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Engineered nanoparticles of titanium dioxide (TiO₂): Uptake and biological effects in a sea bass cell line

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ABSTRACT

With the rapid development of nanotechnology there has been a corresponding increase in the application of titanium dioxide nanoparticles (TiO₂-NPs) in various consumer and industrial products, consequently their potential health hazards and environmental effects are considered an aspect of great concern.

In the present study, in order to assess the impact of TiO₂-NPs in the marine environment, the biological effects of TiO₂-NPs on a sea bass cell line (DLEC) were investigated. Cells were exposed for 24 h to different concentrations of TiO₂-NPs (1, 8, 40, 200 and 1000 µg/ml) or co-exposed with CdCl₂ (Cd). The effects of UV light irradiation were also investigated in cells treated with TiO₂-NPs and/or Cd. The internalization of TiO₂-NPs and the morphological cell modifications induced by the treatments were examined by transmission and scanning electron microscopy, this latter coupled with energy dispersive X-ray spectroscopy (EDS) for particle element detection. In addition, the effects of controlled exposures were studied evaluating the cytotoxicity, the DNA damage and the expression of inflammatory genes.

Our study indicates that TiO₂-NPs were localized on the cell surface mainly as agglomerates revealed by EDS analysis and that they were uptaken by the cells inducing morphological changes. Photo-activation of TiO₂-NPs and/or co-exposure with Cd affects ATP levels and it contributes to induce acute cellular toxicity in DLEC cells dependent on Ti concentration. The inflammatory potential and the DNA damage, this latter displayed through a caspase-3 independent apoptotic process, were also demonstrated.

Overall our data suggest that the interaction of TiO₂-NPs with marine water contaminants, such as cadmium, and the UV irradiation, may be an additional threat to marine organisms.

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List of abbreviations: ENPs, Engineered nanoparticles; TiO₂-NPs, titanium dioxide nanoparticles; TiO₂, Titanium dioxide; DLEC, sea bass continuous embryonic cell line; Cd, Cadmium Chloride; EDS, energy-dispersive X-ray; ROS, reactive oxygen species; TEM, Transmission Electron Microscopy; FDA, fluorescein di-acetate; SEM, Scanning Electron Microscopy; HO, Hoechst; Ti, titanium; ATP, intracellular adenosine triphosphate.

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1. Introduction

The production of nanomaterials is increasing worldwide. Engineered nanoparticles (ENPs) are used in diverse industrial fields [26] and new applications are constantly arising [9,71,90,106,109,124]. This means that the environmental contamination with ENPs is becoming a major issue [2,27]. In fact, there is a general concern about the potential hazards posed by released ENPs not only toward humans but also with respect to other organisms present in the environment [8,21,45,48,55,58,87].

Between these materials, the metal oxide nanoparticulate, and in particular the nanoparticles of titanium dioxide (TiO₂-NPs) are among those produced at the highest volume. TiO₂ is a versatile

compound that is used in nano-form in a variety of consumer products, such as sunscreens and other cosmetics [122], specialist coatings and paints [39,56], food-processing technology [90] and in industrial photocatalytic processes [40,132]. Thus it has the great potential to be released into the aquatic environment, including surface waters that receive industrial and municipal effluents [60,93]. Moreover, we have to take into account that ENPs' behavior depends on the composition of water constituents; in fact particles may agglomerate and interact with the organic material and natural colloids present in these systems, which in turn will likely affect the ENPs potential ecotoxicity and their bioavailability to aquatic organisms [6,42]. However, relatively little is known about the magnitude, the fate and behavior of nanoparticles entering into the bodies of aquatic organisms and their subsequent biological effects [19,22,25,84,85,119] and regard the possible bioaccumulation in species used for human food.

Therefore, considering that the interaction of nanomaterials with cells can be regarded as a first step in the induction of possible health problems, some *in vitro* studies have focused on elucidating the uptake and biological effects of TiO₂-NPs in cell lines, being the *in vitro* systems the best experimental model for studying toxic mechanisms at the molecular and cellular levels in a controlled environment [11]. Common findings include general cytotoxicity [107,110], induction of an inflammatory response [103], as well as generation of free radicals [24], reactive oxygen species (ROS) and oxidative damage [74,103]. The studies have also shown the ability of TiO₂-NPs to cross cell membranes [34] and induce micronuclei formation and apoptosis [95].

In this context, in order to study the intrinsic hazard potential of TiO₂-NPs that may enter into fish from the aquatic environment, different biological effects of NP-TiO₂ on a sea bass continuous embryonic cell line (DLEC) [10] were investigated. Moreover, as the uptake and localization of nanoparticles are relevant for general cytotoxicity and induction of inflammatory responses, we examined the distribution and internalization of TiO₂-NPs in DLEC cells both by Scanning Electron Microscopy (SEM), coupled with an integrated energy-dispersive X-ray analyzer (EDS) for particle element detection, and by Transmission Electron Microscopy (TEM). In addition, as it is well known that the TiO₂-NPs may bind dangerous substances present in traces in marine water such as cadmium [46,130,133], and can absorb UV light [131], catalyzing the generation of ROS, such as superoxide anion radicals, hydrogen peroxide, free hydroxyl radicals, and singlet oxygen in aqueous media [49,63,64,107], the effects of controlled TiO₂-NPs exposure and combined treatment with UV light and/or CdCl₂ (Cd) were analyzed in term of quantitative parameters related to metabolic functions, morphological modifications, DNA damage and expression of some inflammatory related genes.

2. Materials and methods

2.1. Suspension of TiO₂-NPs

The suspension of the nanosized Titanium Dioxide (TiO₂-NPs), namely Aeroxide® (provided by Eigenmann & Veronelli, Milan, Italy; declared purity: 99.9%), was obtained according to the protocol described by Ref. [1]. Briefly, a stock suspension of 10 mg/ml of TiO₂-NPs, previously characterized by analytical [22] and morphological techniques [84], was added in FBS-free medium and sonicated for 1 min (VCX130, Vibra-Cell, 130 W, Sonics & Materials Inc., USA). The end-point concentrations of TiO₂-NPs for exposure were 1, 8, 40, 200 and 1000 µg/ml.

2.2. Cell culture and treatments

The DLEC cells, a continuous embryonic cell line established from sea bass (*Dicentrarchus labrax* L.) [10], were cultured in flasks (BD Falcon, Tissue culture treated, seal cap) at 22 °C in Leibovitz L-15 medium (Sigma-Aldrich) supplemented with 1% L-glutamine, 100 U/ml penicillin-streptomycin and 10% FBS. The cells were treated according to the experiment schedule which is designated in Table 1. CdCl₂ (Cd) 99% (Sigma-Aldrich) (0.1 µg/ml) nominal concentrations were chosen in accordance with a previous pilot study [84]. Differently, the intensity and the time of the UVA light exposition (30,000 µW/cm² for total 24 min) from five fluorescent 8-Watt UV-A lamps (365 nm) (Spectrolinker™ XL-1000A) were chosen in accordance with results shown in supplemental data section. The samples were analyzed by ATPlite™ assay, SEM, TEM, SCGE analysis and real time PCR as reported in Table 1. In particular, the study focused on 1 µg/ml TiO₂-NPs: this dose was chosen as it is far below the LC50 reported for fish species but still able to induce significant biological responses [127].

2.3. ATPlite assay

The intracellular adenosine triphosphate (ATP), widely accepted as a valid marker of viable cells, was measured by the ATPlite™ assay system (Perkin-Elmer), according to the manufacturer's instructions. DLEC cells were transferred (~10,000/well) to polystyrene 96-microwell plates (Perkin-Elmer) and cultured overnight at 22 °C in FBS-free L-15 medium, then the treatments were performed for 24 h as reported in Table 1. Controls were operated by changing the medium every two days to cultivate the cells at 22 °C (negative control) or by addition of 0.2% NaN₃ (positive control) for 24 h.

The ATP lite assay is based on the production of light caused by the chemical reaction of ATP with added luciferase and D-luciferin. The amount of emitted light, linearly correlated with ATP concentration [20], was measured with a luminometer (Victor II Perkin-Elmer) for 10 min in the dark. Five independent experiments and three replicates per treatment were performed.

2.4. Scanning electron microscopy (SEM) and microanalysis

For SEM analysis, cells (70,000) were seeded on sterile glass coverslips inserted in 24-well cell culture plates (IWAKI, Scitech Div. Asahi Techno Glass). The cells were cultured overnight at 22 °C in FBS-free L-15 medium and then exposed for 24 h to different treatments (Table 1). The control was obtained adding fresh FBS-free medium for 24 h.

After treatments, the samples were fixed overnight at 4 °C with 2.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 (all reagents from Sigma-Aldrich). Samples were washed four times with cacodylate buffer, then post-fixed with 1% osmium tetroxide (Sigma-Aldrich) and 0.15% ruthenium red (Sigma-Aldrich) in 0.1 M cacodylate buffer, pH 7.2, for 1 h at 4 °C. After different washings in distilled water, the samples were dehydrated with a graded acetone series (from 30% to 100%) and then dried with the critical point method, using CO₂ in a Balzers Union CPD 020. Dried coverslips were gold-coated in a sputtering unit equipped with an argon inlet (Balzer Union MD 010) for the observations by SEM (Jeol JSM 6010LA) (Tokyo, Japan). The identity of putative TiO₂-NPs was confirmed using the SEM (Jeol JSM 6010LA) (Tokyo, Japan) in combination with an integrated energy-dispersive X-ray analyzer (EDS) for particle element detection.

Table 1
Experiment schedule.

TiO ₂ -NPs μg/ml	CdCl ₂ (Cd) μg/ml	UV μWatt/cm ²						
1	–	–	TiO ₂	ATP _{lite}	SEM	TEM	SCGE	RT-PCR
1	0.1	–	TiO ₂ +Cd					
–	0.1	–	Cd					
1	–	30,000	TiO ₂ +UV					
1	0.1	30,000	TiO ₂ +Cd+UV					
–	–	30,000	UV					
–	0.1	30,000	Cd+UV					
8	–	–	TiO ₂		SEM			
8	0.1	–	TiO ₂ +Cd					
40	–	–	TiO ₂					
40	0.1	–	TiO ₂ +Cd					
200	–	–	TiO ₂					
200	0.1	–	TiO ₂ +Cd					
1000	–	–	TiO ₂					
1000	0.1	–	TiO ₂ +Cd					
1000	–	30,000	TiO ₂ +UV					
1000	0.1	30,000	TiO ₂ +Cd+UV					

2.5. Transmission electron microscopy (TEM)

For TEM analysis, cells (40,000) were seeded on sterile PET track-etched membrane (3 μm pore size) that were inserted in 24-well cell culture plates (IWAKI, Scitech Div. Asahi Techno Glass). The cells were cultured overnight at 22 °C in FBS-free L-15 medium and then exposed for 24 h to different treatments (Table 1). The control was obtained adding fresh FBS-free medium for 24 h. After treatments, cells were fixed overnight at 4 °C with 2.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 (all reagents from Sigma-Aldrich). Samples were washed four times with cacodylate buffer, then post-fixed with 1% osmium tetroxide (Sigma-Aldrich) and 0.15% ruthenium red (Sigma-Aldrich) in 0.1 M cacodylate buffer at pH 7.2 for 1 h at 4 °C. After different washings in distilled water, the samples were dehydrated with a graded acetone series (from 30% to 100%) and embedded in epon-based resin. The ultrathin sections (60–80 nm) were stained with 1% uranyl acetate and Reynolds lead citrate and then observed by TEM (JEOL 1200 EXII). Micrographs were acquired by the Olympus SIS VELETA CCD camera equipped with the iTEM software.

2.6. SCGE analysis (comet assay)

The standard alkaline (pH > 13) single-cell gel electrophoresis (SCGE), or comet assay, was carried out as described earlier under visible fluorescent light [116]. For the SCGE analysis the cells were cultured in flasks (BD Falcon, 500,000 cells/75 cm²) overnight at 22 °C in FBS-free L-15 medium and then exposed for 24 h to different treatments (Table 1). To include a positive control cells were X-irradiated with 3 Gy at 37 °C with a 250 KV and 6 mA with a Gilardoni MGL 200/8 D X-ray apparatus, at a dose-rate of 60 cGy/min in FBS-free medium. The negative control was obtained adding fresh FBS-free medium for 24 h. All the experiments were performed in triplicate. After the drug treatments, cells were collected and processed for the assay.

