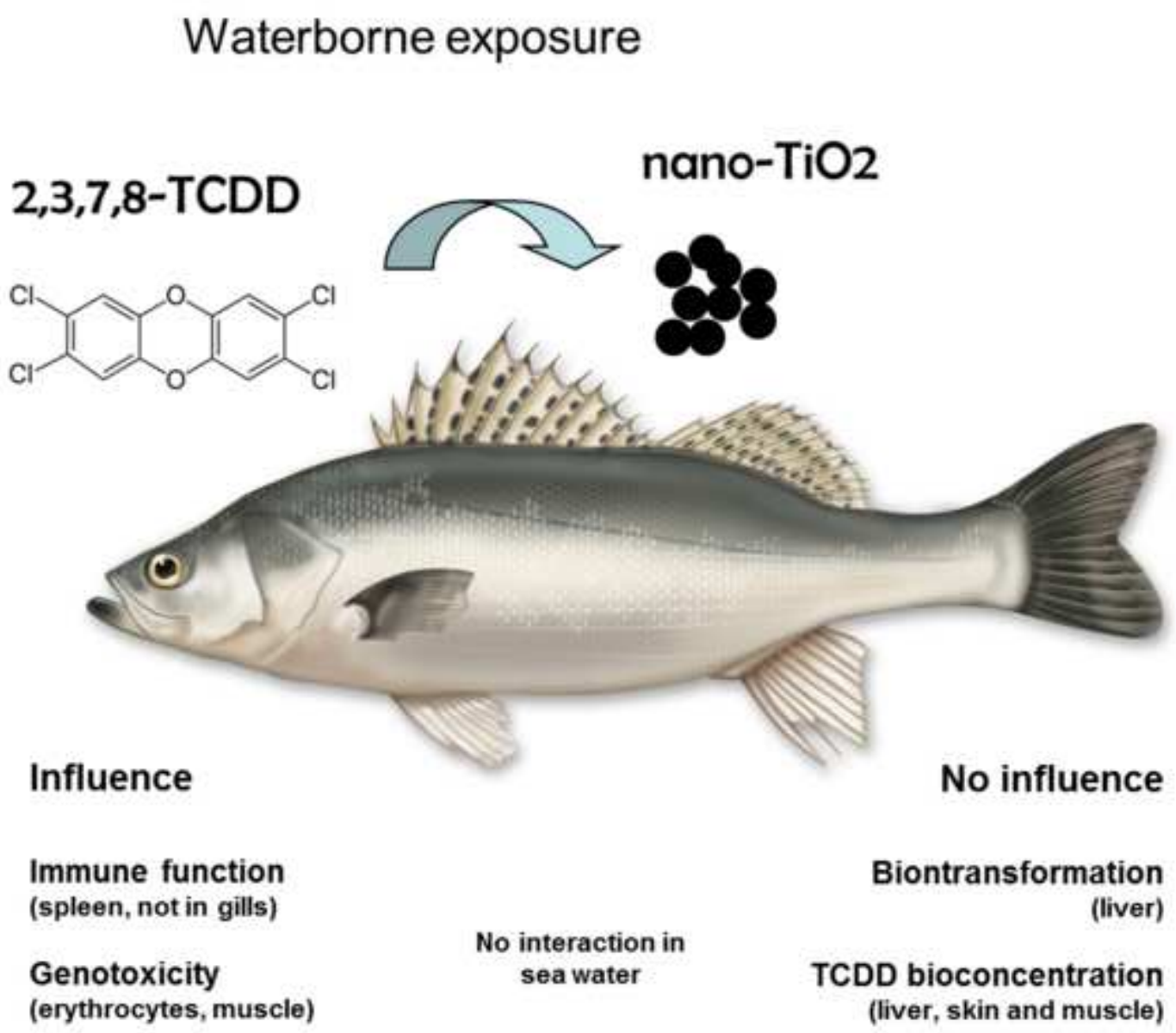
Graphical Abstract (for review)



Highlights

Nano-TiO_2 did not interfere with 2,3,7,8-TCDD detoxification and bioconcentration in liver.

Nano-TiO_2 affected immune response towards 2,3,7,8-TCDD exposure in spleen.

Co-exposure caused a reduction of DNA strand breaks respect to single chemicals exposure.

Nano-TiO_2 alone and combined with 2,3,7,8-TCDD increased MN frequencies in erythrocytes.

Influence of titanium dioxide nanoparticles on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin bioconcentration and toxicity in the marine fish European sea bass (*Dicentrarchus labrax*)

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ABSTRACT

The present study investigated the influence of nano-TiO_2 (1 mg L^{-1}) on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) (46 pg L^{-1}) bioconcentration and toxicity in the European sea bass (*Dicentrarchus labrax*) during 7 days *in vivo* exposure. A multimarkers approach was applied in different organs: detoxification in liver; innate immunity and pro-inflammatory response and adaptive immunity in gills and spleen; genotoxicity in peripheral erythrocytes and muscle. Bioconcentration of 2,3,7,8-TCDD in presence of nano-TiO2 was investigated in liver, skin and muscle as well as interaction between nano-TiO2 and organic pollutants in artificial sea water (ASW). Nano-TiO2 negatively influenced immune response induced by 2,3,7,8-TCDD in spleen but not in gills and reduced the DNA damage induced by 2,3,7,8-TCDD in erythrocytes. nano-TiO2 did not interfere with 2,3,7,8-TCDD detoxification and bioconcentration according to the observed no interaction of the nano-TiO2 with organic pollutants in ASW.

Capsule: The present study provides first evidence on the toxic effects of nano-TiO2 in the marine fish *D. labrax* highlighting nano-TiO2 influence on 2,3,7,8-TCDD bioconcentration and toxicity

Keywords: nano-TiO2; 2,3,7,8-TCDD; CYP1A; immunomodulation; genotoxicity

1. Introduction

Titanium dioxide (nano-TiO_2) is one the most widespread NP used in consumer and personal care products, as well as in many industrial sectors (Robichaud et al., 2009). It is also employed for environmental applications (i.e. nanoremediation) as efficient catalyst and adsorbent of organic contaminants and heavy metals (Karn et al., 2009). Nano-TiO_2 is thus released in huge amount in urban and industrial sewage and it is expected to occur in the aquatic environment at concentration of μg L^{-1} (PEC in water 0.7-16 ug L^{-1}) (Batley et al., 2013). A significant input of nano-TiO_2 from sunscreen products in natural surface waters has been recently reported (Gondykas et al., 2014). From its release into soil and waterways as well as for direct use on maritime technologies, nano-TiO_2 will end up in the sea, which might represents the ultimate sink such to represent an actual risk for marine organisms (Moore, 2006; Delay and Frimmel, 2012; Matranga and Corsi, 2012; Holden et al., 2013). Being listed as possible carcinogen for humans (B2, IARC, 2006), toxicological effects and ecological damage for many marine organisms cannot be excluded and need to be deeply investigated. In freshwater species, immunotoxicity, cytotoxicity and oxidative stress as well as physiological and reproductive alterations, have been well documented (Jovanovic and Palic, 2011, 2012; Menard et al., 2011; Diniz et al., 2013; Hartmann et al., 2013; Ramdsen et al., 2013). Same effects have been already reported in invertebrate marine species (Galloway et al., 2010; Canesi et al., 2012; Barmo et al., 2013; D'agata et al., 2013; Minetto et al., 2014). As for marine fish nano-TiO_2 has shown to induce sub-lethal adverse effects on the early life stages of the brackish species *Oryzias latipes* as premature hatching, pericardial edema and abnormal development (Paterson et al., 2011) while the effects on adults are largely unknown. Two *in vitro* studies on marine mammals clearly showed that nano-TiO_2 cause genotoxicity in bottle-nose dolphin leukocytes (Bernadeschi et al., 2010) and fibroblasts (Frenzilli et al., 2014). Besides toxicity caused by its inherent properties, nano-TiO_2 might also interact with other co-existing environmental pollutants -as metals and organic xenobiotics- thus modifying their availability, bioaccumulation and toxicity. Such effect is reported for freshwater species where adsorption on nano-TiO_2 enhances

