Absorption of Europium chloride from zebrafish (Danio rerio) embryos under experimental conditions

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Abstract. Lanthanides (Ln) have an essential role in the pollution of the environment because of their ecotoxicity. The pollution of Ln significantly increased due to their use in industry and agriculture in the last decade. Europium (Eu) is the most reactive lanthanide by far. This metal is contained in many industries wastes and it may enter the food chain. The biochemical behavior of lanthanides has been extensively studied, but there are limited studies on Eu. It is remarkable that Ln react with biologically chemical compounds, affecting competitively and replacing the basic ions of the cell such as calcium (Ca2+) and magnesium (Mg2+). Based on the international literature, there are not much data on the toxic effects of Eu mainly on aquatic organisms. Exposure of zebrafish embryos to Europium indicated that the absorption of the metal from the embryos was taken place from the earliest stages of their development.

1 Introduction

Lanthanides (Ln) are considered environmental concerns in the past few years because of their releasing into the environment which has been significantly increased due to their use in industry. Lanthanide metals and their compounds are widely useful in a wide variety of applications in chemistry and high technology, worldwide, such as in materials science, medicine and defence. Their applications depend on the chemical and physical properties of metals and their ability to form compounds and complexes. Their uses include alloys, electronics, glass, ceramics, dyes. The compounds of Ln have a significant contribution to their uses as catalysts, phosphors, lasers and materials that serve their magnetic properties. They also include everyday devices such as cell phones, portable DVDs, and laptops [1, 2]. The +3 oxidative state is characteristic of Eu, as of most Ln, both in solid compounds and in aqueous or other solutions. The Eu element combines with all known anions to form a wide range of compounds with many applications (Table 1) [3].

Table 1. Molecular formulas, description and applications of various Eu compounds [3].

Product Name	Formula	Description	Applications
Europium Acetate	$Eu(O_2C_2H_3)_3.xH_2O$	White crystalline or powder	Phosphor; Glass; Ceramics
Europium Carbonate	$Eu_2(CO_3)_3.xH_2O$	White powder	Phosphor; Glass; Ceramics
Europium Chloride	$EuCl_3.6H_2O$	White crystalline	Phosphor; Glass; Ceramics
Europium Fluoride	EuF_3	White crystalline or powder	Phosphor; Glass; Ceramics
Europium Hydroxide	$Eu(OH)_3.xH_2O$	White crystalline	Glass; Neutron absorption
Europium Metal	Eu	Silvery grey lump pieces	Metallurgy; Nuclear industry; Specialty alloy
Europium Nitrate	$Eu(NO_3)_3.6H_2O$	White crystalline	Catalysts; Phosphor; Glass; Ceramics
Europium Oxalate	$Eu_2(C_2O_4)_3.xH_2O$	White crystalline	Phosphor; Glass; Ceramics
Europium Oxide	Eu_2O_3	White powder	Phosphors for lamp, color TV, X-ray and other luminescent materials;

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			Glass; Crystal
Europium Sulfate	$Eu_2(SO_4)_3.8H_2O$	White crystalline	Phosphor; Glass; Ceramics

Europium (Eu) is one of the less abundant Ln elements, but we can't underestimate its toxicity to organisms. It is well known that Eu is never found in nature as the free element, but there are many industries liquid and solid wastes containing Eu toxic elements and they are released into various compartments of the environment, including agricultural land. Thus, toxic elements may enter the food chain, depending on their bioavailability. There is an enough limited number of studies about Eu toxicity to organisms such as plants, mammals, fishes and microorganisms allow a comparison of the ecotoxicity of different Ln under identical experimental or environmental conditions [4]. Exposure of embryos to Europium affects all the parameters of embryonic development resulting in significant dose-dependent mortality, decreased standard length and heart rate as well as in delayed heart formation [5]. Europium complex and europium (III) were respectively enriched by dietary green algae *Platymonas subcordiformi*, and their toxicity to algae were analysed by the 96-h acute toxic experiment. Then, the green algae enriched europium complex and europium salt were respectively fed pearl oysters for 2 months. The results showed that the two Eu compounds can modify pearl structure and colour [6]. Short-term Eu bio uptake fluxes by the freshwater green algae Chlamydomonas Reinhardtian were investigated in the presence and absence of ligands (e.g., malic acid and citric acid) and a second rare earth metal, samarium (Sm). Overall, the results showed that for Chlamydomonas Reinhardtian, Eu was likely to share a common bio uptake pathway; bio uptake of one rare earth element was reduced when another was present, and Ln complexes were bioavailable [7].