As described by Ref. [73]; 20 μl of the cell suspension (5 × 10⁵ cells) were mixed with 80 μl of 0.75% low melting-point agarose in phosphate buffered saline (PBS) at 37 °C and placed on frosted glass microscope slide previously coated with a layer of 1% normal melting-point agarose in PBS. Two slides for each experimental point were then incubated in a lysis solution (2.5 M NaCl, 10 mM Tris-HCl, 100 mM EDTA, pH 10, with 1% Triton and 10%

DMSO freshly added) for 1 day at 4 °C. To allow unwinding of DNA, slides were placed on a horizontal electrophoresis unit and incubated for 15 min with an electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH13). Electrophoresis was performed for 20 min at 25 V (volt per cm) and 300 mA at 4 °C, then the slides were washed in neutralization solution (0.4 M Tris-HCl, pH 7.5) for 5 min and fixed in methanol for 3 min. Slides were stained with ethidium bromide (20 µg/ml) and covered with a coverslip. An automatic image analyser (Comet Assay III, Perceptive Instruments, UK) connected to a fluorescence microscope (Axioskop 2, Zeiss) was used to examine the stained nucleoids. The amount of DNA damage was evaluated using computer-generated tail moment (tm) values and percentages of DNA damage. For each experimental point, 100 cells were scored from two slides for a total of 200 cells.

2.7. Gene expression experiments

The DLEC cells were grown in tissue-culture-treated flasks (BD Falcon, 500,000 cells/75 cm²) with FBS-free L-15 medium. After an overnight incubation at 22 °C, the medium was removed from the flasks and replaced by the FBS-free medium containing the different treatments (Table 1). The cells were exposed for 24 h to different treatments (Table 1) carried out in triplicates at 22 °C. Controls of the experiments were obtained from flasks in which the old medium was replaced by new FBS-free medium for 24 h. The medium was then aspirated and the lysis buffer was added (RNeasy Mini Kit, Qiagen).

2.7.1. Reverse-transcriptase reactions and real time PCR

The used protocol was similar as previously described [92]. The absence of DNA contamination was checked using, in a PCR reaction, sea bass β -actin primers that bracketed an intron (Table 2). For reverse transcription, the BioScript RNase H minus (Bioline) enzyme was used following manufacturer's instructions.

The transcript levels of caspase-3, IL-8 and TGF- β , and rRNA-18s (used as house-keeping gene) were determined with a Mx3000P™ real-time PCR system (Stratagene) equipped with version 2.02 software and using the Brilliant SYBR Green Q-PCR Master Mix (Stratagene) following the manufacturer's instructions. ROX was used as internal passive reference dye since it is not reactive during real-time PCR and therefore can be used to normalize slight differences in the volume of the added real-time PCR reaction mix, transparency of the plastic caps and other sources of well-to-well differences.

Specific PCR primers (Table 2) were designed for the amplification (~200 bp) of the analysed apoptosis and inflammatory-related genes.

In each PCR reaction 10 ng of cDNA template was used. The PCR conditions for caspase-3 were as follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 45 s, 58 °C for 45 s and 72 °C for 45 s. Differently the PCR conditions for IL-8, TGF- β and rRNA-18s were as

follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 45 s, 52 °C for 45 s and 72 °C for 45 s. Reactions were performed in triplicate for each template cDNA, that was replaced with water in all blank reactions. The analysis was carried out using the endpoints method option of the Mx3000P™ software and the fluorescence was collected at the end of each extension stage of amplification. A relative quantification was performed and the untreated cells were used as calibrator (defined as 1.0) for the quantification of transcripts of each gene of interest, performed in separate runs. A normaliser target (rRNA-18s) was used to correct for differences in total cDNA input and the quantitative assessment was based on determination of threshold cycle.

2.8. Statistical analysis

Numerical data are presented in the text as means $\pm$ SD. Homogeneity of variances was tested before data processing. Data from ATPlite™ assay and from experiments on multiple genes expressions were analysed by one-way ANOVA, followed by Bonferroni's test. Data from SCGE analysis (data with non-normal distribution) were analysed by Kruskal-Wallis test followed by Dunn's Multiple Comparison test. Data were analyzed using the GraphPad Prism 4.0 software statistical package. Numerical values obtained by all the treatments were compared to the control (untreated cells), when treatments with X-ray or UV light irradiation were performed, the numerical data were also compared to UV-irradiated cells. The level for accepted statistical significance was $P < 0.05$.

3. Results

3.1. ATP measurements

TiO₂-NPs, dispersed according to the protocol in the M&M section, were investigated for their effect on DLEC intracellular ATP levels. As it is shown in Fig. 1 A, different TiO₂-NPs concentrations (1, 8, 40, 200 and 1000 µg/ml) did not significantly affect the intracellular ATP levels in DLEC cells. However, a significant decrease ($P < 0.001$) of ATP level was found in DLEC cells treated with 0.2% NaN₃ (positive control) compared to the negative control.

As it is known that TiO₂-NPs might also interact with other co-existing environmental pollutants [46,130], as metals and organic xenobiotics, ATPlite™ assay was also performed to evaluate the effect of the combined treatment: TiO₂-NPs + Cd (Table 1). As it is shown in Fig. 1 B, DLEC cells treated with TiO₂-NPs (1000 µg/ml) + Cd and Cd alone showed a significant lower intracellular ATP level compared to control, while when cells were treated with lower concentration of TiO₂-NPs + Cd no significant differences were found compared to the control (Fig. 1 B).

A previous work demonstrated that TiO₂-NPs can be photo-activated [131] and, to study this aspect, DLEC cells were exposed to

Table 2
Primers for Real time PCR analysis.

Gene name	Accession number	Primers sequence (Forward and reverse, 5' → 3')	Size (bp)	Annealing temperature (°C)
Beta-actin	AJ493428	ATGTACGTTGCCATCC GAGATGCCACGCTCTC	550	55
18S Ribosomal RNA	AY831388	CCAACGAGCTGCTGACC CCGTTACCCGTGGTCC	208	52
IL-8	KM225777	GTGCTCTGGCGTTTC CTTCACCCAGGAGC	205	52
TGF-beta	AM421619	GACCTGGGATGGAAGTGG CAGCTGCTCCACCTTGTG	216	52
Caspase-3	DQ345773	CGACGGACAAGAGTCGGAG CATCGCGTTGCCAGCATCC	223	52

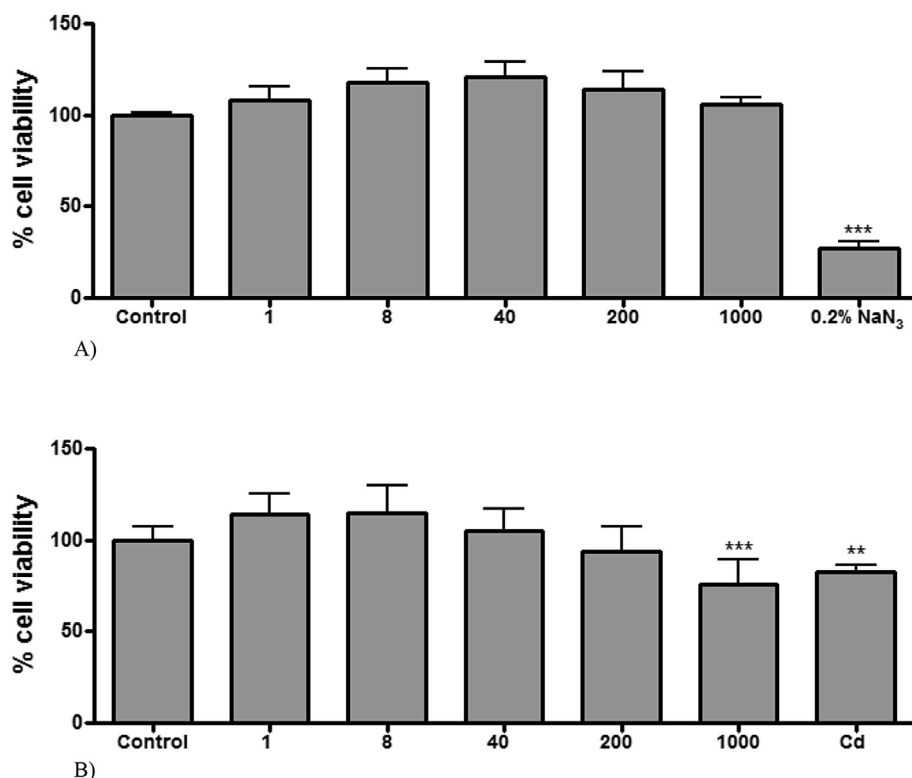


Fig. 1. ATP measurements of DLEC cells exposed to different concentration of TiO₂-NPs and TiO₂-NPs plus Cd. **A.** Viability (ATP content) of DLEC cells exposed to TiO₂-NPs (1, 8, 40, 200 and 1000 µg/ml) and 0.2% NaN₃ (positive control) for 24 h at 22 °C, referred as percent values with respect to untreated control cells. **B.** Viability (ATP content) of DLEC cells exposed to TiO₂-NPs (from 1 µg/ml to 1000 µg/ml) plus Cd (0.1 µg/ml) for 24 h at 22 °C, referred as percent values with respect to untreated control cells. The % of viability is expressed as the mean ± SD from 5 independent experiments (three replicates per treatment). Significantly different from Control: **P < 0.01; ***P < 0.001.

TiO₂-NPs + UV light (Table 1). The intensity and the time of the UV irradiation, which could be used without inducing significant decrease in intracellular ATP levels, were evaluated by *ATPlite*TM assays performed on DLEC cells exposed to increasing doses of UV light with a time of recovery of 24, 48 and 72 h. As the ATP amounts in all the treated and control groups were not significantly affected by the different UV doses, the maximum UV irradiation (30,000 µW/cm² for 24 min) with a time of recovery of 24 h was chosen for the successive experiments (Figure A, supplementary data section).

When the cells were treated with TiO₂-NPs (1 µg/ml) + UV or with TiO₂-NPs (1 µg/ml) + Cd + UV, neither the photoactivation of the nanoparticles nor Cd exposition induced significant changes of the intracellular ATP levels (Fig. 2 A). Differently, the ATP level was significantly affected in cells treated with 1000 µg/ml of photoactivated TiO₂-NPs (Fig. 2 B) when compared to control or UV treatment. The ATP level was also affected in cells treated with 1000 µg/ml of photoactivated TiO₂-NPs + Cd and the decrease was statistically significant. Conversely, the exposure to Cd + UV significantly increased the ATP level in DLEC cells compared to control or UV treatment (Fig. 2 B).

3.2. Cellular morphological modifications

Morphological modifications were observed in the cells exposed to TiO₂-NPs compared to control (Fig. 3A–G). The control cells were flat, showing short protrusions on the cell surface (Fig. 3A–B), while cells treated with TiO₂-NPs (1 µg/ml or 1000 µg/ml) showed smoother cell surface and abundant flocculate material on their surface and filopodia (Fig. 3 D, F). In particular, an increase of cells characterized by a reduction of protrusions density was observed

when DLEC cells were treated with increasing concentration of TiO₂-NPs (Fig. 3 D, F).

Scanning electron microscopy in combination with energy dispersive X-ray spectroscopy (EDS) revealed agglomerates of particles with strong signals in treated cells (1 µg/ml or 1000 µg/ml TiO₂-NPs) (Fig. 3 E, G), but not in control cells (Fig. 3 C). Elemental analysis of the observed agglomerates by EDS showed an X-ray energy peak belonging exclusively to titanium (Ti) (Fig. 3H).

Cells treated with TiO₂-NPs (1 µg/ml) and Cd showed short protrusions and flocculate material on their surface (Fig. 4 A) with strong EDS signals. Round shape and membrane budding were also observed in some cells (data not shown). Differently, the majority of the cells treated with TiO₂-NPs (1000 µg/ml) and Cd appeared destroyed (Fig. 4 B) and showed aggregates of Ti identified by EDS. Morphological modifications were found in cells treated with Cd alone (Fig. 4 C) showing longer protrusions than control cells (Fig. 3 A, B). EDS spectra did not reveal any Ti peak in Cd treated cells as expected.

Cells exposed only to UV showed fusiform shape, and numerous protrusions on the surface (Fig. 4 D). EDS spectra did not reveal any Ti signal as expected. Differently, fusiform cells showing abundant Ti flocculate material on their surface, together with cells having rounded shape and membrane budding were observed after the treatment with TiO₂-NPs (1 µg/ml) photoactivated by UV light (Fig. 4 E, F). Cells treated with TiO₂-NPs (1000 µg/ml) photoactivated by UV light appeared destroyed (Fig. 4 G). In this case elemental map analysis showed Ti signals on the surface.

