1 **Trophic transfer and accumulation of TiO₂ nanoparticles from clamworm**
2 **(*Perinereis aibuhitensis*) to juvenile turbot (*Scophthalmus maximus*) along a**
3 **marine benthic food chain**

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Abstract

In the present work, we investigated the potential benthic trophic transfer of TiO₂ nanoparticles (NPs) from clamworm (*Perinereis aibuhitensis*) to juvenile turbot (*Scophthalmus maximus*) and their related distribution and toxicity. TiO₂ NPs (at 10, 50 and 100 mg/L) could be taken up by clamworms, and mainly accumulated in the lower-digestive tract. TiO₂ NPs were able to transfer from clamworms to juvenile turbot. The accumulation of TiO₂ NPs in juvenile turbot increased with increasing Ti contents in clamworms during the dietary exposure, however, no biomagnification (BMFs, 0.30-0.33) of TiO₂ NPs was observed. For both dietary and waterborne exposure, accumulation of TiO₂ NPs was higher in the gill, intestine and stomach of juvenile turbot, following by skin, liver, and muscle. During dietary exposure at Day 20, the growth of turbot was reduced, and abnormal symptoms of liver and spleen were detected. Moreover, both dietary (50 and 100 mg/L TiO₂ NPs-treated clamworms) and waterborne (100 mg/L TiO₂ NPs) exposures led to significantly lower protein and higher lipid contents, suggesting the nutrition quality reduction of turbot. The findings from this work highlighted the trophic transfer of TiO₂ NPs in marine benthic food chain, leading to the potential negative impact on marine aquaculture and food quality.

Keywords: TiO₂ nanoparticles; Aggregation; Trophic transfer; Benthic organisms; Bioaccumulation; Food safety

1. Introduction

With the development and application of nanotechnology, engineered nanomaterials are widely applied in various fields, with more than 1600 categories of nano-enabled products in the world by 2015 (CPI, 2015). It is estimated that nanotechnology will achieve a \$3 trillion market value by 2020 (Roco et al., 2011). During the production and use of nano-enabled products, nanoparticles (NPs) will be released into the environment through various routes such as atmospheric deposition (Montes et al., 2012), stream and urban runoff (Shandilya et al., 2015), and wastewater treatment plant effluent (Ma et al., 2014), and most likely end up in the ocean. Therefore, estuarine and coastal environments could be a sink for NPs. The NPs released into ocean will aggregate and settle to benthic environments because of high ionic strength and low organic carbon content of seawater (Keller et al., 2010). Thus, the behavior of NPs in benthic environments and related ecological impact will be a major concern.

Benthic organisms, widely present in coastal marine areas, are important parts in marine ecosystems. It is reported that benthic organisms could affect the fate and transport of NPs through bioturbation, uptake, and biotransformation (Montes et al., 2012; Conway et al., 2014). Meanwhile, NPs are able to cause adverse effects to marine benthic organisms such as oyster, sea urchin, and mussel after both waterborne and dietary exposures (Johnson et al., 2015; Wu et al., 2015; Bourgeault et al., 2015). In addition, benthic invertebrates are an important food source for other marine organisms such as benthic vertebrates, marine fish, and seabirds. This prey-predator

relationship is likely to transfer NPs to higher trophic levels, thus posing ecological and economic concerns (Galloway et al., 2010). However, the information on bioaccumulation and distribution of NPs in benthic organisms is scarce. Currently, the food-chain investigations on NPs are mainly focused on freshwater and terrestrial ecosystems (Dalai et al., 2014; Mattsson et al., 2014; Hawthorne, et al., 2014; De la Torre Roche et al., 2015). There are only a few studies investigating the food-chain transport of NPs between marine organisms. Conway et al. (2014) reported that CeO₂ NPs could transfer from phytoplankton to marine mussels. Wang and Wang (2014) demonstrated that the growth and water content of marine medaka were reduced after being fed with high dosages of Ag NPs-contaminated brine shrimp (Ag content, 181 µg/g dry wt.). However, these studies only focused on the food chains in the water column, not the benthic food chain. In fact, most NPs in seawater column could aggregate, precipitate, and accumulate in sediments, therefore leading to greater potential of NPs exposure and bioavailability to benthic organisms than pelagic organisms. Therefore, it is hypothesized that NPs could be transferred through the benthic food chain and accumulated in higher trophic-level organisms. Accumulated NPs are able to cause pathological damage of target organisms/tissues and nutritional compositional changes of higher trophic-level organisms. The bioaccumulation, distribution, and trophic transfer of NPs may be affected by different exposure routes (e.g., dietary and waterborne exposure), which will be investigated in this study.