Early developmental stages of fish are particularly sensitive to water pollution. Heavy metals and Ln may affect various developmental processes during the embryonic period. The eggshell does not fully protect the embryo against metal penetration. The results depend on metal concentration and range from developmental disturbances to death of the embryo. Metals disturb various processes of fish embryonic development and affect the development rate [8, 9]. The toxicity of sulphate in humic, soft freshwater to whitefish (*Coregonus lavaretus*) from fertilization of eggs to hatching i.e., during the critical phases of whitefish early development showed that sulphate loading from industry to freshwaters are increasing in boreal region [10].

The aim of the present study is to estimate the potential Eu absorption from developing embryos of the model freshwater organism Danio rerio.

2 Materials and methods

The maintenance and breeding of zebrafish has been described in detail by Nagel [11]. A breeding stock of non-treated, mature zebrafish is used for egg production. Females and males are kept at a ratio of 1:2 in a glass aquarium filled with charcoal filtered tap water with an oxygen saturation of more than 80 - 95%. The culture conditions are 26 ± 1 °C at a 12-hour day/night light regime. Five to ten females and males were kept separately for 1–2 weeks (the gastric region of females would have dilated due to the eggs), before laying, in special plastic egg storage and collection containers (egg collector tanks). Each tank had a pseudo-bottom (3-4 mm grate) through which the eggs passed and were deposited at the bottom of the vessel without the risk of being eaten by their parents (Figure 1). Zebrafish, (*Danio rerio*), fertilized eggs were collected from spontaneous spawning's according to the procedures described by Westerfield [12]. Eggs collected, from egg collectors, within two hours after spawning were exposed to a range of Eu concentrations (0, 0.05, 0.5, 5, 50, 100, 200, 320, 400 and 500 ppm) in triplicate for each one Europium (III) chloride hexahydrate ($EuCl_3 \cdot 6H_2O$) and incubated at 28.5°C. Additionally, for some concentrations (200, 320, 400 and 500 ppm), the eggs were exposed to the metal only for the first 10 hpf (hrs post fertilization) and placed afterwards into clean fresh water. Dead (non-translucent and dark coloured) eggs were removed and placed in a NaCl saturated solution, before the examination, until they became clear.

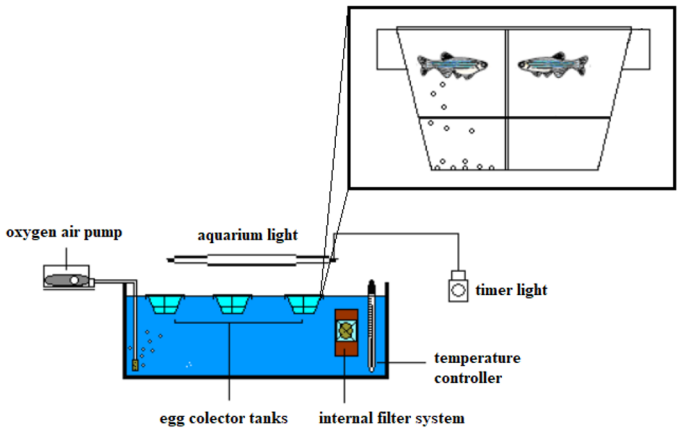

Figure 1. Schematic representation of an aquarium with all its support systems.

In this study, Eu absorption was estimated in a total number of 2318 larvae (Table 2), and the stages that took place during the determination of the metal in the larvae were: (a) Collection and storage of biological material. The larvae immediately after hatching were rinsed twice with distilled water and then frozen at -20° C where they were kept until analysis. (b) Defrosting and weighing the larvae. The larvae were thawed for a few minutes and then their weight was estimated on a precision balance (4th decimal model). (c) Acid digestion (digestion) of biological samples. The acid digestion process involved placing the samples in Teflon - PFA shells and then adding 5 ml of concentrated HNO_3 solution (65%

suprapure), 1 ml H_2O_2 and 4 ml H_2O. The samples were then placed in a P = 400W microwave oven and heated for 20 min. At the end of the heating time the solutions were diluted twice with distilled water to a constant volume of 10 ml. At the same time, blank solutions were prepared similar to the above procedure to minimize the constant (systematic) errors of analysis (bias). (d) Measurement of Eu concentration in the biological sample. For measuring Eu with a toner oven, reference solutions of concentrations of 10, 20 and 50 μg/l were used, which were prepared with 0.2% HNO_3 after successive dilutions from a concentrated standard solution of concentration of 1000 mg/l. Then came the calibration (reference) curve with the absorptions of the standard aqueous solutions of the metal as a function of their concentrations with equation A (peak area) = 0.0027 C(μg/l) + 0.0045.