Fusiform and round shaped cells with membrane budding were observed after TiO₂-NPs (1 µg/ml) photoactivated by UV light and Cd treatment (Fig. 4 H). In addition, flocculate material identified as Ti by EDS analysis was found on the cell surface. Cells treated with

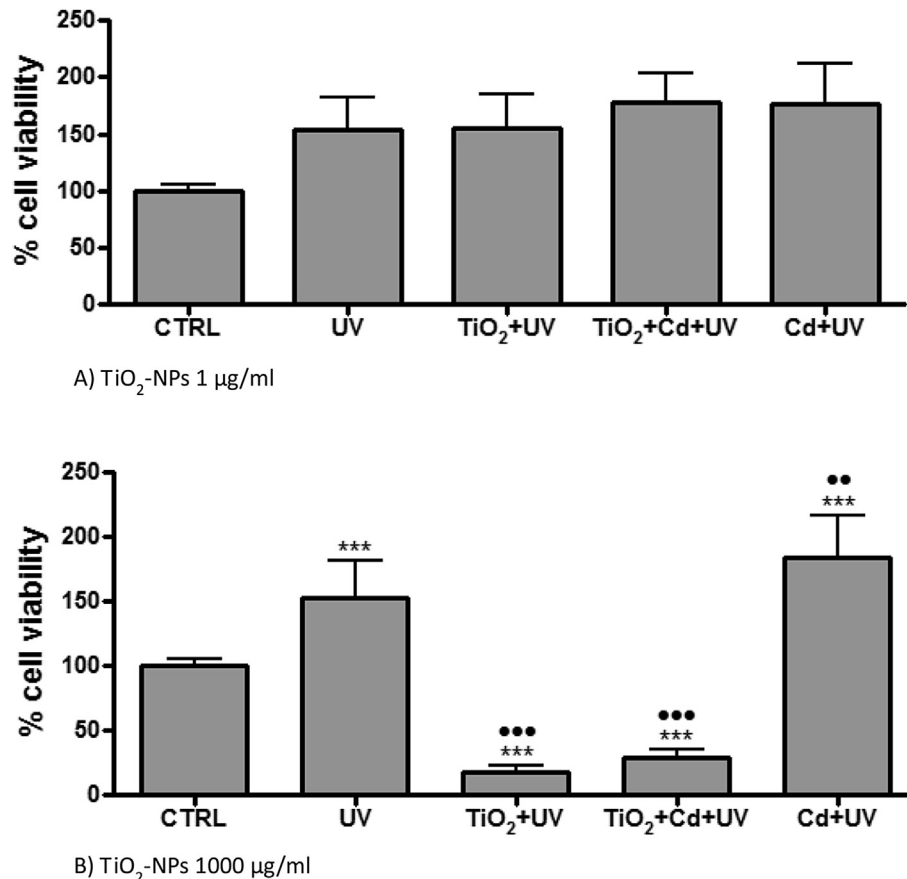


Fig. 2. ATP measurements after TiO₂-NPs photoactivation. **A.** Viability (ATP content) of DLEC cells exposed to UV (30.000 µW/cm² for total 24 min), TiO₂-NPs (1 µg/ml)+UV, TiO₂-NPs (1 µg/ml) +UV + Cd (0.1 µg/ml) or exposed to UV + Cd (0.1 µg/ml), referred as percent values with respect to control cells. **B.** Viability (ATP content) of DLEC cells exposed to UV (30.000 µW/cm² for total 24 min), TiO₂-NPs (1000 µg/ml)+UV, TiO₂-NPs (1000 µg/ml) +UV + Cd (0.1 µg/ml) or exposed to UV + Cd (0.1 µg/ml), referred as percent values with respect to control cells. The % of viability is expressed as the mean ± SD from 5 independent experiments (three replicates per treatment). Significantly different from Control: *** (P < 0.001); significantly different from UV: **P < 0.01; ***P < 0.001.

TiO₂-NPs (1000 µg/ml) photoactivated by UV light and Cd appeared destroyed (Fig. 4 I) and covered by flocculate Ti material. Cell treated with Cd + UV showed fusiform shape and protrusions on the surface; EDS spectra did not reveal any Ti peak as expected.

3.3. Uptake of TiO₂ nanoparticles

TEM analysis of DLEC cells exposed to TiO₂-NPs for 24 h (Table 1) pointed out the presence of agglomerates of nanoparticles on the cell surface that were uptaken by the cells (Fig. 5A–C). At this time, the incorporation of TiO₂-NPs aggregates in the cell was evident (Fig. 5A–B), although small aggregates were rarely found in “coated” pits (Fig. 5 A). Internalized TiO₂-NPs aggregates (Fig. 5B–C) were mostly trapped in vesicles that were distributed across the cytoplasm and in the perinuclear region of nucleus (Fig. 5 C). Control cells did not show any NPs (Fig. 5 D).

When cells were treated with both TiO₂-NPs (1 µg/ml) and Cd for 24 h (Table 1), the areas around the cells were provided with TiO₂-NPs agglomerates and, in addition, TiO₂-NPs appeared in vesicles, disseminated in the cytoplasm (Fig. 5 E). Apparently the Cd treatment did not modify TiO₂-NPs uptake, although portions of the cell membranes were damaged by the treatment (Fig. 5 E). Cells treated with Cd showed plasma membrane integrity and absence of TiO₂-NPs in the cytoplasm (Fig. 5 F). The photoactivation of TiO₂-NPs did not interfere with their uptake by the DLEC cells, while absence of TiO₂-NPs was confirmed in Cd + UV and Cd only treated

cells (data not shown).

3.4. DNA damage

The amount of DNA damage after the different treatments was quantified by comet assay (Fig. 6).

In particular, a significant increase of DNA damage compared to control and UV light was found after TiO₂-NPs + Cd + UV and Cd + UV treatments. Similarly, the damage significantly increased in cells treated with Cd alone when compared to control. Cells treated with X-Ray showed a significant higher amount of DNA damage compared to control or UV.

3.5. mRNA expression of apoptosis and inflammation-related genes

A specific amplification of the expected product related to caspase-3 was not found both in DLEC cells exposed to the different treatments and controls. However, a specific amplicon, using the same primers, was identified in the thymocytes of a sea bass juvenile (data not shown), as the caspase-3 activation is an essential protein in the apoptosis process.

The expression of the inflammation-related genes such as IL-8 and TGF-β (Fig. 7A–D), was quantified in DLEC cells after 24 h of treatments (Table 1).

A significant up-regulation of the IL-8 transcripts compared to the control was quantified in DLEC cells treated with TiO₂-NPs or

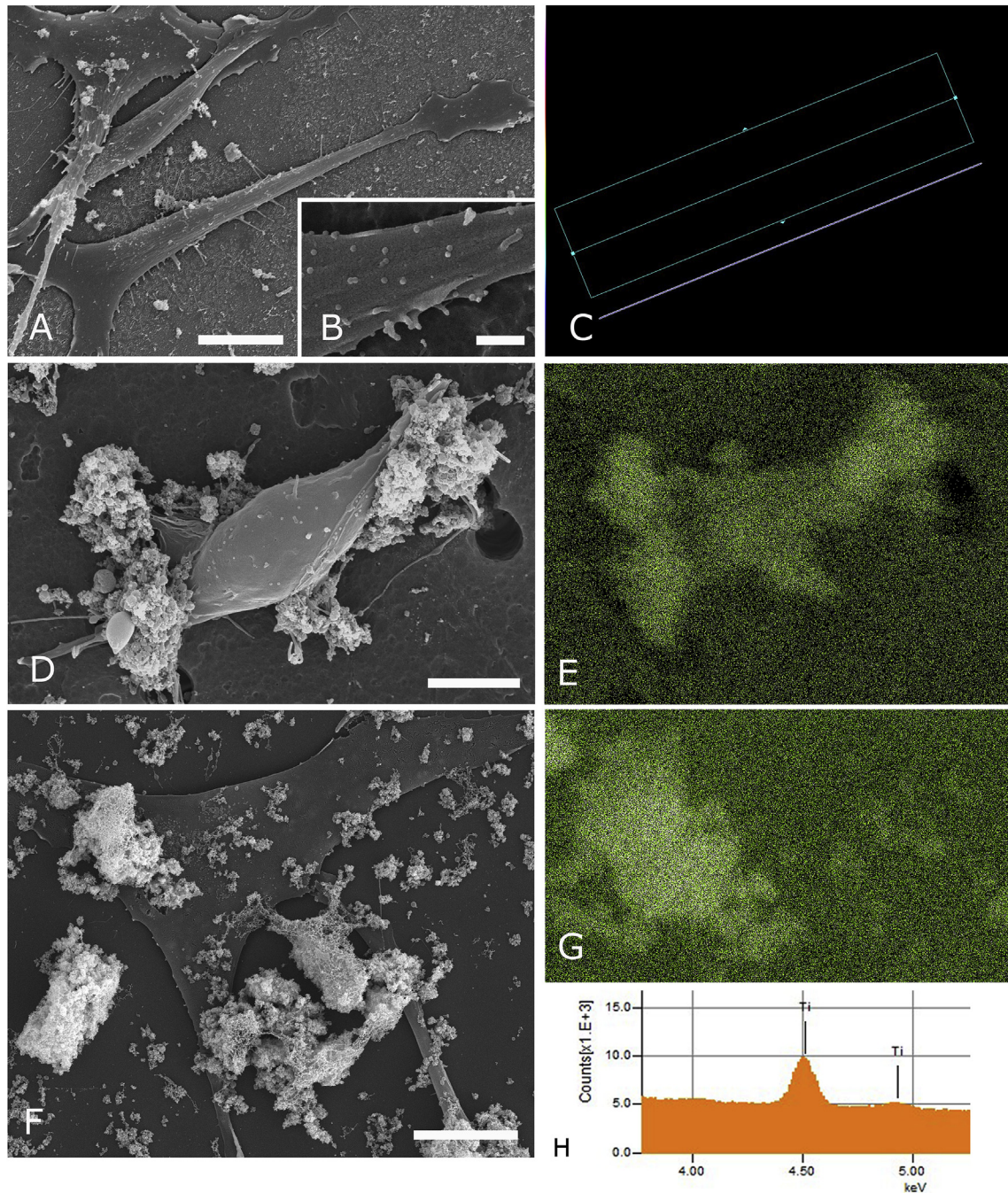


Fig. 3. Morphological modifications and microanalysis of DLEC cells 24 h after the TiO₂-NPs addition. **A.** SEM analysis of DLEC control cells. **B.** Higher magnification showing the DLEC cellular surface. **C.** Absence of EDS signals in DLEC control cells. **D.** TiO₂-NPs (1 µg/ml) treated cell showing smoother surface and abundant flocculate material. **E.** EDS signal in treated cells (1 µg/ml TiO₂-NPs). **F.** TiO₂-NPs (1000 µg/ml) treated cells showing abundant flocculate material on their smooth surface. **G.** EDS signal in treated cells (1000 µg/ml TiO₂-NPs). **H.** EDS analysis showing an X-ray energy peak belonging to titanium (Ti) in TiO₂-NPs treated cells. Bars: **A:** 10; µm **B:** 1 µm; **D:** 5; µm **F:** 10 µm.

TiO₂-NPs + Cd, while a significant down-regulation was found after Cd treatment (Fig. 7 A). After photoactivation, the levels of IL-8 transcripts in cells treated with TiO₂-NPs + UV, TiO₂-NPs + UV + Cd or Cd + UV were significantly different compared to UV. The irradiation of the cells with UV light alone significantly reduced the IL-8 transcripts compared to the control (Fig. 7 B).

The TGF-β levels significantly increased in cells treated with TiO₂-NPs being higher compared to the control (Fig. 7 C). In addition, the level of the TGF-β transcripts after Cd treatment was significantly higher than the control (Fig. 7 C).

After the photoactivation, the levels of TGF-β transcripts (Fig. 7 D) were significantly up-regulated in cells treated with TiO₂-NPs + UV + Cd compared both to the control and UV, while in TiO₂-NPs + UV treated cells the TGF-β levels were down-regulated compared to the control but they were significantly higher compared to UV. Cd + UV treatment significantly increased the TGF-β transcripts when compared to UV. A down regulation of TGF-β transcripts was also found after UV treatment when compared to the control (Fig. 7 D).

