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EFEITOS ECOTOXICOLÓGICOS DE UM NANOMATERIAL MANUFATURADO "INTELIGENTE" EM PEIXE-ZEBRA

ECOTOXICOLOGICAL EFFECTS OF A "SMART" ENGINEERED NANOMATERIAL IN ZEBRAFISH

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Eco-Toxicologia e Análise de Risco, realizada sob a orientação científica do Doutor Roberto Carlos Domingues Martins, Investigador Auxiliar do Departamento de Biologia da Universidade de Aveiro, e coorientação da Doutora Maria Pavlaki, Investigadora do Departamento de Biologia da Universidade de Aveiro.

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Este trabalho é dedicado ao *Danio rerio*.
Que o sacrifício destes exemplares se traduza na proteção dos próximos. E, quem sabe, de muitas outras espécies…

o júri

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palavras-chave Hidróxidos duplos lamelares, dano no DNA, avaliação de risco, *Danio rerio*

resumo

Os hidróxidos duplos lamelares (LDH) são nanoargilas aniónicas, compostas por camadas carregadas positivamente com catiões metálicos divalentes ou trivalentes (por exemplo, Zn^{2+} ou Al^{3+}) e estabilizados por aniões (por exemplo, nitratos) e moléculas de água entre camadas. Estes nanomateriais têm propriedades notáveis, nomeadamente excelente permutabilidade aniónica, efeito de memória, biocompatibilidade, capacidade de resposta a alterações do pH, elevada relação superfície/volume e elevada capacidade de adsorção. Os LDH têm sido considerados nanomateriais manufaturados "inteligentes" e têm recebido um forte interesse em áreas como a medicina, farmacêutica, indústria, entre outras. Estes nanomateriais causam baixa toxicidade em humanos, mamíferos e organismos marinhos. No entanto, os efeitos ecotoxicológicos em organismos de água doce são pouco estudados, lacuna esta que é crítica para fins regulatórios. O peixe-zebra *Danio rerio* é um organismo-modelo de água doce amplamente utilizado na avaliação (eco)toxicológica de inúmeros contaminantes. Existem vários estudos que utilizam o peixe-zebra para avaliar a toxicidade de nanomateriais, principalmente nanopartículas metálicas. O presente estudo visa assim avaliar os efeitos ecotoxicológicos do nanomaterial LDH Zn-Al fases larvares da espécie *Danio rerio*.

Para o efeito, ovos de *Danio rerio* foram expostos a LDH Zn-Al para avaliar a embriotoxicidade e alterações de desenvolvimento, comportamentais, bioquímicas e moleculares (ao nível do DNA). Para a avaliação da embriotoxicidade, os ovos foram expostos a LDH Zn-Al (85 – 1200 mg/L) de acordo com o protocolo OCDE 236, onde a mortalidade, malformações e sucesso de eclosão foram registados diariamente durante 96 h. No ensaio comportamental, os embriões foram expostos a concentrações subletais de LDH Zn-Al (9 – 94 mg/L) durante 120 h. A avaliação da genotoxicidade (ensaio do cometa) e de biomarcadores de neurotoxicidade e de *stress* oxidativo foi realizada após uma exposição de 96 h dos ovos ao nanomaterial (9 – 94 mg/L). O comportamento e destino dos LDH na água do sistema do peixe-zebra foram avaliados através do potencial zeta e tamanho hidrodinâmico das partículas.

No geral, as dispersões de LDH têm tendência para agregação e reduzida estabilidade no meio de exposição. No teste de embriotoxicidade, a concentração de efeito não observada (CENO) foi de 415.2, 244.3 e 143.7 mg/L para os parâmetros de letalidade, eclosão e malformações, respetivamente. Em termos comportamentais, a exposição aos LDH causou diferenças significativas na duração total da locomoção (CEO= 9 mg/L). A exposição aos nanomateriais não causou genotoxicidade, nem diferenças significativas nos parâmetros bioquímicos testados ($p > 0.05$). Em conclusão, os resultados atuais sugerem que o Zn-Al LDH é pouco tóxico para embriões e larvas
de peixe-zebra. Os efeitos observados parecem indicar um modo de atuação iminentemente físico/mecânico, compatível com a tendência para sedimentação em concentrações muito elevadas e pouco realistas (na ordem das dezenas de mg/L).

keywords Layered double hydroxides, DNA damage, hazard assessment, *Danio rerio*

abstract

Layered Double Hydroxides (LDH) are anionic nanoclays, composed of positively-charged layers with divalent or trivalent metal cations (e.g., Zn^{2+} or Al^{3+}) and stabilized by anions (e.g., nitrates) and water molecules in the inter-layers. LDHs have remarkable properties namely excellent anion exchangeability, memory effect, biocompatibility, pH sensitivity, high surface to volume ratio, and high adsorbing capacity. LDHs have been regarded as "smart" engineered nanomaterials and lately received great interest in multiple areas, such as medicine, pharmaceutical, industry, among others. These nanomaterials are of low toxicity to humans, mammals, and marine organisms. However, ecotoxicological effects on freshwater organisms are scarcely studied, which is critical for regulatory purposes. The zebrafish *Danio rerio* is a well-established freshwater model organism that is widely used in toxicological and ecotoxicological assessment of innumerous contaminants. Several studies are using the zebrafish to evaluate the toxicity of nanomaterials, mainly metallic nanoparticles. The present study aimed to assess the ecotoxicological effects of Zn-Al LDH in *Danio rerio*.

Danio rerio eggs were exposed to Zn-Al LDHs to assess developmental, behavioural, biochemical, and molecular (at the DNA level) changes. For the evaluation of embryotoxicity, the eggs were exposed to Zn-Al LDH (85 - 1200 mg/L) according to the OECD 236 protocol, where mortality, malformations, and hatching success were recorded daily for 96 hours. In the behavioural test, the embryos were exposed to sublethal concentrations of Zn-Al LDH (9 - 94 mg/L) for 120 h. The evaluation of genotoxicity (comet assay) and biomarkers of neurotoxicity and oxidative stress after a 96 h exposure of eggs to nanomaterial (9 - 94 mg/L). The behaviour and fate of LDHs in the water of the zebrafish system were characterized through the zeta potential and hydrodynamic particle size.

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AChE	Acetylcholinesterase
ANOVA	One-way analysis of variance
CAT	Catalase
DLS	Dynamic light scattering
EC_{50}	Median effect concentration
ENMs	Engineered nanomaterials
FET	Fish Embryo Toxicity Test
FSW	Fresh water system
GPx	Glutathione peroxidase
GR	Glutathione reductase
GST	Glutathione-*S*-transferase
LC_{50}	Median lethal concentration
LDHs	Layered double hydroxides
LPO	Lipid peroxidation
NM	Nanomaterial
NP	Nanoparticle

CHAPTER 1

GENERAL INTRODUCTION

1. General introduction

1.1. Nanomaterials (NMs)

The physicist Richard Feynman from the California Institute of Technology is considered the pioneer of what is now considered nanotechnology. In a talk given in a meeting of the American Physical Society on December 29, 1959, Feynman described a process by which the ability to manipulate individual atoms and molecules might be developed (Feynman, 1960). Despite this, the term “nanotechnology” only started to be used when Norio Taniguchi, in 1974, distinguished (with a wide variety of applications at the nanometer level) the characteristics of engineering materials (Taniguchi, 1974).

Nanotechnology is transversal to several fields, such as medicine, biology, geosciences, chemistry, physics and materials sciences (Mobasser and Firoozi, 2016). Nanotechnology is, therefore, the technology, engineering and science that, manipulating the molecular and atomic scale (from 1 to 100 nm), allows the production and manipulation of nanomaterials with unique functions and characteristics. According to the European Commission, a nanomaterial can be defined as "a natural, incidental or manufactured material containing particles in an unbound state or as an aggregate or agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm – 100 nm” and/or their area/volume ratio must be greater than 60 m^2/cm^3 (Recommendation 2011/696 EC, EC, 2011). The nanometer is one-billionth of a meter (10^{-9}). But, to have a better perception, think that a nanometer corresponds to one hundred thousand times smaller than the diameter of a human hair (Scott and Chan, 2002). The size of the global nanomaterials market was valued at $ 8.5 billion (7.2 billion euros) in 2019 and is expected to continue to grow from 2020 to 2027 (Grand view research, 2020).

The origin of nanomaterials can be summarized in two type, the natural and the anthropogenic ones. The natural nanomaterials arise through natural processes. The existence of the natural nanoparticles (NPs) in the different nature’s elements (water, air and soil) has been recorded in 10 000 years old in glacial ice cores (Murr *et al.*, 2004). NPs can be produced due to geological and biological mechanisms. Geological processes consist in volcanic eruption and physicochemical weathering (soil erosion and dust storms) (Handy, Owen and Valsami-Jones, 2008). Biological mechanisms include nano-size biological molecules (protein and nucleic acids) and some of the biological NPs are released into the environment directly from the organisms (nucleoprotein exudates from algae) (Bhatt and Tripathi, 2011). The anthropogenic origin is due to

the consequence of human activities and can be classified in two ways, intentional or unintentional. Nanomaterials of intentional anthropogenic origin are obtained through the manipulation of atoms, while those created unintentionally can originate from different mechanisms, such as combustion and refining processes (Griffin *et al.*, 2017).

Currently, there is a wide range of nanomaterials with different chemical compositions, sizes, shapes, among other characteristics, which are produced and commercialized. Nanomaterials can be broadly categorized in nanoparticles, nanoclays and nanoemulsions (Figure 1.1) (Konwar and Ahmed, 2016; Mageswari *et al.*, 2016). The nanoparticles classification can be sub-divided as: (i) carbon-based nanomaterials, including carbon nanotubes, graphene or fullerenes; (ii) inorganic nanoparticles (NPs), which may include mesoporous silica NPs, gold NPs, zinc oxide NPs, titanium dioxide NPs, among many others; (iii) organic nanoparticles, such as dendrimers, liposomes, micelles, among others.

