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Interaction of CdSe/ZnS quantum dots with the marine diatom *Phaeodactylum tricornutum* and the green alga *Dunaliella tertiolecta*: A biophysical approach

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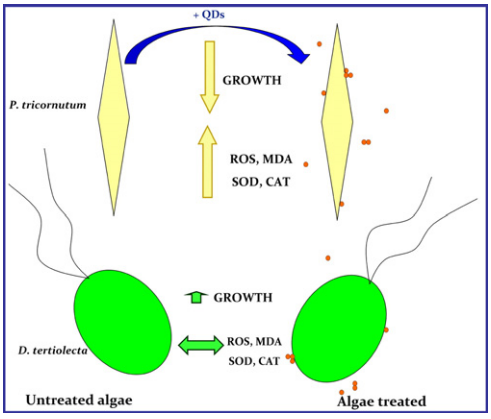
HIGHLIGHTS

- CdSe/ZnS QDs dispersed in seawater exhibited reduced fluorescence emission intensity.
- Confocal microscopy highlighted the presence of QDs on the surface of marine microalgae.
- QDs inhibited the growth rate and increased oxidative stress in *Phaeodactylum tricornutum.*
- QDs slightly stimulated the growth and did not cause oxidative stress in *Dunaliella tertiolecta.*

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, we investigated the interaction of nanoparticles, such as CdSe/ZnS quantum dots (QDs), with the marine diatom *Phaeodactylum tricornutum* and the green alga *Dunaliella tertiolecta*, as biological models in the marine environment. Fluorescence kinetics measurements indicated that 30 min after dispersion in seawater QDs lost the 60% of the initial emission intensity, possibly due to the occurrence of aggregation processes. However, the presence of algae seemed to mitigate this effect. By using confocal microscopy, we highlighted the presence of QDs adsorbed on the surface of both algae, but not inside the cells. The toxicity of QDs was evaluated in terms of inhibition of growth rate, oxidative stress, and lipid peroxidation. QDs in the range of 1–2.5 nM gradually inhibited the growth rate of *P. tricornutum* and increased the oxidative stress, as evinced by the increase in lipid peroxidation, reactive oxygen species (ROS) production and activity of two main antioxidant enzymes (superoxide dismutase and catalase). On the contrary, QDs did not inhibit the growth rate of *D. tertiolecta*, at most a modest stimulation was observed in the range of 0.5–2 nM, suggesting a hormetic response. No effect in the parameters indicating oxidative stress was observed in the green alga. In conclusion our results showed that the biological effects were species-specific.

1. Introduction

Semiconductor nanocrystals or quantum dots (QDs) constitute a promising class of nanomaterials due to their unique size-tunable

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optical properties. QD technology is growing rapidly and it is expected that in the period 2011–2020 its application market will undergo an annual increase of 55% [1]. Three major applications of QDs are now under investigation: as novel materials for photovoltaic technologies, light emitting devices and biological imaging [2]. Owing to their potential increase in the industrial production, new fundamental questions about their environmental fate should be addressed. Since it can be expected that these nanomaterials will be discharged, directly or indirectly, in the aquatic environment, there is an urgent need to understand ecotoxicology of these nanoparticles in natural ecosystems and their effects on aquatic organisms at various trophic levels. Phytoplankton represents the first link of the food chain in natural waters, hence, the knowledge of mechanisms of uptake, accumulation and toxicity of QDs in these unicellular organisms represents a crucial point for understanding their ecotoxicity. Despite the importance of this issue, little is known about the interaction of microalgae with these nanoparticles in the marine environment. Furthermore, the study of the interaction between nanoparticles and living organisms cannot be separated from the knowledge of the chemical behavior of these nanoparticles in a complex medium, such as seawater. The stability of QDs in natural waters is related to their physicochemical properties, such as size, charge, coating, concentration, as well as to environmental factors, such as salinity, the presence of natural organic matter, the solar irradiation [3]. It is expected that the high ionic strength of seawater can effectively shield the repulsive interaction among charged nanoparticles, thereby favoring aggregation processes. In recent years several papers have appeared on the toxicity of QDs to aquatic model organisms, including the freshwater invertebrates *Hydra vulgaris* [4] and *Daphnia magna* [5], zebrafish [6], and the unicellular protozoan *Tetrahymena thermophila* [7]. Few studies focus on the toxicity of QDs to microalgae, showing the induction of oxidative stress [8] and a QD-specific alteration of the transcriptomic profile [9] in the freshwater green alga *Chlamydomonas reinhardtii*. In a study carried out with different strains of freshwater green algae, Worms et al. (2012) highlight the fundamental role of the cell wall in modulating the interaction microalgae-QDs and the capability of the nanoparticles to act as carrier of toxic metals [10]. On the other hand, Zhang et al. (2012) report that extracellular organic matter released by a variety of unicellular marine algae can affect QD stability in a species-specific manner [11]. The interaction between microorganisms and nanoparticles seems to be reciprocal because nanoparticles can affect biological functions, but living organisms can induce transformations in nanoparticles which, in turn, can affect their bioavailability and toxicity. In the present study we investigated the interaction between CdSe/ZnS QDs and two marine microalgae belonging to different phyla taken as models of biological receptors in the marine environment: the diatom *Phaeodactylum tricornutum*, which is characterized by the presence of a rigid siliceous cell wall, and the green alga *Dunaliella tertiolecta*, which lacks a cell wall and is coated with a mucilaginous envelope. Diatoms and green algae are present in a variety of marine habitats and are often the most abundant phytoplanktonic organisms in marine waters.

