The impact of TiO_2 nanoparticles on uptake and toxicity of benzo(a)pyrene in the blue mussel (*Mytilus edulis*)

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Abstract

Nanoparticles are emerging contaminants of concern. Knowledge on their environmental impacts is scarce, especially on their interactive effects with other contaminants. In this study we investigated effects of titanium dioxide nanoparticles (TiO_2NP) on the blue mussel (*Mytilus edulis*) and determined their influence on the bioavailability and toxicity of benzo(a)pyrene (B(a)P), a carcinogenic polyaromatic hydrocarbon (PAH). Blue mussels were exposed to either TiO_2NP (0.2 and 2.0 mg L^{-1}) or B(a)P (20 µg L^{-1}) and to the respective combinations of these two compounds. Aqueous contaminant concentrations, the uptake of Ti and B(a)P into mussel soft tissue, effects on oxidative stress and chromosomal damage were analyzed. The uncoated TiO_2NP agglomerated rapidly in the seawater. The presence of TiO_2NP significantly reduced the bioavailability of B(a)P, shown by lowered B(a)P concentrations in exposure tanks and in mussel tissue. The activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were impacted by the various exposures regimes, indicating oxidative stress in the contaminant exposure groups. While SOD activity was increased only in the 0.2TiO_2NP exposure group, CAT activity was enhanced in both combined exposure groups. The GPx activity was increased only in the groups exposed to the two single compounds. In hemocytes, increased chromosomal damage was detected in mussels exposed to the single compounds, which was further increased after exposure to the combination of compounds. In this study we show that the presence of TiO_2NP in the exposure system reduced B(a)P uptake in blue mussels. However, since most biomarker responses did not decrease despite lower B(a)P uptake in combined exposures, the results suggest that TiO_2NP can act as additional stressor, or potentially alters B(a)P toxicity by activation.

Keywords:

Engineered nanoparticles, Benzo(a)pyrene, Combined exposure, Blue mussel

1 Introduction

The innovative properties of nanomaterials have led to a tremendous increase in their production and usage, turning the nanotechnology based industry into one of the fastest growing industries worldwide (Schmidt, 2009). Due to this increasing use of nano-enabled products, it is assumed that engineered nanoparticles (ENPs) will enter the aquatic environment in quantities that may be of environmental concern (Guzman et al., 2006; Lecoanet and Wiesner, 2004; Nowack and Bucheli, 2007).

Due to their photocatalytic properties TiO_2NP are among the most used and produced ENPs, with applications in building materials, self-cleaning paints and self-sterilizing surfaces, as food additive and in cosmetics (Gottschalk et al., 2009; Quagliarini et al., 2012; Robichaud et al., 2009; Weir et al., 2012). TiO_2NPs have been reported to be released from consumer products such as self-cleaning façade paints, and despite a high removal rate in waste water treatment plants TiO_2NP have been detected in waste water treatment plant effluents, indicating environmental exposure (Kiser et al., 2009; Neal et al., 2011; Westerhoff et al., 2011).

Engineered nanoparticles released from wastewater treatment plants or leaching from consumer products into "pollution hot spots" such as harbors and estuaries adjacent to densely populated areas, will mix with other contaminants. Due to their large surface to volume ratio and thus high reactivity, ENPs can potentially interact with anthropogenic contaminants of concern, and both, carbon-based and metal based ENPs have previously been reported to bind contaminants such as PAHs (Baun et al., 2008; Farkas et al., 2012; Li, et al., 2013 a, b, c). Furthermore, ENPs such as C_{60}, TiO_2NP and iron ENPs have been reported to bind and degrade various chemicals such as brominated organic compounds, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and heavy metals (Karn et al., 2009; Theron et al., 2008; Zhang, 2003), and these ENPs are therefore used for environmental remediation of contaminated sites. In 2010 ENPs were used in approximately 60 remediation sites worldwide (http://www.nanotechproject.org). Environmental cleanup by ENP application involves certain risks associated with their accidental release from remediation sites and their ability to transport contaminants and contaminant breakdown products. Interactions between ENPs and contaminants could alter the environmental behavior and distribution of anthropogenic contaminants of concern.

Engineered nanoparticles can be taken up by aquatic organisms (Tedesco et al., 2010), and are suspected to co-transport adsorbed pollutants, thereby often increasing their toxicity. Evidence for such a facilitated transport of ENP-associated contaminants, coupled with an increased toxicity of the respective contaminants has been reported in recent studies: Altered uptake and effects of different contaminants sorbed to C_{60} were described in *Daphnia magna* and *Pseudokirchnella subcapitata* by Baun and co-workers (Baun et al., 2008). Canesi and coworkers (2014) showed an increased uptake of TCDD in the filter feeding mussel *Mytilus galloprovincialis* in the presence of TiO_2NP. A 20-fold increase in TBT toxicity along with an enhanced uptake of TBT in the presence of TiO_2NP was reported in abalone (*Haliotis diversicolor supertexta*) (Zhu et al., 2011a). Furthermore TiO_2NP were found to accumulate in the digestive tract and on gills of common carp (*Cyprinus carpio*), thereby enhancing the bioavailability of arsenate and cadmium (Sun et al., 2007; Zhang et al., 2007).