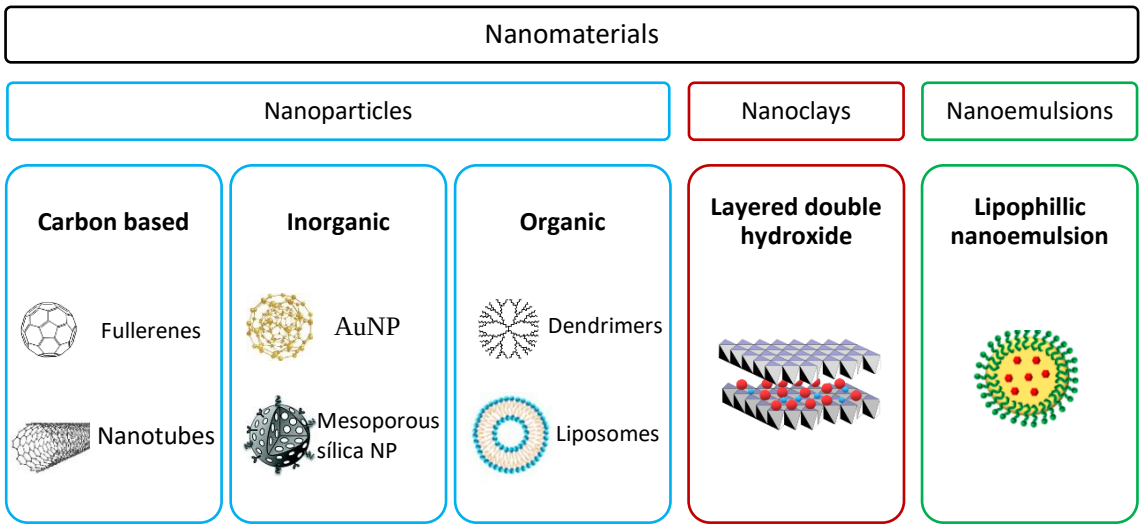

Figure 1.1| Classification and some examples of nanomaterials: nanoparticles (NP), nanoclays and nanoemulsions (adapted from Mageswari *et al.*, 2016).

NMs are used in several products of our daily life, such as sunscreens, detergents, clothing, cosmetics, food, paints, among others, but also in the industry or health sector. NMs have improved medical imaging techniques and are used in therapeutics or treatment (e.g. targeted drug delivery systems, drug therapy, photothermal and hypothermal destruction). NMs have been also proposed or used for environmental applications, such as remediation of water or soil contaminated with hazardous substances or creation of sustainable agroproducts (Wang and O'Hare, 2012; Khan, Saeed and Khan, 2019; Nunes *et al.*, 2019).

While this wide variety of applications benefits users and the economy, humans and ecosystems are constantly exposed to unknown effects. The release of nanomaterials can occur

from point sources (e.g. production facilities and wastewater treatment) or from non-point sources (e.g. wear and tear of materials whose composition consists of nanomaterials). Thus, it is necessary to assess the risks imposed for the use of nanomaterials in commercial products and environmental applications. However, it is only possible to assess these risks after a better understanding of the behaviour, mobility, bioavailability, toxicity and fate of nanomaterials in the environment (Ray, Yu and Fu, 2009; Poel and Robaey, 2017).

1.1.1. Layered double hydroxides (LDHs)

Layered Double Hydroxide (LDHs) have been regarded as "smart" engineered nanomaterials (ENMs) and in the last two decades received a lot of attention from academy and industry. LDHs are anionic nanoclays and can be found in nature or synthesized in laboratory facilities. As a mineral, it was first discovered in Sweden around 1842, but the first patent for a hydrotalcite-like structure was registered only in 1970 (German Patent 2.024.282, 1970).

LDHs are host-guest nanomaterials and their structure consists in two plates composed of mixed metal hydroxides (positively charged) containing divalent (such as Zn^{2+} and Mg^{2+}) and trivalent (such as Al^{3+}) metal cations and between layers containing anions (e.g. nitrates) and water molecules. The structure of LDHs are represented by the formula $[M^{2+}_{1-x}M^{3+}_{x}(OH)_2]A^{n-}_{x/n}\cdot mH_2O$, where M^{2+} and M^{3+} are the corresponding divalent and trivalent metal cations, and A^{n-} is the intercalated anion. Besides, the typical lateral size of LDHs ranges from 20 to 40 nm, thus meeting the definition of a nanomaterial, as previously mentioned (Figure 1.2) (Newman and Jones, 1998).

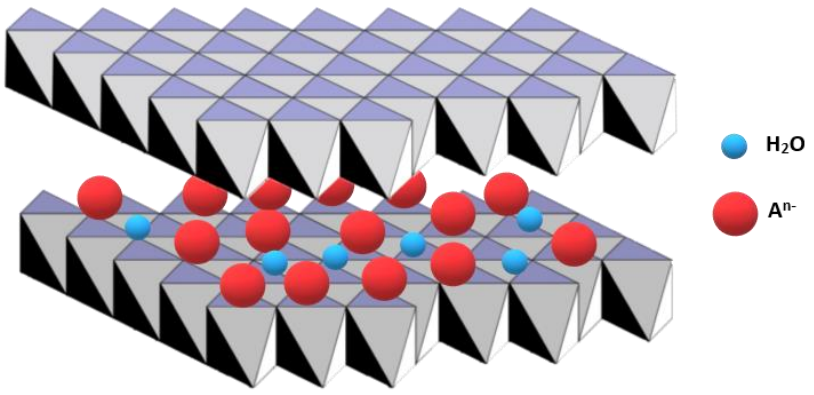

Figure 1.2 | Schematic representation of the interlayer structure of an LDHs (adapted from Kuzmann *et al.*, 2015).

LDHs can be regarded as promising layered nanomaterials due to the remarkable properties such as simple synthesis methodology, excellent anion exchangeability, memory effect, thermal stability, tunable basicity, biocompatibility, pH sensitivity, high surface to volume ratio, high adsorbing capacity, endothermic decomposition, among others (Choy, Oh and Choi, 2011; Mishra *et al.*, 2018; Arrabito *et al.*, 2019).

Due to their impressive characteristics, LDHs have been widely used in several areas, such as catalysis, fuel cells, drug delivery, environmental remediation, analytical extraction, sensing, corrosion, biofouling, flame retardant industry, among many others (Tedim *et al.,* 2010; Zheludkevich, Tedim and Ferreira, 2012; Kalali, Wang and Wang, 2015; Nicotera *et al.*, 2015; Avelelas *et al.,* 2017*;* Baig and Sajid, 2017; Yan *et al.*, 2017; Mishra *et al.*, 2018; Bouali *et al.,* 2020; Tiwari *et al*., 2020; Wan *et al.*, 2021).

1.2. Ecotoxicity

1.2.1. Ecotoxicity of LDHs

As mentioned earlier, the use of LDHs have rapidly increase and that may lead to the occurrence of LDHs in the effluents and the freshwater systems receiving effluent discharge, which has not been reported so far. It may raise ecological and human health concerns about the LDH unknown ecotoxicity effects.

LDHs are an important tool in the field of medicine, being used as nanocarriers for the transport and controlled release of drugs, due to the low toxicity towards humans and other mammalians models (Oh *et al.*, 2006; Choi And Choy, 2011; Kura *et al.*, 2014; Gu *et al.*, 2018).

The Zn-Al LDH have been used as nanocarriers in the field of medicine, as absorbent for water remediation and also in corrosion science (Tedim *et al.,* 2012; Abdolmohammad-Zadeh, Nejati and Ghorbani, 2015; Iftekhar *et al.,* 2018; Pooresmaeil *et al*., 2019; Yasaei *et al*., 2019; Tiwari *et al*., 2020).

Despite this, little is known about the effect of these nanomaterials to freshwater organisms. For marine organisms, there are already some studies in the literature. As can be seen in Table 1.1, Zn-Al LDH-NO_3 cause no acute toxicity on mussels or embryotoxicity in crustaceans and echinoderms (up to a concentration of 100 mg/L) apart from bryozoans *Bugula neritina* (Gutner-Hoch *et al.*,2018; Gutner-Hoch *et al.*, 2019). Likewise, Zn-Al LDH-NO_3 (NOEC=100 mg/L) cause no effects on growth inhibition of microalgae species (*Tetraselmis chuii* and *Phaeodactylum tricornutum*) (Avelelas *et al.*, 2017). On the edible clam *Ruditapes philippinarum*, LDHs exposure

causes inhibition of the neurotransmission marker, acetylcholinesterase (EC_{50} = 97.9 mg/L), however, in the bivalve *Mytilus edulis* caused no oxidative stress or neurotoxicity (until concentrations of 0.1 mg/L) (Avelelas *et al.*, 2017; Martins *et al.*, 2017).

For freshwater organisms, as far as we are concerned, there is only one study on LDHs ecotoxicity, specifically with Cu-Mg-Fe LDH. The 72 h exposure of the green freshwater algae *Scenedesmus quadricauda* to Cu-Mg-Fe LDH causes growth inhibition effects with the EC_{50} estimated at 8.22 mg/L (Ding *et al.*, 2018) (Table 1.1). However, this version of LDHs is not the most used one. Thus, there is a relevant knowledge gap on the aquatic ecotoxicity of such anionic nanoclays.

Table 1.1 | Toxicity data for aquatic organisms exposed to LDHs.