The chemical behavior of QDs was first studied by fluorescence techniques, to get information on the aggregation state of the nanoparticles in seawater. QDs have been proposed as fluorescent labels for biological imaging, thus, we took advantage of their optical properties and used fluorescence confocal microscopy to demonstrate whether QDs can enter into contact with the cells. Biological effects have been investigated in terms of growth rate inhibition and oxidative stress. In particular, we measured the production of reactive oxygen species (ROS), the activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR). Finally, we tested the level of malondialdehyde (MDA) as an index of membrane lipid peroxidation.

2. Materials and methods

2.1. Reagents

Lumidot® CdSe/ZnS, orange quantum dots emitting at 590 nm (5 mg ml^{-1} in toluene), were purchased by Sigma-Aldrich and transferred into aqueous media by encapsulating with the amphiphilic polymer poly(styrene-*co*-maleic anhydride) terminated with cumene (PSMA) and ethanolamine, by following the procedure reported by Lees et al. (2009), with some modifications [12]. The detailed preparation together with the chemical and optical characteristics of water-soluble QDs has been described elsewhere [13]. QD concentration was measured spectrophotometrically using the molar absorption coefficient, $\varepsilon_{569} = 1.6 \times 10^5\ M^{-1}\times cm^{-1}$, provided by the manufacturer. Stable suspensions of water-soluble QDs were stored in the dark at +4 °C for a maximum of 3 months.

Water was purified by a Milli-Q system from Millipore (Vimodrone, Italy). Seawater was collected in an uncontaminated area, 3 miles offshore from the Island of Capraia (Tyrrhenian Sea, Italy), filtered through 0.45 μm membrane filters and stored in the dark at +4 °C. Membrane filters used throughout the experiments were from Millipore.

2.2. Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) measurements were performed using a Leica TCS SP5 inverted confocal microscope (Leica Microsystems AG, Wetzlar, Germany) equipped with two Single Photon Avalanche Diode detectors interfaced with PicoHarp300 correlation system (Picoquant, Berlin, Germany). Excitation was provided by a 561 nm HeNe cw laser (<100 W/cm^2 at sample) and fluorescence emission was collected in both channels by using a bandpass filter peaked at 645 nm and possessing a bandwidth of 75 nm. In all cases, QD solutions in water were freshly prepared and filtered through a 0.2 μm filter before FCS analysis. 30 measurements of 60 s each were performed.

Fluctuation data were analyzed in cross-correlation mode to remove the detectors' afterpulses. Cross-correlation functions were fitted to the analytical autocorrelation accounting for 3D isotropic diffusion of a single moiety [14], as expressed by:

$$G(t) = \frac{1}{\langle N\rangle}\cdot\left(1+\frac{\tau}{\tau_D}\right)^{-1}\cdot\left(1+\left[\frac{r_0}{Z_0}\right]^2\cdot\frac{\tau}{\tau_D}\right)^{-1/2}$$

where τ_D is the characteristic diffusion time during which a molecule resides in the observation volume with an axial (z_0) to lateral (r_0) dimension ratio, and N is the average number of particles in the observation volume. The translational diffusion coefficient D of the QDs was obtained following:

$$\mathrm{D} = \frac{r_0^2}{4\tau_D}.$$

Autocorrelation data fitting was carried out on either the Picoquant Symphotime software or by a code written for IgorPro software (Wavemetrics, Oswego, OR, USA). Cross-correlation traces were fitted for $\tau > 5$ μs, to focus on the sole diffusive behavior of QDs.

2.3. Fluorometric measurements

Fluorescence measurements were performed on CdSe/ZnS QDs in both deionized water and seawater using a Fluoromax-4 Spectrofluorometer (Horiba Jobin Yvon, Milano, Italy). Emission spectra were obtained by exciting the sample at 450 nm. Excitation and emission bandwidths were 5 nm. All spectra were corrected for the instrumental response. Kinetics of QD fluorescence changes in seawater were followed at 590 nm for 30 min, recording every 30 s. A small aliquot of QDs was added under stirring to 3 ml of seawater alone or containing

P. tricornutum or *D. tertiolecta* cells in a cuvette with an optical path of 1 cm. The final CdSe/ZnS QD concentration was 9 nM and the cell density was 10^6 cells ml^{-1}.

2.4. Cultures

The unicellular marine diatom *P. tricornutum* (Bohlin), and the green alga *D. tertiolecta* were obtained from the Culture Collection of Algae and Protozoa, Dunstaffnage Marine Laboratory, UK. Stock cultures were grown in axenic conditions, at 21 ± 1 °C and fluorescent daylight (100 μmol photons$\times m^{-2}\times s^{-1}$) in a 16:8 light–dark cycle. Culture medium was natural seawater enriched with f/2 medium [15] modified as to obtain a f/10 medium for trace metal concentrations. Exponential growth was maintained by inoculating cells into a fresh sterilized medium, weekly.

