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## **Toxicity of Lanthanides on various fish cell lines**

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## **Abstract**

The growing use of Lanthanides in new technologies has increased their anthropogenic releases into the aquatic environment over the last decades. However, knowledge on their ecotoxicological impacts is still incomplete, especially with regard to biological effects of Lanthanides mixtures and the possible regular variation in toxicity along the Lanthanides series. The present study evaluated the individual toxicity of all Lanthanides and the toxicity of mixtures of three of them, namely Neodymium ( $\text{Nd}^{3+}$ ), Gadolinium ( $\text{Gd}^{3+}$ ), and Ytterbium ( $\text{Yb}^{3+}$ ) on *Danio rerio* fibroblast-like cells (ZF4). Individual and mixtures toxicity of Neodymium ( $\text{Nd}^{3+}$ ) and Ytterbium ( $\text{Yb}^{3+}$ ) were also assessed on *Danio rerio* hepatic cells (ZFL) and *Oncorhynchus mykiss* epithelial cells (RTgill-W1). The measured Lanthanide concentrations were close to the nominal ones in the culture media of ZF4, ZFL, and RTgill-W1 cells (85 to 99 %). A toxic impact was observed on the three fish cell lines exposed to all Lanthanides tested individually. RTgill-W1 appeared as the less sensitive cells, compared to the two others. Four Lanthanides, Erbium ( $\text{Er}^{3+}$ ), Thulium ( $\text{Tm}^{3+}$ ), Ytterbium ( $\text{Yb}^{3+}$ ) and Lutetium ( $\text{Lu}^{3+}$ ) showed a higher toxicity than the others on ZF4 cells but no correlation could be established between the toxicity of Lanthanides and the order of the elements within the Lanthanides series. Exposures to binary mixtures highlighted the presence of synergistic effects on cell viability for all cell lines.

**Keywords:** Aquatic ecotoxicology, Lanthanide, Mixture effect, Fish cell line, Cytotoxicity

## 1. Introduction

Lanthanides are a group of fifteen elements from Lanthanum ( $Z = 57$ ) to Lutetium ( $Z = 71$ ). Fourteen of them are stable and one of them, i.e. Promethium ( $Z=61$ ) is a radioactive one. These elements may be divided into three groups: light (Lanthanum to Neodymium), medium (Samarium to Gadolinium), and heavy (Terbium to Lutetium). Due to their chemical and physical properties (Yoshida et al., 1997), Lanthanides are widely used in various fields such as industry (Celik et al., 2015), medical (Cabella et al., 2006), and agriculture (Li et al., 2010). In recent decades, the growing use of Lanthanides has contributed to an increasing release of these compounds into the aquatic environment (Alonso et al., 2012; Zhang et al., 2017). It is generally accepted that surface waters are an important pathway in the transport and distribution of Lanthanides (Verplanck et al., 2010). As a result of these anthropogenic contaminations, the presence of high level Lanthanide concentrations has been detected worldwide from ng to  $\mu\text{g}$  per liter among different aquatic compartments (Bau and Dulski, 1996; Kulaksiz and Bau, 2013; Perrat et al., 2017). Up to now, except for the Netherlands, no other country has set regulatory environmental discharge thresholds of Lanthanides (Sneller et al., 2000).

The current literature on the ecotoxicity of Lanthanides is mostly confined to Lanthanum, Cerium, and Gadolinium, and studies of Lanthanide mixtures remain limited (Herrmann et al., 2016; Romero et al., 2019; Hanana et al., 2021). Until now, the question of a common toxicity profile for Lanthanides is not solved, some authors agree with this assumption (Blinova et al., 2018) while others mentioned a toxicity related to the atomic number (Manusadzianas et al., 2020). Due to the lack of information, no consensus about the Lanthanide ecotoxicity may be established. This lack of consensus emphasized the emerging need to investigate the toxicity of Lanthanides tested alone and in mixtures (Pagano et al., 2015).

Cells are one of the first levels of organization impacted by pollutants (Olabarrieta et al., 2001), and cell culturing is recognized as a sensitive tool for (eco)toxicity assessment. Numerous

studies have used *in vitro* approaches for human health risk assessment of Lanthanides, but few of them have dealt with aquatic ecotoxicological investigations (Pagano et al., 2015).

In this context, the aim of our study was to assess the cellular ecotoxicity of Lanthanides. This evaluation was performed by using three fish cell lines, i.e., ZF4 (ATCC<sup>®</sup> CRL-2050<sup>™</sup>) and ZFL (ATCC<sup>®</sup> CRL-2643<sup>™</sup>) cells from *Danio rerio* and RTgill-W1 (ATCC<sup>®</sup> CRL-2523<sup>™</sup>) cells from *Oncorhynchus mykiss*.

Firstly, the individual toxic effects of all stable Lanthanides were determined on ZF4 cells. Then, three of them, i.e., Neodymium (Nd<sup>3+</sup>), Gadolinium (Gd<sup>3+</sup>), and Ytterbium (Yb<sup>3+</sup>) were tested in mixtures on ZF4 cells and two of them i.e., Neodymium (Nd<sup>3+</sup>) and Ytterbium (Yb<sup>3+</sup>) were tested alone and in mixtures on ZFL and RT-W1 cells. Differences of toxicity between Lanthanides alone, in mixtures, and on the different cell lines are discussed.

## 2. Materials and methods

### 2.1. Chemicals

Stock solutions (5 g/L as salt concentration) were prepared from the following Lanthanide salts: LaCl<sub>3</sub>-7H<sub>2</sub>O, CeCl<sub>3</sub>-7H<sub>2</sub>O, Pr(NO<sub>3</sub>)<sub>3</sub>-6H<sub>2</sub>O, Nd(NO<sub>3</sub>)<sub>3</sub>-6H<sub>2</sub>O, Sm(NO<sub>3</sub>)<sub>3</sub>-6H<sub>2</sub>O, EuCl<sub>3</sub>-6H<sub>2</sub>O, GdCl<sub>3</sub>-6H<sub>2</sub>O, TbCl<sub>3</sub>-6H<sub>2</sub>O, Dy(NO<sub>3</sub>)<sub>3</sub>-6H<sub>2</sub>O, HoCl<sub>3</sub>-6H<sub>2</sub>O, Er(NO<sub>3</sub>)<sub>3</sub>-5H<sub>2</sub>O, Tm(NO<sub>3</sub>)<sub>3</sub>-5H<sub>2</sub>O, YbCl<sub>3</sub>-6H<sub>2</sub>O, and LuCl<sub>3</sub>-6H<sub>2</sub>O, purchased from Sigma Aldrich, St. Louis, Missouri U.S.A. The purity of all salts was about 99.9 %. Two days prior to experiments, exposure concentrations were obtained by diluting the stock solutions in ultrapure water and stored at 4 °C.

### 2.2. Cell culture

Cell culture was performed as previously reported by Parant et al. (2019). Briefly: (a) *Danio rerio* fibroblast-like cells (ZF4; ATCC<sup>®</sup> CRL-2050<sup>™</sup>) were maintained in a humidified chamber (28 °C, 5 % CO<sub>2</sub>) in DMEM: F12 medium (ATCC<sup>®</sup> 30-2006<sup>™</sup>) supplemented with 10 % (v/v)

fetal calf serum (ATCC® 30-2021™). The medium was renewed every 3 days. Cells were passed once a week at  $1 \times 10^6$  cells/flask T<sub>75</sub>; (b) *Danio rerio* hepatic cells (ZFL; ATCC® CRL-2643™) were cultured in mixed medium composed by 50 % (v/v) Leibovitz's L-15 medium (ATCC® 30-2008™), 35 % (v/v) DMEM HG (Gibco® by Life Technologie™, ref: 12100), 15 % (v/v) Ham's F12 (Gibco® by Life Technologie™, ref: 21700), 0.15 g/L (v/v) sodium bicarbonate solution (Sigma Aldrich, ref: 58761), 15 mM (v/v) HEPES solution (Sigma Aldrich, ref: H0887), 0.01 mg/mL (v/v) bovine insulin (Sigma Aldrich, ref: E4127), and 5 % (v/v) inactivated fetal calf serum (ATCC® 30-2020™). Cells were incubated at 28 °C without CO<sub>2</sub> and the medium was renewed every 3 days. Cells were passed twice a week at  $1.25 \times 10^6$  cells/flask T<sub>75</sub>; (c) *Oncorhynchus mykiss* epithelial cells (RTgill-W1; ATCC® CRL-2523™) were maintained at 15 °C without CO<sub>2</sub> in Leibovitz's L-15 medium (Gibco® by Life Technologie™, ref: 11415-064) supplemented with 10 % (v/v) fetal calf serum (Eurobio<sup>Abcys</sup>, ref: CVFSVF00-01). The medium was renewed every 3 days. Cells were passed once a week at  $2.5 \times 10^6$  cells/flask T<sub>75</sub>.

## **2.3. Toxicity assessment of Lanthanides**

### **2.3.1. Toxicity assessment of individual Lanthanides**

ZF4 cells were exposed to the fourteen stable Lanthanides (from Lanthanum to Lutetium except Promethium) at nominal concentrations of 0, 0.5, 5, 50, 500, 1000, 1500, 2000, and 3000 µM during 24, 48, 72, and 96 h, with 4 replicates per condition. For ZFL and RTgill-W1 cells, similar exposure conditions were conducted in the presence of 2 Lanthanides (Nd<sup>3+</sup> and Yb<sup>3+</sup>).

### 2.3.2. Toxicity assessment of Lanthanides mixtures

Frequently described as representative of light, medium and heavy Lanthanides, 3 Lanthanides ( $\text{Nd}^{3+}$ ,  $\text{Gd}^{3+}$ , and  $\text{Yb}^{3+}$ ) were chosen for the toxicity assessment of Lanthanides mixtures on ZF4 cells. Mixtures of  $\text{Nd}^{3+}$  and  $\text{Yb}^{3+}$  were tested on ZFL and RTgill-W1 cells. Nominal Lanthanides concentrations were selected according to EC values (i.e.,  $\text{EC}_5$ ,  $\text{EC}_{10}$ ,  $\text{EC}_{50}$ ) determined on ZF4 cells. Respectively, these concentrations were: 164, 235, and 672  $\mu\text{M}$  for  $\text{Nd}^{3+}$ ; 220, 290, and 849  $\mu\text{M}$  for  $\text{Gd}^{3+}$ ; 149, 209, and 576  $\mu\text{M}$  for  $\text{Yb}^{3+}$ . ZF4 cells were exposed to multiple binary mixtures while ZFL and RTgill-W1 cells were exposed to some of these conditions (Table S1). Experiments were performed for 96 h, repeated three times with six replicates per condition.

### 2.4. Cytotoxicity assay

According to the Mosmann procedure (Mosmann, 1983), cell viability was evaluated using the MTT reduction assay. Fish cell lines were cultured under standard conditions for 6 days prior to experiments. Sub-confluent cells were trypsinized and counted according to the trypan blue exclusion method (0.4 %) (Gibco<sup>®</sup> by Life Technologie<sup>™</sup>, ref: 15250061). ZF4, ZFL, and RTgill-W1 cells were seeded on 96-well plate at initial densities of  $2 \times 10^4$ ,  $1 \times 10^4$ , and  $3.5 \times 10^4$  cells/well in 200  $\mu\text{L}$  of culture medium, respectively. After 96 h of exposure, 20  $\mu\text{L}$  of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide) was added in each well for control and exposed cells. After 3.5 h of incubation, the exposure medium was removed, and formazan salts were dissolved in 100  $\mu\text{L}$  DMSO under agitation for 4 min. Absorbance was measured at 570 nm using a microplate spectrophotometer (Asys Expert Plus). Cell viability was calculated according to the formula: *cell viability (%) = (absorbance of exposed cells/absorbance of control) x 100*.