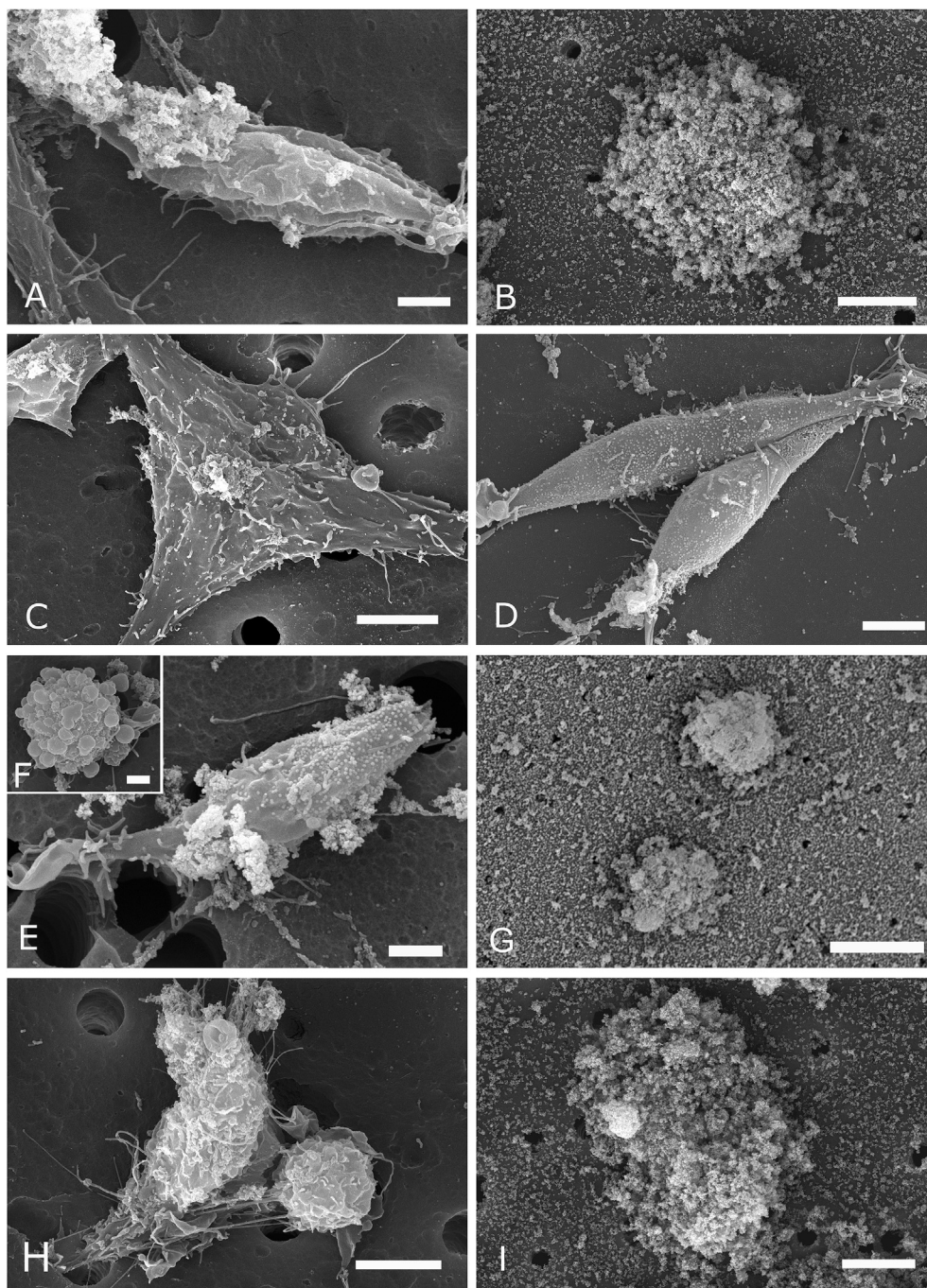


Fig. 4. Morphological modifications and microanalysis of DLEC cells 24 h after TiO_2 -NPs + Cd and TiO_2 -NPs + UV + Cd treatments. **A.** DLEC cells treated with TiO_2 -NPs ($1 \mu\text{g}/\text{ml}$) and Cd showing short protrusions and flocculate material on their surface. **B.** Destroyed cells after TiO_2 -NPs ($1000 \mu\text{g}/\text{ml}$) and Cd treatment. **C.** Cells treated with Cd alone showing protrusions of the cell membrane. **D.** Cells exposed only to UV showing fusiform shape, and numerous bottom-shaped protrusions of the cell membrane. **E.** TiO_2 -NPs + UV treated cell showing fusiform shape and abundant flocculate material on the surface. **F.** Membrane budding observed after the treatment with TiO_2 -NPs ($1 \mu\text{g}/\text{ml}$) photoactivated by UV light. **G.** Destroyed cells after the treatment with TiO_2 -NPs ($1000 \mu\text{g}/\text{ml}$) photoactivated by UV light. **H.** Fusiform and rounded DLEC cells showing flocculate material after TiO_2 -NPs ($1 \mu\text{g}/\text{ml}$) + UV + Cd treatment. **I.** Cell showing destroyed morphology after TiO_2 -NPs ($1000 \mu\text{g}/\text{ml}$) + UV + Cd treatment. Bars: **A:** $2 \mu\text{m}$; **B:** $10 \mu\text{m}$; **C:** $5 \mu\text{m}$; **D:** $5 \mu\text{m}$; **E:** $2 \mu\text{m}$; **F:** $2 \mu\text{m}$; **G:** $10 \mu\text{m}$; **H:** $5 \mu\text{m}$; **I:** $10 \mu\text{m}$.

4. Discussion

The present study investigates the TiO_2 -NPs induced toxicity in a teleost cell line [10]. In addition, it evaluates the capability of TiO_2 -NPs to form aggregates and cross the membranes inducing cell morphological changes, DNA damage and inflammatory responses.

4.1. TiO_2 -NPs photoactivated by UV and Cd co-exposure affect ATP levels in DLEC cells depending on TiO_2 concentration

The ATPlite™ assay revealed that the viability of the DLEC cells was not significantly affected by the different concentration of TiO_2 -NPs ($1, 8, 40, 200, 1000 \mu\text{g}/\text{ml}$), although the compound exhibited a biphasic dose-response curve characteristic of

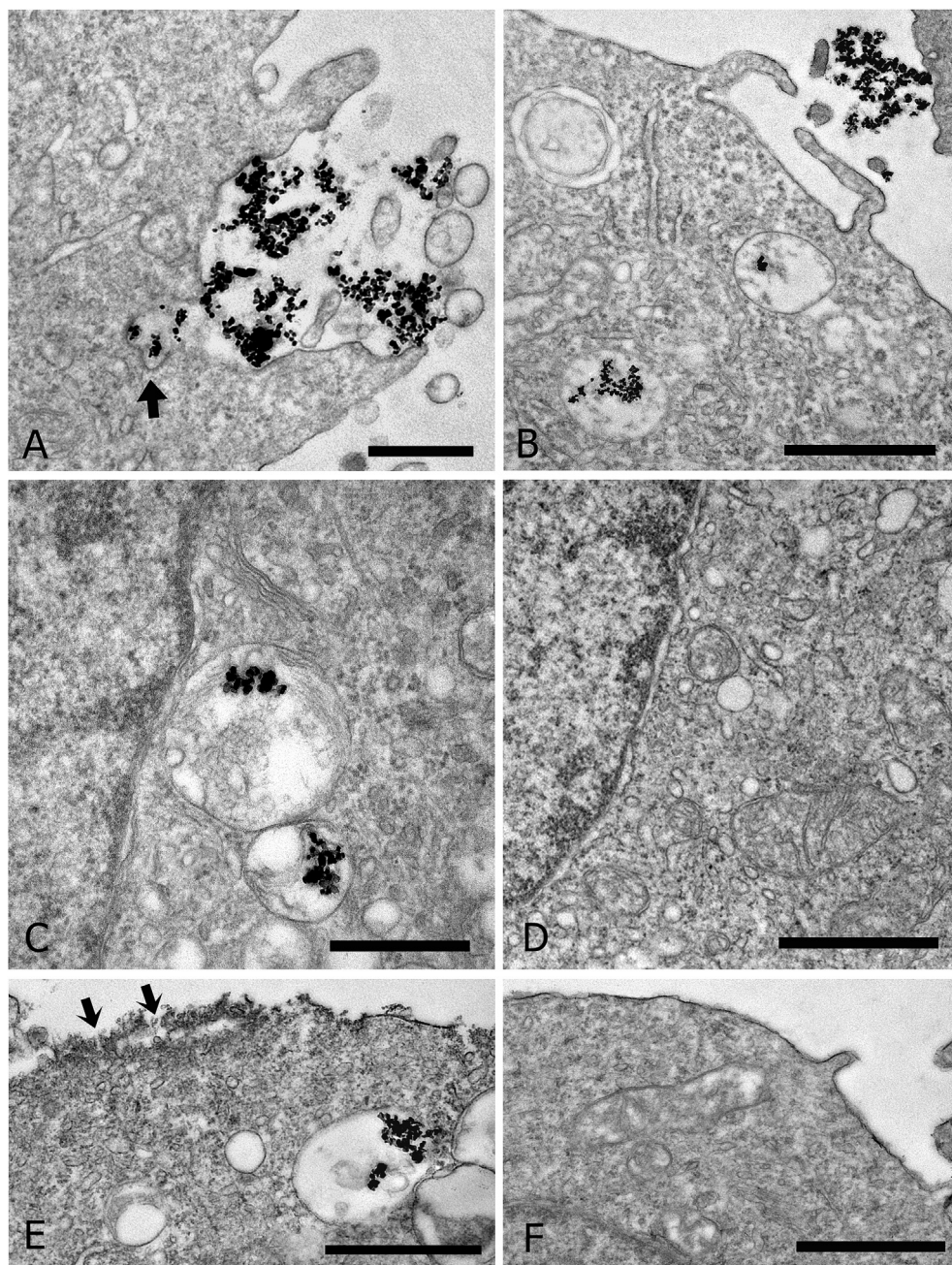


Fig. 5. TEM analysis of DLEC cells 24 h after the TiO_2 -NPs and TiO_2 -NPs plus Cd treatment. **A.** Agglomerates of TiO_2 -NPs (1 $\mu\text{g}/\text{ml}$) internalized by cells and coated pits (arrow). **B.** Cells leading to the incorporation of aggregates inside vesicles. **C.** TiO_2 -NPs aggregates in vesicles localized in the perinuclear region. **D.** Untreated cells. **E.** Portions of the cell membrane damaged by the treatment with TiO_2 -NPs (1 $\mu\text{g}/\text{ml}$) + Cd (arrows) and TiO_2 -NPs in vesicles dispersed in the cytoplasm. **F.** Plasma membrane integrity in Cd treated cells. Bars: **A:** 1 μm ; **B:** 10 μm ; **C:** 1 μm ; **D:** 1 μm ; **E:** 1 μm ; **F:** 1 μm .

hormesis-like effects [76]. The treatment with Cd (0.1 $\mu\text{g}/\text{ml}$), a common aquatic toxic metal pollutant [51,104], which is known for its ability to affect essential cellular processes such as cell division, proliferation, differentiation and apoptosis [78,79,89], significantly reduced the ATP level compared to control. Differently, the co-exposure of TiO_2 -NPs with Cd resulted in increased adverse effects dependent on TiO_2 concentration.

To understand these results, it is important to consider that the uptake/accumulation of Cd in DLEC cells, and consequently its toxicity, might be affected by the presence of TiO_2 -NPs. Studies on different freshwater model species highlighted the interference of

metals in NPs uptake and vice versa [88,99,112] and, in a recent paper [23], it was demonstrated that the co-exposure of Cd with TiO_2 -NPs did not increase, but rather decrease, the Cd content in *Mytilus galloprovincialis* gill cells. The authors suggested that this observation, together with the results on heavy metal detoxification response (metallothionein induction) could indicate the absence of a Trojan horse effect of TiO_2 -NPs toward CdCl_2 , confirming previous data [4].

After photoactivation, the toxicity of TiO_2 -NPs in DLEC cells was dependent on Ti concentration, in fact the ATP level was significantly affected only in cells treated with 1000 $\mu\text{g}/\text{ml}$ TiO_2 -NPs + UV.