Exposure experiments were carried out by inoculating algae from a stock culture (on day 7 of growth) to a fresh medium at an initial cell density of 5×10^4 cells ml^{-1}. Aliquots of 500 ml of this culture were spiked with suitable amounts of CdSe/ZnS QDs, as to obtain a final range of QDs from 0.5 to 2.5 nM. The cultures were allowed to grow for 4 days during the exponential phase. Cell density was measured at the fourth day using a hemocytometer under a microscope or, alternatively, by recording the absorbance of chlorophyll at 680 nm (JASCO V-550 UV/Vis Spectrophotometer, Lecco, Italy). Finally, the growth rate, expressed as doublings day^{-1}, was calculated. In the exposure experiments, a control culture (no QDs added) was always used. Three replicates of the exposure experiments were carried out.

At the end of the exposure, algae from each culture were collected by filtration on 1.2 μm membrane filters (*P. tricornutum*) or by centrifugation at 7000 ×*g* for 10 min (*D. tertiolecta*). In both cases, algae were washed two times with natural seawater, either by performing two successive rinsing of the filter or by re-suspension in seawater and subsequent centrifugation. This procedure was adopted to remove the exposure medium containing QDs. Finally, the harvested algae were re-suspended in 20 ml of seawater, thereby obtaining concentrated algal suspensions, which were subdivided in the following aliquots: 1 ml was used for the measurement of the ROS production, 4 ml were used for the measurement of MDA and the remaining 15 ml for the measurement of the antioxidant enzymatic activities. Each aliquot was filtered or centrifuged again, the medium was discarded and thereafter the algae from 1-ml aliquots were immediately used for ROS measurements, while algae from the 4- and 15-ml aliquots were stored at −70 °C, until analysis.

2.5. Determination of ROS

The production of ROS was measured by following the cellular conversion of the non-fluorescent 2′,7′-dichlorofluorescein diacetate (DCF-DA) to the highly fluorescent compound dichlorofluorescein (DCF) as described by Wang and Joseph (1999) [16]. Harvested algae were transferred to a solution of 10 μM DCF-DA in seawater (3 ml) and left to react for 1 h. The reaction was stopped by filtration or centrifugation, rinsing and re-suspending algae in natural seawater (3 ml). Fluorescence was then determined at 485 nm excitation and 520 nm emission wavelength. Fluorescence emission intensity was normalized to cell density (cell number ml^{-1}) in the cuvette.

2.6. Enzyme assays

The harvested algae were placed in 2 ml of extraction buffer containing 50 mM sodium phosphate buffer (pH 7.0), 1 mM EDTA (ethylenediaminetetraacetic acid, disodium salt), 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1 mM L-ascorbic acid and immediately disrupted by sonication (Sonopuls Ultrasonic Homogenizer, Bandelin) for 3 min with a repeating duty cycle of 0.3 s, in an ice bath. The cell homogenate was centrifuged at 20,000 ×*g* for 30 min at 4 °C and the supernatant was used to measure enzymatic activities.

The activity of SOD was assayed by measuring its ability to inhibit the photoreduction of nitro blue tetrazolium (NBT), according to the method of Beyer and Fridovich (1987) [17]. In this assay, one unit of SOD is defined as the amount required to inhibit the photoreduction of NBT by 50%. The activity of CAT was measured according to Aebi (1974) [18], by monitoring the decomposition of H_2O_2. The enzyme activity was calculated by measuring the decrease in absorbance at 240 nm for 1 min ($\varepsilon = 0.04\ mM^{-1}\times cm^{-1}$). APX activity was determined by following the decrease in absorbance at 290 nm ($\varepsilon = 2.7\ mM^{-1}\times cm^{-1}$) due to ascorbate oxidation [19]. GR activity was determined by following the decrease in absorbance at 340 nm ($\varepsilon = 6.2\ mM^{-1}\times cm^{-1}$) due to the glutathione-dependent NADPH oxidation, according to the modified method of Foyer and Halliwell, 1976 [20]. In the case of CAT, APX and GR, one unit of activity (U) was defined as the amount of enzyme that can transform 1 μmol of substrate per minute. Further experimental details are reported elsewhere [21]. All enzymatic activities were calculated per mg of protein. The protein content in the cell extract was determined according to Bradford (1976) [22] using bovine serum albumin as standard. Enzymatic assays were carried out at 25 °C using the JASCO V-550 UV/Vis Spectrophotometer.

2.7. Lipid peroxidation

The level of lipid peroxidation products in cells of *P. tricornutum* or *D. tertiolecta* was determined by following the method of Heath and Packer (1968) [23], with some modifications as previously described [21]. Briefly, disrupted algae were left to react with thiobarbituric acid and the concentration of malondialdehyde (MDA), which is a secondary end-product of the oxidation of polyunsaturated fatty acids, was measured spectrophotometrically. The absorbance at 532 nm was corrected for non-specific absorption at 600 nm and the MDA concentration was calculated by using an extinction coefficient of 155 $mM^{-1}\times cm^{-1}$.

