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Bioaccumulation and human risk assessment of inorganic nanoparticles in aquaculture species†

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The escalating use of inorganic nanoparticles (NPs) in various applications raises concerns regarding their potential environmental release and subsequent bioaccumulation in the food chain, posing a risk to human health. This study aimed to assess the bioaccumulation potential of titanium dioxide (TiO₂) and silver (Ag) NPs in three commercially relevant aquatic species: sea bream, sea bass, and Japanese carpet shell, and evaluate the associated human health risks through dietary exposure. Bioaccumulation patterns were evaluated in target organs (liver, kidney, and muscle) of sea bream and sea bass following dietary exposure to varying concentrations of NPs (0.25–1.5 mg kg^{−1}) for extended durations (up to 90 days). While moderate bioaccumulation was observed in non-edible organs like kidneys and livers, no significant accumulation was detected in the muscle tissue, even at high exposure levels. Conversely, bioaccumulation of both TiO₂ and Ag NPs was evident in the soft tissues of Japanese carpet shell (maximum concentrations: 2.5 × 10^{−10} g^{−1} for Ag NPs and 8.0 × 10^{−6} g^{−1} for TiO₂ NPs). *In vitro* studies utilizing the Caco-2 human intestinal model revealed limited transcellular transport of NPs from both fish and shellfish muscle tissue (less than 34% for TiO₂ NPs in sea bream and less than 61% and 4% for TiO₂ NPs and Ag NPs, respectively, in Japanese carpet shell). These findings suggest that, while bioaccumulation may occur in certain species and organs, the human health risk associated with dietary exposure to NPs from commonly consumed fish appears to be low due to limited intestinal uptake. However, further research is necessary to elucidate the long-term consequences of chronic exposure and potential health effects.

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Environmental significance

The increase in the use of nanomaterials in several manufactured goods and the potential release of nanomaterials into the environment concerns the scientific community due to the possible risk to humans and the marine ecosystem. We have studied the bioaccumulation of titanium dioxide and silver nanoparticles (TiO₂ NPs and Ag NPs) in aquaculture species such as sea bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*), and Japanese carpet shell (*Ruditapes philippinarum*). Results have revealed that TiO₂ NPs and Ag NPs are not bioaccumulated in fish's flesh, whereas moderate bioaccumulation was observed in the kidneys and liver (non-edible parts). However, Ag NPs and TiO₂ NPs can be bioaccumulated in Japanese carpet shell, although human *in vitro* bioavailability studies have shown low bioavailability.

Introduction

Nanotechnology is now an emerging area of study with many applications in science and technology. According to the European Commission (EC) the term “nanomaterial” embraces all materials specified by EC Recommendation

2022/C229/01 of 10 June 2022 as “a natural, incidental or manufactured material consisting of solid particles that are present, either on their own or as identifiable constituent particles in aggregates or agglomerates, and where 50% or more of these particles in the number”.¹ Based on the StatNano database,² silver nanoparticles (Ag NPs) and titanium dioxide nanoparticles (TiO₂ NPs) are widely used in several industrial sectors such as cosmetics, textile, and medicine. In the case of Ag NPs, about 50% of all nanomaterials-based products used in medicine contain Ag NPs in their formulations.²

Due to the large-scale production and use of NPs their release and presence in the environment is expected. However, the assessment of the true impacts of the presence

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of NPs in the aquatic environment is difficult because the released NPs vary their characteristics when interacting with natural components of aquatic ecosystems. As examples, dissolved compounds in both lake and seawater promote TiO₂ NPs aggregation and sedimentation,³ whereas interactions between Ag NPs and some dissolved organic compounds with oxidative properties promote dissolution (generation of hazardous dissolved ions).⁴ In this regard, some studies have suggested that Ag NPs in environmentally relevant concentrations are not stable in seawater for more than three days.⁵ Since NPs are emerging pollutants, NPs background levels in seawater are nowadays low. Besides, although fish feeding with fish feed containing NPs is not usual, Khosravi-Katuli *et al.*⁶ have reported that animals in aquaculture facilities could be exposed to NPs if nanomaterials are used for seawater treatment and fishpond sterilization, and also by accidental discharges near the catchment area. Therefore, there is concern about the potential effect of NPs in aquaculture species and several research have explored this scenario by performing exposure experiments by keeping the specimens in water containing NPs suspensions.^{7–10} Moreover, cultured animals could be also exposed to NPs by ingestion since NPs could be accidentally or intentionally (use of feed additives) present in some feed's formulations.^{11–13}

Chemical composition, size, shape, surface coating or modification, solubility properties, and other physical and chemical properties are important factors that condition the danger of NPs.^{14,15} To increase the available data about the possible toxicity of NPs, the European Food Safety Agency (EFSA) has published a guidance on the risk assessment of nanomaterials¹⁶ which encompass several assays for nanotoxicity assessment (bio-accessibility and bioavailability, among others). Bioaccumulation can be defined as the build-up of chemicals, usually harmful, in the body of an organism from different exposure sources (mainly water, air, and diet) that are not metabolized or excreted, and consequently accumulate in the organisms over time.¹⁷ Regarding human consumption, the term bio-accessibility refers to the fraction of a compound that is released from the food matrix in the gastrointestinal tract and consequently, is available for absorption. In addition, the term bioavailability names the fraction of a compound that can be taken up by the body, enter circulation and being able to have an active effect.^{16,18} The knowledge of these three parameters enables to assess the risk related to pollutants in the environment and humans. Caco-2 cells are typically used as a model to perform bioavailability studies since they are derived from human colorectal adenocarcinoma and exhibit remarkable morphological and physiological similarities to the human intestine.¹⁹ Previous studies with Caco-2 cultures and Caco-2/HT29 cultures for transcellular transport and uptake of NPs can be found elsewhere.^{20–23}

Regarding marine organisms, the small size and rapid development of zebrafish embryos make these species the preferred candidates for bioaccumulation studies.²⁴ In

addition, some investigations have been conducted in fish organs, such as rainbow trout,²⁵ goldfish,^{26,27} and zebrafish,²⁸ which have shown potential toxicity and effective bioaccumulation after NPs exposure. Other studies have stated that the gills, intestine, liver, and brain are the most affected organs in fish species. Oxidative stress is a potential mechanism of NPs toxicity in fish, as well as the induction of new enzymes involved in antioxidant defences.²⁹ However, the concentration of metals and NPs in the natural environment (especially in the aquatic environment) is quite low, far from the high dose conditions required for acute toxicity testing.