Recent studies have investigated the impacts of TiO_2NP on aquatic organisms such as fish, pelagic and benthic invertebrates (Wallis et al., 2014; Li et al., 2014 a, b, c). Mussels live at the sediment/water interphase and filter large volumes of water, colloids and suspended matter. They are suspension feeders and are therefore highly specialized in the cellular internalization of micro- and nano-scale particles. As a consequence they are particularly susceptible to ENP exposure (Canesi et al., 2012). Previous *in vivo* and *in vitro* studies have reported adverse effects of TiO_2NP on immune system and digestive gland function of *M. galloprovincialis* (Barmo et al., 2013; Canesi et al., 2010). In the present study we examined oxidative stress responses and genotoxic effects of TiO_2NP and B(a)P, a carcinogenic PAH, in single and mixed exposures of these contaminants in the blue mussel *M. edulis.* In addition, TiO_2NP bioavailability and uptake, which is defined as concentration of Ti in tissues after TiO_2NP exposure, as well as B(a)P uptake were determined in both single and mixed exposures to reveal a potential facilitated contaminant uptake or interactive effects of TiO_2NP-B(a)P.

2 Materials and Methods

2.1 Nanoparticles

Uncoated TiO_2NPs were purchased from Particular GmbH (Hannover, Germany). The TiO_2NP were produced by laser ablation in MilliQ water and were provided at a concentration of 500 mg L^{-1} in an aqueous solution without stabilizing agents present. The particle size reported by the manufacturer was 62 nm determined by dynamic light scattering (DLS).

Nanoparticle shape and size were qualitatively determined for the TiO_2NP stock solutions and exposure solutions by transmission electron microscopy (TEM) as follows: 100 µl of each sample was applied on carbon-coated copper grids (200 nm mesh) and the samples were allowed to dry for several minutes to enable TiO_2NP attachment. Remaining liquid was subsequently removed. The nanoparticles were examined with a Zeiss Libra 120 EF TEM (Carl Zeiss AG, Germany) and the particle material was identified by electron energy loss spectroscopy (EELS). The hydrodynamic diameter of TiO_2NPs was determined in MilliQ water by DLS with a N5 submicron Particle Size Analyzer (Beckman Coulter Inc., Brea CA, USA). Samples of 5 mg L^{-1} were prepared and filtered through a 200 nm filter prior to analysis. Measurements were performed at a scattering angle of 90 °. Due to the rapid formation of large aggregates DLS analyses of TiO_2NP in seawater were not performed.

2.2 Husbandry

Mytilus edulis were purchased from a local mussel farm (Snadder & Snaskum AS, Rissa, Norway). The mussels were acclimated in circulating seawater for 14 days in the animal holding facilities at NTNU Sealab (Trondheim, Norway) before being transferred to 1.3 L glass exposure tanks 48 h prior to exposure startup. Each exposure tank housed 15 individuals, adding to a total number of 300 individuals used in the experiment. The exposure tanks were set up as a flow-through system and were continuously supplied with filtered seawater (33.5 ppt) and pressurized air. The temperature was set to constant 10 °C. Mussels were fed throughout the experiment with *Isochrysis galbana* ($8.9*10^8$ algae cells L^{-1}) that were provided constantly via the flow through system. The algae feeding solutions were prepared daily.

2.3 Exposure

The mussels were exposed for 96 h to two concentrations of TiO_2NP: 0.2 mg L^{-1} TiO_2NP ($0.2TiO_2$NP; low dose) and 2 mg L^{-1} TiO_2NP ($2TiO_2$NP; high dose). The nominal exposure

concentration of B(a)P was 20 µg L^{-1}. To investigate combined effects, the mussels were exposed to mixtures of B(a)P and TiO_2NP: 20 µg L^{-1} B(a)P + 0.2 mg L^{-1} TiO_2NP (B(a)P+0.2TiO_2NP), and 20 µg L^{-1} B(a)P + 2 mg L^{-1} TiO_2NP (B(a)P+2TiO_2NP). At the start of the experiment solutions were spiked directly in the tanks. Subsequently, exposure solutions were supplied via the flow through system and adjusted to keep the nominal exposure concentrations as listed above. The stock solutions of B(a)P were prepared by dissolving B(a)P in DMSO. The exposure solutions were prepared daily by diluting the stock solutions in seawater. Control groups received only seawater, and the carrier control groups received only DMSO at the same concentrations as used in the other exposure groups. All exposure conditions were run in triplicate, accounting for 45 individuals per treatment, except for the DMSO exposure, which was run in duplicate, resulting in 30 individuals in this exposure group.

2.4 Exposure validation

Water samples for Ti analysis were taken daily throughout the experiment. Ten mL of water was sampled approximately 5 cm beneath the water surface from each exposure tank to determine concentrations of dispersed TiO_2NP. The samples were preserved in 0.1 M HNO_3 and the total Ti concentration was analyzed with an Element 2 HR-ICP-MS (Thermo Fisher Scientific, Waltham, USA).

