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Title: NANOSIZED TITANIUM DIOXIDE UV FILTER INCREASES MIXTURE TOXICITY WHEN COMBINED WITH PARABENS

Article Type: VSI: Nanotoxicity

Section/Category: Ecotoxicology

Keywords: Inorganic UV filters; Joint toxicity; Nanomaterials; Personal care products; Metal oxide nanoparticles.

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Abstract: To address the concern about the environmental impact of engineered nanoparticles frequently used in the recently marketed personal care and hygiene products (PCPs), we conducted a toxicity assessment and determined the EC50 values of the nanosized inorganic UV filter TiO2 (nano-TiO2), as well as those of the organic UV filter oxybenzone (BP3) and three parabens (methyl, propyl, and benzylparaben) present in most PCPs formulation. The bioassays were carried out through standardized toxicity bioassays on two environmentally relevant aquatic species i.e. Daphnia magna and Phaeodactylum tricornutum. For nano-TiO2 48 h EC50 on D. magna was 3.09 mgl-1 and for parabens ranged from 32.52 to 1.35 mgl-1. The two most toxic compounds on D. magna, nano-TiO2 and benzylparaben (BzP), were further tested with the algae. For nano-TiO2 72 h EC50 value was 2.27 mgl-1 and for BzP it was 10.61 mgl-1.
In addition, D. magna was exposed to selected binary mixtures of the target compounds i.e. nano-TiO2+BP3, nano-TiO2+BzP and BP3+BzP On the endpoint of 48 h, a synergistic action was observed for nano-TiO2+BP3 and nano-TiO2+BzP, but an antagonistic effect occurred in the mixture BP3+BzP. These findings suggest that nano-TiO2 can increase the toxicity of the mixture when combined with other compounds.

Barcelona, 08th August 2019

Dear Editor,

Please find enclosed the manuscript entitled: “Nanosized Titanium Dioxide UV Filter Increases Mixture Toxicity when Combined with Parabens” by Soler de la Vega, Molins-Delgado, Barceló, and Diaz-Cruz, which is submitted with corrections that reviewers have detected for publication in Ecotoxicology and Environmental Safety.

This manuscript is the original work of the authors; it has not been previously published, in whole or in part, is not under consideration by any other journal, and has not been sent to any other publication previously. All authors are aware of, and accept responsibility for the manuscript; additionally, all authors mutually accepted the submission to Ecotoxicology and Environmental Safety. We have edited the submission of the article, the figures are now grouped so as not to exceed the allowed number, we also add the supplementary material.

SUMMARY

In the last few years, concern about the environmental impact of synthetic chemicals used in large volumes for beauty and hygiene has being increasing. Besides, there is little information about the fate and effects of the engineered nanoparticles more frequently used in the recently marketed personal care products. To address this issue, we conducted a toxicity assessment to determine the EC_{50} values of the nanosized inorganic UV filter TiO_2 (nano-TiO_2), as well as those of the organic UV filter oxybenzone (BP3) and three commonly used parabens (methyl, propyl, and benzylparaben), through standardized toxicity assays on two environmentally relevant aquatic species i.e. *Daphnia magna* and

Phaeodactylum tricornutum. The most toxic compounds on *D. magna,* nano-TiO_2 and benzylparaben (BzP), were further tested with the algae.

When combining the target compounds in binary mixtures, we observed that the target compounds displayed greater or lesser toxicity than the sum of the individual toxicities of each compound. For the three mixtures tested on *D. magna*, (nano-TiO_2/BP3, nano-TiO_2/BzP and BP3/BzP), the results of the joint effects varied; synergistic action was observed on the endpoint of 48 h for the mixtures containing TiO_2, nano-TiO_2/BP3 and nano-TiO_2/BzP, but an antagonistic effect occurred in the mixture BP3/BzP. These findings suggest that nano-TiO_2 can increase the toxicity of the mixture when joint with other compounds.

Based on the scope and the outcomes achieved in this study, the authors consider that this manuscript fit in the interest of Ecotoxicology and Environmental Safety readers.

Sincerely yours,

Silvia Diaz-Cruz

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ECOTOXICOLOGY AND ENVIROMENTAL SAFETY

Dear Editor,
Please find enclosed the detailed answer on the comments raised by the reviewers on the Manuscript EES-19-727 entitled: "Nanosized Titanium Dioxide UV Filter Increases Mixture Toxicity when Combined with Parabens" by Soler de la Vega, Molins-Delgado, Barceló, and Diaz-Cruz, which is submitted for publication in Ecotoxicology and Environmental Safety.

We thank the editor for giving us the opportunity to revise our paper and to the reviewers for providing their valuable comments. We carefully considered each point of the comments raised, and have revised our manuscript accordingly. The changes are highlighted in track changes in the revised manuscript.

Reviewers' comments:

Reviewer #1:
Congratulations for work.

We thank the reviewer.

Reviewer #3:
3.1. Testing results are missing to evaluate the overall quality of the toxicity testing such as control mortality…

To most international and national norms or guidelines on the acute D. magna assay, the percentage mortality or immobilization of the organisms in the controls should be 10% at the end of the exposure time, according with our test the range of mortality is between 5-10%. Also, the case for the second validity criterion, namely an oxygen concentration in the controls of at least 2 $mg \cdot l^{-1}$ (ISO, 1996) or 3 $mg \cdot l^{-1}$ (OECD, 2004) at the end of the test, in our laboratory we perform all the experiments according the ISO 1996.
Originally the ISO standard indicated that the 24 h EC50 for potassium dichromate had to be in the range 0.6–1.7 $mg \cdot l^{-1}$ (ISO, 1996), or the maximum 24 h EC50 acceptability to 2.1 $mg \cdot l^{-1}$, before our bioassays, we carry out the experiment for potassium dichromate and the results showed that the 24 h EC50 was 1.16 mg l^{-1}.

3.2. Reference toxicant results (and control charts for how the reference toxicant test results fit into the laboratory performance…

For the reference toxicant results, we based our experiments in bioassays that we performed previously in our laboratory and that derived in published articles. One of them, reported the EC50 for single and mix toxicants: *Daniel Molins-Delgado, Pablo Gago-Ferrero, M. Silvia Díaz-Cruz, Damià Barceló. "Single and joint ecotoxicity data estimation of organic UV filters and nanomaterials toward selected aquatic organisms. Urban groundwater risk assessment",* ***Environmental Research, 145, 126-134 (2016).*** And for the toxicity of BP3, we based our current study on: *P. Gago-Ferrero, M. Badia-*

Fabregat, A. Olivares, B. Piña, P. Blánquez, T. Vicent, G. Caminal, M.S. Díaz-Cruz, D. Barceló. "Evaluation of fungal-and photo-degradation as potential treatments for the removal of sunscreens BP3 and BP1". ***Science of the Total Environment (STOTEN), 427, 355-363 (2012).***

Also we considered: **Molins-Delgado D., Díaz-Cruz M.S., Barceló D. (2014) Introduction: Personal Care Products in the Aquatic Environment. In: Díaz-Cruz M., Barceló D. (eds) Personal Care Products in the Aquatic Environment. The Handbook of Environmental Chemistry, vol 36. Springer.**

3.3. Water quality monitoring during testing (DO, pH, etc)….

For the water quality monitoring during the experiments in our laboratory, values were recorded but not shown. Here we report the range of the results, as required by the reviewer:

Parameter	Threshold criteria
pH	6.9-7.5
Dissolved oxygen (as a percent of the ASV)	≥ 60
Total Hardness (express as mg/L $CaCO_3$	140-320 mgl^{-1}
Temperature	24 – 25°C (regulated by air conditioning)

3.4. Also, no description as to replication of testing and what is meant by having error bars sometimes and not others?

The replicas are considered for calculations of the EC50 values. The replicas were carried out simultaneously and the same procedure was carried out.

The error bars in the graphics is because each symbol corresponds to a single measure (each replicate).

3.5. I also do not think one can make a determination of synergism, etc without having measured concentration results (only nominal). One has no way to judge if this is laboratory precision or a true synergistic or antagonistic response.

In our laboratory, we carry out environmental analysis studies to identify contaminants in a wide variety of samples, including groundwater, surface water, salt water, wastewater, fish, bird eggs, jellyfish, corals, mussels, clams, sediments, soils, sewage sludge, among others. Environmental relevant concentrations of the target contaminants, such as UV filters and parabens, are mostly in the ppt range (ngl^{-1}). We use the most powerful analytical methodology based on Liquid or Gas Chromatography coupled to Tandem Mass Spectrometry (HPLC(GC)-MS/MS). Moreover, the transformation products (biotic-such as human metabolism, abiotic-such as photolysis), present in the environment even at lower concentrations can be identified and characterized, structure and mass determinations by the use of High

Resolution Mass Spectrometry (HRMS), provided by an Orbitrap-MS detector. The error in mass calculation is in the fourth decimal.
For metals determination, as Ti, we use also MS for detection, but in this case with inductively coupled plasma (ICP), ICP-MS.

To perform such analyses, we use many standard solutions of the target compounds in a wide range of concentrations, both to calibrate the systems and to determine the concentrations. We used in the current toxicity tests these standard solutions whose concentrations are calculated from the HPLC-MS/MS analysis (are not nominal concentrations).

As an example, you can know more in deep how we determine parabens in our paper:
D. Molins-Delgado, M.S. Díaz-Cruz, D. Barceló. "Ecological risk assessment associated to the removal of endocrine-disrupting parabens and benzophenone-4 in wastewater treatment". Journal of Hazardous Materials (HAZMAT) 310, 143-151 (2016). DOI:10.1016/j.jhazmat.2016.02.030.*

Finally, to answer why we are deducing the synergism o antagonism, according to many authors, they described synergism or antagonism when the toxicity of the mixture/combination is greater or lower than can be accounted by the cumulative toxicity of the individual toxicants (additive model). When the combination effect is consistent with the sum of the individual drug toxicities, the interaction is additive. This was the assumption to perform our calculations. This was performed according to the combination index (CI), the isobole method, with the two concentrations we calculate by the formula:

$$\frac{d_1}{D_1}+\frac{d_2}{D_2}=1$$

If for a certain effect level, i.e., 50%, there is no interaction between the two drugs in combination, a straight line connects the intercepts (doses) in the x- and y-axes (isobologram equation = 1). However, when the line connecting both doses lies below and to the left of the line of additivity (concave up line), synergism is found (isobologram equation <1). When the line connecting both doses lies above and to the right of the line of additivity (concave-down), antagonism is found (isobologram equation >1), within this calculation we corroborate the synergism or antagonism when we perform the isobolograms in Excel.

