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Titanium dioxide nanoparticles modulate the toxicological response to cadmium in the gills of *Mytilus galloprovincialis*

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Abstract

We investigated the influence of nano-TiO₂ on the response to cadmium in the gills of the marine mussel *Mytilus galloprovincialis* in terms of accumulation and toxicity. Mussels were *in vivo* exposed to nano-TiO₂, CdCl₂, alone and in combination. Several cellular biomarkers were investigated in gills: ABC transport proteins and metallothioneins at gene/protein (*abcb1*, *abcc-like* and *mt-20*) and functional level, GST activity, NO production and DNA damage (Comet assay). Accumulation of total Cd and Titanium in gills as in whole soft tissue was also investigated. Significant responses to Cd exposure were observed in mussel gills as up-regulation of *abcb1* and *mt-20* gene transcription, increases in total MT content, P-gp efflux and GST activity, DNA damage and NO production. Nano-TiO₂ alone increased P-gp efflux activity and NO production. When combined with Cd, nano-TiO₂ reduced the metal-induced effects by significantly lowering *abcb1* gene transcription, GST activity, and DNA damage, whereas additive effects were observed on NO production. A lower concentration of Cd was observed in the gills upon co-exposure. A competitive effect in uptake/ accumulation of nano-TiO₂ and Cd seems to occur in gills. A confirmation is given by the observed absence of adsorption of Cd onto nano-TiO₂ in sea water media.

Keywords: nano-TiO₂, Cadmium, ABC transporters, genotoxicity, NO production

1. Introduction

Titanium dioxide nanoparticles (nano-TiO₂) are one of the most widely used nanomaterial, present in consumer and industrial products [1]. Due to the large production and growing application, nano-TiO₂ is released in huge amounts in urban and industrial sewage and occurs in the aquatic environment [2,3]. From its release into soil and waterways nano-TiO₂ will end up in the sea, where it may represent a risk for ecosystem and human health [4].

Nano-TiO₂ might affect aquatic organisms through its inherent properties, but also by modifying the bioavailability of other aquatic contaminants, including heavy metals [5-12]. Therefore their uptake, organ/tissue distribution and toxicity may be altered by the presence of nano-TiO₂ in the water media [13]. Co-exposure of nano-TiO₂ with Cd has been investigated in different freshwater models leading to conflicting results [7,8,13,14]. In the green algae *Chlamydomonas reinhardtii*, Cd accumulation and toxicity seemed reduced by nano-TiO₂ [8]. In *Daphnia magna* increase Cd bioavailability has been reported in the presence of nano-TiO₂, while no influence was observed on Cd uptake on *Lumbriculus variegatus* [7]. In the zebrafish, nano-TiO₂ enhanced Cd²⁺ bioaccumulation, but this effect was reduced in the presence of humic acid [13]. Overall results suggest that interactions might be species specific and affected by particle properties and behavior in water media. In addition, molecular mechanisms behind such interactions, as changes in Cd accumulation and toxicity in the presence of nano-TiO₂, as well as effects on key cellular pathways are still unknown. The interactions of nano-TiO₂ with Cd should be studied also on marine species, since several factors as for instance salinity, NOM and pH might strongly affect nano-TiO₂ fate and behaviour in sea water. To date, nano-TiO₂ effects on marine organisms has been investigated in terms of physiological and reproductive alterations, immunotoxicity, genotoxicity, cytotoxicity and oxidative stress [2,12,15-20]. Suspension-feeders clearly represent important model organisms to assess the impact of nanoparticles on aquatic biota [21,22]. In the Mediterranean mussel *Mytilus galloprovincialis*, nano-TiO₂ has been reported to affect immune and digestive gland function, including transcription of immune related and antioxidant genes as well as ABC transport proteins efflux [10,15,23,24]. Induced genotoxicity has been reported also in mussel hemocytes as oxidative DNA damage [25]. In this species, a recent study on co-exposure of nano-TiO₂ with Cd revealed complex interactive effects in digestive glands and hemocytes, as well as on embryo development [26]. In the digestive gland, the interactive effects of nano-TiO₂ and Cd were not apparently due to differences in bioavailability/bioaccumulation of Cd in the presence of nano-TiO₂ agglomerates; these effects may result from interactions of either contaminant with both common and distinct targets/mechanisms of action at different levels of biological organization, but also in different organs.

In bivalves the gills represent the first site of entry of soluble chemicals, including metals, from the surrounding environment [27]. Due to their role in the feeding process they might represent the main site of disposal of NPs and their agglomerates. Particles in fact are trapped by the gill sieve and moved towards the labial palps and mouth to the digestive system, whereas those inglobated in mucus are rejected as pseudofaeces [22].

Considering the importance of this tissue as a first barrier against the uptake of contaminants, including NPs, in the present study we investigated the influence of nano-TiO₂ on the bioconcentration and sub-lethal toxicity in the gills of *M. galloprovincialis* *in vivo*.

Key proteins involved in the cellular protective response towards Cd and well established biomarkers of exposure/effects were investigated: ABC transport proteins and metallothioneins both at gene (*abcb1*, *abcc-like* and *mt-20*) and protein function/level, GST activity, NO production and DNA damage (Comet assay). Accumulation of Cd and total Titanium in gills and in whole soft tissue was evaluated. Characterization of the interactions between nano-TiO₂ and Cd in artificial sea water (ASW) was also performed.

2. Methods

2.1 Preparation and characterization of nano- TiO₂

Nanosized Titanium Dioxide (n-TiO₂), namely Aeroxide © (declared purity of 99.9%) was kindly supplied by Eigenmann & Veronelli (Milan, Italy). Stock suspensions (10 mg mL⁻¹) were prepared in Artificial Seawater (ASW) (pH 8, salinity 36 ‰) [28] and in 0.22µm membrane-filtered Natural Seawater (NSW) (pH 7.9, salinity 30 ‰) by sonication at 100 W for 45 min in a cooling ice bath and suitably diluted in different media to obtain working suspensions.

Primary particle diameter and shape were determined through transmission electron microscopy (TEM); Dynamic Light Scattering (DLS) analysis was performed on a Malvern Zetasizer 3000HSa (Malvern Instruments, UK) to evaluate size and z-potential of the suspensions in ASW and NSW. Time-dependent size distributions was investigated through Differential Centrifuge Sedimentation (DCS) with a CPS Disc Centrifuge DC24000 using an 8-24% sucrose gradient. The experiment was repeated for suspensions spiked with CdCl₂ (Merck, Analytical grade) to a final concentration of 0.1 mg L⁻¹ to investigate possible interactive effects between CdCl₂ and nano-TiO₂ in terms of size. The dynamic sedimentation process was monitored using a Cary 6000i UV-Vis NIR Spectrophotometer for suspensions at nano-TiO₂ concentrations (1, 10 and 50 mg L⁻¹) alone and in combination with 0.1 mg L⁻¹ of CdCl₂. Changes in absorbance at 323 nm were measured every 6 min over a period of 360 min.

2.2 Animals and treatments

Mussels (*Mytilus galloprovincialis* Lam.) 4–5 cm long, were purchased from an aquaculture farm (Arborea-OR, Italy) and kept for 3 days in static tanks containing 1 L ASW mussel⁻¹ at 16°C and daily fed with 30 mg mussel⁻¹ Marine Liquifry (Interpet, England). Sea water was changed daily. Stock suspensions of nano-TiO₂ in ASW for both *in vitro* (P-gp efflux assay) and *in vivo* exposure studies were prepared as described above and immediately added to ASW in order to reach the tested concentration. CdCl₂ was diluted in ASW to reach the tested concentrations.

2.2.1 *In vitro* P-gp efflux assay

In vitro P-gp efflux assay was performed using gill punch biopsies (6 mm diameter Acuderm Inc.) and performed according to the method already described [29]. Gill arches from at least 10 untreated individuals were used for obtaining punch biopsies and incubated with nano-TiO₂ (0.1 µg mL⁻¹) or CdCl₂ (0.1 µg mL⁻¹) alone and in combination for 120 min and with ASW. The concentration of nano-TiO₂ was chosen as the lowest concentration able to induce changes in ABC efflux among a dose-dependent concentration curve (from 0.01 to 10 µg mL⁻¹) (data not shown).