Water samples for analysis of B(a)P concentration in the exposure tanks were taken after 24 h, 72 h and 96 h from 2 replicate aquaria in each group. The water samples were transferred into cleaned and burned amber glass bottles with Teflon screw caps. To prevent adsorption of B(a)P to glass surfaces, MeOH was added to reach a final concentration of 10%. The water samples were stored at 4 °C and processed within 7 days after sampling. For B(a)P concentration determination, the water samples were extracted with Chromabond® C18 EC SPE columns (Macherey Nagel, Düren, Germany). Subsequently samples were separated with a Supelco® PAH column at a flow rate of 1 mL and analyzed with a Perkin Elmer Series 200 HPLC with a fluorescence detector (Perkin Elmer, Massachusetts, USA).

2.5 Sampling

Mortality was recorded throughout the experiment. At sampling, total weight, tissue wet weight, width, length and girth of each animal were recorded. Hemolymph samples were withdrawn from the posterior adductor muscle for microscopic determination of DNA damage. Subsequently whole tissues were sampled, weighted and frozen for later analysis. Tissue samples were stored at -20 °C for tissue Ti and B(a)P quantification, or snap-frozen in liquid nitrogen and stored at -80 °C for enzymatic analysis.

2.6 Titanium tissue concentrations

For determination of Ti tissue concentrations after TiO_2NP exposure, mussel samples were freeze dried for 48 h and subsequently homogenized with a Retsch MM 400 homogenizer (Haan, Germany) at a frequency 20 Hz for 90 s. After adding 50% v/v HNO_3, the samples were digested in an Ultra Clave Microwave (Sorisole, Italy). The samples were analyzed for ^{47}Ti with an Element 2 HR-ICP-MS (Thermo Fisher Scientific, Waltham, USA). Ti concentrations were determined in whole animals from the TiO_2NP, combined and control exposure groups (n=9). Additionally, Ti was analyzed in gills, digestive gland and rest tissue from high TiO_2NP exposure group individuals (n=3).

2.7 B(a)P uptake

B(a)P concentrations in whole mussels were analyzed by LC-MS. Samples of 5.0 g mussel homogenate were extracted with acetonitrile with an Agilent Bond Elut QuEChERS AOAC extraction salt packet (p/n 5982-5755) added. Sample cleanup was performed with Bond Elut QuEChERS AOAC Dispersive SPE columns (Agilent, Santa Clara, USA) and subsequent filtering through a 0.45 µm PVDF syringe filter. The sample was separated with an Agilent ZORBAX Eclipse PAH C18 column (Agilent, Santa Clara, USA) at a flow rate of 1 mL min^{-1}. The mass detection analysis was performed with the following parameters: fragmentor 150, gas temperature of electrospray source 350 ºC, gas flow rate 12 L min^{-1} and capillary voltage 3 kV. Single ion was monitored m/z 253 (pseudomolecular ion).

2.8 Enzyme activities

The NADPH cytochrome C reductase (CPR) activity was determined in mussel digestive glands. Digestive glands were dissected from frozen specimens (-80 °C) on ice and were homogenized in ice-cold phosphate buffered saline (PBS). The post mitochondrial fraction was prepared by a double centrifugation step (1000xg and 12.000xg) at 4 °C and was subsequently stored at -80 °C for further analysis. The CPR activity was determined spectrophotometrically, analyzing the reduction of oxidized cytochrome c in the presence of NADPH with a commercial assay kit (Sigma Aldrich, St Louis, Missouri, USA). Enzyme activity is expressed in units mg^{-1} $protein^{-1}$, where 1 unit is defined as reducing 1.0 µmol of oxidized cytochrome c in the presence of 100 µmol NADPH per minute.

The activity of the enzymes SOD, CAT and GPx were analyzed in the post-mitochondrial fraction of mussel digestive glands. Digestive glands were dissected on ice from frozen specimens and homogenized in Tris buffer. The samples were centrifuged twice (1.500xg, 10.000xg) at 4 °C. Samples were stored at -80 °C until analysis.

The peroxidative activity of catalase was determined spectrophotomertically using a slightly modified method developed previously (Johansson and Borg, 1988). Enzymatic activity is expressed as nmol min^{-1} mg^{-1} protein. The SOD activity was determined by a colorimetric method using a SOD assay kit (Sigma-Aldrich, St Louis, Missouri, USA). A standard curve was prepared using superoxide dismutase from bovine erythrocytes (2698 units mg^{-1}) to quantitatively assess the enzyme activity, which is expressed as U mg^{-1} protein. The GPx activity was analyzed using a commercial assay kit (Cayman Chemical, Ann Arbor, Michigan, USA), determining the GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Enzymatic activity is expressed as nmol min^{-1} mg^{-1} protein.