The aim of this research has been the evaluation of Ag NPs and TiO₂ NPs bioaccumulation in cultured fish sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*), and in Japanese carpet shell (*Ruditapes philippinarum*) through controlled-NPs exposure experiments. In addition, human risk assessment of potential consumption of marine products with high Ag NPs and TiO₂ NPs contents has also been studied. To this end, *in vitro* bio-accessibility and bioavailability (Caco-2 cells model for transcellular transport assessment) assays were carried out.

Material and methods

Sea bream, sea bass, and Japanese carpet shell exposure trials and sample preparation

Exposure trials for sea bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*), and Japanese carpet shell (*Ruditapes philippinarum*) with NPs were carried out by personnel qualified in animal experimentation, in authorized facilities of Centro Tecnológico de Acuicultura, CETGA (Ribeira, A Coruña, Spain). All experimental procedures were carried out in accordance with European Union and Spanish Regulations (Council Directive 2010/63/EU (European Union, 2010) and R. D. 53/2013 (BOE, 2013), respectively), for the protection of animals used for experimental purposes.

Regarding assays with sea bass and sea bream, TiO₂ NPs and Ag NPs were incorporated to commercial fish feed pellets (details are given in ESI†) following the method described in previous investigation,³⁰ whereas Japanese carpet shells were fed with microalgae mixture (*Isochrysis galbana* (T-ISO) and *Phaeodactylum tricornutum* (50 : 50, v/v)).

Experiments were performed with fifty individuals in 400 L open circuit tanks (sea bass, average initial weight of 121.6 g), and one hundred and twenty individuals in open circuit 300 L tanks (sea bream, average initial weight of 7.7 g) for 90 days. Sampling was performed each 15 days obtaining exposure times of 0 (experiment beginning), 15, 30, 45, 60, 75, and 90 days. Different exposure NPs concentrations were tested for both cultured species: 0 (control tanks), and 0.25, 0.75 and 1.5 mg kg⁻¹ (concentration referred as the mg of Ag NPs or TiO₂ NPs per kg of fish). Regarding Japanese carpet shell specimens, assays were carried out with forty individuals in 50 L closed circuit tanks. Detailed information



regarding exposure trials and sample preparation can be found in ESI† section.

Microwave assisted acid digestion and enzymatic hydrolysis procedures

Microwave assisted acid digestion was performed for further assessing total silver and titanium contents. The procedure consisted of treating in triplicate approximately 1.000 g of homogenised tissue (muscle + skin from fish and soft tissues from Japanese carpet shell), with a nitric acid/hydrogen peroxide mixture under microwave energy (detailed information can be found in ESI† section). A similar procedure was performed for liver and kidney tissues from fish, although only one replicate (0.1500 g) per specimen was used for digestion (small sample size).

The isolation of Ag NPs and TiO₂ NPs from seafood tissues was performed by enzymatic hydrolysis (pancreatin plus lipase) in accordance with previous developments^{31,32} (detailed information can be found in ESI† section).

Culinary treatments: grilling and boiling

Studies with liver and kidney tissues from fish were discarded since fish's kidney/liver do not offer nutritional interest. Because of the low Ag bioaccumulation in sea bass's muscle + skin and Ti bioaccumulation in sea bream's muscle + skin, the effect of the culinary treatment was only carried out for muscle + skin from sea bream specimens exposed to 1.5 mg TiO₂ NPs kg⁻¹ for 75 days. Sea bream muscle + skin from unexposed specimens (control at 90 days) was also used in the study for comparative purposes. Regarding Japanese carpet shell, the effect of grilling was only tested in samples obtained after 28-day exposure to Ag NPs (0.1 and 1.0 mg kg⁻¹), and in samples from experiments with the maximum TiO₂ NPs dose (1.0 mg kg⁻¹) after 21-day and 28-day exposure.

For all cases, two sample pools were prepared, and total Ti and Ag contents (and TiO₂ NPs and Ag NPs concentrations) were assessed in raw and cooked samples in duplicate (the remaining pooled samples were kept for further bio-accessible and bioavailable experiments). Grilling and boiling were carried out without using oil and spices. In accordance with the literature,³³ grilling treatment was done for 5.0 min, allowing then grilled samples to cool at room temperature. Boiling procedure for sea bream's flesh was carried out by applying heat to 300 mL of ultrapure water inside a cooking pot. Temperature (boiling temperature within the 90–100 °C range) was controlled with a thermometer, and once the boiling temperature was achieved, the fish tissues were immersed a cooked for 10 min. Then, samples were placed onto Petri dishes and left to cool at room temperature and then at 30–40 °C in an oven to dry the cooked sample.

***In vitro* digestion procedure: bio-accessibility assays**

An *in vitro* digestion approach that mimics the environment of the stomach (use of pepsin at acid pH) and intestines (use

of pancreatin and bile salts at neutral pH) was used as a model of human gastrointestinal process.³⁴ Detailed information can be found in ESI† section.

The bio-accessibility ratio was calculated according to eqn (1):¹⁸

$$\% \text{ Bio-accessibility} = \frac{\text{Bio-accessible}}{\text{Total}} \times 100 \quad (1)$$

where \square_{Total} is the total Ti/Ag concentration after microwave assisted acid digestion and ICP-MS assessment or the TiO_2 NPs/Ag NPs number concentration after enzymatic hydrolysis and spICP-MS in sea bream's muscle-skin (raw, grilled and boiled) and Japanese carpet shell (raw and grilled); and $\square_{\text{Bio-accessible}}$ is the total Ti/Ag concentration in the bio-accessible fraction after *in vitro* bio-accessibility and ICP-MS assessment or the TiO_2 NPs/Ag NPs number concentration after *in vitro* bio-accessibility and spICP-MS in sea bream's muscle-skin (raw, grilled and boiled) and Japanese carpet shell (raw and grilled).

Caco-2 cellular transport assays

After Caco-2 monolayer development (see details in ESI† section), cellular transport was performed by loading 1.5 mL of bio-accessible fraction (section 2.4), previously denatured and adjusting the osmolarity at 280–300 mOsm cm², at the basolateral chamber of six-well Transwell® (see details in ESI† section). The transwell plates were then placed in a temperature-controlled environment (37 °C, 95% relative humidity, and 5% CO₂ flow) for 2.0 h (see detail in ESI† section).

The basolateral and apical [2.0 mL of Hanks' Balanced Salt Solution (HBSS)] solutions were carefully removed and kept for analysis. Each bio-accessible fraction was subjected to the cellular transport procedure in triplicate which allows six independent measurements (two bio-accessible fractions per sample). At least two blanks were subjected to the same process in each set of samples.

The bioavailability (transcellular transport across the intestinal epithelium) of total Ti and Ag, and TiO₂ NPs and Ag NPs was obtained by applying eqn (2):¹⁸

$$\% \text{ Transport} = \frac{V_{\text{Basal}}}{V_{\text{Bio-accessible}}} \times 100 \quad (2)$$

where \square_{Basal} is the total Ti/Ag concentration, or TiO₂ NPs/Ag NPs number concentration in the basolateral solution, and $\square_{\text{Bio-accessible}}$ is the total Ti/Ag concentration, or TiO₂ NPs/Ag NPs number concentration in the bio-accessible fraction.

ICP-MS measurements

The determination of the total Ag and Ti contents in the acid digests, bio-accessible fractions and apical and basolateral were performed under the ICP-MS operating conditions detailed in Table 1. Details on instrument components as well as daily performance are given in ESI† section. Rhodium was used as an internal standard for Ag determination under

Table 1 Instrumental conditions and data acquisition used for ICP-MS

Spray chamber type	QuartzCyclonic
PC3x Peltier Cooler System	4 °C
Nebulizer type	Concentric Meinhard™
RF power (W)	1600
Ar plasma gas flow rate (L min ⁻¹)	15
Ar auxiliary gas flow rate (L min ⁻¹)	1.2
Ar nebulizer gas flow (L min ⁻¹)	1.14
Sample loop (μL)	100
Dwell time (ms)	50
Analyte (<i>m/z</i>)	Ag (107)
Internal standard (<i>m/z</i>)	Rh (103)
Mode	KED or collision mode
Helium flow rate (mL min ⁻¹)	4.5
Analyte (<i>m/z</i>)	Ti (131)
Internal standard (<i>m/z</i>)	Sc (45)
Mode	Dynamic reaction cell technology
Ammonia flow rate (mL min ⁻¹)	1.0
Ion-product registered	⁴⁸ Ti(NH)(NH ₃) ₄
Rejection parameter <i>q</i>	0.20
Quadrupole ion deflector (V)	Set for maximum ion transmission

KED work mode using helium at 4.5 mL min⁻¹ as a collision gas, whereas scandium was the selected internal standard for Ti determination under Dynamic Reaction Cell technology by using ammonia at 1.0 mL min⁻¹ as a reaction gas (the ammonia adduct Ti(NH)(NH₃)₄, mass-charge ratio of 131, was recorded). The standard addition method was used for determinations covering range concentrations from 0.1 to 10 μg L⁻¹ for Ag and Ti. The limit of detection and quantification of the method are listed in Table S1 (ESI†).

Single particle-ICP-MS measurements

The determinations for Ag NPs and TiO₂ NPs aiming particle number concentrations and size distributions were

Table 2 Operating conditions for sp-ICP-MS analysis

Analyte (<i>m/z</i>)	Ti (131)
Density (g cm ⁻³)	4.23
Mass fraction	59.90%
Sample flow rate (mL min ⁻¹)	≈0.18
Transport efficiency (%)	≈8%
Dwell time (μs)	100
Mode	Dynamic reaction cell technology
Ammonia flow rate (mL min ⁻¹)	0.75
Ion-product registered	⁴⁸ Ti(NH)(NH ₃) ₄
Rejection parameter <i>q</i>	0.20
Quadrupole ion deflector (V)	Set for maximum ion transmission
Analyte (<i>m/z</i>)	Ag (107)
Density (g cm ⁻³)	10.49
Mass fraction	100%
Sample flow rate (mL min ⁻¹)	≈0.20
Transport efficiency (%)	≈8%
Dwell time (μs)	50
Mode	Standard
Quadrupole ion deflector (V)	Set for maximum ion transmission

performed by ICP-MS operating in the single particle mode (spICP-MS) under operating conditions summarized in Table 2. Details on operating conditions as well as daily performance are given in ESI† section. Transport efficiency (TE%) was assessed by the particle frequency method (see details in ESI† section), resulting in values close to 8.0%.

Calibrations were performed using ultrapure water and 1.0% (v/v) glycerol covering ionic Ti and Ag concentrations within the 0.1–10 μg L⁻¹ range. Several reagent blanks were also analysed throughout the work. Extracts containing TiO₂ NPs and/or Ag NPs were dispersed before analysis by using ultrasound. The limit of detection (number concentration and size) and quantification (number concentration) of the method are listed in Table S1 (ESI†).

Statistical analysis

Fish and clam growth parameters (weight gain (WG), feed conversion ratio (FCR), and specific growth rate (SGR)) were analysed using one-way analysis of variance (ANOVA) following three replicates per treatment (*n* = 3). Statistically significant differences (*p* < 0.05) between groups were identified using Fisher's Least Significant Difference (LSD) *post-hoc* test (STATGRAPHICS Centurion XVI). Mean values with standard deviations are presented in Tables S2 and S3.†

Total Ag and Ti concentrations, as well as Ag NPs and TiO₂ NPs levels, were also analysed by one-way ANOVA after different cooking procedures. Similar to growth parameters, statistically significant differences (*p* < 0.05) were identified.

Results and discussion

Several studies regarding NPs bioaccumulation in biota under controlled laboratory conditions have been reported. The conclusions obtained from these studies depend greatly on the fish/mollusc species under investigation (carp fish,^{7,35,36} rainbow trout,^{9,37} silver barb,¹⁰ Persian sturgeon,³⁸ zebrafish,³⁹ goldfish,^{26,27} turbot,^{30,40} mussel,^{41–43} red swamp crayfish,⁴⁴ clam,^{45–48} and oyster⁴²), as well as the NPs exposure doses (from μg L⁻¹ to mg L⁻¹) and the exposure time (from hours to several days). On the other hand, water salinity^{9,38} and the presence of organic matter^{35,39} have also been found as important parameters affecting the bioaccumulation trends. However, there are two key features to highlight when interpreting the results of these studies. First, the way in which NPs are administered to animals. In most cases, NPs are added to the water in the tanks so, depending on the salinity of the water, the added NPs will be less available for the animals (the higher the salinity of the water, the higher of NPs agglomeration and settlement at the bottom of the tank⁹). A more realistic situation would be NPs integration into the feed to ensure their intake. Second, another important point is the analytical methodology used for NPs assessment. Although the animals were exposed to NPs, most of studies assess the total metal content in the animal's tissues (NPs number concentration is not obtained). Therefore, the determination of total metal contents for



experiments with NPs such as Ag NPs, which are easily ionised during animal metabolism (and when are suspended in water), leads to un-realistic values of NPs bioaccumulation since a great proportion of the measured bioaccumulated fraction encompasses ionic metal.

Bioaccumulation: total Ag and Ti

Sea bass and sea bream. Total Ti concentrations for sea bream, plotted in Fig. 1, and total Ag concentrations for sea

bass (Fig. S1, ESI†), show lower total contents in muscle + skin (within the ng g^{-1} range) than in kidney and liver tissues (concentration within the $\mu\text{g g}^{-1}$ range). This is especially significant for sea bass (Fig. S1, ESI†) which shows bioaccumulation for total Ag quite close to the limit of detection of the method. The highest total Ag contents in sea bass muscle + skin were achieved when exposed to the highest exposure concentration (1.5 mg kg^{-1}) after 60 and 75 exposure days (26.1 ± 1 and $72.6 \pm 50.8 \text{ ng g}^{-1}$, respectively). Total Ti levels in muscle + skin from sea bream are also low (Fig. 1), and the assessed values were between the LOD (83.2 ng g^{-1}) and the LOQ (277 ng g^{-1}) of the method (highest Ag concentrations of 162.9 and 232.8 ng g^{-1} when exposing at 1.5 mg kg^{-1} for 45 and 75 days, respectively). These findings are in good agreement with those previously reported for Ag in fish after Ag NPs and TiO_2 NPs exposure, which have revealed poor bioaccumulation in fish's muscle.^{7,9,26,27,30,36–40}

Regarding liver tissues, Fig. S1 (ESI†) shows that Ag is bioaccumulated from the beginning of the experiment (sampling at 15 days) with maximum bioaccumulation in the middle of the exposure time (between 15 and 60 days) and decreasing at the end of the experiment (sampling at 75 and 90 days) when using the highest exposure doses (0.75 and 1.5 mg kg^{-1}). The maximum Ag bioaccumulation was observed after 30 days of exposure at 0.75 mg kg^{-1} ($4.60 \mu\text{g Ag g}^{-1}$, Fig. S1, ESI†). This trend is also observed for Ti bioaccumulation in sea bream's liver (Fig. 1) although the maximum Ti bioaccumulation was observed after 60 days of exposure at 1.5 mg kg^{-1} ($1.14 \pm 0.09 \mu\text{g Ti g}^{-1}$). Although the exposure time and the fish species were different, maximum bioaccumulation ratios in the middle of the assays have been reported by Xiao *et al.*³⁹ for zebrafish exposed to Ag NPs, and by Ribeiro *et al.*²⁶ for goldfish also exposed to Ag NPs. In general, higher Ag bioaccumulation in sea bass's liver (within the 2.0 – $4.5 \mu\text{g Ag g}^{-1}$ range, Fig. S1 – ESI†) than Ti in sea bream's liver (within the 0.5 – $1.5 \mu\text{g Ti g}^{-1}$ range, Fig. 1) were found, and high bioaccumulation was observed when using the highest exposure doses.

However, clear trends for Ag and Ti bioaccumulation in kidney tissues were not found (Fig. S1 – ESI† and Fig. 1) and similar bioaccumulation concentrations can be observed from the middle to the end of the experiment (within the 15–90 days range in the case of Ag, and between 45 and 90 days for Ti mainly when using the highest dose).

The lower Ag and Ti bioaccumulation at large exposure times is not because fish eat less feed, since the weight of the fish increases according to the time of exposure and feeding (Fig. S2 and S3, ESI†). On the other hand, the evaluation of growth parameters (culture conditions) in fish growth (WG, FCR, and SGR) are similar in all cases (Table S2 and S3, ESI†). No significant differences were observed for any parameter, in both studies. Furthermore, no mortality and anomalous behaviour were registered.

These findings agree with the literature, which shows intestine, liver, kidney, and gills as the target organs for Ag NPs and TiO_2 NPs bioaccumulation. Therefore, moderate-

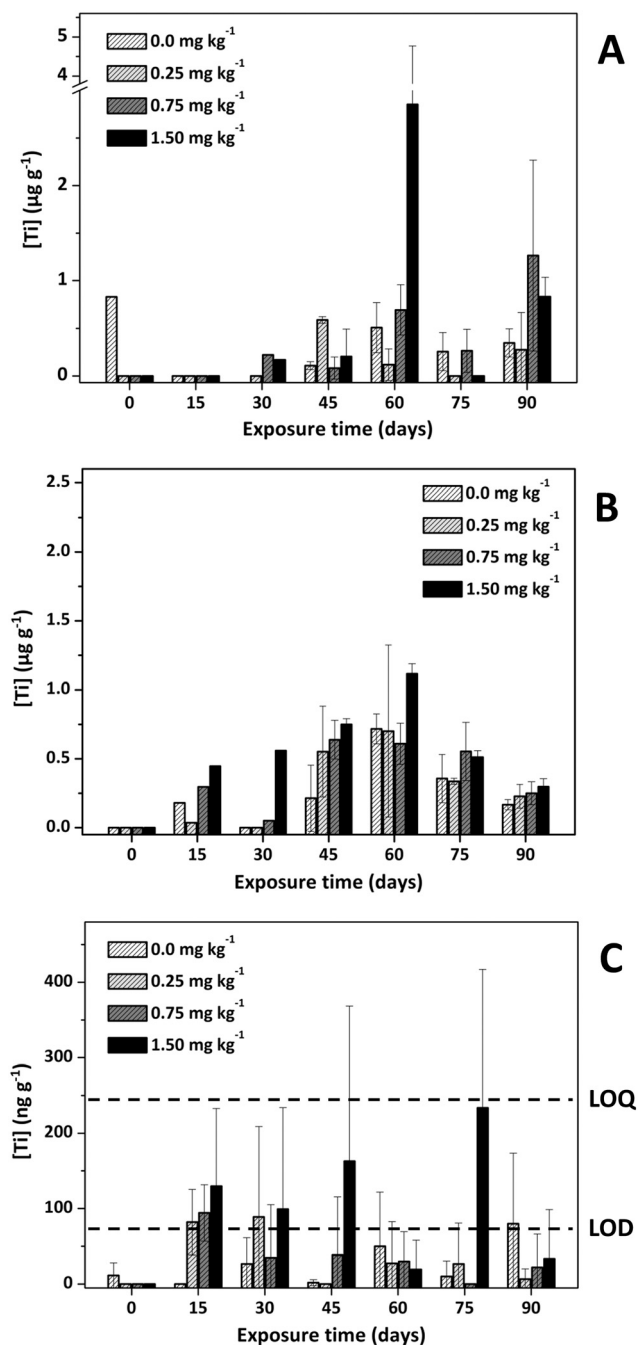


Fig. 1 Titanium concentrations in sea bream kidney (A), liver (B), and muscle + skin (C) after several exposure conditions.



high Ag bioaccumulation has been reported in carp fish's liver^{7,36} and gill,³⁶ rainbow trout's liver and kidney,^{9,37} turbot's liver,³⁰ and in liver, kidney and gill from Persian sturgeon,³⁸ zebrafish³⁹ and goldfish.²⁶ Regarding TiO₂ NPs, exposure assays with goldfish have shown Ti bioaccumulation the intestine and gill,²⁷ whereas liver was the target organ in the case of turbot.^{30,40}

Japanese carpet shell. Fig. 2 shows the total Ag and total Ti concentrations in carpet shells after each exposure condition. In general, higher Ag and Ti bioaccumulation than those measured for sea bass and sea bream were obtained, achieving maximum silver contents of $3.5 \pm 1.0 \mu\text{g g}^{-1}$ and total titanium contents of approximately $5.5 \pm 1.0 \mu\text{g g}^{-1}$. Regarding silver bioaccumulation (Fig. 2A), there were no differences when using exposing to Ag NPs of different concentrations (0.1 and 1.0 mg kg⁻¹), and an increase in the total Ag contents is observed at the beginning of the experiment (sampling at the 7th day) which remains constant until the end of trial (sampling at 28th day). These findings agree with those previously reported for freshwater clam (*Corbicula fluminea*)⁴⁶ and zebra mussel (*Dreissena polymorpha*)⁴³ which have also revealed high Ag bioaccumulation in the soft tissue at the beginning-middle of the experiment, remaining then constant or decreasing at the end of the experiment.

A decrease in weight (Fig. S6, ESI†) and no variation in shell length (Fig. S7, ESI†) was observed for specimens exposed to Ag NPs, where no significant differences were observed (Table S6 and S7, ESI†). The shells from the specimens exposed to the highest Ag NPs dose were found to be brittle. This finding has also been reported by Liu *et al.*⁴⁸ for the freshwater clam *Corbicula fluminea*, and the authors claim that the easily broken shells after exposure at high Ag NPs doses is consequence of induced calcospherite disintegration phenomena (loss of calcium) by Ag NPs. Nevertheless, no mortalities were observed in both clam assays.

Total Ti contents were found to gradually increase along the exposure trial, and higher bioaccumulation (Fig. 2B) was also observed when exposed at the highest TiO₂ NPs dose (1.0 mg kg⁻¹). These findings are quite similar to those reported by Kuehr *et al.*⁴⁶ for the freshwater clam *Corbicula fluminea* using TiO₂ NPs of two different sizes. Comparisons with other studies were not possible since most of them assess Ti bioaccumulation^{41,42,44,45} only at the end of the exposure trials and bioaccumulation trends were not reported.

The weight of the clams exposed to TiO₂ NPs were found to be constant throughout the exposure trial, but shell length increased slightly (Fig. S4, ESI†). No significant differences

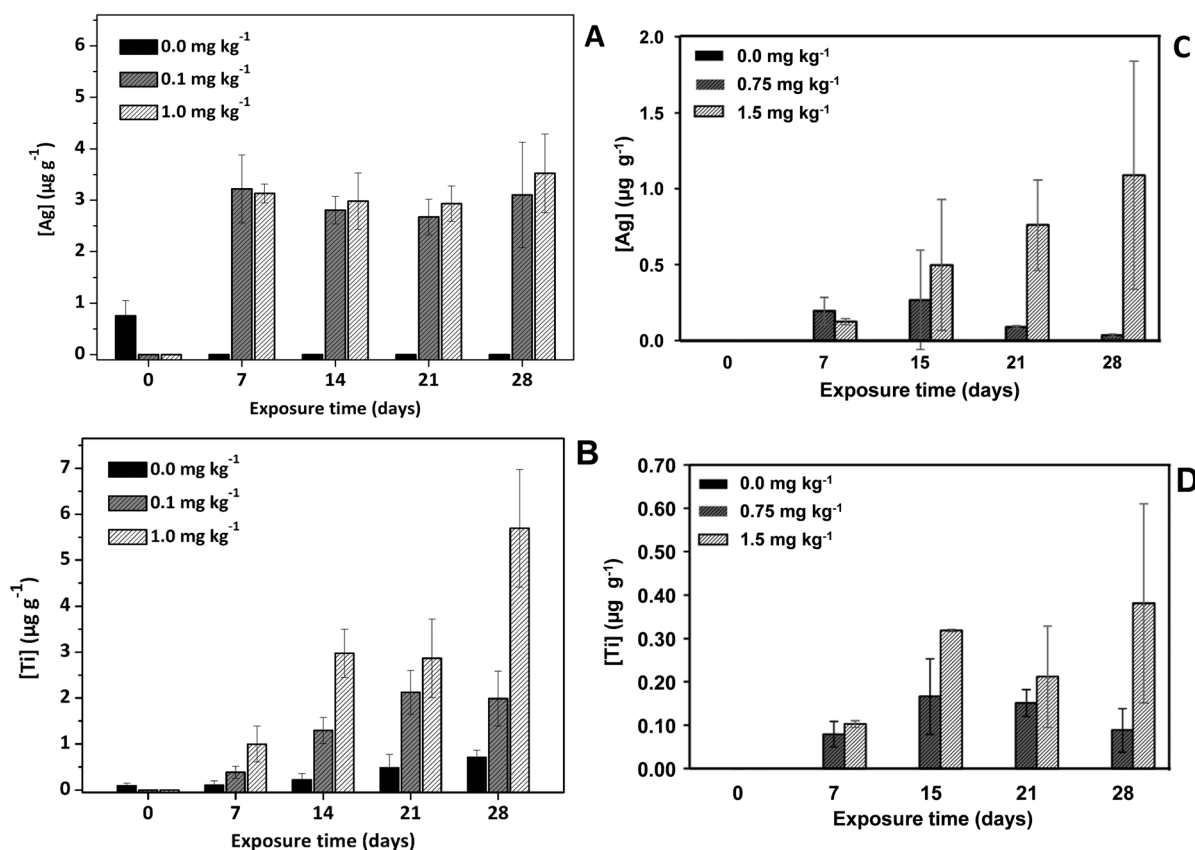


Fig. 2 Silver (A), titanium (B), Ag NPs (expressed as Ag mass) (C), and TiO₂ NPs (expressed as Ti mass) (D) concentrations in Japanese carpet shells after several exposure conditions.



were observed between the weight of clams fed with 0, 0.1 and 1 mg TiO₂ NPs L⁻¹ at any time point (Table S4, ESI†). However, regarding shell length (Fig. S5, ESI†), significant differences were observed between treated and control groups on days 7 and 14, although such differences disappear on days 21 and 28 (Table S6, ESI†).

In conclusion, results suggest that NPs bioaccumulation in Japanese carpet shells depends on the NPs type, and a greater concern is expected for TiO₂ NPs since, in addition to their higher bioaccumulation, TiO₂ NPs tend to bioaccumulate in this mollusc over time.

Bioaccumulation: Ag NPs and TiO₂ NPs

Sea bass and sea bream. As reported by Gallochio *et al.*⁴¹ for mussel exposed to TiO₂ NPs, measurements of total contents do not provide information about metal species (ionic or nanoparticulated forms). Therefore, spICP-MS analysis offers useful information since NPs (particle number concentrations) are obtained. Therefore, fish tissues which showed the highest total Ag and Ti concentrations were further analysed for Ag NPs and TiO₂ NPs by spICP-MS:^{31,32} sea bass's liver and kidney after Ag NPs exposure at 0.25, 0.75 and 1.5 mg kg⁻¹ (Fig. S8, ESI†) and sea bream's muscle + skin, liver, and kidney after TiO₂ NPs exposure at 0.75 and 1.5 mg kg⁻¹ (Fig. 3). Data plotted in Fig. 3 and S8 (ESI†) are referred to Ti and Ag mass (instead of TiO₂ and Ag number concentration), respectively, for avoiding misinterpretations because the agglomeration phenomena of NPs (mainly TiO₂ NPs). This calculation implied the assumption of a spherical shape of TiO₂ NPs and Ag NPs and the use of the mean diameter for assessing the Ti or Ag mass contented in the isolated TiO₂ NPs and Ag NPs.

Findings showed that Ag NPs bioaccumulation in liver was found to be higher than in the kidney, outcomes that agree with results obtained for total Ag in sea bass (Fig. S1, ESI†). Regarding sea bass's liver tissues, a rapid increase in Ag NPs levels (sampling within 15–45 days) was again observed, followed by a clear Ag NPs concentration decrease. Hence, the highest level of Ag NPs concentration was measured for sea bass's liver tissues sampled after 45 days of exposure at 0.75 mg kg⁻¹ ($0.57 \pm 0.00043 \mu\text{g g}^{-1}$, expressed as Ag mass). High Ag NPs concentrations were also observed after 15 days of exposure at 0.25 mg kg⁻¹ ($0.20 \pm 0.00032 \mu\text{g g}^{-1}$, expressed as Ag mass) and at 0.75 mg kg⁻¹ ($0.41 \pm 0.00013 \mu\text{g g}^{-1}$, expressed as Ag mass). Therefore, Ag NPs bioaccumulation in sea bass's liver appears to be higher at low Ag NPs concentrations and at small exposure times. However, results for sea bass's kidney are quite different, and the highest Ag NPs bioaccumulation was observed for long exposure time and also at the highest Ag NPs concentrations (Fig. S8, ESI†). These findings confirm a more reliable interpretation of NPs bioaccumulation when assessing NPs instead of total metal contents.

Regarding Ag NPs size, Table S8 (ESI†) lists the mean Ag NPs sizes in kidney and liver tissues from sea bass after

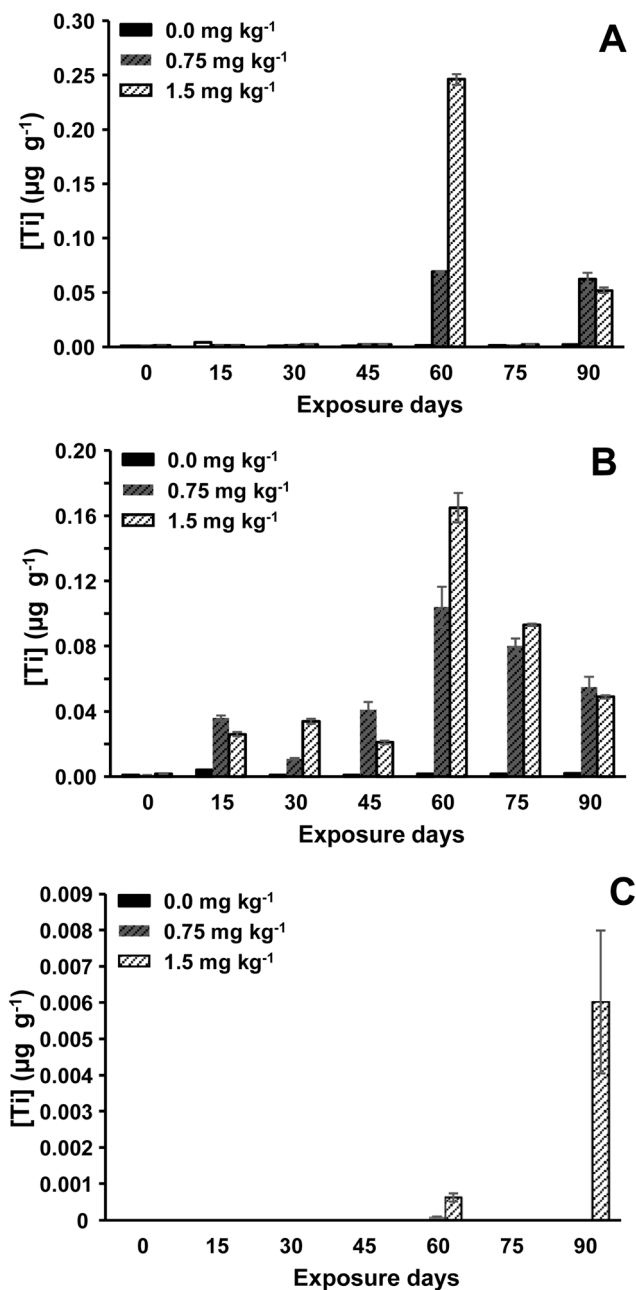


Fig. 3 TiO₂ NPs concentrations (expressed as Ti mass) in sea bream kidney (A), liver (B), and muscle + skin (C).

several Ag NPs doses and exposure times. Mean sizes quite lower than the size used for the exposure assays (100 nm) were measured: mean sizes within the 19–24 nm range for kidney tissue and from 24 to 41 nm in liver tissues. These findings imply that Ag NPs are highly ionized when they are added to the exposure tanks (during the exposure period) and/or during Ag NPs absorption (bioaccumulation) by sea bass specimens.

Lower TiO₂ NPs bioaccumulation in sea bream's liver than that found for Ag NPs bioaccumulation in sea bass was observed, showing values of 10⁶–10⁷ particles per gram for TiO₂ NPs vs. 10⁹ particles per gram for Ag NPs (mass

concentration between 0 and $0.3 \mu\text{g g}^{-1}$ for TiO_2 NPs vs. 0.02 and $0.57 \mu\text{g g}^{-1}$ for Ag NPs). High TiO_2 NPs concentrations were measured in sea bream's liver and kidney tissues than in muscle + skin (Fig. 3), and the highest TiO_2 NPs levels in the liver were noticed after 60- and 75-days exposure at the highest TiO_2 NPs concentrations (0.75 and 1.5 mg kg^{-1}). Similarly, the highest TiO_2 NPs levels in sea bream's kidney tissues were also observed at the end of the experiment (sampling at 60 days) and for exposure at $1.5 \text{ mg TiO}_2 \text{ kg}^{-1}$. On the other hand, TiO_2 NPs assessment in muscle + skin from sea bream was also possible in sea bream specimens exposed to the largest TiO_2 NPs doses and results in Fig. 3 did not show a bioaccumulation trend since the quantified TiO_2 NPs number concentrations were close to the limit of detection of the method ($6.97 \times 10^5 \text{ TiO}_2 \text{ NPs g}^{-1}$ as listed in Table S1, ESI†).

Finally, mean TiO_2 NPs sizes throughout the exposure trial are given in Table S8 (ESI†). The measured mean TiO_2 NPs sizes were higher (from 111 to 141 in kidney tissues and between 106 and 161 nm in liver tissues) than the nominal TiO_2 NPs size used for feeding the seabream specimens. As explained before, the prepared citrate-45 nm TiO_2 NPs shows a high agglomeration degree, which can remain and probably increase when the material is suspended in water and/or is absorbed (bioaccumulated) by the fish. Our results contrast greatly with those reported by Gallochio *et al.*,⁴¹ who have reported unchanged NP sizes for clams exposed to TiO_2 NPs.

Japanese carpet shell. Regarding Ag NPs and TiO_2 NPs bioaccumulation in Japanese carpet shell tissues, Fig. 2C and D shows a higher particle number for both Ag NPs and TiO_2 NPs (and higher Ag and Ti masses) when using the highest exposure dose (1.0 mg kg^{-1}), and similar bioaccumulation trends that that observed for total Ag and total Ti was obtained. Assessed TiO_2 NPs mean sizes in

Japanese carpet shell trials were within the 160–230 nm range (Table S9, ESI†), which is larger than the size of TiO_2 NPs used for the exposure experiments (45 nm). As previously commented for seabream exposure experiments, TiO_2 NPs tend to form agglomerates during the exposure and/or TiO_2 NPs absorption, and high mean sizes are found. In addition, Ag NPs have been found to be highly ionized during the exposure experiment and/or when being absorbed since the mean size of Ag NPs were within the 24–37 nm range (Table S9, ESI†), quite lower than the Ag NPs nominal size used for exposure assays (100 nm).

Effect of the cooking procedure

Culinary processes, such as grilling and boiling, lead to water loss and therefore, the percentage of moisture of the raw samples must be considered to compare the results (total metal and nanoparticles content in the raw and cooked samples). The calculated moisture percentages are listed in Table S10.† Results for total Ti contents in sea bream samples are given in Table 3 and Fig. S9A, ESI†, whereas results for TiO_2 NPs concentrations (expressed as Ag/Ti mass considering the mean size and assuming a solid spherical shape of Ag NPs and TiO_2 NPs) are given in Table 3 (Fig. S9B, ESI† plots the values as number concentrations). Total Ti contents were found to be statistically significant similar (95% confidence interval) when sea bream samples were subjected to grilling (p -value of $0.1873 > 0.05$). However, boiling led to a decrease on total Ti contents (the difference was statistically significant at a 95% confidence interval). Regarding TiO_2 NPs, both culinary processes led to a decrease on the TiO_2 NPs contents (Table 3) and TiO_2 NPs were not detected in the boiled sample.

Table 3 Total Ag and Ti contents and Ag NPs and TiO_2 NPs in raw and cooked samples, and total Ag and Ti contents and Ag NPs and TiO_2 NPs in the bio-accessible fraction

ID sample	Total metal content ^a ($\mu\text{g g}^{-1}$)			Nanoparticles concentration ^{a,b} ($\mu\text{g g}^{-1}$)		
	Sample	Bio-accessible fraction	Bio-accessibility ratio (%)	Sample	Bio-accessible fraction	Bio-accessibility ratio (%)
Japanese carpet shell (Ag) ^c						
CA0.1_28 raw	12.8 ± 3.00	9.70 ± 0.971	76	5.95 ± 1.37	1.00 ± 0.33	17
CA0.1_28 grilled	11.9 ± 1.90	9.27 ± 1.39	78	0.876 ± 0.107	0.570 ± 0.0945	65
CA1.0_28 raw	5.60 ± 2.41	4.33 ± 1.93	77	1.52 ± 0.301	0.270 ± 0.00205	18
CA1.0_28 grilled	10.5 ± 4.84	9.56 ± 2.43	91	0.882 ± 0.0667	0.501 ± 0.0656	57
Japanese carpet shell (Ti) ^d						
CT1.0_21 raw	3.38 ± 0.53	0.696 ± 0.132	21	0.229 ± 0.0526	0.191 ± 0.0226	83
CT1.0_21 grilled	2.95 ± 0.23	0.80 ± 0.16	27	0.0647 ± 0.00390	0.0494 ± 0.00448	76
CT1.0_28 raw	6.07 ± 0.887	0.922 ± 0.0709	15	0.466 ± 0.302	0.289 ± 0.0897	62
CT1.0_28 grilled	4.18 ± 0.962	0.590 ± 0.108	14	0.168 ± 0.0105	0.127 ± 0.00237	76
Sea bream (Ti) ^e						
SBr1.5_90 raw	0.489 ± 0.0212	0.376 ± 0.0752	77	0.124 ± 0.00104	0.125 ± 0.0133	101
SBr1.5_90 grilled	0.861 ± 0.405	0.227 ± 0.0383	27	0.00723 ± 0.00517	0.00766 ± 0.00562	106
SBr1.5_90 boiled	0.222 ± 0.0354	0.0881 ± 0.00762	41	0.0288 ± 0.00353	— ^f	— ^g

^a Expressed as the mean \pm SD of three replicates. ^b Ag NPs/ TiO_2 NPs concentrations expressed as Ag/Ti mass taking into account the mean size and assuming a solid spherical shape of the Ag NPs/ TiO_2 NPs. ^c CA0.1_28 (Japanese carpet shell, 0.1 mg kg^{-1} , 28 days exposure) and CA1.0_28 (Japanese carpet shell, 1.0 mg kg^{-1} , 28 days exposure). ^d CT1.0_21 (Japanese carpet shell, 1.0 mg kg^{-1} , 21 days exposure) and CT1.0_28 (Japanese carpet shell, 1.0 mg kg^{-1} , 28 days exposure). ^e SBr1.5_90 (Sea bream, 1.5 mg kg^{-1} , 90 days exposure). ^f <LOQ. ^g Not calculated.