Similar considerations apply to CdCl₂, that was able to affect ABC efflux at the concentration used, whereas lower concentrations were ineffective (data not shown). Exposed biopsies were collected for the quantification of gene expression or processed for P-gp efflux activity as described below.

2.2.2 *In vivo* exposure experiments

Mussels (at least 15 mussels in quadruplicate for each condition) were exposed for 96 h to either nano-TiO₂ at 0.1 mg L⁻¹ nominal concentration levels, or CdCl₂ 0.1 mg L⁻¹ and to both nano-TiO₂ and CdCl₂ at the same concentrations. The concentrations of n-TiO₂ and CdCl₂ were chosen on the basis of previous data [24,26]. A parallel group of mussels were kept in clean ASW. Sea water was changed daily before addition of the contaminants. Animals were not fed during the experiments. No mortality was observed in different experimental conditions.

After exposure, both gill arches were excised from 40 mussels from each experimental group as described above in order to obtain 4 samples pooled from 10 animals. Punch biopsies were immediately processed for P-gp efflux assay. Ten punch biopsies per experimental point were processed for Comet assay. The remaining gill samples were frozen at -80 °C for subsequent determination of other biomarkers. For each exposure group, whole soft tissues from 10 mussels from each experimental group were removed and stored at -20°C for Ti and Cd residue analysis

2.2.3 *qRT-PCR analysis of abc and mt-20 genes*

Total RNA was isolated from gills pools in Tripure reagent according to the manufacturer's protocol. RNA concentrations were measured using a Traycell spectrophotometer (Eppendorf) at 260 nM. cDNA for RT-PCR was generated from 0.25 µg total RNA from all samples in 20 µl reaction volume using iScript cDNA Synthesis Kit according to the manufacturer's protocol (Biorad, USA). Specific primers for *abcb1*, *abcc-like* genes, *mt-20* and the housekeeping (hk) Elongation Factor *efa* were designed for qRT-PCR using IDTDNA www.idtdna.com. Primer sequences used for qRT-PCR are summarized in Table 1. qRT-PCR was performed in Stratagene 3000xP thermal cycler at parameters described previously [29]. The modulation of mRNA transcription was measured using the $\Delta\Delta C_t$ method [30].

2.2.4 *P-gp efflux activity*

ABC transporter activity assays were performed on gills biopsies [31]. Incubations of 60 gill biopses per experimental group (*in vivo* and *in vitro*) obtained from 4 pools of 10 individuals each were performed in glass dishes in a volume of 30 ml of ASW plus RhB (1 µM) in the dark with gentle shaking. A group was also exposed to the P-gp inhibitor Verapamil (20 µM) as a positive

control. After 90 min incubation biopsies were washed twice and sonicated 20 sec in hypotonic Lysis buffer (10 mM KCl, 1.5 mM MgCl₂, 10 mM Tris pH 7.4). Sonicates were centrifuged at 12,000 rpm for 5 min and 150 µl of the supernatant transferred in 96-well black microplates. RhB fluorescence was measured using Victor3 microplate reader (Wallak) at $\lambda_{\text{Excitation}} = 545 \text{ nm}$ and $\lambda_{\text{Emission}} = 575 \text{ nm}$.

2.2.5 Metallothionein content

The metallothionein tissue content was analyzed as already described [32]. Pooled gills were homogenized in 4 vol. 0.5 M sucrose, 20 mM Tris-HCl, pH 8.6, containing 6 µM leupeptine, 1 mM phenylmethylsulfonyl-fluoride (PMSF), and 0.01% B-mercaptoethanol. Metallothionein concentration in the samples was quantified by spectrophotometric titration of the sulphhydrylic residues using the Ellman's reagent, 5,5-dithiobis-2-nitrobenzoic acid. Metallothionein content ($\mu\text{g protg}^{-1}$ w.w. sample) was calculated against a standard curve of glutathione (GSH).

2.2.6 GST activity

Pooled gills were homogenized in 5 vol. of 20 mM Tris buffer, 0.5 M sucrose, 0,15 M NaCl, pH 7.6 and centrifuged at 500 x g for 15 min at 4 °C. The supernatants were then centrifuged at 12,000 x g for 30 min and utilised for determination of GSH transferase (GST) (E.C. 2.5.1.18) activity using CDNB (chlorodinitrobenzoic acid) as a substrate [33]. Protein content was determined by the BCA (bicinchoninic acid assay) using bovine serum albumin (BSA) as a standard. Spectrophotometric analyses were carried out at 25 °C using a Varian Cary 50 spectrophotometer (Varian, Torino Italy) [33]. All data are reported as percent change with respect to control values.

2.2.7 NO production

Pooled gills were homogenized in 3 vol. of 0.05 M Tris Buffer Solution 2% NaCl, pH 7.4 and centrifuged at 1,000 x g for 15 min at 4°C. The supernatants were then centrifuged at 20,000 x g for 30 min and the resulting supernatants utilized for the spectrophotometric evaluation of nitrite content by the Griess reaction [34]. Absorbance at 540 nm was evaluated by a Varian Cary 50 Bio UV/Visible Spectrophotometer equipped with a microplate reader. Values were corrected for sample blanks readings. Nitrite accumulation ($\mu\text{moles NaNO}_2^{-1}\text{mg sample protein}^{-1}$) was calculated against a standard curve of sodium nitrite (NaNO₂). Protein content was determined according to the bicinchoninic (BCA) method using bovine serum albumin (BSA) as a standard. Average nitrite content in the gill of control mussels was $2 \pm 0.4 \mu\text{moles NaNO}_2^{-1}\text{mg protein}^{-1}$ and data reported as percent of control values.

2.2.8 Comet assay

The genotoxic effects were evaluated in mussel gills as DNA strand breaks (SB) by Single Cell Gel Electrophoresis (or Comet assay). Gill cells were isolated by enzymatic digestion as previously described [35]. The cell suspension obtained was centrifuged at 2000 rpm for 5 min and the pellet used for the Comet assay, the pellet was resuspended in HBSS and cell viability evaluated by Trypan blue exclusion (>95%). Procedures for slide preparation and electrophoresis were carried out according to [36]. Slides were stained with ethidium bromide and observed under a fluorescence microscope (200×). Damaged nuclei were comet shaped due to DNA migration towards the anode. The amount of DNA damage was quantified as the percentage of DNA migrated into the comet tail (% tail DNA) using an image analyzer (Kinetic Imaging Ltd., Komet, Version 5). At least 50 nuclei per slide and 2 slides per sample were scored, for a total of 100 nuclei and the mean calculated.

2.3 Cadmium and Titanium residue in gills and whole soft tissue

Total cadmium and titanium contents were measured by ICP-AES [26] on 4 independent pools of gills and whole soft tissue. Data are expressed as $\mu\text{g g}^{-1}$ dry weight.

2.4 Statistical analysis

Data obtained on gills *in vivo* and *in vitro*, representing the mean \pm SD of at least four samples in triplicate, were analyzed by ANOVA plus Tukey's post-test. For genotoxicity data the effect of exposure, slides, replicates were evaluated by the multifactor analysis of variance (MANOVA). The multiple range test (MRT) was performed in order to detect differences in the tail DNA (%) means among experimental groups. For all data analysis, statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1 Nano- TiO_2 characterization and behaviour in sea water

Typical spheroid irregular shape of nano- TiO_2 (Aeroxide© P25) and primary size of a mean particle diameter of 24 ± 7 nm was confirmed by TEM (Fig. S1). DLS analysis of the particle suspension (10 mg L^{-1}) in ASW confirmed the formation of agglomerates of nano- TiO_2 of micrometric size within the first minute after sonication (Fig. 1), with values of PDI ranging from 0.295 (± 0.028) to 0.383 (± 0.041) and Z-average ranging from 972 (± 35.37) to 1448 (± 25.72) nm (Table 2).