Reviewer #4,

1. Recommendation:
Major Revision

2. Comments to Author:
Manuscript. Number: EES-19-727
Title: NANOSIZED TITANIUM DIOXIDE UV FILTER INCREASES MIXTURETOXICITY WHEN COMBINED WITH PARABENS. Ana C. Soler de la Vega, Daniel Molins-Delgado, Damià Barceló and M. Silvia Díaz-Cruz*

Overview and general recommendation:
The number of nano-based technology Personal Care Products (PCPs) increased a lot these years. Among PCPs, UV filters (UV-Fs) and parabens (PBs) constitute a particular matter of interest because of their ecotoxic effects. The present study determined the EC50 values of the nanosized inorganic UV filter nano-TiO2, as well as those of the organic UV filter BP3 and the preservatives methyl, propyl, and benzyl paraben present in most PCPs formulations. The author also provided a series of toxicity assays on two environmentally relevant organisms Daphnia magna, and Phaeodactylum tricornutum.
According to my opinion, the current manuscript is on a topic of relevance and general interest to the readers of the journal, but major revision should be done. And I explain my concerns in more details below. I ask that the authors specifically address each of my comments one by one in their response.

3. Major comments:
3.1 In figure S2, the author used DLS to determine the size distribution of NPs. But I could not find the data mentioned in P16 line 306 to 311, such as the first one with a size of 886.7 nm and 68.2% of intensity and the second one of 290 nm and 31.8%. Since there was only one curve, what was the meaning of "the first one" and "the second one"? If the author decide to show many DLS results, please show them one by one as Yang et al did (Yang X et al, Water Res. 2013, 47(12):3947-3958).

In Figure S2, there was an error on the information not in the figure, the error was corrected and now the information is just one result for DLS. Thus, the current text is:

`*DLS showed a single population, primary the nano- TiO_2 particles were detected next to agglomerates or aggregates with a mean hydrodynamic diameter of 374.6 nm, with this analysis conclude that the majority of the nano-TiO_2 particles were clustered and aggregation, the surface area of the sample was 150.2 m^2g^{-1}, and the zeta potential was 7.30 mV, as shown in Figure S2*´.

3.2 In P18, Line353-356, did the author mean that PrP had higher toxicity than MeP because of the higher mortality rate of PrP in 48h exposure than that of MeP? I did not agree with this opinion. What was more important according to toxicity was EC50, not the mortality rate, especially when no statistical analysis was done.

In P18, Line 353-356, in this study we compare the toxicity with the EC50. We corrected the paragraph and the final text is:

`*Concerning preservatives, MeP showed low toxicity, with EC50 of 32.52 mgl^{-1}. MeP was the less toxic compound tested (see Figure S3), whereas PrP showed higher toxicity with an EC50 of 3.872 mgl^{-1} (Figure S4). According to Steinberg et al., 2010, the most widely used preservative system consists of a combination of parabens (Steinberg et al., 2010)*´.

3.3 The manuscript was written not very well. Especially the "results and discussion" and "figures and figure legends" parts, many description were confusing. Since Figure 2 and S3~5 were the same type, their figure legends should be consistent, for example, in figure S6, the Y-axis might also be "%Immobilization", which was the same with other legends. What was the meaning of "(b)"in figure S2? And where was the Figure 2A mentioned in P17 line 343? I suggest the authors be more serious in this manuscript.

We corrected all the errors identified. In Figures 2 and S3-5 the Y-axis corresponds to "% immobilization" , because the bioassay is performance with *Daphnia magna*, and in Figures 3 and S6 the legend in the Y-axis is I⁄% because we represent the inhibition rate % of the algae with this symbol. In Figure 2 we corrected the "b" that left over, and in consequence corrected also the sentence mentioning Figure 2A.

4.1 In Table 2, the author should mention what was the meaning of "n.a.".

In Table 2 we added the meaning of n.a. *(not available)*.

4.2 There are many spelling mistakes and wrong descriptions. Such as P7 line 101, "the responsible for", "the" should be deleted; P23 line 485, "diferent" should be "different"; P24 line 516, "exert. for", should be "exert, for". Maybe there are still other spelling mistakes to be corrected. The authors need to carefully check again after completing the revised paper.

We have corrected and revised carefully the entire manuscript thoroughly to correct the spelling mistakes and wrong descriptions.

Barcelona, 08th August 2019

Dear Reviewers,

Please find enclosed in the Correction Reviewer EES_FINAL.docx the detailed answer on the comments raised by the reviewers on the Manuscript EES-19-727 entitled: “Nanosized Titanium Dioxide UV Filter Increases Mixture Toxicity when Combined with Parabens” by Soler de la Vega, Molins-Delgado, Barceló, and Diaz-Cruz, which is submitted for publication in Ecotoxicology and Environmental Safety.

We thank the editor for giving us the opportunity to revise our paper and to the reviewers for providing their valuable comments. We carefully considered each point of the comments raised, and have revised our manuscript accordingly. The changes are highlighted in track changes in the revised manuscript.

Sincerely yours,
Silvia Díaz-Cruz

Highlights

- Estimated EC_{50} for titanium dioxide nanoparticles (nano-TiO_2) and methyl, propyl, and benzylparaben (BzP) toward aquatic organisms were in the mgl^{-1} range**.**

- Nano-TiO_2 and BzP showed the highest toxicity.

- Nano-TiO_2 added to oxybenzone (BP3) and to BzP, provoked synergistic toxic effects.

- An antagonistic action was observed for the mixture BP3+BzP.

NANOSIZED TITANIUM DIOXIDE UV FILTER INCREASES MIXTURE TOXICITY WHEN COMBINED WITH PARABENS

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Abstract

To address the concern about the environmental impact of engineered nanoparticles frequently used in the recently marketed personal care and hygiene products (PCPs), we conducted a toxicity assessment and determined the EC_{50} values of the nanosized inorganic UV filter TiO_2 (nano-TiO_2), as well as those of the organic UV filter oxybenzone (BP3) and three parabens (methyl, propyl, and benzylparaben) present in most PCPs formulation. The bioassays were carried out through standardized toxicity bioassays on two environmentally relevant aquatic species i.e. *Daphnia magna* and *Phaeodactylum tricornutum*. For nano-TiO_2 48 h EC_{50} on *D. magna* was 3.09 mgL^{-1} and for parabens ranged from 32.52 to 1.35 mgL^{-1}. The two most toxic compounds on *D. magna*, nano-TiO_2 and benzylparaben (BzP), were further tested with the algae. For nano-TiO_2 72 h EC_{50} value was 2.27 mgL^{-1} and for BzP it was 10.61 mgL^{-1}.

In addition, *D. magna* was exposed to selected binary mixtures of the target compounds i.e. nano-TiO_2+BP3, nano-TiO_2+BzP and BP3+BzP On the endpoint of 48 h, a synergistic action was observed for nano-TiO_2+BP3 and nano-TiO_2+BzP, but an antagonistic effect occurred in the mixture BP3+BzP. These findings suggest that nano-TiO_2 can increase the toxicity of the mixture when combined with other compounds.

Keywords: UV filters; Joint toxicity; Nanomaterials; Personal care products; Metal oxide nanoparticles.

Highlights

- Estimated EC_{50} for titanium dioxide nanoparticles (nano-TiO_2) and methyl, propyl, and benzylparaben (BzP) toward aquatic organisms were in the mgL^{-1} range**.**

- Nano-TiO_2 and BzP showed the highest toxicity.

- Nano-TiO_2 added to oxybenzone (BP3) and to BzP, provoked synergistic toxic effects.

- An antagonistic action was observed for the mixture BP3+BzP.

Funding sources

This work was funded by the Spanish Ministry of Economy and Competitiveness through the projects SOLAR-2015801004 and ROUSSEAU-CTM2017-89767-C3-1-R and Generalitat de Catalunya (Water and Soil Quality Unit 2017-SGR-1404). A. Soler –Supported by the Mexican CONACyT doctoral grant (409154).

1. Introduction

Personal Care Products (PCPs) are among the leading examples of emerging contaminants of current concern (Ebele et al., 2017). Substances present in these formulations are released routinely in high amounts into the environment and, as they show low degradability, are considered pseudo-persistent contaminants (Molins-Delgado et al., 2015). Most of them are bioactive and lipophilic tending to accumulate in sludge and sediments (Gago-Ferrero et al., 2011b), and bioaccumulate in aquatic organisms (Fent et al., 2010, Gago-Ferrero et al., 2013, Molins-Delgado et al., 2017, Anekwe et al., 2017, Molins-Delgado et al., 2018).

PCPs find their way to natural waters mainly through treated and untreated sewage discharges, in particular in densely populated areas, and by direct introduction, for instance by beachgoers (Gago-Ferrero et al., 2012).

Among PCPs, UV filters (UV-Fs) and parabens (PBs) constitute a particular matter of interest as a result of their ecotoxic effects. UV-Fs are the main ingredients of sunscreens and generally they are used in combination in order to provide enough sun protection by absorbing, reflecting or scattering the UVA and UVB radiations (Díaz-Cruz et al., 2009). The reflection and scattering effects are done by physical UV-Fs, which are inorganic chemicals such as titanium dioxide (TiO_2). Light absorption is accomplished by organic UV-Fs such as oxybenzone (benzophenone 3, BP3) (Gaparro et al., 1998).

