Assessing the effect of different natural dissolved organic matters on the cytotoxicity of titanium dioxide nanoparticles with bacteria

Xiaojia He^{1,*#}, Donee' McAlliser¹, Winfred G. Aker², Huey-min Hwang¹

1. Department of Biology, Jackson State University, Jackson, MS, 39217, USA

2. Environmental Science Ph.D. Program, Jackson State University, Jackson, MS, 39217, USA

Abstract

Titanium dioxide nanoparticles (TiO₂ NPs) are among the most widely manufactured nanomaterials on a global scale. However, prudent and vigilant surveillance, incumbent upon the scientific community with the advent of new technologies, has revealed potentially undesirable effects of TiO₂ NPs on biological systems and the natural environment during their application and discharge. Such effects are likely best evaluated by first assessing the fate of the TiO₂ NPs in natural environments. In this study, the effects of terrestrial humic acid (HA) and tannic acid (TA), two major members of the collective: dissolved organic matter (DOM), on the cytotoxicity of TiO₂ NPs to *E. coli* were investigated in the presence and absence of natural sunlight. Qualitative (TEM) and quantitative (LC₅₀) analyses were employed in this study. In addition, the production of reactive oxygen species (ROS) in the form of •OH was further assessed—as HA or TA increased the production of ROS decreased. The inhibition of bacterial viability in the light treatment groups, with respective treatment organics at concentrations of 10 ppm, was less in TA than in terrestrial HA. SAS was used to analyze the treatment effect of individual factors of light irradiation, DOM, and concentration of TiO₂NPs.

Keywords: TiO₂ nanoparticles; Humic acid; Tannic acid; Cytotoxicity; ROS

* Corresponding author. E-mail: xiaojia.he25@uga.edu

Present Affiliation: Department of Marine Sciences, the University of Georgia, Athens, GA, 30602, USA.

Introduction

Because of the diverse application of engineered nanoparticles in various personal and commercial products, they are produced in large quantities to support the growing global nanotechnology market. The rapid synthesis and use of these engineered nanoparticles in recent years have drawn the attention of the scientific community to their toxicology, health impacts, and their fate in the environment. Titanium dioxide nanoparticles (TiO₂ NPs) are among the most broadly used nanomaterials with applicability in nanomedicine (He et al., 2015a) photocatalysts (He et al., 2016) coating/surfaces, and additives in paints, rubbers and ceramics, (Ma et al., 2009) etc. The fates of nanoparticles are affected by environmental factors such as light, oxidants, and microorganisms, which could exert their influence via chemical or biological modifications (He et al., 2014; He and Hwang, 2014).

Dissolved organic matter (DOM) is composed of a heterogeneous mixture of organic compounds (Mostofa et al., 2013). Humic substances such as fulvic acid and humic acid (HA) are the dominant DOM fractions in freshwater and coastal seawater. Tannic acid (TA) is more dominant in swamps, determined by the brownish-tan coloration (Berner and Berner, 1987). As aquatic systems serve as the primary sink for the released nanomaterials (Gao et al., 2009), it is therefore necessary to understand the fate and ecological effects of these nanomaterials in natural aquatic environments (Dasari and Hwang, 2013; He et al., 2014). It has been reported that both HA (Wiszniewski et al., 2002) and TA (Kruthika et al., 2014) could be degraded by TiO₂ NPs. The implication here then, is that the TiO₂ NPs are themselves affected in their inherent properties by the encounter with these environmental constituents. The interaction between TiO₂ NPs and DOM is critical in determining the toxicity of TiO₂ NPs. However, the toxicological profile of TiO₂ NPs is not completely understood and several concerns have emerged on the potentially undesirable effects of TiO₂ NP properties, in regard to the harmful interactions with biological systems and the environment at the nano-bio-eco interface (He et al., 2014; He et al., 2015b). Hence, this study seeks to elucidate this profile for application in follow-on assessments of environmental impact.

Herein, the effects of selected DOMs on the photoinduced toxicity of TiO₂ NPs to *Escherichia coli* (*E. coli*) *in vitro* are examined. To the best of our knowledge, it is the first report on the role of TA on the phototoxicity of TiO₂ NPs.