## **2.5. Measurement of Lanthanide concentrations**

Lanthanide concentrations were measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) at the Laboratory Mer, Molécules, Santé (MMS, EA 2160) in Nantes, France. Measurements were performed in the culture media of ZF4, ZFL, and RTgill-W1 cells. Each media was supplemented with the individual and mixture concentrations of Lanthanide reported in Table S1. All solutions were prepared both with (biotic) and without (abiotic) cells. Immediately after preparation ( $T_0$ ) and after 96 h ( $T_{96}$ ), half of the medium (representing the total concentration) was acidified to 1 % (v/v) with concentrated  $\text{HNO}_3$  65 %. The remaining medium was filtered on a 0.45  $\mu\text{m}$  nitrocellulose membrane (Millipore, ref: HAWPO4700). The filtrate obtained, representing the dissolved Lanthanides, was acidified to 1 % (v/v) with  $\text{HNO}_3$  65 % and the filter, representing the undissolved Lanthanides, was stored in a tube. All acidified solutions and the filters were stored at 4 °C prior to ICP-MS analyses.

## **2.6. Statistical methods**

### **2.6.1. Analysis of the individual toxicity of Lanthanides**

For each Lanthanide, a dose-response curve was fitted to the viability data using the cumulative distribution function (CDF) of the log-logistic probability distribution. Effective concentrations (EC) inducing 5 % ( $\text{EC}_{5}$ ), 10 % ( $\text{EC}_{10}$ ), and 50 % ( $\text{EC}_{50}$ ) effect on the cell viability with associated 95 % confidence intervals were calculated from these models.

A three-way ANOVA was performed to investigate the effects of Lanthanide, cell type, and time on toxicity, expressed as  $\text{EC}_{50}$ , after log transformation. To this end, the analysis was carried out on the complete factorial design, associated with the factors "Lanthanide" (2 levels:  $\text{Nd}^{3+}$ ,  $\text{Yb}^{3+}$ ) "Cell" (3 levels: ZF4, ZFL, RTgill-W1), and "Time" (4 levels: 24h, 48h, 72h and 96h), respectively.

Analyses were performed using R software (version 4.0.0, DRC package, R Development Core Team, 2020).

### 2.6.2. Analysis of the toxicity of Lanthanides mixtures

The toxicity of Lanthanide mixtures was analyzed using parametric statistical methods. The hypothesis of homogeneity of variances within replicate groups, associated with the different combinations of concentrations considered, was preliminarily tested using the modified Levene test (i.e., the Brown-Forsythe test for the homogeneity of variances).

A weighted log-linear regression model (WLR) was used to describe the variation of cell viability, as a function of two Lanthanide concentrations in each mixture. The model also considered an interaction term, denoted by the sign \* in Equation 1:

$$\text{Log}_{10}(\text{cell viability}) = \beta_0 + \beta_1 \text{Lanthanide 1} + \beta_2 \text{Lanthanide 2} + \beta_{12} \text{Lanthanide 1} * \text{Lanthanide 2}$$

Eq.1

The use of the WLR model is motivated, on the one hand, by its ability to better meet the requirements of parametric statistical methods (i.e., normality and variance homogeneity) and, on the other hand, to consider the multiplicative nature of the toxic effects on cell viability of the Lanthanides in the mixtures. The weight assigned to each data point was the mean cell viability of the assay (i.e., the condition considered) to which it belonged.

The testing of the regression coefficients (i.e.,  $\beta_0$ ,  $\beta_1$ ,  $\beta_2$ , and  $\beta_{12}$ ), and the subsequent construction of the fitted regression models, was carried out following a backward stepwise procedure with a significance threshold set at  $\alpha = 0.01$  for all statistical tests.

The predicted response in each mixture with the WLR model was graphically processed using the response surface methodology (RSM approach). The viability rates calculated from each fitted model were thus represented in both a 3D surface plot and a 2D contour plot. Statistical analyses were performed using R software (version 4.0.0, plot3D package, R Development Core Team, 2020).

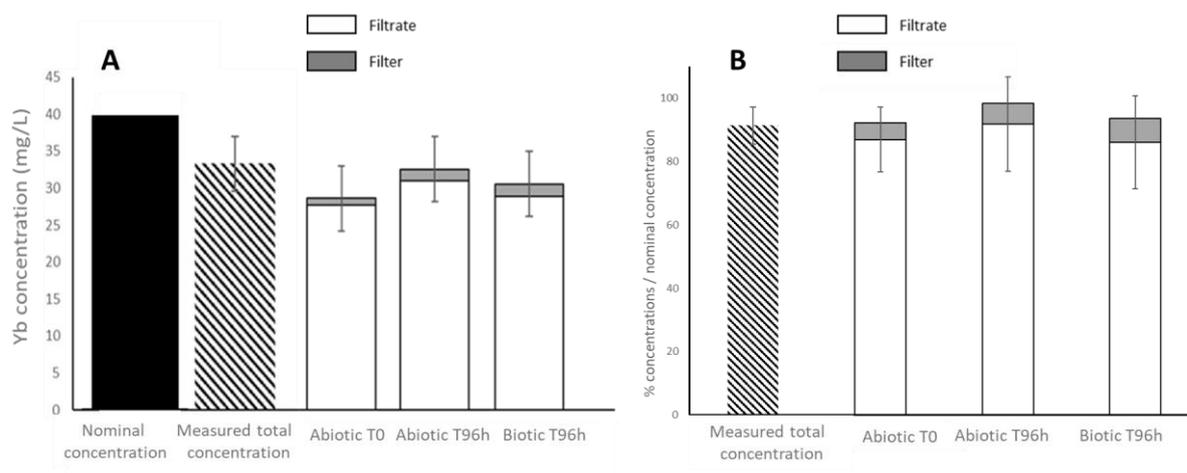
### 3. Results

#### 3.1. Lanthanide concentrations in culture media

As described in Materials and Methods (Table S1) a large panel of concentrations was measured for three Lanthanides ( $\text{Yb}^{3+}$ ,  $\text{Nd}^{3+}$  and  $\text{Gd}^{3+}$ ) after supplementation of the medium ( $T_0$ ) and after 96h ( $T_{96}$ ) of exposure. Fig. 1A shows results obtained for one concentration of  $\text{Yb}^{3+}$  on ZF4 cell medium. These results are representative of those obtained with other Lanthanides for all tested concentrations on ZF4 cells (data not shown). Similar trends were observed for the three Lanthanides in ZFL and RTgills-W1 culture media (Fig. S1).

For all tested conditions regardless of Lanthanides, Lanthanides concentration alone or in mixtures and cell lines, total concentrations measured at  $T_0$  and after  $T_{96}$  were between 85 and 99 % of the nominal ones (Fig. 1B). In the remainder of the paper, Lanthanide concentrations will therefore be expressed as the corresponding nominal values for all results. Regarding quantification in abiotic conditions, the concentration of Lanthanides measured in the unfiltered medium (i.e. “measured total concentration”) was of the same order of magnitude than the one measured in the filtrate, just after supplementation ( $T_0$ ) and after 96 h ( $T_{96\text{h}}$ ) of exposure for all cell culture media. These results suggested that most of the Lanthanides were dissolved in the culture media and that undissolved fraction was negligible (less than 5% of the total measured concentration).

The same trend was observed in the presence of cells (biotic condition) after 96 h of exposure. (Fig.1).



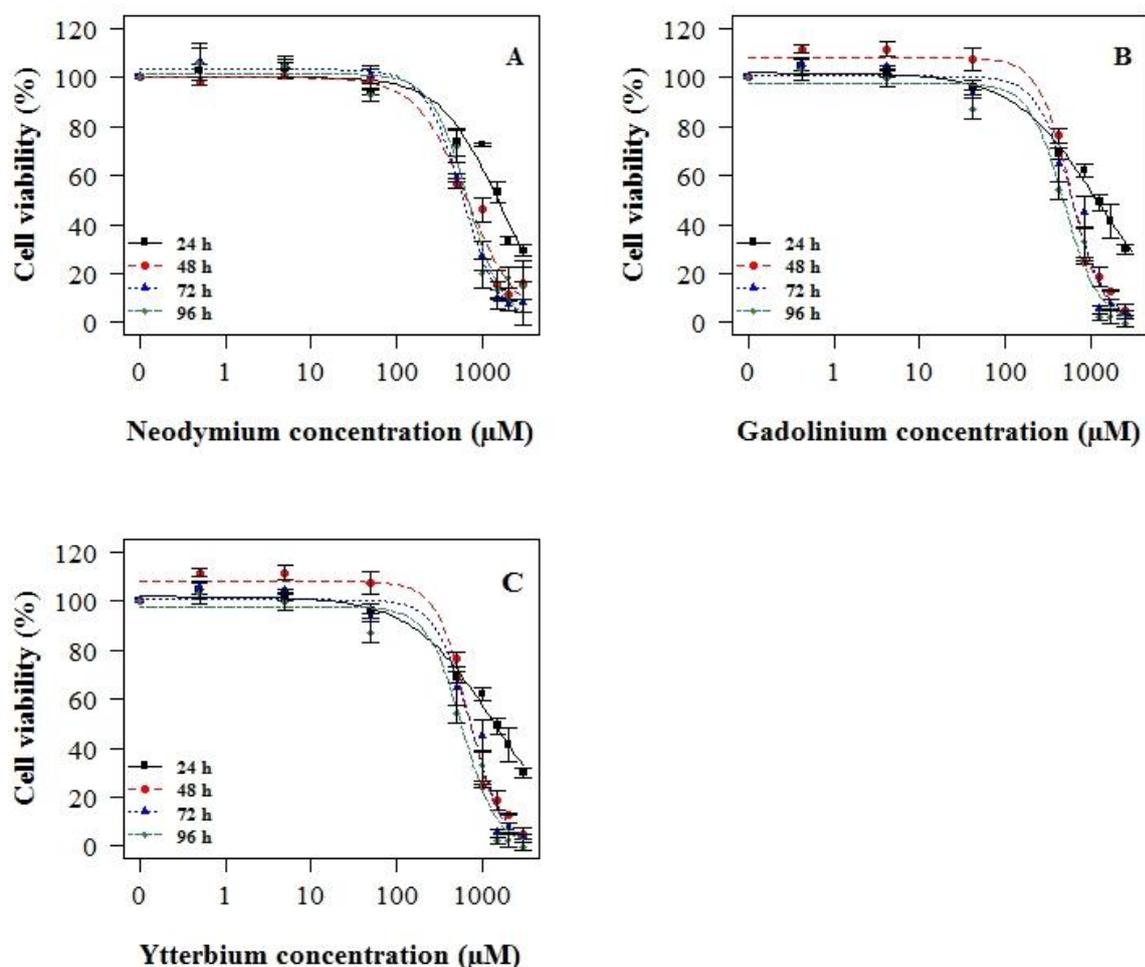
**Fig. 1.** (A) Concentration (mg/L) of Ytterbium ( $\text{Yb}^{3+}$ ) under abiotic and biotic conditions after the supplementation ( $T_0$ ) and after 96 h ( $T_{96h}$ ) of exposure in the media of ZF4 cells. (B) percentage of nominal concentration considering all tested conditions under abiotic and biotic conditions after the supplementation ( $T_0$ ) and after 96 h ( $T_{96h}$ ) of exposure in the media regardless of Lanthanides, Lanthanides concentration (alone or in mixtures) and cell lines. Data are expressed as the mean  $\pm$  one standard deviation ( $n = 3$ ) per condition.

