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Ocean acidification increases the accumulation of titanium dioxide nanoparticles ($nTiO_2$) in edible bivalve mollusks and poses a potential threat to seafood safety

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Large amounts of anthropogenic CO_2 in the atmosphere are taken up by the ocean, which leads to 'ocean acidification' (OA). In addition, the increasing application of nanoparticles inevitably leads to their increased release into the aquatic environment. However, the impact of OA on the bioaccumulation of nanoparticles in marine organisms still remains unknown. This study investigated the effects of OA on the bioaccumulation of a model nanoparticle, titanium dioxide nanoparticles ($nTiO_2$), in three edible bivalves. All species tested accumulated significantly greater amount of $nTiO_2$ in pCO_2-acidified seawater. Furthermore, the potential health threats of realistic $nTiO_2$ quantities accumulated in bivalves under future OA scenarios were evaluated with a mouse assay, which revealed evident organ edema and alterations in hematologic indices and blood chemistry values under future OA scenario (pH at 7.4). Overall, this study suggests that OA would enhance the accumulation of $nTiO_2$ in edible bivalves and may therefore increase the health risk for seafood consumers.

Over the past century, increased CO_2 emissions derived from anthropogenic activities have led to measurable declines in both oceanic pH and the abundance of carbonate ions, a process commonly termed 'ocean acidification'[1]. The average global surface ocean pH has significantly decreased by over 0.1 units since the Industrial Revolution and is expected to further decline by 0.3 units by the end of the century[2,3]. This projected increase in seawater pCO_2 has been identified to exert far-reaching impacts on marine ecosystems and negatively affect a wide range of physiological processes across a large diversity of marine organisms[2,4–10].

Nanotechnology is a rapidly growing field attracting considerable attention owing to its widespread applications in both consumer and industrial products[11,12]. It has been predicted that the global market for nanotechnology products would achieve $3 trillion by 2020[12]. Consequently, this rapid expansion and widespread application would inevitably be accompanied by a heightened release of nanoparticles into the environment, posing potential threats to both environment and human health. For example, the US Environmental Protection Agency (USEPA) has attributed 60,000 deaths per year to the inhalation of atmospheric nanoparticles[13]. Due to precipitation and surface runoff, marine ecosystems are regarded as the ultimate sink for nanoparticles[14,15]. To date, much research has shown that nanoparticle contamination can exert significant impacts on the biological and physiological processes of various marine organisms, including fertilization[16], protein expression[17], immune responses[18], energy budgets[19] and even survival[20]. In addition, previous studies have illustrated that nanoparticles can bio-accumulate in marine invertebrate species and are bio-amplified through the food chain[21–24]. Therefore, consuming nanoparticle-contaminated seafood could be a potential risk for human health. So far, the health consequences of exposure to nanoparticle contaminations have been well studied in mammals[13]. For example, evident liver lesions, kidney pathological alterations[25] and hampered immune responses[26] induced by oral titanium dioxide nanoparticles ($nTiO_2$) exposure have been described in mice. Though toxicity studies of nanoparticles have been conducted *in vivo* in mouse models, excessive doses application in these previous studies have failed to

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Property	$nTiO_2$
Average diameter	30 ± 5 nm
BET surface area	60.65 $m^2 g^{-1}$
Crystal structure	
Purity	99.8%
Hydrodynamic diameter*	
in pH 8.1 seawater	334.8 ± 6.7 nm[a]
in pH 7.8 seawater	439.8 ± 11.2 nm[b]
in pH 7.4 seawater	537.1 ± 13.6 nm[c]
Zeta potential*	
in pH 8.1 seawater	-10.7 ± 0.1 mV[a]
in pH 7.8 seawater	-9.8 ± 0.1 mV[b]
in pH 7.4 seawater	4.9 ± 0.1 mV[c]

Table 1. Physicochemical properties of titanium dioxide nanoparticles (mean ± SE). Mean values that do not share the same superscript were significantly different at $p < 0.05$. *The particle hydrodynamic diameters and zeta potential were tested at a dose of 0.1 mg L^{-1} $nTiO_2$.

precisely reflect the health consequences of consuming nanoparticle-contaminated food[27]. Therefore, the current understanding of nanoparticle-induced food safety issues remains limiting.

It has been demonstrated that water pH can affect surface charging properties and hence the aggregation, potential bioavailability and reactivity of nanoparticles[28–31]. Therefore, theoretical future ocean acidification scenarios may alter the uptake and bioavailability of nanoparticles in marine systems and subsequently determine the critical degree of nanoparticle contaminations. A recent study has indicated that elevated atmospheric CO_2 levels would modify the effects of $nTiO_2$ on the nutritional quality of crops with unknown consequences for human health[32]; whether oceanic pCO_2 increase would aggravate or attenuate the risk of consuming seafood harvested in nanoparticle polluted areas awaits investigation.