Nano- TiO_2 agglomeration and consequent sedimentation were observed in ASW, while suspensions in MilliQ water showed negligible sedimentation (Fig. S2). This was also confirmed by UV-Vis experiments. As expected, 10 and 50 mg L^{-1} suspensions in ASW (as well as in NSW) showed dramatic sedimentation over 6 h, leading to a decrease of about 50% and 80% of the initial

suspended concentration (Fig. S4a,b), while 1 mg L⁻¹ suspensions remained more stable (Fig. 2a). The same behaviour was observed in NSW (Fig. 2a and Fig. S4a,b), while suspensions in MilliQ water did not show any relevant lowering of initial concentrations (Fig. 2a and Fig. S4a,b). No changes in sedimentation rate of 1 mg L⁻¹ of nano-TiO₂ in the presence of CdCl₂ 100 µg L⁻¹ were observed (Fig. 2b) either in ASW, NSW and MilliQ water. On the opposite, at higher nano-TiO₂ concentrations an increased in sedimentation rate over 6 h compared to cadmium free suspensions was observed in MilliQ water, while no changes were observed in ASW and NSW (Figure S4a,b). Since nano-TiO₂ surface charges remain highly unsaturated in MilliQ water, even a small amount of CdCl₂ could enhance particles aggregation and perhaps sedimentation by reducing particle charge. Is thus highly unlikely to find bare nanoparticles of TiO₂ as monomers in high ionic strength (IS) media, suggesting the absence of physico-chemical interactions between nano-TiO₂ and CdCl₂ in ASW. This outcome was confirmed by Differential Centrifugal Sedimentation (DCS) analysis that allowed an in deep investigation of time-dependent size distribution changes (0, 30, 60, 90 and 120 min) with a high level of accuracy and reproducibility, regardless interferences due to large microscale agglomerates.

Fig. 3 (a) clearly showed an agglomerate population of around 120 nm in size in 10 mg L⁻¹ nano-TiO₂ in ASW over the whole experiment, though several variations (from 122.4 to 133.6 nm) are observed time-dependently, leading to shifted peaks, remarkably within the first half an hour. The same sort of experiment was carried out by adding 0.1 mg L⁻¹ of CdCl₂ (Fig. 3b) and the outcomes were essentially the same (112.6 nm at 0 min and 123.5 nm at 120 min), confirming the absence of CdCl₂ influence in size distribution of the TiO₂ agglomerates in ASW. Time-resolved DCS data were obtained at 10 mg L⁻¹ nano-TiO₂ in ASW, since it was not possible to perform DCS at lower concentrations due to background noise perhaps as a consequence of particle instability in ASW.

3.2 Biological responses in gills

3.2.1 ABC transporters gene expression and activity

In gill biopsies exposed *in vitro*, no alteration in ABC transporter gene transcription was observed in any experimental group compared to controls (Fig. 4a). On the contrary, both nano-TiO₂ and CdCl₂, alone and in co-exposure, significantly decreased the accumulation of RhB compared to controls (Fig. 4b). The protective role of ABC transporters against CdCl₂ has been already pointed out in *M. galloprovincialis* [29]. In mammalian models, nano-TiO₂ can attenuate the function of the P-gp, thus potentially affecting cellular disposition of pollutants as well as of endogenous substrates [37,38]. Our results confirm that *in vitro* both CdCl₂ and nano-TiO₂ induced significant reduction in RhB accumulation in gills biopsies; however, no interactive effects were observed with the mixture.

The increased efflux activity was not paralleled by *abcb1* m-RNA abundance in any experimental group, this probably due to the short-term exposure conditions. A different effect was observed upon *in vivo* exposure. While nano-TiO₂ did not affect significantly *abcb1* gene transcription, a significant increase was induced by CdCl₂ alone (up to 2.8-folds with respect to controls), while *abcc-like* gene resulted slightly down-regulated (Fig. 4c). Such increase was not observed in co-exposure conditions (Fig. 4d).

These results further support the role of P-gp in the CdCl₂ defence response and suggests an antagonistic effect by nano-TiO₂.

3.2.2 *mt-20* gene expression and Metallothionein content

In vivo exposure to nano-TiO₂ did not affect *mt-20* gene transcription in mussel's gills. A significant up-regulation of *mt-20* with respect to controls was observed in the gills of mussels exposed to both CdCl₂ alone and combined with nano-TiO₂ (Fig. 5a) A similar trend was also observed for MT protein level; nano-TiO₂ alone did not affect MT content, whereas a significant increase (up to 4 folds) was induced by Cd²⁺, either alone and in combination with nano-TiO₂ (Fig. 5b).

The similar trend observed in *mt-20* gene expression and MT total protein content in gills of mussel exposed to CdCl₂ but also in those in co-exposure confirmed the active response towards CdCl₂ exposure, in line with classical metal-induced transcriptional responses in *Mytilus* [39]. However also in the gills co-exposed, nano-TiO₂ did not affect the metal induced defence response, as previously demonstrated in hemocytes and digestive gland in similar experimental conditions [26].

3.2.3 GST activity and NO production

GST activity and NO production were evaluated as biochemical responses that reflect the gill function. Mussel gills have a high potential for phase II biotransformation through GST, whose activity has been shown to be modulated by certain types of NPs [15]. Nano-TiO₂ did not affect GST activity, whereas a large increase was induced by exposure to CdCl₂ (about +50% with respect to controls; $p \leq 0.05$). However, stimulation of GST was not observed in the gills of mussels co-exposed to nano-TiO₂ and Cd²⁺ (Fig. 6a). Taken together, the results obtained for *abcb1* gene expression and GST activity support the hypothesis of an antagonistic effect of the two contaminants on the detoxification pathways in mussel gills.

NO is an intracellular signalling molecule ubiquitously found in animals: in molluscs, NO is mainly involved in neuromodulation, metamorphosis, and immune response [40]. NO production by mussel immune cells, the hemocytes, represents a sensitive response to chemical exposure, including nano-TiO₂ [24,26]. The results here obtained show that in mussel gills nano-TiO₂ induced

a significant increase in NO production similar to that previously observed in hemocytes (+49%). A larger increase was observed with Cd²⁺ alone (+86%) and an even stronger effect was induced by co-exposure with nano-TiO₂ (+131%) (Fig. 6b). Increased NO production may be partly due to hemocytes infiltrating the tissue, indicating inflammatory processes; however, NO can be also produced by dopaminergic neurons that control ciliary activity of mussel gills. In particular, on *Mytilus*, NO production by dopaminergic neurons inhibits beating of lateral cilia [41], that serve a vital function by generating the water currents that regulate gas exchange, food intake and waste removal. In this light, the results underline how determination of NO production by mussel gills represents a sensitive response to nano-TiO₂ and Cd²⁺, alone and in combination, that can reflect impairment of the physiological function of the tissue.

3.2.4 Genotoxicity

Nano-TiO₂ exposure did not affect DNA integrity in mussel gill cells. On the contrary, CdCl₂ induced a statistically significant increase of DNA strand breaks compared to controls (p<0.05). In co-exposed specimens the DNA damage was reduced to control level (Fig. 7). The genotoxic and carcinogenic potential of Cd has been widely investigated in aquatic organism [27,42,43]. Cd is known to affect genome stability inhibiting DNA repair and unbalancing the cell equilibrium between pro-oxidant forces and antioxidant defenses. Indeed, it is also known to induce oxidative stress by depletion of glutathione (GSH) in association with mitochondrial damage, induction of apoptosis and disruption of calcium signaling [44]. Although the occurrence of oxidized bases was not evaluated, the increased DNA primary damage detected in mussels exposed *in vivo* to Cd might be assumed as the consequence of Cd-related DNA oxidative damage as supported by the increase of GST activity. The ineffectiveness of nano-TiO₂ exposure in term of DNA primary damage induction in mussel was previously reported [10]. However, the same authors documented the elevation of micronucleated cells, suggesting the occurrence of chromosomal alteration, as the consequence of *in vivo* exposure nano-TiO₂.