The bioaccumulation of organic UV-Fs in living organisms is of major concern because many of them and their metabolites can act as endocrine disruptors (Vione et al., 2015). Early studies pointed out that exposure to BP3 caused increased vitellogenin synthesis in *Oryzias latipes*, *Paralichthys californicus* and

84 *Pimephales promelas* (Sapozhnikova et al.; 2005, Schlenk et al., 2005; Kunz et
85 al., 2006). More recently, BP3 has been found to cause coral-cell bleaching,
86 DNA damage, deformity and mortality at environmental significant
87 concentrations (Danovaro et al., 2008; Downs et al., 2016).
88
89 Nowadays, nanotechnology powerfully entered in the production of PCPs
90 (Adawi et al., 2018). According to the definition laid by the European
91 Commissions (EC 2011), the term nanomaterial is designed for a material with
92 50% or more (number based) of the particles in the nano-range, i.e. below 100
93 nm. In daily-use products, nanoparticles (NPs) application has experienced a
94 rapid growth. The number of nano-based technology PCPs increased by almost
95 170% in 5 years, i.e. from 407 in 2012 to 1089 in 2017 (Adawi et al. 2018).
96 NPs of TiO_2 (nano-TiO_2) are the second nanoparticle with wider use worldwide
97 (Lux-Research, 2018) just after nanosized Ag (Bottero and Wiesner, 2010).
98 Currently, nano-TiO_2 is increasingly added as UV-filter in sunscreens and many
99 other PCPs (Piccino et al., 2012, Skin Deep Cosmetics Database, 2018) because
100 its effective UVB radiation attenuation, which is responsible for skin burning.
101 Along with its increasing use, its release into the environment increases and may
102 pose a threat especially to aquatic ecosystems.
103 According to the Organization for Economic Cooperation and Development
104 (OECD) working party on nanomaterials, nano-TiO_2 was included among the 13
105 priority nanomaterials for research and environmental risk assessment (OECD
106 2010).
107 It is well-known that nano-TiO_2 undergoes photocatalytic reactions when
108 exposed to UV radiation, with the consequent generation of reactive oxygen
109 species (ROS) such as $\cdot O_2$, $\cdot OH$ and H_2O_2 (Lewicka et al., 2013); this

photoactivity can lead to environmental toxicity (Moos et al., 2014). Despite nano-TiO_2 in sunscreen formulations are generally coated with organic or inorganic matrices to mitigate these photocatalytic effects, they cannot be completely prevented (Smijs and Pavel, 2011, Morsella et al., 2016).

Several studies reported the bioaccumulation and toxic effects of nano-TiO_2 on algae, marine invertebrates and fish (Balise et al., 2008, Zhu et al., 2010, Johnson et al., 2011, Menard et al., 2011, Wang et al., 2011, Clément et al., 2013, Zhao et al., 2013, Clemente et al., 2014, Minetto et al., 2014). Prolonged exposure of fish to nano-TiO_2 induces biochemical and histopathological alterations in their gills, liver and intestines (Federici et al., 2007, Hao et al., 2009, Johnston et al., 2010, Palaniappan and Pramod, 2010). Exposure to nano-TiO_2 trigger oxidative stress in *D. magna*, fish and mollusks (Federici et al., 2007, Hao et al., 2009, Canesi et al., 2010a, Kim et al., 2010, Xiong et al., 2011) and cause lysosomal instability in *Polychaeta* and mollusks (Canesi et al., 2010a, Galloway et al., 2010). A few studies have shown that the presence of nano-TiO_2 may favor the absorption of other contaminants in fish, such as As and Cd (Sun et al., 2007, Zhang et al., 2007).

Parabens (PBs) are one such group of chemicals which are extensively used as preservatives in a variety of consumer products because of their low production cost and efficiency against a wide spectrum of microorganisms (Soni et al., 2005). As a consequence, they are combined with UV-Fs in sunscreens and in other cosmetic and hygiene products. However, some studies demonstrated their endocrine disrupting activity on fish at environmentally relevant concentrations, for instance to brown trout (Bjerregaard et al. 2008) and medaka (Gonzalez-

Doncel, et al., 2014), being those with longer alkyl chain the more active. In rats, butyl, isobutyl, and benzylparaben demonstrated estrogenic activity (Golden et al. 2005) and both butyl and propylparaben significantly inhibited spermatogenesis (Oishi, 2002). The combination of PBs with other stressors can even boost the hazard, as documented by Handa et al. which reported that PBs potentiated the UVB radiation induced damage in skin keratinocyte by oxidative stress (Handa et al., 2006).

The study of the toxic effects of single chemicals is of importance, as it provides relevant ecotoxicological knowledge but it is, however, insuficient, as in the environment combinations of chemicals inevitably co-occur. PCPs are not an exception, as most studies focus only on the effects of individual chemicals on selected aquatic organisms, and solely a few studies explored the combined effects of mixtures of PCPs (Backhaus et al., 2011, Molins-Delgado et al., 2016). For that reason, their joint effects, potentially producing additive, synergistic or antagonistic toxicity effects remain mostly unknown (Duan et al., 2008). In particular, there is still a lack of data regarding the toxicity of mixtures involving nano-TiO_2 despite its nano-scale size being known to display a "Trojan horse effect" acting as the vehicle for other contaminants transport (Fan et al., 2011; Hartmann et al., 2012; Fang et al., 2015, 2016) and displaying oxidative stress through ROS generation (Lewicka et al., 2013). In particular, oxidative stress on seawater fish and mussels caused by nano-TiO_2 was found to be influenced by salinity and water pH (Huang et al., 2018a, 2018b).

In the present study we aimed at determining the EC_{50} values of the nanosized inorganic UV filter nano-TiO_2, as well as those of the organic UV filter BP3 and

the preservatives methyl, propyl, and benzylparaben present in most PCPs formulations. To this end, a series of toxicity assays on two environmentally relevant organisms i.e. the micro-crustacean *Daphnia magna*, and the alga *Phaeodactylum tricornutum*, were carried out. The generated data allowed evaluating the joint effects on *D. magna* of selected binary mixtures of the target PCPs.

2. Materials and methods

2.1. UV-Fs, PBs and test organisms

Nano-TiO_2, BP3, methylparaben (MeP), propylparaben (PrP) and benzylparaben (BzP) were obtained from Sigma-Aldrich (Munich, Germany) with >98% purity. See Figure S1 in Supplementary material for the chemical structure of the selected compounds.

The toxicity kits for *Daphnia magna* (Daphtoxkit F) and *Phaeodactylum tricornutum* (Marine Algal Toxkit de Microbio Test) were purchased from Microbiotests (Gent, Belgium). A stereomicroscope SZT from VWR (Llinars del Vallés, Spain) and a Jenway 6300 spectrophotometer from Bibby Scientific (Paris, France) were used in the bioassays.

2.2. Physicochemical characterization of nano-TiO_2

In order to determine the size distribution of the commercial standard TiO_2 nanoparticles and to ensure that their size range was in line with that of the particles used in PCP formulations, the physicochemical characterization of the nano-TiO_2 standard was performed by Transmission Electron Microscopy (TEM)

(Hussain and Wahab, 2014). Besides, as the bioassays to be performed need to have a solution/dispersion of the nano-TiO_2 in the test media, the size distribution of the potential aggregates formed was carried out by Dynamic Light Scattering (DLS) (Yadav et al., 2015).

2.2.1. TEM of nano-TiO_2 particles

A nano-TiO_2 sample was prepared with a few drops of ethanol allowed to evaporate in a copper grid at room temperature. The measurements were recorded with a JEOL transmission electron microscope JEM-2100 Plus (JEOL Ltd, Japan). The total body length particle was obtained by using a measuring tool from the IMAGEJ software, used where a line was drawn in the longest part of each particle.

2.2.2. DLS of nano-TiO_2 suspensions

Three different studies at 25°C were completed with nano-TiO_2, suspended in i) fresh water, ii) Daphnis media, and iii) HPLC-grade water, used as the control. For the size measurement trial, 50 μgL^{-1} nano-TiO_2 solutions in all three solvents were made and sonicated before analysis. Measurements were performed in triplicate in a Zetasizer Nano-ZS (Malvern Panalytical, Malvern, UK). The Zetasizer 7.11 software was used to estimate particle mean diameter from the intensity distributions (zeta-average) obtained, which corresponds to the polydispersity index (PDI).

2.3. Bioassays.

2.3.1. Nano-TiO_2 and UV-Fs solutions

The individual standard solutions of the selected chemicals were prepared using a culture medium of each organism considering their solubility in water at 25 ºC. MeP, PrP, BzP, BP3 and nano-TiO_2 test concentrations ranged from their solubility to typical values reported in the literature cited, i.e. 40 mgL^{-1} or 0.05 mgL^{-1}.

2.3.2 Nano-TiO_2 and UV-Fs binary mixtures

Binary mixtures of nano-TiO_2, with BP3 or BzP, were prepared at different concentrations based on the EC_{50} values of nano-TiO_2 and BzP, previously estimated in this work. The concentration values for BP3 were the EC_{50} estimated in our previous work (Molins-Delgado et al., 2016).

2.3.3. Daphnia magna assays

D. magna acute toxicity tests were performed according to the ISO 6341 guideline, in duplicate. First, an artificial fresh water medium was prepared as described by Molins-Delgado et al. by adding 10 mLmL of four different saline solutions provided by the manufacturer containing the needed salts and HPLC-grade water up to a volume of 2 L (Molins-Delgado et al., 2016). A series of vessels containing 10 mLmL of oxygenated medium and the selected chemical were prepared, along with a series of blank control solutions containing only culture medium. Specimens of *D. magna* were then passed from the Petri dish to

the vessels ensuring that in each vessel there were 20 individuals. The prepared vessels containing the neonates were further introduced in an incubator in darkness. At 24, 48 and 72 h, the number of immobilized neonates in each vessel were counted using a stereomicroscope. The number of immobilized neonates was correlated to the compound's concentration, allowing us to determine the corresponding EC_{50} values.

2.3.4. Phaeodactylum tricornutum assays.

The microalgae acute toxicity test was conducted following the ISO/CD 10253 guideline, in duplicate. To prepare the culture medium, the vial kit containing NaCl was poured into a 2 L flask containing 1500 mL of HPLC-grade water, and further stirred until complete salt dissolution. This procedure was repeated with solutions of KCl, $CaCl_2$, $MgCl_2$, $MgSO_4$, $NaHCO_3$, and H_3BO_3, following this sequence of addition. Then, nutrients labelled solution A, 1 mL of solution B and 2 mL of solution C were added in the flask. HPLC-grade water was added up to 2 l and shacked vigorously. To cultivate the algae, one of the two tubes containing the microalgae inoculum was stirred before pouring the contents into one of the pre-cell culture kits. The tube containing the algae was rinsed twice with 7.5 mL culture medium and the cell content pre-culture was transferred to ensure complete transfer of the microalgae. Then, the pre-culture cell was sealed and incubated for 4 days at a controlled temperature of 20 C (+/- 2 °C) with 600 lux illumination in cycles of 8 h of darkness.

2.4. Acute toxicity evaluation

2.4.1 Daphnia magna

Equation (1) was applied to estimate the incidence rates (I) of the target PCPs, where D_0 is the number of initial neonates and D_i the number of the immobilized ones (Molins-Delgado et al., 2016):

$$I = \frac{D_i}{D_0} \times 100 \quad (1)$$

In order to correlate the incidence rates estimated with the concentrations of the target chemicals, Eq. (2) was applied (Molins-Delgado et al., 2016):

$$\mathrm{I} = B + \frac{(T-B)}{(1+10^{\left((LogEC_{50}-X)*H\right)})} \quad (2)$$

where T is the top value of the curve, B is the bottom parameter of the curve, $LogEC_{50}$ is the logarithm of the median effect concentration, X is the logarithm of the concentration of the compound, and H is the Hill coefficient of the curve. The EC_{50} values for the algae were calculated by using this equation.

