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Running title: Oxidative potential of TiO_2 or SiO_2 nanoparticles in A. cepa

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Abstract: The effect of UV-A irradiated or non-irradiated suspensions of agglomerates of TiO_2 or SiO_2 nanoparticles on roots of the onion (*Allium cepa*) has been studied. The reactive potential of TiO_2 nanoparticles which have photocatalytic potential and the non-photocatalytic SiO_2 nanoparticles with the same size of agglomerates was compared. We measured the activity of antioxidant enzyme glutathione reductase (GR), ascorbate peroxidase (A-POD), guaiacol peroxidase (G-POD), catalase (CAT) and lipid peroxidation to assess the oxidative stress in exposed *A. cepa* roots. A wide range of concentrations of nanoparticles was tested (0.1 to 1000 μg/mL). The sizes of agglomerates range in both cases from 300 to 600 nm and the exposure time was 24 h. Adsorption of SiO_2 nanoparticles on the root surface was minimal, but became significant when roots were exposed to TiO_2 agglomerates. No significant biological effects were observed even at high exposure concentrations of SiO_2 and TiO_2 nanoparticles individually. Plants appear to be protected against nanoparticles by the cell wall, which shields the cell membrane from direct contact with the nanoparticles. We discuss the need to supplement conventional phytotoxicity and stress endpoints with measures of plant physiological state when evaluating the safety of nanoparticles.

Keywords: *Allium cepa;* Oxidative stress; TiO_2 nanoparticles; SiO_2 nanoparticles; UV irradiation

INTRODUCTION

Pollution has been an environmental problem since the industrial revolution. Today its range is even bigger due to the increasing human population and new technologies, including nanotechnology. There is growing interest in the effects of unavoidable environmental nanoparticle pollution on plants which are primary producers influencing the entire food chain. The toxic potentials of two widely used nanoparticles, nano-sized silicon dioxide (nano-SiO_2) and nano-sized titanium dioxide (nano-TiO_2) have been reported to be low [1]. In case of TiO_2 nanoparticles however, photoactivation may enhance its biological reactivity [2] and this has not been studied extensively.

Changes in both biotic and abiotic environmental parameters induce responses in exposed organisms. One of the most fundamental reactions to stress conditions is generation of reactive oxygen species (ROS) and consequential oxidative stress. Elevated levels of ROS in cells are handled by ROS scavenging enzymatic and non-enzymatic antioxidants. If such scavenging is absent, oxidative degradation of lipids (lipid peroxidation) and other organic molecules occurs [3].

The main enzymatic ROS scavengers are superoxide dismutase (SOD), ascorbate peroxidase (A-POD), catalase (CAT), glutathione peroxidase (GPX). Together with non-enzymatic antioxidants (ascorbic acid and glutathione) these enzymes constitute efficient machinery for detoxification of O_2^- and H_2O_2 [3]. However, when ROS target lipids, they can initiate the lipid peroxidation process, a chain reaction that produces multiple degradation products, such as malondialdehyde (MDA) and 4-hydroxyalkenals [4]. ROS scavengers and increased lipid peroxidation in biological samples serve as sensitive and reliable biomarkers of oxidative stress.

From an ecotoxicological perspective, TiO_2 nanoparticles have been by far the most extensively studied metal oxide nanoparticles [5]. Nanosized TiO_2 was one of the first widely commercially available nanomaterials and is used in a wide variety of materials and applications, including self-cleaning surface coatings, light-emitting diodes, solar cells, disinfectant sprays, sporting goods, water treatment agents and topical sunscreens [6]. Such widespread use of nanosized TiO_2 can result in significant release of TiO_2 nanoparticles into the environment leading to increased environmental exposure to these nanoparticles. Additionally, nano-TiO_2 in the form of nanopraticulate anatase is known to be a photocatalyst and is capable of undergoing electron transfer reactions under light.

There are reports of positive, negative or sporadic, inconsequential effects of TiO_2 nanoparticles on plants [7-13]. Many of these authors examined the effects of TiO_2 nanoparticles on plants with particular reference to oxidative stress and among these Foltête et al. [10] detected, contrary to expectations, decreased activity of antioxidant enzymes in roots of *Vicia faba*. Similarly, no parameters related to oxidative stress were found in *Triticum aestivum* after 24 hours exposure to TiO_2 nanoparticles [8] but increased lipid peroxidation which was not dose-dependent was reported in roots of *Allium cepa* [12]. Wang et al. [14] reported that TiO_2 nanoparticles transiently induce oxidative stress and some selected stress response genes (sod1, gpx, cat and ptox2) are up-regulated in cells of the green alga *Chlamydomonas reinhardtii*.

Semiconducting TiO_2 nanoparticles are activated by absorption of light of wavelength below 380 nm. There are few data on the effects of TiO_2 nanoparticles in combination with UV radiation on plants. Kim and Lee [15] report that TiO_2 nanoparticles in combination with UV-A irradiation inhibit the photosynthetic activity of the algae *Anabaena, Microcystis* and *Melosira*. Lee and An [16] also report growth inhibition of the alga *Pseudokirchneriella subcapitata* due to TiO_2 nanoparticles but the level of inhibition was the same in all tested irradiation conditions (visible, UV-A and UV-B light). Lei et al. [17] describe significantly decreased ROS formation and lipid peroxidation in spinach chloroplasts after exposure to TiO_2 nanoparticles and UV-B radiation and attributed this to increased activity of the enzymatic antioxidants SOD, CAT, G-POD and A-POD. They concluded that anatase TiO_2 nanoparticles could decrease the oxidative stress in spinach chloroplasts caused by UV-B radiation. Similarly, Hong et al. [18] also report that nano-TiO_2 treatment of spinach could significantly increase the activities of SOD, CAT and POD and decrease the accumulation of reactive oxygen radicals generated by 500 μmol m^{-2} s^{-1} light intensity. The level of the lipid peroxidation product malondialdehyde is thereby reduced, and the stability of a membrane structure of chloroplast exposed to light is maintained.

Nanoparticulate SiO_2 is also a very popular nanomaterial, used in packaging, high-molecule composite materials, ceramics, labelling, imaging, drug delivery, cancer therapy, as a biosensor, and in food and cosmetics [19,20]. SiO_2 nanoparticles have extremely high surface activity and porous structure and because of their adsorption properties, Si-based nanomaterials are expected to be one of the most promising carriers suitable for development of high performance antibacterial materials [21,22].

When compared to data on nano-TiO_2, there is a paucity of reports on the phytotoxicity of SiO_2 nanoparticles. Lee et al. [23] describe the low inhibitory effect of SiO_2 nanoparticles on *Arabidopsis* root elongation, but report no effect on seed germination and leaf number after exposure concentrations ranging up to 4000 mg/L nano-SiO_2. Lin et al. [24] report that nanostructured SiO_2greatly enhances the seedling height, root collar diameter, main root length and the number of lateral roots of *Larix olgensis*. Slomberg and Schoenfisch [25] studied a range of well characterized silica nanoparticles and concluded that they are not phytotoxic to *Arabidopsis thaliana.* However, at the same time they suggest that an indirect negative effect of Si-based nanoparticles is possible due to the adsorption of nutrients by the particles which thus become unavailable for uptake and transport leading to physiological disturbances in the plant.