The finding that nano-TiO₂ co-exposure completely prevents the occurrence of DNA strand breaks associated with Cd exposure suggests that nano-TiO₂ might act as antagonist towards Cd²⁺ induced DNA damage in mussel gill cells.

3.3 Cadmium and Titanium content in gills and whole soft tissues

Mussel exposure to Cd²⁺ resulted in large metal accumulation in the gills, about 150-folds with respect to controls (278 ± 4 μg/Cd²⁺/g d.w.); a slightly lower Cd²⁺ content was observed in mussels co-exposed with nano-TiO₂ (237±3 μg/Cd²⁺/g d.w.) (Fig 8a). Cd²⁺ accumulation was measured also

in whole soft tissue, indicating similar concentrations in mussels exposed to Cd^{2+} alone and in combination with nano- TiO_2 (about 108-folds with respect to controls) (Fig. 8a).

In the gills of nano- TiO_2 -exposed mussels, the Ti content was $0.27 \pm 0.02 \mu\text{g/g d.w.}$, about 2-folds with respect to controls (Fig. 8b); interestingly, co-exposed mussels showed a significantly higher Ti content, about 5-folds with respect to controls (up to $0.8 \pm 0.02 \mu\text{g/Ti/g d.w.}$). Significant accumulation of Ti was also observed in the digestive gland, with lower levels measured in the tissues of mussels co-exposed with Cd^{2+} [26]. We have previously demonstrated that, in the same experimental conditions, only a minor fraction (<10%) of the nominally added nano- TiO_2 is available to the mussels in the suspended form, due to both particle sedimentation and agglomeration/adsorption processes occurring in the presence organic particles produced by the mussels (mucus, fecal pellets, gametes). This would explain the low concentrations of Ti measured in the tissues of mussels exposed to nano- TiO_2 , alone and in combination with Cd^{2+} . However, no increase in Ti content was observed in whole soft tissues of mussel exposed to nano- TiO_2 , either alone or combined with Cd^{2+} (Fig. 8b). In mussels, gills are first target of environmental pollutants acting not only as barrier but also as first temporary storage tissue. Toxicant unmodified or bound to molecules are transferred from gills to the circulatory system and to excretory tissues or to the digestive gland [27]. Taken together, the results obtained in mussel gills and digestive gland in comparison with whole soft tissues, underline the importance of tissue specific accumulation of Ti. Studies on different freshwater model species highlighted the interference of metals in NPs uptake and *vice versa* [11,45,46]. Nevertheless these findings were often conflicting and did not consider tissue-specific accumulation. The results obtained in this work indicate that co-exposure with nano- TiO_2 did not increase, but rather slightly decreased the Cd content in gills. This observation, together with and data on heavy metal detoxication response (metallothionein induction) indicates the absence of a *Trojan horse* effect of nano- TiO_2 towards Cd^{2+} in saltwater media, confirming previous data [26].

With regards to biomarker responses measured from molecular to tissue level, the results indicate that co-exposure did not increase the overall toxicity of either contaminant in mussel gills. However, the results seem to highlight interactive effects of nano- TiO_2 and Cd^{2+} on certain biomarker responses *in vivo* also in this tissue, as previously observed in hemocytes and digestive gland [26]. The observed antagonistic effect of nano- TiO_2 vs Cd^{2+} responses related to detoxication pathways may be of particular importance. Gills in fact are the first site of contaminant entry and detoxication, therefore the co-exposure with nano- TiO_2 may influence the overall disposal, fate and toxicity of contaminants in other tissues.

4. Conclusions

The results of this study provide important information concerning the potential interference of nano-TiO₂ with Cd⁺² responses in gills of the mussel *M. galloprovincialis*. Our results highlighted gills as target organs for both contaminants, alone and in combination. In light of these observations, studies investigating NPs pathways of internalization into the organism as well the internal distribution and accumulation in different compartments, as well as tissue specific biomarker responses, are strongly recommended. The multimarker approach on different tissues allowed to identify sensitive targets of NPs toxicity that might be concealed when considering the effects on the whole organism.

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Appendix A. Supplementary data

Supplementary data associated with article is attached.

Conflicts of interest

Authors declare that there are no conflict of interest.

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Figures Captions

Fig. 1. DLS time-resolved size distribution for nano-TiO₂ in ASW. In the graph are reported 4 distinct measurements of the same suspension (30, 60, 90 and 120 min after sonication) expressed by intensity.

Fig. 2. Size distribution by UV-Vis. Time-dependent UV-Vis analysis of 1 mg L⁻¹ suspensions of nano-TiO₂ in ASW, MilliQ and NSW (a) and with the addition of 0.1 mg L⁻¹ of CdCl₂ (b).

Fig. 3. Size distribution by DCS. DCS analysis of 10 mg L⁻¹ suspensions of nano-TiO₂ in ASW (a) and with the addition of 0.1 mg L⁻¹ of CdCl₂ (b).

Fig. 4. Effects on ABC transporters. *Abcb1* and *abcc-like* genes expression (a, c) and P-gp efflux activity (b,d) in gill biopsies exposed *in vitro* and *in vivo* to nano-TiO₂ (nano-TiO₂ 1 mg L⁻¹) and Cd²⁺ (Cd 0.1 mg L⁻¹), alone and in combination. Data, representing the mean±SD, were analysed by ANOVA plus Tukey's post-test. *Significantly different respect to ctrl # Significantly different respect to Cd single exposure (p< 0.05).

Fig. 5. Effects on Metallothioneins. *mt-20* gene expression (a) shown as fold induction vs control. Total metallothionein content (b) shown as µg/g dry weight (d.w.). Data, representing the mean ± SD, were analysed by ANOVA plus Tukey's post-test. *Significantly different respect to ctrl (p< 0.05) ** (p<0.001).

Fig. 6 GST activity and NO production. GST activity (a) and NO production (b) both shown as % of control. The mean ± SD were analysed by ANOVA plus Tukey's post-test. *Significantly different respect to ctrl (p< 0.05). # Significantly different respect to Cd single exposure. § Significantly different respect to n- TiO₂ single exposure.

Fig. 7. Genotoxicity. DNA damage shown as % of tail DNA. Data represent the mean ± SD. *Significantly different respect to ctrl (p<0.05).

Fig. 8. Cadmium and Titanium content. Total Cadmium (a) and Titanium (b) in mussel gills (white bar) and whole soft tissue (black bar). Data shown as accumulation over control samples. ANOVA plus Tukey's post-test.*Significantly different respect to ctrl (p< 0.05). # Significantly different respect to single exposure.

Table 1. Primers sequences used for the qRT-PCR analyses

gene	Fw 5'-3'	Rev 5'-3'	Amplicon size bp	Annealing T °C	Accession number
<i>abcb1</i>	AAAGCAAGCCCACTGGTATGGT	ATGGCAGTGAAGGCAATGGCAA	162	55	FM999809.2
<i>abcc-like</i>	CCGGTAGGCAGGATTGTAANA	AAGCGCTGTAAGGCCGAATA	194	56	FM999810.2
<i>mt-20</i>	TGTGAAAGTGGCTGCCGA	GTACAGCCACATCCACACGC	80	59	AY566248
<i>efa</i>	TTGCTGTCCGAGACATGAGACA	TCATTCTTGGCCACCGGCTTT	119	55	AB162021.1

Table 2. Time- resolved DLS data for nano-TiO₂ suspensions in ASW at 25°C and $\theta = 173^\circ$ at 10 mg L⁻¹ concentration

Nano-TiO ₂ P25	Time [min]	Z-Ave. D _H [nm]	PDI	Peak 1 D _H [nm]	Peak 2 D _H [nm]
10 mg L ⁻¹ in ASW	30	972 ± 35.37	0.295 ± 0.028	901.5 ± 57.74	5314 ± 291.0
10 mg L ⁻¹ in ASW	60	1073 ± 40.05	0.294 ± 0.007	1003 ± 66.07	5287 ± 107.7
10 mg L ⁻¹ in ASW	90	1448 ± 25.72	0.383 ± 0.041	1159 ± 89.81	5377 ± 4.041
10 mg L ⁻¹ in ASW	120	1134 ± 47.44	0.348 ± 0.045	917.0 ± 16.24	5463 ± 137.2

