Science of the Total Environment 723 (2020) 137974

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Chronic effects of wastewater-borne silver and titanium dioxide nanoparticles on the rainbow trout (*Oncorhynchus mykiss*)

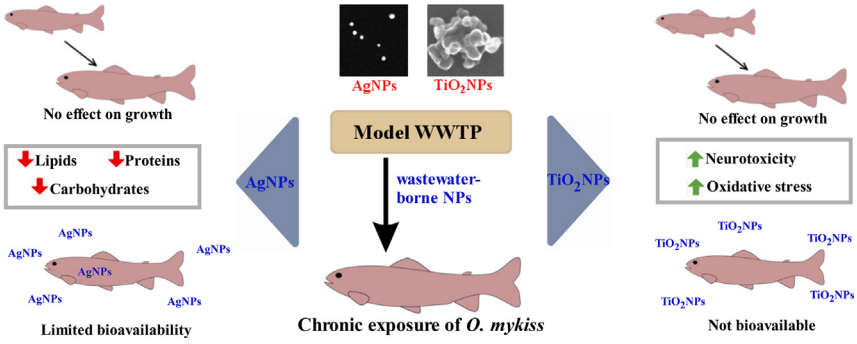
Richard Zeumer [a,b,*,1], Victor Galhano [c,*], Marta S. Monteiro [c], Sebastian Kuehr [a,d], Burkhard Knopf [a], Boris Meisterjahn [a], Amadeu M.V.M. Soares [c], Susana Loureiro [c], Isabel Lopes [c], Christian Schlechtriem [a,b]

[a] Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Department Bioaccumulation and Animal Metabolism, Auf dem Aberg 1, 57392 Schmallenberg, Germany
[b] Institute of Environmental Research (Biology V), RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany
[c] Department of Biology & Centre for Environmental and Marine Studies (CESAM), University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[d] Institute of Biology, University of Siegen, Adolf-Reichwein-Str. 2, 57068 Siegen, Germany

HIGHLIGHTS

- Chronic exposure of rainbow trout to wastewater-borne AgNPs and TiO_2NPs
- Both AgNPs and TiO_2NPs showed no negative effects on fish growth.
- Ag from wastewater-borne AgNPs was less bioavailable than from AgNPs in water.
- Unlike TiO_2NPs, AgNPs depleted energetic reserves in the muscle.
- The WWTP effluent itself increased LPO in gills and energy depletion in the muscle.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 December 2019
Received in revised form 1 March 2020
Accepted 14 March 2020
Available online 18 March 2020

Editor: Damia Barcelo

Keywords:
AgNPs
Biochemical markers
Fish
Growth inhibition
TiO_2NPs
WWTP effluent

ABSTRACT

Even though nanoparticles (NPs) are mostly removed by wastewater treatment plants, wastewater-borne NPs may show an altered toxicity to aquatic organisms. The main objectives of this work were: i) to assess the chronic (28 days) effects of wastewater-borne NPs of silver (AgNPs, 1.4–36.2 μg L^{-1}) and titanium dioxide (TiO_2NPs, 3.1–50.2 μg L^{-1}) at the individual (growth) and biochemical (biomarkers of neurotoxicity, oxidative stress and energy metabolism) levels in rainbow trout *Oncorhynchus mykiss*; and ii) to compare them with their effluent-supplemented and water-dispersed counterparts. The total Ag and Ti levels were determined in several fish organs. The growth of *O. mykiss* was not affected by the NPs in any treatment, except a 29% increase at 5.5 μg L^{-1} of total Ag supplemented to effluents. The Ag level in organs of *O. mykiss* was significantly higher after exposure to water-dispersed AgNPs than their wastewater-borne or effluent-supplemented counterparts. No significant Ti uptake could be observed. Effluent-supplemented TiO_2NPs (50.1 μg L^{-1} Ti) potentially induced neurotoxic effects, indicated by a 24% increase in acetylcholinesterase activity comparatively to controls. Energy reserves were unaffected by TiO_2 treatments, while nearly all AgNP-containing treatments caused a depletion of total lipids, proteins and carbohydrates in the muscle, suggesting an increased energy demand for detoxification

* Corresponding authors at: Faculty of Agriculture, Environment and Chemistry, Dresden University of Applied Sciences, Friedrich-List-Platz 1, 01069 Dresden, Germany; and Department of Biology & Centre for Environmental and Marine Studies (CESAM), University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal.
E-mail addresses: richard.zeumer@htw-dresden.de (R. Zeumer), vgalhano@ua.pt (V. Galhano), mmonteiro@ua.pt (M.S. Monteiro), sebastian.kuehr@ime.fraunhofer.de (S. Kuehr), burkhard.knopf@ime.fraunhofer.de (B. Knopf), boris.meisterjahn@ime.fraunhofer.de (B. Meisterjahn), asoares@ua.pt (A.M.V.M. Soares), sloureiro@ua.pt (S. Loureiro), ilopes@ua.pt (I. Lopes), christian.schlechtriem@ime.fraunhofer.de (C. Schlechtriem).
[1] Present Address: Richard Zeumer, Faculty of Agriculture, Environment and Chemistry, Dresden University of Applied Sciences, Friedrich-List-Platz 1, 01069 Dresden, Germany.

https://doi.org/10.1016/j.scitotenv.2020.137974

processes to cope with AgNPs. Besides NPs, the effluent matrix and dispersing agent (for AgNPs) induced significant effects on energetic reserves and oxidative stress, indicating background toxicity of both treatments at the biochemical level.

Our study is the first to assess chronic effects of wastewater-borne NPs on rainbow trout. While no effects were found at the individual level, several biochemical markers were changed by the NPs exposure. Our results highlight the importance of using complex matrices for a reliable risk assessment of NPs in the aquatic environment.

1. Introduction

The worldwide production of manufactured nanomaterials (MNMs) has increased by >25% since 2005 and led to >1800 consumer products containing MNMs in 2015 (Sun et al., 2017; Vance et al., 2015). These products cover a wide range of applications such as in coatings, paints, pharmaceuticals, cosmetics, textiles, toys and food (He et al., 2019; Thomas et al., 2006). Two of the most widely used nanomaterials are silver nanoparticles (AgNPs) and titanium dioxide nanoparticles (TiO_2NPs), with AgNPs being mainly used due to their antimicrobial properties and therefore being present in medical products, cosmetics and clothing (Vance et al., 2015). On the other hand, TiO_2NPs are present in sanitation, paints, sunscreens, solar cells and used in cancer therapy due to their photocatalytic properties and ability to filter ultraviolet light (Barndõk et al., 2016; Ivanković et al., 2003; Kaegi et al., 2008).

The high usage of AgNPs and TiO_2NPs leads to their release into industrial and urban sewage. In wastewater treatment plants (WWTPs), these NPs are known to adsorb to biosolids in the sewage sludge to a large extent where they can be transformed into insoluble species (*e.g.* Ag_2S) (Kaegi et al., 2011; Kiser et al., 2009). Therefore, only a small fraction of nanoparticles (NPs) enters the aquatic environment following wastewater treatment process. However, the discharge of WWTP effluents can still lead to a significant increase of AgNP and TiO_2NP concentrations in freshwaters. Li et al. (2016) investigated the contribution of WWTP effluents on the AgNP concentration in the river Isar in Germany. Whereas no AgNPs could be measured upstream of the effluent discharge, 2.0–8.6 ng L^{-1} of AgNPs were measured at the discharge point (Li et al., 2016). Regarding TiO_2NPs, information on environmental concentrations in freshwaters receiving WWTP effluents is scarce. Nevertheless, Kiser et al. (2009) detected 17 μg L^{-1} of TiO_2NPs in WWTP tertiary effluents in Arizona, USA. Exposure modelling studies indicated that the predicted concentrations of AgNPs and TiO_2NPs in WWTP effluents in the EU are 42.5 and 3470 ng L^{-1}, respectively, and therefore several magnitudes higher than the predicted environmental concentration for surface waters (0.8 and 15.0 ng L^{-1}, respectively) (Gottschalk et al., 2009).

In the last decade a significant number of studies have explored the toxicity of NPs to aquatic biota (*e.g.* Farkas et al., 2017; Valerio-García et al., 2017; Wu et al., 2017; Baun et al., 2008). The toxicity of AgNPs to aquatic organisms can be attributed to the potential released ions (Ag^+) (Beer et al., 2012), dissolved Ag species, Ag complexes (Ribeiro et al., 2015) and the NPs themselves (Asghari et al., 2012; Navarro et al., 2008). In fish, AgNPs may adsorb to gill tissues, where they cause severe injuries and oxidative stress accompanied by the release of Ag^+ into the blood circulatory system (Scown et al., 2010; Wood et al., 1996). Sulfidized forms of Ag such as Ag_2S are not prone to oxidation and dissolution, and show limited release of free ions (Choi et al., 2009; Rozan et al., 2000).

Few studies have investigated the effects of wastewater-borne AgNPs and TiO_2NPs on aquatic biota (Bruneau et al., 2016; Galhano et al., 2020; Georgantzopoulou et al., 2018; Hartmann et al., 2019; Kühr et al., 2018; Muth-Köhne et al., 2013). In WWTP effluents, transformations like sulfidation, dissolution, aggregation, adsorption and sedimentation may alter bioavailability and impact of AgNPs and TiO_2NPs upon release to aquatic systems (Azimzada et al., 2017; Kiser et al., 2009). Hartmann et al. (2019) performed a multi-generational study with *Daphnia magna* to compare the chronic effects (21 days) of wastewater-borne AgNPs (0.6–10.1 μg L^{-1} measured concentrations) and TiO_2NPs (<6.1–25.2 μg L^{-1} measured concentrations) with the respective ASTM-dispersed counterparts over six consecutive generations. In contrast to ASTM-dispersed AgNPs, which caused a significant reduction of offspring in all six generations, both types of wastewater-borne NPs showed no transgenerational effects on *D. magna* reproduction, while TiO_2NPs showed no effects regardless of the tested matrix. Georgantzopoulou et al. (2018) exposed the rainbow trout gill epithelial cell line RT gill-W1 for 24 h to differently aged (1–5 weeks) WWTP effluents containing AgNPs and TiO_2NPs at 0.7 and 1.2 μg L^{-1}, and a 40% decrease in the epithelial integrity as well as a 2-fold increased formation of reactive oxygen species (ROS) was detected after exposure to 3-weeks old effluents. Muth-Köhne et al. (2013) exposed zebrafish (*Danio rerio*) embryos to AgNPs (0.5–1.5 mg L^{-1} nominal concentrations) and $AgNO_3$ (0.05–0.12 mg L^{-1} nominal concentrations) in ISO water and wastewater-borne AgNPs (0.7–5.5 mg L^{-1} nominal concentrations) for 48 h; the embryos displayed delayed development, tail malformations and oedema in all treatments; the highest embryo toxicity was observed in wastewater-borne AgNPs exposure. In contrast, Bruneau et al. (2016) exposed juvenile rainbow trout for 96 h to AgNPs and $AgNO_3$ at 40 and 4 μg L^{-1}, respectively (nominal concentrations), in municipal wastewater and observed that both forms of silver were bioavailable and induced severe immunotoxic effects. Overall, these studies indicate that wastewater-borne NPs can cause harmful effects to freshwater organisms. Despite the fact that WWTP effluents are one of the major entry paths of NPs into the aquatic environment, information on the biological impact and fate of the wastewater-borne NPs is still lacking, but is crucial for a detailed risk assessment.

Based on these findings, the main aim of this study was to compare the chronic effects of wastewater-borne AgNPs and TiO_2NPs on rainbow trout (*Oncorhynchus mykiss*) to their effluent-supplemented and water-dispersed counterparts to evaluate the impact of WWTP processes on NP toxicity to fish. Therefore, model WWTPs were fed with AgNP or TiO_2NP containing influent and the resulting effluents were used to perform 28-d exposure studies with rainbow trout. The evaluation of effects was performed at the individual (growth) and biochemical (biomarkers of neurotoxicity, oxidative stress and energy budget) levels to gather a comprehensive picture of possible differences between NPs from different treatments.

2. Material and methods

2.1. AgNPs and TiO_2NPs

All studies with AgNPs were performed with NM-300K, an aqueous dispersion of AgNPs which was obtained from the OECD repository of representative manufactured nanomaterials (Totaro et al., 2016). The dispersion presented a nominal Ag concentration of 10.16% (w/w) and the main particle size is 15 nm, with a narrow size distribution (99% < 20 nm) according to transmission electron microscopy (TEM) analyses (Klein et al., 2011). This dispersion is presented as AgNPs within a stabilizing mixture [4% (w/w) each] of two non-ionic surfactants [polyoxyethylene glycerol trioleate (TAGAT® TO) and polyoxyethylene (20) sorbitan mono laurat (Tween® 20)]. The Ag

content and particle number of the NM-300K dispersion were shown to be stable up to 12 months within this dispersing agent (Klein et al., 2011).

TiO_2NPs (NM-105) were also obtained from the OECD repository (Totaro et al., 2016). This uncoated rutile-anatase modification (relation varied between 88.2 : 11.8 and 81.5 : 18.5), with a primary particle size of 10–45 nm, is presented as dry white powder wherein particles have a specific surface area of 46.2–55.5 $m^2\ g^{-1}$ (Rasmussen et al., 2014).

2.2. Model WWTPs

The AgNPs were continuously added to the model WWTP from a previously prepared stock dispersion. Therefore, 8 mL of ultrapure water were added to a glass vial with 2000 mg of NM-300K, resulting in a total Ag concentration of 20 g L^{-1}. The vials were thoroughly shaken by hand for 1 min and sonicated, for 15 min, in a sonication bath (160 W, 144 kJ, Sonorex Super RK 510, Bandelin electronic GmbH & Co. KG, Germany). Subsequently, 3, 15 and 48 mL of this new dispersion were added to polyethylene containers (Züchner GmbH, Germany) with 6 L of ultrapure water to obtain 10, 50 and 160 mg L^{-1} of nominal total Ag, respectively. These stock dispersions were renewed every three days.

Four lab-scale WWTP units (behrotest® Laboratory Sewage Plant KLD 4N, behr Labor-Technik GmbH, Germany) were conducted according to OECD Guideline 303A (OECD, 2001) as described in detail in (Muth-Köhne et al., 2013; Zeumer et al., 2019). In brief, each WWTP (with a total volume of 10 L) consisted of two reactors (non-aerated and aerated) and a secondary clarifier to simulate denitrification, nitrification and sedimentation processes of a full-scale WWTP (see Supporting information, Fig. S1). Every WWTP unit was connected to a tube system (PLP 33; SP04/3.5 K, behr Labor-Technik, Germany), which automatically mixed artificial wastewater (and AgNP stock dispersions when applicable) with tap water, leading to a 10-fold dilution. To compensate this dilution step, the artificial wastewater and the AgNP stock dispersions (10, 50 and 160 mg L^{-1} AgNPs) were previously prepared in 10-fold concentration and stored at 4 °C during the experiments. Finally, the diluted artificial wastewater was pumped into the denitrification reactor of the respective WWTP (see Supporting information, Fig. S1) with a continuous flow of 750 mL h^{-1}, leading to a retention time of 6 h in the WWTPs. For the study with AgNPs, one WWTP was run as a control (without NPs) and 3 different WWTPs were run with a total Ag concentration of 1, 5 and 16 mg L^{-1} in the influent.