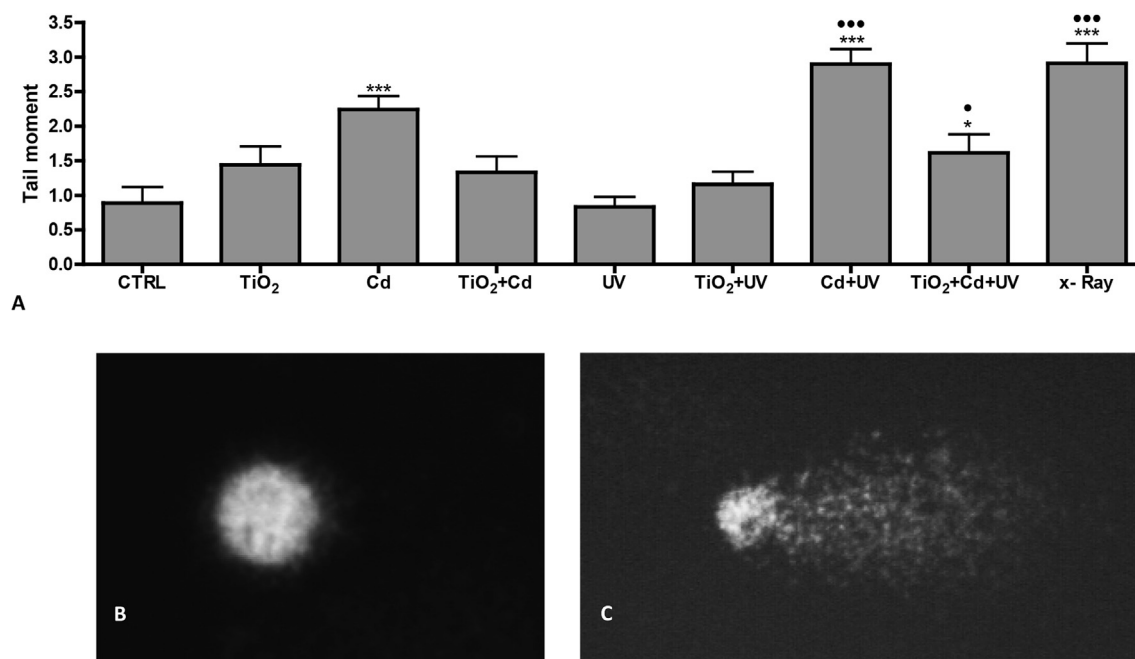


Fig. 6. DNA damage quantified by SCGE analysis (comet assay) after 24 h of treatments. **A.** For the SCGE analysis the cells were exposed to: TiO₂-NPs (1 µg/ml); Cd (0.1 µg/ml); UV (six doses of 5000 µW/cm² for total 24 min) with a recovery of 24 h; TiO₂-NPs (1 µg/ml) + Cd (0.1 µg/ml); only Cd (0.1 µg/ml); TiO₂-NPs (1 µg/ml) + UV; TiO₂-NPs (1 µg/ml)+Cd (0.1 µg/ml) + UV. The control was obtained adding fresh FBS-free medium for 24 h. **B.** Untreated cell **C.** X-Ray treated cell (positive control). Significantly different from Control: *P < 0.05, ***P < 0.001; significantly different from UV: *P < 0.05, ***P < 0.001.

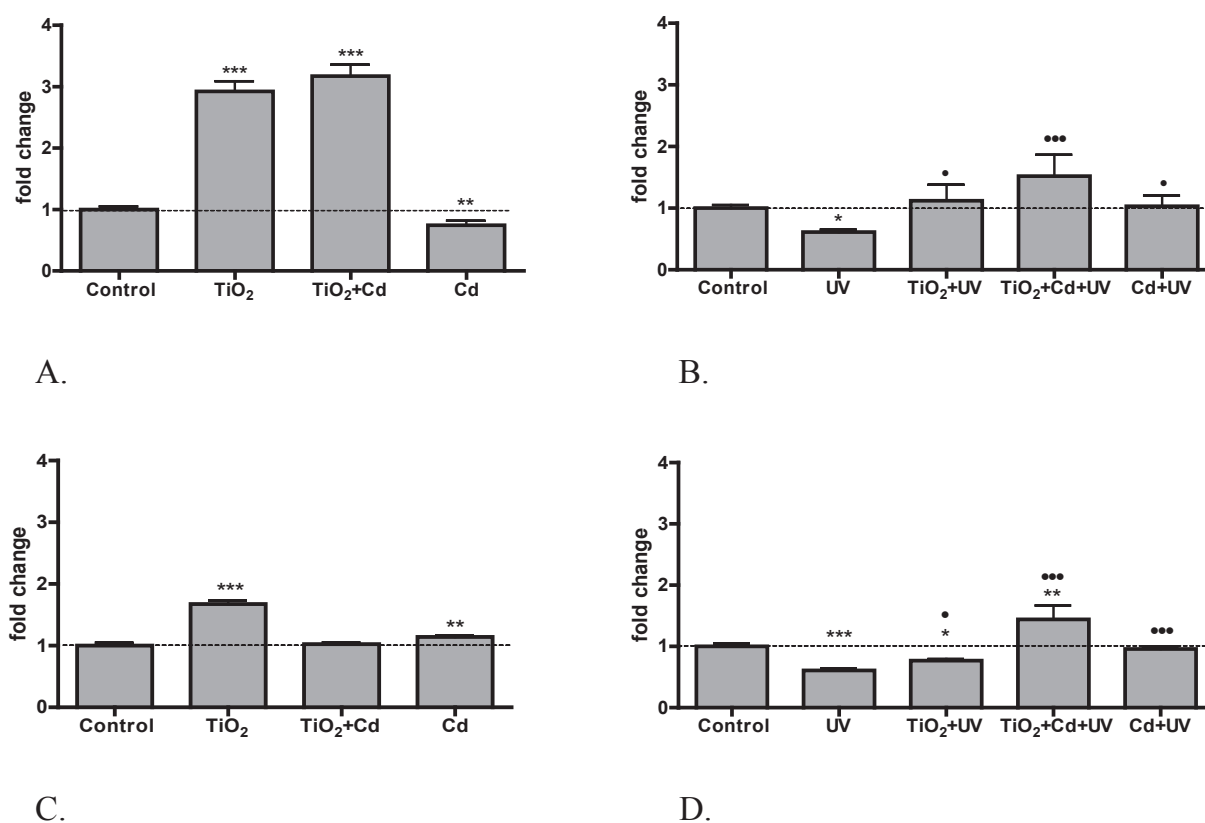


Fig. 7. Q-PCR analysis of inflammation-related genes. **A.** Expression of IL-8 quantified after the exposure of the cells to different treatments: TiO₂-NPs; TiO₂-NPs + Cd; Cd. Control obtained adding fresh FBS-free medium to not treated DLEC cells. **B.** Expression level of IL-8 quantified after the exposure of the cells to different treatments: UV; TiO₂-NPs + UV; TiO₂-NPs + UV + Cd; Cd + UV. Control as above. **C.** Expression level of TGF-β quantified after the exposure of the cells to: TiO₂-NPs; TiO₂-NPs + Cd; Cd. Control as above. **D.** Expression level of TGF-β quantified after the exposure of the cells to: UV; TiO₂-NPs + UV; TiO₂-NPs + UV + Cd; Cd + UV. Control as above. Significantly different from Control: *P < 0.05, **P < 0.01, ***P < 0.001; significantly different from UV: *P < 0.05, ***P < 0.001.

Previous studies reported that nanosized TiO₂ alone (0.1–1000 µg/ml) had little effect on gold fish skin cells, whereas co-exposure with UV caused a significant decrease of cell viability dependent on both the concentration of TiO₂ and the dose of administrated UV [96]. Similarly, the photoactivation and the co-exposure with Cd induced in DLEC cells a TiO₂-NPs dose-dependent decrease of the intracellular ATP levels compared to control.

4.2. TiO₂-NPs induce cellular modifications

By SEM analysis, it was evident that TiO₂-NPs (1 µg/ml and 1000 µg/ml) induced cellular modifications, that need to be considered as a sign of cellular toxicity [1,100]. In particular, TiO₂-NP dose-dependent reduction of cellular protrusions was found in DLEC cells suggesting that nanoparticles could affect fundamental processes of the cells, including cell migration and invasion as previously shown in human epithelial intestinal cells exposed to TiO₂-NPs [33,62]. In human keratinocytes [135], it was demonstrated that TiO₂-NPs can affect extracellular matrix components whose interactions with the cell, mediated by adhesion molecules, are important for the regulation of the cellular shape and for the maintenance of activities such as cell migration, growth, and differentiation [111]. These considerations support the idea that the cellular toxicokinetic of nanoparticles could be focused both on intracellular and extracellular distribution [45]. The irradiation of TiO₂-NPs with UV light further induced cellular modifications. Cells treated with 1 µg/ml TiO₂-NPs irradiated with UV showed plasma membrane blebbing, which is a characteristic of the apoptotic process. Similarly, the co-exposure of TiO₂-NPs with Cd, as previously reported in other cell lines [35,36,113], and the exposure to TiO₂-NPs + UV + Cd induced morphological modifications, like rounded shape and plasma membrane blebbing, suggesting that the photoactivation and the combined exposure with Cd could promote significant rearrangement of the cytoskeleton leading to increased hydrostatic pressure and subsequent detachment of the membrane from the cortex [13]. Longer protrusions compared to control were found in Cd treated cells confirming that Cd induced cellular alterations in DLEC cells as previously showed in different sea bass cell types [35]. When the cells were treated with 1000 µg/ml TiO₂-NPs co-exposed with Cd and/or irradiated with UV light, the toxic effect of the treatments was revealed by the evident morphological damage of the majority of the cells. Such features should be considered as signs of an autolytic necrotic outcome of a complete cellular death program [52,118], when scavengers do not operate. This autolysis was called secondary necrosis by Ref. [126] intending to distinguish this type of cell elimination from “cellular necrosis occurring ab initio”, which should be called “primary necrosis” [37,66,70,83].

4.3. TiO₂-NPs penetrate into the cell via surface folds and invaginations

The events start with the direct contact of TiO₂-NPs with the cell membrane that is suggestive of possible macromolecule internalization mechanisms induced by the nanoparticles [16,29]. Few cases of TiO₂-NPs in “coated” pits that could represent the initial stages of clathrin-dependent receptor-mediated endocytosis finishing in endosomes [53,86] were found in DLEC cells. However, the amount of the “coated” pits was very small suggesting that this mechanism is only occasionally used and that receptor-mediated endocytosis does not provide appreciable internalization of TiO₂-NPs. Our electron microscopic examinations showed formation of small electron dense agglomerations, direct contact of these latter with the cell plasma membrane and successive penetration into the cell via surface folds and invaginations. This could be due to an increase

of the fluidity of cell plasma membrane, allowing it to readily form deep invaginations and providing penetration of the nanoparticles inside the cell. The ability of TiO₂-NPs to destabilize cell membranes after *in vitro* exposure of cell lines has also been noted by other authors [68,108,115]. Moreover, it should be considered that TiO₂-NPs generate free radicals, including oxygenated free radicals and carbon-centered radicals, causing cleavage of C-H bonds in organic molecules that could be the first step of oxidative damage of biological molecules [15,32]. It has been reported that lipid peroxidation may result from interactions between nanoparticles and lipids [59] or from elevated concentrations of ROS which are capable of peroxidising membranes [102] with consequent destabilization [41,123]. After 24 h of exposure TiO₂-NPs seemed to penetrate into the cytoplasm and locate in the peri-region of the nucleus as aggregated particles, which may induce direct interactions between the particles and cellular components, to cause adverse biological responses, as previously reported in cultured human-derived retinal pigment epithelial cells (ARPE-19) after exposure to TiO₂-NPs [134] and in L929 fibroblasts [1]. The role of Cd in cellular uptake of nanoparticles was also investigated, revealing that it not interfere with the internalization process. However, portions of the cell membranes were damaged by the treatment, probably due to Cd that, as reported by several previous reports, could cause lipid peroxidation [50,82,129].

4.4. Photoactivation of TiO₂-NPs and co-exposure with Cd contribute to strengthen the genotoxic effect in DLEC cells

It is known that TiO₂ absorbs UV light, catalyzing the generation of ROS [49,61,63,64], important factors in the apoptotic process, whose excess induces mitochondrial membrane permeability and damages to the respiratory chain, triggering the apoptotic process [52,118]. In DLEC cells the photoactivation of the TiO₂-NPs when co-exposed to Cd contributes to strengthen the genotoxic effect. DNA damage was revealed with the appearance of a prominent comet tail due to irreparable double strand breaks [18,75]. To notice, Cd + UV treatments significantly increased DNA damage in DLEC cells compared to control or UV treatment, although they did not induce a toxic effect on cell viability (ATPlite assay). These results confirmed previous data showing in CHO cells that non-toxic concentrations of cadmium affected the repair of UV-induced DNA damage, providing evidence that the inhibition of DNA repair is an important mechanism of Cd induced mutagenicity and carcinogenicity [30,47]. Similarly, Cd alone induced a significant increase of DNA strand breaks in DLEC cells compared to controls. Cd is also known to induce oxidative stress by depletion of glutathione in association with mitochondrial damage, induction of apoptosis and disruption of calcium signaling [28,114,121].

The ineffectiveness of 1 µg/ml nano-TiO₂ and TiO₂-NPs + UV exposures in term of DNA primary damage induction in DLEC cells suggests that different cell types with various activities could exhibit differential genotoxic responses dependent on TiO₂-NPs concentration. Previous studies demonstrated that much higher TiO₂-NPs concentrations than the ones used in our comet assays resulted in micronuclei formation and apoptosis in Syrian hamster embryo fibroblasts [95], human astrocytes-like astrocytoma U87 cell, normal human fibroblasts [67], goldfish skin (GFSK-S1), rainbow trout gonadal tissue (RTG-2) and rat kidney proximal (NRK-52E) cells [5,96].