1. Materials and methods

1.1 Chemicals

The nanoparticles used in this study were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and Sky Spring Nanomaterials Inc. (Katy, TX, USA) with chemical purity of 98% or higher. HA and TA were purchased from Sigma Aldrich (Sigma-Aldrich Co., USA). HA or TA solutions were prepared in different concentrations with Millipore water. Stock solutions (2000 ppm) were sonicated (FS30, Fisher Scientific, USA) for 30 minutes prior to adding them to make the working solution. TiO₂ NPs were prepared to reach the final concentrations of 0, 1, 2.5, 5 ppm and 250, 500, 750 ppm under light and dark conditions, respectively.

1.2 Characterization

To assess the effect of TA and HA on physicochemical properties of TiO₂NPs, the hydrodynamic size and zeta potential values were obtained via dynamic light scattering (DLS) and phase analysis light scattering (PALS), respectively, using a ZetaSizer Nano ZS (Malvern Instrument, USA) at 25°C and an applied voltage of 120 V. A viscosity of 0.8872 centipoise (cP), a dielectric constant of 78.5, and Henry function of 1.5 were the set parameters. Primary nanoparticle size was determined using a Jeol, JEM 1011 electron microscope working at 100 kV (Joel USA, Inc., MA) equipped with a Gatan camera model 785. Absorbance spectra of the photocatalysts were measured using a UV-vis spectrophotometer (UV-2600, Shimadzu, Japan).

1.3 Preparation of *E. coli*

E. coli was cultured in Luria-Bertani (LB) Broth and placed in a shaker for 18 hours at 37 °C. Following incubation, the culture was washed three times with sterilized physiological saline (0.8%, w/V) in a centrifuge (Eppendorf Centrifuge 5810 R, Eppendorf AG, Germany) at 4°C and 1735×g for 10 minutes. The bacterial suspensions were diluted (10⁶ x dilution factor) and exposed to the TiO₂ in quartz test tubes (ACE Glass Inc., USA).

1.4 Qualitative assay: TEM analysis

In this section, 10 ppm HA or TA (final concentration) was added into the quartz test tube at a final volume of 2 mL. 100 µL diluted bacteria suspension and 75 ppm (final concentration) TiO₂ NPs were added subsequently into the quartz test tubes. Afterwards, the quartz tubes were incubated outdoors in a tub under natural sunlight at noontime during sunny days in Jackson, MS, USA (32° 19' N; 90° 5' W). The incubation temperature for all the tubes was maintained by immersion in a tub of water (~25 ° C). All samples were incubated for a period of 30 min.

Darkness incubation was achieved by wrapping the tubes with aluminum foil at the same temperature maintained by water bath. For each sample, a 20 μ L aliquot was placed on the TEM grid (Ted Pella, USA) and dried at room temperature. TEM measurements were performed on a Jeol, Jem 1011 electron microscope (JEOL Limited, Japan) operating at 100 kV and equipped with a Gatan camera (Gatan Incorporated, USA).

1.5 Quantitative assay: LC₅₀ analysis

HA and TA solutions were prepared to reach the following final concentrations simulating the ambient levels: 10 and 40 ppm. Quartz tubes (13 mL capacity, ACE Glass Inc., USA) were then filled with various amounts of distilled water, bacteria (*E. coli*), DOM (terrestrial HA or TA), and TiO₂ NPs and mixed well. Same as the protocol in qualitative analysis, quartz test tubes with *E. coli*, DOM, and TiO₂ were then exposed to sunlight under the same condition. After exposure of 30 min, aliquots of 100 μ L of all three replicates were used to determine the LC₅₀, at 37°C for 24 hours. LC₅₀ values were calculated using the Probit Method (Hoheisel, Diamond, and Mount, 2012) using GraphPad Prism of GraphPad Software, Inc.

1.6 Determination of reactive oxygen species

The production of hydroxyl radicals ($\bullet$ OH), the reported dominant ROS induced by TiO₂ NPs under full spectrum sunlight, was determined by the fluorescence spectroscopy using terephthalic acid, described previously with slight modification (Ishibashi et al., 2000; He et al., 2015c; He et al., 2016). Terephthalic acid can be specifically hydroxylated by $\bullet$ OH and lead to the formation of fluorescent 2-hydroxyterephthalic acid. Briefly, a stock solution of 5×10^{-3} mol/L terephthalic acid with a concentration of 2×10^{-2} mol/L NaOH was first prepared and stored at 4°C. TiO₂ samples were placed in 5 mL of 5×10^{-4} mol/L terephthalic acid with a concentration of 2×10^3 mol/L NaOH and stirred for 1 hour in the dark. The solution was then exposed to sunlight for 30 minutes. The reaction solution was filtered through a 0.22 μ m PVDF filter (Sigma Aldrich, St. Louis, MO), prior to measurement. The increase of the fluorescence intensity was detected at 425 nm excited by 315 nm light with Horiba Scientific Fluoromax-4 spectrofluorometer (Horiba JobinYvon Inc., USA) equipped with NanoLED pulsed diode light source.