2.9 DNA damage

Micronucleus (MN) frequency was evaluated in hemocyte cells as described elsewhere (Bolognesi and Fenech, 2012). Briefly, a 0.2 mL sample of hemolymph was taken from the posterior adductor muscle and was immediately mixed with 0.2 mL phosphate buffered saline (PBS). The samples were applied onto microscope slides and left in a dark humid chamber to allow cell attachment. Two slides were prepared for each individual. The samples were stained

with 3 % (vol/vol) giemsa. DNA damage was evaluated optically with a Zeiss Axioplan 2 light microscope at a 1000x magnification (Zeiss AG, Oberkochen, Germany). For each individual 2000 cells were scored.

2.10 Protein content

The protein content in the post-mitochondrial fraction of digestive glands was determined with the Bradford method (Bradford, 1976).

2.11 Data analysis

Statistical analysis was performed with SPSS 20.1 (IBM, 2012) and Minitab 17 1.0 (Minitab Inc, 2013). To determine statistically significant differences between exposure groups, ANOVA followed by Games Howell (MN data) or Tukey post hoc test were applied after testing the data for normality and homogeneity of variances. For datasets showing a non-normal distribution Kruskal-Wallis one way analysis of variance on ranks was applied. Significance values were set to $p<0.05$. Graphs were prepared with SigmaPlot 12.0 (Systat software Inc., Chicago, USA).

3 Results

3.1 Aqueous Ti and B(a)P concentrations

In the stock solutions TiO_2NP were mostly present as single particles or loose agglomerates (Fig 1a), with an average hydrodynamic diameter of 146 nm. In seawater particles were present as aggregates (Fig 1b). TiO_2NP was observed to undergo rapid aggregation (Fig 1c) and to sediment on the bottom of the exposure tanks. The TiO_2NP concentrations, determined by HR-ICP-MS as total Ti (Ti<1µm) in the upper layers of the water column, were in average 0.14±0.07 µg L^{-1} and 0.90±1.21 µg L^{-1} for the nominal 0.2 (low) and 2 mg L^{-1} (high) exposure concentration, respectively (Fig 2 a).

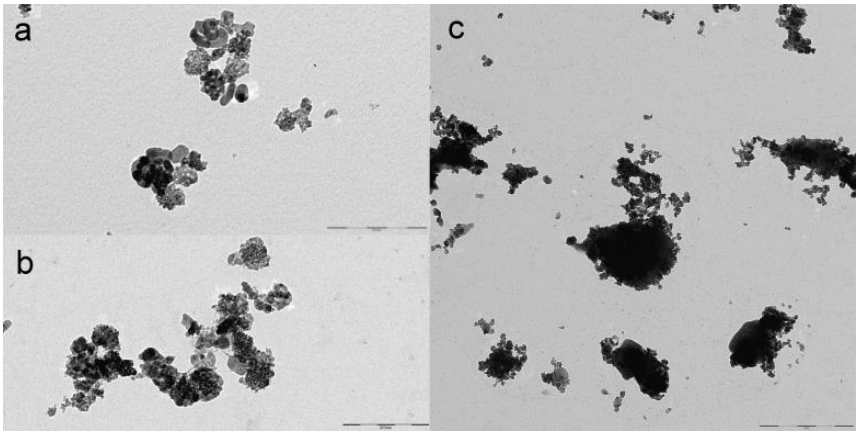

Figure 1: TEM images of TiO_2NP a) in water (stock solution; scale bar 200 nm); b) Single particles in seawater (scale bar 200 nm); c) Large agglomerates in seawater (scale bar 1µm).

The dissolved B(a)P concentrations in the B(a)P exposure tanks were 460±59 ng L^{-1}. In the presence of nanoparticles the dissolved B(a)P concentrations were significantly reduced in a dose dependent manner and were 280±52 ng L^{-1} and 170±49 ng L^{-1} in the B(a)P+0.2TiO_2NP ($p=0.029$) and B(a)P+2TiO_2NP ($p=0.003$) exposure tanks, respectively (Fig 2b).

3.2 Effects on survival and biometry

The mortality rate was low (two out of 300 individuals died; 0.67%), and was not related to contaminant exposures. The mussels had an average total weight of 16.5±3.5 g, and tissue wet weight of 8±1.6 g. Average length, width and height were 5.8±0.4 cm, 2.7±0.2 cm and 2.1±0.2 cm. No significant differences in biometric variables were found between the exposed groups and the control groups after the 96 h exposure.

3.3 Ti and B(a)P in tissues

The uptake of elemental Ti from the TiO_2NP exposures into soft tissue (dry weight, dw) was dependent on the exposure concentrations. Ti tissue concentrations were significantly higher in exposed groups compared than in individuals from the control group, with Ti tissue concentrations of 0.69±0.6 µg g^{-1} and 0.43±0.2 µg g^{-1} for the 0.2TiO_2NP ($p=0.009$) and B(a)P+0.2TiO_2NP ($p=0.036$) exposure groups, and 2.5±1.2 µg g^{-1} and 3.5±2.1 for the 2TiO_2NP ($p<0.001$) and B(a)P+2TiO_2NP ($p<0.001$). Soft tissue concentrations of Ti varied strongly

between individuals (Fig 2c). Ti concentrations in gills, digestive gland and remaining tissue were not found to differ significantly between the groups (data not shown).

