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Mixture toxicity of TiO2 NPs and tetracycline at two trophic levels in the marine ecosystem: Chlorella sp. and Artemia salina

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Mixture Toxicity of TiO_2 NPs and Tetracycline at two trophic levels in the marine ecosystem: *Chlorella* sp. and *Artemia salina*

Abstract

Increasing usage of both nanomaterials and pharmaceuticals and their unabated release to the marine ecosystem pose a serious concern nowadays. The toxicity of the mixture of TiO_2 NPs and tetracycline (TC) in the marine species are not very well covered in prior literature. The current study explores the joint toxic effects of TiO_2 NPs and TC in a simulated marine food chain: *Chlorella* sp. and *A. salina*. *Chlorella* sp. was interacted with pristine TiO_2 NPs (0.05, 05, and 5 mg/L), TC (0.5 mg/L), and their combinations for 48 h. The toxicity induced in *Chlorella* sp. by pristine TiO_2 NPs through oxidative stress and chloroplast damage was not significantly changed in the presence of TC. Principal component analysis for the toxicity parameters revealed a strong association between TiO_2 NPs effects and internalization. In the second trophic level (*A. salina*), the waterborne exposure of TC additively increased the toxicity of TiO_2 NPs. Degradation of TC rather than their adsorption played a major role in their removal from the suspension, resulting in additive toxic effects in both *Chlorella* sp. and *A salina*. Compared to the waterborne exposure, the foodborne exposure of TiO_2 NPs and TC induced lesser toxic effects owing to reduced uptake and accumulation in *A. salina*. Biomagnification results indicate that the dietary transfer of TiO_2 NPs and TC does not pose a serious environmental threat in this two-level marine food chain.

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Keywords: *Artemia salina*; *Chlorella* sp.; Tetracycline; TiO_2 NPs; Trophic transfer

1. Introduction

The use of engineered nanomaterials has increased rapidly over the last decade, resulting in an increasing number of consumer products in the market (Foss Hansen et al., 2016). Titanium dioxide nanoparticles (TiO_2 NPs) are employed in household and industrial applications such as personal care products, pharmaceuticals, sunscreens, papers, paints, and foodstuffs due to their high stability, and transparency to UV and visible light absorption (Braydich-Stolle et al., 2009; Hu et al., 2020; Kotil et al., 2017). The worldwide production and utilization of TiO_2 NPs is expected to reach 2.5 million tons and 8.83 million metric tons, respectively, by 2025 (Leite et al., 2020; Loosli et al., 2019). Because of the massive production and consumption, they inevitably enter the marine ecosystem either directly, during manufacture and use, or indirectly, through sewage sludge and wastewater treatment plants (Luo et al., 2020). There is the potential for TiO_2 NPs to interact with marine species and result in adverse changes to the marine ecosystem, but the potential effects have so far not been well characterized.

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Besides nanomaterials, the marine environment serves as a sink for a plethora of contaminants, including plastics, pharmaceuticals, and pesticides (Saaristo et al., 2018). Thus, in such a complex environment, contaminants will co-exist as mixtures. In recent years, there is a growing concern about the hazards posed by the mixture containing nanomaterial and co-contaminants (Zhuang Wang et al., 2017). Over the last decade, pharmaceuticals have gained increasing attention due to their possible threat to various aquatic organisms. Amidst different pharmaceuticals, antibiotics have broad antibacterial spectrums and are prescribed as over-the-counter medicines in developing countries (Kovalakova et al., 2020; Liu et al., 2018). Tetracycline (TC) is known to be one of the essential antibiotics for use in human and veterinary disease control, as well as agricultural feed additives due to its low cost and broad antibacterial spectrum (Daghrir and Drogui, 2013). TC administered to animals and humans is

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not completely metabolized and the excreta is found to contain the active form of TC (75 %) (Xu et al., 2021). Besides, poor removal of TC by wastewater treatment plants results in their discharge into rivers, which transport it to the marine environment (Xie et al., 2019). Unlike bacteria, there is no specific mode of action of TC in marine organisms. However, it has been reported that TC targets microalgae since they have a ribosomal structure similar to bacteria (Yang et al., 2013). As a result, evaluating the toxic effects of TC on marine biota is quite relevant.

Phytoplankton (microalgae) form an integral part of the marine ecosystem because they produce O_2 and organic molecules, as well as serve as food for other species such as invertebrates and fish. The detrimental effects of contaminants in microalgae can leave an impact on the structural and functional elements of the environment (Ma et al., 2006). *Chlorella* sp. was chosen as the model organism because of its simple cell cycle, and rapid growth. Zooplankton, on the other hand, plays a crucial part in ecological processes by transferring energy from primary producers to secondary consumers. *Artemia salina* is one such zooplankton found in salt lakes and coastal locations across the globe that plays a vital role in the flow of energy through marine food chains (Nunes et al., 2006). It is an excellent model for ecotoxicological investigations owing to several typical characteristics such as ease of culturing, small body size, short breeding cycle, low cost, availability, and strong adaptability to harsh environmental conditions (Yi et al., 2020).

Contaminant exposure in the marine environment is not limited to a single chemical species. Understanding the toxic effects of the mixtures of contaminants may provide insights into their impact on the marine species and the marine ecosystem. A previous study on the combined toxic effects of materials, when present in both nano and bulk form of TiO_2 and triclosan on *Ruditapes philippinarum*, revealed significant alterations in the enzyme activity levels with maximum bioaccumulation of triclosan observed with the nano form of TiO_2

(Sendra et al., 2017). Sendra *et al.* (2018) also explored the mixture effects of erythromycin and CeO_2 NPs on *Phaeodactylum tricornutum* and found the protective role of CeO_2 NPs in the toxicity induced by erythromycin. The genotoxic effects of mixtures containing AuNPs and gemfibrozil on gilt-head seabream (*Sparus aurata*) erythrocytes revealed the antagonistic nature of interaction between the contaminants with the levels of predicted toxicity for the mixture being lesser than the observed toxicity (Barreto et al., 2019).

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Studies related to the combined toxicity of TiO_2 NPs and TC on a single trophic level organisms have already been conducted by our research group, namely in freshwater biota (Iswarya et al., 2017; Roy et al., 2020). Iswarya et al. (2017) reported that in the lower concentrations of TC enhanced the toxicity of TiO2 NPs while the higher concentrations of TC and TiO_2 NPs decreased their toxicity to *Scenedesmus obliquus*. Roy et al. (2020) also studied the combined effect of TC and TiO_2 NPs in *Scenedesmus obliquus* and found that a non-lethal concentration of 0.06 mg/L significantly enhanced the toxicity of both pristine and UV pre-irradiated TiO_2 NPs. However, no prior study exists regarding the joint toxic effects of TiO_2 NPs and TC as well as their biomagnification in a marine food chain (algae-artemia). Moreover, there have been only a handful of studies to date reporting the joint toxicity of the contaminants in a phytoplankton-zooplankton trophic chain (Bergsten-Torralba et al., 2020; Thiagarajan et al., 2021; Yang et al., 2018). The species selected from each habitat might differ taxonomically and play different roles in ecosystems. Multi-level species approaches might be more environmentally relevant, leading to the current study, which tested the combined toxic effects of nanomaterials and pharmaceuticals in algae and artemia. Thus, the objectives of the work were framed to study the joint toxic effects of TiO_2 NPs and TC in (i) *Chlorella* sp.; (ii) *A. salina* (waterborne route); and (iii) *A. salina* (foodborne route).

