a).

In the first hours (24 h), the hetero-aggregation among algae and NP aggregates was favored at lowest concentrations and algae were in fact rapidly surrounded by NP aggregates whereas, at highest concentrations of NPs, the homo aggregation was prevalent and algae resulted not entrapped (fig. S1-S2). The close interaction between algal cells and aggregates provoked a noticeable effect on the cell morphology (fig. S2-S3): cells loss a regular shape and showed a lower turgor with respect to the unexposed ones (fig. S2). This is likely because of this hetero-aggregation that ROS production increased and the cell viability reduced in dependence of exposure time and Zn concentration (fig. 3).

Moreover, when algae came into contact with NP aggregates (fig. S1), the release of toxic ions can occur in proximity of cell membrane/wall (Li et al., 2015; Suman et al., 2015), provoking a direct action inside the cells (Golbamaki et al., 2015). However, the observed increase of DNA damages up to 25 mg Zn/L did not find an immediate correspondence with the effects provoked by an equivalent amount of ionic Zinc (fig. S4). These observations drive to the conclusion that together with the toxic action of ionic zinc, it is also necessary to consider the effects linked to the presence of aggregates in the suspensions as described in other studies, as for example the dissolution in proximity of cell membrane (Manzo et al., 2013a; Manzo et al., 2013b; Matranga and Corsi, 2012).

This suggested a combined action of multiple processes in the resulting effect. The comparison among all the considered endpoint outcomes (genotoxicity/cytotoxicity and growth inhibition) in relation to Zn concentration (fig. 5) highlighted that the 72 h exposure exerted, as first effect, the 50% reduction of cell division (growth inhibition EC50) at value around 2 mg Zn/L. This could be probably due to the particle/ions interaction with protein responsible for regulation of cell cycle events such as DNA replication and cell division (Magdolenova et al., 2013). Starting from 5 mg Zn/L an increasing genotoxicity can be evaluated (fig. 5). It was probable that,

a direct action of Zn ions release near cell membrane/wall provoked Zn accumulation in the cell and consequently the observed DNA damages (Heim et al., 2015). The vitality parameters resulted compromised starting from 10 mg Zn/L (fig. 3b, c) when the DNA repair mechanisms turned out to be insufficient (Rinna et al. 2015). The number of cells in oxidative stress slowly increased from 5 mg Zn/L, becoming more considerable starting from 25 mg Zn/L (fig. 3d) for the probable surpass the cells' ability to compensate by detoxification activities (Guo et al. 2011).

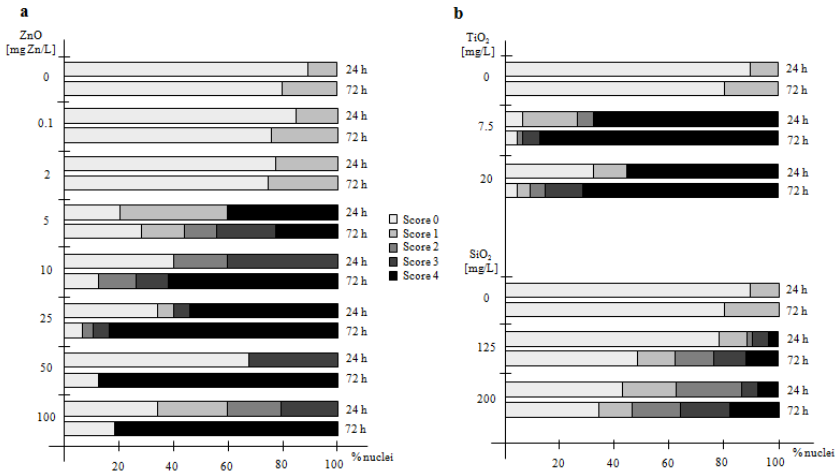

Figure 4. Comet score frequency at different doses of ZnO NP (A) and TiO_2, SiO_2 (B) for different times of exposure (24–72 h). Positive control (H2O2 100 µM) 93% score 4 (24 h) and 95% score 4 (72 h).

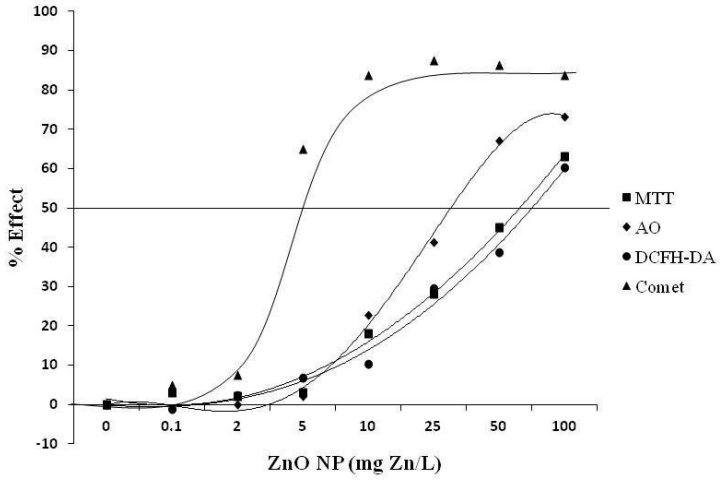

Figure 5. Mutagenic index, cell viability and oxidative stress (% effect) in relation to the concentration corresponding to ecotoxicological parameters of ZnO NP, obtained from (Manzo et al., 2013a, 2015).

TiO_2

In figure 4b, the results of comet assay on microalgae exposed to TiO_2 were shown. After 24 h of exposure, nuclei were already seriously damaged (MI 2.4-3.7). At 7.5 mg/L the highest effect (69% cells with score 4, MI 3.07) was registered, while at 20 mg/L a lower level response (around 50% cells with score 4 and MI of 2.35) was obtained (tab. 3). After 72 h of exposure, the majority of the nuclei (>70%) were categorized as extremely damaged (score 4, see Material and Methods) and showed an undefined morphology of the head region (fig. 2), instead, the other classes (score 0-3) were always poorly represented (<20%) with MI 3.74 at 7.5 mg/L and MI 3.46 at 20 mg/L.

Stating that in seawater, TiO_2 NPs rapidly aggregated up to micron-sized particles (Keller et al. 2010) while dissolution of TiO_2 can be considered negligible, it could be envisaged that the main toxic action was ascribable

to the entrapment of the algal cells by the TiO_2 aggregates (Manzo et al. 2015). This close interaction could induce an oxidative stress as ROS production (Thill et al. 2006; Hartmann et al. 2010).
After the first 24 h of exposure at 7.5 mg/L, concentration that represent the NOEC for algae growth, the probable increment of ROS production inside the algae cell (Jugan et al., 2012; Li et al., 2015), generates free radicals that induces indirect genotoxicity mainly by DNA-adduct formation (Bhattacharya et al., 2009). Additionally the switch on of detoxification activities and DNA repair processes probably occurred. After a longer cell exposure (72 h) while the rank DNA damage for 7.5 mg/L was more or less the same, the genotoxic effect was more evident at highest concentration (20 mg/L) (fig. 2) and was probably due to the loss of cells' ability to detoxify (Guo et al., 2011). This also resulted in evaluable cytotoxic effects and in the reduction (i. e. EC50) or complete inhibition of cell growth (Manzo et al. 2015).
Many studies showed that TiO_2 NPs induce genotoxicity in different organisms (e. g. fish cells, Reeves et al. 2008; Clemente et al. 2013; Earthworms: Hu et al. 2010; plants: Demir et al. 2014; Pakrashi et al. 2014; *Mytilus galloprovincialis*: D'agata et al. 2014) and human cell lines (Bhattacharya et al. 2009; Demir et al. 2013; Ghosh et al. 2013) ranging from 1 mg/L to 1000 mg/L.

SiO_2

Figure 4b showed the results of comet assay for *D. tertiolecta* exposed to SiO_2 NPs. After 24 h, at 125 mg/L, more than 70% of intact nuclei were observed and MI value was 0.55 (tab. 3). Differently, around the 60% of the cells exposed to 200 mg/L showed intact or slightly damaged nuclei (fig. 4b) and a MI value of 1.26. Accordingly, after 24 h algae, although covered by aggregates (as in seawater SiO_2 NPs rapidly aggregated), did not showed consistent toxic effects as viability, and ROS production, independently by concentration (Manzo et al. 2015). After 72 hours, an increasing genotoxic effect was evaluable at both concentrations: approximately 50% of nuclei appeared intact or with a short tail and similar MI values were obtained (1.22; 1.74 at 125 and 200 mg/L respectively) (tab. 3). Analogously, with increasing exposure time (72 h), the SiO_2/cell interaction produced an increment of ROS level in the cell, that provoked an increasing effect at cellular and nuclear level (Manzo et al. 2015).
The genotoxic effect of silica nanoparticles on cells and in particular upon algae are poorly known and the available literature data are often controversial. Some studies reported about the genotoxicity of silica nanoparticles (Gerloff et al. 2009; Yang et al. 2009; Choi et al. 2011) while recent works concluded about the lacking of significant genotoxicity (Singh et al. 2009; Downs et al. 2012; Kain et al. 2012; Lankoff et al. 2013; Golbamaki et al. 2015).
Based on the experimental results, SiO_2 exerted after 72 h a moderate genotoxic action upon algae (MI 1.74 maximum) while at cellular level a long exposure result in evident effect on viability and ROS production that could be the main responsible of cell growth inhibition at 200 mg/L (EC50).
However because genotoxic and oxidative responses measured at 125 mg/L (NOEC) were not so different from those at 200 mg/L (tab. 2) it was very probable that different mechanisms cooccurred in the action of silica particles and that the induced cell growth inhibition was the resultant of balances among anti-ROS responses, DNA damages, chromosome instability, mitosis inhibition. (Yang et al. 2009; Downs et al. 2012)

The different toxicity of ZnO, TiO_2 and SiO_2

In Table 2, the results of ZnO, TiO_2 and SiO_2 genotoxicity (as % effect), cytotoxicity (MTT, AO and oxidative stress as % effect) at EC50 and NOEC values measured for D. tertiolecta population growth inhibition (Manzo et al. 2013a; Manzo et al. 2015) were reported.
ZnO NPs turned out to be the most toxic with the lowest EC50 and NOEC values while SiO_2 was less toxic than TiO_2. On the other hand, based on genotoxicity data (MI, 72 h of exposure) the rank of toxicity was different. At concentration representing EC50 and similarly, at concentration evaluated as NOEC, the order of genotoxicity was TiO_2>SiO_2>ZnO. Actually, TiO_2 and SiO_2 showed similar MI values at both NOEC and EC50 concentrations. Therefore, it could be speculated that at lowest exposure TiO_2 concentration (7.5 mg/L), the algae DNA repair mechanism was still efficient and the exerted damage did not affect the algae growth. A similar trend was observable for SiO_2 (tab. 2).
In the case of ZnO, the adverse effect at 2 mg Zn/L (EC50) was only evaluable at level of cell division inhibition while the genotoxic action was evident only starting from to 5 mg Zn/L (fig. 5). This outcome could be ascribable mainly to the release toxic ions from aggregate of ZnO particle in the proximity of cell membrane

(fig S2-S3-S4) and their action on replication mechanisms together with the capability of cell to recover the DNA damages at low concentration as previously described in the ZnO section.
Considering both parameters of cell viability (MTT and AO), either at EC50 and NOEC values, the rank of toxicity was TiO_2>SiO_2>ZnO while considering the number of cells in oxidative stress, a higher % for SiO_2 respect to TiO_2 was obtained (SiO_2>TiO_2>ZnO). Therefore, SiO_2 exerted a significant oxidative stress that did not result in an extensive DNA damage, reduction of cell vitality, and/or inhibition of algae reproduction and therefore cells activated all the process necessary to maintain the cellular homeostasis in response the external stimuli. In the case of TiO_2, instead it was probable that there was first an action on DNA, that at NOEC did not provoke a loss of vitality neither an inhibition of reproduction, while at EC50 compromised these functions although not as direct response to oxidative stress. The action on DNA could be due to the induction of cellular signals capable to provoke DNA damages, when TiO_2 aggregates remained out of algal cell and to the TiO_2 interaction with DNA molecule in the case of internalization during division processes (Magdolenova et al. 2013) or through holes in cell wall (Navarro et al. 2008; Chen X. et al. 2012; Li et al. 2015).