2.4.2. Phaeodactylum tricornutum.

Cell density (cellmLL^{-1}) was determined by optical density measurements (OD, λ = 670 nm) using a regression line generated by taking measurements every 24, 48 and 72 h, calculating the first average specific growth rate, μ, for each test culture using the equations (3) and (4) (Molins-Delgado et al., 2016):

$$\mu = \frac{\ln N_L - \ln N_0}{t_L - t_0} \quad (3)$$

where N_0 is the initial cell density, t_L is the time of t_e last measure within the exponential growth period, and t_0 is the initial time.

The calculated values were used in the Eq. (4)

$$(4) \quad I\mu i = \frac{\mu c - \mu i}{\mu_c} \times 100$$

where μ_c is the specific growth rate of the control, to obtain the incidence rate. Then, the incidence rates were correlated with the concentrations through Eq. (2).

2.5 Statistical methods

The EC_{50} values for single compounds were determined through linear regression with the Graph Pad Prism4 software (Graph Pad Software, Inc., San Diego, CA, USA).

The mixed doses producing the specified effect were determined from the dose-effect graphs. The mixed doses determined were plotted as the axial points in a Cartesian coordinate plot, termed as isobolograms, with Microsoft® Office Excel and Sigma Plot (Systat Software Inc.). To compare statistical differences between theoretical and experimental average values in the isobolograms, Student's t-test was applied.

3. Results and discussion

3.1. Particle size distribution and aggregation of nano-TiO_2

296 TEM microscopic images revealed that the nano-TiO_2 standard was constituted
297 by spherical primary particles from 8 nm to 50 nm diameter, with an average of
298 19.5 nm. The average experimental value obtained for primary nanoparticle size
299 served not only to confirm the manufacturer's size specification (21 nm), but also
300 to know an overall assessment of its polydispersity, as shown in Figure 1.

301 DLS showed a single population, primary the nano- TiO_2 particles were detected
302 next to agglomerates or aggregates with a mean hydrodynamic diameter of 374.6
303 nm. This indicates that the majority of the nano-TiO_2 particles were clustered and
304 aggregated; the surface area of the sample was 150.2 m^2g^{-1}, and the zeta potential
305 was 7.30 mV, as shown in Figure S2.

306 These results agree with previous observations showing that nano-TiO_2
307 aggregates size increase with pH values close to a zeta potential value near to the
308 point-of-zero-charge, which is around 7, that is the pH of the HPLC-grade water
309 (Guzman et al., 2006, Dunphy-Guzmán et al. 2006, Zhu et al., 2014). MeOH,
310 less polar than water, dispersed quite well the hydrophobic oxide. The presence
311 of ions (Ca^{2+}, Mg^{2+},…) in the *Daphnia*'s media, which can be adsorbed onto the
312 particles surface (modifying its properties and charge) increased the stability of
313 the NPs as a consequence of the repulsion among the particles with the same
314 surface charge, leading to the formation of smaller aggregates.
315
316 The nanoparticle aggregation is an important factor to consider when dealing
317 with NPs. In the natural environment, free nanoparticles tend to aggregate
318 (Adams et al., 2006, Sharma 2009, Romanello and Fidalgo, 2013) becoming less
319 mobile. Understanding this agglomeration phenomenon is fundamental to
320 understand the transport and fate of nano-TiO_2 in the environment. Thus,

aggregate size will determine its transport, by settling out of solution the biggest ones (Sharma 2009). This has further implications in toxicity. For instance, when algae are exposed to nano-TiO_2 the aggregates formed during incubation adsorbed on the algal cells acting as a light screen, impeding their proliferation (Aruoja et al., 2009). Indeed, cytotoxicity of nano-TiO_2 is difficult to assess because the formation of medium-size (microscale) aggregates and precipitates prevent the determination of its dosage for exposure experiments as well as moves from nano- to micro- scale. Thus, cells have to interact with micro sized particles or precipitates of TiO_2, which are larger than cellular components and cannot be so easily absorbed by cellular membranes (Jin et al. 2008).

3.2. Single acute toxicity bioassays.

3.2.1. Daphnia magna.

In *D. magna´*s immobilization test, EC_{50} values after 48 h exposure for the target PBs and nano-TiO_2 ranged from 0.05 to 40 mgL^{-1}, as listed in Table 1. The amount of nano-TiO_2 accumulated in *D. magna* increased with the extended exposure time (see Figure 2). After 48 h, the number of live *D. magna* significantly decreased, in 75% approximately. At 72 h, the 100% of the organisms were immobile. Nano-TiO_2 displayed toxic effects at 3.09 mgL^{-1}, despite significant immobilization was found from 0.5 mgL^{-1} onward. Moreover, we observed that the toxicity drastically increased, about 400-folds, when the exposure time was extended from 24 to 72 h, and an EC_{50} of 0.050 mgL^{-1} was estimated (see Table 1). Zhu et al., observed a similar trend, suggesting that exposure time constitutes a determinant parameter in nano-TiO_2 toxicity (Zhu et al., 2010). L-1L-1L-1

Concerning preservatives, MeP showed low toxicity, with EC_{50} of 32.52 mgl^{-1}. MeP was the less toxic compound tested (see Figure S3), whereas PrP showed higher toxicity with an EC_{50} of 3.872 mgl^{-1} (Figure S4). According to Steinberg et al., 2010, the most widely used preservative system consists of a combination of parabens (Steinberg et al., 2010).″

BzP, in contrast to MeP and PrP, is not frequently used; since previous studies demonstrated that the increase in the alkyl chain length of PBs increases toxicity (Bjerregaard et al. 2008, Nohynek et al. 2010, Gonzalez-Doncel, et al., 2014). As expected, BzP was the most toxic among the PBs tested on *D. magna*, showing an EC_{50} value of 1.354 mgL^{-1}. The dose-response curves are shown in Figure S5. According to Dobbins et al., BzP is one of the most acutely toxic parabens to *D. magna* and *P. promelas* (Dobbins et al., 2009). L-1L-1L-1In our test, the mortality at 24 h was about 25%, but expanding the exposure time to 48 h the mortality increased up to 75% (see Table 1). At 24 h the EC_{50} was 33.53 mgL^{-1}, but at 48 h the value severely decreased up to 1.350 mgL^{-1}.

Despite their potential hazard to the natural ecosystems there are currently no regulation concerning the occurrence of paraben preservatives in the environment or in wastewater. However, they are regulated as food additives by the U.S. Food and Drug Administration (FDA 2018) and as industrial additives by USEPA (USEPA, 2005, 2006), and MeP and PrP are recognized as safe for humans. In the European Union, parabens as food additives are regulated by Regulation (EC) No 1333/2008 (EC, 2008), and in consumers products by

370 Directive 76/768/EEC (EP, 1976). Only MeP is controlled by REACH regulation
371 (EC, 2006).

372

373 **3.2.2. *Phaeodactylum tricornutum*.**

374 Nano-TiO_2 and BzP were the selected compounds to be tested on the algae,
375 because of their higher toxicity observed towards *D. magna*. Four replicates were
376 performed in this case. Table 2 lists the EC_{50} values estimated, which ranged
377 from 0.005 to 30 mgL^{-1}. Nano-TiO_2 showed greater toxicity than BzP.
378 Considering the lowest value of the four replicates (worst case scenario), for
379 nano-TiO_2 an EC_{50} of 2.270 mgL^{-1} was obtained, and for BzP it was 10.61 mgL^{-1}.
380 When comparing the values that could be calculated from EC_{50} for 24, 48 and 72
381 h, it is observed that there is no significant inhibition within the first 48 h, but
382 there is a notorious reduction at 72 h for both compounds (see Figure 3 for nano-
383 TiO_2 and Figure S6 for BzP). This initial time period without toxicity increase
384 could be explained in the case of TiO_2 as the time needed by the NPs to form
385 aggregates and trap the algal cells. During this period, the algae continues
386 growing trying to adapt to the new medium conditions up to a certain time at
387 which the nano–TiO_2 aggregates considerably reduced the light available to the
388 entrapped algal cells inhibiting their growth. These results agree with those by
389 Aruoja et al. reporting the formation of nano–TiO_2 aggregates during incubation,
390 which reduced the availability of light to the algae *Pseudokirchneriella*
391 *subcapitata* (Aruoja et al. 2006). Another plausible explanation involves ROS
392 generation under sunlight (photo-toxicity). The formation of these radicals under
393 UV radiation is well documented (Kim and Lee, 2005; Hong and Otaki, 2005;
394 Armelao et al., 2007), nevertheless, this phenomenon has also been observed in

absence of UV light (Reeves et al. 2008). Another mechanism that nano-TiO_2 can display, leading to the growth inhibition and ultimate starvation of algae, is the sequestration of algal growth medium nutrients, such as Zn and P, by the aggregates formed (hetero-aggregates) leading to the death of algae by starvation (Kuwabara et al., 1986).

As regards the PBs, exposure time does not appear to influence the algae biological response. This moderate toxicity increase observed for BzP could be attributed to a rapid mode of toxicity action, as can be seen in Figure S6.