LDHs	Concentration range (mg/L)	Test organism	Endpoint	Time	NOEC (mg/L)	LOEC (mg/L)	L/EC_{50} ($IC_{95\%}$; mg/L)	References
Zn-Al LDH-NO_3	0.01 – 100	*Ruditapes philippinarum* (bivalve)	Survival	96 h	100	> 100	nd	Martins *et al.*, 2017
			Condition index, Air survival, LPO, GST		100	> 100	nd	
			AChE		0.01	0.1	97.9 (77.8 – 132.7)	
			GSH/GSSG ratio, GR, GPx, CAT		< 0.01	0.01	nd	
	0.01 – 100	*Tetraselmis chuii* (green microalga)	Inhibition growth	96 h	100	>100	> 100	Avelelas *et al.*, 2017
		Phaeodactylum tricornutum (diatom)	Inhibition growth		100	>100	> 100	
	0.001 – 0.1	*Mytilus edulis* (bivalve)	Survival	96 h	0.1	> 0.1	> 0.1	
			LPO, GST, CAT & AChE		0.1	>0.1	nd	
	0.01 – 100	*Mytilus galloprovincialis* (bivalve)	Survival	72 h	100	>100	> 100	Gutner-Hoch *et al.*,2018
		Brachidontes pharaonis (bivalve)	Survival		100	>100	> 100	
	0.000001 – 100	*Bugula neritina* (bryozoan)	Larvae survival	72 h	nd	nd	4.3 (4.2 – 4.4; Red Sea) 9.4 (6.0 – 14.5; Med. Sea)	
	0.001 – 100	*Paracentrotus lividus* (echinoderm)	Pluteus-larvae formation	48 h	100	>100	> 100	Gutner-Hoch *et al.*, 2019
		Artemia salina (crustacean)	Larvae survival	24 h	100	> 100	> 100	
Cu-Mg-Fe LDH	0 – 100	*Scenedesmus quadricauda* (green microalgae)	Inhibition growth	72 h	nd	1.5 (light) 1.8 (dark)	10 (light) (8.2 – 10.0) 25 (dark) (25 – 32)	Ding *et al.*, 2018

1.2.2. Zebrafish

The freshwater fish, *Danio rerio* (also known as zebrafish) is considered a suitable organism for both eco- and toxicology studies and thus, widely used in studies for the assessment of different types of chemical compounds (Aleström, Holter and Nourizadeh-Lillabadi, 2006). *Danio rerio* was firstly described by Hamilton-Buchanan in 1822 and belongs to the *Cyprinidae* freshwater fish family (Spence *et al.,* 2008). The zebrafish is an organism native to the rivers of South Asia and is, currently, found with some frequency in rivers, channels, ponds, ditches and lakes. The zebrafish is a small organism, approximately 1 to 4 cm long, and is easily identified since it has five to seven dark blue lines that extend longitudinally from the operculum to the caudal fin. It is considerably easy to distinguish the male from the female since the latter has a more rounded body shape than the male (Figure 1.3). Its life cycle is very short and this organism reaches reproductive maturity between 3 and 6 months. The fertilization is external, with each female being able to spawn, at a single time, hundreds of eggs (of dimensions approximately equal to 0.7 mm) that have a transparent aspect, which allows (besides observation of the different stages of development) their manipulation. Since this organism has a very rapid embryonic development, its body is developed 24 hours after fertilization and the hatching period happens between 48 and 72 hours after fertilization (Kimmel *et al.,* 1995; Scholz *et al.*, 2008; Spence *et al.,* 2008.).

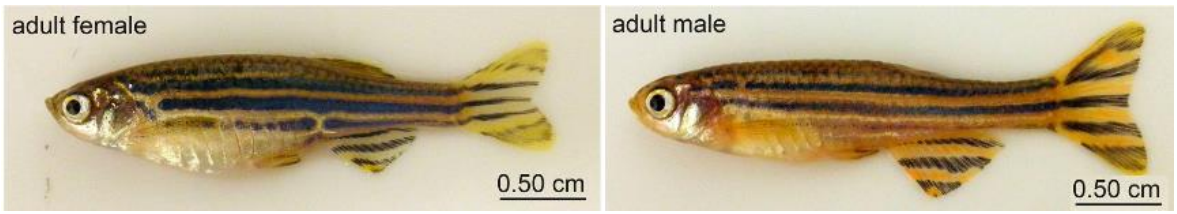

Figure 1.3 | Adult specimens of female and male of zebrafish (*D. rerio)* (Holtzman *et al.*, 2016).

The zebrafish is considered an important model organism and widely used in several areas of study (such as ecotoxicology, developmental biology, neurobiology, neurophysiology, genetics and pharmacology), as previously mentioned. The zebrafish has numerous characteristics that make it an important laboratory tool, such as its translucent appearance, small size, rapid development and the large number of eggs produced at once by the female. Since it is small in size, it does not require a large living space, making it an easy-to-maintain laboratory organism and its associated costs are also affordable (Kimmel *et al.*, 1995; Scholz *et al.*, 2008; Spence *et al.,* 2008). It is considered a model vertebrate organism, so it is possible to make comparisons with other vertebrates, namely humans. The zebrafish genome is completely sequenced, and it is also now

known that this organism shares homologous sequences with the human genome (Howe *et al.*, 2013).

1.2.3. Ecotoxicological effects of nanomaterials in zebrafish

Zebrafish is a model which allows the evaluation of different ecotoxicological endpoints namely lethality, developmental, biochemical and genotoxic alterations and locomotor behaviour, among others, which will help to understand the toxicity induced by a xenobiotic in a more holistic way.

Table 1.2 compiles the ecotoxicological effects caused by the exposure of the zebrafish (*Danio rerio*) to some well-known nanomaterials. As previously mentioned, and as far as we know, there are no studies focused on the ecotoxicological effects of LDHs in zebrafish. In opposition, there are several studies that evaluate the toxicity of nanomaterials (although this is not intended to be an exhaustive literature review), mainly metallic nanoparticles using this model organism. For instance, ZnO NPs demonstrated to have an impact on hatching success, malformations and biochemical markers (Zhu *et al.*, 2008; Bar-Ilan *et al.*, 2013; Kim *et al.*, 2013; Zhao *et al.*, 2013; Brun *et al.*, 2014; Ganesan *et al.*, 2015; Xin *et al.*, 2015; Lu *et al.*, 2016; Zhao *et al.*, 2016; Zhu *et al.*, 2019; Boran and Şaffak, 2020 and Qiang *et al.*, 2020). These nanoparticles show several significant effects at concentrations below 100 mg/L.

Table 1.2 | Toxicity data for *D. rerio* exposed to nanoparticles.

Nanomaterial	Concentration range (mg/L)	Duration	Significative effects observed	Reference
AgNP	0.5 – 1.9	96 h	malformation (LOEC = 0.9 mg/L)	Qiang *et al.*, 2020
	0.5 – 23.1	96 h	malformations (EC_{50} = 5.9 mg/L)	Xin *et al.*, 2015
AuNP	0.08 – 30	120 h	malformation (LOEC= 10 mg/L) behaviour (LOEC = 1 mg/L)	Kim *et al.*, 2013
CuO NP	5 - 80	96 h	mortality (LC_{50}= 64 mg/L) SOD, CAT and GPx decrease (LOEC = 40 mg/L) malformations (LOEC = 40 mg/L)	Ganesan *et al.*, 2015
Al_2O_3 NPs	20 – 500	96 h	mortality (LC_{50}=130.2) DNA damage (LOEC = 100)	Boran and Şaffak, 2020
Multiwall carbon nanotubes	0.8 – 10	72 h	hatching delay (LOEC = 10 mg/L)	Lu *et al.*, 2016
TiO_2 NP	0.00001 – 10	23 days	mortality (LOEC = 0.0001)	Bar-Ilan *et al.*, 2013
	25 – 100	120 h	malformation (LOEC = 50 mg/L) ROS (LOEC = 100 mg/L)	Zhu *et al.*, 2019

ZnO NP	0.2 – 5	96 h	hatching delay (LOEC = 0.2 mg/L)	Brun *et al.*, 2014
	0.1 – 50	96 h	mortality (LC_{50} = 1 mg/L) hatching success (EC_{50} = 2,1)	Zhu *et al.*, 2008
	10 – 120	96 h	hatching success (LOEC = 10 mg/L) MDA (LOEC = 120 mg/L) SOD (LOEC = 90 mg/L)	Zhao *et al.*, 2016
	1 – 100	144 h	hatching success (LOEC = 1 mg/L) malformation (LOEC = 10 mg/L)	Zhao *et al.*, 2013

1.3. Aims

The present study aimed to assess the ecotoxicological effects of Zn-Al LDH using a model organism, the freshwater fish *Danio rerio*. To reach the objective of this work, three questions were addressed.

1. Does Zn-Al LDH exposure cause eco- and genotoxicity to *Danio rerio* embryos and larvae?
2. Does Zn-Al LDH exposure alter the locomotor behaviour of *D. rerio* larvae?
3. Do Zn-Al LDH exposure induce biochemical responses to *D. rerio* larvae?

To respond to the above questions on how Zn-Al-LDH exposure may affect the early stages of *D. rerio*, the following approach was used.

For the first question, the embryo toxicity test (FET) was used to assess if Zn-Al LDH-NO_3 cause ecotoxicological effects on zebrafish embryo and larvae. During and at the end of the FET test, parameters such as mortality, malformations and hatching rate were recorded. Also, to assess the genotoxic effects of Zn-Al LDH-NO_3 (sub-lethal concentrations), the single cell gel electrophoresis assay (comet assay) was used. Regarding the second question, a behaviour assay was performed with zebrafish larvae after they had been exposed to sub-lethal concentrations of Zn-Al LDH-NO_3. Finally, to assess whether Zn-Al LDH-NO_3 induces alterations at an enzymatic level to *D. rerio* larvae different biomarkers were measured. Biomarkers of effect assessed in this study were lipid peroxidation (LPO), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferases (GST) and acetylcholinesterase (AChE).

1.4. Thesis organization

The current thesis is structured in three chapters as:

Chapter 1: General introduction;

Chapter 2: Paper “Ecotoxicological effects of a “smart” engineered nanomaterial in zebrafish”;

Chapter 3: Conclusion and Future Perspectives.

1.5. References

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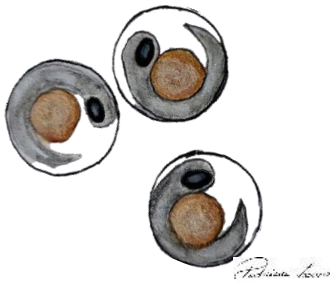
CHAPTER 2

Ecotoxicological effects of a “smart” engineered nanomaterial in zebrafish



2. Ecotoxicological effects of a "smart" engineered nanomaterial in zebrafish

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Abstract

Layered Double Hydroxides (LDHs) are anionic nanoclays, composed by positively-charged layers with divalent or trivalent metal cations (e.g. Zn^{2+} or Al^{3+}) and stabilized by anions (e.g. nitrates) and water molecules in the inter-layers. The vast possibilities of using LDHs can lead to their existence in the ecosystem, which raises ecological concerns. However, little is known about the effect of these nanomaterials on aquatic organisms. The present study aimed to assess the ecotoxicological effects of Zn-Al LDH-NO_3 in zebrafish (*Danio rerio*) early life stages. The endpoints measured were mortality, malformations and hatching rate after exposure of *D. rerio* embryos and larvae to Zn-Al LDH-NO_3 following the OECD 236 guideline. The behavioural, biochemical and molecular (at DNA level) alterations were also assessed using sub-lethal concentrations of Zn-Al LDH-NO_3. No observable effects on mortality were detected at Zn-Al LDH-NO_3 concentrations of 415.2 mg/L, while the LC_{50} value was 559.9 mg/L after 96 h. The tested LDHs caused malformations in *D. rerio* embryos, such as pericardial edema, incomplete yolk sac absorption and tail deformities, with an EC_{50} value of 172.4 mg/L, after 96 h of exposure. Locomotory behaviour in zebrafish larva were affected, during the dark periods, upon Zn-Al LDH-NO_3 exposure. No significant differences were reported between the control (FSW) and different LDHs concentrations for all the biochemical parameters evaluated (AChE, CAT, GPx, GR, GC and LPO). The results obtained in this study suggests that Zn-Al LDH-NO_3 toxicity is low towards zebrafish embryos.