1.7 Statistical analysis

All results were expressed as mean $\pm$ standard deviation (SD). The SAS System for Windows, V 9.0 was used for statistical evaluations. Differences among treatment and control groups were tested by one-way analysis of variance (ANOVA), followed by pair-wise comparisons between

groups using Duncan's test. Differences at $p < 0.05$ were considered significant. The General Linear model in SAS was used to detect main effect and interactions among different parameters. Significant difference in treatments and the interactions among and between the experimental parameters were determined at $p < 0.05$.

2. Results and discussion

2.1 Characterization

Characterization of TiO₂ NPs was accomplished by using transmission electron microscopy (TEM), DLS, and UV–Vis spectroscopy, as shown in **Fig. 1**. The major absorbance region of TiO₂ NPs lies below 400 nm, which is in UV region (**Fig. 1A**). Zeta potential measured with PALS is shown in **Table 1**. A numerical number of zeta potential larger than ± 25 indicates good stabilization of NPs, due to the repulsive forces exceeding attraction forces and preventing aggregation upon ageing (Lu et al., 2008). Thus, the hydrodynamic size (**Fig. 1B, C**) and corresponding zeta potential indicate that in the presence of HA and TA, the particles were approaching the state of moderate stability. In addition, the addition of DOM did not change the primary size of TiO₂ (36.70 ± 2.83 nm).

The hydrodynamic size of nanoparticles can be important for how it behaves in aqueous environments. Size influences nanoparticle uptake, deposition, and clearance (Hwang et al., 2012; He and Hwang, 2014). The agglomeration behavior and surface charge variation of nanoparticle dispersions can have a dramatic effect on both the reactivity of nanomaterial's and their efficiency in contamination treatment (Suttiaponparnit et al., 2011).

2.1 Qualitative assay: TEM analysis

TEM images made it feasible to envision the nano-bio-eco relations between TiO₂, *E. coli*, and DOM. Bacterial samples were exposed to natural sunlight or darkness. The exposure to NPs led to cell disintegration, biofilm disruption, and cell death. To some bacteria, the presence of the TiO₂ NPs resulted in the disruption of bacterial capsule (Chorianopoulos et al., 2011). The added nanoparticles were toxic to the bacteria by virtue of being in contact with the bacterial cells, coupled with internalization or attachment.

As shown in **Fig. 2A, C**, TiO₂ NPs agglomerate in aquatic environments under dark exposure, even in the presence of DOM. This seems inconsistent with our measured zeta potential in **Table 1**, and hydrodynamic size in **Fig. 1**. This behavior might be caused by the

interaction with *E. coli*. The presence of biological organisms could substantially alter the environment surrounding the TiO₂ NPs. In the presence of sunlight, the TEM image shows reduced agglomeration of TiO₂ NPs (**Fig. 2B, D**). This fact suggests that sunlight could stabilize TiO₂ NPs associated with DOM in biological systems. However, it is also observed that the difference between the light and dark exposure of HA-TiO₂ is much greater than TA-TiO₂. It is also noted that DOM-TiO₂ NPs tend to penetrate cell wall in the presence of sunlight (**Fig. 2 E-G**). As shown in **Fig. S1**, *E. coli* undergoing lysing was observed in some cases, suggesting substantial impact of sunlight on DOM-TiO₂.