In this work, clamworm (*Perinereis aibuhitensis*) and turbot (*Scophthalmus maximus*) were selected as the model organisms for benthic invertebrate and benthic

carnivorous fish, respectively. Clamworm is the primary food source for many commercial fish such as turbot, and plays an important role in transferring contaminants to higher trophic levels in marine food webs. Turbot is an important commercial mariculture species. TiO_2 was chosen as the model NPs because of its insolubility (Wang et al., 2009), highest production amount, and modeling concentration in the environment (Liu and Cohen, 2014; Hendren et al., 2011). The objectives of this work were to (1) investigate the bioaccumulation/distribution of TiO_2 NPs in clamworm and their subsequent trophic transfer to juvenile turbot; (2) explore the influence of exposure routes (e.g., dietary and waterborne exposure) on distribution, trophic transfer, and excretion of TiO_2 NPs; and (3) reveal possible biological changes (growth, pathology, and nutritional composition) of juvenile turbot during trophic transfer of TiO_2 NPs. The information provided here will be helpful for understanding the transport and fate of NPs in benthic marine environments and assessing the exposure and health risk of NPs.

2. Materials and methods

2.1. Chemicals

TiO_2 NPs were purchased from Sigma-Aldrich (USA). Individual size and morphology of TiO_2 NPs were characterized using transmission electron microscopy (TEM, H-7650, Hitachi). The surface area of TiO_2 NPs was measured based on adsorption-desorption of N_2 using Autosorb-1 (Quantachrome, USA). A suspension of TiO_2 NPs (100 mg/L) was prepared in ultrapure water and seawater with bath sonication (40 kHz, 30 min) (KQ-500DB, Kunshan ultrasonic instrument Co., China).

The hydrodynamic diameter and zeta potential of TiO₂ NPs were determined by Zetasizer Nano (Nano Series ZS90, Malvern). The TiO₂ suspension (100 mg/L) prepared by seawater was shaken at 18 °C for 24 h, and the concentration of released titanium ions was determined by inductively coupled plasma mass spectrometry (ICP-MS, iCAP Q, Thermo Scientific).

2.2. Benthic marine organisms

Clamworms (*Perinereis aibuhitensis*) were obtained from Qingdao Honzenda Co. (Qingdao, China). The obtained clamworms were acclimated in filtered seawater (18 ± 1 °C) with aeration for 3 days. Then, healthy clamworms with similar weight (2.8 ± 0.3 g) were selected and fasted for another 3 days before exposure. Juvenile turbot (*Scophthalmus maximus*) were obtained from an aquatic market in Qinhuangdao, China. Juvenile turbot were cultured in filtered seawater for 2 weeks before exposure. During culturing, fish were trained to eat clamworms (5% of fish wet weight each time) twice a day (08:00–09:00 and 17:00–18:00), and all the fish (42.5 ± 0.4 g) were fasted for 24 h before exposure. Temperature, pH, and salinity (18 ± 1 °C; 8.0 ± 0.1; 25 ± 1.5 ‰) of seawater were monitored daily in all tanks for both acclimation and subsequent exposure experiments.

2.3. Exposure of clamworm to TiO₂ NPs

A stock suspension of TiO₂ NPs (500 mg/L) was prepared in seawater by bath sonication (40 kHz, 30 min). Then, this stock suspension was diluted to each tank (1 L) with filtered seawater to achieve the final TiO₂ concentrations of 10, 50, and 100 mg/L. For TiO₂ NPs exposure, every 10 pre-cultured clamworms were moved into

each tank which already contained TiO₂ NPs (10, 50 or 100 mg/L) in seawater (1 L) under dark and continuous aeration. After exposure for 24 h, the clamworms were rinsed for four times with ultrapure water to remove the adsorbed TiO₂ NPs, and then stored at -80 °C as food for juvenile turbot feeding. Another experiment was conducted to check if this water rinsing procedure could sufficiently remove the clamworm-adsorbed TiO₂ NPs. Briefly, we exposed the dead clamworms to TiO₂ NPs with different concentrations (0-100 mg/L) for 24 h, and then rinsed with ultrapure water four times before digestion and Ti determination. The result suggested that Ti contents in clamworms showed no significant difference among all the treatments (0, 10, 50, and 100 mg/L TiO₂ NPs) ($p < 0.05$) (Table S1), suggesting that this rinsing procedure was sufficient to remove the adsorbed TiO₂ NPs on the surface of clamworms. For each exposure concentration of TiO₂ NPs, 150 replicates were set up to ensure abundant clamworms for the following food-chain experiments. Further, 10 clamworms were randomly selected for the determination of Ti in whole body and different organs/tissues (i.e., head, upper-digestive tract and lower-digestive tract) of clamworms (Fig. S1). In addition, 10 more clamworms after exposed to TiO₂ NPs (100 mg/L, 24 h) were rinsed and cultured in blank seawater for another 24 h. The feces of clamworms were then collected for further analysis.

2.4. Transfer of TiO₂ NPs from clamworm to juvenile turbot through dietary exposure

Dietary exposure included uptake (20 days) and depuration (7 days) periods. During the uptake period, for each treatment, 36 juvenile turbot in a 300-L tank were fed with TiO₂ NPs-treated clamworm twice a day (08:00–09:00 and 17:00–18:00) (5%

of fish wet weight each time). Every 3 fish were sampled on day 0, 5, 10, 15, and 20, respectively. Then, the whole fish were stored at -80°C for further Ti determination. In addition, on Day 20, the total body weights and lengths of remaining fish in the tanks were measured after fasting for 24 h. Then, fish were euthanized with an ice/water mixture according to the euthanasia guidelines (NIH Guidelines for use of zebrafish, 2011). The liver, stomach, intestines, gill, skin, muscle and blood of fish were immediately excised, rinsed in 0.9% NaCl solution (except blood), and weighed for further histological analysis and Ti measurements. During the depuration period (Day 20-Day 27), all the fish after dietary exposure for 20 days were fed with untreated clamworms. On Day 22, 24, and 27, every 3 fish in each tank were sampled and stored at -80°C for Ti measurement. The remaining fish on Day 27 were fasted for 24 h, and weights and lengths were recorded. For uptake and depuration periods, feces were collected, and the whole volume of TiO_2 NPs-containing seawater (uptake period) or blank seawater (depuration period) in each tank was renewed every day to ensure a clean culturing environment.

