



OPEN ACCESS

EDITED BY

Xuchun Qiu,
Jiangsu University, China

REVIEWED BY

Praxedes Natalia Muñoz,
Catholic University of the North, Chile
Fabrizio Frontalini,
University of Urbino Carlo Bo, Italy

*CORRESPONDENCE

Yurika Ujiié
✉ yujie@kochi-u.ac.jp

†PRESENT ADDRESS

Yuka Inagaki,
Graduate School of Integrated Arts and
Sciences, Kochi University, Kochi, Japan

RECEIVED 03 February 2024

ACCEPTED 17 June 2024

PUBLISHED 01 July 2024

CITATION

Inagaki Y, Ishitani Y, Tame A, Uematsu K,
Tomioka N, Ushikubo T and Ujiié Y (2024)
Foraminiferal detoxification breakdown
induced by fatal levels of TiO₂ nanoparticles.
Front. Mar. Sci. 11:1381247.
doi: 10.3389/fmars.2024.1381247

COPYRIGHT

© 2024 Inagaki, Ishitani, Tame, Uematsu,
Tomioka, Ushikubo and Ujiié. This is an open-
access article distributed under the terms of
the [Creative Commons Attribution License
\(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use, distribution or reproduction
in other forums is permitted, provided the
original author(s) and the copyright owner(s)
are credited and that the original publication
in this journal is cited, in accordance with
accepted academic practice. No use,
distribution or reproduction is permitted
which does not comply with these terms.

Foraminiferal detoxification breakdown induced by fatal levels of TiO₂ nanoparticles

Yuka Inagaki^{1†}, Yoshiyuki Ishitani², Akihiro Tame³,
Katsuyuki Uematsu⁴, Naotaka Tomioka⁵,
Takayuki Ushikubo⁵ and Yurika Ujiié^{1,6*}

¹Department of Biology, Kochi University, Kochi, Japan, ²Institute for Extra-Cutting-Edge Science and Technology Avant-garde Research (X-star), Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Yokosuka, Japan, ³School of Medical Sciences, University of Fukui, Fukui, Japan,

⁴Department of Marine and Earth Sciences, Marine Works Japan Ltd, Yokosuka, Japan, ⁵Kochi Institute for Core Sample Research, X-Star, JAMSTEC, Nankoku, Japan, ⁶Marine Core Research Institute, Kochi University, Nankoku, Japan

The increase discharge of titanium dioxide (TiO₂) nanoparticles, derived from engineered material waste, exerts a detrimental impact on both the marine ecosystem and public health. The cytotoxicity of TiO₂ nanoparticles on marine organisms should be imperatively understood to tackle the urgent concern for the well-being of marine life. Various concentrations of TiO₂ nanoparticles have proven to reach fatal levels in aquatic organisms, requiring a deeper exploration of cytotoxicity. Notably, certain benthic foraminifers, such as *Ammonia veneta*, have been identified as capable of incorporating TiO₂ nanoparticles into vesicles. However, these organisms exhibit a detoxification mechanism through exocytosis, as indicated by previous transcriptomic inferences. This presents the advantage of assessing the tolerance of foraminifers to TiO₂ nanoparticles as pollutants and investigating the long-term effects of cytotoxicity. In this study, we scrutinized the distribution of TiO₂ nanoparticles within cells and the growth rates of individuals in seawater media containing 1, 5, 10, and 50 ppm TiO₂ nanoparticles, comparing the results with a control group over a 5-week period, utilizing *A. veneta* stain. Transmission electron microscopy observations consistently revealed high concentrations of TiO₂ nanoparticles in vesicles, and their expulsion from cells was evident even with exposure to 5 ppm TiO₂ nanoparticles. Under the control and 1 ppm TiO₂ conditions, foraminifers increased their cell volume by adding a calcification chamber to their tests every 1 or 2 days. However, the 5-week culturing experiments demonstrated that foraminifers gradually ceased growing under 5 ppm TiO₂ nanoparticle exposure and exhibited no growth at > 10 ppm concentrations, despite an ample food supply. Consequently, these findings with *A. veneta* suggest that the foraminiferal detoxification system could be disrupted by concentrations exceeding 5 ppm of TiO₂ nanoparticles. The toxic effect of TiO₂ nanoparticles on meiofauna, such as benthic foraminifers, have been poorly understood, though these organisms play an important role in the marine ecosystem. Environmental accumulation of TiO₂

nanoparticles on the coast has already exceeded twenty times more than foraminiferal detoxification level. Future studies focusing on toxic mechanism of TiO₂ nanoparticles are crucial to prevent the breakdown of the marine ecosystem through accelerating discharge of TiO₂ nanoparticles into the ocean.

KEYWORDS

cytotoxicity, detoxification, foraminifera, TiO₂ NPs, fatal level

1 Introduction

Titanium dioxide nanoparticles (TiO₂ NPs) stand as the second most widely employed engineered nanomaterial globally, owing to their advantageous physicochemical properties, notably their photocatalytic capability for the oxidative decomposition of organic matter (Zheng and Nowack, 2021). In recent years, these nanoparticles have found application in sunscreen filters, replacing oxybenzone and octinoxate, to safeguard marine ecosystems (Ammendolia et al., 2022). The escalating demand for TiO₂ NPs has led to the discharge of manufacturing waste into the environment—specifically, soil and groundwater—eventually culminating in the ocean as the ultimate repository. NPs commonly form aggregates in water and sink down to the bottom (Boxall et al., 2007). Indeed, the accumulation of TiO₂ NPs on European coasts was projected to reach up to 123 ppm in 2020, and the current trend indicates an acceleration (Sun et al., 2016; Zheng and Nowack, 2021). This accumulation in the marine environment raises concerns due to its toxicity to aquatic organisms (Moore, 2006; Farré et al., 2009; Delay and Frimmel, 2012; Matranga and Corsi, 2012). Particularly, aggregated nanoparticles are accumulated in the sediments and interacted with sediment-dwelling organisms due to their low mobility (Farré et al., 2009). Experimental studies have previously demonstrated the toxicity of TiO₂ NPs to aquatic organisms, particularly microalgae and macroorganisms (Menard et al., 2011; Minetto et al., 2014; Luo et al., 2020). These studies have assessed the toxic effects through cell stress assays, revealing a Median Effect Concentration (EC50) of 35.3 ppm for algae and a Median Lethal Concentration (LC50) of 5.5–20000 ppm for *Daphnia magna*. Given the observed variability in lethal effects among organisms in exposure experiments with viability examinations, it becomes imperative to extend studies to benthos, encompassing not only macro or microfauna but also benthic foraminifers, to comprehensively understand the impact of TiO₂ NPs on marine ecosystems.

Benthic foraminifers, ubiquitous unicellular meiofauna on the seafloor, particularly in coastal areas (Culver and Buzas, 1999), serve as biomonitoring targets to assess the effects of NPs on marine environments (Ciacci et al., 2019). Foraminifers construct a test using calcium carbonate, sediment particles, or an organic membrane (Goldstein, 1999), with the test growing larger by

adding chambers. Recent studies utilizing single-cell transcriptome analysis and cellular observations from a time-course experiment with the benthic foraminifers *Ammonia* species exposed to 1 ppm TiO₂ NPs for 24 hours have elucidated the cytotoxic mechanism of TiO₂ NPs (Ciacci et al., 2019; Ishitani et al., 2023). Transcriptome analysis with *Ammonia veneta* successfully unveiled the metabolic pathway associated with TiO₂ NPs toxicity and its time-series changes (Ishitani et al., 2023). Foraminifers demonstrated phagocytosis of TiO₂ NPs into their cells, manifesting stress indicators such as the production of reactive oxygen species (ROS) and peroxidized lipids. Intriguingly, they exhibited detoxification, wherein aggregated TiO₂ NPs were encapsulated in ceramide and expelled as waste within 24 hours. This unique detoxification system enables foraminifers to survive in polluted environments, though the long-term cytotoxicity remains unknown. Furthermore, the limitation of TiO₂ NPs concentration that regulates foraminiferal growth or lethality has been never examined. In the other experiments with unicellular diatom, their EC50 and LC50 are ~10 and ~70 ppm of TiO₂ NPs, which are ~20–30 nm in diameter (Hartmann et al., 2010; Clément et al., 2013). Understanding such limitation becomes crucial in light of the rapid accumulation of TiO₂ NPs in marine environments in recent times.