2.3 Quantitative assay: LC₅₀ analysis

Data analysis indicates that light was fundamental in determining the toxicity of TiO₂ NPs in the presence of DOM. The values obtained from plate counting (**Fig. 3**) were used to calculate the LC₅₀ concentrations of TiO₂ NPs in the presence of DOM in **Table 2**. HA at a concentration of 10 ppm had a lower LC₅₀ value than TA in the presence of light, whereas at a concentration of 40 ppm, TA had a lower LC₅₀ value than HA in the presence of light, suggesting higher photo-toxicity. According to our data in characterization, TiO₂ NPs are stabilized by introducing DOM. Stabilization and reduced hydrodynamic size of TiO₂ NPs could largely increase the bioavailability of TiO₂ NPs. In this matter, HA tends to reduce the aggregation status of TiO₂ at a greater extent, which is supported by the zeta potential and hydrodynamic size measurement. This is also in agreement with our observation in the section of qualitative analysis. Such difference in aggregation and subsequent bioavailability may ultimately contribute to different toxicity.

This is also further assessed with TEM analysis in the presence or absence of sunlight in **Fig. 2**, which agrees with our data in **Table 2**. The stabilization of TiO₂ NPs-DOM complex by sunlight irradiation leads to higher toxicity. However, the data also clearly show there is light induced inhibition on the growth of *E. coli*. It was suggested that sunlight radiation could cause rapid decline of *E. coli* and other microorganisms in aquatic habitats (Fujioka et al., 1981).

2.4 SAS analysis: Influence of DOM

In the present study, we compared the levels of cytotoxicity of TiO₂ NPs in the presence of HA and TA. The *p* values of all treatment types and interactions are listed in **Table 3**. Briefly, the viability of *E. coli* was significantly affected by the individual treatment with light irradiation

(LI), type of DOM (HA or TA), and TiO₂ NPs. Among them, the most noteworthy individual factor is the DOM (HA or TA). The SAS analysis of our data confirms that the significant influence on bacterial viability could be due to the type of DOM (either HA or TA) and sunlight. The extent of light inhibition on bacterial viability was higher in the treatments involving higher concentrations of TiO₂ NPs, which corresponds to our LC₅₀ and TEM analyses.

The summarized SAS table (**Table 4**) indicates that under light irradiation, exposure to the TiO₂ NPs inhibited viability more than exposure under the dark condition. Hence it is confirmed that light is an important environmental factor affecting the toxicity of TiO₂ NPs. HA and TA at ambient concentrations (10-40 ppm) reduced TiO₂ NP toxicity; however, the HA and TA treatment groups showed lower viability count under light condition compared to that under the dark condition (**Fig. 3** and **Table 5**), suggesting that the toxicity of this species of NPs by light inhibition did occur in this study.

Significant interactions among several variables in the light irradiation groups may be different from those that occurred under dark conditions. A valuable function of the SAS results is that it can reveal the overall trends with respect to the specific parameters. Although statistical analyses can mathematically indicate interaction between factorially arranged biological and abiotic variables in an ecosystem experiment, interpretation of those interactions in an ecological sense is not straightforward.

2.5 ROS formation

Absorption of light by DOM can initiate various photochemical processes and result in the production of peroxy radicals and hydroxyl radicals, which can damage cellular materials (Balarezo et al., 2002). As shown in **Table 2** and **Fig. 3**, it is apparent that sunlight exposure greatly increases the toxicity of TiO₂-DOM mixture, particularly true for HA. We further investigated the possible mechanism of different DOM with respect to their photo-induced toxicity. Here we use formation of ROS in terms of [•]OH to assess their performance upon exposure to sunlight.

Firstly, we found that the production of [•]OH by pristine HA and TA was negligible in this study (**Fig. 4**). As shown in **Fig. 4**, both HA and TA apparently have a much higher formation of ROS at 10 ppm with relatively high concentration range of TiO₂ (250-750 ppm). There is no significant production of ROS at lower range of concentration (1, 2.5, 5 ppm, $P > 0.05$ compare to DOM alone).

In addition, data suggests that HA-TiO₂ NPs generated much more •OH than TA-TiO₂ NPs at the same concentration. However, our LC₅₀ value in **Table 2** indicates that TA could be more toxic than HA at high concentration (40 ppm). This could be caused by the fact that TA per se is toxic at such high concentration (40 ppm). This could be validated by the fact that lower cell counts (CFU) were observed for TA without TiO₂ at higher concentration ($P < 0.05$). But this is not observed for HA. It is known that a number of polyphenols of TA at high concentration can bind to DNA and this interaction may cause DNA degradation (Labieniec et al., 2003).