69 uptake and retention of Cd^{2+} in carp and Daphnia (Zhang et al., 2007; Hartmann et al., 2010, Hu et
70 al., 2011; Hartmann et al., 2012; Yang et al., 2012), while bioavailability and metabolism of an
71 organic contaminant as BDE209 is enhanced by nano-TiO_2 in zebrafish larvae (Wang et al., 2014).
72 The interaction of nano-TiO_2 with organic pollutants has been also reported in seawater. Enhanced
73 toxicity of TBT was reported in the presence of nano-TiO_2 in marine abalone embryos (Zhu et al.,
74 2011). In our previous study (Canesi et al., 2014) using the marine mussel *Mytilus galloprovincialis*
75 as model species, complex interactions between nano-TiO_2 with 2,3,7,8-tetracholorodibenzo-*p*-
76 dioxin (2,3,7,8-TCDD) were reported on a wide range of molecular and physiological biomarkers
77 measured in hemolymph, gills and digestive gland. The co-exposure with nano-TiO_2 increased
78 accumulation of 2,3,7,8-TCDD in whole soft tissue of mussels. Both synergistic and antagonistic
79 sub-lethal effects were observed depending on cell/tissue type and measured biomarker. A similar
80 study with marine clam *Scapharca subcrenata* showed an enhanced uptake and accumulation of
81 Phenanthrene (PhE) in the presence of nano-TiO_2 based on an high adsorption capability of nano-
82 TiO_2 in seawater (Tian et al., 2014). So far any studies have evaluated this phenomenon in fish
83 species that possess completely different mechanisms of uptake/detoxification/toxicity compared to
84 bivalves.
85 Therefore presence of nano-TiO_2 in marine waters and its potential interaction with organic
86 pollutants highlight the susceptibility of marine organisms and the need of more studies on
87 interactive effects of nano-TiO_2 with existing toxic contaminants in marine waters with particular
88 focus on piscine models.
89 Amon organic pollutants 2,3,7,8-TCDD is one of the most potent carcinogenic chemical, able to
90 elicit a wide spectrum of biological effects following specific cellular pathways (Mandal, 2005;
91 White and Birnbaum, 2009). 2,3,7,8-TCDD and other organochlorines are usually detected in
92 marine organisms (up to pg g^{-1} in fish for 2,3,7,8-TCDD (Greco et al., 2010; Nunes et al., 2011) and
93 biomagnify through trophic webs (Corsolini et al., 2002).

In the present study we investigated the influence of nano-TiO_2 on 2,3,7,8- TCDD bioconcentration and sub-lethal toxicity (detoxification, immunotoxicity, genotoxicity) in the marine fish European sea bass *Dicentrarchus labrax* during 7 days *in vivo* exposure.

A multimarkers approach was applied in different organs: detoxification (CYP1A gene and EROD activity) in liver; innate immunity and pro-inflammatory response (*IL-1β*, *IL-8*, *TNF-α* and *Cox-2*) and adaptive immunity (*IgM*, and *TRβ*) in gills and spleen; DNA primary damage and micronuclei occurrence in peripheral erythrocytes; genomic stability in muscle. Bioconcentration of 2,3,7,8-TCDD in presence of nano-TiO_2 was also investigated in skin, muscle and liver tissues as well as interaction in artificial sea water (ASW) between nano-TiO_2 and organic pollutants.

2.Materials and Methods

2.1 Materials

The nanosized Titanium Dioxide (nano-TiO_2), namely Aeroxide® (declared purity: 99.9%), was kindly supplied by Eigenmann & Veronelli (Milan, Italy). The provided batch was characterized by a combination of analytical techniques (HR-TEM, TEM-EDX, XRD, HR-TEM-SAED, BET, ICP-MS, etc.) as previously described (Barmo et al., 2013). Stock suspension of 1 mg mL^{-1} nano-TiO_2 was prepared by dispersing the NPs in filtered (0.22 μm) artificial sea water (ASW), prepared according to ASTM protocol (2004) at 36‰ salinity, pH 8.3±0.1 and sonicated for 45 min (100W, 50% on/off cycle while cooling the dispersion in an ice bath) with a probe sonicator HD 2070 Bandelin Electronic (Berlin). Nano-TiO_2 solution was freshly prepared prior to use by diluting the stock with ASW and sonicating for 10 min. Size distribution of nano-TiO_2 in ASW suspensions was determined as hydrodynamic diameter by Dynamic Light Scattering (DLS) analysis, performed with a Submicron Particle Sizer Nicomp 370 (Santa Monica, Ca, USA), equipped with a 35 mW He-Ne laser, 632.8 nm laser diode and photodiode detector set to 90°. The 2,3,7,8-TCDD standard solution in dimethyl sulfoxide (DMSO) at 32.2 ± 1.6 mg mL^{-1} was purchased from Wellington Laboratories (Ontario, Canada). Whether not specified, chemicals were purchased from Sigma-

120 Aldrich.

121 **2.2 *In vivo* exposure**

122 Juveniles of European sea bass (*Dicentrarchus labrax*) (7–12 cm TL) were obtained from a local
123 fish farm (Agroittica Toscana, S.r.l., Piombino, Italy) and maintained for 96 h before the experiment
124 in flow-through circulating 100-liters aquaria at 16°C, constant (12:12 light:dark) photoperiod
125 without feeding. Fish husbandry and experimental procedures were conducted within the EU
126 legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU).
127 Stock suspension of nano-TiO_2 ready for use (as above described) were added in each tank (40 L
128 ASW) to reach nominal concentration of 1.0 mg L^{-1}. 10 specimens for each experimental group
129 were exposed as follows: (1) control (ASW); (2) nano-TiO_2 (1 mg L^{-1} in ASW); (3) 2,3,7,8-TCDD
130 (46 pg L^{-1}) (adding DMSO 0.001‰ final concentration); (4) co-exposure of nano-TiO_2 plus 2,3,7,8-
131 TCDD (1 mg L^{-1} and 46 pg L^{-1} respectively and adding DMSO 0.001‰ final concentration); (5)
132 nano-TiO_2 (1 mg L^{-1} adding DMSO 0.001 ‰ final concentration). Both nano-TiO_2 (1 mg L^{-1}) and
133 2,3,7,8-TCDD nominal doses (46 pg L^{-1}) were chosen being far below the LC_{50} reported for fish
134 species but still able to induce a significant biological responses in fish: > 100 mg L^{-1} for nano-TiO_2
135 (Hall Clements et al., 2013; Xiong et al., 2011) and > 5.60 ng L^{-1} for 2,3,7,8-TCDD (Technical
136 Report N°11 2004)(Ortiz Delgado et al., 2008; Ramdsen et al., 2013; Faria et al., 2014).
137 Chemicals were added at the same time in the exposure water. A semi-static experimental condition
138 was chosen and test solution were renewed every 24 h in order to maintain relatively consistent
139 levels of exposure. Fish were not fed during the experiments. After 7 days, peripheral blood was
140 collected from caudal vein with heparinized syringe and processed for genotoxicity assays. Dorsal
141 muscle reduced in small pieces was embedded in ethanol for genomic stability assay. Liver,
142 portions of gills, spleen, muscle and skin were removed and stored at -20°C for 2,3,7,8-TCDD
143 analysis and at -80°C for molecular and biochemical assays.
144