2.8. Confocal microscopy

Imaging of *P. tricornutum* or *D. tertiolecta* cells was carried out using a Leica TCS SL inverted laser scanning confocal microscope (Leica Microsystems AG, Wetzlar, Germany) interfaced with an Ar laser for excitation at 488 nm and a HeNe laser for excitation at 543 nm. A 63 × 1.4 NA numerical aperture oil immersion objective (Leica Microsystems) was adopted. Acquisition parameters were: excitation power 50–200 W at objective and 800 Hz line scanning speed. Transmission images were obtained in differential image contrast mode (Normaski image) by using the same laser source. All images were 512 × 512 pixels in size. Pseudocolors were applied to indicate chlorophyll of microalgae (red) and QDs (green), respectively.

3. Results and discussion

3.1. Fluorescence correlation spectroscopy in deionized water

To characterize the CdSe/ZnS QDs in terms of nanoparticle size and sample heterogeneity we measured the QD hydrodynamic radius (R_h) by using fluorescence correlation spectroscopy (FCS). This technique measures the emission fluctuations generated by fluorescent compounds/particles that diffuse in and out the small illuminated region produced in the confocal microscopy set-up [14]. The temporal pattern of fluorescence fluctuations can be properly analyzed by correlation methods. In more details, the temporal autocorrelation G(t) of the recorded signal is computed and the autocorrelation trace gives access to the diffusional properties of the fluorescent moieties and their concentrations [14]. We should note that reliable G(t) traces

are obtainable only for low concentrations of the fluorescent species, as in such a case large amplitudes of fluctuations (and therefore of G(t)) are detected and proper fitting can be carried out.

Water suspensions of QDs with 1–10 nM concentrations were analyzed by FCS in cross-correlation mode (FCCS) to avoid the influence of detectors' afterpulses on the correlation trace [24]. Low power (<100 W/cm^2 at sample, excitation 561 nm) was always ensured to avoid significant presence of fast and complex photophysical processes typical of QDs [25]. Adopting a simplified perspective, G(t) was fitted to the analytical equation describing the isotropic 3D diffusion of a particle (Fig. S1, Supplementary materials). Notably, we obtained an average diffusion time $t_d = 0.62 \pm 0.11$ ms (30 FCS runs), corresponding to a translational diffusion coefficient $D = 25.0 \pm 4.0$ μm^2/s taking into account the radial size of the confocal spot (0.25 μm). For a rigid sphere, D can be linked to the sphere radius by the Stokes–Einstein relation $D = KT / (6\pi\eta R_h)$. Under this crude assumption, we calculated $R_h = 8.5 \pm 1.5$ nm (T = 293 K, η = 1 cP), a value in good agreement with the expected size of a single QD, thus indicating the minor relevance of large QD aggregates (if present).

3.2. Short-term effect of seawater and microalgae on the fluorescence of QDs

The fluorescence properties of CdSe/ZnS QDs were characterized in seawater in the absence and in the presence of algal cells. Fig. 1A shows that the spectrum shape of QDs in seawater is very similar to that in deionized water with a maximum of the emission at 590 nm. This result suggests that the transfer in seawater does not affect the core size of the nanoparticles. Fluorescence spectra of algae before and after addition of QDs to the culture medium were also measured. Fig. 1B and C shows that, in the absence of QDs, the algae emission was peaked at 680 nm, as expected for chlorophyll *a* emission. On the other hand, after addition of QDs, the spectra exhibited a second fluorescence peak at 590 nm, due to the emission of QDs. The fact that the fluorescence spectrum of QDs is well separated from the one of chlorophyll, allowed to investigate the effect of the presence of algae on the fluorescence intensity of QDs. For this purpose, we followed the kinetics of the change in the fluorescence intensity at 590 nm of a dispersion of QDs in seawater in the absence and in the presence of algae. Fig. 2 shows a sharp decrease in fluorescence intensity when QDs were dispersed in seawater, whereas no change in the emission occurred in deionized water. Thirty minutes after QDs addition in seawater, the fluorescence intensity was reduced by 60% as compared to the initial value. In a previous paper [13], we demonstrated that CdSe/ZnS QDs were almost stable, largely not degraded, 6 h after dispersion in seawater, but underwent an aggregation process. Thereby we can hypothesize that a major cause for the rapid decrease in the emission intensity at 590 nm can be a fluorescence quenching due to the aggregation of nanoparticles, and not to their degradation. However, since the presence of ions in solution may cause changes in the surface states of QDs, possibly affecting their fluorescence quantum yield, a decrease of fluorescence intensity due to the high salinity cannot be excluded [26]. Our results are in agreement with those of Rochira et al. (2007) [27], which show that, at high ionic strength, QD nanoparticles aggregate and the emission fluorescence intensity strongly decreases. Other authors report that fluorescence self-quenching due to the aggregation of QDs is a reversible process, as the disaggregation results in the recovery of the fluorescence intensity [28,29]. In the presence of algae, a smaller decrease in emission intensity with respect to seawater alone was observed. As a matter of fact, in the presence of *P. tricornutum* or *D. tertiolecta* cells (10^6 cells ml^{-1}), the fluorescence intensity after 30 min was reduced, with respect to the initial value, by 50% and 20%, respectively. We attributed this smaller decrease to the interaction with algal cells, which would seem to decrease the aggregation process. When QDs are added to a culture of microalgae, they meet