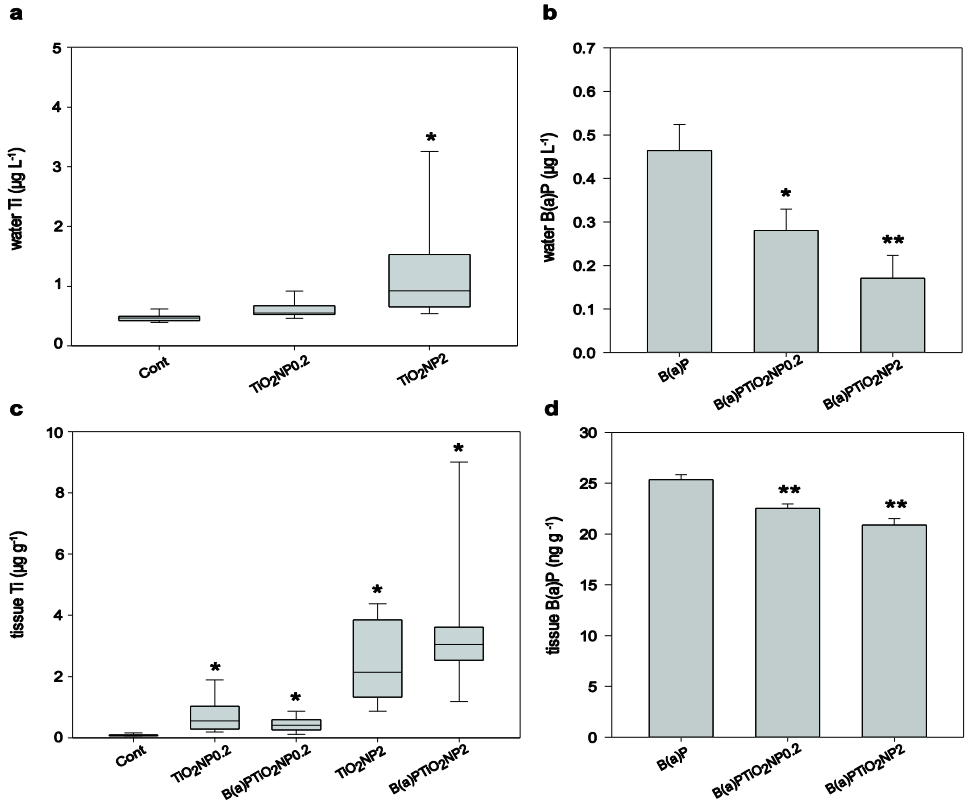

Figure 2: Ti and B(a)P concentrations. a) Aqueous Ti concentration in exposure tanks, median, 10th, 25th, 75th and 90th percentile (*n*=12). b) Aqueous B(a)P concentration in exposure tanks, mean±SE (n=*6*). c) Whole soft tissue Ti concentrations (dw), median, 10th, 25th, 75th and 90th (*n*=9). d) Whole soft tissue B(a)P concentrations (ww), mean±SE (n=*9*). Stars indicate significant differences from control (*p<0.05, **p<0.01).

The presence of TiO_2NP in the exposure tanks reduced the B(a)P tissue concentrations in mussels. Tissue B(a)P concentrations were highest in the group exposed to only B(a)P with 25.4±1.4 ng g^{-1} (wet weight, ww), and significantly lower in the B(a)P+0.2TiO_2NP (p=0.002) and B(a)P+2TiO_2NP (p<0.001) exposures with 22.5±1.3 and 20.9±1.9 ng g^{-1} B(a)P, respectively (Fig 2d).

3.4 Chromosomal damage

The formation of MN in *M. edulis* hemocytes (Fig 3) was determined as a measure of DNA damage. In control cells the average number of MN was 5.4±0.6 MN per 1000 cells.

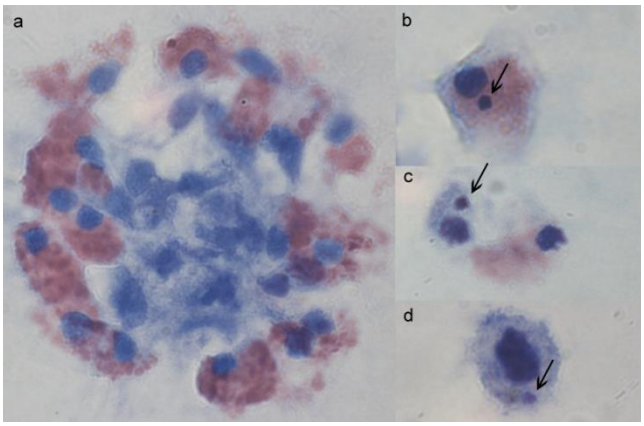

Figure 3: *Mytilus edulis* hemocytes, a) Typical cluster of basophilic and eosinoohilic hemocytes; micronuclei in hemocytes from mussels exposed to B(a)P+2TiO_2NP (b,c) and B(a)P+0.2TiO_2NP (d).