3. Conclusions

In conclusion, we found that TiO₂ NPs appear to be phototoxic, regardless of the presence of DOM. The phototoxicity of HA-TiO₂ NPs is higher than TA-TiO₂ NPs at lower concentration (10 ppm), but lower at higher concentration (40 ppm). ROS production in terms of •OH also suggests a similar trend. This could result from the toxic profile of TA at high concentration. SAS analysis indicates that sunlight and the type of DOM could be crucial in determining the toxicity of TiO₂ NPs. It is noteworthy that sunlight could alter the interaction of TiO₂ NPs at the nano-bio-eco interface. In addition, molar concentration should be used to cross-compare the nanotoxicity in the presence of different DOM. The molar concentration of the added TA ranged from 5.9×10^{-8} to 2.4×10^{-7} mol/L. However, as a mixture of various polymers, it is impractical to compute the molar concentration of HA in this study. Because the chemical characteristics of HA and TA differ with origin, the realistic hazards of TiO₂NPs release could also vary with the type of DOM substance present in a particular environment. Further work has to be done in order to comprehensively investigate the interaction of DOM with TiO₂ NPs and their impact on the toxicity of TiO₂ NPs towards living organisms at the molecular/genetic level.

Acknowledgements

This study was supported by the National Science Foundation-Centers of Research Excellence in Science and Technology (NSF-CREST) (No. HRD-0833178) and the National Institutes of Health (NIH) Research Initiative for Scientific Enhancement (RISE) Program program (No. 5R25GM067122).

References

- Balarezo, A., Jones, V., Yu, H., Hwang, H., 2002. Influence of humic acid on 1-aminopyrene ecotoxicity during solar photolysis process. *Int. J. Mol. Sci.* 3, 1133-1144.
- Berner, E., Berner, R., 2012. *Global water cycle: Geochemistry and environment* (2nd ed.). Princeton University Press, Princeton, New Jersey.
- Chorianopoulos, N.G., Tsoukleris, D.S., Panagou, E.Z., Falaras, P., Nychas, G-J., 2011. Use of titanium dioxide (TiO₂) photocatalysts as alternative means for *Listeria monocytogenes* biofilm disinfection in food processing. *Food Micro.* 28(1), 164-170.
- Dasari, T.P., Hwang, H-M., 2013. Effect of humic acids and sunlight on the cytotoxicity of engineered zinc oxide and titanium dioxide nanoparticles to a river bacterial assemblage. *J. Environ. Sci.* 25(9), 1925–1935.
- Fujioka, R.S., Hashimoto, H.H., Siwak, E.B., Young, R.H., 1981. Effect of sunlight on survival of indicator bacteria in seawater. *Appl. Environ. Microbiol.* 41, 690–696.
- Gao, J., Youn, S., Hovsepyan, A., Llaneza, V.L., Wang, Y., Bitton, G., et al., 2009. Dispersion and toxicity of selected manufactured nanomaterials in natural river water samples: effects of water chemical composition. *Environ. Sci. Technol.* 43(9), 3322-3328.
- He, X., Hwang, H-M., 2014. Engineered TiO₂ nanoparticles: Their fate and effects in natural aquatic environments. In: *Titanium Dioxide: Chemical Properties, Applications and Environmental Effects*, J. Brown (Ed.), Nova Science Publishers, New York (2014), pp. 1–20.
- He, X., Aker, W.G., Leszczynski, J., Hwang, H-M., 2014. Using a holistic approach to assess the impact of engineered nanomaterials inducing toxicity in aquatic systems. *J. Food Drug Anal.* 22(1): 128-46.
- He, X., Aker, W.G., Huang, M-J., Watts, J.D., Hwang, H-M., 2015a. Metal oxide nanomaterials in nanomedicine: Applications in photodynamic therapy and potential toxicity. *Curr. Top. Med. Chem.* 15(18), 1887-1900.
- He, X., Aker, W.G., Fu, P.P., Hwang, H-M., 2015b. Toxicity of engineered metal oxide nanomaterials mediated by nano–bio–eco–interactions: a review and perspective. *Environ. Sci: Nano* 2, 564-582.
- He, X., Sanders, S., Aker, W.G., Lin, Y., Douglas, J., Hwang, H-M., 2015c. Assessing the effects of surface-bound humic acid on the phototoxicity of anatase and rutile TiO₂ nanoparticles *in vitro*. *J. Environ. Sci.* In Press. DOI:10.1016/j.jes.2015.05.028

He, X., Aker, W.G., Pelaez, M., Lin, Y., Dionysiou, D.D., Hwang, H-M. 2016. Assessment of nitrogen–fluorine-codoped TiO₂ under visible light for degradation of BPA: Implication for field remediation. *J. Photochem. Photobiol. A Chem.* 314, 81–92.