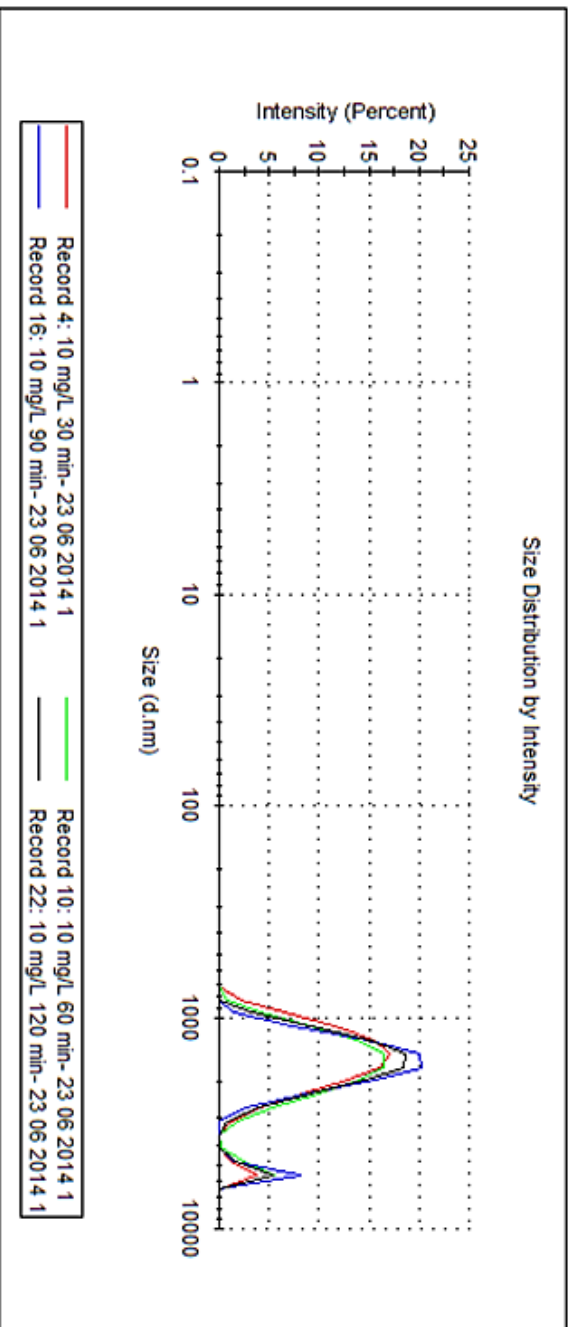


Fig. 1

Fig. 2a

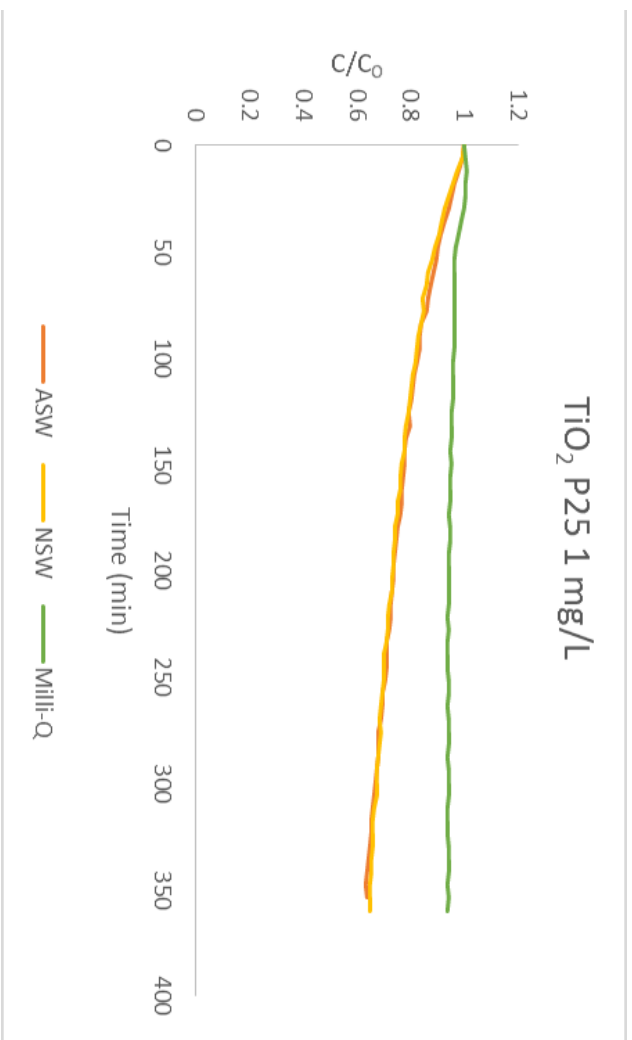


Fig. 2b

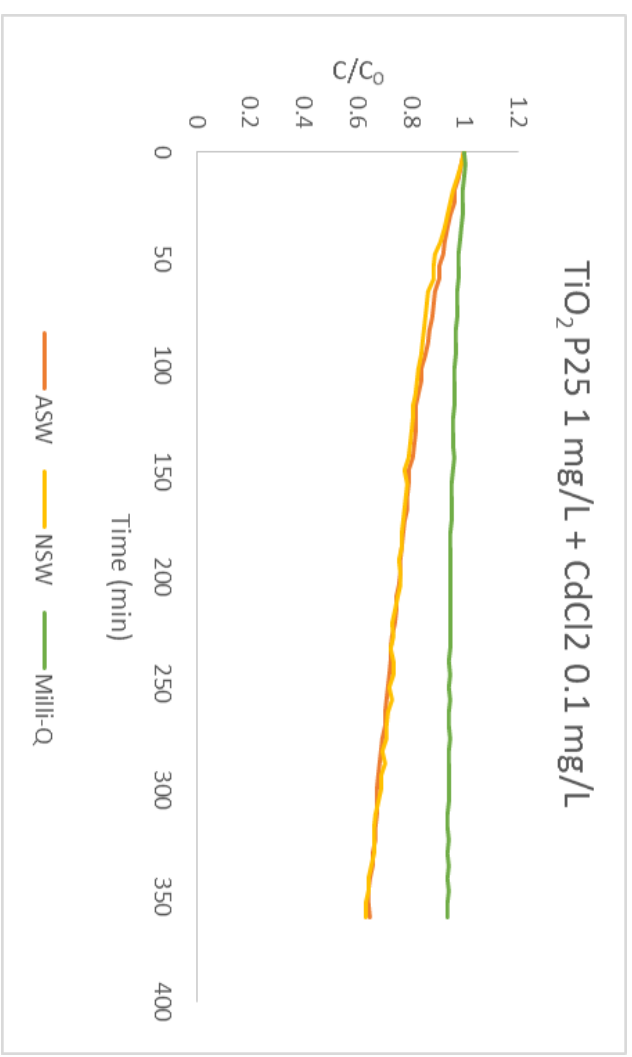


Fig. 3a

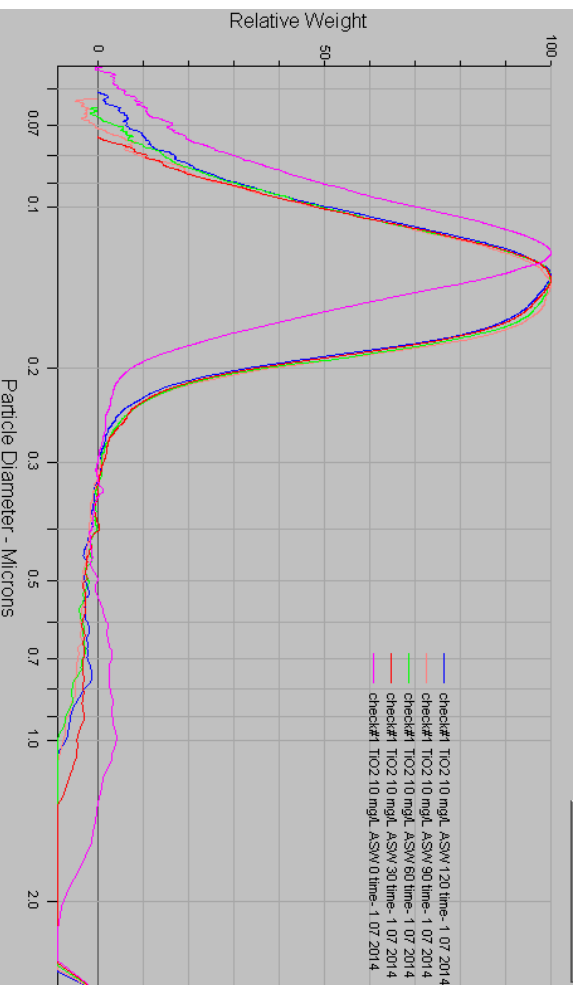