2. Materials and methods

2.1. Materials

Nano powders of titanium (IV) oxide (Aeroxide® P25; CAS No: 13463-67-7; 21 nm), powders of tetracycline antibiotic (CAS No: 60-54-8), and cell-permeable non-fluorescent probe 2′, 7′ dichlorofluorescein diacetate (DCFH-DA) were supplied by Sigma-Aldrich, USA. Other chemicals such as 5, 5′-dithiobis (2-nitrobenzoic acid), trichloroacetic acid (TCA), acetylthiocholine iodide, ethylenediaminetetraacetic acid (EDTA), and thiobarbituric acid (TBA) were offered by Hi-Media Pvt. Ltd., India. *A. salina* cysts were supplied by Ocean Star International Inc., USA.

2.2. Stock preparation

An homogeneous dispersion of TiO_2 NPs (stock concentration-100 mg/L) was prepared in Milli-Q water by sonicating the dispersion for 30 min with a probe sonicator (Sonics, USA). Likewise, a stock concentration of 100 mg/L TC was prepared by completely dissolving the antibiotic powder in Milli-Q water.

2.3. Model organisms

Two model organisms were chosen in this study. The first organism used was *Chlorella* sp. supplied by Central Marine Fisheries Research Institute, Rameswaram, Tamil Nadu, India. Subcultures were regularly grown in sterilized Erlenmeyer flasks containing artificial seawater enriched with Conway medium (Supplementary information). The temperature of the growth chamber was maintained at 23 °C and illuminated with a 3000-lux white fluorescent tube for 16 h and then in the dark for 8 h. The cultures were grown until they entered the log phase, after which they were harvested for toxicity testing. The second organism used in this study was *A. salina*, a marine microcrustacean. They were hatched from *A. salina* cysts before experiments. Around 1 g of dry cysts was added to a round-bottomed tank containing 1 L of natural seawater (filtered and sterilized). The tank was supplied with constant aeration and

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150 lighting (10 W, 1300 lx, and 0.44 mW cm^{-2}), and maintained at room temperature. *A. salina*
151 emerged from the cysts within 24 h, and the hatched nauplii were isolated from the main culture
152 tank and grown in a separate tank with a fresh matrix of natural seawater. Finally, the 48-h old
153 *A. salina* nauplii were collected for use in toxicity experiments.

154 **2.4. Characterization and fate of TiO_2 NPs**

155 The images collected from the transmission electron microscope (HRTEM, FEI
156 TecnaiG2 T20 S-twin, Vellore Institute of Technology, Vellore) were used to identify the size
157 and shape of TiO_2 NPs. For this, TiO_2 NPs dispersed in Milli-Q water were spread over a
158 copper grid and allowed to dry before being observed under the microscope.

159 The particle size of TiO_2 NPs (0.05, 0.5, and 5 mg/L) in filtered ASW was quantified
160 in the absence and presence of 0.5 mg/L TC (0 and 48 h) using NanoBrook 90 Plus Particle
161 Size Analyzer, USA.

162 The sedimentation assay was performed based on the protocol as mentioned in Roy et
163 al. (2016). The absorbance of 5 mg/L TiO_2 NPs in the absence and presence of 0.5 mg/L TC
164 was measured spectrophotometrically and plotted against different time intervals.

165 The fate of TC in the absence and presence of TiO_2 NPs was examined by measuring
166 the degradation of TC using a High-Resolution Liquid Chromatograph Mass Spectrometer
167 (HR-LCMS) (1290 Infinity UHPLC System, Agilent Technologies, USA, Indian Institute of
168 Technology-Bombay).. After 48-h interaction of 0.5 mg/L TC in the absence and presence of
169 5 mg/L TiO_2 NPs, the samples were centrifuged at 12000 rpm for 15 min. The supernatant was
170 filtered using 0.45 µm, 0.1 µm and 3 KDa filters for the complete removal of TiO_2 NPs. The
171 filtered supernatant was then analysed for the degradation of TC using HR-LCMS. For the
172 same supernatant, the adsorption of TC on TiO_2 NPs was determined by measuring the total
173 organic carbon (TOC) content using a TOC analyzer (TOC-L, Shimadzu).

The residual toxicity induced by degraded products of TC was examined in both *Chlorella* sp. and *A. salina*. For this, 0.5 mg/L TC was incubated with 5 mg/L TiO_2 NPs for 48 h, after which the suspension was centrifuged at 12000 rpm for 15 min. The supernatant containing the degraded products of TC was filtered via 0.1 microns and 3 KDa filters. The filtrate containing degraded products of TC was then used to expose *Chlorella* sp. at the cell concentration equal to an optical density (OD) of 0.1 at 610 nm and ten *A. salina* for 48 h and the toxicity was determined by the cell count and immobilization techniques, respectively.

2.5. Toxicity of TiO_2 NPs, and TC to *Chlorella* sp.

2.5.1. Assessment of growth inhibition

For the growth inhibition study, *Chlorella* sp. was collected from their log phase and pelleted down (7000 rpm–10 min–4 ºC). The ASW was added to the pellet to adjust the initial OD to 0.1 at 610 nm. The OD adjusted cultures were taken in sterile glass beakers and subsequently incubated with three concentrations of pristine TiO_2 NPs (0.05, 0.5, and 5 mg/L) and pristine TC (0.5 mg/L). The test concentrations of TiO_2 NPs were selected based on the median effective concentration (6.5 mg/L) computed in our earlier study (Thiagarajan et al., 2019a). The selected concentrations of TiO_2 NPs were less than the median effective concentration of TiO_2 NPs. For tetracycline exposure experiments, the environmentally relevant concentration of 0.5 mg/L was used. In joint experiments, each concentration of pristine TiO_2 NPs (0.05, 0.5, and 5 mg/L) was exposed with 0.5 mg/L of TC to *Chlorella* sp. In addition, *Chlorella* sp. not exposed to either of the contaminants were considered as control. The foregoing interaction was performed under visible light for 48 h in a static condition, with the cultures shaken every 24 h to reduce the precipitation of cells. After 48 h, the decrease in growth was monitored with the help of the Neubauer counting chamber. About 10 µL of algal

197 suspension was loaded into the chamber and individual cells without morphological damages
198 were counted.

199 **2.5.2. Assessment of oxidative stress**

200 Assessment of oxidative radical generation was performed in *Chlorella* sp. exposed to
201 pristine TiO_2 NPs, TC, and their mixture. To evaluate the ROS levels, the contaminant exposed
202 cells were incubated with DCFH-DA dye (100 µM) for 30 min in the dark. Then, the stained
203 cells were examined with a fluorescence spectrophotometer (Cary Eclipse, G9800A; Agilent
204 Technologies, USA) at an excitation-emission wavelength of 485–530 nm.

205 To assess lipid peroxidation, the algal suspensions were centrifuged (7000 rpm–10
206 min–4 ºC). Then, 2 mL of TCA–TBA mixture (2 mL) was added to the pellet, vortexed, and
207 heated to 90 ºC for 30 min. The mixture was then left to cool down and before being centrifuged
208 at 7000 rpm. The absorbance of MDA–TBA abduct (absorbance at 532 nm – absorbance at
209 600 nm) from the supernatant was measured using a microplate reader (xMark, BioRad).