Hwang, H-M., Ray, P.C., Yu, H., He, X., 2012. Toxicology of designer/engineered metallic nanoparticles. In: Luque, R. Varma, R.S. (Eds.), *Sustainable preparation of metal nanoparticles: methods and applications*. Royal Society of Chemistry, Cambridge, UK, pp. 190-212.

Ishibashi, K-i., Fujishima, A., Watanabe, T., Hashimoto, K., 2000. Quantum yields of active oxidative species formed on TiO₂ photocatalyst. *J. Photochem. Photobiol. A.* 134(1-2), 139-142.

Kruthika, N.L., Raju, G.B., Prabhakar, S., 2014. Degradation of tannic acid using TiO₂ nanotubes as electrocatalyst. *J. Nanosci.* 2014, 481023.

Labieniec, M., Gabryelak, T., Falcione, G., 2003. Antioxidant and pro-oxidant effects of tannins in digestive cells of the freshwater mussel *Uniotumidus*. *Mutat. Res.* 539(1-2), 19-28.

Lu, Q., Li, A., Guo, F., Sun, L., Zhao, L., 2008. Experimental study on the surface modification of Y₂O₃: Tm nanoparticles to enhance upconversion fluorescence and weaken aggregation. *Nanotechnology* 19, 145701.

Ma, H., Brennan, A., Diamond, S., 2009. Photocatalytic reactive oxygen species production and phototoxicity of titanium dioxide nanoparticles are dependent on the solar ultraviolet radiation spectrum. *Environ. Tox. Chem.* 31, 2099-2107.

Mostofa, K.M.G., Liu, C-q., Mottaleb, M.A., Wan, G., Ogawa, H., Vione, D., et al., 2013. In: *Dissolved organic matter in natural waters* (Mostofa, K.M.G., Yoshioka, T., Mottaleb, A., Vione, D., (Eds.)), *Photobiogeochemistry of Organic Matter*. Springer Berlin Heidelberg, pp. 1-137.

Suttioponparnit, K., Jiang, J., Sahu, M., Suvachittanont, S., Charinpanitkul, T., Biswas, P., 2011. Role of surface area, primary particle size, and crystal phase on titanium dioxide nanoparticle dispersion properties. *Nanoscale Res. Lett.* 6, 27-34.

Wiszniowski, J., Robert, D., Surmacz-Gorska, J., Miksch, K., Weber, J-V., 2002. Photocatalytic decomposition of humic acids on TiO₂: Part I: Discussion of adsorption and mechanism. *J. Photochem. Photobiol. A Chem.* 152, 267-273.

List of figures

Fig. 1: Physicochemical properties of TiO₂NPs with or without humic acid (HA) or tannic acid (TA). (A) UV-vis spectrum, (B) hydrodynamic size distribution, (C) average hydrodynamic size ($n = 3$) and primary size ($n = 53$), (D), (E) and (F) typical TEM micrograph for pristine TiO₂ NPs, in the presence of HA, and TA, respectively.

Fig. 2: TEM images of *E. coli* exposed to (A-B) HA (10 ppm) or (C-D) TA (10 ppm) with TiO₂ (75 ppm) in the absence (A, C) and presence (B, D) of sunlight. (E, G) are selected images of *E. coli* treated with HA (E) and (TA) in the presence of sunlight. (F, H) are the enlargement of red rectangular region in (E,G), respectively. (G) is the control without nanoparticle. Magnification: (A) 30,000x; (B) 50,000x; (C) 20,000x; (D) 30,000x; (E) 50,000x; (F) 100,000x; (G) 60,000x; (H) 150,000x; (I) 50,000x.

Fig. 3. Effect of DOM (10 and 40 ppm) on the toxicity of TiO₂ NPs to *E. coli* in the presence and absence of sunlight. A vertical bar above each column indicates the standard deviation ($n = 3$). Different letter indicates statistical difference exists.