4. Conclusion

ZnO nanoparticles were largely demonstrated to be toxic to marine microalgae population growth also at concentration not so far from the actual estimate in the seawater. For contributing to the establishment of a protective threshold for marine biota the effects at level of higher sensitivity and lower ecosystem complexity as cellular and genomic level should be determined.
In this work for the first time, the potential ZnO genotoxicity by Comet assay and cytotoxicity of microalgae D. tertiolecta was investigated and the results were evaluated in the light of growth inhibition assessment.
ZnO seemed to exert its toxic action upon algae by a punctual and continuous ion release from aggregates in proximity of algae cell wall. The first interference was at level of the regulation of cell division then resulting in the inhibition of algae population growth while DNA molecule structure and vitality parameters were compromised only at increasing concentration (5 mg Zn/L and 10 mg Zn/L respectively).
The comparison with SiO_2 and TiO_2 toxicity pattern allowed highlighting a different pathways leading to the algae population growth inhibition. For SiO_2 a cascade of effects (ROS production- DNA damages-growth inhibition) were evidenced suggesting a toxicity starting from oxidative stress generation. TiO_2 instead firstly act on DNA structure and, being not soluble in seawater, an internalization during cell division or cell wall destruction could occur together with the activation of cellular signals destabilizing DNA structure. Our study provides some insights into the toxic mechanisms of metal oxide nanoparticles for marine algae however further investigations are still needed to confirm the suggested nano/particle- specific pathways.

Supplementary materials:

Methods:

The algal cells were subjected to light microscope analysis (ZEISS Axioskop 50) for preliminary observation of the nature and extent of the damage, followed by a more specific observation through Focused Ion Beam microscopy. (FEI QUANTA 2 D). Optical observations were carried out on algal cells treated with different ZnO NP concentrations (5-25 and 100 mg Zn/L) at different exposure time (5 h, 24h, 72 h) the most representative images were recorded Axiovision REL 4.8 by Axiocam/cm1 ZEISS).
Before FIB observations algal cells were fixed as described in Li et al 2015. After 72 h of exposure to ZnO NP (100 mg Zn/L) algal cells were centrifugated (4000 rpm 10min). Then the samples were fixed with 3% glutaraldehyde solution in 4◦C for 2 h. The samples were washed with 0.1 M PBS (pH 7.8) by centrifugation (4000rpm, 10 min) three times. Algal cells were fixed with 1% osmium tetroxide for 2 h in 4◦C, and 0.1 M PBS (pH 7.8) was added to wash the cells by centrifugation (3800 rpm, 10 min) three times. The control and treated (10 mg/L) cells were placed on a thin glass slide, air dried and observed under the FIB.

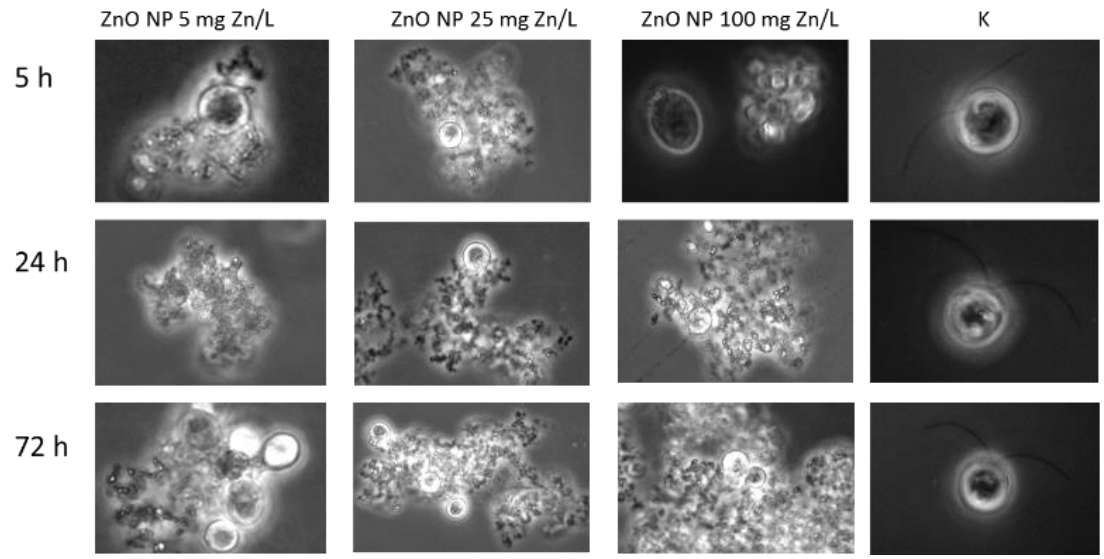

Figure S1. Phase contrast microscopy images (×40) of *D. tertiolecta* cells exposed to selected concentrations of ZnO NP and control at different times of exposure (5 h, 24 h and 72 h).

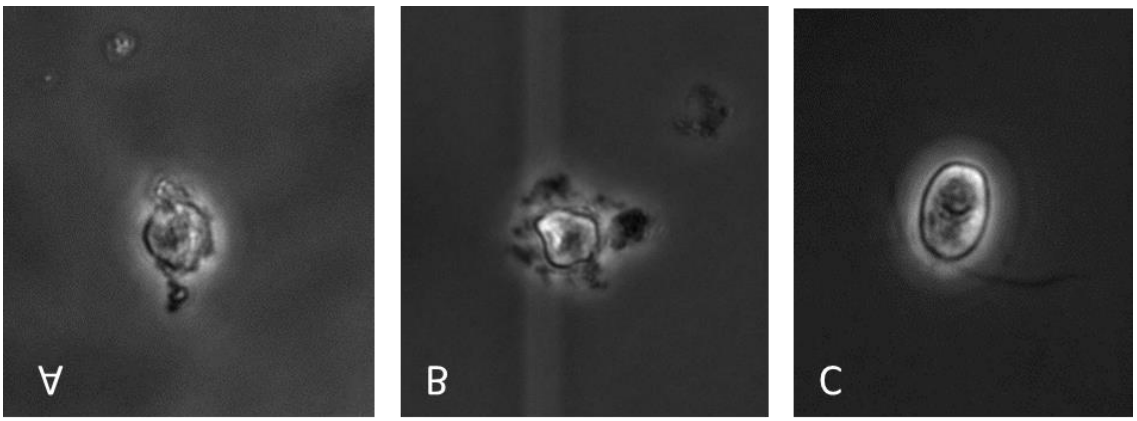

Figure S2. More detailed phase contrast microscopy images (×40) of *D. tertiolecta* cells exposed to ZnO NP 100 mg Zn/L at different exposure time: A (24 h); B (72 h); C (Control)

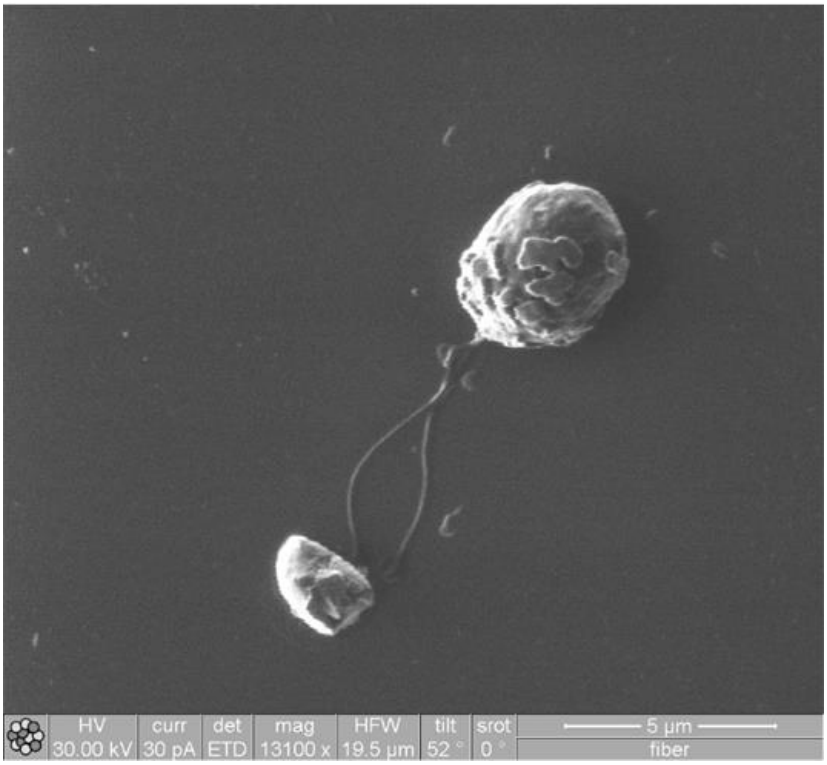

Figure S3. FIB images of *D.tertiolecta* exposed to ZnO NP 100 mg Zn/L for 72 h.

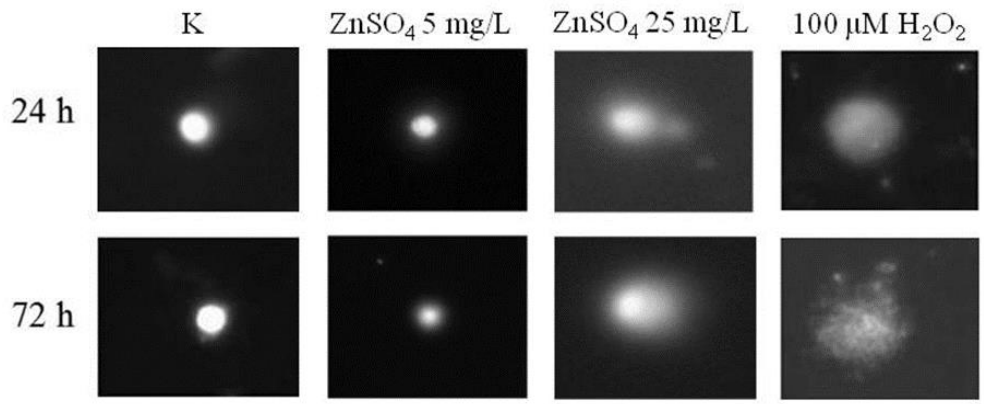

Figure S4. Comet assay microscope images for *D.tertiolecta* exposed to $ZnSO_4$

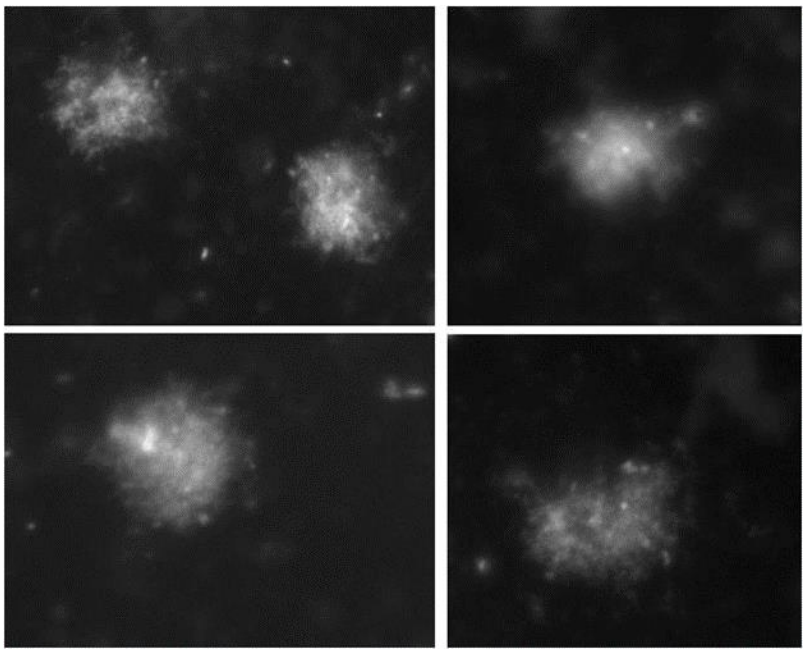

Figure S5. Additional algal cells comet resulting from the exposure (72 h) to ZnO NP 100mg Zn/L.