It has previously been reported that both these particles TiO_2 and SiO_2 provoke oxidative stress in plants [17,25] and in animals [1]. Additionally, dissolution of ions from TiO_2 or SiO_2 nanoparticles does not occur as in metal based particles and the effect on biota therefore can be ascribed to particles and not to dissolved ions [26,27]. When the available data on the effects of TiO_2 or SiO_2 nanoparticles on plants or animals are compared, some significant differences can be seen. For example, much lower effective concentrations were reported for aquatic animals than for plants [1,5]. Various authors have suggested that the cause of such differences can be traced to the plant cell wall which prevents direct contact between nanoparticles and cell membrane [8,28].

The concept of the present study is similar to that conducted on mussels (*Mytilus galloprovincialis)* [1]. In both studies photocatalytically active nanoparticles (nano-TiO_2) and nanoparticles lacking photocatalytic activity (nano-SiO_2) were tested. Onion (*Allium cepa*) plants were incubated for 24 hours in a suspension of either TiO_2 or SiO_2 nanoparticles and subsequently oxidative stress biomarkers were analysed. We also compared the effects of UV-A irradiation on the reactivity of both nanoparticles and investigated their adsorption on the root surface .

We assumed that biochemical stress markers are adequately sensitive to show the oxidative potential of TiO_2 or SiO_2 nanoparticles after 24 hours of exposure to particles in a wide concentration range. We hypothesized that UV-irradiated nanoparticles of TiO_2 may be more biologically potent than non-irradiated TiO_2 and SiO_2 nanoparticles and we expected higher effect concentrations for plants than have been reported for animals.

MATERIALS AND METHODS

Characterisation of nanoparticles

The TiO_2 nanoparticles were supplied by Sigma-Aldrich in the form of a powder with 99.7% purity in anatase crystalline structure with a surface area of 190 to 290 m^2/g. The same batch of particles has been used previously and is described elsewhere [29]. The SiO_2 nanoparticles were provided by Nanologica AB (batch code NNV-001 for nanoValid partners). The surface area of the nano-SiO_2 particles was 860 m^2/g, the pore size 51 Å and the pore volume 1.08 cm^3/g. The shape and size of individual particles were inspected by transmission electron microscopy (TEM) (Jeol 2010F, Jeol 2100 TEM at the Jozef Stefan Institute, Ljubljana, Slovenia). For the TEM investigations the particles were deposited by drying a suspension on a copper-grid-supported, perforated, transparent carbon foil. Secondary characteristics of particles were measured in distilled water, which was also the exposure medium. The zeta potential of the nanoparticles dispersed in water was measured using ZetaPALS (Brookhaven Instruments Corp.) and the size distribution of the particles in the suspensions was determined by dynamic light scattering (DLS) using Fritsch Analisette 12 DynaSizer.

Allium growth and exposure to nanoparticles and UV-A irradiation

The experimental design in the present study was the same as reported previously [30]. Briefly, onion bulbs (*Allium cepa* L.) were grown in distilled water (Millipore Milli-RX 45) at room temperature (23 ± 2°C) for 24 h when roots 1 cm in length were formed. Subsequently, the bulbs with developed roots were exposed for a further 24 h to suspensions with different concentrations of TiO_2 or SiO_2 nanoparticles.

Nanoparticles were suspended in distilled water to prepare stock suspensions with 1000 μg/mL nano-TiO_2 or 1000 μg/mL nano-SiO_2. These stock suspensions were sonicated for 72 hours in an ultrasonic bath (Sonis pio 2GT, Iskra Pio). Suspensions used in exposure experiments were prepared from the stock by dilution with distilled water to give concentrations of 0.1, 1, 10 and 100 μg/mL of either nanoparticle. Control bulbs were grown in pure distilled water. Water was used as the exposure medium in order to reduce the interactions between nanoparticles and test media during the exposure as described by Klančnik et al. [30] and to limit the agglomeration of particles. It is known that nanoparticles agglomerate to a larger extend in aqueous suspensions with increased ionic strength compared to pure water.

Thirty onion bulbs were used in each experiment and five replicates (five bulbs exposed individually) were in each concentration. Four sets of experiments were conducted. In two of these, bulbs were exposed to

TiO_2 nanoparticles and in the other two to SiO_2 nanoparticles. One experimental set of bulbs and each type of nanoparticles was UV-A irradiated (Sylvania light bulb black-light blue F30W/BLB-T8; UV-A spectrum 350 to 400 nm, power 30 W), while the other was not. The irradiation period was 8 h/d of UV-A, followed by 16 h/d of room lighting conditions.

At the end of the 24 h exposure, all roots of each bulb were dried with paper towels, then cut, frozen in liquid N_2 and stored at -80 °C prior to analysis. In experiments with TiO_2 and SiO_2 nanoparticles, some roots were also prepared for SEM/EDX investigation (EDS/WDS Oxford Instruments INCA, Jeol JSM-6500F at the Institute of Metals and Technology, Ljubljana).

Antioxidant enzymes analysis

A sample of ~50 mg of *A. cepa* deeply frozen roots per treatment was homogenized in 1.5 ml of potassium phosphate buffer (100 mM, pH 7.0) and centrifuged (20 min, 16,350 *g*, 4 °C, Eppendorf Centrifuge 5417 R). Supernatants were measured spectrophotometrically (UV-1800 Shimadzu Spectrophotometer).

Protein content in each supernatant was assessed with the BCA Protein Assay Kit (Novagen, USA) using bovine serum albumin as a calibration standard.

Antioxidant enzyme activities were measured according to the modified protocols described by Razinger et al. [31,32]. Briefly, catalase (CAT, EC 1.11.1.6) activity was assessed by measuring the consumption of H_2O_2 at 240 nm (ε=40 $mM^{-1}\cdot cm^{-1}$). The reaction mixture contained 900 µL of potassium phosphate buffer (50 mM, pH 7.0 with 10 mM H_2O_2) and 100 µL of the sample. Guaiacol peroxidase (G-POD, EC 1.11.1.7) activity was measured as the increase of tetraguaiacol absorbance at 470 nm (ε=26.6 $mM^{-1}\cdot cm^{-1}$). The reaction mixture contained 900 µL of potassium phosphate buffer (50 mM, pH 7.0 with 10 mM H_2O_2 and 1% guaiacol) and 100 µL of the sample. Ascorbate peroxidase (A-POD, EC 1.11.1.11) was assesed from the decrease in ascorbate absorbance at 290 nm (ε=2.8 $mM^{-1}\cdot cm^{-1}$). The reaction mixture contained 800 µL of potassium phosphate buffer (50 mM, pH 7.0 with 10 mM H_2O_2 and 5 mM Na-ascorbate) and 200 µL of the sample. Glutathione reductase (GR, EC 1.6.4.2) activity was assayed as the increase of 2-nitro-5-thiobenzoic acid (TNB) absorbance at 412 nm (ε=13.6 $mM^{-1}\cdot cm^{-1}$). The reaction mixture contained 700 µL of potassium phosphate buffer (100 mM, pH 7.6, with 0.15 mM NADPH, 6.3 mM Na-EDTA and 15 mM 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB)), 100 µL of 20 mM glutathione, oxidized form (GSSG) and 200 µL of the sample.