Keywords: Layered double hydroxides, DNA damage, hazard assessment, *Danio rerio*

2.1. Introduction

Layered Double Hydroxides (LDHs) have been regarded as "smart" engineered nanomaterials (ENMs) and in the last decades received a lot of attention both from academics and industries. LDHs are known as hydrotalcite-like systems or anionic clays and are composed by positively charge mixed-metal hydroxide layers intercalated with anions and water molecules (Newman and Jones, 1998). They have peculiar properties like compositional flexibility in cations and anions, excellent anion exchangeability and memory effect. Besides that, LDHs have outstanding physicochemical characteristics, such as good biocompatibility, high chemical stability, pH-dependent, high surface-to-volume ratio and high adsorbing capacity (Choy, Oh and Choi, 2011; Mishra *et al.*, 2018; Arrabito *et al.*, 2019). LDHs have multiple applications, as in catalysis (He *et al.*, 2013), optical science, flame retardants (Kalali, Wang and Wang, 2015), fuel cells (Nicotera *et al.*, 2015), drug delivery (Choi and Choy, 2011), adsorption (Li *et al.*, 2014), nanomaterial engineering and corrosion science (Nalawade *et al.*, 2009; Tedim *et al.,* 2010; Zheludkevich, Tedim and Ferreira, 2012). Furthermore, LDHs have received a lot of attention in the use of removing pollutants from the aquatic environment (e.g., heavy metal, phosphate, nano-scale plastic debris) (Yang *et al.,* 2014; Koilraj, Takaki and Sasaki, 2016; Tiwari *et al.*, 2020). Such a wide possibility of using LDHs can lead to the occurrence of LDHs in the effluents and the freshwater systems receiving an effluent discharge, which has not been reported so far, which raises ecological and human health concerns.

The Zn-Al LDH have been applied in different fields, in the field of medicine used as nanocarriers of drugs, as absorbent for water remediation and also in corrosion science, thus, increasing the risk of their occurrence in the aquatic environment (Tedim *et al.,* 2012; Abdolmohammad-Zadeh, Nejati and Ghorbani, 2015; Iftekhar *et al.,* 2018; Pooresmaeil *et al*., 2019; Yasaei *et al*., 2019; Tiwari *et al*., 2020). On marine organisms, Zn-Al LDH-NO_3 cause no acute toxicity on adult invertebrates (*Ruditapes philippinarum, Mytilus galloprovincialis* and *Brachidontes pharaonis*) or embryotoxicity on larval stages of invertebrates (*Paracentrotus lividus* and *Artemia salina*), nor affect microalgae growth inhibition (*Tetraselmis chuii* and *Phaeodacylum tricornutum*) at concentrations less than 100 mg/L. On the bryozoan *Bugula neritina*, LDHs cause acute toxicity with an LC_{50} of 4.3 mg/L. In terms of physiological biomarkers, it was only observed neurotoxic effects on the edible clam, *Ruditapes philippinarum* (EC_{50} = 97.9) (Martins *et al.,* 2017; Avelelas *et al.,* 2017; Gutner-Hotch *et al.,* 2018; Gutner-Hoch *et al.*, 2019). Lastly, LDHs are shown to be of low toxicity to humans (Choi And Choy, 2011). However, as far as we are concerned, there is only a single ecotoxicological study of LDHs (Cu-Mg-Fe) on freshwater organisms, which is chemically

different from Zn-Al LDH. Cu-Mg-Fe LDH exposure causes a 72 h-IC_{50} value of 8.22 mg/L on the green freshwater algae *Scenedesmus quadricauda* (Ding *et al.*, 2018).

Danio rerio (zebrafish) is a freshwater fish, extensively used as an animal model in several research areas, such as environmental, developmental biology and pharmacology (Canedo and Rocha, 2021; Fontana *et al.*, 2021; Wang *et* al., 2021), since it offers several advantages, such as external and transparent development during embryogenesis, which facilitates the visual inspection of early development progress at stereomicroscope, short life cycle, high offspring production, small size, among others. This fish species model has been widely used to assess the effects of several NPs, namely Ag NP, Au NP, CuO NP, carbon-based nanomaterials, TiO_2 NP, ZnO NP, Al_2O_3 NPs, among others (Kim *et al.*, 2013; Xin *et al.*, 2015; Ganesan *et al.*, 2015; Lu *et al.*, 2016; Zhao *et al.*, 2016; Zhu *et al.*, 2019; Boran and Şaffak, 2020; Qiang *et al.*, 2020).

The ecotoxicological evaluation in freshwater organisms, particularly on fish, is critical for regulatory purposes, since the nanomaterial Zn-Al LDH-NO_3 is already in the market. Considering the wide-variety of applications of this stimuli-responsive nanomaterials, Zn-Al LDH may end up in the aquatic environment, through direct leaching from coatings applied in recreational vessels to lakes or rivers and/or via effluents from wastewater treatment plants, raising ecological and human health concerns. In order to cover this gap knowledge, the present study aims at assessing the short and long-term effects of Zn-Al LDH-NO_3 in *Danio rerio*, using a battery of tests to evaluate eventual developmental, behavioural, genotoxic and biochemical alterations.

2.2. Materials and Methods

2.2.1. Test chemicals

In the present study, the nanomaterial Zn-Al LDH-NO_3 (hereinafter as Zn-Al LDH) was synthetized by co-precipitation and kindly provided by Smallmatek, Lda. (Aveiro, Portugal). Details on the synthesis method and nanomaterial characterization are provided in Martins *et al.* (2017) and the percentage of zinc present in the Zn-Al LDH is 33.08% and aluminium is 7.38%. Fresh stock solutions were prepared using fish system water (FSW, further info on composition of FSW are presented by Almeida *et al*. (2019)) for each test.

The chemicals used in the biomarker's and comet assay, trichloroacetic acid (TCA; CAS: 76-03-9; 99%); Tris hydrochloride (Tris-HCl; CAS: 1185-53-1; 99%); Hydrogen peroxide (H_2O_2; CAS: 7722-84-1; 35%); ethylene diamine tetra-acetic acid (EDTA; CAS: 84256-90-6; 90%); Sodium azide (NaN_3; CAS: 26628-22-8; 99%); L-Glutathione reduced (GSH; CAS: 70-18-8; 98%); β-Nicotinamide

adenine dinucleotide phosphate (NADPH; CAS: 2646-71-1; 93%); L-Glutathione oxidized (GSSG; CAS: 27025-41-8; 98%); Diethylenetriaminepentaacetic acid (DTPA; CAS: 67-43-6; 99%); Acetylthiocholine chloride (DTNB; CAS: 6050-81-3; 99%); Diethylenetriaminepentaacetic acid (DTPA; CAS: 67-43-6; 98%); 2-Thiobarbituric acid (TBA: CAS: 504-17-6; 98%); Dimethyl sulfoxide (DMSO; CAS: 67-68-5 ; 99%); 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI; CAS: 350.25 ; 95%); Triton™ X-100 (Triton; CAS: 9002-93-1); Sodium hydroxide (NAOH; CAS: 1310-73-2; 97%); Sodium chloride (NaCl; CAS: 7647-14-5) were purchased from Sigma-Aldrich (The Netherlands), with the exception of the Bradford reagent, which was purchased from Bio-Rad (Germany).

2.2.2. Environmental fate and behaviour assessment of Zn-Al LDH

The environmental fate and behaviour of Zn-Al LDH in zebrafish FSW were assessed through dynamic light scattering (DLS) and zeta potential (ζP) measurements using a Zetasizer Nano-ZS (Malvern Instruments, UK), following the recommendations of the OECD 318 standard guideline. Two relevant concentrations of Zn-Al LDH (10 and 100 mg/L) were prepared in FSW, and each dispersion was then sonicated for 30 min in an ultrasonic bath (Selecta; 40 kHz) immediately before the measurements. These concentrations were selected based on the range of sublethal concentration used in the different ecotoxicological exposures. Each dispersion was kept in the room where the FET were realized to mimic the exposure scenario (with the same temperature and photoperiod conditions). Measurements were made at 0, 24, 48, 72 and 96 h in triplicate (samples were not agitated to mimic the exposure scenario).

2.2.3. Test organism

The organisms of the species *Danio rerio* (zebrafish, wild-type AB) used in this study were kept and supplied by the Department of Biology of the University of Aveiro. Adult organisms were kept under controlled conditions, in a ZebTEC recirculation system (Tecniplast). The zebrafish were kept in tap water filtered with activated charcoal and reverse osmosis, complemented with "Instant Ocean Synthetic Sea Salt" (Spectrum Brands, USA) at a 0.34 mg/L salinity. The FSW is kept at 27.0 ± 1 °C, dissolved oxygen equal to or greater than 95% saturation, the conductivity of 794 ± 50 µS/cm and the photoperiod was 14 h: 10 h (light: dark). Adult fish were fed twice daily with commercially available artificial diet Gemma Micro 500 (Skretting®, Spain) (Almeida *et al.,* 2019).