### 3.2. Impact of Lanthanides on ZF4 cells

#### 3.2.1. Individual toxicity of 14 Lanthanides on ZF4 cells

The toxicity of the fourteen Lanthanides (from Lanthanum to Lutetium) was assessed on ZF4 cells. Dose-response curves obtained for Neodymium, Gadolinium and Ytterbium for 24 to 96 h of exposure are given in Fig. 2 (dose-response curves for the other Lanthanides are presented in Fig. S2).

ZF4 cell viability was not impacted by the lowest concentrations (from 0.5 to 50  $\mu\text{M}$ ), but decreased for concentrations ranging from 500 to 3000  $\mu\text{M}$ , and 100 % mortality was observed at 3000  $\mu\text{M}$ , from 48h of exposure.



**Fig. 2.** Dose-response curves for the viability of ZF4 cells exposed for 24 to 96 h to Neodymium (A), Gadolinium (B) and Ytterbium (C) at nominal concentrations ranging from 0.5 to 3000  $\mu\text{M}$ . Data are expressed as the mean  $\pm$  one standard deviation ( $n = 3$ ) per condition.

According to  $\text{EC}_{50}$  values, Lanthanides were significantly more toxic (1.3 – 2 times) after 48, 72, and 96 h of exposure (Table 1 and Table S2) when compared to 24 h of exposure. After 96 h of exposure,  $\text{Er}^{3+}$ ,  $\text{Tm}^{3+}$ ,  $\text{Yb}^{3+}$ , and  $\text{Lu}^{3+}$  were the most toxic elements toward ZF4 cells with  $\text{EC}_{50}$  values  $< 600 \mu\text{M}$ , whereas  $\text{Eu}^{3+}$  and  $\text{Tb}^{3+}$  presented the lowest toxicity with  $\text{EC}_{50}$  values  $> 1000 \mu\text{M}$ .

**Table 1.** Effective concentrations (with associated 95 % confidence intervals between square brackets) inducing 50 % (EC<sub>50</sub>) of the effect on ZF4 cell viability. Values were calculated from dose-response curves obtained after 96 h of exposure to fourteen Lanthanides.

Lanthanide	Time (h)	EC <sub>50</sub> [95 % CI]	
			Molarity concentration (μM)
Lanthanum	24	1633 [1430 - 1836]	1188 [1014 - 1363]
	48	1027 [916 - 1139]	994 [845 - 1143]
	72	966 [862 - 1071]	928 [799 - 1056]
	96	864 [760 - 969]	1069 [865 - 1273]
Cerium	24	1546 [1304 - 1788]	1079 [809 - 1349]
	48	907 [792 - 1023]	753 [661 - 845]
	72	835 [700 - 971]	674 [610 - 739]
	96	760 [663 - 857]	668 [593 - 744]
Praseodymium	24	1951 [1318 - 2585]	1131 [978 - 1283]
	48	800 [675 - 924]	936 [778 - 1093]
	72	883 [770 - 996]	805 [683 - 926]
	96	793 [706 - 879]	673 [547 - 799]
Neodymium	24	1504 [1287 - 1722]	731 [638 - 825]
	48	665 [557 - 773]	583 [517 - 649]
	72	583 [525 - 641]	570 [530 - 609]
	96	672 [567 - 776]	480 [449 - 512]
Samarium	24	1479 [1193 - 1766]	1360 [1181 - 1539]
	48	858 [646 - 1071]	777 [659 - 895]
	72	841 [700 - 982]	747 [635 - 860]
	96	823 [696 - 950]	501 [298 - 705]
Europium	24	1500 [1235 - 1765]	1342 [1201 - 1482]
	48	1003 [834 - 1173]	698 [638 - 758]
	72	1053 [911 - 1196]	715 [611 - 818]
	96	1047 [917 - 1178]	576 [488 - 665]
Gadolinium	24	1601 [1319 - 1882]	1145 [1001 - 1289]
	48	919 [758 - 1079]	587 [493 - 680]
	72	890 [747 - 1032]	312 [0 - 650]
	96	849 [717 - 981]	242 [79 - 404]

### 3.2.2. Toxicity of Nd<sup>3+</sup>, Gd<sup>3+</sup>, and Yb<sup>3+</sup> mixtures on ZF4 cells

ZF4 cells were exposed for 96 h to different concentrations of 3 Lanthanides (Nd<sup>3+</sup>, Gd<sup>3+</sup>, and Yb<sup>3+</sup>), tested individually and in binary mixtures. Mixture experiments were conducted using the EC<sub>5</sub>, EC<sub>10</sub>, and EC<sub>50</sub> values calculated on ZF4 cells after 96 h of exposure.

As shown in Table 2, the cell viability was reduced in the presence of Lanthanides for all tested conditions.

**Table 2.** ZF4 cell viability calculated after 96 h of exposure to Neodymium (Nd<sup>3+</sup>), Gadolinium (Gd<sup>3+</sup>), and Ytterbium (Yb<sup>3+</sup>) tested alone and in mixtures. Experiments were repeated three times with six replicates per condition. The percentage of cell viability corresponding to each condition was expressed as the mean ± one standard deviation in parentheses.

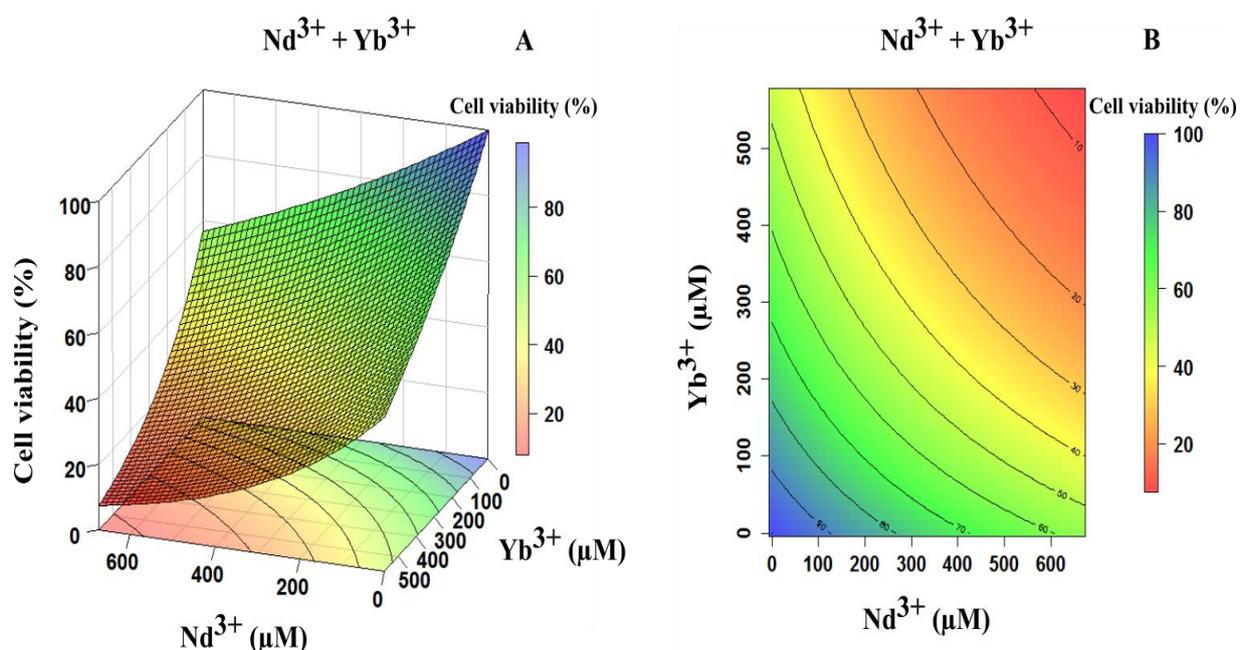
Lanthanide		Mixture Nd <sup>3+</sup> + Yb <sup>3+</sup>	
Condition	Cell viability (%)	Condition	Cell viability (%)
Control	100 (± 1)	EC <sub>5</sub> Nd <sup>3+</sup> + EC <sub>5</sub> Yb <sup>3+</sup>	69 (± 6)
EC <sub>5</sub> Nd <sup>3+</sup> ; 164 μM	93 (± 4)	EC <sub>5</sub> Nd <sup>3+</sup> + EC <sub>10</sub> Yb <sup>3+</sup>	58 (± 11)
EC <sub>5</sub> Gd <sup>3+</sup> ; 220 μM	89 (± 4)	EC <sub>5</sub> Nd <sup>3+</sup> + EC <sub>50</sub> Yb <sup>3+</sup>	30 (± 9)
EC <sub>5</sub> Yb <sup>3+</sup> ; 149 μM	81 (± 2)	EC <sub>10</sub> Nd <sup>3+</sup> + EC <sub>5</sub> Yb <sup>3+</sup>	58 (± 13)
EC <sub>10</sub> Nd <sup>3+</sup> ; 235μM	85 (± 2)	EC <sub>10</sub> Nd <sup>3+</sup> + EC <sub>10</sub> Yb <sup>3+</sup>	54 (± 7)
EC <sub>10</sub> Gd <sup>3+</sup> ; 290 μM	80 (± 4)	EC <sub>50</sub> Nd <sup>3+</sup> + EC <sub>5</sub> Yb <sup>3+</sup>	44 (± 14)
EC <sub>10</sub> Yb <sup>3+</sup> ; 209 μM	70 (± 5)	EC <sub>50</sub> Nd <sup>3+</sup> + EC <sub>50</sub> Yb <sup>3+</sup>	4 (± 3)
EC <sub>50</sub> Nd <sup>3+</sup> ; 672 μM	48 (± 11)		
EC <sub>50</sub> Gd <sup>3+</sup> ; 849 μM	51 (± 7)		
EC <sub>50</sub> Yb <sup>3+</sup> ; 576 μM	51 (± 5)		
Mixture Nd <sup>3+</sup> + Gd <sup>3+</sup>		Mixture Gd <sup>3+</sup> + Yb <sup>3+</sup>	
Condition	Cell viability (%)	Condition	Cell viability (%)
EC <sub>5</sub> Nd <sup>3+</sup> + EC <sub>5</sub> Gd <sup>3+</sup>	80 (± 4)	EC <sub>5</sub> Gd <sup>3+</sup> + EC <sub>5</sub> Yb <sup>3+</sup>	61 (± 5)