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3.3 Growth inhibition of three species of marine microalgae exposed to different sizes of Ag NPs and to coating agent PVP/PEI

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Abstract

Microalgae are at the base of aquatic food chains, being starting points of biomagnification processes that transport increased amounts of accumulated contaminants into food chains. Ag NPs are increasingly used due to their antimicrobial properties, therefore their presence in aquatic ecosystems is expected to grow. We evaluated the potential toxic effects of PVP/PEI coated 5 nm Ag NPs and of uncoated 47 nm Ag NPs to three species of marine microalgae with the aim of assessing their relative sensitivity. The selected species *Isochrysis galbana* (Prymnesiophyceae), *Tetraselmis suecica* (Chlorophyceae) and *Phaeodactilum tricornutum* (Bacillariophyceae) showed cell wall differences: the diatom *P. tricornutum* has an elaborate and thick cell wall, *T. suecica* cell wall is composed of coalesced rigid carbohydrate scales while *I. galbana* has a relatively soft cell coating. Microalgae were exposed for 72 h to PVP/PEI 5 nm Ag NPs (0.00001-100 mg Ag/L) or to 47 nm Ag NPs (1-10 mg Ag/L) following the OECD 201 guideline. In parallel, the toxicity of PVP/PEI was also evaluated. Both types of NPs formed aggregates in seawater, larger for 47 nm Ag NPs than for 5 nm Ag NPs. The release of Ag ions by PVP/PEI 5 nm Ag NPs was around 10 times higher than by 47 nm Ag NPs. Ag NPs were able to interact with algal cells surface, as shown by microscope observations. There were clear differences in sensitivity towards PVP/PEI 5 nm Ag NPs among species. *T. suecica* was about 10 times more sensitive (EC50 0.0052 mg Ag/L) than *I. galbana* (EC50 0.039 mg Ag/L) and *P. tricornutum* (EC50 0.06 mg Ag/L). PVP/PEI alone also showed effect to algae indicating that it contributed significantly to the toxicity of 5 nm Ag NP suspensions. Ag NPs of 47 nm resulted less toxic than 5 nm Ag NPs, probably due to differences in size, dispersant and consequent dissolution and aggregation behavior. *P. tricornutum* was slightly less sensitive (EC50 4.72 mg Ag/L) than *T. suecica* (EC50 4.1 mg Ag/L) and *I. galbana* (EC50 3.30 mg Ag/L) which agrees well with the presence of a resistant silicified cell wall in the diatom. The mechanisms leading to observed inhibitory effects on algae growth need further studies.

Introduction

Silver nanoparticles (Ag NPs) are among the most used metallic nanoparticles in consumer products (Rejeski et al. 2008; Marin et al. 2015) due to their unique physicochemical characteristics, such as high conductivity, scattering, chemical stability and catalytic activity (Johari et al. 2013) as well as their antimicrobial properties (Mohan et al. 2007; Wijnhoven et al. 2009). As the market for silver containing nano-functionalized products is significantly increasing worldwide (Boxall et al. 2007; Blaser et al. 2008; Thio et al. 2012), release of Ag NPs to the environment, including aquatic systems, is becoming relevant and is expected to grow up in the next decades (Tiede et al. 2009; Fabrega et al. 2011; Moreno-Garrido et al. 2015). Once in the aquatic environment, it is likely that Ag NPs become a source of (Ag NP) aggregates and of dissolved silver ions (Ag (I)), that could produce adverse effects in aquatic organisms.

Toxicity of Ag NPs to different freshwater and saltwater organisms belonging to different trophic levels has been widely reported (Fabrega et al. 2011; Wang et al. 2012; Ribeiro et al. 2014; Dorobantu et al. 2015; Gambardella et al. 2015). However, as recently reviewed by Moreno-Garrido and co-workers (2015), data on the toxicity on microalgae, and especially on microalgae from marine or estuarine environments, remain scarce and scattered. Current literature shows a wide variation of results on the toxicity of Ag NPs to microalgae, that vary in as much as five orders of magnitude of EC50 values (Lee et al. 2005; Navarro et al. 2008; Kennedy et al. 2010; Burchardt et al. 2012; He et al. 2012; Ribeiro et al. 2014), possibly in relation to differential algae sensitivity and to different factors including Ag NP size and presence of coating agents. Although the coating agents used for NP stabilization are an integral part of the NPs, still scarce attention is devoted to their potential contribution to the overall toxicity of NPs (El Badawy et al. 2011; Katsumiti et al. 2014a; Zhang et al. 2014; Katsumiti et al. 2015; Navarro et al. 2015).

As microscopic primary producers, microalgae are the first target organisms for most of the pollutants present in aquatic systems. They constitute the base of aquatic food chains, being potential starting points of biomagnification processes. Therefore, marine microalgae, which are widely distributed in coastal ecosystems (Behrenfeld et al. 2006) and are particularly susceptible to pollutants (Miglietta et al. 2011; Manzo et al. 2014) can be regarded as suitable indicators for marine water pollution by NPs (Aruoja et al. 2009). The evaluation of NP effects upon marine phytoplankton is indeed a necessary step to predict their potential impact on the whole ecosystems they support. Different species of microalgae could show differences in sensitivity towards NP toxicity depending on their structural and physiological characteristics. *Phaeodactilum tricornutum* (Bacillariophyceae) is a widespread pennate diatom (Rushforth et al. 1988; Francius et al. 2008) commonly used for assessing effects of NPs (Baker et al. 2014; Castro-Bugallo et al. 2014; Moreno Garrido et al. 2015). The cell wall is formed by two valves overlapping in the girdle band region but in opposition to other diatoms, *Phaeodactylum* is very poor in silica, being the cell wall essentially composed of organic compounds. This diatom showed different sensitivities to Ag NPs and in particular a 50% growth inhibition (IC50) has been recorded at 2380 ± 1880 and 3690 ± 2380 μg/L for citrate-capped (14 nm) and PVP-capped (15 nm) Ag NPs, respectively (Angel et al. 2013) while a EC50 of around 930 μg/L for citrate Ag NPs has also been reported (Moreno-Garrido et al. 2015).
Tetraselmis suecica is an elliptical microalgae of the Chlorophyceae (Prasinophyceae) class ranging up to 12 μm in length while *Isochrysis galbana* (Prymnesiophyceae) is the smallest (4-7 μm) among the three algae tested in this study and is widely cultured to feed bivalves in the aquaculture industry. Both algae show a peculiar cell wall structure: *T. suecica* is enclosed in a rigid polysaccharidic cell wall or theca (Becker et al. 1994) while *I. galbana* has a relatively soft cell coating composed of coalesced carbohydrate scales (Zhu and Lee, 1997). Although both algae are generally used as model organisms for toxicity assessment of NPs (Miller et al. 2012; Minetto et al. 2014), to the best of our knowledge no literature is available about Ag NPs effects on these species.
In this study, the effects of PVP/PEI coated 5 nm Ag NPs and of uncoated 47 nm Ag NPs upon three marine microalgae (*P. tricornutum, T. suecica and I. galbana*) were evaluated with the aim of assessing their relative sensitivity. Population growth rate alterations as well as growth inhibition were evaluated and the No Observed Effect Concentration (NOEC), the Lowest Observed Effect Concentration (LOEC) and EC50 values were calculated. Interactions of algae cells surface with Ag NPs were also studied by microscopy analysis. Moreover, the potential toxic effects caused by the PVP/PEI coating agent were determined in order to understand the potential contribution of the coating agent to the overall toxicity of Ag NP suspensions.

Materials and methods

Obtainment and characterization of Ag NPs

Uncoated Ag NPs and Ag NPs coated with PVP/PEI (Poly N-vinyl-2-pirrolidone + Poly ethyleneimine; 77%:23%) were purchased as a stable aqueous suspension from Nanogap (Galicia, Spain). According to the manufacturers' information, PVP/PEI coated AgNPs showed an average size of 5.08 ± 2.03 nm and a zeta potential of +18.6 ± 7.9 mV, while uncoated Ag NPs presented an average size of roughly 47.2±15.6 nm in distilled water (DW). Particle size distribution of PVP/PEI coated 5 nm Ag NPs and of uncoated 47 nm Ag NPs in artificial seawater (ASW, ASTM 1998) was analyzed daily by Dynamic Light Scattering (DLS) using a Zetasizer Nano Z (Malvern Instruments Ltd., Worcestershire, UK) during the 72 h of the ecotoxicological assay. Dissolution of both types of Ag NPs was assessed in ASW as described by Katsumiti et al. (2014b).
Briefly, 10 ml samples of the Ag NP suspensions were prepared at a concentration of 10 mg/L and filled into dialyzer tubes (Spectra/ Por® Float-A-Lyzer; MWCO 0.1-0.5 kDa). The sample-filled dialyzers were then immersed in 1 L ASW. Samples (3 replicates of 1 ml) were extracted at 24, 48 and 72 h from the solution and analyzed for Ag ions using ICP-MS (Thermo X2 series) after 100- fold dilution using external calibration. Differences obtained for replicate samples were consistently lower than 5 % (R.S.D).

Organisms

P. tricornutum (Bacillariophyceae: Naviculales), *T. suecica* (Chlorophyceae: volvocales) and *I. galbana* (Prymnesiophyceae: Isochrysidales) were maintained in sterilized standard medium (Guillard, 1975) made with ASW.
Microalgae were incubated under cool continuous white fluorescent lights until the log phase growth prevailed (about 58 μmol photons m−2 s−1 at 24±1°C with aeration from 5 to 7 days) to provide enough inocula for experiments. Cell density was measured by a hemocytometer.

Toxicity test

Algae growth inhibition test

Algae bioassays were performed according to IRSA-CNR (IRSA-CNR, 1978). All glassware was rinsed with Milli-Q purified water and autoclaved before use. Microalgae (with a final density of 104 cells/ml) were first filtered (0.22 μm) and rinsed three times with filtered autoclaved ASW.
Then, microalgae were added to each treatment and control (standard culture media, Guillard medium) together with nutrients. Stock suspensions of PVP/PEI 5nm Ag NPs and 47 nm Ag NPs were prepared by dispersing the primary stock solution into ASW to the final concentration of 100 mg/L. Stock dispersions were properly diluted at concentrations ranging 0.00001-100 mg Ag/L for PVP/PEI 5nm Ag NPs and 1-10 mg Ag/L for 47nm Ag NPs. Concentration ranges were selected based on the literature (Navarro et al. 2008; Oukarroum et al. 2012; He et al. 2012) and on preliminary toxicity experiments performed with each NP type.
The PVP/PEI stock solution was diluted at concentrations ranging 0.0001-1040 mg/L (the same concentration range present in the 5nm Ag NP suspensions). After dilution, all the suspensions were briefly vortexed before the addition of micronutrients and test organisms.
Test plates (10 ml) were kept in a growth chamber constantly illuminated with a white fluorescent lamp (enhanced irradiation between 400 and 500 nm), at a temperature of 24 ± 1 °C for 3 days.
Growth inhibition was expressed as percentage effect with respect to controls.

