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Short and long-term effects of nanobiomaterials in fish cell lines. Applicability of RTgill-W1

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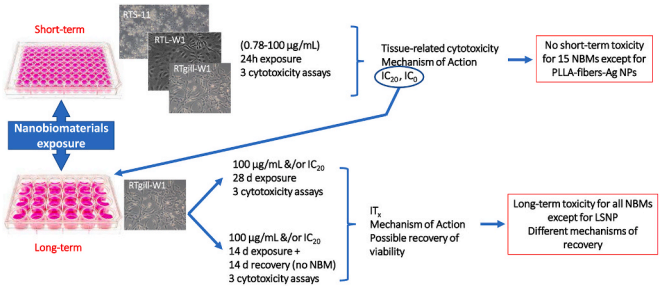
HIGHLIGHTS

- The RTgill-W1 is useful to screen short- & long-term toxicities of nanobiomaterials.
- NBMs are not acutely toxic but induced long-term effects.
- Depending on the NBM tested, different patterns of recovery were observed.
- Results indicated different mechanisms of toxic action for the fifteen NBMs.

GRAPHICAL ABSTRACT



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ABSTRACT

Nanobiomaterials (NBMs) are nanostructured materials for biomedical applications that can reach aquatic organisms. The short and long-term effects of these emerging contaminants are unknown in fish. The RTgill-W1 cell line has been proposed as a model to predict the acute toxicity of chemicals to fish (OECD Test Guideline nº 249). We assessed the applicability of this cell line to study the short and long-term toxicity of 15 NBMs based on hydroxyapatites (HA), lipid (LSNP/LNP), gold, iron oxide, carbon, poly L-Lactide acid (PLLA) fibers with Ag and poly (lactide-co-glycolide) acid. Two more rainbow trout cell lines (RTL-W1, from liver, and RTS-11, from spleen) were exposed, to identify possible sensitivity differences among cells. Exposures to a range of concentrations (0.78–100 µg/mL) lasted for 24 h. Additionally, the RTgill-W1 was used to perform long-term (28 d exposure) and recovery (14 d exposure/14 d recovery) assays. Cells were exposed to the 24 h-IC_{20} and/or to 100 µg/mL. A triple cytotoxicity assay was conducted. After 24 h, only PLLA Fibers-Ag showed cytotoxicity (IC_{50} < 100 µg/mL). However, the NBMs in general provoked concentration-dependent effects after long-term exposures, except the LSNPs. A recovery of viability was only observed for AuNPs, AuNRods, Fe_3O_4PEG-PLGA, MgHA-Collag_Scaffolds, Ti-HA and TiHA-Alg NPs.These results evidenced the need to test the long-term toxicity of NBMs and showed differences in cytotoxicity probably associated to different mechanisms of toxic action. The RTgill-W1 was useful to screen short and long-term toxicities of NBMs and appears as a promiseful model to assess possible toxicity of NBMs in fish.

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1. Introduction

Nanobiomaterials (NBMs) are nanostructured materials for biomedical applications. The interface between biomaterials and nanotechnology has created enormous opportunities for improving the prevention, diagnosis and treatment of diseases. The use of NBMs is receiving much attention because of their specific interaction with the cell membranes and their applications, which include drug delivery system with remote activation, cell therapy, gene therapy, tissue regeneration, etc. However, it should be considered that NBMs or their degradation products are potentially cytotoxic and their assessment is imperative before they are used in clinical applications (Motskin et al., 2009). Moreover, their production and use also imply the risk of release to the environment. Specially, aquatic systems are of particular relevance to nanomaterial testing, because engineered nanomaterials (ENMs, including NBMs) have been predicted to arrive in natural waters via multiple routes of release (wastewater treatment plant effluent, run-off from agricultural and urban areas, and deposition by rain) (Geitner et al., 2020).

Among the NBMs, hydroxyapatite nanoparticles (HA-NP) have excellent prospects in nanomedicine with multifunctional therapeutic approaches. Hydroxyapatite is a calcium phosphate, structurally and chemically similar to the mineral phase of human bone and teeth. Indeed, this material is a bioceramic compound that possesses the ability to activate bone regrowth and bond directly with regenerated bone, becoming an indispensable biomaterial across the medical fields (Agbeboh et al., 2020). HA-NPs have been proposed as a drug delivery vehicle due to its high biocompatibility and bioactivity, being successfully applied in bone tissue engineering and regenerative medicine, as well as in the industry of cosmetics and hygiene products (Zhao et al., 2013a; Oberbek et al., 2018). This includes the slow delivery of different substances such as growth factors, antibiotics, anticancer drugs, enzymes and antigens (Motskin et al., 2009). Solid lipid nanoparticles (SLNP) are another kind of materials that, during the last few decades, have gained attention due to their good release profile and targeted drug delivery with excellent physical stability (Jacob et al., 2022). Good deals of studies on SLNPs have been done to study the therapeutic properties of phytochemicals with anticancer properties encapsulated in SLNPs. Some of these phytochemicals showed that their encapsulation within SLNPs enhances their bioavailability and increases their therapeutic efficacy (Mahato et al., 2021). Au NPs are also commonly used for drug delivery and their excretion when they are used in nanocarrier drug delivery systems should be considered (Su et al., 2019). Regarding the reactivity of NPs, recent research has pointed out the influence of magnetic NP coating on the nanoparticle intracellular fate and biodegradation in a cell-type bias (Portilla et al., 2022).

Some ENMs like nano-TiO_2 and nano-ZnO show the most prominent flows after their release from production, manufacturing, and consumption (PMC) to wastewater (and further to wastewater treatment plants, WWTPs). This is because the major applications for these two ENM are in cosmetics. Other ENMs like nano-Ag, have the major flows from PMC to landfill and to wastewater (Sun et al., 2016). These pathways can be followed by medical products, which can be released in wastewater and end up in WWTPs.

The potential hazard of environmental pollutants in aquatic systems is normally evaluated by performing acute and chronic toxicity assays in fish. In this sense, the most common performed assay is the fish acute toxicity test as the information provided is required by different regulatory agencies as a first step to evaluate the aquatic toxicity of different substances such as chemicals (Regulation (EC)_1907/2006, 2006), biocides (Regulation (EU)_528/2012, 2012) or plant protection products (Regulation (EC)_1107/2009, 2009). In the last years, the 3R's (Refinement, Reduction and Replacement) principle and the animal welfare regulation (e.g. Directive, 2010/63/EU on the protection of animals used for scientific purposes) have been followed as much as possible and several research groups have been working on looking for and validating alternative tests to replace the in vivo tests. Specifically, to try to replace the acute toxicity test in fish, an effort has been made to validate a cytotoxicity assay in the fish cell line RTgill-W1 as a model to predict this toxicity in fish (Tanneberger et al., 2013; Natsch et al., 2018; Fischer et al., 2019). These studies conducted to the publication in 2021 of the OECD test guideline (TG) No. 249 (OECD, 2021). In general, it has been reported that cytotoxicity assays in fish cell lines are a good preliminary approach for fish acute toxicity screening of chemicals before performing assays in vivo (Tanneberger et al., 2013; Natsch et al., 2018; Fischer et al., 2019; Hernández-Moreno et al., 2022). However, there can be some limitations depending on the substance assayed, as it is also recognized in the OECD TG No. 249. These limitations could be applied to new substances such as the nanomaterials.