It needs to be noticed that in samples co-exposed with TiO₂-NPs and Cd the DNA damage was not different compared to control suggesting that TiO₂-NPs might act as an antagonist like previously reported in *Mytilus galloprovincialis* gill cells [23].

4.5. Caspase-3 is not an integral part of the apoptotic response induced by TiO₂-NPs both under UV or Cd exposure

To elucidate the mechanisms of cell death induced by TiO₂-NPs, the signaling pathways involved in apoptosis were also investigated. It is well known that caspase-3 is one of the main executioner caspases in the apoptotic pathway, cleaving and inactivating a number of molecules and largely contributing to the apoptotic phenotype and the dismantling of the apoptotic cells. Studies involving caspase-3 knockout mice have described that the presence of caspase-3 is essential for chromatin condensation and DNA degradation in apoptosis, although is not required for γ -irradiation-induced apoptosis in mouse embryonic stem cells [125]. In fish, information on the apoptotic process is relatively scarce and caspase-3 genes only recently started to be sequenced in zebrafish [128], rainbow trout [98] and in sea bass [80]. Interestingly, in DLEC cells caspase-3 specific transcripts were not found suggesting that the apoptotic response induced by TiO₂-NPs, both under UV or Cd exposure, could be caspase-3 independent. In this regard, Trouiller et al. [117] suggested that TiO₂-NPs might damage DNA through a direct chemical interaction with the DNA phosphate group or indirectly via oxidative stress and/or inflammatory responses. As in our observations TiO₂-NPs were never found inside the nucleus, it seems probable that TiO₂-NPs could cause DNA damage indirectly through generation of ROS [31,52,57,96,118] and/or inflammatory processes [14,38].

4.6. Inflammatory potential of TiO₂-NPs

Whether or not TiO₂-NPs would induce inflammation is a controversial issue. In a *in vitro* study [91], it was suggested that TiO₂-NPs ananase was a stronger inducer of intracellular ROS and that, by the induction of ROS, the expression of inflammation-related genes was also increased in a concentration-dependent manner. In our study the expression of inflammation-related genes such as IL-8 and TGF- β was up-regulated in 1 μ g/ml TiO₂-NPs treated cells revealing the inflammatory potential of TiO₂-NPs, as previously showed by Ref. [14] in the absence of UV irradiation. Recent studies, reported that TiO₂-NPs in human lymphocytes influenced the expression of genes that encode biomarkers of inflammation, such as IL-8 [7], and that they were potent inducers of TGF- β expression in human pulmonary fibroblasts, partly via an IL-1 β -dependent mechanism [3]. In our model the co-exposure of TiO₂-NPs with Cd up-regulated the IL-8 transcripts, while TGF- β levels were not affected. In addition, the expression of IL-8 decreased while, on the contrary, TGF- β increased after the treatment with Cd alone. These data suggest that Cd may modulate the immune responses as previously demonstrated ([69,136]. It should be noted that in cells irradiated with UV light alone the IL-8 and TGF- β m-RNA expression was significantly down regulated confirming the potentially harmful impact of UV radiation on fish immune functions [54,77,101]. UV light is known for its immunosuppressive properties, which are demonstrated by the inhibition of cellular immune reactions and by the exacerbation of infectious diseases [12,65]. UV light induces the release of immunosuppressive cytokines [105], but obviously it can also interfere with the biological effects of cytokines. The photoactivation of TiO₂-NPs co-exposed with Cd modulated the IL-8 and TGF- β levels showing effects that are not easy to understand without further studies.

4.7. Conclusions

Our study demonstrated that TiO₂-NPs contact and cross the membranes of DLEC cells. Moreover, the irradiation of TiO₂-NPs with UV light and the co-exposure with Cd contribute to induce

morphological changes, acute cellular toxicity, up-regulation of inflammatory related genes and DNA damage through a caspase-3 independent apoptotic process. These results suggest that the interaction of TiO₂-NPs with marine water contaminants, such as cadmium, triggered by the UV irradiation, need to be taken into consideration as potentially harmful to marine organisms.

Conflict of interests

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.fsi.2017.01.044>.

References

- [1] Z.E. Allouni, J.P. Hø, M.A. Cauqui, N.R. Gjerdet, M.R. Cimpan, Role of physicochemical characteristics in the uptake of TiO₂ nanoparticles by fibroblasts, *Toxicol. Vitro* 26 (2012) 469–479.
- [2] R.J. Aitken, M.Q. Chaudhry, A.B.A. Boxall, M. Hull, Manufacture and use of nanomaterials: current status in the UK and global trends, *Occup. Med.* 56 (2006) 300–306.
- [3] L. Armand, M. Dagouassat, E. Belade, A. Simon-Deckers, S. Le Gouvello, C. Tharabat, C. Duprez, P. Andujar, J.C. Pairon, J. Boczkowski, S. Lanone, Titanium dioxide nanoparticles induce matrix metalloproteinase 1 in human pulmonary fibroblasts partly via an interleukin-1 β -dependent mechanism, *Am. J. Respir. Cell Mol. Biol.* 48 (3) (2013) 354–363.
- [4] T. Balbi, A. Smerilli, R. Fabbri, C. Ciacci, M. Montagna, E. Grasselli, A. Brunelli, G. Pojana, A. Marcomini, G. Gallo, L. Canesi, Co-exposure to n-TiO₂ and Cd²⁺ results in interactive effects on biomarker responses but not in increased toxicity in the marine bivalve *M. galloprovincialis*, *Sci. Tot. Environ.* 493 (2014) 355–364.
- [5] S. Barillet, A. Simon-Deckers, N. Herlin-Boime, M. Mayne-L'Hermite, C. Reynaud, D. Cassio, B. Gouget, M. Carrière, Toxicological consequences of TiO₂, SiC nanoparticles and multi-walled carbon nanotubes exposure in several mammalian cell types: an *in vitro* study, *J. Nanopart. Res.* 12 (2010) 61–73.
- [6] A. Baun, N.B. Hartmann, K. Grieger, K.O. Kusk, Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing, *Ecotoxicol.* 17 (2008) 387–395.
- [7] L.A. Baranova, E.V. Zhornik, I.D. Volotovskii, Influence of silver and titanium dioxide nanoparticles on the expression of genes of biomarkers of inflammatory responses and apoptosis, *Biofizika* 60 (2) (2015) 234–241.
- [8] O. Bondarenko, K. Juganson, A. Ivask, K. Kasemets, M. Mortimer, A. Kahru, Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells *in vitro*: a critical review, *Arch. Toxicol.* 87 (2013) 1181–1200.
- [9] E.L. Bradley, L. Castle, Q. Chaudry, Application of nanomaterials in food packaging with a consideration of opportunities for developing countries, *Trends. Food Sci. Tech.* 22 (2011) 604–610.
- [10] F. Buonocore, A. Libertini, D. Prugnoli, M. Mazzini, G. Scapigliati, Production and characterization of a continuous embryonic cell line from sea bass (*Dicentrarchus labrax* L.), *Mar. Biotechnol.* 8 (2006) 80–85.
- [11] A. Castano, N. Bols, T. Braunbeck, P. Dierickx, M. Halder, B. Isomaa, et al., The use of fish cells in ecotoxicology, *ATLA* 31 (2003) 317–351.
- [12] R.S. Chapman, K.D. Cooper, E.C. De Fabo, J.E. Frederick, K.N. Gelatt, S.P. Hammond, P. Hersey, H.S. Koren, R.D. Ley, F. Noonan, Solar ultraviolet radiation and the risk of infectious disease: summary of a workshop, *Photochem Photobiol.* 61 (1995) 223–247.
- [13] G.T. Charras, A short history of blebbing, *J. Microsc.* 231 (3) (2008) 466–478.
- [14] H.W. Chen, S.F. Su, C.T. Chien, et al., Titanium dioxide nanoparticles induce emphysema-like lung injury in mice, *FASEB J.* 20 (2006) 2393–2395.
- [15] X. Chen, S.S. Mao, Titanium dioxide nanomaterials: synthesis, properties, forms, and applications, *Chem. Rev.* 107 (2007) 2891–2959.
- [16] B.D. Chithrani, A.A. Ghazani, W.C. Chan, Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells, *Nano Lett.* 6 (2006) 662–668.
- [17] M.R. Cimpan, R. Matre, L.I. Cressey, B. Tysnes, S.A. Lie, B.T. Gjertsen, N. Skaug, The effect of heat- and auto-polymerized denture base polymers on clonogenicity, apoptosis, and necrosis in fibroblasts: denture base polymers induce apoptosis and necrosis, *Acta Odontol. Scand.* 58 (2000) 217–228.