2.5. Accumulation and distribution of TiO_2 NPs in juvenile turbot through waterborne exposure

For the waterborne exposure, fish were exposed to waterborne TiO_2 NPs (10 mg/L) for 20 days (uptake period) and followed by 7 days of depuration period. For both uptake and depuration periods, fish were fed with normal (without TiO_2 NPs-treated) clamworms (5% of fish wet weight each time, twice a day). The time points for fish sampling and the following dissection procedures were the same to that

in the dietary exposure.

2.6. Assessment of health status and histological analysis of juvenile turbot

The pathological changes of fish after uptake period of dietary exposure were analyzed. Briefly, fresh organs of intestine, liver, and spleen sampled from juvenile turbot were fixed in 10% neutral formalin buffer solution. The samples were further processed for paraffin embedding, sectioning, and Hematoxylin and Eosin (H&E) staining. In addition, livers were stained with Masson's trichrome to detect hepatic fibrosis. All the sections were then observed with an optical microscope (BX51T-PHD-J11, Olympus, Japan) for histological analysis.

Growth parameters of fish after 27-day dietary or waterborne exposure were calculated as follows (Wang et al., 2015):

Weight gain rate (WGR, %) = $100 \times [(FBW - IBW) / IBW]$, where IBW (g) and FBW (g) are initial body weight and final body weight (after 27-day dietary or waterborne exposure), respectively.

Specific growth rate (SGR, %/d) = $100 \times (\ln (FBW) - \ln (IBW)) / \text{days}$.

Survival rate (SR, %) = $100 \times (\text{the number of live fish after exposure} / \text{the number of live fish before exposure})$.

Morbidity (%) = $100 \times (\text{the number of sick fish after exposure} / \text{initial healthy fish number})$.

Condition factor (CF, %) = $100 \times (\text{fresh body weight, g}) / (\text{body length, cm})^3$.

Hepatosomatic index, (HSI, %) = $100 \times (\text{fresh liver weight} / \text{fresh body weight})$.

Viserosomatic index, (VSI, %) = $100 \times (\text{fresh visceral weight} / \text{fresh body weight})$.

2.7. *The fish body nutritional composition assays*

The dry matter (DM), crude protein, crude lipid of whole body, and livers of fish were analyzed according to Association of Official Analytical Chemist (AOAC, 1995). For DM, the whole fish were dried to constant weight at 105 °C, while livers were vacuum-freeze dried (Labconco, USA). The dry samples of whole body and livers were grounded, and the crude protein and crude lipid in whole body and livers were determined using Kjeldahl and Soxhlet methods, respectively.

2.8. *Ti determination*

The samples of whole body of clamworms, and organs/tissues and feces of turbot were freeze dried. All dried samples were then acid-digested by microwave digestion (MARSPress, CEM, USA) following the approach of Zhu et al. (2010). Ti contents in the digested samples were determined by ICP-MS. To analyze the food chain transfer of TiO₂ NPs from clamworm to juvenile turbot, bioaccumulation factor (BCF) and biomagnification factor (BMF) were calculated as follows:

$$\text{BCF} = \text{Ti concentration in fish body} / \text{Ti concentration in seawater}$$

$$\text{BMF} = \text{Ti concentration in fish body} / \text{Ti concentration in clamworm}$$

2.9. *Statistical Analysis*

All statistical analyses were performed using STATISTICA 6.0. All the data were subjected to one-way analysis of variance (ANOVA) followed by a Newman-Keuls multiple comparison test. A level of $p < 0.05$ was considered statistically significant.

3. **Results and discussion**

3.1. Uptake of TiO₂ NPs by clamworm

TEM image in Fig. S2A shows that TiO₂ NPs were globular, with the individual size around 20 nm. Surface area of TiO₂ NPs was 39.2 m²/g. The hydrodynamic diameters of TiO₂ NPs in DI water and seawater were 260 and 1660 nm, respectively (Table S2), suggesting that TiO₂ NPs tended to aggregate in seawater. This aggregation behavior could lead to the sedimentation of TiO₂ NPs, which was supported by our light microscopic image in Fig. S2B.