210 **2.5.3. Assessment of chloroplast parameters**

211 The maximum quantum yield of the photosystem II (PSII) was measured for all the
212 treatment groups using MINI-PAM, Photosynthesis Yield Analyzer, Walz, Germany.
213 Following 48-h exposure, the samples were acclimatized to the dark condition for 15 min.
214 Then, 40 µL of culture was exposed to high-intensity actinic light, which recorded the Fv/Fm
215 ratio.

216 The efficiency of photosynthetic apparatus can be monitored by measuring the
217 autofluorescence of Chlorophyll-a (Chl-a) and the same was done using the protocol described
218 previously (Machado and Soares, 2015). After the exposure of pristine TiO_2 NPs, TC, and their
219 combination to *Chlorella* sp. for 48 h, about 200 µL of culture was examined at an excitation-

220 emission wavelength of 485–680 nm using a spectrofluorometer (Cary Eclipse, G9800A;
221 Agilent Technologies, USA).

222 **2.5.4 Quantification of internalized Ti**

223 The amount of Ti internalized in *Chlorella* sp. was quantified following the 48-h
224 exposure to TiO_2 NPs in the absence and presence of TC. The cultures were centrifuged at
225 7000 rpm and the loosely-bound nanoparticles on the surface of microalgae were washed away
226 with EDTA. The wet weight of the pellet (~80-160 mg) was noted. The pellet was transferred
227 to a Teflon digestion vessel and added with 10 mL of concentrated HNO for digestion using a
228 microwave digestion system (Anton Paar, Multiwave GO). The digestion procedure included
229 a 15 min temperature ramp to attain the desired temperature of 200 ºC, which was then held
230 for 25 min. After digestion, equal volume of Milli-Q water (10 mL) was added to dilute the
231 samples. Eventually, the amount of Ti internalized was determined using an Inductively
232 Coupled Plasma-Mass Spectrometer, Agilent (ICP-MS). The analysis was performed at Glens
233 Innovation Labs Pvt. Ltd., and the instrument had a detection limit of 1 µg/L.

234 **2.6. Waterborne exposure of *A. salina* to TiO_2 NPs, and TC**

235 **2.6.1. Assessment of mortality**

236 Mortality experiments on *A. salina* were carried out in a sterile glass beaker containing
237 10 mL ASW. Ten nauplii were added to the beaker containing three concentrations of pristine
238 TiO_2 NPs (0.05, 0.5, and 5 mg/L) and pristine TC (0.5 mg/L) and incubated for 48 h under
239 static condition and constant illumination. The test concentrations of TiO_2 NPs were selected
240 based on the median effective concentration (3 mg/L) computed for the range of concentrations
241 (0.25, 0.5, 1, 2, 4, 8, and 16 mg/L) in our earlier study (Bhuvaneshwari et al., 2018). The
242 concentrations of TiO_2 NPs were selected in such a way that two concentrations were less than,
243 and one closet to the median effective concentration of TiO_2 NPs. For tetracycline exposure

experiments, the environmentally relevant concentration of 0.5 mg/L was used. Likewise, in the joint experiments, ten nauplii were added to the beaker containing a combination of TiO_2 NPs (0.05, 0.5, and 5 mg/L) and TC (0.5 mg/L) and their interactions observed for 48 h under similar exposure conditions. In addition, a control sample was kept that was devoid of any contaminant treatment. Three replicate beakers were kept for each treatment. External feed and aeration were not offered during the experiments. Following 48-h of incubation, the nauplii were placed under the microscope to examine their movements. Considering the immobilized nauplii with no movement as dead, the number of viable nauplii was counted.

2.6.2. Assessment of oxidative stress

To evaluate the ROS levels, the contaminant exposed nauplii were incubated with DCFH-DA dye (10 µM) for 30 min in the dark. Then, the dye incubated animals were rinsed in Milli-Q water to remove surface-bound contaminants and subsequently homogenized in pH 7.4 phosphate buffer. The homogenate was centrifuged at 13000 rpm for 15 min and the obtained supernatant was examined with a fluorescence spectrophotometer (Cary Eclipse, G9800A; Agilent Technologies, USA) at an excitation-emission wavelength of 485–530 nm.

Similarly, lipid peroxidation in *A. salina* after treatment with pristine TiO_2 NPs, TC, and their mixture was quantified (Piotrowska-Niczyporuk et al., 2012). *A. salina* nauplii from all the treatment groups were homogenized using a probe sonicator for 30 s in the presence of ice to avoid enzyme degradation. The homogenate was centrifuged at 7000 rpm for 10 min at 4 ºC. Then, 2 mL of TCA–TBA mixture was added to the tissue homogenate, vortexed, and heated to 90 ºC for 30 min. The mixture was then left to cool down and before being centrifuged at 7000 rpm. The absorbance of MDA–TBA abduct (absorbance at 532 nm – absorbance at 600 nm) from the supernatant was measured using a microplate reader (xMark, BioRad).

2.6.3. Estimation of Cholinesterase activity

The activity of Cholinesterase (ChE) enzyme in *A. salina* was estimated using the previously described protocol (Cavion et al., 2020). *A. salina* nauplii from all the treatment groups were homogenized using a probe sonicator for 30 s in the presence of ice to avoid enzyme degradation. The homogenate was centrifuged at 7000 rpm for 10 min at 4 ºC. The supernatant (50 µL) was mixed with 10 µL of acetylthiocholine iodide (10 mM), 10 µL of 5, 5′-dithiobis (2-nitrobenzoic acid) (5 mM), and 30 µL of pH 7.4 potassium phosphate buffer (100 mM). For blank reading, potassium phosphate buffer was used in place of 5, 5′-dithiobis (2-nitrobenzoic acid). The absorbance of the mixture was measured at 405 nm every minute for 3 min using a microplate reader (xMark, BioRad).

2.6.4. Quantification of internalized and accumulated Ti

The amount of Ti internalized and accumulated in *A. salina* was quantified following the 48-h exposure to TiO_2 NPs in the absence and presence of TC. To quantify the amount of internalized Ti, around 150 live nauplii were separated and rinsed in water. Then, the wet weight of the nauplii was noted. The nauplii were transferred to a Teflon digestion vessel and added with 10 mL of concentrated HNO_3 for digestion using a microwave digestion system. The digestion procedure included a 15 min temperature ramp to attain the desired temperature of 200 ºC, which was then held for 25 min. After digestion, 5 mL of Milli-Q water was added to dilute the samples. The digested samples were analyzed with ICP-MS to determine the concentration of internalized Ti.

To quantify the amount of accumulated Ti, the 48-h interacted *A. salina* was separated and transferred to a 100 mL sterile beaker containing 30 mL of fresh ASW to allow the discharge of particles from *A. salina*. After the depuration period of 24 h, the wet weight of the nauplii was noted. Subsequently, the nauplii were transferred to a Teflon digestion vessel and added with 10 mL of concentrated HNO_3 for digestion using a microwave digestion

system. The digestion procedure included a 15 min temperature ramp to attain the desired temperature of 200 ºC, which was then held for 25 min. After digestion, 5 mL of Milli-Q water was added to dilute the samples. The digested samples were analyzed with ICP-MS to determine the concentration of accumulated Ti.