145 **2.3 Quantification of 2,3,7,8-TCDD in fish tissues**

146 Small portions of skin, muscle and liver of exposed fish were pooled using 10 organisms per
147 experimental group (range weight of pools 0.42-4.87 g) and analysed in duplicate according to
148 Method EPA 1613 (US EPA, 1994). Briefly, samples were extracted in duplicate using a Dionex
149 Accelerated Solvent Extractor (ASE) with 60 mL of toluene (US EPA 3545A method revision B,
150 US EPA (1996) and a Soxhlet system (*n*-hexane-dichloromethane v/v 1:3, 12 h).
151 A labelled standard (13C12-2,3,7,8-PCDD, Cambridge Isotope Laboratories) suitable for the EPA
152 1613 method was used for the isotopic dilution. The extract was cleaned up using a Power Prep
153 System TM (Fluid Management Systems Inc.) with silica, alumina and carbon packed columns
154 (PX-21) (Software DMS 6000).
155 The 2,3,7,8-TCDD was identified and quantified using a Trace 2000 GC equipped with an AS
156 Autosampler (Thermo Finnigan) according to Corsolini et al. (2007). IDL was estimated at 0.2 pg
157 μL^{-1} and LOD at 0.001 ng g^{-1} wet weight (wet wt). Results were confirmed by HR-GCMS using a
158 Thermo Scientific DFS HRMS instrument equipped with two Thermo Scientific Trace 1310 GC
159 and Thermo Scientific TriPlus RSH robotic sampler, following a method described by Domotorova
160 et al. (2012). The LOQ of the 2,3,7,8-TCDD was 0.00014 ng μL^{-1}. One blank every set of five
161 samples was analysed throughout the procedure to check for interference and laboratory
162 contamination; blanks were <LOD (0.001 ng g^{-1} wet wt and 0.05 pg μL^{-1} for GC/MS and HR
163 GC/MS, respectively). Reported concentrations were blank-corrected. The accuracy and precision
164 of the procedure were tested using an inter-calibration exercise (X CIND, 2010). A certified
165 reference material was used (WMF-01: Freeze-Dried Fish Tissue for Organic Contaminant
166 Analysis, Wellington Laboratories). Results are given on wet wt basis.
167
168 **2.4 nano-TiO_2 interaction with 2,3,7,8-TCDD**
169 Chemical interaction between nano-TiO_2 Aeroxide® and 2,3,7,8-TCDD was investigated by
170 adsorption spectroscopy (Perkin Elmer Lambda 40 UV-Vis) and by Nuclear Magnetic Resonance
171 spectroscopy (NMR) (Varian Unity 400 NMR), operating at 400 and 100.2 MHz for ^{1}H and ^{13}C,

respectively. Spectrophotometry measurements were carried out using 10x10 mm light path quartz cuvettes (Hellma Analytics, GmbH & Co., Germany) for all samples, except for the 2,3,7,8-TCDD samples, using 10x4 mm light path, due to the low volume amount. After scanning the 200-900 nm range for each sample, UV-Vis measurements were performed between 200 and 500 nm against ultrahigh-pure water produced by a MilliQ water purifier system (Millipore, Bedford, MA, USA) and DMSO (0.001‰ final concentration in ASW). All NMR experiments were done in deuterated water and DMSO-d_6 with the probe at ambient temperature (23°C).

Taking into account the Lambert-Beer law, experimental conditions for UV-Vis analyses were selected to obtain a linear relationship between absorbance and concentration of each analyte as follows: ASW, 10^{-7}M DMSO (0.001‰) in ASW, 1 mg L^{-1} nano-TiO_2 adding 10^{-7}M DMSO in ASW, 10^{-4}M 2,3,7,8-TCDD adding 10^{-7}M DMSO, 1 mg L^{-1} nano-TiO_2 plus 10^{-4}M 2,3,7,8-TCDD adding DMSO. Before running experiments, nano-TiO_2 was sonicated 10 min at 100W with an UP-200H Hielscher (HielscherUltrasonics GmbH, Teltow, Germany) ultrasonic probe. Naphthalene and 1,3-benzodioxole were also investigated as model compounds of 2,3,7,8-TCDD under the same experimental conditions, except for the UV-Vis tested concentrations that were 10^{-3}M for both chemicals.

2.5 Detoxification and immune genes expression by quantitative real-time PCR

RNA was isolated from 50 mg of liver, gills and spleen tissues, using Tripure reagent following the manufacturer's protocol and including a DNAse treatment (Ambion, USA) according to the manufacturer's instructions. RNA concentrations were measured using a tray cells spectrophotometer (Eppendorf, Milano Italy) at 260 nm Abs. RNA quality was confirmed on 1% agarose gel that showed discrete 18S and 28S rRNA bands.

cDNA for q-PCR was generated with 0.5 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 liver detoxification as *Cyp1a*, immunoregulatory genes as *IL-1β*, *IL-8*, *TNF-α*, *Cox-2*, *IgM*, and *Tcrβ* and the housekeeping *r18S* were designed for q-PCR using IDTDNA (www.idtdna.com). Primer sequences used for q-PCR are listed in table S.1. Q-PCR was performed using Stratagene 3000xP thermal cycler. Each amplification reaction contained 10 µl SYBRGreen® (Biorad, USA), 0.75 µl of Forward and Reverse primers 10 µM and 1 µl cDNA in 25 µl total volume. The cycling parameters were: 3 min denaturation at 95°C, 40 cycles at 95°C for 44 s, annealing at 55 °C for 60 s, elongation at 72°C for 60 s. PCR efficiency for each primer pair was determined from a standard curve using dilutions of pooled cDNA. The modulation of mRNA transcription in exposed groups respect to controls was measured using the ΔΔCt method (Pfaffl et al. 2001).

2.6 EROD activity

The citosolic liver fractions were obtained as described previously (Della Torre et al. 2012). EROD activity was measured according to the fluorimetric method adapted for 96 well microplate (Eggens and Galgani, 1992). Total proteins were measured according to Bradford (1976) using a Shimadzu UV-160A visible recording spectrometer and BSA as standard. EROD activity was expressed as nmol min^{-1} mg $prot^{-1}$.

2.7 Genotoxicity and genomic stability

Erythrocytes obtained from peripheral blood of European sea bass were processed for the evaluation of DNA integrity by the Comet assay and apoptosis by the diffusion assay (Frenzilli et al. 2008, 2009). Due to the high levels of alkali labile sites in functionally highly condensed chromatin characteristic of erythrocyte nuclei, in order to detect only single strand breaks the mild-alkaline version of the Comet assay (at pH 12.1) was applied following the method of Frenzilli et al. (2004). Four slides per animal were set up (for diffusion and Comet assay), 50 cells per slide were scored and the mean was calculated. The amount of DNA damage was evaluated as the percentage