After exposure to 10, 50, and 100 mg/L of TiO₂ NPs for 24 h, Ti contents in clamworms were 108 ± 14, 159 ± 12, and 194 ± 17 µg/g dry wt., respectively (Fig. 1A), showing that Ti accumulation in clamworms was TiO₂ NPs concentration-dependent. The BCFs of Ti were calculated as 10.8, 3.18, and 1.94 for exposed TiO₂ NPs at 10, 50, and 100 mg/L, respectively (Table S3). TiO₂ NPs in seawater were mainly internalized by clamworms through mouth to digestive tract. Therefore, distribution of TiO₂ NPs in different parts (head, upper-digestive tract, and lower-digestive tract) of clamworms was investigated. Ti mainly accumulated in the lower-digestive tract (59.4%) after exposure for 24 h (Fig. 1B), indicating that TiO₂ NPs certainly entered the intestines, and some NPs were likely to be excreted. This was supported by the image of feces (Fig. S3), in which the feces appeared white in color when clamworms were treated with TiO₂ NPs. TiO₂ NPs cannot be dissolved in seawater (Table S2), which is supported by our previous study (Wang et al., 2009). Clamworm, a stomachless organism, cannot provide an extreme (e.g., highly acidic) environment to enhance NPs dissolution. Therefore, the detected Ti in clamworms

should be in the form of TiO₂ NPs. Although some TiO₂ NPs were excreted with feces, the high accumulation (> 100 µg/g dry wt.) was still observed. These TiO₂ NPs in the clamworms could be transferred to their predators at higher trophic levels.

3.2. Uptake of TiO₂ NPs by juvenile turbot through dietary and waterborne exposure

Accumulation of TiO₂ NPs in juvenile turbot during dietary and waterborne TiO₂ NPs exposure was investigated as a function of exposure times (Fig. 2). For dietary exposure, juvenile turbot were fed with clamworms that were pre-treated with TiO₂ NPs at 10, 50 and 100 mg/L (defined as 10DE, 50DE, and 100DE, respectively). Ti contents in fish gradually increased with increasing exposure times during the uptake period (0-20 days). At the end of uptake period (Day 20), Ti contents of fish in the 10DE, 50DE, and 100DE treatments reached the maximum values of 32.7 ± 3.8 , 53.0 ± 6.9 , and 59.3 ± 3.4 µg/g, respectively, suggesting that the body burden of TiO₂ NPs in the predator turbot was positively affected by TiO₂ contents in food (clamworms). During the depuration period (Day 20-Day 27), Ti contents rapidly decreased and then reached the final Ti contents of 6.5-13.5 µg/g at Day 27.

Upon waterborne exposure (10 mg/L TiO₂ NPs, 10WE), Ti content in juvenile turbot at the end of uptake period (Day 20) was much higher than that after dietary exposure (10DE, 50DE, and 100DE) ($p < 0.05$) (Fig. 2). During the depuration period (Days 20-27), Ti contents in fish (10WE) decreased faster than that upon dietary exposure over time, showing that free TiO₂ NPs in seawater column were more readily excreted in comparison to TiO₂ NPs in food (clamworms). The reason may be that TiO₂ NPs immobilized in clamworms required digestion in turbot before excretion

while free TiO₂ NPs could be directly excreted from turbot through the digestive tract. At the beginning of depuration (Day 20), a certain amount of TiO₂ NPs might be still adsorbed/bound on the skin of turbot after rinsing. These adsorbed TiO₂ NPs could be gradually desorbed from skin of turbot during 7-day depuration, which may be another reason for the sharp decrease of Ti contents in fish in the 10WE treatment. On Day 27, there was no significant difference between waterborne (10WE) and dietary exposure (10DE) ($p>0.05$).

It is of importance to explore the biomagnification of NPs in food chain (Klaine et al., 2008). The biomagnification of a contaminant in a food web occurs when BMF was higher than 1 (Kelly et al., 2007). Table S4 shows that the BMFs of juvenile turbot fed with 10, 50 and 100 mg/L TiO₂ NPs-treated clamworms were 0.30, 0.33, and 0.32, respectively, clearly suggesting that no biomagnifications of TiO₂ NPs occurred along this food chain. It is reported that TiO₂ NPs (Zhu et al., 2010) and CdSe/ZnS quantum dots (Lewinski et al., 2011) could also be trophically transferred from daphnia to zebrafish in freshwater system, but biomagnifications did not occur, which is in agreement with our findings in marine systems. Only one report observed the biomagnification of NPs (CdSe quantum dots) in aquatic food chain, in which cadmium concentration in the protozoa predator (*Tetrahymena thermophila*) was 5 times higher than that in the bacterial prey (*Pseudomonas aeruginosa*) (Werlin et al., 2011). Organism species may be responsible for the above conflicting findings on NPs transfer, which needs further investigation. The surface properties of NPs could be important to its biomagnification within the food chain. Hydrophobic organic

contaminants such as dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs) tended to accumulate in lipids and fats of organisms (Mader et al., 1996). However, most NPs such as TiO_2 are relatively hydrophilic, which could be a reason for the low BMFs during food chain transfer.