Compared with other nanoparticles (Cu, Ag, Fe, ZnO nanoparticles, etc.), $nTiO_2$ has the highest model-predicted concentrations in the environment, which may reach as high as several mg/L in the aquatic system[33,34]. Therefore, $nTiO_2$ has been widely used as a representative nanoparticle in ecotoxicological studies[35–37]. With little known about food safety risk induced by nanoparticle contamination, specifically under future ocean acidification scenarios and at environmentally realistic concentrations, the present study was conducted using $nTiO_2$ as a model nanoparticle with three edible bivalve species to address the following questions: (1) Does elevated pCO_2 alter the ingestion-related physicochemical properties of nanoparticle in seawater? (2) How will nanoparticle accumulation in bivalves be affected by future ocean acidification scenarios? and (3) How and to what extent elevated pCO_2 in seawater will affect human's risk via consuming seafood contaminated with environmentally realistic nanoparticle concentrations.

Results

Effects of ocean acidification on the ingestion-related physicochemical properties of $nTiO_2$. Compared to the particle sizes (334.8 nm in diameter) in seawater at an ambient pH of 8.1, larger sizes of $nTiO_2$ (439.8 and 537.1 nm in diameter at pH of 7.8 and 7.4, respectively) were detected in pCO_2 acidified seawater. The zeta potentials of $nTiO_2$ (the charge of $nTiO_2$ in relation to the surrounding conditions) increased with decreasing seawater pH, being most stable in seawater at pH 8.1 due to the large negative zeta potential (Table 1). Since it has been reported that nanoparticles in larger aggregates are easier to be ingested[38], the particle size alteration detected, which may partially result from zeta potential change, implies increased likelihood of $nTiO_2$ to be ingested by bivalves under ocean acidification scenarios.

Effects of ocean acidification on the accumulation of $nTiO_2$ in various tissues of three edible bivalve species. The $nTiO_2$ contents accumulated in the gills, foot and mantles of blood clam (*Tegillarca granosa*), hard clam (*Meretrix meretrix*), and venus clam (*Cyclina sinensis*) after a 21-day 100 μg/L $nTiO_2$ exposure at pH 7.4 and 7.8 were about 1.34 and 1.16 times greater than those raised in the ambient pH of 8.1, respectively (Fig. 1, $p < 0.05$). This finding suggests that ocean acidification increases the accumulation of $nTiO_2$ in the bivalve species (Table S1).

The impacts of ocean acidification on seafood safety: mouse assays. Upon feeding with 2.5 mg/kg $nTiO_2$ per day, an equivalent dose simulating the intake of $nTiO_2$ through consuming contaminated bivalves raised under pH 7.4, the numbers of white blood cell (WBC) and lymphocytes (Lym) in mice were significantly greater than those under ambient pH of 8.1 (Table 2). Similarly, the levels of alanine transaminase (ALT), alkaline phosphatase (ALP), and creatinine (Crea), as well as the ALT/aspartate transaminase (AST) ratio were significantly induced upon feeding with an equivalent amount of $nTiO_2$ accumulated in the bivalves raised under ocean acidification scenarios (Table 3). Evident liver edema indicated by sinusoidal expansion (arrows in Fig. 2a) and inflammatory cell infiltration (or hydropic degeneration) (circles in Fig. 2a) was detected in mice fed with 2.5 mg/kg $nTiO_2$ daily (Fig. 2a). In addition, inflammatory cell infiltration and slight swelling of renal tubular epithelial cells (circles in Fig. 2b) were also observed in the kidneys of these mice.