Table 2. The number of larvae per concentration.

Eu concentration (ppm)	Number of larvae	
	Exposure time	
	10 hpf	72 hpf
0	0	140
0.05	0	157
0.5	0	213
5.0	0	214
50	0	190
100	0	195
200	0	143
320	0	196
200	231	0
320	196	0
400	149	0
500	142	0

3 Results

Based on the importance of embryonic mortality that occurred at concentrations of 100 ppm Eu and above, the hatching rate was analyzed only with live and successfully hatched embryos. In the continuous exposure of 72 hpf (hours post fertilization) at the highest concentrations of metal used the hatching time was extended to 53 - 57 hpf in relation to the control group, i.e., about two twenty-four hours more (≈ 42 hpf) while in the 10 hpf exposure of embryos to the metal, which the concentrations were even higher, the hatching time increased even more (98 - 116 hpf). In both exposures of 10 and 72 hpf, the mean-hatching time increased to approximately 83 hpf, where extensive fetal mortality was observed. Thus, continuous exposure of embryos to the Eu significantly increased the median-hatching time (Figure 2) as well as the total duration of hatching (Mantel-Cox, $p<0.001$) where continuous exposure at 72 hpf showed a longer hatching delay (Figure 2).

The results of this study, when determining the Eu in biological samples of larvae, showed that it is possible the detection of the metal.

The absorption of Eu by the fetal tissues was studied in detail for seven metal concentrations and showed a positive increase with the increased concentration of the metal throughout the 72 hpf exposure (Figure 3).

Furthermore, Eu detection was possible in larvae samples when they were exposed to the metal development and after the embryos were transferred to the water which was free from metal. The absorption of the metal during embryonic development was studied at four different concentrations of Eu (ppm). The concentration of metal (μg/g) in the larvae also showed a positive increase depending on the increase in the concentration capacity of the solution (ppm) (Figure 4).

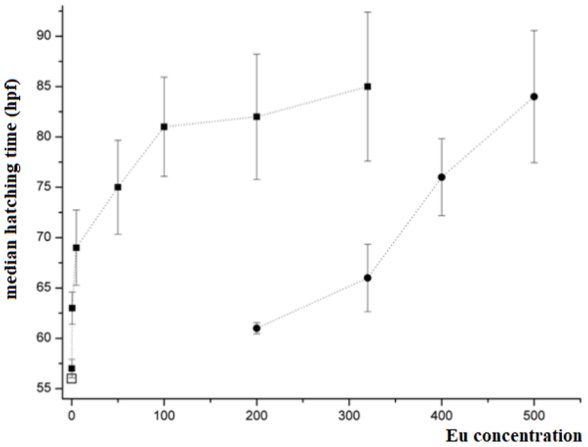

Figure 2. Mean hatching time curves at different Eu concentrations with mean time points (95% confidence limits). The 72 hpf exposure is shown in black squares, while the 10 hpf exposure is shown in black circles.

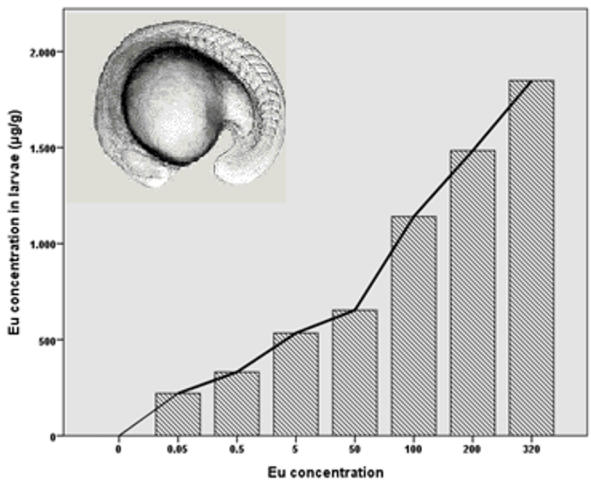

Figure 3. Eu absorption from the zebrafish larvae within 72 hpf exposure.