3.3. Distribution of TiO_2 NPs in different organs/tissues of juvenile turbot after dietary and waterborne exposure

The distribution of TiO_2 NPs in different organs/tissues of juvenile turbot was investigated after dietary and waterborne exposures for 20 days (Fig. 3). For all the treatments (10DE, 50DE, 100DE, and 10WE), Ti contents followed the order: gill > intestine > stomach > skin > liver > muscle. For dietary exposure, Ti contents in a given organ/tissue increased with increasing Ti contents in clamworms (108 ± 14 , 159 ± 12 , and 194 ± 17 $\mu\text{g/g}$ dry wt. for 10DE, 50DE, and 100 DE, respectively), suggesting that TiO_2 NPs accumulation in organs/tissues of fish predator was dependent on their contents in the clamworm prey. For waterborne exposure (10WE), Ti contents in gill and skin were significantly higher than those of all the DE treatments. This is probably because TiO_2 NPs could directly contact gill and skin of juvenile turbot without the immobilization in clamworms as in the DE system.

The highest Ti level was detected in gills of juvenile turbot for both dietary and waterborne exposures (Fig. 3A), followed by digestive tract (intestine and stomach) (Fig. 3B, 3C), showing that gill and mouth were major routes for NPs uptake for exposures. Ti contents in gills were 55.0 ± 5.6 , 90.2 ± 7.7 , 95.2 ± 7.8 , and 130 ± 7 $\mu\text{g/g}$ for 10DE, 50DE, 100DE, and 10WE treatments, respectively. Gills with a high

surface area could be an initial site for NPs accumulation (Zhao et al., 2011; Jang et al., 2014). In addition, TiO₂ NPs in seawater were mainly in the form of relatively big aggregates (Fig. S2B), therefore, a large amount of TiO₂ NPs could accumulate in gills without much further transport to other organs/tissues. For dietary exposure, TiO₂ NPs could be taken up along with clamworms by turbot and then be accumulated in stomach and intestine after the digestion process. During waterborne exposure, a high amount of TiO₂ NPs was also detected in digestive tract because turbot drink seawater to balance osmotic stress.

Interestingly, Ti was detected in the blood of juvenile turbot for all the treatments (53-111 ng/mL) (Fig. 3D). This indicated that TiO₂ NPs could be absorbed by capillary vessels in digestive tract and/or gills, thus being migrated to bloodstream. Although TiO₂ NPs formed aggregates in seawater, individual or aggregated TiO₂ NPs could still be transferred from gills to bloodstream. Johnston et al. (2010) found that TiO₂ NPs were able to go across the epithelial membrane in the gill of rainbow trout. Epithelium cells in gastrointestinal tract were also reported as an important route for transfer of Ag NPs to blood and liver tissue of rainbow trout (Scown et al., 2010). The presence of TiO₂ NPs in bloodstream suggests that TiO₂ NPs were able to enter the circulatory system of turbot.

After entering bloodstream and circulatory system, TiO₂ NPs were able to accumulate in other organs/tissues such as livers and muscle (Fig. 3E, 3F). Macrophages located in the liver have the ability to remove NPs and other foreign particles from the bloodstream (Panagi et al., 2001). After reaching liver tissues, TiO₂

NPs with individual size (~20 nm) smaller than the pores of liver fenestrae (about 100 nm) could be taken up by the liver (Dziendzikowska et al., 2012). Ti contents in livers (0.7-1.7 µg/g) were much lower than gills and intestines (Fig. 3) although liver is commonly recognized as a target organ and an important line of defense against NPs. After waterborne exposure of TiO₂ NPs (100 mg/L) for 20 days, Ti content (1.2 µg/g) in the liver of juvenile turbot was much lower than Cu content (~200 µg/g) in the same organ of carps after treated with CuO NPs at the same concentration (Zhao et al., 2011). In addition to the difference of tested fish (juvenile turbot *versus* juvenile carp), possible explanations for this accumulation difference could be (1) much higher aggregation of NPs in seawater than that in tap water; and/or (2) TiO₂ NPs are insoluble while CuO NPs could be partially dissolved in both tap water and carp bodies. Ti contents in muscle of juvenile turbot were in the range of 0.5-0.6 µg/g for both dietary and waterborne exposures (Fig. 3F), which were the lowest in all the tested organs/tissues, and thus, turbot muscle was not the main target organ/tissue for NPs accumulation. However, turbot is an important commercial fish species, and the trophic transfer of TiO₂ NPs in juvenile turbot highlights potential risk to human food safety.

Ti was also detected in the skin sample (14-33 µg/g dry wt.) of juvenile turbot after dietary and waterborne exposures (Fig. 3G). The nonlinear response of the detected Ti in turbot skin as a function of exposed TiO₂ NPs concentrations could be partially caused by possible nonlinear adsorption/binding of TiO₂ NPs on skin, which may be influenced by the charge properties of TiO₂ NPs (positively charged, Table S2)

and instability of TiO₂ suspensions in seawater. In addition, TiO₂ NPs could be irreversibly trapped or/and internalized in skin. The renewal of TiO₂ NPs-containing seawater (once a day) in each tank during the exposure may further increase the amount of TiO₂ NPs that were irreversibly trapped/ internalized in the skin. Mucus could act as effective barriers to prevent the penetration and attack of NPs (Zhao et al., 2011; Xiao et al., 2015). This may be an important reason for low Ti level in the muscle of turbot. In addition, the skin samples for Ti determination were pre-rinsed several times with 0.9% NaCl solution, indicating that the adsorbed TiO₂ NPs cannot be completely removed from skin by this rinsing procedure. Thus, both adsorbed and internalized TiO₂ NPs could be transferred to the organisms at higher trophic level. As a scaleless fish, skin and muscle are sometimes eaten together by people, thus causing further risk to food safety.