223 of DNA migrating out of the nucleus by the use of an image analyzer (Komet 5.0 Software, Kinetic
224 Imaging Ltd.), connected to the fluorescent microscope.
225 Slide preparation, lysing and staining for diffusion assay were carried out as described for Comet
226 assay. Visualization of not migrated DNA was performed under a fluorescent microscope (Jenaval,
227 Zeiss).
228 The RAPD (Random Amplified Polymorphism DNA)-PCR technique was used to evaluate the
229 genomic stability in muscle tissue of European sea bass according to the methods already reported
230 by Rocco et al. (2012) and Atienzar and Jha (2006). RAPD-PCR was performed in final reaction
231 volume of 25 μL containing Taq DNA recombinant polymerase (2.5 units) nucleotides (dNTPs) (0.4
232 mM), DNA (40 ng) and the primer 6 (5'-d[CCCTCAGCA]-3') (5 pmol μL^{-1}) (Zhiyi and Haowen,
233 2004; Rocco et al., 2014). The reaction products were analyzed by means of electrophoresis on 2%
234 agarose gel and examined after gel staining with ethidium bromide. The electrophoretic profiles
235 obtained by RAPD-PCR were used to evaluate the percentage of Genome Template Stability (GTS,
236 %) as following: GTS = (1-*a*/*n*)*100, where *a* is the average number of polymorphic bands detected
237 in each exposed sample and *n* the number of total bands in the un-treated samples. Polymorphism in
238 RAPD profiles included disappearance of bands and appearance of new bands with respect to the
239 control. The average was calculated for each experimental group. Changes in these values were
240 considered as a percentage of their controls (set to 100%).
241 The micronucleus (MN) test was carried out according to Frenzilli et al. (2008). A total of 4000
242 erythrocytes per specimen were scored in coded slides (at least two slides) to determine the MN
243 frequency.
244
245 **2.8 Statistical analysis**
246 Data from gene expression analysis was processed using Rest 2008 V2.0.7 software
247 (http://www.gene-quantification.de/rest.html). STATGRAPHICS Plus for Windows, version 5.1 was
248 used. Multifactor analysis of variance (MANOVA) or multiple regression analysis (MRA) was

249 performed, taking into account experimental groups. Multiple range test (MRT) was used (P <0.05)
250 to detect differences in DNA migration among experimental groups.
251
252 **3.Results and discussion**
253 **3.1 nano-TiO_2 characterization and interaction with 2,3,7,8-TCDD in ASW**
254 TEM images of Aeroxide® nano-TiO_2 P25 showed size distribution ranging approx. from 10 to 65
255 nm, 27 nm average (90% of the particles from 15 to 47 nm, Figure S1), with shape partly irregular
256 and semi-spherical (Figure S2). According to the manufacturer, the main crystallographic phases
257 obtained by XRD were anatase (86.5%) and rutile (13.5%), with 21 nm crystallite size. BET
258 analysis showed a specific surface area of 54±0.2 $m^2 g^{-1}$, with a pore size of 0.2 ml g^{-1}. Investigation
259 regarding inorganic impurities carried out by ICP-MS showed the presence of Mg (323 ± 57 μg g^{-1})
260 and Na (24 ± 6 μ g g^{-1}).
261 nano-TiO_2 characterization in ASW has been performed as described in our previous study (Canesi
262 et al., 2014). As far the concentration at 1 mg L^{-1}, agglomeration occurred immediately after sample
263 preparation with an hydrodynamic diameter in the range of 350 ± 41 nm, remaining almost stable
264 within 24 h of exposure. Comparison between absorption spectra of experimental groups did not
265 shown any significant variation in terms of spectral shifting or increase/decrease of 2,3,7,8-TCDD
266 signal intensity among the following samples: (1) control in ASW; (2) nano-TiO_2 (1 mg L^{-1}); (3)
267 nano-TiO_2 (1 mg L^{-1}) adding DMSO (0.001‰ final concentration); (4) 2,3,7,8-TCDD (10^{-4}M)
268 adding DMSO (0.001‰ final concentration) and nano-TiO_2 (1 mg L^{-1}) plus 2,3,7,8-TCDD (10^{-4}M)
269 adding DMSO (0.001‰ final concentration).
270 NMR analysis did not show any shift of ^{1}H and ^{13}C over 24 h. Therefore, under the tested
271 conditions, the results obtained with both UV-Vis and NMR spectroscopy suggested no interaction
272 between nano-TiO_2 and 2,3,7,8-TCDD in ASW. In addition, a further investigation with the same
273 techniques to study any interaction between nano-TiO_2 in ASW and, separately, Naphthalene and
274 1,3-benzodioxole, showed negative results. Recently, the adsorption on nano-TiO_2 of two organic

toxicants, i.e. Decabromodiphenyl ether (BDE-209) and Phenanthrene, was highlighted by Wang et al. (2014) and Tian et al. (2014), respectively, modifying the physical state of samples by centrifugation with high speeds (12000xg and 3000xg, respectively). In order to obtain the sorption kinetics for each of the two organic compounds with nano-TiO_2, the supernatants were analysed via GC-MS (Wang et al., 2014) and via HPLC (Tian et al., 2014).

3.2 2,3,7,8-TCDD in fish tissues

The 2,3,7,8-TCDD concentrations in the European sea bass tissues were <0.001-65.97 ng g^{-1} wet wt in the liver, 0.33-22.63 ng g^{-1} wet wt in the skin, and 0.11-9.2 ng g^{-1} wet wt in the muscle of control and co-exposed fish, respectively (Figure 1). Liver is confirmed to be a target tissue for 2,3,7,8-TCDD accumulation in fish (Greco et al., 2010).

Skin, muscle and liver samples showed a slightly higher 2,3,7,8-TCDD levels when the specimens were co-exposed with nano-TiO_2, but values were not significantly different. This result is in line with the absence of physico-chemical interaction between nano-TiO_2 and 2,3,7,8-TCDD. The presence of nano-TiO_2 might affect the contaminant mobility due to distinctive adsorption properties toward certain organic contaminants thus facilitating their transport into internal tissues, as also reported for other NPs (Pan and Xing, 2008). The role of nano-TiO_2 as carrier of organic contaminant in fact was already reported in freshwater species including fish (Zhu et al., 2011; Wang et al., 2014) and marine invertebrates (Tian et al., 2014; Canesi et al., 2014). Evidence to date indicates that TiO2-NPs are not appreciably absorbed in fish during aqueous exposure (Federici et al., 2007; Ramdsen et al., 2013) so it is unlikely that the NPs are absorbed in this study. However, it could be that TiO2 facilitate transport of 2,3,7,8-TCDD to the surfaces of external tissues and thereby increases absorption of 2,3,7,8-TCDD when brought in close proximity to epithelial surfaces. This does not appear to be the case as 2,3,7,8-TCDD concentrations in tissues did not differ in the presence of TiO2-NPs. The high levels of 2,3,7,8-TCDD measured in exposed fish either in single and in co-exposure condition suggests instead that once in the water the 2,3,7,8-

301 TCDD partitioned directly into the fish without associating with the nano-TiO_2. With particular
302 regard to the observed *Trojan horse* effects in marine mussels, 96h of exposure might be sufficient
303 to elicit a significant higher uptake of 2,3,7,8-TCDD in mussels co-exposed to nano-TiO_2 while
304 waterborne 7 days of exposure might be too short to detect any effect in fish.
305
306 **3.3 Detoxification**
307 After 7 days of exposure 2,3,7,8-TCDD caused a significant induction of liver detoxification in
308 terms of *cyp1a* gene expression and EROD activity (Figure 2). nano-TiO_2 alone did not affect
309 neither gene expression nor enzyme activity (Figure 2). Even co-exposure with nano-TiO_2 did not
310 affect the induction caused by 2,3,7,8-TCDD at both gene and enzyme levels, albeit a slight
311 decrease (but not significant) is observed when compared to European sea bass exposed to single
312 2,3,7,8-TCDD. As expected 2,3,7,8-TCDD exposure is able to induce a significant activation of
313 liver detoxification in European sea bass in agreement with our previous study (Della Torre et al.
314 2014 *in press*).
315 Once inside the body, 2,3,7,8-TCDD undergoes active detoxification via Aryl Hydrocarbon
316 Receptor (*AhR*) pathway and with the involvement of CYP1A. In fish as in mammals, the
317 detoxification involved different phases. The cytochrome P450 system –and CYP1A in particular-
318 plays the dominant role in the phase I of detoxification and the induction of CYP1A is considered a
319 specific 2,3,7,8-TCDD-induced response (van der Oost et al., 2003).
320 Concerning nano-TiO_2, our results clearly indicate that it does not affect detoxification pathways
321 induced by 2,3,7,8-TCDD in liver of European sea bass during 7 days of *in vivo* exposure. Such
322 result is also confirmed by chemical analysis showing that the co-exposure with nano-TiO_2 does not
323 affect 2,3,7,8-TCDD accumulation in liver. An interaction of nano-TiO_2 with detoxification system
324 has been reported in mammalian models, where nano-TiO_2 increased *cyp1a* gene expression in mice
325 hepatocytes exposed for 60 days (Cui et al., 2010). Different exposure route as well as animal