Fig. 4. ROS production in terms of $\bullet\text{OH}$ in HA (A) and TA (B) treatment groups exposed to sunlight. Arrow lines indicate ROS formation in descending order aligning with graphic data.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

List of tables

Table 1. Zeta potential of TiO₂ NPs with humic acid (HA) or tannic acid (TA) at various concentrations

Groups	Zeta potential (mV)	pH
100 ppm TiO ₂	-(14.633±0.21)	7.62
100 ppm TiO ₂ + 10 ppm HA	-(38.77 ±1.17)	7.26
100 ppm TiO ₂ + 40 ppm HA	-(37.95 ±1.34)	6.98
100 ppm TiO ₂ + 10 ppm TA	-(26.9±0.46)	7.28
100 ppm TiO ₂ + 40 ppm TA	-(30.5±0.87)	7.41

Data are presented as mean ±S.D. ($n = 3$).

HA: HUMIC ACID; TA: tannic acid.

Table 2. LC₅₀ concentrations for the *E. coli* exposed to DOM with TiO₂ NPs under light and dark conditions for 30 min.

DOM	TiO ₂ NPs			
	LC ₅₀ (ppm, light)	R^2	LC ₅₀ (ppm, dark)	R^2
Humic Acid (10 ppm)	596.6	0.92	2041.3	0.84
Humic Acid (40 ppm)	974.5	0.95	1993.6	0.87
Tannic Acid (10 ppm)	677.7	0.81	691.0	0.81
Tannic Acid (40 ppm)	690.3	0.89	759.0	0.82

Table 3: Summarized SAS p values of main effects and interactions among all independent variables in the study with TiO₂NPs

Variable source	p Value	
	Terrestrial HA	TA
LI	<0.0001	<0.0001
DOM	<0.0001	<0.0001
TiO ₂	0.0006	0.0280
LI*DOM	<0.0001	<0.0001

LI* TiO ₂	0.0036	0.0002
DOM* TiO ₂	<0.0001	0.0064
LI*DOM* TiO ₂	<0.0001	<0.0001

LI: factor of light; DOM: factor of dissolved organic matter (DOM); LI*DOM: combinational factor of light and DOM; LI* TiO₂: combinational factor of light and TiO₂; DOM* TiO₂: combinational factor of DOM and TiO₂; LI*DOM* TiO₂: combinational factor of light, DOM and TiO₂.

Table 4: Summarized SAS *p* and F values of the study with TiO₂NPs in the presence of humic acid or tannic acid treated samples

Variable Source	DF	Type I sum of squares	Mean square	F Value	<i>p</i> Value
Humic acid treatment					
LI	1	51384.52083	51384.52083	238.60	<.0001
HA	2	5159.46833	2579.73417	11.98	<.0001
TiO ₂	6	5966.38668	994.39778	4.62	0.0006
LI-HA	2	5406.62324	2703.31162	12.55	<.0001
LI-TiO ₂	6	4730.06805	788.34467	3.66	0.0036
HA- TiO ₂	6	12615.25677	2102.54280	9.76	<.0001
LI-HA-TiO ₂	6	13137.69421	2189.61570	10.17	<.0001
Tannic acid treatment					
LI	1	381680.3495	381680.3495	791.26	<.0001
TA	2	23426.2754	11713.1377	24.28	<.0001
TiO ₂	6	7396.5073	1232.7512	2.56	0.0280
LI-TA	2	69547.4056	34773.7028	72.09	<.0001
LI-TiO ₂	6	15130.3843	2521.7307	5.23	0.0002
TA- TiO ₂	6	9674.7658	1612.4610	3.34	0.0064
LI-TA-TiO ₂	6	26424.6186	4404.1031	9.13	<.0001

LI: factor of light; HA: factor of humic acid; LI*HA: combinational factor of light and HA; LI* TiO₂: combinational factor of light and TiO₂; HA* TiO₂: combinational factor of HA and TiO₂; LI*HA* TiO₂: combinational factor of light, HA and TiO₂.

Table 5: LSMEANS Data of HA and TA.

Light Irradiation	HA (ppm)	LSMEAN (CFU/mL)	TA(ppm)	LSMEAN (CFU/mL)
Dark	10	203.1	10	322.8
Light	10	139.7	10	137.98
Dark	40	202	40	238.8
Light	40	167.2	40	168.57

Final CFU conversation factor is 2×10^7