2.2.4. Ecotoxicity

2.2.4.1. Zebrafish embryo toxicity test (FET)

To assess the toxic effects of Zn-Al LDH in terms of mortality and development of zebrafish embryos, the OCDE 236 guideline on fish embryo toxicity test (FET) was followed (OCDE, 2013). The day before the test initiation, a physical barrier was placed in the aquaria to separate males and females. Spawning occurred once the physical separation was removed, and fish were allowed to mate for 30 min to 1h before the beginning of the assay. Afterwards, the eggs produced were collected and checked under a stereomicroscope (Stereoscopic Zoom Microscope - SMZ 1500, Nikon). Eggs with cleavage irregularities, injuries, or other kinds of malformations were discarded following the OECD 236 guideline. Approximately at 2 – 3 hours post fertilization (hpf) zebrafish embryos were exposed to different concentrations of Zn-Al LDH (85 – 1200 mg/L; dilution factor of: 1.7) and negative control (only FSW). For the exposure, 24-well plates were used with 10 eggs per concentration (one egg per well in 2 mL of exposure media) and internal control for up to 96 h. Treatments were carried out in triplicate. The medium was partially renewed after 48 hours. The development of zebrafish was observed under a stereoscopic microscope (Stereoscopic Zoom Microscope - SMZ 1500, Nikon) and the following parameters were recorded: hatching, tail deformation, equilibrium (embryos unable to keep an upright position), yolk and/or heart oedema and sac absorption. The physical-chemical data (pH and conductivity) were measured at the beginning and the end of the exposure. The pH ranged from 7.3 ± 0.3 to 7.6 ± 0.1 at the beginning and end of the experiment, respectively, and the conductivity in the beginning was 790 ± 3 (µs/cm) and 833 ± 6 (µs/cm) at the end of the exposure test.

2.2.4.2. Behavioural assay

In order to assess the behavioural changes caused by Zn-Al LDH exposure, a total of 40 eggs were exposed to sub-lethal concentrations according to the FET results, ranging from 8.92 to 93.68 mg LDH/L (dilution factor of: 1.8), in 24-well plates. After 120 hours of exposure, the organisms were observed with the aid of a stereoscopic microscope (Stereoscopic Zoom Microscope - SMZ 1500, Nikon) and those without malformations were selected for the behaviour analysis assay. A total of 12 larvae from each treatment were randomly placed in a 96-well plate and introduced into the Viewpoint Zebrabox® (Viewpoint Life Science, Lyon, France). The test consisted of alternating periods of light and dark, lasting 5 minutes each, with a total of 2 light-dark cycles (20 minutes of tracking) after a 15-minute light acclimation period of the exposed larvae to the ZebraBox system.

This behaviour analysis was carried out in triplicate. The video tracking configuration of the Zebrabox tracked the total movement of the fish over the light and dark cycles duration. The methodology described was based on published procedures by Almeida *et al* (2019).

2.2.4.3. Biomarkers of oxidative stress and neurotoxicity

To assess the biochemical effects caused by Zn-Al LDH exposure, a total of 20 eggs were exposed in triplicate to sub-lethal concentration, from 8.92 to 93.68 mg LDH/L (dilution factor of: 1.8), in 24-well plates. After 120 hours of exposure, twenty larvae per treatment were immediately transferred to an eppendorf, cryopreserved in liquid nitrogen and stored at -80°C until analysis. The samples were homogenized in 1 ml of phosphate buffer (0.1 M, pH 7.4). Each replicate was homogenized using an ultrasonic homogenizer and cooled in ice during the process. After homogenization of the organisms, a 150 µL aliquot of the homogenate was separated for immediate lipid peroxidation (LPO) determination. The rest of the homogenate was then centrifuged at 10,000 *g* for 20 min (4 °C) to isolate the post-mitochondrial supernatant (PMS) and stored at −80 °C until the determination of glutathione-S-transferase (GSTs), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), and acetylcholinesterase (AChE) enzymatic activities.

LPO assessment was based on the spectrophotometric quantification of thiobarbituric acid reactive substances (TBARS), according to the protocols described by Ohkawa *et al* (1979) and Bird and Draper (1984), adapted for microplates. To the samples separate for LPO analysis was added 12% TCA, Tris-HCl (60 mM) with DTPA (0.1 mM) and 0.73% TBA. Absorbance was measured at 535 nm and the results were expressed as nmol of TBARS per mg protein.

The protein content (PROT) of the samples was determined through the spectrophotometric method of Bradford adapted to microplates (wavelength 595 nm) (Brandford, 1976). The Bio-Rad protein detection kit and bovine gamma globulin as the standard was used. Before each enzymatic determination, PROT of each sample was normalized to around 0.5 mg/mL, except from the AChE samples that were normalized to 0.2 – 0.3 mg/mL (Guilhermino *et al.,* 1996).

CAT activity was determined by the method Clairborne (1985). 15 µL of supernatant which was added to 135 µL of phosphate buffer (0.05 M, pH 7.0) and 150 µL of H_2O_2 (0.03 M) (adapted to microplates). Then, the measured in microplate reader at 240 nm and the results were expressed as µmol of substrate hydrolyzed per min per mg protein.

Glutathione peroxidase (GPx) activity was calculated by the method of Mohandas (1984), adapted for microplates. 15 µL of supernatant was added to 282 µL of a solution composed by phosphate buffer (0.05 M, pH 7.0), EDTA (1mM), Sodium azide (1 mM), GR, GSH (4 mM) was also added NADPH (0.8 mM) and to start the reaction 3 µL of H_2O_2 (0.5 M) was added. Absorbance was measured for 3 min at 340 nm and the results were expressed as µmol of substrate hydrolyzed per mg protein.

GR activity was determined according to the methodology described by Cribb (1989). The methodology was adapted for microplates using 15 µL of PMS and 285 µL of the reaction solution (composed by NADPH, GSSG and DTPA). Absorbance was measured for 1 min at 340 nm and the results were expressed as µmol of substrate hydrolyzed per mg protein.

AChE activity was spectrophotometrically determined following method described by Ellman *et al.* (1961) adapted for microplates (Guilhermino *et al.,* 1996). Activity was determined using 50 µL of PMS and 250 µL of reaction solution (acetylthiocholine (0.075 M), DTNB in potassium-phosphate buffer (0.1 M, pH 7.2). Absorbance was measured at 414 nm for 10 min after starting the reaction and then repeated twice every 5 min. Results were expressed as nmol of substrate hydrolyzed per min per mg protein.

GST activity was determined according to the methodology described by Habig *et al.* (1974). The methodology was adapted for microplates using 100 µL of PMS and 20 µL of the reaction solution (composed by potassium-phosphate buffer (0.1 M, pH 6.5), CDNB (10 nM) and GSH (10 mM)). The absorbance was measured at 340 nm every 20 s for 5 minutes and the results were expressed as nmol of hydrolyzed substrate per minute per mg of protein.

2.2.4.4. Single cell gel electrophoresis assay (comet assay)

In order to evaluate the DNA damage instigated by Zn-Al LDH exposure, a total of 10 eggs per replicate with three replicates per treatment were exposed to sub-lethal concentration, from 8.92 to 93.68 mg LDH/L (dilution factor of: 1.8), in 24-well plates. After 96 hours of exposure, only larvae with normal development showing no malformations were selected for the comet assay. The following methodology was adapted by the experimental procedure proposed by Pavlaki *et al.* (2016). The positive control was performed at 96h, exposing 10 fish larvae to 0.1% (v/v) hydrogen peroxide (H_2O_2) for 1 hour in the dark. All organisms were sampled and placed in 900 µL of phosphate buffered saline (PBS) with 20 mM ethylene diamine tetra-acetic acid (EDTA) and 10% of dimethyl sulfoxide (DMSO). The organisms were mechanically disintegrated with an appropriate

micro-pestle and then were centrifuged at 200 *g* for 10 min at 4 °C. After removing the supernatant, the remaining cell medium was resuspended and 20 µL were then mixed with 140 µL of 1% low-melting point agarose (0.5% at 37 °C) and finally spread onto microscope slides, previously coated with 1% normal melting point agarose. After, the slides were placed on ice for 10 min to solidify and lastly, immersed in cold lysing solution (10 mM Tris-HCl, 100 mM EDTA acid, 2.5 NaCl, 10% DMSO, 1% Triton X-100 at pH 10) for 4 h at 4 °C, in the dark. After the cell lysis time was over, the slides were transferred to the electrophoresis device. Before starting electrophoresis, the slides were kept immersed in the electrophoresis solution (10 M of NaOH (pH 10) and 200 mM Na_2 EDTA) for 20 minutes to allow the DNA to denature and unwind. For the electrophoresis, an electric current of 300 mA (25 Volts) was applied for 15 min. Afterwards, slides were washed with neutralization solution (0.4 M Tris-HCl in UP water with pH 7.5, 4 °C). Lastly, slides were dehydrated with absolute ethanol for 10 s and left to dry for 24 hours. On the day of visualization, the slides were stained with 80 µL of DAPI and observed under a fluorescence microscope (Carl Zeiss Axio Scope A1 fluorescence microscope), with a 400x magnification. To analyse the DNA damage (comet length, comet height, tail DNA, tail moment and tail olive moment), approximately 50 cells were examined per slide with the CometScore software®.

2.2.5. Statistical analysis

Data normality and homoscedasticity were tested using the Shapiro–Wilk and the Levene tests ($p < 0.05$), respectively. The genotoxicity parameters for comet length, comet height, tail DNA, tail moment and tail olive moment were calculated using the CometScore software. Statistical differences between the control and each treatment were analysed by a one-way ANOVA, followed by Dunnett's multiple comparison tests whenever significant differences were observed ($p < 0.05$). The no observed effect concentration (NOEC) was therefore derived. When data normality and homoscedasticity were not verified, and data transformation did not correct normality or homoscedasticity, a non-parametric Kruskal–Wallis one-way analysis of variance on ranks was used followed by the Dunn's test when significant differences were found ($p < 0.05$). The statistical analyses were carried out using IBM SPSS statistic software. The LC_{50} values (i.e., the concentration that caused 50% mortality) were calculated by non-linear regression with the software GraphPad Prism v.6.0. The non-linear regression equation that best fits to the data was chosen, considering the R^2 value, the absolute sum of squares and the 95% confidence intervals.