Growth rate determination

During the experiments, growth rate was determined daily by counting microalgae with a Burker chamber under a light microscope. Growth rate was calculated according to the equation described by Xiong et al. (2005):

$$U = (\ln Nt - \ln N0) / (t - t0)$$

where U (cell number/h) is the growth rate; Nt and N0 mean the quantity of cells at times t and 0, respectively,where t means any exposure time (h) and t0 means the initial time of the treatment (h).

Microscope observations

Observations on the interactions of Ag NPs with microalgae were performed through light microscope analysis (ZEISS Axioskop 50) followed by more detailed observations through Focused Ion Beam (FIB) microscopy (FEI QUANTA 2 D). Before FIB observations, microalgae were fixed as described in Li et al. (2015). Briefly, after 72 h of exposure, microalgae were centrifuged (4000 rpm for 10min) and then samples were fixed with a 3% glutaraldehyde solution at 4°C for 2 h.
Samples were then washed three times with 0.1 M PBS (pH 7.8) by centrifugation (4000 rpm for 10 min). Microalgae were fixed with 1% osmium tetroxide for 2 h at 4°C and finally 0.1 M PBS (pH 7.8) was added to wash the cells by centrifugation (3800 rpm for 10 min, three times). Control and treated (10 mg Ag/L) microalgae were placed on a thin glass slide, air dried and observed under the FIB.

Data analysis

Statistical analyses for growth inhibition as well as for NOEC and LOEC values were conducted using the statistical package SPSS v.19 (SPSS Inc., IBM Company, Chicago, USA). Prior to the analysis, data were tested for normality (Kolmogorov-Smirnov normality test) and homogeneity of variances (Levene's test). Significant differences ($p<0.05$) with respect to controls were set based on analysis of variance (ANOVA) and the multiple comparisons Dunnett's test. EC50 values were calculated using the Linear Interpolation Method (Inhibition Concentration procedure or ICp) (US EPA, 1993) and the bootstrap method was used to obtain 95% confidence intervals

Results

Physicochemical characterization

PVP/PEI coated 5 nm Ag NPs dispersed in ASW at 10 mg/L immediately reached a mean size of around 100 nm according to DLS measurements (Figure 1).The mean size remained stable up to 24h (100 nm), while after 48-72 h aggregates were slightly smaller (around 90 nm, Figure 1). Zeta potential values for PVP/PEI 5 nm Ag NPs in ASW ranged from -5 mV to +0.2 mV. Uncoated 47 nm Ag NPs tended to aggregate rapidly in ASW reaching a mean size of about 880 nm that turned to be slightly smaller at 24 h (~600 nm) (Figure 2). The largest size was achieved after 48 h (~3000 nm) and aggregates were smaller (around 1400 nm) after 72 h (Figure 2). Zeta potential in ASW was around -8 mV.

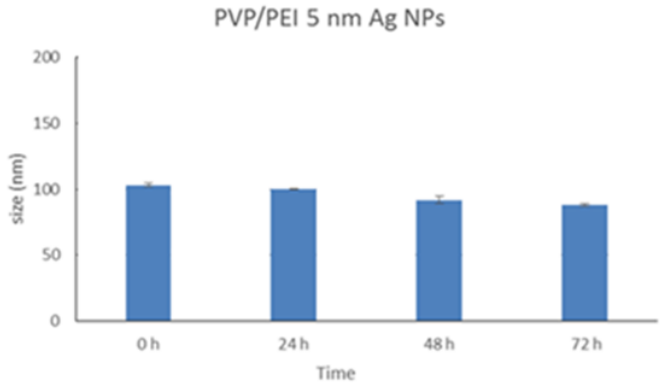

Figure 1: Mean size (±SD) of aggregates of PVP/PEI 5 nm Ag NPs (10 mg/L) during the 3 days algae bioassay.

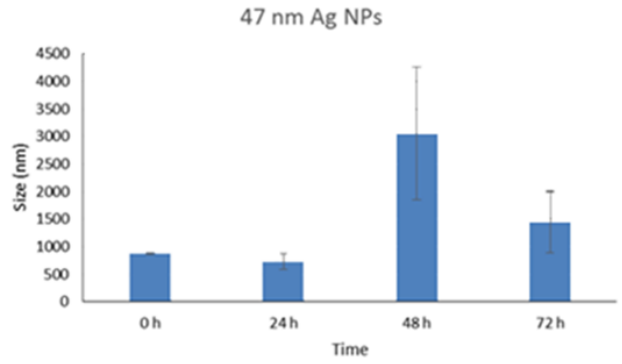

Figure 2: Mean size (±SD) of aggregates of 47 nm Ag NPs (10 mg/L) during the 3 days algae bioassay

Dissolution of both types of Ag NPs in ASW was detected along the 72 hours of experimentation (Table 1). In general, the release of Ag ions by PVP/PEI coated 5 nm Ag NPs was around 10 times higher than that by uncoated 47 nm Ag NPs (Table 1). After 24 hours, PVP/PEI coated 5 nm Ag NPs released around 20% of Ag ions while only near 1.5% of the uncoated 47 nm Ag NPs was converted into ionic Ag (Table 1). At 72 hours, dissolution of PVP/PEI coated 5 nm Ag NPs increased to 29.6% and for uncoated 47 nm Ag NPs to 3.4% (Table 1).

Table 1. Release of Ag ions as % of total starting material of Ag NPs (10 mg/L) in ASW.

Time (h)	**PVP/PEI 5 nm Ag NPs**	**47 nm Ag NPs**
0	0	0.65
12	10.13	0.83
24	20.20	1.52
48	25.63	3.04
72	29.66	3.41

Toxic effects of PVP/PEI 5nm Ag NPs and coating agent PVP/PEI

Figure 3 shows the effects of PVP/PEI 5 nm Ag NPs and of the coating agent PVP/PEI on the growth of selected microalgae. Main ecotoxicological parameters evidenced clear differences insensitivity among studied species (Table 2). For PVP/PEI 5 nm Ag NPs, the effects were significant at the lowest concentration for all three microalgae. In fact, the NOEC was below 0.00001 mg Ag/L and the LOEC was always represented by the lowest concentration tested (Table 2). Based on the EC50 values obtained, *T. suecica* was about 10 times more sensitive (EC50 0.0052 mg Ag/L) than *I. galbana* (EC50 0.039 mg Ag/L) and *P. tricornutum* (EC50 0.06 mg Ag/L). This greater sensitivity of *T. suecica* was also evidenced by the analysis of the growth rates reported in Table 3. In fact, for *T. suecica* significant growth rate inhibition was measured starting from 0.01 mg Ag/L whereas the growth rates for *I. galbana* and *P. tricornutum* were not significantly different from controls up to 0.1 mg Ag/L (Table 3).

Table 2. Ecotoxicological parameters (EC50; NOEC; LOEC) for the three microalgae exposed to PVP/PEI 5 nm Ag NPs and PVP/PEI coating agent.

PVP/PEI 5 nm Ag NPs (mg/L)	***I. galbana***	***P. tricornutum***	***T. suecica***
EC50 [confidence limits]	0.039 [0.035-0.043]	0.06 [0.047-0.077]	0.0052 [0.0022-0.0080]
NOEC	<0.00001	<0.00001	<0.00001
LOEC	0.00001	0.00001	0.00001
PVP/PEI (mg/L)			
EC50 [confidence limits]	0.03 [0.009-0.055]	0.83 [0.69-0.94]	0.004 [0.0009-0.007]
NOEC	<0.0001	0.0001	0.001
LOEC	0.0001	0.001	0.01

The coating agent PVP/PEI alone inhibited markedly the growth of the three microalgae (Figure 3 and Table 4). The highest EC50 value (0.83 mg/L corresponding to 0.083 mg Ag/L in PVP/PEI 5 nm Ag NP suspension) was registered for P. tricornutum. T. suecica resulted the most sensitive with a EC50 value of 0.004 mg/L (= 0.0004 mg Ag/L in PVP-PEI 5 nm Ag NP suspension), while for I. galbana an intermediate EC50 value was obtained (0.03 mg/L corresponding to 0.003 mg Ag/L in PVP/PEI 5 nm Ag NP suspension). Thus, PVP/PEI alone was toxic to algae indicating that it contributed significantly to the toxicity of PVP/PEI 5 nm Ag NP suspensions.

The observations by light microscopy and by FIB of the three microalgae exposed to PVP/PEI 5 nm Ag NPs (10 mg Ag/L) are reported in Figure 5a and Figure 6a. PVP/PEI 5 nm Ag NPs seemed to interact with algae mainly by entrapping them in a network of hetero-aggregates without changing their shape or morphology. Similarly algae exposed to PVP/PEI alone appeared entirely covered by the polymeric matrix. Moreover, FIB images allowed a more detailed observation of algae surface which resulted completely covered and surrounded by PVP/PEI 5 nm Ag NPs aggregates. In the case of *P. tricornutum* and *I. galbana* this coverage did not result in alterations of cell morphology, while for *T. suecica* a change in shape and a loss of cell turgor, with respect to the control, could be observed.

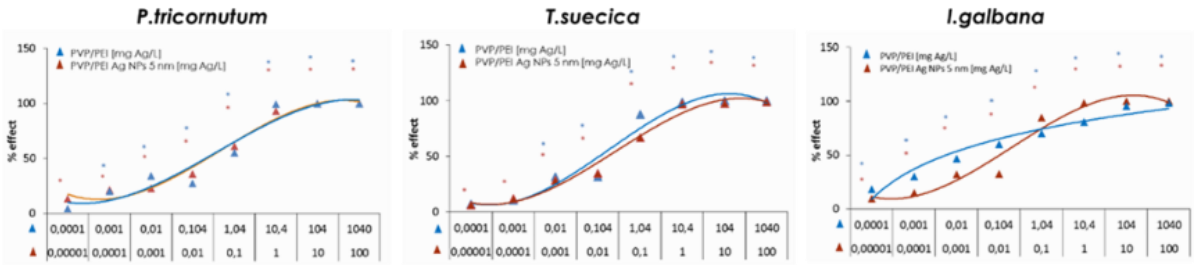

Figure 3: Toxic effects of different concentrations of PVP/PEI 5 nm Ag NPs (red triangle) and PVP/PEI (blue triangle) upon *P. tricornutum*, *T. suecica* and *I. galbana*. Asterisks indicate statistically significant differences with respect to controls ($p<0.05$).

Table 4. Growth rate (mean ± SD) for the three microalgae exposed to PVP/PEI at the same concentration range present in the 5 nm Ag NP suspensions. Asterisks indicate statistically significant differences with respect to controls ($p<0.05$).

PVP/PEI (mg/L)	***I. galbana***	***P. tricornutum***	***T. suecica***
Control	1.53±0.03	1.71±0.05	0.99±0.04
0.0001	1.51±0.03	1.69±0.04	0.98±0.03
0.001	1.49±0.05	1.63±0.05	0.82±0.04
0.01	1.42±0.06	1.57±0.03	0.51±0.02*
0.104	1.39±0.04	1.60±0.02	0.56±0.06*
1.04	1.16±0.02*	1.44±0.04*	0.36±0.04*
10.4	1.10±0.06*	0*	0*
104	0*	0*	0*
1040	0*	0*	0*

Toxic effects of 47 nm Ag NPs

Overall, 47 nm Ag NPs were less toxic than PVP/PEI 5 nm Ag NPs for the three microalgae tested. *P. tricornutum* was slightly less sensitive (EC50 4.72 mg Ag/L) than T. suecica (EC50 4.1 mg Ag/L) and *I. galbana* (EC50 3.30 mg Ag/L) (Table 5). *T. suecica* showed a peculiar inverted U-shaped dose-response curve (Figure 4). For the three species tested, the decrease in growth rate was maximal at 5 mg Ag/L and then growth rate values increased at 7.5 and 10 mg Ag/L (Figure 4, Table 6).
Figures 5b and 6b show light microscopy and FIB images obtained for the three microalgae exposed to 47 nm Ag NPs (10 mg Ag/L). Light microscopy images showed that 47 nm Ag NPs interacted by surrounding algae surface without entrapping them in hetero-aggregates. Algae surfaces were observed in greater detail by FIB microscopy: algae cells resulted covered by 47 nm Ag NP aggregates but no signs of cell morphology alterations and plasma membrane damages could be highlighted.