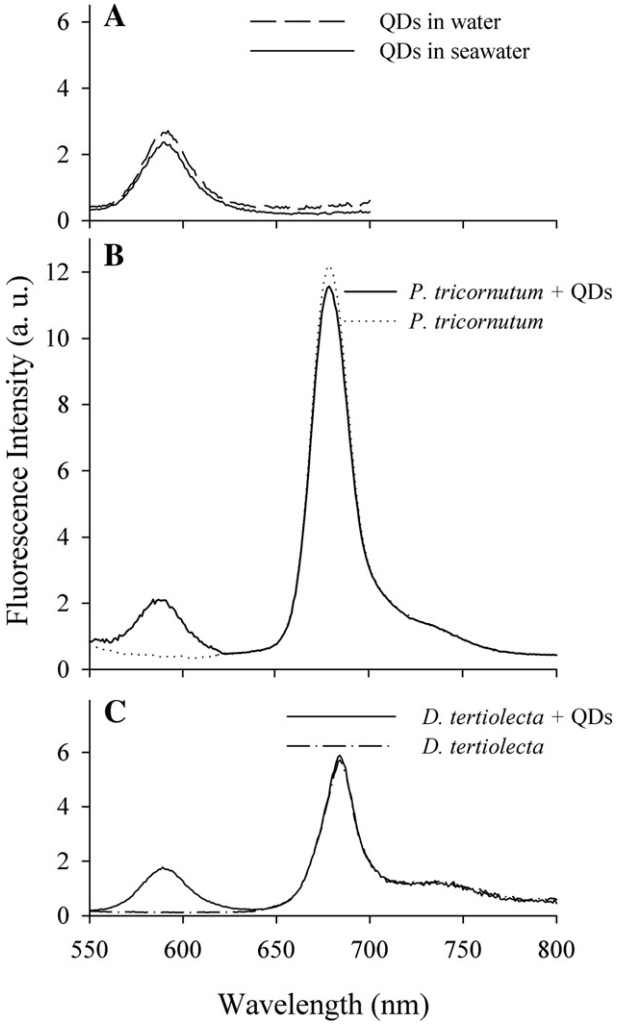

Fig. 1. Normalized fluorescence emission spectra of CdSe/ZnS QDs in deionized water and in seawater (A). Comparison of fluorescence emission spectra of cultures of *Phaeodactylum tricornutum* (B) or *Dunaliella tertiolecta* (C) before and after addition of QDs. [QDs] = 9.2 nM, cell density = 1.4×10^6 cells ml^{-1}. Excitation wavelength was 450 nm.

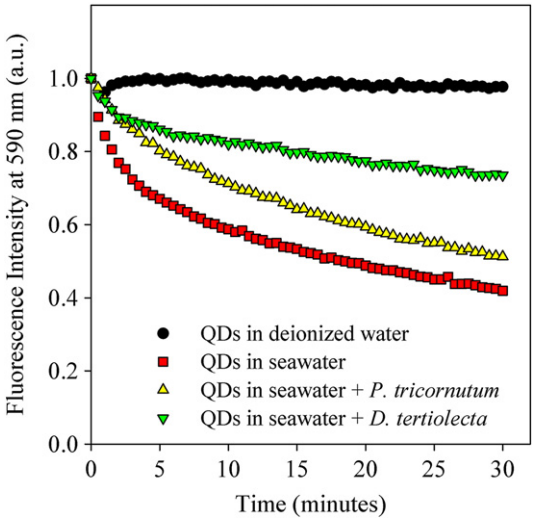

Fig. 2. Kinetics of fluorescence intensity change after QD addition in seawater, in the absence and in the presence of *Phaeodactylum tricornutum* or *Dunaliella tertiolecta* cells. The time-course of fluorescence intensity of QDs in deionized water is shown for comparison. [QDs] = 9.2 nM, cell density = 1×10^6 cells ml^{-1}. Excitation wavelength was 450 nm, emission wavelength was 590 nm.

a complex mixture of cells and their extracellular products, which could modify the QD surface chemistry and alter their intrinsic properties. Recent papers have appeared in the literature on the effect of microalgae and of their extracellular products on the stability of QDs in the marine environment [11,30] and on the fluorescence of QDs under freshwater conditions [31]. All these results suggest the occurrence of biologically-induced alterations of the properties of QDs.