2.7. Foodborne exposure of *A. salina* to TiO_2 NPs and TC

Before foodborne experiments, 10 mL of 0.1 OD *Chlorella* sp. was exposed to three test concentrations of pristine TiO_2 NPs (0.05, 0.5, and 5 mg/L), TC (0.5 mg/L), and their combination for 48 h in a 100 mL sterile glass beaker under static condition and constant illumination for 48 h.

2.7.1. Assessment of mortality and ingestion rate

To assess mortality, ten nauplii were fed with contaminated pellets in a sterile beaker and incubated for 48 h under static condition and constant illumination. Following 48-h of incubation, the nauplii were placed under the microscope to examine their movements. Considering the immobilized nauplii with no movement as dead, the number of viable nauplii was counted. Eventually, the mortality rate in *A. salina* was determined.

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In addition to the mortality tests, the ingestion of algae by *A. salina* was calculated. For this, *Chlorella* sp. was exposed to pristine TiO_2 NPs (0.05, 0.5, and 5 mg/L), TC (0.5 mg/L), and their mixture for 48 h. After the exposure period, the cell numbers were enumerated by loading 10 µL of algal suspension into the Neubauer chamber. Then, the samples were centrifuged at 7000 rpm and the pellet was fed to *A. salina*. After 48 h, the suspension was agitated, and 10 µL of algal suspension was loaded into the Neubauer chamber. Cell numbers were counted once again and the ingestion rate was calculated using the following formula:

$$I = (Cc-Ce).(V/Nt)$$

316 where I is the ingestion rate (number of algal cells ingested per artemia per h); Cc is the final
317 algal concentration in control samples; Ce is the final algal concentration in the test samples; t
318 is the duration of the experiment in hours; V and N are the respective volume (mL) and number
319 of *A. salina* in test samples.

320 **2.7.2. Quantification of internalization and accumulation of Ti**

321 The amount of Ti internalized and accumulated in *A. salina* was quantified following
322 the 48-h exposure to contaminated *Chlorella* sp. as feed. To quantify the amount of internalized
323 Ti, around 150 live nauplii were separated and rinsed in water. Then, the wet weight of the
324 nauplii was noted and subsequently digested in 10 mL of concentrated HNO_3 using a
325 microwave digestion system. The digested samples were analyzed with ICP-MS to determine
326 the concentration of internalized Ti.

327 To quantify the amount of accumulated Ti, after 48-h, the *A. salina* was separated and
328 transferred to a 100 mL sterile beaker containing 30 mL of fresh ASW to allow the discharge
329 of particles from *A. salina*. After the depuration period of 24 h, the wet weight of the nauplii
330 was noted and subsequently digested in 10 mL of concentrated HNO_3 using a microwave
331 digestion system. The digested samples were analyzed with ICP-MS to determine the
332 concentration of accumulated Ti.

333 **2.7.3. Calculation of BMF**

334 The BMF of TiO_2 NPs with and without TC was computed to assess their transfer across
335 the trophic levels. The BMF was derived by taking the ratio of Ti concentration in artemia to
336 the concentration in algae.

337 **2.8. Data analysis**

All experiments employed three replicates (n=3) and the results are reported as mean ± standard error. GraphPad Prism 6 was used to plot the graph and conduct the statistical analysis of the data. Statistical variances in the data were analyzed by Two-way analysis of variance and Bonferroni post-test.

3. Results

3.1. Characterization of TiO_2 NPs

From the TEM image (Fig. S1, supplementary information), the primary particle size of TiO_2 NPs was estimated to be in the range of 20-25 nm and the shape of the NPs was spherical and cuboidal. From our earlier study, the X-ray diffraction analyses revealed the crystalline nature of NPs, which included both anatase and rutile phases (Thiagarajan et al., 2019b). Contact angle measurements (14.7 º ± 1.53 º) demonstrated that TiO_2 NPs were hydrophilic (Thiagarajan et al., 2019b).

3.2. Stability of TiO_2 NPs in the presence of TC

The stability of TiO_2 NPs (0.05, 0.5, and 5 mg/L) in ASW was determined by measuring their effective diameter (0 and 48 h) in the presence and absence of 0.5 mg/L TC. The 0th h results revealed the effective diameter of 0.05, 0.5, and 5 mg/L pristine TiO_2 NPs to be 297.84 ± 13.28, 315.75 ± 4.33, and 352.09 ± 4.40 nm respectively, which significantly ($p < 0.05$) increased to 1251.61 ± 12.52, 1682.64 ± 116.00, and 2670.24 ± 30.33 nm respectively by the end of 48 h.

After mixing with 0.5 mg/L TC, the effective diameter of 0.05, 0.5, and 5 mg/L TiO_2 NPs at 0th h was 314.83 ± 3.77, 319.90 ± 1.14, and 369.91 ± 8.88 nm respectively, which significantly ($p < 0.05$) increased to 1491.95 ± 30.12, 1883.81 ± 66.13, and 2834.23 ± 231.45 nm respectively by the end of 48 h. It was noticed that the addition of TC did not significantly increase ($p > 0.05$) the effective diameter of TiO_2 NPs.

In addition to particle size measurements, the sedimentation of 5 mg/L TiO_2 NPs in the absence and presence of 0.5 mg/L TC was evaluated (Fig. S2, supplementary information). With an increase in time, the absorbance of TiO_2 NPs in the absence and presence of TC decreased indicating settling in the medium. Similar to DLS measurements, the presence of TC did not significantly alter the sedimentation of TiO_2 NPs ($p > 0.05$).

3.3. Fate of TC in the presence of TiO_2 NPs

HR-LCMS analysis was performed to study the possible degradation of TC in the presence and absence of TiO_2 NPs. The HR-LCMS spectra of 0.5 mg/L TC alone is provided in Fig. S3A (supplementary information). The analysis of 0.5 mg/L TC alone exhibited a sharp and high-intensity peak at 445.15 m/z, which confirmed the presence of TC in the suspension. Besides, the small intensity peak at 227.17 and 340.25 m/z indicate a slight degradation of TC. On the other hand, analysis of 0.5 mg/L TC with 5 mg/L TiO_2 NPs (Fig. S3B, supplementary information) revealed the intensity of the TC peak (445.15 m/z) to be considerably less compared to the intensity of peaks of the degraded compounds (340.25, 227.17, and 175.01 m/z), confirming TC degradation. Moreover, the intensity of TC peak in the absence of TiO_2 NPs was greater compared to the intensity of TC peak in the presence of TiO_2 NPs, confirming TC degradation in the presence of TiO_2 NPs.

The TOC content in the solution was measured to determine how much TC was adsorbed on TiO_2 NPs. The TOC content for 0.5 mg/L TC at 0th h was 5.19 ± 0.11 mg/L. The TOC content of 0.5 mg/L TC in the presence of 5 mg/L TiO_2 NPs, on the other hand, remained unaltered after 48 h ($p > 0.05$) and the value was found to be 5.19 ± 0.05 mg/L. No change in the TOC content possibly indicates negligible adsorption of TC on TiO_2 NPs.