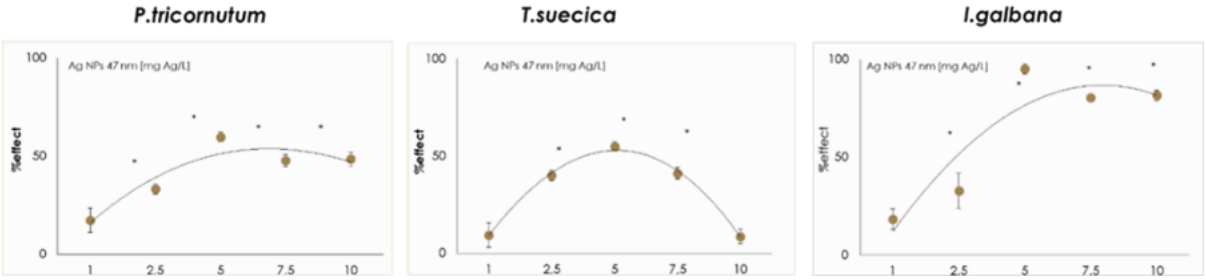

Figure 4: Toxic effects of different concentrations of 47 nm Ag NPs upon *P. tricornutum*, T. suecica and *I. galbana*. Asterisks indicate statistically significant differences with respect to controls ($p<0.05$).

Table 5.Ecotoxicological parameters (EC50; NOEC; LOEC) for the three algae exposed to 47 nm Ag NPs

47 nm Ag NP (mg/L)	***I. galbana***	***P. tricornutum***	***T. suecica***
EC50 [confidence limits]	3.3 [2.9-3.6]	4.72 [4.60-4.90]	4.1 [3-6.2]
NOEC	1	1	1
LOEC	2.5	2.5	2.5

Table 6. Growth rate (mean ± SD) for the three microalgae exposed to 47 nm Ag NPs. Asterisks indicate statistically significant differences with respect to controls ($p<0.05$).

47 nm Ag NPs (mg/L)	***I.galbana***	***P.tricornutum***	***T.suecica***
Control	1.10±0.02	1.26±0.02	0.71±0.03
1	1.03±0.01	1.19±0.04	0.68±0.04
2.5	0.97±0.05	1.12±0.06	0.54±0.05
5	0.09±0.01*	0.95±0.03*	0.44±0.04
7.5	0.56±0.04*	1.04±0.07*	0.54±0.04
10	0.54±0.04*	1.04±0.03*	0.68±0.03

Discussion

In this study, the effects of two different types of Ag NPs upon three different species of marine microalgae were evaluated with the aim of assessing their relative sensitivity. Obtained results can contribute to the understanding of effects of nanomaterials in the marine aquatic environment. We assessed the toxicity of PVP/PEI coated 5 nm Ag NPs and of uncoated 47 nm Ag NPs. Since it has been reported that coating agents contribute to the overall toxicity of NPs (El Badawy et al. 2011; Nguyen et al. 2013; Katsumiti et al. 2014a; Katsumiti et al. 2015; Navarro et al. 2015), the effects of PVP/PEI on the three microalgae were also evaluated.

Physicochemical characterization

DLS measurements of PVP/PEI coated 5 nm Ag NPs dispersed in ASW clearly showed hydrodynamic sizes larger than their primary size. PVP and PEI are both good NP stabilizers (Dai and Bruening, 2002; Zhang et al. 2010), although aggregation of primary particles is likely to occur in high ionic strength media such as seawater (Thio et al. 2012). Already in the first minutes NPs reached a mean size of around 100 nm that appeared to be balanced by steric hindrance and particles were stable up to 24 h, decreasing then their size to 90 nm after 48-72 h. Similar relative stability was reported for PVP-coated Ag NPs in SW by other authors (Angel at al. 2013). Considering that polymeric coating enhance particles stability (Lin et al. 2012), the absence of PVP/PEI in 47 nm Ag NP suspensions led to a sharp phenomenon of aggregation: DLS measurements in ASW indicated that particles tend to rapidly aggregated reaching the size of 3000 nm after 72 h.

Dispersions of PVP/PEI 5 nm Ag NPs showed Z-potential values ranging from -5 mV to +0.2 mV.

Generally, PVP-coated Ag NPs are nearly neutral in seawater (Thio et al. 2012) and thus, measured values are possibly a consequence of the positively charged PEI present in the capping mixture as electrosterical stabilizer agent. As recently reported in the OECD dossier on silver nanoparticles (OECD, 2015) a positive Z-potential value often generates an enhanced Ag NP toxicity since the reduced electrostatic barrier allows a more probable cell-particle interaction. In this study, the slight positive charges of PVP/PEI 5 nm Ag NPs could play an attractive role on the negatively charged algae surfaces allowing a greater toxicity respect to negatively charged 47 nm Ag NPs (-8 mV).

The NP dissolution rate is generally not affected by the presence of coating agents (Angel et al. 2013), but it was mainly influenced by the NP primary size (Ho et al. 2010). Accordingly, we found that dissolution rate from PVP/PEI 5 nm Ag NPs was 10 fold higher than dissolution rate measured for suspensions of uncoated 47 nm Ag NPs. Similarly, it was reported a dissolution rate for 5.4 nm Ag-NPs of about 9 times higher than that for 20.5 nm Ag-NPs (Ho et al. 2010).

On the other hand, in environmental and biological solutions containing chloride ions, AgCl complexes (and some times silver carbonate and silver phosphate) will form from the dissolved silver ions. Therefore, due to the high salinity of seawater, dissolved silver is likely to be less toxic, respect to low salinity waters, because silver speciation may have been dominated by AgCl complexes that are less toxic than free silver due to their lower bioavailability (Lee et al. 2005). For example, Angel et al. (2013) reported that only 34% of the total silver present at the start of the test dissolved in seawater. At the same way, we found that after 72 h the 29% of total silver was released by PVP/PEI 5 nm Ag NP while only 3% of total silver was released by 47 nm Ag NPs.

Toxic effects upon microalgae

By comparing the dose-response curves obtained for uncoated and coated Ag NPs, different levels of toxicity were clearly detectable. Microalgae exposed to uncoated 47 nm Ag NPs showed EC50 values of several orders of magnitude higher (3-4 mg Ag/L) than those obtained by the algae exposed to PVP/PEI coated 5 nm Ag NPs (0.0052-0.06 mg Ag/L). The different algal sensitivity could be ascribable to a combined effect of particle size and occurrence or not of coating agent PVP/ PEI once suspended in saline medium (Miao et al. 2009; He et al. 2012; Gambardella et al. 2015).
In the present study, the three algae, when exposed to PVP/PEI 5 nm Ag NP, were less sensitive respect to *Thalassiosira weissflogii* (Miao et al. 2009) exposed to PVP Ag 10 nm NP (EC50 1-2 *10-12 M), but more sensitive respect to *D. tertiolecta* (EC50 0.9 mg/L) and *S. costatum* (EC50 3.1 mg/L) treated with uncoated 10 nm Ag NP (Gambardella et al. 2015). Instead, when *I. galbana*, *T. suecica* and *P. tricornutum* were exposed to 40 nm Ag NPs, minor sensitivity respect to C. marina (EC50 0.2 mg/L, He et al. 2012). In the case of P. tricornutum the obtained EC50 values were not directly comparable with that ones obtained for this species and other diatoms in previous works (Angel et al. 2013; Moreno-Garrido et al. 2015) due to difference in NP sizes used (14-15 nm) and in capping agent composition.
The nano aggregates of coated Ag NPs (less than 100 nm) resulted more available to microalgae in comparison to the larger ones produced by uncoated NPs. This greater availability resulted in a closer interaction with microalgae surfaces that appeared entrapped in a network of large heteroaggregates of PVP/PEI coated 5 nm Ag NPs, as could be highlighted by light microscopy and FIB microscopy.

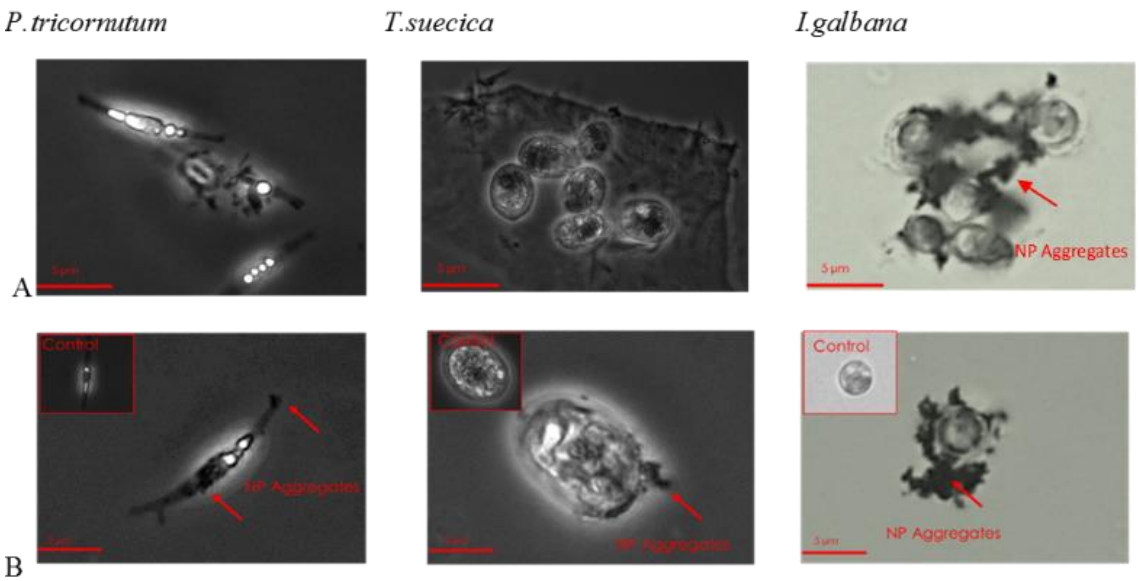

Figure 5: Micrographs obtained by light microscopy of *P.tricornutum*, *T.suecica* and *I. galbana* exposed to 10 mg/L PVP/PEI 5 nm Ag NPs (A) and 47 nm Ag NPs (B). Control cells are shown in the insets

It seems that aggregates covering cell surfaces can induce a certain inhibition of the photosynthetic activity of microalgae due to the reduction of light availability (Navarro et al. 2008; Wei et al. 2010; Manzo et al. 2015). On the other hand, based on light microscope observations, aggregates of uncoated Ag NPs mainly entrapped few algae cells at time resulting in generally small formations. This was also observable in FIB images in which only few aggregates were found surrounding the algae cell surfaces.