Regarding Japanese carpet shells (Table 3 and Fig. S10, ESI†), grilling was not found to change the total Ti and Ag contents [the ANOVA test at a 95% significant level showed *p*-values higher than 0.05 (0.6833 and 0.1916 for total Ag, and 0.2669 and 0.0666 for total Ti)]. However, TiO₂ NPs and Ag NPs (expressed as Ag/Ti mass) were lower in grilled samples than those calculated in the raw ones (Table 3).

Human oral bio-accessibility from exposed sea bream and Japanese carpet shell

The total Ti contents in the bio-accessible fractions (Fig. S9A, ESI†) are lower than those found in sea bream's muscle + skin sample (Table 3), which implies a bio-accessibility ratio of 77% in raw sea bream flesh, and lower bio-accessibility ratios (41% and 27%) for cooked sea bream flesh. In general, we can conclude that the fraction of Ti that can be released from the matrix sample under gastro-intestinal conditions is moderate, which is a positive issue regarding human risk assessment. However, results are quite different for TiO₂ NPs, and therefore bio-accessible TiO₂ NPs, expressed as Ti mass considering the mean size and a solid spherical shape of the TiO₂ NPs, in raw and grilled sea bream's flesh led to bio-accessible ratios of 100% (101 and 106% for raw and grilled samples, Table 3).

Regarding Japanese carpet shell (Table 3 and Fig. S10, ESI†), moderate bio-accessibility ratios (within the 15–21% and 14–27% ranges for raw and grilled shellfish, respectively) were observed for total Ti, whereas higher bio-accessibilities ratios were obtained for total Ag (76–77% for raw shellfish and 78–91% for grilled pooled samples). Results for Ag NPs bio-accessibility (concentrations referred to Ag mass, Table 3 and Fig. S10, ESI†) were found to be lower in raw shellfish than in grilled samples, and bio-accessibility ratios of 17 and 18% (raw samples) against 35 and 57% (grilled samples) were obtained. However, TiO₂ NPs bio-accessibility (expressed as Ti mass) gave similar bio-accessibility ratios for raw and grilled samples (62 and 83% for raw shellfish, and 76% for grilled seafood, as listed in Table 3).

Human oral bioavailability (transcellular transport) from exposed sea bream and Japanese carpet shell

Transcellular transport ratios (bioavailability ratios) for TiO₂ NPs, expressed as Ti mass considering the mean size and a solid spherical shape of the TiO₂ NP, were 8 and 34% for raw and grilled sea bream, respectively, whereas total Ti transcellular transport ratios were quite higher (87 ± 11 and 67 ± 9% for raw and grilled sea bream samples, respectively). Regarding Japanese carpet shell tissues exposed to TiO₂ NPs (1.0 mg kg⁻¹ and 21-day exposure, and 1.0 mg kg⁻¹ and 28-day exposure), Ti transcellular transport ratios were moderate (within the 37–66% and 20–33% ranges for raw and grilled samples, respectively) when assessing total Ti, and also when considering TiO₂ NPs (within the 44–61% for raw shellfish and from 34 to 55% for grilled samples). Lower transcellular transport ratios were achieved for Ag in raw and grilled

samples: from 7 to 9% for total Ag, and within the 3–4% for Ag NPs. The culinary treatment (grilling) does not appear to alter the transcellular transport ratio for Ag: between 15 to 20% for total Ag, and within the 0.2–1% for Ag NPs.

As listed in Table S11, ESI†, Ag NPs sizes are quite similar in the bio-accessible and basolateral fractions (from 30 to 37 nm in the bio-accessible fraction and from 21 to 33 nm in the basolateral solutions). However, the size of TiO₂ NPs is dependent on the environment and TiO₂ NPs sizes are quite higher in the basolateral fractions (from 110 to 147 nm) than in the bio-accessible fractions (within the 73–121 nm range). The agglomeration phenomena must be favoured by the substances present in the basolateral liquid.

Limitations and future directions

The main limitations of the topic addressed in this work lie in the lack of a standardized protocol to carry out NPs exposure trials. Differences on NPs bioaccumulation ratios can be attributed to the NPs administration procedure. Nanoparticles are directly added to the water in the experimental tanks in most of reviewed papers, being less available to the individuals than when NPs are present in the feed (feed pellets for feeding fish, or microalgae previously exposed to NPs for feeding mollusc). In addition, standardized conditions (doses and exposure times) are needed for more realistic comparisons.

Another issue to highlight is the potential differences raised from assessing total metal contents or NPs (particle number concentrations). Most research use ICP-MS that give total metals content (ionic plus nanoparticulate species) and therefore they do not give a realistic measure of NPs bioaccumulation itself (NPs can be ionized in the feed tanks or once they are incorporated by the biota).

Finally, there are not studies of human oral bioavailability of NPs, and studies, mainly using cellular models that offer a more realistic approximation of bioavailability, are needed for an accurate NPs risk assessment. Other important issue is the potential interaction of NPs with other food components which can change the bioavailability ratios. A recent study by Li *et al.*⁴⁹ has shown that polyphenols human oral bioavailability is reduced in the presence of TiO₂ NPs.

Conclusions

This study investigated the bioaccumulation potential of nanoparticles in commonly consumed fish and shellfish. Controlled dietary exposure revealed moderate bioaccumulation of NPs in non-edible organs (kidneys and livers) of sea bass and sea bream, but minimal presence in their muscle (edible flesh) even at high doses and extended durations (up to 90 days). Conversely, Japanese carpet shells accumulated NPs in their soft tissues. However, this is unlikely to pose a significant health risk under normal environmental conditions, as *in vitro* studies showed limited human uptake (bioavailability) of NPs from both fish and shellfish, even after cooking. Bioavailability from fish muscle



was below 67% for total titanium and 34% for titanium dioxide nanoparticles (TiO₂ NPs). Similarly, bioavailability from shellfish was generally low, except for TiO₂ NPs in cooked Japanese carpet shells, which showed comparable levels to total titanium (around 33%). These findings suggest minimal risk for human consumers of fish exposed to environmental NPs. However, shellfish exposed to very high NP concentrations (exceeding realistic environmental levels) may warrant further investigation.

Research involving human participants and/or animals

The activities performed in the current research (fasting and sacrifice), are included in the article 1.5f of the Council Directive 2010/63/EU regarding the protection of animals used for experimental purposes, as they are considered practices not likely to cause pain, suffering, distress, or lasting harm equivalent to, or higher than, that caused by the introduction of a needle. Therefore, there was no need to have a specific approval by the competent Spanish authority to complete these experiments. Nevertheless, fish were sacrificed by personnel qualified in animal experimentation, in accordance Spanish Ministerial Order ECC/566/2015.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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