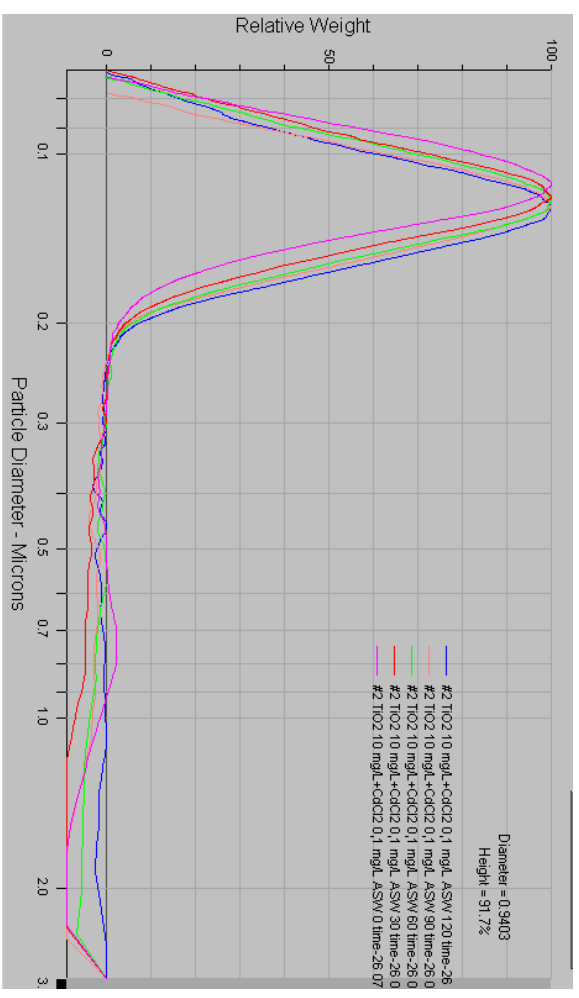


Fig. 3b



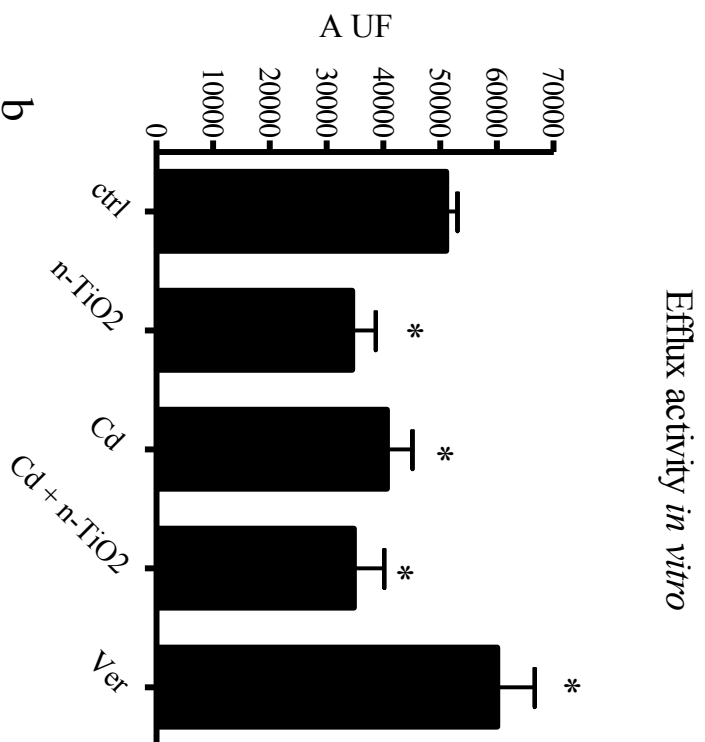
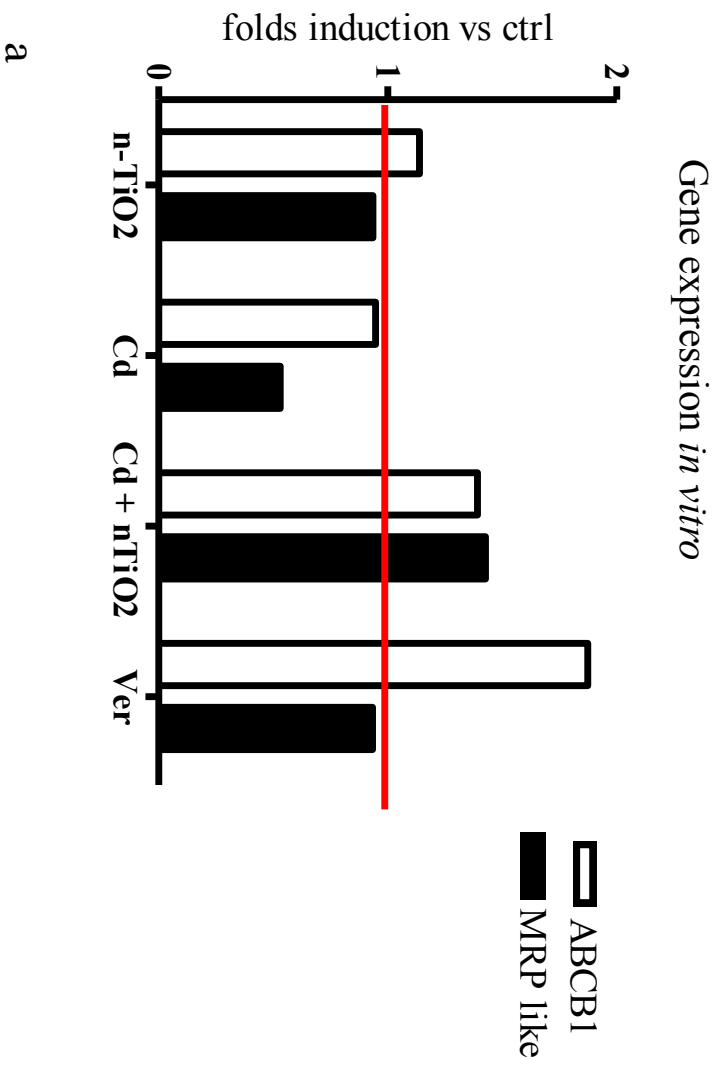
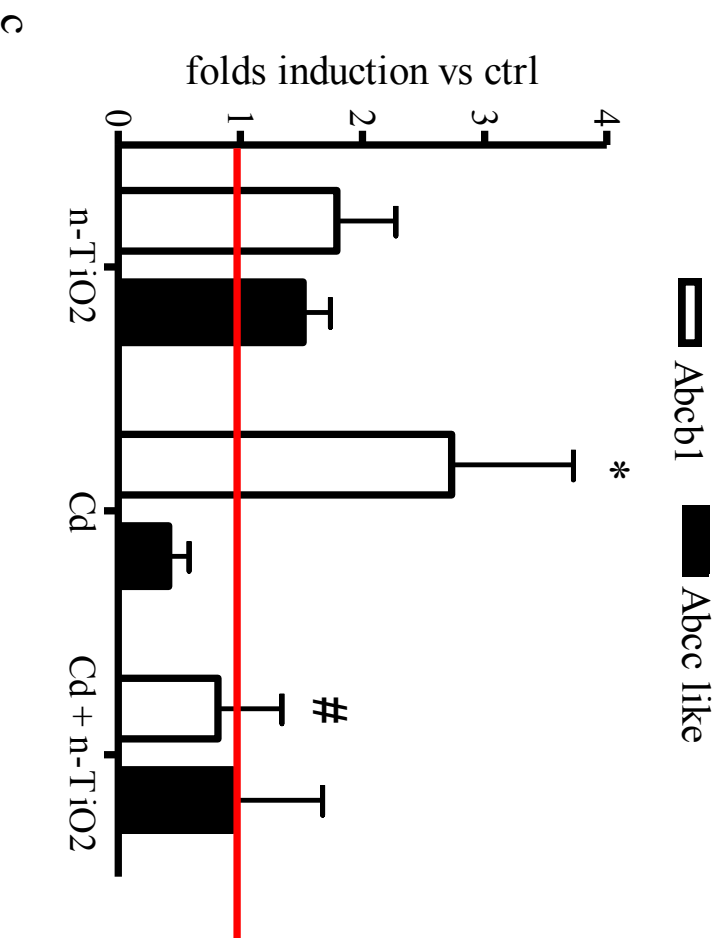


Fig. 4 (a,b)

Gene expression *in vivo*



Efflux activity *in vivo*

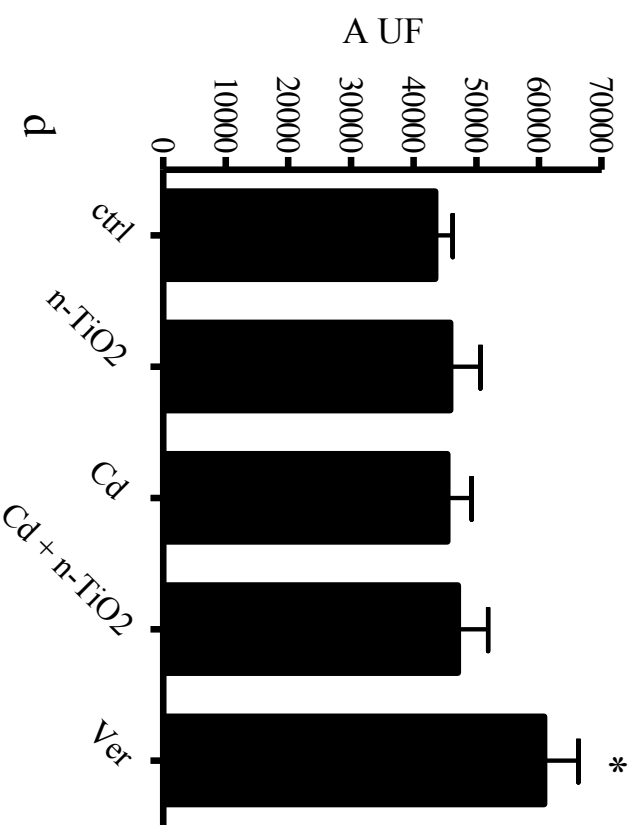
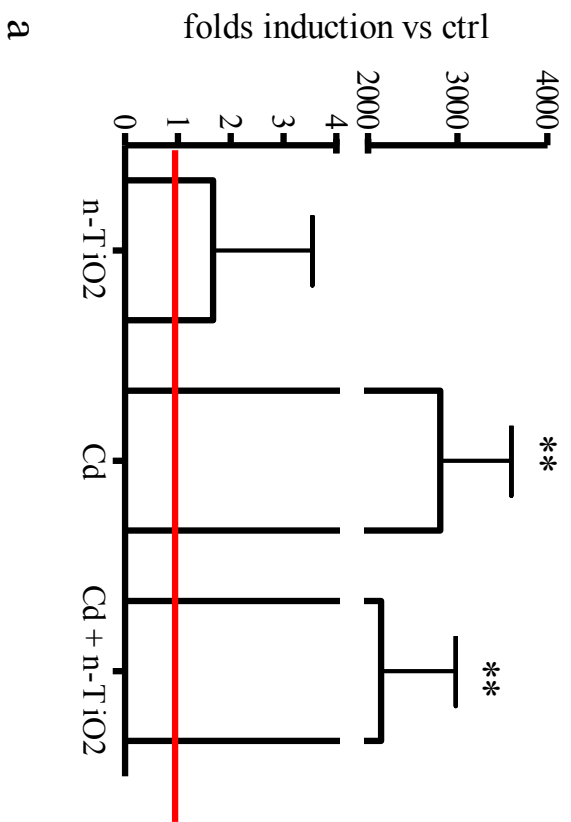


Fig. 4 (c,d)

MT-20 gene expression



MT content

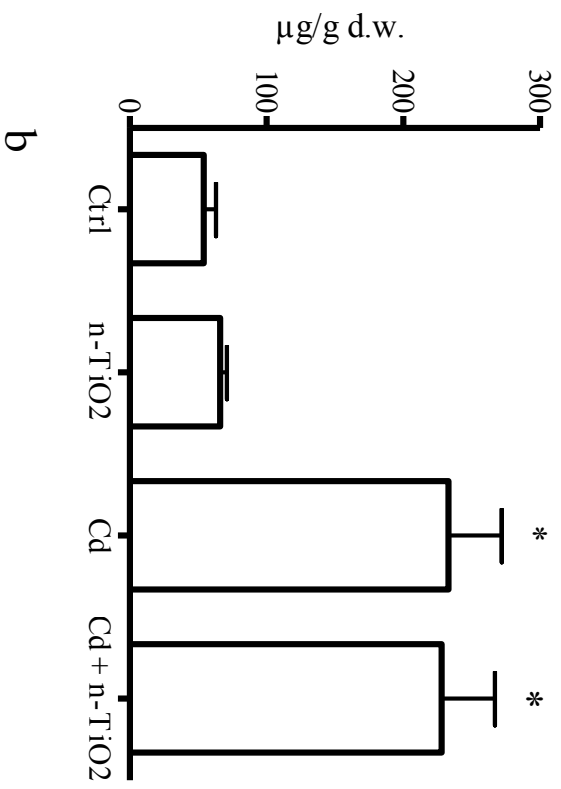
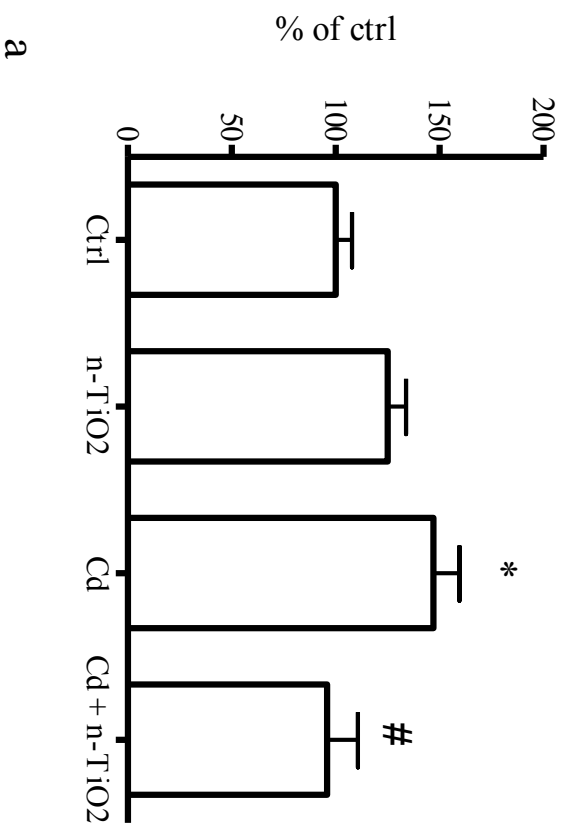


Fig. 5 (a,b)

GST



Nitrite accumulation

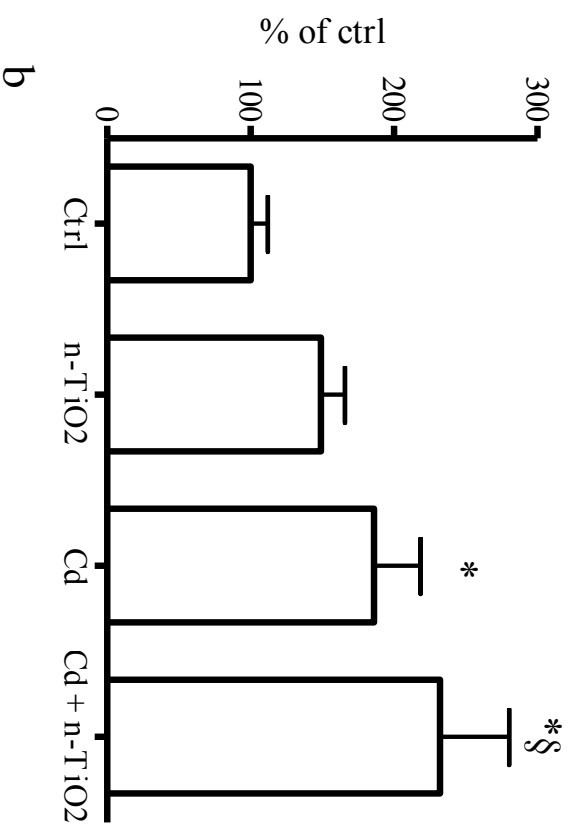


Fig. 6 (a,b)

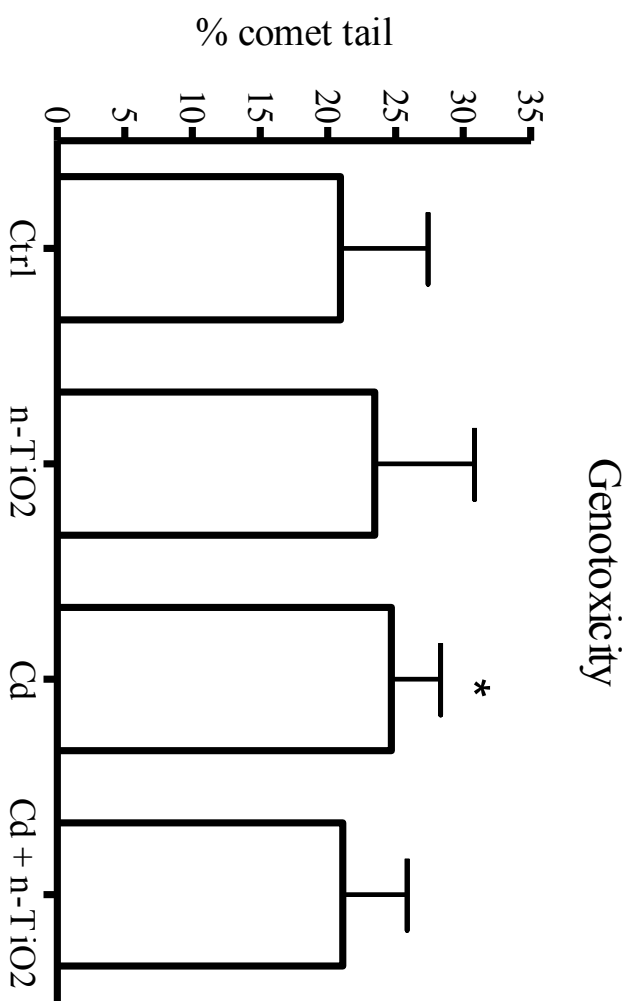


Fig. 7

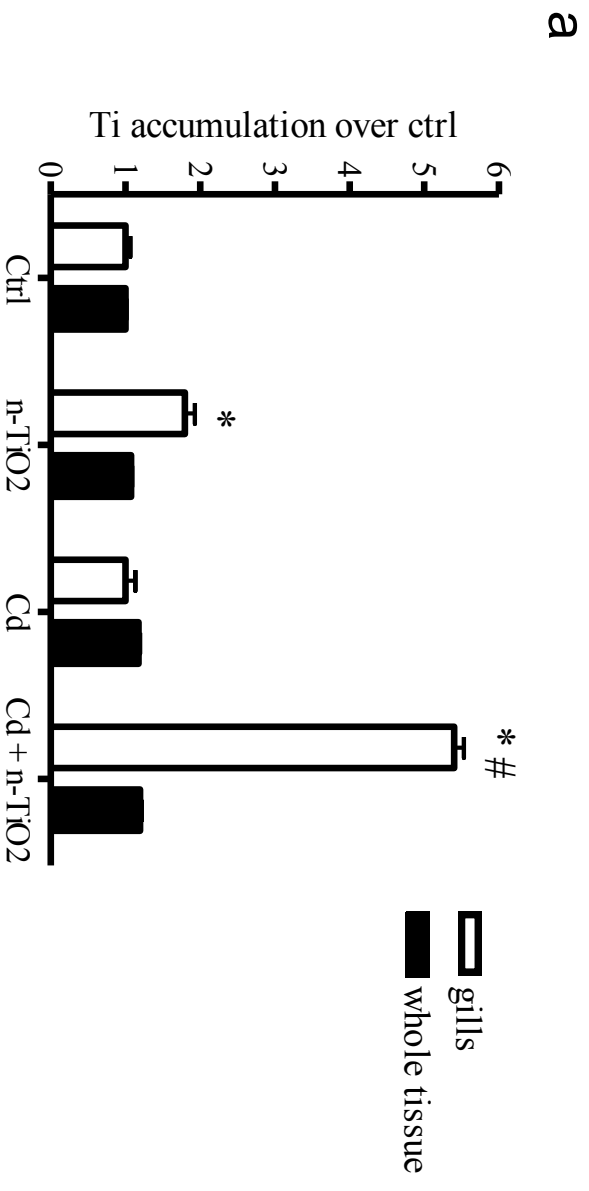
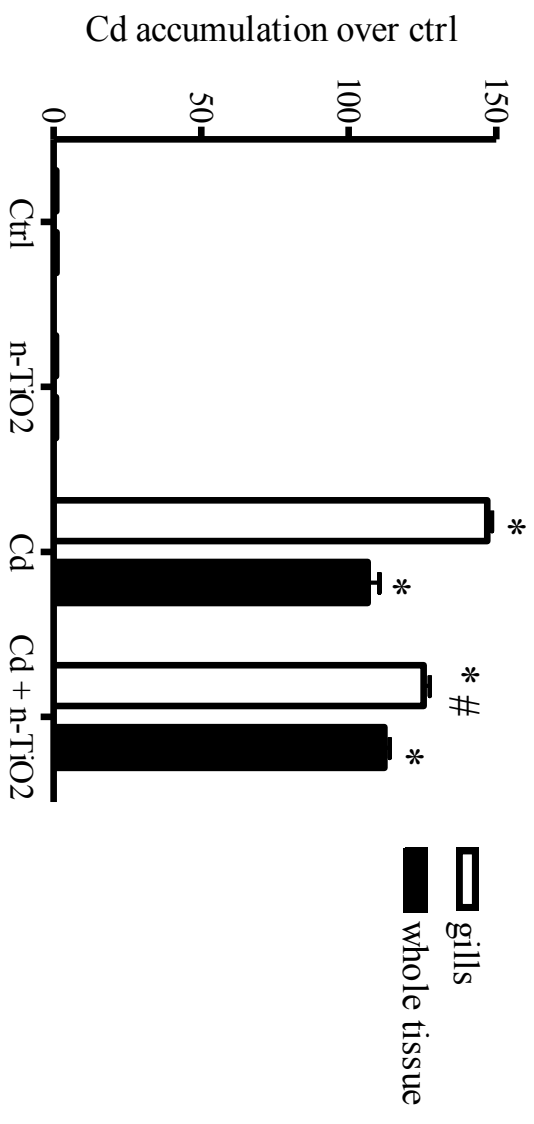


Fig. 8 (a,b)