326 models might account for the differences observed suggesting the need of more studies for better
327 addressing such interaction considering the key role of *cyp1a* in the 2,3,7,8-TCDD detoxification.
328
329 **3.4 Immunomodulation**
330 A modulation (up-regulation) of the gene transcripts associated with innate and adaptive responses
331 has been observed in gills in the following order respect to control group: nano-TiO_2> 2,3,7,8-
332 TCDD > nano-TiO_2 (DMSO) > nano-TiO_2 plus 2,3,7,8-TCDD, the latter comparable to controls
333 (Figure 3). A different transcription profile of genes has been observed in the spleen with 2,3,7,8-
334 TCDD up-regulating all genes and in particular *IL-1β*. nano-TiO_2 alone induced only a slight
335 modulation but down regulate both innate and acquired immunity genes in the presence of 2,3,7,8-
336 TCDD (Figure 4). The results are not statistically significant due to the high inter-individual genetic
337 variability of the immune responses, but a clear trend can be seen.
338 The observed effect of nano-TiO_2 in gills is in agreement with previous investigations where it has
339 been shown to induce the respiratory burst in fathead minnow neutrophils *in vitro* (Jovanovic et al.,
340 2011) and, therefore, it should affect *IL-8* expression that primes the respiratory burst by activating
341 the phosphorylation of different molecules involved in this process. Moreover, both carbon-based
342 and metallic NPs have been shown to interfere with macrophages in rainbow trout (Klaper et al.
343 2010; Jovanovic and Palic, 2012), where they are able to induce *in vitro IL-1β* transcription.
344 Regarding 2,3,7,8-TCDD effects on spleen, induction of *Cox-2* expression has been observed in
345 exposed medaka embryos (*Oryzias latipes*) (Dong et al., 2010) and, as far as we know, our data
346 represent first observations showing up regulation *in vivo* of the immune system by 2,3,7,8-TCDD
347 in fish.
348 In general, our results show measurable effects of 2,3,7,8-TCDD, nano-TiO_2, and their combination
349 on transcription levels of selected immunoregulatory genes, and a tissue difference on measured
350 effects. In the gills, a mucosal tissue at direct contact with water and thus with potential pathogens,
351 we observed a hierarchy in the effects caused by -2,3,7,8-TCDD, nano-TiO_2 and their co-exposure.

352 Indeed a local immune response can be mounted in gill tissue after a pathogen invasion (Pennacchi
353 et al., 2013), and a contaminant-induced immune depression may render animal prone to
354 pathologies. Some immuno-modulatory effects have been observed in DMSO exposed groups
355 perhaps due to membranes chaotropic effects. Concentration used in the present study (10^{-7}M final
356 concentration) should have not caused any damage in gill structural integrity according to previous
357 studies (Kais et al., 2013). By the way, a DMSO interaction with gill membranes, able to affect
358 nano-TiO_2 uptake and the observed toxicity in gills but not in spleen, cannot be excluded. The
359 spleen, an internal non-mucosal tissue might be more subjected to specific pathways of exposure,
360 including retention of NPs able to cause an immunotoxic response. Rainbow trout fed with nano-
361 TiO_2 was shown to accumulate these NPs after eight weeks of exposure compared with other organs
362 (Ramsden et al., 2009). This observation, together with our findings showing a general down-
363 regulation of immunoregulatory genes expression in European sea bass spleen, reinforce the
364 hypothesis that spleen can be employed as a target tissue to measure nano-TiO_2 effects on immuno-
365 physiology.
366 The observed differences in immune responses between the two tissues can be explained by their
367 different physiological functions and route of exposure to chemicals as direct up-take from gills and
368 through the circulatory system in the spleen (Jovanovic and Palic, 2012).
369 The observed modulation of nano-TiO_2 on the immune responses as well as the antagonist behavior
370 with 2,3,7,8-TCDD stimulates more research on this topic considering the key role played by the
371 immune system for fish physiological homeostasis. This aspect has to be taken into account and
372 could represent a great environmental concern for fish living in sea water, as it has been
373 demonstrated that water contaminants, like nano-TiO_2, could potentially interfere with the fish
374 disease resistance and make, as an example, fathead minnows more susceptible to pathogens, as
375 *Aeromonas hydrophila* (Jovanovic and Palic, 2012).
376
377 **3.5 Genotoxicity and genomic stability**

No statistically significant induction of DNA strand breaks was observed after nano-TiO_2 and 2,3,7,8-TCDD exposure by Comet assay (Figure 5a). The only significant increase of DNA primary damage was detected in erythrocytes of fish exposed to nano-TiO_2 (DMSO) compared to controls (Figure 5a). This effect was also observed in terms of immunomodulation, likely suggesting the ability of DMSO to interfere with nano-TiO_2 toxic response in gills. nano-TiO_2 was actually found to induce oxidative DNA damage in freshwater fish cells (Reeves et al., 2008) and to be phototoxic through ROS activation in zebrafish embryos (Faria et al., 2014) and in medaka (Ma et al., 2012). No increase in DNA breaks was previously reported after *in vivo* exposure to nano-TiO_2 in other animal models (Lindberg et al., 2012; Naya et al., 2012). The only studies dealing with marine vertebrate species showed the ability of nano-TiO_2 to cause genotoxicity *in vitro* in bottle-nose dolphin leukocytes (Bernardeschi et al., 2010) and fibroblasts (Frenzilli et al., 2014). Co-exposure with 2,3,7,8-TCDD caused a reduction of DNA damage in comparison with single chemicals exposure, speaking in favor of an antagonistic effect.

RAPD PCR results showed that both nano-TiO_2 and 2,3,7,8-TCDD are genotoxic. RAPD-PCR (Figure 5b) polymorphic pattern of nano-TiO_2 exposed fish showed the appearance of four new bands: 320, 460, 500 and 650 bp and the disappearance of only one band at 480 bp respect to the control (Figure 5b). nano-TiO_2 (with the addition of DMSO) showed the appearance of two new bands at 320, and 500bp and the disappearance of only one band at 480 bp respect to the control. 2,3,7,8-TCDD exhibited only two new bands at 500 and 650 bp compared to controls. Co-exposure to nano-TiO_2 plus 2,3,7,8-TCDD showed the appearance of a band at 500 bp and the disappearance of the band at 700 bp respect to control. All exposed groups caused a decrease in genome template stability (GTS%) respect to controls. A decreasing stability was observed in the following order: 35% by nano-TiO_2 alone and co-exposed with 2,3,7,8-TCDD, 33% by nano-TiO_2 (DMSO) and 30% by 2,3,7,8-TCDD alone.

Neither diffusion assay nor cytogenetic analysis shown an increase in the frequency of apoptotic cells compared to controls (data not shown). On the contrary, MN test revealed the presence of a

chromosomal damage in European sea bass erythrocytes exposed to nano-TiO_2 alone and in combination with 2,3,7,8-TCDD (Figure 5c). These features confirm our recent findings on the Mediterranean mussel (Canesi et al., 2014) and suggest a common mechanism accounting for the similar genotoxic response to nano-TiO_2 and 2,3,7,8-TCDD in European sea bass and Mediterranean mussel *M. galloprovincialis*.

4.Conclusions

Results highlighted for the first time the influence of nano-TiO_2 on 2,3,7,8-TCDD pathway in fish showing mostly antagonistic effects in selected organs (muscle, spleen and gills). We demonstrate that nano-TiO_2 could affect immune response towards 2,3,7,8-TCDD in spleen but not interfere with detoxification and bioconcentration in liver and other organs. The observed absence of physico-chemical interaction between nano-TiO_2 and 2,3,7,8-TCDD in ASW might suggest that the antagonism might occur inside the body and/or in specific organs rather than in the sea water media. In agreement with previous findings, our results showed that nano-TiO_2 at 1 mg L^{-1} is able to elicit significant biological responses also in marine fish species affecting the expression of immunoregulatory genes in gills and producing chromosomal damages in peripheral blood erythrocytes. By measuring several endpoints of toxicity in different organs (detoxification, immune response and genotoxicity), we demonstrated that co-exposure of nano-TiO_2 with existing toxic pollutants present in the marine environment need to be fully investigated in order to prevent any unexpected behaviour and consequent toxicity to marine biota. Therefore further research is recommended in order to better understand the influence of nano-TiO_2 on the bioavailability and toxicity of other toxic pollutants with particular regard to those highly present and bioaccumulated in marine biota as dioxins and more generally POPs.

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Supporting Information

Table S1 showing Primer sequences used for q-PCR and Figures S1 and S2 on nano-TiO_2 Aeroxide® characterization.

Figure Captions

Figure 1. Bioaccumulation. 2,3,7,8-TCDD concentrations in the tissues of the European sea bass exposed to 2,3,7,8-TCDD (TCDD 46 pg L^{-1}) and to nano-TiO_2 (n-TiO_2 1 mg L^{-1}) plus 2,3,7,8-TCDD. Values <LOD (0.001) were reported as LOD/2.

Figure 2. Effects on CYP1A. Expression of *Cyp1a* gene and related EROD enzymatic activity in European sea bass exposed for 7 days to nano-TiO_2 (n-TiO_2 1 mg L^{-1}) and 2,3,7,8-TCDD (TCDD 46 pg L^{-1}) alone and in combination. Results are presented as mean ± s.d. (N = 7). Different letters indicate significant differences ($p < 0.05$) between the experimental groups.

Figure 3. Immunomodulation in gills. Expression of genes linked to innate immunity and pro-inflammatory response and adapted immunity in gills of European sea bass exposed for 7 days to nano-TiO_2 (n-TiO_2 1 mg L^{-1}) and 2,3,7,8-TCDD (TCDD 46 pg L^{-1}) alone and in combination. Results are presented as mean ± s.d. (N = 7).

Figure 4. Immunomodulation in spleen. Expression of genes linked to innate immunity and pro-inflammatory response and adapted immunity in spleen of European sea bass exposed for 7 days to nano-TiO_2 (n-TiO_2 1 mg L^{-1}) and 2,3,7,8-TCDD (TCDD 46 pg L^{-1}) alone and in combination. Results are presented as mean ± s.d. (N = 7).

Figure 5. Genotoxicity. DNA damage (% tail DNA) (a), RAPD-PCR (b) and micronuclei frequencies (c) evaluated in European sea bass exposed for 7 days to nano-TiO_2 (n-TiO_2 1 mg L^{-1}) and 2,3,7,8-TCDD (TCDD 46 pg/ L^{-1}) alone and in combination. Results are presented as mean ± s.d. (N = 10). Different letters indicate significant differences ($p< 0.05$) between the experimental groups. In RAPD-PCR analysis the controls showed bands at: 200, 240, 270, 290, 400, 480, 600, 700 and 800 bp.

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Figure
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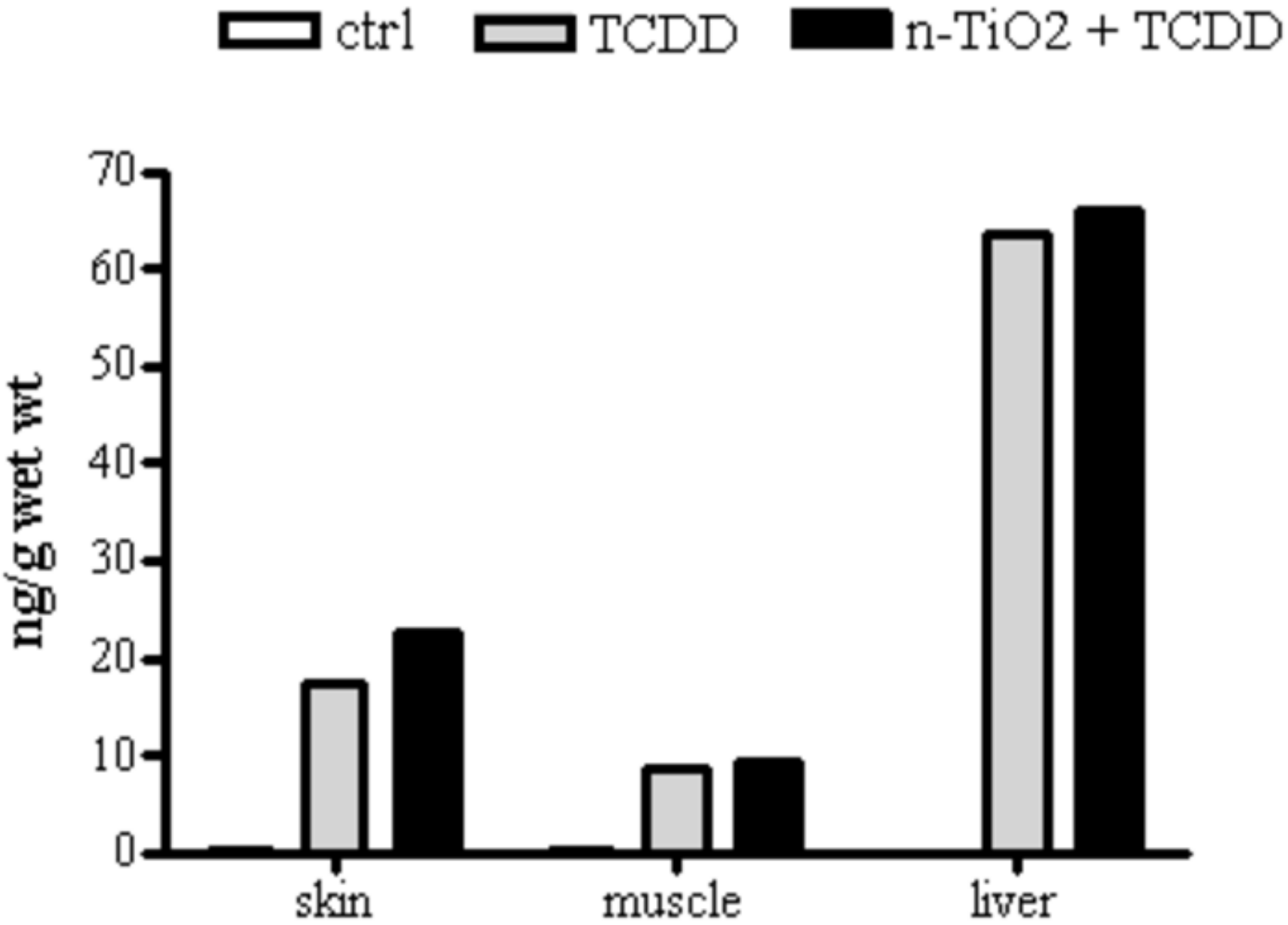

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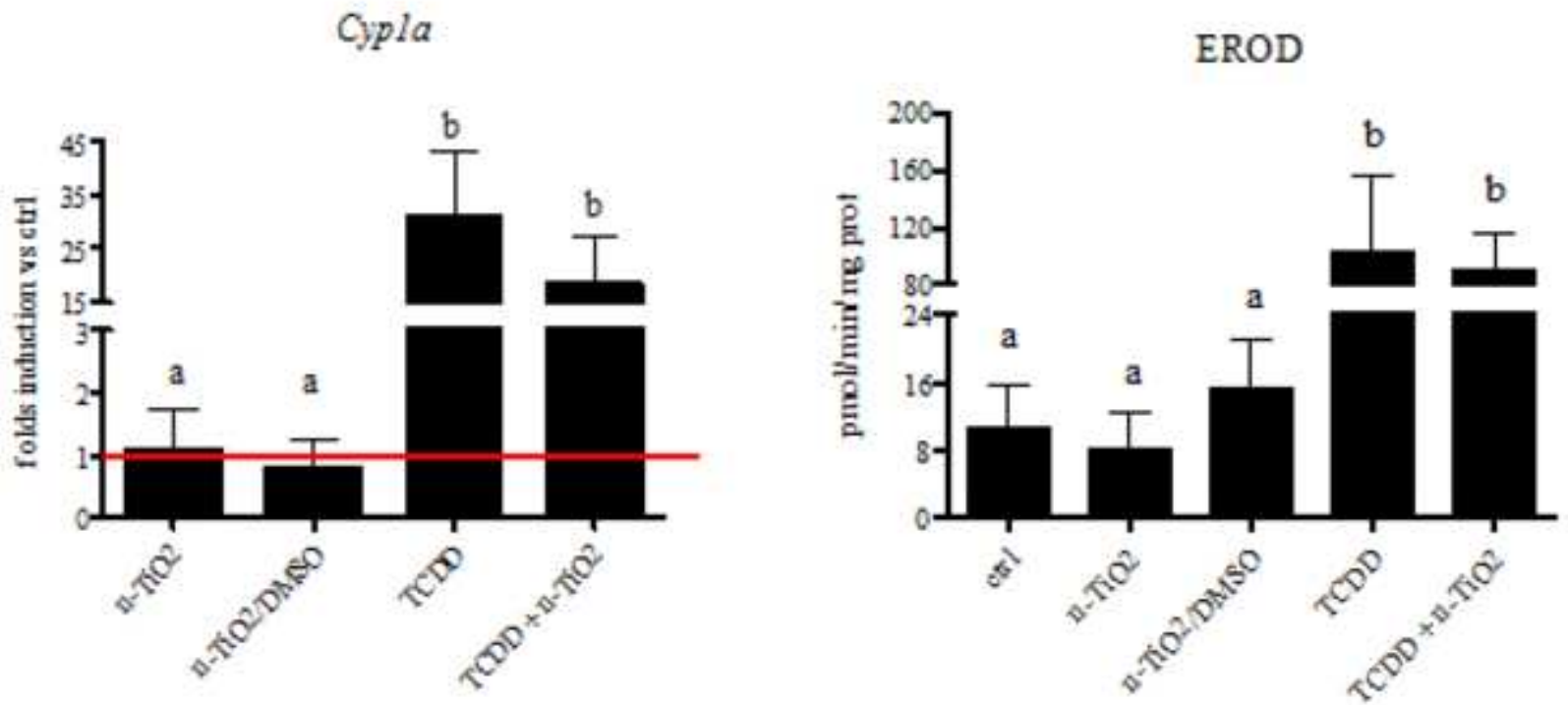

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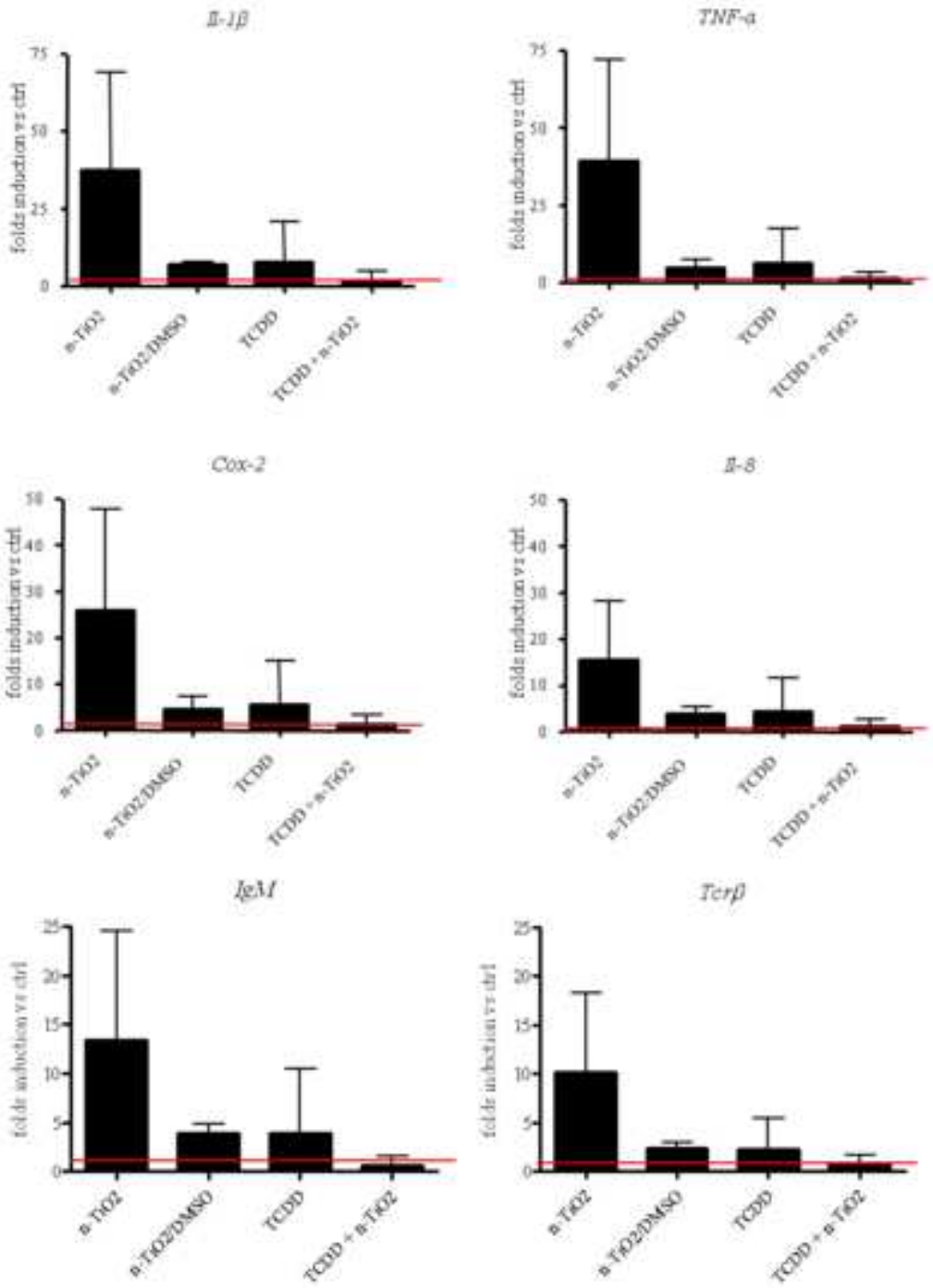