Moreover, the feces of turbot were collected for dietary and waterborne exposures, and the detected Ti contents in feces were much higher than that in all the tested organs/tissues (Fig. 3H), showing that a fraction of TiO₂ NPs internalized by fish was eliminated with feces. The quick elimination of TiO₂ NPs with feces could be a reason for the low BMFs of TiO₂ NPs as listed in Table S4. It was reported that TiO₂ NPs were readily taken in and excreted from zebrafish, and the half-life of TiO₂ NPs in zebrafish body was 1-2 days via food chain (Zhu et al., 2010). The bowel movement of organisms could also be promoted after exposure to NPs-containing food. Conway et al. (2014) found that the fecal production was doubled when marine mussels were exposed directly to free CeO₂ NPs or CeO₂ NPs adsorbed on food

(phytoplankton) in comparison with the unexposed control. It should be noted that TiO₂ NPs in feces could remain available to turbot themselves. In the present work, the fecal matters of turbot were easily broken apart under aeration, thus leading to the re-exposure of TiO₂ NPs and/or their aggregates to turbot, which could explain the high Ti content of turbot gills in the dietary exposure condition (Fig. 3A). In addition, the feces of turbot and other fish are the food of some benthic organisms (e.g., crab mussel), thus providing a chance for further exposure if NPs-containing feces deposited on the seafloor.

3.4. Assessment of health status and histological analysis of juvenile turbot

Upon dietary (10DE, 50DE, and 100DE) and waterborne (10WE) exposures for 20 days, health of juvenile turbot was assessed. The SR of juvenile turbot in each treatment was 100% (Table 1). However, the growth (WGR and SGR) of turbot after dietary exposure gradually decreased with increasing Ti contents in clamworms, and significant reduction of WGR and SGR was observed for 100DE treatment ($p < 0.05$). In addition, the growth inhibition of fish after waterborne exposure (10WE) was stronger than that in the 10DE and 50DE treatments while showing insignificant difference with 100DE treatment. Moreover, morbidity of turbot was determined, and the order followed: 10WE > 100DE > 50DE > 10DE > control (Table 1). Abnormal behaviors of fish were observed after dietary and waterborne exposures. For example, the turbot attempted to swim on the upper layer of seawater after exposure (data not shown). Pathological changes such as hemorrhage, enteric redmouths, fin rot (Fig. S4) were observed on turbot after dietary exposure (100DE).

These symptoms are mainly caused by bacteria (Pridgeon and Klesius, 2012), indicating that the resistance of turbot against external stress decreased after exposure.

The abnormal symptoms of specific organs/tissues after dietary exposure (100DE) were further observed. The bile in normal gallbladder was green while it became black after dietary exposure (Fig. 4A, 4B). Bile is a fluid involved in the digestive process in intestine. In this case, the black bile indicates gastrointestinal function disorders. Spleen appeared swollen and hyperemic compared with the normal spleen, indicating possible splenic dysfunction (Figs. 4C and 4D). Further light microscopy observation of tissue sections showed obvious pathology changes in liver and spleen (Fig. 5). For liver, fibrosis was obvious in portal area and central vein for 100DE treatment (Fig. 5B). Congestion (Fig. 5D) and inflammatory cell infiltration (Fig. 5E) were also increased in livers after 100DE exposure. For spleen, hyperemia, fatty degeneration and necrosis of splenocytes were obviously increased (Fig. 5G, 5H). It is reported that the changes of spleen morphology of rainbow trout such as decrease in area of red pulp and increase in number of melanomacrophages were observed after waterborne exposure to TiO₂ NPs for 14 days (Boyle et al., 2013). In the same study, liver morphology change was minor with mild lipidosis and increased melanomacrophage deposits. These histological changes are different from that observed in the present study (Fig. 5), indicating that the toxicity mechanism for dietary exposure is different from waterborne exposure. There are three possible reasons for the histological changes of fish after TiO₂ NPs exposure: (1) Excessive

accumulation of reactive oxygen species (ROS) directly induced by TiO₂ NPs (Xiong et al., 2011). Although TiO₂ NPs immobilized in clamworms were not illuminated by sunlight, the TiO₂ NPs released from feces may be under sunlight illumination, thus possibly generating ROS. (2) Oxidative stress driven by hypoxia (Boyle et al., 2013). The accumulation of TiO₂ NPs in gill tissues (Fig. 3A) could lead to the injury of gill tissues, thus causing hypoxia respiratory and hypoxia-driven oxidative stress in various organs such as liver and spleen. (3) Direct physical damage. The internalized TiO₂ NPs in organs/tissues could physically damage their histological structures.