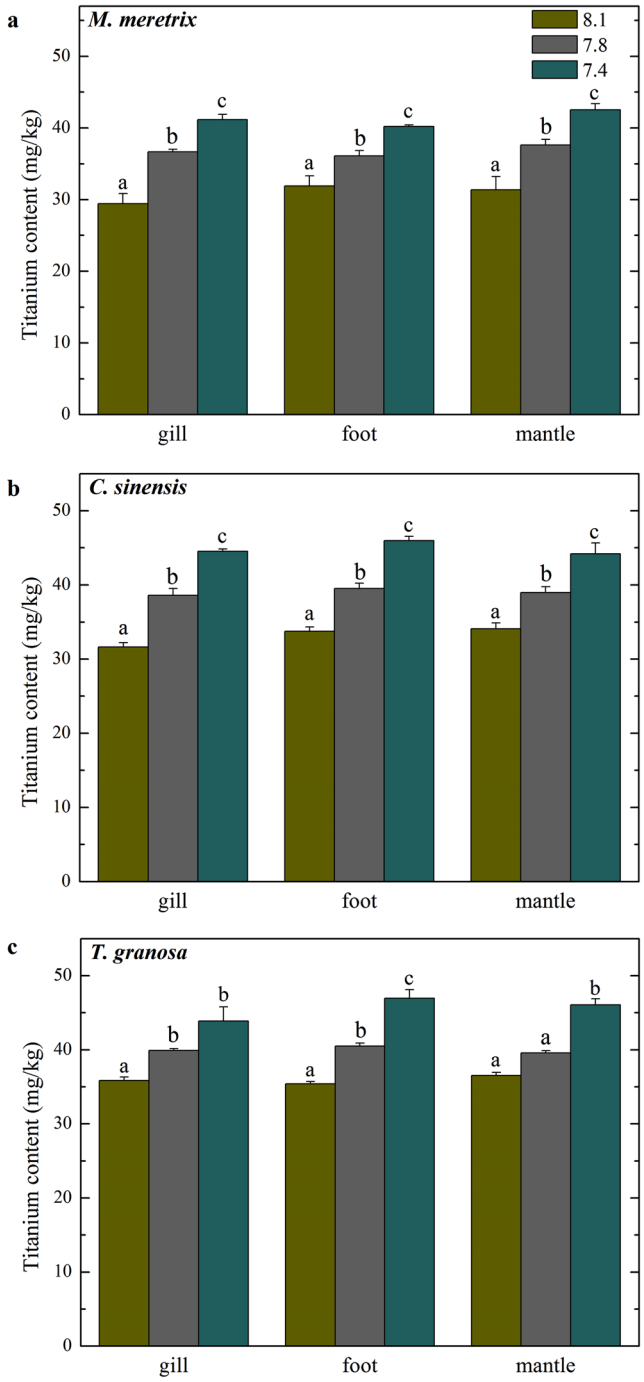

Figure 1. TiO_2 concentration (mean ± SE) (mg/kg, dry wet) in different tissues of (**a**) *M. meretrix*, (**b**) *C. sinensis*, and (**c**) *T. granosa* reared in different pCO_2 levels (21 days exposure). Mean values that do not share the same superscript were significantly different at $p < 0.05$.

The body weights of mice increased continuously throughout the experiment (Fig. S1), and no significant differences were detected among groups fed with different amounts of $nTiO_2$ (equivalent intake doses through consuming contaminated bivalves raised under ambient and two ocean acidification scenarios, respectively) (Table S2). The coefficients of heart, liver, spleen, lung, and kidney with body weight did not significantly differ among groups (Table S2). No obvious pathological changes were detected in the heart, spleen and lung of mice exposed to various tested doses of $nTiO_2$ (Fig. S2).

Group	Exposure dose	WBC (10^9/L)	Lym (10^9/L)	Mon (10^9/L)	Gran (10^9/L)	Mon %	RBC (10^{12}/L)	MCH (pg)
pH 8.1	1.5 mg/kg BW	9.9 ± 1.3^a	7.0 ± 0.7^a	0.3 ± 0.1^a	2.6 ± 0.6^a	3.4 ± 0.4^a	8.6 ± 0.9^a	16.5 ± 0.4^a
pH 7.8	2 mg/kg BW	10.3 ± 0.6^a	6.9 ± 0.2^b	0.4 ± 0.1^a	2.9 ± 0.4^a	3.8 ± 0.4^a	8.7 ± 0.9^a	17.2 ± 0.2^a
pH 7.4	2.5 mg/kg BW	15.9 ± 1.2^b	12.5 ± 1.1^c	0.4 ± 0.0^a	3.1 ± 0.2^a	2.6 ± 0.2^a	9.7 ± 0.6^a	16.9 ± 0.4^a
Group	**Exposure dose**	**RDW %**	**Gran %**	**Lym %**	**Hgb (g/L)**	**HCT %**	**MCV (fl)**	**MCHC (g/L)**
pH 8.1	1.5 mg/kg BW	12.6 ± 0.4^a	25.6 ± 2.7^a	71.0 ± 2.9^a	142.2 ± 14.5^a	42.8 ± 4.2^a	50.1 ± 1.2^a	330.6 ± 2.5^a
pH 7.8	2 mg/kg BW	12.3 ± 0.4^a	28.3 ± 2.5^a	67.8 ± 2.7^a	151.2 ± 16.3^a	45.9 ± 5.0^a	52.5 ± 0.7^a	328.8 ± 1.4^a
pH 7.4	2.5 mg/kg BW	12.3 ± 0.8^a	19.3 ± 1.6^a	78.1 ± 1.5^a	163.2 ± 7.4^a	49.8 ± 3.1^a	51.5 ± 0.9^a	329.7 ± 5.4^a

Table 2. Hematologic indices of mice after oral exposure to $nTiO_2$ at different exposure doses corresponding to daily intake of $nTiO_2$-contaminated seafood at different pCO_2 levels for 30 days (mean ± SE). WBC, white blood cell; Lym, lymphocytes; Mon, monocytes; Gran, granulocytes; RBC, red blood cells; MCH, mean corpuscular hemoglobin; RDW, red cell distribution width; Hgb, hemoglobin; MCV, Mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration. Mean values that do not share the same superscript were significantly different at $p < 0.05$.