The guideline provided by the Commission Directive 93/67/EEC allows classifying chemicals within 4 toxic categories in function of the EC_{50} values as: harmful (>100 mgL^{-1}), moderate toxic (10-100 mgL^{-1}), toxic (1-10 mgL^{-1}), and very toxic (<1 mgL^{-1}). Considering the tests performed in this study, exposure time for EC_{50} classification was taken at 48 h and 72 h for *D. magna* and *P. tricornutum*, respectively. Thus, our results indicate that nano-TiO_2 and BzP are toxic to *D. magna* and from toxic to very toxic to *P. tricornutum,* PrP is toxic and MeP moderate toxic to *D. magna.*

Table 3, compiles the toxicity data on three sensitive and environmentally relevant species, i.e. fish, crustacean, and algae, as reported in the literature. The comparison shows that our estimated EC_{50} values are similar to those determined for other chemicals (Hernando et al., 2003, Zlámalová Gargošová et al., 2013). In the case of nano-TiO_2 (see Table 3), a different behavior is observed depending on the species; it is more toxic to algae than BzP, whereas the opposite occurs for crustacean and fish. This effect could be due to the different mechanism of contaminant access to the organisms as the one described in this work for the algae *P. tricornutum*. Nano-TiO_2 adsorbs onto algal surfaces impeding the food

420 access, shielding the light or sequestering media nutrients, whereas in crustacean
421 and fish the ingestion drives the uptake (oral route). Indeed, the algal species
422 chosen, its cell size and morphology, determine its cell surface area, which in
423 turn defines the adsorptive capacity of the algae, and thus larger spherical cells
424 show lower adsorption capacity (Taylor et al., 1998). This rational can be behind
425 the difference observed between the EC_{50} values for nano-TiO_2 determined in
426 this study and that reported by Aruoja et al. (2009) towards *Pseudokirchneriella*
427 *subcapitata* (see Table 3). In addition, the different chemical composition of the
428 culture media in the bioassays can favor NPs aggregation of different size, as
429 demonstrated in the DSL experiments (see section 2), which will facilitate or
430 hinder nano-TiO_2 access.
431
432 Whereas for algae cellular uptake of NPs is an unexpected scenario, for fish and
433 *Daphnids*, and likely other NPs-ingesting organisms, the ingestion and
434 subsequent particle-related toxicity of internalized TiO_2 nanoparticles appear to
435 be behind its toxicity. Internalization of nano-TiO_2 has already been documented
436 by TME analysis of the bacterium *Salmonella typhimurium* exposed to 50 nm
437 TiO_2 particles. Despite the specific uptake mechanisms not being determined, it
438 was hypothesized that the formation of micelles or protein coating on NPs would
439 be the responsible (Kumar et al, 2011). Some studies have pointed out that
440 dissolution also exerts an important role in metallic NPs ecotoxicity. A variable
441 fraction of the metal ions from the metal NPs is dispersed in the media, and can
442 exert toxic properties. This is the case of nano-ZnO, whose particles and released
443 Zn ions showed comparable toxicity in several organisms (Bondarenko et al.,
444 2013). Similarly, it was found that nano-CuO toxic effects correlated with the
445 soluble fraction of CuO (Bondarenko et al., 2012, Lin et al 2013).

446 Considering that nanomaterials are known to influence cellular metabolic
447 processes by boosting ROS generation in exposed organisms (Poljšak et al.
448 2011), and that TiO_2, spontaneously produce ROS at the NPs surface due to their
449 chemical composition and properties (Jassby et al. 2012), the observed toxicity
450 of nano-TiO_2 in the bioassays conducted could be also attributed to the oxidative
451 stress caused in the organisms via alterations of the intracellular redox
452 homeostasis (anti-oxidants *vs* pro-oxidants species) (Xia et al. 2006).
453

454 ***3.3. D. magna acute mixture toxicity assays***

455 For these series of mixture toxicity tests, BP3 was included considering its
456 extended use as UV-F and documented toxicity and endocrine disruption activity
457 (Molins-Delgado et al., 2017). Thus, binary mixtures of nano-TiO_2, BzP and BP3
458 were investigated.

459 The concentration addition approach for the mixtures containing nano-
460 TiO_2 predicts lower mixture toxicity than the experimentally observed, thus
461 suggesting a synergistic effect through the addition of nano-TiO_2 to the organic
462 UV-F and paraben solutions. The bioavailability of BP3 might be increased by
463 the presence of the metal NPs. The UV-F could be adsorbed onto the large
464 surface provided by the TiO_2 aggregates and more efficiently transported to the
465 microorganism cells. Another feasible explanation would be the transformation
466 of BP3 into a more toxic product under the influence of the ROS generated by
467 the nano-TiO_2 at the experimental conditions. Despite yet the toxicity of the BP3
468 transformation products formed was not investigated, it is known that ·OH plays
469 an important role in the transformation of BP3 and of its main human metabolite,
470 4-hydroxy benzophenone (Li et al.2016).

As we can confirm through the mixture' isobolograms represented in Figure 4, nano-TiO_2 in combination with BzP or BP3 displays higher toxic effect than that of the individual compounds; it appears that nano-TiO_2 increases the cumulative toxicity of the resulting mixture when combined with other chemicals. When two or more chemicals simultaneously interact with an organism up to six processes can be affected: bioavailability, uptake, internal transportation, binding at the target site, metabolization, and excretion. The interactions so far studied are usually caused by interactions involving more than one of these processes (Cedergreen, 2014).

Concerning the mixture BzP+BP3 as shown in Figure 4, the tentative explanation for its different behavior might be associated to two different toxicity action modes displayed by BZP depending on its concentration level. At lower concentrations mechanisms for BZP and BP3 would be opposite, thus leading to an antagonistic effect, but at higher concentrations, when the ratios between BZP and BP3 are equal or > 1, identical mechanisms would be displayed by the two compounds in the mixture, then observing additivity. Another plausible explanation would be related to the transport rate of BZT towards its molecular target in the crustacean, less impeded at higher concentrations. The surface topography dose-response representations for the three mixtures are ahown in Figure S7.

4. Conclusions

We conducted a series of bioassays with the two UV filters, nanosized TiO_2 and BP3, and three paraben preservatives, namely MeP, PrP and BzP, towards two environmentally relevant aquatic organisms *Daphnia magna* and *Phaeodactylum tricornutum*, in order to determinate their toxicity. Nano-TiO_2 and BzP, with EC_{50} values of 1.35 mgL^{-1} and 3.09 mgL^{-1}, respectively, showed the highest toxicities towards *D. magna*, whereas the MeP was the least toxic demonstrating toxicity only after 48 h exposure with an EC_{50} of 32.51 mgL^{-1}. For the bioassays with *P. tricornutum*, an initial lag period with non-observed toxicity was identified for nano-TiO_2 as the time required for the TiO_2 NPs to form aggregates, and thus, shielding the light and sequestering the medium nutrients. However, other toxicity mechanisms such as oxidative stress caused by ROS generation cannot be ruled out. Small variations in toxicity with exposure time were observed for BzT as the result of a very fast toxic action.

For the bioassays of binary mixtures on *D. magna*, our results showed increased toxicity for the mixtures containing nano-TiO_2 with respect to individual toxicities. This could be explained by the increased bioavailability of BP3 or/and by its transformation products into a more toxic compound under the influence of the ROS generated by nano-TiO_2. Increased bioavailability and further accumulation of the chemical could be attributed to the Trojan horse effect that nanomaterials may exert. A different behavior was shown by the mixture BzP+BP3. First, an antagonistic effect occurred to became additive as the concentration ratios between BzP and BP3 increased. Such effect's change could be due to the potential of BzT to display two different toxicity mechanisms or to a potentiation of transport rate toward its target. More likely, the toxicity

observed may be the result of a combination of the factors discussed here. Our findings manifest that due to the differences encountered in the toxicity displayed among the model organisms, one single bioassay cannot predict the ecotoxicological effects of chemicals, in particular nanomaterials. Moreover, biotests on individual substances neither can provide the real picture of ecotoxicity. Thus, to carry out proper risk assessments, a battery of tests with model organisms at different levels of biological organization as well as mixtures of chemicals should be applied.

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FIGURE CAPTIONS

Figure 1: TEM observation of two different magnification images for titanium dioxide, scale bars for insets: a) 100 nm and b) 50 nm.

Figure 2: Fitted sigmoidal dose-response (Log [nano-TiO_2]–immobilization) curves from single-exposure bioassays on *D. magna*. (two replicates). Plot shows the observed activity in the organism at different exposure time, 24, 48 h and 72 h.

Figure 3: Fitted sigmoidal dose-response (Log [mixture nano-TiO_2]–immobilization) curves from single-exposure bioassays to *Phaeodactylum tricornutum* (four replicates). Observed activity in the algae at different exposure time 24, 48 and 72 h.

Figure 4: Response surface with arithmetic axes (3D surface topography) dose-response curve for the mixtures nano-TiO_2+BzP, nano-TiO_2+BP3, and BzP+BP3.

945 **TABLE HEADERS**

946 **Table 1:** EC_{50} average (n=2) concentration values for single compounds tested
947 towards *Daphnia magna*, at 24, 48 and 72 h exposure time.

948 **Table 2:** Average (n=4) EC_{50} values for target single compounds tested towards
949 *Phaeodactylum tricornutum* at 24, 48 and 72 h exposure time. n.a.: not available.

950 **Table 3:** Comparison of the two most toxic compounds in the single toxicity
951 bioassay EC_{50} values with others studies.

952

953

Table

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Compound	Test criteria	EC_{50} (mgl^{-1})		
		24 h	48 h	72 h
Nano-TiO_2		20.00	3.090	0.050
MeP		54.04	32.52	8.893
PrP		18.66	3.800	2.340
BzP		33.63	1.350	0.570

Table

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Organisms	Species	Compound	EC50 (mgl^{-1})	Time exposure	Reference
Algae	*Pseudokirchneriella subcapitata*	Nano-TiO_2	5.83	72h	Aruoja et al., 2009
	Phaeodactylum tricornutum	Nano-TiO_2	2.27	72h	This study
	Phaeodactylum tricornutum	BzP	10.6	72h	This study
Crustaceans	*Daphnia magna*	Ethylhexyl methoxycinnamate	3.4	48h	Molins-Delgado et al., 2016
	Daphnia magna	Propanil	1.65	48h	Moore et al., 1998
	Daphnia magna	Dichlofluanid	1.33	48h	Hernando et al., 2003
	Daphnia magna	Galaxolide	1.12	48h	Gargošová et al., 2013
	Daphnia magna	Tonalide	1.33	48h	Gargošová et al., 2013
	Daphnia magna	Nano-TiO_2	3.09	48h	This study
	Daphnia magna	MeP	32.5	48h	This study
	Daphnia magna	PrP	3.8	48h	This study
	Daphnia magna	BzP	1.35	48h	This study
Fishes	*Oryzias latipes*	TiO2	8.5	48h	Li et al., 2014
	Oryzias latipes	BzP	0.73	96h	Yamamoto et al.,20011
	Zebrafish	Nano-TiO_2	4.92	96h	Xiong et al., 2011
	Pimephales promelas	BzP	3.3	48h	Dobbins et al., 2009