In this study, we investigated the threshold limits of TiO₂ NPs concentrations for the survival of the benthic foraminifer *A. veneta*. The foraminifer was cultured for five weeks with exposures to 1, 5, 10, and 50 ppm TiO₂ NPs, and the potential for long-term survival was assessed based on individual growth rates, evaluated through the increased number of chambers, and the distribution of intracellular TiO₂ NPs. Our investigations suggest that foraminiferal growth is gradually inhibited in rising of TiO₂ NPs concentration most likely due to the breakdown of detoxification.

2 Materials and methods

2.1 TiO₂ NPs – source, characterization, and preparation

AEROXIDE®TiO₂, a mixture of rutile and anatase crystals with a nominal diameter of 25 nm (Evonik, Germany), was utilized in the

present study. The stock suspension (1000 mg/L) of TiO₂ NPs was prepared using AEROXIDE®TiO₂ powder in Milli-Q water and sonicated using an NR-50M ultrasonic homogenizer (Microtec, Japan) at 50 W for 60 min with 2 s on/off cycles placed in ice to prevent aggregation. Stock suspensions were shielded from light and stored at -20°C. For each experiment, the thawed stock suspension was similarly sonicated for approximately 60 min to disperse the TiO₂ particles, which tend to clump due to their cohesive nature, bringing them to a particle size of 25 nm. The resulting dispersion was diluted with artificial sea water (ASW), which was filtered through a 0.2µm mesh membrane filter (ADVANTEC®, Japan) of 35ppt Coral Pro Salt (Red Sea, USA) in Milli-Q water, according to the experimental conditions.

2.2 Culture experiments of foraminiferal strain *Ammonia veneta*

The study employed specimens derived from the culture strain *A. veneta* (Ishitani et al., 2023), which has been maintained in ASW at 23°C under a 12:12 hour light/dark cycle with weekly feedings of fresh *Dunaliella salina* (NIES-2257). This species forms a calcium carbonate test, which is sequentially increased by the maturity phase prior to reproduction. Adult (ca. 10 chambers) specimens of *A. veneta* showing reticulopodia extension and activity were randomly selected from subcultures, and three individuals were transferred to each petri dish (Aznor Petri Dish φ55 × 17 mm Azwan, Japan) containing 10 mL of either TiO₂ NPs seawater medium or ASW (control). For each medium condition, three replicated petri dishes were prepared and incubated at 23°C under a 12:12 h light/dark cycle for five weeks. All cultures per experiment were run in parallel.

Four different experimental conditions were set for TiO₂ NPs concentrations, as well as the frequency of medium changes, feeding frequency, and amount of food (Table 1). In both culture experiments 1 and 2, TiO₂ NPs seawater media were set up at 1, 10, and 50 ppm, in addition to the control. The frequency of medium changes differed between culture experiments 1 and 2: once a week in Experiment 1 and twice a week in Experiment 2. At each time, 80 µL of food was added to each petri dish. In culture experiment 3, TiO₂ NPs seawater media were set up at 1, 5, and 10 ppm, in addition to the control, with weekly medium changes and with feeding 40 µL of food and increased feeding to a total of 80 µL per week through addition of 20 µL food every few days. Culture experiment 4 involved TiO₂ NPs seawater media at 5 and 10 ppm, in addition to the control, with weekly medium changes with 80 µL of food and increased feeding to a total of

160 µL per week through addition of 40 µL food every few days. In experiments 2 and 4, the number of feedings and the amount of food were increased to keep double the amount of fresh *D. salina* in the petri dishes per week (Table 1).

At the beginning of culture experiments, we photographed each individual using an inverted research microscope (IX73 Olympus, Japan) with a microscope digital camera (DS-Ri2 Nikon Japan) and assigned an individual identification number. We photographed each of all studied individual weekly to check for signs of life or death, regarding the observations: extension and movement of reticulopodia and the movement of the individual to escape from light emission of the inverted microscope for a few minutes. Specimens, which lacked these three observation points, were categorized as dead. Individual growth was evaluated by the number of chamber increases from the previous week.

We firstly tested normality and variances for total numbers of increased chambers for five weeks at each culture experimental condition with Kolmogorov-Smirnov and F test in R v.4.0.2 (R Core Team, 2021), respectively. According to the normality and variances, we conducted the Student-t and Man-Whitney-U tests in R v.4.0.2. The *p* values of multiple comparisons were adjusted with the Bonferroni correction.

2.3 Culture experiment of *Dunaliella salina*

We independently conducted a culture experiment of *D. salina*, a food source for foraminifers, to examine cell divisions in TiO₂ NPs seawater medium. We prepared seven replicates for each of the 10 mL culture media with 1, 5, and 10 ppm TiO₂ NPs and ASW (control) in petri dishes and added 80 µL of *D. salina* to each of the 28 dishes. During the 6-day culture experiments under the same conditions as foraminiferal experiments, we examined the cell density of *D. salina* in each of the four different media daily. All 10 mL medium was transferred into a 15 mL centrifuge tube, and the petri dish was rinsed with 3 mL ASW to recover all remaining cells. After centrifugation at 3,300 rpm for 10 min at 23°C using a micro high-speed centrifuge (CF 16RX II Hitachi, Japan), the pellet of *D. salina* and TiO₂ NPs was suspended with 30 µL of ASW and transferred to a 1.5 ml tube. We immediately added 0.5% xanthan gum solution to a final concentration of 0.01% to inhibit the motility of *D. salina*. Three replicates were made, each of which was mounted with 10 µL of cell suspension to a cell counter (AS ONE, Japan). Following the manufacturer's protocol of the cell counter, we counted the number of cells derived from the control

TABLE 1 Conditions of foraminiferal culture experiments.

| | TiO ₂ NPs concentration (ppm) | Frequency of medium change (per week) | Frequency of feeding (per week) | Feeding volume for each time (µL) | Total volume of food per week (µL) |
|--------------|--|---------------------------------------|---------------------------------|-----------------------------------|------------------------------------|
| Experiment 1 | 1, 10, 50 | 1 | 1 | 80 | 80 |
| Experiment 2 | 1, 10, 50 | 2 | 2 | 80, 80 | 160 |
| Experiment 3 | 1, 5, 10 | 1 | 3 | 40, 20, 20 | 80 |
| Experiment 4 | 5, 10 | 1 | 3 | 80, 40, 40 | 160 |

and 1 ppm samples using a stereomicroscope (SMZ18 Nikon, Japan) equipped with an inclined-angle lens tube. As TiO₂ NPs aggregations in the 5 and 10 ppm overlapped on *D. salina* and avoided observation, we captured the autofluorescence of *D. salina* with a digital camera (DS-Ri2 Nikon, Japan) using fluorescence observation (U-HGLGPS Olympus Japan, Excitation filter: BP530-550, Barrier filter: BA575IF) equipped with an inverted research microscope (IX73 Olympus, Japan) and counted the cell numbers.

2.4 Scanning electron microscope observation of foraminiferal tests

We used six specimens: two for control and four for the 10 ppm TiO₂ NPs treatment. Two out of four 10 ppm TiO₂ NPs treated specimens formed a new chamber during the culture experiment, while the other two showed no growth. Each test was cleaned with a brush in water and dried in air. These tests were placed on carbon tape and glued to a stage where carbon deposition was performed with the carbon coater (JEE-420 JEOL, Japan). The surface structure and element analysis of foraminiferal tests were conducted with a field emission SEM equipped with an energy dispersive X-ray spectrometer (EF-SEM-EDS) (JSM-6500F JEOL, Japan).