3.3. Confocal microscopy

In order to establish the association with algae, a fluorescence imaging study of *P. tricornutum* (Fig. 3, first column) and *D. tertiolecta* (Fig. 3, second column) cells exposed to 2 nM QDs for 5 h was carried out. The figure shows representative pseudotransmission (DIC) images of algae (Fig. 3A–B), along with confocal fluorescence images at emission typical of QDs (Fig. 3C–D; green pseudocolor scale), and at emission typical of chlorophyll, but not of QDs (Fig. 3E–F; red pseudocolor scale). Remarkably, the confocal fluorescence images show the presence of bright spots in the green channel (signaled by white arrows), located on the cell surface of both microalgae (Fig. 3C–D). These bright spots are not visible in the red channel, although some chlorophyll emission is still visible inside the cell in the green channel, on account of the large concentration of chlorophyll in the chloroplasts. Thus, the bright spots cannot be attributed to cross-talk of chlorophyll emission in the green channel and are likely due to QDs adhered to the surfaces. This finding is strongly supported by the inspection of the emission spectra of the fluorescent spots and of the internal chlorophyll reservoirs measured in confocal spectral mode (Fig. 3G). Clearly, the spots' emission resembles closely the spectrum of CdSe/ZnS QDs, with an emission at 590 nm (Fig. 3H). Instead, fluorescence spectra measured at the level of chloroplasts did not evidence peaks at 590 nm. These data indicate that QDs interact with *P. tricornutum* and *D. tertiolecta* cells by binding to the cell surface, thus providing evidence of a direct contact with the cell. Under our experimental conditions, we were not able to detect fluorescent spots inside the cells. Prior studies on imaging of unicellular algae-QDs have shown the occurrence of QDs adsorbed to the algal surface, but, to our knowledge, no study has shown the internalization of intact QDs [32,33]. Instead, the uptake of QDs has been shown in cultured mammalian [34,35] and plant [36] cells, as well as in protozoans [7]. So far, the mechanisms of internalization of nanoparticles are not well known, but it is believed it occurs via endocytosis, at least in most eukaryotic cells [37]. Nevertheless in plants, algae and fungi the cell wall can act as a barrier or a filter for the entry of nanoparticles [38]. In the present paper, we showed the direct contact of QDs with the cell surface, which is preliminary to any effect to the microalgae and does not exclude the possibility of physical or chemical damage to the algal cell.