The MN formation in hemocytes was significantly higher in groups exposed to B(a)P ($p=0.028$), low and high TiO_2NP ($p<0.001$), and in the two groups exposed to the mixtures of both contaminants ($p<0.001$) compared to control groups (Fig 4). The MN formation was slightly, although not significantly higher, in the two TiO_2NP groups and in the two mixed exposure groups as compared to the B(a)P exposure group.

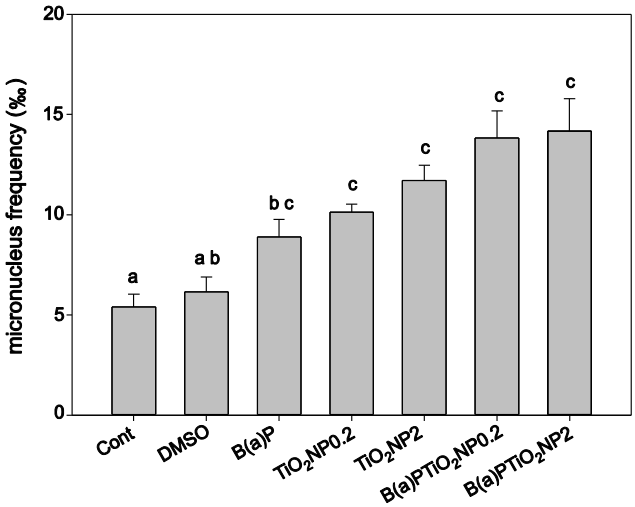

Figure 4: Micronucleus frequency (‰) in *M. edulis* hemocytes. Different letters indicate statistically significant differences between treatment groups ($p<0.05$). Mean±SE; DMSO, n=7; 0.2TiO_2NP, n=13; B(a)P, 2TiO_2NP, B(a)P+0.2TiO_2NP, n=14; control, B(a)P+2TiO_2NP, n=15.

3.5 Effects on enzyme activities

Antioxidant enzyme activities were differentially impacted by the exposures. Superoxide dismutase activity was significantly higher only in the 0.2TiO_2NP exposure group compared to untreated controls ($p=0.020$) and the B(a)P exposed group ($p=0.013$) (Fig 5a). The activity of CAT was not significantly altered by the presence of B(a)P or TiO_2NP in the single exposure groups (Fig 5b), although the mean CAT activity was roughly 50% higher in the combined exposure treatments compared to the untreated control group. CAT activity was significantly higher in the combined exposure groups compared to the DMSO controls ($p=0.021$; $p=0.013$) and B(a)P exposed groups ($p=0.042$). Glutathione peroxide activity was significantly enhanced in the single exposures, B(a)P ($p=0.009$), 0.2TiO_2NP ($p<0.001$) and 2TiO_2NP ($p=0.033$) compared to the untreated control group (Fig 5c). In general, the combined exposure groups featured a slightly lower activity compared to the single exposure groups, and slightly higher activities as compared to the control groups, although the latter trends were not statistically significant.

The NADPH cytochrome c activity was not altered significantly by any treatment (data not shown).