2.5 Transmission electron microscope observation of cells

Ammonia veneta specimens, cultured in the 5 ppm TiO₂ NPs medium, were fixed with 2.5% glutaraldehyde in filtered artificial sea water (FASW) for longer than 24 hours at 4°C. The fixed specimens were embedded in 1% aqueous agarose and then decalcified with 0.2% EGTA in 0.81 mol/L aqueous sucrose solution (pH 7.0) for several days. The specimens were rinsed with FASW, then postfixed with 2% osmium tetroxide in FASW for 2 hours at 4°C, dehydrated in a graded ethanol series, and embedded in epoxy resin (Quetol 651). Ultrathin sections (100 nm for observation and 200 nm for analysis) were cut using a diamond knife on an Ultracut S ultramicrotome (Leica Reichert, Germany), stained with 2% aqueous uranyl acetate and lead staining solution (0.3% lead nitrate and 0.3% lead acetate), and examined using TEM equipped with an energy dispersive X-ray spectrometer (TEM-EDS) (FEI Tecnai G2 20, USA) operated at 200 kV. Elemental mapping was performed in scanning TEM (STEM) mode.

3 Results

3.1 Cell observation and viability of *Ammonia veneta*

Living specimens of *A. veneta* are filled with cytoplasm inside the test and extend fine reticulopodia radially from the aperture and pores of the test to capture food particles in their surroundings (Figure 1). In 1 ppm TiO₂ NPs seawater medium, TiO₂ NPs aggregate up to ~2 μm within 24 hours and accumulate with

other wastes on the bottom (Ishitani et al., 2023). TiO₂ NPs is aggregating faster in high TiO₂ NPs concentration: over 1 μm in hydrodynamic diameter within 1 hour in 5 and 10 ppm TiO₂ NPs seawater and over 15 μm in 50 ppm TiO₂ NPs seawater (Morelli et al., 2018; Reyes et al., 2021; Palmeira-Pinto et al., 2023). These aggregates visibly settled at the bottom of the petri dish as brownish substances during the culture experiment with TiO₂ NPs seawater media (Figure 1). However, these depositions were not observed in the surrounding areas of living *A. veneta*, particularly around the reticulopodia extensions (Figure 1). At the onset of the culture experiment, all studied specimens extended fine reticulopodia. In the control and 1 ppm TiO₂ NPs seawater medium, specimens showed reticulopodia activity and also added new chambers and grew cytoplasm, which typically filled the interior of the test. Under TiO₂ NPs seawater media concentrations greater than 10 ppm, reticulopodia were retracted, and cytoplasm continuously shrank (Figures 2A–D). After reduction of cytoplasm, any reticulopodia activity and movement of specimens were not observed. In these conditions, aggregations containing TiO₂ NPs were observed around foraminiferal specimens.

Holes appeared on the surface of the tests in some specimens exposed to TiO₂ NPs seawater media concentrations higher than 10 ppm (Figure 3). After a 5-week experiment, additional holes were observed on the umbilical side of the test.

3.2 Foraminifera culture experiments

In Experiment 1, control specimens increased by an average of ~2 chambers every week, reaching a total of ~9 chambers over five weeks (Figure 4A; Supplementary Table S1). One out of nine

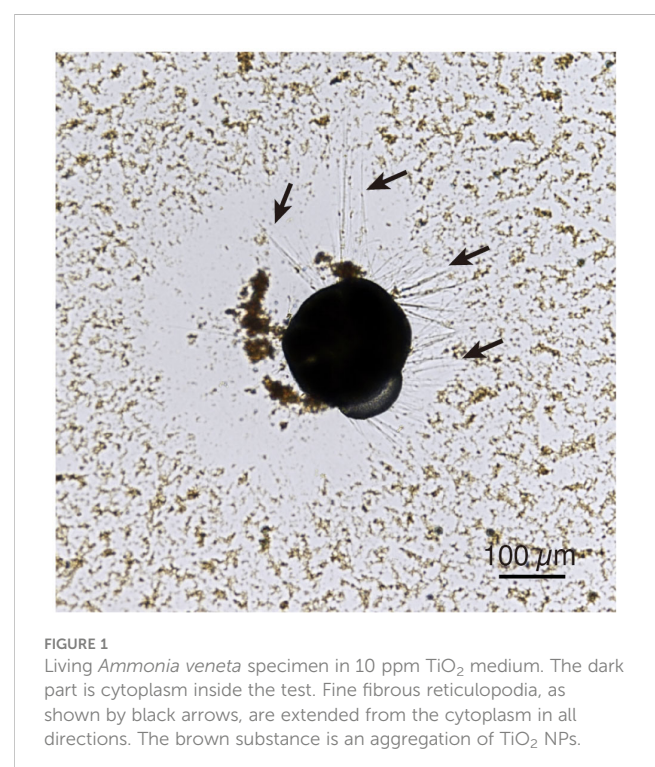


FIGURE 1
Living *Ammonia veneta* specimen in 10 ppm TiO₂ medium. The dark part is cytoplasm inside the test. Fine fibrous reticulopodia, as shown by black arrows, are extended from the cytoplasm in all directions. The brown substance is an aggregation of TiO₂ NPs.

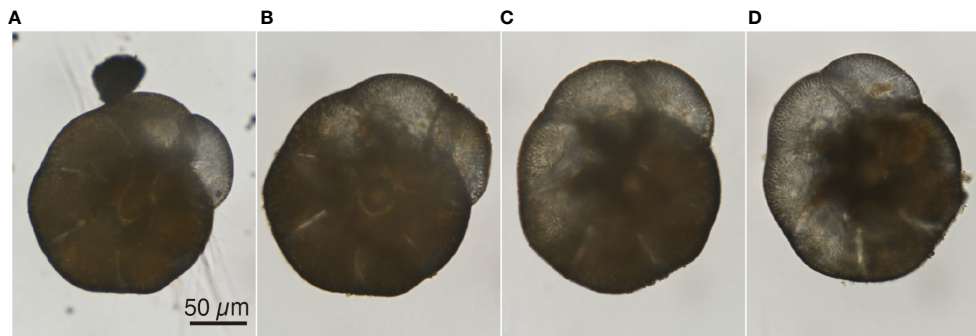


FIGURE 2

Light microscopy images of an *Ammonia veneta* specimen exposed to 10 ppm TiO_2 medium for four weeks. (A) Reticulopodia were extended from the cytoplasm at the onset of experiment. (B) There were no reticulopodia, and cytoplasm was reduced after a week. (C) Cytoplasm was retracted from the previous week. (D) Cytoplasm completely shrank in the fourth week.

control specimens reproduced in the third week. By the end of the 5-week experiment, three specimens had reproduced clones, forming an average of ~ 20 chambers during their entire lifespan. In the 1 ppm TiO_2 treatment, eight out of nine specimens increased by ~ 1 chamber per week, although five specimens died after 3 and 5 weeks, respectively. In the 10 ppm TiO_2 treatment, six out of nine specimens increased by ~ 1 chamber per week, but all specimens ceased growth by the third week and died by the fourth week. In the 50 ppm TiO_2 treatment, only one specimen increased by 1 chamber in the first week, but all specimens died by the fourth week. The total number of increased chambers over the 5-week experiment showed a significant difference between the control specimens and the 10 and 50 ppm TiO_2 treated specimens (Figure 4B).

In Experiments 2, all specimens, both in the control and the 1 ppm TiO_2 treatment, increased by 6–8 chambers over 5 weeks



FIGURE 3

Light microscopy image of the umbilical side of an *Ammonia veneta* specimen exposed to 50 ppm TiO_2 medium for five weeks. Three big holes were opened on the surface of the test, as shown by the black arrows.

(Figure 5A; Supplementary Table S1). During the experiment, four and five specimens reproduced clones in the control and the 1 ppm TiO_2 treatment, respectively. However, in both the 10 and 50 ppm TiO_2 treatments, all specimens showed no growth and died by the fourth week. The total number of increased chambers over the 5-week experiment was the same between the control and the 1 ppm TiO_2 treated specimens, but there were significant differences between the controls and the 10 and 50 ppm TiO_2 treatments (Figure 5B).