Furthermore, in vitro experiments are usually conducted for short exposure times, which do not reflect real-life situations with continuous release of products over a long period. The controversial results shown by most of the in vitro studies concerning a direct impact of NPs have been related to the short-term exposure approach followed in the cells experiments, suggesting a not realistic information from an environmental point of view (Geffroy et al., 2011). Information provided by long-term experiments is of special relevance in those cases where acute toxicity was not found but previous experiments denote sublethal effects in vitro or in vivo. Therefore, in vitro systems using long-term exposure protocols, following the recommendations of the in vivo guidelines (e.g. exposure time, species) can be useful as screening tools before performing long-term assays in fish.

Considering all of the above described, the aim of the present study was to evaluate the cytotoxicity of different NBMs following short- or long-term exposures and the applicability of the fish cell line RTgill-W1 to conduct these assays with NBMs. The first step was to perform short-term assays in three different cell lines of a same fish specie with the objective of obtaining a complete dataset of toxicities for different cell organs, of defining which cell lines can be representative of the effect triggered in vivo and of identifying intracellular targets of toxicity in the cells. Secondly, long-term assays were performed to identify possible long-term toxic effects as well as possible recoveries of viability following depuration of the NBMs. Also important was to assess the behaviour of the NBMs once dispersed in the exposure media in order to correlate the size of the NBM with a specific effect.

2. Materials and methods

2.1. Chemicals and nanomaterials

The 15 different nanobiomaterials (NBMs) used are described in Table 1.

Ethanol was purchased from Panreac (Barcelona, Spain) and bovine serum albumin (BSA) from Merck KGaA (Darmstadt, Germany). Fetal bovine serum (FBS), penicillin and streptomycin (P/S) (10,000 U/ml/10 mg/mL) were purchased from Lonza (Barcelona, Spain). The cell culture Leibovitz's L-15 medium (ref.: 11,415–049) was purchased from Thermo Fisher Scientific Inc. (Madrid, Spain). AlamarBlue (AB) reagent was purchased from Invitrogen (ThermoFisher Scientific; Madrid, Spain). Neutral red (NR) solution (0.33%), 5-carboxyfluorescein diacetate-acetoxymethyl ester (CFDA-AM) and glacial acetic acid were from Sigma-Aldrich (Madrid, Spain).

Table 1
Nanobiomaterials used to perform the in vitro assays.

NBM		Brief description	Supplied as	Supplier
Carbon nanoparticle (CNP)		Highly pure carbon particles that are produced by hydrothermal carbonization of saccharides followed by thermal reduction (90–200 nm). Spheroidal shape. Applications: photo-thermal and photodynamic properties if activated with near-infrared light.	Dispersion	UniTorino (Italy)
Fe_3O_4 PEG-PLGA NP		Magnetite NPs coated with Polyethyleneglycol-Poly (lactide-co-glycolide) polymer (43–59 nm) carried with targeting units (Mab, peptides). Applications: contrast agent for theranostic purposes	Dispersion	Colorobbia Consulting SRL (Italy)
Gold NBMs	AuNP	Gold nanoparticles (5–20 nm) with capping agent (citrate). Water based (0.5%wt). Spheroidal. Applications: drug delivery, in vivo imaging.	Dispersion	Colorobbia Consulting SRL (Italy)
	AuNRod	Gold nanorods (800–1080 nm) coated with PEG-PLGA polymer and with a binding of targeting units on the polymeric shell (Mab, peptides …). Fiber-like shape. Application: theranostic purposes.	Dispersion	Colorobbia Consulting SRL (Italy)
	AuNM0330	Gold nanoparticles (14 nm) with capping agent (citrate). Spheroidal. Applications: drug delivery	Dispersion	Joint Research Cetre
Hydroxyapatite-based NBMs	Ca(HA)-P NP	Calcium phosphate nanoparticles (50–100 nm) prepared by means of a neutralization process between calcium hydroxyde and phosphoric acid. Rounded morphology. Applications: Bone fillers, dental implants, drug delivery systems	Powder	ISTEC-CNR (Italy)
	Fe-HA NP	Superparamagnetic carbonated iron doped hydroxyapatite nanoparticles (long axis 75–100 nm, short axis 15–20 nm). Rod-like morphology of CaP NPs with the occurrence of round-shaped NPs of maghemite (5 nm). Applications: Bone fillers, dental implants, drug delivery systems, cell therapy, gene therapy.	Powder	ISTEC-CNR (Italy)
	Ti-HA NP	Calcium phosphate nanoparticles doped with titanium isopropoxide solution (long axis 75–100 nm, short axis 15–20 nm). Rod-like morphology of calcium phosphate NPs with a pore on the surface. Applications: physical sunscreens with enhanced protection from UV radiation; drug delivery, tissue regeneration.	Powder	ISTEC-CNR (Italy)
	TiHA-Alg NP	Functional hybrid NMs. The hydroxyapatite doped with Ti^{4+} was nucleated on alginate fiber through a biomineralization process. The hybrid composite was freeze-dried obtaining a hybrid flakes. The NBM was finally milled (50–100 nm). Rounded in shape. Applications: physical sunscreens with enhanced protection from UV radiation; drug delivery, tissue regeneration.	Powder	ISTEC-CNR (Italy)
	MgHA-Collag_Scaffold	Natural polymers mineralized with Mg/CO_3-doped HA nanocrystals. Bio-hybrid bone scaffolds obtained by heterogeneous nucleation of nano-apatite onto assembling collagen fibers. Applications: 3-D biomimetic scaffold for bone, osteochondral and dental regeneration	Dispersion	Finceramica (Italy)
Lipid NBMs	LSNP-Dis	Water-based colloidal suspension of lipid surfactant nanoparticles (340 nm) in which composition is included melatonin as active pharmaceutical ingredient. Applications: drug delivery.	Dispersion	Nanovector SRL (Italy)
	LSNP-Sol	Water-based colloidal suspension of lipid surfactant nanoparticles (340 nm) in which composition is included melatonin as active pharmaceutical ingredient. This NBM contains xantham gum added as thickening agent. Applications: drug delivery.	Dispersion	Nanovector SRL (Italy)
	LNP	Liposomal excipient for eye drops, consisting in a colloidal water suspension of lipid nanoparticles obtained by emulsification process (170 nm). Applications: ophthalmology (eye drop formulation/intravitreal injection - treatment of retinitis pigmentosa)	Dispersion	Nanovector SRL (Italy)
PLLA Fibers-AgNPs		Electrospun poly L-Lactide (PLLA) micro fibres (~4 μm fibres) used as a composite incorporating Ag NPs. Applications: as antibacterial wound healing patches.	Dispersion	Electrospinning company LTD (United Kingdom)
PLGA-NPs		Poly (lactide-co-glycolide NPs (200 nm). Round morphology. Applications: drug delivery.	Dispersion	CIC biomaGUNE (Spain)

2.2. Preparation and characterization of the tested NPs

2.2.1. Preparation of the exposure medium

NBMs received as powder (Ca(HA)-P, FeHA, TiHA and TiHA-Alg) were prepared by applying the standard operation procedure (SOP) developed under EU NANOGENOTOX Joint Action (Jensen et al., 2011). According to this procedure, the powder was mixed with a solution of BSA in milliQ water at a concentration of 2.56 mg/L. The suspensions were sonicated for 15 min and 30 s in an ice-water bath using a probe sonicator (Vibra cell VCX130, Sonics & Materials Inc., Newtown, CT, USA) at 40% amplitude in continuous mode with a 6 mm microtip. This suspension was diluted directly in the cell exposure medium to obtain the highest concentration used for the assays. All the other NBMs, received as suspensions, were directly dispersed into the appropriated volume of the exposure medium, at the desired highest working concentration.

2.2.2. Dynamic light scattering (DLS) measurements

The hydrodynamic size of the particles in suspension was determined by DLS using a Zetasizer Nano-ZS (Malvern Instruments Ltd., UK). Measurements were performed in the exposure suspensions at the maximum concentrations directly after preparation and after their incubation at the exposure conditions (24 h and 7 days). Medium without particles was used as a control and to record any background signals that may arise from medium components. Before preparing the samples the instrument temperature was set to the corresponding exposure conditions temperature. Four independent measurements were taken with each measurement consisting of six runs of 20 s duration.

2.3. Cell culture and exposure

2.3.1. Short-term assays

To test possible differences in toxicities due to tissue specificities three different fish cell lines derived from rainbow trout (*Oncorhynchus mykiss*) were exposed to a range of concentrations of NBMs for 24 h. The RTL-W1 (CRL-2301, derived from liver) cell line was a generous gift from Drs. Lee and Bols, who obtained it from normal liver tissue (Lee et al., 1993). The RTgill-W1 (CRL-2523, derived from branchial arc) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The RTS-11 (derived from spleen) cells were

developed by Ganassin and Bols (1998) and kindly provided by Dr. Bols. Cells were cultured at 20 °C without CO_2 in Leibovitz's L-15 supplemented with 1% (50,000 Units/500 mL) P/S and FBS (10% for RTL-W1 and RTgill-W1, and 20% for RTS-11).

Cells (100 μL suspension) were seeded in 96 well plates, at a density of 7.5×10^4 cells per well (RTS11) or 2.5×10^4 cells per well (RTL-W1 and RTgill-W1). After 24 h, cells were exposed to all the selected substances in, at least, three individual experiments in 96-well plates in triplicate (Greiner Bio-one GmbH), at a maximum concentration of 100 mg/L of each NBM and half serial dilutions. The range of concentrations tested were 0.78–100 mg/L. A control of vehicle and a control without any treatment (negative control) were included in each plate. In addition, a positive control of sodium dodecyl sulphate (19–144 mg/L) was also included in each plate. Exposure lasted for 24 h, after which the medium was removed, and three cytotoxicity assays were performed to determine the concentration which inhibited the cell viability in 20% or 50% (IC_{20} or IC_{50}).

2.3.2. Long-term assays

The RTgill-W1 cell line was selected to develop the long-term toxicity studies because of their potential to extrapolate results from in vitro to in vivo (OECD, 2021). The study was aimed to evaluate the time needed to reduce by 20% or 50% the cell viability respect to the control (IT_{20} or IT_{50}).

Cells were exposed to a single concentration of 100 mg/L of those NBMs that did not provoque toxicity at such concentration after 24 h exposure. Whereas when an IC_{20} could be calculated, the cells were exposed to 100 mg/L and a concentration close to this IC_{20} value. The exposure was performed in 24 well plates, following the protocol described by Galbis-Martínez et al. (2018), with modifications. Briefly, cells (560 μL suspension) were seeded in 24 well-plates, at a high density (28×10^4 cells per well) and maintained at 20 °C for 24 h to obtain a confluent cell monolayer. Thereafter, cells were exposed to the respective concentration of NBM (in triplicate), including three wells as negative controls (not treated cells) per plate. At days 7, 14 and 21, cells were rinsed with PBS, trypsinized with trypsin-EDTA and ¼ of cells were sub-cultured in a new 24 well plate. They were maintained in L-15 medium without treatment for 24 h to allow them to attach to the bottom of the wells. Thereafter, medium was replaced by new medium with the corresponding NBM. Five plates were initially established for each experiment (n = 3), and cytotoxicity was evaluated after 1, 7, 14, 21 and 28 days, each time in a different plate. In order to evaluate a potential recovery of the viability, the exposure medium was replaced by fresh medium (without NBM) in some replicates after 14 days of exposure to the NBM. This day was selected because, based on the results, the IC_{50} was reached for some NBMs. Therefore, from that time, cells were maintained in separated experiments (exposure and recovery) until the end of the experiment and the recovery was checked after 7 and 14 days of exposure in medium without NBM.

2.4. Cytotoxicity assays

To monitor different endpoints of viability following 24 h exposure to NPs (short-term assays) and 1, 7, 14, 21 and 28 d (long-term assays), a fluorometric-based assay system that facilitates the simultaneous use of three assays was employed. The assays were conducted following the protocol described by Dayeh et al. (2005), with the modifications reported by Lammel et al. (2013). This system includes three different cytotoxicity assays to determine the mechanism of action by which NPs can interfere with cellular homeostasis. The cell viability on the basis of mitochondrial activity (Alamar Blue (AB)); damage to the plasma membrane (5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM)); and the accumulation of neutral red (NR) dye in the lysosomes of viable uninjured cells (neutral red uptake (NRU)). The methodology is explained in supplementary data.

2.4.1. Interference of NBMs with the cytotoxicity assays

Potential interferences of the NBMs with the different cytotoxicity assays were checked following the method reported by Connolly et al. (2015) with modifications. The methodology is explained in supplementary data.

2.5. Internalization of NBMs and cellular alterations

Intracellular accumulation and distribution of NBMs, as well as ultrastructural modifications, were analyzed by transmission electron microscopy (TEM), using ultrathin sections of cells, following the method of Lammel et al. (2013). A JEOL 1010 JEM microscope operating at an acceleration voltage of 100 kV was used. with integrated energy dispersive X-ray (EDX) spectroscopy (Oxford Inca). This study was conducted in the cells exposed for 14 days to the thirteen NBMs used in the long-term toxicity assays and at the end of the recovery period (14 days). The analyses were performed at the Centro Nacional de Microscopía Electrónica (ICTS-CNME, Madrid, Spain).

2.6. Statistical analysis

The raw data of the cytotoxicity assays were corrected subtracting the background fluorescence (cell-free control) and normalized as percentage against the control. All results are presented as mean ± standard deviation (SD) of at least three independent experiments performed in triplicate. The estimation of the concentration-response or the time-response functions and the calculation of the $IC_{20/50}$ and the $IT_{20/50}$ were done by fitting the assay results to a non-linear regression model equation for a logarithmic curve using GraphPad Prism 5. To check for a potential recovery of viability, the values of % of viability obtained in cells after 14 d exposure were used as reference values to make the comparisons with cells that continue being exposed to the NBM (21 and 28 d exposure), and also with cells exposed to medium without NBM (7 and 14 d of recovery). The statistical analysis was performed applying a one-way-ANOVA followed by a post-hoc test (Dunnett's Multiple Comparison Test). Normality and homoscedasticity of all data were checked by the Shapiro–Wilk test and Bartlett's test, respectively.

3. Results

3.1. Characterization of the NBMs in the exposure media

The hydrodynamic size of the fifteen NBMs dispersed in the specific cell exposure medium is shown in Table S1. In general, there were not differences in size related to the FBS content in the cells exposure media. However, TiHA-Alg presented smaller aggregates in presence of higher concentrations of FBS with sizes of 400–500 nm in RTS11 medium (20% FBS) and 814–1063 nm in RTgill-W1/RTL-W1 medium (10% FBS), denoting a potential effect of the serum content in the behaviour of this NBM. Another three NBMs (Fe_3O_4PEG-PLGA, AuNRods and PLGA) showed high polydispersity index (PdI>0.7) when dispersed in medium with 20% of serum. In addition, we could observed that all the NBMs presented a good stability without differences in size at the time of preparation of the dispersion and after 24 h. The stability of the NBMs was also tested after 7 days of preparation (Table S2). In general, the NBMs showed a good stability over time, and no concentration-related differences were observed. Regarding the gold NBMs, the size of the AuNPs aggregates increased with time. Interesting is the case of AuNRods, presenting higher sizes in the dispersions prepared for the long-term assays than in the preparation for the short-term assay. This fact could be explained by the lower concentrations tested for 28 d (10 and 50 μg/mL), which may lead to a higher aggregation.

3.2. Short-term cytotoxicity assays

The NBMs tested do not interfere with the cytotoxicity assays.

According to the IC_{50} values (Table 2), the NBMs were not toxic for the three fish cell lines with the exception of PLLA Fibers-AgNPs, which showed an IC_{50} of 78 μg/mL only for the fish cell line RTS11. In this fish cell line all the NBMs except Ti-HA and TiHA-Alg exerted some toxicity, presenting IC_{20} values higher than 30 μg/mL (Table 2). The 20% of effect was also observed in the RTgill-W1 exposed to the PLLA Fibers-AgNPs. Fe_3O_4PEG-PLGA and LNP also exerted a low toxicity in the other fish cell lines.

There was a different sensitivity to the NBMs exposure depending on the tested cell line, showing always a greater effect the RTS-11 cells. Regarding the endpoints assessed, there was not a general sensitive one. The metabolic endpoint (alamar blue) was mainly affected by CNP and lipid NMs (specifically LNP, where the three cell lines were affected), whereas Fe_3O_4PEG-PLGA and hydroxyapatites (Ca(HA)-P and FeHA) showed a higher effect on the cell membrane impairment (CFDA-AM). PLLA Fibers-AgNPs was the only NBM for which an IC_{50} was calculated for RTS11 at the lysosomal level.

3.3. Long-term cytotoxicity assays

Almost all the NBMs provoked a cytotoxic effect before the end of the exposure period at the concentrations tested (Table 3). Only the NBMs LSNP-Sol and LSNP-Dis confirmed their lack of toxic effect. Table 3 shows the time needed to reduce the viability by 20% or 50% at the three endpoints of toxicity studied. In general, looking at the IT_{50} most of the NBMs produced a similar toxic effect at the three endpoints of toxicity.

Cells presented IT_{50s} of 17.7 ± 5.6 d and 24 ± 0.3 d after exposure to 100 μg/mL of CNP and Fe_3O_4PEG-PLGA, respectively. Among the gold NBMs, it was proven the higher toxicity of AuNRods (50 μg/mL: 9.3 ± 1.2 d) in comparison with AuNP and AuNM0330 (100 μg/mL: 26.8 ± 2.2 d and 23.1 ± 1.3 d, respectively). Differences were found also among the HAs (100 μg/mL), both NBMs containing titanium showed a higher toxicity (Ti-HA: 14.9 ± 1 d and TiHA-Alg: 13 ± 0.5) than MgHA-Collag_Scaffold, Fe-HA and Ca(HA)-P (19.8 ± 4.2, 21.7 ± 1.6 and 25.1 ± 2.8 d, respectively). Respect to the lipid nanoparticles, 100 μg/mL of LNP were cytotoxic after 10.0 ± 2.4 d, whereas the LSNPs were not toxic at all.

3.3.1. Recovery assays

Fig. 1 shows the viability of cells during the last 14 d of the 28 d exposure period, when parallel studies were conducted to compare how the toxicity exerted after 14 d of exposure continues or recovers in presence and absence of the NBMs, respectively. In this sense, the effect

Table 2
Hazard data for fish cell lines after 24 h-exposure to different NBMs. Mean ± standard deviation (μg/mL).

NBM		Cell line	IC_{20} (μg/mL)			IC_{50} (μg/mL)		
			AB	CDFA-AM	NRU	AB	CDFA-AM	NRU
Carbon nanoparticle (CNP)		RTS-11 (n = 4)	79.1 ± 15.2	>100	>100	>100	>100	>100
		RTLW1 (n = 3)	>100	>100	>100	>100	>100	>100
		RTgill-W1 (n = 4)	>100	>100	>100	>100	>100	>100
Fe_3O_4PEG-PLGA NP		RTS-11 (n = 4)	>100	65.2 ± 16.8	>100	>100	>100	>100
		RTLW1 (n = 4)	>100	74.2 ± 7.3	>100	>100	>100	>100
		RTgill-W1 (n = 4)	>100	33.4 ± 6.4	>100	>100	>100	>100
Gold NBMs	AuNP	RTS-11 (n = 4)	56.2 ± 26.8	66.6 ± 25.0	44.6 ± 20.8	>100	>100	>100
		RTLW1 (n = 4)	>100	>100	>100	>100	>100	>100
		RTgill-W1 (n = 4)	>100	>100	>100	>100	>100	>100
	AuNRod	RTS-11 (n = 4)	32.4 ± 19.6	40.9 ± 15.5	48.2 ± 41.4	>100	>100	>100
		RTLW1 (n = 4)	>100	>100	>100	>100	>100	>100
		RTgill-W1 (n = 4)	>100	>100	>100	>100	>100	>100
	AuNM0330	RTS-11 (n = 4)	81.8 ± 16.9	81.0 ± 14.8	78.5 ± 18.6	>100	>100	>100
		RTLW1 (n = 4)	>100	>100	>100	>100	>100	>100
		RTgill-W1 (n = 4)	>100	>100	>100	>100	>100	>100
Hydroxyapatite-based NBMs	Ca(HA)-P NP	RTS-11 (n = 3)	>100	95.3 ± 1.4	>100	>100	>100	>100
		RTLW1 (n = 3)	>100	>100	>100	>100	>100	>100
		RTgill-W1 (n = 3)	>100	>100	>100	>100	>100	>100
	Ti-HA NP	RTS-11 (n = 3)	>100	>100	>100	>100	>100	>100
		RTLW1 (n = 3)	>100	>100	>100	>100	>100	>100
		RTgill-W1 (n = 3)	>100	>100	>100	>100	>100	>100
	Fe-HA NP	RTS-11 (n = 3)	>100	70.1 ± 5.3	>100	>100	>100	>100
		RTLW1 (n = 3)	>100	>100	>100	>100	>100	>100
		RTgill-W1 (n = 3)	>100	>100	>100	>100	>100	>100
	TiHA-Alg NP	RTS-11 (n = 3)	>100	>100	>100	>100	>100	>100
		RTLW1 (n = 3)	>100	>100	>100	>100	>100	>100
		RTgill-W1 (n = 3)	>100	>100	>100	>100	>100	>100
	MgHA-Collag_Scaffold	RTS-11 (n = 3)	>100	>100	87.4 ± 16.1	>100	>100	>100
		RTLW1 (n = 3)	>100	>100	>100	>100	>100	>100
		RTgill-W1 (n = 3)	>100	>100	>100	>100	>100	>100
Lipid NBMs	LSNP-Sol	RTS-11 (n = 3)	53.8 ± 8.5	70.3 ± 28.2	>100	>100	>100	>100
		RTLW1 (n = 3)	>100	>100	>100	>100	>100	>100
		RTgill-W1 (n = 3)	>100	>100	>100	>100	>100	>100
	LSNP-Dis	RTS-11 (n = 4)	37.5 ± 24.8	80.1 ± 41.4	78.6 ± 47.7	>100	>100	>100
		RTLW1 (n = 3)	>100	>100	>100	>100	>100	>100
		RTgill-W1 (n = 3)	>100	>100	>100	>100	>100	>100
	LNP	RTS-11 (n = 4)	78.8 ± 27.3	85.9 ± 51.85	99.7 ± 30.9	>100	>100	>100
		RTLW1 (n = 3)	74.2 ± 14.7	>100	>100	>100	>100	>100
		RTgill-W1 (n = 3)	32.2 ± 13.1	>100	>100	>100	>100	>100
PLLA Fibers-AgNPs		RTS-11 (n = 4)	>100	71.7 ± 15.2	65.4 ± 18.3	>100	>100	78.3 ± 16.9
		RTLW1 (n = 4)	>100	>100	>100	>100	>100	>100
		RTgill-W1 (n = 4)	>100	82.6 ± 13.1	>100	>100	>100	>100
PLGA-NPs		RTS-11 (n = 4)	83.0 ± 10.6	83.2 ± 28.3	>100	>100	>100	>100
		RTLW1 (n = 3)	>100	>100	>100	>100	>100	>100
		RTgill-W1 (n = 4)	>100	>100	>100	>100	>100	>100

Table 3
Time to reach the 20% or 50% inhibition of viability for fish cell lines after a 28 days exposure to different NBMs. Mean ± standard deviation (days).

NBM		µg/mL	IT_{20} (days)			IT_{50} (days)		
			AB	CDFA-AM	NRU	AB	CDFA-AM	NRU
Carbon nanoparticle (CNP)		60	12.7 ± 3.5	16.2 ± 6.4	16.7 ± 3.6	22.7 ± 6.2	27.1 ± 3.7	23 ± 2.4
		100	11 ± 1.1	11.4 ± 5.3	13.8 ± 5.6	18.4 ± 5.5	19.6 ± 6.8	17.7 ± 5.6
Fe_3O_4PEG-PLGA NP		20	25.3 ± 3.0	21.6 ± 3.3	>28	>28	>28	>28
		100	15.6 ± 1.8	1.6 ± 0.8	18.5 ± 1.9	24.0 ± 0.3	>28	26.8 ± 2.7
Gold NBMs	AuNP	50	22.2 ± 3.2	17.5 ± 1.4	24.1 ± 1.1	>28	>28	>28
		100	21.8 ± 3.4	13.4 ± 1.8	24.0 ± 3.0	26.8 ± 2.2	>28	>28
	AuNRod	10	23.2 ± 3.5	21.5 ± 2.7	18.6 ± 3.5	25.7 ± 2.4	26.5 ± 2.7	25.0 ± 1.4
		50	6.4 ± 0.8	10.4 ± 1.9	9.1 ± 2.8	9.3 ± 1.2	12.3 ± 1.6	10.8 ± 3.3
	AuNM0330	80	24.6 ± 8.5	>28	21.4 ± 6.1	>28	>28	27.1 ± 0.9
		100	25.1 ± 2.9	>28	18 ± 2.2	>28	>28	23.1 ± 1.3
Hydroxyapatite-based NBMs	Ca(HA)-P NP	50	>28	27.3 ± 2.2	>28	>28	>28	>28
		100	20.5 ± 1.2	20.7 ± 3.3	21.3 ± 1.1	25.1 ± 2.8	25.8 ± 3.2	27 ± 4.8
	Ti-HA NP	100	11.2 ± 2.7	11.9 ± 1.1	12 ± 2.3	16.5 ± 2.3	14.9 ± 1	14.9 ± 1.4
	Fe-HA NP	50	26.9 ± 0.2	21.9 ± 1.7	25 ± 3.2	27.5 ± 0.4	25.2 ± 3.2	26 ± 3.1
		100	21.6 ± 2.7	18.1 ± 3.4	21.3 ± 0.9	24.2 ± 2	21.7 ± 1.6	22.7 ± 1.4
	TiHA-Alg NP	100	8.4 ± 2.3	10.2 ± 0.8	10.9 ± 2.4	13.7 ± 2.1	13 ± 0.5	13.4 ± 0.8
	MgHA-Collag_Scaffold	50	21 ± 5.1	22.6 ± 6.7	19.8 ± 3.9	24.1 ± 6.2	26.9 ± 8.3	23.5 ± 5
		100	15.7 ± 4.3	15.1 ± 8.6	12.1 ± 4.1	20.9 ± 5.5	>28	19.8 ± 4.2
Lipid NBMs	LSNP-Sol	100	>28	>28	>28	>28	>28	>28
	LSNP-Dis	100	>28	>28	>28	>28	>28	>28
	LNP	20	>28	>28	19.4 ± 10.1	>28	>28	>28
		100	8.9 ± 0.6	11.4 ± 2.1	7.5 ± 0.8	10.7 ± 0.5	12.7 ± 2.2	10.0 ± 2.4

(% of viability decrease) recorded in cells exposed during 14 d to the respective NBM was used as the reference value to stablish comparisons with the % of viability obtained at times 21 and 28 d. The statistical analysis (one-way ANOVA followed by Dunnett's test) was done for cells which continued to be exposed to the NBM from day 14 to day 28 and for the cells for which the exposure to the NBM stopped at day 14. The statistical differences shown in this section relates to the graphs presented in Fig. 1. In general, after removing the NBMs from the exposure medium, a recovery of the cell viability was observed. The cellular damage provoked by the CNP was irreversible, whereas cells exposed to Fe_3O_4PEG-PLGA recovered their viability almost completely except at the lysosomal level. Regarding the gold NBMs, a quick and complete recovery of the fish cell line was attained after removing the AuNP (both concentrations). A slower but also complete recovery was observed for the lower AuNRod concentration (10 µg/mL). Finally, no recovery was possible for cells exposed to 100 µg/mL of AuNRods and to AuNM0330.

Among the HA-based NBMs, the lack of toxic effect after 14 d exposure to Ca(HA)-P did not allow proving a recovery. On the other hand, even when the effect of 100 µg/mL of Fe-HA was almost negligible after 14 d of exposure, it was strong enough as for not allowing the complete recovery of the cells when the NBM was removed. Indeed, a decrease in viability was observed after 14 d of the recovery period ($p < 0.05$ (AB and CFDA-AM) and $p < 0.01$ (NRU)). Regarding the HAs with titanium, they showed the strongest deletereous effect, with decreases in viability during the first 7 d of recovery at the three tested endpoints (significant decrease for NRU: Ti-HA, $p < 0.01$; TiHA-Alg, $p < 0.05$). However, after 14 days without NBM the toxic effect was reverted (no differences between time 14 d exposure and time 14 d recovery). These results suggested a possible greater recovery of viability if the cells would continue to be exposed to clean medium. Cells exposed to MgHA-Collag_Scaffold were not highly damaged after a 14 d exposure to the NBM, however the viability decreased equally in cells which continued to be exposed to the NBM and those exposed to the clean medium. A slight recovery was only observed at the lower concentration (50 µg/mL). Finally, regarding the cells exposed to LNP, there was an increase in viability after removing the NBM at the low concentration (no significant differences found), but the impairment provoked by 100 µg/mL was too high to be reversed.

Altogether, these results indicated that NBMs exerted their toxic effect by different mechanisms of action. Indeed, for some of them no recovery was possible at both concentrations tested (CNP, MgHA-Collag_Scaffold) or at the higher concentration (AuNRods and LNP) but for others a complete recovery was observed (AuNP, TiHA, TiHA-Alg). Finally, it was very curious to observe how the FeHA and AuNM0330 NBMs, which were not toxic after 14 days of exposure, exerted a strong toxicity in the recovery period. This could be the result of sublethal effects which mechanisms promoted a delayed toxicity.

3.3.2. *TEM images*

The internalization of most of the NBMs was confirmed by microscopy through EDX analysis, after 28 d exposure. It was possible to identify titanium, iron, calcium (Fig. S1) and phosphate after exposure to the hydroxyapatites. However, even though collagen fibres were detected in a single cell exposed to MgHA-Collag_Scaff, Mg was not identified. For these NBMs, the bad quality of the cells did not allow confirming the presence of metals in cells after the recovery period. Gold (Fig. S1) was identified in cells exposed to AuNRods, AuNPs and AuNM0330, and also, but to a lesser extent, in cells after the recovery period.

4. Discussion

The main aim of this study was to evaluate the potential hazard of different NBMs for fish cell lines after short and long-term exposures. To reach this aim, different fish cell lines were exposed to the NBMs under study, paying special attention to the RTgill-W1 which was recently presented as a validated screening cell line to predict the acute toxicity of chemicals in fish. To our knowledge, up to date, in vivo and in vitro toxicity assays with the selected NBMs have only been performed in mammals and there is a need to evaluate their toxicity to aquatic species.

The specific properties of NMs, regarding structure, chemistry, mechanics, magnetism and biological interactions make them of special and growing interest to be used in nanomedicine as, for example, active ingredients, carriers for targeted drug delivery or excipients (Mohanraj and Chen, 2007). All the particles tested in the present study where manufactured with medical purposes. Specifically, the main application of the selected NBMs is their use as drug delivery systems with remote activation, cell therapy and gene therapy. Thus, they are expected to be internalized in the cells. They are designed to safely reach their targets and specifically release their cargo at the site of disease for enhanced therapeutic effects, thereby increasing the drug tissue bioavailability (Kumar et al., 2013). It is, therefore, important to obtain NBMs that demonstrate their stability over time. Altogether our results of

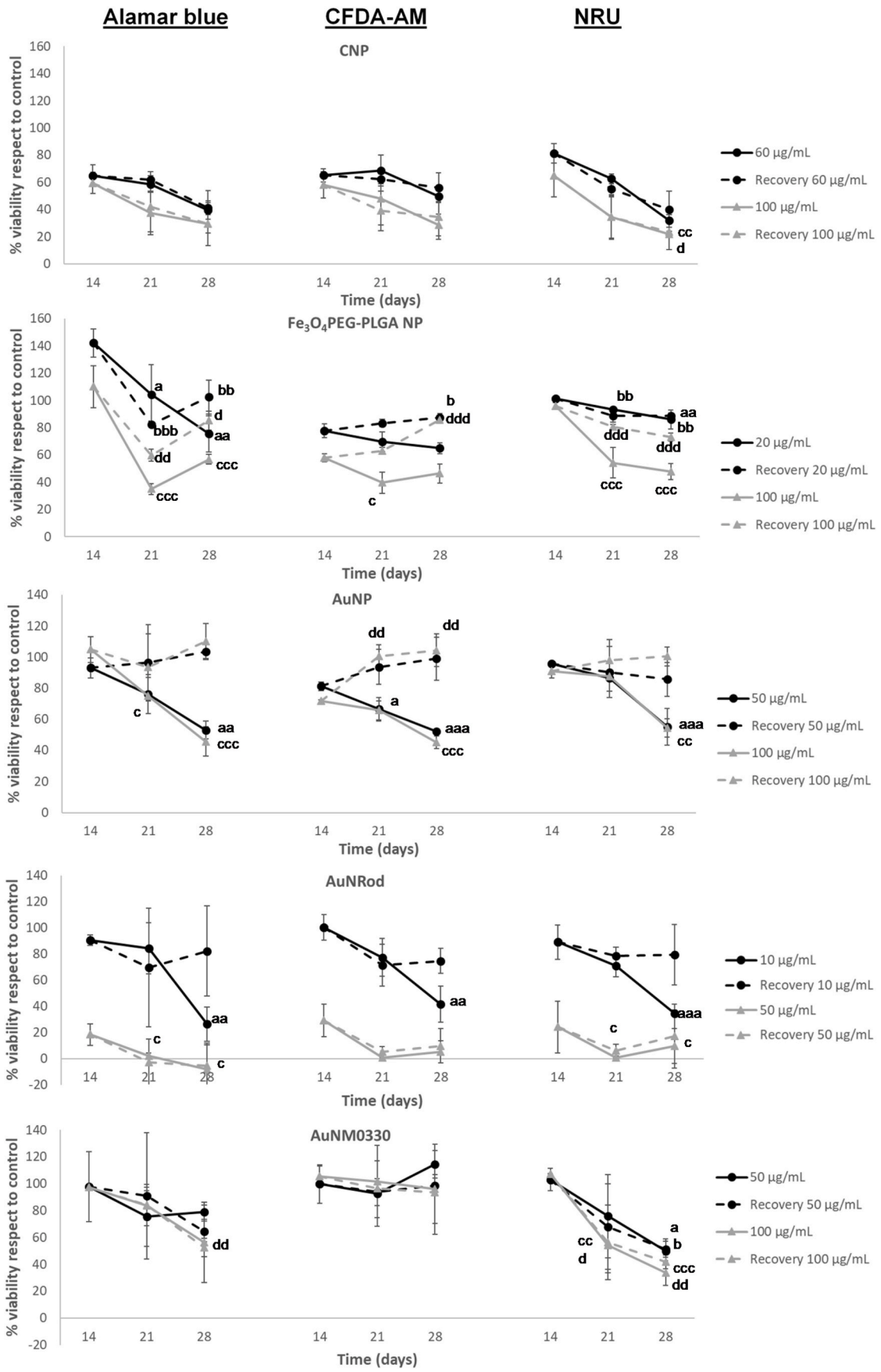

Fig. 1. Viability of cells during the last 14 d of the 28 d exposure period, showing the continuation of exposure to NBM (solid line) and the withdrawal of the NBM (dashed line). Results shown for the three endpoints tested (mitochondrial activity, cell membrane and lysosomes). Mean ± standard deviation (n = 3)). Letters

denote significant differences at time 21 d or 28 d respect to time 14 d. Letters "a" and "b" relate to the distinct low treatment and recovery, while "c" and "d" relate to the high treatment and recovery, respectively. Single letters: $p < 0.05$; double letters: $p < 0.01$; triple letters: $p < 0.001$.

Fig. 1 (cont.): Viability of cells during the last 14 d of the 28 d exposure period, showing the continuation of exposure to NBM (solid line) and the withdrawal of the NBM (dashed line). Results shown for the three endpoints tested (mitochondrial activity, cell membrane and lysosomes). Mean ± standard deviation ($n = 3$). Letters denote significant differences at time 21 d or 28 d respect to time 14 d. Letters "a" and "b" relate to the distinct low treatment and recovery, while "c" and "d" relate to the high treatment and recovery, respectively. Single letters: $p < 0.05$; double letters: $p < 0.01$; triple letters: $p < 0.001$.

characterization, confirm the stability of the selected NBMs up to seven days after preparation. The lipid NMs have been highlighted among these delivery systems because of their high physical stability (Ridolfi et al., 2011). Not only the core NP, but the coating also influences the reactivity and stability of the particle. For instance, it has been described that AuNPs stabilized with citrate are very sensitive to the change of concentration and time assay compared to starch and gum arabic stabilized gold nanoparticles (Vijayakumar and Ganesan, 2012). This could also be observed in our results, with AuNPs (caped with citrate), which showed increased sizes over time, but not with AuNM0330, also caped with citrate, which presented a high stability.

The short-term exposure assays indicated a low risk for fish cell lines exposed to NBMs. Only the PLLA Fibers-AgNPs presented an IC_{50} of 78 μg/mL. The lack of acute toxicity of the materials tested was expected, at least desired, since they are designed for biomedical devices. The safety of the hydroxyapatites was also observed by Mondal et al. (2012), after exposure of RAW macrophage-like cells to hydroxyapatite powder (80–90 nm). These authors found that although the cells were attached to the hydroxyapatite surface no toxic effect was observed.

In this study we also studied the long-term toxicity of these materials and the possible recovery of the effects. We found that all the NBMs except LSNP-Sol and LSNP-Dis produced a decrease in the viability of the fish cell line. We could establish the following decreasing ranking of toxicities according to the IT_{50}: AuNRod > LNP > TiHA-Alg > Ti-HA > CNP > MgHA-Collag_Scaffold > Fe-HA > AuNM0330 > Fe_3O_4PEG-PLGA > Ca(HA)-P > AuNP > LSNP(-Sol and -Dis).

The relevance of the long-term exposure assays developed in vitro with fish cell lines, and specifically with RTgill-W1, resides on the proven correlations that exist between the in vitro and in vivo hazard data. Several approaches were developed to try to establish correlations, using different families of chemicals, between the results obtained in fish cell lines and in fish (Fisher et al., 2011; Tanneberger et al., 2013; Natsch et al., 2018; Hernández-Moreno et al., 2022). Some of these results derived in the publication of the OECD TG 249 already mentioned. The OECD test guideline No. 215 fish juvenile growth test is designed to study any effects on growth after a 28 day-exposure to a chemical. The decrease of cellular viability related to different endpoints of toxicity could also be correlated with the in vivo sublethal effects such as growth decrease, and further studies should be conducted to estimate the value of the long-term studies in fish cell lines as predictive of effects observed in this long-term test in fish. Previous studies developed long-term exposure assays in different fish cell lines to assess the toxicity of NMs (Galbis-Martínez et al., 2018; Hund-Rinke et al., 2021). Galbis-Martínez et al. (2018) selected the RTL-W1 as the target fish cell line to be exposed for 21 d to CuO NPs. In the present study we selected the RTgill-W1 as recommended by OECD TG 249 and the exposure period was selected taking into account the recommendation of 28 d for the fish juvenile growth test OECD TG 215 (OECD, 2000). Previous experiments developed with artificial effluents contaminated with the AuNPs used in the present study, showed that the RTS-11 cell line is not suitable to perform long-term assays for more than 14 d (Hund-Rinke et al., 2021).

The sublethal effects provoked after prolonged exposures by concentrations resulting in non-acute-toxicity have been reported several times. For example, a Fe_3O_4 NP showed no immobility in the daphnid *Ceriodaphnia silvestrii Daday* (EC_{50} > 100 μg/mL) after 48 h, whereas chronic exposure affected the number of eggs (7-d IC_{20}: 6.69 μg Fe/mL) and neonates (7-d IC_{20}: 3.75 μg Fe/mL) (Gebara et al., 2019). Our results (Table 3) showed a toxic effect after 21 d of exposure to both NBM containing iron (20% of effect on the cell membrane after 1.6 d for Fe_3O_4PEG-PLGA and 18.1 d for Fe-HA). To our knowledge, there are not too many in vivo assays using juvenile fish to evaluate the toxicity after acute or long-term exposure to NBMs. Some NMs have been assayed in cells and fish, as needle-(nHA-ND) and rod-shaped (nHA-RD) hydroxyapatites NPs, which toxicity was evaluated in catfish B-cells and T-cells and in vivo in zebrafish embryos (Zhao et al., 2013b). Authors did not find cytotoxicity after 24 h exposure to both NPs (10–300 μg/mL), but lower metabolic activity was observed with 30 μg/mL of nHA-ND. In the embryos, 300 μg/mL of nHA-ND provoked axial deformations in zebrafish after 120 h and 3 μg/mL of both types of nHA caused the highest hatching inhibition. The results obtained after long-term exposure to the five HA-based NBMs in the present study, could suggest, at least, a sublethal effect in fish after prolonged exposure.

The effect of AuNPs (12 and 50 nm) has been studied in the zebrafish after long-term dietary exposure (36 or 60 d) to very low doses (from 36 to 106 ng gold/fish/day). AuNP exposure resulted in various sublethal effects, with alteration at the gen level, detoxification processes, apoptosis, mitochondrial metabolism and oxidative stress (Geffroy et al., 2011). The mitochondrial dysfunctions observed in zebrafish were also observed in the present study after 26.8 d of exposure to 100 μg AuNP/mL.

Our approach is also reporting information about potential recoveries of viability that can be found once the NBM is removed, to resemble the depuration that fish can suffer when pollutants are removed from the water resources. This recovery study was of great interest as it allowed the identification of different mechanisms of toxicity depending on the NBM. Indeed, the toxic effect produced after 14 days exposure could not be reversed for some NBMs or concentrations and for those NBMs less toxic, the recoveries were different depending on the NBM. We could identify fast and slow recoveries as well as partial or complete recoveries. In general, the characterization of the mechanistic pathways followed by the NBMs once in contact with cells is of high relevance. In fact, several of these can involve cytotoxicity related with the adherence to the cell membrane, degradation and subsequent release of cytotoxic degradation products. Therefore, the use of multiple cytotoxicity assays is highly recommended, to know the specific endpoint and mechanism of action for each NBM. Moreover, the internalization of the NBM by the cells should be considered, with the potential intracellular degradation and subsequent toxic effects inside them (Ridolfi et al., 2011). In the present study, it was possible to observe the presence of some NBMs inside the organelles, even at the end of the recovery period (Fig. S1). The amount of AuNRods present, and observable, inside the cells can explain the high toxicity observed during the long-term exposure assays, with a similar result in the case of AuNM0330.

5. Conclusions

The results evidenced the need to test the long-term toxicity of NBMs and showed different mechanisms of toxic action. The most sensitive cell line was the RTS-11 although it was not appropriated as a model to test long-term toxicities. In contrast, the RTgill-W1 was useful to screen short and long-term toxicities of NBMs and appears as a promiseful model to predict the short and long-term toxicity of NBMs in fish. Although the extrapolation of these in vitro results to fish should be investigated further, they suggest that the tested NBMs could suppose a risk for fish after a long-term exposure. The sublethal effects after short-term exposures should also be assessed in fish.

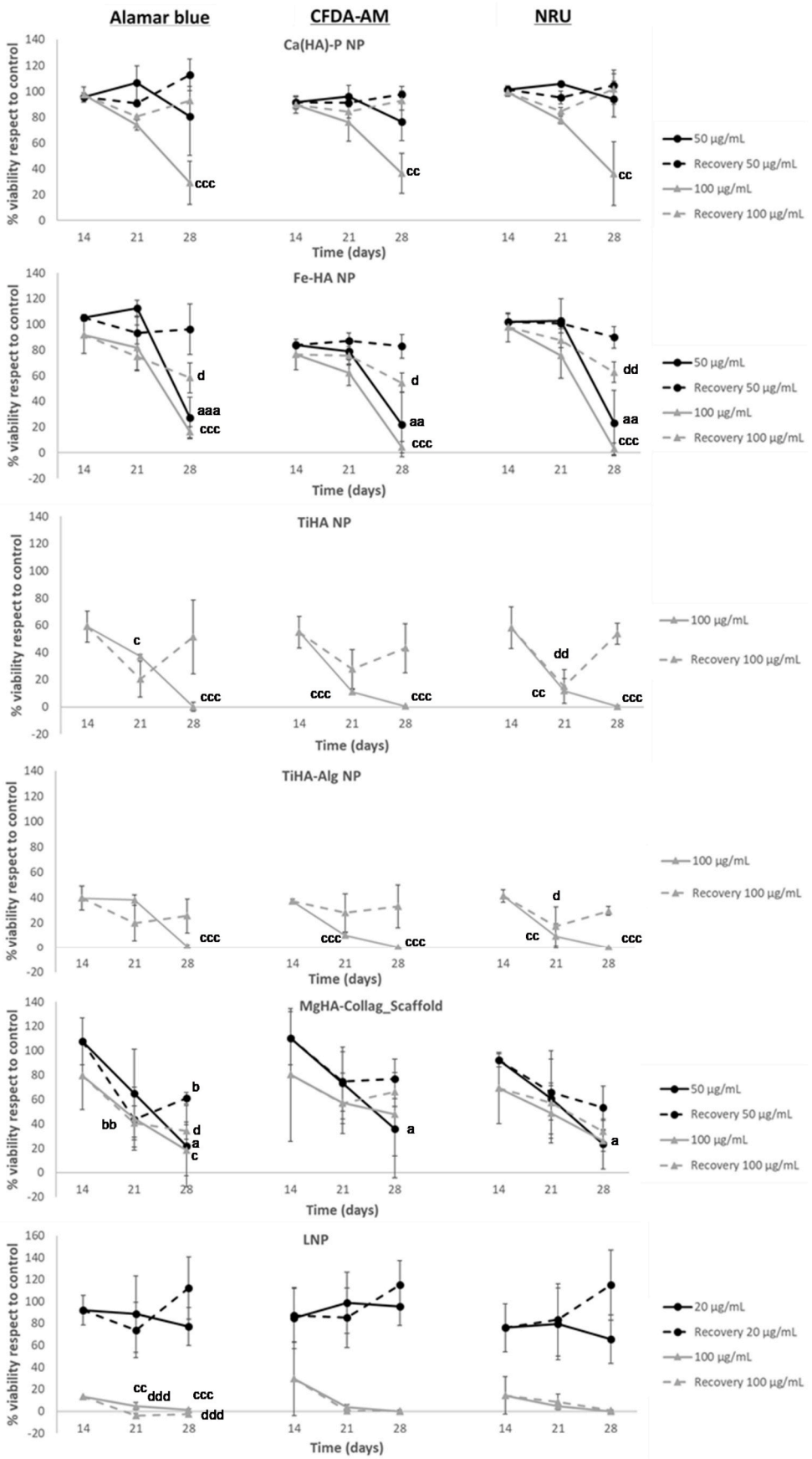

Fig. 1. (*continued*).

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.136636.

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