3.4. Impact of TiO_2 NPs and TC in *Chlorella* sp.

3.4.1. Growth Inhibition

Fig. 1A depicts the growth inhibition induced by pristine TiO_2 NPs, TC, and their mixture in *Chlorella* sp. 48-h interaction with individual concentrations (0.5 and 5 mg/L) of TiO_2 NPs produced a concentration-dependent effect on the growth of *Chlorella* sp. that varied significantly with control ($p < 0.05$). Likewise, 0.5 mg/L of TC also induced a significant growth inhibition compared to control ($p < 0.05$). In joint experiment, the addition of TC significantly enhanced the growth inhibition induced by TiO_2 NPs as compared to control ($p < 0.05$). Besides, the growth inhibition induced by 5 mg/L TiO_2 NPs together with TC significantly increased when compared to the growth inhibition induced by 5 mg/L pristine TiO_2 NPs ($p < 0.05$).

The growth inhibition by the degraded products of TC formed through interaction of 0.5 mg/L of TC with 5 mg/L TiO_2 NPs was assessed in *Chlorella* sp. The percentage of growth inhibition induced by degraded products of TC in *Chlorella* sp. was found to be 4.80 ± 0.48 %, which was lower than the growth inhibition induced by 0.5 mg/L TC alone (7.10 ± 1.91 %).

3.4.2. Oxidative damage

The ROS generated in *Chlorella* sp. treated with pristine TiO_2 NPs, TC, and their combination is depicted in Fig. 1B. Compared to the control, a lower concentration (0.05 mg/L) of TiO_2 NPs did not induce a significant increase in ROS generation, whereas 0.5, and 5 mg/L of TiO_2 NPs significantly increased ($p < 0.05$) the ROS formation in *Chlorella* sp. Besides, 0.5 mg/L TC also significantly altered ($p < 0.05$) the ROS responses in *Chlorella* sp. In the joint experiment, the addition of TC to 0.5, and 5 mg/L of TiO_2 NPs significantly increased ($p < 0.05$) the ROS responses in *Chlorella* sp. as compared to the control. However, no significant difference ($p > 0.05$) in ROS formation was observed between mixture and respective concentrations of pristine TiO_2 NPs.

409 The lipid peroxidation in *Chlorella* sp. after exposure to pristine TiO_2 NPs, TC, and
410 their mixture is depicted in Fig. 1C. Only 5 mg/L pristine TiO_2 NPs significantly induced lipid
411 peroxidation in *Chlorella* sp. when compared with control ($p < 0.05$). Treatment with 0.5 mg/L
412 TC, on the other hand, had no significant difference ($p > 0.05$) in lipid peroxidation of *Chlorella*
413 sp. as compared to the control. In the combined tests, the addition of TC to 0.5, and 5 mg/L
414 TiO_2 NPs significantly increased the lipid peroxidation in *Chlorella* sp. when compared with
415 control ($p < 0.05$). However, no significant difference ($p > 0.05$) in lipid peroxidation was
416 observed between mixture and respective concentrations of pristine TiO_2 NPs.

417 **3.4.3. Chloroplast parameters**

418 The maximum quantum yield of PSII in the treated samples was determined (Fig. 2A).
419 *Chlorella* sp. treated with pristine TiO_2 NPs and TC exhibited a significant reduction in the
420 quantum yield relative to control ($p < 0.05$), except for 0.05 mg/L pristine TiO_2 NPs. Likewise,
421 the inclusion of TC to three concentrations (0.05, 0.5, and 5 mg/L) of TiO_2 NPs significantly
422 reduced the quantum yield relative to control ($p < 0.05$). However, the reduction in quantum
423 yield observed between pristine TiO_2 NPs and TiO_2 NPs with TC varied significantly only for
424 the following combinations: 0.05 mg/L TiO_2 NPs vs 0.05 mg/L TiO_2 NPs with 0.5 mg/L TC
425 ($p <0.05$).

426 The autofluorescence of Chl-a from cell suspensions treated with pristine TiO_2 NPs,
427 TC, and their mixture is depicted in Fig. 2B. Three concentrations of pristine TiO_2 NPs and
428 one concentration of TC did not significantly alter the Chl-a autofluorescence in *Chlorella* sp.
429 relative to the control ($p > 0.05$). In the joint study, the addition of 0.5 mg/L TC to the 5 mg/L
430 TiO_2 NPs significantly decreased ($p < 0.05$) Chl-a autofluorescence with respect to control

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433 **3.4.4. Ti internalization**

434 Figure 3 depicts the amount of Ti internalized by *Chlorella* sp. after treatment with
435 TiO_2 NPs in the absence and presence of TC. Pristine TiO_2 NPs produced a dose-dependent
436 increase in the concentration of Ti inside *Chlorella* sp. Likewise, in the joint study, the addition
437 of TC to TiO_2 NPs produced a concentration-dependent increase in the internalization of Ti.
438 Moreover, there was no statistical significance in Ti internalization for pristine TiO_2 NPs and
439 TiO_2 NPs together with TC ($p > 0.05$).

440 **3.4.5. Principal component analysis**

441 To figure out the correlations between different parameters tested during the microalgal
442 study, PCA was performed. Figure 7A shows the components PC1 and PC2 collectively
443 interpreting 97.67% of data variability. For the highest concentration of pristine TiO_2 NPs (5
444 mg/L), parameters such as growth inhibition and uptake lie in the same quadrant suggesting
445 their correlation. For lower concentrations (0.05, and 0.5 mg/L) of pristine TiO_2 NPs,
446 parameters such as effective quantum yield, and Chl-a autofluorescence cluster together
447 indicating their correlation. On the other hand, none of the parameters lie in the quadrant of 0.5
448 mg/L TC suggesting no correlation of parameters tested. However, for the highest mixture
449 concentration containing 5 mg/L TiO_2 NPs and 0.5 mg/L TC, ROS and lipid peroxidation were
450 clustered together indicating their correlation.

451 **3.5. Impact of waterborne exposure of TiO_2 NPs and TC to *A. salina***

452 **3.5.1. Mortality**

453 Figure 4A depicts the mortality of *A. salina* upon aqueous exposure of pristine TiO_2
454 NPs, TC, and their combinations. Pristine concentrations of TiO_2 NPs induced dose-dependent
455 mortality in *A. salina* that varied significantly with control only at 5 mg/L TiO_2 NPs ($p < 0.05$).
456 Similarly, mortality induced by TC was also significant ($p < 0.05$). In the combined experiment,

TC significantly enhanced the toxicity of all three concentrations of TiO_2 NPs (0.05, 0.5, and 5 mg/L) when compared with control ($p < 0.05$).

Toxicity assessment of degraded products of TC in *A. salina* was carried out, and the percentage of mortality induced by degraded products of TC in *A. salina* was found to be 17.85 ± 3.57 %, which was less than the mortality induced by 0.5 mg/L TC alone (28.57 ± 3.57 %).