In the experiment with TiO_2NPs, two WWTP were run, one as a control and one with a nominal total concentration of 3.0 mg L^{-1} Ti. Since NM-105 does not contain any dispersing agent, dispersions were prepared freshly twice a day and added directly to the lab-scale WWTP (Fig. S1). For such, 50 mL of ultrapure water were added to a glass vial with 45.0 mg of NM-105, thoroughly shaken by hand for 1 min, sonicated for 15 min (160 W, 144 kJ, Sonorex Super RK 510, Bandelin electronic GmbH & Co. KG, Germany) and added directly to the denitrification reactor to reach a nominal influent Ti concentration of 3.0 mg L^1.

The room temperature was kept within 20–25 °C. The oxygen concentration in the nitrification reactor was automatically monitored and kept constant at 2–4.5 mg L^{-1}. The concentrations of ammonium, nitrite and nitrate in the effluents were photometrically measured (NANOCOLOR® 500D, Macherey-Nagel, Germany) at least once per week. The pH values in the denitrification and the nitrification reactors were monitored daily and stayed within 6.8–7.7 throughout the experiments. After test start, a running-in phase without the addition of NPs lasting 6–11 days was carried out until the WWTP reached stable conditions, which are characterized by a dissolved organic carbon (measured with TOC-V CPH Total Carbon Analyzer, Shimadzu, Japan) elimination rate of >80% (OECD, 2001) and constant ammonium, nitrite and nitrate concentrations in the effluent. Subsequently, the AgNPs or TiO_2NPs were added to the influent for 8 days, as described above. For the last 40 h of these 8 days, the effluents were collected separately in 30 L polyethylene containers (Züchner GmbH, Germany) and stored in the dark at 4 °C for 5 weeks until the beginning of the fish exposure experiments.

2.3. Rainbow trout exposure to NPs

2.3.1. Model species and maintenance

Rainbow trout (*Oncorhynchus mykiss*) juveniles were obtained from Fischzucht Störk (Bad Saulgau, Germany) and maintained in 200–250 L tanks with copper ion-reduced tap water, in a flow-through system with constant aeration (oxygen saturation >60%) at 14 ± 2 °C and a 16/8 h light/dark photoperiod. The fish were fed with commercially available food (Inicio Plus®, BioMar, Denmark) at a rate of 4% of their body weight per day. These conditions (aeration, temperature, light, feeding) were also used in the juvenile growth test.

Fish maintenance, handling and experimental procedures were previously approved by an ethical committee on animal experiments (LANUV NRW, Germany, permit number: 84–02.04.2016.A312).

2.3.2. Juvenile growth test

The chronic exposure was performed with juvenile rainbow trout according to OECD test guideline No. 215 (OECD, 2000). The animals (10 fish per tank, 1 tank per treatment) were exposed in 10 L tanks, over 28 days, to (i) effluents from model WWTPs with NP-treated influent (*i.e.* wastewater-borne NPs); (ii) NPs manually added to control effluents from WWTPs (*i.e.* wastewater-supplemented NPs); and (iii) NPs added to dilution water (*i.e.* water-dispersed NPs). Copper ion-reduced tap water (for physicochemical properties see Supporting information, Table S2) was used as dilution water. All effluents used in both treatments (i) and (ii) were diluted 10-fold with copper ion-reduced tap water to simulate the dilution effect of the receiving waters of a full-scale WWTP. All treatments used in the exposure experiments are summarized in Table 1. In the case of TiO_2NP experiments, the effluent of the TiO_2NP-treated WWTP was diluted 1:1, 1:2 and 1:4 with TiO_2-free effluent in order to achieve three concentrations of wastewater-borne TiO_2NP (see Table 1), before the 10-fold dilution with tap water.

For the experimental exposures of fish with AgNPs, the stock dispersions used for the preparation of NPs tested concentrations were prepared with 300 mL of NM-300K (approx. 30 mL Ag) in 30 mL of ultrapure water in plastic centrifuge vials (polypropylene, Sarstedt, Germany). The dispersions were thoroughly shaken by hand and sonicated for 10 min (2 min effective, 200 W, 24 kJ, pulsation pause ratio of 0.2/0.8) by indirect probe sonication (Cup Horn BB6, Bandelin electronic GmbH & Co. KG, Germany). The stock dispersions were renewed every two days. Three controls were used in the AgNPs exposure experiments: dilution water as a negative control (DW); NP-free effluent (EFF); and the control for the dispersing agent (DA) present in the NM-300K. The DA contained the same concentration of dispersing agent as used in the highest tested AgNP concentration (36.2 µg L^{-1}) in order to identify possible harmful effects caused by the DA in relation to DW treatment.

For the fish exposure experiments with TiO_2NPs, stock dispersions were prepared with 30 mg of NM-105 in 30 mL of ultrapure water in plastic centrifuge vials (polypropylene, Sarstedt, Germany). The dispersions were renewed every two days and handled similarly to those of AgNPs.

Before the beginning of the exposure experiments, an acclimation period of 14 days was performed to fulfill the test guideline criterion (<5% mortality) (OECD, 2000). Prior to weighing the fish, food was withheld from the animals for 24 h. Before the beginning of the test, fish were weighed [1.0 ± 0.1 and 1.3 ± 0.6 g (average of 10 individuals ± standard deviation) in exposure experiments with AgNPs and TiO_2NPs, respectively], pooled and distributed randomly between the respective tanks. Fish were weighed again on days 14 and 28 to recalculate the amount of feed (based on the feeding rate described in

Table 1
Composition and total Ag and Ti concentrations in the different exposure treatments used in fish chronic studies.

Experiments	Treatments (media and nanoparticles)	Dilution ratio		TWA (μg L^{-1})
		Dilution with Effluent	Dilution with Dilution Water	
AgNPs	Dilution water control (DW)	n/r	n/r	–
	WWTP effluent control (EFF)	n/r	1:10	–
	Dispersing agent control (DA)	n/r	n/r	–
	Wastewater-borne AgNPs	n/r	1:10	1.4, 3.4, 28.8
	AgNPs-supplemented effluents	n/r	1:10	2.4, 5.5, 28.0
	Water-dispersed AgNPs	n/r	n/r	1.6, 5.4, 36.2
TiO_2NPs	Dilution water control (DW)	n/r	n/r	2.8*
	WWTP effluent control (EFF)	n/r	1:10	3.5*
	Wastewater-borne TiO_2NPs	1:1, 1:2, 1:4	1:10	3.1*, 3.8*, 4.0*
	TiO_2NPs-supplemented effluents	n/r	1:10	15.1, 27.6, 50.1
	Water-dispersed TiO_2NPs	n/r	n/r	12.7, 22.9, 42.8

TWA – time weighted average total Ag or Ti concentration. Ti values marked with "*" were statistically not different ($p > 0.05$; ANOVA followed by Dunnett's *post-hoc* test) to the digestion blank (2.9 μg L^{-1} total Ti). The limits of quantification were 0.028 μg L^{-1} and 0.386 μg L^{-1} for total Ag and Ti, respectively. n/r – not required. WWTP – wastewater treatment plant.

Section 2.3.1) and to determine the growth increment. During the semi-static exposure period (change of media at 48 h intervals), animals were fed at a rate corresponding to 4% of body weight per day. During exposure, behaviour and mortality of animals and the water conditions (concentrations of ammonium, nitrate and nitrite, pH and water temperature) were monitored periodically. The pH was maintained constant (7.7 ± 0.1) throughout the test duration. Growth rates were calculated for the complete exposure period (28 d) by using Eq. (1), which was based on the 'pseudo' specific growth rate adapted from the OECD test guideline No. 215 (OECD, 2000):

$$r = \frac{\log_e W_{28} - \log_e \overline{W}_0}{28\,d} \times 100 \quad (1)$$

where: r = growth rate (d^{-1}); W_0, W_{28}= weight (g) of an individual fish at times 0 and 28 days, respectively; $\log_e W_0$ = logarithm of the average of the values W_0 at day 0; $\log_e W_{28}$= logarithm of the weight of an individual fish at day 28.

On day 28, fish were sacrificed on ice by a percussive blow to the head followed by the dislocation of the neck according to the directive of the EU on animal welfare (EU, 2010). The fish organs, *viz.* liver, gills, muscle, stomach, intestine, brain and carcass were dissected on ice. The liver, gills, muscle and brain were immediately snap frozen in liquid N_2 and stored at −80 °C until processing for biochemical analyses (Section 2.6). The intestine, stomach and carcass were snap frozen in liquid N_2 without any buffer and stored at −20 °C until processing for total Ag and Ti determination (Section 2.5.2). The obtained NP levels in the organs and carcass were used to calculate the Ag transfer factor by dividing the Ag content in the respective organ by the Ag concentration of the test medium.

2.4. Nanoparticle characterization

2.4.1. Scanning transmission electron microscopy

The characterization of the NM-300K stock suspension by scanning transmission electron microscopy (STEM) in combination with high-angle annular dark field (HAADF) and energy dispersive X-ray (EDX) detectors was part of a parallel study (Zeumer et al., 2019) and is presented again in the supporting information for consistency (see Supporting information, Fig. S3). In a similar way, the NM-105 stock dispersion was characterized in this study by STEM (for a detailed description of the methodologies see Supporting information).

Hartmann et al. (2019) characterized wastewater-borne AgNPs in effluent material which was prepared under identical conditions as the effluent applied in this study. Likewise, the characterization of the wastewater-borne TiO_2NPs applied in this study was already presented by Hartmann et al. (2019), who extracted the TiO_2NPs by cloud-point extraction and characterized them by STEM in combination with HAADF and EDX detectors.

2.4.2. Dynamic light scattering

The hydrodynamic diameter (DZ) of AgNPs and TiO_2NPs was characterized using a zetasizer (Zetasizer Nano Series, Malvern Instruments Ltd., UK). For this, the NPs were dispersed and measured in ultrapure water, copper ion-reduced tap water, and in WWTP influent to detect potential effects of the media on the DZ of the NPs. Measurement of dynamic light scattering (DLS) was carried out as described in the supporting information.

2.5. Determination of total Ag and Ti

2.5.1. Preparation of aqueous samples

Aliquots of 20 mL were taken periodically from the effluent of the WWTPs as well as from test media during exposure experiments for determination of total Ag and Ti concentrations (Table 1). Aliquots were taken in duplicate from the collected effluent in polyethylene vials (Sarstedt, Germany). During the exposure experiments, aqueous samples from test media were taken before and after (24 h and 48 h) exposure at least twice per week. All samples were acidified with 0.2 mL nitric acid (69%, Suprapur®, Carl Roth, Germany). The sample preparation for total Ag analysis was performed according to Kraas et al. (2017). Prior to measurements, 5 mL of aqua regia were added to each sample for 24 h and subsequently transferred to polypropylene vials (Sarstedt, Germany). The original polyethylene storage vials were rinsed twice with 10 mL of aqua regia to prevent sorption of AgNPs. After 48 h, samples were made up to 50 mL with ultrapure water.

Prior to Ti measurements, 4 mL of aqueous samples were mixed with 0.8 mL of 69% nitric acid and 0.2 mL of hydrofluoric acid (40%, Suprapur®, Merck, Germany) and digested in a microwave UltraClave II (MLS GmbH, Germany; 25 min heating up to 220 °C, 30 min on 220 °C, max. pressure 80 bar). After digestion, 1 mL of boric acid (4%, Merck, Germany) was added and vials filled up to 15 mL with ultrapure water.

To check for potential Ag or Ti background levels caused by the digestion procedure, three digestion blanks (4 mL ultrapure water) were added to every digestion process and treated identically as the aqueous samples. The water used was purified using an ELGA Pure Lab Ultra water purification system (>18 MΩcm).

2.5.2. Preparation of fish samples

The total Ag and Ti levels were determined in fish stomach, intestine and carcass. For total Ag determination, 5 mL of 69% nitric acid were added to the fish samples. Due to their size, carcass samples from exposure experiments with TiO_2NPs were manually ground in a zirconium oxide mortar under constant cooling by liquid nitrogen, and then freeze dried (Alpha 1–2 LDplus, Christ, Germany). Subsamples of 200 mg

ground carcass were used for total Ti determination. Subsequently, 4.8 mL (stomach and intestine) or 0.8 mL (carcass) of 69% nitric acid, and 0.2 mL of 40% hydrofluoric acid were added to the samples.

All samples for Ag and Ti determination were digested in the same microwave under the same conditions as described in Section 2.5.1. After digestion, the Ag samples were made up to 15 mL with ultrapure water. The Ti samples were complexed with 1 mL of boric acid 4% and made up to 15 mL with ultrapure water. As described in Section 2.5.1 digestion blanks were set up to check for potential Ag or Ti background levels.

2.5.3. Quantitative total Ag and Ti analysis

Total Ag was determined by ICP-MS (Agilent7700, Agilent Technologies, Germany) as described by Zeumer et al. (2019).

Total Ti was measured by ICP-OES (Agilent 720, Agilent technologies, Germany) set at 334.941 nm. For the preparation of matrix adjusted calibration standards and stock solutions, a commercially available Ti ICP standard containing 1000 mg L^{-1} Ti in 10% (v/v) nitric acid (Merck, Germany) was used. The calibration function was calculated by the ICP-OES software (Agilent MassHunter workstation) by using a linear regression. Likewise, the limit of detection was calculated by the ICP-OES software and the limit of quantification was calculated as 3 times the limit of detection. All samples were measured in triplicate (internal measurements). In all measurements, quality control samples were prepared independently from the calibration samples and certified reference materials (TMDA 52.4, Environment Canada) were measured in parallel to validate the calibration. Results are expressed as total Ag and total Ti in aqueous (μg L^{-1}) and fish samples(μg kg^{-1}).

2.6. Biochemical marker assays

A battery of biochemical marker assays was performed to assess the potential effects of NPs on rainbow trout. The analyses were performed in the brain (acetylcholinesterase, AChE), gills (catalase, CAT; glutathione *S*-transferase, GST; lipid peroxidation, LPO), liver (superoxide dismutase, SOD; CAT; GST; LPO) and muscle [lactate dehydrogenase, LDH; energy related parameters (total lipids, total carbohydrates and total proteins, energy available – E_a, energy consumption – E_c, and cellular energy allocation - CEA)]. These markers were selected based on previous studies where AgNPs and TiO_2NPs induced significant effects, at the biochemical level, in aquatic species (*e.g.* Galhano et al., 2020) and especially in fish (*e.g.* Miranda et al., 2016; Khan et al., 2017; Valerio-García et al., 2017; Shobana et al., 2018).

After thawing on ice, all samples ($N = 8$–10 and $N = 4$–10 in AgNPs and TiO_2NPs experiments, respectively) were homogenized in specific homogenization buffers (see Supporting information, Table S3). Subsequently, an aliquot of 150 μL of the resulting homogenate was transferred to a microtube with 4 μL 2,6-Di-*tert*-butyl-4-methylphenol for LPO determination. The remaining homogenate was centrifuged at 10,000 ×*g* for 20 min at 4 °C to separate the post-mitochondrial supernatant. Then, this fraction was divided into aliquots and further diluted in each specific homogenization buffer (Table S3) whenever necessary for the subsequent quantification of each biochemical marker (for a detailed description of the used methodologies see supporting information). In the present study, the NPs revealed no interference in biochemical markers measurements when tested at the maximum concentrations found in rainbow trout tissues. The absorbance spectra of AgNPs and TiO_2NPs are presented in supplementary information (Fig. S5A and B).

2.7. Statistical analysis

Before comparing between treatments, data was tested for normality (Shapiro-Wilk) and homogeneity of variance (Levene's), and outliers were also inferred from the data showing normal distribution using the Grubbs' test (SigmaPlot for Windows, v. 14, Systat Software, Inc., USA; OriginPro 2017, OriginLab Corp., USA; and SQS 2013,Version 1.00 by J. Klein and G. Wachter). Independent *t*-tests (derived from Student's *t*-test with two-tailed *p* values) and one-way analyses of variance (ANOVA) followed by Dunnett's *post-hoc* test or Kruskal-Wallis followed by Dunn's *post-hoc* test were used to determine differences between treatments and respective controls. The α-level was set at 0.05. All results were expressed as means ± standard errors (SE). Time weighted average concentrations (TWA) of Ag and Ti in test media of the different treatments (Table 1) were calculated according to OECD test guideline No. 211 (OECD, 2012).

3. Results and discussion

3.1. Total Ag and Ti concentrations in WWTP effluents and test media

The effluents collected from the model WWTPs with AgNP-treated influent, contained 50.7, 222.0 and 475.7 μg L^{-1} of Ag; after storage and 10-fold dilution, the resulting exposure concentrations of wastewater-borne AgNPs in fish experiments were 1.4, 3.4 and 28.8 μg L^{-1}, respectively (Table 1). These concentrations are in the same range as the AgNPs supplemented to the effluent (2.4–28.0 μg L^{-1} Ag) or water-dispersed AgNPs (1.6–36.2 μg L^{-1} Ag) as reported in Table 1.

The effluents collected from the model WWTP with TiO_2NP-treated influent, contained 130.7 μg L^{-1} Ti. The 5-week storage of the effluents and the following dilution steps led to Ti concentrations (3.1, 3.8 and 4.0 μg L^{-1}, Table 1), which were not statistically different ($p > 0.05$, ANOVA followed by Dunnett's *post-hoc* test) to the 2.9 ± 1.9 μg L^{-1} measured in the digestion blanks (ultrapure water). Similar Ti concentrations were also found in DW (2.8 μg L^{-1}) and EFF (3.5 μg L^{-1}) controls ($p > 0.05$, ANOVA followed by Dunnett's *post-hoc* test). This similarity was most probably due to a possible background contamination of the reagents used in the digestion process, since no Ti could be measured in the undigested ultrapure water (<limit of quantification of 0.386 μg L^{-1}) which was used as blank samples for Ti analysis. For this reason, precise measurements of Ti concentrations ≤4.0 μg L^{-1} in the tested effluents were not possible. Nevertheless, to allow a comparison of the effects of the different treatments on *O. mykiss*, the effluents which probably contained low levels of Ti (≤4 μg L^{-1}) were not excluded from the study.

The ranges of Ti in TiO_2NPs-supplemented effluent or water were similar, with 15.1–50.1 μg L^{-1} Ti and 12.7–42.8 μg L^{-1} Ti, respectively. The Ti concentrations measured in the different treatments are presented in Table 1.

3.2. Nanoparticles characterization

3.2.1. Stock dispersions of pristine NPs

The STEM analyses of the AgNP stock dispersion were presented in a previous study (Zeumer et al., 2019) and showed well dispersed AgNPs with a mean particle size of 18.3 ± 3.1 nm (Supporting information, Fig. S3A), while EDX analyses of individual particles showed a dominant signal of Ag (Lα), next to Cu (Kα) which was related to signals resulting from the Cu grid (Supporting information, Fig. S3B). In ultrapure and tap water, AgNPs presented a zeta potential of −5.1 and −22.8 mV, respectively (Zetasizer Nano Series, Malvern Instruments Ltd., UK).

The TiO_2NPs in the stock dispersion were present as large agglomerates on the TEM grids (Supporting information, Fig. S4A). The mean particle size obtained was 28.1 ± 7.2 nm and therefore within the range of 10–45 nm stated by Rasmussen et al. (2014) for NM-105. Elemental analyses of individual particles showed a dominant signal of Ti (Kα), next to Cu (Kα) which was related to signals resulting from the Cu grid (Supporting information, Fig. S4B). The zeta potential of TiO_2NPs in ultrapure and tap water was shown to be 20.3 and −17.1 mV, respectively (Zetasizer Nano Series, Malvern Instruments Ltd., UK).

3.2.2. NPs in WWTP effluents

The STEM analyses of the wastewater-borne AgNPs prepared identically as described in this study showed that the particle size was not changed during the passage on the WWTP, while the EDX revealed an association of wastewater-borne AgNPs with sulphur (S), indicating their potential sulfidation due to the ratio of the EDX signals of S and Ag of around 2:1 (Hartmann et al., 2019). The sulfidation of AgNPs within WWTPs is well known (*e.g.* Kaegi et al., 2011; Kampe et al., 2018). The sulfidation of the surface or the whole AgNPs causes a strong decrease in the release of Ag^+ due to surface passivation that may alter the potential toxicity of wastewater-borne AgNPs (Liu and Hurt, 2010). In other words, pristine (water-dispersed) and wastewater-borne AgNPs represent different main exposure forms to aquatic organisms: (*i*) potentially high release of Ag^+ and a reactive surface at the pristine AgNPs; and (*ii*) low Ag^+ release and reactivity on the surface of the potentially completely sulfidized and thus passivated wastewater-borne AgNPs (Liu and Hurt, 2010). Most probably, the AgNPs supplemented to the effluent or dispersed in water may suffer less transformation processes compared to those observed on wastewater-borne AgNPs.

The STEM analyses of wastewater-borne TiO_2NPs used in this study were already presented in Hartmann et al. (2019) and showed that TiO_2NPs formed aggregates of several 100 nm. Even though no transformation of the TiO_2NPs could be observed, several organic compounds from the effluents associated with the TiO_2NPs and detected as C, O and N signals by STEM analysis were found (Hartmann et al., 2019). This might have influenced the bioavailability of TiO_2NPs and in turn potentially modulated their toxicity at individual and biochemical levels (Farkas et al., 2015; Zhu et al., 2011).

The TiO_2NPs were chemically not altered while passing through the WWTP, hence no alterations were expected for effluent-supplemented or water-dispersed TiO_2NPs.

3.2.3. Size of nanoparticles

The AgNPs and TiO_2NPs were measured in ultrapure water at different concentrations to characterize the pristine particles for their hydrodynamic diameter (see Supporting information, Table S1). Z-average values of 58.0 to 65.3 nm were found for nominal Ag concentrations of 100 and 1000 μg L^{-1}, respectively. These values were within the size range mentioned by Klein et al. (2011) for NM-300K. For NM-105, the *Z*-average values of 775.5 and 1476 nm were obtained with nominal concentrations of 100 and 1000 μg L^{-1} TiO_2, respectively, whereby high polydispersity indexes of 0.8 and 1.0 indicated polydispersity and thus minimal suitability. This is in accordance with Rasmussen et al. (2014), who obtained Z-average values of 554.9 nm or even >1 μm due to a high polydispersity of the NM-105 suspensions.

To obtain further information on the behaviour of the NPs in the tested media, the materials were also measured in water (Cu ion-reduced tap water) and WWTP influent. In those media, the obtained results were similar to those measured in ultrapure water (Supporting information, Table S1). At low nominal Ag concentrations (10 and 1 μg L^{-1}), high attenuator values (9 and 11) and implausible high Z-Average values (up to 2236 nm) indicate that the concentrations were too low to be measured with reliability. For NM-105, high attenuator values of 9 and 11 were obtained with 1000 and 100 μg L^{-1} TiO_2, respectively. As explained in the supporting information, DLS measurements on NPs were not carried out with effluent. The wastewater-borne NPs

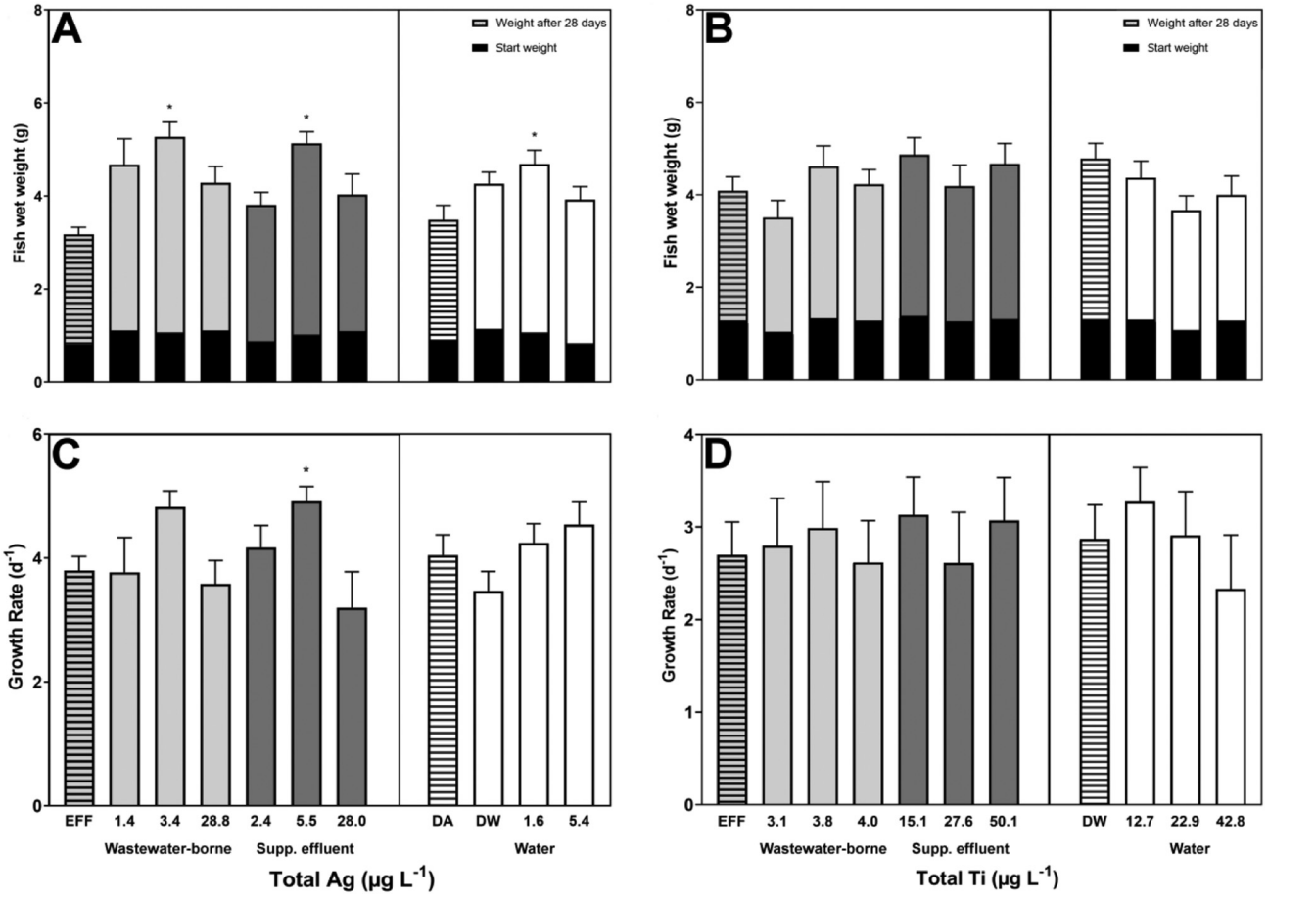


Fig. 1. Effect of wastewater-borne, effluent-supplemented (Supp. effluent) and water-dispersed (Water) AgNPs (**A**, **C**) and TiO_2NPs (**B**, **D**) on growth of *Oncorhynchus mykiss* after 28 d of exposure. DW, EFF and DA are dilution water, effluent and dispersing agent controls, respectively. **A**, **B**: Wet weight of fish at start and end of the experiment. **C**, **D**: Fish growth rate. Bars represent mean ± SE of 10 organisms. Asterisks represent significant differences ($p \leq 0.05$) comparatively to the respective controls (EFF or DW), according to one-way ANOVA followed by Dunnett's *post-hoc* test.

were present at total concentrations between 1.4 and 28.8 μg L^{-1} for Ag and between 3.1 and 4.0 μg L^{-1} for Ti and were therefore too low to receive reliable DLS measurement results.

3.3. Effects of AgNPs and TiO_2NPs on the growth of O. mykiss

Few *in vivo* studies have investigated chronic effects of AgNPs and their released ions in rainbow trout (*e.g.* Clark et al., 2018; Johari et al., 2015; Scown et al., 2010). Scown et al. (2010) exposed juvenile rainbow trout during 10 days to AgNPs (9.4–35.5 μg L^{-1}) of different particle sizes (10 and 35 nm). These authors didn't report any mortality or behavioural changes in fish exposed to Ag concentrations of about 35.5 μg L^{-1} in dechlorinated tap water. In contrast, in the present study, exposure of rainbow trout to 36.2 μg Ag L^{-1} in water-dispersed AgNPs treatment lead to observable changes in swimming behaviour (uncoordinated movements in lateral position) and a mortality of 20% after five days of exposure. However, AgNPs supplemented to effluent at 28.0 μg Ag L^{-1} didn't affect mortality and behaviour of the fish, suggesting that the effluent mitigated the impact of the AgNPs on the rainbow trout, probably due to the adsorption of the particles to suspended materials and their potential sulfidation (see Section 3.2.2 and Hartmann et al., 2019).

Considering the effects of AgNPs on fish weight, fish exposed to the intermediate Ag concentrations, in the three different media, showed a significantly higher average weight ($p \leq 0.05$) compared to the respective controls (Fig. 1A). However, except of the fish exposed to the effluent-supplemented AgNPs with 5.5 μg L^{-1} Ag, which showed a significantly higher growth rate (4.9 d^{-1}, 29% increase) compared to EFF, no further differences in the growth rates were observed (Fig. 1C). Positive effects of AgNPs on growth of rainbow trout were shown by Brauner and Wood (2002), who observed an earlier hatching as well as larvae with higher length and weight relative to the control, after exposure of rainbow trout larvae to 1.0 μg L^{-1} total Ag (as $AgNO_3$) for 37 days. However, since the exposure to 28.0 μg L^{-1} led to a slightly decreased growth rate compared to EFF (even though not significant, $p > 0.05$), this growth-supporting effect might be attributed to hormetic effects at low total Ag concentrations (Calabrese, 2004).

In the present work, no effects on mortality, behaviour and growth were observed in rainbow trout exposed to each of the TiO_2NPs treatments (Fig. 1B and D). This is in accordance with Ramsden et al. (2009), who exposed rainbow trout to 10 and 100 mg kg^{-1} food-borne TiO_2NPs (21 nm, 25% anatase, 75% rutile) for 8 weeks and did not observe any effects on growth, feeding and the blood circular system. Federici et al. (2007) observed a thickening of the lamellae and the formation of oedema in the gills after a 14-d exposure of rainbow trout to 100–1000 μg L^{-1} TiO_2NPs (21 nm, 25% anatase, 75% rutile). However, these concentrations are considerably higher than those used in the present study (3.1–50.1 μg L^{-1} Ti; Table 1). It was stated that TiO_2NPs are probably not a major ionoregulatory toxicant for fish, since minor effects on the main electrolytes (Na^+, K^+ and Ca^{2+}) and tissue moisture content were found (Federici et al., 2007). Hence, these findings and our results indicate, that TiO_2NPs at environmentally relevant concentrations of Ti (≤50 μg L^{-1}) have no impact on the growth of rainbow trout.

3.4. Ag and Ti levels in O. mykiss

The Ag and Ti levels were analysed in carcass, stomach and intestine at the end of the 28-d exposure period (Fig. 2). The Ag content determined in all fish organs increased with increasing Ag concentration in the three different tested treatments (wastewater-borne, effluent-supplemented and water-dispersed AgNPs; Fig. 2A, C, E). In general, the highest Ag content was obtained in the intestine, with a maximum Ag content of 582 μg kg^{-1} measured for animals exposed to 28 μg L^{-1} AgNPs supplemented to the effluent (Fig. 2E). The elevated Ag levels determined in the digestive tract were most probably caused by oral uptake of AgNPs or Ag^+ attached to food pellets or in the form of aggregates, as described for several NPs (Johnston et al., 2010; Scown et al., 2010). Nevertheless, the presence of Ag in the carcass indicates that Ag was taken up by fish through crossing the intestinal or gill epithelia, most probably in the form of Ag^+. Effectively, it has been shown that AgNPs and Ag^+ are taken up by the gills and transported *via* the blood to other internal organs like the liver (Scown et al., 2010; Wood et al., 1996). In a previous study, we investigated the uptake and bioavailability of Ag dispersed in water, wastewater and planktonic-borne AgNPs (NM-300K) in a 28-d bioaccumulation experiment with rainbow trout (Zeumer et al., 2019). While a limited Ag uptake into the carcass was observed following dietary exposure (121.5 μg kg^{-1} total Ag), the exposure of fish to 12.4 μg L^{-1} total Ag from water dispersions led to high Ag levels in the gills and carcass of fish, indicating a minor role of the digestive tract and a major role of the gill in the Ag uptake route (Zeumer et al., 2019). In the present study, we also calculated Ag transfer factors (see Supporting information, Fig. S2) for the fish organs. Generally, the highest transfer factors (9.7–10.0 L kg^{-1} in carcass, 6.3–6.4 L kg^{-1} in stomach, 32.7–42.9 L kg^{-1} in intestine) were found in animals exposed to water-dispersed AgNPs regardless of the Ag concentration in the water. In comparison to the water-dispersed treatment, the Ag transfer factors after exposure to wastewater-borne AgNPs (0.8–1.0 L kg^{-1} in carcass, 1.2–2.0 L kg^{-1} in stomach, 4.2–19.9 L kg^{-1} in intestine) or effluent supplemented AgNPs (1.3–3.9 L kg^{-1} in carcass, 1.5–3.1 L kg^{-1} in stomach, 13.3–20.8 L kg^{-1} in intestine) were relatively lower. In effluents, the AgNPs were probably mostly transformed to Ag_2S as inferred by Hartmann et al. (2019); these authors showed that Ag is associated with sulphur. Therefore, in our study, the Ag derived from wastewater-borne and effluent supplemented AgNPs was probably less bioavailable for rainbow trout due to potential sulfidation.

Regarding the Ti levels in fish organs after TiO_2NPs exposure, no significant differences to the respective EFF and DW controls were observed for any of the treatments (Fig. 2B, D, F). In fact, we obtained relatively high Ti concentrations within the analysed organs of the EFF (159.2, 170.8 and 2943.9 μg kg dry $weight^{-1}$ in carcass, stomach and intestine, respectively) and DW controls (119.7, 263.6 and 1782.5 μg kg wet $weight^{-1}$ in carcass, stomach and intestine, respectively). Likewise, Federici et al. (2007) observed 200–400 nmol Ti g dry $weight^{-1}$ (equal to 9580 μg Ti kg dry $weight^{-1}$) in the muscle of untreated (dechlorinated tap water) rainbow trout, while Ramsden et al. (2009) detected 1–8 nmol Ti g dry $weight^{-1}$ (equal to 48–380 μg kg dry $weight^{-1}$) in several tissues of rainbow trout from the control (dechlorinated tap water). Taking into account these findings, the Ti values obtained in our study indicate a natural Ti background in fish, which might depend on the natural Ti levels of the region where fish were obtained and the used commercial fish food. Further, this high natural Ti background might have hidden a potential uptake of Ti from TiO_2NP treatments. Nevertheless, Johnston et al. (2010) observed no uptake of TiO_2 into the tissues of rainbow trout after a 9-d aqueous exposure to 5000 μg L^{-1} TiO_2 (34 nm) and a 21-d dietary exposure to 300 mg g^{-1} TiO_2. These authors stated that the aggregation and/or association of TiO_2NPs with biological material (*e.g.* suspended solids in WWTP effluents) limit their bioavailability even if these aggregates are taken up orally. Therefore, it seems that TiO_2NPs administered through water, either as wastewater-borne, effluent-supplemented or water-dispersed, are not bioavailable to rainbow trout.

3.5. Effects on biochemical markers in O. mykiss

3.5.1. Effects of the effluent

The effects of the effluent itself (EFF) were also assessed at the biochemical level in fish and some alterations were perceived. For instance, in the gills, there was a significant 214% increase of LPO levels in effluent control comparatively to DW control ($p < 0.05$, AgNPs experiment, Fig. 3), suggesting lipid oxidative damage in these organs.

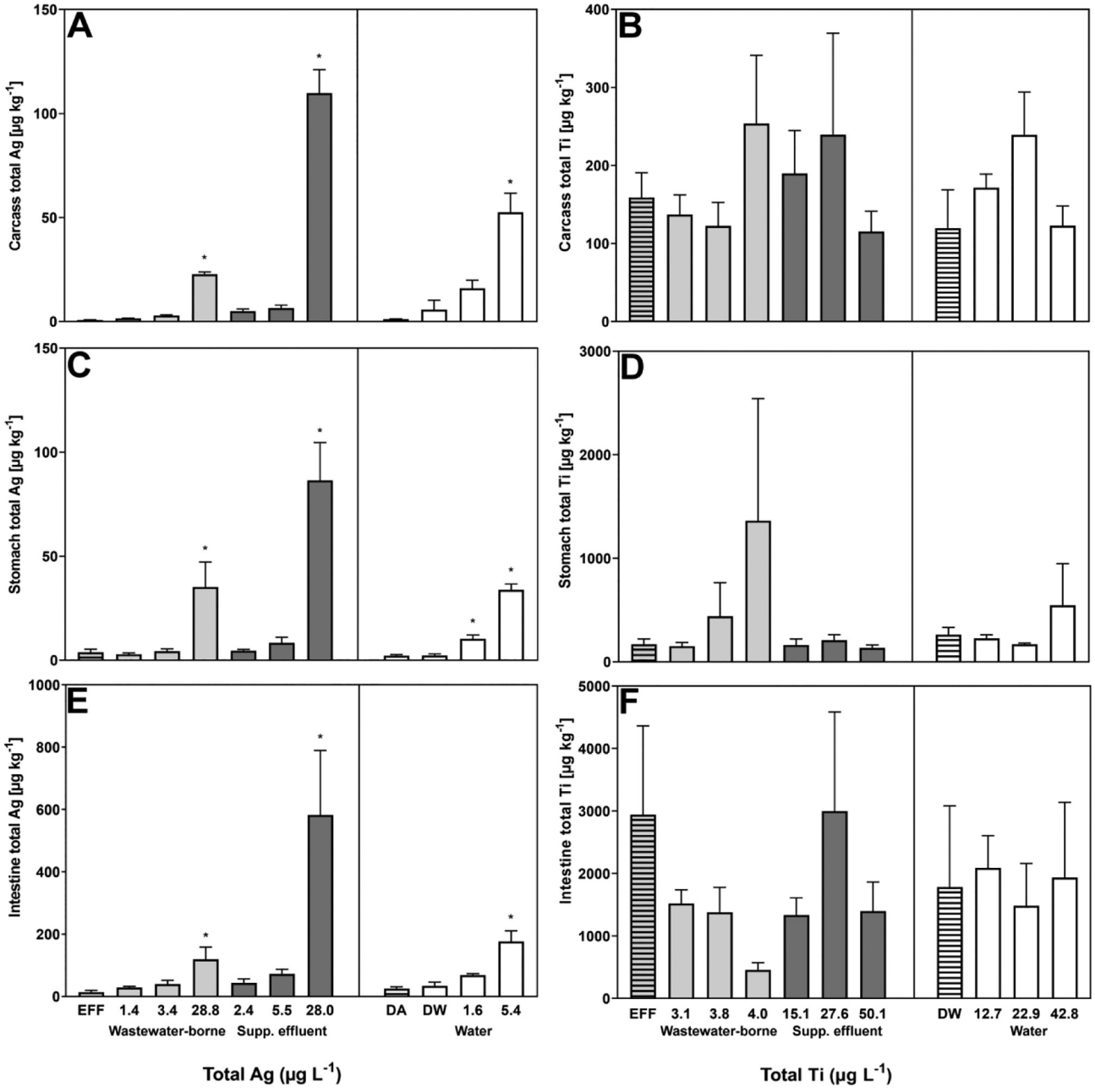


Fig. 2. Total Ag and Ti levels in *Oncorhynchus mykiss* organs after 28 d exposure to wastewater-borne, effluent-supplemented (Supp. effluent) and water-dispersed (Water) AgNPs (**A**, **C**, **E**) and TiO_2NPs (**B**, **D**, **F**). DW, EFF and DA are dilution water, effluent and dispersing agent controls, respectively. (**A**, **B**), (**C**, **D**) and (**E**, **F**) represent Ag and Ti levels (µg kg^{-1} wet weight) in carcass, stomach and intestine, respectively. Bars are mean ± SE of five (**A**, **C**, **E**) and four (**B**, **D**, **F**) organisms. Asterisks represent statistically significant differences ($p \leq 0.05$) relatively to the respective controls, according to Dunnett's *post-hoc* test.

In the muscle, total protein levels and CEA decreased significantly by 20% and 12% (respectively) in EFF comparatively to DW ($p < 0.05$, TiO_2NPs experiment, Supporting information, Table S4). The WWTP effluents are complex matrices constituted by a mixture of various substances, including some potential contaminants. Even though chemical analyses allowed us to record the presence of Ag and Ti in effluent-based treatments (Table 1), the occurrence of additional compounds in these treatments, including the EFF controls, should not be neglected. Notwithstanding, these additional constituents in the EFF controls, like *e.g.* natural organic matter and/or other dissolved organic and inorganic molecules, alone or combined with mineral colloids, were probably responsible for the observed effects (Meinelt et al., 2008).

3.5.2. Effects of the dispersing agent

In order to verify the potential influence of the dispersant agent in NM-300K on rainbow trout biochemical markers, a dispersant control was included in the experimental design. In our study, there was a significant increase above DW control of 73 and 65% in the activity of GST and LPO levels, respectively, in gills of fish exposed to DA ($p < 0.05$, AgNPs experiment Fig. 3E and F, respectively), thus suggesting an effect of DA by inducing detoxification mechanisms and enhancing oxidative damage of lipids. Since the dispersant constituents have lipophilic properties, they could interact with the lipid moiety of the cell membrane, thus leading to a potential increase in the uptake of NPs into the cell contributing to the overall toxicity of the mixture (Deng et al., 2017; Handy et al., 2012). In an experiment with waterborne AgNPs, Clark

et al. (2018) observed that the number of blood erythroblasts of rainbow trout juveniles was significantly higher in dispersant control solutions (similar mixture; 0.001% of 4% of each chemical) comparatively to negative control after 4 days. Nevertheless, other studies showed that the dispersing agent used for increasing NM-300K solubilization did not have any relevant effects on other biota [see *e.g.* Muth-Köhne et al., 2013 for zebrafish (*D. rerio*) embryos; McKee et al., 2017 for 9–12 d old springtail (*Folsomia candida*); Kühr et al., 2018 for amphipod (*Hyalella azteca*) adults]. However, the toxicity and adverse effects of synthetic dispersants used to stabilize AgNPs remain inconclusive and further studies are necessary to assess the potential side-effects of these compounds on fish.

3.5.3. Effects of AgNPs exposure

3.5.3.1. AChE in the brain. There was no change in the activity of AChE in the brain of *O. mykiss* exposed to all AgNP-containing treatments (Fig. 3A). Our results are consistent with Klingelfus et al. (2017), who showed that 0.2–20 μg L^{-1} of PVP coated AgNPs (20 nm), administered through prey as food, had no effect on AChE activity in the brain of *Hopalias intermedius* after 96 h and 40 d exposure. However, the biochemical responses related to the synthesis and/or inhibition of AChE activity after exposure to AgNPs need further clarification, since they may vary according to type and size of NPs, dispersing agent or coating, and the prevailing experimental conditions.

3.5.3.2. Oxidative stress in liver and gills. In comparison to the controls, there was an increase in the activity of hepatic SOD and CAT (Fig. 3C, G) of fish exposed to 1.4 μg L^{-1} of wastewater-borne AgNPs (132% and 129%, respectively) and 5.4 μg L^{-1} of water-dispersed AgNPs (42% and 87%, respectively), thus indicating an increase of oxidative stress in fish liver exposed to these treatments. Furthermore, as depicted in Fig. 3H, fish exposed to wastewater-borne AgNPs at the lowest concentration, corresponding to 1.4 μg L^{-1} of total Ag, showed a significant 55% increase of hepatic GST comparatively to EFF. Similarly, the exposure of *Oryzias latipes* embryos to 125–250 and 6.2–1000 μg L^{-1} of PVP-stabilized AgNPs (29.9 ± 5.0 nm) resulted in an increase of SOD activity and a decrease in the levels of reduced glutathione after 7 d (Wu and Zhou, 2012). The increase of CAT activity in the liver of fish exposed to almost all of the AgNPs-containing treatments (Fig. 3G) was associated with the absence of changes in the hepatic LPO levels (Fig. 3I), thus suggesting that the liver used the antioxidative defence capacities to prevent the oxidative damage. This is particularly relevant in fish exposed to either 1.4 μg L^{-1} of wastewater-borne AgNPs or 5.4 μg L^{-1} of water-dispersed AgNPs, since the respective associated detoxification mechanisms in the liver also increased at these tested concentrations (GST, Fig. 3H). In the gills, the CAT activity increased by 76 and 69% with 1.4 and 2.4 μg L^{-1} of wastewater-borne and effluent-supplemented AgNPs, respectively (Fig. 3D). The increase of CAT activity in the gills could be associated with an effective antioxidant defence response, which eventually compensates the inactivation of other antioxidant enzymes like *e.g.* GST (Fig. 3E). Effectively, a decrease of GST

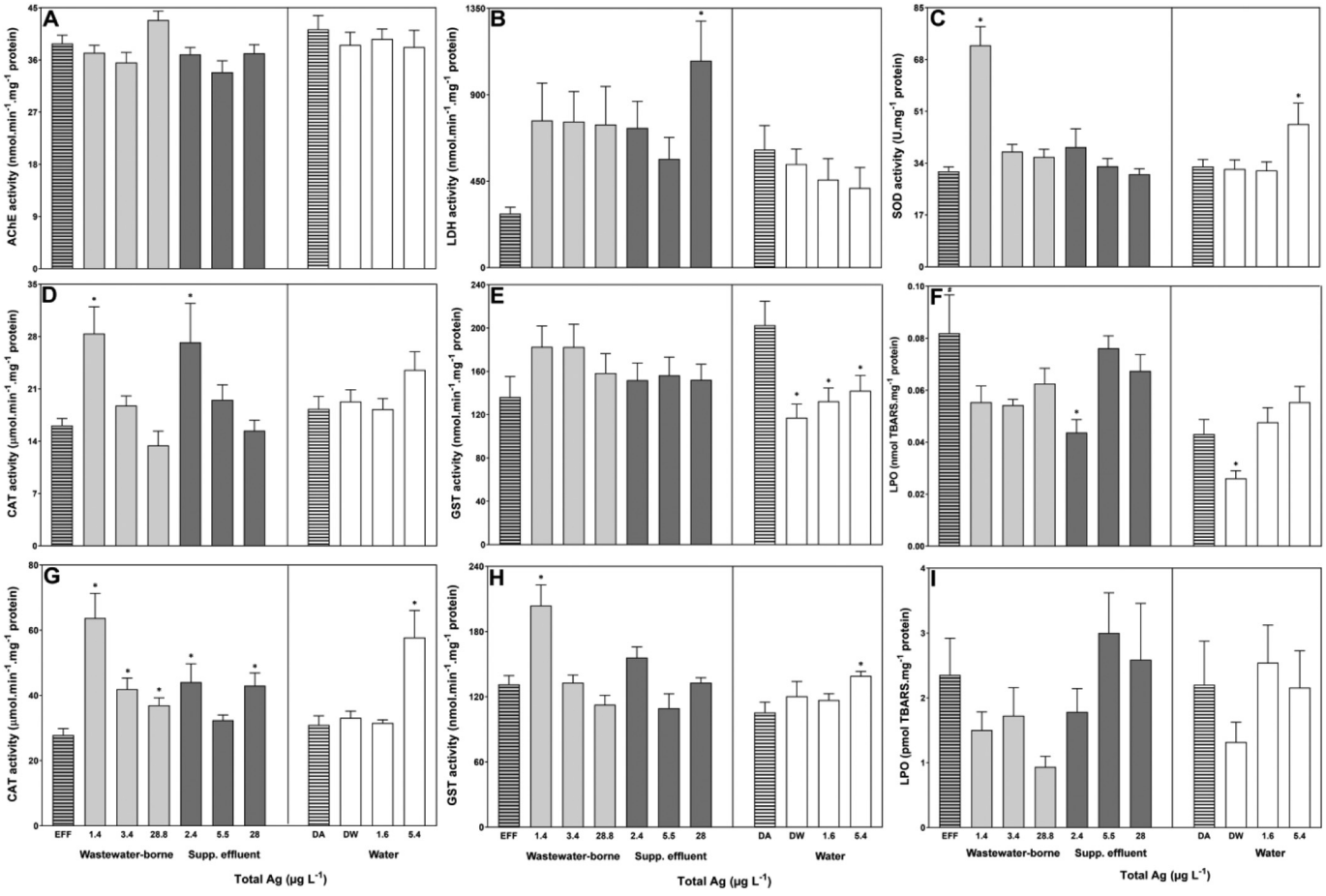


Fig. 3. Effects of wastewater-borne, effluent-supplemented and water-dispersed AgNPs on enzymatic activities of (**A**) brain acetylcholinesterase (AChE), (**B**) muscle lactate dehydrogenase (LDH), (**C**) liver superoxide dismutase (SOD), gills (**D**) and liver (**G**) catalase (CAT), gills (**E**) and liver (**H**) glutathione *S*-transferase (GST); and lipid peroxidation (LPO) levels in gills (**F**) and liver (**I**) of *O. mykiss* after 28 d exposure. DW, EFF and DA are dilution water, effluent and dispersing agent controls, respectively. Bars represent means of 8–10 organisms ± SE. Asterisks represent statistical differences ($p < 0.05$) relatively to the respective controls according to one-way ANOVA followed by Dunnett's *post-hoc* test (data was $\log_{10}$ transformed when necessary) or Kruskal-Wallis followed by Dunn's *post-hoc* test. Number sign represents statistical difference ($p < 0.05$) between DW and EFF according to the independent *t*-test.

activity in the gills by 35 and 30% with 1.6 and 5.4 μg L^{-1} of water-dispersed AgNPs was respectively observed (Fig. 3E). These results are in agreement with Khan et al. (2017), who showed a decrease of GST activity in the gills of the Indian major carp (*Labeo rohita*), after 14 or 28 d of exposure to 55 mg L^{-1} of AgNPs (~18 nm). Working with environmentally relevant concentrations, Shobana et al. (2018) noticed a significant decrease of GST activity in the gills of *L. rohita* exposed to 2.5 or 5 μg L^{-1} of AgNPs (20–50 nm) for 21 d. Furthermore, these authors noticed an increment of GST activity in the liver of carp with 5 μg L^{-1} of AgNPs after the same exposure time, which is also concordant with our results.

In general, the LPO levels in the gills and liver of fish exposed to all AgNP-containing treatments were unchanged, with the only exception of a significant reduction in the gills by 53% with 2.4 μg L^{-1} of effluent-supplemented AgNPs (Fig. 3F, I), suggesting an absence of oxidative damage of lipids in the tissues of both organs. These results contrast with previous studies, which demonstrated the potential oxidative damage of AgNPs to fish after chronic exposures (*e.g.* Khan et al., 2017). Notwithstanding, our results are supported by Scown et al. (2010), which showed that 10 and 100 μg L^{-1} of AgNPs (10 and 35 nm) dispersed in tap water for 10 d had no effects on the LPO levels of gills and liver of rainbow trout juveniles. These authors explained the absence of oxidative damage in the analysed tissues by the presence of a mucous layer surrounding the gill epithelia, thereby impeding the uptake of AgNPs. Effectively, in our study, the effects of AgNPs on the antioxidant enzymes were observed in both organs, and moreover, Ag levels in fish carcass further prove that Ag^{+} was effectively internalized. However, a suitable explanation for the insignificant variation of LPO levels in fish with almost all AgNPs-containing treatments can be related to: (i) the exposure time, which was not long enough to cause lipid damage at low AgNP concentrations and/or; (ii) the overall antioxidative defence system could have effectively mitigated the oxidative stress caused by AgNPs.

3.5.3.3. LDH and energy related parameters in the muscle. The available information about the potential effects of metallic NPs on the anaerobic metabolism of fish is scarce. A significant increase of LDH activity (283% compared to EFF) was observed in the muscle of rainbow trout exposed to the highest AgNP concentration (28 μg L^{-1} Ag) supplemented to the effluent (Fig. 3B), thereby pointing to an increase of the anaerobic metabolism in fish exposed to this treatment. Probably, the high energy demand exerted by muscle could not be met only by respiration, as confirmed by the reduced E_c value (0.004 ± 0.000 mJ·mg^{-1} FW·min^{-1}) observed with this treatment (Table 2). In other words, fish exposed to 28 μg L^{-1} of AgNPs supplemented to the effluent induced a significant respiratory dysfunction and the metabolism was most probably shifted towards the anaerobic glycolytic pathway. Furthermore, the increase of lactate following exposure to 28 μg L^{-1} Ag was probably not enough to replenish the glucose levels *via* muscle gluconeogenesis (Ale et al., 2018; Lacave et al., 2018), since carbohydrate levels were also greatly reduced (42% compared to EFF) at this concentration (Table 2). Another possible explanation for the observed effects of AgNPs on LDH activity could also be related with a potential inhibition of the oxygen and carbon dioxide exchange mechanisms in the gills, leading to the use of alternative (anaerobic) pathways in the same or alternative tissues (*e.g.* muscle), in order to cope with the imposed nanochemical stress (Lee et al., 2012; Valerio-García et al., 2017). Notwithstanding, our results are in accordance with Wu and Zhou (2012), who demonstrated that the LDH activity of medaka (*O. latipes*) embryos increased in a time-dependent and concentration-dependent manner with 62.5–1000 μg L^{-1} of AgNPs after 9 days of exposure. Relatively to energy related parameters in rainbow trout muscle, there was a significant depletion comparatively to controls in each of the fractions of total lipids, proteins, and carbohydrates in almost all AgNP-containing treatments (Table 2), thus contributing to the overall decrease of the E_a: (i) Ag from wastewater-borne AgNPs (72 and 67% with 3.4 and 28.8 μg L^{-1} Ag, respectively); (ii) effluent-supplemented AgNPs (44, 63 and 37% with 2.4, 5.5 and 28 μg L^{-1} Ag, respectively); and (iii) water-dispersed AgNPs (57 and 48% with 1.6 and 5.4 μg L^{-1} Ag, respectively). The depletion of the energy reserves in the muscle was probably a result of the drainage of these macromolecules to the associated detoxification mechanisms due to the metabolic dysfunction that eventually occurred in another organs/tissues.

Few data are currently available on the inhibition of synthesis of lipids (and/or increase of their degradation) in fish exposed to AgNPs. Notwithstanding, a decrease in the content of these biomolecules has been related to an increase in the energetic request associated with the stress imposed by AgNPs (Valerio-García et al., 2017). Although not statistically significant, a decrease of 14 and 18% of total lipids was observed in *Prochilodus lineatus* muscle respectively exposed to 2.5 and 25 μg L^{-1} of AgNPs (nanArgen®, 20–40 nm) for 15 d (Ale et al., 2018). In another recent study, Lacave et al. (2017) observed that several genes related to lipid transport and lipid localization were regulated in the liver of *D. rerio* adults [daily fed with brine shrimp larvae previously exposed to 100 ng L^{-1} and 100 μg L^{-1} of AgNPs (PVP/PEI coated, 8.08 nm) for 3 days] after 21 days of fish exposure to AgNPs. An excessive accumulation of lipid droplets in fish hepatocytes after 28 d of exposure to 1.5 mg L^{-1} of AgNPs (<100 nm) was confirmed in rainbow trout by Ostaszewska et al. (2018), thus indicating a reduced fatty acid utilization and/or impaired lipid catabolism in the affected cells. Regardless of the different mobilization pathways and the specific organs

Table 2
Effects of wastewater-borne, effluent-supplemented (Supp. effluent) and water-dispersed (Water) AgNPs on the energy related parameters in the muscle of *O. mykiss* after 28 d exposure.

Parameters	Controls			Wastewater-borne			Supp. effluent			Water	
	DW	EFF	DA	1.4	3.4	28.8	2.4	5.5	28	1.6	5.4
Lipids (mJ mg^{-1} FW)	59 ± 8.7	72 ± 11.7	54 ± 6.7	51 ± 15.8	18 ± 0.1*	21 ± 3.9*	39 ± 7.0*	17 ± 0.8*	34 ± 5.7*	29 ± 4.8*	23 ± 2.0*
Carbohydrates (mJ mg^{-1} FW)	53 ± 6.4	65 ± 9.0	43 ± 6.3	47 ± 12.4	16 ± 1.9*	23 ± 2.6*	31 ± 4.3*	23 ± 2.2*	38 ± 8.0*	20 ± 2.1*	28 ± 4.7
Proteins (mJ mg^{-1} FW)	1440 ± 193.0	1543 ± 209.8	1057 ± 166.3	1155 ± 300.5	433 ± 39.8*	591 ± 72.5*	708 ± 82.5*	484 ± 39.9*	803 ± 168.5*	635 ± 101.8*	682 ± 70.5
E_a (mJ mg^{-1} FW)	1448 ± 191.3	1390 ± 172.3	1156 ± 175.5	1028 ± 253.1	393 ± 39.4*	453 ± 49.0*	771 ± 88.2*	521 ± 45.6*	875 ± 175.5*	592 ± 50.1*	722 ± 86.9*
E_c (mJ mg^{-1} FW min^{-1})	0.013 ± 0.004	0.014 ± 0.004	0.018 ± 0.003	0.013 ± 0.001	0.004 ± 0.000	0.013 ± 0.002	0.012 ± 0.002	0.006 ± 0.001	0.004 ± 0.000*	0.024 ± 0.003	0.011 ± 0.002
CEA	83,568 ± 8408	70,479 ± 9016	91,367 ± 22,239	56,167 ± 782	38,686 ± 10,494*	17,682 ± 1708*	63,111 ± 9415	83,057 ± 22,939*	100,375 ± 7820*	78,245 ± 17,584	52,086 ± 9971

E_a – energy available; E_c – energy consumption; CEA – cellular energy allocation. DW, EFF and DA are dilution water, effluent and dispersing agent controls, respectively. Values represent means of 8–10 independent experiments ± SE. Asterisks represent significant differences ($p < 0.05$) according to one-way ANOVA followed by Dunnett's *post-hoc* test (data was $\log_{10}$ transformed when necessary) or Kruskal-Wallis followed by Dunn's *post-hoc* test.

responsible the for accumulation of lipid reserves, it seems that the depletion of total lipids in rainbow trout in our study could have been induced by the high energy demand of the whole organism through a yet unknown mechanism of disturbance of lipid metabolism (Cedervall et al., 2012; Valerio-García et al., 2017).

The overall reduction of total carbohydrate content observed in this study with almost all tested treatments (Table 2) could be indicative of an increase of the fish energetic demands used for detoxification processes in order to cope with AgNPs. A reduction of 25% of total carbohydrates was recently observed in the muscle of the goodeid fish (*Chapalichthys pardalis*) adults exposed to 4.1 mg L^{-1} of PVP-AgNPs (20 nm) for 21 days (Valerio-García et al., 2017). Similarly, a reduction of 53% of muscle carbohydrates was recently demonstrated in catfish (*Mystus gulio*) fingerlings exposed to 0.4 mg L^{-1} of AgNPs for 15 days (Abirami et al., 2017). Even though much lower concentrations were tested in our study, it can be hypothesized that the observed depletion of total carbohydrates could be associated with a reduction of the muscle glucose levels due to a high energy demand imposed by AgNPs (Abirami et al., 2017; Valerio-García et al., 2017).

With few exceptions, all AgNP-containing treatments caused a significant decrease of total proteins comparatively to controls in the muscle (Table 2). As stated for total lipids and total carbohydrates, the decrease of total proteins can be related to an increase in the energy cost required by fish in order to maintain homeostasis under chemical stress, since tissue protein content depends on the dynamic equilibrium between synthesis and degradation mechanisms of these macromolecules (Ale et al., 2018). A significant reduction of 60% of total protein content was recently noticed in the muscle of *P. lineatus* juveniles treated with 25 μg L^{-1} of AgNPs (nanArgen®, 20–40 nm) for 15 days (Ale et al., 2018). In spite of a potential mobilization of amino acids to cope with the nanochemical stress, some studies have effectively shown that AgNPs can disrupt protein structure and enzyme function through binding of Ag^{+} to negatively charged thiol groups of amino acids, peptides and proteins, thus causing disturbances of energy homeostasis (Ravindran et al., 2012).

3.5.4. Effects of TiO_2NPs

3.5.4.1. AChE in the brain. An increase of AChE activity of 24% was found in rainbow trout brain with 50.1 μg L^{-1} Ti supplemented effluent comparatively to controls, but no alterations were found in fish exposed to water-dispersed TiO_2NPs (Fig. 4A). Sheng et al. (2016) also found alterations in other neurotransmission markers in a 45-d experiment with *D. rerio* adults exposed to anatase TiO_2NPs. Effectively, these authors observed a concentration dependent decrease of other neurotransmitters with important functions in brain development and cognitional function, *viz.* norepinephrine, dopamine and 5-hydroxytryptamine, with 10–40 μg L^{-1} of TiO_2NPs (anatase, 5–6.5 nm) dispersed in zebrafish culture medium (Sheng et al., 2016). Xia et al. (2017) observed an increase of AChE activity in gills and digestive gland of the marine scallop *Chlamys farreri* by the factor of 4.43 and 1.74, respectively, compared to the control after 14-d exposure to 1 mg L^{-1} TiO_2NPs (98.6% anatase, 1.4% rutile), but also stated that the involved mechanism stayed unclear.

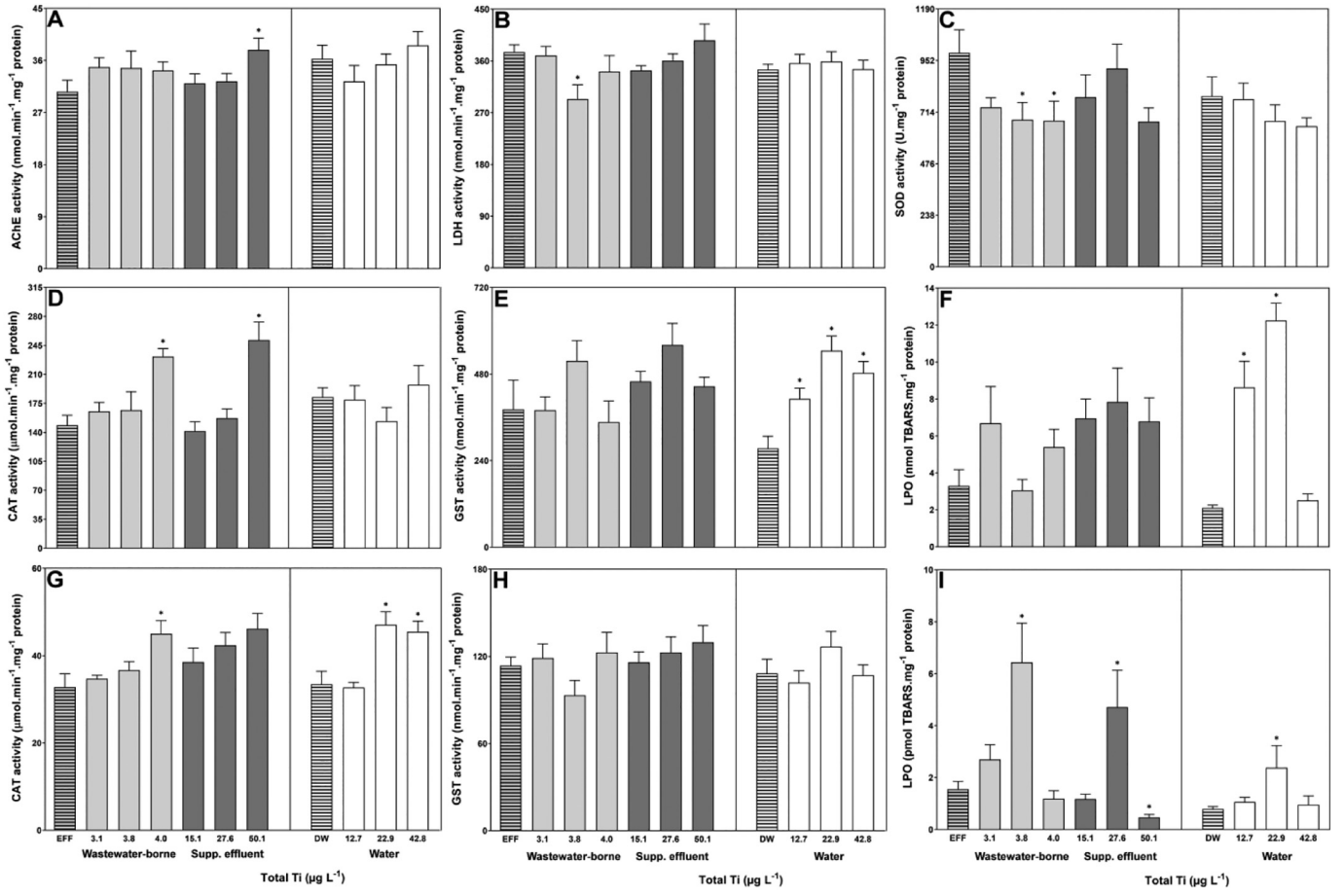


Fig. 4. Effect of wastewater-borne, effluent-supplemented (Supp. effluent) and water-dispersed (Water) TiO_2NPs on enzymatic activities of (**A**) brain acetylcholinesterase (AChE), (**B**) muscle lactate dehydrogenase (LDH), (**C**) liver superoxide dismutase (SOD), gills (**D**) and liver (**G**) catalase (CAT), gills (**E**) and liver (**H**) glutathione *S*-transferase (GST); lipid peroxidation (LPO) levels in gills (**F**) and liver (**I**) of *O. mykiss* after 28 d exposure. DW, EFF and DA are dilution water, effluent and dispersing agent controls, respectively. Bars represent means of 4–10 independent experiments ± SE. Asterisks represent statistical differences ($p < 0.05$) according to one-way ANOVA followed by Dunnett's *post-hoc* test (data was $\log_{10}$ transformed when necessary) or Kruskal-Wallis followed by Dunn's *post-hoc* test.

Together, these findings suggest that the chronic exposure of fish to TiO_2NPs can lead to neurological alterations when fish are exposed in aqueous suspensions with certain levels of organic matter. Furthermore, the AChE activity in the brain of *P. lineatus* juveniles remained constant after 30 d of exposure to 0.1–10 μg L^{-1} of TiO_2NPs (21 nm) dispersed in tap water (Miranda et al., 2016), which is in accordance with our results obtained for *O. mykiss* exposed to water-dispersed TiO_2NPs (Fig. 4A), and thus confirming that the neurotoxicological effects of TiO_2NPs were absent in exposure media without any organic load. On the other hand, the absence of effects of wastewater-borne TiO_2NPs in fish AChE activity (Fig. 4A) could be explained by the lower total Ti levels measured in these treatments.

3.5.4.2. Oxidative stress in liver and gills. The evaluation of the potential induction of oxidative stress by TiO_2NPs in exposed fish is particularly important, since it is known that TiO_2NPs are photoactive and produce ROS (Bermejo-Nogales et al., 2017). In the gills, the significant increase of GST activity (50–99% above controls) and LPO levels (210–382% above controls) in water-dispersed TiO_2NPs treatments respectively indicate oxidative stress and oxidative damage (Fig. 4E, F). However, the CAT activity remained unchanged in the gills of fish exposed to all water-dispersed TiO_2NPs (Fig. 4D). Notwithstanding, CAT activity increased by 56 and 69%, with 4.0 and 50.1 μg L^{-1} of total Ti present in wastewater-borne and effluent-supplemented treatments, respectively (Fig. 4D).

In the present study, the decrease of liver SOD activity by 31 and 32% with 3.8 and 4.0 μg L^{-1} of wastewater-borne TiO_2NPs (Fig. 4C) respectively, could be indicative of an impairment of the antioxidant defence system. For instance, Hao et al. (2009) found that the activity of the hepatic SOD of juvenile carp (*Cyprinus carpio*) showed a significant decrease after 8-d exposure to 50–200 mg L^{-1} of TiO_2NPs (rutile, 50 nm). On the other hand, in the present study, the hepatic CAT activity increased with the highest Ti concentrations resulting from wastewater-borne TiO_2NPs (4.0 μg L^{-1}, 37% increase above controls) and water-dispersed TiO_2NPs (22.9 and 42.8 μg L^{-1}, 41 and 36% increase above controls, respectively), thus indicating oxidative stress in this organ (Fig. 4G). Furthermore, a significant increase of LPO levels in liver (203–315%) of fish exposed to wastewater-borne, effluent-supplemented and water-dispersed TiO_2NPs, denote hepatic oxidative damage induced by TiO_2NPs irrespective of the exposure matrix.

Overall, the obtained results suggest that: (i) the antioxidant defence system of *O. mykiss* was activated in order to prevent cellular damage during TiO_2NP exposure; (ii) different adaptative mechanisms operated in the gills and liver in order to counteract the increased oxidative stress; and (iii) despite these mechanisms, oxidative damage occurred in both organs of fish exposed to different treatments. Accordingly, the obtained results could be explained by each one of the main mechanisms (or their combination), which ultimately lead to toxicity of TiO_2NPs on rainbow trout: (i) photocatalytic activity (phototoxicity) of TiO_2NPs; (ii) physical stress associated with the size and surface properties of TiO_2NPs (cytotoxicity); and/or (iii) capacity of TiO_2NPs to adsorb other potential xenobiotics present in the exposure media (Callaghan and MacCormack, 2017; Clemente et al., 2015). Despite of these mechanisms, the occurrence of oxidative stress in rainbow trout could be most probably related to the direct production of ROS by TiO_2NPs (with or without irradiation), which, ultimately, may be a result of the hypoxia induced by the adsorption of TiO_2NPs to the gills, thereby causing the occlusion of this organ (Boyle et al., 2013). In brief, the interpretation of oxidative stress mechanisms caused by Ti present as TiO_2NPs on fish are complex and should be interpreted with caution, since it depends of several factors like *e.g.* the exposure media, formulation type and experimental conditions.

3.5.4.3. LDH and energy related parameters in the muscle. The LDH activity (Fig. 4B) and almost all of energy related parameters (Table S4) remained unchanged in the muscle of fish exposed to all TiO_2NP-containing treatments, with only few exceptions. The only significant alteration on LDH was a 22% decrease of its activity with 3.8 μg L^{-1} of wastewater-borne TiO_2NPs (Fig. 4B), which could be explained by a binding mechanism of Ti or TiO_2NPs to the LDH protein, with the consequent enzymatic inactivation, as proposed by Zaqout et al. (2012). Nevertheless, this mechanism needs to be further confirmed, since these authors used a purified form of LDH and much higher concentrations of TiO_2NPs (Zaqout et al., 2012).

Contrary to AgNP experiments, the energy reserves in fish were not affected by exposure to the media containing TiO_2NPs. This is consistent with the findings of Ramsden et al. (2009), which showed that rainbow trout's carcass composition at the end of an 8-week dietary experiment with 100 mg kg^{-1} TiO_2NPs (Aeroxide® P25, anatase-rutile, 21 nm) remained practically unchanged, thus confirming the absence of any significant variations in the major energy reserves of fish. In other experiment with *P. lineatus*, it was noticed that fish exposed to 1–50 mg L^{-1} of TiO_2NPs (Aeroxide® P25, anatase-rutile, 21 nm) for 14 d did not either display changes in aerobic respiration nor did present hypo- or hyperventilation (Do Carmo et al., 2018). Although few studies have demonstrated that TiO_2NPs caused respiratory distress associated with oxidative stress on fish (*e.g.* Boyle et al., 2013; Federici et al., 2007; Hao et al., 2009; Ramsden et al., 2009), none of them considered environmentally-relevant concentrations after long periods of time (chronic; 28 d) like those presented herein.

4. Conclusions

In our study we combined the production of AgNP- and TiO_2NP-containing effluents from model WWTPs with the investigation of several endpoints in *O. mykiss* after chronic exposure. It was shown that AgNPs and TiO_2NPs, with concentrations <52 μg L^{-1} did not inhibit growth in rainbow trout. The analyses of total Ag and total Ti levels in fish organs indicate a lower bioavailability of Ag from wastewater-borne AgNPs to rainbow trout compared to their water-dispersed counterparts due to their potential sulfidation in the WWTP. No uptake of Ti from TiO_2NPs in any of the tested treatments was observed. Hence, although both NPs did not inhibit the growth of rainbow trout at the tested concentrations, regardless of the source of NPs, their passage through WWTPs seemed to lower AgNPs bioavailability to fish, based on the measured Ag concentrations in the organs. Despite these findings at the individual level, the changes at the biochemical level, assessed through several biochemical markers following exposure to AgNPs or TiO_2NPs, have shown a different pattern of the organism's reaction to contaminations with each type of NPs. Regarding AChE, none of the AgNP-containing treatments induced a change in this biochemical marker, while the highest tested Ti concentration (50.1 μg L^{-1}) of TiO_2NPs supplemented to the effluent induced a significant increase in AChE activity. This suggests a potentially neurotoxic effect caused by the combination of TiO_2NPs with other substances from the effluent matrix. On the other hand, TiO_2NPs had no effect on the energetic reserves of the fish, while total proteins, lipids and carbohydrates were depleted following exposure to nearly all AgNP-containing treatments. Besides, our results revealed substantial effects of the effluent matrix itself at the biochemical level, thereby causing oxidative stress and alterations in energy reserves.

Essentially, our study shows that wastewater-borne NPs still pose a risk to freshwater fish thereby affecting the animals at the biochemical level. However, the interactions and synergetic effects of NPs with potentially harmful substances present in complex natural matrices, like the effluents from WWTPs, are still unknown and should be addressed in future studies. This will allow the establishment of a more reliable risk assessment of AgNPs and TiO_2NPs at environmentally relevant concentrations in the aquatic environment. Likewise, the localization of wastewater-borne NPs in fish tissues and organs should be performed in future studies, in order to investigate whether the NPs themselves

were taken up into the tissues or if the observed effects were purely based on ions as herein assessed.

CRediT authorship contribution statement

Richard Zeumer: Investigation, Writing - original draft, Writing - review & editing, Visualization.**Victor Galhano:** Investigation, Writing - original draft, Writing - review & editing, Visualization. **Marta S. Monteiro:** Investigation, Writing - original draft, Writing - review & editing, Visualization. **Sebastian Kuehr:** Investigation, Writing - review & editing. **Burkhard Knopf:** Methodology. **Boris Meisterjahn:** Methodology. **Amadeu M.V.M. Soares:** Supervision. **Susana Loureiro:** Writing - review & editing, Supervision. **Isabel Lopes:** Methodology, Writing - review & editing, Project administration. **Christian Schlechtriem:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration.

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.

Acknowledgements

This work was funded by the ERA-NET SIINN project FENOMENO (*Fate and Effect of Wastewater-borne Manufactured Nanomaterials in Aquatic Ecosystems*; Grant No.: 03XP0005A/03XP0005B). Thanks are due to FCT/MCTES for the financial support to CESAM (UIDB/50017/2020 + UIDP/50017/2020), through national funds. The work of VG and MSM is funded by national funds (OE), through FCT – Fundação para a Ciência e a Tecnologia, I.P., in the scope of the framework contract foreseen in the numbers 4, 5 and 6 of the Article 23, of the Decree-Law 57/2016, of August 29, changed by Law 57/2017, of July 19. Further funding of the project FENOMENO by the Federal Ministry of Education and Research (BMBF) is also gratefully acknowledged. We express our gratitude to Ralf Kaegi for the provision of STEM analyses and acknowledge the support from the Scientific Center for Optical and Electron Microscopy (ScopeM, ETH-Zurich, Switzerland) and from the Electron Microscopy Center at Empa (Swiss Federal Institute for Materials Science and Technology, Duebendorf, Switzerland). A special thanks also to Verena Kosfeld, Dana Esser and Anna Schauerte for their technical support and to Virginia Schraps, Laura Föckeler, Jona Seidel and Kevin Ladage for their help in the analytical determinations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.137974.

References

Abirami, T., Jose, A.G.R., Govindarajulu, B., Karthikeyan, J., 2017. Ecotoxicology of green synthesized silver nanoparticles on fresh water fish *Mystus gulio*. Int J Pharm Pharm Sci 9 (11), 192–198. https://doi.org/10.22159/ijpps.2017v9i11.21627.

Ale, A., Rossi, A.S., Bacchetta, C., Gervasio, S., de la Torre, F.R., Cazenave, J., 2018. Integrative assessment of silver nanoparticles toxicity in *Prochilodus lineatus* fish. Ecol. Indic. 93, 1190–1198. https://doi.org/10.1016/j.ecolind.2018.06.023.

Asghari, S., Johari, S.A., Lee, J.H., Kim, Y.S., Jeon, Y.B., Choi, H.J., Moon, M.C., Yu, I.J., 2012. Toxicity of various silver nanoparticles compared to silver ions in *Daphnia magna*. J. Nanobiotechnology 10, 14. https://doi.org/10.1186/1477-3155-10-14.

Azimzada, A., Tufenkji, N., Wilkinson, K.J., 2017. Transformations of silver nanoparticles in wastewater effluents: links to Ag bioavailability. Environ. Sci.-Nano 4, 1339–1349. https://doi.org/10.1039/C7EN00093F.

Barndõk, H., Hermosilla, D., Han, C., Dionysiou, D.D., Negro, C., Blanco, Á., 2016. Degradation of 1, 4-dioxane from industrial wastewater by solar photocatalysis using immobilized NF-TiO_2 composite with monodisperse TiO_2 nanoparticles. Appl. Catal. B Environ. 180, 44–52. https://doi.org/10.1016/j.apcatb.2015.06.015.

Baun, A., Hartmann, N.B., Grieger, K., Kusk, K.O., 2008. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. Ecotoxicology 17 (5), 387–395. https://doi.org/10.1007/s10646-008-0208-y.

Beer, C., Foldbjerg, R., Hayashi, Y., Sutherland, D.S., Autrup, H., 2012. Toxicity of silver nanoparticles - nanoparticle or silver ion? Toxicol. Lett. 208, 286–292. https://doi.org/10.1016/j.toxlet.2011.11.002.

Bermejo-Nogales, A., Connolly, M., Rosenkranz, P., Fernández-Cruz, M.L., Navas, J.M., 2017. Negligible cytotoxicity induced by different titanium dioxide nanoparticles in fish cell lines. Ecotox. Environ. Safe. 138, 309–319. https://doi.org/10.1016/j.ecoenv.2016.12.039.

Boyle, D., Al-Bairuty, G.A., Ramsden, C.S., Sloman, K.A., Henry, T.B., Handy, R.D., 2013. Subtle alterations in swimming speed distributions of rainbow trout exposed to titanium dioxide nanoparticles are associated with gill rather than brain injury. Aquat. Toxicol. 126, 116–127. https://doi.org/10.1016/j.aquatox.2012.10.006.

Brauner, C.J., Wood, C.M., 2002. Ionoregulatory development and the effect of chronic silver exposure on grwoth, survival, and sublethal indicators of toxicity in early life stages of rainbow trout (*Oncorhynchus mykiss*). J. Comp. Physiol. B. 172 (2), 153–162. https://doi.org/10.1007/s00360-001-0238-8.

Bruneau, A., Turcotte, P., Pilote, M., Gagné, F., Gagnon, C., 2016. Fate of silver nanoparticles in wastewater and immunotoxic effects on rainbow trout. Aquat. Toxicol. 174, 70–81. https://doi.org/10.1016/j.aquatox.2016.02.013.

Calabrese, E.J., 2004. Hormesis: a revolution in toxicology, risk assessment and medicine. EMBO Rep. 5, S37–S40. https://doi.org/10.1038/sj.embor.7400222.

Callaghan, N.I., MacCormack, T.J., 2017. Ecophysiological perspectives on engineered nanomaterial toxicity in fish and crustaceans. Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 193, 30–41. https://doi.org/10.1016/j.cbpc.2016.12.007.

Cedervall, T., Hansson, L.-A., Lard, M., Frohm, B., Linse, S., 2012. Food chain transport of nanoparticles affects behaviour and fat metabolism in fish. PLoS One 7. https://doi.org/10.1371/journal.pone.0032254.

Choi, O., Cleuenger, T.E., Deng, B., Surampalli, R.Y., Ross, J.L., Hu, Z., 2009. Role of sulfide and ligand strength in controlling nanosilver toxicity. Water Res. 43, 1879–1886. https://doi.org/10.1016/j.watres.2009.01.029.

Clark, N.J., Shaw, B.J., Handy, R.D., 2018. Low hazard of silver nanoparticles and silver nitrate to the haematopoietic system of rainbow trout. Ecotox. Environ. Safe. 152, 121–131. https://doi.org/10.1016/j.ecoenv.2018.01.030.

Clemente, Z., Castro, V.L., Feitosa, L.O., Lima, R., Jonsson, C.M., Maia, A.H.N., Fraceto, L.F., 2015. Biomarker evaluation in fish after prolonged exposure to nano-TiO_2: influence of illumination conditions and crystal phase. J. Nanosci. Nanotechnol. 15, 5424–5433. https://doi.org/10.1166/jnn.2015.10021.

Deng, R., Lin, D., Zhu, L., Majumdar, S., White, J.C., Gardea-Torresdey, J.L., Xing, B., 2017. Nanoparticle interactions with co-existing contaminants: joint toxicity, bioaccumulation and risk. Nanotoxicology 11, 591–612. https://doi.org/10.1080/17435390.2017.1343404.

Do Carmo, T.L.L., Azevedo, V.C., de Siqueira, P.R., Galvão, T.D., dos Santos, F.A., dos Reis Martinez, C.B., Appoloni, C.R., Fernandes, M.N., 2018. Reactive oxygen species and other biochemical and morphological biomarkers in the gills and kidneys of the Neotropical freshwater fish, *Prochilodus lineatus*, exposed to titanium dioxide (TiO_2) nanoparticles. Environ. Sci. Poll. Res. 25, 22963–22976. https://doi.org/10.1007/s11356-018-2393-4.

EU, 2010. Directive 2010/63/EU of the European Parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes. Off. J. Eur. Union L276, 33–79.

Farkas, J., Bergum, S., Nilsen, E.W., Olsen, A.J., Salaberria, I., Ciesielski, T.M., Bączek, T., Konieczna, L., Salvenmoser, W., Jenssen, B.M., 2015. The impact of TiO_2 nanoparticles on uptake and toxicity of benzo(a)pyrene in the blue mussel (*Mytilus edulis*). Sci. Tot. Environ. 511, 469–476. https://doi.org/10.1016/j.scitotenv.2014.12.084.

Farkas, J., Salaberria, I., Styrishave, B., Staňková, R., Ciesielski, T.M., Olsen, A.J., Posch, W., Flaten, T.P., Krøkje, Å., Salvenmoser, W., Jenssen, B.M., 2017. Exposure of juvenile turbot (*Scophthalmus maximus*) to silver nanoparticles and 17α-ethinylestradiol mixtures: implications for contaminant uptake and plasma steroid hormone levels. Environ. Poll. 220, 328–336. https://doi.org/10.1016/j.envpol.2016.09.067.

Federici, G., Shaw, B.J., Handy, R.D., 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): gill injury, oxidative stress, and other physiological effects. Aquat. Toxicol. 84, 415–430. https://doi.org/10.1016/j.aquatox.2007.07.009.

Galhano, V., Hartmann, S., Monteiro, M.S., Zeumer, R., Mozhayeva, D., Steinhoff, B., Müller, K., Prenzel, K., Kunze, J., Kuhnert, K.-D., Schönherr, H., Engelhard, C., Schlechtriem, C., Loureiro, S., Soares, A.M.V.M., Witte, K., Lopes, I., 2020. Impact of wastewater-borne nanoparticles of silver and titanium dioxide in the swimming behaviour and biochemical markers of *Daphnia magna*: an integrated approach. Aquat. Toxicol. 220, 105404. https://doi.org/10.1016/j.aquatox.2020.105404.

Georgantzopoulou, A., Carvalho, P.A., Vogelsang, C., Tilahun, M., Ndungu, K., Booth, A.M., Thomas, K.V., Macken, A., 2018. Ecotoxicological effects of transformed silver and titanium dioxide nanoparticles in the effluent from a lab-scale wastewater treatment system. Environ. Sci. Technol. 52, 9431–9441. https://doi.org/10.1021/acs.est.8b01663.

Gottschalk, F., Sonderer, T., Scholz, R.W., Nowack, B., 2009. Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, fullerenes) for different regions. Environ. Sci. Technol. 43, 9216–9222. https://doi.org/10.1021/es9015553.

Handy, R.D., Cornelis, G., Fernandes, T., Tsyusko, O., Decho, A., Sabo-Attwood, T., Metcalfe, C., Steevens, J.A., Klaine, S.J., Koelmans, A.A., Horne, N., 2012. Ecotoxicity test methods for engineered nanomaterials: practical experiences and recommendations from the bench. Environ. Toxicol. Chem. 31, 15–31. https://doi.org/10.1002/etc.70.

Hao, L.H., Wang, Z.Y., Xing, B.S., 2009. Effect of sub-acute exposure to TiO_2 nanoparticles on oxidative stress and histopathological changes in juvenile carp (*Cyprinus carpio*). J. Environ. Sci. 21, 1459–1466. https://doi.org/10.1016/S1001-0742(08)62440-7.

Hartmann, S., Louch, R., Zeumer, R., Steinhoff, B., Mozhayeva, D., Engelhard, C., Schönherr, H., Schlechtriem, C., Witte, K., 2019. Comparative multi-generation study on long-term effects of pristine and wastewater-borne silver and titanium dioxide nanoparticles on key lifecycle parameters in *Daphnia magna*. NanoImpact 14, 100163. https://doi.org/10.1016/j.impact.2019.100163.

He, X., Deng, H., Hwang, H., 2019. The current application of nanotechnology in food and agriculture. J. Food Drug Anal. 27 (1), 1–21. https://doi.org/10.1016/j.jfda.2018.12.002.

Ivanković, S., Gotić, M., Jurin, M., Musić, S., 2003. Photokilling squamous carcinoma cells SCCVII with ultrafine particles of selected metal oxides. J. Sol-Gel Sci. Technol. 27 (2), 225–233. https://doi.org/10.1023/A:1023715004575.

Johari, S.A., Kalbassi, M.R., Yu, I.J., Lee, J.H., 2015. Chronic effect of waterborne silver nanoparticles on rainbow trout (*Oncorhynchus mykiss*): histopathology and bioaccumulation. Comp. Clin. Pathol. 24, 995–1007. https://doi.org/10.1007/s00580-014-2019-2.

Johnston, B.D., Scown, T.M., Moger, J., Cumberland, S.A., Baalousha, M., Linge, K., van Aerle, R., Jarvis, K., Lead, J.R., Tyler, C.R., 2010. Bioavailability of nanoscale metal oxides TiO_2, CeO_2, and ZnO to fish. Environ. Sci. Technol. 44, 1144–1151. https://doi.org/10.1021/es901971a.

Kaegi, R., Ulrich, A., Sinnet, B., Vonbank, R., Wichser, A., Zuleeg, S., Simmler, H., Brunner, S., Vonmont, H., Burkhardt, M., Boller, M., 2008. Synthetic TiO_2 nanoparticle emission from exterior facades into the aquatic environment. Environ. Pollut. 156 (2), 233–239. https://doi.org/10.1016/j.envpol.2008.08.004.

Kaegi, R., Voegelin, A., Sinnet, B., Zuleeg, S., Hagendorfer, H., Burkhardt, M., Siegrist, H., 2011. Behavior of metallic silver nanoparticles in a pilot wastewater treatment plant. Environ. Sci. Technol. 45, 3902–3908. https://doi.org/10.1021/es1041892.

Kampe, S., Kaegi, R., Schlich, K., Wasmuth, C., Hollert, H., Schlechtriem, C., 2018. Silver nanoparticles in sewage sludge: bioavailability of sulfidized silver to the terrestrial isopod *Porcellio scaber*. Environ. Toxicol. Chem. 37, 1606–1613. https://doi.org/10.1002/etc.4102.

Khan, M.S., Qureshi, N.A., Jabeen, F., 2017. Assessment of toxicity in fresh water fish *Labeo rohita* treated with silver nanoparticles. Appl. Nanosci. 7, 167–179. https://doi.org/10.1007/s13204-017-0559-x.

Kiser, M.A., Westerhoff, P., Benn, T., Wang, Y., Pérez-Rivera, J., Hristovski, K., 2009. Titanium nanomaterial removal and release from wastewater treatment plants. Environ. Sci. Technol. 43, 6757–6763. https://doi.org/10.1021/es901102n.

Klein, C.L., Comero, S., Stahlmecke, B., Romazanov, J., Kuhlbusch, T.A.J., Van Doren, E., De Temmerman, P.-J., Mast, J., Wick, P., Krug, H., Locoro, G., Hund-Rinke, K., Kördel, W., Friedrichs, S., Maier, G., Werner, J., Linsinger, T., Gawlik, B.M., 2011. NM-Series of Representative Manufactured Nanomaterials: NM-300 Silver Characterization, Stability, Homogeneity. JRC Scientific and Technical ReportsEuropean Commision, Joint Reseach Centre, Institute for Health and Consumer Protection. Publications Office of the European Union, Luxembourg https://doi.org/10.2788/23079 EUR 24693 EN-2011. 86 pp.

Klingelfus, T., Lirola, J.R., Silva, L.F.O., Disner, G.R., Vicentini, M., Nadaline, M.J.B., Robles, J.C.Z., Trein, L.M., Voigt, C.L., Silva de Assis, H.C., Mela, M., Lerne, D.M., Cestari, M.M., 2017. Acute and long-term effects of trophic exposure to silver nanospheres in the central nervous system of a neotropical fish *Hoplias intermedius*. Neurotoxicology 63, 146–154. https://doi.org/10.1016/j.neuro.2017.10.003.

Kraas, M., Schlich, K., Knopf, B., Wege, F., Kägi, R., Terytze, K., Hund-Rinke, K., 2017. Long-term effects of sulfidized silver nanoparticles in sewage sludge on soil microflora. Environ. Toxicol. Chem. 36, 3305–3313. https://doi.org/10.1002/etc.3904.

Kühr, S., Schneider, S., Meisterjahn, B., Schlich, K., Hund-Rinke, K., Schlechtriem, C., 2018. Silver nanoparticles in sewage treatment plant effluents: chronic effects and accumulation of silver in the freshwater amphipod *Hyalella azteca*. Environ. Sci. Eur. 30, 11. https://doi.org/10.1186/s12302-018-0137-1.

Lacave, J.M., Fanjul, A., Bilbao, E., Gutierrez, N., Barrio, I., Arostegui, I., Cajaraville, M.P., Orbea, A., 2017. Acute toxicity, bioaccumulation and effects of dietary transfer of silver from brine shrimp exposed to PVP/PEI-coated silver nanoparticles to zebrafish. Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 199, 69–80. https://doi.org/10.1016/j.cbpc.2017.03.008.

Lacave, J.M., Vicario-Parés, U., Bilbao, E., Gilliland, D., Mura, F., Dini, L., Cajaraville, M.P., Orbea, A., 2018. Waterborne exposure of adult zebrafish to silver nanoparticles and to ionic silver results in differential silver accumulation and effects at cellular and molecular levels. Sci. Tot. Environ. 642, 1209–1220. https://doi.org/10.1016/j.scitotenv.2018.06.128.

Lee, B.C., Duong, C.N., Cho, J., Lee, J., Kim, K., Seo, Y., Kim, P., Choi, K., Yoon, J., 2012. Toxicity of citrate-capped silver nanoparticles in common carp (*Cyprinus carpio*). J. Biomed. Biotechnol., 14 https://doi.org/10.1155/2012/262670.

Li, L., Stoiber, M., Wimmer, A., Xu, Z., Lindenblatt, C., Helmreich, B., Schuster, M., 2016. To what extent can full-scale wastewater treatment plant effluent influence the occurrence of silver-based nanoparticles in surface waters? Environ. Sci. Technol. 50 (12), 6327–6333. https://doi.org/10.1021/acs.est.6b00694.

Liu, J., Hurt, R.H., 2010. Ion release kinetics and particle persistence in aqueous nano-silver colloids. Environ. Sci. Technol. 44 (6), 2169–2175. https://doi.org/10.1021/es9035557.

McKee, M.S., Engelke, M., Zhang, X., Lesnikov, E., Köser, J., Eickhorst, T., Filser, J., 2017. Collembola reproduction decreases with aging of silver nanoparticles in a sewage sludge-treated soil. Front. Environ. Sci. 5, 19. https://doi.org/10.3389/fenvs.2017.00019.

Meinelt, T., Schreckenbach, K., Pietrock, M., Heidrich, S., Steinberg, C.E.W., 2008. Humic substances - part 1: dissolved humic substances (HS) in aquaculture and ornamental fish breeding. Environ. Sci. Poll. Res. 15, 17–22. https://doi.org/10.1065/espr2007.08.448.

Miranda, R.R., da Silveira, A.L.R.D., de Jesus, I.P., Grötzner, S.R., Voigt, C.L., Campos, S.X., Garcia, J.R.E., Randi, M.A.F., Oliveira Ribeiro, C.A., Filipak Neto, F., 2016. Effects of realistic concentrations of TiO_2 and ZnO nanoparticles in *Prochilodus lineatus* juvenile fish. Environ. Sci. Poll. Res. 23, 5179–5188. https://doi.org/10.1007/s11356-015-5732-8.

Muth-Köhne, E., Sonnack, L., Schlich, K., Hischen, F., Baumgartner, W., Hund-Rinke, K., Schäfers, C., Fenske, M., 2013. The toxicity of silver nanoparticles to zebrafish embryos increases through sewage treatment processes. Ecotoxicology 22, 1264–1277. https://doi.org/10.1007/s10646-013-1114-5.

Navarro, E., Piccapietra, F., Wagner, B., Marconi, F., Kaegi, R., Odzak, N., Sigg, L., Behra, R., 2008. Toxicity of silver nanoparticles to *Chlamydomonas reinhardtii*. Environ. Sci. Technol. 42, 8959–8964. https://doi.org/10.1021/es801785m.

OECD, 2000. Test No. 215: Fish, Juvenile Growth Test. OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing Paris https://doi.org/10.1787/9789264070202-en.

OECD, 2001. Test No. 303: Simulation Test - Aerobic Sewage Treatment – A: Activated Sludge Units; 303B: Biofilms, OECD Guidelines for the Testing of Chemicals, Section 3. OECD Publishing, Paris https://doi.org/10.1787/9789264070424-en.

OECD, 2012. Test No. 211: *Daphnia magna* Reproduction Test, OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, Paris https://doi.org/10.1787/9789264185203-en.

Ostaszewska, T., Śliwiński, J., Kamaszewski, M., Sysa, P., Chojnacki, M., 2018. Cytotoxicity of silver and copper nanoparticles on rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Environ. Sci. Poll. Res. 25, 908–915. https://doi.org/10.1007/s11356-017-0494-0.

Ramsden, C.S., Smith, T.J., Shaw, B.J., Handy, R.D., 2009. Dietary exposure to titanium dioxide nanoparticles in rainbow trout, (*Oncorhynchus mykiss*): no effect on growth, but subtle biochemical disturbances in the brain. Ecotoxicology 18, 939–951. https://doi.org/10.1007/s10646-009-0357-7.

Rasmussen, K., Mast, J., De Temmerman, P.-J., Verleysen, E., Waegeneers, N., Van Steen, F., Pizzolon, J.C., De Temmerman, L., Van Doren, E., Jensen, K.A., Birkedal, R., Levin, M., Nielsen, S.H., Koponen, I.K., Clausen, P.A., Kofoed-Sørensen, V., Kembouche, Y., Thieriet, N., Spalla, O., Giuot, C., Rousset, D., Witschger, O., Bau, S., Bianchi, B., Motzkus, C., Shivachev, B., Dimova, L., Nikolova, R., Nihtianova, D., Tarassov, M., Petrov, O., Bakardjieva, S., Gilliland, D., Pianella, F., Ceccone, G., Spampinato, V., Cotogno, G., Gibson, P., Gaillard, C., Mech, A., 2014. Titanium Dioxide, NM-100, NM-201, NM-102, NM-103, NM-104, NM-105: Characterisation and Physico-chemical Properties. JRC Repository: NM-series of Representative Manufactured Nanomaterials. JRC Science and Policy ReportsEuropean Commision, Joint Reseach Centre, Institute for Health and Consumer Protection. Publications Office of the European Union, Luxembourg https://doi.org/10.2788/79554 EUR 26637 EN. 210 pp.

Ravindran, A., Chandrasekaran, N., Mukherjee, A., 2012. Studies on differential behavior of silver nanoparticles towards thiol containing amino acids. Curr. Nanosci. 8, 141–149. https://doi.org/10.2174/157341312799362331.

Ribeiro, F., Gallego-Urrea, J.A., Goodhead, R.M., Van Gestel, C.A., Moger, J., Soares, A.M.V.M., Loureiro, S., 2015. Uptake and elimination kinetics of silver nanoparticles and silver nitrate by *Raphidocelis subcapitata*: the influence of silver behaviour in solution. Nanotoxicology 9 (6), 686–695. https://doi.org/10.3109/17435390.2014.963724.

Rozan, T.F., Lassman, M.E., Ridge, D.P., Luther III, G.W., 2000. Evidence for iron, copper and zinc complexation as multinuclear sulphide clusters in oxic rivers. Nature 406, 879–882. https://doi.org/10.1038/35022561.

Scown, T.M., Santos, E.M., Johnston, B.D., Gaiser, B., Baalousha, M., Mitov, S., Lead, J.R., Stone, V., Fernandes, T.F., Jepson, M., van Aerle, R., Tyler, C.R., 2010. Effects of aqueous exposure to silver nanoparticles of different sizes in rainbow trout. Toxicol. Sci. 115, 521–534. https://doi.org/10.1093/toxsci/kfq076.

Sheng, L., Wang, L., Su, M., Zhao, X., Hu, R., Yu, X., Hong, J., Liu, D., Xu, B., Zhu, Y., Wang, H., Hong, F., 2016. Mechanism of TiO_2 nanoparticle-induced neurotoxicity in zebrafish (*Danio rerio*). Environ. Toxicol. 31, 163–175. https://doi.org/10.1002/tox.22031.

Shobana, C., Rangasamy, B., Surendran, S., Selvan, R.K., Ramesh, M., 2018. Green synthesized silver nanoparticles and their impact on the antioxidant response and histology of Indian major carp *Labeo rohita*, with combined response surface methodology analysis. J. Clust. Sci. 29, 267–279. https://doi.org/10.1007/s10876-017-1328-4.

Sun, T., Mitrano, D.M., Bornhöft, N.A., Scheringer, M., Hungerbuhler, K., Nowack, B., 2017. Envisioning nano release dynamics in a changing world: using dynamic probabilistic modeling to assess future environmental emissions of engineered nanomaterials. Environ. Sci. Technol. 51, 2854–2863. https://doi.org/10.1021/acs.est.6b05702.

Thomas, T., Thomas, K., Sadrieh, N., Savage, N., Adair, P., Bronaugh, R., 2006. Research strategies for safety evaluation of nanomaterials, part VII: evaluating consumer exposure to nanoscale materials. Toxicol. Sci. 91 (1), 14–19. https://doi.org/10.1093/toxsci/kfj129.

Totaro, S., Cotogno, G., Rasmussen, K., Pianella, F., Roncaglia, M., Olsson, H., Riego Sintes, J.M., Crutzen, H.P., 2016. The JRC nanomaterials repository: a unique facility providing representative test materials for nanoEHS research. Reg. Toxicol. Pharmacol. 81, 334–340. https://doi.org/10.1016/j.yrtph.2016.08.008.

Valerio-García, R.C., Carbajal-Hernández, A.L., Martínez-Ruíz, E.B., Jarquín-Díaz, V.H., Haro-Pérez, C., Martínez-Jerónimo, F., 2017. Exposure to silver nanoparticles produces oxidative stress and affects macromolecular and metabolic biomarkers in the goodeid fish *Chapalichthys pardalis*. Sci. Tot. Environ. 583, 308–318. https://doi.org/10.1016/j.scitotenv.2017.01.070.

Vance, M.E., Kuiken, T., Vejerano, E.P., McGinnis, S.P., Hochella, M.F., Rejeski, D., Hull, M.S., 2015. Nanotechnology in the real world: redeveloping the nanomaterial consumer products inventory. Beilstein J. Nanotechnol. 6, 1769–1780. https://doi.org/10.3762/bjnano.6.181.

Wood, C.M., Hogstrand, C., Galvez, F., Munger, R.S., 1996. The physiology of waterborne silver toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*). 1. The effects of ionic Ag^+. Aquat. Toxicol. 35, 93–109. https://doi.org/10.1016/0166-445X(96)00003-3.

Wu, F., Harper, B.J., Harper, S.L., 2017. Differential dissolution and toxicity of surface functionalized silver nanoparticles in small-scale microcosms: impacts of community complexity. Environ. Sci.: Nano 4 (2), 359–372. https://doi.org/10.1039/C6EN00324A.

Wu, Y., Zhou, Q., 2012. Dose- and time-related changes in aerobic metabolism, chorionic disruption, and oxidative stress in embryonic medaka (*Oryzias latipes*): underlying mechanisms for silver nanoparticle developmental toxicity. Aquat. Toxicol. 124, 238–246. https://doi.org/10.1016/j.aquatox.2012.08.009.

Xia, B., Zhu, L., Han, Q., Sun, X., Chen, B., Qu, K., 2017. Effects of TiO_2 nanoparticles at predicted environmental relevant concentration on the marine scallop *Chlamys farreri* : an integrated biomarker approach. Environ. Toxicol. Pharmacol. 50, 128–135. https://doi.org/10.1016/j.etap.2017.01.016.

Zaqout, M.S.K., Sumizawa, T., Igisu, H., Wilson, D., Myojo, T., Ueno, S., 2012. Binding of titanium dioxide nanoparticles to lactate dehydrogenase. Environ. Health Prev. Med. 17, 341–345. https://doi.org/10.1007/s12199-011-0245-7.

Zeumer, R., Hermsen, L., Kaegi, R., Kühr, S., Knopf, B., Schlechtriem, C., 2019. Bioavailability of silver from wastewater and food borne silver nanoparticles in the rainbow trout *Oncorhynchus mykiss*. Sci. Tot. Environ. 706, 135695. https://doi.org/10.1016/j.scitotenv.2019.135695.

Zhu, X., Zhou, J., Cai, Z., 2011. TiO_2 nanoparticles in the marine environment: impact on the toxicity of tributyltin to abalone (*Haliotis diversicolor supertexta*) embryos. Environ. Sci. Technol. 45 (8), 3753–3758. https://doi.org/10.1021/es103779h.