Injury of liver and spleen could affect the growth and metabolism of fish, which explains the growth inhibition upon dietary (100DE) and waterborne (10WE) exposures (Table 1). In addition, it is reported that severe impairment of the digestion tract was observed when rainbow trout were exposed to 0.5 mg/L TiO₂ NPs for 14 days (Federici et al., 2007). The increase of intestine length (as an indicator of intestine development) was reduced when marine medaka was fed with Ag NPs-treated artemia (Wang and Wang, 2014). We thus investigated intestine morphology of turbot after both dietary (100DE) and waterborne (10WE) exposures. Different from intestinal changes reported by Wang and Wang (2014), all the parameters including fold height, enterocyte height, and microvillus height of intestines in the present work were not significantly different between 100DE/10WE treatment and the unexposed treatment (Table S5).

3.5. Nutritional composition of juvenile turbot after exposure

The biometric parameters and nutrition composition of juvenile turbot after

436 trophic transfer were analyzed. As shown in Table 1, CF, and VSI of juvenile turbot
437 showed no significant differences among all the treatments ($p>0.05$). However, HSI
438 which indicates the stress-response of livers, was significantly increased for the 50DE,
439 100DE and 10WE treatments in comparison with control and 10DE groups ($p<0.05$),
440 which was consistent with the abnormal symptoms of liver (e.g., congestion, and
441 inflammatory cell infiltration) (Fig. 5). For the whole body, the moisture did not show
442 any significant difference among all the treatments. However, crude protein content in
443 turbot was significantly decreased while crude lipid was increased after 50DE,
444 100DE and 10WE exposures (Table 1), indicating that the nutritional composition
445 was altered upon exposure. The moisture, crude protein, and crude lipid in livers were
446 also determined. Liver moisture and crude protein did not significantly change for
447 both dietary (10DE-100DE) and waterborne exposure (10WE). Interestingly, the
448 crude lipids were significantly increased with increasing Ti content in clamworms
449 upon dietary exposure. This indicated that TiO_2 NPs during trophic transfer affected
450 the metabolism of fat in the liver, which could potentially result in fatty liver. The
451 liver is an important organ for nutrient metabolism (e.g., carbohydrate, lipid, protein
452 and vitamins). HSI value commonly showed a positive correlation with liver
453 metabolism of nutrients (e.g., lipids) in grass carp (Gao et al., 2010). The abnormal
454 metabolism of liver, as indicated by increasing HSI values, may be thus responsible
455 for the above nutritional compositional changes of the whole turbot after exposure
456 (50DE, 100DE and 10WE). In addition, protein content is one of most important
457 nutritional indexes for turbot and other commercial fish. Reduction of protein content

was observed for 50DE, 100DE and 10WE treatments, showing that the nutritional quality of fish decreased during trophic transfer of TiO₂ NPs. Previous studies on trophic transfer of NPs mainly focused on their bioaccumulation and toxicity (Mattsson et al., 2014; Wang and Wang., 2014; Conway et al., 2014). To our knowledge, this is the first report highlighting the nutrition quality of organisms upon trophic transfer of NPs through food chain.

4. Conclusions and environmental implications

As a type of persistent NP, TiO₂ is insoluble in seawater. Also, it is reported that TiO₂ NPs could be still inert and undergo no lattice modification in simulated gastric fluids (pH 1.2) and intracellular compartments (Veronesi et al., 2012). Therefore, TiO₂ NPs rather than ionic Ti could indeed undergo trophic transfer and accumulation from clamworm to juvenile turbot in the present work. Biomagnification of TiO₂ NPs was not observed in this marine benthic food chain, and the [internalized](#) TiO₂ NPs could be partially eliminated by turbot, which is good for both turbot and higher trophic level organisms. However, food safety would be a concern because Ti was detected in muscle, blood, and other various organs/tissues for both dietary and waterborne exposure [conditions](#). TiO₂ NPs in feces of both turbot and clamworm will return to food chain because feces could be naturally degraded or taken up directly by some benthic organisms (e.g., crab mussel). For juvenile turbot, reduction of growth and nutritional quality were observed for both dietary and waterborne exposure. It is well known that turbot is an important commercial fish in Europe and Asia. In 2012, farmed turbot production in Europe was as high as 12,676 tons and valued [80 million](#)

USD (Abate et al., 2015). Thus, potential NPs exposure should receive attention in aquaculture as the nutritional quality reduction of turbot could lower the economic benefits and affect the long-term development of aquaculture. In addition, it is recently reported that for accumulation and trophic transfer of CeO₂ NPs in the terrestrial food chain (zucchini to crickets) was greater than equivalent bulk materials (Hawthorne et al., 2014). In the marine benthic environment, both nano and bulk particles would be highly aggregated, thus, the size-dependent trophic transfer may not be obvious. However, distribution and accumulation of NPs in marine organisms at higher trophic level (e.g., turbot in the present study) could be size-dependent because the particles with smaller individual size would readily transfer from initially-exposed organs/tissues to circulatory systems (e.g., bloodstream). In addition, the trophic transfer of well-dispersed NPs in marine food chain deserves further investigation. To our knowledge, this is the first report on trophic transfer of NPs in a marine benthic food chain, and the information provided here should be helpful for better understanding the fate, transport, and transfer of NPs in marine environments.

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Appendix, Supplementary material

Four figures and five tables. Supplementary data associated with the article can be found in the online version, at <http://www.sciencedirect.com>.