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3.5.2. Oxidative damage

The ROS generated in *A. salina* after exposure to pristine TiO_2 NPs, TC, and their mixture is depicted in Fig. 4B. Lower concentration of 0.05 mg/L pristine TiO_2 NPs did not induce significant ROS formation ($p > 0.05$), whereas 0.5, and 5 mg/L TiO_2 NPs significantly enhanced the ROS responses in *A. salina* ($p < 0.05$). Besides, 0.5 mg/L of TC also significantly varied the ROS responses in *A. salina* ($p < 0.05$). In the combined experiment, the addition of TC to 0.5, and 5 mg/L of TiO_2 NPs significantly enhanced the ROS responses in *A. salina* ($p < 0.05$). Moreover, there existed no statistical significance in ROS responses between pristine TiO_2 NPs and TiO_2 NPs together with TC ($p > 0.05$).

The lipid peroxidation in *A. salina* after exposure to pristine TiO_2 NPs, TC, and their mixture is depicted in Fig. 4C. Lipid peroxidation induced by pristine TiO_2 NPs and TC did not vary significantly with control ($p > 0.05$). Likewise, in the combined experiment, lipid peroxidation induced was statistically insignificant with control ($p > 0.05$), except for 0.5 mg/L TC with 5 mg/L TiO_2 NPs. Furthermore, there existed no difference in the lipid peroxidation between pristine TiO_2 NPs and TiO_2 NPs together with TC ($p > 0.05$).

3.5.3. ChE activity

The activity of ChE in *A. salina* incubated with TiO_2 NPs, TC, and their combination are denoted in Fig. 5. A dose-dependent rise in ChE activity was noted upon exposure to pristine TiO_2 NPs that varied insignificantly with control ($p > 0.05$). Likewise, treatment with

0.5 mg/L TC also produced an insignificant rise in ChE activity with control ($p > 0.05$). In the combined experiment, the rise in ChE activity was noted to be dose-dependent for all the mixture concentrations that varied significantly with control for 0.5 mg/L TC with 5 mg/L TiO_2 NPs ($p < 0.05$).

3.5.4. Internalization and accumulation

In the presence of TC, the internalization of TiO_2 NPs was also dose-dependent and varied significantly with control ($p < 0.05$) for the following concentration: 0.5 mg/L TC with 5 mg/L TiO_2 NPs.

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The internalization of TiO_2 NPs in the absence and presence of TC is displayed in Fig. 6A. Internalization of TiO_2 NPs into *A. salina* was dose-dependent and varied significantly with control only at 5 mg/L TiO_2 NPs ($p < 0.05$). Likewise, in the presence of TC, the internalization of TiO_2 NPs was also dose-dependent and varied significantly with control for 0.5 mg/L TC with 5 mg/L TiO_2 NPs ($p < 0.05$).

The accumulation of TiO_2 NPs in the absence and presence of TC is displayed in Fig. 6B. A dose-dependent increase in TiO_2 NPs accumulation was observed in the absence and presence of TC ($p > 0.05$). Nevertheless, there existed a statistical significance between internalization and accumulation for 5 mg/L TiO_2 NPs with and without TC ($p < 0.05$).

3.5.5. Principal component analysis

To figure out the correlations between different parameters tested during waterborne exposure, PCA was performed. Figure 7B shows the components PC1 and PC2 collectively interpreting 99.99% of data variability. For the highest concentration of pristine TiO_2 NPs (5 mg/L), parameters such as mortality, ROS, and lipid peroxidation lie in the same quadrant suggesting their correlation. Whereas no correlation in parameters was observed for the lower concentrations (0.05, and 0.5 mg/L) of pristine TiO_2 NPs and 0.5 mg/L TC. The addition of 0.5

mg/L of TC to 0.05, and 0.5 mg/L of TiO_2 NPs, on the other hand, resulted in a correlation between uptake and accumulation of Ti in *A. salina*.

3.6. Impact of foodborne exposure of TiO_2 NPs and TC to *A. salina*

The mortality experienced in *A. salina* fed with contaminated *Chlorella* sp. was very low compared to that in case of waterborne exposure. Almost all the groups experienced a mortality of less than 10 % ($p > 0.05$). Besides mortality, the ingestion rate in *A. salina* was measured (Fig. S6). The ingestion rate of *A. salina* decreased with the increasing concentration of TiO_2 NPs with and without TC. However, the ingestion rate was lower in the presence of TC.

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The internalization of TiO_2 NPs in the absence and presence of TC from contaminated algae is displayed in Fig. 6C. Internalization of TiO_2 NPs into *A. salina* increased with increasing doses of TiO_2 NPs and varied significantly with control at 5 mg/L TiO_2 NPs ($p < 0.05$). Likewise, in the presence of TC, the internalization of TiO_2 NPs was also dose-dependent and was significantly different from the control for 0.5 mg/L TC with 5 mg/L TiO_2 NPs ($p < 0.05$).

The accumulation of TiO_2 NPs in the absence and presence of TC from contaminated algae is displayed in Fig. 6D. Accumulation of TiO_2 NPs (with and without TC) in *A. salina* was independent of their concentration and was found to be not significantly different from the control ($p > 0.05$). Nevertheless, there existed a statistical significance between internalization and accumulation from contaminated *Chlorella* sp. for 5 mg/L TiO_2 NPs with and without TC ($p < 0.05$).

The BMF of TiO_2 NPs was estimated and presented in Table 1. BMF of TiO_2 NPs increased with increasing concentrations of TiO_2 NPs and did not vary significantly in the

absence and presence of TC ($p > 0.05$). Moreover, all the measured BMF values were less than one that indicating negligible magnification of TiO_2 NPs between *Chlorella* sp. and *A. salina*.

4. Discussion

4.1. Fate of TiO_2 NPs and TC in ASW

The changes in the effective diameter of pristine TiO_2 NPs demonstrated their rapid aggregation in the ASW. The high electrolyte content in ASW partly shields the surface charge of the nanoparticles, which compresses the electric double layer. Particles experience Brownian motion, which causes them to collide and form aggregates (Morelli et al., 2018). The optical images (Fig. S4 in Supplementary information) of the TiO_2 NPs confirm the formation of the homo-aggregates in the ASW medium. Such homo-aggregates form quite rapidly and remain suspended in the water column for a few minutes before beginning to settle (Hsiung et al., 2016). As the micron-sized homo-aggregates settle, their residence time and bioavailability in the top layer of the suspension would decrease (Fig. S2). However, the aggregation of TiO_2 NPs was not significantly affected by the presence of TC. This may be attributed to the rapid adsorption-desorption mechanism indicated as follows: At first, TC could rapidly adsorb on the surface of TiO_2 NPs, followed by the degradation of TC by $\bullet O_2^-$ radical. Finally, desorption of intermediate/degraded products of TC from the surface of TiO_2 NPs into the medium might have minimal impact on the size of TiO_2 NPs. The nanoparticles can also form hetero-aggregates with the microalgae as depicted in Fig. S5. The formation of such hetero-aggregates may further exacerbate the settling and deposition process, thus reducing the availability of the NPs (Thiagarajan et al., 2021).