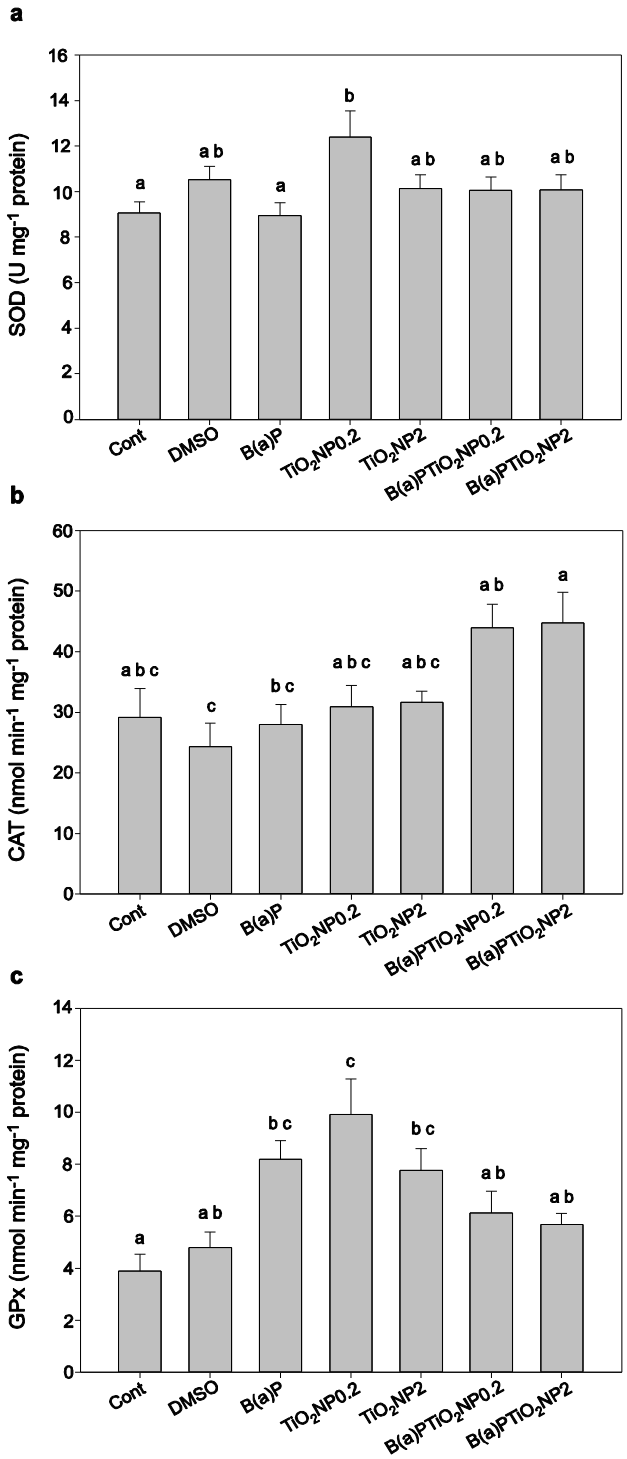

Figure 5: Antioxidative stress enzyme activities in *M. edulis* digestive gland tissue, a) SOD activity; b) CAT activity; c) GPx activity. Different letters indicate statistically significant differences between treatment groups ($p<0.05$). Mean±SE. DMSO, n=6; TiO_2NP, n=8; control, B(a)P, B(a)P+TiO_2NP, n=9.

4 Discussion

In the present study we observed a rapid aggregation and flocculation of TiO_2NP in seawater, and sedimentation of TiO_2NP onto the bottom of the exposure tanks. This was reflected in low measured Ti concentrations in the water column compared to the nominal concentrations. This is in accordance with previous studies that showed the aggregation of uncoated ENPs in environments with higher ionic strength, and aggregates formed by uncoated TiO_2NP are assumed to be highly bioavailable for filter feeding organisms such as *M. edulis* (Canesi et al., 2012; Christian et al., 2008).

The uptake of Ti into *M. edulis*, defined as Ti tissue concentrations, appeared to be dose dependent. However, Ti concentrations in whole soft tissues, gills and digestive glands were in the low µg g^{-1} range, even in the high TiO_2NP exposure group. The relatively large variation in Ti tissue concentrations between individuals in some of the exposure groups can possibly be explained by the positioning of individuals in the exposure tanks. Individuals located at the bottom of the tanks were likely experiencing higher TiO_2NP exposure from settling particles, compared to individuals attached to the tank walls higher up. The Ti concentration in whole soft tissue (0.7 µg g^{-1} dw) in the low TiO_2NP exposure group herein was comparable to Ti concentrations reported in *M. galloprovincialis* gill tissues (approximately 0.5 µg g^{-1} dw) after a 0.1 mg L^{-1} TiO_2NP exposure (Canesi et al., 2014). In contrast to Canesi and coworkers (2014), who reported higher Ti accumulation in digestive glands compared to gills and other tissues, we found no significant differences in Ti concentrations between gill, digestive gland and rest of the mussel soft tissue.

The presence of TiO_2NP in water caused lower concentrations of dissolved B(a)P in the exposure tanks, as well as in *M. edulis* soft tissues. This indicates interactions between TiO_2NP and B(a)P. Due to its hydrophobicity, B(a)P absorbs to particulate matter in aqueous environments (Karickhoff, 1984), and TiO_2NP, featuring a high surface area could act as adsorption surface for B(a)P. Indeed, previous studies have reported adsorption of PAHs, such as phenanthrene, fluoranthene, and chrysene, to C_{60} and gold ENPs (Baun et al., 2008; Farkas et al., 2012; Hu et al., 2008). Consequently, by adhesion to aggregating and settling TiO_2NP, B(a)P could partly be removed from the water column and hence become less bioavailable. Furthermore, since TiO_2NP

can produce hydroxyl radicals in water and has been shown to enhance the degradation of several PAHs, including B(a)P, it is possible that TiO_2NP caused an oxidative breakdown of B(a)P (Ireland et al., 1995; Zhang et al., 2008). However, it should be noted that a TiO_2NP catalyzed degradation of B(a)P was not assessed in the present study, and further investigations on those processes are needed.

Whereas we found that exposure to B(a)P in the presence of TiO_2NP resulted in reduced B(a)P tissue concentrations, several previous reports have described increased uptake of organic contaminants after co-exposure to ENPs. For example, Canesi and co-workers (2014) reported that TiO_2NP enhanced the accumulation of 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) in *M. galloprovincialis*, and suggested that TiO_2NP acted as co-transporter. In a study that investigated the influence of TiO_2NP on TBT effects in abalone (*Haliotis diversicolor supertexta*), Zhu and coworkers (2010) described an enhanced uptake of TBT in the presence of TiO_2NP. ENPs also appear to affect the uptake of metals and chemical elements in aquatic organisms. In *D. magna*, co-exposure to TiO_2NP enhanced the uptake of Ag and As, but reduced the uptake of Cu by a factor of 14 (Rosenfeldt et al., 2014). Furthermore, higher accumulation of As and Cd was reported in carp (*Cyprinus carpio*) when exposed in combination with TiO_2NP (Sun et al., 2007; Zhang et al., 2007).