- [19] V.L. Colvin, The potential environmental impact of engineered nanomaterials, *Nat. Biotechnol.* 21 (2003) 1166–1170.
- [20] I.A. Cree, P.E. Andreotti, Measurement of cytotoxicity by ATP-based luminescence assay in primary cell cultures and cell lines, *Toxicol. Vitro* 11 (1997) 553–556.
- [21] J. Curtis, M. Greenberg, J. Kester, S. Phillips, G. Krieger, Nanotechnology and nanotoxicology: a primer for clinicians, *Toxicol. Rev.* 25 (2006) 245–260.
- [22] C. Della Torre, F. Buonocore, G. Frenzilli, S. Corsolini, A. Brunelli, P. Guidi, A. Kocan, M. Mariottini, F. Mottola, M. Nigro, K. Pozo, E. Randelli, M.L. Vannuccini, S. Picchiotti, M. Santonastaso, V. Scarcelli, S. Focardi, A. Marcomini, L. Rocco, G. Scapigliati, I. Corsi, Influence of titanium dioxide nanoparticles on 2,3,7,8-tetrachlorodibenzo-p-dioxin bioconcentration and toxicity in the marine fish European sea bass (*Dicentrarchus labrax*), *Environ. Poll.* 196 (2015a) 185–193.
- [23] C. Della Torre, T. Balbi, G. Grassi, G. Frenzilli, M. Bernardeschi, A. Smerilli, P. Guidi, L. Canesi, M. Nigro, F. Monaci, V. Scarcelli, L. Rocco, S. Focardi, M. Monopoli, I. Corsi, Titanium dioxide nanoparticles modulate the toxicological response to cadmium in the gills of *Mytilus galloprovincialis*, *J. Hazard. Mat.* 297 (2015b) 92–100.
- [24] K. Donaldson, P.H. Beswick, P.S. Gilmour, Free radical activity associated with the surface of particles: a unifying factor in determining biological activity? *Toxicol. Lett.* 88 (1996) 293–298.
- [25] A. Dowling, R. Clift, N. Grobert, D. Hutton, R. Oliver, O. O'Neill, J. Pethica, N. Pidgeon, J. Porritt, J. Ryan, et al., in: *Nanoscience and Nanotechnologies: Opportunities and Uncertainties*, The Royal Society, The Royal Academy of Engineering, London, UK, 2004.
- [26] K.A. Dunphy Guzman, J.F. Finnegan, J.F. Banfield, Influence of surface potential on aggregation and transport of titania nanoparticles, *Environ. Sci. Technol.* 40 (2006) 7688–7693.
- [27] M. Dusinska, Z. Magdolenova, L.M. Fjellsbø, Toxicological aspects for nanomaterials in humans. Chapter 1D. Oupicky, in: M. Ogris (Ed.), *Nanotechnology for Nucleic Acid Delivery, Methods in Molecular Biology*, Vol. 948, Humana Press, Springer BWF, 2013, pp. 1–12.
- [28] C. Emmanouil, T.M.T. Sheehan, J.K. Chipman, Macromolecule oxidation and DNA repair in mussel (*Mytilus edulis* L.) gill following exposure to Cd and Cr(VI), *Aquat. Toxicol.* 82 (2007) 27–35.
- [29] M. Farré, K. Gajda-Schranz, L. Kantiani, D. Barcelo, Ecotoxicity and analysis of nanomaterials in the aquatic environment, *Anal. Bioanal. Chem.* 393 (2009) 81–95.
- [30] T. Fatur, T.T. Lah, M. Filipic, Cadmium inhibits repair of UV-, methyl methanesulfonate- and N-methyl-N-nitrosourea-induced DNA damage in Chinese hamster ovary cells, *Mut. Res.* 529 (2003) 109–116.
- [31] G. Federici, B.J. Shaw, R.D. Handy, Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): gill injury, oxidative stress, and other physiological effects, *Aquat. Toxicol.* 84 (2007) 415–430.
- [32] I. Fenoglio, G. Greco, S. Livraghi, B. Fubini, Non-UV-induced radical reactions at the surface of TiO₂ nanoparticles that may trigger toxic responses, *Chem. Eur. J.* 15 (2009) 4614–4621.
- [33] P.A. Gagliardi, A. Puliafito, L. Di Blasio, F. Chianale, D. Somale, G. Seano, F. Federico Bussolino, L. Primo, Real-time monitoring of cell protrusion dynamics by impedance responses, *Sci. Rep.* 15 (5) (2015) 10206.
- [34] M. Geiser, B. Rothen-Rutishauser, N. Kapp, S. Schurch, W. Kreyling, H. Schulz, M. Semmler, V. Im Hoff, J. Heyder, P. Gehr, Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and cultured cells, *Environ. Health Perspect.* 113 (2005) 1555–1560.
- [35] L. Giari, M. Manera, E. Simoni, B.S. Dezfali, Cellular alterations in different organs of European sea bass *Dicentrarchus labrax* (L.) exposed to cadmium, *Chemosphere* 67 (2007) 1171–1181.
- [36] P.L. Goering, M.P. Waalkes, C.D. Klaassen, Toxicology of cadmium, *Toxicol. Met.* 115 (1995) 189–214.
- [37] P. Golstein, G. Kroemer, Cell death by necrosis: towards a molecular definition, *Trends biochem. Sci.* 32 (2007) 37–43.
- [38] V.H. Grassian, P.T. O'Shaughnessy, A. Adamcakova-Dodd, J.M. Pettibone, P.S. Thorne, Inhalation exposure study of titanium dioxide nanoparticles with a primary particle size of 2 to 5 nm, *Environ. Health Perspect.* 115 (2007) 397–402.
- [39] M. Guarino, A. Costa, M. Porro, Photocatalytic TiO₂ coating—to reduce ammonia and greenhouse gases concentration and emission from animal husbandries, *Bioresour. Technol.* 99 (2008) 2650–2658.
- [40] C. Guillard, J. Disdier, C. Monnet, J. Dussaud, S. Malato, J. Blanco, M.I. Maldonado, J.M. Herrmann, Solar efficiency of a new deposited titania photocatalyst: chlorophenol, pesticide and dye removal applications, *Appl. Catal. B* 46 (2003) 319–332.
- [41] J.R. Gurr, A.S.S. Wang, C.H. Chen, K.Y. Jan, Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells, *Toxicology* 213 (2005) 66–73.
- [42] R.D. Handy, T.B. Henry, T.M. Scown, B.D. Johnston, C.R. Tyler, Manufactured nanoparticles: their uptake and effects on fish – a mechanistic analysis, *Ecotoxicology* 17 (2008) 396–409.
- [45] R.A. Hardman, A toxicological review of quantum dots: toxicity depends on physicochemical and environmental factors, *Environ. Health Perspect.* 114 (2006) 165–172.
- [46] N.B. Hartmann, F. Von der Kammer, T. Hofmann, M. Baalousha, S. Otfoellung, A. Baun, Algal testing of titanium dioxide nanoparticles—Testing considerations, inhibitory effects and modification of cadmium bioavailability, *Toxicol* 269 (2010) 190–197.
- [47] A. Hartwig, D. Beyersmann, Comutagenicity and inhibition of DNA repair by metal ions in mammalian cells, *Biol. Trace Elem. Res.* 21 (1989) 359–365.
- [48] M. Heinlaan, A. Ivask, I. Blinova, H.C. Dubourguier, A. Kahru, Toxicity of nanosized and bulk ZnO, CuO and TiO₂ to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*, *Chemosphere* 71 (2008) 1308–1316.
- [49] K. Hirakawa, M. Mori, M. Yoshida, S. Oikawa, S. Kawanishi, Photo-irradiated titanium dioxide catalyzes site specific DNA damage via generation of hydrogen peroxide, *Free Radic. Res.* 38 (2004) 439–447.
- [50] C.O. Ikediobi, V.L. Badisa, L.T. Ayuk-Takem, L.M. Latinwo, J. West, Response of antioxidant enzymes and redox metabolites to cadmium-induced oxidative stress in CRL-1439 normal rat liver cells, *Int. J. Mol. Med.* 14 (2004) 87–92.
- [51] J. Iliopoulou-Georgoudaki, N. Kotsanis, Toxic effects of cadmium and mercury in rainbow trout (*Oncorhynchus mykiss*): a short-term bioassay, *B. Environ. Contam. Toxicol.* 66 (2001) 77–85.
- [52] P. Jezek, L. Hlavatá, Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism, *Int. J. Biochem. Cell. Biol.* 37 (2005) 2478–2503.
- [53] M. Jovic, M. Sharma, J. Rahajeng, S. Caplan, The early endosome: a busy sorting station for proteins at the Crossroads, *Histol. Histopathol.* 25 (2010) 99–112.
- [54] E.I. Jokinen, H.M. Salo, S.E. Markkula, A.K. Immonen, T.M. Aaltonen, Ultra-violet B irradiation modulates the immune system of fish (*Rutilus rutilus*, Cyprinidae). Part III: Lymphocytes, *Photochem Photobiol.* 73 (5) (2001) 505–512.
- [55] V.E. Kagan, H. Bayer, A.A. Shvedova, Nanomedicine and nanotoxicology: two sides of the same coin, *Nanomedicine* 1 (2005) 313–316.
- [56] V. Kandavelu, H. Kastien, K.R. Thampi, Photocatalytic degradation of isothiazolin-3-ones in water and emulsion paints containing nanocrystalline TiO₂ and ZnO catalysts, *Appl. Catal. B* 48 (2004) 101–111.
- [57] S.J. Kang, B.M. Kim, Y.J. Lee, H.W. Chung, Titanium dioxide nanoparticles trigger p53-mediated damage response in peripheral blood lymphocytes, *Env. Mol. Mutagen.* 49 (2008) 300–405.
- [58] H.M. Kipen, D.L. Laskin, Smaller is not always better: nanotechnology yields nanotoxicology, *Am. J. Physiol.-Lung Cell Mol. Physiol.* 289 (2005) L696–L697.
- [59] Kiwi, Nadtochenko, New evidence for TiO₂ photocatalysis during bilayer lipid peroxidation, *J. Phys. Chem. B* 108 (2004) 17675–17684.
- [60] S.J. Klaine, P.J.J. Alvarez, G.E. Batley, T.F. Fernandes, R.D. Handy, D.Y. Lyon, S. Mahendra, M.J. McLaughlin, J.R. Lead, Nanomaterials in the environment: behavior, fate, bioavailability, and effects, *Environ. Sci. Technol.* 27 (2008) 1825–1851.
- [61] J. Kockler, M. Oelgemöller, S. Robertson, B.D. Glass, Influence of titanium dioxide particle size on the photostability of the chemical UV-filters butyl methoxy dibenzoylmethane and octocrylene in a microemulsion, *Cosmetics* 1 (2014) 128–139.
- [62] B.A. Koeneman, Y. Zhang, P. Westerhoff, Y. Chen, J.C. Crittenden, D.G. Capco, Toxicity and cellular responses of intestinal cells exposed to titanium dioxide, *Cell Biol. Toxicol.* 26 (2010) 225–238.
- [63] R. Konaka, E. Kasahara, W.C. Dunlap, Y. Yamamoto, K.C. Chien, M. Inoue, Irradiation of titanium dioxide generates both singlet oxygen and superoxide anion, *Free Radic. Biol. Med.* 27 (1999) 294–300.
- [64] R. Konaka, E. Kasahara, W.C. Dunlap, Y. Yamamoto, K.C. Chien, M. Inoue, Ultraviolet irradiation of titanium dioxide in aqueous dispersion generates singlet oxygen, *Redox Rep.* 6 (2001) 319–325.
- [65] M.L. Kriepke, Photoimmunology, *Photochem Photobiol.* 52 (1990) 919–924.
- [66] G. Kroemer, B. Dallaporta, M. Resche-Rigon, The mitochondrial death/life regulator in apoptosis and necrosis, *Annu. Rev. Physiol.* 60 (1998) 619–642.
- [67] J.C.K. Lai, M.B. Lai, S. Jandhyam, et al., Exposure to titanium dioxide and other metallic oxide nanoparticles induces cytotoxicity on human neural cells and fibroblasts, *Int. J. Nanomed.* 3 (2008) 533–545.
- [68] Y.S. Lee, S. Yoon, H.J. Yoon, K. Lee, H.K. Yoon, J.H. Lee, C.W. Song, Inhibitor of differentiation 1 (Id1) expression attenuates the degree of TiO₂-induced cytotoxicity in H1299 non-small cell lung cancer cells, *Toxicol. Lett.* 189 (2009) 191–199.
- [69] E.K. Leffel, C. Wolf, A. Poklis, J. White, Drinking water exposure to cadmium, an environmental contaminant, results in the exacerbation of autoimmune disease in the murine model, *Toxicology* 188 (2003) 233–250.
- [70] M. Leist, B. Single, A.F. Castoldi, S. Kuhnle, P. Nicotera, Intracellular ATP concentration: a switch deciding between apoptosis and necrosis, *J. Exp. Med.* 185 (1997) 1481–1486.
- [71] Q. Li, S. Mahendra, D.Y. Lyon, L. Brunet, M.V. Liga, D. Li, Antimicrobial nanomaterials for water disinfection and microbial control: potential applications and implications, *Water Res.* 42 (2008) 4591–4602.
- [73] C. Lorenti Garcia, S. Filippi, P. Mosesso, M. Calvani, R. Nicolai, L. Mosconi, F. Palitti, The protective effect of L-carnitine in peripheral blood human lymphocytes exposed to oxidative agents, *Mutagenesis* 21 (2006) 21–27.
- [74] T.C. Long, J. Tajuba, P. Sama, N. Saleh, C. Swartz, J. Parker, S. Hester, G.V. Lowry, B. Veronesi, Nanosize titanium dioxide stimulates reactive oxygen species in brain microglia and damages neurons *in vitro*, *Environ. Health Perspect.* 115 (2007) 1631–1637.
- [75] O.M. Lukandu, T. Bredholt, E. Neppelberg, B.T. Gjertsen, A.C. Johannessen, O.K. Vintermyr, D.E. Costea, Early loss of mitochondrial inner transmembrane potential in khat-induced cell death of primary normal human oral cells, *Toxicol* 263 (2009) 108–116.