HR-LCMS results demonstrated that TC degraded in the presence of TiO_2 NPs under visible light, resulting in the formation of intermediate compounds (Fig. S3B). Negligible degradation of TC in the presence of visible light could be attributed to the photolysis

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phenomenon (Jiao et al., 2008). On the other hand, the degradation of TC in the presence of TiO_2 NPs could be attributed to the photocatalytic effect of TiO_2 NPs. Besides, the TOC measurements revealed no change in the values between pristine TC at 0th h, and TC with TiO_2 NPs at 48th h, confirming negligible mineralization of TC. Similar to our findings, Wu et al. (2020) found no mineralization of TC by TiO_2 NPs under visible light condition. The mechanism of visible light photocatalytic degradation of TC over TiO_2 NPs may be proposed as follows: initially, TC may adsorb over TiO_2 NPs, and the excitation of surface-adsorbed TC by visible light may result in the transfer of electron from the lowest unoccupied molecular orbital of TC into the conduction band of TiO_2 NPs. Eventually, electrons in the conduction band of TiO_2 may transfer to an adsorbed O_2 to form an $\bullet O_2^-$ radical, which potentially might degrade TC (Wu et al., 2020). The toxicity of intermediate products of TC in *Chlorella* sp. and *A. salina* was lower than the toxicity induced by TC alone, showing that the intermediate products were less toxic than the parent compound. Similar to our results, Ravikumar et al. (2019) reported lower toxic effects of degraded products of TC after interaction with NiFe nanoparticles on bacterial and algal species.

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4.2. Toxic effects in *Chlorella* sp.

Pristine TiO_2 NPs induced dose-dependent acute toxicity in *Chlorella* sp. (Fig. 1A). Aggregates of TiO_2 NPs formed in ASW are prone to sorb on the surface of *Chlorella* sp., causing cell wall damage and increasing the likelihood of NPs passage into the cell (Ozkaleli and Erdem, 2018). The NPs associated with the algae was dose-dependent (Fig. 3). PCA also demonstrated that the toxicity and association of TiO_2 NPs with algae to be positively correlated (Fig. S7A). Besides the membrane damage, the sorption of TiO_2 NPs on the surface of *Chlorella* sp. (shading effect) can inhibit light and restrict nutrient availability for photosynthesis (Xia et al., 2015). On the other hand, the mechanism by which TC induces toxic effects in microalgae is not quite clear. Although microalgae are not target organisms, the

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presence of chloroplast ribosomes that are similar to the bacterial ribosomal RNA makes them a potential target for TC (Gray, 1992). Our results agree well with the findings from Roy et al. (2020), where lower doses of TC enhanced the toxicity of the TiO_2 NPs to freshwater microalgae *Scenedesmus obliquus*. A small increase in the association of TiO_2 NPs in the presence of TC (Fig. 3) can be explained by exacerbated damage to the cell wall of *Chlorella* sp.

The amount of ROS and lipid peroxidation induced in *Chlorella* sp. was dose-dependent upon treatment with pristine TiO_2 NPs (Fig. 1B and C). TiO_2 NPs are photo-active and aggregates of the NPs can come in contact with *Chlorella* sp. that generates ROS, damages the cell membrane, and enhances the membrane permeability as well TiO_2 NPs internalization (Lei et al., 2016; Xia et al., 2015). Besides, the localization of NPs in the chloroplasts and mitochondria could promote the formation of oxidative radicals (Aravantinou et al., 2017; Li et al., 2015). Our previous research (Thiagarajan et al., 2019c) found that chloroplasts and mitochondria were the major sites of ROS production in *Chlorella* sp. ROS formation in chloroplasts can damage the photosynthetic apparatus that decreases the autofluorescence of chlorophyll a, as well as the maximum quantum yield of PSII, as observed in our study (Fig. 2A and B). TC, on the other hand, also increased the ROS and lipid peroxidation in *Chlorella* sp. (Fig. 1B and C). This is consistent with the findings of Xu et al. (2019), wherein the ROS induced by 10 mg/L TC in freshwater *Chlorella vulgaris* was significantly higher than the control group. It was also reported that the low concentrations of TC induced cell membrane damage that decreased the membrane integrity and increased the permeability to TC. It is known that TC can inhibit protein synthesis in the chloroplast that decrease the quantum yield of PSII, increase oxidative stress, and cause detrimental effects to chlorophyll synthesis (Seoane et al., 2014). In joint experiments, TiO_2 NPs and TC may have additively increased

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the levels of ROS and lipid peroxidation, thereby decreasing the autofluorescence of chlorophyll a and the maximum quantum yield of PSII.

4.3. Waterborne exposure effects to *A. salina*

Pristine TiO_2 NPs induced dose-dependent acute toxicity in *A. salina*, which was in line with the previous findings of Thiagarajan et al. (2020). Accumulation of TiO_2 NPs in the gut is considered as one of the mechanisms by which TiO_2 NPs may induce mortality in *A. salina*. This is because *A. salina* can consume particles as large as 50 µm in size (Ates et al., 2013). As evident from the DLS results, the effective diameter of TiO_2 NPs falls within the range of 50 µm for *A. salina* to ingest the aggregated TiO_2 NPs. Thus, ingestion was the most likely route for TiO_2 NPs to enter causing toxicity in *A. salina*. Ingestion of TiO_2 NPs was confirmed by their uptake and accumulation as depicted in Fig. 6A and 6B. Here, uptake was more as compared to the accumulation that indicates the depuration of TiO_2 NPs from the body of *A. salina*. Since waterborne exposures are conducted without food supplements, the recognition of NPs as feed by *A. salina* can result in their increasing concentrations in the gut. Toxicity induced by TC in *A. salina* was significantly enhanced as compared to the control (Fig. 4A). In contrast to our findings, Metcalf et al. (2002) reported that TC was not found to be toxic up to 64 h (LC_{50} > 20 µg/mL) of interaction with *A. salina*. In general, TC binds reversibly to the 30S subunit of ribosomal RNA in eukaryotic organisms, where it interferes with the binding of aminoacyl tRNA to the "acceptor" site and inhibits protein translation in the host. Eukaryotic cells, unlike most bacteria, lack an uptake mechanism and hence the effects of TC is bacterial-specific (Sanchez et al., 2020). However, the mechanism of action of TC is specifically unavailable for *A. salina*. Overall, the toxicity could depend on the ingestion and bioaccumulation rate of TC in *A. salina*. Similar to the results obtained in the tests with the microalgae, the toxic effects of TiO_2 NPs in *A. salina* were additively increased by TC. This might be attributed to the ingestion of both TC and TiO_2 NPs that might have imparted their

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individual effects in *A. salina*. Once again, the uptake and accumulation data confirm the assimilation of TiO_2 NPs by *A. salina*.

Ingestion and accumulation of TiO_2 NPs and TC in the gut of *A. salina* could alter the gut microbiota, thereby jeopardizing their health. The gut microbiota aids in the regulation of multiple physiological activities (nutrient absorption and energy metabolism) and contributes to the overall well-being of the host (Evariste et al., 2019). Chen et al. (2018) studied the chronic and combined effects of TiO_2 NPs and bisphenol A on the dynamics of gut microbiota in zebrafish. Co-exposure to TiO_2 NPs and bisphenol A significantly altered the gut microbial composition by lowering the abundance of normal metabolic bacteria in the gut. With a lack of clear evidence on the combined effects of nanoparticles and pharmaceuticals on gut microbiota, more study is warranted in the future to elucidate their impact.