Table

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Chemical		Toxicity of Phaeodactylum tricornutum mgl-1		
		24 hr	48 hr	72 hr
TiO2	1	7,81	26,68	14,51
	2	8,05	19,26	17,08
	3	8,32	32,82	3,49
	4	7,88	13,66	2,27
Bz P	1	21,90	n.a.	10,61
	2	n.a.	28,54	14,32
	3	n.a.	n.a.	15,78
	4	0,20	n.a.	10,72

n.a.: not available

Figure1
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Figure 1

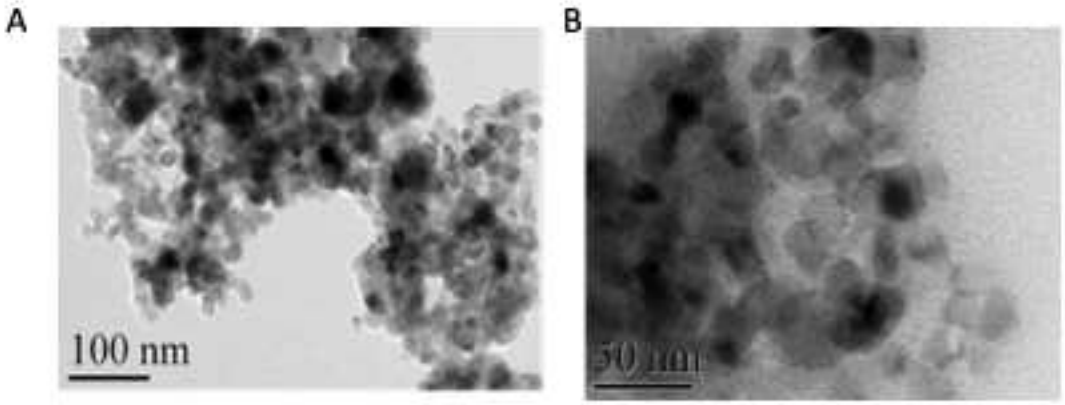

Figure3

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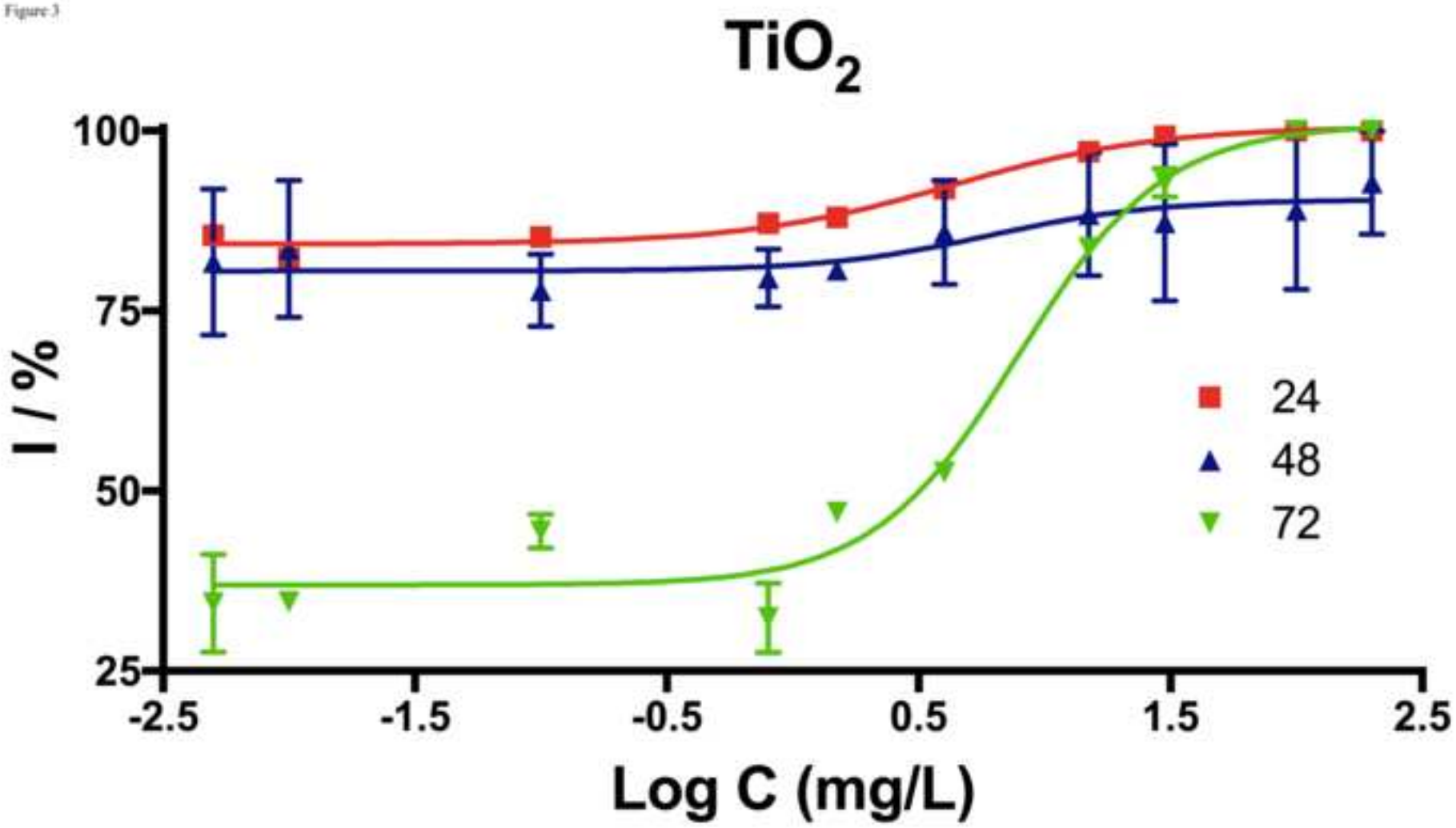

Figure2

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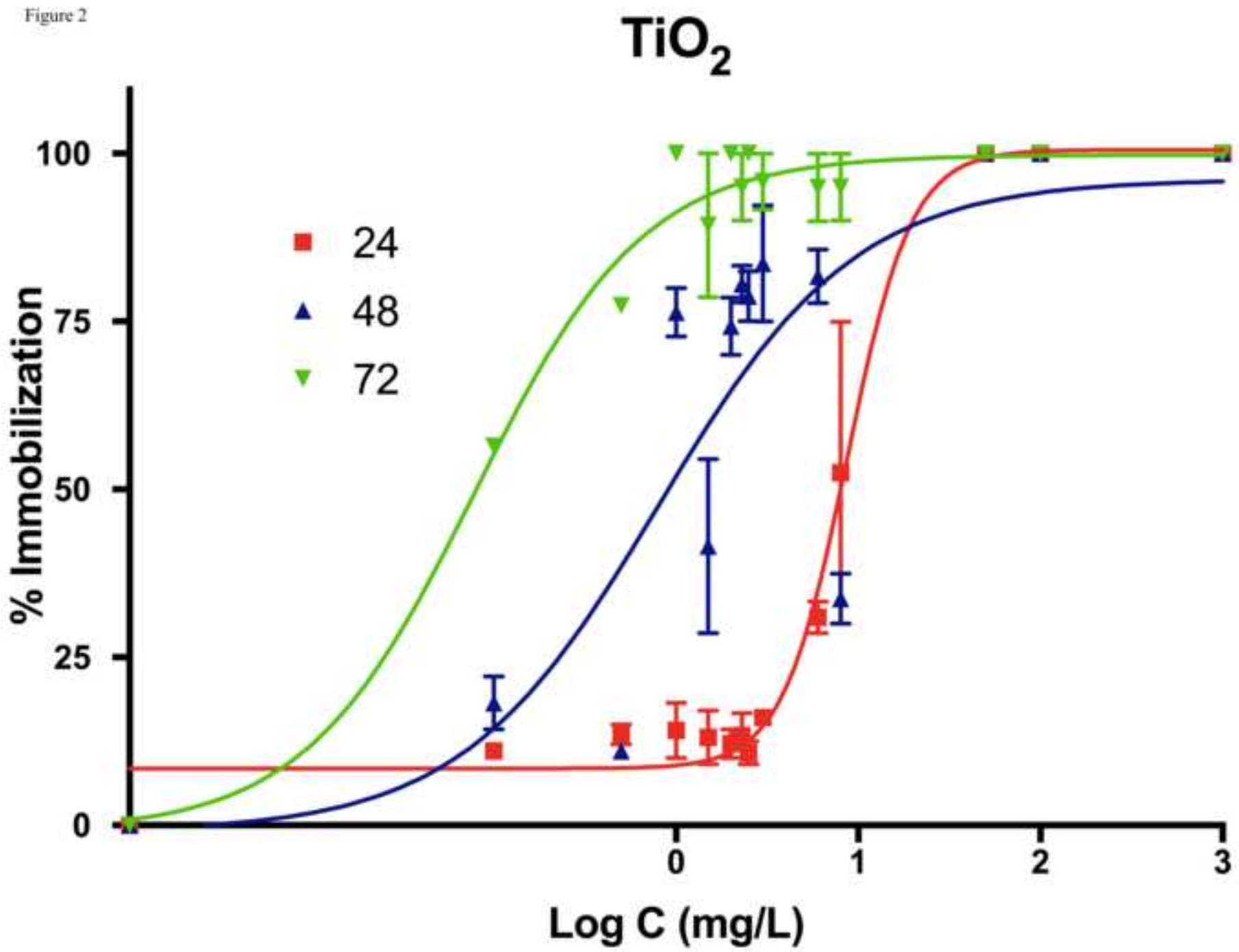

Figure4

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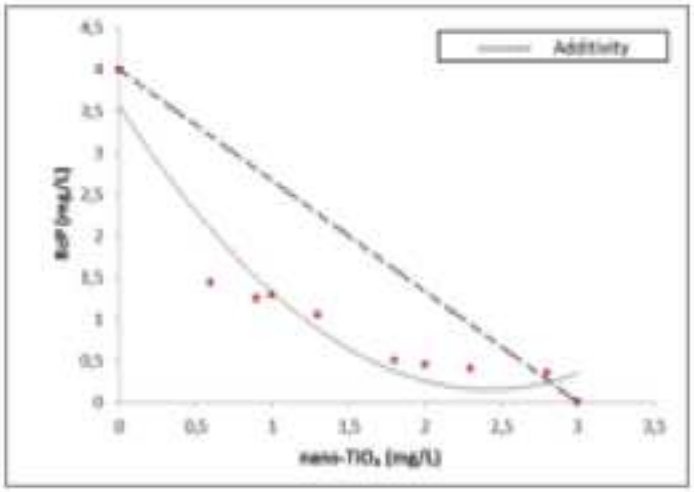