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1 **Table**

2 **Table 1.** Growth performance, biometric parameters and nutritional compositions of
3 juvenile turbot after dietary and waterborne TiO₂ NPs exposures (%).

	Control	10DE	50DE	100DE	10WE
Growth performance					
WGR	6.03±0.08 ^a	6.00±0.23 ^a	5.47±0.42 ^{ab}	4.70±0.43 ^b	4.78±0.12 ^b
SGR	0.29±0.00 ^a	0.29±0.01 ^a	0.27±0.02 ^{ab}	0.23±0.02 ^b	0.23±0.01 ^b
SR	100	100	100	100	100
Morbidity	5.56±4.81 ^c	19.4±4.81 ^b	22.2±4.81 ^b	33.3±8.33 ^a	36.1±4.81 ^a
Biometric parameters					
CF	3.16±0.16 ^a	3.17±0.17 ^a	3.16±0.19 ^a	3.15±0.17 ^a	3.15±0.09 ^a
VSI	4.36±0.14 ^a	4.34±0.22 ^a	4.29±0.29 ^a	4.39±0.20 ^a	4.42±0.12 ^a
HSI	1.36±0.08 ^b	1.37±0.07 ^b	1.69±0.15 ^a	1.66±0.14 ^a	1.73±0.07 ^a
Whole body					
Moisture	78.5±0.6 ^a	78.5±0.4 ^a	78.9±0.5 ^a	78.6±0.6 ^a	78.1±0.7 ^a
Crude protein	14.1±0.1 ^a	13.9±0.1 ^a	13.5±0.1 ^b	13.6±0.1 ^b	13.4±0.1 ^b ^c
Crude lipid	4.16±0.12 ^b	4.28±0.04 ^b	4.86±0.06 ^a	4.80±0.06 ^a	4.71±0.10 ^a
Liver					
Moisture	74.9±1.1 ^a	75.1±1.9 ^a	74.9±0.7 ^a	74.9±0.6 ^a	74.7±1.3 ^a
Crude protein	10.5±0.4 ^a	10.2±0.4 ^a	10.2±0.1 ^a	10.0±0.1 ^a	10.0±0.2 ^a
Crude lipid	14.3±0.2 ^c	14.3±0.1 ^c	15.2±0.2 ^b	16.0±0.3 ^a	16.4±0.5 ^a

4 Note: For a given parameter, different letters (a-c) behind the values indicate significant difference
5 among dietary and waterborne exposures (n=3, *p*<0.05).

1 **Figures**

2
3 **Fig. 1.** Distribution of TiO₂ NPs in clamworms. (A) Ti contents in clamworm after
4 exposure to TiO₂ NPs (0, 10, 50 and 100 mg/L) for 24 h. (B) The distribution of TiO₂
5 in different parts of clamworm after exposure to TiO₂ NPs (100 mg/L) for 24 h. In
6 panel A, bars with different letters indicate significant difference among different
7 treatments (n=3, *p*<0.05). In panel B, the values on the figure are the percentages of Ti
8 contents in different parts of clamworm.

10
11 **Fig. 2.** Accumulation of TiO₂ NPs in the whole body of juvenile turbot after dietary or
12 waterborne exposures. Both dietary and waterborne exposures include a uptake period
13 (Day 0-Day 20) and a depuration period (Day 20-Day 27). For a given exposure time,
14 * indicates significant difference between dietary (10DE) and waterborne (10WE)
15 exposures (n=3, *p*<0.05).

Fig. 3. Ti content in (A) gill, (B) intestines, (C) stomach, (D) blood (unit, ng/mL), (E) liver, (F) muscle, (G) skin, and (H) feces of juvenile turbot after dietary (10DE, 50DE, and 100DE) or waterborne (10WE) exposure for 20 days (feces for 27 days). It should be noted that the unit of Ti content in blood (ng/mL) is different from the Ti contents

in other organs/tissues ($\mu\text{g/g}$ dry wt.). For a given organ/tissue, different letters represent significant difference among different treatments ($n=3$, $p<0.05$).

Fig. 4. Gallbladder and spleen of juvenile turbot after dietary exposure (100DE) for 20 days. (A) Gallbladder of normal juvenile turbot; (B) Gallbladder of juvenile turbot after dietary exposure; (C) Spleen of normal juvenile turbot; (D) Spleen of juvenile turbot after dietary exposure. Bile in gallbladder became black while spleen was swollen and hyperaemic after dietary exposure.

Fig. 5. Pathological changes in liver and spleen of juvenile turbot after dietary exposure (100DE) for 20 days. (A, C) Liver of unexposed juvenile turbot; (B, D, E) Liver of juvenile turbot after dietary exposure; (F) Spleen of unexposed juvenile turbot; (G, H) Spleen of juvenile turbot after dietary exposure. Yellow arrows in (B), (D) and (E) indicate that fibrosis increased obviously in portal area and central vein, congestion, and inflammatory cell infiltration of liver, respectively. Yellow arrows in (G) and (H) indicate congestion of spleen, and fatty degeneration and necrosis, respectively.

Graphic Abstract: