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Bioaccumulation patterns in aquaculture mussels and turbots exposed to different sizes of TiO_2NPs

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ABSTRACT

Titanium dioxide nanoparticles (TiO_2NPs) are widely used in industry, leading to their presence in the marine environment where they can interact with and harm marine life. This study examines the impact and bioaccumulation of TiO_2NPs in aquaculture mussels and turbot species.

Mussels were exposed for 28 days, and turbot for 90 days, to different concentrations of citrate-coated TiO_2NPs (25 and 5 nm). Inductively coupled plasma mass spectrometry (ICP-MS) and single-particle-ICP-MS (SP-ICP-MS) were used for Ti determination, and TiO_2NPs content and size distribution determination.

In mussels TiO_2NPs concentrations reached $2.28 \times 10^8 \pm 5.84 \times 10^7$ NPs g^{-1} after exposure to 1.0 mg L^{-1} of 25 nm TiO_2NPs for 28 days, and $4.79 \times 10^8 \pm 2.36 \times 10^8$ NPs g^{-1} for 1.0 mg L^{-1} of 5 nm TiO_2NPs at 21 days, respectively. Bioaccumulation was also observed in mussel shells, which became fragile, with the highest Ti concentration reaching 12.4 ± 3.5 μg g^{-1} d.w. In turbot, the highest Ti concentration was found in the liver, reaching 1.6 ± 0.4 μg g^{-1} w.w. at 90 days to the highest dose of 5 nm TiO_2NPs. Furthermore, turbots expelled Ti through the feces, reaching 41.0 ± 6.6 μg g^{-1} d.w.

The results show the safety of turbot consumption and highlight the need for a correct depuration process of mussels before commercialization.

1. Introduction

Titanium dioxide nanoparticles (TiO_2NPs) are one of the most employed metallic oxide NPs due to their stability, anticorrosion, antibacterial, and catalytic properties. TiO_2NPs are commonly found in papers, plastics, paints, medicines, pharmaceuticals and personal care products like sunscreens, toothpaste or even soaps (Gallocchio et al., 2020). Besides, TiO_2NPs have innovative applications in solar cells, photocatalytic water purification systems, and photodynamic tumor therapy (Abd El-Atti et al., 2019; Gornati et al., 2016). Until February 2022, TiO_2 was commonly used in the EU as a food additive (coded as E171) for color and consistency. However, EFSA concluded that it could no longer be considered safe and the European Commission implemented a regulation to ban its use (European Commission, 2022; Younes et al., 2021).

Due to the wide use and dissemination of TiO_2NPs around the world, they can enter marine systems and interact with biota, these being potential sinks for NPs (Gallocchio et al., 2020). The study of the interaction and behavior of TiO_2NPs with living beings is still a challenge because little is known about NP bioavailability, mode of uptake, ingestion rates, and actual internal exposure concentrations (Canesi et al., 2012). To understand the bioaccumulation, biomagnification, trophic transfer to the food chain and potential toxicity of TiO_2 in marine species, several aspects must be considered. The kind of organism tested, the size and the coating of the particle, the matrix of dispersion (natural water, seawater or even brackish water), the presence of organic matter, or the route of NPs exposure (NPs in water suspension or mixed and homogenized in food) are variables to consider. In order to assess bioaccumulation in fish, the Organization for Economic Co-operation and Development (OECD) Test Guideline 305 (TG 305) is

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the current gold standard. It takes into account both water exposure and food exposure (Gallocchio et al., 2020; Mona et al., 2023; OECD, 2008); however, there is still a lack of studies in the bibliography that use the dietary exposure route to NPs instead of the aqueous route (Mona et al., 2023).

According to the Apromar report on aquaculture in Spain, Europe and the World in 2024 (APROMAR, 2024), Mytilus sp. is the most important benthic bivalve mollusk species in Europe and it is harvested mainly in the Atlantic area. Mussels are considered targets for monitoring aquatic pollutants due to their capacity to filter high amounts of seawater, processing microalgae, bacteria, sediments, particulates, and natural nanoparticles (Canesi et al., 2012; Larios et al., 2018). Some studies in literature were found regarding the effects of the exposure to TiO_2NPs in different species of mussels including M. Coruscus, M. edulis and M. galloprovincialis, usually after exposures of several days. These effects were assessed in terms of modifications to their physiology and ability to reproduce as well as their immunotoxicity, genotoxicity, cytotoxicity, metabolic reactions, and production of oxidative stress (Canesi et al., 2010a; Canesi et al., 2010b; Minetto et al., 2014). Thus, embriotoxicity probably associated to the photocatalytic properties of nanosized titanium dioxide was observed in M. Galloprovincialis 48h-old larvae, and the growth retard and impact of the malformations depended on the concentration with a non linear response and on light exposure (Libralato et al., 2013). Exposure to TiO_2NPs caused alterations in the activities of antioxidant enzymes in the gill, muscle and digestive glands of M. Coruscus. This kind of particles also stimulated the production of oxygen radicals and triggered the release of lysosomal enzymes in hemocytes of the marine mussel M. galloprovincialis. (Lopes Rocha et al., 2015; Roma et al., 2020).

Regarding bioaccumulation, Larios et al. (2018) measured the concentration of Ti in Dreissena polymorpha (zebra mussel) by inductively coupled plasma-optical emission spectrometry (ICP-OES). The zebra mussel was fed with Chlorella mixed with 0.35, 0.7, and 3.5 mg L^{-1} of TiO_2NPs for 3 days. The results showed Ti accumulation with the exposure time (until 36 h); it was observed slightly more accumulation of Ti when it was mixed with the fed microalgae, and the amount of Ti in the sediments was higher in the presence of mussels due to their filtration activity (Larios et al., 2018). Gallocchio et al. (2020) reported the bioaccumulation of titanium in Mytilus galloprovincialis after 4 exposure days to 0.25 and 2.5 mg L^{-1} of TiO_2NPs or 1.6 mg L^{-1} of ionic Ti. SP-ICP-MS results showed the presence of TiO_2NPs in the treated mussels, but they were able to eliminate more than 70 % of the particles after 3 days of depuration (Gallocchio et al., 2020).

On the other hand, Scophthalmus maximus (Turbot) is a benthic carnivore flat fish (Wang et al., 2016), highly prized in Spain, and that was produced in tenth place by tons in Europe in 2023 (APROMAR, 2024). Some reports were found in the literature regarding TiO_2NPs bioaccumulation in fish. The most used species were the freshwater zebrafish (Danio rerio)(Asztemborska et al., 2018; Fang et al., 2016; Lu et al., 2022; Ren et al., 2018; Zhu et al., 2010), rainbow trout (Oncorhynchus mykiss) (Connolly et al., 2022; Federici et al., 2007; Johnston et al., 2010), and carp (Cyprinus carpio)(Zhang et al., 2007). Connolly et al. (2022) evaluated the influence of the citrate and polyethylene glycol coatings used to stabilize nanoparticles of TiO_2NPs and CeO_2NPs, and observed a higher uptake of the coated NPs in the tissues of rainbow trout. Coating also influenced distribution and the depuration profile. However, fewer studies were found for fish from marine and brackish water. Turbot bioaccumulation assays were performed to evaluate aqueous, dietary exposure, and trophic transfer (Mona et al., 2023). Wang et al. (2016) studied the trophic transfer from the benthic marine clamworm (Perinereis aibuhitensis) exposed to 10, 50, and 100 mg L^{-1} TiO_2NPs to juvenile turbots, and the waterborne exposure of turbot to 100 mg L^{-1} TiO_2NPs. Uptake and depuration periods were 20 and 7 days, respectively, and total Ti was determined by ICP-MS. No biomagnification of Ti was observed through the food chain, and the bioaccumulation was higher in the gill, intestine and stomach of juvenile turbots, followed by skin, liver, and muscle.

To the best of our knowledge, no reports were found about the study of TiO_2NPs bioaccumulation in Mytilus edulis (mussel) and Scophthalmus maximus (turbot) through the diet during long exposure periods (28 and 90 days, respectively) and using single particle-inductively coupled plasma-mass spectrometry (SP-ICP-MS) for TiO_2NPs concentration and size distribution determination. Thus, the main goal of this research work is to study the in-vivo bioaccumulation of different sizes (5 and 25 nm) of citrate-coated TiO_2NPs in two of the most important aquaculture species farmed in the Atlantic area. Total Ti and TiO_2NPs concentration and size distributions in Mytilus edulis and Scophthalmus maximus were determined by inductively coupled plasma-mass spectrometry (ICP-MS) and single-particle-ICP-MS (SP-ICP-MS), respectively.

2. Materials and methods

2.1. Citrate-coating of TiO_2NPs

The TiO_2NPs used for the in vivo bioaccumulation tests were commercially available and used without any further purification. The 25 nm TiO_2NPs and 5 nm TiO_2NPs powders were from Sigma Aldrich (citrate P-25 TiO_2NPs, Darmstadt, Germany; a mixture of rutile and anatase), and Nanostructured & Amorphous Materials, Inc. (Katy, TX, USA; anatase, 5 nm size, 99 %), respectively. To ensure the stability of TiO_2NPs, citrate was selected as their coating. The NPs stock dispersions were prepared in ultrapure water. To create citrate-5 nm TiO_2NPs and citrate-25 nm TiO_2NPs, trisodium citrate dihydrate from Sigma-Aldrich was mixed with TiO_2 powder at weight ratios of 1:1.5 for 5 nm and 1:0.8 for 25 nm, respectively. An ultrasonic probe (Branson Disintegrator Ultrasonic Mod. 450) was used for 30 min (30 s pulse on /5 s pulse off) to disperse NPs in citrate. Both sizes of citrate-TiO_2NPs suspensions had a final concentration of 15.5 g L^{-1}. The characterization results of these particles by transmission electron microscopy (TEM) showed that there were no significant changes in size upon adding the citrate coating to the particles. The sizes measured were 8.6 $\pm$ 1 and 29 $\pm$ 1 nm for the 5 and 25 nm particles, respectively.

2.2. Bioaccumulation study design

Mussels (Mytilus edulis) and Turbot (Scophthalmus maximus) were selected for the in vivo bioaccumulation test. The cultivation of these aquaculture species took place in the Atlantic region: mussels were cultivated in the Indigo Rock Marine Research Center (Cork, Ireland), while turbots were cultured in the Technological Center of Aquaculture of Galicia (CETGA,Galicia, Spain).

The bioaccumulation experiments were performed in mussels and turbot exposed to 25 nm TiO_2NPs (Trial 1), and in mussels and turbot exposed to 5 nm TiO_2NPs (Trial 2).

2.2.1. Feeding and exposure of mussels to TiO_2NPs

For mussel trials, TiO_2NPs (5 and 25 nm, citrate-coated) were dispersed in phytoplankton (*Tisochrysis lutea*, T-iso) before feeding. A 30 mL solution containing 3 mL of phytoplankton and the corresponding concentration of TiO_2NPs (0.10 or 1.0 mg L^{-1}) or citrate coating (in the case of solvent control tanks) was added once per week.

Mussels (*M. edulis)* were cultured in aerated 40-L pond systems. Seawater for the experiments was properly filtered before use in the tanks. The bioaccumulation experiments lasted 28 days. Each trial consisted of twelve tanks: three control tanks (no exposure to nanoparticles), three solvent control tanks (with addition of citrate), three low-exposure tanks (0.10 mg L^{-1} TiO_2NPs), and three high-exposure tanks (1.0 mg L^{-1} TiO_2NPs). Mussels were exposed to 12 h of white light and 12 h of darkness at 16 $\pm$ 1 °C. Water parameters (pH, temperature, dissolved O_2, and salinity) were monitored daily, while conductivity, ammonium, and nitrites were measured twice a week. Mussels

were fed with zooplankton (copepods and rotifers) on Monday, Tuesday, Thursday, and Friday. A complete water change was performed before particle addition on Wednesdays with a 30 mL solution of phytoplankton, and the corresponding TiO_2NPs concentration or citrate coating (in the case of solvent tanks).

Sampling was carried out every seven days (five times in total). Each trial consisted of 12 tanks: three control tanks (A, B, and C), three tanks exposed to citrate coating (D, E, and F), three tanks exposed to 0.10 mg L^{-1} TiO_2NPs (G, H, and I), and three tanks exposed to 1.0 mg L^{-1} TiO_2NPs (J, K, and L). From each tank three mussel replicates were sampled at five different sampling times (0, 7, 14, 21, and 28 days), resulting in a total of 180 samples per 25 nm TiO_2NPs and 180 samples for 5 nm TiO_2NPs (3 replicates x 12 tanks x 5 sampling times x 2 trials = 360 samples). There were enough mussels in the tanks to carry out the sampling and the amount of food was not excessive as mussels were removed during the experiment. Mussel flesh was separated from shells and samples were stored at –20 °C until analysis.

2.2.2. Feeding and exposure to TiO_2NPs (turbots)

In the case of turbot trials, TiO_2NPs were mixed with micronized calcium carbonate (C.T.S. España, S.L.) to promote dispersion and were incorporated into commercial pellets (Biomar Iberia S.A.). The feed was prepared to reach the exposure doses of 0.25, 0.75, and 1.5 mg TiO_2NPs kg^{-1} fish per day.

S. maximus were cultivated in an open-flow system with 500-L tanks with a flow of 500 L per hour. Incoming seawater was mechanically filtered and disinfected using UV treatment before entering the tanks. The trials lasted 90 days at 14 ± 2 °C. Each experiment consisted of twelve tanks: three control tanks (no TiO_2NPs exposure), three tanks exposed to 0.25 mg kg^{-1} fish per day, three tanks exposed to 0.75 mg kg^{-1} fish per day, and three tanks exposed to 1.5 mg kg^{-1} fish per day. Juvenile turbots had an average body weight of 45 g (25 nm trial) and 73 g (5 nm trial). Fish were fed four times per day at a rate of 1–2.5 %, depending on body weight and sampling time.

Turbot samples were randomly collected every 15 days from at least two tanks per exposure level, resulting in 150 samples per trial. Turbots were euthanized with iced water, dissected into liver, kidney, and muscle with skin. After that, they were stored at –20 °C until analysis.

2.3. Microwave-assisted acid digestion and total Ti determination by ICP-MS

Microwave-assisted acid digestion was employed to process the aquaculture samples before total Ti determination by ICP-MS. Wet mussel and turbot samples (one gram weighed on an analytical balance) were ground and mixed with 4.0 mL of ultrapure water (18 MΩ cm resistivity) obtained from a Milli-Q® IQ70033 (Millipore Co., Bedford, MA, USA), 3.0 mL of 69 % (w/w) nitric acid (SUPRAPUR®, Sigma Aldrich, Darmstadt Germany), and 1.0 mL of 33 % (w/v) hydrogen peroxide (ACS, ISO., AppliChem, Barcelona, Spain). Then, the samples were submitted to the microwave-assisted acid digestion protocol described previously by López-Mayán et al. (2022). An Ethos Plus microwave lab station from Milestone (Bergamo, Italy), equipped with a temperature and pressure control probe, and 100 mL Teflon vessels, was used for sample digestion. The program lasts 34 min at 850 W. The first step consists of heating from room temperature to 90 °C (ramp of 4 min), then heating from 90 to 140 °C (ramp of 5 min), then from 140 to 200 °C (ramp of 5 min), and finally maintaining the reactors at 200ºC for 20 min. After cooling, the acid digests were made up to 25 mL with ultrapure water and stored before ICP-MS analysis. Each seaweed sample was subjected to microwave-assisted acid digestion in triplicate and two reagent blanks were prepared for each set of sample pre-treatment. The digested samples were diluted with ultrapure water 10–25 times before ICP-MS analysis, using the conditions listed in Table 1S (electronic supplementary information).

The ICP-MS equipment (NexION® 2000 spectrometer equipped with a Single Cell Micro DX autosampler, Perkin Elmer, Waltham, USA) uses Ar and NH_3 as nebulization and reaction gases, respectively, both with a 99.999 % purity (Nipon Gases, Madrid, Spain). Calibration was performed using the standard addition method to avoid the matrix effects with an ionic Ti standard (Perkin Elmer) in the range of 0–15.0 μg L^{-1}. To minimize interferences in the determination of Ti, the Dynamic Reaction Cell (DRC) with ammonia as reaction gas (flow rate 1.0 mL min^{-1}), was used. The purpose of ammonia gas is to form the Ti-NH_3 cluster $[^{48}Ti^{14}N^1H(^{14}N^1H_3)_4^+]$ of *m/z* 131 to avoid the main interference present in aquaculture species (^{48}Ca) during the determination of the analyte (Stephan, 2018).

2.4. Enzymatic extractions and determination of TiO_2NPs by SP-ICP-MS

Enzymatic extractions were employed to extract the 25 and 5 nm TiO_2NPs from the aquaculture matrix before TiO_2NPs determination by SP-ICP-MS. The enzymatic extraction was selected due to its mild conditions and to avoid the size and shape change of TiO_2NPs during extraction (Taboada-lópez et al., 2019). The pancreatin and lipase from Candida rugose (both from Sigma Aldrich) mixture was selected to extract the particles due to their ability to solubilize fats from fish and mussel samples. The methodology used was previously optimized by Taboada-López et al.(2019), where a volume of 7.5 mL of 8.0 g L^{-1} of each enzyme solution in a buffer of 0.20 M NaH_2PO_4/0.20 M NaOH (pH=7.4) was added to a one gram of sample (weighed on an analytical balance) for extraction. Three replicate extractions per sample along with two blanks were performed when the amount of sample was enough. The mixtures were submitted to overnight incubation at 37 ºC and 200 rpm in a Boxcult temperature-controlled incubation chamber (Stuart Scientific, Surrey, UK) placed on a Rotabit orbital-rocking platform shaker from J.P. Selecta (Barcelona, Spain). Finally, the extracts were filtered with Minisart NML hydrophilic non-sterile 5 μm filter disks (Sartorius, Goettingen, Germany), diluted with 1 % (v/v) glycerol, and analyzed by SP-ICP-MS. Table 1S (b) shows the specific operational conditions that change when the single particle mode is operating on the ICP-MS. TiO_2NPs concentrations and size distributions were determined by the Syngistix™ Nano 2.5 software (Perkin Elmer). The transport efficiency (TE %) of SP-ICP-MS was calculated by the software based on the measured of the sample flow rate, the analysis of an ionic gold stock standard (1000 mg L^{-1} in 2 M HCl, Merck, USA) calibrating in the range 0–3.0 μg L^{-1}, and the certified reference material of PEG-COOH Au Nanospheres (49.6 nm by TEM, 9.89 $\times 10^4$ particles mL^{-1}) from NanoComposix (San Diego, USA). The experimental TE % was between 8 % and 12 %. The ionic Ti calibrations between 0 and 5.0 μg L^{-1} were performed with an ionic Ti standard (Perkin Elmer) in ultrapure water due to the high dilution of sample extracts. The mussels and turbot enzymatic extracts were diluted with 1 % (v/v) glycerol 500 times before SP-ICP-MS analysis. The experimental flow rates and transport efficiencies were between 0.19 and 0.22 mL min^{-1}, and between 7.3 % and 10.7 %, respectively. Finally, the software obtained TiO_2NPs concentrations and size distributions in the diluted extracts, and Microsoft Excel was used to calculate the concentrations in aquaculture samples.

2.5. Statistical analysis

To study the bioaccumulation effects in both mussels and turbot, a statistical analysis was performed using Statgraphics Centurion XVIII (Warrenton, USA). ANOVA tests were carried out to determine whether significant differences existed between means, e.g. each type of tank (control, solvent, 0.10, or 1.0 mg L^{-1} TiO_2NPs) across the different exposure times (0, 7, 14, 21, and 28 days) in mussels, and among tank types (0, 0.25, 0.75, and 1.5 mg kg^{-1} fish per day) across exposure times in turbot. After ANOVA tests, a multiple range comparison was conducted as post-hoc test to identify which groups had statistically different means. The Fisher's Least Significant Difference (LSD) was calculated to compare all pairs of means. This LSD value is obtained

using the t-value, the mean square error and sample size. If the absolute difference between two means is greater than the LSD value, those means are considered statistically different.

3. Results and discussion

3.1. Validation of analysis

The total concentration of titanium in mussel and turbot samples was determined following the methodology detailed in Section 2.3. Titanium dioxide nanoparticles in the sample extracts were quantified by SP-ICP-MS as described in Section 2.4. Prior to sample analysis, the analytical performance was thoroughly evaluated for mussels and turbot in terms of calibration, sensitivity, and recovery.

3.1.1. Mussels

Calibration using the standard addition method was used due to the matrix effect in a calibration range from (0.25–25 μg L^{-1}) with correlation coefficients of 0.999. The limit of detection (LOD) and limit of quantification (LOQ) resulted in 0.0025 and 0.0083 μg g^{-1} w.w. (wet weight), respectively, and they were calculated as 3σ/m and 10σ/m (where σ is the standard deviation of ten measurements of a blank of digestion, and m is the slope of the standard addition calibration graph). The accuracy of the method was evaluated through the analytical recovery, which resulted in 117 ± 5, 109 ± 3, and 118 ± 4 % for 0.5, 5.0, and 10.0 μg L^{-1} of Ti added (n = 5). The precision was also evaluated through the repeatability of ICP-MS measurements of Ti in the diluted digests, with RSD < 4 % (n = 10).

Laborda et al., 2020(Laborda et al., 2020) criteria were used to calculate the limits of detection in single-particle mode. The LOD_{number} resulted in 1.99 × 10^6 TiO_2NPs L^{-1} in the extract and 7.46 × 10^7 TiO_2NPs g^{-1} in the sample. The LOD_{size} using the 3σ and the more restrictive 5σ criteria resulted in 24 and 28 nm, respectively.

3.1.2. Turbot

The standard addition calibration method was also employed, and the analytical performance was also guaranteed for total Ti in turbot. The LOD and LOQ were 0.0014 and 0.0045 μg g^{-1}, respectively. The average analytical recovery was 98 ± 3 % (n = 5), and the repeatability of the measurements achieved RSD< 2 % (n = 5).

For the analysis of TiO_2NPs the LOD_{number} achieved was 1.19 × 10^6 NPs L^{-1} and 4.46 × 10^6 NPs g^{-1} (referred to the 500 times diluted sample), and the LOD_{size} were 42 and 36 nm for the 5σ and 3σ criteria, respectively.

3.2. Mussel bioaccumulation tests

3.2.1. Total Ti in mussels

Total Ti was determined in mussels exposed to 25 nm TiO_2NPs (Trial 1) and to 5 nm TiO_2NPs (Trial 2). Fig. 1(a), corresponding to Trial 1, shows the intertank average Ti concentrations in control tanks, tanks exposed to the nanoparticle citrate coating, and tanks exposed to 0.10 mg L^{-1} of 25 nm TiO_2NPs for 28 days. Different letters indicate significant differences over time after ANOVA and a multiple range test. No bioaccumulation was found in control tanks within the exposure days, reaching the highest average basal Ti concentration for control tanks at 21 exposure days, being 0.06 ± 0.02 μg g^{-1} w.w. The one-way ANOVA test showed a P-value in control tanks higher than 0.05; then, no statistical differences were found between the concentrations in control tanks at different exposure days at a 95 % confidence level. In the same way, no bioaccumulation was found in solvent citrate tanks, reaching the highest average Ti concentration at 21 exposure days, being 0.05 ± 0.01 μg g^{-1} w.w. These small changes were attributed to the natural variability among the different specimens at such small concentrations of the element.

The intertank average Ti concentrations, in tanks exposed to

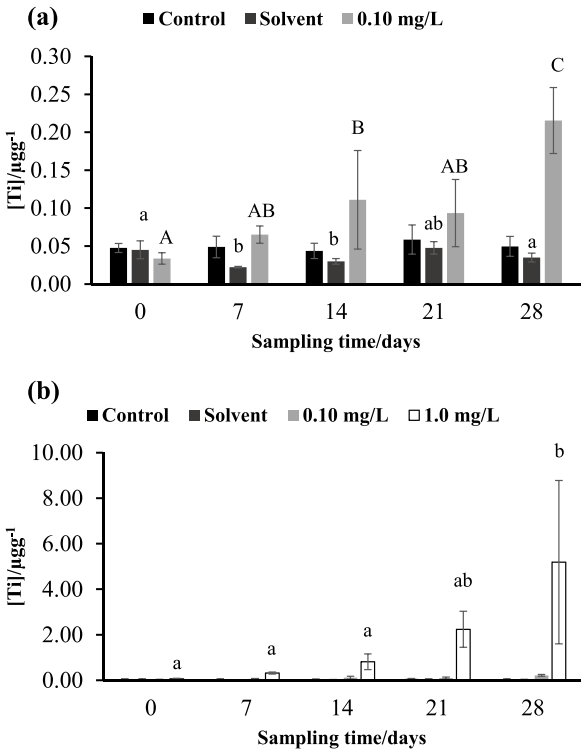

Fig. 1. Ti bioaccumulation in mussels exposed to 25 nm TiO_2NPs: Fig. 1(a) Control, citrate solvent, and 0.10 mg L^{-1} tanks. Fig. 1(b) Control, citrate solvent, 0.10 and 1.0 mg L^{-1} tanks. Different letters indicate significant differences over time after ANOVA and a multiple range test (lowercase for solvent and 1.0 mg L^{-1}, uppercase for 0.10 mg L^{-1}).

0.10 mg L^{-1} of 25 nm TiO_2NPs, increased with the exposure time until 0.22 ± 0.04 μg g^{-1} w.w. at 28 days. Statistical differences (P-value<0.05) between the concentration in mussels exposed at different exposure days were observed. The multiple range test showed statistical differences between concentrations at days 0 and 14 and 28, 7 and 28 days, 14 and 28 days, and 21 and 28 days. Fig. 1(b), corresponding to Trial 1, also shows the intertank average Ti concentrations in tanks exposed to 1.0 mg L^{-1} of 25 nm TiO_2NPs (average of concentrations in mussels from tanks J, K, and L) for 28 days. Ti concentration increased with time reaching 5.19 ± 3.19 μg g^{-1} w.w. at 28 days. Statistical differences among the concentration of mussels exposed to this concentration as a function of time were also observed (P-value<0.05).

Fig. 2(a), corresponding to Trial 2, shows the intertank average Ti concentrations in mussels from control tanks (average of tanks A, B, and C), tanks exposed to the nanoparticle citrate coating (average of D, E, and F), and tanks exposed to 0.10 mg L^{-1} of 5 nm TiO_2NPs (average of G, H, and I) for 28 days. No bioaccumulation was found in control and solvent tanks with the exposure days. The Ti concentration in control and solvent tanks reached 0.39 ± 0.15 and 0.26 ± 0.04 μg g^{-1} w.w., both at 28 days, respectively. No statistical differences between Ti concentrations in control and solvent tanks as a function of exposure time (P-value>0.05) were found. Total Ti concentration in mussels exposed to 0.10 mg L^{-1} of 5 nm TiO_2NPs reached a maximum mean value of 1.51 ± 0.53 μg g^{-1} w.w. of Ti after 28 days of exposure. However, no statistical differences were found among the concentrations (P-value>0.05), probably due to the great variability of the concentrations in the specimens.

Fig. 2(b), corresponding to Trial 2, also shows the intertank average Ti concentration in mussels from tanks exposed to 1.0 mg L^{-1} of 5 nm TiO_2NPs (average of J, K, and L) for 28 days. The concentration of the element increased with exposure time until 21 days, reaching 4.76 ± 0.86 μg g^{-1} w.w. of Ti. Statistical differences between the

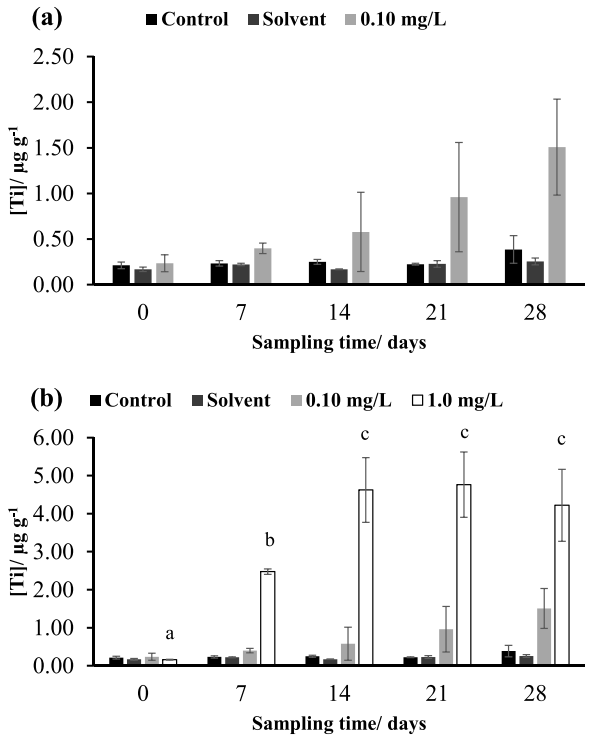

Fig. 2. Ti bioaccumulation in mussels exposed to 5 nm TiO_2NPs for 28 days. (a). Control, citrate solvent, and 0.10 mg L^{-1} tanks. (b) Control, citrate solvent, 0.10 and 1.0 mg L^{-1} tanks.

concentrations of mussels exposed to 1.0 mg L^{-1} as a function of time were found (P-value<0.05). The concentration remained approximately constant at 14, 21, and 28 days. Figs. 1S and 2(S) (supplementary electronic information) show total Ti concentrations found in all the control, solvent, 0.10 mg L^{-1} and 1.0 mg L^{-1} tanks exposed to 25 and 5 nm TiO_2NPs, respectively. These graphs present data for each tank separately, showing the average concentration of three mussels per tank and exposure day. All tanks were maintained under identical conditions (feeding, light, and water parameters). The water of the tanks was aerated, filtered and disinfected to avoid external contamination and ensure consistent experimental conditions without TiO_2NPs gradient through the water column. However, the results showed that mussels cannot assimilate the same amount of Ti, even under identical environmental conditions. Consequently, some tanks may display slightly higher or lower mean Ti values than expected, and relatively high intertank standard deviations were observed, reflecting the inherent biological variability associated with working with living organisms. Additionally, a potential nanoparticle concentration gradient may have formed, possibly due to TiO_2NPs aggregation processes, which was not assessed in the present study. Investigating this aspect in future experiments would be valuable to reduce variability and obtain more homogeneous bioaccumulation responses with lower standard deviations.

In summary, the results showed that mussels bioaccumulate Ti when they are exposed to both sizes of TiO_2NPs. The results also showed that the concentration of bioaccumulated Ti increased with the exposure dose. Total Ti concentrations were higher in mussels exposed to 1.0 mg L^{-1} than in mussels exposed to 0.10 mg L^{-1} for both sizes. Statistical analysis showed significant differences between Ti concentrations in mussels exposed to 0.10 and 1.0 mg L^{-1}. Although at the end of the bioaccumulation assays the total Ti concentration reached in both trials was of the same order of magnitude, the bioaccumulation in mussels exposed to 5 nm TiO_2NPs occurred earlier, with a stabilization of the concentration at 14 days after exposure at a dose of 1.0 mg L^{-1}. Particle size is one of the most important properties of nanomaterials and can affect their bioavailability (Lopes Rocha et al., 2015). Moreover, according to Connolly et al. (2022) citrate coating increased the uptake of TiO_2NPs by rainbow trout, and the removal of the tissues was also faster during depuration. The smaller size of 5 nm NPs and the bigger surface area of the citrate coating could accelerate the bioaccumulation in living organisms.

In a previous study, Gallocchio et al. (2020) evaluated the accumulation of TiO_2NPs in Mytillus galloprovincialis grown in artificial seawater and exposed to ionic Ti (concentration in the tank of 1.6 mg L^{-1}) or TiO_2NPs (0.25 or 2.5 mg L^{-1}) for 4 days. The authors observed that the concentration of Ti (209–1119 µg kg^{-1}) in exposed mussels was proportional to the dose and independent of the form (ionic or nanoparticulate). The concentrations found in this study were of the same order as those obtained in the present work.

The estimated daily intake (EDI) of Ti can be calculated according to the following equation (Xu et al., 2020):

EDI = (Concentration of metal×Average daily ingestion rate)/Body weight.

The value of EDI was calculated for the maximum average concentration of Ti determined (5.19 µg g^{-1} w.w.), an average ingestion rate of 0.33 g mussel per day in Spain for adults between 18 and 74 years old ("ENALIA 2 Survey. National Food Survey on adults, the elderly and pregnant women" 2016), and a body weight of 70 kg. The value obtained was 0.0245 µg of Ti per day and kg of body weight, lower than the EDI (0.84–1.34) calculated by Xu et al. (2020)(Xu et al., 2020) for mollusk consumption, and much lower than the adverse effect level of the element proposed by Lim et al. (2018)(Lim et al., 2018) (6.25 $\times 10^4$ µg per kg BW and day). However, depuration is still important to minimize the potential effects of nanoparticles, which may also serve as vectors for introducing other pollutants into living organisms. Sami (2024) reviewed the literature on the depuration of bivalve mollusks following metal accumulation, collecting information on the techniques used, influencing factors, results, and considering possible elimination mechanisms due to the presence of specific ion channels and membrane transporters. Thus, Saha et al. (2006) observed a high degree of organ specificity in the regulation of metal levels in bivalves in the surrounding water, with greater metal accumulation in the gills and mantle, attributed to the ion exchange properties of the mucous layer present in these organs. Moreover, different authors studied the ability of several species to reduce metal concentrations after bioaccumulation: Mytilus edulis to eliminate Cu and Zn from antifouling paints (Turner et al., 2009), Pinna nobilis showing different patterns to eliminate metals (Cd, Pb, Mn, Zn, and Fe) from target tissues (Jebali et al., 2014), and Mytilus galloprovincialis and Callista chione to eliminate Cd and Ni after ten days of depuration (Chalkiadaki et al., 2014).

3.2.2. *Total Ti in mussel shells*

Weakening and fractures were observed in some mussel shells exposed to NPs in Trial 2 (bioaccumulation of mussels exposed to 5 nm TiO_2NPs). Approximately fifty percent of shells were damaged and cracked on the bottom of the tank from day 7 of exposure to both 0.10 and 1.0 mg L^{-1} TiO_2NPs; however, no differences in shell fragility were observed between the exposure concentrations. Therefore, shells were also sampled from that day onwards. Shi et al.(2020) had observed that the presence of TiO_2NPs decreased the byssal attachment strength of Mytilus coruscus after 10 days of exposure to a concentration of Ti as low as 100 µg L^{-1}. Then, a study was carried out to assess the presence of titanium in the shells. Shells from one control tank (tank A), one solvent tank (tank D), one of 0.10 mg L^{-1} of 5 nm TiO_2NPs tank (tank G), and one of 1.0 mg L^{-1} of 5 nm TiO_2NPs tank (tank J), were sampled at 7, 14, 21, and 28 days. The shells were washed with ultrapure water, dried, and pulverized in a zirconia ball mill (Retsch (Haan, Germany). The pulverized samples were submitted, in triplicate, to microwave-assisted acid digestion and the ICP-MS analysis following the process described in Section 2.3. Fig. 3 shows the variation of Ti concentration with exposure time in the four tanks studied. The highest Ti concentration was found in shells of mussels exposed at 1.0 mg L^{-1} for 21 days,

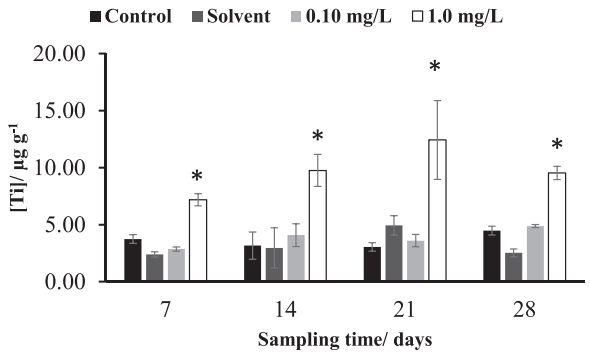

Fig. 3. Ti bioaccumulation in mussel shells exposed to 5 nm TiO_2NPs. "*" indicates significant differences between the high dose (1.0 mg L^{-1}) and the other groups on the same day.

reaching 12.4 ± 3.5 µg g^{-1} d.w. The multiple range tests showed that the concentrations of Ti in shells of mussels exposed to 1.0 mg L^{-1} were higher than those in the other tanks, including control and solvent tanks that could be considered as background level. Thus, it can be concluded that the shells of mussels bioaccumulated Ti after exposure to the high dose of TiO_2NPs, and it could be the reason for the weakening and breakage of those shells.

3.2.3. *TiO_2NPs in mussels*

Pancreatin-lipase extractions were performed only in mussels from tanks exposed to 0.10 and 1.0 mg L^{-1} of TiO_2NPs (NPs of both sizes). Fig. 4(a) shows the average intertank TiO_2NPs concentrations in mussels exposed to both concentrations of 25 nm TiO_2NPs for 28 exposure days. The highest NPs concentrations found were $1.15 \times 10^8 \pm 2.84 \times 10^7$ and $2.28 \times 10^8 \pm 5.84 \times 10^7$ TiO_2NPs g^{-1} for 0.10 and 1.0 mg L^{-1} of TiO_2NPs L^{-1}, respectively, both at the 28th day.

No statistical differences were found between the TiO_2NPs concentration at different exposure days with a 95 % of confidence level for mussels exposed to 0.10 mg L^{-1}. However, significative differences among TiO_2NPs concentrations at different exposure days were observed for mussels exposed to 1.0 mg L^{-1}. Therefore, the concentrations of TiO_2NPs in the samples increased with time, especially at the high exposure dose.

Fig. 4(b) and 4 (c) show the most frequent and the mean sizes of nanoparticles detected in mussels exposed to 25 nm TiO_2NPs for 28 days. For mussels exposed to 0.10 mg L^{-1} (Fig. 4(b)), the most frequent and mean sizes ranged between 60 ± 1 nm (0 days) and 62 ± 4 nm (28 days), and from 81 ± 2 nm (0 days) to 85 ± 10 nm (28 days), respectively. On the other hand, in mussels exposed to 1.0 mg L^{-1} (Fig. 4(c)), the most frequent and mean sizes ranged between 55 ± 4 nm (0 days) and 57 ± 2 nm (28 days), and from 70 ± 5 nm (0 days) to 74 ± 1 nm (28 days), respectively. No significant statistical differences were detected in the modal size of TiO_2NPs in mussels over the exposure period (at either 0.10 or 1.0 mg L^{-1}), nor were significant differences observed in the most frequent size when mussels were exposed to both concentrations of TiO_2NPs. Moreover, different concentrations did not affect the size of the particles.

Fig. 5(a) shows the average intertank TiO_2NPs concentrations in mussels exposed to 1.0 mg L^{-1} of 5 nm TiO_2NPs for 28 exposure days. In Trial 2 the enzymatic extractions in mussels exposed to 0.10 mg L^{-1} TiO_2NPs resulted in values lower than LOD_{number} ($<7.46 \times 10^7$ TiO_2NPs g^{-1}). TiO_2NPs content in mussels exposed to the high dose increased with the exposure time, reaching $4.79 \times 10^8 \pm 2.63 \times 10^8$ TiO_2NPs g^{-1} at 21 exposure days and no statistical differences were found between concentrations at different exposure days. Fig. 5(b) shows the most frequent and the mean sizes of nanoparticles detected in mussels exposed to 1.0 mg L^{-1} for 28 days. The most frequent and mean sizes were between 59 ± 7 nm (0 days) and 45 ± 1 nm (28 days), and

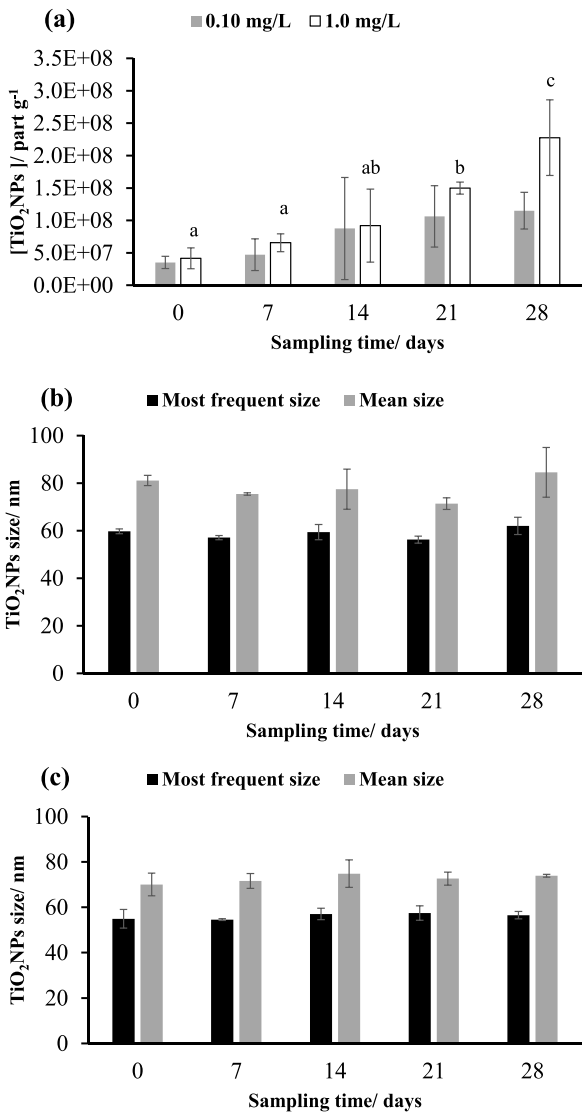

Fig. 4. Bioaccumulation in mussels exposed to 25 nm TiO_2NPs at 0.10 and 1.0 mg L^{-1} . (a). TiO_2NP concentration, (b) most frequent and mean particle size of TiO_2NPs in the 0.10 mg L^{-1} exposure group, (c) most frequent and mean particle size of TiO_2NPs in the 1.0 mg L^{-1} exposure group.

between 96 ± 4 nm (0 days) and 60 ± 1 nm (28 days), respectively. Although a slight decrease in the most frequent sizes can be observed with the exposure times, the one-way ANOVA test did not show significant differences (at 95 % of confidence level) for most frequent sizes between the exposure days. Mussel samples were rinsed with ultrapure water to remove TiO_2NPs adhered to the surface. However, after extraction, SP-ICP-MS analysis does not allow discrimination between particles adhered to the mussel surface and those internalized. Although this distinction is not relevant from a nutritional perspective, as mussels are consumed whole, electron microscopy studies would be valuable in future work to determine the exact localization of the nanoparticles.

The sizes measured for both trials were different from the theoretical primary sizes of 25 and 5 nm TiO_2NPs. This could be explained by the common aggregation phenomenon of TiO_2NPs. The citrate-coated TiO_2NPs aggregation was previously tested by Dynamic Light Scattering after alkaline extractions and SP-ICP-MS analysis in our research group (López-Mayán et al., 2023). TiO_2NPs of 25 and 5 nm presented aggregation in contact with ultrapure water, and even more in presence of seawater, where the TiO_2NPs formed large micrometer aggregates (128 ± 2.9 and 65 ± 14 nm for 25 and 5 nm TiO_2NPs respectively, in

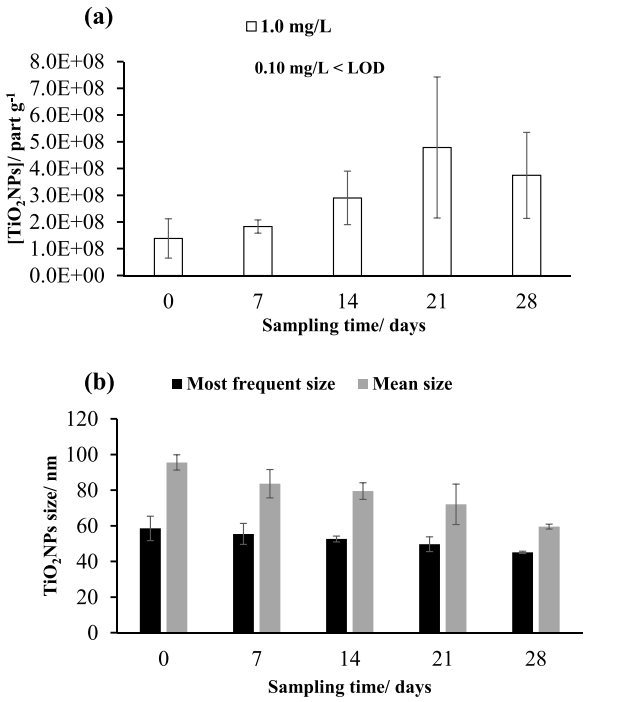

Fig. 5. Bioaccumulation in mussels exposed to 5 nm TiO_2NPs at 1.0 mg L^{-1}. Intertank average: (a) TiO_2NP concentration, (b) most frequent and mean particle size of TiO_2NPs.