In Experiment 3, all specimens, both in the control and the 1 ppm TiO_2 treatment, increased by ~ 8 chambers over 5 weeks (Figure 6A; Supplementary Table S1). During this experiment, one specimen reproduced clones in each of the control and the 1 ppm TiO_2 treatments, but some specimens died. In the 5 ppm TiO_2 treatment, three out of nine specimens increased by ~ 1 chamber per week, but all had stopped growing and died by the third week. In the 10 ppm TiO_2 treatment, all individuals showed no growth and died by the third week. The total number of increased chambers over the 5-week experiment was the same between the control and the 1 ppm TiO_2 treated specimens, but there were significant differences between the controls and the 5 and 10 ppm TiO_2 treatments (Figure 6B).

In Experiment 4, eight out of nine control specimens increased by ~ 8 chambers over 5 weeks (Figure 7A; Supplementary Table S1). At the end of the experiment, three specimens reproduced clones. In the 5 ppm TiO_2 treatment, four out of nine specimens increased by ~ 1 chamber per week, but they stopped growing by the third week and all died by the fifth week. In the 10 ppm TiO_2 treatment, only two specimens increased by one chamber in the first week, but all individuals died by the third week. The total number of increased chambers over the 5-week experiment showed significant differences between the controls and the 5 and 10 ppm TiO_2 treatments, as well as those of Experiment 3 (Figure 7B).

3.3 Surface structure of *Ammonia veneta* test

Morphological differences between control and 10 ppm TiO_2 treated specimens were observed on the surface of the tests, but

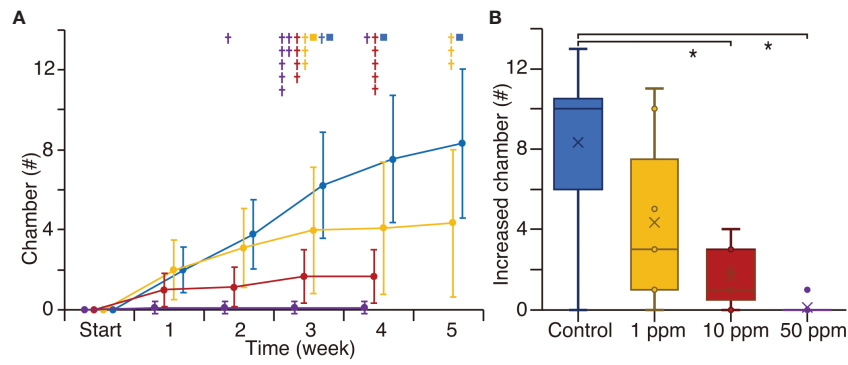


FIGURE 4

The number of increased chambers in culture experiment 1. Blue, yellow, red, and purple colors show the control, 1 ppm, 10 ppm, and 50 ppm TiO₂ treatments, respectively. (A) The average of additional numbers of chambers every week during the 5-week experiment. Crosses indicate dead specimen and squares show clonal reproduction. (B) Box plot of the number of increased chambers for five weeks. Asterisks show significant differences ($p < 0.0125$ with the Bonferroni correction).

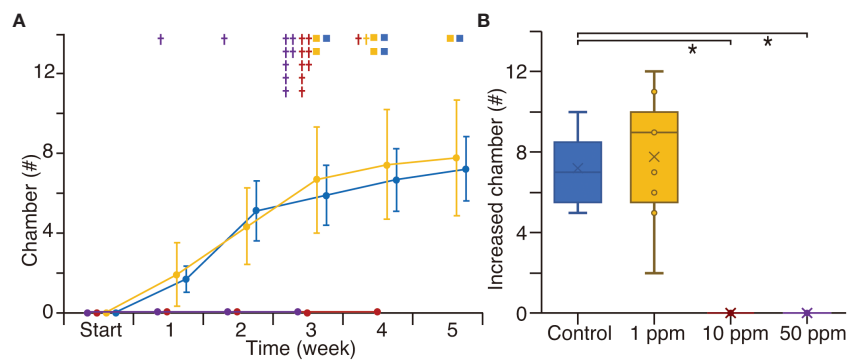


FIGURE 5

The number of increased chambers in the culture experiment 2. The manner of plots is the same as the in Figure 4.

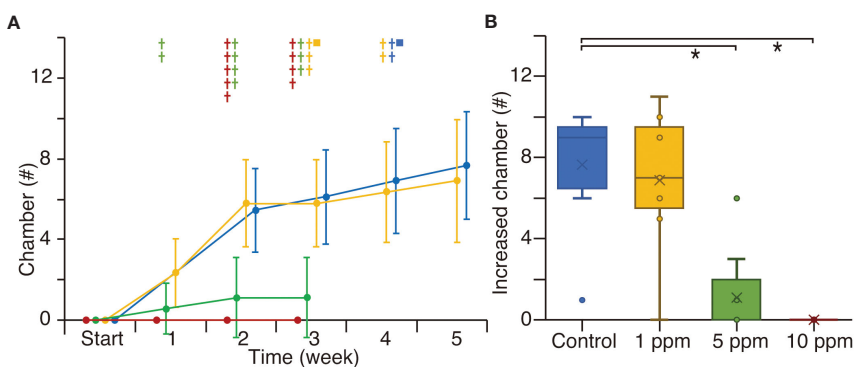


FIGURE 6

The number of increased chambers in the culture experiment 3. Green color indicates the 5 ppm TiO₂ treatment. The other manners of plots are same as Figure 4. The control data for the first week was absent.

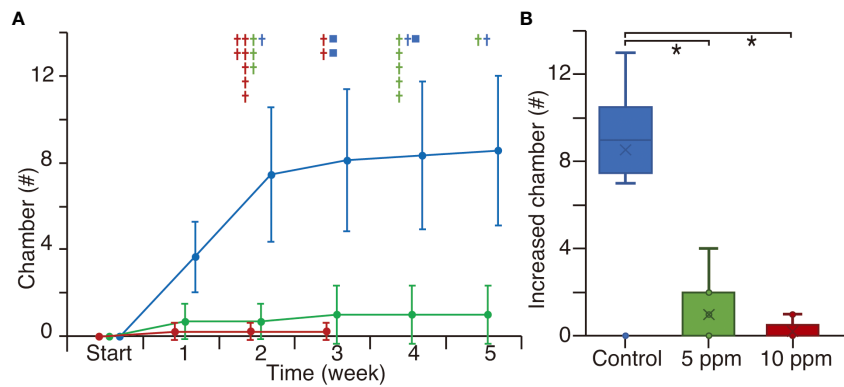


FIGURE 7

The number of increased chambers in culture experiment 4. Blue, green, and red show the control, 5 ppm, and 10 ppm TiO₂ treatments, respectively. The other manners of plots are the same as in Figure 4. Significant differences are $p < 0.0167$ with the Bonferroni correction.

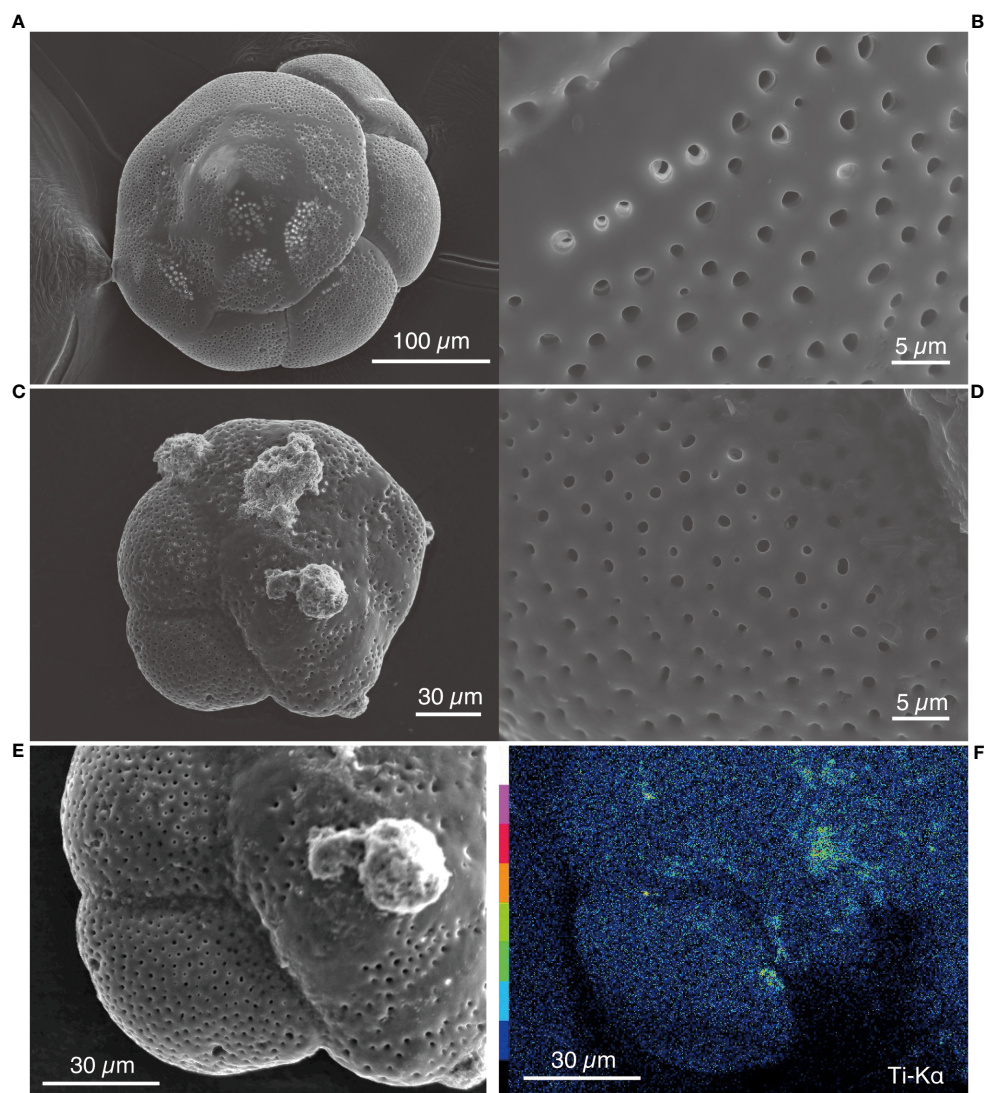


FIGURE 8

SEM images and EDS elemental map. (A) SEM image of the control specimen. (B) The surface test structure of the control specimen. (C) SEM image of a specimen cultured in 10 ppm TiO₂ seawater medium. (D) The surface test structure of the 10 ppm TiO₂ treated specimen. (E) SEM image around a last chamber, which was formed during a culture experiment in 10 ppm TiO₂ medium. (F) EDS elemental map showed the distribution of titanium on the surface of the test (E).

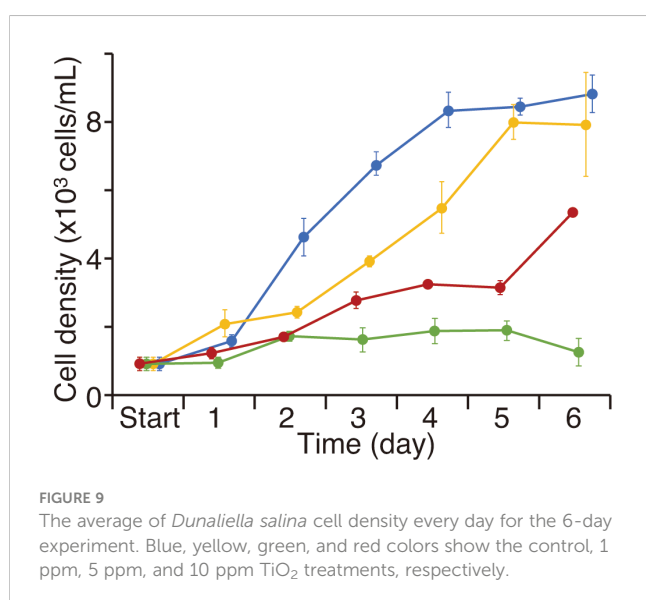
pores were clearly opened on both specimens (Figures 8A–D; Supplementary Figure S1). The test surface was smooth on control specimens but slightly rough on 10 ppm TiO₂ treated specimens (Figures 8B, D). Moreover, some adhesive materials were observed on the test surface of TiO₂ treated specimens, even when cleaned with brushes. These adhesive materials were often observed from the 10 ppm TiO₂ treated specimens, which showed no growth during the culture experiment (Supplementary Figure S1). The SEM-EDS mapping showed a trace amount of titanium from adhesive materials and on the surface of the test but not covering the aperture and pores of the test (Figures 8E, F).

3.4 Increase of *Dunaliella salina* cell density

Time-series changes in *D. salina* cell density were recorded for each culture condition, including control and 1, 5, and 10 ppm TiO₂ seawater media, over a 6-day period. In the control, *D. salina* cell density continuously increased for four days, reaching a stationary phase thereafter (Figure 9). Over the 6-day experiment, the cell density was approximately 10 times higher than the initial density. In the 1 ppm TiO₂ seawater medium, cell density started a slow increase by the second day, significantly rising afterward and reaching a level comparable to the control by the fifth day. However, in the 5 and 10 ppm TiO₂ seawater media, cell densities slightly increased but remained at 2000 cells/mL throughout the experiment. By the end of the experiment, cell densities in the 5 and 10 ppm TiO₂ seawater media were less than half of those in the control and the 1 ppm seawater medium.

3.5 TiO₂ NPs in *Ammonia veneta* cell

Angular-shaped grains with distinct contours were observed in vesicles distributed in the latest three chambers by TEM observations (Figures 10A–C; Supplementary Figure S2). These



grains, several tens of nanometers in size, exhibited a darker contrast than the surrounding organics and resin, indicating a heavier and more electronically dense element. STEM-EDS analysis detected titanium X-ray peaks from these angular grains (Figures 10D–F), but no titanium peaks were detected from the background (Figures 10G, H). Additionally, STEM-EDS mapping identified abundant titanium-bearing grains in the vesicles, each tens of nanometers in size (Figures 11A, B).

Aggregates of angular grains covered with light-contrast materials were found along the cell, relevant to the surface of the decalcified test (Figure 12A). STEM-EDS analysis detected titanium X-ray peaks from these grains, as well as those in vesicles (Figure 12B), while no titanium peaks were detected from the background (Figure 12C).

4 Discussion

We successfully demonstrated the viability of the clonally reproduced strain of the foraminifer based on the activity of the reticulopodia and chamber formation under microscopic observation (Figures 1–3). *Ammonia veneta* mostly survived for the first three weeks under TiO₂ NPs-containing media, even at high TiO₂ NPs concentrations exceeding 10 ppm (Figures 4–7). TiO₂ NPs did not physically impede the extension of reticulopodia by covering the pores and aperture on the test, as shown by the SEM image (Figures 8C, D). Few TiO₂ NPs were observed in the adhesive materials and on the test surface of 10 ppm-treated specimens (Figures 8E, F). The adhesive materials were often found on the shell surface of the 10 ppm-treated specimens, which stopped reticulopodia activity in the early stage of culture experiments and had no growth of chamber (Supplementary Figure S1). Aggregated TiO₂ NPs might be attached to the shell surface, when reticulopodia did not extend covering over the shell. Although TiO₂ NPs did not physically prevent the extension of reticulopodia, specimens in high concentrations of TiO₂ medium over 5 ppm exhibited no extended reticulopodia and shrunk cytoplasm without chamber formation, leading to their demise (Figures 2, 4–7). The control and 1 ppm TiO₂ treated specimens showed a gradual increase in chambers up to ~20 in total until the reproduction stage. The methods for counting new chambers in addition to reticulopodia activity have been validated to estimate foraminiferal viability and health in response to anthropogenic pollution (Ben-Eliahu et al., 2020; Losada Ros et al., 2020). Our observation based on the test and cytoplasm including reticulopodia is consistent with the trend of chamber increase over time and is useful for estimating the viability and health of foraminifers.

All four foraminiferal culture experiments showed that the control specimens increased by ~8 chambers and grew into maturity from the young form with ~10 chambers during five weeks (Figures 4–7). The specimens exposed to 1 ppm TiO₂ medium showed a slightly low growth rate in Experiment 1, though there was no significant difference from the controls (Figure 4B). When we modified feeding frequency and amount of food in Experiments 2 and 3, the growth rate in 1 ppm TiO₂ treatment was the same as the controls, and some specimens

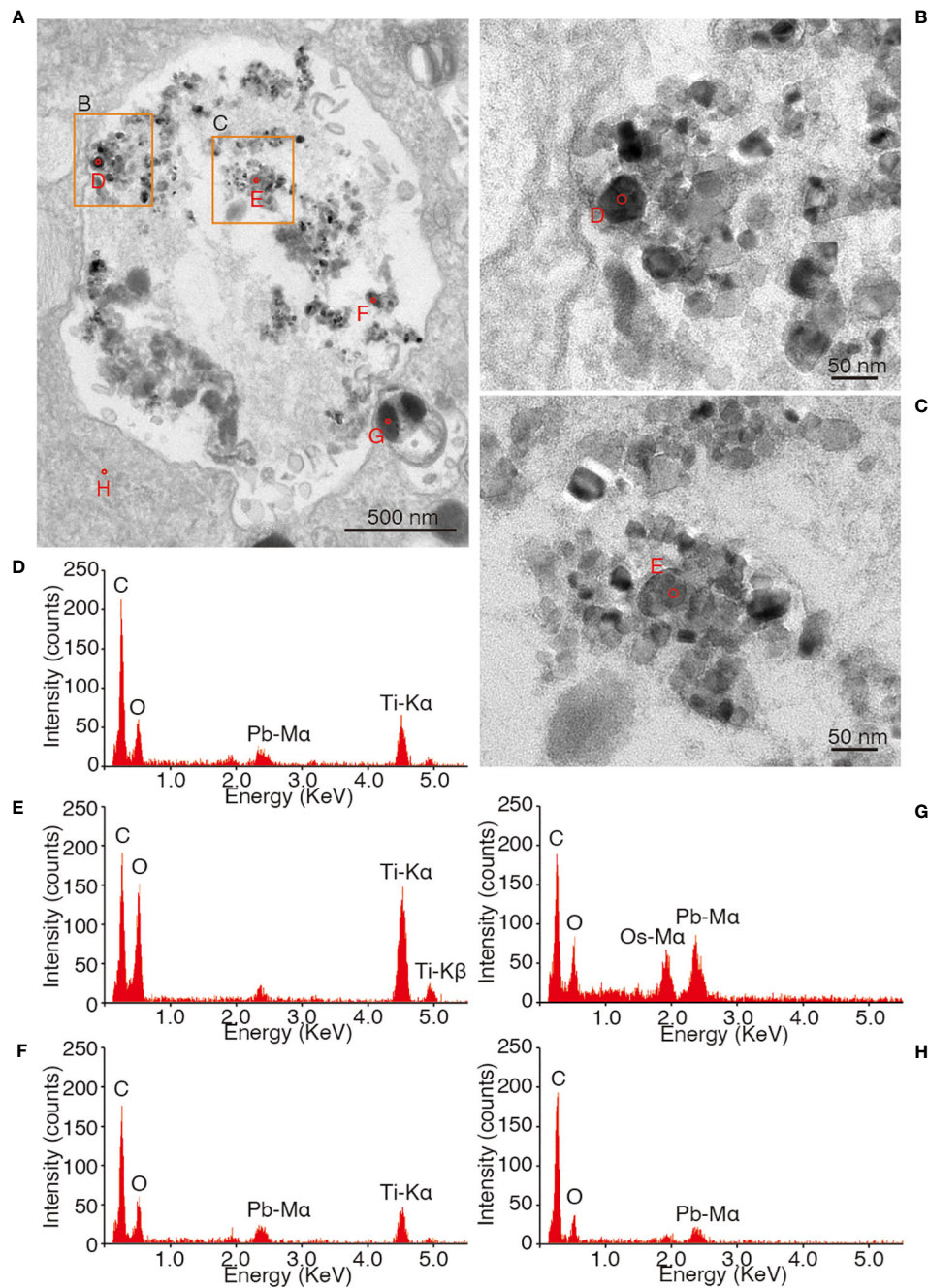


FIGURE 10

Sectioning images and EDS qualitative spectra of the vesicle, which was placed in the second last chamber of *Ammonia veneta* cultured in a 5 ppm TiO_2 seawater medium. The images are contrast-inverted transmission electron micrographs for better visualization of microstructure. (A) Heavier and electronic dense grains in dark color were distributed in the vesicle. Orange squares correspond to (B, C). Red circles show the spots for EDS analysis. (B, C) Heavier and electronic dense grains were ~ 50 nm in size with an angular shape. (D–H) Qualitative spectra of EDS analysis at each of the five spots shown in (A). (D–F) are associated with small angular grains, (G) shows an electronic dense body, and (H) places the cytosol as a background.

reproduced clones (Figures 5B, 6B). The foraminiferal life cycle has been maintained under a 1 ppm TiO_2 NPs-contained condition, in congruence with the long-term culturing experiment of the previous study (Ishitani et al., 2023). However, the specimens exposed to high TiO_2 NPs concentrations greater than 10 ppm died without growth for four weeks, despite increases of feeding frequency and food volume (Figures 4–7). This indicates that 10

ppm TiO_2 NPs concentration is fatal for foraminifers. Furthermore, we found hole-opening on the test surface of specimens exposed to 10 and 50 ppm TiO_2 media during the experiments (Figure 3). It is thought that foraminifers melt their own tests under conditions of strong stress (Buzas-Stephens and Buzas, 2005), suggesting that high TiO_2 NPs concentrations lead foraminifers to strong stress conditions and cell death.

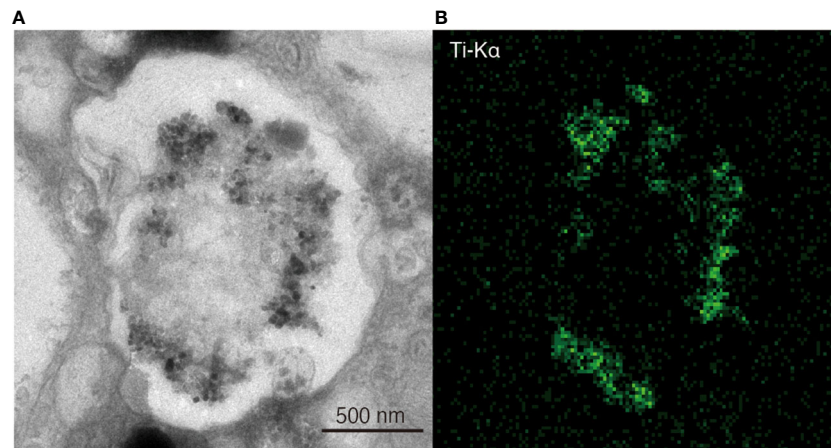


FIGURE 11

Sectioning images and EDS elemental map of the vesicle, which placed in the second last chamber of *Ammonia veneta* cultured in 5 ppm TiO_2 seawater medium. (A) Contrast-inverted TEM image of vesicle. (B) Distribution of titanium with green color by the EDS elemental map in the same area of the TEM image.

In Experiment 3, most specimens stopped growing in 5 ppm TiO_2 medium during the 5-week experiments (Figure 6A). This is possibly caused by a lack of food, *D. salina*. The culture experiment with *D. salina* in TiO_2 media showed that the cell densities of *D. salina* in the 5 and 10 ppm TiO_2 media were lower than half of those in the control and the 1 ppm TiO_2 medium for six days (Figure 9). This result has indicated that the feeding volume in Experiment 3 could not be enough for foraminiferal specimens. However, *A. veneta* specimens stopped growing in 5 ppm TiO_2 medium, even though we increased food volume in Experiment 4 (Figure 7A). Therefore, feeding is not a direct cause to prevent growth of

foraminifers. The growth of some microalgae is suppressed in media with metallic NPs as well as TiO_2 NPs because of physicochemical effects such as inhibition of photosynthesis by packed TiO_2 NPs aggregation and oxidative stress, which damage the cell membrane (Chen et al., 2012; Li et al., 2015; Wang et al., 2016; Ghazaei and Shariati, 2020). The studied specimens have extended reticulopodia at any concentration of TiO_2 treatments and seem to take surroundings including TiO_2 NPs via protoplasmic streaming of reticulopodia without organismal selection (Figure 1). TEM observation of *A. veneta* cells derived from 5 ppm TiO_2 treatment congruently demonstrates uptake of TiO_2 NPs into

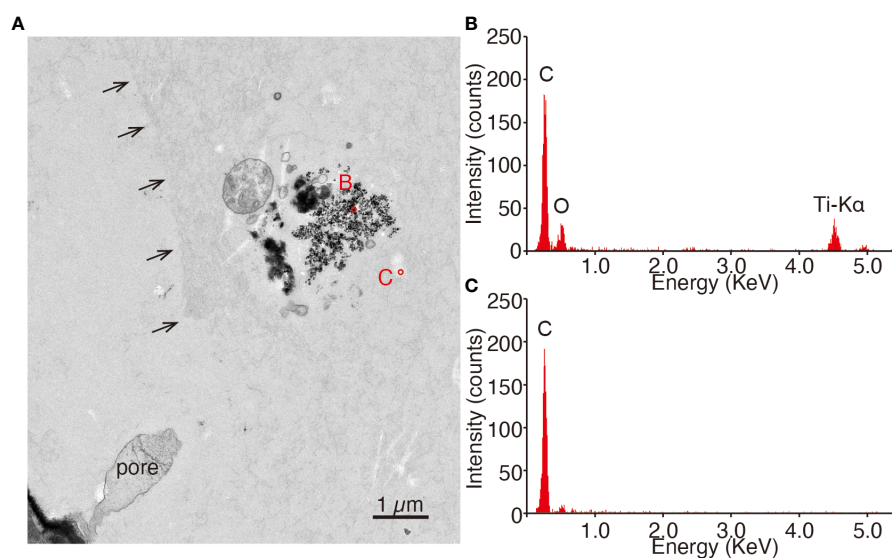


FIGURE 12

Sectioning images and EDS qualitative spectra of expelled materials placed outside of the decalcified test. (A) Contrast-inverted TEM images. The structure of the pore, as shown by the black arrows, remained. Electronically dense grains were distributed near the pore. Circles show the spot of EDS analysis. (B, C) Qualitative spectra of EDS analysis at the spot shown in (A). (B) is associated with a small angular grain, and (C) is a background.

vesicles (Figures 10A–C, 12B). These specimens could take TiO₂ NPs via phagocytosis because of fast aggregation of TiO₂ NPs in high concentrations more than 5 ppm (Morelli et al., 2018; Reyes et al., 2021; Palmeira-Pinto et al., 2023). This is congruent with the previous transcriptomic study, which estimated uptake of TiO₂ NPs via endocytosis or phagocytosis based on the metabolic pathway in 1 ppm TiO₂ treatment (Ishitani et al., 2023). This previous study showed that captured TiO₂ NPs induce the Fenton reaction in vesicles, resulting in ROS production, though such TiO₂ NPs are encapsulated in ceramide and these capsules are expelled from the cell as mucus after 24 hours (Ishitani et al., 2023). The light-contrast materials, including titanium, around the test could be leftovers of waste excreted by foraminifers (Figure 12B). These observations suggest that the foraminiferal metabolic system is acting even in high concentrations of TiO₂ NPs at least 5 ppm condition. This could be caused by two possibilities: a) ROS production is accelerated due to regulation of genes related to stress or releasing toxic substances, and b) overexpression of genes related to releasing toxic substances inhibits cell growth leading to cell death. Future studies will be required to examine the functional changes of detoxification under high TiO₂ NPs concentration environments.

The lethal level of our studied organism, *A. veneta*, is lower than that of microalgae such as green algae and diatoms and those of microorganisms, whose EC50 and LC50 were applicable at concentrations higher than 10 ppm TiO₂ NPs (Menard et al., 2011; Minetto et al., 2014). Our results suggest that the previous examination for EC50 and LC50 could be underestimated than the actual effect of TiO₂ NPs concentration on marine organisms. The previous studies have used different types of TiO₂ NPs: rutile and anatase types with different size ranging from ~10 to ~100 nm. Such material specificity provides different surface charge and other physical characteristics leading to various toxicities reported as cell death by rutile type, while cell necrosis and cell membrane damage by anatase type (Kose et al., 2020). It is still unclear what processes in the metabolic system are affected by the physical characteristics of TiO₂ NPs leading to toxicity. Our study, conducted with same type and size of TiO₂ NPs as those of the transcriptome study (Ishitani et al., 2023), presented that foraminiferal detoxification is possibly disturbed by overdose (> 5 ppm) of TiO₂ NPs. Toxic mechanisms are likely changed associating with concentration of nanoparticles shown by this study, as well as size of nanoparticles. Understanding of both cellular and metabolic mechanisms is important to unveil toxic effects of nanoparticles on various organisms in sets of nanoparticle types. The concentration around 5 ppm of TiO₂ NPs, a limit of foraminiferal detoxification, is much lower than ~120 ppm potentially accumulated on European coasts (Sun et al., 2016; Zheng and Nowack, 2021), though nanoparticle pollution has been invisibly but certainly spread out over marine environment. Considering sedimentation of nanoparticles after aggregation, benthic fauna is dominantly exposed to pollutants. Long-term experiments are crucial to examine an extended cytotoxicity of marine organisms including meiofauna as happen in nature. These studies will help to estimate toxic tolerance of marine organisms to nanoparticles for sustaining marine ecosystem under a harmful impact of nanoparticles pollution.

5 Conclusions

This study succeeded to conduct long-term culture experiments based on the exposure of a marine foraminiferal strain, *A. veneta*, to 1, 5, 10, and 50 ppm TiO₂ NPs. This species had been known to have a detoxification mechanism for TiO₂ NPs by the previous transcriptome analysis. Our TEM observations showed that these foraminifers took TiO₂ NPs into vesicles and ejected some of them even at 5 ppm TiO₂ NPs exposure. However, foraminifers stopped growing in the early stage of 5-week culture experiments under 5 ppm TiO₂ NPs exposure despite the availability of food. These findings indicate that the foraminiferal detoxification system could be disrupted by over 5 ppm TiO₂ NPs. The demand for TiO₂ NPs has grown and their concentration is estimated at up to 120 ppm around the European coast nowadays. Such acceleration of discharging TiO₂ NPs to the ocean could moderate the foraminiferal detoxification ability leading a breakdown of marine ecosystem, because meio-benthos such as foraminifers play an important role for marine food chain. Future studies will be required to examine toxic mechanisms of various marine organisms, for understanding an effect of nanoparticles to the entire marine ecosystem including meiofauna.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/[Supplementary Material](#).

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

YIn: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. YIs: Writing – original draft, Resources, Investigation. AT: Writing – review & editing, Investigation. KU: Writing – review & editing, Investigation. NT: Writing – review & editing, Investigation. TU: Writing – review & editing, Investigation. YU: Writing – original draft, Project administration, Funding acquisition, Conceptualization.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work is supported by ESPEC Foundation for Global Environment Research and Technology and Japan Society for the Promotion of Science (JSPS) grant (20H02016).

Acknowledgments

We thank M. Kakimoto, K. Kukita, I. Ikuma, and K. Miura from Kochi University for their help with the culture experiments.

Conflict of interest

Author KU was employed by Marine Works Japan Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated

References

- Ammendolia, C., Hofkirchner, C., Plener, J., Bussi eres, A., Schneider, M. J., Young, J. J., et al. (2022). Non-operative treatment for lumbar spinal stenosis with neurogenic claudication: an updated systematic review. *BMJ Open* 12, e057724. doi: 10.1136/bmjopen-2021-057724
- Ben-Eliahu, N., Herut, B., Rahav, E., and Abramovich, S. (2020). Shell growth of large benthic foraminifera under heavy metals pollution: Implications for geochemical monitoring of coastal environments. *Int. J. Environ. Res. Public Health* 17, 3741. doi: 10.3390/ijerph17103741
- Boxall, A. B., Tiede, K., and Chaudhry, Q. (2007). Engineered nanomaterials in soils and water: how do they behave and could they pose a risk to human health? *Nanomed. (Lond.)* 2, 919–927. doi: 10.2217/17435889.2.6.919
- Buzas-Stephens, P., and Buzas, M. A. (2005). Population dynamics and dissolution of foraminifera in Nueces Bay, Texas. *J. Foraminiferal Res.* 35, 248–258. doi: 10.2113/35.3.248
- Chen, L., Zhou, L., Liu, Y., Deng, S., Wu, H., and Wang, G. (2012). Toxicological effects of nanometer titanium dioxide (nano-TiO₂) on *Chlamydomonas reinhardtii*. *Ecotoxicol. Environ. Saf.* 84, 155–162. doi: 10.1016/j.ecoenv.2012.07.019
- Ciacchi, C., Grimmelmont, M. V., Corsi, I., Bergami, E., Curzi, D., Burini, D., et al. (2019). Nanoparticle-biological interactions in a marine benthic foraminifer. *Sci. Rep.* 9, 19441. doi: 10.1038/s41598-019-56037-2
- Cl ement, L., Hurel, C., and Marmier, N. (2013). Toxicity of TiO₂ nanoparticles to cladocerans, algae, rotifers and plants - effects of size and crystalline structure. *Chemosphere* 90, 1083–1090. doi: 10.1016/j.chemosphere.2012.09.013
- Culver, S. J., and Buzas, M. A. (1999). "Biogeography of neritic benthic foraminifera." in *Modern Foraminifera*, eds. K. Barun and G. Sen (Dordrecht: Springer Netherlands). doi: 10.1007/0-306-48104-9_6
- Delay, M., and Frimmel, F. H. (2012). Nanoparticles in aquatic systems. *Anal. Bioanal. Chem.* 402, 583–592. doi: 10.1007/s00216-011-5443-z
- Farr e, M., Gajda-Schrantz, K., Kantiani, L., and Barcel o, D. (2009). Ecotoxicity and analysis of nanomaterials in the aquatic environment. *Anal. Bioanal. Chem.* 393, 81–95. doi: 10.1007/s00216-008-2458-1
- Ghazaei, F., and Shariati, M. (2020). Effects of titanium nanoparticles on the photosynthesis, respiration, and physiological parameters in *Dunaliella salina* and *Dunaliella tertiolecta*. *Protoplasma* 257, 75–88. doi: 10.1007/s00709-019-01420-z
- Goldstein, S. T. (1999). "Foraminifera: A biological overview," in *Modern Foraminifera*, eds. K. Barun and G. Sen (Dordrecht: Springer Netherlands). doi: 10.1007/0-306-48104-9_3
- Hartmann, N. B., Von der Kammer, F., Hofmann, T., Baalousha, M., Ottofuelling, S., and Baun, A. (2010). Algal testing of titanium dioxide nanoparticles - testing considerations, inhibitory effects and modification of cadmium bioavailability. *Toxicology* 269, 190–197. doi: 10.1016/j.tox.2009.08.008
- Ishitani, Y., Ciacchi, C., Ujiie, Y., Tame, A., Tiboni, M., Tanifuji, G., et al. (2023). Fascinating strategies of marine benthic organisms to cope with emerging pollutant: Titanium dioxide nanoparticles. *Environ. Pollut.* 330, 121538. doi: 10.1016/j.envpol.2023.121538
- Kose, O., Tomatis, M., Leclerc, L., Belblidia, N. B., Hochepeid, J. F., Turci, F., et al. (2020). Impact of the physicochemical features of TiO₂ nanoparticles on their *in vitro* toxicity. *Chem. Res. Toxicol.* 33, 2324–2337. doi: 10.1021/acs.chemrestox.0c00106
- Li, F., Liang, Z., Zheng, X., Zhao, W., Wu, M., and Wang, Z. (2015). Toxicity of nano-TiO₂ on algae and the site of reactive oxygen species production. *Aquat. Toxicol.* 158, 1–13. doi: 10.1016/j.aquatox.2014.10.014
- Losada Ros, M. T., Al-Enezi, E., Cesarini, E., Canonico, B., Bucci, C., Alves Martins, M. V., et al. (2020). Assessing the cadmium effects on the benthic foraminifer *Ammonia cf. parkinsoniana*: An acute toxicity test. *Water* 12, 1018. doi: 10.3390/w12041018
- Luo, Z., Li, Z., Xie, Z., Sokolova, I. M., Song, L., Peijnenburg, W. J. G. M., et al. (2020). Rethinking nano-TiO₂ safety: overview of toxic effects in humans and aquatic animals. *Small* 16, e2002019. doi: 10.1002/smll.202002019
- Matranga, V., and Corsi, I. (2012). Toxic effects of engineered nanoparticles in the marine environment: model organisms and molecular approaches. *Mar. Environ. Res.* 76, 32–40. doi: 10.1016/j.marenvres.2012.01.006
- Menard, A., Drobne, D., and Jemec, A. (2011). Ecotoxicity of nanosized TiO₂. Review of *in vivo* data. *Environ. Pollut.* 159, 677–684. doi: 10.1016/j.envpol.2010.11.027
- Minetto, D., Libralato, G., and Ghirardini, A. V. (2014). Ecotoxicity of engineered TiO₂ nanoparticles to saltwater organisms: an overview. *Environ. Int.* 66, 18–27. doi: 10.1016/j.envint.2014.01.012
- Moore, M. N. (2006). Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? *Environ. Int.* 32, 967–976. doi: 10.1016/j.envint.2006.06.014
- Morelli, E., Gabellieri, E., Bonomini, A., Tognotti, D., Grassi, G., and Corsi, I. (2018). TiO₂ nanoparticles in seawater: Aggregation and interactions with the green alga *Dunaliella tertiolecta*. *Ecotoxicol. Environ. Saf.* 148, 184–193. doi: 10.1016/j.ecoenv.2017.10.024
- Palmeira-Pinto, L., Emerenciano, A. K., Bergami, E., Joviano, W. R., Rosa, A. R., Neves, C. L., et al. (2023). Alterations induced by titanium dioxide nanoparticles (nano-TiO₂) in fertilization and embryonic and larval development of the tropical sea urchin *Lytechinus variegatus*. *Mar. Environ. Res.* 188, 106016. doi: 10.1016/j.marenvres.2023.106016
- R Core Team (2021). *R: A language and environment for statistical computing* (Vienna, Austria: R Foundation for Statistical Computing). Available at: <https://www.R-project.org/>.
- Reyes, J. P., Lagdameo, J. D., Celorico, J. R., Almeda, R. A., Peralta, M. M., and Basilia, B. A. (2021). Aquatic toxicity studies of titanium dioxide and silver nanoparticles using *Artemia franciscana* Nauplii and *Daphnia magna*. *Mater. Sci. Eng. A.* 11, 107–113. doi: 10.17265/2161-6213/2021.10-12.001
- Sun, T. Y., Bornh of, N. A., Hungerb uhler, K., and Nowack, B. (2016). Dynamic probabilistic modeling of environmental emissions of engineered nanomaterials. *Environ. Sci. Technol.* 50, 4701–4711. doi: 10.1021/acs.est.5b05828
- Wang, Y., Zhu, X., Lao, Y., Lv, X., Tao, Y., Huang, B., et al. (2016). TiO₂ nanoparticles in the marine environment: Physical effects responsible for the toxicity on algae *Phaeodactylum tricornutum*. *Sci. Total Environ.* 565, 818–826. doi: 10.1016/j.scitotenv.2016.03.164
- Zheng, Y., and Nowack, B. (2021). Size-specific, dynamic, probabilistic material flow analysis of titanium dioxide releases into the environment. *Environ. Sci. Technol.* 55, 2392–2402. doi: 10.1021/acs.est.0c07446

organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2024.1381247/full#supplementary-material>

SUPPLEMENTARY FIGURE 1
SEM images and EDS analyses of control and 10 ppm TiO₂ treated specimens.

SUPPLEMENTARY FIGURE 2
TEM images of vesicles in a 5 ppm TiO₂ treated specimen.

SUPPLEMENTARY TABLE 1
The number of increased chambers in foraminiferal culture experiments 1–4.