Lipid peroxidation analysis

Lipid peroxidation was indirectly estimated from the formation of malondialdehyde (MDA). 50 mg of *A. cepa* deeply frozen roots were homogenized in 1.5 mL of thiobarbituric acid/trichloracetic acid (TBA/TCA) reagent, consisted of 0.3% (w/v) 2-thiobarbituric acid and 10% (w/v) trichloroacetic acid. After homogenization, samples were incubated at 95 °C for 30 min, then chilled on ice and centrifuged (20 min, 16,350 *g*, 4 °C, Eppendorf Centrifuge 5417 R). The absorbance of the supernatant was measured at 535 nm and 600 nm. The concentration of MDA was calculated using the extinction coefficient ε=155 $mM^{-1} \cdot cm^{-1}$ [32].

Root preparation for scanning electron microscopy (SEM)

Roots exposed to nano-TiO_2 or nano-SiO_2 were investigated by EDX/SEM. After exposure, roots were cut and transferred to the fixative containing 2.5% glutaraldehyde, 0.4% paraformaldehyde and 0.1 M sodium phosphate buffer (pH 7.2). Primary aldehyde fixation was followed by postfixation with 1% OsO_4. The fixed roots were dehydrated in series of ethanol and dried with hexamethyldisilizane. After drying, roots were mounted on holders with silver paint (SPI) and carbon sputtered (Sputter coater SCD 050, BAL-TEC). Energy dispersive X-ray analysis (EDX) was used to establish the chemical composition of the material on the root surface.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (Ver. 4.0) and Microsoft Excel 2007. The significance of the difference between exposed and control plants was tested by One-way ANOVA and Dunnett's multiple comparison test. The significance of difference between both types of nanoparticles and light conditions was tested by Two-way ANOVA and Bonferoni multiple comparison test. The level of significance was accepted at *p* values lower than 0.05 and is denoted in the Figures by an asterisk.

RESULTS

Nanoparticles' characteristics

TEM revealed that the TiO_2 is in a form of agglomerates of primary nanoparticles, several hundreds of nm in size (Figure 1A). The primary particle size of the TiO_2 nanoparticles as observed by TEM was approximately 10 nm. The globular, mesoporous, amorphous SiO_2 nanoparticles had diameters ranging from approximately 60 to 250 nm (Figure 1B).

The suspension of nano-SiO_2 sedimented more slowly than the suspension of nano-TiO_2. The superior stability of the nano-SiO_2 suspension is consistent with its much higher, negative zeta potential (Table 1). The silica surface is always terminated with silanol (-Si-OH) groups, which provide a relatively strong negative surface charge at neutral pH. The electro-kinetic measurements showed a zeta potential of -27 mV for the nano-SiO_2 suspension and -9 mV for the suspension of nano-TiO_2. However, the DLS measurement showed comparable hydrodynamic size of the particles in both suspensions (Figure 2). The majority of particles had a size which was between 300 and 600 nm. Some larger agglomerates with size up to 2 μm were also detected while even larger agglomerates most probably sedimented before the completion of the DLS measurement.

Nano-TiO_2 and nano-SiO_2 attached to the root surface

The SEM imaging revealed intense adsorption of agglomerates of TiO_2 nanoparticles on the roots incubated in the suspension with nano-TiO_2 (Figure 3 A, B). Significant adsorption was not expected in the case of the nano-SiO_2 suspension because of the large primary particle size (Figure 2 C, D). This was confirmed by SEM. Energy dispersive X-ray analysis (EDX) revealed chemical composition of deposits adhered to the roots (Figure 4). Titanium was detected only on TiO_2-exposed roots while silicon was found on all tested roots but its content was very low on control and SiO_2-exposed roots (Table 2).

Effects of suspensions of TiO_2 or SiO_2 nanoparticles on A. cepa *roots*

The exposure of *A. cepa* to suspensions of TiO_2 or SiO_2 nanoparticles had no effect on the measured activity of antioxidative enzymes and on lipid peroxidation (Figure 5). The interaction between particle concentration and particle type, calculated with Two-way ANOVA revealed no detectable statistically significant differences in enzyme activities or MDA content between control and treated roots (p value > 0.05). Using additional statistical post-tests, significant differences were detected only in GR activity and MDA content as follows. GR activity in roots exposed to 1 and 1000 μg/mL nano-TiO_2 was 38.6% and 49.4% higher than in roots exposed to the same concentration of nano-SiO_2 (p value < 0.05 and 0.01 respectively) (Figure 5). The MDA content in roots exposed to 1000 μg/mL nano-SiO_2 was 73% higher than in roots exposed to the same concentration of nano-TiO_2 (p value < 0.01) (Figure 5).

An additional set of experiments was conducted to test the hypothesis that nano-TiO_2 is more biologically potent when irradiated with UV-A. The same biochemical parameters were measured as in experiments without UV-A irradiation (Figure 6). The enzyme activity of G-POD, A-POD and GR increased

when roots were also irradiated but decreased in the case of CAT (*p* value calculated with One-way ANOVA was 0.042 for G-POD, 0.001 for A-POD, < 0.0001 for GR and 0.0008 for CAT). When interaction between particle concentration and irradiation was tested by Two-way ANOVA the only significant difference was detected for CAT activity ($p = 0.048$). Lipid peroxidation expressed as MDA content was not affected by nano-TiO_2 or by UV-A irradiation.

Similar results to TiO_2 studies were obtained when roots were co-exposed to nano-SiO_2 and UV-A irradiation. The activities of antioxidant enzymes and lipid peroxidation showed no significant change (data not shown).

DISCUSSION

We provide experimental evidence that neither UV-A irradiated or non-irradiated suspensions of TiO_2 or SiO_2 nanoparticles (both at concentrations from 0.1 to 1000 μg/mL) exhibit oxidative potential on *Allium cepa* roots after 24 h of incubation.