Group	Exposure dose	ALT	AST	ALT/AST	ALP	Crea	Bun
pH 8.1	1.5 mg/kg BW	28.9 ± 1.4^a	93.7 ± 7.4^a	0.31 ± 0.01^a	77.9 ± 5.5^a	33.6 ± 0.3^a	17.2 ± 0.9^a
pH 7.8	2 mg/kg BW	34.0 ± 2.3^a	103.5 ± 3.9^a	0.33 ± 0.01^a	110.0 ± 4.8^b	34.2 ± 0.4^a	18.0 ± 0.6^a
pH 7.4	2.5 mg/kg BW	42.0 ± 3.1^b	106.5 ± 3.2^a	0.39 ± 0.02^b	108.9 ± 6.5^b	35.3 ± 0.4^b	18.1 ± 0.5^a

Table 3. Blood chemistry values of mice after oral exposure to $nTiO_2$ at different exposure doses corresponding to daily intake of $nTiO_2$-contaminated seafood at different pCO_2 levels for 30 days (mean ± SE). ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; Crea, creatinine. Mean values that do not share the same superscript were significantly different at $p < 0.05$.

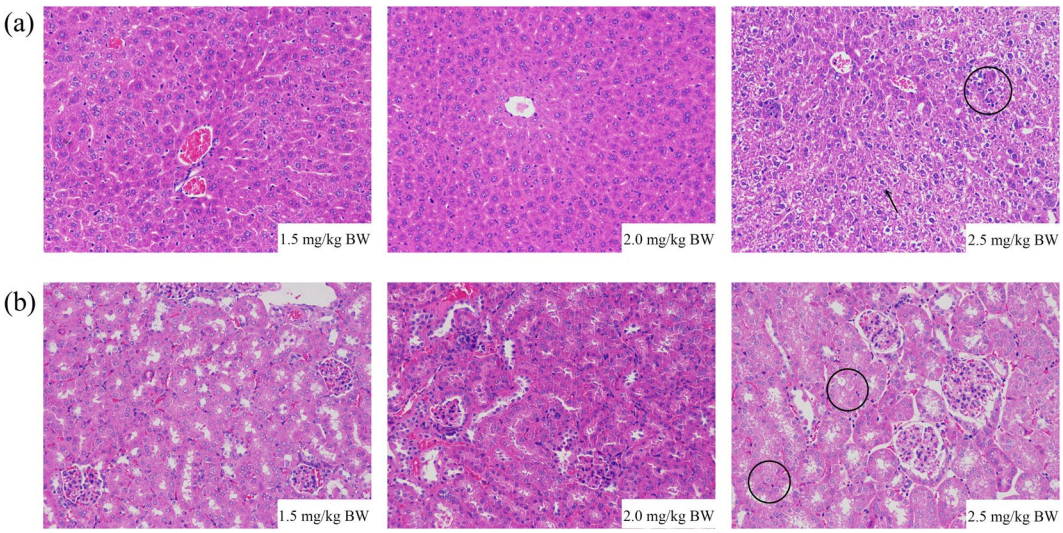

Figure 2. Representative histological photomicrographs of liver (**b**) and kidney (**b**) in mice after exposure to $nTiO_2$ at different doses corresponding to daily intake of $nTiO_2$-contaminated seafood at different pCO_2 levels for 30 days (HE × 200). Three mice in each group were used for histological examination (n = 3). (**a**) Inflammatory cell infiltration (circles) and edema (arrows) were observed in the 2.5 mg/kg BW group. (**b**) Renal tubular epithelial cells were slightly swollen (circles) in the 2.5 mg/kg BW group. No obvious pathological changes were found in the heart, spleen and lung among the different treatment groups.

Discussion

Seafood, including edible bivalves, contributes almost 20% of animal proteins for over three billion people in the world[39]. However, the presence of contaminants in seafood poses threats to consumers' health and therefore draws increasing attention from both the scientific community and regulatory authorities, such as the Food and Agriculture Organization (FAO)[39], the Food and Drug Administration (FDA)[40] and the European Food Safety Authority (EFSA)[41]. The fast growth of the production and application of nanoparticles in a variety of products

will inevitably add onto the release of nanoparticles into the environment, which can accumulate in marine organisms, including commercial seafood species, posing potential threats to human health[22,24,42]. Although some evidence suggests that seafood safety may worsen under future climate scenarios[7,43], the plausible effects of future ocean acidification scenarios on the safety of seafood with respect to nanoparticle contaminations have been largely overlooked.