- [76] M.P. Mattson, Hormesis defined, *Ageing Res. Rev.* 7 (1) (2008) 1–7.
- [77] S.E. Markkula, H.M. Salo, A.K. Immonen, E.I. Jokinen, Effects of short- and long-term ultraviolet B irradiation on the immune system of the common carp (*Cyprinus carpio*), *Photochem Photobiol.* 81 (3) (2005) 595–602.
- [78] P. Morcillo, D. Romero, J. Meseguer, M.Á. Esteban, A. Cuesta, Cytotoxicity and alterations at transcriptional level caused by metals on fish erythrocytes *in vitro*, *Environ. Sci. Pollut. Res. Int.* 23 (12) (2016a) 12312–12322.
- [79] P. Morcillo, J. Meseguer, M.Á. Esteban, A. Cuesta, In vitro effects of metals on isolated head-kidney and blood leucocytes of the teleost fish *Sparus aurata* L. and *Dicentrarchus labrax* L., *Fish. Shellfish Immunol.* 54 (2016b) 77–85.
- [80] D.S. Nascimento, A. Vale, A.M. Tomas, J. Zou, C.J. Secombes, et al., Cloning, promoter analysis and expression in response to bacterial exposure of sea bass (*Dicentrarchus labrax* L.) interleukin-12 p40 and p35 subunits, *Mol. Immunol.* 44 (2007) 2277–2291.
- [82] A.A. Newairy, A.S. El-Sharaky, M.M. Badrelddeen, S.M. Eweda, S.A. Sheweita, The hepatoprotective effects of selenium against cadmium toxicity in rats, *Toxicol* 242 (2007) 23–30.
- [83] P. Nicotera, M. Leist, E. Ferrando-May, Intracellular ATP, a switch in the decision between apoptosis and necrosis, *Toxicol. Lett.* 102–103 (1998) 139–142.
- [84] M. Nigro, M. Bernardeschi, D. Costagliola, C. Della Torre, G. Frenzilli, P. Guidi, P. Lucchesi, F. Mottola, M. Santonastaso, V. Scarcelli, F. Monaci, I. Corsi, V. Stingo, L. Rocco, n-TiO₂ and CdCl₂ Co-exposure to titanium dioxide nanoparticles and cadmium: genomic, DNA and chromosomal damage evaluation in the marine fish European sea bass (*Dicentrarchus labrax*), *Aquat. Toxicol.* 25 (168) (2015) 72–77.
- [85] G. Oberdörster, A. Maynard, K. Donaldson, V. Castranova, J. Fitzpatrick, K. Ausman, J. Carter, B. Karn, W. Kreyling, D. Lai, et al., Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy, *Part. Fibre Toxicol.* 2 (2005) 8.
- [86] N.S. Parkar, B.S. Akpa, L.C. Nitsche, L.E. Wedgewood, A.T. Place, M.S. Sverdllov, O. Chaga, R.D. Minshall, Vesicle formation and endocytosis: function, machinery, mechanisms, and modeling, *Antioxid. Redox. Signal* 11 (2009) 1301–1312.
- [87] K. Parivar, N. Hayati Rudbari, R. Khanbabaei, M. Khaleghi, The effect of nano-titanium dioxide on limb bud development of NMRI mouse embryo *in vivo*, *Cell J. Summer* 17 (2015) 296–303.
- [88] S. Pavagadhi, M. Sathishkumar, R. Balasubramanian, Uptake of Ag and TiO₂ nanoparticles by zebrafish embryos in the presence of other contaminants in the aquatic environment, *Water Res.* 55 (2014) 280–291.
- [89] S. Petanidis, M. Hadzopoulou-Cladaras, A. Salifoglou, Cadmium modulates H-ras expression and caspase-3 apoptotic cell death in breast cancer epithelial MCF-7 cells, *J. Inorg. Biochem.* 121 (2013) 100–107.
- [90] R. Peters, P. Brandhoff, S. Weigel, H. Marvin, H. Bouwmeester, K. Aschberger, et al., Inventory of Nanotechnology Applications in the Agricultural, Feed and Food Sector External Scientific Report, CFT/EFSA/FEED/2012/01, EFSA supporting publication, 2014, pp. 1–125. EN-621.
- [91] J. Petkovic, B. Zegura, M. Stevanovic, N. Drnovsek, D. Uskokovic, S. Novak, M. Filipic, DNA damage and alterations in expression of DNA damage responsive genes induced by TiO₂ nanoparticles in human hepatoma HepG2 cells, *Nanotoxicol* 5 (2011) 341–353.
- [92] S. Picchiatti, A.M. Fausto, E. Randelli, O. Carnevali, A.R. Taddei, F. Buonocore, G. Scapigliati, L. Abelli, Early treatment with *Lactobacillus delbrueckii* strain induces an increase in intestinal T-cells and granulocytes and modulates immune-related genes of larval *Dicentrarchus labrax* (L.), *Fish. Shellfish Immunol.* 26 (2009) 368–376.
- [93] M.J. Pitkethly, Nanomaterials—the driving force, *NanoToday* 7 (2004) 20–29.
- [95] Q. Rahman, M. Lohani, E. Dopp, H. Pemsel, L. Jonas, D.G. Weiss, D. Schiffmann, Evidence that ultrafine titanium dioxide induces micronuclei and apoptosis in Syrian hamster embryo fibroblasts, *Environ. Health Perspect.* 110 (2002) 797–800.
- [96] J.F. Reeves, S.J. Davies, N.J.F. Dodd, A.N. Jha, Hydroxyl radicals (·OH) are associated with titanium dioxide (TiO₂) nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells, *Mut. Res.* 640 (2008) 113–122.
- [98] V. Rojas, F. Guzman, C. Valenzuela, S.H. Marshall, L. Mercado, Development of a caspase-3 antibody as a tool for detecting apoptosis in cells from rainbow trout (*Oncorhynchus mykiss*) Electron, *J. Biotechnol.* 15 (5) (2012). ISSN 0717–3458.
- [99] R.R. Rosenfeldt, F. Seiz, R. Schulz, M. Bundschuh, Heavy metal uptake and toxicity in the presence of titanium dioxide nanoparticles: a factorial approach using *Daphnia magna*, *Environ. Sci. Technol.* 48 (2014) 6965–6972.
- [100] E.I. Ryabchikova, N.A. Mazurkova, N.V. Shikina, Z.R. Ismagilov, The crystalline forms of titanium dioxide nanoparticles affect their interactions with individual cells, *J. Med. CBR* 8 (2010).
- [101] H.M. Salo, E.I. Jokinen, S.E. Markkula, T.M. Aaltonen, H.T. Penttilä, Comparative effects of UVA and UVB irradiation on the immune system of fish, *J. Photochem Photobiol. B* 56 (2–3) (2000) 154–162.
- [102] C.M. Sayes, A.M. Gobin, K.D. Ausman, J. Mendez, J.L. West, V.L. Colvin, Nano-C₆₀ cytotoxicity is due to lipid peroxidation, *Biomaterials* 26 (2005) 7587–7595.
- [103] C.M. Sayes, R. Wahi, P.A. Kurian, Y.P. Liu, J.L. West, K.D. Ausman, D.B. Warheit, V.L. Colvin, Correlating nanoscale titania structure with toxicity: a cytotoxicity and inflammatory response study with human dermal fibroblasts and human lung epithelial cells, *Toxicol. Sci.* 92 (2006) 174–185.
- [104] I.R. Schultz, E.L. Peters, M.C. Newman, Toxicokinetics and disposition of inorganic mercury and cadmium in channel catfish after intravascular administration, *Toxicol. Appl. Pharmacol.* 140 (1996) 39–50.
- [105] T. Schwarz, A. Urbanski, T.A. Luger, in: T.A. Luger, T. Schwarz (Eds.), *Epidermal Growth Factors and Cytokines*, Dekker, New York, 1993, pp. 453–473.
- [106] B.S. Sekhon, Food nanotechnology – an overview, *Nanotechnol. Sci. Appl.* 3 (2010) 1–15.
- [107] H. Shi, R. Magaye, V. Castranova, J. Zhao, Titanium dioxide nanoparticles: a review of current toxicological data, *Part. Fibre Toxicol.* 15 (2013) 10–15.
- [108] A. Simon-Deckers, B. Gouget, M. Mayne-L'Hermite, N. Herlin-Boime, C. Reynaud, M. Carriere, In vitro investigation of oxide nanoparticle and carbon nanotube toxicity and intracellular accumulation in A549 human pneumocytes, *Toxicology* 253 (2008) 137–146.
- [109] B. Smolkova, N. El Yamani, A.R. Collins, A.C. Gutleb, M. Dusinska, Nanoparticles in food. Epigenetic changes induced by nanomaterials and possible impact on health Food and Chemical, *Toxicology* 77 (2015) 64–73.
- [110] K.F. Soto, A. Carrasco, T.G. Powell, K.M. Garza, L.E. Murr, Comparative *in vitro* cytotoxicity assessment of some manufactured nanoparticle materials characterized by transmission electron microscopy, *J. Nano. Res.* 7 (2005) 145–169.
- [111] B. Steffensen, L. Häkkinen, H. Larjava, Proteolytic events of wound-healing-coordinated interactions among matrix metalloproteinases (MMPs), integrins, and extracellular matrix molecules, *Crit. Rev. Oral Biol. Med.* 12 (2001) 373–398.
- [112] C. Tan, W.-X. Wang, Modification of metal bioaccumulation and toxicity in *Daphnia magna* by Titanium dioxide nanoparticles, *Environ. Pollut.* 186 (2014) 34–42.
- [113] S. Thophon, M. Kruatrachue, E.S. Upatham, P. Pokethitiyook, S. Sahaphong, S. Jaritkhan, Histopathological alterations of white seabass, *Lates calcarifer*, in acute and subchronic cadmium exposure, *Environ. Pollut.* 121 (2003) 307–320.
- [114] F. Thévenod, Cadmium and cellular signaling cascades: to be or not to be? *Toxicol. Appl. Pharmacol.* 238 (2009) 221–239.
- [115] P. Thevenot, J. Cho, D. Wavhal, R.B. Timmons, L.P. Tang, Surface chemistry influences cancer killing effect of TiO₂ nanoparticles, *Nanomed-Nanotechnol* 4 (2008) 226–236.
- [116] R.R. Tice, E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J.C. Ryu, Y.F. Sasaki, Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing, *Environ. Mol. Mutagen* 3 (2000) 206–211.
- [117] B. Trouiller, R. Reliene, A. Westbrook, P. Solaimani, R.H. Schiestl, Titanium dioxide nanoparticles induce DNA damage and genetic instability *in vivo* in mice, *Cancer Res.* 69 (2009) 8784–8789.
- [118] M. Valko, C.J. Rhodes, J. Moncol, M. Izakovic, M. Mazur, Free radicals, metals and antioxidants in oxidative stress-induced cancer, *Chem. Biol. Interact.* 160 (2006) 1–40.
- [119] M.L. Vannuccini, G. Grassi, M.J. Leaver, I. Corsi, Combination effects of nano-TiO₂ and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on biotransformation gene expression in the liver of European sea bass *Dicentrarchus labrax*, *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.* 176–177 (2015) 71–78.
- [121] F. Vincent-Hubert, A. Arini, C. Gourlay-Francé, Early genotoxic effects in gill cells and haemocytes of *Dreissena polymorpha* exposed to cadmium, B[a]P and a combination of B[a]P and Cd, *Mutat. Res.* 723 (2011) 26–35.
- [122] G. Wakefield, J. Stott, J. Hock, Sunscreens and cosmetics containing manganese doped titanium oxide nanoparticles, *SOFW J.* 131 (2005) 46–51.
- [123] H.H. Wang, R.L. Wick, B.S. Xing, Toxicity of nanoparticulate and bulk ZnO, Al₂O₃ and TiO₂ to the nematode *Caenorhabditis elegans*, *Environ. Pollut.* 157 (2009) 1171–1177.
- [124] C. Wei, M. Yamato, W. Wei, X. Zhao, K. Tsumoto, T. Yoshimura, et al., Genetic nanomedicine and tissue engineering, *Med. Clin. North Am.* 91 (2007) 889–898.
- [125] M. Woo, R. Hakem, M.S. Soengas, et al., Essential contribution of caspase 3/ CPP32 to apoptosis and its associated nuclear changes, *Genes Dev.* 12 (1998) 806–819.
- [126] A.H. Wyllie, J.F. Kerr, A.R. Currie, Cell death: the significance of apoptosis, *Int. Rev. Cytol.* 68 (1980) 251–306.
- [127] D. Xiong, T. Fang, L. Yu, X. Sima, W. Zhu, Effects of nano-scale TiO₂, ZnO and their bulk counterparts on zebrafish: acute toxicity, oxidative stress and oxidative damage, *Sci. Total Environ.* 409 (2011) 1444–1452.
- [128] T. Yabu, S. Kishi, T. Okazaki, M. Yamashita, Characterization of zebrafish caspase-3 and induction of apoptosis through ceramide generation in fish fathead minnow tailbud cells and zebrafish embryo, *Biochem. J.* 360 (2001) 39–47.
- [129] N. Yadav, S. Khandelwal, Effect of Picroliv on cadmium-induced hepatic and renal damage in the rat, *Hum. Exp. Toxicol.* 25 (2006) 581–591.
- [130] W.W. Yang, A.J. Miao, L.Y. Yang, Cd²⁺ toxicity to a green alga *Chlamydomonas reinhardtii* as influenced by its adsorption on TiO₂ engineered nanoparticles, *Plos One* 7 (2012) 1–8.
- [131] J.J. Yin, J. Liu, M. Ehrenshaft, J.E. Roberts, P.P. Fu, R.P. Mason, B. Zhao, Phototoxicity of nano titanium dioxides in HaCaT keratinocytes Generation of reactive oxygen species and cell damage, *Toxicol. Appl. Pharmacol.* 263 (1) (2012) 81–88.
- [132] T. Zhang, L. You, Y. Zhang, Photocatalytic reduction of p-chloronitrobenzene on illuminated nano-titanium dioxide particles, *Dyes Pigments* 68 (2006)

- 95–100.
- [133] X. Zhang, H. Sun, Z. Zhang, Q. Niu, Y. Chen, J.C. Crittenden, Enhanced bio-accumulation of cadmium in carp in the presence of titanium dioxide nanoparticles, *Chemosphere* 67 (2007) 160–166.
- [134] R.M. Zucker, E.J. Massaro, K.M. Sanders, L.L. Degn, W.K. Boyes, Detection of TiO₂ nanoparticles in cells by flow cytometry, *Cytom. A* 77 (2010) 677–685.
- [135] K. Fujita, M. Horie, H. Kato, S. Endoh, M. Suzuki, A. Nakamura, A. Miyauchi, K. Yamamoto, S. Kinugasa, K. Nishio, Y. Yoshida, H. Iwahashi, J. Nakanishi, Effects of ultrafine TiO₂ particles on gene expression profile in human keratinocytes without illumination: involvement of extracellular matrix and cell adhesion, *Toxicol. Lett.* 15 (2009) 109–117.
- [136] Z. Krocova, A. Macela, M. Kroca, L. Hernychova, The immunomodulatory effect(s) of lead and cadmium on the cells of immune system in vitro, *Toxicol. Vitro* 14 (2000) 33–40.