In our study, primary particle size for nanocrystalline anatase TiO_2 was ~10 nm while their agglomerates were a few hundred nm in size, and further agglomerate when suspended in water. The size of the majority of agglomerates in water was from 300 to 600 nm (Table 1, Figure 2). The primary particle size of amorphous SiO_2 nanoparticles was one order of magnitude larger than the nano-TiO_2 and was comparable to that of the TiO_2 agglomerates. The size of the SiO_2 agglomerates in the water suspension was also in the same size range as nano-TiO_2 (Table 1, Figure 2). Similar TiO_2 nanoparticles were used to test growth inhibition of alga *Pseudokirchneriella subcapitata* [16] and accumulation, translocation and impact of TiO_2 nanoparticles in wheat (*Triticum aestivum* spp.) [8]. Larue et al. [8] found size-dependent accumulation of TiO_2 nanoparticles in roots and root-to-shoot translocation of particles when the particle size was ≤ 36 nm. These authors suggest that nanoparticle accumulation in the root could be ascribed to increased cell wall porosity which results in transport of nanoparticles from the surface into the deeper root tissues *via* the apoplast. Cell wall porosity can increase directly *via* interactions between nanoparticles and pectin matrix or indirectly by local ROS generation. In the same study the production of H_2O_2 increased insignificantly.

The TiO_2 and SiO_2 nanoparticles used in the present study proved to be non-toxic after short-time exposure. Enzyme activity and lipid peroxidation was similar in TiO_2- and SiO_2-treated roots and remained close to the control values (Figure 5). The only significant increase was measured in GR activity and MDA

content at selected TiO_2 concentrations but the effect was dose-independent. Non-specific increase of MDA in *A. cepa* roots exposed to 4 mM TiO_2 was reported also by Ghosh [12]. Non-toxic effects of TiO_2 were also demonstrated for *Salix* sp. [7], *Zea mays* [28], *Triticum aestivum* [8] and some other common crop plants [9].

Decreased activity of antioxidant enzymes in roots of *Vicia faba* [10] as well as increased enzyme activity in spinach chloroplasts [17] has been reported. Song et al. [33] found oxidative stress response after incubating *Lemna minor* in a suspension of nano-TiO_2 for 7 days. Landa et al. [34] reported that only mild changes in gene expression of *Arabidopsis thaliana* were observed upon 7 days exposure to TiO_2 nanoparticles at a concentration of 100 mg/L. These changes resulted in up- and down-regulation of genes involved mainly in responses to biotic and abiotic stimuli. No other effects were found in roots but despite this, it is important not to overlook the possibility of indirect effects from exposure of roots to TiO_2 nanoparticles.

As expected, we also failed to find oxidative potential of agglomerates of nano-SiO_2 measured with conventional oxidative stress biomarkers. This is consistent with reports provided by other authors who also found low toxic potential of SiO_2 nanoparticles [24,35]. As was shown for TiO_2, Slomberg and Schoenfisch [25] pointed to indirect effect of SiO_2 nanoparticles on plants either by pH alteration of the growing medium after addition of nanoparticles or by adsorption of nutrients by the particles, thus making them unavailable for the uptake and transport leading to physiological disturbances in the exposed plants.

However, we proved that the adsorption of nano-TiO_2 agglomerates on root surface (Figure 3) was as found by Asli and Neumann [28]. They showed that in *Zea mays* rapid inhibition of leaf growth and transpiration occur in plants grown in hydroponic solution with TiO_2 nanoparticles. They surmised that reduced water availability was caused by external nanoparticles and the associated leaf responses appeared to involve a rapid physical inhibition of apoplastic flow through nanosized pores among the fibrils in the root cell walls rather than the toxic effects of nanoparticles. Other authors have provided evidence that suspensions of nanosized materials such as large polymer molecules, China ink pigments or gold nanoparticles might reduce water flow into plant cells and tissues by accumulating in the cell walls [36,37]. It was also shown that some nanoparticles (Si, Au, CdSe) inhibit the ezyme activity of purified enzyme lactate dehydogenase because they change its structure [38]. The inhibiotion of enzymes by nanoparticles during the test might lead to false negative or false positive results therefore the authors suggest caution when interpreting the results of nanotoxicology testing methods. We think that post-exposure inhibition was not the case in our study because

no differences in the enzyme activity were observed in a range of tested concentrations of nanoparticles. If post-exposure inhibition during the tests occured it is expected to be dose related. In our study, the centrifugation process most likely removed nanoparticles from the root surface but their concentration in the supernatant was too low to influence the results of the subsequent biochemical assays.

A similar comparative study on the oxidative stress potential of TiO_2 and SiO_2 nanoparticles was conducted by Canesi et al. [1] on mussels (*Mytilus galloprovincialis*). In this case, mussels were exposed to suspensions of nano-TiO_2 (primary particle size 22 nm and size of agglomerates in a range 50 to 1600 nm) or nano-SiO_2 (primary particle size 12 nm and size of agglomerates in a range 150 to 1600 nm) for 24 hours. The oxidative potential of both TiO_2 and SiO_2 nanoparticles was confirmed by increased activity of enzymatic antioxidant catalase and by significant destabilisation of the lysosomal membrane. Concentrations causing such effects were up to 5 mg/L. Concentrations tested in our study were in the range 0.1 to 1000 mg/L but we failed to detect the oxidative potential of nano-TiO_2 (primary particle size 15 nm and size of agglomerates in the range 300 to 600 nm) or nano-SiO_2 (primary particle size 100 nm and size of agglomerates in the range 300 to 600 nm). This indicates significant differences in the effective concentration of particles with the same secondary characteristics when oxidative stress related biomarkers are measured after 24 h of exposure. The differences in response to nanoparticle exposure between plants and animals could be attributed to the cell structure. Plants, fungi and bacteria have cell walls which constitute a primary site for interactions and a barrier for the entrance of nanoparticles into the cell [28,37]. A similar explanation was suggested in case of water plant *Lemna minor* where TiO_2 nanoparticles adhered to leaves but no cellular internalisation was observed [39] and in cell wall free mutant green alga *Chlamydomonas reinhardtii* with higher accumulation rate of carbonate coated silver nanoparticles [36]. In the second set of experiments, the co-exposure to nanoparticles and UV-A irradiation was tested. The fact that TiO_2 is photoactivated by UV-A is well known but only limited research have been reported on the effect of co-exposure to living organisms. In the present study, the enzyme activity increased mainly when UV-A irradiation was applied but the overall effect of double exposure (nanoparticles and UV-A) was insignificant and similar to that in control plants (Figure 6). On the other hand, photoactivation of TiO_2-coated beads with UV-A caused rapid decrease of photosynthetic activity in the algae *Anabaena, Microcystis* and *Melosira* leading to altered and reduced growth after longer exposure time of 8 days [15]. In the study reported by Tiano et al. [40] the effect of modified TiO_2 nanoparticles and UV-A was tested on human skin

fibroblast. The results showed that anatase TiO_2 particles retained photocatalytic activity and reduced cell viability as shown by various effects including DPPH photobleaching, deoxyribose degradation, DNA damage and ROS formation. Contrary to these findings, Lei et al. [17] described that TiO_2 nanoparticles decreased the oxidative stress caused by UV-B radiation in spinach chloroplasts where accumulation of ROS decreased due to the elevated activities of antioxidant enzymes.