Coating agents are chemicals (such as polymers and surfactants) used in the synthesis of Ag NPs to prevent their aggregation through electrostatic repulsion, steric repulsion or both (Phenrat et al. 2008; Hotze et al. 2010). In the case of silver, the most prevalent capping agents are citrate and polyvinylpyrrolidone (PVP) (El Badawy et al. 2011). The PVP-AgNPs are stabilized through the steric repulsion caused by the adsorption of PVP on the particle surface (Zhang and Zhang, 2014). The PEI-Ag NPs are electrosterically stabilized due to the adsorption of the PEI on the particle surface (El Badawy et al. 2010). In general, no toxic effects have been previously reported for PVP upon marine microalgae (Moreno-Garrido et al. 2015) while a clear toxic effect has been reported for PEI for different organisms (El Badawy et al. 2011; Ivask et al. 2014). However, to the best of our knowledge, there is no literature available upon the use of these two polymers together. In the present study, algae exposed to the coating agent PVP/PEI alone showed an evident growth inhibition effect similar to that observed for coated NPs, but a different behaviour could be distinguished for the three algae. In particular, in the case of *I. galbana* and *T. suecica*, the coating agent alone resulted more toxic than PVP/PEI coated Ag NP suspensions at PVP/PEI equivalent concentrations. We can speculate that once algae were added to the PVP/PEI suspension, an immediate steric interaction occurred due to the stabilizing nature of the agent itself. This could provoke the inclusion of microalgae in the polymeric matrices with a subsequent clear toxic effect, especially in the case of microalgae without a resistant cell wall (*I. galbana* and *T. suecica*).

Similarly, a possible role of the coating agent in the overall toxicity to algae of different metal NPs as well as some evidences for different organic coating agents has been previously reported by some authors (El Badawy et al. 2011, Moreno-Garrido et al. 2015, Navarro et al. 2015).

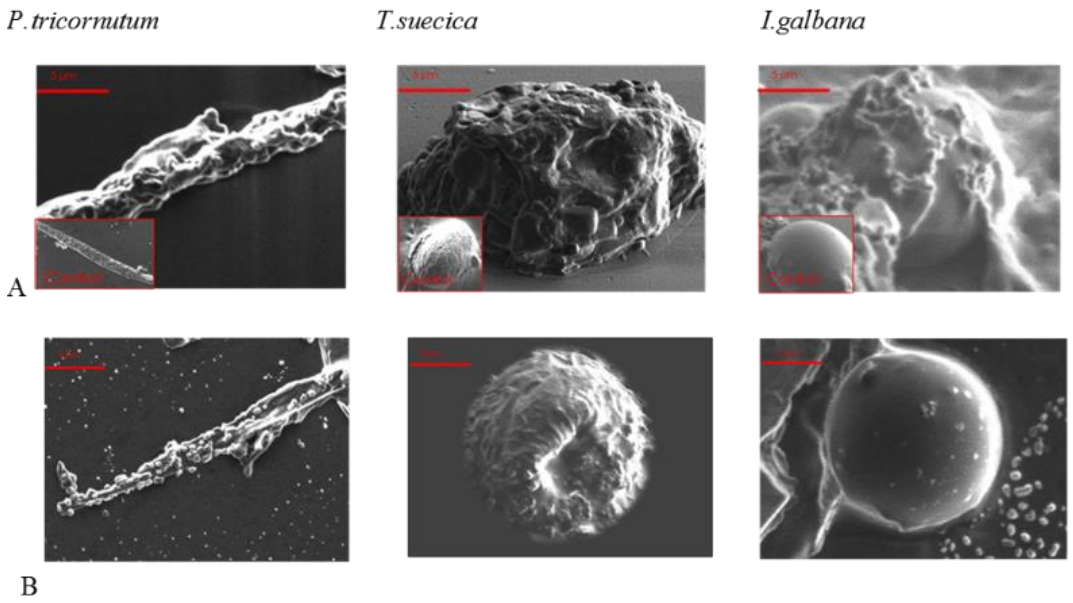

Figure 6: FIB micrographs of *P. tricornutum*, *T. suecica* and *I. galbana* exposed to 10 mg/L PVP/PEI 5 nm Ag NPs (A) and 47 nm Ag NPs (B). Control cells are shown in the insets.

The three selected microalgae differed in sensitivity towards tested NPs; *T. suecica* and *I. galbana* were more sensitive than *P. tricornutum* to both NPs as well as to the coating agent. The higher sensitivity of *I. galbana* and *T. suecica* could be due to their cell wall characteristics. These algae have no distinct or mineralized cell walls (Throndsen et al. 1993; Zhu et al. 1997) and therefore, they may be more vulnerable compared to other taxa with different cell wall properties. Thus, the resistant cell wall of *P. tricornutum* seemed to limit the toxic effects of coated and uncoated Ag NPs and of the coating agent. Additionally, small microalgae have been

found to be more sensitive than diatoms (Helbling et al. 1994; Karentz et al. 1994; Holm-Hansen et al. 1997). This higher sensitivity has been related to their larger surface area to volume ratio compared to larger microalgae (Quigg et al. 2006; Levy et al. 2007). Moreover, due to their dimensions, small microalgae may be covered faster by NPs. Finally, algae motility is a key process that should be taken into account: it allows a greater interaction between algae and NP aggregates but it could also be a very sensitive process that could be affected. Mobile algae cells are widely distributed in culture medium, thus increasing their contact time with Ag NP aggregates, with respect to immobile diatoms that tend to settle at the bottom of the well (Zhou et al. 2015).

Conclusions

Ag NPs of 47 nm resulted less toxic than PVP/PEI coated Ag NPs of 5 nm for the three microalgae species. This is possibly related to the fact that 5 nm Ag NPs formed smaller aggregates that could be more available to cells when compared to aggregates of 47 nm Ag NPs. An additional reason could be that PVP/PEI 5 nm Ag NPs released around 10 times more Ag ions than 47 nm Ag NPs.

PVP/PEI alone was also toxic to algae indicating that it contributed significantly to the toxicity of PVP/PEI 5 nm Ag NP suspensions. The mechanisms leading to observed inhibitory effects on algae growth remain to be explored and need further studies. Finally, of the three species tested, *T. suecica* was the most sensitive to PVP/PEI 5 nm Ag NP exposure while the diatom *P. tricornutum* was most resistant, highlighting the influence of algae cell surface characteristics on NP toxicity.

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4. General discussion

In this work, the ecotoxicity of ZnO, SiO_2 TiO_2 (section 3.1-3.2), and of coated and uncoated Ag (Chapter 3.3) NPs upon marine microalgae were studied using different approaches. Selected NPs varied in chemical composition, size, shape, crystal structure and presence of coating agent. To evaluate the bioavailability to microalgae, NPs were characterized in testing media (i.e. seawater) and all main results, such as size distribution, Z-potential, ion release, sedimentation were summarized in Table 1.

Overall results of this work evidenced how the toxic effects were strictly related to the metal containing NPs and then to their different physic-chemical behavior in a complex matrix like seawater.

In the main, these physicochemical features highlighted the instability of all tested nanomaterials in seawater, which result in the formation of large, micrometric aggregates with specific sedimentation trend also in relation to the initial particle concentrations. The Z-potential magnitudes indicated indeed that the repulsive energy among the particles was smaller than van der Waals attraction energy, and so the particles showed a marked tendency to flocculate. All these parameters influenced the, availability and then toxicity and the mode of action of the different nanoparticles.

Therefore, it could be assumed that the Ag 5 nm is the most toxic metal followed by ZnO, TiO_2 while SiO_2 was the least toxic metal. These results are in line with findings of other studies upon different organisms. Katsumiti et al. (2014) founds the same rank of toxicity upon hemocytes and gill cells of *Mytilus galloprovincialis*. Other authors found that ZnO was more toxic, respect to TiO_2.and SiO_2 upon 4 different microalgae species (Miller et al. 2012) or upon two different bacteria (Adams et al., 2006)

The following section discusses the key findings of this thesis and aimed to make recommendations for future investigations of nanomaterial effect in marine environment for a reliable hazard assessment.

4.1 The role of dissolution in NP toxic action for algae

The dissolution is an important process to take into account in the NP toxicity as it largely influences the mode of action and the impact on human and environmental health. The NP potential to dissolve influences their persistence in the environment and acts as a critical factor that determines the diverse biological responses. Dissolution can delivery highly toxic ions, as in the case of NPs composed of elements, which, in solution, are known to be toxic (such as Zn^{2+}, Cu^{2+}, Cd^{2+} and Ag^{+} (Brunner et al., 2006; Xia et al., 2008).

The comparison among different metal bearing NPs in seawater, showed TiO_2 and SiO_2 NPs as almost insoluble, while ZnO and Ag NPs were instead soluble. Dissolution of ions from metal bearing NPs such as ZnO and Ag appears to be a major driver of toxicity for many aquatic organisms. The toxicity of ZnO NPs was previously attributed to the dissolution of ionic Zn in freshwater (Aruoja et al., 2009; Franklin et al., 2007; Heinlaan et al., 2008), seawater (Wong et al., 2010; Miller et al., 2010) and in cell culture media (Xia et al., 2008). Similarly in many studies (Lok et al., 2007; Navarro et al., 2008) the dissolution of Ag ions was suggested as the responsible for Ag NP toxicity.

In the present work, we confirmed that dissolution plays a role in NPs toxicity since the most toxic NPs were those with the highest capability of toxic ion release: Ag NPs 5 nm and ZnO NPs (see table 1).

In the case of Ag NPs (Section 3.3), it has been found that algae population growth inhibition was positively related to the Ag ion release. The higher was the dissolution rate measured (PVP/PEI 5 nm Ag NPs respect to the uncoated 47 nm Ag NPs) the higher was the toxic effect observed.

For ZnO NPs (Section 3.2) the comparison among all the considered endpoint outcomes (genotoxicity/cytotoxicity and growth inhibition) highlighted that a direct action of Zn ion release near algae cellular membrane/wall could provoke Zn accumulation in the cell and consequently DNA damages (Heim et al., 2015). In the case of the ZnO NPs the toxic effect is not solely ascribable to ion releasing but is also related to the particles aggregation process in the medium.

4.2 NP Size dependent toxicity

In this work we found that also other NP properties such as the pristine size could influence the toxicity to microalgae cells. Comparing the two types of Ag NPs tested, smaller NPs (PVP/PEI 5nm) were more ecotoxic than the larger ones (Ag 47 nm). Microalgae exposed to uncoated 47 nm Ag NPs showed EC50 values of several orders of magnitude higher (3-4 mg Ag/L) than those obtained by the algae exposed to PVP/PEI coated 5 nm Ag NPs (0.0052-0.06 mg Ag/L). The different algal sensitivity could be ascribable to a combined effect of particle size and occurrence or not of coating agent PVP/PEI once suspended in saline medium (Miao et al. 2009; He et al. 2012; Gambardella et al. 2015).

Size dependent toxicity was also reported in other studies. For example Clement and co-authors reported that TiO_2 NPs (15 nm; 25 nm; 32 nm) were more toxic toward different organisms (microalgae, daphnia, rotifer and plants) respect to microsized TiO_2 (44 µm). For Ag NPs different researcher reported about silver size-dependent toxicity (Jiang et al., 2008; Zhang et al., 2014; Gliga et al., 2014) in particular Carlson et al. (2008) found that Ag NPs 15 nm and 30 nm were more toxic respect Ag NPs 55 nm. Passagne et al. (2012) reported that toxicity was size- and time-dependent, with 20 nm SiO_2 NPs being more cytotoxic than larger ones (50 nm).
Regarding ZnO NPs in different studies a higher toxicity of nano ZnO with respect to bulk was observed for different organisms(Heinlaan et al., 2008, Ji et al., 2011, Jiang et al., 2009 and Wong et al., 2010). In particular in Manzo et al 2013 bulk ZnO (>100nm) resulted less toxic than nano ZnO (<100nm) upon the green algae *D. tertiolecta*.
In most of these studies the small size and the relatively large surface have been suggested to result in increased toxicity when compared to particles in micrometer size. There is a general consensus on that ≤30 nm NPs are more toxic than larger ones due to their dramatic changes in behavior that enhance their reactivity (Auffan et al., 2009). Particles of 30 nm in size show less than 20% of the constituent atoms in their surface while particles of 10 nm show approximately 35-40% of the atoms localized at their surface (Auffan et al., 2009). Therefore the higher toxicity of NPs ≤ 30 nm respect larger ones was attributed to the considerable changes in NP property that enhance their reactivity (Auffan et al., 2009).