2.3. Results

2.3.1. Environmental fate and behaviour assessment of Zn-Al LDH

DLS measurements of Zn-Al LDH tested showed a very high polydispersity index (PdI > 0.8), highlighting the heterogeneity and the possible presence of large aggregates/agglomerates or even sedimentation. The hydrodynamic size of the suspended particles was based on the average value (n = 3) of intensity distribution (peak 1) of each measurement, instead of the Z-average diameter. The DLS data shows that hydrodynamic size increase with the time regardless the exposure concentration (10 and 100 mg/L). The 100 mg/L dispersion tend to form larger aggregates in time (830 nm at 96 h) than the dispersion of 10 mg/L (340 nm at 96 h) (Figure 2.1).

The zeta potential values ranging between -12.2 and 5.3 mV. For the lower concentration (10 mg/L) the values of zeta potential were negative (-12.2 to -9.4 mV) and for the concentration of 100 mg/L the zeta potential values were positive (2.9 to 5.3 mV) (Figure 2.2).

Table 2.1 reports the parameters variation over time (0 – 96 h) according to the OECD 318. Particle size variation (DLS data) was greater than 20% in both concentrations meaning that both dispersions are unstable over time. Zeta potential varied 44% between the beginning and end of the measurements period in the concentration of 100 mg LDH/L denoting that this dispersion is also unstable according to this parameter. The lowest tested concentration (10 mg/L) had a zeta potential variation lower than 20%, which, according to the criteria of the OECD 318 protocol, can be considered a stable dispersion.

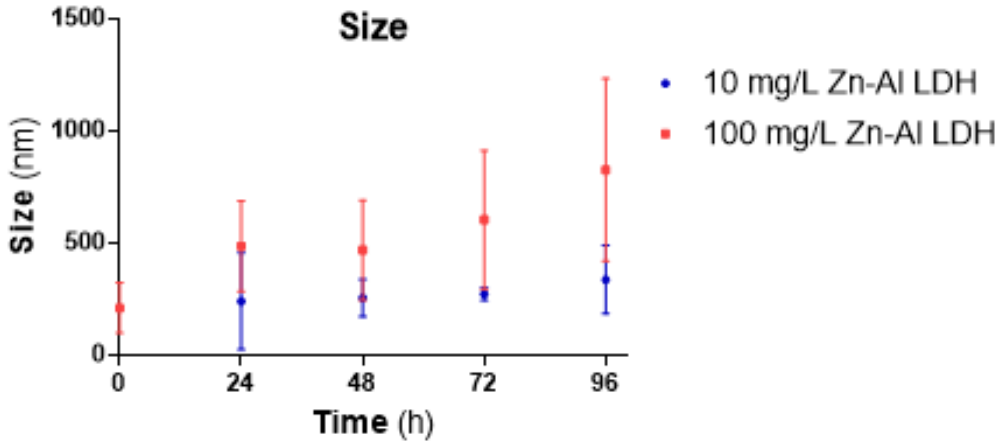

Figure 2.1| Hydrodynamic size (mean ± standard deviation) in nanometers (nm) (based on Pk1 mean values) of the two dispersion of Zn-Al LDH (10 and 100 mg/L) in zebrafish FSW during different timepoints (0, 24, 48, 72 and 96 hours, h).

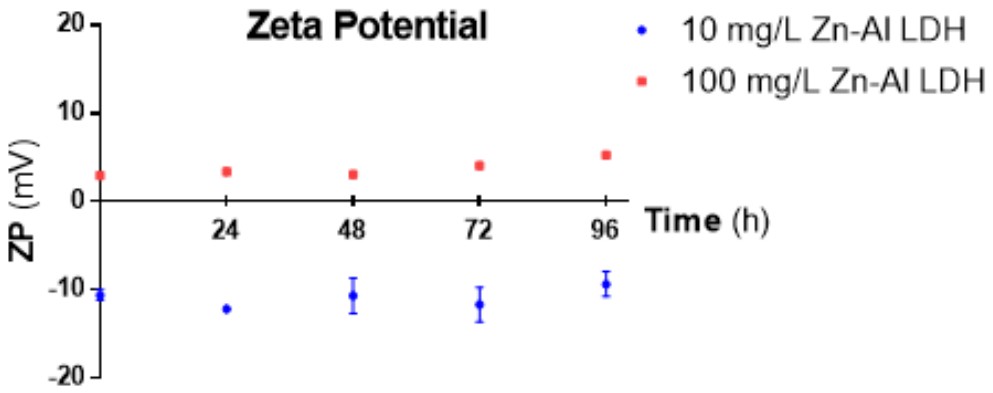

Figure 2.2| Zeta potential (mean ± standard deviation) in millivolts (mV) of the two dispersion of Zn-Al LDH (10 and 100 mg/L) in zebrafish FSW during different timepoints (0, 24, 48, 72 and 96 hours, h).

Table 2.1| Zeta potential (ZP) and dynamic light scattering (DLS) variation over time (from 0 to 96 h) measured in dispersions of Zn-Al LDH (10 and 100 mg/L). Dispersion stability can be inferred when values are lower than ± 20% (OECD 318).

	ZP	DLS
10 mg Zn-Al LDH/L	-13%	28%
100 mg Zn-Al LDH/L	44%	74%

2.3.2. Ecotoxicity

Control organisms showed normal development. The concentration that caused 50% mortality (LC_{50}) for Zn-Al LDH after 96 h of exposure was set at 551.9 mg Zn-Al LDH/L ($CI_{95\%}$ = 467.1 – 652.0) and the no observed effects concentration (NOEC) was 415.2 mg Zn-Al LDH/L.

After 48 h of exposure, the control presented 100% hatching success. The hatching success decreased with increasing Zn-Al LDH concentrations. The EC_{50} was calculated at 801.2 mg Zn-Al LDH

/L ($Cl_{95\%}$ = 551.6 – 1050.8 mg/L) and NOEC at 244.3 mg/L. For malformations, the no observed effects concentration (NOEC) was 143.7 mg Zn-Al LDH/L (Table 2.2).

Table 2.2| Median lethal and effect concentrations (L/EC_{50}) and lowest tested concentration with observed effects (LOEC) for Zn-Al LDH (mg/L) and Zn (represent 33.1% of Zn-Al LDH) and Al (represent 7.4% of Zn-Al LDH) after a 96 h exposure to *Danio rerio* (n.d.- not determined).

	LOEC (mg/L)			**L/EC_{50}** (mg/L)		
	Zn-Al LDH	**Zn**	**Al**	**Zn-Al LDH**	**Zn**	**Al**
Survival	705.8	233.5	52.1	551.9	182.6	40.7
Hatching success	415.2	137.4	30.6	801.2	265.0	59.1
Malformation	244.3	80.8	18.0	n.d.	n.d	n.d

During the exposure assay, it was observed adhesion of nanomaterials to the chorion of the embryo (Figure 2.3). Figure 2.3 shows the embryo during the exposure at 72 h at the low and the high concentration tested.

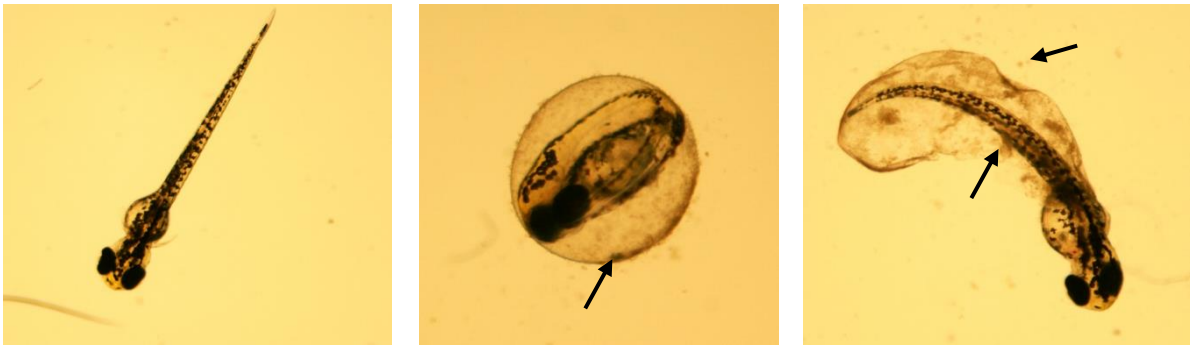

Figure 2.3| Zebrafish embryo exposure to the control (A) and to the concentration of 84.5 mg Zn-Al LDH/L (B) and 1200 mg Zn-Al LDH/L (C). The arrow in figure B and C show the adhesion of particles and/or aggregates/agglomerates of Zn-Al LDH.

2.3.3. Behaviour assay

Figure 2.4 shows the total distance (mean ± standard error; mm) covered during the two alternating cycles of light/dark by *Danio rerio* larvae after 120 h exposure to Zn-Al LDH. The total distance recorded by larvae exposed to all treatments in both dark cycles was significantly lower (Dunnett's, $p < 0.05$) compared to control organisms, except for the higher concentration on the first dark cycle (Figure 2.4). Lastly, no significant decrease in total distance was observed in both light cycles when compared to the control, except at 16 mg/L and 52 mg/L during the first and second light cycle, respectfully (Figure 2.4).

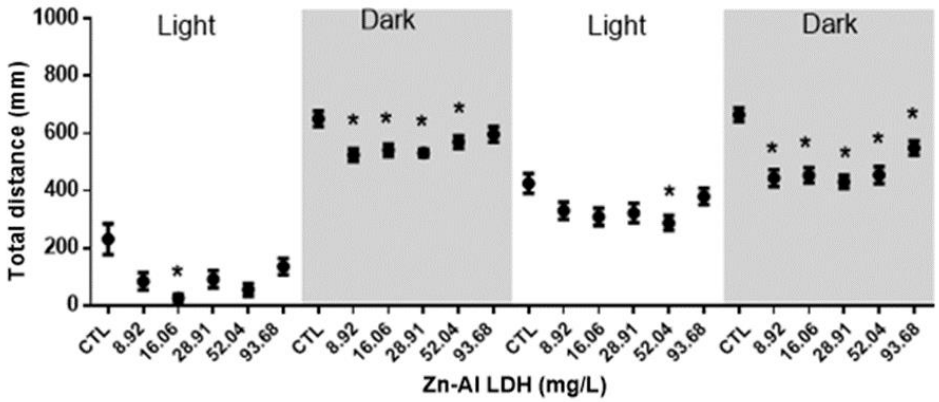

Figure 2.4| Total swimming (mean ± standard error) in millimeters (mm) covered during two alternating cycles of light/dark by *Danio rerio* larvae after 120 h exposure to Zn-Al LDH.

Figure 2.5 shows the total duration of locomotion (mean ± standard error; s) during two alternating cycles of light/dark by *Danio rerio* larvae after 120 h of exposure to Zn-Al LDH. A significant decrease in the total duration of locomotion was detected in all exposure concentrations on the second dark cycle, as well as in the concentration of 16 mg/L in the first light cycle when compared to the control (Figure 2.5).

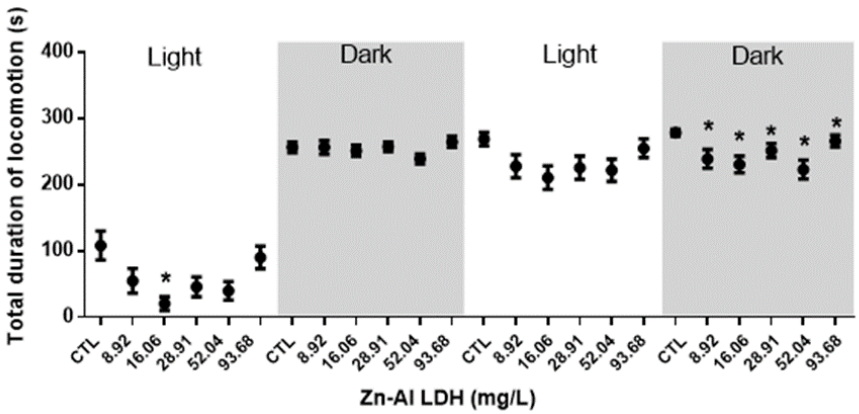

Figure 2.5| Total duration of locomotion (mean ± standard error) in seconds (s) during two alternating cycles of light/dark by *Danio rerio* larvae after 120 h exposure to Zn-Al LDH.

2.3.4. Biomarkers of oxidative stress and neurotoxicity

Globally, no significant biochemical differences were found between the control and all LDHs treatments (Dunnett test; $p > 0.05$). Despite not statistically significant, some patterns were seen, namely an increase of the antioxidant enzymes (GPx and GR as low as 16.1 mg/L; CAT at 28.9 mg/L) followed by membranes lipoperoxidation (TBARS levels) at 28.9 mg/L. The detoxification enzyme GST and the neurotoxicity biomarker AChE do not significantly change along the range of exposure concentrations. Biochemical responses induced by Zn-Al LDH are shown in Figure 2.6.

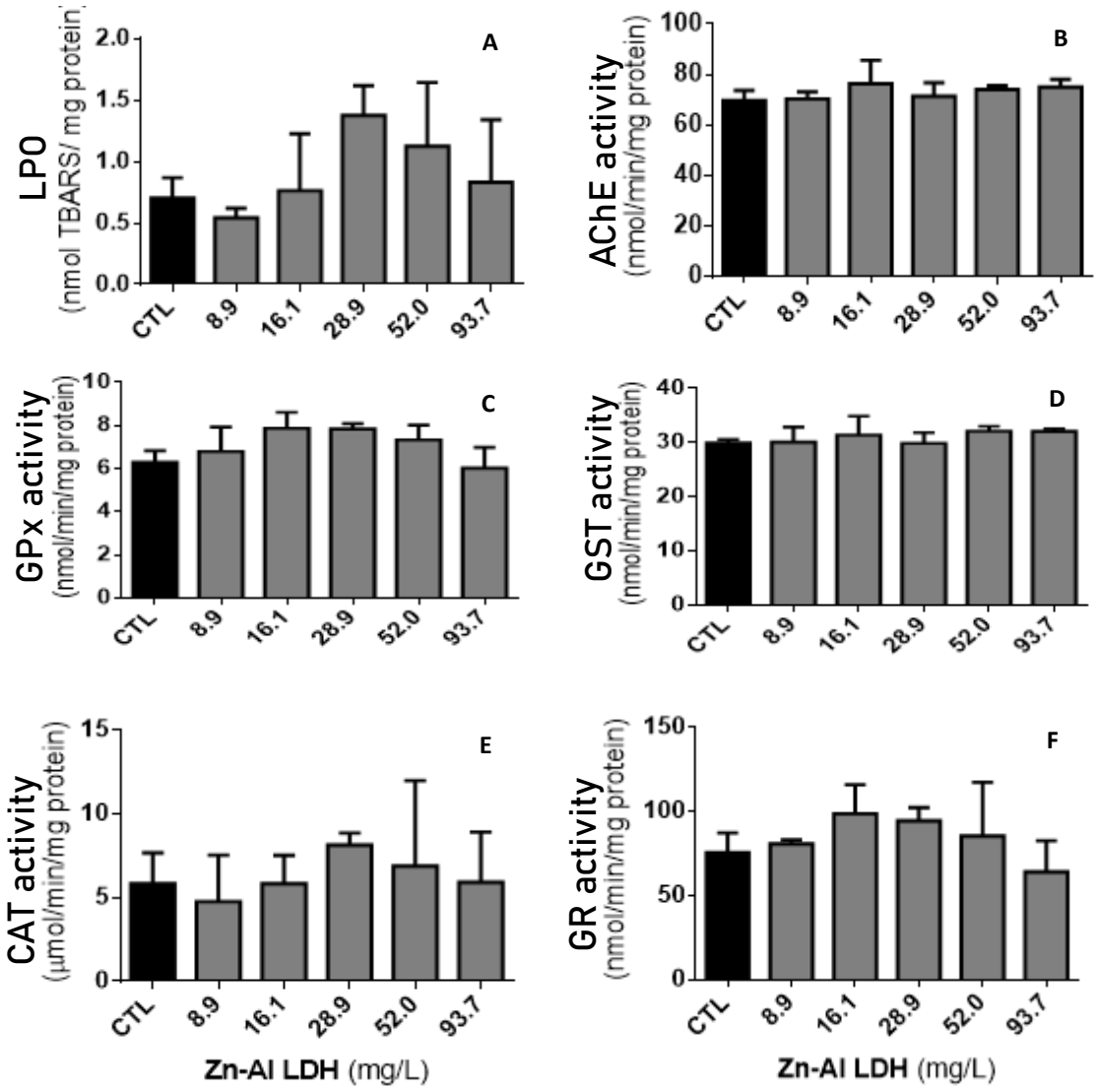

Figure 2.6| Biochemical endpoints measured in *Danio rerio* larvae after 120 h of exposure to 8.9 – 93.7 mg/L of Zn-Al LDH (mean ± standard deviation): lipid peroxidation (LPO; A) and enzymatic activity of acetylcholinesterase (AChE; B); glutathione peroxidase (GPx; C); glutathione-*S*-transferases (GSTs; D); catalase (CAT; E); glutathione reductase (GR; F). CTL – negative control.

2.3.5. Single cell gel electrophoresis assay (comet assay)

DNA damage induced by Zn-Al LDH is shown in Figure 2.7, namely tail DNA, tail moment, tail olive moment, comet length and comet height. The negative control presents a low value of DNA damage for the parameter of percentage of tail DNA (14.5% ± 3.2%) while the positive control (H_2O_2) shows a high value of tail DNA (29.4% ± 20.6%) thus validating the assay. No significant

differences (Dunnett's; $p > 0.05$) in the tested parameters were found in all exposure concentrations when compared to the control (only FSW).

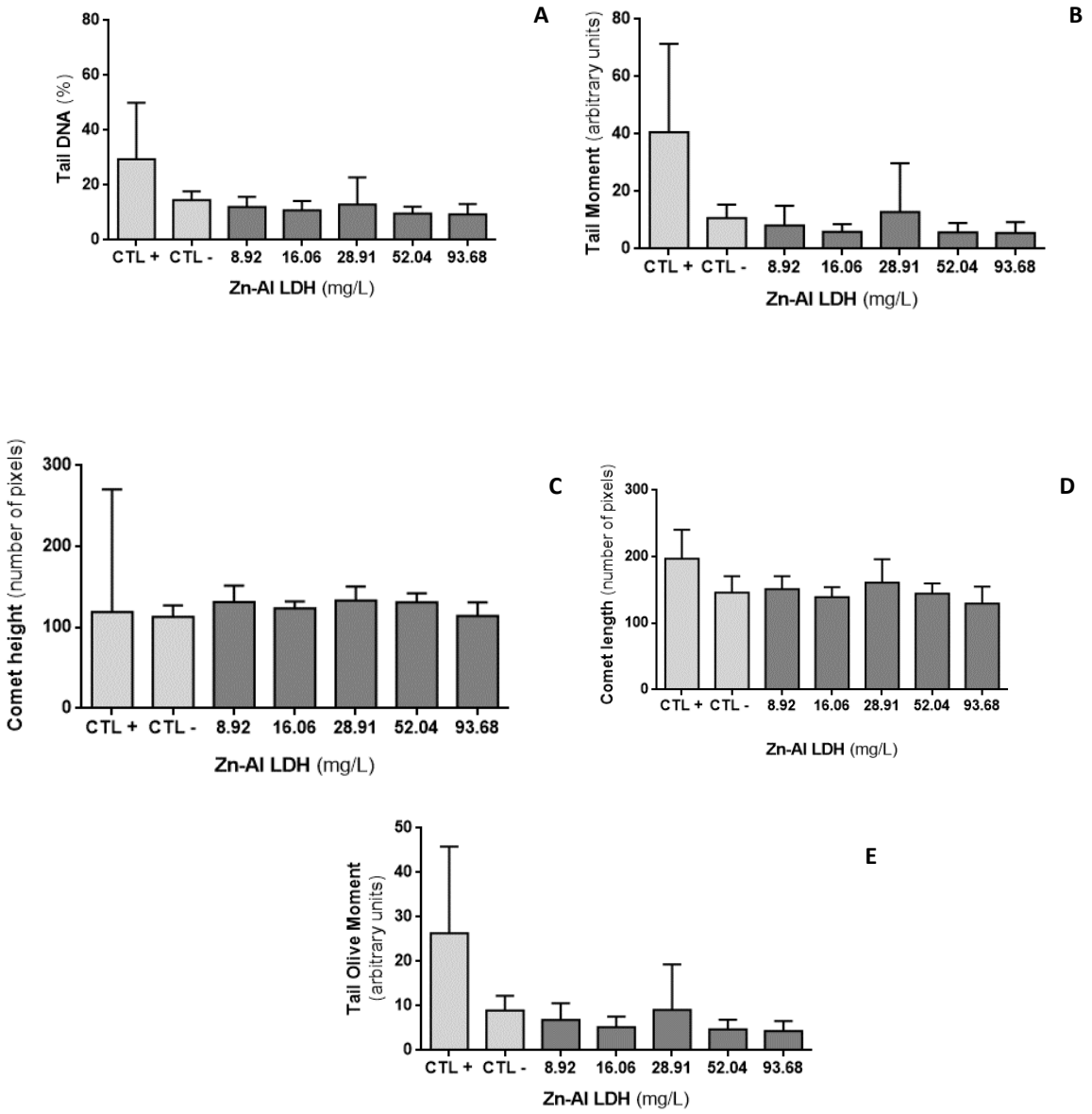

Figure 2.7| Genotoxic effects in Danio rerio larvae after 96 h exposure to Zn-Al LDH (8.92 – 93.68 mg/L) determined by comet assay. Data is expressed as DNA content in the tail (A), intensity of tail moment (B), comet height (C), comet length (D), and intensity of tail olive moment (E) (mean ± standard deviation). Values are expressed as percentage (A), arbitrary units (B and E) and number of pixels (C and D). Positive (CTL+; H_2O_2) and negative (CTL-; only fish system water) controls are indicated in light grey bars.

2.4. Discussion

The wide-range of nano-based products and the intense use of nanomaterials have been raising environmental concerns (Bundschuh *et al.,* 2018). LDHs are "smart" nanomaterials with anionic exchange capacity which makes them very attractive for industrial and academic purposes, such as nanocarrier, functional additive for coatings or concrete, flame retardant or environmental remediation. However, the ecotoxicity of LDHs, which are already in the market, is scarcely understood, particularly towards freshwater organisms. Thus, the present study aimed to fill in part of such knowledge gap by assessing the environmental behaviour and fate as well as ecotoxicity towards the model fish species *Danio rerio*, thus providing key data for a future regulatory process. In the present study, 100 mg/L LDH dispersions were unstable and tended to form large aggregates/agglomerates over time, similarly to other engineered nanomaterials (e.g. Figueiredo *et al.*, 2019). The FSW has a low salt content explaining the electrical conductivity close to 800 µS/cm, five orders of magnitude higher than ultra-pure water (0.055 µS/cm) and ~70-fold lower than the typical conductivity of seawater (53 mS/cm) (Assiry *et al*., 2010). Aqueous dispersions that are ion-rich are known to cause agglomeration of NMs influencing their toxicity (Murdock *et al.,* 2008). This process can change their physicochemical properties and may reduce exposure to suspended NMs (OECD GD 317, 2020) making them less available to cause toxicity.

Zebrafish have been used to assess the toxic effects of a wide-range of nanoparticles (e.g. Lu *et al.,* 2016; Zhu *et al.*, 2019; Qiang *et al.,* 2020). Although Zn-Al LDH is chemically and structurally different of such studied nanoparticles, some chemical similarity can be found with ZnO NPs and Al_2O_3 NPs. Zhu *et al.* (2008) evaluated the effects on the development of zebrafish embryos and larvae after exposure to ZnO NPs and determined a LC_{50} of 1.4 mg Zn/L and an EC_{50} of 1.6 mg Zn/L in terms of hatching success. Boran and Şaffak (2020) also determined a LC_{50} of 68.9 mg Al/L in Al_2O_3 NP exposure and LC_{50} of 3.3 mg Al/L from ionic Al^{3+}. These values are lower than the present findings demonstrating that Zn-Al LDH are less toxic nanostructured forms comparing ZnO NPs and Al_2O_3 NPs. Survival, hatching and malformations were significantly affected only at concentrations higher than 415, 244 and 144 mg/L of Zn-Al LDH, respectively, well above the recommend threshold for the highest test concentration (100 mg/L), according to the OECD guidance document to test NMs toxicity (e.g. OECD GD317, 2020). According to the toxicity categories adapted for nanomaterials by Blaise *et al*. (2008), based on the European Commission Directive 93/67/EEC, Zn-Al LDH can be classified as non-toxic nanomaterial. This is fully in agreement with other studies performed with marine organisms in lower trophic levels, namely invertebrates and photosynthetic species (Avelelas *et al*., 2017; Martins *et al*., 2017; Gutner-Hoch *et al*., 2018, Gutner-Hoch *et al*., 2019).

Taking into consideration the acute and developmental (non-)toxicity of LDHs, an important question remained unaddressed on whether LDHs can cause sub-lethal toxicity. The first approach of the present study was to assess possible behavioural changes caused by LDHs exposure. It is known that changes in the swimming behaviour of zebrafish can influence the prey-predator behaviour and can cause a decrease in the ability to compete for food (Domingues *et al.,* 2016). Results from this study concluded that the total distance and duration of zebrafish larvae movement in the dark were affected by Zn-Al LDH exposure causing larvae hypoactivity an effect that can be explained by the aggregation followed by NMs sedimentation in the well plate. The LOEC for the locomotory behaviour was 2.9 mg Zn/L in this study. Previously, Chen *et al*. (2014), reported similar values in zebrafish larvae when exposed to zinc oxide nanoparticles, with a LOEC value of 3.9 mg Zn/L. In addition, the decrease in the total swimming distance moved herein, suggests that Zn-Al LDH exposure can cause depression-like behaviour in the fish larvae. Similarly, Li *et al.* (2017) found the similar behavioural and depression inducing effects when adult zebrafish were exposure to SiO_2 nanoparticles.

Regarding, oxidative stress, neurotoxicity and genotoxicity after observing behavioural changes to zebrafish exposed to sub-lethal concentrations of Zn-Al LDH is of utmost importance. Alterations at a molecular level can be observed at concentrations where no physiological responses can be observed. DNA damage and biochemical changes can highlight the activation of some metabolic pathways after exposure to xenobiotics which can have consequences on the physiological performance and ultimately on organism's survival. The present study concluded that Zn-Al LDH exposure caused no genotoxicity, oxidative stress or changes on enzymes of the antioxidant and detoxification systems and AChE related to neurotransmission impairment. Boran and Saffak (2020) demonstrated a LOEC value of 26.5 mg Al/L from Al_2O_3 NPs and a LOEC of 15.0 mg Al/L from ionic Al^{3+}. This may highlight that the nanomaterial may act differently in function of the tested species, ecology or life traits (e.g. freshwater/marine, pelagic/benthic, feeding strategies, age).

The absence of significant sub-cellular effects together with the behavioural impairment may be associated to the toxicity mechanism: more physical/mechanic than specific "nano-chemistry"-based effects, closely related to the LDHs tendency to aggregate and sink in time and their instability in the exposure media. LDH particles, or aggregates/agglomerates formed during the exposure period, seem to be too large to be internalized. Furthermore, partial dissolution of metals from LDH over time (Gomes *et al.,* 2020) is unlikely to cause a chemical-driven toxicity in the studied parameters. In fact, during the exposure assay it was possible to see LDHs around the

chorion of the zebrafish embryo (cf. Figure 2.1). The zebrafish chorion plays an important role as a first barrier against the harmful effects of several nanomaterials, namely AgNPs (Chen *et al.,* 2020), SiO_2 NPs (Vranic *et al.,* 2019), mesoporous silica NPs (Paatero *et al*., 2017), among others. The zebrafish chorion pores are critical for the exchange of oxygen and nutrients between the aqueous environment and the embryo (Qiang *et al*., 2020). The pore's size of zebrafish chorion is 0.77 µm (Chen *et al*., 2020), while bigger agglomerates can easily cover/overlap the pores and impair their regular function. In this study, the hydrodynamic size observed for the concentration of 100 mg/L of Zn-Al LDH at 48 h and 72 h were 471.5 and 606.6 nm, respectively. The hydrodynamic size that this nanomaterial can reach and the fact that can occur adhesion of LDH's agglomerates in the chorion of zebrafish, may support the hypotheses of a mechanical effect. Martins *et al.* (2017) also suggested that these nanoclays can cause toxicity through a mechanical/physical barrier once larger particles of Zn-Al LDH can be retained in the gills of the clam *Ruditapes philippinarum* and cause depletion of oxygen with all cascade of effects.

2.5. Conclusion

The present study demonstrated that Zn-Al LDH caused no genotoxic or oxidative stress effects to *Danio rerio* larvae to concentrations up to 93.7 mg/L. The behaviour observed combined with the absence of sub-cellular effects may highlight a mechanical-driven effect of these nanoclays, which need further studies. The present findings suggest that Zn-Al LDH is low toxic to zebrafish embryos and larvae. This study is an important step to cover the gap knowledge of nanomaterial toxicology for freshwater organisms and to the ecotoxicological evaluation of the Zn-Al LDH for regulatory purposes.

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CHAPTER 3

Conclusion and Future Perspectives

3. Conclusion and Future Perspectives

The aquatic environment is considered a sink for various environmental contaminants, particularly nanomaterials, due to industrialization, agricultural productivity and also the discharge of urban effluents.

Zn-Al layered double hydroxides (Zn-Al LDH) are widely used and can reach freshwater ecosystems through direct release (e.g., coatings) or wastewater treatment effluents. Despite Zn-Al LDH are already in the market, their ecotoxicological effects on freshwater organisms are not yet documented which is critical for regulatory purposes. Since the ecotoxicological effects of such nanomaterial in freshwater organisms are scarcely studied, this work took a small step towards the important assessment of the ecotoxicity of this innovative nanomaterial using a widely used model freshwater fish species.

The present study concluded that Zn-Al LDH-NO_3 can be considered low toxic since they cause mortality at concentrations higher than 100 mg/L (LC_{50} >> 100 mg/L) and induce no genotoxicity, neurotoxicity and oxidative stress effects on *Danio rerio* larvae. However and even though it was observed an absence of subcellular effects, in combination with the significant changes observed and measured in the behaviour, it may indicate a mechanical effect of this nanoclay to the organism studied.

Further studies are still needed to validate the mode of action of Zn-Al LDHs. The present findings are critical for a future risk assessment of Zn-Al LDH together with data from other freshwater species (e.g. microalgae and crustaceans) and probably confirm the eco-friendly properties of this nanomaterial.