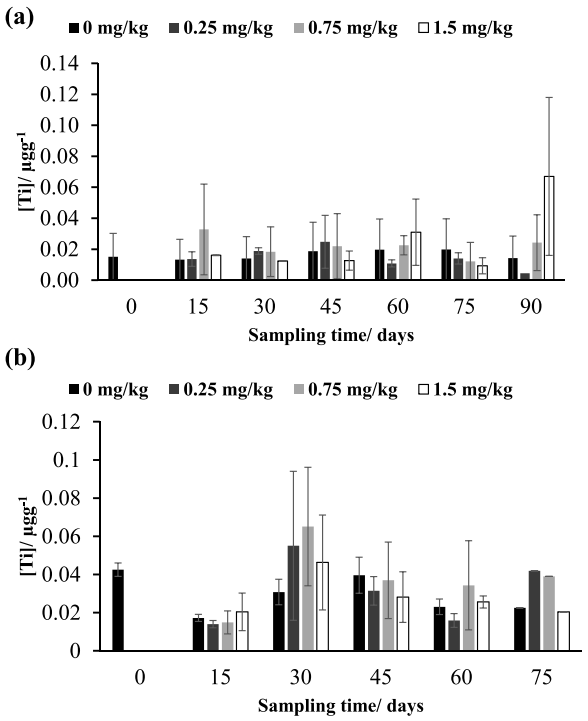

Fig. 6. Ti bioaccumulation in muscle with skin of turbot exposed to TiO_2NPs at 0, 0.25, 0.75, and 1.5 mg kg^{-1} fish and day. (a) Exposure to 25 nm TiO_2NPs. (b) Exposure to 5 nm TiO_2NPs.

ultrapure water; 3168 ± 1089 and 3320 ± 1248 nm for 25 and 5 nm TiO_2NPs respectively, in artificial seawater). Once the particles were internalized in the mussel matrix, the size (determined by SP-ICP-MS) changed again. This could be due to a stabilization effect during the enzymatic extraction to form smaller TiO_2NPs aggregates (mean sizes between 70 and 75 nm and between 60 and 100 nm for TiO_2NPs of 25 and 5 nm).

Fig. 3(S) shows a histogram example of SP-ICP-MS corresponding to mussels exposed to a dose of 1.0 mg L^{-1} of 25 nm TiO_2NPs for 21 days. This size distribution is narrower than that obtained by Gallochio et al. (2020) in samples exposed to 2.5 mg L^{-1} of nanosize titanium, and enzymatically digested with proteinase K, Tris buffer and 1 % Triton X-100. In their study about Mytillus galloprovincialis, Gallocchio et al. (2020) observed by SP-ICP-MS that the specimens accumulated NPs with average sizes of 93–111 nm. The size of the distributions was like that of pristine TiO_2NPs administered in the in vivo experiments. In the case of mussels exposed to ionic Ti, they also observed aggregation with NPs of an average size of 55 nm. In the case of offshore aquaculture mussels, Xu et al. (2020)(Xu et al., 2020) determined that the mean size of TiO_2NPs in the muscle of the specimens studied was 70.6 ± 12.7 nm.

3.3. Turbot bioaccumulation tests

3.3.1. Total Ti in turbot

Total Ti concentrations were determined in the liver, kidney, and muscle with skin in Trial 1 (juvenile turbots fed with 25 nm TiO_2NPs), and Trial 2 (juvenile turbots fed with 5 nm TiO_2NPs). Ti concentrations were lower than LOQ (<0.0045 mg kg^{-1}) in the case of the kidney, for all exposure doses and days. In the case of muscle with skin (Fig. 6), the amount of Ti found in the samples was approximately the basal Ti concentration. In both trials, only Ti bioaccumulation was observed in the liver (Fig. 7).

Fig. 6(a) and (b) show total Ti concentrations in muscle with skin of turbots exposed to 0, 0.25, 0.75, and 1.5 mg TiO_2NPs kg^{-1} fish and day of 25 nm for 90 days, and 5 nm for 75 days, respectively. No statistical differences were found among Ti concentrations as a function of exposure time, and among Ti concentrations as a function of exposure dose for both trials. Therefore, no bioaccumulation was observed in turbot muscle.

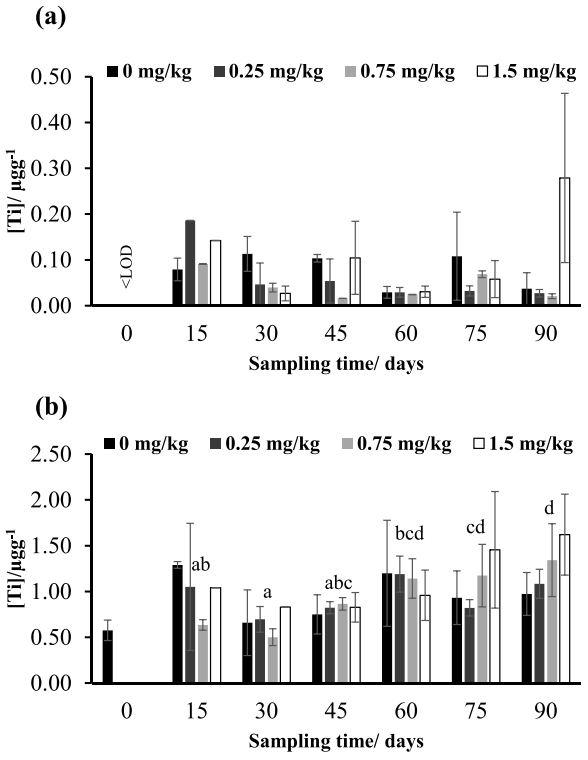

Fig. 7. Ti bioaccumulation in liver of turbots exposed to TiO_2NPs at 0, 0.25, 0.75, and 1.5 mg kg^{-1} fish and day. (a) Exposure to 25 nm TiO_2NPs. (b) Exposure to 5 nm TiO_2NPs.

Fig. 7(a) and (b) show total Ti concentration in turbot liver. Different letters indicate significant differences over time at 0.75 mg kg^{-1} (ANOVA, $P < 0.05$ and multiple range test). Higher Ti concentrations were found in turbot exposed to 5 nm TiO_2NPs (Fig. 7 (b)) than in turbot exposed to 25 nm TiO_2NPs (Fig. 7(a)). The highest Ti concentrations reached in the liver of turbot exposed to 25 and 5 nm of TiO_2NPs were 0.28 ± 0.18 and 1.6 ± 0.4 μg g^{-1} w.w., respectively, measured after 90 days of exposure to the highest dose. In the case of turbot fed with 25 nm TiO_2NPs, no statistical differences were found between Ti concentrations at the different exposure times. However, statistical differences were found at different exposure times in the liver from turbots exposed to 0.75 mg kg^{-1} of TiO_2NPs (P-value<0.05) for turbot fed with 5 nm TiO_2NPs. (Fig. 7(b)). The multiple range test provided significant differences between 15 days and 90 days, and between 30 days and 60, 75, and 90 days. Therefore, it seems that there is a slight increase in the concentration of Ti in the liver from turbots exposed to nanoparticles of 5 nm, and that is difficult to observe due to the variability of the samples.