4.3 The role of NP aggregation in toxic effect

The aggregation status is a key factor affecting the NP availability, uptake and toxicity. Physicochemical parameters of testing media such as pH and ionic strength can lead to a different precipitation of NPs and consequently to lower bioavailability for biota (Baun et al., 2008). NPs are known to rapidly aggregate and precipitate in high ionic strength media such as seawater (Canesi et al., 2010; Brunelli et al., 2013).
NP once in seawater gave place to two different aggregation phenomenon homoaggregation (aggregation among NPs) and heteroaggregation (aggregation between NPs and organisms or natural organic matter (NOM). In surface water, whether the NPs will undergo homoaggregation rather than heteroaggregation with inorganic colloids depends on the respective kinetics of these competing scenarios. High NP concentration will increase the homoaggregation rate, while high colloid concentration or presence of organisms will likely favor heteroaggregation (Labille et al., 2015)
In the case of ZnO (470-1040 nm) the hetero-aggregation among algae and NP aggregates was favored at lowest concentrations and algae were in fact rapidly surrounded by NP aggregates whereas, at highest concentrations of NPs, the homo aggregation was prevalent and algae resulted not entrapped. The close interaction between algal cells and aggregates provoked a noticeable effect on the cell morphology: cells loss a regular shape and turgor. Regarding SiO_2 NPs (1300-1800 nm), within 24 h from dispersion in seawater, mainly aggregates with sizes around 600 nm were present in the suspension independently from the starting concentration. (Table 1). This indicates that SiO_2 formed a stable population of homoaggregates and that, by increasing the SiO_2 concentration, only the overall number of these aggregates increased. Independently by the tested concentration, the algae were covered by aggregates; however, no clear toxic effects upon algal cell number, viability, and ROS production were evident. On the other hand, it is likely that the cell surfaces covering by the aggregates can induce a certain inhibition of the photosynthetic activity due to the reduction of the light availability (Navarro et al. 2008; Wei et al. 2010). This sharp tendency to a strong heteroagglomerations between SiO_2 aggregates and algal cells was also reported, in experimental conditions (i.e., pH and IS) close to ours, in a recent work by Ma et al. (2015).
The size of the TiO_2 aggregates rapidly increased in seawater to a few microns. However, increasing the particle concentrations increases the probability of collisions not only between particles (homoaggregation) affecting the aggregation rate but also between aggregates and algal cells, thus increasing the number of potential cells injured by the (nano)material. This heteroaggregation phenomenon was previously described (Wang et al. 2008, Li et al. 2015, Ma et al. 2015, Xia et al. 2015), and the rate of agglomeration seemed to be faster in the presence of algae (Sadiq et al. 2011). Since it was observed that the TiO_2 aggregates were already few microns sized in these first hours, it could be envisaged that the first step of the toxic action is the entrapment of the algal cells by means of very large particle aggregates. Then, the close interaction between aggregates and cell membranes induced an oxidative stress as ROS production (Thill et al. 2006; Hartmann et al. 2010). The NP aggregates of coated Ag NPs (less than 100 nm) resulted more available to microalgae in comparison to the larger ones produced by uncoated NPs. This greater availability resulted in a closer interaction with microalgae surfaces

that appeared entrapped in a network of large heteroaggregates of PVP/PEI coated 5 nm Ag NPs. It seems that aggregates covering cell surfaces can induce a certain inhibition of the photosynthetic activity of microalgae due to the reduction of light availability (Navarro et al. 2008; Wei et al. 2010). On the other hand, based on light microscopy observations, aggregates of uncoated Ag NPs also entrapped few algae cells at a time, resulting in generally small formations.

4.4 The presence of coating agent could influence the toxicity

The modifications of NP surface chemistry (e.g. Coating agents) or the presence of additives in the NPs preparation (e.g. surfactants) are used to increase the stability of NPs in suspension but may influence their biological reactivity. The NP modifications can influence significantly NP dissolution and can promote toxicity by its own chemistry (Falck et al., 2009; Mano et al., 2012) increasing the production of ROS and producing oxidative stress (Mano et al., 2012). Although the coating agents used for NP stabilization (PEG, PVA, PVP, PEI) are an integral part of the NPs, still scarce attention is devoted to their potential contribution to the overall toxicity of NPs (El Badawy et al. 2011; Katsumiti et al. 2014a; Zhang et al. 2014; Katsumiti et al. 2015; Navarro et al. 2015). Although various surfactants (Triton x, Tween) are commonly applied to stabilize metal NP their toxicity upon different organisms still lacking (Kvıtek et al 2008).

Coating agents are chemicals (such as polymers and surfactants) used in the synthesis of NPs to prevent their aggregation through electrostatic repulsion, steric repulsion or both (Phenrat et al. 2008; Hotze et al. 2010). In the case of silver, the most prevalent capping agents are citrate and polyvinylpyrrolidone (PVP) (El Badawy et al. 2011). The PVP-AgNPs are stabilized through the steric repulsion caused by the adsorption of PVP on the particle surface (Zhang and Zhang, 2014). The PEI-Ag NPs are electrosterically stabilized due to the adsorption of the PEI on the particle surface (El Badawy et al. 2010). In general, no toxic effects have been previously reported for PVP upon marine microalgae (Moreno-Garrido et al. 2015) while a clear toxic effect has been reported for PEI for different organisms (El Badawy et al. 2011; Ivask et al. 2014). However, to the best of our knowledge, there is no literature available upon the use of these two polymers together.

In the present study, algae exposed to the coating agent PVP/PEI alone showed an evident growth inhibition effect similar to that observed for coated NPs, but a different behavior could be distinguished for the three algae. We can speculate that once algae were added to the PVP/PEI suspension, an immediate steric interaction occurred due to the stabilizing nature of the agent itself. This could provoke the inclusion of microalgae in the polymeric matrices with a subsequent clear toxic effect, especially in the case of microalgae without a resistant cell wall. So it could be concluded that the presence of the coating agents could influence the NP toxicity.

4.5 Different algae sensitivity

In marine coastal ecosystems, microalgae play a key role as primary producers and, being at the base of the aquatic food web, any modification of their growth could affect higher trophic levels (Rioboo et al., 2007). Additionally, phytoplankton represents an excellent aquatic model for the study of the effects of pollutant exposure at population level (C. Chen et al. 2012), due to a short generation time and high sensitivities. The evaluation of NP effects upon marine phytoplankton is indeed a necessary step to predict their potential impact on the whole ecosystems they support. Different species of microalgae could show differences in sensitivity towards NP toxicity depending on their structural and physiological characteristics. The selected microalgae belong to different classes *Phaeodactilum tricornutum* (Bacillariophyceae) is a widespread pennate diatom (Rushforth et al. 1988; Francius et al. 2008) commonly used for assessing effects of NPs (Baker et al. 2014; Castro-Bugallo et al. 2014; Moreno Garrido et al. 2015). The cell wall is formed by two valves overlapping in the girdle band region but in opposition to other diatoms, *Phaeodactylum* is very poor in silica, being the cell wall essentially composed of organic compounds. *Tetraselmis suecica* is an elliptical microalgae of the *Chlorophyceae* (*Prasinophyceae*) class ranging up to 12 µm in length while *Isochrysis galbana* (*Prymnesiophyceae*)is the smallest (4-7 µm) among the three algae tested in this study and is widely cultured to feed bivalves in the aquaculture industry. Both algae show a peculiar cell wall structure: *T. suecica* is enclosed in a rigid polysaccharidic cell wall or theca (Becker et al. 1994) while *I. galbana* has a relatively soft cell coating composed of coalesced carbohydrate scales (Zhu and Lee, 1997). Although both algae are generally used as model organisms for toxicity assessment of NPs (Miller et al. 2012; Minetto et al. 2014). The results of this thesis (chapter 4) showed that these three microalgae differed in sensitivity toward tested NPs; *T. suecica*, *I. galbana* and *D. tertiolecta* were more sensitive than *P. tricornutum*. The higher sensitivity of *I. galbana*, *D.*

tertiolecta and *T. suecica* could be due to their cell wall characteristics. These algae have no distinct or mineralized cell walls (Throndsen et al. 1993; Zhu et al. 1997) and therefore, they may be more vulnerable compared to other taxa with different cell wall properties. Thus, the resistant cell wall of *P. tricornutum* seemed to limit the toxic effects of NPs and of the coating agent. see cap. Additionally, small microalgae have been found to be more sensitive than diatoms (Helbling et al. 1994; Karentz et al. 1994; Holm-Hansen et al. 1997). This higher sensitivity has been related to their larger surface area to volume ratio compared to larger microalgae (Quigg et al. 2006; Levy et al. 2007). Moreover, due to their dimensions, small microalgae may be covered faster by NPs. Finally, algae motility is a key process that should be taken into account: it allows a greater interaction between algae and NP aggregates but it could also be a very sensitive process that could be affected. Mobile algae cells are widely distributed in culture medium, thus increasing their contact time with NP aggregates, with respect to immobile diatoms that tend to settle at the bottom of the well (Zhou et al. 2015).

4.6 Suitability of genotoxic and cytotoxic assays to evaluate NP toxicity mechanisms

The action of nanoparticles (NPs) upon microalgae is usually evaluated by parameters that integrate and reflect sublethal effects at population level such as growth rate, biomass, chlorophyll fluorescence and primary production (Aruoja et al., 2009; Ji et al., 2011; Chen X. et al. 2012; Ma et al., 2013). However to shed light on the NP mode of toxic action it could be useful to combine the investigation of the cellular response, as cell viability, ROS production, with genotoxicity as DNA damage (Dalai et al., 2013; Gunawan et al., 2013; Bhuvaneshwari et al., 2015; Demir et al., 2014; Golbamaki et al., 2015; Suman et al., 2015). The results of this thesis evidenced the usefulness of considering different parameters and endpoint (Cell viability, Oxidative stress, DNA damage, algae population growth inhibition) to understand the mechanisms behind NP toxicity. By carrying out different assays, it could be possible to observe not only the effects but even all the cascade events that conduct to the toxicity.

In this thesis for the first time the evaluation of NP genotoxicity was performed using the comet assay upon microalgae cells. Due to a high growth rate, phytoplankton offers the possibility to study the trans-generational effects of NPs exposure. With a view to obtaining a better insight into the long-term consequences of genotoxicity at population level, it appeared so valuable to evaluate the genotoxicity of different NPs on microalgae through comet assay.

Despite other genotoxicity tests (e.g. micronucleus test), the comet assay is applicable to any kind of eukaryotic cell and it is independent of cell proliferation or cell cycle status. This method, generally used to test NPs, as well as other genotoxic agents (Kumar et al., 2011; Shukla et al., 2011) is a suitable tool for measuring primary DNA damage also in microalgae (Akcha et al., 2008; Prado et al., 2009).

From the genotoxic evaluation herein performed it could be established that the Comet Assay is a useful method to assess not only the NP genotoxicity but also to understand their mode of action.

Table 1. NPs physic-chemical characterization

NPs	[NPs] (mg/L)	Size (nm)	Z-pot (mV)	pH	Ion release (mg/L)
SiO_2	125	1300 ±100	-12.15±0.63	7.8	negligible
	200	1800±180	-10.31±0.81	7.9	negligible
TiO_2	7.5	1300±110	-10.7±0.28	8.0	negligible
	20	1350±115	-9.4±0.35	8.0	negligible
ZnO	1	470±45	-10.35±0.83	8.0	--
	5	1040±70	-10.51±1.43	8.0	
	10				3.5±0.5
PVP/PEI Ag 5nm	10	100	-5	7.8	2
Ag 47 nm	10	600	-8	7.8	1.52

5. Conclusion

In conclusion it could be assumed that many different factors should be taken into account in the understanding the mechanisms underlying NP toxicity.

The NP ecotoxicity could not be only ascribed to ion release but it is also dependent to the NP behavior (aggregation trends, sedimentation process) together with all the transformation that could have place in a complex matrix like seawater.

In fig, 1 the mode of action of ZnO, SiO_2 and TiO_2 are graphically summarized on the bases of results presented in this thesis. ZnO NP exerted its toxic action upon algae by a punctual and continuous ion release from aggregates in proximity of algae cell wall. The first interference was at level of the regulation of cell division then resulting in the inhibition of algae population growth while DNA molecule structure and vitality parameters were compromised only at increasing concentration (5 mg Zn/L and 10 mg Zn/L respectively). The comparison with SiO_2 and TiO_2 toxicity pattern allowed highlighting different pathways leading to the algae population growth inhibition. For SiO_2 a cascade of effects (ROS production–DNAdamages– growth inhibition) was evidenced suggesting a toxicity starting from oxidative stress generation. TiO_2 instead firstly act on DNA structure and, being not soluble in seawater, an internalization during cell division or cell wall destruction could occur together with the activation of cellular signals destabilizing DNA structure. In figure 2 the results obtained for Ag NP are summarized In the case of the Ag NP Ag NPs of 47 nm resulted less toxic than PVP/PEI coated Ag NPs of 5 nm for the three microalgae species. This is possibly related to the fact that 5 nm Ag NPs formed smaller aggregates that could be more available to cells when compared to aggregates of 47 nm Ag NPs. An additional reason could be that PVP/PEI 5 nm Ag NPs released around 10 times more Ag ions than 47 nm Ag NPs. PVP/PEI alone was also toxic to algae indicating that it contributed significantly to the toxicity of PVP/PEI 5 nm Ag NP suspensions. It could be speculated that the aggregation and the dissolution play a key role in the toxicity thus microalgae surfaces that appeared entrapped in a network of large heteroaggregates. It seems that aggregates covering cell surfaces can induce a certain inhibition of the photosynthetic activity of microalgae due to the reduction of light availability. However the mechanisms leading to observed inhibitory effects on algae growth remain to be explored and need further studies.

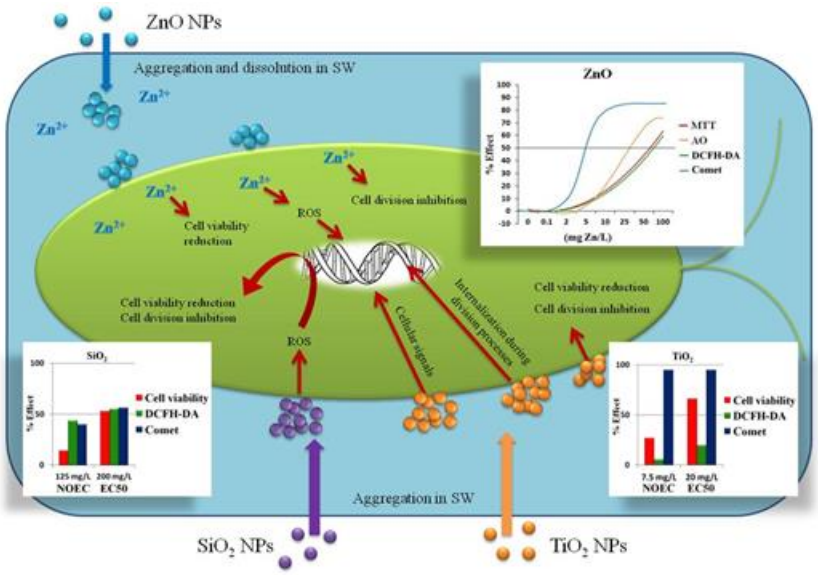

Figure 1: The diverse mode of action of ZnO NPs, SiO_2 and TiO_2 upon microalgae

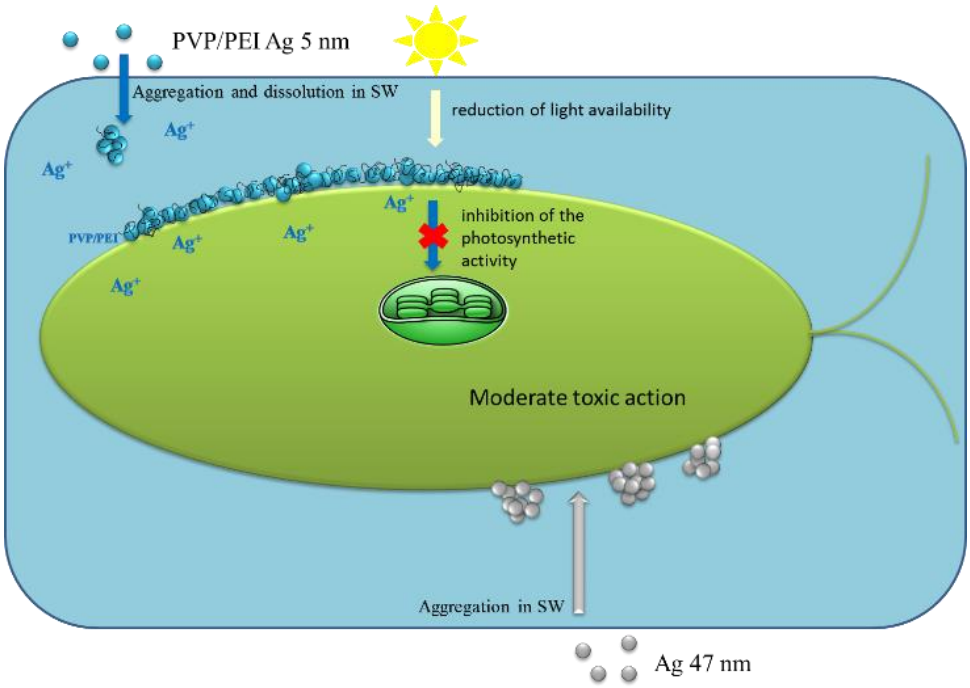

Figure 2: Toxic effect of PVP/PEI Ag NP 5 nm and Ag 47 nm upon microalgae

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Appendix 1 List of publications and participation in conferences

1. Graziani G, **Schiavo S**, Nicolai MA, Buono S, Fogliano V, Pinto G, Pollio A (2013) Microalgae as human food: Chemical and nutritional characteristics of the thermo-acidophilic microalga *Galdieria sulphuraria*. Food and function DOI: 10.1039/c2fo30198a

2. Manzo S, **Schiavo S,** Aleksi P Tabaku A (2014) Application of a toxicity test battery integrated index for a first screening of the ecotoxicological threat posed by ports and harbors in the southern Adriatic Sea (Italy). Environmental Monitoring and Assessment 186 (11). DOI: 10.1007/s10661-014-3915-2.

3. Manzo S, Ansanelli G, Parrella L, Di Landa G, Massanisso P, **Schiavo S**, Minopoli C, Lanza B, Boggia R, Aleksie P and Tabaku A (2014) First evaluation of the threat posed by antifouling biocides in the Southern Adriatic Sea. Environmental Science Processes & Impacts, 16, 1981

4. Manzo S, Ansanelli G, Monetti F, Parrella L, Di Landa G, Massanisso P, **Schiavo S**, Minopoli C, Lanza B, Rimauro J, Salluzzo A, Aleksi P & Tabaku A (2014) CARISMA Project: an example of integrated approach in the study of the adverse effects posed by antifouling agents in the Southern Adriatic Sea. ENEA Journal Energia ambiente e innovazione. Pag 80-88. DOI: 10.12910/EAI2014-49.

5. Manzo S, Buono S, Rametta G, Miglietta M, **Schiavo S**, Di Francia G (2015) The diverse toxic effect of SiO_2 and TiO_2 nanoparticles toward the marine microalgae *Dunaliella tertiolecta*. Environ. Sci. Pollut. R DOI:10.1007/s11356-015-4790-2.

6. Tammaro M, Salluzzo A, Rimauro J, **Schiavo S**, Manzo S (2016) Experimental investigation to evaluate the potential environmental hazards of photovoltaic panels. J Hazard Mater 306:395-405. doi:10.1016/j.jhazmat.2015.12.018.

7. **Schiavo S,** Oliviero M, Miglietta M, Rametta G, Manzo S (2016) Genotoxic and cytotoxic effects of ZnO nanoparticles for *Dunaliella tertiolecta* and comparison with SiO_2 and TiO_2 effects at population growth inhibition levels. Sci Total Environ 550:619–627

8. Li J, **Schiavo S,** Rametta G, Miglietta ML, La Ferrara V, Wu C, Manzo S (2016) Comparative toxicity of nano ZnO, bulk ZnO and Zn salt towards marine algae *Tetraselmis suecica* and *Phaeodactylum tricornutum*. Environmental Science and Pollution Research **(Under review)**.

9. **Schiavo S**, Duroudier N, Bilbao E, Mikolaczyk M, Schäfer J, Cajaraville MP & Manzo S. 2016. Growth inhibition of three species of marine microalgae exposed to different sizes of Ag NPs and to coating agent PVP/PEI. Aquat Toxicol. **(Under review).**

10. Li J, **Schiavo S**, Wu B, Yao W, Rametta G, Miglietta ML, Wu C, Manzo S (2016) Tissues zinc uptake and histological damages of Mediterranean mussel exposed to ZnO nanoparticles. (Submission reparing).

11. Li J, **Schiavo S**, Dong X, Rametta G, Miglietta ML, Wu C, Manzo S (2016) Active expression of DNA damage-responsive genes and antioxidant enzymes genes in Mytilus galloprovincialis exposed to ZnO nanoparticles. (Submission preparing).

12. Li J, **Schiavo S**, Dong X, Wu C, Manzo S (2016) Active expression of DNA damage-responsive genes and antioxidant enzymes genes in *Mytilus galloprovincialis* exposed to Zn salt. (Submission preparing).

1. Manzo S., Ansanelli G., Chiavarini S., Di Landa G., Lanza B., Massanisso P., Monopoli C., Parrella L., Salluzzo A., **Schiavo S**., Tabaku A., Aleski P., Lazo P. Caratterizzazione (chimico fisica ecotossicologica) ed analisi rischio ecologico di biocidi antivegetativi nel sud del Mar Adriatico Progetto CARISMA. Ricerca e applicazione di metodologie ecotossicologiche in ambienti acquatici e matrici contaminate - **Giornate di Studio - 5a edizione, Livorno; 11/2012**

2. Manzo S., Ansanelli G., Di Landa G., Salluzzo A., Minopoli C., Lanza B., Rimauro J., Massanisso P., Parrella L., **Schiavo S.,** Aleski P., Tabaku A., Monetti F. Il progetto carisma: un esempio di monitoraggio ambientale integrato per la definizione del rischio ecologico da agenti antivegetativi. ISPRA - **Giornate di Studio 6° Edizione – Ricerca e applicazione di metodologie ecotossicologiche in ambienti acquatici e matrici contaminate, Livorno (Italy); 11/2014**

3. Manzo S., Cirino P., **Schiavo S.**, Oliviero M., Ciaravolo M., Paglialonga A. I ricci di mare nella ricerca ecotossicologica, possibili strategie per la disponibilità continua di gameti. Risultati preliminari. ISPRA - **Giornate di Studio 6° Edizione – Ricerca e applicazione di metodologie ecotossicologiche in ambienti acquatici e matrici contaminate, Livorno (Italy); 11/2014**

4. **Schiavo S.,** Duroudier N., Bilbao E., Cajaraville M. P. and Manzo S. Conference paper and poster: Growth inhibition of three species of microalgae exposed to different sizes of Ag NPs and to coating agent PVP/PEI. **18 th International Symposium on Pollutant Responses In Marine Organisms (PRIMO) Norway 24-27 May 2015.**