In the present study, exposure to TiO_2NP alone appeared to induce GPx and SOD activity in the *M. edulis* digestive gland indicating that ROS production increased due to the TiO_2NP exposure. However, those effects were not strictly dose dependent, as induction in SOD activity was detected in the low TiO_2NP exposure group but not in the high TiO_2NP exposure group. In contrast, GPx activity was increased in both TiO_2NP exposure groups, although induction was highest the low TiO_2NP exposure. Similar variable dose-dependent responses in GPx and SOD activities with inductions at exposure to low concentrations of TiO_2NP but not at higher exposure concentrations have previously been reported in *M. galloprovencialis* and abalone (Barmo et al., 2013; Zhu et al., 2011a; Zhu et al., 2011b).

The exposure of *M. edulis* to B(a)P caused no NAHPH cytochrome c activity changes in the digestive gland tissue as compared to the control group, which is in contrast to previous studies

(Livingstone, 1988; Livingstone and Farrar, 1984; Michel et al., 1993). Furthermore, exposure to B(a)P alone did not affect SOD and CAT activities as compared to the control groups. However, we detected an elevated enzymatic activity of GPx following exposure to B(a)P alone. These findings are in agreement with those reported by Cheung and coworkers (2004) who found no correlations between B(a)P exposure and SOD and CAT activities, but a significant correlation between B(a)P exposure and GPx activity in greenlipped mussel (*Perna viridis*) digestive glands. Orbea and co-workers (2002) also reported increased GPx activity in *M. galloprovincialis* after B(a)P exposure.

Although the CAT activity in the combined exposure groups (TiO_2NP and B(a)P) did not differ from that in the control group, the CAT activity was significantly higher in the highest combined exposure group than in the group exposed to B(a)P alone. In contrast, while GPx activity was enhanced in both the single exposure groups (TiO_2NP and B(a)P) compared to the control group, no such changes were observed in the combined exposure groups. In general GPx activity decreased in high TiO_2NP and combined exposures. This suggests that the increase in ROS production can lead to reduced activity of certain antioxidant enzymes (Livingstone, 2003; Zhang et al., 2004). Previous studies on interactions between these antioxidant enzymes reported that CAT plays a minor role in H_2O_2 metabolism when H_2O_2 is produced at low rates, but a larger role when H_2O_2 production is enhanced (Barata et al., 2005; Jones et al., 1981).

Despite the varying effects on enzymatic biomarkers of the digestive gland, a clear increase was observed in chromosomal damage in hemocytes in the present study. All exposure groups showed a higher frequency in micronuclei compared to the control. The micronucleus frequency was about 50 % higher in the combined exposure groups as compared to the B(a)P exposure group. The increased chromosomal damage, despite the reduced B(a)P tissue concentrations in the presence of TiO_2NP, suggest that TiO_2NP may have potentiated or activated the genotoxic effects of B(a)P. Dass (1994) proposed a TiO_2NP mediated photo-catalysis of PAHs in water leading to the production of PAH metabolites. One possible reaction pathway involves the production of hydroxyl radicals on the TiO_2NP surface (Hirano et al., 2005, Xia et al., 2006), which could further react with PAHs and molecular oxygen to ultimately produce PAH-quinone

leading to increased toxicity. However, the formation of PAH-quinones in water was not analyzed in the present study, and further research is needed on these processes.

The analyzed endpoints showed a different response to combined exposures in this study. This is in agreement with previous studies. Canesi and coworkers (2014) investigated a wide range of biomarkers after exposure of *M. galloprovincialis* to TiO_2NP and 2,3,7,8-TCDD, alone and in combination, and found that both antagonistic and synergistic effects occurred depending on the tissue and investigated biomarker. Baun and co-workers (2008) reported an increase of phenanthrene toxicity (EC_{50}) in the presence of C-60 and a decreased toxicity of pentachlorophenol on *D. magna* and *P. subcapitata*. Rosenfeldt and coworkers (2014) found an increased toxicity of Ag, while toxicities of As and Cu were reduced in the presence of TiO_2NP. The results of these studies indicate that the mode of combinatory effect is dependent on the investigated endpoints and studied contaminants.

5 Conclusions

The present study showed that exposure to TiO_2NP alone can cause oxidative stress responses and chromosomal damage in *M. edulis.* In addition, TiO_2NP altered the distribution, bioavailability and toxicity of B(a)P. Despite that the combined exposure to TiO_2NP and B(a)P reduced the tissue uptake of B(a)P, most endpoints did not indicate reduced, but rather increased toxicity, as in the case of CAT and chromosomal damage. Hence, this study indicates that NPs may cause additional stress during co-exposure with B(a)P despite of reduced B(a)P tissue concentrations. This maybe due to TiO_2NP-induced breakdown of B(a)P into reactive products. Further research is needed in this field in order to reveal the underlying mechanisms at play.

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