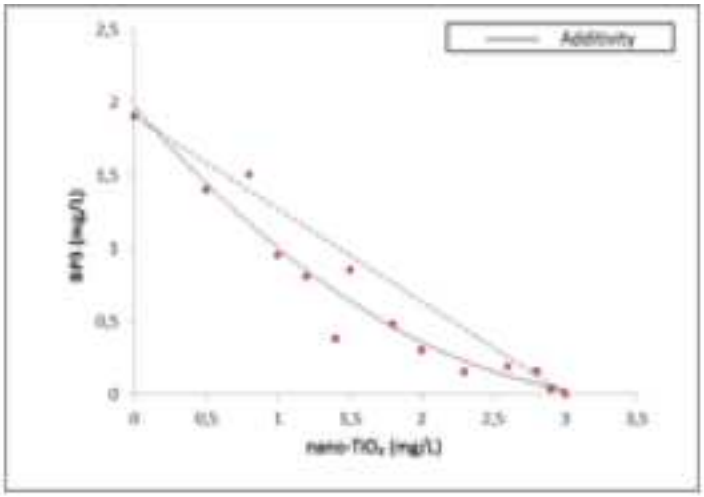

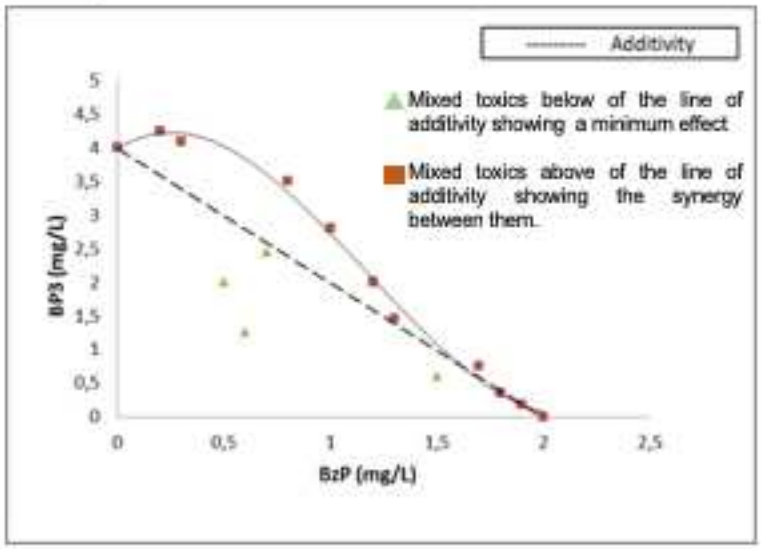

Figure 4

NANOSIZED TITANIUM DIOXIDE UV FILTER INCREASES MIXTURE TOXICITY WHEN COMBINED WITH PARABENS

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Supplementary material

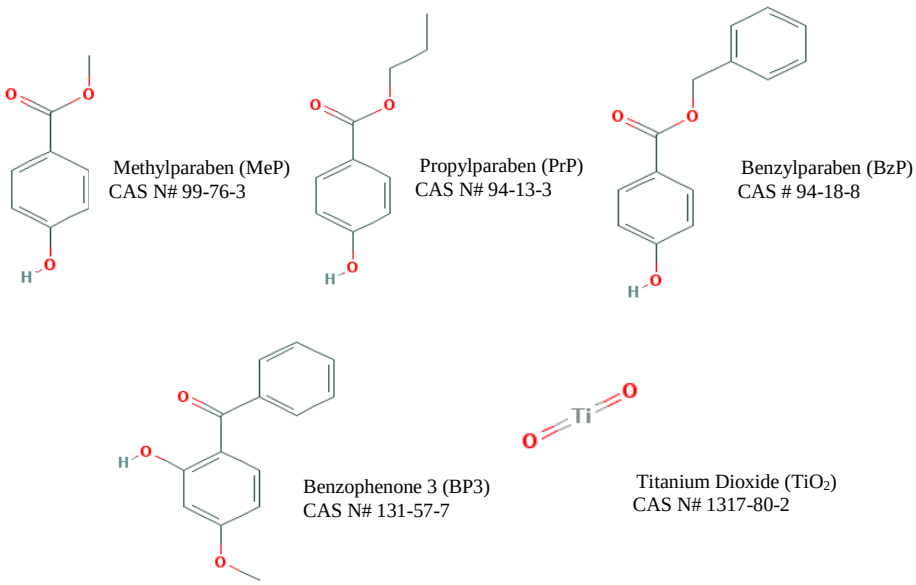

Figure S1. Chemical structures of the three parabens and two UV filters investigated in the present study.

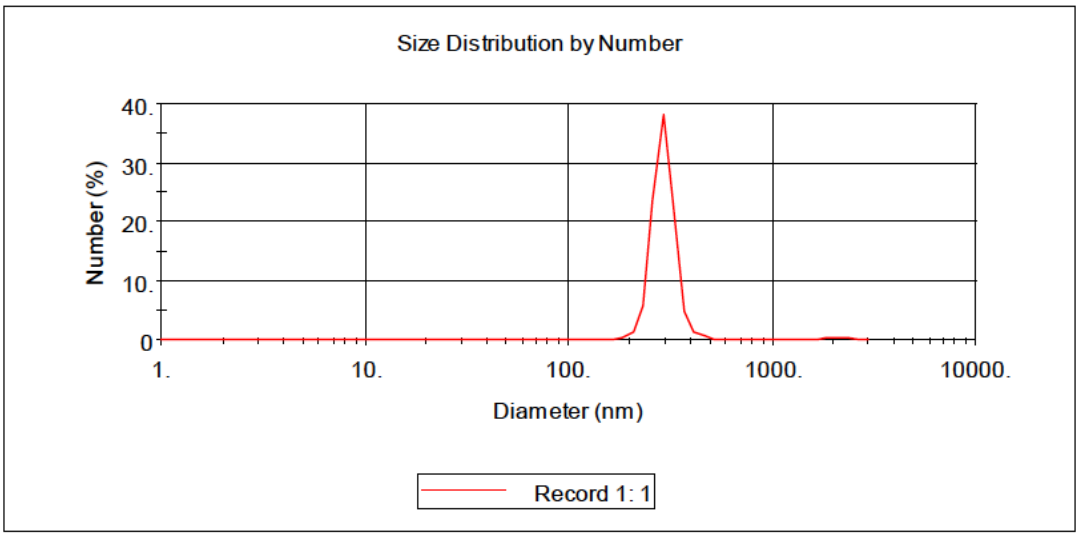

Figure S2. Hydrodynamic diameter distribution of nano-TiO_2 in *Daphnia* media solvent using DLS characterization.

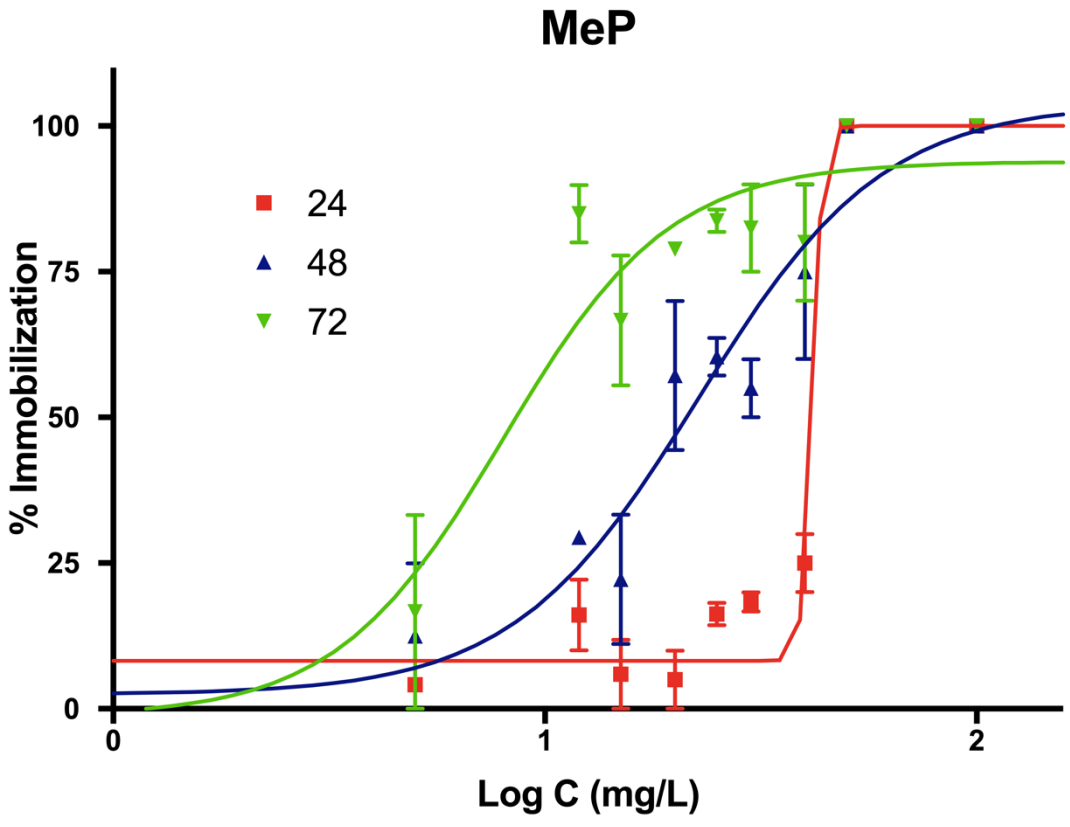

Figure S3. Fitted sigmoidal dose-response for duplicate analyses, Log [MeP]–immobilization, show observed activity in the organism at different exposure time, 24, 48 and 72 h., with two replicates for this compound.