The present study showed that future ocean acidification scenarios will lead to an increased accumulation of $nTiO_2$ in marine edible bivalves, which may be due to a synergic effect of pCO_2-driven ocean acidification on the uptake of $nTiO_2$ from the environment and the exclusion of $nTiO_2$ out of the body of bivalves. First, ocean acidification may induce $nTiO_2$ accumulation by changing the aggregate size of $nTiO_2$ in seawater. In aqueous solution, nanoparticles tend to aggregate forming large particles and the extent of aggregation is dependent on various factors including the surface charge of nanoparticle and the pH value of medium[29–31]. It has been shown that marine bivalves capture and ingest larger nanoparticle aggregates more efficiently than those in suspension[38,44]. For example, particles larger than 4 μm in diameter can be easily ingested by bivalve species through the process of filter feeding. However, the nanoparticle uptake efficiency of bivalves through this filtration process decreases asymptotically with the decreasing particle size of the nanoparticle[45]. According to previous studies, the pH at the point of zero charge (pH_{zpc}) of nanoparticles has important implications for their aggregation, as electrostatic repulsion between nanoparticles of similar potential decreases when the solution pH approaches the pH_{zpc}[29,31,35]. Our data showed that the zeta potential of $nTiO_2$ approach the critical pH_{zpc} value of 0 in pCO_2-acidified seawater, which subsequently reduced the electrostatic repulsion between $nTiO_2$ favoring the formation of larger aggregates. Therefore, enhanced $nTiO_2$ accumulation under future ocean acidification scenarios could be partially due to easier ingestion of bigger aggregates by marine bivalves.

Second, the increase of $nTiO_2$ accumulation in bivalves could also be attributed to an increase in $nTiO_2$ uptake through other pathways. Theoretically, all substances in the marine environment could diffuse directly into the body via damaged tissues[46]. As increased pCO_2 can bring about severe tissue damage to marine organisms[47,48], ocean acidification may facilitate the entry of nanoparticles into marine organisms by causing tissue lesion. In addition, nanoparticles can also enter the extrapallial fluid between the soft tissues and shell of marine bivalves through incorporation into the nacreous layer[49]. As mantle and labial palps have been confirmed as the target organs for the internalization of nanoparticles in marine invertebrates[50], nanoparticles deposited on the shell surfaces may be transported into the extrapallial fluid and subsequently accumulate in the body. Since ocean acidification can result in shell dissolution and nanoparticle deposition acceleration[5,51], increases in shell permeability and nanoparticle deposition on the shell surface induced by pCO_2 acidification would facilitate the uptake of $nTiO_2$ through the shell-extrapallial fluid pathway.

Third, since some of the nanoparticles ingested will be excreted from the body[52], a hampered $nTiO_2$ exclusion induced by ocean acidification may also account for the increased $nTiO_2$ accumulation. The exclusion of most exogenous substances is an energy-consuming process, and it has been shown that ocean acidification may constrain energy availability for toxicant metabolism in marine invertebrates[7,53]. Therefore, the exclusion of nanoparticle could be reduced to some extent in elevated pCO_2 conditions, leading to an increase in nanoparticle accumulation.

Previous investigations on the impacts of nanoparticles on food safety, including $nTiO_2$, were generally conducted with excessive doses[36]. In addition, although it has been suggested that nanoparticles can accumulate in marine organisms such as gastropods[54], amphipods[20], bivalves[42] and fish[24], the health consequences of oral exposure to nanoparticle-contaminated seafood at environmentally realistic concentrations remains unknown. Our data showed that a 30-day oral exposure to $nTiO_2$ at the equivalent doses of seafood consumption under future ocean acidification scenarios can threaten health in terms of organ lesion and alterations in hematologic indices and blood chemistry values. For instance, increased WBC and lymphocyte counts indicated an inflammatory reaction upon feeding with $nTiO_2$ at the dose equivalent to oral intake via consuming contaminated seafood under future ocean acidification scenarios[55]. In addition, evident injuries were detected in liver since the organ is the main target organ for nanoparticles via oral exposure[56–59]. Similar kidney and hepatic injuries in mice after gastrointestinal $nTiO_2$ exposure has been observed previously, and it was suggested that these injuries are associated with alterations in inflammatory cytokine expression and reduction in detoxification of $nTiO_2$[60].

According to our results obtained, ocean acidification would induce the bioaccumulation of $nTiO_2$ in marine edible bivalves and cause liver and kidney injuries in mice at the equivalent dose of seafood consumption, indicating an increased health risk by consuming seafood under future ocean acidification scenarios. We speculate that this health risk could be underestimated, given the relatively short accumulation duration (30 days) examined in the present study. In addition, both ocean acidification and nanoparticles have been reported to facilitate the accumulation of other toxic pollutants through complex interactions[9,61], further increasing seafood safety risk. Though it is generally accepted that the concentrations of nanoparticles in the environment will grow steadily and reach as high as mg/L level in water system[33], little is known about its environmental concentrations. Future studies should consider long-term effects of nanoparticles and pH in the context of future ocean acidification as well as populations in different geographic locations.

Methods

This study was carried out in three steps namely collection of bivalves from Yueqing Bay (Wenzhou, China) and acclimation in the Qingjiang Station of Zhejiang Mariculture Research Institute (Wenzhou, China) in July 2016, followed by a bioaccumulation experiment under different pH scenarios (pH 7.8 and 7.4, representing ocean acidification scenarios predicted by IPCC in 2100 and 2300, respectively) during August 2016, and mice toxicology assays performed in Zhejiang University (Hangzhou, China) during September 2016 to evaluate human's risk of consuming seafood contaminated with nanoparticle.

Target pH	T (°C)	Sal (‰)	pH_{NBS}	TA (μmol/kg)	pCO_2 (μatm)	DIC (μmol/kg)	Ωara	Ωcal
8.1	25.3 ± 0.4	21.3 ± 0.3	8.10 ± 0.03	2074 ± 6	581 ± 13	1912 ± 6	2.31 ± 0.03	3.65 ± 0.05
7.8	25.1 ± 0.3	21.7 ± 0.3	7.82 ± 0.03	2094 ± 11	1188 ± 25	2026 ± 4	1.27 ± 0.02	2.00 ± 0.04
7.4	25.2 ± 0.2	21.4 ± 0.3	7.41 ± 0.02	2073 ± 9	3140 ± 22	2153 ± 10	0.54 ± 0.01	0.85 ± 0.02

Table 4. Carbonate chemistry variables of seawater during the experiment (mean ± SE). T: temperature; Sal: salinity; TA: total alkalinity; pCO_2: CO_2 partial pressure; DIC: dissolved inorganic carbon; Ωara: aragonite saturation state; and Ωcal: calcite saturation state.

Collection and acclimation of bivalves. Adult *T. granosa* (29.6 ± 3.5 mm), *M. meretrix* (50.6 ± 4.7 mm) and *C. sinensis* (36.0 ± 4.4 mm) of similar shell length outside their breeding seasons were obtained from Yueqing Bay (Wenzhou, China). In order to determine the TiO_2 concentration in these bivalve species from sampling sites, 10 individuals of each species were analyzed and TiO_2 concentrations in all bivalve species were found to be under detection limits (0.3 ng/mg). To obtain the background concentration of TiO_2 in the seawater, 10 mL of seawater samples was analyzed and TiO_2 concentrations in these samples were 2.7 ± 0.4 μg/L. The TiO_2 concentrations in both tissues and seawater samples were determined following the methods of the National Standard of China (Determination of titanium in foods, GB5009.246-2016)[62]. Prior to experiments, bivalves were acclimatized in sand-filtered seawater (pH 8.10 ± 0.03, salinity 21.3 ± 0.6‰) at an ambient water temperature 25.3 ± 0.5 °C with constant aeration and a natural light/dark cycle for at least 7 days. Bivalves were fed daily with microalgae (*Tetraselmis chuii*) at the satiation feed rate during the acclimation, and selected bivalves were cultured overnight in filtered seawater without feeding before the exposure experiment.

***p*CO_2-driven seawater acidification.** Sand-filtered seawater from the clam-sampling site, Yueqing Bay, was used throughout the experiment. To simulate the present and near-future projections of the Intergovernmental Panel on Climate Change (IPCC), one ambient present pH (pH at 8.1) and two lower pH levels (pH 7.8 and 7.4, representing ocean acidification scenarios in 2100 and 2300, respectively) were used in the present study[2,3]. Seawater at different pH values was achieved and maintained by continuous aeration with CO_2-air mixture with corresponding predicted pCO_2, which was obtained by mixing CO_2-free air and pure CO_2 gas at known flow rates using flow controllers (Fig. S3)[7,9]. Throughout the experiment, pH calibrated with standard US National Bureau of Standards buffers (pH_{NBS}), salinity, temperature, total alkalinity (TA) and carbonate system parameters including dissolved inorganic carbon (DIC), aragonite saturation state (Ωara) and calcite saturation state (Ωcal) were monitored daily. Seawater pH was measured with a Sartorius PB-10 pH meter (Sartorius, Germany) with an accuracy of ±0.01. Salinity was measured using a conductivity meter (Multi 3410 WTW, Germany) with an accuracy of ±0.5% measurements. Total alkalinity measurements were performed by potentiometric titration with an automatic titrator system (SMTitrino 702, Metrohm). Throughout the experiment, seawater pH_{NBS} was measured and adjusted by pH/ORP controllers (PC-2110, House, China; pH fluctuations were controlled within 0.01 units) to maintain the desired pH. The carbonate system parameters were calculated from the measured pH, salinity, temperature and TA values using the open-source program CO2SYS, as described previously[10]. The pH fluctuations were within 0.01 units over the 21 days exposure in all tanks. Seawater carbonate chemistry parameters in each tank measured and calculated daily for all treatments during the exposure were summarized in Table 4.

Characterization of nanoparticles. In this study, $nTiO_2$ were purchased from Shanghai Klamar Reagent Co. Ltd, China, and the size and shape of the $nTiO_2$ particles were determined using transmission electron microscopy (TEM, JEM-1230, JEOL, Tokyo, Japan). Crystal structure of the particles was identified using X-ray powder diffractometry (XRD, Rigaku D/MAX 2550/PC, Tokyo, Japan), and surface area was measured through Brunauer-Emmett-Teller (BET) adsorption measurements (Micromeritic TriStarII 3020, Micrometritics Instrument Corp., Norcross, GA). The majority of $nTiO_2$ used in this study was anatase crystals with irregular shapes and a surface area of 60.65 m^2/g (Fig. S4 and Table 1). To minimize weighing errors and ensure concentration accuracy, a stock solution of 1 g/L $nTiO_2$ was prepared daily by dispersing the $nTiO_2$ in ultrapure water followed by sonication for 15 mins[57,63,64]. Test solutions of $nTiO_2$ were prepared immediately prior to use by diluting the stock solution with 0.1-μm membrane-filtered seawater (pH 8.10, salinity 21.3‰). Particle hydrodynamic diameter and zeta potential of $nTiO_2$ in seawater at different pH values (ambient pH 8.1, pH 7.8 and 7.4) were tested with the Zetasizer Nano ZS90 (Malvern Instruments Ltd, Malvern, UK).

Bioaccumulation experiment. Since $nTiO_2$ is mostly introduced into the environment via sewage discharge and it remains about 100 μg/L $nTiO_2$ in the waste water effluents after treatment[33,36], seawater in polluted areas could be contaminated at equivalent magnitudes, especially when the steady increase of environmental $nTiO_2$ is taken into account. Therefore, 100 μg/L was chosen to simulate the environmental concentration of $nTiO_2$ in the polluted areas in this study. After one week of acclimation, bivalves (90 individuals for each species) were randomly assigned into plastic tanks, with a total seawater volume of 30 L containing approximately 100 μg/L $nTiO_2$ and maintained under the desired pH levels. In total, 27 experimental tanks and 270 bivalves (10 individuals per tank × 3 replicate tanks × 3 pH levels × 3 species) were used in the present study. Bivalves were fed with *T. chuii* twice a day during the experiment and the seawater was changed daily with corresponding pre-acidified seawater containing the desired concentration of $nTiO_2$. No mortality was observed throughout the experimental period.

	background	pH 8.1	pH 7.8	pH 7.4
Target concentration	0	100	100	100
Ti concentration	2.7 ± 0.4	99.5 ± 3.5	97.3 ± 1.7	98.3 ± 1.7

Table 5. Background and working Ti concentration (μg/L) at different pH levels (mean ± SE).

Following that of Johnston[65], Hooper[66], and Gaiser[67], in the present study an exposure time of 21 days was adopted to avoid the effect of stress syndrome. After 21-day exposure to 100 μg/L $nTiO_2$ at different pH levels, five live individuals of each species were randomly taken from each tank and purged in sand-filtered seawater overnight. After rinsing with ultrapure water, the bivalve individuals were dissected on ice and the gill, mantle and foot muscle of each individual were peeled off and stored separately at −20 °C for TiO_2 residue analysis. TiO_2 concentrations in the various tissues were calculated and expressed in mg/kg dry weight for each individual. Similarly, the entire soft body of bivalves was peeled off to determine TiO_2 concentration (expressed in mg/kg wet weight), which was later used to calculate the equivalent oral exposure dose of $nTiO_2$ used in the mice toxicology assays.

Content analysis of $nTiO_2$. To obtain working concentration of $nTiO_2$ in the seawater for each experimental group (pH at 8.1, 7.8, and 7.4, respectively), 1 mL of seawater samples (three replicates for each experimental group) were taken after seawater renewal. In addition, approximately 0.1–0.3 g of each tissue or the entire soft body were used to determine the amount of accumulated $nTiO_2$. The contents of TiO_2 in both tissue and seawater samples were determined following that of the National Standard of China (GB5009.246-2016)[62]. In brief, the water and tissue samples were digested in ultrapure nitric acid overnight. After adding 0.5 mL H_2O_2, mixtures were heated with an electric heating plate until samples were completely digested, and the remaining nitric acid was removed until colorless and clear solutions were achieved. The solutions were diluted to 3 mL with 2% nitric acid and used for titanium concentration measurement using inductively coupled plasma atomic emission spectrometry (ICP-MS, PE NexION 300X, USA). Indium (20 ng/ml) was taken as the internal standard and the detection limit of titanium was 0.074 ng/ml. The background and working concentrations of $nTiO_2$ in seawater during the 21-day experiment are listed in Table 5.

Mice toxicology assays. Healthy Kunming (KM) male mice (8 weeks old) were purchased from the Experimental Animals Center of Zhejiang University and housed in plastic laboratory animal cages in room conditions (20 ± 2 °C, 60 ± 10% relative humidity, under a 12 h light/dark cycle) for a week. During the acclimation, a commercial pellet diet and deionized water were available *ad libitum*. After 7 days of acclimation, 15 adult mice were equally divided into 3 treatment groups (n = 5): the pH 8.1 exposed group, the pH 7.8 exposed group, and the pH 7.4 exposed group. All experiments were approved by the Animal Care Committee of Zhejiang University and all methods were performed in accordance with the Guidelines for the Care and Use of Animals for Research and Teaching at Zhejiang University.

The obtained mean values of the amount of $nTiO_2$ accumulated in *M. meretrix*, the lowest of the three bivalve species, were used to calculate oral exposure doses. According to previous dietary surveys and guidelines[68,69], 150 g/person, equivalent to approximately 3 g/body weight (BW)/day, was used as the amount of daily intake of seafood. Based on the $nTiO_2$ concentrations detected in bivalve *M. meretrix* in the present study (5.05, 6.77, and 8.30 mg/kg at pH 8.1, 7.8 and 7.4, respectively), the daily intake of dietary $nTiO_2$ through consuming $nTiO_2$ contaminated seafood raised under pH 8.1, 7.8, and 7.4 was estimated to be 0.015, 0.020, and 0.025 mg/kg, respectively. Taking the interspecies extrapolation into consideration[70], a 100-fold dose of the human exposure was used for mice assays (1.5, 2.0, and 2.5 mg/kg BW, respectively). After 15-min sonication in ultrapure water, $nTiO_2$ suspensions were given to mice by gavage once a day for 30 consecutive days. During the 30-day experiment, body weight was recorded every 5 days and any symptom or mortality was observed and recorded daily. After 30 days of $nTiO_2$ exposure, all mice were weighed and sacrificed after anesthetization. Blood samples were collected from the femoral artery in the groin area. Serum was obtained by centrifuging blood at 3000 rpm for 15 min. Tissues and organs such as heart, kidney, spleen, lung and liver were excised and weighed. Tissue samples for histopathologic examination were fixed in 10% neutral buffered formalin.

After weighing, the coefficients of various tissues to the body weight were calculated as the ratio of tissues (wet weight, mg) to body weight (g). Blood component parameters were determined with an auto hematology analyzer (BC-2800Vet, Shenzhen, China). Liver function was evaluated based on the serum levels of ALT, AST and ALP. Nephrotoxicity was determined by blood urea nitrogen (BUN) and Crea, which were determined using an automated biochemical analyzer (Hitachi 7170 A, Tokyo, Japan). All histopathological observations were performed according to standard laboratory procedures. Tissues were embedded in paraffin, sliced into 5-μm thicknesses and placed onto glass slides. After hematoxylin-eosin (HE) staining, the slides were examined and images were taken using an optical microscope (Nikon Eclipse Ci-L, Tokyo, Japan). The identity and analysis of the pathology slides were blind to the pathologist.

Statistical analyses. Differences in hydrodynamic diameter and zeta potential of $nTiO_2$ in seawater at different pH levels (pH 8.1, 7.8, and 7.4) were compared by one-way analyses of variance (One-way ANOVAs) followed by post-hoc Tukey tests using OriginPro 9.0.

The $nTiO_2$ concentrations accumulated in individuals were assessed using a linear mixed effects model with treatment pH as a fixed factor and the treatment tank as a random factor. In total, nine linear mixed effects models were performed for each tissue and species investigated (3 species × 3 tissues) using 'R' statistical package lme4 (R Development Core Team, 2012).

Differences in hematologic indices and blood chemistry values of the mice after oral exposure to different $nTiO_2$ doses were evaluated using one-way ANOVAs followed by post-hoc Tukey tests using OriginPro 9.0. Percentage data (e.g. percentages of monocytes, granulocytes and lymphocytes) were arcsine-square root transformed prior to analysis to meet the assumption of a normal distribution[71].

For all analyses, Levene's test and Shapiro-Wilk's test were performed using OriginPro 9.0 to verify homogeneity of variance and normality, respectively. All data were presented as mean ± standard error (SE) and a p-value at $p < 0.05$ was taken as statistically significant.

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Author Contributions

W.S., Y.H., C.G., X.G.Z. and G.X.L. contributed to all aspects of the paper, including study design, statistical analysis and writing and revisions. S.J.Z., W.H.S. and Y.C.W. contributed to the design of the study, to substantive analysis of the results and to revisions of the paper.

Additional Information

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