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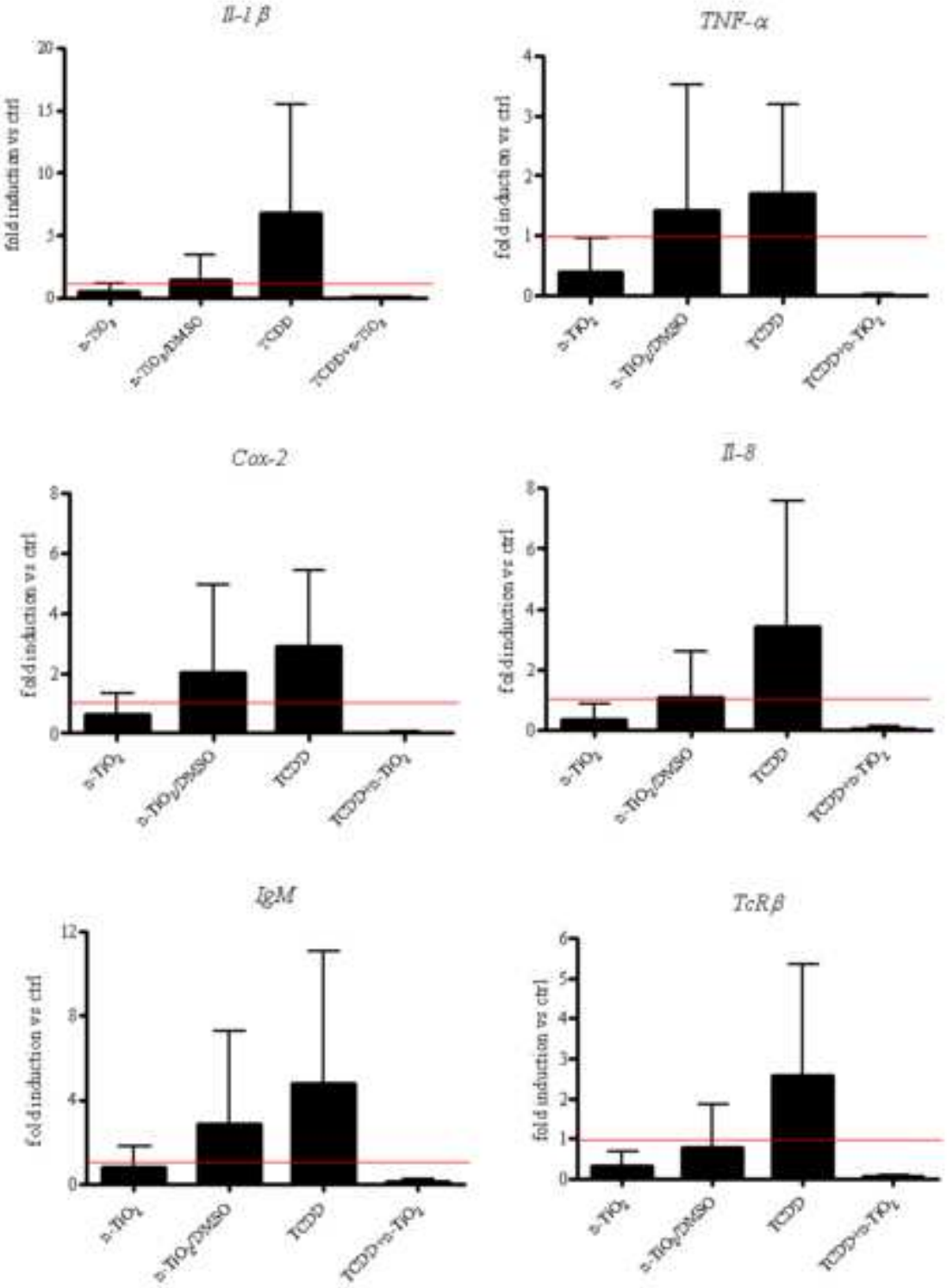

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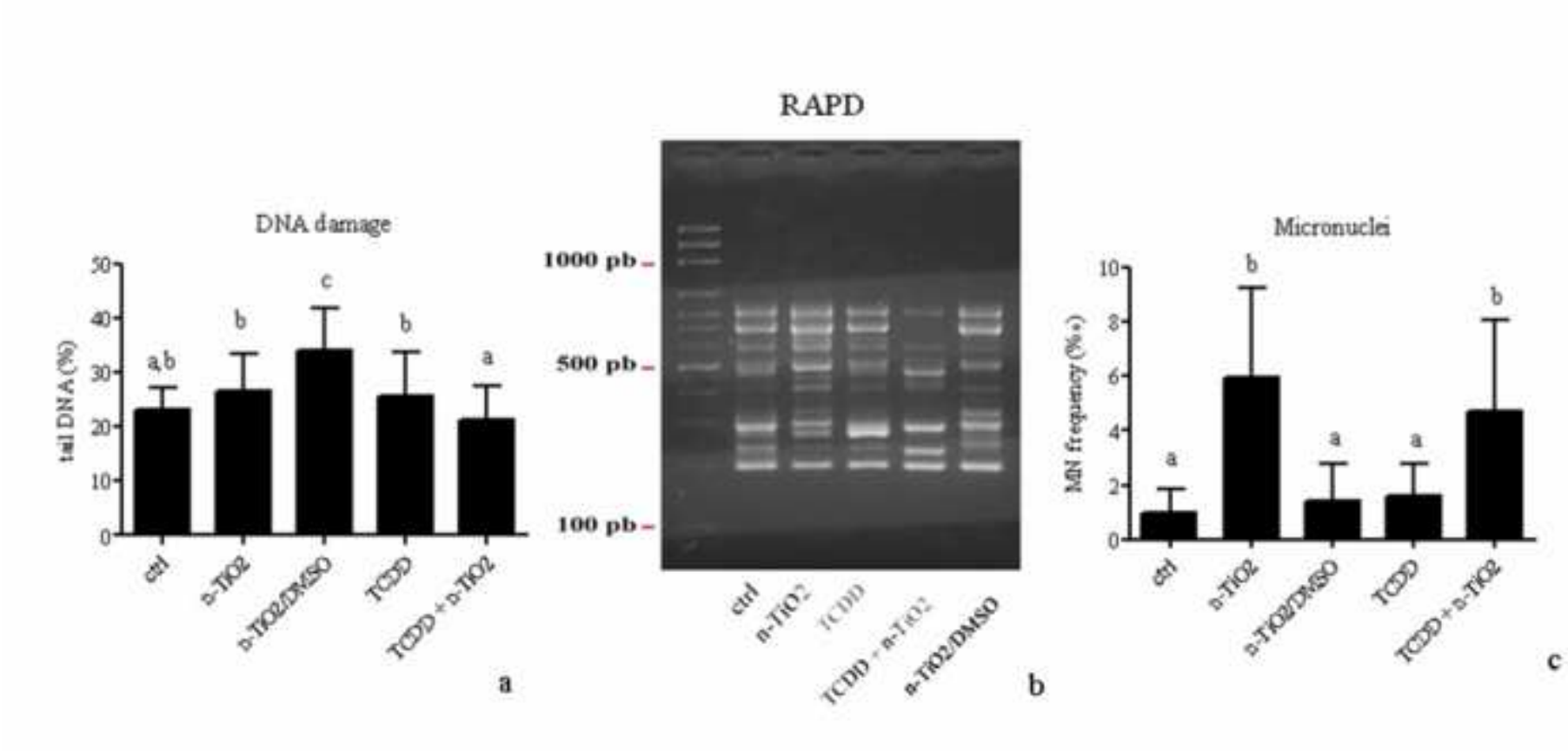

Supplementary Material

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