Based on the study presented here and our previous study [30] we conclude that TiO_2 and SiO_2 nanoparticles do not cause significant changes in oxidative status after short exposure time. Also co-exposure to UV-A irradiation fails to cause toxic effects. Further research with additional markers and longer exposure times is needed to assess the physiological potential of nanoparticles like physical inhibition of water flow throughout the plant.

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Figure 1. TEM micrographs of TiO_2 nanoparticles (A) and SiO_2 nanoparticles (B). The scale bars indicate 100 nm.

Figure 2. Number and volume weighted size distribution of TiO_2 (A, B) and SiO_2 (C, D) particles in suspensions at a concentration of 10 μg/mL, measured with DLS.

Figure 3. SEM micrographs of roots exposed to TiO_2 (A) or SiO_2 nanoparticles (suspensions with concentration 1000 μg/mL) (C) and control roots grown in distilled water (E). Root surface covered with suspension of TiO_2 (B) or SiO_2 (D) nanoparticles and control roots (F) with marked areas where EDX spectra were taken (values in Table 2). The scale bars in Figures A, C and E indicate 50 μm and in Figures B, D and F indicate 20 μm.

Figure 4. EDX spectrum of control (A) and TiO_2 exposed (B) root (area of Spectrum 1 marked in Figure 3F and 3B respectively). Among other elements the presence of Ti, Si and Os (which was used for root fixaton) is detected.

Figure 5. Specific enzyme activity (SEA) of guaiacol peroxidase (G-POD), ascorbate peroxidase (A-POD), glutathione reductase (GR) and catalase (CAT) and MDA content in roots exposed to suspensions with different concentrations of TiO_2 (•) and SiO_2 (□) nanoparticles. Separate data and the median values (N=5) are shown as a solid line for TiO_2 and a dashed line for SiO_2. The level of significance was accepted at p value < 0.05 (*) and p value <0.01 (**).

Figure 6. Specific enzyme activity (SEA) of guaiacol peroxidase (G-POD), ascorbate peroxidase (A-POD), glutathione reductase (GR) and catalase (CAT) and MDA content in roots exposed to suspensions with different concentrations of nano-TiO_2 and no UV-A irradiation (•) and UV-A co-exposed (□). Separate data and the median values (N=5) are shown as a solid line for no UV and a dashed line for UV-A irradiated plants. The level of significance was accepted at p value < 0.05 (*) and p value < 0.01 (**).

Table 1. Zeta (ζ) potential values and results of DLS measurements for both tested suspensions at a concentration of 10 µg/mL

Nanoparticles suspension	pH-value	ζ-potencial[a] (mV)	Size distribution (nm)
SiO_2	6.2	-27±3	300 – 600
TiO_2	7.3	-9±5	300 - 600

[a] Mean value ± error is presented for the zeta potential.

Table 2. EDX spectra analysis of root surface shown as weight %

	Control		TiO_2[a]		SiO_2[b]	
Measurement spot	Ti (%)	Si (%)	Ti (%)	Si (%)	Ti (%)	Si (%)
1	0	0.89	29.53	1.04	0	9.60
2		0.88	18.11	1.08		1.49
3		0.91	0.79	0.66		1.43
4			27.62	1.06		5.35
5			9.87	1.02		
6			5.22	0.93		
7			14.39	1.58		
8			3.92			
mean±SE	0.00±0.00	0.89±0.01	13.68±3.81	1.05±0.10	0.00±0.00	4.47±1.94

[a] Ti and Si presence (shown as weight %) on the roots exposed to 1000 µg/mL TiO_2.

[b] Ti and Si presence (shown as weight %) on the roots exposed to 1000 µg/mL SiO_2.