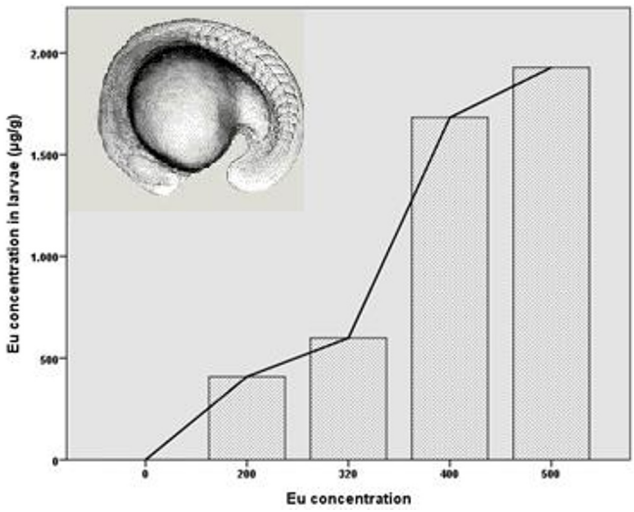

Figure 4. Eu absorption from the *zebrafish* larvae within 10 hpf exposure.

In both cases, the Eu concentration (µg/g) in the larvae also showed a positive increase with the concentration of the solution (ppm).

4 Conclusion and discussion

The effects of metals on fish are related to their uptake and accumulation by the organism, resulting in metal-induced disturbances in the structure and function of various tissues and organs. Early developmental stages of fish, including when the embryos are protected by the eggshell (chorion), are particularly sensitive to intoxication. The chorion is permeable to water, ions and salts but not to molecules of higher molecular weight. Its functional role is not only to be a natural, protective mechanism for developing embryos from their external environment (but to some extent unless the environment is highly toxic) but also to maintain a difference in ionic potential between internal and external environments [13, 8].

The data from the present study also show that hatchability is one of the most sensitive biological parameters in the presence of pollutant metal. Hatching, based on the number of live and successfully hatched embryos, was significantly affected by the toxic effect of Eu. The hatching time was significantly extended by almost two 24 hours after continuous exposure. References have shown that fish embryos grown in toxic heavy metal environments such as Cu, Cd, Pb showed very low hatchability which was proportional to the concentration due to inhibition of the function of specialized hatching glands embryos needed to break the eggshell and release the larvae. In general, pre-hatching fish embryos develop special hatching glands that rest on their head and are associated with hatching. These glands produce chorionase, a protein enzyme necessary for the breakdown of the eggshell during the hatching process. However, many metals can have serious effects on the growth and function of these glands, which can disrupt transcription and translation processes, resulting in reduced protein synthesis, including chorionase. The surface of the glands due to the presence of metals shrinks whenever the glands become non-functional [8].

The absorption of Eu from the embryos was taken place from the earliest stages of their development. This was confirmed by the metal in the embryo's tissues after the first 10 hpf exposure and their growth in free-metal water. Thus, the egg as the first embryonic defence mechanism, in the early stages after fertilization, does not seem to restrict the entry of metal into it regardless of the time of exposure. During the first stages of embryonic development, immediately after fertilization, swelling of the egg is observed due to the absorption of water from the external aquatic environment. In this phase, if the environment is loaded with heavy metal ions or Ln, then the egg due to the entry of metal receives a strong influence of ions, which can change the permeability of the eggshell and the structure of the chorion. A common parameter of these two effects is the competitive action of pollutant metals and essential metals of egg biomolecules, such as Ca^{2+} ions. Probably the main biological causes that seem to have contributed to the entry and incorporation of Eu in eggs during the toxic effect of the metal, in the early stages of development, are chorion and Ca^{2+} ions.

The chorion may not be a significant limiting factor for the metal to enter the egg, with the majority of Eu^{3+} ions entering the egg. The ultimate destination of Eu ions is their incorporation into embryonic tissue, acting competitively and replacing Ca^{2+} ions, as well as other important ions such as Mg^{+2}, at specific binding sites. Ca^{2+} ions are important because they play a key role in hatching, embryonic development, and organogenesis. Freshwater teleosts in both adulthood and larval age live in environments poorer in Ca^{2+} ions relative to the intracellular concentration. The competitive behavior of Eu against Mg^{2+} ions is not as strong as that of Ca^{2+} ions, but it is possible. This is due to the fact that heavy Ln due to their ionic size are more similar to that of Mg^{2+} ions [14], thus inhibiting metabolic pathways of nutrition and growth during embryonic differentiation [15].

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