3.3.2. Total Ti in turbot feces

The presence of Ti in feces collected from aquaculture tanks was also studied. The samples were dried in a laboratory oven and then homogenized and digested using the microwave-assisted acid digestion method used for turbot samples described in Section 2.3. By the end of the experiment, the feces were collected in both Trials at different exposure TiO_2NPs doses (0, 0.25, 0.75, and 1.5). Fig. 8(a) and 8 (b) show the linear increase of Ti in feces with the increasing dose of 25 and 5 nm TiO_2NPs nanoparticles, respectively. Ti concentration increased with time, and the highest concentrations measured were 11.9 ± 1.7 and 41.0 ± 6.6 μg g^{-1} w.w. for an equivalent dose of 1.5 mg kg^{-1} TiO_2NPs fish and day of 25 and 5 nm, respectively.

As in mussels, the highest concentrations of Ti were found in Trial 2, and this fact agrees with the results obtained for turbot liver, with a bigger accumulation and subsequent excretion of Ti observed for 5 nm nanoparticles. The feces results indicate that most of Ti is excreted through the turbot feces and only a bit is accumulated in the turbot liver.

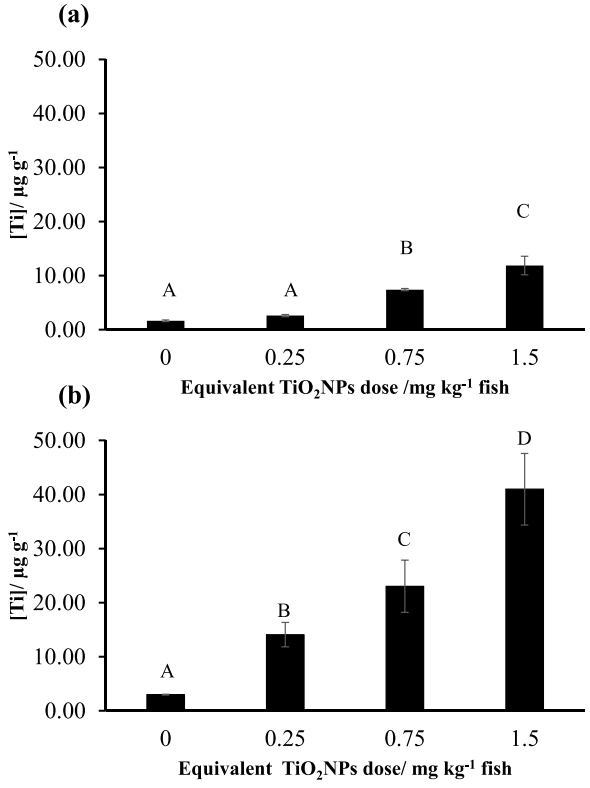

Fig. 8. Ti concentration in feces of turbot exposed to TiO_2NPs (mg Kg^{-1} fish and day). (a) Exposure to 25 nm TiO_2NPs. (b) Exposure to 5 nm TiO_2NPs.

3.3.3. TiO_2NPs in turbot

Since total Ti in the kidney was lower than the LOQ, and total Ti in muscle was present at the basal Ti concentration, the enzymatic extractions were performed only in the liver of turbot specimens exposed to 25 and 5 nm TiO_2NPs. No peaks corresponding to NPs were detected in SP-ICP-MS monitoring in any liver sample; thus, all the concentrations in the samples were lower than 4.46×10^6 NPs g^{-1}. This indicates that the accumulation in the liver is only related to ionic Ti, metabolizing NPs into ionic Ti or TiO_2NPs lower in size than 42 nm.

Even when the concentration of Ti or nanoparticles did not increase with time in turbot (Fig. 6), other effects of the exposure were observed. Thus, the weight gain of the specimens was lower at the highest exposure dose of 25 nm TiO_2NPs compared to the control turbot. The initial mean weight of juvenile turbot was 45.0 ± 4.5 g ($n = 10$). After 90 days of exposure, the mean weight of fish in the control tanks was 184.5 ± 31.5 g ($n = 12$), whereas those exposed to 1.5 mg kg^{-1} of 25 nm TiO_2NPs averaged 153.8 ± 32.1 g ($n = 12$). A one-way ANOVA test followed by a Multiple Range Test indicated a statistically significant reduction in weight in the exposed group compared to control group after 90 days of exposure ($p < 0.05$).

Exposure to NPs through the diet decreases the dispersibility problems of the nanomaterials and facilitates the control of the exposure doses (Mona et al., 2023). As an example, Wang et al. (2016) (Wang et al., 2016) observed a fast aggregation and sedimentation of TiO_2 NPs in seawater used for feeding juvenile turbots. However, a similar pattern of accumulation of Ti was found for both types of exposure (aqueous or through the diet of treated clamworms), with specimens showing a higher concentration of the element in gills, intestines and stomach, followed by skin, liver and muscle. After 20 days, histological analysis by microscopy showed pathological changes in the tissue of the liver and spleen(Wang et al., 2016). Alterations in the liver were also observed by our research group in juvenile turbots of this study after 14 days of exposure to citrate-coated 25 nm TiO_2NPs (Araújo et al., 2022). Proteomic results showed Differentially expressed proteins (DEPs) in turbot livers exposed to 25 nm TiO_2NPs (*lower concentration*), where they could be related with metabolic pathways, purine metabolism, drug metabolism, and pyrimidine metabolism. The number of lipid droplets in hepatocytes decreased but their diameters increased, and proteomic analysis revealed lipid and energy metabolism modifications (Araújo et al., 2022). Then, the active contribution of the fish liver for detoxification was confirmed, whereas the analysis of whole mussels seems more helpful as a sentinel organism for TiO_2NPs in environmental research.

4. Conclusions

This research work studied the bioaccumulation of TiO_2NPs in an aquaculture benthic bivalve (Mytilus edulis) and a benthic predator (Scophthalmus maximus). The bioaccumulation assays using different sizes of nanoparticles (25 nm and 5 nm) and different exposure concentrations were carried out during long exposure times (until 90 days in the case of fish). Mussels bioaccumulated Ti and TiO_2NPs of 25 and 5 nm, with the concentrations increasing with the dose and exposure time. The total Ti content in fractured and weakened shells of mussels exposed to 5 nm TiO_2NPs was also determined, resulting in a higher amount of the element in tanks of mussels exposed to high doses in comparison to the control tanks. The bioaccumulation of Ti in mussels highlights the need for purification periods of these filtering organisms to ensure the food quality of this highly consumed product around the world. Ti was also determined in the muscle with skin, kidney and liver of turbot, and the highest Ti concentrations were found in the liver, the un-edible part of this appreciated flat fish. Finally, the fact that the muscle tissue does not accumulate Ti, and that the element is excreted

through the feces shows that turbot can be considered safe for human consumption.

CRediT authorship contribution statement

María Vázquez: Resources, Methodology. **Natalia Mallo:** Resources, Methodology. **Santiago Cabaleiro:** Resources, Funding acquisition, Conceptualization. **Antonio Moreda-Piñeiro:** Project administration, Methodology, Funding acquisition, Conceptualization. **Pilar Bermejo-Barrera:** Project administration, Funding acquisition, Conceptualization. **Julie Maguire:** Resources, Conceptualization. **Mick Mackey:** Resources, Methodology. **María Carmen Barciela-Alonso:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Data curation. **Juan José López-Mayán:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Elena Peña-Vázquez:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2025.108790.

Data availability

Data will be made available on request.

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