SE = standard error

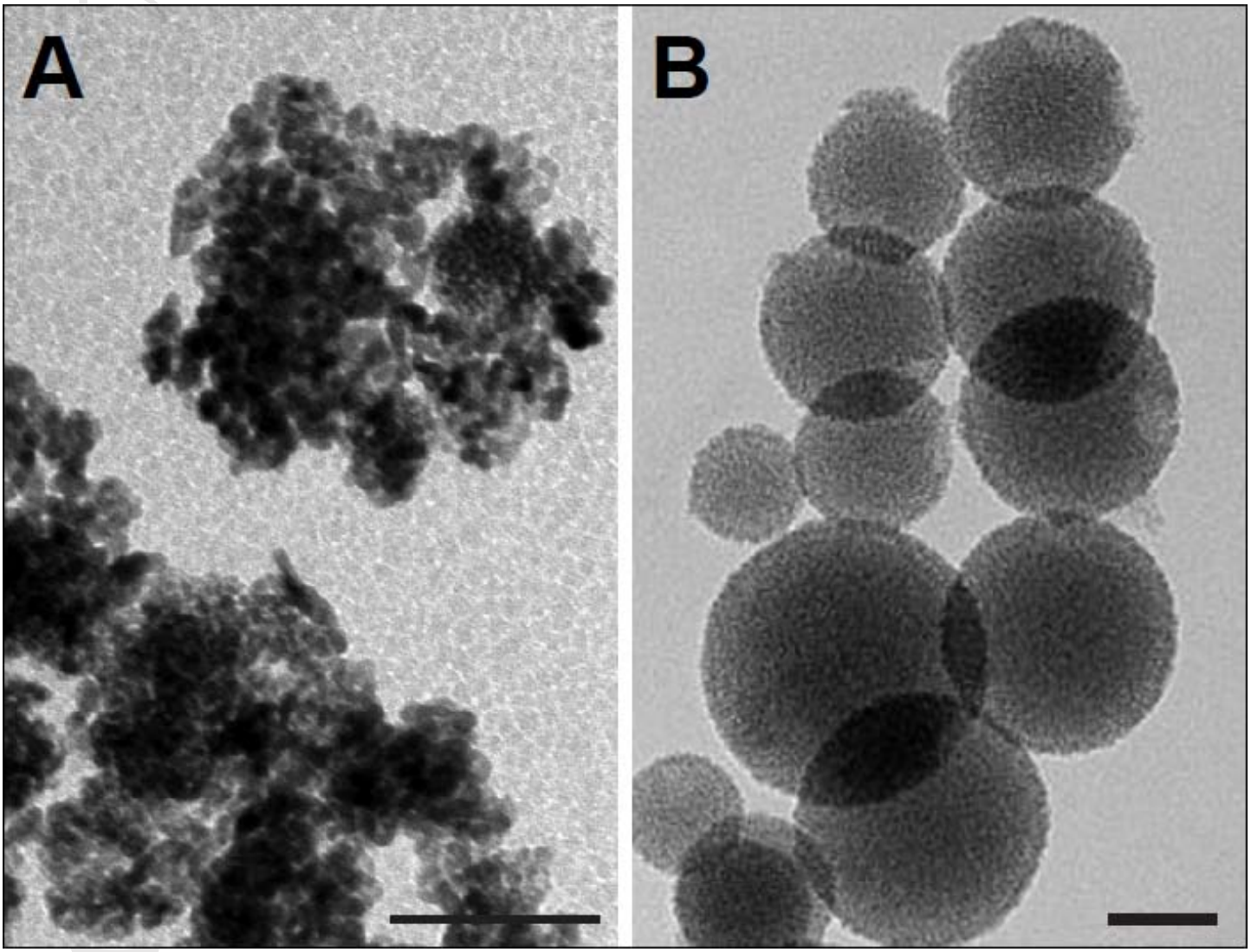

Figure 1

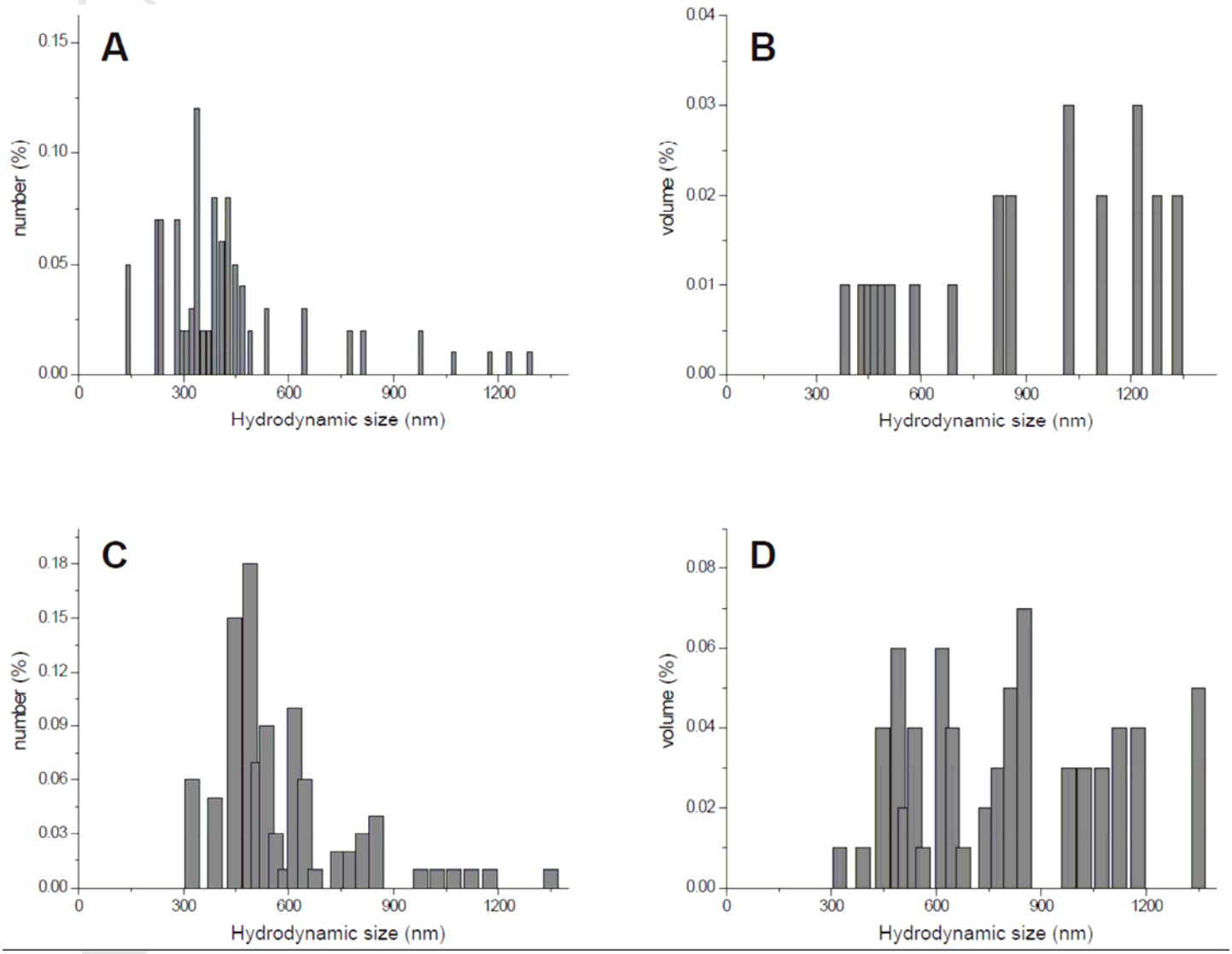

Figure 2

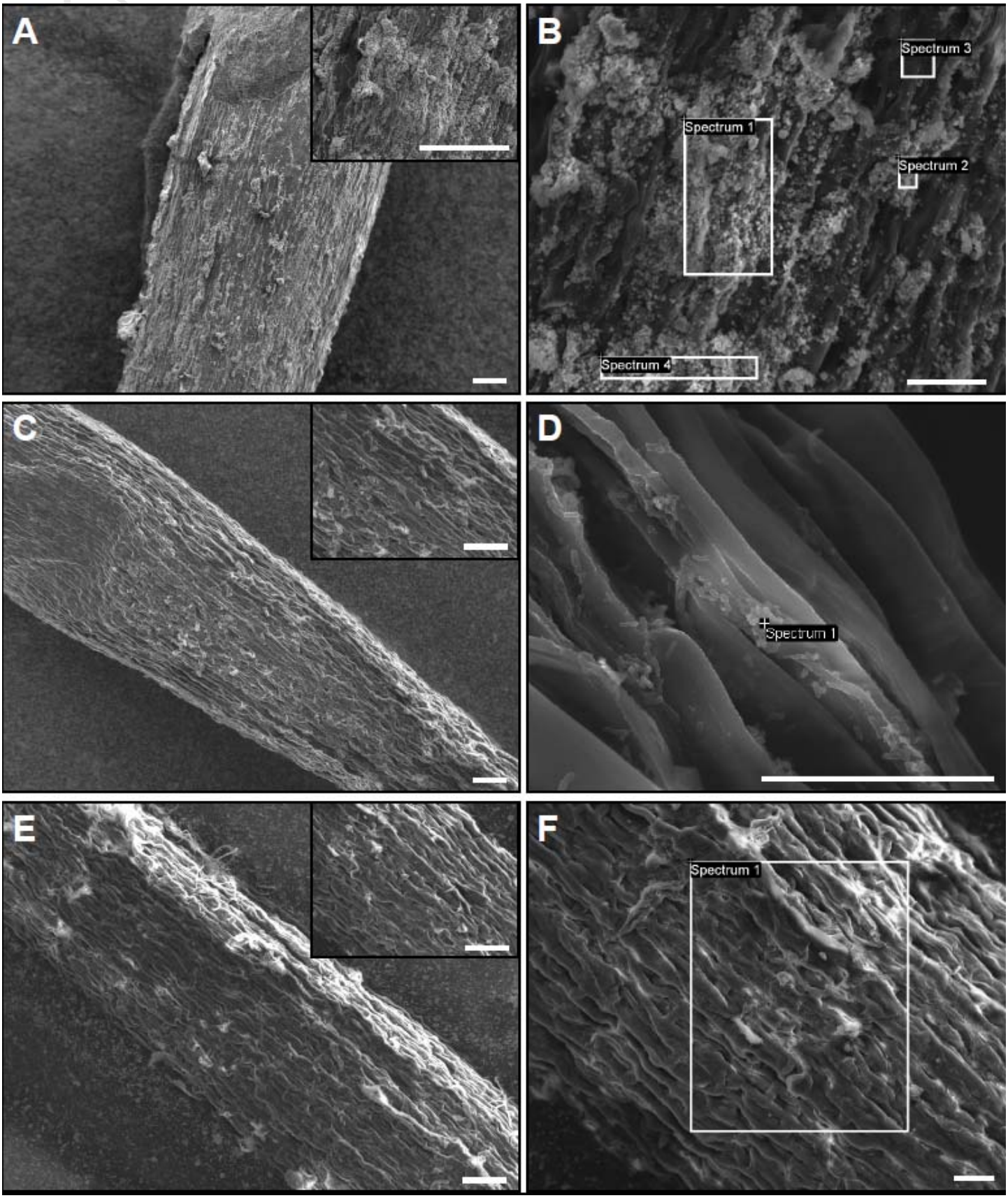
A
B
Spectrum 3
Spectrum 1
Spectrum 2
Spectrum 4
C
D
Spectrum 1
E
F
Spectrum 1