ROS induced by the pristine TiO_2 NPs in *A. salina* was concentration-dependent (Fig. 4B). The photocatalytic activity of TiO_2 NPs results in the generation of oxidative radicals such as H_2O_2, OH, O^{2-}, and $^1O_2$. Bhuvaneshwari et al. (2017) too reported the generation of ROS in *A. salina* treated with increasing doses of TiO_2 NPs (10-160 mg/L). As per Rekulapally et al. (2019), the gut accumulation of TiO_2 NPs might have resulted in mortality and ROS production. Excessive ROS production can disrupt the lipid membrane (Fig. 4C) and induce membrane damage, resulting in oxidative damage to protein and DNA, reducing cell defence mechanisms, and ultimately cell death. TC, on the other hand, also induced ROS and lipid peroxidation through waterborne exposure. There is no evidence in the literature to substantiate the generation of ROS and lipid peroxidation in *A. salina* after TC exposure. Our finding is in agreement with the previous reports wherein the exposure of TC to zebrafish embryos resulted in the superfluous generation of ROS and consequently triggered apoptosis (Zhang et al., 2015).

The activity of ChE was evaluated to assess the biochemically-mediated neurotoxicity in *A. salina* (Fig. 5). ChE activity induces both physiological and behavioural consequences in *A. salina*. Inhibition of ChE activity by pollutants results in excess levels of acetylcholine at nerve endings. However, in this study, the activity of ChE was not significantly different from the control upon exposure to TiO_2 NPs, and TC, which indicates no impairment in ChE activity. This was in line with the study reported by Cavion et al. (2020) wherein the interaction of *Artemia franciscana* with graphene oxide produced no significant difference in ChE activity. However, the upregulation of ChE activity at higher mixture concentration (5 mg/L TiO_2 NPs with 0.5 mg/L TC) could be related to greater accumulation that might have induced internal tissue damage, causing the levels of the biochemical enzyme to change (Yang et al., 2018).

4.4. Foodborne exposure effects to *A. salina*

Toxicity induced via foodborne exposure of TiO_2 NPs, TC, and their combination was lower compared to the waterborne exposure of the contaminants. This could be attributed to the preferential ingestion pattern of *A. salina* towards the uncontaminated *Chlorella* sp. From the ingestion studies (Fig S6), the ingestion of contaminated *Chlorella* sp. by *A. salina* was in the following order: Control > 0.05 > 0.5 > 5 mg/L both in the absence and presence of TC. This indicates that *A. salina* consumed more algal cells when the algae were less contaminated. As crustaceans prefer morphologically intact and non-clumped algal cells as prey, an increase in the concentrations of the contaminants increases the number of contaminated algae in the suspension and lowers the ingestion rate (Dalai et al., 2014). Thus, *A. salina* is forced to consume a limited amount of food, which can lead to starvation and chronic repercussions (Schiavo et al., 2018).

Such a preferential ingestion approach of *A. salina* resulted in lower uptake and accumulation of NPs than that in case of waterborne exposure (Fig. 6C and D). Our results are

683 in accordance with Bhuvaneshwari et al. (2018), wherein the exposure of TiO_2 NPs through
684 aqueous route to *A. salina* produced higher mortality, uptake, and accumulation compared to
685 the foodborne exposure. Compared to the uptake of TiO_2 NPs, the concentration of TiO_2 NPs
686 accumulated in *A. salina* after depuration for 24 h indicates the elimination of TiO_2 NPs. Yang
687 et al. (2018) reported a rapid elimination of unassimilated chemicals from the gut of *A. salina*.
688 Similarly in our study, the quick removal of contaminated *Chlorella* sp. from the body of *A.*
689 *salina* before digestion might have reduced the concentration of absorbed and accumulated
690 TiO_2 NPs.

691 The BMF of TiO_2 NPs was less than one for all pristine and mixture concentrations,
692 indicating negligible transfer/magnification between *Chlorella* sp. and *A. salina*. Zhenyu Wang
693 et al. (2017) investigated the effect of feeding 1 mg/L TiO_2 NPs contaminated *Nitzschia*
694 *closterium* (microalgae) to *Chlamys farreri* (scallops) and found BMF values greater than 1 in
695 multiple tissues (gill, digestive gland, and mantle), indicating the occurrence of
696 biomagnification. Additionally, the BMF of TiO_2 NPs following the addition of TC did not
697 change significantly from the BMF obtained in the absence of TC, demonstrating that TC does
698 not affect the trophic transfer of TiO_2 NPs.

699 **5. Conclusions**

700 The current study investigates the two-level trophic toxicity of TiO_2 NPs and TC in
701 *Chlorella* sp. and *A. salina* through a range of bio-assays. Toxicity induced in *Chlorella* sp. did
702 not differ significantly between pristine TiO_2 NPs and mixture containing TiO_2 NPs and TC,
703 showing that the two pollutants did not interact significantly. Likewise, the exposure of TC
704 through waterborne route additively increased the toxic effects of TiO_2 NPs in *A. salina*. Both
705 adsorption and degradation played a major role in the removal of TC from the suspension that
706 resulted in additive toxic effects in both *Chlorella* sp. and *A. salina*. However, during the

foodborne exposure, the uptake and accumulation of TiO_2 NPS were lower, indicating the lower toxic effects of contaminants than through the waterborne exposure. Notably, TiO_2 NPs were not biomagnified between *Chlorella* sp. and *A. salina* regardless of the presence of TC, indicating possibly lower environmental threat of these contaminants in this two-level food chain.

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Figure Captions:

Fig. 1: Percentage of (A) growth inhibited, (B) oxidative radicals generated, and (C) lipid peroxidation induced in *Chlorella* sp. treated with pristine TiO_2 NPs (0.05, 0.5, and 5 mg/L) or TC (0.5 mg/L) or their mixture. (Note: The statistical variance between experimental groups and control is represented as '*', whereas the symbol 'γ' ($p<0.001$) indicates that the toxicity difference in the absence and presence of TC is significantly different).

Fig. 2: The effect of pristine TiO_2 NPs (0.05, 0.5, and 5 mg/L) or TC (0.5 mg/L) or their mixture on (A) maximum quantum yield of PSII, and (B) Chl-a fluorescence of *Chlorella* sp. (Note: The statistical variance between experimental groups and control is represented as '*').

Fig. 3: Concentration of Ti associated with *Chlorella* sp. treated with 0.05, 0.5, and 5 mg/L of TiO_2 NPs in the absence and presence of 0.5 mg/L TC. (Note: The statistical variance between experimental groups and control is indicated as '*').

Fig. 4: Percentage of (A) Mortality, (B) oxidative radicals generated, and (C) lipid peroxidation induced in *A. salina* treated with pristine TiO_2 NPs (0.05, 0.5, and 5 mg/L) or TC (0.5 mg/L) or their mixture. (Note: The statistical variance between experimental groups and control is indicated as '*').

Fig. 5: ChE activity in *A. salina* treated with pristine TiO_2 NPs (0.05, 0.5, and 5 mg/L) or TC (0.5 mg/L) or their mixture. (Note: The statistical variance between experimental groups and control is indicated as '*').

Fig. 6: Concentration of Ti (A) taken up by *A. salina* via waterborne exposure, (B) accumulated in *A. salina* via waterborne exposure, (C) taken up by *A. salina* via foodborne exposure, (D) accumulated in *A. salina* via foodborne exposure. (Note: The statistical variance between experimental groups and control is indicated as '*').

Table caption:

Table 1: Biomagnification factor of TiO_2 NPs in the absence and presence of TC.