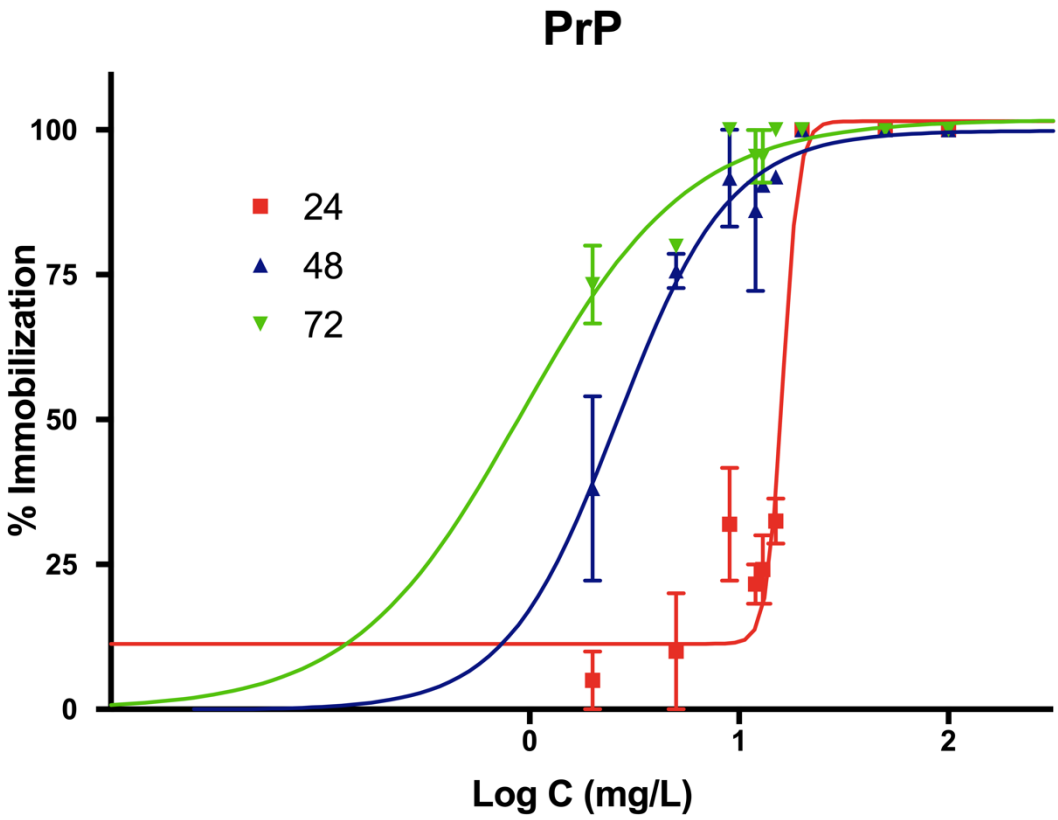

Figure S4. Log [PrP]–immobilization curves from single-exposure bioassays on *D. magna*, respectively. Plot show observed activity in the organism at different exposure time, 24, 48 and 72 h., with two replicates for this compound.

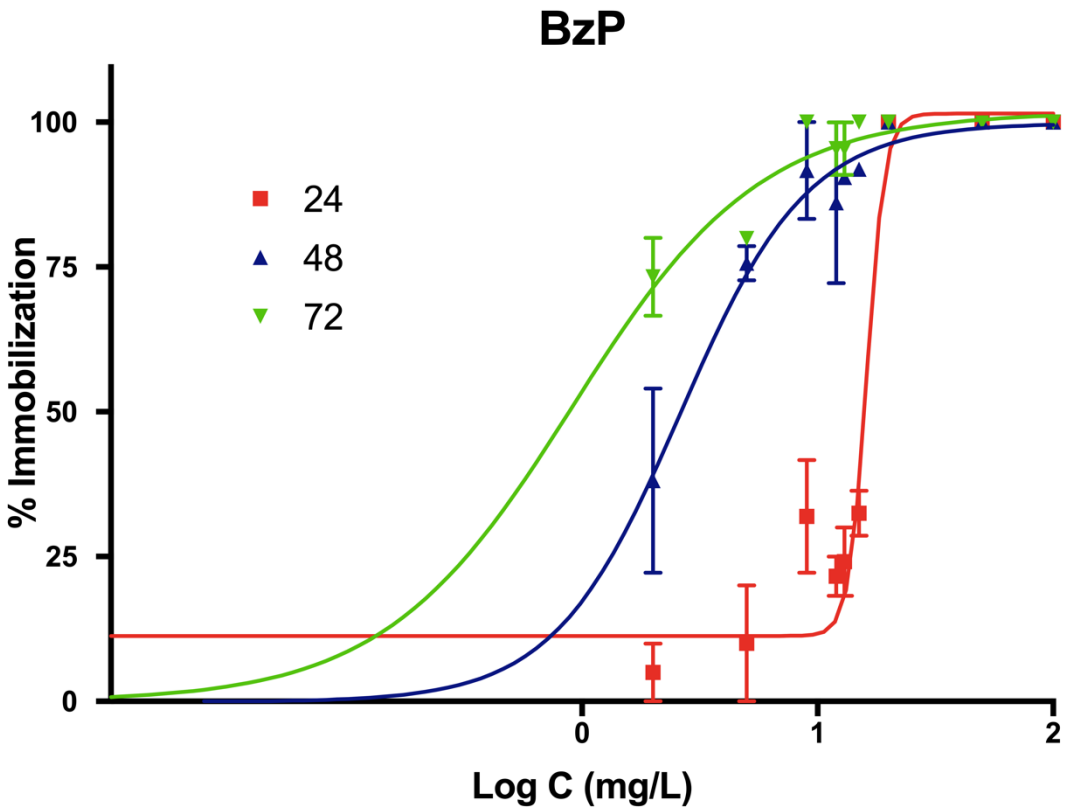

Figure S5. Fitted sigmoidal dose-response for duplicate analyses (Log [BzP]–immobilization) curves from single-exposure bioassays on *D magna*. This compound is the second most toxic compound, in the bioassay. Plot show observed activity in the organism at different exposure time, 24, 48 and 72 h., with two replicates for BzP.

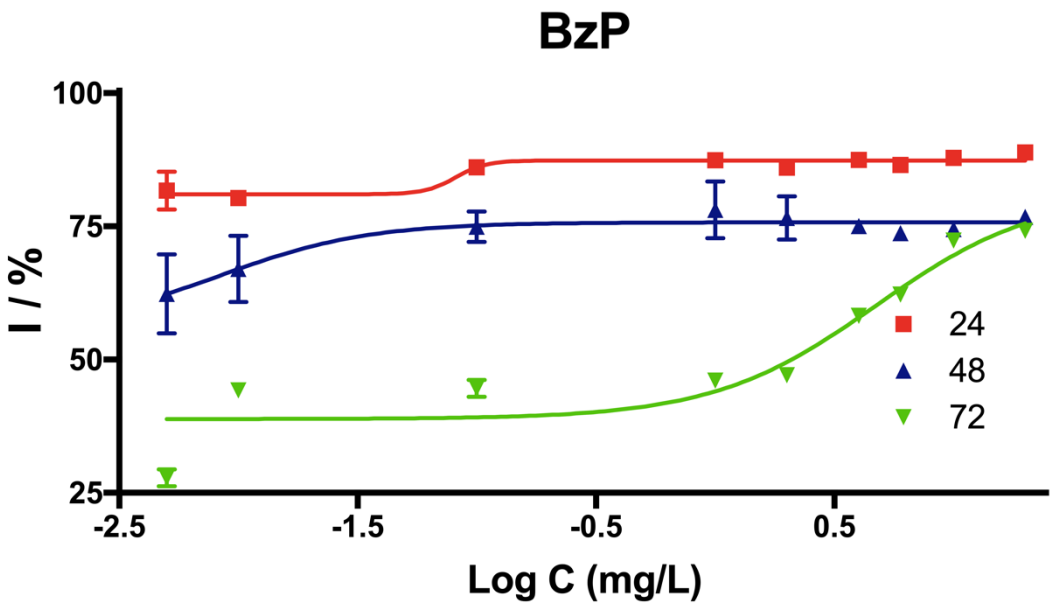

Figure S6. Fitted sigmoidal dose-response (Log [mixture BzP]–immobilization) curves from single-exposure bioassays to *Phaeodactylum tricornutum* (four replicates). Observed activity in the algae at different exposure time 24, 48 and 72 h.

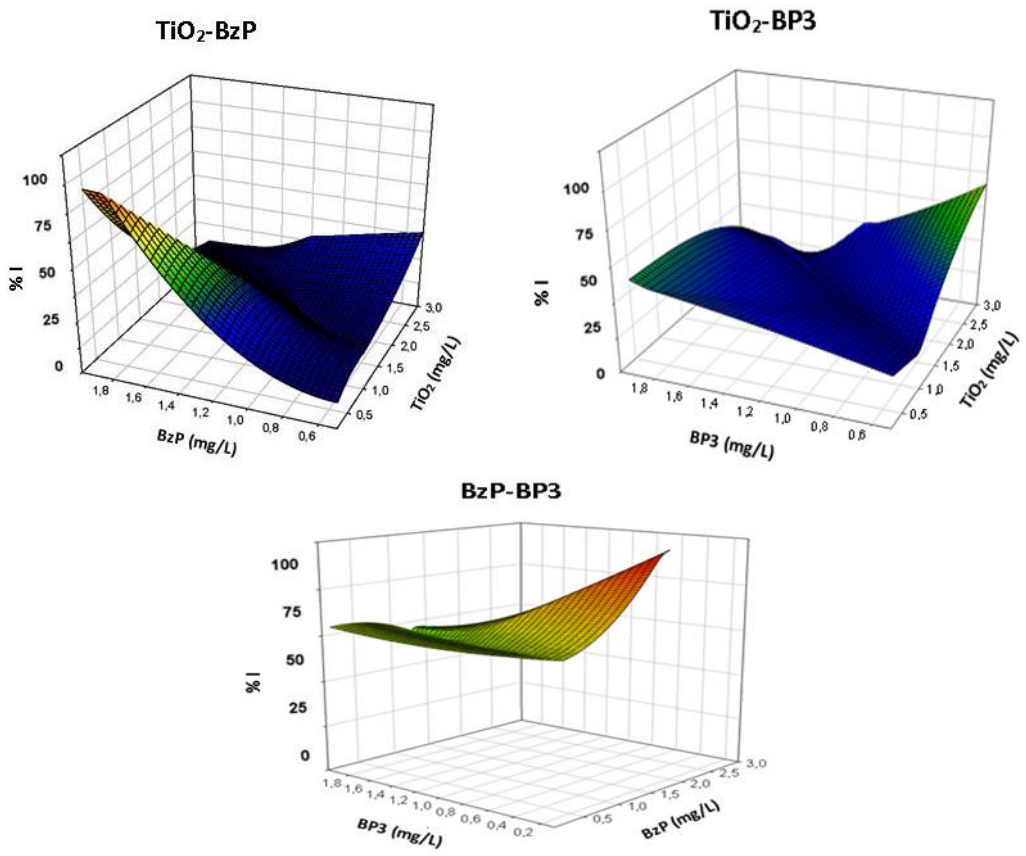

Figure S7: Response surface with arithmetic axes (3D surface topography) dose-response curve for the mixtures nano-TiO_2+BzP, nano-TiO_2+BP3, and BzP+BP3.