Figure 3

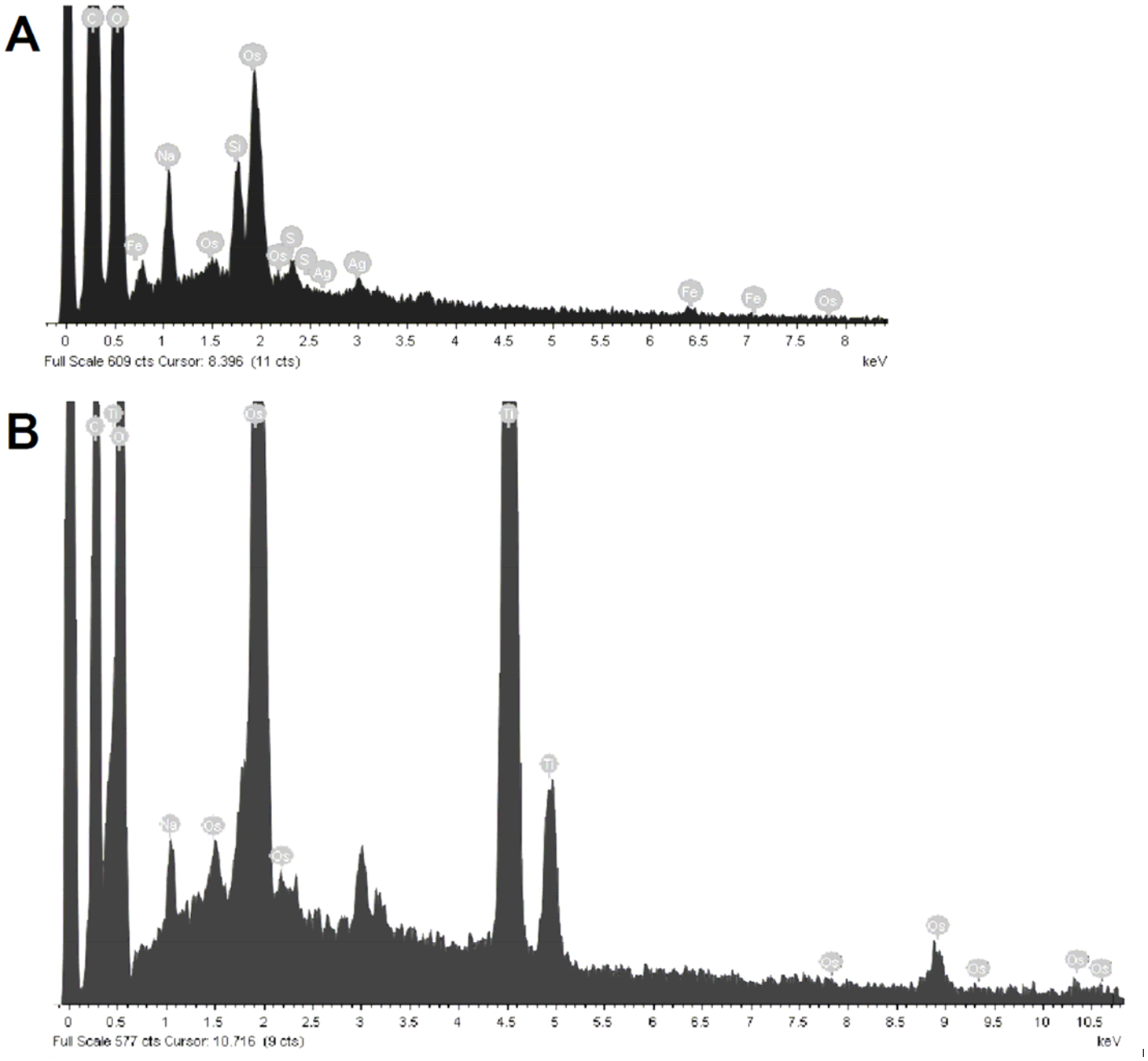

Figure 4