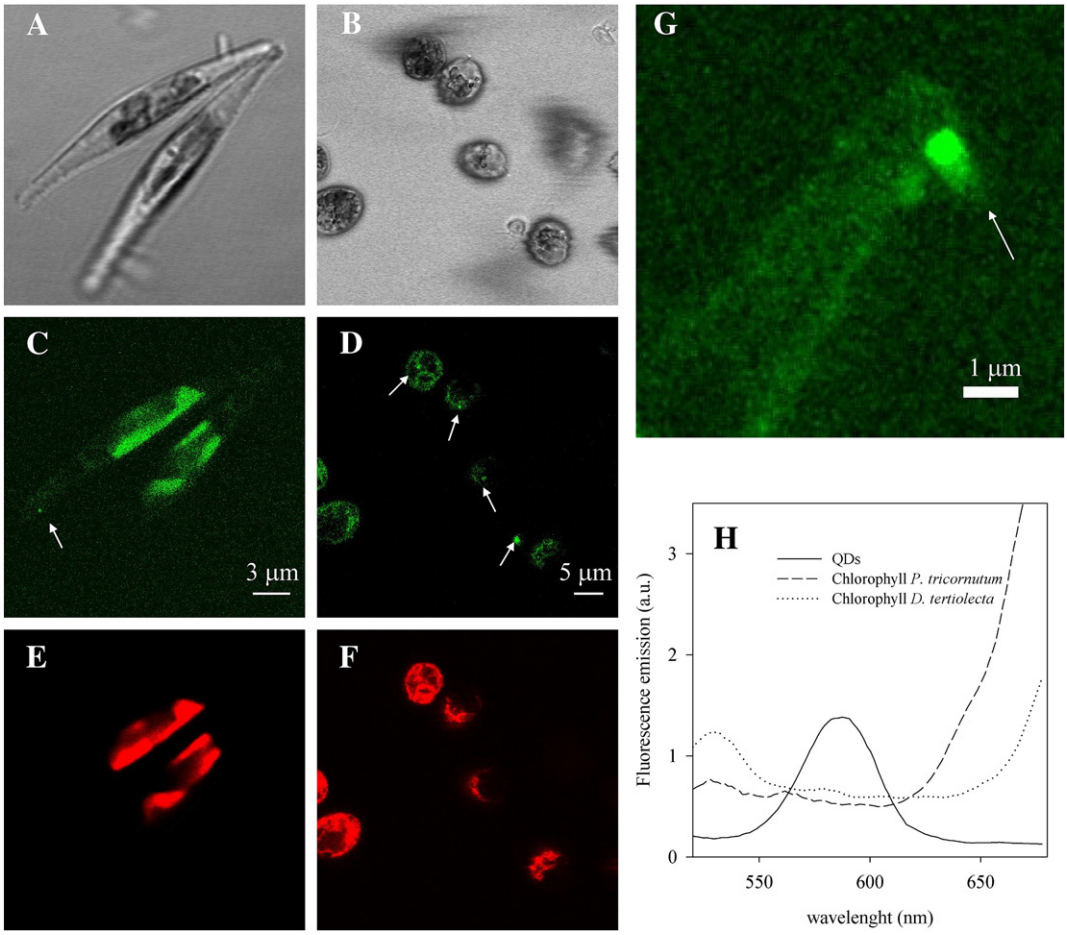

Fig. 3. Confocal micrographs of *Phaeodactylum tricornutum* (panels A, C, E) or *Dunaliella tertiolecta* (panels B, D, F) cells exposed to 2 nM QDs for 5 h. Pseudotransmission images of the cells (A and B); confocal fluorescence images, at λ = 590 nm showing QDs emission (green pseudocolor scale) (C and D); confocal fluorescence images at λ = 680 nm showing chlorophyll emission (red pseudocolor scale) (E and F); detail of the image of QDs adsorbed on a cell of *P. tricornutum*, average projection of a λ series of confocal imaging covering from 500 to 700 nm band width (G). Emission spectra of chloroplasts and of QDs (H). QDs are indicated by a white arrow.

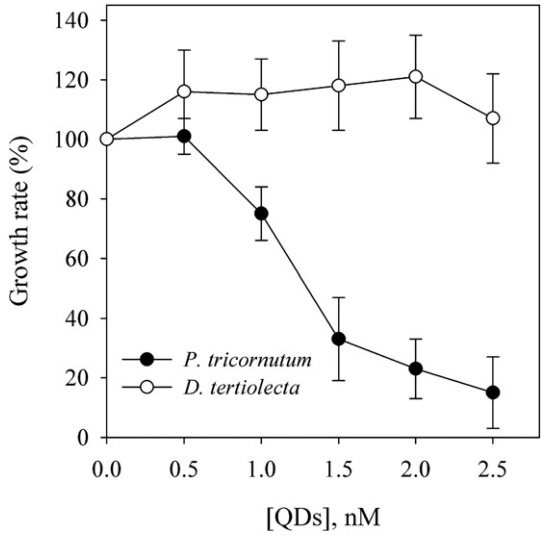

Fig. 4. Percentage of growth rate (μ, doublings day^{-1}) of cultures of *Phaeodactylum tricornutum* or *Dunaliella tertiolecta* exposed for 4 days to increasing concentrations of CdSe/ZnS QDs, with respect to the control culture. Initial cell density = 5×10^4 cells ml^{-1}.

3.4. Effect of QDs exposure on the growth rate

The effect of QDs on the growth rate (μ, doublings day^{-1}) of *P. tricornutum* and *D. tertiolecta* was investigated by growing cells for 4 days in culture media at increasing QD concentrations, in the range from 0.5 to 2.5 nM (initial cell density 5×10^4 cells ml^{-1}). The growth rate of the control culture of *P. tricornutum* was 1.3 ± 0.2 doublings day^{-1} (n = 3). In the range of QDs from 1 to 2.5 nM the growth rate gradually decreased (Fig. 4) and, at the maximum dose ([QDs] = 2.5 nM), the growth rate was 0.22 ± 0.2 doublings day^{-1} (n = 3). It was extrapolated that the 50% inhibition of the growth rate occurred at [QDs] = 1.3 nM. The growth rate of the control culture of the green alga *D. tertiolecta* was 0.57 ± 0.07 doublings day^{-1} (n = 3). No inhibition of growth rate was measured for the entire range of QD concentrations (Fig. 4), rather a stimulatory effect (16% increase of μ) was observed for [QDs] ≤ 2 nM. A similar transient positive effect on growth rate was previously observed in *P. tricornutum* cells exposed to [QDs] ≤ 0.2 nM [13], and was attributed to a hormetic response. This effect is reported in the literature for experiments in vivo with nanoparticles [39], including QDs [7]. Hormesis is considered as a modest stimulation of the affected function (in our case, growth rate) at low doses and inhibition of the same at high doses, and is attributed to an adaptive response to toxicants [40]. Since the hormetic response consists in an overcompensation of the organism to an agent recognized as toxic [41], this response can be considered a signal of cellular stress. This result confirms the occurring of some interaction between algae and QDs.

3.5. Oxidative stress

The above results suggest that nanoparticles, although they can be largely aggregated, interact with algal cells in a manner dependent on the species considered. In order to describe the cellular response to the interaction with QDs, we evaluated the level of cellular ROS, along with other biochemical parameters of oxidative stress such as the level of MDA, and the activity of the main antioxidant enzymes, SOD, CAT, APX, and GR (Table 1). Our results show a dose-dependent increase in ROS production in *P. tricornutum*, but not in *D. tertiolecta* cells. At the maximum QD concentration, the ROS level in *P. tricornutum* was approximately three-fold higher than in the control. In agreement with the higher level of ROS, *P. tricornutum* cells enhanced the activity of two main antioxidant enzymes (SOD and CAT), but not of APX and GR, as already reported for the same alga at lower QD doses [13]. The level of MDA, a biochemical indicator of lipid peroxidation, also increased in concomitance with ROS, thus showing that QDs induced an impairment of the structure and function of the cellular membrane system. On the contrary, no increase in cellular ROS concentration was observed in *D. tertiolecta*, and the large variability of the data hinders further speculations to be done. As expected, the antioxidant enzymatic activities and the MDA levels were not affected in *D. tertiolecta* cells (Table 1). Taken together, our results suggest that the cellular level of ROS is a good indicator of the cellular stress and of the toxic effects of QDs. The occurrence of toxicity due to ROS induced by QDs has been described by other authors in microalgae such as *C. reinhardtii* [8], in bacteria [42], in suspension cultures of *Medicago sativa* [36] and in mammalian cell cultures [43,44].