$EC_5 Nd^{3+} + EC_{10} Gd^{3+}$	75 ( $\pm$ 6)	$EC_5 Gd^{3+} + EC_{10} Yb^{3+}$	53 ( $\pm$ 8)
$EC_5 Nd^{3+} + EC_{50} Gd^{3+}$	31 ( $\pm$ 2)	$EC_5 Gd^{3+} + EC_{50} Yb^{3+}$	43 ( $\pm$ 6)
$EC_{10} Nd^{3+} + EC_5 Gd^{3+}$	76 ( $\pm$ 4)	$EC_{10} Gd^{3+} + EC_5 Yb^{3+}$	54 ( $\pm$ 7)
$EC_{10} Nd^{3+} + EC_{10} Gd^{3+}$	69 ( $\pm$ 2)	$EC_{10} Gd^{3+} + EC_{10} Yb^{3+}$	49 ( $\pm$ 4)
$EC_{50} Nd^{3+} + EC_5 Gd^{3+}$	22 ( $\pm$ 9)	$EC_{50} Gd^{3+} + EC_5 Yb^{3+}$	40 ( $\pm$ 10)
$EC_{50} Nd^{3+} + EC_{50} Gd^{3+}$	4 ( $\pm$ 4)	$EC_{50} Gd^{3+} + EC_{50} Yb^{3+}$	12 ( $\pm$ 11)

To understand this toxicity, the combined effects of Lanthanides in mixtures were studied. The assumption of homogeneity of the variance of the data within the various conditions was not rejected for any mixture examined (Levene test,  $p > 0.05$ ). Thus, a WLR analysis was performed using all available viability data. The fitted models presented in Table S3 express the variation of cell viability for each mixture studied according to the regression model in Eq.1. The values resulting from the fitting procedure (i.e., F-test,  $p < 10^{-4}$ ;  $r^2 > 0.88$ ; residual coefficient of variation  $< 15\%$ ) suggested both high explanatory power and good predictive capacity of the fitted models. Moreover, all regression coefficients in these models were statistically significant ( $p < 0.01$ ) and negative. This indicates a toxic effect of each of the two Lanthanides, as well as the existence of a synergistic effect for each of the binary mixtures considered.

The predicted combined effect of each mixture was explored using the RSM approach. Thus, the effects for the  $Nd^{3+}$  and  $Yb^{3+}$  mixture were represented on the 3D plot of the regression surface (Fig. 3A), as well as by the 2D survival level contours, i.e., the isobologram in Fig. 3B. The synergistic effect, which means that the combination of two lanthanides shows a higher toxicity than expected from the individual components, can be seen from the isobologram in Fig. 3B. Indeed, in the presence of an interaction effect, the isoboles are curved, while they would be straight lines in the absence of interaction. Furthermore, the curvature of the lines (i.e., concavity or convexity) helps to decide between antagonism and synergy, respectively. Here, the convexity of the isoboles in Fig. 3B reflects a synergistic effect. The same phenomenon was observed for the other mixtures examined in the present study (Fig. S3).

Finally, the fitted model, presented in Table S3, show that this synergistic effect is more pronounced at higher concentrations.



**Fig. 3.** (A) Three-dimensional response surface plot (3D) showing the combined effect of the Neodymium ( $\text{Nd}^{3+}$ ) and Ytterbium ( $\text{Yb}^{3+}$ ) mixtures on the viability of ZF4 cells after 96 h of exposure; (B) Two-dimensional level contour plots (2D), i.e. isobologram showing the relationship between combinations of Neodymium ( $\text{Nd}^{3+}$ ) and Ytterbium ( $\text{Yb}^{3+}$ ) concentrations to attain predetermined values of ZF4 cell viability after 96 h of exposure (print in color).

### 3.3 - Impact of Lanthanides on ZFL and RTgill-W1 cells

#### 3.3.1 - Individual Toxicity of $\text{Nd}^{3+}$ and $\text{Yb}^{3+}$ on ZFL and RTgill-W1 cells

Cells were exposed to  $\text{Nd}^{3+}$  and  $\text{Yb}^{3+}$  under the same conditions as ZF4 cells. The responses of ZFL (Fig. S4) and RTgill-W1 (Fig. S5) cells were like those observed for ZF4 cells. Indeed, results showed no effect of the lowest Lanthanide concentrations (from 0.5 to 50  $\mu\text{M}$ ) and a gradual decrease in cell viability for Lanthanide concentrations ranging from 500 to 3000  $\mu\text{M}$ .

The three-way ANOVA, performed on the data in Table 3, leads to the following conclusions:

(i) there is no statistically significant difference between the mean  $EC_{50}$  of the two Lanthanides considered ( $p>0.30$ ); (ii) there are differences between the three cell lines: specifically, the mean  $EC_{50}$  value observed for RTgill-W1 cells is significantly higher than the corresponding values of ZF4 and ZFL cells ( $p<10^{-3}$ ), which are not statistically different from each other; and (iii) the mean  $EC_{50s}$  observed for all factors decrease with time ( $p<10^{-3}$ ).

Thus, based on  $EC_{50}$  values, the toxicity of Lanthanides increased from 24 to 96 h for all three cell lines (Table 3) RTgill-W1 cells seemed to be less sensitive than ZF4 and ZFL cells, from 48h and especially after 96h of exposure. Based on  $EC_{50}$  values,  $Nd^{3+}$  was clearly more toxic for ZFL cells compared to ZF4 and RTgill-W1 cells after 96h of exposure.

**Table 3.** Effective concentrations (with associated 95 % confidence intervals in brackets) inducing 50 % (EC<sub>50</sub>) loss in cell viability in ZF4, ZFL, and RTgill-W1 cell lines exposed to Neodymium and Ytterbium. Values were calculated from dose-response curves after 24, 48, 72, and 96 h of exposure to Neodymium and Ytterbium.

Lanthanide	Time (h)	EC <sub>50</sub> [95 % CI]		
		ZF4	ZFL	RTgill-W1
Neodymium	24	1504 [1287 - 1722]	1666 [1405 - 1926]	1509 [1392 - 1625]
	48	665 [557 - 773]	616 [506 - 727]	1420 [1207 - 1633]
	72	583 [525 - 641]	566 [511 - 620]	1018 [865 - 1170]
	96	672 [567 - 776]	461 [417 - 505]	799 [697 - 901]
Ytterbium	24	1342 [1201 - 1482]	1359 [1160 - 1558]	1494 [1348 - 1640]
	48	698 [638 - 758]	952 [827 - 1078]	1558 [1343 - 1773]
	72	715 [611 - 818]	630 [529 - 732]	964 [849 - 1081]
	96	576 [488 - 665]	565 [498 - 632]	840 [774 - 906]

### 3.3.2 - Toxicity of Nd<sup>3+</sup> and Yb<sup>3+</sup> mixtures on ZFL and RTgill-W1 cells

To determine whether the toxicity induced by the Lanthanide mixtures on ZF4 cells varied among cell lines, ZFL and RTgill-W1 cells were exposed for 96 h to different Nd<sup>3+</sup> and Yb<sup>3+</sup> concentrations, tested alone and in mixtures. The tested concentrations are EC<sub>5</sub>, EC<sub>10</sub> and EC<sub>50</sub> values obtained on ZF4 cells.

As shown in Table 4, the viability of ZFL and RTgill-W1 cells was reduced in the presence of Lanthanides. For these experiments, it may be also noted that the RTgill-W1 cells were less sensitive, with higher viability of cells exposed to Lanthanides tested individually or in binary mixtures, compared to ZFL cells (two-sided Wilcoxon signed-rank test;  $p \leq 0.05$ ).

**Table 4.** ZFL and RTgill-W1 cell viability calculated after 96 h of exposure to Neodymium (Nd<sup>3+</sup>) and Ytterbium (Yb<sup>3+</sup>), tested alone and in mixtures. Experiments were repeated three times with six replicates per condition. The percentage of cell viability corresponding to each condition was expressed as the mean  $\pm$  one standard deviation in parentheses.

	Condition	Cell line	
		ZFL	RTgill-W1
Lanthanide	Control	100 ( $\pm$ 4)	100 ( $\pm$ 5)
	EC <sub>5</sub> Nd <sup>3+</sup> : 164 $\mu$ M	74 ( $\pm$ 6)	99 ( $\pm$ 10)
	EC <sub>50</sub> Nd <sup>3+</sup> : 672 $\mu$ M	48 ( $\pm$ 6)	63 ( $\pm$ 5)
	EC <sub>5</sub> Yb <sup>3+</sup> : 149 $\mu$ M	79 ( $\pm$ 13)	100 ( $\pm$ 6)
	EC <sub>50</sub> Yb <sup>3+</sup> : 576 $\mu$ M	50 ( $\pm$ 11)	72 ( $\pm$ 9)
Mixture Nd <sup>3+</sup> + Yb <sup>3+</sup>	EC <sub>5</sub> Nd <sup>3+</sup> + EC <sub>5</sub> Yb <sup>3+</sup>	70 ( $\pm$ 15)	82 ( $\pm$ 5)
	EC <sub>50</sub> Nd <sup>3+</sup> + EC <sub>50</sub> Yb <sup>3+</sup>	1 ( $\pm$ 1)	39 ( $\pm$ 9)

Furthermore, the combined effect of Nd<sup>3+</sup> and Yb<sup>3+</sup> in the mixture was assessed both on ZFL and RTgill-W1 cells by performing a WLR analysis, as previously described for ZF4 cells.

The values obtained from the fitting procedure (i.e., F-test,  $p < 10^{-4}$ ;  $r^2 > 0.88$ , residual coefficient of variation  $< 15\%$ ) indicated a good fit of the models to the experimental data, as well as high explanatory power and good predictive ability. As shown in Table S4, all regression coefficients of the fitted models were statistically significant ( $p < 0.01$ ) and negative. This result led to the same conclusion as for ZF4 cells, namely that a synergistic effect existed in addition to the direct effects of Nd<sup>3+</sup> and Yb<sup>3+</sup> on both-ZFL and RTgill-W1 cells.

As observed on ZFL (Fig. S6A) and RTgill-W1 cells (Fig. S6C), the 3D surface plots showed that for a given Nd<sup>3+</sup> concentration, the toxicity increased with the Yb<sup>3+</sup> concentration, and *vice versa*. However, the toxicity of Lanthanide mixtures was lower for RTgill-W1 cells, with cell viability ranging from 45 to 100 % (Fig. S6C) than for ZFL cells (Fig. 6A).

Furthermore, in addition to the fitted models in Table S4, which highlighted the presence of a synergistic effect, the 2D contour plots, i.e., the isobolograms in Fig. S6B and Fig. S6D, allow

visualization of this effect for both cell lines. However, the synergistic effect appears to be more pronounced for ZFL cells than for RTgill-W1 cells, as the convexity of the isoboles is more marked in Fig. S6B than in Fig. S6D, respectively

#### **4. Discussion**

In aquatic ecotoxicology, the bioavailability of Lanthanides remains an important issue to understand their toxicity and assess their environmental risk. Because Lanthanides can form precipitates in the presence of carbonates, phosphates, and sulfates in culture medium (Petersen et al., 1974; Ng et al., 2014), Ng et al. (2014) suggested that their toxicity could be linked to indirect effects caused by such precipitates, as previously demonstrated by Petersen et al. (1974). The analytical results conducted on total medium spiked with lanthanides and on the filtrates showed that more than 95% of the Lanthanides were in solution, suggesting that, in our medium, the precipitation of Lanthanides was negligible. Nevertheless, the mechanical impact of aggregates, even in small amounts, could not be excluded. Then, it would be of great interest to assess precisely the size of Lanthanides aggregates and then to evaluate the impact of inert aggregates with such size on the cell viability. As previously described by Bergna et al. (1994), silicon dioxide can be organized as aggregates with a size slightly higher than 0.45  $\mu\text{m}$  (corresponding of the filtering size of our filters) and could be used for such experiments. A preliminary experiment was conducted in this way (data not shown) and did not show any toxicity on ZF4 cells at concentrations close to the Lanthanides one. Therefore, it might be assumed that the observed toxicity on fish cell lines during our experiments was mainly induced by the direct effects of Lanthanides, even in the presence of tiny amounts of aggregates.

Concentration-dependent effects of Lanthanides were reported *in vitro* on the cell viability of human (Rucki et al., 2020), rat (Yang et al., 2012), and fish cell lines (Laville et al., 2004), but also *in vivo* using bioassays with aquatic organisms (Gonzalez et al., 2015; Romero et al., 2019;

Blinova et al., 2020). Laville et al. (2004) used the MTT assay to investigate Gadolinium toxicity on primary hepatocyte cells from *Oncorhynchus mykiss* and hepatocyte cell line (PLHC-1) from *Poeciliopsis lucida*. These authors observed a decrease in the cell viability, after 24 h of exposure to Gadolinium concentrations ranging from 7  $\mu\text{M}$  to 1 mM. Our results agree with these observations. For all exposure durations, concentrations-response curves showed Lanthanide toxicity on ZF4 cells, leading to 100 % mortality at the highest tested concentrations.

Although several adverse effects of Lanthanides have been previously reported, no consensus exists as to regular changes in their toxicity along the Lanthanides series. Some authors (Blinova et al., 2018) suggested that Lanthanides should be considered as a uniform group with all elements exhibiting similar toxicity, while others mentioned that their toxicity appears to change as a function of atomic number (Manusadzianas et al., 2020). Our results are not in agreement with Blinova et al. (2018) nor with Manusadzianas et al. (2020). Indeed, the impact observed on ZF4 cells shows a high toxicity concerning the four heaviest Lanthanides ( $\text{Er}^{3+}$ ,  $\text{Tm}^{3+}$ ,  $\text{Yb}^{3+}$ , and  $\text{Lu}^{3+}$ ) but no correlation appears by the comparison of  $\text{EC}_{50}$  of light and intermediate Lanthanides depending on their atomic number. Two of them ( $\text{Eu}^{3+}$  and  $\text{Tb}^{3+}$ ) are clearly less toxic when the others display a toxicity of the same order. These observations underlined the need to assess not only  $\text{La}^{3+}$ ,  $\text{Ce}^{3+}$ , and  $\text{Gd}^{3+}$  as frequently mentioned in the current literature, but also the other Lanthanides, especially the heavy ones, which are the most toxic but the less studied (Pagano et al., 2015; Herrmann et al., 2016).

Our results clearly show that, in our experimental conditions, all Lanthanides do not induce the same toxicity without displaying any clear relation between the toxicity and the order of the elements within the Lanthanides series. From a molecular point of view, the observed differences of toxicity between Lanthanides could be explained by their abilities to compete

differently within cells with essential metallic ions such as Calcium (Ansoborlo et al., 2006; Bresson et al., 2011).

During our experiments, 8 Lanthanides were used as chloride salts ( $\text{Yb}^{3+}$ ,  $\text{Lu}^{3+}$ ,  $\text{La}^{3+}$ ,  $\text{Ce}^{3+}$ ,  $\text{Eu}^{3+}$ ,  $\text{Gd}^{3+}$ ,  $\text{Tb}^{3+}$  and  $\text{Ho}^{3+}$ ) when 6 of them were used as nitrate salts ( $\text{Er}^{3+}$ ,  $\text{Tm}^{3+}$ ,  $\text{Pr}^{3+}$ ,  $\text{Nd}^{3+}$ ,  $\text{Sm}^{3+}$  and  $\text{Dy}^{3+}$ ). It is interesting to note that no link was observed between the salt form used and the toxicity observed. For instance, between the four Lanthanides displaying the highest toxicity ( $\text{Yb}^{3+}$ ,  $\text{Lu}^{3+}$ ,  $\text{Er}^{3+}$ ,  $\text{Tm}^{3+}$ ) two of them were used as chloride salts and the two others were used as nitrate salts.

As previously described in the literature, environmental concentrations of Lanthanides in rivers range from 1 to several ng/L with some spot area showing sometimes high concentrations close to several  $\mu\text{g/L}$  (Kulaksız and Bau, 2013, Parant et al. 2018). EC50 values measured on ZF4 cells range from 40 to 160 mg/L. The large difference between the range of these values shows that even if a cellular toxicity of Lanthanides is observed during our experiments, the risk of cellular toxicity in aquatic organisms in the current situation is low.

Mixture studies are qualified as a more realistic point of view to determine the effects of pollutants in aquatic systems (Altenburger et al., 2018). In environmental toxicology, to predict and understand the biological responses induced by the effects of mixtures (i.e., synergism, additivity or antagonism) is one of the greatest challenges, and at the same time an essential aspect of ecotoxicological risk assessment (Norwood et al., 2003). To date, few studies were carried out to assess the impacts of Lanthanide mixtures using various organisms such bacteria, algae, rotifers, daphnids, ostracods, and fish (Hao et al., 1997; Tai et al., 2010; Tan et al., 2017; Blinova et al., 2018; Romero et al., 2019; Hanana et al., 2021). In these studies, different mixture toxicities were observed, according to Lanthanide compositions. Tai et al. (2010) showed an additive effect for the mixture of fourteen Lanthanides on *Skeletetonema costatum*, while a synergistic effect was found on *Chlamydomonas reinhardtii* in the presence of  $\text{Sm}^{3+}$ ,

La<sup>3+</sup>, Ce<sup>3+</sup>, and Eu<sup>3+</sup> mixture (Tan et al., 2017). In another study on the Ce<sup>3+</sup>, Gd<sup>3+</sup>, and Lu<sup>3+</sup> mixtures, Romero et al. (2019) showed synergistic effects on bacteria and algae, and antagonistic ones on rotifers, by means on standardized bioassays. Based on these observations, it is a challenge to analyze the effects of Lanthanide mixtures at the organism level due to the diversity of studied endpoints, biological variations, stress factors, and sensitivity of the selected organisms (Liess et al., 2016). Moreover, possible invalid data interpretations may result due to a lack of statistical analysis of the interaction effects (Kerr and Meador, 1996). The combination of studies at the cellular level with statistical and modelling methods could be an interesting approach to overcome these limitations. The results of the WLR analysis and the RSM approach used in the present study showed no antagonist effects for all mixtures tested whatever the cell line used. Dose-response curves showed concentration-dependent effects of Lanthanides on cell viability, as well as synergistic effects for each of the five mixtures examined mixture, which were more pronounced at higher concentrations after 96 h of exposure.

Our results are in accordance with Jonker et al. (2005), namely synergistic effect becoming more marked at high pollutant concentrations. Although synergistic effect was observed and confirmed by statistical tests for each of the five mixtures examined in this study, we must acknowledge that, at this time, we have no mechanistic or biological explanation to offer for interpreting synergism. Further studies are therefore needed to confirm these observations and eventually clarify this phenomenon. We suggest that statistical and modelling methods could be alternative and more powerful tools to predict the toxicity of Lanthanide mixtures compared with the classical models of concentration addition (Lowe and Muischeck, 1926) and independent action (Bliss, 1939).

Three fish cell lines were used to evaluate Nd<sup>3+</sup>, Gd<sup>3+</sup>, and Yb<sup>3+</sup> toxicity. RTgill-W1 appeared as the less sensitive cells, compared to the two others, exposed on Lanthanides alone as well as

in mixture. Gills are the first tissues in contact with the external medium and possess potentially more efficient defense mechanisms against external aggressions (Perrat et al., 2017), which can make them less sensitive than other cellular models. No clear difference of sensitivity in both individual or mixture toxicity of Lanthanides could be observed between ZF4 and ZFL cells. Our results suggested that the *in vitro* approaches to assess the biological responses of living organisms exposed to abnormal Lanthanide concentrations in aquatic systems can be useful to compare the sensitivity of cells with various tissue origins.

## 5. Conclusion

Lanthanides are frequently employed in new technologies and are considered as emerging pollutants due to their growing release into the aquatic environment. Increased risks of interaction with living organisms can therefore be expected. In an innovative way, the present study is conducted from an aquatic environmental point of view by evaluating the cellular impact of Lanthanides individually and in mixtures. However, it is still uncertain whether all Lanthanides have the same toxicity, regarding few studies in the literature carried out on the toxicity of some elements. Our results highlighted that  $\text{Nd}^{3+}$ ,  $\text{Gd}^{3+}$ , and  $\text{Yb}^{3+}$  have a toxic impact on fish cell lines but could not be considered as representatives of the toxicity of the other light, medium, and heavy Lanthanides, respectively. The evaluation of the toxicity of the fourteen Lanthanides on ZF4 cells showed, on the one hand, that the heavy Lanthanides (i.e.,  $\text{Er}^{3+}$ ,  $\text{Tm}^{3+}$ ,  $\text{Yb}^{3+}$ , and  $\text{Lu}^{3+}$ ) were the most toxic and, on the other hand, that  $\text{Eu}^{3+}$  and  $\text{Tb}^{3+}$ , one medium and one light Lanthanide, were the least toxic elements. More generally, these observations did not show the existence of a relationship between increasing toxicity and atomic mass of lanthanides. Thus, our results support the need to pursue more investigations on the toxicity of all the fourteen Lanthanides to better understand their intracellular behavior (specific abilities

to bind with biomolecules and to compete with essential ions) in order to elucidate the reasons of differences in toxicity among individual elements.

The analysis of Lanthanide mixtures highlighted synergistic effects in all tests conducted with the three cell lines from two fish models. Synergistic effects became stronger at high Lanthanide concentrations confirming the need of further research on the toxic potential of their mixtures in aquatic environments. The use of *in vitro* approaches coupled with statistical and modelling methods appears to be an effective way to assess the ecotoxicological risk of Lanthanides and their mixtures.

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### **Ethics approval**

The cellular models ZF4 (ATCC® CRL-2050™) and ZFL (ATCC® CRL-2643™) cells from *Danio rerio* and RTgill-W1 (ATCC® CRL-2523™) cells from *Oncorhynchus mykiss* do not benefit from any particular protection status. No legal professional qualification is currently required for their experimental use in the laboratory.

### **CRedit authorship contribution statement**

**Emmanuel Fleurbaix:** Conceptualization, Methodology, Investigation, Resources, Validation, Formal analysis, Writing – original draft. **Marc Parant:** Conceptualization, Writing – original draft, Supervision. **Armand Maul:** Formal statistic and modelling analysis, Writing – original

draft. **Carole Cossu-Leguille:** Conceptualization, Supervision, Writing – original draft, Funding acquisition.

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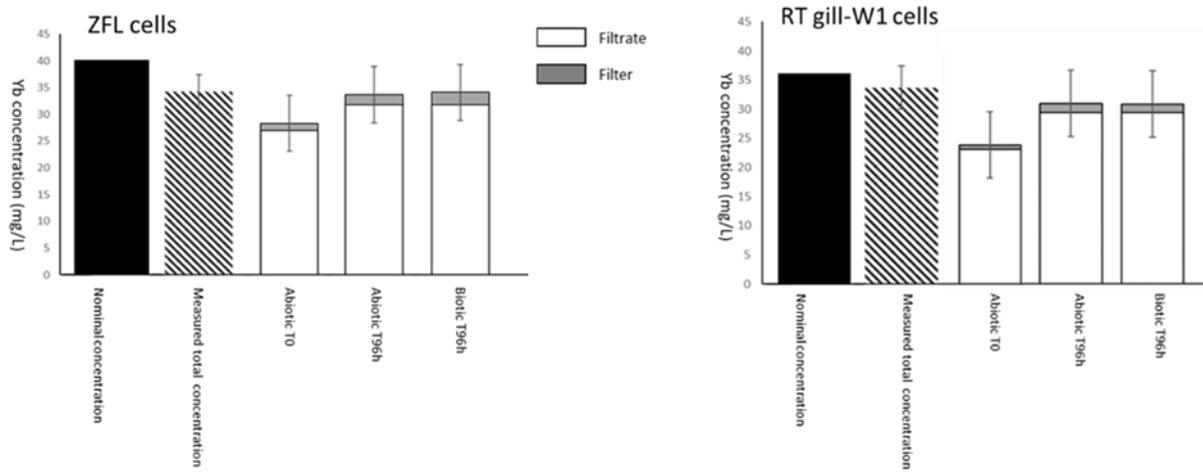
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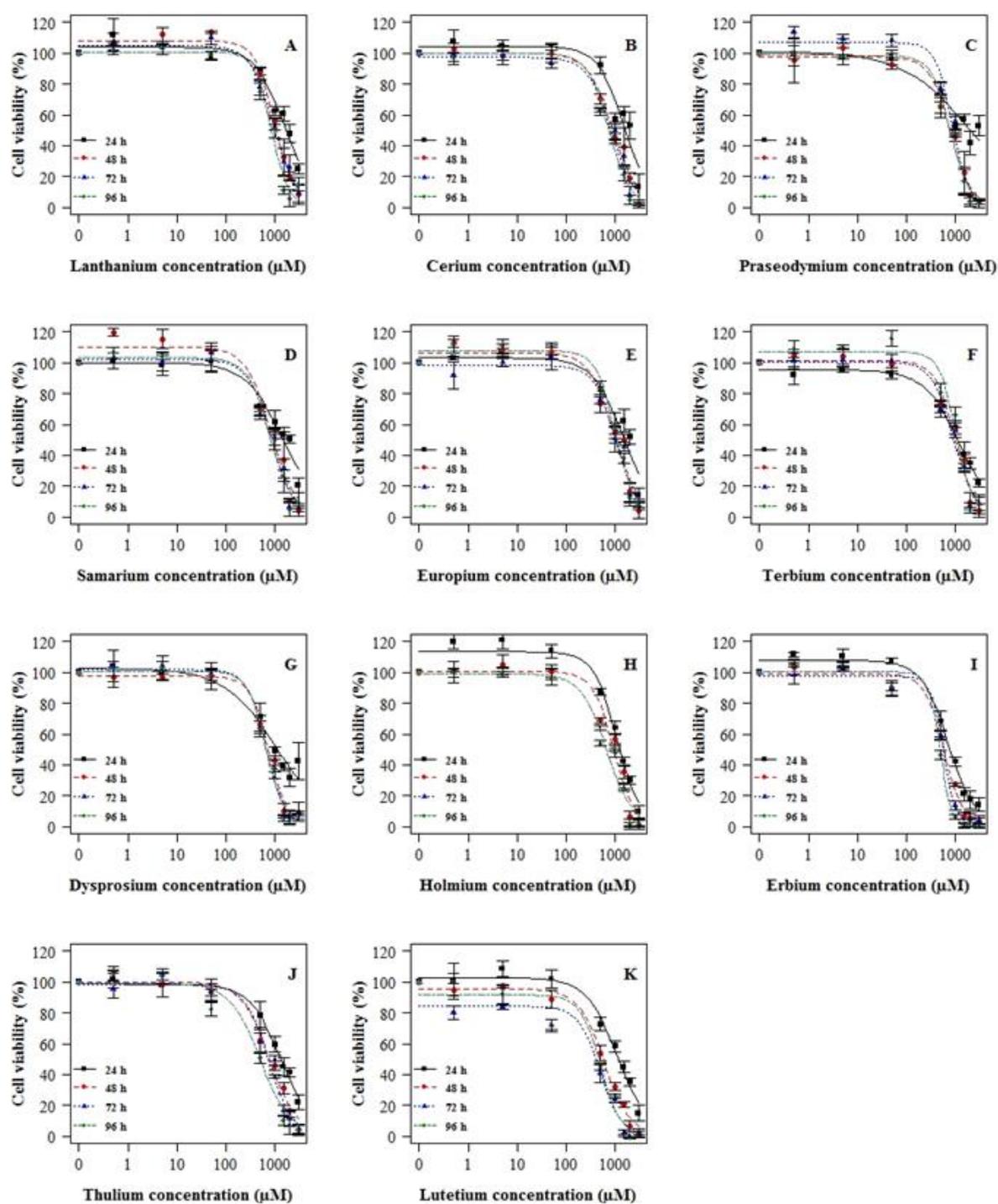
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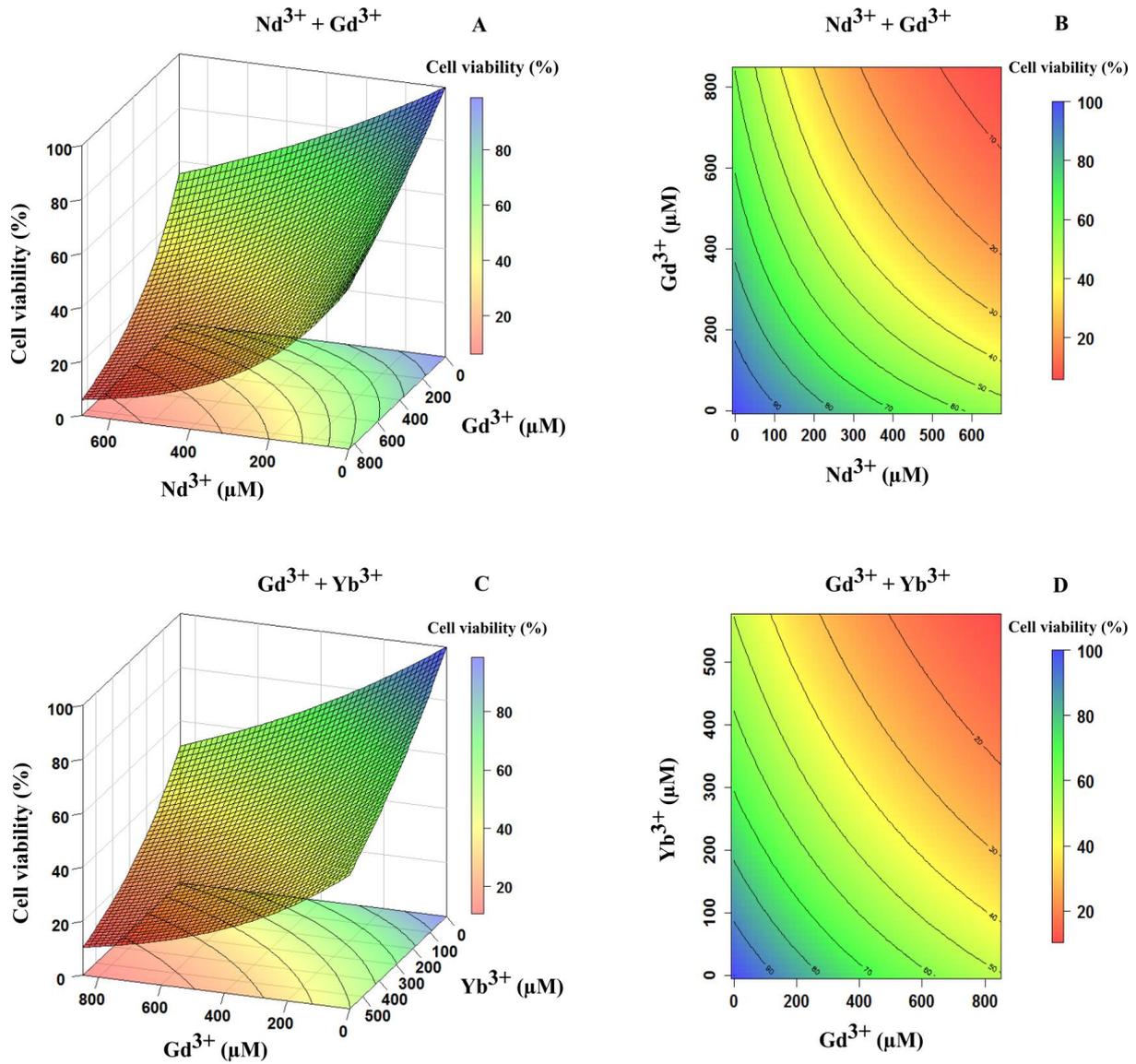
## Supplementary Data



**Fig. S1.** Concentration (mg/L) of Ytterbium ( $\text{Yb}^{3+}$ ) under abiotic and biotic conditions after the supplementation ( $T_0$ ) and after 96 h ( $T_{96h}$ ) of exposure in the media of ZFL and RTgill-W1 cells. Data are expressed as the mean  $\pm$  one standard deviation ( $n = 3$ ) per condition.



**Fig. S2.** Dose-response curves for the viability of ZF4 cells exposed for 24 to 96 h to Lanthanum (A), Cerium (B), Praseodymium (C), Samarium (D), Europium (E), Terbium (F), Dysprosium (G), Holmium (H), Erbium (I), Thulium (J) and Lutetium (K) at nominal concentrations ranging from 0.5 to 3000  $\mu\text{M}$ . Data are expressed as the mean  $\pm$  one standard deviation (n = 3) per condition.



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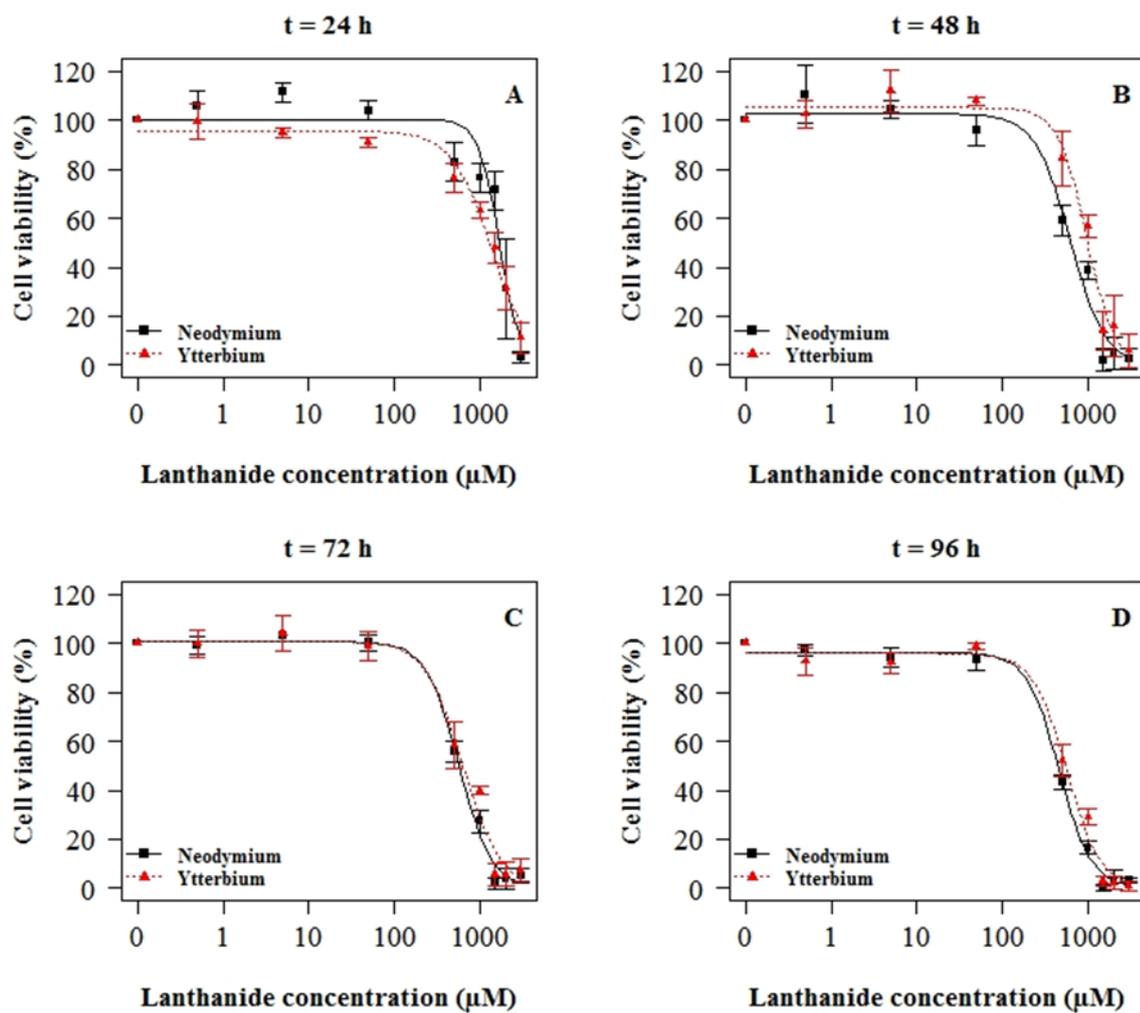
3 **Fig. S3.** Three-dimensional response surface plot (3D) showing the effect of Neodymium ( $\text{Nd}^{3+}$ ) and Gadolinium  
 4 ( $\text{Gd}^{3+}$ ) (A), Gadolinium ( $\text{Gd}^{3+}$ ) and Ytterbium ( $\text{Yb}^{3+}$ ) (C), mixture on the viability of ZF4 cells after 96 h of  
 5 exposure; Two-dimensional level contour plot (2D) showing the relationship between various combinations of  
 6 Neodymium ( $\text{Nd}^{3+}$ ) and Gadolinium ( $\text{Gd}^{3+}$ ) (B), Gadolinium ( $\text{Gd}^{3+}$ ) and Ytterbium ( $\text{Yb}^{3+}$ ) (D) concentrations to  
 7 attain predetermined values of ZF4 cell viability after 96 h of exposure (print in color).

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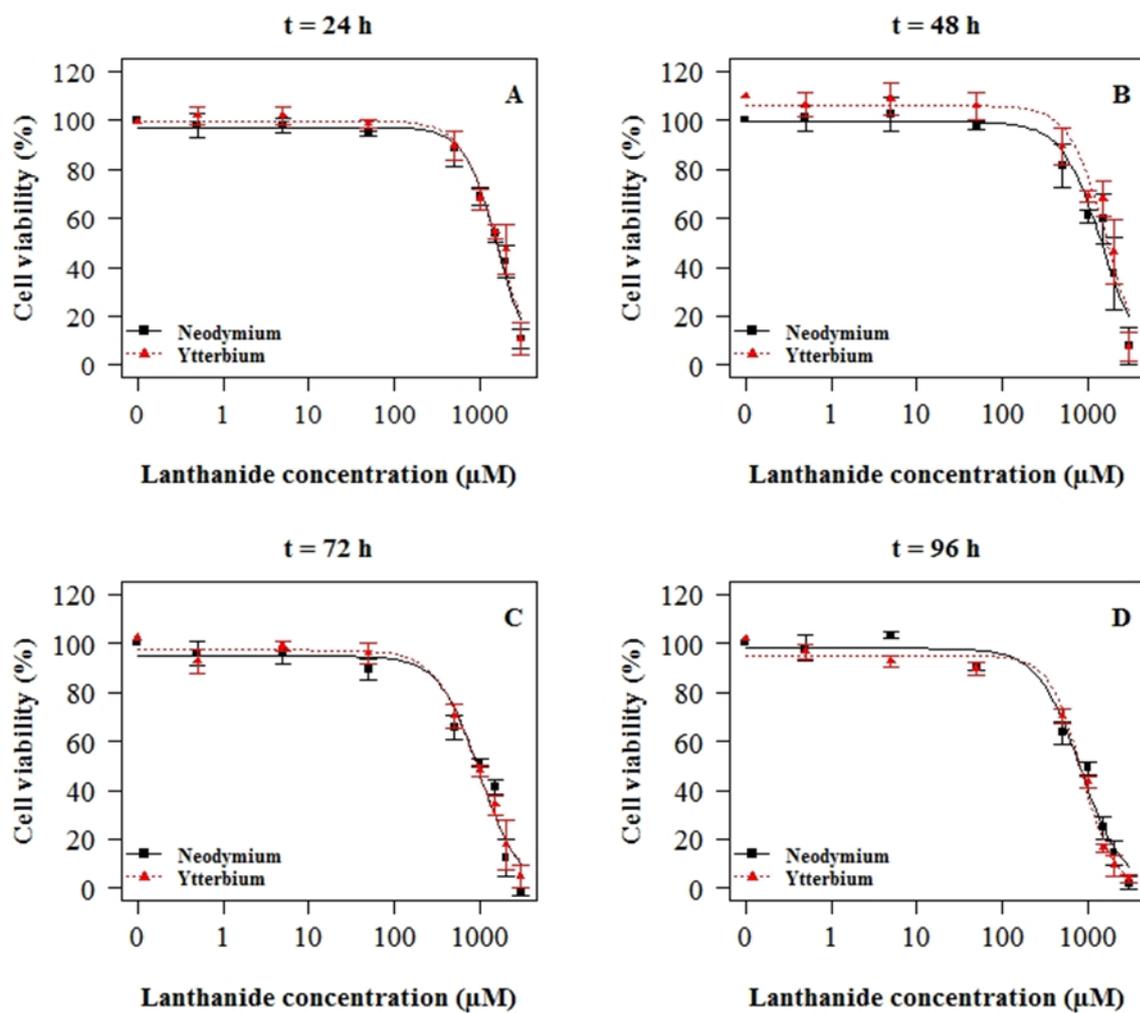
**Fig. S4.** Dose-response curves fitted on the viability data of ZFL cells exposed to Neodymium ( $\text{Nd}^{3+}$ ) and Ytterbium ( $\text{Yb}^{3+}$ ) at nominal concentrations ranging from 0.5 to 3000  $\mu\text{M}$  for 24 (A), 48 (B), 72 (C), and 96 h (D).

Data are expressed as the mean  $\pm$  the standard deviation ( $n = 4$ ) per condition.

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19 **Fig. S5.** Dose-response curves fitted on the viability data of RTgill-W1 cells exposed to Neodymium ( $\text{Nd}^{3+}$ ) and

20 Ytterbium ( $\text{Yb}^{3+}$ ) at nominal concentrations ranging from 0.5 to 3000  $\mu\text{M}$  for 24 (A), 48 (B), 72 (C), and 96 h (D).

21 Data are expressed as the mean  $\pm$  the standard deviation ( $n = 4$ ) per condition.

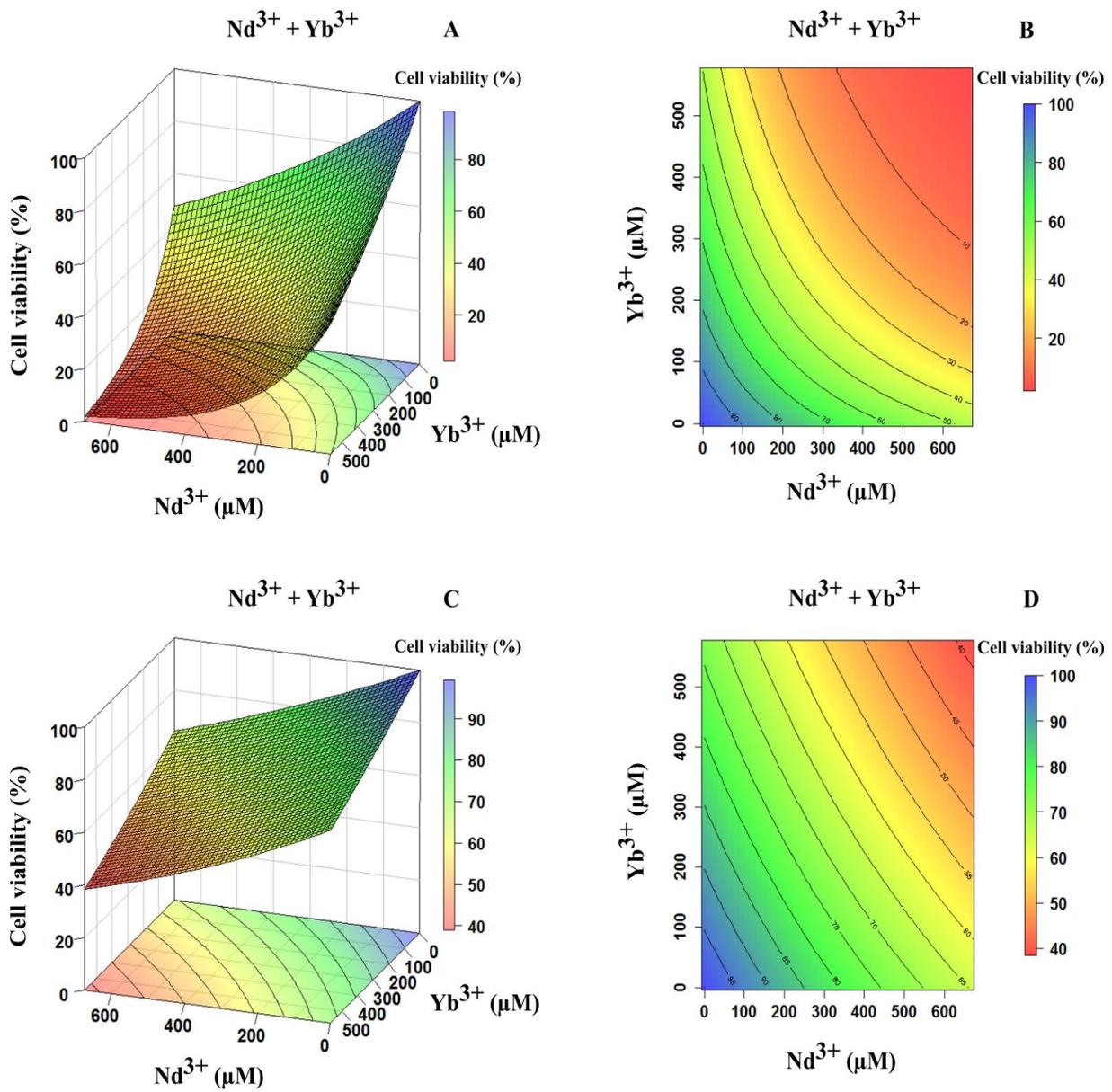
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29 **Fig. S6.** Three-dimensional response surface plots (3D) showing the combined effect of Neodymium ( $\text{Nd}^{3+}$ ) and

30 Ytterbium ( $\text{Yb}^{3+}$ ) mixtures on the viability of ZFL (A) and RTgill-W1 (C) cells after 96 h of exposure. Two-

31 dimensional contour plots (2D) i.e. isobolograms showing the relationship between combinations of Neodymium

32 ( $\text{Nd}^{3+}$ ) and Ytterbium ( $\text{Yb}^{3+}$ ) concentrations to attain predetermined values of ZFL (B) and RTgill-W1 (D) cell

33 viability after 96 h of exposure (print in color).