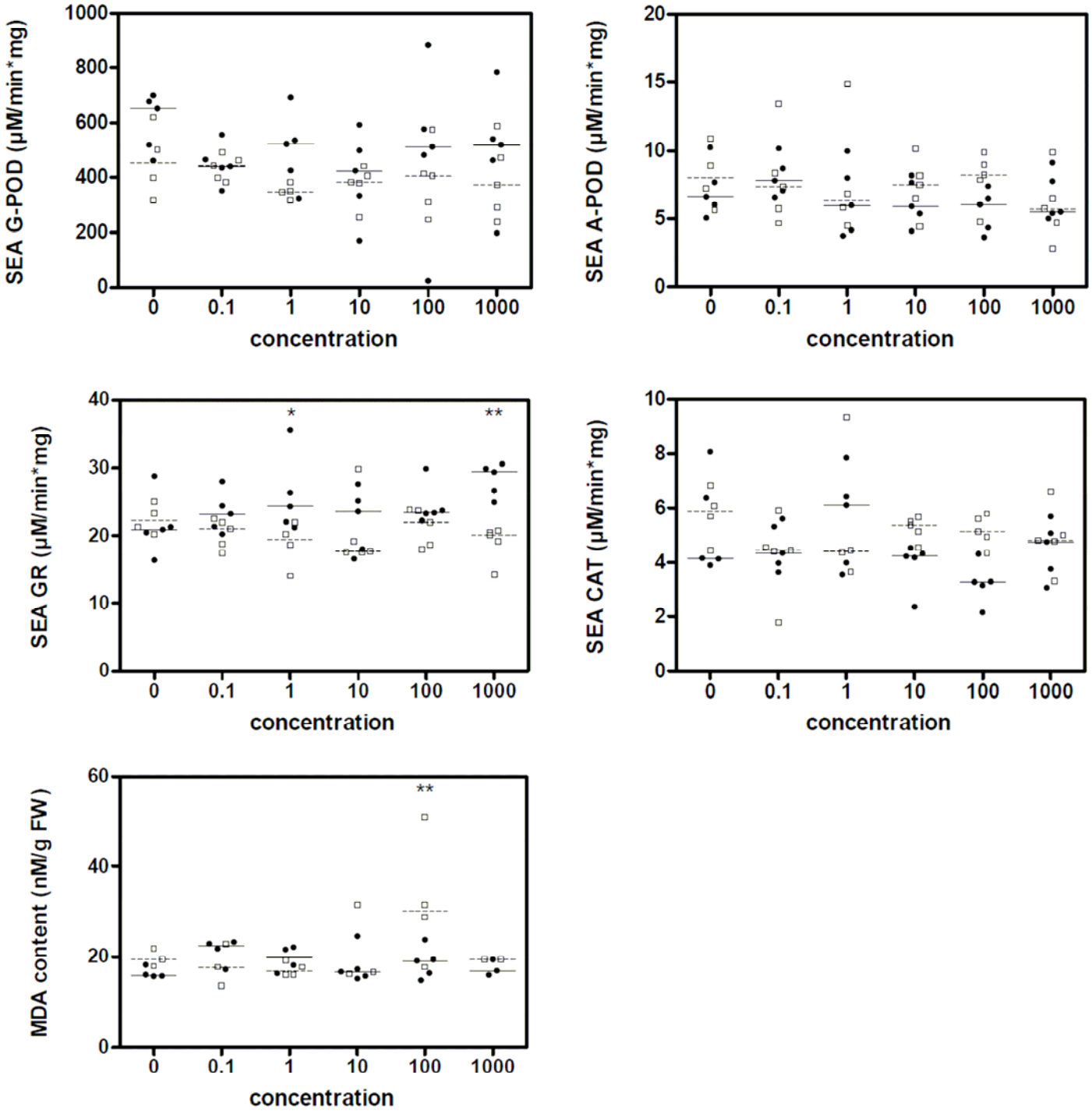

Figure 5

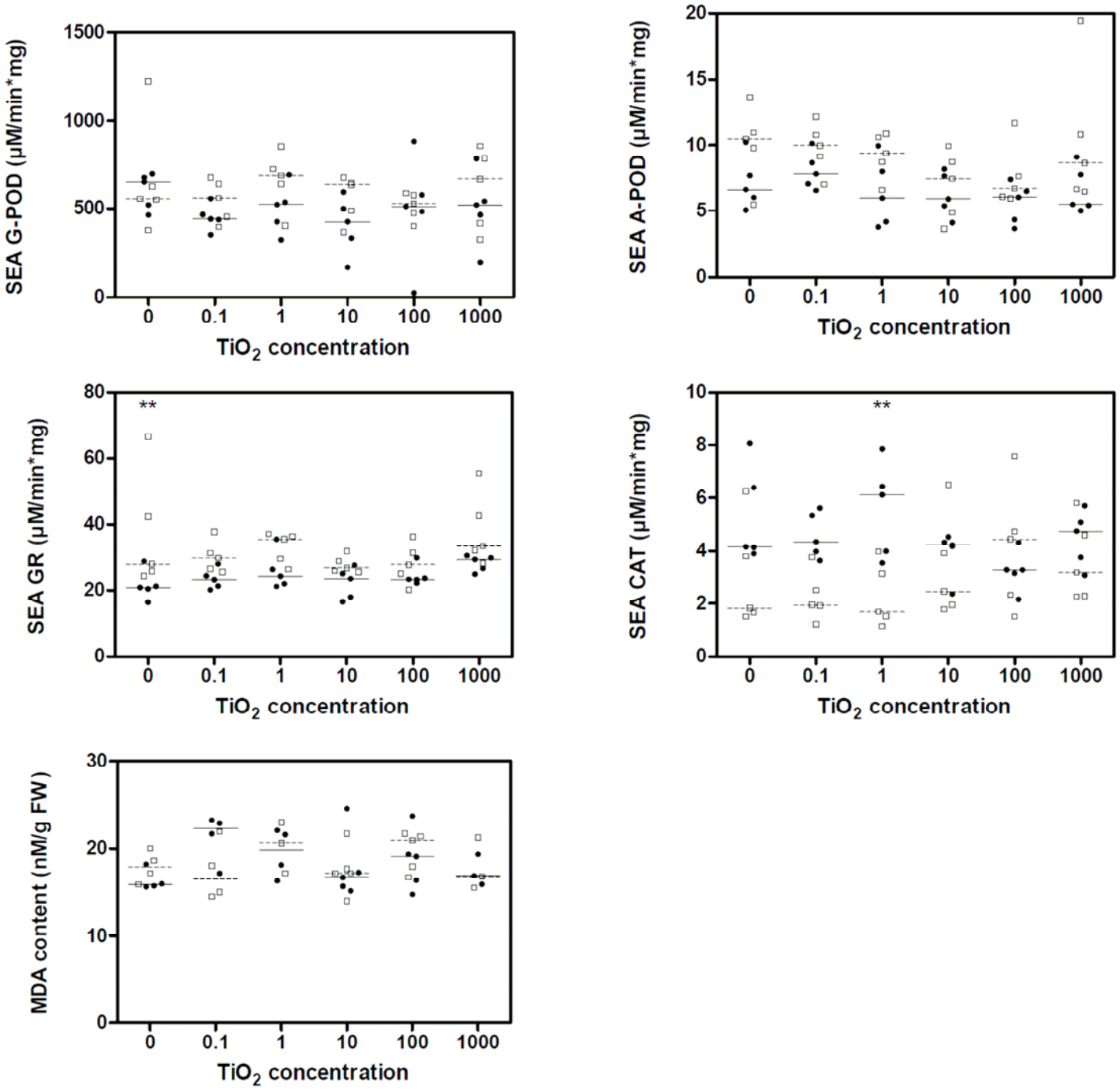

Figure 6