4. Conclusions

This study provided some important insights on the interaction of QDs with marine microalgae. We demonstrated that the effect of these nanoparticles on toxicity of algae depends on the species investigated. We observed that in *P. tricornutum* the toxic effects, such as inhibition of growth rate and increase in lipid peroxidation, were correlated with the increase in the cellular ROS production and the activation of at least two antioxidant enzymes, SOD and CAT. We can reasonably hypothesize that these effects are due to the simple contact with the cell surface, which could be sufficient to stimulate the production of ROS and subsequent toxicity. The aggregation of

Table 1
Oxidative parameters of *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* grown for 96 h in seawater added with CdSe/ZnS QDs. F_{520} is the DCF fluorescence intensity at 520 nm (arbitrary units) and N is the cell density (cell number ml^{-1}).

[QDs] (nM)	ROS level F_{520}/N	SOD activity U/mg protein	CAT activity U/mg protein	APX activity U/mg protein	GR activity U/mg protein	[MDA] nmol/10^9 cells
P. tricornutum						
0	0.030 ± 0.003	28.62 ± 10	2.74 ± 1.0	0.52 ± 0.12	0.047 ± 0.010	15.4 ± 0.5
0.5	0.033 ± 0.013	30.00 ± 8	2.90 ± 1.1	0.63 ± 0.20	0.062 ± 0.006	22.8 ± 2.3
1.0	0.066 ± 0.017	33.00 ± 5	3.80 ± 0.5	0.24 ± 0.10	0.058 ± 0.007	23.0 ± 4.4
1.5	0.050 ± 0.015	42.96 ± 12	3.75 ± 0.3	0.37 ± 0.03	0.034 ± 0.005	34.9 ± 5.5
2.0	0.055 ± 0.012	59.72 ± 14	5.00 ± 0.6	0.43 ± 0.04	0.026 ± 0.005	31.9 ± 3.2
2.5	0.090 ± 0.008	66.12 ± 10	8.81 ± 0.7	0.57 ± 0.10	0.045 ± 0.009	46.1 ± 5.7
D. tertiolecta						
0	0.39 ± 0.10	28.70 ± 2	12.10 ± 1.8	0.29 ± 0.22	0.220 ± 0.02	47.3 ± 5.9
0.5	0.34 ± 0.13	28.80 ± 2	12.50 ± 2.0	0.42 ± 0.22	0.260 ± 0.20	45.8 ± 11.2
1.0	0.25 ± 0.08	29.70 ± 6	11.80 ± 2.5	0.29 ± 0.14	0.290 ± 0.10	41.8 ± 4.8
1.5	0.27 ± 0.07	30.90 ± 2	12.30 ± 1.6	0.33 ± 0.10	0.250 ± 0.08	42.7 ± 7.6
2.0	0.29 ± 0.12	35.00 ± 6	12.40 ± 0.1	0.31 ± 0.4	0.270 ± 0.13	43.8 ± 6.2
2.5	0.34 ± 0.14	22.60 ± 5	12.40 ± 7.0	0.32 ± 0.4	0.260 ± 0.07	45.2 ± 6.6

the nanoparticles seemed not to hinder the contact with algae. By using confocal microscopy we demonstrated the presence of fluorescent spots of QDs adsorbed on the cell surface of *P. tricornutum* and of *D. tertiolecta*, upon 5 h of exposure. Since the cell wall is the primary barrier with the external medium of unicellular algae, it is supposed to have a fundamental role in protecting cells from internalization of contaminants, including nanoparticles. While *P. tricornutum* has a rigid porous cell wall, with pores large enough to allow the contact of adsorbed QDs with the membrane, *D. tertiolecta* lacks a cell wall and is protected only by a mucilaginous envelop, which could prevent the direct contact with the plasmatic membrane. Our results can be also explained taking into account the behavior of these species in the marine environment. *P. tricornutum*, although is considered a planktonic diatom, can be found in the upper part of sediments together with other benthic species. Indeed this species has been used as a standard organism in marine sediments toxicity tests [45]. On the other hand, *D. tertiolecta* being a biflagellate green alga, is considered a strictly planktonic alga, which lives suspended in the water column. Thus, we can speculate that the propensity of the *P. tricornutum* cells to go down to the bottom of the flask can facilitate their interaction with the aggregates of nanoparticles, likely deposited on the bottom.

For a better comprehension of the impact of nanoparticles in natural marine ecosystems, further investigations are in progress to understand the dynamics of aggregation and/or stabilization of these nanoparticles in cultures of phytoplankton under long term exposure.

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