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39 **Table S1.** Nominal Lanthanide concentrations tested alone and in mixtures on ZF4 cells. Bold text represents the  
 40 conditions tested on ZFL and RTgill-W1 cells.

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Individual exposures			
	Nd <sup>3+</sup>	Gd <sup>3+</sup>	Yb <sup>3+</sup>
EC <sub>5</sub> :	<b>164 μM</b>	220 μM	<b>149 μM</b>
EC <sub>10</sub> :	235 μM	290 μM	209 μM
EC <sub>50</sub> :	<b>672 μM</b>	849 μM	<b>576 μM</b>
Mixture exposures			
	Nd <sup>3+</sup> + Gd <sup>3+</sup>	Nd <sup>3+</sup> + Yb <sup>3+</sup>	Gd <sup>3+</sup> + Yb <sup>3+</sup>
	EC <sub>5</sub> Nd <sup>3+</sup> + EC <sub>5</sub> Gd <sup>3+</sup>	<b>EC<sub>5</sub> Nd<sup>3+</sup> + EC<sub>5</sub> Yb<sup>3+</sup></b>	EC <sub>5</sub> Gd <sup>3+</sup> + EC <sub>5</sub> Yb <sup>3+</sup>
	EC <sub>5</sub> Nd <sup>3+</sup> + EC <sub>10</sub> Gd <sup>3+</sup>	EC <sub>5</sub> Nd <sup>3+</sup> + EC <sub>10</sub> Yb <sup>3+</sup>	EC <sub>5</sub> Gd <sup>3+</sup> + EC <sub>10</sub> Yb <sup>3+</sup>
	EC <sub>5</sub> Nd <sup>3+</sup> + EC <sub>50</sub> Gd <sup>3+</sup>	EC <sub>5</sub> Nd <sup>3+</sup> + EC <sub>50</sub> Yb <sup>3+</sup>	EC <sub>5</sub> Gd <sup>3+</sup> + EC <sub>50</sub> Yb <sup>3+</sup>
	EC <sub>10</sub> Nd <sup>3+</sup> + EC <sub>5</sub> Gd <sup>3+</sup>	EC <sub>10</sub> Nd <sup>3+</sup> + EC <sub>5</sub> Yb <sup>3+</sup>	EC <sub>10</sub> Gd <sup>3+</sup> + EC <sub>5</sub> Yb <sup>3+</sup>
	EC <sub>10</sub> Nd <sup>3+</sup> + EC <sub>10</sub> Gd <sup>3+</sup>	EC <sub>10</sub> Nd <sup>3+</sup> + EC <sub>10</sub> Yb <sup>3+</sup>	EC <sub>10</sub> Gd <sup>3+</sup> + EC <sub>10</sub> Yb <sup>3+</sup>
	EC <sub>50</sub> Nd <sup>3+</sup> + EC <sub>5</sub> Gd <sup>3+</sup>	EC <sub>50</sub> Nd <sup>3+</sup> + EC <sub>5</sub> Yb <sup>3+</sup>	EC <sub>50</sub> Gd <sup>3+</sup> + EC <sub>5</sub> Yb <sup>3+</sup>
	EC <sub>50</sub> Nd <sup>3+</sup> + EC <sub>50</sub> Gd <sup>3+</sup>	<b>EC<sub>50</sub> Nd<sup>3+</sup> + EC<sub>50</sub> Yb<sup>3+</sup></b>	EC <sub>50</sub> Gd <sup>3+</sup> + EC <sub>50</sub> Yb <sup>3+</sup>

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46 **Table S2.** Effective concentrations inducing 5 % (EC<sub>5</sub>) and 10 % (EC<sub>10</sub>) effect on ZF4 cell viability with associated  
 47 95 % confidence intervals between square brackets. Values are calculated from dose-response curves fitted  
 48 obtained for 24, 48, 72, and 96 h of exposure to fourteen Lanthanides.

Lanthanide	Time (h)	EC <sub>5</sub> [95 % CI]	EC <sub>10</sub> [95 % CI]
		Molarity concentration (μM)	
Lanthanum	24	213 [103 - 323]	357 [215 - 499]
	48	263 [172 - 354]	371 [269 - 474]
	72	190 [123 - 258]	288 [206 - 369]
	96	261 [162 - 359]	353 [245 - 461]
Cerium	24	263 [111 - 415]	413 [277 - 598]
	48	162 [95 - 229]	251 [167 - 334]
	72	154 [74 - 235]	237 [137 - 337]
	96	167 [104 - 230]	245 [170 - 320]
Praseodymium	24	22 [0 - 56]	69 [0 - 148]
	48	182 [95 - 269]	265 [163 - 367]
	72	279 [175 - 384]	374 [260 - 488]
	96	206 [141 - 271]	290 [215 - 364]
Neodymium	24	151 [44 - 259]	271 [123 - 419]
	48	89 [39 - 138]	148 [82 - 214]
	72	137 [93 - 181]	197 [146 - 249]
	96	164 [65 - 264]	235 [123 - 347]
Samarium	24	111 [13 - 209]	214 [69 - 359]
	48	154 [31 - 277]	238 [85 - 392]
	72	167 [80 - 255]	252 [146 - 358]
	96	175 [90 - 259]	259 [158 - 360]
Europium	24	182 [52 - 312]	310 [138 - 483]
	48	183 [80 - 286]	282 [155 - 408]
	72	226 [126 - 326]	334 [216 - 452]
	96	278 [165 - 391]	389 [262 - 516]
Gadolinium	24	114 [27 - 201]	223 [95 - 351]
	48	106 [33 - 178]	183 [83 - 282]
	72	202 [95 - 310]	295 [169 - 420]
	96	220 [94 - 309]	290 [166 - 415]

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	24	98 [33 - 162]	184 [89 - 279]
	48	247 [128 - 365]	351 [215 - 487]
Terbium	72	204 [113 - 295]	300 [191 - 408]
	96	340 [118 - 563]	455 [216 - 694]
	24	32 [0 - 70]	79 [34 - 149]
	48	196 [125 - 268]	276 [195 - 358]
Dysprosium	72	175 [125 - 226]	247 [190 - 304]
	96	183 [123 - 243]	254 [187 - 321]
	24	236 [127 - 345]	352 [223 - 480]
	48	219 [104 - 334]	317 [183 - 451]
Holmium	72	186 [104 - 269]	270 [173 - 368]
	96	110 [46 - 173]	174 [92 - 256]
	24	116 [66 - 167]	186 [121 - 250]
	48	149 [95 - 203]	210 [149 - 272]
Erbium	72	231 [180 - 281]	290 [240 - 340]
	96	212 [146 - 278]	261 [198 - 324]
	24	148 [63 - 233]	260 [145 - 376]
	48	109 [51 - 166]	179 [103 - 255]
Thulium	72	140 [72 - 208]	214 [131 - 298]
	96	60 [0 - 143]	102 [0 - 219]
	24	55 [26 - 84]	124 [73 - 174]
	48	203 [148 - 257]	278 [218 - 337]
Ytterbium	72	187 [108 - 265]	262 [172 - 352]
	96	148 [80 - 216]	209 [131 - 287]
	24	132 [67 - 198]	229 [140 - 318]
	48	94 [45 - 144]	150 [86 - 213]
Lutetium	72	27 [0 - 112]	51 [0 - 181]
	96	14 [0 - 38]	29 [0 - 70]

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52 **Table S3.** WLR analysis on each binary mixture data (n = 42) from ZF4 cells. A backward stepwise procedure was performed with  $\alpha = 0.01$ .

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Mixture	Fitted model	F-test p-value	r <sup>2</sup>
Nd <sup>3+</sup> + Gd <sup>3+</sup>	$\text{Log}_{10}(\text{cell viability}) = -3.902 \times 10^{-4} \text{Nd}^{3+} - 2.252 \times 10^{-4} \text{Gd}^{3+} - 1.106 \times 10^{-6} \text{Nd}^{3+} * \text{Gd}^{3+}$	$< 10^{-4}$	0.885
Nd <sup>3+</sup> + Yb <sup>3+</sup>	$\text{Log}_{10}(\text{cell viability}) = -3.648 \times 10^{-4} \text{Nd}^{3+} - 5.664 \times 10^{-4} \text{Yb}^{3+} - 1.432 \times 10^{-6} \text{Nd}^{3+} * \text{Yb}^{3+}$	$< 10^{-4}$	0.927
Gd <sup>3+</sup> + Yb <sup>3+</sup>	$\text{Log}_{10}(\text{cell viability}) = -2.922 \times 10^{-4} \text{Gd}^{3+} - 4.350 \times 10^{-4} \text{Yb}^{3+} - 6.754 \times 10^{-6} \text{Gd}^{3+} * \text{Yb}^{3+}$	$< 10^{-4}$	0.931

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**Table S4.** WLR analysis on each binary mixture data (n = 42) from ZFL and RTgill-W1 cells. A backward stepwise procedure with  $\alpha = 0.01$ .

Cell line	Mixture	Fitted model	F-test	
			p-value	r <sup>2</sup>
ZFL	Nd <sup>3+</sup> + Yb <sup>3+</sup>	Log <sub>10</sub> (cell viability) = -4.783 x 10 <sup>-4</sup> Nd <sup>3+</sup> -5.272 x 10 <sup>-4</sup> Yb <sup>3+</sup> -2.793 x 10 <sup>-6</sup> Nd <sup>3+</sup> * Yb <sup>3+</sup>	<10 <sup>-4</sup>	0.88
				7
RTgill-W1	Nd <sup>3+</sup> + Yb <sup>3+</sup>	Log <sub>10</sub> (cell viability) = -2.838 x 10 <sup>-4</sup> Nd <sup>3+</sup> -2.335 x 10 <sup>-4</sup> Yb <sup>3+</sup> -2.326 x 10 <sup>-7</sup> Nd <sup>3+</sup> * Yb <sup>3+</sup>	<10 <sup>-4</sup>	0.97
				0